ACEROLA (MALPIGHIA EMARGINATA DC): PHENOLIC PROFILING, ANTIOXIDANT CAPACITY, ANTIMICROBIAL PROPERTY, TOXICOLOGICAL SCREENING, AND COLOR STABILITY

By

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To GOD for keeping me healthy throughout this study cycle and for my entire life
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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
AGE	Advanced Glycation End
CVD	Cardiovascular Disease
DAD	Diode Array Detector
DNA	Deoxyribonucleic Acid
DPPH	Diphenyl Picrylhydrazyl
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
HPLC	High Performance Liquid Chromatography
LDL	Low Density Lipoprotein
MDR	Multi Drug Resistance
MHA	Mueller Hinton Agar
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
ORAC	Oxygen Radical Absorbance Capacity
PDA	Photodiode Array
SAS	Statistical Analysis System
SPE	Solid Phase Extraction
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

Abstract of Dissertation Presented to the Graduate School
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ACEROLA (*MALPIGHIA EMARGINATA* DC): PHENOLIC PROFILING, ANTIOXIDANT CAPACITY, ANTIMICROBIAL PROPERTY, TOXICOLOGICAL SCREENING, AND COLOR STABILITY

By

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This study aimed to characterize acerola fruit based on phenolic profiling, antioxidant and antimicrobial properties, toxicological evaluation, and color stability. Three specific objectives were pursued: separate, identify, and quantify the phenolic compounds; perform the antioxidant, antimicrobial, and toxicological evaluations of the phenolic extracts; study the color stability of acerola anthocyanin extracts in the presence of ascorbic acid (AA).

Acerola fruits were grouped by levels of maturity. Phenolic compounds were fractionated by SPE into anthocyanins and non-anthocyanins, separated and identified by HPLC-DAD-MS^2. The antioxidant capacity (AOC) was assayed by ORAC and DPPH. The antimicrobial property was determined by the disk diffusion method, and the toxicological screening was assessed by the Ames mutagenicity test. Color stability was examined by monitoring the anthocyanins degradation in acerola anthocyanin-containing AA extracts over time. The effect of AA on anthocyanins and color loss was also studied in AA-free açai extracts to which AA was added at levels that are similar to AA content of the acerola anthocyanin extracts. Pure anthocyanin model systems

composed of free cyanidin and cyanidin-3-rhamnoside with added amounts of AA were assessed.

Two anthocyanins, cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside and various types of non-anthocyanin phenolics including caffeic, chlorogenic, ferulic and p-coumaric acid derivatives and some catechin derivatives were identified in acerola fruit. Total antioxidant capacity expressed by ORAC were higher in immature fruits (43.5 mmol $TEkg^{-1}$) when compared with fruits at intermediate (36.5 mmole $TEkg^{-1}$) and complete (36.2 mmol $TEkg^{-1}$) stages of maturity. The phenolic fractions contributed 7.1-36.5 % while AA accounted for 18-39 % of total AOC. The flavonoid fractions of the fruit displayed antimicrobial potential against *S. aureus*. The results suggest that the phenolic fractions did not contribute to mutagenicity and are possibly suitable for use as food supplements. The detrimental effect of AA on anthocyanins and color was obvious in all the systems regardless of the storage conditions, resulting in increased L*, decreased a* and C* values.

Acerola may be promoted as a healthy foodstuff based on its high antioxidant potential. Future studies to stabilize the color of acerola anthocyanin extracts should be oriented toward the stability of both anthocyanin and AA.

CHAPTER 1
INTRODUCTION

In the last few decades there has been a growing trend in the consumption of tropical fruits and according to the Food and Agriculture Organization (FAO), the world production and the trade of tropical fruits is expected to expand over the next decade (FAO 2003). The less-developed countries produce 98 % of the total production of tropical fruit, while the industrialized countries and major markets are responsible for 80 % of the world import trade. While the major tropical fruits (mango, pineapple, papaya, and avocado) are the dominant tropical fruits produced worldwide, the market share of the group that are called “minor tropical” fruits (lychee, guava, passion fruit, acerola) have been expanding rapidly in recent years (FAO 2003).

Tropical fruits are appreciated by the consumer because of their diversity of aroma, flavor, and their nutritional value, both real and perceived. And among the minor tropical fruits, acerola appears potentially attractive because of its very high vitamin C content, its appealing red color at complete maturity, and its biological activity.

Acerola is grown in most of the Caribbean countries, in Central America and in Brazil, and is a tropical shrub (Matta and others 2004). At complete maturity it has a red color, a unique aroma, and is extremely rich in ascorbic acid. Acerola is also grown (although not at a large scale) in Florida and in Hawaii. Acerola cultivation is straightforward in these locations, and the production is relatively cost-effective (i.e. not requiring many production inputs), making it a perfect fit for low income farmers.

The high levels of ascorbic acid make acerola one of the world’s best natural sources of this vitamin. Depending on different factors such as cultivar, level of maturation, and climate (Matta and others 2004), the ascorbic acid levels range from

1000-4500 mg/100g of fruit on a fresh weight basis. Owing to its carotenoid content (371-1881 mg/100g) (De Rosso and Mercadante 2005) and the presence of phenolic compounds such as anthocyanins, and non-anthocyanin phenolics, acerola is considered as a functional food. Acerola was recently placed in the “super fruits” category together with other fruits such as Maqui berry, Indian gooseberry, guarana, seabuckthorn and the like. Given the health promoting potential of carotenoids and phenolic compounds, acerola has become very popular among people that are health conscious (Hanamura and others 2006). Recent experiments with various acerola preparations have suggested diverse biological activities including anticarcinogenic activity against lung cancer (Nagamine and others 2002), inhibitory action against nitric oxide production (Wakabayashi and others 2003), tumor-specific cytotoxic activity, and multidrug resistance reversal activity (Motohashi and others 2004), antihyperglycemic (Hanamura and others 2006), prevention of dyslipidemia and its complications (Barbalho and others 2011), and antigenotoxicity (Nunes and others 2011).

The attractive red color of acerola fruit is due to anthocyanin pigments. Vendramini and Trugo (2004) reported total anthocyanins of 37 mg/100g acerola skin, making acerola potentially usable as a food colorant. In addition to its colorant properties anthocyanins have been proven to demonstrate anti-inflammatory effects, protection against radiation, and inhibition of low density lipoprotein (LDL) oxidation (Wang and others 1997; Seeram and Nair 2002). The biological properties of anthocyanins depend on its structural scheme such as degree of glycosylation and the amount of hydroxyl groups attached to the B-ring (Kong and others 2003). Therefore it is very important to determine the structure of the anthocyanins in acerola fruit. The anthocyanin

composition of acerola fruit has already been reported but the results are inconsistent. In addition, the anthocyanin contents reported by previous experimenters are also discrepant probably because those experiments have been conducted on different and sometimes unknown varieties. Vendramini and Trugo (2004) reported malvidin 3,5-diglucoside, cyanidin-3-glucoside, and pelargonidin as major anthocyanins in an unidentified acerola variety. Hanamura and others (2005) and De Britto and others (2007) identified cyanidin-3-α-O-rhamnoside and pelargonidin-3-α-O-rhamnoside while reporting no free anthocyanidin in acerola fruit. The research of De Brito and others (2007) was conducted on two varieties: Acerola II47/1 and another variety called "Roxhina" while the variety used by Hanamura and others was not mentioned. De Rosso and others (2008) reported two anthocyanin hexosides: cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside, and two free anthocyanidins: cyanidin and pelargonidin in two different varieties: "Waldi Cati 30" and "Olivier". It is worth mentioning that in addition to variety and method differences between the information available, acerola is indiscriminately referred to as *Malpighia punicifolia* L, *Malpighia glabra* L, or *Malpighia emarginata* DC; however, these names are synonymous and the most common one for all acerola clones, genotypes and varieties is *Malpighia emarginata* DC (De Rosso and others 2008).

Acerola also contains non-anthocyanin phenolic compounds such as phenolic acids and flavonoids. However these groups of phenolic compounds are poorly investigated. Very few reports exist in this area and most of them are not peer reviewed. Compounds like chlorogenic, ferulic, and caffeic acids were reported as main phenolic acids in acerola fruit (Vendramini and Trugo 2004; Righetto and Netto 2005), however

the identification of compounds was made by solely comparing their retention time and their spectral information. The identification of phenolic compounds based on those parameters may not be accurate because compounds with similar or closely related chemical structures may have similar spectral characteristics. Therefore, it is necessary to use more powerful techniques to elucidate the structure of phenolic compounds in acerola fruit.

One of the problems the acerola growers are facing is the high perishability of this fruit at complete maturity. According to Vendramini and Trugo (2000), the fruits last only three days at room temperature. This perishability is thought to be caused by the climacteric nature of the fruits (Sean-Carrington and King 2002). Shortly after harvest (3-4 days) the fruit loses its attractive red color and turns to a dull yellowish color that is often seen by the consumer as index of poor quality, therefore limiting the market potential of the fruit. It is believed that the color instability of the fruit is due to interaction between two important elements in the chemical composition of the fruit: ascorbic acid and anthocyanins. It has been hypothesized that ascorbic acid degrades anthocyanin in model systems but the mechanisms proposed by previous experimenters are inconsistent. We believe that it is important to investigate the potential role of ascorbic acid in the degradation of anthocyanin in acerola which will probably help to propose ways to stabilize the color or to even develop acerola-based products with better color.

The overall aim of this study was to characterize acerola fruit based on phenolic profiling, antioxidant and antimicrobial properties, toxicological evaluation, and color stability. Three specific objectives were set: (1) Identify and quantify anthocyanins and non-anthocyanin phenolic compounds, (2) Perform the antioxidant, antimicrobial, and

toxicological evaluation of phenolic extracts from acerola fruit and, (3) Study the color stability of acerola fruit by monitoring anthocyanin-ascorbic acid interactions in the acerola anthocyanin extracts and provide recommendations for preserving the color of fresh and processed acerola products.

CHAPTER 2
LITERATURE REVIEW

Acerola (*Malpighia emarginata* DC)

Acerola (*Malpighia emarginata* DC. Syn. *Malpighia punicifolia*, L) is a plant originating in Central America that has spread to South America including Brazil, and the Caribbean due to its good adaptation to soil and climate. This shrub is grown in tropical and subtropical areas from the southern end of Texas, through Mexico and Central America to northern South America and throughout the Caribbean especially in Barbados, Trinidad & Tobago, Haiti, and Puerto-Rico. The tree has also been introduced widely into tropical regions of Asia and Africa. The perennial tree bears a red fruit known by the common names Barbados cherry, West Indian cherry especially in the English-speaking Caribbean countries or simply cherry in Haiti. However, the name acerola, as it is called in Puerto Rico is becoming more and more popular and is the name that will be used throughout this dissertation.

Characteristics, Production, Harvest, Post-Harvest Handling and Market Requirements

Acerola trees may reach an average height of 3–5 m with a short slender trunk that is 0.5–1 m high, and 7–10 cm in diameter. The fruit is small (1–4 cm diameter) and weighs 2–15 g. The thin skin is green during the first development stage but turns orange to orange-red at the intermediate stage of maturity and become bright red at complete maturity. Figure 2-1 portrays the three color stages of acerola fruit: green immature, semi-ripe (partially mature), and fully ripe (mature). The flesh is usually of a reddish-yellow hue, although some varieties with deep red skins have also a dark red pulp.

Regardless of the size of the fruit, the three winged seeds are large in comparison to the flesh, but due to their light and pithy nature, they represent only 20 % of the weight (Miller and others 1961). The tree produces fruits 3–4 times a year and each plant produces 20–30 kg fruit per year (Mezadri and others 2006).

There has been commercial cultivation of acerola in some regions of the Americas, but it is only in the last three decades that Brazil began to exploit it commercially. Currently, the world's largest producer, consumer and exporter of acerola products is Brazil which commercializes it in the forms of juice, marmalades, frozen concentrates, jam, and liquors. Other plantations of commercial importance are in Florida and Hawaii.

The color of the peel has been used traditionally as an index of ripeness and therefore, as the main criterion used to determine the harvest date of the fruit. Another method of assessing the ripeness of acerola includes the measurement of sugar/acid ratio in the fruit. However the use of peel color as an index of maturity represents a more practical option especially in field situations where laboratory analyses may not be available. The harvest date depends on the intended use of the fruit. In the case of freezing or processing into pulp or juice, fruits must be red in color but firm enough to tolerate handling. The fruit quality is high at this stage of maturation, that is, sugar content is high and acidity is low. Fruits may be picked at the beginning of maturation for use in the production of powdered products such as pharmaceuticals or concentrates for food enrichment, where the vitamin C content is the most important characteristic. The acerola fruits have high metabolic activity after harvest and the achievement of the ripening occurs rapidly (3–4 weeks after flowering), causing it to be

too perishable for the fresh market (Alves and others 1999). Consequently, the fruits must be frozen or processed quickly. The mature fruit lasts only 2–3 days at room temperature (Vendramini and Trugo 2000). However, the shelf life of the mature acerola fruit maybe improved to over three days at room temperature when wrapped in PVC film. In addition, storage at 8 ^{o}C and 85–90 % relative humidity with PVC wrapping increased shelf life to a week (Alves and others 1995). The CO_2 behavior displayed by the fruit especially at the intermediate and complete stage of ripeness suggested a climacteric behavior. According to Carrington and King (2002), the fruit has a very high respiratory rate (900 mL CO_2 $kg^{-1}h^{-1}$) but with a low rate of peak ethylene production (3 µL C_2H_4 $kg^{-1}h^{-1}$); the high respiration rate is thought to be in part responsible for the perishable nature of the fruit. One of the problems faced by acerola producers is the great sensitivity of the mature fruits during picking, packing, processing and/or distribution. The skin of the mature fruit is thin and fragile, and therefore can be easily damaged by even a very small impact. If the skin is damaged, the pulp of the fruit deteriorates rapidly. To alleviate the problem of the delicate skin, people who have experience in handling acerola fruits for international trade suggest that the harvest be made by hand. This will also help to exclude flowers and immature fruits that are present simultaneously with the mature fruits on the acerola tree. However, the main drawback of hand picking is that it raises the cost of labor (Alves and others 1999). Post-harvest stability of the fruit is also affected by solar radiation; exposure of the fruit to solar radiation for more than 4 hours after harvest leads to substantial loss of vitamin C (Alves and others 1999). Consequently, it is suggested that the harvest of the fruit be

made in the early hours of the morning, before the temperature increases to levels that can be detrimental to the mature fruits.

The standard requirements for acerola fruit intended for international trade are not well established. Brazilian producers experienced in international trade suggest that buyers require fruit with at least 7^{o} Brix for Europe, or 7.5^{o} Brix for Japan and about 1000 mg vitamin C per 100 g of fruits in Europe and the United States (Alves and others 1999). Japan is the most important market for acerola products, followed by the United States and Europe. In Germany, France and Hungary, fruit is used primarily for juice while in the United States it is used by the pharmaceutical industry.

Food and Other Uses of Acerola Fruit

Due to the relatively small size of the fruit and its relatively large seeds, the consumption of the fruit in the raw stage has a limited fresh market. The fruits have been incorporated into commercial fruit juices and energy drinks and are an increasingly attractive additive due to its current interest in developing products with health related properties. Fruits are used to enhance the vitamin C content of other fruits poor in this nutrient like apples, bananas, passion fruit, and pears. A product formulated with 65 % green coconut water, 15 % pineapple, and 20 % acerola pulp presented the characteristics for a new commercial product (Da Silva Pereira and others 2009). In some cases the fruit maybe cooked, strained to remove seeds and the resulting sauce or puree is utilized as topping on cake, pudding, ice cream or sliced bananas, or used in other culinary products. In a recent study an acerola ice cream has been developed and was proven to be suitable for the delivery of vitamin C and bifidobacterium strains, while maintaining excellent viability and acceptable sensory characteristics (Favaro-Trindade and others 2006) .

Acerola juice, sweetened or unsweetened, due to its high ascorbic acid content maybe used to prevent browning of fruits such as banana slices, fruit salad, and will at the same time improve the ascorbic acid content of the product (Miller and others 1961).

The fruits may be made into syrup or, with the addition of pectin, excellent jelly, jam, and other preserves. Cooking causes the bright-red color to change to brownish-red. The pasteurization process during the canning of the juice changes the color to orange-red or yellow, and packing in tin cans brings on further color deterioration. It was found that enamel-lined cans lead to a better preservation of color. Wine made from acerola in the State of Hawaii was found to retain 60 % ascorbic acid (Monton 1987).

Due to its very high vitamin C content, green acerola fruit has been extracted for use in dietary supplements. However, the high cost associated with the cultivation of the fruit seems to limit the expansion of this market. At immature stages the fruit may also be used as a source of pectin in confections or as an enriched source of dietary fiber (Rufino and others 2010; Schreckinger and others 2010).

Physico-Chemical Properties and Nutritional Value of Acerola Fruit

Protein, Fat and Carbohydrate

The physico-chemical properties of acerola fruit and its nutritional value depend on several factors including: environmental conditions, growing location, cultural practices, the stage of maturation, and processing and storage (Mezadri and others 2006). One hundred gram of acerola fruit contains approximately 90.6-92.4 g of water, 0.21-1.20 g of protein, 0.23-0.80 g of fat, and 3.57-7.80 g of carbohydrate (Table 2-1). The main sugars in mature acerola fruit are fructose and glucose, with small amounts of sucrose (Mezadri and others 2006). The fruits from wild varieties of acerola such as those grown in the Caribbean islands are considerably tart, probably because of their low sugar and

their high ascorbic acid contents. Recent genetic improvements have led to the development of new cultivars with higher sugar and lower ascorbic acid values such as the varieties “Florida Sweet” and “Will #2” developed in Homestead, Florida. The sweeter acerola varieties, although containing a much lower vitamin C tend to be more popular in the fresh markets and juicing operations. The mature fruit contains approximately 8.98 % total carbohydrates.

Vitamins and Minerals

Acerola fruit is one of the most significant sources of vitamin C obtained from plant material, and this vitamin plays a significant role in both the nutrition and the chemistry of this fruit. Reports from various sources indicated that the vitamin C content of acerola can range from 695–4827 mg/100 g of fruit (Table 2-1). The vitamin C content is affected by the ripening process and by the region in which the fruit is grown. Itoo and others (1990) reported reduction in the vitamin C content of acerola fruit grown in three different geographic regions (Nago, Naze, and Ibusuki) in Japan. From immature stage to full maturity, the vitamin C content decreased from 3.20 g/100g–1.83 g/100g, 2.78 g/100g–1.75 g/100g, and 2.15 g/100g–1.45 g/100g respectively for regions Nago, Naze, and Ibusuki. Similarly, a reduction of about 50 % vitamin C has been observed by Vendramini and Trugo (2000) as the fruit ripened. This decrease in the vitamin C content has been ascribed to ascorbic acid oxidase enzyme (Butt 1980). The activity of this enzyme seems to be more intense in the mature fruits compared to the immature ones. Other authors however attributed the decrease in vitamin C content to biochemical oxidation. This hypothesis has been verified when the compound 3-hydroxy-2-pirone, an oxidative breakdown product of ascorbic acid was detected only in the aroma profile of mature acerola fruits (Vendramini and Trugo 2000).

Given the high concentration of vitamin C in the green (immature) acerola fruits, it is used by some nutraceutical companies as a source of vitamin C in dietary supplements. However, the high cost associated with the cultivation of the fruit seems to limit the expansion of this market. Nevertheless, the utilization of the immature acerola is preferred when there is a need to develop products with high vitamin C content and when the flavor or the aroma characteristics of the fruit are not of interest.

Besides maturity, post-harvest handling and storage conditions can substantially impact vitamin C and the shelf life of acerola fruit. The information available in the field of acerola processing, although scarce, suggests that the vitamin C content begins to decrease about 4 hours after the harvest (Alves and others 1995). One possible way to reduce post-harvest loss of vitamin C is frozen storage at -18 ^{o}C which not only reduced vitamin C loss but also preserved some of the sensory qualities of the harvested material (Maciel and others 1999).

Besides vitamin C, carotene (0.41 mg/100g), vitamins B6 (8.70 mg/100g), B_2 (0.07 mg/100g), and B1 (0.02 mg/100) and niacin (0.34 mg/100g) were reported in acerola fruit, but at levels below that recommended by the USRDA (Miller and others 1961; Johnson 2003).

Major macro minerals in acerola fruits include phosphorus (17.1 mg/100g), calcium (11.7 mg/100g) and iron (0.22 mg/100g) (Table 2-1). The micro minerals content of this fruit, such as zinc, selenium and copper are not well studied.

pH, Acidity, Soluble Solids, and Organic Acids

Acerola is a very acidic fruit, and as for other components of the fruit, the pH also varies with the stage of maturity. The pH value fluctuates from 3.60–3.70, the acidity

expressed in grams malic acid equivalent per 100 g fruit ranges from 1.04–1.87, and the total soluble solid varies from 7.70–9.20 g (Table 2-1).

Although acerola is commonly called a cherry, its odor and flavor are more like that of tart apples than cherries. The organic acids in acerola in order of predominance are malic (0.25–0.38 g/100g), citric (0.01–0.03 g/100g), and tartaric acids (0.002–0.01 g/100g). Malic acid represents 32 % of the total acids in mature acerola fruits and 12 % in immature fruits (Righetto and Netto 2005). Another experiment reported that malic acid accounted for up to 20 % of the acidity found in acerola fruit (Asenjo 1980). In general, the organic acids perform important functions in the metabolic process of fruits. They are directly involved in growth, maturation, and senescence; they also influence the growth of microorganisms in fruit juices, and therefore affect the shelf life of the product; they may also participate in the synthesis of phenolic compounds and are important in the development of the characteristic flavor of the fruit (Ulrich 1970).

Phytochemicals in Acerola

The term phytochemical refers to bioactive non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods that have been associated with reducing the risk of major chronic diseases. In the literature, more than 5000 phytochemicals have been reported in fruits, vegetables, and grains, but large numbers remain unknown and need to be identified before the health benefits of phytochemicals in whole foods can be fully understood (Liu 2003). Convincing evidence implies that the benefits of phytochemicals in fruits, vegetables and whole grains may be even greater than is currently thought because the oxidative stress generated by free radicals is involved in the cause of a wide range of chronic diseases (Ames and Gold 1991). Because phytochemicals differ largely in composition and ratio from fruits to vegetables to grains,

and often have complimentary mechanisms to one another, it is thought that one should consume a wide variety of these plant-based foods (Liu and Felice 2007).

The phytochemicals include phenolic compounds, carotenoids, alkaloids, nitrogen-containing compounds, and organosulfur containing compounds. The area of phytochemicals in acerola fruit is poorly documented; the most studied so far are the phenolic compounds and the carotenoids. Therefore, more emphasis will be put on phenolic compounds and the carotenoids in this literature review.

Phenolic Compounds in Acerola

Phenolic compounds in foods originate from one of the main classes of secondary metabolites in plants. They are particularly important for plant metabolism and have also become important for humans due to their health characteristics, particularly related to their antioxidant power. Phenolic compounds are also important because of their contribution to the sensory quality of fruits, which may be changed during the technological processes used in the production of juice and other derived products (Fernandez and others 1992). Phenolic compounds in foods may be categorized into simple phenols, phenolic acids (hydroxybenzoic and hydroxycinnamic acid derivatives), flavonoids, stibenes, lignans, and tannins (Shahidi and Ho 2007). In foods, phenolics may occur in esterified, free or insoluble-bound forms. The phenolic compounds identified in acerola fruits are depicted in Figure 2-2. Due to lack of published data, only flavonoids and phenolic acids in acerola are discussed in this literature review.

Phenolic compounds in foods form a large group of secondary plant metabolites which vary in chemical structure and reactivity. All plant phenolic compounds have one characteristic in common, an aromatic ring carrying one or more hydroxyl groups. The chemical structure may vary greatly from simple phenols to highly complex polymerized

compounds like tannins. Several thousand of these natural compounds have been identified in plants, with a large diversity in their structural features (Harborne and Williams 2000) which contrast them from one another. The vast majority of dietary phenolic compounds, often defined as polyphenols, originate from plant foods (Scalbert and Williamson 2000). Their occurrence in animal tissues and non-plant materials is almost entirely due to ingestion of plant sources (Shahidi and Naczk 1995). In plants, phenolic compounds exert interesting physiological attributes, such as protecting against ultraviolet radiation, pathogens and predators, imparting color and flavor, and helping growth and reproduction (Bravo 1998; Harborne and Williams 2000; Heim and others 2002).

Phenolic compounds can be classified according to the structural characteristics of their carbon frame. The main classes of natural polyphenols include phenolic acids and derivatives, flavonoids, lignans, and stilbenes, coumarins, tannins and lignans (Shahidi and Naczk 2003).

Phenolic compounds occur mostly as conjugates with sugars, glucuronic or galacturonic acids, and sometimes with other phenols that are attached to hydroxyl groups or, less often, aromatic carbon atoms. The most common sugar moiety is glucose while residues such as galactose, rhamnose, xylose or arabinose residues, are also often found (Bravo 1998). The structural differences of phenolic compounds lead in a wide variety of phytochemicals ingested by humans. In this section, the more abundant classes of dietary phenolic compounds will be discussed; with a particular focus on those that occur in acerola fruit.

Flavonoids

In late 1930s, the Szent-Gyorgyi group observed that some flavonoids enhanced the biological activity of ascorbic and could cure scorbutic pigs; and recommend that the flavonoids be considered as P vitamin (Rusznyak and Szent-Györgyi 1936). However, even though humans or animals never show the ability to synthesize flavonoids, this class of phenolic compounds has never been proven to show the usual properties of a true vitamin; therefore their classification as a vitamin has never been validated (Kuhnau 1976). Even so, the health promoting effect of flavonoids is unanimously recognized. Lampe (1999) believes that the health benefits of flavonoids may be enough to justify a semi-essential status for these groups of phenolic compounds.

Flavonoids are the most abundant class of phenolic compound in the diet (Harborne and Williams 2000). They are present in edible fruits, leafy vegetables, roots, tubers, spices, legumes, tea, coffee, chocolate, and red wine. Flavonoids are commonly classified into seven groups: flavones, flavanones, flavonols, isoflavones, flavanols and anthocyanidins (Liu and Felice 2007). They are frequently found in nature as conjugates in glycosylated or esterified forms but can occur as aglycones, especially from the consequences of food processing. Many different glycosides can be found in nature as more than 80 different sugars have been discovered bound to flavonoids (Hollman and Arts 2000). All flavonoids are characterized by the flavan nucleus, a structure composed of two benzene rings (A and B) connected by an oxygen-containing pyran ring (C) (Kuhnau 1976). The flavonoids are grouped into the subclasses of flavones, isoflavones, flavanols, flavonols, flavanones, anthocyanins, and proanthocyanidins, and this categorization is based on the degree of oxidation of the C-ring, the hydroxylation

pattern of the nucleus, and the substituent at carbon 3 (Scalbert and Williamson 2000). Flavonols like quercetin are ubiquitous in edible plants, isoflavones are found strictly in other foodstuffs, flavonols, flavanols, and anthocyanins are abundant in the human diet, while flavones and isoflavones are less common (Scalbert and Williamson 2000).

Flavonoids are excellent antioxidant and their antioxidant capacity depends of their structure. Rice-Evans and others (1997) studied the structural requisite for effective antioxidant action of flavonoids and phenolic acids. Due to their reduction potential, polyphenols can protect endogenous antioxidants. This property is similar to that exhibited by ascorbic acid which exerts a vitamin E recycling ability. For example phenolic acids can effectively remove free radicals in model systems (Laranjinha and others 1994; Chen and Ho 1997), delay lipid oxidation, spare vitamin E, and regenerate tocopherol from its tocopheroxyl radical in human LDL, erythrocyte membrane hosts, and monocytic cells (Laranjinha and others 1995; Nardini and others 1995; Nardini and others 1998; Laranjinha and Cadenas 1999; Liao and Yin 2000). It was proven in a rat model system that caffeic acid spared vitamin E and increased the resistance of LDL towards oxidative stress (Nardini and others 1997). Lotito and Frei (2004) demonstrated in vitro and in vivo that flavonoids and phenolic acids from apples delay the oxidation of ascorbic acid and α-tocopherol in blood plasma.

In the last few years, efforts have been made to study flavonoids in acerola; flavonols such as quercetin and kaempferol have been isolated in acerola (Vendramini and Trugo 2004; Hanamura and others 2006), however no quantitative data is currently available. Acerola anthocyanins, however, have been relatively well-studied. This

subgroup of flavonoids is very important because they are responsible for the attractive red color of the acerola fruits at complete maturity.

Anthocyanins

Anthocyanins include a group of natural pigments that are responsible for a wide range of color in the plant world including blue, purple, violet, and magenta. Chemically, anthocyanins are polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium also known as flavylium salt. For quite a long time the safety of synthetic pigments has been a subject of concern which fosters an increased interest in the use of naturally occurring coloring compounds such as anthocyanin (Francis 1984). However, the stability of anthocyanin in food is also a major problem. Anthocyanins demonstrate greatest stability in acidic media, but are generally unstable and degrade by one of several possible mechanisms to form colorless and insoluble brown pigments (Jackman and others 1984). The changes in anthocyanins usually occur during processing and storage; therefore, thorough knowledge of the factors that affect the stability of anthocyanins and their degradation mechanism is important if these pigments are to be utilized in the manufacture of food products.

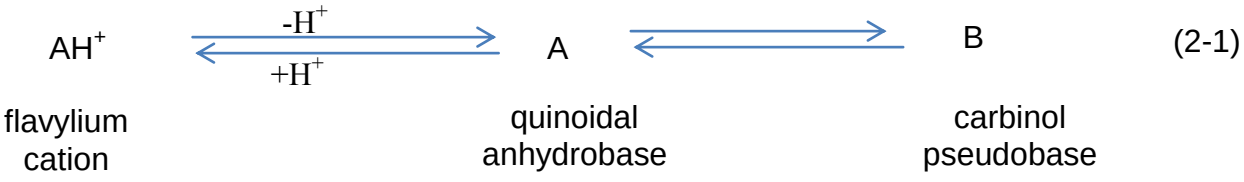
A number of factors influence the stability of the anthocyanin pigment including pH, temperature, sugars, metal ions, co-pigments, and ascorbic acid. Another important factor that may influence the stability of anthocyanin is an internal factor, also called the structural effect. Given the objectives of this study, the structural effect of pH and Vitamin C will be discussed.

The anthocyanins are structurally characterized by C_6-C_3-C_6 carbon skeleton. All their biosynthetic origin is similar to other naturally occurring flavonoids; they differ from these compounds by showing strong absorption in the visible range of the spectrum. In

addition to the various external factors, the stability of anthocyanins is a function of their inherent molecular structure. All the naturally occurring anthocyanins are glucosides of mainly six anthocyanin aglycone also called anthocyanidins, these being polyhydroxy and polymethoxy derivatives of flavylium salts. The aglycone moiety is highly reactive and this reactivity is responsible for diverse structural modifications that anthocyanins undergo under acidic conditions (Timberlake and Bridle 1967; Brouillard 1982). Another aspect of the anthocyanin structure that may influence its stability is the position of attachment of the sugars, acyl, and methoxy groups on the pigment molecules (Timberlake 1980). Because anthocyanidins are in general unstable and less soluble in aqueous media than anthocyanins, it is assumed that glycosylation confers stability and solubility to the anthocyanin molecule (Harbone 1979). Jurd (1972) showed that loss of the glycosyl moiety at the 3-position is accompanied by rapid decomposition of the aglycone in model systems, with irreversible loss of color. If a second site in the anthocyanin molecule is glycosylated, it is often located at position five (Brouillard 1982). Each glycosidic substitution is associated with a characteristic bathochromic shift (shift to a longer wavelength) (Harbone 1958). According to Brouillard (1982), a free hydroxyl group at any of 5, 7, 4’ positions is essential for the formation of a quinoidal (anhydrobase) structure. This structure is derived from the flavylium cation form of the anthocyanin, by loss of acidic hydroxyl hydrogen, generally above pH 3. The quinoidal structure of anthocyanins is primarily responsible for the pigmentation of flower and fruit tissues. The flavylium cation, from which the quinoidal structure is derived, is relatively stable under acidic pH (1-3). This cation has been described as a heterocyclic carboxonium cation with its positive charge delocalized over the entire structure giving

rise to six contributing resonance forms. According to Bendz and others (1967), the highest partial positive charges occur at the 2 and 4 positions of the flavylium cation. The stability of the cation is highly dependent upon nucleophilic attack at either position, by such compounds as water and sulfite ions (Bendz and others 1967). In addition, protons of hydroxyl groups at positions 5, 7, 4' may be easily removed with only slight increases in pH. Under acidic conditions, the color of anthocyanins is determined largely by the degree of hydroxylation in the B-ring of the aglycone, the greater the substitution the bluer the color (Asen 1977). Thus glycosides of delphinidin are bluer than those of cyanidin, which themselves are bluer than those of pelargonidin (Jackman and others 1984).

pH has a marked effect of the color of anthocyanin solutions. Anthocyanins behave somewhat like pH indicators as a result of their amphoteric nature. Below pH 3, anthocyanin solutions display their most intense red coloration. When the pH of such solutions is raised, their red color normally fades to the point where they appear colorless in the pH range of 4 to 5. Further increases in pH of anthocyanin solutions give rise to purple and blue, and these, upon storage or heat treatment, have been observed to change in pigmentation from blue to yellow (Jackman and others 1984). According to prior studies the observed changes in pigmentation with variations in pH may be attributed to the equilibrium reaction scheme presented in Equation 2-1 (Jurd 1963).



(2-1)

In this scheme, under acidic conditions there is equilibrium between the flavylium cation (AH^+) and the carbinol pseudobase (B) form of the anthocyanin, with the supposed existence of a transient species, the quinoidal anhydrobase (A) structure obtained by the deprotonation of the flavylium cation. Pigment solutions above pH 7, held for an extended period or at elevated temperatures, were presumed to gradually change in pigmentation from blue to yellow as an indirect result of the formation of a chalcone (C) structure via ring fission of the anhydrobase (Hrazdina and Franzese 1974). The existence of the chalcone structure was postulated by Markakis and others (1957) in studies on the thermal degradation of anthocyanin. The chalcone has been described as a colorless compound; however, its ionized form is associated with a pale yellow color (Brouillard 1982).

Timberlake (1980) and Brouillard (1982) suggested that the distribution of the four different anthocyanin structures at a particular pH, under equilibrium conditions, may lead to some interesting conclusions. Research by Brouillard and Delaporte (1978) has shown that in very acid media (pH 0.5), the red flavylium cation exists as the only species at equilibrium. With an increase in solution pH, both the concentration of this species and the pigmentation of the solution decrease as the cation hydrate to the colorless carbinol base.

The formation of colorless chalcone and blue quinoidal anhydrobase begins at a pH slightly below that corresponding to the pK characteristic of the equilibrium between the flavylium cation and carbinol base and continues to increase with increasing pH at the expense of the red flavylium cation. In the pH range of 4 to 5, the concentrations of

the two colored anthocyanin forms tend to be very small, their total color effect contributing little to the pigmentation of the solution.

The possible involvement of ascorbic acid in anthocyanin instability has been pointed out in 1940 by Battie and in the late 1940s by Pederson and others (1947). The two authors were among the first to observe the concurrent disappearance of ascorbic acid and anthocyanin in stored fruit juices and suggested possible interaction between the two compounds. Oxygen and metal ions are essential requirements in the decolorization of anthocyanin by ascorbic acid (Sondheimer 1953; Timberlake1960; king and others 1980). It is reported that cranberry juice pigments degraded more rapidly when the greatest amount of ascorbic acid and oxygen were present. Under ascorbic acid conditions, the addition of transition metal for example copper ions stimulates the destruction of ascorbic acid and anthocyanin in their mutual presence. Under these conditions, hydrogen peroxide (H_2O_2) produced by copper catalyzed breakdown of ascorbic acid is believed to be the cause of the degradation (Timberlake 1960). Copper ions were found to accelerate and flavonols such as quercetin and quercitrin to slow down the degradation of both cranberry anthocyanin and ascorbic acid in model systems when they are present simultaneously (Shrikande and Francis 1974). However, in the model system containing no ascorbic acid, no anthocyanin degradation was noticed suggesting that ascorbic acid plays a role in anthocyanins break down. Recently, in a more contradictory result, Garzón and Worlstad (2002) found that fortification of strawberry juice with ascorbic acid at certain levels increase the half-life of the pigment. In contrast, Rababah and others (2005) found that the addition of 1 %

ascorbic acid increases the lightness and decreases the redness and yellowness color values of fresh strawberry, peach, apple slices and puree from them.

The mechanism of degradation of anthocyanin by ascorbic acid has been investigated. However, the results are subjected to debate and up to now a resolution has yet to be found.

When ascorbic acid is oxidized in the presence of copper, hydrogen peroxide (H_2O_2) is produced; and since H_2O_2 is as an anthocyanin bleacher, it is believed that the ascorbic acid-induced anthocyanin degradation is mediated by H_2O_2 (Markakis and others 1957). Jurd and others (1972) speculated that ascorbic acid degrades anthocyanins by a mechanism involving direct condensation of the ascorbic acid to the position 4 on the flavylium structure. However, the authors did not provide experimental evidence. Because β-diketone dimedone condenses very readily with flavylium salts, and assuming that ascorbic acid has similar structure, the authors speculated that a similar condensation reaction may justify the observed effect of this substance.

The direct condensation hypothesis is discarded by other experimenters. According to Lacobucci and Sweeny (1983), color bleaching of anthocyanin by ascorbic acid occurs by cleavage of the pyrilium ring of the anthocyanin molecule. Garcia-Viguera and Bridle (1999) studied the color stability of anthocyanin and flavylium salt with ascorbic acid. They found that ascorbic acid had a destructive effect on the flavylium salts even when the position 4 is substituted. They concluded that the degradation is more likely to occur via a free radical mechanism proposed by Lacobucci and Sweeny (1983) rather than by direct condensation as proposed by Jurd and others (1972). Garcia-Viguera (1999) made some observations that let them to question the

direct condensation hypothesis: (1) the loss of color happened slowly rather than instantaneously; red color does not return immediately upon acidification, (2) no new UV observing compound were seen in HPLC analysis nor were any change in λmax that could be an indication of formation of new compounds, (3) the degradation effect of ascorbic acid was seen of the flavylium salt even when the position 4 was substituted.

Another hypothesis was tentatively explained by Meschter and others (1953). According to this hypothesis, dehydroascorbic acid which is a product of ascorbic acid oxidation can also decolorize anthocyanins, but no mechanism has been proposed.

In acidic solution, malvidin-3-5-diglucoside was oxidized faster than its acylated counterpart (Hrazdina and Franzese 1974), an effect attributed to reduced activity of the C_2 position and/or steric hindrance. The influence of ascorbic acid is slightly greater for a monoglucoside solution than a diglucoside solution .The presence of anthocyanins and the nature of these compounds influence the rate of degradation of ascorbic acid at pH 2.35 (Garcia-Viguera and Briddle 1999).

Anthocyanins provide some level of protection toward ascorbic acid. Garcia-Viguera and Briddle (1999) reported total loss of ascorbic acid after 15 days in the presence of malvidin-3-glucoside, while 5 % remains after 17 days in the presence of the diglucoside. At low pH, the predominant anthocyanin form is the flavylium cation, known to be more active as a free radical scavenger (Garcia-Viguera and Briddle 1999). Decrease in chroma value means loss of red color, lower contribution of the a^* value to the red color observed. The type of flavylium showed no significant influence on the rate loss of ascorbic acid (less than 3 % AA left after 10 days for 4-phenyl and 0 % left for 4-H and 4-CH_3 (Garcia-Viguera and Bridle 1999).

Phenolic Acids

Phenolic acids are divided into three subclasses the hydroxycinnamic acids and their derivatives which are the most important subclass, the benzoic acid derivatives, and the hydrolysable tannins. These compounds differ in patterns of hydroxylation and methylation of their aromatic rings. Figure 2-2 shows the chemical structures of some of these compounds. Some common examples of hydroxycinnamic acids are p-coumaric, ferulic, and caffeic acids. Of these hydroxycinnamic acids, caffeic acid is thought to be the most abundant in fruits and vegetables (Shahidi and Naczk 1995) and also the human diet (Clifford 2000). Hydroxycinnamic acids are generally present in the bound form and rarely present in free form. Bound forms of hydroxycinnamic acids are esters of hydroxyacids like quinic, shikimic, and tartaric acids as well as their sugar derivatives. The quantitatively most important conjugate of caffeic acid is its ester with quinic acid, 5-caffeoylquinic acid also known as chlorogenic acid. Hydroxycinnamic acids are generally present in the bound form and rarely present in free form. The presence of chlorogenic acid in many foods of plant origin including apples, apricots, blackberries (Herrmann 1973) and carrots (Babic and Amiot 1993) has been reported. Especially high concentrations of phenolic acid are found in coffee, apples, citrus fruits and juices, and the bran of cereal grains. Excessive coffee drinkers may achieve a daily consumption of phenolic acids in excess of 1 g (Clifford 2000). The intake of caffeic acid alone was reported to be up to 983 mg per day in a southern German population, but also as low as 5 mg per day in some individuals.

Hydroxybenzoic acids are commonly present in bound form. They are components of complex structures such as lignans and hydrolysable tannins. Hydroxybenzoic acids are also found in the forms of organic acids and derivatives of sugar (Schuster and

Herrmann 1985). The content of hydroxybenzoic acids in foods of plant origin is generally low, except for blackberry, raspberry, blackcurrant, red currant, and strawberry, as well as vegetables such as onions and horseradishes in which the content of hydroxyl benzoic acid partially protocatechuic acid, p-hydroxybenzoic acid, and gallic acids may be very high.

In acerola fruit, p-coumaric and ferulic acids were identified as two major phenolic acids (Vendramini and Trugo 2004). The same authors identified chromatographic peaks corresponding to chlorogenic and caffeic acids. In addition, benzoic acid derivatives like gallic, and syringic acids have also been reported in acerola (Righetto and others 2005).

Carotenoids

Carotenoids are yellow, orange, and red pigments present in many fruits and vegetables. More than 600 carotenoids have been identified in nature and approximately 20 are present in quantifiable amount in human serum (Cooper and others 1999). Of the six major dietary carotenoids found in human serum (β-carotene, α-carotene, β-cryptoxanthin, lycopene, lutein, and zeaxanthin (Bendich 1989) only lycopene has not been identified in acerola fruit. De Rosso and Mercadante (2005) studied the carotenoids composition of two Brazilian acerola genotypes and reported β-carotene (265–1669 µg/100g), lutein (37.6–101 µg/100g), β-cryptoxanthin (16.3–56.5 µg/100g) and α-carotene (7.80–59.3 µg/100g) as major carotenoids in both acerola genotypes. Other types of dietary carotenoids of less quantitative importance including: neoxanthin+neochrome, violaxanthin, luteoxanthin, 5,6,5’,6’-diepoxy-β-cryptoxanthin, 5,6-epoxy-β-cryptoxanthin, 5,8-epoxy-β-cryptoxanthin, zeinoxanthin, 5,6,5’,6’-diepoxy-β-carotene, 5,8-epoxy-β-carotene were also reported. In another experiment, Lima and

others (2005) reported the total carotenoid contents in acerola fruit cultivated in Brazil at different stages of maturity and different weather conditions. The results showed that the levels of carotenoids were very low in green fruit and then greatly increased as the fruit matured (Table 2-2); changes that were thought to reflect degradation of chlorophylls with a concomitant rise in carotenoids (Alves and others 1995). In addition, a higher level of carotenoids was reported for mature fruits harvested in the rainy season compared to those harvested in the dry season (Table 2-2). These data show that the carotenoids content vary according to environmental conditions such as harvest season and stage of maturity.

Occurrence of Anthocyanins in Acerola Fruit

Acerola fruit has a very attractive red color at full maturity due the presence of anthocyanin pigments. Recently, the anthocyanins in acerola have been characterized and quantified. However, there are some discrepancies in the results. Anthocyanins from two different acerola cultivars (Waldy and Olivier) have been extracted using 0.5 % HCL in methanol and identify by liquid chromatography, mass spectrometry (LC-MS). With 76–78 % of the total anthocyanin, cyanidin-3-rhamnoside represented the major anthocyanin in acerola fruit followed by pelargonidin-3-rhamnoside (13–16 %), cyanidin (6–8 %), and pelargonidin (2–3 %) (De Rosso and others 2008). While those results seem to be more or less consistent with the results obtained by Hanamura and others (2005) who identified cyanidin-3-α-O-rhamnoside and pelargonidin-3-α-O-rhamoside in acerola by NMR, they were less in agreement with another study in which the authors used different extraction and analytical techniques. Vendramini and Trugo (2004) identified three types of anthocyanins in acerola by means of chromatographic and spectral data, finding only malvidin 3, 5-diglucoside, cyanidin-3-glucoside, and free

pelargonidin. Recently in our lab, we identified only two anthocyanins, cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside in acerola fruit from the variety Florida Sweet grown in Florida; cyanidin-3-rhamnoside being the most abundant form (Delva and Goodrich 2010). In contrast to the results obtained by Hanamura and others (2005) or Vendramini and Trugo (2004) no anthocyanin aglycons (anthocyanidins) were identified under our experimental conditions. The kind of anthocyanin present in acerola fruit is variety dependent; some varieties may contain free anthocyanin aglycon while other varieties may not. Therefore it is important to mention the type of variety used in anthocyanin-related studies of acerola fruit.

With regards to the total anthocyanin, amounts of 6.5–7.6 mg/100g and 7.9–8.4 mg/100g acerola pulp for the varieties Waldy and Olivier respectively have been reported (De Rosso and others 2008). Total anthocyanin in the pulp of the two varieties mentioned above can be considered low when compared to fruits known as good sources of anthocyanin such as red raspberry (cultivar Jewel) (197.2 mg/100g), blackberry (cultivar Chester Thornless) (153 mg/100g), and mulberry (14.7–272 mg/100 g) (Wang and Lin 2000; Liu and others 2004). Conversely, acerola skin is much more concentrated in anthocyanin; the amount of 37.5 mg/100g of ripe acerola skin has been reported (Vendramini and Trugo 2004). This anthocyanin content is higher than that found in red cabbage (25 mg/100g), plum (2–25 mg/100g), strawberries (15–35 mg/100g) and banana bracts (32 mg/100g FW) (Timberlake 1988; Pazmino-Duran and others 2001). Therefore, acerola skin, which is considered as a byproduct of the acerola pulp production, may be used as a commercial source of natural pigment.

Anthocyanin, Ascorbic Acid and Color Stability of Acerola

Attractive color is a very important sensory attribute for the consumer of fruits and processed food products derived from them. Unfortunately the appealing red color of fresh acerola fruit at complete maturity is not maintained through processing and storage.

Anthocyanin-containing products during processing and storage are prone to color change resulting from the effect of anthocyanin degradation and brown pigment formation. When color changes occur extensively, visually unacceptable products result. Under normal processing conditions, a dull, brownish, pigment often appears which is often perceived by the consumer as indicative of poor quality and implies the spoilage of the fruit. Therefore, the instability of the color has a negative impact on the market potential of the fruit.

Acerola anthocyanin is very unstable and its degradation is responsible for the loss in red color of the frozen acerola pulp and processed juice. Although losses occur throughout the entire processing system, the main problem occurs during the commercial storage of these products. Because acerola anthocyanins appear to be more unstable than anthocyanins found in other fruits, the instability is thought to be due to structural differences in the individual anthocyanins. It has been reported that the color of some foodstuffs containing anthocyanins that are rich in cyanidin or delphinidin aglycones is less stable than foods containing anthocyanins that are rich in petunidin or malvidin aglycones. Using a different hypothesis, it has been demonstrated in two separate model systems that the presence of elevated concentrations of ascorbic acid may be the main cause of the low stability of acerola anthocyanins, which occurs mainly due to the direct condensation of ascorbic acid on carbon 4 of the anthocyanin, resulting

in losses of both compounds (De Rosso and Mercadante 2007). The mechanism by which ascorbic acid may degrade anthocyanin is still unclear with further research needed for clarification of the degradation mechanism.

Acerola Components and Potential Health Benefits

The consumption of fruits and vegetables is considered to be beneficial to health. Many recent clinical studies support the fact that increased consumption of fruits and vegetables is beneficial for the prevention of cancer (Steinmetz and Potter 1991; Block and others 1992; Margetts and others 1994; Steinmetz and Potter 1996) and cardiovascular disease (Ness and Powles 1997). An increase in consumption of fruits and vegetables to 400 g or five portions a day has been promoted by national and international bodies inferring that such a change would reduce the incidence of both cancer and cardiovascular disease (James and others 1988).

Due to the worldwide increase in obesity, the United States Department of Health and Human services suggests increasing the consumption of fruits and vegetables as an effective strategy for weight management. According to Tohill (2007), fruits and vegetables have high water and dietary fiber contents and their consumption contribute to satiety and reduce energy intake. Nutrients such as dietary fiber, vitamins, and bioactive phytonutrients such as phenolic compounds, dietary carotenoids, and lignans are held responsible for the anti-cancer activity and other health-related benefits provided by fruits and vegetables (Van't Veer and others 2000). Acerola fruit is an outstanding source of vitamin C, a good source of phenolic antioxidant especially anthocyanins and phenolic acids, dietary carotenoids and fiber. Most of the acerola’s biological activity is due to the high antioxidant power of the polyphenols, vitamin C and potential carotenoids.

Vitamin C is a very significant water soluble antioxidant in biological fluids (Frei and others 1989; Frei and others 1990). It readily scavenges reactive oxygen and nitrogen species, such as superoxide and hydroperoxyl radicals, singlet oxygen, ozone, peroxynitrite, nitrogen dioxide, nitroxide radicals, and hydrochlorous acid (Halliwell 1996), therefore efficiently sparing other substrates from oxidative damage. Acute deficiency of vitamin C contributes to scurvy, expressed by blood vessel fragility, connective tissue damage, fatigue, and ultimately death (Li and Schellhorn 2007).

Some observational studies report a negative correlation between dietary intake of vitamin C, taken alone or in combination with other antioxidant vitamins, and the risk of cardiovascular complications (Taniyama and Griendling 2003, Salonen and others 2000; Salonen and others 2003), although this correlation was not seen in randomized controlled trials (Willett and Stampfer 2001; Stanner and others 2004). Ascorbic acid has the ability to regenerate vitamin E by rapidly reacting with tocopherol radical and reducing it back to its original form (Nagoaka and others 2007). In addition, it was found in cigarette smokers that the pace of the blood vitamin E oxidation cause by increased oxidative stress is significantly reduced by vitamin C supplementation (Bruno and others 2005), suggesting a vitamin E recycling function of vitamin C and potential cooperative relationship between the two vitamins.

The transport mechanism of vitamin C imposes a physiological restriction on its bioavailability that is attainable by oral consumption (Padayatty and others 2004). Recent investigations suggest that ascorbic acid from natural sources is more readily absorbed by the human body than the synthetically-produced vitamin C. In a double blind experiment, it was found that vitamin C in acerola powder is 1.63 times more bio-

available in the human body than vitamin C synthetically produced (Tang 1995). Studies showed that infants consuming apple juice supplemented with acerola juice exhibited above average or average growth and development for their age and weight (Johnson 2003). Vitamin C levels in the blood were above average for all infants after the acerola juice was introduced in the diet. No allergic reaction was observed during this study, suggesting that acerola juice combined with apple is a good alternative to orange juice as a source of vitamin C in the diet.

In a controversial study it was shown that administration of a high dose of ascorbic acid improved the survival of patients with last stage cancer (Cameron and Campbell 1974; Cameron and Pauling 1976). Their results led to the suggestion of using megadoses of vitamin C to combat degenerative diseases, including cancer and CVD. Vitamin C may protect against cancer through several mechanisms in addition to inhibition of DNA oxidation. One potential mechanism is chemoprotection against mutagenic compounds such as nitrosamines (Tannenbaum and others 1991; Hecht 1997). Although there still is doubt on the result of this study, acerola fruit with its very high ascorbic acid content represents an appealing option for the use of mega dose ascorbic acid in cancer therapy.

Vitamin C also acts as a coantioxidant by regenerating α-tocopherol (vitamin E) from α-tocopheroxyl-radical produced via scavenging of lipid-soluble radicals (Bowry and others 1995; Packer and Fuchs 1997). This is an important function because in vitro experiments have shown that α-tocopherol can act as a prooxidant in the absence of coantioxidants such as vitamin C (Bowry and others 1995; Neuzil and others 1997).

Numerous studies in humans have investigated the effects of the oxidazability of LDL by vitamin C supplementation in combination with vitamin E or β-carotene or both (Frei and McCall 2000). Studies have been carried out in smokers (Steinberg and Chait 1998; Nyyssönen and others 1994), non-smokers (Jialal and Grundy 1993) and persons with hypercholesterolemia or cardiovascular disease (Rifici and Khachadurian 1993; Gilligan and others 1994). In all cases, a significant reduction in LDL oxidizability was observed.

The potential health promoting effect of ascorbic acid is due to the fact that this vitamin is a powerful antioxidant. Research conducted on the antioxidant potential of acerola fruit and acerola-based drinks always mention the potential contribution of ascorbic acid although this contribution has never been quantified to the very best of our knowledge.

The antioxidant activity of acerola fruit and acerola juices has been investigated. Low density lipoprotein (LDL) has been incubated with acerola extract containing 25 and 51 µM equivalents of ascorbic acid in the presence and absence of 0.1 µM genistein/daidzein equivalents of soy extract or 0.2 µM coumestrol/apigenin equivalents of alfalfa extract. It was found that the inhibition of copper-mediated LDL oxidation by soy extract was significantly enhanced in the presence of 25 µM acerola extract, and an even higher inhibition was reported with 51 µM acerola extract (Hwang and others 2001). In the same experiment, it was also demonstrated that low density lipoprotein radical ($LDL^{\cdot}$) formation is decreased in cells pretreated with soy, alfalfa, and acerola extracts. Pretreatments with soy, alfalfa, and acerola extracts noticeably reduced the extent to which 100 µg/mL of LDL was converted to $LDL^{\cdot}$ after 24 h. The protective

effect of these extracts is thought to be related to the presence of flavonoids in soy and alfalfa extracts and to the presence of ascorbic acid in acerola extract, which may act synergistically as antioxidants. More recently the antioxidant activity of juice extracts from mature and immature acerola fruit and of a concentrated juice from immature acerola fruit was evaluated with the objective of assessing the potential of immature and mature acerola used in food formulations. It was demonstrated that all acerola juices tested exhibited antioxidant activity in a lipid model system consisting of methyl linoleate. The oxidation curves of the methyl linoleate in the presence of acerola extracts (fractionated or not with a solid phase extraction (SPE) column) showed an increase in the rate of the oxidation after 48 h, which was an indication of the start of the propagation of the oxidation process. The antioxidant power of samples collected after 48 h indicated the highest antioxidant activity (72.1 %) for the immature concentrated juice extract followed by mature acerola and immature acerola juice extract at 68.5 % and 52.7 % respectively. Alpha-tocophenol which was used as a standard antioxidant exhibited an antioxidant capacity of 86.2 % (Righetto and others 2005). The fact that the mature acerola extract which contained smaller amounts of ascorbic acid and total phenolics exhibited higher antioxidant capacity than the immature acerola juice extract allowed the authors to speculate that phenolic compounds and/or other constituents with antioxidant activity rather than the total phenolics or vitamin C content were responsible for this activity. Since ferulic acid was detected in mature fruit, the higher antioxidant activity may be related to the presence of ferulic acid. In fact, due to its ability to prevent the autooxidation of oils, ferulic acid has been largely utilized as a food preservative (Trombino and others 2004).

Dietary flavonoids and other plant phenolics have been found to have strong antioxidant capacity, and antimicrobial and anti-inflammatory effects (Huang and others 1992). They are also known for their health promoting effect by reducing the risk of cardiovascular diseases (CVD) and cancer (Temple 2000). Caffeic acid is thought to be the most abundant phenolic acid in the diet. Especially high concentrations are found in coffee, apples, and the bran of cereal grains. Excessive coffee drinkers may achieve a daily consumption of phenolic acids in excess of 1 g (Clifford 2000). The intake of caffeic acid alone was reported to be up to 983 mg per day in a Southern German population, but also as low as 5 mg per day in some individuals (Frank 2004). Phenolic acids have been reported to efficiently scavenge free radicals in various model systems. In a rat model, caffeic acid spared vitamin E and enhanced the resistance of LDL towards oxidative stress (Nardini and others 1997). It has been proven that phenolic acids in apple delayed the oxidation of ascorbic acid in blood plasma (Lotito and Frei 2004); but no increase resistance to oxidation of endogenous antioxidants was found in blood plasma collected from volunteers up to 4 hours after the consumption of five apples.

The evaluation of acerola fruit as a source of phenolic antioxidant has been conducted. Also biological activity of a phenolic extract from acerola fruit has been studied.

Total phenolic compounds have been quantified in 12 different acerola genotypes harvested during the dry and rainy seasons at three ripening stages in Brazil. The phenolic concentration decreased as the fruits ripened. For the 12 different genotypes evaluated, it was reported that mature fruits harvested in the dry season showed higher

total phenolic contents than those harvested in the rainy season (Lima and others 2005). For one genotype, 1703 mg/100g and 930 mg/100g catechin equivalent (fresh weight) were reported for mature acerola harvested in the dry and rainy seasons, respectively. A possible explanation is that the rainfall may dilute the cellular juice, and therefore, decrease the total phenolic level (Lima and others 2005). In the same year however, Righetto and others (2005) quantified the total phenolics in acerola and found that the mature acerola juice had 1.35 mg of catechin/g juice (57 mg of catechin/g dry material), and the immature acerola juice exhibited a level of 3.8 mg/g juice (24.5 mg of catechin/g of dry matter). Other authors reported similar results for berries and other fruits (Kähkönen and others 1999; Kähkönen and others 2001). Hanamura and others (2005) isolated three polyphenols from acerola fruit: cyanidin-3-α-O-rhamnoside and pelargonidin-3-α-O-rhamnoside as anthocyanins, and quercetin-3-α-O-rhamnoside (quercetin) as flavonol. These acerola polyphenols were found to have radical scavenging activities and inhibitory effects on both α-glucosidase and advance glycation end (AGE) production, which are both closely related to diabetes mellitus and its complications. Recently, Hanamura and others (2006) reported that crude acerola polyphenol inhibits glucose uptake in Caco-2 cells in a dose-dependent fashion by adding acerola with an IC_{50} value of nearly 0.2 mg/L. In addition, crude acerola polyphenols significantly suppress the glucose and maltose plasma levels after administering both glucose and maltose to ICR mice suggesting that crude acerola polyphenols had a preventive effect on hyperglycemia in the postprandial state by a mechanism that includes either the suppression of the intestinal glucose transport or the inhibition of α-glucosidase (Hanamura and others 2006).

In a relatively recent study, Motohashi and others (2004) investigated acerola extracts for cytotoxic, antibacterial, and antifungal activities and demonstrated that acerola extracts contained various biocidal substances. Cytotoxic activity against normal and tumor cells, antibacterial activity, radical quenching effect, multi drug resistance (MDR) reversal activity and other activities were enriched by fractionation with organic solvent extraction, and silica gel or reversed phase column chromatography. Among the biological effect tested, the reversal multidrug resistance in tumor cells was the most impressive. The authors found that some acerola fractions such as hexane-acetone fraction, acetone fraction, both from fresh acerola and a hexane-acetone fraction from dried acerola powder showed highest tumor-specific cytotoxic activity. In addition, those fractions of acerola inhibited the P-glycoprotein (PgP) function in the MDR cancer cell more effectively than did verapamil (a drug used in cell biology as an inhibitor of drug efflux pump proteins such as P-glucoprotein) alone, thus improving the efficacy as a cancer chemotherapeutic treatment. Based on these results and the overall biological activity, the authors stated that acerola extract is not only a good candidate as a new type of MDR reversal agent but may also be applied in the future for cancer therapy. Multidrug resistance is one of the major obstables to long term successful cancer chemotherapy. The use of MDR reversal (MDRR) agents is a promising approach to overcome undesired MDR. In another experiment, Nagamine and others (2002) reported that acerola extract at 70 mg/kg body weight and 700 mg/kg body weight reduced the number of 4-methylnitrosamino-1-3-pyridyl-1-butanone (NNK)-initiated cells at the initiation stage in mice. NNK is a potent carcinogen formed from nicotine during tobacco processing and cigarette smoking (Hecht 1997). Because it has

been shown in an epidemiological study that the risk of tobacco-induced lung cancer is lower in persons receiving high intake of vitamin C, the authors speculated that vitamin C might contribute to a part of acerola extract-dependent inhibition of the initiation. However, it was also observed that the inhibition reached a plateau at the lowest level of acerola extract (70 mg/kg body weight), suggesting that other factors might play significant roles in the suppression of the initiation. In fact, as presented earlier, acerola may be a good source of carotenoids especially when it is cultivated in rainy season and harvested in the mature state (De Rosso and Mercadante 2005). Therefore, as suggested in the publication of Nagamine and others (2002), carotenoids in the acerola extract may also participate in the inhibition process against NNK-induced lung tumorigenesis through the suppression of the initiation stage.

Besides ascorbic acid and dietary phenolics, carotenoids represent a significant group of phytochemicals in acerola fruit. Godoy and Rodriguez-Amaya (1994) reported that acerola has a higher vitamin A value (64 Retinol Equivalents, RE/100g) compared to some other fruits cultivated in Brazil such as nectarine (47 RE/100g) or peach cultivar (Cv Diamante) (58 RE/100g). In another experiment, Lima and others (2005) reported the total carotenoid contents in 12 different acerola genotypes cultivated at the active Germplasm Bank in the Federal Rural University of Pernambuco, Brazil at different stages of maturity and different weather conditions. For a given genotype, the levels of carotenoids were very low in green fruit and then greatly increased as the fruit matured; changes that were thought to reflect degradation of chlorophylls with a concomitant rise in carotenoids (Alves and others 1995). Within different genotypes, the carotenoid content was higher in some genotypes compared to others. In addition, a higher level of

carotenoids was reported for mature fruits harvested in the rainy season compared to those harvested in the dry season. The relatively high carotenoid (particularly β-carotene) content of acerola can be used to promote the consumption of acerola fruit and acerola-based drinks and other products as healthy foodstuffs. In fact, carotenoids have been studied widely and proven to show diverse beneficial effect on human health. β-carotene is believed to have antioxidant activity. It has been shown to exhibit radical trapping behavior only at partial pressures of oxygen significantly less than in normal air (Burton and Ingold 1984). β-carotene appears to potentially act against angiogenesis in vivo (using male C57BL/6 mice as well as B16F-10 cells) and in vitro (using rat aortic ring assay, human umbilical vein endothelial cell proliferation, migration, and tube formation) (Guruvayoorappan and others 2007). Angiogenesis is the formation of new blood vessels out of the preexisting vascular network and involves a sequence of events that are important for some pathological processes including the growth of tumor and metastasis. There is a growing body of evidence, including, in vivo, in vitro and epidemiological studies supporting the claim that lutein and zeaxanthin contribute to health and delay age related macular degeneration of the eyes and, to a lesser extent, cancer and heart diseases (Snodderly 1995; Rong and others 2007). Another epidemiological study conducted in the Pacific Island indicated that people with higher intake of β-carotene, α-carotene, and lutein had the lowest risk of lung cancer (Le Marchand and others 1993). Lutein showed anti-tumor promoting activity in a two stage carcinogenesis experiment in lung of ddY mice; β-cryptoxanthin showed anti-tumor promoting activity in two stage carcinogenesis in skin of IRC mice (Nishino and others 2002). Bishayee and others (2000) showed that carotenoids can inhibit the initiation

stage of the tumorigenic process in rat liver carcinogenesis initiated by a single injection of diethylnitrosamine (200 mg/kg) followed by promotion with phenobarbital (0.05 %) in a basal diet.

Dietary Phenolic Compounds and Contribution to Human Health

Phenolic compounds in foods form a large group of secondary plant metabolites which vary in chemical structure and reactivity. All plant phenolic compounds have one characteristic in common, an aromatic ring carrying one or more hydroxyl groups. The chemical structure may vary greatly from simple phenols to highly complex polymerized compounds like tannins. Several thousand of these natural compounds have been identified in plants, with a large diversity in their structural features (Harborne and Williams 2000) which contrast them from one another. The vast majority of dietary phenolic compounds, often defined as polyphenols, originate from plant foods (Scalbert and Williamson 2000). Their occurrence in animal tissues and non-plant materials is due to ingestion of plant foods (Shahidi and Naczk 1995). In plants, phenolic compounds exert essential physiological functions, such as protecting against ultraviolet radiation, pathogens and predators, contributing to their color and flavor, and facilitating growth and reproduction (Bravo 1998; Harborne and Williams 2000; Heim and others 2002).

Toxicological Safety of Acerola Phenolic Compounds

Fruits' polyphenols display interesting functional characteristics that impart benefits to human health. Those functional properties include action against virus, hyperglycemia, and hypersensitivity. As it was described in previous sections of this chapter, acerola fruit contains previously isolated and identified polyphenols such as anthocyanins (cyanidin-3-rhamnoside, pelargonidin-3-rhamnoside) and flavonols such

as quercetin (quercetin-3-α-Orhamnoside) (Hanamura and others 2005). The same authors demonstrated that those polyphenols exert radical scavenging power and potential anti-hyperglycemic activity particularly with regards to diabetes mellitus. A new type of polyphenolic constituent isolated in green acerola fruit and arbitrarily named “aceronidin” was shown to display radical scavenging capacity (Kawaguchi and others 2007). The identification of this new type of phenolic compound may contribute to the enhanced health benefits of acerola polyphenols. It may also raise concern about the safety of acerola phenolic extracts. Phenolic extracts from other sources have been evaluated for their biological properties and their toxicological safety. Yamane and others (1996) showed that epigallocatechin gallate (EGCG) and green tea extracts inhibited chemical carcinogenesis of the gastrointestinal tract in rodent. In the same research, green tea extract was shown to be non-toxic and its clinical use showed no harmful effect. It was also reported that procyanidins from grapes were not mutagenic in Salmonella mutagenesis assay system (Yu and Swamitan 1987). It was also reported that aplephenon, a commercial apple polyphenolic extract, displays little mutagenicity at high concentration 2500 µg/plate and chromosomal aberration tests and micronucleus tests exhibited no significant mutagenicity (Shoji and others 2004).

However, reports on the toxicology of acerola polyphenols both in vivo, in vitro, and in animal studies are very scarce to the best of our knowledge. Hanamura and Aoki (2008) evaluated the toxicological safety of acerola polyphenols in rats and they suggested that the minimum lethal dose maybe higher than 2000 mg/kg. In addition, the authors reported no abnormal clinical signs regarding the administration of acerola polyphenols in the experimental subjects. While this research provide some promising

results with regard to the safety of acerola polyphenols, it is clear that more research needs to be conducted in this area.

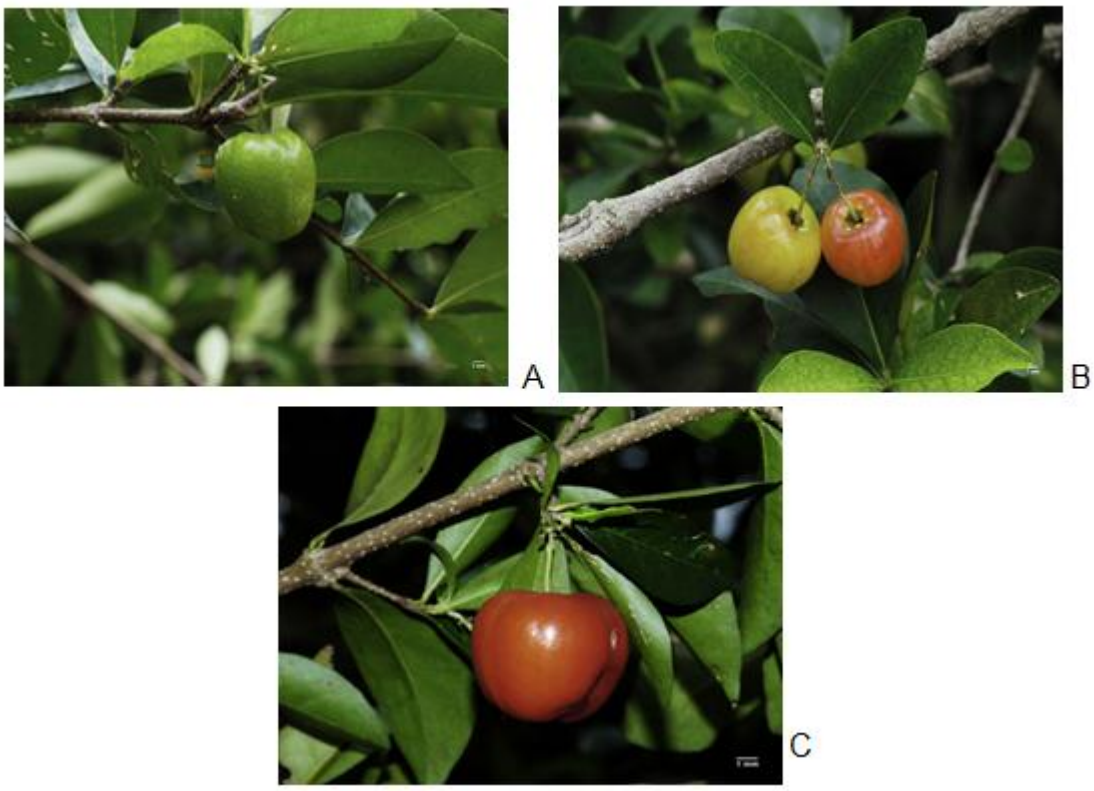
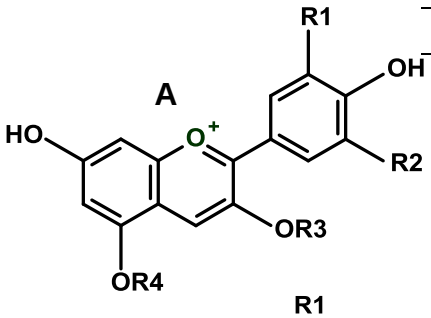
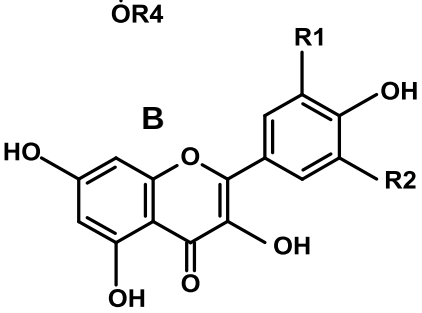


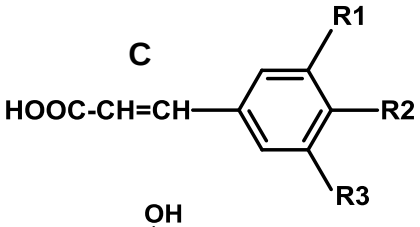
Figure 2-1. Acerola at various stages of maturity. A: immature stage (green); B: intermediate stage (orange or orange-red); C: mature stage (red).



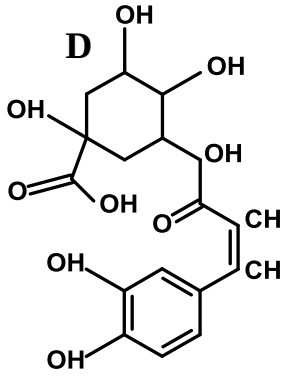
Anthocyanins	R1	R2	R3	R4
Malvidin 3,5 diglycoside	OCH3	OCH3	glucose	glucose
Cyanidin-3-glucoside	OH	Glucose	glucose	glucose
Pelargonidin	H	H	H	H
Cyanidin-3-rhamnoside	OH	H	rhamnose	H



Flavonols	R1	R2
Quercetin	OH	H
Kaempferol	H	H



Phenolic acids	R1	R2	R3
Caffeic acid	H	OH	OH
p-coumaric acid	H	OH	H
Ferulic acid	H	OH	OCH3



Chlorogenic acid

Figure 2-2. Structure of phenolic compounds in acerola fruit: A, anthocyanins; B, flanonols; C, chlorogenic acid; D, phenolic acids.

Table 2-1. Nutritional value of acerola fruit

Nutrient	Content for 100 g acerola fruit	Authors
Water	90.6–92.4 g	Mezadri and others (2006)
Protein	0.21–0.80 g	Mezadri and others (2006)
	0.90–1.20 g	Vendramini and Trugo (2000)
Fat	0.23–0.80 g	Mezadri and others (2006)
Carbohydrate	3.57–7.80 g	Mezadri and others (2006)
	4.30–4.40 g	Vendramini and Trugo (2006)
Fructose	0.25–0.38 g	
Glucose	2.14–3.33 g	Righetto and others (2005)
Sucrose	0.02 g	
Vitamin C	970–1900 mg	Righetto and others (2005)
	1074–2164 mg	Vendramini and Trugo (2000)
	695–4827 mg	(Righetto and others 2005)
		Mezadri and others (2006)
Vitamin B6	8.70 mg	
Vitamin B2	0.07 mg	Mezadri and others (2006)
Vitamin B1	0.02 mg	
Phosphorus	17.1 mg	
Calcium	11.7 mg	Mezadri and others (2006)
Iron	0.22 mg	
Ash	0.40 g	Mezadri and others (2006)
Dietary fiber	3.00 g	Mezadri and others (2006)
Soluble solid	7.70–9.20 g	Vendramini and Trugo (2000)
Acidity*	1.04–1.87 g	Mezadri and others (2006)
pH	3.60–3.70	Vendramini and Trugo (2000)
Malic acid	0.25–0.38 g	
Citric acid	0.01–0.03g	Righetto and others (2005)
Tartaric	0.002–0.01 g	

*Acidity expressed in gram malic acid equivalent

Table 2-2. Phytonutrient content of acerola fruit

Phytonutrients	Content for 100 g acerola fruit	Authors
Anthocyanins	3.79–59.74 mg	Mezadri and others (2006)
	6.5–8.4 mg	De Rosso and others (2008)
	37.5 mg	Vendramini and Trugo (2004)
Total carotenoids	32–323[a] µg	Lima and others (2005)
	100–352[b] µg	
	75–419[c] µg	
	147–589[d] µg	
	940–3100[e] µg	
	1410–4060[f] µg	
β-carotene	371–1881 µg	De Rosso and Mercadante (2005)
	265.5–1669 µg	De Rosso and Mercadante (2005)
	536.55 µg	Mezadri and others (2006)
trans-β-carotene	340 µg	Godoy and Rodriguez-Amaya(1994)
α-carotene	7.8–59.3 µg	De Rosso and Mercadante (2005) Lima and others (2005)
Lutein	37.6–100.7 µg	De Rosso and Mercadante (2005)
	99.21 µg	Mezadri and others (2006)
β-Cryptoxanthin	16.3–56.5 µg	De Rosso and Mercadante (2005)
	417.46 µg	Mezadri and others (2006)
trans-β-cryptoxanthin	40 µg	Godoy and Rodriguez-Amaya (1994)
Violaxanthin	395.3 µg	Mezadri and others (2006)
Vitamin A value	46.2–283 RE*	De Rosso and Mercadante (2005) Godoy and Rodriguez-Amaya (1994)

* RE: Retinol equivalent.
a, b: green acerola fruit cultivated in dry and rainy season respectively.
c, d: half mature acerola fruit cultivated in dry and rainy seasons respectively.
e, f: mature acerola fruit cultivated in dry and rainy seasons respectively.

CHAPTER 3
IDENTIFICATION AND QUANTIFICATION OF PHENOLIC COMPOUNDS IN ACEROLA FRUITS AND JUICES

Overview

Acerola is a tropical shrub grown in the Americas that produces a deep-red cherry-like fruit called differently (acerola, Barbados cherry, West Indian, cherry, etc.) depending on the region. This fruit is particularly known for its very high vitamin C content and has become very attractive especially among people that are health-conscious (Hanamura and others 2006). Recent investigations gave indication of some interesting biological activity of acerola fruit extract such as anticarcinogenic effect against lung cancer (Nagamine and others 2002), inhibition of nitric oxide production (Wakabayashi and others 2003), antimicrobial properties, and tumor specific cytotoxic effect (Motohashi and others 2004). These effects are thought to be attributable to the presence of phytonutrients other than vitamin C such as carotenoids and phenolic compounds (Hanamura and others 2006).

Phenolic compounds are very important groups of secondary metabolites in plants. They play a significant role in the nutritional and sensory characteristics of different fruits and vegetables. Over the years, fruits and vegetables containing phenolic compounds have received considerable attention due to their potential biological and health promoting effects (Ahmed and Beigh 2009; Cartea and others 2011).

Anthocyanins are brightly colored polyphenolic pigments responsible for the red color of acerola fruit. The visual impact of anthocyanins associated with their potential health benefits make then potentially attractive as natural food colorants. The beneficial health-related effect linked with anthocyanin intake may include: a reduced risk of heart disease (Sumner and others 2005), protection against obesity and low blood glucose

(Jayaprakasam and others 2006), enhancement of memory (Andres-Lacueva and others 2005), and the protection against fetal brain tissue (Loren and others 2005). Anthocyanins are known as good antioxidants which may explain the health advantage they deliver (De Brito and others 2007). Kong and others (2003) described the protection efficiency of anthocyanins as a function of the chemical structure of the molecule, such as degree of glycosylation, and number of hydroxyl groups in the B-ring. Therefore, the determination of anthocyanins structure in foods and food products is a topic of interest. Recently, anthocyanins from acerola fruits have been reported; however the results are very inconsistent. De Rosso and others (2008) identified two anthocyanin hexosides: cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside, and two free anthocyanidins: cyanidin and pelargonidin by HPLC-PDA-MS/MS. Hanamura and others (2005) identified cyanidin-3-α-O-rhamnoside and pelargonidin-3-α-O-rhamoside in acerola by NMR, but reported no free anthocyanidins. Vendramini and Trugo (2004) identified malvidin 3, 5-diglucoside, cyanidin-3-glucoside, and pelargonidin by means of chromatography and spectral data. In addition to those conflicting results, the identification of anthocyanins in the acerola variety used in this experiment (the variety Florida sweet) has never been reported to the best of our knowledge. Moreover, quantitative analysis of anthocyanins based on concentration basis is lacking. The quantification analysis performed by De Rosso and others (2008) was based on peak area percent of the identified compounds. Another gap of knowledge is that the non-anthocyanin phenolics profile in both edible and non-edible portions of acerola fruit and in acerola juice is not well understood. Vendramini and Trugo (2004) identified p-coumaric and ferulic acids as two major phenolic acids in acerola. The same authors

identified chromatographic peaks corresponding to chlorogenic and caffeic acids. Furthermore, benzoic acid derivatives like gallic, and syringic acids have also been reported in acerola (Righeto and others 2005; El-Malak and others 2010). The objective of this study was to identify and quantify phenolic compounds in acerola fruit.

Materials and Methods

Chemicals

Cyanidin chloride (≥95 % purity), pelargonidin chloride, ferulic (99 % purity), p-coumaric (≥98 % purity), caffeic, gallic, and chlorogenic acids, Saint-Louis, MO were purchased. 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) 6-hydroxy-2-5-7-8 tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Wacko Chemicals, Bellwood, RD, USA. L (+)-ascorbic acid (99 % purity) was obtained from Acros Organic, NJ, USA. All other reagents were purchased from Fisher (Fair Lawn, NJ). Methanol, ethyl acetate, and acetonitrile were of HPLC grade; all other solvents were of analytical grade.

Fruits-Harvesting, and Separation into Different Maturity Stages

Acerola fruits from the variety Florida Sweet were donated by Elson's Exotic Farm in Davie, South Florida and by several growers in Vero Beach, Central Florida. The fruits were packed in Ziploc bags and transported the same day to the pilot plant of the Food Science and Human Nutrition Department at the University of Florida. The fruits were grouped into three different stages of maturity based on the visual color of the peel. The green, orange-red, and deep red fruits were selected as the initial, intermediate, and complete stages of maturity respectively. The visual categorization was instrumentally and statistically validated using a machine vision method described in Yagiz and others (2009). The machine vision system was composed of a light box

and a Nikon D200 digital color camera (Nikon Corp., Tokyo, Japan) coupled with a computer having a fire wire connection. The camera settings were the following: 36 mm focal length, ISO 100 sensitivity, 1/3s F/11 shutter speed, −1 eV exposure compensation and direct sunlight white balance. A computer program was created to collect images and to obtain color results based on lightness (L^*), redness (a^*) and yellowness (b^*) values of the fruits. The fruits were placed in the light box and the digital camera captured a picture of the fruits for each treatment. The machine vision system was calibrated with a standard red plate ($L^* = 51.1$, $a^* = 50.0$, $b^* = 24.0$) from Labsphere (North Sutton, NH, USA). Average L^*, a^*, b^* values of each fruit's surface area was calculated using a color analysis program.

Firmness analysis was performed as an additional means of validating the grouping of the fruits by stages of maturity. The firmness analysis was performed using a TA.XT Plus texture analyzer (Texture Technologies Corp., Scarsdale, NY). The experimental condition was as follows: compression to 3 mm using a 35 mm diameter cylinder Perspex probe at a test speed of 2 mm/sec. The results were obtained in terms of kilogram force (kgf). The lower the force applied means the softer the fruit.

The categorization of the fruits by stage of maturity was statistically validated by performing a one-way analysis of variance with the Duncan pair wise comparison test of the mean L^*, a^*, b^*, and softness values. The statistical analysis was performed using the SAS (statistical analysis system, SAS Institute Inc., Cary, NC, USA).

Separation of the Fruits into Edible and Non-Edible Portions

The fruits were separated into edible portions (skin+pulp) and non-edible portion (seed). In addition to the fruits, frozen single strength acerola juice was purchased from ITI Tropicals, Lawrenceville, NJ. The fruit portions and the juice were kept at -20 °C until

needed. The edible and non-edible portions of the fruits were packed in new Ziploc bags in 100 g increment and freeze-dried; the frozen single strength juice was also freeze-dried. The freeze-dried material was finely ground using a Waring Blender (model 51BL31, Torrington, CT, USA) and stored at -20 ^{o}C prior to extraction.

Extraction of the Phenolic Compounds

Fresh fruits

The extraction and fractionation of the phenolic compounds were achieved according to the method described in Kim and Lee (2002) with necessary modifications. Briefly, 100 g edible portion of fruit was mixed with 100 mL of methanol in a beaker. The content was transferred into a chilled blender and immediately macerated at high speed for 3 min. The ground material was crushed and returned to the original beaker with 50 ml of 80 % aqueous methanol, placed in an ice bath for 20 min and sonicated every 5 min at low temperature. After 20 min the mixture was passed through two strainers with different pore sizes. The residues were re-extracted with 100 mL of absolute methanol. The liquid extract was filtered through a Whatman no. 2 filter paper. The filtrates were combined and transferred to a 1000 mL round-bottom evaporating flask with 40 mL of 80 % aqueous methanol. The methanol was evaporated in a rotary evaporator under vacuum at 30 ^{o}C to a volume of 20-25 mL. The concentrated extract was dissolved to a volume of 100 mL with deionized water and stored at -20 ^{o}C until fractionation.

Freeze-dried samples

Ground freeze-dried powder from 100 g of fruits was mixed with 100 mL of 80 % aqueous methanol in a 500 mL Erlenmeyer flask which was immersed into an ultrasonic bath and sonicated for 20 min at room temperature and periodic shaking; the temperature of the ultrasonic bath was kept low throughout the extraction process. The

mixture was strained and filtered through a Whatman no. 2 filter paper by vacuum suction using a chilled Buchner Funnel. The filter cakes were re-extracted with 100 mL of 80 % aqueous methanol and the filtrates were combined and transferred into a 1000 mL round-bottom evaporating flask with 50 mL of 80 % aqueous methanol and evaporated at 40 ^{o}C using a rotary evaporator until a volume of 10-30 mL was reached. The concentrate was solubilized into a 100 mL volume with deionized water, flushed with nitrogen and stored at -20 ^{o}C.

Fractionation of the Crude Aqueous Phenolic Extract into Anthocyanin and Non-Anthocyanin

Twenty (20) certified C_{18} Sep-Pak cartridges were precondition by sequentially passing 6 mL of ethyl acetate, 6 mL of absolute methanol and 6 mL of 0.01 N HCl into each cartridge. The aqueous phenolic extract was filtered through a 0.45 Millipore filter and 5 mL was loaded onto each cartridge. The cartridges were washed with 6 mL of 0.01 N aqueous HCl to remove sugars, acids, vitamin C and other water-soluble compounds. The cartridges were allowed to dry for 10 min under vacuum. Each cartridge was rinsed with 10 mL of ethyl acetate to elute the non-anthocyanin phenolic compounds and the eluates were combined in a 200 mL round-bottom flask. The adsorbed anthocyanins from each cartridge were eluted with 3 mL of acidified methanol and the eluates were combined in a 50 mL round-bottom flask. The ethyl acetate from the non-anthocyanin fraction was removed under reduced pressure at 20 ^{o}C using a rotary evaporator and the methanol in the anthocyanin fraction was removed under the same condition but at 40 ^{o}C. Each fraction was dissolved in 15 mL deionized water, flushed with nitrogen and stored at low temperature for later use.

Solid Phase Extraction of the Non-anthocyanin Phenolics

Three (3) SPE cartridges were preconditioned for the fractionation of the non-anthocyanin phenolics into neutral fraction by sequentially passing 2 mL of absolute methanol and 2 mL of deionized water. The aqueous phenolic extract was filtered through a 0.45 µm PVDF filter and the pH was adjusted to 7.0 with concentrated NaOH. The extract was passed through the preconditioned cartridge to absorb the neutral phenolic compounds and acidic fractions. For neutral phenolic compounds, the cartridges were preconditioned; for the acidic fraction, 2 mL of 0.01N HCl was used instead of deionized water. The aqueous phenolic extract was adjusted to pH 7.0 with concentrated sodium hydroxide and passed through the cartridges preconditioned for neutral. Three (3) more cartridges were preconditioned for the acidic fractions by sequentially passing 2 mL of methanol and 2 mL of 0.01N HCl. The pH of the effluent portion from the neutral cartridges was adjusted to pH 2.0 using 1N HCl and passed through the acidic cartridges to absorb the acidic phenolic compounds. Both the acidic and the neutral fractions were eluted with 5 mL of absolute methanol. The solvent was removed at 30 ^{o}C using a rotary evaporator. The fractions were solubilized with 5 mL of deionized water, sonicated to remove dissolved oxygen and stored at very low temperature for HPLC analysis.

HPLC Analysis of the Phenolic Compounds

Anthocyanins

Chromatographic analyses were performed on an Agilent 1200 series HPLC system (Agilent, Palo Alto, CA) coupled with an autosampler/injector and diode array detector (DAD). A Zorbax Stablebond Analytical SB-C_{18} column (4.6 mm x 250 mm, 5 µm, Agilent Technologies, Rising Sun, MD) was used for separation, the elution was

performed using mobile phase A (1 % formic acid aqueous solution) and mobile phase B (100 % methanol). UV-vis spectra were scanned from 220 to 600 nm on a diode array detector with detection wavelength of 520 nm. The flow rate was 1 mL/min, and the following convex gradient was used: 5 to 20 % B from 0 to 2 min, 20 to 30 % B from 2 to 5 min, 30 to 45 % B from 5 to 10 min, 45 to 55 % B from 10 to 15 min, 55 to 70 % B from 15 to 30 min, isocratic (70 % B) from 30 to 32 min, followed by re-equilibration of the column for 3 min for the next run.

Electrospray mass spectrometry was performed with a HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The anthocyanins are positively charged therefore, the mass spectrometer was operated in positive ionization mode. Other experimental conditions for the mass spectrometer were as follows: nebulizer, 45 psi; dry gas, 11.0 L/min; dry temperature, 350 ^{o}C; ion trap, scan from m/z 100 to 2200; smart parameter setting (SPS), compound stability, 50 %; trap drive level, 60 %. The mass spectrometer was operated in auto MS/MS mode to capture and fragments the most abundant ion in full scan mass spectra. The identification of the anthocyanin was based on mass spectral information, chromatography of pure standards when available, and UV-vis spectra of the diode array detector. Individual anthocyanin contents was calculated using standard calibration curves, cyanindin-3-rhamoside was calculated as cyanindin equivalent while pelargonidin-3-rhamnoside was expressed as pelargonidin equivalent.

Acidic and neutral phenolic fractions

The acidic and the neutral fractions were diluted 1:1 (v/v) with methanol and filtered through a 0.45 µm filter. Chromatographic analyses were performed on an Agilent 1200 series HPLC system (Agilent, Palo Alto, CA) coupled with an

autosampler/injector and diode array detector (DAD). A Zorbax Stablebond Analytical SB-C_{18} column (4.6 mm x 250 mm, 5 µm, Agilent Technologies, Rising Sun, MD) was used for separation.

The separations were performed by gradient elution of increasing concentration of acetonitrile in acidified water at a flow rate of 0.2 mL/min. The starting eluent (A) and the gradient former (B) consisted of water and acetonitrile, respectively, both containing 1.0 % (v/v) formic acid, and the elution was performed by a multisegment gradient, according to the program described in Nicoletti and others (2008) and summarized in Table 3-2. The DAD detector was set at 280 nm for the neutral phenolic compounds or 320 nm for acidic phenolic compounds.

Results and Discussions

Validation of the Categorization of the Fruits by Stage of Maturity

To validate the grouping of the fruits by stage of maturity, the mean values of certain color quality parameters mainly the lightness (L^*) yellowness (b^*), and redness (a^*) and the softness were statistically compared using a Duncan multiple range test performed by the Statistical Analysis Software (SAS). The SAS program used and the SAS output are presented in Appendix B (Tables B-1 and B-2). Table 3-1 shows that the L^* values increased from the immature to the intermediate stage of the fruit; a decrease in the L^* was seen from the intermediate to the complete stage of development of the fruit. The a^* values increased from the immature to the fully mature stage; while the yellowness (b^*) decreased as the fruit ripened. The softness of the fruit followed the following trend: softness fully mature fruit > softness fully intermediate>softness immature. Statistical analysis showed significant difference ($p<0.05$) between the parameters within a given stage of maturity; therefore validating

the visual categorization of the fruit by stage of maturity made after harvest. The observed changes in the parameters (increase L^*, decrease a*, and decrease b*, and decrease softness) translate the complex biochemical transformation that takes place during the ripening process (Vendramini and Trugo 2000).

Anthocyanins Identification and Quantification

The anthocyanin analysis was performed only on the edible portions of the fully mature (red) fruits. Two chromatographic peaks were detected in the partially purified anthocyanin fractions from fruits grown in Davie (Ace-DA), Vero Beach (Ace-VE) and the frozen single strength acerola juice (FSSAJ) by HPLC-DAD-MS^2. The chromatograms of the identified anthocyanins are shown in Figure A-1 (Appendix A), and the characteristics of the peaks are given in Table 3-3. The peaks obtained from different samples showed similar mass spectral characteristics. The molecular ion of peak 1, respectively from Ace-DA, Ace-VE, and the FSSAJ was found at a mass-to-charge ratio (m/z) 433 and product ions at m/z 287. The presence of these ions suggests that the aglycon cyanindin was glycosylated with a deoxyhexose due to the lost of 146 u (433-287), this peak was tentatively identified as cyanidin-3-rhamnoside. The Molecular ions of peak 2 was found at m/z 417, and fragment ions at 271 ($M-146^+$) corresponding to aglycone pelargonidin with the loss of one molecule of hexose. Therefore, peak 2 was tentatively identified as pelargonidin-3-rhamnoside. The results show that acerola fruits grown in Davie, those grown in Vero Beach, and the juice which was processed from an unknown variety have similar anthocyanin profile. Cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside are the only anthocyanins identified in the samples. The identification of cyanidin-3-rhamnoside and pelargonidin-3-rhamoside is in agreement with the information reported in the literature (Hanamura and others 2005;

De Brito 2007, De Rosso and others 2008). However, malvidin-3-5-di-glucoside, malvidin-3-glucoside, free cyanidin and free pelargonidin reported in the literature (Vendramini and Trugo 2004; De Rosso and others 2005) were not identified under the conditions that this experiment was conducted.

Each individual anthocyanin detected was quantified using a standard calibration curve from 0-50 µg/mL of pure standard compound; the results are presented in Table 3-3. Due to unavailability of cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside standards, the individual anthocyanin contents were expressed as cyanidin or pelargonidin equivalents. The results show that the concentration of cyanidin-3-rhamoside in Ace-DA sample is 2.67 mg/100g FW and nearly is twice as high as the concentration of pelargonidin-3-rhamnoside, 1.34 mg/100 mg FW in the same sample. In contrast, the concentration of cyanidin-3-rhamnoside in the Ace-VE sample (8.47 mg/100g) is just a little bit higher than the concentration of pelargonidin-3-rhamnoside (6.52 mg/100g). The results also show that the fruits collected in Vero Beach exhibited a higher individual anthocyanin content than those collected in Davie. The cyanidin-3-rhamnoside content in Vero Beach sample on a fresh weight basis was 8.47 mg/100g, about three times the cyanidin-3-rhamnoside content in the samples grown in Davie. In addition, pelargonidin-3-rhamnoside content in the Vero Beach sample was more than four times as high as the content found in samples collected in Davie.

While the individual anthocyanin content in acerola fruit appears to be low when compared to other fruits, there is no doubt that these pigments are responsible for the appealing red color of acerola fruit.

Non-Anthocyanin Compounds

Method development

The effect of different parameters affecting the efficacy of the extraction and the performance of the diode array detection (DAD) of the polyphenols was considered during this experiment. The extraction and isolation of the polyphenols in acerola fruit were accomplished according to a protocol developed by Kim and Lee (2002) with minor but necessary adjustments. Two types of extraction were performed, a dry extraction where freeze-dried powdered acerola fruits where used and a wet extraction in which frozen fruits were extracted directly. In the case of the dry material, aqueous methanol combined with sonication was used for the extraction. The cavitation generated by ultrasound when used in aqueous methanol extraction helps to enhance mass transfer rate and favors higher production yield with less extraction time and solvent usage (Kim and Lee 2002). The disadvantage of using the freeze-dried extraction is that freeze-drying is a very expensive process where a freeze drier is not always available. The sample preparation in which the polyphenols in the fresh material are extracted using absolute methanol and homogenization is simple although time consuming. Following the extraction, the crude polyphenolic extracts were fractionated into anthocyanin and non-anthocyanin fractions. In our preliminary experiments HPLC separation of the non-anthocyanin phenolic compounds lead to very crowded chromatograms due to too many interfering peaks such as sugars, organic acid and most importantly ascorbic acid in the case of acerola fruit. Therefore, the non-anthocyanin phenolic compounds were further fractionated into neutral and acidic fractions based on the fact that polyphenolic acids are completely ionized at pH 7.0 and un-ionized at pH 2.0 Kim and Lee (2002).

After the extraction and the purification steps the samples were analyzed by HPLC. The separation was performed using an Agilent HPLC system composed of a DAD detector, and the separation was carried out on a Zorbax Stablebond Analytical SB-C_{18} column (4.6 mm x 250 mm) operating under a gradient of elution mode composed of water containing 1 % formic acid (solvent A) and acetonitrile containing 1 % formic acid (solvent B). One percent formic acid was added in the mobile phase in order to keep carboxyl and hydroxyl groups of the analytes in their protonated form and help to minimize peak broadening (Nicoletti and others 2008).

Validation

The validation of a chromatographic method is usually judged based on the precision, linearity, and accuracy; only the precision of this method was measured. The identification of the phenolic compounds in acerola fruits was accomplished on the basis of their retention time and visible spectra collected with the DAD detector. The identification of each peak was tentatively confirmed by ESI-MS^n detection. The parameters set for the ESI-MS^n are described in the materials and methods section, almost all phenolic compounds produced mass spectra with the base peak corresponding to the so-called molecular ion.

The precision of the method was evaluated in terms of interday repeatability of the retention time for most of the standard phenolic compounds used in this study. The standard phenolic compounds were analyzed in sextuplicate over six consecutive days. The results are expressed as mean values, standard deviation, and relative standard deviation of the retention times. Table 3-4 show very small interday variability in the retention times of the standard phenolic compounds analyzed.

Identification by HPLC-ESI-MS^3

Two samples of chromatograms of the acidic and neutral fractions of the phenolic compounds in the edible portion of the acerola fruit are shown in Appendix A (Figure A-2 and Figure A-3). Because the phenolic compounds at the different maturity stages are not qualitatively different, only chromatograms of samples from the full stage of maturity are presented. The chromatograms were captured at 320 nm and 280 nm for acidic and neutral fractions respectively. However, most of the acidic phenolic compounds could also be detected at 280 nm. The acidic fraction is mostly composed of phenolic acids which are derivatives of hydroxycinnamic or hydroxybenzoic acids while flavonols and to a lesser extent flavan-3-ols are the predominant groups of phenolic compounds identified in the neutral fractions.

The identification of the phenolic compounds in the different samples was based on mass spectral data and chromatography of pure standards (when available), and the published literature data. All samples combined, a total of twelve (12) phenolic compounds were tentatively identified. Among the twelve compounds, 4 of them are reported for the first time that include two flavan-3-ols, epicatechin and another epicatechin derivative identified only in the edible portions at complete maturity, free ellagic acid and another ellagic acid derivative identified only in the non-edible portions (seed) of the mature fruit, and another phenolic compound in the subclass of stilbene: resveratrol hexoside, only identified in the edible portion of the fruit at full maturity.

Hydroxycinnamic acid derivatives

Compound 22 from Table 3-5 and Figure A-2 (Appendix A) had a mass-to-charge ratio (m/z) 377 $[M+Cl^-]$ ion indicating that a chloride-based adduct was fragmented to give rise to a base peak at m/z 341 $[M-H]^-$. This compound was further fragmented to

give another ion at m/z 179 probably after the removal of a molecule of hexose. This compound was tentatively identified as a derivative of caffeic acid. Other hydroxycinnamic acid derivatives identified include derivatives of clorogenic, ferulic and p-coumaric acids (Table 3-5).

Caffeic acid is known as one of the most abundant phenolic acids in fruits and vegetables, and in the human diet (Shahidi and Naczk 1995; Clifford 2000). Chlorogenic acid is one of the most abundant conjugates of caffeic acid and it has been reported in fruits such as apple, apricots, blackberries (Herrmann 1973) and in carrots (Babic and Amiot 1993). p-coumaric acid and ferulic acid were reported as main phenolic acids in acerola fruit (Vendramini and Trugo 2004). The authors also identified chromatographic peaks corresponding to chlorogenic acids. In contrast, benzoic acid derivatives like gallic, and syringic acids reported in the literature (Righetto and others 2005, El Malak and others 2010) were not found under the conditions of this experiment.

Flavan-3-ols

Compound 16 (Table 3-5) and Figure A-3 (Appendix A) shows m/z at 289 $[M-H]^-$. Although we did not see the characteristic base peak usually seen at m/z 245 at MS^2, however at MS^3 a minor ion was observed at m/z 205 probably from the cleavage of ring A from the flavan-3-ol molecule, this compound was tentatively identified as epicatechin. Compound 46 with m/z 453 $[M-H]^-$ was fragmented and shows one product ion at m/z 289 probably from the cleavage of the epicatechin molecule from the mother ion; therefore this compound was tentatively identified as an epicatechin derivative. Epicatechin and its derivatives were not previously reported in acerola fruit and our identification is tentative with further experiments needed to confirm the presence of epicatechin and its derivative in mature acerola fruit.

Flavonols

Flavonols are a very important subclass of flavonoids in acerola fruit. In this research, the flavonols identified are all glycosides: quercetin-3-α-O-rhamnoside (compound 55, Table 3-5), kaemferol-3-rhamnoside (compound, 54 Table 3-5) and another kaempferol derivative (compound 41, Table 3-5). Compound 41 showed a parent ion at m/z 593 $[M-H]^-$ which was fragmented to give a base peak at m/z 285 corresponding to the loss of an hexose molecule. Further degradation of this ion showed another product at m/z 211. This compound was identified as a kaempferol derivative. Compound 55 showed a parent at m/z 463$[M-H]^-$, the dissociation of this ion lead to a fragment ion at m/z 301 (MS^2) which in turn was degraded into fragments observed at m/z 277, 271, 179 typical of quercetin fragmentation observed in the literature. Compound 55 was therefore identified as quercetin-3-α-O-rhamnoside. In addition, compound 54 was also identified as kaempferol derivative on the basis of mass data and the chromatography of pure standard. The flavonols identified in this experiment are in line with literature data. The presence of quercetin and kaempferol in acerola fruit has been mentioned in at least two previous experiments. Vendrami and Trugo (2004) showed by means of chromatography and spectral data two chromatographic peaks having similar characteristics with quercetin and kaempferol. The existence of a quercetin hexoside (quercetin-3-α-O-rhamnoside) was detected and identified in acerola fruit by nuclear magnetic resonance (NMR) (Hanamura and others 2005).

Ellagic acid and stilbene

Free ellagic acid (compound 26 Table 3-5) as well as a potential derivative of ellagic acid (compound 33, Table 3-5) was tentatively identified in the non-edible portion

(seed) of the mature fruit. Due to lack of a standard compound, we could not confirm the identification of this compound. Free ellagic acid as well as other derivatives of ellagic acid has been reported in seeds of muscadine grape (Sandhu and Gu 2010).

A compound having similar spectral characteristics with resveratrol hexoside was observed in the edible portion of the fruits at complete maturity, but as for the ellagic acid and its derivative, the identification was not confirmed.

Summary

Our results show that all stages of maturity for the acerola variety Florida Sweet contained two types of anthocyanins: cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside, and various groups of non-anthocyanin phenolic compounds including hydroxycinnamic acid derivatives, flavonols, flavan-3-ols, ellagic acid and stilbene. While the identification of some of the non-anthocyanin phenolics was not confirmed, the chromatograms and the mass spectral data generated can be used as fingerprint for future research in this area. Because anthocyanins and non-anthocyanin phenolic compounds display interesting health promoting effects, their presence in the acerola fruit may contribute to the expansion of this fruit to markets of exotic tropical fruit.

Table 3-1. Color characteristic and the hardness of acerola fruit at different stages of maturity

Maturity stage	L^*	a^*	b^*	Hardness (kgf)
Immature (green)	52.09[a]	-8.30[c]	40.66[a]	6.03[a]
Intermediate (Orange)	52.39[a]	19.49[b]	38.33[b]	3.20[b]
Mature (red)	43.80[b]	38.60[a]	31.33[c]	2.21[c]

L^*: lightness (0 indicate black, 100 indicates white); a^*: redness or greenness (positive values indicate red, negative values indicate green); b^*: yellowness or blueness (positive values indicate yellow, negative values indicate blue).
Values in a column followed by different letters are significantly different ($P \leq 0.05$) according to the Duncan test.

Table 3-2. Solvent gradient for reversed-phase HPLC analysis of the neutral and acidic fractions of the phenolic compounds[a]

Time (min)	Solvent A (%)	Solvent (%)
0	95	5
3	95	5
15	91	9
27	86.5	13.5
32	86.5	13.5
42	81.5	18.5
44	81.5	18.5
51	77.5	22.5
55	70	30
56	60	40
57	95	5

[a]Solvent A, 1:99 % (v/v) formic acid/water; solvent B, 1:99 % (v/v) formic acid/acetonitrile

Table 3-3. Identification of anthocyanin using HPLC-ESI/MS/MS

Sample	Peak	t_R	ʎmax (nm)	$[M^+]$ (m/z)	[MS/MS] (m/z)	Compound	Content*
Ace-DA	1	12.6	280, 520	433	287	Cyanidin-3-rhamnoside	2.67
	2	13.5	270, 508	417	271	Pelargonidin-3-rhamnoside	1.34
Ace-VE	1	12.6	280, 520	433	287	Cyanidin-3-rhamnoside	8.47
	2	13.6	270, 506	417	271	Pelargonidin-3-rhamnoside	6.52
FSAJ	1	11.9	280, 520	433	287	Cyanidin-3-rhamnoside	3.49
	2	13.0	270, 506	417	271	Pelargonidin-3-rhamnoside	NQ

* Anthocyanin content in mg/100g FW
Ace-DA: Sample from the cultivar Florida Sweet grown in Davie
Ace-VE: Sample from the cultivar Florida Sweet grown in Vero Beach
FSSAJ: Frozen single strength acerola juice
NQ: Not quantified

Table 3-4. Interday precision

Peak no.	Analyte	Repeatability (n=6) Retention time (min) Average	SD	RSD %
1	Gallic	8.31	0.03	0.31
2	Protocatechuic acid	15.77	0.09	0.60
3	Chlorogenic acid	28.48	0.21	0.74
4	Caffeic acid	31.19	0.15	0.49
5	Syringic acid	35.92	0.05	0.13
6	Ferulic acid	44.41	0.33	0.75
7	p-coumaric	51.62	0.24	0.46
8	Sinapic	54.43	0.25	0.47

Table 3-5. Retention times and mass spectrometric data of non-anthocyanin phenolic compounds in fruit determined by HPLC-ESI-MS^2, all stages of maturity included

Cpd No.	t_R (min)	Mwt	MS^1 (m/z)	MS^2 (m/z)	MS^3 (m/z)	Identification (tentative)
			Acidic Fraction			
Hydroxycinnamic acid derivatives[a]*						
22	31.9	342	377[M+Cl^-]$^-$	[f]341, 279, 149	179	Caffeic acid derivative
20	28.8	345	344[M-H]$^-$	[f]181	163	Chlorogenic acid derivative
31	44.2	356	355[M-H]$^-$	193 337, 265, 209		Ferulic acid hexoside
36	51.7	522	521[M-H]$^-$	[f]503, 415	459, 415, 307, 265, 221	p-coumaric acid hexoside derivative
			Neutral Fraction			
Flavan-3-ols[b]						
48	29.1	454	453[M-H]$^-$	[f]289, 433	[f]127, 271	Epicatechin derivative
16	16.8	290	289[M-H]-	[f]271, [f]209, 113	253, 197, [f]203, 113	(+)--Epicatechin
Flavonols[c]						
55	41	464	463[M-H]$^-$	[f]301, 273	271, 255, [f]179	Quercetin-3-α-O-rhamnoside
54	38.5	432	431[M-H]$^-$	[f]285	255, 229, 195, 174	Kaemferol-3-rhamnoside
41	21.7	594	593[M-H]$^-$	534, 533, 288, [f]285	211	Kaemferol derivatives
Conjugate of Ellagic acid[d]						
26	36.0	302	301[M-H]$^-$	284, [f]257		Ellargic acid
33	47.3	434	433[M-H]-	[f]301, 177, 133	396, 340, 265, 219, 177, [f]133	Ellagic acid derivative
Stilbenes[e]						
56	41.9	390	389[M-H]-	[f]371, 297, 221, 177, 133	327, 283, 221, 197, [f]133	Resveratrol hexoside

[a, c]identified in edible portion of fruits at both immature and mature stages, [b,e]identified in edible portion only at full maturity,[d]identified only in non-edible portion (seed) of mature fruits.[f] most intense product ions.

CHAPTER 4
ANTIOXIDANT ACTIVITY, ANTIMICROBIAL PROPERTIES, AND TOXICOLOGICAL SCREENING OF PHENOLIC EXTRACTS FROM ACEROLA (MALPIGHIA EMARGINATA DC) FRUIT

Overview

In recent years, the relationship between food and good health has become a very important issue. Many common foods are now considered “functional” foods, which in addition to fulfilling the basic nutritional needs should be able to provide additional physiological benefits, such as preventing or delaying the occurrence of chronic diseases in human (Kaur and Kapoor 2001). Research in the field of food science and nutrition has been focusing on the development of food products with higher nutritional values, and the evaluation of foods for their health promoting potential (Nicoli and others 1999). Recently, phytochemicals in fruits and vegetables have attracted a great deal of attention mainly owing to their role in the prevention of degenerative diseases caused by oxidative stress. Oxidative stress has been defined as a disturbance in the equilibrium status of pro-oxidant/antioxidant systems in intact cells resulting in oxidative damage to nucleic acids, lipids, proteins and carbohydrates (Thomas 1994). Oxidative stress releases free oxygen radicals in the body, and is involved in a number of disorders including heart disease, cataracts, cancers, rheumatism, ageing and many other auto-immune diseases (Kaur and Kapoor 2001). Phytochemicals act as antioxidant compounds and are very effective free radical scavengers.

Epidemiological evidence has shown correlation of dietary patterns with the prevention of non-transmissible chronic diseases such as cancer and cardiovascular disease. Many clinical studies have consistently demonstrated positive correlations between the consumption of fruits and vegetables and the reduction rate of heart

disease mortality, certain forms of cancer and other types of degenerative disorders (Steinmetz and Potter 1991; Steinmetz and Potter 1996; Block and others 1992; Margetts and others 1994; Ness and Powell 1997). This is due to the fact that fruits and vegetables contain different class of phytochemical compounds such as vitamin C, vitamin E, dietary fiber, dietary phenolic compounds and dietary carotenoids. For all the reasons indicated above there is an increased interest in the evaluation of the antioxidant activity in fruits and vegetables, and there is a plethora of publication in this area (Wada and Ou 2002; Kolayli and others 2003; Chinnici and others 2004; Silva and other 2004; Roesler and others 2006). The consumption of exotic tropical fruits has increased on both domestic and international markets due to increase recognition of its nutritional and health promoting effects.

Acerola is a shrub grown in tropical and subtropical areas from the southern end of Texas, through Mexico and Central America to northern South America and throughout the Caribbean. It has also been introduced widely into tropical areas of Asia especially in the Island of Okinawa in Japan, and in Africa. The tree bears a soft, red fruit that can be consumed fresh or processed for use as an ingredient in a variety of foods including commercial fruit juices, and energy drinks. In Germany, France, and Hungary, the fruit is used primarily for juice while in the United States it is utilized by the supplement and pharmaceutical industries as a rich source of vitamin C. Acerola is therefore an exotic fruit that has excellent agro-industrial potential and represents an appealing economic prospect for growers to reach niche markets created by consumers' demand for exotic products rich in nutrients for maintaining health and preventing degenerative diseases (Alves and others 2008). Recent research showed that in addition to vitamin C, acerola

fruit may be a good source of phytochemicals such as anthocyanins (De Rosso and others 2008; Delva and Goodrich 2010), non-anthocyanin phenolic compounds (Vendramini and Trugo 2004; Hanamura and others 2005), and dietary carotenoids (De Rosso and Mercadante 2005; Lima and others 2005). The presence of those compounds is indicative of potential high antioxidant capacity. Using a linoleic acid model system it was shown that acerola juice exhibited high antioxidant capacity (Righetto and others 2005). It was also demonstrated that acerola extracts have the ability to enhance the antioxidant capacity of soy and alfalfa extracts in a variety of low density lipoprotein oxidation systems (Hwang and others 2001). However, not only published data in this area is scarce, but the majority of research reported does not mention the variety of acerola used; given that the antioxidant capacity is dependent on the variety, it becomes difficult to compare results across laboratories.

Food spoilage and food poisoning by microbes represent a serious problem that has not yet been satisfactorily controlled in spite of the powerful preservation techniques available. The antibiotic resistance by pathogenic organisms to conventional drugs has compelled the search for novel therapeutic methods. In addition, consumer's preferences for foods that are prepared without preservatives of chemical origin have driven the search for natural surrogates providing sufficiently long shelf life of foods and a high level of safety with regards to foodborne microorganisms. Previous studies have indicated that medicinal plants are one of the best resources for the isolation and development of new bioactive compounds (Mohan and others 2008). In addition, plant-derived preparations have drawn the attention of people worldwide because of their fewer side effects and lesser toxicity in comparison to synthetic drugs (Jain and others

2011). There are a wide range of antimicrobial compounds occurring naturally and that play a significant role in the defense of different kinds of living organisms (Rauha and others 2000). The phenolic compounds represent a large group of secondary metabolites that are widespread in superior plants. Attention has been paid to their antimicrobial activity, but no impressive proof of their efficacy has been found (Rauha and others 2000). Scientific information about the antimicrobial property of acerola fruit is very scarce. A publication by Motohashi and others (2004) reported that hexane and ethyl acetate extracts from acerola fruit showed relatively high antibacterial activity particularly against Gram-positive bacteria such as *Staphyloccocus epidermidis* (ATCC 1228). However, since the experiment was not conducted on purified extracts, the nature of the active compounds is not well known.

To evaluate the ability of acerola fruit phenolic extracts to be used as a dietary supplement, it is important to perform its toxicological evaluation. The Food and Drug Administration (FDA) recommended a list of toxicological tests to the food industry. Tests that are relying on genetic toxicity such as the bacterial reverse mutation test are among the tests recommended to evaluate all chemicals for their toxicological safety.

The Ames mutagenicity assay employs different strains of *Salmonella typhimurium* which were mutated for different sensitivity towards different types of DNA-damaging chemical mutagens. In contrast to common *Salmonella*, the ability to synthesize biotin from histidine is lacking in these mutants. Because biotin is required for their growth and development the mutant *Salmonella strains* lose their capacity to grow in an environment where biotin is a limiting factor. However, the growth capacity can be restored in case of reverse mutation which may be caused by exposure to mutagenic

compounds (Maron and Ames 1983). In the Ames test, a glucose minimal agar plate with top agar having a very small amount of histidine is used in order to produce an environment deficient in histidine and biotin.

Acerola fruit at incomplete, intermediate and complete stage of maturity contain phenolic compounds. Acerola phenolic extracts have demonstrated interesting biological activity. However, very limited information is available on the toxicological evaluation of the acerola phenolic extracts. This lack of information on the toxicology of the acerola phenolic extracts limits their use as a food supplement. Acerola phenolic extract contain both simple and polyphenol, and potentially some non-identified compounds. It is important to perform the toxicological evaluation of acerola phenolic extracts in order to assess if they are safe to be used as dietary supplement.

In a tier approach suggested for general screening, two strains of S*almonella Typhimurium* (TA 98 and TA 100) are recommended to be employed in the initial step. Results are usually presented as mean of revertant colonies per plate ± standard deviation. The mutagenicity was determined by a method described in Mortelmans and Zeiger (2000). In this method, the mutagenicity is determined by setting up fold increase as a cut-off point. In general, when there is a 2-3 fold increase in the number of colonies from the negative control, the extract is considered mutagenic (Mortelmans and Zeiger 2000).

This study had three major objectives. (1). To evaluate the total antioxidant value of acerola fruit, and, given the importance of ascorbic acid and phenolic compounds in this fruit, we assess their contribution in the antioxidant activity of the fruit. (2). To investigate the antimicrobial potential of phenolic fractions from acerola fruit. To achieve

this goal, three different microbial species were used. Among the species investigated, *Staphylococcus aureus* is one of the most common gram-positive bacteria causing food poisoning (Rauha and others 2000). *Escherichia coli*, which are well understood indicator organisms was chosen as a representative of Gram negative bacteria. *Pseudomonas putida*, also a gram negative organism was used as a representative of spoilage microorganisms. (3). To perform preliminary screening of acerola phenolic fractions (anthocyanins, flavonols and phenolic, and phenolic acids) for mutagenicity based on the Ames mutagenicity test.

Materials and Methods

Chemicals and Biological Media

Folin-Ciocalteu phenol reagent was purchased from MP Biochemicals, LLC. 1,1-diphenyl-2-picrylhydrazil (DPPH), Fluorescein (free acid), and 6-hydroxy-2-5-7-8 tetramethylchroman-2-carboxylic acid (Trolox) were from Sigma-Aldrich. 2-2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals. Glucose was obtained from Difco (Houston, TX, USA). Tryptic soy agar (TSA) was acquired from Bacto (Bexton, Dickinson and company, Sparks, MD, USA). Agar power was provided by Fisher Scientific. Donomycine, top agar supplemented with 0.6 % L-histidine and D-biotin, Vogel-Bonner E salts (VB salts 50x). Dubelco sodium phosphate buffer, 0.1 mM, pH 7.0 was obtained from Sigma (Saint Louis, MO, USA).

Bacterial Strains

To perform the Ames mutagenicity test, cultures from two strains of *Salmonella Typhimurium*, TA 98, and TA 100 were employed. For the antimicrobial activity assay by the disc diffusion method, cultures of *E. coli* (ATCC 25922), *Staphylococcus aureus*

(ATCC 29247), and *Pseudomonas putida* (ATCC 12633) were used as test organisms. They were all purchased from the American Type Culture Collection (VA, U.S.A.).

Extraction and Fractionation of the Phenolic Compounds

The extraction and fractionation of the phenolic compounds were accomplished according to the method by Kim and Lee (2002) as described in Chapter 3 (see materials and methods section of Chapter 3).

Total Phenolics Analysis

The crude water extracts was diluted to proper strength for analysis. The total phenolic content was determined using the Folin & Ciocalteu assay (Singleton and Rossi 1965). The extracts were mixed with diluted Folin-Ciocalteu reagent and 15 % sodium carbonate. Absorption at 765 nm was measured in a microplate reader (SPECTRAmax 190 Molecular Devices, Sunnyvale, CA) after incubation for 30 min at room temperature. The results were expressed as milligrams gallic acid equivalents per kilogram of fresh weight (mg of GAE/kg) for the fruits or milligram of gallic acid equivalent per liter (mg of GAE/L) for the juice, using a standard curve generated with 100-600 mg of gallic acid per liter.

Antioxidant Capacity Assays

One of the objectives of this research was to determine the contribution of phenolic compounds to the total antioxidant activity of the acerola fruit. Total antioxidant capacity was determined on the acerola phenolic extracts (crude water extract). After the fractionation of the phenolic compounds, the antioxidant capacity of each fraction was determined by the ORAC and DPPH assays.

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was conducted according to a modified method of Sandhu and Gu (2010). The assay was conducted on a Spectra XMS Gemini plate reader (Molecular Devices, Sunnyvale, CA). In summary, 50 µL of standard and samples were added to the designated wells of a 96-well black plate. This was followed by the addition of 100 µL of fluorescein (20 nM). The mixture was incubated at 37 ^{o}C for 10 min before the addition of 50 µL of the free radical AAPH. The fluorescence was monitored using 485 nm excitation and 530 nm emission at 1 min intervals for 40 min. Trolox was used to generate a standard curve. The antioxidant capacity of the samples was expressed as mmol Trolox equivalent (TE) per kg (mmol of TE/kg) on a fresh weight basis.

DPPH (2-2'-Diphenyl-1-picrylhydrazyl) Assay

The DPPH assay was conducted according to Sandhu and Gu (2010). Fifty µL samples was mixed with 950 µL DPPH solution. The mixture was incubated for 60 minutes in the dark. Fifty (50) µL Trolox solutions were added to 950 µL DPPH solution to generate a standard curve. Fifty (50) µL MeOH was mixed with 950 µL DPPH working solution and used as a control. After the incubation, 200 µL of mixture was pipetted into a 96 well plate and the plate was read in a spectrophotometer at 515 nm. The result was expressed as mmol Trolox equivalent per kilogram fresh weight (mmol TE/kg).

Determination of Ascorbic Acid in the Extract

The AA analysis was conducted according to the method described in Lee and Coates (1999) with necessary modifications. Briefly, the acerola crude extracts and single strength juice were diluted to proper strength using potassium phosphate monobasic (KH_2PO_4) solution (pH 2.4).The diluted extract was filtered through a 0.45

µm Nylon filter and analyzed by HPLC, The ascorbic acid content in the extracts was determined using a standard calibration curve with concentration of 0-60 µg/mL ascorbic acid. The ascorbic acid content was expressed in mg ascorbic acid per 100 g sample on a fresh weight basis.

The HPLC analysis was conducted on a Dionex model P680 liquid chromatograph equipped with a Dionex model AS-100 automated sample injector, and a Dionex model 100 photodiode array (PDA) detector set at 254 nm. A Dionex model Acclaim® C_{18} column (4.6 mm x 250 mm, 5 µm) operated at ambient temperature was used. A 0.2 M potassium phosphate monobasic (KH_2PO_4) (Merck) in deionized water solution was used as the mobile phase with a flow rate of 1.0 mL/min. The pH of the mobile phase was adjusted to 2.4 with phosphoric acid (H_3PO_4).

Antimicrobial Activity

Sample Preparation for Antimicrobial Test

Concentrated stock of freeze-dried acerola phenolic extracts were reconstituted with sterile deionized water to achieve diluted concentrations of 0.25, 2.5, and 25 mg/mL. During preliminary experiments, only selected samples from the concentration of 25 mg/mL showed some types of antimicrobial activity. Therefore, the full scale antimicrobial assay was carried out only on that concentration.

The Disk Diffusion Test

The antimicrobial activity of the acerola phenolic extracts was conducted using the Kerby-Bauer disk diffusion susceptibility protocol (Hudzicki 2009). One day prior to the inoculum preparation, the microorganisms were sub-cultured. Using a sterile inoculating loop, five well-defined colonies were touched and suspended in 6 mL of sterile tryptic soy broth (TSB) and incubated until a cell density of $1x10^8$ cfu/mL was achieved. The

density was monitored according to the McFarland standard (absorbance measured with a spectrophotometer was approximately 0.1 at 625 nm). The suspension was used within 15 min of preparation. A sterile swab was dipped into the inoculum tube; the swab was rotated against the side of the tube to remove excess of liquid. The dry surface of the Mueller-Hinton agar (MHA) plate was inoculated by streaking the swab three times over the entire surface of the agar. Twenty (20) µL of acerola phenolic extract was impregnated onto sterile 6 mm diameter blank paper discs (final amount of 500 µg of phenolic compounds). The discs were allowed to rest in an aseptic hood until complete dryness and placed in triplicate onto the surface of the pre-treated agar plates. Prepared antibiotic disks of penicillin and ampicillin were used as positive control while discs impregnated with reagent alcohol were used as negative control. The plates were inverted and incubated at 37 ^{o}C for 24 hours for *E. coli* and *S. aureus*, and at room temperature for 24 hours for *P. putida*. Following the incubation, the inhibition zone sites were measured to the nearest millimeter using a ruler, and recorded. The experiment was then repeated at least two times.

Interpretation of the Results

When a known concentration of an antimicrobial compound is applied on a disc and the disc is placed on the surface of an MHA plate, there is immediate movement of water from the agar to the disc. And subsequently, the antimicrobial compound begins to diffuse into the surrounding agar. The rate of the diffusion of the antimicrobial compound through the agar is dependent on the diffusion and the solubility properties of the antimicrobial compound in the agar and the molecular weight of the compound (Bauer and others 1996). If the agar plate has been inoculated with a suspension of the testing microorganism before the disc has been placed on its surface, the growth of the

micro-organism and the diffusion of the antimicrobial compounds take place simultaneously. The growth occurs in the presence of antimicrobial compounds when the bacteria reach a critical mass and can overpower the inhibitory effect of the antimicrobial compound (Hudzicki 2009). The diameter of the inhibition zone is a function of concentration, potency and diffusion coefficient. One of the methods used to interprete the result is described in Rauha and others (2000). In this method, the inhibition zone (i.z.) of the sample is compared to that of the negative controls (antibiotics). Briefly when i.z. sample < i.z. reagent alcohol + 1 mm, the samples is considered to exhibit no antimicrobial activity, when i.z. sample is 1-3 mm > i.z. reagent alcohol, the sample is considered to have a slight antimicrobial activity, samples having i.z. between 3–4 mm > i.z. reagent alcohol, the sample is considered having moderate antimicrobial properties, when i.z. samples is 4–10 mm > i.z. reagent alcohol, the sample is said to have clear antimicrobial property; finally when sample i.z. > i.z. reagent alcohol + 10 mm, the sample is considered to have strong antimicrobial property.

Ames Mutagenicity Test

The Ames mutagenicity test was conducted according to the procedure available in Mortelmans and Zeiger (2000) with necessary modifications. In summary, glucose minimal agar plates were prepared by aseptically adding 50 mL of sterile glucose solution, 20 mL sterile VB salt solution and 930 mL of sterile agar at 65^{o}C; the mixture was then mixed with a magnetic stirrer. Twenty five (25) mL of the agar medium was poured into each of the 100 x 15 mm petri dishes; all the manipulations were conducted aseptically. The bacterial cultures were grown on TSA and five (5) well defined colonies were selected and inoculated in 25 mL of TSB broth in the flask. The flasks were placed

in a shaking water bath at 37 ^{o}C until a desired culture density of 1-2x10^{9} colony forming units (cfu)/mL was reached after nearly 16 hours. The density of the culture was monitored spectrophometrically at 660 nm and the absorbance at the desired density was between 1.2-1.4. Two (2) mL of sterile agar containing 0.6 % histidine and biotin were transferred to aseptic glass tubes and kept in water bath at 48 ^{o}C until needed.

The Ames test was conducted on the three phenolic fractions of acerola fruit (anthocyanins, flavonols and phenolic acids) at three different concentrations: 0.25, 2.5, and 25 mg/mL. The test was performed by pipetting aseptically 0.5 mL of 0.1 mM sodium phosphate buffer pH 7.4, 20 µL of acerola phenolic fraction (corresponding to amounts of 5, 50, and 500 µg phenolic compounds per plate), 0.1 mL of overnight salmonella culture into top agar and vortexed. The mixture was quickly poured and evenly distributed onto the surface of the GM agar mixture. Following the solidification of the surface of the agar, the plates were inverted and incubated at 37 ^{o}C for 48 hours.

For all the samples, sterile deionized water was chosen as negative control, daunomycin at a concentration of 60 µg/mL was used as a positive control for assay with TA 98, and sodium azide at a concentration of 60 µg/mL was chosen as positive control for assay with TA 100; all experiments were conducted at least in duplicate.

After an incubation time of 48 hours, the number of colonies were counted and recorded, and the background of each sample dish was also compared to the negative control in the absence of the background.

Statistical Analysis

The effect of maturity on the total phenolic index, antioxidant capacity, and vitamin C content was studied by performing a one-way analysis of variance with the Duncan multiple range test comparing the mean values within each type of extraction (fresh or

freeze dried extraction). The statistical analysis was performed using SAS (Statistical Analysis System, SAS Institute Inc., Cary, NC). The SAS program codes used and the SAS output are presented in Appendix C (Tables C-1 and C-2). Total phenolics, DPPH, ORAC, and vitamin C values are expressed as means plus or minus the standard deviation. The mutagenic dose response of acerola phenolic fractions to the *S. typhimurium* strains are also expressed as means plus or minus the standard deviation.

Results and Discussion

Total Phenolics, Total Antioxidant Capacity and Vitamin C Content

As shown in Table 4-1, all the acerola samples analyzed, no matter the treatments (stage of maturity, conditions of extraction, edible or non-edible portion of the fruits) show higher total phenolic values than other tropical fruits such as mango (16.4 $mgkg^{-1}$), pineapple (13.4 $mgkg^{-1}$) (Gorinstein and others 1999); and other food products like virgin olive oil (3000 $mgkg^{-1}$) (Gallina-Toschi and others 2005), and honey (3500 mg kg^{-1}) (Gheldof and others 2002). For the fruits grown in Davie, the edible portions from the immature fruits show higher total phenolic values than the edible fractions obtained from the mature fruits (Table 4-1). The total phenolic value decreases from the immature stage to the intermediate stage and increased again as the fruits reached the full maturity stage. The same trend is also observed for the fruits cultivated in Vero Beach. Righetto and others (2005) reported a decrease in total phenolic content of acerola juice from the immature to the mature stages. Extracts obtained from the freeze-dried powder showed a higher total phenolic index. This is because the powdered plant material maximizes polyphenolic extraction due to its high surface contact area with solvent and easy destruction of biological cell walls (Kim and Lee 2002). The total phenolics were also determined in the non-edible fraction of the fruits; only seeds from mature fruits

were considered and only the freeze-dried extraction was performed. Overall, the total phenolic content of the seeds is higher than the phenolic content of the edible portion of the fruits. On a fresh weight basis, the seed from the fruits grown in Vero Beach showed a higher total phenolics value (18155 mg GAE kg^{-1}) than all the other edible samples. It is also important to mention that acerola juice purchased from a supplier shows higher total phenolics value even higher than some of the edible portions of the fruit.

The antioxidant capacity was performed by ORAC and DPPH assays, the results are gathered in Table 4-1. For the edible portion of the fruits and regardless of the condition of extraction, the ORAC values vary from 79-43.5 mM TE kg^{-1}, 62-36 mM TE kg^{-1}, 53-36 mM TE kg^{-1} respectively for immature, intermediate and mature stage of ripeness. ORAC values of acerola samples were higher than values reported in the literature for cauliflower (17.7 mM), strawberry (15.4 mM) and spinach (12.6 mM) (Cao and others1996).The DPPH values vary from 251-95 mM TE kg^{-1}, 142-54.4 mM TE kg^{-1}, 53-36 mM TE kg^{-1} respectively for immature, intermediate and mature stage of ripeness these values are higher than the DPPH values reported for wine, green tea infusion, and pomegranate (Fogliano and others 1999; Prior and Cao 2000; Gil and others 2000).

The results also show that regardless of the extraction technique applied and the stage of maturity, fruits grown in Vero Beach show higher antioxidant capacity (expressed by ORAC or DPPH) than those grown in Davie. As it was observed for the total phenolic index, freeze-dried samples show higher DPPH scavenging capacity than fresh extraction. The DPPH value of the samples decreased as the fruit goes from the immature to complete maturity. At complete maturity, the seeds from the fruits grown in Vero Beach show a much higher DPPH scavenging capacity nearly 148 (mmol kg^{-1} FW)

than other samples. The ORAC value follows a similar trend; it is decreasing as the fruits ripen. At complete maturity, the non-edible fractions from the fruits grown in Vero Beach exhibited much higher ORAC value (85 mmol TE kg^{-1} FW) than the other samples analyzed.

Table 4-1 shows AA content of the different samples analyzed; the AA analysis was carried out only in the edible fractions of the fruits. The vitamin C content ranged from 1161-1744 g/100g, 970-1049 g/100g, and 405-987 g/100g respectively for immature, intermediate, and mature stages. These values are slightly lower than those reported by Vendramini and Trugo (2000), but higher that those reported by Mezadri and others (2008). These results are however much higher than the AA contents reported for other fruits or fruit juices such as orange juice (0.516 g L^{-1}), grapefruit juice (0.274 g L^{-1}) or lemon juice (0.327 g L^{-1}) (Ashoor and others 1984).

The fruits grown in Vero Beach exhibited lower vitamin C content than those grown in Davie. For a given growing location, the AA content decreases as the fruit ripens. As for the fruits grown in Davie, the AA content decreases from 1744 mg/kg FW at the immature stage (green), to 1049 mg/kg FW at the intermediate stage, and reached 987 mg/kg FW (nearly 43 % decrease) at complete maturity. Vendramini and Trugo (2000) reported a 50 % reduction of AA from the green to the red fruits and explained the loss of AA by biochemical oxidation.

Contribution of Phenolic Compounds and AA to the Antioxidant Capacity of Acerola Fruit

One of the main objectives of this study was to investigate the contribution of different phenolic fractions, and AA to the antioxidant capacity of acerola fruit. The procedure includes the determination of the total antioxidant of the crude extract before

fractionation, the determination of ascorbic acid, and the fractionation of the crude extract into anthocyanins (F1), flavonoids (F2), and phenolic acids (F3) as described in earlier sections. Following the fractionation of the phenolic compounds, the antioxidant capacity of each fraction was determined and the contribution of each fraction to the total antioxidant capacity was calculated. The contribution of AA to the total antioxidant capacity of the samples was also accomplished by performing the antioxidant activity of AA solutions having similar concentrations to the analyzed samples.

Table 4-2 shows that the antioxidant capacity of the phenolic fractions are in the following order: anthocyanins<phenolic acids<flavonols: (F1 < F3 < F2). Depending on the growing location, stage of maturity, and types of extraction, the phenolic fractions contributed 7.1 %-36.5 % of the antioxidant activity expressed by ORAC. The contribution of AA accounted for 18-39 % to the total activity. This contribution of AA is much lower than the values reported in the literature. Vitamin C was reported to contribute 65-100 % of the antioxidant potential of beverages derived from citrus fruit but less than 5 % in apple and pineapple juices (Gardner and others 2000). The relatively low contribution of phenolic compounds and AA to the total antioxidant power of acerola fruit is probably the evidence that the antioxidant activity of acerola is built upon the complementary action of its different components as suggested by Righetto and others (2005) rather than the independent action of each individual compound. Lower contribution of phenolic fractions and AA to the antioxidant capacity of acerola fruit is a probable indication that other compounds could play a significant role in its overall antioxidant power. Other compounds that may contribute to the antioxidant activity of acerola fruit include vitamin A and non-vitamin A carotenoids which acerola is

a good source of (De Rosso and Mercadante 2005; Lima and others 2005) and also a novel class of flavonoid compounds named aceronidin recently isolated in acerola fruit and whose DPPH scavenging power was reported to be superior to that of α-tocopherol (Kawaguchi and others 2007).

Antimicrobial Properties

The antimicrobial properties of the phenolic extract were evaluated by the disc diffusion method. Tables 4-3, 4-4, and 4-5 show the inhibition by the phenolic fractions. The entire activities correspondent to sample amount of 500 µg. The phenolic extracts in the concentration tested showed limited antimicrobial activity against the bacterial strains tested. The anthocyanin fractions (F1) (Table 4-3) did not demonstrate antimicrobial activity. In contrast, two extracts from the flavonoids fraction (F2) show moderate or clear antimicrobial properties. For instance, freeze dried edible portion of mature fruits from Davie showed moderate antimicrobial activity while freeze dried edible portions of immature and freeze dried non-edible portion of the fruit from Davie both showed clear antimicrobial activity against the strain of *S. aureus* used in this experiment (Table 4-4). For the phenolic acid fraction (F3), only the non-edible portion of the fruits shows some moderate activity against *S. aureus* (Table 4-4). Overall the flavonoids show more activity especially against *S. aureus* than the other phenolic fractions. The main flavonoid in acerola fruit has been identified as quercetin-3-α-O-rhamnoside (Hanamura and others 2005); and various quercetin glucosides have been identified as the active antimicrobial compounds in plant extracts especially against *S. aureus* (Rhamaswamy and others 1972; Khanna and others 1980; Rauha and others 2000). It is also important to point out that all the fractions (F1, F2, and F3), did not demonstrate activity against *E. coli* and *P. putida* strains tested in this experiment. This

is not different from the results reported by Motohashi and others (2004) who reported good antioxidant properties of acerola extracts against bacteria such as: *S. epidermidis* (ATCC 12228), but almost no activity against Gram-negative bacteria such as *E. coli* (ATCC 25925) and *P. aeruginosa* (ATCC 27853).

Overall, under the conditions that this experiment was conducted, the flavonoids fraction of the fruits shows relatively good antioxidant activity against *S. aureus*. It is important to mention that the antimicrobial test was performed using a 6 mm disc that has a very limited loading capacity. After the application of 25-30 µL liquid sample corresponding to 500 µg of active compound, the disc apparently reached its limit. The investigation of the antimicrobial properties of acerola phenolic extracts using other methods is therefore necessary in order to generate more information on the antimicrobial potential of acerola phenolic extracts.

Ames Mutagenicity Assay

Acerola phenolic extracts were screened for mutagenicity. Three (3) types of phenolic fractions were studied: anthocyanins, phenolic acids and the flavonols fractions. The Ames mutagenicity test was conducted on the two mutant strains of *Salmonella typhimurium* TA 98 and TA 100. The results, expressed in terms of the number of revertant colonies grown on the glucose minimum agar with histidine limited top agar is shown in Table 4-6, Table 4-7, and Table 4-8 respectively for anthocyanin, flavonol and phenolic acid fractions. Each phenolic fraction was tested at 3 three different concentrations: 5, 50, and 500 µg per plate. For the three types of phenolic fractions tested, regardless of the conditions: type of extraction, stage of maturity, the number of revertant colonies at the tested concentrations was lower than that of the negative control. Only a few samples present a number of revertant colonies higher

than that of the negative control, one example of such samples is the anthocyanin fraction from the fresh edible portion of acerola fruit at intermediate stage of maturity on the *S. typhimurium* strain T100. More importantly, in all the samples, the number of revertant colonies were not at least two fold higher than the negative control, suggesting that the phenolic fractions in acerola fruit, regardless of their nature did not contribute to mutagenicity

Because the number of revertant colonies was not at least 2 fold higher than that of the negative control, it can be concluded that the phenolic compound fractions are not mutagenic. The toxicological evaluation of acerola phenolic extract has never been conducted using the Ames mutagenic test. The concentration (5, 50. 500 µg per plate) corresponds to the chronic, subchronic and the acute levels used by Hanamura and Aoki (2008). The results reported are similar to those reported by Hanamura and Aoki (2008) who showed no toxic effect for acerola extract a concentration as high as 2000 ppm in rats.

Summary

Acerola fruits exhibit high total phenolics value with significant antioxidant capacity expressed by both the ORAC and DPPH methods. Ascorbic acid accounted for higher contribution in the overall antioxidant capacity of the fruit, but still much lower than expected considering the high ascorbic acid content of the fruit. Other antioxidant compounds such as carotenoids and newly isolated flavonoids may also contribute in the overall antioxidant capacity of the fruit. Overall this study demonstrates the antioxidant potential of the fruit, but more research needs to be conducted in order to better understand the contribution of compounds other than AA and phenolic compounds in the antioxidant capacity of the fruit. The results also show the

antimicrobial potential of the flavonoids fraction of the fruit particularly against *S. aureus.* However, further research using different assays is needed for a thorough assessment of the antimicrobial potential of the acerola phenolic extracts. The results show that no matter the method of extraction (freeze dried or fresh) and the stage of maturity (green, red, or intermediate) the phenolic fractions did not contribute to mutagenicity.

Table 4-1. Total phenolic index, total antioxidant value and vitamin C content of acerola sample

Sample	TPI*	ORAC**	DPPH***	Vit. C****
		Fruits grown in Davie		
Fresh extraction				
ED-G	9403[a]	43.5[a] ± 0.02	95.0[a] ± 0.30	1744[a]
ED-O	7944[c]	36.5[b] ± 0.10	54.7[b] ± 0.15	1049[b]
ED-R	8340[b]	36.2[b] ± 0.05	40.7[c] ± 0.35	987[c]
Freeze dried extraction				
ED-G	16285[a]	48.0[a] ± 0.04	136.0[a]	1161[a]
ED-O	12915[b]	39.7[b] ± 0.07	106.0[b]	970[b]
ED-R	18155[c]	40.0[b] ± 0.02	74.0[c]	405[c]
NED	18155	85.0 ± 0.50	147.0	NQ
FSSJ	12562	85.0 ± 0.02	30.0	921
		Fruits grown in Vero Beach		
Fresh extraction				
ED-G	11317[a]	79.0[a] ± 0.30	251.0[a]	1238[a]
ED-O	10191[b]	62.0[b]	142.0[b]	894[b]
ED-R	10283[c]	53.0[c] ± 0.04	101.0[c]	470[c]

ED-G: edible portion green; ED-O: edible portion orange red; ED-R: edible portion red; NED: non-edible portion (seed); FSSJ: frozen single strength juice.

*TPI: total phenolic index in mg GAE/kg; **ORAC value in mmol TE/kg, ***DPPH value expressed in mmol TE/kg; ****Vitamin C content in mg per 100g.

NQ: not quantified.

Within each type of extraction, values in a column followed by different letters are significantly different (P≤0.05) according to the Duncan multiple range test.

All results are expressed on fresh weight basis.

Table 4-2. Contribution of phenolic fractions and AA in the total antioxidant value expressed by ORAC

Sample	F1	F2	F3	Sum (F1+F2+F3)	$ORAC_{AA}$	Tot. ORAC[a] sample	% Contribution Phenolics	% Contribution AA
				Fruit grown in Davie				
Fresh extraction								
ED-G	N/A	4.20	1.18	5.38	17.1	43.5	12.4	39.2
ED-O	1.64	2.17	0.44	4.25	12.8	36.5	11.6	35.0
ED-R	1.77	2.63	0.94	5.34	13.8	36.2	7.10	38.1
Freeze dried extraction								
ED-G	N/A	8.90	1.60	10.5	12.2	48.1	21.8	25.3
ED-O	6.05	6.88	1.58	14.5	13.6	39.7	36.5	34.3
ED-R	6.03	6.28	2.30	14.6	14.9	40.0	36.5	37.4
NED	3.37	6.11	1.05	10.5	N/A	85.0	12.3	NQ
FSSJ	4.19	4.90	0.09	9.20	17.9	85.2	10.8	21.1
				Fruit grown in Vero Beach				
Fresh extraction								
ED-G	N/A	5.38	0.52	5.90	14.3	79.3	7.44	18.1
ED-O	2.26	6.03	0.54	8.83	14.2	61.2	14.4	23.2
ED-R	1.63	2.76	0.65	5.04	12.2	53.0	9.50	23.0

F1: anthocyanin, F2: flavonols, F3: phenolic acids.
ED-G: edible portion green; ED-O: edible portion orange red; ED-R: edible portion red; NED: non-edible portion (seed); FSSJ: frozen single strength juice.
[a]total ORAC value in mmol TE/kg on a fresh weight basis.
NQ: not quantified.

Table 4-3. Antimicrobial effect of anthocyanin fractions from acerola fruit; sample amount 500 µg (n=2)

Anthocyanin fraction	*Staphylococcus aureus* 29247	*E. coli* 25922	*Pseudomonas putida* ATCC 12633
	Fruit grown in Davie		
Fresh Extraction			
ED–R	-	-	-
ED–O	-	-	-
Freeze-dried extraction			
ED–R	-	-	-
ED–O	-	-	-
Non-edible freeze-dried			
NED	-	-	-
	Fruit grown in Vero Beach		
Fresh extraction			
ED–R	-	-	-
ED–O	-	-	-
FSSJ	-	-	-
Reference/activity	Ampicillin +	Ampicillin +++	Penicillin -

-: No antimicrobial activity, inhibition zone (i.z) of sample < i.z. reagent alcohol plus 1 mm; +: moderate antimicrobial activity, i.z. of sample 3-4 mm > i.z. reagent alcohol; ++: clear antimicrobial activity, i.z of sample 4-10 mm > i.z. reagent alcohol; +++: strong antimicrobial activity, i.z of sample > i.z. of distilled water plus 10 mm.
ED-G: edible portion green; ED-O: edible portion orange red; ED-R: edible portion red; NED: non-edible portion (seed); FSSJ: frozen single strength juice.

Table 4-4. Antimicrobial effect of flavonoids fractions from acerola fruit; sample amount 500 µg (n=2)

Flavonoid fractions	*Staphylococcus aureus* 29247	E. *coli* 25922	*Pseudomonas putida* ATCC 12633
	Fruits grown in Davie		
Fresh extraction			
ED–R	-	-	-
ED–O	-	-	-
ED–G	-	-	-
Freeze-dried extraction			
ED–R	+	-	-
ED–O	-	-	-
ED–G	++	-	-
Non-edible freeze dried			
NED–R (seed)	++	-	-
	Fruits grown in Vero Beach		
Fresh extraction			
ED–R	-	-	-
ED–O	-	-	-
ED–G	-	-	-
FSSJ	-	-	-
Reference/activity	Ampicillin +	Ampicillin +++	Penicillin -

-: No antimicrobial activity, inhibition zone (i.z) of sample < i.z. reagent alcohol plus 1 mm; +: moderate antimicrobial activity, i.z. of sample 3-4 mm > i.z. reagent alcohol; ++: clear antimicrobial activity, i.z of sample 4-10 mm > i.z. reagent alcohol; +++: strong antimicrobial activity, i.z of sample > i.z. of distilled water plus10 mm.
ED-G: edible portion green; ED-O: edible portion orange red; ED-R: edible portion red; NED: non-edible portion (seed); FSSJ: frozen single strength juice.

Table 4-5. Antimicrobial effect of phenolic acid fractions from acerola fruit; sample amount 500 µg (n=2)

phenolic acid fractions	*Staphylococcus aureus* 29247	*E. coli* 25922	*Pseudomonas putida* ATCC 12633
	Fruits grown on Davie		
Fresh extraction			
ED–R	-	-	-
ED–O	-	-	-
ED–G	-	-	-
Freeze-dried extraction			
ED–R	-	-	-
ED–O	-	-	-
ED–G	-	-	-
Non-edible freeze dried			
NED	+	-	-
	Fruit grown in Vero Beach		
Fresh extraction			
ED–R	-	-	-
ED–O	-	-	-
ED–G	-	-	-
FSSJ	-	-	-
Reference/activity	Ampicillin +	Ampicillin +++	Penicillin -

-: No antimicrobial activity, inhibition zone (i.z) of sample < i.z. reagent alcohol plus 1 mm; +: moderate antimicrobial activity, i.z. of sample 3-4 mm > i.z. reagent alcohol; ++: clear antimicrobial activity, i.z of sample 4-10 mm > i.z. reagent alcohol; +++: strong antimicrobial activity, i.z of sample > i.z. of distilled water plus10 mm.
ED-G: edible portion green; ED-O: edible portion orange red; ED-R: edible portion red; NED: non-edible portion (seed); FSSJ: frozen single strength juice.

Table 4-6. Mutagenic dose response of acerola anthocyanin fraction to *S. Typhimurium* (TA98 and TA100) as represented by mean number of revertant colonies (CFU/plate) (n=2)

	Number of colonies (CFU/Plate	
	TA 98	TA 100
Dose level (µg/plate)		
Fruits grown in Davie		
Fresh extraction		
Sterile DI water (NC)	35.0 ± 8.00	15.0 ± 4.00
Daunomycine (60 µg/plate)	400 ± 20.0	-
Sodium azide (PC)	-	998 ± 125
5 µg ED–R	26.0 ± 3.00	10.0 ± 4.50
5 µg ED–O	21.5 ± 5.10	12.0 ± 2.00
25 µg ED–R	15.0 ± 5.00	2.70 ± 3.00
25 µg ED–O	11.0 ± 2.70	4.70 ± 2.50
50 µg ED–R	5.00 ± 0.00	8.00 ± 3.00
50 µg ED–O	7.50 ± 2.00	6.00 ± 1.00
500 µg ED–R	2.00 ± 1.20	0.00 ± 0.00
500 µg ED–O	3.10 ± 0.00	0.00 ± 0.00
Freeze-dried extraction		
5 µg ED–R	21.0 ± 7.00	16.4 ± 4.00
5 µg ED–O	17.0 ± 3.00	12.2 ± 6.00
25 µg ED–R	17.0 ± 7.00	11.0 ± 3.00
25 µg ED–O	12.4 ± 5.00	8.40 ± 2.00
50 µg ED–R	11.0 ± 7.00	2.00 ± 0.00
50 µg ED–O	13.1 ± 4.00	19.0 ± 3.0
500 µg ED–R	6.70 ± 2.00	4.50 ± 1.50
500 µg ED–O	9.50 ± 5.00	7.00 ± 0.00
Non-edible freeze-dried		
5 µg NED	27.0 ± 8.20	16.0 ± 4.00
50 µg NED	12.5 ± 3.00	8.20 ± 1.50
500 µg NED	4.00 ± 0.00	4.00 ± 2.50
Fruit grown in Vero Beach		
Fresh extraction		
5 µg ED-R	17.0 ± 6.50	11.5 ± 7.10
5 µg ED-O	13.0 ± 2.50	21.0 ± 9.00
5 µg FSSJ	15.5 ± 0.00	10.0 ± 0.00
50 µg ED-R	7.00 ± 2.00	9.10 ± 5.00
50 µg ED-O	10.3 ± 6.50	5.70 ± 2.00
50 µg FSSJ	13.5 ± 2.50	11.0 ± 1.00
500 µg ED-R	5.00 ± 3.50	0.00 ± 0.00
500 µg ED-O	8.30 ± 1.00	7.00 ± 1.00
500 µg FSSJ	10.2 ± 2.00	6.50 ± 4.50

Values are presented as mean ± SD of at least two experiments. Two fold or more of number of revertant colonies is an indicator of mutagenicity; NC: negative control; PC: positive control.

Table 4-7. Mutagenic dose response of acerola flavonols fraction to *S. Typhimurium* (TA98 and TA100) as represented by mean number of revertant colonies (CFU/plate) (n=2)

	Number of colonies (CFU/Plate)	
	TA 98	TA 100
Dose level (µg/plate)		
Fruits grown in Davie		
Fresh extraction		
Without metabolic activation		
Sterile deionized water (NC)	35.0 ± 6.00	10.0
Daunomycine (60 µg/plate; PC)	1500	-
Sodium azide (60 µg/plate; PC)	-	128 ± 15.0
5 µg ED–R	31.5 ± 6.00	18.5 ± 2.00
5 µg ED–O	28.5 ± 7.00	16.5 ± 5.00
5 µg ED-G	27.0 ± 1.00	16.0 ± 1.00
25 µg ED–R	24.5 ± 4.50	12.5 ± 4.50
25 µg ED–O	18.7 ± 1.00	12.7 ± 1.00
25 µg ED–G	20.0 ± 8.00	12.0 ± 2.00
50 µg ED–R	15.4 ± 2.00	9.50 ± 1.00
50 µg ED–O	15.7 ± 7.50	11.0 ± 5.00
50 µg ED–G	17.0 ± 4.50	7.00 ± 4.50
500 µg ED–R	11.5 ± 2.50	4.70 ± 2.00
500 µg ED–O	8.00 ± 1.00	3.00 ± 1.00
500 µg ED–G	8.30 ± 2.40	2.70 ± 2.00
Freeze-dried extraction		
5 µg ED–R	29.5 ± 4.00	18.5 ± 6.00
5 µg ED–O	30.5 ± 7.00	19.5 ± 4.60
5 µg ED–G	37.0 ± 2.00	19.0 ± 1.00
25 µg ED–R	25.0 ± 11.0	14.5 ± 5.40
25 µg ED–O	27.0 ± 2.50	13.3 ± 3.00
25 µg ED–G	23.0 ± 10.0	14.0 ± 5.00
50 µg ED–R	14.4 ± 2.00	11.5 ± 2.00
50 µg ED–O	16.7 ± 5.70	13.0 ± 2.00
50 µg ED–G	17.0 ± 1.50	7.00 ± 4.50
500 µg ED–R	9.50 ± 3.00	5.70 ± 2.00
500 µg ED–O	13.0 ± 0.00	5.60 ± 0.00
500 µg ED–G	10.3 ± 0.00	3.70 ± 1.00
Non-edible freeze-dried		
5 µg NED	25.0 ± 0.00	18.5 ± 6.00
50 µg NED	16.8 ± 2.50	19.5 ± 4.60
500 NED	11.7 ± 4.00	19.5 ± 4.60

Table 4-7. Continued.

	Number of colonies (CFU/Plate)	
	TA 98	TA 100
	Fruit grown in Vero Beach	
Fresh extraction		
5 µg ED-R	24.5 ± 3.00	15.0 ± 4.00
5 µg ED-O	27.5 ± 6.00	16.5 ± 3.60
5 µg ED-G	30.0 ± 3.00	14.0 ± 2.00
5 µg FSSJ	23.2 ± 5.10	13.10 ± 1.00
50 µg ED-R	20.0 ± 6.00	8.30 ± 2.00
50 µg ED-O	14.0 ± 2.00	9.10 ± 2.00
50 µg ED-G	19.7 ± 5.00	8.50 ± 2.00
50 µg FSSJ	17.0 ± 5.00	13.0 ± 2.00
500 µg ED-R	10.7 ± 0.00	7.00 ± 2.50
500 µg ED-O	12.5 ± 5.00	4.70 ± 1.00
500 µg ED-G	10.0 ± 0.00	3.60 ± 0.00
500 µg FSSJ	9.70 ± 0.00	2.90 ± 2.00

Values are presented as mean ± SD of at least two experiments. Two fold or more of number of revertant colonies is an indicator of mutagenicity; NC: negative control; PC: positive control.

Table 4-8. Mutagenic dose response of acerola phenolic acid fraction to *S. Typhimurium* (TA98 and TA100) as represented by mean number of revertant colonies (CFU/plate) (n=2)

	Number of colonies (CFU/Plate	
	TA 98	TA 100
Dose level (µg/plate)		
Fruits grown in Davie		
Fresh extraction		
Without metabolic activation		
Sterile deionized water (NC)	28.0 ± 6.00	10.0 ± 2.00
Daunomycine (60µg/plate; PC)	300 ±15.0	
Sodium azide (60 µg/plate; PC)	-	1150
5 µg ED–R	30.0 ± 0.00	12.4 ± 0.00
5 µg ED–O	27.7 ± 7.10	11.1 ± 2.50
5 µg ED-G	27.0 ± 5.00	10.0 ± 0.00
25 µg ED–R	17.5 ± 2.60	6.70 ± 2.10
25 µg ED–O	16.2 ± 1.00	6.00 ± 2.00
25 µg ED–G	18.7 ± 2.60	6.00 ± 2.00
50 µg ED–R	12.7 ± 1.00	5.00 ± 1.00
50 µg ED–O	14.4 ± 0.00	4.50 ± 0.00
50 µg ED–G	13.6 ± 2.50	4.70 ± 3.20
500 µg ED–R	2.50 ± 1.70	2.00 ± 1.60
500 µg ED–O	3.70 ± 0.00	2.70 ± 2.30
500 µg ED–G	4.70 ± 4.50	2.00 ± 1.00
Freeze-dried extraction		
5 µg ED–R	35.0 ± 1.40	15.0 ± 1.00
5 µg ED–O	40.5 ± 3.20	17.5 ± 2.10
5 µg ED–G	37.1 ± 2.20	16.4 ± 4.20
25 µg ED–R	21.4 ± 3.50	11.5 ± 5.30
25 µg ED–O	24.7± 4.00	9.70 ± 0.00
25 µg ED–G	26.8 ± 1.00	10.6 ± 0.00
50 µg ED–R	19.1 ± 1.20	7.40 ± 0.00
50 µg ED–O	18.2 ± 4.00	6.80 ± 1.00
50 µg ED–G	16.7 ± 2.00	7.00 ± 3.20
500 µg ED–R	7.80 ± 1.00	2.70 ± 0.00
500 µg ED–O	8.90 ± 0.00	2.00 ± 0.00
500 µg ED–G	7.20 ± 0.00	3.00 ± 1.00
Non-edible freeze-dried		
5 µg NED	32.7 ± 1.00	18.2 ± 2.10
50 µg NED	25.0 ± 4.50	10.8 ± 2.30
500 µg NED	15.2 ± 0.00	5.00 ± 3.10

Table 4-8. Continued.

	Number of colonies (CFU/Plate	
	TA 98	TA 100
Fruit grown in Vero Beach		
Fresh extraction		
5 µg ED-R	20.0 ± 5.10	14.8 ± 4.80
5 µg ED-O	24.4 ± 0.00	12.7 ± 1.70
5 µg ED-G	24.2 ± 2.00	13.5 ± 2.50
5 µg FSSJ	19.5 ± 4.80	13.7 ± 2.10
50 µg ED-R	15.6 ± 2.60	8.90 ± 2.00
50 µg ED-O	15.0 ± 0.00	7.90 ± 0.00
50 µg ED-G	18.7 ± 4.80	9.10 ± 4.00
50 FSSJ	14.0 ± 2.70	8.70 ± 1.70
500 µg ED-R	7.20 ± 1.20	2.10 ± 0.00
500 µg ED-O	6.70 ± 0.00	4.30 ± 1.30
500 µg ED-G	5.00 ± 1.00	4.10 ± 0.00
500 FSSJ	4.60 ± 0.00	3.80 ± 1.20

Values are presented as mean ± SD of at least two experiments. Two fold or more of number of revertant colonies is an indicator of mutagenicity; NC: negative control; PC: positive control.

CHAPTER 5
EFFECT OF DIFFERENT ASCORBIC ACID CONCENTRATIONS ON THE COLOR STABILITY OF ANTHOCYANIN EXTRACTS FROM ACEROLA (*MALPIGHIA EMARGINATA* DC) FRUITS

Overview

Acerola tree belongs to the Malpighiaceae family. This tree gives fruit that has a smooth and thin skin, a soft pulp, and an exceptional bright red color at complete maturity. The attractive red color of acerola is due to the presence of anthocyanin pigment which we identified in the variety Florida Sweet used in this study as cyanidin-3-rhamnoside, the major kind; and pelargonidin-3-rhamnoside, the minor type. One of the problems the acerola growers are facing is the high perishability of this fruit at complete maturity. Shortly after harvest (3-4 days) the fruit losses its attractive red color and turns to a dull yellowish color that is often seen by the consumer as index of poor quality, therefore limiting the market potential of the fruit. The low stability of acerola anthocyanins is also a problem during processing and storage of the acerola juice. Preventing the degradation of anthocyanin can therefore be beneficial to both the acerola growers, processors and ultimately the consumers of acerola juice or related products.

During processing and storage, food products that contain anthocyanin are prone to color degradation occurring as the result of the conjoined effect of anthocyanin degradation and the formation of brown pigment (Abers and Wrolstad 1979; Skrede and others 1983).

The type of anthocyanins of fruit is dependent on the variety (Timberlake and Briddle 1982), and according to Markakis (1982), the type of anthocyanin may affect the resistance to color change. The stability of anthocyanin in foods maybe affected by

several factors including the chemical structure of the pigment; for instance diglucosidic substitution is known to impart more stability to the molecule than monoglucosidic (Markakis 1982; Mazza and Miniati 1993). Other elements in the composition of the fruit may also affect the stability of anthocyanin pigment such as phenolic compounds and ascorbic acid (Timberlake and Bridle 1982).

Acerola fruit when compared with other fruits is relatively low in anthocyanin pigment, but very high in ascorbic acid. It has been proven that when anthocyanin and ascorbic acid are present in the same system, depending on the conditions, one may degrade the other. It is suggested that the high vitamin C content of acerola fruit maybe the cause of the red color instability in this fruit. The mechanism of degradation of anthocyanin by ascorbic acid has been investigated. However, the results are subjected to debate and up to now a mechanism has yet to be found. Two theories exist: (1) When ascorbic acid is oxidized in the presence of copper, hydrogen peroxide (H_2O_2) is produced; and since H_2O_2 is an anthocyanin bleacher, it is believed that the ascorbic acid-induced anthocyanin degradation is mediated by H_2O_2 (Markakis 1982). Jurd (1972) speculated that "…ascorbic acid degrades anthocyanins by a mechanism involving direct condensation of the ascorbic acid to the position 4 on the flavylium structure." Both theories suffer from lack of experimental evidence. In this experiment, the objective is to study the color stability of acerola anthocyanin in the presence of ascorbic acid while additionally further explaining the degradation kinetics of anthocyanin in the presence of ascorbic acid in a model system and the mechanism by which ascorbic acid may degrade anthocyanin.

To understand the kinetics of anthocyanin degradation, anthocyanins were extracted from acerola fruits, and the stability of those extracts were monitored over time and compared with an açai anthocyanin model system in which ascorbic acid was added to levels that match the ascorbic acid contents in acerola anthocyanin extracts; this model was proposed by De Rosso and others (2007).

Materials and Methods

To understand the kinetics of anthocyanin degradation, anthocyanins were extracted from acerola fruits, and the stability of those extracts were monitored over time and compared with an açai anthocyanin model system in which ascorbic acid was added to levels that match the ascorbic acid contents in acerola anthocyanin extracts; this model was proposed by De Rosso and others (2007). Açai is a good model because like acerola, it contains monoglucosylated anthocyanins. Pure anthocyanin model systems with added ascorbic acid were also developed and the possible formation of anthocyanin breakdown products was monitored by spectrophotometry.

Acerola Fruit and Açai Puree

Acerola fruits from the variety Florida Sweet (FSW) were harvested from different trees on the Elson's Exotic Farm in Davie (DA), South Florida and in different backyards in Vero Beach (VE), Central Florida. The fruits were manually harvested and transported to the Food Science and Human Nutrition Department at the University of Florida. Upon arrival, the fruits were washed with clean water and separated into edible portion (pulp+ skin) containing the anthocyanins and non-edible portion containing the seeds were discarded. The edible portions of the fruits were stored in a freezer at -20 ^{o}C until needed for analysis. Frozen açai puree was donated by ITI Tropicals (Lawrenceville, NJ). The puree was stored in a freezer at -20 ^{o}C for later use.

Preparation of the Anthocyanin Extracts

Edible portions of fruits, 353 g, and 362 g respectively for fruit collected in Davie (Ace-DA) and Vero Beach (Ace-VE) and açai puree, 344 g were blended with 500 mL 0.1 % HCl in methanol and allowed to stay overnight in a refrigerator. The mixtures were strained and centrifuged (*4000 g*, 4 ^{o}C, and 10 min) to obtain an extract free of sediment. The extract was then concentrated in a Buchi rotary evaporator at low temperature (~30 ^{o}C) to evaporate the methanol; the concentrated extract was stored at -20 ^{o}C until needed.

Development of the Model Systems

The anthocyanin model systems were developed in citrate-phosphate buffer, pH 2.5. The buffered crude acerola extracts had AA contents of 288 mg/100 mL and 97 mg/100 mL for Ace-DA, and Ace-VE samples respectively. Therefore, 288 mg AA/100 mL was added to the açai extract to match the AA level in Ace-DA samples. Similarly, 97 mg AA/100 mL was added to açai extract to simulate the AA content in Ace-VE samples. In another separate treatment, half of the AA content of each type of acerola (144 mg in the case of Ace-DA or 48 mg in the case of Ace-VE) was added to the açai extracts. A similar model system was used by De Rosso and Mercadante (2007). The crude anthocyanin extracts were diluted and scanned from 400 and 700 nm to obtain the wavelength of the maximum absorption. The wavelengths of maximum absorption were 505, and 517 nm for diluted acerola and açai solutions respectively. All absorbance readings were made against the dilution buffer as a blank. Spectrophotometric measurements were conducted using a DU® 730 Life Science UV-Vis spectrophotometer (Beckman Coulter®). The solutions were distributed in glass

tubes in 20 mL increments and stored under two different conditions (light or dark at 20 ^{o}C ± 1).

The major anthocyanin in the acerola variety used in this experiment is cyanidin-3-O-rhamnoside. Therefore, a model system was developed with this particular type of anthocyanin. Cyanidin-3-O-rhamnoside chloride (HPLC grade, purity ≥ 90 %), and free cyanidin (HPLC grade, purity ≥ 96 %) were purchased from Extrasynthèse Genay Cedex, (Lyon, France) and stored at -15 ^{o}C until needed. Anthocyanin solutions having absorbance values ca 1.5 were prepared in citrate-phosphate buffer (pH 2.5) with 0.1 % sodium benzoate, using cyanidin-3-O-rhamnoside (1.19 mg/L) and cyanidin (1.45 mg/L) with initial absorbance value of 1.0. To a portion of each solution, ascorbic was added to give final concentration of 330 mg/L (Garcia-Viguera and Bridle 1999). The anthocyanin solutions were scanned from 400-700 nm to determine the wavelength of maximum absorption which was 520 nm for both cyanidin and cyanidin-3-O-rhamnoside. Reaction mixtures (20 mL) were placed in tubes in 20 mL increment and stored in darkness at 20 ^{o}C.

Stability and Visual Color Attributes of the Anthocyanin Extracts

The stability of the anthocyanins in the different systems was monitored periodically by spectrophotometry, measuring changes in absorption at maximum wavelength (λmax). The zero-time absorbance value was considered as the initial absorbance. The anthocyanin retention for each time period was calculated as a percentage of the zero time-time absorbance readings taken at 100 % retention (Özkan 2002).

Absorbance readings at 700 nm were recorded to correct for turbidity. Browning index, a reading of the changes in browning compounds was determined as follows:

ABS_{420}-ABS_{700})/($ABS_{\lambda max}$-ABS_{700}) (Reyes and Cisneros-Zevallos 2007). Changes in the color of the anthocyanin solutions were determined using the CIELAB system, using the Color Quest XE colorimeter (Hunter Lab., Reston, United States) equipped with light source D65 and observation angle of 10^{o}. Color parameters lightness (L^{*}), redness (a^{*}) and yellowness (b^{*}) were read. Other parameters such as chroma value ($C^{*}= [(a^{*})^{2}+ (b^{*})^{2}]^{1/2}$) and hue angle ($h= \arctan (b^{*}/a^{*})$) were calculated. These parameters were calculated because $L^{*}a^{*}b^{*}$ coordinates do not directly express hue and chroma and are difficult to translate independently (Reyes and Cisneros-Zevallos 2007).

Determination of Ascorbic Acid

The AA analysis was conducted according to the method described in Lee and Coates (1999) with necessary modifications. Briefly, the samples were diluted to proper strength using potassium phosphate monobasic (KH_2PO_4) solution (pH 2.4).The diluted samples was filtered through a 0.45 µm Nylon filter and analyzed by HPLC, the ascorbic acid content was determined using a standard calibration curve with concentration of 0-60 µg/mL ascorbic acid.

The HPLC analysis was conducted on a Dionex model P680 liquid chromatograph equipped with a Dionex model AS-100 automated sample injector, and a Diomex model 100 photodiode array (PDA) detector set at 254 nm. A Dionex model Acclaim® (4.6 mm x 250 mm, 5 µm) C_{18} column operated at ambient temperature was used. A 0.2 M potassium phosphate monobasic (KH_2PO_4) (Merck) in deionized water solution was used as the mobile phase with a flow rate of 1.0 mL/min. The pH of the mobile phase was adjusted to 2.4 with phosphoric acid (H_3PO_4).

Kinetics Calculations

The degradation kinetics for anthocyanins assuming first order could be modeled using the Equation 5-1.

$$\log [A]_t = -2.303\ kt + \log [A]_0 \quad (5\text{-}1)$$

But since the disappearance of the anthocyanin over time was monitored by UV-visible spectrophotometry, the concentration was replaced by the absorbance (A) in the Equation 5-1. We assumed that both anthocyanin and potential anthocyanin degradation product absorb at the monitored wavelength, therefore the final absorbance (absorbance read at the final storage time) is non-zero. Under these conditions, the Equation 5-2 presented in Billo (2001) was used.

$$\ln|A_t - A_{inf}| = -kt + \ln|A_i - A_{inf}| \quad (5\text{-}2)$$

Where A_i is the initial absorbance reading and A_{inf} is the absorbance value when the reaction is assumed complete. The first order behavior was verified by the straight line fit of the data shown in Figure 5-1.

Results and Discussion

Effect of Ascorbic Acid on the Stability of the Anthocyanin Extracts

Among all the samples studied in this experiment, the degradation of anthocyanins followed a first order kinetics. Açai extracts showed a greater stability than did the acerola samples. Açai samples in which 288 mg AA was added showed greater stability than Ace-VE samples (containing lower AA contents), but similar stability with the Ace-DA samples (containing higher AA acid contents). It is important to note that for both the dark and light samples near the end of storage, the residual anthocyanins in the Ace-DA sample became unexpectedly higher than the residual anthocyanin in Açai+144 mg AA, Ace-VE and Açai+288 mg AA (Figure 5-2). This is due to the development of more

brown pigments in Ace-DA samples as a result of higher AA content than the other samples. Under light, the addition of 48, 97, 144, or 288 mg AA to the açai anthocyanin solution lead to a 3.7, 3.07, 5.2, or 6.48-fold increase in the degradation rate constant respectively when compared with non-enriched açai solution (Table 5-1). Under dark, the same trend was also observed, the degradation rate constant increased with increasing level of AA fortification. Fortification levels of 48, 97, 144, or 288 mg AA lead to 3.34, 3.78, 4.19, and 5.21-fold increase in the degradation rate. The degradation rate constant for anthocyanin from Ace-DA is 1.27 times as high as that of Ace-VE under the presence of light. Under light, the degradation rate constant of the anthocyanin solutions from Ace-DA is nearly similar to the degradation rate constant of açai sample enriched with 288 mg AA. Higher stability of açai anthocyanins can be ascribed to the presence of much higher total flavonoids which may protect anthocyanin through intermolecular copigmentation (Mazza and Brouillard 1990). The protective effect of flavonoids like quercetin and quercitrin against the deleterious effect of AA on cranberry anthocyanin has been reported (Shrikhande and Francis 1974).

Regardless of the system considered, addition of AA decreased the half-life of anthocyanins. Lower half lives are reported for samples enriched with higher amounts of AA. Under light, the addition of 288 mg AA decreased the half-life from 104 hours (no AA added) to as low as 16 hours. Under light or in darkness, anthocyanin extract from Ace-VE showed a higher stability than Ace-DA. For example in the presence of light, the half-life of the anthocyanin solutions from Ace-VE was 26.9 hours while the half-life of anthocyanin of extract from Ace-DA was nearly 16 hours. This is due to the fact that Ace-VE sample contains less ascorbic acid than Ace-DA samples.

Effect of Ascorbic Acid on the Stability of the Pure Anthocyanin Solution

The rate of decrease of the anthocyanin is faster in samples containing ascorbic acid especially at the start (the first 5 hours) of the experiment. The influence of ascorbic acid is greater for the cyanidin aglycone than its corresponding monoglucoside (cyanidin-3-O-rhamnoside). After storage for nearly 36 hours, the percentage loss of cyanidin with AA (39 % remain) or without added ascorbic acid (62 % remain) is much higher than that of cyanidin-3-O-rhamnoside with AA (65 % remain) or without AA (89 % remain) (Figure 5-3). The greater stability of cyanidin-3-O-rhamnoside over cyanidin corroborates the fact that glucosidic substitution confers more stability to the anthocyanin molecule (Markakis 1982; Mazza and Miniati 1993). Also as another proof of lower stability, a higher browning index was observed in the pure cyanidin system (data not shown).

Effect of Light

In the acerola model systems, the anthocyanin degradation was expectedly faster for samples stored in the presence of light than those stored in darkness (Figure 5-4). This tendency was similar for both acerola fruits grown in Davie, in which the ascorbic acid content is higher, and acerola fruits grown in Vero Beach Florida. Overall, the deleterious effect of light appears to be more intense on acerola anthocyanin, this tendency however was reversed with increasing açai fortification with ascorbic acid. No matter the system considered, Ace-VE, Ace-DA, Açai+48 mgAA, Açai+97mgAA, Açai+144mgAA, or Açai+288mgAA, storage in darkness lead to a higher percentage of anthocyanin remaining near the end of storage (Figure 5-4, panels A, B, and C). This is not surprising since light is one of the factors usually involved in anthocyanin degradation (Markakis 1982).

Color Stability of the Different System

Noticeable changes were observed in $L^*a^*b^*$, hue and chroma values for all the extracts, confirming the degradation of visual parameters in the anthocyanin extracts over time. The changes however were more obvious in acerola anthocyanin extracts containing higher levels of ascorbic acid and in açai extracts enriched with higher levels of ascorbic acid. The lightness (L^*) is the intensity of the luminosity transmitted by the solution. The linear behavior of LnL^* over time as shown in Figure 5-5 proved that the anthocyanin degradation in all the systems followed a first order pattern. The increase of L^* values under light or in darkness is related to the formation of translucent extracts due to color fading (Reyes and Cisneros-Zevallos 2007).

The redness (a^*) (Figure 5-6) and the yellowness (b^*) (Figure 5-7) values in all the samples decreased. Redness (a^*) value decreased at a much faster pace in Ace-DA samples than the Ace-VE samples. Ace-DA samples stored under light or under dark at 20 ^oC showed more than 96 % decrease in a^* value after 120 hours storage time. The same tendency was also observed for açai samples enriched with ascorbic acid (Table 5-2). The color parameters a^* and b^* decrease quicker with the addition of ascorbic acid in the açai model systems. For a given storage condition (under light or in darkness) açai samples enriched with 97 mg AA exhibited higher percent decrease of a^* and b^* value than açai sample enriched with 48 mg AA (Table 5-2). It is also important to point out that initial color parameters a^* and b^* of the açai samples appeared to be degraded at a slower pace compared to a^* and b^* from the acerola anthocyanin extracts. This could be related to the higher total flavonoids content in açai samples which may confer some level of protection of anthocyanins against the potential harmful effect of ascorbic

acid. The fact that a^* and b^* decrease faster in the Ace-DA in comparison with Ace-VE anthocyanin extracts maybe due to the higher ascorbic acid content in the former.

In the CIELAB color space, the hue parameter (h) is the angle made by the parameters b^*(yellow) and a^*(red). It usually defines specific colors which include yellow, red, blue, green or any combination of these colors (Gonnet 1998). During storage, in the presence of light or under dark, the hue angle in all the samples increased over the 120 hour period of time (Figure 5-8). The most spectacular increase was observed in the Ace-DA samples while only minor increase was observed in Ace-VE samples and in açai solutions enriched with AA (48 mg AA/100 mL or 97 mg AA/100 mL). The difference in the behavior of h values in all the model systems during storage under different conditions (light, dark) indicates that the color was changing from orange-red to yellow during storage of Ace-DA samples whereas the tonality in the Ace-VE samples and the AA-enriched açai systems remained red. The increase of h value has been previously reported in strawberry syrup fortified with AA (Skrede and others 1992); and in aqueous extracts of purple and red-flesh potatoes where the increase in the hue angle was associated to the formation of yellow chalcone species (Reyes and Cisneros-Zevallos 2007) .The chroma (C^*) is another parameter that is usually used to describe color intensity. In all the samples, the C^* values also decreased over the course of storage (Figure 5-9), confirming that intensive degradation of anthocyanin occurred. Decrease in chroma is often associated to degradation of monomeric anthocyanin (Reyes and Cisneros-Zevallos 2007).The detrimental effect of AA enrichment on the color was obvious in all the systems regardless of the storage conditions, resulting in the increased L^*, decreased a^* and C^* values over time.

Another parameter used in the assessment of color is the overall color difference (ΔE^*). Figure 5-10 shows that the overall color difference for the model systems showed the following order: ΔE^*Ace-Ve > ΔE^*Ace-Da > ΔE^*Açai + 97mg AA > ΔE^*Açai + 48mg AA. The final values of ΔE^* were, 26.3, and 28.4 for Ace-Ve sample stored under light and dark at 20^{o}C respectively, and the values for the Ace-Da system were 32.5, and 30.4. In the açai system, the final values of ΔE^* were 22.8, 23.8 and 29.2, 31.6 for açai +48 mg AA and açai+97 mg AA under light and dark storage respectively. These results show that storage under light produce more color difference than sample stored in darkness.

Degradation of Ascorbic Acid over Time

The change in ascorbic acid content either in the acerola anthocyanin solution where this compound is naturally present, or in the açai systems where the ascorbic acid was added was monitored by high performance liquid chromatography HPLC. The ascorbic acid was measured before and at the end of the storage period. Table 5-3 shows that the degradation of ascorbic acid occurred faster in the presence of light. Lower percent decrease was observed in açai samples enriched with AA when compared with the acerola samples, probably because flavonoids in açai confer some level of protection towards the AA. In summary anthocyanins from açai extracts show greater stability in the presence of ascorbic acid. Addition of ascorbic acid negatively influences the color parameters in açai extracts. Acerola anthocyanin extracts having highest AA content showed highest degradation rate constant and lower half-life.

Some Discussion on the Type of Reaction that May Take Place between Anthocyanin and Ascorbic Acid

The results suggest that AA play a significant role in the instability of acerola and açai anthocyanin. Now the question is what type of reaction took place between the anthocyanin and the ascorbic acid molecule. The flavylium nucleus of the anthocyanin molecule lacks electron and is therefore very susceptible to nucleophilic attack. It has been proven that flavylium salts readily condenses with β-diketone dimedone to yield colorless 4-substituted adducts, and based on the similarities of the AA structure to dimedone, Jurd (1972) speculated that a similar condensation reaction may occur. Based on the results generated in this experiment and certain observations made especially in the pure anthocyanin model system, conclusion similar to that of Garcia-Viguera and Bridle (1999) can be drawn. It is improbable that direct condensation between anthocyanin and AA occurred because the red color faded away rather slowly in both the acerola anthocyanin solutions, and açai extracts, and the pure anthocyanin model systems, suggesting that the reaction that took place was not spontaneous. Timberlake and Bridle (1968) described a spontaneous condensation reaction between SO_2 (a nucleophilic agent like ascorbic acid) and anthocyanin. Another observation that argues against the direct condensation theory is that in the pure cyanidin and cyanidin-3-O-rhamnoside model systems, no changes were seen in the wavelength scans. The spectrophotometric profile of cyanidin+AA at time zero is similar to the profile obtained at the middle of the storage period; and the same trend was also observed in the scanning profile of cyanidin-3-O-rhamnoside model system (Figure 5-11). Therefore, the degradation of anthocyanin through a free radical mechanism as proposed by Lacobucci and Sweeny (1983) and supported by Garcia-Viguera and Bridle (1999) is

more likely. In the free radical mechanism theory, it is believed that ascorbic acid activates molecular oxygen by producing free radical that leads to the cleavage of the flavylium ring.

Summary

The results show that ascorbic acid plays an important role in the degradation of anthocyanin in acerola fruit. However, the degradation seems to be promoted by the degradation products of ascorbic acid. Therefore, until the mechanism of this degradation is fully elucidated, it is important to store acerola fruit or its related products under conditions that favor the stability of ascorbic acid.

Table 5-1. Degradation rate constant and the half-life for anthocyanin in different systems citrate-phosphate buffer pH 2.5

Samples	k_{obs} (h^{-1})	Half-life (hr)	R^2
Ace-VE-Light	3.43×10^{-2}	27.0	0.99
Ace-VE-Dark	6.20×10^{-2}	26.0	0.98
Ace-DA-Light	4.35×10^{-2}	16.0	0.95
Ace-DA-Dark	4.34×10^{-2}	16.0	0.98
Açai-NoAA-Light	6.60×10^{-3}	104	0.81
Açai-NoAA-Dark	8.20×10^{-3}	84.0	0.84
Açai+48mgAA-Light	2.45×10^{-2}	28.0	0.99
Açai+48mgAA-Dark	2.74×10^{-2}	25.0	0.93
Açai+97mgAA-Light	2.03×10^{-2}	23.0	0.96
Açai+97mgAA-Dark	3.10×10^{-2}	22.0	0.98
Açai+144mgAA-Light	3.43×10^{-2}	20.0	0.96
Açai+144mgAA-Dark	3.44×10^{-2}	20.0	0.96
Açai+288mgAA-Light	4.28×10^{-2}	16.0	0.94
Açai+288mgAA-Dark	4.40×10^{-2}	16.0	0.96

Ace-VE-Light: acerola anthocyanins extract from fruits harvested in Vero Beach and stored under light; Ace-VE-Dark: acerola anthocyanins extract from fruits harvested in Vero Beach and stored in darkness; Ace-DA-Light: acerola anthocyanins extract from fruits harvested in Davie and stored under light;Ace-DA-Dark: acerola anthocyanins extract from fruits harvested in Davie and stored in darkness; Açai-NoAA-Light: açai anthocyanins extract with no added ascorbic acid and stored under light; Açai-NoAA-Dark: açai anthocyanins extract with no added ascorbic acid and stored in darkness; Açai+48mgAA-Light :açai anthocyanins extract enriched with 48 mg/100 mL ascorbic acid and stored under light; Açai+48mgAA-Dark: açai anthocyanins extract enriched with 48 mg/100 mL ascorbic acid and stored in darkness; Açai+97mgAA-Light: açai anthocyanins extract enriched with 97mg/100mL ascorbic acid and stored under light; Açai+97mgAA-Dark: açai anthocyanins extract enriched with 97mg/100 mL ascorbic acid and stored in darkness; Açai+144mgAA-Light: açai anthocyanins extract enriched with 144mg/100 mL ascorbic acid and stored under light; Açai+144mgAA-Dark: açai anthocyanins extract enriched with 144mg/100 mL ascorbic acid and stored in darkness; Açai+288mgAA-Light: açai anthocyanins extract enriched with 288mg/100 mL ascorbic acid and stored under light; Açai+288mgAA-Dark: açai anthocyanins extract enriched with 288mg/100 mL ascorbic acid and stored in darkness.

Table 5-2. Changes in color parameters (a^* and b^*) for initial and final storage time

Samples	Initial time (T_0)	Light 120 h	Dark (120 h)
a^*value			
Ace-Ve	35.8	14.0 (60.9)	12.74 (64.5)
Ace-Da	22.8	0.82 (96.4)	0.71 (97.0)
Açai+ 48mg AA	43.3	27.2 (37.3)	25.18 (41.8)
Açai+ 97mg AA	42.0	19.8 (51.7)	16.51 (59.8)
b^*value			
Ace-Ve	22.6	9.56 (58.0)	11.3 (50.4)
Ace-Da	27.3	14.9 (45.4)	13.2 (51.6)
Açai+ 48mg AA	15.8	13.9 (11.9)	14.4 (8.79)
Açai+ 97mg AA	16.8	14.1 (16.1)	14.6 (13.4)

Value within parentheses represent the percent decrease of the a^* and b^* values after 120 h of storage under different conditions

Ace-VE: acerola anthocyanins extract from fruits harvested in Vero Beach; Ace-DA: acerola anthocyanins extract from fruits harvested in Davie; Açai+48mgAA: açai anthocyanins extract enriched with 48 mg/100mL ascorbic acid; Açai+97mgAA: açai anthocyanins extract enriched with 97mg/100mL ascorbic acid.

Table 5-3. Ascorbic acid degradation in acerola and AA-fortified açai

Samples	Acid ascorbic content (mg/100mL)*	
	T_0	T_f=(120h)
Ace-VE-Light	97.0	22.4 (77)
Ace-VE-Dark	97.0	29.7 (69)
Ace-DA-Light	288	169 (23)
Ace-DA-Dark	288	169 (41)
Açai+48mgAA-Light	48.0	41.6 (13)
Açai+48mgAA-Dark	48.0	41.0 (15)
Açai+97mgAA-Light	97.0	57.0 (41)
Açai+97mg AA-Dark	97.0	64.7 (33)
Açai+144mgAA-Light	144	79.2 (50)
Açai+144mgAA-Dark	144	87.0 (39)
Açai+288mgAA-Light	288	96.5 (67)
Açai+288mgAA-Dark	288	102 (64)

*Values in parenthesis represent % decrease of AA

Ace-VE-Light: acerola anthocyanins extract from fruits harvested in Vero Beach and stored under light; Ace-VE-Dark: acerola anthocyanins extract from fruits harvested in Vero Beach and stored in darkness; Ace-DA-Light: acerola anthocyanins extract from fruits harvested in Davie and stored under light; Ace-DA-Dark: acerola anthocyanins extract from fruits harvested in Davie and stored in darkness; Açai+48mgAA-Light :açai anthocyanins extract enriched with 48 mg/100mL ascorbic acid and stored under light; Açai+48mgAA-Dark: açai anthocyanins extract enriched with 48 mg/100mL ascorbic acid and stored in darkness; Açai+97mgAA-Light: açai anthocyanins extract enriched with 97 mg/100mL ascorbic acid and stored under light; Açai+97mgAA-Dark: açai anthocyanins extract enriched with 97mg/100mL ascorbic acid and stored in darkness; Açai+144mgAA-Light: açai anthocyanins extract enriched with 144 mg/100mL ascorbic acid and stored under light; Açai+144mgAA-Dark: açai anthocyanins extract enriched with 144 mg/100mL ascorbic acid and stored in darkness; Açai+288mgAA-Light: açai anthocyanins extract enriched with 288 mg/100mL ascorbic acid and stored under light; Açai+288mgAA-Dark: açai anthocyanins extract enriched with 288 mg/100mL ascorbic acid and stored in darkness.

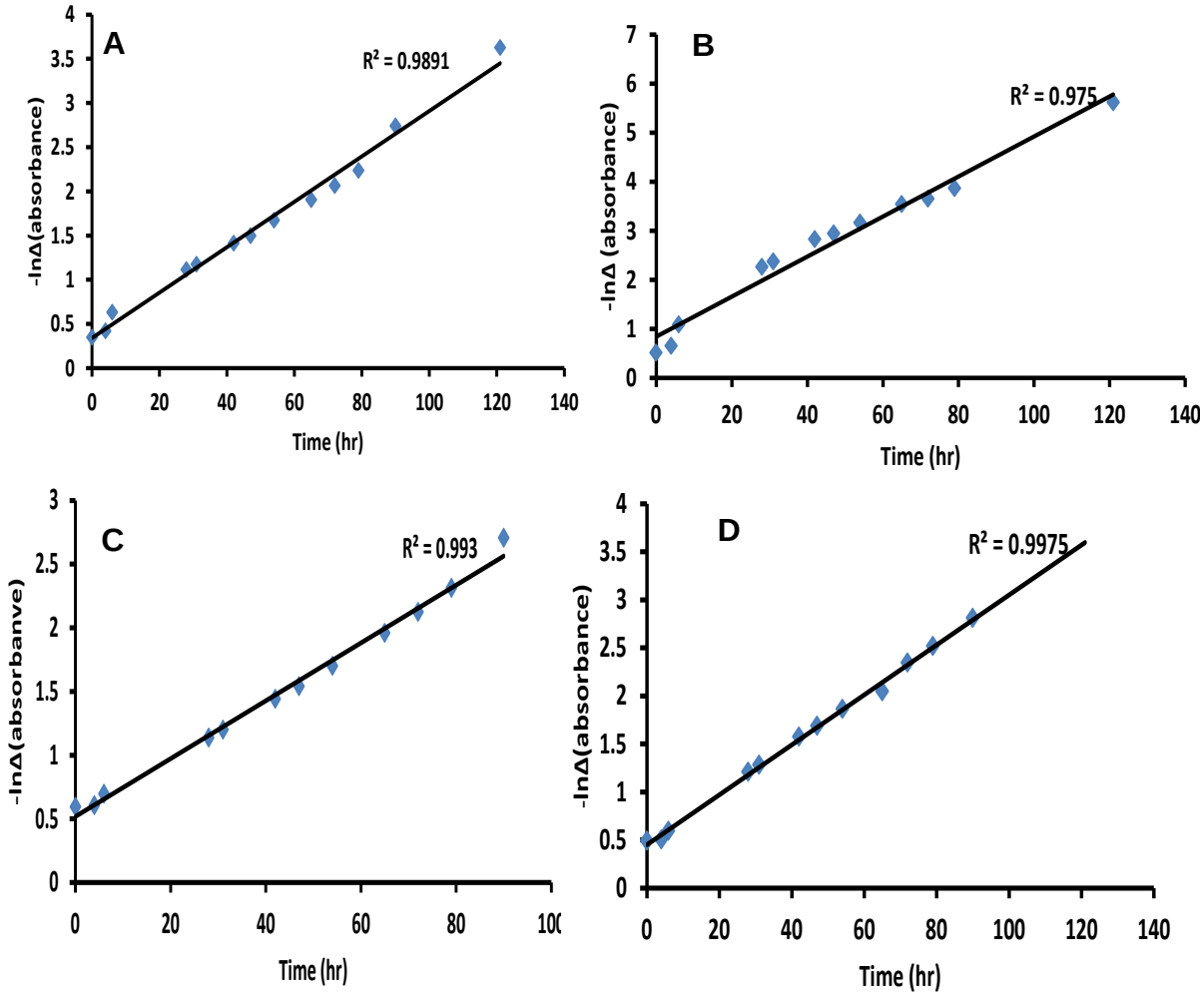


Figure 5-1. First order plot for some selected anthocyanins extracts during storage under light at 20^{o}C: A: Ace-VE-light; B: Ace-DA-light; C: Açai+48AA-light; D: Açai+97AA-light.

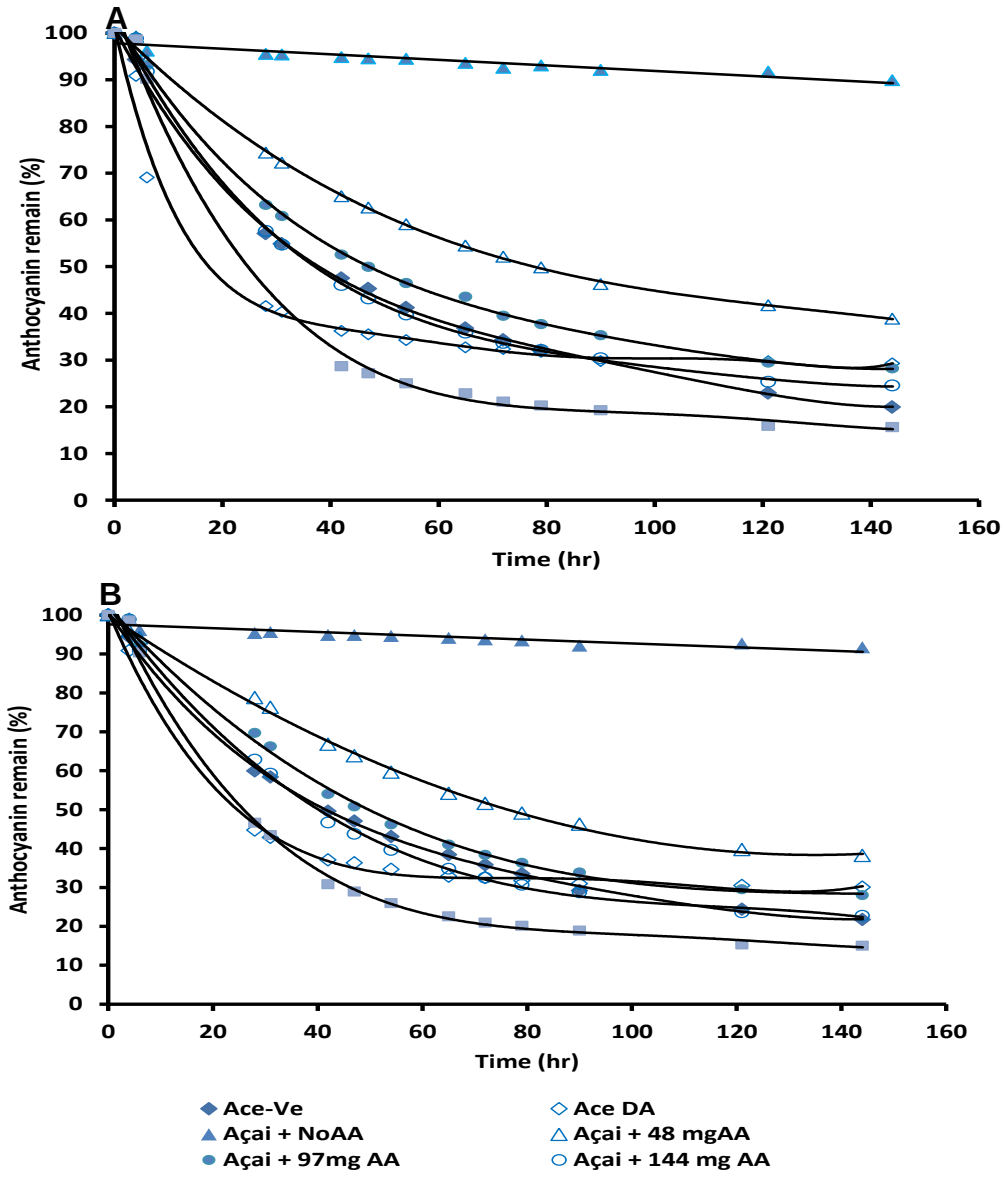


Figure 5-2. Degradation curves of anthocyanin from acerola fruit and açai spiked with ascorbic acid at different level and stored under light (A) and in darkness (B) at 20 ^{o}C; in citrate buffer pH 2.5. Ace-VE: acerola anthocyanins extract from fruits harvested in Vero Beach; Ace-DA: acerola anthocyanins extract from fruits harvested in Davie; Açai-NoAA: açai anthocyanins extract with no added ascorbic acid; Açai+48mgAA: açai anthocyanins extract enriched with 48 mg/100mL ascorbic acid; Açai+97mgAA: açai anthocyanins extract enriched with 97 mg/100 mL ascorbic acid.

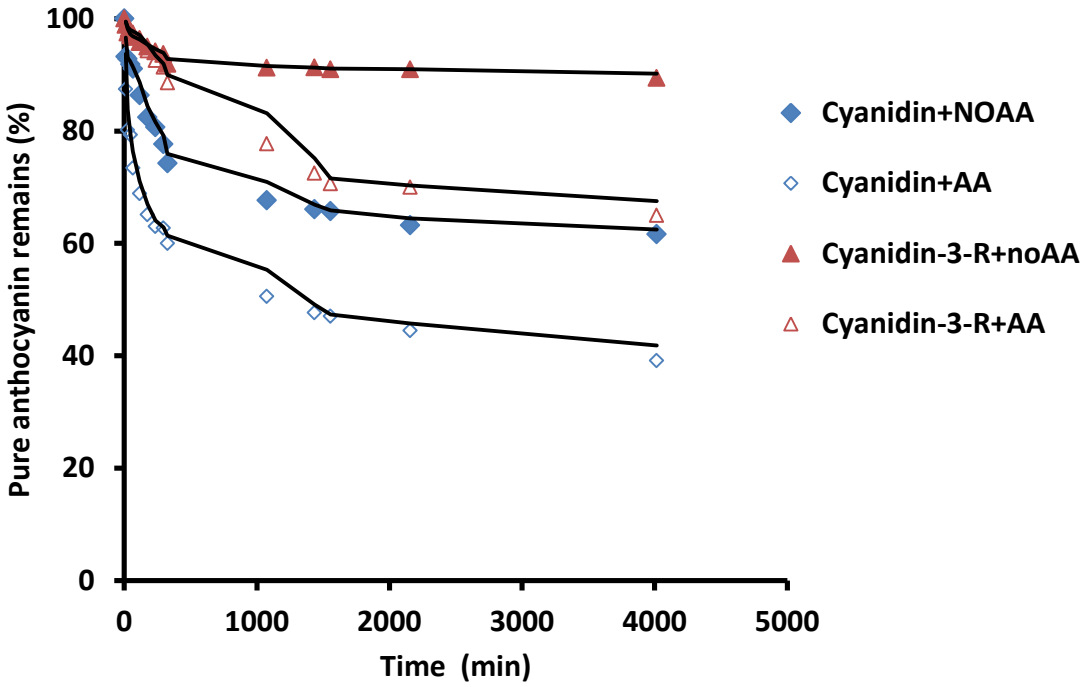


Figure 5-3. Degradation curves of anthocyanin from pure cyanidin and cyanidin-O-rhamnoside spiked with ascorbic acid and store in darkness in citrate buffer pH 2.5. Cyanidin+NoAA: Cyanidin with no added ascorbic acid; Cyanidin+AA: cyanidin enriched with ascorbic acid; Cyanidin-3-R+NoAA: cyanidin-3-rhamnoside with no added ascorbic acid; Cyanidin-3-R+AA: Cyanidin-3-rhamnoside enriched with ascorbic acid.

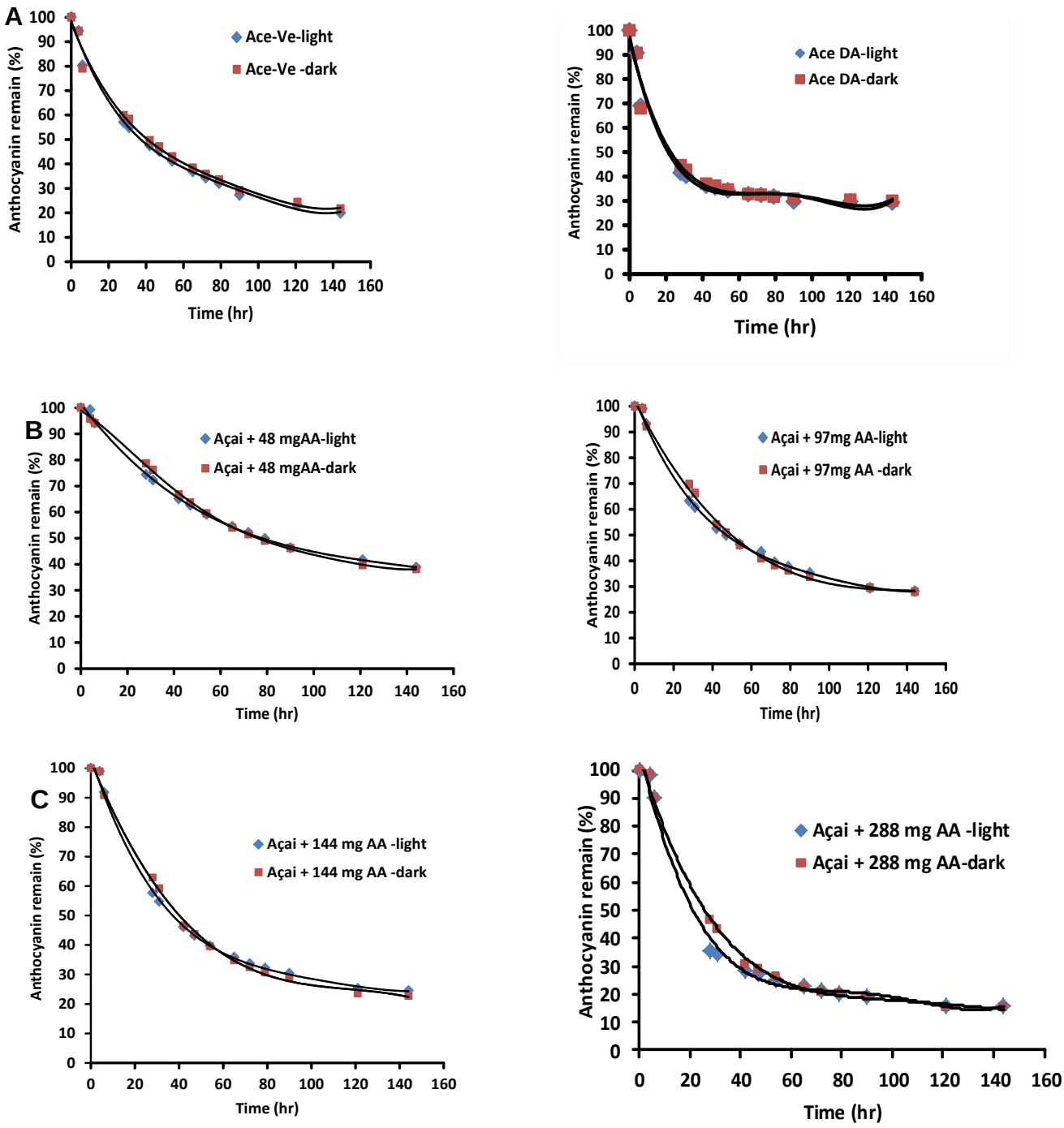


Figure 5-4. Behavior of the different systems stored in the presence or in the absence of light, Panel A: Ace-VE and Ace-DA, Panel B: Açai+48mgAA and Açai+97mgAA, Panel C: Açai+144mgAA and Açai+288mgAA.

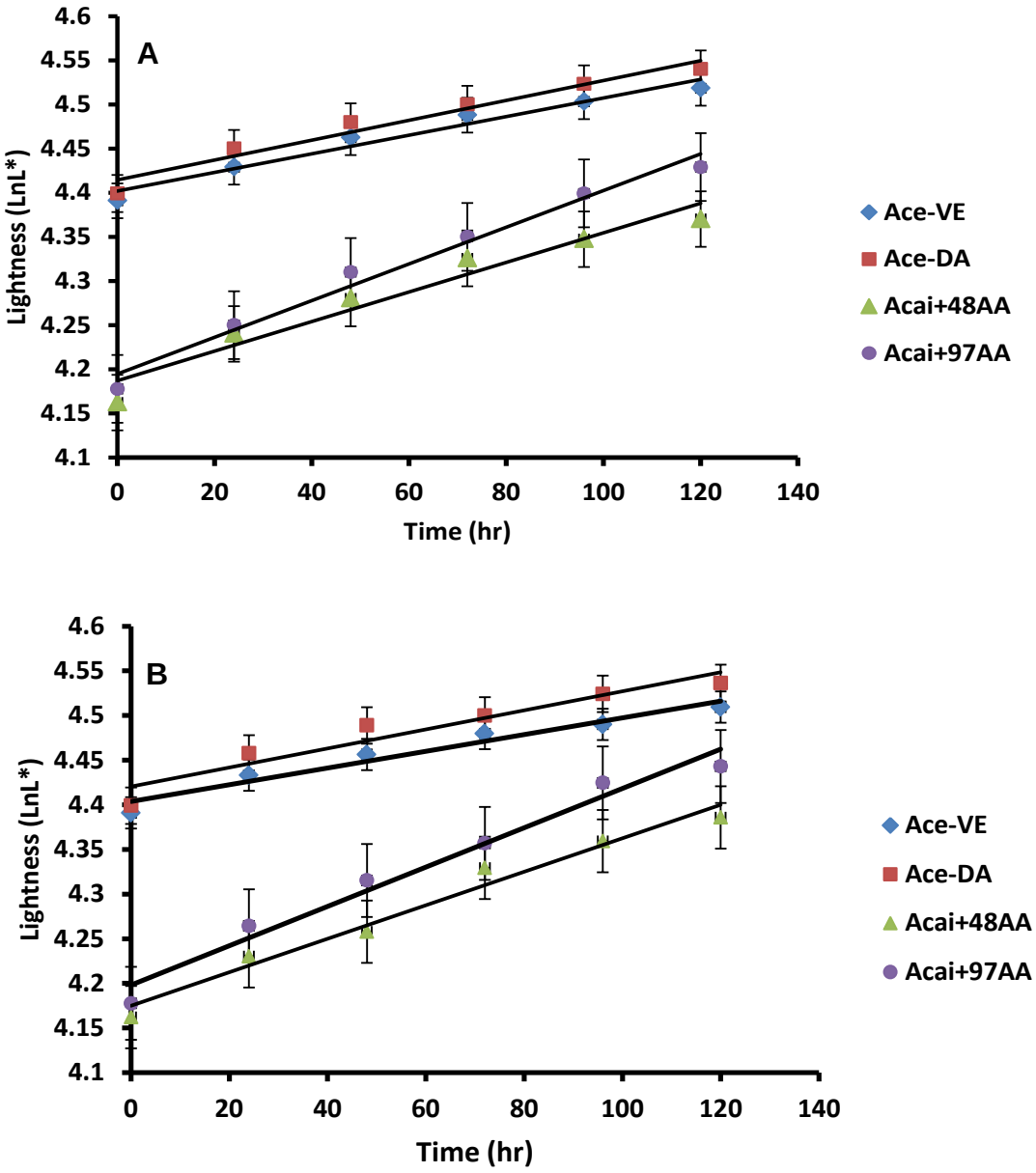


Figure 5-5. Evolution of the lightness (L*) value for acerola extract and the açai systems enriched with ascorbic acid of anthocyanin extracts in phosphate-citrate buffer, pH 2.5 stored under light at 20 ^{o}C (A), under dark at 20 ^{o}C (B). Ace-VE: acerola anthocyanins extract from fruits harvested in Vero Beach; Ace-DA: acerola anthocyanins extract from fruits harvested in Davie; Açai+48mgAA: açai anthocyanins extract enriched with 48 mg/100 mL ascorbic acid; Açai+97mgAA: açai anthocyanins extract enriched with 97 mg/100 mL ascorbic acid. Result presented as mean plus or minus standard deviation.

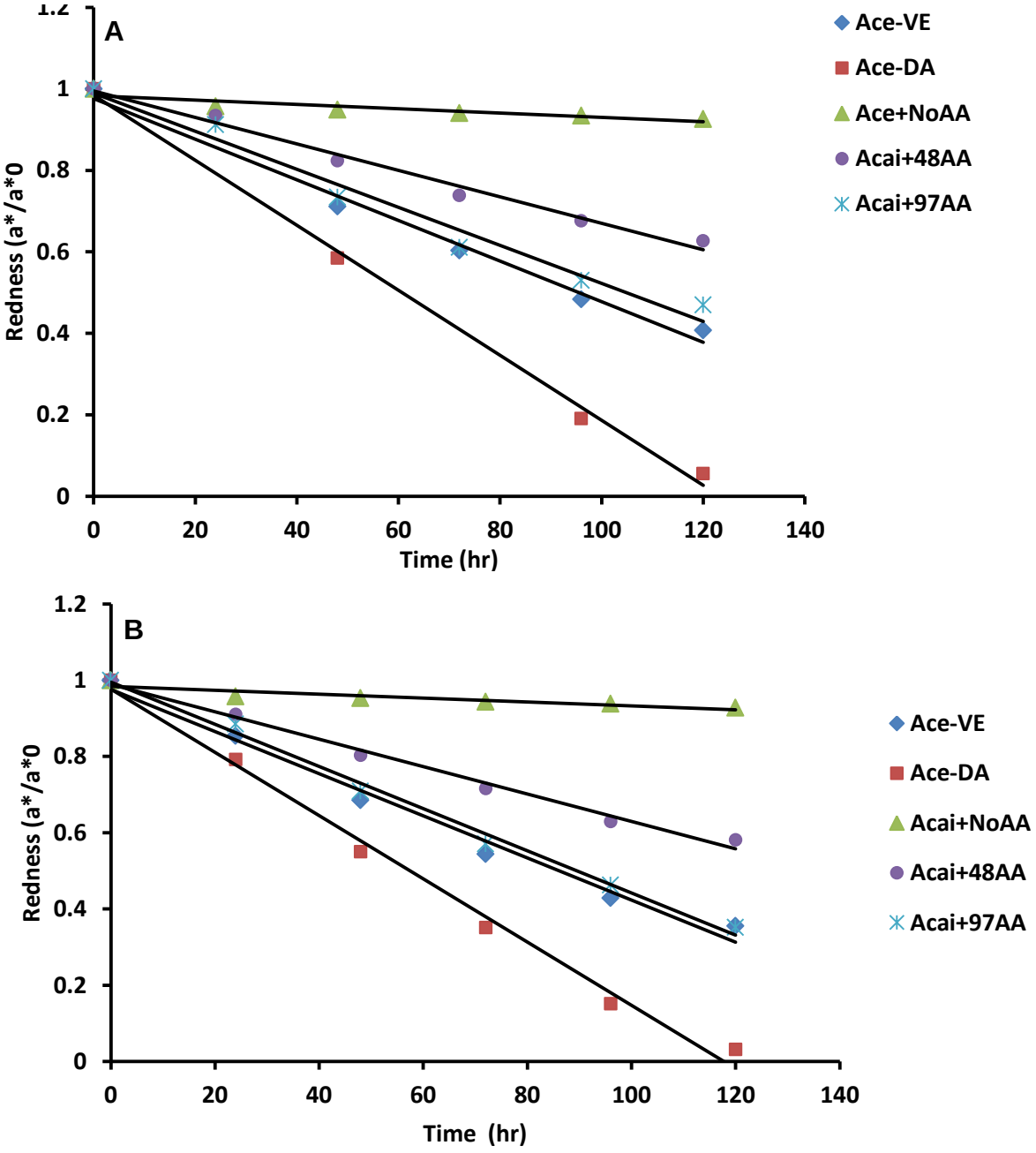


Figure 5-6. Changes in the color parameters a^*/a_0^* value for the anthocyanin extracts from acerola and the açai in phosphate buffer solutions at pH 2.5 in the presence (A) or the absence (B) of light. Ace-VE: acerola anthocyanins extract from fruits harvested in Vero Beach; Ace-DA: acerola anthocyanins extract from fruits harvested in Davie; Açai+48mgAA: açai anthocyanins extract enriched with 48 mg/100 mL ascorbic acid; Açai+97mgAA: açai anthocyanins extract enriched with 97mg/100 mL ascorbic acid. Result presented as mean plus or minus standard deviation.

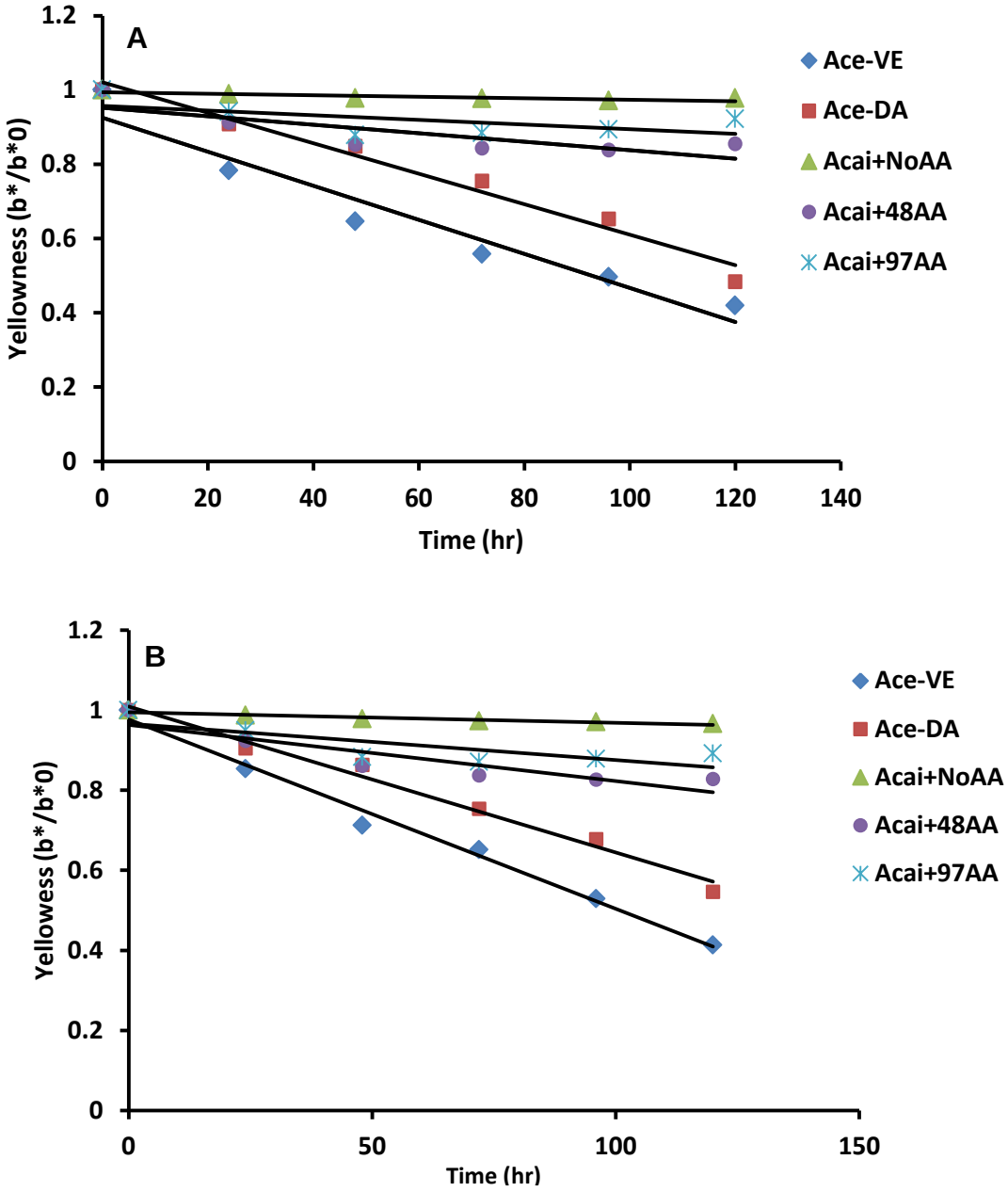


Figure 5-7. Changes in the color parameters b^*/b_0^* value for the anthocyanin extracts from acerola and the açai in phosphate buffer solutions at pH 2.5 in the presence (A) or the absence (B) of light. Ace-VE: acerola anthocyanins extract from fruits harvested in Vero Beach; Ace-DA: acerola anthocyanins extract from fruits harvested in Davie; Açai+48mgAA: açai anthocyanins extract enriched with 48 mg/100 mL ascorbic acid; Açai+97mgAA: açai anthocyanins extract enriched with 97 mg/100 mL ascorbic acid. Result presented as mean plus or minus standard deviation.

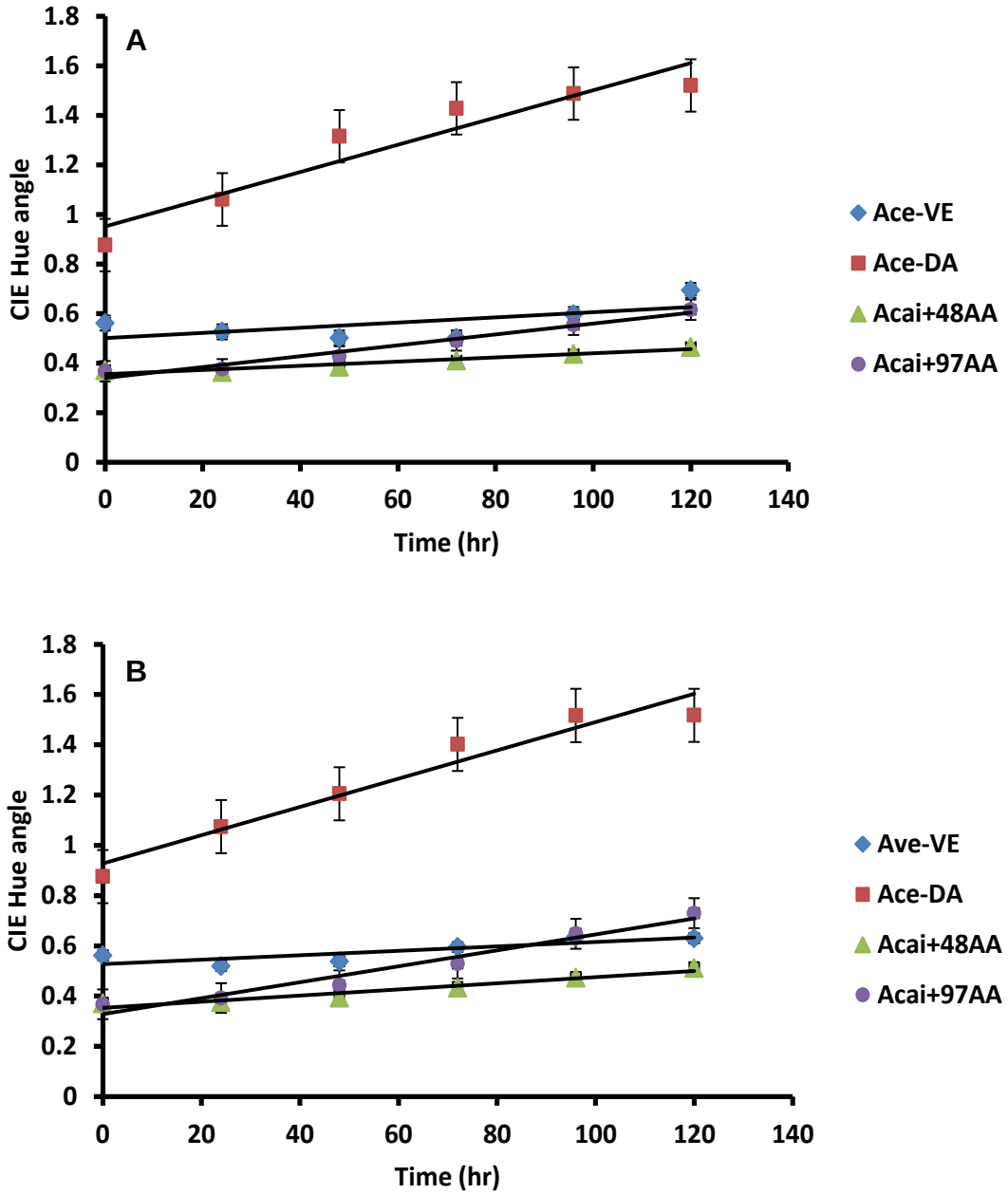


Figure 5-8. Evolution of the hue value for acerola and açai systems enriched with ascorbic acid of anthocyanin extracts in phosphate-citrate buffer, pH 2.5 stored under light at 20 ^{o}C (A), under dark at 20 ^{o}C (B). Ace-VE: acerola anthocyanins extract from fruits harvested in Vero Beach; Ace-DA: acerola anthocyanins extract from fruits harvested in Davie; Açai+48mgAA: açai anthocyanins extract enriched with 48 mg/100 mL ascorbic acid; Açai+97mgAA: açai anthocyanins extract enriched with 97 mg/100 mL ascorbic acid. Result presented as mean plus or minus standard deviation.

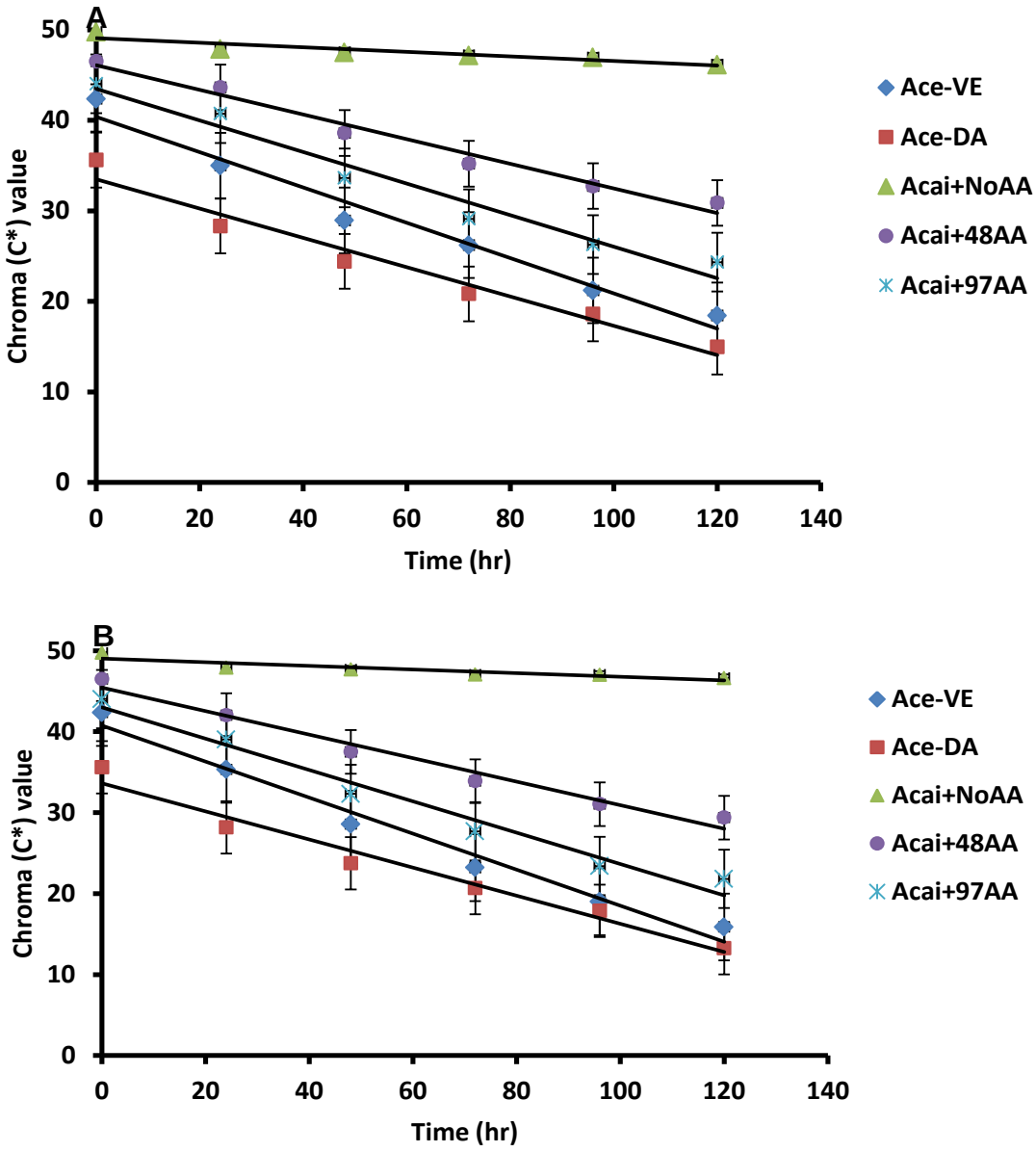


Figure 5-9. Evolution of the chroma (C*) value for acerola and açai systems enriched with ascorbic acid of anthocyanin extracts in phosphate-citrate buffer, pH 2.5 stored under light at 20 ^{o}C (A), under dark at 20 ^{o}C (B). Ace-VE: acerola anthocyanins extract from fruits harvested in Vero Beach; Ace-DA: acerola anthocyanins extract from fruits harvested in Davie; Açai+48mgAA: açai anthocyanins extract enriched with 48 mg/100 mL ascorbic acid; Açai+97mgAA: açai anthocyanins extract enriched with 97 mg/100 mL ascorbic acid. Result presented as mean plus or minus standard deviation.

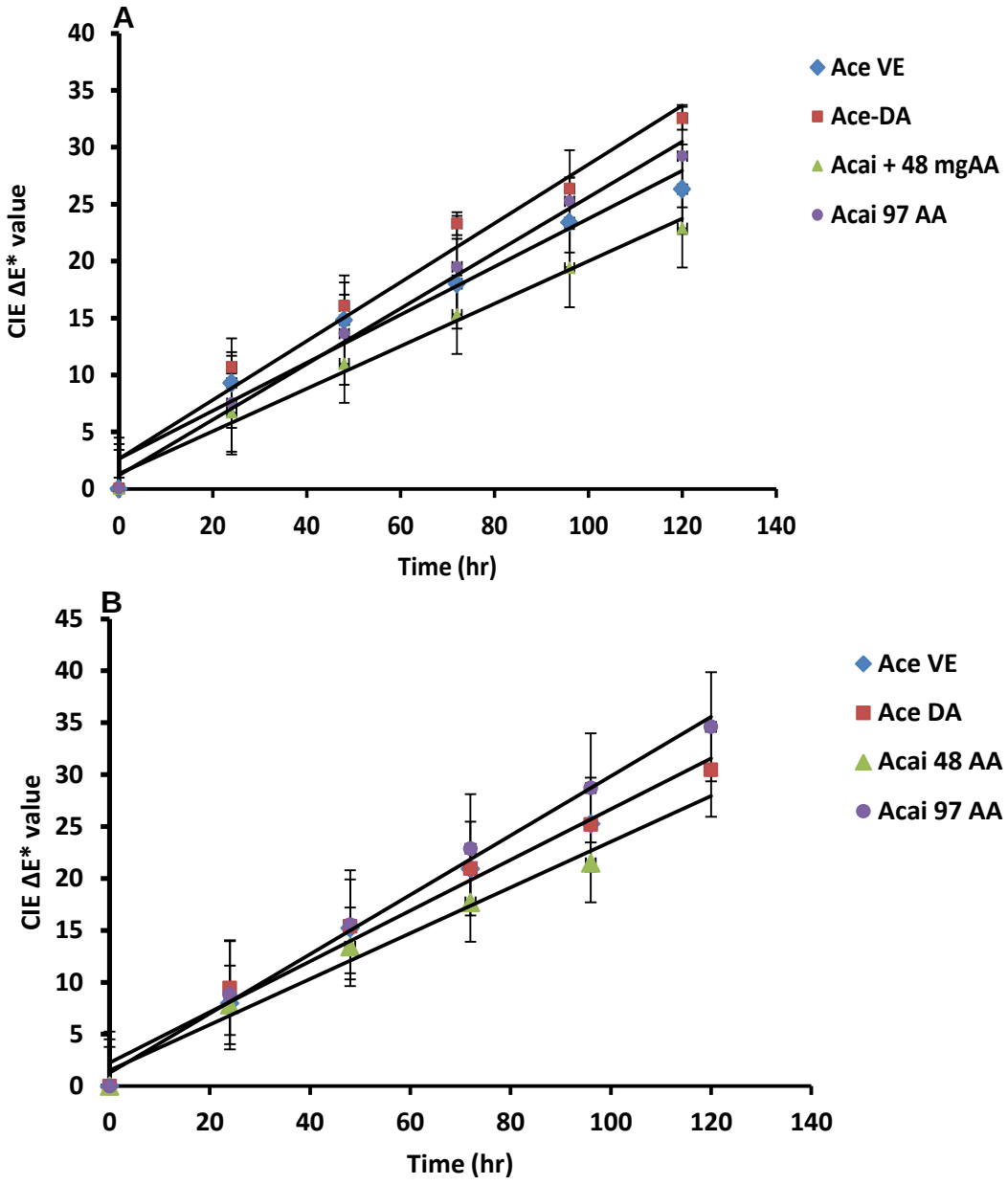


Figure 5-10. Evolution of the parameter color difference (ΔE*) value for acerola and açai systems enriched with ascorbic acid of anthocyanin extracts in phosphate-citrate buffer, pH 2.5 stored under light at 20 ^{o}C (A), under dark at 20 ^{o}C (B). Ace-VE: acerola anthocyanins extract from fruits harvested in Vero Beach; Ace-DA: acerola anthocyanins extract from fruits harvested in Davie; Açai+48mgAA: açai anthocyanins extract enriched with 48 mg/100 mL ascorbic acid; Açai+97mgAA: açai anthocyanins extract enriched with 97 mg/100 mL ascorbic acid. Result presented as mean plus or minus standard deviation.

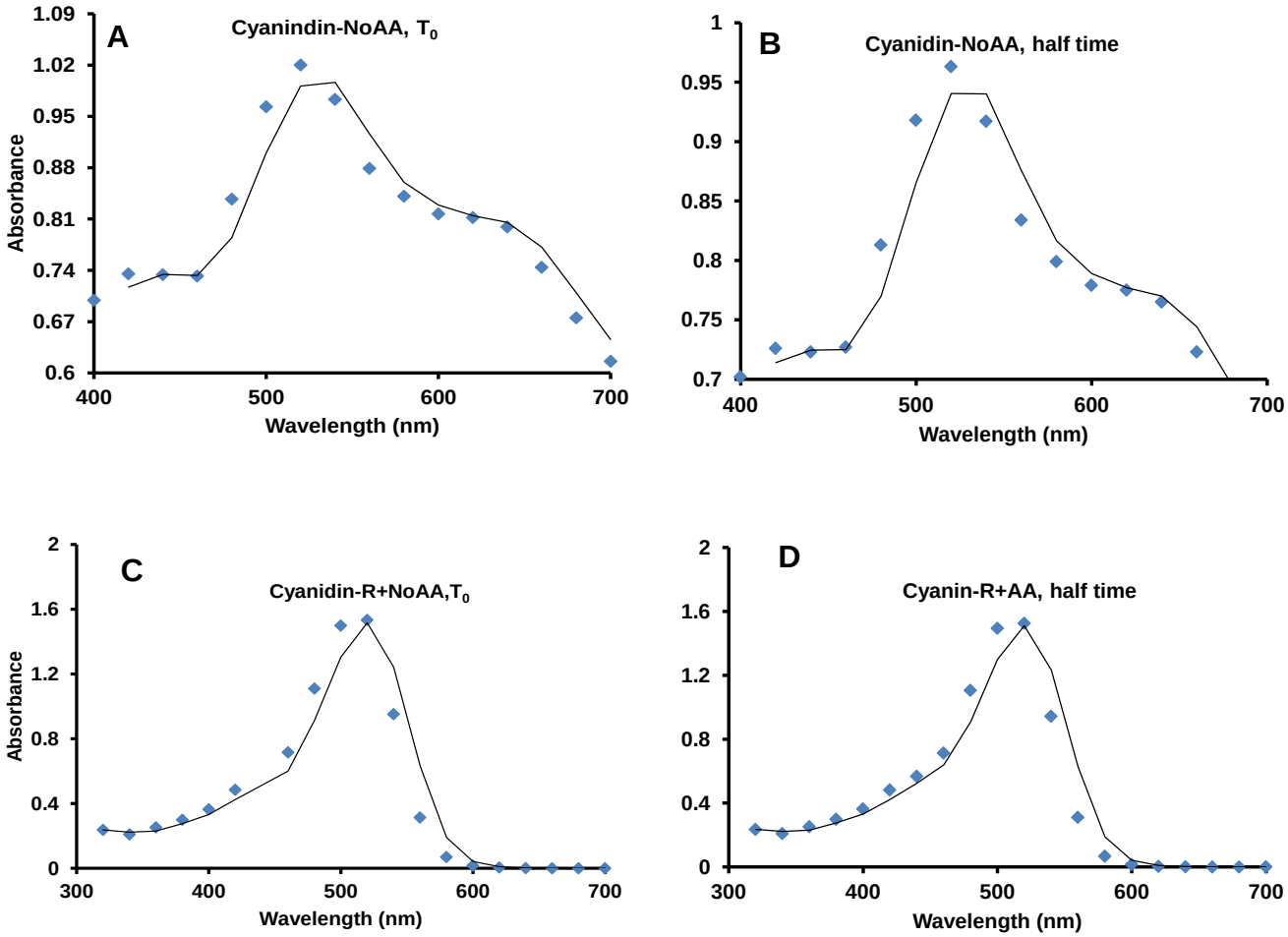


Figure 5-11. Spectrophotometric profile of selected samples in the pure anthocyanin solutions with added ascorbic acid at pH 2.5. A: cyanidin+NoAA, T_0; B: cyanidin+AA, halftime; C: cyanidin-3-O-R+NoAA, T_0; D: cyanidin-3-O-R+AA, half time. Cyanidin+NoAA: Cyanidin with no added ascorbic acid; Cyanidin+AA: cyaniding enriched with ascorbic acid; Cyanidin-3-R+NoAA: cyanidin-3-rhamnoside with no added ascorbic acid; Cyanidin-3-R+AA: Cyanidin-3-rhamnoside enriched with ascorbic acid.

CHAPTER 6
CONCLUSIONS

In this study, the phenolic profile, the antioxidant capacity, the antimicrobial property, the toxicological screening, and the color stability of acerola fruit were examined. The acerola fruits were from the variety ‘Florida Sweet’ grown in two geographic locations in Florida. Two types of anthocyanins: cyanidin-3-O-rhamnoside and pelargonidin-3-O-rhamnoside were identified. The non-anthocyanin phenolic compounds identified include various types of phenolic acids, and some neutral phenolic compounds such as: quercetin, quercerin-3-rhamnoside, (+)-- epicatechin, and resveratrol. Resveratrol, and (+)--epicatechin were reported for the first time in acerola fruit. The non-edible (seed) portion of the fruits showed exceptionally high total phenolic contents. Overall phenolic extracts at the concentration considered (25 mg/mL) showed limited antimicrobial properties against the microbial strains tested. However, selected flavonol extracts (especially from seeds) show antimicrobial effects against *S. aureus*. Using an AA-free açai anthocyanin model system (açai) it was indicated that AA was the main cause of anthocyanin degradation in acerola anthocyanin extracts. The mechanism of the degradation is still not completely understood, however results and observations suggest that direct condensation of AA on 4-position of flavylium cation of the anthocyanin molecule is unlikely.

Although some phenolic compounds including anthocyanins and non-anthocyanin phenolics were identified, chromatograms of the non-anthocyanin phenolic compounds contained many unidentified peaks. Therefeore, the use of new analytical and more informative analytical techniques such as nuclear magnetic resonance (NMR) could help to identify the unidentified compounds. Regarding the antimicrobial testing, the

results demonstrate limited antimicrobial properties at the concentration tested. The antimicrobial property was conducted on 6 mm diameter discs with limited sample retention capacity. It would be important for future research in this area to use disc having higher retention. In addition, the antimicrobial activity of the acerola phenolic extracts was conducted only on three strains of microorganisms; it would be important to test the effect of the acerola phenolic fractions on a much larger number of bacterial strains to gather more information that would help to better assess the antimicrobial potential of acerola phenolic extracts.

Regarding the effect of ascorbic acid on the anthocyanins and color loss of the acerola anthocyanin extracts the overall conclusion is that the degradation of anthocyanin is more likely to occur through a free radical mechanism rather than by direct condensation between ascorbic acid and the anthocyanin molecule. Given the co-existence of the two compounds in the same systems, the stabilization of anthocyanin in acerola fruit or its derived products would be a difficult task. Therefore until the mechanism is elucidated, it is important to store the anthocyanin extracts under conditions (packaging, temperature, etc.) that favor the stability of both anthocyanin and ascorbic acid. Another potential solution to improve the stability of anthocyanin in acerola extract would be the addition of polyphenolic compounds to anthocyanin solutions as copigment. This method has been reported to improve the stability of anthocyanin during storage in model and fruit juice systems (Brenes and others 2005; Talcott and others 2005). As the addition of pure phenolic compounds is not applicable in the food industry, the general method is the use of phenolic extracts from natural sources to stabilize anthocyanins. For instance, Pozo-Insfran and others (2007)

reported that the addition partially purified rosemary and thyme phenolic extracts as copigment increase Muscadine grape juice color, antioxidant capacity, and also reduced phytochemical losses during high hydrostatic pressure processing and storage. The application of natural polyphenolic extracts to stabilize the anthocyanin in acerola extracts and juices could be an interesting area of research where question like the practical commercial levels that have no effect on the flavor and other sensory attributes of acerola extracts or juices could be addressed.

APPENDIX A
HPLC-DAD CHROMATOGRAMS OF THE NON-ANTHOCYANIN PHENOLIC COMPOUNDS DETECTED ACEROLA FRUIT

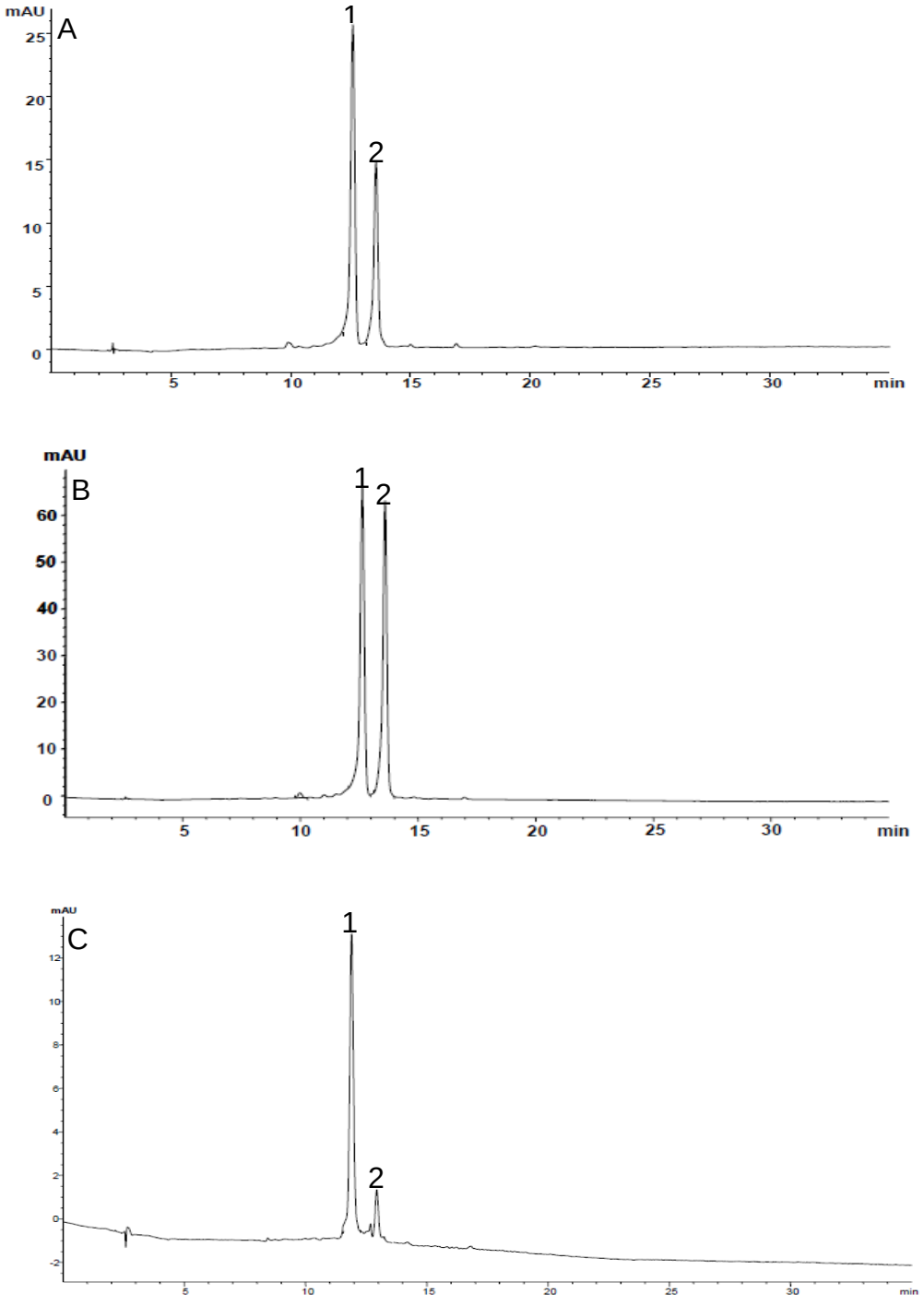


Figure A-1. HPLC-DDA chromatogram for partially purified acerola anthocyanin extracts Ace-DA (A), Ace-VE (B), and frozen single strength juice (FSSJ) (C) acerola juice at 520 nm. Peak identification is given in Table 3-3

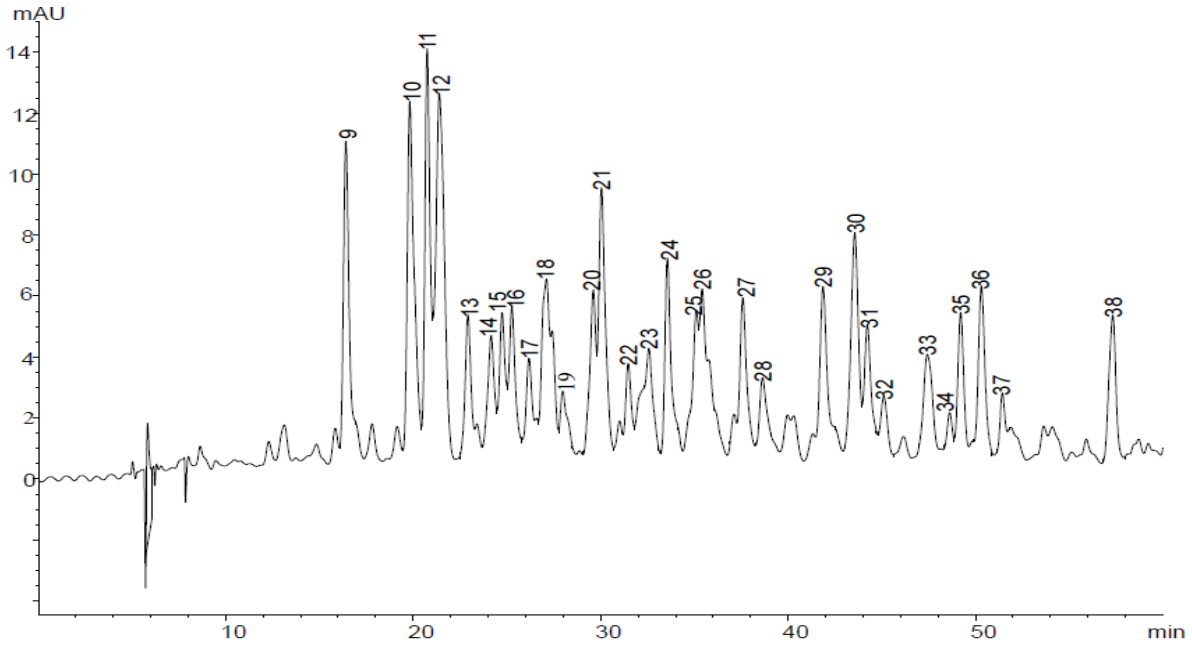


Figure A-2. Sample chromatogram of the acidic fraction of phenolic compounds detected in edible portion of acerola fruit, detection wavelength: 320 nm

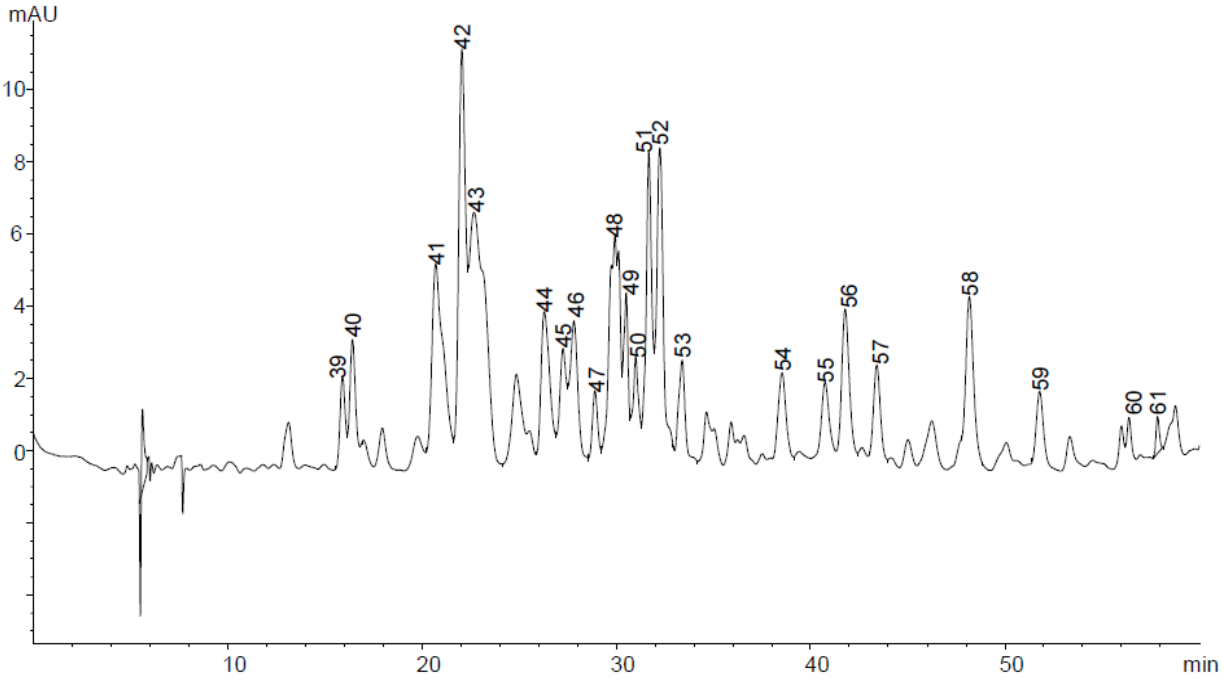


Figure A-3. Sample chromatogram of the neutral fraction of phenolic compounds detected in edible portion of acerola fruit, detection wavelength: 280 nm

APPENDIX B
STATISTICAL ANALYSIS OF THE COLOR AND SOFTNESS OF THE DATA COLLECTED AT THE THREE STAGES OF MATURITY

Table B-1. SAS software code used for the statistical analysis of peel color ($L^*a^*b^*$) and softness (H) parameters using the Duncan multiple range test

```
Data experiment;
Input maturity $ sample L* a* b* H @@;
Datalines;
1 1 50.09 -8.30 40.60 5.99
1 2 52.09 -8.31 40.59 6.03
1 3 54.09 -8.31 40.66 6.03
2 1 52.39 19.24 38.00 3.19
2 2 52.45 19.48 38.66 3.20
2 3 52.69 19.59 38.31 3.23
3 1 43.80 38.60 31.37 2.21
3 2 43.78 38.67 31.40 2.26
3 3 43.60 39.00 31.33 2.30
;
Proc glm;
Class maturity sample;
Model L* = maturity sample;
Means maturity/Duncan;
Proc glm;
Class maturity sample;
Model a*= maturity sample;
Means maturity/Duncan;
Proc glm;
Class maturity sample;
Model b* = maturity sample;
Means maturity/Duncan;
Proc glm;
Class maturity sample;
Model H = maturity sample;
Means maturity/Duncan;
Run;
```

Table B-2. SAS software output used for the statistical analysis of peel color (L, a, b) and softness (H) parameters using the Duncan multiple range test

The GLM Procedure
Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value
Model	4	150.0703778	37.5175944	28.46
Error	4	5.2729778	1.3182444	
Corrected Total	8	155.3433556		

Coeff Var	Root MSE	L Mean
2.322202	1.148148	49.44222

Source	DF	Type I SS	Mean Square	F Value	Pr > F
maturity	2	147.2686889	73.6343444	55.86	0.0012
sample	2	2.8016889	1.4008444	1.06	0.4264

Source	DF	Type III SS	Mean Square	F Value	Pr > F
maturity	2	147.2686889	73.6343444	55.86	0.0012
sample	2	2.8016889	1.4008444	1.06	0.4264

The GLM Procedure
Duncan's Multiple Range Test for L

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	1.318244

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	maturity
A	52.5100	3	2
A			
A	52.0900	3	1
B	43.7267	3	3

Table B-2. Continued.

The GLM Procedure
Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	3358.004644	839.501161	53264.1	<.0001
Error	4	0.063044	0.015761		
Corrected Total	8	3358.067689			

R-Square	Coeff Var	Root MSE	a Mean
0.999981	0.754971	0.125543	16.62889

Source	DF	Type I SS	Mean Square	F Value	Pr > F
maturity	2	3357.912289	1678.956144	106525	<.0001
sample	2	0.092356	0.046178	2.93	0.1646

Source	DF	Type III SS	Mean Square	F Value	Pr > F
maturity	2	3357.912289	1678.956144	106525	<.0001
sample	2	0.092356	0.046178	2.93	0.1646

The GLM Procedure
Duncan's Multiple Range Test for a

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha		0.05
Error Degrees of Freedom		4
Error Mean Square		0.015761
Number of Means	2	3
Critical Range	.2846	.2908

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	maturity
A	38.7567	3	3
B	19.4367	3	2
C	-8.3067	3	1

Table B-2. Continued.

The GLM Procedure
Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	139.2941778	34.8235444	952.04	<.0001
Error	4	0.1463111	0.0365778		
Corrected Total	8	139.4404889			

R-Square	Coeff Var	Root MSE	b Mean
0.998951	0.520149	0.191253	36.76889

Source	DF	Type I SS	Mean Square	F Value	Pr > F
maturity	2	139.2170889	69.6085444	1903.03	<.0001
sample	2	0.0770889	0.0385444	1.05	0.4289

Source	DF	Type III SS	Mean Square	F Value	Pr > F
maturity	2	139.2170889	69.6085444	1903.03	<.0001
sample	2	0.0770889	0.0385444	1.05	0.4289

The GLM Procedure
Duncan's Multiple Range Test for b

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha		0.05
Error Degrees of Freedom		4
Error Mean Square		0.036578
Number of Means	2	3
Critical Range	.4336	.4431

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	maturity
A	40.6167	3	1
B	38.3233	3	2
C	31.3667	3	3

Table B-2. Continued.

The GLM Procedure
Dependent Variable: H

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	22.94106667	5.73526667	20242.1	<.0001
Error	4	0.00113333	0.00028333		
Corrected Total	8	22.94220000			

R-Square	Coeff Var	Root MSE	H Mean
0.999951	0.439874	0.016833	3.826667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
maturity	2	22.93620000	11.46810000	40475.6	<.0001
sample	2	0.00486667	0.00243333	8.59	0.0357

Source	DF	Type III SS	Mean Square	F Value	Pr > F
maturity	2	22.93620000	11.46810000	40475.6	<.0001
sample	2	0.00486667	0.00243333	8.59	0.0357

The GLM Procedure
Duncan's Multiple Range Test for H

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05	
Error Degrees of Freedom	4	
Error Mean Square	0.000283	
Number of Means	2	3
Critical Range	.03816	.03899

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	maturity
A	6.01667	3	1
B	3.20667	3	2
C	2.25667	3	3

APPENDIX C
STATISTICAL ANALYSIS OF THE TOTAL ANTIOXIDANT AND VITAMIC DATA COLLECTED FOR THE FRUITS GROWN IN DAVIE, FLORIDA

Table C-1. SAS software code used for the statistical analysis of parameters (TPI, ORAC, DPPH, ORAC, vit. C) using the Duncan multiple range test

```
Data experiment;
Input extraction maturity $ sample TPI ORAC DPPH VitC @@;
Datalines;
1 1 1 9403 43.5 90 1745
1 1 2 9406 43.5 90 1743
1 1 3 9406 43.5 105 1744
1 2 1 7944 36.5 54.77 1049
1 2 2 7945 36.5 54.77 1049
1 2 3 7947 36.5 54.77 1049
1 3 1 8342 36.2 41.75 987
1 3 2 8340 36.2 39.75 980
1 3 3 8338 36.2 39.75 994
2 1 1 16280 48.0 134.0 1162.0
2 1 2 16280 48.0 138.0 1160.0
2 1 3 16295 48.0 136.0 1161.0
2 2 1 12917 39.7 106.0 966.0
2 2 2 12915 39.7 106.0 972.0
2 2 3 12913 39.7 106.0 972.0
2 3 1 11942 40.0 78.0 407.0
2 3 2 11942 40.0 71.0 405.0
2 3 3 11942 40.0 73.0 403.0
;
Proc glm;
Class extraction maturity sample;
Model TPI = extraction maturity sample;
Means extraction maturity/Duncan;
Proc glm;
Class extraction maturity sample;
Model ORAC = extraction maturity sample;
Means extraction maturity/Duncan;
Proc glm;
Class extraction maturity sample;
Model DPPH = extraction maturity sample;
Means extraction maturity/Duncan;
Proc glm;
Class extraction maturity sample;
Model VitC = extraction maturity sample;
Means extraction maturity/Duncan;
```

Table C-2. SAS software output used for the statistical analysis of parameters (TPI, ORAC, DPPH and Vit.C) using the Duncan multiple range test

The GLM Procedure
Dependent Variable: TPI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	145830326.9	29166065.4	43.04	<.0001
Error	12	8132742.7	677728.6		
Corrected Total	17	153963069.6			

R-Square	Coeff Var	Root MSE	TPI Mean
0.947177	7.390818	823.2427	11138.72

Source	DF	Type I SS	Mean Square	F Value	Pr > F
extraction	1	119377001.4	119377001.4	176.14	<.0001
maturity	2	26453306.8	13226653.4	19.52	0.0002
sample	2	18.8	9.4	0.00	1.0000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
extraction	1	119377001.4	119377001.4	176.14	<.0001
maturity	2	26453306.8	13226653.4	19.52	0.0002
sample	2	18.8	9.4	0.00	1.0000

The GLM Procedure
Duncan's Multiple Range Test for TPI

Alpha	0.05	
Error Degrees of Freedom	12	
Error Mean Square	677728.6	
Number of Means	2	3
Critical Range	1036	1084

Duncan Grouping	Mean	N	maturity
A	12845.0	6	1
B	10430.2	6	2
B	10141.0	6	3

Table C-2. Continued.

The GLM Procedure
Dependent Variable: ORAC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	300.2150000	60.0430000	567.34	<.0001
Error	12	1.2700000	0.1058333		
Corrected Total	17	301.4850000			

R-Square	Coeff Var	Root MSE	ORAC Mean
0.995788	0.800296	0.325320	40.65000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
extraction	1	66.1250000	66.1250000	624.80	<.0001
maturity	2	234.0900000	117.0450000	1105.94	<.0001
sample	2	0.0000000	0.0000000	0.00	1.0000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
extraction	1	66.1250000	66.1250000	624.80	<.0001
maturity	2	234.0900000	117.0450000	1105.94	<.0001

The GLM Procedure
Duncan's Multiple Range Test for ORAC

Alpha	0.05	
Error Degrees of Freedom	12	
Error Mean Square	0.105833	
Number of Means	2	3
Critical Range	.4092	.4283

Duncan Grouping	Mean	N	maturity
A	45.7500	6	1
B	38.1000	6	3
B	38.1000	6	2

Table C-2. Continued.

The GLM Procedure
Dependent Variable: DPPH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	18270.22088	3654.04418	108.87	<.0001
Error	12	402.75457	33.56288		
Corrected Total	17	18672.97544			

R-Square	Coeff Var	Root MSE	DPPH Mean
0.978431	6.867049	5.793348	84.36444

Source	DF	Type I SS	Mean Square	F Value	Pr > F
extraction	1	7914.49742	7914.49742	235.81	<.0001
maturity	2	10336.27901	5168.13951	153.98	<.0001
sample	2	19.44444	9.72222	0.29	0.7536

Source	DF	Type III SS	Mean Square	F Value	Pr > F
extraction	1	7914.49742	7914.49742	235.81	<.0001
maturity	2	10336.27901	5168.13951	153.98	<.0001
sample	2	19.44444	9.72222	0.29	0.7536

The GLM Procedure
Duncan's Multiple Range Test for DPPH

Alpha	0.05	
Error Degrees of Freedom	12	
Error Mean Square	33.56288	
Number of Means	2	3
Critical Range	7.287	7.628

Duncan Grouping	Mean	N	maturity
A	115.500	6	1
B	80.385	6	2
C	57.208	6	3

Table C-2. Continued.

The GLM Procedure
Dependent Variable: VitC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2507431.333	501486.267	23.73	<.0001
Error	12	253630.667	21135.889		
Corrected Total	17	2761062.000			

R-Square	Coeff Var	Root MSE	VitC Mean
0.908140	13.81082	145.3819	1052.667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
extraction	1	773768.000	773768.000	36.61	<.0001
maturity	2	1733647.000	866823.500	41.01	<.0001
sample	2	16.333	8.167	0.00	0.9996

Source	DF	Type III SS	Mean Square	F Value	Pr > F
extraction	1	773768.000	773768.000	36.61	<.0001
maturity	2	1733647.000	866823.500	41.01	<.0001
sample	2	16.333	8.167	0.00	0.9996

The GLM Procedure
Duncan's Multiple Range Test for VitC

Alpha		0.05
Error Degrees of Freedom		12
Error Mean Square		21135.89
Number of Means	2	3
Critical Range	182.9	191.4

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	maturity
A	1452.50	6	1
B	1009.50	6	2

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BIOGRAPHICAL SKETCH

Lemâne Delva received his bachelor's degree in 2000 from the College of Agriculture and Veterinary Medicine of the State University of Haiti, majoring in Animal Sciences. After obtaining his bachelor's degree, he worked as a teaching assistant at his home University for nearly one and a half year. In 2003, he was granted a scholarship form the International Cooperation and Development Fund (ICDF) and went to the National Pintung University of Science and Technology in Taiwan, where two years later he graduated with a master's degree in Food Science. He returned to Haiti in 2005 and started to work as research assistant and instructor at his home University; he kept that University position for two years. In summer 2007, he came to the United States as a Fulbright grantee to pursue a doctoral degree in Food Science at the Department of Food Science and Human Nutrition of the University of Florida.

Under the mentorship of Dr. Renée Goodrich Schneider, Lemâne received his Ph.D. in Food Science and Human Nutrition with a Minor in Agricultural Education and Communication in the fall of 2012.