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Supercritical fluid extraction of limonoids and naringin from grapefruit (*Citrus paradisi* Macf.) seeds

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Abstract

Limonoids and naringin were extracted from grapefruit (*Citrus paradisi* Macf.) seeds by a supercritical carbon dioxide (SC-CO_2) extraction technique. Prominent limonoids such as limonin and limonin-17-β-D-glucopyranoside (LG) and flavonoid naringin were quantified for process optimization. Extraction was completed in two stages. In stage one less polar limonin was extracted using SC-CO_2 while in stage two extraction of high polar LG and naringin were completed using SC-CO_2 modified by ethanol as co-solvent. Extraction parameters were studied using Box–Behnken experiment design. Response surface analysis (RSA) of the data was completed to determine effects of variables on extraction efficiency. Highest yield of limonin (6.3 mg/g seeds) was achieved at 48.3 MPa pressure, 50 °C temperature and 60 min of extraction time where as highest yield of LG (0.62 mg/g seeds) was achieved at 41.4 MPa pressure, 60 °C temperature and 30% ethanol concentration in 40 min. Highest yield of naringin (0.2 mg/g seeds) was achieved at 41.4 MPa pressure, 50 °C temperature and 20% ethanol concentration in 40 min. In all the experiments, mobile phase flow rate was kept constant at 5.0 L/min. The results demonstrated environmentally friendly, practical application of supercritical CO_2 extraction of limonoids and flavonoids from grapefruit seeds.

Keywords: Citrus; Bioactive compounds; Box–Behnken design; Limonin; Limonin-17-β-D-glucopyranoside; Naringin

1. Introduction

Limonoids and flavonoids are important constituents of citrus which have beneficial effects on human health. Limonoids are a unique natural product group with highly oxygenated triterpenoid backbones found only in members of the Rutaceae and Meliaceae families whereas flavonoids are one of the most diverse groups of plant secondary metabolites from both structural and functional points of view (Fig. 1). The food and commercial applications of these groups of compounds have been investigated, and research in biological activities of limonoids and flavonoids for their potential cancer prevention properties have been increased (Hasegawa, Berhow, & Manner, 2000; Lam, Hasegawa, Bergstorm, Lam, & Kenney, 2000; Lam, Li, & Hasegawa, 1989; Scambia et al., 1994).

As research on citrus limonoids has progressed, it has shown that these compounds have biological functionality in plants and possibly in humans as well. Studies in our lab (Poulose, Harris, & Patil, 2005; Tian, Miller, Ahmad, Tang, & Patil, 2001; Vanamala et al., 2006) and elsewhere (Guthrie et al., 2002; Lam et al., 1989, 2000; Miller, Taylor, Berry, Zimmerman, & Hasegawa, 2000) have shown that citrus limonoids can induce the detoxifying enzyme glutathione S-transferase in the liver of mice and rats, inhibit the formation of chemically induced neoplasia in the oral cavity, forestomach, small intestine, colon, lung and skin of laboratory animals and also inhibit the proliferation of breast cancer cells grown in culture. Recent results in our

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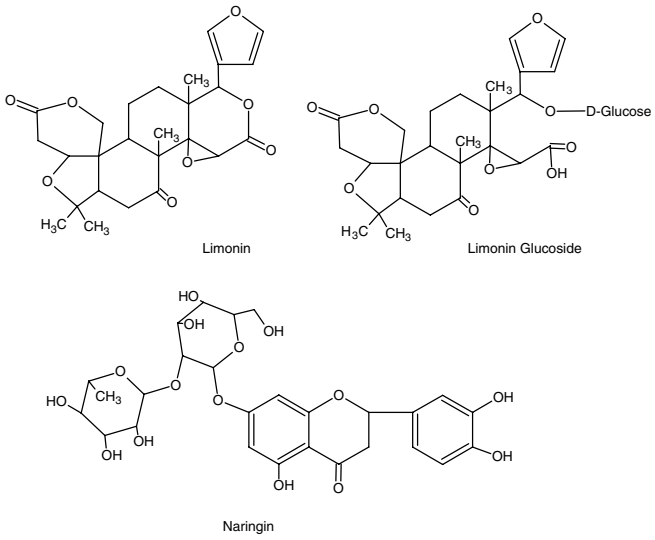

Fig. 1. Structures of limonin, LG and naringin.

lab (Deyhim et al., 2006; Jayaprakasha & Patil, 2007; Yu, 2004; Yu et al., 2005) and elsewhere (Battinelli et al., 2003; Kurowska, Manthey, & Hasegawa, 2000) demonstrated that citrus limonoids have the ability of lowering LDL cholesterol, help reduce the risk of atherosclerosis, positively affect bone strength, inducing apoptosis and anti HIV activity.

Flavonoids are a group of polyphenolic compounds with a multitude of health benefits. Citrus flavonoids have been shown to impede the proliferation of human lymphocytes, and exhibit activity against cancer cell lines such as A-549 lung carcinoma, MCF-7 breast carcinoma, HT-colon adenocarcinoma and MLM melanoma (Scambia et al., 1994). Dietary flavonoids also been associated with reduced risk of coronary heart disease (Bronner & Beecher, 1995; Hollman, Hertog, & Katan, 1996; Soares & Hotchkiss, 1998).

Despite increasing demand for citrus limonoids and flavonoids with documented and potential health benefits, lack of well-defined environmentally friendly and economical extraction and purification methods have precluded commercial development of this market (Braddock & Cadwallader, 1992). For this reason improved methods of extraction and purification will be of interest to the citrus industry as it seeks to capture its share of the dietary phytochemical/human health benefits.

Previous reports indicated feasibility of extracting limonoids and flavonoids from citrus juice and its byproducts-molasses, peels and seeds (Braddock & Bryan, 2001; Jayaprakasha, Brodbelt, Bhat, & Patil, 2006; Jayaprakasha, Patil, & Bhat, 2006; Mandadi, Jayaprakasha, Bhat, & Patil, 2007; Matthews, Rouseff, Manlan, & Norman, 1990; Miyake et al., 1991, 2000; Ozaki et al., 1995; Raman, Cho, Brodbelt, & Patil, 2005). Grapefruit seeds are a good source of limonoids since the amount of limonoid aglycones and glucosides in grapefruit seeds could be up to 1% (Braddock & Bryan, 2001). Most of these studies were focused on the use of organic solvents, resins and enzymatic methods.

Considering the increasing environmental and consumer concerns about the use of organic solvents in the extraction of natural products, there has been a growing interest in alternative extraction techniques such as supercritical fluid extraction (SFE) using supercritical carbon dioxide (SC-CO_2) (Modey, Mulholland, & Raynor, 1996). Applications of SFE in the food industry have been reviewed (Rozzi & Singh, 2002; Sihvonen, Hietaniemi, & Huopalahti, 1999).

Recent studies of the extraction of citrus using SC-CO_2 have been reported that limonoids and flavonoids could be extracted from citrus seeds and byproducts (Giannuzzo, Boggetti, Nazareno, & Mishima, 2003; Miyake et al., 2000). However, to the best of our knowledge very little information is available on the extraction of limonoids and flavonoids from grapefruit (*Citrus paradisi* Macf.) seeds using supercritical fluid techniques. Further the optimum operating parameters to maximize the extraction yield is not yet reported.

The current study has focused on SC-CO_2 extraction of limonoids and flavonoids from grapefruit seeds and determination of the optimum operating parameters.

2. Materials and methods

2.1. Materials

Grapefruit seeds were obtained from fruits harvested at the Texas A&M University–Kingsville Citrus Center, Weslaco, TX. Kernels inside the shells of dry seeds were removed and shells were collected, ground and used for extraction. High purity CO_2 (99.9%, Air Liquide America Corp., Augusta, GA) was used for supercritical fluids extraction (SFE). All solvents used were of HPLC grade and obtained from Fisher Scientific, Atlanta, GA. Limonin, limonin 17-β-D-glucopyranoside (LG) and naringin standards were prepared and purified according to the established procedures. Purified compounds were identified by ^{13}C NMR using Varian Inova 300 (Palo Alto, CA) and mass spectroscopy using ThermoFinnigan System (San Jose, CA) (Manners, Hasegawa, Barnett, & Wong, 2000; Poulose et al., 2005; Raman, Jayaprakasha, Brodbelt, Cho, & Patil, 2004; Tian et al., 2001; Tian, Li, Barbacci, Schwartz, & Patil, 2003).

2.2. Extraction

SFE of limonoids and naringin from grapefruit seeds was done in two stages. In first stage, limonoid aglycones were extracted using SC-CO_2 followed by second stage extraction of limonoid glucosides and naringin using SC-CO_2 and ethanol as a co-solvent. All experiments were done at a constant flow rate of 5.0 L/min.

During first stage of extraction, raw material (35 g) was filled in the extraction thimble and SC-CO_2 was passed over at extraction conditions. Upon exiting the thimble, extract was passed through a micrometering valve that

reduced the pressure to one atmosphere. The extract was then admitted into a collection vessel to trap the solute while CO_2 exited the system through a gas meter at ambient pressure.

The second stage extractions were completed using SC-CO_2 and ethanol. Ethanol was delivered using a syringe pump (Model 260D, Isco Inc., Lincoln, NE). Percent ratio of ethanol in the mobile phase was varied while keeping flow rate constant.

The extraction vessel was installed inside a temperature-controlled oven (Model 3119-005 Instron, Canton, MA), with a 5 m length coil of tubing inside the oven to preheat the CO_2 before contacting with the sample. The sample temperature was monitored with a thermocouple inserted through a thermocouple well installed on the wall of the extraction vessel, and that extended into the center of the vessel.

The collection vessel consisted of a glass test tube (200 ml) contained within a pressurized and temperature-controlled cell maintained at 40 °C. The extract from the extraction thimble entered the collection vessel via a tube that extended into the bottom of the vessel. The opening at the tip of the tube was welded shut and multiple 1 mm diameter holes were drilled on the side at 1 cm intervals up to 10 cm from the tip. This permitted the extract to enter the collection vessel directed towards the wall. The aliquots of extract in the collection vessel were taken for analysis of limonoids and naringin.

2.3. Experiment design

Extraction of limonoids and naringin from grapefruit seeds was optimized by varying operating parameters according to the Box–Behnken design (3^3 factorial). Box–Behnken design is an independent quadratic design in which the treatment combinations are multiple of the edge of the process space and the center. Box–Behnken designs thus place points on the midpoints of the edges of the (hyper-) cubical design region, as well as points at the center. This design uses three levels of each factor which makes them appealing when the factors are quantitative but the set of achievable values is small. Although it has limited capability for orthogonal blocking compared to central composite design, for three factors, Box–Behnken design requires fewer experiments. Three variables selected for first stage extraction viz. pressure (34.5 MPa, 41.4 MPa and 48.3 MPa), temperature (40, 50 and 60 °C) and time of extraction (20, 40 and 60 min) whereas during second stage extraction three variables selected were pressure (34.5, 41.4 and 48.3 MPa), temperature (40, 50 and 60 °C) and % of co-solvent (10%, 20% and 30%). All the experiments were done in duplicate and yields were averaged.

2.4. Analytical methods

All the extracts were analyzed by HPLC (HPLC-UV2000, Thermo Hypersil-Keystone Company, Bellefonte, PA, USA) equipped with Waters (Milford, MA, USA) Spherisorb ODS column (250 mm × 4.6 mm). Limonin, LG and naringin were quantified in all the extracts to determine the efficiency of each extraction experiment (Li, 2002; Raman et al., 2004, 2005; Yu et al., 2005).

2.5. Statistical analysis

The data collected was analyzed using the response surface analysis (RSA) procedure (SAS® for Windows® 9.0 version). The data from the Box–Behnken Statistical Design was used to calculate and model the optimum conditions for both extraction stages.

3. Results and discussion

3.1. Effect of feeding mode on extraction yield

During experimentation the mobile phase can be passed either in upward or downward direction depending on the substance being processed. However, upward flow is normally preferred as it eliminates channeling effect and gives even distribution. In the present study, both upward and downward flow extraction experiments were completed and yields were compared. The extraction parameters and yield are presented in Table 1. The extraction yield of limonin was higher with bottom feeding of CO_2 compared to top feeding. However, in case of top feeding of CO_2–ethanol resulted in better extraction yield of LG than bottom feeding. This result is difficult to explain since the nature of mass transfer kinetics depends not only upon temperature and pressure but also on where and how the solutes are bound to raw material matrix and its mechanism of release and transport within the solid matrix (Mukhopadhyay, 2000).

3.2. Limonin extraction

A total of 15 experiments were conducted to optimize the extraction conditions. Table 2 summarizes the experiment design and corresponding yield data for limonin. Response surface analysis (RSA) of the data in Table 2 demonstrate that the relationship between limonin yield and pressure, temperature and time of extraction is quadratic with very good regression coefficient ($R^2 = 0.99$). Eq. (1) shows

Table 1
Effects of SFE feeding mode on extraction yield

Mode	Limonin yield (mg/g)[a]	LG yield (mg/g)[b]
Top feeding	4.3, 4.7	0.48, 0.54
Bottom feeding	5.2, 5.4	0.41, 0.45

[a] Extraction conditions were pressure 41.4 MPa, temperature 50 °C, extraction time 40 min and flow rate 5.0 L/min.
[b] Extraction conditions were pressure 41.4 MPa, temperature 50 °C, 20% ethanol, extraction time 40 min and flow rate 5.0 L/min.

Table 2
Supercritical CO_2 extraction of limonin from grapefruit seeds: extraction conditions and yield

Run	Pressure (MPa)	Temperature (°C)	Time (min)	Limonin yield (mg/g dry seeds)
1	34.5	40	40	2.8, 3.2
2	48.3	40	40	4.1, 4.3
3	34.5	60	40	3.3, 3.7
4	48.3	60	40	4.5, 4.7
5	34.5	50	20	1.8, 2.2
6	48.3	50	20	3.5, 4.1
7	34.5	50	60	4.3, 4.9
8	48.3	50	60	6.1, 6.5
9	41.4	40	20	2.4, 2.6
10	41.4	60	20	3.6, 4.2
11	41.4	40	60	5.3, 5.7
12	41.4	60	60	4.7, 5.1
13	41.4	50	40	5.2, 5.4
14	41.4	50	40	5.1, 5.5
15	41.4	50	40	4.9, 5.3

relation between limonin yield with extraction parameters pressure, temperature and time of extraction

$$Y = -55.0625 + 0.8613X_1 + 0.2644X_2 + 1.4348X_3 - 0.0073X_1^2 - 0.0009X_2^2 - 0.0158X_3^2 - 0.0025X_1X_2 - 0.0004X_1X_3 - 0.0002X_2X_3, \quad (1)$$

where Y is the extraction yield, X_1 is the temperature, X_2 is the time of extraction and X_3 is the pressure.

It can be seen that limonin yield depends more on pressure variations followed by temperature variation. Dependence of yield on extraction time is least. The RSA showed that extraction yield first increased and then decreased when extraction pressure increased with system temperature (Fig. 2a). This could be due to the density change of SC-CO_2 (Mukhopadhyay, 2000).

The extraction conditions that gave the maximum yield of limonin (6.3 mg/g dried seeds) consisted of pressure 48.3 MPa, temperature 50 °C and time of extraction 60 min. A maximum predicted yield determined by RSA was of 6.8 mg/g seed was at the pressure 46 MPa, temperature 43 °C and extraction time of 90 min.

3.3. LG extraction

Table 3 gives the experiment design and corresponding yield data for LG. Experimental results shows that the pressure 48.3 MPa, temperature 50 °C and 30% ethanol produced the maximum extraction yield of LG (0.62 mg/g seeds). The RSA of the data in Table 3 also demonstrated a high regression value ($R^2 = 0.99$), which supported our hypothesis that the relationship between extraction yield and pressure, temperature and molar concentration of ethanol was quadratic. Eq. (2) shows the relation between LG yield and extraction parameters pressure, temperature and % of ethanol

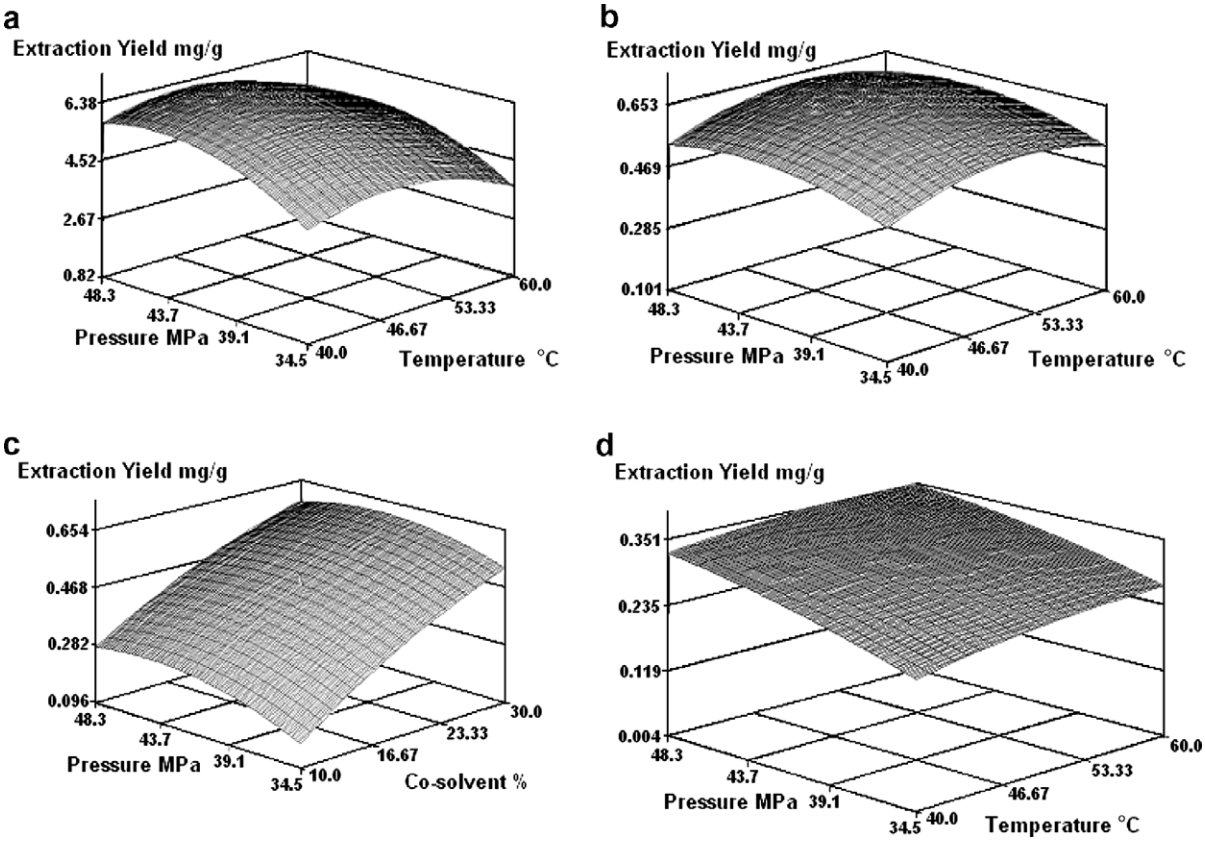

Fig. 2. Response surface for operating parameter effects on extraction yield.

Table 3
Supercritical CO_2 extraction of LG and naringin from grapefruit seeds: extraction conditions and yield

Run	Pressure (MPa)	Temperature (°C)	Ethanol (%)	LG yield (mg/g dry sample)	Naringin yield (mg/g dry sample)
1	34.5	40	20	0.31, 0.33	0.12, 0.14
2	48.3	40	20	0.38, 0.42	0.05, 0.09
3	34.5	60	20	0.36, 0.4	0.06, 0.08
4	48.3	60	20	0.44, 0.48	0.08, 0.1
5	34.5	50	10	0.17, 0.23	0.01, 0.03
6	48.3	50	10	0.27, 0.33	0.01, 0.01
7	34.5	50	30	0.56, 0.58	0.08, 0.12
8	48.3	50	30	0.6, 0.64	0.16, 0.2
9	41.4	40	10	0.18, 0.22	0.03, 0.05
10	41.4	60	10	0.25, 0.31	0.02, 0.02
11	41.4	40	30	0.53, 0.57	0.2, 0.4
12	41.4	60	30	0.59, 0.63	0.08, 0.1
13	41.4	50	20	0.47, 0.51	0.15, 0.25
14	41.4	50	20	0.48, 0.53	0.2, 0.2
15	41.4	50	20	0.52, 0.54	0.17, 0.19

$$Y = -4.1538 + 0.0705X_1 + 0.0406X_2 + 0.1027X_3 - 0.00066X_1^2 - 0.00034X_2^2 - 0.0011X_3^2 - 0.00005X_1X_2 - 0.00018X_2X_3, \quad (2)$$

where Y is the extraction yield, X_1 is the temperature, X_2 is the % ethanol and X_3 is the pressure.

Eq. (2) shows that two crucial factors during LG extraction are pressure and temperature where LG yield depends more on pressure variations followed by temperature variation.

In addition, it seems that the experimental design included the optimal conditions for the maximize yield. Fig. 2b shows variation of yield with pressure and temperature. However, in the limonoid glucosides extraction, an additional factor (co-solvent) influenced the extraction yield. Fig. 2c shows the relationship between the RSA generated extraction yield and pressure and co-solvent percentage. The extraction yield increased with co-solvent percentage until the solubility of limonoid glucosides in the solvent system was achieved. The RSA predicted a maximum yield of 0.73 mg/g defatted seeds at a pressure of 42 MPa, temperature of 52 °C and 45% ethanol. The RSA optimum appears to be desirable in the extraction of LG compared to any of the individual treatments in the experimental design.

3.4. Naringin extraction

Table 3 summarizes the experimental design and corresponding yield data for naringin. On quantitative analysis it was observed that the maximum extraction yield of 0.2 mg naringin/g seeds was obtained at the conditions of pressure 41.4 MPa, temperature 50 °C and 20% ethanol.

Response surface analysis (RSA) of the naringin yield data in Table 3 also demonstrated a high regression value ($R^2 = 0.99$), which supported our hypothesis that the relationship between extraction yield and pressure, temperature and molar concentration of ethanol was quadratic. Eq. (3) shows relation between naringin yield and extraction parameters pressure, temperature and % of ethanol

$$Y = -0.88975 + 0.01336X_1 - 0.00072X_2 + 0.02351X_3 - 0.00010X_1^2 + 0.00006X_2^2 - 0.00022X_3^2 + 0.00008X_1X_2 - 0.00009X_1X_3 + 0.00018X_2X_3, \quad (3)$$

where Y is the extraction yield, X_1 is the temperature, X_2 is the % ethanol and X_3 is the pressure.

Eq. (3) clearly shows that crucial factor during extraction of naringin was pressure followed by temperature. Fig. 2d shows dependence of extraction yield with respect to pressure and temperature.

The naringin extraction results indicated that the region selected for extraction in this experimental design is more appropriate for limonin and LG than naringin. Previous study showed that ideal extraction conditions for extraction of naringin from peels of grapefruit are temperature of 40–60 °C, ethanol % of 5–15%, and pressure of 9.5 MPa (Giannuzzo et al., 2003). In the present study, the pressure conditions were too high for naringin extraction, and naringin under these conditions may be considered as a byproduct.

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