POSTHARVEST TREATMENT EFFECTS ON HEALTH PROMOTING PROPERTIES AND FLAVONOID GENES OF GRAPEFRUIT

A Dissertation

by

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ABSTRACT

Certain naturally occurring compounds present in grapefruit have shown several health promoting properties such as anticancer, antioxidant, antimicrobial and cardioprotective. However, the levels of these naturally occurring compounds are influenced by several preharvest and postharvest factors. The primary objective of the current research is to determine the effects of different postharvest treatments on naturally occurring compounds present in grapefruit.

In the first and second studies, effect of ethylene degreening on 'Star Ruby' and 'Rio Red' grapefruit natural compounds was investigated. Degreening helped to improve grapefruit color while maintaining the health promoting compounds. Significant influence of ethylene treatment was observed on flavonoids and furocoumarins. The third and fourth study examined the changes in 'Star Ruby' and 'Rio Red' grapefruit under cold storage and with low temperature conditioning treatment. Conditioning the fruits prior to cold storage reduced the incidence of chilling injury and conditioned fruits had similar or higher levels of health promoting compounds compared to fruits stored under cold storage without conditioning. However, results suggest that for short term storage of few weeks, storing fruits at 11ºC was better for retention of most naturally occurring compounds.

The fifth study focused on use of modified atmosphere packaging (MAP) to maintain grapefruit quality and nutritional properties. Two MAP films, micro-perforated (modified oxygen, carbon dioxide and humidity levels) and macro-perforated (modified

humidity only), were investigated for their influence on naturally occurring compounds and fruit quality under a prolonged storage period. MAP treatments did not have significant effect on ascorbic acid, limonoids and fruit quality parameters such as total soluble solids, acidity, fruit taste, decay and disorders. Based on our research, use of MAP films is recommended to maintain fruit quality and health promoting compounds.

The sixth and seventh studies focused on variation in health promoting compounds, specifically, flavonoid pathway gene expression and volatile compounds present in 'Rio Red' grapefruit during fruit development and maturity. Overall expression of flavonoid pathway genes and related flavonoid content decreased as the fruits developed and matured, with the levels being highest in immature fruits harvested in June. Levels of limonene decreased as the fruits developed from June to April; while, nootkatone levels increased with fruit development and maturity. The eighth study investigated the effect of different ethylene concentrations on flavonoid pathway gene expression and related flavonoids in grapefruit. Significant effect of ethylene concentration was observed on flavonoids and furocoumarins as well as the genes involved in the flavonoid biosynthesis.

Overall, significant influence of different postharvest treatments was observed on grapefruit health promoting compounds. We believe that this research will be helpful to the citrus industry to optimize the postharvest treatments in order to maximize their benefits in regards to fruit quality and nutritional value.

DEDICATION

I would like to dedicate this dissertation at the lotus feet of the most merciful Lord Sri Krishna, Srimati Radharani, Lord Chaitanya and Lord Nityananda.

To His Divine Grace A.C. Bhaktivedanta Swami Srila Prabhupada, Founder Acharya of International Society for Krishna Consciousness (ISKCON).

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CHAPTER I

INTRODUCTION

Grapefruit (*Citrus paradisi* Macf.) is a cross between pummelo and sweet orange. The origin of grapefruit is dated back to early 18th century which was first discovered in Jamaica, and known as the Forbidden fruit. Later, it was introduced in Florida in early 19th century.[1] Grapefruit was earlier considered a spontaneous sport of pummelo and given botanical name as *Citrus paradisi* by James Macfayden in 1837. In 1948, it was found to be an accidental hybrid between pummelo and orange.[2]

Grapefruit is one of the important citrus crops accounting for nearly 7% of total global Citrus trade (NASS, USDA 2015) with total 6 million metric tons production in 2015-2016.[3] United States ranked second in grapefruit production and fourth in export in the world in 2016.[4] In USA, it is mainly grown in Florida, Texas, California, and Arizona. According 2015-2016 data, 27 % of total grapefruit acreage in the United States is in Texas, with a total of 17,100 acres.[3] The majority of citrus cultivation in Texas is located in Lower Rio Grande Valley and mainly consists of grapefruits (nearly 70%) and oranges.[5] All grapefruit varieties have been evolved from white fleshed grapefruit with most of them developed and released from Florida and Texas. Duncan is the oldest white fleshed grapefruit cultivar originating from Florida.[1] Marsh seedless, also originating from Florida, is one of the most important white fleshed cultivar which is seedless, unlike Duncan.[1] Pigmented grapefruit cultivars were discovered as mutations on white fleshed cultivars. Foster, a bud sport mutation, and Thompson, a sport limb on Marsh seedless were first pigmented grapefruit cultivars reported.[1] However, they were

replaced by deep red flesh cultivars, which were developed in Texas. Hudson (a bud sport of Foster) was irradiated with to induce seedless resulted in development of deep red variety which was released as Star Ruby in 1970.[1, 2] Another new cultivar, Rio Red grapefruit, which is a bud sport mutant having red flesh color was released in 1985.[1]

Grapefruit contains a wide array of health promoting compounds (naturally occuring compounds) including vitamin C, carotenoids, limonoids, flavonoids, furocoumarins, folic acid, phenolics, etc. Several studies have reported the various health promoting properties of these naturally occurring compounds such as cholesterol lowering property, anti-oxidant activity, anti-cancer activity, neuroprotective properties, anti-inflammatory activity, and protection against cardiovascular diseases and osteoporosis.[6, 7] These secondary metabolites were affected by various pre-harvest and postharvest factors such as cultivar, growing location, climatic conditions, fertilizer application, sunlight availability, diseases and pests, postharvest treatments, storage period and temperature, etc.[8, 9] Various post-harvest treatments are practiced by citrus industry to prolong the shelf life, improve quality and reduce incidence of diseases and disorders in the fruits. Most common postharvest treatments used are ethylene degreening, quarantine treatments, temperature conditioning and modified atmosphere packaging. Furthermore, it is essential to understand the changes quality parameters including health promoting components in grapefruit during fruit development and maturity.

Objectives

1. To determine the effect of ethylene degreening on levels of certain naturally

occurring compounds such as ascorbic acid, carotenoids, limonoids, flavonoids and furocoumarins in Star Ruby and Rio Red grapefruit.

2. To determine the effect low temperature conditioning on health promoting compounds present in Star Ruby and Rio Red grapefruit.
3. To investigate the influence of modified atmosphere packaging (MAP) on the health promoting compounds present in Star Ruby grapefruit.
4. To determine variation in key flavonoid biosynthetic enzymes and naturally occurring compounds present in grapefruit during fruit development and maturity
5. To determine changes in grapefruit juice volatiles during different stages of fruit development and maturity.
6. To determine the influence of degreening using different ethylene concentrations on the key enzymes involved in grapefruit flavonoid pathway and the health promoting compounds.

CHAPTER II

LITERATURE REVIEW

Ethylene degreening

Ethylene is a gaseous plant hormone commonly known as ripening hormone. Role of ethylene in senescence was discovered in nineteenth century when illumination gas leaking from street lights caused premature leaf fall of nearby trees. In 1901, Neljubov a Russian plant physiologist observed triple response in etiolated pea seedlings growing in greenhouse.[10] He later found that the gas released from the illuminating lights was ethylene which caused the triple response in pea seedlings.[10] In 1934, Gane reported that ethylene is synthesized by plants and was proposed as a fruit ripening hormone by Crocker in 1935.[10] Since then researchers have been focused on effect of ethylene on ripening of various fruits such as tomatoes, apples, bananas and other fruits.[11-14] Peel color is important factor in determining the fruit ripeness and maturity; however, in non-climacteric fruits, such as citrus, peel color usually does not represent fruit maturity. Citrus fruit is a hesperidium consisting of two parts: exocarp and endocarp. The exocarp is further divided into flavedo and albedo. External peel ripening and internal pulp ripening are usually different physiological processes in citrus that occur simultaneously.[15] Early season citrus fruits harvested in October-November are mature but yet retain green peel color.

Natural ripening process in citrus is initiated by cold temperature, especially minimum night temperature by triggering ethylene biosynthesis.[15] In tropical climate night temperature may not reach required low temperature to change peel color.

Furthermore, chlorophyll degradation and carotenoid accumulation in peel is affected by warm temperature.[15] Degreening of early season citrus fruits using ethylene gas helps to achieve uniform red/orange peel color. Ethylene increases chlorophyllase activity resulting in degradation of chlorophyll from the chloroplasts in peel.[16, 17]

Various factors influence the degreening treatment such as ethylene concentration, temperature, relative humidity, carbon dioxide level and degreening time.[18, 19] Previous studies mainly investigated peel color changes due to ethylene degreening. Carotenoids, namely lycopene are influenced by temperature, with temperatures above 30 °C inhibiting the synthesis; while, β-carotene synthesis remain unaffected.[18, 20] The optimum degreening temperature for citrus fruits ranges from 15 to 25 °C.[18, 20] Usually the duration for degreening treatment is determined by the color development of fruits. Duration of degreening cannot be reduced by using higher concentrations of ethylene.[19] The recommendations for degreening rooms are temperature ranging from 20 to 28 °C, ethylene concentration of 5 ppm, and relative humidity ranging from 92 to 95% [21]. It is also recommended to have CO_2 level in degreening rooms to be less than 0.1% and at least one air change per hour. Furthermore, the degreening duration should not exceed more than 72 hours.[21]

Ethylene is reported to influence various enzymes involved in biosynthesis of health promoting compounds. Phenylalanine ammonia-lyase enzyme (*PAL*) involved in phenylpropanoid pathway is reported to increase with ethylene application.[22, 23] Ethylene produced after wounding in citrus peel discs as well as exogenous ethylene applied to intact grapefruits were able induce *de novo* synthesis of *PAL* enzyme.[23] Chalcone

synthase (*CHS*), the first enzyme in flavonoid pathway is also reported to increase with exogenous ethylene application.[24] In strawberry application of abscisic acid increased ethylene production and consequently *PAL* activity resulting in higher phenolics and anthocyanin production.[25]

Chilling injury in citrus

Fruits and vegetables are stored at low temperature to enhance storage period, reduce disease incidence and also as quarantine treatment before exporting to other countries.[26, 27] However, fruits and vegetables from tropical and sub-tropical regions are susceptible to chilling injury.[28] Chilling injury (CI) in citrus fruits is a common occurrence when fruits are stored below optimum temperature which varies according to the species. Grapefruit, limes and lemons and shamouti oranges are found to be more susceptible to CI. Grapefruit when stored at 6°C is more susceptible to CI as compared to temperatures such as 2 °C and 4 °C.[29] Most common symptoms of CI are peel pitting, brown staining of flavedo and albedo, surface lesions and water soaked tissues.[30] Grapefruit showed water soaking when stored at 0 °C.[29]

Several postharvest treatments have been recommended to reduce the incidence of CI. Most common amongst them are temperature conditioning, hot water dips (HWD), intermittent warming, modified atmosphere packaging and use of growth regulators. Short period (2-3 min) HWD were effective in controlling CI and postharvest decay of citrus fruits namely grapefruit, oroblanco, lemon and kumquat.[31] In Star Ruby grapefruit, hot water brushing for 20 s at 59 or 62 °C was effective in reducing the incidence of CI by 42 and 58% respectively after 6 weeks of storage at 2 °C followed by

subsequent 1 week storage at 20 °C.[32] In addition, the treatment also helped to reduce the postharvest decay caused by green mold (*Penicillium digitatum*).[33] Intermittent warming by interrupting cold storage with warm storage period is reported to delay CI incidence as well as enhance resistance to CI in citrus fruits.[34, 35]

Temperature conditioning

Temperature conditioning is one of the effective methods commercially practiced to reduce CI in citrus fruits. Fruits can be conditioned at higher temperature for short period (high-temperature conditioning) or at lower temperature for longer period (low-temperature conditioning).[36] High temperature conditioning in citrus is done by storing fruits at 37 °C for 1-3 days.[37, 38] In low temperature conditioning citrus fruits are stored at 16 °C for 7 days.[27, 36]

Several studies have been conducted to elucidate the effect of conditioning treatment on various enzymes and mechanisms. Squalene content in epicuticular wax was reported to be inversely related to CI in grapefruit.[39] Oxidative stress is also proposed as a factor causing cold temperature damage. Putrescine, a polyamine found in citrus was reported to increase with chilling tolerance in grapefruits stored at lower temperature, suggesting its role in inducing chilling tolerance in grapefruits.[40] However, the increase in polyamines levels was not maintained after the removal of fruits from low temperature.[41] Increase in ethylene biosynthesis and *PAL* enzyme activity as a result of CI is well documented and studied.[22, 42] In Fortune mandarin, *PAL* enzyme is induced with CI, and is considered as marker for determining sensitivity to CI.[43] Conditioning fruits and their subsequent storage temperature had significant effect on PAL activity in

Fortune mandarin.[43]

Modified atmosphere packaging

Modified atmosphere packaging (MAP) helps to extend the shelf life of the fresh produce by modifying the oxygen, carbon dioxide and humidity inside the package. Oxygen levels are reduced and carbon dioxide levels are increased to slow down the respiration and metabolic processes.[44] In addition, low oxygen and higher level of carbon dioxide also reduces ethylene production.[45] MAP can be classified as active and passive depending upon gas composition modified within the package. In active MAP, mixture of predetermined gases are introduced within the package depending upon the commodity. In passive MAP, the packaging film modulates the gases within the package, namely oxygen and carbon dioxide exchange due to respiration and gas diffusion through the film.[46] Individual seal packing and 'bag in box' are common MAP techniques used for fruits.[47] Perforations on the films used for MAP help to control the gas exchange and humidity. Micro-perforated films have controlled gas permeability which assist in maintaining proper oxygen and carbon –dioxide concentration within the package.[48, 49] Furthermore, MAP helps to control postharvest diseases and disorders such as chilling injury, stem end rind breakdown, etc.[30, 47] Both modified atmosphere and high humidity under MAP play role in reducing the disorders in citrus fruits.[30] In Navel oranges, high humidity achieved using MAP reduced CI and albedo breakdown significantly.[50]

Health promoting compounds

Health promoting compounds are secondary metabolites produced by plants which are not essential for their growth and development but play important role against biotic and abiotic stresses, attracting pollinators and seed dispersing organisms, wound healing, protection against UV radiation, defense against herbivores, pathogens and pests.[51, 52] Several studies reported the health beneficial properties of naturally occurring compounds present in fruits and vegetables.[53, 54] Increase in intake of fruits and vegetables has been linked to prevention of certain chronic diseases such as coronary heart disease, cancer, stroke, hypertension, etc.[55] These health promoting compounds have shown to be effective against several chronic diseases accounting to their anti-oxidant, anti-inflammatory, anti-proliferative, anti-mutagenic, anti-tumor and anti-angiogenic properties.[56, 57] Vitamin C, carotenoids, flavonoids, limonoids, furocoumarins and phenolics are most common naturally occurring compounds present in grapefruit.

Vitamin C

Vitamin C, commonly known as an antiscorbutic factor, is an important micronutrient that is obtained only from food. Citrus fruits are considered a good source of vitamin C.[58] It is a water-soluble compound which is unstable in aqueous solutions. Vitamin C constitutes of ascorbic acid and dehydroascorbic acid and is biosynthesized from mannose in plants.[59] Various factors such as heat, light, storage temperature and storage time affect ascorbic acid content in fruits and vegetables.[60] Oxidation of ascorbic acid results in formation of dehydroascorbic acid.[61] Vitamin C is an excellent antioxidant and helps in scavenging free radicals thus preventing degenerative diseases.[62-64] Many

studies have reported health-promoting effects of ascorbic acid, such as its role in treatment of atherosclerosis,[65, 66] in inducing apoptosis in human gastric cancer cells,[67] and in preventing coronary heart disease.[68] High dose ascorbate was used in 1970s to treat cancer patients. However, no beneficial effects of ascorbate were observed.[69] In recent years reevaluation of ascorbate for cancer treatment is being considered as previous studies used oral administration instead of intravenous.[70] Intravenous administration of ascorbate helps in attaining high doses in order to maintain ascorbate levels in plasma that have cytotoxic effect on tumor cells.[71, 72] More clinical trials and meta-analysis will help in determining the role of ascorbate in cancer treatment. Several postharvest treatments have been reported to affect vitamin C content in fruits and vegetables. Storage temperature during grapefruit shipping and marketing conditions is one of the important factor influencing vitamin C levels.[27, 73]

Carotenoids

Carotenoids are lipid-soluble color pigments that are found in plants and are synthesized in plastids (chloroplasts and chromoplasts).[74, 75] Carotenoids are tetraterpenoid compounds containing 40 carbon atoms formed by condensation of 8 isoprene units. In plants, carotenoids contribute to different colors such as red, orange and yellow. Carotenoids are commonly classified as xanthophylls (containing oxygen atoms) and carotenes (containing only carbon and hydrogen atoms).[74, 76] Examples of carotenes are lycopene, β-carotene and α-carotene, whereas lutein, zeaxanthin, b-cryptoxanthin and astaxanthin are among the most common xanthophylls. Lycopene and β-carotene are the main carotenoids present in pink flesh grapefruit.[77-79] The pink flesh

color of pink grapefruit varieties is due to presence of lycopene in juice vesicles. Phytofluene and zeta-carotene are also reported to be present in lesser amounts in grapefruit juice vessels.[78-80] Carotenoids have antioxidant activity,[81] and are shown to reduce risk of prostate and breast cancer,[82] induce apoptosis,[83] and lower LDL cholesterol levels.[84] Carotenoids in citrus peel have been widely studied after ethylene treatment.[18, 20] However, effect of ethylene treatment and storage under market simulated conditions have not been investigated in grapefruit.[85]

Limonoids

Limonoids are class of oxygenated triterpenoids commonly found in plants of Rutaceae and Meliaceae families.[86] The first limonoid was isolated from navel orange in 1938 and was called 'limonin'.[87] In 1949, Emerson reported limonin to be the bitter principle in navel orange juice.[88] Limonoids occur in citrus as aglycones and glucosides.[89, 90] As the fruit matures, limonoid aglycones are converted into non-bitter limonoid glucosides with help of UDP-D-glucose:limonoid glucosyltransferase (limonoid glucosyltransferase) enzyme.[91-93] Until now, 44 limonoid aglycones and 18 limonoid glucosides have been identified from Citrus species and their hybrids.[94] Nomilin is the main precursor of limonoids in fruits and is synthesized in stem and later transported to fruits.[95] Grapefruit contain limonoids such as limonin, limonin glucoside, nomilin and deacetyl nomilinic acid glucoside etc.[96] Limonoids have shown to induce glutathione S-transferase activity (GST) in liver and intestinal mucosa of mice and rats,[97-99] which helps in detoxifying carcinogenic compounds. Induction of GST activity is mainly due to the presence of furan ring present on C17 of limonoids.[100] In addition,

limonoids reported to possess anti-cancer activity,[94, 101, 102] anti-microbial activity,[103] anti-quorum sensing activity,[96] anti-malarial activity,[103] anti-feedant activity[102] and cholesterol lowering properties.[102] Limonoids are affected by various pre and postharvest factors such as production systems and storage temperature.[104] However, very little information is available in relation to the effect of postharvest treatments on limonoids in grapefruit.

Flavonoids

Flavonoids are secondary metabolites derived from phenylpropanoid pathway. They are grouped into six different classes on basis of their molecular structure as flavones, isoflavones, flavonols, flavanols (flavans), flavanones and anthocyanidins.[57, 105] Citrus fruits contain five flavonoids excluding isoflavones. Grapefruit is a good source of flavonoids namely flavanones which are present in aglycone and glycoside forms. Flavonone glycosides are most commonly present in grapefruit and are further grouped as neohesperidose and rutinosides based on their sugar moiety.[106] Neohesperidoses are usually bitter and include naringin, poncirin and neohespeidin,[107, 108] while rutinosides are tasteless and include narirutin and didymin.[106] Naringin is the most abundant flavanone and principle bitter component present in grapefruit.[106]

Flavonoids have ability to reduce risk of cancer, cardiovascular and other degenerative diseases.[109, 110] Flavonoids are polyphenolic compounds commonly found in wide variety of plants. They possess antioxidant activity and radical scavenging activity due to their ability to donate hydrogen atoms.[105, 111, 112] Flavonoids are considered as potential chemopreventative agents since they inhibit DNA damage,[113, 114]

carcinogenesis,[115] angiogenesis,[115] tumor development[116, 117] and cell proliferation.[118] Few studies have investigated effect of irradiation and processing effect on grapefruit flavonoids.[119, 120] However degreening, conditioning and MAP treatment effect on limonoids in grapefruit pulp have not yet been studied.

Furocoumarins

Coumarins are another class of secondary metabolites formed through the phenylpropanoid pathway. Coumarins were first isolated from tonka bean (*Dipteryx odorata* Wild., Fabaceae) in 1820.[121] The name was derived from vernacular name of tonka bean 'Coumarou'. Coumarins belong to the benzopyrone family, having a benzene ring attached to a pyrone ring.[122] They are classified into four types: simple coumarins lacking fused ring systems, furanocoumarins containing 7-oxygen atom in five membered furan ring attached to benzene ring, pyranocoumarins containing 7-oxygen atom in six-membered furan ring attached to the benzene ring and the pyrone-substituted coumarins.[123, 124] Coumarin biosynthesis is linked to other secondary metabolites such as flavonoids and lignins through L-phenylalanine via the shikimate pathway.[124]

Furocoumarins are naturally occurring compounds containing a furan ring attached to coumarin.[124] Furocoumarins are further divided into two types, linear and angular. Linear furocoumarins have furan ring attached to 6,7 positions while in angular furocoumarins, the furan ring is attached at 7,8 position.[124] Umbelliferone is the main precursor for all furocoumarins.[124, 125] Furocoumarins are mainly found in Rutaceae and Apiaceae (Umbelliferae) families. The common furocoumarins occurring in grapefruit are bergamottin, 6',7'-dihydroxybergamottin and their dimers.[126, 127] Furocoumarins

affect bioavailability of certain drugs, which is usually referred as 'Grapefruit juice-drug interaction'.[128] Initially increase in oral bioavailability of these drugs was due to naringin present in grapefruit juice.[128] However, recent research suggest that not only naringin, but also other bioactive compounds, namely furocoumarins, play major role in drug interaction effects by inhibiting intestinal CYP3A4.[128-132] CYP3A4 is an intestinal protein and an isoform of CYP450, which primarily metabolizes the drugs and increases drug concentrations in plasma.[133] Furocoumarins have shown inhibitory effect on CYP 3A4 below 10 µM concentration.[60] It is warranted to study how different postharvest treatments affect the furocoumarins in grapefruit. Seasonal variation and juice processing techniques have been reported to affect the furocoumarins content in grapefruits.[8] However, further investigation on influence of other postharvest practices influence on furocoumarins is required.

Volatile oils

Volatile oils in citrus were first noticed in the sixteenth century, when Conrad Gesner mentioned distilled essential oils from oranges and lemons, followed by Jacques Besson.[134] Giovanni Battista della Porta (1589) was, however, first to describe in his work *Magiae naturalis* the distillation process to obtain essential oils from citrus peels.[134] It was not until early twentieth century, before the First World War, that machines to extract essential oil were used.[134]

Fruit aroma and flavor is generated through complex mixture of wide array of compounds from different classes and chemical groups such as alcohols, aldehydes, esters, terpenoids etc. Volatile compounds are generally influenced by various factors

such as ripening stage, cultivar, and preharvest and postharvest conditions.[135] Ethylene, a gaseous plant hormone known for its role in ripening and senescence, is also reported to affect the volatile compounds profiles.[136, 137] The most common grapefruit volatiles reported have been limonene and nootkatone. Nootkatone is considered as a senescence indicator as its levels increase with fruit maturity and senescence.[138]

Flavonoid biosynthesis pathway

Grapefruits are a good source of flavonoids and furocoumarins which are derived from the phenylpropanoid pathway.[139] *PAL* is the first enzyme of phenylpropanoid pathway while *CHS* is the first committed enzyme of flavonoid biosynthesis. Chalcone isomerase (*CHI*) which converts naringenin chalcone to naringenin (aglycone) and 1,2-rhamnosyl transferase (*2RT*) which converts naringenin-7-O-glucoside to naringin (glucoside) are the downstream enzymes in the grapefruit flavonoid pathway.[140] The common precursor for flavonoids and furocoumarins biosynthesis, 4-coumaroyl-CoA, is derived from L-phenylalanine through the phenylpropanoid pathway.[125, 139] *PAL* enzyme has been reported to be affected by various postharvest treatments and conditions. Ethylene is one of the most common factor influencing *PAL* enzyme.[22, 42] Irradiation of citrus fruits also increases *PAL* activity leading to increased phenolic content.[141] Elicitors from *Alternaria carthami* are reported to increase the activities of *PAL* and 4-coumarate: CoA ligase (*4CL*); however, no effect was observed on enzymes involved in flavonoid pathway.[142] Furthermore, furocoumarins were increased via *PAL* and *4CL* by external elicitors,[142-144] which can be due to preferential activation of enzymes involved in furocoumarin biosynthesis. Several postharvest factors are reported to influence the

enzymes involved in the flavonoid pathway especially *PAL* enzyme.[22, 42, 43, 145] *CHS* and *CHI* expression was studied in Satsuma mandarin and were found to regulate the accumulation of flavonoids during fruit maturation.[146] *2RT* has been isolated and studied in pummelo and 'Cara Cara' navel orange.[147, 148] *2RT* has been reported to be functional only in bitter citrus species such as pummelo.[147, 148] Even though *2RT* expression is high in 'Cara Cara' navel orange, accumulation of bitter flavanones such as naringin, poncirin and neohesperidin is not observed suggesting non-functionality of the gene.[148] However, these flavonoid biosynthesis genes have not yet been isolated and studied in grapefruit. Considering the high content of both bitter flavanones (especially naringin) and furocoumarins in grapefruit, studying the expression of these flavonoid pathway genes is warranted.

CHAPTER III

DEGREENING AND POSTHARVEST STORAGE INFLUENCES 'STAR RUBY' GRAPEFRUIT (*Citrus paradisi* Macf) HEALTH PROMOTING COMPOUNDS*

Introduction

Fruits and vegetables are rich sources of bioactive compounds that possess anti-oxidant, anti-inflammatory and anti-proliferative activities.[149] Consumption of a diet rich in naturally occuring compounds is effective in reducing the risks of various cancers, such as breast cancer, prostate cancer and lung cancer, along with cardiovascular diseases.[149-151] These health-promoting compounds in fruits and vegetables are influenced by various preharvest and postharvest factors.[73] Fruits and vegetables continue their metabolic activities after harvest and also undergo various biotic and abiotic stresses leading to variations in natural compounds, before the produce reaches consumers.[152] Therefore, it is important to study the effects of commercially practiced postharvest treatments on bioactive compounds in fruits and vegetables.

Several postharvest treatments are used to improve the attractiveness, quality and shelf-life of citrus fruits. Generally, growers use ripening ratio as an indicator for harvesting early season citrus fruits that are mature and acceptable for consumption except for their green peel color. However, consumers often relate peel color to ripeness, and consider green fruits undesirable. To overcome this problem, early season mature

*Reprinted with permission from "Degreening and postharvest storage influences 'Star Ruby' grapefruit (*Citrus paradisi* Macf.) bioactives" by Chaudhary, P.; Jayaprakasha, G. K.; Porat, R.; Patil, B. S., 2012, *Food Chemistry. 135*, 1667-1675. Copyright [2012] Elsevier

grapefruits are treated with ethylene, which results in an attractive uniform red/orange peel color.

Previous studies on citrus degreening were primarily focused on optimization of ethylene concentration, degreening temperature and time,[18, 19] along with the effect of ethylene on carotenoids in the peel and juice vesicles.[18, 85, 153, 154] Matsumoto et al. studied carotenoid accumulation in flavedo and in juice vesicles of Satsuma mandarin at different temperatures (5, 20 and 30 °C) and ethylene concentrations (10 and 1000 μL/L).[85] Ethylene application at 20 °C enhanced carotenoid synthesis in flavedo; however, it had no significant influence on carotenoids present in juice vesicles.[85]

Grapefruit is a rich source of health promoting compounds, containing carotenoids, limonoids, flavonoids, ascorbic acid, furocoumarins, folic acid, pectins, phenolics and dietary fiber. Limonoids are reported to possess various health promoting properties *in vitro* and *in vivo.*[155] Flavonoids and furocoumarins are important classes of secondary metabolites present in citrus, which are derived from the phenylpropanoid pathway. Flavonoids are polyphenolic compounds well known for their antioxidant properties and prevention of chronic diseases.[110] Ethylene was reported to increase the levels of phenylalanine ammonia-lyase (PAL), a key enzyme in the phenylpropanoid pathway.[23] Therefore, it is critical to understand the influence of postharvest ethylene treatment on the health promoting compounds present in grapefruit juice vesicles. In the present study, the influence of ethylene treatment on limonoids, flavonoids, furocoumarins, ascorbic acid and carotenoids in grapefruit juice vesicles was investigated. To the best of our knowledge, this study represents the first examination of

the effect of ethylene on nomilin, deacetyl nomilinic acid glucoside (DNAG), 6,7-dihydroxybergamottin and bergamottin in Star Ruby grapefruit.

Materials and methods

Chemicals

ACS grade solvents were used for extraction and high performance liquid chromatography (HPLC) grade solvents were used for quantitative analysis; solvents were obtained from Fisher Scientific (Fisher Scientific Research, Pittsburgh, PA, USA). Butylated hydroxytoluene (BHT), lycopene, β-carotene, narirutin, naringin, didymin, poncirin, limonin, nomilin, and 6, 7-dihydroxybergamottin were procured from Sigma Aldrich Co. (St. Louis, MO, USA). Deacetyl nomilinic acid glucoside (DNAG) was purified in the lab according our published methods.[156]

Plant material

Star Ruby grapefruits were harvested from a commercial grove on the south coast of Israel on November 18, 2008 and transported to a commercial citrus packing house.

Degreening treatment

Fifteen boxes containing 40 grapefruits per box were degreened for 60 h with 2 ppm of ethylene at a constant temperature of 20 °C in a commercial citrus packing house. The control included 15 boxes of grapefruits stored at 20 °C without ethylene treatment. After degreening, both control (non-degreened) and degreened fruits were rinsed with 2% OPP (2-Phenylphenol / ortho-phenylphenol), dipped for 20 sec in 400 ppm hot imazalil solution. The grapefruits were waxed with a commercial polyethylene-

based wax solution (Zivdar, Safe-Pack cooperation Ltd., Kfar Saba, Israel) containing 800 ppm of imazalil and 400 ppm of thiabendazole (TBZ) at the packing house and then transferred to the Dept. of Postharvest Science at the Volcani center (Bet Dagan, Israel). Each treatment included 3 replications.

Storage study

Fruits were stored under simulated market conditions, with initial 21 days of storage at 10 °C to simulate the shipment period and were later transferred to room temperature (20 °C) for 14 days to simulate retail store conditions. Samples were collected in triplicates at an interval of 7 days over a period of 35 days. Juice samples were prepared by blending three peeled whole fruits and were used for further quality analysis. To quantify bioactive compounds such as carotenoids, limonoids, flavonoids and furocoumarins, juice samples were lyophilized and shipped to the Vegetable and Fruit Improvement Center (Texas A&M University, College Station, Texas, USA).

Fruit quality analysis

Fruit quality analysis, including total soluble solids (TSS), acidity, color measurements, weight loss, disease and decay evaluation, were conducted at the Volcani Center, Israel. The juice TSS content was determined with a Model PAL-1 digital refractometer (Atago, Tokyo, Japan), and acidity percentages were measured by titration to pH 8.3 with 0.1 M NaOH by means of a Model CH-9101 automatic titrator (Metrohm, Herisau, Switzerland). Each measurement comprised of five replications, each replication prepared from three individual fruits.

For color measurements, 15 fruits per treatment were circled with a black marker on their equatorial side, and the peel color within these circles was determined weekly by measuring the hue angle with a Chromo Meter, model CR-200 (Minolta, Tokyo, Japan); a hue angle of ~90° represents yellow, ~60° orange, and ~30° red color.

Fruit weight loss was evaluated by weighing 15 fruits per treatment before (Time 0) and after the storage, and the percentage of weight lost was calculated. Evaluation of decay and blossom-end clearing (BEC) incidence was performed by determining the total number of fruits manifesting the disease symptoms, and the results were expressed as percentages of the total number of fruits in each treatment. Additionally, fruit softness was measured as the degree of deformation (mm) after exerting 2 kg pressure on the equatorial side of the fruit.

Sensory analysis

The effect of ethylene degreening treatment on fruit sensory quality was evaluated at 28 days after storage (three weeks of cold storage and a subsequent one week at room temperature). Sensory analysis was conducted at the Volcani Center, Israel. Fruits were peeled, and separated segments were cut into halves and placed into covered glass cups. Each treatment included a mixture of cut segments prepared from five individual fruits. Fruit taste was evaluated by a sensory panel consisting of 10 members, five males and five females, aged between 25 to 62 years. Each panelist assessed the various attributes of three samples, according to an unstructured 100 mm scale, with anchor points at 'very weak' and 'very strong' for each attribute, and sensory

data were recorded as distances (mm) from the origin. The samples were identified by means of randomly assigned three-digit codes.

Ascorbic acid analysis

Ascorbic acid quantification was conducted in Israel to avoid degradation during freeze drying. Ascorbic acid levels in Star Ruby grapefruit juice were determined by titration with 2,6-dichlorophenol indophenol,[157] and comparing the titration volumes with a 0.1% ascorbic acid standard (Sigma-Aldrich, St. Louis, MO). The results were expressed as mg of ascorbic acid per 100 ml of juice.

Quantification of carotenoids

Reconstituted freeze dried juice samples (1 g + 5 ml water) were subsequently extracted for three times with 15 ml chloroform containing 0.2% BHT. Water was added to freeze dried samples prior to extraction to increase extractability. The organic layer was collected, pooled and filtered using Whatman grade 1 filter paper. All the extractions were carried out in the dark under yellow light to avoid degradation of carotenoids. The extracts were further subjected to HPLC analysis using an Agilent HPLC 1200 Series (Foster City, CA, USA) system consisting of a solvent degasser, quaternary pump, autosampler, column, oven and diode array detector. A Devosil 3 µl RP-Aqueous C 30 (150 × 4.6 mm) column was used along with a Devosil 5 µl RP - Aqueous (10 × 4 mm) a guard column (Phenomenex, Torrance, CA, USA). Gradient mobile phase consisting of acetonitrile (A) and methyl-*tert*-butyl ether (B) was used for separation.[158] Initially solvent A was maintained at 60% for 7 min, then decreased linearly to 50% A up to 12 min, 30% A up to 15 min and 0% A up to 20 min. Sample

(10 µl) was injected at the flow rate of 0.8 ml/min. The column was equilibrated in between runs for 2 min and oven temperature was maintained at 15 °C. The β-carotene and lycopene were detected at 450 nm and quantified using external standard calibration.

Determination of limonoids, flavonoids and furocoumarins

Sample preparation for limonoids, flavonoids and furocoumarins

Reconstituted freeze dried juice (1 g + 5 ml water) was extracted according to a previously published method with slight modifications, using 15 ml of ethyl acetate.[8] The organic layer was separated and the residue was extracted successively in two cycles. The extracts obtained were pooled, filtered using Whatman grade 1 filter paper and concentrated to dryness. The dried residue was then reconstituted with acetone, filtered (0.45µm PTFE filter) and analyzed using HPLC.

HPLC analysis for limonoids and flavonoids

Limonoids and flavonoids were analyzed using our previous method.[159] A Waters HPLC (Milford, MA, USA), spectra model with a PDA detector (2996) coupled with binary HPLC pump 1525 and 717 plus auto sampler was used. The separations were carried out on a C-18, 5 µm particle size Gemini column (250 mm × 4.6 mm i.d.) purchased from Phenomenex (Torrance, CA, USA). The peaks were detected at 210 nm and 280 nm and analysis was carried out by Empower 2 software (Waters, Milford, MA, USA). A gradient mobile phase of 0.03 M phosphoric acid (A) and acetonitrile (B) with 1ml/min flow rate was used. Each sample was analyzed three times and each treatment had three replications.

HPLC analysis for furocoumarins

Furocoumarins were analyzed using our previous method.[120] The analytical HPLC system consisted of Perkin-Elmer series 200 pump, PDA detector (235C) and an autosampler (Perkin-Elmer, Norwalk, CT, USA). The separation was carried out on a C-18, 5 µm particle size Gemini column (250 mm × 4.6 mm i.d.) with a guard cartridge purchased from Phenomenex (Torrance, CA, USA). A gradient mobile phase of 0.03 M phosphoric acid (A) and acetonitrile (B) with 1ml/min flow rate was used. The peaks were detected at 320 nm and analysis was carried out by Turbochrom software (Perkin-Elmer, Norwalk, CT, USA). Each sample was analyzed three times and each treatment had three replications.

Determination of total phenolics

Total phenolics content in methanol: water extracts was determined using a previously published method with slight modifications and the results were expressed as catechin equivalents.[160] Freeze dried juice samples (1 g) were extracted twice with methanol: water (80:20). The extracts were pooled together and further used for quantification of total phenolics. Different concentrations (25, 50, 100, 150, 200, 250, 300 µg) of catechin and methanol: water extracts of samples (100 µl) were pipetted into separate test tubes and the volume was adjusted to 10 ml using distilled water. One-fold diluted Folin-Ciocalteu reagent (0.5 ml) was added to all tubes and the samples were incubated at 25 °C for 10 min. Sodium carbonate solution 1ml (240 g/L) was added to all tubes, vortexed and allowed to stand for 20 min at 25 °C. The assay control was prepared without adding any standard or sample. All standards and samples were

pipetted in triplicate into 96 well plates separately. Absorbance was measured at 760 nm using Synergy^{TM} HT Multi-Mode Microplate Reader (BioTek, Instruments, Winooski, VT) at 25 °C. The regression equation obtained from catechin values and the dilution factor were applied to determine the total phenolics present in the samples. The results were expressed as mM Trolox equivalent per g of dry weight of samples.

DPPH radical scavenging activity

Radical scavenging activity of Star Ruby methanol: water extracts was measured using a previously published method[161] with slight modifications. Freeze dried juice samples (1 g) were extracted twice with methanol: water (80:20). The extracts were pooled together and used for DPPH assays. Various concentrations (0.15, 0.3, 0.45, 0.6, 1.125, 1.5 µg/ 100 µl) of ascorbic acid and methanol: water (80:20) extracts (10 µl) were pipetted into 96 well plates in triplicate. The volume of each well was adjusted to 100 µl using methanol. Methanolic solution of DPPH (180 µl) was added to all the wells and incubated for 30 minutes at 25 °C. Absorbance was measured at 515 nm using a Synergy^{TM} HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT) at 25 °C. The assay control was prepared using the same procedure but without ascorbic acid or sample. Radical scavenging activity was expressed as mg of ascorbic acid equivalent per g of dry weight sample.

Statistical analysis

One way analysis of variance (ANOVA) was performed using PASW Statistics 18 software (© SPSS Inc. 2009). Significant differences were tested using a general

linear model and means were compared using Tukey's HSD test at 5% probability level ($P < 0.05$). The results are expressed as means ± SE.

Results and discussion

Influence of ethylene degreening on fruit quality and external appearance

Star Ruby grapefruits were harvested at the color break stage (November 18, 2008), when the fruits had a green peel with yellow and red patches. Degreening accelerated color development, resulting in a much more attractive fruits with yellow/red external color (**Figure 1A**). In contrast, the internal color of the fruits was already red at harvest time and the degreening treatment did not have any further effect. The effect of ethylene degreening on the fruit internal and external color was further evaluated by measuring their hue angles (**Table 1**); yellow color has a hue angle of ~90° and red color has a hue angle of ~30°. At the day of harvest (Time-0) the hue angle of peels was 80° ± 2.9. Degreened fruits had lower hue angles (65.5° ± 5.4) for the peels as compared to the non-degreened fruits (81.5° ± 8.7), at the beginning of the storage period (0 days). The hue angle of peels of degreened fruits remained more or less constant during the entire storage period, while it decreased slightly in non-degreened fruits to 77.1º ± 5.3 at the end of 35 days of storage. On the other hand, significant variations were not found in the hue angles of the juice vesicles, which were already red (24º - 28°) at the time of harvest in both the treatments.

Ethylene degreening effects on total soluble solids (TSS) and acidity levels were studied. Degreening treatment had no significant effect on the juice TSS levels.

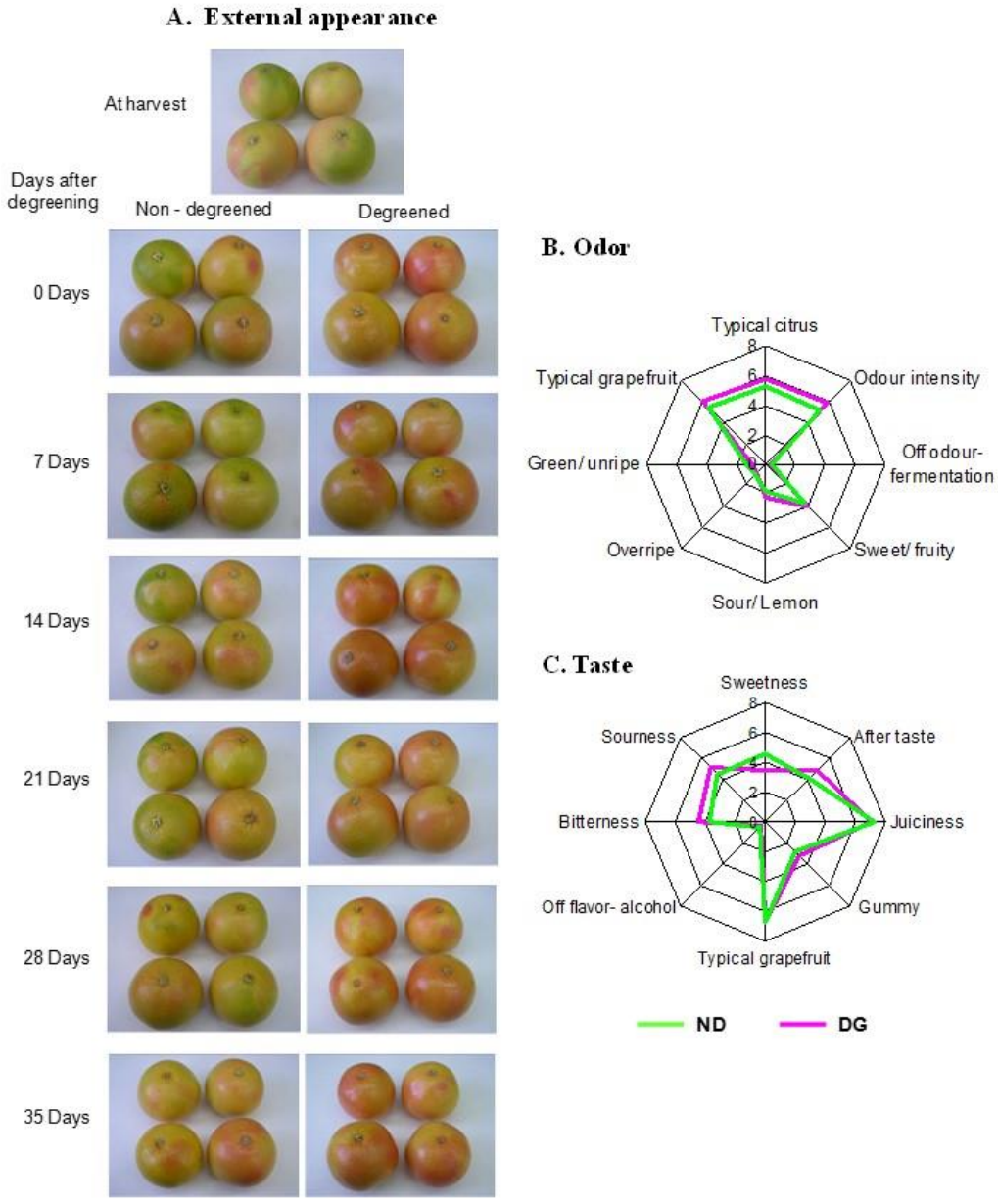

Figure 1. Sensory analysis and external appearance of non-degreened (ND) and degreened (DG) Star Ruby grapefruits after 3 weeks of storage at 10 °C with subsequent one week storage at 20 °C; (A) indicates fruit odor, (B) indicates fruit taste whereas (C) indicates external appearance of fruits. Fruit flavor was evaluated by a trained sensory panel consisting of 10 members**.**

Table 1. Effects of ethylene degreening on peel and juice color of Star Ruby grapefruit

Storage (days)	Peel color (°h)		Juice color (°h)		[a]
	ND	DG	ND	DG	
T-0	80.0 ± 2.9	80.0 ± 2.9	24.5 ± 3.2	24.5 ± 3.2	
0	81.5 ± 8.7	65.5 ± 5.4	24.6 ± 1.7	28.0 ± 3.9	
7	81.3 ± 7.6	65.1 ± 5.1	21.8 ± 2.4	25.8 ± 1.8	
14	80.5 ± 8.0	65.8 ± 4.8	27.6 ± 5.5	28.3 ± 4.6	
21	79.5 ± 7.7	66.9 ± 4.9	24.6 ± 2.7	27.4 ± 3.5	
28	78.8 ± 6.9	65.7 ± 5.4	22.8 ± 3.9	23.1 ± 2.8	
35	77.1 ± 5.3	65.4 ± 4.9	24.7 ± 2.7	29.7 ± 4.7	

Fruits were stored for 21 days at 10 °C and then transferred to 20 °C for 2 weeks. Data represents means ± S.E, n=15 fruits. (ND = non-degreened, DG = degreened).

Degreened fruits had significantly ($P < 0.05$) higher acidity levels as compared to non-degreened fruits at 35 days after storage (**Table 2**). Acidity levels declined to 1.9 % in non-degreened fruits but remained at 2.6% in degreened fruits at 35 days after storage (**Table 2**). Overall, there was no significant difference between both treatments from 0 days to 28 days of storage. Non-degreened fruits had a significantly higher ripening ratio at 35 days after storage.

Degreening had no significant effect on fruit weight and fruit softening during cold storage. The weight loss was minimal (less than 1%) during the first 21 days of cold storage at 10 °C, but increased in both non-degreened and degreened fruits upon transfer to room temperature (**Table 3**). Weight loss was significantly higher in non-degreened fruits as compared to the degreened fruits at 28 and 35 days after storage. Similarly, fruit softness (measured as the degree of deformation after pressure of 2 kg)

did not change during cold storage (10 °C) but increased in both non-degreened and degreened fruits after 28 and 35 days of storage (data not shown).

In both degreened and non-degreened fruit, minor amounts of decay developed during the storage period (**Table 3**). At the end of 5 weeks of simulated market storage, overall 1% decay in non-degreened fruits and 1.5% decay in degreened fruits were observed. In the current experiment, no visual external peel disorders were detected on fruits from either treatment (data not shown). Nevertheless, blossom-end clearing (BEC) symptoms manifested as internal browning of the albedo tissue at the blossom end were

Table 2. Influence of ethylene degreening on TSS, acidity and ripening ratio in juice of Star Ruby grapefruit.

Storage	TSS (%)		Acidity (%)		TSS/Acidity	
(days)	ND	DG	ND	DG	ND	DG
T-0	11.75 ± 0.1 a	11.75 ± 0.1 a	2.56 ± 0.1 a	2.56 ± 0.1 a	4.60 ± 0.1 a	4.60 ± 0.1 a
0	11.55 ± 0.3 a	11.38 ± 0.3 a	2.31 ± 0.0 a	2.53 ± 0.0 a	5.01 ± 0.1 a	4.50 ± 0.1 a
7	10.93 ± 0.2 a	10.53 ± .2 a	2.46 ± 0.1 a	2.39 ± 0.1 a	4.45 ± 0.1 a	4.41 ± 0.1 a
14	10.83 ± 0.3 a	10.17 ± 0.3 a	2.44 ± 0.0 a	2.54 ± 0.0 a	4.44 ± 0.1 a	4.00 ± 0.1 a
21	11.08 ± 0.3 a	11.10 ± 0.3 a	2.35 ± 0.1 a	2.56 ± 0.1 a	4.73 ± 0.2 a	4.34 ± 0.2 a
28	11.28 ± 0.2 a	10.90 ± 0.2 a	2.34 ± 0.1 a	2.53 ± 0.1 a	4.83 ± 0.2 a	4.31 ± 0.2 a
35	10.80 ± 0.2 a	11.18 ± 0.2 a	1.88 ± 0.1 a	2.58 ± 0.1 b	5.76 ± 0.2 a	4.35 ± 0.2 b

[a] Fruits were stored for three weeks at 10 °C and then transferred to 20 °C for two weeks. Data represent means ± S.E, n=5 samples, each sample prepared from three fruits. Same letter indicates no significant differences between treatments for each parameter. (ND = non-degreened, DG = degreened).

observed. Overall, BEC symptoms were 4.5% and 11.5% for non-degreened and degreened fruits respectively after 5 weeks of storage (**Table 3**).

Fruit taste and aroma

The effect of ethylene degreening on grapefruit taste and odor was evaluated by a sensory panel after four weeks of storage. Interestingly, the ethylene degreening treatment had no effect on either fruit odor or taste (**Figure 1B and 1C**). Furthermore, ethylene degreening did not cause any 'over ripe' or 'off flavor' sensations and it barely affected the 'bitterness' (**Figure 1B and 1C**).

Table 3. Ethylene degreening effect on weight loss (%), decay (%) and blossom-end clearing (BEC %) of Star Ruby grapefruit.

Storage	Weight loss (%)		Decay (%)		BEC (%)	
(days)	ND	DG	ND	DG	ND	DG
7	0.39 ± 0.2 a	0.37 ± 0.2 a	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.00a	0.00 ± 0.0 a
14	0.69 ± 0.0 a	0.60 ± 0.0 a	0.00 ± 0.0 a	0.50 ± 0.4 a	0.00 ± 0.00a	0.00 ± 0.0 a
21	0.93 ± 0.0 a	0.85 ± 0.0 a	0.00 ± 0.0 a	0.50 ± 0.4 a	0.00 ± 0.00a	0.00 ± 0.0 a
28	2.15 ± 0.1 a	1.90 ± 0.1 b	0.50 ± 0.8 a	1.50 ± 0.8 a	0.00 ± 0.00a	0.00 ± 0.0 a
35	3.76 ± 0.1 a	3.26 ± 0.1 b	1.00 ± 0.8 a	1.50 ± 0.8 a	4.50 ± 2.74a	11.50 ± 2.7 a

*Fruits were stored for three weeks at 10 °C and then transferred to 20 °C for two weeks. Data represent means ± S.E. Same letter indicates no significant differences between treatments for each parameter. n=15 for weight loss (%); n= 5 replications for decay(%) and BEC(%), each replication containing 40 fruits. (ND = non-degreened, DG = degreened).

Influence of degreening on ascorbic acid, carotenoids, limonoids, flavonoids and furocoumarins

Bioactive compounds such as ascorbic acid, limonoids, flavonoids and furocoumarins were analyzed after ethylene degreening treatment. Ascorbic acid levels were significantly ($P < 0.05$) higher in non-degreened fruits as compared to degreened fruits at 14 days after storage (**Figure 2A**). However, no significant difference between the treatments was observed in ascorbic acid levels at 0, 7, 21, 28 and 35 days of storage. Indeed, ascorbic acid was stable during the storage period. At the end of the storage period, both non-degreened and degreened fruits had similar levels of ascorbic acid. Ascorbic acid is one of the important health promoting compounds which is found abundantly in citrus. Previous studies have directly correlated degradation of ascorbic acid due to storage temperature and storage period.[60, 162]

β-carotene was significantly ($P < 0.05$) higher in degreened fruits at 14 and 21 days after storage, while it was higher in non-degreened fruits at 28 days after storage (**Figure 2A**). However, at the end of the storage period of 35 days, no significant difference was observed between both the treatments. β-carotene levels increased gradually up to 35 days of storage while lycopene levels were retained at initial levels at the end of 35 days of storage. Lycopene was significantly higher in non-degreened fruits at 7 days of storage. Nevertheless, at the end of the storage period of 35 days, no significant difference in lycopene levels was observed between the treatments. A recent study demonstrated that ethylene treatment increased carotenoids in the peels of Satsuma mandarin; however, ethylene had no significant effect on carotenoids in juice vesicles of

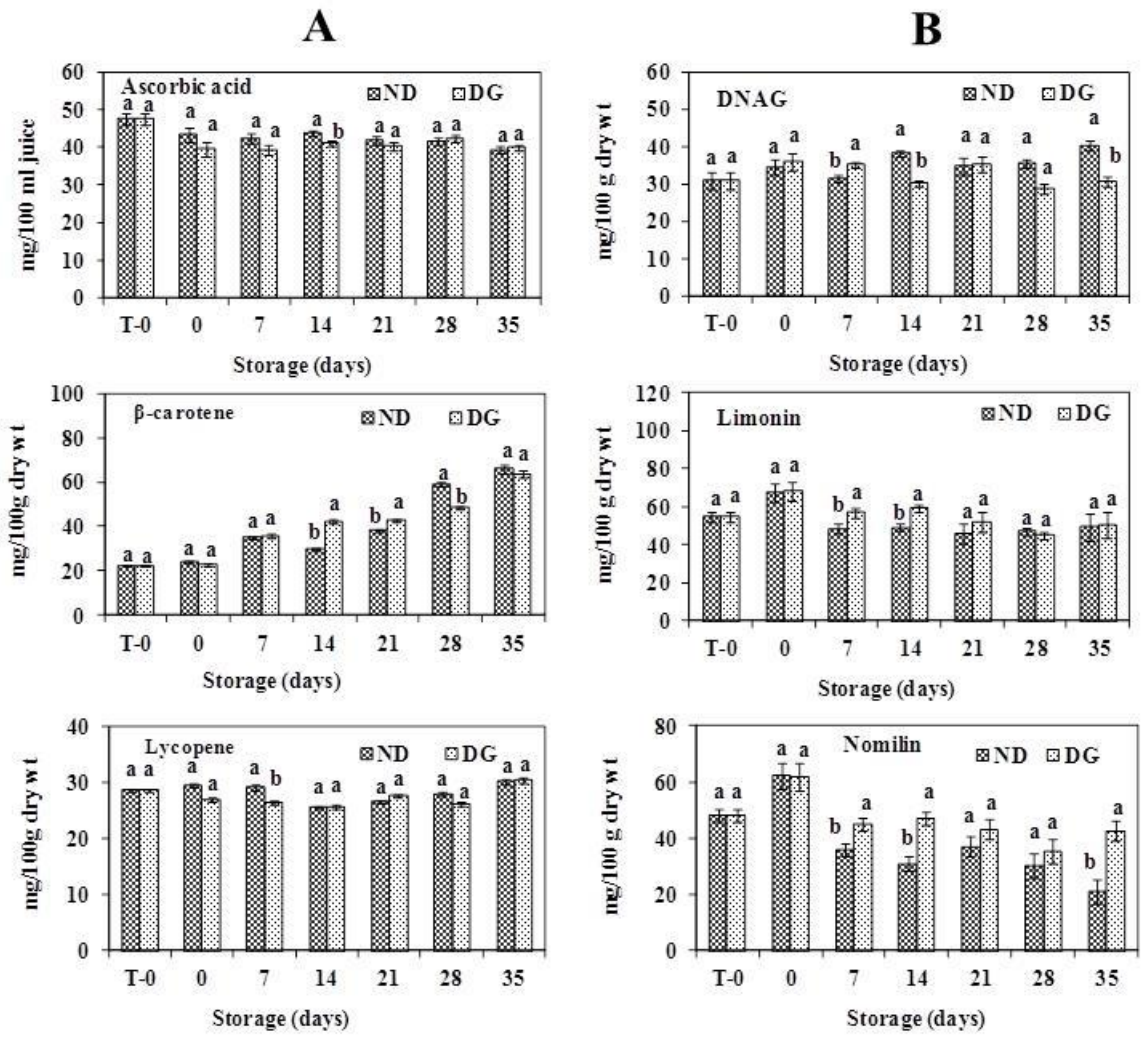

Figure 2. (A) Variations in ascorbic acid, β-carotene and lycopene (B) Variations in limonoids in the juice of non-degreened (ND) and degreened (DG) Star Ruby grapefruits. Fruits were stored for three weeks at 10 °C and then transferred to 20 °C for two weeks. Data are expressed as means ± S.E. of three samples, each sample prepared from three fruits. Means with the same letter indicate no significant differences between treatments ($P < 0.05$).

fruits stored at 20 °C and 5 °C.[85]

Three limonoids, DNAG, limonin and nomilin, were analyzed by HPLC. Deacetyl nomilinic acid (DNA) is a precursor of nomilin, while DNAG is a glucoside of DNA.[163] Two separate pathways were suggested for DNAG and nomilin.[86] Both DNA and nomilin are biosynthesized in stems and then transported to other plant parts, including fruits.[86] Furthermore, nomilin is a precursor of limonin and other limonoids (**Figure 3A**). In the present study, nomilin levels were significantly higher ($P < 0.05$) in degreened fruits than non-degreened fruits at 7, 14 and 35 days of storage (**Figure 2B**). DNAG levels were significantly higher ($P < 0.05$) in degreened fruits at 7 days of storage (**Figure 2B**). However, non-degreened fruits showed higher DNAG levels at 14 and 35 days after storage. At the end of 35 days of storage, degreened fruits had higher nomilin, whereas non-degreened fruits had higher DNAG levels.

It is possible that the opposite effects of ethylene on DNAG and nomilin could be due to conversion of DNA to either DNAG or nomilin. Limonin levels were significantly higher in degreened fruits at 7 and 14 days of storage, but no significant difference was observed between treatments at 35 days of storage. A previous study showed that ethylene and temperature inversely affected limonin levels in citrus fruit;[164] however, ethylene level (20 ppm) used in the study[164] was higher than the ethylene levels used in commercial degreening process.

Furocoumarins such as 6,7-dihydroxybergamottin (DHB) and bergamottin were quantified (**Figure 3B**). Degreened fruits had significantly ($P < 0.05$) higher contents of DHB at 7 and 14 days of storage. Bergamottin content was also higher in degreened

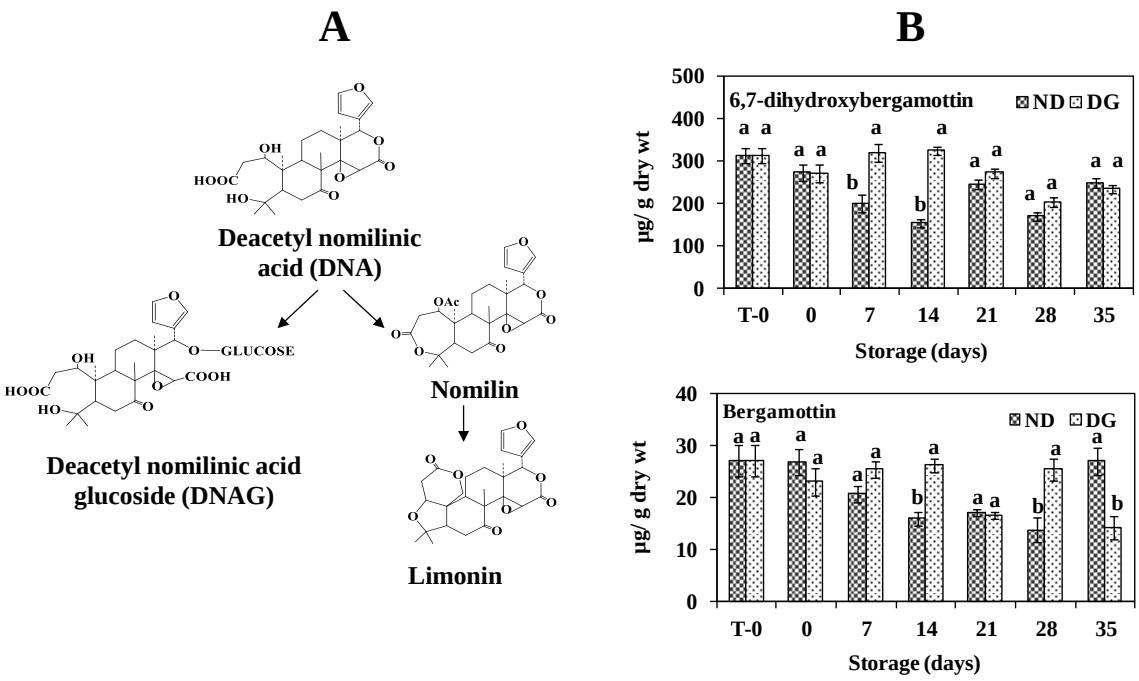

Figure 3. (A) Biosynthetic pathway of nomilin, limonin and deacetyl nomilinic glucoside (DNAG). (B) Levels of furocoumarins in non-degreened (ND) and degreened (DG) Star Ruby grapefruits. Fruits were stored for three weeks at 10 °C and then transferred to 20 °C for two weeks. Data are expressed as means ± S.E. of three samples, each sample prepared from three fruits. Means with the same letter indicate no significant differences between treatments for each bioactive compound ($P < 0.05$).

fruits at 14 and 28 days after storage. No significant difference was observed between treatments in all three furocoumarins at time 0, 0 days and 21 days after storage. At the end of 35 days of storage, bergamottin was significantly higher in non-degreened fruits, however no significant difference was observed in DHB between treatments.

Flavonoids such as narirutin, naringin, neohesperidin, didymin and poncirin were quantified. Narirutin levels were significantly ($P < 0.05$) higher in non-degreened fruits in comparison with degreened fruits at 14 and 35 days after storage (**Figure 4**). Naringin, a glucoside of naringenin, and poncirin were higher in non-degreened fruits than degreened fruits at 35 days after storage. No significant difference was observed in neohesperidin levels in both treatments during the storage period, while didymin was significantly higher in non-degreened fruits at 14 days after storage. Overall flavonoids were higher in non-degreened fruits at 14 and 35 days after storage. Previous reports have suggested that ethylene increases the activity of PAL,[23] which is an important enzyme in the flavonoid pathway. However, in the present experiment, higher flavonoids were observed in non-ethylene treated fruits.

Flavonoids and furocoumarins have a common precursor, 4-coumaroyl-CoA, which is derived from L-phenylalanine through the phenylpropanoid pathway.[139] PAL plays an important role in the phenylpropanoid pathway and increases with ethylene levels.[23] In the present study, higher narirutin and didymin levels were observed at 14 days after storage in non-degreened fruits, while furocoumarins were higher in degreened fruits at 7 and 14 days after storage. This further suggests conversion of 4-coumaroyl-CoA into flavonoids in non-degreened fruits, while in degreened fruits 4-coumaroyl-CoA may be converted into furocoumarins.

A previous study reported that elicitors from *Alternaria carthami* increased activities of PAL and 4-coumarate: CoA ligase (4CL), two enzymes involved in the phenylpropanoid pathway; however, elicitors had no effect on enzymes involved in the

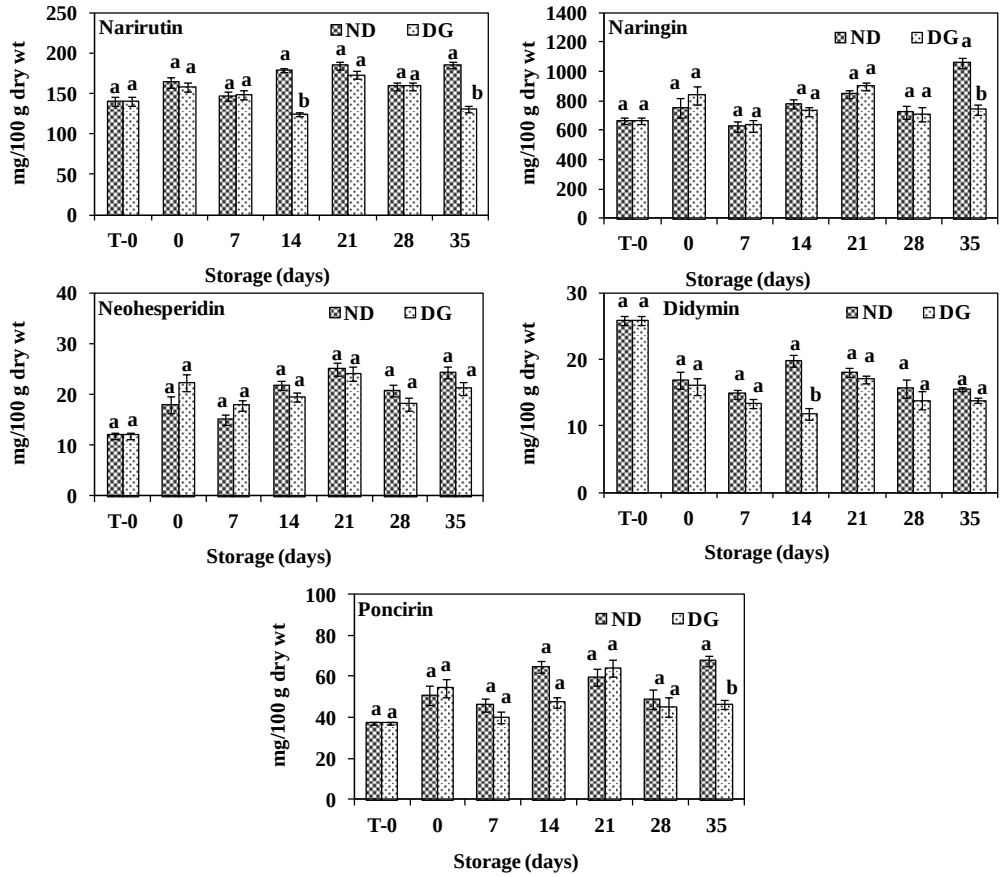

Figure 4. Influence of ethylene degreening on flavonoids in Star Ruby grapefruits (non-degreened = ND, degreened = DG). Fruits were stored for three weeks at 10 °C and then transferred to 20 °C for two weeks. Data represents means ± S.E. of three samples, each sample prepared from three fruits. Means with the same letter indicate no significant differences between treatments ($P < 0.05$).

flavonoid pathway.[142] In addition, external elicitors increased furocoumarins via PAL and 4CL,[142-144] suggesting preferential activation of enzymes involved in the furocoumarin pathway over the flavonoid pathway. Hahlbrock et al. suggested that PAL

and 4CL may have additional roles other than synthesis of flavonoids and lignin.[144] S-adenosylmethionine (SAM) is an intermediate in ethylene biosynthesis. It is also involved in the furocoumarin pathway via S-adenosyl-L-methionine:xanthotoxol O-methyltransferase (XMT) and S-adenosyl-L-methionine: bergaptol O-methyltransfersase (BMT), which are enzymes involved in the furocoumarin pathway.[165] This suggestive evidence links SAM to the furocoumarin pathway.[139] It is possible that ethylene application may shift the balance of enzymes more towards the furocoumarin pathway instead of the flavonoid pathway, which might have resulted in higher furocoumarins and lower flavonoids in degreened fruits. However, more evidence and further studies are required to prove the effect of ethylene on the furocoumarin and flavonoid pathways.

Effect of degreening on total phenolics and radical scavenging activity

Overall phenolics increased in both treatments during the storage period (**Figure 5A**). No significant difference ($P < 0.05$) was observed in total phenolics content between both treatments during storage period. Flavonoids are one of the important components of total phenolics. However, there are other phenolic compounds, such as hydroxybenzoic acids and hydroxycinnamic acids, which are present in grapefruits.[166] Naringin accounted for approximately 75% of the total flavonoids quantified, and was higher in non-degreened fruits than degreened fruits at 35 days after storage (**Figure 4**).

Radical scavenging activity of all the samples was measured using the DPPH assay. There was no significant effect of degreening treatment on radical scavenging activity (**Figure 5B**). In addition, the storage period had no detrimental effect on radical scavenging activity, which was retained at initial levels throughout the storage period.

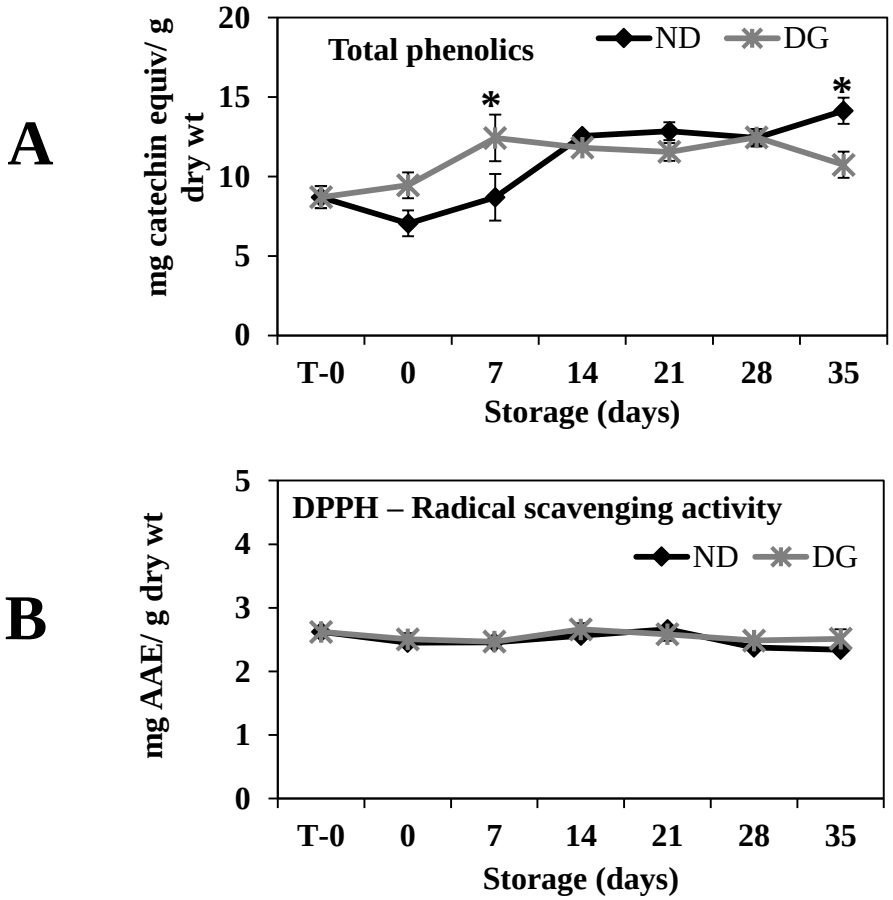

Figure 5. Influence of ethylene degreening on total phenolics (A) and radical scavenging activity (B) in Star Ruby grapefruits (non-degreened = ND, degreened = DG). Fruits were stored for three weeks at 10 °C and then transferred to 20 °C for two weeks. Data represents means ± S.E. of three samples, each sample prepared from three fruits. Means with (*) indicate significant differences between treatments ($P < 0.05$).

Radical scavenging activity is a combined effect of different antioxidants present in fruits including ascorbic acid, flavonoids and carotenoids.[167] Radical scavenging activity

helps to quench free radicals present in the body, preventing DNA damage and other chronic diseases.

Conclusion

Overall, degreening treatment improved fruit peel color and appearance with no significant effect on Star Ruby grapefruit taste and quality. Interestingly, ethylene-treated fruits showed higher levels of nomilin and lower levels of DNAG at 35 days after storage. Based on the above results, it can be concluded that ethylene treatment had no significant influence on levels of ascorbic acid, carotenoids, total phenolics and radical scavenging activity; while it had differential effects on limonoids, coumarins and flavonoids. Therefore, degreening treatment can be used to enhance early season grapefruit peel color, with minimal effect on nutritional quality.

CHAPTER IV

THE EFFECT OF ETHYLENE DEGREENING ON THE HEALTH PROMOTING COMPOUNDS OF 'RIO RED' GRAPEFRUIT*

Introduction

Several studies have shown the potential health benefits of fruits and vegetables and their important roles in human health.[168] The 'My Plate' food guide developed by the USDA also emphasizes increasing the consumption of fruits and vegetables.[169] Increased awareness about the role of naturally occurring compounds in improving health, has increased consumer demand and broadened the market for functional foods.[170, 171] The media and food processing companies have increased their interest in fruits and vegetables with higher levels of antioxidants, promoting fruits such as pomegranates, acai berries, and blueberries. Citrus is a major fruit crop that is eaten fresh and used in processing. Citrus fruits are rich sources of vitamin C; moreover, recent research has focused on other citrus health promoting compounds. Several previous studies used cell culture and animal models to demonstrate various health-promoting properties of citrus natural compounds, including anti-inflammatory,[172] anti-proliferative effect on human neuroblastoma cells,[173] anti-carcinogenic activity in human colon, breast, pancreatic, prostate cancer cells,[94] cholesterol lowering[174] and cardioprotective effects.[175]

Rio Red, a bud sport mutant having red flesh, is the main grapefruit variety

*Reprinted with permission from "Ethylene degreening modulates health promoting phytochemicals in Rio Red grapefruit." by Chaudhary, P. R.; Jayaprakasha, G. K.; Patil, B. S., 2015, *Food Chemistry. 188*, 77-83. Copyright [2015] Elsevier

grown in the Rio Grande Valley in South Texas. Grapefruit contains several health beneficial secondary metabolites including lycopene, β-carotene, limonoids, flavonoids, ascorbic acid, folic acid, sterols, volatiles and furocoumarins which are influenced by various postharvest treatments.[120] One of the most common postharvest treatment in early season grapefruits and other citrus fruits is ethylene degreening. The gaseous hormone ethylene regulates many physiological responses in plants and is commonly known as the ripening hormone, due to its role in fruit ripening. Cold temperatures, especially minimum night temperatures,[15] trigger ethylene production and initiate normal ripening in citrus. The Star Ruby and Rio Red grapefruit cultivars require temperatures below 13-14 ºC to begin the natural degreening of the peel.[176] During the early season (October), the temperature may not fall below the required level to initiate the color change. In addition, warm temperature interferes with chlorophyll degradation and carotenoid accumulation.[15] Thus, early season grapefruits, harvested in October and November, are often degreened using ethylene gas to change their peel color from green to orange / red. Moreover, ethylene degreening will affect different metabolic pathways and the levels of certain health promoting compounds will vary significantly. In our previous study significant amount of natural compounds were influenced in the edible part of Star Ruby grapefruit after ethylene degreening.[177] Thus, it is imperative to study the influence of ethylene on the health beneficial compounds in the juice sacs of 'Rio Red' grapefruit. Therefore, the main objective of the current study was to investigate the effect of artificial ethylene degreening on the levels of health promoting compounds,

including ascorbic acid, carotenoids, limonoids, flavonoids, and furocoumarins present in ‘Rio Red’ grapefruit juice vesicles.

Materials and methods

Plant materials

Rio Red grapefruits of uniform size were harvested from three different blocks (replications) from a commercial grove in the Rio Grande Valley in South Texas (November 19, 2008).

Chemicals

Reagent-grade butylated hydroxytoluene (BHT), metaphosphoric acid, L-ascorbic acid, lycopene, β-carotene, narirutin, naringin, didymin, poncirin, limonin, and 6’, 7’-dihydroxybergamottin (DHB) were procured from Sigma Aldrich Co. (St. Louis, MO, USA). Sodium hydroxide was purchased from EMD Chemicals (Gibbstown, NJ, USA). Analytical grade solvents were used for quantitative analysis (Fisher Scientific Research, Pittsburgh, PA, USA).

Degreening treatment

Fruits collected from each block (400 fruits) were randomly assigned to two groups of 200 fruits; one group was used for ethylene treatment (degreened fruits) and other was used as a control without any ethylene treatment (non-degreened fruits). Fruits were degreened with 3.5 µL/L (ppm) ethylene for 72 hours at 21 °C and 80% relative humidity in commercial degreening room.

At the packing house, both non-degreened (control) and degreened fruits were passed through treatments consisting of: a dump sprayer line with 0.02% chlorine

buffered at pH 7.0, a high pressure washing system with 0.02% chlorine at pH 7.0, pre-drier brushes, a spray with 0.085 mL/L peracetic acid. The grapefruits were waxed with Decco Pearl Lustr (Decco, Cerexagri Inc. Monrovia, CA) containing 2 g/kg imazalil and 3.5 g/kg thiabendazole and then transferred to the Vegetable and Fruit Improvement Center, Texas A&M University, College Station (Texas, USA). Grapefruits were stored under market-simulated conditions with 3 weeks at 11 °C followed by 2 weeks of storage at 21 °C. Samples were collected every 7 days. Each treatment had three replications containing 200 fruits per replication (fruits collected from 3 different blocks). Furthermore, from each replication, three subsamples were prepared (n = 3 replications × 3 subsamples = 9). In the current study all parameters (except for peel color) had single sample set for day 0 analysis.

Juice sample preparation

Juice subsamples were prepared by blending three peeled grapefruits. The juice samples were stored at -80 ºC until further phytochemical analysis. All phytochemical analysis and fruit quality parameters (except peel color) were conducted on the juice sacs/juice samples of Rio Red grapefruit.

Peel color measurements

The peel color of the non-degreened and degreened fruits was measured with a Minolta CR-400 Chroma Meter (Konica Minolta Sensing, Inc., Osaka, Japan). Before recording the sample measurements, the instrument was calibrated every week, using the white calibration plate (Calibration Plate CR-A43, Minolta Cameras, Osaka, Japan). Peel color was measured for 90 grapefruits (30 fruits per replication, for 3 replications) per

treatment. Fruits were circled with a black marker on their equatorial side (three readings per fruit) and the hue angle was measured within these circles at weekly intervals. The results were expressed as hue angles, with a hue angle of 90º indicating yellow, 60º indicating orange, and 30º indicating red color.[177]

Total soluble solids and titratable acidity

Total soluble solids (TSS) were measured using hand refractometer (American Optical Corp., South Bridge, MA, USA) and expressed as ºBrix. A DL 22 Food and Beverage analyzer (Mettler Toledo, Columbus, OH, USA) was used to measure the titratable acidity of juice. Grapefruit juice (5 mL) was mixed with 50 mL of nanopure water and titrated against 0.1 N NaOH.

Ascorbic acid determination

Ascorbic acid was extracted using meta-phosphoric acid according to our previously developed method.[178] Extracted samples were injected into an HPLC for ascorbic acid determination at 254 nm.[178] Each sample was injected three times and the ascorbic acid contents were expressed as mg/ 100 mL.

Carotenoid analysis

Extraction of carotenoids was done using chloroform, as per our previously established method.[177] BHT was added to chloroform (0.2%) to prevent oxidation of carotenoids. All extractions were conducted in the dark, using yellow light to avoid degradation of carotenoids. An Agilent HPLC 1200 Series (Foster City, CA, USA) system consisting of a solvent degasser, quaternary pump, autosampler, column, oven, and diode array detector was used for quantification. A Gemini 5 µm C-18 column (250

mm × 4.6 mm i.d.) (Phenomenex, Torrance, CA, USA) with a guard cartridge was used for separating carotenoids. Elution was carried out using mobile phase of acetonitrile (A) and isopropyl alcohol (B). Carotenoids were detected at 450 nm and quantified using external standard calibration. Three injections per sample were carried out.

Analysis of limonoids, flavonoids, and furocoumarins

Sample preparation

Each juice sample (10 g) was extracted using 15 mL of ethyl acetate by vortexing and homogenizing for 5 min.[177] The organic layer was separated and the residue was re-extracted twice. All extracts were pooled and the solvent was evaporated to dryness. The dried residue was reconstituted with acetone, filtered using 0.45µm PTFE filter, and further analyzed by HPLC for limonoids, flavonoids, and furocoumarins.

Quantification of limonoids and flavonoids using HPLC

Simultaneous analysis of limonoids and flavonoids was conducted with a Waters HPLC (Milford, MA, USA), spectra model with a PDA detector (2996) coupled with binary HPLC pump 1525 and 717 plus auto sampler. [177] The chromatographic separations were accomplished on a Gemini 5 µm C-18 column (250 mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA). The limonoids were detected at 210 nm and the flavonoids were detected at 280 nm. The entire chromatographic separation was performed with a gradient mobile phase of 0.03 M phosphoric acid (A) and acetonitrile (B) with 1 mL/min flow rate. Each sample was injected three times.

Quantification of furocoumarins using HPLC

Furocoumarin namely, 6,7-dihydroxybergamottin (DHB) was analyzed using a Perkin Elmer HPLC system consisting of a series 200 pump, PDA detector (235C) and autosampler (Perkin-Elmer, Norwalk, CT, USA).[120] The separations were carried out on a C-18, Gemini 5 µm column (250 mm × 4.6 mm i.d.) with a guard cartridge from Phenomenex (Torrance, CA, USA) and the peaks were detected at 320 nm. The mobile phase consisted of 0.03 M phosphoric acid (A) and acetonitrile (B) with 10 µL injection volume at the flow rate of 1 mL/min. The gradient started with 10 % B which was linearly increased to 60% in 7 min and held isocratic for 2 min, 65% in 3 min and held for 1 min, 90% in 7 min and back to initial 10% B in 3 min and equilibrated for 2 min before next injection. Each sample was injected three times.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) using PASW Statistics 18 software (SPSS Inc.). General linear model was used to test significant differences and means were compared using Tukey's HSD test at 5% probability level. The results were expressed as means ± SE.

Results and discussion

Effect of degreening on peel color

Peel color change was measured in present study as it is an important parameter to assess the effect of degreening treatment. Degreening treatment helps increase the degradation of chlorophyll and the accumulation of carotenoids in the peel. The peel color of the fruits was measured as hue angle (**Table 4**); a hue angle of 90º indicates

yellow fruits, 60º indicates orange fruits, and 30º indicates red fruits. In current study grapefruits were divided into two groups a) degreening (with ethylene) and b) non-degreening (control) treatments at the time of harvest (Day 0), and measured for their hue angle at Day 0 and at an interval of 7 days during the storage period. Degreened fruits had a higher hue angle (83.00º ± 1.16) than non-degreened fruits (75.64º ± 1.16) on the day of harvest (Day 0). After degreening with 3.5 µL/L ethylene, the hue angle decreased sharply in degreened fruits (61.58º ± 0.89). Degreened fruits had uniform peel color as compared with non-degreened fruits. Significant differences in peel color were observed at 7 and 14 days of storage. Several other previous studies have reported improvement in peel color of citrus fruits after degreening.[18, 20, 179] In the current study, we observed a significant effect of ethylene degreening on peel color, making the early season degreened fruits more appealing and uniform in color. Since consumers use color as the most important attribute when selecting fruits; this makes it necessary to degreen early-season grapefruits.

Effect of degreening on TSS, total acidity and ripening ratio

We observed no significant effect of ethylene degreening on TSS, total acidity, or ripening ratio (**Table 4**). TSS increased in both treatments during storage, whereas total acidity decreased slightly in both treatments at the end of 35 days of storage. Our previous study in Star Ruby grapefruit gave similar results.[177] Another study on Satsuma mandarin, Star Ruby grapefruit, and navel orange showed that ethylene did not affect TSS, acidity, or ripening ratio [180].

Effect of degreening on ascorbic acid

In the present study, ascorbic acid levels gradually increased during storage in both non-degreened and degreened fruits (**Table 4**). The levels of ascorbic acid were significantly lower in degreened fruits at 7 days (29.5 mg/100 mL) and 21 days (37.76 mg/100 mL), as compared to non-degreened fruits (36.69 mg/100 mL and 39.52 mg/100 mL, respectively). Interestingly, at the end of 35 days of storage, ascorbic acid levels were significantly higher in degreened fruits as compared to non-degreened fruits. However, no significant difference was observed between treatments at 14 and 28 days of storage. Similar results were observed in our previous study in Star Ruby grapefruit, with degreened fruits having less ascorbic acid than controls at 14 days of storage.[177]

Ascorbic acid is one of the most abundant antioxidants in citrus fruits and is affected by various factors such as production system, storage, and harvest time.[104] A previous study by Huang et al. reported an increase in ascorbic acid content in Cara Cara navel oranges during storage.[181] In another study, degreening with ethylene slightly increased L-dehydroascorbic acid in mandarins; however the increase was not significant.[182] An increase in ascorbic acid can be attributed to the increase in transcript levels of the gene encoding L-galactose-1-phosphate phosphatase (*GPP*), which is a key enzyme regulating the ascorbic acid biosynthesis.[183, 184] Ethylene treatment was reported to increase *GPP* transcript levels in tomato.[185] Overall, degreening treatment helped to retain the ascorbic acid contents in Rio Red grapefruit.

Effect of degreening on carotenoids

Carotenoids, namely lycopene and β-carotene, produce the pink/red color of red grapefruit.[79] β-carotene levels increased sharply at 14 days in both treatments (**Table 4**). However, β-carotene content decreased at 21 days, then remained constant during the remaining storage period. β-carotene levels were significantly higher in non-degreened fruits at 7 and 28 days of storage. No significant difference was observed between the treatments at 14, 21, and 35 days of storage.

Lycopene contents showed a similar trend during the storage period (**Table 4**). Lycopene levels increased sharply at 14 days of storage and were significantly higher in non-degreened fruits at 7, 21, and 28 days as compared to degreened fruits. However, the treatments showed no significant difference at 35 days of storage. Lycopene levels in juice vesicles are reported to be higher in early-season grapefruit and decrease as the harvest season progresses.[186] Several previous studies have mainly focused on carotenoids in peel, while only a few have studied the effect of ethylene on juice vesicles. Ethylene treatment was also reported to increase carotenoids in the peel of citrus fruits.[20, 187] In Ponkan mandarin, ethylene treatment preferentially increased the accumulation of orange carotenoids (β-carotene and β-cryptoxanthin) while decreasing the accumulation of yellow carotenoids (lutein, violaxanthin and 9-*cis*-violaxanthin) in the peel.[187] In another study, exogenous ethylene treatment in Satsuma mandarin up-regulated upstream carotenoid biosynthesis genes and carotenoid cleavage dioxygenases, leading to lower violaxanthin content in peel.[188] However, another study observed no effect of ethylene treatment on carotenoids in juice vesicles of Satsuma mandarin.[85] In

Table 4. Effect of ethylene degreening on peel color, TSS, total acidity, and ripening ratio, ascorbic acid, β-carotene and lycopene of Rio Red grapefruit. Grapefruits were stored for three weeks at 11 °C and then transferred to 21 °C for two weeks.

		Storage days					
Parameters	**Treatment**	**0**	**7**	**14**	**21**	**28**	**35**
Peel color (hue angle, in degrees)	**ND**	75.64 ± 1.16 b	69.52 ± 0.88 a	69.58 ± 0.97 a	66.09 ± 0.98 a	66.98 ± 0.95 a	65.69 ± 1.01 a
	DG	83.00 ± 1.16 a	61.58 ± 0.89 b	65.12 ± 0.97 b	63.94 ± 0.98 a	64.62 ± 0.97 a	65.74 ± 1.01 a
TSS (%)	**ND**	9.60 ± 0.15 a	9.73 ± 0.11 a	10.10 ± 0.15 a	9.97 ± 0.34 a	9.96 ± 0.21 a	10.37 ± 0.19 a
	DG	9.60 ± 0.15 a	9.83 ± 0.11 a	10.44 ± 0.15 a	9.63 ± 0.34 a	9.87 ± 0.19 a	10.07 ± 0.19 a
Total acidity (%)	**ND**	1.12 ± 0.04 a	1.01 ± 0.02 a	1.00 ± 0.03 a	0.90 ± 0.04 a	0.97 ± 0.05 a	1.04 ± 0.05 a
	DG	1.12 ± 0.04 a	1.04 ± 0.02 a	1.05 ± 0.03 a	0.96 ± 0.04 a	0.98 ± 0.05 a	0.96 ± 0.05 a
Ripening ratio (TSS/acidity)	**ND**	8.63 ± 0.39 a	9.65 ± 0.24 a	10.10 ± 0.25 a	11.11 ± 0.47 a	10.35 ± 0.43 a	10.05 ± 0.64 a
	DG	8.63 ± 0.39 a	9.52 ± 0.24 a	9.97 ± 0.27 a	10.26 ± 0.47 a	10.19 ± 0.39 a	10.66 ± 0.64 b
Ascorbic acid (mg / 100 mL)	**ND**	34.56 ± 0.36 a	36.69 ± 0.35 a	37.18 ± 0.64 a	39.52 ± 0.57 a	36.97 ± 0.39 a	38.27 ± 0.45 b
	DG	34.56 ± 0.36 a	29.50 ± 0.29 b	38.61 ± 0.64 a	37.76 ± 0.57 b	37.65 ± 0.40 a	39.59 ± 0.46 a
β-carotene (mg/100g fresh wt)	**ND**	2.09 ± 0.05 a	2.49 ± 0.07 a	3.51 ± 0.13 a	2.49 ± 0.08 a	2.49 ± 0.06 a	2.64 ± 0.11 a
	DG	2.09 ± 0.05 a	2.19 ± 0.07 b	3.73 ± 0.14 a	2.42 ± 0.08 a	2.15 ± 0.06 b	2.85 ± 0.11 a
Lycopene (mg/100g fresh wt)	**ND**	2.82 ± 0.06 a	2.85 ± 0.08 a	3.65 ± 0.10 a	2.81 ± 0.06 a	2.45 ± 0.03 a	2.32 ± 0.04 a
	DG	2.82 ± 0.06 a	2.28 ± 0.09 b	3.67 ± 0.11 a	2.33 ± 0.06 b	2.21 ± 0.03 b	2.33 ± 0.05 a

*Data represents means ± S.E, (Peel color: n = 90 fruits; TSS, acidity and ripening ratio: n = 6 juice samples; Ascorbic acid, β-carotene and lycopene: n = 9 juice samples. Each juice sample prepared from three fruits). Different letters denote significant differences ($P < 0.05$) between treatments for each parameter at each storage period. (ND = non-degreened, DG = degreened). All parameters except for peel color had single sample set for day 0 analysis (common samples for both treatments).

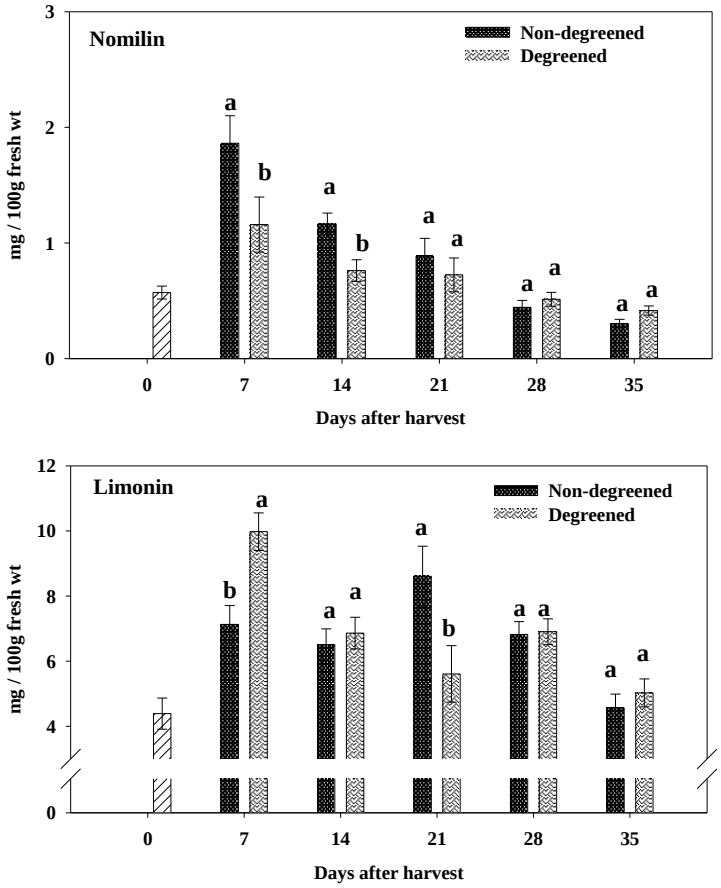

Figure 6. Changes in limonoids in non-degreened and degreened Rio Red grapefruits. Fruits were stored for three weeks at 11 °C and then transferred to 21 °C for two weeks. Data represents means ± S.E. of nine juice samples (n=9), each sample prepared from three fruits. Means with different letters denote significant differences ($P < 0.05$) between treatments at each storage period.

previous study in Star Ruby grapefruit, similar results were observed, with non-degreened fruits having higher carotenoids at 7 and 28 days; however, the treatments showed no significant difference at 35 days of storage.[177]

Effect of degreening on limonoids

Limonoids, triterpenoids commonly found in members of the Rutaceae family, include limonin and nomilin, the bitter-tasting components of citrus fruits.[189] In the current study, nomilin and limonin increased sharply in both treatments at 7 days after harvest (**Figure 6**). Limonin levels were significantly higher in degreened fruits at 7 days, whereas nomilin was significantly higher in non-degreened fruits at 7 and 14 days after harvest. Nomilin levels decreased gradually in both treatments after 7 days of storage, with no significant difference observed between treatments at 21, 28, and 35 days of storage. Limonin in both treatments was higher during storage until 28 days, as compared to initial levels at 0 days. Nevertheless, at 35 days after storage, limonin levels were similar to the initial levels. Limonin was significantly higher in non-degreened fruits at 21 days of storage, but levels of limonin showed no significant difference in both treatments at 14, 28, and 35 days of storage.

In a recent study in Thai pummelo, ethylene treatment had no effect on juice vesicles, due to the thick rind.[190] However, Rio Red grapefruits have thinner rinds than pummelos. Our previous study in Star Ruby grapefruit showed similar increases in levels of limonin and nomilin immediately after storage.[177] In addition, nomilin and limonin levels were higher in degreened fruits during storage of Star Ruby grapefruit. Interestingly, in the present study in Rio Red grapefruit, nomilin was higher in non-degreened fruits and limonin was higher in degreened fruits specifically at 7 days after harvest. However, the ethylene concentration used and the storage intervals differed in both studies. Nomilin is the precursor of limonin and can affect the levels of limonin.[86]

In addition, ethylene is reported to accelerate limonoid metabolism in grapefruit.[164] This is the first study to report the effect of ethylene degreening on limonoids in juice vesicles of Rio Red grapefruit.

Effect of degreening on flavonoids and furocoumarins

Naringin is the major flavonoid in grapefruit, followed by narirutin, poncirin, didymin, and neohesperidin (**Figure 7**). Degreened fruits had significantly higher levels of all flavonoids at 7 days after harvest and more narirutin and poncirin at 14 days, but no significant difference in the levels of naringin, neohesperidin, and didymin compared to controls. In addition, we found no significant difference in levels of narirutin, naringin, and didymin between the treatments at 21, 28, and 35 days after harvest, whereas neohesperidin and poncirin levels were higher in non-degreened fruits. Flavonoids increased sharply at 7 days after harvest, and then gradually decreased up to 21 days, in both treatments. Flavonoids increased again at 28 days in both treatments, possibly resulting from transferring the fruits to room temperature. However, in the second week at room temperature, at 35 days after harvest, flavonoids decreased in both treatments.

Furocoumarins are naturally occurring compounds containing a furan ring attached to coumarin.[124] Furocoumarins in grapefruit interact with some orally administered drugs, increasing their bioavailability.[133] In the current study, 6,7-dihydroxybergamottin (DHB) levels were significantly lower at 7 and 35 days in degreened fruits; however, at 14 and 21 days the DHB contents were significantly higher in degreened fruits (**Figure 8**). This is the first study to report the effect of ethylene

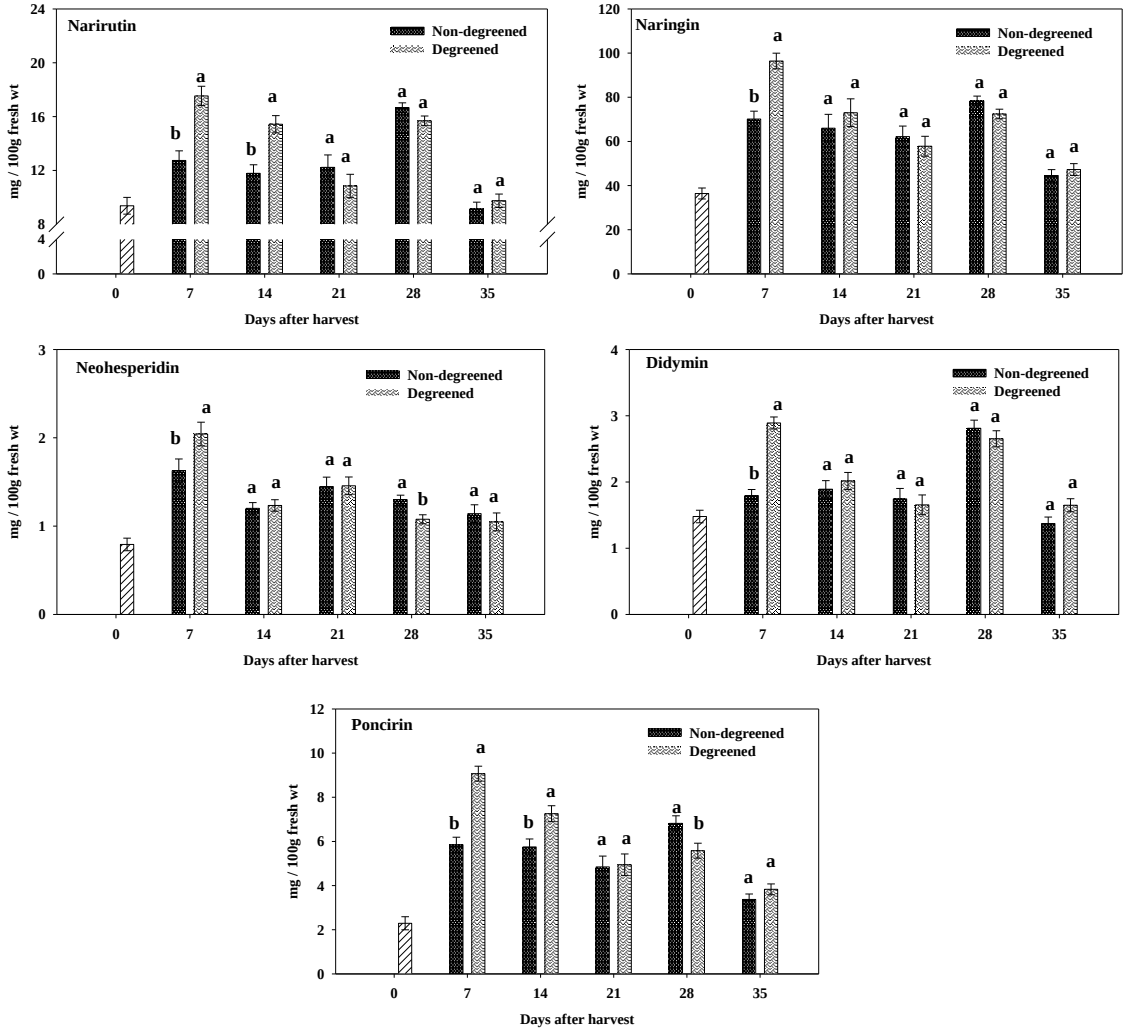

Figure 7. Changes in flavonoids in non-degreened and degreened Rio Red grapefruits. Fruits were stored for three weeks at 11 °C and then transferred to 21 °C for two weeks. Data represents means ± S.E. of nine juice samples (n=9), each sample prepared from three fruits. Means with different letters denote significant differences ($P < 0.05$) between treatments at each storage period.

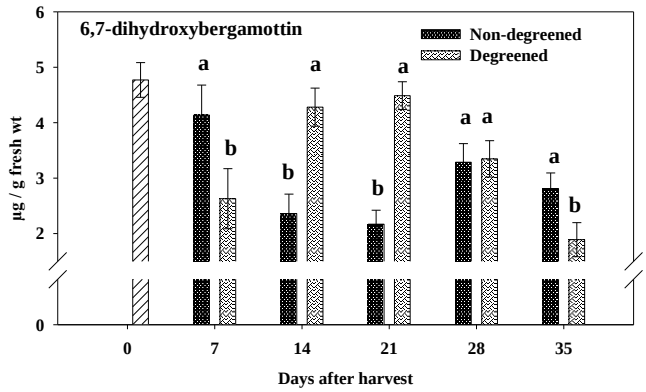

Figure 8. Changes in furocoumarins (6,7-dihydroxybergamottin) in non-degreened and degreened Rio Red grapefruits. Fruits were stored for three weeks at 11 °C and then transferred to 21 °C for two weeks. Data represents means ± S.E. of nine juice samples (n=9), each sample prepared from three fruits. Means with different letters denote significant differences ($P < 0.05$) between treatments at each storage period.

degreening on furocoumarins in Rio Red grapefruit. In Star Ruby grapefruit, degreening increased the levels of furocoumarins during storage.[177]

Interestingly, we observed an inverse relationship between flavonoids and furocoumarins at 7 days after harvest, where degreened fruits had higher content of flavonoids but lower content of furocoumarins. Degreened fruits also showed a delayed increase in furocoumarins at 14 and 21 days, possibly due to an effect of ethylene on the common phenylpropanoid pathway for biosynthesis of flavonoids and furocoumarins. Ethylene treatment increased the levels of the phenylalanine ammonia lyase (PAL) enzyme, which plays crucial role in the phenylpropanoid pathway.[22] However, once the citrus fruits are removed from ethylene gas, PAL returns to its initial levels.[23] The

complicated phenylpropanoid biosynthesis pathway produces flavonoids, furocoumarins, tannins, and lignins, and will require further study to understand effect of ethylene on the enzymes involved in the pathway.

Conclusion

Degreening improved the peel color of grapefruit without affecting total soluble solids or acidity of juice. Degreened fruits had significantly more ascorbic acid after 35 days of storage. Degreening had no significant effect on the levels of carotenoids, limonoids and flavonoids as compared to the non-degreened fruits, at 35 days of storage. However, at 7 days, degreened fruits had more limonin and flavonoids, and less furocoumarin, namely 6,7-dihydroxybergamottin. Overall, ethylene treatment had a significant effect on the phytochemical contents of Rio Red grapefruit, especially at 7 days of storage.

CHAPTER V

LOW TEMPERATURE CONDITIONING REDUCES CHILLING INJURY WHILE MAINTAINING QUALITY AND CERTAIN BIOACTIVE COMPOUNDS OF 'STAR RUBY' GRAPEFRUIT*

Introduction

Quality is an important attribute which influences marketability of fruits and vegetables. Storing fresh produce at low temperature is commonly practiced to enhance storage life, reduce storage losses and to retain the quality of fruits and vegetables by slowing their rate of metabolic activities. However, tropical and subtropical fruits are sensitive to low temperature storage and develop chilling injuries (CI) when stored at low temperature for prolonged period. Among the different citrus fruits, lemons, limes and grapefruits are highly susceptible to CI.[191] The most common symptoms of CI manifested in citrus fruits are internal discoloration, browning of flavedo and albedo, pitting, surface lesions, and water soaked tissues.[30, 192] Grapefruit (*Citrus paradisi* Macf.) develops CI (pitting or brown staining of flavedo) when stored at temperatures below 10 °C. Early season and late season grapefruits are more sensitive to CI as compared to those harvested in the midseason.[193]

Various treatments used in our lab and by others, including the temperature conditioning treatment,[32] intermittent warming,[194] use of different waxes and

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vegetable oils[195] as well as modified atmosphere packaging[30] are reported to reduce CI incidence. Pre-storage temperature conditioning is one of the most common treatments used to prevent the incidence of CI, by increasing the cold stress tolerance. Conditioning treatment of citrus fruits to enhance fruit quality has been extensively studied.[27, 32, 196, 197] In this treatment fruits are cured at relatively higher temperature prior to cold storage. Grapefruits are usually conditioned at either 21 °C for 3 days or at 16 °C for 7 days.[196, 197] Conditioning fruits at 16 °C for 7 days was reported to be better as compared to 21 °C for 7 days in minimizing incidence of CI in grapefruits stored at 1 °C.[197]

Recent study showed that high-temperature conditioning at 37 °C for 1-2 days had no negative effect on flavonoids, vitamin C and antioxidant capacity in the chilling sensitive 'Fortune' mandarin pulp.[37] The treatment was conducted taking into consideration the single (16 days at 1.5 °C) and double (32 days at 1.5 °C) quarantine treatments required for eradication of the Mediterranean fruit fly, with maximum storage period of 32 days. However, influence of low-temperature conditioning (7 days at 16 °C) on the bioactive compounds including limonoids, furocoumarins and carotenoids in 'Star Ruby' grapefruit juice vesicles during prolonged cold storage has not been investigated.

Grapefruit contains diverse class of bioactive compounds such as limonoids, flavonoids, furocoumarins and vitamins. These bioactive compounds help in reducing the risks from various chronic disorders such as cardiovascular diseases, cancer and inflammation, by protecting against the free radicals.[56, 102] Previous studies in our laboratory have demonstrated that limonoids and flavonoids can inhibit the growth of human neuroblastoma,[173] colonic adenocarcinoma cells[173] and oral carcinogenesis.[198] In

addition, limonoids and flavonoids also enhance the levels of phase – II detoxifying enzymes such as glutathione-S-transferase and NAD(P)H: quinone reductase.[199, 200] Therefore, it is essential to understand the influence of storage period, storage temperature and low-temperature conditioning treatment on the contents of bioactive compounds present in grapefruit juice vesicles. The present study reports changes in the levels of bioactive compounds such as ascorbic acid, carotenoids, limonoids, flavonoids and furocoumarins present in the juice vesicles of 'Star Ruby' grapefruit stored for 16 weeks at 11 °C, 2 °C and in preconditioned fruits (7 days at 16 °C) stored at 2 °C. To the best of our knowledge, this is first report on the influence of low-temperature conditioning treatment and prolonged cold storage on limonoids and furocoumarins present in 'Star Ruby' grapefruit juice vesicles.

Materials and methods

Chemicals

The solvents used for the extractions were of ACS-grade, while HPLC-grade solvents were used for quantitative analysis (Fisher Scientific Research, Pittsburgh, PA, USA). Butylated hydroxytoluene (BHT), lycopene, β-carotene, narirutin, naringin, didymin, poncirin, limonin, nomilin, 6', 7'-dihydroxybergamottin (DHB) and bergamottin were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). Deacetyl nomilinic acid glucoside (DNAG) was purified in the lab according to our previously published methods.[156]

Plant material

Star Ruby grapefruits were purchased in April, 2009 from a commercial packinghouse at the Hachula valley in North Israel. The fruits were harvested at optimal maturity and were further washed, sorted, dipped in 1,000 µL L^{-1} Imazalil fungicide and coated with commercial 'Zivdar' polyethylene-based wax emulsion (Safe-Pack Ltd., Kfar Saba, Israel), in the packinghouse, as per the commercial practice.

Storage and conditioning treatment

Grapefruits were divided into three lots, one for each temperature treatment, 11 °C, 2 °C, and conditioning treatment (CD) in which fruits were subjected to 16 °C for 7 days, followed by storage at 2 °C. The grapefruits in all three treatments were stored for period of 16 weeks and the samples were collected at an interval of 4 weeks, with subsequent one week storage at 20 °C to simulate shelf life conditions. Juice samples, prepared by homogenizing three peeled fruits, were used for quality analysis. Additionally, juice samples were lyophilized and sent to the Vegetable and Fruit Improvement Center, Texas A&M University, College Station for quantification of bioactive compounds.

Fruit quality analysis

A model PAL-1 digital refractometer (Atago, Tokyo, Japan) was used to determine the total soluble solids (TSS), while acidity percentages were measured by titration to pH 8.3 with 0.1 M NaOH by means of an automatic titrator model CH-9101 (Metrohm, Herisau, Switzerland). Each measurement comprised of five replications, where each replication was prepared from three fruits.

Color measurements were taken using a Chromo Meter, model CR-200 (Minolta, Tokyo, Japan). Fruits (n = 15 per treatment) were circled with a black marker on their equatorial side, and the peel color within these circles was determined by measuring their hue angle; a hue angle of ~90° represents yellow, ~60° orange, and ~30° red color.

Fruit weight loss was evaluated at an interval of 4 weeks by weighing 10 fruits per treatment before and after the storage, and calculating their percentages of weight loss.

Evaluation of decay and chilling injury

Decay incidence was determined as the number of fruits manifesting decay symptoms (mainly green mold) in each treatment after each storage interval as compared to the total amount of fruit, and expressed as decay percentage. All treatments included three replications, with each replication containing fifteen fruit.

CI was evaluated by sorting the fruit after each storage interval into four categories according to their peel damage severity: none (score 0; no pitting), slight (score 1, a few scattered pits), moderate (score 2; pitting covering up to 30% of the fruit surface), and severe (score 3, extensive pitting covering >30% of the fruit surface). Overall CI incidence was determined as the total number of fruits manifesting CI symptoms in each treatment after each storage interval as compared to the total amount of fruit, and expressed as CI percentage. All treatments included three replications, with each replication containing fifteen fruit.

Sensory analysis

Fruit sensory quality was evaluated at 4 weeks storage intervals with subsequent one week storage in shelf life conditions at 20 °C. Separated segments of peeled grapefruits were

cut into halves and placed into covered glass cups. Each treatment included a mixture of cut segments prepared from five individual fruits. Fruit taste was evaluated by a sensory panel consisting of 10 members; five males and five females, aged between 25 to 62 years. Each panelist assessed the various attributes of three samples, based on an unstructured 100 mm scale, with anchor points 'very weak' and 'very strong' for each attribute. Sensory data were recorded as distances (mm) from the origin. The samples were identified by means of randomly assigned three-digit codes.

Ascorbic acid determination

Ascorbic acid content in fruits was determined by titrating the juice with 2,6-dichlorophenolindophenol[157] and comparing the titration volumes with 0.1% ascorbic acid (Sigma-Aldrich, St. Louis, MO). The results were expressed as mg of ascorbic acid per 100 mL of juice.

Carotenoids analysis

Sample preparation for carotenoid analysis was carried out according to our previously published method.[177] Water (5 mL) was added to the freeze dried juice samples (1 g) in order to increase their extraction efficiency. Reconstituted juice samples were further extracted with 15 mL chloroform containing BHT (0.2 %). Extraction was carried out by vortexing the mixture of sample and chloroform for 2 min. Organic layer was collected and the residue was further extracted twice. The three extracts from each sample were pooled, filtered using Whatman grade 1 paper, and used for HPLC analysis. Volume of the extracts was measured for calculating dilution factor. The extractions were performed in dark using yellow light to avoid degradation of carotenoids.

The Agilent HPLC 1200 Series (Foster City, CA, USA) system consisting of a solvent degasser, quaternary pump, autosampler, column, oven and diode array detector was used for quantification. Carotenoids were eluted through a 250 × 4.6 mm reverse-phase C-18, Gemini 5 µm column with a guard cartridge (Phenomenex, Torrance, CA, USA). Elution was carried out at a flow rate of 0.8 ml/min with a 10 µL injection volume using mobile phase of acetonitrile (A) and isopropyl alcohol (B); gradient started with 50% A at 0 min, 30% A at 7 min, 50% A at 12 min and 50% A at 15 min. Oven temperature was maintained at 15 °C and column was equilibrated for 2 min in between the runs. Carotenoids were detected at 450 nm and quantified using external standard calibration.

Quantification of limonoids, flavonoids and furocoumarins

Sample preparation

Sample preparation for limonoids, flavonoids and furocoumarins was conducted in accordance to our previous published method.[177] Water (5 mL) was added to the freeze dried juice (1 g), and was extracted using 15 mL of ethyl acetate by vortexing for 2 min. Organic layer was separated and the residue was extracted twice. Extracts from each sample were pooled and the solvent was evaporated to dryness. The dried residue was reconstituted with acetone, filtered using 0.45µm PTFE filter and further analyzed for limonoids, flavonoids and furocoumarins using HPLC.

HPLC analysis of limonoids and flavonoids

A Waters HPLC (Milford, MA, USA), spectra model consisting of a PDA detector (2996) coupled with binary HPLC pump 1525 and 717 plus auto sampler was

used for quantification of limonoids and flavonoids. The chromatographic separations were accomplished on a C-18, 5 µm Gemini column (250mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA). Limonoids were detected at 210 nm while flavonoids were detected at 280 nm. Data analysis was carried out using Empower pro software. The entire chromatographic separation was performed at a gradient mobile phase of 0.03 M phosphoric acid (A) and acetonitrile (B) with 1 mL/min flow rate. Each sample was injected thrice and each treatment was triplicated.

HPLC analysis of furocoumarins

Furocoumarins were analyzed using our previous method with slight modifications.[120] Perkin Elmer HPLC system consisting of series 200 pump, a PDA detector (235C) and an autosampler (Perkin-Elmer, Norwalk, CT, USA) was used for analysis. The separations were carried out on a C-18, 5 µm Gemini column (250mm × 4.6 mm i.d.) with a guard cartridge from Phenomenex (Torrance, CA, USA). Peaks were detected at 320 nm and the data was integrated by Turbochrom software (Perkin-Elmer, Norwalk, CT, USA). Gradient mobile phase consisted of 0.03 M phosphoric acid (A) and acetonitrile (B) with 10 µl injection volume at the flow rate of 1 mL/min. Each sample was analyzed thrice and each treatment was triplicated.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) using PASW Statistics 18 software (© SPSS Inc. 2009). General linear model was used to test significant differences and the sample means were compared using Tukey's HSD

test at 5% significance level. The results are expressed as means ± SE (unless otherwise stated).

Results and discussion

Visual appearance and fruit color

At the beginning of storage (0 weeks), the fruits were sound, attractive and had external yellow/red color. During prolonged storage at 11 °C, grapefruits became orange/red and appeared to be over-ripe, mainly after 12 and 16 weeks of storage. In contrast, fruits stored at the low temperature of 2 °C, either with or without application of conditioning treatment, remained fresh, attractive and retained their original yellow/red color during the entire storage period. Unlike the marked differences in the external appearance of grapefruits, no significant difference was observed in the internal color following the storage at various temperature regimes. The observed external color change at 11 °C can be attributed to development of carotenoids at intermediate temperatures. Previous reports have shown that temperatures ranging between 15-25 °C are optimum for development of carotenoids in citrus peels.[18] In Palmer navel orange intermediate temperatures between 11 – 15 °C showed better carotenoid accumulation as compared to lower temperatures.[201]

Total soluble solids and acid percentages

The TSS levels of 'Star Ruby' grapefruits were 10.5% at harvest and remained more or less constant during the entire 16 weeks of storage period (**Table 5**). In contrast, acidity levels decreased from 1.3% at harvest to 0.9-1.0% after prolonged storage.

Overall, the observed changes in TSS and acidity levels resulted in a slight increase in the ripening

Table 5. Effect of storage temperature and duration on TSS, acidity, ripening ratio and flavor of 'Star Ruby' grapefruit. Fruits were stored for 4, 8, 12 and 16 weeks at 11 or 2 °C or conditioned (CD) and then transferred to shelf life conditions at 20 °C for 1 week.

	Storage duration (weeks)				
	0	**4**	**8**	**12**	**16**
TSS (%)					
11 °C	10.5 ± 0.1 a	11.3 ± 0.3 a	9.3 ± 0.2 b	10.3 ± 0.3 a	10.1 ± 0.4 a
2 °C	10.5 ± 0.1 a	10.6 ± 0.3 a	10.2 ± 0.2 a	10.3 ± 0.3 a	10.5 ± 0.4 a
CD	10.5 ± 0.1 a	11.2 ± 0.3 a	9.0 ± 0.2 b	10.2 ± 0.3 a	10.6 ± 0.4 a
Acidity (%)					
11 °C	1.3 ± 0.1 a	1.1 ± 0.1 a	1.2 ± 0.1 a	1. 0 ± 0.1 a	0.9 ± 0.1 a
2 °C	1.3 ± 0.1 a	1.1 ± 0.1 a	1.2 ± 0.1 a	1.0 ± 0.1 a	1.0 ± 0.1 a
CD	1.3 ± 0.1 a	1.0 ± 0.1 a	1.2 ± 0.1 a	1.0 ± 0.1 a	1.0 ± 0.1 a
TSS/acid ratio					
11 °C	8.1 ± 0.4 a	10.4 ± 0.3 a	7.9 ± 0.5 a	10.6 ± 0.4 a	11.3 ± 1.0 a
2 °C	8.1 ± 0.4 a	9.6 ± 0.3 a	8.5 ± 0.5 a	9.8 ± 0.4 a	10.5 ± 1.0 a
CD	8.1 ± 0.4 a	10.8 ± 0.3 a	8.6 ± 0.5 a	10.0 ± 0.4 a	11.2 ± 1.0 a
Taste score (0-10)					
11 °C	9.0 ± 0.0 a	8.2 ± 0.4 a	6.5 ± 0.4 b	6.3 ± 1.0 b	6.1 ± 1.2 b
2 °C	9.0 ± 0.0 a	8.4 ± 0.4 a	8.0 ± 0.4 a	7.1 ± 1.2 ab	7.0 ± 0.9 ab
CD	9.0 ± 0.0 a	8.4 ± 0.4 a	8.0 ± 0.4 a	7.9 ± 1.2 a	7.9 ± 0.7 a

Data are expressed as means ± S.E. Means with same letter indicate no significant differences between treatments ($P < 0.05$).

ratio from 8.1 at harvest up to 11.3 after 16 weeks of cold storage. No significant differences were observed in the TSS, acidity and ripening ratio among all three treatments during the 16 weeks storage period; except at 8 weeks where grapefruits stored at 2 °C had significantly higher *(P< 0.05)* TSS content than the other two treatments.

Decay and chilling injury

One of the most important objectives during postharvest storage is to reduce the storage loss by minimizing decay development. It was found that grapefruits stored at the optimum temperature of 11 °C, showed a gradual increase in the decay development reaching 4, 11 and 24% after 8, 12 and 16 weeks, respectively (**Figure 9**). In contrast, grapefruits stored at the low temperature of 2 °C, with or without application of conditioning treatment, had remarkable reduction in decay development, which did not exceed 4% at any time during storage (**Figure 9**).

To reduce appearance of CI, grapefruit is usually stored at a minimum safe temperature of 11°C. The CI symptoms, visualized as dark sunken areas of collapsed peel tissue, were negligible or absent during prolonged storage at 11°C (**Figure 9**). However, CI increased to 24% during prolonged storage at the low temperature of 2 °C by the end of 16 weeks of storage. Application of a pre-storage conditioning treatment (7 days at 16 °C) before transferring to cold storage was effective in reducing development of CI to 0, 2, 4 and 12% after 4, 8, 12 and 16 weeks of cold storage, respectively (**Figure 9**). The CI was higher in non-conditioned fruits stored at 2ºC than CD fruits, whereas fruits stored at 11 ºC showed no CI symptoms. Overall, our current

results are consistent with other previous studies which also demonstrate reduction in incidence of CI in CD fruits during the cold storage.[32, 197]

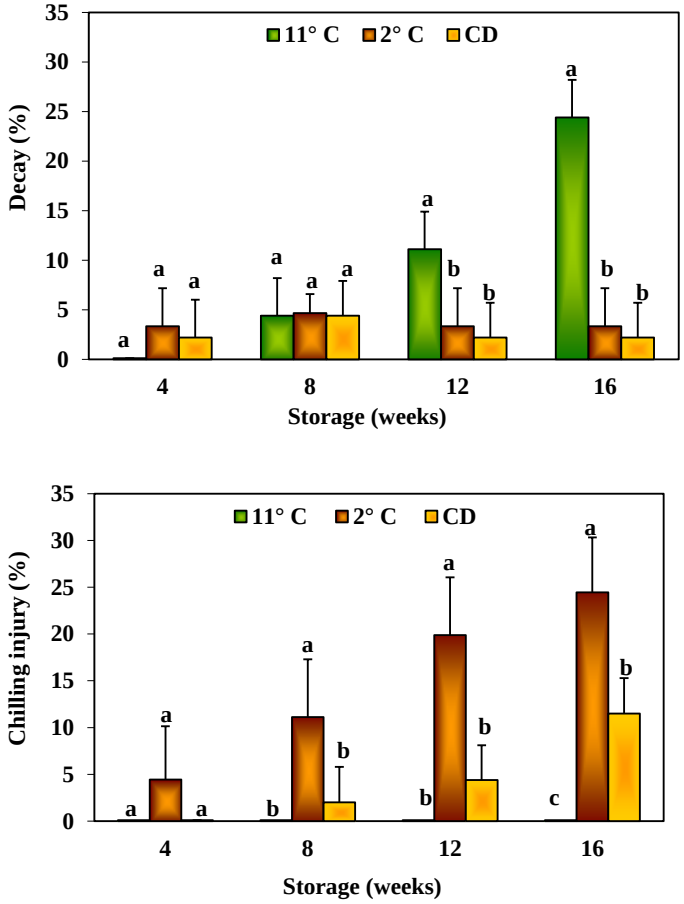

Figure 9. Effects of storage temperatures and duration on development of decay and chilling injuries in 'Star Ruby' grapefruit. Fruits were stored for 4, 8, 12 or 16 weeks at 11 °C or 2 °C or conditioned (CD) and then transferred to shelf life conditions at 20 °C. Data are means ± S.D. of three replications, each including fifteen fruit. Means with same letter indicate no significant differences between treatments ($P < 0.05$).

Fruit taste preference

The grapefruits used in the current experiment had relatively low acid levels and were rated as very tasty at the day of harvest (taste score = 9) (**Table 5**). Fruits stored at 11 °C, remained tasty upto 4 weeks, however fruit taste preference decreased to 6.5, 6.3 and 6.1 after 8, 12 and 16 weeks, respectively. At 2 °C, the fruits remained tasty upto 8 weeks (taste score ≥ 8); nevertheless, the taste score decreased to 7.1, 7.0 after 12 and 16 weeks of storage respectively. The CD fruits remained tasty during the entire 16 weeks of postharvest storage period. To summarize, the conditioning treatment helped to retain fruit taste during the entire storage period; unlike the fruits stored at 11 °C and 2 °C, which retained taste only up to 4 and 8 weeks, respectively.

Ascorbic acid

Ascorbic acid content of 'Star Ruby' grapefruits at beginning of storage was 28 mg/100 ml of juice (**Figure 10**), which was retained during the first 4 weeks of cold storage. However, ascorbic acid levels sharply declined in fruits stored at 11 °C to 21, 14 and 12 mg/100 mL of juice after 8, 12 and 16 weeks, respectively. On the other hand, ascorbic acid levels in fruits stored at 2 °C, with or without application of conditioning treatment, remained stable (~28 mg/100 ml juice) up to 8 weeks after harvest. Nevertheless, ascorbic acid levels in fruits stored at 2 °C and CD fruits decreased markedly, with no significant differences observed among the three treatments after 16 weeks of storage (**Figure 10**).

Ascorbic acid is reported to be temperature sensitive and more specifically decreases at high temperatures as well as with increasing postharvest storage period.[60,

[162] Our results are in accordance with the previous report, demonstrating that the low-temperature storage (2 °C with or without conditioning treatment) helped to retain ascorbic acid for a longer period.[202]

Carotenoids

Levels of both β – carotene and lycopene were significantly higher (P< 0.05 %) in fruits stored at 11 °C than those stored at 2 °C and CD fruits after 16 weeks of storage (**Figure 10**). β-carotene was lower in CD fruits after 4 and 8 weeks of storage than the other two treatments; whereas, no significant difference was observed between fruits stored at 11 °C and 2 °C (**Figure 10**). After 16 weeks of storage increase in the levels of β-carotene was observed in fruits stored at 11 °C and CD fruits; while at 2 °C, the levels of β-carotene decreased. On the other hand, lycopene levels decreased in all the three treatments with increase in the storage period. The CD fruits had lower levels of lycopene at 4 and 8 weeks after storage than the other two treatments. No significant difference was observed between fruits stored at 11 °C and 2 °C at 4 weeks after storage; however, lycopene levels were higher in fruits stored at 11 °C than those stored at 2 °C at 8 weeks. Recent study reported decrease in levels of carotenoids in juice sacs of Satsuma mandarins stored at 5 °C than those stored at 20 °C; therefore, suggesting the accumulation of carotenoids in citrus species to be temperature sensitive and tissue specific [85] . Additionally, our results provide further evidence that β-carotene (at 16 weeks) and lycopene levels (at 8 and 16 weeks) were higher in fruits stored at 11°C in comparison to fruits stored at 2 °C and CD fruits.

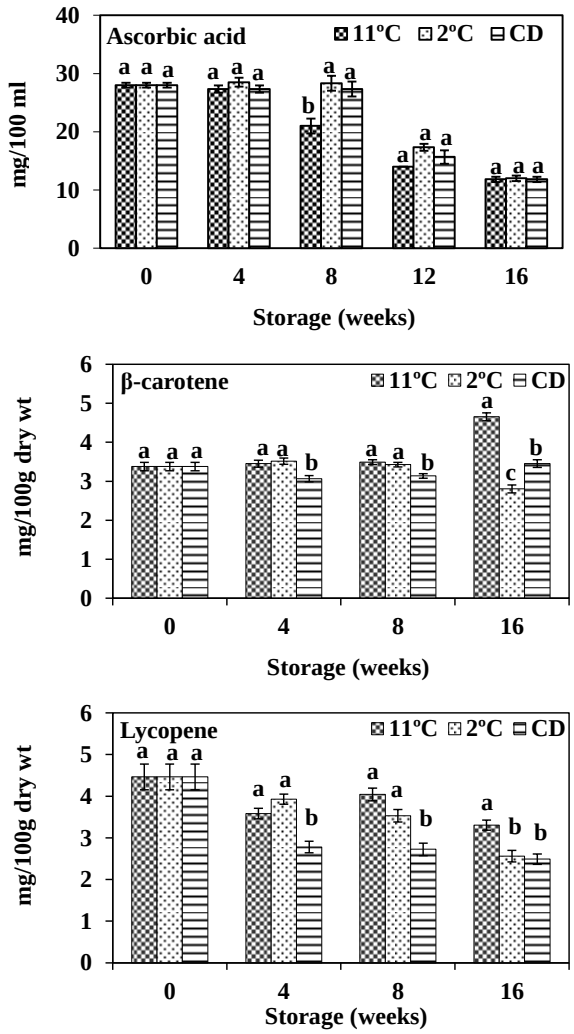

Figure 10. Levels of ascorbic acid, β-carotene and lycopene in juice of Star Ruby grapefruit at different storage temperatures. Fruits were stored for 4, 8, 12 or 16 weeks at 11 or 2 °C or conditioned (CD) and then transferred to shelf life conditions at 20°C. Data are means ± S.E. of three replications, each replication prepared from three individual fruits. Means with same letter indicate no significant differences between treatments ($P < 0.05$).

Limonoids

Limonoids such as deacetyl nomilinic acid glucoside (DNAG), limonin and nomilin were quantified and expressed as mg/ 100 g dry weight. DNAG was significantly higher (P<0.05) in fruits stored at 11 °C after 16 weeks of storage as compared to fruits stored at 2°C and CD fruits (**Figure 11**). However, no significant difference was observed between fruits stored at 2 °C and CD fruits after 16 weeks of storage. Two fold increases in DNAG levels was observed after 16 weeks of storage as compared to initial levels in fruits stored at 11 °C, while DNAG levels were retained in fruits stored at 2 °C and CD fruits after 16 weeks of storage period.

Limonin content in all the three storage treatments decreased during storage period of 16 weeks. Limonin content of fruits stored at 2 °C (77.29 mg/ 100 g) was significantly higher (P< 0.05) after 16 weeks of storage than the fruits stored at 11 °C (45.19 mg/ 100 g) and CD fruits (46.31 mg/ 100 g), with no significant difference observed between fruits stored at 11 °C and CD fruits (**Figure 11**). Nomilin levels sharply decreased from the beginning of storage (0 weeks) to 4 weeks of storage in all three treatments, with no significant difference observed among the three treatments during the entire storage period (**Figure 11**). This is first study to report the effect of low-temperature conditioning on limonoids in comparison with storage temperature of 11 °C and 2 °C.

Flavonoids and furocoumarins

Flavonoids such as narirutin, naringin, didymin and poncirin were quantified and expressed as mg/ 100 g dry weight (**Figure 12**). All flavonoids were significantly higher

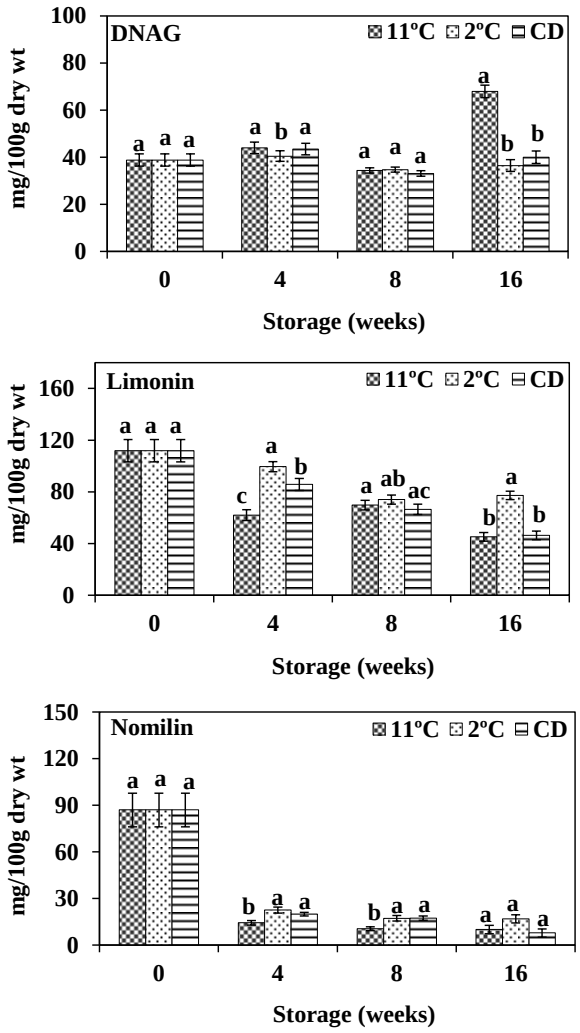

Figure 11. Variations in limonoids content of 'Star Ruby' grapefruit. Fruits were stored for 4, 8 and 16 weeks at 11 °C, 2 °C or conditioned (CD) and then transferred to shelf life conditions at 20 °C for 1 week. Data are means ± S.E. of three replications, each replication prepared from three individual fruits. Means with same letter indicate no significant differences between treatments ($P < 0.05$).

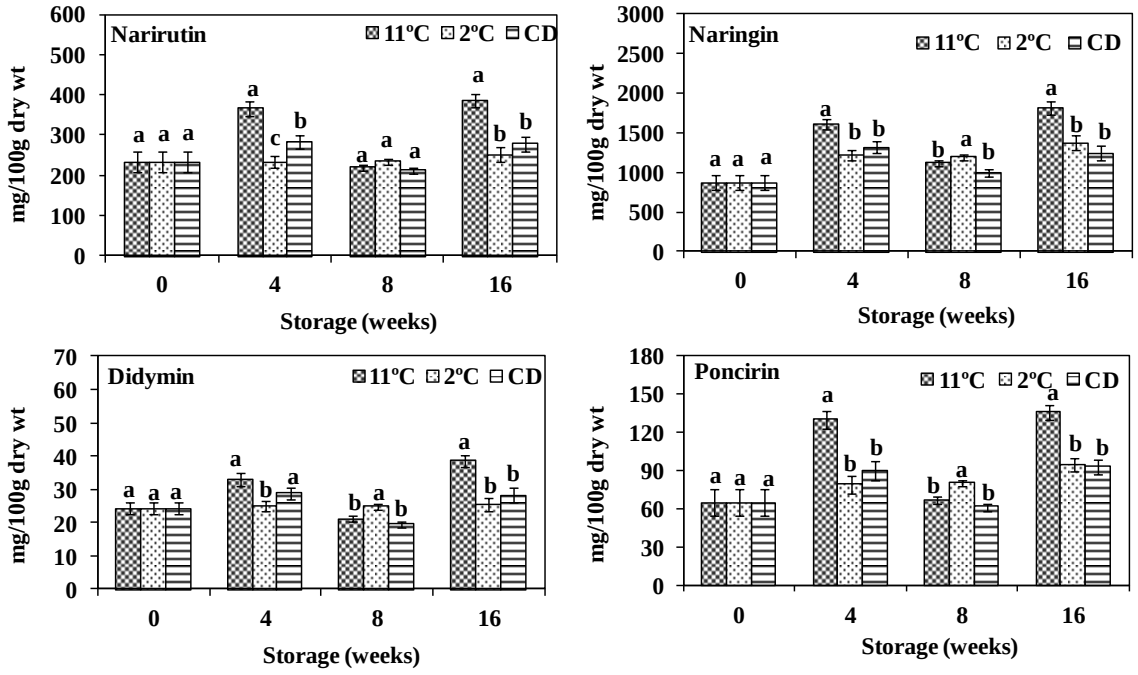

Figure 12. Influence of storage temperature and duration on flavonoids content in juice of 'Star Ruby' grapefruit. Fruits were stored for 4, 8 and 16 weeks at 11 °C or 2 °C or conditioned (CD) and then transferred to shelf-life conditions at 20 °C for 1 week. Data are means ± S.E. of three replications, each replication prepared from three individual fruits. Means with same letter indicate no significant differences between treatments ($P< 0.05$).

($P< 0.05$) in the fruits stored at 11 °C after 4 and 16 weeks of storage, except didymin, which was higher in the fruits stored at 11 °C only at 16 weeks. The CD fruits had higher narirutin and didymin than the fruits stored at 2 ºC at 4 weeks after storage. However, levels of naringin, didymin and poncirin increased in the fruits stored at 2 ºC at 8 weeks

of storage than the other two treatments. No significant difference was observed in flavonoids content between fruits stored at 2 ºC and CD fruits after 16 weeks storage. Overall, the levels of flavonoids were higher in all three treatments at the end of 16 weeks storage period as compared to initial levels. This increase in flavonoids could be

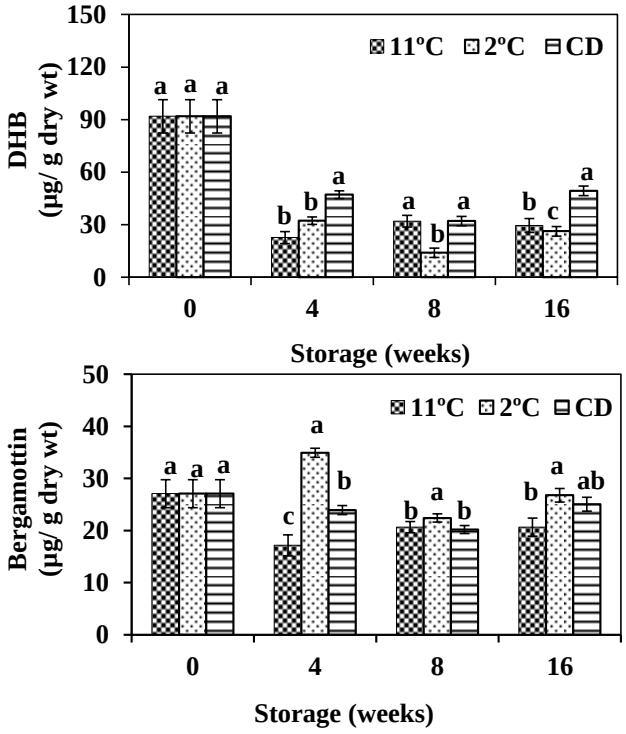

Figure 13. Furocoumarins - DHB (6',7'-dihydroxybergamottin) and bergamottin levels in juice of 'Star Ruby' grapefruits stored for 4, 8 and 16 weeks at 11 °C, 2 °C or conditioned (CD) with subsequent 1 week storage at 20 °C. Data are means ± S.E. of 3 replications, each prepared from 3 different fruits. Means with same letter indicate no significant differences between treatments ($P < 0.05$).

attributed to changes in phenylalanine ammonia-lyase (PAL) activity and temperature stress. The CI incidence is reported to increase ethylene production and PAL activity in citrus peels which are considered as defensive mechanisms during the development of CI symptoms.[38, 203] Citrus fruits exposed to conditioning treatment had higher PAL activity in peels both during conditioning treatment as well as after being transferred to the low temperatures.[42]

Furocoumarins such as 6', 7'-dihydroxybergamottin (DHB) and bergamottin were also quantified and the results were expressed as µg/ g dry weight (**Figure 13**). A significant difference was observed after 4 weeks where CD fruits had significantly higher levels (47.1 µg/ g) of DHB, whereas the fruits stored at 11 °C and 2 °C had 32.3 µg/ g and 22.6 µg/ g respectively (**Figure 13**). After 16 weeks of storage CD fruits had significantly higher levels of DHB with no significant difference observed between fruits stored at 11 °C and 2 °C. The levels of DHB decreased significantly during storage in all the three treatments. On the other hand, bergamottin levels were significantly ($P< 0.05$) higher in fruits stored at 2 °C after 4 weeks than the other treatments, while significant difference was observed between fruits stored at 11 °C and CD fruits after 4 weeks of storage. At the end of 16 weeks of storage, bergamottin levels were similar in the fruits stored at 2 °C and CD fruits, while the fruits stored at 11 °C had lower levels of bergamottin (**Figure 13**).

In phenylpropanoid pathway, PAL is a common precursor for both flavonoids and furocoumarins, which are synthesized from separate pathways branching out from 4-coumaroyl-CoA.[139] Elicitor treated parsley showed PAL induction; however, acetyl-Co-A carboxylase and chalcone synthase (the enzymes involved in flavonoid pathway) were not induced.[142] In addition, elicitor treatment induced dimethylallyldiphosphate:

Umbelliferone dimethylallyltransferase, which is involved in coumarins pathway [142]. On the other hand, UV-light is reported to induce PAL and the enzymes involved in flavonoids pathway, but not the enzymes involved in coumarins pathway.[204] Therefore, cold storage and low-temperature conditioning can also induce different responses in flavonoid and furocoumarins pathway.

Conclusion

Our results implicate that commercial application of low-temperature conditioning treatment is recommended to improve postharvest storage performance and to maintain certain bioactive compounds during prolonged cold storage of grapefruit. However, for a short storage period, 11 °C temperature is preferable.

CHAPTER VI

EFFECT OF STORAGE TEMPERATURE AND LOW-TEMPERATURE CONDITIONING ON HEALTH PROMOTING COMPOUNDS IN 'RIO RED' GRAPEFRUIT*

Introduction

The Mexican fruit fly (*Anastrepha ludens* Loew) is a quarantine pest of citrus, and lays eggs inside the fruit. The larvae feed on the fruit flesh and their presence limits marketing and export of the citrus fruits. To be exported, fruits must meet phytosanitary requirements and undergo quarantine treatments such as fumigation with methyl bromide,[205] heat treatment,[206] cold treatment,[207] or irradiation.[208] However, methyl bromide is a class I ozone-depleting chemical, indicating the need for alternative techniques. Low-temperature quarantine treatment, where fruits are stored at a temperature below the thermal tolerance of the pests, provides a good alternative. Temperature also affects respiration and other metabolic activities in fruits and vegetables, possibly affecting their quality and shelf-life.

Grapefruit must be stored below 5 °C for disinfestation; however, certain tropical and subtropical fruits, develop chilling injury (CI) when stored at low temperature. Various factors affect the incidence of CI in citrus, such as variety, harvest time, fruit maturity, temperature, and waxing.[209] For example, a previous study reported that an increase in temperature during the winter increased the chilling tolerance in grapefruits

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after harvest.[210] Also, fruits harvested from the exterior of the canopy are more susceptible to CI.[211] The main factor affecting CI of grapefruit is moisture loss from the fruits[211] and therefore, treatments that reduce moisture loss, such as waxing,[212] can reduce the incidence of CI.

Temperature conditioning before the fruits are stored at low temperature can also prevent CI. The influence of conditioning treatment on citrus peels and fruit quality has been studied extensively.[41, 213, 214] Porat et al reported conditioning grapefruit at 16 °C for 7 days before cold storage effectively minimizes chilling injury.[32, 215] Several studies on low temperature conditioning treatment effect on molecular mechanisms involved in chilling tolerance have been conducted.[215, 216] However, the effect of low temperature conditioning on Rio Red grapefruit health promoting compounds present in juice vesicles has not been investigated. The natural compounds predominantly present in grapefruit are ascorbic acid, carotenoids, limonoids, flavonoids, and furocoumarins; dietary intake of these health promoting compounds reportedly reduces the risks of certain chronic diseases.[217] Therefore, maintaining the levels of these compounds during postharvest storage has important implications for human health. Studies on the effect of temperature and storage period on the natural compounds in the juice vesicles, as well as on the maintenance of quality of grapefruit, will provide key information for maintaining phytochemical contents. The present study investigated the influence of cold storage and low-temperature conditioning on levels of health promoting compounds in Rio Red grapefruit, examining levels of ascorbic acid, carotenoids, limonoids, flavonoids, furocoumarins, total phenolics, and radical-scavenging activity during a 12 week

storage period.

Materials and methods

Plant material

Rio Red grapefruits of uniform size were harvested on February 10, 2010 from three different blocks (250 fruits from each block) from a commercial grove in the Rio Grande Valley in South Texas.

Chemicals

Sodium hydroxide, L-ascorbic acid, butylated hydroxytoluene (BHT), lycopene, β-carotene, narirutin, naringin, neohesperidin, didymin, poncirin, limonin, 6', 7'-dihydroxybergamottin (DHB), catechin, Folin-Ciocalteu reagent, sodium carbonate, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma Aldrich Co. (St. Louis, MO, USA). Analytical grade solvents were obtained from Fisher Scientific Research (Pittsburgh, PA, USA).

Treatment and storage

Approximately 250 grapefruits were harvested from each block (replication) and were further divided into three lots of 80 fruits for each temperature treatment, 11 °C, 5 °C, and conditioning treatment (CD) in which fruits were conditioned at 16 °C for 7 days and then stored at 5 °C. Fruits in all the three treatments were stored for 12 weeks at 90% relative humidity. Three juice subsamples were collected at an interval of three weeks from each replication. Juice samples (subsamples) were prepared by blending three peeled grapefruits and were stored at -80 °C until further analysis (n=9 per treatment, 3 replications × 3 subsamples).

Total soluble solids and total acidity

Total soluble solids (TSS) was measured using a hand refractometer (American Optical Corp., South Bridge, MA, USA) and results were expressed as °Brix. A DL 22 Food and Beverage analyzer (Mettler Toledo, Columbus, OH, USA) was used to measure the total acidity of juice. Grapefruit juice (5 mL) was mixed with 50 mL of nanopure water and titrated with 0.1 N sodium hydroxide and total acidity was expressed as percent citric acid. Ripening ratio was calculated as the ratio of TSS/total acidity. Each treatment had three replications containing two samples each (n=6).

Chilling injury index

Chilling injury was evaluated and expressed as CI index.[32] Grapefruits were sorted into four groups based on their severity of CI: score 0 (no pitting), score 1 (a few scattered pits), score 2 (pitting covering up to 30% of the fruit surface), and score 3 (extensive pitting covering more than 30% of the fruit surface). The CI index for each treatment was further calculated by multiplying the number of fruits in each category by their score and dividing the sum of each treatment by the total number of fruits assessed. All treatments included three replications, each containing 10 fruits (n=30).

Ascorbic acid determination

Ascorbic acid was extracted and quantified using liquid chromatography according to previous method.[178] Each sample was analyzed three times and the ascorbic acid contents were expressed as mg/100 mL juice.

Carotenoids analysis

Extraction of carotenoids was performed according to a previously published method with slight modifications.[177] Juice samples (10 g) were extracted using chloroform (15 mL) containing BHT (0.2%). An Agilent HPLC 1200 Series (Foster City, CA, USA) system consisting of a solvent degasser, quaternary pump, autosampler, column, oven, and diode array detector was used for quantification. A C-18, Gemini 5 µm column (250 mm × 4.6 mm i.d.) with a guard cartridge was used (Phenomenex, Torrance, CA, USA). Elution was carried out using a gradient mobile phase of acetonitrile (A) and isopropyl alcohol (B). Carotenoids were detected at 450 nm and quantified using external standard calibration.

Quantification of limonoids, flavonoids, and furocoumarins

Sample preparation

Extraction was carried out according to our previously published method with slight modification.[177] Each juice sample (10 g) was extracted using 15 mL of ethyl acetate on a shaker for 3 hours. The organic layer was separated and the residue was extracted twice. All extracts were pooled and the solvent was evaporated to dryness. The dried residue was reconstituted with 4 mL acetone, filtered using a 0.45 µm PTFE filter, and further analyzed for limonoids, flavonoids and furocoumarins using HPLC.

Quantification of limonoids and flavonoids using HPLC

Limonoids and flavonoids were quantified simultaneously using Waters HPLC (Milford, MA, USA), spectra model with a PDA detector (2996) coupled with a binary HPLC pump 1525 and 717 plus auto sampler. The chromatographic separations were

conducted on a C-18, Gemini 5 µm column (250 mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA). Limonoids were detected at 210 nm and flavonoids were detected at 280 nm. The entire chromatographic separation was performed with a gradient mobile phase of 0.03 M phosphoric acid (A) and acetonitrile (B) with 1 mL/min flow rate. Each sample was injected three times.

Quantification of furocoumarins using HPLC

Furocoumarins were analyzed using our previously described method.[177] Each sample was analyzed in triplicate and the results were expressed as µg/100 g fresh weight.

Determination of total phenolics and radical-scavenging activity

Sample preparation

Juice samples (10 g) were extracted twice with 20 mL methanol on a shaker for three hours. The extracts from each sample were pooled, filtered using Whatman grade 1 filter paper, and further used for quantification of total phenolics and radical-scavenging activity using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Volume of the extracts was measured for calculating dilution factor.

Total phenolics

The total phenolics contents of methanol extracts were determined using our previously published method and the results were expressed as catechin equivalents.[177]

Radical-scavenging activity

Radical-scavenging activity of Rio Red grapefruit methanol extracts was measured according to our previously published method, using the 2,2-diphenyl-1-

picrylhydrazyl (DPPH) assay.[177] Radical-scavenging activity was expressed as mg of ascorbic acid equivalent per g of fresh sample weight.

Statistical analysis

One way analysis of variance (ANOVA) was performed using PASW Statistics 18 software (SPSS Inc.). A general linear model was used to test significant differences and means were compared using Tukey's HSD test at 5% probability level. The results were expressed as means ± SE.

Results and discussion

Total soluble solids and titratable acidity

In the current study, we observed no significant effect of storage temperature on total soluble solids (**Table 6**), which remained constant in all three treatments throughout the storage period. We observed a slight decrease in total acidity in all three treatments with increasing storage period. Consequently, ripening index in all treatments increased with storage, due to the slight decrease in acidity (**Table 6**). A decrease in total acidity during storage is commonly observed and is attributed to consumption of organic acids for energy production.[218] Previous studies showed similar results, where temperature and storage did not affect TSS (sugar), but reduced the acidity.[32] The sugar-to-acidity ratio (ripening index) is one of the most important factors influencing the taste, and determining the harvest time of the fruits.

Incidence of chilling injury

Grapefruit develops CI, which manifests as pitting or brown staining of the flavedo when stored below 10 °C (**Figure 14**). In current study, no CI symptoms were

seen stored at 5 °C had severe CI with CI indices of 0.33, 0.63, 1.43, and 1.83 at 3, 6, 9, and 12 weeks of storage, respectively (**Figure 15**). Conditioning grapefruits at 16 °C for 7 days prior to cold storage significantly reduced and delayed the incidence of CI. Conditioned fruits (CD) showed no CI symptoms until 9 weeks of storage and had significantly lower CI indices (0.30 and 0.73 at 9 and 12 weeks of storage, respectively) as compared to non-conditioned fruits stored at 5 °C. CD fruits had CI indices nearly 4.5- and 2.5-fold lower those of non-conditioned fruits stored at 5 °C at 9 and 12 weeks of storage, respectively. Our results agree with previous studies where temperature conditioning delayed and reduced CI in Star Ruby grapefruit.[27, 219] In conclusion, conditioned fruits can be stored up to 9 weeks with minimal incidence of CI.

Table 6. TSS, acidity and TSS/Acidity ratio (ripening ratio) of Rio Red grapefruit stored for 0, 3, 6, 9 and 12 weeks at 11 °C, 5 °C, or conditioned (CD)

	Storage duration (weeks)				
	0	**3**	**6**	**9**	**12**
TSS (%)					
11°C	11.70 ± 0.11 a	11.20 ± 0.22 a	11.27 ± 0.27 a	11.23 ± 0.23 a	11.30 ± 0.20 a
5 °C	11.70 ± 0.11 a	11.36 ± 0.24 a	11.23 ± 0.27 a	11.37 ± 0.23 a	11.67 ± 0.20 a
CD	11.70 ± 0.11 a	11.73 ± 0.22 a	11.63 ± 0.27 b	11.6 ± 0.23 a	11.97 ± 0.20 a
Acidity (%)					
11 °C	1.03 ± 0.02 a	0.96 ± 0.05 a	0.96 ± 0.05 a	0.95 ± 0.05 a	0.85 ± 0.05 a
5 °C	1.03 ± 0.02 a	1.06 ± 0.06 a	1.00 ± 0.05 a	1.01 ± 0.05 a	0.92 ± 0.05 a
CD	1.03 ± 0.02 a	1.02 ± 0.05 a	0.99 ± 0.05 a	0.99 ± 0.06 a	0.92 ± 0.05 a
Ripening index					
11 °C	11.4 ± 0.32 a	11.68 ± 0.36 a	11.76 ± 0.39 b	11.87 ± 0.38 a	13.51 ± 0.61 a
5 °C	11.4 ± 0.32 a	10.89 ± 0.40 a	11.34 ± 0.39 a	11.39 ± 0.38 a	12.89 ± 0.61 a
CD	11.4 ± 0.32 a	11.65 ± 0.36 a	11.79 ± 0.39 a	11.81 ± 0.46 a	13.09 ± 0.61 a

*Data represent means ± S.E. of three replications, each replication containing two samples prepared from three individual fruits (n=6). Means with different letters indicate significant differences between treatments for each time period ($P< 0.05$).

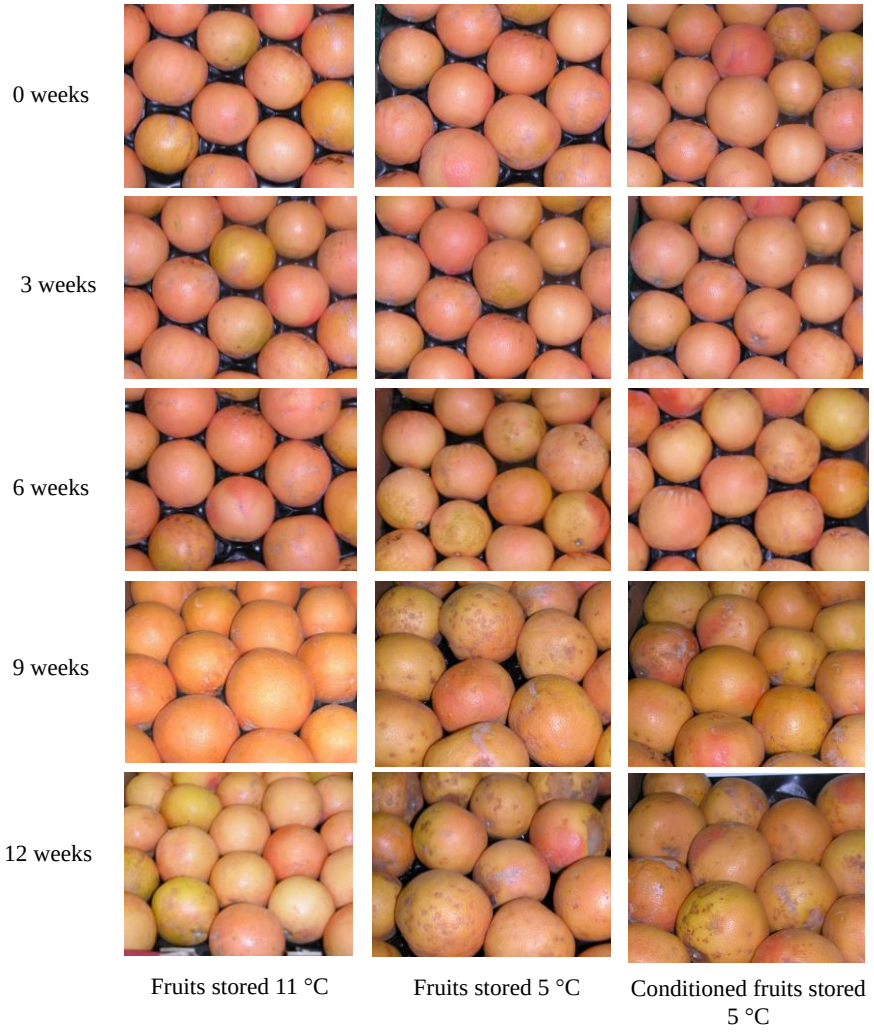

Figure 14. External appearance of 'Rio Red' grapefruits observed at 0, 3, 6, 9, and 12 weeks of storage at 11 °C, 5 °C, or conditioned fruits stored at 5 °C.

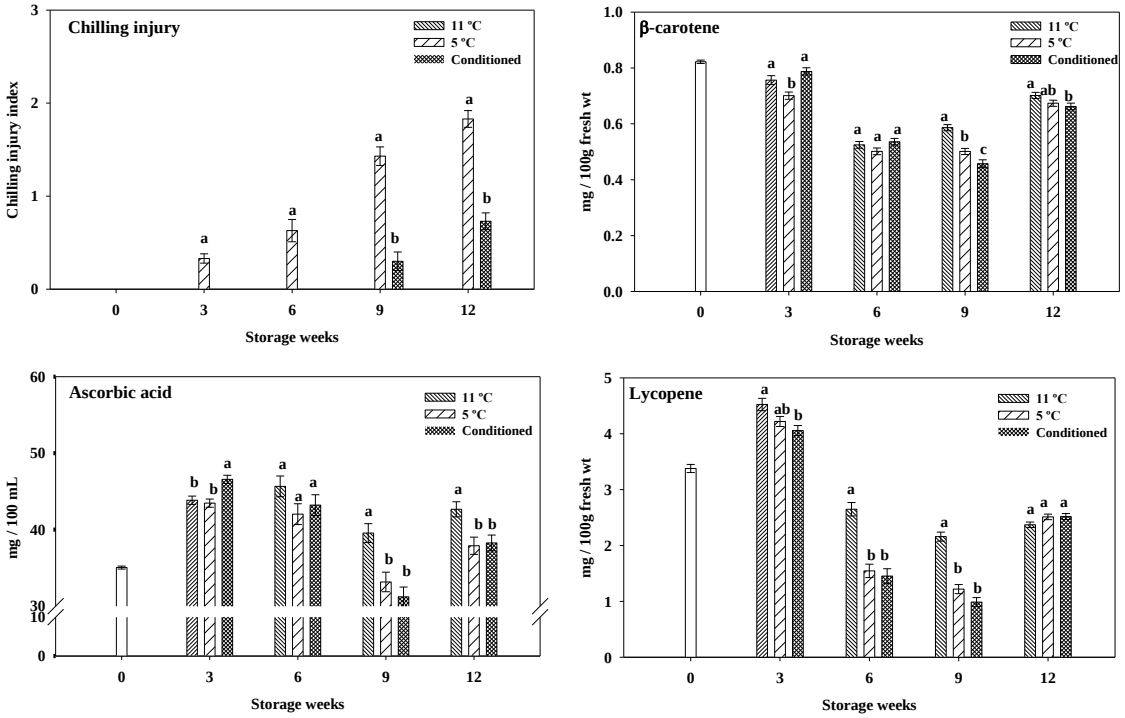

Figure 15. Chilling injury index and levels of ascorbic acid, β-carotene, and lycopene in pulp of 'Rio Red' grapefruit stored at 11 °C, 5 °C, and in conditioned (CD) fruits. Data represent means ± S.E. of three replications, each replication containing three samples (n=30 for CI index study, each replication containing 10 fruits). Means with different letters indicate significant differences between treatments at each time period ($P< 0.05$).

Ascorbic acid

Regulating temperature during postharvest operations is one of the most important factors in maintaining fruit quality and extending the shelf-life. Ascorbic acid is significantly influenced by storage temperature in fruits and vegetables. In present study we investigated the effect of different storage temperatures and conditioning

treatment in grapefruit ascorbic acid. Ascorbic acid levels increased during cold storage (5 °C and CD) at 3 weeks of storage and then gradually decreased to initial levels at 9 weeks of storage (**Figure 15**). Ascorbic acid levels in the fruits stored at 11 °C gradually increased up to 6 weeks, decreased at 9 weeks, and increased again at 12 weeks of storage. CD fruits had higher ascorbic acid levels at 3 weeks of storage; however, fruits stored at 11 °C had significantly higher levels at 9 and 12 weeks of storage. No difference was observed between the two cold-storage treatments at 6, 9 and 12 weeks of storage. Ascorbic acid is one of the most important anti-oxidants in plants, acting to counter biotic and abiotic stress by detoxifying reactive oxygen species produced under stress, with the help of the ascorbate - glutathione cycle.[220] In citrus fruits, ascorbic acid usually degrades with increasing storage temperature and period.[221] Chilling injury causes accelerated loss of ascorbic acid in susceptible crops.[60] Besides, CI leads to cellular and oxidative stress, which can affect the levels of ascorbic acid.[222] Ascorbic acid levels decreased in cucumbers with CI.[223] In the current study, after 9 weeks, CI incidence increased in both cold storage treatments, which could have led to the decrease in ascorbic acid contents. In addition, other studies also reported that cold storage decreased the ascorbic acid contents in citrus fruits.[162, 224] In previous study, ascorbic acid levels Star Ruby grapefruit decreased with increase in storage period.[225]

Carotenoids

Carotenoids in citrus are influenced by storage temperature and are differentially regulated in different tissues.[226] In current study β-carotene and lycopene were quantified in juice vesicles during cold storage period (**Figure 15**). β-carotene decreased

in all three treatments up to 9 weeks of storage and increased at 12 weeks of storage. After 9 weeks of storage, fruits stored at 11 °C had significantly higher β-carotene, followed by fruits stored at 5 °C and CD fruits. We observed a small increase in lycopene levels in all treatments at 3 weeks of storage; however, after 3 weeks, the levels gradually decreased up to 9 weeks of storage. Lycopene levels were significantly higher in fruits stored at 11 °C at 6 and 9 weeks of storage, with no significant difference observed in fruits stored at cold temperature. Nevertheless, we observed no significant difference in lycopene levels between treatments after 12 weeks of storage. Carotenoid biosynthesis in citrus fruits is temperature-dependent, with temperatures of 15-25 °C allowing the most carotenoid production.[18] Storage temperatures below 5 °C affect carotenoid biosynthesis and cause carotenoid degradation.[201, 226] In the current study, decrease in carotenoids, mainly lycopene, was more in fruits stored at low temperatures. However, the effect of storage temperature on carotenoid biosynthesis is more prominent in citrus peel than in the pulp.[226, 227] In contrast to our results, in Cara Cara navel orange fruits stored at 4 °C for 35 days, the total carotenoid contents increased, including lycopene, in the pulp as compared with the fruits stored at 20 °C.[226] Also, no effect of storage temperature was observed on the carotenoids in the pulp of Satsuma mandarin.[85]

Limonoids

Limonin and nomilin were quantified in the current study (**Figure 16 A**). Limonin levels were higher in CD fruits at 6 and 12 weeks, followed by fruits stored at 5 °C and 11 °C. At 3 weeks of storage, no significant differences were observed in limonin content among the three treatments. Limonin levels were overall maintained in

conditioned fruits during the storage period, but they decreased in other two treatments. Nomilin levels decreased in all treatments during the storage period. No significant differences were observed in nomilin levels among the treatments at 6 and 9 weeks of storage. Fruits stored at 11 °C had higher nomilin contet at 3 and 12 weeks of storage as compared to other two treatments. Limonin and nomilin are limonoid aglycones that have tissue-specific synthesis and accumulation.[228] Nomilin is also the precursor of limonin in the limonoid biosynthetic pathway, which can affect the levels of limonoids during storage. As fruit matures, limonoid aglycones are converted into non-bitter limonoid glucosides by limonoid glucosyltransferase.[93]

Furocoumarins

Furocoumarins, namely 6,7-dihydroxybergamottin (DHB) and bergamottin, were quantified in the current study (**Figure 16 B**). Levels of both furocoumarins decreased in all treatments at 12 weeks of storage as compared to the initial storage period. We observed no significant differences in DHB levels among the treatments at 6, 9, and 12 weeks of storage. While, bergamottin levels did not significantly differ among the treatments at 3, 9, and 12 weeks of storage. Furocoumarins are synthesized in response to stress and are linked to other secondary metabolites such as flavonoids and lignins through L-phenylalanine *via* the shikimate pathway. In addition, several pre- and post-harvest factors influence the levels of furocoumarins in grapefruit.[8, 177] As compared to DHB, bergamottin is more stable in grapefruit which was also observed in the current study, where bergamottin degraded relatively less than DHB in all treatments.[8] White flesh grapefruits have higher furocoumarins than red varieties.[8] Both DHB and

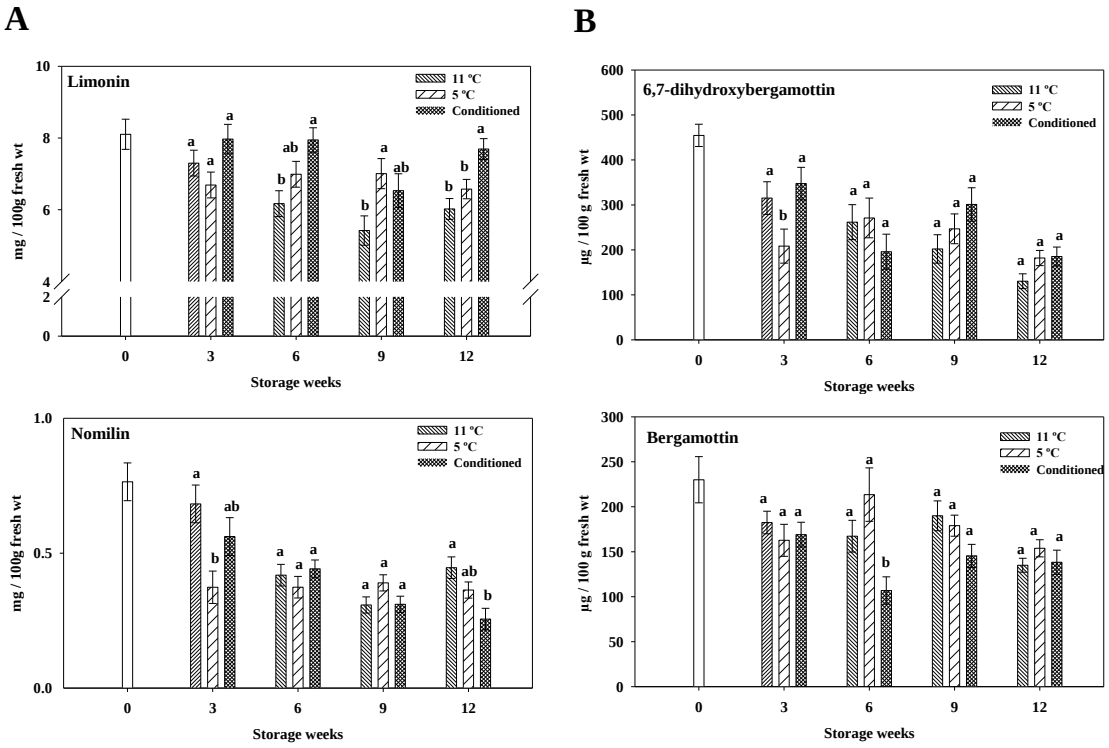

Figure 16. Variation in limonoid (A) and furocoumarin (B) contents of 'Rio Red' grapefruit. Fruits were stored for 0, 3, 6, 9, and 12 weeks at 11 °C, 5 °C, or conditioned (CD). Data represent means ± S.E. of three replications, each replication containing three samples. Means with different letters indicate significant differences between treatments at each time period ($P < 0.05$).

bergamottin can strongly inhibit *CYP3A4 enzymes*, which causes drug interactions, with DHB being more potent than bergamottin.[133]

Flavonoids

In the present study, five flavonoids namely narirutin, naringin, neohesperidin, didymin, and poncirin were quantified (**Figure 17 A and B**). Naringin is the main bitter flavonoid present in grapefruit juice, along with poncirin and neohesperidin. Naringin and narirutin were the major flavonoids detected. Narirutin, naringin, neohesperidin and poncirin levels did not differ significantly among the treatments at 6 and 9 weeks of storage (**Table 7**). Naringin, neohesperidin and didymin were lower in fruits stored at 5 °C at 3 and 12 weeks of storage while CD fruits showed significantly higher levels followed by fruits stored at 11 °C. Overall, flavonoids were at similar or at higher levels in CD fruits as compared to other two treatments. Previous studies have reported induction of the enzymes involved in the phenylpropanoid pathway, especially phenylalanine ammonia-lyase under stress conditions.[42, 229] Flavonoids are polyphenols biosynthesized from the phenylpropanoid pathway and have been reported to be affected by storage temperature.[224] The significant increase in most of the flavonoids in CD fruits, especially at 3 weeks, can be attributed to change in storage temperature during conditioning (16 °C) and during storage (5 °C), by influencing the flavonoid metabolism.

Total phenolics

Total phenolics were significantly higher in CD fruits at 3 weeks of storage as compared to other treatments (**Figure 17 C**). However, at 6 and 12 weeks of storage, fruits stored at 5°C had higher phenolics followed by conditioned fruits. This could be due to an increase in CI in fruits stored at cold temperatures. Phenolic compounds in grapefruit

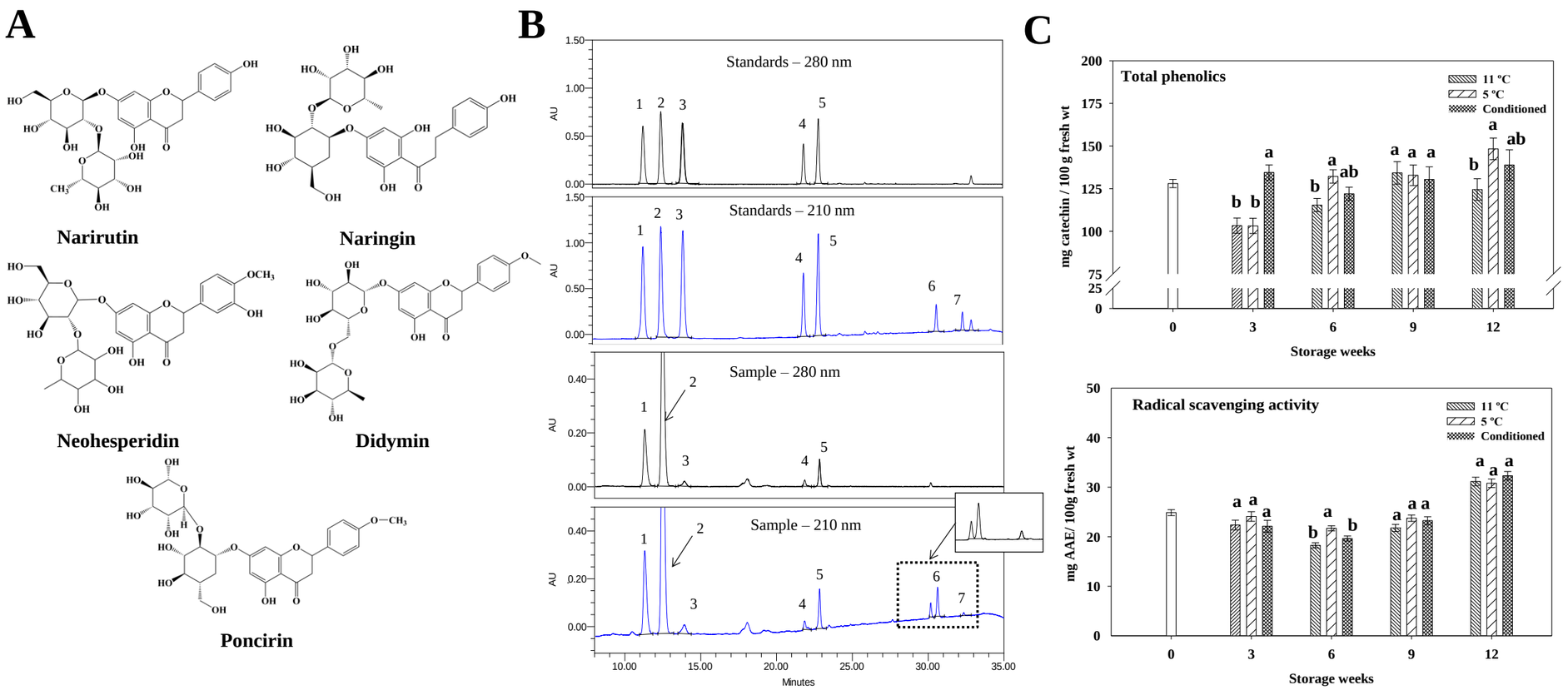

Figure 17. (A) Structures of flavonoids detected in grapefruit juice. (B) HPLC chromatogram of limonoids and flavonoids analyzed at 210 and 280 nm, respectively. Peak 1– narirutin, 2-naringin, 3-neohesperidin, 4- didymin, 5- poncirin, 6- limonin, 7- nomilin. (C) Total phenolics contents and radical-scavenging activity of methanol extracts of Rio Red grapefruit stored for 0, 3, 6, 9, and 12 weeks at 11 °C, 5 °C and in conditioned fruits stored at 5°C. Data represent means ± S.E. of three replications, each replication containing three samples. Means with different letters indicate significant differences between treatments at each time period ($P < 0.05$).

Table 7. Influence of storage temperature and duration on flavonoid content in juice of 'Rio Red' grapefruit. Fruits were stored for 0, 3, 6, 9, and 12 weeks at 11 or 5 °C or conditioned (CD)

	Weeks of storage				
	0	**3**	**6**	**9**	**12**
Narirutin					
11 °C	13.88 ± 0.37 a	14.60 ± 0.41 a	15.28 ± 0.54 a	14.28 ± 0.82 a	16.06 ± 0.49 a
5 °C	13.88 ± 0.37 a	13.28 ± 0.41 b	15.69 ± 0.55 a	14.06 ± 0.82 a	14.70 ± 0.46 a
CD	13.88 ± 0.37 a	15.14 ± 0.50 a	15.84 ± 0.54 a	14.72 ± 0.92 a	15.29 ± 0.48 a
Naringin					
11 °C	68.28 ± 1.90 a	77.54 ± 1.82 b	80.38 ± 2.39 a	70.29 ± 4.43 a	76.72 ± 2.13 a
5 °C	68.28 ± 1.90 a	70.98 ± 1.82 c	76.69 ± 2.39 a	65.36 ± 4.27 a	68.73 ± 1.89 b
CD	68.28 ± 1.90 a	85.31 ± 2.04 a	75.70 ± 2.24 a	69.42 ± 5.05 a	76.25 ± 2.13 a
Neohesper.					
11 °C	2.33 ± 0.09 a	2.37 ± 0.08 ab	2.73 ± 0.08 a	2.50 ± 0.16 a	2.70 ± 0.07 a
5 °C	2.33 ± 0.09 a	2.11 ± 0.08 b	2.51 ± 0.08 a	2.34 ± 0.16 a	2.40 ± 0.06 b
CD	2.33 ± 0.09 a	2.44 ± 0.09 a	2.66 ± 0.07 a	2.37 ± 0.17 a	2.51 ± 0.06 ab
Didymin					
11 °C	2.49 ± 0.10 a	2.42 ± 0.08 b	2.36 ± 0.10 b	2.34 ± 0.23 a	2.59 ± 0.08 a
5 °C	2.49 ± 0.10 a	2.17 ± 0.08 c	2.50 ± 0.10 ab	2.62 ± 0.23 a	2.33 ± 0.07 b
CD	2.49 ± 0.09 a	2.73 ± 0.09 a	2.74 ± 0.10 a	2.41 ± 0.25 a	2.62 ± 0.07 a
Poncirin					
11 °C	9.42 ± 0.30 a	10.62 ± 0.38 a	10.35 ± 0.52 a	9.24 ± 0.42 a	9.32 ± 0.43 a
5 °C	9.42 ± 0.30 a	9.46 ± 0.38 b	9.84 ± 0.52 a	8.23 ± 0.42 a	9.13 ± 0.41 a
CD	9.42 ± 0.30 a	12.03 ± 0.46 a	10.36 ± 0.52 a	9.31 ± 0.47 a	10.34 ± 0.42 a

*Data represent means ± S.E. of three replications, each replication containing three samples. Means with different letter indicate significant differences between treatments at each time period ($P< 0.05$).

mainly comprise flavanone glucosides, which are influenced by different abiotic and biotic stresses.[104] The fruits stored at 5 °C had higher CI, followed by CD fruits, and total phenolics showed a similar trend. Our previous study in Star Ruby grapefruit also showed an increase in total phenolics during storage.[177]

Radical-scavenging activity

Radical-scavenging activity was measured using a DPPH assay (**Figure 17 C**). Fruits stored at 5 °C had higher radical-scavenging activity at 6 weeks of storage. However, we observed no significant differences among the different treatments at 3, 9, and 12 weeks of storage. Antioxidant activity of all three treatments increased at 12 weeks of storage. Ascorbic acid and phenolics are the main contributors to antioxidant activity in citrus fruits, with ascorbic acid contributing more than 65% of total antioxidant activity.[64] In addition, a synergistic effect of phenolics and ascorbic acid can influence antioxidant activity.[230]

Conclusion

Low-temperature conditioning treatment can effectively reduce the incidence of CI. Overall, conditioning treatment did not affect most of the health promoting compounds present at the end of 12 weeks of storage. Fruits stored at 5 °C without conditioning had lower content of health promoting compounds at 3 weeks of storage. However at 12 weeks of storage, all three treatments showed similar levels of lycopene, narirutin, poncirin, and furocoumarins. Generally, conditioning treatment maintained the levels of most of the secondary metabolites assessed as compared to non-conditioned fruits stored at 5 °C. Storage period significantly affected all health promoting

compounds. Low-temperature conditioning of the fruits prior to quarantine treatments or cold storage is recommended to prevent CI and to maintain health promoting natural compounds.

CHAPTER VII

INFLUENCE OF MODIFIED ATMOSPHERE PACKAGING ON 'STAR RUBY' GRAPEFRUIT HEALTH PROMOTING COMPOUNDS*

Introduction

Recent decades have seen an increase in the use of modified atmosphere packaging (MAP) for storage of fresh and minimally processed fruits and vegetables. MAP is a postharvest packaging method that modifies the micro-atmosphere around the fruit or other produce by lowering O_2 levels and increasing CO_2 and humidity levels.[231] Low O_2 and high CO_2 delay senescence and slow down the metabolic activity of the fruit, which in turn improves shelf life, reduces postharvest losses, and maintains quality. Reduced O_2 prevents the growth of aerobic pathogens, reduces respiration, and retards oxidation and browning reactions. Increased CO_2 reduces the rate of respiration and metabolic activities.[232, 233]

MAP also controls humidity to prevent moisture loss and pathogen growth. Moisture loss from fruit causes weight loss, changes in taste, and development of problems such as peel pitting, chilling injury, and rind disorders, especially in citrus.[211] In citrus fruits, use of MAP has reduced the incidence of peel disorders and chilling injury.[30] Furthermore, MAP can be used as an alternative to fungicide and pesticide treatments, to minimize the incidence of disease during storage. During storage

*Reprinted with permission from "Influence of modified atmosphere packaging on 'Star Ruby' grapefruit phytochemicals." by Chaudhary, P. R.; Jayaprakasha, G. K.; Porat, R.; Patil, B. S., 2015, *Journal of Agricultural and Food Chemistry. 63* (3), 1020-1028 Copyright [2015] American Chemical Society.

MAP can be achieved either by individual seal-packaging or by bulk packaging (bag in box) of fruits.[47]

The use of advanced techniques to improve fruit quality and shelf life has increased, but very little is known about the effects of such treatments on the phytochemical content of fruits. In recent years, consumers have become increasingly aware of the health benefits of fruits and the role of various naturally occurring compounds in reducing risks of certain diseases. These compounds are secondary plant metabolites that protect plants from various biotic and abiotic stresses. In humans, these compounds also have certain health-promoting properties.[234] For example, natural compounds have several demonstrated activities that protect against chronic diseases such as cancer, including induction of apoptosis, cell cycle arrest, cell differentiation, and induction of phase-II enzymes, along with anti-proliferative activity.[101, 200, 235] Flavonoids reportedly reduce the risk of cancer, cardiovascular disease, and other degenerative diseases.[57] Limonoids, commonly present in plants from the Rutaceae and Meliaceae families, have anti-tumor and anti-cancer properties.[94, 101]

A few studies have examined the effect of MAP on health promoting compounds in fruits, including plums,[236] strawberry,[237] tomato,[238] and others; however, the effect on natural compounds in citrus fruits has not been explored. In plums, MAP delayed ripening and development of phenolics and carotenoids during storage.[236] A recent study suggested that MAP and an edible coating of chitosan enhanced the phenolic contents of ready-to-eat carrot sticks.[239] The main objective of the current study was to examine the effect of modified atmosphere conditions on the levels of secondary metabolites present

in grapefruit juice vesicles during prolonged storage. To the best of our knowledge, this is the first study to report the effect of MAP on the levels of health promoting compounds such as ascorbic acid, carotenoids, limonoids, flavonoids, and furocoumarins in 'Star Ruby' grapefruit during prolonged storage.

Materials and methods

Plant materials

'Star Ruby' fruits of uniform size were harvested from a commercial grove on the south coast of Israel (January, 26th 2010). Each treatment included four cartons of 10 kg grapefruits, i.e. 40 kg fruit per treatment. In the current study "bag in box" method of MAP was used. Fruits were stored at 10 °C for 16 weeks, followed by one week of storage at shelf conditions (20 °C) with the following treatments: a) Control (untreated); b) Micro-perforated Xtend® bag (MIPB; modified atmosphere and humidity); c) Macro-perforated Xtend® bag (MAPB; modified humidity). The relative humidity in the 10°C cold storage room was between 80-85%. The optimum humidity for postharvest storage of citrus fruit is about ~90-95%.

The MIPB and MAPB liners used included 20 µm thick Xtend® films (StePac LA, Tefen, Israel), in which the total area of pores as a percentage of the film surface area were 0.002 for MIPB and 0.06% for MAPB. The water vapor transmission rate of the film was 18×10^{-10}mol s^{-1} m^{-2} Pa^{-1}. The O_2 and CO_2 concentrations in the atmosphere surrounding the untreated control fruit were similar to those of the regular air (0.1-0.3% CO_2 and 20.5-21.0% O_2) (**Table 8**). In the MAPB, the atmospheric gas concentrations were also similar to those of regular air. In contrast, the fruit in the MIPB

experienced a modified atmosphere with constant levels of 3.5% CO_2 and 18.2% O_2. The relative humidity inside both perforated bags was about 97-98% as compared with ~80% for the control untreated fruit.

Fruit samples were collected at intervals of 4 weeks and were further stored for one week at room temperature (20 °C) before analysis, to simulate market conditions. Three juice samples per treatment at each time interval were prepared by blending 3 peeled fruits from different cartons, i.e. a total of 9 fruits for each timepoint. The juice samples were lyophilized and sent to the Vegetable and Fruit Improvement Center (Texas A&M University, College Station, Texas, USA) for quantification ofsecondary metabolites.

Chemicals

Butylated hydroxytoluene (BHT), lycopene, β-carotene, narirutin, naringin, didymin, poncirin, limonin, and 6', 7'-dihydroxybergamottin (DHB) were procured from Sigma Aldrich Co. (St. Louis, MO, USA). Analytical grade solvents used for quantitative analysis were obtained from Fisher Scientific Research (Pittsburgh, PA, USA).

Fruit quality analysis

Fruit quality parameters such as total soluble solids (TSS), titratable acidity, ripening ratio (TSS/acidity), weight loss, and disease incidence were measured. TSS was determined using a digital refractometer (Model PAL-1, Atago, Tokyo, Japan) and is expressed as ºBrix. Titratable acidity was determined by titrating the juice with 0.1 M NaOH to pH 8.3 using a Model CH-9101 automatic titrator (Metrohm, Herisau,

Switzerland). Each measurement included five replications and each replication included three fruits. Weight loss was determined by weighing fruits (n=10) at intervals of 4 weeks (plus the subsequent one week of storage at 20°C) and was expressed as percent weight loss compared to the pre-storage weight. Decay incidence was determined by monitoring the number of fruits with disease symptoms and was expressed as the percentage of decayed fruit per carton.

Sensory analysis

Fruit sensory quality was evaluated at 4-week intervals (plus the subsequent one week of storage at 20 °C). Fruits were peeled, and the separated segments were cut in halves and placed into covered glass cups. Each treatment included a mixture of cut segments from five different fruits. Sensory evaluations were conducted by a trained panel consisting of five males and five females aged between 25 and 62 years, who were well trained and experienced in tasting citrus fruit. The sensory training included open panel sessions with tasting of reference standards. Each panelist assessed the various attributes of the three samples, according to an unstructured 100-mm scale, with anchor points 'very weak' and 'very strong' for each attribute, and the sensory data were recorded as distances (mm) from the origin. Overall flavor preference was recorded on a hedonic scale of 1 to 10. The samples were identified by randomly assigned three-digit codes.

Quantification of ascorbic acid

Direct titration of juice with 2,6-dichlorophenolindophenol (DCIP) was used to determine ascorbic acid content by measuring the reduction of DCIP with ascorbic acid in an acidic solution.[157] Titration volumes were compared with 0.1% ascorbic acid

(Sigma-Aldrich, St. Louis, MO) and the results were expressed as mg of ascorbic acid per 100 mL of juice.

Quantification of carotenoids

Carotenoids were extracted and analyzed using a previously described method.[177] Freeze-dried samples (1 g + 5 mL water) were extracted using chloroform (15 mL) containing BHT (0.2 %). The reconstituted juice was sequentially extracted three times under yellow light. The organic layer was allowed to separate and extracts from each sample were pooled, filtered using Whatman grade 1 filter paper, and subjected to HPLC analysis. Carotenoids were quantified by HPLC using a Gemini C-18, 5 µm column (250 mm × 4.6 mm i.d.) with a guard cartridge (Phenomenex, Torrance, CA, USA) on a Agilent 1200 series HPLC (Foster City, CA, USA). Acetonitrile (A) and isopropyl alcohol (B) were used for the mobile phase at the flow rate of 0.8 mL/min with 10 µL injection volume. External standards were used to quantify β-carotene and lycopene at 450 nm wavelength.

Quantification of limonoids, furocoumarins and flavonoids

Sample preparation

Extraction of samples was carried out according to our previously published method.[177] Each freeze dried juice sample was reconstituted (1 g + 5 mL water) and extracted with 15 ml of ethyl acetate on a shaker for four hours. The organic layer was separated and the residue was extracted twice, pooled, and the solvent was evaporated to dryness. The dried residue was reconstituted with acetone (4 mL), filtered using a 0.45µm PTFE filter and analyzed by HPLC for limonoids, flavonoids, and

furocoumarins. In the current study, a common extraction solvent was used for measuring the levels of limonoids, flavonoids, and furocoumarins, to allow comparison and minimize the extraction errors caused by using different solvents.

Quantification of limonoids and flavonoids with HPLC

Waters HPLC (Milford, MA, USA), spectra model with a photodiode array detector (2996), a binary HPLC pump 1525, and a 717 plus autosampler, was used for quantification of limonoids and flavonoids. The chromatographic separations were performed according to a previously published method on a Gemini C-18, 5 µm column (250 mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA).[177] The limonoids and flavonoids were detected at 210 and 280 nm, respectively. Data analysis was carried out using the Empower pro software. The chromatographic separation was performed using a gradient mobile phase of 0.03 M phosphoric acid (A) and acetonitrile (B) with 1 mL/min flow rate. Each sample was injected three times and all measurements were conducted in triplicate.

Confirmation of limonoids and flavonoids by liquid chromatography - mass spectrometry

The structures of limonoids and flavonoids in samples were confirmed by ultra-high performance liquid chromatography-time of flight-mass spectrometry (LC-QTOF-MS) (maXis impact, Bruker Daltonics, Billerica, MA). Limonoids and flavonoids were separated on an Eclipse Plus C18 LC-MS column (2.1 ×100 mm), 1.8 µm partical size connected to Agilent 1290 UPLC instrument (Agilent, Santa Clara, CA) at 70 °C with a flow rate of 0.2 mL/min using binary gradient elution of 0.1% formic acid and

acetonitrile. Compounds were separated by a gradient of 98% A to 10% in 0-9 min followed by equilibration for 2 min before the next injection. The 1290 UPLC is equipped with auto sampler, binary pump, solvent degasser, thermostatted column compartment and diode-array detector (DAD). The LC peaks were monitored at 210 nm and 280 nm for limonoids and flavonoids respectively. Mass spectral analyses were performed using the ESI-Q-TOF mass spectrometer equipped with an electrospray ionization. Both positive and negative modes were used for ionization. Mass spectrometer conditions are as follows, MS scan range 50–1000 m/z; end plate offset−500 V; capillary voltage 3000 V, nebulizer gas (N_2) 4 bar; dry gas (N_2) flow rate 12 L/min; dry gas temperature: 200 ºC; ion transfer conditions funnel RF: 200 Vpp; multiple RF: 200 Vpp; quadruple low mass 55 m/z; collision energy 5.0 eV; collision RF 600 Vpp; ion cooler RF 50–250 Vpp ramping; transfer time 121µs; pre-pulse storage time 1µs. Calibration was done before each run through a loop injector of 20 µL of 10 mM sodium formate. The Bruker Compass Data analysis 4.1 software was used for data acquisition.

Quantification of furocoumarins with HPLC

Furocoumarins were analyzed using our previously described method, with slight modifications.[120] A Perkin Elmer HPLC system consisting of a series 200 pump, PDA detector (235C) and autosampler (Norwalk, CT, USA), was used for analysis. The separations were carried out on a Gemini C-18, 5 µm column (250 mm × 4.6 mm i.d.) with a guard cartridge from Phenomenex (Torrance, CA, USA). The peaks were detected at 320 nm and the data were integrated using Turbochrom software (Perkin-Elmer,

Norwalk, CT, USA). The gradient mobile phase consisted of 0.03 M phosphoric acid (A) and acetonitrile (B) with 10 µL injection volume at the flow rate of 1 mL/min. Each sample was analyzed three times and all measurements were conducted in triplicate.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with PASW Statistics 18 software (SPSS Inc. 2009). A general linear model was used to test significant differences and means were compared using Tukey's HSD test at the 5% probability level. The results were expressed as means ± SE.

Results and discussion

Weight loss

Overall, we observed no significantly detrimental weight loss in any of the three treatments, but in the control fruits, weight loss gradually increased during the postharvest storage from 1.7% after 4 weeks to 4.2% after 16 weeks of storage (**Table 8**). MIPB and MAPB significantly ($P< 0.05$) reduced weight loss during the 16-weeks storage period. Humidity, temperature, and storage period strongly affect weight loss in fruits.[240] Moisture loss from the peel, a primary reason for weight loss in citrus fruits, can lead to peel disorders and affect the fruit's appearance.[241, 242] In the current experiment, the reduction in weight loss observed in grapefruits stored in MAP can be attributed to reduced transpiration due to the high humidity maintained in the MAP. Similar results were reported in previous experiments where individual film wrapping[242] and use of box liner bags[30] for storage of citrus fruits significantly reduced weight loss.

Table 8. Effect of MAP on carbon dioxide (%), oxygen (%) and weight loss (%) in 'Star Ruby' grapefruit. Fruits were stored for 4, 8, 12, and 16 weeks in micro-perforated and macro-perforated bags at 10 °C, followed by one week of storage at shelf conditions (20 °C).

Weeks	**Carbon dioxide (%)**			**Oxygen (%)**			**Weight Loss (%)**		
	Control	**Micro**	**Macro**	**Control**	**Micro**	**Macro**	**Control**	**Micro**	**Macro**
4	0.10 ± 0.18a	2.97 ± 0.18b	0.40 ± 0.18a	21.00 ± 0.13a	18.83 ± 0.13b	20.60 ± 0.13a	1.70 ± 0.07a	1.11 ± 0.07b	1.18 ± 0.07b
8	0.20 ± 0.04a	3.53 ± 0.04b	0.30 ± 0.04a	20.70 ± 0.02a	18.30 ± 0.02b	20.13 ± 0.02c	2.04 ± 0.08a	1.47 ± 0.08b	1.53 ± 0.08b
12	0.30 ± 0.13a	3.10 ± 0.09b	0.37 ± 0.07a	20.00 ± 0.09a	18.20 ± 0.07b	20.03 ± 0.05a	3.08 ± 0.16a	2.12 ± 0.16b	2.17 ± 0.16b
16	0.30 ± 0.27a	3.47 ± 0.16b	0.50 ± 0.16a	20.40 ± 0.09a	18.40 ± 0.08b	20.10 ± 0.08a	4.24± 0.20a	3.29 ± 0.20b	3.74 ± 0.20b

*Data are means ± S.E. of 3 replications, each from 3 different fruits. Same letter between treatments indicates no significant differences ($P < 0.05$). n=10 for weight loss (%).

Visual appearance and color

At the time of harvest (Jan. 26, 2010), the fruits were attractive, with an external yellowish color with red cheeks (**Figure 18**). During the prolonged storage at 10 °C the control fruits gradually became more reddish as compared to time zero (at harvest). Packing the fruits in perforated bags slightly delayed the development of external color. Low oxygen and high carbon dioxide levels inhibit ethylene production and action,[243, 244] which may cause the delay in peel color development in grapefruits stored in MAP.[245] Modified atmosphere storage delays ripening and color development in plums,[246] avocado,[247] and in Marsh grapefruit.[242] With respect to internal appearance, the fruit flesh was red at harvest, and no further remarkable changes occurred during storage.

Juice TSS and acid percentages

Juice TSS was 12.2% at harvest and remained mostly constant during postharvest storage in all treatments (**Table 9**). By contrast, acidity levels were 1.9% at harvest, and slightly decreased to 1.6-1.7% after prolonged storage. Overall, the observed changes in juice TSS and acidity levels resulted in a slight increase in ripening ratio from 6.5 at harvest to 8.0 after 16 weeks of storage. Fruit stored in perforated bags showed only a slight reduction in acidity levels. Similar results were observed in previous studies where MAP had no significant effect on TSS and acidity levels in citrus fruits.[30, 248]

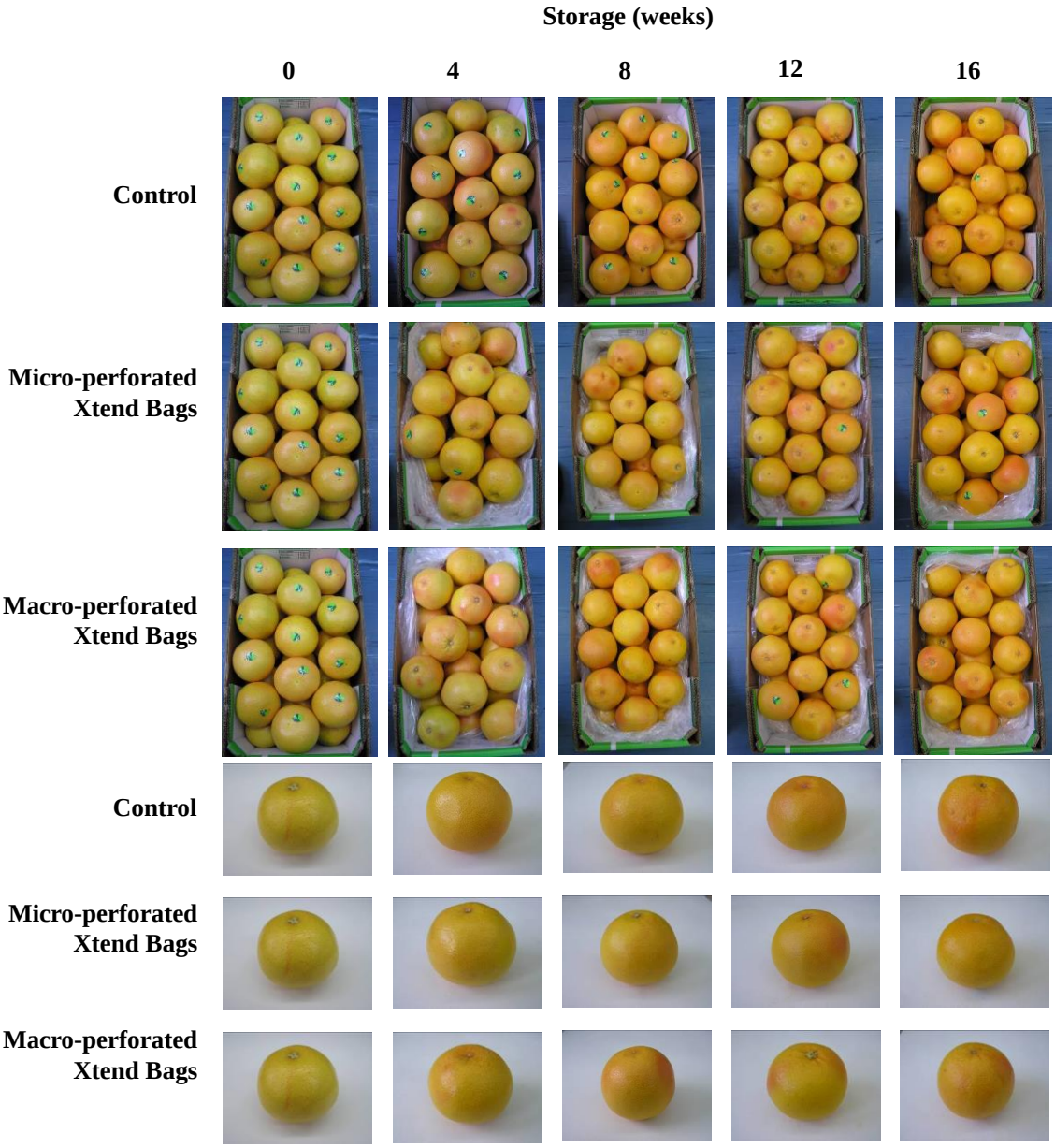

Figure 18. Effect of MAP on the appearance of 'Star Ruby' grapefruit. Fruit were untreated or kept in perforated bags and pictures were taken after 4, 8, 12, and 16 weeks of storage at 10 °C, followed by one week of storage at shelf conditions (20 °C).

Table 9. Effect of MAP on TSS, acidity (%), TSS/Acidity ratio and taste score in 'Star Ruby' grapefruit. Fruits were stored for 4, 8, 12, and 16 weeks in micro-perforated and macro-perforated bags at 10 °C, followed by one week of storage at shelf conditions (20 °C)

	Storage duration (weeks)				
	0	**4**	**8**	**12**	**16**
TSS (%)					
Control	12.2 ± 0.2 a	12.2 ± 0.4 a	11.6 ± 0.3 a	11.8 ± 0.1 ab	11.6 ± 0.3 a
Micro	12.2 ± 0.2 a	10.7 ± 0.4 a	12.3 ± 0.3 a	11.9 ± 0.1 a	12.5 ± 0.3 a
Macro	12.2 ± 0.2 a	11.4 ± 0.4 a	10.3 ± 0.3 b	11.3 ± 0.1 b	12.2 ± 0.3 a
Acidity (%)					
Control	2.0 ± 0.1 a	1.9 ± 0.1 a	2.0 ± 0.1 a	1.8 ± 0.0 a	1.7 ± 0.1 a
Micro	2.0 ± 0.1 a	1.6 ± 0.1 a	1.9 ± 0.1 a	1.7 ± 0.0 a	1.6 ± 0.1 a
Macro	2.0 ± 0.1 a	1.6 ± 0.1 a	1.7 ± 0.1 b	1.7 ± 0.0 a	1.6 ± 0.1 a
Ripening ratio					
Control	6.3 ± 0.2 a	6.5 ± 0.4 a	5.8 ± 0.2 b	6.5 ± 0.2 a	6.8 ± 0.3 a
Micro	6.3 ± 0.2 a	6.8 ± 0.4 a	6.6 ± 0.2 a	7.0 ± 0.2 a	8.0 ± 0.3 a
Macro	6.3 ± 0.2 a	7.1 ± 0.4 a	6.2 ± 0.2 ab	6.6 ± 0.2 a	7.5 ± 0.3 a
Taste score					
Control	8.8 ± 0.5 a	8.1 ± 1.1 a	8.1 ± 0.6 b	8.0 ± 0.9 a	7.4 ± 1.2 a
Micro	8.8 ± 0.5 a	7.9 ± 0.7 a	8.9 ± 0.7 a	8.1 ± 0.7 a	7.1 ± 1.6 a
Macro	8.8 ± 0.5 a	7.7 ± 1.0 a	8.7 ± 1.0 a	7.8 ± 0.9 a	7.6 ± 0.8 a

*Data are means ± S.E. of 3 replications, each from 3 different fruits. Same letter within the group indicates no significant differences between treatments ($P < 0.05$)

Decay development

The incidence of decay was very low throughout the course of the experiment, and after 16 weeks of storage only 3-4% decay was observed in all the treatments (data

not shown). Thus, the increased humidity in the MAP did not increase the incidence of decay. MAP is recommended as it reduces various rind disorders in citrus fruits including oranges, tangerines and grapefruit.[30, 47]

Fruit taste

At harvest, fruits achieved a high taste score of 8.8 on a scale of 1 to 10 (**Table 9**). Although the fruit taste remained good during the entire experiment, the taste score gradually decreased to 7.2-7.5 after 16 weeks of storage. Overall, storing the fruits in MAP had no significant effect on fruit taste or sensory acceptability (**Table 9**). Taste is an important factor in determining the quality of fruits, especially in fruits stored for prolonged periods. Sugars (TSS) and acids are the primary factors affecting taste, and MAP did not alter these in the current study. Other studies conducted using MAP have reported retention of taste for longer periods than control [249]. However, care must be taken to avoid very high CO_2 (>7-8%) or low O_2 (<14-15%), which can lead to anaerobic conditions and result in off-flavors.[30]

Ascorbic acid

Ascorbic acid content was 40 mg per 100 mL juice at harvest and was retained during postharvest storage in all the three treatments (**Figure 19**). MAP had no significant effect on ascorbic acid levels during the storage period. Ascorbic acid levels increased in all three treatments at 8 weeks after storage; nevertheless, at the end of the 16-weeks storage period, fruits in all three treatments had ascorbic acid levels similar to initial levels. Ascorbic acid is a major antioxidant present in citrus fruits.[62, 250] In Valencia oranges, coating with different commercial waxes that modified their internal

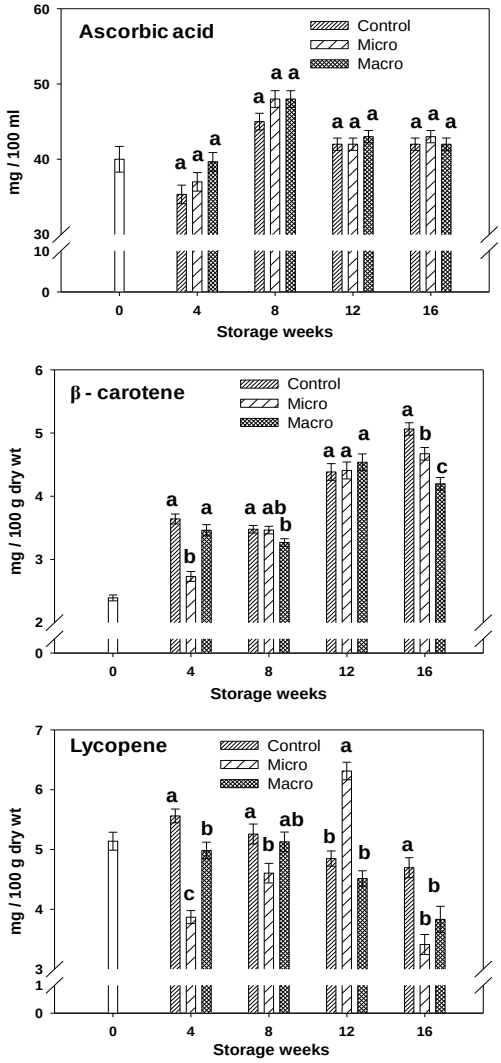

Figure 19. Effect of MAP on ascorbic acid and carotenoid contents of 'Star Ruby' grapefruit. Fruits were stored for 4, 8, 12, and 16 weeks in micro-perforated and macro-perforated bags at 10 °C, followed by one week of storage at shelf conditions (20 °C). Data are represented as means ± S.E. of 3 replications, each from 3 different fruits. Same letter between treatments indicates no significant differences ($P < 0.05$).

O_2 and CO_2 produced no significant difference in ascorbic acid levels between coated and non-coated fruits.[251] However, Valencia oranges also showed a decrease in ascorbic acid with increasing storage period.[251] A decrease in ascorbic acid with storage time, even with MAP, was reported in Chinese cabbage.[252] Ascorbic acid was higher in broccoli stored in MAP due to its low autoxidation[253] and MAP did not affect ascorbate peroxidase, the major enzyme involved in ascorbic acid degradation, in broccoli. However, in the present study, no significant differences were seen in ascorbic acid contents during storage period, regardless of the treatment.

Carotenoids

β-carotene levels gradually increased in grapefruit pulp during the storage period in all the three treatments (**Figure 19**). At the end of 16 weeks of storage, β-carotene levels were higher in control fruits, followed by fruits stored in MIPB and MAPB. In addition, lycopene was significantly higher in control fruits at 4 and 16 weeks of storage. Fruits stored in MAPB had higher lycopene contents than fruit stored in MIPB at 4 weeks of storage; however, no significant difference was observed between MIPB and MAPB fruits at 8 and 16 weeks of storage. A sharp increase in lycopene levels was observed in the fruits stored in MIPB at 12 weeks of storage, though lycopene decreased after 16 weeks of storage. The current results indicate that MAP delayed color development in peel and carotenoid development especially β-carotene in fruit flesh. In plums, storage in MAP delayed anthocyanin and carotenoid development.[236, 254] Another study conducted on shredded carrots showed similar results, where MAP (95% O_2 and 5% CO_2) reduced the carotenoid contents of both orange and purple carrots during

storage.[255] Being non-climacteric in nature, mature citrus fruit produce low levels of endogenous ethylene.[256] However, MAP affects ethylene biosynthesis and action, and thus may delay carotenoid development.

Limonoids and furocoumarins

Limonin levels increased sharply after 4 weeks of storage in all three treatments (**Figure 20**), but limonin was higher in control and in MIPB at 4 and 8 weeks after storage. However, control fruits had lower levels of limonin than MAP-treated fruits at 12 weeks of storage. No significant difference in limonin was observed among the treatments after 16 weeks of storage. LC-MS analysis was conducted to further confirm the increase in limonin observed at 0 days and 4 weeks. The total ion chromatogram (TIC) of mass spectral analysis of ethyl acetate extracts were illustrated in **Figure 21 A** and mass spectrum of limonin shows the negative molecular ion m/z 469.1849 $[M+1]^-$ (mass error 1.39 ppm) (**Figure 21 B**). The limonin levels were found to be more than two-fold higher at 4 weeks, compared to at 0 days. In citrus fruits, limonin is the most abundant aglycone followed by nomilin.[257] Limonin is one of the compounds responsible for the bitter taste of grapefruit. Nomilin is the precursor of limonin, and limonin is further converted into limonin glucoside.[95]

Furocoumarin, namely DHB, was quantified in the current study. DHB levels decreased in all three treatments with longer storage, after 4 weeks (**Figure 20**). No significant difference ($P< 0.05$) was observed in DHB levels among all the three treatments up to 4 weeks of storage. At eight weeks of storage, DHB was significantly lower in fruits stored in MIPB, but control and fruits stored in MAPB showed no

difference. Fruits stored in MIPB then showed higher DHB levels at 12 weeks of storage than fruits in the other two treatments. Nevertheless, after 16 weeks of storage, no

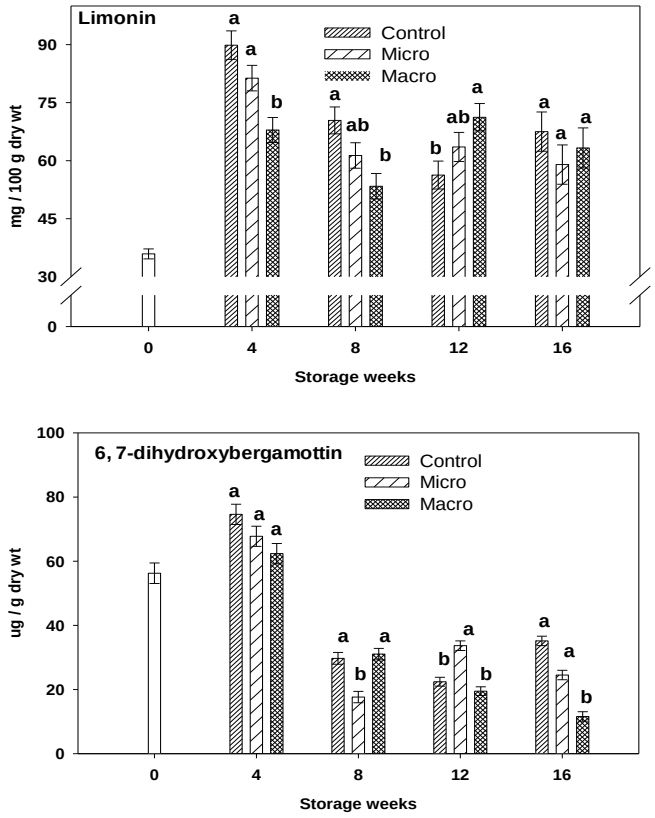

Figure 20. Effect of MAP on limonin (limonoid) and DHB (furocoumarin) contents of 'Star Ruby' grapefruit. Fruits were stored for 4, 8, 12, and 16 weeks in micro-perforated and macro-perforated bags at 10 °C, followed by one week of storage at shelf conditions (20 °C). Data are represented as means ± S.E. of 3 replications, each from 3 different fruits. Same letter between treatments indicates no significant differences ($P < 0.05$).

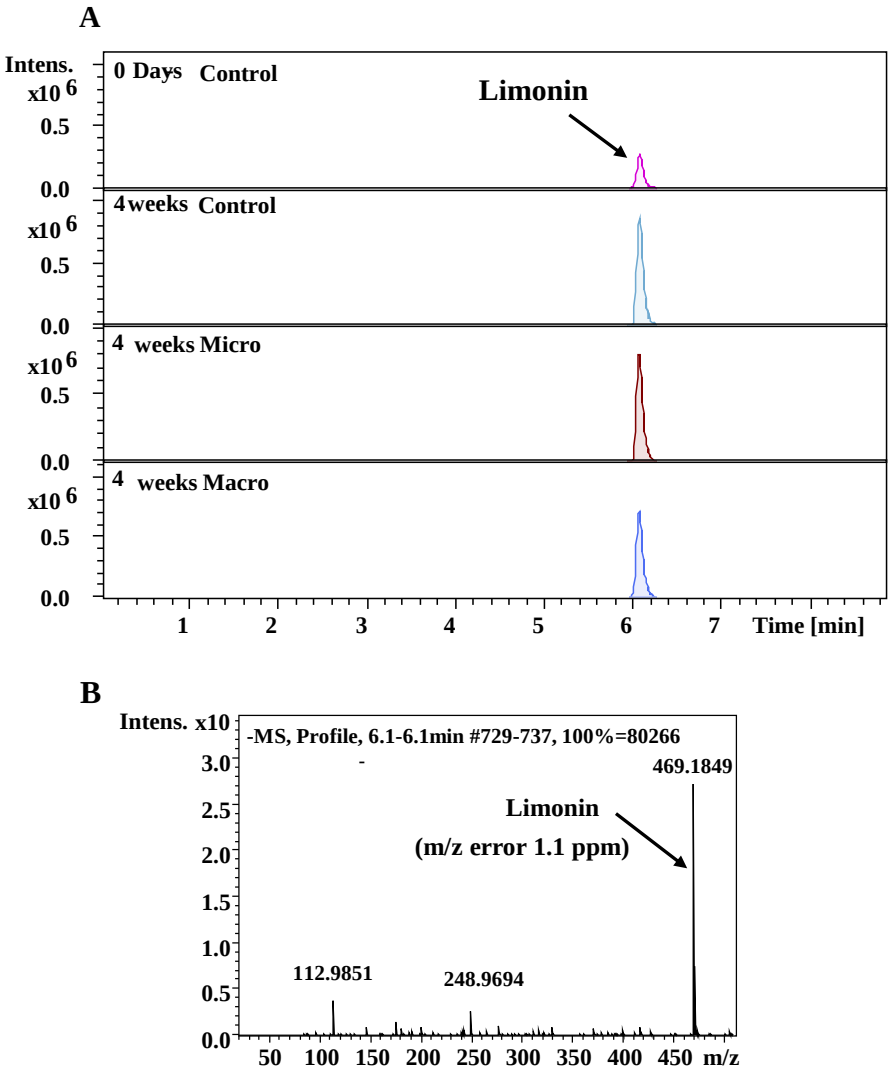

Figure 21. (A). Extracted ion chromatograms of limonin in control grapefruits and fruits stored in micro-perforated and macro-perforated bags at 10 °C for 0 days or 4 weeks, followed by one week of storage at shelf conditions (20 °C). (B) ESI–MS spectrum of limonin fragment detected by negative ionization mode.

significant difference was seen between control and the fruits stored in MIPB, while the fruits stored in MAPB showed lower DHB levels. Furocoumarins are derived from the phenylpropanoid pathway, with umbelliferone being their main precursor.[258]

Furocoumarins are affected by various factors such as environmental conditions, variety, season, storage period, and postharvest treatment.[8, 120, 259] A previous study by Girennavar et al reported a decrease in DHB levels during storage when Rio Red grapefruits were stored at 9ºC for 45 days.[8] The current study is the first to report the effect of MAP on limonoids and furocoumarins in 'Star Ruby' grapefruit.

Flavonoids

The four flavonoids (narirutin, naringin, didymin, and poncirin) showed similar trends during storage (**Figure 22**). Their levels decreased gradually with storage up to 8 weeks, with a sharp increase at 12 weeks of storage in all three treatments. However, at the end of the 16-weeks storage period, initial flavonoid levels were maintained in fruits stored in MAPB, while flavonoids levels decreased slightly in the other two treatments. The sharp increase in flavonoids in all three treatments can be a response to physiological stress during storage. No significant difference was observed in narirutin and didymin levels at 8 weeks of storage in all three treatments. Naringin, didymin and poncirin levels were higher in the control and in fruits stored in MAPB at 4 weeks of storage. At eight weeks of storage, controls and fruits stored in MIPB had higher naringin and poncirin levels in comparison to the fruits stored in MAPB.

In addition, mass spectral experiments were performed using electrospray ionization operated in negative mode for zero day control and 4 weeks samples of

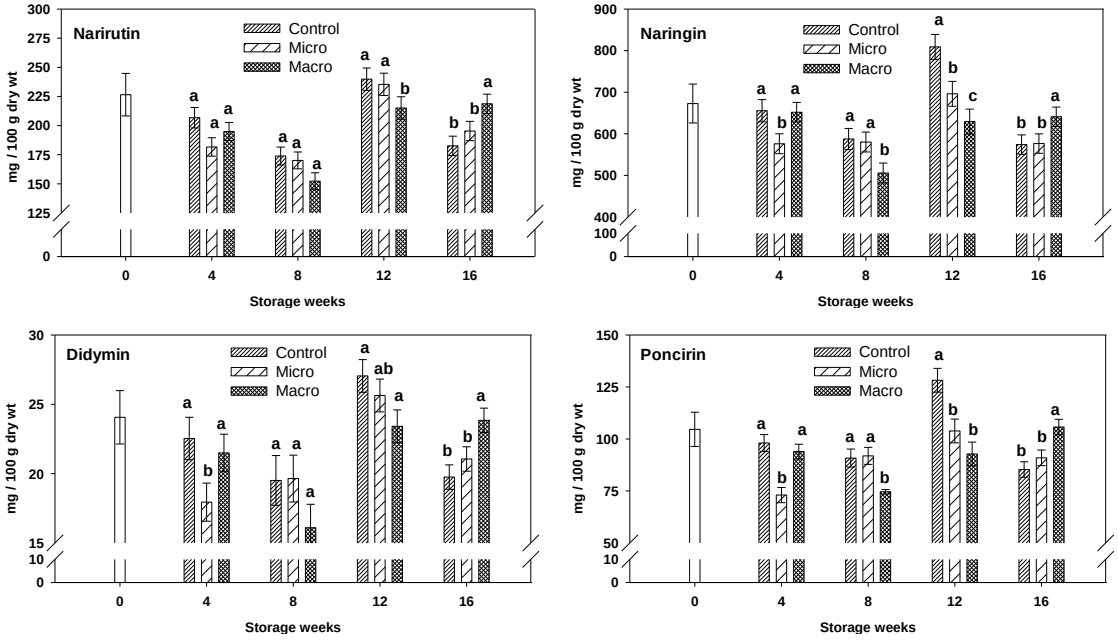

Figure 22. Effect of MAP on flavonoid contents of 'Star Ruby' grapefruit. Fruits were stored for 4, 8, 12 and 16 weeks in micro-perforated and macro-perforated bags at 10 °C, followed by one week of storage at shelf conditions (20 °C). Data are represented as means ± S.E. of 3 replications, each from 3 different fruits. Same letter between treatments indicates no significant differences ($P < 0.05$).

control, micro-perforated and macro-perforated samples to validate the structural confirmation using high resolution mass spectra. **Figure 23 A** UPLC separation for four flavonoids eluted at 3.8, 3.9, 4.6 and 4.7 min. **Figure 23 B** depicts the negative molecular ion mass spectra of narirutin, m/z 579.1676 (m/z error - 5.6 ppm), naringin m/z 579.1705 (m/z error 0.57 ppm), didymin m/z 593.1970 (error 4.7ppm), and poncirin m/z 593.1860 (error 3.5 ppm). A mass error of the molecular ion of each flavonoid is

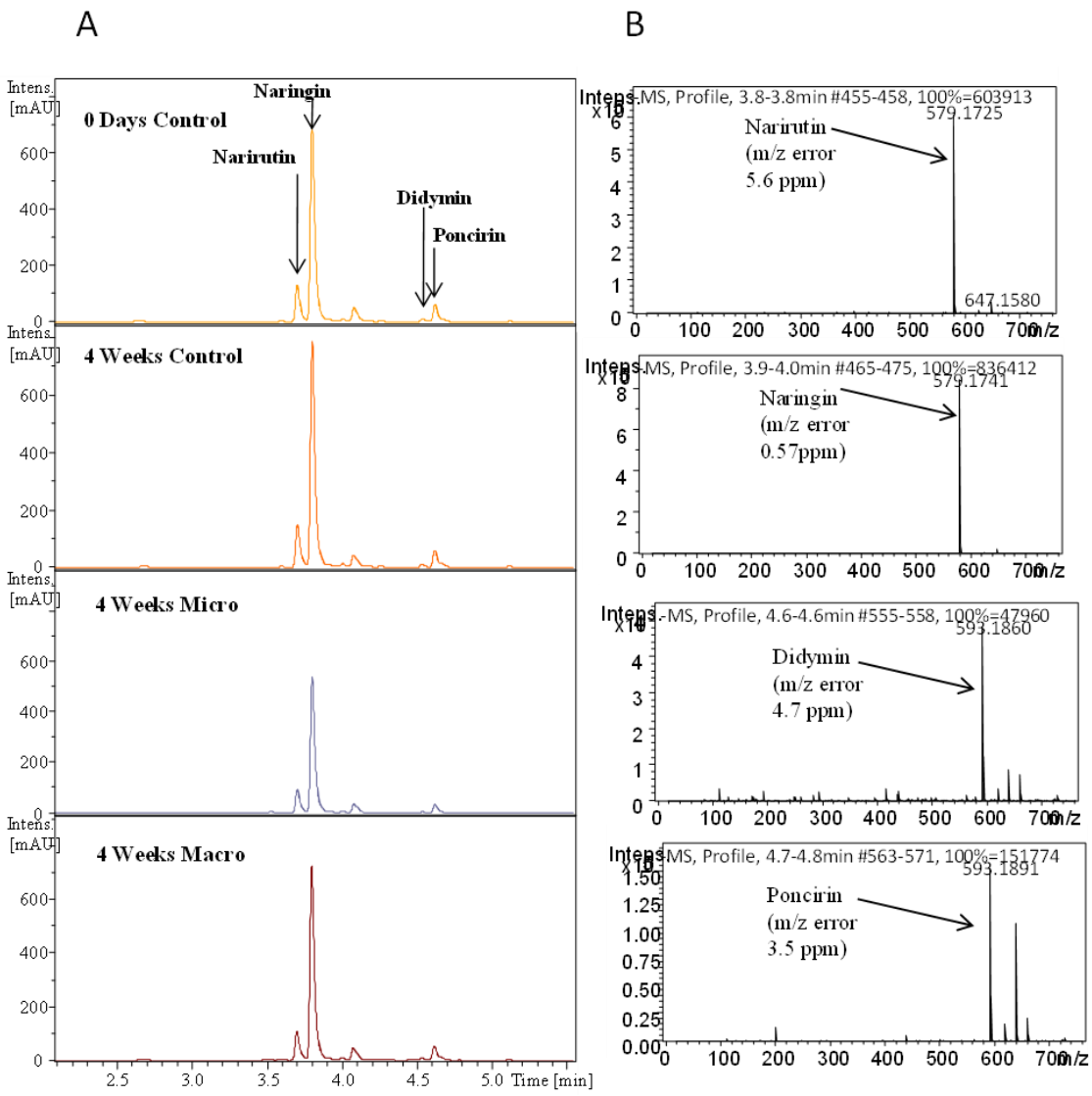

Figure 23. Representative LC-MS total ion chromatogram of flavonoids of control grapefruits and fruits stored in micro-perforated and macro-perforated bags at 10 °C followed by one week of storage at shelf conditions (20 °C). (B) Negative mode ESI–MS spectra of narirutin, naringin, didymin and poncirin. A mass error of the molecular ion is less than 5 ppm, which confirms the positive identification of measured mass compared to theoretical mass.

less than 5 ppm, which confirms the positive identification of measured mass compared to theoretical mass.

Flavonoids are phenolic compounds derived from the phenylpropanoid pathway and phenylalanine ammonia lyase (PAL) is the first enzyme in this pathway. Various biotic and abiotic factors including ethylene, chilling injury, irradiation, and low temperature affect the activity of PAL.[23, 260, 261] A previous study in plum reported a delay in increase of total phenolics when fruits were stored in MAP for up to 35 days.[236] Delays in ripening or ethylene inhibition due to MAP can affect flavonoid biosynthesis. In addition, grapefruits contain furocoumarins, phenylpropanoid compounds that can influence flavonoid biosynthesis. Further research on biosynthesis of flavonoid and furocoumarins is warranted to help us understand the mechanisms acting under different treatments and storage conditions.

Conclusion

MAP can be used as a valuable approach to maintain postharvest quality of grapefruits and reduce the occurrence of disease and rind disorders. The present study demonstrates that among the different health promoting compounds evaluated, MAP has a significant effect on carotenoids, flavanoids and furcoumarins. Overall, MAP produced no detrimental effects on fruit weight, color development, sensory taste, TSS, acidity and ripening ratio. In addition, we observed no significant effects of MAP on the levels of ascorbic acid and limonoids among all three treatments at 16 weeks of storage. Further studies on grapefruits stored at temperatures below10 ºC will examine the effect of MAP on chilling injury and changes in secondary metabolites.

CHAPTER VIII

VARIATION IN KEY FLAVONOID BIOSYNTHETIC ENZYMES AND HEALTH PROMOTING COMPOUNDS IN RIO RED GRAPEFRUIT (*Citrus paradisi* Macf) DURING FRUIT DEVELOPMENT*

Introduction

Plants produce several secondary metabolites to protect themselves from biotic and abiotic stresses. These secondary metabolites are classified on the basis of their structure, biosynthesis, and their functional groups. Flavonoids are class of secondary metabolites which occur ubiquitously in plants, and are classified in six different groups on the basis of their molecular structure: flavones, iso-flavones, flavonols, flavanones, anthocyanidins, and flavanols (catechins).[105]

Citrus fruits are rich sources of flavanones, which are present in different fruit parts including rind (flavedo), albedo, juice sacs, lamella, and seeds.[167] Flavanones are synthesized in response to different stresses. Naringin, narirutin, hesperidin, quercetin, eriocitrin, neohesperidin, didymin, poncirin etc., are common flavanones from different citrus species. These compounds are good antioxidants, chelating agents, and can benefit human health in different ways.[57, 262] For example, flavonoids inhibit DNA damage,[113, 114] tumor development,[117] and cell proliferation,[118] thus potentially acting as chemopreventative agents for different cancers.

*Reprinted with permission from "Variation in key flavonoid biosynthetic enzymes and phytochemicals in 'Rio Red' grapefruit (*Citrus paradisi* Macf.) during fruit development." by Chaudhary, P. R.; Bang, H.; Jayaprakasha, G. K.; Patil, B. S., 2016, *Journal of Agricultural and Food Chemistry. 64*, 9022-9032 Copyright [2016] American Chemical Society.

Flavonoids are biosynthesized through the phenylpropanoid pathway, which is a common pathway for tannins, flavonoids, lignins, stilbenes, and coumarins. Phenylalanine is the common precursor, marking as the first step in the phenylpropanoid pathway. Phenylalanine ammonia-lyase (*PAL*), the first enzyme involved in phenylpropanoid pathway, converts phenylalanine into cinnamate. Chalcone synthase (*CHS*), the first enzyme in flavonoid biosynthesis, converts 4-coumaroyl-coA into naringenin chalcone, which is further converted into naringenin (flavanone) by chalcone isomerase (*CHI*).[263] Naringenin is the main precursor for all flavanone aglycones which is further converted into naringenin 7-O-glucoside with help of 7-O-glucosyltransferase. The conversion of naringenin-7-O-glucoside into naringin is catalyzed by 1,2-rhamnosyltransferase (*2RT*); while, 1,6-rhamnosyltransferase (*6RT)* catalyzes the conversion of naringenin-7-O-glucoside to narirutin.[148]

Several factors influence *PAL* expression in citrus fruits, including pathogen attack,[264] irradiation,[141] ethylene and chilling injury.[22, 42] Moriguchi et al. isolated *CHS* from *Citrus sinensis* (Osb.) and *CHI* from *Citrus unshiu* (Mark.), and studied their expression in citrus fruits during development.[265, 266] However, these genes have not yet been isolated and studied in grapefruit (*Citrus paradisi*, Macf.). In the current study, we isolated genes corresponding to *PAL*, *CHS*, *CHI*, and *2RT* from immature grapefruits and studied their expression in juice vesicles of Rio Red grapefruit. This is the first study to isolate the main genes involved in flavanone biosynthesis from grapefruit and understanding the expression pattern during fruit development.

In addition, grapefruit also contains other secondary metabolites such as carotenoids, vitamin C, limonoids, and furocoumarins which are reported to have several health promoting effects. Grapefruit juice drug interactions are mainly attributed to increase in bioavailability of drugs due to inhibition of cytochrome P450 enzyme family by furocoumarins.[133] Therefore, further information regarding accumulation of naturally occurring compounds in the pulp during fruit development and maturity is highly desirable to understand how these processes are regulated in grapefruit. In current study, other health promoting compounds including vitamin C, carotenoids, limonoids and furocoumarins, were also examined by analyzing fruits harvested at different developmental and maturity stages. The current study will be useful not only to guide further research studying the main mechanisms regulating the secondary metabolites biosynthesis, but also for the industry, paving the way for its continuous search of new sources of naturally occurring compounds for different uses.

Materials and methods

Plant material

For cloning experiment, immature Rio Red grapefruits were harvested in June 2012 from TAMUK-Citrus Center, Weslaco, Texas. For maturity study, fruits were harvested at different developmental stages at intervals of 75 days in June (Stage I, cell division), August (Stage II, cell expansion), November (Stage III, maturity, early season), January (Stage III, maturity, mid-season), and April (Stage III, maturity, late season) in 2012-2013 (**Figure 24A**). Fruits were harvested from three different trees from different blocks representing three replications. Four fruits from each trees were

used for gene expression and phytochemical analysis. Juice samples for phytochemical analysis were prepared by blending individual peeled grapefruits and were stored at -80 °C until further analysis.

Chemicals

Reagent grade butylated hydroxytoluene (BHT), metaphosphoric acid, tris (2-carboxy ethyl) phosphine hydrochloride, L-ascorbic acid, lycopene, β-carotene, narirutin, naringin, neohesperidin, didymin, poncirin, limonin, 6', 7'-dihydroxybergamottin (DHB), bergamottin and auraptene were procured from Sigma Aldrich Co. (St. Louis, MO, USA). Analytical grade solvents were obtained from Fisher Scientific Research (Pittsburgh, PA, USA).

Vitamin C quantification

Vitamin C was extracted and quantified using liquid chromatography according to optimized protocol.[178] Each sample was analyzed three times and the total ascorbic acid contents were expressed as mg/100 mL juice.

Carotenoid analysis

Extraction of carotenoids was performed according to a previously published method with slight modifications.[177] Juice samples (10 g) were extracted using chloroform (15 mL) containing BHT (0.2%). An Agilent HPLC 1200 Series (Foster City, CA, USA) system consisting of a solvent degasser, quaternary pump, autosampler, column, oven, and diode array detector was used for quantification. A C-18, Gemini 5 µm column (250 mm × 4.6 mm i.d.) with a guard cartridge was used (Phenomenex, Torrance, CA, USA). Elution was carried out using a gradient mobile phase of

acetonitrile (A) and isopropyl alcohol (B). Carotenoids were detected at 450 nm and quantified using external standard calibration.

Limonoids, flavonoids, and furocoumarins analysis

Sample preparation

Extraction was carried out according to previously published method with slight modification.[177] Freeze dried juice samples (1 g + 5 mL water) were extracted ethyl acetate (15 mL) on a shaker for 3 hours. The organic layer was separated and the residue was extracted twice. All extracts were pooled and the solvent was evaporated to dryness. The dried residue was reconstituted with 4 mL acetone, filtered using a 0.45 µm PTFE filter, and further analyzed for limonoids, flavonoids, and furocoumarins using HPLC.

Quantification of limonoids and flavonoids using HPLC

Limonoids and flavonoids were quantified simultaneously using a Waters HPLC (Milford, MA, USA), spectra model with a PDA detector (2996) coupled with a binary HPLC pump 1525 and 717 plus auto sampler. The chromatographic separations were conducted on a C-18, Gemini 5 µm column (250 mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA). Limonoids were detected at 210 nm and flavonoids were detected at 280 nm.[177] The entire chromatographic separation was performed with a gradient mobile phase of 0.03 M phosphoric acid (A) and acetonitrile (B) with 1 mL/min flow rate. Each sample was injected three times.

Quantification of furocoumarins using HPLC

Furocoumarins were analyzed using previously described method.[177] Each sample was analyzed in triplicate and the results were expressed as µg/g dry weight.

RNA isolation and cDNA synthesis

Total RNA was isolated from juice vesicles of grapefruit with the RNeasy plant mini kit (Qiagen, Valencia, CA, USA). First-strand cDNAs were synthesized using the Advantage RT-for-PCR kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions.

Gene cloning. SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) was used to synthesize *PAL*, *CHS*, *CHI*, and *2RT* first strand cDNAs from immature Rio Red grapefruit. Forward and reverse degenerate primers were designed using available gene sequences through NCBI (http://www.ncbi.nlm.nih.gov/) from citrus species and other crops using Blockmaker and codehop software (**Table 10**). High fidelity and proof reading Advantage® 2 Polymerase (Clontech, CA, USA) was used for RACE cDNA amplification pcr. After purification, PCR products were cloned in the TOPO vector (Invitrogen, Carlsbad, CA, USA). Colonies were picked and amplified using same primers and Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA), and then sequenced in both directions. Full-length sequences were aligned using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/-clustalw2/).

Southern blot analysis

Genomic DNA was extracted from immature grapefruits using the CTAB method with slight modifications.[267] Genomic DNA was digested to completion with the restriction enzymes *Eco*RI and *Hind*III for *PAL* and *2RT* and with *Ava*I, *Bsa*I and *Xba*I for *CHS* and *CHI*. The digested DNA was then subjected to southern blot analysis under

high stringency. DIG-labeled probes were synthesized using DIG PCR Probe Synthesis kit (Roche Diagnostics Corp, Indianapolis, IN USA).

Table 10. Primers used for cDNA cloning and real-time PCR

Gene detected	Primer sequence (5'-3;) Cloning
PAL	Forward: TGCTGTTGGTTCTGGCCTGGCTTC
	Reverse: GGTGAACTCGGGCTTGCCGBDCATNACYTC
CHS	Forward: CGGCGCCGACTACCAGCTCA
	Reverse: CACTCAACACTTGACGTGTGGCCTTCA
CHI	Forward: CCGGCAAATGGAAGGGGAAGACT
	Reverse: CCGATCATCGACTCCAGTACTGCCTCA Reverse: GGCAATGCAATTTTCGGCAACCT
2RT	Forward: TGGGCACATAGCTCCACACCTTGA
	Reverse: CTGCTGCCCATGGTTGGAACAAAT
Gene detected	Primer sequence (5'-3;) qPCR
PAL	Forward: CTTTGAGTTGCAGCCTAAGGAGGGTCT
	Reverse: GGGCATAACGATCCTGTTTCGGC
CHS	Forward: CCCTGCCGATACTCATCTTGATTCT
	Reverse: TAGACTCCACTTGGTCCAGAATTGC
CHI	Forward: GAATCCGTTGAGTTCTTCAGAGACG
	Reverse: GCAACACCATCTTTCGGTATTGAAC
2RT	Forward: ATCCCAGCCTGAAATACCCTTTCTT
	Reverse: TGGAAGGAAAGTACTCACTGCCAAA
Actin	Forward: GAACGGGAAATTGTCCGTGACATGA
	Reverse: CTGGCCATCAGGCAGCTCATAGTTC

Real-time PCR analysis

Quantitative real-time PCR was performed on a CFX96 Real Time System (Bio-Rad, Hercules, CA, USA), using the SSO Advanced SYBR Green Supermix (Bio-Rad, USA). Reaction mix and conditions followed the manufacturer's instructions. The protocol for all genes analyzed consisted of 30 s at 95 °C for pre-incubation, then 40 cycles of 5 s 95 °C for denaturation, 30 s at 61 °C for annealing, and 10 s at 72 °C for

extension. Fluorescence intensity data were acquired during the extension time. For expression measurements, we used the Bio-Rad CFX manager Software release (Bio-Rad, USA) and calculated expression levels relative to values of a reference sample using the Relative Expression Software Tool. Normalization was performed using the expression levels of the *Actin* gene. For all genes analyzed, the reference sample was the expression value obtained in juice sacs of immature fruit harvested in June. Results are the average of three replicates.

Statistical analysis

One way analysis of variance (ANOVA) was performed using PASW Statistics 18 software (SPSS Inc.). A general linear model was used to test significant differences and means were compared using Tukey's HSD test at 5% probability level. The results were expressed as means ± SE.

Results and discussion

Vitamin C quantification

Vitamin C is one of the most important and abundant anti-oxidants present in grapefruit. Several studies have reported its health benefits. In the present study, vitamin C levels decreased in Rio Red grapefruit as the fruits matured (**Figure 24 B**). Immature fruits harvested in June had significantly higher levels than the mature fruits, with nearly two-fold higher vitamin C contents, compared to the other harvests. Fruits attain maximum size and maturity by November and start ripening. Vitamin C levels were maintained during maturation from November to April. Higher levels of dehydroascorbic acid (DHA) were observed in immature fruits harvested in June (25.64

mg/ 100 mL juice). However, very low levels (< 2 mg/100 mL juice) of DHA were detected as the fruits developed and matured from August to April **(Figure 24 B**). In the present study, fresh juice was used for analysis to avoid any degradation during freeze-drying. The water content in mature fruits was higher than in immature fruits, which can lead to lower concentrations of vitamin C due to dilution. However, even after calculating the dry-weight equivalent, immature fruits showed higher vitamin C than mature fruits. A previous study in Valencia orange and lemon showed similar decreases in vitamin C per gram as the fruits matured.[167] Similar results were reported in four citrus species (mandarin, clementine, orange and grapefruit) where decrease in ascorbic acid and DHA levels were observed in pulp as the fruits developed and matured, with highest levels found in fruits harvested in June.[268] Unlike in the current study, where the vitamin C content remained stable in mature fruits, our previous study showed mature Rio Red grapefruits from November (early season) had higher vitamin C than in April.[269] Vitamin C levels in fruits are affected by several factors including de novo biosynthesis, degradation and recycling of monodehydroascorbate and dehydroascorbate to ascorbic acid.[268]

Carotenoids analysis

Citrus fruits contain more number of carotenoids than any other fruit crop. However, only three species of citrus accumulate lycopene and β-carotene in pulp, namely orange mutants such as Hong Anliu and Cara Cara, red-fleshed grapefruits, and pummelo. In the current study, lycopene and β-carotene showed different accumulation patterns in the juice vesicles (**Figure 24 B**). Both carotenoids were significantly low in

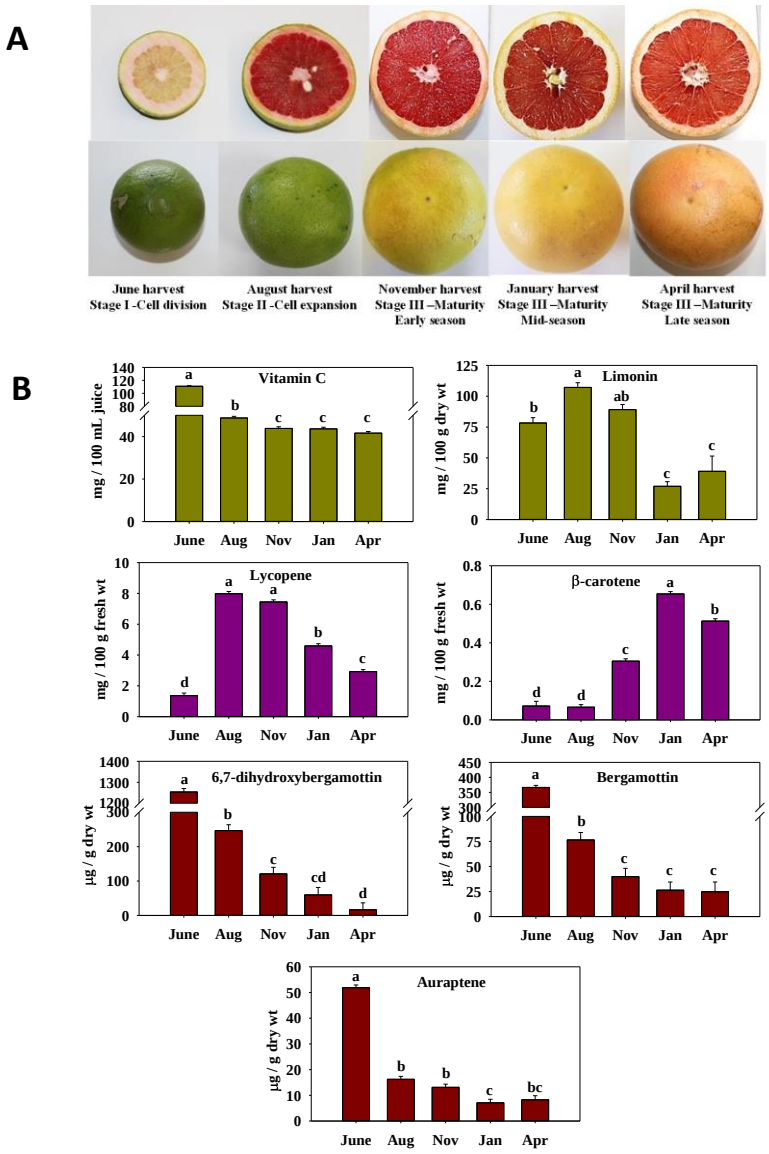

Figure 24. Grapefruits harvested at different developmental and maturity stages. B. Changes in vitamin C, limonoids (limonin), carotenoids (lycopene, and β-carotene), and furocoumarins (6,7-dihydroxybergamottin, bergamottin and auraptene) in pulp during grapefruit development and maturation from June to April. Data represent means ± S.E. of three replications, each replication containing four samples. Means with different letters indicate significant differences at each time period ($P< 0.05$).

fruits harvested in June. Lycopene levels sharply increased in immature fruits harvested in August and then gradually decreased with maturity from November to April. By contrast, β-carotene levels gradually increased until mid-season (January) and then significantly decreased in late-season fruits (April). Lycopene content was significantly higher in August and November fruits, while it was lowest in immature fruits harvested in June. On other hand, β-carotene was higher in January and lower in fruits harvested in June and August. Lycopene levels were higher than β –carotene levels throughout the study. Lycopene β-cyclase is a key regulatory enzyme that catalyzes conversion of lycopene into β-carotene[270] and lycopene accumulation in grapefruit has been proposed to be linked to a lower expression and functionality of β-cyclase enzymes.[271] There was rapid accumulation of lycopene in immature fruits from June to August. During maturation, lycopene degraded gradually while β-carotene accumulated during maturation in early- and mid-season fruits (November to January) but later decreased in late-season fruits (April). Previous studies in grapefruit have reported decrease in lycopene during different harvest seasons as the fruits ripened.[186, 269] In a recent study in Yuzu (*Citrus junos Sieb* ex Tanaka), Kjool (*Citrus unshiu* Marcow), and Dangyooja (*Citrus grandis* Osbeck), similar trends were observed, with lycopene decreasing and β-carotene increasing with maturity.[167]

Limonoids analysis

Limonin is one of the bitter components of grapefruit and generally decreases with fruit maturity, thus reducing the bitterness. In current study, limonin was detected in fruits at all stages of maturity (**Figure 24 B**). Limonin levels increased significantly in

immature fruits from June to August, but decreased markedly from November as the fruits matured. The levels of limonin were found to be significantly higher in immature and early-season fruits (November), as compared to mid- (January) and late-season (April) harvest. For example, in mid-season fruits, limonin was detected only in few samples. Similarly, nomilin was detected only in the immature fruits (June and August, data not shown). Hasegawa et. al. studied the biosynthesis and accumulation of limonin in citrus and reported that both immature and mature fruits synthesize limonin from nomilin, obacuanone, and obacunoate.[95] Non-bitter limonin monolactone (namely limonoate A- ring lactone) is reported to be converted to bitter dilactone limonin when it comes in contact with citrus juice.[272, 273] Limonoate A- ring lactone is reported to decrease as the fruits matured in navel oranges.[93]

Furocoumarins quantification

Furocoumarins and coumarins are phenylpropanoids present in grapefruit pulp and peel.[274] In the current study, we detected two furocoumarins in grapefruit pulp, 6, 7-dihydroxybergamottin (DHB) and bergamottin; we also detected one coumarin, auraptene (**Figure 24 B**). All three compounds were significantly higher in immature fruits and gradually decreased as the fruit matured. Immature fruits harvested in June had significantly higher DHB (~5-fold), bergamottin (~ 4-fold) and auraptene (~ 4-fold) than the fruits harvested in August. Furthermore, DHB and bergamottin were significantly higher in August than in the remaining three harvests. DHB levels decreased nearly 75-fold from the earliest harvest in June (1253.9 µg/g) to the late-season harvest in April (16.5 µg/g), bergamottin decreased 15-fold from June (366.5

µg/g) to April (24.8 µg/g), and auraptene decreased 6-fold from June (51.9 µg/g) to April (8.3 µg/g). Furocoumarins are phytoalexins, which protect from pests and diseases. A previous study in Rio Red grapefruit reported similar trends, where DHB and bergamottin decreased from early season (November) to late season (May).[120] Auraptene is a prenylated coumarin found in higher concentrations in citrus (grapefruit) peel and peel oil as compared to juice sacs; auraptene also has anti-tumor properties.[275] In Chinotto (*Citrus* × *myrtifolia* Raf.) furocoumarins, namely bergapten and epoxybegamottin, also decreased with fruit ripening.[276]

Quantification of flavonoids

The levels of different flavanones in the fruits at different stages were measured (**Figure 25**). Grapefruit contains mainly flavanones, which include rutinosides and neohesperidoses. Rutinosides are tasteless; while, neohesperidoses impart bitter taste. In grapefruit, naringin, poncirin, and neohesperidin are neohesperidoses; while, narirutin and didymin are rutinosides. Narirutin, didymin and neohesperidin levels were several fold higher in the pulp of immature grapefruits collected in June, relative to the levels in the pulp of fruits collected at other time points. The levels of these compounds decreased significantly in immature fruits collected in August, and in mature fruits.

The accumulation of naringin and poncirin in grapefruit pulp differs from other flavanones described above. Naringin and poncirin levels in fruit pulp gradually

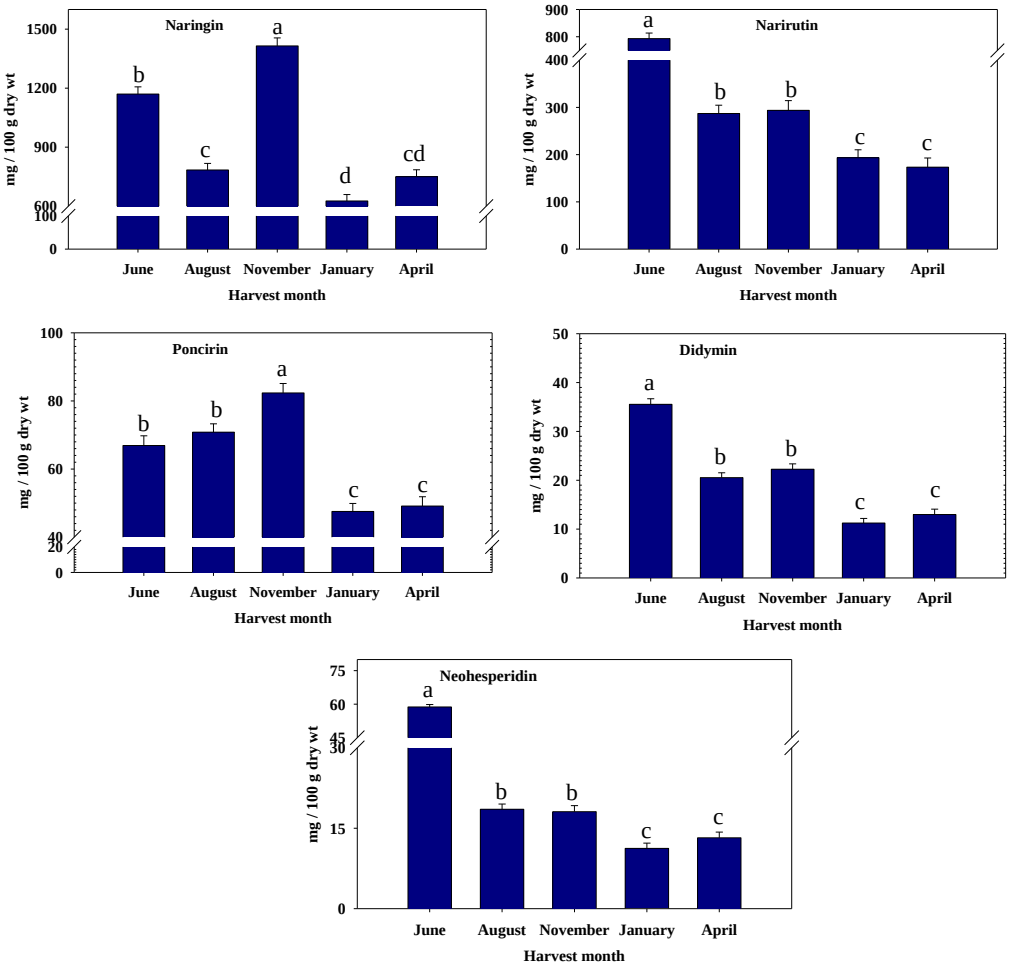

Figure 25. Variation in flavonoid levels in grapefruit pulp during development and maturation from June to April. Data represent means ± S.E. of three replications, each replication containing four samples. Means with different letters indicate significant differences at each time period ($P< 0.05$).

increased until November, but declined sharply during maturity from January. The grapefruit flavonoid profile is distinct from that of other citrus species, as grapefruit predominantly contains naringin, in higher concentrations than in other citrus species.[277]

Citrus fruits containing naringin are also reported to contain neohesperidin, which could be related to the biosynthetic pathway (**Figure 26**).[277] In the present study, naringin was the most abundant flavanone and accounted for more than 50% of total flavonoids (**Figure 25**). Similar results were previously reported by Hagen et al in Ruby Red grapefruit, where decreases in flavanone glycosides including narirutin, naringin, neohesperidin, didymin and poncirin were observed with fruit maturity.[108] Previous studies have suggested that naringin is higher in young tissues as compared to mature tissues in grapefruit.[278] In yuzu citrus, both hesperidin and naringin are present and their levels decreased in pulp and increased in peel with fruit maturity.[279] Another study in Satsuma mandarin reported decrease in flavanones with fruit maturity, with hesperidin and narirutin being the major flavonoids detected.[266]

Naringin is the major characteristic flavanone present in bitter tasting citrus fruits such as grapefruit and pummelo and is not found in other non-bitter citrus species such as sweet orange and mandarin. Previous studies have focused on characterization of *6RT* leading to biosynthesis of non-bitter rutinosides such as narirutin and hesperidin in other citrus varieties [148]. However, not much attention has been given to temporal expression of *2RT* in bitter citrus species such as grapefruit. Therefore, to further understand the different biochemical steps involved in production of the bitter component, genes involved in naringin biosynthesis were cloned and their expression during fruit development all the way to maturity was studied.

Sequence analysis of PAL, CHS, CHI, and 2RT

Four main genes involved in naringin biosynthesis were selected based on their regulatory role. *PAL* catalyzes the first committed step in phenylpropanoid pathway by deaminating L-phenylalanine to trans-cinnamic acid (**Figure 26**). *CHS* condenses three malonyl Co-A molecules with one molecule of 4-coumaroyl Co-A to synthesize naringenin chalcone, which is further isomerized to naringenin with help of *CHI*. Naringenin is glucosylated at seventh position by *7-O-glucosyltransferase* and further glucosylated to form either bitter naringin by *2RT* or into tasteless narirutin by *6RT*. We selected to study the biosynthesis pathway of naringin, it being highest in content among flavanones and imparting bitter taste which is distinct to grapefruit and pummelo fruits. PCR products of *PAL*, *CHS*, *CHI*, and *2RT* were sequenced individually after extraction, purification and cloning. The open reading frames for *PAL*, *CHS*, *CHI*, and *2RT* were 1467, 1203, 669, and 1359 bp, respectively. Multiple copies of *PAL*, *CHS* and *CHI* were detected having differences in nucleotide and deduced amino acid sequences (**Figure 27-28**). Total 13 colonies were picked and sequenced for *PAL*, out of which *PAL1* had four colonies, *PAL2* had one colony and *PAL3* had three colonies. While only partial sequences were obtained from other colonies (data not included). Three amino acid differences were observed among *PAL1*, *PAL2* and *PAL3* (**Figure 27 A**). *PAL1* and *PAL2* have three nucleotide differences and one amino acid difference.

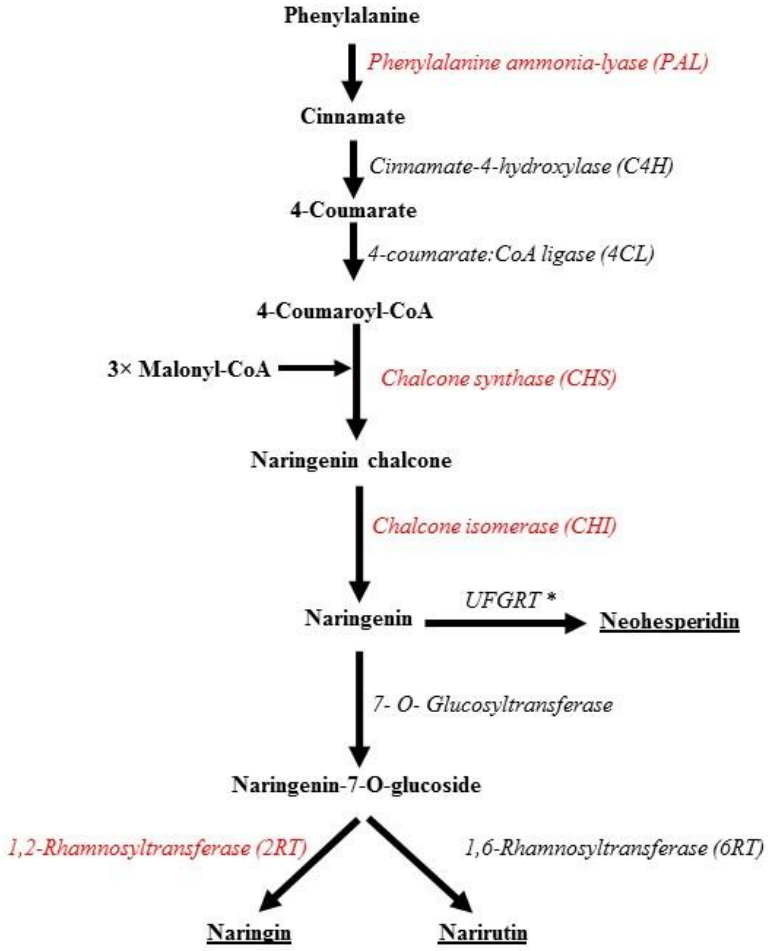

Figure 26. Flavonoid biosynthesis pathway in grapefruit. (*UFGRT - UDP-rhamnose flavanone glucoside rhamnosyltransferase). Genes involved in naringin biosynthesis were cloned (highlighted in red) and their expression during developmental to maturity stages was studied.

While, *PAL3* has fifteen nucleotide differences and two amino acid differences with *PAL1* and *PAL2*. The deduced amino acid sequence of grapefruit *PAL* (**Table 11**) showed significant sequence identity to *Citrus clementina* × *reticulata FPAL1*

(CAB42793.1) (96%) and *FPal2* (CAB42794.1) (99-99%), *Arabidopsis thaliana* (AAC18870.1) (85%), *Vitis vinifera* (AEX32790.1) (89%), Oryza sativa (NP_001047481.1) (73%) and *Prunus salicina* (AFP24940.1) (86%). The grapefruit *PAL* sequence has higher amino acid sequence similarity to *FPAL2* than *FPAL1* of *Citrus clementina* × *reticulata.*

Total 16 colonies were picked and sequenced for *CHS* out of which *CHS1* had five colonies, *CHS2* had four colonies and remaining other isoforms had single colony each. In *CHS,* copies from *CHS1* to *CHS4* have only nucleotide differences and no amino acid differences (**Figure 27 B**). *CHS3* (partial cds) obtained from gDNA has an intron between two exons, which is most common form of alternate splicing in plants [280] and is observed in *CHS* genes from other crops.[281-283] While, copies from *CHS6* to *CHS9* have differences in amino acid sequences. *CHS1* has deletion of ten nucleotides, while *CHS5* has deletion of two nucleotides leading to difference in amino acids at the end of the sequence. This deletion is not observed in *Citrus maxima* (ACX37403.1) or *Citrus sinensis* (BAA81663.1; BAA81664.1). Further, there is single amino acid substitution in *CHS6, CHS7* and *CHS8* while double amino acid substitution in *CHS9*. Comparison of deduced amino acid sequence of grapefruit *CHS* indicated 99% identity with the *CHS* from *Citrus maxima* cultivar Feng wei (ACX37403.1), which is female parent of grapefruit (**Table 11**). On other hand, grapefruit *CHS* had higher amino acid sequence similarity with *CitCHS2* (BAA81664.1) (~98%) as compared to *CitCHS1* (BAA81663.1) (~87%) of its male parent crop, *Citrus sinensis*.

Table 11. Deduced amino acid sequence identity between *PAL, CHS, CHI, 2RT* cDNA clones from grapefruit and other plant species

Grapefruit	Other plant species					
PAL	*C. cl-ret-FPAl1*	*C.ccl-ret-FPAL2*	*A. thaliana*	*V. vinifera*	*O. sativa (Jap)*	*P. salicina*
1	96	99	85	89	73	86
2	96	98	85	89	73	86
3	96	98	85	89	73	86
CHS	*C. maxima*	*C. sinensis CHS1*	*C. sinensis CHS2*	*A. thaliana*	*V. vinifera*	*O. sativa*
1	99	86	99	85	89	85
2	99	86	99	85	89	85
3	99	89	99	88	91	85
4	99	87	99	87	89	84
5	99	87	98	87	89	85
6	99	87	98	87	89	85
7	99	87	98	87	89	85
8	99	87	98	86	89	84
9	98	86	97	86	88	83
CHI	*C. maxima*	*C. sinensis*	*A. thaliana*	*V. vinifera*	*O. sativa*	*P. vulgaris*
1A	99	100	68	76	61	56
1B	96	100	68	76	61	56
1C	96	100	68	76	61	56
2	97	99	68	76	61	56
3	100	96	68	75	62	55
4	99	97	68	75	62	55
2RT	*C. maxima*	*C. max Feng.*				
1	100	99				

**C. paradisi – Citrus paradisi, C. maxima – Citrus maxima, C. jamberi – Citrus jamberi, C. unshiu – Citrus unshiu, C. sinensis – Citrus sinensis, C. cl-ret – Citrus clementina × reticulata, A. thaliana – Arabidopsis thaliana, V. vinifera – Vitis vinifera, O. sativa – Oryza sativa (japonica), P. vulgaris – Phaseolus vulgaris, P. salicina – Prunus salicina, Citrus maxima* cultivar Fenghuangyou.

Identity of grapefruit CHS isoforms is > 80% with *Arabidopsis thaliana* (NP_196897.1), *Vitis vinifera* (AAB72091.1) and *Oryza sativa* (BAB39764.1).

Total 24 colonies were picked and sequenced for *CHI* out of which *CHI1A* had ten colonies, *CHI3A* had nine colonies, while *CHI1B*, *CHI1C*, *CHI2*, *CHI3B* and *CHI4* had one colony each. In *CHI* copies, *CHI1 A*, *B* and *C* have only nucleotide differences and no differences in amino acid sequence. *CHI1* (*A,B,C*) deduced amino acid sequences have complete similarity with *Citrus sinensis* (BAA36552.1) (**Table 11**).

CHI1A nucleotide sequence (ORF) has complete similarity with *Citrus sinensis* nucleotide sequence (AB011794.1). *CHI2* has just one amino acid and two nucleotide differences with *CHI1* and *Citrus sinensis* sequence (**Figure 28 A**). While, deduced amino acid sequence and nucleotide sequence (ORF) of *CHI3* has complete similarity with *Citrus maxima* cultivar Feng wei (ADB92596.1). *CHI4* has one amino acid and three nucleotide differences with *CHI3* and *Citrus maxima* sequence. Total there are six amino acid differences between *CHI1* and *CHI3* same as the differences between sequences of *Citrus sinensis* (AB011794.1) and *Citrus maxima* (ADB92596.1). Grapefruit is a cross between *Citrus maxima* and *Citrus sinensis* and therefore, *CHI* copies having exact similarities with both species are found in the grapefruit genome. Furthermore, grapefruit *CHI* isoforms showed 68% identity with *Arabidiopsis thaliana* (AAA32766.1), 76% identity with *Vitis vinifera* (CAA53577.1), 61% identity with *Oryza sativa* (AAM13448.1), and 56% identity with *Phaseolus vulgaris* (CAA78763.1) (**Table 11**).

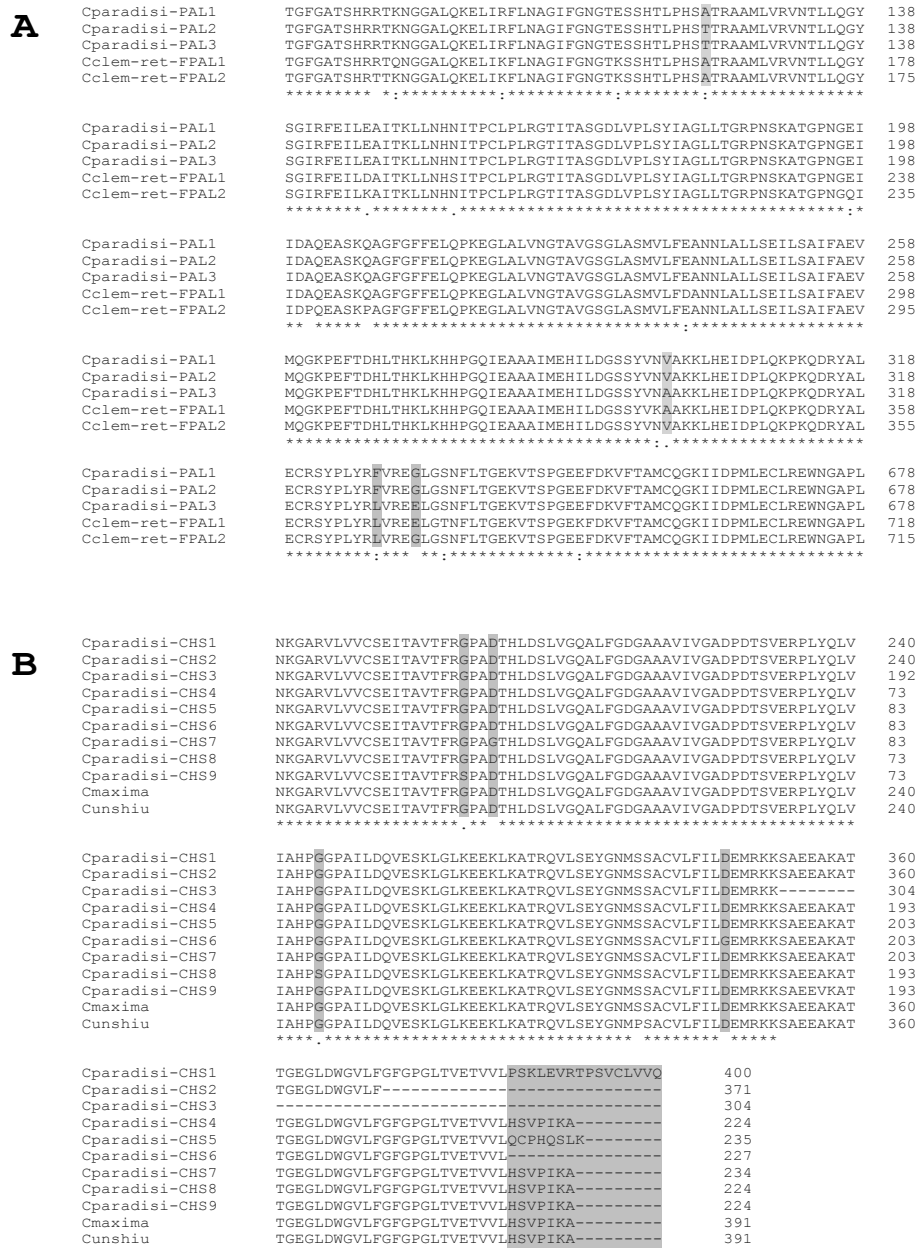

Figure 27. Amino acid sequence alignment of different copies of the *PAL* (A) and *CHS* (B) genes in grapefruit with other species - *Citrus clementina* × *Citrus reticulata (FPAL1 and FPAL2), Citrus maxima, Citrus unshiu.* Differences among deduced amino acid sequences within grapefruit isoforms isolated in the current study are indicated by highlights.

Total 12 colonies were picked and sequenced for *2RT*. Grapefruit *2RT* deduced amino acid sequence showed 100% similarity with *Citrus maxima* (AAL06646.2), while it had one amino acid and nucleotide difference with *2RT* of *Citrus maxima* cultivar Fenghuangyou (ACX70154.1) (**Figure 28 B, Table 11**).

Southern blot analysis

To determine the copy number of these genes in grapefruit, we performed Southern blot analysis on genomic DNA (**Figure 29**). This analysis showed four major bands for *PAL* under *Hind*III, suggesting that *PAL* also has a multi-gene family in citrus. In tomato 26 copies of *PAL* gene were isolated in diploid genome, with the high number of copies present due to duplication; however, most of them were inactive due to gene silencing [284]. Several multiple isoforms help to extend regulatory flexibility under different developmental and stress conditions.

In *CHS* two major bands were detected with *Ava*I restriction enzyme while just one band was seen under *Xba*I (weak band) and *Bsa*I (strong band), indicating two copies in the genome. Only one major band was detected for *CHI* under *Xba*I and indicating a single gene encoding *CHI* in grapefruit. In *2RT* single strong band was seen under *Ecor*I while two bands were seen under *Hind*III restriction enzyme. However, in current study multiple copies of *PAL*, *CHS* and *CHI* were identified after cloning and sequencing. In *PAL*, *CHS*, and *CHI* have been reported to be multigene families in grape (*Vitis vinifera*),[285] bilberry (*Vaccinium myrtillus*),[286] tomato (*Solanum lycopersicum*),[284] and petunia (*Petunia hybrida).*[282, 287] Multiple copies of genes are common and can be attributed to their duplication and redundancy[283] or their activation under different

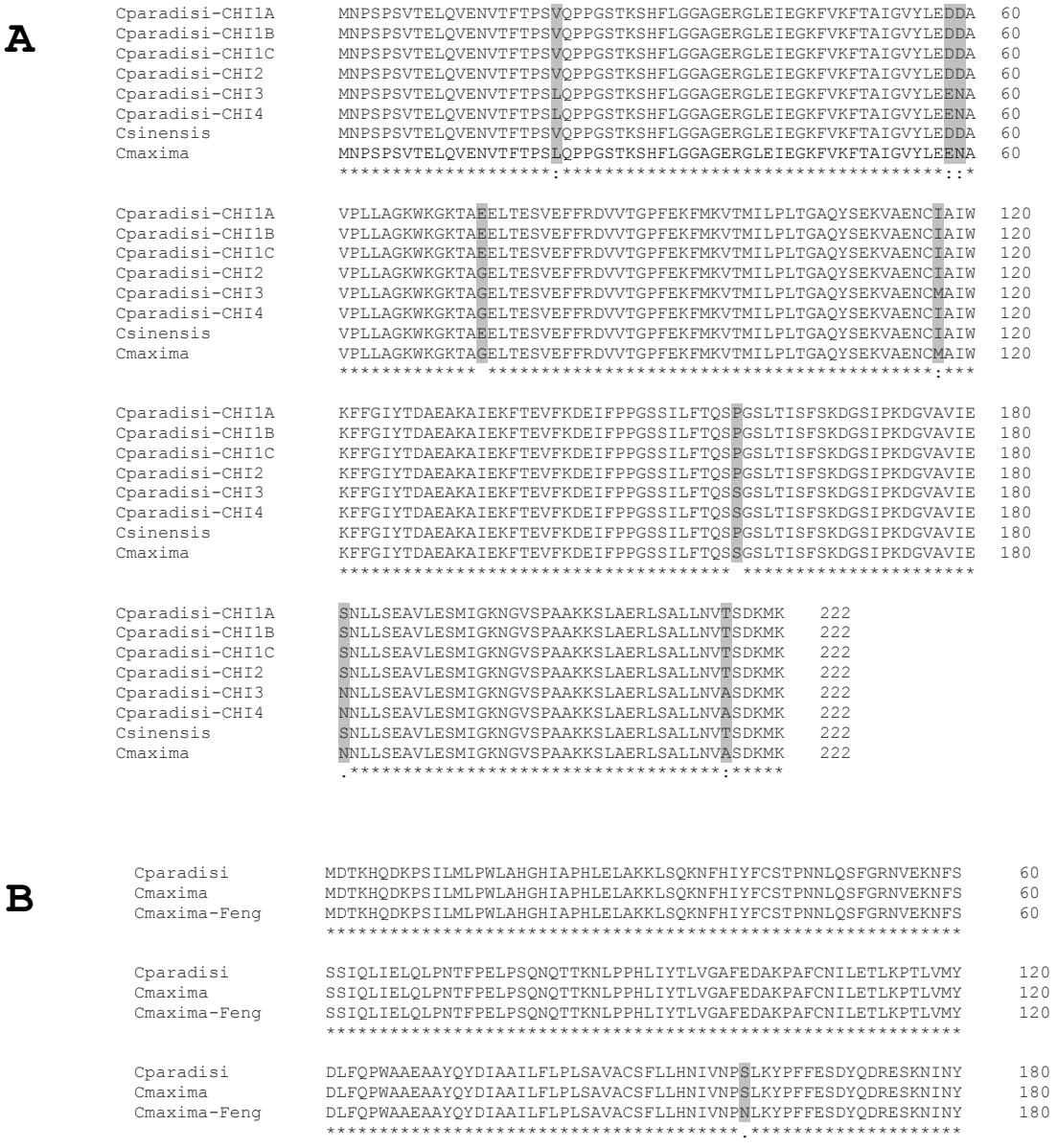

Figure 28. Amino acid sequence alignment of different copies of the *CHI* (A) and *2RT* (B) genes in grapefruit with other species - Citrus *sinensis, Citrus maxima*, *Citrus maxima* cultivar Fenghuangyou. Differences among deduced amino acid sequences within grapefruit isoforms isolated in the current study are indicated by highlights.

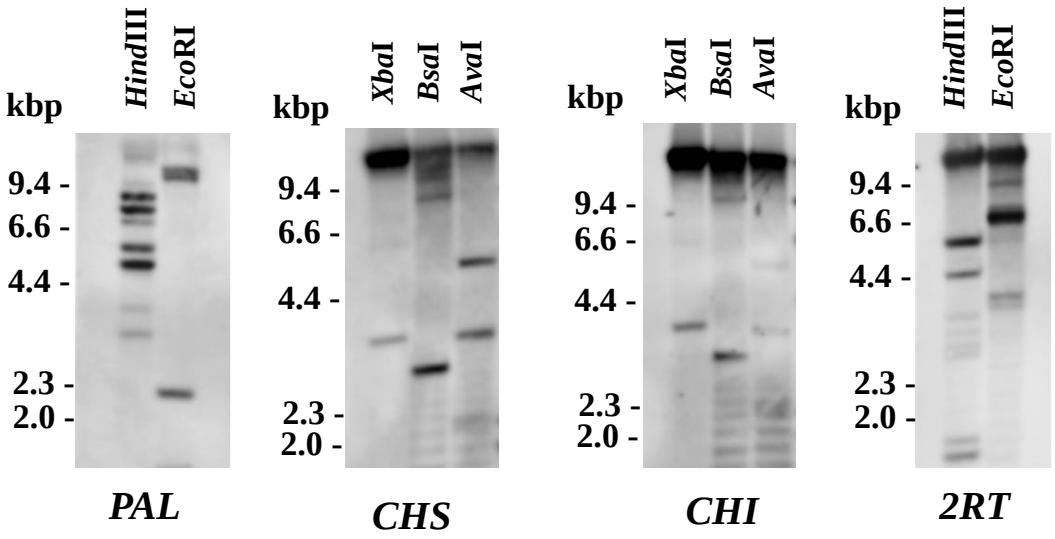

Figure 29. Southern blot analysis of genomic DNA digested with different restriction enzymes. *Hind*III, *Eco*RI, *Xba*I, *Bsa*I, *Ava*I. The blots were hybridized with the cDNA clones for *PAL*, *2RT*, *CHS*, and *CHI*. Positions of molecular mass markers are shown on the left.

stress conditions and in different tissues.[288] Less number of bands as compared to clones identified can be due to only one restriction site present and therefore at least two and possibly more copies of *CHS* and *CHI* genes can be present in grapefruit.

Flavonoid gene expression

To understand molecular mechanism underlying flavanone composition in grapefruit, expression of *PAL*, *CHS*, *CHI*, and *2RT* genes was measured during fruit development and maturation (**Figure 30**). In general, expression of *PAL*, *CHS*, and *CHI* was significantly higher in pulp of immature grapefruit harvested in June as compared to

the mature, early-, mid-, and late-season fruits harvested in November, January, and April respectively. In contrast, expression of *2RT* was significantly higher in early- and mid-season fruits compared to immature and late-season fruits. The key step where the flavanones are converted into either tasteless rutinosides is catalyzed by *6RT* or into bitter neohesperidoses is catalyzed by *2RT*. In a recent study, Chen et al. studied expression of both *2RT* and *6RT* in sweet orange tissues (*C. sinensis*).[148] Despite having higher expression of *2RT*, its functional inability resulted in very low levels of naringin, poncirin for detection in fruit tissues of 'Anliu' and 'Honganliu' sweet oranges. Therefore, higher expression of *2RT* does not warranty higher levels of naringin. In current study, *PAL*, *CHS* and *CHI* have higher activity in early developmental stages which may lead to higher accumulation of precursor naringenin in young fruitlets. In later maturity stages, *2RT* expression is higher which can be linked to higher content of naringin and poncirin in November. However, in January even though the expression of *2RT* is high, no transient increase in naringin levels is observed which can be attributed to less availability of naringenin due to low expression of the upstream genes – *PAL*, *CHS* and *CHI*. Further, *PAL* and *CHS* are key flux regulating steps in flavonoid pathway and their low expression leads to reduced levels of downstream compounds. *PAL* is the first step and main flux point for phenylpropanoid pathway and flavonoid synthesis,[289] and is also rate limiting flux point for other phenylpropanoid secondary metabolites such as chlorogenic acid.[290] On the other hand, *CHS* is the first committed step in flavonoid synthesis and drives the carbon flux to the flavonoid pathway. In strawberry, antisense *CHS* reduced the anthocyanins accumulation and increased accumulation of other

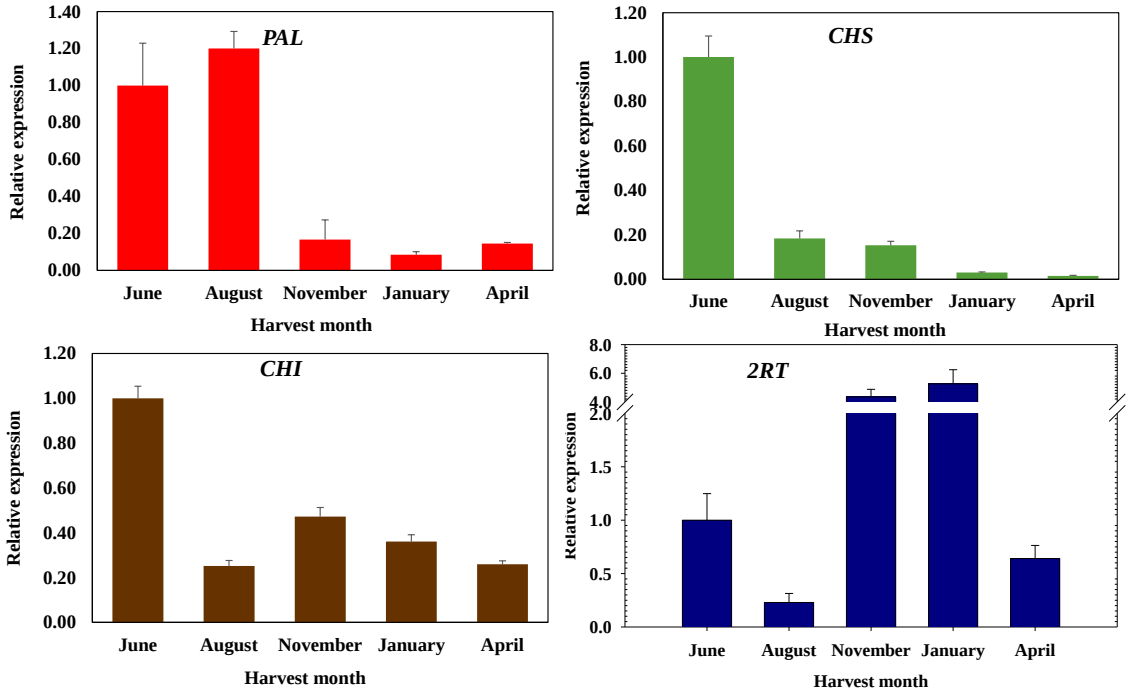

Figure 30. Relative expression levels of *PAL*, *CHS*, *CHI*, and *2RT* genes were detected by qRT-PCR in grapefruit pulp during fruit development and maturation. Data represent means ± S.E.M. of three replications, each replication containing four samples.

phenylpropanoid products by redirecting the flux into branch pathways.[291] On other hand, even though *PAL*, *CHS* and *CHI* levels are high in June and August, it is not corresponding to higher accumulation of naringin in August, which may be due to low expression of *2RT* in August. Therefore, *2RT* expression can be the bottle neck for naringin accumulation. Overall it can be concluded that naringin biosynthesis is regulated by availability of precursor compound naringenin and thus indirectly on *PAL*, *CHS* and *CHI*.

Furthermore, in red fleshed citrus fruits carotenoid and flavanone pathway are reported to be interrelated. Red fleshed citrus fruits are reported to contain higher levels of flavonoids in immature stages which gradually decreased as fruits matured.[148] In our previous study white grapefruit and pummelo varieties had higher naringin content as compared to red/pink fleshed grapefruit varieties.[259] Carotenoid pathway may have influence on flavonoid biosynthesis and needs to be further investigated.

Conclusion

This is first study in grapefruit (*Citrus paradisi*) to report the isolation of genes corresponding to *PAL*, *CHS*, *CHI*, and *2RT*, which encode enzymes from the flavonoid biosynthetic pathway, and to examine their expression in different tissues of grapefruit. Relative mRNA levels of genes encoding upstream enzymes, namely *PAL*, *CHS*, and *CHI*, were higher in immature fruits, whereas *2RT*, which encodes a downstream enzyme, had higher mRNA levels in mature grapefruits. Most of the flavonoids, vitamin C, furocoumarins, limonin, and lycopene had higher levels in immature fruits, but decreased as fruits ripened. Nevertheless, out of the marketable fruit stages, early-season grapefruits harvested in November contained more health beneficial compounds, compared with mid- and late-season grapefruits harvested in January and April respectively, and therefore are best for consumption. Naringin did not exactly follow the pattern of *2RT* or of *PAL*, *CHS*, and *CHI* expression, indicating that the four genes may have complementary effects on the level of naringin.

CHAPTER IX

COMPARATIVE ANALYSIS OF VOLATILE PROFILES OF GRAPEFRUIT AT DEVELOPMENTAL TO MATURITY STAGES

Introduction

In plants, volatile compounds occur in remarkable diversity and are important contributors to the flavor and aroma of fruits, consequently affecting the fruit sensory qualities. Citrus is commonly consumed around the world in both fresh and processed forms. Citrus fruits contain several volatile components, each with a distinct flavor and aroma. Citrus volatiles include oil soluble compounds commonly found in the peel and water soluble compounds commonly found in the juice.[292] Volatile compounds present in citrus fruits include monoterpenes and sesquiterpenes. The major component limonene occurs ubiquitously in citrus oils.[293] Grapefruit (*Citrus paradisi* Macf.) is an important citrus crop cultivated in the USA. Grapefruit volatile oil can be distinguished from other citrus volatile oils by its specific sulphur-containing compounds, such as 1-p-menthene-8-thiol,[294] hydrogen sulfide,[295] and methyl sulfide,[296] along with the sesquiterpene nootkatone,[297-299] which contribute to the aroma of grapefruit juice.

Volatile compounds also have potential uses in beverages, confections, desserts, cosmetics, perfumes, air fresheners, cleaning products, aromatherapy oils, as insect repellents, and in medicine.[300] Citrus volatiles have anti-microbial activities and other health-promoting properties.[301, 302] For example, recent work has extensively examined the role of volatile oils in cancer prevention.[303, 304] Limonene and β-

caryophyllene have chemo-preventative functions, such as induction of glutathione-S-transferase activity and uridine diphosphoglucuronosyl transferase activity in the small intestine and liver of mice.[305, 306] In addition, limonene is also reported to have chemotherapeutic activity against pancreatic, mammary and prostatic tumors.[307] Limonene helps to block the initiation and suppress the promotion and progression of mammary and liver cancer.[308] Therefore, dietary intake of these volatile components may help prevent cancer.[309]

Fruits undergo several physio-chemical changes during development and maturation, including change in size, shape, color, taste, flavor and aroma, which act as indicators for fruit maturity and harvest time. During different developmental stages the profile of volatile components vary in order to protect immature fruits from pests and herbivores and later to help mature fruits to attract seed dispersing animals.[310] Few studies have investigated the influence of developmental stages on volatiles in citrus peel.[311, 312] However, the effect of maturity stages on volatiles present in Rio Red grapefruit juice vesicles remain to be investigated. It is warranted to study changes in volatiles present in grapefruit juice vesicles instead of peel as they have health benefits and affect the flavor and aroma. Therefore, in current study volatile compounds present in juice vesicles of Rio Red grapefruits harvested at different developmental and maturity stages were collected by headspace solid phase micro-extraction (HS-SPME) and analyzed using gas chromatography-mass spectrometry (GC-MS).

Materials and methods

Chemicals

Analytical grade hexanol, limonene, α-copaene, β-caryophyllene, α-humulene, valencene, β-caryophyllene oxide, nootkatone, standard hydrocarbons, perillyl alcohol and SPME fibers were obtained from Sigma Aldrich Co. (St. Louis, MO, USA).

Plant material

Rio Red grapefruits were harvested from varietal block in Citrus Center, Texas A&M University-Kingsville (Weslaco, Texas), in June, August, November (early harvest), January (mid-harvest) and April (late harvest) during 2012-2013 at an interval of 75 days. Fruits were harvested from two to three trees from three different blocks constituting three replications. Each replication consisted of three juice samples, which were prepared by blending three peeled fruits per juice sample. Total 9 samples comprising of 27 fruits were analyzed for each harvest stage to reduce variation (n = 9).

Total soluble solids and titratable acidity

Total soluble solids (TSS) were measured using hand refractometer (American Optical Corp., South Bridge, MA, USA) and expressed as ºBrix. A DL 22 Food and Beverage analyzer (Mettler Toledo, Columbus, OH, USA) was used to measure the titratable acidity of juice. Grapefruit juice (5 mL) was mixed with 50 mL of nanopure water and titrated against 0.1 N NaOH. Acidity was expressed as percent. Ripening ratio represents ratio of TSS to percent acidity.

Sample preparation for GC-MS analysis

Perillyl alcohol, which is not present in grapefruit juice was used as an internal standard for relative quantification of volatile compounds. One gram of juice samples were weighed into 20 mL GC-MS headspace vials and 5 µL of 250 ppm perillyl alcohol was added to all juice samples. The vails were sealed with aluminum caps and vortexed for uniform distribution of standard within the juice. Standard regression equation was obtained for internal standard by analyzing serially diluted solutions. Volatile compounds were quantified by comparing the peak areas to that of internal standard and using the regression equation. Values were expressed as µg/100 g fresh weight.

Extraction of volatiles using SPME and identification by GC-MS

Grapefruit volatiles were analyzed by HS-SPME-GC-MS using a Thermo Scientific Triplus autosampler, Trace Ultra GC, and DSQ II mass spectrometer (Thermo Finnigan, Thermo Fisher Scientific, Inc., San Jose, CA, USA). One gram of juice samples were weighed into 20 mL GC-MS headspace vails. The vails were sealed with aluminum caps. Headspace – solid phase micro-extraction was conducted using 50/30 µm divinylbenzene /carboxen / polydimethylsiloxane (DVB/CAR/PDMS) fiber. The SPME fiber was initially conditioned in the GC injector at 225 °C for 1 h according to manufacturer's recommendations. The vial containing sample was preheated at 60 °C while agitating for 30 seconds. The SPME fiber was then exposed to the HS of the vial for 5 min at 60 °C for the adsorption of volatiles. The SPME fiber was introduced into the inlet of the GC for 2 min at 225 °C to desorb the volatile

compounds in splitless mode. Post-conditioning was done for 12 min after each sample. The injector port temperature was maintained at 225 °C, while the column temperature was ramped twice as follows: 50 °C for 1 min and increased to 170 °C at the rate of 6 °C/min, further raised to 225 °C at the rate of 25 °C/min and held for 1 min. Volatile compounds were separated on a fused silica Zebron ZB-WAXPlus capillary column (30 m × 0.25 mm, 0.25 micron film (Phenomenex, CA, USA) coated with bonded 100% polyethylene glycol. Helium was used as a carrier gas at a flow rate of 1 ml/min and run time was 24 min. The ion source temperature was maintained at 285 °C. The ionization voltage was 70 eV, the mass range was 45-400 amu and the scan rate was 12.82 scans/sec. The relative area was calculated using Thermo xcalibur software version 2.2 SP1.48. The volatiles were identified on the basis of their retention indices, which were calculated by injecting hydrocarbons (C8 – C24) under the same program and operating conditions according to the published method.[313] The volatile components were identified by comparing kovats indices (KI), retention times of authentic standards, and matching the spectral fragmentation patterns in Wiley library database and published mass spectra.[314-316] Limonene, α-copaene, β-caryophyllene, α-humulene, valencene, β-caryophyllene oxide and nootkatone peaks were further confirmed by spiking the authentic standards, retention times and mass spectra. Total 9 samples (each sample prepared from 3 fruits) were analyzed for each harvest stage to reduce variation.

Statistical analysis

One way analysis of variance (ANOVA) was performed using PASW Statistics 18 software (© SPSS Inc. 2009). Significant differences were tested using general linear model and means were compared using Tukey's HSD test at 5% probability level. The results are expressed as means ± SE.

Results and discussion

Fruit quality measurements

The TSS levels were found to be significantly ($P \leq 0.05$) lower during first two stages of harvest (immature stage, **Table 12**). The levels increased as the fruits matured with no significant differences observed amongst November, January and April harvest. Total acidity (%) reduced with fruit development with highest levels observed in June while lowest levels observed in April harvest. This resulted in increase in ripening ratio from June to April. Increase in TSS and decrease in acidity is commonly observed in fruits as the fruit develops and matures.

Volatile compounds analysis

In the present study we examined the effects of fruit development and maturity stages on grapefruit juice volatiles. Total 25 of volatile compounds from grapefruit juice were identified including major components such as limonene, α-copaene, β-caryophyllene, α-humulene, δ-cadinene and nootkatone (**Figure 31, Table 13**). Hydrocarbons accounted for nearly 98% while oxygenated compounds accounted for < 2% (**Table 13**). In total one acyclic, three monocyclic, seven bicyclic and one tricyclic hydrocarbon; while, four acyclic and two bicyclic oxygenated compounds were

Table 12. Total soluble solids, acidity (%) and ripening ratio for grapefruits harvested at different developmental and maturity stages.

Harvest stage	TSS (°Brix)	Acidity (%)	Ripening ratio
June	11.61 ± 0.12 b	1.78 ± 0.06 a	6.57 ± 0.24 d
August	11.84 ± 0.07 b	1.35 ± 0.0.03 b	8.82 ± 0.24 c
November	13.17 ± 0.32 a	1.23 ± 0.05 bc	10.81 ± 0.40 b
January	13.43 ± 0.33 a	1.12 ± 0.05 cd	12.09 ± 0.36 b
April	13.19 ± 0.25 a	0.96 ± 0.04 d	13.84 ± 0.49 a

Data represent means ± S.E. of nine juice samples (n=9). Means with different letters denote significant differences ($P < 0.05$) between harvest stages.

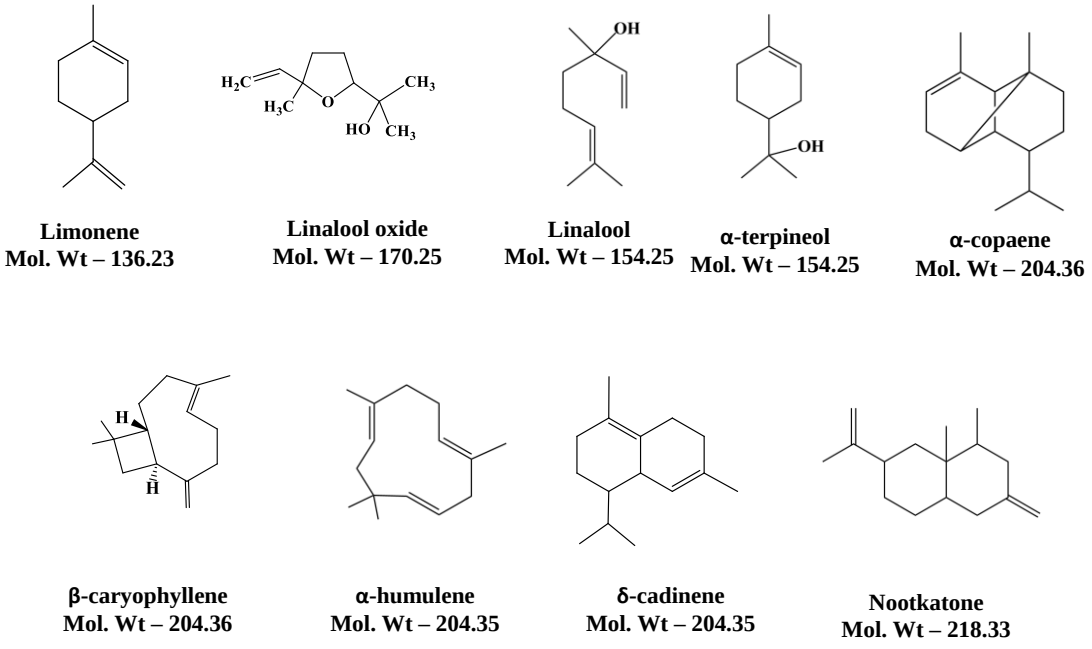

Figure 31. Structures of monoterpenes and sesquiterpenes present in grapefruit juice.

identified. Further the hydrocarbons were classified into monoterpenes (two compound) and sesquiterpenes (ten compounds). Monoterpene hydrocarbons mainly consisted of limonene and β-ocimene while sesquiterpene hydrocarbons consisted of α-cubebene, α-copaene, β-cubebene, α-bergamotene, β-elemene, β-caryophyllene, α-humulene, germacrene-d, valencene, α-muurolene and δ-cadinene, amongst which β-caryophyllene was found to be the most prominent component (**Table 13**). Oxygenated compounds comprised of aldehydes (four compounds), ketones (two compounds), alcohols (four compounds), aldehydes (five compounds) and oxides (two compounds). Oxygenated hydrocarbons included aliphatic aldehyde (octanal, nonanal, decanal), monoterpene alcohol (linalool, α-terpineol, carveol and nerol), monoterpene aldehyde (perillaldehyde), monoterpene ketone (geranyl acetone) and monoterpene oxide (linalool oxide); while the sesquiterpenes included oxide (β-caryophyllene oxide) and ketone (nootkatone). Monoterpenes are generally synthesized from merthylerythritol 4-phosphate (MEP) pathway while sequiterpenes are synthesized from mevalonic acid (MVA) pathway from farnesyl pyrophosphate.[317]

Limonene, an important citrus aroma compound, has been reported to possess health promoting anti-cancer properties such as anti-inflammatory, anti-tumor, and apoptosis-promoting activities.[302, 318] A decreasing trend was markedly observed with limonene decreasing from June to April, with lowest levels observed in January and April. Limonene is reported to increase in satsuma peel while decreasing in the juice as the fruit matures.[319] In previous study by Barboni et al,[320] maturity affected the volatile composition of juices obtained from different citrus fruits including mandarin,

Table 13. Juice volatile composition of grapefruits harvested at different maturity stages analyzed by GC-MS[x]

				Perillyl alcohol equivalent (µg/100 g fresh weight)				
No	**Compounds**	**Lit KI[z]**	**Cal KI**	**June**	**August**	**November**	**January**	**April**
1	Limonene[y]	1201	1208	22305.59 ± 2804.29 a	14094.01 ± 2119.8 ab	13114.21 ± 2295.77 b	8994.77 ± 1474.65 b	5962.16 ± 1399.77 b
2	β-ocimene	1258	1255	ND	76.25 ± 15.28 a	46.70 ± 10.87 ab	49.95 ± 6.87 ab	28.22 ± 4.98 b
3	Octanal	1298	1292	ND	21.67 ± 3.81 a	21.28 ± 4.83 a	17.86 ± 4.54 a	13.61 ± 4.88 a
4	Nonanal	1383	1391	ND	17.52 ± 3.85 a	22.84 ± 4.00 a	16.11 ± 3.02 a	9.98 ± 1.91 a
5	Linalool oxide	1453	1446	22.08 ± 4.62 a	6.30 ± 1.06 b	7.37 ± 3.61 b	10.57 ± 4.06 b	Tr
6	α-cubebene	1468	1463	191.80 ± 37.30 a	90.00 ± 18.21 b	37.54 ± 5.72 bc	15.86 ± 1.95 bc	11.04 ± 1.48 c
7	α-copaene[y]	1499	1500	831.96 ±148.58 a	143.02 ± 27.18 b	49.84 ± 7.70 b	28.30 ± 4.30 b	19.20 ± 3.89 b
8	Decanal	1501	1502	ND	119.65 ± 20.84 a	60.01 ± 9.65 b	49.58 ± 9.33 b	23.08 ± 5.28 b
9	β-cubebene	1550	1533	426.09 ± 85.14 a	53.18 ± 10.92 b	17.92 ± 3.18 b	8.30 ± 1.08 b	4.80 ± 0.80 b
10	Linalool	1553	1550	ND	71.00 ± 13.53 a	24.50 ± 3.41 b	26.50 ±2.79 b	11.40 ± 2.78 b
11	α-bergamotene	1589	1584	ND	11.81 ± 5.84 a	Tr	59.73 ± 14.87 a	37.20 ± 9.16 a
12	β-elemene	1598	1588	160.95 ± 30.65 a	35.97 ± 5.09 b	17.44 ± 3.03 b	32.85 ± 20.73 b	11.92 ±1.43 b
13	β-caryophyllene[y]	1609	1595	8191.73 ± 1063.86 a	1859.22 ± 388.43 b	1072.28 ± 164.09 b	622.91 ± 85.17 b	414.20 ± 44.77 b
14	Humulene[y]	1660	1674	1089.69 ± 166.50 a	295.31 ± 70.90 b	127.48 ± 21.10 b	72.06 ± 10.30 b	41.22 ± 5.09 b
15	α-terpineol	1703	1703	ND	38.54 ± 8.41 a	10.54 ± 2.20 ab	13.52 ± 2.20 ab	4.58 ± 1.89 b
16	Germacrene D	1704	1714	199.30 ± 60.95 a	37.71 ± 9.84 b	11.45 ± 2.33 b	8.63 ± 1.86 b	3.77 ± 0.63 b
17	Valencene[y]	1714	1724	ND	3.40 ± 1.22 b	5.61 ± 2.64 b	10.07 ± 1.85 b	23.40 ± 2.44 a
18	α-muurolene	1730	1730	89.92 ± 19.62 a	32.65 ± 11.94 b	13.03 ± 4.00 b	37.20 ± 11.32 b	25.83 ± 5.69 b
19	δ-cadinene	1766	1760	715.60 ± 141.72 a	216.70 ± 42.00 b	88.71 ± 17.68 b	55.41 ± 8.85 b	37.80 ± 5.06 b
20	Perillaldehyde	1794	1784	46.41 ± 6.58 a	30.58 ± 5.14 ab	21.86 ± 2.74 b	17.49 ± 1.54 b	12.58 ± 0.77 b
21	Carveol	1841	1841	11.67 ± 4.63 a	8.27 1.65 a	4.51 ± 1.51 a	10.19 ± 3.35 a	2.27 ± 0.72 a
22	Nerol	1853	1853	ND	7.13 ± 1.39 a	4.77 ± 0.89 a	3.17 ± 0.82 a	3.75 ± 1.55 a
23	Geranyl acetone	1860	1860	Tr	4.25 ± 0.67 a	2.98 ± 0.42 ab	2.04 ± 0.21 b	2.62 ± 0.12 ab
24	β-caryophyllene oxide[y]	1999	1989	91.57 ± 11.56 a	37.95 ± 11.71 b	12.63 ± 1.81 b	12.93 ± 2.19 b	14.36 ± 2.69 b
25	Nootkatone[y]	2515	2514	ND	Tr	1.74 ± 0.87 b	19.90 ± 4.15 b	56.42 ± 6.31 a

Data represent means ± S.E. of nine juice samples (n=9). Means with different letters denote significant differences ($P < 0.05$) between harvest stages.

[x]Volatile compounds were identified by mass spectra and KI values, [y]Confirmed by co-injection with authentic standards.

[z]Literature Kovats indices (KI) were compared to published papers [314-316].

(ND- not detected, Tr – Trace amount).

clementine and their hybrids. Limonene was reported to decrease as the fruit matures in juices prepared from peeled fruits as compared to the juices prepared from the whole fruit (including peel) where the limonene levels remained stable.[320] In current study, limonene and β-caryophyllene were the two main components which accounted for nearly 87 to 94% of the total volatiles. β-caryophyllene levels also decreased with fruit maturity, with fruits harvested in June showing significantly ($P \leq 0.05$) higher levels than other harvest times. Sharp decrease in β-caryophyllene was observed from June to August. The levels further decreased gradually from August till April with no significant differences observed.

Sesquiterpene hydrocarbon α-cubebene, α-copaene, β-cubebene, α-bergamotene, β-elemene, β-caryophyllene, α-humulene, germacrene-d, α-muurolene and δ-cadinene showed similar trend with highest levels observed in June harvest and the content decreasing as the fruits matured up to April. On other hand aldehydes namely octanal, nonanal and decanal; monoterpene alcohols namely linalool, α-terpineol and nerol were not detected in immature fruits harvested in June. Nevertheless, these compounds also followed similar pattern with their levels being highest in immature fruits harvested in August and lowest in mature fruits harvested in April.

Nootkatone levels increased sharply and were significantly higher ($P < 0.05$) in later stages of maturity mainly January and April (**Table 13**). Nootkatone levels were not detected in June harvest and were low in fruits harvested in August and November. Alomost threefold difference was observed in Nootkatone content in January and April harvested fruits. Valencene levels also showed similar trend and were not detected in

June while they were low in August and November harvest. Nootkatone is an important component of grapefruit volatiles and imparts a distinct grapefruit aroma.[321] Valencene is considered as the precursor of nootkatone and the levels of both the compounds have been reported to increase as the fruit matures.[322] Similar increase of nootkatone and valencene was observed in the present study as the fruit matured and ripened. Therefore, nootkatone is often considered as a senescence indicator of grapefruit.[323, 324]

In addition, other minor terpene oxygenated compounds were identified in the juice samples namely, linalool oxide, β-caryophyllene oxide, carveol and perillaldehyde. Perillaldehyde content decreased with maturity, while no particular trend was observed in linalool oxide and β-caryophyllene oxide. Geranyl acetone and α-panasinsen which were identified in current study have been previously reported by Miyazaki et al in tangerine hybrids.[325] Levels of geranyl acetone decreased while α-panasinsen increased as the fruits matured (**Table 13**). Sulphur containing compounds such as 1-p-menthene-8-thiol have been reported in grapefruit using flame photometric detectors.[326] However in present study sulphur containing compounds were not detected.

Conclusion

Overall developmental and maturity stages had significant effect volatile components with most of the compounds decreasing as the fruit developed and matured from June to April. Terpene hydrocarbons constituted major volatiles followed by sesquiterpene hydrocarbons. Nootkatone and valencene, levels increased with fruit maturity. It can be concluded that as the fruits matured several diverse group of volatile

components decreased and nootkatone the main grapefruit aroma compound increased. Thus indicating the ripening and harvest time for fruits.

CHAPTER X

EFFECT OF ETHYLENE TREATMENT ON HEALTH PROMOTING COMPOUNDS AND FLAVONOID-RELATED GENE EXPRESSION IN RIO RED GRAPEFRUIT (C*itrus paradisi* Macf)

Introduction

Plants have developed complex mechanisms involving molecular signaling pathways, hormones, secondary metabolites, and programmed cell death for defense against biotic and abiotic stresses. Plant hormones play important roles in growth, development, and controlling metabolic activities. Abscisic acid, jasmonic acid, salicylic acid, and ethylene are the chief plant hormones involved in major biotic and abiotic stress induced signaling pathways. Ethylene regulates diverse metabolic processes such as ripening, senescence, and flower induction in plants. Ethylene biosynthesis increases in response to pathogen attack, hypoxia, and wounding.[327-329] In fruit ripening, an increase in ethylene biosynthesis induces key changes in fruit texture, aroma, and color. Ethylene accelerates chlorophyll degradation and accumulation of carotenoids. The use of ethylene to induce ripening and improve peel color is widely practiced in cultivation of various climacteric and non-climacteric fruits. Grapefruits (*Citrus paradisi* Macf) harvested in the early season (Oct – Dec) fetch higher prices compared with later in the season; however these fruits require degreening using ethylene to obtain a uniform, marketable peel color. Furthermore, ethylene can influence the biosynthesis of several secondary metabolites, including carotenoids and flavonoids.

Exogenous ethylene can affect several enzymes involved in plant metabolic pathways. Phenylalanine ammonia lyase (*PAL*), an important enzyme in the shikimate pathway, is the first enzyme in the phenylpropanoid pathway. A previous study reported that *PAL* expression increased in the flavedo in response to ethylene in citrus.[23] Concurrent increases in ethylene and *PAL* occur under different stress conditions (wounding, radiation, chilling injury)[22, 145, 330] and in response to pathogen elicitors,[331] confirming that ethylene significantly induces *de novo PAL* synthesis in citrus flavedo. Induction of genes involved in both ethylene and the phenylpropanoid pathway confer resistance in citrus fruits against pathogens.[332]

Chalcone synthase (*CHS*) catalyzes first committed step in flavonoid biosynthesis and is reported to be induced by ethylene in grape berries.[24] Chalcone isomerase (*CHI*) and 1,2-rhamnosyl transferase (*2RT*) are downstream enzymes in the flavonoid pathway. However, the effect of ethylene on the genes encoding these key flavonoid enzyme in grapefruit remains unclear. Grapefruit is a rich source of flavonoids, mainly flavanones; therefore, the effect of ethylene treatment on the expression of *PAL*, *CHS*, *CHI*, and *2RT* requires further investigation, especially in the juice vesicles which are consumed.

In addition to flavonoids, ethylene also influences other secondary metabolites in grapefruit, such as carotenoids, vitamin C, and limonoids. The effect of ethylene on carotenoids in citrus peels has been extensively studied.[85, 153, 187] The main objective of the current study was to investigate the effect of ethylene on health promoting compounds and flavonoid-related genes in grapefruit juice sacs during market-simulated

storage conditions, as well as to study the correlation between gene expression profiles and flavanone accumulation. Genes involved in flavonoid biosynthesis such as *PAL*, *CHS*, *CHI*, and *2RT* were studied for the first time in grapefruit. Key health promoting compounds, namely vitamin C, carotenoids, limonoids, flavonoids, and furocoumarins were quantified using high performance liquid chromatography.

Materials and methods

Plant materials

Rio Red grapefruits were harvested in October 2012 from the Citrus Center, Weslaco, Texas. Fruits of uniform size and color were harvested from different blocks (three trees each). Fruits were brought to the Vegetable and Fruit Improvement Center, Texas A&M University, College Station (USA) for further treatment.

Chemicals

L-ascorbic acid, reagent-grade butylated hydroxytoluene (BHT), metaphosphoric acid, tris (2-carboxy ethyl) phosphine hydrochloride, β-carotene, lycopene, narirutin, naringin, didymin, poncirin, neohesperidin, limonin, 6,7-dihydroxybergamottin (DHB), bergamottin, and auraptene were procured from Sigma Aldrich Co. (St. Louis, MO, USA). Analytical grade solvents were obtained from Fisher Scientific Research (Pittsburgh, PA, USA).

Degreening treatment and storage

Fruits were washed and dried, then divided into three groups for ethylene treatment (degreened) and control treatment with air (non-degreened). The two sets of degreened fruits (5 ppm and 10 ppm) were treated with 5 µL/L and 10 µL/L ethylene gas

at 85 to 90% humidity at room temperature for 72 hours (3 days) respectively, while non-degreened fruits were exposed to air and stored at similar conditions for 72 hours. Fruit juice samples were collected before the treatment, at day zero (D0) and each day during degreening treatment (D1, D2 and D3).

After the treatment, grapefruits were stored under market-simulated conditions for 35 days, with the last degreening day being the first storage day (D3/S0). Samples were collected at 7-day intervals. Fruits were stored for 3 weeks (S0, S7, S14 and S21) at 11°C followed by 2 weeks (S28 and S35) at room temperature (~21°C). Each treatment had three replications containing 100 fruits per replication (fruits collected from 3 different blocks). Furthermore, from each replication, three subsamples were prepared (n = 9, 3 replications × 3 subsamples). Subsamples were prepared by blending individual peeled grapefruits and were stored at -80°C until further analysis.

Peel color measurements

The peel color of the non-degreened and degreened fruits was measured with a Minolta CR-400 Chroma Meter (Konica Minolta Sensing, Inc., Osaka, Japan). Before recording the sample measurements, the instrument was calibrated every week, using the white calibration plate (Calibration Plate CR-A43, Minolta Cameras, Osaka, Japan). Total 30 fruits were used for peel color measurement with 10 fruits per replication for 3 replications, per treatment (n=30). Readings were taken on equatorial sides of the fruits by marking circles using black marker (three readings per fruit) and the hue angle was measured within these circles at weekly intervals. The results were expressed as hue

angles, with a hue angle of 90º representing yellow, 60º representing orange, and 30º representing red color.[177]

Vitamin C quantification

Vitamin C was extracted and quantified using liquid chromatography according to our optimized protocol.[178] Each sample was analyzed three times and the ascorbic acid contents were expressed as mg/100 mL juice.

Carotenoid analysis

Extraction of carotenoids was performed according to a previously published method with slight modifications.[177] Juice samples (10 g) were extracted using chloroform (15 mL) containing butylated hydroxytoluene (0.2%). An Agilent HPLC 1200 Series (Foster City, CA, USA) system consisting of a solvent degasser, quaternary pump, autosampler, column, oven, and diode array detector was used for quantification. A C-18, Gemini 5 µm column (250 mm × 4.6 mm i.d.) with a guard cartridge was used (Phenomenex, Torrance, CA, USA). Elution was carried out using a gradient mobile phase of acetonitrile (A) and isopropyl alcohol (B). Carotenoids were detected at 450 nm and quantified using external standard calibration.

Quantification of limonoids, flavonoids, and furocoumarins

Sample preparation

Extraction was carried out according to our previously published method with slight modification.[177] Each juice sample (10 g) was extracted using 15 mL of ethyl acetate on a shaker for 3 hours. The organic layer was separated and the residue was extracted twice. All extracts were pooled and the solvent was evaporated to dryness. The

dried residue was reconstituted with 4 mL acetone, filtered using a 0.45 µm PTFE filter, and further analyzed for limonoids, flavonoids, and furocoumarins using HPLC.

Quantification of limonoids and flavonoids using HPLC

Limonoids and flavonoids were quantified simultaneously using a Waters HPLC (Milford, MA, USA), spectra model with a PDA detector (2996) coupled with a binary HPLC pump 1525 and 717 plus auto sampler. The chromatographic separations were conducted on a C-18, Gemini 5 µm column (250 mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA). Limonoids were detected at 210 nm and flavonoids were detected at 280 nm. The entire chromatographic separation was performed with a gradient mobile phase of 0.03 M phosphoric acid (A) and acetonitrile (B) with 1 mL/min flow rate. Each sample was injected three times.

Quantification of furocoumarins using HPLC

Furocoumarins were analyzed using our previously described method.[177] Each sample was analyzed in triplicate and the results were expressed as µg/100 g fresh weight.

RNA isolation and cDNA synthesis

The RNeasy plant mini kit (Qiagen, Valencia, CA, USA) was used for isolation of total RNA from grapefruit juice vesicles. First-strand cDNAs were synthesized using the Advantage RT-for-PCR kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions.

Real-time PCR

Quantitative real-time PCR was performed on a CFX96 Real Time System (Bio-Rad, Hercules, CA, USA), using the SSO Advanced SYBR Green Supermix (Bio-Rad,

USA). Primers were designed based on the genes isolated from grapefruit in our previous study (data unpublished). The reaction mix and conditions were prepared following the manufacturer's instructions. The amplification protocol for all genes analyzed consisted of 30 s at 95 °C , then 40 cycles of 5 s at 95 °C, 30 s at 61 °C, and 10 s at 72 °C. Fluorescence intensity data were acquired during the extension time. For expression measurements, we used the Bio-Rad CFX manager 3.1 software (Bio-Rad, USA) and calculated expression levels relative to values of a reference sample using the Relative Expression Software Tool. Normalization was performed using the expression levels of the *Actin* gene. For all genes analyzed, the reference sample was the expression value obtained in juice sacs of immature fruit harvested in June. Results are the average of three replicates.

Statistical analysis

Statistical analysis was conducted using one way analysis of variance (ANOVA) using PASW Statistics 18 software (SPSS Inc.). A general linear model was used to test significant differences and means were compared using Tukey's HSD test at 5% probability level. The results were expressed as means ± SE.

Results and discussion

Peel color measurement

Degreening fruits with ethylene for three days had significant effect on peel color. Fruits under both degreening treatments developed reddish yellow, while non-degreened fruits retained their green peel color (**Figure 32**). Hue angle measurements showed significant differences between non-degreened and degreened fruits throughout

the storage period (**Table 14**). As the hue angle decreases the peel color appears to be more orange/red. After three days of degreening treatment (D3) sharp decrease in hue

Figure 32. Peel color changes in non-degreened and fruits under 5 ppm and 10 ppm ethylene degreening treatment at beginning (Day 0) and after three days ethylene treatment. Non-degreened fruits were held under air at same conditions as ethylene degreeneed fruits.

angle was observed from ~107° to ~85° in both degreening treatments. Where as in non-degreened fruits hue angle gradually decreased throughout storage and reached ~85° after 21 days (S21) of storage. At end of 35 days (S35) significant differences was observed only between non-degreened and fruits under 10 ppm ethylene treatment. No significant differences were observed between the peel color of both degreening treatments throughout the degreening and storage period. Several studies have reported changes in citrus peel color after ethylene treatment.[20, 85, 153] This study confirms that increasing ethylene concentration has no significant effect on improving peel color or reducing degreening time as reported previously.[19]

Table 14. Peel color readings measured in °hue of non-degreened and degreened fruits (5 ppm and 10 ppm ethylene) stored under market simulated conditions for three weeks at 11 °C and then transferred to 21 °C for two weeks

Days	Non-degreened	Deg 5 ppm	Deg 10 ppm
D0	106.33 ± 0.84 a	105.58 ± 0.84 a	107.88 ± 0.84 a
D3/S0	100.25 ± 0.78 a	84.63 ± 0.78 b	85.27 ± 0.78 b
S7	94.32 ± 0.76 a	81.19 ± 0.76 b	81.90 ± 0.77 b
S14	90.94 ± 0.72 a	80.33 ± 0.72 b	81.06 ± 0.72 b
S21	85.14 ± 0.72 a	77.95 ± 0.72 b	78.51 ± 0.72 b
S28	81.74 ± 0.79 a	76.69 ± 0.78 b	76.79 ± 0.77 b
S35	79.38 ± 0.96 a	76.34 ± 0.96 ab	75.75 ± 0.89 b

*The data represent means ± S.E. of 30 fruits (n=30, except at S28, n=27 and at S35 n=18). Means with different letters denote significant differences ($P < 0.05$) between treatments at each storage period.

Vitamin C analysis

Vitamin C is the main anti-oxidant component of citrus fruits. Vitamin C (total ascorbic acid) comprises ascorbic acid (AA) and its oxidized form, dehydroascorbic acid (DHA). DHA is converted back to ascorbic acid by dehydroascorbic acid reductase (DHAR).[59] In current study ascorbic acid and total ascorbic acid were analyzed. Vitamin C was significantly higher in non-degreened fruits at S7 as compared to both degreening treatments (**Figure 33**). While, fruits degreened at 10 ppm had lower vitamin C levels after 3 days of degreening (D3) and fruits degreened at 5 ppm had lower levels after S35 as compared to other two treatments. There was a sharp decrease in vitamin C levels in all treatments at day 3 after harvest (D3). Nevertheless, the levels increased in all treatments at S7 after transferring the fruits to storage at 11 °C. No significant differences were observed among the three treatments during the remaining storage period. At the end of the 35 days of storage (S35), the levels of vitamin C were similar to the initial levels at harvest (D0) in non-degreened and fruits degreened at 10 ppm. On other hand, ascorbic acid levels were higher in non-degreened fruits than both degreening treatments at D1, S7, S21 and S35 time periods. No significant difference was observed in ascorbic acid levels between both degreening treatments throughout the study. Both degreening treatments had lower while non-degreened fruits had higher ascorbic acid levels at S35 as compared to initial harvest levels (D0).

In spinach, ethylene treatment rapidly decreased AA synthesis, increased AA oxidation and decreased the recovery of AA from DHA by reducing DHAR activity [333].

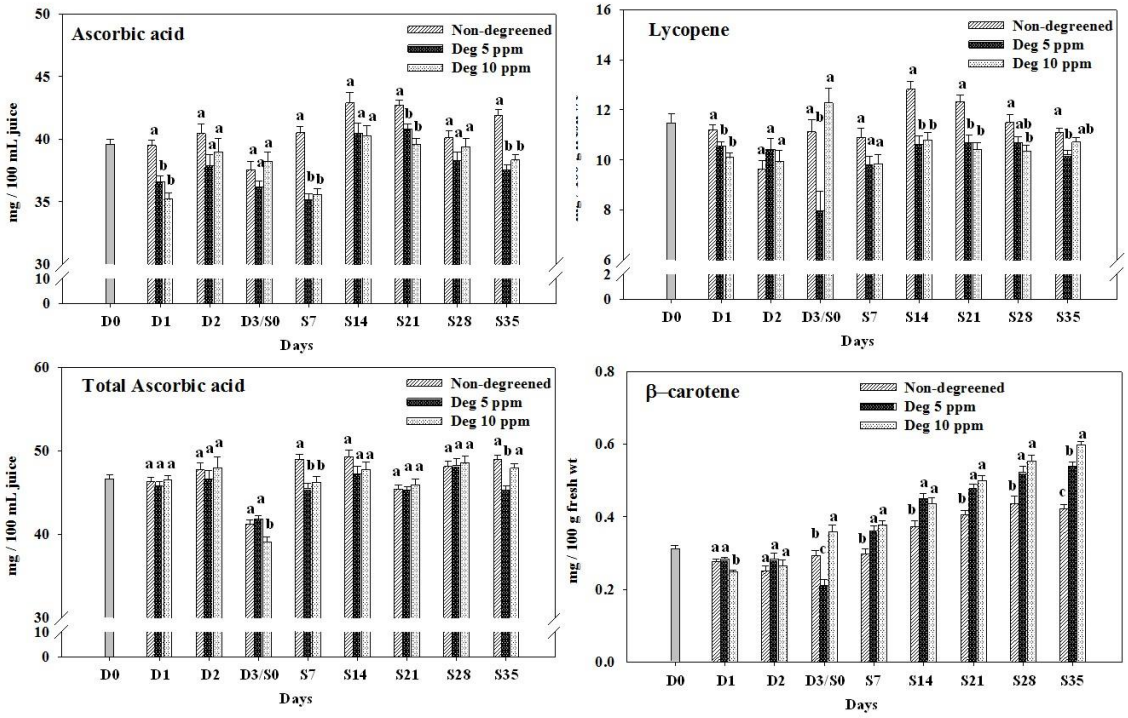

Figure 33. Changes in vitamin C and carotenoids in non-degreened and degreened Rio Red grapefruits. Fruits were stored for three weeks at 11 °C and then transferred to 21°C for two weeks. Data represent means ± S.E. of nine juice samples (n=9). Means with different letters denote significant differences ($P < 0.05$) between treatments at each storage period.

In the current study, DHA levels were higher in degreened grapefruits throughout the treatment and storage period as compared to non-degreened fruits (data not shown). The decrease in ascorbic acid in degreened fruits may be related to the increase in oxidation of AA and reduced DHAR activity. Conversion of DHA to ascorbic acid may be inhibited by ethylene treatment leading to low ascorbic acid levels in degreened fruits.

Whereas, vitamin C levels are affected only by decreased synthesis of ascorbic acid. In our previous study, degreened fruits had lower vitamin C contents after removal from ethylene and storage at 11 °C.[334] Our results are in agreement with previous studies showing that ethylene reduced vitamin C content. However, another study in navelina oranges and several clementine cultivars reported no effect of ethylene on vitamin C contents during storage.[335] Ascorbic acid biosynthesis in plants is a complex process having alternative pathways, and vitamin C levels change with the hour of day, plant tissue, as well as cellular compartment.[59] Therefore, comprehensive study of the effect of ethylene degreening on the ascorbic acid biosynthesis pathway is warranted.

Carotenoids analysis

In the present study, we compared the carotenoid contents of non-degreened and degreened (5 ppm and 10 ppm) fruit juice vesicles after ethylene treatment (**Figure 33**). Lycopene was significantly higher in non-degreened fruits as compared to degreened fruits during treatment and storage, except at degreening day 2 (D2) and storage day 7 (S7). At D3 fruits degreened using 5 ppm ethylene had lower lycopene content than other two treatments, with no significant difference observed between non-degreened and fruits degreened using 10 ppm ethylene. At S28 and S35 no significant difference was observed in lycopene content between degreened fruits. Lycopene levels increased in non-degreened fruits after S14 and then gradually decreased during remaining storage period. By contrast, lycopene in fruits degreened at 5 ppm decreased sharply during degreening treatment at D3 and then gradually increased during storage until day 21 and was maintained for the rest of the storage period. In 10 ppm treatment, sharp increase in

lycopene was observed after 3 days of degreening (D3); however, after transferring fruits for storage under market conditions the levels decreased and were maintained for rest of storage period. In all treatments, β-carotene levels increased gradually during storage after the treatments. Degreened fruits had significantly higher levels of β-carotene than non-degreened from S7 upto S35. After 3 days of degreening period, fruits under 5 ppm treatment had significantly lower levels followed by non degreened fruits as compared to fruits under 10 ppm ethylene treatment. While after 35 days of storage under market stimulated conditions (S35), fruits degreened with 10 ppm ethylene had significantly higher β-carotene levels than fruits degreened with 5 ppm ethylene and non-degreened fruits.

Citrus species contain the largest number of carotenoids amongst all fruits. Grapefruit has pink-colored pulp, mainly due to the presence of lycopene and β-carotene. Higher accumulation of lycopene in grapefruit can be attributed to decreased activity of chromoplast-specific lycopene β-cyclase 2; this prevents conversion of lycopene to downstream β-carotene.[336] The effect of ethylene on the flavedo is reported to be more pronounced than its effect on juice sacs[85] and ethylene increases carotenoid contents in flavedo.[153] In a study by Mayuoni et al, ethylene increased the transcript levels of carotenoid-related genes in flavedo but had no significant effect on their expression levels in flesh.[337] However, in our current study as well as a previous study in Rio Red grapefruit, ethylene-treated fruits had lower lycopene contents.[334] In grapefruit, lycopene and β-carotene are synthesized independently by different fruit tissues, even after harvest.[338] The current results suggest that ethylene may have a role in upregulating

the downstream enzymes in the carotenoid pathway, mainly lycopene β- cyclase 2, leading to higher β-carotene and lower lycopene accumulation in pulp of degreened fruits.

Limonoids quantification

Limonin was the only limonoid detected and quantified in the current study (**Figure 34**). Non-degreened fruits had significantly higher limonin levels than both degreening treatments at D3 and S14, and higher levels than 10 ppm treatment at D2 and S7. By contrast, both degreening treatments had higher levels of limonin at the end of storage S35, and with fruits degreened at 5 ppm having higher levels than other treatments at S21 and S28. Limonin gradually increased in non-degreened fruits during the first 3 days (D1 to D3) of treatment and remained high until S14. However, limonin decreased sharply in non-degreened fruits at S21 of storage and then remained even during the remaining storage period. Overall limonin levels were lower in all treatments after 35 days of market simulated storage (S35) as compared to initial levels after harvest (D0). Sharp decrease in was observed in fruits degreened with 10 ppm ethylene after day 2 of treatment and the levels remained low until S7. However, after S14 limonin levels increased in 10 ppm treatment and were overall maintained during remaining storage period.

Whereas in fruits degreened with 5 ppm ethylene, limonin was more or less retained during the entire storage period as upto S28 compared to initial levels at day harvest (D0). Treatment with ethylene is reported accelerate de-bittering process, by preventing conversion of limonoate A-ring lactone (LARL) into limonin. [339] Treating

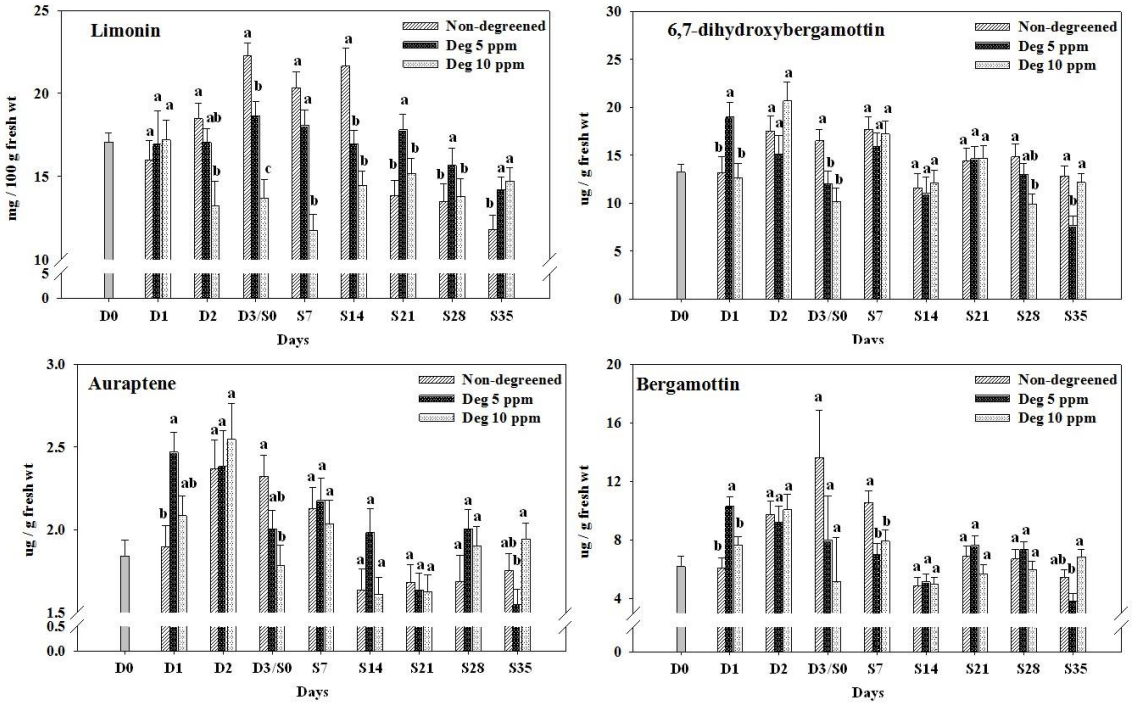

Figure 34. Levels of limonin, coumarin (auraptene) and furocoumarins (6,7-dihydroxybergamottin and bergamottin) in non-degreened and degreened Rio Red grapefruits. Fruits were stored for three weeks at 11°C and then transferred to 21°C for two weeks. Data represent means ± S.E. of nine juice samples (n=9). Means with different letters denote significant differences ($P < 0.05$) between treatments at each storage period.

citrus fruits, including grapefruit, with 20 ppm ethylene for 3 hr increased limonoate A-ring lactone metabolism resulting in reduced substrate for limonin formation.[339] In Thai pummelo, reduction in limonin content was reported after ethylene treatment, and was influenced by both ethylene concentration and duration of treatment.[190] In our previous

study, degreening fruits had higher levels of limonin at certain storage intervals and after 35 days of storage.[177, 334] Overall, ethylene and storage period influenced limonin contents in grapefruit.

Furocoumarins quantification

In the current study, furocoumarins 6,7-dihydroxybergamottin (DHB) and bergamottin, as well as the coumarin auraptene were quantified (**Figure 34**). A sharp increase in all three compounds was observed in fruits degreened at 5 ppm at D1 as compared other treatments which had levels similar to initial harvest levels (D0). Non-degreened fruits had significantly higher content of DHB as compared to both degreening treatments at D3, while they had higher levels than 10 ppm and 5 ppm treatment at S28 and S35 storage interval respectively. On other hand, bergamottin levels were higher in non-degreened fruits as compared degreened fruits at S7 of storage. No significant differences were observed between treatments for all three compounds during the remaining storage period. Overall the levels of furocoumarins were maintained in non-degreened fruits throughout the storage period, but the levels were relatively lower in degreened fruits as compared to initial levels at day 0. In our previous studies, non-degreened fruit had higher furocoumarins at the end of 35 days of storage.[177, 334]

Coumarins, including furocoumarins, are phytoalexins biosynthesized from the phenylpropanoid pathway. Furocoumarins contain a furan ring fused to the coumarin structure and grapefruit contains mainly DHB and bergamottin. DHB and bergamottin are the most common furocoumarins present in grapefruit and pummelo species of

citrus. [340] Auraptene is commonly found in citrus peel and juices[341] and has anti-inflammatory, neuroprotective, and other health beneficial properties.[342] DHB and bergamottin are reported to inhibit cytochrome P450 isoenzymes, causing drug interactions and resulting in increased bioavailability of certain drugs.[343] This drug interaction can help increase the bioavailability of poorly absorbed drugs, but it restricts consumption of grapefruit by the elderly. The effect of ethylene on biosynthesis of furocoumarins has not been investigated thoroughly. In mandarin, ethylene was shown to increase the transcription of the gene encoding O-methyltransferase, an important enzyme in the flavonoid and furocoumarin pathways.[337] *S*-adenosyl-*L*-methionine (SAM) a substrate in the ethylene pathway, acts as a methyl donor to numerous secondary metabolites in reactions that are catalyzed by methyltransferases. SAM: bergaptol methyltransferase is involved in the furocoumarin pathway[125] and SAM: flavonoid 4'-*O*-methyltransferase is involved in conversion of naringenin to ponciretin (poncirin precursor) in the flavonoid pathway.[344] There may be an interaction between the SAM: O-methyltransferases in the flavonoid and furocoumarin pathways and SAM in the ethylene pathway. Coumarins in bean are reported to induce ethylene synthesis, which in turn affects inhibitory action of coumarins on hypocotyl hook opening.[345] Since all three pathways (flavonoid, furocoumarin, and ethylene) are linked to the common precursor SAM, further studies on the ethylene pathway and SAM-related methyltransferases will be necessary to further understand the effect of exogenous ethylene.

Flavonoid quantification and flavonoid-related gene expression

Citrus fruits contain five groups of flavonoids: flavones, flavanones, flavonols, flavans, and anthocyanins.[57] Of these, flavanones are most abundant in grapefruit and present in both aglycone and glycoside forms.[346] In the current study, narirutin, naringin, neohesperidin, didymin, and poncirin were detected in grapefruit juice vesicles, with naringin having the highest concentration (**Figure 35**). A sharp increase in all flavonoids was observed in non-degreened fruits after first week of storage (S7), to concentrations significantly higher than those observed in degreened fruits (except in neohespedidin). Fruits degreened with 10 ppm ethylene showed marked increase and had significantly higher levels of didymin and poncirin after first day of degreening (D1) and narirutin and naringin after 3 days of degreening (D3) as compared to non-degreened and fruits degreened with 5 ppm of ethylene. On other hand, after the fruits were transferred to storage at 11 °C significantly higher levels of all four flavonoids except neohesperidin were observed in non-degreened fruits as compared to both degreening treatments at S7 of storage. At the end of 35 days (S35) of storage, all the flavonoids levels were similar to the levels detected at harvest (D0) in non-degreened and fruits degreened with 5 ppm ethylene, while fruits under 10 ppm ethylene treatment had significantly higher levels as compared to both treatments (except neohesperidin).

Higher ethylene concentration had significant effect of flavonoids and furocoumarins after S35. Several factors have been reported to influence flavonoids during storage in grapefruit, including temperature, storage period, and postharvest treatments.[119] A minor increase in flavonoid contents was observed in sweet oranges

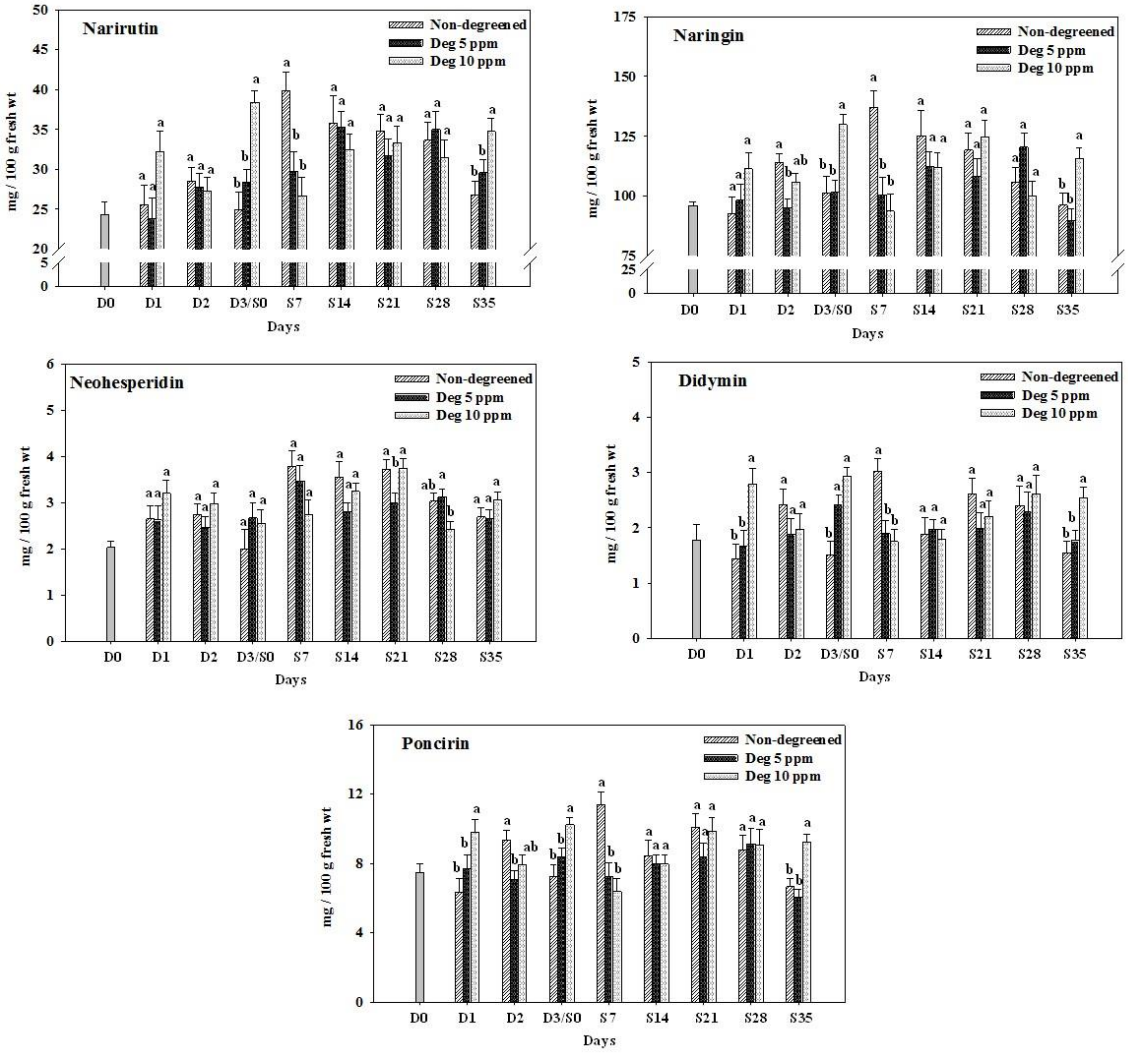

Figure 35. Variation in flavonoids in non-degreened and degreened Rio Red grapefruits. Fruits were stored for three weeks at 11 °C and then transferred to 21 °C for two weeks. The data represent means ± S.E. of nine juice samples (n=9). Means with different letters denote significant differences ($P < 0.05$) between treatments at each storage period.

after treatment with ethylene.[347] In a study conducted on commercial citrus fruits

cultivated in Spain, ethylene had no significant effect on the levels of flavanones except in 'Clemenrubi' and 'Clemenpons' clementines, which showed higher contents of flavanones.[335] In our previous study, ethylene treatment decreased the levels of flavonoids in Star Ruby grapefruit, but it increased the levels in Rio Red grapefruit at certain times during storage.[177, 334] However, both studies observed no significant effect of ethylene on flavonoids during overall storage.

Ethylene was reported to increase the levels of flavonoids (anthocyanins) in grape berries by triggering the expression of genes involved in the anthocyanin pathway.[24] To further investigate the effect of ethylene on genes involved in the flavonoid pathway, we measured the expression of *PAL*, *CHS*, *CHI*, and *2RT* in juice vesicles during ethylene treatment and 35-day storage (**Figure 36**). All four genes showed different expression patterns during treatment and storage. Transcript levels of *PAL* increased gradually in all treatments during storage up to S21 and then decreased after the fruits were transferred to room temperature at S28. A sharp decrease in *PAL* expression was seen in fruits degreened with 10 ppm ethylene at S14 of storage. During the initial treatment (D1 to D3) *PAL* expression was significantly lower in degreened fruits compared with non-degreened fruits, which retained the initial levels at harvest (D0). After the fruits were transferred to 11 °C, *PAL* transcript levels increased in all treatments, with non-degreened fruits having significantly higher levels followed by 5 ppm and 10 ppm ethylene treated fruits at S14 of storage.

CHS transcript levels decreased in all treatments during the storage period (S7 to S35) as compared to initial harvest levels (D0) (**Figure 36**). A marked increase in *CHS*

transcript levels was observed at D1 in the non-degreened fruits, but decreased to initial harvest levels at D2 and D3 of the treatment. By contrast, in degreened fruits, *CHS* transcript levels were significantly lower than in non-degreened fruits during ethylene treatment, with 5 ppm treated fruits having higher levels at D2 and 10 ppm treated fruits having higher levels at D3. No significant differences were observed in *CHS* transcript levels between the non-degreened and 5 ppm treated fruits during entire storage, except

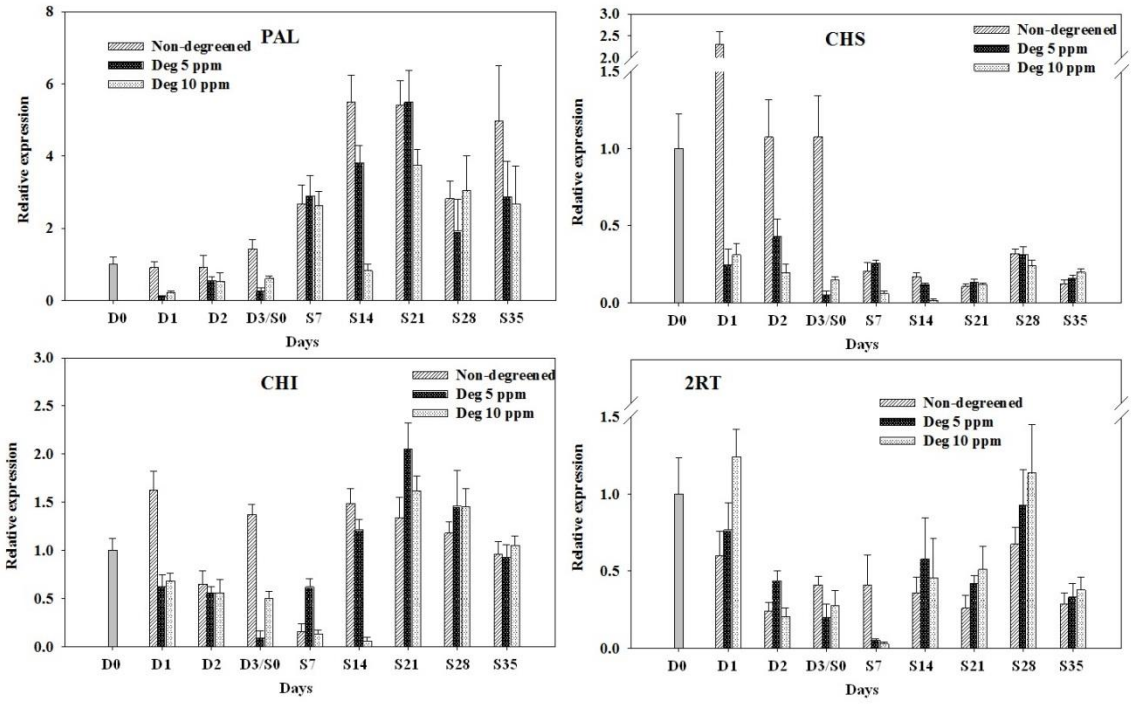

Figure 36. Relative transcript levels of *PAL*, *CHS*, *CHI*, and *2RT* genes detected by qRT-PCR in non-degreened and degreened grapefruit pulp. Fruits were stored for three weeks at 11 °C and then transferred to 21 °C for two weeks. Data represent means ± S.E.M. of three replications, each replication containing four samples.

at S14. On other hand, fruits treated with 10 ppm ethylene had significantly lower *CHS* expression compared to other two treatments at S7 and S14. After removing the fruits to room temperature, *CHS* transcript levels increased at 28 days in the all treatments and then reduced at end at S35.

The expression patterns of *CHI* were similar to *PAL* in fruits degreened with 5 ppm ethylene. *CHI* transcript levels gradually decreased during the ethylene treatment in fruits degreened with 5 ppm ethylene, while in 10 ppm degreening treatment the *CHI* levels continued to decrease upto S14. *CHI* levels gradually increased in 5 ppm treated fruits when transferred to storage at 11 °C up to 21 days and then decreased after removing to room temperature (S28 – S35). On other hand, fruits under 10 ppm treatment showed marked increase in CHI expression at S21 which decreased after transferring fruit to room temperature at S28-S35. In non-degreened fruits, *CHI* transcript levels were significantly higher than in degreened fruits at D1 and D3 of the treatment period. At day 7 of storage, non-degreened fruits had significantly lower *CHI* expression, but the levels increased sharply at S14 and then decreased gradually during the remaining storage period. Fruits under 5 ppm treatment had significantly higher *CHI* transcript levels at S7 as compared to other treatments, at S14 as compared to 10 ppm treatment and at S21 as compared to non-degreened fruits.

The transcript levels of *2RT* in fruits degreened with 10 ppm were significantly higher at D1, while fruits degreened with 5 ppm ethylene had higher levels at D2 than other two treatments. *2RT* levels significantly decreased in both degreened treatments and were significantly lower than non-degreened fruits at S7. However, no significant

differences were observed during the remaining storage period among the three treatments. Overall, *PAL* expression was higher in both degreened and non-degreened fruits at S35, while *CHS* and *2RT* transcript levels were lower and *CHI* transcript levels were retained, in comparison to their respective levels at harvest (D0). *PAL* expression increased under cold storage, whereas *CHS* expression was higher at room temperature.

Citrus fruits generally have low levels of endogenous ethylene produced; however, under stress conditions ethylene levels increased in wounded or infected tissues.[348] Both auto-catalytic and auto-inhibitory effects of exogenous ethylene have been observed in citrus fruits. Increased activities of *PAL* and other key enzymes of the phenylpropanoid biosynthesis pathway have been reported in peel and juice vesicles of citrus fruits after ethylene treatment.[23, 337] However, in the current study, ethylene treatment significantly reduced the expression of the genes involved in the flavonoid pathway, especially during treatment and initial storage weeks. Furthermore, ethylene concentration used in degreening also influenced the expression of genes. The effect of exogenous ethylene is tissue specific and exogenous ethylene is reported to auto-inhibit the production of endogenous ethylene by suppressing formation of 1-aminocyclopropane-1-carboxylic acid, thus limiting ethylene biosynthesis.[349] Other studies in citrus peels have reported increased *PAL* activity with exogenous ethylene application.[23] However, after removal from ethylene the *PAL* activity decreased.

Both *PAL* and *CHI* activity increased after treatment with the ethylene-generating compound ethephon in Fuji apples and were mainly correlated with the increase in ethylene concentration in the apples.[350] Mayuoni et al. suggested two factors

affecting transcript levels during ethylene degreening, namely ethylene exposure and storage temperature during treatment.[337] In the current study, we observed effects of both ethylene and storage temperature on flavonoids and related genes. This suggests that exogenous ethylene plays a significant role in regulation of the genes involved in the phenylpropanoid pathway.

Conclusion

Degreening fruits with different ethylene concentrations had no effect on peel color change and degreening duration. Fruits degreened with 10 ppm had significantly higher content of most of the secondary metabolites measured as compared to 5 ppm treated fruits. *PAL* and *CHI* expression increased, while *CHS* and *2RT* expression decreased after 35 days of storage, as compared to initial expression levels. Ethylene is an important postharvest treatment for marketing early season grapefruits and also influences health beneficial compounds. The current study helps to broaden our understanding of the molecular mechanisms involved in flavonoid biosynthesis and the effect of ethylene on flavonoid-related genes. In conclusion, degreening fruits with 10 ppm ethylene is recommended to maintain the health promoting compounds of grapefruits while enhancing the peel color.

CHAPTER XI

CONCLUSIONS

Grapefruit is one of the important citrus fruit crop both in terms of market value as well as health beneficial properties. Postharvest treatments are critical to improve and maintain the quality of fruits under market-simulated conditions. Degreening of early season grapefruits is the most commonly utilized treatment to change green peel color to an attractive orange/red. In current study, we observed significant effects of ethylene degreening on health promoting compounds present in grapefruit as well as on the expression of genes involved in the flavonoid pathway. Ethylene treatment resulted in lower levels of health promoting compounds after the grapefruits were stored under market conditions for 1-2 weeks. However, after 35 days of storage no significant changes were seen in most of the compounds studied in both degreened and non-degreened grapefruits. Based on our study, degreening grapefruits with 10 ppm ethylene is recommended to retain the certain health promoting compounds during storage. Fruits treated with 10 ppm ethylene had higher content of vitamin C, flavonoids, carotenoids and furocoumarins after 35 days of storage.

Conditioning grapefruits for 7 days at 16 ºC helped to reduce chilling injury and decay in grapefruits. Further, conditioning treatment also helped to maintain the levels of health promoting compounds present in both ‘Star Ruby’ and ‘Rio Red’ grapefruits. Star Ruby fruits stored at 11 ºC had higher levels of health promoting compounds; however, fruit taste preference decreased after 4 weeks storage. Storing grapefruits at low temperature (2 ºC) helped to retain fruit taste for a longer period as compared to fruits

stored at 11 ºC. Therefore it can be concluded that conditioning treatment can maintain fruit quality, taste, and health promoting compounds, while reducing the incidence of chilling injury in grapefruit. For short storage period of 4-6 weeks, grapefruits can be stored at 11 ºC.

In the modified atmosphere packaging (MAP) study, fruits stored in micro-perforated bags had modified carbon dioxide, oxygen and humidity levels, while macro-perforated bags modified only humidity levels. MAP reduced weight loss and had no significant effect on ascorbic acid, limonoids and fruit quality parameters. Grapefruits stored in micro-perforated bags had similar or higher contents of health promoting compounds as compared to control and fruits stored in macro-perforated bags. Micro-perforated bags are more suitable to maintain quality and phytochemicals during prolonged storage conditions.

To further understand the accumulation of various health promoting compounds, grapefruits were harvested during different stages of development and maturity in June, August, November, January and April, and were analyzed for phytochemical content and volatile compounds. Furthermore, genes involved in flavonoid biosynthesis, namely *PAL*, *CHS*, *CHI* and *2RT* were cloned for the first time in grapefruit. Flavonoids (except naringin and poncirin), vitamin C and furocoumarins gradually decreased from June to April as the fruits developed and matured. Lycopene was highest in August – November, while β-carotene increased as fruits matured. *PAL*, *CHS* and *CHI* had higher expression in immature fruits whereas *2RT* expression was higher in mature fruits. In general, phytochemicals decreased as the fruits developed and matured. Fruits harvested in

November had highest content of phytochemicals as compared to other matured marketable fruits (January and April). Variation in accumulation of volatile components was observed during different fruit developmental stages. As the fruits ripened several volatile oil components decreased while nootkatone content increased which is considered as the senescence indicator. Limonene and β-caryophyllene were the most abundant components present throughout the study at all developmental stages.

On the basis of our ethylene degreening studies in Star Ruby and Rio Red grapefruit, we further investigated the effect of different ethylene concentrations on health promoting compounds and expression of certain genes of the flavonoid biosynthesis pathway. Fruits degreened with 10 ppm ethylene had higher levels of lycopene, vitamin C, 6,7-dihydroxybergamottin, flavonoids and β-carotene as compared to fruits degreened with 5 ppm ethylene. PAL, CHS and CHI expression was significantly reduced during ethylene treatment as compared to non-degreened fruits. Overall, 10 ppm ethylene treatment is recommended for degreening grapefruits in order to maintain and increase the content of health promoting compounds.

These outcomes and findings can be utilized to optimize current citrus industry postharvest treatments to maximize fruit quality and health promoting properties. Further studies can be conducted to study the influence of ethylene treatment on furocoumarins pathway to modulate the levels of furocoumarins. Isolation of grapefruit flavonoid pathway genes will be valuable for future research to study the effect of other preharvest or postharvest factors influencing their expression, and can be applied to increase the flavonoid content.

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