PRODUCTION SYSTEMS AND PROCESSING EFFECT ON PHYTOCHEMICALS IN CITRUS FRUITS AND THEIR ANALYTICAL AND ISOLATION METHODS

A Dissertation

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RAM MOHAN UCKOO

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Approved by:

Chair of Committee,	Bhimanagouda S. Patil
Committee Members,	Shad D. Nelson
	G.K. Jayaprakasha
	Leonardo Lombardini
	Suresh Pillai
Head of Department,	Daniel Lineberger

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ABSTRACT

The emerging scientific evidences on the role of food components in prevention of several chronic diseases are the momentum for shifting from a traditional focus on production to enhancement of nutritional quality. To further understand the role of these phytochemicals this dissertation describes the development of rapid analytical and isolation methods, and the effect of production systems and processing techniques on the levels of phytochemicals in citrus fruits.

In the first study, a simultaneous high performance liquid chromatography (HPLC) method for the rapid analysis of amines and organic acids was developed. The simultaneous extraction and analysis of samples provides an economical method for analyzing a large number of samples. In the second study, rapid separation method of potent health beneficial phytochemicals such as polymethoxyflavones from citrus peels using flash chromatography was developed. Using the developed method, five polymethoxyflavones were separated and isolated with high purity in gram level quantity. In the third study, the levels of phytochemicals in organically and conventionally grown lemons and their storage at market simulated conditions were determined. Results suggest that organically produced citrus fruits have higher content of organic acids and flavonoids than conventionally produced. The fourth and fifth study determined the influence of household processing (blending, juicing, hand squeezing techniques) and emerging processing (high pressure processing [HPP], thermal processing) on the phytochemicals content of 'Rio Red' grapefruits. Fruits processed by

blending had significantly higher levels of flavonoids, furocoumarins and limonin compared to juicing and hand squeezing, while HPP enabled in extending the shelf life of the processed juice without any adverse effects. Therefore, consuming grapefruit juice processed by blending may provide higher levels of health beneficial phytochemicals. The sixth study describes a rapid flash chromatography method for isolation of PMFs and furocoumarins from citrus industrial by products such as peel oil. In the seventh study the developed method was applied to isolate 10 different phytochemicals from an unexplored citrus species, Miaray mandarin (*Citrus miaray* TAN.). Among them, the 5,7,8,3',4' pentamethoxyflavone was isolated for the first time from the genus *Citrus*.

DEDICATION

I would like to dedicate this dissertation and my entire academic experience to my family who taught me the value of education. To my father, Chandra Mohan Uckoo, who always supported, encouraged, and motivated me. I owe my entire education to his advice and motivation. I know it would be impossible for me to be as selfless person as he is, but I will try my best. 'Nana', you are and always will be the best and the greatest father in the universe. To my mother, Shiva Rani for her immense love and encouragement. 'Mamma', you were the reason I smiled when things got bad. To my brothers, Vishnu anna and Krishna anna, for being the pillars of support, in every aspect of life, joy and sorrow, right from the early days that I have memory of. Thank you for all the love and support that you have given me throughout the years. To my sisters-in-law, Gayatri and Sreedevi, nieces, Pravalika and Omna and my nephew, Rithvik for their love and support. To my in-laws and Chandana for their love and patience.

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NOMENCLATURE

HPLC	High performance liquid chromatography
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
HPP	High pressure processing
TP	Thermal processing
FC	Flash chromatography
PMF	Polymethoxyflavone
LRGV	Lower Rio Grande Valley

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1. INTRODUCTION

Citrus is a plant genus as well as a common name for a group of fruits which belong to the family Rutaceae. The unique taste, texture and the wide range of health benefits associated with consumption of citrus fruits have led it to be one of the most consumed fruits in the world. The total world production of citrus for the harvest season 2010/11 was approximately 54 million metric tons [1]. Besides the commonly known vitamins such as vitamin C, citrus fruits contain a wide array of phytochemicals considered to have several health promoting properties. These phytochemicals are naturally synthesized by plants whose primary role is protection from various pests and diseases. Due to genetic, environmental and physio-chemical changes there are numerous phytochemicals synthesized in varying contents and structures in different citrus species. They are classified into different groups based on their structural similarity. Some of the major phytochemicals present in citrus are flavonoids, carotenoids, limonoids, amines, furocoumarins, folate, and pectin. The structural diversity of these phytochemicals confers several bioactive properties, among which anti-oxidant, anti-cancer, anti-inflammatory, anti-proliferative, and prevention of cardio vascular diseases are well known. To further elucidate and understand their role in human health benefits, a comprehensive study of the factors affecting their biochemical variation is essential.

Some of the factors that influence the levels of phytochemicals are pre-harvest and post-harvest conditions such as production systems and processing techniques. To

analyze and study these phytochemicals affected by the pre-harvest and post-harvest factors, efficient chromatographic techniques are necessary. Similarly, isolation and characterization of phytochemicals from understudied citrus species, and industrial by products will enable in conducting large scale *in vivo* studies.

1.1 Objectives

1) To develop a rapid HPLC method for the separation and determination of amines and organic acids in citrus fruits.
2) To develop a rapid flash chromatography method for separation of polymethoxyflavones from citrus fruits and confirm the identity by spectral analysis using NMR and mass spectra.
3) To determine the influence of organic and conventional production systems on the levels of phytochemicals in Meyer lemon (*Citrus meyeri* TAN.)
4) To quantify the content of flavonoids, limonoids, furocoumarins, organic acids, and evaluate the quality parameters (acidity and total soluble solids) in grapefruit juice processed by blending, hand squeezing and juicing techniques.
5) To evaluate the influence of non-thermal processing technique such as high pressure processing technique and thermal processing technique on the levels of phyochemicals present in grapefruits juice and the shelf life during storage.

6) To develop an efficient separation and isolation furocoumarins and polymethoxyflavones from citrus juice processing industrial by product using flash chromatography.

7) To evaluate the potential for isolation of unexplored polymethoxyflavones from understudied Miaray mandarin and evaluate the biofilm inhibitory activity using *Vibrio harveyi*.

8) To separate and purify polymethoxyflavones from ‘Clementine’ and ‘Blood orange’ citrus fruits.

2. REVIEW OF LITERATURE*

Citrus trees are evergreen, and prefer a moderate climatic condition with optimum temperatures ranging in between 20 ºC and 35 ºC [2]. It is believed to have been originated in Asia and then spread to other continents [3]. The plants are large shrubs reaching to a height of 5–15 m, with thorny or thornless shoots and alternately arranged leaves with an entire margin. The flowers are solitary or in small corymbs, each flower is 2–4 cm in diameter, with 4-5 white petals and numerous stamens with a strong scent. The fruit is a hesperidium, a specialized berry, globose to elongated, 4–30 cm long and 4–20 cm diameter, with a leathery rind surrounding segments filled with pulp vesicles. Due to sexual hybridization among number of species and intraspecific hybrids, a large genetic variation in the citrus group exists [4].

2.1 Amines

The majority of the citrus fruits are eaten fresh, processed as juice, or the sections are utilized in fruit salads, gelatins, puddings, or on cakes. They contain flavonoids, and phenyl-ethyl-amines, which collectively contribute to antioxidant activity.

Apart from flavonoids citrus fruits are characterized by the presence of folate, dietary fiber and other bioactive components such as carotenoids and limonoids, with limonin and nomilin predominant. The roles of flavonoids, limonoids and carotenoids have been elucidated as health maintaining compounds with several beneficial properties. Flavonoids transfer electron free radicals, chelate metal catalysts [4], inhibit oxidases [5], promote differentiation [6], and are modulators of tyrosine kinases [7]. Numerous epidemiological studies suggest the protective effects of flavonoids against cardiovascular diseases, cancers, and other age-related diseases [8]. Similarly, limonoids have been well documented for their part in antioxidant activity, detoxification of carcinogens and harmful chemicals, stimulation of the immune system, effects on cell differentiation, increase apoptosis of cancer cells and decrease cell proliferation. Citrus is also a good source of phenyl-ethyl-amines such as octopomine, synephrine and tyramine [9-11].

In recent years, there has been an increased attention towards the study of amines particularly synephrine, a predominant amine present in citrus species. The role of synephrine as well as other amines such as octopomine and tyramine has been well studied as a potent anti-obesity compounds. Synephrine is a sympathetic α-adrenergic [12] agonist similar to phenylephrine [13] and suppresses appetite much like ephedrine, causing weight reduction. These agents act on the central nervous system to reduce the appetite thus causing the feeling of fullness [14]. In animal studies synephrine has shown to raise blood pressure when administered intravenous [9]. However no variation in hemodynamics of normotensive human adults was noticed in a clinical trial evaluating

sour orange juice, which has predominant synephrine content [10]. Due to thermogenic and adrogeneric properties of these amines, many formulations and extracts are being promoted as weight reducing agents. Moreover, the ban of ephedrine containing substance by FDA [11] has resulted in higher consumption of these dietary supplements which show relatively less health risks. Many herbal formulations derived from sour orange extracts are promoted due to the high levels of amines present in them. Ascorbic and citric acid both characteristic of citrus species have been well documented on their antioxidant, anticancer properties [18, 19]. Several epidemiological studies have shown correlation between ascorbic acid and health benefits [15, 16]. Both the acids collectively contribute towards the flavor of citrus fruit [17] hence, a vital fruit maturity and quality indicator. Due to the concerns regarding the bioactivity of amines and the economical attributes of quality and flavor associated with the organic acids, an analytical method for quantifying these in the citrus fruits is necessary. Several high performance liquid chromatography (HPLC) methods have been reported for quantification of these compounds in dietary supplements [5-7] and citrus fruits [8-10]. Similarly, several methods have been reported for analysis of organic acids such as, spectrophotometric [11, 12] calorimetric [13-15], and chromatographic methods [15-17].

2.2 Separation and isolation of polymethoxyflavones

An integral part of studying bioactive components of citrus is to identify new compounds with potent antiproliferative and antioxidant activities. The correlation

between citrus phytochemicals with anti-tumor activity necessitates the need for determining the identity of bioactive compounds that inhibit tumor cell growth that may play a role in cancer prevention and therapy. Studies involving limonoids displayed an array of biological activities such as anti-cancer activity in laboratory animals and cultured human breast cancer cells [18, 19]. Previous results from laboratory studies indicate that extracts containing high levels of obacunone 17β-d-glucopyranoside, nomilinic acid 17β-d-glucopyranoside and small amounts of nomilin 17β-d-glucopyranoside and limonin 17β-dglucopyranoside are cytotoxic to MCF-7 cells [20, 21]. Similarly, both aglycones and glucosides of limonoids have shown significant inhibitory activity against cancerous tumors while inducing glutathione-S-transferase activity in animals [22]. In a model for oral carcinogenesis, limonin 17β-d-glucopyranoside and aglycones such as limonin and limonin carboxymethoxime were found to have significant activity [18, 21, 23]. Results from these studies suggest that further research is essential in order to analyze the role of bioactive compounds in their pure form and to elucidate their structural relationship in different cancer models.

Several chromatographic methods have been used for isolation and purification of these compounds. Phytochemicals such as limonoid glucosides have been isolated by open column chromatography [24, 25] and preparative chromatography [26] as well as by a combination of these techniques. Partial purification of the limonoid glucosides using DEAE Sephacel and XAD resin columns prior to preparative chromatographic separation has been reported [24, 25]. Medium pressure liquid chromatography has also been used for separation, but a major limitation is large amounts of raw material needs to

be processed using equivalent amounts of solvents to obtain sufficient yield of limonoids [27]. Additionally different solvents are required for extraction and purification of limonoids with different polarities [28]. Though the use of preparative chromatography is ideal for obtaining pure standard compounds, it is tedious and time-consuming to obtain the large quantities of the respective compounds which are essential for animal model studies and for human clinical trials. The major challenge in isolating and purifying phytochemicals from citrus raw material is their low abundance. Hence, rapid and simple separation methodologies are essential to isolate and purify the phytochemicals from extracts that are obtained from citrus seeds and peel. Some of these phytochemicals include limonoids [29, 30], flavonoids [31, 32], carotenoids, phenolic acids [33], organic acids [34], furocoumarins [35, 36], and amines [37]. Due to rapid hybridization and mutations along with polyploidy nature of citrus led to development of several varieties. These genetical variations might have resulted in characteristic changes in the levels of phytochemicals. Chemotaxonomy was also proposed for the classification of the citrus genus based on the variation of limonoids [38]. Similarly, the variation in composition of polymethoxyflavones was also used as a basis for chemotaxonomic classification of the *Citrus* genus [39].

Polymethoxyflavones are a group of flavonoids with two or more methoxy groups. There are approximately more than 25 PMFs reported from citrus among which the major occurring are tangeretin (5,6,7,8,4′-pentamethoxyflavone), heptamethoxyflavone (3,5,6,7,8,3',4'-heptamethoxylflavone), nobiletin (5,6,7,8,3,4'-hexamethoxyflavone), tetramethoxyflavone (5,6,7,4'-tetramethoxyflavanone) and sinensitin (5,6,7,3',4'-

pentamethoxyflavone) [40]. While limited literature is available on the evolution of PMFs in citrus, methoxylation of flavone or flavanone aglycones was proposed as a pathway for biosynthesis of PMFs in oranges [41]. They occur in leaves, peel and juice but are mainly localized in the peels of the citrus fruits. Studies suggest that the concentration of PMFs varies based on maturity and species of citrus [42, 43]. In plants, PMFs are considered to be protective against disease causing pathogens [44-46]. On human health perspective, PMFs were investigated since early 19^{th} century and implicated in several health benefits such as antiproliferative [47], anticancer [48-51], anti-inflammatory [52], antilipogenic and antimutagenic [53] activity. A comprehensive review explaining the multitude of health benefits of PMFs was reported by Li *et al.* ,[54]. Due to their relevant role in health benefits, PMFs were isolated from different species and parts of citrus. The isolation of PMFs was achieved by using several extraction and isolation methodologies.

2.2.1 Extraction methods

Polymethoxyflavones are low polar compounds and can be extracted using non polar solvents such as hexane and polar solvents including water [47], ethanol and methanol [55-57]. Moreover, these compounds were extracted from various parts of citrus such as the peel, leaves and cold pressed oil. Raman et al.,[58] reported extraction of *C. reticulata* peels using non polar hexane solvent followed by treating with 10% sodium hydroxide solution. The mixture was later extracted with diethyl ether, washed

with water and subjected to adsorptive separation using cation exchange resin Dowex 50WX2 to yield nobiletin and tangeretin. Chaliha et al., [59] reported extraction of *Citrus jambhiri* peels using petroleum ether solvent in a Soxhlet apparatus for separation of PMFs. Jayaprakasha et al., [60] reported extraction of *Citrus reticulata* (Blanco Coorg Mandarin) using hexane and chloroform successively in a Soxhlet apparatus. The extracts were subjected to further separation using silica gel column chromatography for isolation of desmethylnobiletin, nobiletin and tangeretin. Miyake [61] reported the extraction efficiency of PMFs using ethanol and aqueous solution of ethanol at various proportions (5%, 25%, 50%, 75% and 100%). Among the evaluated ratios, 75% ethanol in water and 100% ethanol resulted in 100% extraction efficiency. Moreover, extraction of PMFs was influenced by the temperature of the solvent. Extraction of citrus fruits using hot 25% ethanol in water resulted in higher content of PMFs in comparison to water, 5% and 25% ethanol aqueous solution under cold and hot water and 5% ethanol in water. Similarly, these compounds were extracted from peels of *C. reticulata* Blanco *cv.* Ponkan by refluxing with 75% ethanol for 3 h [62]. The ethanol solution was concentrated and further extracted with dichloromethane to yield PMFs rich fraction. Wang et al., [63] reported extraction of PMFs from dried peel powder of *C. reticulata* by refluxing with 75% (v/v) ethanol for 10 h. The extract was concentrated and extracted with chloroform to yield mixture of PMFs. Individual PMFs were separated by column chromatography using chloroform:acetone (9:2, v/v). Chen and Montanari [64] reported extraction of PMFs from leaves of Dancy tangerine using methanol:chloroform (1:1). The extract was further subjected to separation using combination of flash C_{18} column

chromatography and C_{18} preparative HPLC for separating individual PMFs. In a recent report [65], hexane was suggested as a better solvent in comparison to chloroform and methanol solvents for Soxhlet extraction of dried peel powder of Cleopatra mandarin (*C. reshni*). Soxhlet extraction by hexane yielded extract with low occurrence of flavonoid glucosides and higher content of PMFs.

2.2.2 Supercritical fluid extraction

Apart from citrus peel and leaves, cold pressed oil is a rich source of PMFs. Extraction of these compounds from the precipitate of winterized (storing the oil at 20 ºC for long duration of time) citrus peel oil was commonly reported [66-69]. These compounds were also extracted from peel oil extract using super critical fluid extraction [66]. Recently, the optimum conditions for extraction of nobiletin and tangeretin from *C. depressa* Hayata by supercritical CO_2 was developed by comparing various combinations of pressure and percentage of modifier ethanol solvent. Optimum extraction was achieved by ethanol (85%) as a modifier in supercritical CO_2 maintained at 80 °C and 30 MPa of pressure. Also, the % yield of PMFs by SFE was 107% as compared to conventional solvent extraction yielding 100% [70].

Yao et al., [71] reported extraction of PMFs from dried peels of *C. sinensis* Osbeck by enzymatic hydrolysis. The dried peel powder (100 g) was extracted exhaustively using 95% ethanol (1,500 mL) and 5% cellulase at 60º C for 2.5 h. The extract was concentrated and treated with diethyl ether (200 mL × 3) and washed with 0.4% sodium

hydroxide solution until the extract turned colorless. The clear diethyl ether extract was collected, concentrated, and freeze-dried to obtain crude PMFs (564 mg). In addition to the traditional extraction methodologies, an advanced technology such as microwave-assisted extraction has also been reported for extraction of PMFs. Dried peels of *Citrus yuko* Hort. ex Tanaka were refluxed using microwave for 2.5 min to 5 min with methanol yielding 0.12% and 0.10% of tangeretin and nobiletin, respectively [55].

2.2.3 Separation methods

Tangeretin was the first PMF isolated from Tangerine (*Citrus nobilis deliciosa*) oil by Nelson [72]. Nobiletin was isolated from the peels of Chinese mandarin oranges (*C. nobilis*) by Tseng [73]. Tetramethoxyflavone and heptamethoxyflavone were reported and identified by Swift [74] in the neutral fraction of orange peel oil. Sinensitin was isolated by Born [75], and named by Swift [76]. Although all major PMFs were isolated by the late 1960s, extensive isolation was triggered by the implication of these compounds in the several health beneficial properties and consumers' interest in natural products. PMFs were separated using thin layer chromatography (TLC), preparative high performance liquid chromatography (prep-HPLC), supercritical fluid chromatography (SFC), and high speed counter current chromatography (HSCCC).

2.2.4 Preparative thin layer chromatography (prep-TLC)

Among the reported separation methods of PMFs, prep-TLC is the most economical. It is relatively low in cost and does not require sophisticated instrumentation. However, this method is limited by the low amount of sample loaded and yield. Successive separations may be required for obtaining pure PMFs. Del Rio et al., [77] reported separation of these compounds from peel oil of various citrus fruits. Citrus oil was mixed with 2-propanol and distilled water in a decantation funnel and extracted with hexane. The 2-proponol/water phase was concentrated, mixed with water and liquid-liquid extraction was conducted using benzene. The organic phase was separated, concentrated and dehydrated by adding anhydrous sodium sulfate. The extract was placed on a TLC plate containing silica and eluted with benzene:acetone (3:1, v/v). The separated compounds were visualized by their fluorescence and the individual bands were collected and analyzed by HPLC and mass spectrometry.

Machida and Osawa [78] reported the isolation of PMFs from the peels of *C. hassaku* using a combination of column chromatography and prep-TLC. Citrus peels were extracted by ethanol under reflux and concentrated. The extract was partitioned between ether and water. The residue was separated on silica gel using benzene-acetone mixture. The components that tested positive in Mg-HCl test were further fractionated by prep-TLC yielding 8 different PMFs.

2.2.5 Preparative-HPLC

Increased interest in investigating the biological activity of PMFs and advancement in chromatographic techniques led to exploring isolation of PMFs using prep-HPLC. Chen at al., [69] reported the separation of these compounds from cold pressed Dancy tangerine peel oil solids using prep-HPLC. The procedure involved a combination of normal phase chromatography and C_{18} prep-HPLC. The dried tangerine oil solids were loaded to an open silica gel column and eluted with increasing polarity gradient of benzene/ethyl acetate, ethyl acetate, ethyl acetate/2-propanol and 2-propanol. The fractions with similar PMFs were pooled and purified using C_{18} prep-HPLC with a gradient mobile phase of methanol/water and ethanol/water. The procedure was applied for separation of PMFs from Dancy tangerine leaves leading to the isolation of pure compounds [64]. However, use of solvents such as benzene for isolation studies should be avoided due to their carcinogenic and mutagenic properties. Li et al., [79] reported a gram-scale isolation method of nobiletin using a combination of normal phase flash chromatography and prep-HPLC. The procedure involved initial separation of orange peel extract using silica gel flash column eluted with a gradient solvent system of ethyl acetate and hexanes. The collected fractions containing nobiletin and 5,6,7,4′-tetramethoxyflavone were concentrated and further separated on a Regis chiral column connected to a prep-HPLC. The solvent system consisted of 35% ethanol and 65% hexanes with a flow rate set at 85 mL/min resulting in isolation of gram amounts of

nobiletin and 5,6,7,4′-tetramethoxyflavone. Similar procedure was further applied for isolation of other PMFs from cold-pressed orange peel oil [80].

2.2.6 Supercritical fluid chromatography

This method is one of the ideal methods for separation of PMFs. This method involves use of pressure and temperature combinations maintained at critical point of the mobile phase used. Moreover, the absence of permanent adsorptive loss of sample on to the stationary phase which is commonly noticed in column chromatography makes this method advantageous. Among the various mobile phases used for SFC, CO_2 along with methanol seems to be ideal for separation of PMFs. This method was initially used for analyzing the authenticity of citrus oils by quantification of PMFs [81]. The separation of PMFs was conducted using CO_2 as mobile phase and methanol as a polar modifier. In an another report, hydroxy- and methoxy-flavones were separated by supercritical CO_2 chromatography on capillary columns using flame ionization and Fourier transform infrared (FT-IR) spectroscopy detection [82]. Recently, a large scale isolation method of four PMFs such as nobiletin, tangeretin, 3,5,6,7,8,3′,4′-heptamethoxyflavone and 5,6,7,4′-tetramethoxyflavone was reported using a combination of normal phase flash column separation and SFC separation [66]. The raw material used for the separation was crude sweet orange peel extract. The extract was separated on a silica gel flash column using a gradient mobile phase. Individual fractions were analyzed by LC–ESI-MS and TLC and grouped into 6 groups. The groups that had high concentration of

PMFs were subjected to SFC separation using mobile phase of CO_2 and methanol. The separated peaks were collected as individual fractions to obtain pure PMFs.

2.2.7 High speed counter current chromatography (HSCCC)

This is a chromatography technique in which liquid–liquid partition is used as a strategy for separations and unlike other chromatographic techniques does not use any solid support matrix. Due to the characteristic absence of solid support matrix there is no loss of samples by adsorption. This method was first reported as efficient method for the preparative isolation and purification of polymethoxylated flavones from tangerine peel extracts [83]. Tangerine peels were extracted by light petroleum, concentrated and frozen. The sediment was dried and injected to the HSCCC in 15 mL sample injections. The separations were conducted using a two-phase solvent system composed of *n*-hexane, ethyl acetate, methanol and water (1:0.8:1:1) (v/v). The effluent was monitored with a UV detector at 254 nm and peak fractions were collected according to the elution profile. Similar peaks were pooled and four PMFs nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, tangeretin and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone were isolated in milligram quantity.

2.2.8 Flash chromatography (FC)

This method is also called as medium pressure liquid chromatography which is a rapid technique of column chromatography. The regulated application of medium pressure enables separation of compounds using large sample volumes, thereby yielding high quantity of pure compounds. Recent technological advances have also enabled conducting separations with online detection and robotic fraction collectors. These advancements have enabled in development of large scale separation of PMFs. Dried peels of Cleopatra mandarin and Marrs sweet orange (*Citrus sinensis* L. Osbeck) fruits were powdered and extracted using a non-polar solvent in a Soxhlet. The extract was concentrated, impregnated with silica gel and subjected to separation using flash chromatography. A gradient solvent system was used for separation and the eluent was monitored at wavelengths of 254 nm & 340 nm. Individual peaks were collected in fractions and pooled after analyzing by HPLC. The isolated compounds were identified as sinensitin, tetramethoxyflavone, nobiletin, and tangeretin using NMR and mass spectrometry [65, 84].

2.2.9 Identification and structure elucidation

Identification of PMFs is challenging due to their close similarity in structures and as well as molecular weight. This necessitates use of proper tools and techniques to determine their exact structure. Until late 70's, infrared spectroscopic studies coupled

with degradation and synthetic studies were commonly used for elucidating the structure of PMFs. Although degradation and synthetic studies were used for structural analysis, IR analysis played an important role in confirmation of the structure of PMFs. One such example can be given as the ambiguity in the flavonol constitution of a compound synthesized by Goldsworth and Robinson [85]. The compound was considered identical to tangeretin as suggested by degradation and synthetic studies which was later proved to be different from that of the actual structure of tangeretin given as 5,6,7,8,4′-pentamethoxyflavone confirmed by the IR spectroscopy [86]. These early investigations on the structure of isolated PMFs were comprehensively reviewed by Sarin and Sheshadri [87].

In the modern era of chemistry, nuclear magnetic resonance (NMR) [57, 60, 63-65, 69, 78, 80, 88, 89] is used for accurate structure elucidation. The importance of ^{13}C NMR and its application for identification of flavonoids was reviewed by Agrawal [90]. However, this method is limited by the requirement of large quantity of purified compounds. Other spectral analysis techniques used for identification of PMFs were gas chromatography- mass spectrometry (GC-MS) [91, 92], and LC-MS [93, 94]. These techniques provide valuable information in regards to the compounds molecular weight along with the fragment ions. The advantage of minimal sample requirement for identification provides a valuable tool for structure elucidation. Raman et al., [58] reported identification of nobiletin and tangeretin using mass spectrometry in negative electronspray ionization (ESI) mode. The structures were further elucidated by collisional activated dissociation (CAD) to generate fragmentation patterns of the

deprotonated flavones. Wang and Zhang [93] reported positive electronspray ionization tandem mass spectrometry of PMFs. Although NMR and MS studies provide structure information of individual isolated compounds, these methods are limited in application for identification of components in crude extracts. Recently, Weber et al., [67] reported the LC-NMR method for identification PMFs present in residues from molecular distillation of cold pressed peel oils of *C. sinensis.* The individual PMFs were initially separated using HPLC followed by NMR analysis conducted in the stop-flow mode.

2.3 Influence of pre-harvest factors on phytochemicals

In Texas, the majority of citrus acreage is present in Lower Rio Grande Valley (LRGV) consisting of approximately 27,000 acres, with Rio Red grapefruit comprising of almost 70%. Most LRGV citrus growers use flood irrigation for their orchards (Swetlik, 1992). Due to rapid urban development and semi arid conditions there has been a decline in irrigation water supplies. Recently, we observed significant differences in citrus yield among drip, microjet spray and flood irrigation systems (Uckoo et al., 2005). Low water use system had higher irrigation use efficiency (IUE), the ratio of yield to supplemental irrigation applied during the cropping season (Iglesias and Minguez, 1997). In spite of the differences in the yield, the variation of phytochemicals remains an unanswered question. Very little information is available on the effect of pre-harvest factors on variation of phytonutrients in citrus (Patil et al., 2006). Various eco-physiological responses may also be the result of different biosynthetic pathways for the

numerous phytonutrients (Monika Schreiner. 2005). The content of phytonutrients depends both quantitatively and qualitatively on their genetic information, as well as environmental factors including water and mineral nutrition. All of these factors are responsible for the wide variation in the formation and content level of phytochemicals. To increase the intake of health-promoting phytochemicals via the consumption of fresh fruit along with their derived products, comprehensive monitoring of the pre- harvest influences including production systems on the contents of phytochemicals is necessary.

2.4 Processing techniques

2.4.1 Household processing techniques

The United States of America accounts for approximately 15% of the total world citrus production [1, 95]. Among citrus fruits, red grapefruits are distinct with unique sensory quality of sweet and tart taste in addition to red coloration of the juice segments. Grapefruits contain several phytochemicals such as flavonoids, carotenoids, limonoids, organic acids, pectin, and folate [31, 96, 97]. The major flavonoids present in grapefruit are narirutin, naringin, hesperidin, neohesperidin, didymin and poncirin. These phytochemicals were extensively studied using *in vitro* and *in vivo* models to understand their role in human health benefits. Previous studies suggest that these phytochemicals have anti-inflammatory, anti-proliferative, anti-carcinogenic and antimicrobial properties [31]. Naringin (naringenin-7-O-neohesperidoside), a flavonoid glucoside, is one of the

major bitter compounds and significantly contributes to the juice sensory taste quality. Additionally, flavonoids have characteristic presence of hydroxyl groups which makes these compounds potent antioxidants. Optimum intake of antioxidants is positively correlated with health benefits such as prevention of certain cancers and cardiovascular diseases [98]. In a clinical trial, naringin was suggested to have lipid lowering properties and also increased the erythrocyte antioxidant enzyme activities in hypercholesterolemic subjects [99]. Similarly, results from animal studies suggest limonoids such as limonin present in grapefruit have anti-inflammatory activity, induce gluthathion-S-transferase activity and help in inhibiting carcinogenesis [22, 100, 101]. On the other hand, studies suggest limonin to have several health benefits such as inhibitory effect on HIV-1 replication in cell culture systems [102], anti-inflammatory [103] and anti-cancer activity [104]. Additionally, grapefruits are also a good source of citric acid and ascorbic acid. Both of these organic acids prevent non-enzymatic browning as well as contribute towards the antioxidant capacity of the fruit [105, 106]. Therefore, optimum dietary intake of these phytochemicals is essential for maintaining ideal health.

Grapefruits also contain furocoumarins such as bergamottin, 5-methoxy-7 gernoxycoumarin (5-M-7-GC) and dihydroxybergamottin (DHB). They are known to increase the bioavailability of orally administered drugs by inhibiting CYP 450 enzymes [107]. Although this activity is speculative [108], other reports suggest that these phytochemicals are beneficial and have antitumor activity which may help to protect from cancer [109, 110]. Therefore, to obtain optimum levels of grapefruit

phytochemicals, evaluation of processing techniques that may influence their content is essential.

Grapefruits have a relishing taste and are popularly eaten fresh. The outer rind of the fruit is leathery and not consumable. Hence, the fruits are either peeled and blended or cut in half and the edible segments are juiced for consumption of fresh juice. These household processing techniques may result in extraction of juice with varying amounts of the phytochemicals.

2.4.2 Industrial processing techniques

Currently, there are limited reports on evaluation of the phytochemicals content in grapefruits processed by different household techniques. It was reported that the levels of naringin, naringenin and bergapten in grapefruit juice processed by various processing methods varied significantly. However, phytochemicals such as limonoids and organic acids were not reported [111]. In an another study it was reported that the industrial and laboratorial processed grapefruit juice had significant variation in levels of furocoumarins [112]. The levels of health promoting bioactive compounds vary significantly by variety, maturity, cultivation practices, environment, storage and processing methods [112, 113]. To provide optimal health benefits from grapefruit consumption, novel processing techniques must be selected, which may prevent degradation of these compounds while also maintaining the taste and visual appeal of citrus juice.

In recent years, technological advances in processing have resulted in the development of novel techniques such as high pressure processing (HPP). This method of non-thermal processing provides several advantages over traditional thermal processing (TP). High pressure processing inactivates pathogens, inhibits degradative enzymes, and prevents the degradation of antioxidants [114]. Unlike TP, the moderate temperatures used in HPP maintain the texture, flavors, nutrients, and other sensory quality attributes of the product [115, 116]. These benefits have led to the implementation of HPP in several fruit and vegetable processing industries [117]. Reports suggest that HPP of orange juice maintained its acceptability to consumers for up to 12 weeks of storage at 4 ^{o}C without any significant variation in odor and flavor profiles [118]. HPP was also shown to produce significant inactivation of pectin methylesterase activity and reduction of microorganisms in orange juice [119]. In an another study, HPP was found to be an excellent technique for maintaining the levels of ascorbic acid and anthocyanins, which are both potent antioxidants, in blood orange juice [120]. Very few studies of this emerging technique have been reported examining the effects of HPP on bioactive compounds in orange juice [118, 121].

3. DEVELOPMENT OF RAPID SIMULTANEOUS HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR ANALYSIS OF AMINES AND ORGANIC ACIDS*

Citrus fruits contain numerous naturally occurring compounds including amines. Synephrine, octopomine and tyramine are commonly occurring amines in citrus (Fig. 1). In recent years, an increased attention towards the importance of amines was observed due to their potential role in obesity prevention [122, 123]. Synephrine is chemically similar to ephedrine based on which, several formulations and extracts with synephrine are currently being promoted as weight reducing dietary supplements. Moreover, the ban of ephedrine containing substance by Food & Drug Administration [124] seems to have resulted in higher consumption of dietary supplements containing amines due to their relatively less health risks [125]. In addition to amines, citrus fruits contain a high content of organic acids such as ascorbic and citric acid (Fig. 1). Ascorbic acid is a potent antioxidant, anti-proliferative and anti-scurvy agent [126-128]. Organic acids not only influence browning in citrus juices but also have a major role in quality control in the citrus processing industry [129, 130]. Considering bioactivity of amines, several analytical methods were reported for determining their content in *Citrus aurantium* fruits [10, 131, 132] and dietary supplements [133-135].

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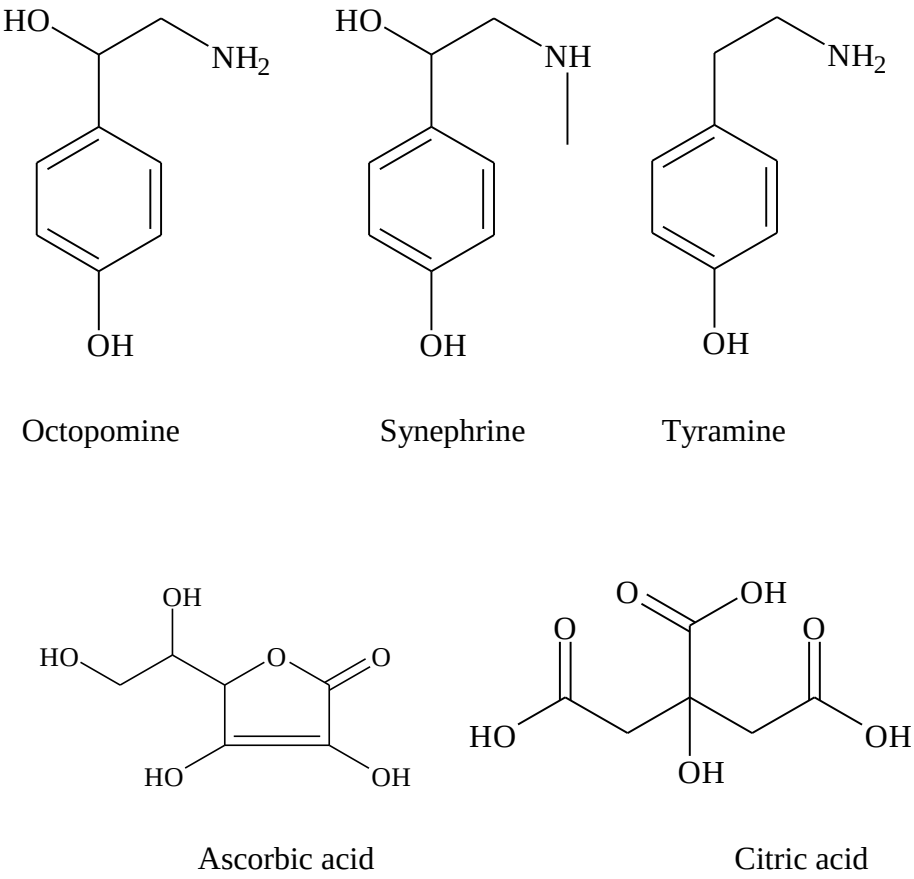

Fig. 1. Structures of amines and organic acids.

amines present in other citrus species have been reported. In a report evaluating the content of amines in orange juices, an ion pair agent was used for separation using a μBondapak C_{18} column [131]. The juice sample was initially purified on a C_{18} reverse-phase (Sep-Pak) cartridges, followed by separations of synephrine and octopomine using an isocratic elution of 0.1 M acetate buffer and acetonitrile (91:9) with UV detection of 275 nm. Tyramine and other amines were analyzed separately using a gradient of 0.1 M acetate buffer and acetonitrile with a run time of 71 minutes. Use of dual methods for separation of amines is time consuming and may not be economically viable. A 'green'

HPLC technique for analysis of octopomine, synephrine and tyramine using ionic mobile phase was reported by Tang [80]. While this method enabled separation of amines, use of pyridinium and methylimidazolium salts in the mobile phase for routine purpose may not be safe due to their toxicity [132]. Moreover, due to poor retention of amines on the column, buffers have been commonly used in mobile phase for separation of amines [133-135]. Recently, Pellati [136] analyzed aqueous extract of sour orange sample using a pentafluorophenylpropyl stationary phase without clear baseline separation between synephrine and tyramine.

Similarly, several methods were described for quantification of organic acids using different transduction systems such as spectrophotometer [137, 138], colorimeter [13, 14], and HPLC [139-142]. However, a simultaneous method with separation of both amines and organic acids would be of vital interest to monitor quality control in citrus processing industry. Quantification of these quality parameters may also enhance the consumer preference for citrus consumption. Prior research in our lab has demonstrated efficient HPLC quantification methods of citrus bioactives such as limonoids and glucaric acid [143-145]. To the best of our knowledge, currently an economical simultaneous technique for the extraction and separation of amines and organic acids is not available. Moreover, a simultaneous method will be less time consuming and economical.

Since both amines and organic acids are highly polar, we hypothesize that the use of a polar solvent would enable in simultaneous extraction. The objective of this study

was to develop a rapid HPLC method for the separation and determination of amines and organic acids in citrus fruits.

3.1 Materials and methods

3.1.1 Reagents and standards

Octopomine, synephrine, tyramine, citric acid, and HPLC grade phosphoric acid were purchased from Sigma-Aldrich (St. Louis, MO). L-Ascorbic acid was purchased from Mallinckrodt (Paris, KY). Nanopure water (NANOpure, Barnstead/Thermolyne, Dubuque, IA) was used for the sample preparation and HPLC analysis. The standard amines such as octopomine, synephrine, and tyramine were prepared in nanopure water to obtain 1 mg/mL stock solution. Citric acid was also dissolved in nanopure water. All the standards were sonicated for 30 s and serial dilutions were made with nanopure water. Ascorbic acid was prepared in 3% meta-phosphoric acid.

3.1.2 Fruit samples

Six different species and two varieties of citrus fruits such as Marrs sweet orange (*Citrus sinensis* Tan.), Rio Red (*C. paradisi* Macf.), Red fleshed pummelo (*C. grandis* Tan.), Meyer lemon (*C. limon* Tan.), Nova tangerine (*C. reticulata* Tan.), Ugli tangelo (*C. reticulata* x *C. paradisi*), and Wekiwa tangelo (*C. reticulata* x *C. paradisi*), were

harvested in the month of November 2008 from Texas A&M-Kingsville Citrus Center (Weslaco, TX). Clementine fruits (*C. clementina*) were harvested in the month of November 2008 from Placer County, CA.

3.1.3 Instrumentation

The HPLC system consisted of a Waters 1525 HPLC series (Milford, MA, USA) connected to a Waters 2996 PDA detector and Waters 717 autosampler. The columns evaluated for optimum separations of amines and organic acids were Xbridge C_{18} (3.5 µm, 4.6 mm × 150 mm i.d.) from Waters (Milford, MA, USA), Gemini C_{18} (5 µm, 250 mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA), and Luna C_{18} (5 µm, 250 mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA). An isocratic mobile phase of 3 mM phosphoric acid was used at a flow rate of 1.0 mL/min. The sample injection volume for the analysis of amines and organic acids was 10 µL. The amines and organic acids were detected at 223 nm and 254 nm respectively. Chromatographic data was collected and processed using Empower2 software (Waters-Milford, MA).

3.1.4 Sample preparation

Fruit samples of clementine mandarins and meyer lemons were peeled, blended for 3 min and homogenized for 30 s using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA). Two solvents such as water and 3% meta phosphoric acid

were used to optimize extraction efficiency of amines and organic acids. An aliquot of 10 mL of homogenized juice sample was diluted with 30 mL of water in a centrifuge tube and mixed for 15 min. Three milliliters of diluted sample was filtered under vacuum using a 0.45 µm membrane filter (Millipore Corp., Bedford, MA, USA). The residue was re-extracted with 1 mL of solvent and filtered. The procedure was repeated for another two times using 1mL of solvent each time. Filtrate from all the extractions were pooled and 10 µL was injected to HPLC for analysis. Similarly, four extractions were performed using 3 % meta phosphoric acid and analyzed by HPLC. The above extraction was conducted at 4 °C using an ice bath to prevent degradation of ascorbic acid. The sample extracts were stored at -80º C until analyzed.

3.1.5 Recovery and repeatability

To validate the sample preparation procedure, recovery studies were performed by adding known concentration of standard mixture of amines and organic acids to meyer lemon and clementine mandarin juice samples. These two species were selected based on high and low concentrations of the analyzed compounds present naturally. Meyer lemon juice (10 mL) was fortified by adding 0.40 mg of octopomine, 0.50 mg of synephrine, 0.2 mg of tyramine, 120 mg of citric acid and 0.25 mg of ascorbic acid. To evaluate the dependence of recovery on the concentration, Clementine mandarin juice was fortified with two different concentration levels of standard amines and organic acids. A 10 mL aliquot of clementine mandarin juice was fortified with 1.00 mg of

octopomine, 1.00 mg of synephrine, 0.40 mg of tyramine, 0.50 mg of ascorbic acid and 260 mg of citric acid. Similarly, for evaluation of different level of concentration, 0.50 mg of octopomine, 0.50 mg of synephrine, 0.20 mg of tyramine, 0.25 mg of ascorbic acid and 130 mg of citric acid was added to 10 mL of Clementine mandarin juice. After addition of standards to the respective juice samples, the volume was made up to 40 mL by adding 3% meta-phosphoric acid and extracted using the resultant optimized extraction procedure and analyzed by HPLC. This analysis was evaluated on a different day using a different set of samples. Repeatability was expressed as the relative standard deviation (% RSD) and was determined by repeating the extraction procedure and analysis five times.

3.1.6 Precision and ruggedness

The precision of the HPLC system was determined by evaluating inter-day and intra-day injections of standard amines and organic acids. Six injections were performed for each day within three consecutive days. The % RSD of the retention times was evaluated for all the injections. The ruggedness of the analytical method was evaluated by varying the HPLC systems and keeping all the other parameters such as column: Xbridge C_{18} column (3.5 µm, 4.6 mm X 150 mm i.d.), mobile phase (0.03 mM phosphoric acid), flow rate (1 mL/min) and detection constant. The two HPLC systems Waters 1525 HPLC series (Milford, MA, USA) and Agilent 1200 Series (Foster City,

CA, USA) were evaluated for the separation of amines and organic acids using the developed method.

3.1.7 Quantification of amines and organic acids in citrus samples

An aliquot of 10 µL microlitres of each sample was injected onto HPLC for the analysis of amines and organic acids. The elution and quantification of the target compounds was carried out using the optimized method. The concentration of the respective compound was calculated using the regression equation and dilution factor. The concentration of amines and ascorbic acid are represented as µg mL^{-1}, and citric acid is expressed as mg mL^{-1} of juice.

3.1.8 Mass spectral analysis

The individual peaks were collected from HPLC and subjected to mass spectral analysis. The analyses of octopomine, synephrine and citric acid were performed on MDS-Sciex QSTAR Pulsar*i*quadrupole-time-of-flight (QqTOF) mass spectrometer (Toronto, Ontario, Canada). Analysis were performed under following conditions; collision gas: nitrogen, curtain gas: 20 psi, ion spray voltage: 4500 V, declustering potential: 10 V, focusing potential: 220 V, second declustering potential10 V, ion release delay: 11 µs, ion release width: 10 µs, resolution ion energy: 1 V, detector (MCP): 2150 V, and syringe pump flow: 7 µL min^{-1}. Mass spectral analysis of tyramine and ascorbic

acid was performed on LCQ™ Deca (Thermoscientific) ion trap mass spectrometer. Ionization was done using the atmospheric chemical ionization (APCI) source. The source heater temperature was set at 450 ºC, sheath gas flow was maintained at 80 units and auxiliary gas flow was set to 10 units, The discharge current: 4.5 µA, capillary temperature: 150º C, capillary voltage: 46 V, and tube lens offset was 10 V. Amines and ascorbic acid were analyzed by positive mode and citric acid was analyzed in negative mode.

3.1.9 Statistical analysis

Data was analyzed using the General Linear Model (GLM) procedure with the Walter-Duncan K-ratio t-test (SAS, 2007). The analysis of variance differentiates the means by assigning different letters to the treatment means that are significantly different at the 95% level of probability ($P \leq 0.05$). The tests of linearity for the calibration equations and the P-P plots were determined by using regression function in PASW Statistics 18, Version 18.0.0 (SPSS, Inc., Chicago, IL).

3.2 Results and discussion

3.2.1 Method development

Three C_{18} columns such as Xbridge , Gemini, and Luna in combination with organic solvents (acetonitrile, methanol) and modifiers (phosphoric acid, trichloroacetic acid, acetic acid) were evaluated for rapid separation of amines and organic acids in citrus juice. Due to the poor retention of the compounds on the column because of their high polarity, water seemed to be more ideal mobile phase. Use of acetonitrile, methanol and modifiers such as trichloroacetic acid and acetic acid did not yield optimum separations. In early trials, using water as a mobile phase resulted in peak tailing and poor separation of the compounds. The peak tailing may be due to the interaction between amines and the silanols on the surface of stationary phase [80]. Using (3 mM) phosphoric acid as a modifier coupled with a wide range of pH compatible Xbridge column reduced the peak tailing and clear separations of amines and organic acids were observed (Fig. 2).

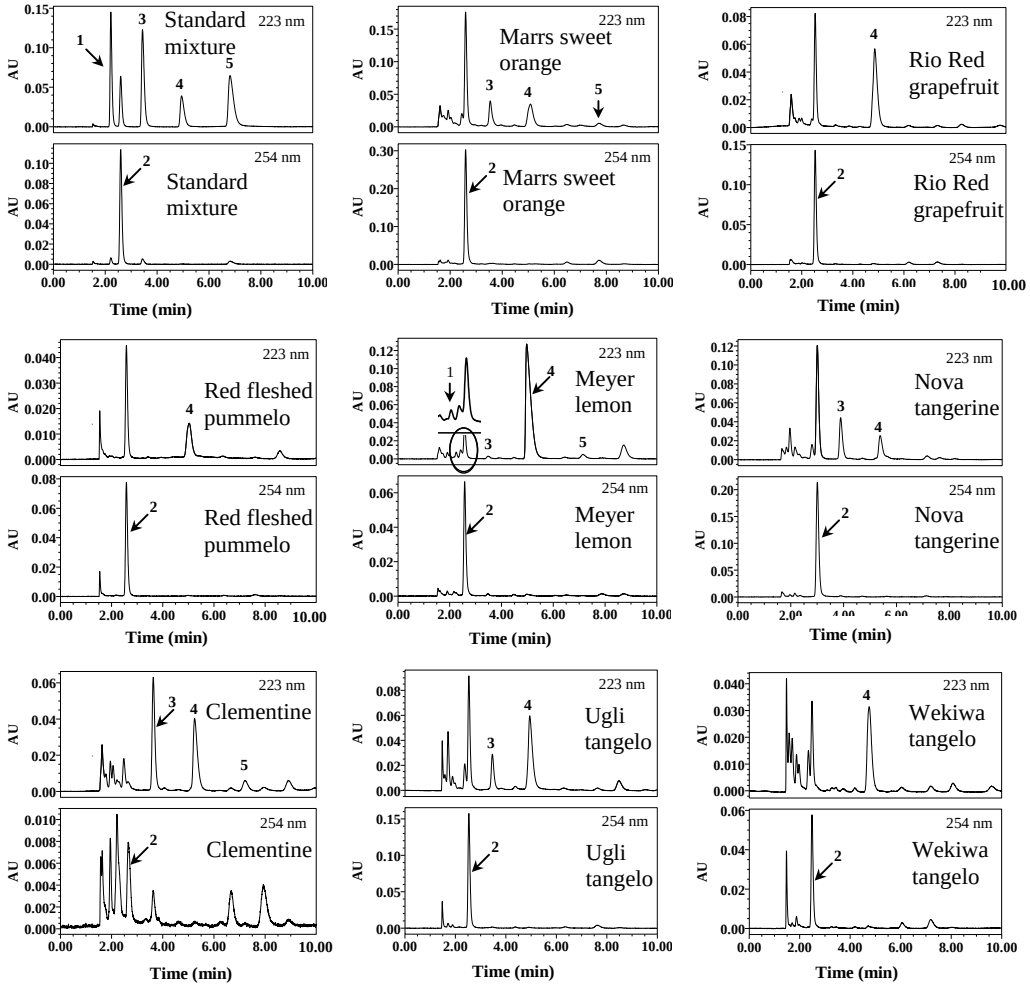

Fig. 2. HPLC chromatograms of standard mixture of amines and organic acids as well as different citrus species juice (Peak identification: 1, (±)-octopomine; 2, ascorbic acid; 3, (±)-synephrine 4, citric acid 5, tyramine: Detection, PDA at 223 & 254nm).

3.2.2 Sample extraction procedure

Homogenized Clementine mandarin and Meyer lemon juice was extracted with water and compared with 3% meta-phosphoric acid extract to determine the optimum extraction procedure for simultaneous analysis of amines and organic acids. In Clementine mandarin juice, synephrine, tyramine, ascorbic acid and citric acid were detected. The extraction efficiency for octopomine was determined using Meyer lemon juice. Sample extraction with 3% meta-phosphoric acid resulted significantly higher content of synephrine, tyramine, ascorbic acid and citric acid in comparison with water. No significant difference in the octopomine content was noticed between 3% meta-phosphoric acid and water extraction. Therefore, 3% meta-phosphoric acid seems to be an ideal solvent for simultaneous extraction of both amines and organic acids (Fig. 3). Previous analytical methods suggest, water as an ideal solvent for optimum extraction of amines [136, 146]. For optimum simultaneous extraction of organic acids, water as a solvent is a limiting factor since ascorbic acid is highly unstable and requires acidic medium for stability [147]. Although both amines and organic acids are soluble in 3% meta-phosphoric acid, the dense matrix of citrus juice limits optimum extraction in a single step. Results from monitoring successive extractions of the unfiltered residue of the juice suggest that re-extraction of the residue with 3 mL of 3% meta-phosphoric acid was optimum for complete extraction of amines and organic acids (Fig. 4).

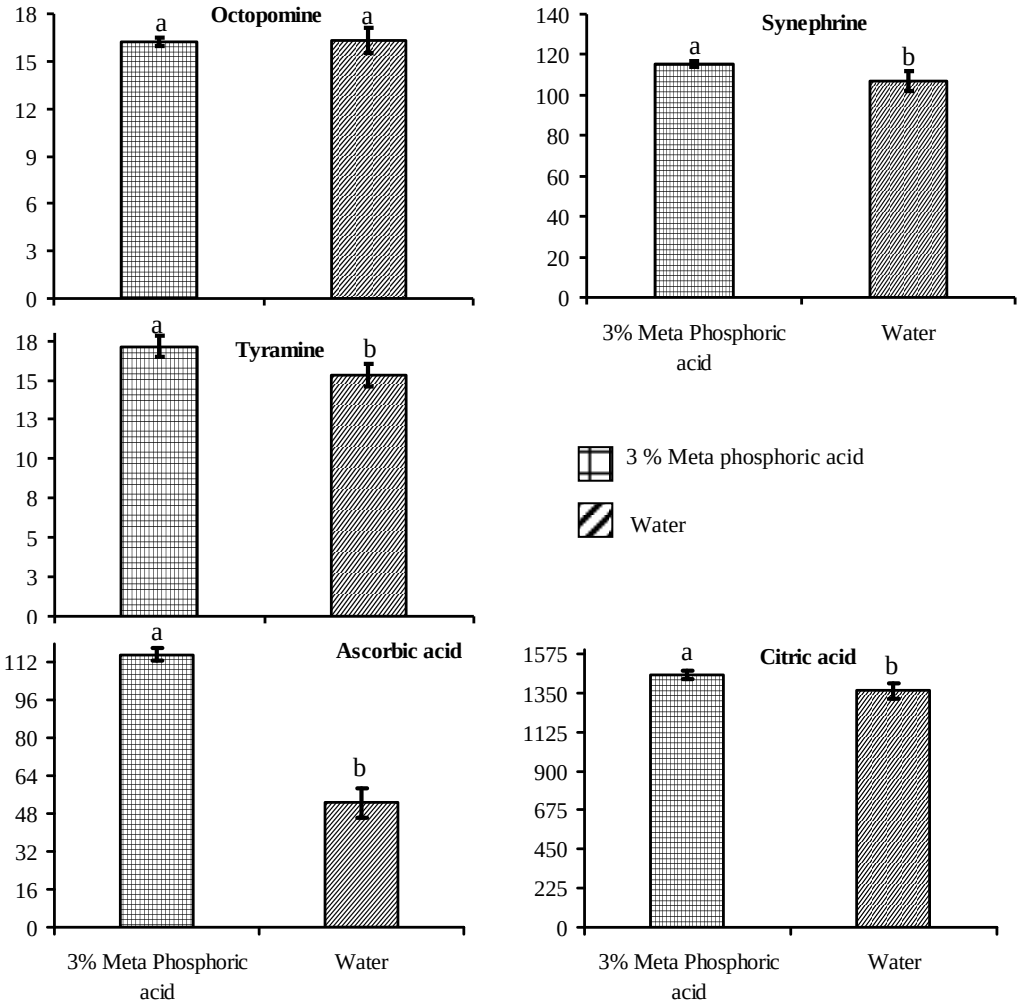

Fig. 3. Extraction of amines and organic acids by two solvents. Octopomine and ascorbic acid were quantified in Meyer lemon juice. Synephrine, tyramine and citric acid were quantified in Clementine mandarin juice. Different letters indicate significant differences at $P<0.05$ and similar letters indicate no significant differences $P<0.05$.

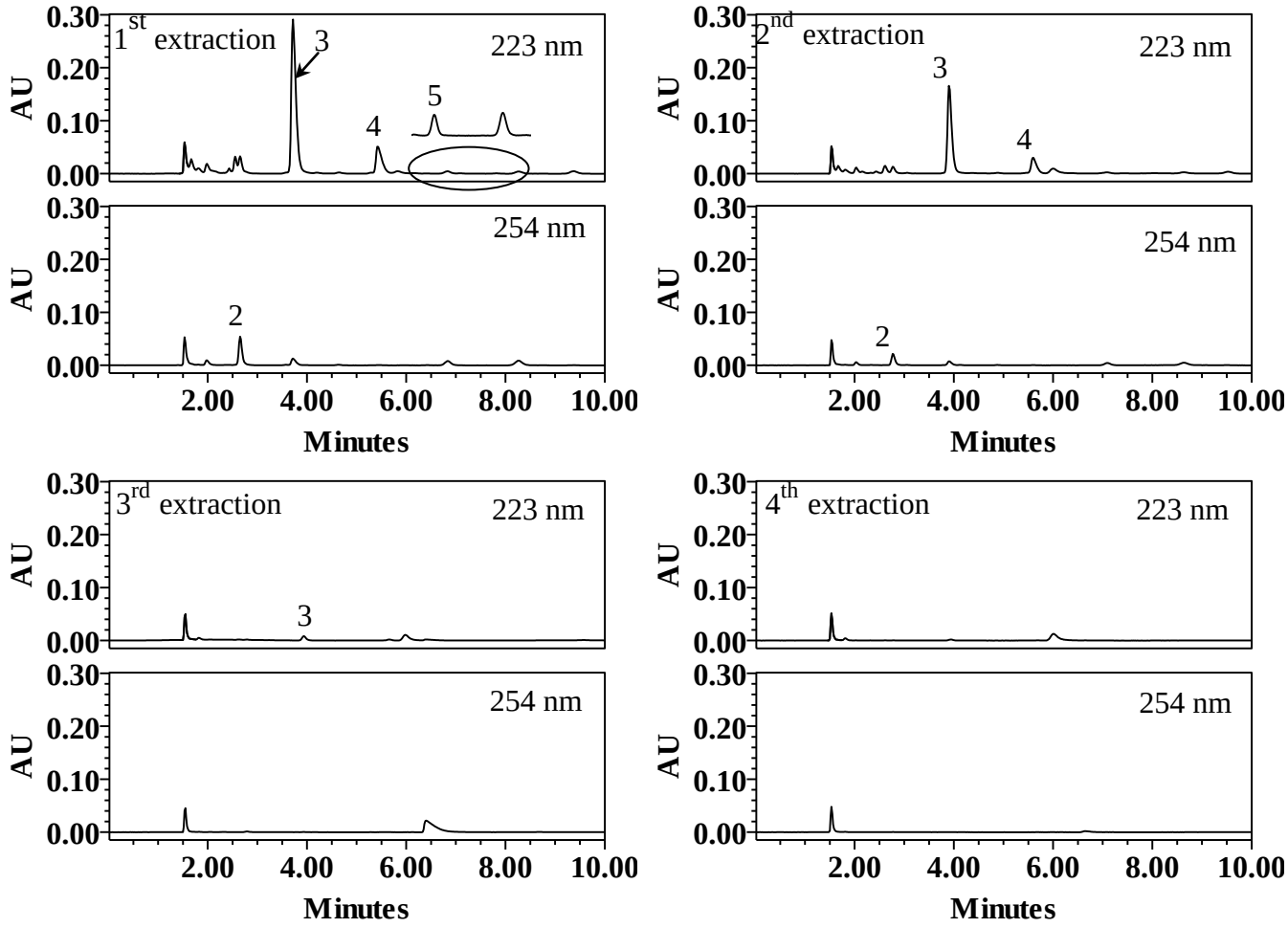

Fig.4. HPLC chromatograms of Clementine mandarin juice extract samples analyzed after repeated extractions using 3 % meta phosphoric acid. The chromatograms demonstrate that three repeated extractions are ideal for complete extraction of amines and organic acids. Peak identification (2) L- ascorbic acid; (3) (±)-synephrine; (4) citric acid; (5) tyramine.

3.3 Method validation

3.3.1 Linearity, LOD and LOQ

Linear curves for all the standards (octopomine, synephrine, tyramine, ascorbic acid) were prepared using six concentrations ranging from 9.8 ng - 312.5 ng and citric acid ranging 1.25 - 40 µg. with triplicate injections. The linear curves were obtained by plotting the standard concentration as a function of peak area obtained from HPLC analysis (Fig. 5). Good linear relationship and correlation coefficients were observed with their peak area responses. The correlation coefficient (R^2) of amines and organic acids were found to be > 0.9992 (Table 1). The residual plots corresponding to the respective compound linearity plot indicated random distribution of residuals (Fig. 6). Similarly, normal probability plots (P-P plots) were approximately linear for all the calibrations of the analyzed compounds (Fig. 7). The t-test ($P < 0.05$) also confirmed that there was no statistically significant difference in the predicted and observed values. The limit of detection or sensitivity was measured by injecting serial diluted standard solutions, considering the signal-to-noise ratio (3:1). The limit of quantification (LOQ) was determined as the lowest concentration which can be determined with an accuracy and precision of >95%. The LOD for the amines as well as ascorbic acid was determined as 5 ng while 63 µg was for citric acid. The LOQ for amines and ascorbic acid was determined to be 9.8 ng while 125 µg was for citric acid. The low LOD and LOQ values confirm that the method developed was sensitive to detect and quantify samples.

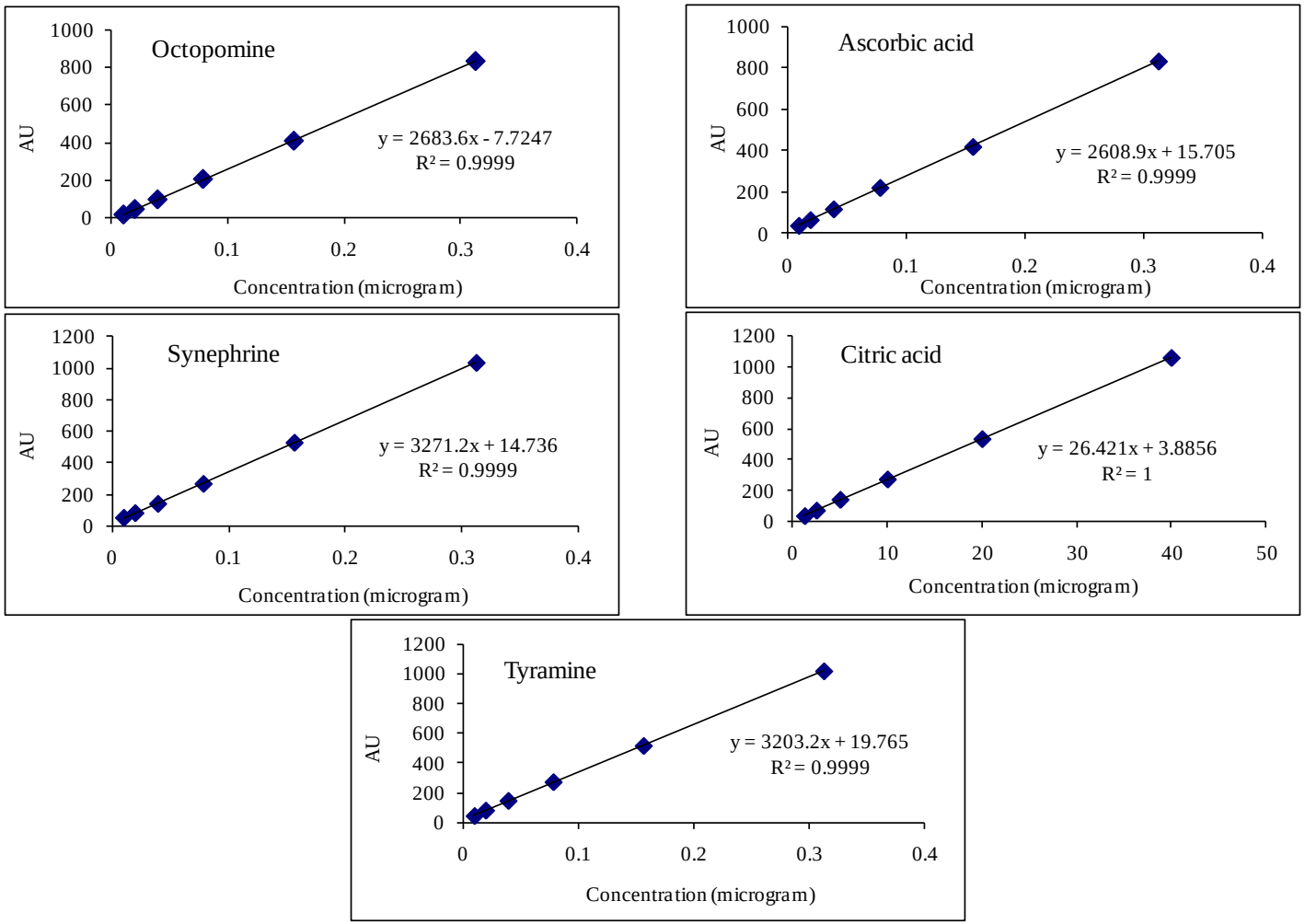

Fig. 5. Linear regression and correlation statistics of the analyzed compounds.

Table 1. Linear ranges, coefficient of determination (R^2) limit of quantification (LOQ) and limit of detection (LOD) of the amines and organic acids.

Compounds	Regression equation	R^2	Linear range (ng)	LOQ (ng)	LOD (ng)
Octopomine	y = 2683.6x - 7.7247	0.9999	9.8 - 312.5	9.8	5
Synephrine	y = 3271.2x + 14.736	0.9999	9.8 - 312.5	9.8	5
Tyramine	y = 3203.2x + 19.765	0.9999	9.8 - 312.5	9.8	5
Ascorbic acid	y = 2608.9x + 15.705	0.9999	9.8 - 312.5	9.8	5
Citric acid*	y = 26.421x + 3.8856	1.0000	1.25 - 40.0*	1.25*	0.63*

x = concentration of the respective compounds
y = peak area (AU)

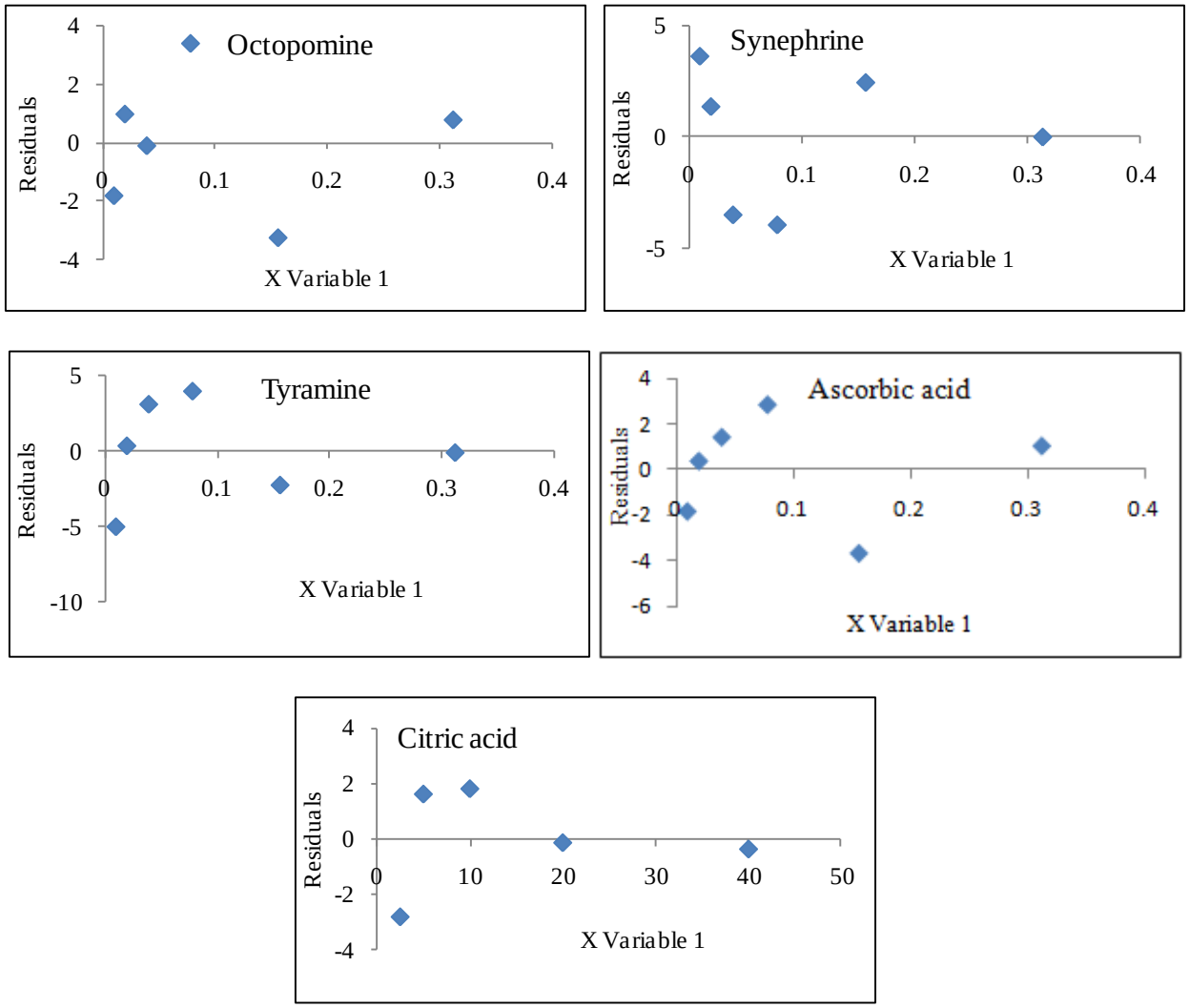

Fig. 6. Residual plots of corresponding linear calibrations of the analyzed compounds.

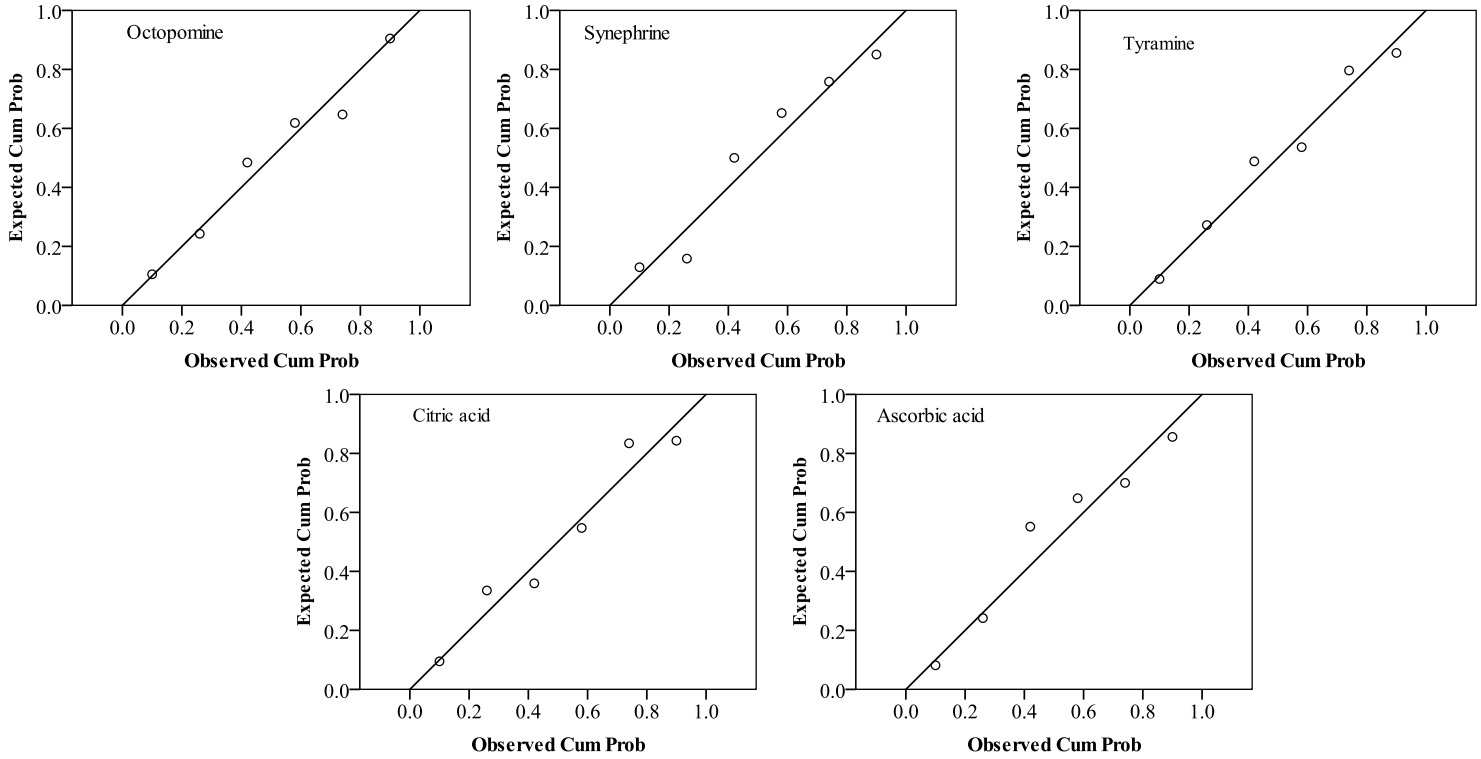

Fig. 7. Normal probability P-P plots of corresponding linear calibrations of the analyzed compounds.

containing low concentrations of amines and organic acids. Peaks from the sample were identified by comparing the UV spectra and retention time (t_R) with those obtained from the individual standard samples. The results were confirmed by spiking the sample with standards for detection of peak enhancement.

3.3.2 Recovery and repeatability

To evaluate the recovery test, known concentration of standard solutions was added to the Clementine mandarin and Meyer lemon juice. This fortified sample mixture was extracted and analyzed by the optimized HPLC method. Results obtained from the recovery analysis are summarized in Table 2. The mean recovery of the analytes was compared with the actual quantity of the analytes present in the sample. The recovery percentage for the analytes ranged between 84.01-117.28%, indicating the reliability and accuracy of the developed method. The %RSD for the recovery of all the amines ranged between 0.50 and 4.56. Among the analyzed organic acids, citric acid had a low %RSD of 0.16 in the recovery analysis for Meyer lemon juice, whereas %RSD of ascorbic acid ranged in between 10.56 and 1.05 for recovery analysis in Meyer lemon and Clementine, respectively. The low %RSD obtained in the recovery of standard amines and organic acids evaluated in two different concentrations added to the Clementine mandarin juice further validate the sample extraction procedure.

Repeatability of the extraction procedure was determined by repeating the extraction procedure five times using the same Clementine sample and analyzed by

Table 2. Recovery studies of amines and organic acids from citrus juices[a].

Variety Compound	Actual amount present in the juice sample (mg 10 mL^{-1})	Amount of standard added to the sample (mg 10 mL^{-1})	Amount expected (mg 10 mL^{-1})	Amount determined (mg 10 mL^{-1})	Recovery (%)	RSD[b] (%)
Meyer Lemon						
Octopomine	0.16 ± 0.01	0.40	0.56	0.55	98.71	3.03
Synephrine	0.01 ± 0.00	0.50	0.51	0.46	90.89	3.30
Tyramine	0.10 ± 0.00	0.20	0.30	0.31	102.24	1.90
Ascorbic acid	0.88 ± 0.01	0.25	1.13	0.95	84.01	10.56
Citric acid	598.99 ± 5.38	120.00	718.99	700.00	97.57	0.16
Clementine						
Octopomine	ND	1.00	1.00	0.86	86.24	1.01
Synephrine	1.24 ± 0.04	1.00	2.24	1.99	88.63	0.50
Tyramine	0.15 ± 0.01	0.40	0.59	0.55	93.21	4.25
Ascorbic acid	0.19 ± 0.01	0.50	0.70	0.59	90.45	4.46
Citric acid	142.58 ± 3.38	260.00	402.58	472.15	117.28	1.73
Octopomine	ND	0.50	0.50	0.48	96.82	0.89
Synephrine	1.24 ± 0.04	0.50	1.74	1.66	95.41	1.45
Tyramine	0.15 ± 0.01	0.20	0.39	0.57	93.33	3.72
Ascorbic acid	0.19 ± 0.01	0.25	0.40	0.37	92.71	1.05
Citric acid	142.58 ± 3.38	130.00	267.58	311.57	114.30	0.41

[a]Results are mean ± standard deviation values of three replications of each sample.
[b]RSD (%) = Relative standard deviation; (standard deviation / mean) × 100.
ND: Not detected

HPLC. The RSD (%) values for synephrine, tyramine, ascorbic acid and citric acid were determined to be 1.22, 3.73, 1.74 and 9.94 respectively. The high RSD (9.94%) for citric acid could be due to its presence in high concentration in samples. Further diluting the sample was not ideal since octopomine and tyramine could not be detected due to low concentrations. The low RSD (%) of values for other compounds demonstrated good repeatability. Thus, the method could be used for quantification of both high concentrations of amines and organic acids.

3.3.3 Precision and ruggedness

The precision of the HPLC system was determined by evaluating inter-day and intra-day injections of standard solution consisting of octopomine, synephrine, tyramine, citric acid and ascorbic acid (Table 3). The RSD of the retention times for intra-day ranged in between 0.5% and 1% for all the compounds and the inter-day variation ranged in between 1.2% and 3.5%.

The ruggedness of the present analytical method was evaluated by varying the HPLC systems without changing sample extraction procedure (Table 4). We did not observe any change in the resolution of the peaks evaluated for the same column**.** For both of the HPLC systems tested, the RSD (%) were calculated to range between 0.15 to 1.14%. The results from the tests of precision and ruggedness demonstrate that the method is precise and rugged and could be used for analysis of commercial samples.

Table 3**.** Intra-day and inter-day variation for retention time of amines and organic acid.

Compound	Intra-day precision[a]						Inter-day precision[b]	
	Day 1		Day 2		Day 3			
	tR (min)	RSD (%)[c]	tR (min)	RSD (%)[c]	tR (min)	RSD (%)[c]	tR (min)	RSD (%)[c]
Octopomine	2.16	0.9	2.21	0.9	2.18	0.5	2.19	1.2
Ascorbic acid	2.50	1.1	2.59	1.1	2.62	0.7	2.56	3.5
Synephrine	3.32	0.9	3.44	0.9	3.39	0.7	3.38	1.8
Citric acid	4.75	1.0	4.96	1.0	4.84	0.6	4.85	2.2
Tyramine	6.52	1.0	6.83	1.0	6.73	0.7	6.69	2.3

[a]Results are mean values of four separate injections of standard sample within each day. [b]Results are mean values of injections of standard sample in three consecutive days. [c]RSD (%) = Relative standard deviation; (standard deviation / mean) × 100.

Table 4. Retention (t_R) time's and RSD (%) of amines and organic acids for ruggedness[a].

Compound	Waters 1525 Xbridge		Agilent 1200 Xbridge	
	tR (min)	RSD (%)[b]	tR (min)	RSD (%)[b]
Octopomine	2.16	0.21	1.95	0.21
Ascorbic acid	2.45	0.19	2.17	0.28
Synephrine	3.32	0.21	2.84	0.48
Citric acid	4.74	0.40	3.83	0.91
Tyramine	6.52	0.55	5.38	1.14

[a]Results are mean values of five separate injections of sample for each individual HPLC system. [b]RSD (%) = Relative standard deviation; (standard deviation / mean) × 100.

3.3.4 Analysis of citrus fruits samples

The developed optimized method was used for quantification of amines and organic acids in six different species and two varieties of citrus. All the samples were extracted and analyzed in triplicate. The HPLC chromatograms of the analyzed citrus species are presented in Fig. 2. Table 5 demonstrates variation of amines and organic acids among citrus species. Octopomine was detected only in the Meyer lemon (16.29 μg mL^{-1}). Synephrine was the predominant amine in most of the analyzed citrus species, but was not detected in grapefruit, pummelo and Wekiwa tangelo. Clementine mandarin had the highest content (114 μg mL^{-1}) of synephrine while Meyer lemon had the lowest content (2.75 μg mL^{-1}). In both grapefruit and red fleshed pummelo, amines were not detected. However, it was interesting to note the presence of synephrine in Ugli tangelo variety (46.88 μg mL^{-1}) and its absence in Wekiwa tangelo. Tangelos are a hybrid between tangerine (*C. nobilis var*. deliciosa) and grapefruit (*C. paradisi*). The absence of synephrine in Wekiwa tangelo may be due to hereditary characteristic of the parent crosses. Wekiwa tangelo is a cross between grapefruit and Sampson tangelo [148], and based on our analysis amines were not detected in grapefruits. Tyramine was detected in Clementine (17.0 μg mL^{-1}), Marrs sweet orange (4.82 μg mL^{-1}) and Meyer lemon (9.22 μg mL^{-1}). Among organic acids, citric acid was the predominant of the two with the high concentration determined in Meyer lemon (52.94 mg mL^{-1}), which is characteristic of acidic fruits. Pummelo had low citric acid content (5.44 mg mL^{-1}) which seems to be the

Table 5. Content of amines and organic acids in eight citrus juice samples.

Species (common name)	Octopomine	Synephrine	Tyramine	Ascorbic acid	Citric acid
	($\mu g\ mL^{-1}$) [a]				($mg\ mL^{-1}$) [a]
C. sinensis Tan. (Marrs sweet orange)	ND	85.17 ± 2.69	4.82 ± 2.87	565.21 ± 6.90	15.28 ± 0.34
C. paradisi Macf. (Rio Red grapefruit)	ND	ND	ND	250.82 ± 6.27	21.89 ± 1.89
C. grandis Tan. (Red fleshed pummelo)	ND	ND	ND	137.16 ± 1.90	5.44 ± 0.14
C. limon Tan. (Meyer lemon)	16.29 ± 0.26	2.75 ± 0.60	9.22 ± 0.44	115.23 ± 2.81	52.94 ± 1.11
C. reticulata Tan. (Nova tangerine)	ND	78.28 ± 6.36	ND	363.60 ± 4.23	7.31 ± 0.43
C. clementina Tan. (Clementine)	ND	114.61 ± 2.89	17.00 ± 0.72	16.08 ± 3.06	14.42 ± 0.47
C. reticulata X *C. paradisi* (Ugli Tangelo)	ND	46.88 ± 5.78	ND	262.32 ± 0.26	19.92 ± 1.12
C. reticulata X *C. paradisi* (Wekiwa Tangelo)	ND	ND	ND	95.97 ± 1.98	11.66 ± 0.17

[a] Data presented are mean ± standard deviation values of three replications of each sample.
ND: Not detected

less tart variety among the analyzed citrus species. Marrs sweet orange had high ascorbic acid content (565.21 µg mL^{-1}) followed by Nova tangerine (363.60 µg mL^{-1}).

3.3.5 Mass spectral analysis

The identity of pure peaks collected from HPLC peaks were confirmed by the mass spectral analyses (Fig. 8). The mass spectrum of octopomine shows a molecular ion $[M+H]^+$ at *m/z* 154.08, an intense adduct ion $[M+H-H_2O]+$ at *m/z* 136.07. Synephrine generated molecular ion $[M+H]^+$ at *m/z* 168.10 and prominent product ions as a result of loss of H_2O, $[M+H-H_2O]^+$. Tyramine generated molecular ion $[M+H]^+$ at *m/z* 138.03 and an intense adduct by the loss of NH_3, $[M+H-NH_3]^+$ at *m/z* 121.21 from protonated tyramine molecule. The mass spectra of ascorbic acid and citric acid shows a molecular ion $[M+H]^+$ at *m/z* 177.0, and $[M-H]^+$ at *m/z* 191.07 respectively.

3.4 Conclusion

For the first time, a rapid simultaneous separation as well as determination of amines and organic acids in citrus juice was achieved. The developed HPLC method demonstrates that, 3% meta phosphoric acid can be used for simultaneous extraction of organic acids and amines. The method is precise and rugged combined with high recovery and repeatability. The simultaneous extraction and analysis of samples provides an economical method for analysis of large number of samples in short duration of time.

Thus, this method has potential of being applied as an analytical technique for quality control in citrus fruits processing industries.

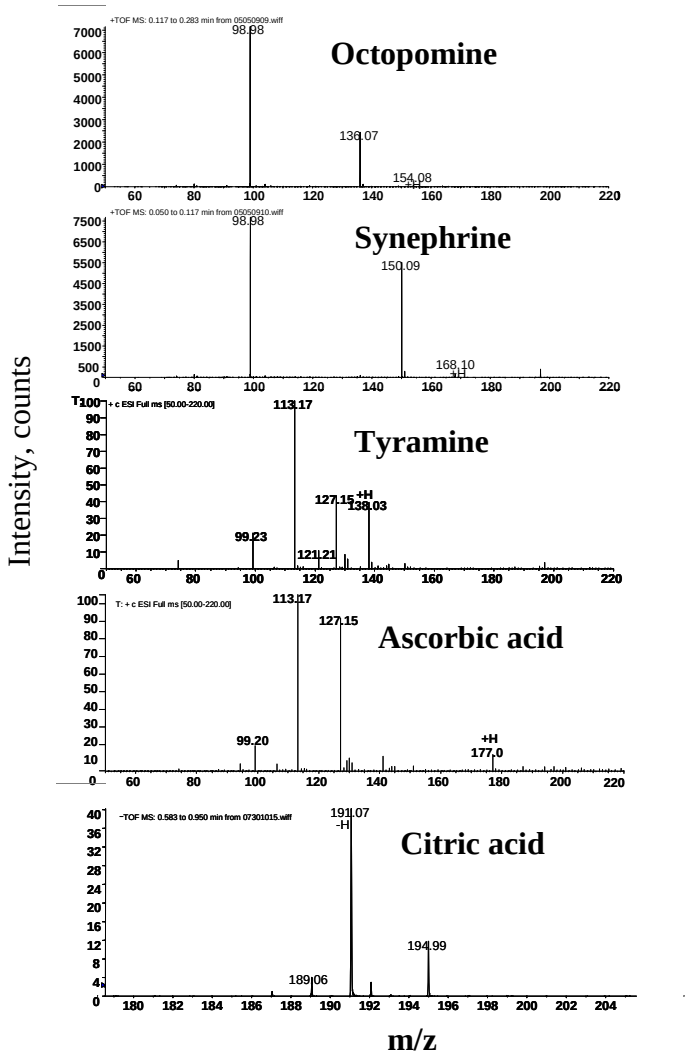

Fig. 8. Mass spectra of amines and organic acids. Each compound was collected from HPLC and analyzed by mass spectra.

4. RAPID SEPARATION METHOD OF POLYMETHOXYFLAVONES FROM CITRUS USING FLASH CHROMATOGRAPHY*

Citrus fruits contain several bioactive compounds such as flavonoids, limonoids, carotenoids, amines, organic acids, sterols and furocoumarins [31, 37, 96, 149-152]. Among these, polymethoxyflavones (PMFs) a group of flavonoids are unique to citrus species and are present mainly in the fruit peel. These compounds have two or more methoxyl's on their basic flavonoid structure. During recent years, PMFs have been demonstrated for multiple benefits. They are an important component of plant defensive mechanism against various disease causing pathogens [77]. They are known to occur in varying proportions in different citrus species. Hence, are used as marker compounds for detection of adulteration in citrus juices [153]. They also contribute towards the taste attribute of citrus juice with an estimated taste threshold level ranging from 15 ppm to 46 ppm [154]. Due to their role in quality control of citrus juices, they were extensively studied for development of methods for quantification in citrus juices and its byproducts [81, 89, 144, 155-157].Several studies have demonstrated the role of PMFs in human health perspective as anti-inflammatory [52, 158], anti-carcinogenic [49, 159], anti-fungal [44] and potent inhibitory agents of P-glycoprotein [160, 161].

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Considering the potential use of these bioactive compounds as a chemopreventive agent based on *in vitro* studies [49], large quantities of these compounds are required to conduct further *in vivo* experiments both in animals and humans. Separation and purification of PMFs is challenging due to the complexity of plant materials. Several strategies have been reported for the separation of these compounds using various methodologies. Among them, combination of column chromatography and preparative-high performance liquid chromatography (Prep-HPLC) was commonly used for the separation of PMFs [63, 162, 163]. Although column chromatography using silica gel is traditionally used for separation of bioactive components, the main disadvantage of this method are, it is time consuming, laborious and requires large volumes of solvents. Furthermore, a scalable method was proposed using a combination of normal phase chromatography and super critical fluid chromatography (SFC) for separation of PMFs from orange peel extracts (cold pressed oil) [66]. The extract was initially fractionated using a FC and followed by SFC separation of the fractions which had high PMFs concentration. The use of high capital requirement for the instruments may not be a viable economical strategy. In an another report, PMFs were isolated from tangerine peels in milligrams quantity using high speed counter current chromatography (HSCCC) with high purity [83]. The method was limited by the time consumed for each separation which was 5.5 h. In a recent report [64], leaves of Dancy tangerine were used as a source for separation of PMFs using a combination of vacuum flash silica gel chromatography and flash C_{18} column chromatography. Collecting and processing of leaves in a large

quantity could be cumbersome, as well as not feasible as an ideal raw material for large scale separation of PMFs.

The limitations of the reported methods include high capital input, high time requirement, need for successive separations by a combination of instruments. These limitations warranted to explore an efficient rapid method for separation and purification of PMFs. Considering the need for an economically viable method to separate large quantity of PMFs, a rapid efficient separation method is critical. In this context, a study was conducted to develop a rapid flash method for separation of PMFs from citrus and confirm the identity by spectral analysis using NMR and mass spectra. To the best of our knowledge, this is the first report on separation and identification of PMFs from Cleopatra mandarin and Marrs sweet orange.

4.1 Materials and methods

4.1.1 Reagents and instrumentation

Solvents used for analysis were HPLCgrade and were obtained from Fisher Scientific (Pittsburgh, PA, USA). Nanopure water (NANOpure, Barnstead/Thermolyne, Dubuque, IA, USA) was used for HPLC analysis. The solvents used for flash chromatography were analytical grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Nobiletin and tangeretin were purchased from ChromaDex Inc. (Irvine, CA, USA). The separation of PMFs was carried out on an automated flash chromatography

system (Combiflash® Rf , Teledyne Isco, Lincoln, NE, USA). Silica gel (particle size 35–60 μm) flash columns (40 g, 220 g, and 330 g) were purchased from ISCO Inc. (RediSep® Rf ISCO Inc., Lincoln, NE, USA).

4.1.2 Plant materials

Mature fruits of *Citrus reshni* Hort. ex Tan. (Cleopatra mandarin) and *Citrus sinensis* L. Osbeck (Marrs sweet orange) were harvested in the month of November 2009 from Texas A&M University-Kingsville Citrus Center orchard (Weslaco, TX). The peels were separated and dried to obtain ≤ 5% moisture. The peels were blended to obtain (40-60 mesh size powder in a Vita-prep™ blender (Vita-Mix Corporation, Cleveland, OH, USA).

4.1.3 Selection of solvent for extraction of PMFs

To evaluate the ideal solvent for extraction of PMFs, 10 g of Marrs sweet orange peel powder was loaded to a Soxhlet type apparatus and extracted at 70 - 80º C with different solvents (600 mL) such as hexane (low polar), chloroform (medium polar) and methanol (polar) for 3 h each separately. Each solvent extraction was replicated three times. The solvent extracts were concentrated by rotary evaporator and dried. The dried extracts were dissolved in dimethyl sulfoxide (DMSO) and filtered using a 0.45 micron membrane filter and 10 μL was injected to HPLC.

4.1.4 Liquid chromatography

The HPLC system consisted of a Waters 1525 HPLC series (Milford, MA, USA) connected with a PDA detector. A Gemini C_{18} column (5 µm, 4.6 mm × 250 mm i.d.) (Phenomenex, Torrance, CA, USA) was used for the separations. A gradient mobile phase of 4% acetic acid (A) and acetonitrile (B) was used for the separations at a flow rate of 0.9 mL/min. Initially, elution was started with a gradient of 5% B followed by linear increase to 50% in 5 min, returned back to 5% in 5 min and held for 5 min. Injection volume was set at 10 µL and the PMFs were detected at 280 nm and 340 nm. Chromatographic data was collected and processed using Empower2 software (Waters, Milford, MA, USA).

4.1.5 Extraction of PMFs from citrus peels

The powdered peels of Cleopatra mandarin (1.7 Kg) and Marrs sweet orange (0.8 Kg) were extracted in a Soxhlet type apparatus using hexane for 8 h maintained at a temperature of 70 ºC to 80 ºC. The extract was concentrated by a rotary evaporator (Rotavapor RE11, Buchi, Switzerland) which yielded 44 g and 16 g of Cleopatra mandarin and Marrs sweet orange extracts, respectively. The concentrated extracts were impregnated with silica gel and subjected to flash chromatography separation.

4.1.6 Separation of PMFs by flash chromatography

The silica gel impregnated hexane extracts of Cleopatra mandarin peel (5 g) and Marrs sweet orange (5 g) were subjected to flash chromatography on a silica gel (particle size 35–60 μm) flash column 40g. The column was equilibrated with hexane for three column volumes prior to separations. PMFs were separated in 40 min gradient program of solvent A (hexane) and solvent B (acetone): 100% A held for 4 min, linearly increased to 10% B over 1 min, held for 7.5 min, linearly increased to 40% B over 3.5 min, held for 14 min, linearly increased to 100% B over 6 min and held for 2 min, then finally returned to the initial conditions and held for 2 min. The flow rate was maintained at 40 mL/min and individual fractions were collected by monitoring the eluting analytes at 254 nm and 340 nm. Two major peaks were observed (Fig. 9A) with retention time (RT) of peak A and peak B at 21 min and 24.5 min respectively. Fifteen fractions were collected for each FC separation of Cleopatra mandarin and analyzed by HPLC. The fraction numbers 8-9 (peak A) yielded compound 1 and fractions10-13 (peak B) yielded compound 2.

In FC separation of Marrs sweet orange, total of 19 fractions were collected with 3 major peaks (Fig. 9B). Fractions 12-14 (Peak B) and 15-19 (Peak C) after pooling yielded compound 2 and 3 respectively. The fractions 1-11 and the eluent collected after fraction 19 was pooled together and dried for cross column separation.

4.1.7 Separation of minor PMFs from Marrs sweet orange

The pooled fractions (Fr 1-11) and the eluent after fraction 19 were concentrated and impregnated with silica gel (particle size 35–60 μm) and subjected to flash chromatography on a 40 g column. The separation was conducted using a 33 min gradient program of hexane (solvent A) and acetone (solvent B) which was as follows: isocratic A held for 3 min, followed by linear increase to 100% B over 26 min and held for 4 min. Individual peaks were monitored by the online detector set at 254 nm and 340 nm. Two major peaks (Peak A and Peak D) were observed and pooled based on HPLC analysis (Fig. 10). Further concentration of the pooled fractions of peak A and peak D resulted in crystallization of compound 1 and 4 respectively. The thin colorless crystals were washed with hexane and collected.

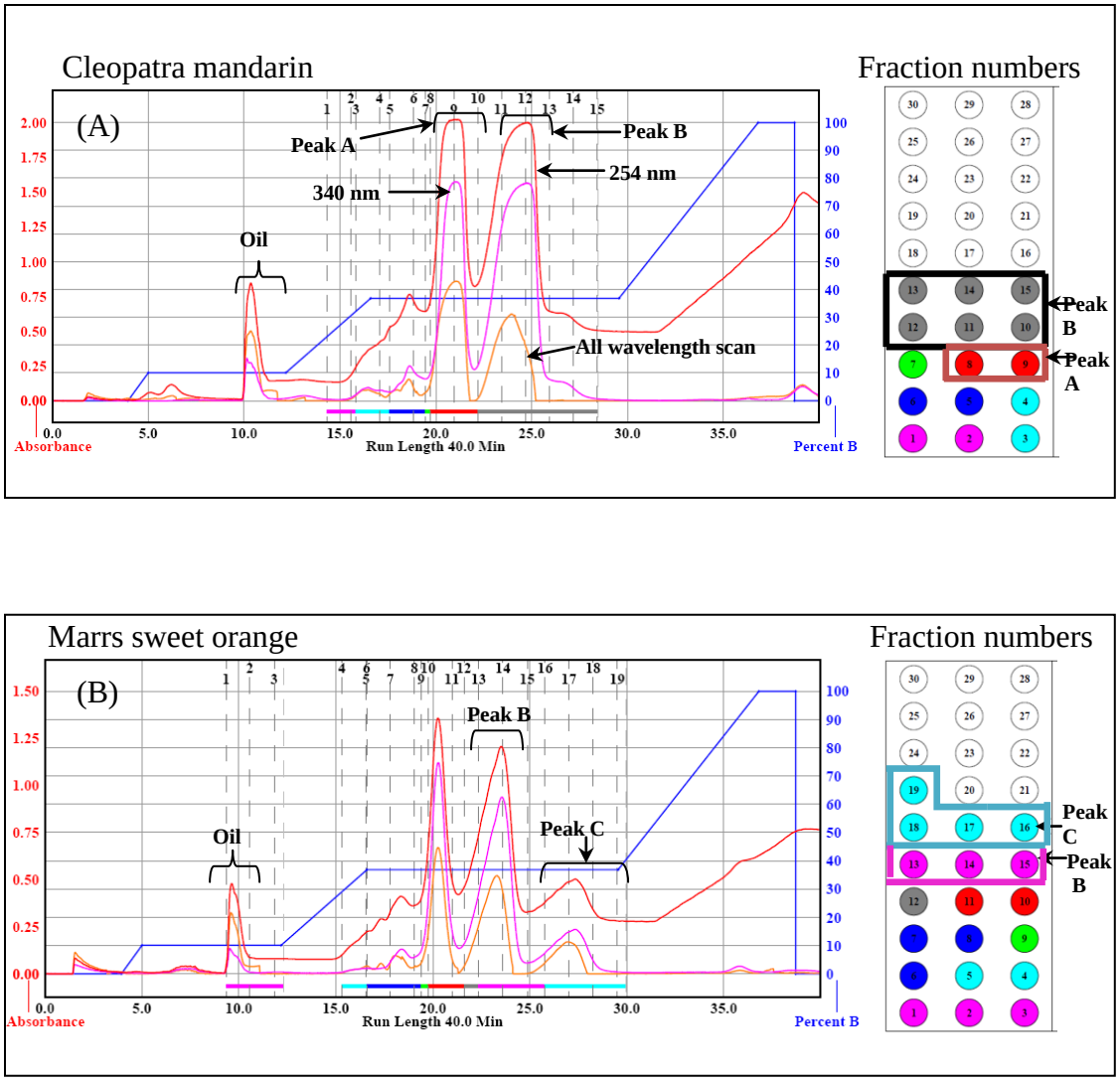

Fig. 9. Flash chromatograms of Cleopatra mandarin and Marrs sweet orange hexane extracts (5 g) separated on a 40 g silica column. Individual peaks collected for each separation are indicated with same color with respective fraction numbers. The line graph colored red, purple and orange correspond to the absorbance monitored at wavelengths of 254 nm, 340 nm and all wavelength scan.

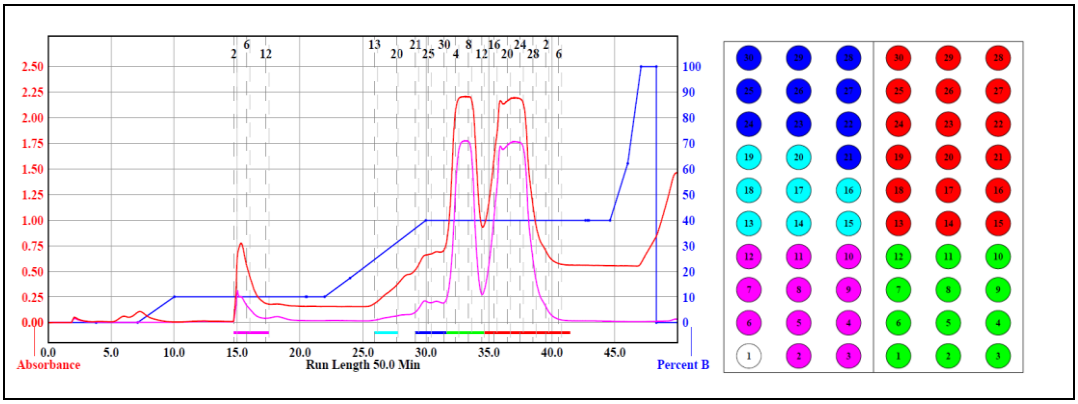

Fig. 10. Flash chromatogram of Cleopatra mandarin extract (35 g) separated on a 330 g silica column. Individual peaks collected for each separation are indicated with similar color with respective fraction number.

4.1.8 Repeatability and robustness

The FC method was evaluated for repeatability by evaluating the separation procedure three times using the same instrument parameters and analyzed for RSD % yield of the isolated PMFs. The developed method was also evaluated for robustness by altering the size of flash column from 40 g to 330 g. Impregnated hexane extract (35 g) of Cleopatra mandarin with silica gel was used for this analysis. The gradient used for the separation was programmed proportional to the 40 g column. The separation was conducted using a 50 min gradient program of hexane (solvent A) and acetone (solvent B) as follows: isocratic A held for 7 min, followed by linearly increased to 10% B over 3 min, held for 12.5 min, linearly increased to 40% B over 7.5 min, held for 15 min, linearly increased to 100% B over 2.5 min, maintained for 1 min and returned back to

100% A. The flow rate was set at 200 mL/min and individual fractions were collected by monitoring the eluting analytes at 254 nm and 340 nm. A total of 63 fractions were collected for the flash chromatography separation with two major peaks collected in fractions 32-41 and 46-56 (Fig. 11). The individual fractions collected were analyzed by HPLC and pooled based on peak similarity to obtain compound 1 and 2.

4.1.9. Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (Maldi-TOF-MS) analysis.

The samples for MS analysis were prepared by dissolving the isolated compounds in acetonitrile and mixed with 2′, 4′, 6′-trihydroxyacetophenone (THAP) matrix. A 0.5 µL of the matrix mixture was spotted on a MALDI sample plate and air-dried. MALDI-TOF mass spectra were acquired using Voyager DE-Pro (Applied Biosystems, CA) mass spectrometer in positive reflector ion mode. After time-delayed extraction of 275 nsec, the ions were accelerated to 20 kV for TOF mass spectrometric analysis. A total of 100 laser shots were acquired with the signal averaged per mass spectrum.

4.1.10 NMR analysis

^{1}H and attached proton test (APT) spectra were recorded at 400 MHz and 100 MHz respectively by FT NMR (JEOL USA, Inc., MA, USA).

4.2 Results and discussion

4.2.1 Extraction of PMFs from citrus peels

Polymethoxyflavones were reported to be extracted using various methods such as Soxhlet extraction [60], supercritical fluid extraction [70] and microwave assisted extraction [55]. Among these extraction methods, Soxhlet extraction was selected due to its ability to extract large sample volumes. Citrus peels are a rich source of flavanones such as naringin and hesperidin in comparison to PMFs which occur in relatively low concentration. To determine the ideal solvent for extraction of PMFs, dried peel powder of Cleopatra mandarin was extracted with different solvents such as hexane, chloroform and methanol followed by HPLC analysis. Highest polarity solvent yielded maximum yield of 33.9 ± 0.02. Hexane and chloroform extraction resulted in % yield of 1.21 ± 0.10 and 2.24 ± 0.26, respectively. The HPLC chromatograms (Fig. 11) of the analyzed extracts demonstrate the selective extraction of PMFs by hexane. Quantitative analysis of PMFs such as nobiletin (NOB) and tangeretin (TAG) present in the various solvent extracts demonstrate that hexane [NOB: 42.77 ± 0.66 (mg/g); TAG: 56.11 ± 0.39 (mg/g)] and methanol [NOB: 51.72 ± 0.77 (mg/g); TAG: 45.15 ± 0.66 (mg/g)] extracts had relatively similar content where as chloroform extract [NOB: 113.63 ± 0.46 (mg/g); TAG: 109.75 ± 0.56 (mg/g)] had higher levels. Due to high polarity of methanol and chloroform, flavonoids such as hesperidin, didymin, hesperitin along with PMFs were extracted. The complex mixtures of methanol and chloroform make it unsuitable for

rapid separation and may require several purification techniques and solvents for the separation of high purity PMFs. Unlike other flavonoids, the absence of hydroxyls and glucose moieties in PMFs make them less polar. Hence, low polar hexane solvent was selected as an optimum solvent for the extraction of PMFs. Subsequently, the peel powders of Cleopatra mandarin and Marrs sweet orange were extracted with hexane and concentrated under vacuum. The dried hexane extracts of Cleopatra mandarin and Marrs sweet orange, were dissolved in hexane and impregnated with silica gel and used for flash chromatography separation.

4.2.2 Identification and characterization

The isolated compounds were analyzed by HPLC, MS and their structures were determined by NMR spectra. HPLC chromatograms of the four isolated compounds are given in Fig. 12. The separations of PMFs were conducted on a Gemini C_{18} column using a gradient of 4% acetic acid (96:4 (v/v); pH 2.45) and acetonitrile. The ability of the column to enable separations using a wide range of pH (1-12) enabled good separations of low polar PMFs within 15 min of runtime. The absence of other peaks demonstrates the purity of the isolated compounds. The UV spectra of the isolated compounds were evaluated to determine the identity (Fig. 12). The absorption maxima (λ_{max}) of Compound 1 was observed to be 271.0 , 324.5 nm, Compound 2: 248.5, 335.2 nm, Compound 3: 242.6, 330.5 nm and Compound 4: 269.8, 337.6 nm.

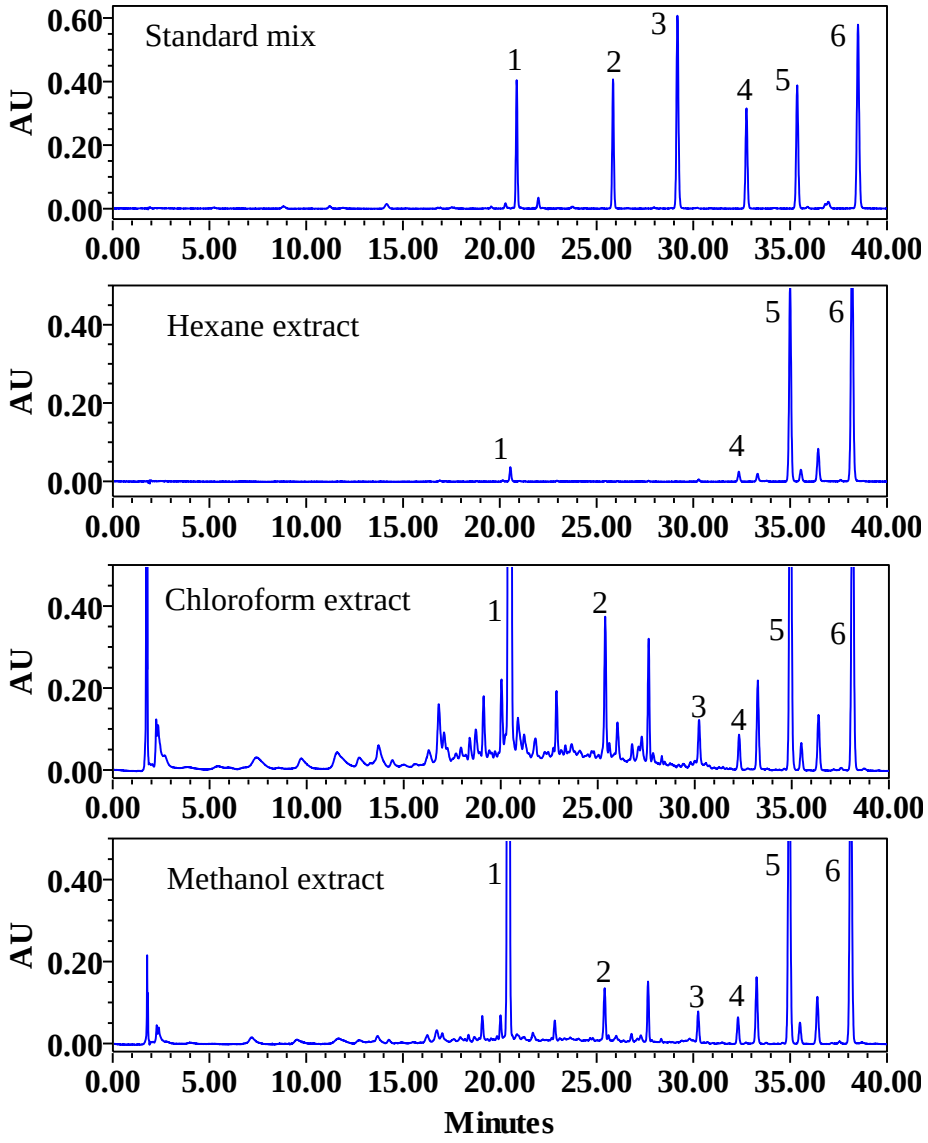

Fig. 11. HPLC chromatograms of standard flavonoids and different solvent extracts of Cleopatra peel. Separations were conducted on a Gemini C_{18} column, 10 µl injection at ambient temperature, detection at 280 nm, flow rate 1 mL/min, gradient elution 4% acetic acid (Solvent A) and acetonitrile (Solvent B), linear gradient 5% to 50% B in 35 min and 50 % to 95 % B in 5 min. Peak (1) Hesperidin; (2) Didymin; (3) Hesperitin; (4) Sinensitin; (5) Nobiletin; (6) Tangeretin.

Figure 13, depicts the positive-ion mode MALDI-TOF spectra of the isolated compounds 1-4. The mass spectrum of isolated compounds 1, 2, 3 and 4 shows a molecular ion $[M+H]^+$ at *m/z* 373.04, 403.20, 373.17 and 343.61, respectively. ^1H and APT spectra of the isolated compounds are given in Fig. 14 and Fig. 15 respectively. Results from the spectral analysis of the isolated compounds 1, 2, 3 and 4 confirm the identity of the compounds as tangeretin, nobiletin, sinensitin and tetramethoxyflavone, respectively. The chemical shifts are in confirmation with the reported values [58, 63, 163].

4.2.3 Repeatability and robustness of the flash chromatography method

Polymethoxyflavones are low polar compounds. Hence, require low polar solvents for flash chromatography separations on polar silica gel stationary phase. The medium back pressure levels (50 psi -200 psi) attained during the flash chromatography make it unsuitable for use of medium polar solvents such as ethyl acetate (EtOAc) and chloroform (CH_3Cl_3) which result in co-elution of PMFs along with oils present in citrus peel extracts. Using a gradient elution of hexane (90 %) and acetone (10 %) enabled optimum separation of oils and PMFs from citrus peels while subsequently individual PMFs were separated after linear increase of acetone to 40 % (Fig. 11).

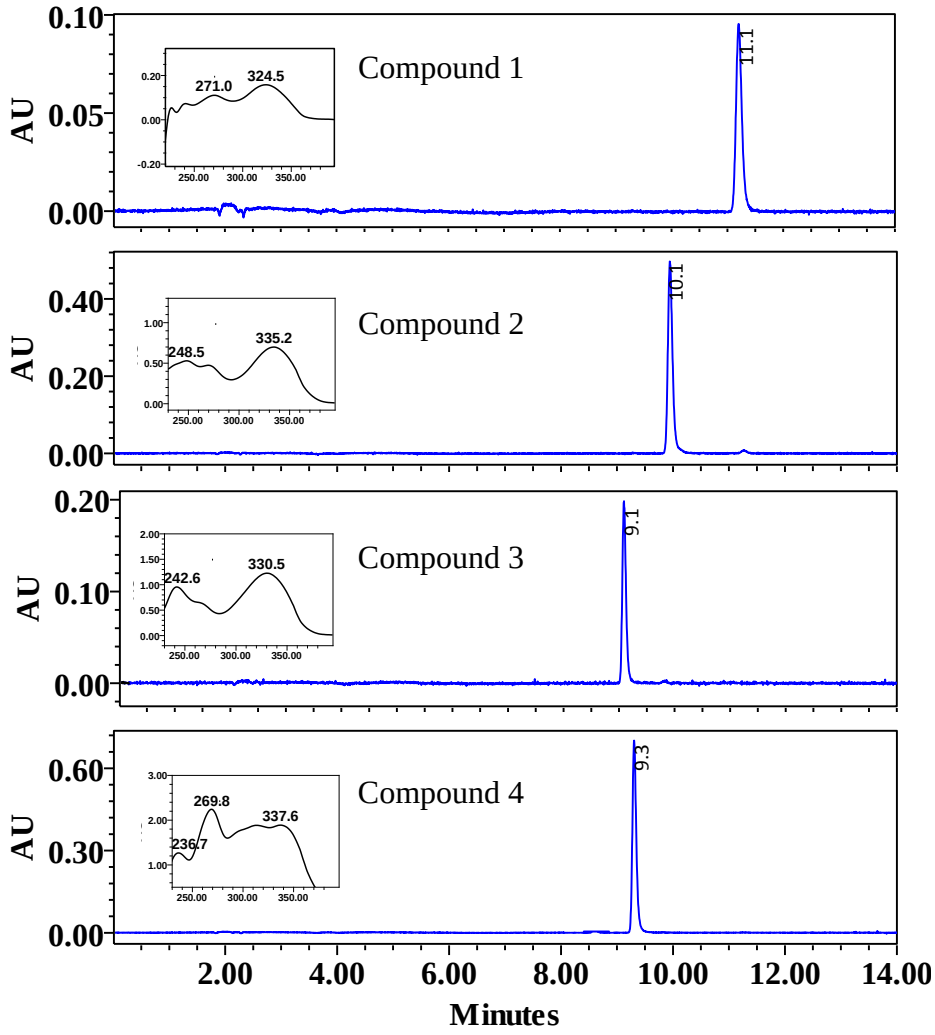

Fig. 12. HPLC chromatograms the purified compounds (1 - 4) along with UV spectra.

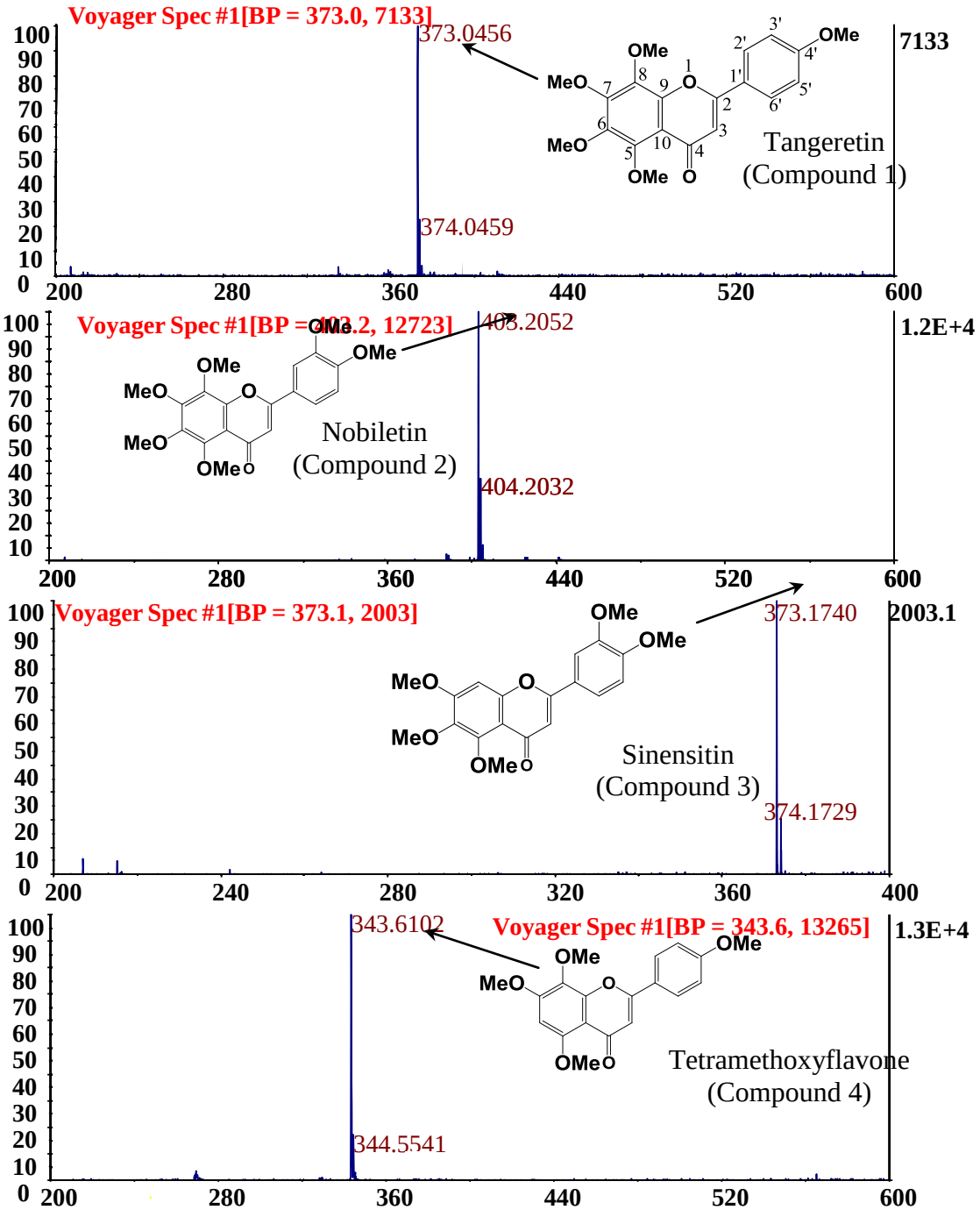

Fig. 13. Positive MALDI-TOF mass spectra and the structures of compounds isolated from Cleopatra mandarin and Marrs sweet orange.

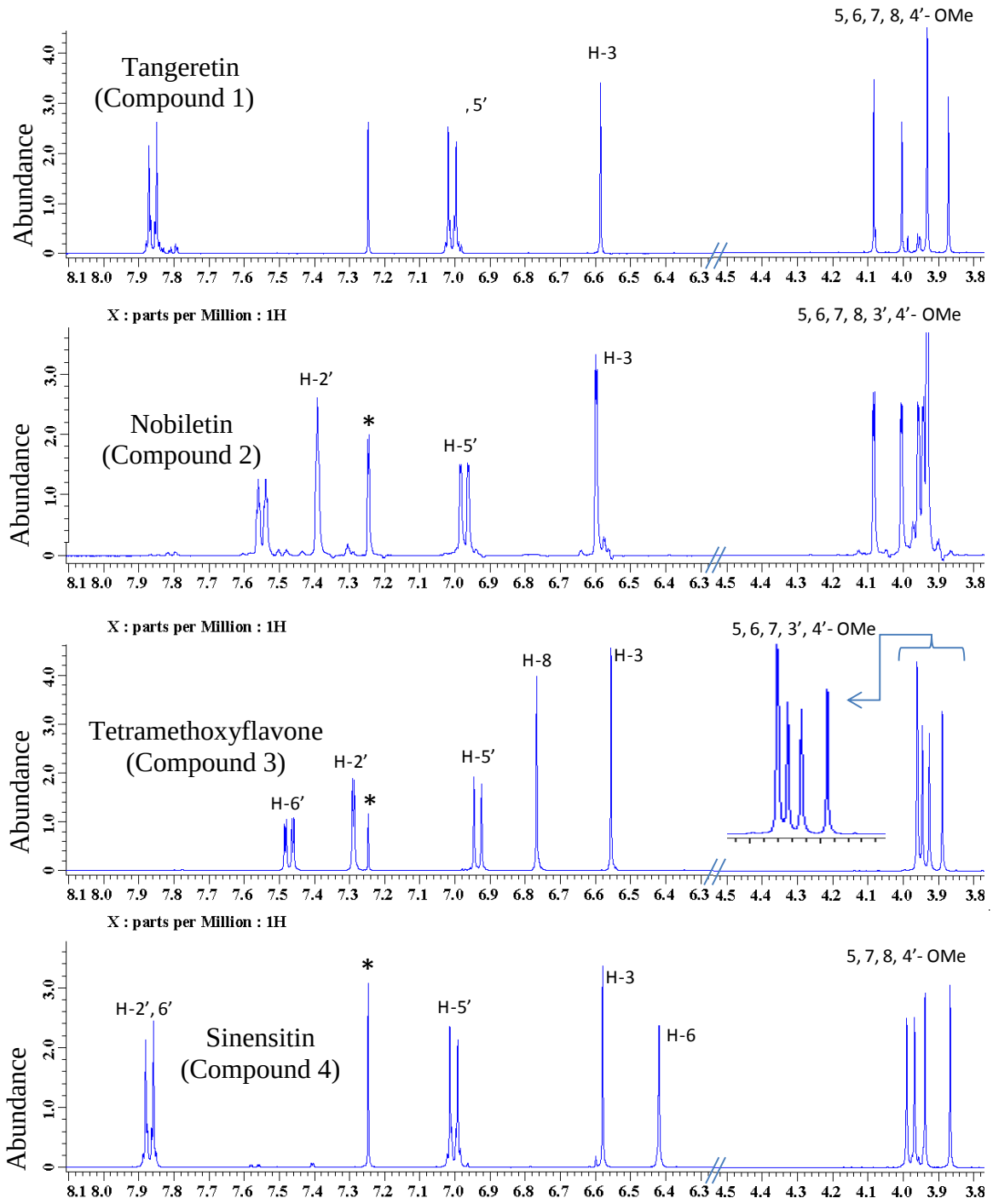

Fig. 14. ^{1}H NMR spectra (δ_H in $CDCl_3$) of the isolated compounds.

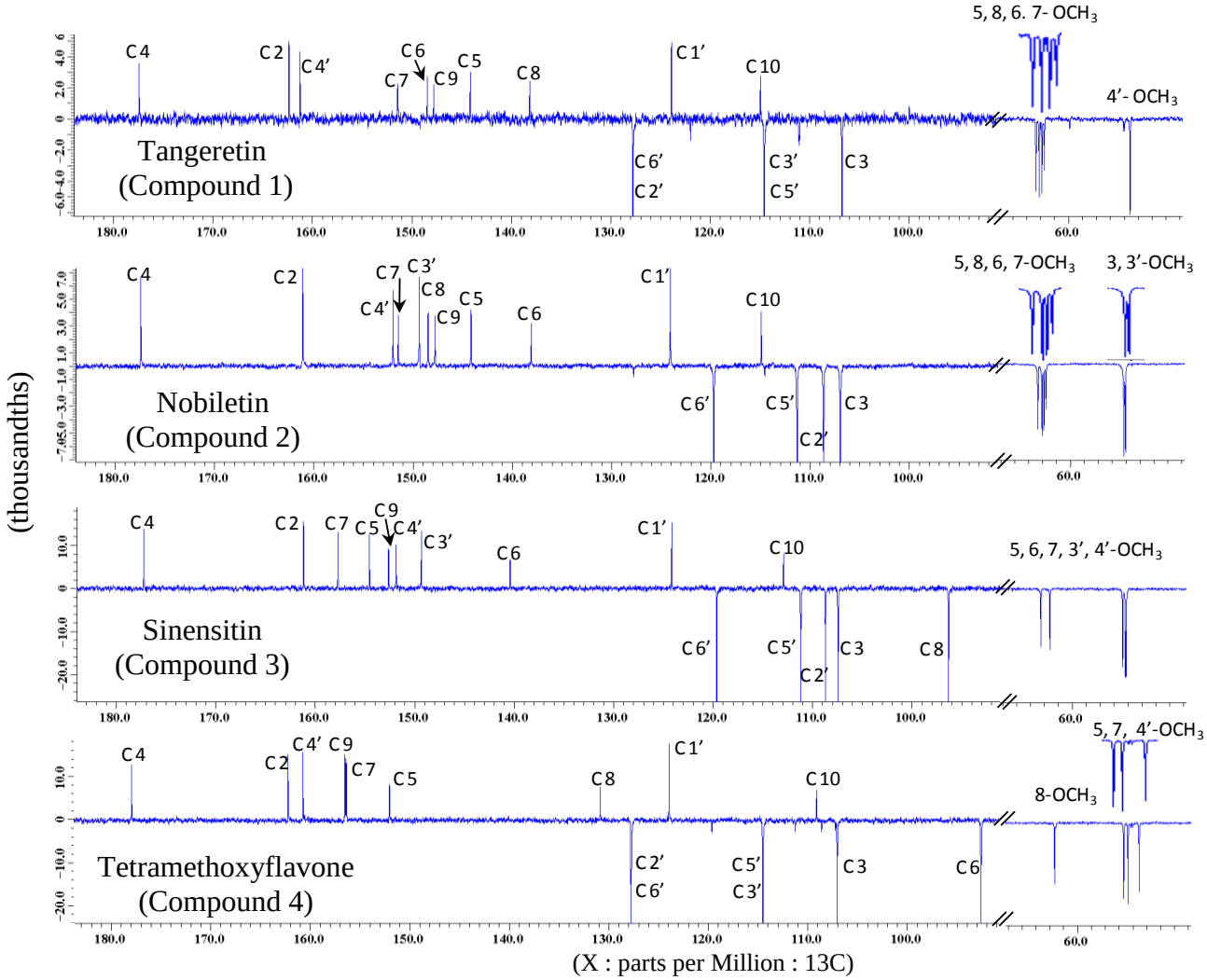

Fig. 15. ^{13}C NMR spectra (δ_C in $CDCl_3$) of the isolated compounds (1 – 4).

The developed flash method was validated by the tests of repeatability and robustness. The test of repeatability is an important criterion for method development, especially in silica based stationary phase. The availability of pre packaged silica columns reduces the time consumption in column preparation. This enables separations of compounds with little variation. To demonstrate the repeatability of the separation methodology, three individual separations with three prepackaged silica columns (40 g) were used to isolate PMFs from Cleopatra and Marrs sweet orange extracts. All the parameters such as flow rate, detection wavelength, amount of sample (5 g) and solvent gradient for all the separations were maintained same. Individual fractions of 50 mL were collected in the fraction collector. The yields of compounds obtained from the separations are summarized in Table 6. Flash chromatography of Cleopatra mandarin extract yielded a total weight of 621 mg of tangeretin as well as 660 mg of compound nobiletin with an average yield of 207 mg and 220 mg respectively. The calculated RSD (%) of 2.79 for tangeretin and 9.63 for nobiletin suggests that the method is repeatable with little variation. Similar low RSD (%) of 12.91 and 3.08 were obtained for Marrs sweet orange extracts yielding cumulatively 280 mg of nobiletin and 150 mg of sinensitin with an average of 93 mg and 50 mg, respectively. In all the separations similar peak patterns were obtained. The cross column separation of Marrs sweet orange pooled fractions resulted in separation of tangeretin (111 mg) and tetramethoxyflavone (90 mg) (Fig. 16). The method was further validated by testing the robustness. To examine the robustness of the method 35 g of silica impregnated with Cleopatra peel hexane extract was subjected to flash chromatography on a 330 g prepackaged silica gel column. The

gradient elution of solvents was programmed proportional to the replicated test. The resultant chromatogram obtained from the FC separation was similar to the chromatogram observed for the 40 g silica column. A total of 63 fractions were collected with two major peaks collected in fractions 32-41 and 46-56. Pooling of these fractions and subsequent concentration by evaporating the solvents yielded 1.45 g of tangeretin and 1.29 g of nobiletin.

4.3 Conclusion

A rapid and efficient separation of PMFs from citrus peels using flash chromatography was developed. Using the developed method, four PMFs were successfully separated with high purity in gram level quantity. The purified PMFs were identified and characterized as tangeretin, nobiletin, tetramethoxyflavone and sinensitin by spectroscopic studies such as HPLC, MS and NMR. Hence, this method is viable for rapid and large scale separation of PMFs. The developed FC method can enable utilization of citrus by-products such as peels for separation of economically important PMFs, which could add value addition to the citrus processing industry.

Table 6. Yields of separated compounds (mg) from citrus using flash chromatography.

Citrus species	Column	Sinensitin	Nobiletin	Tangeretin	Total Yield	Total Avg. yield
		Mean (% RSD)a			(mg)	(%)b
Cleopatra	40 g	-	219.63 (9.63)	207.17 (2.79)	658.90	5.76
Cleopatra	330 g	-	1290.00	1450.00	2740.00	-
Marrs sweet	40 g	49.67 (3.08)	93.33 (12.91)	-	429.00	4.23

[a] Average of three individual chromatographic separations. % RSD = Relative standard deviation; (standard deviation / mean) × 100. [b] Average yield of isolated PMFs calculated as a percent weight of the crude extract.

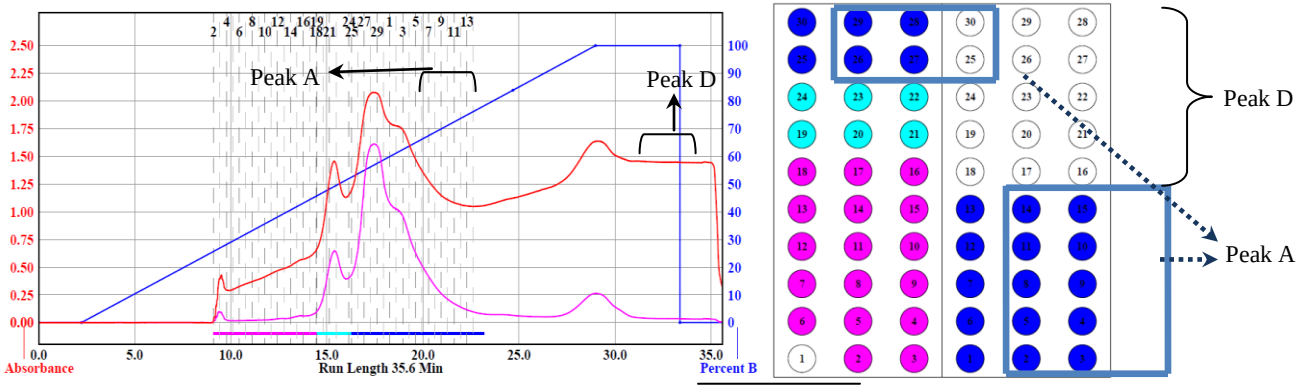

Fig. 16. Flash chromatogram of cross column separation of Marrs sweet orange on a 40 g silica column.

5. INFLUENCE OF ORGANIC AND CONVENTIONAL PRODUCTION SYSTEMS ON THE LEVELS OF PHYTOCHEMICALS IN MEYER LEMON (*Citrus meyeri* TAN)

Lemons are among the most commonly consumed citrus fruits in worldwide. The United States ranks fifth in the world in lemon production with an estimated acreage of approximately 63,000 acres [164, 165]. Among citrus fruits, lemons have high citric acid content rendering them unpalatable. Therefore, lemons are consumed along with other food materials, used as garnish as well as juiced to make lemonades [166]. These citrus fruits are known to have high levels of both organic acids and health promoting compounds also known as phytonutrients such as dietary fiber, pectin, flavonoids, and limonoids [167, 168]. Citrus flavonoids are associated with health beneficial properties such as antioxidant, anti-proliferative, anti-inflammatory and coronary heart disease prevention [169-171]. Furthermore, hesperidin a flavonoid present in lemons was tested in human clinical trials and implicated as a potential component for control of bleeding from acute internal haemorrhoids [172]. Lemons have also been reported to contain amines such as octopomine, synephrine and tyramine [173]. Amines are metabolized to form epinephrine or norepinephrine due to which several formulations and extracts are promoted as weight reducing dietary supplements with amines as the main ingredient [174].

Accumulating evidence on the role of phytochemicals on health benefits and their increase in consumer's interest in healthy foods led to development of strategies

that could enhance the levels of phytonutrients. The content of phytochemicals depends both quantitatively and qualitatively on their genetic information and as well as other environmental factors including water and mineral nutrition [113, 175]. Therefore, both pre harvest and post harvest factors including genetics and environmental factors cause wide variation in the levels of phytochemicals. However, little information is available on the effect of pre-harvest factors on variation of phytochemicals in lemons. Production system is one of the major pre-harvest factor that could influence the phytochemicals content [176, 177]. Production systems such as organic farming has been gradually increasing due to the changes in consumer preferences [178]. Sales of organic citrus have increased at an annual rate of 20% since the year 1990 [179]. Organic farming is a form of cultivation practice that integrates basic cultivation practices such as crop rotation, green manure, compost, biological pest control, and mechanical cultivation to sustain productivity and control pests. Due to lack of use of synthetic fertilizers, pesticides and growth regulators in organic production system various biotic and abiotic stresses seems to enhance synthesis of polyphenolic components to provide plant defense mechanism [180]. Currently, information related to the effect of these production systems on health promoting phytochemicals in lemons is very limited. Determination of levels of phytochemicals could help us to better understand the role of physio-chemical changes in production systems.

5.1 Materials and methods

5.1.1 Plant material and experimental design

During 2008-2010, a field experiment was conducted to evaluate the influence of organic and conventional management practices on the phytonutrients content of Meyer lemon fruits. The orchards were located at the Lower Rio Grande Valley (LRGV) of South Texas. Conventional citrus orchard was located at Texas A&M University-Kingsville, Citrus Center in Weslaco, and an organic orchard- South Texas Organics (Mission, TX, USA), certified by the Department of Agriculture (USDA) located approximately 24 miles apart, were selected for evaluating conventional and organic management practices. Both the orchards were grown under flood irrigation practice with a common irrigation source, the Rio Grande River. Trees were spaced 4.6 m × 7.3 m with a planting density of approximately 300 trees ha^{-1}. Five fruit trees were grouped as a replicate and three replications were used for each individual production system. Agronomic operations nutrient management and weather data for both the experimental orchards were monitored and recorded (Table 7 and 8). Mature Meyer lemon fruits of uniform size and shape were harvested in the month of November 2008 and in February 2010 from both the experimental orchards. The fruits were washed with clean water, air dried and packed in cardboard boxes. The boxes were stored at the optimum temperature of 10º C in an automated thermostat regulated refrigerator. Fruits were periodically inspected at a time interval of two days for any decay of fruits. For analysis of

phytonutrients, 24 fruits from each replicate of organic and conventional were randomly sampled at 4, 11, 18 and 25 days after storage.

5.1.2 Chemicals and reagents

Octopomine, synephrine, tyramine, citric acid, meta-phosphoric acid, Folin–Ciocalteu reagent and HPLC grade phosphoric acid were purchased from Sigma-Aldrich (St. Louis, MO). L-Ascorbic acid was purchased from Mallinckrodt (Paris, KY, USA). Authentic reference standards of narirutin, didymin, hesperidin, were purchased from ChromaDex Inc. (Irvine, CA, USA). Nanopure water (NANOpure, Barnstead/Thermolyne Corp. Dubuque, IA) was used for the sample preparation and HPLC analysis. Acetonitrile (HPLC grade) and dimethylformamide (HPLC grade) were purchased from Fisher scientific (Pittsburgh, PA). Glacial acetic acid (>99.5%) was purchased from Fluka (Milwaukee, WI).

Table 7. Cumulative potential evapotranspiration, temperature (maximum and minimum), relative humidity, solar radiation, rainfall and irrigation applied at the two field locations during the harvest seasons.

Cultivation	Year	PET (cm)	T max (c)	T min (c)	RH min (%)	Solar mJ/m2	Rain (cm)	Irrigation (cm)
Conventional	2008	146.38	29	18	48	20.07	79.04	76.20
	2010	190.78	28	17	48	22.71	61.72	101.60
Organic	2008	202.11	30	19	36	17.45	20.32	63.50
	2010	217.53	28	17	39	16.13	36.68	88.90

Table 8. Fertilization, compost application, mineral nutrients, insecticides and weedicide applied in conventional and organic lemon orchards.

Application	Rate	Conventional[a]	Organic[a]
Fertilization			
Fertilizer	46-0-0	100.00	-
Compost	-	-	8000.00
Compost brew	-	-	379.00*
Gypsum	Mixed in solution for ground application	-	6.13
Manganese		-	4.00
Magnesium sulfate		-	0.45
Zinc		-	0.45
Copper		-	0.25
Insect control			
Agrimek	-	0.45	-
Vendex	-	0.23	-
Envidor + Micromite	4 intervals	0.28	-
Sulfur spray	-	-	11.00*
Weed control		2.27	-
Simazine + Diuron	-	0.45 + 1.35	-
Cultural practice	(harrowing)	-	6-7

[a]Agricultural inputs represented as Kg/acre.
*Units measured in liters/acre

5.1.3 Extraction of phenyl ethyl amines and organic acids.

The amines and organic acids were analyzed using the developed method reported earlier in our lab. Eight fruits were grouped as a sub sample with three sub samples in each replication of individual treatment. Fruits from each sub sample were peeled and blended using a house hold blender (Vita-prep™, Cleveland, OH). The blended juice was homogenized for 30 sec using a Polytron homogenizer (Brinkmann Instruments

Inc., Westbury, NY, USA). Three percent meta phosphoric acid (MPA) was used for extraction of amines and organic acids. In brief, 10 g of the homogenized juice sample was diluted with 30 mL of 3% MPA in a centrifuge tube and vigorously mixed. Three milliliters of sample mixture was filtered under vacuum using a 0.45 µm membrane filter (Millipore Corp., Bedford, MA, USA). The unfiltered juice residue was re-extracted with 3 mL of MPA in successive volumes of 1 mL each. All the extracts were pooled and 10 µL was injected to the HPLC for analysis. The HPLC system consisting of a Waters 1525 HPLC series (Milford, MA) connected to a PDA detector was used. A Xbridge C18 column (3.54 µm, 4.6 mm × 150 mm i.d.) from Waters (Milford, MA) was used for all the separations. Elution was carried out at ambient temperature using the mobile phase comprised of 3 mM phosphoric acid under isocratic condition. The flow rate was set at 1.0 mL/min, and detection was set at dual wavelengths of λ223 nm and λ254 nm with a total analysis time of 10 min. Three injections were performed for each sample. Peaks were identified on the basis of comparing and matching the UV spectra as well as the retention time (tR) of the individual standards. The results were further validated by spiking the sample extracts with pure standards.

5.1.4 Extraction of flavonoids and identification

Ten grams of blended juice sample was mixed with 10 mL of dimethyl formamide (DMF) in a 50 mL centrifuge tube and homogenized for 30 sec using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA). The homogenized

juice was placed on a shaker for 3 hrs and later filtered to collect the extract separately. The procedure was repeated two more times and all the extracts were pooled together. The extract was filtered using 0.45 μm membrane filter and 10 μL clear filtrate was injected to the HPLC for analysis. The HPLC system consisting of a Waters 1525 HPLC series (Milford, MA) connected to a PDA detector was used. Flavonoids were separated on Xbridge C-18 column (3.54 μm, 4.6 mm × 150 mm i.d.) from Waters (Milford, MA) and detected at 280 nm. The solvent system of acetonitrile (MeCN)/ water plus 4% acetic acid gradient starting at 15% and ending at 50% MeCN gradient was used. Flavonoids were identified by comparing their UV spectra and retention times with those of standards. Quantification of flavonoids was done by using known concentrations of external standards from the commercial source and all samples were run in triplicates. To further validate the identification of flavonoids, homogenized lemon juice sample (10 mL) was extracted by methanol in three successive steps consisting of 10 mL each. All the solvent extracts were pooled, filtered by 0.45 μm membrane filter and analyzed by LC-MS. The LC system consisted of Finnigan Surveyor plus (West Palm Beach, FL) coupled to a mass spectrometer- Ion Trap (LCQ-DECA, ThermoFinnigan). The flavonones were separated on a Aquasil, C-18 column (2.1 × 150 mm, 3 μm) (Keystone-Hypersil, Bellefonte, PA) using a gradient mobile phase of 0.1 % formic acid (A) and acetonitrile (B), maintained at a flow rate of 0.2 mL/min. The gradient conditions consisted of linear change of (A) from 95% to 67% in 9 min followed by linear change to 40% in 8 min and finally returned to 95% in four

min. The mass spectrometer was operated using electron spray ionization in negative ion mode (ESI-) with the spray voltage set at 3.5 kV.

5.1.5 Total phenolics assay

The concentration of total phenolics in the extracts was determined using Folin–Ciocalteau colorimetric method reported by Negi and Jayaprakasha [181] with some modifications and the results expressed as catechin equivalents. Ten grams of lyophilized fruit juice of each replicate of individual treatment was extracted exhaustively with 500 mL of methanol in Soxhlet type apparatus for 8 h. The extract was concentrated by roto-evaporation (Buchi Rotavapor; Büchi Labortechnik, Flawil, Switzerland) and lyophilized in a freeze dryer (Labconco Freezone 4.5; Labconco Corp., Kansas City, MO).The freeze dried methanol extract was dissolved in a solvent mixture of methanol and water (80:20) v/v) to obtain a concentration of 5 mg/ mL. Calibration curves were prepared for the working solutions of catechin (10, 20, 30, 40, 50, 75 and 100 µg) of standard by diluted in solvent mixture of methanol and water (80:20). Briefly, the dissolved sample extracts (100 µL) and standard concentrations of catechin were taken in test tubes and the volume was adjusted to 10 ml by addition of distilled water. One mL of 1-fold diluted Folin–Ciocalteu reagent and 1 ml of 7.5% sodium carbonate solution was be added to all the tubes. The resultant samples were incubated for 30 min at room temperature and the absorbance measured at 760 nm using a

spectrophotometer. The estimation of total phenolics in all the extracts were carried out in triplicate and the mean results presented as a relative measure of catechin.

5.1.6 Soil, leaf and juice mineral analysis

Soil core samples from the upper 30 cm depth were collected from three different rows of organic and conventional systems in the harvest seasons 2008 and 2010. For leaf nutrient analysis, mature leaves were randomly harvested from trees in three rows for both treatments. The harvested leaf samples were washed with 1% hydrochloric acid solution, air dried, and analyzed for macro and micro nutrients. For juice mineral analysis, fruits harvested in both harvest seasons from the three replicates in each treatment were processed at 4, 11, 18 and 25 days after storage and juice was collected. The juice samples were homogenized and freeze dried in a freeze dryer (LabConco, Kansas City, MO). The lyophilized juice samples were blended to a fine powder, sieved and submitted for analysis. All the mineral analysis was conducted at Texas A&M University's Soil, Water and Forage Testing Laboratory at College Station, Texas. Nitrate nitrogen in soil and plant material (NO_{3}-) was extracted using 1 N potassium chloride (KCl) solution. Nitrate was determined by reduction of nitrite (NO_{-2}) to nitrate using a cadmium column followed by spectrophotometric measurement. The micronutrients in soil (Cu, Fe, Mn and Zn) were extracted using a 0.005 M DTPA, 0.01 M $CaCl_2$ and 0.10 M triethanolamine solution. The analytes were determined by inductive coupled plasma−atomic emission spectroscopy (ICP-AES) (Spectro Genesis,

Deutschland, Germany). Phosphorus, K, Ca, Mg, Na and S were extracted using the Mehlich III extractant and determined by ICP. For determining micronutrients (B, Ca, Cu, Fe, K, Mg, Mn, Na, P, S, and Zn) in plant materials the tissue samples were digested overnight at room temperature in concentrated nitric acid. After digestion the samples were heated to 125^{o} C for 4 h and subjected to ICP analysis.

5.1.7 Statistical analysis

Analysis of statistical differences between treatment groups was conducted using a general linear model (GLM) procedure of SAS (SAS Institute, North Carolina, USA). Mean comparisons were made using Duncan multiple range test with significant differences of means at the 95% confidence level ($P \leq 0.05$).

5.2 Results and discussion

5.2.1 Phenyl ethyl amines and organic acids content.

Among the detected amines, octopomine was the predominant amine present in both organic and conventionally grown lemons. In both the harvest years conventionally grown lemons had a significantly higher content of octopomine. No significant ($P \leq 0.05$) differences were noticed in the contents of synephrine and tyramine among organic and conventionally grown lemons (Table 9). The fruits harvested in 2008 had a higher

content of amines compared to the fruits harvested in 2010. Amines such as octopomine, synephrine and tyramine are compounds containing nitrogen functionality. These compounds are considered as end products of nitrogen metabolism,[182] due to which these compounds are also studied as indicators for evaluating the authenticity of organic and conventional citrus [183]. The significant variation in the levels of these amines among organic and conventionally produced lemons could be due to the variation in the availability of soil nitrogen to the plants. Nitrogen management is an important factor of "Best Management Practices" in citrus crop production and prior studies have demonstrated the positive correlation between nitrogen fertilization and yield [184]. The readily available N through inorganic fertilizer application in the conventional practice resulted in higher accumulation of these compounds. In the storage analysis, no significant variation was noticed among all the amines for both the treatments. These results suggest that storage of lemons at 10º C could be an ideal strategy for maintaining the levels of amines in lemons.

Ascorbic acid content was significantly high ($P \leq 0.05$) in organically grown lemons in comparison to conventionally grown in the 2008 harvest year (Table 10). The ascorbic acid content of organic fruits ranged from 272.25 mg/100g to 309.75 mg/100g whereas the conventionally grown fruit ranged in between 194.49 mg/100g and 243.12 mg/100g. Our results are consistent with previous results [185-187]. However in the 2010 harvest, organically grown lemons had a relatively lower content of ascorbic acid ranging from 189.25 mg/100g to 198.17 mg/100g.

Table 9. Octopomine, Synephrine and Tyramine content of Meyer lemons cultivated under organic and conventional management practices harvested in 2008 and 2010 analyzed at different intervals of storage (days after harvest)*.

Cultivation practice	Days after harvest	mg/100 g					
		Harvest year 2008			Harvest year 2010		
		Octopomine	Synephrine	Tyramine	Octopomine	Synephrine	Tyramine
Conventional	4	22.48 a	10.93 a	16.34 a	6.13 a	3.80 a	4.41 a
Organic	4	17.88 b	10.39 a	16.83 a	5.53 b	3.81 a	4.04 b
Conventional	11	19.23 b	12.41 a	15.29 b	6.77 a	3.82 a	4.71 a
Organic	11	21.49 a	12.26 a	18.67 a	5.39 b	4.49 a	4.45 a
Conventional	18	19.92 a	11.98 a	15.15 a	5.88 a	3.28 b	4.74 a
Organic	18	16.96 b	11.81 a	14.94 a	5.48 a	4.40 a	4.54 a
Conventional	25	19.31 a	10.80 a	15.57 a	5.35 a	3.81 a	5.63 a
Organic	25	17.20 b	11.34 a	14.46 b	4.04 b	3.86 a	4.31 b

* Each value is an average of three replications. Mean separations within each harvest year and for similar storage interval between organic and conventional by Duncan's multiple range test at $P \leq 0.05$.

Furthermore, conventionally grown lemons had a higher ascorbic acid in early harvest fruit during 2008 than the late harvest of 2010. The variation in ascorbic acid could be due to the difference in the stage of maturity at harvest. Mature citrus fruits harvested late in the season have lower concentration of ascorbic acid in contrast to the early harvested which have higher concentration [188]. In the storage study, a gradual trend of decline in ascorbic acid content was noticed with a loss of 13% and 12 % in conventional and organically grown lemons, respectively in the harvest year 2008.

Table 10. Ascorbic acid and citric acid content of Meyer lemons cultivated under organic and conventional management practices harvested in 2008 and 2010 analyzed at different intervals of storage (days after harvest)*.

Cultivation practice	Days after harvest	Harvest year 2008		Harvest year 2010	
		Ascorbic acid	Citric acid	Ascorbic acid	Citric acid
		(mg/100 g)	(g/100 g)	(mg/100 g)	(g/100 g)
Conventional	4	243.12 B	75.08 a	196.74 a	54.08 b
Organic	4	309.75 A	53.61 b	198.17 a	50.79 a
Conventional	11	216.73 B	71.68 a	199.99 b	53.81 a
Organic	11	320.93 A	50.93 b	208.50 a	51.60 a
Conventional	18	194.49 B	69.70 a	171.18 b	47.64 b
Organic	18	306.66 A	54.48 b	208.50 a	51.60 a
Conventional	25	212.44 B	72.11 a	194.92 a	57.10 a
Organic	25	272.25 A	52.29 b	191.85 a	54.10 a

*Each value is average over three replications. Mean separations within each harvest year and for similar storage interval between organic and conventional by Duncan's multiple range test at $P \leq 0.05$.

However in the 2010 the loss in ascorbic acid during storage was minimal in both organic and conventional production systems. The content of citric acid, a tart flavoring compound, was significantly ($P \leq 0.05$) higher in conventional than organic fruit throughout the storage period in 2008 harvest (Table 10). The content ranged from 69.68 g/100g to 74.95 g/100g in conventional lemons during the storage period of 25 days. Similar trend was also noticed in 2010 harvest year with conventional fruits having significantly higher citric acid content at the 4 days after harvest and 25 days after storage. Both organic acids are important quality parameters in citrus processing industries and also contribute to the citrus flavor. The presence of high content of ascorbic acid in organic fruits suggest that organic cultivation practice could be used as a tool for obtaining higher levels of organic acids.

5.2.2 Analysis of flavonoids

Lemons are a good source of flavonoids and characteristically contain high amounts of these health promoting compounds. Flavonoids such as narirutin, hesperidin and didymin were detected in the lemon juice and among these, the level of hesperidin was higher the other flavonoids. The identity of the detected flavonoids in HPLC analysis was confirmed by LC-MS analysis using ESI in negative ion mode. Figure 17 illustrates the LC-MS spectra obtained from the $[M-H]^-$ ions for the three flavonoids narirutin, hesperidin and didymin. Quantification of these flavonoids by HPLC suggests that organic fruits had significantly higher levels of hesperidin and didymin in both the

harvest years (Table 11). Narirutin was relatively low in concentration ranging from 0.97 mg/100g to 1.63 mg/100g in both the harvest years for organic and conventional lemons. These results suggest that lemons are a good source of dietary intake of flavonoids such as hesperidin. Flavonoids are polyphenolic compounds which are implicated in the plant defensive mechanism against disease and pests. Cumulative evidence suggest that hesperidin, narirutin and didymin have antioxidant properties *in vivo* [189], inhibit bone loss [190] and possess antimicrobial properties [191]. It is possible that the higher level of certain phytonutrients (hesperidin, didymin) in lemons grown under organic production system compared to conventional system could be due to the effect of nutrient stress conditions. The limitation in nutrient availability observed in the soil and leaf analysis in organic field could have resulted in enhanced synthesis of phytonutrients. In contrast, the fertilizer application in conventional orchard could have resulted in less nutrient stress which corresponds to low accumulation of these compounds.

5.2.3 Total phenolic content

In both the harvest years organic lemons (5.54 – 7.00g/100 g of juice dry weight) had higher levels of total phenolic content as compared to conventional lemons (Fig.18). However, in storage analysis no significant ($P \leq 0.05$) variation was noticed among the treatments in both harvest years. These results suggest that lemons have other phenolic compounds apart from flavonoids, and organic acids contributing towards the total

phenolic content. Phenolic compounds contribute significantly towards the fruit quality and health beneficial properties.

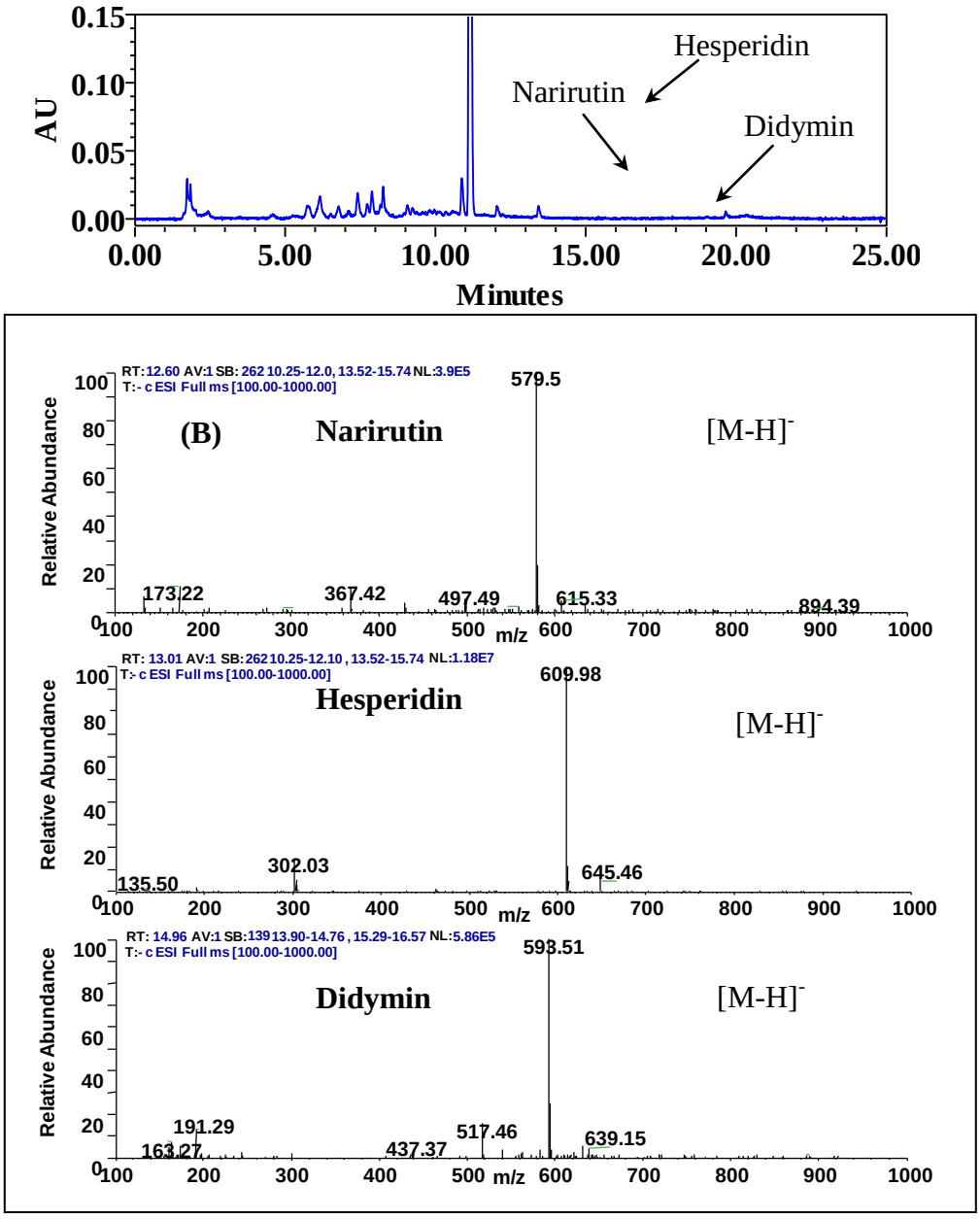

Fig. 17. (A) HPLC chromatogram of Meyer lemon juice, (B) Negative ion mass spectra of flavonoids detected in Meyer lemon juice extracts analyzed by LC-MS.

Table 11. Flavonoids: Narirutin, Hesperidin and didymin content of Meyer lemons cultivated under organic and conventional management practices harvested in 2008 and 2010 analyzed after different intervals of storage (days after harvest)*.

Cultivation practice	Days after harvest	mg/100 g											
		Harvest year 2008						Harvest year 2010					
		Narirutin		Hesperidin		Didymin		Narirutin		Hesperidin		Didymin	
Conventional	4	1.11	a	101.99	b	18.44	b	1.43	a	126.66	B	34.99	b
Organic	4	1.14	a	133.86	a	27.84	a	1.63	a	150.11	A	45.12	a
Conventional	11	1.32	a	93.90	b	16.70	b	1.26	a	119.82	B	35.50	b
Organic	11	1.44	a	155.19	a	32.28	a	1.33	a	145.32	A	43.61	a
Conventional	18	1.07	a	90.23	b	18.51	b	1.43	a	137.79	B	40.10	b
Organic	18	1.26	a	129.65	a	31.28	a	1.31	a	151.72	A	46.76	a
Conventional	25	0.97	a	81.69	b	14.01	b	1.37	a	151.40	A	47.80	b
Organic	25	1.01	a	114.65	a	27.81	a	1.57	a	148.29	A	52.72	a

*Each value is average over three replications. Mean separations within each harvest year and for similar storage interval between organic and conventional by Duncan's multiple range test at $P \leq 0.05$.

Lemon polyphenols were evaluated to inhibit diet induced obesity in animal studies [192]. Estimation of the total phenolic content provides a measure of the reducing capacity of the various bioactive compounds. Results from the current study suggest that lemon fruits cultivated by organic or conventional production systems are a good source of phenolic compounds. Moreover, storage at 10º C aids in maintaining the levels of phenolic content in both organic and conventionally produced lemon fruits.

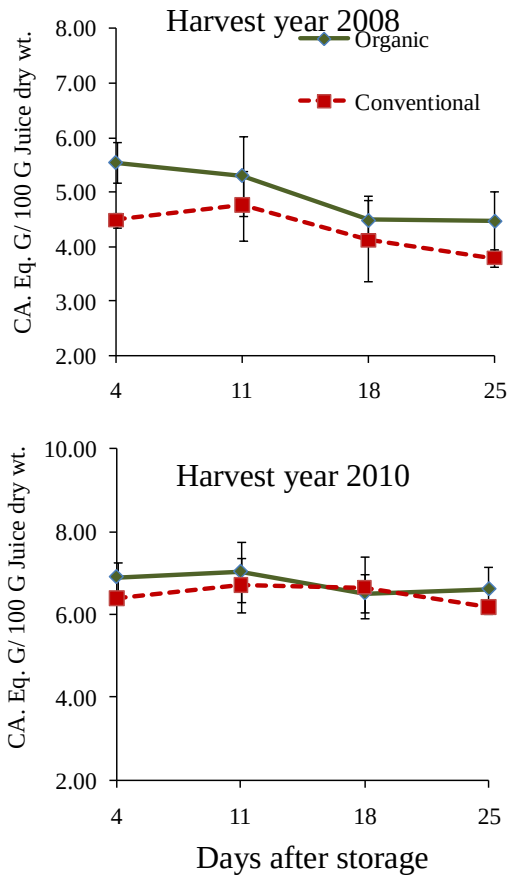

Fig. 18. Total phenolic content expressed as catechin equivalent (g /100 g of Juice dry wt.) of meyer lemon grown under organic and conventional management practices at 4, 11 week, 18 and 25 days after storage.

5.2.4 Soil, leaf and juice mineral analysis

The nutrient composition of the compost brew and the compost applied to the organic lemon orchard as well as the soil nutrient analysis of conventional and organic orchard are presented in Table 12. In both the harvest years, organic orchards had low NO^{3-} content (9.0 % - 2.0 %) in comparison to the conventional orchard (12.0 % and 4.2 %). The low NO^{3-} content in organic orchard soil could be due to the absence of synthetic fertilization while in the conventional orchard, synthetic fertilizers were applied during both the harvest years. Similar trend of high content in the levels of Ca, Mg, Fe and Zn were noticed in the conventional orchard soil in comparison to the soil from organic orchard. Organic cultivation practice limits the availability of essential macronutrients due to the reduced mineralization capacity by the soil organisms [193]. Moreover, the high temperature conditions prevalent in the south Texas result in high soil microbial activity resulting in low organic matter content [194]. Soil amendments such as the application of fertilization and compost are important factors for maintaining good plant health and also to obtain optimum yield [195]. Our previous results related to long term evaluation of compost application on citrus suggest the positive correlation between compost application and root hair growth [196]. Therefore, compost application enables efficient water uptake and as well as nutrient uptake. Citrus crops require periodic nutrient management practices through fertilization to replenish the nutrients lost through the high yield of fruits harvested annually. Studies also suggest the positive correlation between fertilization and yield of citrus [184]. Hence, in conventional

production system, inorganic fertilizers are commonly applied to obtain high fruit yields. In contrast, organic orchards substitute the application of fertilizers through the application of organic amendments such as compost and compost brew. Compost brew is a nutrient culture obtained from agitating, aerating and extraction of compost using Earth tea brewer (Sustainable Agricultural Technol. Cottage grove, OR). The compost brew applied contains both macro and micro nutrients, among these nutrients the brew contained higher amounts of micro nutrients (Fe: 128% and Zn: 1676%) as compared to macro nutrients (NO^{3-}: 0.02%, P: 0.01%, K: 0.09%). These results suggest the soil nutrient content in organic and conventional was different.

In juice, mineral and nutrient analysis of organic and conventional lemons, significant differences were noticed for the content of total N (Fig. 19), P, K, Ca, Mg and protein (Table 13). However, no significant differences were noticed for the content of Zn, Cu, Fe and Mn. The high content of N observed in the conventional lemons could be due to readily available N applied as a fertilizer (46-0-0). This trend was noticed in both the harvest years. In storage analysis no major differences was noticed, suggesting that lemon juice nutrients are not influenced by storage at 10º C in both the production systems.

Table 12. Nutrient analysis of soil amendments and soil in upper 45 cm of the soil in conventional and organic lemon orchards.

Cultivation	NO_3^-	P	K	Ca	Mg	Na	Zn	Fe
	----------%----------							
Organic								
Compost Brew	0.02	0.01	0.09	0.03	0.01	0.03	0.58	6.11
Compost	3.29	0.75	1.59	0.77	0.50	0.29	127.92	1676.05
	%	----------ppm----------						
Soil: 2008	9.00	83.00	345.33	2236.00	413.33	202.00	0.53	4.52
Soil :2010	1.96	100.00	348.46	3139.23	322.38	88.37	0.82	3.16
Conventional								
Soil: 2008	12.00	34.00	408.00	6435.00	519.00	241.00	0.80	8.55
Soil: 2010	4.24	51.49	261.93	5261.23	389.14	146.23	2.98	4.52

Table 13. Juice mineral and nutrient analysis of Meyer lemons (dry weight basis) cultivated under organic and conventional management practices harvested and analyzed at different intervals of storage (days after harvest)*.

Cultivation	Days after harvest	P		K		Ca		Mg		Na		Zn		Fe		Cu		Mn		Protein	
		ppm																			
2008 harvest																					
Conventional	4	1574.99	a	11249.97	b	3347.12	b	959.58	a	608.47	a	8.38	a	11.59	a	4.22	a	11.47	a	6.17	a
Organic	4	1801.24	a	13145.77	a	3885.30	a	1004.09	a	542.47	a	8.91	a	13.24	a	3.87	a	11.94	a	4.42	b
Conventional	11	1691.16	a	11348.00	b	3330.54	b	998.48	a	503.97	a	8.91	a	12.35	a	3.71	a	12.01	a	6.72	a
Organic	11	1731.46	a	12369.20	a	4401.43	a	933.44	a	473.41	a	7.93	a	12.40	a	3.45	a	11.78	a	2.54	b
Conventional	18	1760.44	a	12054.87	b	3470.11	b	1107.87	a	586.76	a	10.05	a	13.38	a	4.13	a	12.29	a	6.58	a
Organic	18	1967.35	a	13621.27	a	4608.05	a	1130.64	a	454.30	b	8.74	a	12.77	a	3.91	a	12.72	a	4.82	b
Conventional	25	1582.35	b	11323.83	b	3308.21	b	1038.72	a	514.88	a	9.63	a	12.55	a	3.85	a	11.95	a	6.47	a
Organic	25	1962.93	a	13197.77	a	4292.78	a	1092.48	a	546.91	a	8.68	a	13.14	a	4.27	a	11.94	a	5.36	b
2010 harvest																					
Conventional	4	1716.51	b	11159.67	b	3149.05	a	856.80	b	307.31	a	5.52	a	7.46	a	2.89	a	4.65	a	5.70	a
Organic	4	2052.17	a	13573.80	a	3631.57	a	983.05	a	187.92	a	5.08	a	9.13	a	2.95	a	3.88	a	5.88	a
Conventional	11	1619.36	b	11050.20	b	3124.65	b	894.46	a	273.79	a	5.30	a	8.06	a	2.89	a	4.93	a	6.24	a
Organic	11	2009.17	a	13534.73	a	3922.16	a	955.39	a	219.02	a	5.29	a	9.00	a	3.02	a	3.72	b	5.89	a
Conventional	18	1726.18	b	11143.67	b	3061.39	b	820.69	b	473.16	a	6.00	a	7.41	a	3.09	a	4.30	a	5.77	a
Organic	18	2067.28	a	13236.97	a	4204.88	a	961.51	a	243.00	b	4.99	a	8.43	a	2.98	a	3.76	a	5.39	a
Conventional	25	1696.34	b	10951.64	b	2975.73	b	815.62	b	508.36	a	5.71	a	7.93	a	3.50	a	4.26	a	5.92	a
Organic	25	2038.49	a	13038.63	a	4171.51	a	957.08	a	277.82	b	5.00	a	9.75	a	2.99	a	3.77	a	5.46	a

∗Each value is average over three replications. Mean separations within each harvest year and for similar storage interval between organic and conventioanl by Duncan's multiple range test at P ≤0.05.

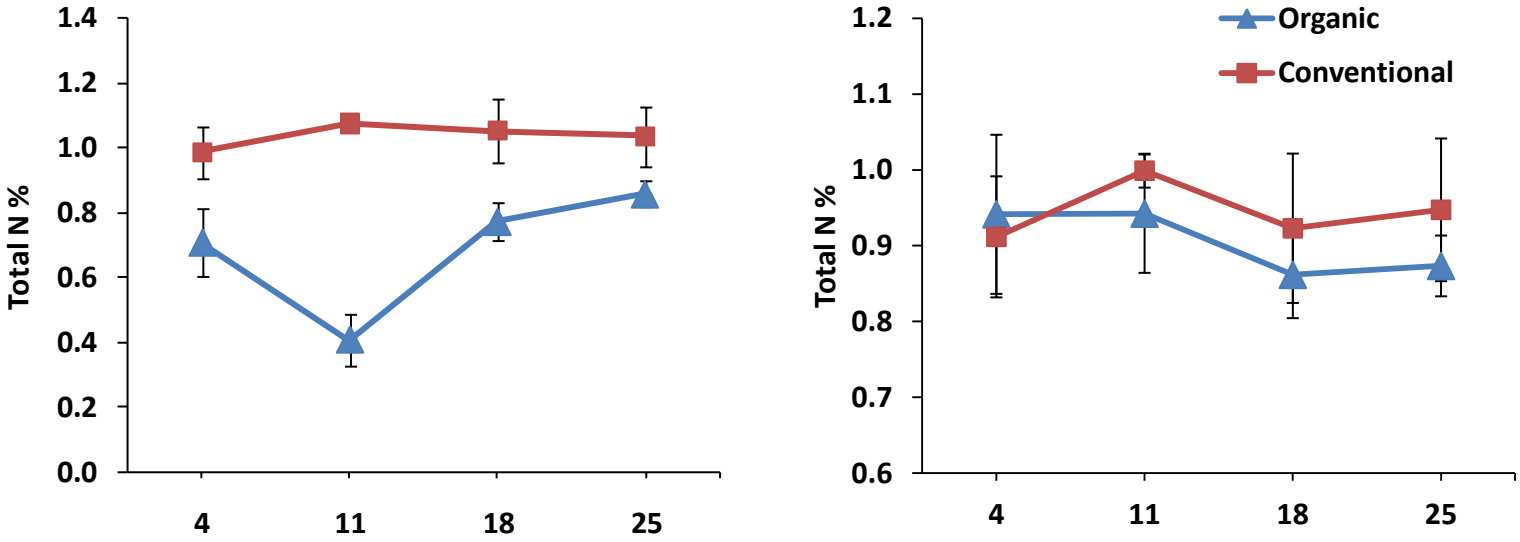

Fig. 19. Total N% of Meyer lemons juice cultivated under organic and conventional management practices harvested in (a) 2008 and (b) 2010 analyzed after different intervals of storage (days after harvest).

5.3 Conclusion

The results from this study suggest that Meyer lemons are a good source of phytochemicals and production systems could be used as an effective tool for modulating their content. Organically produced lemons have higher content of organic acids and flavonoids than conventionally produced. No variation in the content of amines was noticed in lemons produced organically and conventionally. Storage at 10º C helps in maintaining the levels of these phytochemicals without any major adverse affects. Further long term multi location field studies are required to validate the variation of phytochemicals content in Meyer lemon.

6. INFLUENCE OF HOUSEHOLD PROCESSING TECHNIQUES ON GRAPEFRUIT (*Citrus paradisi* MACFAD) PHYTOCHEMICALS*

Among citrus fruits, red grapefruits are distinct with unique sensory quality of sweet and tart taste in addition to red coloration of the juice segments. Grapefruits contain several phytochemicals such as flavonoids, carotenoids, limonoids, organic acids, pectin, and folate [31, 96, 97]. The major flavonoids present in grapefruit are narirutin, naringin, hesperidin, neohesperidin, didymin and poncirin. These phytochemicals were extensively studied using *in vitro* and *in vivo* models to understand their role in human health benefits. Previous studies suggest that these phytochemicals have anti-inflammatory, anti proliferative, anti carcinogenic and antimicrobial properties [31]. Naringin (naringenin-7-O-neohesperidoside) a flavonoid glucoside is one of the major bitter compounds and significantly contributes to the juice sensory taste quality. Additionally, flavonoids have characteristic presence of hydroxyl groups which makes these compounds potent antioxidants. Optimum intake of antioxidants is positively correlated with health benefits such as prevention of certain cancers and cardiovascular diseases [98]. In a clinical trial, naringin was suggested to have lipid lowering property and also increased the erythrocyte antioxidant enzyme activities in hypercholesterolemic subjects [99].

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*Reprinted with permission from "*Grapefruit (Citrus paradisi* Macfad*) Phytochemicals Composition Is Modulated by Household Processing Techniques*", by Ram M. Uckoo, Guddadarangavvanahally K. Jayaprakasha, V. M. Balasubramaniam, Bhimanagouda S. Patil, 2012, *Journal of Food Science*, 77, C921-926, USA. Copyright [2012] by John Wiley and Sons.

Similarly, studies suggest limonoids have anti inflammatory activity, induce gluthathion-S-transferase activity and help in inhibiting carcinogenesis [22, 100, 101]. On the other hand, studies suggest limonin to have inhibitory effect on HIV-1 replication in cell culture systems [102], anti-inflammatory [103] and anti-cancer activity [104]. Additionally, grapefruits are a good source of citric acid and ascorbic acid. Both of these organic acids prevent non enzymatic browning and contribute towards the antioxidant capacity of the fruit [105, 106]. Therefore, optimum dietary intake of these phytochemicals is essential for maintaining ideal health. Grapefruits also contain furocoumarins such as bergamottin, 5-methoxy-7 gernoxycoumarin (5-M-7-GC) and dihydroxybergamottin (DHB). They are known to increase the bioavailability of orally administered drugs by inhibiting CYP 450 enzymes [107]. Although this activity is speculative [108], other reports suggest that these phytochemicals are beneficial and have antitumor activity which may help to protect from cancer [109, 110]. Therefore, to obtain optimum levels of grapefruit phytochemicals, evaluation of processing techniques that may influence their content is essential. Grapefruits have a relishing taste and are popularly eaten fresh. The outer rind of the fruit is leathery and not consumable. Hence, the fruits are either peeled and blended or cut in half and the edible segments are juiced for consumption of fresh juice. These household processing techniques may result in extraction of juice with varying amounts of the phytochemicals. Currently, there are limited reports on evaluation of the phytochemicals content in grapefruits processed by different household techniques. It was reported that the levels of naringin, naringenin and bergapten in grapefruit juice processed by various processing methods varied

significantly. However, phytochemicals such as limonoids and organic acids were not reported [111]. In an another study it was reported that the industrial and laboratorial processed grapefruit juice had significant variation in levels of furocoumarins [112].

Determination of the variation in the levels of grapefruit phytochemicals due to different household processing techniques would be of interest to consumers to obtain higher levels of these compounds. Therefore, the objectives of this study were to quantify the content of flavonoids, limonoids, furocoumarins, organic acids, and evaluate the quality parameters (acidity and total soluble solids) in grapefruit juice processed by blending, hand squeezing and juicing techniques.

6.1 Materials and methods

6.1.1 Chemicals and reagents

HPLC grade acetic acid (>99.5%) and phosphoric acid were purchased from Fluka (Buchi, Switzerland). Narirutin, didymin, hesperidin, were purchased from ChromaDex Inc. (Irvine, CA, USA) whereas meta-phosphoric acid, ascorbic acid, citric acid, naringin, neohesperidin, poncirin and limonin were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile, ethyl acetate and dimethylformamide were purchased from Fisher scientific (Pittsburgh, PA).

6.1.2 Plant material

Mature Texas ‘Rio Red’ grapefruits of uniform size, color and shape were purchased from local grocery store in College station, TX. Each replication consisted of three fruits and 10 such replications were made with a total of 30 fruits. The fruits were stored at 9º C for a period of 18 h before processing.

6.1.3 Household processing of grapefruits

For evaluating the different juice processing methods, each individual fruit was horizontally cut in two equal halves. One half was used for juice extraction using citrus juicer (White-Westing house citrus juicer, Mansfield, OH) while the remaining half fruit was further sliced into two equal halves. One quarter was peeled to separate the segment membranes and the other quarter was hand squeezed to obtain juice. Care was taken to avoid any seeds and the white albedo layer in the samples. The peeled segment membranes were blended in a blender (Vitaprep, Cleveland, OH) to obtain juice. Therefore, each fruit was juiced using three treatment procedures of juicing, squeezing and blending. The blending processing was conducted for 3 minutes for each replicate sample at a speed set at approximately $\geq$ 24,000 rpm. Both the squeezing and juicing treatments had a strainer enabling minimal occurrence of any whole juice vesicles. The juice samples thus obtained were immediately extracted using different solvents for the analysis of phytochemicals content and juice quality parameters.

6.1.4 Acidity and total soluble solids analysis

The titratable acidity of the juice was determined in accordance to the reported method of Nelson et al., [197]. Each treatment juice solution was analyzed for acidity using a computer-controlled, automated pH titration system (Mettler Toledo DL50 Titrator, Schwerzenbach, Switzerland). The pH electrode (Mettler Toledo DG115 SE, Greifensee, Switzerland) was calibrated with pH buffers: 4.0, 7.0, and 10.0 (Fisher Scientific, Fair Lawn, NJ). Total soluble solids (TSS) was determined using a hand held refractrometer (BRIX50 model 137531L0, Leico Microsystems Inc., Buffalo, NY).

6.1.5 Determination of flavonoids

Flavonoid analysis was conducted using the method reported earlier by Vanamala et al., [198] with some modifications. Briefly, 10 mL of grapefruit juice was mixed with 10 mL of dimethyl formamide (DMF) in a 50 mL centrifuge tube and homogenized for 30 s using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA). The homogenized juice was sonicated for 1 h and filtered. The procedure was further repeated two times and all the extracts were pooled together. The extract volume was adjusted to 40 mL by adding DMF. The pooled extract was filtered using 0.45 μm membrane and 10 μL was injected to the HPLC. Flavonoid analysis was conducted using a HPLC system consisting of a Waters 1525 HPLC series (Milford, MA) connected to a PDA detector. Flavonoids were separated on an Xbridge C_{18} column

(3.54 µm, 150 mm × 4.6 mm, i.d.) from Waters (Milford, MA) and detected at λ 280 nm. The gradient mobile phase consisted of (A) 4% acetic acid in water and (B) acetonitrile (MeCN). The elution started at 15% of A and ended at 50% of B in 20 min. Flavonoids were identified by comparing their UV spectra and retention times with those of standards. Quantification of the flavonoids was performed by injecting known concentrations of standards obtained from the commercial source and all samples were analyzed in triplicate.

The identities of the separated flavonoids were validated by LC-MS analysis. The LC system consisted of Finnigan Surveyor plus (West Palm174 Beach, FL, USA) coupled to a mass spectrometer- Ion Trap (LCQ-DECA, ThermoFinnigan). The flavonones were separated on a Aquasil, C_{18} column (3 µm, 150 × 2.1 mm, i.d.) (Keystone-Hypersil, Bellefonte, PA) using a gradient mobile phase of 0.1 % formic acid (A) and acetonitrile (B), maintained at a flow rate of 0.2 mL/min. The elution started with 5% (B), linearly increased to 33% in 9 min, followed by linear increase to 60% in 8 min and finally linearly returned to initial conditions of 5% (B) in 4 min. The mass spectrometer was operated using electron spray ionization in negative ion mode (ESI-) with the spray voltage set at 3.5 kV.

6.1.6 Quantification of limonoids and furocoumarins

Limonoid analysis was conducted using the previously established method with some modifications [144]. Ten mL of homogenized grapefruit juice was extracted with

20 mL of ethyl acetate (EtOAc) in a 50 mL centrifuge tube. The sample was vigorously mixed by vortexing for 3 min and shaking overnight. After extracting the organic layer was separated and collected separately. The procedure was repeated two more times and all the extracts were pooled and transferred to a beaker. The pooled extract was evaporated until completely dry and later reconstituted with 2 mL of DMF. The reconstituted DMF extract was filtered using 0.45 μm membrane and 10 μL of the clear filtrate was injected to the HPLC. Limonoid analysis was conducted using a HPLC system consisting of a Waters 1525 HPLC series (Milford, MA) connected to a PDA detector. Limonoids were separated on an Xbridge C_{18} column (3.54 μm, 150 mm × 4.6 mm, i.d.) from Waters (Milford, MA) and detected at λ 210 nm. The solvent system consisted of 3 mM phosphoric acid (A) in water/ MeCN (B), starting at 15% and ending at 85% MeCN. Limonin was detected at λ 210 nm and identified by comparing the UV spectra and retention time with the known standard. Quantification was done by using known concentrations of standard limonin and all samples were analyzed in triplicate.

Analysis of furocoumarins were conducted using a PerkinElmer (Boston, MA, USA) Series 200 pump coupled with a PerkinElmer Series 200 autosampler and a PerkinElmer Series 200 UV–vis detector using a C_{18} Phenomenex Gemini series column (Torrence, CA, USA), 5 μm particle size, (250 mm × 4.6 mm, i.d.). A gradient mobile phase of 3 mM phosphoric acid in water (A) and acetonitrile (B) was maintained at a flow rate of 1 mL/min. Initially, the elution was started with a gradient of 10% B followed by a linear increase to 60% in 7 min, held for 2 min, linearly increased to 65% in 3 min, held for 1 min, linearly increased to 90% in 7 min, returned back to 10% in

3 min and held for 2 min. Furocoumarins were detected at a wavelength of λ 320 nm. For quantification known concentrations of DHB, bergamottin and 5-M-7-GC previously isolated were analyzed by HPLC for calibrations [107].

6.1.7 Determination of ascorbic acid and citric acid

Simultaneous analysis of ascorbic acid and citric acid was conducted using the previously reported method [37]. Briefly, 10 mL of homogenized juice sample was diluted with 30 mL of 3% meta phosphoric acid (MPA) in a centrifuge tube and mixed for 15 min. Three milliliters of the diluted sample was filtered under vacuum using a 0.45 μm membrane filter (Millipore Corp., Bedford, MA, USA). The residue was re-extracted with 1 mL of 3% MPA and filtered. The procedure was repeated for another two times using 1 mL of 3% MPA each time. The filtrate from all the extractions was pooled and 10 μL was injected to HPLC. The HPLC system consisted of a Waters 1525 HPLC series (Milford, MA, USA) connected to a Waters 2996 PDA detector and Waters 717 auto sampler. The organic acids were separated on Xbridge C_{18} column (3.5 μm, 150 mm × 4.6 mm, i.d.) from Waters (Milford, MA) and citric acid was detected at λ 223 nm while ascorbic acid was detected at λ 254 nm.

6.1.8 Statistical analysis

Data was analyzed using the General Linear Model (GLM) with the Walter-Duncan K-ratio t-test procedure of SAS (version 9.2; SAS Inst. Inc., Cary, N.C., U.S.A.). The analysis of variance differentiates the means by assigning different letters to the treatment means that are significantly different at the 95% level of probability ($P \leq 0.05$). Graphical presentations of the results were made with SigmaPlot 11.0 software program (Systat Software Inc., San Jose, USA)

6.2 Results and discussion

6.2.1 Juice acidity, TSS and yield

The processing techniques evaluated in this investigation did not significantly influence the levels of juice acidity. The juice processed by blending, juicing and hand squeezing had a mean acidity content of 1.36 %, 1.61 % and 1.50 % respectively. The relatively low levels of acidity present in the blended juice could be due to the higher content of pulp and segment membranes. Similar to juice acidity no significant variation in the TSS was also observed but blended juice (12.73 %) had relatively higher brix content as compared to juicing (11.48 %) and hand squeezing (11.86 %).

The volume of juice obtained from each processing treatment was also evaluated. Each replicate sample (comprising of three fruits) resulted in a volume of approximately

150 mL, 140 mL and 420 mL of juice by blending, squeezing and juicing treatments respectively. The higher yield (%) obtained in blending is due to the presence of the segment membranes.

6.2.2 Quantitative analysis of flavonoids in juice processed by different household method

Six flavonoids such as narirutin, naringin, hesperidin, neohesperidin, didymin and poncirin were detected and identified by HPLC analysis (Fig. 20). The identity of the flavonoids was further validated by LC-MS analysis (Fig. 21). Clear separations were observed for all the detected flavonoids. Significantly higher ($P < 0.05$) levels of all flavonoids were present in blended juice as compared to juice obtained by juicing and hand squeezing (Table 14). Naringin was the major flavonoid present in all the three juice processing methods. Approximately, 7 fold higher content of naringin was detected in the blended juice (160.79 mg/100 mL) than the juice processed by juicing (26.25 mg/100 mL) and hand squeezing (22.51 mg/100 mL). Similarly, narirutin and poncirin were also significantly higher in blended juice with a mean content of 15.12 and 16.81 mg/100 mL, respectively. Blended juice had the highest pulp content which corresponds to the maximum levels of naringin. Moreover, peel and segment membranes have higher concentration of flavonoids [199] and due to which the blended juice had higher content in contrast to the juice processed using juicer and hand squeezing techniques. Therefore

blending of grapefruits, which is a common household processing method could be a good strategy to obtain significantly higher levels of flavonoids than juicing and hand squeezing.

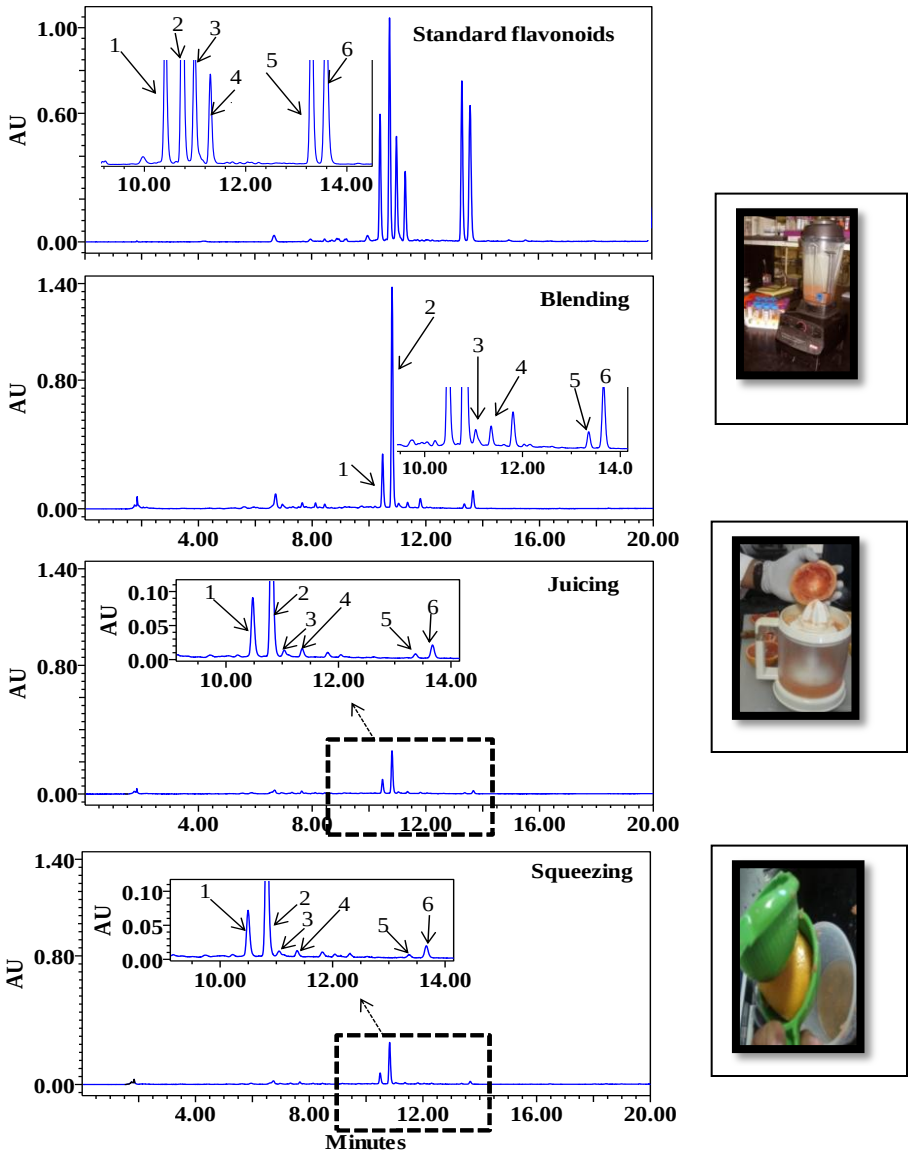

Fig. 20. HPLC chromatograms of grapefruit juice obtained by different processing methods. Peak identification: (1) Narirutin, (2) Naringin, (3) Hesperidin, (4) Neohesperidin, (5) Didymin and (6) Poncirin.

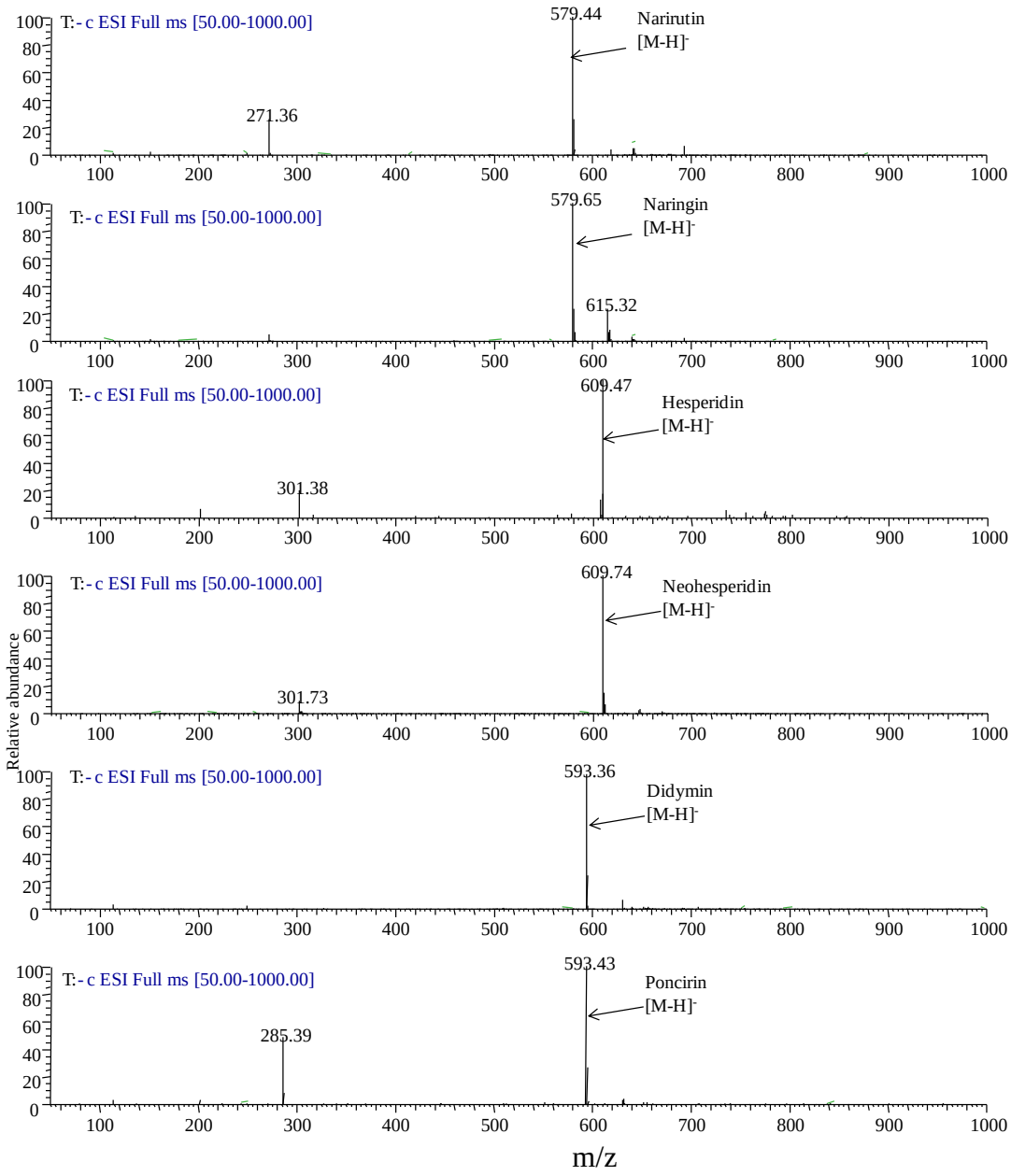

Fig. 21. Mass spectra of flavonoids in negative ion mode obtained by LC-MS analysis of grapefruit juice.

Table 14. Levels of phytochemicals (mg/100 mL) present in grapefruit juice processed by different household processing techniques*.

Phytochemicals	Blending	Juicing	Squeezing
Flavonoids			
Narirutin	15.12 ± 1.76 a	3.40 ± 0.67 b	2.36 ± 0.62 c
Naringin	160.80 ± 18.68 a	26.25 ± 5.44 b	22.51 ± 7.91 b
Hesperidin	7.17 ± 0.96 a	2.84 ± 0.35 b	2.29 ± 0.60 b
Neohesperidin	9.69 ± 1.45 a	3.03 ± 0.57 b	2.20 ± 0.55 c
Didymin	1.38 ± 0.19 a	0.21 ± 0.14 b	0.09 ± 0.14 b
Poncirin	16.82 ± 2.08 a	2.84 ± 0.58 b	2.33 ± 0.78 b
Limonoids			
Limonin	245.58 ± 35.77 a	8.75 ± 4.18 b	15.07 ± 7.61 c
Furocoumarins			
DHB	0.20 ± 0.06 b	0.10 ± 0.03 c	0.38 ± 0.15 a
Bergamottin	0.22 ± 0.05 a	0.06 ± 0.02 c	0.12 ± 0.06 b
5-M-7 GC	0.26 ± 0.06 a	0.07 ± 0.02 c	0.13 ± 0.07 b
Organic acids			
Ascorbic acid	13.44 ± 1.52 b	16.76 ± 3.01 a	15.70 ± 1.48 ab
Citric acid	809.70 ± 90.56 a	664.45 ± 127.31 ab	606.08 ± 73.91 b

* Different letters indicate significant differences at $P < 0.05$ and similar letters indicate no significant differences at $P < 0.05$ for individual phytochemical contrasted between the processing techniques.
± Standard deviation calculated for the three replications.

6.2.3 Quantitative analysis of bitter limonin in different juice processing methods

Limonin is a bitter triterpenoid having a taste threshold of 0.6 mg/ 100 mL [200]. Due to its bitter taste, this phytochemical is considered as a key component for determining the quality of grapefruit juice in the processing industry. Moreover, consumers prefer grapefruit juice which is less bitter. Clear separation of limonin was obtained by HPLC analysis and quantified (Fig. 22). Significant variation in the limonin content was observed in the three processing methods (Table 14). Grapefruit processed by blending had significantly higher limonin content (2.45 mg/ 100 mL) than the juicer (0.14 mg/ 100 mL) and hand squeezed juice (0.09 mg/ 100 mL). The higher levels of limonin detected in the juice processed by blending technique could be due to the occurrence of limonin in the segment membranes which were extracted during the blending process. In contrast, the juice processed by juicing and hand squeezing technique had lower levels of limonin in the juice. These results indicate that processing grapefruits by juicing and hand squeezing could result in these juices being relatively less bitter than the blended juice.

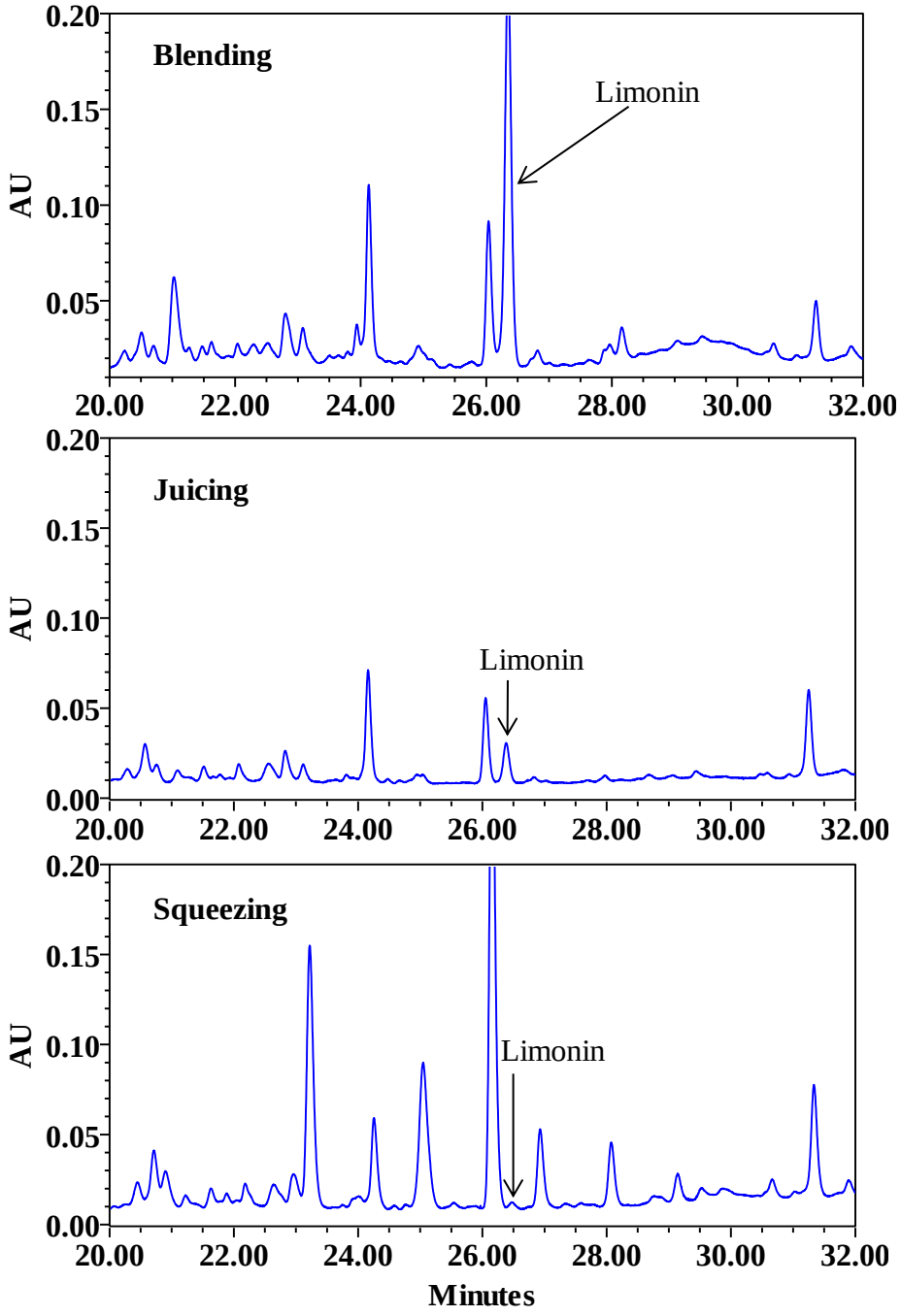

Fig. 22. HPLC chromatograms of grapefruit processed using different household techniques analyzed for determination of limonin content.

6.2.4 Quantitative analysis of furocoumarins

Grapefruit furocoumarins have several biological activities such as anti-tumor activity [110], blocking tumor necrosis factor [201], inhibit carcinogen metabolizing enzymes [202]. They are biologically active even at nano molar concentrations. Therefore, it is very important to understand their levels as affected by different processing techniques. Figure 23 illustrates the different chromatograms for furocoumarins analysis of the processing methods. Among the different processing techniques, grapefruit juice processed by hand squeezing had significantly high levels of DHB (0.38 mg/100 mL) than juice processed by blending (0.20 mg/100 mL) and juicing (0.10 mg/100 mL) (Table 14). Bergamottin and 5-M-7-GC were analyzed to be present in significantly high content in juice processed by blending (bergamottin: 0.22 mg/100 mL, 5-M-7-GC: 0.25 mg/100 mL) than juice extracted by juicing and hand squeezing. These results suggest that blending may be a better juice processing technique to obtain high levels of furocoumarins.

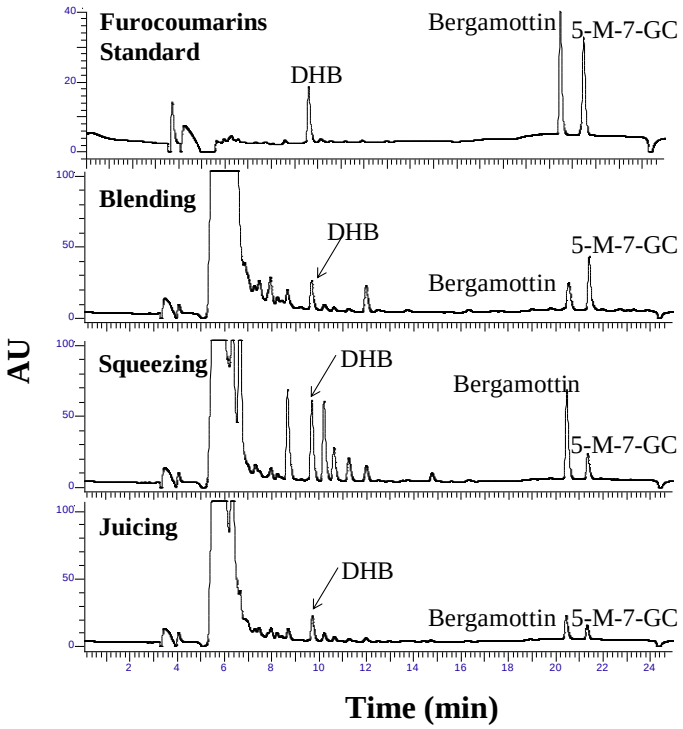

Fig. 23. HPLC chromatograms of grapefruit depicting the presence of furocoumarins in juice obtained by different household processing techniques.

6.2.5 Quantitative analysis of ascorbic and citric acid in different household processing methods

Figure 24 illustrates the different chromatograms for furocoumarins analysis of the processing methods. Juice processed by blending technique had significantly lower levels of ascorbic acid content (13.43 mg/100 mL) in comparison to juicing (16.76 mg/100 mL) and hand squeezing (15.69 mg/100 mL) (Table 14). In contrast, juice

processed by blending (809.70 mg/100 mL) had significantly higher content of citric acid as compared to juice processed by squeezing technique (606.08 mg/100 mL). No significant variation in the levels of citric acid was observed in juice processed by juicing (673.14 mg/100 mL) and squeezing techniques. This could be due to the segment membranes and pulp present in the blended juice, which causes decrease in the relative proportion of soluble content in the juice. Both citric acid and ascorbic acid are highly soluble and this property enables juicing and hand squeezed juice to have relatively similar content. Ascorbic acid and citric acid are the major organic acids characteristic of citrus fruits. These acids cumulatively contribute towards the taste sensory attribute. Among these two, ascorbic acid is a potent antioxidant with several health benefits. Due to its role in health maintaining properties consumers are more interested in consuming food with high ascorbic acid content. However, ascorbic acid is sensitive to temperature and readily oxidizes to form dehydroascorbic acid. This oxidation process is prevented in acidic conditions, which is contributed by citric acid. Citric acid is a weak acid, but due to its occurrence in high content enables in preventing the oxidation of ascorbic acid. Thus, the content of citric acid is indirectly important for antioxidant activity of grapefruit juice.

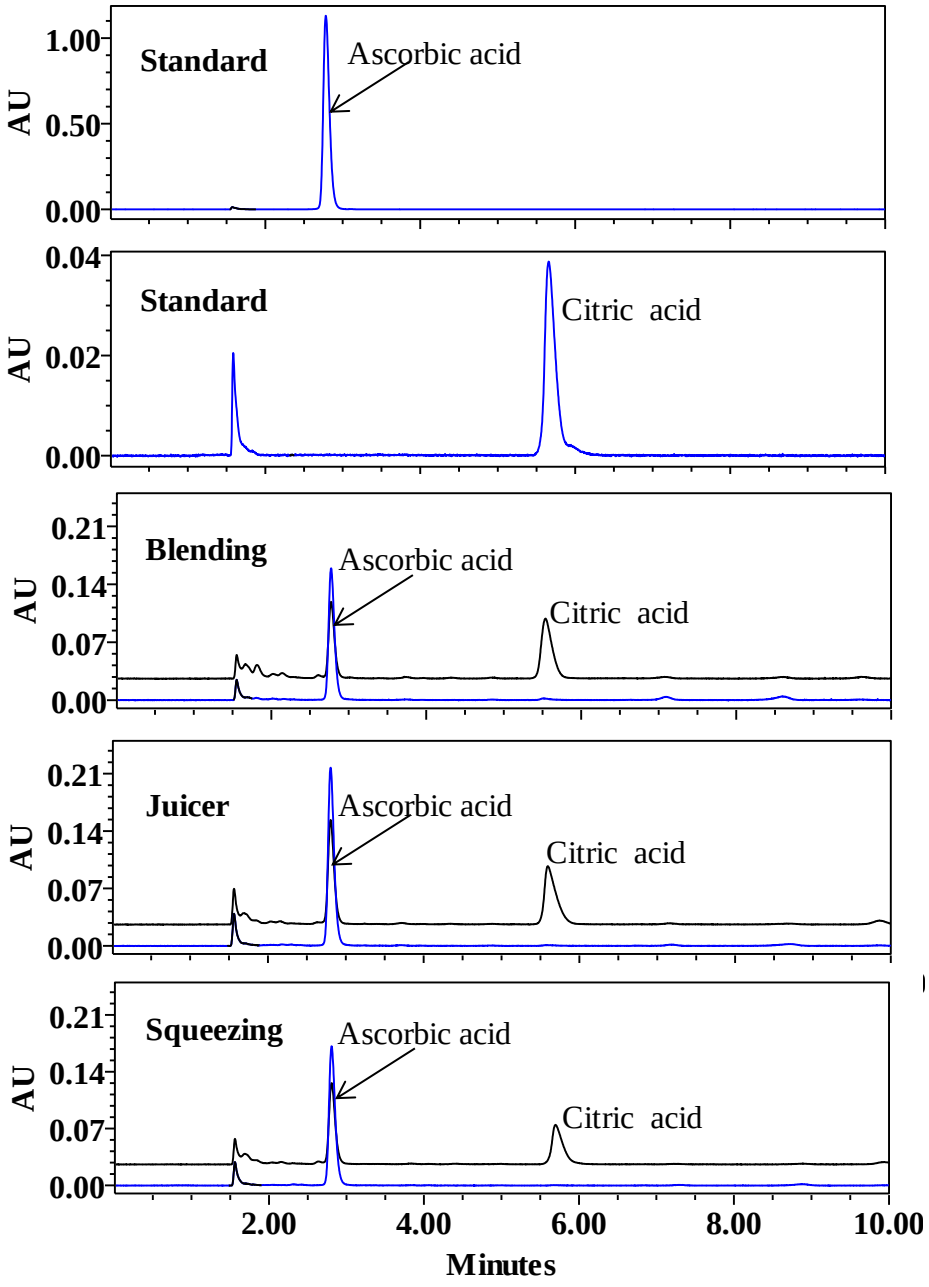

Fig. 24. HPLC chromatogram of grapefruit processed using different household techniques analyzed for determination of ascorbic acid and citric acid content.

6.3 Conclusion

Processing whole edible part of grapefruit by blending resulted in high levels of flavonoids, limonin and citric acid. These phytochemicals may cumulatively contribute to the health maintaining properties. Significantly low levels of phytochemicals were present in juice processed by juicing and squeezing techniques. Results from this study could help the consumers make a better choice for obtaining higher levels of health maintaining phytochemicals. Further studies are required to evaluate the consumer's acceptance of these processing techniques with respect to sensory qualities affected by varying contents of phytochemicals.

7. EFFECT OF INDUSTRIAL APPLICABLE THERMAL AND NON-THERMAL HIGH PRESSURE PROCESSING METHODS ON THE LEVELS OF PHYTOCHEMICALS IN GRAPEFRUIT (*Citrus paradisi* MACFAD) JUICE*

Consumption of citrus fruits and citrus products is associated with various health benefits including prevention of chronic diseases. Citrus fruits are among the most popular fruits consumed and cultivated, with an estimated worldwide production of 51.4 million metric tons [1]. California, Florida and Texas are the major citrus growing states in the U.S. and more than 90% of the citrus fruits are processed for juice production [203]. The large amount of production could be attributed to consumer's demand based on its taste, high nutritional value and health-promoting properties [171, 204]. Among the several species of citrus fruits, Rio Red grapefruits (*Citrus paradisi* var Macfad) are unique, having red pigment in the juice vesicles and relatively high levels of bioactive compounds [37, 205]. The major bioactive compounds present in grapefruits include flavonoids, limonoids, carotenoids, furocoumarins and organic acids [40, 97, 149]. Our recent research demonstrated that certain compounds play a major role in key health-promoting biological properties such as antioxidant, anti-proliferative, anti-inflammatory, anti-carcinogenic and anti-microbial activities [22, 100, 171].

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*Reprinted with permission from "*High pressure processing controls microbial growth and minimally alters the levels of health promoting compounds in grapefruit (Citrus paradisi* Macfad*) juice"*, by Ram M. Uckoo, Guddadarangavvanahally K. Jayaprakasha, Bhimanagouda S. Patil, 2012, *Innovative Food Science & Emerging Technologies* http://dx.doi.org/10.1016/j.ifset.2012.11.010, USA. Copyright [2012] by Elsevier.

The levels of health promoting bioactive compounds vary significantly by variety, maturity, cultivation practices, environment, storage and processing methods [112, 113]. In recent years, technological advances in processing have resulted in the development of novel techniques such as high pressure processing (HPP). This method of non-thermal processing provides several advantages over traditional thermal processing (TP). High pressure processing inactivates pathogens, inhibits degradative enzymes, and prevents the degradation of antioxidants [114]. Unlike TP, the moderate temperatures used in HPP maintain the texture, flavors, nutrients, and other sensory quality attributes of the product [115, 116]. These benefits have led to the implementation of HPP in several fruit and vegetable processing industries [117]. Reports suggest that HPP of orange juice maintained its acceptability to consumers for up to 12 weeks of storage at 4^{o} C without any significant variation in odor and flavor profiles [118]. HPP was also shown to produce significant inactivation of pectin methylesterase activity and reduction of microorganisms in orange juice [119]. In an another study, HPP was found to be an excellent technique for maintaining the levels of ascorbic acid and anthocyanins, which are both potent antioxidants, in blood orange juice [120]. Very few studies of this emerging technique have been reported examining the effects of HPP on bioactive compounds in orange juice [118, 121]. However, there are no reports on the effects of HPP on the levels of bioactive compounds in grapefruit juice.

Measuring the levels of bioactive compounds in grapefruits processed by HPP, to determine whether this method can effectively maintain the health-promoting properties of grapefruit juice as well as, or better than, current processing methods, would benefit

both consumers and juice processing industries. In this study, the effects of two processing techniques (HPP and TP) on the levels of bioactive compounds in grapefruit juice were evaluated, in comparison with control, during 21 days of storage. Additionally, the shelf life of the treated juice stored at 4^{o} C was determined by microbial count after 28 days. To the best of our knowledge, this is the first report on comparative analysis of bioactive compounds in grapefruit juice processed by HPP and TP and stored at refrigerated conditions.

7.1 Materials and methods

7.1.1 Chemicals and reagents

Phosphoric acid (HPLC grade) was purchased from Fluka (Milwaukee, WI). Narirutin, didymin, poncirin, and neohesperidin were purchased from ChromaDex Inc. (Irvine, CA, USA). Meta-phosphoric acid, ascorbic acid, citric acid, naringin, and limonin were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (HPLC grade), ethyl acetate and acetone were purchased from Fisher scientific (Pittsburgh, PA). Nanopure water (NANOpure, Barnstead/Thermolyne Corp. Dubuque, IA) was used for sample preparation and HPLC analysis.

7.1.2 Plant material and packaging

Mature ‘Rio Red’ grapefruits (*Citrus paradisi* var Macfad) of uniform size, color and shape were purchased from a local grocery store in Columbus, OH. The fruits were washed with water, sliced equatorially and juiced using a Proctor Silex 66332RH Juicit citrus juicer (Southern Pines, NC). Following extraction, the juice was passed through a 20 mesh sieve (Fisher Scientific Co., IL) to separate any large pulp material. One hundred grams of juice was packaged into each 15 × 7 cm EVOH flexible pouch (Thompson Equipment and Supply, Cincinnati, OH) and sealed using a hand impulse sealer (MP-16C, Midwest Pacific, J.J. Elmer Corp., St. Louis, MO), removing as much air as possible. The samples were then cooled to 4° C and held overnight prior to processing. The packaged juice samples were further subjected to TP, HPP and control (no processing) treatments as described below.

7.1.3 Thermal processing

Packed juice samples were heated to 85° C and held for 45 s in a steam jacketed kettle, then immediately cooled to 4° C in an ice-water bath. Temperature of the samples was monitored using a brass stuffing box (Ecklund-Harrison, Fort Myers, FL) and a T-type thermocouple (Omega Engineering, Inc., Stamford, CT). Half of the day 0 samples were placed into a blast freezer (-40° C) and storage samples were placed in a 4° C walk-in cooler. The processed refrigerated and frozen samples were shipped under

refrigerated conditions by overnight shipment from Ohio State University, Columbus, OH (OSU) to the Vegetable and Fruit Improvement Center at Texas A&M University, College Station, TX (TAMU) for analysis of specific bioactive compounds.

7.1.4 High pressure processing

Juice samples were maintained at 4° C in a refrigerator prior to pressure processing. The samples were loaded into a cylindrical stainless steel loading basket (11.0 cm × 65.5 cm) insulated with 0.5 cm polytetrafluoroethylene (PTFE) and filled with USP kosher propylene glycol at 4° C (Brenntag Mid-South, Inc., St. Louis, MO). The basket was then loaded into the pressure chamber of a Stansted Iso-Lab FPG11500 (Stansted Fluid Power LTD, Stansted, Essex, UK), with vessel temperature maintained at 15° C. Temperature of the samples was monitored using a brass stuffing box (Ecklund-Harrison, Fort Myers, FL) and a T-type thermocouple (Omega Engineering, Inc., Stamford, CT) at 5 cm from the top and 5 cm from the bottom of the loading basket. The system was pressurized to 402 ± 1.9 MPa with a pressure come-up time of 120 s and held for 3 min. Sample temperature under pressure was maintained at 31.8 ± 0.5° C from both monitoring positions. The vessel was depressurized to ambient pressure (90 s), the samples were unloaded and the packages rinsed. Additional details of the high pressure processing equipment are described elsewhere [206]. One half of the initial day 0 storage samples were placed into a blast freezer (-40° C) in preparation for frozen shipment for analysis. Storage study samples were placed in a 4° C walk-in

cooler. The processed refrigerated and frozen samples were shipped under refrigerated conditions using overnight shipment from OSU to TAMU for analysis of bioactive compounds.

7.1.5 Storage

The grapefruit juice pouches processed by TP, HPP and control treatment received at TAMU were stored in a refrigerator maintained at 4° C and analyzed for phytochemicals at 0, 7, 14, and 21 days after storage. Each treatment consisted of three replications (individual pouches containing 100 mL juice). All samples for day 0 storage analysis were extracted and analyzed by HPLC on the same day (approximately 24 h after treatment) that the shipment was received. Sample pouches for both treatments and control were also stored at OSU for 28 days and maintained at 4° C for microbial and pH analysis.

7.1.6 Color measurement

The color of the treated juice was measured with a tristimulus reflectance colorimeter -Minolta CR-400 Chroma Meter (Konica Minolta Sensing, Inc., Osaka, Japan). The instrument was calibrated using the white calibration plate (Calibration Plate CR-A43, Minolta Cameras, Osaka, Japan) before the measurement. Color measurements were recorded for all the replicates of the two treatments and the control. In brief, 30 mL

of homogenous juice was transferred into the liquid tester of the Minolta CR-400 Chroma Meter for measurement. Color was expressed in Hunter Lab units, L* indicating lightness (varying from 0, black, to 100, white), *a** indicating red-green (varying from -60, green, to +60, red) and *b** indicating yellow-blue (varying from -60, blue, to +60, yellow).

7.1.7 Analysis of organic acids

Ascorbic acid and citric acid were simultaneously extracted and analyzed using a previously reported method from our lab, with minor modifications (Uckoo, Jayaprakasha, Nelson, & Patil, 2011). Briefly, 10 mL of fresh juice was diluted with 10 mL of 3% metaphosphoric acid, in a centrifuge tube and mixed for 15 min. The diluted sample was centrifuged at 10,000 rpm for 10 minutes and the clear supernatant was filtered through a 0.45 µm membrane filter (Millipore Corp., Bedford, MA, USA). Ten µL was injected into the HPLC for analysis. The HPLC system consisted of a Waters 1525 HPLC series (Milford, MA, USA) connected to a Waters 2996 PDA detector and Waters 717 autosampler. The mobile phase consisted of isocratic 3 mM phosphoric acid in water maintained at a flow rate of 1 mL/min. Ascorbic acid and citric acid were separated on a C_{18}, Phenomenex Gemini series column (Torrence, CA, USA), 5 µm particle size, (250 mm × 4.6 mm). Peak separation of ascorbic acid and citric acid was monitored at λ_{254} nm and λ_{223} nm respectively. Chromatographic data were collected and processed using Empower2 software (Waters, Milford, MA, USA).

7.1.8 Determination of flavonoids and limonoids

Flavonoid analysis was conducted using a method previously reported from our lab, with some modifications [198]. Briefly, 10 mL of grapefruit juice was mixed with 30 mL of methanol in a 50 mL centrifuge tube and homogenized for 30 s using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA). The homogenized juice was sonicated for 1 h and filtered. The extract volume was adjusted to 40 mL by adding MeOH. The pooled extract was filtered using 0.45 μm membrane and 10 μL was injected to the HPLC. Separations were conducted on an HPLC system consisting of a Waters 1525 HPLC series (Milford, MA) connected to a PDA detector. The limonoids and flavonoids were separated on a C_{18}, Phenomenex Gemini series column (Torrence, CA, USA), 5 μm particle size, (250 mm × 4.6 mm). The gradient solvent system consisted of 3 mM phosphoric acid in water/ MeCN gradient starting at 15% and ending at 85% MeCN. Limonoids (deacetyl nomilinicacid glucoside-DNAG and limonin) and flavonoids were detected at λ_{210} nm and λ_{280} nm respectively. The separated flavonoids and limonoids were identified by comparing their UV spectra and retention time with known standards. Chromatographic data were collected and processed using Empower2 software (Waters, Milford, MA, USA). Quantification was done by comparison to known concentrations of standard flavonoids and limonoids.

7.1.9 Analysis of furocoumarins

Ten mL of homogenized grapefruit juice was extracted with 20 mL of ethyl acetate in a 50 mL centrifuge tube. The sample was vigorously mixed on a mechanical shaker for 8 h, followed by separation of the organic layer. The procedure was repeated two more times (1 h each) and all the extracts were pooled and transferred to a beaker. The pooled extract was evaporated until completely dry and reconstituted with 1.5 mL of acetone under sonication. The reconstituted extracts were analyzed for furocoumarins using a Finnigan Surveyor plus HPLC system (West Palm Beach, FL, USA) equipped with a PDA plus detector coupled with a quaternary LC pump and a surveyor plus auto-sampler. The furocoumarins were separated on a C_{18}, Phenomenex Gemini series column (Torrence, CA, USA), 5 µm particle size, (250 mm × 4.6 mm). Furocoumarins were detected at a wavelength of λ_{320} nm and the analysis was carried out using Chromquest 5.0 software. A gradient mobile phase of 3 mM phosphoric acid in water (A) and acetonitrile (B), maintained at a flow rate of 1 mL/min was used for the separations. The elution was started with a gradient of 10% B followed by a linear increase to 60% in 7 min, held for 2 min, linearly increased to 65% in 3 min, held for 1 min, linearly increased to 90% in 7 min, returned back to 10% in 3 min and held for 2 min. For quantification, known concentrations of dihydroxybergamottin (DHB), bergamottin and 5-gernyloxy-7-methoxycoumarin (5-G-7-MC) previously isolated in our lab were analyzed by HPLC for calibration [107].

7.1.10 Analysis of carotenoids

Ten mL of homogenized grapefruit juice was extracted with 10 mL of chloroform (CH_3Cl_3) in a 50 mL centrifuge tube. The samples were vigorously agitated on a shaker at 200 rpm. The samples were kept in the dark, covered with a black cloth, and maintained at 4^oC using crushed ice to avoid any degradation of the carotenoids. After 1 h of shaking, the organic layer was separated, filtered and measured. Ten µL of the filtered extract was injected into the HPLC for carotenoid analysis. Analysis was performed on an Agilent Series 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA), equipped with a vacuum degasser, a quaternary pump, an autosampler and a diode array detector, connected to Agilent ChemStation software. Carotenoids were separated on a C_{18} Phenomenex Gemini series column (Torrence, CA), 5 µm particle size (250 mm × 4.6 mm). The peak separations of β-carotene and all-*trans*-lycopene were monitored at λ_{450} nm and λ_{470} nm respectively. The binary solvent system used was 3 mM phosphoric acid (solvent A, pH 3.0) and acetonitrile (solvent B) maintained at a flow-rate of 1 mL/min and the injection volume was 5 µl. For quantification known concentrations of standard β-carotene and all-*trans*-lycopene were analyzed by HPLC for calibration.

7.1.11 Microbial analysis

The total plate counts of the untreated and treated juice samples were determined using the spread plate method on trypticase soy agar (TSA; Becton, Dickinson and Co., Sparks, MD, USA) incubated at 37°C for 72 h. The yeast and mold count of the juice was also measured using the spread plate method on potato dextrose agar (PDA; Becton, Dickinson and Co., Sparks, MD, USA) adjusted to pH 3.5 with 10% tartaric acid, after incubation at 25°C for 5 days. To prepare the dilutions, each of the duplicate pouches was opened under aseptic conditions. One mL of juice was taken from each pouch and mixed with 9 mL of sterile 0.1% (w/v) peptone water. Serial dilutions were prepared in 0.1 % (w/v) peptone water. The detection limit of the plating method was 10 CFU/mL.

7.1.12 pH measurement

The pH of untreated, HPP and TP treated juice samples was determined at day 0 and 28 using a Denver Instruments Model 215 pH meter (Bohemia, NY). The pH meter was calibrated using pH 4 and pH 10 standard stock solutions (Fisher Scientific Co., IL) prior to analysis.

7.1.13 Statistical analysis

Data were analyzed using the General Linear Model (GLM) with the Walter-Duncan K-ratio t-test procedure of SAS (version 9.2; SAS Inst. Inc., Cary, N.C., U.S.A.). The analysis of variance differentiates the means by assigning different letters to the treatment means that are significantly different at the 95% level of probability ($P \leq 0.05$). Graphical presentations of the results were made with SigmaPlot 11.0 software program (Systat Software Inc., San Jose, USA).

7.2 Results and discussion

7.2.1 Color

Color is a major quality criterion for consumer preference and is measured as a parameter of juice quality. The change in color also acts as an indicator for determining degradation of phytochemicals, such as reduction of ascorbic acid [207]. Figure 25 illustrates the change in L^*, a^* and b^* of the HPP, TP and control juices. A gradual trend of decrease in L^*, a^* and b^* were noticed as the storage time increased. The maximum rate of decrease in a^* and b^* was noticed at 7 days after storage. During the remainder of the storage period, both a^* and b^* remained unaltered. Among the processing treatments, HPP enabled the juice to maintain L^*, a^* and b^* with no significant ($P < 0.05$) variation as compared to the control treatment. However in TP juice, both a^* and

*b** had significant ($P < 0.05$) variation in comparison to the untreated control juice. These results suggest that storage and TP had a significant impact on grapefruit juice color. Grapefruit juice treated with TP had significantly higher *L** and *b** values throughout the storage period, suggesting that higher temperature negatively affects the color of the juice. Furthermore, the TP treated juice visually appeared to be considerably paler colored than the HPP and control (Figure 26). Similar results were also reported by Lee and Coates [208], evaluating the effect of TP on color of Red grapefruits. TP of oranges and carrots significantly decreased the color parameter *a** during storage, but HPP produced minimal changes in color [209, 210]. In an another study, an increase in the intensity of HPP treatment on pomegranate juice significantly decreased the color parameters in comparison to untreated juice [211]. Our results provide further evidence of the benefits of HPP treatment in maintaining the color of fruit juices and indicate that HPP can be used as an alternative to TP to maintain the fresh-like color of grapefruit juice preferred by consumers.

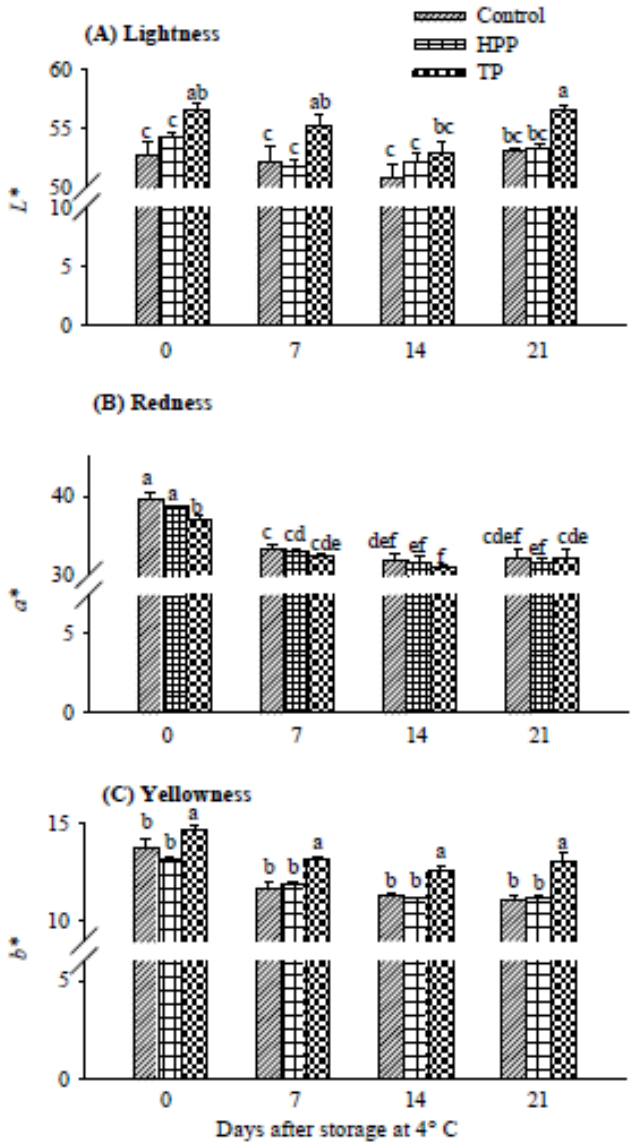

Fig. 25. Color parameters (*L*a*b** values) of grapefruit juice untreated (control) and treated with HPP and TP techniques. All values are means ± SD of three replicates. Bars with the same letter are not significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100). Bars with different letters are significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100).

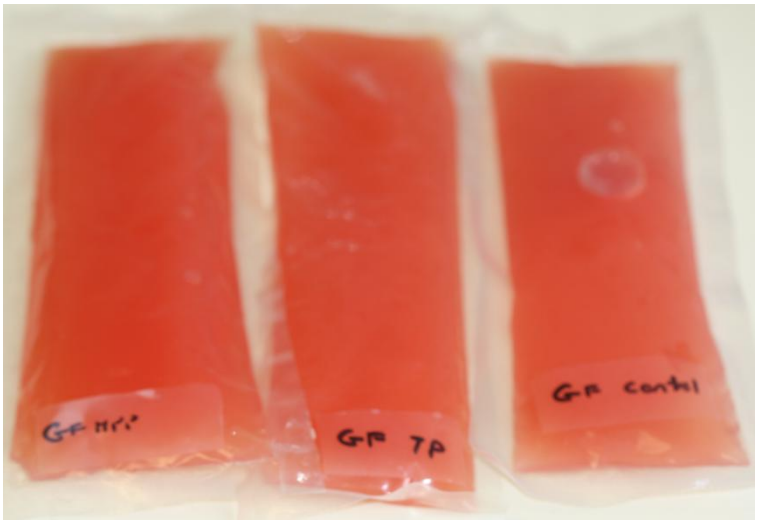

Fig. 26. Grapefruit juice pouches treated with HPP (GF HPP), Thermal processing (GF TP) and untreated (GF Control) after 21 days of storage at 4^{o} C.

7.2.2 Organic acids

Citrus fruits are a good source of organic acids such as ascorbic acid and citric acid. These two organic acids collectively contribute to the taste and health benefits of citrus fruits [212]. Ascorbic acid is a potent antioxidant and essential for scavenging radical singlet O^{-} and OH^{-} ions [213]. Maintaining the optimum contents of ascorbic acid in grapefruit juice is important for preventing damage caused by reactive oxygen. Figure 27A and 27B illustrate the levels of ascorbic acid and citric acid in grapefruit juice processed by different treatments and control. No significant ($P \leq 0.05$) variation in the citric acid levels were noticed among control and HPP treated juice during the storage period. However, both processing and storage significantly affected the ascorbic acid contents of juice. Among the processing treatments, HPP juice had significantly higher

levels of ascorbic acid than TP juice at 0 and 7 days after storage. However, no significant effects on levels of ascorbic acid were observed in the HPP and TP treated juice samples after refrigerated storage for 14 and 21 days. TP samples had significantly lower ascorbic acid contents compared to the control juice throughout the storage period. Furthermore, a gradual decrease in ascorbic acid contents during storage was observed in both HPP and TP juice, and in control juice samples. The significant change in the levels of ascorbic acid due to HPP and TP treatment as compared to control at 0 and 7 days after storage could be due to degradation of ascorbic acid by moderate-high temperatures during processing. The absence of significant differences at 14 and 21 days of storage in both treatments could be due to the gradual acclimatization of the juice with the storage conditions as well as absence of any air. Similarly, packaging material and inactivation of oxidative enzymes such as polyphenol oxidase may also be contributing factors [214]. Ascorbic acid is an antioxidant with high reactivity and low stability. These factors could have caused the gradual decline in the levels of ascorbic acid during storage. A similar trend in ascorbic acid contents during refrigerated storage was reported in blood orange juice [215] and concentrates of various citrus juices at elevated temperatures [216]. Similarly, Bull et al., (2004) reported a decrease in ascorbic acid in valencia and navel orange juice treated with HPP or TP. Polydera et al., [217] reported that HPP orange juice (500 MPa, at 35° C for 5 min) maintained its ascorbic acid contents better than TP juice (80° C, 30 s). In other non acidic fruits such as strawberry, HPP treatment at 600 MPa preserved more (94%) of the ascorbic acid, compared to TP at 70° C

(77.4%). Overall, results from this study and other reported studies suggest that HPP is a comparable processing technique that helps in maintaining ascorbic acid contents.

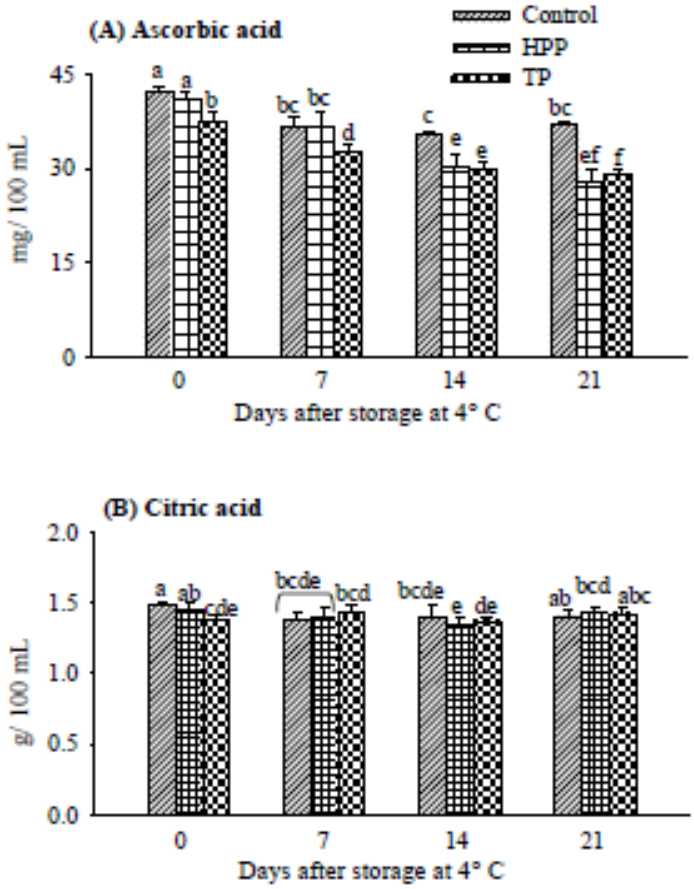

Fig. 27. (A) Ascorbic acid (mg/100 mL) and (B) citric acid (g/100 mL) contents of grapefruit juice untreated (control) and treated with HPP and TP, analyzed after storage for different times. All values are means ± SD of three replicates. Bars with the same letter are not significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100). Bars with different letters are significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100).

7.2.3 Flavonoids and limonoids

Grapefruits are a good source of flavanones, a group of flavonoids that are unique to citrus species and well known for several health promoting properties [218]. Five different flavanones such as narirutin, naringin, neohesperidin, didymin and poncirin were detected in the grapefruit juices. Processing treatments and storage demonstrated significant effects on the levels of these bioactive compounds (Table 15). Naringin and narirutin were the predominant whereas, neohesperidin, didymin and poncirin were comparatively lower in concentration. In general, processing grapefruit juice with HPP and TP had a significant ($P < 0.05$) influence on the levels of flavonoids, which was most distinct at 7 days after storage. On the initial day (day 0) of storage, no significant variations in the levels of flavonoids were found among both the processing treatments and the control. At 7 days after storage, significantly ($P < 0.05$) higher levels of narirutin (9.76 mg/100 mL) and naringin, (26.69 mg/100 mL) were measured in the TP juice than the control (narirutin: 0.73 mg/100 mL, naringin: 25.16 mg/100 mL) and HPP (narirutin: 0.61 mg/100 mL and naringin: 23.58 mg/100 mL) juice. A similar trend of increase in the mean levels of neohesperidin (1.40 mg/100 mL), didymin (0.51 mg/100 mL) and poncirin (0.77 mg/100 mL) was observed in TP juice at 7 days after storage as compared to their levels at 0 days. However at 14 and 21 days after storage, no significant change in the levels of narirutin, naringin, neohesperidin and pocirin was found in the treatments and control. HPP did not cause any significant change in the levels of neohesperidin, didymin and poncirin with respect to the control treatment during the entire storage

period. Flavonoids are thermally stable and do not degrade during storage. Previous studies have reported similar results with approximately no changes in the levels during chilled storage of orange juice processed by HPP and TP treatments [219]. Naringin, poncirin and neohesperidin are bitter-tasting compounds and collectively contribute to the characteristic flavor of grapefruit juice [220-222]. Flavor, an important parameter, influences both overall quality and consumers' preference for grapefruit juice. Due to the economic significance of these health promoting compounds, maintaining optimum levels is an important criterion for the juice processing industries [223]. Results from the current study suggest that HPP could be used as an alternate non-thermal processing technique for long-term storage of fresh grapefruit juice without any adverse effect on the levels of flavonoids.

Limonoids are triterpenoids that contribute substantially to the sensory qualities of grapefruit juice [25]. In the HPLC analysis of grapefruit juice, two limonoids, limonin and DNAG, were detected. Processing and storage produced significant changes in limonin contents (Fig. 28). The levels of limonin were significantly higher at 7 days after storage in treated and control samples, as compared to 0 days and 14 days. A similar trend was also noticed in the levels of DNAG. TP juice had significantly higher levels of DNAG (21.56 mg/100 mL) than control (16.77 mg/100 mL) and HPP (18.06 mg/100 mL) after 7 days of storage. No significant changes in levels of limonoids were observed between control and HPP treatment at 0, 7 and 14 days of storage suggesting that limonoids are stabile during processing. Limonoids are potential anticancer agents that were studied in *in vitro* for their ability to inhibit proliferation of colon [224],

neuroblastoma [29] and breast cancer cells [225]. Due to the potential health benefits associated with the dietary intake of limonoids, maintaining their levels in grapefruit juice is critical. Results from this study suggest that HPP helps maintain the levels of limonoids similar to TP.

Table 15. Levels of flavonoids (mg/100 mL) in grapefruit juice untreated (control) and treated with HPP and TP techniques analyzed after storage at 4º C*.

	Storage (Days)	Control	HPP	TP
Narirutin	0	7.01 ± 0.25 d	7.01 ± 0.32 d	8.01 ± 0.56 bcd
	7	9.05 ± 1.75 ab	8.42 ± 0.74 bc	9.76 ± 1.92 a
	14	7.71 ± 0.21 cd	7.83 ± 0.55 cd	8.77 ± 0.84 abc
	21	8.52 ± 0.23 bc	7.90 ± 0.16 bcd	7.67 ± 0.52 cd
Naringin	0	21.26 ± 0.39 d	21.04 ± 0.61 d	23.24 ± 1.33 bcd
	7	25.16 ± 3.82 ab	23.58 ± 1.57 bcd	26.69 ± 4.39 a
	14	22.34 ± 0.46 cd	22.37 ± 1.20 cd	24.77 ± 1.94 abc
	21	23.68 ± 0.36 bcd	22.59 ± 0.55 bcd	21.99 ± 0.99 d
Neohesperidin	0	0.79 ± 0.18 d	0.82 ± 0.10 d	0.96 ± 0.20 cd
	7	1.23 ± 0.58 abc	1.47 ± 0.26 a	1.40 ± 0.49 ab
	14	0.78 ± 0.18 d	0.76 ± 0.10 d	0.87 ± 0.11 cd
	21	1.01 ± 0.14 cd	1.10 ± 0.17 bcd	1.05 ± 0.15 bcd
Didymin	0	0.43 ± 0.11 abc	0.48 ± 0.08 abc	0.41 ± 0.08 abc
	7	0.49 ± 0.19 ab	0.41 ± 0.09 ab	0.51 ± 0.20 ab
	14	0.39 ± 0.07 bc	0.31 ± 0.10 bc	0.57 ± 0.13 a
	21	0.43 ± 0.11 abc	0.35 ± 0.09 abc	0.35 ± 0.05 bc
Poncirin	0	0.61 ± 0.15 bc	0.56 ± 0.05 c	0.66 ± 0.07 abc
	7	0.73 ± 0.15 ab	0.61 ± 0.08 bc	0.77 ± 0.20 a
	14	0.60 ± 0.11 bc	0.59 ± 0.03 bc	0.69 ± 0.09 abc
	21	0.59 ± 0.06 bc	0.61 ± 0.05 bc	0.58 ± 0.08 c

* All values are means ± SD of three replicates. Means in the same column and row for individual phytochemical followed by the same letter are not significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100). Means in the same column

and row for individual phytochemical followed by different letters are significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100).

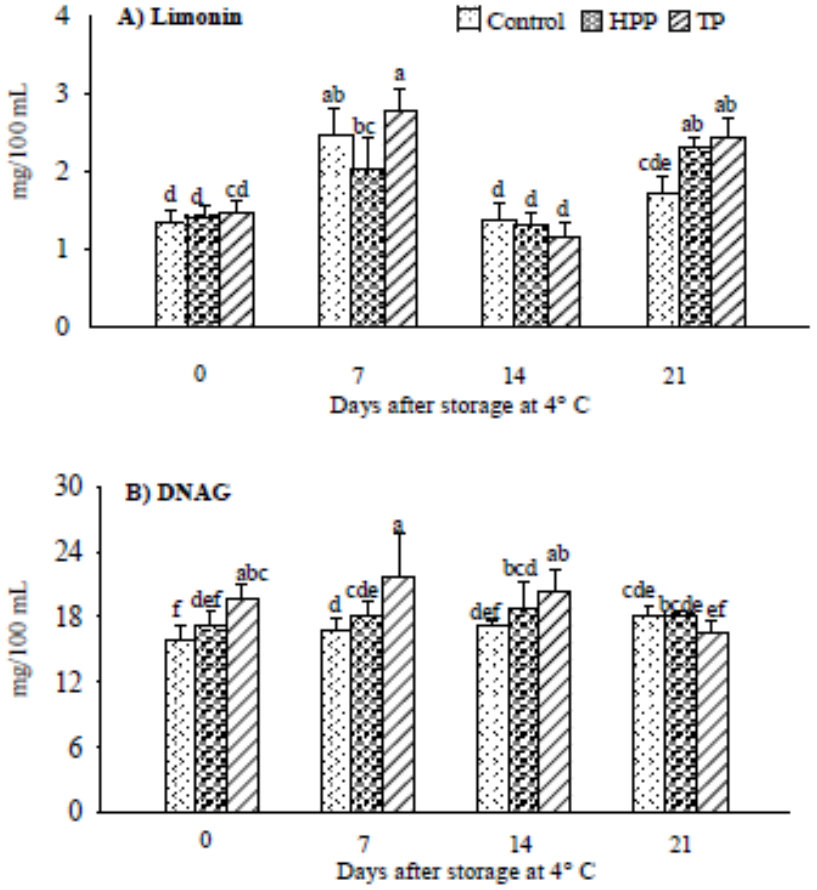

Fig. 28. Limonoids, A) Limonin and B) Deacetyl nomilinicacid glucoside (DNAG) content (mg/100mL) of grapefruit juice untreated (control) and treated with HPP or TP analyzed after storage for different times. All values are means ± SD of three replicates. Bars with the same letter are not significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100). Bars with different letters are significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100).

7.2.4 Furocoumarins

Three furocoumarins, DHB, bergamottin and 5-G-7-MC, were detected in the grapefruit juice samples. Processing grapefruit by HPP and TP had a significant effect on the levels of furocoumarins (Table 16). The levels of furocoumarins (DHB and 5-G-7-MC) in both HPP and TP treated juice significantly decreased after 14 days of storage as compared to 0 days of storage. No significant change in the levels of furocoumarins was found during storage of HPP and control samples. Thermal processed juice at 21 days after storage had significantly higher levels of bergamottin and 5-G-7-MC as compared to 14 days after treatment. These results suggest that processing treatments did not have any adverse effect on the levels of furocoumarins but that levels can vary during the storage period.

Table 16. Levels of furocoumarins (mg/100 mL) in grapefruit juice untreated (control) and treated with HPP and TP techniques analyzed after storage at 4º C *.

	Storage (Days)	Control	HPP	TP
DHB	0	0.56 ± 0.04 c	0.62 ± 0.03 ab	0.62 ± 0.09 ab
	7	0.62 ± 0.07 ab	0.58 ± 0.09 b	0.67 ± 0.10 a
	14	0.44 ± 0.05 d	0.44 ± 0.02 d	0.37 ± 0.06 e
	21	0.47 ± 0.06 d	0.47 ± 0.02 d	0.45 ± 0.08 d
Bergamottin	0	0.09 ± 0.01 cd	0.11 ± 0.02 bc	0.10 ± 0.01 cd
	7	0.09 ± 0.03 cd	0.11 ± 0.03 bc	0.10 ± 0.01 cd
	14	0.09 ± 0.02 cd	0.08 ± 0.01 d	0.07 ± 0.02 d
	21	0.08 ± 0.02 d	0.13 ± 0.03 ab	0.15 ± 0.08 a
5-G-7-MC	0	0.38 ± 0.02 bc	0.42 ± 0.03 ab	0.42 ± 0.04 ab
	7	0.38 ± 0.09 bc	0.42 ± 0.10 ab	0.41 ± 0.03 ab
	14	0.35 ± 0.03 c	0.34 ± 0.01 c	0.29 ± 0.03 d
	21	0.34 ± 0.05 c	0.35 ± 0.04 c	0.43 ± 0.13 a

* All values are means ± SD of three replicates. Means in the same column and row for individual phytochemical followed by the same letter are not significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100). Means in the same column and row for individual phytochemical followed by different letters are significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100).

7.2.5 Carotenoids

All-trans-lycopene and β-carotene were the two carotenoids detected in grapefruit juice. The levels of lycopene were significantly lower in the TP treated juice than the control juice at 7 and 14 days after storage (Fig. 29). No significant variation in the contents of lycopene was found at 0 days of storage among the processing treatments.

However, at 7 days after storage both HPP and TP treated juice had significantly lower levels of lycopene as compared to the control. In TP juice, the levels of lycopene were significantly lower at 7, 14 and 21 days after storage than at 0 days after storage. In the control, no significant variations were found at 0, 7 and 14 days after storage. At 21 days after storage the contents of lycopene were significantly lower in the treated and control samples. A similar trend was observed in the levels of β-carotene during storage. The levels of β-carotene were significantly lower at 21 days after storage than 0 and 7 days after storage. Carotenoids are thermally unstable compounds and degrade during storage due to structural changes such as isomerization and oxidation. Refrigerated storage can prevent these changes and help maintain carotenoid levels [226]. Our results are similar to previous reports on the effect of HPP and TP on the levels of carotenoids in different vegetables [227]. However, other reports on lycopene suggest that pressure treatment increased the extractability of lycopene from tomato juice [206], tomato products [228] and watermelon juice [229]. The observed differences in lycopene contents could be due to differences in sample matrices, pH and storage conditions.

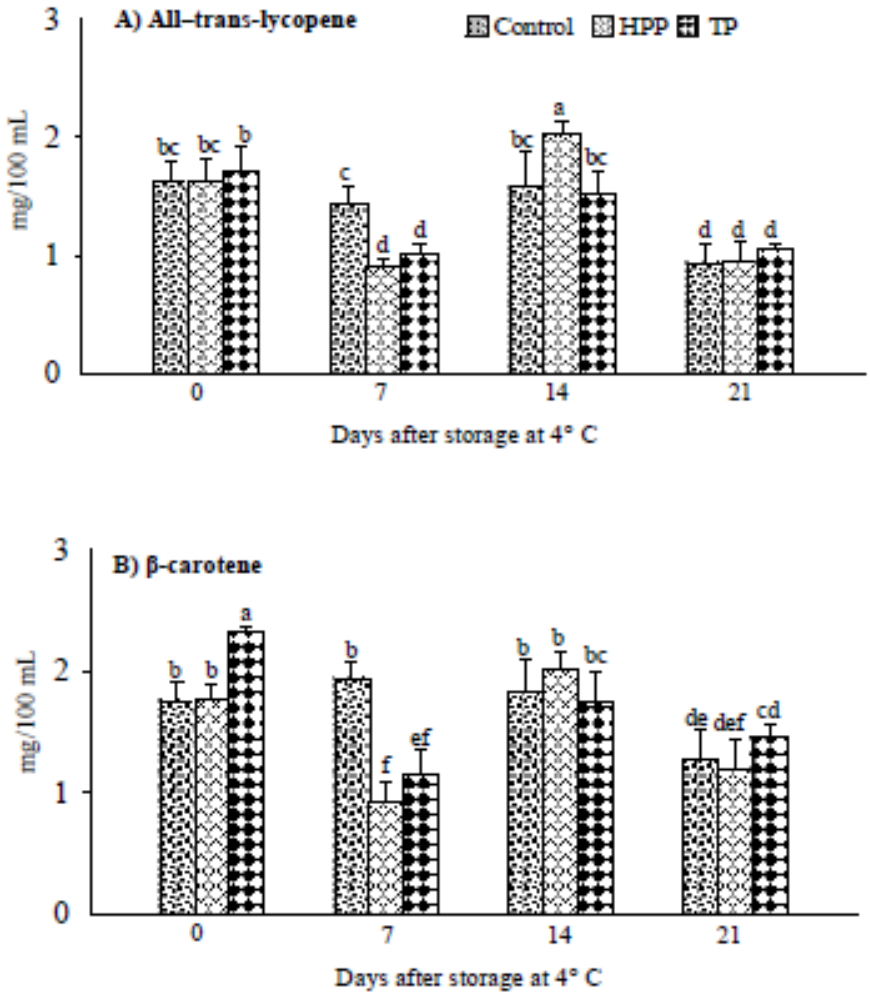

Fig. 29. Carotenoids A) Lycopene and B) β-carotene contents (mg/100mL) of grapefruit juice untreated (control) and treated with HPP and TP techniques analyzed after storage for different times. All values are means ± SD of three replicates. Bars with same letter are not significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100). Bars graphs with different letters are significantly different as judged by the Waller-Duncan k-ratio t-test (K ratio = 100).

7.2.6 Microbial analyses

Treated and untreated grapefruit juice samples were analyzed for total aerobic plate count and yeast and molds. Total aerobic plate count of the control samples at day 0 indicated 2.0 ± 0.3 Log CFU/ml (Table 17); HPP and TP samples were below the detection limit (< 1.0 Log CFU/ml). After 28 days of refrigerated storage (4° C), samples showed no signs of microbial growth (<1.0 Log CFU/ml) for HPP and TP treated samples. The untreated juice samples also showed microbial growth at less than the detection limit. Yeast and mold analyses indicated an initial 2.1 ± 0.1 Log CFU/ml for the control samples; HPP and TP samples were below the detection limit. Similar to the total plate count, there was no indication of growth of yeasts or molds during 28 days of refrigerated storage (4° C), and a decline in growth of the untreated samples to less than the detection limit. Traditionally, TP is applied for microbial inactivation and preventing spoilage. Unlike HPP treatment, the effect of TP is not uniform and is time dependent [230]. The long time duration required for optimum treatment by TP, along with high temperatures, often generates undesirable off flavors in juices. The rapid and innovative HPP method provides a better alternative with a minimal temperature requirement for uniform treatment application. Previous studies have reported the effect of HPP in controlling a wide spectrum of food borne pathogens depends on the amount of pressure applied [231]. While HPP inactivated the majority of the microbial growth below the detection limit at the applied pressure (402 ± 1.9 MPa), further studies are required to determine the optimum level of pressure required.

Table 17. Microbial analyses of grapefruit juice samples (Log CFU/mL), untreated (control), treated with HPP and TP for 0 and 28 days storage (4° C).

	Storage (Days)	Control	HPP	TP
TPC	0	2.0 ± 0.3	<1.0 ± 0.0*	<1.0 ± 0.0*
	28	<1.0 ± 0.0*	<1.0 ± 0.0*	<1.0 ± 0.0*
Y&M	0	2.1 ± 0.1	<1.0 ± 0.0*	<1.0 ± 0.0*
	28	<1.0 ± 0.0*	<1.0 ± 0.0*	<1.0 ± 0.0*

* Indicates values below the detection limit of (<1.0 Log CFU/ml)

7.2.7 pH determination

The pH of the untreated juice was higher than that of both HPP and TP juice samples (Table 18) at day 0. After 28 days of refrigerated (4° C) storage, a slight decrease in pH of the untreated control sample was observed (3.58 ± 0.04 to 3.47 ± 0.03). However, both HPP and TP juice samples demonstrated a stable pH throughout refrigerated storage. Maintaining pH is an important criterion for both quality and stability of health beneficial compounds such as ascorbic acid. Citrus fruits are acidic fruits and have a relatively low pH, which prevents degradation of ascorbic acid [232]. The relatively stable pH observed in the HPP treatment compared to control during 28 days of storage suggests that the processing treatment had no adverse effect.

Table 18. pH of grapefruit juice samples, untreated (control), treated with HPP and TP for 0 and 28 days storage (4° C).

Storage (Days)	Control	HPP	TP
0	3.58 ± 0.04	3.15 ± 0.06	2.99 ± 0.03
28	3.47 ± 0.03	3.12 ± 0.05	2.97 ± 0.03

7.3 Conclusion

It is essential that processing techniques to be commercialized demonstrate that they maintain or increase the levels of health promoting compounds. In general, HPP treatment maintained the levels of health promoting compounds in grapefruit juice comparably to TP treatment when the product was stored at 4º C. The emerging HPP technology also maintained the visual color quality of the juice, resulting in a fresh-like appearance for long storage periods, unlike TP. The results obtained in this study confirm the potential application of HPP in grapefruit processing industries. Further studies are required to optimize the pressure conditions and assess the organoleptic properties of juice for providing consumer-preferred fresh like nutritious grapefruit juice.

8. SEPARATION AND ISOLATION OF FUROCOUMARINS AND POLYMETHOXYFLAVONES FROM BYPRODUCTS OF CITRUS JUICE PROCESSING INDUSTRY

Citrus cultivation ranks among the top in horticultural crop production worldwide, which is estimated to be around 51.4 million metric tons [1]. The thick inedible leathery peel along with tasteless fibrous carpel membranes contribute to approximately 50% of waste byproducts in citrus processing industries [233, 234]. Therefore, it could be estimated that annually citrus industries generate approximately 25.7 million metric tons of byproducts. Utilization of these byproducts for isolation of biologically active phytochemicals could be beneficial to both consumers and the industry by added economic returns. Furthermore, these phytochemicals can be used to understand their role in human health by researching *in vitro* and *in vivo* studies.

Citrus fruits contain several phytochemicals such as vitamins, carotenoids, flavonoids limonoids, PMFs, amines, organic acids and furocoumarins [37, 97, 144, 171, 235]. Among these, polymethoxyflavones (PMFs) are a group of flavonoids unique to citrus species. They were studied to have several health beneficial properties such as anticancer, anti-inflammatory, antitumor, and antilopegic activities in *in vitro* models [40]. Similar to PMFs, in recent years furocoumarins have received much attention due to their implication in various biological activities. Bergamottin and its isomers are the commonly occurring furocoumarins in grapefruits (*Citrus paradisi* var. Macfad), which are synthesized by plants as a defensive mechanism against pests and diseases. Studies

in our lab and elsewhere suggest that these compounds have anti bacterial properties and inhibit biofilm formation [236, 237]. Additionally, furocoumarins inhibit the CYP 3A4 enzyme activity, which may enhance the bioavailability of certain medications [238]. Majority of these activities are reported based on *in vitro* studies and may require further *in vivo* trials to understand their role in bioavailability [239]. The beneficial properties of furocoumarins and PMFs have led to the development of chromatographic techniques to analyze and quantify them in grapefruit juice and its products [240-242]. To further investigate their biological role in human and animal models isolation of these compounds in large quantity is essential. In the past decade furocoumarins were isolated from grapefruit juice [243], molasses and peel using preparative thin layer chromatography [244], column chromatography [245], preparative HPLC [246] and high speed counter current chromatography (HSCCC) [247] techniques. However, these methods require multi step procedures and yield low quantity of isolated compounds.

Currently there are no efficient strategies reported for separation and isolation of furocoumarins and PMFs from citrus industrial byproducts such as cold pressed grapefruit peel oil (GFO). In continuation of research on citrus phytochemicals in our lab, we have developed isolation methods using wide range of chromatographic techniques such as column chromatography [248], adsorptive chromatography [58], supercritical chromatography [249] and flash chromatography [27]. Flash chromatography provides several advantages over conventional separation methods in terms of the amount of raw material used, rapid separation, online detection, low solvent use and reproducible results. Therefore, based on the industrial and biological

significance in utilization of citrus juice byproduct, an efficient separation and isolation furocoumarins and polymethoxyflavones using flash chromatography was developed.

8.1 Materials and methods

8.1.1 Reagents and materials

Solvents hexane, chloroform and acetone used were analytical grade and obtained from Fisher Scientific (Pittsburgh, PA, USA). HPLC grade acetonitrile and acetic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Nanopure water (NANOpure, Barnstead/Thermolyne, Dubuque, IA, USA) was used for HPLC analysis. The separation of phytochemicals was conducted on an automated flash chromatography system (Combiflash® Rf, Teledyne Isco, Lincoln, NE, USA). Silica gel (particle size 35–60 μm) flash columns (80 g) were purchased from ISCO Inc. (RediSep® Rf ISCO Inc., Lincoln, NE, USA). Rio Red grapefruit oil was obtained from Texas Citrus Exchange (Mission, Texas, USA) in the month of November 2009.

8.1.2 Sample preparation for flash chromatography

Grapefruit peel oil was strained using a muslin cloth to remove any suspended particles. The strained oil (200 mL) was impregnated with silica gel (100 g) and air dried. The dried sample was fractionated on silica gel column using successive elution of

hexane (1 L) and chloroform (1 L). The individual solvent fractions were concentrated under vacuum (Buchi, Switzerland) and analyzed by HPLC for detection of furocoumarins and polymethoxyflavones. For further separation and isolation of pure phytochemicals, the concentrated chloroform extract was impregnated with silica gel and used for flash chromatographic separation.

8.1.3 Flash chromatography separation of furocoumarins and PMFs

A CombiFlash Rf 4x flash chromatography system (Teledyne Isco, Lincoln, NE, USA) with silica gel (particle size 35–60 μm, 80g) column was used for the separations. The column was initially equilibrated with hexane for three column volumes prior to separations. For separations, a 45 min pump program with a solvent gradient of hexane (solvent A) and acetone (solvent B) was used. Initially, 100% A was held for 11 min, linearly increased to 25 % B in 4 min, held for 5 min, linearly increased to 30 % B in 1 min, held for 5 min, linearly increased to 100 % B in 13 min, held for 4 min and returned to 100% A, maintained for 2 min. The flow rate was set at 60 mL/min and individual fractions were collected by monitoring the eluting analytes at λ_{254} nm and λ_{340} nm. Five peaks (A-E) were separated in the flash chromatography. The retention time of the separated peaks were, 15.5 - 17 min (peak A), 17- 20 min (peak B), 20 - 24 min (peak C), 24 - 28 min (peak D) and 28 - 34 min (peak E). A schematic presentation of the separation and isolation process is explained in figure 30. The individual peak fractions were analyzed by HPLC and pooled based on peaks similarity and matching the UV

spectral results. Concentration of the solvent from the pooled fractions of peaks A-E yielded crystallized compounds 1-5 respectively.

8.1.4 HPLC analysis

All the fractions collected by flash chromatography and the individual crystallized compounds were analyzed by HPLC. For analysis of the isolated compounds were dissolved in acetonitrile and injected to HPLC. The HPLC system consisted of a Waters 1525 HPLC series (Milford, MA, USA) connected to a PDA detector. A Gemini C_{18} column (5 µm, 4.6 mm X 250 mm i.d.) from Phenomenex (Torrence, CA) was used for the separations. A gradient mobile phase of 4% ascetic acid and acetonitrile was used for the separations maintained at a flow rate of 1 mL/min. Injection volume was set at 10 µl and the separated compounds were detected at λ280 nm and λ340 nm. Chromatographic data was collected and processed using Empower2 software (Waters- Milford, MA, USA).

8.1.5 Identification

(a). MALDI-TOF-MS analysis. The samples for MS analysis were prepared by dissolving the isolated compounds in acetonitrile and mixed with 2′, 4′, 6′-trihydroxyacetophenone (THAP) matrix. A 0.5 µl of the matrix mixture was spotted on a MALDI sample plate and air-dried. MALDI-TOF mass spectra were acquired using

Voyager DE-Pro (Applied Biosystems, CA) mass spectrometer in positive reflector ion mode. After time-delayed extraction of 275 nsec, the ions were accelerated to 20 kV for TOF mass spectrometric analysis. A total of 100 laser shots were acquired with the signal averaged per mass spectrum.

(b). NMR analysis. The structures of the compounds were confirmed by NMR results in $CDCl_3$.^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz respectively (JEOL USA, Inc., MA, USA). ^{13}C NMR spectral assignments were given on the basis of DEPT spectra.

8.1.6 Repeatability of flash chromatography

To validate the separation method, the procedure was repeated three times while maintaining the instrument parameters as well as same sample size. The separations were conducted on three 80 g silica columns using a gradient of hexane and acetone similar to the procedure explained in section 8.1.3. For each separation, the separated peak fractions were collected, analyzed by HPLC and pooled for crystallization of the compounds. The variation in the retention times of the separated peaks and the yield of the isolated compounds was calculated for percent relative standard deviation (% RSD).

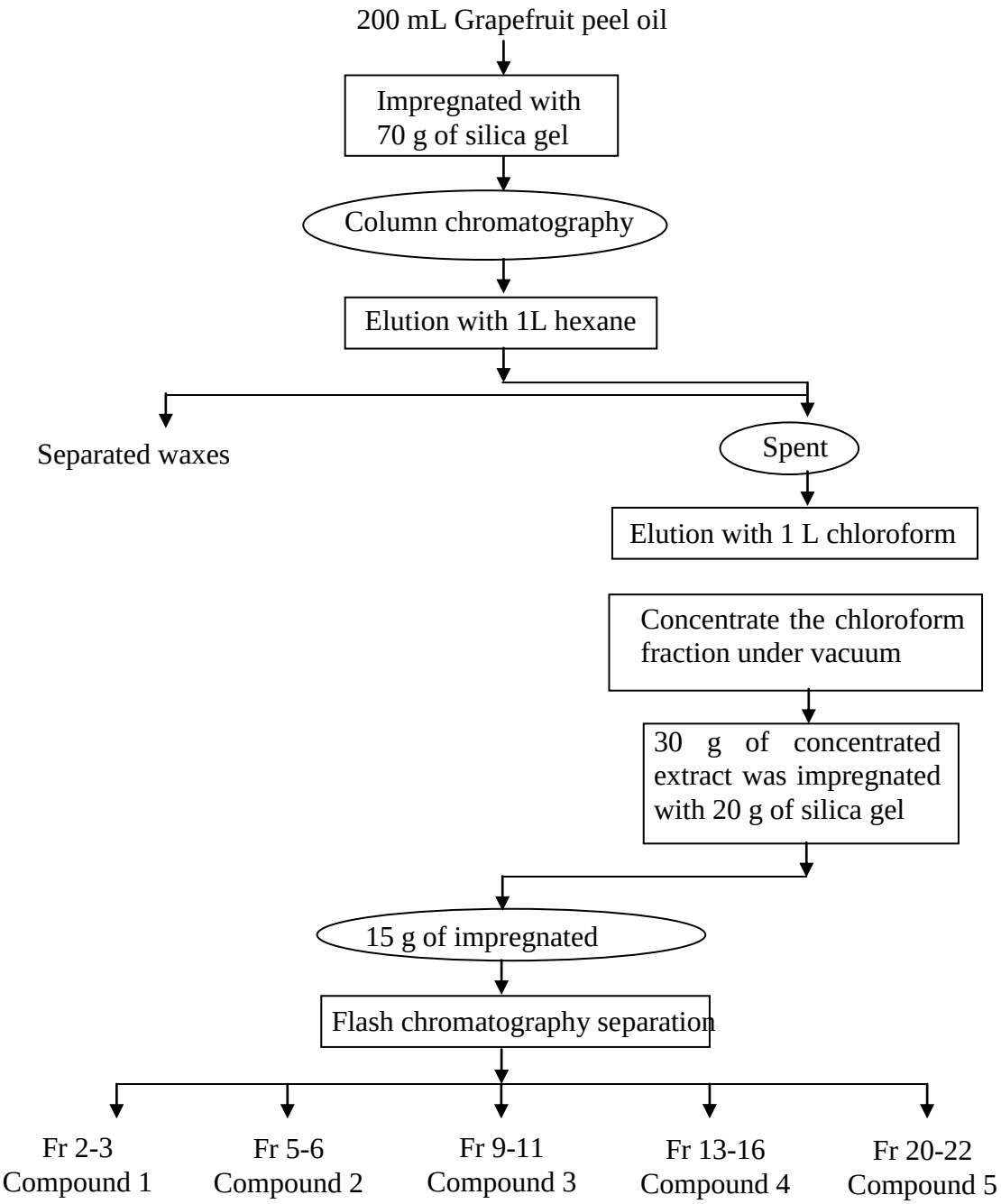

Fig. 30. Schematic presentation of the separation of five compounds from citrus byproduct.

8.2 Results

8.2.1 Fractionation of waxes from citrus oil

The selection of raw material is critical for large scale isolation of minor compounds. Grapefruits have a characteristic thick peel along with high content of waxes in the epicuticular layer. During the expression of GFO from the fruits, these waxes are also extracted. The GFO also contains relatively higher levels of furocoumarins than other fruit parts. Moreover, it contains low content of PMFs unlike orange peel oil which have high content [250]. Therefore, the industrial byproduct cold pressed GFO was selected as a raw material for isolation of these compounds. However, the large content of fixed oils and waxes present in the GFO is a major challenge for separation of pure compounds. To prevent co-elution of the separated compounds with the oils and waxes, the GFO was subjected to fractionation on a silica column using hexane and chloroform solvents. The sequential elution of the impregnated GFO with hexane and chloroform enabled separation of waxes from the compounds with the later eluting the waxes. The chloroform extract containing the compounds was concentrated under vacuum and impregnated with silica. The impregnated sample was further used for separation using rapid flash chromatography.

8.2.2 Identification and characterization

Figure 31 represents the flash chromatogram obtained from the separation of the concentrated chloroform fraction of citrus byproducts. Using the gradient elution of hexane and acetone resulted in separation of 5 peak separations. Since the compounds were less polar, a step gradient was used to enable separations without overlapping and co-elution of the compounds. To achieve good separation, selection of solvents and their gradient is important. Although the choice of solvents is large in normal phase chromatography, selection of solvents is influenced by the desired wavelength for monitoring the separations and the solvent absorption maxima. Solvents such as ethyl acetate and chloroform both medium polar solvents adsorb UV light in the wavelength range of λ_{200} to λ_{245} nm which may interfere with the absorption of UV light by the eluting compounds and as well elute the compounds without separation. To limit the interference and obtain good separation, a gradient of hexane (low polar) and acetone (mid polar) was used. Hexane adsorbs light in the wavelength range of λ_{195} to λ_{225} nm while acetone has a range of λ_{250} to λ_{270} nm. Monitoring the separations at λ_{254} nm and λ_{340} nm enabled to avoid interference by solvent absorption. Figure 32 represents the HPLC chromatograms obtained from the analysis of the isolated compounds along with the crude GFO used for separation. The isolated compounds were tested to be pure, evident from the absence of any other peaks. The UV spectra of the individual peaks were used to determine the partial identity of the compounds. The absorption maxima (λ_{max}) of compound 1 was observed to be 298.3, 325.7 nm, compound 2: 222.6, 267.4,

312.6 nm, compound 3: 271.0, 322.1 nm, compound 4: 254.4, 340.0 nm and compound 5: 249.7, 271.0, 337.6. The positive-ion mode MALDI-TOF spectra of the isolated compounds 1, 2, and 4 are provided as supporting information. The MALDI-TOF spectra obtained for compound 3 and 5 are similar to our earlier reported results [97]. The mass spectrum of isolated compounds 1-5 have molecular ions $[M+H]^+$ at *m/z* 339.04, 217.61, 373.04, 433.17, and 403.21, respectively. Further, results from the NMR spectral analysis of the isolated compounds (1-5) confirmed as bergamottin, bergapten, 5,6,7,8,4'-pentamethoxyflavone (tangeretin), 3,5,6,7,8,3',4'- heptamethoxyflavone (HMF) and 5,6,7,8,3',4'-hexamethoxyflavone (nobiletin) (Fig. 33) [35, 40, 58, 63, 163].

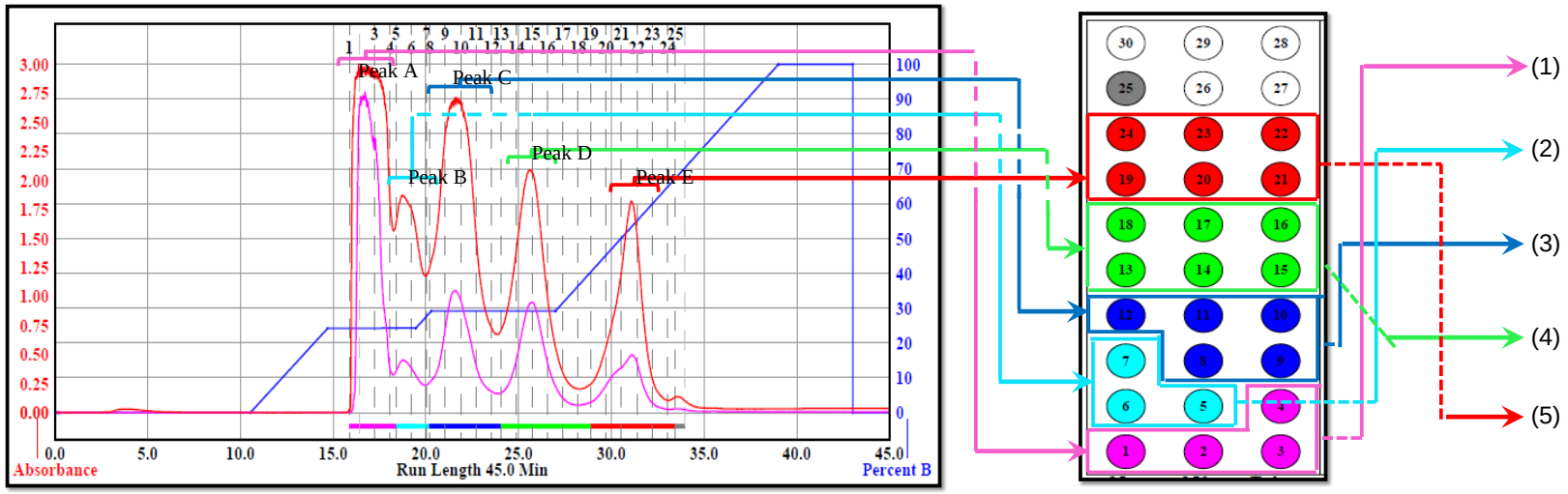

Fig. 31. Flash chromatogram obtained from the separation of chloroform extract of byproduct, Mobile phase used, hexane (A) and acetone (B), flow rate 60 mL/min, detection at λ_{254} and λ_{340} nm. The separated peaks marked as same color were collected in fractions and pooled to obtain pure compounds 1-5.

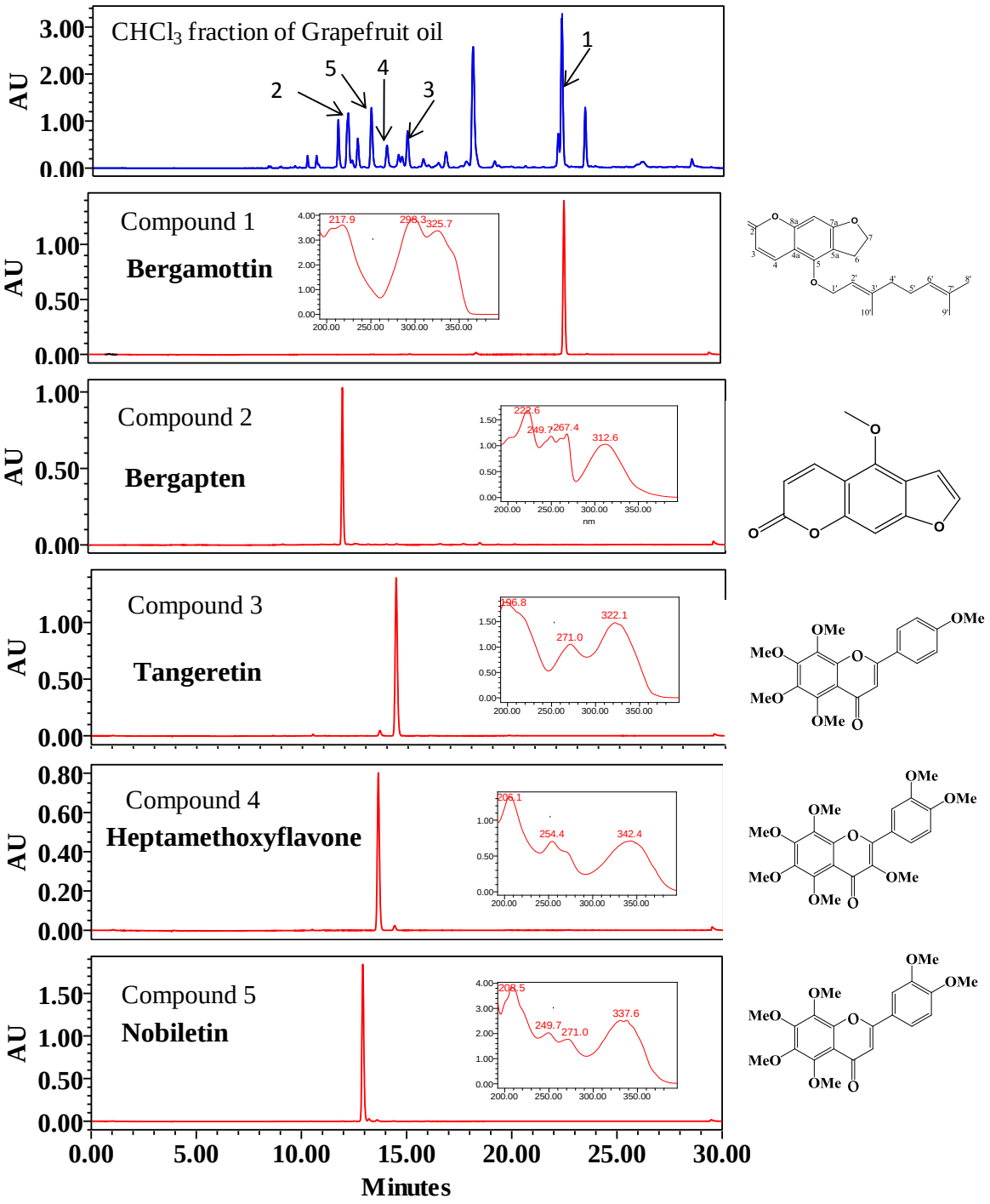

Fig. 32. HPLC chromatograms of grapefruit peel oil and the isolated compounds (1-5) along with their UV spectra as well as structures.

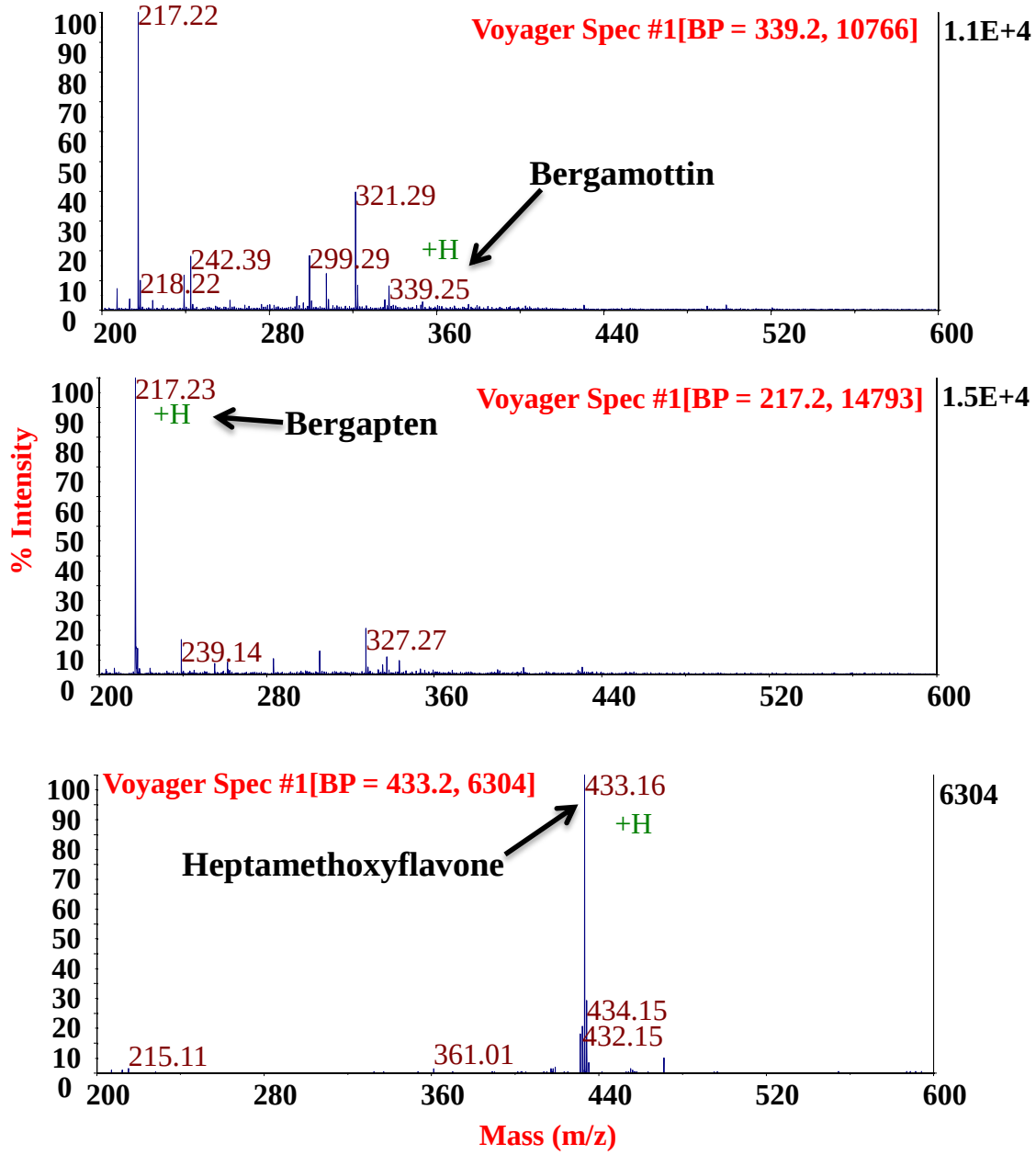

Fig. 33. Positive MALDI-TOF mass spectra of furocoumarins and PMF separated and isolated from citrus byproducts.

8.2.3 Repeatability of the flash separation

The developed flash chromatography method was validated by the test of repeatability. To demonstrate the repeatability of the separation methodology, three individual separations with prepackaged silica columns (80 g) were used. In all the three replications the parameters such as flow rate, detection wavelength, amount of sample (15 g) and solvent gradient were maintained same. All three replications had similar retention times for the separated peaks with less than 4% relative standard deviation. The average yield of the isolated compounds such as bergamottin, bergapten, tangeretin, HMF, and nobiletin were 513 mg, 19.67 mg, 13.67 mg, 21.33 mg and 12.33 mg, respectively. The total yield of the isolated compounds from the three replications were, bergamottin (1.54 g), bergapten (59 mg), tangeretin (41 mg), HMF (64 mg), and nobiletin (37 mg). Relatively low RSD % was noticed in the yield of the isolated compounds ranging between 5.53 for bergapten and 20.12 for nobiletin. Bergamottin, tangeretin and HMF had a RSD% of 8.81, 14.95 and 12.38, respectively. The percent yield of the isolated compounds calculated as a relative measure to the amount of sample used was 0.80 %, 0.03 %, 0.02 %, 0.03 % and 0.02 % for bergamottin, bergapten, tangeretin, HMF, and nobiletin, respectively. The low RSD % for the retention times and yield of the isolated compounds using the developed flash chromatography method suggests that the developed method is repeatable and could be used for large scale isolation of furocoumarins and PMFs from grapefruit byproducts.

8.3 Discussion

Furocoumarins are naturally occurring compounds structurally consisting of a furan ring attached to a coumarin. These compounds have received attention primarily due to their modulating effect on cytochrome P450 enzymes [251]. Similarly, polymethoxyflavones are also biologically potent phytochemicals known to have several health beneficial properties such as anticancer, antiproliferative, anti-inflammatory activities [40]. However, there are very few *in vivo* studies which may be due to the lack of availability of these phytochemicals with high purity. Therefore, we developed an efficient method for large scale isolation of furocoumarins and PMFs from industrial byproduct which could enable further biological studies.

Isolation of furocoumarins was reported from several citrus species using a wide range of chromatographic techniques. Manthey et al., reported isolation of furocoumarins from grapefruit juice retinate using a combination of normal phase silica chromatography, preparative thin layer chromatography, and preparative HPLC [243]. In an another study, GFO was subjected to successive normal phase silica flash chromatography for isolation of 7-O-geranyl-coumarin and a mixture of bergamottin epoxide derivative and coumarin epoxide derivative [237]. While these studies report identification of the isolated phytochemicals, information related to yield and repeatability of the separation procedure has not been provided. The automated flash chromatography described in the current developed method provides several advantages such as ability to simultaneously monitor in real time the separation of peaks and alter

the solvent gradient, flexibility in the sample size, as well as obtain phytochemicals with high purity.

8.4 Conclusion

Two furocoumarins, bergamottin and bergapten along with 3 polymethoxyflavones, tangeretin, heptamethoxyflavone and nobiletin were separated from peel oil, a citrus industrial byproduct by flash chromatography method. A two-phase gradient solvent system consisting of hexane and acetone was used for efficient flash separation of the compounds. The overall process for separation of the compounds is simple and rapid. The identity of the compounds was confirmed by spectral analysis using HPLC, MALDI-TOF and NMR analysis. The yield, repeatability and purity of the compounds suggest the developed method could be used for large scale isolation. The method may help in providing value added products from citrus industrial byproducts and be beneficial for further research on biological activities using the isolated compounds.

9. ISOLATION OF PHYTOCHEMICALS FROM MIARAY MANDARIN (*Citrus miaray* TAN) AND THEIR BIOFILM INHIBITORY ACTIVITY IN *Vibrio harveyi*

Mandarins (*Citrus reticulata*) are a group of citrus cultivars which have loose peel, bright orange-red color and juicy succulent segment membranes. They are among the most rapidly increasing citrus varieties in terms of production and consumption worldwide [252]. They are native to South East Asia and are commercially cultivated in the temperate regions of the world [253]. It is estimated that the annual production of mandarins in the world could be approximately 20.3 million metric tons [1]. Due to the economical benefits in cultivation of mandarins, they are extensively studied for enhancing the yield and quality. Additionally, these fruits have high diversity due to hybridization and somatic mutations [254]. These factors have led to significant phenotypic and genetic variations resulting in numerous species. Among them, Miaray mandarin (*Citrus miaray* Tan.) is a mandarin species used as a rootstock for citrus propagation [255]. The fruits of this species are round bright yellow colored and have a diameter of 5 – 7 cm with sour tasting juicy segment membranes. There are only few scientific reports on this species and a majority of these are limited to evaluation of genetic heritability. Studies suggest they are unique with distinct genotype characteristics in comparison to other mandarin species [254].

Prior research from our lab, we have reported the development of chromatographic methods for analysis of amines in different mandarin species and isolation of polymethoxyflavones from mandarin and orange species [97]. Our results suggest that

mandarins are a good source of polymethoxyflavones and could be utilized as a potential source for large scale isolation. Polymethoxyflavones are a group of flavonoids which have three or more methoxyl moiety's attached to their basic flavone structure. These molecules were extensively studied for their biological properties such as anticancer, anti-lipogenic, antitumor, antiviral and anti-inflammatory [68, 159]. They primarily occur in the fruit peels and to a lesser extent in the juice. Although there are several reports on antibacterial activity of citrus peel extracts, [60, 256] there are limited studies evaluating individual PMFs. In a recent study two PMFs such as nobiletin and tangeretin were tested for their inhibitory activity on six strains of microorganisms including *Escherichia coli*, *Staphylococcus sp.*, and *Salmonella typhi* [257]. The study concluded that nobiletin and tangeretin exhibited low antimicrobial activities. However, advances on elucidation of bacterial cell-cell communication pathways suggest that inhibition of the gene expression in response to cell density signaling could also enable attenuation of bacterial pathogenicity. This mechanism of action was demonstrated by our research group using several citrus limonoids and flavonoids [258-260]. Among these studies we have also evaluated the potential biofilm inhibitory activity of 3',4',5,6,7-pentamethoxyflavone (sinensitin) which demonstrated significant inhibition in a dose dependent manner. There are approximately 28 different polymethoxyflavones reported in citrus, each structurally distinct from each other in the number and position of the methoxyls attached to the flavone backbone [40]. These structural variations could have a wide range of inhibitory activity of bacterial biofilm and could aid in determining novel agents for effective control of bacterial pathogenesis.

Therefore, due to the absence of reports on bioactive compounds analysis present in Miaray mandarin and the potential for isolation of unexplored polymethoxyflavones, we conducted chromatographic analysis of peels using hyphenated chromatographic technique. The isolated PMFs were evaluated by spectral analysis using high performance liquid chromatography (HPLC), Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (Maldi-TOF) and nuclear magnetic spectroscopy (NMR) for identification. The isolated PMFs were further evaluated for determining the biofilm inhibitory activity using *V. harveyi*. Results from this study could enable development of strategies using PMFs for preventing bacterial pathogenicity.

9.1 Materials and methods

9.1.1 Reagents and instrumentation

Solvents used for analysis were HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA, USA). Nanopure water (NANOpure, Barnstead/Thermolyne, Dubuque, IA, USA) was used for HPLC analysis. The solvents used for hyphenated chromatography were analytical grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). The separation of PMFs was carried out on an hyphenated chromatography system (Combiflash® Rf , Teledyne Isco, Lincoln, NE, USA). Silica gel (particle size

35–60 µm) columns (220 g) were purchased from ISCO Inc. (RediSep® Rf ISCO Inc., Lincoln, NE, USA).

9.1.2 Plant material

Mature *Citrus miaray* mandarin fruits (Fig. 34) were harvested in the month of December, 2010 from Texas A&M University-Kingsville Citrus Center orchard (Weslaco, TX). A voucher specimen (#249960) has been submitted in the S.M. Tracy Herbarium, Texas A&M University (College Station, TX). The peels were separated and dried to obtain ≤ 5% moisture. The peels were blended to obtain 40-60 mesh size powder in a Vita-prep™ blender (Vita-Mix Corporation, Cleveland, OH, USA).

Fig. 34. Fruits of Miaray mandarin

9.1.3 Soxhlet extraction of peels

Eight hundred grams of dried peel powder was loaded to a Soxhlet type apparatus and extracted with hexane and chloroform sequentially for 16 h each. The extracts were concentrated to yield 31 g and 23 g of concentrated hexane and chloroform extracts respectively.

9.1.4 Sample preparation for chromatographic separation

The concentrated hexane extract was dissolved in hexane and impregnated with silica gel (20 g) to obtain fine slurry. The mixture was further evaporated under vacuum to make a free-flowing powder. The impregnated sample was used for separation. Similar procedure was followed for the chloroform extract.

9.1.5 Flash chromatography

The chromatography system Teledyne Isco CombiFlash Rf 4x system (Lincoln, NE) equipped with a silica gel (particle size 35–60 μm) column from ISCO Inc. was used. Binary solvent system of hexane (solvent A) and acetone (solvent B) was used with a flow rate of 150 mL/min. The column was eluted with 100% hexane for 3 column volumes after injection followed by a gradient with acetone. Individual fractions were

further separated and collected by monitoring the eluting analytes at λ_{254} nm, λ_{340} nm and absorption spectra.

9.1.6 Separation of PMFs from hexane extract

The silica gel impregnated hexane extracts of Miaray mandarin were subjected to hyphenated chromatography on a silica gel (particle size 35–60 μm) column 220g. The column was equilibrated with hexane for three column volumes prior to separations. PMFs were separated in 65 min gradient program of solvent A (hexane) and solvent B (acetone): 100% A held for 4 min, linearly increased to 10% B over 1 min, held for 12 min, linearly increased to 40% B over 6 min, held for 14 min, linearly increased to 100% B over 6 min and held for 12 min, then finally returned to the initial conditions and held for 10 min. The flow rate was maintained at 150 mL/min and individual fractions were collected by monitoring the eluting analytes at λ_{210} nm and λ_{340} nm. Seven major peaks (A - G) were observed (Fig. 35 A) and collected in individual fractions. The retention time of the separated peaks were, peak A: 22.5 - 25 min, peak B: 25 - 29.5 min, peak C: 29.5 - 34 min, peak D: 34 - 37.5 min, peak E: 39 - 42 min, peak F: 42 - 45 min, peak G: 45 - 50 min. The individual peak fractions were further analyzed by HPLC and pooled based on their similarity in retention time and matching the UV spectral analysis. Evaporation of the solvent from the pooled fractions of peak A-G yielded in crystallization of compounds 1-7 respectively.

9.1.7 Separation of PMFs in chloroform extract

The silica gel impregnated chloroform extract was subjected to hyphenated chromatography on a silica gel (particle size 35–60 μm) column 220 g. The column was equilibrated with hexane for three column volumes prior to separations. PMFs were separated in 75 min gradient program of solvent A (hexane) and solvent B (acetone): 100% A held for 6 min, linearly increased to 10% B over 4 min, held for 15 min, linearly increased to 40% B over 5 min, held for 15.5 min, linearly increased to 60% B over 5 min, held for 4.5 min, linearly increased to 100% B over 6 min and held for 12 min, then finally returned to the initial conditions and held for 2 min. The flow rate was maintained at 150 mL/min and individual fractions were collected by monitoring the eluting analytes at λ_{210} nm and λ_{340} nm. Seven major peaks (H-N) were observed (Fig. 35 B) and collected in individual fractions. The retention time of the separated peaks were, peak H: 30- 32.5 min, peak I: 32.5 - 35 min, peak J: 35 - 39 min, peak K: 39 - 44 min, peak L: 51 – 55 min, peak M: 55 - 59 min and peak N: 62 - 70 min. Twenty μl of each fraction was diluted with 500 μl of acetone and analyzed by HPLC for detection of PMFs and further pooling of fractions with similar compounds. Evaporation of the solvent from the pooled fractions of peak H-N yielded in crystallization of compounds which were collected and analyzed for identification.

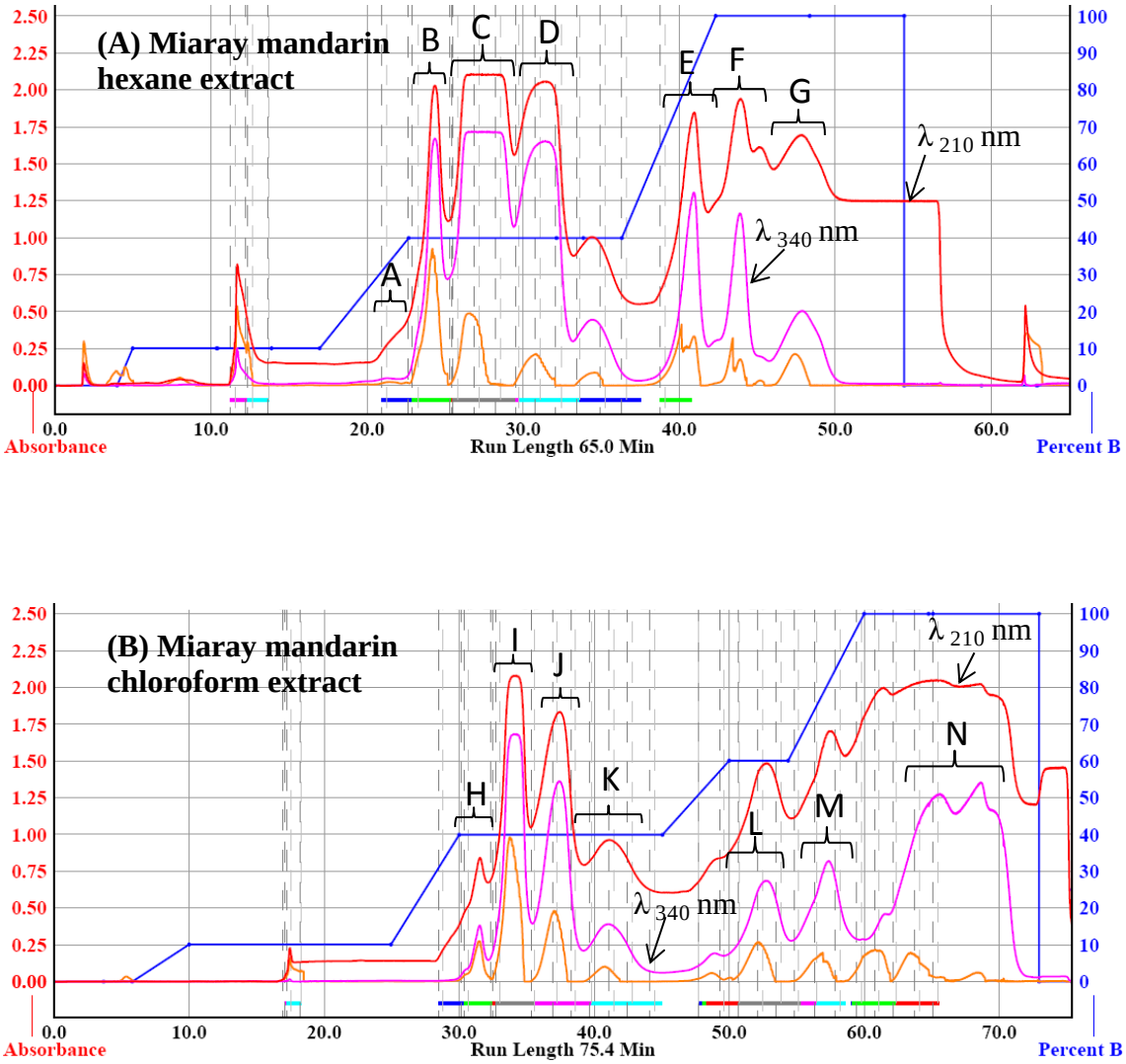

Fig. 35. Chromatogram obtained by the separation of (A) hexane and (B) chloroform extracts of Miaray mandarin peel using silica gel columns.

9.1.8 Liquid chromatography

The HPLC system consisted of a Waters 1525 HPLC series (Milford, MA, USA) connected to a photo diode array (PDA) detector. A Gemini C_{18} column (3 µm, 250 × 4.6 mm) (Phenomenex, Torrance, CA, USA) was used for the separations. A gradient mobile phase of 3 mM phosphoric acid (A) and acetonitrile (B) was used for the separations at a flow rate of 1 mL/min. Initially, elution was started with a gradient of 5% B followed by linear increase to 50% in 20 min, returned back to 5% in 5 min. Injection volume was set at 10 µL and the PMFs were detected at 280 nm and 340 nm. Chromatographic data was collected and processed using Empower2 software (Waters, Milford, MA, USA).

9.1.9 Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (Maldi-TOF-MS) analysis.

The samples for MS analysis were prepared by dissolving the isolated compounds in acetonitrile and mixed with 2′,4′,6′-trihydroxyacetophenone (THAP) matrix. A 0.5 µL of the matrix mixture was spotted on a MALDI sample plate and air-dried. MALDI-TOF mass spectra were acquired using Voyager DE-Pro (Applied Biosystems, CA) mass spectrometer in positive reflector ion mode. After time-delayed extraction of 275 nsec, the ions were accelerated to 20 kV for TOF mass spectrometric analysis. A total of 100 laser shots were acquired with the signal averaged per mass spectrum.

9.1.10 NMR analysis

^{1}H and attached proton test (APT) spectra were recorded at 400 MHz and 100 MHz respectively by FT NMR (JEOL USA, Inc., MA, USA).

9.1.11 Bacterial strains and media

V. harveyi strains BB170 (luxN : : Tn5), BB886 (luxPQ : :Tn5), BB120 (wild-type), JAF483 (luxO D47A), JAF553 (luxU H58A), and BNL258 (hfq : : Tn5lacZ) were kindly provided by B. L. Bassler (Princeton University, Princeton, NJ, USA) [261-264]. *Escherichia coli* #5, an environmental isolate was used as a positive control for autoinducer-2 (AI-2) activity [265]. Autoinducer bioassay (AB) or Luria Marine (LM) media were used to culture the *V. harveyi* strains [266].

9.1.12 Growth assay

Overnight cultures of *V. harveyi* BB120 were diluted 100-fold in AB media and treated with polymethoxyflavones (100 µM) or an equivalent volume of DMSO. The cultures were grown for 16 h and OD600 was measured every 15 min by using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments). The instrument was set to maintain a temperature of 30 ºC and plates were constantly shaken at medium

speed between readings. The data are presented as the mean of three biological replicates.

9.1.13 Bioluminescence assay

The bioluminescence was measured using the method described previously from our lab [258]. In brief, *E. coli* #5 and *V. harveyi* BB120 were cultured overnight in Luria–Bertani (LB) and LM media, respectively to obtain high concentrations of autoinducer activity. The overnight cultures were centrifuged at 10,000 r.p.m. for 10 min in a micro centrifuge, followed by filtration using a 0.2 mm pore-size membrane filter. The clear filtered cell-free culture supernatants (CFSs) obtained was later stored at -20º C until use. Inhibition of autoinducer [harveyi autoinducer (HAI) and AI-2]-mediated bioluminescence were measured in a 96-well plate assay. The final concentrations of PMFs tested were 12.5, 25 and 50 µM. Diluted (2500-fold) overnight cultures (900 µL) of reporter strains BB886 (for HAI) and BB170 (for AI-2) were incubated with 5 µl CFS, 0.5 µl PMFs or DMSO, and 4.5 µl sterile AB medium at 30 ºC with shaking at 100 r.p.m. Light production was measured by a Victor2 1420 multilabel counter (Beckman Coulter) in luminescence mode. The values were recorded as relative light units and used in calculation [266].

9.1.14 Biofilm assay

The biofilm assay was conducted using the method described previously from our lab [260]. Briefly, an overnight culture of *V. harveyi* BB120 was diluted 1:50 in LM media and 190 µl of this fresh culture was incubated with 7 µl sterile medium and 0.5 µl DMSO or PMFs (12.5, 25, 50 µM) dissolved in DMSO. The biofilm mass was quantified by washing with phosphate buffer (0.1 M, pH 7.4), followed by staining with 0.3% crystal violet (Fisher) for 20 min. The dye associated with biofilm was dissolved with 200 µl of 33% acetic acid, and A570 was measured. The mean ± standard deviation of three biological replicates is presented.

9.2 Results and discussion

9.2.1 Soxhlet extraction

Successive extractions of mandarin peels using low polar hexane solvent and medium polar chloroform solvents in a Soxhlet type apparatus yielded a percent yield of 3.9% and 2.9% of concentrated extract, respectively. HPLC analysis of the extracts suggests the extracts contain high levels of polymethoxyflavones. Additionally, the chromatograms suggest the successive extractions by hexane and chloroform enabled fractionation of low polar and medium polar molecules respectively. For further isolation and purification of individual polymethoxyflavones the extracts were

impregnated with silica gel and subjected to hyphenated chromatography separation using silica columns.

9.2.2 Flash chromatographic separation of hexane extract

The step gradient elution using hexane and acetone enabled in clear separation of individual PMFs (Fig. 35A). The initial isocratic elution using 10% acetone separated the oil from the crude extract. After separating the oils, the linear change in gradient elution to 60: 40 ratio of hexane: acetone resulted in good separations of the low polar PMFs while medium polar PMFs were separated by gradual increase in the solvent polarity to 100% acetone. The pooled fractions based on similarity by HPLC analysis were pooled and concentrated by evaporation. The concentrated peak (A – G) fractions yielded crystallized compounds which were collected and subjected to spectral analysis for identification and purity analysis.

9.2.3 Flash chromatographic separation of chloroform extract

The impregnated chloroform extract was subjected to hyphenated chromatographic separation using a step gradient of hexane and acetone solvents with a total run time of 75 min. To enable good separations of medium polar PMFs from low polar PMFs an additional step gradient of 40: 60 hexane: acetone was used followed by linear increase to 100% acetone. The step gradient elution resulted in separation of peaks

H-N (Fig. 35B). The fractions collected for individual peaks were analyzed by HPLC and concentrated to obtain crystallized compounds. These were further subjected to spectral analysis using MS and NMR for identification and structure elucidation.

9.2.4 Identification and characterization

The identification and structural elucidation of the isolated compounds from Miaray mandarin was conducted using spectral analysis by HPLC, MS and NMR. The chromatograms of HPLC analysis for the isolated compounds are presented in figure 36. The absence of other peaks demonstrates the purity of the isolated compounds. The absorption spectra of the compounds were analyzed using a PDA detector. All the isolated compounds had distinct UV maxima in the range of λ_{325}- λ_{353} which is characteristic of polymethoxyflavones. The results are in confirmation with the earlier reports. [63, 71, 97] The mass spectrum of isolated compounds **1-10** shows a molecular ion $[M+H]^+$ at *m/z* 359, 373, 433, 403, 373, 470, 343, 313, 343, and 373 respectively. The MALDI-TOF spectra of compounds **1**, **5**, **6**, **8** and **10** are presented in the Fig 37. The APT and ^{1}H NMR spectra of the isolated compounds **1**, **5**, **6**, **8**, and **10** are given in Fig. 38 and Fig. 39. The APT and ^{1}H NMR spectra of the compounds **2**, **3**, **4**, **7** and **9** were similar to those reported [97, 267]. Results from the spectral analysis of the isolated compounds confirm the identity of the isolated compounds as 5-hydroxy 3,7,3',4' tetramethoxyflavone (**1**), 5,6,7,8,4' pentamethoxyflavone (tangeretin) (**2**), 3,5,6,7,8,3',4' heptamethoxyflavone (**3**), 5,6,7,8,3',4' hexamethoxyflavone (nobiletin) (**4**),

3,5,7,8,3',4' hexamethoxyflavone (**5**), 3,5,7,3',4' pentamethoxyflavone (pentamethyl quercetin) (**6**), limonin (**7**), 5,7,4' trimethoxyflavone (**8**), 5,7,8,4' tetramethoxyflavone (**9**) and 5,7,8,3',4' pentamethoxyflavone (**10**), respectively. The results are in confirmation with the reported values [63, 163, 268]. The yield of the isolated compounds were measured to be: **1**-18 mg, **2**-76 mg, **3**-8628 mg, **4**-2012 mg, **5**-210 mg, **6**-121 mg, **7**-1077 mg, **8**-79 mg, **9**-810 mg and **10**-820 mg. To the best of our knowledge this is the first report of the isolation of 3,5,7,3',4' pentamethyl quercetin from the genus *Citrus*. This compound was earlier reported in a review article describing the various compounds present in citrus species by Manthey et al.[269]. In a personal communication, Manthey did confirm that the source of 3,5,7,3',4' pentamethoxyflavone was not from a citrus species.

9.2.5 Microbial activity of citrus polymethoxyflavones

Growth: The kinetic growth curve was calculated by recording the OD_{570} for time period of 16 h at optimal temperature. Figure 40 illustrates the growth kinetics of *V. harveyi* BB120 after treating with the 50 µM concentration of eight PMFs. The sigmoid bacterial growth curve observed indicates the PMFs do not have any inhibitory property on the bacterial growth at the 50 µM concentration. Similar results were also observed in other reports evaluating antimicrobial activity of PMFs (nobiletin and tangeretin) which had MIC of $\geq$ 1600 µg/mL [257] suggesting that these compounds are not antibacterial.

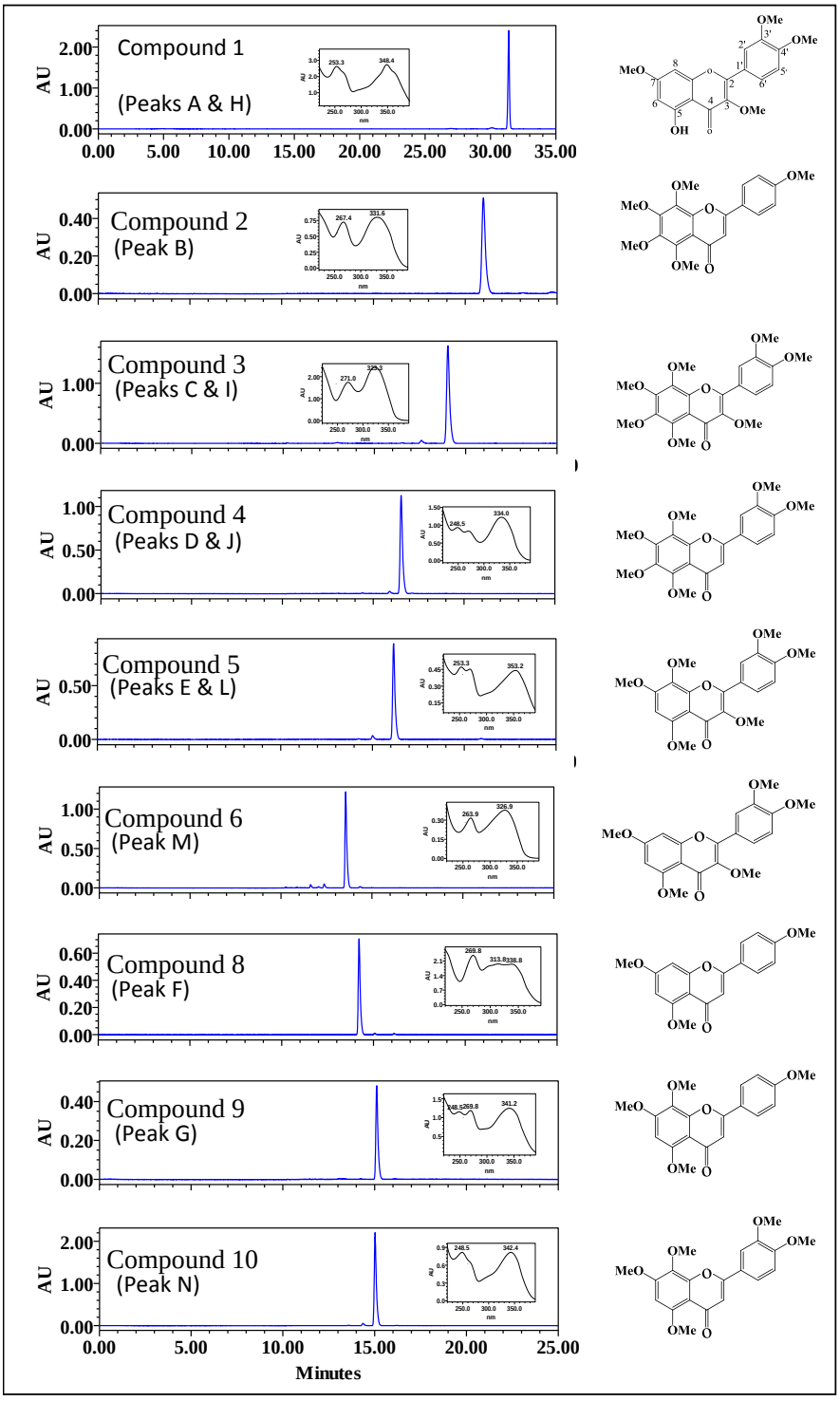

Fig. 36. HPLC chromatograms of isolated compounds and there UV spectra.

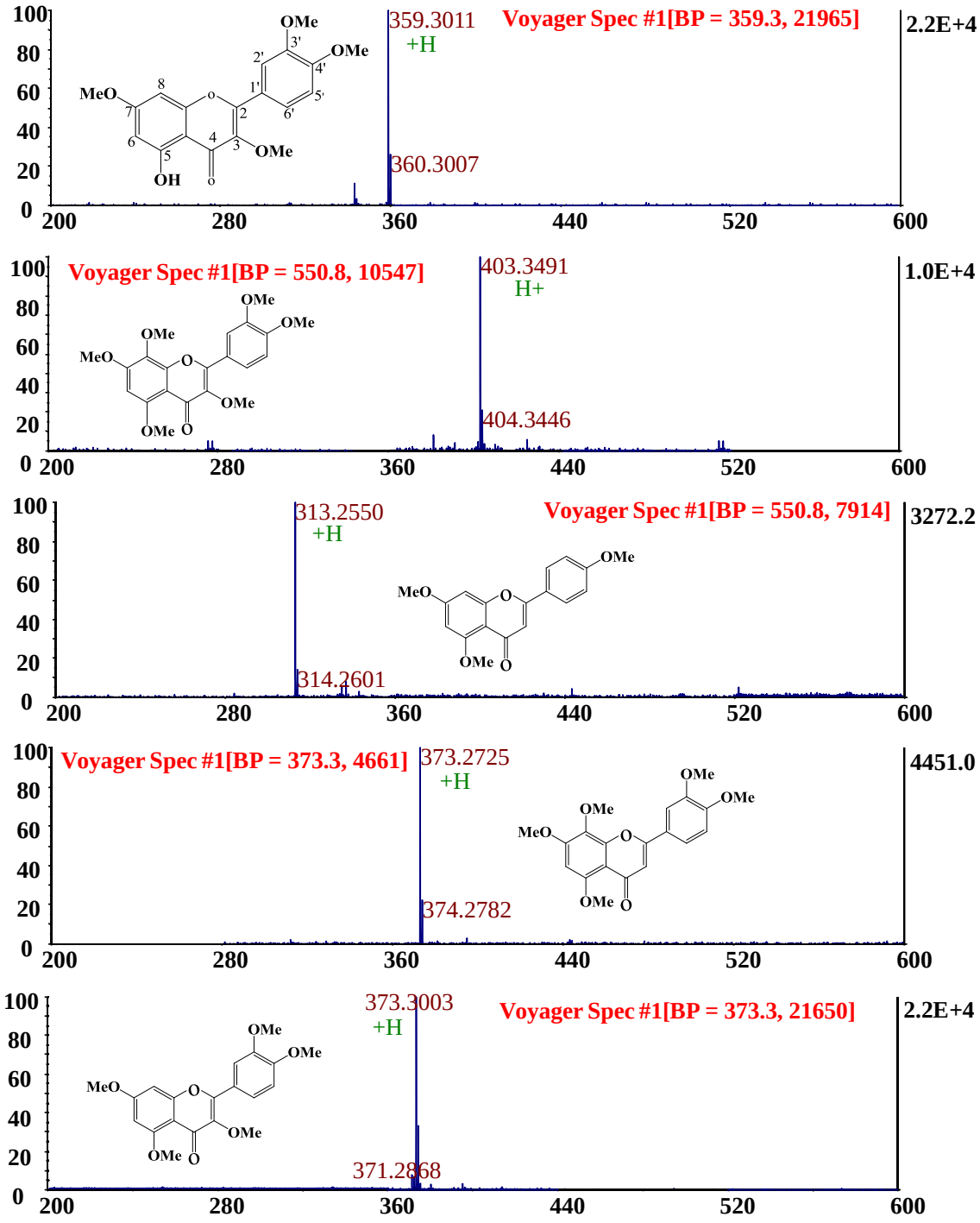

Fig. 37. Maldi TOF of the isolated compounds.

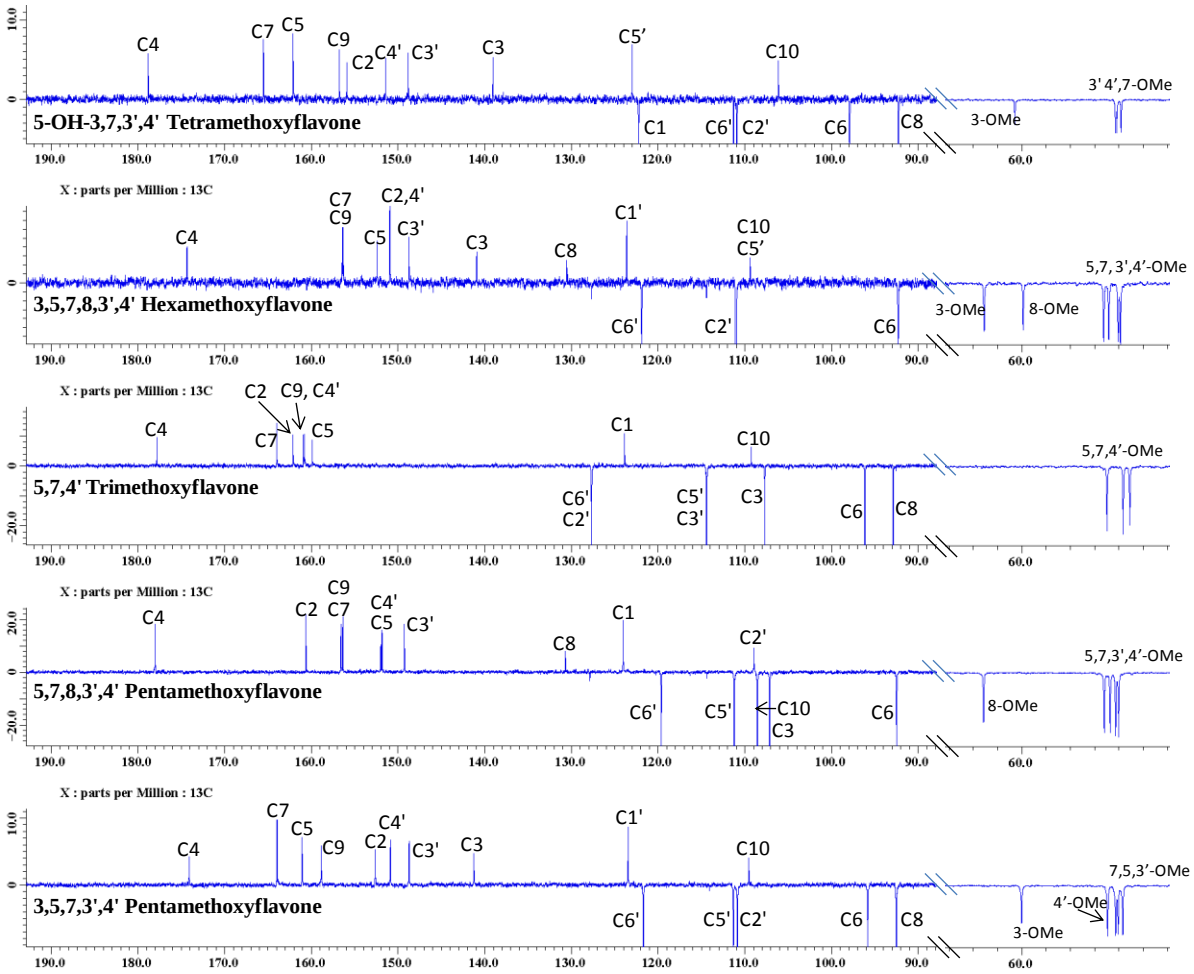

Fig. 38. Attached proton test (APT) spectra of the isolated compounds.

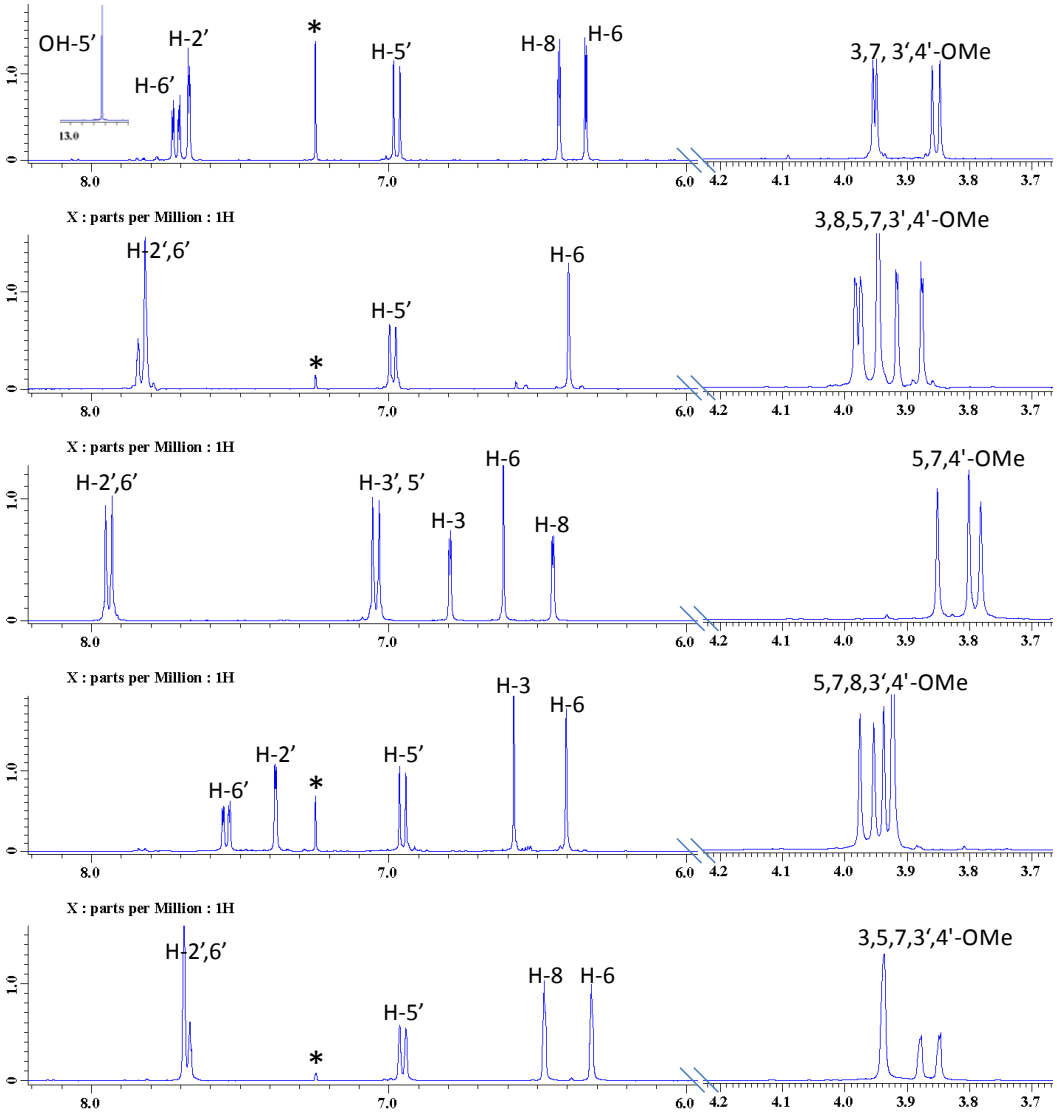

Fig. 39. [1] H NMR of the isolated compounds

*Biofilm and bioluminescence inhibitory activity***:** Figures 41A and 41B illustrate the results obtained from the analysis of bioluminescence in BB886 and BB170 strains of *V. harveyi*. Among the evaluated PMFs only heptamethoxyflavone and 3,5,7,8,3',4' hexamethoxyflavone interfered the AI-2 mediated bioluminescence in BB170 strain.

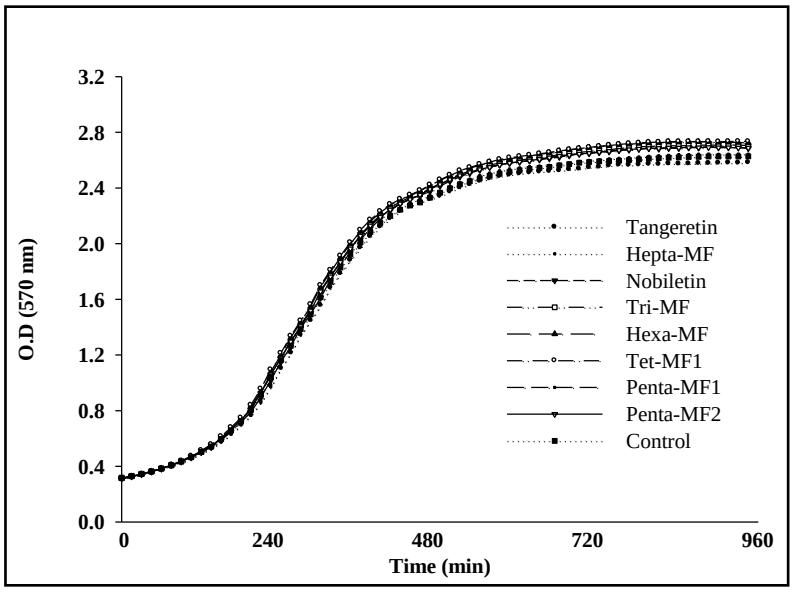

Fig. 40. Growth curve of *V. harveyi* BB120 in presence of PMFs.

The treatment of PMFs on BB886 strain did not have inhibitory activity greater than 40% with respect to control. The inhibition of bioluminescence suggests that PMFs may have the ability to interfere with biofilm formation mediated by QS. Corresponding to the bioluminescence interference only heptamethoxyflavone and 3,5,7,8,3',4' hexamethoxyflavone inhibited biofilm significantly in a dose dependent manner (Fig. 42). Approximately 61% (with respect to control) of biofilm was inhibited by 3,5,7,8,3',4' hexamethoxyflavone at a concentration of 50 µM. The IC_{50} value of 3,5,7,8,3',4' hexamethoxyflavone was calculated to be 37.38 µM. Although heptamethoxyflavone inhibited the biofilm, the percent inhibition was approximately 35% at the maximum evaluated concentration of 50 µM. Other PMFs did not have greater than 35% inhibition of biofilm with respect to control. Biofilm is extracellular polymeric matrix formed by a surface adherent colony of bacteria for protecting

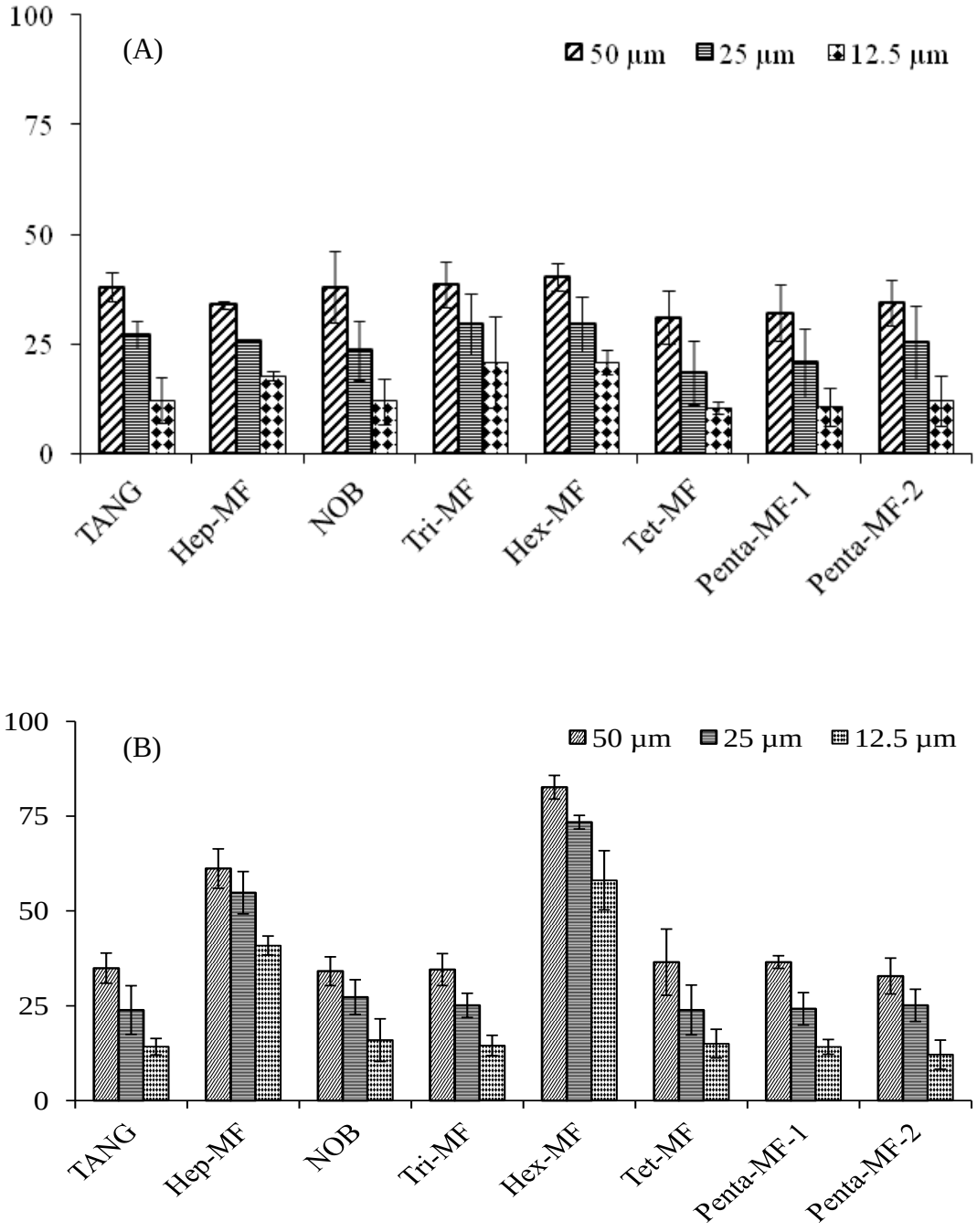

Fig. 41. AI-2 and HAI-1 induced bioluminescence inhibition in *V.harveyi* BB886 (A) and BB170 in presence of polymethoxyflavones respectively (B).

themselves from adverse environmental conditions. Control of biofilm is a serious concern among medical fraternity especially related to medical devices which seem to spread large number of hospital acquired infections. Furthermore, bacterial infections in medical implants are difficult to control due to the protective biofilm [270]. Formation of biofilm is a product of several factors among which the mechanism of cell-cell signaling known as quorum sensing (QS) is well established. It is a population dependent and mediated through the concentration of 'hormone-like' signaling molecules synthesized by bacteria which are known as autoinducers [271]. The QS also regulates bioluminescence production in *V. harveyi* and its signal transduction pathway has been well studied. Analyzing the bioluminescence production in specific mutant strains after treatment with PMFs enable us to understand the possible pathway through which the compounds inhibit the biofilm formation. Furthermore, constitutively luminescent *V. harveyi* mutants were investigated after treatment with 50µm of 3,5,7,8,3',4' hexamethoxyflavone. The results from the treatment are presented in figure 43. An increase in bioluminescence in the luxO mutant strain of *V. harveyi* JAF483 was noticed while no change in the bioluminescence was noticed in the hfq mutant BNL258. These results indicate that 3,5,7,8,3',4' hexamethoxyflavone selectively inhibits QS by interfering with the AI-2 system. Further studies are required to evaluate the ability of PMFs to interfere with QS mediated biofilm formation in human pathogens.

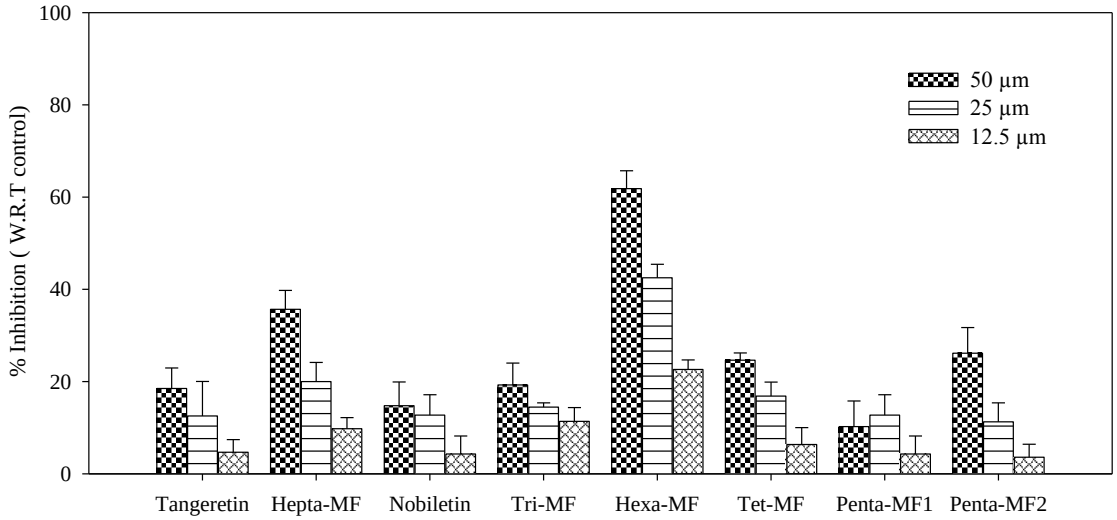

Fig. 42. Inhibition of *V. harveyi* BB120 biofilm after treatment with PMFs.

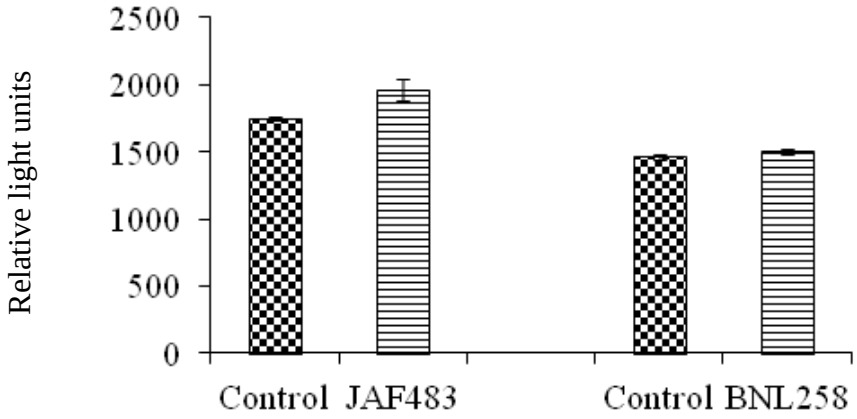

Fig. 43. Bioluminescence inhibition in *V.harveyi* mutants JAF483 (*luxO*) and BNL258 (*hfq*) by hexamethoxyflavone (50 µm).

9.3 Conclusion

Ten bioactive compounds in gram level quantities were successfully isolated from Miaray mandarin using hyphenated chromatography and their identities were confirmed by spectral analysis using Maldi-TOF and NMR. Among the isolated compounds 3,5,7,3',4' pentamethoxyflavone is being reported for the first time in the genus *Citrus*. Among the evaluated PMFs, 3,5,7,8,3',4' hexamethoxyflavone demonstrated to have inhibitory activity on autoinducer-mediated cell–cell signaling and biofilm formation in *V. harveyi*. Further research is required to understand the role of PMFs in inhibition of biofilm in pathogenic bacteria.

10. ISOLATION OF POLYMETHOXYFLAVONES FROM CLEMENTINE MANDARIN (*Citrus clementina*) AND BLOOD ORANGE (*Citrus sinensis* L.)

Among the several species and varieties in the Citrus genus, Clementine mandarins and Blood Oranges are unique with distinct taste and phenotypic characteristics. Clementine mandarin is one of the most popular and fastest expanding mandarin varieties during the past three decades. Also, Blood oranges unlike other common orange fruits, have high content of anthocyanins resulting in these fruits appear bright red in color and have an astringent like taste. Due to their appealing sensory and aesthetic qualities, these fruits are commercially ranked among the top citrus varieties in production and consumption. These fruits are well suited for tropical and temperate climatic conditions and are popularly grown in countries such as China, Japan, Europe, Turkey, Morocco, South Korea and United States. In the past five years due to the increase in area under cultivation the total production in these countries increased by one million metric tons. On the other hand, Clementine mandarins have a loose easy to peel rind, and contain sweet juicy succulent segment membranes. These characteristics have resulted in an increase in the consumption and as well as the production of these two fruits. It is estimated that the production of mandarins increased from 15 million tonnes in 1997-1999 to 17 million tonnes in 2010.

These fruits are primarily consumed fresh and are a potent source of several phytochemicals such as limonoids, flavonoids, carotenoids, anthocyanins, amines and organic acids. These phytochemicals cumulatively contribute towards the health

maintaining properties of Clementine mandarin and Blood orange fruits. Prior research in our lab and elsewhere has demonstrated the antioxidant properties of blood orange juice [205, 272]. We recently reported the occurrence of amines such as synephrine and tyramine in high content in Clementine mandarin as compared to other citrus fruits.

Apart from these phytonutrients they are also reported to contain polymethoxyflavones (PMFs), a group of flavonoids with three or more methoxyls attached to the basic flavone structure. Studies suggest that these molecules have anticancer, anti-inflammatory activities. However, there are no reports on the isolation of polymethoxyflavones from these fruits. In our quest to explore the potential health beneficial bioactive compounds of citrus, several analytical and isolation techniques of citrus bioactive compounds were earlier reported [88, 152, 248, 268, 273-275]. In our earlier reported method for separation of polymethoxy flavones, a rapid flash chromatography method was developed with high yield and purity [97]. Furthermore based on the current trends in production of these fruits, utilization of peels for isolation of health promoting components would be highly beneficial to both the citrus industry and human health.

In this context, a study was conducted for the separation and purification of PMFs. The four PMFs isolated in this study are tangeretin, 3,5,6,7,8,3´,4´heptamethoxyflavone, nobiletin & sinensitin. The structures of purified compounds have been confirmed on the basis of NMR studies and mass spectrometry. To the best of our knowledge, this is the first report on isolation of PMFs from Clementine and blood orange peels using flash chromatography.

10.1 Materials and methods

10.1.1 Reagents and instrumentation

Solvents used for analysis were HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA, USA). Nanopure water (NANOpure, Barnstead/Thermolyne, Dubuque, IA, USA) was used for HPLC analysis. The solvents used for flash chromatography were analytical grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). The separation of PMFs was carried out on an automated flash chromatography system (Combiflash® Rf , Teledyne Isco, Lincoln, NE, USA). Silica gel (particle size 35–60 μm) flash columns (220 g) were purchased from ISCO Inc. (RediSep® Rf ISCO Inc., Lincoln, NE, USA).

10.1.2 Plant material and soxhlet extraction

Mature Clementine (*C. clementina*) mandarin fruits and Blood orange were harvested in the month of November, 2008 from Texas A&M University-Kingsville Citrus Center orchard (Weslaco, TX). The peels were separated and dried to obtain ≤ 5% moisture. The peels were blended to obtain (40-60 mesh size powder in a Vita-prep™ blender (Vita-Mix Corporation, Cleveland, OH, USA). 1.8 kg of Clementine and 0.6 kg of dried Blood orange peel powder was loaded on to Soxhlet type apparatus and

extracted with hexane for 16 hrs. The extract was concentrated to yield 23.8 g and 14.5 g of concentrated extract respectively.

10.1.3 Sample preparation for flash chromatography separation

The concentrated hexane extracts of Clementine mandarin and Blood orange were dissolved in hexane and mixed with silica gel 17 g and 7.5 g respectively. The samples were mixed thoroughly to make a fine slurry and the mixtures were evaporated under vacuum to make a free-flowing powder. The silica impregnated samples were further used for flash chromatography.

10.1.4 Flash chromatography

An automated flash chromatography system Teledyne Isco CombiFlash Rf 4x system (Lincoln, NE) equipped with a silica gel (particle size 35–60 μm) flash column from ISCO Inc. was used. Binary solvent system of hexane (solvent A) and acetone (solvent B) were used in a gradiant. The column was eluted with 100% hexane for 3 column volumes after injection followed by a gradient with acetone. Individual fractions were collected by monitoring the eluting analytes at 254 nm, 340 nm and absorption spectra.

10.1.5 Separation of PMFs from Clementine mandarin extract

The silica gel impregnated hexane extracts of clementine mandarin were subjected to flash chromatography on a silica gel (particle size 35–60 μm) flash column 120g. The column was equilibrated with hexane for three column volumes prior to separations. PMFs were separated in 35 min gradient program of solvent A (hexane) and solvent B (acetone) maintained at a flow rate of 85 ml/min: 100% A held for 2 min, linearly increased to 5% B over 2 min, held for 10 min, linearly increased to 100% B over 16.5 min, held for 5 min, then finally returned to the initial conditions and held for 2 min. The flow rate was maintained at 150 mL/min and individual fractions were collected by monitoring the eluting analytes at 254 nm and 340 nm. Four major peaks (A - D) were observed (Fig. 44) and collected in individual fractions. The retention time of the separated peaks were, peak A: 17.0 – 18.5 min, peak B: 18.6 – 20.3 min, peak C: 20.4 – 21.5 min, and peak D: 21.5 – 23.0 min. The individual peak fractions were analyzed by HPLC and pooled based on peak similarity and matching the UV spectral analysis. Evaporation of the solvent from the pooled fractions of peak A-D yielded compounds 4 respectively.

10.1.6 Separation of PMFs from Blood orange extracts

The silica gel impregnated extracts was subjected to flash chromatography on a silica gel (particle size 35–60 μm) flash column 120 g. The column was equilibrated

with hexane for three column volumes prior to separations. PMFs were separated in 50 min gradient program of solvent A (hexane) and solvent B (acetone) maintained at a flow rate of 85ml/min. Briefly, 100% A held for 5 min, linearly increased to 20% B over 5 min, held for 5 min, linearly increased to 50% B over 22.5 min, linearly increased to 100% B over 7.5 min, held for 2.0 min, then finally returned to the initial conditions and held for 2 min. The flow rate was maintained at 150 mL/min and individual fractions were collected by monitoring the eluting analytes at 254 nm and 340 nm. Eight major peaks (A - H) were observed (Fig. 45) and collected in individual fractions. The retention time of the separated peaks were, peak E: 23.0 - 25.5 min, peak F: 25.6 – 29.5 min and peak G: 29.6 – 32.5 min. The individual peak fractions were analyzed by HPLC and pooled based on peak similarity and matching the UV spectral analysis. Evaporation of the solvent from the pooled fractions of peak E-G yielded 3 compounds respectively.

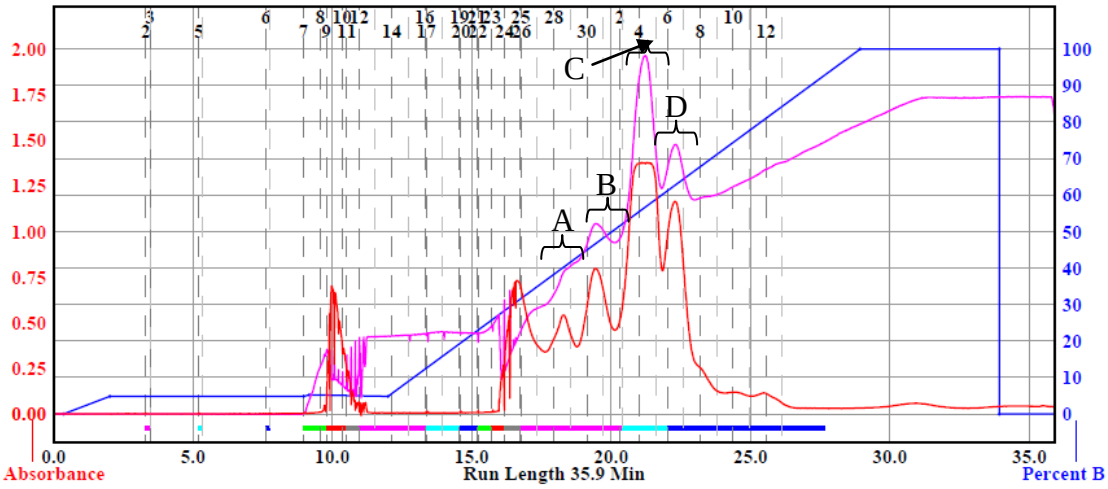

Fig. 44. Chromatogram of the automated flash chromatography system monitored at 280 nm and 340 nm for the separation and isolation of PMFs from hexane extract of Clementine peel.

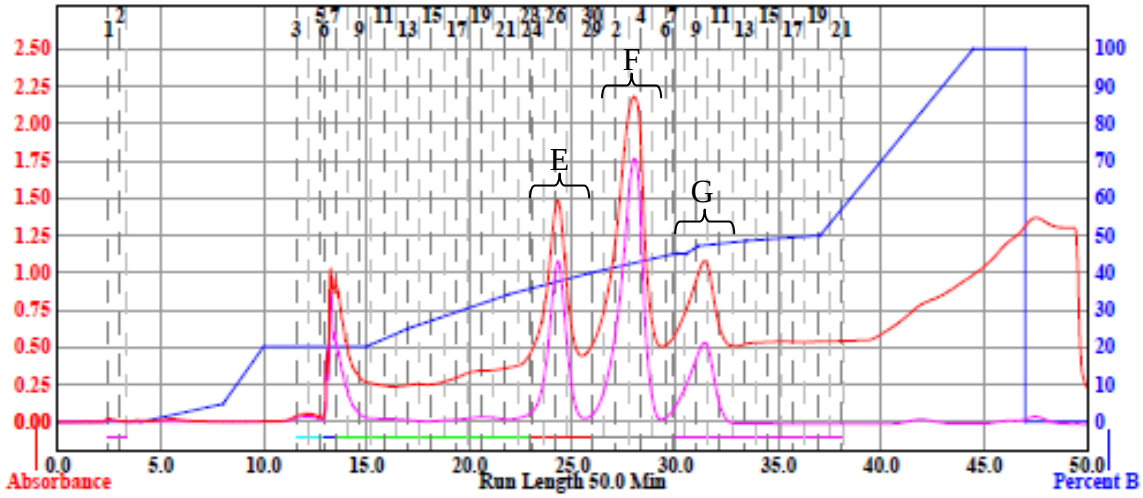

Fig. 45. Chromatogram of the automated flash chromatography system monitored at 280 nm and 340 nm for the separation and isolation of PMFs from hexane extract of Blood orange peel.

10.1.7 Liquid chromatography

The HPLC system consisted of a Waters 1525 HPLC series (Milford, MA, USA) connected with a PDA detector. A Gemini C_{18} column (3 µm, 250 × 4.6 mm mm i.d.) (Phenomenex, Torrance, CA, USA) was used for the separations. A gradient mobile phase of 3 mM phosphoric acid (A) and acetonitrile (B) was used for the separations at a flow rate of 1 mL/min. Initially, elution was started with a gradient of 5% B followed by linear increase to 50% in 20 min, returned back to 5% in 5 min. Injection volume was set at 10 µL and the PMFs were detected at 280 nm and 340 nm. Chromatographic data was collected and processed using Empower2 software (Waters, Milford, MA, USA).

10.1.8 Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (Maldi-TOF-MS) analysis.

The samples for MS analysis were prepared by dissolving the isolated compounds in acetonitrile and mixed with 2′, 4′, 6′-trihydroxyacetophenone (THAP) matrix. A 0.5 µL of the matrix mixture was spotted on a MALDI sample plate and air-dried. MALDI-TOF mass spectra were acquired using Voyager DE-Pro (Applied Biosystems, CA) mass spectrometer in positive reflector ion mode. After time-delayed extraction of 275 nsec, the ions were accelerated to 20 kV for TOF mass spectrometric analysis. A total of 100 laser shots were acquired with the signal averaged per mass spectrum.

10.1.9 NMR analysis

^{1}H and attached proton test (APT) spectra were recorded at 400 MHz and 100 MHz respectively by FT NMR (JEOL USA, Inc., MA, USA).

10.2 Results and discussion

Citrus peels are a rich source of flavonoids such as naringin and hesperidin which occur at high concentrations in comparison to PMFs. Soxhlet extraction of Clementine mandarin and Blood orange peels by hexane, enabled in selective separation of PMFs from other flavonoids. The isolated compounds were separated and analyzed by HPLC, MS and there structure determined by NMR. Based on the spectral analysis the crystallized compounds from peaks A yielded compound 1, peaks B and E yielded compound 2, peaks C and F yielded compound 3 and peaks D and G yielded compound 4. Figure 46 depicts the chromatogram of HPLC analysis of the isolated compounds. The absence of other peaks demonstrates the purity of the isolated compounds. Furthermore the identity of the compounds was confirmed by UV spectra (Fig. 47) and mass spectral analysis. The mass spectrum of isolated compound 1, had a molecular ion $[M+H]^{+}$ at *m/z* 373. Compound 2, 3 and 4 generated molecular ion $[M+H]^{+}$ at *m/z* 403, 433 and 373 respectively. ^{1}H and ^{13}C NMR spectra of the isolated compounds are in conformation with our earlier reported results [97]. In conclusion the isolated compounds from the 23g of hexane extract of Clementine mandarin were identified and measured to be 15 mg of

tangeretin (5,6,7,8,4′-pentamethoxyflavone), 0.42 g of 3,5,6,7,8,3´,4´-heptamethoxyflavone, 0.37 g nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone, and 40 mg of sinensitin (5,6,7,3′,4′-pentamethoxyflavone). The compounds isolated from blood orange peels were 35 mg of 3,5,6,7,8,3´,4´- heptamethoxyflavone, 37 mg nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), and 97 mg of sinensitin (5,6,7,3′,4′-pentamethoxyflavone).

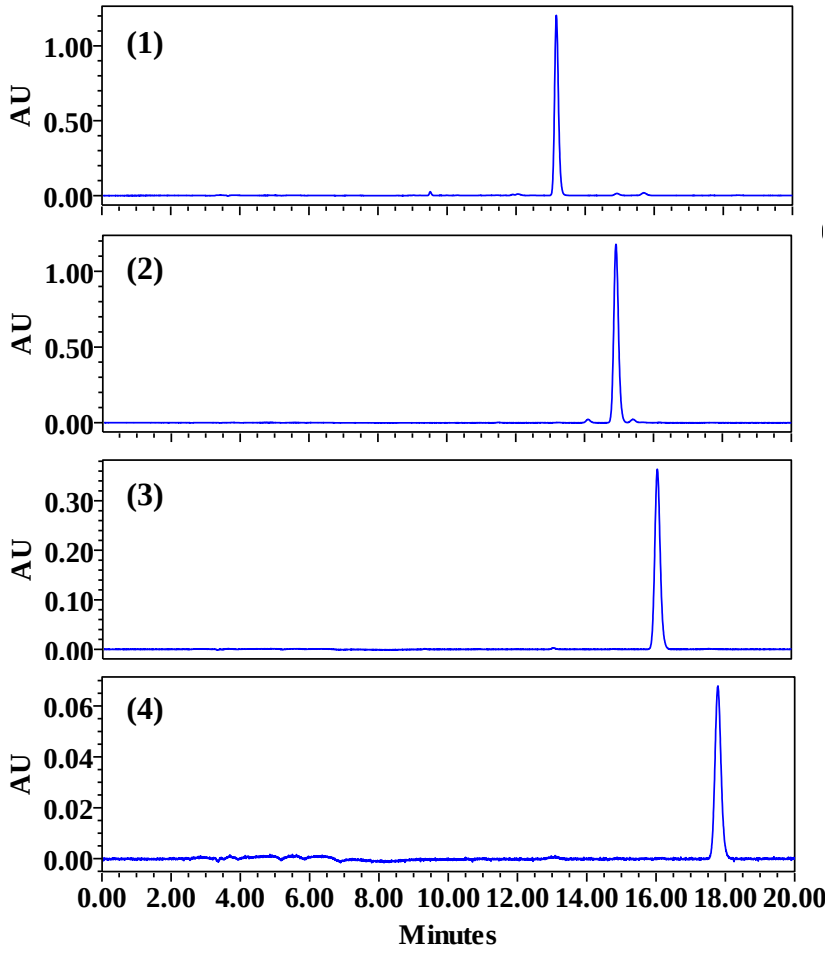

Fig. 46. HPLC chromatograms of the isolated compounds: 1) Tangeretin 2) 3,5,6,7,8,3´,4´ heptamethoxyflavone 3) Nobiletin and 4) Sinensitin

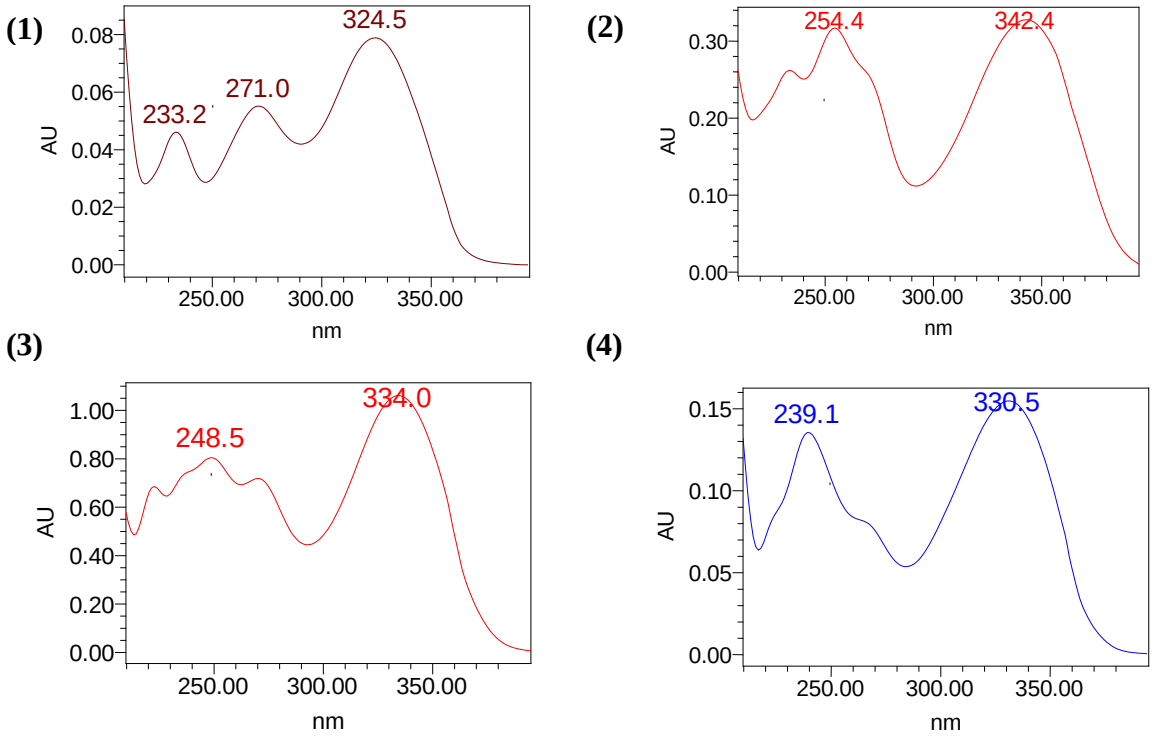

Fig. 47. UV spectra of the isolated compounds 1) Tangeretin 2) 3,5,6,7,8,3´,4´ heptamethoxyflavone 3) Nobiletin and 4) Sinensitin.

10.3 Conclusion

Four polymethoxyflavones were separated and isolated from citrus peels. Flash chromatography of Clementine mandarin and Blood orange peels resulted in isolation of multi milligram quantities of potent compounds that could help further studies in understanding there health beneficial properties.

11. SUMMARY AND CONCLUSION

Citrus fruits are a rich source of wide array of phytochemicals and essential vitamins. These phytochemicals were found to have several human health beneficial properties. To obtain optimum level of these phytochemicals analytical and isolation methods are necessary to quantify and conduct further *in vitro* and *in vivo* studies. Similarly, factors such as production systems and processing techniques also have an effect on the levels of phytochemicals. In the present study a rapid simultaneous separation, as well as determination of amines and organic acids in citrus juice, was achieved. The developed HPLC method demonstrated that 3% meta phosphoric acid can be used for simultaneous extraction of organic acids and amines. The method is precise and rugged combined with high recovery and repeatability. The simultaneous extraction and analysis of samples provides an economical method for analysis of large number of samples in short duration of time. The developed method was applied for determining the effect of production systems on the levels of amines and organic acids along with other phytochemicals in Meyer lemons. Organically produced lemons had higher levels of organic acids and flavonoids than conventionally produced lemons. No significant variation in the levels of amines was noticed in lemons produced organically and conventionally. Storage at 10 ºC helps in maintaining the levels of these phytochemicals without any major adverse affects. Further long term, multi-location field studies are required to validate the variation of phytochemicals content in Meyer lemon.

The studies on method development for isolation of phytochemicals from citrus peels and industrial by products resulted in isolation of limonoids, polymethoxyflavones and furocoumarins. The developed flash chromatographic method is rapid and capable of efficient separation of PMFs from citrus peels. The method was tested to be robust and repeatable. Using the developed method, four PMFs were successfully separated with high purity in gram level quantity. The purified PMFs were identified and characterized as tangeretin, nobiletin, tetramethoxyflavone and sinensitin by spectroscopic studies such as HPLC, MS and NMR. Hence, this method is viable for rapid and large scale separation of PMFs. The developed FC method can enable utilization of citrus by-products such as peels for separation of economically important PMFs, which could add value addition to the citrus processing industry. Similarly, a method was developed for isolation of two furocoumarins, bergamottin and bergapten, along with 3 polymethoxyflavones, tangeretin, heptamethoxyflavone and nobiletin from grapefruit peel oil that is a citrus industrial byproduct. A two-phase gradient solvent system consisting of hexane and acetone was used for efficient flash separation of the compounds. The overall process for separation of the compounds is simple and rapid. The yield, repeatability and purity of the compounds suggest the developed method could be used for large scale isolation. The method may help in providing value added products from citrus industrial byproducts and be beneficial for further research on biological activities using the isolated compounds. The study of isolation of phytochemicals from understudied Miaray mandarin, Clementine mandarin and Blood Orange resulted in isolation of 10 bioactive compounds in gram level quantities. Among

the isolated compounds 3,5,7,3',4' pentamethoxyflavone is reported for the first time in the genus *Citrus.* Among the isolated PMFs, 3,5,7,8,3',4' hexamethoxyflavone demonstrated to have inhibitory activity on autoinducer-mediated cell–cell signaling and biofilm formation in *V. harveyi*. Further research is required to understand the role of PMFs in inhibition of biofilm in pathogenic bacteria.

In the evaluation of the household processing techniques, blending whole edible part of grapefruit resulted in high levels of flavonoids, limonin and citric acid. These phytochemicals may cumulatively contribute to health maintaining properties. Significantly low levels of phytochemicals were present in juice processed by juicing and squeezing techniques than by blending. Results from this study could help the consumers make a better choice for obtaining higher levels of health maintaining phytochemicals in their diet. Furthermore, among the evaluated industrial processing techniques, high pressure processing (HPP) of grapefruit juice maintained the levels of phytochemicals comparable to that of thermal processing (TP) during storage at 4º C. The emerging HPP technology also maintained the visual color quality of the juice, resulting in a fresh-like appearance for longer storage periods, unlike TP. The results obtained in this study confirm the potential application of HPP in grapefruit processing industries. Further studies are required to evaluate the consumer's acceptance of these processing techniques with respect to sensory qualities affected by varying contents of phytochemicals.

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