FACTORS INFLUENCING THE STABILITY AND MARKETABILITY OF A NOVEL, PHYTOCHEMICAL-RICH OIL FROM THE AÇAI PALM FRUIT (*Euterpe oleracea* Mart.)

A Dissertation

by

CHRISTOPHER EDWARD DUNCAN

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

DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Food Science and Technology

Factors Influencing the Stability and Marketability of a Novel, Phytochemical-Rich Oil from the Açai Palm Fruit (*Euterpe oleracea* Mart.)

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ABSTRACT

Factors Influencing the Stability and Marketability of a Novel, Phytochemical-Rich Oil from the Açai Palm Fruit (*Euterpe oleracea* Mart.). (December 2010)

Christopher Edward Duncan, B.S., University of Florida

Chair of Advisory Committee: Dr. Stephen T. Talcott

The açai palm fruit has recently become the focus of numerous research endeavors due to its extraordinary antioxidant content. However, little is known about the fruit's phytochemical rich oil, which is a by-product of the açai pulp. Therefore, the aim of this study was to investigate the phytochemical content of açai oil and its relation to oxidative stability.

A total of 206 mg/kg of chlorophylls, which included chlorophyll a as well as four chlorophyll derivatives were tentatively identified by HPLC in crude açai oil (CAO). Two predominant carotenoids (216 mg/kg β-carotene and 177 mg/kg lutein) were also characterized in addition to α-tocopherol (645 mg/kg). Initial investigations into oil stability focused upon the photooxidation of açai oil due to its significant chlorophyll content and findings demonstrated that the increases of nonanal were observed when phospholipids and polyphenolics were reduced. Subsequently, investigations into the interactions between phospholipids and lipophilic antioxidants and their contributions to the stability of açai oil were also assessed by isolating phytochemicals and selectively reconstituting the oil. Findings demonstrated a potential

relationship between phospholipids and lipophilic antioxidants, but this was not conclusive.

Advances in the processing of the açai pulp by-product created from the clarification process yielded a partially refined açai oil (RAO). The stability of both RAO and CAO as a result of autoxidation were compared to other common food oils (olive, canola, and soybean) and primary and secondary oxidation data suggested CAO was most stable. The difference in the stability of the two açai oils was also investigated by blending these oils and assessing oxidation. An increased stability was demonstrated in the blended RAO, which suggested a significant antioxidant contribution from the CAO. With such similar lipophilic compositions in CAO and RAO, it is theorized that the driving factor behind the stability of CAO can be attributed to its water soluble antioxidant content.

While further investigations are required to fully comprehend the interactions of açai oil phytochemicals, these experiments provide insight into the phytochemical content and stability of açai oil. The understanding and information obtained in these studies is geared at increasing the marketability of açai oil as a food ingredient.

NOMENCLATURE

CAO	Crude Açai Oil
RAO	Partially Refined Açai Oil
HPLC	High Performance Liquid Chromatography
GC	Gas Chromatography
SPME	Solid-Phase Micro Extraction

DEDICATION

To all those who inspired and supported me throughout my life

ACKNOWLEDGEMENTS

I first would like to thank my advisor and mentor, Dr. Steve Talcott, for his inspiration and encouragement throughout the last decade. His insight, guidance, and support have helped mold me into the person and scientist that I am today. I also would like to acknowledge my committee members, Dr. Matt Taylor, Dr. Tommy McDonald, and Dr. Mian Riaz, for their guidance and support during my time here at Texas A&M. I am very grateful to have had the opportunity to work with you all.

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My passion for science began at an in early age and I attribute that in large part to my high school science teacher and mentor, Mr. Craig Gates. Thank you for constantly pushing me to achieve more. I owe my deepest gratitude to my parents, Chuck and Carmela, and my brother, Nick. Thank you for continually encouraging me to achieve my goals and always supporting me no matter where the winds of change may carry me. I could not have asked for a better family.

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1. INTRODUCTION

One of the most economically important crops of the highly fruitful Amazon Rain Forest is the açai palm, *Euterpe oleracea* Mart. Açai has gained popularity over the recent decade due to its unique chemical composition and purported health benefits (Córdova-Fraga and others 2004; Del Pozo-Insfran and others 2006; Galotta and others 2008; Hassimotto and others 2005; Lichtenthäler and others 2005; Mertens-Talcott and others 2007; Pacheco-Palencia and others 2008b; Rocha and others 2007). It can be found in a myriad of food products ranging from beverages to jams and jellies to cereal bars. It is often sold as a food ingredient in a variety of forms including pulp, clarified juice, and freeze dried powders. A large portion of current açai research has been devoted to the pulp and juice obtained from the açai palm fruit. Yet, it is often overlooked that the pulp has significant (6-11%) lipid content (Anderson 1988; Silva 1996). In many commercial applications, however, these lipids are removed in the juice clarification process and subsequently discarded as waste. Thus, there is potential to create a value added product, but a comprehension of açai oil and its chemistry must first be gained.

Recently, the advent of a patent-pending extraction protocol has led to the creation of a high polyphenolic, crude açai oil derived from the açai pulp processing waste stream (Talcott 2008). Açai oil is commonly used in cosmetic applications, yet its use in the food industry has not yet reached its full potential. The appearance of açai oil

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This dissertation follows the style of the Journal of Food Science.

is unlike any other food grade oil due to its dark green hue and viscosity. A number of early investigations focused on the basic chemical characteristics of the oil found in açai pulp (Lubrano and others 1994; Muñiz-Miret and others 1996; Rogez 2000), but little is known about the phytochemical content and stability of the oil.

Açai oil is unique in many aspects, most noticeably in its dark green color due to its high content of chlorophyll, which is a known photosensitizer. The presence of such a high content of chlorophyll could quickly lead to quality losses due to photooxidation. Numerous studies have detailed the photooxidation of oils that contain chlorophyll, such as olive oil (Kiritsakis and Dugan 1985; Fakourelis and others 1987; Gutiérrez-Rosales and others 1992); however, a typical olive oil contains approximately up to 15 mg/kg of total chlorophylls (Minguez-Mosquera and others 1990a), while the chlorophyll content of açai oil is thought to be significantly higher (206 mg/kg). Given the extraordinary content of this photosensitizer, it is critical to gain an understanding of the behavior of açai oil when exposed to light.

In addition to photooxidation, autoxidation is a major concern of any lipid matrix. Autoxidation does not rely upon the presence of a sensitizer and is often fueled by temperature abuse. Much like olive oil (534 mg/kg) (Satue and others 1995), açai oil also contains a myriad of water soluble polyphenolics (approximately 250 to 2,000 mg/kg). While the thermal stability of açai oil polyphenolics has been briefly studied (Pacheco-Palencia and others 2008), no investigations into the oxidative stability of the oil have been conducted. Moreover, the contributions of these phytochemicals to the stability of the oil are not completely understood. The lipophilic antioxidant profile of

açai oil is also not fully elucidated, which could contribute to the stability of the oil. Thus, it is imperative to examine the behavior of açai oil under oxidative conditions in order to understand the contributions of the açai oil phytochemicals to the oil's stability.

Gaining an understanding of açai oil, particularly its chemical properties and oxidative stability will provide insight into its potential uses in the food industry. The aim of this dissertation is to investigate the chemical properties behind this novel fruit oil obtained as a co-product of the açai juice clarification process. The information obtained in this research will provide avenues for the oil to be marketed uniquely as a superior cosmetic and supplemental product. In the end, a value added product can be obtained from a waste stream that is otherwise discarded thereby promoting the açai industry as a whole. Furthermore, investigating how this phytochemically rich oil behaves under oxidative conditions could lead to a better understanding of phytochemical interactions in unrefined oils.

The specific objectives of this dissertation are:

Objective I. To determine the stability of açai oil when subjected to environmental conditions that promote photooxidation

Objective II. To determine the autoxidative stability of açai oils in comparison to other common food grade oils

Objective III. To determine the role of natural phospholipids and lipophilic antioxidants on the stability of açai oil

Objective IV. To understand the reasons behind the difference in stability of two açai oils that vary in phytochemical content

2. LITERATURE REVIEW

2.1 Açai

The açai palm (*Euterpe oleracea* Martius) is native to regions of South America, particularly Brazil. Within the country of Brazil, the açai palm is commercially grown in the states of Pará, Amazonas, Tocantins, Maranhão, and Amapá (Strudwick and Sobel 1988) with the Pará state yielding an annual production of approximately 130,000 tons of fruit (Vera de Rosso and others 2008). The main source of heart of palm in Brazil is from the açai palm accounting for a domestic and export value of $300 million annually (Pollack and others 1995). The açai palm is a monoecious palm that can range from 4 to 30 m in height and grows natively in the estuaries of the Amazon (Muñiz-Miret and others 1996; Strudwick and Sobel 1988). The tree is multi-stemmed, sometimes having up to 45 slender trunks in various stages of growth and fructification (Lichtenthaler 2004). The fruit of the açai palm are globose in shape (1.5 cm in diameter) and turn from green to purple with maturity, producing a dark purple color at full maturity (Strudwick and Sobel 1988). The palm trees are harvested from July to December and generally yield up to 4 bunches containing 3 to 6 kg of fruit each (Del Pozo-Insfran and others 2006).

Açai fruit is a staple food for people native to this region of Brazil and is not only consumed as a food, but for its medicinal purposes as well (Brondízio and others 2002). Rural medicinal uses of açai include the use of its sap as a haemostatic and its oils as a anti-diarrheal agent (Plotkin and Balick 1984). However, the fruit is more commonly

made into a thick beverage for regular consumption. Macerating the berries in water results in a thick porous puree (Strudwick and Sobel 1988), which is consumed nearly on a daily basis by people indigenous to the Amazon region, particularly in the poorer regions (Anderson 1988). This beverage is often referred to as “vinho de açai” (açai wine), even though it is not fermented (Galotta and others 2008). Depending upon the creaminess and thickness of the resultant beverage, açai is often divided amongst three classifications (in descending order of thickness): açai grosso, açai medio, and açai fino (Lichtenthaler 2004).

2.1.1 Açai Oil

A significant portion of the açai fruit is the seed (~80%) (Rogez 2000), which is inedible; however, beyond that is a thin fibrous layer covered by a layer of deep purple pulp (Del Pozo-Insfran and others 2006; Strudwick and Sobel 1988). The outer layer of pulp beyond the fibrous material contains a significant amount of oil. Studies have reported the lipid content of açai pulp to range from 5.9% (Silva 1996) to 11.0% (Anderson 1988). Previous studies have reported the fatty acid composition of açai oil to be 60% oleic acid, 22% palmitic acid, 12% linoleic acid, and 6% of palmitoleic and stearic acids in addition to other fatty acids in trace amounts, as well as sterols including β-sitosterol, stigmasterol, δ5-avenasterol, campesterol, and cholesterol (Lubrano and others 1994). Numerous polyphenolics were also identified in the oil including vanillic acid (1,616 mg/kg), syringic acid (1,073 mg/kg), *p*-hydroxybenzoic acid (892 mg/kg), protocatechuic acid (630 mg/kg), ferulic acid (101 mg/kg), and (+)-catechin (66.7

mg/kg) (Pacheco-Palencia and others 2008). Procyanidin oligomers (3,120 mg/kg) have also been quantified in the oil (Pacheco-Palencia and others 2008). The stability of these compounds in açai oil were also studied and it was determined that phenolic acids degraded up to 16% after 10 weeks of storage at 20 or 30 °C and up to 33% loss at 40 °C (Pacheco-Palencia and others 2008). Procyanidin oligomers were more susceptible to degradation during the heat treatments displaying losses of 23% at 20 °C, 39% at 30 °C, and 74% at 40 °C (Pacheco-Palencia and others 2008). The antioxidant capacity (21.5 μmol Trolox equivalents/g) of water soluble extracts from açai oil has also been previously assessed (Pacheco-Palencia and others 2008).

While açai pulp and juice has come to the forefront of many research investigations, little research has been conducted on the oil itself. Açai pulp is often regarded for its antioxidant content and purported health benefits (Brondízio and others 2002; Del Pozo-Insfran and others 2004; Gallori and others 2004; Lichtenthäler and others 2005; Pacheco-Palencia 2006; Pacheco-Palencia and others 2007a), yet it is unknown if açai oil is also phytochemically rich. Furthermore, the major concern of any lipid system is its susceptibility to oxidation, which is directly influenced by its antioxidant content. Investigations into the phytochemical content and oxidative stability of açai oil are required to fully understand its uses in the food industry and improve the marketability of this novel tropical fruit oil.

2.2 Lipid Oxidation in Oils

During the storage and processing of edible oils, oxidation can occur through two different chemical mechanisms, autoxidation and photosensitized oxidation (Choe and Min 2006). Autoxidation occurs through three well-studied steps: initiation, propagation, and termination. Initiation of oxidation occurs when a free radical is produced through a number of different reactions, which may include thermal disassociation, peroxide decomposition, or metal catalysis (Frankel 1980). Propagation is a process by which free radicals oxidize other fatty acids to create another free radical (McClements and Decker 2008). Figure 2-1 depicts typical initiation and propagation cycles of autoxidation.

In addition to autoxidation, photooxidation of oils can occur with exposure to light. While both photooxidation and autoxidation can both lead to the deterioration of lipids and the creation of off-odors, the mechanisms behind these reactions are slightly different. Photooxidation is not a free radical mediated reaction, but rather it is driven by the creation of singlet oxygen, a reactive oxygen species (ROS). Singlet oxygen is a non-radical, electrophilic molecule requiring very little activation energy (22.5 kcal/mol above ground state) to react with a myriad of non-radical, electron–rich compounds (Min and Boff 2002). Figure 2-2 diagrams the two mechanisms (Type I and II) that can lead to photosensitized oxidation. The Type I mechanism occurs when a sensitizer reacts directly with another compound such as a fatty acid to initiate a free radical chain reaction (Foote and Denny 1968). The Type II pathway involves the conversion of triplet oxygen to singlet oxygen by way of energy transfer from the sensitizer (Sharman and

others 2000). Yet, despite the differences in mechanism between these pathways, the end results are the same (peroxide formation).

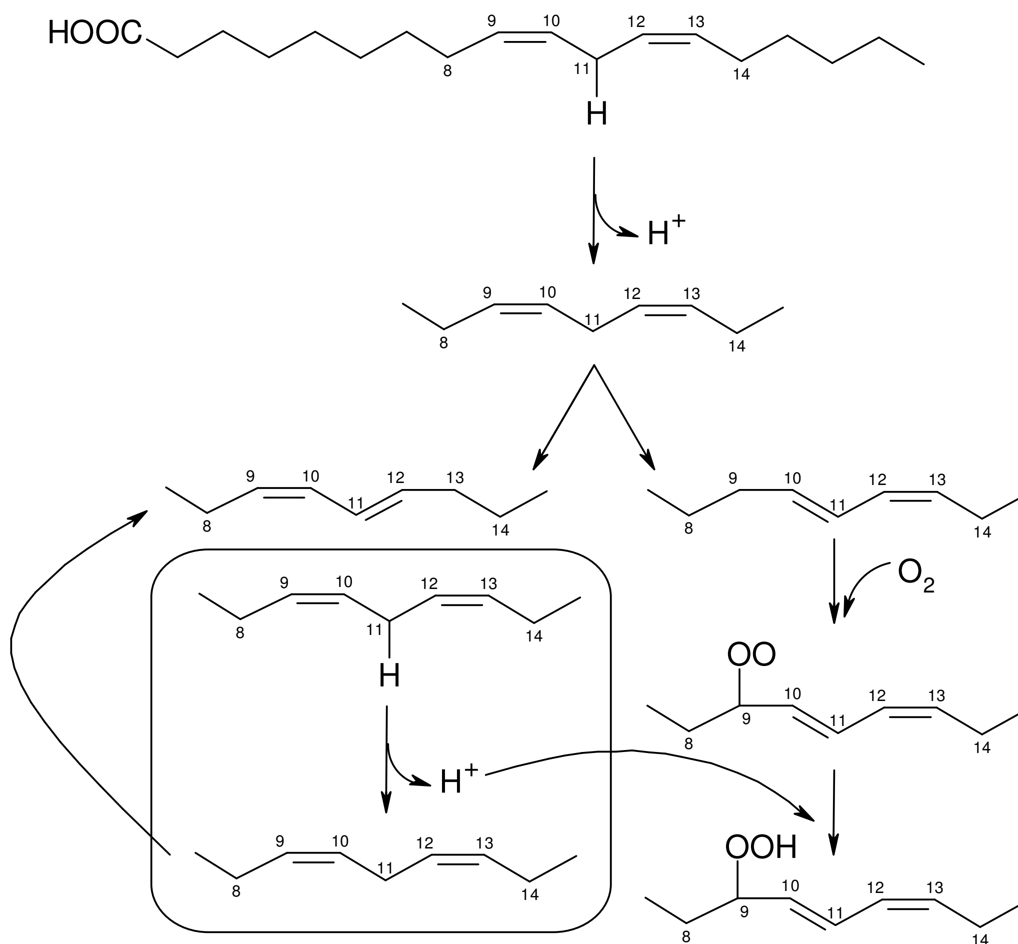


Figure 2-1. General schematic of lipid oxidation (initiation and propagation steps) of linoleic acid (Frankel 1984).

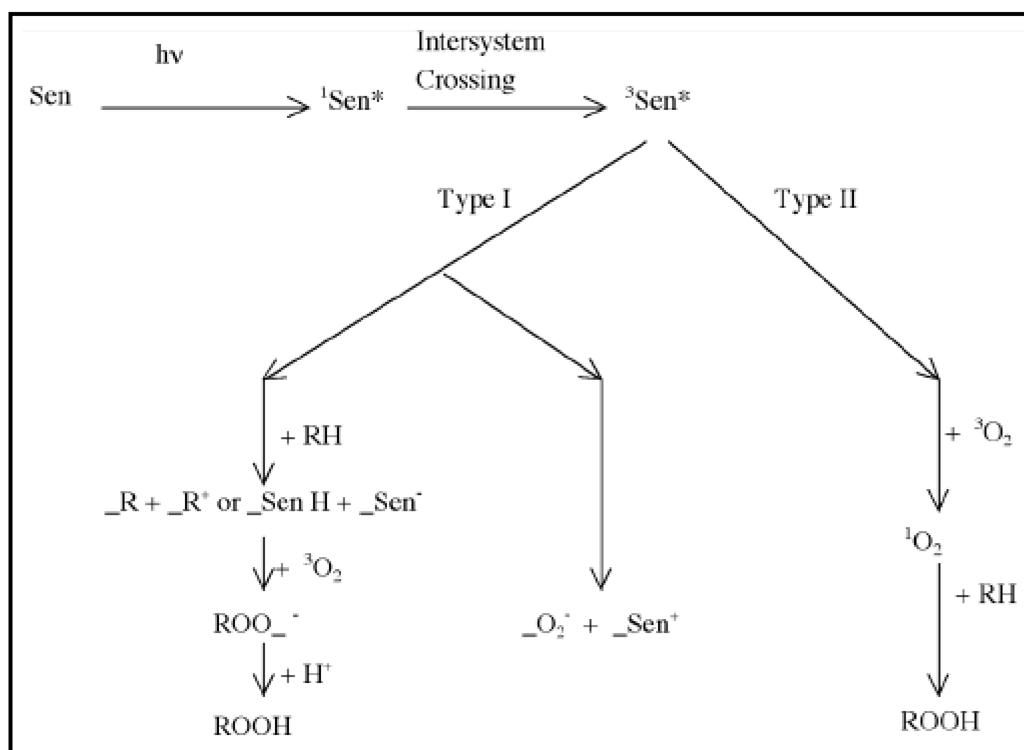


Figure 2-2. Mechanism by which the excited triplet sensitizer (^{3}Sen*) is formed and the two corresponding pathways that lead to peroxyl radical formation (Sharman and others 2000).

Once singlet oxygen has been formed it will only have a lifespan of approximately 50 to 70 µs, depending on the food matrix (Min and Boff 2002). Even though both autoxidation and photooxidation can occur by means of ROS, the mechanism behind the electrophilic attack of singlet oxygen on lipids is different than that of other ROS. In autoxidation, ROS will attack adjacent to the double bond in the lipid molecule. However, in singlet oxygen driven oxidation, the attack will occur

directly at the double bond forming a lipoperoxide. Figure 2-3 diagrams the electrophilic attack of singlet oxygen on linoleic acid.

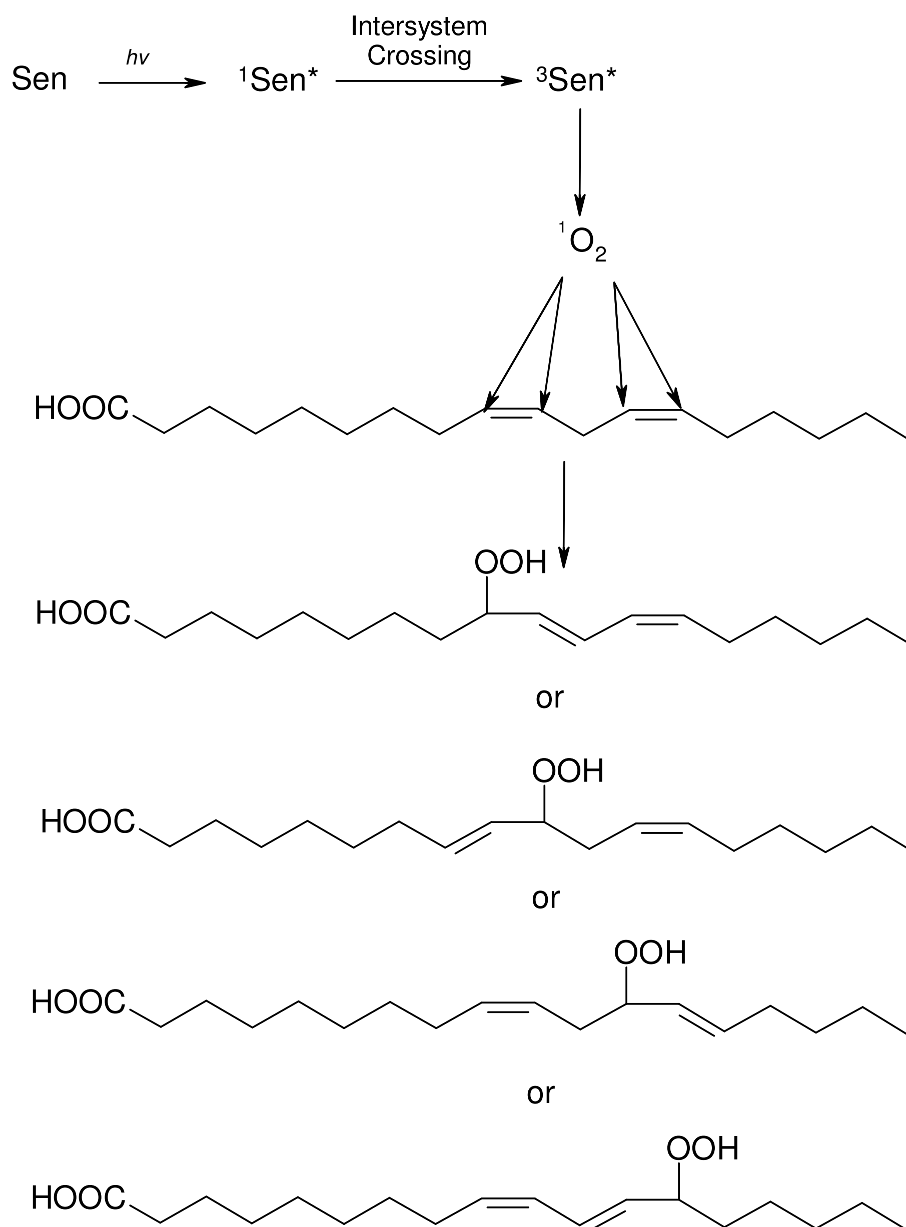


Figure 2-3. Possible outcomes of a nucleophilic attack of singlet oxygen on linoleic acid (Damodaran and others 2008).

Once primary lipid oxidation products (i.e. hydroperoxides) are formed, they can react once more with oxygen to form a myriad of secondary products such as epoxyhydroperoxides, ketohydroperoxides, dihydroperoxides, cyclic peroxides, and bicyclic endoperoxides (Frankel 1984). Numerous studies have investigated the pathways that lead to the creation of secondary oxidation products (Frankel 1984; Frankel and others 1982; Frankel and Neff 1983). Secondary oxidation products are commonly associated with off-odors and off-flavors that are responsible for quality defects in many lipid containing products. Thus, understanding the factors that affect the production of these oxidation products is crucial to maintaining quality of lipids.

2.2.1 Oxidation of Soybean Oil

Due to the economic importance and prominence of soybean oil processing in the United States, a plethora of research has been conducted on its oxidation and factors that both prevent and promote oxidation. Of particular interest is the effects of oil refining, bleaching, and deodorizing (RBD). Kwon and others (1984) demonstrated that all processing steps during the processing of crude soybean oil were detrimental to oxidative stability. These findings were also supported by Jung and others (1989a) and Yoon and Kim (1994). Sherwin (1978) reported that during the refining process many of the antioxidant compounds, including tocopherols, are removed from the oil.

Generally speaking, extracted oils are often subjected to degumming, alkali refining, bleaching, and deodorizing processes post-extraction. All of these processes have an effect on the oxidative stability of the oil. Of the aforementioned processes,

deodorizing is the least detrimental to oxidative stability of soybean oil followed by degumming, refining, and bleaching (Jung and others 1989a; Going 1968). However, a different trend was observed in the oxidation of rice bran oil where degummed oil was the most stable treatment followed by bleached and deodorized (no significant difference between the two treatments), and then alkali-refined oil (Yoon and Kim 1994).

2.2.2 Oxidation of Olive Oil

Numerous studies exist pertaining to the oxidative stability of olive oil, which is perhaps one of the most studied food grade oils (Bouaziz and others 2008; Calligaris and others 2006; Essid and others 2006; Fakourelis and others 1987; García and others 2006; Gutiérrez-Rosales and others 1992; Kiritsakis and Dugan 1985; Psomiadou and Tsimidou 2002; Satue and others 1995). Olive oil is unique as a result of its high content of minor polar compounds. Crude olive oil has been shown to contain phenolic compounds such as hydroxytyrosol, tyrosol, and catechol in various concentrations depending on the level of refining (García and others 2006; Owen and others 2000). Crude, unrefined olive oil has been reported to contain 534 mg/kg (reported as gallic acid equivalents) total phenolics (Satue and others 1995). It is difficult to directly compare olive oil to many of the popular food oils (soybean, canola, etc.) due to their extensive refining processes. Though virgin olive oil is traditionally consumed unrefined, a large portion of olive oil is refined in order to make it more acceptable for consumption (García and others 2006). As a result of the refining process, it has been

shown that olive oil can see a marked decrease in phenolic compounds, thereby decreasing its shelf stability (García and others 2006).

Investigations in the oxidative stability of olive oil under photooxidative conditions showed that β-carotene played a significant role in the prevention of chlorophyll-sensitized oxidation (Fakourelis and others 1987). Kiritsakis and Dugan (1985) described the losses of β-carotene and α-tocopherol in olive oil subjected to light and spiked with chlorophyll and attributed these losses to photooxidation. Furthermore, it was demonstrated in this study that β-carotene was more effective at preventing photooxidation in comparison to α-tocopherol. Another study demonstrated contradictorily that chlorophylls *a* and *b* did not play a significant role in the photooxidative stability of olive oil (Gutiérrez-Rosales and others 1992).

Studies have also investigated the stability of olive oil under autoxidative conditions. One study reported that the losses in α-tocopherol and carotenoids over 24 months of storage were proportional to the losses of polar phenolic compounds (Psomiadou and Tsimidou 2002). Moreover, phenolics were shown to act most efficiently at preventing the formation of primary oxidation; however, tocopherols were more effective at preventing the degradation of primary to secondary oxidation products (Satue and others 1995).

2.3 Industrial Oil Processing

The process of removing oil from a commodity and preparing it to be sold on the market often contains a number of key steps, which include degumming, refining,

bleaching, and deodorizing. These steps are taken to improve the organoleptic and physical qualities of the oils. However, this processes often results in the loss of many phytochemicals that enhance oil stability (Jung and others 1989a). Degumming is a process aimed at the removal of phospholipids and gums, which can form unwanted sediment in the oil (Braae 1976). Perhaps the most important of the processes is alkali refining, which is done to remove free fatty acids that can have adverse effects on oil quality (Carr 1976). Bleaching is a process that includes the removal of pigments to improve the appearance of the oil (Jung and others 1989b). The deodorizing process is usually the final step in oil refining and is geared at removing impurities, in particular volatile compounds, which can improve oil stability and flavor (Brekke 1980c).

2.3.1 Degumming

The main purpose of the degumming process is to remove phospholipids and gums, which can have an adverse effect on the physical and organoleptic properties of oil. Failure to degum oils, particularly extracted oils can result in sediment formation often obstructing flow in storage tanks (Braae 1976). Furthermore, presence of phospholipids may promote oxidation during the refining process as well as promote discoloration during the deodorization process (Liu 1999). Hydration of phospholipids present will help to partition them from the oil (Brekke 1980b). Upon hydration, phospholipids as well as any gums present form gels, which have higher specific gravities than the oil and then agglomerate and precipitate from solution (Anderson 1962). In general, the process of degumming soybean oil begins by mixing the oil with

water (approximately 1 to 3% of the oil's total mass) and agitating the mixture for 30 to 60 min at 70˚C (Brekke 1980b). The oil is then centrifuged at a temperature between 50 and 70˚C to aid in the separation of the two phases (Carr 1976). The quantity of water utilized to degum oil is crucial, and, ideally, it should be approximately 75% of the phospholipid content by weight (Swern 1964). If too little water is added then not all phospholipids will be removed from the oil; however, if too much water is added a third phase consisting of excess oil in an emulsified state will form in between the water and oil phases following centrifugation (Brekke 1980b). Furthermore, numerous degumming operations are continuous where the oil is heated to approximately 80˚C (176˚F) and water is added prior to centrifugation (Braae 1976; Carr 1976). Other methods of degumming such as phosphoric acid pre-treatment (Braae 1976) and post-treatment (De Greyt and Kellens 2000) and acetic anhydride (Myers 1957) methods are also used industrially.

2.3.2 Refining

The purpose of refining a food-grade oil is to separate triacylglycerides from free fatty acids, residual phospholipids and gums, natural colorants, insoluble matter, settlings, and other unsaponifiable matter (De Greyt and Kellens 2000). Commonly, alkali treatment with caustic soda is used industrially to accomplish this task. The alkali reacts with free fatty acids present in the oil to form soaps; phospholipids and gums coagulate in the presence of alkali; the undesired colorants are either absorbed in the phospholipid/gum coagulation or degraded by alkali; and the insoluble matter is also

coagulated with the phospholipid/gum complex (Mounts 1980). It is common practice to pre-treat crude oil with phosphoric acid prior to alkali refining to remove non-hydratable phospholipids (Liu 1999).

Once oil has been degummed and is ready to be refined it will be pumped into a tank and held at a temperature of 38˚C (110˚F) for 8 to 24 hours (Mounts 1980). The free fatty acid content is then analyzed, which determines the amount of caustic soda required to refine the crude oil (Braae 1976). Upon mixing of the caustic soda and crude oil, the temperature is raised to approximately 75-82˚C (160-180˚F) and then separated in a centrifuge (Mounts 1980). In order to remove any remaining soap residue, the caustic refined oil is heated to 88˚C (190˚F), then washed with soft water at 93˚C (200˚F), and centrifuged again to separate the phases (Mounts 1980).

2.3.3 Bleaching

Though the main goal of bleaching is to remove the coloring compounds naturally present in oil, it also aids as the final removal step of residual soaps, phospholipids, and metals (De Greyt and Kellens 2000; Brekke 1980a). Historically, heat-activated bleaching clays have been used to accomplish this task, but in recent times the industry has moved towards acid-activated clays because of their stability and efficiency (De Greyt and Kellens 2000). The clays act by adsorbing the unwanted components of the oil on their surface through Van der Waals attraction forces (De Greyt and Kellens 2000). Bleaching times and conditions are specific to the characteristics and desired quality of the oil. However, underbleaching an oil can lead to

a green discoloration when the oil is deodorized (Armstrong and Ireland 1958). Furthermore, overbleaching an oil can promote color reversion in addition to unnecessary processing costs (Brekke 1980a).

2.3.4 Deodorizing

The final step in many conventional oil processing applications is deodorization. This process entails deaeration, heating, deodorization, and cooling of the oil (De Greyt and Kellens 2000). The purpose of deodorization is to remove various flavor and odor compounds that can contribute adversely to oil quality (Brekke 1980c). After the oil is filled into a stainless steel tank, heated up to 220 to 260˚C (428-500˚F), and held under a pressure of 2 to 4 mbar, approximately 0.5 to 3% sparging steam is bubbled through the oil (De Greyt and Kellens 2000).

2.4 Phospholipids

Phospholipids are a class of lipids in which the fatty acid at the *sn*-3 position is substituted with a phosphate group. The simplest phospholipid is phosphatidic acid (PA), which contains an –OH on the phosphate group (McClements and Decker 2008). Other variations include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidylserine (PS) as indicated in Figure 2-4. The key feature of phospholipids is their amphipathic nature, meaning they contain both polar and non-polar regions. This presence of the polar phosphate group provides phospholipids with interfacial surface activity that allows

phospholipids to arrange in bilayers and is a crucial feature of cell membrane fluidity (McClements and Decker 2008).

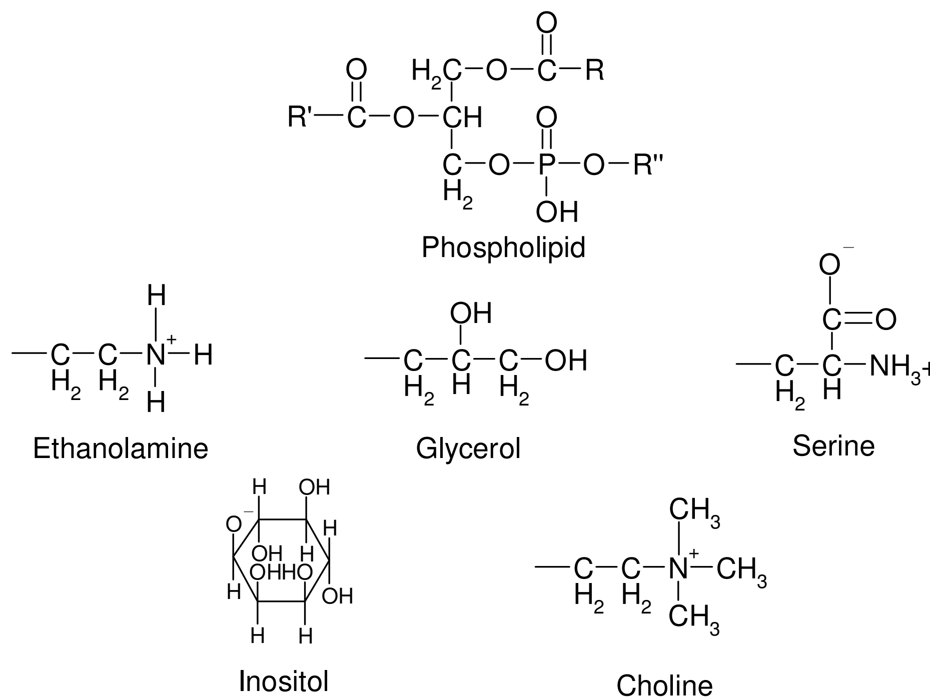


Figure 2-4. Basic structure of a phospholipid and the various head group substitutions (R"). R and R' represent various fatty acid substitutions that can occur at the *sn*-1 and *sn*-2 positions, respectively (McClements and Decker 2008).

As a result of their amphipathic nature, phospholipids are used widely in the food industry as emulsifiers. Phospholipids are also found naturally in many biological commodities including oil seeds. Typically, soybean oil contains approximately 1 to 3% phospholipids (De Greyt and Kellens 2000). In crude oils, phospholipids can exist in either the free hydratable or non-hydratable forms (De Greyt and Kellens 2000). PC and PE both exist as hydratable phospholipids while PA and PE can exist as non-hydratable magnesium and/or calcium salts (De Greyt and Kellens 2000).

Phospholipids are known to exist in many oils; however, no research has been reported that shows the existence of phospholipids in açai oil. It is hypothesized that the phospholipid content is rather high in açai oil due to its high content of non-triacylglycerols (~8%). Furthermore, if phospholipids are present in açai oil, their role in terms of interacting with other phytochemicals or preventing oxidation are not understood. Since açai oil will more than likely be marketed as a crude oil, it is imperative that there should be a comprehension of the interactions of phospholipids in açai oil, if they are present.

2.5 Carotenoids in Oil

Carotenoids are a class of polyenes that range in color from red to yellow, of which nearly 600 have been identified (McClements and Decker 2008). Chemically, carotenoids are composed of two tetraterpene units that are joined by the tail ends (Rao and Agarwal 1999). Carotenoids can be divided into two groups based upon structure as hydrocarbon-based carotenes and oxygenated xanthophylls. Carotenes are hydrocarbon molecules, such as lycopene and β-carotene while oxygenated xanthophylls are composed of a variety of carotene derivatives including hydroxyl, epoxy, aldehyde, and keto groups (Schwartz and others 2008). These pigments are commonly found in oils such as palm (Ooi and others 1994), olive (Minguez-Mosquera and others 1990b), and soybean (Jung and Min 1991). Figure 2-5 diagrams a simplified pathway to obtain many of the most predominant carotenoids in nature.

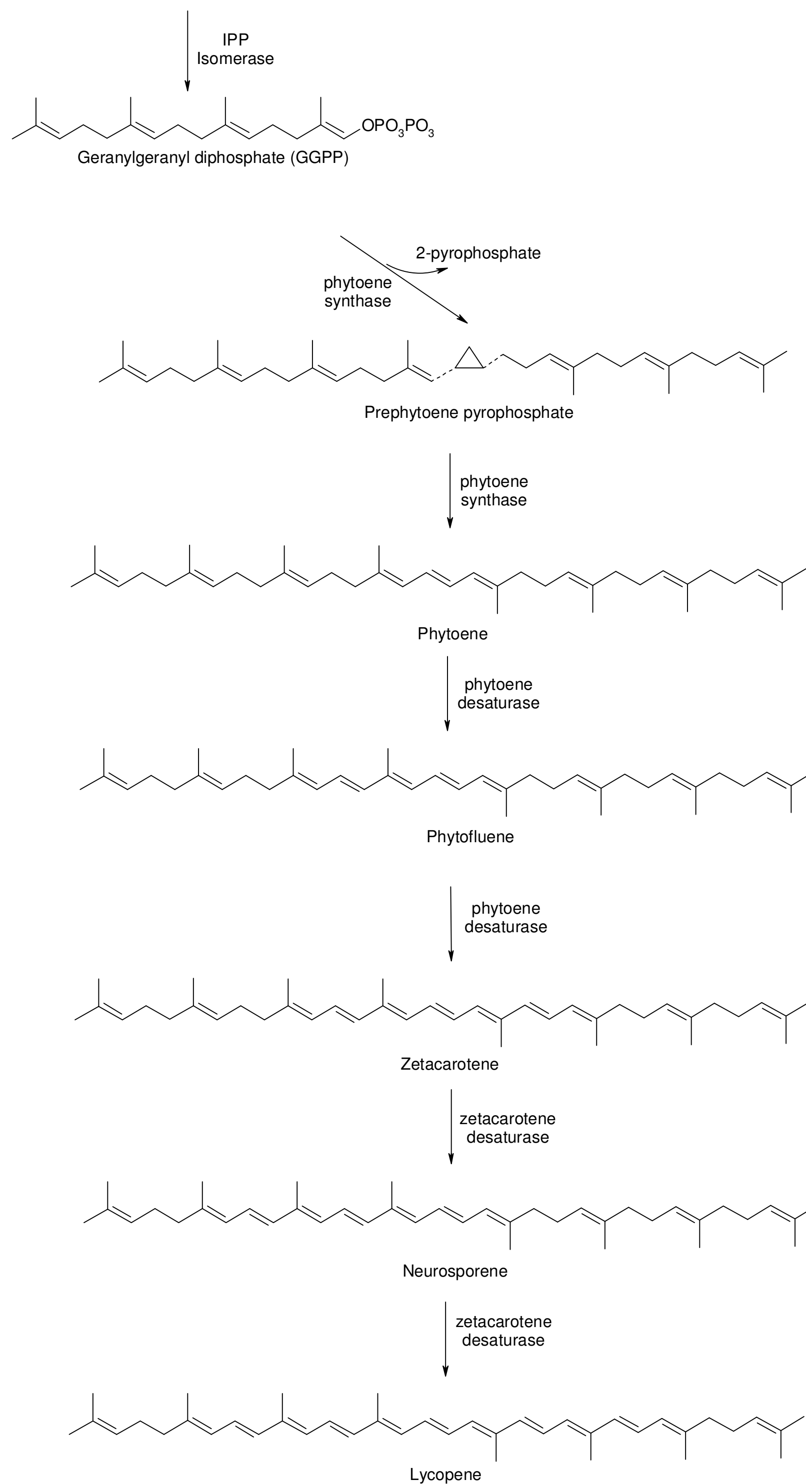


Figure 2-5. Biosynthetic pathway that leads to the synthesis of carotenoids.

lycopene
epsilon cyclase

lycopene
beta cyclase

Delta-carotene

Gamma-carotene

lycopene
beta cyclase

lycopene
beta cyclase

Alpha-carotene

Beta-carotene

alpha-carotene
hydrolase

beta-carotene
hydrolase

OH

HO

Lutein

HO

Beta-cryptoxanthin

beta-carotene
hydrolasee

beta-carotene
hydrolase

OH

Zeinoxanthin

OH

HO

Zeaxanthin

OH

HO

O

Capsanthin

OH

O

HO

Antheraxanthin

OH

HO

O

Capsanthin 5,6 epoxide

OH

O

O

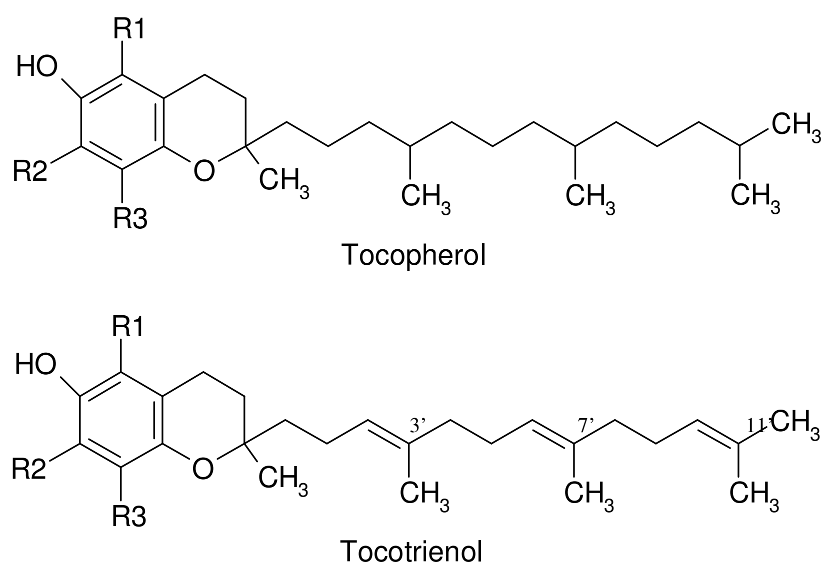
Violaxanthin

Figure 2-5. Continued

Carotenoids are ubiquitous in many crude oils. Yet no research has been published on the carotenoid content of açai oil. Investigations are required to determine if carotenoids are present in açai oil and, if so, what contributions they have to the stability of the oil.

2.6 Tocopherols and Tocotrienols

Tocopherols and tocotrienols constitute a class of non-polar compounds that are commonly prevalent in many food oils. Structurally, the difference in tocopherols and tocotrienols are in the three double bonds at the 3', 7', and 11' position on the side chain (Figure 2-6).



Form	R1	R2	R3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3

Figure 2-6. Structure of the various forms of tocopherols and tocotrienols.

Of the various forms of tocopherols and tocotrienols, α-tocopherol (the true form of Vitamin E) is the most antioxidant potent, while all other forms also exhibit Vitamin E-like activity (McClements and Decker 2008). Additionally, all non-esterified forms of both tocopherols and tocotrienols have the ability to act as lipophilic antioxidants by donating the H^+ of the hydroxyl group on the phenolic ring (McClements and Decker 2008). Much like mechanisms behind other phenolic antioxidants, the reaction of tocopherols and tocotrienols with a free radical creates another radical, which is relatively stable due to resonance of the unpaired electron across the phenolic ring (McClements and Decker 2008).

The presence of tocopherols and tocotrienols in food grade oils has been widely studied. Concentrations of α-tocopherol in refined, bleached, and deodorized (RBD) soybean oil have been reported by numerous studies to range from 12 to 72 mg/kg (Carpenter 1979; Chase and others 1994; Warner and Mounts 1990). Meanwhile, soybean oil has also been reported to contain 60 to 92 mg/kg of γ-tocopherol and 23 to 43 mg/kg of δ-tocopherol (Carpenter 1979; Chase and others 1994; Warner and Mounts 1990). While tocopherols are present in many oils, it is unknown whether they are present in açai oil. Further investigations should lead to a better understanding of the tocopherol content of açai oil and its role as an antioxidant.

2.7 Synergistic Effect of Phospholipids and Tocopherols on Lipid Oxidation

Tocopherols and phospholipids are important components naturally present in foods and both are commonly used as food ingredients. The relationship between

oxidation and phospholipid content in altering the rate of oxidation was nominally examined (Bandarra and others 1999; Hamilton and others 1998; Hildebrand and others 1984), but a mechanism for their interaction has not been elucidated. Bandarra and others (1999) demonstrated that the combination of PE and α-tocopherol in sardine oil resulted in lower concentrations of primary oxidation products. An enhanced effect between tocopherols and phospholipids as well as the superior efficacy of PE and PI was confirmed by Hildebrand and others. (1984). Combinations of α-tocopherol and lecithin in fish oil have also been shown to significantly decrease the occurrence of lipid oxidation (Hamilton and others 1998).

While a number of theories have been proposed as the driving mechanisms behind the synergistic effects of phospholipids and tocopherols, no definitive mechanism has been confirmed. Bandarra and others (1999) suggested that this synergistic effect could be due to a simultaneous antioxidant mechanism involving Maillard reaction products. It has also been suggested that increased fatty acid chain length on the phospholipids in the presence of tocopherol may also increase the induction period of oxidation (Koga and Terao 1995). Frankel and others (1994) described the effectiveness of antioxidants in bulk oils to be dependent upon the interfacial interactions, which could explain the efficacy of the phenolic-containing tocopherol. There is also evidence that this enhanced effect could potentially be due to a hydrogen atom transfer mechanism involving the amine group of the phosphate moiety on the phospholipid and tocopherol (Hildebrand and others 1984; Koga and Terao 1995). Though there are a number of different theories relating the enhanced antioxidant efficacy of tocopherols in the

presence of phospholipids, more investigations are needed to completely understand this relationship in bulk oils.

3. CHEMICAL CHANGES IN CRUDE AÇAI OIL INDUCED BY PHOTOOXIDATION

3.1 Introduction

The açai palm is native to South America and particularly prominent within the state of Pará located in Brazil (Vera de Rosso and others 2008). Açai has become one of the main export commodities for this region due to the emergent international market for açai pulp and related products (Brondízio and others 2002). Subsequently, a considerable amount of research has been conducted on açai pulp and its water-soluble constituents (Choi and others 1998; Del Pozo-Insfran and others 2004; José Dalton Cruz and Paula Vanessa da Silva e 2007; Lichtenthäler and others 2005; Pacheco-Palencia 2006; Pacheco-Palencia and others 2007a; Pacheco-Palencia and others 2007b), yet little is known of its lipophilic constituents and their resultant stability. However, it is often not realized the pulp created from this fruit can contain anywhere from 5.9% (Silva 1996) to 11.0% (Anderson 1988) lipids.

Many commercial açai processing plants in the United States incorporate clarification steps into the processing of açai pulp prior its use in beverages. In this clarification process, oil and other insoluble solids are removed from the pulp and can be later extracted or separated to obtain açai oil. When extracted using an organic solvent such as ethanol, the resultant crude oil is exceptionally dark green in color and is highly viscous due to its chlorophyll and phospholipid content, respectively. Previous studies have reported the fatty acid composition of açai oil to be approximately 56% oleic acid,

24% palmitic acid, 12% linoleic acid, and less than 6% of palmitoleic, stearic, and other fatty acids in trace amounts (Lubrano and others 1994; Schauss and others 2006). Extracting oil in this manner resulted in a food grade oil that contained over 1400 mg/kg of water-soluble polyphenolics that included vanillic, syringic, *p*-hydroxybenzoic, protocatechuic, and ferulic acids as well as (+)-catechin and numerous procyanidin oligomers (Talcott 2008; Pacheco-Palencia and others 2008).

It is widely known that chlorophyll can catalyze photo-induced oxidation in lipid systems serving as a photo-sensitizer that can react with ground state or triplet oxygen ($^3O_2$) to yield singlet oxygen ($^1O_2$), an extremely damaging reactive oxygen specie (Rawls and Van Santen 1970). Singlet oxygen is a highly reactive nucleophilic molecule with a lifespan of 50 to 700 μs (Min and Boff 2002). Since açai oil contains an abundance of chlorophyll it was hypothesized that that oil would be relatively unstable under photooxidative conditions. However, the counter role of natural antioxidants in the prevention of oxidation has not been investigated.

Little is know about the chemical properties of açai oil particularly in regards to lipophilic phytochemicals and oxidative stability. Only one study has addressed the oxidative stability of açai oil and found that no changes in peroxide value over 10 weeks were detected in oils stored in the dark at 20, 30, or 40˚C (Pacheco-Palencia and others 2008); however, photooxidation was not assessed. Schauss and others (2006) described the lipophilic components of freeze-dried açai powder to include 3 sterols (β-sitosterol, campesterol, and sigmasterol). Meanwhile, previous investigations into açai oil stability

focused on the polyphenolics, which demonstrated minor losses (<10%) after 20 min at 170˚C (Pacheco-Palencia and others 2008).

Since açai oil is rich in numerous other phytochemicals including and abundance of chlorophylls and carotenoids, understanding the photooxidative stability of the oil is crucial to promoting the use of açai oil in the food industry. Thus, the aim of this study was to examine the role of phytochemicals, both hydrophilic and lipophilic, in the prevention of light induced oxidation of açai oil.

3.2 Material and Methods

3.2.1 Açai Oil and Oil Refinement

Açai pulp (Yakima Fruit Works, Yakima, WA) was processed into clarified açai juice with the use of a rotary drum vacuum filtration system that utilized diatomaceous earth as the filter aid. From this filter cake, açai oil was extracted utilizing a patent pending extraction protocol (Pacheco-Palencia and others 2008; Talcott 2008). Briefly, CAO was extracted from the filter cake with denatured food grade ethanol (SDA35A), which is a mixture of ethanol and ethyl acetate (95:5) along with the residual water from the açai pulp. The polarity of SDA35A allows for polar (i.e. phenolic acid, procyanindins) and non-polar (triglycerides, phospholipids) phytochemicals to be simultaneously extracted from the filter cake. Upon solvent and water removal, a water-in-oil emulsion system is created with high residual hydrophilic compounds and phospholipids.

For this experiment, CAO was used as the control for this experiment. Three oil refining treatments were subsequently evaluated to determine the role of oil refining on açai oil oxidation. The crude oil was degummed by the addition of water (80˚C; 1:1 v/v) and the mixture held at 80˚C for 10 min under constant agitation prior to immediate centrifugation (Degummed). The aqueous phase containing phospholipids and other polar constituents was discarded. Polyphenolics were removed from a second aliquot of oil by first dissolving in methanol and hexanes (9:2 v/v) to which water was then added at a 10% v/v ratio and the mixture centrifuged to separate the organic phase. This extraction was repeated 3 times and the residual hexane was removed under reduced pressure at 50˚C to produce a Phenolic Extracted oil. Lastly, an aliquot of açai oil was Stripped of its chlorophyll, carotenoids, tocopherols, and other phytochemicals by dissolving oil in hexane (1:1 v/v) and mixing with activated carbon (20:1 v/w; Activated Darco® G-60, Fisher Scientific, Pittsburgh, PA). The mixture was stirred for 1 hour prior to centrifugation to remove carbon, and the process was repeated three times prior to vacuum filtration through filter paper (Fisherbrand P8, Fisher Scientific, Pittsburgh, PA) and solvent likewise removed under reduced pressure at 50°C to create a Stripped açai oil.

3.2.2 Oil Storage

The original CAO and the three oil refining treatments (3.5 mL) were stored in 9-mL borosilicate test vials with polystyrene screw caps. For each treatment, the tube headspace was either flushed with 100% nitrogen for 1 min prior to sealing or sealed

with ambient air (oxygen exposure). Oil samples were stored in the same chamber at 50°C under both light and dark conditions, created by complete shading from light. Vials subjected to light exposure were placed horizontally at a distance of 10 cm from four fluorescent light bulbs (10,000 lux) inside a closed temperature controlled chamber at 40 ± 2˚C (Figure 3-1). Meanwhile, vials without light exposure were placed inside an opaque air-permeable box inside the same chamber. Vials were manually rotated every 2 days to assure even light exposure and individual vials were removed every 5 days for 25 days.

Figure 3-1. Experimental set-up for oils exposed to light for 25 days. Oils were placed 10cm directly under four fluorescent lights (10,000 lux).

3.2.3 Polyphenolics

Water soluble phenolics were isolated from the oil and oil treatments by first dissolving 0.1g of oil in methanol:hexanes (9:2 v/v). Following vigorous mixing, 1 mL of deionized water was added and then the mixture was centrifuged to partition the organic and aqueous phases. The aqueous phase (hydrophilic isolate) was then removed and stored at -20˚C until analysis. Individual polyphenolics were separated and characterized by reversed phase HPLC according to the conditions of Pacheco-Palencia et al. (2007) using a Waters 2695 Alliance HPLC system (Milford, MA). Separations were conducted on a Dionex Acclaim 120 (4.6 x 250 mm; Sunnyvale, CA) column with detection at 280 nm using a Waters 2996 PDA detector. Mobile phases consisted of water (phase A) and a 60:40 methanol and water (phase B), both adjusted to pH 2.4 with *o*-phosphoric acid. The gradient solvent program ran phase B from 0% to 60% in 20 min; 60% to 100% in 20 min; 100% for 7 min; 100% to 0% in 3 min and final conditions were held for 2 min at a flow rate of 0.8 mL/min. Polyphenolic compounds were identified by UV/VIS spectroscopic interpretation compared to authentic standards of *p*-hydroxybenzoic, ferulic, syringic, and vanillic acids (Sigma-Aldrich, St Louis, MO).

3.2.4 Lipophilic Phytochemicals

Carotenoids, tocopherols, and chlorophylls were separated and characterized by normal phase HPLC using a modified method of Psomiadou & Tsimidou (Psomiadou and Tsimidou 1998). Separations were conducted on a Waters 2695 Alliance HPLC system (Milford, MA) using a Supelco LiChrospher Si-60 (4.6 x 250 mm; Bellefonte,

PA) column with detection from 280 to 700 nm using a Waters 2996 PDA detector. Mobile phases consisted of hexane:propanol (99:1 v/v; Phase A) and 100% propanol (Phase B) at a flow rate of 0.8 mL/min. The gradient was 0% Phase B for 8 min, 0 to 5% Phase B in 8 min, 5% Phase B for an additional 8 min, 5 to 0% Phase B for 2 min, and finally held isocratic for 4 min. Tocopherols (294 nm), carotenoids (453 nm), and chlorophylls (430 nm) were compared to spectral data and relative retention times as described by Psomiadou & Tsimidou (1998). Compounds were identified by retention time and UV/VIS spectroscopic interpretation compared to authentic standards of α-tocopherol (Sigma-Aldrich, Santa Clara, CA) and β-carotene (Sigma-Aldrich, Santa Clara, CA) and quantified in equivalents of each. Chlorophylls were quantified in equivalents of chlorophyll *a*, which was isolated from spinach (10 g) by acetone extraction. The acetone was removed by rotary evaporation at 40˚C and the extract was redissolved in hexane prior to being loaded onto a open glass chromatography column (1.5 x 10 cm) packed with 3 g of silica gel and washed with hexanes (Purasil, 60A 230-400 Mesh ASTM, Whatman, Piscataway, NJ). Impurities were eluted with hexanes followed by acetone to elute mixed chlorophylls enriched with chlorophyll *a* (Jubert and Bailey 2007). The volume was then reduced by rotary evaporation and the concentration was determined by spectrophotometrically at 668 nm, using $\varepsilon_{668} = 575{,}000\ M^{-1}cm^{-1}$ (Pierre and others 1997) prior to injection in the HPLC to assure purity.

3.2.5 Phospholipids

Total phospholipid concentration was quantified based upon a modified colorimetric method of Vaskovsky and Kostetsky (1968) and Totani and others (1982). The chromogenic reagent was prepared by dissolving ammonium molybdate (1.6 g) in 12 mL of hot water to yield solution 1. An 8 mL aliquot of solution 1 was then shaken for 30 min with 0.1 g of Tin (II) chloride and 25 µL of hydrochloric acid to give solution 2. To the remainder of solution I, 20 mL concentrated sulfuric acid was slowly added, which was then added to solution to 2 to yield the chromogenic reagent.

Phospholipids were extracted from Crude oil with acetic acid (1:1 v/v) and vigorously vortexed for 30 s. A 100 µL aliquot of the extract was then mixed with 400 µL of chloroform in a screw cap vial and then 100 µL of the chromogenic reagent was added. This mixture was immediately capped and placed in boiling water for 1 min. Following cooling, 5 mL of chloroform was added to the samples and then samples were centrifuged (2000 x *g* for 10 min). The absorbance of the Prussian-blue phospholipid complex was then recorded on a spectrophotometer at 735 nm. Samples were compared to a standard curve of Degummed oil spiked with lecithin.

3.2.6 Secondary Lipid Oxidation Products

Determination of secondary oxidation products were determined by GC-MS using a solid phase micro-extraction (SPME) headspace device. Analysis was conducted using a ThermoElectron Trace GC Ultra (Waltham, MA) equipped with a TriPlus Autosampler and a DSQII mass spectrometer. The autosampler was fitted with a

Carboxen™/poly-dimethylsiloxane (CAR/PDMS) StableFlex™ SPME fiber (Supelco, Bellefonte, PA). Açai oil samples (0.1 g) were incubated for 3 min at 80˚C and allowed to adsorb from the headspace onto the fiber for 5 min. The fiber was desorbed onto a DB-5 column (30 m x 0.53 mm x 5µm film thickness, J&W Scientific, Agilent Technologies, Santa Clara, CA) in splitless mode for 1.25 min at 220˚C using helium as the carrier gas (2.0 mL/min). The column was held at 40˚C for 3 min followed by a linear ramp at 7˚C/min to 300˚C where it was held isothermal for 4 min. The mass spectrometer was operated in electron impact (EI) ionization mode at 70eV with the source temperature at 200˚C. Mass spectral data was acquired from *m/z* 35-300 and secondary oxidation products identified by mass spectral interpretation and comparison to authentic standards (Sigma-Aldrich, St. Louis, MO).

3.2.7 Statistical Analysis

Data was analyzed as a 4 x 5 x 4 full factorial that included four oils analyzed at five sampling times and four storage conditions. Data represent the mean of triplicate determinations. Multiple linear regression, Pearson correlation coefficients and analysis of variance were conducted using JMP software Version 5 (SAS Institute, 2002), with mean separation performed by the LSD test ($P<0.05$).

3.3 Results and Discussion

3.3.1 Phytochemicals Present in CAO

Visually, crude, unaltered açai oil appears to be dark green in color due to its remarkable chlorophyll content. Chlorophyll *a* and four unidentified chlorophyll derivatives were tentatively identified by HPLC spectral comparisons to a standard of chlorophyll *a* (Table 3-1). The concentration of total chlorophylls present in CAO was 206 mg/kg which is 13-fold higher than extra virgin olive oils reported in literature (Gutiérrez-Rosales and others 1992; Minguez-Mosquera and others 1990a). Furthermore, the predominant chlorophyll present in CAO is chlorophyll *a* (66 mg/kg). In comparison, virgin olive oil has been shown to contain anywhere from 0 to 9.5 mg/kg of chlorophyll *a* (Minguez-Mosquera and others 1990a; Fakourelis and others 1987; Gutiérrez-Rosales and others 1992).

Table 3-1. Changes in the concentration (mg/kg) of chlorophyll *a* and four chlorophyll derivatives over 25 days of storage for Crude, Degummed, and Polyphenolic Extracted Açai oil subjected to light and oxygen exposure.

	Storage Time											
	Day 0		**Day 5**		**Day 10**		**Day 15**		**Day 20**		**Day 25**	
Crude												
Chlorophyll *a*[1]	65.6	a[2]	34.8	c	45.0	a	48.1	b	36.5	c	29.1	c
Chlorophyll derivative 1	54.8	a	5.76	b	0	c	0	c	0	c	0	c
Chlorophyll derivative 2	19.2	a	3.04	c	1.95	c	0	d	0	d	7.01	b
Chlorophyll derivative 3	35.6	a	11.6	c	7.95	c	14.7	bc	15.8	b	0	d
Chlorophyll derivative 4	31.6	a	17.0	b	11.4	b	18.9	b	18.3	b	6.27	c
Degummed												
Chlorophyll *a*	66.9	a	45.0	abc	25.7	cd	52.2	ab	38.5	bcd	20.1	d
Chlorophyll derivative 1	64.5	a	3.92	B	0	c	0	c	0	c	0	c
Chlorophyll derivative 2	20.3	a	2.21	C	0.991	de	1.21	cd	0	e	5.58	b
Chlorophyll derivative 3	37.3	a	9.92	C	2.98	d	13.8	bc	19.1	b	0	d
Chlorophyll derivative 4	31.8	a	14.4	B	3.39	c	19.2	b	15.4	b	5.06	c
Polyphenolic Extracted												
Chlorophyll *a*	65.9	a	27.4	C	32.4	b	29.4	bc	31.5	b	17.3	d
Chlorophyll derivative 1	70.5	a	11.2	b	0	c	0	c	0	c	0	c
Chlorophyll derivative 2	20.2	a	1.34	e	7.54	b	6.35	c	7.37	b	4.47	d
Chlorophyll derivative 3	36.7	a	11.8	d	10.8	d	14.7	c	23.5	b	8.47	e
Chlorophyll derivative 4	31.1	a	14.6	c	14.6	c	22.6	b	14.1	c	6.28	d

[1]All compounds were measured by HPLC and quantified in equivalents of chlorophyll *a*. [2]Values with similar letters within each row are not significantly different (P>0.05)

The carotenoid content of CAO was 393 mg/kg, and was masked from visible color detection by the chorophylls present. Two predominant carotenoids were tentatively identified based on spectral comparisons as β-carotene and lutein (Figure 3-2). β-carotene was present at 177 mg/kg and lutein at 216 mg/kg, both quantified in equivalents of β-carotene. Prior studies reported that virgin olive oil contained 2.5 to 11 mg/kg β-carotene with other minor carotenoids at less than 1 mg/kg (Fakourelis and others 1987; Minguez-Mosquera and others 1990a). Other minor carotenoids were detected in açai oil; however, they were not quantified in this study.

Though often numerous forms of tocopherol are found in oils, only α-tocopherol (645 mg/kg) was identified in açai oil. This concentration of α-tocopherol was appreciably higher than other food oils such as crude palm at 140 to 161 (Pocklington and Dieffenbacher 1988), virgin olive oil at 100 to 284 (Gimeno and others 2000; Psomiadou and Tsimidou 1998), and soybean oil at 80 to 87 mg/kg (Pocklington and Dieffenbacher 1988).

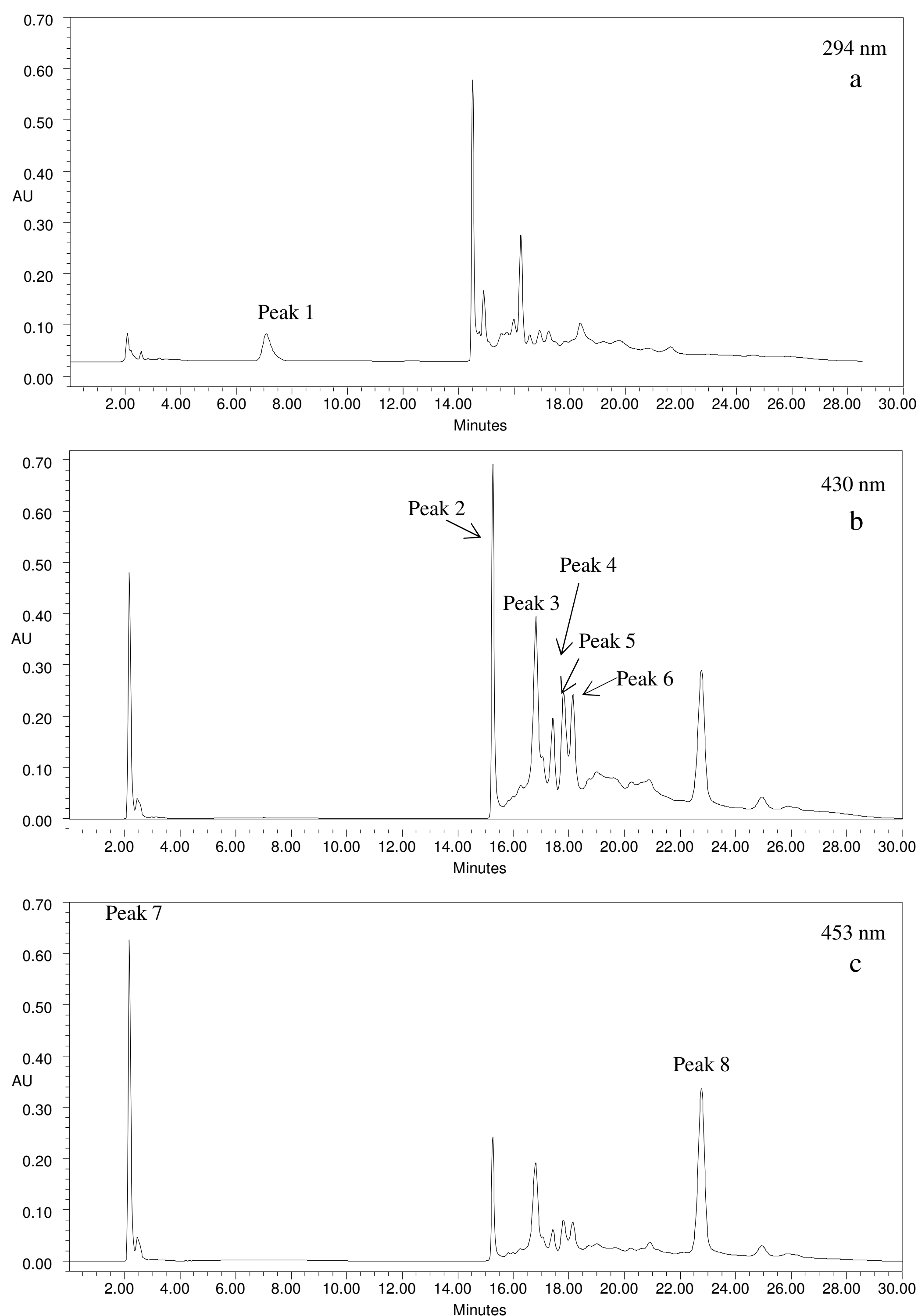


Figure 3-2. HPLC chromatograms at 294 nm (α-tocopherol) (a), 430 nm (chlorophylls) (b), and 453 nm (carotenoids) (c) of fresh crude açai oil by normal phase HPLC. Peak assignments: 1, α-tocopherol; 2, chlorophyll a; 3-6, chlorophyll derivatives; 7, β-carotene; 8, lutein.

3.3.2 Açai Oil and Oil Treatments

As with most crude oils, açai oil contains phospholipids, which are known to be significant contributors to the viscosity of the food oils (Sinram 1986). To monitor the effects of phospholipids on açai oil stability, a degumming treatment was performed. Industrially, oils are commonly degummed to prevent oil cloudiness and assure the production of an oil sediment free oil (Carr 1976; Choukri and others 2001). The degumming process removed 55% of the phospholipids, while losses were of only 7.9% were observed as a result of the polyphenolic extraction process. No lipophilic antioxidants were removed during degumming of açai oil, however losses of polyphenolics (39% on average) were observed. To assess the role of polyphenolics in the oxidation of açai oil, a polyphenolic removal process was employed, which removed an average of 52% of the polyphenolics present in the crude oil. The final treatment was Stripped açai oil, which contained only triacylglycerols and no other lipophilic or hydrophilic compounds.

3.3.3 Changes in Lipophilic Antioxidants Exposed to Light and Oxygen

To determine phytochemical changes under conditions of photooxidation, oil treatments were stored with exposure to intense light and atmospheric air. The presence of oxygen coupled with light exposure are known factors in the progression of singlet oxygen-induced lipid oxidation. Lipophilic phytochemicals such as carotenoids (Mordi 1993) and α-tocopherol (Fukuzawa and Gebicki 1983) are known antioxidants and can easily be degraded. Understanding the roles of these phytochemicals on the oxidation of

açai oil under photooxidative conditions is essential to comprehend the stability of the oil.

Overall, for all three treatments a decreasing trend in β-carotene was observed over time (Figure 3-3). Losses after 25 days storage were greater for the Degummed oil as opposed to the Crude and Polyphenolic Extracted oils (81.6, 44.2, and 53.3%, respectively). Losses of lutein were more marked in Degummed açai oil after storage (99.5%); however, losses of 72.7% and 78.2% were observed in the Crude and Polyphenolic Extracted oils, respectively. The effect of the degumming treatment on both carotenoids was significant and suggested that there may be a potential relationship between these carotenoids and phospholipids under photooxidative conditions. Research into the relationship between carotenoids and phospholipids in reverse micelles made from lecithin theorized that β-carotene may end cap active agents and result in altered rates of oxidation (Cirkel and others 1999). The removal of phospholipids from the oil could have played a significant role in the ability of carotenoids to quench singlet oxygen due to their supposed interactions with phospholipid micelles.

A significant decrease in concentration of α-tocopherol occurred during the first 5 days of storage for the Polyphenolic Extracted (77.5% or 479 mg/kg), Degummed (77.3% or 475 mg/kg), and Crude (62.2% or 402 mg/kg) oils, while changes after day 5 were insignificant for all oils. These results suggested that tocopherol may be preferentially oxidized to protect lipids. Both experimental observations and theoretical predictions made by Buettner (1993) confirm the preferential oxidation of α-tocopherol in lipid systems.

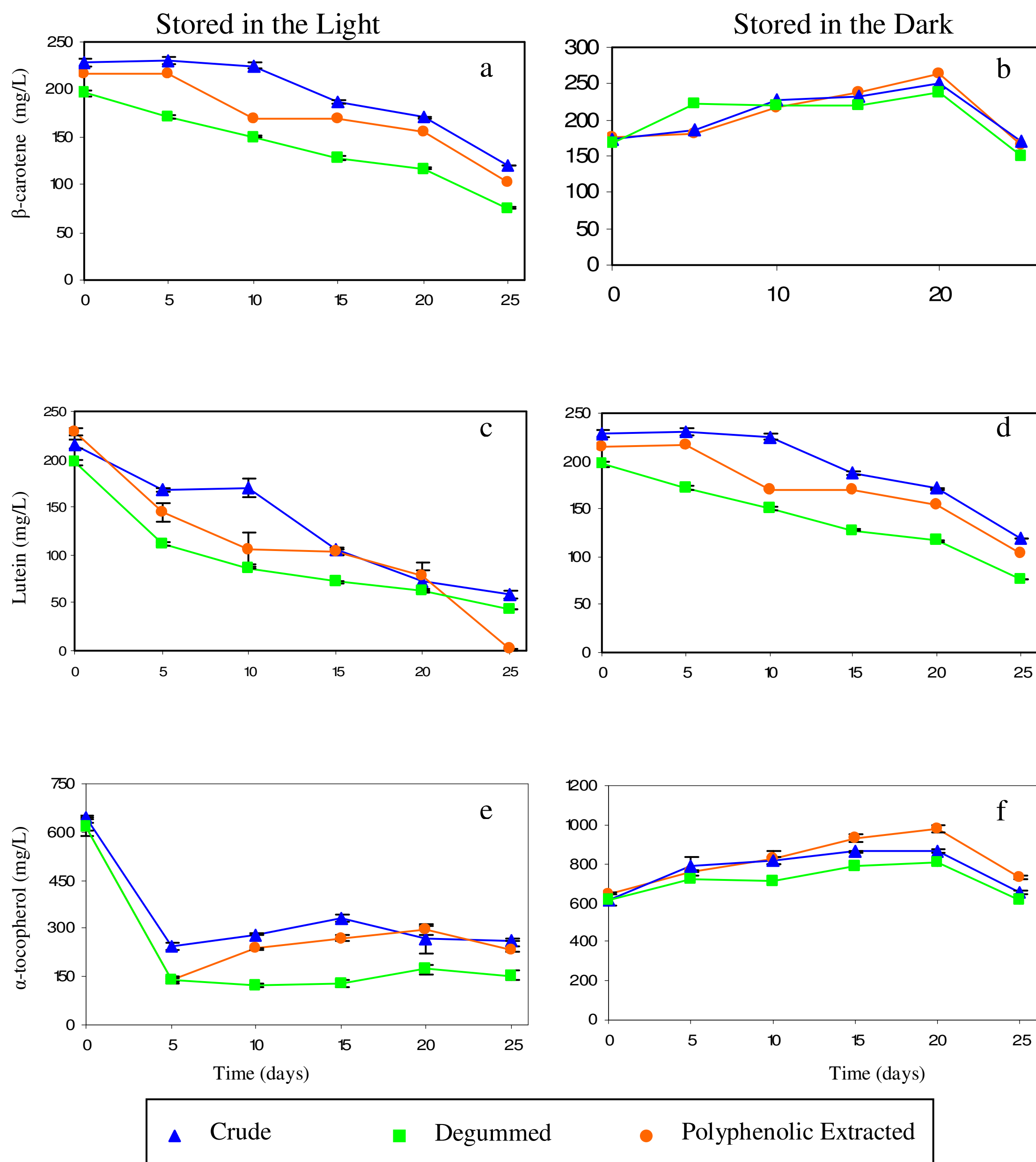


Figure 3-3. Changes in concentrations (mg/L) of β-carotene (a-b), lutein (c-d), and α-tocopherol (e-f) over 25 days of storage. The left column represents oil treatments stored in the light with oxygen while the right column represents oil treatments stored in the dark with oxygen. Stripped açai oil did not contain any carotenoids or α-tocopherol.

3.3.4 Changes in Lipophilic Antioxidants Exposed to Oxygen and without Light

Since energy transfer in the form of light is the driving force behind photooxidation, changes observed in the presence of oxygen and under dark storage conditions are mainly attributed to autoxidation. Through the first 20 days of storage, β-carotene increased by 49.3, 47.0, and 39.7%, respectively in the Crude, Degummed, and Polyphenolic Extracted oils followed by a similar decrease over the last 5 days (Figure 3-3). The mechanism for this observed increase was not elucidated in this investigation, but supporting trials in attempt to elucidate causative factors were included in Appendix A.

In a similar response as observed with ß-carotene, concentration changes were observed with α-tocopherol over the first 20 days of storage resulting in 52.1, 41.1, and 30.2% increase for the Crude, Degummed, and Polyphenolic Extracted oils, respectively. Furthermore, subsequent decreases were observed in all three oils in the final 5 days where only the Crude oil was different (12.8% higher) than the day 0 concentration. Constant losses of lutein occurred in the Polyphenolic Extracted oil over the extent of the study with a final loss of 61.4% at day 25. Decreasing trends in lutein after day 5 and 10 in the Crude and Degummed oils were also observed with overall losses of 47.6 and 52.2%, respectively. These decreasing trends in lutein over storage were also observed in olive oil (Psomiadou and Tsimidou 1998) and the ability of lutein to quench singlet oxygen was demonstrated in soybean oil (Lee and Min 1990).

3.3.5 Changes in Lipophilic Antioxidants Exposed to Light and without Oxygen

Prior to storage, the headspace of oils to be stored without oxygen present were flushed with nitrogen to determine the effects of anaerobic conditions on photooxidation. While oils were stored in glass vials with polystyrene caps, it was assumed that the diffusion of oxygen into the vial did not occur, which was supported by the overall statistically significant difference ($p < 0.01$) in oils stored in the presence and absence of oxygen. However, while the headspace of each anaerobic vial was flushed with nitrogen prior to storage, the oil may still have contained dissolved oxygen, which could have contributed to oxidation reactions. Thus, oxidation could still occur in a system with a nitrogen flushed headspace as observed in this experiment.

Both the Crude and Degummed oil exhibited an increase (17.5 and 13.4%, respectively) in β-carotene over the first 20 days of storage and then markedly decreased within the last 5 days (22.5 and 24.1% as compared to Day 0, respectively) while no significant changes in β-carotene concentration occurred in the Polyphenolic Extracted oils. Decreases in lutein over 25 day storage occurred in all oils where losses of 81.8, 65.4, and 67.6% were observed in the Crude, Degummed, and Polyphenolic Extracted oils, respectively. Meanwhile, losses (40.0, 37.0, and 43.3%) were also observed in α-tocopherol for the Crude, Degummed and Polyphenolic Extracted oils over the extent of storage.

3.3.6 Changes in Lipophilic Antioxidants without Oxygen or Light Exposure

The behavior of oils stored in the absence of headspace oxygen and light exposure was examined to further understand the changes under photooxidative conditions. The changes observed in oils stored without headspace oxygen or light were markedly different than those observed in the presence of light and oxygen. The relative changes of β-carotene over from day 0 to 25 of storage were insignificant for all oils. However, losses of 48.1, 45.3, and 59.7% (Crude, Degummed, and Polyphenolic Extracted oils, respectively) in lutein occurred over the 25 days of storage. Increases of 20.1 and 9.8% were observed in α-tocopherol for the Crude and Degummed oils while no changes were noted in the Polyphenolic Extracted oil from day 0 to 25.

3.3.7 Chlorophylls

Chlorophyll *a* and four unidentified chlorophyll derivatives were tentatively identified by HPLC spectral comparisons to a standard of chlorophyll *a* and their relative changes determined during storage under photooxidative conditions (Table 3-1). Losses of total chlorophyll were apparent over time for the Crude, Degummed, and Polyphenolic Extracted oils (54.4, 60.4, and 65.8%, respectively) stored in photooxidative conditions. A decrease of 55.6, 70.0, and 73.7% in chlorophyll *a*, the predominant chlorophyll, over time occurred in the Crude, Degummed, and Polyphenolic Extracted oil, respectively, when subjected to light exposure. Losses in chlorophyll *a* over storage in photooxidative conditions were also observed in virgin olive oil (Gutiérrez-Rosales and others 1992).

3.3.8 Polyphenolics

Açai oil is characterized by its high water-soluble polyphenolic content, which includes vanillic acid, syringic acid, *p*-hydroxybenzoic acid, protocatechuic acid, ferulic acid, and (+)-catechin, in addition to procyanidin oligomers (Pacheco-Palencia and others 2008). Other oils such as olive oil contain polar components such as catechol, apegenin, luteolin; however, these compounds were shown to be reduced during the refining process (García and others 2006). Other studies reported the presence of protocatechuic acid, syringic acid, tyrosol, *p*-hydroxyphenylacetic acid, *o*-coumaric acid, *p*-coumaric acid, *p*-hydroxybenzoic acid and vanillic acid in virgin olive oil (Papadopoulos and Boskou 1991). In these investigations, minimal changes to predominant polyphenolics such as vanillic acid, syringic acid, and *p*-hydroxybenzoic acid were observed with storage in all treatments. This trend was not supported in olive oil, where losses of phenolic compounds were found to correlate with lipid oxidation (Baldioli and others 1996). Furthermore, decreases in vanillic acid (44%), syringic acid (43%), p-hydroxybenzoic acid (37%), and ferulic acid (33%) were observed in the Degummed as compared to the CAO. This potentially suggests that the presence of phospholipids may play a role in the protection of water-soluble antioxidants in lipophilic systems.

3.3.9 Secondary Oxidation Products

GC-MS coupled with SPME was utilized to identify changes in secondary oxidation products. Two secondary oxidation products, nonanal and hexanal, were

analyzed to understand the changes due to photooxidation. Significant increases in nonanal (Figure 3-4) occurred in all oils exposed to light and oxygen as compared to oils kept in the dark with oxygen, while no differences in hexanal (Figure 3-5) in any oil were observed. This trend suggested that nonanal was the major secondary oxidation product of photooxidized açai oil which is supported by the investigations of Frankel and others (1982) based upon the fatty acid composition of açai oil.

Increases in oils stored in light (regardless of headspace composition) were greater than those stored in the dark. Over the 25-day storage a 2.5-fold increase in nonanal in the crude açai oil stored under photooxidative conditions was observed and likewise an increase in nonanal was present in Polyphenolic Extracted (2.9-fold), Degummed (3.6-fold) and Stripped (3.9-fold) açai oils by comparison. The most notable increase occurred in the Degummed oil stored under photooxidative conditions where the final concentration of nonanal after 25 days was 13.9 mg/kg. While human thresholds of detection for many secondary oxidation products vary widely, nonanal has a relatively high odor threshold of 13.5 mg/kg, while its taste threshold is 0.32 mg/kg (Meijboom 1964).

After 25 days of storage with exposure to light and oxygen, the Polyphenolic Extracted oil contained 8.03 mg/kg of nonanal in comparison to 13.9 mg/kg in the Degummed oil. While both the Degummed and the Polyphenolic Extracted oils contain reduced polyphenolics (39 and 52%, respectively), the major difference in chemistry between the two oils is the phospholipid content. With the lower phospholipid content of the Degummed açai oil and the higher concentration of nonanal it can be hypothesized

that phospholipids may be associated with decreased photooxidation. Studies have suggested that phospholipids can play a role in decreasing oxidation possibly through their interactions with tocopherol (Bandarra and others 1999; Hildebrand and others 1984; Koga and Terao 1995). However, the underlying mechanism behind the influence of phospholipids is not fully understood and more work is required to comprehend its association with decreased levels of oxidation.

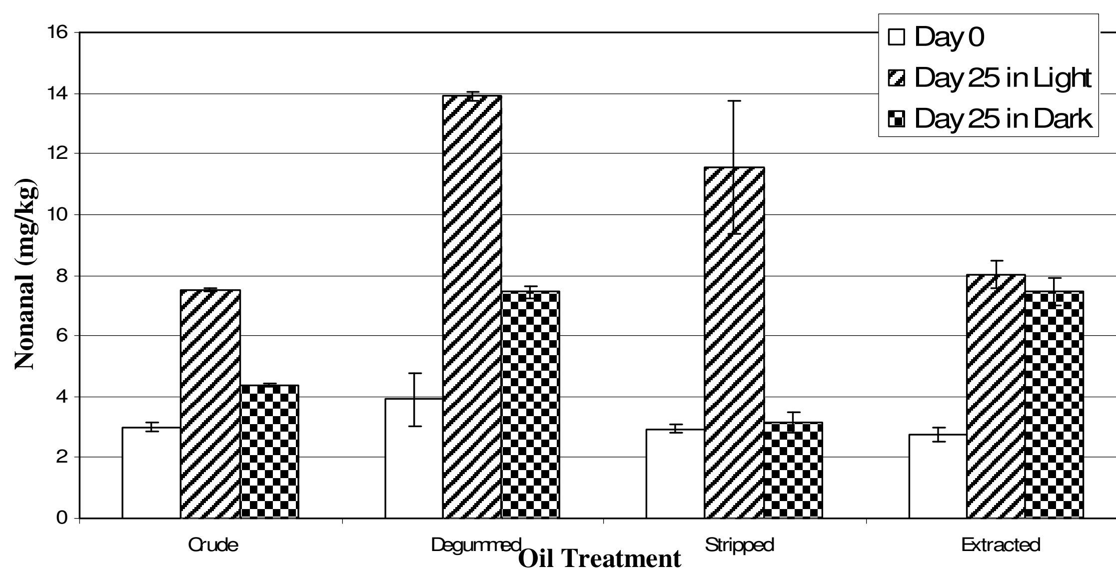


Figure 3-4. Concentration of nonanal, a secondary oxidation product, in Crude, Degummed, Stripped, and Polyphenolic Extracted oils before and after 25 days of storage in the presence of oxygen (n=3).

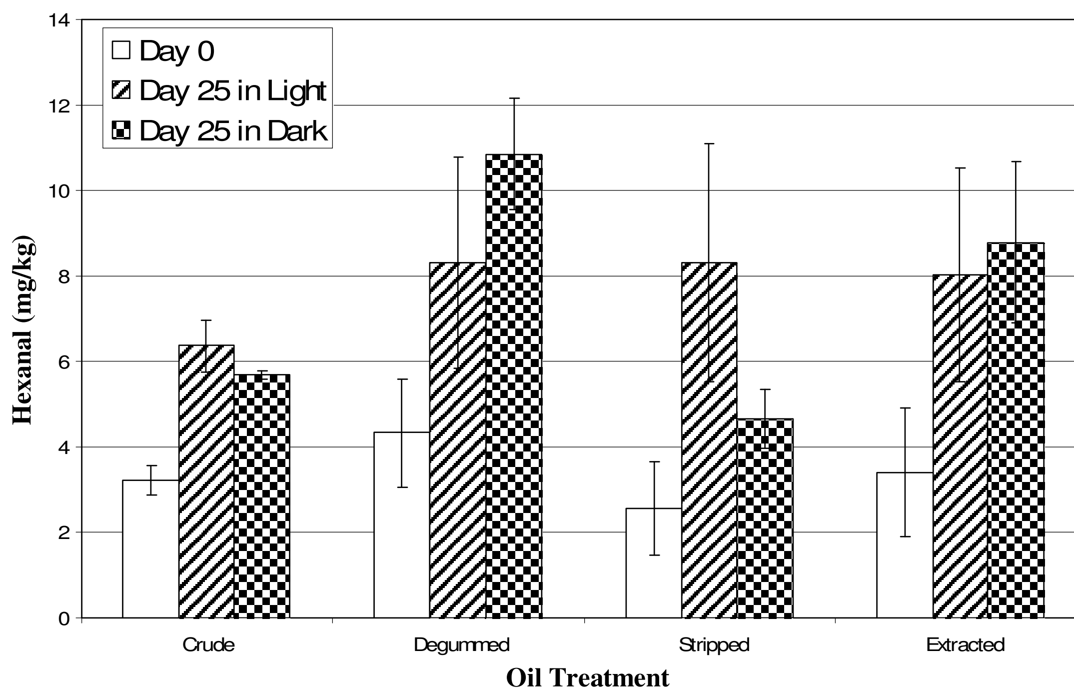


Figure 3-5. Concentration of hexanal, a secondary oxidation product, in Crude, Degummed, Stripped, and Polyphenolic Extracted oils before and after 25 days of storage in the presence of oxygen (n=3).

3.4 Conclusions

A total of 206 mg/kg of chlorophylls, which included chlorophyll *a* as well as four chlorophyll derivatives, were tentatively identified by HPLC in crude açai oil. Two predominant carotenoids (216 mg/kg β-carotene and 177 mg/kg lutein) were also characterized in addition to α-tocopherol (645 mg/kg). The presence of water soluble phenolics such as vanillic, syringic, ferulic, and *p*-hydroxybenzoic acids were in alignment with previous studies (Pacheco-Palencia and others 2008).

The storage of Crude, Degummed, and Polyphenolic Extracted oils also provided insight on the involvement of phospholipids and polyphenolics on the oxidation of açai oil. Exposure to photooxidative conditions led to decreases in β-carotene, lutein, and α-tocopherol in all oil treatments. Meanwhile, increases were noted under conditions of no light exposure and the presence of headspace oxygen. Decreases in polyphenolics over storage were more evident in the Degummed oil, potentially suggesting an interaction between phospholipids and polyphenolics.

Changes in hexanal content appeared to be more significant in oils stored in the dark, while photooxidized oils correlated more with nonanal concentration. Increases in nonanal were noted in all oils over storage with the most significant increases occurring in the Degummed oils. The reduced phospholipid content of the Degummed oil suggested that phospholipids may play a role in the photooxidation of açai oil. While phospholipids are known to interact with many other phytochemicals, further investigation into this theory is still required. The information gained from this investigation provides a better understanding of the chemistry of açai oil and allows for more avenues of its uses to be explored.

4. THE ROLE OF NATURALLY PRESENT ANTIOXIDANTS AND PHOSPHOLIPIDS ON THE OXIDATIVE STABILITY OF CRUDE AÇAI OIL

4.1 Introduction

Açai fruit is a staple in the diets of many people native to Amazonia. Since 2000 when açai was first imported on a large scale into the United States, the açai palm has come into the focus of the scientific community as a result of its purported health-related benefits due to its rich nutrient and phytochemical content. Research efforts have mainly centered on the water-soluble phytochemical content of the fruit, in particular the anthocyanins and procyanindins (Pacheco-Palencia 2006; Pacheco-Palencia and others 2007b). However, it is often not realized the pulp created from this fruit can contain anywhere from 5.9% to 11.0% lipids (Lubrano and others 1994). Furthermore, recent industrial advancements in the extraction of this oil yields a food grade oil rich in carotenoids, tocopherol, and phospholipids (Talcott 2008).

Photooxidation is a concern for many food and lipid systems and processing, packaging, and storage conditions are often selected to minimize its severity, particularly when known photosensitizers are present. In the case of açai oil, its dark green color is due to the presence of several forms of chlorophyll (Section 3) that are likely to act as prooxidants, therefore it is imperative to understand the role of naturally occurring antioxidants in relation to other oil matrix constituents in maintaining and preserving the quality of açai oil. For example, carotenoids are known as good singlet oxygen quenchers in the prevention of photooxidation (Lee and Min 1988; O'Neil and Schwartz

1995; Van Rooyen and others 2008; Fakourelis and others 1987; Warner and Frankel 1987), while tocopherols are also widely known to mitigate lipid oxidation (Terao and Matsushita 1986; Yang and others 2002).

Previous investigations showed the stability of açai oil to be relatively higher than many common food grade oils (Section 4). Research also suggests a beneficial relationship between lipophilic antioxidants, such as α-tocopherol, and phospholipids content and the mitigation of lipid oxidation (Bandarra and others 1999; Hildebrand and others 1984; Koga and Terao 1995). A previous investigation into the photooxidative stability of açai oil determined that losses of the predominant carotenoid present were not significantly detected until 20 days of storage (10,000 lux). Moreover, losses in lutein were observed steadily over time, while α-tocopherol was degraded (73% loss) within the first 5 days. As noted in Section 3, the process of oil degumming (removing phospholipids) leads to an increased level of photooxidation in the oil. Furthermore, developing an understanding of the roles of each phytochemical class in the stability of açai oil will enhance the marketability of the oil. This study was designed to investigate the potential relationship between phospholipids and antioxidants and the roles they play in the prevention of photooxidation of açai oil.

4.2 Material and Methods

4.2.1 Phytochemical Isolations

Crude açai oil (CAO) was extracted from a water-insoluble filter cake by-product from the açai pulp clarification process (Talcott 2008) as defined in Section 3.2.1 and phytochemical isolates separated from the oil in effort to evaluate their contributions to the stability of the initial oil (Figure 4-1). A fraction enriched in carotenoids and α-tocopherol was isolated from the oil utilizing an open glass chromatography column (5 x 25 cm) packed with 125 g of silica gel (Purasil, 60A 230-400 Mesh ASTM, Whatman, Piscataway, NJ). Five-hundred grams of crude açai oil was loaded onto the column pre-washed with hexanes and the carotenoids and a-tocopherol eluted with 1 L of hexanes under 10 PSI vacuum pressure. The chlorophylls that remained adsorbed to the silica were subsequently eluted with 1 L of 2-propanol under vacuum. Solvent for both isolates was removed by rotary evaporation at 40˚C and the isolate re-dissolved in methanol.

Phospholipids were isolated from açai oil based upon the methods of Carelli & others (1997) using Hypersep Diol 5 g cartridges (Thermo Scientific, Waltham, MA). One hundred gram aliquots of oil were dissolved in 250 mL of chloroform and loaded onto a cartridge prepared by washing with 10mL of chloroform followed by 10 mL of hexanes. Phospholipids were eluted by flushing the cartridge twice with 50 mL aliquots of 100% methanol and the combined extracts desolvated by rotary evaporation and re-dissolved in methanol.

Stripped açai oil (500 g) was prepared by dissolving oil in 1 L of hexane with 100 g of activated carbon and stirring for 1 hr to remove naturally occurring

antioxidants. The mixture was vacuum filtered through a filter paper (Fisherbrand P8, Fisher Scientific, Pittsburgh, PA) and the process repeated twice more. Trace amounts of residual carbon was removed by centrifugation (2000 x *g* for 15 min), and the solvent subsequently removed by rotary evaporation at 40˚C.

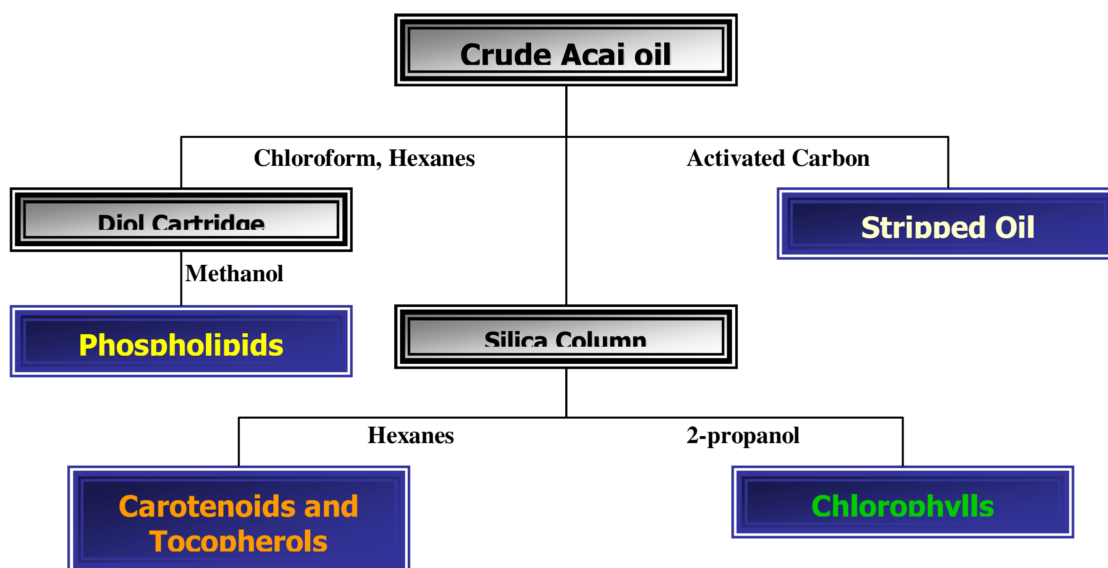


Figure 4-1. Diagram of isolation techniques to obtain phospholipid, chlorophyll, and antioxidant fractions. The blue shaded boxes represent the various isolates used to reconstitute the oils.

4.2.2 Oil Reconstitution

To identify phytochemical contributors to the oxidative stability of CAO, nine different açai oil combinations were constituted that differed in phytochemical composition. Each combination was made from the stripped açai oil as a base oil, and each oil (other than the stripped oil control, Oil A) contained the chlorophyll isolate in equivalent concentrations. Oils fractions were combined according to Figure 4-2, vigorously vortexed for 15 min, and residual solvents removed by rotary evaporation at 40°C. After solvent removal, the oils were once more vigorously vortexed prior to storage. Table 4-1 details the concentrations at which each isolate was added to the reconstituted oils.

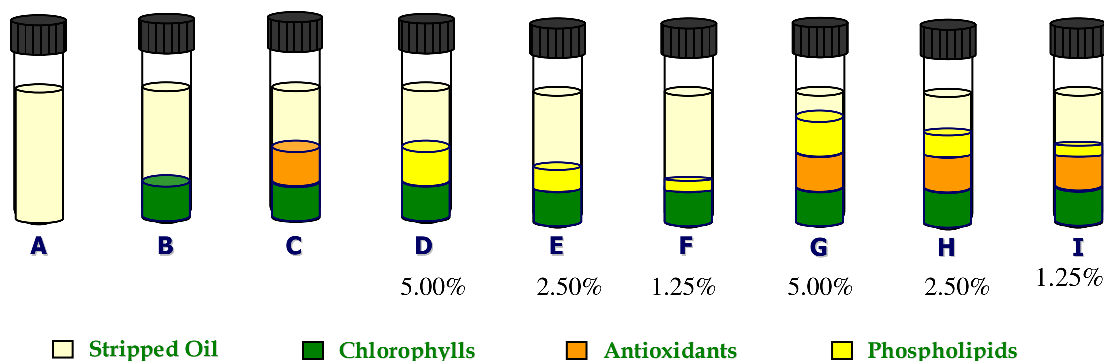


Figure 4-2. Composition of Oils A-I. The legend details the color code for each fraction. Phospholipids were dosed into the appropriate oils at high, medium, and low concentrations (5.00, 2.5, and 1.25%). Antioxidants (carotenoids and α-tocopherol) and chlorophylls were added into the appropriate oils as depicted.

Table 4-1. Concentrations of the phytochemicals added into the various reconstituted oils.

Oil	Tocopherol (mg/kg)	Carotenoids (mg/kg)	Phospholipids (%)	Chlorophyll (mg/kg)
A	0	0	0	0
B	0	0	0	65
C	365	175	0	65
D	0	0	5.00	65
E	0	0	2.50	65
F	0	0	1.25	65
G	365	175	5.00	65
H	365	175	2.50	65
I	365	175	1.25	65

4.2.3 Lipophilic Antioxidants

Carotenoids and tocopherols were separated and quantified according to the methods outlined in Section 3.2.4.

4.2.4 Secondary Lipid Oxidation Product (Nonanal)

Nonanal was quantified according to the methods outlined in Section 3.2.5.

4.2.5 Storage Conditions

Oil samples (1.5 g) were stored in 9-mL screw cap (polypropylene), borosilicate clear vials, with ambient air in the headspace. Vials were stored 10 cm directly under four fluorescent bulbs (10,000 lux) inside a closed temperature controlled chamber at 30 ± 2 °C as shown in Figure 4-3. Samples were rotated every 2 days to assure even light exposure and individual vials were removed every 5 days for 20 days. Oils were stored at -20˚C until analysis.

Figure 4-3. Experimental conditions for açai oil fractions. Oils were placed 10 cm directly under four fluorescent bulbs (10,000 lux) for 20 days.

4.2.6 Statistical Analysis

Data was analyzed as a 5 x 5 x 4 full factorial compared to the control, which included five oil varieties analyzed at five sampling times and at four storage conditions. Data represent the means of triplicate determinations. Multiple linear regression, Pearson

correlation coefficients and analysis of variance, were conducted using JMP software Version 5 (SAS Institute, 2002), with means separation performed by the LSD test ($P<0.05$).

4.3 Results and Discussion

Photooxidation of food oils can result in appreciable decreases to the shelf life of edible oils, especially in the presence of a strong photosensitizer, such as chlorophyll. During the photooxidation process, ground state triplet oxygen is transformed into highly reactive singlet oxygen through energy transfer from a photosensitzer (Min and Boff 2002). Singlet oxygen then can react with double bonds present in lipids to create peroxides, which can quickly lead to the quality deterioration of the oil (Carlsson and others 1976; Neff and others 1993). While Section 3 detailed the stability of crude açai oil subjected to photooxidative conditions, the reasons for the oils' relative stability are still unknown.

As demonstrated in Section 3, açai oil is rich in chlorophylls, phospholipids, and lipophilic antioxidants, such as α-tocopherol and carotenoids. To investigate the relationship between phospholipids and antioxidants (α-tocopherol and carotenoids), açai oil was fractionated into isolates of phospholipids, antioxidants, and chlorophylls. Nine oils were then selectively reconstituted with stripped açai oil in an attempt to comprehend the oxidative stability of crude açai oil. While all oils contained stripped açai oil as the base, oils B through I each contained 65 mg/kg of chlorophyll to aid in inducing photooxidation. As shown in Figure 4-4, the oils with the antioxidant fractions

(Oils C, G, H, and I) each contained 175 mg/kg of carotenoids and 365 mg/kg of α-tocopherol. Oils C through I contained various levels of phospholipids (see Table 2), which were added at levels presumed to be above the critical micelle concentration (CMC). When phospholipids are present in lipophilic systems at concentrations above the CMC, they will form reverse micelles which will encapsulate hydrophilic compounds (Aliotta and others 2002). In order to induce the formation of these reverse micelles, oils were thoroughly vortexed; however, the existence of reverse micelles in the reconstituted oils could not be confirmed. The presence of reverse micelles in lipid systems has been shown to interact with both α-tocopherol (Bandarra and others 1999) and β-carotene (Cirkel and others 1999). In order to investigate these interactions, oils were selectively reconstituted in an effort to recreate the reverse micelles presumed to be present in crude açai oil.

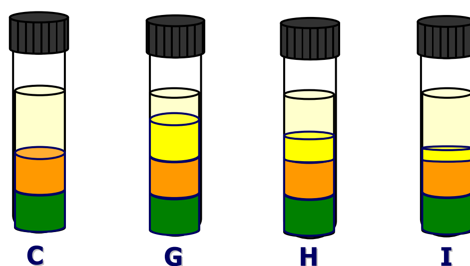


Figure 4-4. Composition of Oils C, G, H, and I (oils containing the antioxidant fraction).

4.3.1 Changes in Carotenoids during Storage

Carotenoids are widely known to act as singlet oxygen quenchers by transferring energy from the excited oxygen molecule to the carotenoids and then dissipating that excess energy through heat (Choe and Min 2006). Two compounds in the antioxidant isolate were tentatively classified as carotenoids (Figure 4-5) based on their spectral similarities to a β-carotene standard, previous studies (Section 3), and published literature (Psomiadou and Tsimidou 1998). Previous investigations into the photooxidative stability of açai oil demonstrated that the predominant carotenoid present, β-carotene, was stable throughout 20 days of storage (10,000 lux) and then decreased to 55.8% of the initial concentration in the final 5 days. This trend was more sudden in this experiment where oils were held under identical conditions (with oxygen and light exposure at 10,000 lux). Losses in total carotenoids reached 69.4, 87.9, 81.0, and 73.1% in the first five days of storage Oils C, G, H, and I, respectively (Figure 4-6). By the day 20 of storage overall decreases of 91.7, 97.3, 77.4, and 99.7% were observed in Oils C, G, H, and I, respectively. These trends were inconclusive and were not supported by previous investigations with unaltered crude açai oil (Section 3). This discrepancy between the behavior of carotenoids in this study as compared to unaltered crude açai oil in Section 3 could potentially be due to a disruption of the native açai oil matrix and certain interactions present in the unaltered oil that are not fully understood.

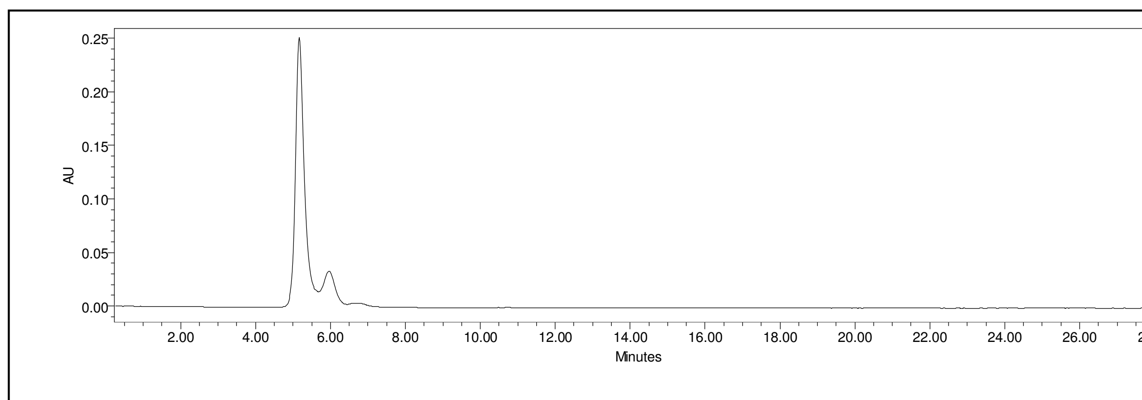


Figure 4-5. Normal phase HPLC chromatogram of typical oil containing antioxidants at 453 nm (carotenoids). Two compounds were tentatively identified as carotenoids by spectral similarities to a β-carotene standard as described in Section 3.2.4.

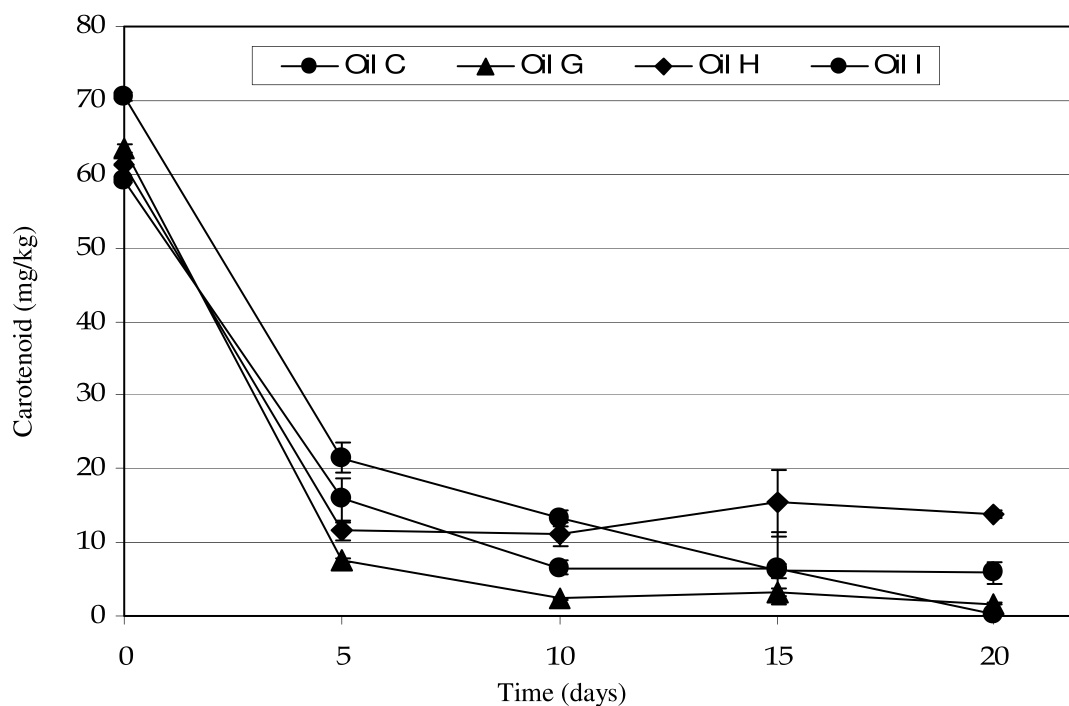


Figure 4-6. Changes in total carotenoids as a result of photooxidation in the oils containing the antioxidant isolate and varying concentrations of phospholipids (0, 5.00, 2.50, and 1.25% for Oils C, G, H, and I, respectively.

4.3.2 Changes in Tocopherol during Storage

Tocopherol is naturally found in many food oils and has been previously shown to be present in açai oil (Section 3). Research has shown that tocopherol is highly reactive towards singlet oxygen and can act by preventing the formation of this reactive oxygen species as well as prevent the attack of singlet oxygen on lipids (Frankel 1991). In this study, all α-tocopherol was degraded by day 5 in all antioxidant containing oils as seen in Figure 4-7. This trend followed earlier observations as seen in Section 3 where 95% of α-tocopherol was lost in the first 5 days of storage under identical conditions.

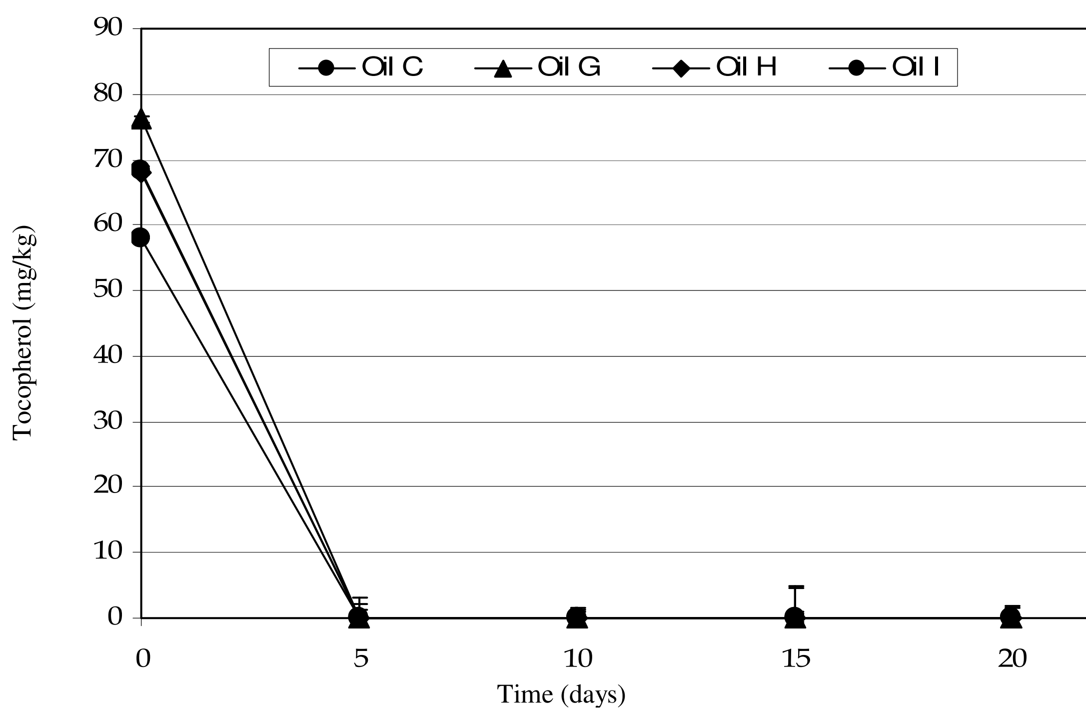


Figure 4-7. Changes in α-tocopherol as a result of photooxidation in the oils containing the antioxidant isolate and varying concentrations of phospholipids (0, 5.00, 2.50, and 1.25% for Oils C, G, H, and I, respectively.

4.3.3 Changes in Nonanal during Storage in Oils without Phospholipids

While photooxidation of oil may yield a myriad of oxidation products, previous studies have shown that nonanal is the most suitable marker of secondary oxidation products induced by photooxidation in açai oil (Section 3). Figure 4-8 shows the composition of all oils that do not contain phospholipids. As expected, nonanal increased during storage in all oils. An increase of 60.9% in nonanal occurred in the stripped oil (Oil A), which did not contain any chlorophyll; however, a marked increase of 702.8% occurred in the oil containing only chlorophyll (Oil B). The differences represent the increases in nonanal due to photosensitized oxidation, which was anticipated considering the extraordinary content of chlorophyll. Meanwhile, there was a significant increase in nonanal for Oil C (oil with antioxidants) during storage, final concentrations of nonanal were less than the oils that did not contain antioxidants (Oils A, B, D, E, and F). Furthermore, over the 20 days of storage, nonanal in Oil C increased from 0.027 to 0.27 mg of nonanal per kg of oil with the largest increase (0.24 mg/kg) occurring in the first 5 days. Yet, the largest decrease in lipophilic antioxidants for Oil C occurred during the initial 5 days of storage as well.

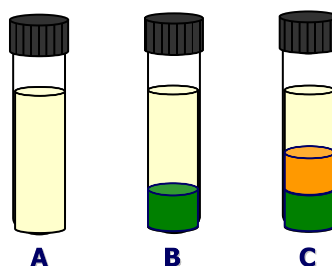


Figure 4-8. Composition of the three control oils (stripped, chlorophyll only, and chlorophyll and antioxidants).

4.3.4 Changes in Nonanal during Storage in Oils with Phospholipids

In industrial oil processing, the main objective of the degumming process is to remove phospholipids. As noted in Section 3, degummed oil demonstrated a marked increase in nonanal over storage in comparison to crude açai oil. Figure 4-9 shows the composition of all oils containing phospholipids in this investigation. Increases in nonanal for oils containing phospholipids and no antioxidants (Oil D, E, and F) were not significantly different than increases observed in the oil containing only chlorophyll (Oil B). This data suggests that phospholipids alone do not play a significant role in the prevention of lipid oxidation. Furthermore, all oils containing phospholipids and no antioxidants were not significantly different in nonanal concentration after 20 days of storage, suggesting that oil oxidation is not dependent on phospholipid concentration alone. While certain studies have shown a potential antioxidant effect of phospholipids due to their ability to chelate metals (Pokorný 1991), that was not observed in this experiment.

Research has suggested a beneficial relationship between phospholipids and lipophilic antioxidants in the prevention of lipid oxidation (Bandarra and others 1999; Koga and Terao 1995). In particular, numerous investigations demonstrated that tocopherols and phospholipids have exhibited a synergistic relationship in terms of preventing oxidation. The exact mechanism behind this synergistic effect is not yet known, however, theories focus upon the ability of phospholipids to regenerate oxidized tocopherols (Hildebrand and others 1984; Koga and Terao 1995).

Oil G (antioxidants and high phospholipids) demonstrated the smallest increases in nonanal during the 20 days of storage (Figure 4-10). The final concentration of nonanal for Oil G (0.16 mg/kg) was significantly lower than for the oil containing antioxidants only (0.27 mg/kg). Oils H (antioxidants and medium phospholipids) and I (antioxidants and low phospholipids) were not significantly different than the oil containing only antioxidants. This data suggests that while there appears to be a relationship between phospholipids, lipophilic antioxidants and decreased oxidation, the relationship is concentration dependent. The oils with added phospholipids at the high level contained a final concentration of 5.0% phospholipids, which is higher than many oils natively contain. Furthermore, açai oil has been shown to contain 0.9% phospholipids. Therefore, while phospholipids and lipophilic antioxidants were shown to reduce nonanal concentrations in reconstituted oil, it is unclear if this is the main reason for the oxidative stability of crude açai oil. More investigations need to be conducted in order to determine if phospholipids and lipophilic antioxidants are responsible for the oxidative stability of crude açai oil.

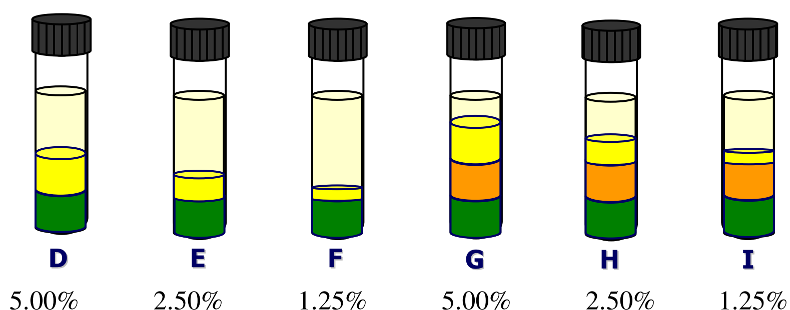


Figure 4-9. Composition of all oils containing phospholipids (concentrations listed below oils) Oils G, H, and I also contain equal concentrations of antioxidants.

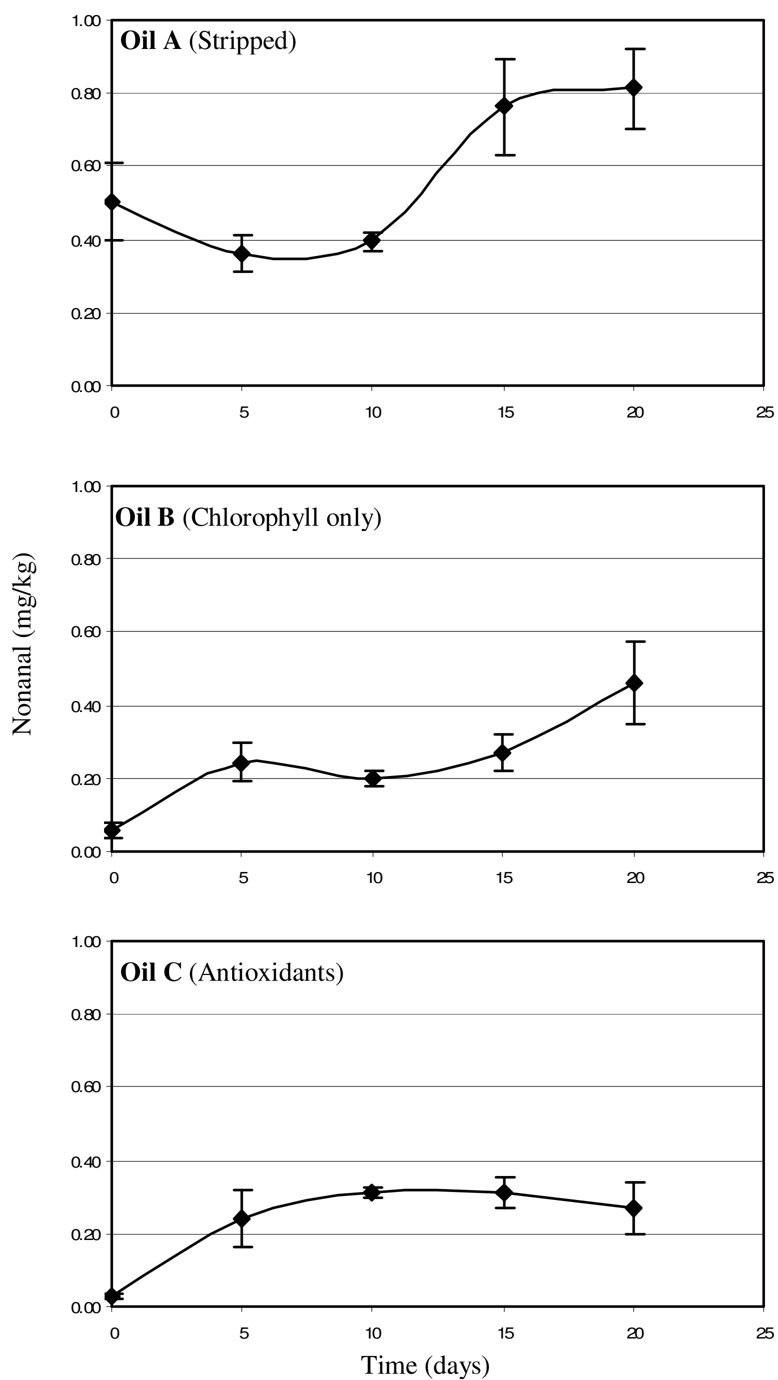


Figure 4-10. Changes in nonanal in the oil treatments as induced by storage under photooxidative conditions. PL = phospholipid.

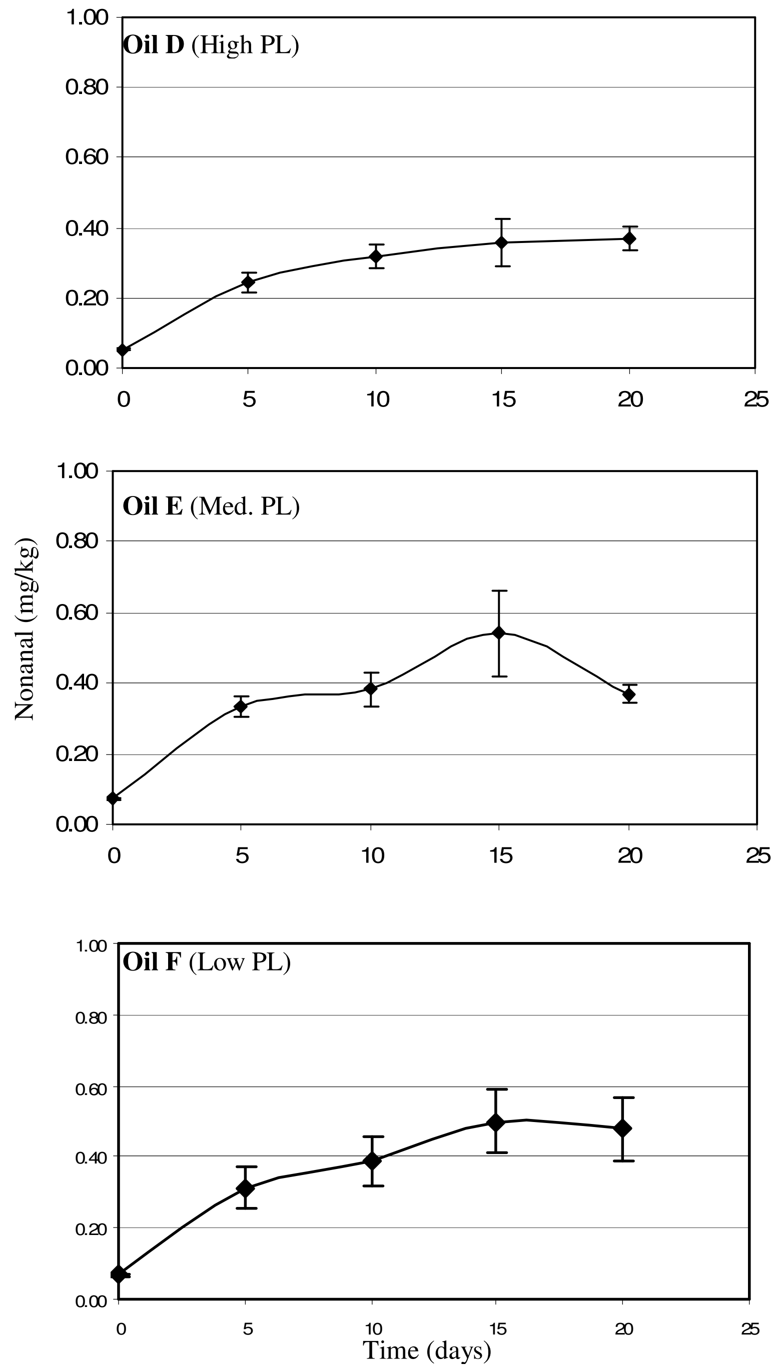


Figure 4-10. Continued.

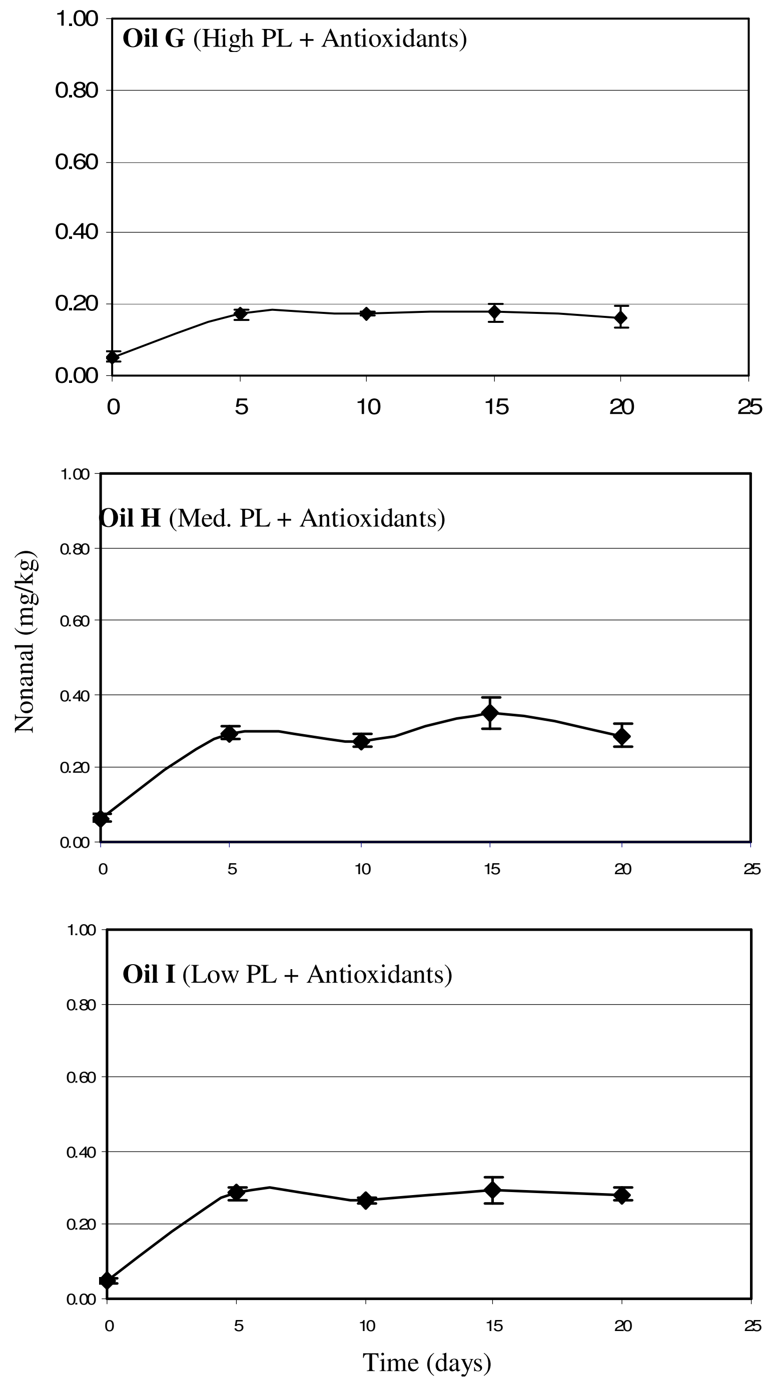


Figure 4-10. Continued.

4.4 Conclusions

Investigations into the photooxidative stability of açai oil demonstrated that phospholipids alone did not play a significant role in the stability of the carotenoids during photooxidation. However, oils containing lipophilic antioxidants and a high concentration of phospholipids showed a significantly smaller increase in nonanal than oil with antioxidants alone. This data supports previous research published detailing the synergistic effects of phospholipids and lipophilic antioxidants on the stability of lipids. Nevertheless, due to the concentration of phospholipids necessary to see a significant decrease in oxidation in this study, it is questionable if the interaction between phospholipids and lipophilic antioxidants are responsible for the stability of açai oil. In this experiment, the contributions of water soluble antioxidants were not examined. More research, including investigations into the contributions of hydrophilic antioxidants, is needed before the reasons for the stability of açai oil can be more closely identified.

One of the most significant observations as a result of this study concerned the behavior of the reconstituted oils as compared to unaltered crude açai oil. It was observed that the behavior of the carotenoids in the reconstituted oils was different than previously conducted studies on crude açai oil. Losses of the predominant carotenoid were observed immediately in this study, while no significant losses were seen in unaltered crude açai oil until 20 days of storage. With its remarkable hydrophilic and lipophilic phytochemical content, açai oil is a unique matrix. The interactions between all of these components, particularly phospholipids and the other phytochemicals may

have been disrupted during the isolation process. It is difficult to determine if the reconstituted oils are truly representative of the chemical interactions that occur in crude açai oil. Further investigations into isolating and reconstituting açai oil in a more authentic manner have not been successful. It is unclear what is responsible for the difference in behavior between the unaltered and reconstituted oil, however it is theorized that phospholipid reverse micelles might play a role. While it is difficult to assess the formation of reverse micelles in a complicated matrix, Section 7 will focus on comprehending these interactions in a model system. Yet, more research is required in order to fully comprehend the interactions within CAO.

5. PHYTOCHEMICAL AND OXIDATIVE CHANGES IN AÇAI AND OTHER COMMON OILS INDUCED BY AUTOXIDATION

5.1 Introduction

Açai oil is most commonly obtained as a co-product of açai pulp clarification processes whereby pulp is processed into oil-free food and beverage ingredients. Previous studies have shown açai pulp to contain approximately 5.9% (Silva 1996) to 11.0% (Anderson 1988) lipids. The removal of the oil from the diatomaceous earth filter cake utilized to clarify açai pulp yields a viscous crude oil, which is dark green to black in color (L* = 20.20, a* = -14.71, b* = 19.11) with a distinct fruity aroma. Investigations have shown the fatty acid composition of crude açai oil (CAO) to be similar to that of olive oil with a composition of 60% oleic acid, 22% palmitic acid, 12% linoleic acid, and 6% of palmitoleic and stearic acids in addition to other fatty acids in trace amounts (Lubrano and others 1994). CAO is rich in hydrophilic phytochemicals including polyphenolics such as vanillic acid (1,616 mg/kg), syringic acid (1,073 mg/kg), *p*-hydroxybenzoic acid (892 mg/kg), protocatechuic acid (630 mg/kg), ferulic acid (101 mg/kg), and (+)-catechin (66.7 mg/kg) (Pacheco-Palencia and others 2008). Furthermore, as described in Section 3, the oil has been shown to contain β-carotene (393 mg/kg) and α-tocopherol (695 mg/kg) in significant quantities.

Additional industrial developments have lead to the creation of açai oil with reduced levels of phospholipids and hydrophilic polyphenolics, namely partially refined açai oil (RAO). Like CAO, RAO is a co-product of açai pulp processing. Visually, RAO

is slightly less viscous and light green in color (L* = 14.38, a* = 22.88, b* = -0.93). The lipid composition (including lipophilic phytochemicals) for both RAO and CAO are nearly identical, however, RAO is approximately 5.5-fold lower in total soluble phenolics and 3-fold lower in phospholipids. While research has looked the interactions of oils containing phospholipids and antioxidants previously, the relationship of these compounds to oil stability is still not fully understood (Bandarra and others 1999; Cirkel and others 1999; Hildebrand and others 1984; Huang and others 1994; Frankel and others 1994). Thus, given the compositional similarities of lipophilic compounds and the differences in concentrations in hydrophilic compounds in RAO and CAO, investigations into their relative stabilities will provide insight into the contributions of these compounds in preventing oxidation. The aim of this investigation is to compare the oxidative stability and phytochemical content of CAO and RAO to other common food oils (soybean, canola, and olive). This is the first known study comparing known food oils to açai oil in measuring relative rates of oxidation.

5.2 Materials and Methods

5.2.1 Açai and Other Commercial Oils

CAO was extracted using a hydroalcoholic solution from the water-insoluble filter cake that remained from a commercial açai pulp clarification process (Talcott 2008). RAO was produced by agitating the filter cake with hot water (70˚C) until phase separation was evident, decanting the oil phase, and washing with hot water once more prior to centrifugation to remove residual water. Highly refined, commercial cooking

oils were obtained for canola and soybean oil along with Extra Virgin Olive oil (all Hill Country Farms brand) each obtained from local grocery stores.

5.2.2 Lipophilic Antioxidants

Carotenoids and tocopherols were separated and quantified according to the methods outlined in Section 3.2.4.

5.2.3 Primary Lipid Oxidation Products

The concentration of lipid peroxides were assessed for all samples based upon a modified method of the American Oil Chemists' Society (Firestone 1997). Samples of oil (4 g) were dissolved in 20 mL of a 3:2 acetic acid-chloroform mixture. Following mixing, 500 µL of a saturated potassium iodide solution was added to the sample. After one minute of vortexing (3000 x *g*), 20 mL of deionized water was added. The sample was then vigorously vortexed for 1 min and then centrifuged for 3 min at 3000 x *g* to separate the bilayer. The aqueous (top layer) was then decanted and 2 mL of starch indicator solution (Fisher Scientific, Pittsburgh, PA) was added. While under constant agitation, the mixture was then titrated with 0.01 N sodium thiosulfate until the mixture was colorless. The data are reported as milliequivalents of peroxides per kg of oil.

5.2.4 Secondary Lipid Oxidation Product (Nonanal)

Nonanal was measured as the predominant secondary oxidation product of lipids by GC-MS with the aid of a SPME headspace device. Analysis was conducted using a

ThermoElectron Trace GC Ultra (Waltham, MA) equipped with a TriPlus Autosampler and a DSQII mass spectrometer. The autosampler was fitted with a Carboxen™/polydimethylsiloxane (CAR/PDMS) StableFlex™ SPME fiber (Supelco, Bellefonte, PA). Açai oil samples (100 mg in 10 mL deionized water) were incubated for 3 min at 80˚C and allowed to adsorb from the headspace onto the fiber for 5 min. The fiber was desorbed onto a DB-5 column (30 m x 0.53 mm x 5µm film thickness, J&W Scientific, Agilent Technologies, Santa Clara, CA) in splitless mode for 1.25 min at 220˚C using helium as the carrier gas (2.0 mL/min). The column was held at 40˚C for 3 min followed by a linear ramp at 7˚C/min to 300˚C where it was held isothermal for 4 min. The mass spectrometer was operated in electron impact (EI) ionization mode at 70eV with the source temperature at 200˚C. Mass spectral data was acquired from *m/z* 35-300 and nonanal identified by mass spectral interpretation and comparison to an authentic standard.

5.2.5 Chlorophylls

As discussed in Section 3, chlorophyll *a* is the predominant chlorophyll pigment in açai oil, similar to olive oil (Minguez-Mosquera and others 1990a). Changes in total chlorophyll over time were expressed in equivalents of chlorophyll *a* using the spectrophotometric method of Valentina and others (2004). Oil samples were diluted 150x in n-hexanes and absorbance determined at 663 nm for quantification using a molar extinction coefficient of $9.01 \times 10^4\ M^{-1}\ cm^{-1}$.

5.2.6 Storage Conditions

Oil samples (10 g) were stored in clear 40-mL screw cap vials without headspace modification. Vials were stored vertically in a temperature regulated chamber at 63 ± 2˚C (Malcolmson and others 1994) and individual vials were removed from storage every 3 days for 18 days and then stored at -20˚C until analysis.

5.2.7 Statistical Analysis

Data were analyzed as a 5 x 7 full factorial that included five oil varieties analyzed at seven sampling times. Data represent the mean of triplicate determination. Multiple linear regression, Pearson correlation coefficients and analysis of variance, were conducted using JMP software Version 5 (SAS Institute, 2002), with mean separation performed by the LSD test ($P<0.05$).

5.3 Results and Discussion

5.3.1 CAO vs. RAO

CAO and RAO are value-added products of the açai pulp clarification process. While the fatty acid compositions of CAO and RAO are nearly identical, there are certain compositional differences that are a direct result of the extraction process. Both oils originated from the diatomaceous earth filter cake created from açai pulp clarification. CAO was extracted from the filter cake with denatured food grade ethanol (SDA35A), which is a mixture of ethanol and ethyl acetate (95:5) along with the residual water from the açai pulp. The polarity of SDA35A allows for polar (i.e. phenolic acid,

procyanindins) and non-polar (triglycerides, phospholipids) phytochemicals to be simultaneously extracted from the filter cake. Upon solvent and water removal, a water-in-oil emulsion system is created with high residual hydrophilic compounds and phospholipids. Conversely, RAO is removed from the filter cake with water followed by a physical separation to remove residual water, leaving lower amounts of residual phospholipids and polar phytochemicals as a result of the water addition analogous to a refining step.

5.3.2 Changes in Tocopherols during Storage

Tocopherols are an important class of lipophilic antioxidants that occur naturally in food oils and are a common food additive to retard oxidation reactions. In this study α-tocopherol was monitored over time in an effort to understand its contributions to the stability of the various oils. Initial concentrations of α-tocopherol in soybean and canola oil (59.1 and 115 mg/kg, respectively) decreased to zero in the first 3 days of storage of the soybean oil and the first 6 days for canola oil (Figure 5-1). Decreases in α-tocopherol over time were also observed in RAO where tocopherol decreased from 279 to 65.2 mg/kg over the 18 days of storage.

In olive oil, tocopherol increased 68.3% over the 20 days of storage. Increases in tocopherol over storage were also observed in CAO where the concentration of tocopherol increased by nearly 4-fold. This trend was also seen in previous investigations where açai oil was stored in accelerated storage conditions (Section 3). The synthesis of tocopherols in processed oils is not possible, however, it is theorized

that the regeneration of tocopherol during storage may have occurred. The synergistic effects of tocopherols and phospholipids were studied previously (Bandarra and others 1999; Hamilton and others 1998; Hildebrand and others 1984; Koga and Terao, 1995; Terao and Matsushita, 1986) and these investigations showed that phospholipid addition to oils containing α-tocopherol suppressed oxidation in a mechanism that was not elucidated (Bandarra and others 1999). It is theorized that after tocopherols act to reduce reactive oxygen species, phospholipids may help regenerate tocopherol by hydrogen transfer from the amino group (Hildebrand and others 1984; Koga and Terao 1995). Furthermore, certain phospholipids, such as phosphatidylinositol and phosphatidylethanolamine, have been shown to be more efficient in enhancing the effectiveness of tocopherol as compared to other common phospholipids (Hildebrand and others 1984). Thus, it is hypothesized that a potential explanation for the increase in tocopherol observed in CAO and olive oil in this experiment could be due to the regeneration of oxidized tocopherol over time. Moreover, this phenomena has been observed in other investigations where α-tocopherol has been regenerated by reducing agents such as ascorbic acid (Terao and Matsushita 1986; Bandarra and others 1999). Another plausible explanation for this increase is a physical interaction between phospholipids and a-tocopherol. Tocopherol was previously thought to accumulate at the interfacial region of a reverse micelle because they contain both hydrophilic and lipophilic groups (Huang and others 1994). This 3-dimensional structural arrangement may allow for entrapment of a-tocopherol at its interface, sequentially releasing tocopherol during storage that in turn helped to prevent lipid oxidation.

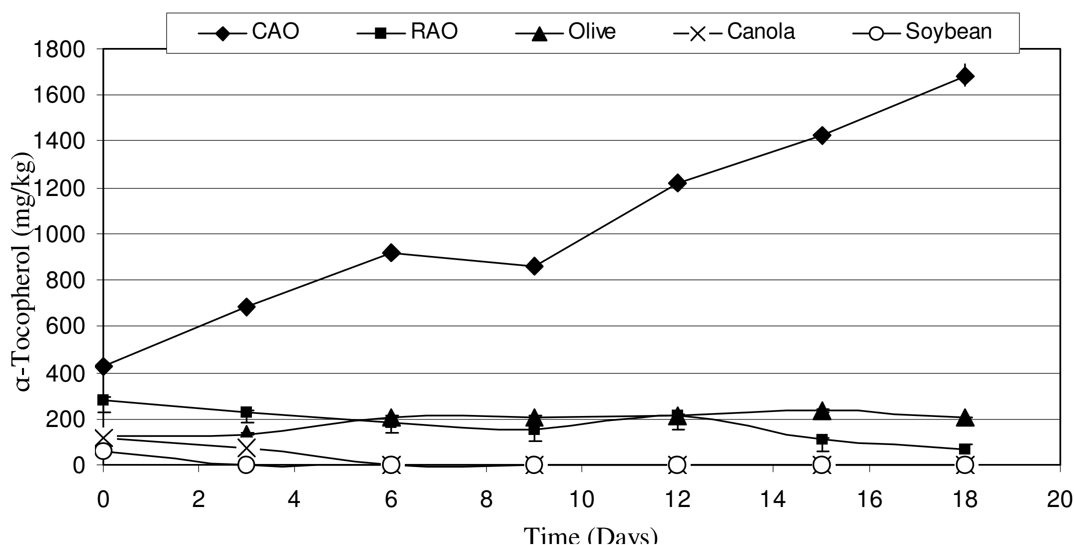


Figure 5-1. Tocopherol content of various oils stored at 63˚C for 18 days

5.3.3 Changes in Carotenoids during Time

No carotenoids were observed in the soybean, olive, or canola oils. One major carotenoid, β-carotene, in both RAO and CAO was indentified. In RAO, a slight decrease occurred over the 18 days of storage (9.10%) with the most significant losses occurring during the initial 3 days (Figure 5-2). Trend in the CAO demonstrated a marked increase (89.8%) in carotenoids content over the extent of storage. Like tocopherol, this increase may result from an association of the carotenoid to the interfacial region of the reverse micelle. Research has shown that carotenoids can act at the micellar interface of emulsion systems (Montenegro and others 2009), which demonstrates their association with phospholipids. Another investigation described the

association of carotenoids to reverse micelles as “end-capping” molecules that act at the interfacial region of the reverse micelle (Cirkel and others 1999). While the mechanism behind this interaction between phospholipid reverse micelles and carotenoids is not fully understood, current data suggest that these carotenoids are protected from the oxidizing conditions of high temperature storage and are potentially released into solution from the reverse micelle interface.

5.3.4 Changes in Chlorophyll during Storage

The chlorophyll content of the oil directly impacts the visual appearance and therefore overall quality of oils. The degradation of chlorophyll was monitored over time by spectrophotometric analysis. No significant losses in total chlorophyll were observed

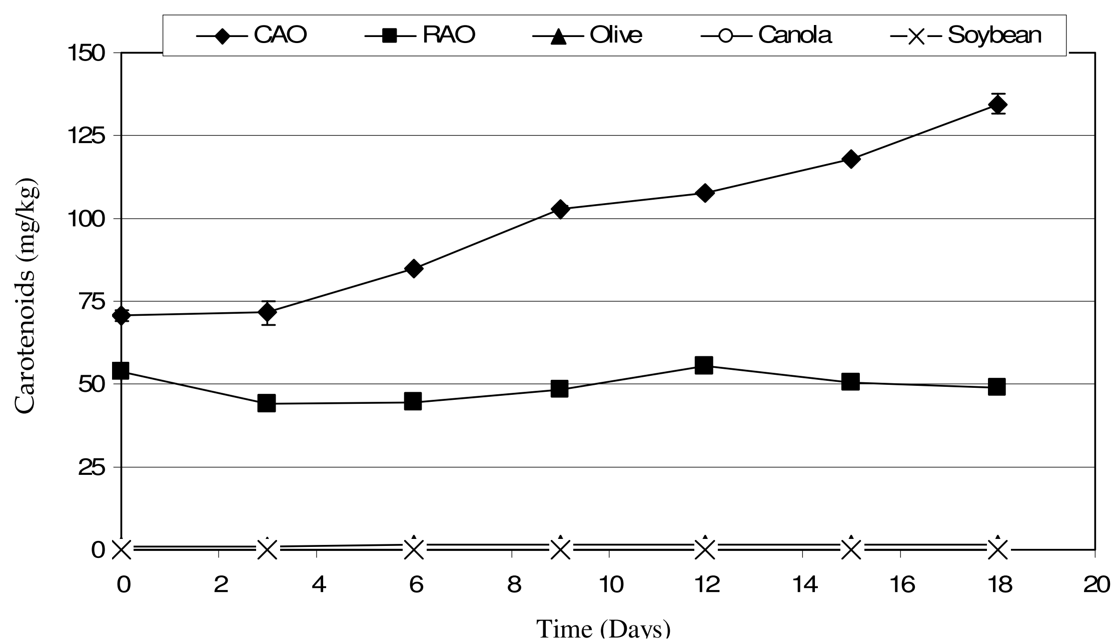


Figure 5-2. Carotenoid content of various oils stored at 63˚C for 18 days.

in either of the chlorophyll containing oils (CAO and RAO) over time. This trend was not observed in açai oil subjected to photooxidative conditions (Section 3) most probably due to the active role chlorophylls play in photooxidation. This stagnant trend in chlorophylls in this investigation could be due to the lack of sensitivity of the spectrophotometric analysis or the possible conversion of certain chlorophylls to other chlorophylls.

5.3.5 Changes in Peroxides during Storage

Peroxide formation was measured during storage as an assessment of lipid oxidation. An increase in peroxide value over time is a strong indication of the propagation cycle in free radical induced oxidation, which rapidly decreases oil quality (Frankel 1991). The highly refined commercial cooking oils from soybeans and canola were expected to rapidly oxidize under the conditions of storage and served as a positive control for the rapid deterioration of oils during storage. For soybean oil, a marked increase in peroxide value (51.8 milliequivalents of peroxides per kg) was observed during storage (Figure 5-3) whereas its oxidation progressed more rapidly after day 12 with a 3-fold increase in the final 6 days. Increases in peroxides for canola oil followed a similar trend as soybean oil over 18 days with an increase from 1.42 to 40.7 milliequivalents of peroxides per kg.

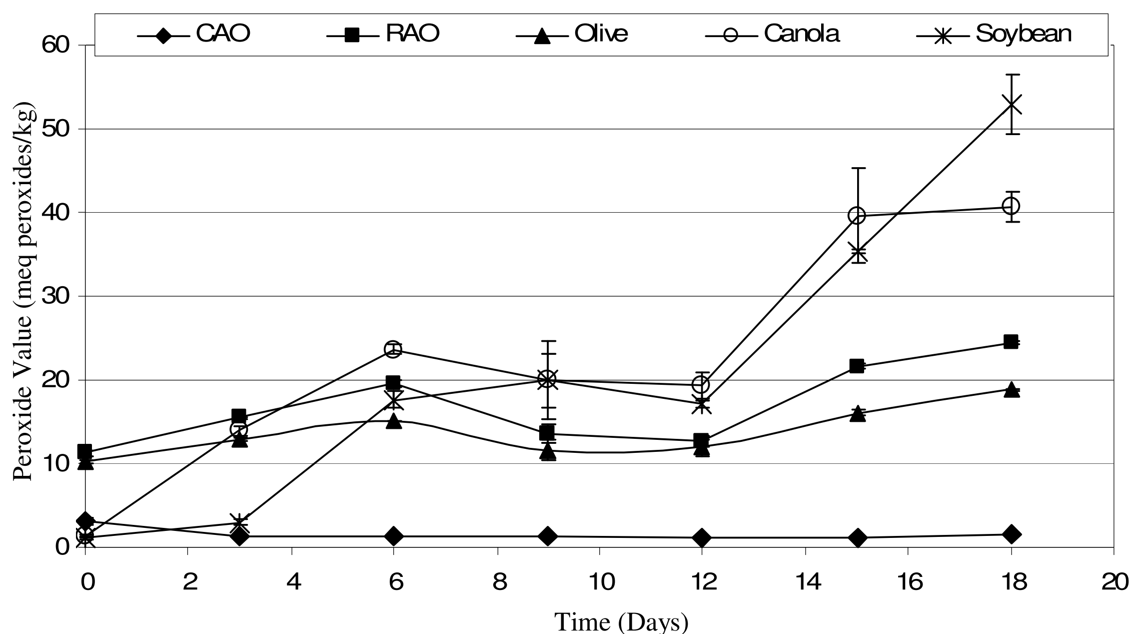


Figure 5-3. Primary oxidation products of various oils stored at 63˚C for 18 days.

Other than its organoleptic attributes, extra virgin olive oil is widely known to experience minimal refining that results in high concentrations of chlorophyll, polyphenolics, and lipophilic antioxidants generally associated with its relative stability over other refined oils (Velasco and Dobarganes 2002). Studies have reported virgin olive oils to contain between approximately 150 and 700 mg/kg of total soluble phenolics depending upon a number of factors, such as cultivar and degree of maturation (Visioli and Galli 2002) and while a myriad of water soluble phenolics exist in olive oil the predominant compounds include hydroxytryrosol, tyrosol, and oleuropein (Brunelleschi and others 2007; Papadopoulos and Boskou, 1991; Visioli and Galli, 2002; Deiana and others 2007). During storage in these trials, olive oil exhibited lower

peroxide formation than both canola and soybean oils. Over 18 days of storage, the peroxide value increased 83.8% with the major increase (11.7 to 18.8 milliequivalents per kg) occurred in the last 9 days.

As a result of the extraction process to obtain CAO, the oil is rich in both hydrophilic and lipophilic antioxidants. These antioxidants may play a significant role in the stability of CAO compared to highly refined oils yet should compare somewhat directly with an unrefined oil such as extra virgin olive oil. During storage a slight decrease (1.92 milliequivalents of peroxides per kg) in peroxides occurred over the fist three days, however, peroxide value remained constant thereafter. After 18 days of accelerated storage conditions, the peroxide value of the crude açai oil did not surpass the initial value of 3.16 milliequivalents of peroxides per kg. The rich phytochemical content of CAO was hypothesized as a critical factor in the preventing the onset of lipid oxidation. On the contrary, a 116% increase in peroxides in the RAO occurred over the 18 days of storage, reaching a final peroxide value of 24.4 milliequivalents of peroxides per kg. Increases were more pronounced between days 9 and 18, which is similar to the trend observed with olive oil. These trends suggest that the antioxidants played a role in prolonging the induction period; however could not retard the onset of lipid oxidation.

5.3.6 Changes in Nonanal during Storage

Secondary oxidation products are mainly aldehydes and short chain hydrocarbons that result from β-scission reactions that follow lipid oxidation. These degradation products often impart rancid odors, which in trace amounts can significantly

decrease the quality of oils. Commonly, certain aldehydes, such as hexanal, nonanal, and pentanal, are tracked over storage to gain an understanding about the progression of lipid oxidation. As discussed in Section 3, investigations into the markers of secondary oxidation products of açai oil led to the determination that nonanal is the degradation product most associated with lipid oxidation. The determination that nonanal is the most suitable marker for secondary oxidation in açai oil was also supported by the data obtained in this study.

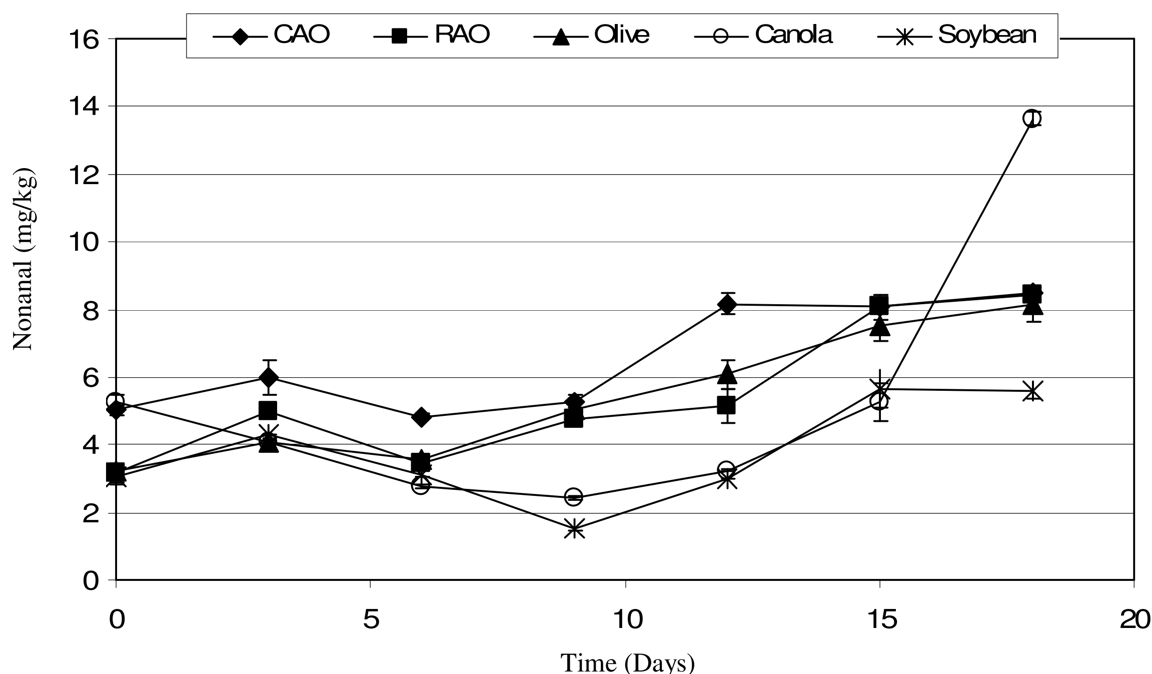


Figure 5-4. Nonanal concentration of various oils stored at 63˚C for 18 days.

As seen in Figure 5-4, a net increase in nonanal was observed in all oils over the 18 days of storage at 63°C. Nonanal concentrations of CAO increased 69.1% (5.01 to 8.47 mg/kg) over this time period, the smallest increase among the oils compared to the

most extreme change in RAO, which increased 165%. Increases of 151, 158, and 84.6% were observed in olive, canola, and soybean oils, respectively. While soybean oil exhibited the largest increase in peroxides, its increase in nonanal was relatively small. This discrepancy could be explained by examining the fatty acid composition of soybean oil. As discussed by Frankel and others (1984), the conversion of primary to secondary oxidation products is a process that is often dependent on fatty acid composition. Soybean oil has been reported to contain elevated levels of linoleic acid (54%) and lower levels of oleic acid (23%) (Mounts and others 1988), whereas all other oils studied in this experiment are composed of 60% or greater oleic acid (Warner and Mounts, 1993; Gutfinger, 1981). While a myriad of secondary oxidation products can be formed from primary oxidation product degradation, nonanal was selected as a marker for this study because of previous investigations (Section 3) into oxidation of açai oil, an oil high in oleic acid. With such a different fatty acid composition the secondary oxidation products generated in soybean may be different than that of the other high oleic acid oils. Thus, secondary oxidation may still be occurring in the soybean oil, but it was not observed in the analysis performed in this experiment.

5.3.7 Theory for Difference in Behaviors of RAO and CAO

Though the fatty acid composition of RAO and CAO were identical, the concentration of hydrophilic antioxidants and phospholipids varied significantly. The phospholipid content of CAO and RAO was 0.9 and 0.3%, respectively. It is hypothesized that as a result of the higher phospholipid content as well as the processing

method, RAO contains 331 mg/kg water soluble phenolics as compared to 1840 mg/kg in CAO.

Given the compositional similarity of CAO and RAO, the stability of CAO was likely attributable to an elevated concentration of lipophilic antioxidants or higher concentration of water soluble phenolics. While higher concentrations of lipophilic antioxidants are known to enhance the stability of oils (Farombi and Britton 1999; Hirayama and others 1994; Huang and others 1994; Kamal-Eldin and Appelqvist 1996; Romero and others 2007; Terao and Matsushita 1986; Yang and others 2002), the relative difference between the RAO and CAO was small. Meanwhile, it has been reported that in lipophilic systems containing emulsions, the more effective antioxidants are water-soluble rather than lipophilic (Frankel and others 1994). In the CAO, the use of SDA35A allowed for the incorporation of water soluble phytochemicals into the miscella. As the solvent is removed, the incorporation of water soluble phenolics is presumed to be reliant upon the incorporation into phospholipid reverse micelles (Majid and others 2000). Thus, it is theorized that the exceptional content of water soluble antioxidants is a result of the extraction process combined with naturally present phospholipids.

5.4 Conclusions

Primary and secondary oxidation data suggests that CAO is highly stable in relation to the others oils examined in this study. RAO, which contains lower concentrations of phospholipids and water soluble phenolics, exhibited less stability than

the CAO; however it was still more stable than other oils examined. Determining the exact reason for the stability of CAO proved to be challenging. Significant increases in the two predominant lipophilic antioxidants occurred over the entire storage period. These increases could be due to potential interactions with phospholipids. Furthermore, contributions from water soluble antioxidants were not assessed in this study. Investigations into the contributions of the water soluble components of açai oil could help determine the reason for the stability of CAO.

6. THE OXIDATIVE STABILITY OF BLENDED AÇAI OILS

6.1 Introduction

Recent advancements in the processing of açai pulp by-product have lead to the creation of two distinctly different açai oils: partially-refined açai oil (RAO) and crude açai oil (CAO). CAO is hydroalcoholic extract of the diatomaceous earth filter cake from the açai pulp clarification process (Talcott 2008). RAO is a hot water-rendered oil, also obtained from the açai pulp clarification process. As a result of the process used to extract CAO, it has a unique chemical composition. While its fatty acid composition is very similar to other common oils such as olive oil (Lubrano and others 1994; Gutfinger 1981), its antioxidant chemistry is distinctive. As described in Section 3, CAO has been shown to be rich in lipophilic phytochemicals such as carotenoids and tocopherol, as well as hydrophilic phytochemicals such as phenolic acids and procyanidins (Pacheco-Palencia and others 2008). Moreover, it has been hypothesized that phospholipid could play a role in the incorporation of these antioxidants into the açai oil matrix (Section 4).

Previous research has demonstrated that RAO and CAO behave differently under accelerated storage conditions. As observed in Section 5, the peroxide value of CAO did not increase over 18 days of storage at 63˚C, while the peroxide value of RAO steadily increased over time. Compositionally, RAO and CAO are identical in fatty acid composition and both contain significant quantities of lipophilic antioxidants. The major differences in the two oils lie in phospholipid (0.9% for CAO, 0.3% for RAO) and total soluble phenolic (approximately 1800 mg/kg for CAO, 400 mg/kg for RAO) content.

Thus, it was hypothesized that these compositional dissimilarities are the key factors behind the difference in oxidative stability in the two oils.

The aim of these experiments was to determine if the chemistry of CAO could alter the oxidative stability of less stable oils in an effort to demonstrate the superior antioxidant potential of unaltered CAO. Understanding the role of CAO in the presence of oxidized oils is a key step in identifying the contributors to the stability of CAO itself.

6.2 Material and Methods

6.2.1 Crude and Refined Açai Oil Blends (Experiment I)

CAO was extracted from a water-insoluble filter cake that was used to clarify açai pulp in a commercial application utilizing a patent pending extraction protocol (Talcott 2008). RAO was produced by agitating the water-insoluble filter cake by product of the açai juice clarification process with hot water at 70˚C until phase separation was evident. Following decanting, the oil was washed with hot water once more and centrifuged until separated. The oil mixtures were blended at various ratios (1:3, 1:1, 3:1, CAO:RAO) by continuous mixing for 1 hr.

6.2.2 Crude Açai Oil Blends with Oxidized Canola Oil (Experiment II)

CAO was blended with oxidized canola oil (Canola Harvest, Canbra Foods Ltd., Lethbridge, AB, Canada; PV = 25 milliequivalents of peroxides per kg) by continuous mixing for 1 hr. The oils were combined at 3 ratios (1:9, 1:1, and 9:1, Canola:CAO) prior to storage.

6.2.3 Accelerated Storage

Oil samples (10 g) were stored in 40-mL screw cap, clear vials, with ambient air in the headspace. Samples were stored vertically in a temperature regulated chamber at 63 ± 2˚C. For the experiment I, individual vials were taken from the chamber every 3 days for 15 days and stored at -20˚C until further analysis. For Experiment II, individual vials were taken from the chamber after 24 hrs of storage.

6.2.4 Primary Lipid Oxidation Products

Peroxide value was assessed by the methods described in Section 5.2.3.

6.2.5 Total Soluble Phenolics by the Folin-Ciocalteu Assay

A modified version of the Folin-Ciocalteu assay (Swain and Hillis 1959) was used to analyze contributions of the water-soluble components to the total reducing capacity of the oils. Oil (0.1 g) was dissolved in 1 mL hexanes and mixed vigorously for 30 sec. Then, 1 mL of Folin-Ciocalteu reagent (2 N) was added followed by vigorous mixing for 30 sec. One mL of sodium carbonate (0.5 N) was vigorously mixed (30 sec.) in exactly 3 minutes after the Folin-Ciocalteu reagent was added. Exactly 7 minutes after the addition of the sodium carbonate, 5 mL of deionized water was added and the mixture was centrifuged (1000 rpm for 5 min). Aliquots were taken from the bottom layer and absorbencies were compared to a standard curve (mg/L) of gallic acid at 726 nm.

6.2.6 Statistical Analysis

Data for Experiment I was analyzed as a 5 x 6 full factorial that included five oil blends analyzed at six sampling times. Data for Experiment II was analyzed as a 4 x 2 factorial that included four oil blends analyzed at 2 sampling times. Data represent the mean of triplicate determination. Multiple linear regression, Pearson correlation coefficients and analysis of variance, were conducted using JMP software Version 5 (SAS Institute, 2002), with mean separation performed by the LSD test ($P<0.05$).

6.3 Results and Discussion

6.3.1 Changes in Primary Oxidation Products of Blended Açai Oils (Experiment I)

The formation of peroxides is a key indicator of the oxidation of lipids. Peroxide values were monitored in this experiment to assess the ability of CAO, which was blended into less stable oils, to act as an antioxidant and thereby retard the formation of peroxides. Initial peroxide values for CAO and RAO were 2.5 and 17.5 milliequivalents of peroxides per kg, respectively. The initial values for the blended oils were linear within this range. Figure 6-1 details the trends in peroxide values over storage. Peroxide value decreased over storage in all blended açai oils. Over the 15 days of storage, decreases of 66.7, 70.0, and 75.6% were observed in the oils containing 25, 50, and 75% CAO. The decrease in peroxides over time in the blended oils demonstrated the ability of antioxidants present in CAO to quench radicals. It is uncommon to add antioxidants to oils that have begun to oxidize; however, the phytochemical content of CAO has shown

an ability to act against free radicals in a propagation cycle. Therefore, this data provides insight into CAO and its remarkable stability over storage in extreme conditions.

The major phytochemical differences between these two açai oils are in phospholipid and total soluble phenolic content. From previous investigations it was demonstrated that contributions from phospholipids alone to the oxidative stability of açai oil is minimal (Section 4). Therefore, it can be theorized that the main contributor to the stability of CAO and CAO blends are the water soluble antioxidants. This theory is supported by Frankel and others (1994), who stated that the contributions of hydrophilic antioxidants in an emulsion system are crucial to the stability of the system. It is proposed that the underlying mechanism behind the effectiveness of hydrophilic antioxidants in water-in-oil emulsions is explained by their affinities towards the interfacial regions of the emulsion system (Frankel and others 1994).

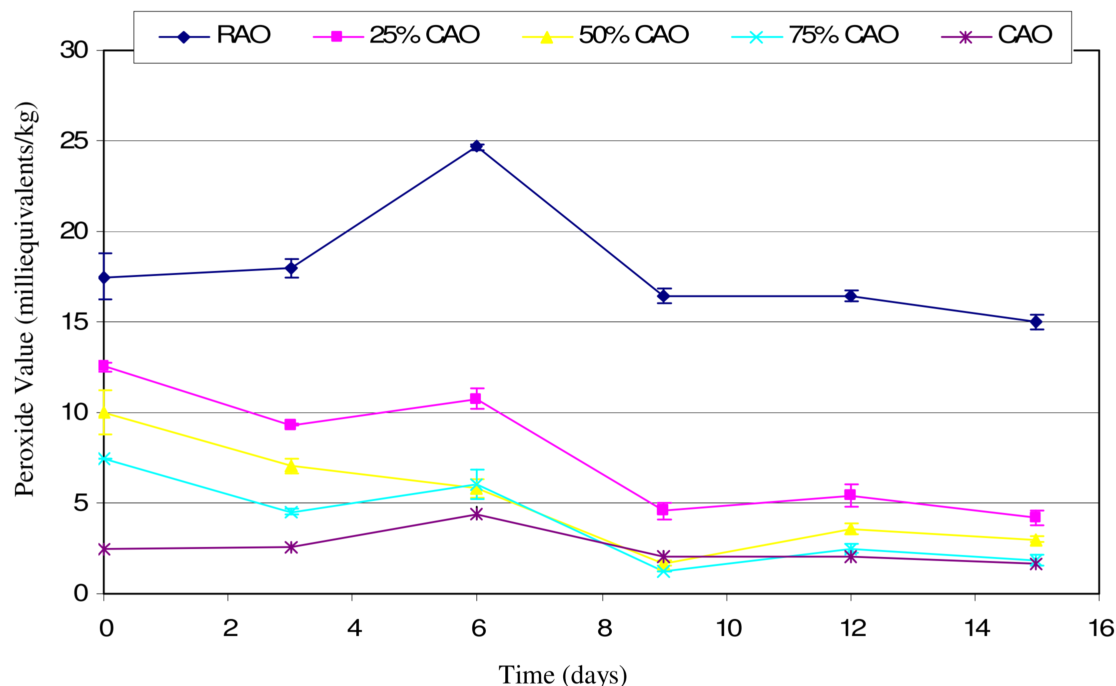


Figure 6-1. Changes in the primary oxidation products in blended açai oils over time.

6.3.2 Changes in Total Soluble Phenolics in Blended Açai Oil (Experiment I)

With such a significant soluble phenolic content, it is imperative to understand the changes in total soluble phenolics of açai oil over time. Investigations have indentified a myriad of water soluble phenolic in CAO including vanillic acid, syringic acid, *p*-hydroxybenzoic acid, protocatechuic acid, ferulic acid, (+)-catechin, and procyanidin oligomers (Pacheco-Palencia and others 2008). Figure 6-2 details the trends in total soluble phenolics over accelerated storage. Decreases of 26.9, 15.2, 19.0% were observed over 15 days of storage in the three blended oils (75, 50, 25% CAO,

respectively). Losses of 34.9% were recorded in the CAO, while a slight increase in total soluble phenolics occurred in RAO.

Given the differences in the chemistry and stability of CAO and RAO, it can be deduced that the loss in total soluble phenolics is responsible for the stability of CAO. This theory can be supported by examining the stability of olive oil, which also contains water soluble phenolics. Numerous studies have been conducted that attribute the stability of olive oil in part to its water soluble phenolic content, which has been shown to be as high as 531 mg/kg (Papadopoulos and Boskou 1991; Psomiadou and Tsimidou 2002; Satue and others 1995). Furthermore, a linear correlation between oxidative stability and polyphenol content has been demonstrated in olive oil (Gutierrez and others 1977). Nergiz and Ünal (1991) also reported that the differences in olive oil processing lead to reduced polyphenolic content, which was responsible for lower stability.

6.3.3 Changes in Primary Oxidation Products of Açai Oil Blended with Oxidized Oil (Experiment II)

A key step in gaining a perspective on the potential mechanism behind the stability of CAO is to understand the interaction between the phytochemicals present in CAO and peroxides is. Blending CAO with an oil of lower stability, such as RAO, was shown to increase overall stability of the blended oil. However, it is unknown if the addition of CAO to an oxidized oil reduced the concentration of primary oxidation products immediately.

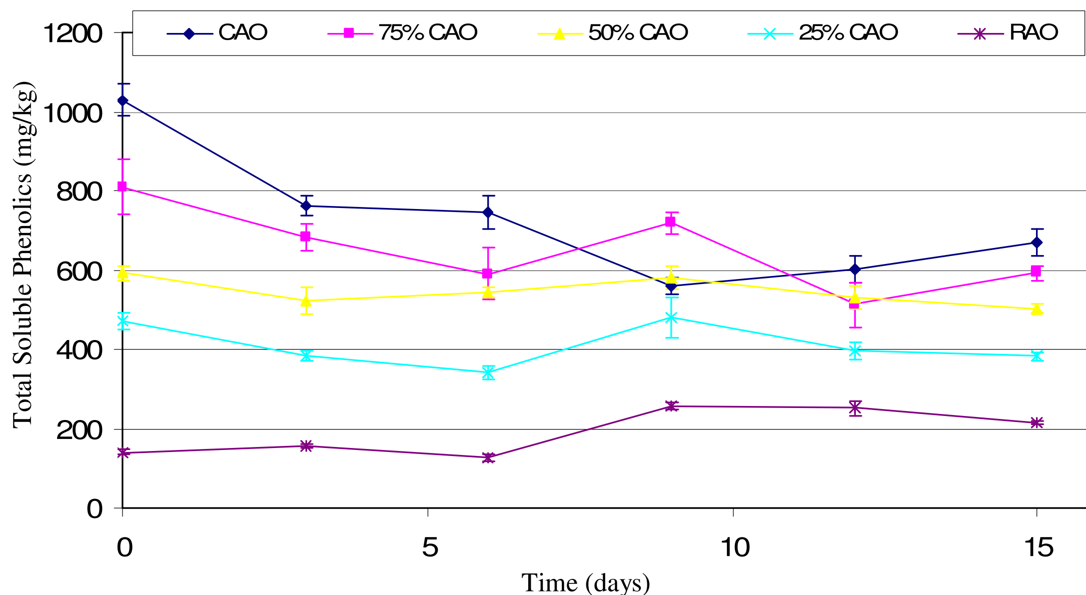


Figure 6-2. Changes in total soluble phenolics in blended açai oils over time.

Figure 6-3 details the changes in blends of CAO and oxidized canola oil over 24 hrs storage. An increase (2.3 milliequivalents of peroxides per kg) in peroxide formation occurred in the canola oil over 24 hrs of accelerated storage. Meanwhile, decreases of 1.7 and 2.4 milliequivalents of peroxides per kg were observed in the 10 and 50% CAO blends, respectively. No significant change occurred in the 10% CAO, which demonstrates the potency of the antioxidants present in CAO. It is widely known that antioxidants retard the formation of primary oxidations products, yet adding antioxidants to oxidized oils does not rejuvenate the oil (Sherwin 1978). In this experiment, the addition of CAO to oxidized canola oil decreased the concentration of peroxides. Given these findings and the findings of experiment I, it can be theorized that the water soluble phenolic content is responsible for this antioxidant effect. The extraordinary content of

water soluble phenolics present in CAO can be attributed to the presence of phospholipid reverse micelle.

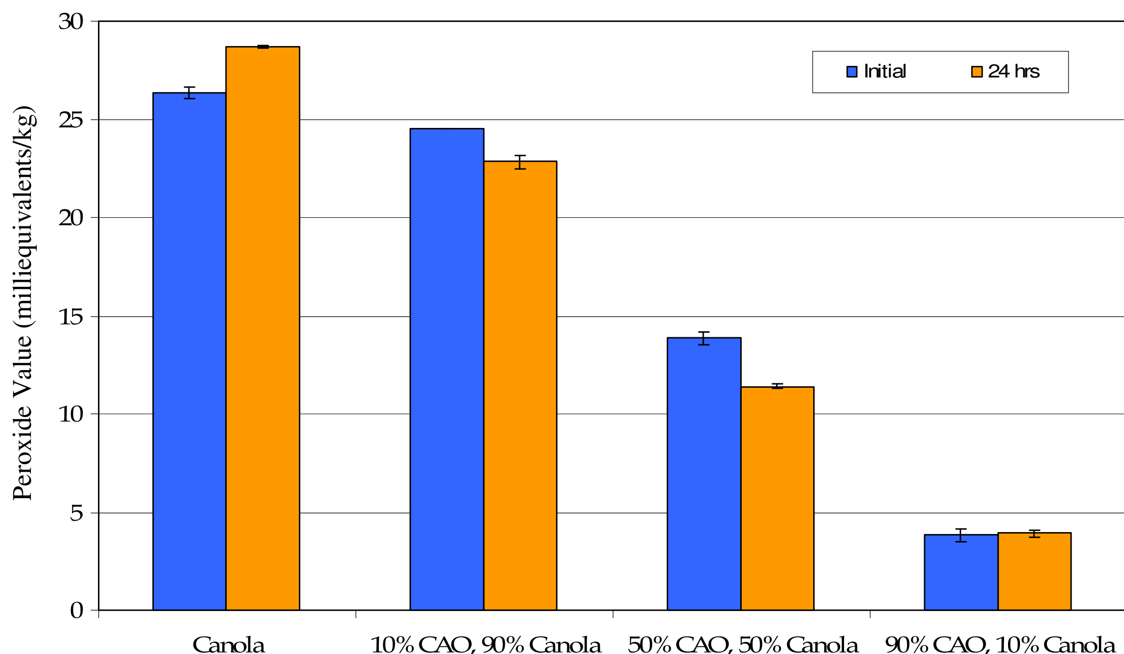


Figure 6-3. Changes in the primary oxidation products in an oxidized oil blended with açai oil over time.

6.4 Conclusions

The addition of CAO to RAO lowered peroxide values of the blended oils over time. This data suggests that there is a significant antioxidant contribution from the CAO. Given the compositional similarities of the two açai oils, it can be theorized that the driving factor behind the stability of CAO can be attributed to its water soluble antioxidant content. Additionally, the addition of CAO to oxidized oil also lowered peroxides within 24 hrs of storage. While it is not common for antioxidants to rejuvenate oils once they have begun to oxidize, the unique chemistry of CAO was shown to lower

peroxide formation of oxidized canola oil. The lower occurrence of peroxide formation is indicative of the high antioxidant activity of CAO. Furthermore, the unique chemistry of CAO is mainly due to the inclusion of water soluble phenolics in phospholipid reverse micelles, which is theorized to account in part for the remarkable stability of CAO.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Açai oil is a by-product of the açai pulp clarification process, which yields clarified juice and an oil-rich diatomaceous earth filter cake. This filter cake is then subjected to a patent pending extraction process to yield a phytochemical-rich crude açai oil (CAO). Initial investigations into CAO focused upon the photooxidative stability of the oil due to its significant chlorophyll content. Findings demonstrated that the stability of CAO was higher than other common oils. Observations also showed that the stability of the oil is decreased when phospholipids and polyphenolics are removed.

Investigations into the interactions between phospholipids and lipophilic antioxidants and their contributions to the stability of CAO were also assessed by isolating phytochemicals and selectively reconstituting the oil. This study demonstrated that lipophilic antioxidants in the presence of phospholipids reduced oxidation as compared to phospholipids or lipophilic antioxidants alone, suggesting a potential synergistic relationship. However, it is difficult to draw accurate conclusions from this experiment since the reconstituted oils did not exhibit the same oxidative stability as CAO. More research was conducted to attempt to more accurately reconstruct the oil, yet attempts were not successful.

Advances in the processing of the diatomaceous earth filter cake by product created from the açai pulp clarification process yielded a partially refined açai oil (RAO). Investigations into the auto-oxidative stability of both RAO and CAO showed

that their stability in relation to other common oils (olive, canola, and soybean) was higher. Moreover, primary and secondary oxidation data suggests that CAO is more stable than RAO. This difference in the stability of the two açai oils is theorized to be a direct result of the main phytochemical differences in the oils, namely, the phospholipid and water soluble antioxidant content.

Due the difference in stability between RAO and CAO, the stability of blends of these two oils was studied. The stability of RAO was observed to increase when blended in various proportions with CAO. Blending CAO to RAO was shown to decrease peroxide values over time, which suggested a significant antioxidant contribution from the CAO. With such similar lipophilic compositions in CAO and RAO, it is theorized that the driving factor behind the stability of CAO can be attributed to its water soluble antioxidant content. It was also demonstrated that the addition of CAO to oxidized oil lowered peroxides within 24 hrs of storage. This antioxidant effect can be attributed to the unique composition of CAO, particularly the significant content of water soluble phenolics contained in phospholipid reverse micelles.

The understanding and information obtained in these studies is geared at increasing the marketability of açai oil as a food ingredient. It was demonstrated that the unique phytochemical content of açai oil increased the stability of other oils including oils which are already oxidized. Due to the cost of açai oil, the most economical use may be as an oil that is blended with other, less stable oils. Blending açai oil with other oils will provide stability as well as increase the antioxidant potential of the oil.

7.2 Recommendations

A better understanding of the physical and chemical interactions of all components of açai oil would be beneficial to completely comprehend the stability of açai oil. While conducting the research presented here in this dissertation, numerous hindrances arose as a result of the açai oil matrix. The significant hydrophilic phytochemical content of açai oil creates an inherently complex matrix. As a result, many common analytical methods geared for refined oils are not possible with açai oil due to various interferences. The development of analytical methods that are more closely tailored to the complexities of the açai oil matrix would aid in the analysis of the oil.

The ability to understand the formation of the phospholipid reverse micelles during the oil extraction would also lead to a better understanding of the oil. Numerous attempts were made to mimic the composition and extraction of açai oil in various model systems; however, efforts were not successful. Findings suggested that if components of the oil are selectively isolated and the oil is reconstituted it does not behave the same as the original oil (Section 5). Furthermore, constructing the oil of commercially extracted components and obtaining similar stability to CAO also was not successful. Identifying a method by which the oil can be isolated and reconstituted while maintaining the stability of the oil would help to better understand the phytochemical contributions to the stability of the oil.

Future investigations into the oxidative stability of CAO should focus upon identifying the factors that promote the inclusion of water soluble phytochemicals in the

açai oil matrix. Investigating the formation of reverse micelles and the factors that affect their physical and chemical interactions could also provide insight into the stability of the oil. Eventually, this knowledge could lead to increasing stability of common food grade oils by incorporating a antioxidant rich reverse micelle system as observed in CAO.

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APPENDIX A. INTERACTION BETWEEN LIPOPHILIC PHYTOCHEMICALS AND PHOSPHOLIPID REVERSE MICELLES

The analytical interactions between phospholipids and lipophilic antioxidants were assessed to better understand the findings in Sections 3, 4, and 5.

A.1 Material and Methods

A.1.1 Formation of Reverse Micelles in the Presence of Lipophilic Antioxidants

A 2% soy lecithin (Alfa Aesar, Ward Hill, MA) solution in hexanes (5 mL) was mixed thoroughly (15 min at 2000 x *g*) with 5 mL of soybean oil, 10 μL of deionized water, and varying concentrations of α-tocopherol, β-carotene, or both. Oils contained five concentrations of α-tocopherol (100, 200, 300, 400, and 500 mg/kg), five concentrations of β-carotene (20, 40, 60, 80, and 100 mg/kg) and an inverse gradient of both were created to asses dose-dependent effects (n=1).

A.1.2 Storage of Oils

Oils were stored in 15 mL clear screw cap test tubes at 63˚C for 10 days.

A.1.3 Lipophilic Phytochemicals Determined by HPLC

Lipophilic phytochemicals (α-tocopherol and β-carotene) were determined according to the methods described in Section 3.2.2.

A.2 Results and Discussion

A.2.1 Increases in β-Carotene and α-Tocopherol due to Phospholipid Interaction

Previous data has demonstrated an increase in carotenoids during the storage of açai oil. These investigations were designed to understand the potential interactions of lipophilic phytochemicals and phospholipid reverse micelles over time. Initial analysis of β-carotene was made immediately after mixing, while final analysis was made after 10 days of storage at 63˚C. Initial concentrations were lower than expected, however, a 20% increases in concentration on average was observed for β-carotene after storage. These increases observed over storage could potentially be due to the same factors that are responsible for the increases in β-carotene observed in Sections 3, 4, and 5. Figure A-1 diagrams the changes in β-carotene in the oils in which the only lipophilic antioxidants was β-carotene.

Figure A-2 indicates changes observed in the oils with added α-tocopherol. Increases were also observed for α-tocopherol after 10 days storage. However, as the concentration of α-tocopherol added increased the experimental values deviated more from the spiked values. These findings provide insight into the increases observed in α-tocopherol as described in Section 5. Figure A-3 details the changes in the oils which contained both β-carotene and α-tocopherol in inverse proportions. These results exhibited similar trends to the oils containing just one lipophilic antioxidant, which suggested that there is no interfacial competition between α-tocopherol and β-carotene.

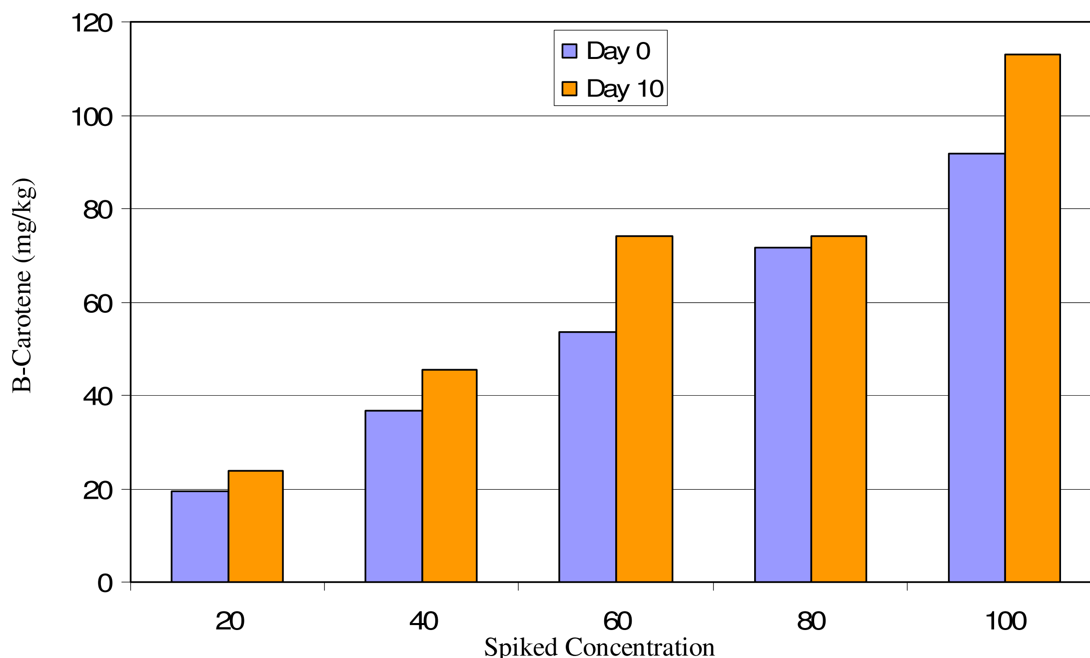

Figure A-1. Changes in β-carotene concentration after 10 days of storage in an oil and reverse micelle solution (n=1).

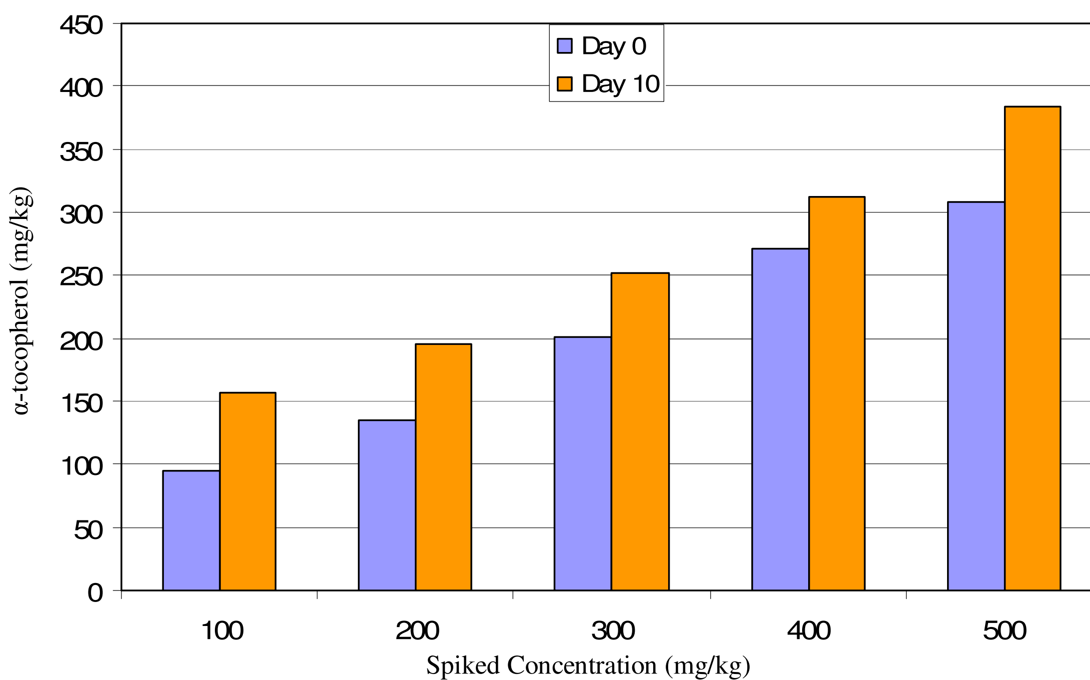

Figure A-2. Changes in α-tocopherol concentration after 10 days of storage in an oil and reverse micelle solution (n=1).

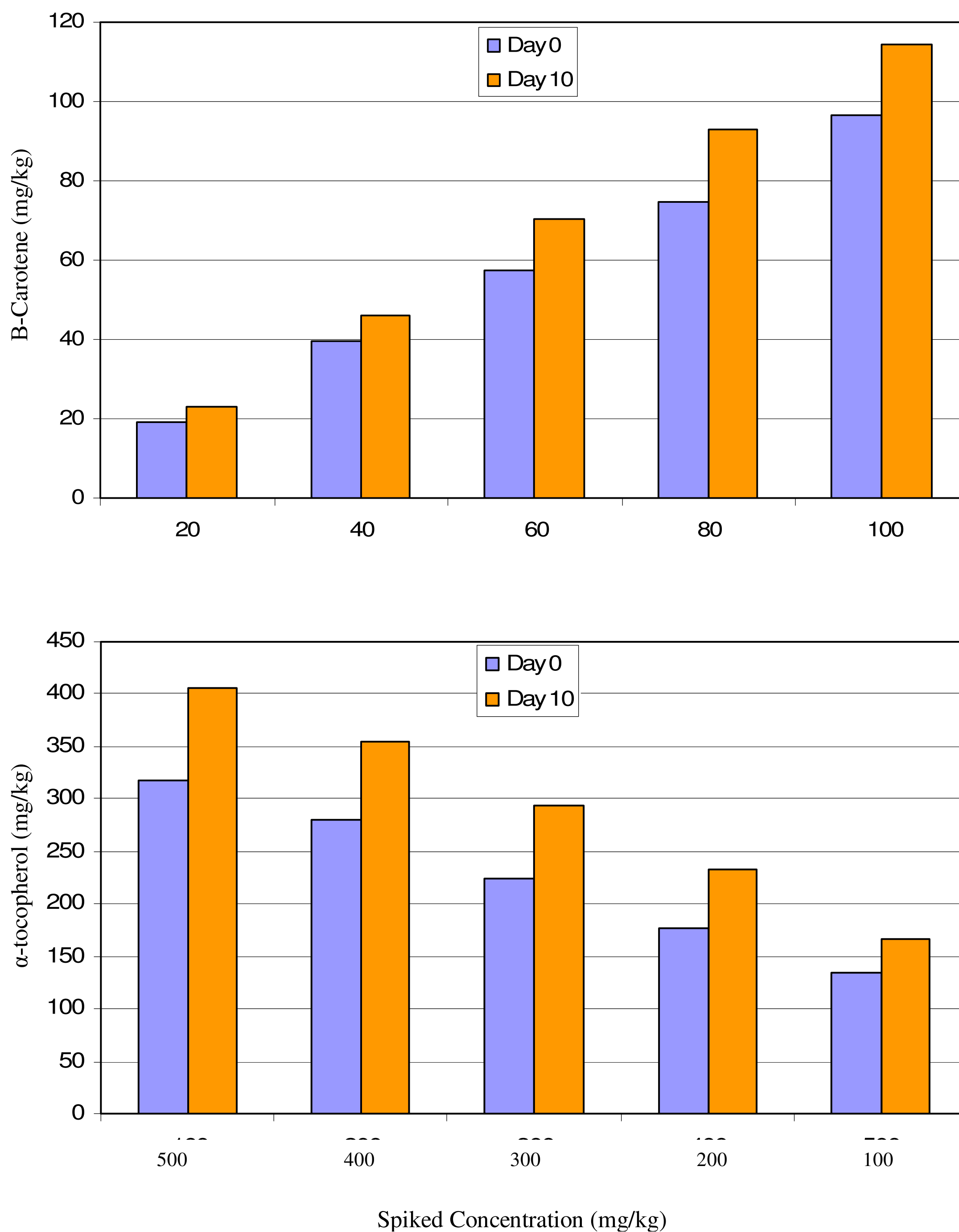

Figure A-3. Changes in α-tocopherol and β-carotene concentration after 10 days of storage in an oil and reverse micelle solution (both lipophilic antioxidants were spiked in the same oil) (n=1).

The addition of phospholipids to an oil matrix, especially in the presence of small quantities of water should facilitate the formation of reverse micelles (Aliotta and others 2002). When amphipathic molecules, such as phospholipids, assemble in a lipophilic system they often form 3-dimensional structures that orient with the lipophilic regions of the molecule facing outward (Hauser 1993). Moreover, the hydrophilic components of the system are often encapsulated in the reverse micelle due to its core's relative hydrophilic nature. Research has shown that carotenoids can act as "end-cap active agents" drastically affecting the viscoelastic properties of the reverse micelle system (Cirkel and others 1999). Furthermore, Montenegro and others (2009) demonstrated that the spectral properties of carotenoids were shifted when present in matrix that included a reverse micelle, which suggested that carotenoids interact with the micellar interface. Investigations have also suggested that the interaction of carotenoids at reverse micelle interfaces could be due to the presence of –OH and –COOH groups that can interact with the hydrophilic regions of the reverse micelle (Correa and others 2001). This interaction is demonstrated in Figure A-4. Meanwhile, A synergistic effect between tocopherol and phospholipids has been investigated on multiple occasions, yet the interaction between the two compounds is not completely understood (Bandarra and others 1999; Hildebrand and others 1984; Dziedzic and Hudson 1984).

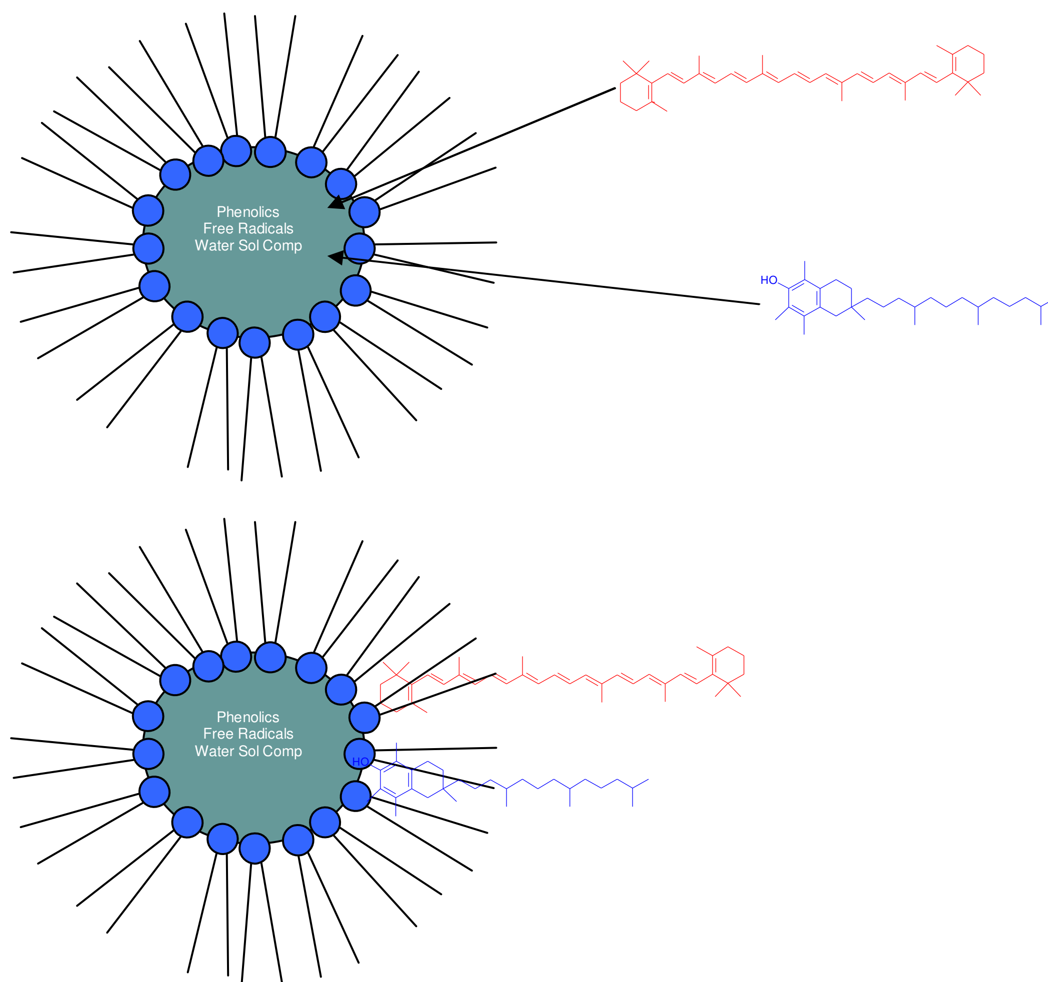

Figure A-4. Theoretical diagram of the interactions between phospholipid reverse micelles (blue spheres with tails) and α-tocopherol (blue structure) and carotenoids (red structure).

While it is not possible to synthesize carotenoids and tocopherols in this model system, it can be presumed that the increase in lipophilic compounds over storage is due to the interference in detection of those compounds during the initial analysis. One

possible explanation for this interference could be the interaction of these compounds with phospholipid micelles. In a separate analysis, a 17% decrease was observed in detector response of a mixture of 2% phospholipids, oil, and β-carotene as compared to an equivalent concentration of β-carotene in oil. As shown by Cirkel et. al. (1999), β-carotene is thought to interact with the interfacial region of reverse micelles, effectively altering the viscosity of the system. Over storage at elevated temperatures, the fluidity and integrity of a phospholipid reverse micelles is known to fluctuate (Schurtenberger and others 1989). Moreover, it is unknown how the interactions between lipophilic antioxidants and phospholipids change during storage. One possible explanation for the perceived increase in lipophilic antioxidants over storage could be the subsequent release from the interfacial region of the reverse micelle, which would allow for those compounds to be detected by chromatographic analyses. However, this theory could not successfully be confirmed through analytical attempts.

APPENDIX B. LINEARITY OF ß-CAROTENE STANDARD CURVE IN THE PRESENCE OF PHOSPHOLIPID REVERSE MICELLES

The linearity of a β-carotene standard curve in the presence and absence of phospholipids was investigated to assure that there were no analytical interferences between phospholipids and carotenoids. This investigation was geared towards supporting data in Sections 4, 5, and 6.

B.1 Material and Methods

B.1.1 Analytical Interaction between Phospholipids and β-Carotene

The linearity of a β-carotene standard curve in oil and hexanes was examined in the presence and absence of phospholipids. Ten concentrations of β-carotene ranging from 1 to 13 mg/kg were mixed (15 min at 2,000 x *g*) with and without 1% lecithin into 10 g soybean oil in hexanes (1:1) containing 50 µL of water. The oils were then analyzed 6 hours after mixing.

B.1.2 Lipophilic Phytochemicals

Lipophilic phytochemicals (α-tocopherol and β-carotene) were determined according to the methods described in section 3.2.4.

B.2 Results and Discussion

A number of investigations have prepared reverse micelle systems in the presence of water and carotenoids (Montenegro and others 2009; Cirkel and others 1999; Aliotta and others 2002). The preparation of a reverse micelle system, particularly in oil, is dependent upon the concentration of water, among other factors (Aliotta and others 2002). Furthermore, the critical micelle concentration (CMC) must be surpassed to yield the formation of micelles (Fulton and Smith 1988). While both of these parameters are unknown for the model system in this experiment, the concentrations used were modeled after previous investigations into β-carotene and reverse micelle interactions (Cirkel and others 1999).

The linearity of β-carotene in oil with and without phospholipids was assessed to identify if analytical interferences may exist. The linearity of the mixtures was examined over concentrations ranging from 1 to 13 mg/kg β-carotene. Figure B-1 details the linearity of both mixtures. In both instances, R^2 values of 0.999 or greater were obtained assuring that the actual concentrations of β-carotene coincided with the intended, spiked concentrations. Furthermore, though there was a slight divergence of trend lines as the concentrations increased, the slopes were within 5%. This difference is no significant enough to demonstrate that there is a definitive interference from phospholipids during the HPLC analysis of β-carotene.

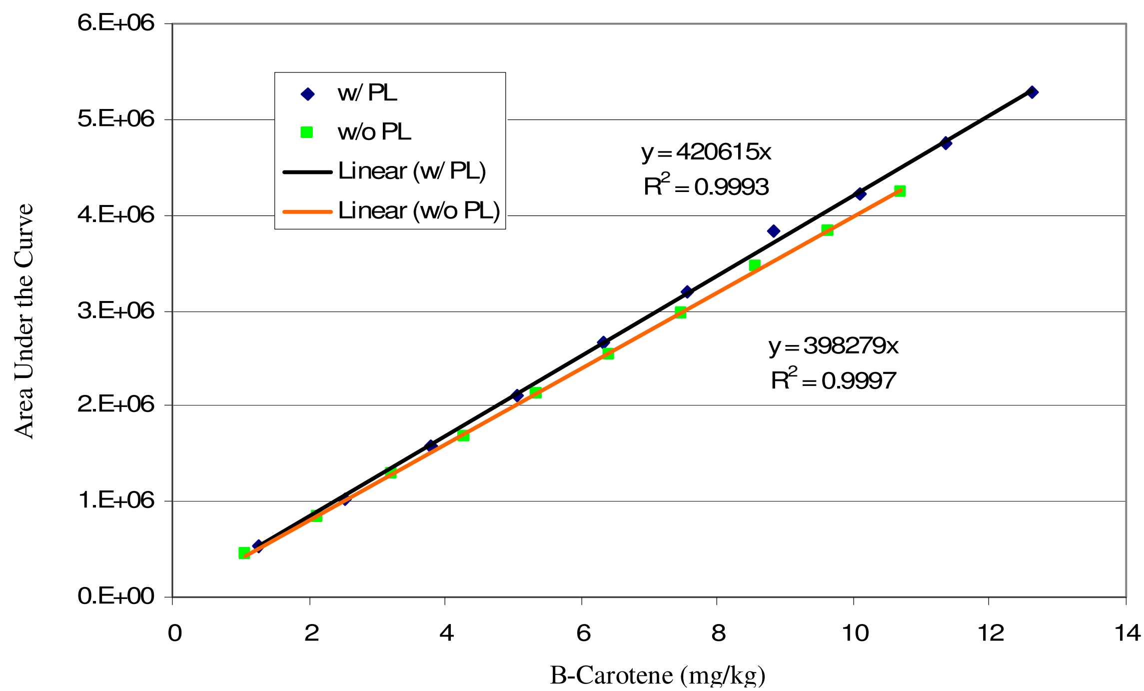

Figure B-1. Standard curve of β-carotene in the presence and absence of phospholipids.

VITA

Christopher Edward Duncan graduated Summa Cum Laude from the University of Florida with his Bachelor of Science in food science and human nutrition in May of 2006. While completing his undergraduate requirements at the University of Florida, Chris worked as a student researcher studying phytochemicals and antioxidants in numerous fruit and vegetable commodities. Following graduation, Chris began a year-long internship with the Kellogg Co. in Battle Creek, MI where he worked in the Food Chemistry Department developing analytical methods. At the conclusion of the internship, he came to Texas A&M University, to begin his doctoral program in food science and technology. Chris' research interests focused on lipid oxidation and phytochemicals. While at Texas A&M, Chris was an active in a number of organizations and held the positions of Product Development Team Leader, President of the Food Science Graduate Student Association, Captain of the College Bowl Team, and Area Representative for the IFT Student Association.

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