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Effects of Modifiers on the Supercritical CO_2 Extraction of Glycyrrhizin from Licorice and the Morphology of Licorice Tissue after Extraction

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Abstract Optimal conditions for the supercritical carbon dioxide ($scCO_2$) extraction of glycyrrhizin from licorice (*Glycyrrhiza glabra*) were investigated, with an emphasis on the types and levels of modifiers. The morphology of the licorice tissue remaining after the $scCO_2$ extraction of glycyrrhizin was examined by scanning electron microscopy, coupled with measurements of absolute density. Conventional organic solvent extraction was also carried out for purpose of quantitative comparison. At 50 MPa and 60°C glycyrrhizin could not be extracted with pure $scCO_2$, while a considerable amount of glycyrrhizin was extracted when water was added to $scCO_2$ as a modifier. The highest recovery was found to be about 97% when 70% aqueous methanol was added to $scCO_2$ at a concentration of 15%. The optimal pressure and temperature for the supercritical fluid extraction of glycyrrhizin were observed to be 30 MPa and 60°C, respectively. Under these conditions, the percentage recovery of glycyrrhizin attained a maximum value of 102.67 ± 1.13% within 60 min. Furthermore, in the case of $scCO_2$ modified with 70% aqueous methanol, the licorice tissue obtained after extraction was found to be severely degraded by excessive swelling, and the absolute density of the licorice residues was observed to be the highest.

Keywords: supercritical carbon dioxide, extraction, licorice, glycyrrhizin

INTRODUCTION

The licorice plant (*Glycyrrhiza glabra*) is a perennial herb of the *Leguminosae* family, the roots of which contain 25~30% starch, 3~10% D-glucose and sucrose, 3~5% glycyrrhizin, and traces of flavonoids, saponoids, sterols, amino acids, *etc*. Glycyrrhizin is one of the main active components in licorice and usually occurs in the form of combined calcium and potassium salts within its roots [1]. Glycyrrhizin and licorice extracts are widely used in the food industry as sweeteners (50~60 times sweeter than sucrose) and flavoring agents as well as foaming and emulsifying agents [2,3]. Glycyrrhizin has also been used in various pharmaceutical products, due to its bioactive characteristics such as anti-ulcer, anti-carcinogenic, anti-allergic, and anti-inflammatory activities [4-6]. Conventional extraction methods, using environmentally hostile organic solvents, are sufficiently effective in the extraction of glycyrrhizin from licorice. However, these methods usually require large quantities of toxic solvents, long extraction times and high extraction temperatures, although a microwave-assisted extraction method has recently been proposed to reduce both extraction time and the amount of toxic solvents necessary [7].

Since the 1960s, supercritical fluid extraction (SFE) technology has been used as an environmentally benign alternative to the conventional extraction methods [8]. Of all the gases and liquids thus far studied, carbon dioxide is generally the most widely used fluid for SFE applications due to its low critical temperature and pressure, non-toxicity, non-flammability and low cost [9-11]. In order to extract polar compounds from plant materials with non-polar supercritical CO_2 ($scCO_2$), its polarity must be modified by the judicious application of polar solvents as co-solvents or modifiers. The addition of polar modifiers can increase the polarity of $scCO_2$, and significantly enhance extraction efficiency, resulting in faster extractions [12]. Recently, many studies have been performed to extract and separate biologically active components from natural products using $scCO_2$ modified by various polar co-solvents such as methanol, ethanol, mix-

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tures of methanol and water, basified methanol with the addition of 10% diethylamine, caprylic acid methyl ester, *etc.* [13-17]. It has also been found that modifiers help to increase the interior volume and surface area of plant matrices by destroying or swelling them, thus resulting in significant increases in extraction efficiency [18,19]. Fahmy *et al.* [18] reported that the swelling of plant matrices through treatment with $scCO_2$ modified with water considerably augments the extraction efficiency of target materials from plant materials.

The main objective of this study was to investigate the optimal extraction conditions for various process parameters such as pressure, temperature, and the types and amount of polar modifiers for the SFE of glycyrrhizin from licorice. The influence of various CO_2-based extraction fluids on the morphology of the licorice tissue remaining after SFE was also examined by scanning electron microscopy and measurements of absolute density.

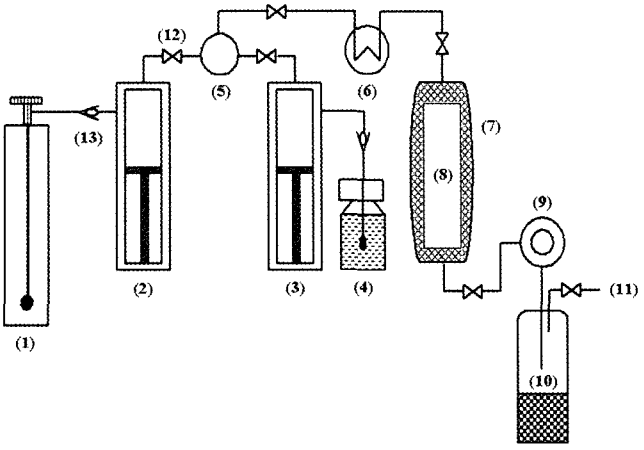

Fig. 1. A schematic diagram for the ISCO SFX 3560 used in this study (1: liquid CO_2 storage tank, 2: syringe pump for CO_2, 3: syringe pump for modifier, 4: modifier tank, 5: mixing zone, 6: pre-heating exchanger, 7: high pressure chamber, 8: extraction cartridge, 9: restrictor, 10: collection vial, 11: CO_2 venting, 12: valve, 13: check valve).

MATERIALS AND METHODS

Materials

Dried licorice roots were pulverized prior to extraction by a Waring blender (Dynamic Corp., Hartford, USA) and then sieved through 30 and 60 mesh sieves. The ground samples were packed in polypropylene bags and stored at 4°C until further analysis.

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

Organic Solvent Extraction

The organic solvent extraction of glycyrrhizin from licorice was performed under selected extraction conditions in our previous study [20]. A mixture of dry licorice and 30% (v/v) aqueous methanol with a weight ratio of 1:30 was placed in an extraction vessel and shaken at 40°C for 6 h. During this period, the extraction solvent was exchanged with the same volume of fresh organic solvent every 3 h. The licorice extracts were centrifuged at 3,000 rpm for 20 min. The supernatant was analyzed by high-performance liquid chromatography (HPLC, M616LC, Waters, USA) after filtration. According to the results of the organic solvent extraction, the maximum extraction yield for glycyrrhizin, which was used later to calculate the percentage recovery, was 4.51 ± 0.77 wt% relative to the weight of the dry sample.

Supercritical Fluid Extraction

Extractions were performed with an ISCO SFX 3560 supercritical fluid extractor (ISCO, Lincoln, USA) equipped with two syringe pumps (100DX, ISCO) at various temperatures (40~120°C) and pressures (11~50 MPa), a schematic diagram of which is shown in Fig. 1. 2 g dry licorice sample was loaded into a 10 mL extraction cell, and both CO_2 and a modifier were supplied to the extractor by each syringe pump. The flow rate was fixed at 3 mL/min, and every extract obtained was collected in a 20-mL vial. A static extraction was carried out for 15 min and then followed by a 120-min dynamic extraction.

HPLC Analysis

The HPLC apparatus used in this study was composed of a 616 controller, a 996 photodiode array UV detector operating at 254 nm, a 515 HPLC pump, and a TM 717 Plus autosampler (M616LC, Waters, USA). A sample solution was injected through a 20 μL loop and separated in the CapCell PAK C18 UG120 S-5 (250 mm × 4.6 mm i.d., Shiseido, Japan) at 40°C. Isocratic elution was performed with a 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 shown in Fig. 2 was established by using a standard solution of ammonium glycyrrhizinate. The percentage recovery was calculated as the ratio of the extraction yield of glycyrrhizin by SFE to the maximum extraction yield of glycyrrhizin obtained from the organic solvent extraction.

Changes in the Morphology of Licorice Tissue

Supercritical Drying

The licorice residues remaining after the SFE of glycyrrhizin were dried to less than 10% of the original moisture content by a supercritical drying method [21]. When employing 70% aqueous methanol as a modifier, the moisture in the wet licorice residues was extracted

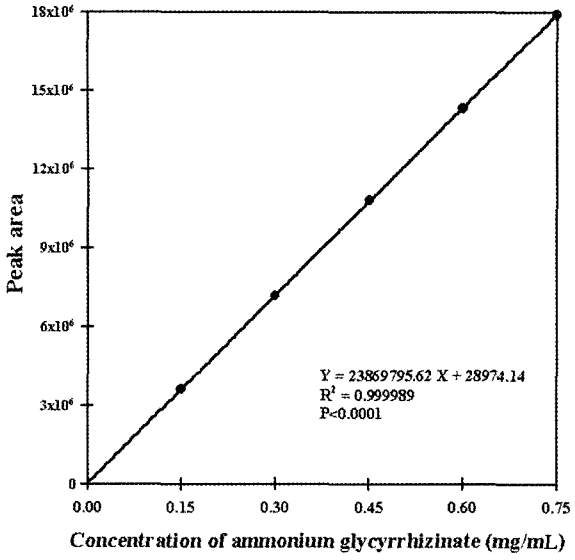

Fig. 2. Calibration curve for ammonium glycyrrhizinate.

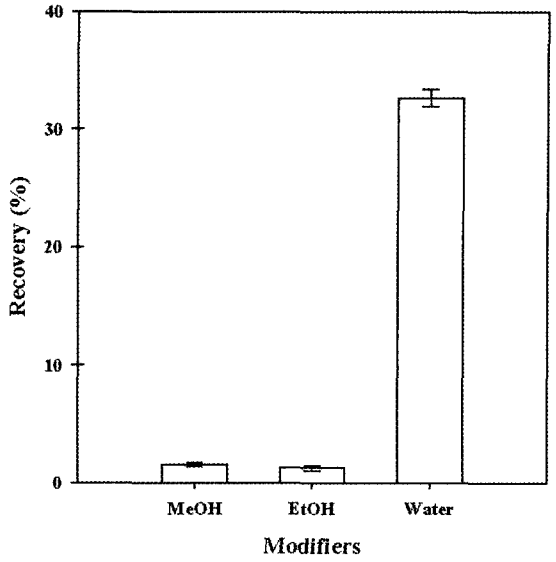

Fig. 3. Effects of various modifiers on the SFE of glycyrrhizin from licorice. SFE conditions: 50 MPa, 60°C, CO_2 flow rate of 3 mL/min, 10% volume of modifier, and 15-min static followed by 120-min dynamic extractions.

with $scCO_2$ containing 15% (v/v) methanol for 70 min at 30 MPa and 60°C, and the drying of the licorice residues was then completed by removing the residual methanol with pure $scCO_2$ under identical conditions. Not only was the flow rate of supercritical fluids maintained at 3 mL/min, but also no glycyrrhizin was extracted from the licorice residues during the supercritical drying process.

Scanning Electron Microscopy and Absolute Density Analysis

Dried licorice residues were applied to the non-sticky side of an aluminum tape attached to a brass disc. The specimens were coated with gold/palladium (60/40) using a sputter coater. The morphology of these samples was analyzed using a scanning electron microscope (JSM-5200, JEOL, Japan).

The absolute density (g/cm^3) of the licorice residues was measured by a gas pycnometer (Accupyc 2375, Micromeritics Instrument, USA). Ten replicate density measurements were carried out for each sample, and the results were expressed as the mean ± standard deviation.

Statistical Analysis

All the data obtained in this study were analyzed by Duncan's multiple range test using SAS (SAS Institute, NC, USA). The significant difference was established within a 5% level of significance ($P<0.05$).

RESULTS AND DISCUSSION

Effect of Modifiers on SFE of Glycyrrhizin from Licorice

The percentage recovery of glycyrrhizin from licorice was found to be 0.04 ± 0.004% when pure $scCO_2$ was used as an extraction solvent at 50 MPa and 60°C. Such a low degree of recovery is attributable to the high polarity of glycyrrhizin, since highly polar or ionic compounds cannot be extracted with non-polar $scCO_2$ [18]. The application of polar modifiers such as methanol, ethanol and water is one of the simplest, yet most effective, ways to increase the polarity of $scCO_2$. The experimental results for the percentage recovery of glycyrrhizin obtained from the addition of 10% (v/v) of various polar modifiers to $scCO_2$ are shown in Fig. 3. Extractions were performed at 50 MPa, 60°C, and a CO_2 flow rate of 3 mL/min with 15-min static followed by 120-min dynamic extractions. Among these three modifiers, the highest recovery (32.66 ± 0.77%) was observed with water, while both methanol and ethanol were found to have little influence on the recovery of glycyrrhizin (1.55 ± 0.18% and 1.24 ± 0.25%, respectively). Although the addition of water to $scCO_2$ as a modifier enhanced the recovery significantly, the flow rate of the water-modified supercritical fluid was observed to be very unstable during extraction. According to Jackson *et al.* [22], the solubility of water in $scCO_2$ is only 0.5% (v/v) at 34.4 MPa and 75°C. Therefore, the unstable flow of water-modified $scCO_2$ might be attributable to the trickling phenomenon caused by phase separation in the extractor.

In order to ameliorate the limitations of water as a modifier, a variety of methanol-water mixtures of different concentrations ranging from 0 to 100% (v/v) were employed as modifiers and the effects of water contents in the aqueous methanol solutions on the SFE of glycyrrhizin was examined under identical conditions. As clearly seen in Fig. 4, the percentage recovery increased with increasing water concentration of the aqueous methanol solution up to 70%, and then decreased abruptly above

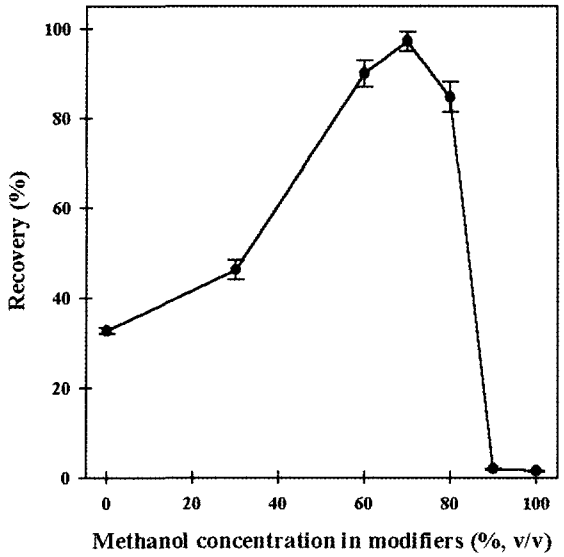

Fig. 4. Effects of methanol concentration in the methanol-water mixtures on the SFE of glycyrrhizin from licorice. SFE conditions: 50 MPa, 60°C, CO_2 flow rate of 3 mL/min, 10% volume of modifier, and 15-min static followed by 120-min dynamic extractions.

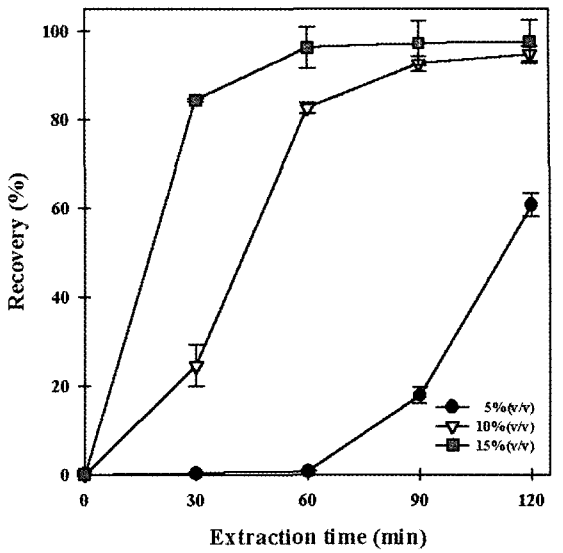

Fig. 5. Variations in the percentage recovery with the dynamic extraction time for different amounts of 70% aqueous methanol added to $scCO_2$. SFE conditions: 50 MPa, 60°C, CO_2 flow rate of 3 mL/min, and 15-min static followed by 120-min dynamic extractions.

80%. The highest recovery was found to be 97.22 ± 2.17% when 70% aqueous methanol was used as a modifier, while the lowest recovery was observed to be 1.45 ± 0.16% for pure methanol. These observations are consistent with the previous experimental results of Janicot *et al.* [23] and Lin *et al.* [24]. Janicot *et al.* reported that the extraction yield of alkaloids from plants increases with increasing water content in a supercritical fluid composed of carbon dioxide, methanol, and water. Lin *et al.* [14] showed that the extraction yield of flavonoids from plant materials with 70% aqueous methanol was higher than that obtained with pure methanol.

The effect of the amount of 70% aqueous methanol added to the $scCO_2$ on the SFE of glycyrrhizin is shown in Fig. 5. The total recovery of glycyrrhizin after a 120-min extraction at 50 MPa and 60°C was 60.77 ± 2.62, 94.88 ± 1.68 and 97.63 ± 4.95%, respectively, at concentrations of 5, 10, and 15% (v/v) relative to CO_2. When the concentration of the 70% aqueous methanol was 5%, most of the glycyrrhizin present in the licorice was extracted after a 60-min dynamic extraction. Although the difference in total recovery obtained at concentrations of 10% and 15% was not significant, given the 5% level of significance, employing a 15% modifier concentration reduced the extraction time to attain the same recovery by about 30 min compared with the case of 10% modifier concentration.

Optimal Process Conditions for the SFE of Glycyrrhizin from Licorice

Optimal process conditions such as pressure, temperature and extraction time for the SFE of glycyrrhizin from licorice were determined using $scCO_2$ modified with a 15% volume of 70% aqueous methanol. The effects of the extraction pressure on the glycyrrhizin recovery at 60°C are summarized in Table 1. An initial extraction pressure of 11 MPa yielded 88.08 ± 1.98% of recovery, and the recovery increased to 99.87 ± 2.23% as the pressure increased to 30 MPa. However, further increases in pressure up to 50 MPa were found to exert little influence on the recovery of glycyrrhizin. These results are consistent with the observations of Pathumthip *et al.* [24] that there exists an optimal extraction pressure for the $scCO_2$ extraction of bioactive components from plant materials, although Floch *et al.* [13] argued that the extraction yield increases with increasing pressure without any significant decrease in the yield.

Fig. 6 illustrates the effects of extraction temperature

Table 1. The effect of pressure on the total recovery of glycyrrhizin at 60°C and a CO_2 flow rate of 3 mL/min with 15-min static followed by 120-min dynamic extractions

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

* Means with different letters in a column are significantly different as determined by Duncan's multiple range test ($P<0.05$).

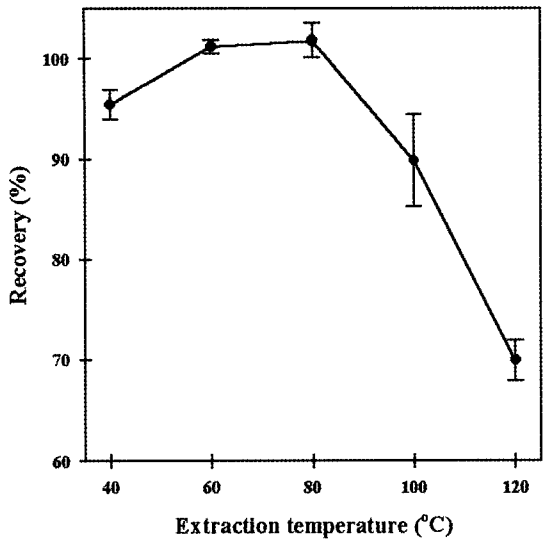

Fig. 6. Effect of extraction temperature on the total recovery of glycyrrhizin from licorice. SFE conditions: 30 MPa, CO_2 flow rate of 3 mL/min, 15% volume of 70% aqueous methanol, and 15-min static followed by 120-min dynamic extractions.

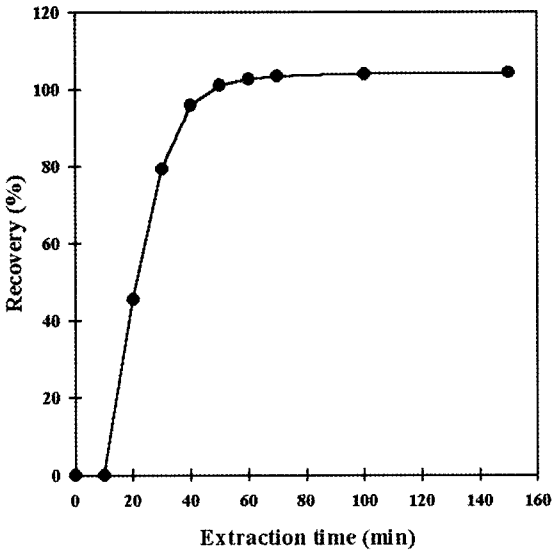

Fig. 7. Influence of extraction time on the recovery of glycyrrhizin from licorice. SFE conditions: 30 MPa, 60°C, CO_2 flow rate of 3 mL/min, and 15% volume of 70% aqueous methanol.

on the SFE of glycyrrhizin at 30 MPa in a range between 40°C and 120°C. As the temperature increased from 40°C to 80°C, the recovery of glycyrrhizin gradually increased and reached a maximum recovery of 101.83 ± 1.70% at 80°C. However, with further increases in temperature from 80°C to 120°C, the percentage recovery dramatically decreased, reaching a value of 70.01 ± 2.02% at 120°C. This might be due to reduction of the $scCO_2$ density and the degradation of glycyrrhizin at very high extraction temperatures. On the contrary, as explained by Floch *et al.* [13], the extraction yield increased with temperature in a moderately high temperature range due to easy desorption of the target component from the sample matrices.

Table 2. The recovery of glycyrrhizin and the absolute density of licorice residues remained after SFE under optimal extraction conditions*

Extraction solvent	Absolute density (g/cm^3)	Recovery (%)
Original licorice sample	$1.4684 \pm 0.0031^{a**}$	-
$scCO_2$	1.4639 ± 0.0016^{b}	0.04 ± 0.004^{a}
$scCO_2$ + methanol	1.4540 ± 0.0021^{c}	1.87 ± 0.25^{b}
$scCO_2$ + 70% (v/v) aqueous methanol	1.5505 ± 0.0061^{d}	101.69 ± 0.98^{c}

* Means with different letters in columns are significantly different as determined by Duncan's multiple range test ($P<0.05$).

** Extraction conditions are 30 MPa, 60°C, CO_2 flow rate of 3 mL/min, 15% volume of modifier, and 60-min dynamic extraction.

The influence of extraction time on the SFE of glycyrrhizin was evaluated at 30 MPa and 60°C using $scCO_2$ modified with a 15% volume of 70% aqueous methanol. As shown in Fig. 7, a maximum recovery of 102.67 ± 1.13% was attained within 60 min, but further increases in extraction time did not result in any significant effect on the recovery of glycyrrhizin.

Changes in the Morphology of Licorice Tissue after Extraction

The observations mentioned in the previous sections showed that the addition of aqueous methanol to $scCO_2$ could significantly increase the extraction efficiency. Therefore, it was reasonable to investigate the influences of various polar modifiers on morphological changes in the licorice tissue occurring during SFE; the results are shown in Fig. 8 and Table 2.

Neither pure $scCO_2$ nor methanol-modified $scCO_2$ had any significant effect on the licorice tissue and, consequently, its absolute density, whereas the licorice tissue remaining after SFE was found to be seriously degraded (see Fig. 8(d)), and its absolute density was observed to be highest (1.5505 ± 0.0061 g/cm^3) when 70% aqueous methanol was used as a modifier. Here it should be noted that the highest recovery (101.69 ± 0.98%) could be achieved under these extraction conditions. According to Fahmy *et al.* [18], the extraction yield increases as the degree of swelling in plant samples caused by either the water added directly to the samples or the water present in the modifiers increases. Therefore, our experimental results might be attributed to the following: the cell membranes of the licorice tissue were largely destroyed due to the excessive swelling of the licorice tissue caused by the mixture of $scCO_2$ and aqueous methanol, which

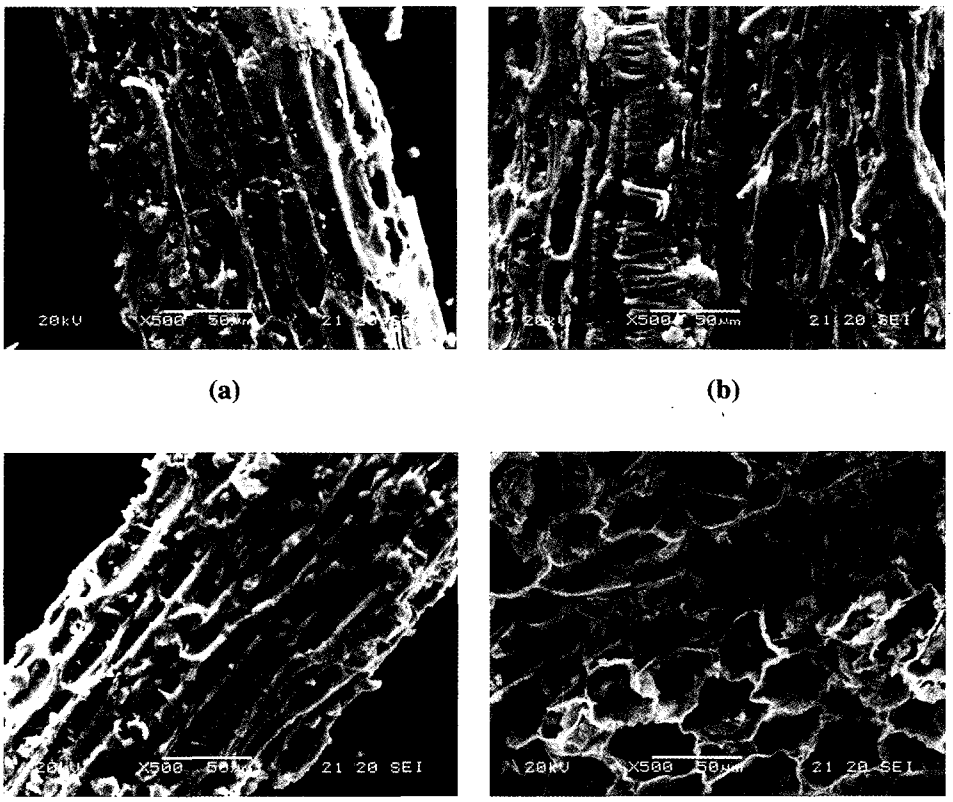

Fig. 8. SEM micrographs of the licorice tissues remaining after SFE: (a) original licorice tissue, (b) licorice tissue extracted with pure $scCO_2$, (c) licorice tissue extracted with a mixture of $scCO_2$/methanol (85/15, v/v), and (d) licorice tissue extracted with a mixture of $scCO_2$/70% aqueous methanol (85/15, v/v). SFE conditions: 30 MPa, 60°C, CO_2 flow rate of 3 mL/min, and 60 min dynamic extraction.

made it much easier to extract glycyrrhizin and other low-density components present in the licorice tissue cells.

CONCLUSION

In this study, optimal conditions for the SFE of glycyrrhizin from licorice were investigated using pure and polar solvent-modified $scCO_2$. Most of glycyrrhizin could be extracted from licorice in 60 min by adding a 15% volume of 70% aqueous methanol, as a modifier, to $scCO_2$. Optimal process conditions for the extraction pressure and temperature were found to be 30 MPa and 60°C, respectively. Furthermore, it was shown that a significant improvement in the extraction efficiency might result from the degradation of the licorice tissue, due to the excessive swelling caused by the $scCO_2$ modified with 70% aqueous methanol. Consequently, it could be concluded that the SFE of glycyrrhizin from licorice using $scCO_2$ modified with aqueous methanol is a much better method than conventional organic solvent extraction when the extraction efficiency and the required amount of environmentally hostile organic solvent are considered.

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