Supercritical Fluid Extraction of Fungal Oil Using CO_2, N_2O, CHF_3 and SF_6

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The extraction of oil from fungi (*Mortierella ramanniana* var. *angulispora*) was studied using carbon dioxide (CO_2), nitrous oxide (N_2O), trifluoromethane (CHF_3) and sulfur hexafluoride (SF_6) under supercritical conditions. The oil solubility was highest in SC-N_2O followed by SC-CO_2, while both SC-CHF_3 and SC-SF_6 showed poorer solvent power. The recorded oil solubilities at 333 K and 24.5 MPa were 2.3 wt% in N_2O, 0.48 wt% in CO_2, 0.0099 wt% in CHF_3 and 0.0012 wt% in SF_6.

The oil solubilities in SC-N_2O and SC-CO_2 were measured over the pressure range 15.7–29.4 MPa and at temperatures ranging from 313–353 K. N_2O always showed greater solvent power than did CO_2 at the same temperature and pressure. The solvent power of a supercritical fluid increases with density at a given temperature, and increases with temperature at constant density.

The change in neutral lipid composition of the extracted oil with the extraction ratio was measured. Free fatty acids or diglycerides were extracted more easily than triglycerides or sterol esters. The change in fatty acid composition was also measured. The proportion of γ-linolenic acid in the extract remained constant throughout the extraction.

KEY WORDS: Carbon dioxide, extraction, fungal oil, γ-linolenic acid, neutral lipids, nitrous oxide, solubility, sulfur hexafluoride, supercritical fluids, trifluoromethane.

Polyunsaturated fatty acids such as γ-linolenic acid and eicosapentaenoic acid have been found to play an important role *in vivo* as precursors of prostaglandins or leukotriens (1,2). In recent years, attempts have been made to produce polyunsaturated fatty acids using microorganisms (3,4). This is because microorganisms (as compared with animals or plants) have many advantages, such as high growth rate and simple culture conditions. It was found that the fungi producing γ-linolenic acid could be cultivated at high cell density by using glucose as the main carbon source (5). This technique could be useful in the production of γ-linolenic acid since one of the only sources of this fatty acid is evening primrose seeds. Furthermore, this method made it possible to produce γ-linolenic acid in arbitrary quantities at any time.

Supercritical fluid extraction (SFE) has received much attention in various fields, such as the food industry and pharmacology, because supercritical fluids (SCFs) show higher selectivity to solutes and can be removed easily from the samples by reducing the pressure. Carbon dioxide (CO_2) is the fluid most commonly employed for SFE as solvent because of its low toxicity, safety and low cost. Solubility data of lipids in SC-CO_2 have been reported by many researchers (6–9); however, data for SCFs have only been reported rarely (10).

In this work, CO_2, nitrous oxide (N_2O), trifluoromethane (CHF_3) and sulfur hexafluoride (SF_6) were used for SFE of fungal lipids containing γ-linolenic acid, and the oil solubility in each fluid was measured. The effects of flow rate, temperature and pressure on the solubility were also studied. Further, the neutral lipid composition and the fatty acid composition of the extracted oil were measured and compared with those of the feed.

MATERIALS AND METHODS

Materials. Mortierella ramanniana var. *angulispora* (IFO 8187) was cultivated as previously reported (5). The fungi were cultivated with forced aeration in a 3 L jar fermenter. Initial glucose concentration was 200 g/L, and culture temperature was 303°K. The harvested cells were treated at 393°K for 10 min in an autoclave to deactivate the intracellular lipases causing the hydrolysis of lipids. The change in the fatty acid composition before and after the treatment was not observed. The lipids are stored in the cells, and the hard cell wall prevents the permeation of lipids. Thus, it is necessary to crush the cells in order to extract the produced lipids. A ball mill was utilized for this pretreatment. The cells were crushed for 3 hr in about two-fold ethanol, followed by filtration. Most of the polar lipids and some of the neutral lipids were extracted into ethanol by this treatment, while the major amount of neutral lipids remained in the cells. The cells were also dehydrated during the crushing. This may improve the extraction because water has been reported to inhibit the oil extraction from fungal cells (11). The crushed cells were used as the sample for SFE after residual ethanol was evaporated. The sample contained 6% water by weight. The neutral lipids content and polar lipids content in the sample were 41.0 and 1.2 wt%, respectively. The neutral lipids were mainly composed of free fatty acids, free sterols, diglycerides, triglycerides and sterol esters; the concentrations of these components were 1.1, 6.4, 6.2, 82.5 and 3.7 wt%, respectively. The fatty acid composition of the fungal lipid fraction was: myristic acid, 1.3 wt%; palmitic acid, 30.3 wt%; palmitoleic acid, 1.3 wt%; stearic acid, 5.5 wt%; oleic acid, 46.1 wt%; linoleic acid, 8.5 wt%; γ-linolenic acid, 6.3 wt%; and others, 0.7 wt%.

Extraction. A schematic diagram of the supercritical fluid extraction apparatus is shown in Figure 1. The equipment (Atsuryoku Kiki Engineering Co., Tokyo, Japan) had a cylindrical extraction cell with a volume of 120 ml. The pressure was set by the pressure regulating valve and the flow rate of the fluid was regulated by the needle valve. The total volume of consumed fluid was measured by the volumetric flow

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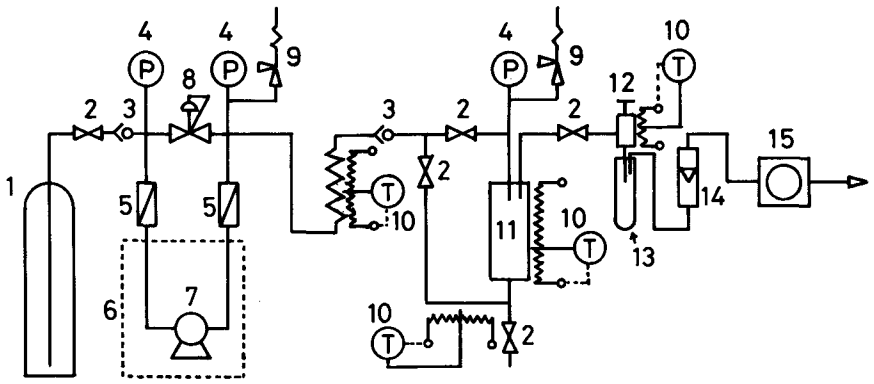

FIG. 1. Supercritical fluid extraction apparatus.

meter. Twenty or thirty grams of sample was weighed accurately and supplied into the extraction cell. The extraction cell was purged with the fluid at atmospheric pressure and left for 1 hr at the desired temperature and pressure without fluid effusion. Then the extraction was carried out with the needle valve open. The extracted lipids were reprecipitated due to pressure reduction at the outlet of the needle valve and collected into test tubes. When the extract accumulated to 0.5–1.5 g, the test tube was replaced by a new one. Each extracted oil was weighed, and the neutral lipid composition and fatty acid composition in it were measured.

Analysis. Neutral lipids were chromatographed on thin-layer chromatography (TLC) plates (20 × 20 cm; 0.25 mm coating) with the following solvent systems: benzene/diethyl ether/ethanol/28% ammonium hydroxide (50:40:2:0.5, v/v) (solvent 1) and hexane/diethyl ether (94:6) (solvent 2). At first the solvent front was allowed to run a distance of 12 cm from the origin in solvent 1, then the plates were developed in solvent 2 in the same direction until the solvent front ran within 1 cm of the top of the plate (12). Quantitative analysis was performed by a densitometric method. A small amount of each extracted lipid was taken up for determining the fatty acid composition. The sample was esterified using 0.5N NaOH/methanol and 7% BF_3/methanol according to the method of Metcalf (13), and quantitatively analyzed utilizing a gas-chromatograph equipped with an FID. A glass column (3 mm i.d. × 2 m) packed with 20% DEGS on chromosorb WAW was used.

RESULTS AND DISCUSSION

The extraction curves with SC-CO_2, SC-N_2O, SC-CHF_3 and SC-SF_6 are shown in Figure 2. The degree of extraction is plotted as ordinate and the consumed fluid

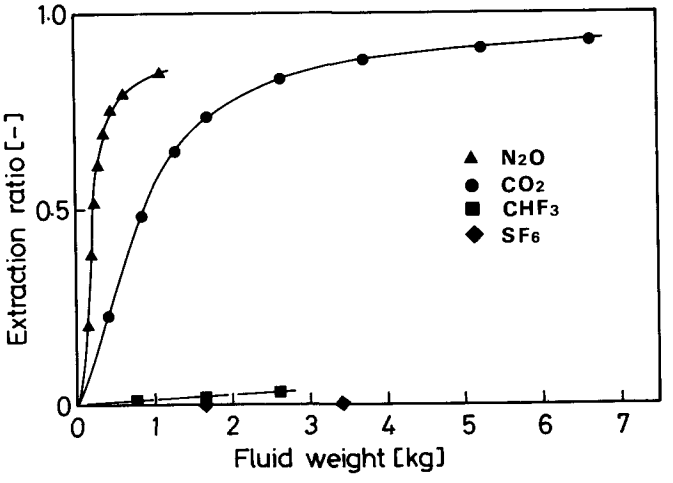

FIG. 2. Relationship between extraction ratio and weight of consumed fluids. Extraction conditions: temperature, 333°K; pressure, 24.5 MPa; sample weight, 20.0 g; and flow rate, 0.11 mol/min.

weight as abscissa. Each loaded sample weighed 20.0 g, and the extraction was performed at a temperature of 333°K and a pressure of 24.5 MPa. SC-N_2O showed the highest solvent power for the fungal oil, and SC-CHF_3 and SC-SF_6 showed much poorer solvent power than either SC-N_2O or SC-CO_2. The concentration of the oil in each fluid collected during the initial linear portion of the extraction curve corresponds to its solubility, provided that the fluid becomes saturated through the vessel. The oil solubilities in each SCF under the above conditions are presented in Table 1 with the critical parameters and characteristics of the fluids.

TABLE 1

Critical Conditions and Characteristics of CO_2, N_2O, CHF_3 and SF_6, and Oil Solubilities in Each Fluid

Fluid	T_c (K)	P_c (MPa)	Characteristic	Oil solubility[a] (wt%)
CO_2	304.2	7.37	nontoxic	0.48
N_2O	309.6	7.24	narcotic	2.3
CHF_3	299.1	4.83	stable	0.0099
SF_6	318.7	3.76	insulating	0.0012

[a]See Figure 2 for extraction conditions.

The oil solubility in SC-N_2O was five times greater than in SC-CO_2. The oil solubilities in SC-CHF_3 and SF_6 were three orders of magnitude smaller than in SC-N_2O. This wide range of solvent power is interesting considering that all four of these fluids are inorganic and come to supercritical conditions at similar temperatures.

In recent years the solubility parameter concept has been used often to explain the solvent power of SCFs (14,15). The oil solubilities in each fluid are plotted in Figure 3 vs the solubility parameter values calculated according to Allada's method (14). Figure 3 shows that a higher solubility parameter leads to higher oil solubility. Carbon dioxide and N_2O have similar physical properties such as molecular weight, critical temperature and critical pressure as shown in Table 1. The solubility parameter is calculated, taking into account only the physical properties and the state of the solvent, so CO_2 and N_2O have similar solubility parameter values at the same temperature and pressure. The difference in solvent power between CO_2 and N_2O is apparently due to the difference in affinity between the oil and each fluid. That is to say, N_2O shows a higher affinity for the fungal oil than does CO_2. It is concluded that N_2O is the most suitable fluid for SFE of the fungal oil.

The effect of flow rate on extraction efficiency was also studied. The flow rate of the solvent is an important operating condition. High flow rate sometimes results in low oil solubility. This is ascribed to the fluid not becoming saturated with the oil during its passage in the vessel. The effect of flow rate on SFE was studied. The amount of lipid dissolved per gram of CO_2 is plotted in Figure 4 vs the extraction ratio at the flow rates of 2.0, 5.0 and 8.4 g-CO_2/min. The extraction was performed at 333°K and 24.5 MPa. Extraction efficiency at the flow rate of 8.4 g-CO_2/min was lower than that at 2.0 or 5.0 g-CO_2/min. The flow rates of 2.0 and 5.0 g-CO_2/min showed very similar extraction efficiencies. A higher flow rate is preferable for practical use, since a higher flow rate leads to shorter extraction time for a fixed extraction efficiency.

Figure 4 also shows that the extraction efficiency decreased rapidly above an extraction ratio of 0.4. This is caused by the mass transfer resistance of fungi particles. Lipids in the layer close to the surface are easily extracted, while extraction is more difficult for the part closer to the center. Crushing samples into finer powder is an effective treatment for reducing the influence of the mass transfer resistance (7,16).

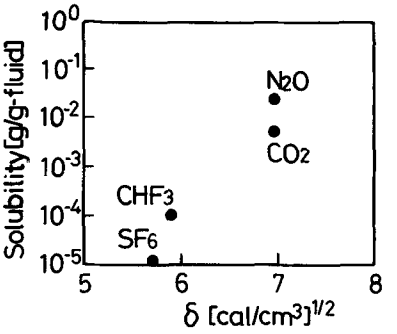

FIG. 3. Relationship between oil solubility and solubility parameters of CO_2, N_2O, CHF_3 and SF_6. See Figure 1 for extraction conditions.

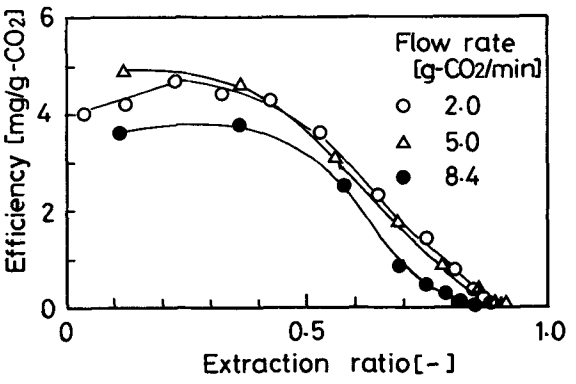

FIG. 4. Change in extraction efficiency with extraction ratio at the flow rates of 2.0, 5.0 and 8.4 g/min. Extraction conditions: fluid, CO_2; temperature, 333°K; pressure, 24.5 mPa; and sample weight, 20.0 g.

The effect of temperature and pressure on oil solubility in SC-CO_2 and SC-N_2O was also investigated. As shown in Figure 2, SC-N_2O and SC-CO_2 showed good solvent power for the fungal oil. The effect of temperature and pressure on oil solubility was investigated using these fluids as solvents. The solubilities at various pressures were measured at temperatures of 313, 333 and 353°K, and plotted in Figure 5. The data were obtained at a flow rate of about 5.0 g fluid/min. The solubility in Figure 5 was defined as the amount of oil dissolved per gram of CO_2 or N_2O in the range of low extraction ratio where the extraction efficiency was shown to be constant as described above. However, it should be noted that the solubility was not necessarily equal to the real solubility as measured under equilibrium conditions. At the same temperature and pressure, the solvent power of SC-N_2O was 5–12 times higher than that of SC-CO_2. The solubility increased with pressure in the pressure range below 29.4 MPa for both CO_2 and N_2O, with the curves gradually levelling off.

The same data were plotted in Figure 6 with temperature as the abscissa to show the effect of temperature on oil solubility. Figure 6 shows that the oil solubility decreased with temperature under isobaric con-

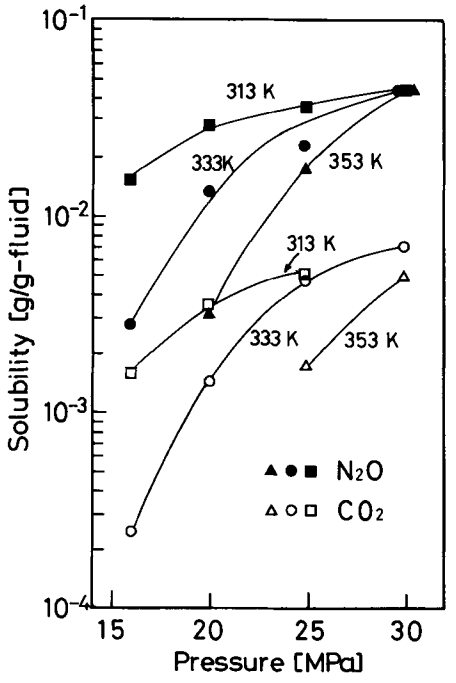

FIG. 5. Effect of pressure on oil solubility in supercritical CO_2 and N_2O at 313, 333 and 353°K.

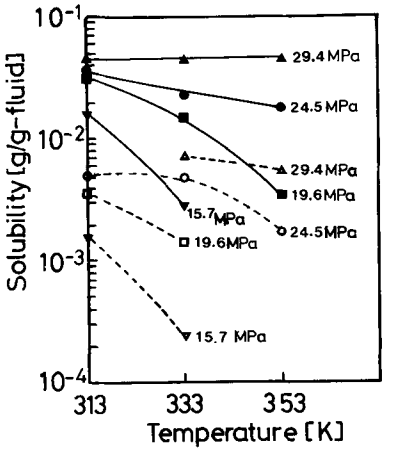

FIG. 6. Effect of temperature on oil solubility in supercritical CO_2 and N_2O at 15.7, 19.6, 24.5 and 29.4 MPa. The dashed curves are for CO_2 and the solid curves are for N_2O.

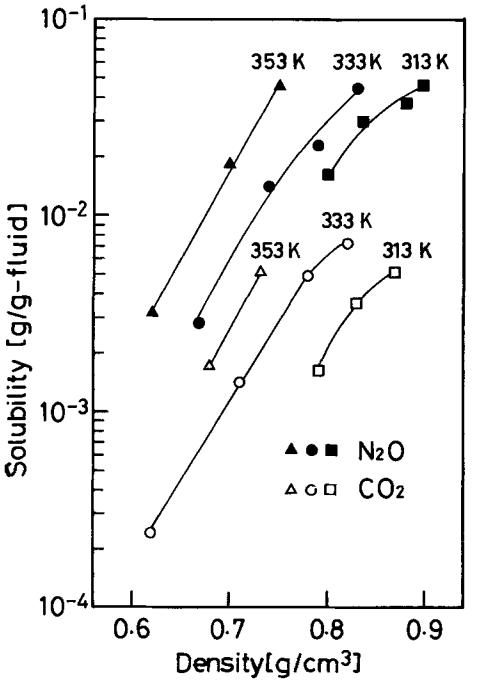

FIG. 7. Relationship between oil solubility and fluid density at 313, 333 and 353°K.

ditions. This tendency was more pronounced at lower pressures, because the fluid density changes with temperature more strongly at lower pressures. Figure 6 also shows that N_2O gave a constant value of oil solubility of about 4.5×10^{-2} g/g-fluid at a pressure of 29.4 MPa regardless of temperature. A few reports have described the phenomenon that the effect of temperature on the solubility of a substance in SCFs apparently disappears at a certain pressure (17,18). Such behavior is attributed to the reciprocal effect of temperature on the solubility. A rise in temperature leads to an increase in vapor pressure of the solute, so the concentration in the supercritical phase rises. On the other hand, a rise in temperature at constant pressure causes a decrease in fluid density, so the solvent power of the fluid decreases. At high pressures the former effect is dominant, while at moderate pressures the latter effect prevails.

Although pressure is one of the important parameters describing the state of fluids, density is a more preferable parameter for discussing the solvent power of SCFs. This is because dissolution arises from the mutual interaction between solvent molecules and solute molecules. The above data were plotted vs fluid density in Figure 7. It is apparent that higher temperatures led to larger oil solubility for constant fluid density. The logarithm of oil solubilities increased linearly with fluid density below 0.8 g/cm^3, while at higher density the slope of each solubility line decreased. Figure 7 indicates that high fluid density at higher temperatures should induce larger oil solubility. While this result means that extraction under higher pressure conditions is more advantageous, the operating cost must be taken into account for practical use. A high pressure-proof apparatus is expensive and operation under higher pressure conditions becomes more complicated.

In Figure 8 the neutral lipid composition of the extract is plotted vs the extraction ratio. CO_2 was used for this experiment at a temperature of 333°K and a pressure of 24.5 MPa. The triglyceride content was lower than that of the original lipid in the initial stage of the extraction, and it gradually increased with the extraction ratio. Conversely, the concentration of components with lower molecular weight, such as free fatty acids or diglycerides, decreased with the extraction

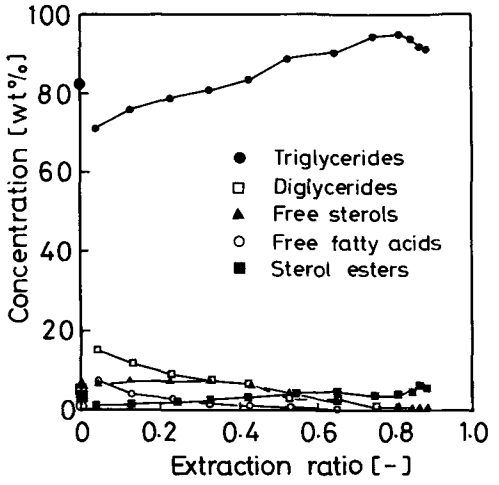

FIG. 8. Change in neutral lipid composition with extraction ratio. Extraction conditions: fluid, CO_2; temperature, 333°K; pressure, 24.5 MPa; sample weight, 20.0 g; and flow rate, 2.0 g/min. Keys on the ordinate represent the neutral lipid composition in the original lipid.

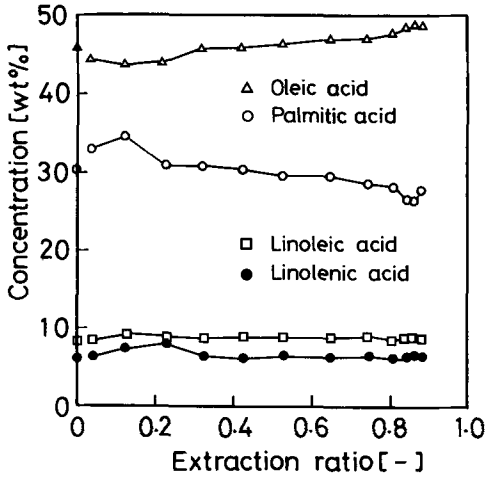

FIG. 9. Change in fatty acid composition with extraction ratio. See Figure 8 for extraction conditions. Keys on the ordinate represent the fatty acid composition in the original lipid.

ratio. This result means that components with lower molecular weight are extracted more easily. Similar results have been reported in the literature (19,20). This result suggests that SFE could be used for oil reforming such as deacidification.

The change in fatty acid composition with extraction ratio is shown in Figure 9. Extraction conditions were identical to those described for Figure 8. The ratio of oleic acid showed a gradual increase with the extraction ratio, while the ratio of palmitic acid showed the opposite tendency. The ratio of γ-linolenic acid remained fairly constant during the extraction, so it would be difficult to concentrate γ-linolenic acid by SFE. The fatty acid composition of the oil extracted using SC-N_2O was also measured—in this case the ratio of γ-linolenic acid hardly changed during the extraction. So the use of other separation techniques, such as liquid chromatography or supercritical fluid chromatography, may be necessary in order to concentrate specific fatty acids in neutral lipids.

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[Received August 11, 1989; accepted March 23, 1990]