INFLUENCE OF THERMAL POSTHARVEST STRESS ON MANGO \textit{(Magnifera indica)} POLYPHENOLICS DURING RIPENING

By

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I wish to dedicate this thesis to my daughter, Emily Faith-Lounds Singleton and nieces: Karly Brianne, Chloe Marie, Kelsey LeeAnn, Olivia Tatum and Kendall Elizabeth Lounds. May this accomplishment serve to inspire you to set your goals high, never give up pursuit of your dreams and always remember that through God anything is possible. I love you all dearly.
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Mangoes are considered one of the most preferred fruits of the world because of their attractive color, delicious taste and excellent nutritional properties. Abundant in antioxidants, mangoes are among the many fruits consumed for their potential health benefits including anticancer and antiviral activities and reduced risk of cardiac disease associated with antioxidant activities (AOX) of polyphenolic and carotenoid compounds. Postharvest studies have been pursued to optimize shelf stability of fresh mango fruit for world distribution, but no investigations have addressed the effects these treatments have on synthesis, concentration, and retention of polyphenolic compounds. This study identified polyphenolics present at various maturity stages of mango fruit subjected to thermal quarantine treatments followed by variable storage temperatures using high performance liquid chromatography (HPLC). Gallotannins were hydrolyzed with β-glucosidase and tannase to quantify free and bound gallic acid (GA). Polyphenolic
fractions were assessed for their chemical activities using the oxygen radical absorbance capacity (ORAC) and Folin-Ciocalteu assays. Hot water dips accelerated ripening of mangoes and increased 100 μg/g total soluble phenolics and 10 μM Trolox equivalence/mL antioxidant capacity of polyphenolic compounds in ripe fruit. Cold storage caused chilling injury, a result of postharvest abiotic stress resulting in loss of gallic acid, gallotannins, total soluble phenolics and antioxidant capacity.
Mango (*Magnifera indica*) is a tropical tree and its fruit are considered a staple food in many areas of the world. Consumption of mangoes has increased over the past decade, becoming one of the most favored tropical fruits. Mangoes were ranked second to bananas among fruit crops and fifth overall in the world in terms of tropical and subtropical agricultural production in 1996 (Gil et al., 2000; Olle et al., 1996), and are currently ranked second to pineapple in quantity and value (Food and Agriculture Organization [FAO], 2003). Though they are grown around the world, India is the leading world producer, providing over 60% of the world’s supply (FAO, 2000; Mitra and Baldwin, 1997; Olle et al., 1996).

Mangoes are highly perishable tropical fruit, with a shelf life of 2-4 weeks at 10-15°C (Yahia, 1998), limiting their availability in fresh markets. Shelf stability and physical and chemical quality of mangoes can be threatened by required insect quarantine treatments, chilling temperatures and fungal rot, as these conditions may induce abiotic or biotic stress to the fruit (Cisneros-Zevallos, 2003). Postharvest physiologists have extensively studied postharvest treatments to identify optimal conditions for the retention of fruit quality, enabling these fruit to be shipped to distant markets.

Phenolic compounds increase under environmental stress, playing a vital role in plant survival. They provide cell-wall support materials (lignin), function as inhibitory compounds in competition among plants (allelopathy), and act as signal molecules in
plant defense mechanisms against invasive stressors of microorganisms, excessive UV light, drought and wounding.

The chemical composition of fruit and thus their health benefits to consumers are largely dependent on the maturity of the fruit and the degree of processing applied prior to consumption. Generally, fruit will reach their highest levels of sugars, ascorbic acid, soluble solids, fats and pectin, and their lowest levels of acidity and phenolic acids as they ripen (Bulk et al., 1997; Joseph and Aworh, 1991). These ripening related changes provide ripe fruit with essential vitamins and minerals needed in the human diet. Thermal preservation of fruit serves to lengthen the shelf life, providing the desired commodity regardless of fresh fruit availability. These processes, however, often degrade ascorbic acid and oxidize fat soluble vitamins, reducing the overall health benefit to consumers. Fresh mangoes are considered to be rich in vitamins A (80.3% DRI per 100 g) and C (95.3% DRI per 100 g), and are a significant source of folate, calcium, magnesium, phosphorus and potassium (Cyberdiet, as referred by the USDA website).

Recent awareness of the role antioxidants play in the promotion of health, due to their ability to act as chemoprotective agents (Hertog et al., 1992; Nicoli et al., 1997; Teissedre and Waterhouse, 2000), has contributed to the rise in consumer demand for fresh fruits. Epidemiological studies have linked numerous food polyphenolic compounds with the reduction in the risk of various diseases, attributed to their natural antioxidants ability to act as free-radical scavengers. Green tea extracts, containing catechins, possess strong antioxidant activity in protecting against lipid peroxidation and free radical generation in neonatal rat cardiomyocytes cultures (Toschi et al., 2000). Curcumin, a constituent of curry spice, prevented tumorigensis through inducing apoptosis in newly
emerging cancer cells as they promoted the formation of reactive oxygen species (Kelly et al., 2001). An in vitro study of flavonoids from various food sources demonstrated their ability to inhibit lipoprotein oxidation (Vinson et al., 1995a, b), a precursor for coronary heart disease (CHD). Later, in 1999, total flavonoid intake was associated with the reduced risk of coronary heart disease in postmenopausal women (Yochum et al., 2000). Neochlorogenic and chlorogenic acid, found in prune extracts, have been reported to significantly inhibit oxidation of lipids in human low density lipoproteins (LDL) (Donovan et al., 1998). Sufficient evidence exists between in vivo and in vitro studies to support the role antioxidant polyphenolic compounds play in the prevention of disease and thus promotion of health.

Consumer demand for mangoes has caused a dramatic increase in fresh mango availability throughout the year, with the US importing $157 million (262,298 tons) of fresh mango in 2001 (United States Department of Agriculture-Agricultural Marketing Service [USDA-AMS], 2003), up 185% from 1995 (141,684 tons). Abiotic stress has been reported to cause biochemical and physiological changes in ripening fruit. Specifically, environmental stress of excessive UV light has been reported to stimulate the synthesis of flavonoids, as phenolic compounds are thought to help attenuate the amount of light reaching plant photosynthetic cells (Beggs et al., 1985; Britton, 1983; Macheix et al., 1990). The influence of thermal insect quarantine and cold storage postharvest treatments on the development of polyphenolic compounds in mango fruit has not been reported. Understanding the effects these commercial practices have on potential health benefits of radical-scavenging polyphenolic compounds will enable fruit

handlers (growers, processors, distributors, consumers) to provide the fresh fruit market with fruit abundant in antioxidant polyphenolic compounds.

It is hypothesized that the applications of thermal quarantine and cold storage postharvest treatments will induce changes in polyphenolic compounds as these treatments may stimulate a stress-induced response that elicits a chemical defense mechanism. The objectives for this study were

1. To isolate and identify polyphenolic compounds in mango fruit at varying levels of maturity and to assess their stability and radical scavenging activities.

2. To observe physicochemical changes in mango phytochemicals subjected to various postharvest treatments.

3. To address industrial applications for postharvest handling of mango fruit that would insure optimal AOX for a fresh foods market.
Mango Market

Mango originated in the Indo-Burmese region of Southeast Asia, and is now produced by several countries worldwide. India is the world leader in production, with China, Mexico, Thailand, Philippines, Pakistan, Nigeria, Indonesia, Brazil and Egypt providing up to 40% of the market (in descending order) (FAO, 1999). World production reached 23,852,000 tons in 1999, 1.2 million tons higher than 1995 production (DA-AMAS, 2003). Consumer demand for mangoes is expected to continue to increase throughout the next decade.

The United States (US) produces less than 1% (2,270 metric tons in 1998) of the world production of mangoes, Florida providing 97% with a crop value of $5.8 million (FAO, 1999). Prior to hurricane Andrew in 1992, Florida’s production of mangoes reached 22,000 metric tons. The devastation of this storm has lead to the US being the largest importer of fresh mango (Department of Agriculture-Agribusiness and Marketing Assistance Service [DA-AMAS], 2003), with the US market supplied primarily by Mexico with off-season fruit from Central and South Americas.

The fresh mango market in the US has rapidly increased over the past decade, with a fresh market value of $157 million (2001). Consumption of mangoes has increased from 0.21 pounds per capita (1979) to 0.51 pounds (1989) and was projected to reach 1.8 pounds per capita in 2000 (USDA-AMS, 2003). Record levels in the US reached 235,000 metric ton of fresh mango imported in 2001 (USDA-AMS, 2003).
Hundreds of mango cultivars exist throughout the world, differing in size, shape and external color. In the US, the Tommy Atkins variety is the most popular, followed by Keitt, Kent and Haden. Tommy Atkins, marketed for its eye-appeal, is considered to be medium sized, weighing roughly 1 pound, has a beautiful-firm exterior, is finely fibrous and has a mild flavor compared with other varieties.

**Mango Postharvest Handling**

The trade of mango has been significantly limited due to their short shelf life and highly perishable nature (Gil et al., 2000). Harvest maturity and postharvest storage conditions are commonly altered to lengthen the shelf life of fruit for extended markets. Time of harvest, storage temperature and atmospheric conditions are all key factors in postharvest physiology of fruit.

A climacteric fruit, mangoes are harvested at a mature, unripe (green) stage as they will naturally ripen off the tree. Harvesting mangoes prematurely will prevent fruit from reaching full ripeness. Harvesting fruit at stages beyond mature green will reduce their shelf stability and shorten their fresh market life. Kalra et al. (1995) reported that mature ripe mangoes perished within 6 days under ambient conditions.

Postharvest observations of mango have been used to understand the physiological changes due to environmental conditions. The rate of respiration and ripening, development of pigments, flavor compounds, polyphenolics, sugars, fruit quality, and postharvest diseases are all affected by handling procedures following harvest (Baldwin et al., 1999; El Ansari et al., 1969; Fuchs et al., 1980; Fuchs et al., 1989; Gil et al., 2000; Jacobi and Wong, 1991; Jacobi et al., 2000; Jacobi et al., 2001a, b; Lakshminarayana et al., 1970; Lakshminarayana, 1973; Lizada, 1991; Medlicott et al., 1988; Medlicott et al., 1990; Mercandante and Rodriguez-Amaya, 1998; Muda et al., 1995; Parmar and
Chundawat, 1988; Roy and Joshi, 1988; Singh and Chundawat, 1991; Tandon and Kalra, 1983). Because most mangoes must be exported to distant markets around the world, they are often subjected to various postharvest treatments (cold storage (CS), modified atmosphere, controlled atmosphere, thermal quarantines, fungicidal sprays, etc.) intended to lengthen their shelf life and prevent the spread of invasive pests that cause economic and environmental harm to US agriculture.

Thermal Quarantine

Thermal quarantine treatments are heat disinfection treatments used as a viable non-chemical control method for the prevention of invasive pests, specifically the Mediterranean fruit fly (Ceratitis capitata) and the Mexican fruit fly (Anastrepha ludens), in mango around the world. Prior to their implementation in 1992 by USDA-Animal and Plant Health Inspection Service (APHIS), ethylene dibromide fumigation was the standard disinfection treatment for mango (Paull and Armstrong, 1994; Sharp et al., 1994). Three thermal quarantine treatments (vapor heat treatment (VHT), forced hot-air treatment (FHAT) and hot water immersion treatment (HWT)) are used worldwide, but only VHT (6 hour treatment) and HWT (60-110 minute treatment) are currently accepted by the US, of which all imported mango from a non-fly free zone must undergo prior to their entrance (Jacobi et al., 2001b). HWT is the most preferred quarantine treatment because it is easily adaptable by growers and produce distributors, uses short treatment times, is reliable and accurate in the monitoring of fruit temperatures, is efficient in killing surface decay organisms, and cleanses fruit surface during treatment (Jacobi et al., 2001b; Shellie and Mangan, 2000).

HWT is a hot water dip consisting of fruit immersion in water held at 43-46°C, according to the USDA-APHIS Treatment Schedule in Table 2-1 (USDA-APHIS, 2002).
Table 2-1 USDA-APHIS treatment schedule of required dip time based on fruit origin taken from USDA-APHIS (2002).

<table>
<thead>
<tr>
<th>Origin of fruit</th>
<th>Shape of fruit</th>
<th>Weight of fruit (grams)</th>
<th>Dip time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerto Rico, U.S. Virgin Islands, or West Indies</td>
<td>Flat, elongated varieties(^1)</td>
<td>Up to 400</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400 to 570</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Rounded varieties(^2)</td>
<td>Up to 500</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500-700</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>701-900</td>
<td>110</td>
</tr>
<tr>
<td>Mexico or Central America</td>
<td>Flat, elongated varieties(^1)</td>
<td>Up to 375</td>
<td>65</td>
</tr>
<tr>
<td>(North of and including Costa Rica)</td>
<td></td>
<td>400 to 570</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Rounded varieties(^2)</td>
<td>Up to 500</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 to 700</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>701 to 900</td>
<td>110</td>
</tr>
<tr>
<td>Panama, South America or West Indies</td>
<td>Flat, elongated varieties(^1)</td>
<td>Up to 375</td>
<td>65</td>
</tr>
<tr>
<td>islands of Aruba, Bonaire, Curacao, Margarita, Tortuga, or Trinidad and Tobago</td>
<td></td>
<td>375 to 570</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Rounded varieties(^2)</td>
<td>Up to 425</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>425 to 650</td>
<td>90</td>
</tr>
</tbody>
</table>

\(^1\)Cultivars: ‘Frances,’ ‘Carrot,’ ‘Zill,’ ‘Ataulfo,’ ‘Carabao,’ ‘Irwin,’ and ‘Manila.’

\(^2\)Cultivars: ‘Tommy Atkins,’ ‘Kent,’ ‘Hayden,’ and ‘Keitt.’

Temperatures above 46°C have been reported to produce excessive fruit damage (Sharp, 1994) including skin scalding, abnormal erratic yellow patches of color development with ripening, accelerated skin color development (yellowing), damaged lenticels (dark halo of tissue surround lenticel pores), accelerated respiration rates during pre-climacteric period and development of storage disease such as anthracnose and stem end rot (Jacobi and Wong, 1991; Jacobi and Wong, 1992; Jacobi et al., 2000; Jacobi et al., 2001a, b; Joyce et al., 1993; Paull, 1994; Singh and Chundawat, 1991). These HWT induced injuries are often referred to as heat injuries (HI) as they are a result of hot bath temperatures. HI reported in ‘Tommy Atkins’ include darkened lenticels with HWT of 46°C for 120 minutes and 49°C for 60 minutes (Spalding et al., 1988) and skin scalding with HWT of
42-48°C for 30-90 minutes (Smith and Chin, 1989). Under optimal HWT conditions (dependent upon fruit size and shape), studies have reported HWT to accelerate the rate of ripening (Jacobi and Giles, 1997; Jacobi and Wong, 1991, 1992; Jacobi et al., 1996, 1998, 2000, 2001a; McCollum et al., 1993) and promote the uniformity of color development in the mango peel of ‘Tommy Atkins’ fruit (Jacobi et al., 1998).

Cold Storage

Cold storage of mango is used to prolong shelf life by slowing the metabolic rate of fruit. Chaplin et al. (1991) was successful in the application of CS at 15°C to ‘Kensington’ mango for 4 weeks, with acceptable ripening and quality indices upon ripening. However, because mango are of tropical origin, their storage conditions are limited to those with temperatures greater than their critical minimum temperature of 10°C (~10-16°C depending on the cultivar and maturity or ripeness stage) in order to prevent chilling injury (CI). CI occurs as a result of disruption in cell wall membrane functions, changing the flow of cellular fluids in and out of the cell, causing the leakage of metabolites such as amino acids, sugars, and mineral salts (Wills et al., 1981). Symptoms of CI have been reported in mango as brown discoloration of the skin, pitting and breakdown of pulp (Lizada, 1991), lenticel spotting (Pesis et al., 2000), uneven ripening (Lederman et al., 1997), grey skin scald and softening of the tissues (Kalra et al., 1995) and electrolyte leakage (Fuchs et al., 1989). The degree of CI symptoms is often measured by indices (Equation 2.1) that compare the number of fruit at a particular injury level to the total number of fruit in each treatment (Lederman et al., 1997; Pesis et al., 2000). Symptoms of these injuries generally develop after removal from the chilling


\[
\text{CI index} = \frac{(\text{Injury level}) \times (\text{Number of fruits at the level})}{(\text{Total number of fruits in the treatment})} \quad \text{Equation 2.1}
\]

temperature to non-chilling temperatures, as the development of symptoms is very slow at low, injurious temperatures. Reductions in shelf life, total soluble solids and tissue softening have been associated with CI symptoms (Chaplin et al., 1991; Lederman, 1997; Medlicott et al., 1990). Chaplin et al. (1991) stored mature green ‘Kensington’ mangoes at 1, 5, 10 or 15°C for 1, 2, 3, or 4 weeks, and found that CI symptoms increased with duration of storage and with decreases in temperature. Lederman (1997) applied CS treatments of 0, 2, 5, 14, and 20°C to mature green ‘Keitt’ mangoes, with CS optimum at 14°C as CI symptoms in fruit stored below 14°C prevented fruit ripening. Pesis et al. (2000) stored mature green ‘Tommy Atkins’ mangoes at 12°C and found slight CI symptom of red spots around lenticels. However, in an earlier study by Medlicott et al. (1990), ‘Amelie,’ ‘Tommy Atkins’ and ‘Keitt,’ mature green mangoes stored at 12°C for 21 days failed to reach full ripeness. These differences in chilling sensitivity with CS at 12°C demonstrate variation among maturities, cultivars, fruit season and/or growing location. CI of mature green ‘Tommy Atkins’ fruit stored at 12°C has been reduced by modified atmosphere of ~5% CO\(_2\) and ~10% O\(_2\) and modified humidity packaging using Xtend® films and polyethylene following 3 weeks of CS and 1 week at 20°C (Pesis et al., 2000). Additionally, CI symptoms have been reduced and/or prevented with pre-conditioning of mature green fruit using intermittent warming techniques of 38°C for 24 or 48 hours prior to CS of 5°C for 11 days (McCollum et al., 1993) and with storage at 27-34°C until ripe with subsequent CS of 5, 10, or 15°C (Thomas and Joshi, 1988). Pre-conditioned fruit in both of these studies had an extended shelf life of 1-2 weeks.
attributed to the fruit’s ability to withstand reduced temperatures that in turn slow respiration rates.

The goal of postharvest storage regimes for fresh fruit distribution is to create a set of conditions conducive to extending shelf life while reducing or eliminating conditions deleterious to consumer appeal. The use of postharvest techniques may biochemically alter fruit tissue, as respiration rates increase or decrease, affecting the natural ripening process. These biochemical changes in ripening may be responsible for potential alterations in radical scavenging compounds due to an elicited biological response to stress when stored above or below optimum temperatures. As a result, postharvest treatments may have a new role as a food additive process, improving potential health benefits of mango.

**Mango Ripening**

Mango is a climacteric fruit that is harvested at a physiologically mature-green stage and allowed to ripen for fresh market. The ripening of mango fruit involves many chemical and physiological changes as the climacteric peak of respiration is reached. Unripe fruit are characterized by their firm texture, high starch content, high organic acid concentrations and subsequent low pH.

During ripening, starch is hydrolyzed by amylase and converted to sugars (Modi and Reddy, 1967) as Shashirekha and Patwardhan (1976) have demonstrated an increase in glucose (420-4200 mg/100g), fructose (560-4300 mg/100g) and sucrose (16 to 4400 mg/100g) during ripening of ‘Badami’ mangoes. Meanwhile, cell wall carbohydrates (pectin and hemicellulose) are enzymatically hydrolyzed causing textural softening of mango pulp. Polygalacturonase (PG) and β-D-galacturonase are commonly regarded as
being responsible for tissue softening, as enzyme levels increase simultaneously with pectin depolymerization (Ali et al., 1995, Muda et al., 1995).

Organic acids are crucial to the ripening of mango as they are primary respiratory substrates (Baqui et al., 1974; Matoo and Modi, 1970; Modi and Reddy, 1967) over glucose. Though several organic acids have been identified in mango (Table 2-2), only citrate, malate and succinate serve as respiration substrates. As climacteric fruit,

Table 2-2 Organic acids measured in mango fruit

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>mg/100g</th>
<th>Cultivar</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unripe</td>
<td>Ripe</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>4730</td>
<td>360</td>
<td>Keitt</td>
</tr>
<tr>
<td></td>
<td>2250</td>
<td>244</td>
<td>Badami</td>
</tr>
<tr>
<td></td>
<td>3200</td>
<td>280</td>
<td>Unknown</td>
</tr>
<tr>
<td>Malate</td>
<td>2700</td>
<td>55.0</td>
<td>Keitt</td>
</tr>
<tr>
<td></td>
<td>24.0</td>
<td>96.0</td>
<td>Badami</td>
</tr>
<tr>
<td></td>
<td>894</td>
<td>14.0</td>
<td>Unknown</td>
</tr>
<tr>
<td>Succinate</td>
<td>316</td>
<td>40.0</td>
<td>Badami</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>--</td>
<td>30.0</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>73.0</td>
<td>55.0</td>
<td>Wild</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>90.0</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>25.3</td>
<td>16.4</td>
<td>Unknown</td>
</tr>
<tr>
<td>Folate</td>
<td>--</td>
<td>&gt;5</td>
<td>Unknown</td>
</tr>
<tr>
<td>Uronates</td>
<td>11.0</td>
<td>20.0</td>
<td>Badami</td>
</tr>
</tbody>
</table>

the highest respiration rate in ripening mango fruit occurs at the climacteric peak, paralleling the sigmoidal ethylene production pattern. Once fruit have fully ripened, they begin the senescence process with respiration rates reaching their lowest levels while ethylene productions drop to hardly detectible (Ketsa et al., 1999, McCollum et al., 1993).

Carotenoids are responsible for the attractive skin and flesh color of ripe mangoes (Medlicott et al., 1986) and may be used as a visual indicator of fruit maturity and ripeness as pigments shift from green to yellows and oranges. As a result, total carotenoids in mango flesh have been reported to increase from 12.3 to 38.0 µg/g in
‘Keitt’ and 17.0 to 51.2 µg/g in ‘Tommy Atkins’ (Mercandante et al., 1998).

Development of carotenoids provide ripe mango with an attractive and enticing color.

**Mango Polyphenolics**

Mango polyphenolic compounds have been separated and identified using various extraction and separation techniques for the past 30 years. Authors of the first publication relating to polyphenolics present in mango used two-dimensional paper chromatography to separate polyphenolic compounds extracted with acetone, ether, ethyl acetate, and methanol (El Ansari et al., 1969). They were successful in the identification of ellagic acid, gallic acid (GA), $m$-digallic acid, $m$-trigallic acids, isoquercitin, mangiferin, quercetin and gallotannin (GT) from the variety ‘Rumani.’ Saeed et al. (1976) also utilized two-dimensional paper chromatography to identify mango polyphenolic compounds extracted with water and ethyl acetate, and tentatively confirmed similar polyphenolics. More recent studies have shown a preference for the separation of phenolic compounds using HPLC due to its speed, resolution, and sensitivity. Schieber et al. (2000) identified, but did not quantify, seven glycosides of quercetin (quercetin 3-arabanose-glucose (peltatoside), quercetin 3-galactose (hyperoside), quercetin 3-glucose (isoquercitrin), quercetin (pentose), quercetin 3-arabonose (avicularin), quercetin 3-rhamanose (quercitrin), and quercetin (aglycon)), four glycosides of kaempferol (kaempferol (hexose), kaempferol 3-glucose (astragalin), kaempferol (pentose), kaempferol (aglycon)), GA, caffeic acid, protocatechuic acid, $p$-coumaric acid, mangiferin, and GT through extractions with various organic solvents and HPLC-MS.

Mango polyphenolics are found throughout the fruit, and change during fruit growth and ripening. El Ansari et al. (1969) reported noticeable differences in polyphenolic
compounds present in pulp at various developmental stages, with biosynthesis of \textit{m}\-trigallic acid, quercetin, and isoquercetin during fruit ripening.

Polyphenolic compounds commonly serve as a protective mechanism in plants, warding off predator and microbiological attack. Many factors affect polyphenolic concentrations, including cultivar differences, growing conditions, maturity, and postharvest handling of fruit (Häkkinen and Törrönen, 2000; Lakshminarayana et al., 1970; Selvaraj and Kumar, 1989; Wang and Lin, 2000). Abiotic stresses such as excessive UV, heat and chilling temperatures, wounding and drought that are introduced before or after fruit harvest may affect the biosynthetic pathways of secondary metabolites (Cisneros-Zevallos, 2003), as fruit attempt to protect themselves. Under normal ripening conditions, mango polyphenol content is highest during fruit growth, decreasing with ripening (Lakshminarayana et al., 1970, Selvaraj and Kumar, 1989). These observations indicate a correlation between fruit ripening and loss of secondary metabolic substrates. However, mango polyphenolic content may increase under conditions of abiotic stress as phenolic compounds have been reported to increase following CI of spinach leaves (Howard et al., 2002) and jicama root (Cantwell et al., 2002), wound healing of potato tubers (Bernards et al., 1995; Borg-Olivier and Monties, 1993; Ramamurthy et al., 1992) and iceberg lettuce (Kang and Saltveit, 2002) and heat shock of iceberg lettuce (Loaiza-Velarde et al., 1997, Saltveit, 2000) and soybean (Graham and Graham, 1996).

**Polyphenolics as Antioxidants**

Polyphenolic compounds such as benzoic acids, flavonoids, cinnamic acids and tannins possess an aromatic ring bearing hydroxyl substituents that will readily take part in hydrogen bonding unless sterically hindered, allowing them to donate hydrogen ions to
free radicals. These hydrogen donations classify them as antioxidants in many *in vivo* and *in vitro* systems because of their ability to scavenge free radicals. Their function in biological systems is to delay or prevent the oxidation of important biological components that are a part of the life cycle. The efficiency of a given polyphenolic compound in acting as an antioxidant, is dependent on the number and/or position of hydroxyl groups available for donation (Natella et al., 1999, Arora et al., 1998, De Whalley et al., 1990). Polyphenolic compounds in fruit and fruit products (such as wine, grapes, prune juice, strawberries, cranberries and apples) may, therefore, be linked to prevention of degenerative diseases due to their antioxidant activity. Epidemiological studies indicate that fruit phenolic phytochemicals are capable of reducing the risks of cardiovascular disease, stroke, and atherosclerosis (Kelly et al., 2001, Hertog et al., 1993), through the prevention of cellular oxidative damage. Their antioxidant activities also have the potential to inhibit oxidative damage to cellular DNA, preventing mutagenesis and tumorigenesis (Kelly et al., 2001). Acting as antioxidants on LDL free radicals, they can lower the amount of oxidized LDL cholesterol, which in turn can reduce the risk of coronary atherosclerosis (De Whalley et al., 1990; Gorinstein et al., 1999; Hertog et al., 1993; Vinson and Hontz, 1995; Vinson et al., 1995, 1999). Though the majority of *in vivo* studies concerning plant polyphenolics have focused on flavonoids in rodents and human serum, the basis for their function as antioxidants apply to other polyphenolic compounds and should be explored for *in vivo* plant antioxidant properties associated with *in vivo* human antioxidant activity.

**Gallic Acid and Gallotannins**

Gallic acid and GT have been topics of interest for over 100 years as early scientists found them abundant in various plant foods. Strecker (1854), Fischer and
Freudnberg (1913), and Perkin and Everest (1918) were among the first to successfully hydrolyze GT, suggesting they consisted of a single polyol unit (mainly glucose) and multiple GA units (reviewed by Nierenstein et al., 1925 and Russell, 1935). These discoveries were milestones in the world of plant tannins, allowing for the understanding of their role in plants and how they are synthesized.

Gallic acid, chemically denoted as 3,4,5-trihydroxybenzoic acid, is thought to be derived from the dehydrogenation of 5-dehydroshikimic acid, an early intermediate of the shikimic acid pathway (Grundhöfer et al., 2001). Gallic acid has been associated with phytotoxicity and antifungal activity and has been of great interest in the potential prevention of atherosclerosis (Abella, 1997). Gallic acid can undergo esterification, attaching to glucose, which is transferred by uridine-5’-diphosphate, to eventually form GT. The process is thought to start with the formation of β-glucogallin (a

![Figure 2.1 Illustration of gallotannin biosynthesis with initial formation of β-glucogallin (2) to the final formation of 1, 2, 3, 4, 5-pentagalloylglucose (6) which is thought to be the precursor in formation of complex tannins (Grundhöfer et al., 2001).](image)

monogalloylate) that can then serve as an acyl donor/acceptor in a series of transgalloylation steps successively leading to the formation of larger GT: 1, 6-digalloyl-
β-D-glucopyranose, 1, 2, 6-trigalloyl-β-D-glucopyranose, 1, 2, 3, 6-tetragalloyl-β-D-glucopyranose and 1, 2, 3, 4, 6-pentagalloyl-β-D-glucopyranose (β-PGG). These rather simple GT can form more complex GT when additional galloyl groups attach to free phenolic hydroxyls of β-PGG via a m-depsidic bond (Grundhöfer et al., 2001, Quideau and Feldman, 1996) as illustrated in Figures 2-1 and 2-2.

Figure 2.2 Gallic acid depside bond (Mueller-Harvey, 2001).

Gallic acid and GT, abundantly found in plants and foods, have been widely studied for their role as free radical scavengers. Gallic acid acts as an antioxidant through the donation of hydrogen ions, stabilizing free radicals. Gallic acid is thought to be an active antioxidant because it possesses three hydroxy groups with capacity for hydrogen donation. Gallotannins have the ability to possess multiple GA compounds, but its efficiency as an antioxidant is dependent upon the position of galloyl groups (Hatano et al., 1990). For example, Kimura et al. (1984) found 1, 3, 6-trigalloyl-β-D-glucose to have highest antioxidant effect on lipid peroxidation in mitochondria and microsomes of liver, while 3, 4, 6-trigalloyl-β-glucose was least effective. Gallic acid, however, can act as a pro-oxidant, inducing apoptosis of human promyelocytic leukemia HL-60 cells, while GT
were effective antioxidants (Sakagami et al., 1997). Their results concerning GT
antioxidant effects support the theory of antioxidant activity and the number of hydroxyl
groups in a compound. The pro-oxidant activity of GA in this system contradicts radical
scavenging properties demonstrated by antioxidant assays.
CHAPTER 3
RIPENING ASSOCIATED CHANGES IN SOLUBLE POLYPHENOLIC
COMPOUNDS AND ANTIOXIDANT ACTIVITY OF MANGO FRUIT

Introduction

Mango fruit are frequently subjected to postharvest treatments involving mild applications of heat (thermal quarantine) and cold (low temperature storage) conditions, enabling their distribution to distant markets. The application of these treatments may present environmental conditions of stress for fruit as visible signs of heat injury (HI) and chilling injury (CI) have been described (Chaplin et al., 1991; Fuchs et al., 1989; Jacobi and Wong, 1991, 1992; Joyce et al., 1993, 2000, 2001a, b; Kalra et al., 1995; Lederman, 1997; Lizada, 1991; Medlicott et al., 1990; Paull, 1994; Pesis et al., 2000; Sharp, 1994; Singh and Chundawat, 1991; Smith and Chin, 1989; Spalding et al., 1998). These injuries imply postharvest treatments may cause abiotic stress on fruit, especially under conditions below or above their optimum for ripening. The duration and severity of a given stress imposed on the fruit directly influences its ability to survive. Fruit may compensate for environmental stress by eliciting protective mechanisms, allowing them to reach ripeness. Under severe conditions, fruit may fail to survive, resulting in accelerated senescence.

Thermal quarantine applications such as HWT for mango fruit have been reported to cause HI in fruit subjected to temperatures above the required thermal quarantine temperature of 46°C. ‘Tommy Atkins’ fruit subjected to HWT of 46°C for 120 minutes or 49°C for 60 minutes have been reported to develop darkened lenticels (Spalding et al.,
1988) and HWT of 42-48°C for 30-90 minutes have resulted in skin scalding of ‘Tommy Atkins’ (Smith and Chin, 1989). The severity of these HI is related to HWT temperature and duration of treatment, implying fruit tolerance of higher temperatures may be possible with short treatment times.

‘Tommy Atkins’ has been reported to develop symptoms of CI during storage below 12°C, including lenticel spotting, decrease in pH, increase in tissue browning and a decrease in skin color development with ripening (Medlicott et al., 1990). Treatment duration and temperature impact the severity of these symptoms as storage of ‘Tommy Atkins’ at 8 and 10°C has resulted in severe CI as fruit failed to ripen (Medlicott et al., 1990). ‘Tommy Atkins’ fruit have been successfully stored up to 21 days at 12°C, with favorable ripening qualities and minimal CI symptoms (Medlicott et al., 1990, Pesis et al., 2000).

The intent of this study was to assess the effects of abiotic postharvest stress on mango polyphenolic compounds throughout ripening. Abiotic postharvest stress was assessed using high temperature/short time thermal quarantine HWT and short time CS below optimum for ripening. Fruit were continuously assessed for fresh market acceptability with notation of HI and CI. The effects of abiotic stress in the synthesis of polyphenolic compounds were chemically analyzed using HPLC and Folin-Ciocalteu assay, with radical scavenging properties assessed by the ORAC assay.

Materials and Methods

Fruit Preparation and Treatment

Mango fruit (cv. Tommy Atkins) were provided through contact with Dr. Jonathan Crane on June 11, 2002. Fruit were harvested from a single grove at color-break maturity from growers near Homestead, FL and were sorted for uniform maturity to reduce
variation. Fruit were transported on the day of harvest to the Food Science and Human Nutrition Department at the University of Florida and divided randomly into four groups for postharvest treatment application. The first group was immersed into a hot water bath of 50°C for 60 minutes to simulate an accelerated thermal quarantine for insect disinfection treatment similar to the USDA-APHIS requirements for imported mango fruit, while the second group remained untreated as a control. Following treatment, both control and HWT fruit were divided further for storage at either 5 or 23°C. After 8 days of storage at 5°C, both HWT and control fruit were transferred to 23°C and allowed to ripen. Triplicate samples of five fruit were analyzed after 0, 4, 8, 12, 16, 20 and 24 days from each of the four treatments. Treatment groups stored at 23°C were analyzed through day 20, as day 24 fruit showed signs of senescence with severe breakdown of tissue. Fruit were manually peeled and pulp was cut away from the seed and blended in a kitchen scale food processor until a smooth puree was obtained. These samples were analyzed for total and individual soluble phenolics, and antioxidant capacity. All data are reported on a dry weight basis.

**Subjective Fruit Quality**

Mango fruit were subjectively observed for their quality changes during ripening, as a result of postharvest applications. Ripening was assessed by the pigmentation changes in the mango peel. Heat injury was assessed by signs of skin scalding and bruising. Chilling injury was identified by signs of pitting, lenticel spotting and shrivel.

**Polyphenolic Extraction**

A 3 g sample of fruit pulp from each treatment was homogenized with a tissue homogenizer in an equal volume (w/v) of extraction solution (methanol (MeOH)) for 1
minute and centrifuged. The soluble phenolic extract was filtered through a Whatman 0.45 µM filter disc and frozen at -20°C until time of analysis.

**Analysis and Quantification of Polyphenolic Concentration**

Methanolic phenolic extracts were analyzed on a Waters Alliance 2690 HPLC (Waters Corp., Milford, MA) dual column system using a Supelcosil LC-18 column, 250 x 4.6 mm and a Waters Spherisorb C-18 ODS (5µm) column, 4.6 x 250 mm. Polyphenolic compounds were detected using a Waters 996 photodiode array (PDA) detector. Individual soluble polyphenolic compounds were separated with gradient mobile phases of acidified water (98:2, water:acetic acid) and (68:30:2, water:acetonitrile:acetic acid) (Talcott et al., 2000) at a flow rate of 0.8 mL/min with PDA detection at 280 nm. UV spectral properties (200-400 nm) and retention time of each compound were compared to that of authentic standards obtained (Sigma Chemical Co., St. Louis, USA). Gallic acid, GT, and p-OH-benzoic acid were quantified with a GA standard curve, and are reported as GA equivalents (GAE).

Total soluble phenolics (TSP) were measured by the Folin-Ciocalteu assay. 100µL of each methanolic extract were mixed with 100µL Folin reagent for 3 minutes to allow reduction of phosphomolybdic acid by phenolic compounds. 100µL of 1N sodium carbonate were then added as an alkali to form chromophore, with a visible blue color. After 7 minutes, the test solutions were brought up to 1 mL total volume with water and read spectrophotometrically using a Beckman DU 640 Spectrophotometer at 726nm after 1 hour. Absorbance values were assessed for quantification of TSP with a GA standard curve and expressed as GAE according to Swain and Hillis (1959).
Quantification of Antioxidant Capacity

Antioxidant capacity was measured using the ORAC (oxygen radical absorbance capacity) assay first described by Cao et al. (1995) and later modified by Ou et al. (2002) with the use of fluorescein as the fluorescent probe (Talcott et al., 2003). A 40x dilution of methanolic extracts with a pH neutral ORAC buffer (61.6:38.9 v/v, 0.75 M K$_2$HPO$_4$ and 0.75M NaH$_2$PO$_4$) was used throughout the study and compared to a standard curve representing 6.25, 12.5, 25 and 50 µM of α-Trolox, a water soluble form of vitamin E. Solvent effects were accounted for when determining antioxidant capacity through the comparison of two blanks, one containing only ORAC buffer and the other containing 50:50, water:MeOH. This experiment was performed at 37°C and fluorescent readings were taken every 2 minutes for 70 minutes using a 96-well Molecular Devices fmax® fluorescent microplate reader (485 nm excitation and 538 nm emission) and fluorescent decay curves were calculated using Microsoft Excel.

Ascorbic Acid Extraction and Quantification

Ascorbic acid was extracted from 2 g of mango puree in 5 mL 3% citric acid buffer (2:5 w/v), uniformly homogenized for 1 minute and centrifuged. Supernatant was diluted 2-fold with citric acid buffer prior to separation using a Waters 2690 HPLC (Waters Corp., Milford, MA) on a Waters Spherisorb C-18 ODS (5µm) column, 4.6 x 250 mm with an isocratic mobile phase adapted from Gökmen et al. (2000).

Moisture Determination

Moisture analysis was performed by placing a 5 g sample of mango pulp puree in an aluminum pan and drying to a constant weight in a Precision Economy Oven at 135°C for 2 hours with method adapted from Association of Analytical Communities (AOAC) Official Method 920.149149(c).
Statistical Analysis

Statistical analyses were performed using JMP 5 Statistical Software (SAS Institute, 2002). Data represent the mean and standard deviation from triplicate analyses. Analysis of variance, Pearson correlation, and mean separation was conducted using Duncan’s multiple-range test (P<0.05). LSD tests (P<0.05) were employed to determine the effect of HWT and CS treatment on assay values.

Results and Discussion

Ripening Associated Changes in Antioxidant Compounds

Effects on polyphenolic concentrations

Activity of the shikimic acid pathway due to both biotic and abiotic stress of various fruits and vegetables has been shown to appreciably alter the concentrations of antioxidant polyphenolic compounds. Biosynthesis of GA in ripening fruit has not been

![Graph](image)

Figure 3-1 Polyphenolic changes (µg/g GAE) of methanolic fractions obtained from ripening mango fruit. Measurements were taken at Day 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.
determined, but has been assumed to increase as the shikimic acid pathway is stimulated, producing various polyphenolic compounds (Neish, 1964). Gallotannins synthesis during fruit ripening has not been reported as its synthesis and role in the plant is still not understood. Changes in free GA and GT concentrations due to ripening were minor, with no significant difference between means of fruit at days 0 and 24 (Figure 3-1). These data suggest that GA and GT are not synthesized during ripening.

*p*-Hydroxy-benzoic acid is synthesized via the shikimic acid pathway by means of phenylalanine ammonia lyase (PAL). Concentrations of this phenolic compound should decrease during ripening as suggested by the observations that PAL activity declines in peaches (Kubota et al., 2001). Ripening of mango fruit showed no significant changes in *p*-hydroxy-benzoic acid concentrations, indicating that PAL is not active in mature green fruit and throughout the ripening process (Table 3-1 and Figure 3-2).

### Table 3-1 Tentative identification of mango polyphenolics based on retention time and UV-Visible spectral properties using HPLC and authentic standards.

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Spectral Properties</th>
<th>Tentative Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.3</td>
<td>228.9, 271.3</td>
<td>?</td>
</tr>
<tr>
<td>13.5</td>
<td>228.9, 280.8</td>
<td>Soluble protein</td>
</tr>
<tr>
<td>16.5</td>
<td>228.9, 271.3</td>
<td>Gallic acid</td>
</tr>
<tr>
<td>22.4</td>
<td>224.2, 257.2, 299.8</td>
<td><em>p</em>-OH-Benzoic derivative</td>
</tr>
<tr>
<td>23.4</td>
<td>224.2, 257.2</td>
<td><em>p</em>-OH-Benzoic acid</td>
</tr>
<tr>
<td>31.3</td>
<td>224.2, 271.3</td>
<td>Gallotannin</td>
</tr>
<tr>
<td>35.1</td>
<td>233.6, 290.3, 314.0</td>
<td><em>p</em>-Coumaric acid ester</td>
</tr>
<tr>
<td>36.6</td>
<td>210.1, 276.1, 337.8, 374.8</td>
<td>Gallotannin</td>
</tr>
<tr>
<td>37.6</td>
<td>224.2, 233.6, 299.8, 384.2</td>
<td>Gallotannin</td>
</tr>
<tr>
<td>41.8</td>
<td>224.2, 266.6</td>
<td>Gallotannin</td>
</tr>
<tr>
<td>48.1</td>
<td>219.5, 280.8, 374.6</td>
<td>Tryptophan</td>
</tr>
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<td>51.1</td>
<td>214.8, 228.9, 276.1</td>
<td>Gallotannin</td>
</tr>
<tr>
<td>62.1</td>
<td>219.5, 233.6, 314.0, 389.0</td>
<td><em>p</em>-Coumaric acid ester</td>
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</tbody>
</table>
Figure 3-2  HPLC chromatograms of polyphenolic compounds in methanolic mango fractions from unripe (A) (Day 0) and ripe (B) (Day 20) fruit. Identification of compounds was made by comparing retention time and/or spectral properties to authentic standards. Tentative peak identifications include 1) gallic acid, 2) gallotannins and 3) \( p \)-hydroxy-benzoic acid.

**Effects on ascorbic acid**

Ascorbic acid analysis was attempted utilizing methods of Gökmen et al. (2000) as its presence in mango has been previously reported. This organic acid, however, could not be identified as chromatograms produced by this assay generated a peak with retention time (5.5 minutes) very similar to an authentic standard of L-AsA (5.2 minutes) with differing spectral properties (Figure 3-3). Additionally, the retention time of this
unknown compound did not match those of malic acid (4.4 minutes) or citric acid (6.4 minutes), two primary organic acids previously measured in mango fruit. It is presumed that this chromatographic peak is a result of AsA co-eluting with a water-soluble UV visible substance.

Figure 3-3  HPLC chromatogram (A) and spectral properties of (B) ascorbic acid and (C) unknown peak of 3% citric acid soluble organic acid in mango as measured using assay for ascorbic acid identification described by Gökmen et al. (2000).

Effects on total soluble phenolics

Changes in TSP during fruit ripening have been associated with pigment development of anthocyanins in fruit tissue such as blackberry TSP decrease while raspberries increase (Wang and Lin, 2000). Colorless phenolics in mango were attributed
to an increase in TSP of ‘Keaw’ during ripening (Gorinstein et al., 1999), as carotenoids are not detected by this assay. Apparent changes in TSP during fruit maturation can be attributed to sugar and ascorbic acid synthesis and degradation, as they interfere with the Folin-Ciocalteu assay. These data (Figure 3-4) suggest chemical changes in fruit pulp associated with ripening directly influence TSP as days 12 and 16 fruit had significantly lower TSP concentrations than days 0, 4, 20 and 24. Subjective quality observations of fruit ripening coincide with signs of ripening in mango peel color development from green to yellow and orange. These data suggest mango phenolic compounds, as measured by Folin-Ciocalteu assay, behave similarly to raspberry fruit (Wang and Lin, 2000) with highest TSP in green and ripe berries with lowest concentrations in pink fruit. TSP changes, however, are often associated with PAL activity as with ‘Splendour’ and
‘Granny Smith’ apples (Lister et al., 1996) and loquat fruit (Ding et al., 2001), which have their highest concentrations of phenolic compounds and PAL activity in unripe and almost ripe stages, with lowest levels in between. Mango TSP changes appear to be the result of phenolic compounds other than GA, GT, and \( p \)-hydroxy-benzoic acid.

**Effects on polyphenolic antioxidant activity**

Antioxidant capacity of polyphenolic compounds significantly increased as fruit ripened, with average values across treatments of 27.9 to 54.2 µM TE/g dry weight fruit (Figure 3-5). These data resemble trends reported in raspberry fruit by Wang and Lin (2002), with highest ORAC levels in green and ripe fruit and lowest in pink fruit. These observations support those of TSP, indicating polyphenolic substrate(s) are changing during the ripening process.

![Antioxidant capacity (µM Trolox equivalents/g) of phytochemical fractions obtained from mango fruit during ripening. Measurements were taken at Days 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.](image)

Figure 3-5  Antioxidant capacity (µM Trolox equivalents/g) of phytochemical fractions obtained from mango fruit during ripening. Measurements were taken at Days 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.
Moisture Content

Figure 3-6 illustrates moisture loss of mango fruit for all treatment groups during ripening. Moisture loss is generally associated with fruit respiration; however, CI symptoms may result in additional moisture loss due to wilting. Losses greater than 3% in fruit moisture (Kader, 2002) are considered significant, reducing the quality of fruit in a fresh market. Moisture loss in mango subjected to HWT showed 1-2% loss during ripening with application of CS resembling similar loss. These changes in moisture loss are comparable to those of Jacobi et al. (2000) in ‘Kensington’ mango fruit held at 47°C core temperature for 15 minutes. Minor significant differences in moisture loss due to CS treatment were noticed in mango fruit, which parallels with CI symptoms noticed in CS fruit at days 20 and 24.

![Figure 3-6 Percentage of moisture loss in fruit from days 0 to 20 of storage in control and hot water immersion treatment of mango stored at 5 and 23°C. Average values and standard error bars of triplicate samples for all treatments are represented.](image-url)
Overall Effect of Hot Water Treatment

The application of HWT to mango has not previously been studied for its influence on mango polyphenolics. It has, however, been reported to increase the rate of ripening as the application of heat may increase respiration rates (Jacobi and Wong, 1992, Smith and Chin, 1989, Spalding et al., 1988). Mild heat treatments may cause fruit to produce elevated endogenous levels of ethylene, accelerating the ripening process. The application of heat as a stress, however, may cause inhibition of ACC synthase and ACC oxidase enzymes (Lurie, 1998), which could result in early fruit senescence without ever ripening. The influence of HWT on fruit ripening was evident as completion of ripening, determined by yellow peels, was reached by day 16, 4 days prior to non-HWT fruit. These accelerated ripening observations support hot water responses previously reported.

![Graph showing changes in free gallic acid during ripening](image)

**Figure 3-7** Hot water treatment induced changes in gallic acid during ripening for fruit sampled at Days 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.
Free GA concentrations in HWT mango fruit were not significantly different in ripe mango (Figure 3-7). However, initial differences were noticed in unripe (day 0, 4, and 8) fruit pulp, as free GA concentrations were significantly lower in HWT fruit. This occurrence indicates an immediate response by fruit to HWT prior to reaching ripeness.

![Graph showing gallotannins (µg/g GAE) over time with and without HWT treatment.](image)

Figure 3-8  Hot water treatment induced changes in gallotannins during ripening for fruit sampled at Days 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.

The application of HWT did not induce any changes in GT and p-hydroxy-benzoic acid concentrations compared with non-HWT fruit (Figures 3-8 and 3-9). These data indicate fruit were not inclined to form GT or synthesize p-hydroxy-benzoic acid in response to the stress of hot water temperatures. TSP did not show evidence of HWT conditions posing a stress on fruit (Figure 3-10), as values did not significantly change during ripening due to treatment. Additionally, antioxidant capacity of mango fruit was slightly improved with HWT application throughout ripening (Figure 3-11); suggesting HWT can improve potential health benefits of mango. These data suggest the application of HWT
at 50°C to color break fruit enhances GA and polyphenolic peroxyl radical scavenging activities. However, initial losses in free GA and antioxidant capacity were observed in day 0 fruit, indicating HWT at 50°C caused a stress response in fruit resulting in polyphenolic losses.

Figure 3-9  Hot water treatment induced changes in $p$-hydroxy-benzoic acid during ripening for fruit sampled at Days 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.

Figure 3-10  Hot water treatment induced changes in total soluble phenolics during ripening for fruit sampled at Days 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.
Overall Effect of Cold Storage Treatment

Cold storage of fruit is used to lower fruit respiratory rates, extending their shelf life for extended marketing. The use of CS in this study effectively extended mango shelf life as fruit stored at 23°C for the entire ripening process had a shelf life of 20 days, 4 days shorter than fruit subjected to CS at 5°C for the initial 8 days of this study. Storage of mangoes in temperatures below optimum for ripening is avoided due to mango chill sensitivity, resulting in irreversible injuries that may cause rapid senescence of fruit. Storage of mangoes in chilling temperatures produced signs of CI, with symptoms of pitting, peel and tissue browning, wilting, lenticel spotting, and arrest in peel pigment development noticed in day 16, 20 and 24 fruit.

Application of chilling temperatures to mangoes significantly influenced free GA and TSP concentrations in fruit after 8 or 12 days of ripening respectively (Figures 3-12...
and 3-15), with antioxidant capacity effects noticed after 16 days of storage (Figure 3-16). Decreased concentrations of GA and antioxidant capacity disagree with Cantwell et al. (2002) and Kang and Salveit (2002) who associated CI with increased phenolic concentrations.

Figure 3-12 Cold storage induced changes in gallic acid during ripening as fruit were measured at Days 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.

Figure 3-13 Cold storage induced changes in gallotannins during ripening as fruit were measured at Days 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.
Figure 3-14 Cold storage induced changes in $p$-hydroxy-benzoic acid during ripening as fruit were measured at Days 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.

Figure 3-15 Cold storage induced changes in total soluble phenolics during ripening of mango fruit. Measurements were taken at Days 0, 4, 8, 12, 16, 20 and 24.
Figure 3-16. Cold storage induced changes in oxygen radical absorbance capacity during ripening of mango fruit. Measurements were taken at Days 0, 4, 8, 12, 16, 20 and 24. Average values and standard error bars of triplicate samples for all treatments are represented.

**Conclusions**

The application of abiotic postharvest stress to mango fruit in the forms of accelerated thermal quarantine HWT and cold storage produced intriguing results. HWT at 50°C for 60 minutes did not adversely affect levels of GA, GT, and \( p \)-hydroxy-benzoic acid in ripe fruit. Initial GA losses were noticed immediately following application of HWT, with no significant difference in ripe fruit. Likewise, TSP and ORAC values slightly increased due to HWT. These observations imply that the application of an accelerated thermal quarantine slightly improves potential health benefits of mango fruit to consumers in the fresh market, although signs of heat injuries may be present. Mango fruit were affected by CI due to the 5°C for 8 days CS treatment. Decreases in GA, TSP, and ORAC concentrations were attributed to CI. These observations indicate that heat and cold abiotic stress to unripe mango influence polyphenolic concentrations during ripening.
CHAPTER 4
THERMAL QUARANTINE ASSOCIATED CHANGES IN HYDROLYSABLE POLYPHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF MANGO FRUIT

Introduction

The effectiveness of thermal quarantine treatments for insect disinfection of mango is based on the combination of time and temperature with current USDA-APHIS requirements calling for core temperatures to reach 43°C for a minimum of 60 minutes. Although fruit heat tolerance varies by species, stage of fruit maturity, fruit size, environmental factors, and the method of heat application, (Jacobi et al., 2001b) these treatments have been proven effective for eliminating invasive pests on mangoes entering the US. The influence of HWT on ripening of mango fruit can be characterized as inhibiting, promoting or disrupting characteristic fruit ripening, resulting in symptoms ranging from accelerated yellowing of fruit skin (ripe stage) to accelerated fruit senescence, bypassing ripening. In study 1 (Chapter 3) GA was immediately affected by an accelerated thermal quarantine treatment, suggesting metabolic changes were taking place in mango fruit as a result of HWT at 50°C for 60 minutes. HWT-induced physiological changes were assessed in mango fruit subjected to USDA-APHIS thermal quarantine. B-glucosidase and tannase hydrolysis of GT was employed to characterize the role of GA and GT as antioxidants measured by Folin-Ciocalteu and ORAC assays.
Materials and Methods

Fruit Preparation and Treatment

Mango fruit (cv. Tommy Atkins) were obtained at color-break maturity on June 21, 2003 from Lyons Farms in Homestead, FL. Fruit were transported on the day of harvest to the Horticultural Sciences Department at the University of Florida where they were held at 14°C for 2 days. Following storage, fruit were sorted by maturity (determined by peel color development) and weight. Forty fruit weighing between 465 and 530 g with peels that were green, with some patches of purple and red, were selected for this study to replicate fruit maturity in previous study (Chapter 3). These selected fruit were divided into four groups, five fruit per group. Fruit from three groups were subject to a quarantine treatment of required temperature (46°C), with varying lengths of time (70, 90 and 110 minutes). Hot water immersion treatments were applied with fruit, in mesh bags, entirely submerged in laboratory scale fruit heating system (Model HWH-2, Gaffney Engineering, Gainesville, FL). Internal pulp temperatures were measured to verify that treatment conditions met those required by USDA-APHIS. The fourth group remained untreated to serve as the control. Following quarantine treatment, fruit were cooled to 20°C and transported to the Food Science and Human Nutrition Department at the University of Florida where 20 fruit were immediately peeled and pureed for storage at –20°C until time of analysis. The remaining 20 fruit were allowed to ripen for 4 days at 23°C for subjective ripening evaluation. Mango puree was analyzed for total and individual soluble phenolics, antioxidant capacity, color, total soluble solids, moisture and organic acids. All data are reported on a dry weight basis.
Polyphenolic Extraction

Polyphenolic composition of mango purees in a methanolic extract was analyzed as previously described with the modification that mango puree (3 g) was extracted with 100% MeOH under filtration until tissue was colorless. MeOH was then evaporated and polyphenolics dissolved in 6 mL of water with the aid of a sonic water bath.

Polyphenolic Hydrolysis

Each polyphenolic extract was hydrolyzed with an enzyme mixture of (53.4 mg) almond β-glucosidase (12.1 units/mg; 1 µmol glucose/minute at pH 5.0 and 37°C) (Sigma, St. Louis, MO) and (53.4 mg) Aspergillus oryzae tannase (50 units/mg; 1 µmol tannin/minute at pH 5.5 and 30-40°C) (Wako Pure Chemical Industries, Japan) mixture. Hydrolysis took place at 30°C in the absence of light. Hydrolysis was terminated after 5 hours by placing samples in a 100°C water bath until they reached 90°C. Following hydrolysis, samples were extracted with ethyl acetate and hydrated in water:MeOH (50:50) solution for analysis of individual and total soluble phenolics via HPLC and Folin-Ciocalteu assays and additionally assayed for antioxidant capacity (ORAC assay) according to methods outlined in Chapter 3.

Total Soluble Solids (Brix °)

Total soluble solids were measured using an Abbe Mark II digital refractometer (Leica Inc., Buffalo, NY) by placing 0.5 g mango pulp puree on the lens and reading the sample for temperature corrected Brix.

Statistical Analysis Methods

Experimental data were evaluated in triplicate, as previously described with each data point representing the mean with standard error bars. LSD test (P<0.05) was
employed to determine the effect of HWT on assay values using JMP 5 Software (SAS Institute, 2002).

**Results and Discussion**

**Influence of Hot Water Treatment**

**HWT-Induced fruit injury**

Mango fruit subjected to thermal quarantine treatments showed no visible signs of HI or acceleration in skin color development as noted in Study 1. Additionally, no HWT-induced injuries were noticed in mature green or ripe fruit. The application of HWT at 46°C for 70, 90 or 110 minutes had no heat-induced physiological damage that would prevent its distribution in the fresh market.

**Effects on total soluble solids (Brix °)**

Total soluble solids increase as mango fruit ripen, due to the hydrolysis of starch to sugars. Reported in % and °Brix, mango fruit range from approximately 9 to 21% during ripening (Thomas and Joshi, 1988). TSS values for mature green fruit in this study resembled those of unripe mature-green fruit with a mean value of 10.1°. TSS change due to HWT as illustrated in Figure 4-1.

![Figure 4-1](image.png)

Figure 4-1  HWT-induced effects in total soluble solids following hot water treatment of unripe fruit at 46°C.
**Effects on polyphenolic concentrations**

Polyphenolic changes in unripe mango due to HWT required by USDA-APHIS were not expected, as discussed in Chapter 3, as these conditions are considered mild heat treatments. Free GA was not significantly affected by HWT at 46°C for 70, 90 or 110 minutes, as these concentrations were not significantly different from control fruit (Figure 4-2). Treatment affects on polyphenolic composition were significantly affected as GT concentrations increased in fruit subjected to the longest HWT treatment (110 minutes) (Figure 4-2). These data indicate that the required time and temperature requirements for imported mango thermal quarantine treatments do not significantly effect the concentrations of GA. Extended treatment time (110 minutes), however, demonstrated fruit response to heat stress, resulting in increased GT concentrations. TSP and antioxidant capacity were not affected by HWT (Figure 4-2 and 4-3).

![Figure 4-2 Polyphenolic concentrations of mature green mango following HWT at 46°C for 0, 70, 90, and 110 minutes. Average values and standard error bars of triplicate samples for all treatments are represented.](image-url)
Overall Effect of Hydrolysis

Hydrolysis of GT with β-glucosidase and tannase was effective in releasing GA compounds from polyl units as GA concentrations increased by 65.2%, on average. GT hydrolysis is assumed to be complete as GT peaks disappeared in HPLC chromatograms following hydrolysis (Figures 4-4 and 4-5).
Figure 4-5 Changes in gallic acid concentrations due to enzymatic hydrolysis following hot water treatment (46°C) of mango for 0, 70, 90 and 110 minutes.

**Effects on Total Soluble Phenolics**

Methanolic soluble reducing compounds as measured by TSP were not expected to change due to HWT at 46°C as no changes were observed in day 0 fruit from Study 1. TSP was not significantly affected by HWT, indicating that polyphenolic compounds

Figure 4-6 Hot water treatment induced changes in mango total soluble phenolics as a result of β-glucosidase and tannase hydrolysis.
were stable under the required thermal quarantine treatment. Significant losses in TSP occurred following hydrolysis, indicating bound GA in the form of GT has a 5.60-fold increase in reducing capacity than free GA (Figure 4-6).

**Effects on Antioxidant Capacity**

Antioxidant capacities of $\beta$-glucosidase and tannase hydrolyzed fractions were not expected to significantly differ from the original fraction as Kikuzaki et al. (2000) reported nearly equivalent radical-scavenging properties between GA and galloglucosides. Antioxidant capacity of fruit decreased 40% following hydrolysis (Figure 4-7), supporting TSP data and suggesting bound GA in the form of GT is a better peroxyl radical scavenger than free GA.

![Graph showing changes in antioxidant capacity before and after hydrolysis](image)

Figure 4-7  Hot water treatment induced changes in mango antioxidant capacity as a result of $\beta$-glucosidase and tannase hydrolysis.
Conclusions

Chemical composition of mango fruit did not change as a result of required thermal quarantine treatment of 46°C for 90 minutes. Treatment duration had no implications on chemical composition, but showed significant effects on GT concentrations. Hydrolysis of GT was successful using an enzyme mixture of β-glucosidase and tannase, which produced D-glucose and quantifiable GA. Significant losses in reducing and antioxidant capacity of mango extracts following enzymatic hydrolysis were observed indicating bound GA in the form of GT is a better radical scavenger than free GA. Mass spectrometric analysis of polyphenolic compounds may indicate the role of galloyl ester positioning on polyol molecule and AOX.
CHAPTER 5
SUMMARY AND CONCLUSIONS

Postharvest treatments to fruit may benefit consumers through stress-induced biosynthesis of antioxidant phenolic compounds. The application of hot water thermal quarantine treatments slightly improved antioxidant phenolic compounds in ripe mango, suggesting domestic growers would benefit from the use of hot water baths. Hot water treatments utilizing an elevated temperature (50°C) resulted in significant antioxidant increases of mango polyphenolics. Cold storage of mango at chilling temperature resulted in chilling injuries, but did not promote the formation of phenolic compounds as a defense mechanism. Hot water and cold storage treatments should be further explored utilizing non-threatening temperatures for longer periods of time to evaluate potential roles of these treatments as positive abiotic stressors to mango fruit.
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BIographiesketch

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