Increase in the γ-Linolenic Acid Content by Solvent Winterization of Fungal Oil Extracted from *Mortierella* Genus

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The fungal oil extracted from *Mortierella ramanniana* var. *angulispora* (IFO 8187) was solvent winterized in order to raise the content of γ-linolenic acid (GLA). Effects of winterization conditions (solvent, oil concentration in the solvent and temperature) and changes of glyceride compositions were discussed. The fungal oil was separated into four diglycerides and 17 triglycerides (TG) with high performance liquid chromatography. The predominant species were POO, POP and LOP, whose contents were 24.4, 22.9 and 9.4% of the total TG, respectively. Ethanol at 4°C gave the highest GLA content of 10.5% in spite of lower yield than with acetone at -20°C. The highest separation efficiency for GLA (η_{GLA}) was 0.27 with acetone at -20°C and 10% oil concentration, resulting in 8.3% of GLA from the fungal oil at 5.7% GLA. In case of lower oil concentration at 5-20%, η_{GLA} showed higher in the following order: acetone (-20°C) > *n*-hexane (-20°C) > acetone (4°C) > petroleum ether (-20°C). The winterization process also proved to be effective for the separation of TG type, Sa_2U (Sa; saturated fatty acid; U, unsaturated fatty acid) into the crystallized fraction and SaU_2 into the liquid fraction. Acetone at -20°C showed higher separation efficiency for triunsaturated TG than the other solvents.

KEY WORDS: Crystallization, fungal oil, glyceride composition, γ-linolenic acid (GLA), *Mortierella ramanniana*, separation efficiency, Triglyceride composition, winterization.

γ-Linolenic acid (6,9,12-octadecatrienoic acid-GLA) has attracted increasing interest, because the Δ6-desaturase reaction from linoleic acid (18:2) to GLA (18:3) is generally thought to be the rate limiting step in the biosynthetic pathway of (n-6) fatty acid to arachidonate (1-4). Since GLA was found in a *Mortierella* genus (5), we tried to get high GLA productivity with *Mortierella ramanniana* var. *angulispora* (IFO 8187) using two stage repeated batch cultivation (6). Increase in GLA content has been tried by screening a mutant grown at a low temperature (7). Some trials of increasing GLA content for extracted fungal oil have been done using adsorption to zeolites (8) or supercritical fluid chromatography (9,10). The present research aimed firstly to obtain higher GLA content of fungal oil using solvent winterization. Solvent winterization of sunflower seed oil (11,12) and effect of operational variables on the performance of the winterization (13) have been reported. Besides increasing the content of GLA, the present paper also tries to clarify the effect of winterization conditions (solvent, oil concentration in solvents and temperature) on various type of triglyceride (TG) and diglyceride (DG) and the separation efficiency for GLA (η_{GLA}) and TG types.

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EXPERIMENTAL PROCEDURES

Materials. The fungal oil extracted from *Mortierella ramanniana* var. *angulispora* IFO 8187, grown under the cultural conditions as described (6), was used throughout the study. The solvents utilized for winterization were *n*-hexane, petroleum ether (boiling point 30-60°C), acetone, ethanol and chloroform, all of the analytical grade.

Winterization procedure. A fixed amount of the fungal oil was mixed with the appropriate amount of solvent in centrifugal tubes to make 5, 10, 20 and 40% oil solutions by weight and cooled to 4°C or -20°C for 24 hr or more until crystallization was complete. Separation of the crystallized fraction (CF) from the liquid fractions (LF) was carried out in Kubota Centrifuge KR/200A (Kubota Enshinki Co., Tokyo, Japan) previously cooled to the same temperature. Centrifugation was done at 8,000 rpm (6,000 g) for 10 min followed by decantation of the liquid fraction. The amounts of each resulting oil fraction was determined gravimetrically.

High performance liquid chromatography (HPLC). Separation and analysis of the TGs and DGs were carried out with HPLC using a Shimadzu Liquid Chromatograph LC-3A (Shimadzu Co., Kyoto, Japan) containing a 10 μL loop injector with a reverse phase column (Zorbax ODS, 250 mm × 6.2 mm I.D., DuPont Co., Wilmington, NC). The separated components were detected using a Shimadzu Refractive Index Detector RID-3A. To determine the best separation of the TGs and DGs, combination of acetone/acetonitrile at a ration of 4:1 (v/v) as mobile phase were investigated. Samples in chloroform were injected and the HPLC was run isocratically at a flow rate of 1.0 mL/min. Triglyceride and DG standards, tripalmitin, triolein, trilinolein and 1,2-diolein, were obtained from Serdary Research Laboratories Inc. (Ontario, Canada). Evening primrose oil, soybean oil and palm oil were used as TG mixture standards and their retention volume served as basis for the plots of log retention volume vs theoretical carbon number (TCN)(14-17).

Thin-layer chromatography (TLC). The lipid classes of the fungal oil and its separated fractions (CF, LF) were determined with TLC using the one-dimensional double development procedure of Freeman and West (18), with some modification (19) using two solvent systems. Composition of solvent system I was benzene/diethyl ether/ethanol/28% ammonium water (50:40:2:0.5, v/v/v/v), and that of solvent system II was *n*-hexane/diethyl ether (94:6, v/v), respectively. TLC plates were 0.25 mm in thickness, 200 × 200 mm, precoated with Silica Gel 60 (Merck, A.G., Darmstadt, West Germany). Quantitative analysis of the lipids separated on TLC was performed by charring with 13% H_2SO_4 solution containing 2% $CuSO_4$ and spot densities were measured with a Shimadzu Densitometer CS-910 with a zigzag scanning mode (20).

Gas liquid chromatography (GLC). Fatty acid composition of fungal oil and its separated fractions (CF, LF)

TABLE 1

Lipid Composition of Fungal Oil of *M. ramanniana* var. *angulispora*

Lipid class	Composition (%)
Triglyceride	81.8
1,3-Diglyceride	3.4
1,2-Diglyceride	13.7
Sterol ester	0.4
Free sterol	0.1
Free fatty acid	0.6

TABLE 2

Fatty Acid Compositions of Fungal Oil of *M. ramanniana* var. *angulispora* Analysed With GLC and HPLC

Fatty acid	GLC[a] (%)	HPLC[b] Mean ± S.D. (%)
16:0	31.9	32.7 ± 0.7
16.1	1.2	ND[c]
18:0	4.5	5.4 ± 0.2
18:1	46.0	47.3 ± 0.8
18:2	9.2	9.3 ± 0.7
18:3 (n-6)	5.7	4.9 ± 0.4

[a]GLC analysis of FAME.

[b]The compositions obtained by calculation from HPLC analysis of TG and DG compositions of separated fraction after winterization process (number of samples-20).

[c]Not detected.

were also analyzed as methyl esters (FAME) according to the method of Metcalfe and Schmitz (21). The esters were determined using a Shimadzu GC-4CM PF Gas Chromatograph equipped with a flame ionization detector and fitted with a 2.0 m × 3 mm I.D. glass column packed with 20% diethylene glycol succinate polyester on Chromosorb WAW (Supelco Inc., Bellefonte, PA). The column was operated isothermally at 190°C, injector and detector temperature at 220°C. Helium was used as carrier gas to 2kg/cm^2 and flow rate of 40 mL/min.

RESULTS AND DISCUSSION

Lipid composition of the fungal oil. Analysis of the fungal oil from *Mortierella ramanniana* var. *angulispora* by TLC (Table 1) showed that the oil consisted mainly of TGs and 1,2-DGs. Analysis of the fatty acids in major quantities as shown in the left column of Table 2. Content of GLA was 5.7% based on total fatty acids.

Triglyceride and DG composition were analyzed by HPLC. The solvent mixture of acetone/acetonitrile at a ratio of 4:1 (v/v) gave a good separation of TG mixture standards as well as fungal oil. The critical pairs with the same partition numbers (PN, in Table 3), such as OOO (54:3), POO (52:2) and POP (50:1), were clearly separated with the present method using the TG mixture standards (L, linoleic; O, oleic; S, stearic; and P, palmitic. The designation LOP, POP, etc., does not imply the TG LOP, but a mixture of all isomers—LOP, OLP and OPL). Identification of each TG was based on comparison of retention volume and TCN (15) of both the TG of the mixture standards and the TG of the fungal oil. The theoretical carbon number was calculated from the following formula:

$$TCN = PN - (\Sigma_i^3 U_i)$$

where U_i is a factor determined from several standards or TG mixture and was found to be 0.65 for O, 0.85 for L, 0.2 for GLA and 0.0 for saturated acyl groups, $\Sigma_i^3 U_i$, the total U_i of individual fatty acids present in the TG.

The peaks of chromatogram of fungal oil shown in Figure 1 were identified as follows. Coincidence of retention volume of major peaks such as peak 13 and peaks 16 to 20 between TG mixture standards and fungal oil gave identification of these peaks, from which TCN relation was obtained (Fig 2A; Table 3). Other peaks from peak 5 to peak 21 were identified using the TCN relation. Peak 5 to peak 21 were identified as 17 TGs, GLALL, GLALP, LLO, GLAOO, GLALS, GLAOP, LOO, LLS, LOP, LPP, GLASP, OOO, POO, POP, SOO, SOP and SOS, in the order of elution. Peak 1 to peak 4 deviated from the line in Figure 2A, and peak 3 had the same retention volume as standard DG of 1,2-diolein. Retention volume and TCN of peaks of 1 to 4 also showed the same relationship to TG as shown in Figure 2B. The peaks of 1 to 4, therefore, were identified as DGs, (i.e., GLAO, OL, OO and PO, in the order of elution).

TABLE 3

Partition Number (PN) and Theoretical Carbon Number (TCN) of Triglycerides (TG) and Diglycerides (DG)

Glyceride	TC[a]:DB[b]	PN[c]	TCN[d]
Triglyceride			
GLALL[e]	54:7	40	38.1
GLALP	52:5	42	41.0
LLO	54:5	44	41.7
GLAOO	54:5	44	42.5
GLALS	54:5	44	43.0
GLAOP	52:4	44	43.2
LOO	54:4	46	43.9
LLS	54:4	46	44.3
LOP	52:3	46	44.5
LPP	50:2	46	45.2
GLASP	52:3	46	45.8
OOO	54:3	48	46.1
POO	52:2	48	46.7
POP	50:1	48	47.4
SOO	54:2	50	48.7
SOP	52:1	50	49.4
SOS	54:1	52	51.4
Diglyceride			
GLAO	36:4	28	27.0
LO	36:3	30	28.5
OO	36:2	32	30.7
PO	34:1	32	31.4

[a]Total carbon number.

[b]Number of double bonds per molecule.

[c]PN = TC - 2 × DB.

[d]TCN = PN - (Σ_i^3Ui). (Ui, 0, 0.65; L, 0.85, GLA, 0.2).

[e]GLA, γ-linolenate; L, linoleate; O, oleate; S, stearate; and P, palmitate. The order of designation does not indicate the separation of positional isomers.

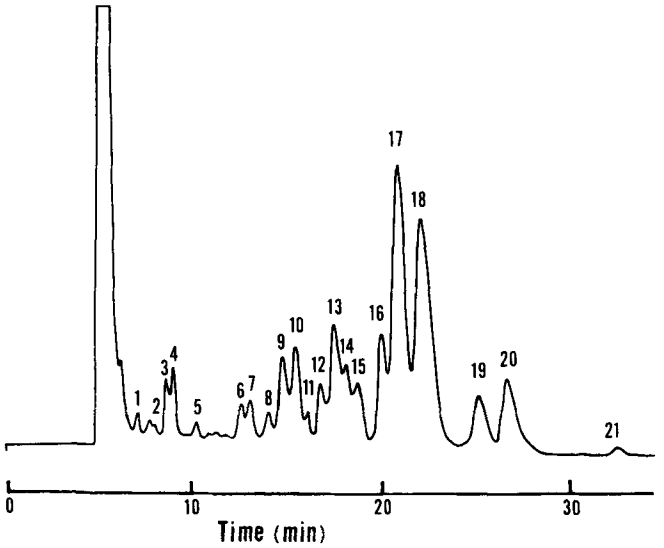

FIG. 1. HPLC chromatogram of the fungal oil from *M. ramanniana* var. *angulispora*. Column, Zorbax ODS (250 mm × 6.4 mm I.D.); and solvent system, acetone/acetonitrile (4:1) at a flow rate of 1.0 mL/min. Peaks were identified as 4 diglycerides and 17 triglycerides, 1, *GLAO* 2, *OL*; 3, *00*; 4, PO; 5, GLALL; 6, GLALP; 7, LLO; 8, GLAOO; 9, GLALS; 10, GLAOP; 11, LOO; 12, LLS; 13, LOP; 14, LPP; 15, GLASP; 16, OOO; 17, POO; 18, POP; 19, SOO; 20, SOP; and 21, SOS.

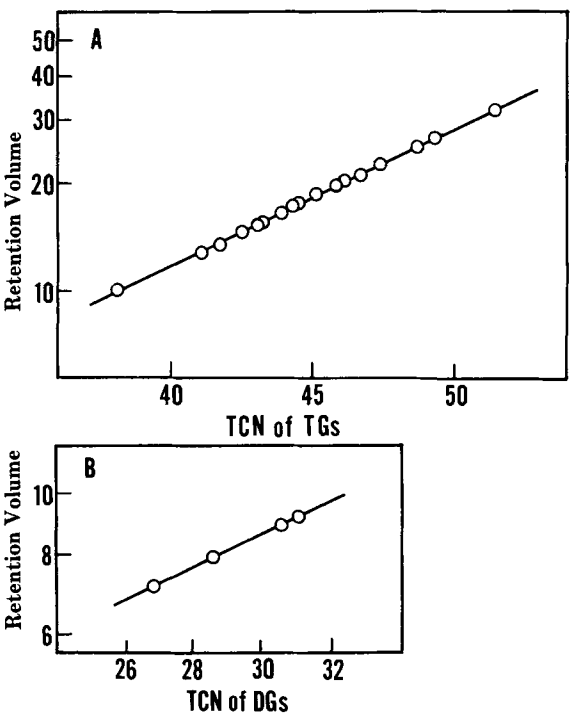

FIG. 2. Theoretical carbon number of triglycerides (TGs)(A) and diglycerides (DGs)(B) vs their retention volume of fungal oil.

Glyceride composition of the fungal oil and its CF and LF are shown in Table 4. The predominant species were POO, POP and LOP, whose contents were 24.4, 22.9 and 9.4% of the total TG, respectively. In the right column of Table 2, the fatty acid composition from HPLC analysis is shown, indicating the comparison with that from GLC analysis of FAME. The fatty acid composition from HPLC was obtained by calculation from TG and DG compositions of the fungal oil and of CF and LF in various conditions (number of samples—20) and expressed as mean ± SD. Analysis of HPLC gave a good agreement with that of GLC, which indicated validity of the identification of each TG and DG by HPLC analysis, though separation of each peak in Figure 1 was not necessarily good because of many types of TGs.

Winterization of the fungal oil. Table 5 summarizes the yields of fraction (Y_f), lipid composition, GLA content and yield of GLA (Y_{GLA}) of the CF and LF of the fungal oil after winterization.

Increase in oil concentration in solvents, as well as decrease in the winterization temperature, decreased Y_f in LF. Triglyceride was concentrated into CF, but DG was concentrated into LF. Ethanol provided the highest concentration of TG into CF (92.3% of lipid) in spite of high yield of CF (73.7%). The high concentration of TG seems to be bought about by high polarity of ethanol and very low TG solubility in ethanol.

γ-Linolenic acid contents of LF (6.0–10.5%), were higher than the original oil (5.7%), owing to comparatively high solubility of GLA. The fractions which had more than 10% GLA content in LF, however, showed both very low yield of LF (18.3% with acetone at -20°C and 26.3% with ethanol at 4°C) and relatively low Y_{GLA} at less than 50%. In order to evaluate the effectiveness of separation including GLA yield, separation efficiency for the substance S into fraction LF [(η_S) defined as the following equation] is used:

$$\eta_S=(Y_{LF}x_1 / 100x_f) - (Y_{LF}(1 - x_1 / 100) / (100 - x_f))$$
$$= (x_f - x_c)(x_1 - x_f) / x_f(1 - x_f / 100)(x_1 - x_c)$$

where Y_{LF} is the yield of fraction LF (%); x_f, content of S in fungal oil (%); x_1, content of S in fraction LF (%); and x_c content of S in fraction CF (%).

Figure 3 shows the dependence of η_{GLA} on oil concentration in solvent (%). Higher winterization temperature decreased η_{GLA} and oil concentration of 40% showed the least η_{GLA} except for petroleum ether. Effect of winterization temperature on η_{GLA} revealed that lower temperature was advantageous for concentration of GLA. The highest η_{GLA} (0.27) was obtained with acetone at -20°C and at 10% oil concentration in the solvent. Acetone, at -20°C and at less at 20% of oil concentrations, indicated comparably higher η_{GLA}. In case of lower oil concentration (5–20%), η_{GLA} generally showed higher in the following order: acetone (-20°C) > *n*-hexane (-20°C) > acetone (4°C) > petroleum ether (-20°C). Oil concentration at 5%, ethanol (4°C) showed high value of η_{GLA}, it seems to be affected by lower solubility of TG without GLA.

Among the main TG mentioned above, disaturated TGs (Sa_2U; Sa, saturated fatty acid; and U, unsaturated fatty acid; POP, POS, SOS, etc.) were concentrated into CF, whereas diunsaturated TGs (SaU_2, OOP, LOP, LLS, etc.) and triunsaturated TGs (U_3, GLALL, LLO, OOO, etc.) were concentrated into LF. These results indicated that the winterization was effective for the separation of Sa_2U and SaU_2. As for the concentration of GLA, most TGs containing GLA were separated into LF.

Figure 4 shows the separation efficiencies for η_{Sa_2U} into CF, η_{SaU_2} and η_{U_3} into LF, respectively, vs oil concentration in solvents. Separation efficiency for Sa_2U showed high value (0.7–0.8) with *n*-hexane throughout the oil concentration examined, and η_{Sa_2U} showed relatively high values

TABLE 4

Comparative Glyceride Composition of the Fungal Oil and Separated Fractions with Winterization Process

Solvent	Temp (°C)	C[a] (%)	F[b]	Triglyceride (%) GLALL	GLALP	LLO	GLAOO	GLALS	GLAOP	LOO	LLS	LOP	LPP	GLASP	OOO	POO	POP	SOO	SOP	SOS	Diglyceride (%) GLAO	OL	OO	PO
—	—	—	Fungal oil	2.7	1.7	2.5	1.5	4.3	5.6	1.0	2.7	9.4	3.8	2.0	5.8	24.4	22.9	2.6	5.5	1.2	6.3	12.5	27.1	54.1
Hxn[c]	-20	5	CF	—	—	—	—	—	0.6	—	—	0.7	2.0	3.4	0.8	5.4	63.2	—	19.1	4.2	—	—	23.8	76.2
			LF	2.5	1.7	2.7	1.2	5.2	7.0	1.2	3.6	14.7	3.5	1.4	8.2	31.2	9.0	5.0	1.7	—	7.0	15.8	31.6	45.6
		10	CF	—	—	—	—	—	0.6	—	—	0.9	1.9	3.7	0.9	5.5	63.6	—	18.7	4.1	—	—	18.2	81.8
			LF	2.4	2.1	3.2	1.6	5.6	7.5	1.3	4.1	15.0	3.5	1.3	8.6	33.3	4.8	4.4	0.7	—	8.8	14.0	31.6	45.6
		20	CF	—	—	—	—	0.5	0.9	—	0.2	1.8	3.4	4.3	1.1	8.6	58.1	1.1	16.5	3.3	—	25.8	16.1	58.1
			LF	2.5	2.2	3.1	1.2	6.0	8.8	1.4	4.4	20.6	—	—	8.8	31.8	3.9	4.8	0.5	—	8.3	15.0	30.0	46.7
		40	CF	—	0.9	0.8	—	2.3	3.1	—	1.0	3.7	7.5	—	2.6	10.7	47.4	3.0	13.5	2.7	—	—	—	98.0
			LF	2.2	2.5	3.6	1.7	5.7	8.0	1.6	4.3	20.7	—	—	8.9	31.1	3.7	4.7	1.3	—	7.4	14.8	27.8	50.0
	4	40	CF	—	0.7	1.1	0.5	2.5	3.1	—	0.3	0.8	2.0	0.6	4.5	16.8	47.3	1.9	16.8	1.0	—	—	32.0	68.0
			LF	1.6	1.9	2.9	1.4	5.1	7.1	1.4	3.8	13.5	3.8	2.2	8.0	30.0	10.5	3.4	3.4	—	8.6	13.8	29.3	48.3
Pet. ether[d]	-20	5	CF	—	—	—	—	0.1	0.9	0.5	1.7	0.6	0.9	2.8	0.5	2.2	63.6	—	21.4	4.6	—	—	—	99.9
			LF	2.6	2.1	3.6	2.1	5.2	6.8	1.6	3.5	13.7	4.5	2.4	7.5	28.1	11.0	3.4	1.7	—	7.4	14.8	29.6	48.2
		10	CF	0.2	0.4	0.7	—	0.2	1.3	1.7	2.9	0.2	—	—	1.1	5.0	61.3	0.6	20.2	3.7	—	—	—	99.9
			LF	1.5	2.0	2.5	1.1	5.0	6.3	0.8	3.2	12.9	4.4	1.8	7.4	30.3	12.5	4.3	2.9	—	5.3	14.0	33.3	47.4
		20	CF	0.3	0.6	2.3	0.6	0.9	1.4	0.3	0.5	1.9	2.6	3.1	1.6	7.9	54.5	0.7	17.2	3.4	—	30.8	25.6	43.6
			LF	1.7	2.4	3.6	1.6	6.0	7.1	1.6	3.8	13.8	2.7	1.7	6.9	27.5	12.8	3.7	2.5	—	5.8	13.4	30.8	50.0
		40	CF	—	0.4	0.7	0.7	1.6	2.6	0.8	1.3	4.1	2.8	3.5	2.4	11.5	49.1	1.3	13.7	3.5	—	—	39.1	60.9
			LF	2.6	1.5	2.7	1.1	4.8	6.8	1.0	3.2	17.3	—	—	7.9	30.9	13.4	3.8	3.0	—	6.7	13.3	26.7	53.3
Atn[e]	-20	5	CF	—	—	—	—	0.9	1.7	0.7	0.3	1.8	2.3	2.7	1.7	19.4	51.5	2.3	14.7	—	—	—	36.4	63.6
			LF	1.7	3.1	4.2	2.1	6.9	8.9	1.2	4.4	17.5	—	0.7	10.0	29.8	3.6	4.3	—	—	6.8	12.3	28.8	52.1
		10	CF	—	0.4	0.7	—	1.3	2.0	0.9	1.0	6.7	4.7	3.5	2.1	18.9	43.6	1.9	11.4	0.3	—	—	38.5	61.5
			LF	2.1	2.9	3.8	1.5	7.3	9.2	0.8	5.0	18.9	—	0.8	10.9	30.6	0.7	4.9	—	—	5.9	12.9	30.6	50.6
		20	CF	—	0.5	0.9	0.4	2.8	3.9	1.2	1.6	6.3	4.6	2.8	3.3	23.3	35.1	3.2	9.6	0.4	—	—	38.5	61.5
			LF	2.3	3.7	4.8	1.8	8.2	9.6	0.7	5.1	16.3	—	—	12.5	28.6	1.2	3.7	—	—	8.7	7.8	30.1	53.4
		40	CF	—	0.9	1.9	0.5	3.8	4.8	0.6	1.3	6.7	1.4	—	6.0	28.2	29.6	4.3	9.0	0.7	—	16.7	30.0	53.3
			LF	2.7	4.1	5.4	2.6	8.2	10.3	—	7.0	18.0	—	1.2	11.3	23.0	1.7	2.8	—	—	7.3	15.4	32.0	45.3
	4	5	CF	—	0.6	0.9	0.5	2.1	2.7	0.7	1.4	4.6	4.2	2.8	3.2	15.1	44.3	1.9	13.0	2.0	—	12.0	32.0	56.0
			LF	2.4	2.8	3.4	1.3	6.3	8.0	1.2	3.7	15.8	3.1	1.4	8.7	32.9	3.7	4.6	—	—	6.0	14.9	31.1	47.8
		10	CF	—	0.8	1.2	0.8	2.4	2.9	1.0	1.4	4.3	4.3	3.0	3.0	13.9	44.3	1.7	12.7	2.0	—	17.4	30.4	52.2
			LF	2.5	2.5	3.6	1.4	6.1	8.2	1.5	4.2	18.7	—	1.6	9.3	33.6	2.5	4.3	—	—	8.5	14.1	29.6	47.9
		20	CF	—	0.7	1.0	0.6	2.5	3.2	0.8	1.8	6.7	3.8	3.0	4.5	18.5	39.5	2.4	10.8	—	—	16.0	32.0	52.0
			LF	2.4	2.2	3.2	1.5	6.5	8.8	1.3	4.6	18.9	—	1.1	9.6	32.6	1.9	4.4	—	—	7.3	15.8	29.3	47.6
		40	CF	0.4	1.0	1.4	0.8	3.3	5.0	1.2	2.4	8.5	2.9	2.4	4.8	20.2	32.1	2.7	9.9	0.9	—	13.9	30.6	55.5
			LF	2.8	2.4	3.3	1.4	6.0	7.7	1.2	4.0	18.9	—	1.0	9.0	33.5	3.1	4.4	—	1.0	9.7	17.1	29.3	43.9
EtOH[f]	4	5	CF	—	0.6	1.5	0.7	2.7	3.9	1.1	1.4	9.6	4.3	2.5	3.5	27.0	29.8	2.7	7.5	1.0	—	25.0	33.3	41.7
			LF	2.5	5.6	5.5	3.1	10.3	10.6	—	9.6	14.5	—	—	16.4	16.2	1.6	—	3.3	—	8.6	15.3	20.3	56.1
Chl[g]	-20	40	CF	—	0.8	1.3	2.2	2.2	3.6	—	1.2	5.3	1.1	5.1	4.4	17.1	43.7	—	11.9	—	—	—	34.6	65.4
			LF	1.2	1.7	2.4	1.5	5.0	6.4	1.1	3.6	12.3	3.4	2.0	7.7	29.6	13.2	3.9	3.0	—	9.8	8.2	31.1	50.9

[a]Concentration of oil in solvent (%w).

[b]The fraction separated with winterization process (CF, crystallized fraction; LF, liquid fraction).

[c]Hexane.

[d]Petroleum ether.

[e]Acetone.

[f]Ethanol.

[g]Chloroform.

TABLE 5

Yields, Lipid Compositon and γ-Linolenic Acid Content of the Winterized Fraction of the Fungal Oil

Solvent	Temp. (°C)	C[a] (%)	F[b]	Y_f[c] (%)	Lipid composition TG	1,3-DG	1,2-DG	GLA[d] (%)	Y_{GLA}[e] (%)
Hxn[f]	-20	5	CF	24.8	87.2	2.7	10.0	1.7	7.3
			LF	75.2	75.5	4.3	18.4	7.1	92.7
		10	CF	31.8	88.4	2.8	8.6	1.8	10.1
			LF	68.2	75.3	4.2	19.0	7.5	89.9
		20	CF	36.0	87.6	3.4	8.8	2.0	12.9
			LF	64.0	75.1	3.9	19.2	7.6	87.1
		40	CF	41.6	84.9	4.0	10.1	3.0	22.2
			LF	58.4	73.7	6.5	18.3	7.5	77.8
	4	40	CF	27.5	86.9	4.4	8.3	3.5	15.9
			LF	72.5	79.5	5.4	14.7	7.0	84.1
Pet. ether[g]	-20	5	CF	19.9	93.0	1.4	5.6	1.4	4.8
			LF	80.1	80.0	3.7	15.4	6.9	95.2
		10	CF	21.4	87.9	2.9	9.2	2.0	7.2
			LF	78.6	78.3	4.0	16.7	7.0	92.8
		20	CF	20.7	88.3	2.9	8.8	2.4	8.4
			LF	79.3	78.0	4.2	16.6	6.8	91.6
		40	CF	27.0	86.4	2.6	11.0	3.0	13.9
			LF	73.0	79.1	3.9	15.7	6.9	86.1
Atn[h]	-20	5	CF	44.4	92.4	2.3	5.0	2.7	21.0
			LF	55.6	72.1	4.4	21.9	8.1	79.0
		10	CF	49.7	89.1	2.0	8.4	2.7	24.3
			LF	50.3	73.3	5.2	19.6	8.3	75.7
		20	CF	63.8	90.9	0.8	8.0	3.6	39.6
			LF	36.2	70.7	5.9	20.7	9.7	60.4
		40	CF	81.7	87.6	2.1	10.0	4.7	67.6
			LF	18.3	62.5	6.9	29.5	10.1	32.4
	4	5	CF	46.4	92.5	1.1	6.0	3.7	29.9
			LF	53.6	73.5	5.7	18.8	7.5	70.1
		10	CF	46.8	90.5	2.3	6.9	3.5	29.1
			LF	53.2	76.9	4.2	17.4	7.5	70.9
		20	CF	56.8	88.6	2.3	8.7	4.2	40.8
			LF	43.2	73.1	4.9	19.8	8.0	59.2
		40	CF	67.3	84.5	3.5	11.5	4.8	55.6
			LF	32.7	74.2	4.6	19.7	7.9	44.4
EtOH[i]	4	5	CF	73.7	92.3	2.0	4.9	3.9	51.0
			LF	26.3	53.5	7.8	30.5	10.5	49.0
Chl[j]	-20	40	CF	33.4	86.1	2.8	10.3	4.0	25.1
			LF	66.6	78.7	2.5	17.8	6.0	74.9

[a]Concentration of oil in solvent (%w).
[b]The fraction separated with winterization process (CF, crystallized fraction; LF, liquid fraction).
[c]Yield of each separated fraction
[d]Content of γ-linolenic acid by GLC analysis.
[e]Yield of γ-linolenic acid.
[f]Hexane.
[g]Petroleum ether.
[h]Acetone.
[i]Ethanol.
[j]Chloroform.

(> 0.5) with all the solvents examined in the range of oil concentration less than 20%. Disaturated TGs, Sa_2U and especially Sa_2O such as POP, POS and SOS are the main components of cocoa butter, which has a melting point of approximately 35°C (22). The present method, therefore, is an excellent one to obtain a cocoa butter like fat into CF. Higher η_{SaU_2} was obtained with *n*-hexane than with any other solvent. In case of U_3, however, low oil concentration in acetone (-20°C) and ethanol showed high η_{U_3} which was correlated to the high η_{GLA} with the solvent.

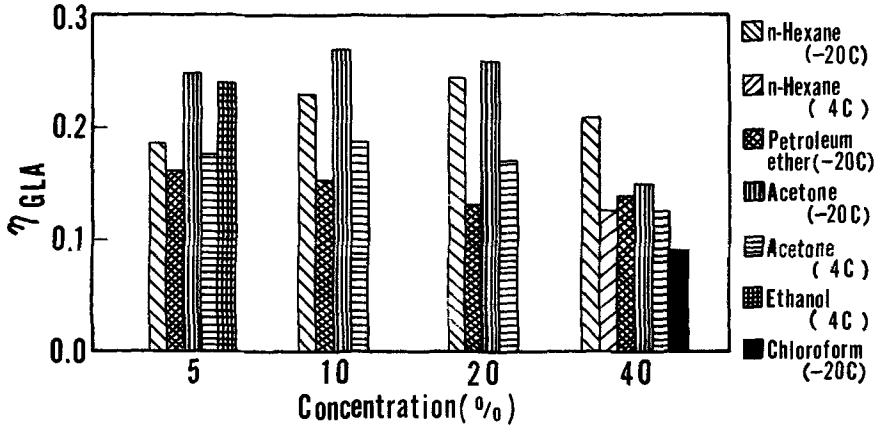

FIG. 3. Effect of oil concentration in solvent on separation efficiency for γ-linolenic acid (η_{GLA}).

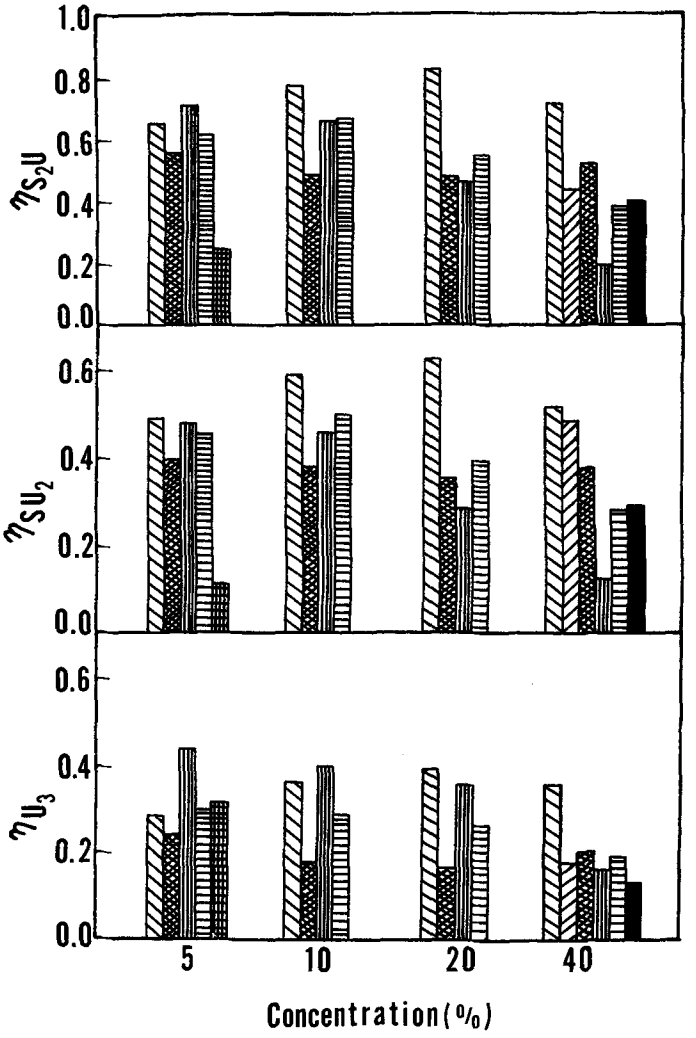

FIG. 4. Effect of oil concentration in solvent on separation efficiency (η)for triglyceride type of Sa_2U, $SaU_2$and U_3. Condition of each bar was the same as in Fig. 3.

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