Large-Scale Purification of γ-Linolenic Acid by Selective Esterification Using *Rhizopus delemar* Lipase

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ABSTRACT: γ-Linolenic acid (GLA) is a physiologically valuable fatty acid, and is desired as a medicine, but a useful method available for industrial purification has not been established. Thus, large-scale purification was attempted by a combination of enzymatic reactions and distillation. An oil containing 45% GLA (GLA45 oil) produced by selective hydrolysis of borage oil was used as a starting material. GLA45 oil was hydrolyzed at 35°C in a mixture containing 33% water and 250 U/g-reaction mixture of *Pseudomonas* sp. lipase; 91.5% hydrolysis was attained after 24 h. Film distillation of the dehydrated reaction mixture separated free fatty acids (FFA; acid value 199) with a recovery of 94.5%. The FFA were selectively esterified at 30°C for 16 h with two molar equivalents of lauryl alcohol and 50 U/g of *Rhizopus delemar* lipase in a mixture containing 20% water. The esterification extent was 52%, and the GLA content in the FFA fraction was raised to 89.5%. FFA and lauryl esters were not separated by film distillation, but the FFA-rich fraction contaminated with 18% lauryl esters was recovered by simple distillation. To further increase the GLA content, the FFA-rich fraction was selectively esterified again under similar conditions. As a result, the GLA content in the FFA fraction was raised to 97.3% at 15.2% esterification. After simple distillation of the reaction mixture, lauryl esters contaminating the FFA-rich fraction were completely eliminated by urea adduct fractionation. When 10 kg of GLA45 oil was used as a starting material, 2.07 kg of FFA with 98.6% GLA was obtained with a recovery of 49.4% of the initial content.
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KEY WORDS: Borage oil, distillation, hydrolysis, γ-linolenic acid, *Pseudomonas* sp. lipase, purification, *Rhizopus delemar* lipase, selective esterification, urea adduct.

γ-Linolenic acid (GLA, 18:3n-6) is biosynthesized from linoleic acid (18:2n-6) by Δ6-desaturase, which is the rate-limiting factor in the essential fatty acid cascade (1,2). GLA is also an intermediate precursor of local hormones (prostaglandins, thromboxanes, and leukotrienes) (3). These hormones participate in the development and regulation of immunological and inflammatory response, together with eicosanoids derived from n-3 series of polyunsaturated fatty acids (4). Thus, an abnormal fatty acid profile is implicated in impairment of the immune function, and pathogenesis associated with inflammatory, autoimmune, and neoplastic diseases (5). For example, patients with atopic eczema were shown to have higher levels of linoleic acid and lower levels of GLA, suggesting impaired Δ6-desaturase activity (6,7). In a number of clinical trials, dietary supplementation with GLA increased epidermal levels of eicosanoid precursors (8) and produced significant decreases in itching and antihistamine use (9,10). Furthermore, GLA-containing oil was effective for curing rheumatoid arthritis (11,12) and multiple sclerosis (13). These studies were conducted with GLA-containing oil for use as health food and in infant formula (14) and, hence, the highly purified GLA has been desired as a medicine.

Studies in the last decade have proposed several methods for GLA enrichment; urea adduct formation (15), separation on Y-zeolite (16), solvent winterization (17), and selective enzymatic hydrolysis or esterification (18–22). However, these methods result in low GLA content. The use of a large amount of *n*-hexane and/or the high cost has prevented their adoption as industrial-scale purification methods. In addition, because GLA-containing oils (borage, blackcurrant, and evening primrose oils) contain a large amount of 18-carbon-length fatty acids, rectification cannot be expected to work as a large-scale purification method. Quite recently, we purified docosahexaenoic acid (22:6n-3) to 90% purity by hydrolyzing tuna oil with *Pseudomonas* lipase, followed by selective esterification of the resulting free fatty acids (FFA) with lauryl alcohol using *Rhizopus delemar* lipase (two-step enzymatic method) (23,24). Furthermore, GLA was purified from borage oil to 94% purity in a laboratory scale by a similar procedure (22). The two-step enzymatic method would be suitable for the large-scale purification of GLA for the following reasons: (i) FFA can be separated from the reaction mixture of enzymatic hydrolysis of GLA-containing oil by distillation;(ii) the reaction mixture of selective esterification consists of lauryl alcohol (molecular weight, 186), GLA (278), and lauryl esters (>460), and these compounds also may be separated by distillation. In this paper, we describe how GLA can be effectively purified by a combination of the enzymatic hydrolysis of 45% GLA-containing oil, selective esterification of the resulting FFA with lauryl alcohol, and distillation.

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MATERIALS AND METHODS

Oil and alcohol. The oil containing 45% GLA (GLA45 oil), which was produced by selective hydrolysis of borage oil, was a commercial product of the Nippon Synthetic Chemical Industry Co. Ltd. (Osaka, Japan). Lauryl alcohol (Kalcol 2098) was of industrial grade from Kao Corp. (Tokyo, Japan).

Lipases. Pseudomonas sp. lipase (Lipase-PS) and *R. delemar* lipase (Lipase-D) were purchased from Amano Pharmaceutical Co. Ltd. (Aichi, Japan). Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical Ind. Co., Osaka, Japan) with 50 mM KOH as described previously (25). The reaction was carried out at 30°C for 30 min with stirring at 500 rpm. One unit (U) of lipase activity was defined as the amount of enzyme that liberated 1 μmol of fatty acid per minute.

Reactions. Hydrolysis of GLA45 oil was performed at 35°C for 24 h in a mixture containing 33% water and 250 U/g of *Pseudomonas* lipase. Esterification of FFA was performed at 30°C for 16 h in a mixture containing two molar equivalents of lauryl alcohol, 20% water, and 50 or 70 U/g of *Rhizopus* lipase. The molar amount of FFA was calculated from the acid value. The hydrolysis extent was calculated from the acid value of the reaction mixture and the saponification value of GLA45 oil, 183. The esterification extent was calculated on the basis of the amount of fatty acid consumed during the reaction.

Large-scale reactions were carried out using a 2-L reactor (TBR-2-3; Oriental Yeast Co., Ltd., Tokyo, Japan) and a 30-L reactor (Mituwa Co. Ltd., Osaka, Japan). Their agitation speeds were 500 and 200 rpm, respectively.

Distillation. Film distillation was carried out at 150 to 160°C and 0.05 mmHg using a molecular distillation apparatus (MS-150; Nippon Sharyo Ltd., Aichi, Japan) to separate FFA and glycerides. Simple distillation was performed by supplying a small amount of nitrogen gas through a capillary tube to separate FFA from the reaction mixture of esterification of FFA with lauryl alcohol.

Extraction of FFA with n-*hexane.* The extraction of FFA from the mixture of FFA/glycerides or FFA/lauryl alcohol/lauryl esters with *n*-hexane was carried out as described previously (26). Glycerides, lauryl alcohol, and lauryl esters were extracted with 100 mL *n*-hexane after adding 70 mL of 0.5 N KOH (20% ethanol solution) into 5 g of mixture containing FFA. FFA in the water layer was extracted with 100 mL *n*-hexane after returning to acidic pH (<pH 2) with HCl.

Urea adduct fractionation. Urea (20 g) was dissolved at 50°C in 100 mL methanol and 2.5 mL water with stirring, and 20 g of a mixture of FFA/lauryl esters was then added gradually. After the mixture was completely dissolved, the temperature of the solution was gradually decreased to 25, 15, and 5°C. The precipitate was removed with filter paper and washed with urea-saturated methanol. The volume of the filtrate was reduced to 50 mL by evaporation, and FFA was then extracted with 150 mL *n*-hexane after adding 100 mL of 0.2 N HCl. Large-scale fractionation was performed by almost the same procedure. A mixture of FFA/lauryl esters (400 g) was gradually dissolved at 50°C in a solution of 2 L methanol and 50 mL water, and the temperature was gradually decreased to 5°C. After removing the precipitate, the volume of the filtrate was reduced to *ca*. 700 mL, and 300 mL of 0.2 N HCl was then added. The oil layer (FFA fraction) was washed with 300 mL water three times.

Analysis. Fatty acids in glycerides were methylated at 75°C in methanol for 15 min with Na-methylate as methylating reagent, and FFA were methylated at 75°C in 5% HCl/methanol for 3 h. These methyl esters were analyzed with a Hewlett-Packard 5890 plus gas chromatograph (Avondale, PA) connected to a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) as described previously (26). The contents of lauryl alcohol, FFA, and lauryl esters were analyzed based on the area of their peaks separated using a DB-5 capillary column (0.25 mm × 10 m, J&W Scientific) according to our previous paper (27).

RESULTS AND DISCUSSION

Preparation of FFA originating from GLA45 oil. Because *Pseudomonas* sp. lipase (Lipase-PS) was found to hydrolyze GLA45 oil more strongly than LIPOSAM (*Pseudomonas* sp. lipase; Showa Denko K.K., Tokyo, Japan) used in the previous study, the former was used in the present work. Several factors affecting the hydrolysis (enzyme amount, water content, temperature, and reaction period) were investigated, and the reaction conditions were set as follows: a mixture consisting of borage oil/water (2:1, w/w) and 250 U/g-reaction mixture of the lipase was stirred at 35°C for 24 h.

When 1200 g of GLA45 oil was hydrolyzed under the above conditions, the hydrolysis extent reached 90% after 24 h. The oil layer was dehydrated, and film distillation was then performed at 150°C and 0.05 mm Hg. As shown in Table 1, distillate-1 was obtained (822 g; acid value 199). The glyceride-free FFA originating from GLA45 oil showed an acid value of 200, suggesting that the distillate-1 was high-purity FFA. Because the acid value of the residue was still high (108), distillation was repeated at 160°C. The second cycle of distillation gave 63 g of distillate-2 (acid value 193) and 123 g of residue (acid value 58). Distillate-1 and -2 were combined (GLA45-FFA; GLA content 46.5%), and used as substrates of the following selective esterification.

Film distillation was found to be very effective for the separation of FFA from a mixture of FFA/glycerides. In addition, the residue (glycerides) can be used as a substrate for further hydrolysis. Thus, the loss of GLA was considered very small in the industrial preparation of FFA from GLA45.

Selective esterification of GLA45-FFA with lauryl alcohol. Our preliminary experiment showed that the content and recovery of GLA in the FFA fraction depended mainly on the esterification extent. To set the reaction conditions of the selective esterification, the reaction was conducted at 30°C for 16 h in a mixture consisting of GLA45-FFA/lauryl alcohol (1:2, mol/mol), 20% water, and various amounts of *Rhizopus* lipase. Figure 1 shows the relationships of the content and re-

TABLE 1
Film Distillation of Hydrolysis Products of GLA45 Oil with *Pseudomonas* sp. Lipase

	Acid value	Weight (g)	Amount of FFA[a] (g)	Amount of GLA in FFA fraction (g)
Hydrolysis				
Before	183[b]	1200	1098[c]	495[d]
After[e]	180	1030	927	431
Distillation				
Distillate-1[f]	199	822	819	381
Distillate-2[g]	193	63	61	28
Residue	58	123	38	n.t.[h]

[a]The amount of free fatty acid (FFA) was calculated from its acid value, 200.
[b]Saponification value of GLA45 oil.
[c]Amount of fatty acid contained in GLA45 oil.
[d]Amount of γ-linolenic acid (GLA) contained in GLA45 oil.
[e]Dehydrated reaction mixture after the hydrolysis of GLA45 oil. The hydrolysis was conducted as described in the text.
[f]Distillation was performed at 150°C and 0.05 mm Hg.
[g]Distillation was performed at 160°C and 0.05 mm Hg.
[h]n.t., not tested. GLA45 oil, oil containing 45% GLA.

covery of GLA in the FFA fraction to the esterification extent. The GLA content in the FFA fraction was raised with increasing esterification extent, but the limit value was 90%. The recovery of GLA in the FFA fraction decreased significantly at above 50% esterification. When the esterification extent was 50 to 55%, the GLA content was raised to 90% with a recovery of 85 to 90%. This esterification extent was attained using 50 U/g-reaction mixture of the lipase.

Separation of FFA from the reaction mixture after esterification of GLA45-FFA. A reaction mixture containing 600 g

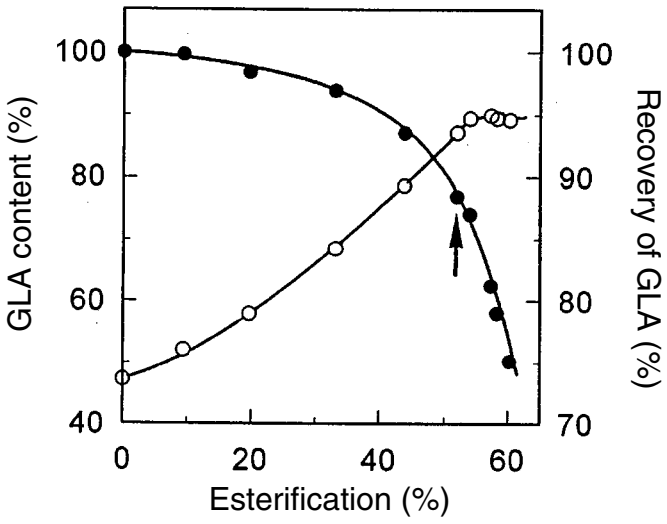

FIG. 1. Selective esterification of GLA45-free fatty acids (FFA) with lauryl alcohol and different amounts of *Rhizopus delemar* lipase. A mixture consisting of 4.8 g GLA45-FFA/lauryl alcohol (1:2, mol/mol), 1.2 g water, and 10 to 400 U/g-reaction mixture of *Rhizopus* lipase was incubated at 30°C for 16 h with stirring at 500 rpm. Arrow indicates the reaction with 50 U/g of lipase. ○, Content of γ-linolenic acid (GLA) in the FFA fraction; ●, recovery of GLA in the FFA fraction. The recovery was calculated by assuming that all FFA was recovered by the extraction with *n*-hexane.

TABLE 2
Simple Distillation of Reaction Mixture After Selective Esterification

	Acid value	Weight (g)	Amount of FFA[a] (g)	Amount of GLA in FFA fraction (g)
Esterification				
Before	86	1390	597	278
After[b]	40	1310	262	238
Distillation				
Distillate-1[c]	3	557	8	n.t.
Distillate-2[d]	157	312	245	223
Residue-2[d]	3	419	7	n.t.

[a]The amount of FFA was calculated from its acid value, 200. See Table 1 for abbreviations.
[b]Reaction mixture was dehydrated after the reaction. The selective esterification was conducted as described in the text.
[c]Distillation was performed at 105°C and 0.2 mm Hg.
[d]Distillation was performed at 185°C and 0.2 mm Hg.

(2.13 mol) of GLA45-FFA, 792 g (4.26 mol) of lauryl alcohol, 348 g of water, and 50 U/g of *Rhizopus* lipase was stirred at 30°C for 16 h. The esterification extent was 53.5%, and the GLA content in the FFA fraction reached 90.9%. The result showed that a large-scale reaction can be performed under the same conditions as those of the small-scale reaction.

Because film distillation was not effective to separate FFA and lauryl esters, simple distillation was employed (Table 2). The distillation of the dehydrated reaction mixture (1310 g) at 105°C and 0.2 mm Hg gave 557 g of distillate-1 which was composed of 98.7% lauryl alcohol and 1.3% FFA. The distillation temperature was then raised to 185°C, and 312 g of distillate-2 was obtained. The composition of the distillate-2 was lauryl alcohol/FFA/lauryl esters (5.6:78.9:15.5, w/w/w). The calculation based on the acid value showed that 93.5% of FFA were recovered in this fraction. The GLA content in the FFA fraction of the distillate-2 was 91.0%, and 93.8% of GLA in the reaction mixture was recovered by the distillation. The amount of residue was 419 g, and 98.5% of the fraction was lauryl esters.

Repeated selective esterification. The GLA content in the FFA fraction was raised to 91.0% by the single selective esterification. To further increase the GLA content, FFA recovered from the reaction mixture was esterified again with lauryl alcohol. Because 15.5% of lauryl esters were present in the FFA fraction obtained by simple distillation (Table 2, distillate-2), we examined the effect of the ester contaminants on the repeated esterification (Fig. 2). The lauryl ester-free FFA was prepared by eliminating lauryl esters from the distillate-2 with *n*-hexane. When the resulting FFA was esterified for 16 h with lauryl alcohol and 70 U/g-reaction mixture of *Rhizopus* lipase, the GLA content in the FFA fraction was raised to 98.7% at the esterification extent of 15.8% (Fig. 2B). The GLA recovery in the FFA fraction was 91.3%. Even though the esterification extent was increased by using a larger amount of lipase, the content of GLA was not increased and its recovery was decreased. On the other hand, when FFA contaminated with lauryl esters (distillate-2) was esterified under the same conditions, the GLA content was raised to

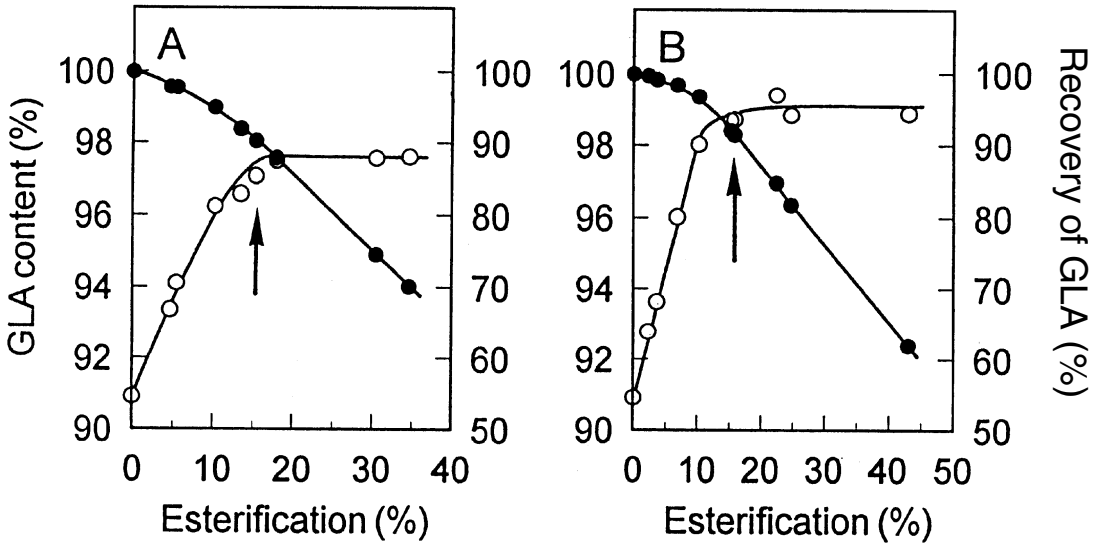

FIG. 2. Selective esterification of FFA obtained by single esterification with lauryl alcohol. The FFA contaminated with 15.5% lauryl esters (A) and the lauryl ester-free FFA (B) were esterified at 30°C for 16 h with two molar equivalents of lauryl alcohol in 6 g of reaction mixture containing 20% water and 5 to 400 U/g-reaction mixture of *Rhizopus lipase*. Arrows indicate the reactions with 70 U/g of lipase. ○, content of GLA in the FFA fraction; ●, recovery of GLA in the FFA fraction. The recovery was calculated by assuming that all FFA were recovered by the extraction with *n*-hexane. See Figure 1 for abbreviations.

97.1% at the esterification extent of 15.4% (Fig. 2A). The recovery of GLA in the FFA fraction was 90.3%. The GLA content in the FFA fraction was not raised by the further increase of the esterification extent, as shown in the esterification of lauryl ester-free FFA. These results show that lauryl ester contaminants had little effect on the increase of GLA content in FFA fraction.

Elimination of lauryl esters by urea adduct fractionation. Urea adduct fractionation was attempted to eliminate lauryl esters from the FFA fraction obtained by simple distillation (Table 2, distillate-2). After 20 g of distillate-2 was dissolved at 50°C in a methanol solution containing urea, the temperature was gradually decreased to 25, 15, and 5°C with stirring. The urea adduct precipitates were removed by filtration. Table 3 shows the amounts of lauryl alcohol, FFA, and lauryl esters in the filtrate fraction. When the temperature was decreased to 25°C, all the lauryl esters were precipitated as urea adduct complexes and 14.7 g of FFA was recovered in the filtrate. The recovery of FFA in the filtrate was almost the same even when the temperature was decreased to 5°C. More lauryl alcohol was, however, eliminated by decreasing the temperature to 5°C. In addition, the GLA content in the FFA fraction was increased from 91.0 to 92.1%, because part of the saturated and monoenoic acids was removed.

TABLE 3
Urea Adduct Fractionation of a Mixture of FFA and Lauryl Esters[a]

Temperature	Acid value	Lauryl alcohol (g)	FFA (g)	Lauryl esters (g)
Before fractionation[b]	157	1.1	15.8	3.1
25°C	188	1.0	14.7	n.d.[c]
15°C	189	0.8	13.8	n.d.[c]
5°C	193	0.5	14.0	n.d.[c]

[a]Urea adduct fractionation was performed according to Materials and Methods section. See Table 1 for abbreviation.
[b]Distillate-2 in Table 2
[c]n.d., not detected.

Large-scale purification of GLA. On the basis of the results described above, we attempted the purification of GLA from 10 kg of GLA45 oil (Table 4). Because the saponification value of GLA45 oil was 183 and the GLA content was 45.1%, the amounts of FFA and GLA in the oil were estimated to be

TABLE 4
Large-Scale Purification of GLA from GLA45 Oil

Step	Weight (kg)	Amount of FFA[a] (kg)	GLA in FFA fraction: Content (%)	GLA in FFA fraction: Amount (kg)	GLA in FFA fraction: Recovery (%)
GLA45 oil	10	9.15[b]	45.1[c]	4.13[c]	100
Hydrolysis[d]	8.69	7.95	46.3	3.68	89.1
Distillation[e]	7.55	7.51	46.3	3.48	84.2
Esterification[d]	16.54	3.41	89.5	3.05	73.9
Distillation[f]	3.87	3.15	89.4	2.82	68.2
Esterification[d]	7.61	2.53	97.3	2.46	59.6
Distillation[f]	2.67	2.28	98.1	2.24	54.2
Urea fractionation[g]	2.09	2.07	98.6	2.04	49.4

[a]The amount of FFA was calculated from its acid value, 200. See Table 1 for abbreviations.
[b]The amount of fatty acid in GLA45 oil.
[c]The content and amount of GLA in GLA45 oil.
[d]Reaction was conducted as described in the text, and the oil layer recovered was dehydrated.
[e]The first and second cycles of distillations were performed at 150°C and 0.05 mm Hg and at 160°C and 0.05 mm Hg, respectively. The distillates obtained by two cycles of distillations were combined.
[f]The distillate at 185°C and 0.2 mm Hg was recovered after removing the distillate at 105°C and 0.2 mm Hg.
[g]Carried out according to Materials and Methods section.

9.15 and 4.13 kg, respectively. The oil was hydrolyzed at 35°C for 24 h in a mixture containing 5 kg water and 250 U/g of *Pseudomonas* lipase. After the hydrolysis, 8.69 kg of the dehydrated oil layer was recovered and the acid value was 183. Thus, the FFA amount in the oil layer was calculated to be 7.95 kg, showing that the hydrolysis extent was 91.5% (7.95/8.69). Because the GLA content in the FFA fraction was 46.3%, 89.1% of GLA in the oil was recovered as FFA by the hydrolysis. The FFA fraction separated by film distillation showed an acid value of 199, and the recovery of GLA was 94.5%. The resulting FFA were esterified at 30°C for 16 h in a mixture containing 20% water with two molar equivalents of lauryl alcohol and 50 U/g of *Rhizopus* lipase. The GLA content in the FFA fraction was raised to 89.5% at the esterification extent of 52.0%. Simple distillation of the dehydrated reaction mixture separated 3.87 kg of FFA fraction, in which 18.5% of lauryl esters were present, because the acid value of the FFA was 163. The recoveries of GLA by esterification and distillation were 87.6 and 92.5%, respectively. The FFA containing lauryl esters were allowed to react again with two molar equivalents of lauryl alcohol and 70 U/g of *Rhizopus* lipase. The GLA content in the FFA fraction was raised to 97.3% at the esterification extent of 15.2%. The FFA fraction recovered by distillation was contaminated with 13.5% of lauryl esters and 1.1% of lauryl alcohol, and the esters were completely removed by urea adduct fractionation, although 0.8% of lauryl alcohol remained. The FFA with 98.6% GLA were prepared with a recovery of 49.4% of the initial content of GLA45 oil by a series of the purification procedures.

Large-scale purification of GLA was achieved by a combination of enzymatic reaction and distillation. In general, distillation can fractionate a desired component in a high yield. Actually, the distillations of the reaction mixtures after hydrolysis, esterification, and repeated esterification recovered 94.5, 92.4, and 90.1% of FFA, respectively. The simple distillation of the reaction mixture after selective esterification was performed at 0.2 mm Hg using a laboratory-scale apparatus. Because an industrial scale of rectification can be operated under higher vacuum, FFA and lauryl esters may be completely separated. Thus, urea adduct fractionation may be unnecessary for industrial purification.

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