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Optimization of flavanones extraction by modulating differential solvent densities and centrifuge temperatures

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ABSTRACT

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

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1. Introduction

Flavanones have a great potential for antioxidant activity and contributes to various traits such as color and flavor in fruits and vegetables [1]. Flavonoids are classified into flavones, flavanones and flavanols based on their structure and these occur as aglycones and glycosides [1]. In the late 1960s, Albach and Redman classified genus *Citrus* chemotaxonomically based on the type and quantity of major flavonoid glucosides (bitter neohesperidosides and non-bitter rutinosides) present in *Citrus* leaves and fruits [2]. Chemotaxonomically, grapefruit is considered as a hybrid among *Citrus* species due to the occurrence of both bitter (naringin, neohesperidin, poncirin) and non-bitter (narirutin, didymin) flavonoids [3]. Hence, accurate quantification of flavonoids is absolutely necessary for taxonomic evaluations.

Previously published reports demonstrated variations among grapefruit flavanone levels due to genotype, season, growing conditions, storage and also differences in sample preparation procedures (Table 1). However, the resulted flavanone variations could be either due to the treatments or experimental artifacts. According to Luthria et al., approximately 30% of analytical errors originate during sample preparation; therefore, identification and optimization of factors influencing the sample preparation are critical for accurate quantification [4–6]. Due to a wide structural diversity, flavonoids have different physicochemical properties that makes it virtually impossible to fully extract them in a single extraction step [7]. Despite the obvious difficulties in extraction, sample preparation methods can be optimized by focusing one or two specific classes of flavonoids with similar properties. This approach not only improves the extraction efficiency but also reduces the extraction time. Moreover, the goal of optimized extraction procedure is to obtain a uniformly rich extract devoid of matrix interferences [8].

Since flavanones are relatively non-labile compared to vitamin C and carotenoids, robust analytical methods can be employed for extraction of flavanones [3,9,10]. The problems during extraction are better understood when real samples are used rather than model standard matrices [11]. Solubility and mass transfer of the analytes of interest not only depend on physicochemical properties of the compounds themselves but also greatly influenced by the other non-specific analytes in the matrix [12]. Therefore, these aspects require a detailed investigation in fruits due to their com-

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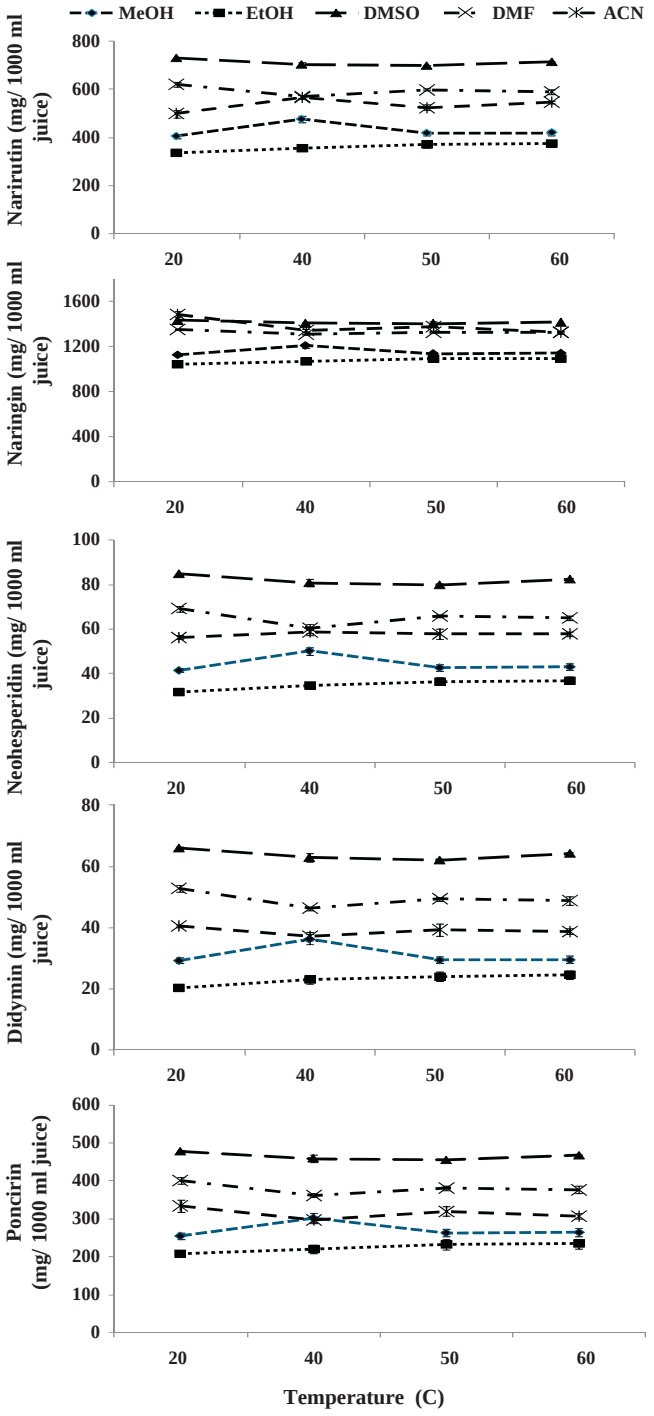

Fig. 1. Extraction efficiency of grapefruit flavanones with various solvents such as methanol (MeOH), ethanol (EtOH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF) and acetonitrile (ACN) at different temperatures ranging from 20, 40, 50 and 60 °C. The data presented is mean ± standard deviation values of three individual samples.

plex matrices. Traditionally, flavanones have been extracted with aqueous solvents from freeze-dried samples to increase the tissue permeability to the solvent [5]. However, addition of water is not required when extracted directly from fruit juice [13]. Maceration or blending, centrifugation and filtration are three commonly followed extraction steps for citrus flavanones [13]. In case of higher sample volumes and number, the centrifugation step is highly advantageous before filtration because this reduces the total filtration cost prior to HPLC analysis. Nevertheless, the physical phenomenon behind flavanone extraction with different solvents and their distribution in a miscible solvent mixture after centrifugation has not been investigated. Furthermore, variations in flavanone levels in the same extract after centrifugation were not observed in previous flavanone methods [14–17]. In addition to these extraction steps, the influence of other factors such as solvent, extraction cycles and sample to solvent ratio can play a key role in accurate quantification of flavanones. Furthermore, the commonly used extraction procedures such as microwave extraction and sonication were also evaluated. The objective of the present study was to evaluate the extraction efficiency of various solvents, temperature, centrifugal speed, and centrifuge temperature, number of extraction cycles, microwave extraction and sonication on grapefruit flavanones.

2. Experimental

2.1. Plant materials

Rio red grapefruits were harvested in November 2007, washed in a commercial packing shed in Mission, Texas. The fruits were peeled and blended for 3 min in a Vita-Prep blender obtained from Vita-Mix food services (Cleveland, OH, USA). The same blended juice was used for all the analysis except in Section 2.3.4, where the fruits were harvested from February 2010. The fruit juice was stored at −80 °C until all the experiments were conducted.

2.2. Chemicals and instrumentation

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

2.3. Factors affecting sample preparation

2.3.1. Solvent and heat

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

2.3.2. Solvent and centrifuge speed

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

Table 1
Sample preparation methods and reported naringin levels from grapefruit on fresh weight (FW) or dry weight (DW) basis.

Variety	Location	Sample preparation	Extraction solvent	Naringin quantity[a]	Reference
Rio red	South Texas	5 g of pulp + 20 mL DMF, 1.5 mL aliquot centrifuged at 7500 × *g*, supernatant analyzed in HPLC	DMF	1500/FW	Vanamala et al. [14]
Ruby Red	NA	Juice: solvent; 400 μL:400 μL (v/v), vortex – 5 min, sonication – 15 min at 60 °C. centrifugation, 2000 rpm	Methanol	626.2/FW	Desiderio et al. [17]
Local market[b]	South Portugal	Centrifugation – 8000 rpm, 15 min; supernatant filtered; dilution with sodium acetate buffer (0.02 M); pH 4	No solvent	476.82/FW	Ribeiro and Ribeiro [29]
NA	NA	Juice extractor; centrifugation – 7200 rpm, 10 min supernatant filtered; the aliquot diluted with borate buffer (60 mM)	No solvent	44.6/FW	Wu et al. [15]
Pink mash	Florida	25 mL juice + 20 mL DMF heated at 90 °C for 10 min adjusted volume to 50 mL, centrifuged at 2500 × *g* for 10 min	DMF	428/FW	Mouly et al. [16]
Red blush	Italy	Centrifugation, pellet suspended in water and extracted 3 times similarly and pooled all extracts. A Sep-Pak cartridge was used for flavonoid separations	Methanol	4600–5240/DW	Del Caro et al. [30]
Rio red	Texas	Juice is mixed with DMSO 1:1 (v/v) and centrifuged	DMSO	1200/FW	Patil et al. [31]
Rio red	Texas	Pulp filtered through cheese cloth, centrifuged, separated the flavonoid fraction on Sep-Pak C-18 cartridge	Methanol	129/FW	Lester et al. [32]
Duncan	Texas	Freeze dried sample extracted 4 times with 1:1 mixture of DMSO and methanol. The extract was further mixed with DMSO at 1:1 (v/v) and centrifuged	DMSO	382/FW	Berhow et al. [33]
Ruby red	Florida	Pulp filtered through cheese cloth, centrifuged, separated the flavonoid fraction on Sep-Pak C-18 cartridge	Methanol	124/FW	Rouseff et al. [3]
Rio red/Texas	Texas	1 mL juice mixed with 2 mL methanol and filtered	Methanol	2200/FW	Girennavar et al. [34]

NA: not available information.
[a] All values are presented in ppm.
[b] Source of material.

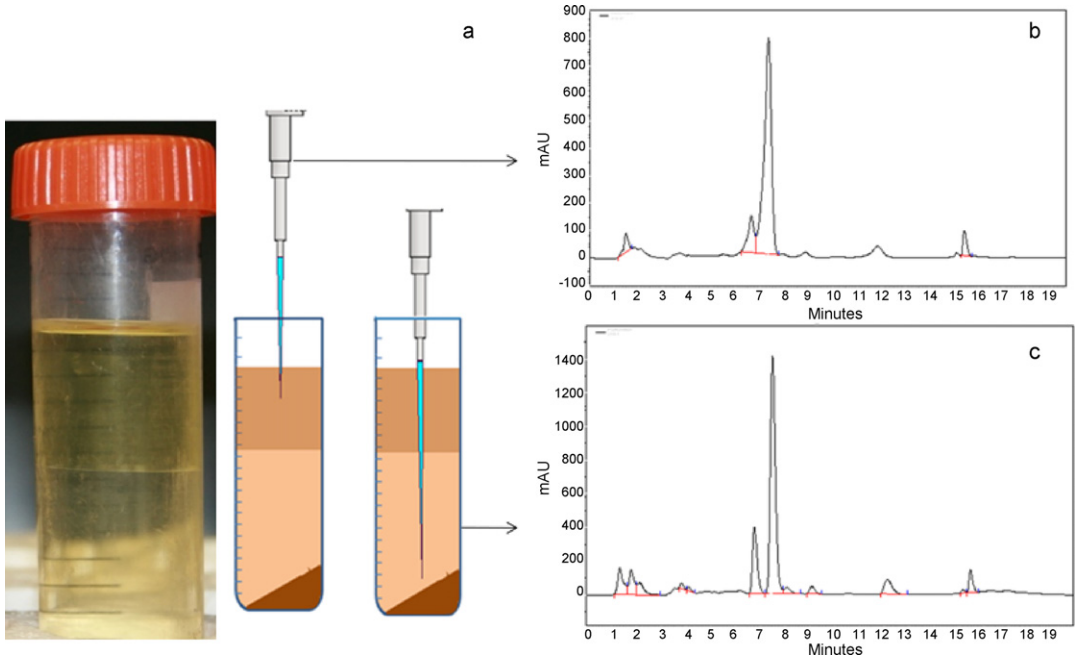

Fig. 2. The extraction of flavanones using centrifugation (a) phase separations observed in the ACN extracts of grapefruit juice after centrifugation at 6720 × *g*. (b) HPLC chromatogram of the top layer. (c) Bottom layer.

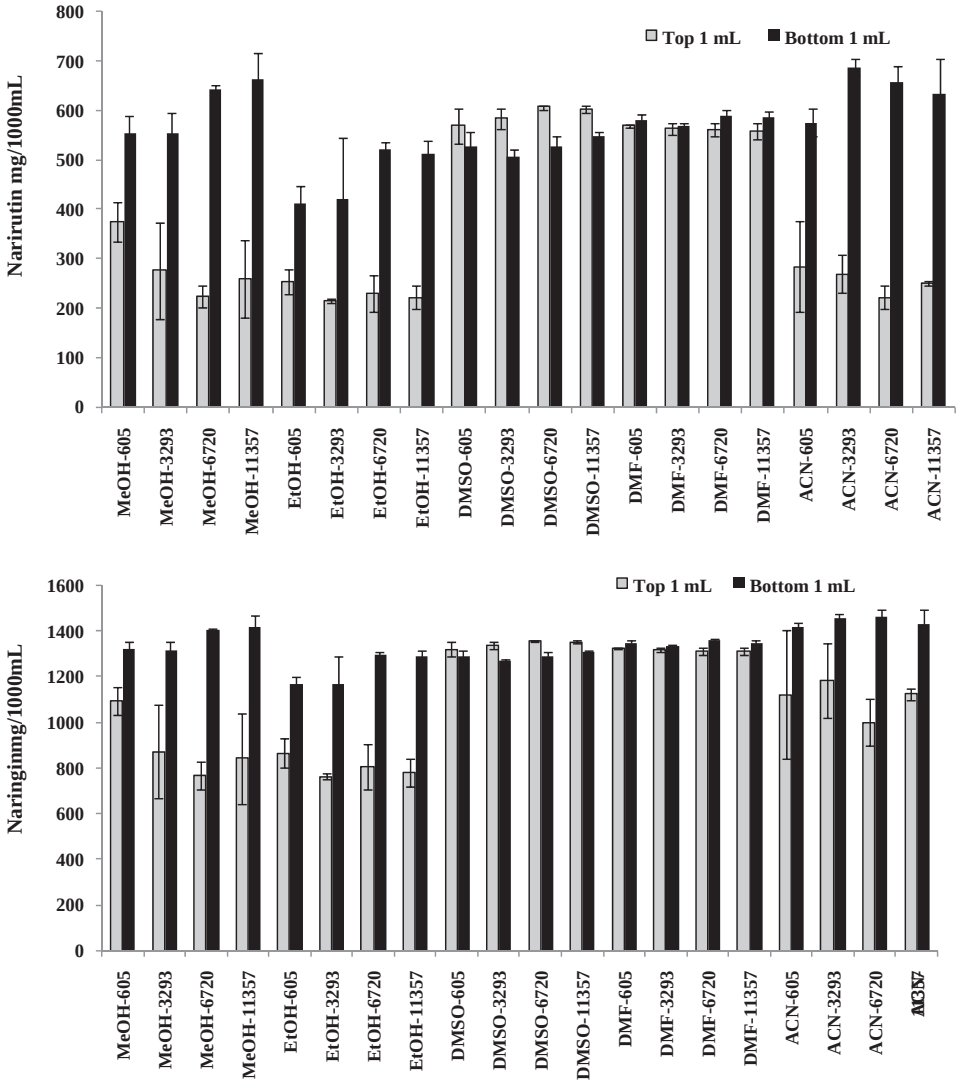

Fig. 3. Narirutin and naringin levels from 1 mL top and bottom of centrifuge tube with five different solvents namely methanol (MeoH), ethanol (EtOH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF) and acetonitrile (ACN) at various centrifugal speeds 605, 3293, 6720 and 11357 × *g*. The data presented is mean ± standard deviation values of three individual grapefruit samples.

2.3.3. Solvent and centrifuge temperature

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

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

2.3.4. Extraction cycles

Grapefruit juice (3 mL) was extracted with 3 mL of DMSO in a centrifuge tube. The sample mixture was vortexed for 5 s, centrifuged at 6720 × *g* for 10 min and the supernatant was analyzed in HPLC. The residue was extracted two times with 3 mL DMSO, filtered through 0.45 μm acrodisc filter and analyzed separately.

2.3.5. Sonication and heat

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

2.3.6. Microwave extraction and solvent ratio

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

2.4. HPLC and LC–MS analysis

The HPLC analysis of five grapefruit flavanones (narirutin, naringin, neohesperidin, didymin and poncirin) were separated in

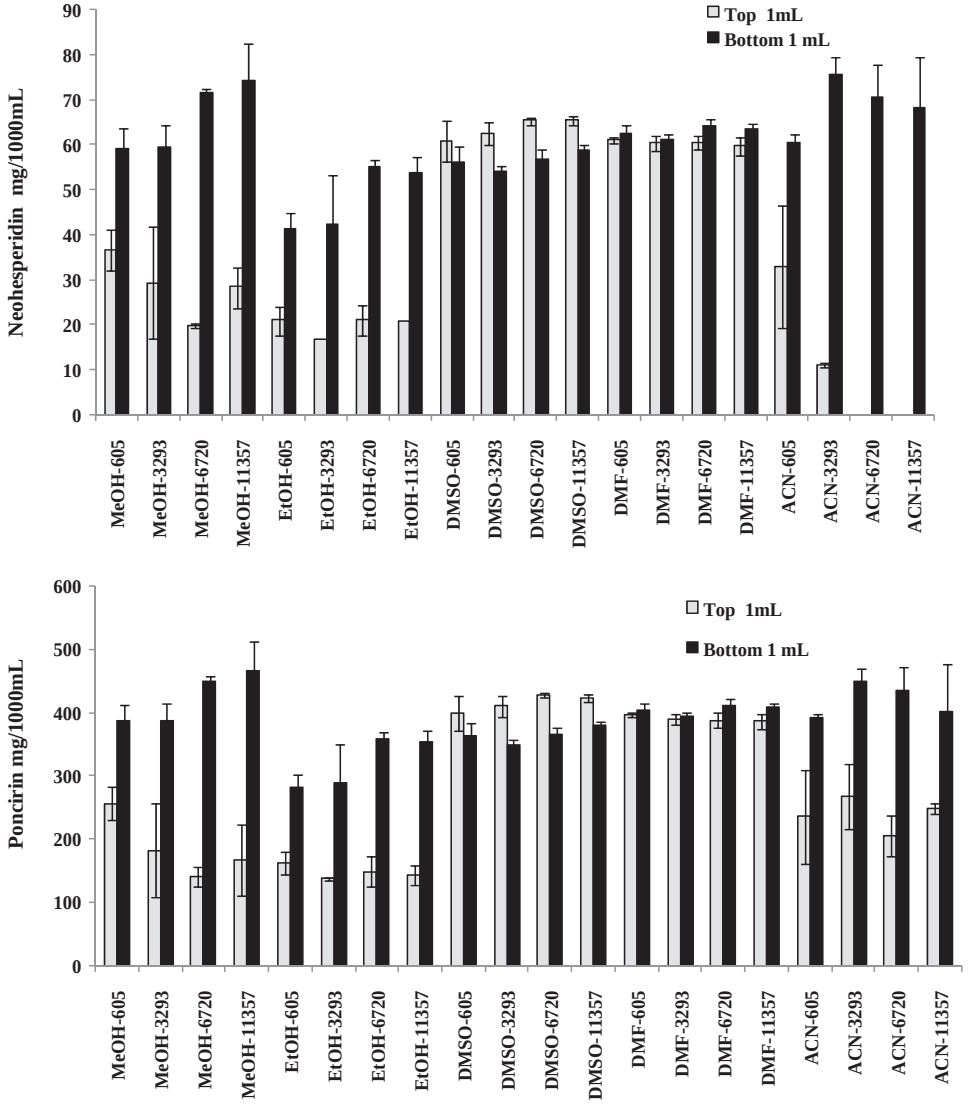

Fig. 4. Neohesperidin and poncirin levels from 1 mL top and bottom of centrifuge tube with different solvents namely methanol (MeoH), ethanol (EtOH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF) and acetonitrile (ACN) at various centrifugal speeds 605, 3293, 6720 and 11357 × *g*. The data presented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

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

The identity of flavonones was confirmed by a LC–MS analysis (Finnigan, LCQ Deca XP, West Palm Beach, FL, USA). The flavanones were separated on an Aquasil, C-18 column (2.1 × 150 mm, 3 μm). The flavanones were separated using a binary solvent gradient of (A). 0.1% formic acid and (B). ACN. The grapefruit samples were run at 0–2.6 min, 95% A; 2.6–11.6 min, 83% A; 11.6–15.0 min, 80%; 15.0–17.6 min, 75% A; 17.6–19.6 min, 95% A and ended with 95% A at 25 min at a 0.2 mL/min flow rate. All five flavanones eluting from LC column were identified using electron spray ionization (ESI) in negative mode. The operating capillary temperature was 250 °C and capillary voltage was maintained at −15 V. The sheath gas and the auxiliary gas (nitrogen) were maintained at 60 and 20 au, respectively with an applied voltage of 3.0 kV.

2.5. Statistical analysis

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

3. Results and discussion

3.1. Effect of solvent and heat

In the present study, various solvents such as MeOH, EtOH, ACN, DMSO and DMF were used for extraction of flavanones from

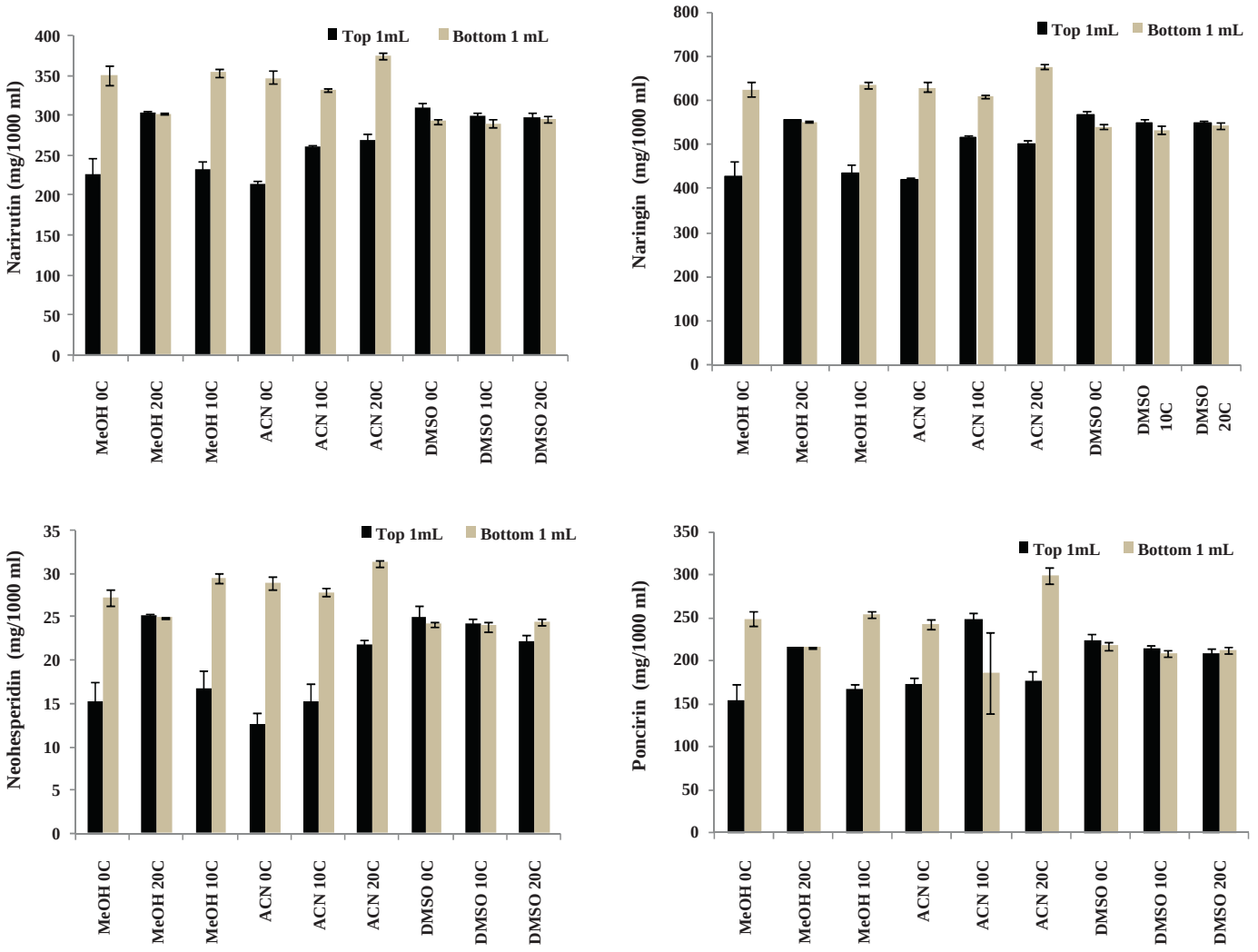

Fig. 5. Naringin, narirutin, neohesperidin and poncirin levels in grapefruits extracted from top and bottom 1 mL of centrifuge tube with different solvents such as methanol (MeOH), acetonitrile (ACN) and dimethyl sulfoxide (DMSO) at centrifuge rotor temperatures, 0, 10 and 20 °C. The histograms represent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

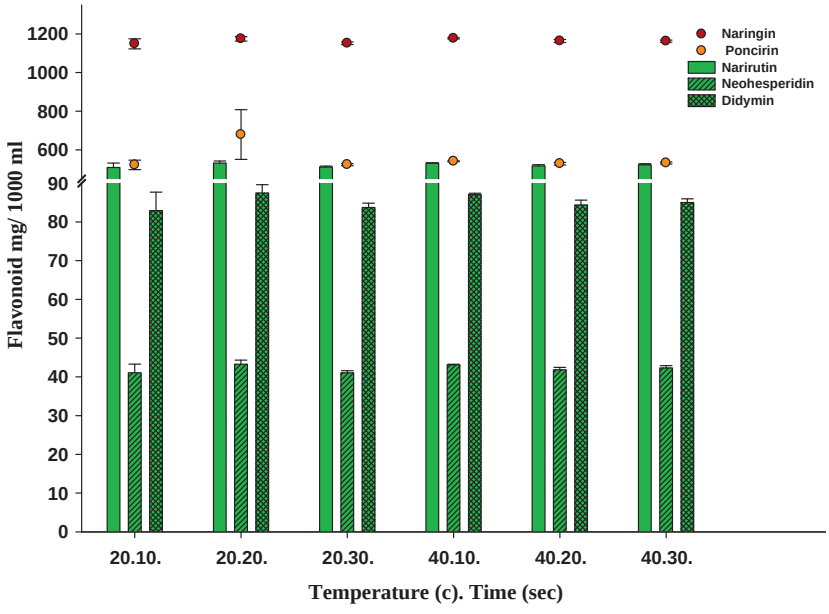

Fig. 6. Five grapefruit flavanones were extracted using dimethyl sulfoxide at three different sonication times namely 10, 20 and 30 min at 20 or 40 °C. The histograms represent mean ± standard deviations of three individual grapefruit samples.

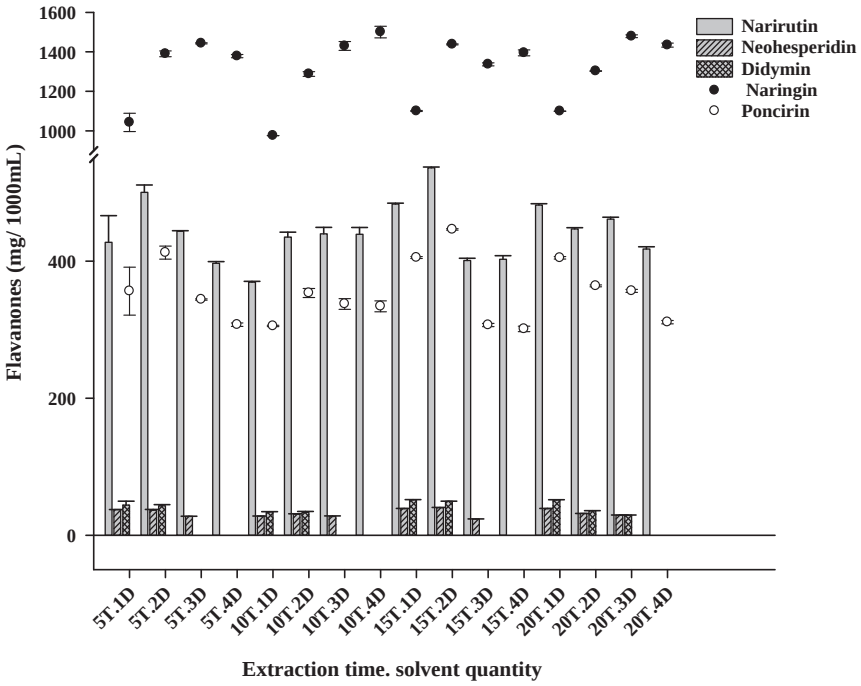

Fig. 7. Influence of extraction time (T in sec) on grapefruit flavanone extraction in microwave and sample to solvent volume 1, 2, 3, 4 dilutions (D). All the samples were extracted with dimethyl sulfoxide in triplicates.

grapefruit juice at 20, 40, 50 and 60 °C. Except naringin, relatively higher levels of narirutin, neohesperidin, didymin and poncirin were extracted with DMSO (polarity index 7.2) than other solvents. Solvent polarity (DMSO > DMF > ACN > MeOH > EtOH) [19] had a greater effect on flavanone extraction compared to temperature.

The results presented in Fig. 1 suggest that high temperatures did not significantly increase the naringin levels (1413 mg/1000 mL juice with DMSO). Similar findings from previous study strongly suggested that sample heating is not essential for grapefruit flavanone extraction [21]. In general, extraction temperature increases diffusion coefficient (mass transfer rate) and solubility [1,20,22]. On the other hand, an increase in the extraction solvent to sample ratio can further enhance extraction.

These findings suggest that extraction solvent is one of the critical factors for optimizing extraction methods. Since fruit matrix consists of a complex mixture of biological components that interact with solvents and other extraction factors during flavanone extraction [13], they are addressed in the following experiments.

3.2. Influence of solvent and centrifugation

DMSO (713 mg/1000 mL) and DMF (590 mg/1000 mL) extracts had higher concentrations of narirutin than ACN (547 mg/1000 mL), MeOH (420 mg/1000 mL) and EtOH (375 mg/1000 mL) extracts (Fig. 1). However, the phase separations in ACN extracts led us to conduct further investigation of flavanone levels at different heights in centrifuge tubes after centrifugation (Fig. 2a). In the present experiment, flavanones were extracted at various centrifuge speeds such as 605, 3293, 6720, 11357 × *g* with MeOH, EtOH, ACN, DMSO or DMF at 0 °C, and flavanones quantities were analyzed from 1 mL top and bottom phases (Fig. 2a) of extracts. Flavanones extracted at different centrifuge speeds contributed to minor flavanone variations whereas major variations were observed among

Table 2
The levels of individual grapefruit flavanones (mg/1000 mL) obtained by sequential extraction with DMSO[a].

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

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

Table 3
Regression equations, coefficient of determination (r^2), linear range, limit of quantification (LOQ) and limit of detection (LOD) of various grapefruit flavanones analyzed in HPLC.

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

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

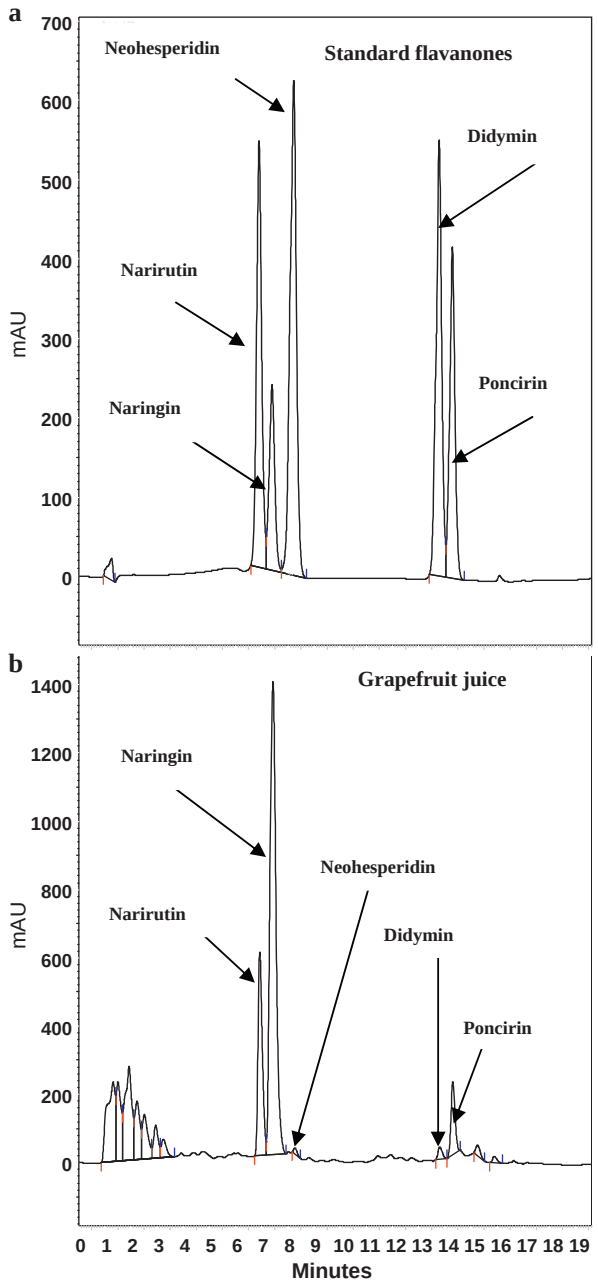

Fig. 8. The HPLC chromatograms of (a) flavanone standards and (b) grapefruit flavanones separated on a C-18 Hypersil gold column (100 mm × 4.6 mm i.d. with 5 μm particle size) and eluted with a gradient mobile phase of 3 mM phosphoric acid and 100% acetonitrile.

different solvent extracts (Figs. 3 and 4). Therefore, in all the following experiments 6720 × *g* was consistently used for centrifugation.

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

When grapefruit juice was extracted with ACN, the phase separations occurred in the extracts because of the interaction of ACN with water present in the juice sample. The striations observed in Fig. 2a were possible because of the anomalous behavior of ACN in the presence of water (present in juice) at low temperatures. Zarzycki et al. [23] suggested that the phase separations occur when water concentration in ACN ranged from 31 to 89%.

Consequently, more hydrophobic compounds (sterols and carotenoids) diffused into the top layer, thus two phases were observed. The top layer of ACN extract affected the HPLC peak resolution (Fig. 2b) as compared to the bottom layer (Fig. 2c). To confirm the peak purity of narirutin and naringin, LC–MS analysis was performed at different time frames during the elution of the compounds and the results were presented in Figs. S1 and S2.

According to Durling et al. [24], ratio of 40–60% aqueous ethanol and methanol in water can be considered as hydroalcoholic solvent extractions. In the current experiment, extractions from grapefruit juice with MeOH and EtOH simulates hydroalcoholic extractions because the final extraction solvent constitutes of water obtained from juice and MeOH or EtOH. In hydroalcoholic extraction, the visible striations were not observed because both these solvents can potentially form better hydrogen bonding with water.

Flavanone levels in 1 mL top and bottom of DMSO and DMF extracts were not different suggesting that flavanones were homogenously distributed in the centrifuge tube (Figs. 3 and 4). For several decades, MeOH was invariably used as a default extraction solvent for flavonoid and phenolics from fruits and vegetables. However, in the present study MeOH extracts (hydroalcoholic extractions) not only showed lower extraction efficiency but also showed variable flavanone quantities in top and bottom aliquots in the centrifuge tube after centrifugation.

3.3. Influence of solvent and centrifuge temperature

In previous studies, centrifuge temperature during sample preparation was not considered critical during flavanone extraction. In the current experiment, the importance of the centrifuge temperature during centrifuge extraction was demonstrated. When grapefruit juice was extracted with ACN in centrifuge, two clear phase separations were observed at 0 and 10 °C but not at 20 °C. However, variations in the flavanone levels in the top and bottom 1 mL of centrifuge tubes were found to be significantly different at three different centrifuge temperatures (Fig. 5). Though MeOH extracts did not show phase separations at all the three temperatures tested (data not shown), the flavanone levels were different at 0 and 10 °C (Fig. 5). The MeOH extracts from 20 °C centrifuge temperature did not show any difference in the flavanone levels from the top and bottom 1 mL. In case of DMSO extracts, flavanone levels in the top and bottom 1 mL of centrifuge tubes were not significantly different in all the centrifuge temperatures tested. The differential densities in the extraction mixture were observed because the individual solvent components (ACN and water in the juice) showed different response towards applied temperature and centrifugal force.

3.4. Extraction cycles

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

3.5. Effect of heat and sonication

In this study, DMSO was used to extract flavanones from grapefruit juice. Extractions were conducted at 20 and 40 °C in a

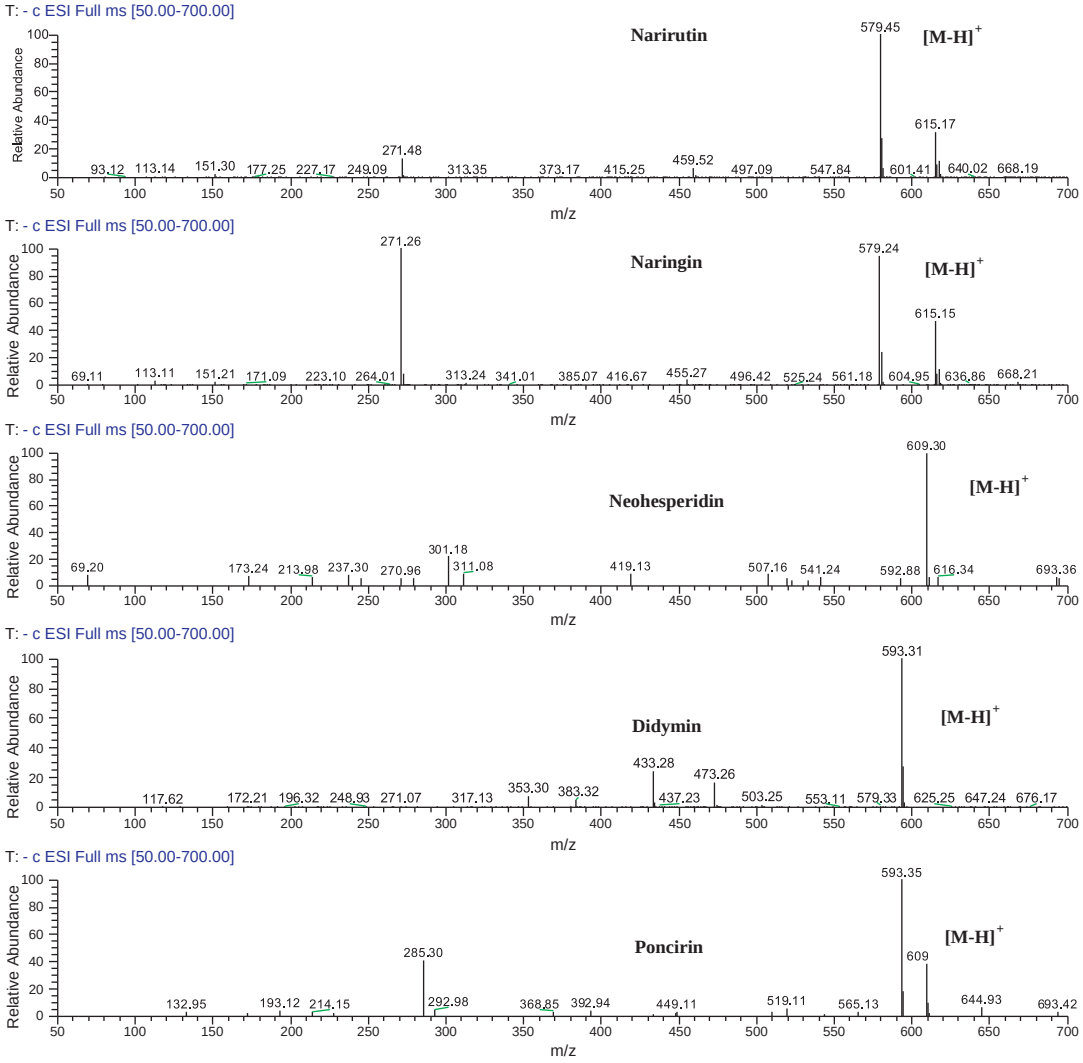

Fig. 9. Mass spectra of five grapefruit flavanones through LC–MS in electron spray ionization (ESI) negative mode.

sonicator for 10, 20, and 30 min. Although slightly higher levels of narirutin, naringin, neohesperidin and didymin were found in treatments 20 °C sonicated for 20 min and 40 °C sonicated for 10 min than other treatments, the change in levels due to sonication treatment were not significantly different (Fig. 6). The increase in extraction time did not always have higher levels of bioactive compounds [25]. Extraction of flavanones from the grapefruit juice was conducted by the phenomenon called cavitation, where the sample mixture is subjected to ultrasonic waves. The cavitation process can scour the tissues and allow the mass transfer of flavanones into the solvent [26]. Finally, once the extraction solvent is saturated with flavanones, further mass transfer from juice to solvent is negligible [25].

3.6. Effect of microwaves and juice to solvent ratio

Four extraction times (5, 10, 15 and 20 s) were used for extraction using microwaves with DMSO. Narirutin and poncirin levels were relatively higher when extracted for 15 s. The optimum levels of naringin (1383 mg/1000 mL), narirutin (536 mg/1000 mL) and poncirin (447 mg/1000 mL) were observed when one part of the juice was extracted with two parts of the solvent (Fig. 7). Further 3 and 4 times dilutions of sample with solvent had limited detection of minor flavanones (neohesperidin and didymin). The role of solvent quantity in the flavanone extraction is extremely critical [1]. Higher sample to solvent ratio may stop the mass transfer of the analyte due to solvent saturation [27]. On the other hand, higher levels of solvent to sample ratio increased the extraction efficiency, yet it had detection problems for neohesperidin and didymin. Therefore optimum sample to solvent ratio improves the overall extraction efficiency.

Since microwaves have both electric and magnetic fields, the sample and solvent mixture is possibly heated by two different mechanisms such as dipolar rotation and ionic conduction when exposed to microwaves [28]. The heating of the sample expands the cell contents and causes disruption in the cell walls.

3.7. Flavanone separation and identification

In the current HPLC method, narirutin, naringin, neohesperidin, didymin and poncirin eluted at 7.2, 7.9, 9.7, 15.2 and 15.5 min, respectively (Fig. 8). The regression equations, coefficient of determination, limit of quantification and limit of detection were given in Table 3. The five grapefruit flavanones were identified by ESI, negative mode, LC/MS (Fig. 9). The mass spectra of narirutin and naringin showed a molecular ion $[M-H]^+$ at *m/z* 579.4 and 579.2 respectively. While, neohesperidin showed a molecular ion $[M-H]^+$ at *m/z* 609.3. Both didymin and poncirin generated $[M-H]^+$ at *m/z* 593.3.

4. Conclusion

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.03.075.

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