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Supercritical fluid extraction of grape seed oil and subsequent separation of free fatty acids by high-speed counter-current chromatography

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Abstract

Supercritical fluid extraction (SFE) of grape seed oil was performed to study the effect of various parameters such as pressure, temperature and the particle size of the sample on the yield and composition of oil using an analytical-scale SFE system. Then the extraction was scaled up by 125 times using a preparative SFE system under the optimized conditions of high pressure (30–40 MPa) and low temperature (35–40 °C) with medium particle size (20–40 mesh). The maximum yield of the oil can reach 6.2% with pure supercritical CO_2 and 4.0% more oil can be obtained by adding 10% of ethanol as modifier. The unsaturated fatty acids (UFSs) make up about 70% in the oil on the basis of free fatty acids. The grape seed oil was then subjected to separation and purification for free fatty acids after saponification by high-speed counter-current chromatography coupled with evaporative light scattering detection (ELSD). The separation of 1.0 g of oil can yield about 430 mg pure linoleic acid at 99% purity. The fatty acids were analyzed by HPLC–ELSD.

Keywords: Supercritical fluid extraction; Counter-current chromatography; Preparative chromatography; Oils; Grapes; Fatty acids

1. Introduction

Grape seeds contain about 14–17% of oil. The main interest in grape seed oil lies in its high contents of unsaturated fatty acids (UFAs) such as linoleic acid (72–76%, w/w), which exceeds those in safflower oil (70–72%), sunflower oil (60–62%), and corn oil (about 52%) [1]. In addition, grape seed oil contains a large amount of tannins, i.e. the oligomeric proanthocyanosides (OPCs), at levels 1000-fold higher than in other seed oils [2]. This makes grape seed oil more resistant to peroxidation. Studies have shown that grape seed oil exhibits many pharmaceutical activities, such as properties against the oxidation of low-density lipoproteins, prevention of thrombosis, inhabitation of cardiovascular diseases, reduction of cholesterol in serum, dilation of blood vessel, and regulation of autonomic nerve. Grape seed oil has been served as high-quality nutritional oil for infants and elderly people or healthy oil for aircrews in the world [3].

Meanwhile, grape seed oil can also be used for the production of conjugated linoleic acid (CLA), which is a mixture of positional and configurational isomers of $C_{18:2}$ fatty acid. Many studies have reported that synthetic CLA is an effective agent for inhibiting mammary, colon, forestomach, and skin carcinogenesis in experimental models, due to its modulation of lymphocyte and macrophage activities. Recent clinical and in vivo experimental data disclosed novel biological effects of CLA, e.g. the anti-atherogenic and anti-hyperinsulinemic activities. Therefore, CLA may be effectively used as a nutritional supplement in combination with the food antioxidant [1].

Grape seed oil consists mainly of triglycerides, triacylglycerols of fatty acid. Supercritical CO_2 is a promising solvent for extraction and fractionation of edible oils containing labile UFAs, since the extraction can be carried out at low temperature. Besides, the supercritical fluid extrac-

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tion (SFE) offers many other favorable features over the traditional techniques, like steam distillation and solvent extraction, due to the fact that it uses a clean, inexpensive, non-flammable and non-toxic solvent.

Several reports have studied the SFE of edible oil [4,5], essential oils and related products from different raw materials, as reviewed by Reverchon [6]. However, few studies have been focused on the extraction of oil from grape seeds [7,8]. In this work, the effects of pressure, temperature and particle size on the yield and composition of grape seed oil were investigated using an analytical scale (10 ml) SFE system. Then, the process was scaled up on a preparative scale (2 l) system. Subsequently, the separation and purification of free fatty acids from the grape seed oil was performed by high-speed counter-current chromatography (HSCCC) coupled with evaporative light scattering detection (ELSD). HPLC–ELSD was used for analysis of fatty acids.

Fatty acids in oil samples are usually analyzed as fatty acid methyl esters by GC–flame ionization detection (FID) after saponification and methylation. Here the analyses of fatty acids were performed using an HPLC system coupled with ELCD directly after saponification. We feel that latter is simpler as further methylation is not necessary. In addition, the sensitivity of ELSD is high enough for the detection of most of the free fatty acids from grape seeds oil. The methods established for the extraction, analytical and preparative separations of grape seed oil in this paper could be served as a reference for oil samples from other sources.

2. Experimental

2.1. Reagents

Carbon dioxide (CO_2, 99.95% purity) was obtained from Beijing Analytical Instrument Factory. All solvents and other chemicals including ethanol, methanol, diethyl ether, sulfuric acid, potassium hydroxide, anhydrous sodium sulfate, hexane, heptane, light petroleum (bp 60–90 °C), ethyl acetate, acetonitrile, and acetic acid were of analytical grade, while methanol used for HPLC was of HPLC grade, all of them being purchased from Beijing Chemical Factory (Beijing, China).

Standards of linoleic acid (99%), oleic acid (92%), palmitic acid (99.5%), and stearic acid (99%) were from Chem Service (West Chester, USA), while γ-linolenic acid (99%) was from Sigma (St. Louis, MO).

Grape seeds were bought locally, and were dry and clean without any skin.

2.2. Optimization of SFE conditions

An analytical-scale SFE system used for optimizing extraction conditions was designed and fabricated in our laboratory. The volume of extraction cell was 10 ml. As listed in Table 1, an orthogonal test $L_9(3)^3$ was designed where three processing parameters including particle size of ground grape seeds, temperature, and pressure of extraction were considered to be the major factors for effective extraction. In each test, 4 g of milled and sieved grape seed sample were placed into the extraction cell. CO_2 with purity of 99.95% was used as a solvent without modifier. After 1 h of static extraction (no liquid flow), the sample was subjected to dynamic extraction for 1 h by flowing liquid CO_2 at a rate of 0.4 ml/min. The extract was trapped into a collection vessel containing about 15 ml of ethanol, and then analyzed after concentration.

Table 1
$L_9(3)^3$ orthogonal test design

Test no.	A		B		C	
		Particle size (mesh)		Temperature (°C)		Pressure (MPa)
1	A_1	10–20	B_1	35	C_1	20
2	A_1	10–20	B_2	40	C_2	25
3	A_1	10–20	B_3	45	C_3	30
4	A_2	20–40	B_1	35	C_2	25
5	A_2	20–40	B_2	40	C_3	30
6	A_2	20–40	B_3	45	C_1	20
7	A_3	40–60	B_1	35	C_3	30
8	A_3	40–60	B_2	40	C_1	20
9	A_3	40–60	B_3	45	C_2	25

2.3. Scaling-up SFE

Under the optimized SFE conditions determined above, the extraction was scaled up by about 125-fold using the same preparative-scale SFE system as used in our previous study [9]. A 500 g amount of grape seed sample was placed into an extraction vessel with a 2-l capacity, and extracted statically for 0.5 h and dynamically for 3 h. The flow-rate of CO_2 was set at 4 l/h and the extract in supercritical fluid was depressed directly into two separate vessels. The extract from grape seed was light-yellow oil. After saponification, the oil sample was subjected to HSCCC separation for free fatty acids.

2.4. HSCCC separation procedure

The present studies employed two different HSCCC units, i.e. a Model GS20 analytical HSCCC system and a Model GS10A2 preparative HSCCC system both manufactured by Beijing Institute of New Technology Application, Beijing, China. For the analytical model, the multilayer coil separation column was prepared by winding 0.8 mm i.d. PTFE tubing coaxially onto a spool-shaped column holder where the β value ranged from 0.4 to 0.72. The total capacity was 35 ml. For the preparative model, the multilayer coil was prepared by winding 1.6 mm i.d. PTFE tubing coaxially onto a spool-shaped column holder. The β value ranged from 0.5 to 0.75, and the total capacity was 230 ml. The detection of

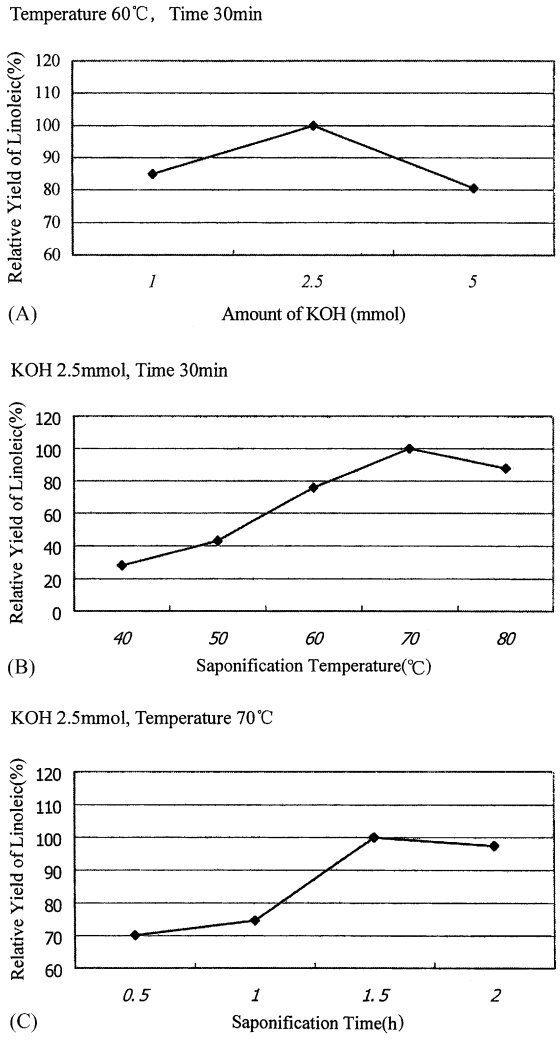

Fig. 1. Impacts of different saponification conditions on the recovery of linoleic acid from grape seeds by SCF separation.

fatty acids was achieved by connecting the tail outlet of the coiled column to a model 75 evaporative light scattering detector (Sedere, France) through a split valve. An on-line filter was used before the ELSD system to eliminate particulates.

A two-phase solvent system composed of heptane–acetonitrile–acetic acid–methanol was used for the separation of free fatty acids extracted from grape seeds.

The solvent system was thoroughly equilibrated in a separatory funnel and two phases separated shortly before use. In each separation, the coiled column was first entirely filled with the upper stationary phase, and then the lower mobile phase was pumped into the column at a flow-rate of 1 ml/min under 1800 rpm of column rotation for analytical HSCCC and at 2 ml/min under 800 rpm for preparative HSCCC. After the mobile phase front emerged and hydrodynamic equilibrium was established, the outlet of the coil was connected to the ELSD system. Then the sample solution (sample dissolved in the mobile phase) was injected through the sample loop. Peak fractions were collected according to the recorded elution profile.

2.5. Saponification and HPLC analysis

Fatty acids in the grape seed oil were analyzed using a Shimadzu LC-10A system after saponification. Experimental conditions are as follows: column, Phenomenex Luna C_{18} (150 mm × 4.6 mm i.d.); temperature, 35 °C; mobile phase, methanol with 1% HAc–water with 1% HAc (95:5, v/v); flow-rate, 1 ml/min; detector, ELSD.

Meanwhile the influence of the saponification processing parameters including the concentration of KOH, temperature and reaction time on the yield of free fatty acids was tested. In each test, 100 mg of oil was sampled and saponified with KOH in methanol, and then the solution was acidified with sulfuric acid–water (1:4, v/v) and extracted with diethyl ether. After concentration, the extract was analyzed for fatty acids by HPLC–ELSD.

3. Results and discussion

3.1. Optimization of saponification conditions

Fatty acids exist mainly in the form of triglycerides in the grape seed oil. Because of the lack of UV absorption,

Table 2
$L_9(3)^3$ test results

Test no.	A	B	C	Oil yield (%)[a]	UFAs (%)[b]			SFAs (%)[b]		
	1	2	3		Linoleic acid	Oleic acid	Total	Paltimic acid	Stearic acid	Total
1	A_1	B_1	C_1	1.9	54.8	12.7	67.5	4.9	2.3	7.2
2	A_1	B_2	C_2	2.2	51.9	11.0	62.9	3.7	1.5	5.2
3	A_1	B_3	C_3	3.8	57.2	12.3	69.5	3.8	2.2	6.0
4	A_2	B_1	C_2	3.5	56.6	11.2	67.8	3.9	2.2	6.1
5	A_2	B_2	C_3	5.1	63.2	14.2	77.4	4.1	2.1	6.2
6	A_2	B_3	C_1	1.7	48.0	9.6	57.6	3.6	2.0	5.6
7	A_3	B_1	C_3	4.2	60.4	11.6	72.0	3.8	1.5	5.3
8	A_3	B_2	C_1	2.1	49.6	10.4	60.0	3.8	1.3	5.1
9	A_3	B_3	C_2	3.7	48.6	9.4	58.0	3.4	1.4	4.8

[a] The amount of oil/sample mass.
[b] The amount of UFAs and SFAs in oil on the basis of free fatty acids.

Table 3
Analysis of $L_9(3)^3$ test results

	Oil yield (%)			Total UFAs (%)			Total SFAs (%)		
	A	B	C	A	B	C	A	B	C
K_1	7.9[a]	9.6	5.7	199.9	207.3	185.1	18.4	18.6	17.9
K_2	10.3	9.4	9.4	202.8	200.3	188.7	17.9	16.5	16.1
K_3	10.0	9.2	13.1	190.0	185.1	218.9	15.2	16.4	17.5
k_1	2.6[b]	3.2	1.9	66.6	69.1	61.7	6.1	6.2	6.0
k_2	3.4	3.1	3.1	67.6	66.8	62.9	6.0	5.5	5.4
k_3	3.3	3.1	4.4	63.3	61.7	73.0	5.1	5.5	5.8
R	0.8[c]	0.1	2.5	4.3	7.4	11.3	1.0	0.7	0.6
Optimal level	A_2	B_1	C_3	A_2	B_1	C_3	A_1	B_1	C_1

[a] $K_i^A = \sum$ oil yield at A_i.
[b] $k_i^A = K_i^A/3$.
[c] $R^A = \max\{k_i^A\} - \min\{k_i^A\}$.

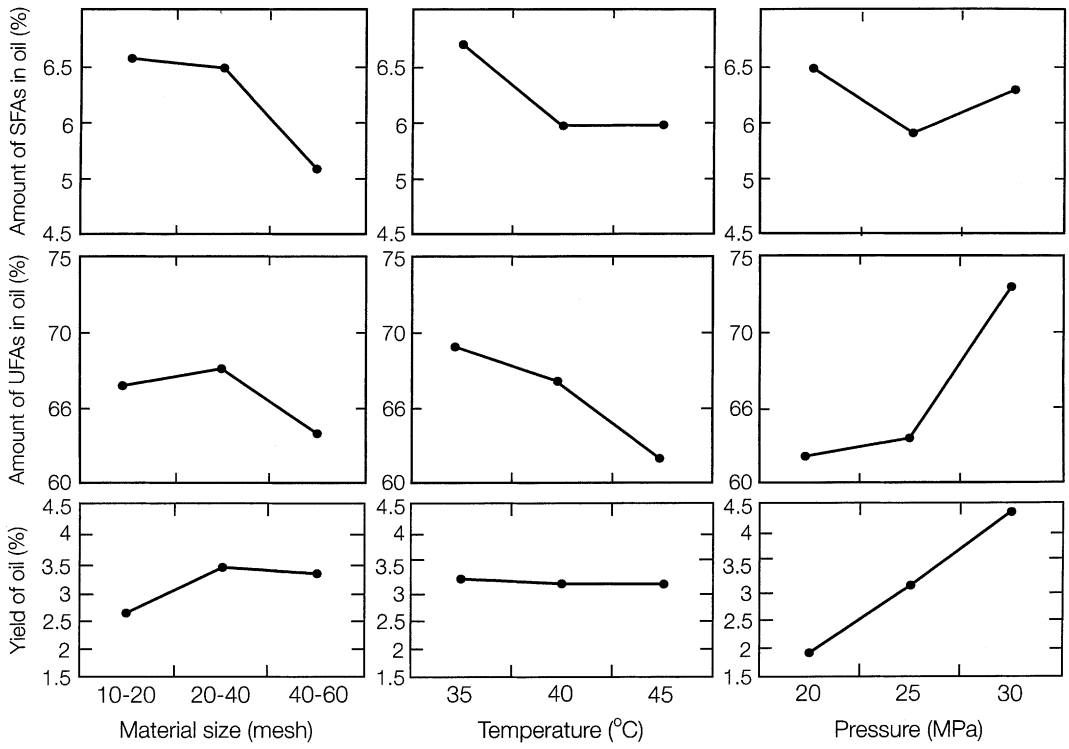

Fig. 2. Effects of temperature, pressure, and particle size on the yield and composition of grape seed oil by SCF separation.

fatty acids are usually analyzed by GC–FID after saponification followed by methylation. Here the analyses of fatty acids were performed using an HPLC system coupled with ELSD directly after saponification. Fig. 1 shows the impacts of different saponification conditions on the recovery of linoleic acid. Based on the results of the above studies, the optimum saponification condition was determined for an aliquot of 100 mg oil sample as 2.5 mmol KOH at 70 °C for 1.5 h, which was used in the following experiments.

Table 4
Results of scale-up SFE test

Test no.	Pressure (MPa)	Temperature (°C)	Oil yield (%)	UFAs (%)			SFAs (%)		
				Linoleic acid	Oleic acid	Total	Paltimic acid	Stearic acid	Total
1	30	35	2.5	57.7	14.2	71.9	3.7	1.3	5.0
2	30	40	3.1	58.6	14.3	72.9	3.7	3.9	7.6
3	40	40	6.2	55.9	12.9	68.8	2.8	1.4	4.2

Table 5
Comparison of extraction efficiency between SFE and solvent extraction

Extraction	Oil yield (%)	UFAs (%)			SFAs (%)		
		Linoleic acid	Oleic acid	Total	Paltimic acid	Stearic acid	Total
SFE	6.2	55.9	12.9	68.8	2.8	1.4	4.2
Solvent	10.6	57.1	13.8	69.9	3.1	1.4	4.5

Table 6
Comparison of extraction efficiency by supercritical CO_2 and supercritical CO_2 with modifier

Extraction	Oil yield (%)	UFAs (%)			SFAs (%)		
		Linoleic acid	Oleic acid	Total	Palmitic acid	Stearic acid	Total
Supercritical CO_2	6.2	55.9	12.9	68.8	2.8	1.4	4.2
Supercritical CO_2 + ethanol[a]	4.0	57.0	12.8	69.8	3.0	1.5	4.5

[a] The sample after extraction with pure supercritical CO_2 was subjected to further extraction with supercritical CO_2 modified with 10% of ethanol.

3.2. Optimization of particle size, temperature and pressure for maximizing SFE efficiency

The grape seed oil obtained from each test in analytical SFE was quantitatively analyzed for the yield of oil, and the amount of each UFA and saturated fatty acid (SFA) by the HPLC method as mentioned above. Results of the $L_9(3)^3$ tests presented in Table 2 revealed that the maximum yield of oil was about 5%, and in the oil, the total amount of UFAs including linoleic and oleic acids accounted for

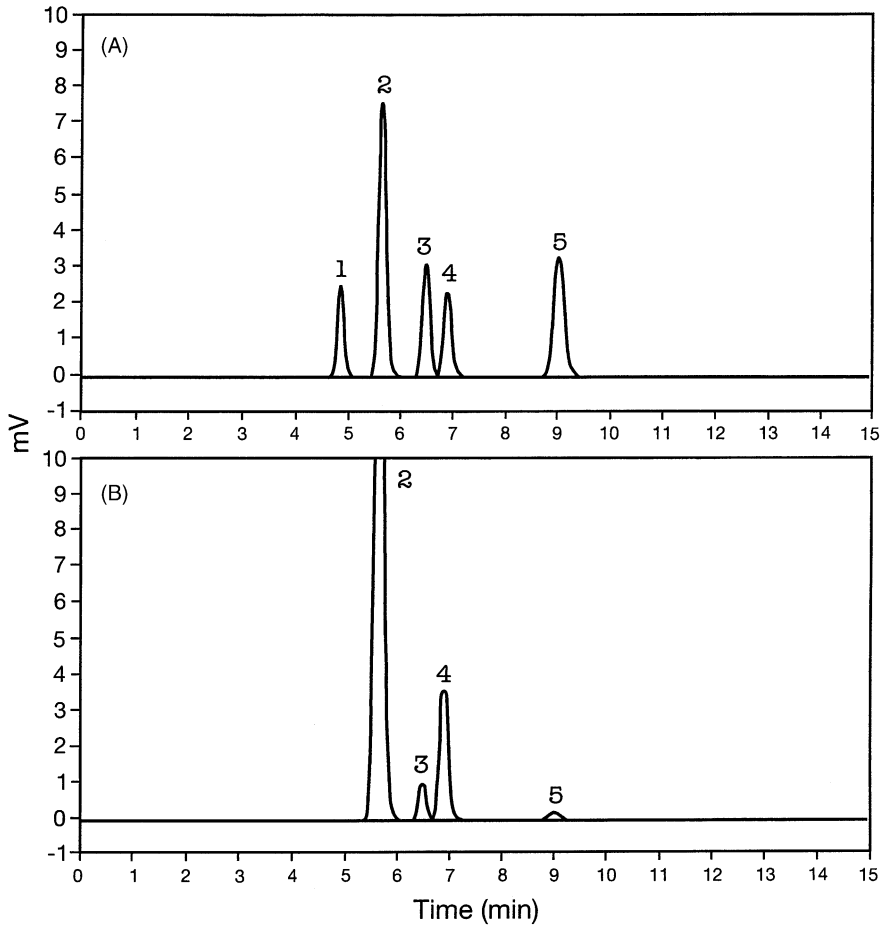

Fig. 3. HPLC chromatograms of free fatty acids in (A) a mixture standards and (B) a sample of grape seed oil. Sample: (A) 1 = γ-linolenic acid (4.12 μg), 2 = linoleic acid (9.95 μg), 3 = palmitic acid (5.78 μg), 4 = oleic acid (4.66 μg), 5 = stearic acid (10.83 μg); (B) 100 mg oil sample, diluted with 50 ml methanol after saponification and concentration, injection volume: 10 μl. The other experimental conditions are as follows: instrument: Shimadzu LC-10A; column: Phenomenex Luna C_{18} (150 mm × 4.6 mm i.d.); temperature: 35 °C; mobile phase: methanol with 1% HAc–water with 1% HAc (95:5, v/v); flow-rate: 1 ml/min; detection: ELSD. (Note: This chromatogram is for demonstration, and a smaller amount of sample was injected for quantitative determination.)

58–77%, while that of SFAs including palmitic and stearic acids accounted for 5–7% on the basis of free fatty acids.

The results in Table 2 were transformed to Table 3 after orthogonal analysis. Pressure was found to be the most important factor, where higher pressure increases significantly the yield of oil and the amount of UFAs in the oil as illustrated in Fig. 2. Although temperature did not influence the yield of oil, higher temperature seems unfavorable for the extraction of UFAs. As the particle size was reduced, the yield of the oil increased, reaching the maximum at 20–40 mesh, and then decreased. This can be attributed to the fact that the oil release was enhanced as the particle size decreases until the release way was blocked by fine particles. Finally, the optimum conditions of 30 MPa, 35 °C, and 20–40 mesh particle size were used for SFE of grape seed oil.

3.3. Preparative-scale SFE

Based on the above SFE condition, the extraction was scaled up by about 125-fold under three different pressure and temperature conditions described in Table 4. The results indicated again that higher pressure is beneficial to the yield of oil. When the pressure was increased from 30 to 40 MPa, the yield of oil was increased more than twofold reaching the maximum of 6.2%. Under the higher pressure, the influence of temperature to the yield of oil was rather trivial in the range of 35–40 °C. And generally, under the high pressure and relatively low temperature, their influence on the composition of oil were also very small for the total amount of UFAs accounted for about 70%.

3.4. Comparison of SFE with traditional solvent extraction

A 25 g amount of 20–40 mesh grape seed sample was soxhlet-extracted with 300 ml hexane for 6 h. The yield of oil by solvent extraction was found to be 10.6% (see Table 5). This indicates that the SFE efficiency is about 60% of that of solvent extraction, while the color and fatty acid composition are almost the same. This result is still acceptable as only pure CO_2 was used as supercritical fluid.

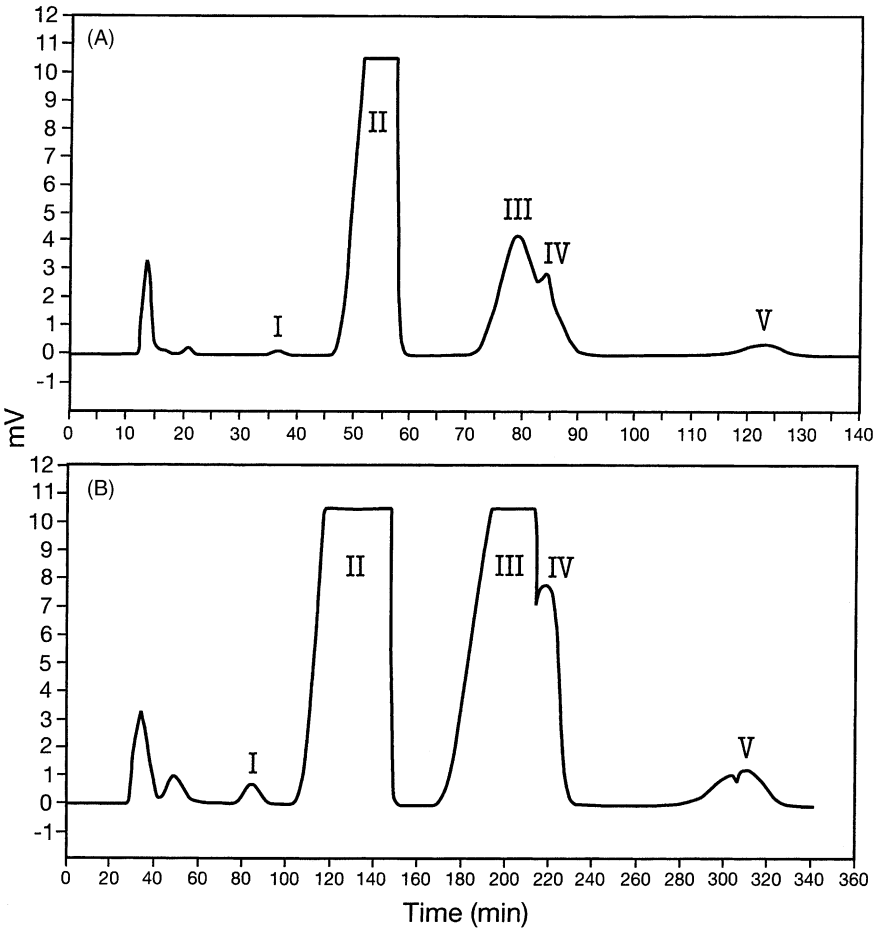

Fig. 4. Separation profiles of free fatty acids from grape seed oil by analytical and preparative HSCCC with ELSD. The experimental conditions are as follows: instrument: multilayer coil planet centrifuge, Model GS20 analytical unit (A) and GS10A2 preparative unit (B); column: 0.8 mm i.d. PTFE tubing with 35 ml capacity (A) and 1.6 mm i.d. PTFE tubing with 230 ml capacity; solvent system: heptane–acetonitrile–acetic acid–methanol (4:5:1:1, v/v); mobile phase: lower aqueous phase; flow-rate: 1 ml/min (A) and 2 ml/min (B); detection: ELSD.

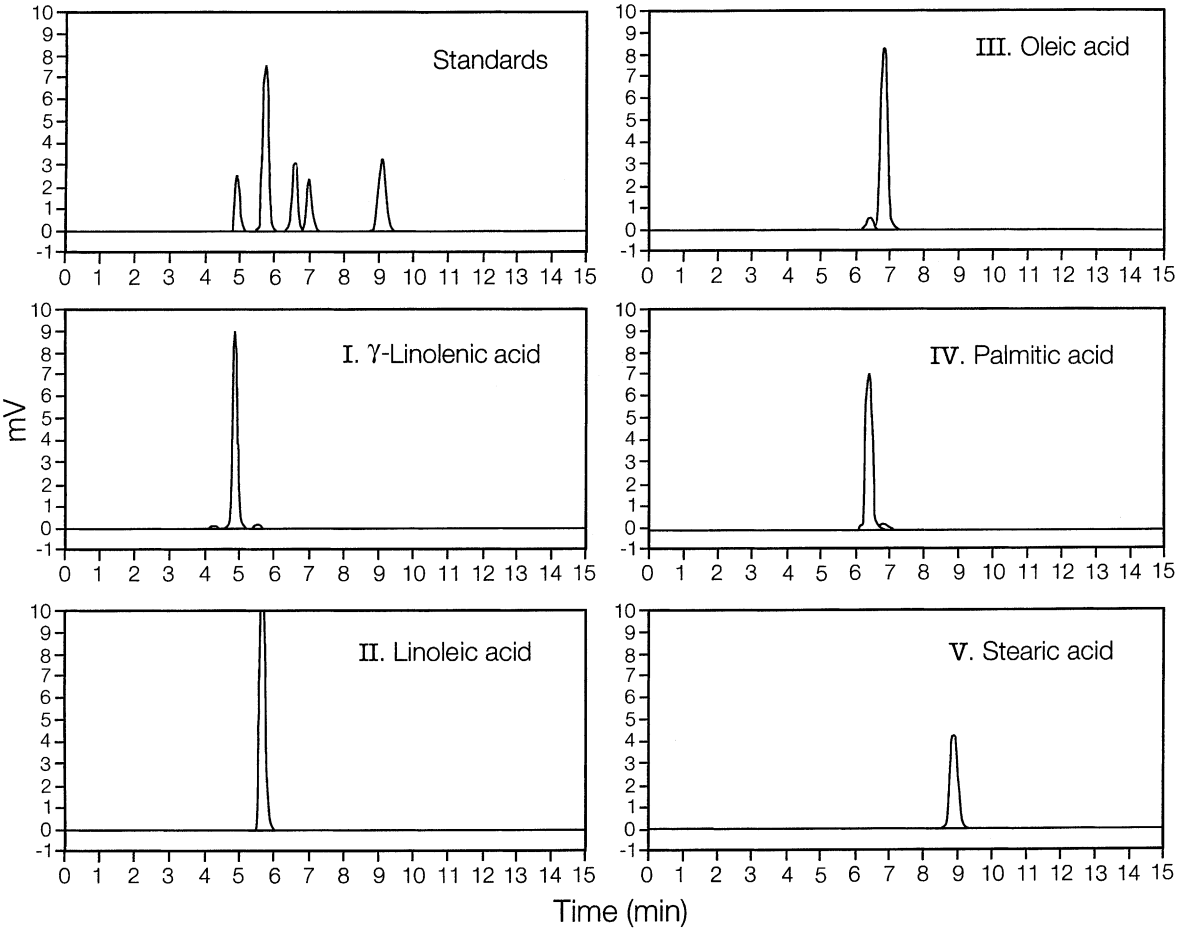

Fig. 5. HPLC analysis of individual fatty acids separated from grape seed oil by HSCCC. After separation by HSCCC, each peak fraction was collected and concentrated, and then a small amount of each fraction was dissolved in about 1 ml methanol and injected into HPLC in 5–20 μl to examine the purity. Otherwise the analytical conditions are same as those described in Fig. 3.

3.5. Effect of modifier to the yield of grape seed oil

After extraction by pure supercritical CO_2 for 3 h at 40 MPa and 40 °C, the sample was subjected to further extraction with supercritical CO_2 modified with 10% of ethanol for 2 h, and 4.0% more oil with almost the same composition was obtained (see Table 6). This made the yield of oil from SFE up to the same level of solvent extraction. But the color of oil became dark-green instead of light-yellow. This may be attributed to the extraction of OPCs from grape seeds. The purification of these compounds may become our future work in the study of bioactive compounds from the grape seeds.

3.6. HSCCC separation of free fatty acids

The fatty acids in grape seed oil exist mainly in the form of triglycerides. The separation for free fatty acids was carried out by HSCCC coupled with ELSD after saponification and extraction. Since fatty acids are a group of lipophilic compounds, several hydrophobic two-phase solvent systems including heptane–methanol, heptane–methanol–acetic acid, heptane–acetonitrile–acetic acid, heptane–acetonitrile–acetic acid–methanol were tested for the separation of fatty acids using analytical HSCCC. Among those, a two-phase solvent system composed of heptane–acetonitrile–acetic acid-methanol (4:5:1:1, v/v) was found to be the best choice and used for the separation of free fatty acids extracted from grape seed oil.

Fig. 3 shows HPLC chromatograms of a mixture of the fatty acid standards (A) and a sample of grape seed oil (B). HPLC analysis indicated that four fatty acids including linoleic acid, oleic acid, palmitic acid, and stearic acid were the main fatty acids in the grape seed oil, where linoleic acid were most abundant. Fig. 4 displays the separation profiles of free fatty acids from grape seed oil by analytical (A) and preparative (B) HSCCC. HPLC analysis revealed that five individual fatty acids including γ-linolenic acid (which was not detected from the mixture) were isolated (see Fig. 5). All the fatty acids are of 95–99% HPLC purity. The separation of 1.0 g of oil can yield about 430 mg pure linoleic acid at 99% purity.

4. Conclusion

The overall results indicated that the extraction of grape seed oil is successfully performed using supercritical CO_2 without a modifier. The yield of oil depends on the pressure and temperature applied during extraction as well as the particle size of the sample seeds. The extraction efficiency by supercritical CO_2 is about 60% of that obtained by the traditional solvent under the optimized SFE condition, which can be improved to the same level by adding ethanol at 10% although the color of oil becomes darker probably due to the contamination of proanthocyanidin. There is not much difference found in the composition of fatty acids between oils from SFE and solvent extraction as analyzed by HPLC coupled with ELSD. Individual fatty acids with purity of over 95% were obtained by HSCCC separation. The methods set up in this paper will be used for the analysis and separation of fatty acids from other sources.

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