OPTIMIZATION AND ISOLATION OF GRAPEFRUIT SECONDARY METABOLITES AND THEIR CHANGES DUE TO PRODUCTION SYSTEMS AND STORAGE

A Dissertation

by

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ABSTRACT

Grapefruits have shown a multitude of health promoting properties owing to their secondary metabolites. Modulation of production systems to increase the levels of nutrient content (secondary metabolites) in fruits and vegetables is a topic of intense scientific debate. The goal of this present research is to understand the influence of production systems and storage on grapefruit secondary metabolites and to identify and purify potentially bioactive grapefruit secondary metabolites.

The first and second studies encompass the optimization of extraction procedures for the accurate quantification of flavanones and vitamin C respectively. The grapefruit flavanones were best extracted using two times dimethyl sulfoxide with the grapefruit sample volumes. Three percent meta phosphoric acid is the best extraction solvent and 5mM of TCEP is the best reducing agent for the quantification of vitamin C in grapefruit. The optimized extraction procedures were used for the quantification of grapefruit flavanoids and vitamin C.

The third and fourth studies encompass the influence of production systems (organic or conventional) and storage on various groups of grapefruit secondary metabolites and their antioxidant properties. Vitamin C, limonoids and flavonoids were found to be higher in organic grapefruits compared to conventional grapefruits in the November 2008 harvest. However, there were no significant differences observed in the above mentioned secondary metabolites in the February 2010 harvest. In general, during storage the vitamin C losses were minor while limonoids and carotenoids losses were

significant. In the 2010 sample, flavonoid levels increased during storage. The total phenolics and total antioxidant (DPPH) showed trends similar to flavanones during storage. The results of these studies suggest that organic production (might have) caused a small increase in the levels of a few secondary metabolites. However, it was the harvest season that had a greater impact that probably masked the effect of production systems in the 2010 sample.

The fifth study focused on the isolation and purification of grapefruit minor bioactive compounds. Seven coumarins and two polymethoxy flavones including Meranzin and pranferin were purified from grapefruit byproducts such as grapefruit oil and peels using solvent partitioning and flash chromatography. The purified dihydroxy bergamottin was used as a standard in the quantification of coumarins from organic and conventional grapefruits. In future, pure coumarins, especially meranzin and pranferin, produced using various isolation techniques needs to be studied to understand the mechanism of drug interaction.

DEDICATION

To

My father (Seshagiri Rao), mother (R.Lakshmi), wife (Padma) and friends for their unconditional love and support

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Background

Citrus, a *Rutaceae* family member, is a rich source of various secondary metabolites such as vitamin C, polyphenols (flavonoids, anthocyanins, phenolic acids), carotenoids, terpenoids and pectin. Due to a wide genetic diversity among citrus plants, a greater degree of variations occurs in their secondary metabolites. Therefore, each species is unique in terms of composition and quantities of these secondary metabolites. Citrus fruits have a multitude of health promoting properties owing to secondary metabolites. Citrus is widely cultivated in the subtropical regions of the world. In the US, citrus is mainly grown in the states of Florida, California and Texas. In 2011, the US occupied second place in world citrus production, next to Brazil, with 7.96 million tons of orange and 1.11 million tons of grapefruit [1].

Grapefruit (*Citrus paradisi Macf.*) is one of the major commercial citrus crops grown in the subtropical regions of the United States. The red-colored varieties such as the ‘Rio Red’ grown in Texas, are particularly rich in health promoting bioactive compounds such as vitamin C, limonoids and carotenoids [2,3]. Additionally, grapefruits contain non-nutritive bioactive compounds such as flavanones and phenolics essential for good health. Grapefruit flavanones demonstrate a wide range of biological activities against several age-related diseases and have been well-studied for their preventive properties against heart diseases [4,5]. Owing to this, the American Heart Association

(AHA) has given a "healthy heart check" symbol for several commercially available grapefruit juices [6]. In addition, several *in-vitro* studies have also demonstrated grapefruit's anti-cancer properties [7-9].

1.2 Production systems and post harvest storage

Plants regulate their gene expression to survive unfavorable environmental conditions leading to varying levels of secondary metabolites even among the same species. Production systems and post harvest storage too affect gene expression and can be used to modulate secondary metabolite levels. Hence, understanding the influence of different production systems and post harvest storage on grapefruit secondary metabolites becomes significant to horticulturists. Conventional production systems use large quantities of water and fertilizer for higher yields. Additionally, the crops use high doses of pesticides for plant protection. In contrast, organic agricultural production is in harmony with the ecology by not using GMOs, synthetic pesticides, antibiotics, growth hormones, chemical fertilizers and sewage sludge [10]. The nutrient supply for organic crops is mostly obtained from compost applications. Also, nitrogen fixing plants (legumes) can supply essential nutrients to crop. The organic grower should abide by the USDA's national organic program standards [10]. For growers, organic agriculture can yield higher valued produce and higher profits. Research conducted in several fruit and vegetables showed organic produce with better quality compared to conventional produce because the former is devoid of pesticide residues [10].

Several recent reports discussed the impact of plant nutrient status (nitrogen, phosphorus and potassium) and their influence on the levels of various secondary metabolites [11-13]. According to these studies, the nitrogen deficiency upregulated phenyl propanoid pathway, while it downregulated carotenoid pathway. Other studies suggested that the lack of protection from pesticides in organic produce can trigger secondary metabolites production and subsequently protect the plant against pests [14]. Due to the above reasons it could be hypothesized that organic grapefruits have higher levels of secondary metabolites compared to conventional grapefruits.

In addition to different production practices, post-harvest procedures including storage duration and storage temperature can have a significant influence on secondary metabolite levels [15-17]. These effects can vary depending on the type of secondary metabolites, plant species as well as plant organ or tissue. Furthermore, the effects of production system, storage and harvest time on grapefruit bioactives are still unclear [18].

1.3 Secondary metabolites

Plants produce a wide variety of chemical intermediates known as secondary metabolites during the biosynthesis of carbohydrates, proteins and lipids. The secondary metabolites are categorized into carotenoids, coumarins, flavonoids, limonoids and organic acids based on their structure in plants [19]. They are mainly involved in maintaining plant health. Secondary metabolites have evolved to evade, fight and survive various biotic and abiotic components of nature through different metabolic

processes [20]. These secondary metabolites are chemically bound to cell walls or localized in cytoplasm and vacuoles. Although produced by plants for their own protection, they have a wider application in human health.

In literature, there are numerous citings of using plant secondary metabolites in traditional medicine. For example, an ancient Greek medication, theriak, used to cure several diseases and poisoning, consisted of approximately 64 plant secondary metabolites and opium as its major ingredients [21]. Identification of secondary metabolites from the plant extracts is a two-stage process and has long been studied in the field of pharmacy and phytochemistry. The first stage is screening the extracts from different plant tissues such as fruits, bark, leaves or roots. The extracts with promising results are further evaluated. Usually, the crude extracts comprise of 10-100 compounds. The second stage is purification and characterization of these compounds from the extracts constitutes the second stage. In this stage, various chromatographic and spectroscopic methods are used to purify, characterize and quantify novel molecules.

1.4 Grapefruit secondary metabolites

1.4.1 Vitamin C

Vitamin C is a water soluble organic acid that plays a major role in the biosynthesis of collagen, norepinephrine, peptide hormones, and tyrosine [22]. Vitamin C was also attributed as a cure for scurvy, defense against cellular oxidation, prevention of cancer and suppression of free radicals produced during the metabolic processes [23,24]. Under oxidative stress, ascorbic acid (AA) is converted to dehydroascorbic acid

(DHA) by losing two protons [25]. However, due to the reversible nature of this reaction, the DHA is then recycled into AA by dehydroascorbate reductase (DHAR) in different cells such as erythrocytes, hepatocytes and smooth muscle cells [26]. Although AA biosynthetic pathway exists in all plants and higher animals, humans and a few other animals lost the ability to synthesize AA due to a mutation in the gene L-gulono-1, 4-lactone oxidase [27]. Due to this inability to synthesize AA, fruits and vegetables are the only sources of this nutrient in humans [28]. Currently, the recommended daily allowance (RDA) of vitamin C for women and men are 75 and 90 mg/day, respectively [29]. As fruits and vegetables are the only source of vitamin C, an accurate quantification of AA and DHA is warranted.

1.4.2 Limonoids

Limonoids are chemically oxygenated tetrano-triterpenoids present in *Rutaceae* and *Meliaceae* family. Limonin, the first identified among limonoids, was well-known for its bitter principle. Limonoids are well known for their antifeedant properties produced in fruits, leaves and seeds. Recently, citrus limonoids have been found to significantly reduce the incidence of colon cancer in rats [30]. The five major groups of enzymes that catalyze the biosynthesis of citrus limonoids are:

1. Enzymes that are solely involved in the synthesis of nomilin in stem tissues (phloem)
2. Enzymes that synthesize limonin from nomilin in leaves, stems, fruit juice sacs, fruit peel and seeds
3. Limonoid D-ring hydrolase that catalyzes lactonization of D-ring in seeds

4. UDP-glucosidase that mediates conversion of aglycon limonoids to glycosides
5. β-glucosidase enzymes hydrolyze glycosides and liberate limonoid aglycons and during seed germination

Contrary to limonin biosynthesis, nomilinate A-ring lactone, and deacetylnomilinic acid are produced only in the stem tissues. Further, nomilin synthesized in the stems is translocated into leaves, fruits and seeds. After translocation, the other limonoid aglycons are synthesized. The limonoid aglycons are later converted into limonoid glycosides in different plant tissues (except stems) until the fruits are harvested.

1.4.3 Carotenoids

β-carotene and lycopene are the two major carotenoids that contributes to grapefruit red color [31]. It was established that β-carotene is one of the most efficient scavenger of singlet oxygen, making it a very interesting molecule in cancer prevention studies because of its antioxidant mechanism [32]. Lycopene is implicated in prevention of prostate cancer. This was strongly supported by a study conducted by *Giovannucci et al.* on tomato products consumption and prostate cancer risk [31]. Lycopene occurs upstream of β-carotene in the biosynthetic pathway.

1.4.4 Flavanones

Polyphenols consist of lignins, anthocyanins and flavonoids that contribute to approximately 40% of total organic carbon in the biosphere. Lignins are polymeric

polyphenols that reinforce the plant cell walls against physical strain in large trees, while anthocyanins, impart colors to flowers, fruits and leaves.

Flavonoids are commonly found throughout the in the plant kingdom and 6,467 compounds were identified till date [33]. Flavonoids are produced in different parts of plants such as flowers, fruits, seeds and leaves. They are involved in plant signaling, defense against microbes, UV radiation, and also a feeding deterrence. The flavanones are built on C_6-C_3-C_6 skeletal structure and owing to their structural similarity with phenolic groups, the polarity of flavonoids range from medium to high. The medium polar compounds include polymethoxy flavones, while the polar compounds include flavanones.

Flavonoids have a great potential for antioxidant activity [34]. Flavonoids are classified into flavones, flavanones and flavanols based on their structure and occur as aglycones and glycosides[34].

1.4.5 Coumarins

Coumarins occur in fruits, flowers, leaves, roots, seed coats and stems. These are mainly plant defense compounds that have antimicrobial, anti-feedant and UV screening properties. Coumarins form photoaducts by intercalating between DNA base pairs in herbivores [35] and are often used in the treatment of skin diseases such as psoriasis and vitiligo [36,37]. Additionally, coumarins exhibit antimicrobial [38,39], cytotoxicity [40,41] and neuroprotection properties [42,43].

Previous studies suggested that grapefruit coumarins inhibit the popular cytochrome p-450 family of enzymes [44-46], which include CYP 3A4 (drug metabolizing enzyme) and CYP 1B1 (pro-carcinogen activator) [47]. Grapefruit coumarins are structurally diverse and occur in extremely low concentrations ranging from 0.06 ppm to 1ppm [48,49]. Certain grapefruit coumarins have demonstrated high biological activity, therefore, only studies using pure compounds can reveal the mechanisms involved in grapefruit drug interactions [44].

1.5 Analytical techniques

Various sample preparation and analysis tools are available to identify and quantify the secondary metabolites. Currently available detection techniques are extremely sensitive and capable of identifying secondary metabolites in the range of picomoles to femtomoles [50]. In some instances, due to the high variability among species, secondary metabolite profiling is necessary before quantifying.

Sample preparation, chromatographic separation and identification are the three major steps involved in the analysis of these compounds.

1.5.1 Sample preparation

Extracting a sample uniformly and enriching all components free from interference of matrix is the primary goal of sample preparation. A wide range of sample preparation and extraction procedures were used based on the type of sample matrix and analyte properties. Selection of extraction method and solvent (extraction media) are

critical for the right sample preparation. According to *Luthria et al*. approximately 30% of analytical errors originate during sample preparation; therefore, identification and optimization of factors influencing sample preparation are critical for an accurate quantification [51-53]. For example, hydrophilic compounds such as flavanones and phenols are extracted using methanol or dimethylsulfoxide while the hydrophobic compounds such as carotenoids and coumarins are extracted using non polar solvents such as hexane and chloroform. Furthermore, the extraction method depends upon the type of sample matrix and the location of analytes in the sample.

1.5.2 Chromatographic separations and detection

The invention of chromatography and its subsequent development find use in many fields including pharmaceutics, agriculture, food and pesticide. High pressure liquid chromatography (HPLC) has been in use for the identification and quantification of plant secondary metabolites for the past few decades. HPLC coupled with a PDA detector has been the most widely used instrument in the field of agriculture. However, in recent years, HPLC coupled with a Mass Spectrometer (MS) detection has increased the accuracy and sensitivity of the analytical methods. For purification purposes, preparative HPLCs are available to isolate the target compounds. For large scale purification of plant bioactives, flash chromatography is widely used in the field of agriculture and drug discovery. HPLC is used to quantify different groups of plant secondary metabolites such as flavonoids, carotenoids, limonoids (triterpenoids) and vitamins on a regular basis.

Gas chromatography (GC) is another instrument invented prior to HPLC and is commonly used for the analysis of volatile compounds of plant origin. The plant flavoring compounds such as monoterpenes, ethers, esters and drug interacting compounds such as coumarins are regularly analyzed in GC. An MS detector can be linked to GC identify new volatile compounds from plants. Mass spectral detection and identification is based on the type of ionization, type of mass analyzer and detector.

Based on the current research, we hypothesized the following objectives for the dissertation.

- To optimization of extraction procedures for grapefruit flavanone quantification
- To optimization of quantification technique for grapefruit vitamin C
- To evaluate the influence of organic and conventional production, and storage on grapefruit vitamin C, carotenoids and limonoids
- To evaluate the influence of organic and conventional production on grapefruit coumarins, flavanones, total phenols and antioxidant properties
- Purify and identify grapefruit minor secondary metabolites with potential bioactivity

CHAPTER II

OPTIMIZATION OF FLAVANONES EXTRACTION BY MODULATING DIFFERENTIAL SOLVENT DENSITIES AND CENTRIFUGE TEMPERATURES*

2.1 Synopsis

Understanding the factors influencing extraction is critical for knowledge in sample preparation. The present study was focused on the extraction parameters such as solvent, heat, centrifugal speed, centrifuge temperature, sample to solvent ratio, extraction cycles, sonication time, microwave time and their interactions on sample preparation was investigated. The five flavanones were analyzed in a high performance liquid chromatography (HPLC) and later identified by liquid chromatography and mass spectrometry (LC-MS). The flavanones eluted by a binary mobile phase with 0.03% phosphoric acid and acetonitrile in 20 minutes and detected at 280 nm, and later identified by mass spectral analysis. Dimethylsulfoxide (DMSO) and dimethyl formamide (DMF) had optimum extraction levels of narirutin, naringin, neohesperidin, didymin and poncirin compared to methanol (MeOH), ethanol (EtOH) and acetonitrile (ACN). Centrifuge temperature has significant effect on flavanone distribution in the extracts. The DMSO and DMF extracts had homogeneous distribution of flavanones compared to MeOH, EtOH and ACN after centrifugation. Furthermore, ACN showed

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clear phase separations due to differential densities in the extract after centrifugation. The number of extraction cycles significantly increased the flavanone levels during extraction. Modulating the sample to solvent ratio has increased naringin quantity in the extracts. Current research provides critical information on the role of centrifuge temperature, extraction solvent and their interactions on flavanone distribution in extracts; the influence of key parameters such as sample to solvent ratio and extraction cycles on sample preparation was discussed.

2.2 Introduction

Flavanones have a great potential for antioxidant activity and contribute to various traits such as color and flavor in fruits and vegetables [54]. Flavonoids are classified into flavones, flavanones and flavanols based on their structure and these occur as aglycones and glycosides [54]. In the late 1960s, Albach and Redman classified genus *Citrus* chemotaxonomically based on the type and the quantity of major flavonoid glucosides (bitter neohesperidosides and non-bitter rutinosides) present in their leaves and fruits [55]. Chemotaxonomically, grapefruit is considered a hybrid among *Citrus* species due to the occurrence of both bitter (naringin, neohesperidin, poncirin) and non-bitter (narirutin, didymin) flavonoids [56]. Hence, accurate quantification of flavonoids is absolutely necessary for taxonomic evaluations.

Previously published reports demonstrated variations among grapefruit flavanone levels due to genotype, season, growing conditions, storage and also differences in sample preparation procedures (Table. 2.1). However, the resulted flavanone variations

Table.2.1. Sample preparation methods and reported naringin levels from grapefruit on fresh weight (FW) or dry weight (DW) basis.

Variety	Location	Sample preparation	Extraction solvent	Naringin Quantity[a]	Reference
Rio red	South Texas	5 g of pulp +20 mL DMF, 1.5 mL aliquot centrifuged at 7500 × *g*, supernatant analyzed in HPLC	DMF	1500/ FW	Vanamala et al.[66]
Ruby red	NA	Juice: solvent; 400 μL: 400 μL (V/V), vortex-5min, Sonication-15 min at 60 °C. centrifugation , 2000 rpm	Methanol	626.2/ FW	Desiderio et al.[69]
Local market[b]	South Portugal	Centrifugation-8000 rpm, 15 min; supernatant filtered; dilution with sodium acetate buffer(0.02 M); pH 4	No solvent	476.82/ FW	Ribeiro et al.[79]
NA	NA	Juice extractor; centrifugation- 7200 rpm, 10 min supernatant filtered; the aliquot diluted with borate buffer (60 mM)	No solvent	44.6/ FW	Wu et al.[67]
Pink mash	Florida	25 mL juice +20 mL DMF heated at 90 °C for 10 min. adjusted volume to 50 mL , centrifuged at 2500 × *g* for 10 min	DMF	428/ FW	Mouly et al.[68]
Red blush	Italy	Centrifugation, pellet suspended in water and extracted 3 times similarly and pooled all extracts. A Sep-pak cartridge was used for flavonoid separations	Methanol	4600-5240/ DW	Del Caro et al.[80]

Table.2.1. Continued

Variety	Location	Sample preparation	Extraction solvent	Naringin Quantity[a]	Reference
Rio red	Texas	Juice is mixed with DMSO 1:1 (v/v) and centrifuged	DMSO	1200/ FW	Patil et al.[81]
Rio red	Texas	Pulp filtered through cheese cloth , centrifuged, separated the flavonoid fraction on Sep-Pek C-18 cartridge	Methanol	129/ FW	Lester et al.[82]
Duncan	Texas	Freeze dried sample extracted 4 times with 1: 1 mixture of DMSO and methanol. The extract was further mixed with DMSO at 1:1v/v and centrifuged	DMSO	382/FW	Berhow et al.[83]
Ruby red	Florida	Pulp filtered through cheese cloth , centrifuged, separated the flavonoid fraction on Sep-Pek c-18 cartridge	Methanol	124/FW	Rouseff et al.[56]
Rio red/Texas	Texas	1 mL juice mixed with 2 mL methanol and filtered.	Methanol	2200/F W	Girennavar et al.[84]

NA = not available information.

[a] All values are presented in ppm.

[b] Source of material.

could be either due to the treatments or experimental artifacts. According to Luthria et al., approximately 30 % of analytical errors originate during sample preparation; therefore, identification and optimization of factors influencing the sample preparation are critical for accurate quantification [57-59]. Due to a wide structural diversity, flavonoids have different physicochemical properties which makes it virtually impossible to fully extract them in a single extraction step [60]. Despite the obvious difficulties in extraction, sample preparation methods can be optimized by focusing on one or two specific classes of flavonoids with similar properties. This approach not only improves the extraction efficiency but also reduces the extraction time. Moreover, the goal of optimized extraction procedure is to obtain a uniformly rich extract devoid of matrix interferences [61].

Since flavanones are relatively non-labile compared to vitamin C and carotenoids, robust analytical methods can be employed for extraction of flavanones [56,62,63]. The problems during extraction are better understood when real samples are used rather than model standard matrices [64]. Solubility and mass transfer of the analytes of interest not only depend on physicochemical properties of the compounds themselves but also greatly influenced by the other non-specific analytes in the matrix [65]. Therefore, these aspects require a detailed investigation in fruits due to their complex matrices. Traditionally, flavanones have been extracted with aqueous solvents from freeze dried samples to increase the tissue permeability to the solvent [58]. However, addition of water is not required when extracted directly from fruit juice [50]. Maceration or blending, centrifugation and filtration are three commonly followed extraction steps for

citrus flavanones [50]. In case of higher sample volumes and number, the centrifugation step is highly advantageous before filtration because this reduces the total filtration cost prior to HPLC analysis. Nevertheless, the physical phenomenon behind flavanone extraction with different solvents and their distribution in a miscible solvent mixture after centrifugation was not investigated. Furthermore, variations in flavanone levels in the same extract after centrifugation were not observed in previous flavanone methods [66-69]. In addition to these extraction steps, the influence of other factors such as solvent, extraction cycles and sample to solvent ratio plays a key role for accurate quantification of flavanones. Furthermore, the commonly used extraction procedures such as microwave extraction and sonication were also evaluated. The objective of the present study is to evaluate the extraction efficiency of various solvents, temperature, centrifugal speed, and centrifuge temperature, number of extraction cycles, microwave extraction and sonication on grapefruit flavanones.

2.3 Experimental

2.3.1 Plant materials

Rio red grapefruits were harvested in November 2007, washed in a commercial packing shed in Mission, Texas. The fruits were peeled and blended for 3 min in a Vita-Prep blender obtained from Vita-Mix food services (Cleveland, OH, USA). The same blended juice was used for all the analysis except in extraction cycles experiment, where the fruits were harvested from February 2010. The fruit juice was stored in -80 °C until all the experiments were conducted.

2.3.2 Chemicals and instrumentation

Narirutin, naringin, neohesperidin, didymin and poncirin standards were purchased from Sigma Aldrich (St. Louis, MO, USA). For sample preparation, 5 mL BD syringe, 0.45 µm acrodisc 25 mm syringe filters were purchased from Fisher Scientific (Fair Lawn, NJ, USA). High performance liquid chromatographic grade methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), dimethyformamide (DMF) solvents were obtained from Fisher Scientific (Fair Lawn, NJ, USA) and ACS grade dimethyl sulfoxide (DMSO) was purchased from Mallinckrodt Inc. (Phillipsburg, NJ, USA). HPLC grade phosphoric acid was purchased from EMD Inc. (Gibbstown, NJ, USA). The samples were centrifuged in the Beckman model J2-21 high speed centrifuge (Beckman Instruments, Fullerton, CA, USA).

2.3.3 Factors affecting sample preparation

2.3.3.1 Solvent and heat

Grapefruit juice was extracted with solvents, such as MeOH, EtOH, ACN, DMSO and DMF. Extraction solvent (3 mL) was added to 3 mL of grapefruit juice and the mixture was vortexed for 5 sec. The sample and solvent mixture was then heated in a hot water bath set at different temperatures (20, 40, 50 or 60 °C) for 30 min and later the samples were centrifuged at $4301 \times g$ for 10 min. The extraction temperatures above the boiling points of the solvents were avoided. Centrifuge supernatant was filtered with 0.45 µm *acrodisc* syringe filter into amber glass vial and analyzed using HPLC.

2.3.3.2 Solvent and centrifuge speed

Grapefruit juice (3 mL) was extracted with 3 mL of MeOH, EtOH, ACN, DMSO and DMF. The sample mixture was vortexed for 5 sec and centrifuged for 605, 3293, 6720 and 11357 × *g* at 0°C for 10 min. Two aliquots (from centrifuged sample) of 1 mL each were taken from top and bottom of the centrifuge tube, and analyzed separately to determine flavanone concentrations from grapefruit extracts.

2.3.3.3 Solvent and centrifuge temperature

The grapefruit juice (3 mL) was mixed with 3mL solvent (MeOH, ACN and DMSO) and vortexed for 5 sec and centrifuged at 6720 × *g* and extracted separately by maintaining the rotor temperature at 0, 10 and 20°C for 10 min.

These centrifuge temperatures were selected for explaining any possible trends in top and bottom 1 mL of extracts. Since EtOH and DMF showed similar extraction pattern as MeOH and DMSO they were not used in this experiment. The samples were prepared by filtering 1 mL from top and bottom of the centrifuge tube without disturbing the extracts. Later, the samples were analyzed using HPLC.

2.3.3.4 Extraction cycles

Grapefruit juice (3 mL) was extracted with 3 mL of DMSO in a centrifuge tube. The sample mixture was vortexed for 5 sec, centrifuged at 6720 × *g* for 10 min and the

supernatant was analyzed using HPLC. The residue was extracted two times with 3 mL DMSO, filtered through 0.45 μm acrodisc filter and analyzed separately.

2.3.3.5 Sonication and heat

Since DMSO was found to be a better solvent for flavanone extraction, the other solvents were not used in this method. The extracts were prepared by mixing 3 mL of grapefruit juice and 3 mL of DMSO. All extractions were carried out in a Cole Parmer (42 kHz and 180 W) ultrasonic cleaning bath. The mixtures were vortexed for 5 min and the extraction was continued for 10, 20 or 30 min in a sonicator at room temperature (20°C) and 40°C. Further, the samples were centrifuged at 6720 × *g* for 10 min and the aliquots were analyzed using HPLC.

2.3.3.6 Microwave extraction and solvent ratio

The extractions were carried out on a Sharp carousel microwave (Mahwah, NJ, US). The flavanones were extracted with DMSO in a microwave for 5, 10, 15 and 20 sec. The extractions were conducted for 10, 15 and 20 sec by pausing for 2 min after every 5 sec with different ratios of sample to DMSO at 1:1, 1:2, 1:3 and 1:4 (v/v). The extraction temperature obtained from 1:1, 1:2, 1:3 and 1:4 (v/v) of sample to DMSO ranged from 50-60, 65-80, 65-75 and 70-80 °C respectively. Later, the extracts are passed through 0.45 μm filters and analyzed using HPLC.

2.3.4 HPLC and LC-MS analysis

The five grapefruit flavanones (narirutin, naringin, neohesperidin, didymin and poncirin) were separated in 20 min using a Finnigan Surveyor plus HPLC (West Palm Beach, FL, USA) according to our previous publication [2]. The HPLC system was equipped with a PDA plus detector coupled with a quaternary LC pump plus system and a surveyor plus auto-sampler (25 µL sample loop with valco fittings). The flavonones separation was carried out on a C-18, Hypersil gold column (100 mm x 4.6 mm i.d. and 5 µm particle size). The peaks were detected at 280 nm and the analysis was carried out by Chromquest 5.0 software. Chromatographic separation was performed using a gradient mobile phase consisting of (A) aqueous phosphoric acid (3 mM) and (B) ACN. The flavanones were separated as the following elution solvent gradient: 0 - 4.5 min, 80 % A; 4.5 - 11.6 min, 70% A; 11.6 – 13.0 min, 42% A; and 13.0-19.6 min, 80% A. All samples were filtered 0.45 µm filters and 5 µL was injected into HPLC.

The identity of flavonones was confirmed by a LC-MS analysis (Finnigan, LCQ Deca XP, West Palm Beach, FL, USA). The flavanones were separated on an Aquasil, C-18 column (2.1 ×150 mm, 3 µm). The flavanones were separated using a binary solvent gradient of (A). 0.1 % formic acid and (B). ACN. The grapefruit samples were run at 0-2.6 min, 95% A; 2.6-11.6 min, 83% A; 11.6-15.0 min, 80%; 15.0-17.6 min, 75 % A; 17.6-19.6 min, 95% A and ended with 95% A at 25 min at a 0.2 mL/ min flow rate. All five flavanones eluting from LC column were identified using electron spray ionization (ESI) in negative mode. The operating capillary temperature was 250 °C and capillary

voltage was maintained at -15 V. The sheath gas and the auxiliary gas (nitrogen) were maintained at 60 and 20 au, respectively with applied voltage 3.0 kV.

2.3.5 Statistical analysis

All the samples were prepared in triplicates and analyzed three times in HPLC. The peak areas were exported to a spreadsheet and the quantities of the flavanones were calculated by applying regression equation and dilution factor. Finally, the data processing and statistical analysis of the data was performed by SPSS version 16.0 (SPSS Inc., USA) software program. ANOVA was performed to analyze mean variations among treatments and results were expressed in mean ±SD.

2.4 Results and discussion

2.4.1 Effect of solvent and heat

In the present study, various solvents such as MeOH, EtOH, ACN, DMSO and DMF were used for extraction of flavanones from grapefruit juice at 20, 40, 50 and 60 °C. Except naringin, relatively higher levels of narirutin, neohesperidin, didymin and poncirin were extracted with DMSO (polarity index 7.2) than other solvents. Solvent polarity (DMSO> DMF> ACN > MeOH> EtOH) [70] had a greater effect on flavanone extraction compared to temperature.

The results presented in Fig. 2.1 suggested high temperature did not significantly increase the naringin levels (1413 mg/ 1000 mL juice with DMSO). Similar findings from previous study strongly suggested that sample heating is not essential for grapefruit

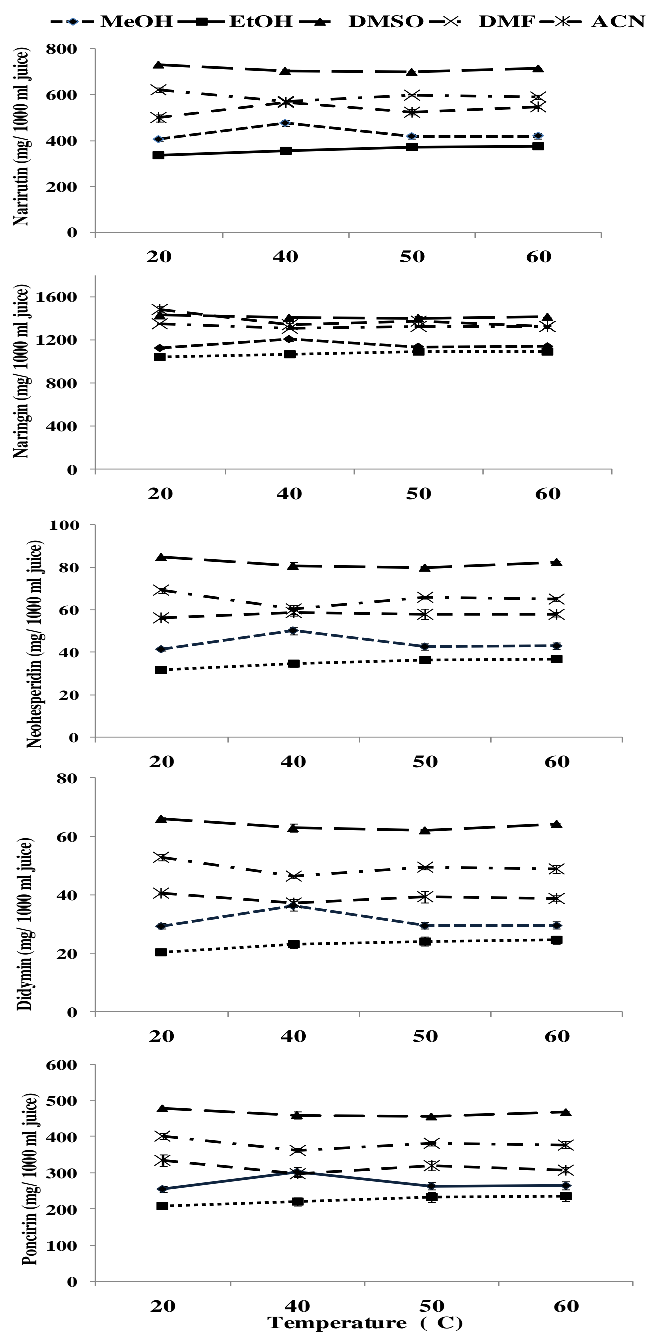

Fig. 2.1. Extraction efficiency of grapefruit flavanones with various solvents such as methanol (MeOH), ethanol (EtOH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF) and acetonitrile (ACN) at different temperatures ranging from 20, 40, 50 and 60 ˚C. The data presented is mean ± standard deviation values of three individual samples.

flavanone extraction [71]. In general, extraction temperature increases the diffusion coefficient (mass transfer rate) and solubility [72]. On the other hand, an increase in the extraction solvent to sample ratio can further enhance extraction.

These findings suggest that extraction solvent is one of the critical factors for optimizing extraction methods. Since fruit matrix consists of a complex mixture of biological components that interact with solvents and other extraction factors during flavanone extraction [50], they are addressed in the following experiments.

2.4.2 Influence of solvent and centrifugation

DMSO (713 mg/ 1000 mL) and DMF (590 mg/ 1000 mL) extracts had higher concentrations of narirutin than ACN (547 mg/ 1000 mL), MeOH (420 mg/ 1000 mL) and EtOH (375 mg/ 1000 mL) extracts (Fig. 2.1). However, the phase separations in ACN extracts led us to conduct further investigation of flavanone levels at different heights in centrifuge tubes after centrifugation (Fig. 2.2a). In the present experiment, flavanones were extracted at various centrifuge speeds such as 605, 3293, 6720, 11357 × *g* with MeOH, EtOH, ACN, DMSO or DMF at 0°C, and their quantities were analyzed from 1mL top, and bottom extracts of centrifuge tubes. Flavanones extracted at different centrifuge speeds contributed to minor flavanone variations where as major variations were observed among different solvent extracts (Fig. 2.3 and 2.4). Therefore, in all the following experiments 6720 × *g* was consistently used for centrifugation.

Interestingly, the quantities of flavanones in the extracts obtained from 1 mL top or bottom of the same centrifuge tube were different. The concentration of narirutin in the

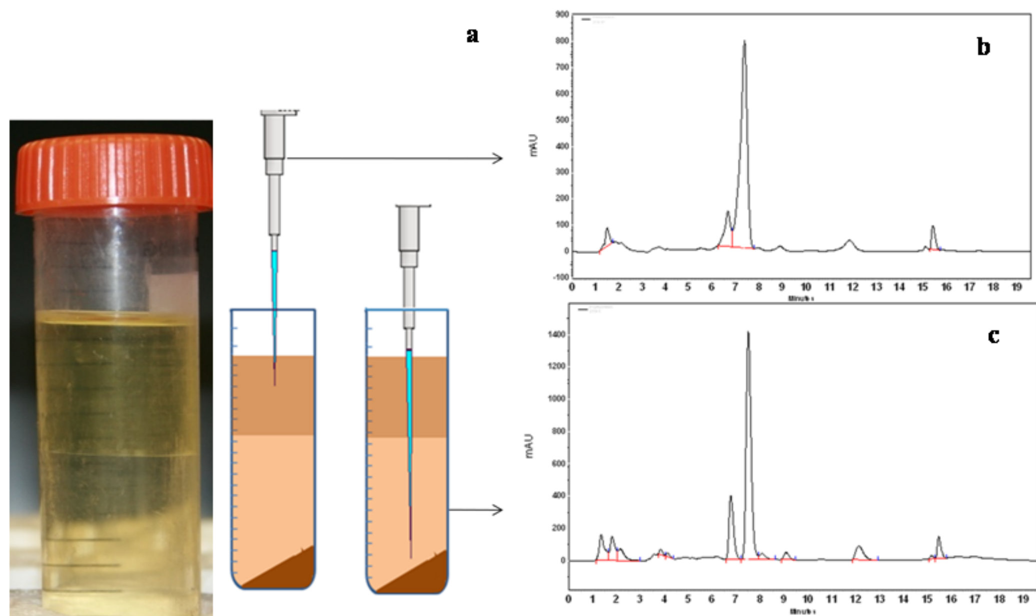

Fig. 2.2. The extraction of flavanones using centrifugation (a) phase separations observed in the ACN extracts of grapefruit juice after centrifugation at 6720 g. (b) HPLC chromatogram of the top layer. (c) Bottom layer.

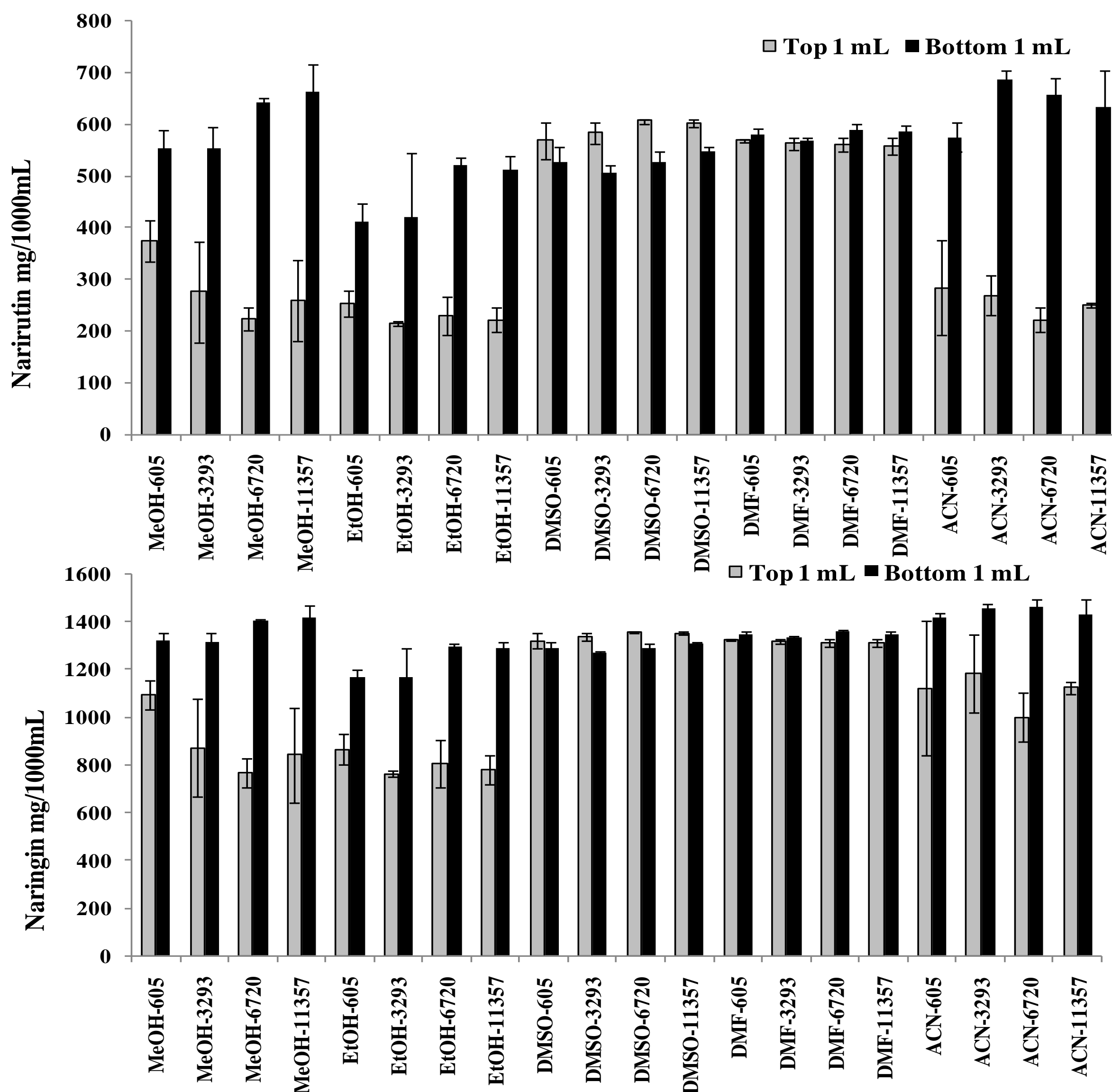

Fig. 2.3. Narirutin and naringin levels from 1 ml top and bottom of centrifuge tube with five different solvents namely methanol (MeoH), ethanol (EtOH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF) and acetonitrile (ACN) at various centrifugal speeds 605, 3293, 6720 and 11357 × *g*. The data presented is mean ± standard deviation values of three individual grapefruit samples.

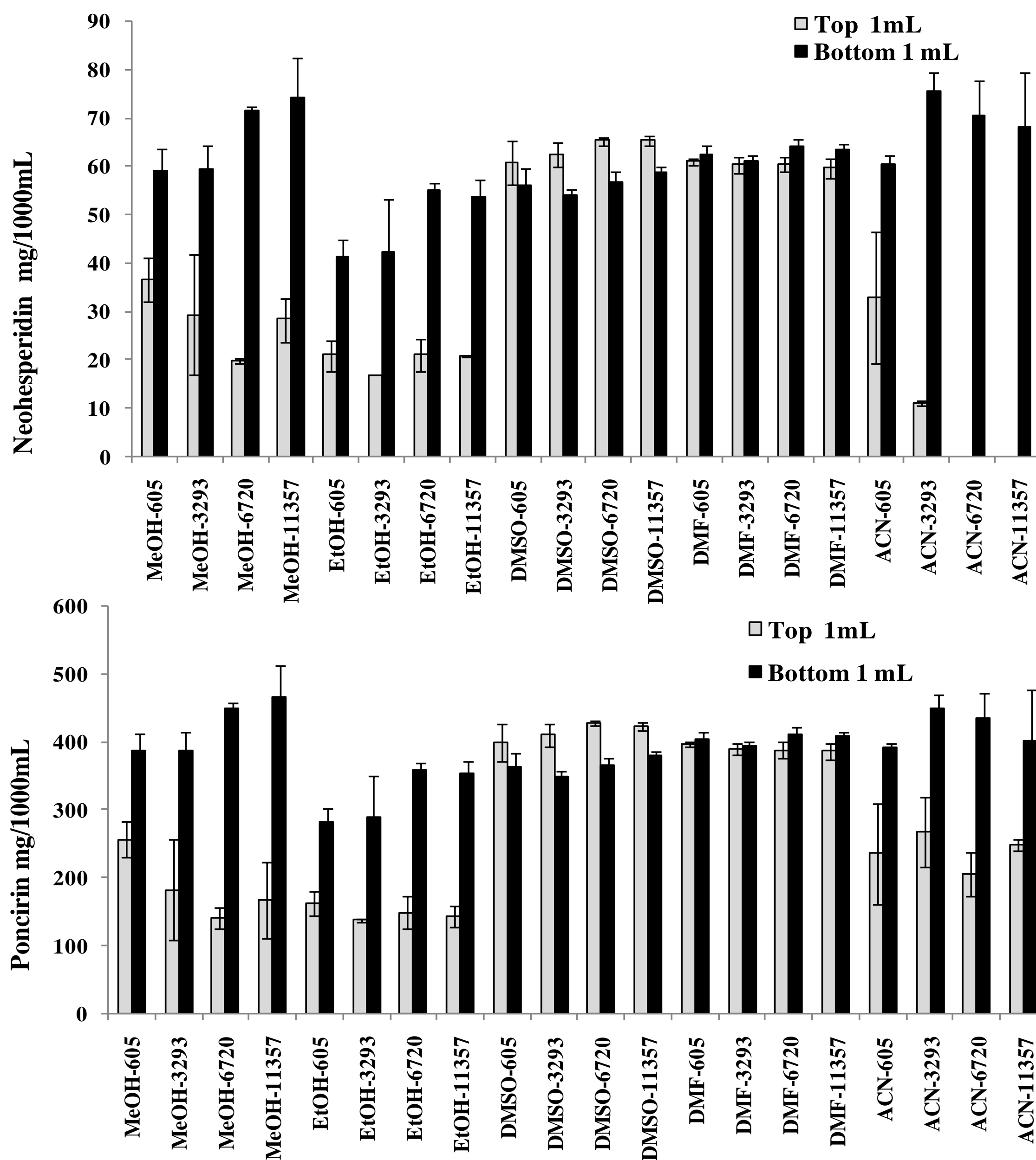

Fig.2. 4. Neohesperidin and poncirin levels from 1 ml top and bottom of centrifuge tube with different solvents namely methanol (MeOH), ethanol (EtOH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF) and acetonitrile (ACN) at various centrifugal speeds 605, 3293, 6720 and 11357 × *g*. The data presented is mean ± standard deviation values of three individual grapefruit samples.

methanol extract from bottom 1 ml was 195 % higher than that of top 1 mL of the supernatant when extracted at 11357 × *g*. Similar trends were observed in EtOH and ACN extracts. The ACN extracts obtained from all centrifugal speeds clearly separated into two distinct phases which was not reported in earlier flavanone studies.

When grapefruit juice was extracted with ACN, the phase separations occurred in the extracts because of the interaction of ACN with the water present in juice sample. The striations observed in Fig. 2.2a were possible because of the anomalous behavior of ACN in the presence of water (present in juice) at low temperatures. Zarzycki et al., [73] suggested that the phase separations occur when water concentration in ACN ranged from 31 to 89%.

Consequently, more hydrophobic compounds (sterols and carotenoids) diffused into the top layer, thus two phases were observed. The top layer of ACN extract affected HPLC peak resolution (Fig. 2.2b) as compared to bottom layer (Fig. 2.2c). According to Durling et al [74] , ratio of 40-60 % aqueous ethanol and methanol in water can be considered as hydroalcoholic solvent extractions. In the current experiment, extractions from grapefruit juice with MeOH and EtOH simulates hydroalcoholic extractions because the final extraction solvent constitutes of water obtained from juice and MeOH or EtOH. In hydroalcoholic extraction, the visible striations were not observed potentially because both these solvents can form better hydrogen bonding with water.

Flavanone levels in 1 mL top and bottom of DMSO and DMF extracts were not different suggesting that flavanones were homogenously distributed in the centrifuge tube (Fig. 2.3 and 2.4). For several decades, MeOH was invariably used as a default

extraction of flavonoid and phenolics from fruits and vegetables. However, in the present study MeOH extracts (hydroalcoholic extractions) not only showed lower extraction efficiency but also showed variable flavanone quantities in top and bottom aliquots in the centrifuge tube after centrifugation.

2.4.3 Influence of solvent and centrifuge temperature

In previous studies, centrifuge temperature during sample preparation was not considered critical during flavanone extraction. In the current experiment, the importance of the centrifuge temperature during centrifuge extraction was demonstrated. When grapefruit juice was extracted with ACN in centrifuge, two clear phase separations were observed at 0°C and 10°C temperatures but not at 20°C. However, variations in the flavanones in top and bottom 1 mL of centrifuge tube were found to be significantly different at three different centrifuge temperatures (Fig.2.5). Though MeOH extracts did not show phase separations at all the three temperatures tested (data not shown), the flavanone levels were different at 0°C and 10°C (Fig. 2.5). The MeOH extracts from 20°C centrifuge temperature did not show any differences in the flavanone levels from top and bottom 1 mL. In case of DMSO extracts, flavanone levels in top and bottom 1 mL of centrifuge tubes were not significantly different in all the centrifuge temperatures tested. The differential densities in the extraction mixture were observed because the individual solvent components (ACN and water in juice) showed different response towards applied temperature and centrifugal force.

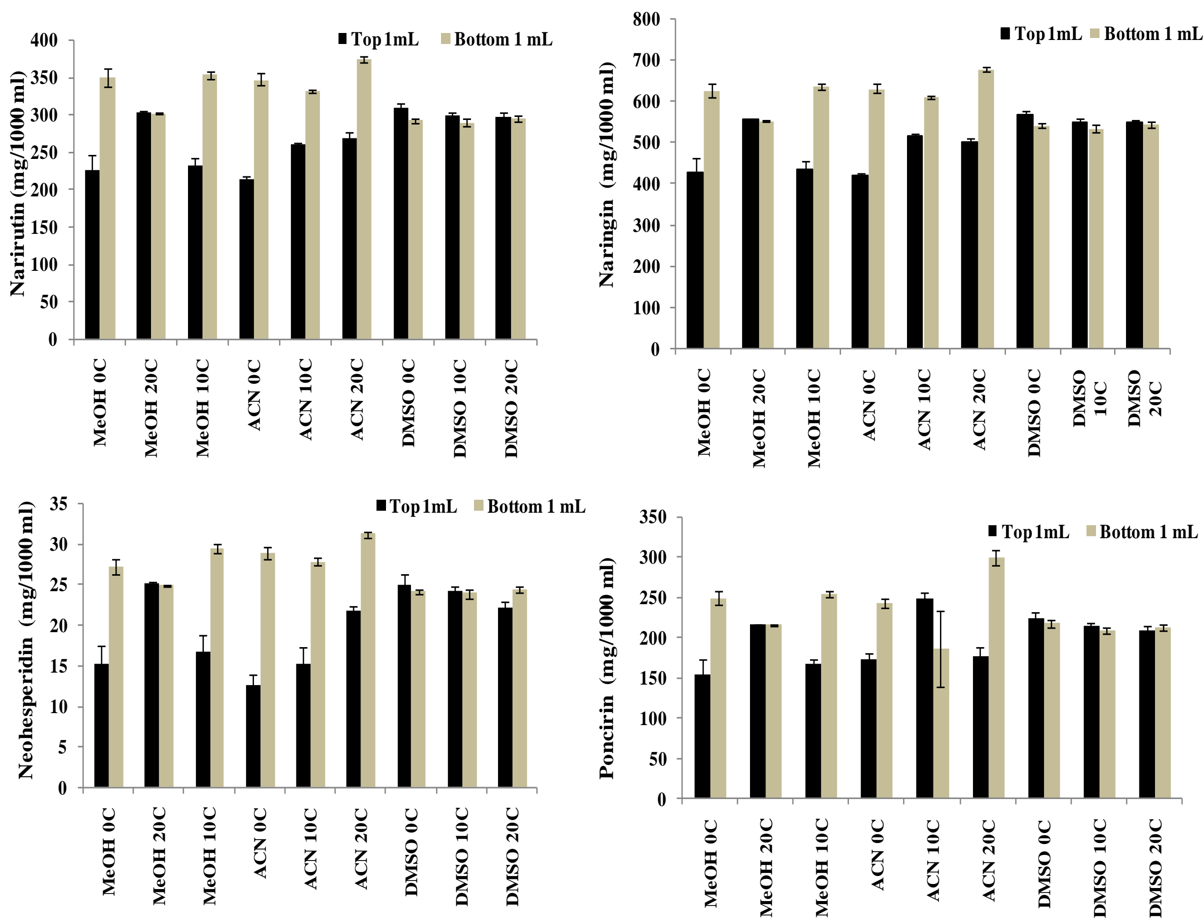

Fig.2. 5. Narirutin, naringin, neohesperidin and poncirin levels in grapefruits extracted from top and bottom 1 ml of centrifuge tube with different solvents such as methanol (MeOH), acetonitrile (ACN) and dimethyl sulfoxide (DMSO) at centrifuge rotor temperatures, 0, 10 and 20°C. The histograms represent mean ± standard deviations of three individual grapefruit samples.

2.4.4 Extraction cycles

In this experiment, DMSO was selected for extractions because it did not show significant variations in the flavanone levels in top and bottom 1 mL of centrifuge tube irrespective of centrifuge temperature. DMSO was used to extract grapefruit flavanones three times sequentially. The results from the current study suggest that 71 % (Table 2.2) of flavanones were extracted from the 1st extraction cycle while 20 % and 8 % of grapefruit flavanones were extracted from 2nd and 3rd cycle, respectively.

2.4.5 Effect of heat and sonication

In this study, DMSO was used to extract flavanones from grapefruit juice. Extractions were conducted at 20 and 40 °C in a sonicator for 10, 20, and 30 min. Although slightly higher levels of narirutin, naringin, neohesperidin and didymin were found in treatments 20 °C sonicated for 20 min and 40 °C sonicated for 10 min than other treatments, the change in levels due to sonication treatment were not significantly different (Fig. 2.6). The increase in extraction time did not always have higher levels of bioactive compounds [75]. Extraction of flavanones from the grapefruit juice was conducted by the phenomenon called cavitation, where the sample mixture is subjected to ultrasonic waves. The cavitation process can scour the tissues and allows the mass transfer of flavanones into the solvent [76]. Finally, once the extraction solvent is saturated with flavanones, further mass transfer from juice to solvent is negligible [75].

Table 2.2. The levels of individual grapefruit flavanones (mg/1000 mL) obtained by sequential extraction with DMSO[a].

Extraction Cycle	Narirutin	Naringin	Neohesperidin	Poncirin
1st extract	387±8.81	1199±24.19	38±0.99	281±7.41
2nd extract	111±10.60	354±33.48	9±1.38	72±8.08
3rd extract	46±1.28	143±9.37	3±0.17	32±1.74

[a] The results are expressed in mean ± standard deviation values of three grapefruit samples.

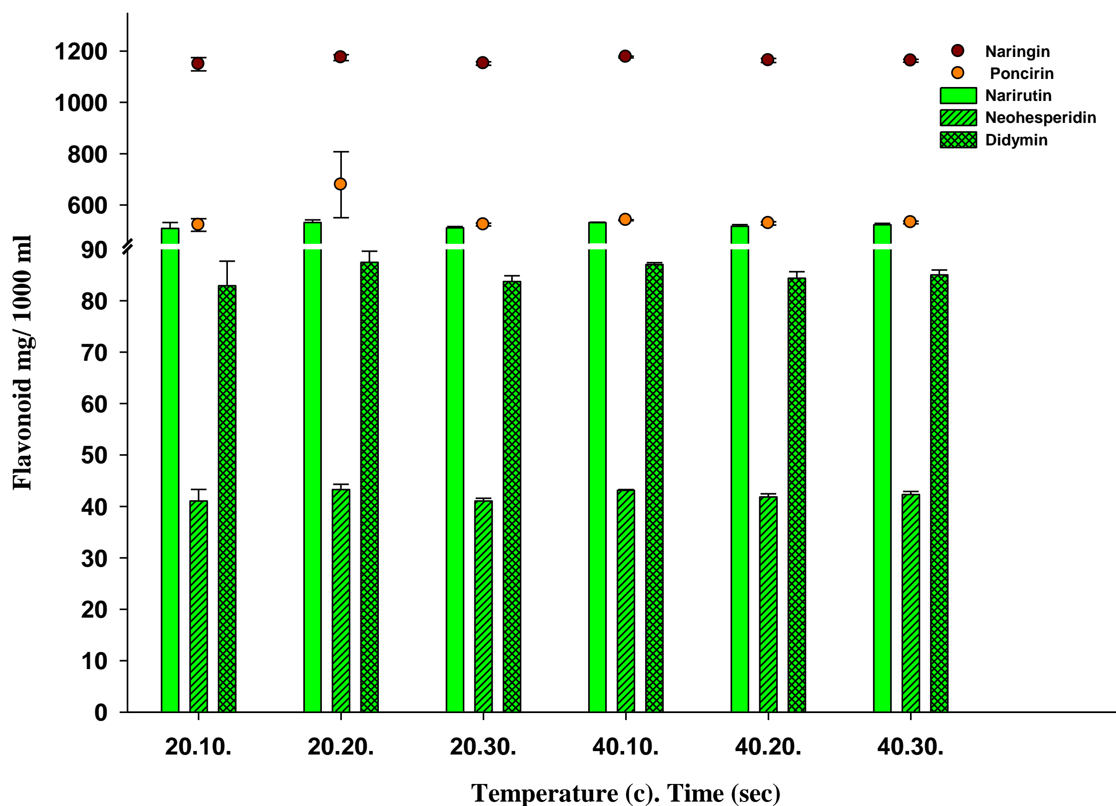

Fig. 2.6. Five grapefruit flavanones were extracted using dimethyl sulfoxide at three different sonication times namely 10, 20 and 30 min at 20 or 40 ˚C. The histograms represent mean ± standard deviations of three individual grapefruit samples.

2.4.6 Effect of microwaves and juice to solvent ratio

Four extraction times (5, 10, 15 and 20 sec) were used for extraction using microwaves with DMSO. Narirutin and poncirin levels were relatively higher when extracted for 15 sec. The optimum levels of naringin (1383 mg/1000 mL), narirutin (536 mg/1000 mL) and poncirin (447 mg/1000 mL) were observed when one part of the juice was extracted with two parts of the solvent (Fig. 2.7). Further 3 and 4 times dilutions of sample with solvent had limited detection of minor flavanones (neohesperidin and didymin). The role of solvent quantity in the flavanone extraction is extremely critical [54]. Higher sample to solvent ratio may stop the mass transfer of the analyte due to solvent saturation [77]. On the other hand, higher levels of solvent to sample ratio increased the extraction efficiency, yet it had detection problems of neohesperidin and didymin therefore optimum sample to solvent ratio improves the overall extraction efficiency.

Since microwaves have both electric and magnetic fields, the sample and solvent mixture is possibly heated by two different mechanisms such as dipolar rotation and ionic conduction when exposed to microwaves [78]. The heating of the sample expands the cell contents and causes disruption in the cell walls.

2.4.7 Flavanone separation and identification

In the current HPLC method narirutin, naringin, neohesperidin, didymin and poncirin eluted at 7.2, 7.9, 9.7, 15.2 and 15.5 min, respectively (Fig. 2.8). The regression equations, coefficient of determination, limit of quantification and limit of detection

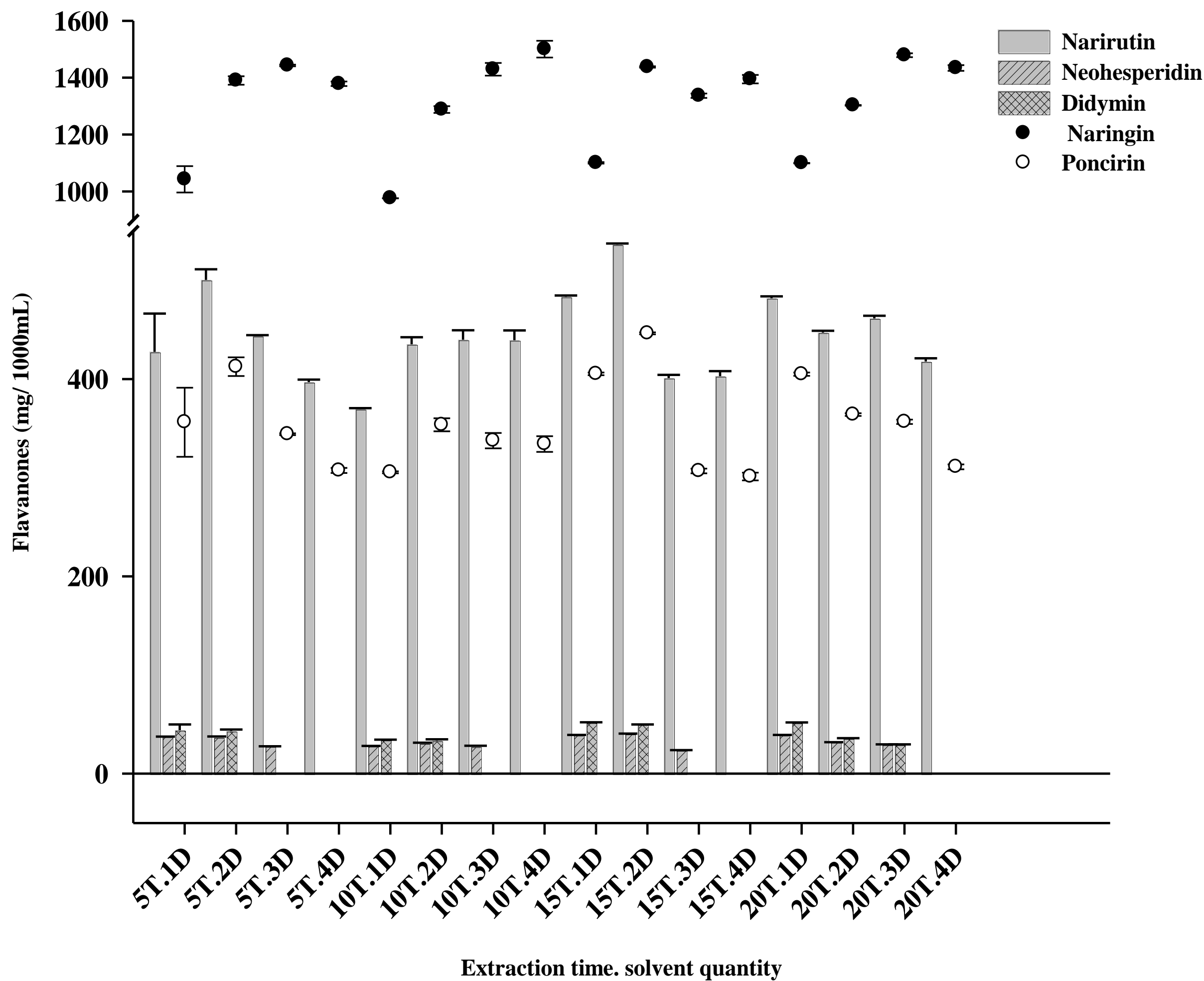

Fig. 2.7. Influence of extraction time (T in sec) on grapefruit flavanone extraction in microwave and sample to solvent volume 1, 2, 3, 4 dilutions (D). All the samples were extracted with dimethyl sulfoxide in triplicates.

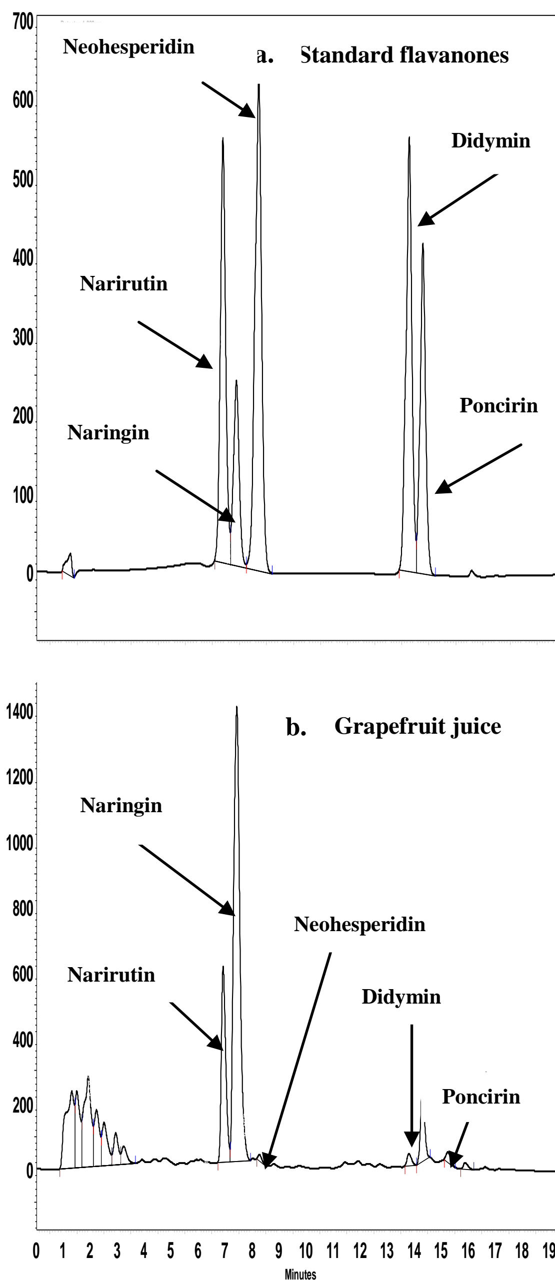

Fig. 2.8. The HPLC chromatograms of (a) flavanone standards and (b) grapefruit flavanones separated on a C-18 Hypersil gold column (100 mm x 4.6 mm i.d. with 5 µm article size) and eluted with a gradient mobile phase of 3 mM phosphoric acid and 100% acetonitrile.

were given in Table 2.3. The five grapefruit flavanones were identified by ESI, negative mode, LC/MS (Fig. 2.9). The mass spectra of narirutin and naringin showed a molecular ion $[M-H]^+$ at *m/z* 579.4 and 579.2 respectively. While, neohesperidin showed a molecular ion $[M-H]^+$ at *m/z* 609.3. Both didymin and poncirin generated $[M-H]^+$ at *m/z* 593.3.

To the best of our knowledge, this is the first report on variations of flavanone levels due to the interaction of solvents such as MeOH, EtOH and ACN with different centrifugation temperatures. Though the solvents used are miscible with water, flavanone distribution in these extracts was not homogenous after centrifugation. However, homogenous flavanone distribution was only found in DMSO and DMF extracts. The current study has opened a new area of research with respect to solvent, centrifuge temperature and flavanone migration in different phases in the field of sample preparation methods for bioactives. Among the various factors evaluated, the influence of solvent, extraction cycles and sample to solvent ratio had a major impact on accurate quantification. Though sonication and microwave extractions are two commonly used extraction methods for plant bioactives, understanding their interaction with different physical factors during extraction are critical for optimization of extraction procedures.

Table 2.3. Regression equations, coefficient of determination (r^2), linear range, limit of quantification (LOQ) and limit of detection (LOD) of various grapefruit flavanones analyzed in HPLC.

Flavanones	**Regression equation**	r^2	**Linear range (µg)**	**LOQ (µg)**	**LOD (µg)**
Narirutin	y=5E+06x + 26542	0.997	0.09-3.00	0.09	0.02
Naringin	y=7E+06x + 80994	0.996	0.31-2.50	0.31	0.02
Neohesperidin	y=6E+06x + 24221	0.998	0.07-2.50	0.07	0.03
Didymin	y=2E+06x + 54733	0.986	0.15-5.00	0.15	0.06
Poncirin	y=2E+06x + 50099	0.989	0.15-5.00	0.15	0.02

x: Concentration of the flavanone in the sample.
y: The peak area in terms of mAU.

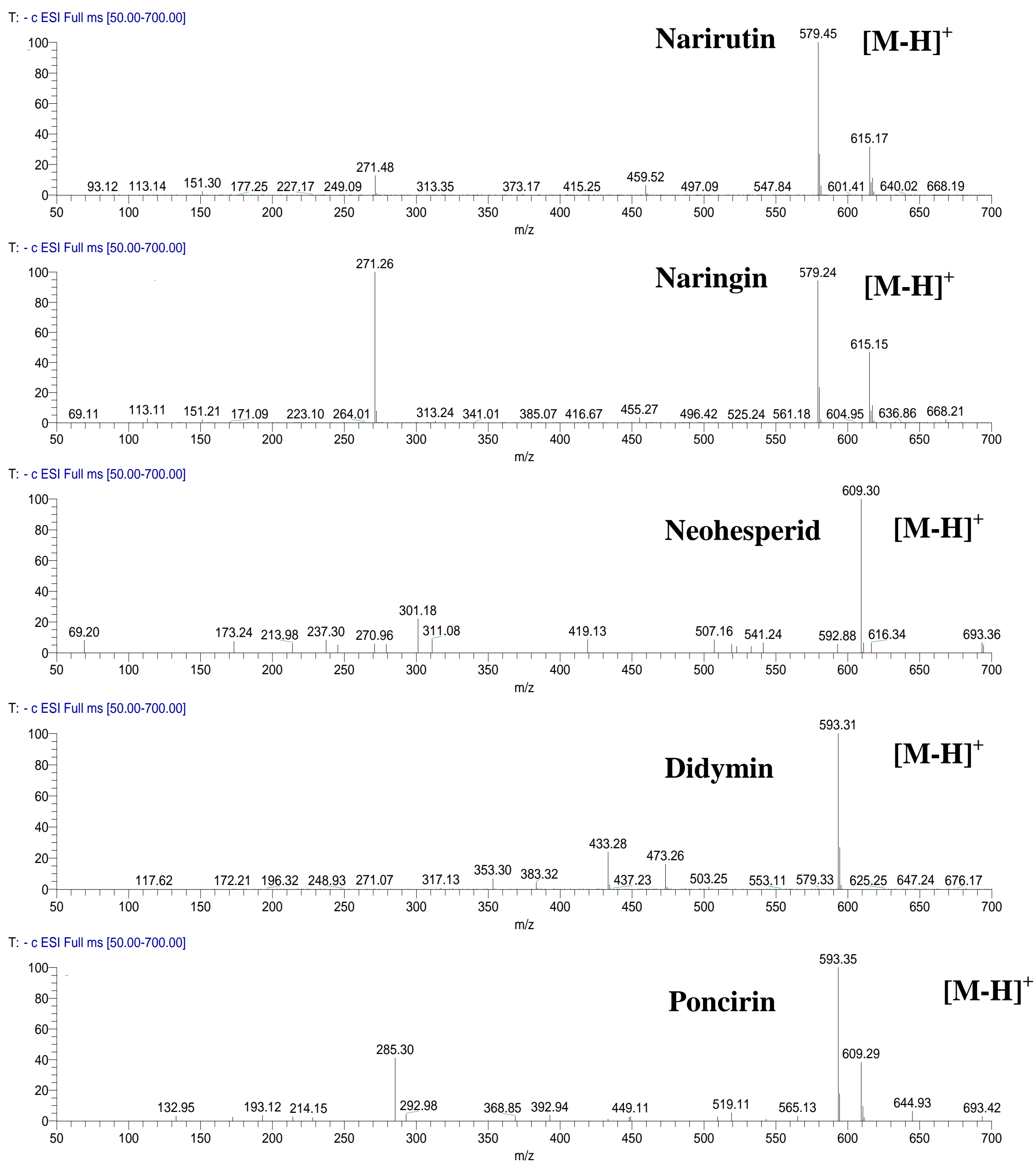

Fig.2.9. Mass spectra of five grapefruit flavanones through LC-MS in electron spray ionization (ESI) negative mode.

CHAPTER III

AN IMPROVED SAMPLE PREPARATION METHOD FOR QUANTIFICATION OF ASCORBIC ACID AND DEHYDROASCORBIC ACID BY HPLC*

3.1 Synopsis

Ascorbic acid (AA) and dehydroascorbic acid (DHA) are reduced and oxidized forms of vitamin C, which are ubiquitously found in various fruits and vegetables. The present study has evaluated and optimized various factors responsible for AA and DHA stability in grapefruit samples. Furthermore, the optimized method was used to quantify these compounds in different fruits and vegetables. The AA stability in the samples was evaluated by extracting grapefruit juice using 1, 3 and 5 g/100 mL meta-phosphoric acid (MPA) and trichloro-acetic acid (TCA). The AA levels were stable in grapefruit samples extracted with 1, 3 and 5 g/100 mL MPA, whereas TCA extracts showed degradation in 48 h. Among the three reducing agents studied, tris (2-carboxy ethyl) phosphine hydrochloride (TCEP) has efficiently converted DHA at all concentrations and the samples were stable for 48 h at 2.5 mmol/L TCEP. At lower pH favored complete conversion of DHA by TCEP than dithiothreitol. Among various fruits and vegetables analyzed, the highest levels of AA (260.1 mg/100 g) were observed in guava and DHA (58.6 mg/100 g) in parsley samples. The current optimized method prevents the degradation of AA and DHA from fruit and vegetable samples stored at room

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temperature for two days.

3.2 Introduction

Fruits and vegetables being a major source of vitamin C, accurate quantification of AA and DHA is warranted. Fruits and vegetables have complex matrices with numerous nonspecific compounds that interfere with quantification of AA and DHA [85,86]. Moreover, AA and DHA are highly unstable compounds at milder conditions such as pH 7 and room temperature [87]. Therefore, quantification of these compounds in fruits and vegetables without degradation losses is challenging.

Spectrophotometry analysis [88] used to be most popular analytical method among HPLC [89], capillary zone electrophoresis [90] and voltametry [91] for AA and DHA analysis. However, recently HPLC gained popularity due to its high throughput and accuracy over spectrophotometry.

AA determination by HPLC is more accurate than spectrophotometric analysis, but lack of conjugated double bonds in DHA minimizes its absorption in UV spectra [92-94]. DHA absorbs wavelength at 185 nm and thus limits its quantification in the UV range [95]. Despite the absorption constraints of DHA, several studies were conducted to develop a method for simultaneous determination of AA and DHA using HPLC equipped with a photo-diode array (PDA) detector [96]. Recently, a new method was developed for simultaneous quantification of AA and DHA using a charged aerosol detector [97]. Yet, this detection method is less common for the analysis of a wide array of bioactives present in fruits and vegetables on a regular basis.

Accurate quantification of DHA was made possible in the UV region by pre-column derivatization technique [98]. However, these sample preparation methods for pre-column derivatization involved multi-step reactions and used high concentration buffers (soluble solids) in mobile phase, thus could limit the reproducibility of the method and cause crystallization in solvent lines [99,100]. Although studies related to DHA quantification with pre-column derivatization exist, little information is known about the factors affecting the stability of AA and DHA in real samples.

Traditionally, AA and DHA were extracted using trichloro acetic acid (TCA) and metaphosphoric acid (MPA) [85,101]. Yet, the stability of AA in TCA and MPA extracts was not compared after extraction from real samples.

DHA quantification was commonly conducted through pre column derivatization using dithiothreitol (DTT) and β-mercaptoethanol (BME) [95,102]. Recently, tris (2-carboxy ethyl) phosphine hydrochloride (TCEP) was used to analyze DHA in milk and plasma samples [85,99,103]. Also, very few reports provided critical information on reaction kinetics of TCEP and standard DHA [104,105]. However, studies involving method development requires optimization of these experimental parameters in real samples rather than working on standard matrices.

The objective of the current study is to optimize the sample stability parameters in grapefruit for accurate analysis of vitamin C (AA and DHA). The experimental parameters such as extraction solvents, reducing agents and pH for sample stability in grapefruit are optimized. Finally the optimal conditions were used to determine vitamin C content in various fruits and vegetables.

3.3 Experimental

3.3.1 Plant materials

Fresh fruits (kiwi, guava, strawberries, star fruit and citrus fruits) and vegetables (parsley, Italian parsley and cabbage) were purchased from a local store. Grapefruits were harvested at Texas A&M University- Kingsville, citrus center, Weslaco, TX. After harvesting, the fruits were boxed and transported to vegetable and fruit improvement center, College Station, TX.

3.3.2 Reagents

Chemicals such as dihydrogen ammonium phosphate, β-mercapto ethanol (BME) and dithiothreitol (DTT) were purchased from Acros Chemicals (Morris Plains, NJ, USA). L-ascorbic acid, tris (2-carboxy ethyl) phosphine hydrochloride (TCEP), metaphosphoric acid (MPA) and trichloro acetic acid (TCA) were purchased from Sigma Chemicals (St. Louis, MO, USA) and orthophosphoric acid was obtained from EMD Chemicals (Gibbstown, NJ, USA).

3.3.3 Optimization of extraction solvents and extraction efficiency

Grapefruits were harvested in February 2010 from Texas A&M University-Kingsville, citrus center, Weslaco, TX. Ascorbic acid was extracted with three different concentrations (1, 3 and 5 g/100 mL) of MPA and TCA. A 0.75 g of grapefruit juice was mixed with 0.75 mL of extraction solvent in a micro centrifuge tube, vortexed for 5 s and centrifuged at 4500 x *g* (Marathon 16 KM centrifuge, Fisher Scientific, Hanover

Park, IL, USA) for 10 min. The supernatant was passed through 0.45 µm acrodisc syringe filters (Fisher Scientific, Hanover Park, IL, USA) and analyzed by HPLC at 0, 12, 24, 36, and 48 h at room temperature to determine the inter and intra-day AA stability.

The extraction efficiency of the current method was compared with the reported method [106]. Three milliliters of 3 g/100 mL MPA was added to 3 mL of grapefruit juice, vortexed for 30 s and the mixture was carefully passed through a 0.45 µ filter under vacuum. The residue was further re-extracted two times using 3 g/100 mL MPA and analyzed by HPLC.

3.3.4 Reduction of DHA

For DHA analysis, TCEP was used for reduction and compared with the commonly used reducing agents namely BME and DTT. Ten mmol/L of BME was prepared by adding 7 µl of BME (14.3 mol/L) to 10 mL of nano-pure water. Similarly, 15.42 mg and 28.66 mg of DTT and TCEP were dissolved in 10 mL of nano-pure water separately to prepare 10 mmol/L solutions. The stock solutions were stored at 4 °C and serial dilutions were prepared just before sample reduction. Grapefruit juice (0.75 g) was mixed with 3 g/100 mL MPA (0.75 mL) in eppendrof tubes and centrifuged at 4500 x *g* for 10 min. The sample aliquot (300 µl) was treated with 1.25, 2.5, 5, and 10 mmol/L concentrations of BME, DTT or TCEP (300 µl) separately. After 30 min, the samples were injected into HPLC for TA analysis. The sample DHA was calculated as the difference between free AA and TA.

The effect of extraction solvents on total ascorbic acid (TA) stability was further studied by mixing 600 µL of 3 g/100 mL MPA and TCA extracts separately with 600 µL of 5 mmol/L of TCEP and the sample mixture was analyzed in HPLC at zero and 36 h.

3.3.5 Determination of optimal pH for DHA analysis

The samples were prepared from grapefruits harvested in November 2007. Optimum sample pH for DHA analysis was determined by using 5 mmol/L concentration of TCEP and DTT for sample reduction. One normal hydrochloric acid and sodium hydroxide were used to adjust sample pH to 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, and 6. Later, the samples were centrifuged at 4500 x *g* for 10 min and the aliquot was filtered with 0.45 µ acrodisc filter and 5 µl of sample was injected for HPLC analysis.

3.3.6 Inter-day and intra-day stability of total ascorbic acid

Grapefruit samples were extracted with 3 g/100 mL MPA at pH 2.4. The sample aliquots were reduced with five different concentrations (0.312, 0.625, 1.25, 2.5, 5 mmol/L) of TCEP and DTT separately. The samples were allowed to stay at room temperature for 30 min for complete reduction and then analyzed by HPLC at 0, 12, 24, 36 and 48 h to determine the inter and intra-day AA stability.

3.3.7 Recovery studies

The accuracy of sample preparation was evaluated by conducting the recovery studies in grapefruit, guava and parsley samples. Stock solutions were prepared by dissolving a known quantity of AA standards (0.075, 0.15 and 3 mg) in 10 mL of 3 g/100 mL MPA and stored at 4ºC. The AA standards (0.75 mL) were added to 0.75 mL of grapefruit and guava samples before extraction. In case of parsley, 49 g of sample was blended along with 49 ml of MPA consisting different concentrations (0.075, 0.15 and 3 mg/ 10 mL) of AA. The sample mixtures were centrifuged at 4,500 x *g* and passed through a 0.45 µ acrodisc filter. All the recovery studies were conducted by extracting three individual samples and analyzed in HPLC.

3.3.8 Chromatograpic conditions

The current chromatoraphic method was modified from previously published report [107]. Ascorbic acid was analyzed using a Thermo Finigan, Spectra system (Wathman, MA, USA), with a PDA detector (spectra system UV6000 LP) coupled with a quaternary pump system P4000 and an AS3000 auto sampler. The separation was carried out using a C_{18}, spherisorb column (150 mm x 4.6 mm i.d. and 3 µm particle size) and the run time was 10 min. The AA peak was detected at 254 nm and the analysis was carried out by Chromquest 4.0 version software. The entire chromatographic separation was performed at an isocratic mobile phase of 0.01 mol/L dihydrogen ammonium phosphate maintained at pH 2.6 and 1 mL/min flow rate. All the samples

were prepared in triplicates and injected three times in HPLC with a 5 µl injection volume.

3.3.9 Calibration and regression equation

The linearity of the calibration curve was determined by injecting the standards within the working range of the samples. Six serial dilutions such as 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 µg/10µL were prepared from 1.25 µg/10µL standard AA in 3 g/100 mL MPA and injected thrice in HPLC. The calibration graph was prepared by plotting peak area against the corresponding standard AA concentrations.

3.3.10 Mass spectral analysis

An aliquot of 0.75 g of guava was extracted with 0.75 mL of 3 g/100 mL glacial acetic acid. The mixture was centrifuged and the supernatant was filtered with 0.45 µ acrodisc disposable filter. Twenty micro-liters of the supernatant was injected in to a HPLC and the AA fractions were collected. The collected fractions were taken for mass spectral analysis. The electron impact ionization mass spectra (EI-MS) of ascorbic acid were performed using a GC-MS equipped with a direct insertion probe (DIP) (Thermo Fisher Scientific, Waltham, MA). The probe temperature was maintained at 280 °C under 70 eV EI conditions and DIP-MS analyses was conducted with a DSQ II, quadrupole mass spectrometer to record mass spectra.

3.3.11 Extraction of AA and DHA from fruits and vegetables

The fruits and vegetables known for their vitamin C content such as kiwi, guava, strawberries, star fruit, parsley, Italian parsley and cabbage were blended with 3 g/100 mL MPA at 1:1 (g:g) for 3 min and homogenized for 30 s. For citrus fruits, 3 g/100 mL MPA was added during centrifugation. Later, the homogenized juice was extracted in eppendrof tube and centrifuged at 4500 x g for 10 min. The aliquot was passed through 0.45 µ acrodisc filter and injected into the HPLC system for analysis of AA. For DHA quantification, the filtered aliquots (0.5 mL) were treated with 0.5 mL of 5 mmol/L concentration TCEP for 30 min at room temperature and analyzed in HPLC.

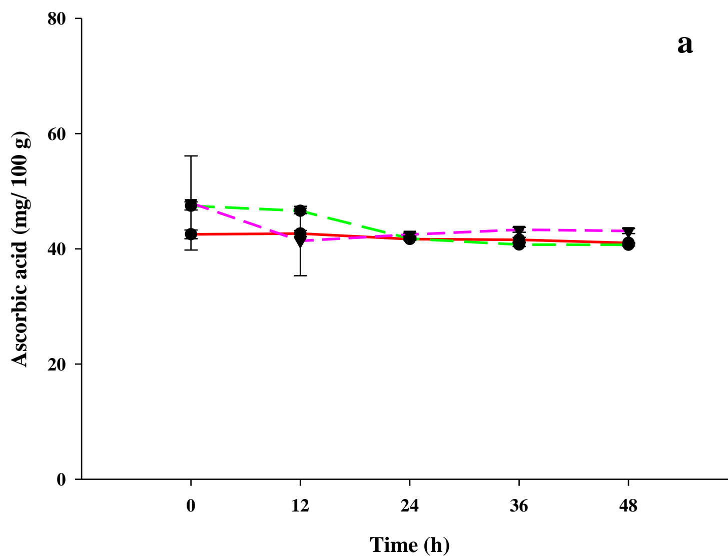
3.3.12 Data analysis

The sample analysis was carried out in triplicates and the results were presented as mean ± SD. The levels of AA and DHA were calculated using the regression equation obtained from the calibration graph. The data was analyzed by ANOVA using statistical software program version 16.0 (SPSS Inc., USA) at $p \leq 0.05$. The mean variations of sample triplicates and graphs were plotted by *Sigmaplot* 11.0 version software.

3.4 Results and discussion

3.4.1 Extraction and stability of AA

Among the two extraction solvents (MPA and TCA) used, AA was stable in MPA compared to TCA extracts (Fig. 3.1a). No significant loss was observed in AA levels of MPA extracts (1, 3 and 5 g/100 mL) stored at room temperature in 48 h. While,



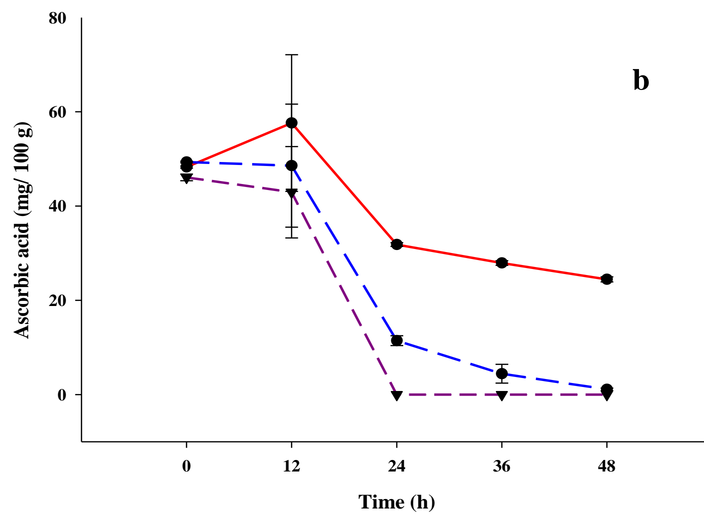


Fig.3.1. Influence of solvents on extractability of ascorbic acid from grapefruit juice. **(a)** inter-day and intra-day stability of AA in 1 ( ), 3 ( ) and 5 ( )g/100 mL metaphosphoric acid (MPA) extracts; **(b)** inter-day and intra-day stability of AA in 1 ( ), 3 ( ) and 5 ( )g/100 mL trichloroacetic acid (TCA) extracts.

AA completely degraded in 3 and 5 g/100 mL TCA extracts and partially degraded in 1 g/100 mL TCA extracts in 48 h (Fig. 3.1b). AA was stable up to 48 h in MPA extracts mainly due to the inhibition of ascorbate oxidaze, metal catalysts and precipitation of proteins by MPA [98]. Although 1, 3 and 5 g/100 mL MPA showed no significant differences in AA stability, only 3 g/100 mL MPA was consistently used for following experiments to prevent possible degradation of AA in mildly acidic samples (vegetables).

The difference between the current extraction method for AA analysis and the first extraction of the reported method was minor (Fig. 3.2). Furthermore, the standard error bars on the reported method (4.96 mg/ 100g) were greater compared to the current method (1.36 mg/100g). This could be possible due to longer exposure of extract to a steady stream of air during vacuum filtration. Therefore, we continued to use the current extraction method in all the following experiments.

3.4.2 Reducing agent selection and optimization

BME and DTT are the two commonly used reducing agents for DHA analysis by derivatization [25,108]. In the present experiment, the reducing efficiency of BME, DTT and TCEP was evaluated at four different concentrations (1.25, 2.5, 5 and 10 mmol/L). The reducing efficiency of TCEP (11.45 ± 0.11 mg/100g of DHA) was consistent at all four concentrations tested (Table 3.1). Complete reduction of DHA (11.38 ± 0.23 mg/100g) was obtained only at the highest DTT concentration, while BME could not completely reduce DHA at any of the four concentrations tested. Furthermore,

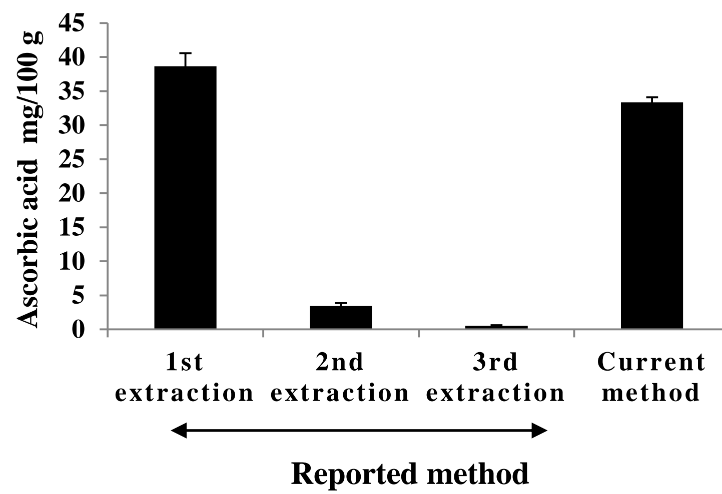


Fig. 3.2. Extraction efficiency of the current method with the reported method

Table 3.1. Reduction efficiency of tris (2-carboxy ethyl) phosphine hydrochloride (TECP), β- mercaptoethanol (BME), and dithiothreitol (DTT) at different concentrations on dehydroascorbicaicd in the grapefruit samples.

Concentration	**Dehydroascorbic acid[a] (mg/100 g)**		
(mmol/L)	**TCEP**	**BME**	**DTT**
1.25	11.23±0.07	7.40±0.08	9.05±0.07
2.50	11.28±0.09	6.80±0.18	9.79±0.13
5.00	11.28±0.08	7.39±0.10	10.29±0.12
10.00	11.45±0.11	6.69±0.08	11.38±0.23

[a]All the results are average of three individual samples ± standard deviation and expressed in fresh weight basis.

the efficiency of DTT and BME declined at lower concentrations. Reduction of DHA was a 1st order reaction and therefore, reagent concentration played a significant role in complete DHA reduction [104].

BME is a volatile liquid with an offensive odor while DTT is relatively unstable and less efficient at lower pH (< 3) [104,109]. Additionally, DTT is readily oxidizable by metal ions such as Fe^{+3} and Ni^{+2} [102,110]. TCEP is a non volatile, odorless and comparatively less expensive reducing agent [110]. Unlike DTT, the applications of TCEP in the field of biochemistry were not popular until recently [102]. Although in recent years, few studies used TECP for DHA reduction in plasma, fruits and vegetables, it was not directly studied for optimizing extraction methods in real fruit samples to improve sample stability. L-cysteine, another common reducing agent, was not used in this study because it can reduce DHA only at pH 7 [111].

As TCEP showed higher reducing efficiency compared to other reducing agents tested; only TCEP was selected to reduce the grapefruit samples extracted with MPA and TCA. In the presence of TCEP, TA was found stable at all concentrations of MPA and TCA extracts (Fig. 3.3). No significant differences were found among the treatments. In the present experiment (Fig. 3.3), the levels of AA in grapefruit were higher than the rest of the studies (Table 3.2 and 3.3) presented in this paper possibly due to the seasonal effect on grapefruit production [82]. Later, the reducing efficiency of TCEP and DTT was evaluated at different sample pH.

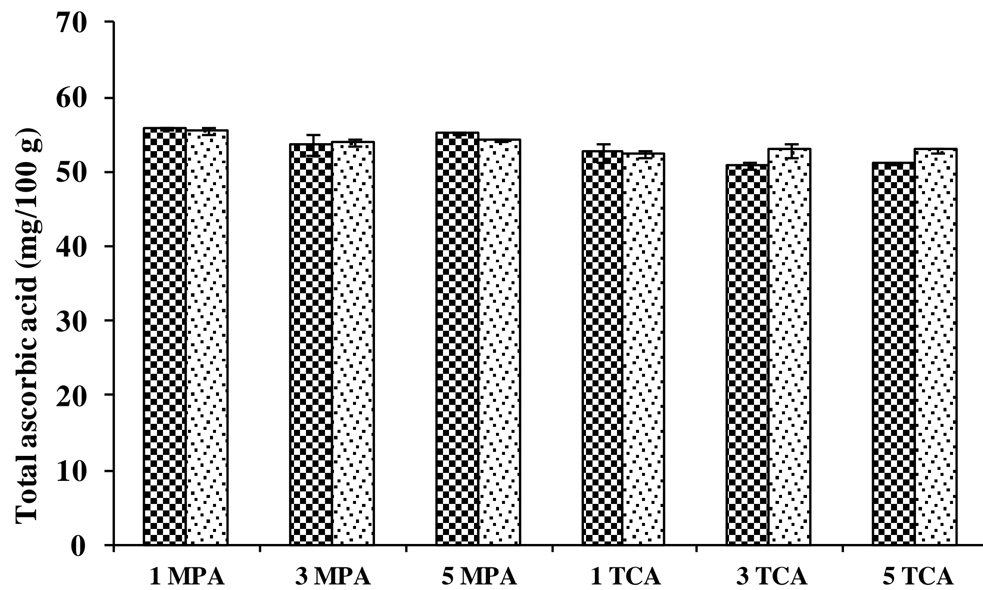


Fig. 3.3. Grapefruit total ascorbic acid extracted with 1, 3, 5 g/100mL metaphosphoric acid (MPA) and trichloro acetic acid (TCA) at zero hour (⊠) and 36 h (⊡) after conversion of DHA to AA using 5 mmol/L of tris(2-carboxy ethyl) phosphine hydrochloride.

Table 3.2. Recoveries of ascorbic acid (mg/100 g juice) in grapefruit, guava and parsley extracted with 3 g/100 mL MPA.

Sample	Original ascorbic acid	Added standard ascorbic acid	Expected quantity	Observed quantity	Recovary (g /100 g)
Conventional Grapefruit	30.1	30	60.1	57.60±0.50	95.84
		15	45.1	44.97±0.31	99.73
		7.5	37.6	38.10±0.22	101.33
Guava	260.105	30	290.105	328.76±11.29	113.33
		15	275.105	292.19±14.29	106.21
		7.5	267.605	277.37±23.44	103.65
Parsley	28.116	30	58.116	54.68±6.8	94.09
		15	43.116	41.49±0.30	96.24
		7.5	35.616	32.04±1.55	89.97

[a]All the values are average of three individual samples ± standard deviation and expressed in fresh weight basis.

Table 3.3. Ascorbic acid, dehydroascorbicacid and total ascorbic acid levels (mg/100 g) in various fruits and vegetables extracted with 3 g/100 mL MPA.[a]

Fruits/vegetables	**AA**	**DHA**	**TA**
Kiwi	93.40±01.92	22.04±01.96	115.45±01.08
Parsley	35.84±02.44	58.66±16.42	94.50±14.04
Italian parsley	26.84±00.28	26.19±00.24	53.04±00.43
Cabbage	45.46±01.41	43.22±00.71	88.68±02.11
Strawberries	48.87±02.72	38.34±01.94	87.22±03.00
Lime	38.96±01.28	16.41±00.34	55.38±00.32
Lemon	55.50±00.44	16.45±00.81	71.96±00.71
Starfruit	18.65±00.31	22.59±00.14	41.24±00.27
Organic grapefruit	36.68±01.05	12.28±00.99	48.71±00.11
Conventional grapefruit	30.67±01.84	12.17±00.59	42.88±01.09
Orange Varieties			
Temple orange	64.27±03.08	14.78±01.93	79.05±00.59
OL marsh white	21.15±00.88	27.82±00.48	48.98±00.58
Shimouti orange	36.46±02.43	27.84±00.76	64.31±00.53
OL pine apple	24.22±00.30	31.59±00.31	55.81±00.24
Thornton tangelo	38.56±01.15	25.06±00.43	63.62±00.57

[a]All the results are average of three individual samples ± standard deviation and expressed in fresh weight basis.

3.4.3 Optimization of sample pH and TA stability

The half-life of DHA at pH 7 is two minutes [89] and pK_1 of AA is 4.2 [104]. Therefore, a sample with pH < 4.2 can prevent rapid degradation of AA and DHA. In the current study, reducing efficiency of TCEP and DTT were evaluated at various pH levels ranging from 2 to 6 with 0.5 increments. A pH < 2 was not used in this experiment because the column stationary phase is unstable at such a low pH [112]. Higher DHA yields were recorded in the samples reduced by TCEP at pH 2 (Fig. 3.4). These results are also in accordance with previously published report [104]. Optimization of sample pH is critical for AA and DHA stability and also to increase the reducing efficiency of TCEP.

Inter and intra-day stability of AA and DHA together was obtained by analyzing TA for every 12 h over a 48 h period. Since BME showed lower reducing efficiency, it was not considered for evaluation in this experiment. The sample reduction was carried out with TCEP and DTT (Fig. 3.5a and 3.5b) at five different concentrations. TA was stable for 48 h in the samples reduced by TCEP at 2.5 and 5 mmol/L concentrations, while reduction was incomplete at all concentrations of DTT. In this study an efficient extraction with aqueous solvent modifier (MPA) and reducing agent (TCEP) together prevented oxidation of TA.

3.4.4 Chromatography, method validation

The quantification of DHA was performed by calculating the difference between TA and original AA before derivatization (Fig. 3.6a). In the present chromatographic

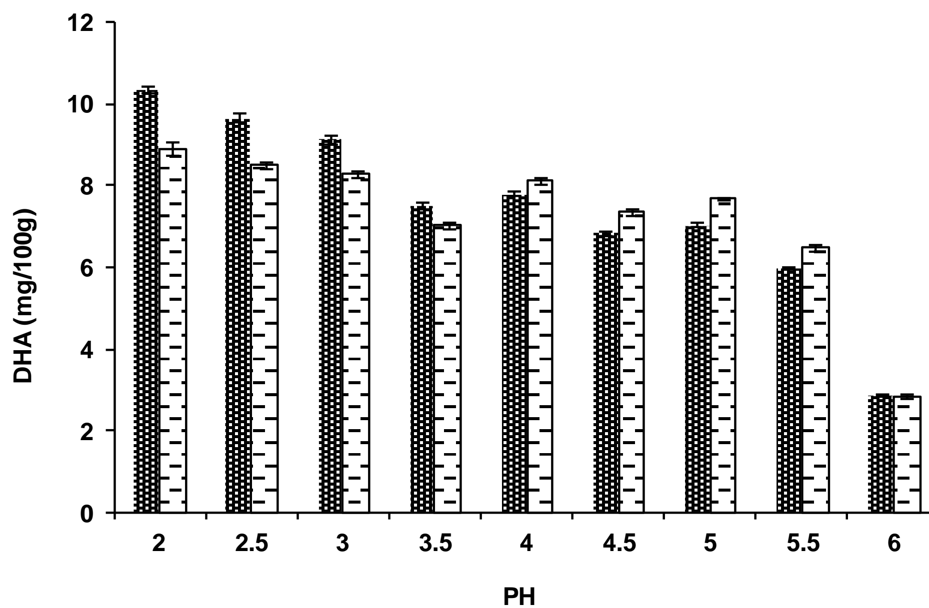
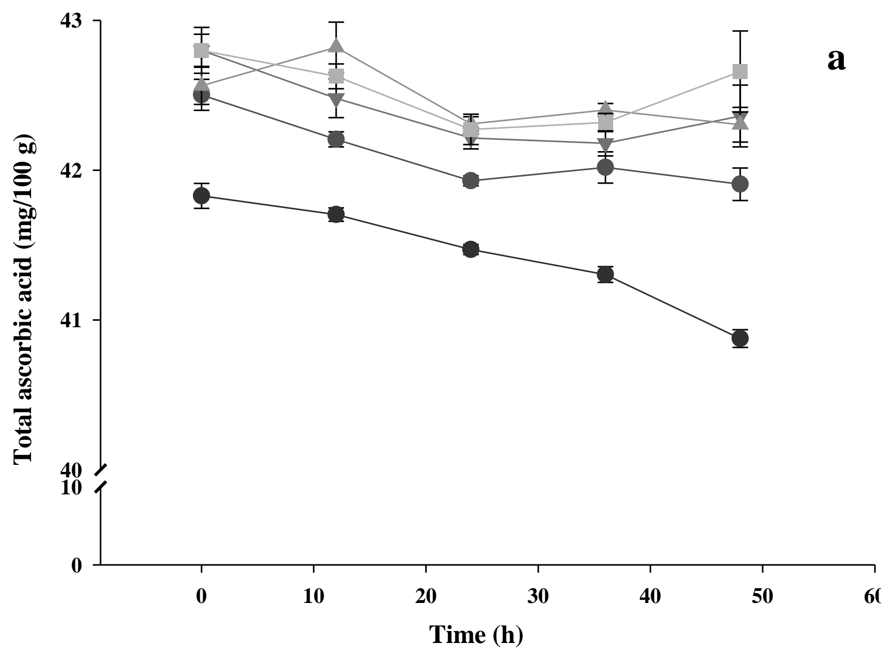


Fig. 3.4. Reduction of dehydroascorbic acid by tris (2-carboxy ethyl) phosphine hydrochloride (TCEP) and dithiothreitol (DTT) at various pH ranging from 2 to 6.



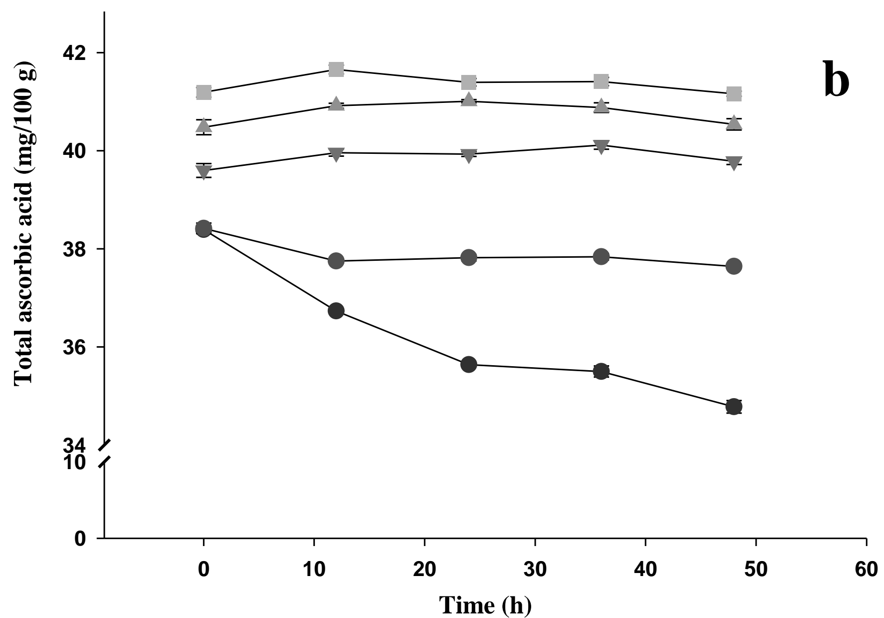


Fig. 3.5. Inter and intra-day degradation rates of total ascorbic acid at room temperature with tris (2-carboxy ethyl) phosphine hydrochloride (TCEP) and dithiothreitol (DTT) at ●0.312, ● 0.625, ▼1.250, ▲ 2.500, ■5.000 mmol/L.

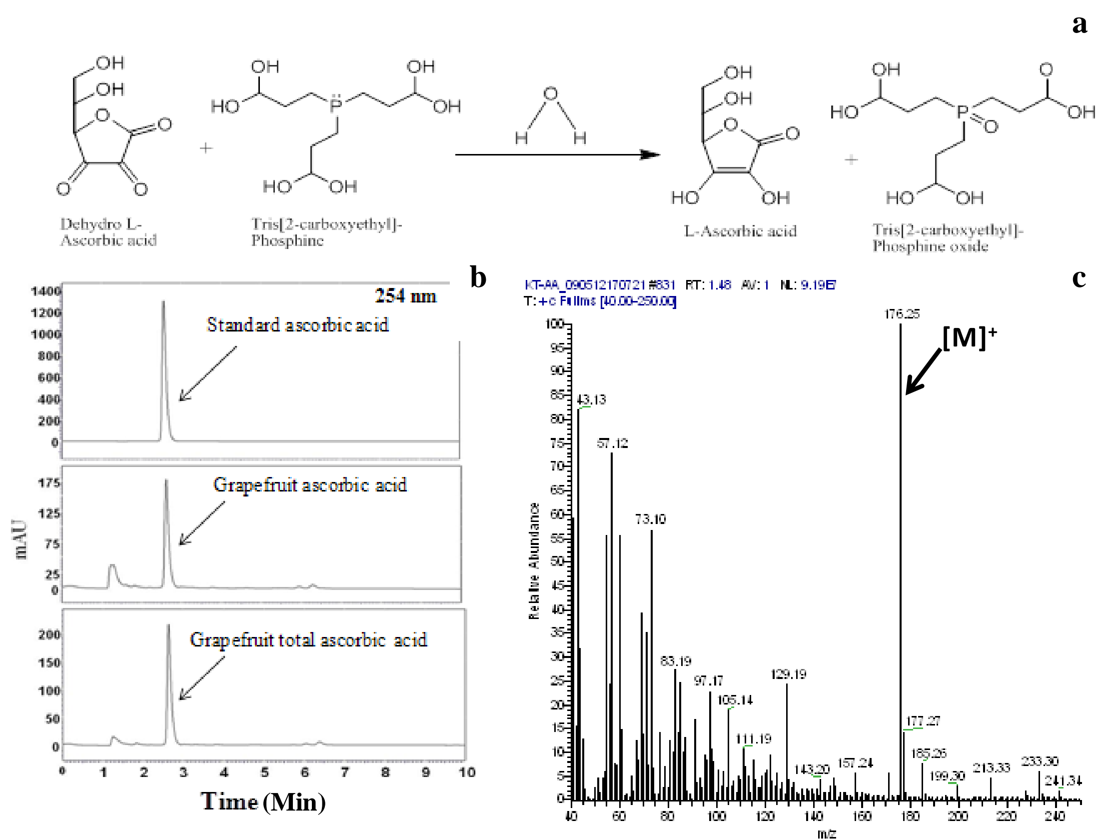


Fig. 3.6. (a) The schematic representation of reduction of dehydroascorbic acid to ascorbic acid facilitated by tris (2-carboxy ethyl) phosphine hydrochloride (b) HPLC separations of standard ascorbic acid, ascorbic acid from grapefruit and total ascorbic acid in grapefruit monitored at 254 nm (c) Mass spectrum of ascorbic acid fraction of a guava sample. The analysis was conducted by a direct insertion probe (DIP) in electron impact ionization (EI) mode.

method, AA was eluted at 3.5 min (Fig. 3.6b) and detected at 254 nm. Since guava showed higher AA levels, the identity of AA was confirmed by collecting the AA peak fraction in guava sample using HPLC followed by mass spectrometry. The Fig. 3.6c confirms the presence of AA molecular ion $[M]^+$ recorded at m/z 176.25. The method was validated by injecting 7 concentrations ranging from 0.019 µg to 1.25 µg with a regression equation y = 2862 x + 8.88 and coefficient of determination (r^2) ≥ 0.999 (Fig. 3.7a). Furthermore, linearity was confirmed by residual plot and normal probability plot (*P-P* plot) given in Fig. 3.7b, 3.7c. The precision of the method was calculating the inter day and intraday variation of grapefruit sample that resulted in ≤ 0.003 CV. Recovery studies were carried out to evaluate accuracy of the method by spiking grapefruit (optimized sample), guava (high AA content) and parsley samples (leaf matrix) with standard AA. The results from the recovery tests are presented in table 3.2. The mean recoveries of the samples ranged from 90 to 113.33 g/ 100g indicating the reliability and accuracy of the optimized method.

3.4.5 Applicability

Fruits and vegetables with different sample types including leafy matrices (parsley, Italian parsley and cabbage) were selected for AA and DHA analysis. The Kiwi fruit contained highest concentration of AA (93.4 mg/100 g) and parsley contained highest DHA (58.6 mg/100 g) concentration (Table 3.3). Among the two parsley

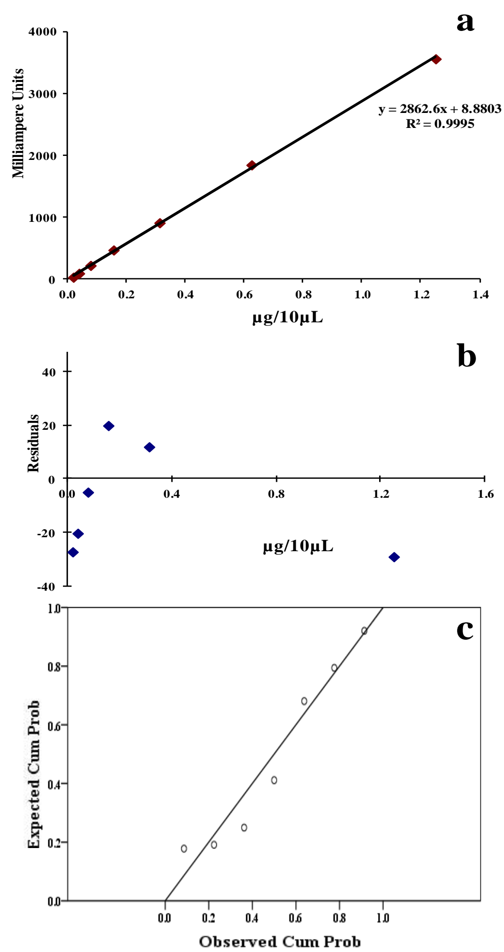


Fig. 3.7. Linearity of standard ascorbic acid (a) calibration curve and regression equation (b) residual plot of the predicted values and (c) normal probability plot (*P*-*P* plot).

varieties analyzed, DHA was higher in flat leaf parsley *(Petroselinum crispum var. neapolitanum)* than that of curly leaved Italian Parsley *(Petroselinum crispum var. crispum).* Among oranges, Temple orange had the highest AA (64.28 mg/100 g) and OL pineapple had highest the DHA (31.59 mg/100 g). Our observed values of AA and DHA were comparatively higher than the previously reported concentrations except for parsley [89,113]. In the study conducted by Gokmen et al., deionized water was used as an extraction solvent in which vitamin C is highly unstable [89]. In another study conducted by Thompson et al., the samples were extracted with 3g/100 mL MPA and later DHA reduction was done with DTT [113]. From Fig.3.4, DTT is not as efficient as TCEP in reducing DHA at $pH < 3.5$. This could be a possible explanation for the reported lower DHA levels. It is also possible that higher values in AA and DHA could be due to the use of an efficient reducing agent and/or variations in the cultivars and season. The current optimized method took less than 30 min for sample preparation and analysis. Hence, the optimized method is commercially viable for analysis of AA and DHA in numerous samples.

Ascorbic acid and TA were more stable in MPA than TCA extracts. Unlike DTT and BME, TCEP has completely converted DHA to TA and maintained sample stability even at lower concentrations. Furthermore, optimization of experimental conditions facilitated better understanding and significance of extraction solvent, pH and reducing agent in stability of AA and DHA in samples. The optimized method reflects higher values of AA and DHA due to the use of efficient reducing agent at a lower pH. The

developed method is rapid and can be adopted for analyzing numerous samples without degradation of vitamin C for two days at room temperature.

CHAPTER IV

PRODUCTION SYSTEMS AND STORAGE TEMPERATURE INFLUENCE GRAPEFRUIT VITAMIN C, LIMONOIDS AND CAROTENOIDS*

4.1 Synopsis

Concentrations of grapefruit (cv. 'Rio Red'; *Citrus paradisi Macf.*) bioactives grown under organic and conventional production systems were evaluated at various storage temperatures. The first experiment was conducted in Nov 2008 and the second experiment was conducted in Feb 2011 using commercial production, and packing procedures. The harvested grapefruits were stored at 23 °C room temperature (RT) or 9 ºC for four weeks and analyzed for vitamin C, limonoids and carotenoids at the end of each week using HPLC. Vitamin C levels were higher in organically grown grapefruits (418 mg/L) compared to conventionally grown grapefruits (392 mg/L) at zero days after harvest in the first experiment. However, production system did not significantly affect vitamin C levels in the second experiment. During storage at RT, the vitamin C degradation losses ranged from 7-18% for organically produced grapefruits and 0-3% for conventional grapefruits in both the experiments. In the first experiment at harvest, organic grapefruits had 77% higher ($p \leq 0.05$) nomilin than conventionally produced

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grapefruits whereas grapefruits grown under the conventional production system had two-fold higher lycopene compared to organic grapefruits.

In the second experiment, both β-carotene and lycopene levels were significantly ($p \leq 0.05$) higher in conventionally produced grapefruits than in organic grapefruits. Overall, conventional production significantly increased grapefruit carotenoid levels in both the experiments. In general, storage temperature (room temperature and 9 ºC) had minimal effects on vitamin C degradation but significantly degraded carotenoids in the first experiment.

4.2 Introduction

Grapefruit (*Citrus paradisi Macf*) is one of the major commercial citrus crops grown in the subtropical regions of the United States including Florida, Texas and California for fresh market as well as processing. The red-colored varieties such as 'Rio Red' grown in Texas are particularly rich in bioactive compounds (health promoting compounds) such as vitamin C, limonoids and carotenoids [2,3]. While genetics plays a key role in determining the levels of bioactive compounds in fruits and vegetables, cultural practices such as fertilization, pesticide use, and environmental factors such as temperature can also significantly impact bioactive concentrations. In recent years, the role of production systems, especially the effect of organic versus conventional practices on bioactive properties of fruits and vegetables has been a topic of intense public debate and research. Organic fruits and vegetables are perceived by most consumers to be safer

than conventionally grown produce [114]. This perception has been partly responsible for the dramatic rise in sales of organic produce in the US from $3.6 billion in 1997 to $21.1billion in 2008 [115]. Organic produce, according to the US regulations, is grown under conditions devoid of synthetic pesticides, growth hormones (GH), antibiotics, chemical fertilizers, genetically modified organisms and sewage sludge [10]. Organically grown produce generally attracts approximately 73-108% higher prices compared to the conventionally grown foods in the fresh food market [116]. While organic agriculture has traditionally focused on risk reduction of chemical residues and heavy metals, recent studies have indicated that organic production practices such as fertilization may also influence the content of health promoting compounds [117-119].

Unlike the synthetic fertilizers used in conventional production systems, most organic supplements have slow nutrient release properties. This slow availability of nutrients and the resulting changes in photo-assimilate partitioning may lead to preferential accumulation of secondary metabolites that have bioactive properties [120],[121]. Previous reports suggested that nitrogen availability to plants may have an inverse relationship with vitamin C content and a positive influence on β-carotene levels[122], other studies also suggested that minimal synthetic chemical use in also organic production may increase the nutrient quality of fruits and vegetables [82,123]. In addition to different production practices, several post harvest procedures including storage duration and storage temperature can have a significant influence on levels of bioactive compounds in fruits and vegetables [16,17,124]. These effects are expected to

vary depending on the type of bioactive compound, plant species, organ and tissue. The effects of production system and storage temperature on grapefruit bioactives were poorly understood.

Vitamin C, limonoids and carotenoids are the grapefruit bioactives that primarily contribute to the fruit's sensory attributes such as flavor and color in addition to its health promoting properties [125-127]. Vitamin C occurs as both ascorbic acid (reduced form) and dehydroascorbic acid (oxidized form) in the fruit at the time of harvest and storage. Consumption of both forms is beneficial to human health due to their anti-scorbutic properties [128,129]. Therefore, vitamin C analysis is critical as the levels of ascorbic acid and dehydroascorbic acid continuously interchange during storage. Limonin and nomilin, the two major limonoid aglycons in grapefruit have demonstrated to have anti-carcinogenic properties and also responsible for grapefruit bitterness [130,131]. Lycopene and β-carotene are two major antioxidant carotenoids that contribute to flesh color of grapefruit. The information related to production systems and post harvest handling practices on the levels of these compounds in grapefruit is still scanty. The objectives of the current study were to investigate the influence of organic and conventional grapefruit production systems and simulate postharvest storage conditions on grapefruit on health promoting bioactive compounds.

4.3 Methods and materials

4.3.1 Chemicals

L-ascorbic acid, tris (2-carboxy ethyl) phosphine hydrochloride (TCEP) and metaphosphoric acid (MPA) were purchased from Sigma Chemicals (St. Louis, MO, USA), orthophosphoric acid was obtained from EMD chemicals (Gibbstown, NJ, USA) and dihydrogen ammonium phosphate was obtained from Acros Chemicals (Morris Plains, NJ, US) for ascorbic acid analysis. Potassium chloride and nitric acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). β-carotene, lycopene and tert-Butylmethyl ether were purchased from Sigma Chemicals (St. Louis, MO, USA), while HPLC grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and sodium hydroxide was purchased from EMD chemicals (Gibbstown, NJ, USA).

4.3.2 Orchard selection

In both organic and conventional orchards, the grapefruit trees were planted in 1990. Organic grapefruits were harvested from the South Texas Organics (Mission, TX, USA) while conventional grapefruits were harvested from the Rio Queen Citrus Farms (Mission, TX, USA). The certified organic Rio Red grapefruit orchard, South Texas Organics (latitude 26° 29' N, longitude 98° 38' W, lat, elevation 60 m) is located three miles away from the conventional grapefruit orchard, Rio Queen Citrus (latitude 26° 26' N, longitude 98° 38' W, lat, elevation 60 m). Rio Red grapefruits with uniform color (without patches of green and red), size 48 (10 cm in diameter) and maturity were

selected from four quadrants of the trees. The first experiment (E1) was conducted in Nov 2008 and the second experiment (E2) in Feb 2010. The seven day mean precipitation, temperature, potential evapo-transpiration, solar radiation, relative humidity and temperature (max and min) were obtained from the weather station located at Weslaco, TX . Due to the similarities in soil type (sandy loam), climate and source of irrigation (Rio Grande River) in the two production systems (Table 4.1), fruits were compared for their nutrient quantity produced from organic and conventional management systems under common storage conditions.

4.3.3 Harvest, storage and processing

Adjacent trees of five in a row (block) were randomly selected and three such blocks were selected from each production system. The harvest was started around early morning and completed by noon, followed by washing, waxing (carnauba wax for conventional grapefruits and Decco Natur™ 550 wax for organic grapefruits) and packing of the fruits in the respective packing sheds. The process from harvest was concluded within the same day. After packing, the fruits were shipped overnight to the Vegetable and Fruit Improvement Center, Texas A&M University, College Station, TX. Fruits were stored at 23 ºC RT (room temperature) and 9 ºC (cold storage) for the next four weeks. The cold storage and normal temperature storage rooms were maintained at 95% and 65% RH respectively. Furthermore, weekly storage weight loss and fruit decay were measured during storage at room temperature and 9 ºC.

Table 4.1. Farm inputs in organic and conventional grapefruit orchards for fertilization, insect and weed control

Production System	Inputs	Rate of application during growing season/ acre	Number of Applications
Organic	Compost	N-47.5 Kg, P-29.5 Kg, K-18 Kg	1
	Compost Spray Formula	N-9 Kg	3
	Micronutrient Spray	Ca-1.3 Kg, Mn-1.3 Kg, Mg-2.2 Kg, Zn-0.9 Kg	2
	Sulfur Spray	5.4 Kg	5
	Pest control	DesX (fatty acid)-5.6 L, Safe Tside (Vegetable oil)-5.6 L	3
	Flood Irrigation	15 cm	4
Conventional	Inorganic fertilizer	N-32- 87 L	1
	Herbicides	Krover IDF-1.13 Kg, Simazine 90 DF-0.9 Kg, Diuron 80 DF- 0.58 Kg, Glyphosate-spot application if necessary, Bucanneer Plus- 60 mL, Bronc Max- 60 mL	1
	Insect Control	Vendex-1.13 Kg, Danitol-0.5 L, Gem-0.25 L	1
	Fertilizer Spay	Foli Gro- Booster fertilizer	2
	Fungicide	Kocide 3000-1.8 Kg	1
	Flood Irrigation	12 cm	5

In this experiment, blocks were considered as replications in each of organic and conventional production systems. A set of 27 fruits (3 fruits × 3 samples × 3 blocks) were collected from room temperature and 9 °C storage of organic grapefruit lot (a total of 54 fruits) each week during storage. Similar procedure was followed for sample collection from conventional grapefruits during storage. A total of 108 fruits were processed during each week of storage for grapefruit bioactive analyses. The fruits were collected on 0th, 7th, 14th, 21st and 28th day after the harvest. Three fruits were peeled and blended using a Vita Prep blender (Cleveland, Ohio, USA) to prepare a single grapefruit sample and three samples were prepared from each block.

4.3.4 Juice and soil mineral analysis

The grapefruit juice and soil samples were analyzed at the soil, water and forage testing lab, (College Station, TX). The nitrite nitrogen was extracted from grapefruit juice using 1N potassium chloride (KCl) solution on a reciprocal shaker for 30 min followed by nitrite to nitrate reduction through a cadmium column in a colorimetric apparatus (FIA lab Instruments Inc., Bellevue, WA, USA). Furthermore, the nitrate nitrogen of the sample was quantified in soil and grapefruit [132]. Other grapefruit juice minerals were quantified in Inductive Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES), (Spectro Genesis, Deutschland, Germany). After digesting the juice samples in concentrated nitric acid, they were allowed to stay overnight at room temperature [133]. The digested samples were heated up to 125°C for four hours and after cooling

and sample dilution, the intensity of the ion response was measured in ICP-AES. For other soil minerals, the extractions were conducted using Mehlich III reagent and analyzed in an ICP [134].

4.3.5 Sensory analysis

Rio Red grapefruits stored for four weeks in the E1 were evaluated for sensory attributes such as sweetness, sourness, tartness, and their overall acceptability. On the day of the experiment, the fruits were taken out from cold storage (9°C) and washed with tap water. For flavor analysis, the fruits were cut into four quarters and placed in plastic bowls for evaluation. Fruits were also evaluated based on a previously established protocol for color, roughness and overall appearance [3]. A 41 member untrained sensory panel evaluated the grapefruits. Additionally, a nine centimeter hedonic scale was constructed similar to a published report [135]. The non structured hedonic scale was anchored at 0, 3, 6 and 9 cm respectively but numbers are not provided on the scale to prevent the panelist from selecting a specific number on the scale. The panelists were given clear verbal instructions and also evaluation sheets before being allowed to enter the booth. The grapefruit samples with a three digit code were placed in front of the panelists for evaluation. The panelists were provided with bottled water and unsalted crackers to remove the residual flavor between evaluations. The panelists were asked to place a vertical line across the hedonic scale to indicate the intensity of each attribute.

Later, quantitation was performed by measuring the distance between zero and the vertical line.

4.3.6 Titratable acidity and total soluble solids

The titratable acidity of the fruits was analyzed using a DL 22 Food and Beverage analyzer, Mettler Toledo, (Columbus, OH, USA). Grapefruit juice (5 g) was taken and mixed with 45 ml of nanopure water and titrated against 0.1 N NaOH. The total soluble solids were analyzed using a hand refractometer (American Optical Corporation, South bridge, MA, USA).

4.3.7 Bioactive compounds analysis

4.3.7.1 Vitamin C analysis

Sample preparation and analysis of vitamin C followed the same procedure as the above reported method.[136] The grapefruit samples were analyzed using a HPLC (Thermo Finnigan, Spectra system, Wathman, MA, USA), equipped with a PDA detector (UV6000 LP) coupled with a quaternary pump system (P4000) and an auto sampler (AS3000). Rio Red grapefruit juice samples (0.75 ml) were mixed with 0.75 ml of 3 % metaphosphoric acid, vortexed for 5 sec and centrifuged at 10,000 rpm for 10 min. The supernatant was passed through a 0.45 μm acrodisc syringe filter. A 300 μL of the filtered aliquot was mixed with 300 μL of 10 mM tris (2-carboxyethyl) phosphine hydrochloride to reduce sample dehydroascorbic acid to ascorbic acid. The resulting

solution was analyzed for vitamin C (total ascorbic acid) using HPLC. The peak separation was carried out in a C-18, Spherisorb column (150 mm x 4.6 mm i.d. and 3 µm particle size) using an isocratic mobile phase of 10 mM ammonium dihydrogen phosphate buffer with a flow rate of 1 ml/min. Each sample was analyzed twice in the HPLC with a 5µL injection volume. The total ascorbic acid peak was detected at 254 nm and the data was analyzed using Chromquest 4.0. Standard ascorbic acid concentrations of 5, 2.5, 1.25, 0.625, 0.3125, 0.156 and 0.078 µg were injected into HPLC to calculate the regression equation. The final ascorbic acid levels were expressed in mg/100g of grapefruit juice.

4.3.7.2 Limonoid analysis

Sample preparation for limonoid analysis was modified from the previously reported method.[137] Rio Red grapefruit juice (10 g) was extracted with 20 ml of ethyl acetate on a shaker for 12 h. The organic fraction of the mixture was separated and the residual juice was re-extracted with 10 ml of ethyl acetate for 2 h. Both the organic fractions were combined and evaporated to dryness. The dried extract was then reconstituted in 5 ml of DMSO. One milliliter of the resultant extract was passed through a 0.45 µm acrodisc syringe filter into an amber glass vial and 10 µl was injected into HPLC.

Separation of limonoids was performed using a Finnigan Surveyor Plus HPLC system (West Palm Beach, FL, USA). The HPLC system was equipped with a PDA

Surveyor Plus, detector coupled with a quaternary LC Pump Plus system, a Surveyor Plus auto-sampler (25 µl sample loop with valco fittings) and a C-18, PFP, kinetex column (100 mm x 4.6 mm i.d. and 2.6 µm particle size) (Philadelphia, PA,USA). Pure limonin and nomilin standards were obtained according to established procedures [138]. Peaks were detected at 210 nm and the analysis was carried out using Chromquest 5.0. Chromatographic separations were performed with a gradient mobile phase consisting of 3 mM phosphoric acid prepared in nanopure water (A) and 100 % acetonitrile (B). Limonoids were eluted with the following solvent gradient: started with 80 % A; 0.1-7.00 min gradient reached 75 % A; 7-12.00 min an isocratic of 75% A; 12-16.00 min the gradient reached 70% A; 16-25.00 min the gradient reached 50% A; 25-30.00 min gradient reached 40% A; and the method had five minutes of equilibration at the end of the run. The sample injection volume for the analysis is 10 µL. The identity of the limonin and nomilin in the samples was obtained by matching with the retention times of pure standards. Each sample was analyzed three times in HPLC. The limonoid concentrations were expressed in µg/ g of grapefruit juice.

4.3.7.3 Carotenoid analysis

The carotenoid analysis method was modified from a previously published report [139]. Grapefruit juice samples (5 g) were extracted with 20 ml of chloroform [140]. The extractions were conducted in orange light to prevent any possible carotenoid degradation. The moisture from the samples was removed by adding sodium carbonate

to the extracts. The samples devoid of moisture were used for the HPLC analysis. A 10 μL of sample volume was injected into HPLC for carotenoid analysis. Carotenoid separations were carried out on an YMC C-30 column (Milford, MA, USA). The elution of carotenoids occurred with the following mobile phase gradient constituting methanol (A) and tert-butyl ether (B). The carotenoids were eluted as follows 0.1- 10 min, 85% A; 10-18 min, 20% A and in 18-25 min gradient combination reached to 100% B. The column was equilibrated for 5 min with 85% A before successive injections and detected at 465nm wavelength with the aid of a tungsten lamp (PDA detector). The samples in the auto-sampler were maintained at 6 °C throughout the analysis. The two carotenoids present in grapefruit were identified as β-carotene (retention time 9.1 min) and lycopene (retention time 15.5 min) by running the standard carotenoids. The calibration curves for the standard β-carotene and lycopene were prepared by injecting six serial dilutions ranging from 0.3 μg to 0.007 μg /10 μL injection volume. Furthermore, each sample was analyzed three times in HPLC and the carotenoid levels were expressed in μg/g.

4.3.8 Data analysis

Data was processed and analyzed using the statistical software program SPSS version 16.0 (SPSS Inc., USA). A general linear model was used to analyze the variations of grapefruit bioactives between production systems and two storage temperatures for four weeks. The means and standard errors obtained from the outputs after performing the analysis of variance were presented. A split-split plot design

including production system as the main plot factor and storage temperature as subplot factor 1 and storage time as subplot factor 2 were used in the analysis. In this experiment, blocks were used as replications and the treatment means were separated by tukey's test at a significance level of $p \leq 0.05$. The compositional variations that occurred at harvest and storage were expressed on a fresh weight basis in order to have a better representation of actual concentrations experienced by the consumer.

4.4 Results and discussion

4.4.1 Sensory evaluation and weight loss

For sensory evaluation, taste parameters such as sweetness, sourness, tartness and overall acceptability were evaluated and no significant differences ($p \leq 0.05$) were observed between the organic and conventional grapefruits (Table 4.2). The sensory evaluation results were consistent with TA and TSS (Table 4.3) levels in the organic and conventional grapefruits. Furthermore, the overall appearance of the organic and conventional grapefruits was not significantly different ($p \leq 0.05$) with the fruit color being an exception. Conventional grapefruits had a brighter red colored flesh compared to organic grapefruits.

Table 4.2. Sensory evaluation of grapefruit grown under organic and conventional production systems.

Production system	Color	Roughness	Appearance acceptability	Sweetness	Sourness	Tartness	Flavor acceptability
Organic	3.76±1.8[b]	5.16±1.7	4.35±1.9	4.86±2.2	5.57±1.9	4.84±1.9	5.60±2.0
Conventional	5.26±1.9[a]	4.78±2.2	4.64±1.8	5.44±2.2	5.08±2.2	5.15±2.4	5.37±2.4

The values are expressed as mean ± SD, (n = 41) on a scale on 9.

a, b depicts the significant differences at $P \leq 0.05$ and the values without ***a*** or ***b*** means that there is no significant difference between the treatments.

Table 4.3. Titratable acidity of organic and conventional grapefruit expressed in g/L (FW) and total soluble solids of organic and conventional grapefruit expressed in °Brix[†].

		First experiment				Second experiment			
		RT		9 °C		RT		9 °C	
		Organic	Conventional	Organic	Conventional	Organic	Conventional	Organic	Conventional
Titratable	**Week 0**	9.57±1.19	10.67±0.75	9.57±1.19	10.67±0.75	9.22±1.10	10.23±0.71	9.22±1.10	10.23±0.71
	Week 1	10.19±0.60	10.05±0.61	10.99±0.20	9.62±0.09	11.27±1.01	10.89±0.47	10.68±1.16	10.87±1.52
	Week 2	11.51±1.80	10.50±0.87	11.42±1.68	9.48±0.62	11.22±1.66	10.55±0.99	10.75±1.60	11.07±1.75
	Week 3	10.31±1.64	10.03±0.15	9.67±0.81	9.93±0.55	12.46±2.06	10.89±2.19	10.53±0.51	10.91±2.13
	Week 4	11.81±1.70	10.88±0.72	10.72±1.16	10.54±0.49	11.65±0.99	10.70±0.47	10.47±1.24	-
Total soluble	**Week 0**	9.60±0.40	10.10±0.40	9.60±0.40	10.10±0.40	10.71±0.30	11.56±0.61	10.71±0.27	11.56±0.61
	Week1	10.30±0.20	10.30±0.20	9.80±0.60	10.50±0.60	10.76±0.50	11.73±0.49	11.00±0.56	11.56±0.74
	Week2	10.50±0.60	10.60±0.40	10.20±0.70	10.30±0.40	10.70±0.50	11.82±0.58	10.90±0.43	11.53±0.46
	Week3	10.00±0.60	10.50±0.40	9.70±0.30	10.40±0.40	11.41±0.50	11.53±0.45	11.30±0.58	11.92±0.47
	Week4	10.10±0.60	10.40±0.30	9.70±0.40	9.90±0.40	11.18±0.20	11.40±0.65	10.78±0.82	11.42±0.44

[†]The values are expressed as mean ± SD.

The percentage of moisture lost during storage was slightly higher in the E2 compared to the E1. Furthermore, the moisture lost at room temperature storage was higher than that of cold storage due to lower (65%) relative humidity. The moisture loss in the E2 for organic grapefruits at the end of four week storage was 4.2 % and 16.2% at 9°C and RT respectively. The moisture loss for conventional grapefruits at room temperature in E2 was 11.1% while in the E1 it was 6.2%. The corresponding percentage of fruit decay in the E2 (13.5%) was greater than the E1 (8.1%). Unlike organic grapefruits, conventional grapefruits were coated with carnauba (shiny) wax which probably reduced moisture losses during storage.

4.4.2 Vitamin C analysis

In the E1, organic grapefruits showed significantly higher levels of vitamin C over conventional grapefruit at zero days after harvest (Table 4.4). However, the vitamin C levels ranged from 25.74 to 61.99 mg/100g in organic grapefruits and 26.06 to 64.80 mg/100g in conventional grapefruit in both experiments. It seems vitamin C levels at harvest had an inverse relation to the levels of nitrate nitrogen (NO_3-N) content of the respective soils (Table 4.5). Lower levels of NO_3-N in organic grapefruit orchard soils

Table 4.4. Changes in the vitamin C† levels of organic and conventionally produced grapefruit juice during four week storage at room temperature (RT) and 9 °C from first experiment (E1) and second experiment (E2).

Year	**2008**		**2010**	
Storage (week)	**Organic (mg/100 g)**	**Conventional (mg/100 g)**	**Organic (mg/100 g)**	**Conventional (mg/100 g)**
		Room temperature (RT)		
0	41.85±0.07[a]	39.25±0.07[b]	40.98±1.74	44.59±1.71
1	26.87±0.22[a]	26.06±0.22[b]	61.99±0.33[a]	64.80±0.33[b]
2	47.39±0.04[a]	43.77±0.04[b]	39.20±1.30	41.63±1.23
3	36.96±0.05[a]	37.24±0.05[b]	36.03±0.94	36.86±0.54
4	38.88±0.10[a]	38.09±0.10[b]	41.14±1.33[a]	36.62±1.33[b]
		Cold storage (9 °C)		
0	41.85±0.07[a]	39.25±0.07[b]	40.98±1.74	44.59±1.71
1	25.74±0.10[a]	26.30±0.10[b]	60.62±0.50[b]	63.44±0.47[a]
2	45.90±0.05[a]	44.20±0.05[b]	39.87±0.93	39.31±0.87
3	37.62±0.05[a]	36.48±0.04[b]	35.07±1.25	37.98±1.19
4	39.52±0.04[a]	37.39±0.04[b]	38.56±0.62[b]	33.63±0.65[a]

†The values are expressed as mean ± SD; $n = 9$ per each treatment and reported on fresh weight basis. The letters ***a, b*** depicts the significant differences at $P \leq 0.05$, between organic and conventional production systems. The values without ***a*** or ***b*** means that there is no significant difference between the treatments.

Table 4.5. Grapefruit micronutrients (ppm) from organic and conventional production system and soil nutrient analysis of organic and conventional grapefruit orchards.

Experiment	Production system	NO_3-N†	P	K	Ca	Mg	S	Na	Zn	Fe	Cu	Mn
		Juice mineral analysis of organic and conventional grapefruit										
First experiment	**Organic**	0.08	61.62	1979.78	324.27	86.00	NA	352.78	0.58	3.99	0.60	0.70
	Conventional	0.10	48.72	1977.13	274.83	83.95	NA	280.19	0.67	14.70	0.67	0.67
Second experiment	**Organic**	0.10	243.92	3146.92	472.80	110.98	NA	53.16	0.60	10.93	0.27	0.93
	Conventional	0.11	208.14	2428.37	276.78	75.33	NA	44.96	0.26	4.32	0.23	0.20
		Soil micro nutrient analysis of organic and conventional grapefruit orchards										
First experiment	**Organic**	8.00	108.00	335.00	2012.00	410.00	28.00	NA				
	Conventional	20.00	61.00	310.00	5568.00	463.00	32.00					
Second experiment	**Organic**	3.00	87.00	283.00	4016.00	399.00	20.00					
	Conventional	2.00	67.00	610.00	7866.00	785.00	29.00					

† values are expressed in percentage for juice mineral analysis.

could have caused higher grapefruit vitamin C content. Additionally, the lower vitamin C levels in conventional grapefruits could be due to the dilution effect, a secondary response to increased vegetative growth because of excess plant available soil nitrogen [121,141]. Although P, K, Ca, Mg and Na were higher in organic grapefruits, only nitrate nitrogen have been linked to vitamin C levels in previous studies [121].

In the E2, vitamin C levels were not significantly different at zero days after harvest in organic and conventionally grown grapefruits. These vitamin C levels (25.74 to 64.80 mg/100g) are in accordance with previously reported study [136]. Additionally, higher levels of ascorbic acid levels were shown in organically produced grapefruits in a previous study [82].

In the E1, the vitamin C lost during grapefruit storage (both organic and conventional) at RT was significantly higher than at 9 ºC. However, similar pattern was not observed in the E2. The vitamin C degradation was minimal in both organic and conventional grapefruits during RT and 9 ºC (Table 4.4). Variability in vitamin C degradation and accumulation is a very common phenomenon observed among fruits and vegetables during storage [16,142-144]. In plants, vitamin C is the first line of defense against oxidative stress that occurs due to increased respiration in storage [141]. Therefore vitamin C decreased immediately in the first week of storage in the E1. Although first week of storage showed vitamin C degradation, their levels reached to original concentrations by second week of storage. Normally fruits tend to maintain their vitamin C levels by denovo biosynthesis during storage [145,146]. However, prolonged

storage periods would decrease vitamin C content due to excessive free radical accumulation as a result of increased respiration. In case of E2, the vitamin C levels increased immediately in the first week of storage but their levels reached to their original concentrations (concentrations at the time of harvest) in second week. Generally, the fruits harvested in February had softer tissue (thinner cell walls) than those harvested in November [147] which could have contributed to more glucose 6-phosphate, a major substrate for vitamin C biosynthesis [148]. In a previous study, the cell wall softening is related to apoplastic ascorbic acid concentrations [148]. In some fruits including strawberries, vitamin C biosynthesis occurs as the fruit cell-wall degrades during ripening process [149]. It seems that in the E2 harvest time could have contributed to an immediate increase in ascorbic acid in the first week of storage.

In the E2, grapefruit orchards continuously experienced cooler temperatures (60 °F) over a period of three months before commencement of harvest. The cold weather could have contributed to the higher vitamin C levels [141]. Overall the results of this study demonstrated that vitamin C levels in fruits and vegetables are highly influenced by various factors including production system, storage and time of harvest.

4.4.3 Limonoid analysis

Limonoids are anti-feedants that are primarily produced by plants as a response to pests and diseases. Although organic grapefruits had similar quality attributes (TSS, TA and taste) as conventional grapefruits, they showed higher levels of total limonoid

concentrations in juice at zero days after harvest. Nomilin but not limonin levels were significantly higher in organic grapefruit compared to conventional grapefruits in the E1 (Table 4.6). Plants exposed to biotic stress could have increased the levels of phytoalexins and this could have possibly increased the levels of limonoids in organic grapefruit [120]. The levels of these compounds (21.57-94.82 µg/g of limonin and 1.69 - 31.78 µg/g of nomilin) in grapefruits are in accordance with the reported levels [84] and demonstrate that biosynthesis of limonin and nomilin in grapefruits is a complementary but not continuous process [150].

In plants, nomilin (limonoid aglycon substrate) is synthesized in the stem tissues and translocated to fruits [151,152]. Later, the nomilin accumulated in the fruits is used for the biosynthesis of other limonoid aglycons including limonin. This is probably the main cause for lower concentrations of nomilin compared to limonin in all grapefruits after harvest (Table 4.6). Although there are no major differences observed between different storage temperatures, the concentrations of limonin and nomilin have significantly decreased in the fruits from both the production systems at room temperature and 9°C storage. Additionally, after harvest, the accumulation of nomilin in fruit tissues has stopped. However, the remaining nomilin is continuously used up for biosynthesis of limonoid aglycons [151,152].

In the E2, there were no major differences in organic and conventional grapefruit limonoid concentrations at harvest. The colder temperatures that prevailed prior to second harvest might have decreased the biotic stress on the plant which could have lead

Table 4.6. Concentrations of limonoids from organic and conventional grapefruit during four week storage at RT (23 °C) and 9 °C reported on fresh weight basis.

		Limonin (μg/g)					Nomilin (μg/g)				
	Storage (week)	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**
1st experiment	**Conventional RT**	89.91 ± 3.04^{a}	71.71 ± 3.04^{b}	71.182 ± 3.04^{b}	72.53 ± 3.24^{b}	72.91 ± 3.04^{b}	18.65 ± 0.92^{a}	13.33 ± 0.92^{bc}	11.42 ± 0.94^{bc}	14.79 ± 0.98^{ab}	0.974 ± 0.92^{c}
	Organic RT	94.82 ± 3.30^{a}	90.07 ± 3.30^{a}	75.25 ± 3.30^{b}	87.25 ± 3.30^{ab}	82.07 ± 3.30^{ab}	31.78 ± 1.41^{a}	23.19 ± 1.41^{b}	15.84 ± 1.41^{c}	9.36 ± 1.41^{d}	14.57 ± 1.41^{cd}
	Conventional 9C	89.91 ± 3.60^{a}	77.51 ± 3.60^{ab}	72.23 ± 3.60^{bc}	55.80 ± 5.00^{c}	71.87 ± 3.60^{bc}	18.65 ± 0.82^{b}	13.18 ± 0.82^{c}	10.65 ± 0.82^{cd}	25.19 ± 1.15^{a}	7.52 ± 0.82^{d}
	Organic 9C	94.82 ± 3.22^{a}	85.45 ± 3.22^{ab}	80.84 ± 3.22^{b}	82.47 ± 3.22^{ab}	80.58 ± 3.22^{b}	31.78 ± 1.60^{a}	20.55 ± 1.60^{bc}	22.38 ± 1.70^{b}	10.12 ± 1.65^{d}	14.96 ± 1.60^{cd}
2nd experiment	**Conventional RT**	42.92 ± 1.98^{b}	25.48 ± 2.89^{c}	49.64 ± 2.80^{ab}	24.29 ± 2.89^{c}	57.48 ± 3.94^{a}	3.85 ± 0.82^{ab}	2.21 ± 0.80^{b}	6.72 ± 0.77^{a}	3.60 ± 0.80^{ab}	3.96 ± 0.77^{ab}
	Organic RT	40.87 ± 2.09^{a}	26.85 ± 2.09^{b}	41.46 ± 2.09^{a}	21.57 ± 2.53^{b}	38.02 ± 2.24^{a}	3.35 ± 0.48^{bc}	2.06 ± 0.48^{c}	5.18 ± 0.48^{ab}	3.91 ± 0.61^{ab}	5.85 ± 0.51^{a}
	Conventional 9C	42.92 ± 1.98^{b}	27.07 ± 1.85^{b}	44.54 ± 1.85^{b}	29.22 ± 1.91^{b}	62.64 ± 2.14^{a}	3.77 ± 0.23^{b}	2.65 ± 0.22^{c}	6.23 ± 0.22^{b}	4.71 ± 0.23^{a}	1.69 ± 0.22^{d}
	Organic 9C	40.87 ± 1.78^{a}	23.39 ± 1.78^{b}	45.52 ± 1.78^{a}	26.81 ± 1.78^{b}	42.33 ± 1.83^{a}	3.35 ± 0.58^{bc}	2.16 ± 0.58^{c}	5.57 ± 0.58^{b}	4.95 ± 0.64^{b}	8.98 ± 0.76^{a}

The mean ± SD of limonin and nomilin were expressed in (μg/g) that resulted from analysis of nine individual samples.

a, b, c and ***d*** depicts the significant differences ($P \leq 0.05$) of limonin and nomilin during grapefruit storage.

to the moderate levels of limonoids in organic and conventional grapefruits. Furthermore, the E2 showed lower levels of limonin and nomilin due to possible glucosidation of limonoid alycons [153].

4.4.4 Carotenoid analysis

Grapefruits grown under a conventional system had higher β-carotene and lycopene levels than organic grapefruits in both experiments at zero days after harvest (Table 4.7). In the E1, lycopene was more than two-fold higher in conventional grapefruits compared to organic grapefruits. These presented carotenoid levels (1.23 - 4.51 µg/g of β-carotene and 4.35 - 26.13 µg/g lycopene) are in agreement with a previously published report [82]. In a carrot study, the variations in carotenoid content due to different production systems demonstrated that higher plant available nitrogen in conventional production significantly increased β-carotene levels [122].

Degradation losses of carotenoids were greater during storage in both organic (57.5%) and conventional grapefruits (53%) in the E1. In the E1, carotenoids were more stable at RT compared to 9°C. Furthermore, similar degradation losses of carotenoids were not observed in the E2 for conventional grapefruit at RT while carotenoids in organic grapefruits were more stable. Previous studies have demonstrated that harvest time[82,154] and storage conditions had a tremendous influence on citrus carotenoids [155].

Table 4.7. Storage variations† in (a) β-carotene and (b) Lycopene from grapefruit harvested from organic and conventional production systems reported on fresh weight basis.

	Storage (week)	β-carotene (µg/g)				Lycopene (µg/g)			
		RT		9C		RT		9C	
		Conventional	Organic	Conventional	Organic	Conventional	Organic	Conventional	Organic
1st experiment	0	3.57±0.05[a]	2.61±0.05[b]	3.57±0.05[a]	2.61±0.05[b]	25.87±0.23[a]	12.52±0.22[b]	25.87±0.23[a]	12.52±0.22[b]
	1	5.05±0.15	5.27±0.12	4.69±0.03[a]	2.68±0.02[b]	23.07±0.83[a]	26.13±0.67[b]	25.51±0.28[a]	16.69±0.25[b]
	2	3.82±0.13[a]	3.49±0.15[b]	3.47±0.06[a]	4.74±0.11[b]	18.00±0.50[a]	11.46±0.52[b]	19.96±0.08[a]	15.46±0.08[b]
	3	4.90±0.05[a]	3.50±0.05[b]	3.61±0.02[a]	1.94±0.02[b]	15.63±0.20[a]	9.63±0.20[b]	15.57±0.26[a]	8.80±0.26[b]
	4	4.51±0.15[a]	2.89±0.16[b]	2.00±0.08[a]	1.23±0.11[b]	11.17±0.21[a]	6.15±0.23[b]	8.08±0.23[a]	4.35±0.23[b]
2nd experiment	0	4.23±0.04[a]	2.5±0.04[b]	4.23±0.04[a]	2.50±0.04[b]	11.64±0.18[a]	7.54±0.19[b]	11.64±0.18[a]	7.54±0.19[b]
	1	2.69±0.08	2.90±0.08	3.71±0.03[a]	2.57±0.02[b]	6.27±0.24[a]	8.73±0.22[b]	8.92±0.27	8.55±0.27
	2	4.25±0.07[a]	3.80±0.08[b]	4.58±0.05[a]	3.73±0.05[b]	11.78±0.28[a]	10.32±0.34[b]	12.16±0.19	12.61±0.19
	3	5.97±0.05[a]	6.32±0.05[b]	4.17±0.02[a]	4.63±0.02[b]	15.49±0.24[a]	17.31±0.23[b]	11.31±0.15[a]	16.34±0.15[b]
	4	4.31±0.04[a]	2.40±0.04[b]	2.43±0.02	2.53±0.03	12.40±0.06[a]	4.52±0.07[b]	8.22±0.06[a]	5.85±0.09[b]

†The values are expressed as mean ± SD; $n = 9$ per each treatment. The letters ***a, b*** depicts the significant differences at $P \leq 0.05$, between organic and conventional production systems. The values without ***a*** or ***b*** means that there is no significant difference between the treatments.

In the current study, carotenoid levels were greatly influenced by harvest time in the first and E2. Another study showed that harvest time had significantly influenced lycopene biosynthesis in citrus fruits [156]. β-carotene is converted to violaxanthin that occurs downstream in carotenoid biosynthesis as the harvest time progressed in Satsuma mandarin [127].

In conclusion, vitamin C, limonoids and carotenoids were differentially influenced by production system, storage conditions and harvest time. Vitamin C levels were inversely related to soil nitrate nitrogen content in the two production systems. The vitamin C loss during storage was minimal in both organic and conventional grapefruits. Production system and month of harvest have influenced both limonin and nomilin contents in the grapefruit. Limonoid aglycon levels were generally higher in 2008 than in 2010 perhaps due to the effect of harvest times. Lycopene and β-carotene were higher in conventional than organic grapefruits in both the experiments. It is likely that cooler temperatures at the time of harvest might have caused the variation in carotenoids.

CHAPTER V

PRODUCTION SYSTEMS AND POSTHARVEST STORAGE CONDITIONS INFLUENCE GRAPEFRUIT COUMARINS, FLAVANONES AND ANTIOXIDANT ACTIVITY

5.1 Synopsis

Grapefruits are an important source of heterocyclic oxygenated bioactive compounds including coumarins, flavanones and antioxidant activity. The grapefruits were harvested in November 2008 (first experiment, E-1) and February 2010 (second experiment, E-2) and kept at room temperature (RT) and 9°C for four weeks (postharvest). Grapefruits grown under organic or conventional production systems were analyzed for coumarins and flavanones using high performance liquid chromatography (HPLC), and DPPH radical scavenging activity using a micro-plate reader.

Higher levels of 6, 7 dihydroxy bergamottin (DHB) and 5 methoxy 7-geranoxy coumarins (GC) were observed in conventional grapefruits during second, third and fourth week at 9°C. Narirutin levels were significantly higher ($P \leq 0.05$) in organic grapefruits in E-1 at first, third and fourth week after harvest, while no significant differences were observed in the E-2 except in fruits immediately after harvest. Naringin levels were not significantly different between organic and conventional grapefruits in E-1. Organic production systems did not show significant ($P \leq 0.05$) influence on DPPH

radical scavenging activity. The DPPH radical scavenging activity of fruit extracts was correlated with flavanones.

Grapefruits had higher levels of DHB and GC than organic grapefruits after harvest. The flavanone levels were higher in organic grapefruits in E-1. The individual experiments have greatly contributed to a wide spread non significant levels of flavanones that was also pronounced in DPPH radical scavenging activity. These effects are probably due to the change of weather conditions around the harvest period and plant nitrogen levels.

5.2 Introduction

Grapefruits contain a host of non-nutritive bioactive compounds such as flavanones and phenolics essential for maintaining good health. Grapefruit flavanones have demonstrated a wide range of biological activities against several age-related diseases, and flavanones as a class have been well-studied for their preventative properties against heart diseases.[4],[5] The American Heart Association has given a "healthy heart check" symbol for several commercially available grapefruit juices due to their preventive properties against coronary heart disease.[6,157] Similarly, several in-vitro studies have also demonstrated grapefruit's anti-cancer properties.[7,9,158] Despite these health benefits, grapefruit consumption was not recommended for patients under certain medications due to its potential for drug interactions.

Grapefruit coumarins interact with several drug classes including antiepileptics, antihistamines, antimalarials, antiarrthmics, cardiovascular agents (verapamil,

amlodipine, felodipine and nicardipine), statins (atorvastatin, lovastatin and simvastatin), corticosteroids and several others.[159-171] These reports on grapefruit drug interactions have led to consumer apprehension over grapefruit consumption and health benefits. Therefore researchers have developed novel techniques such as employing edible fungi [172], fruit irradiation [173] and breeding new grapefruit cultivars [174] for lower concentrations of coumarins in juice.

Flavanones are primarily produced in different parts of plants such as flowers, fruits, seeds and leaves. Flavanones, play a key molecule in plant signaling, defense against microbes and ultra violet radiation, and also a feeding deterrence in plants. Furthermore, plant genetics and growing conditions (climatic and production systems) could interact and greatly influence grapefruit flavanone and coumarin levels. Previous reports suggest that conventionally produced grapefruits had higher levels of coumarins compared to organic grapefruits.[175,176] The levels of flavanones and coumarins vary in many plants species and are highly dependent on growing environment. Therefore, modulation of the pre-harvest factors to affect the levels of these compounds before they reach the consumer would be of greater interest. The objective of the current study is to understand the variability in levels of grapefruit coumarins, flavanones and antioxidant activity in grapefruits produced by organic and conventional production systems before reaching consumer.

5.3 Materials and methods

5.3.1 Fruit harvest and storage

Rio Red grapefruits with uniform color, size and maturity were selected from four quadrants of trees. The harvesting and sampling followed the same protocol as our previously published organic grapefruit study. [177] The first experiment (E-1) was conducted in Nov 2008 and the second experiment (E-2) was conducted in Feb 2010. Organic grapefruits were harvested from South Texas Organics Citrus orchards (Mission, TX, USA) while conventional grapefruits were harvested from Rio Queen Citrus Farms (Mission, TX, USA). The certified organic Rio Red grapefruit orchard is located three miles away from the conventional orchard. The fruits were harvested in the morning, processed and packed by noon. The packed grapefruits were shipped overnight to Texas A&M University, College Station, TX. The grapefruits were kept at room temperature (RT) and 9°C for four weeks. The fruits were collected every week from grapefruit boxes and analyzed for coumarins, flavanones, and antioxidant capacity. Every week, 27 grapefruits were taken out from storage boxes to prepare nine grapefruit samples for each treatment by blending three individual fruits together. Although the fruits are kept for four weeks, this study compared the levels of coumarins, and DPPH radical scavenging activity of only organic and conventional grapefruits. However, the study is not considered to investigate storage trends because the samples prepared. The weather, crop production data, and sampling methods were presented in our previous publication.[177]

5.3.2 Coumarin analysis

Ten grams of grapefruit pulp was taken in a 50 ml centrifuge tube and extracted with 20 mL of ethyl acetate. The organic fraction was separated and the residual juice is re-extracted with 10 mL of ethyl acetate. The two organic extracts were pooled and evaporated to dryness. The extract was reconstituted with five milliliters of DMSO. The grapefruit coumarins were separated and the identified by LC-MS analysis (Finnigan, LCQ Deca XP, West Palm Beach, FL, USA). The coumarins were separated on Aquasil, C-18 column (2.1 ×150 mm, 3μm) using a binary solvent gradient of (A) 0.1% formic acid and (B) methanol with 0.1% formic acid. The elution started with 10 % B and reached 50 % B in 0 – 4.0 min; a step gradient of 50-60% B, 4.0 – 7.0 min; an isocratic of 60% B, 7.0 – 9.0 min; step gradient of 60-65% B, 9-12 min; isocratic of 65 % B, 12-13min; a step gradient of 65-75%, 13-15 min, a step gradient of 75-100%, 15-20 min, isocratic of 100% from 20-25 min, a step gradient of 100-10%, 25-27 min and isocratic of 10% from 27-29 min. The sample was ionized using atmospheric chemical ionization (APCI) in positive mode in a Thermofinnigan LCQ DECA (San Jose, CA). The capillary inlet temperature was maintained at 250°C, and vaporization temperature was 450°C. The corona discharge current was 5μA in a positive mode.

Coumarins were quantified after modification of our previous reported method.[49] The analysis was conducted using an analytical HPLC system consisting of a Perkin-Elmer series 200 pump, PDA detector and an autosampler (Perkin-Elmer, Norwalk, CT, USA). The separation was carried out on a C-18, 5 μm Gemini column (250 mm × 4.6

mm i.d.) attached with a guard column (Phenomenex, Torrance, CA, USA). The sample coumarins were eluted by a gradient mobile phase of 0.03 M phosphoric acid (A) and acetonitrile (B) with a constant flow rate of 1mL/min throughout the analysis. The peak detection was carried out at 320 nm and analysis was carried out by Turbo chrome software (Perkin-Elmer, Norwalk, CT, USA). Each sample was analyzed three times and each treatment had nine samples.

5.3.3 Flavanone analysis

The sample preparation for flavanone analysis was conducted according to the previously published method.[178] Rio Red grapefruit juice (3 ml) was extracted with 6 mL of DMSO and the mixture was vortexed for 5 sec. Later, the samples were centrifuged at 4600 rpm for 10 min. Approximately one milliliter of supernatant from the centrifuge tube was passed through a 0.45 µm acrodisc syringe filter into an amber glass vial and 6 µL was injected into the HPLC. The separation of flavanone was performed using a Finnigan Surveyor Plus, HPLC system (West Palm Beach, FL, USA). The HPLC system was equipped with a PDA Plus detector coupled with a quaternary LC Pump Plus system, a Surveyor Plus auto-sampler (25 µL sample loop with valco fittings). A C-18 Hypersil Gold column (100 mm x 4.6 mm i.d. and 5 µm particle size) was used to separate all five flavanones from grapefruit juice. The standard flavanones were purchased from Sigma Chemicals (St. Louis., MO, USA). Peaks were detected at 280 nm, and the analysis was carried out using Chromquest 5.0 software.

Chromatographic separations were performed with a gradient mobile phase consisting of 3 mM phosphoric acid prepared in nanopure water (A) and 100 % acetonitrile (B). The flavanones were eluted with the following solvent gradient: 0 - 4.5 min, 80 % A; 11.6 min, 70% A; 13 min, 42% A; and 19.6 min, 80% A. The column was equilibrated for 5 min before successive injections.

5.3.4 Sample preparation for grapefruit antioxidant activity

Ten grams of grapefruit juice were mixed with 20 ml of methanol and extracted for 12 h on a mechanical shaker. The organic layer of the extract was separated and the residual pulp was re-extracted with 10 mL of methanol for 2 h. Both the methanol extracts were pooled and stored at -20 ºC until all the assays were completed.

5.3.5 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH assay was conducted according to our recent publication .[179] The radical scavenging activity of the methanol extracts were tested for $DPPH^{\bullet}$ scavenging activity. Forty milligrams of DPPH solution was freshly prepared in 1000 mL of methanol. The standard ascorbic acid solutions were prepared in nanopure water at different (0.30, 0.6, 0.9, 1.2, 1.5, 1.8, 2.25 and 3µg) concentrations. The grapefruit sample (40 µL) was pipetted into 96 well plates and volume was adjusted to 100 µL with methanol and 180 µL of DPPH was added into each well. The assay plate was read in KC 4 microplate

reader at 515 nm for 30 min. The results were expressed as µg of ascorbic acid equivalents /g of sample.

5.3.6 Oxygen radical absorbance capacity (ORAC) fluorescene assay

Antioxidant capacity of the organic and conventional grapefruit extracts were evaluated using the ORAC-fluorescene method. The ORAC assay was performed according to our publication.[180] Ten microliters of grapefruit methanol extracts were pipetted into 96 well plates. The sample volume was later adjusted to 40 µL in each well using phosphate buffer. The instrument was preheated to 40ºC and 200 µL of fluorescene and 20 µL of APPH was added using dispenser of Synergy HT micro plate reader (Winooski, VT, US). The fluorescence was read at an excitation and absorption of 485 and 535 nm respectively 1h for every 5 min interval.

5.4 Results and discussion

5.4.1 Identification and quantification of grapefruit coumarins

The grapefruit coumarins were identified using the standard coumarins obtained from our previous purification studies.[176] The two grapefruit coumarins were identified as 6, 7, dihydroxy bergamottin (DHB) and Geranyl coumarin (GC) by their protonated molecular ions $[M+H]^+$ at 373.15 and 329 m/z respectively (Fig. 5.1). In E-1 (Fig. 5.2), at harvest, organic grapefruits had same levels of DHB levels as conventional grapefruits ($P \leq 0.05$). In E-2, the DHB levels were higher in conventional grapefruits as compared to organic grapefruits in 2nd and 3rd and 4th week of storage (Fig.5.3). The results from E-1 and E-2 were not in complete agreement with a previously reported study on organic grapefruit production.[175] Similarly, the GC levels in conventional grapefruits at week four were higher than organic grapefruits (Fig.5 2). In general, the

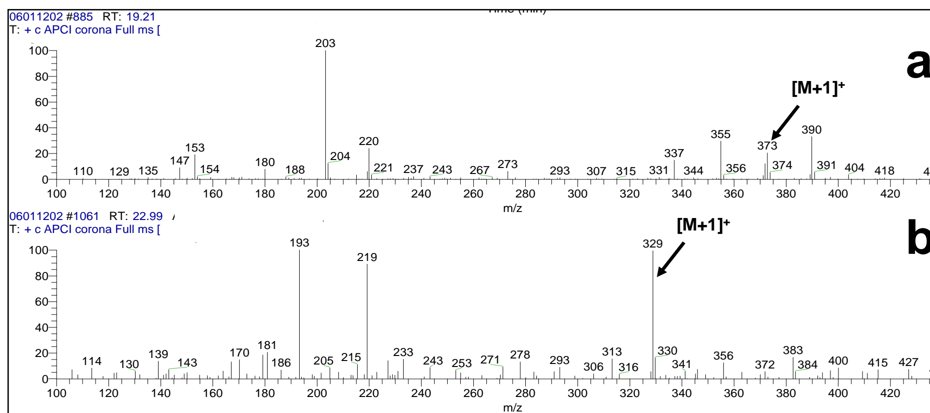


Fig 5.1. The Mass spectral analysis of (a) 6, 7 dihydroxybergamottin and (b) 5-methoxy 7- geranoxy coumarin eluted from LCMS and identified using a APCI-TOF.

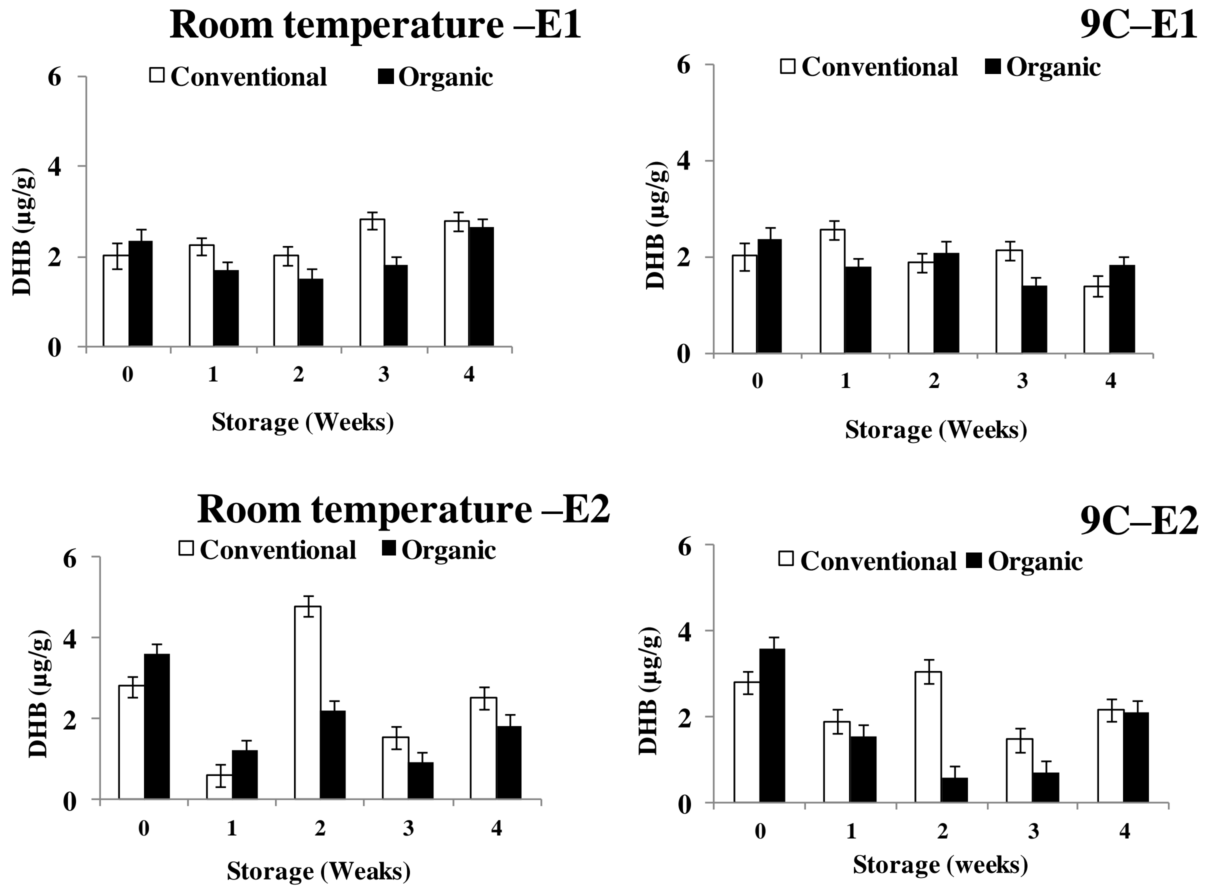
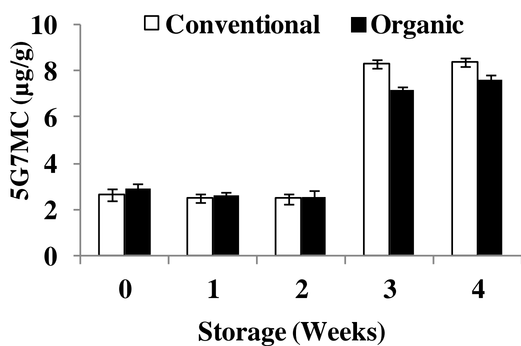
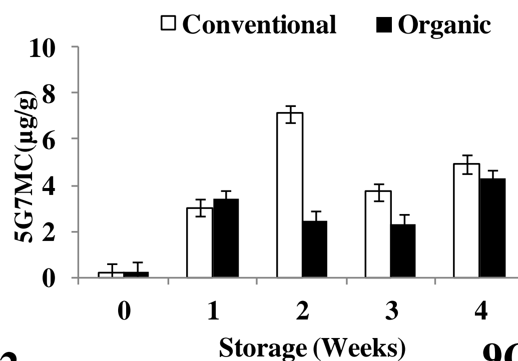


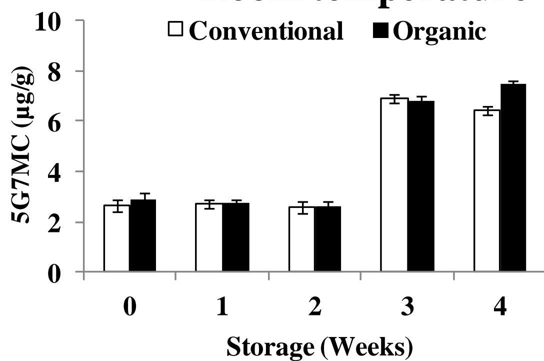
Fig 5.2. DHB levels from organic and conventional grapefruit from nine individual samples (each sample is a mixture of three fruits) processed in E-1 and E-2 at room temperature and 9°C.

Room temperature –E1 **9C–E1**





Room temperature –E2 **9C–E2**



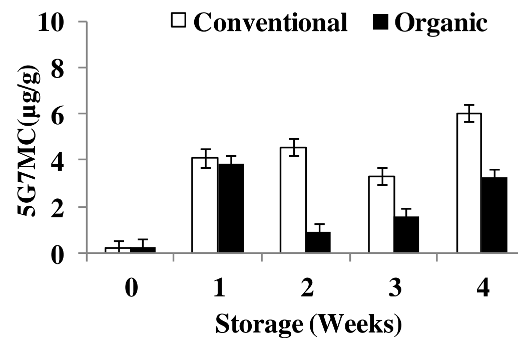


Fig 5.3. 5,Geranoxy, 7 Methoxy Coumarin levels from organic and conventional grapefruit from nine individual samples (each sample is a mixture of three fruits) processed in E-1 and E-2 at room temperature and 9°C.

conventional fruits of E2 had higher levels of DHB and GC than organic grapefruits in week two. Although, coumarins and flavanones partly shared the same biosynthetic pathway, currently there is no direct evidence showing that the enzymes involved in these pathways are influenced by different production and storage conditions.

5.4.2 Flavanone content

The levels of grapefruit flavanones were determined immediately after harvest for four weeks. Five major grapefruit flavanones such as narirutin, naringin, neohesperidin, didymin and poncirin were determined. Narirutin and naringin together accounted for about 90% of the total flavanones present in grapefruits. Narirutin levels were higher in organic grapefruits compared to conventional fruits at E-1 in first, third and fourth week (Table 5.1). In E-2, naringin levels were higher only in second and third weeks in organic grapefruits. However, narirutin levels remained non significant in all four weeks except at the time of harvest.

5.4.3 Antioxidant activity

The antioxidant capacity of the grapefruit extracts were evaluated by two commonly used methods: antioxidant methods such as DPPH radical scavenging activity and ORAC. The effect of production systems and storage temperatures on the fruit's radical scavenging capacity using DPPH was shown in Fig.5. 4. In E-1, second week at RT (0.09± 0.006 mg/g ascorbic acid equivalents) and third week at 9°C (0.09± 0.006

mg/g ascorbic acid equivalents), the organic grapefruits had higher radical scavenging activity than conventional grapefruits. In E-2, production system did not have significant influence on antioxidant activity.

In Fig. 5.5, the radical scavenging activity using ORAC were expressed in trolox equivalents (mM/L). In E-1, there are no significant differences in antioxidant levels of organic and conventional grapefruits in all weeks except for second week. Additionally, in E-2, the grapefruits from both organic and conventional production did not have significant differences. Flavonoids, ascorbic acid and carotenoids are the four different groups of bioactive compounds that occur in grapefruit which primarily contribute to antioxidant activity. Since the analysis was done using methanolic extracts, it is more likely that the antioxidant capacity was mainly due to flavanones and phenolic acids.

In E-1, the levels of flavanones are in correlation with ORAC (R= 0.400, $p \leq 0.01$) values. Also DPPH and ORAC have shown significant correlation (R= 0.322, $p \leq 0.05$) for all four weeks of storage. In E-2, flavanone levels were in correlation with DPPH (R=0.441, $p \leq 0.01$). From these results, it is observed that the mechanism of free radical scavenging for both DPPH and ORAC are different.

Table 5. 1 Concentrations of flavanones from organic and conventional grapefruit during four week storage at RT (23 °C) and 9°C. The mean ±SD of flavanones were expressed in (mg/1000g) that resulted from analysis of nine individual samples.

		First experiment				Second experiment			
		RT		9 C		RT		9 C	
Flavanone	Week	Conventional	Organic	Conventional	Organic	Conventional	Organic	Conventional	Organic
Narirutin	0	465.95±62.35	588.88±62.35	465.95±62.35	588.88±62.35	333.50±8.91	261.07±8.91	333.50±8.91	261.07±8.91
	1	597.55±35.35	850.65±35.35	619.33±47.20	760.51±47.20	337.73±33.21	388.76±33.21	314.07±14.70	431.94±14.70
	2	619.83±82.56	839.96±82.56	593.99±41.73	735.17±41.73	878.82±21.22	841.45±25.45	540.31±6.86	460.31±8.25
	3	488.84±29.95	705.98±29.95	574.70±54.34	629.78±54.34	515.43±11.08	509.97±11.08	578.67±13.70	488.46±13.70
	4	473.24±37.74	754.96±37.74	543.35±42.99	698.15±42.99	522.87±14.70	540.75±14.70	505.34±24.37	509.96±22.56
Naringin	0	928.42±81.79	773.81±81.79	928.42±81.79	773.81±81.79	657.45±21.19	585.64±21.19	657.45±21.19	585.64±21.19
	1	1205.90±44.71	1215.09±44.71	1189.64±68.52	1119.62±68.52	613.79±43.53	732.56±43.53	574.47±15.40	785.01±15.40
	2	1218.2±131.10	1189.53±131.10	1135.05±87.04	1067.23±87.04	1503.41±44.38	1628.14±53.23	1193.97±18.03	1249.87±21.68
	3	966.27±62.12	1033.15±62.12	1172.85±108.67	990.99±108.67	1212.39±62.21	900.17±62.21	1375.34±18.23	770.35±18.23
	4	1007.83±54.41	1025.80±54.41	1110.22±73.23	890.02±73.23	801.25±10.67	803.03±10.67	809.26± 14.37	790.07±13.28
Neohesperidin	0	16.37±1.99	18.36±1.99	16.37±1.99	18.36±1.99	22.70±0.85	13.88±0.85	22.70±0.85	13.88±0.85
	1	22.85±2.01	30.29±2.01	22.47±1.63	24.67±1.63	19.30±3.46	24.53±3.46	17.79±1.34	28.80±1.34
	2	23.07±3.32	30.13±3.32	21.82±2.71	28.08±2.71	41.43±1.14	37.57±1.37	35.88±1.01	31.06±1.21
	3	15.60±1.51	20.15±1.51	19.81±3.13	19.06±3.13	34.85±0.57	30.44±0.57	36.31±1.01	29.97±1.01
	4	15.88±1.58	23.25±1.58	18.72±2.09	22.29±2.09	44.26±1.76	41.46±1.76	37.65±2.16	36.30±2.00
Didymin	0	56.42±8.42	67.74±8.42	56.42±8.42	67.74±8.42	58.70±3.14	49.67±3.14	58.70±3.14	49.67±3.14
	1	78.67±7.92	108.68±7.92	69.69±9.45	98.86±9.45	63.57±8.93	80.25±8.93	64.24±4.93	91.78±4.93
	2	69.36±11.49	110.68±11.49	63.64±8.16	99.38±8.16	139.84±6.31	129.96±7.56	100.64±2.57	80.21±3.08
	3	78.04±13.24	86.77±13.24	63.45±8.98	71.28±8.98	77.42±2.52	96.39±2.52	100.67±2.57	103.88±2.57
	4	48.47±7.43	96.63±7.43	61.06±8.68	88.30±8.68	104.07±5.00	102.25±5.00	98.28±5.86	98.63±5.42
Poncirin	0	131.58±16.43	104.19±16.43	131.58±16.43	104.19±16.43	259.60±10.69	223.76±10.69	259.60±10.69	223.76±10.69
	1	176.01±12.51	184.56±12.51	156.93±14.02	170.53±14.02	254.12±31.78	348.06±31.78	264.45±18.92	395.86±18.92
	2	165.41±19.68	192.06±19.68	147.24±16.64	179.79±16.64	362.50±25.82	377.56±30.97	420.95±10.72	400.36±12.88
	3	135.64±10.72	158.29±10.72	164.96±15.67	134.96±14.31	354.81±11.96	452.52±11.96	450.12±10.47	474.00±10.47
	4	131.60±10.26	159.33±10.26	148.04±12.44	160.02±17.59	434.38±20.56	418.38±20.56	466.22±21.31	421.04±19.73

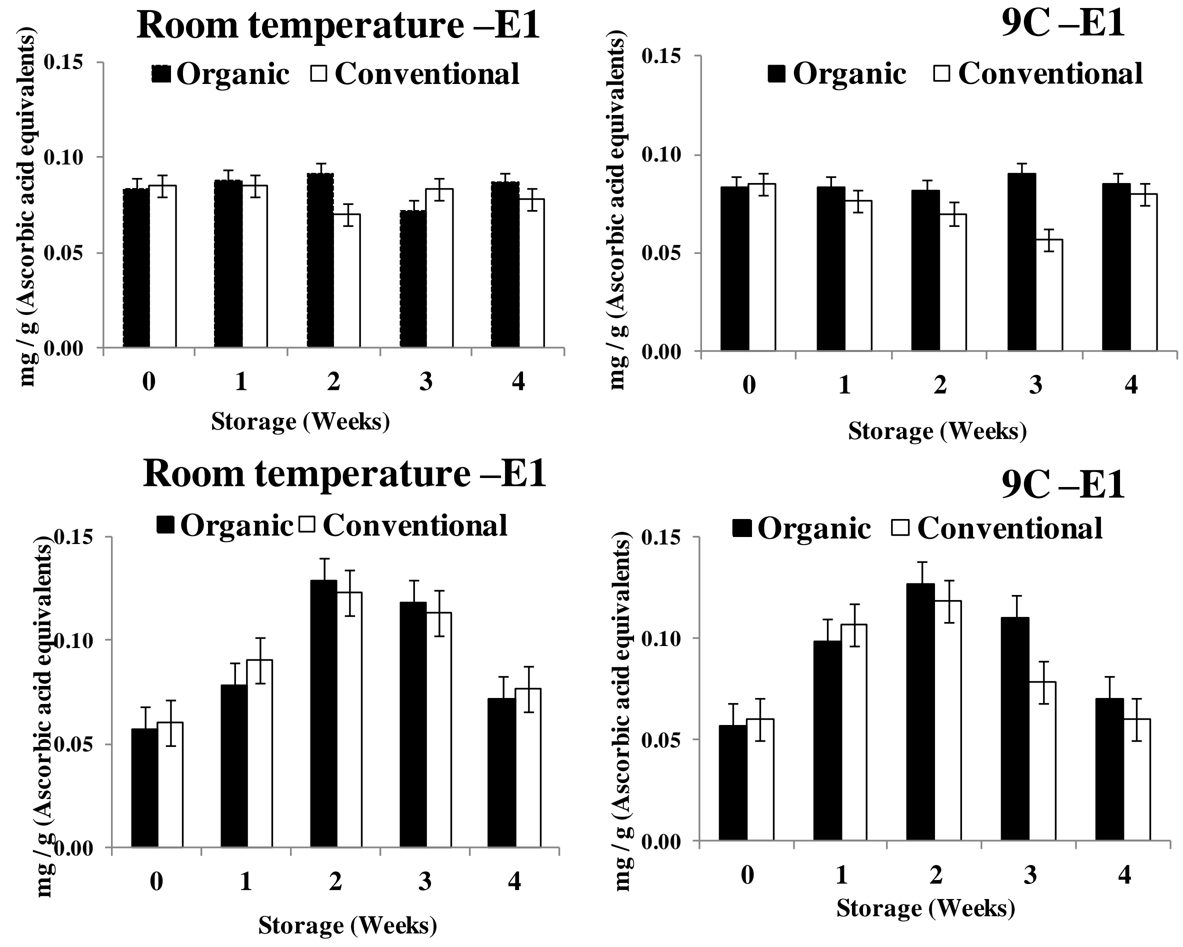


Fig 5.4. Antioxidant capacity (DPPH) of organic and conventional grapefruits in E-1 and E-2 at room temperature and 9°C.

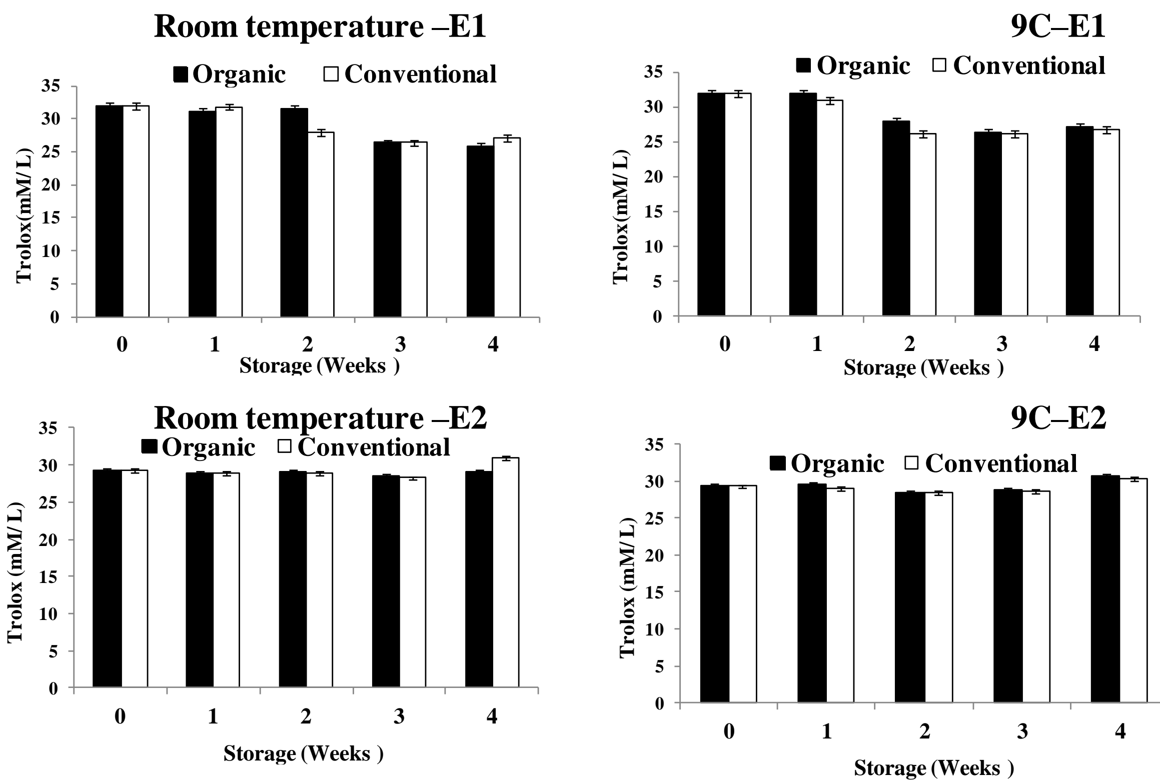


Fig 5.5 Antioxidant capacity (ORAC) of organic and conventional grapefruits in E-1 and E-2 at Room temperature and 9°C.

CHAPTER VI

PURIFICATION OF COUMARINS FROM GRAPEFRUIT BY SOLVENT PARTITIONING AND A FLASH LIQUID CHROMATOGRAPHY

6.1 Synopsis

Grapefruit coumarins have demonstrated antimicrobial and cytotoxic properties; purifying these compounds is essential for systematic biological studies to decipher their bioactive properties. In the current study, we describe a method for isolating and purifying recalcitrant coumarins from grapefruit oil and peel. Grapefruit oil was fractionated into volatile and non-volatile fractions (NVF) by vacuum distillation. The NVF was partitioned into hexane soluble and hexane insoluble fractions (HIF). The NVF-HIF was subjected to a hyphenated chromatographic method (HCM) to obtain six compounds. Alternatively, grapefruit peel was extracted with hexane using a Soxhlet-type apparatus, then concentrated, and purified using HCM which yielded five compounds. The structures of purified coumarins were elucidated by ^{1}H and attached proton test ^{13}C NMR spectral methods and identified as pranferin (**1**), meranzin (**2**), bergapten (**3**), dihydroxybergamottin (**4**), osthol (**7**) and marmin (**9**). Compounds (**5**), (**6**) and (**8**) were identified using HPLC as heptamethoxy flavone, bergamottin and nobilitin respectively. The compounds 1-6 were purified from NVF-HIF of grapefruit oil while compounds 3 and 6-9 were purified from grapefruit peel. The current method has selectively separated bergamottin from minor bioactives before subjecting to a

successful HCM. This is the first report on solvent partitioning and rapid HCM of grapefruit oil to obtain pranferin and meranzin.

6.2 Introduction

Coumarins are heterocyclic oxygenated plant bioactives that occur ubiquitously in the *Rutaceae, Umbelliferae, Asteraceae, Fabaceae, Rosaceae, Solanacea* and *Moraceae* families with approximately 200 coumarins identified from *Rutaceae* alone [181-183]. Grapefruits contain numerous structurally diverse coumarins that occur in very low concentrations (<1ppm) [48,49] and have shown biological activity against several chronic diseases [36,37]. The biological activity of most of these compounds is not yet fully understood, partially because of the difficulty in isolating and purifying them for clinical assays [44].

Grapefruit coumarins are found concentrated in oil glands of grapefruit peel, most likely enter the human food chain during processing when fruit is squeezed [184-186]. However, purification of coumarins has proved challenging compared to other bioactive compounds due to their low concentrations. Previously, several analytical approaches were used for purification of grapefruit coumarins, including open column chromatography, preparative HPLC and high-speed counter current chromatography [44,184,187]. However, manually packed gravity columns could lead to inadequate separations. Additionally, purification using gravity columns increase the experimental run time and quantity of solvent usage. Furthermore, the chemical composition of gravity columns fractions have to be analyzed by TLC or HPLC. Thus, this technique

will be tedious and cumbersome. The use of preparative HPLC for separation of coumarins in several purification experiments has overcome the challenges faced due to open column purification methods. However, sample loading capacity is limited to obtain compounds for biological activities. Unlike adsorption chromatography (gravity column separation, preparative HPLC), the compounds were lost due to adsorption are minimal in counter current chromatography. Therefore, the principles of counter current chromatography can be efficiently used for separation of natural compounds.

Flash chromatography coupled with a UV detector system is emerged as hyphenated chromatographic method (HCM) for the rapid separation and identification of targeted compounds [188] that can provide a solution to achieve reasonable separations due to its simplicity and low cost operation [189]. Furthermore, pre-purification of the target compounds can also the improve purification of low-abundance minor compounds [185,190], either by removing interfering major compounds, or by concentrating the minor compounds of interest. The objective of the current study was to isolate minor coumarins in grapefruits by enriching them using pre-purification techniques (based on their polarity). Furthermore, purification of coumarins was conducted by HCM, and their structures were elucidated using spectroscopic studies.

6.3 Experimental

6.3.1 Plant material and solvents

Cold-pressed grapefruit oil was obtained from the Texas Citrus Exchange (Mission, TX, USA). Rio Red grapefruits were harvested from Rio Queen Citrus Farms

(Mission, TX, USA). The grapefruit peels were air dried to brittleness under shade for 48 h. Dried peels were ground to fine powder in a Vita Prep blender (Cleveland, OH, USA) to obtain 40-60 mesh size particles. High performance liquid chromatography grade hexane, acetone and methanol were obtained from Fisher Scientific (Hanover Park, IL, USA). Nanopure water, obtained from Barnstead, Nanopure purification system (Markham, Ontario, Canada) was used for all separations on a C_{18} column.

6.3.2 Enrichment of coumarins in grapefruit oils

One hundred and twenty-seven milliliters of non-volatile fraction (NVF) were obtained from cold pressed red grapefruit oil (900 ml) through vacuum distillation at 10 mbar vacuum and 60°C. The NVF was mixed with 400 ml of hexane and kept on a shaker for 5 h. Later, the hexane soluble fraction was separated from the dense hexane-insoluble fraction (HIF) in a separating funnel. The resulting HIF was dried in a water bath at 60 °C until all the hexane was evaporated. Finally, the HIF yielded 45 g; 25 g of the HIF was used for subsequent purification by HCM.

6.3.3 Selective isolation of minor grapefruit coumarins

Four liters of grapefruit oil were fractionated to yield 303.3 g of NVF by vacuum distillation at 60 °C and 10 mbar. The NVF was extracted with 1000 ml of hexane for 12 h on a shaker and allowed to separate into distinct layers to selectively isolate the major bioactive compound. The NVF fraction was extracted with hexane three more times and the nonvolatile hexane insoluble fractions were obtained (35.3g).

6.3.4 Grapefruit oil (HIF) purification

Purification of grapefruit bioactives was performed using a HCM system (Teledyne ISCO., Thousand Oaks, CA, USA). Approximately 25 g of HIF was directly loaded onto a C-18 reversed phase flash column (40μ particle size, 200 x 60 mm and 360 g), (RediSep® Rf ISCO., Inc) in a liquid-solid injection mode. The elution was carried out using a binary solvent gradient consisting of nanopure water (A) and methanol (B). Separation of the compounds was performed with an elution gradient as follows: 0-28 min, a step gradient 0-45% B; 28-40 min, 45-50% B; 40-70 min, isocratic of 50% B; 70-75 min, 50-60% B, 75-85 min, isocratic of 60% B; 85-90 min, 60-70% B; 90-140 min, isocratic of 70% B; 140-160 min, 70-80% B; 160-170 min, isocratic 80% B; 170-180 min, 80-90% B; 160-180 min, 80-90% B; 180-210 min, isocratic of 90% B; 210-220 min, 100% B; 220-270 min, 100% B. The flow rate was maintained at 55 ml/min. Chromatographic separations were monitored at 240 and 320 nm (Fig. 6.1a). Two hundred and ten fractions of 50 ml each were collected and analyzed by HPLC. The fractions 1-19, 19-30, 55-58, 68-71, 79-80, 161-165 were pooled (based on their HPLC peaks), concentrated, and crystallized to obtain compounds **3** (25.2 mg), **4** (634.7 mg), **5** (52 mg) and **6** (2699 mg) respectively.

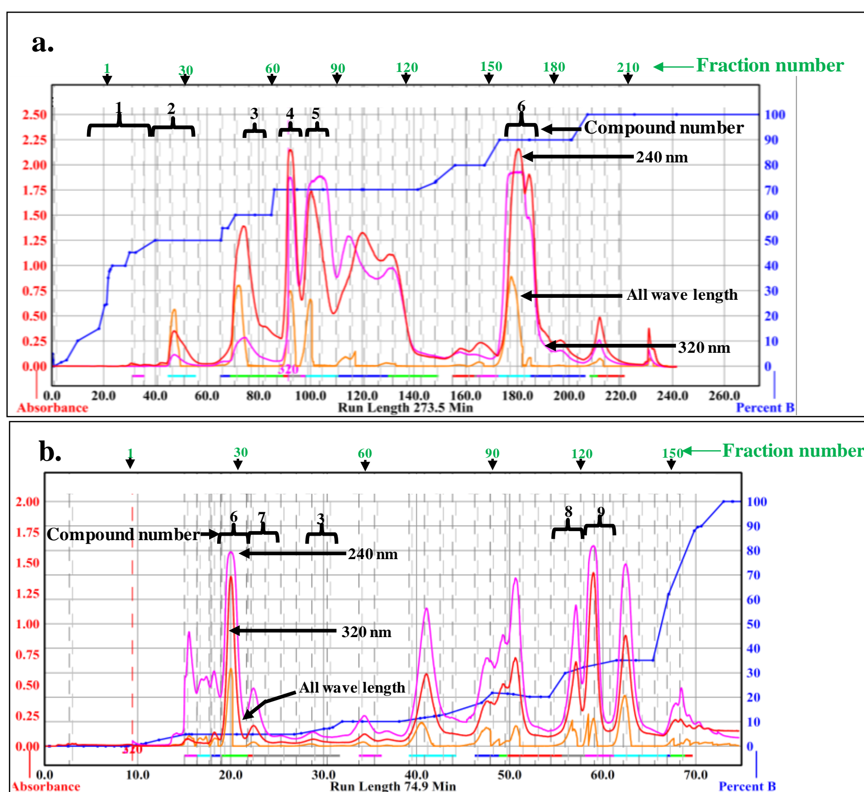


Fig. 6.1. Hyphenated Chromatographic method for grapefruit bioactives monitored at 240 nm and 320 nm wavelengths (a) Hexane insoluble fraction (HIF) was separated on a C_{18} column using a gradient mobile phase consisting of nanopure water and methanol. The numbers 1-6 indicate the compounds purified from grapefruit oil. (b) Grapefruit peel hexane extract was separated on a silica column using a gradient mobile phase consisting of hexane and acetone and the compounds 3, 6-9 were purified. Different color traces indicate absorbance at different wavelengths (red, 240 nm; orange, all wavelengths; purple, 320 nm).

6.3.5 Re-crystallization of compounds 1 and 2

The fractions were dissolved in 5 ml of hexane and a few drops of acetone to ensure complete solubility of compounds at 70°C. The mixture was allowed to cool at room temperature to obtain crystallization. The crystals were recovered and washed with hexane and small quantities of acetone to remove all impurities adhering to the crystals. The recrystallization process was conducted for 2-3 times until pure colorless crystals of compounds **1** (93.4 mg) and **2** (278.9 mg) were recovered.

6.3.6 Soxhlet extraction

Grapefruit peel powder (2100 g) was extracted with 8000 ml of hexane for 16 h in a Soxhlet type apparatus. The extract was filtered and concentrated under vacuum to obtain crude extract (30 g).

6.3.7 Grapefruit peel hexane extract purification

Thirteen grams of hexane extract was impregnated with 12 g of silica gel. Impregnated samples were loaded into a flash loading cartridge and connected to a 120 g normal phase silica column. Purification of the hexane extract was performed using a gradient mobile phase consisting of hexane (A) and acetone (B) and elution was performed as follows, isocratic flow of 0% B, 0-10.0 min; a step gradient of 0-5% B, 10.0-15.0 min; isocratic of 10% B, 15-32 min; isocratic of 10% B, 32.0-40.0 min; step gradient of 10-20% B, 40-48 min; isocratic of 20% B, 48-55 min; a step gradient of 20-30%, 55-58 min, a step gradient of 30-35%, 58-62 min, isocratic of 35%, 62-68 min, a step gradient of 35-90%, 65-70 min. The separations were monitored at 240 nm and 320

nm (Fig. 6.1b). A total of 155 fractions were collected and fractions 22-23, 47-53, 31-36, 109-119, and 120-125 were pooled based on TLC and HPLC analysis and resulted in compounds **3** (8.7 mg), **6** (155 mg), **7** (21.6 mg), **8** (21 mg) and **9** (56.9 mg) respectively.

6.3.8 HPLC analysis

Compounds **1** to **9** were analyzed using HPLC for purity and identification. The run-time of the analysis was 60 min, using a Finnigan Surveyor Plus HPLC (Austin, TX, USA). The HPLC system included a PDA plus detector coupled with a quaternary LC Pump Plus and a surveyor plus auto-sampler. Separations were carried out on a C-18, Luna column (100 mm x 4.6 mm i.d. and 3 µm particle size). The peaks were detected at 320 nm and the analysis was conducted using Chromquest version 5.0. Chromatographic separations were performed using a gradient mobile phase consisting of MeOH (A) and water (B), with the following isocratic solvent of 55% A, 0-20 min; a step gradient mobile phase of 55-100% A, 20-55 min; and a step gradient mobile phase of 100-55% A at 55-60 min.

6.3.9 Gas chromatography -Mass spec and atmospheric pressure chemical ionization

Compounds **1**, **3** and **7** were analyzed in a GC-MS (Thermo Finnigan, Austin, USA) with Rtx-5-Sil MS column (Bellefonte, PA, USA) with 30 m x 0.25 mm i.d. and 1µm particle size. Helium was used as a carrier gas with a constant flow rate of 1.2 ml/min. The injector port temperature was 220 °C, and the oven temperature was programmed to reach an initial temperature of 70 °C in 1 min, then increase to 350 °C in

5 min and then maintain at isothermal conditions for 10 min. The sample injection volume was 0.1µL in a split-less mode. Mass spectra were recorded at 70 eV with *m/z* ranging from 50 to 400 mass units and the temperature of the ion source was maintained at 280 °C for compounds.

The compound 4 had higher flash point (temperature required for vaporization) compared to compounds 1, 3 and 7 hence we used atmospheric pressure chemical ionization (APCI) instead of GCMS. Positive mode in APCI was used to identify compound **4** and the analysis was done in Thermofinnigan LCQ DECA (San Jose, CA). The solvent used was methanol, the flow rate was maintained at 200 µl/min, the capillary inlet temperature was maintained at 250 °C and the vaporization temperature was 450 ^{o}C. The corona discharge current was 5 µA in a positive mode.

6.3.10 Nuclear magnetic resonance spectra

NMR spectra of compounds **1** and **9** were recorded in acetone-d_6 and compounds **2**, **3**, **4** and **7** were recorded in $CDCl_3$. ^{1}H and ^{13}C attached proton test results (APT) were recorded at 400 and 100 MHz respectively using JEOL ECS 400 instrument (JEOL USA, Inc., MA, USA).

6.4 Results and discussion

6.4.1 Enrichment of minor coumarins

Cold pressed grapefruit volatile oil is rich (85%) in d-limonene. This was removed by vacuum distillation to enrich minor components at temperature (70 °C) under vacuum (2 mbar) to obtain the NVF. Non-volatiles constituted about 8.9% (w/w)

of total cold pressed grapefruit oil. In NVF, many major peaks including bergamottin obscured minor peaks. Prior to HCM separation, selective partitioning of minor bioactives from NVF further concentrated the target compounds. Therefore, the grapefruit oil NVF was partitioned with hexane to obtain NVF-HIF.

HPLC- PDA scans ranging from 200 to 360 nm showed that the hexane soluble fraction (HSF) had only a few compounds, unlike the HIF, which had many (Fig. 6.2). HPLC chromatograms showed an increase in minor nonvolatile peaks due to partitioning of grapefruit NVF.

6.4.2 Sequential partitioning of minor coumarins

The NVF cold pressed grapefruit oil is a rich source (30.74%) of bergamottin, another major NVF component that can interfere with isolation of minor components. To isolate minor coumarins, grapefruit oil was partitioned four times, successively, with hexane to remove the bergamottin. The bergamottin levels during each partition stage were 25.1, 13.0, 8.6 and 5.7%. According to Sticher, the combination of a compound's properties such as solubility, volatility and stability should be carefully modulated to isolate target compounds before successful purification using chromatographic techniques [189]. In this experiment, the partitioning method was used to selectively remove the major compound, which obscured minor compounds, to enrich the minor compounds present in grapefruit oil. In general, purification of coumarins from grapefruits using open column chromatography is tedious and labor intensive. Therefore, countercurrent chromatography techniques, where compounds are partitioned based on their solubility, have become a useful alternative to open column chromatography [191].

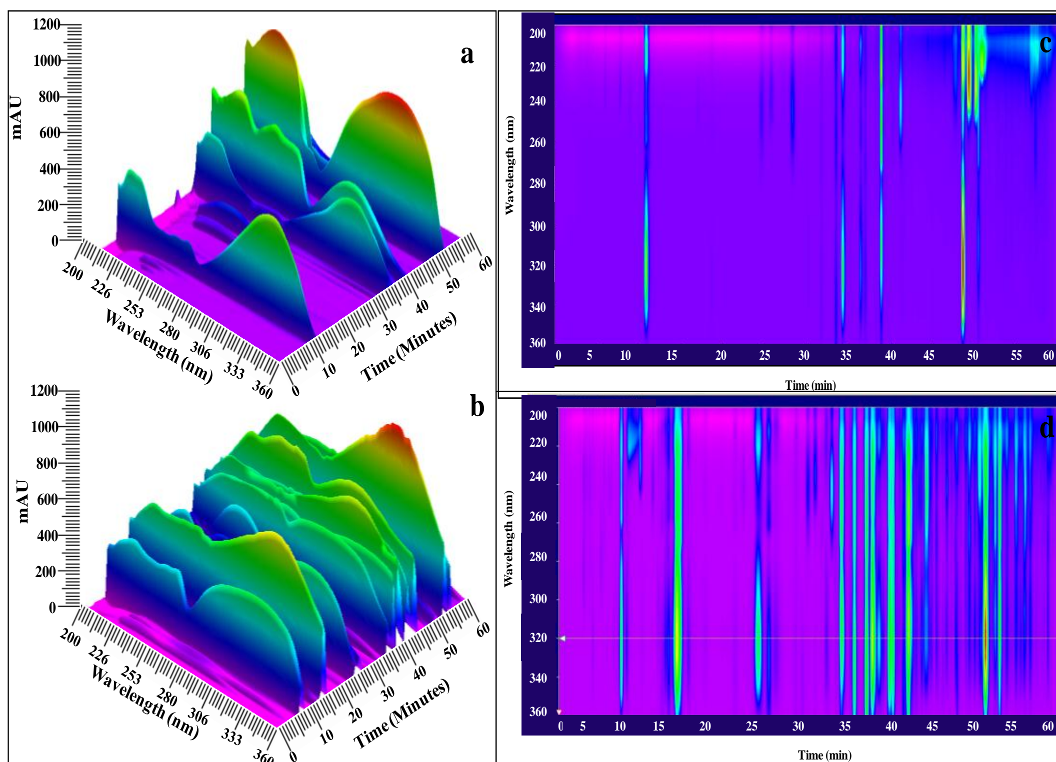


Fig. 6.2. HPLC analysis showing photo diode array detector's three dimensional scans of (**a**) hexane soluble fraction (HSF) and (**b**) hexane insoluble fraction (HIF), and two dimensional UV scan ranging from 200 to 360 nm (**c**) HSF and (**d**) HIF

This is a very useful technique for the separation and purification of natural compounds from complex extracts consisting of analytes that show a wide range of polarities and concentrations. However, the major pitfall in countercurrent chromatography is its lower separation efficiency due to band broadening. In the current study, we used a partitioning technique that works on the same principle as countercurrent chromatography for the separation of medium polar and non-polar compounds from grapefruit oil-NVF. Subsequently, the phase that was rich in minor compounds was purified using HCM.

6.4.3 Identification and characterization

Compounds **1** (0.032 %), **2** (0.05 %), **3** (0.005 %), **4** (0.134 %), **5** (0.011 %) and **6** (0.565 %) were isolated by HCM from grapefruit oil and compounds **3** (0.001%), **6** (0.017%), **7** (0.002%), **8** (0.002%) and **9** (0.006%) were isolated from grapefruit peel extract. The yield percent for each compound was calculated with respect to the quantity present in the raw material; compound purity was analyzed and compounds were identified by HPLC (Fig. 6.3 and 6.4). The crystalline fractions of compounds **1**, **2**, **3**, **4**, **7** and **9** were also characterized and identified by ^{1}H NMR and ^{13}C APT spectral analysis; the chemical shifts are given in Fig. 6.5 and 6.6, respectively. Finally, the chemical structures of these compounds were elucidated and identified as pranferin (**1**), meranzin (**2**), bergapten (**3**), dihydroxy bergamottin (**4**), osthol (**7**) and marmin (**9**). The chemical shifts of these compounds were matched to reported values [192-199].

Further, compounds **5**, **6** and **8** were identified as heptamethoxy flavones, bergamottin and nobilitin by comparison with the known standard compounds obtained

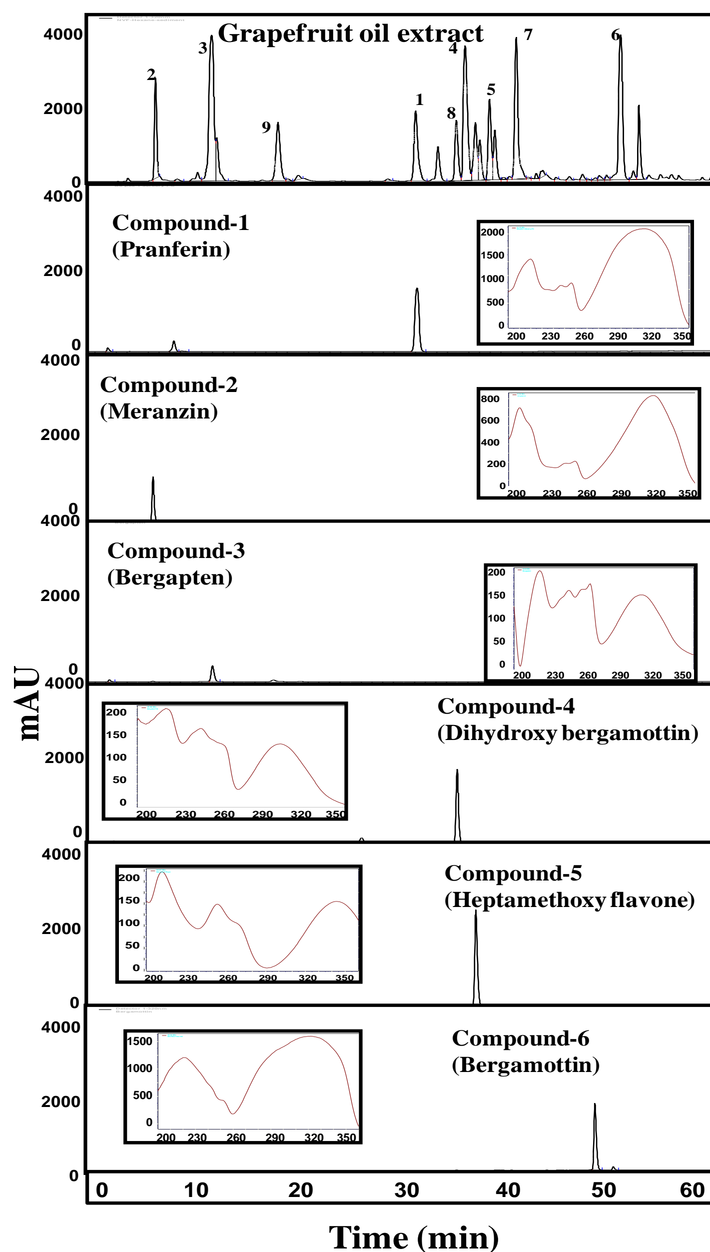


Fig. 6.3. HPLC chromatograms of purified compounds from cold pressed grapefruit oil (**1**, **2**, **3**, **4**, **5** and **6**) and grapefruit peel hexane extract (**3 and 6**) using a reversed phase flash chromatography

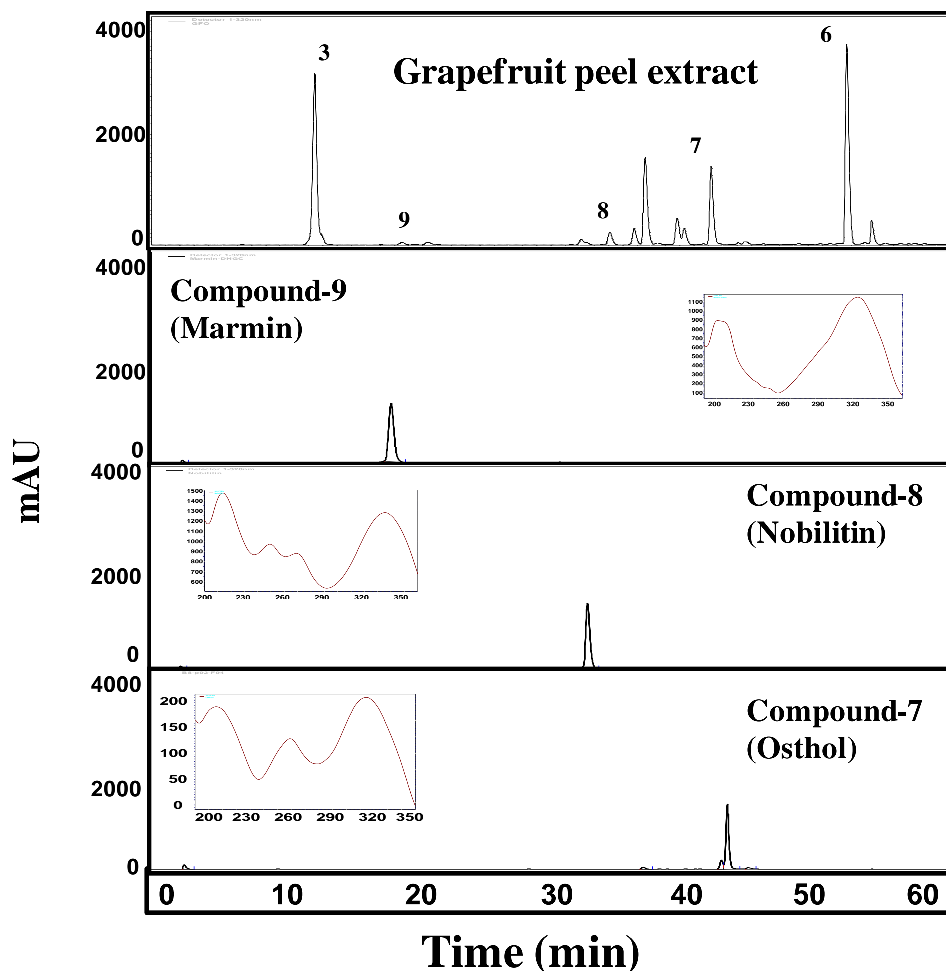


Fig. 6.4. HPLC chromatograms of purified grapefruit peel bioactives using a normal phase flash chromatography

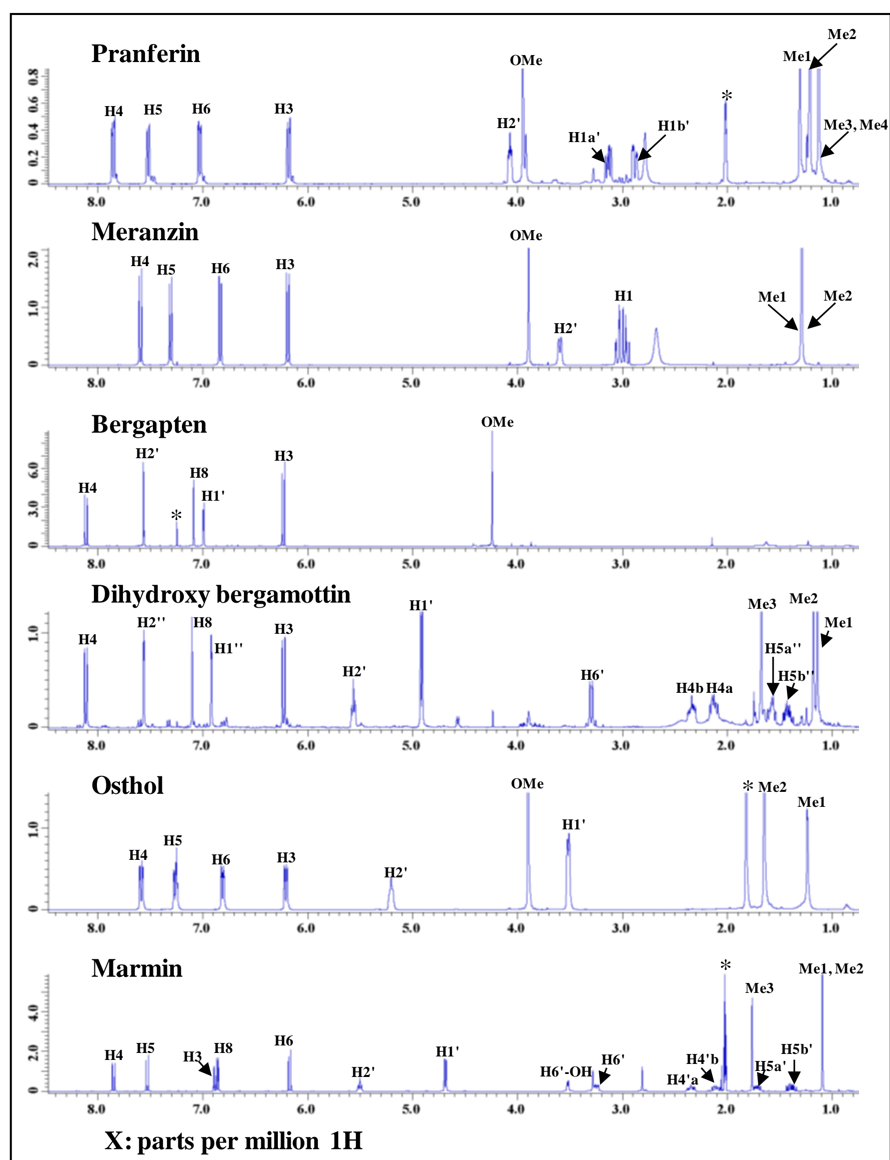


Fig. 6.5. ^{1}H NMR chemical shifts of, pranferin (**1**), meranzin (**2**), bergapten (**3**), dihydroxy bergamottin (**4**), osthol (**7**) and marmin (**9**).

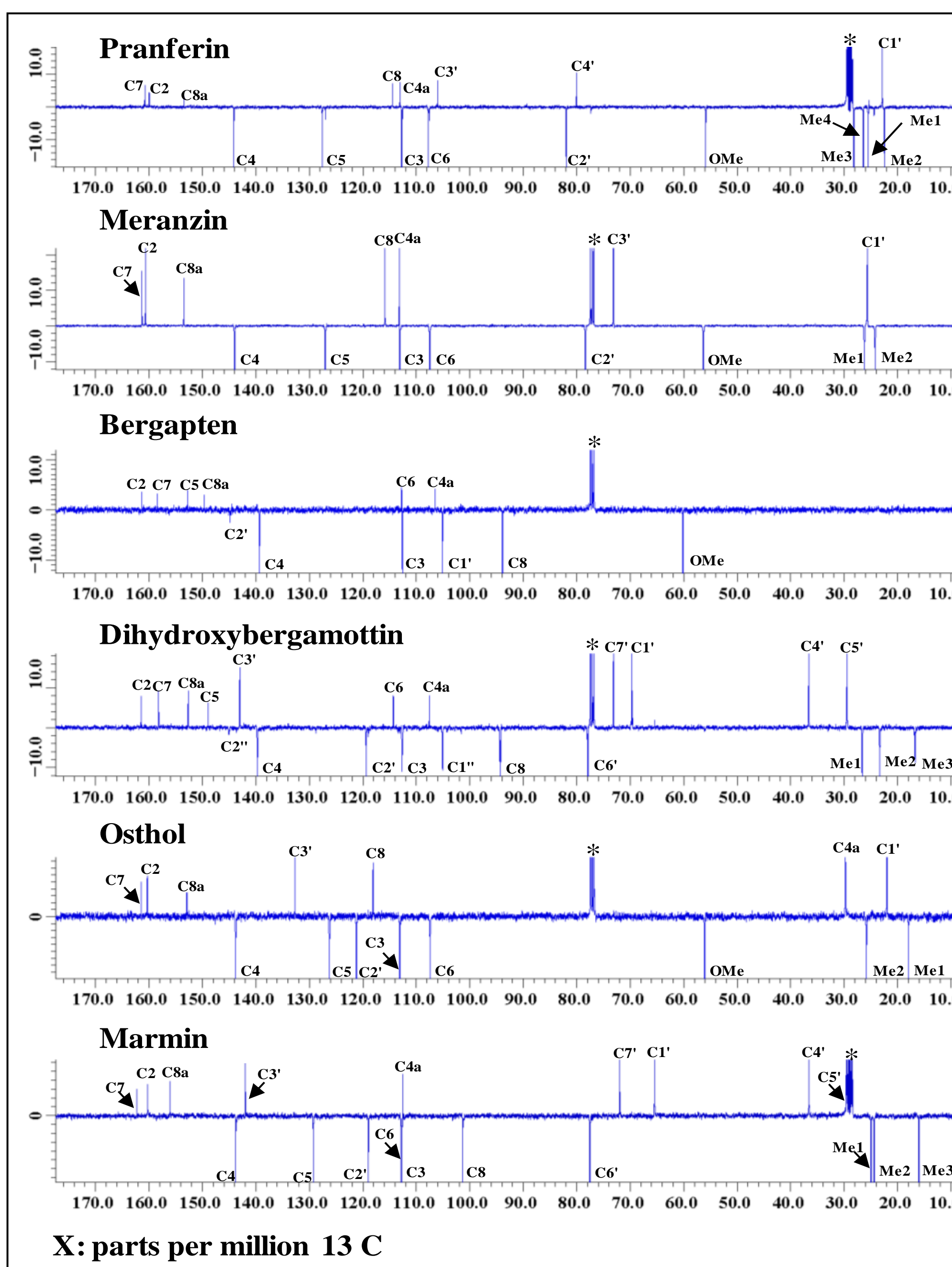


Fig. 6.6. Attached proton test (APT) spectra of pranferin (**1**), meranzin (**2**), bergapten (**3**), dihydroxy bergamottin (**4**), osthol (**7**) and marmin (**9**).

from previous studies from our group [44,200]. The identity of the crystalline compounds **1**, **3** and **7** were confirmed by GC-MS as pranferin, bergapten and osthol respectively (Fig. 6.7). Compound **4** was identified as dihydroxybergamottin, which showed a protonated molecular ion $[M+H]^+$ at 373.15 (Fig. 6.7) in APCI-MS. The structures of all nine purified compounds are presented in Fig. 6.8.

6.4.4 Role of normal phase and reversed phase in grapefruit extract purification

The current study reports the role of normal and reversed phase separations for purification of grapefruit extracts. In general for any chromatographic separation, the peaks that elute at the end of the run tend to have low resolution. The purification of minor compounds, such as meranzin and pranferin, was possible because they eluted earlier than begamottin (major coumarin) on a C-18 column. However, in reversed phase separations, minor components such as meranzin and pranferin eluted before bergamottin, thus eliminating possible contamination due to the major compound present in the complex sample matrix. This observation explains the preferential use of reverse phase columns for purification of minor grapefruit bioactives. This is the first report of identification and purification of meranzin and pranferin from grapefruit oil by HCM after enriching the extracts successively by pre-purification steps.

The present study investigated the potential utility of solvent partitioning for separating polar and non-polar compounds from cold press grapefruit oil. The pre-purification step was employed to enrich minor bioactives. The present method was used for selective separate bergamottin from the hexane extract. Further, grapefruit byproducts were purified by both normal and reversed phase HCM to obtain seven

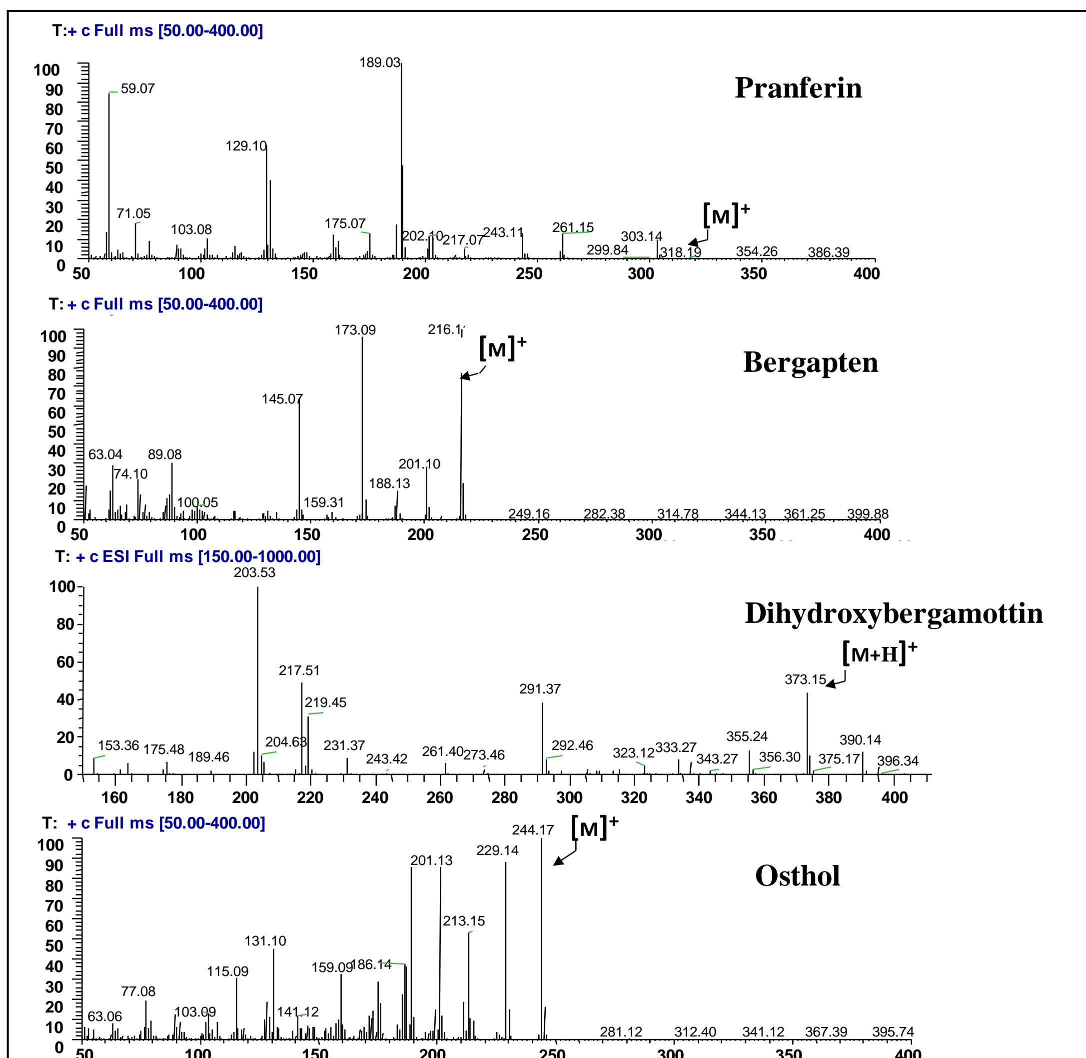


Fig.6. 7. Mass spectral analysis of pranferin, bergapten, osthol and dihydroxybergamottin purified from grapefruit oils using flash liquid chromatography.

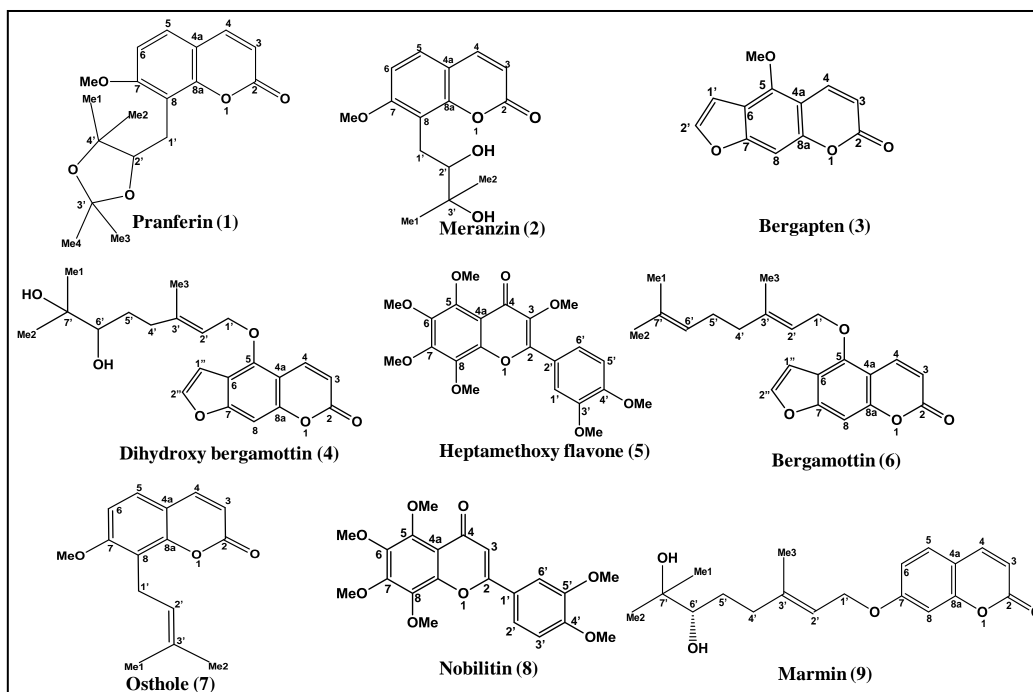


Fig.6. 8. Structures of nine compounds purified from grapefruit cold pressed oil and peel hexane extract.

coumarins and two polymethoxy flavones. Meranzin and pranferin were two minor coumarins purified from grapefruit oil for the first time after solvent partitioning and using HCM.

CHAPTER VII

SUMMARY AND CONCLUSION

Grapefruit secondary metabolites have shown unique health beneficial properties in several *in-vitro* and *in-vivo* studies. As the consumers become increasingly conscious of the nutrients derived from healthy foods, understanding the variations in the levels of these nutrients in fruits and vegetables is of greater significance to horticulturists. The basic knowledge produced from these investigations would contribute to the production of various secondary metabolite rich foods, promoting healthy communities.

Accurate quantification methods are necessary to understand the changes in grapefruit secondary metabolites obtained from different production systems. Since, accurate quantification requires optimization of various extraction steps due to a vast compositional variation (percent water, cell wall material or percent of fiber and spatial occurrence of secondary metabolites) among plant samples. Currently, several optimized analytical methods are available in the literature for quantifying limonoids, coumarins and carotenoids [49,137,140], but not for grapefruit flavanones or vitamin C. In view of that, we optimized the extraction procedures for the accurate quantification of flavanones and vitamin C [136,178].

The grapefruit flavanones were best extracted with DMSO using a 1:2 sample to solvent ratio. In the case of vitamin C, 3% MPA was the best solvent modifier for extraction and 5mM TCEP was the best reducing agent to derivatize DHA to AA at 2.5 pH. These optimized methods were used to quantify the levels of grapefruit flavanones

and vitamin C, along with several other secondary metabolites in grapefruits produced under different production systems and storage temperatures.

Vitamin C and nomilin levels were higher in organic grapefruits compared to conventional grapefruits in E1. Vitamin C levels were inversely related to soil nitrate nitrogen content in the two production systems. The loss of vitamin C loss during storage was minimal in both organic and conventional grapefruits. However, lycopene and β-carotene levels were higher in conventional grapefruits compared to organic in both E1 and E2. Carotenoid levels were generally higher in E1 than in E2, which may be due to the effect of harvest time. Carotenoid levels were higher in conventional than in organic grapefruits in both the experiments. It is likely that the cooler temperatures at the time of harvest might have caused the variation in carotenoids in E1 and E2.

The DHB levels were significantly higher in conventional grapefruits during E2 storage while, narirutin levels were higher in organic grapefruits in E1. The harvest period could be the reason for the same flavanone levels in organic and conventional grapefruits in E2. The total phenolics also followed trends similar to that of flavanones. During storage, a strong correlation was observed between flavonoid levels to the total phenolics. In E1, the total phenolics were in consonance with ORAC while in E2, the total phenolics were also correlating with the DPPH radical scavenging activity.

The present research encompasses information on several parameters including productions systems, time of harvest, and storage conditions, which influence grapefruit bioactive compounds. However, the underlying mechanisms that cause these variations

due to plant nutrition, environmental factors, biotic and abiotic stress, plant growth and their interactions need to be addressed in further studies.

At the current state of knowledge, the required (curative) levels of all secondary metabolites are not completely established except for few popular secondary metabolites such as ascorbic acid (90 mg/day). In the prospect of human health, higher levels (2-4 mg/100 g) of vitamin C in organic grapefruits in E1 storage (November harvested fruits) may not be biologically relevant (curative effects). For instance, the other secondary metabolites such as limonoids, the levels were higher in organic E1 but their required levels in humans was not yet established. As the season prolonged, the differences in the levels between organic and conventional grapefruits decreased. Furthermore, each plant species respond differently to changes in production systems by producing different secondary metabolites at various levels. The environment and season seem to have a greater impact on plant secondary metabolites than production system alone. Therefore, it is not definitive that organic produce would always contain higher levels of secondary metabolites than conventional produce at all times. It appears that there is a need for consumer education on the current state of research on organic foods rather generalizing the outcomes of few studies conducted under a set of conditions.

Grapefruit coumarins are primarily produced by grapefruits to ward off herbivores and infections caused by bacteria and fungi. The coumarins are popular secondary metabolites for their drug interaction properties and lower concentrations in fruits. Isolation and purification of coumarins is critical for quantification and for understanding the drug interaction mechanisms. The present study investigated the

potential of solvent partitioning in separating polar and non-polar compounds from cold press grapefruit oil. The pre-purification step was employed to enrich minor bioactives. The present method was used for the selective separation of bergamottin from the hexane extract. Further, grapefruit byproducts were purified by both normal and reversed phase HCM to obtain seven coumarins and two polymethoxy flavones. Meranzin and pranferin were the two minor coumarins purified from grapefruit oil for the first time after solvent partitioning and using HCM. The purified DHB was used as a standard in the quantification of coumarins from organic and conventional grapefruits. In future, pure coumarins produced using various isolation techniques will be studied to understand the mechanisms of drug interaction especially for meranzin and pranferin.

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