J Am Oil Chem Soc (2010) 87:1221–1226
DOI 10.1007/s11746-010-1602-1

ORIGINAL PAPER

Supercritical Carbon Dioxide Extraction of *Microula sikkimensis* Seed Oil

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Received: 25 December 2009 / Revised: 30 April 2010 / Accepted: 30 April 2010 / Published online: 20 May 2010

Abstract The seed oil of *Microula sikkimensis* had been intensively studied due to its pharmacological actions. In the present study, seed oil of *Microula sikkimensis* was extracted using supercritical fluid extraction (SFE). Determinations of the extracts composition were performed by gas chromatography (GC). An orthogonal array design (OAD), OA_9 (3^4), was employed for optimization of the supercritical fluid extraction of the compound with regard to the various parameters. Four factors, namely pressure (21.0–27.0 MPa), the dynamic extraction time, temperature, and CO_2 flow rate of the supercritical fluid, were studied and optimized by a three-level OAD. The effects of the parameters on the yield of seed oil were studied using analysis of variance (ANOVA). The results revealed that the pressure had a significant effect on the yield of seed oil ($p < 0.05$), while the other three factors, i.e., CO_2 flow rate, dynamic extraction time and temperature, were not identified as significant factors under the selected conditions based on ANOVA. The results show that the best values for the extraction condition of seed oil was pressure 24.0 MPa, extraction time 3 h, temperature 45 °C and a CO_2 flow rate 20 L/h in the 20-L vessel.

Keywords *Microula sikkimensis* · Supercritical fluid extraction · Orthogonal array design

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Introduction

Microula sikkimensis, an endemic oil plant species has multiple applications for medicine, food and fodder production. It belongs to the genus *Microula* Benth. of the family Boraginaceae, which is a biennial herb rich in γ-linolenic acid. As a stenotopic species, it is mainly confined to specific habitats on the eastern rim of the Qinghai-Tibetan Plateau [1]. The seed oil content of *Microula sikkimensis* was 45%, and unsaturated fatty acids was 86% of this, the essential fatty acid content was 43%, and the γ-linolenic content acid was 8.1% close to the values in *Oenothera biennis*. It also contains α-linolenic and other unsaturated fatty acids [2]. Previous studies indicated that the seed oil was nonpoisonous [3], that it could significantly decrease cholesterol and triglycerides in blood serum, and it could increase the ratio between total cholesterol and high-density lipoprotein, prevent the accumulation of atheroma, preserve the structural integrity of biological membranes and inner membranes of vessels, and alleviate high blood fat [4, 5].

At present, about 80 species are known to contain some γ-linolenic acid, but only the seed oil of *Oenothera biennis* has become a commercial product [6–8]. As a new source of oil crops, *Oenothera biennis* had been widely planted in 15 countries over the world. The seed oil content of *Microula sikkimensis* is twice as high as that of *Oenothera* *biennis* [9–11], but commercial production is still on a very small scale. Therefore, the objectives of the present study were to extract seed oil from *Microula sikkimensis* and to investigate the optimization of the extraction process. Supercritical fluid extraction (SFE) with supercritical carbon dioxide (SC-CO_2) is an alternative method for the extraction of oils from natural products and has received considerable attention [12–14]. SFE was used for extraction from grape seeds, soybean, amaranth seeds, hazelnuts, and so on [15–18]. But there is no information on the SC-CO_2 extraction of oil from *Microula sikkimensis.*

CO_2 is a cheap, non-toxic, environmentally–friendly solvent and allows SFE at temperatures near room temperature and relatively low pressures. In combination with the fact that CO_2 immediately evaporates when brought into atmospheric conditions, the oil obtained is free from chemical and thermal degradation compounds and from solvent residues. As a process, SFE offers numerous potential advantages over conventional extraction processes, including reduced extraction time, reduced organic solvent volume, and more selective extractions [19]. In fact, CO_2 extracts are generally recognized as safe (GRAS) to be used in food products [20]. Therefore, SFE may serve as a very promising technology in food and pharmaceutical processing [21].

Orthogonal array design (OAD) is a fractional factorial designed series of trials assigned by an orthogonal array. The results of OAD experiments can be treated by analysis of variance (ANOVA) and direct observation analysis. The advantages of OAD included:

– It can minimize assay numbers and time to keep the experimental cost at a minimum level,
– the optimum parameters obtained from the laboratory can be utilized for large scale of production.

This method has been adopted in different areas for the optimization of the analytical procedure, also in SFE for the optimal procedure of extraction of target compounds from various samples.

Materials and Methods

Plant Material

Seeds of *Microula sikkimensis* from the Qinghai province were obtained from the Northwest Institute of Plateau Biology of the Chinese Academy of Science. The dried seeds were ground in a rotary mill and then sieved (20–30 mesh). The standards for fatty acid analysis were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. Petroleum ether for Soxhlet extraction was of analytical grade and was purchased from Enji Chemical Reagent Co., Ltd. CO_2 (food grade) was from the Dongsheng Gas Ltd.

Supercritical Fluid Extraction

A supercritical fluid extractor Spe-ed SFE-2 (Applied Separation, USA) was used. It operates with two pumps, a master pump fitted with a cooling jacket on the pump head and a second pump (Knauer pump, model K-501, Berlin, Germany) for the addition of organic modifier. The Spe-ed SFE is a screening system extractor and is capable of pressures up to 68.0 MPa and temperatures up to 240 °C, static and dynamic extraction with flow from 0 to 10 L/min (gaseous carbon dioxide at atmospheric pressure) and extraction vessels from 1 to 20 L. The temperature-controlled chamber and the master pump were set to the desired temperature and pressure. A metering valve is used to vary the CO_2 flow rate. The separator was maintained at 5 MPa to stabilize the expanded mixture. A glass vial with a rubber plug at the top was placed there to separate the mixture of CO_2 and the extract. The collection condition was at room temperature and atmospheric pressure.

Extractions were performed at 35–55 °C, with a pressure range of 21.0–27.0 MPa and a CO_2 flow rate range of 17.0–23.0 L/h. Approximately 5 kg of sample were placed into the 20-L extraction vessel. The extraction vessel was then placed in the extractor and was allowed to equilibrate to the desired temperature. Upon reaching the desired temperature, pressurization was initiated, and the CO_2 flowed through the extraction vessel from the bottom to the top. The extracted oil was separated from oil-rich CO_2, and was collected in the glass vial glass vials with a rubber plug at the top. In all cases, the restrictor temperature was set at 55 °C. The extracted oil was weighed with an analytical balance at a specific time interval as per the experimental design. The extraction yield was determined, based on the amount of the extracted oil by weight of the sample.

$$\text{Extraction Yield }(\%) = \frac{\text{mass of extracted oil}}{\text{mass of sample}} \times 100\%$$

The schematic diagram of apparatus used for SC-CO_2 extractions is shown in Fig. 1.

Experimental Design and Data Analysis

OAD was used to arrange the experiments and to obtain the optimization of the extraction process for seed oil from *Microula sikkimensis*. The effects of extraction pressure, CO_2 flow rate, extraction temperature and time on the extraction yield were investigated by a four-factor, three-level OAD with an OA_9 (3^4) matrix without considering the interactions between the parameters. Factors and levels tested are reported in Table 1. The experiments of single

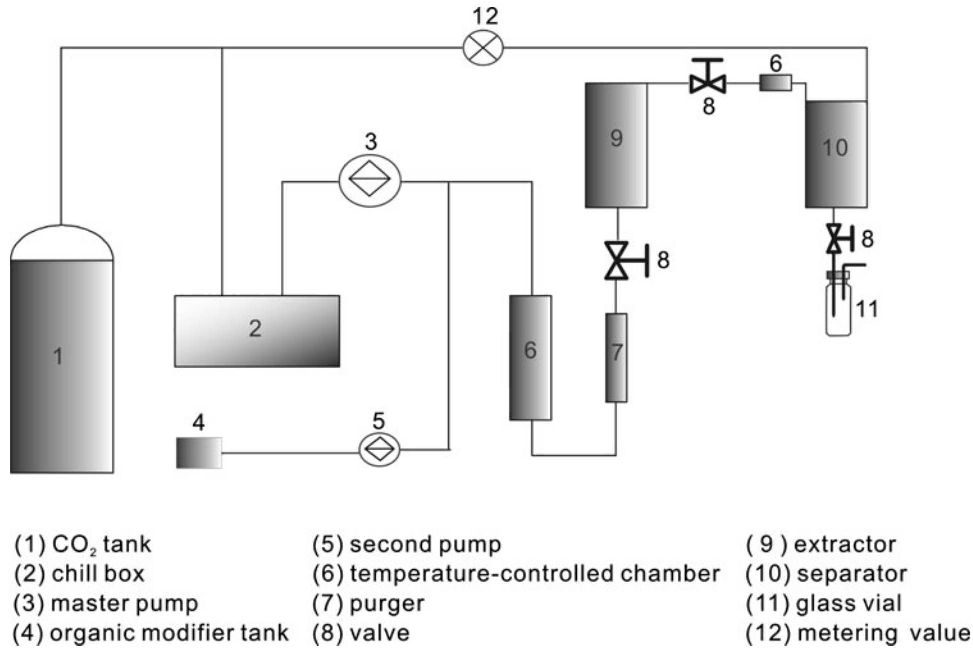

Fig. 1 Schematic diagram of apparatus for SC-CO_2 extraction of *Microula sikkimensis* seed oil

Table 1 The factors and levels of the orthogonal array design

Level	Factors			
	P (MPa)	*t* (h)	*T* (°C)	*F* (L/h)
1	21	2	35	17
2	24	3	45	20
3	27	4	55	23

parameter were repeated in triplicate and the extraction yields were average values. The seed oil was also extracted using Soxhlet extraction from *Microula sikkimensis.* ANOVA were performed by using SPSS software package (version 16.0) to identify significant differences between samples.

Soxhlet extraction was performed at temperature (30–60 °C) using petroleum ether as the solvent for 8 h [2].

Gas Chromatography Analysis

Fatty acid methyl esters (FAME) were prepared and analyzed using a fatty acid methyl method (Xu, Gao, Liu, Wang, & Zhao, 2008). The analysis of the FAME was performed on an Agilent 6890 GC equipped with a fused silica capillary column (30 m × 0.25 mm i.d., 0.32 µm film thickness, Agilent Technologies, Folsom, CA, USA) coated with polyethylglycol (PEG). The sample (1 µl) was injected with a split ratio of 100:1 and the inlet temperature was set at 280 °C. The detector temperature was set at 300 °C. The initial oven temperature was 170 °C. This temperature was maintained for 14 min and then increased at a rate of 10 °C/min to 250 °C, which was held for 8 min. Nitrogen was used as the carrier gas at a flow rate of 1.0 ml/min. The FAME peaks were identified using FAME standards. Each sample was analyzed three times. The composition of the fatty acids was calculated from their peak areas.

Results and Discussion

Fatty Acid Composition of Seed Oil

The seed oil of *Microula sikkimensis* is extremely rich in γ-linolenic acid at a level twice as high as that of *Oenothera biennis* [9–11]. The fatty acid composition of seed oils extracted by SFE and by Soxhlet extraction were determined by GC. The yield of the Soxhlet extraction was 40.0%. However, SFE only took 37.5% of the time. As shown in Table 2, levels of both γ-linolenic acid and α-linolenic acid from SFE were higher than that from Soxhlet extraction. In the present study, the results showed that SFE provides a safer, more efficient process as substitute for conventional extraction processes using liquid solvents like Soxhlet extraction and possibly *Microula sikkimensis* can be a substitute for *Oenothera biennis* as a source of γ-linolenic acid.

The Effect of Factors on Extraction Yield

Effect of Pressure

SFE was performed at three different pressures (21.0, 24.0, and 27.0 MPa) in order to evaluate the effect of pressure on the yield of seed oil. This showed that the yield of seed oil is influenced remarkably, with a mean yield from 27.6% at

Table 2 Fatty acid composition of *Microula sikkimensis* seed oil

Chemical formula	Fatty acid composition	Content (%) ($n = 3$)	
		Soxhlet extraction	SFE
$C_{16}H_{32}O_2$	Palmitic acid (hexadecanoic acid)	7.618	7.168
$C_{18}H_{36}O_2$	Stearic acid (octadecanoic acid)	2.636	1.776*
$C_{18}H_{34}O_2$	Oleic acid (*cis*-9-octadecenoic)	35.372	33.891*
$C_{18}H_{32}O_2$	Linoleic acid (*cis*,*cis*-9,12-octadecadienoic)	20.53	20.691
$C_{18}H_{30}O_2$	γ-linolenic acid (*cis*,*cis*,*cis*-6,9,12-octadecatrienoic)	6.94	7.412*
$C_{18}H_{30}O_2$	α- linolenic acid (*cis*,*cis*,*cis*-9,12,15-octadecatrienoic)	14.06	18.018*
$C_{18}H_{28}O_2$	Stearidonic acid (*cis*,*cis*,*cis*,*cis*-6,9,12,15-octadecatetraenoic)	5.364	5.133
$C_{20}H_{38}O_2$	Gondoic acid (*cis*-11- eicosenoic acid)	5.403	5.249
–	Unknown	2.077	0.662*

*Significant difference at $p = 0.05$ level under different treatments

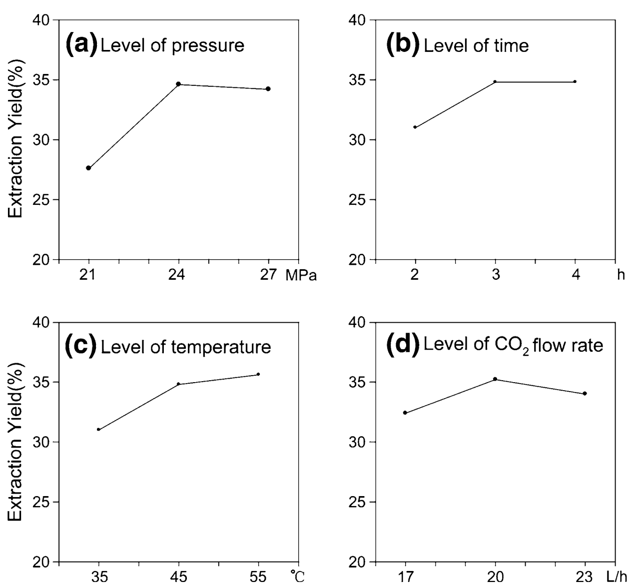

Fig. 2 The effect of each parameter on the extraction yield of *Microula sikkimensis* seed oil. **a** Pressure; **b** Time; **c** Temperature; **d** CO_2 flow rate

Table 3 Orthogonal array design matrix OA_9 (3^4) and experimental results

Trial no.	A	B	C	D	Yield ($n = 3$)
1	21	2	35	17	0.192
2	21	3	45	20	0.284
3	21	4	55	23	0.264
4	24	3	35	23	0.356
5	24	4	45	17	0.372
6	24	2	55	20	0.316
7	27	4	35	20	0.308
8	27	2	45	23	0.33
9	27	3	55	17	0.338
K_1^a	0.247	0.279	0.285	0.301	
K_2	0.348	0.331	0.329	0.303	
K_3	0.325	0.315	0.306	0.317	
R	0.101	0.052	0.043	0.016	

[a] $K_i^A = \sum(\text{Yield at Ai})/3$, the mean values of yields for a certain factor at each level with standard deviation

21.0 MPa to 34.6% at 24.0 MPa. The difference is about 7%. It was observed that the effect of pressure on the yield of seed oil was higher than the other factors. The peak of the yield appeared at 24.0 MPa. Increasing pressure could increase the solubility of seed oil in the solvent [22]. However, increasing pressure did not improve the yield of seed oil between 24.0 and 27.0 MPa (Fig. 2a). ANOVA shows that the best extraction pressure was 24.0 MPa in the experiment regarding the single parameter of pressure.

Effect of Extraction Time

The yield from the extraction can be influenced by the extraction time. Shorter extraction times could cause incomplete extraction and longer extraction times could waste time and energy [23]. In this experiment, the effect of extraction time on the extraction of seed oil is shown in Fig. 2b. As can be seen, with an extraction time of 3 h, the extraction yield has risen to about 34.8%. However, a further increase in the extraction time improves the extraction efficiency only slightly. The increase in yield is about 3.8% between 2 and 3 h, but there was no variation between 3 and 4 h. Obviously, an extraction time of 3 h can extract most of seed oil. ANOVA shows that extraction time is not a significant factor for the extraction yield when the extraction time was between 3 and 4 h.

Effect of Temperature

Increasing temperature decreased the density of solubility to diminish the solubility of object in the SFC and

Table 4 ANOVA analysis of four parameters for SFE of *Microula sikkimensis* seed oil

Source	Sum of Squares	*df*	Mean Square	*F*	*p* value	Type of effect
Corrected Model	0.023[a]	6	0.004	17.064	0.056	
A (pressure)	0.017	2	0.008	37.216	0.026*	Significant
B (time)	0.003	2	0.001	6.181	0.139	
C (temperature)	0.004	2	0.002	7.795	0.114	
D (CO_2 flow rate)	–	2	–	–	–	

[a] $R^2 = 0.981$ (adjusted $R^2 = 0.923$)

–, not applicable

*Significant difference at the $p = 0.05$ level with different treatments

increased diffusivity of object in the SFC [24]. Increasing temperature was studied in the present study. The temperature range was chosen from 45 to 55 °C. Figure 2c shows the effect of temperature (35, 45 and 55 °C) on the yield of seed oil. The extraction yield increased with increasing temperature. The change of temperature enhanced the mean yield from about 34.8% at 45 °C to 34.9% at 55 °C and therefore the increase was very small. Obviously, an extraction temperature 45 °C can extract most of the seed oil and that the yield is increased only marginally by increasing the temperature further. ANOVA shows that temperature is not a significant factor for the extraction yield when the temperature is between 45 and 55 °C.

Effect of CO_2 Flow Rate

The effect of the CO_2 flow rate was investigated in this study. The CO_2 flow rate had an effect on seed oil yields from 0.324 at 17.0 L/h to 0.352 at 20.0 L/h. The matrix appeared at 20.0 L/h ($p < 0.05$), and then the yield was decreased by increasing CO_2 flow rate. An excessively high CO_2 flow rate prevented the sample from thoroughly mixing in the SFC [25]. ANOVA analysis showed that the CO_2 flow rate was not a significant factor for the extraction yield when the CO_2 flow rate was between 20.0 and 23.0 L/h (Fig. 2d).

The Optimal Extraction Conditions

The experiment revealed that each of the four factors exerted an influence on the yield of seed oil in the selected ranges, among which pressure was identified as a significant factor based on ANOVA with a confidence of 95%. Optimum values of these factors for extraction of seed oil from *Microula sikkimensis* under the experimental conditions are $A_2B_2C_2D_2$: pressure 24.0 MPa, extraction time 3 h, temperature 45 °C and CO_2 flow rate 20 L/h (Table 3). In order to prove that the extraction conditions on the yield obtained were optimal, ANOVA analysis of the four parameters was carried out, see Table 4.

Conclusions

The effects of the four factors, namely CO_2 flow rate, extraction time, temperature and pressure on the yield were observed at pressures from 21.0 to 27.0 MPa, at temperatures from 35 to 55 °C, at extraction times from 2 to 4 h and at CO_2 flow rates from 17.0 to 23.0 L/h. The results showed that the extraction pressure had a significant ($p < 0.05$) effect on the yield of seed oil during supercritical extraction, however, the other three factors seem to have little effect ($p > 0.05$) on the result. The mean yield, based on the calculation of the OAD, varied at range from 19.2 to 35.6%. The optimum values of the factors inside the experimental domain considered were: pressure 24.0 MPa, extraction time 3 h, temperature 45 °C and a CO_2 flow rate 20 L/h.

Acknowledgments The authors wish to express their thanks for the financial support from the Fund of the Chinese Academy of Science and Technology of Qinghai.

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