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Effects of Modifiers on the Supercritical CO_2 Extraction of Licorice (*Glycyrrhiza glabra*) and the Morphology of Licorice Tissue

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Abstract Optimal extraction conditions such as pressures, temperatures, and modifiers on glycyrrhizin extraction from licorice were investigated using supercritical CO_2 (SC-CO_2) at 3 mL/min flow rate. Morphology of licorice tissue, after glycyrrhizin extraction, was examined by SEM, and absolute density (g/cm^3) measurement and glycyrrhizin content were determined by HPLC. Pure SC-CO_2 had no effect on glycyrrhizin extraction, but recovery of glycyrrhizin (32.66±0.77%) was enhanced when water was used as modifier. The highest recovery was 97.22±2.17% when 70% (v/v) aqueous methanol was added to 15% (v/v) SC-CO_2 at 50 MPa and 60°C. Under optimal extraction conditions, 30 MPa pressure and 60°C heating temperature, glycyrrhizin recovery reached maximum (102.67±1.13%) within 60 min. Licorice tissue was severely damaged by excessive swelling, and absolute density of licorice residues was highest when aqueous methanol was used as a modifier.

Key words: supercritical CO_2 extraction, modifier, licorice, glycyrrhizin

Introduction

Glycyrrhiza glabra (licorice) is a herb of the *Leguminosae* family. Its root contains 25-30% starch, 3-10% D-glucose and sucrose, 3-5% glycyrrhizin, and trace amounts of flavonoids, saponoids, sterols, and amino acid, with glycyrrhizin, generally found in a calcium and potassium-combined salt form, being the main active components (1). Glycyrrhizin and licorice extract have been used extensively in food industry as sweeteners (50-60 times sweeter than sucrose), flavor correctors, foaming-agents, and emulsifying agents (2-3). They are also used in pharmaceutical products owing to their bioactive characteristics such as anti-ulcer, anti-cancergenic, anti-allergic, and anti-inflammatory activities (4-6). A traditional extraction method using organic solvents is effective for the extraction of glycyrrhizin from licorice; however, longer extraction time and higher extraction temperature have been employed, and the toxic solvents can remain in the products. Recently, a microwave-assisted extraction method has been used to reduce the extraction time and solvent usage (7).

Since the 1960's, supercritical fluid extraction (SFE) technology has been used as an alternative method (8). Carbon dioxide generally is one of the most desirable solvents for SFE owing to its relatively low critical temperature and pressure, non-toxicity, low cost, and non-flammability (9). The non-polar supercritical CO_2 (SC-CO_2), for use in the extraction of polar compounds, should be modified by adding polar solvents as co-solvents or modifiers. The addition of modifiers increases the polarity of SC-CO_2, and enhances the extraction efficiency significantly, resulting in decreased extraction time (10). Many studies have been reported to extract and separate biologically active components from natural products using SC-CO_2 modified with various modifiers such as methanol, ethanol, the mixture of methanol and water, methanol basified with 10% diethylamine, caprylic acid methyl ester, and so on (11-16). Another role of modifiers is to increase the interior volume and surface area of the matrices by destructing or swelling matrices. This interaction of the modifier and matrix results in improving the extraction efficiency (17-18). Fahmy *et al.* (17) reported that matrix swelling by SC-CO_2 with water as a modifier increases the extraction efficiency of the target materials from plant tissues.

The objective of this study was to investigate the optimal processing conditions such as extraction pressure, temperature, and types and amount of modifiers for the highly polar glycyrrhizin extraction from licorice by SC-CO_2, and to determine the effects of various extraction fluids on licorice tissue during SFE under optimal extraction conditions.

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Materials and Methods

Materials Dried roots of *G. glabra* were pulverized, prior to extraction, using a Warning blender (Dynamic Corp., Hartford, USA) and sieved with 30- and 60-mesh sieves. The ground samples were packed in a polypropylene bag and stored at 4°C.

The following chemicals were used to extract and analyze the glycyrrhizin: carbon dioxide (99.995%, Dongmin Special Gases, Pyoungtaek, Korea), HPLC-grade methanol, ethanol, and acetonitrile (Fisher Scientific, Pittsburg, PA, USA), ammonium glycyrrhizinate (75%) and phosphoric acid (86%) (Sigma, St. Louis, MO, USA).

Organic solvent extraction Organic solvent extraction of glycyrrhizin from licorice was conducted under the selected extraction conditions of a previous study (19). Mixture (1:30, w/w) of the licorice sample and 30% (v/v)

aqueous methanol was shaken at 40°C for 6 hr with the extraction solvent changed every 3 hr. The licorice extracts were centrifuged at 3,000 rpm for 20 min, and the supernatant was analyzed by HPLC after filtration. Based on the results of the organic solvent extraction, the maximum extraction yield used to calculate the recovery was 4.51± 0.77% (weight of glycyrrhizin/weight of licorice).

Supercritical fluid extraction (SFE) SFE was performed using a supercritical fluid extractor (SFX 3560, ISCO, Lincoln, NE, USA) with two ISCO syringe pumps (100 DX) for CO_2 and a modifier at various temperatures (40-120°C) and pressures (11-50 MPa) (Fig. 1). Dried sample (about 2 g) was put into 10-mL extraction cell, and both CO_2 and an aqueous modifier were supplied by each syringe pump to an extractor. The flow rate was fixed at 3 mL/min, and each extract was collected in a 20-mL vial.

HPLC analysis HPLC was composed of a 616 controller, 996 photodiode array UV detector operated at 254 nm, 515 HPLC pump, and TM 717 plus autosampler (M616LC system, Waters, Milford, MA, USA). Sample solution was injected through a 20-μL loop and separated on the CapCell PAK C18 UG120 S-5 μm (250 mm × 4.6 mm i.d, Shiseido, Tokyo, Japan) at 40°C. Isocratic elution was performed with water-acetonitrile (62:38, v/v) mobile phase (pH 2.5, H_3PO_4) at a flow rate of 1.2 mL/min.

The calibration curve for glycyrrhizin was established using a standard solution of ammonium glycyrrhizinate (Fig. 2). A recovery (%) was calculated as the extraction yield of glycyrrhizin by SEF divided by the organic solvent extraction.

Supercritical CO_2 drying of licorice tissue after extracting Licorice residues after glycyrrhizin extraction were dried to less than 10% (w.b) moisture content using supercritical CO_2 (20). Briefly, the moisture from damp licorice residues was removed by SC-CO_2 with methanol for 70 min, and methanol remaining in the dehydrated licorice residues was eliminated using pure SC-CO_2 for 40 min at 30 MPa and 60°C. During the drying process, the flow rate of supercritical fluids was maintained at 3 mL/min. Additional glycyrrhizin was not extracted from the licorice residues.

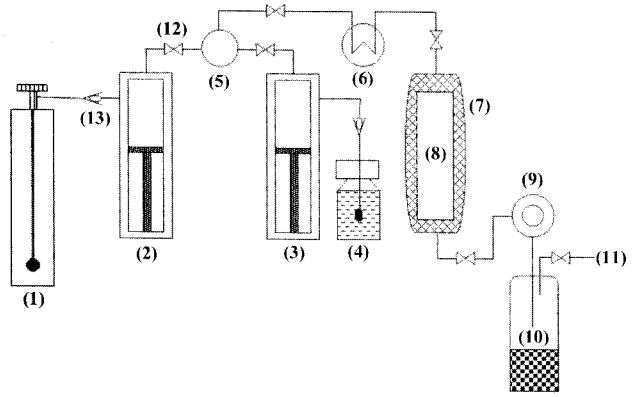

Fig. 1. A Schematic flow diagram of ISCO SFX 3560 ((1) Liquid CO_2 storage tank, (2) syringe pump for CO_2, (3) syringe pump for co-solvent, (4) co-solvent tank, (5) mixing zone, (6) preheating exchanger, (7) high pressure chamber, (8) extraction cartridge, (9) restrictor, (10) collection vial, (11) CO_2 venting, (12) valve, (13) check valve).

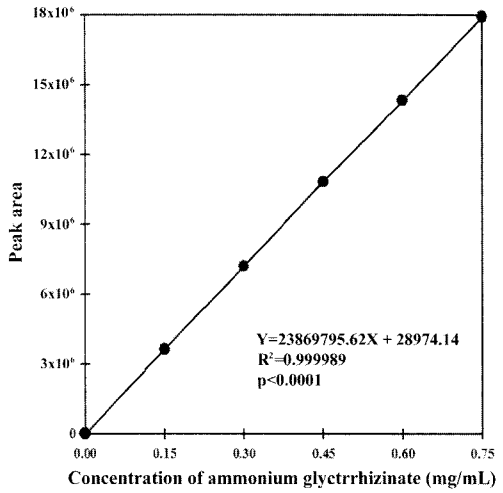

Fig. 2. Calibration curve of ammonium glycyrrhizinate.

Scanning electron microscopy and absolute density Dried licorice residues were applied onto the non-sticky side of aluminum tape attached to a brass disc. The specimens were coated in a sputter coater with gold/palladium (60/40). The prepared samples were observed using a scanning electron microscope (JSM-5200, JEON, Tokyo, Japan) at 20 kV.

Absolute density (g/cm^3) of the dried licorice residues was measured using a gas pycnometer (Accupyc 2375, Micromeritics Instrument Co., Norcross, GA, USA), and experiments were carried out in ten times. The results were expressed as means±standard deviation.

Statistical analysis Data were analyzed by Duncans multiple range test using SAS (SAS Institute Inc., Cary, NC, USA). Significant difference was established at $p< 0.05$.

Results and Discussion

Effect of modifiers on the extraction of glycyrrhizin from licorice Recovery of glycyrrhizin from licorice was 0.04±0.004% when pure SC-CO_2 was used at 50 MPa and 60°C. This low recovery might be due to the high polarity of glycyrrhizin, which prevented the extraction with non-polar SC-CO_2 (17). The results of addition of 10 % (v/v) polar modifiers such as pure methanol, ethanol, and water to SC-CO_2 are shown in Fig. 3. Extractions were performed at 50 MPa and 60°C at 3 mL/min flow rate of CO_2 and 15 min static extraction time, followed by 120 min dynamic extraction time. High recovery (32.66± 0.77%) was obtained when water was used as a modifier; however, both pure methanol and ethanol had little effects on the recovery (1.55±0.18% and 1.24±0.25%, respectively). Although the use of water as a modifier resulted in the increased recovery, the flow rate of supercritical fluid was unstable during the extraction. According to Jackson *et al.* (21), the solubility of water in SC-CO_2 was only 0.5% (v/

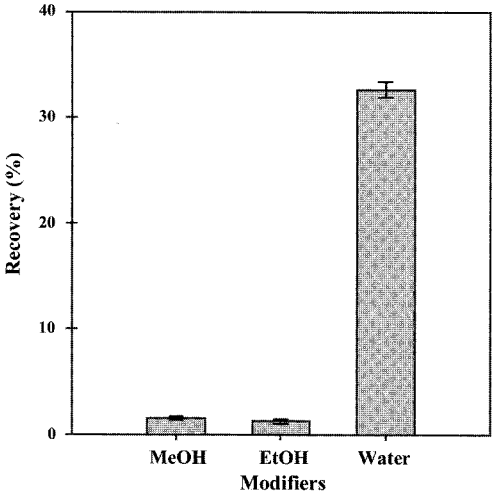

Fig. 3. Effects of various modifiers on the extraction of glycyrrhizin from licorice. SFE condition: 50 MPa, 60°C, flow rate of 3 mL/min, 10% (v/v) of modifier, 15 min static, and 120 min dynamic extraction.

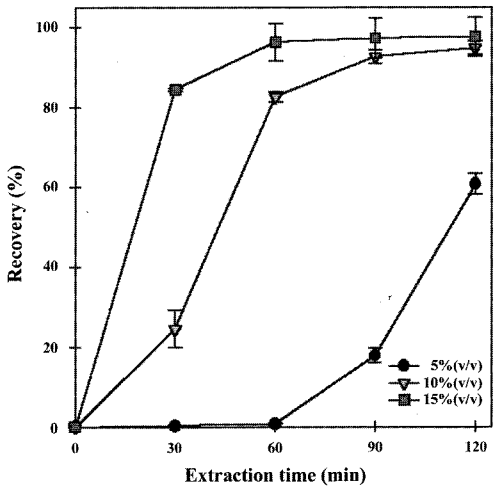

Fig. 5. Changes in the yield of extraction with dynamic extraction time from different amounts of 70% (v/v) aqueous methanol in CO_2. SFE condition: 50 MPa, 60°C, flow rate of 3 mL/min, 15 min static, and 120 min dynamic extraction.

v) at 34.4 MPa and 75°C. Thus, the unstable flow rate caused by higher water content in SC-CO_2 might have resulted from the trickling phenomenon by phase separation within the extractor.

To overcome the limitation of pure water as a modifier, the effect of aqueous methanol concentration from 0 to 100% (v/v) on the extraction efficiency was examined under the same extracting conditions of pure SC-CO_2, 50 MPa and 60°C (Fig. 4). The recovery increased with increasing concentration of aqueous methanol up to 70% (v/v) and thereafter significantly decreased. The highest recovery was 97.22±2.17% at 70% (v/v) aqueous methanol, while the lowest recovery (1.45±0.16%) was obtained with pure methanol as a modifier. Janicot *et al.* (22) reported that the extraction yield of alkaloids from plants increased with increasing water content in the extraction fluids, which were a mixture of carbon dioxide, methanol, and water. In addition, Lin *et al.* (12) reported that the extracting yield of flavonoids from plants with 70% methanol modifier was higher than that with pure methanol only.

The concentration effect of modifier [70% (v/v) aqueous methanol] in SC-CO_2 is shown in Fig. 5. Total recoveries of glycyrrhizin after 120 min extraction time were 60.77±2.62, 94.88±1.68, and 97.63±4.95% at 5, 10, and 15% of 70% (v/v) aqueous methanol, respectively. When 70% (v/v) aqueous methanol was added at 5% (v/v) to SC-CO_2, most of the glycyrrhizin were extracted after 60 min dynamic extraction time. Although total recoveries for both 10 and 15% of 70% (v/v) aqueous methanol were not significantly different ($p<0.05$), the concentration of 15% (v/v) could shorten the extraction time by 30 min, reaching recovery comparable to that of the 10% (v/v) concentration.

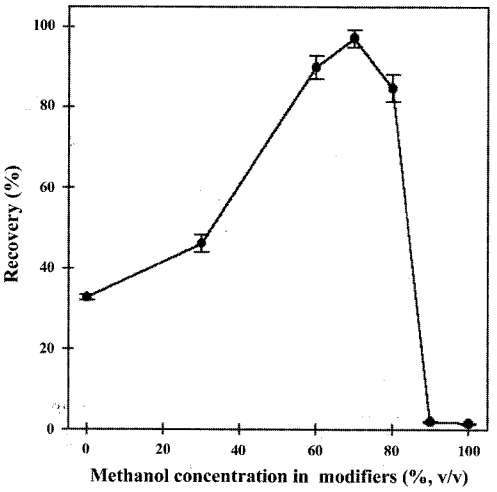

Fig. 4. Effect of modifiers of different methanol concentrations on the extraction of glycyrrhizin from licorice. SFE condition: 50 MPa, 60°C, flow rate of 3 mL/min, 10% (v/v) of modifier, 15 min static, and 120 min dynamic extraction.

Selection of process conditions for the extraction of glycyrrhizin from licorice Optimal process conditions such as pressure, temperature, and extraction time were obtained using SC-CO_2 modified with 15% (v/v) of 70% (v/v) aqueous methanol. The initial pressure (11 MPa) produced 88.08±1.98% recovery and enhanced to 99.87±2.23% at 30 MPa (Table 1). However, further increase in the pressure up to 50 MPa did not show any significant differences in recoveries. Pathumthip *et al.* (23) reported the existence of the optimal extraction pressure in the

Table 1. Total recoveries of glycyrrhizin from licorice at various extraction pressures[1)]

Extraction pressure (MPa)	Recovery (%)
11	88.08 ± 1.98[a2)]
20	96.00 ± 1.00[b]
30	99.87 ± 2.23[c]
40	98.41 ± 1.85[c]
50	97.33 ± 0.59[c]

[1)]Operation conditions are 60°C, flow rate of 3 mL/min, 15% (v/v) of 70% (v/v) aqueous methanol, 15 min static, and 120 min dynamic extraction.

[2)]Means with different letters within a column are significantly different ($p<0.05$).

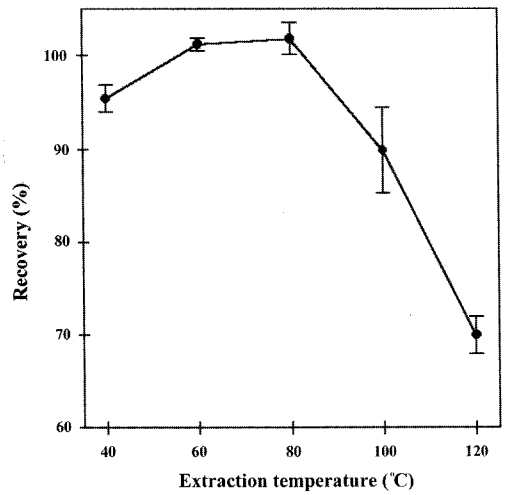

Fig. 6. Effect of temperature on total extraction yield of glycyrrhizin from licorice. SFE condition: 30 MPa, flow rate of 3 mL/min, 15% (v/v) of 70% (v/v) aqueous methanol, 15 min static, and 120 min dynamic extraction.

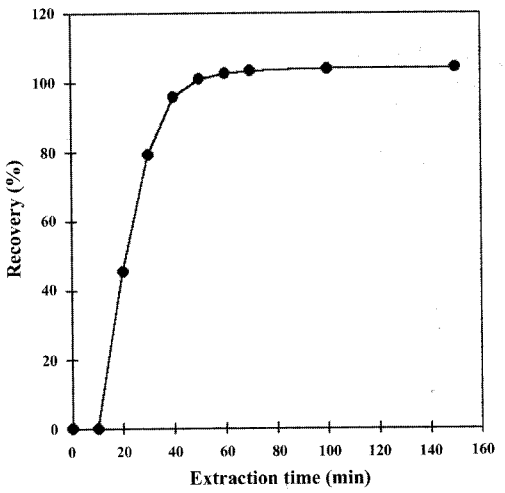

Fig. 7. Influence of the extraction time on the extraction glycyrrhizin from licorice. SFE condition: 30 MPa, 60°C, flow rate of 3 mL/min, and 15% (v/v) of 70% (v/v) aqueous methanol.

supercritical CO_2 extraction of bioactive component from plants, whereas Floch *et al.* (11) showed that extraction yield increased with increasing extraction pressure.

Figure 6 shows the effect of temperature on the extraction of glycyrrhizin at 30 MPa and temperature ranges from 40°C to 120°C. As the temperature increased from 40°C to 80°C, the recovery of glycyrrhizin gradually increased and reached the maximum recovery of 101.83±1.70% at 80°C. However, further increasing the temperature from 80 °C up to 120°C decreased the recovery dramatically, reaching 70.01±2.02% at 120°C. This might be due to the reduction of the SC-CO_2 density and the degradation of glycyrrhizin at the extraction temperature. On the contrary, Floch *et al.* (11) reported that the extraction yield increased due to easy desorption of the target components from a sample matrix at high extraction temperature.

The influence of extraction time using SC-CO_2 with 15% (v/v) modifier [70% (v/v) aqueous methanol] at 30 MPa and 60°C is shown in Fig. 7. The maximum recovery of 102.67±1.13% was reached within 60 min; however, further increase in the extraction time did not significantly affect the recovery of glycyrrhizin.

Changes of licorice tissue by various extraction fluids Results of this study confirmed that the extraction efficiency and rate could be improved using SC-CO_2 with aqueous methanol. Accordingly, the influence of modifiers on the structure of licorice tissues was examined using pure SC-CO_2 and SC-CO_2 with pure and 70% (v/v) aqueous methanol. The results are shown in Fig. 8 and Table 2.

Both pure SC-CO_2 and SC-CO_2 with methanol did not affect the structure of licorice tissue and the absolute density of licorice residues. However, when 70% (v/v) aqueous methanol was used as a modifier, not only were licorice tissues extensively broken down (Fig. 8 (a-d)), but the absolute density (1.5505±0.0061 g/cm^3) and highest recovery (101.69±0.98%) were obtained. Fahmy *et al.* (17) reported that the extraction yield increased as plant samples swelled by the addition of water, which acted as a good swelling agent. In conclusion, this study demonstrated that glycyrrhizin and low-density components of licorice tissue cell could be easily extracted through the destruction of licorice tissue by excessive swelling attributed to the mixture of SC-CO_2 and aqueous methanol. In conclusion, this study investigated not only the optimal extraction conditions of glycyrrhizin, which was not extracted by pure SC-CO_2 but also the influence of modifiers on licorice tissue. A large amount of glycyrrhizin from licorice was recovered by the addition of 15% of 70% (v/v) aqueous methanol to SC-CO_2 over the extracting periods of 60 min. The optimal process conditions for pressure and temperature were 30 MPa and 60°C, respectively. SC-CO_2 with 70% (v/v) aqueous methanol may improve the extraction efficiency by breaking licorice tissue resulting from the excessive swelling. In addition, the extraction of

Table 2. Recoveries of glycyrrhizin from licorice and absolute densities of licorice residues after the extraction under optimal extracting conditions

Extraction solvent	Absolute density (g/cm^3)	Recovery (%)
Original licorice sample	1.4684 ± 0.0031[a2)]	-
SC-CO_2	1.4639 ± 0.0016[b]	0.04 ± 0.004[a]
SC-CO_2 + methanol	1.4540 ± 0.0021[c]	1.87 ± 0.25[b]
SC-CO_2 + 70% (v/v) aqueous methanol	1.5505 ± 0.0061[d]	101.69 ± 0.98[c]

[1)]Operation condition are 30 MPa, 60°C, flow rate of 3 mL/min, 15% (v/v) of modifier, and 60 min dynamic extraction.
[2)]Means with different letters within a column are significantly different ($p<0.05$).

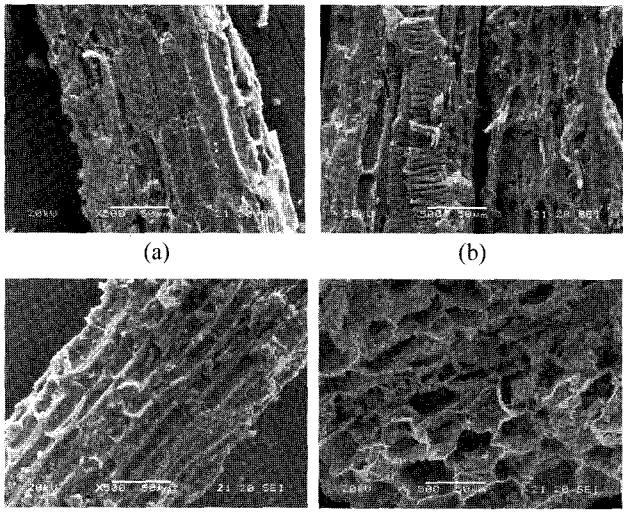

Fig. 8. The SEM of dried liquorice tissues after extracting: (a) original licorice tissue, (b) licorice tissue extracted by supercritical CO_2, (c) licorice tissue extracted by supercritical CO_2/methanol (85/15, v/v), and (d) licorice tissue extracted by supercritical CO_2/70% (v/v) aqueous methanol (85/15, v/v). SFE condition: 30 MPa, 60°C, flow rate of 3 mL/min, and 60 min.

glycyrrhizin from licorice using SC-CO_2 with an aqueous modifier gave better results than traditional organic solvent extraction in terms of the extraction yield, extraction time, and the consumption of organic solvent.

Acknowledgments

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