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Supercritical Fluid Extraction of Limonoid Glucosides from Grapefruit Molasses

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Limonoid glucosides (primarily limonin 17-β-D-glucopyranoside, LG) were extracted from grapefruit molasses by supercritical fluid extraction using a supercritical carbon dioxide−ethanol (SC CO_2−ethanol) system. Extraction conditions to maximize the yield of LG were determined by varying pressure, temperature, ethanol concentration, and extraction time. The highest yield of LG at 0.61 mg/g molasses was obtained at a pressure 48.3 MPa, a temperature of 50 °C, 10% ethanol (X_{Eth} = 0.1), and 40 min of extraction time at a flow rate of 5.0 L/min. The results demonstrated that SC CO_2 extraction of limonoid glucosides from grapefruit molasses has practical significance for commercial production.

KEYWORDS: Citrus; health; limonin 17-β-D-glucopyranoside; Box−Behnken design; response surface analysis

INTRODUCTION

Modern medicinal chemists have been searching for complex molecules that have beneficial biological activity. Several modern drugs have been developed using plant secondary metabolites. Citrus limonoids are potential secondary metabolites primarily found in *Rutales*, especially in the families *Meliaceae* and *Rutaceae*. Limonoids are structurally classified in two groups viz. limonoid aglycones and limonoid glucosides. It is well-known that prominent aglycones (mainly limonin and nomilin) impart bitterness to citrus juices and their presence in the juice has been a problem for the citrus juice industry for a long time. On the other hand, limonoid glucosides are water soluble, tasteless, abundant in citrus, and are safe natural compounds to consume (*1*). Structures of typical limonoids are given in **Figure 1**.

Accumulated evidence suggests that citrus limonoids have biological functionality in plants and possibly in humans as well (*2*). This group of compounds has been reported to induce the activity of the detoxifying enzyme glutathione S-transferase in the liver of mice and rats (*3*), inhibit the formation of chemically induced neoplasia in the oral cavity, forestomach, small intestine, colon, lung, and skin of laboratory animals (*4*−*6*), and also inhibit the proliferation of breast cancer cells grown in culture (*7*, *8*). Our recent results demonstrated that citrus limonoid glucosides have the ability to induce caspase 3/7 activity, suggesting that limonoid glucosides were capable of inducing apoptosis (*9*). A recent study also demonstrated limonin and nomilin inhibition of HIV-1 replication in vitro studies (*10*).

Studies with HepG2 cells indicated that citrus limonoids (especially limonin) were partly responsible for lowering low-density lipoprotein (LDL) cholesterol (*11*). Furthermore, our recent animal studies showed that citrus limonoids (limonin and limonin 17-β-D-glucopyranoside, LG) significantly decrease the LDL/high-density lipoprotein cholesterol ratio, increase the LDL particle diameter, and reduce the susceptibility of LDL to oxidation, which is thought to reduce the risk of atherosclerosis (*12*). Furthermore, our studies provided evidence that certain limonoids and citrus extracts show antioxidant activity in vitro study (*13*, *14*). The notion was also demonstrated that an increase in serum antioxidant status by orange juice and grapefruit juice positively affects bone strength independent of bone density (*15*).

With the increasing interest in the bioactive functions of limonoids, the demand for these chemicals has significantly increased (*16*). Because the limonoid glucosides are not available commercially, extraction and purification of limonoids from juice processing plants byproducts could increase the potential value of the citrus crop. It was reported that molasses contains 18% of total limonoid glucosides present in whole fruits (*17*). Thus, citrus byproducts such as molasses are excellent sources for limonoid glucosides. Furthermore, molasses being liquid is the best potential industrial source for the extraction of limonoid glucosides.

As compared to traditional extraction with liquid organic solvents, extraction with supercritical (SC) fluids such as SC CO_2 has been of interest due to its environmental compatibility (*17*, *18*). Besides, SC CO_2 has several other advantages such

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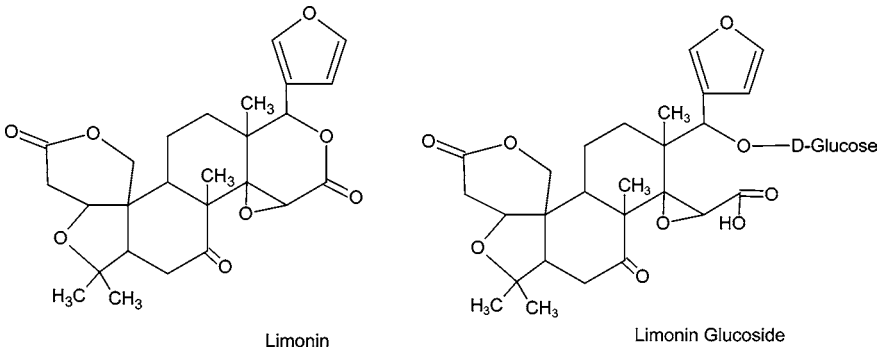

Figure 1. Typical structure of citrus limonoids.

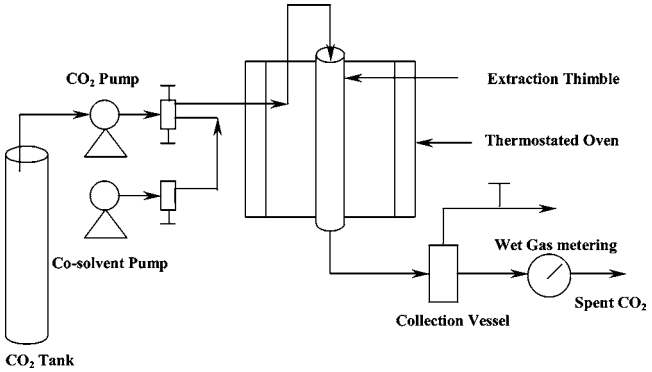

Figure 2. Schematics of the pilot scale SFE unit used in this study.

as nontoxicity, nonflammability, inability to leave residual chemical, and low/moderate operating temperatures and pressures (*19*). SC CO_2 has thus been widely used in the food processing industry. Advantages of SC fluid extraction technique and benefits in the food industry were recently reviewed (*19*, *20*).

Recent studies reported that SC CO_2 could be used to remove limonoid aglycones and concentrate limonoid glucosides in citrus juices (*17*, *21*−*23*). Traditional methods to extract limonoid glucosides from citrus molasses for analytical purposes have also been explored (*24*, *25*). However, very little information is available on the optimization of operating parameters and other factors influencing the extraction. Our research focused on SC CO_2 and cosolvent extraction of limonoid glucosides from grapefruit molasses and attempted to optimize the operating parameters for SC CO_2 and ethanol extraction of these bioactive compounds.

MATERIALS AND METHODS

Materials. Wet grapefruit molasses was obtained from Texas Citrus Exchange (Mission, TX). All solvents used were of high-performance liquid chromatography (HPLC) grade and were obtained from Fisher Scientific (Atlanta, GA). High-purity CO_2 (99.9%, Air Liquide America Corp., Augusta, GA) was used for supercritical fluid extraction (SFE). The standard of LG was prepared and purified (≥95% pure) according to the procedures in our lab (*7*, *26*). Purified compound was identified by MS and NMR.

SC CO_2 Extraction. Limonoid glucosides were extracted from the wet grapefruit molasses by one-step extraction using SC CO_2−ethanol cosolvent. A schematic diagram of SFE is illustrated in **Figure 2**. Wet grapefruit molasses (60 g) was loaded into the extraction thimble of a pilot-scale SFE apparatus. Glass wool was placed at both ends of the thimble to prevent plugging of the cap frits. The SC fluid was a mixture of CO_2 (5.0 L/min at 1 atm and 25 °C) and ethanol (0.015 L/min ≡ 0.59 X_{Eth}). Carbon dioxide was pumped through an air-driven nonlubricating gas booster (model AGD-62-C, Double Acting Single Stack, Haskel Inc., Burbank, CA), and ethanol was pumped through a syringe pump (model 260D, Isco Inc., Lincoln, NE). Pressurized CO_2 and ethanol were allowed to pass through the extraction thimble. The extraction thimble was installed inside a temperature-controlled oven (model 3119-005, Instron, Canton, MA), and a 5 m length coil of tubing inside the oven preheated the CO_2 and ethanol before entering the extraction thimble. The sample temperature was monitored with a thermocouple installed on the extraction thimble wall that extended into the center of the vessel. The thermocouple output was recorded by a digital data logger (Hydra 2635A, Fluke, Everett, WA).

Upon exiting the thimble, the extract passed through a micrometering valve that reduced the pressure to atmospheric pressure, and finally, the extract entered a collection vessel. The collection vessel consisted of a glass test tube (200 mL) contained in 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 entry of extract into the collection vessel to be directed toward the wall.

Aliquots from the extract were analyzed for limonoid glucosides. Residual molasses was finally discarded. Carbon dioxide exited the system to the surrounding air through a gas meter at 1 atm. Thus, the reported volumetric flow rate was that of CO_2 gas at 1 atm and 25 °C.

Experiment Design. Various operation parameters were investigated to extract limonoids. It is known that SFE efficiency depends mainly on pressure and temperature variations as these factors change the density of SC solvent. Cosolvent percentage is also an important variable as selectivity of extracting solvent changes with change in polarity. Thus, to optimize the process for SC CO_2 extraction, these three parameters were altered and the yield of LG was evaluated as a function of specific parameters. Furthermore, the effect of changes in one parameter on other parameters and their combined effects on the total yield were studied. To collect the maximum possible information from the minimum number of experiments, a Box−Behnken experiment design was used. A Box−Behnken design is an independent quadratic design in which the treatment combinations are multiples of the edges of the process space and the center. Although it has limited capability for orthogonal blocking as compared to central composite design, for three factors, the Box−Behnken design requires fewer experiments.

The three factors chosen to optimize the process were pressure, temperature, and percent cosolvent. The optimal extraction conditions were identified by operating the SFE unit at 34.5 (5000 psi), 41.4 (6000 psi), and 48.3 MPa (7000 psi) pressures, at each of the three temperatures of 40, 50, and 60 °C with ethanol at the molar fraction of 10, 20, and 30%, and a SC CO_2−ethanol flow rate of 5.0 L/min.

To determine the effect of operating time on the extraction yield, experiments were carried out at 41.4 MPa pressure, 50 °C temperature, 20% ethanol, and 5.0 L/min SC CO_2−ethanol flow rate for 20, 40, 60, and 80 min, respectively.

Limonoid Glucosides Determination. SFE extracts were analyzed by HPLC (HPLC-UV2000, Thermo Hypersil-Keystone Co., United States) equipped with a Waters Spherisorb ODS column (250 mm × 4.6 mm). The column was eluted using a linear gradient flowing at a rate of 1 mL/min starting with 10% acetonitrile in 0.03 mM phosphoric acid and ending with 24% acetonitrile in 0.03 mM phosphoric acid for

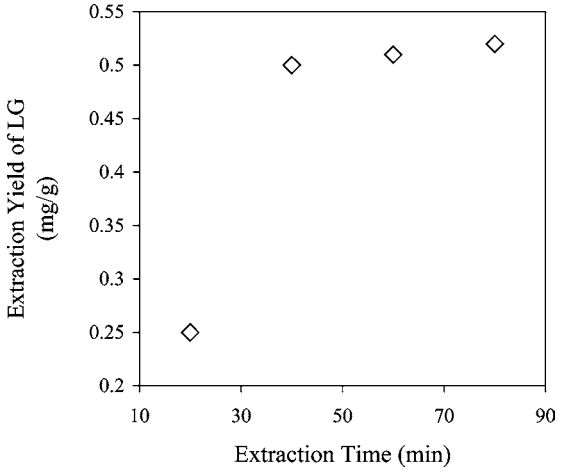

Figure 3. Effects of time on the extraction yield.

80 min. Injection volumes of 20 μL with limonoids were detected at UV 210 nm (*27*).

Statistical Analysis. The data collected were analyzed using the response surface analysis (RSA) procedures (SAS for Windows, version 9.0).

RESULTS AND DISCUSSION

Extraction of limonoid glucosides from molasses is a single-step process; however, many variables affect the extraction yield. Variables such as pressure, temperature, time of extraction, polarity of SC solvent, and SC solvent flow rate are main variables that directly affect the yield. Pressure and temperature are key variables in the SC extraction process, which determines the density of SC solvent and thus efficiency, whereas the polarity of the solvent determines selectivity toward particular groups of solutes (*28*). Carbon dioxide (CO_2) is a readily available gas with favorable properties as a SC fluid. However, when the chemical structure and orientation of atoms in CO_2 molecule are considered, it has two oxygen atoms attached to a carbon but in a perfectly symmetrical manner. This makes SC CO_2 a nonpolar/low polar solvent, which can be used to extract nonpolar/low polar solutes. Because glucosidation of limonoids increases the polarity of limonoids, it is necessary to extract limonoid glucosides from citrus molasses using a more polar solvent such as SC CO_2 modified by ethanol.

The flow rate of the SC solvent also influences the extraction yield. Previous work from our laboratory demonstrated that the time to exhaustive solute extraction was a function of the CO_2 flow rate (*29*). Generally, a higher flow rate led to waste of CO_2 and a lower flow rate led to inefficient extraction. Thus, on the basis of previous work, we chose the medium CO_2 flow rate of 5.0 L/min in this study in all of the experiments.

The time of extraction is also an important variable. To determine the time of extraction, experiments were carried out at 41.4 MPa pressure, 50 °C temperature, 20% ethanol, and a 5.0 L/min SC CO_2−ethanol flow rate for 20, 40, 60, and 80 min, respectively. **Figure 3** demonstrates the results of these experiments. It was observed that extraction yield increases with increasing extraction time from 20 to 40 min. Beyond 40 min, a plateau was observed with no significant difference in yield with extraction times of 60 and 80 min. Therefore, 40 min was chosen as the treatment time for all subsequent extractions.

The effects of the three key factors, pressure, temperature, and cosolvent molar percentage, were studied for the optimization of extraction process. The experimental limits for these studies were pressures within the range of 34.5−48.3 MPa, temperatures between 40 and 60 °C, and cosolvent concentrations of 10−30% ethanol. The total amount of LG in grapefruit molasses was determined as 0.65 mg/g, and the extraction yield from each experiment was used as a dependent variable. **Table 1** gives the experimental data. The extraction experiment at a pressure of 48.3 MPa, a temperature of 50 °C, and 10% ethanol produced the maximal yield of 0.61 mg LG/g molasses.

Table 1. SC CO_2 Extraction of Limonin Glucoside (LG) from Grapefruit Molasses: Extraction Conditions and Yields (CO_2 Flow Rate, ~5 L/min; Extraction Time, 40 min)

run	pressure (MPa)	temp (°C)	ethanol (%)	yield (mg/g wet sample)	yield (% LG from molasses)
1	34.5	40	20	0.20[a]	30.8
2	48.3	40	20	0.36	55.4
3	34.5	60	20	0.50	76.9
4	48.3	60	20	0.56	86.2
5	34.5	50	10	0.52	80.0
6	48.3	50	10	0.61	93.8
7	34.5	50	30	0.43	66.2
8	48.3	50	30	0.55	84.6
9	41.4	40	10	0.45	69.2
10	41.4	60	10	0.53	81.5
11	41.4	40	30	0.46	70.8
12	41.4	60	30	0.48	73.8
13	41.4	50	20	0.51	78.5
14	41.4	50	20	0.49	75.4
15	41.4	50	20	0.50	76.9

[a] Values are the duplicate extraction yields.

RSA of the data in **Table 1** 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. Equation 1 shows a relation between LG yield and extraction parameters

$$Y = -3.3913 + 0.0968X_1 - 0.0199X_2 + 0.0650X_3 - 0.0007X_1^2 + 0.0005X_2^2 - 0.0005X_3^2 - 0.0002X_1X_2 - 0.0004X_1X_3 + 0.0001X_2X_3 \quad (1)$$

where Y is the extraction yield, X_1 is the temperature, X_2 is the cosolvent percentage, and X_3 is the pressure.

It can be seen that all three factors are crucial for extraction yield. The LG yield depends more on temperature variations followed by pressure variation. Dependence on percent of entrainer is less but not negligible.

RSA of the data in **Table 1** produced the prediction model between the extraction yield and the three key factors illustrated in **Figures 4**−**6**. **Figure 4** shows that extraction yield of LG increases till a maximum value before dropping as a function of extraction pressure, which increases with the system temperature. This effect could be explained by the density change of the mixture of SC CO_2 and ethanol. In general, a higher SC fluid density allows for greater solute dissolution and a higher solute concentration at saturation.

Figure 5 demonstrates that the extraction yield of LG decreased as the molar percentage of the cosolvent ethanol increased from 10 to 30% or when the extraction pressure decreased from 48.3 to 34.5 MPa. This response may be specific with the current system. As molasses contains a large amount of water and the SC CO_2−ethanol system is immiscible with water, two phases must be present in the extractor. During the extraction process, limonoid glucosides are extracted by solvent−solvent extraction; thus, the highest extraction yield was obtained

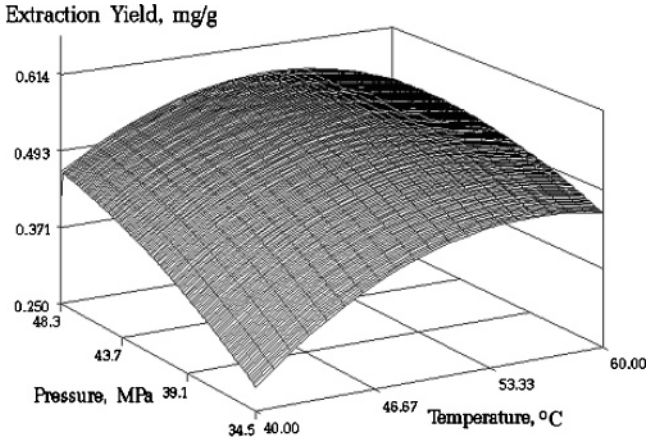

Figure 4. Response surface estimated for the extraction yield effects of pressure and temperature.

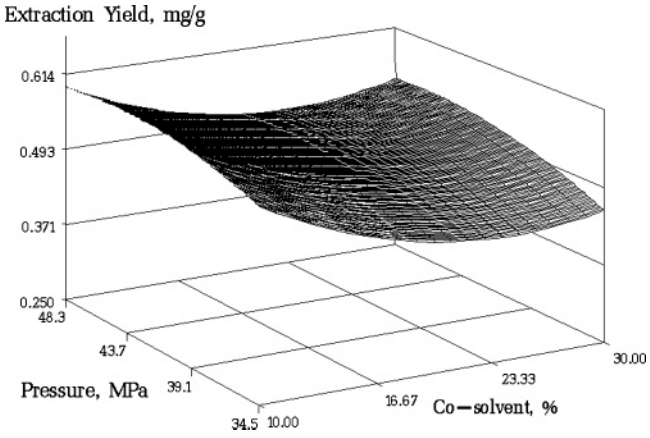

Figure 5. Response surface estimated for the extraction yield effects of pressure and cosolvent percentage.

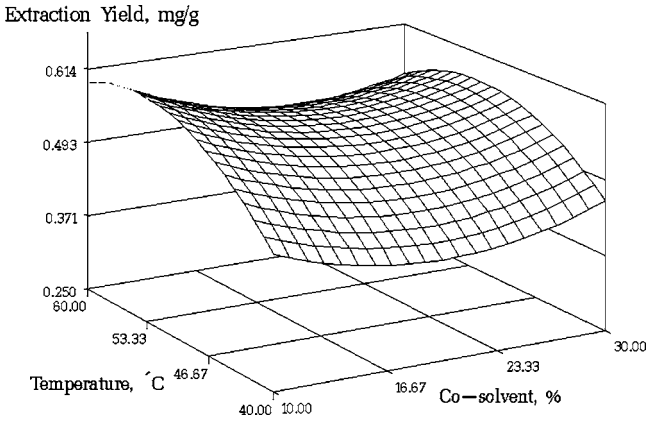

Figure 6. Response surface estimated for the extraction yield effects of temperature and cosolvent percentage.

when the partition ration was favorable for more limonoid glucosides in the SC CO_2−ethanol system.

In **Figure 6**, it can be seen that the extraction yield increased first and then decreased with the increase of both temperature and ethanol percentage. This behavior also can be explained by the partition ratio. The highest extraction yield is observed when the partition ratio was favorable for more limonoid glucosides in the SC CO_2−ethanol system.

Finally, RSA predicted optimum conditions for the extraction of LG at 48.2 MPa, 53 °C, and 22% ethanol with the predicted value of 0.54 mg/g; however, when compared with the trials listed in the **Table 1**, the conditions under trial 6 are more desirable and practical. Therefore, the optimized condition for the extraction of LG can be determined as 48.3 MPa, 50 °C, 10% ethanol, 40 min, and a 5.0 L/min SC CO_2−ethanol flow rate.

In conclusion, limonoid glucosides were extracted successfully from grapefruit molasses, a byproduct from the citrus juice industry, using SC CO_2 and ethanol as a cosolvent. Extraction parameters were optimized using RSA as 48.3 MPa, 50 °C, 10% ethanol, 40 min, and a 5.0 L/min SC CO_2−ethanol flow rate with the extraction yield of 0.61 mg limonoid glucosides/g grapefruit molasses. The results suggest the viability of SFE for the commercial production of limonoid glucosides.

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