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Supercritical Fluid Extraction of Seed Oil from Chinese Licorice (*Glycyrrhiza uralensis* Fisch.): Chemical Composition and Antibacterial Activity

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ABSTRACT

Seed oil from *Glycyrrhiza uralensis* Fisch. was extracted by supercritical fluid (CO_2) extraction. The oil was analysed by GC-MS after methylation. Compounds were identified according to their mass spectra (EI, 70 eV) by comparison with authentic reference substances and literature data. Five fatty acids were identified, with linoleic acid (24.3%) and α-linolenic acid (25.51%) being the main constituents. The effects of extraction time, pressure, temperature and CO_2 flow rate on seed oil yield were investigated. Results showed that, under dynamic extraction time of 1.5 h, pressure 30 MPa, temperature 50 °C and CO_2 flux of 10 kg h^{-1}, the oil yield was 2.09% (m/m). The method was efficient for the extraction of this oil. Antibacterial activity of the oil was checked against Gram-negative and Gram-positive strains, evaluated by investigation of MIC and MBC. The seed oil of *Glycyrrhiza uralensis* did not exhibit an antibacterial effect against the tested strains in our test system.

KEYWORDS

Fatty acid, methylation, GC-MS, SCFE-CO_2, *Glycyrrhiza uralensis* Fisch., antibacterial activity.

1. Introduction

Glycyrrhiza uralensis Fisch. is the most important species of the genus *Glycyrrhiza* in China. Its roots and stolons have been employed as very important components in many traditional medicinal herb prescriptions as well as flavouring and sweetening agents for a long time.[1]

The constituents and activities of the crude drug have been studied by several investigators.[2–4] Recently, it has been found to be a potential therapeutic herb against SARS, chronic hepatitis and AIDS[5,6] and this has called for more attention to this herb. However, we have never read any reports on the fatty acid composition in the seed oil of this species.

Supercritical fluid extraction (SCFE) has received increasing attention in a variety of fields in recent years. It is regarded as a promising alternative technique to steam distillation and solvent extraction for the extraction of natural products.[7] The combined liquid-like solvating capabilities and gas-like transport properties of supercritical fluids make them suitable for the extraction of diffusion-controlled matrices such as plant tissues. Moreover, the solvating power of the supercritical fluid can be manipulated by changing the pressure and/or the temperature. Carbon dioxide, the most commonly used supercritical fluid, has the additional advantages of being non-flammable, fairly non-toxic, cost-effective and easily removed from the extract after decompression, and due to its relatively low critical temperature (31.1 °C), thermal sample decomposition is reduced.

Often SCF-CO_2 extraction methods involve the investigation of many variables, which may affect the efficiency of extraction.[8] The selection of these variables and their levels is complicated.[9] Among them the extraction time, pressure, temperature and CO_2 flux are generally considered as the most important factors.[10]

The aims of our research were to investigate the most efficient SCF extraction for seed oil from *Glycyrrhiza uralensis,* to analyse the chemical composition by GC-MS and to evaluate the antibacterial activity by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

We hope this can provide evidence for more exploitation and utilization of licorice resource as healthy food.

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2. Experimental

2.1. Plant Material

Seeds of *Glycyrrhiza uralensis* were purchased in the Nei Meng Province of China in September 2002 and identified by N. Shaoquan in our laboratory. The drying temperature was 30 °C for 24 h. Linoleic acid and α-linolenic acid standards were obtained from Sigma-Aldrich, USA.

2.2. Supercritical Fluid Extraction

A HA121-50.01 SCFE system (Hua An, Nan Tong, China) was used to perform the supercritical fluid extraction. The mass of the sample used for supercritical fluid extraction was 180 g, with particle size 40–60 mesh, separation pressure 5.8 MPa and temperature 40 °C, similar to that of material used by conventional producers.[11]

SCFE-CO_2 was conducted at extraction times of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h, extraction pressures of 15, 20, 25, 30 and 35 MPa, temperatures of 40, 45, 50,55 and 60 °C and CO_2 flow rates of 6, 8, 10, 12 and 14 kg h^{-1} while other parameters were kept constant.

2.3. Pretreatment of the Extracted Oil

The seed oil was pretreated by methyl ester before injection. That is, 10 mL of a 10:1 (v/v) methanol and sulphuric acid mixture was added to 1 g of extracted oil, and then esterified for 3 h in a water bath at 60 °C. After 3 h, the esterification was cooled

down to room temperature, 10 mL of distilled water was added and the reaction terminated by adding 7 mL of chloroform three times for extraction. Anhydrous sodium sulphate was used to absorb the remaining water that the chloroform layer had retained. The oil was then stored at –20 °C until analysed.

2.4. Gas Chromatography–Mass Spectrometry Analysis conditions

The analysis of the oil sample was run on a TRACE MS GC-MS system (Finnigan Systems, Fremont, USA) on a DB-5MS capillary column (30 m × 0.25 mm i.d.; film thickness 0.25 μm). The oven temperature was programmed at 50 °C for 1 min, then to 280 °C at 20 °C min^{-1} and subsequently held isothermal until analysis was completed. The injection port temperature was 200°C and the detector temperature was 200 °C. A sample of 0.2 μL of a 50% solution of the oil in ether was injected with a split ratio of 1:20 with a solvent delay of 3 min. The carrier gas was helium (99.99999) at a rate of 1 mL min^{-1}. The ionization of the sample components was performed in the EI mode (70 eV). The percentage compositions of the individual components were computed from the GC peak areas without any correction for the relative response factors.

2.5. Antibacterial Activity

2.5.1. *Preparation of Test Seed Oil*

The seed oil was tested in geometrical dilution from 2% (v/v) to 0.1313% (v/v) in a sterile 96-well microtitre plate. The oil was emulsified in physiological saline solution (0.9% NaCl) containing 1% Tween 80 as an emulsifier. The final Tween concentration in the test system was 0.5% in all wells. Therefore the growth control contained 1.5% ethanol and 0.5% Tween.

2.5.2. *Preparation of the Bacterial Inoculum*

Bacteria were incubated overnight in Iso-Sensitest Broth (Oxoid) at 37 °C. After 20 ± 2 h the inocula were adjusted to approx. 5 × 10^5 colony forming units (CFU) per mL. The colony number was verified by the spiral plater counting method.

2.5.3. *Preparation and Incubation of the Microtitre Plate*

Each well of the plate was filled with 100 μL test or control solution and 100 μL bacterial inoculum. The inoculated plates were inoculated at 37 °C for 20 ± 2 h.

2.5.4. *Determination of MIC and MBC*

Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) used a modified broth microdilution method according to the German DIN regulation 58940-7. The MIC is defined as the lowest concentration of the antibacterial agent that inhibits visible growth of bacteria, i.e. a turbidity or precipitation of bacteria at the bottom of the wells. The MBC was determined by seeding 10 μL from each well on an agar plate which was then incubated for a further 24 h at 37 °C. The MBC was the lowest concentration without colony growth on the agar plate. Because the test solutions were unstable emulsions, the wells of the microtitre plate showed a turbidity and precipitation even without bacterial growth, which impaired the correct reading of the MIC. This problem was solved by adding of 10 μL of the redox-reagent p-iodonitrotetrazolium-violet (0.5% in aqueous solution) to each well. The presence of growing bacteria turns the colourless salt into the red formazan. The MIC could be read as the lowest concentration of the test substance which stayed colourless after addition of the dye.

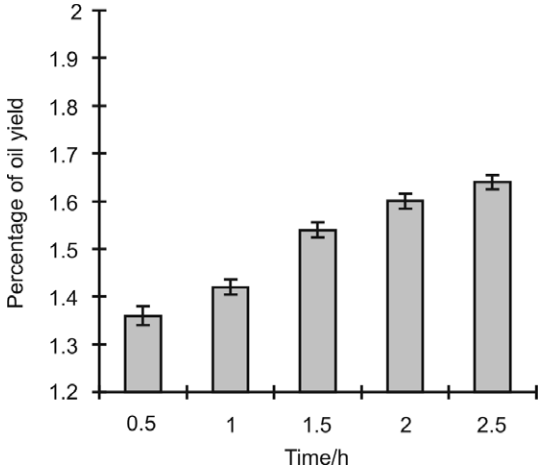

Fig. 1 Effect of extraction time.

3. Results and Discussion

3.1. Supercritical Fluid Extraction

The optimization of the experimental conditions is a crucial step in the development of a SCFE method because various parameters potentially affect the extraction process. In fact, extraction time, pressure, temperature and flow rate of CO_2 are generally considered as the most important factors. The optimization was carried out step-by-step.[8] The optimal supercritical CO_2 extraction conditions were decided by using various levels of these parameters.

The effect of extraction time, at the selected constant extraction pressure of 25 MPa, temperature of 50 °C and flow rate of 10 kg h^{-1} was studied. The extraction times were 0.5, 1.0, 1.5, 2.0 and 2.5 h. It was observed that there were no significant differences in the amounts of extracted oil. 1.5 h was selected for further experiments.

The oil yields were measured at five different pressures (15, 20, 25, 30 and 35 MPa) and at a constant temperature of 50 °C and flow rate of 10 kg h^{-1}. The yield increased significantly with pressure. With increasing pressure, the density and solvent power of supercritical CO_2 increased, along with enhanced dissolution and solvent mass transferring efficiency. Taking operation safety and power consumption into account, 30 MPa was chosen for further studies.

The extraction temperature was studied in the range of 40 °C to 60 °C at a constant pressure of 30 MPa and flow rate of 10 kg h^{-1}. On one hand, the solubility increased at elevated temperature,[12]

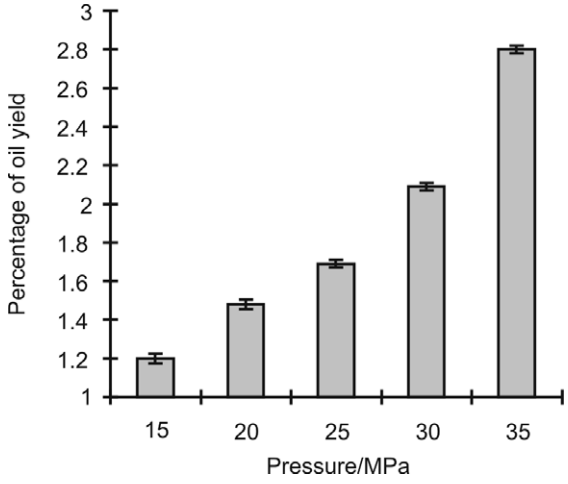

Fig. 2 Effect of extraction pressure.

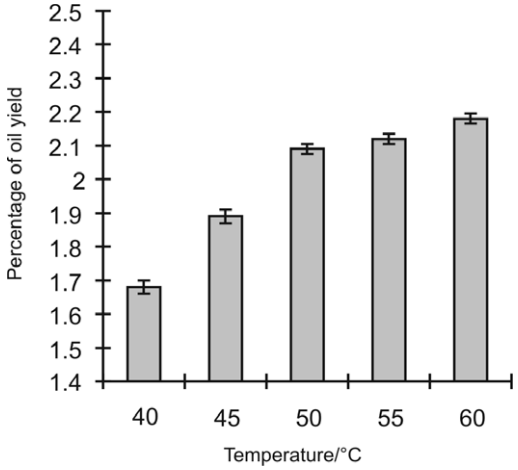

Fig. 3 Effect of extraction temperature.

on the other hand, the density and dissolution ability of supercritical CO_2 decreased with increasing temperature. The oil yield increased with increasing temperatures in our test. Above 50 °C, the change was slight. Considering the decomposition of high-temperature sensitive substances, we selected a temperature of 50 °C.

The flow rate was studied in the range of 6 to 14 kg h^{-1} at a constant pressure of 30 MPa and temperature of 50 °C. It was found that the oil yield increased with increasing CO_2 flow rate. Because of the increasing CO_2 flow rate, the mass transfer and dissolution ability of supercritical CO_2 increased. Cost also increases with higher flow rate. In view of cost and efficiency, we selected 10 kg h^{-1}.

3.2. Qualitative and Quantitative Analyses

Most constituents were identified by comparison of their mass spectra with those stored in the NIST MS databases, by comparison with authentic reference substances and literature data.[13] Relative component concentrations were obtained directly from GC peak areas by a normalization method.

The components of the seed oil composition and their percentages are given in Table 1, where the components are listed in order of elution on the DB-5MS column.

Twenty peaks were identified, which represented 70.37% of the total GC peak areas. Fatty acids represented 61.84% of total peak areas, and unsaturated fatty acids mainly consisted of linoleic acid (24.3%) and α-linolenic acid (25.51%). The two fatty acids have many activities, and cannot be synthesized by the human body. They have to be obtained from the daily diet.

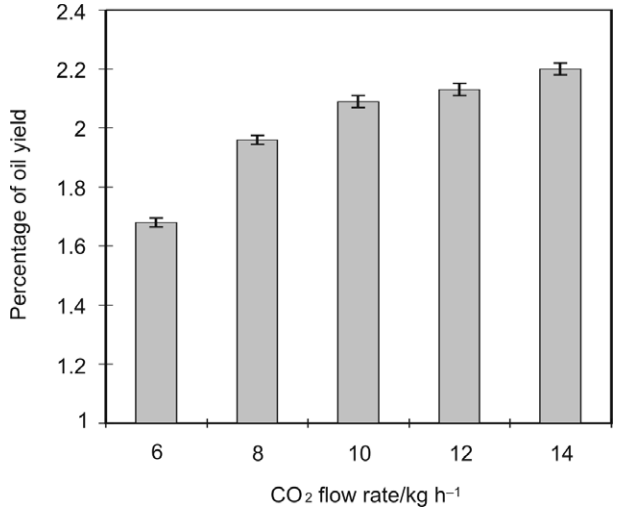

Fig. 4 Effect of CO_2 flow rate.

Table 1 Analytical results for fatty acids and other compounds in seed oil from *Glycyrrhiza uralensis* Fisch.

No.	Retention time/min	Compound	Area/%
1	4.03	1,3-Dimethyl-benzene	0.19
2	5.41	Decane	0.21
3	5.74	o-Cymene	0.25
4	6.49	Undecane	0.26
5	7.52	Dodecane	0.33
6	8.49	Tridecane	0.45
7	9.41	Tetradecane	0.61
8	10.28	Pentadecane	0.82
9	11.10	Hexadecane	0.73
10	11.88	Heptadecane	1.03
11	12.02	1,2-Benzenedicarboxylic acid, butyl ester	1.09
12	12.20	Octadecane	0.07
13	13.33	Nonadecane	1.11
14	13.50	Hexadecanoic acid	7.98
15	14.00	Eicosane	0.81
16	14.64	Linoleic acid	24.30
17	14.68	α-Linolenic acid	25.51
18	14.81	Octadecanoic acid	3.02
19	15.03	Linoleic acid ethyl ester	1.18
20	15.26	Docosane	0.48

At the same time, we identified some volatile compounds. Most of them were identical with formerly identified volatile compounds from this species, for example, alkanes.[13] We can therefore analyse fatty acids and some volatile constituents simultaneously by this method.

3.3. Antibacterial Activity of Seed Oil

The difficulty in testing essential oils in standard test media is that the pure oil is not soluble in water. The lipophilic oil is on the top of the test medium.[14] Therefore, we used Tween 80 as an emulsifer to solve this problem. The final Tween 80 concentration was 0.5% (v/v), which neither inhibits the growth of any bacteria nor affects the visual determination of the MIC.

The MIC and MBC results are given in Tables 2 and 3. Obviously, the seed oil did not exhibit an antibacterial effect against the tested strains in our test system, because all MICs and MBCs are higher than 2%.

In an aqueous test system the phase separation may affect the effectiveness of the oil against bacteria. Because of the high lipophilicity of the test substance, other test systems might be more suitable.

4. Conclusions

A method for the extraction of seed oil from *Glycyrrhiza uralensis* Fisch. using supercritical CO_2 was optimized. The oil

Table 2 Antibacterial activity of seed oil of *Glycyrrhiza uralensis* Fisch. against Gram-positive bacteria.

Bacterial strain	MIC% (v/v)	MBC% (v/v)
Listeria monocytogenes ATCC 15313	>2	>2
Enterococcus faecium ATCC 11229	>2	>2
Staphylococcus aureus ATCC 6538	>2	>2
Staphylococcus aureus NCTC 10442	>2	>2
Staphylococcus epidermidis ATCC 49134	>2	>2
Staphylococcus saprophyticus ATCC 15305	>2	>2

Table 3 Antibacterial activity of seed oil of *Glycyrrhiza uralensis* Fisch. against Gram-negative bacteria.

Bacterial strain	MIC% (v/v)	MBC% (v/v)
Enterobacter aerogenes ATCC 13048	>2	>2
Escherichia coli ATCC 11229	>2	>2
Klebsiella pneumoniae ATCC 10031	>2	>2
Proteus mirabilis ATCC 14153	>2	>2
Pseudomonas aeruginosa ATCC 15442	>2	>2
Shigella flexneri ATCC 29903	>2	>2

yield was 2.09% under optimal conditions. Identification and qualification of the seed oil components by GC-MS showed that it is rich in unsaturated fatty acids (>49.81%), so it could be used as food additive or in health food. The antibacterial activity of the oil is not strong, and further research is needed in order to obtain significant results about the antimicrobial activities of supercritical extracts in other systems. The antioxidative capacity of the oil towards LDL oxidation will be evaluated later.

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