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Enzymatic synthesis of glycerides from DHA-enriched PUFA ethyl ester by glycerolysis under vacuum

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Abstract

Pseudomonas lipase immobilized on $CaCO_3$ powder was used for the glycerolysis of $n-3$ polyunsaturated fatty acid ethyl esters to prepare nutritionally valuable glycerides. The initial ethyl ester contained 65 or 99% docosahexaenoic acid ethyl ester (DHAEE) which is very unstable and readily oxidized. The process performance was intensified by: (1) using *Pseudomonas* lipase which has good specificity for docosahexaenoic acid (DHA), (2) shifting the reaction equilibrium by evaporation of the resulted ethanol under vacuum and (3) enhancing the enzyme operational stability by immobilization on $CaCO_3$ powder. Under these conditions, over 90% conversion of DHAEE was achieved in 5 h and the oxidative deterioration of DHA was avoided. The final product contained 53% partial glyceride and, thus, had good emulsifying power. The catalyst was reused 5 times showing a very good stability in this system. Other lipases were tried for this reaction and different glyceride compositions were obtained depending on the enzyme specificity for the 1(3)-position of glycerol.

Keywords: *Pseudomonas* lipase; Glycerolysis; DHA ethyl ester; $CaCO_3$; Immobilized enzyme; DHA glycerides; Vacuum

1. Introduction

Recently, $n-3$ polyunsaturated fatty acids ($n-3$ PUFA) have attracted great interest for their nutritional and pharmaceutical applications [1,2]. It was demonstrated that dietary fats high in $n-3$ PUFA, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have positive preventing effects on different cardiovascular disorders and related diseases.

Fish oils containing 30% of $n-3$ PUFA, which are combined in mixed triacylglycerols (TAG) with ordinary fatty acids, are the main source of $n-3$ PUFA [3]. They cannot be separated directly by molecular distillation because of their relatively low stability. Their multiple double bonds are easily oxidized and polymerized at high temperatures. Therefore, TAG are transformed into ethyl or methyl esters which have lower boiling points and can be more easily separated by vacuum distillation. $n-3$ PUFA ethyl or methyl esters are the most common commercially available forms of high grade purity PUFA.

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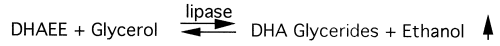
$n-3$ PUFA ethyl esters are extensively marketed as a dietary supplement in Japan. However, it was demonstrated that the TAG form was adsorbed more effectively and thus glycerides were the most desirable form as nutritional additives [4].

Immobilized lipases seem to have great potential in the substitution of inorganic catalysts in the syntheses of these compounds. Mild reaction conditions, easy separation of the components of the reaction mixture and avoidance of toxic compounds as catalysts are some of their advantages.

Esterification of free $n-3$ PUFA with glycerol in the presence or absence of organic solvent with lipases as biocatalysts was employed by several groups of researchers [5–7]. Li and Ward [5] obtained glycerides from a $n-3$ PUFA concentrate containing 80% EPA and DHA, and glycerol in hexane. The final products contained 65–75% partial glycerides and 12–18% TAG with a good incorporation of $n-3$ PUFA and 12–17% unreacted free fatty acids (FFA). The main disadvantage of this method is the use of large quantities of solvents as reaction medium and for product separation. When the reaction was carried out without organic solvent [6,7] using an excess of glycerol as reactant and reaction medium, the product contained 15% unreacted FFA, 45% TAG and 40% partial glycerides.

In both studies, the degradation of reaction mixture components is not mentioned, although FFA are the most easily degraded among the PUFA derivatives (this can be one reason why the FFA form of PUFA is usually not accepted in food, although they are the most promptly digested [4]). Serious degradation is anticipated due to the long reaction times at normal pressure even though mild temperatures were employed.

The purpose of our work is to enzymatically synthesize glycerides which are rich in DHA and have a high content of partial glycerides. These mixtures which have enhanced nutritional properties and good emulsifying power conferred by partial glycerides can be used as valuable additives in food products.



Scheme 1. Synthesis of DHA glycerides by glycerolysis of DHAEE.

Glycerolysis of DHAEE under low pressure is our approach for obtaining these glycerides (Scheme 1). The use of organic solvent is avoided by working with an excess of glycerol as reaction medium and reactant. The vacuum applied to the system ensures both high reaction rates and conversions and also prevents the oxidative degradation of the oil. Enzyme immobilization makes the reuse of enzyme feasible.

2. Experimental

2.1. Materials

4,7,10,13,16,19-Docosahexaenoic acid ethyl ester (all *Z* form): DHAEE (65%) and DHAEE (99%) were gifts from Nippon Suisan Co., Tokyo. DHAEE (65%) contained the following acyl species: 0.7% of C21-5, 5.1% of C22:1, 2.1% of C22:4, 17.1% of C22:5, 64.5 of C22:6 (DHA) and 10.5% of other acyl species. DHAEE (99%) contained more than 99% of C22:6 (DHA). Commercially available lipases were obtained from the following companies: *Pseudomonas* sp. KWI-56 lipase, culture supernatant solution (14000 U/ml hydrolytic activity) from Kurita Water Industries, Tokyo; pure *Chromobacterium viscosum* lipase (1183 U/mg hydrolytic activity), from Asahi Chemical Industry, Tokyo; *Mucor miehei* lipase (6 U/mg hydrolytic activity), from Novo Nordisk Ind., Denmark and *Rhizopus delemar* lipase (37 U/mg hydrolytic activity), from Seikagaku Kogyo, Tokyo. $CaCO_3$ (Softon 3200), which is widely used as an additive in the food industry, was a gift from Shiraishi Calcium, Osaka.

2.2. Enzyme immobilization

2.0 g $CaCO_3$ powder was added to 5 ml lipase solution that contained approximately

8000 U enzyme/ml (*Chromobacterium viscosum* or *Rhizopus delemar* lipases) or 14 000 U/ml *Pseudomonas* lipase (culture supernatant solution was used without dilution) and stirred using a magnetic bar at 300 rpm for 1 h at room temperature. Afterwards, 20 ml chilled acetone was added and the suspension was filtered through a Buchner funnel. The immobilized enzyme was washed on the filter paper with another 20 ml aliquot of chilled acetone and dried under vacuum to constant weight.

2.3. Assay of lipase hydrolytic activity

Hydrolytic activity was measured by the olive oil emulsion method without addition of surfactants as described in a previous article [8].

One activity unit is defined as the amount of enzyme which liberates 1 μmol of free fatty acid per min at 37°C.

2.4. Glycerolysis

The reaction mixture contained 1.5 mmol DHAEE, 75 mmol glycerol, 4500 U immobilized enzyme and 3% water (wt%). Unless otherwise specified, *Pseudomonas* lipase immobilized on $CaCO_3$ and DHAEE (65%) were used. The reaction mixture containing DHAEE and glycerol was mixed for 10 min in the reaction vessel and then water was added (to become 3% in the total reaction mixture) and mixed for a further 10 min to emulsify the mixture. The reactor was made of a glass vessel with a glass jacket through which water (25°C) was recirculated. The reaction mixture was mixed using a magnetic stirrer. The reaction vessel was connected to a vacuum pump via a Pirani gauge (type AVP 202N13, Okano Works, Osaka) and the pressure was adjusted with a needle valve. The reaction was started by adding the immobilized enzyme and immediately the reaction vessel was connected to the vacuum pump. The reaction conditions were: 5 mmHg pressure (unless otherwise stated), 300 rpm agitation speed and 25°C.

2.5. Analyses of the reaction mixture composition

During the reaction, samples were intermittently withdrawn from the reaction vessel and dissolved in 0.3 ml chloroform/methanol (2:1). The immobilized enzyme was separated by centrifugation at 15 000 rpm for 2 min. The removal of glycerol from the supernatant solution was achieved by extraction with water (0.2 ml). For an effective extraction of partial glycerides (soluble in water to some extent), the water layer was extracted twice with 0.3 ml chloroform each time. The total chloroform extract (approximately 0.9 ml) was concentrated to dryness by a N_2 stream. The concentrate was analyzed by a thin-layer chromatography/flame ionization detector (Iatroscan MK-5, Iatron Laboratories, Tokyo) using Chromarod S III quartz rods. All the components of the reaction mixture were successfully separated by two consecutive developments of the rods in two different solvents. The first development was done in hexane/ethyl ether/acetic acid (87:13:0.2) solvent for 10 cm at 5°C. After drying at room temperature, the rods were scanned partially. 3 cm of the rods from the origin remained unscanned. DHAEE, TAG and FFA, in this order, were effectively separated. The second development was performed with chloroform/acetone (98:2) solvent for 10 cm at room temperature. After drying the rods were scanned entirely. 1,3-Diacylglycerol (1,3-DAG), 1,2-diacylglycerol (1,2-DAG), 2-monoacylglycerol (2-MAG) and 1-monoacylglycerol (1-MAG) in the order of decreasing retention factors (R_f) were separated. The rods were scanned under the following conditions: hydrogen flow rate, 0.16 l/min; air flow rate, 2.0 l/min and scanning speed, 30 s/scan. Peak areas were calculated using an integrator (SIC Chromatocorder 12, System Instruments Co., Tokyo). The results were expressed as percentage of peak areas of the reaction mixture's components on a glycerol-free basis and may vary slightly from the actual weight percentages [9].

Ethyl alcohol concentration in the reaction mixture was determined by an enzymatic UV method [10].

Water concentration was determined with a Karl–Fischer moisture meter (MKS-1, Kyoto Electronics, Kyoto).

2.6. Operational stability of the immobilized enzyme

The initial reaction mixture for the first use of catalyst contained 3 mmol DHAEE (65%), 150 mmol glycerol, 0.6 g immobilized lipase (3200 U/mmol DHAEE) and 5% water. This water concentration was used in order to avoid the enzyme inactivation in the later stage of the process due to the loss of water. The product was separated from the reaction mixture by extraction with 10 ml of hexane three times. The remaining hexane was removed from the glycerol phase containing the biocatalyst by evaporation under vacuum. For each cycle the experiment was scaled down with respect to the weight of the recovered glycerol phase. Fresh substrate was added and a new reaction began.

3. Results and discussion

3.1. Choice of lipases for glycerolysis

It is well known that usually, lipases exhibit a low specificity for $n-3$ PUFA and this property was used for selective hydrolysis of fish oils when glycerides enriched in PUFA were obtained [11] or for purification of DHA by selective esterification of fish oil hydrolysates [12]. Therefore, finding a lipase with a good specificity for this kind of fatty acids is of major importance.

Pseudomonas lipase immobilized on $CaCO_3$ was used successfully in our laboratory for 1-MAG production by glycerolysis of olive oil [8]. This lipase has no positional specificity in glyceride synthesis and has a good resistance towards organic solvents [8,13]. For these reasons immobilized *Pseudomonas* lipase was used in most of our experiments (Fig. 1).

Three other lipases were tried for the glycerolysis of DHAEE under 5 mmHg vacuum (Fig. 2). *Chromobacterium viscosum* lipase immobilized on $CaCO_3$ has no positional specificity and acted similarly to *Pseudomonas* lipase (the product had almost the same composition of glycerides), but the reaction was slower. *Mucor miehei* lipase had a very low hydrolytic activity (6 U/mg) and could not be immobilized. It was used as an aqueous solution and the water content of the initial reaction mixture was higher than usual (8.6%) because of the large amount of enzyme preparation needed. Even in these conditions, it showed a good activity. Due to the enzyme preference for the 1(3)-position of glycerol, the product at 12 h contained: 10.68% unreacted DHAEE, 13.24% TAG, 3.55% FFA, 39.56% 1,3-DAG, 3.47% 1,2-DAG and 29.30% 1-MAG. Partial glycerides counted for more than 70% and thus the product had very good emulsifying properties. *Rhizopus delemar* lipase is well known for its very low activity for DHA [12] and, thus, to avoid any doubt related to the enzyme specificity towards the acyl species in the ethyl ester mixture, DHAEE (99%) was used. The enzyme

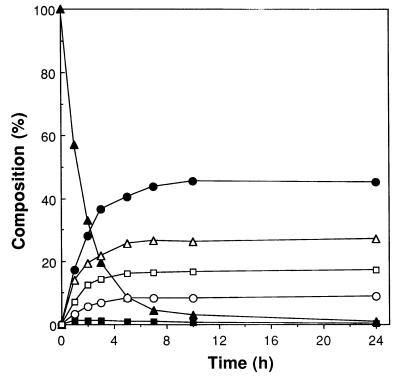


Fig. 1. Glycerolysis of DHAEE at 5 mmHg pressure. The initial reaction mixture contained: 2 mmol DHAEE, 100 mmol glycerol, 0.565 g catalyst (4000 U catalyst/mmol DHAEE) and 3% water. DHAEE (▲), TAG (●), FFA (■), 1,3-DAG (△), 1,2-DAG (○) and 1-MAG (□).

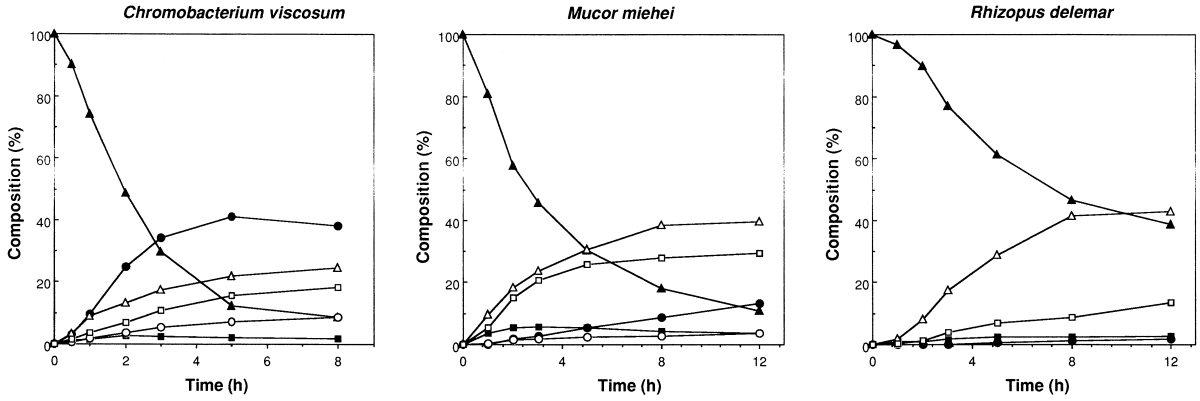


Fig. 2. Comparative reaction time courses at 5 mmHg pressure for three lipases with different specificities: DHAEE (▲), TAG (●), FFA (■), 1,3-DAG (△), 1,2-DAG (○) and 1-MAG (□). *Chromobacterium viscosum*: 4000 U/mmol DHAEE immobilized lipase (14 U/mg) were used. *Mucor miehei*: because of the low activity of the free lipase (6 U/mg hydrolytic activity) which made immobilization impossible, the enzyme (3000 U/mmol DHAEE) was added as a solution and the initial water content of the reaction mixture was 8.6%. $CaCO_3$ powder (200 mg/mmol DHAEE) was added separately. *Rhizopus delemar*: 3000 U/mmol DHAEE of immobilized lipase (16.6 U/mg hydrolytic activity) and DHAEE (99%) were used.

exhibited a very good 1(3)-specificity (1,3-DAG and 1-MAG contents combined were approximately 95% among the reaction products at 12 h), but obviously, the low specificity for DHA caused the low reaction rate.

These data demonstrate that in this system, the choice of enzyme can lead to different glyceride compositions depending on the designed use of the product.

3.2. Glycerolysis at normal pressure

At normal pressure (Fig. 3), the reaction rate was high during the first hour (29% conversion of DHAEE). This fact shows that the enzyme has a good activity. Afterwards, the reaction became very slow and the equilibrium was reached after 5 h at approximately 38% consumption of the initial DHAEE. The reaction was accompanied by rapid degradation of the oil, which was visualized by the enlargement of the peaks around the origin in TLC/FID analysis. At 5 h of reaction time, the content of degradation products became significant affecting the reaction yield and possibly the enzyme activity.

3.3. Effect of vacuum on glycerolysis

Vacuum has a double effect in this system: it helps to hinder the oil degradation and pushes the reaction equilibrium towards a high DHAEE conversion.

An appropriate value of vacuum is necessary for the effective removal of formed ethanol

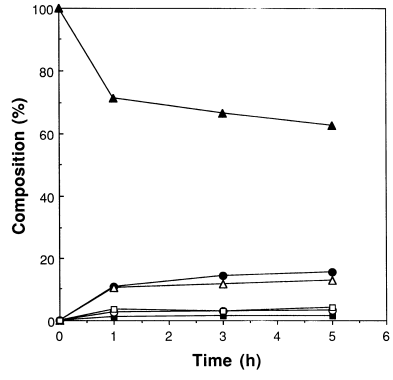


Fig. 3. Glycerolysis of DHAEE at normal pressure. The initial reaction mixture contained 2 mmol DHAEE, 100 mmol glycerol and 0.565 g catalyst (4000 U catalyst/mmol DHAEE) and 3% water. DHAEE (▲), TAG (●), FFA (■), 1,3-DAG (△), 1,2-DAG (○) and 1-MAG (□).

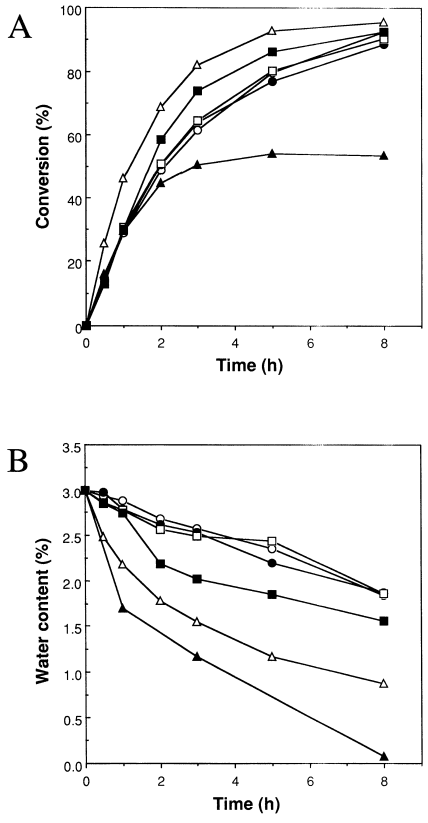


Fig. 4. Effect of vacuum: (A) on reaction time-course and (B) on water removal. The initial reaction mixture contained 3200 U catalyst (200 mg)/mmol DHAEE. Time-course of DHAEE conversion and water removal at: 20 mmHg (○), 15 mmHg (●), 10 mmHg (□), 7 mmHg (■), 5 mmHg (△) and 3 mmHg (▲).

from the reaction mixture and thus for shifting the reaction equilibrium towards complete consumption of the substrate (Scheme 1). Ethanol and water are the only volatile components in this system and ethanol is removed as a mixture with water. The reaction time course and the time course of water removal were followed at different values of vacuum (Fig. 4A and B).

From 20 to 10 mmHg, water and implicitly the formed ethanol were removed only by spontaneous evaporation. This is the reason why the curves of the reaction time course and water removal were very close for this interval of pressure (Fig. 4A and B). 38% of the initial amount of water was evaporated after 8 h.

At 7 mmHg, the vacuum become more efficient increasing not only the reaction rate, but also the rate of water removal. The mixture of water and ethanol reached its boiling point in this system at 5 mmHg. This lead to the efficient removal of ethanol which accelerated the reaction rate and made possible the almost complete conversion of DHAEE in 8 h of reaction time. However, 70% of the initial amount of water was also removed and water content reached a dangerous level (0.9%) at which the enzyme might be inactive (Fig. 4B and Table 2).

Water was removed too quickly at 3 mmHg and this fact resulted in the early inactivation of enzyme. No improvement in the reaction rate was observed, even in the first hour of reaction. At 3 h, water content reached the limit for enzyme inactivation (Fig. 4B and Table 2) and the reaction stopped after 5 h at only 54% conversion of the substrate (Fig. 4A). The enzyme inactivation was reversible and full recovery was observed when 3% water was added to the previous reaction mixture after 8 h of reaction time at 3 mmHg. The reaction was contin-

Table 1
Initial rates and reaction mixture compositions at 24 h of reaction time for different glycerol to DHAEE molar ratios

Glycerol/DHAEE (molar)	Initial reaction rate[a] (wt%/h)	Composition of final reaction mixture (%)					
		1,3-DAG	1,2-DAG	1-MAG	TAG	DHAEE	FFA
25[b]	18.57	22.6	6.65	9.41	31.7	28.19	1.45
50	42.77	27.09	8.83	17.33	45.29	1.04	0.42
75	52.27	23.22	8.03	18.64	48.5	1.01	0.59

[a] The initial reaction rate was defined as the initial rate of conversion of ethyl ester: (100 − wt% DHAEE)/h.
[b] The reaction did not reach the equilibrium in 24 h of reaction time.

Table 2
Effect of water content on initial reaction rate and FFA formation rate

Water content (%)	Initial reaction rate[a] (wt%/h)	Initial FFA formation rate[b] (wt%/h)
1	3.11	0.84
1.5	16.37	1.09
3	46.04	1.62
5	40.13	3.44

[a]The initial reaction rate was defined as the initial rate of conversion of DHAEE: (100 − wt% DHAEE)/h.
[b]Initial FFA formation rate is the change in FFA content (wt%) per h.

ued at 5 mmHg and the substrate was almost completely consumed after 16 h (98.5% DHAEE conversion).

Vacuum is a very important parameter for the process and special precautions have to be taken when choosing its value because the effect of shifting the reaction equilibrium is counterbalanced by the water removal which affects the enzyme activity.

A vacuum value of 5 mmHg conferred good performances for glycerolysis (Fig. 1). The higher reaction rates assured a consumption of more than 90% of DHAEE after 5 h of reaction time. The FFA content remained at a low value throughout the reaction time. At 24 h, 99% of DHAEE was converted into glycerides and the FFA content was less than 0.5%. The final content of the partial glycerides reached 53%, which means that the product had good emulsifying properties. 2-MAG were not detected among the glycerides.

Ethanol was removed effectively from the reaction mixture (95% of the formed ethanol, estimated from the consumption of DHAEE, was removed after 10 h), but also half of the initial amount of water. The latter might result in decreasing the enzyme activity and its eventual inactivation.

3.4. Optimization of other parameters

The molar ratio of glycerol to DHAEE was optimized with respect to high reaction rates and yields of partial glycerides (Table 1). A molar ratio of 50/1 gave a good initial reaction rate and approximately 53% partial glycerides in the product at 24 h. A further increase in the ratio to 75/1 did not lead to a higher content of the partial glycerides although the initial reaction rate was higher. For these reasons, 50/1 molar ratio of glycerol to DHAEE was used for all the experiments.

The optimum amount of the catalyst added to the reaction mixture was 3000 U catalyst/mmol DHAEE. The optimum content of $CaCO_3$ particles in the reaction mixture was 300 mg/mmol DHAEE.

A certain amount of water in the reaction mixture is necessary for a good enzyme activity, but this content should keep the hydrolysis at a low value. At 1% water in the initial reaction mixture the enzyme was almost inactive (Table 2). At higher contents the reaction rate increased gradually and reached the maximum at

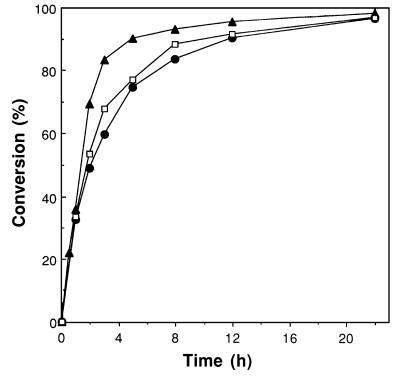


Fig. 5. Reusability of biocatalyst. Time-courses of the conversion during: cycle 1 (▲), cycle 3 (●) and cycle 5 (□). Conditions as described in Section 2.6.

3% water. Further increase did not improve the reaction rate, but the free fatty acid content doubled due to hydrolysis.

3.5. Operational stability of immobilized enzyme

The operational stability of the immobilized enzyme is very important for the production costs. The reusability of the immobilized enzyme was studied over five consecutive batch reactions. After 5 cycles of utilization, the reaction rate and DHAEE conversion after 24 h remained almost unchanged (Fig. 5).

The major purpose of this study was to obtain basic data to attain a high yield and high productivity of the lipase-catalyzed conversion of DHAEE to partial DHA glycerides for further industrial implementation. A bioreactor system with two ancillary units: one for the evaporation of the ethanol resulted from glycerolysis and the other for the adjustment of the water content of the reaction mixture could be a promising configuration [14].

Acknowledgements

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References

[1] A.P. Simopoulos, R.R. Kifer, R.E. Martin (Eds.), Health Effect of Polyunsaturated Fatty Acids in Seafoods, Academic Press, London, 1986.
[2] J.E. Kinsella, Seafoods and Fish Oils in Human Health and Disease, Marcel Dekker, New York, 1987.
[3] F.D. Gunstone, J.L. Harwood, F.B. Padley, in: Chapman and Hall (Eds.), The Lipid Handbook, 2nd ed., University Press, Cambridge, 1994, ch. 1, p. 5.
[4] L.D. Lawson, B.G. Hughes, Biochem. Biophys. Res. Commun. 152 (1988) 328.
[5] Z.Y. Li, O.P. Ward, J. Am. Oil Chem. Soc. 70 (1993) 745.
[6] K. Osada, K. Takahashi, M. Hatano, J. Jpn. Oil Chem. Soc. 39 (1990) 50.
[7] K. Osada, K. Takahashi, M. Hatano, J. Am. Oil Chem. Soc. 67 (1990) 21.
[8] R. Rosu, Y. Uozaki, Y. Iwasaki, T. Yamane, J. Am. Oil Chem. Soc. 74 (1997) 445.
[9] T. Tatara, T. Fujii, T. Kawase, M. Minagawa, Lipids 18 (1983) 732.
[10] H.O. Beutler, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, vol. 6, 3rd ed., Verlag Chemie, Weinheim, 1984, pp. 598–606.
[11] A. Sugihara, Y. Shimada, N. Takada, T. Nagao, Y. Tominaga, J. Ferment. Bioeng. 82 (1996) 498.
[12] Y. Shimada, A. Sugihara, H. Nakano, T. Kuramoto, T. Nagao, M. Gemba, Y. Tominaga, J. Am. Oil Chem. Soc. 74 (1997) 97.
[13] T. Iizumi, K. Nakamura, T. Fukase, Agric. Biol. Chem. 54 (1990) 1253.
[14] Y. Yoshida, M. Kawase, C. Yamaguchi, T. Yamane, J. Am. Oil Chem. Soc. 74 (1997) 261.