QUANTITATIVE CHEMICAL ANALYSIS OF FLAVONOIDs IN PURPLE FRUITED SELECTIONS OF PITANGA

(*Eugenia uniflora L.*)

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We certify that we have read this Thesis and that, in our opinion, it is satisfactory in scope and quality as a Thesis for the degree of Master of Science in Molecular Biosciences and Bioengineering.

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Dedication Page

This thesis is dedicated to my mother, father, sister, and grandmother who always throughout my life have supported me and believed in my potential. I am forever grateful for your love and strength and friendship. Thank you.
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I would like to sincerely thank my professor Jon-Paul Bingham for advising and motivating me through the past three years of graduate school. I am very grateful for Dr. John Griffis, who provided his guidance, support, and the opportunity to study the pitanga fruit. I have a great respect and gratitude for the help I have received from each one of my committee members, instructors and professors, and for the support and camaraderie of the Bingham Laboratory. I would like to thank my previous undergraduate research mentor from the University of South Florida; Dr. Bill J. Baker. Working with you and the Baker Lab members inspired me to continue on to graduate school. Thank you for providing such an excellent environment and experience. I would like to thank my colleagues; Jeff Milisen, Elizabeth Mahi, Liz Andrews and Pavlos Anastasiadis for support and advice.
Abstract

A variety of flavonoids have been quantified from purple-fruited seedling selections of pitanga (*Eugenia uniflora* L.). Flavonoids are widely known as antioxidants that function as photoprotectants in plants. Flavonoids have previously exhibited anticancer, antioxidant, and cardioprotectant effects. High Performance Liquid Chromatography was utilized to investigate the composition of flavonoids present in fruits of *E. uniflora*. Methods of quantification were improved using pre-purification methods to remove undesired compounds that may interfere with analyses. Lipophilic compounds were removed by a hexane/water partition. Pitanga extracts were passed through a preconditioned Waters Sep-Pak™ C₁₈ column. Anthocyanins and flavonols were selectively separated using ethyl acetate and 0.1N HCl MeOH. A Waters XBridge™ column facilitated selective retention, resolution, and high sensitivity. The pitanga fruits are underutilized as possible sources of dietary antioxidants and relatively unknown to the public. These results will be useful to exemplify the pitanga as a nutritionally desirable crop for consumption.
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List of Abbreviations and Symbols

API: atmospheric pressure ionization
C₈: octasilyl bonded-phase
C₁₈: octadecylsilyl bonded-phase
CF-FAB: continuous-flow fast-atom bombardment
CID: Collision Induced Dissociation
EGCG: Epigallocatechin gallate
ES: electrospray
ESI: Electrospray Ionization
HIV: Human Immunodeficiency Virus
HPLC: High Performance Liquid Chromatography
HSV-I: Herpes Simplex Virus I
IgE: Immunoglobulin E
KEGG database: Kyoto Encyclopedia of Genes and Genomes database
MAE: Microwave-assisted extraction
MeOH: MeOH
MS: Mass Spectrometry
MPO: myeloperoxidase
NADPH: Nicotinamide adenine dinucleotide phosphate
NMR: Nuclear Magnetic Resonance
Nm: Nanometers (10⁻⁹ meter)
ODS: octadecylsilyl bonded-phase
P450: Cytochrome P450, P: pigment, 450: reflects wavelength of the absorption maximum of the enzyme when it is in the reduced state and complexed with CO
PLE: Pressurized liquid extraction
ROS: reactive oxygen species
SAR: Structure-Activity Relations
SCF: stem cell factor
SFE: supercritical fluid extraction
SPE: solid-phase extraction
THF: tetrahydrofuran
TLC: thin-layer chromatography
TSP: Thermospray
UAE: Ultrasound-assisted extraction
UV: ultraviolet
Chapter 1: Literature Review on Flavonoids

1.1 Chemical Structure

Flavonoids are secondary metabolites produced by all vascular plants. More than 8150 structurally diverse flavonoids have been identified (Andersen & Markham, 2006) all of which possess a common polyphenol backbone consisting of two benzene rings linked through a heterocyclic pyran ring (Figure 1.1). Their naming and sub-classification is based on substituents present around this base structure (Table 1.1). Common examples of subclasses of flavonoids are flavones, flavonols and their glycosides, isoflavones, flavanones, chalcones, anthocyanins, and proanthocyanidins (Figure 1.2 and Table 1.1). Both flavonoids and tocopherols (Vitamin E) possess a chromane ring (De Groot & Raven, 1998).

![Figure 1.1: Polyphenol Backbone: carbon skeleton of flavonoid chemical structure](image)

Figure 1.1: Polyphenol Backbone: carbon skeleton of flavonoid chemical structure
Table 1.1 Chemical Properties of Flavonoids

<table>
<thead>
<tr>
<th>Flavonoid Class Member</th>
<th>Example</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Food Source</th>
<th>Amount **</th>
<th>Lambda Max *</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonol</td>
<td>Kaempferol</td>
<td>H</td>
<td>H</td>
<td></td>
<td>Kale</td>
<td>26.74</td>
<td>265, 365</td>
<td>Slightly sol. in water, soluble in hot alcohol, ether or alkalies</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>OH</td>
<td>H</td>
<td></td>
<td>Onions c</td>
<td>19.36</td>
<td>258, 375</td>
<td>Abs. alcohol, GAA*</td>
</tr>
<tr>
<td>Anthocyanin</td>
<td>Delphinidin</td>
<td>OH</td>
<td>OH</td>
<td></td>
<td>Blueberry</td>
<td>29.54</td>
<td>544 *</td>
<td>MeOH, ethanol, ethyl acetate Dil. Acid, alcohol, sodium carbonate solution</td>
</tr>
<tr>
<td></td>
<td>Cyanidin</td>
<td>H</td>
<td>OH</td>
<td></td>
<td>Elderberry</td>
<td>794.2</td>
<td>535 *</td>
<td>Sparingly soluble in water, soluble in abs. alcohol, MeOH</td>
</tr>
<tr>
<td></td>
<td>Malvinidin</td>
<td>OMe</td>
<td>OMe</td>
<td></td>
<td>Blueberry</td>
<td>49.21</td>
<td>542 *</td>
<td></td>
</tr>
<tr>
<td>Flavonone</td>
<td>Eriodictyol</td>
<td>OH</td>
<td>OH</td>
<td></td>
<td>Lemon j</td>
<td>4.88</td>
<td>290, 326</td>
<td>Sparingly sol. in boiling water, hot alcohol, ether, GAA*; sol. in dil alkalies</td>
</tr>
<tr>
<td>Flavon-3-ol</td>
<td>(+)-Catechin</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>Black grapes</td>
<td>11.99</td>
<td>278 *</td>
<td>Acetone, glacial acetic acid, hot water, alcohol</td>
</tr>
<tr>
<td></td>
<td>(-)-Epicatechin</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>Dark chocolate</td>
<td>41.5</td>
<td>278 *</td>
<td>Acetone, glacial acetic acid, hot water, alcohol</td>
</tr>
</tbody>
</table>

* in MeOHic HCl, = cooked, = in ethanol, = juice, = yellow, GAA* = glacial acetic acid, **=(mg/100g), n=(nm)

Overview of common flavonoid groups by structure, substituents, food sources, UV lambda maximum, and solubility properties
1.2 The oxidation-reduction potential of flavonoids

Flavonoids possess the ability to quench reactive oxygen species, as well as chelate to free metals. This is achieved by donating an electron or a hydrogen atom to a free radical such as a hydroxyl radical (• OH) or lipid peroxyl radical (LOO •) (Rice-Evans, Miller, & Paganga, 1996).

\[ \text{Fl} - \text{OH} + \text{R}^* \rightarrow \text{Fl} - \text{O}^* + \text{RH} \]

The antioxidant and prooxidant reactivity increases with the number of hydroxyl substitutions present in the backbone structure. The di-OH substitution at the 3’ and 4’
position showed to possess the greatest peroxyl radical absorption activity (Cao, Sofic, & Prior, 1997).

Myricetin, quercetin, rutin and catechin are a few examples of flavonoids which have been thoroughly studied for their ability to scavenge reactive oxygen species *in vitro* (Chen, Li, Zao, Zeng, & Xin, 1989; Laughton, Halliwell, Evans, & Hoult, 1989; Bors & Saran, 1987; Scott & Slatter, 1981). Bors, Heller, Michel, & Saran, (1990) studied and identified various flavonoids which scavenge reactive oxygen species. They were the first to propose function groups responsible for antioxidant activity.

In biological systems, the chemical structure of flavonoids determines where it will be potentially localized and useful. Since flavonoids are lipophilic in structure they are capable of infiltrating lipid bilayers (Terao, 2009). Flavonoids have shown to inhibit the oxidation of unsaturated fatty acids in membranes *in vitro* (Simpson & Uri, 1956). The flavonols kaempferol and quercetin demonstrated high scavenging activity against linoleic acid peroxyl radicals (Erben-Russ, Bors, & Saran, 1987).

### 1.3 Biological Function in Plant

Flavonoids are proposed to have evolved alongside vascular plants for the past billion years (Swain, 1975). These compounds are synthesized in all vascularized plants from the amino acids phenylalanine and tyrosine. These compounds are not synthesized in animals (De Groot & Raven, 1998). In plants, flavonoids are synthesized via the phenylpropanoid pathway in a multienzyme complex at the cytoplasmic side of the
endoplasmic reticulum (Stafford, 1974; Winkel, 1999). The flavonoid biosynthetic pathway has been characterized for a variety of organisms including *Arabidopsis thaliana* (thale cress), *Vitis vinifera* (wine grape), and *Zea mays* (maize). The detailed pathway is described at KEGG database (Kanehisa, Goto, Sato, Furumichi, & Tanabe, 2012; Kanehisa & Goto, KEGG: Kyoto Encyclopedia of Genes and Genomes, 2000). In fruits such as the grape, flavonoids are localized in the vacuoles and in the hypodermal tissues (Calderon, Florenciano, Munoz, & Barcelo, 1992). Flavonols and tannins are also transported for storage to the cell wall of the fruit as well as the seed (Braidot, et al., 2008). Flavonols localize in greater quantities on the surface of the fruit upon UV induction (Schmelzer, Jahnen, & Hahlbrock, 1988; Shirley, 1996).

Flavonoids possess a breadth of functions most integral for plant operation. Flavonoids attract animals to pollinate and spread seeds, signal Rhizobium bacteria to initiate nitrogen fixation, signal reabsorption process when cells in leaves are deficient in minerals (Gould & Lister, 2006; Firmin, Wilson, Rossen, & Johnston, 1986). Flavonoids function as antioxidants, pigments inhibitors of enzymes and light screens as well as provide protection from pathogens (Harborne, Mabry, & Mabry, 1975; Smith & Banks, 1986).

Numerous variables regulate the quantity of flavonoids produced in vascular plants. Plant hormones such as ethylene increase flavonoid production while gibberellic acid and inhibitors of ethylene receptors decrease flavonoid synthesis (Jeong, Goto-Yamamoto, Kobayashi, & Esaka, 2004; Deikman & Hammer, 1995; Dan & Lee, 2004). Wounding and infection have also shown to decrease flavonoid production
(Slomczynski, Nakas, & Tanenbaum, 1995). External influences such as excessive fertilizers, insufficient light on fruits and extreme temperatures can cause a lowering of flavonoid production (Downey, Harvey, & Robinson, 2004). All of these factors should be considered and documented when experimentally quantifying flavonoid content in fruits. Flavonoid production could be maximized by optimizing mineral content, ensuring full sunlight exposure to fruits, as well as minimizing pathogen contact and wounding to the plant.

1.4 Biological Function in Human

1.4.1 Flavonoids in the diet

Flavonoids are found in many of the foods we eat on a daily basis. The estimated consumption of flavonoids in the US diet is on average approximately 150-200 mg/day (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). Flavonoids are present in fruits, vegetables, nuts, herbs, flowers, and spices. They can also be found in beverages such as tea, red wine, and unfiltered beer. An extensive database has been complied by the USDA reviewing the literature up to 2007 on flavonoids quantified in food products (U. S. Department of Agriculture, Agricultural Research Service, 2011).

This survey of research contains various limiting criteria to ensure a level of accuracy and consistency with the present data. Hollman & Katan, 1998 has shown that
the glycosylated form of flavonoids to be more readily absorbed in the body than the aglycone. Once ingested, flavonoids are metabolized by various bacterial enzymes into smaller metabolites. These products relative to the variety of flavonoids present are much fewer (Rechner, et al., 2004). Flavonoids can be hydrolyzed by glycosidases of microbes present in the gut (Baba, Furuta, Fujioka, & Goromaru, 1983). They can also be converted by dehydroxylation, demethylation and broken down into smaller fragments (Cermak, Breves, Lupke, & Wolffram, 2006). The gut metabolism of flavonoids is of great importance to the biological affects to the body and will be discussed in greater detail in the later section of this chapter. After absorption in the gut, Manach et al. (2005) investigated data from 97 previous studies on flavonoid bioavailability. In the plasma, flavonoid metabolites ranged from 0 to 4.0 µmol/L, when the equivalent of 50 mg algylcone was ingested. A varying urine excretion of 0.3%-43% was seen, depending on flavonoid type. The best absorbed flavonoids were found to be gallic acid and isoflavones. The least absorbed flavonoids were found to be proanthocyanidins, galloylated tea catechins, and anthocyanins.

1.4.2 Biological benefits of flavonoids

Flavonoids have experimentally exhibited a wide array of protective effects including anti-inflammatory, anti-allergic, anti-oxidant, antiviral, and anticancer activity, all of which are discussed in further detail. While there is still much more to understand
about the biological affects in humans, the initial *in vitro* and animal model studies have revealed a breadth of beneficial activity. Since these compounds are broken down in the gut to smaller molecules and complexed in foods with a variety of other bioactive compounds, it remains a challenging task to elucidate their overall health effects on various diseases. A variety of research has been conducted, and is also ongoing to further understand the flavonoids role in the body.

1.4.3 Anti-inflammatory

Flavonoids based on their structure and antioxidant capacity have been studied for the potential uses as anti-inflammatory agents. A study conducted by Stewart, et al. (2008) observed a significant decrease in inflammatory markers in mice after 8 weeks when a high fat diet was supplemented with the flavonol, Quercetin. Both *in vitro* and *in vivo* down-regulation was observed in the nuclear factor-kappa B pathway without affecting the kinase activity of the c-Jun N-terminal. This resulted in a lower expression of cytokine and inducible nitric oxide synthase (in rat model of colitis). This result was similar to experimental model using cell culture which observed a Quercetin induced inhibition of interleukin-1-induced transcriptional expression of monocyte chemoattractant protein-1 in glomerular cells by suppression of nuclear factor-kappa B (Ishikawa, Sugiyama, Stylianou, & Kitamura, 1999).
Another mechanism by which flavonoids may decrease inflammation is through inhibition of neutrophil myeloperoxidase (MPO). This enzyme is ultimately responsible for producing reactive chlorinated intermediates such as HOCl and the hypochlorite ion (OCl\textsubscript{2}) in neutrophils (Pincemail, Deby, Thirion, de Bruyn, & Goutier, 1988). A study by Winterbourn, 1985 found Quercetin to be a stronger inhibitor of MPO than a current specific inhibitor of MPO and methimazole.

1.4.4 Anti-allergic Activity

Flavonoids have been studied for their anti-allergic activity since the 1950s. Schoenkerman & Justice (1952) investigated the supplementation of an antihistamine with a flavonoid (Rutin). These compounds exhibited a synergistic benefit to patients suffering from allergy diseases. Mast cells function as regulators of allergic response in the body. These cells have a strong affinity for binding IgE and contain a large concentration of histamine with in their cell (Prussin & Metcalfe, 2003). As a result these cells function in pathogenesis of allergic asthma, anaphylaxis, urticaria, rhinoconjunctivitis, and possibly rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis (Gali, 1993; Theoharides, 1996).

Flavonoids have been investigated for their ability to interfere and down regulate mast cells. Baicaein and some derivatives exhibited inhibition of mast cell growth (Nagai, Osuga, & Koda, 1975). In rat peritoneal mast cells, genistein inhibited
the release of histamine, when induced by stem cell factor (SCF) (Nagai, et al., 1995). Flavonols; Quercetin, Kaempferol, and myricetin inhibited the release of mast cell histamine in rats (Park, et al., 2008). Flavonoids have also exhibited anti-allergenic activity in animal models. For example, an oral dose of Quercetin provided to guinea pigs inhibited bronchial obstruction and hyperactivity when previously agitated by antigen in aerosol form (Dorsch, et al., 1992). Flavonoids supplemented with proteoglycans (an inhibitor of mast cell secretion) have recently been patented in the US as a treatment for diseases involving mast cell activation (Theoharides, 2003; Theoharides, Alexandrakis, Kempuraj, & Lytinas, 2001).

1.4.5 Anti-oxidant effects of Flavonoids

Flavonoids are compounds which are chemically capable of reducing and chelating to metals as well as quenching reactive oxygen species (ROS) (Wilson, Price-Jones, & Hughes, 1976; Torel, Cillard, & Cillard, 1986; Kandaswami & Middleton, 1994). Whether these compounds actually function as antioxidants in vivo has been a topic of much debate. Flavonoids are highly metabolized in the body and have an absorption that is lower than other compounds with traditional antioxidant activity. These compounds however are present in a quantity capable of affecting enzymes, transcription factors and receptors.
Flavonoids have exhibited the ability to modulate a variety of cell signaling pathways including protein and lipid kinases (Williams, Spencer, & Rice-Evans, 2004). One way which flavonoids can detoxify the body utilizing cell signaling is through up-regulation of phase II detoxification enzymes (Kong, et al., 2001). These compounds also interfere and decrease ROS production at the surface of neutrophils through inhibition of NADPH oxidase and neutrophil MPO (Tauber, Fay, & Marletta, 1984; Stolc, 1979). Quercetin specifically, has shown to be a strong inhibitor of neutrophil degranulation as well as the production of O_2 radical. (Pagonis, Tauber, Pavlotsky, & Simons, 1986; Blackburn, Heck, & Wallace, 1987).

Diseases such as Parkinson’s Disease may be modulated by flavonoids and their ability to alter cell signaling pathways as well. Meng, Munishkina, Fink, & Uversky 2010, tested 48 flavonoids for the inhibition of α-synuclein aggregations in vitro. A number of these inhibited fibril formation and aggregation and a few also exhibited strong inhibition which disaggregated the fibrils. The current hypothesis of inhibition was found to be; stabilization of the unfolded state of the α-synuclein protein.

1.4.6 Antiviral Activity of Flavonoids

Flavonoids have been studied since the 1940s for their antiviral capacity (Selway, 1986). Structure-Activity Relations revealed that the 4’-hydroxyl and 3-methoxyl groups, as well as 5 position substitution and multiple substitutions on the A ring are
essential for strong antiviral activity against rhino- and poliomyelitis viruses (De Meyer, et al., 1991). Antipicomavirus activity was identified from a flavonoid isolated from the Chinese medicinal herb *Agastache folium* (Ishitsuka, Ohsawa, Ohiwa, Umeda, & Suhara, 1982). A novel plant flavonoid polymer, SP-303, exhibited activity against two strains of Herpes Simplex Virus I and Herpes Simplex Virus II, by inhibiting the virus from penetration into cells (EC50 = 2.1 +/- 0.2 μM) (Barnard, Smee, Huffman, Meyerson, & Sidwell, 1993). Quercetin, Naringin, Hesperetin, and Catechin have been studied in cell culture monolayers for their ability to affect replication and render HSV-I, polio virus type 1, parainfluenza virus type 3, and respiratory syncytial virus infectivity. In this study, Quercetin exhibited a concentration-dependent reduction in the virus’s ability to infect cell monolayers, and reduced intracellular virus replication (Kaul, Middelton, & Ogra, 1985). Flavonoids have been studied for their antiviral capacity against Human Immunodeficiency Virus (HIV) (Ng, Huang, Fong, & Yeung, 1997). One study conducted by (Gerdin & Srensso, 1983) tested 28 flavonoids and found flavans to be the most selective in inhibiting HIV-1 and HIV-2.

### 1.4.8 Flavonoids and Cancer

Studies have been conducted on the health benefits related to a diet rich in fruits and vegetables. A review by Block, Patterson, & Subar (1992) summarizes the epidemiological correlation of this diet to cancer incidence. As flavonoids are present
in fruits and vegetables to a great extent these compounds are implicated as one of the contributing factors to these health effects (Block, Patterson, & Subar, 1992). A variety of flavonoids have shown isolated anticancer activity both in vitro and in vivo. The flavonoid luteolin inhibited cancer angiogenesis as well as endothelial cell survival and growth by targeting phosphatidylinositol 3'-kinase activity (Bagali, et al., 2004).

Flavonoids (64) were investigated for antimutagenic activity from mutagens such as heterocyclic amines in cooked food. Of these 64 compounds tested, the flavones containing a carbonyl functional group on the C-4 possessed the greatest antimutagenic activity (Edenharder, von Petersdorff, & Rauscher, 1993). Colon cancer in rats (induced by azoMeOH) was inhibited by administration of the flavonol, Quercetin (Deschner, Ruperto, Wong, & Newmark, 1991; 1993). Quercetin also exhibited the ability to stop cell cycle in proliferating lymphoid cells (Reed, Tanaka, & Cuddy, 1992). The major flavonoid component in green tea, Epigallocatechin Gallate (EGCG) has exhibited a variety of anticancer activities. EGCG has shown suppression of carcinogenic toxicity possibly by indirect induction of P450 IA1 and IA2 isozymes in the intestines, to speed up elimination and breakdown of precarcinogenic compounds present in the diet (Hayatsu, et al., 1992). In mice with experimentally induced skin papillomas, EGCG caused cancer regression and growth inhibition (Wang, et al., 1992). When EGCG was administered to mouse B16 melanoma cell lines, cancer metastasis was inhibited (Taniguchi, et al., 1992).

In epidemiological case-control studies, flavonoid intake has been associated with lower risk of a variety of cancers; lung (Le Marchand, Murphy, Hankin, Wilkens, &
Results from epidemiological prospective cohort studies have been less straightforward. Some studies have found a correlation to cancer risk related to baseline dietary flavonoid intake and some have not identified a correlation between cancer risk and flavonoids. Inverse links were observed for total cancer incidence, lung cancer (Knektl, et al., 1997), colorectal cancer (Arts, Jacobs, Gross, Harnack, & Folsom, 2001), and prostate cancer (Knelt, et al., 2002). In some studies, no correlation was observed for example; colorectal cancer (Lin, et al., 2006), Catechins and epithelial cancer (Arts, Hollman, Bueno De Mesquita, Feskens, & Kromhout, 2001), and overall cancer risk in the Zutphen Elderly Study (Hertog, Feskens, Hollman, Katan, & Kromhout, 1994).

1.4.9 Gut Metabolism of Flavonoids

The human gut microflora plays a very important role in digestion and metabolism of flavonoids. These compounds in the small intestine can be absorbed if present as aglycones or glucosides. If absorbed, they are quickly metabolized and
excreted out of the body through the bile and urine (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). Flavonoids which remain in the gut and reach colonic bacteria are broken down into smaller metabolites and absorbed into the body. However, the variety of metabolites produced by the colonic microflora is entirely dependent on the individual bacteria present, and varies greatly from person to person (Rechner, et al., 2004). One example is the isoflavonone metabolite Equol (Setchel, Brown, & Lydeking-Olsen, 2002). This compound is metabolized from the flavonoid Daidzein, found in soy. It is produced entirely by gut bacteria and has shown to possess anti-cancer activity by binding to estrogen receptor, without causing activation (antiandrogenic). These effects are much more potent than the reactant from which it came (Daidzein) as well as other flavonoids found in soy. Equol is not metabolized by all adults; about one-third to one-half of the population is capable of this biotransformation (Yuan, Wang, & Liu, 2007).

In summary, most of the data and correlations found on flavonoids has been the result of in vitro studies. These do not fully prove the compounds will also have the same effect in the body (in vivo). Epidemiological observational studies also exhibit a beneficial relationship between flavonoid intake and improved health. However further clinical trials are required to show a causal relationship between flavonoids and improved outcome of a diseased state. Another factor which is important to consider is the dose required to experience a beneficial biological effect. Some relationships have been observed at high doses of flavonoid which may be difficult to ingest on a regular basis. This is important to consider when determining whether a correlation between
intake and effect is relevant to the public. One example of this is the effect of quercetin on lowering blood pressure. This effect can be experienced from a quercetin intake of a 1095 mg single dose and a four week regimen of 730 mg quercetin/day (Larson, Symons, & Jalili, 2012). In order to achieve this effect through diet, from a food which contains a large amount of quercetin (33mg/100g wet weight) you would need to consume approximately 18.4 onions for the initial 1095 mg dose, and 12 onions daily (180g/onion) (U. S. Department of Agriculture, Agricultural Research Service, 2011). This quantity would be difficult to accomplish on a daily basis.

1.5 Folk and Ethnobotanical Uses

The Guarani people of South America were the first to utilize the pitanga for medicinal treatments in the 15th Century (Alonso, 1998). Many people now utilize the leaves as a traditional medicine treatment for various treatments. The pitanga is used in Nigeria as an early treatment to Malaria (Agbedahunsi & Aladesanmi, 1993), in Madeira for treatment of bronchitis and influenza (Rivera & Obon, 1995). The leaves of *E. uniflora* have been investigated based on folk medicine usage and experimentally exhibit anti-inflammatory and diuretic effects (Schalpoval, Silveira, Miranda, Alice, & Henriques, 1994). Paraguan folk medicine exploit extracts from the leaves as a treatment for gout. The flavonoids present in leaf extract experimentally inhibit xanthine oxidase, decreasing production of uric acid in the body. Though the fruits
contain the flavonoids as well, the leaves alone were utilized as a tea for this treatment (Schmeda-Hirschmann, Theoduloz, Franco, Ferro, & Rojas de Arias, 1987).

1.6 Methods of Extraction

A variety of methods have been utilized for the extraction of flavonoids. For these methods the plant material is typically dried to a powder using a lyophilizer or frozen. Flavonoids, if left fresh, will be degraded by enzymes present in the plant (Andersen & Markham, 2006). The solvent chosen is based on the class of flavonoids desired. See Table 1.1 for an overview of individual flavonoid class solubilities. The presence of sugars on flavonoids greatly increases flavonoid’s water solubility, as a result, can be extracted in aqueous alcohol along with the more polar aglycones. Isoflavones, flavonols, methylated flavones and flavonones are non-polar and can be extracted with like solvents such as; ethyl acetate, diethyl ether, dichloromethane, and chloroform. Other methods for extraction of flavonoids less commonly employed than liquid extractions are Soxhlet apparatus extraction, sequential solvent extraction, supercritical fluid extraction, pressurized liquid extraction, Ultrasound-assisted extraction, and Microwave-assisted extraction.

Soxhlet apparatus (Soxhlet, 1879) extraction involves vaporization of the extraction solvent in an enclosed glass apparatus which contains above it the solid extract (Figure 1.3). This extraction works for heat labile, non-volatile compounds. This
method separates compounds from impurities by using an extraction solvent which does not solubilize impurities.

Sequential solvent extraction involves the extraction of increasingly polar compounds. The most non-polar solvent and class of compounds desired is extracted first, increasing the solvent polarity with each subsequent extraction, to select for further classes of compounds (Finkelman, et al., 1990).

A super critical fluid extraction utilizes thermodynamics properties of making a super critical liquid (most often CO₂) as the extracting solvent. This method is beneficial for food and pharmaceutical industry being non-toxic, nonflammable and relatively inexpensive for large scale extractions (Brunner, 2005; Sahena, et al., 2009). Pressurized liquid extraction (PLE) utilizes high pressure and temperature to increase solubility and
solvent diffusivity. PLE provides shorter extraction times and decreased solvent used (Dawidowicz, Wianowska, Gawdzik, & Smolarz, 2003). Ultrasound-assisted extraction utilizes the phenomenon of cavitation, which breaks cell walls and allows solvent to solubilize compounds present within the core of the extract’s cells (Cravotto, et al., 2008). Microwave-assisted extraction (MAE) is a rapid extraction method which uses microwave energy to extract samples in solvent (Kaufmann & Christen, 2002).

1.7 Methods of Separation

Initial methods of phenolic separation included thin-layer chromatography (TLC), polyamide chromatography, and paper electrophoresis. While TLC still remains a method of preparative separation, both other methods are no longer used for flavonoid separation. For qualitative and quantitative separation the most commonly used method is high performance liquid chromatography (HPLC). When connected to a second apparatus for detection the identification and quantification process can be rapidly achieved. HPLC has been coupled to nuclear magnetic resonance (NMR), mass spectrometry (MS), and ultraviolet (UV) detectors (Andersen & Markham, 2006).

Initial methods to prepare samples for HPLC analysis involve a pre-purification to remove impurities and or separate classes of flavonoids. One of the first methods used was precipitation using lead acetate, followed by acidification. Since not all flavonoids would precipitate or co-precipitation could occur this method was eventually phased out (Andersen & Markham, 2006). Other methods employed include a solvent partition
or preparative column separation using a variety of column types (for example Sephadex LH-20, anion exchange resin). Extract is adsorbed on to solid matrix and eluted using a like solvent to flavonoids (MeOH-water gradient for example) (Hostettmann, Marston, & Hostettmann, 1998). Filtration and liquid-liquid extraction (Tura & Robards, 2002) as well as solid-phase extraction (SPE) are useful sample pre-purification methods for initial separation. SPE provides rapid, economical, and sensitive results for pre-purification of flavonoids. Once extract is adsorbed on to the cartridge, sugars can be removed using aqueous MeOH and fractions of flavonoid classes can be separated and collected. Previous studies have used SPE to separate phenolic acids and flavonoids from wines and fruit juice (Wang & Sporns, 2001), as well as to fractionate flavonol glycosides and phenolics into neutral and acidic components before HPLC (Chen, Zuo, & Deng, 2001).

These semi-purification methods of extract prior to further treatment avert the possibility of undesired chemical reactions. Lipophilic compounds are removed using a water and hexane partition. Anthocyanins and flavonols are isolated based on their characteristic affinities for different solvents on a Sep-Pak™ C_{18} Waters column. This separation step can prevent undesired addition reactions, previously shown to occur with anthocyanins and flavonols in acidic conditions. Reactions such as these have shown to occur forming a variety of anthocyanin-flavonol polymers in wine when present in acidic conditions (Salas, et al., 2004). This decreases the possibility of underestimation of the quantity of flavonoids present.
1.8 Methods of Identification and Quantification

Flavonoids in fruit are present in the glycosylated form to make more soluble for vacuole storage (Taiz & Zeiger, 1991; Heller & Forkman, 1986). Numerous combinations of sugars on various parts of the flavonoid present a challenge for identification of the base flavonoid. To simplify the process, sugars are removed by acid hydrolysis prior to identification. The type and number of sugars present affects the required length of hydrolysis and concentration of acid used. This must be optimized for compounds present. Due to the heat and acidic conditions of the hydrolysis there is potential for undesired side reactions between impurities and flavonoids. For that reason, there is a great imperative to optimize the hydrolysis time necessary for each fraction to minimize these reactions.

Upon hydrolysis the most common method of analysis for flavonoids is utilizing HPLC methods of separation and identification. For quantitative and qualitative analytical HPLC of flavonoids the solvent, stationary phase and gradient must be optimized for extract prior to analysis.

Elution of flavonoid classes varies greatly between classes and glycoslyations present. Flavone C-glycosides will elute before flavone O-glycosides. Unsaturation present on C2-C3 of flavones causes longer retention time when compared to flavanones. Anthocyanins change structure and charge at varying pH and temperature. As a result, these compounds must be analyzed at constant pH and temperature for
accurate results. Optimal conditions (pH >2) push anthocyanins to form their flavylium form and provide the best resolution by lowering peak tailing (Andersen & Markham, 2006). Acetonitrile-water or MeOH-water gradients are frequently used without the addition of acids. Some analyses have utilized tetrahydrofuran (THF), isopropanol, or n-propanol. The addition of acid modifiers to solvents will improve peaks and decrease tailing through suppression of phenolic hydroxyl ions (Andersen & Markham, 2006). A review by Merken & Beecher, 2000 overviews the HPLC conditions of flavones, flavonols, flavanones, isoflavones, anthocyanidins, catechins, and sugars present in food products. Stationary phases for flavonoid identification are usually some variety of reverse phased material. A great number of these experiments utilize octadecylylsilyl bonded-phase (ODS, RP-18, or C<sub>18</sub>) HPLC particles. Some studies have used octasilyl bonded–phase (RP-8 or C<sub>8</sub>) but this is less common. On these solid phases flavonoids bound to sugars will elute before aglycones. Flavonoids containing more hydroxyl groups will also elute first when compared to less hydroxylated counterparts (Andersen & Markham, 2006).

When HPLC is coupled to UV or MS, a variety of information can be revealed as to the identity of individual flavonoids. The most often studied method is HPLC coupled to UV. Various classes of flavonoids have individual absorbance maxima and can be identified by class by observing absorbance over a broad range of wavelengths. For example, the absorbance maximum for flavones is measured at 270 nm, 370 nm for flavonols, 290 nm for flavanones, 520 nm for anthocyanins, and 210 nm or 280 nm for catechins, and 520 nm for anthocyanins (Andersen & Markham, 2006). The structure
can be determined by comparison to standard known flavonoid compounds or by addition of a shift reagent after separation on HPLC. For example, various shifts in UV have been observed and documented for quercitrin after adding 5 different shift reagents. These shifts are characteristic to quercitrin and aid in the structure elucidation (Ducrey, Wolfender, Martson, & Hostettmann, 1995).

HPLC coupled to MS can provide a great deal of information and accuracy with regards to sugars present on flavonoids as well as provide a great deal of sensitivity and information on purity of each individual peak isolated. The combination of MS and HPLC however does present a few obstacles as the conditions of HPLC are polar opposite of required conditions of MS. Mass Spectrometry conditions require low flow rates, gas-phase, high vacuum and high temperature. HPLC operates instead under liquid-phase, high flow rate and low temperature. Thermospray (TSP), continuous-flow fast-atom bombardment (CF-FAB), and electrospray (ES) are capable of measuring ionization of small nonpolar aglycones to highly polar molecules (200-2000 MW range) (Wolfender, Rodriguez, Hostetttmann, & Wagner-Redeker, 1995). Both TSP and CF-FAB require the source to be under vacuum, the ion source in ES is present at atmospheric pressure.

The utilization of atmospheric pressure ionization (API) increases ease of use and sensitivity to MS methods (Andersen & Markham, 2006). TSP and ES are soft ionization methods which will provide molecular mass of the parent compound but not fragmentation patterns. Collision-induced dissociation (CID) or collision-activated dissociation methods will provide fragments and patterns of fragmentation which can
be analyzed for structure determination (Cole, 1997). Fragmentation patterns of various flavonoids have been characterized and reported utilizing CID-MS (Lin, Ng, & Yang, 1993; Li & Claeys, 1994; Grayer, Kite, Abou-Zaid, & Archer, 2000).

1.9 Methods of Quantification

The most widely utilized and accurate method for flavonoid quantification is achieved by coupling of HPLC with a Photo Diode Array Detector (PDA). This method provides a direct quantification from samples injected on to HPLC column. Flavonoids in fruits, vegetables and beverages have been quantified by (Justesen, Kruthsen, & Leth, 1998) utilizing this method. In this method external standards were employed for quantification, combined into a stock mixture at known concentrations. Peak areas were integrated to determine the aglycones flavonoid quantity. Standard calibration curves were prepared by plotting absorbance versus concentration over a linear concentration range.

1.10 Overall Aims of Research

The aim of this study was to develop a directed extraction for flavonoids, optimal hydrolysis, pre-purification and HPLC methodologies to accurately identify flavonoids in the pitanga.
Though previous research has identified flavonols present in the pitanga. They have not identified the other classes of flavonoids are thought to be present in this type of fruit. This method provides a through quantification for the identification of both flavonols and anthocyanins present in the pitanga fruit. Individual HPLC gradients for flavonols and anthocyanins were established and used for identification by comparison to known flavonoid standards. HPLC was equipped with a PDA and monitored at wavelengths of 520nm for anthocyanins and 370nm flavonols.

The specific aims of research are: (1) To develop a method for optimal quantification of flavonoids in pitanga fruits; (2) To identify and quantify flavonols and anthocyanins found in the pitanga fruit, and; (3) To analyze the composition present between ripe and unripe collections of the fruits. Due to the color of the fruit (red to purple) the pitanga is hypothesized to contain flavonoids. The pitanga fruit is believed to contain more flavonols and anthocyanins at the ripe stage of fruit growth, as they possess more color at this stage.

1.11 Importance of Study

Previous studies have shown the pitanga fruit possesses a variety of beneficial compounds. As previously discussed, the pitanga has been employed for a variety of health remedies. This may be due to the plethora of bioactive compounds present in the leaf and fruit of the plant. The leaves contain flavonoids, terpenoid oils, condensed
and hydrolysable tannins, steroids and triterpenoids (Amorim, Lima, Hovell, Miranda, & Rezende, 2009). The fruit of the pitanga contains a variety of flavonoids, carotenoids, vitamin C, monoterpenes as well as other volatile compounds which have been previously isolated in the pitanga leaf (Oliveira, Lopes, Cabral, & Eberlin, 2006). The red to purple color present in the fruit, suggests that a variety of flavonoids are present based on the characteristic color of anthocyanins (purple to blue) and flavonols (yellow) (Herbert, 1989).

![Purple and Red Zill Dark Pitanga](image)

Figure 1.4: Purple and Red Zill Dark Pitanga: Example images of coloration for red and purple pitanga

Due to the increasing interest in the fruit, preliminary identification of flavonoids has been recently studied by Celli, Pereira-Netto, & Beta (2011) and Hoffmann-Ribani, Huber, & Rodriguez-Amaya (2009). However these results did not include the identification of the anthocyanins, which are also a class of flavonoids present in the fruit. These studies have not fully addressed the difficulties of quantifying flavonoids present in a fruit.

A review by Egert & Rimbach (2011) overviews the importance of flavonoids from complex foods as opposed to supplement forms. The effects provided by whole fruits and vegetables containing a complex mixture of secondary metabolites, minerals
and fiber is not comparable to a single isolated compound provided at a higher dose. Especially for the case of flavonoids, a higher quantity may actually cause adverse health effects. Flavonoids in high dose supplement form have previously inhibited metabolism and excretion of other drugs, decreased trace element bioavailability, impaired folate absorption, and inhibited the transport of vitamin C (Cermak, 2008).

High concentrations of flavonoids found in cranberry juice (600 mL/day) inhibit the metabolism of the anticoagulant medication Warfarin (Yamazaki & Shimada, 1997; He, Kunze, & Trager, 1995), causing therapeutic failure and bleeding complications. Yet, smaller amounts of cranberry juice are not expected to cause this same side effect (Aston, Lodolce, & Shapiro, 2006; Zikria, Goldman, & Ansell, 2010).

The importance of accurately identifying flavonoids and bioactive compounds in food sources is of great necessity. Many epidemiological researchers are looking at consumption of whole foods when determining correlations to risk of developing certain diseases. When correlating health benefits, researchers refer to previously reported quantitative data on the given food’s composition. When these values are inaccurately reported, overestimation or underestimation can occur and lend to mistaken interpretations. This study will provide an accurate method for flavonoid analysis of the pitanga as well as other fruits which contain flavonols and anthocyanins. The results will further highlight the pitanga as a beneficial fruit for consumption.
Chapter 2: Methods

2.1 Fruit Collection and Processing

Pitanga fruit was collected from Kona, Hawaii. Each fruit collected was a self-cross of the *Eugenia uniflora* 'Zill Dark' variety. The samples were collected at stages of “Ripe” and “Unripe” and labeled accordingly. Fruits were immediately frozen and shipped to Oahu for lyophilization and dry mass recorded. Samples manually ground in liquid nitrogen.

2.2 Extraction Development

Originally tested method of extraction involved a simultaneous extraction and hydrolysis. This technique was determined to result in lower concentrations of flavonoids when compared to isolated extraction and hydrolysis. Ultimately extraction method was adapted from a previously optimized extraction by Kajdzanoska, Petreska, & Stefova (2011).

This method compared various solvents commonly used and determined pure acetone to extract most flavonols. Similarly, MeOH /water/HCl 80:18:2 v/v/v was previously determined to extract the maximum amount of anthocyanins.
Approximately 1.0 g of fruit was weighed and extracted 10 mL of acetone supplemented with 1.0 g/100 mL ascorbic acid. Extract was vortexed for 30 sec, sonicated in an ice bath for 20 min, then centrifuged for 5 min at 4000 rpm and repeated 3 times. Plant material was allowed to air dry and subsequently extracted with ethanol: water: acetic acid mixture (10:9:1 v/v) supplemented with 1.0 g/100 mL ascorbic acid using the aforementioned methods. See Figure 2.1 for visual of overall extraction method.

**Figure 2.1: Extraction Flowchart:** step by step method of extraction; collect pitanga from “Zill Dark” variety trees, freeze dry fruit, extract one gram of sample in acetone (3X) then in aqueous acidic ethanol (3X).

### 2.3 Pre-purification Development

Various pre-purification methods were enacted to reduce side reactions between flavonoid types and other classes of compounds. The acetone extract was dried by rotary evaporation at 30°C and pre-purified with a hexane-water partition to
remove lipophilic compounds following protocol from Handbook of Food Analytical Chemistry. Briefly, dried extract was resolubilized in 5 mL hexane and 10 mL water. Mixture was vortexed and centrifuged at 4000 rpm for 5 min. The hexane layer was pipetted from top layer of the partition. This process was repeated 2 more times to ensure removal of lipophilics. Water partition containing flavonoids was reserved and used for further analysis. Water fraction was further diluted in 25 mL of water. This extract was loaded on a Sep-Pak™ C18 silica cartridge (part No 51910, Waters, Milford, MA) preconditioned using 10 mL ethyl acetate, 10 mL MeOH, 10 mL 0.01 N HCl. After the extract was loaded on column, 6 mL 0.01 N HCl was passed through column to remove free sugars. The column was dried for 30 min by attaching directly to a stream of N2 gas. Flavonoids other than anthocyanins were eluted using 6 mL ethyl acetate. Anthocyanins are insoluble in ethyl acetate and remained bound to the solid phase. These anthocyanins were subsequently eluted using 0.1% v/v MeOH/ HCl. Ethanolic extracts were pre-purified by loading onto two combined Sep-Pak™ C18 silica cartridges and eluted using previous method aforementioned for flavonols. All solvents were passed through columns at the recommended flow rate of 1 mL per min.

These pre-purification steps result in a more accurate quantification as well as an unambiguous HPLC chromatogram. All collected fractions were dried by rotary evaporation at 30°C in preparation for hydrolysis. See Figure 2.2 for visual of overall pre-separation method.
Figure 2.2: Pre-purification Flowchart: pre-purification steps involved for each extract (acetone and aqueous acidic ethanol).

2.4 Hydrolysis Development

In order to simplify the flavonoid identification sugars were removed by acid hydrolysis prior to HPLC separation. The following methods were adapted from Hertog et al. (1992a). Pre-purified fractions were hydrolyzed at 95°C in 1.2 M HCl 50% v/v aq. MeOH.
Figure 2.3 Hydrolysis: Optimized hydrolysis method adapted from Hertog, Hollman, & Venema, 1992, provides method to remove sugar from flavonoid backbone.

Hydrolysis times were optimized for maximal quantification and minimal degradation by comparison of HPLC retention times to standards. The ethyl acetate elutant from the acetone extract was hydrolyzed for 60 min. The acidic MeOH elutant from the acetone extract was hydrolyzed for 80 and 120 min. The ethyl acetate elutant from the ethanolic extract was hydrolyzed for 60 and 80 min. The acidic MeOH elutant from the ethanolic extract was hydrolyzed for 4 hours and 30 min (see Table 2.1). Hydrolysis times were optimized by comparison to standard retention times. Time was selected for maximal quantity of each compound with minimal degradation. Degradation was identified from the appearance of new peaks and a decrease in height for flavonoid peak of interest.
Table 2.1 Optimized Hydrolysis Times

<table>
<thead>
<tr>
<th>Extraction Solvent</th>
<th>Sep-Pak™ C₁₈ fraction</th>
<th>Flavonoids Class eluted from column</th>
<th>Hydrolysis Time Required (Min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>Ethyl Acetate</td>
<td>Flavonols</td>
<td>60</td>
</tr>
<tr>
<td>Acetone</td>
<td>Acidic MeOH</td>
<td>Anthocyanins</td>
<td>80 &amp; 120</td>
</tr>
<tr>
<td>Acidic Ethanol</td>
<td>Ethyl Acetate</td>
<td>Flavonols</td>
<td>60 &amp; 80</td>
</tr>
<tr>
<td>Acidic Ethanol</td>
<td>Acidic MeOH</td>
<td>Anthocyanins</td>
<td>270</td>
</tr>
</tbody>
</table>

Hydrolysis method optimized varying time for each pre-purified sample fraction, two extractions were further purified on a Sep-Pak™ C₁₈ column for flavonols using ethyl acetate, and for anthocyanins using acidic MeOH.

2.5 Flavonoid Standards

Dry powdered standards of myricetin, kaempferol, quercetin, quercitrin were purchased from Indofine Chemical Company (NJ, USA). The standards of pelagonidin, peonidin, malvinidin, cyanidin, and delphinidin were purchased from Apin Chemicals Limited (Abingdon, UK). Nano-pure water was used through these experiments. HPLC grade MeOH, reagent grade; formic acid, acetic acid, hydrochloric acid and ethanol were purchased from Fischer Scientific.

Flavonol standards included kaempferol, myricetin, quercetin, and quercitrin. Stock solutions of flavonols standards, at 0.6 mg/mL, were dissolved in HPLC grade MeOH, and stored -20 ° C. Due to degradation of standards, as monitored by UV-vis spectrometry (Hertog, Hollman, & Venema, 1992) myricetin standard was reconstituted from dry powered stock after 1 month, other flavonols replaced after 3 months. Anthocyanin standards included peonidin, pelagonidin, malvinidin, delphinidin, and
cyanidin. Stock solutions of anthocyanin standards, at 1.0 mg/mL, were dissolved in HPLC grade MeOH, and stored -20 °C.

2.6 HPLC Method Development

Chromatographic separation was carried out on a high performance liquid chromatographer (HPLC) Waters 2695 (Waters Corp., Milford, MA, USA). This was equipped with a PDA Waters 996 (Waters Corp.) and an autosampler (Waters Corp.). The column used was a 2.1 mm x 20 mm i.d., 3.5 µm, XBridge RP C18 column (Waters Corp., Milford, MA, USA). Injection volumes used were 10 µL and 50 µL of sample that was loaded and injected by the autosampler. Samples were eluted with a gradient mobile phase consisting of A (10% v/v formic acid in water) and B (MeOH) at a constant flow rate of 0.6 mL/min. Unhydrolyzed fractions were reserved and injected for comparison. All fractions to be analyzed were diluted with 10% v/v aqueous formic acid. Gradient elution for flavonols began isocratically at 0% B for 5 min. Linear gradient continued from 0 to 25% B in 40 min, from 25 to 100% B in 5 min, from 100 to 0% B in 5 min, 0% B continued for 5 min. The linear gradient for analysis of anthocyanins was programmed from 10% B for 1 min, from 10 to 25% B for 12 min to 12.5 min, from 25 to 10% B in 2 min. Gradients were returned to initial conditions over 5 min. UV-Vis absorbance spectra were monitored from 200 to 600 nm. Peak area was
analyzed for quantification at 370 and 520 nm. All sample runs were equilibrated to initial solvent gradient for 5 min prior to injection.

2.7 Flavonoid Identification and Quantification

Flavonols and anthocyanin aglycones were identified by comparison to known standards. The fractions that were injected on to HPLC were pre-purified for flavonoid isolation as well as subjected to hydrolysis reactions. This provided a prior verification in the identification process that flavonoids are being examined in the chromatograms of interest. The UV wavelength examined was chosen because it is the lambda maximum for the given class of flavonoids. HPLC chromatograms were compared before and after hydrolysis reaction and retention times shifted from the polar region (where glycosylated flavonoids would be located) to the more non-polar region of the chromatogram (where aglycones would be retained). Peaks changed location after the hydrolysis reaction was complete to match retention with known standards.

All samples and standards injected on to the column contained an identical solvent composition of 1:1 Solvent A (10% formic acid) and 1.2 M HCl in 50% v/v MeOH. All flavonol standards were combined from stock solutions in MeOH (1.0 mg/mL) to make a stock mixture. The stock mixture was diluted to make a range of concentration for injection (1.5-30 ng/μL). All anthocyanin standards were combined from stock solutions in MeOH (1.0 mg/mL) to make a stock mixture. The stock mixture was diluted
to make a range of concentration for injection (0.3-8 ng/μL). Individual calibration
curves were constructed for each standard at both 10 and 50μL injection volumes.
Matching calibration curves were used for given injection volume of sample compared
to standard for accuracy to ensure relative equivalent amount was injected.
Quantitation was accomplished utilizing HPLC-PDA where peak area for each standard
was integrated from HPLC-PDA chromatograms at 520 nm for anthocyanins and 370 nm
for flavonols. These quantities were plotted against concentration to create a linear
trend line.

2.8 Limits of Detection and Quantification

Minimum limits of detection (LOD) was calculated from the standard deviation
of the y-intercepts from 3 trials of flavonoid standard calibration curve run over a
relevant range of concentrations using y-intercept average in the formula LOD= (3.3*SD/slope).

Minimum limits of quantification (LOQ) was calculated from the standard
deviation of the y-intercepts from 3 trials of flavonoid standard calibration curve run
over a relevant range of concentrations using y-intercept average in the formula LOQ= (10*SD/slope). This method was previously described by The International Conference
on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for
Chapter 3: Results

3.1 Extraction

A two-fold extraction was conducted using acetone as the first solvent and acidic aqueous ethanol as the second extract. The color of the liquid acetone extract after three subsequent extractions was golden-yellow. The solid pitanga extract remained reddish purple in color even after three acetone extractions. Refer to Figure 3.1 and 3.2 for pictures of extracts. This pitanga extract was then subjected to second extraction using 10:9:1 v/v Ethanol: H₂O: Acetic Acid. The color observed from the liquid extract was fuchsia to purple.
3.2 Pre-purification

The acetone extract was dried by rotary evaporation and a hexane: water partition removed lipophilic compounds such as fats and carotenoids which could potentially interfere with the desired flavonoids. The fractionation process was observed during loading of extracts and elution of both flavonols and anthocyanins. When retained extract was rinsed with ethyl acetate a yellow color was observed in the eluting solution. When acidic MeOH solvent was passed over extract loaded on the Sep-Pak™ C₁₈ column a blue to pink color was observed in the eluting solution. Upon removal of the anthocyanins using acidic MeOH, the column was clear of observable color. The use of pre-purification using Sep-Pak™ C₁₈ columns provided fractions containing individual classes of flavonoids.

3.3 Hydrolysis

The hydrolysis methods were adapted from Hertog, Hollman, & Venema (1992) and provided a starting point for optimization of each individual fraction. The ethyl acetate elutant which was hydrolyzed beyond 60 min exhibited new smaller peaks and a decrease in the flavonol peaks of interest. The acidic MeOH elutant from the acetone extract hydrolyzed longer than 120 min contained smaller new peaks in chromatogram and lower peak height for anthocyanin peaks of interest. The ethyl acetate elutant from the ethanolic extract hydrolyzed longer than 80 min showed a decrease in observable
flavonols and appearance of new peaks in chromatogram. The acidic MeOH elutant from the ethanolic extract hydrolyzed longer than 4 hours and 30 min exhibited a decrease in observable anthocyanin peaks of interest and appearance of small new peaks in chromatogram. See Table 2.1 for hydrolysis times selected for each fraction.

3.4 HPLC Chromatograms

Figure 3.3 is an example chromatogram of flavonol standards compared to hydrolyzed unknown flavonols identified in pitanga extract. Flavonols myricetin (My), quercitrin (Qu 3-rha), quercetin (Qu), and kaempferol (K) were injected onto the column at identical concentrations and injection volumes. These pure compounds were compared to the hydrolyzed pitanga extract resulting in two major peaks that matched retention times of the flavonoid standards of myricetin (My) and quercetin (Qu).
Figure 3.3 Chromatogram of Hydrolyzed Flavonols: HPLC chromatogram example of flavonol standards compared to hydrolyzed flavonols identified in pitanga extract. Flavonols; myricetin (My), quercitrin (Qu 3-rha), quercetin (Qu), and kaempferol (K) were injected onto the column at 30 ng/µL and 50 µL injection volume. These pure compounds were compared to the hydrolyzed pitanga extract with identical injection volume of 50 µL. Solvents utilized for HPLC separation; 10% formic acid in water (solvent A) and MeOH (solvent B). Gradient elution for flavonols began isocratically at 0% B for 5 min. Linear gradient continued from 0 to 25% B in 40 min, from 25 to 100% B in 5 min, from 100 to 0% B in 5 min, 0% B continued for 5 min.

In Figure 3.4, the unhydrolyzed extract was compared to flavonol standards for the identification of the glycosylated flavonol, quercitrin (Qu 3-rha) in the pitanga extracts. Small quantities of quercitrin were identified in the unhydrolyzed flavonol fractions of pitanga extracts by comparison to known quercitrin standard. When the
sample fraction was not subjected to hydrolysis reaction (which would remove sugars from the flavonoid backbone) quercitrin was able to be identified by HPLC.

**Figure 3.4 Chromatogram of Unhydrolyzed Flavonols:** HPLC chromatogram example of flavonol standards compared to unhydrolyzed flavonols identified in pitanga extract. Flavonols; myricetin (My), quercitrin (Qu 3-rha), quercetin (Qu), and kaempferol (K) were injected onto the column at 30 ng/μL and 50 μL injection volume. These pure compounds were compared to the unhydrolyzed pitanga extract with identical injection volume of 50 μL. Solvents utilized for HPLC gradient; 10% formic acid in water (solvent A) and MeOH (solvent B). Gradient elution for flavonols began isocratically at 0% B for 5 min. Linear gradient continued from 0 to 25% B in 40 min, from 25 to 100% B in 5 min, from 100 to 0% B in 5 min, 0% B continued for 5 min.
Figure 3.5 Chromatogram of Hydrolyzed Anthocyanins: HPLC chromatogram example of anthocyanin standards compared to hydrolyzed anthocyanins identified in pitanga extract. Anthocyanins; delphinidin (Dp), cyanindin (Cy), pelargonidin (Pg), peonidin (Pn), and malvinidin (Mv) were all injected at 8 ng/µL with an injection volume of 50 µL. Solvents utilized for HPLC gradient; 10% formic acid in water (solvent A) and MeOH (solvent B). Gradient elution for anthocyanins consisted of a gradient of 10% MeOH for 1 min, from 10 to 25% MeOH for 12 min to 12.5 min, from 25 to 10% MeOH in 2 min.

The chromatogram in Figure 3.5 is an example trial of anthocyanin standards which were compared to unknown anthocyanins. These unknown anthocyanins in pitanga extracts were identified by identical retention times to anthocyanin standards. Anthocyanins; delphinidin (Dp), cyanindin (Cy), pelargonidin (Pg), peonidin (Pn), and malvinidin (Mv) were all injected at identical concentrations (0.3 ng/µL- 8 ng/µL) and
injection volumes (10 μL and 50 μL). These pure compounds were compared to pitanga extract resulting in two peaks that matched retention times of the flavonoid standards. These peaks were the major peaks observed for this given extract injected, their retention times were identical to delphinidin (Dp) and cyanidin (Cy).

HPLC profiles were optimized by injecting known flavonoid standards on to column and determining conditions which provided a short run time while allowing a reasonable distance between each flavonoid peak to ensure optimal separation of individual compounds. Flow rates between 0.6 and 1.0 mL/min were examined. A flow rate of 0.6 mL/min provided the best separation within a reasonable time. Total time of analysis was 14 min for anthocyanin separation and 50 min for flavonol separation. Actual flavonol peaks were eluted within 20 min however the last 30 min ensured complete removal of other compounds injected on to column which could potentially affect future condition of column if left out of procedure.

3.5 Flavonoid Identification and Quantification

Two classes of flavonoids were analyzed (flavonols and anthocyanins) in two separate extracts from freeze dried pitanga fruit samples. The samples were collected at ripe and unripe stages of growth. Identification of flavonoids was accomplished by comparison to standard pure compounds (0.3 ng/μL - 30 ng/μL); based on retention time and their
characteristic UV absorption maximum using High Performance Liquid Chromatography methods as discussed in Methods.

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Ripeness</th>
<th>N</th>
<th>Mean (µg/g dried extract)</th>
<th>Standard Deviation (µg/g dried extract)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myricetin</td>
<td>Unripe</td>
<td>9</td>
<td>422.6</td>
<td>±79.30</td>
<td>0.0005 **</td>
</tr>
<tr>
<td></td>
<td>Ripe</td>
<td>15</td>
<td>231.3</td>
<td>±124.54</td>
<td></td>
</tr>
<tr>
<td>Quercitrin</td>
<td>Unripe</td>
<td>9</td>
<td>26.95</td>
<td>±12.69</td>
<td>0.0186 **</td>
</tr>
<tr>
<td></td>
<td>Ripe</td>
<td>15</td>
<td>16.41</td>
<td>±7.75</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>Unripe</td>
<td>9</td>
<td>129.6</td>
<td>±36.63</td>
<td>0.0686 *</td>
</tr>
<tr>
<td></td>
<td>Ripe</td>
<td>15</td>
<td>97.71</td>
<td>±41.0</td>
<td></td>
</tr>
<tr>
<td>Kaempferol</td>
<td>Unripe</td>
<td>7</td>
<td>3.9</td>
<td>±2.06</td>
<td>0.0207 **</td>
</tr>
<tr>
<td></td>
<td>Ripe</td>
<td>11</td>
<td>1.98</td>
<td>±1.13</td>
<td></td>
</tr>
<tr>
<td>Delphinidin</td>
<td>Unripe</td>
<td>9</td>
<td>18.9</td>
<td>±14.34</td>
<td>0.0080 **</td>
</tr>
<tr>
<td></td>
<td>Ripe</td>
<td>15</td>
<td>40.35</td>
<td>±20.07</td>
<td></td>
</tr>
<tr>
<td>Cyanidin</td>
<td>Unripe</td>
<td>9</td>
<td>33.83</td>
<td>±20.28</td>
<td>0.002 **</td>
</tr>
<tr>
<td></td>
<td>Ripe</td>
<td>15</td>
<td>68.95</td>
<td>±28.96</td>
<td></td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>Unripe</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ripe</td>
<td>1</td>
<td>0.0163</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Peonidin</td>
<td>Unripe</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ripe</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Malvinidin</td>
<td>Unripe</td>
<td>6</td>
<td>1.21</td>
<td>±1.16</td>
<td>0.2967 *</td>
</tr>
<tr>
<td></td>
<td>Ripe</td>
<td>9</td>
<td>0.65</td>
<td>±0.37</td>
<td></td>
</tr>
</tbody>
</table>

N= number of sample trials, CL= confidence limit, P value < 0.05 is statistically significant,*=p value>0.05 not statistically significant,**=p value <0.05 statistically significant, ND= not detected, - = not determined

Table of ripe and unripe quantities of flavonols and anthocyanins identified in purple pitanga fruit extracts, standard deviation, n value included. Student’s T-Tests for determining statistical significance of difference between flavonoids quantified in ripe and unripe fruits.

The results corresponding to quantities of 9 flavonoids investigated by HPLC in pitanga extracts (2) are summarized in Table 3.1 and Figures 3.6-3.7 for ripe and unripe fruits collected which contained both flavonols and anthocyanins. The chromatograms
in Figure 3.3-3.5 show the retention times matching sample extracts to standards as well as overall composition of the individual extracts at their characteristic wavelengths.

### 3.6 Limits of Detection and Quantification

Table 3.2 presents regression line equations (y=mx+b), correlation coefficients (R²), and limits of detection and quantification for all flavonoids. LOD for flavonols were calculated to be 0.98, 1.57, 1.22, 0.65 ng/µL for myricetin, quercitrin, quercetin, and kaempferol respectively. LOQ for flavonols were calculated to be 2.96, 4.76, 3.68, 1.97 ng/µL for myricetin, quercitrin, quercetin, and kaempferol respectively. LOD for anthocyanins were calculated to be; 0.47, 0.47, 0.49, 0.48, and 0.48 ng/µL for delphinidin, cyanidin, pelargonidin, peonidin, and malvinidin respectively. LOQ for anthocyanins were calculated to be; 1.42, 1.41, 1.48, 1.45, and 1.44 ng/µL for delphinidin, cyanidin, pelargonidin, peonidin, and malvinidin respectively (Table 3.2).

**Table 3.2 Limit of Detection and Quantification of Flavonoids**

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>LOD (ng/µL)</th>
<th>LOQ (ng/µL)</th>
<th>y=mx+b</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myricetin</td>
<td>0.98</td>
<td>2.96</td>
<td>y = 39404x + 1002.9</td>
<td>0.9979</td>
</tr>
<tr>
<td>Quercitrin</td>
<td>1.57</td>
<td>4.76</td>
<td>y = 16780x + 4485.4</td>
<td>0.9987</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.22</td>
<td>3.68</td>
<td>y = 74807x + 89474</td>
<td>0.978</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.65</td>
<td>1.97</td>
<td>y = 45007x + 29330</td>
<td>0.9901</td>
</tr>
<tr>
<td>Delphnidin</td>
<td>0.47</td>
<td>1.42</td>
<td>y = 99695x + 3057.2</td>
<td>0.9999</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>---</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Cyanindin</td>
<td>0.47</td>
<td>1.41</td>
<td>y = 94254x + 2279.1</td>
<td>0.9999</td>
</tr>
<tr>
<td>Pelagonidin</td>
<td>0.49</td>
<td>1.48</td>
<td>y = 177235x + 6856.6</td>
<td>0.9999</td>
</tr>
<tr>
<td>Peonidin</td>
<td>0.48</td>
<td>1.45</td>
<td>y = 108419x + 2701.8</td>
<td>0.9999</td>
</tr>
<tr>
<td>Malvinidin</td>
<td>0.48</td>
<td>1.44</td>
<td>y=75611x + 1369.1</td>
<td>1</td>
</tr>
</tbody>
</table>

Limits of detection and quantification for flavonols and anthocyanins quantified in pitanga fruits, trend lines for calibration curves and $R^2$.

3.7 Statistical Analysis: Ripe versus Unripe Flavonoid Quantification

SAS software version 9.3 was used for statistical analysis. Student’s T-tests were used to find significant differences between the quantity of a given flavonoid found in ripe and unripe pitanga fruits. P values for flavonoids were determined for flavonoid quantity in both ripe and unripe fruit extracts. Five out of seven flavonoids were found to be statistically significantly different in quantity of flavonoid when compared. Myricetin, quercitrin, kaempferol, delphinidin and cyanidin contained p values that were statistically significant ($p < 0.05$) (see Table 3.1). The p value < 0.05 indicate a statistically significant difference in given flavonoid quantity between the unripe and ripe fruit extract examined.
3.8 Concentration Changes of Flavonols and Anthocyanins in Pitanga fruit During Ripening

Both flavonols and anthocyanins were identified in pitanga fruit extracts at ripe and unripe periods of collection. Unripe fruit was collected from the plant when the fruit exhibited a reddish color (see Figure 1.5) and had not fully darkened to the purple to black color (see Figure 1.4). Ripe fruit was picked at the darkest stage of ripening when it appeared purple to black in color. See Table 3.1 and Figure 3.5 for quantities of individual flavonols at ripe and unripe stages of collection.

Figure 3.6 Flavonol Ripening Trends: Line graph of flavonols identified and quantified in ripe
and unripe pitanga fruits. Each flavonol identified is labeled by color of line graph. From top to bottom, myricetin decreases from unripe to ripe; 422.62 \( \mu \text{g myricetin/g dried fruit extract (unripe)} \) to 218.61 \( \mu \text{g myricetin/g dried fruit (ripe)} \). Quercetin decreases from unripe to ripe; 129.57 \( \mu \text{g quercetin/g dried fruit extract (unripe)} \) to 93.75 \( \mu \text{g quercetin/g dried fruit (ripe)} \). Quercitrin decreases from unripe to ripe; 26.95 \( \mu \text{g quercitrin/g dried fruit extract (unripe)} \) to 16.14 \( \mu \text{g quercitrin/g dried fruit (ripe)} \). Kaempferol decreases from unripe to ripe; 3.9\( \mu \text{g Kaempferol/g dried fruit extract (unripe)} \) to 1.98 \( \mu \text{g Kaempferol/g dried fruit (ripe)} \).

**Figure 3.7 Anthocyanin Ripening Trends:** Line graph of Anthocyanins quantified in ripe and unripe pitanga fruits. Each anthocyanins identified is labeled by color of line graph. From top to bottom, cyanidin increases from unripe to ripe; 33.83 \( \mu \text{g cyanidin/g dried fruit extract (unripe)} \) to 69.95 \( \mu \text{g cyanidin/g dried fruit (ripe)} \). Delphinidin quantity increases from unripe to ripe; 18.9 \( \mu \text{g delphinidin/g dried fruit extract (unripe)} \) to 40.35 \( \mu \text{g delphinidin/g dried fruit (ripe)} \). Malvinidin decreases from unripe to ripe; 1.21 \( \mu \text{g Malvinidin/g dried fruit extract (unripe)} \) to 0.65 \( \mu \text{g Malvinidin/g dried fruit (ripe)} \).
The major flavonol present in fruit collected at unripe stage of growth was Myricetin (422.62 ± 79.30 µg/g dry fruit) (See Table 3.1), quercetin was also found in significant quantity at 129.57 ± 36.63 µg/g dry fruit, followed by quercitrin (26.95 ± 12.69 µg/g dry fruit) and kaempferol (3.9 ± 2.06 µg/g dry fruit). The major anthocyanin found in fruits collected at unripe stage of growth was cyanidin (33.38 ± 20.28 µg/g dry fruit). Delphinidin was found (18.9 ± 14.34 µg/g dry fruit) as well as malvinidin (1.21 ± 1.16 µg/g dry fruit). Pelargonidin and peonidin standards were employed and not found in any sample extracts when compared to standard retention times.

The major flavonol present in fruit collected at the ripe stage of growth was myricetin (231.31 ± 124.54 µg/g dry fruit). Quercetin was also found in moderate amount (93.75 ± 41.0 µg/g dry fruit), followed by quercitrin (16.14 ± 7.75 µg/g dry fruit) and kaempferol (1.98 ± 1.13 µg/g dry fruit). The major anthocyanin found in fruits collected at ripe stage of growth was cyanidin (68.95 ± 28.96 µg/g dry fruit). Delphinidin was found (40.35 ± 20.07 µg/g dry fruit) as well as malvinidin (0.65 ± 0.37 µg/g dry fruit). Pelargonidin and peonidin standards were employed and not found in sample extracts when compared to standard retention times. Pelargonidin was detected in only one sample of ripe fruit at a very small quantity (0.16 µg/g dry fruit).
4.1 Extraction

Extraction methods used in flavonoid studies typically do not utilize multiple solvents. Most methods use a single solvent for extraction. When observing the extraction with both solvents it was evident that a majority of anthocyanins remained after the first extraction. This was based on the observation that the freeze-dried fruit still contained a bright pink color in the solid extract. Upon the second extraction this color was removed into the solvent mixture. This second extract was later identified by HPLC to contain anthocyanins. This method provided a more thorough extraction by utilizing two solvents which both removed a great deal of flavonoids from the freeze-dried pitanga fruits.

The acetone extract after 3 extractions, was yellow in color. This extract matched the color observed for that of flavonols and was later verified by HPLC to contain these compounds. The extraction directed towards anthocyanins was conducted using 10:9:1 v/v Ethanol: H$_2$O: Acetic Acid. The color observed from this extract corresponded to the color of anthocyanins (fuchsia to purple). This suggested a majority of the compounds extracted from this dried extract were anthocyanins. This was later verified by HPLC to contain these anthocyanins. The non-polar extraction solvent (acetone) was used first to remove flavonols and lipophilic compounds from the outer epidermal cells (Koes, Quattrocchio, & Mol, 1994; Shirley, 1996). This aided in the maximal removal of the
aqueous polar anthocyanins stored in the vacuoles (Charriere-Ladreix & Tissut, 1981) of the cell being removed second using acidic ethanolic extract.

4.2 Pre-purification

The acetone extract after being dried by rotary evaporation was subjected to a hexane: water partition. The hexane layer which was discarded contained a white waxy layer as well as an orange color in the hexane solvent. This suggests that carotenoids (orange in color) may have been removed from the extract. This procedure appears to have removed lipophilic compounds (example: fats and carotenoids) which could potentially interfere with the desired flavonoids. Pre-separation of anthocyanins and flavonols isolated the flavonoid classes which could potentially react together during the acidic hydrolysis reaction. The yellow color which eluted during the ethyl acetate column rinse was indicative of the flavonols being separated from the overall extract based on their UV absorption maximum and characteristic yellow color. The blue to fuchsia color which eluted during the acidic MeOH column wash indicated that anthocyanins were being removed from the C18 pre-column from the overall mixture of compounds loaded on to the column. This pre-purification method has also been successfully employed by (Oszmianski, 1990) purifying polyphenols found in red grapes. This method of pre-purification is not commonly used for flavonoid identification. However, these steps greatly improve the method and analysis by; eliminating possible side reactions and degradation which can occur from a longer than necessary hydrolysis.
4.3 Hydrolysis

Having both anthocyanins and flavonols separated prior to HPLC and hydrolysis allowed for a more controlled hydrolysis reaction with minimal degradation to undesired compounds. The ethyl acetate elutant from acetone extract was hydrolyzed for 60 min. This fraction appeared to contain either the most hydrolysable flavonols or the lowest quantity of glycosylated flavonols, as the lowest time was required for full removal of sugars. The acidic MeOH elutant from the acetone extract was hydrolyzed for 120 min. These anthocyanins appeared to contain glycosylations which are more difficult to remove than the flavonols extracted from the more non-polar acetone extract. The ethyl acetate elutant from the ethanolic extract was hydrolyzed for 80 min. This fraction appeared to contain glycosylations which required more time to remove than the fraction isolated from the acetone extract and purified for flavonols. The hydrolysis time was 20 min longer than flavonols extracted using acetone. It could be possible that the flavonols in this more polar extract (aqueous acidic ethanol) contained multiple glycosylations. As these compounds were extracted using a more polar solvent, and flavonols with multiple sugars would be more polar in structure as well. The acidic MeOH elutant from the aqueous acidic ethanol extract required 4 hours and 30 min for complete removal of sugars. This fraction may have contained anthocyanins with multiple glycosylation or sugars which required a longer reaction time to remove.
Flavonols in this study overall required a shorter hydrolysis reaction time when compared to the anthocyanins. The more polar extraction fractions seemed to contain a greater quantity of sugars present on flavonoid backbones.

4.4 HPLC Chromatograms

As seen in Figures 3.1-3.3 sample extracts contained flavonoids which matched retention times to known standards. In Figure 3.3 sample pitanga extract was pre-purified for flavonols and hydrolyzed for 60 min. The two main peaks which appeared when examined at the wavelength of 370 nm were myricetin followed by quercetin. See Figures 4.1 and 4.2 for reference to chemical structure and degree of hydroxylation. Myricetin being more polar in structure by one hydroxyl group eluted from the column before quercetin.

![Figure 4.1 Myricetin (CAS 529-44-2)](image1)
![Figure 4.2 Quercetin (CAS 117-39-5)](image2)

Above is the structure of flavonols; myricetin and quercetin for comparison of degree of hydroxylation affecting the order of retention on HPLC.
In Figure 3.4, the unhydrolyzed extract was compared to flavonol standards for the identification of quercitrin (Qu 3-rha) in the pitanga extracts. Quercitrin containing one glycosylation possessed a retention time which was shorter than that of its deglycosylated counterpart quercetin. See Figures 4.2 and 4.3 for comparison of quercetin structure to quercitrin. As the glycosylated form of the compound (quercitrin) would be more polar in chemical property it is logical that it would elute from the column first. The more polar compounds will have a greater affinity to the liquid phase which is also polar and lesser affinity to the solid phase which is non-polar in character.

![Quercitrin structure](image)

**Figure 4.3 Quercitrin (CAS 522-12-3)**

Above is the structure of the flavonol; quercitrin for comparison of degree of hydroxylation which affected order of retention on HPLC.

The chromatogram in Figure 3.5 is a representative trial of anthocyanin standards which were compared to unknown anthocyanins. These peaks were the major peaks observed for this given extract injected, their retention times were identical to delphinidin and cyanidin. Malvinidin was also identified in some of the extracts but
not all (9 unripe, 6 ripe samples). The first compound to elute from the column was delphinidin followed by cyanidin. See Figures 4.4 and 4.5 for reference to chemical structure and degree of hydroxylation. Delphinidin being more polar in structure by one hydroxyl group eluted from the column before cyanidin.

![Delphinidin and Cyanidin Structures](image)

**Figure 4.4 Delphinidin (CAS 528-53-0)  Figure 4.5 Cyanidin (CAS 528-58-5)**

Above is the structure of the anthocyanins; delphinidin and cyanidin for comparison of degree of hydroxylation affecting order of retention on HPLC.

### 4.5 Flavonoid Identification and Quantification

The unknown flavonols and anthocyanins identified in pitanga extracts were identified by identical retention times to standards. When these pure compounds were compared to hydrolyzed pitanga extract pre-purified for flavonols or anthocyanins they resulted in two major peaks with identical retention times to pure the flavonoid standards; delphinidin (Dp), cyanindin (Cy) for anthocyanins and myricetin and
quercetin for flavonols. Their quantities were found by extrapolation from matching calibration curves with identical injection volumes.

Similar methods were employed by Hoffmann-Ribani, Huber, & Rodriguez-Amaya, (2009) and three flavonols were identified in undefined varieties of garden and supermarket pitanga (*E. uniflora* L.). From garden variety pitanga; Myricetin (3.7 ± 0.4 mg/100g fresh weight), quercetin (6.2 ± 0.9 mg/100g fresh weight), and Kaempferol (0.4 ± 0.0 mg/100g fresh weight) was found. From supermarket variety pitanga; Myricetin (3.1 ± 0.4 mg/100g fresh weight), quercetin (5.5 ± 1.0 mg/100g fresh weight), and Kaempferol (0.4 ± 0.1 mg/100g fresh weight) was quantified in 3 samples. The results from Hoffmann-Ribani et al. (2009) were documented in wet weight and not freeze-dried weight as currently reported, therefore an accurate comparison between these quantities can only be estimated.

The wet weight quantities were estimated by massing 9 Zill Dark pitanga fruits at ripe stage of growth. The wet mass was recorded for each fruit, then freeze-dried and massed. From this an average wet mass/dry mass was used to convert each sample’s freeze-dried mass to wet mass. This was then calculated as the mass per quantity flavonoid isolated, to give an idea of how our results compare to previously published quantities of flavonols identified in the pitanga fruit. From these calculations, the ripe Zill Dark pitanga contained 3.6 ± 2.0 mg/100g fresh weight of myricetin, (n=15). The ripe fruit contained 1.5 ± 0.6 mg/100g fresh weight of quercetin, (n=15), 0.3± 0.1mg/100g fresh weight (n=15) of quercitrin, and 0.03± 0.02 mg/100g fresh weight of kaempferol, (n=11). The quantity of Myricetin is very similar in the ripe Zill Dark pitanga when
compared to the previous work conducted, however all other masses have a great deal of variation. This may be due to variation in growth conditions between Brazil and Hawaii, HI as well as the number of samples which were studied. The results published by Hoffmann-Ribani et al. (2009) examined only 3-4 samples, where as our results analyzed 7-15 samples for each flavonoid identified.

4.6 Limits of Detection and Quantification

Excellent linearity was observed for all flavonoids peak areas versus concentrations (0.3-8 ng/μL for anthocyanins, 1.5-30 ng/μL for flavonols). Anthocyanins appeared to have a more sensitive level of detection and quantification than flavonols by about a factor of 3 for all flavonols except Kaempferol. See Table 3.2 for numerical results of LOD, LOQ, regression line equations (y=mx+b), and correlation coefficients (R²). The current method appears to be sensitive with a relatively low limit of detection and quantification in the range of ng/μL volume injected.

4.7 Concentration Changes of Flavonols and Anthocyanins in Pitanga fruit

During Ripening

Overall the concentrations of flavonols were greater in unripe fruit when compared to ripe fruits see Table 3.1 and Figure 3.6 for comparison. There was a two-
fold increase in the quantity of anthocyanins in ripe fruits when compared to unripe (see Figure 3.7). This result is consistent with the increase in purple color observed in the fruit during the ripening process. There appears to be a correlation in this study between the flavonol quantity decreasing and anthocyanin quantity during the ripening process. These results are consistent with observed bilberry fruit ripening by (Jaakola, et al., 2002) where flavonol content was highest at beginning of ripening and decreased at ripest stage. Anthocyanins were not identified in fruit until middle of ripening period and increased until reaching ripest stage.

One hypothesis for the early accumulation of flavonols could be related to their role in UV protection on the epidermal layers of the fruit and plant. Flavonols and flavones accrue on the epidermal surface of plants after being signaled by UV induction (Shirley, 1996; Schmelzer, Jahnen, & Hahlbrock, 1988; Koes, Quattrocchio, & Mol, 1994). The flavonoid biosynthetic genes responsible for flavonol and flavone production are up-regulated after UV induction (Kubasek, et al., 1992; Schmelzer, Jahnen, & Hahlbrock, 1988; van Tunen, et al., 1988). This could explain why the concentration is higher in early stages and could become depleted by degradation from UV light. Studies on a variety of plant species including; Petunia (Ryan, Swinny, Markham, & Winefield, 2002), Arabidopsis (Ryan, Swinny, Winefield, & Markham, 2001), Vicia faba (Rozema, et al., 2002), and Oryza sativa (Marham, et al., (1998) found UV light to favorably up-regulate synthesis of flavonols containing a higher quantity of hydroxyl groups. These hydroxyl groups confer a greater degree of antioxidant capacity for the plant as a protection against UV light damage (Ryan, Swinny, Winefield, & Markham, 2001; Smith &
Markham, 1998). This may explain why the quantity of Quercetin identified in pitanga extracts was greater when compared to that of Kaempferol in pitanga extracts. See Table 1.1 and Figures 4.1-4.5 to compare degree of hydroxylation of flavonols and anthocyanins. Refer to Figure 3.6 and Table 3.1 for flavonol quantities.

Anthocyanins are also believed to function as antioxidants (Takahama, 1988) but instead of accumulating in the epidermal layers like the flavonols, they localize mostly in the vacuoles and cytoplasm of the cell. These compounds may instead function as a counter to oxidative stress and reactive oxygen species produced within the fruit during growth (Charriere-Ladreix & Tissut, 1981). As these activities would be most necessary upon fruit ripening, they may be synthesized in greater quantities towards the later stages of fruit growth.
Chapter 5: Conclusions

Four flavonols and three anthocyanins were identified in the extracts of purple fruited pitanga (*E. uniflora* L.). Overall the concentration of flavonols found at unripe stages was higher than in ripe fruit. The overall concentration of anthocyanins was found to be higher in ripe fruits than in unripe fruits. Cyanidin was the major anthocyanin identified in both ripe and unripe extracts pitanga fruits. Myricetin was found to be the major flavonol identified in both ripe and unripe extracts of pitanga fruits.

The current method discussed provides a rapid analysis of individual flavonoid classes. The dual optimized extraction allows for maximum removal of flavonols and anthocyanins. The pre-purification step using a hexane: water partition removes a great deal of impurities from the extract mixture. Removal of these impurities prevents them from reacting with desired flavonoids and or being permanently retained on to the reverse phase column. The Sep-Pak™ *C*$_{18}$ fractionation method effectively isolated flavonols and anthocyanins for a more controlled hydrolysis and HPLC analysis. The hydrolysis optimization for each pre-purified fraction minimized degradation and maximized quantification for each flavonoid examined.

These results show that both ripe and unripe selections of the pitanga are a rich source of flavonols and anthocyanins, as well as a valuable addition to the human diet.
This work can be utilized to inform the public and epidemiological studies on the flavonoid content of the purple fruited selections of the pitanga fruit as well as the unripe to ripe variations in flavonol and anthocyanin content.

The Zill Dark variety of pitanga fruit grown on Kona, HI as of 2011 is being sold in local grocery stores and farmers markets. The containers are labeled describing their bioactive content including flavonoids and Vitamin C. This helps to inform the consumer and sell the fruit. The pitanga has also been made into jam and sold in a variety of stores on Maui, Hawaii, and Oahu. The juice has been combined into a variety of foods, drinks and fermentation products such as local wine, beer, and kombucha.
Bibliography


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