Studies of the Fatty Acid Specificity of the Lipase from *Rhizomucor miehei* Toward 20:1n-9, 20:5n-3, 22:1n-9 and 22:6n-3

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ABSTRACT: The fatty acid specificity of the lipase from *Rhizomucor miehei* toward 20:1n-9, 20:5n-3, 22:1n-9 and 22:6n-3 has been determined by comparing the alcoholysis (by *n*-propanol) of various mixtures of C_{20} and C_{22} fatty acids (FFA) or the corresponding ethyl esters (FAEE) in *n*-heptane. For all the fatty acids examined, the degree of conversion was much higher when using FFA rather than FAEE. When comparing the experiments with either single FAEE or FAEE mixtures, it was found for all four fatty acids that the degree of conversion depended on whether the FAEE was alone or together with other fatty acids in the reaction mixture. The lipase showed a strong specificity toward 20:1n-9, whereas the polyunsaturated fatty acids were much poorer substrates, especially 22:6n-3. The degrees of conversion for the two n-3 fatty acids show a clear preference for 20:5n-3 over 22:6n-3, not only when present alone but also in the different mixtures examined. The results obtained in the present experiments therefore suggest that when using the lipase from *R. miehei* for enrichment of fish oils with n-3 fatty acids, it should not only be possible to diminish the content of 20:1 and 22:1 present in the outer positions in the triacylglycerols, but also to incorporate relatively more 20:5n-3 than 22:6n-3 into the triglycerides.

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KEY WORDS: Docosahexaenoic acid, eicosapentaenoic acid, fatty acid specificity, interesterification, lipase, long-chain monoenoic acid, marine oil fatty acids, organic solvents, reaction kinetics, *Rhizomucor miehei*.

In recent years, health aspects of marine fat have been studied extensively. This interest was initiated by epidemiological studies of the relation between coronary heart diseases (CHD) and fat intake by Greenland Eskimos, carried out by Bang and Dyerberg (1), who found a decreased incidence of CHD in this population compared to Danes.

The beneficial effect has been attributed to the intake of n-3 polyunsaturated fatty acids (n-3 PUFA): 20:5n-3 (eicosapentaenoic acid, EPA) and 22:6n-3 (docosahexaenoic acid, DHA) in particular, which are characteristic of marine fat. Marine oil may also contain appreciable amounts of 20:1 and 22:1 fatty acids, and investigations with different animal species have shown that these fatty acids may transiently accumulate in their heart triacylglycerols.

Compared to Danes, Greenland Eskimos consume relatively higher amounts of marine fat and thereby higher amounts of 20:1 and 22:1, which also are reflected in their plasma fatty acid distribution (2). At the same time, the frequency of CHD is low; therefore, it is not likely that ingestion of the long-chain monoenoic fatty acids from fish oil involves a health risk (1).

EPA and DHA are bound in triacylglycerols and in an amount up to 30% in most fish oils. A higher concentration of EPA and DHA cannot be obtained by simple physical fractionations due to the great variety of fatty acid combinations in the fish oil triacylglycerols. However, by using an n-3 PUFA concentrate achieved by urea complexation (3) as a source of free fatty acids (FFA), natural fish oils have been enriched to a level of above 60–65% n-3 PUFA by interesterification with a specific lipase from *Rhizomucor miehei* (4,5).

Because many of the marine fish oils contain reasonable amounts of 20:1 and 22:1 fatty acids, it is of great interest to elucidate whether the lipase from *R. miehei* (Lipozyme; Novo-Nordisk, A/S Bagsvaerd, Denmark), commercially available, shows any preference among the C_{20} and C_{22} fatty acids.

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are enzymes that catalyze hydrolysis of the ester bonds of triacylglycerols (6). The specificities of lipases are the determining factors for the possible enrichment of marine triacylglycerols with nutritionally important n-3 fatty acids. As pointed out by Deleuze *et al.* (7), the competition may be examined by comparing the extent of alcoholysis with *n*-propanol. In the present work, we have compared the rate of alcoholysis (by *n*-propanol) for various mixtures of C_{20} and C_{22} fatty acids or the corresponding ethyl esters (FAEE) in *n*-heptane (8). By means of the reaction kinetics, described by Deleuze *et al.* (7), the usefulness of the *R. miehei* lipase (Lipozyme) was evaluated in relation to interesterification of fish oils and n-3 PUFA.

EXPERIMENTAL PROCEDURES

Materials. Lipozyme, an insoluble immobilized preparation of the lipase from *R. miehei*, was kindly provided by Novo-

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Nordisk A/S. The activity of the lipase was 30 BIU (BIU: one batch interesterification unit is defined as 1 μmol of palmitic acid incorporated into triolein per minute). The enzyme preparation was insoluble in the solvents used in this study; it contained 10% water, which is necessary for optimal activity (9).

The following FFA and FAEE were purchased from Nu-Chek-Prep Inc. (Elysian, MN): 22:6n-3 (purity >97%), 20:1n-9 (purity >99%), 22:1n-9 (purity >99%) and 17:0 (purity >99%). 20:5n-3 FAEE (purity >95%) was kindly donated by Dr. S. Galloway, Southeast Fisheries Center (Charleston, SC). The FFA of 20:5n-3 was prepared by saponification of the ethyl ester (10). The ethyl esters (FAEE) are included in this experiment because they occur in the urea concentrates commonly used in enzymatic interesterification of fish oils (3,5).

The solvents *n*-heptane, diethyl ether, chloroform, glacial acetic acid and *n*-propanol were of analytical grade and purchased from Merck Inc. (Darmstadt, Germany).

Experimental design. The relative rates of esterification of various fatty acids are determined by a modification of the procedure described by Rangheard *et al.* (8). The relative esterification rates for different fatty acid species were determined by reacting a 0.1 M solution of FFA or FAEE, respectively, with a final concentration of 2 M of *n*-propanol. The suspension was incubated at 37°C under maximal reciprocal agitation.

To simulate the competition during interesterification of normal triacylglycerol mixtures, mixtures of selected FAEE also were included. The reaction was carried out under nitrogen and in the dark to avoid degradation of the PUFA by oxidation. At regular time intervals, 0.2-mL aliquots were withdrawn from the reaction mixture by a syringe, and the formation of fatty acid propyl esters (FAPE), as well as the remaining FFA or FAEE, were analyzed by gas–liquid chromatography (GLC). The reaction mixtures were incubated at 37°C for 48 h to achieve a reasonable degree of conversion for 22:6n-3 (5).

Product isolation. In experiments with FFA, a separation procedure was developed for FAPE and FFA on a small silica column (Bond Elut; Varian Associates, Harbor City, CA). FAPE and FFA were eluated with *n*-heptane/diethylether 97:3 (vol/vol) and chloroform/acetic acid 99:1 (vol/vol), respectively (5).

Gas-chromatographic analysis. Ethyl, methyl and propyl esters of fatty acids were separated by GLC in a Hewlett-Packard HP 5890 gas chromatograph (Palo Alto, CA) with a flame-ionization detector (240°C) and a split injector (240°C). The column used was a fused-silica capillary SP2330 30 m × 0.25 mm i.d.; film thickness, 0.2 μm; Supelco Inc., St. Louis, MO). Helium was the carrier gas (1 mL/min; split, 1:10).

The temperature program was as follows: initial temperature 140°C, then increased to 200°C at 3°C/min, held for 1 min at 200°C and then increased at 3°C/min to 220°C and finally held for 9 min. Quantitative data were obtained by integrating of peak areas with a Hewlett-Packard integrator (model HP 3396A).

Calculation. Reaction kinetics were calculated from the degrees of conversion (C_n) of the FFA or FAEE (of a fatty acid containing *n* carbon atoms) into FAPE, as described by Rangheard *et al.* (8). The following equation was used:

$$C_n = (A_p/PM_p)/(A_p/PM_p + A_x/PM_x) \quad [1]$$

where A_p and A_x are the respective areas of the FAPE and FFA or FAEE peaks in GLC, and PM_p and PM_x the corresponding molecular weights of the esters or fatty acids.

Statistical analysis. Data were interpreted by one-way analysis of variance. Significance was expressed at the $P \leq 0.05$ level (11).

RESULTS AND DISCUSSION

Lipase-catalyzed reactions in organic solvents, such as ester synthesis and transesterification, follow classical Michaelis-Menten kinetics with the intermediate formation of an acyl–enzyme complex (7,12).

When two substrates (A_1 and A_2) are present at the same time in the reaction mixture, a simple parameter, the competitive factor (a) as proposed by Deleuze *et al.* (7), can be used to describe the kinetics of the reaction:

$$\alpha = \log(1 - C_{ref})/\log(1 - C_n) \quad [2]$$

where C_{ref} is the degree of conversion of the substrate chosen as reference, and C_n the degree of conversion of the sample.

By definition, the competitive factor for the reference substrate is equal to 1, and all other competitive factors are higher than 1. Rangheard *et al.* (8) defined $1/\alpha$ for a substrate as a convenient measure of the specificity for this substrate.

In our experiments, the general reactions can be described as:

$$\text{FAEE: } RCOOC_2H_5 + C_3H_7OH \rightleftarrows RCOOC_3H_7 + C_2H_5OH \quad [3]$$

$$\text{FFA: } RCOOH + C_3H_7OH \rightleftarrows RCOOC_3H_7 + H_2O \quad [4]$$

A high concentration of *n*-propanol was used to shift the equilibrium to the formation of propyl esters and to increase the reaction rate (8). To test the fatty acid specificity and whether the fatty acids should be used as free acids (FFA) or esters (FAEE), the series of experiments described in Table 1 were conducted.

In experiment no. 11, the ratios between 20:1, 22:1, 20:5 and 22:6 resembled a menhaden (*Brevoortia tyrannus*) oil, which is characterized by low concentrations of the long-chain monounsaturated fatty acids 20:1 and 22:1. By contrast, in experiment no. 12, the ratios between 20:1, 22:1, 20:5 and 22:6 resembled sandeel (*Ammodytes lancea*) oil with relatively high amounts of the long-chain monoenoic fatty acids.

In Figure 1, the time-dependent degrees of conversion are shown for the four fatty acids 20:1n-9, 20:5n-3, 22:1n-9 and

TABLE 1
Experimental Design

Experiment no.	Fatty acid (FFA or FAEE)[a]
1	20:5n-3, FFA
2	20:5n-3, FAEE
3	20:1n-9, FFA
4	20:1n-9, FAEE
5	22:6n-3, FFA
6	22:6n-3, FAEE
7	22:1n-9, FFA
8	22:1n-9, FAEE
9	20:1n-9/20:5n-3, FAEE 1:1
10	22:1n-9/22:6n-3, FAEE 1:1
11	20:5/22:6/20:1/22:1, FAEE 1:0.5:0.1:0.02
12	20:5/22:6/20:1/22:1, FAEE 1:1.3:0.4:0.9
13	20:5/22:6/20:1/22:1, FAEE 1:1:1:1

[a]FFA, free fatty acid; FAEE, fatty acid ethyl ester.

22:6n-3, respectively. The highest degree of conversion after 48 h was observed for 20:1n-9 and 22:1n-9 in the experiments with a single FFA in the reaction mixture (Experiments 1, 3, 5 and 7; see Table 1). For 20:1n-9 ethyl ester, alone or in mixtures, high degrees of incorporation also were observed (Experiments 4, 9–13, see Table 1).

For all the fatty acid mentioned above, the degree of conversion was much higher when using FFA than FAEE (alone or in mixture), which could be statistically proven by an analysis of variance, assuming that the experimental errors are randomly and normally distributed (11).

When comparing the experiments with either single FAEE (Experiments 2, 4, 6 and 8) or FAEE mixtures (Experiments 9–13), statistical evidence confirmed that the degree of conversion depended on whether the fatty acid was used alone or in mixture with other fatty acids.

The competition between the various fatty acids in the esterification process has been calculated by the formulas developed by Rangheard *et al.* (8) for C_{18} fatty acids. By use of their approach, the 20:1n-9 fatty acid was found to have the highest degree of conversion and, therefore, was chosen as

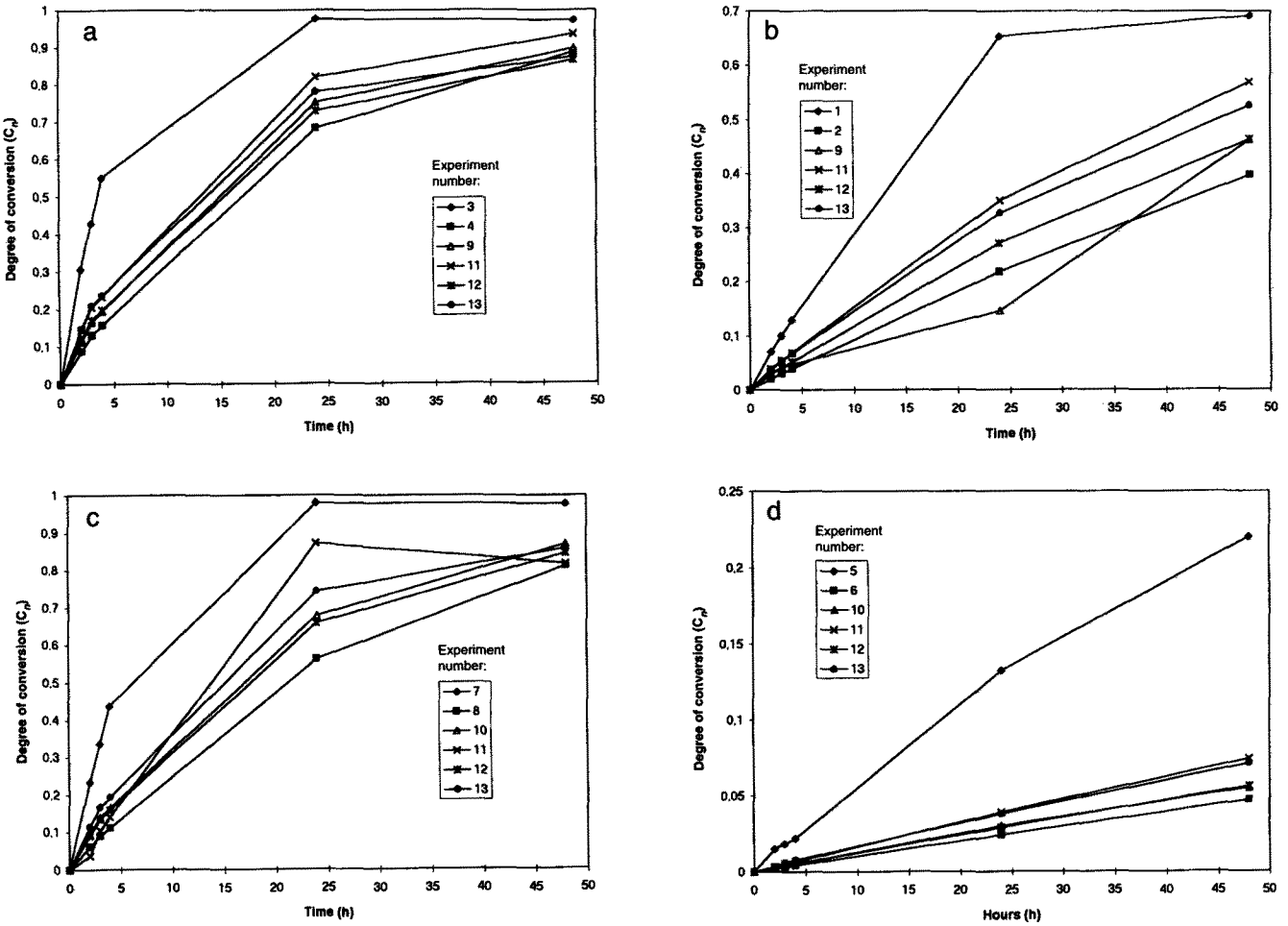

FIG. 1. Time-dependent degree of conversions (C_n) for the fatty acids examined: (a) 20:1n-9, (b) 20:5n-3, (c) 22:1n-9, (d) 22:6n-3. The experimental mixtures are described in Table 1.

the reference. Rangheard *et al.* (8) have shown that, as long as the degree of conversion is lower than 30% and the *n*-propanol is in excess in the reaction mixture, it is possible to calculate the competitive factor by using Equation 2.

The competitive factors related to the different experiments are shown in Table 2; they are calculated after 2 h of incubation, when the degree of conversion is less than 0.3 for all experiments.

Table 2 shows that the competitive factors (relative to 20:1n-9) for 20:5n-3 and 22:1n-9 remain practically constant in the different experiments, which is in accordance with data of Rangheard *et al.* (8) for C_{18}-fatty acids. By contrast, the competitive factor for 22:6n-3 varied considerably between the different experiments. A closer examination of the 22:6n-3 ethyl ester used in these experiments showed that it was mildly oxidized, and this circumstance may explain the fluctuation in the competitive factors. This factor should, therefore, be judged from the experiment with the FFA of 22:6n-3.

The specificity of the lipase from *R. miehei* (Lipozyme) defined as $1/\alpha$ by Rangheard *et al.* (8), where a is the competitive factor, is illustrated in Figure 2. The term specificity is an expression of the discrimination for substrates competing for the active site of an enzyme.

In all experiments, after two hours of incubation, the lipase showed the highest preference for 20:1n-9, which was therefore used as the reference compound. The lipase had a lesser preference toward 22:1n-9, whereas the PUFA were much poorer substrates, especially 22:6n-3. The lipase specificity decreased with increasing unsaturation, when dealing with 1, 5 and 6 double bonds.

Rangheard *et al.* (8) have shown that the lipase from *R. miehei* has a slightly higher activity toward 18:1n-9 compared to the corresponding fatty acids with two and three double bonds, which are nearly equal. At the same time, a higher specificity toward the unsaturated fatty acids than toward the corresponding saturated fatty acid was observed.

The reduced reactivity of 20:5n-3 and 22:6n-3 found in our experiments may be due to steric hindrance effected by the three-dimensional structure of the fatty acid or the position of the double bond near the carboxyl group.

The lipase from *R. miehei* seems ideal, when fish oils with monoenoic acids in the outer positions are reacted with n-3 concentrates. The urea fractionation eliminates the long-chain monoenoic acids, and in the reacting triacylglycerol, the original monoenoic acids in the outer positions are replaced by n-3 fatty acids.

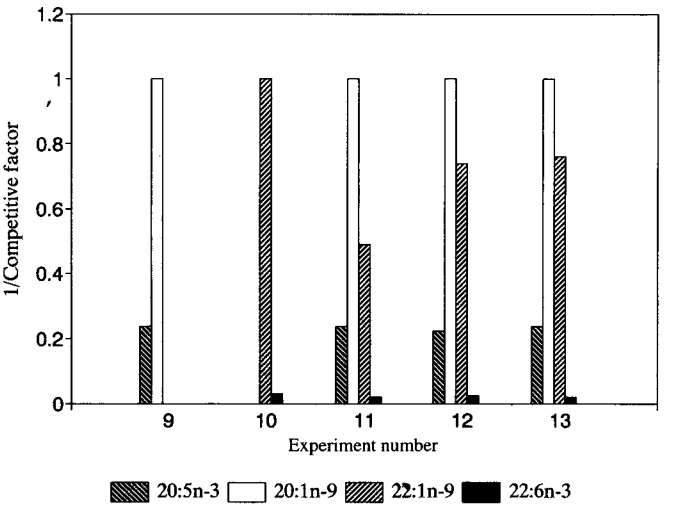

FIG. 2. The fatty acid specificity of the lipase from *Rhizomucor miehei* in experiments 9 to 13. Data are calculated with 20:1n-9 as reference compound.

Further investigations (Pedersen and Hølmer, unpublished results) have indicated that 20:1 and 22:1 primarily are situated in the *sn*-1 and *sn*-3 positions in triacylglycerols from various fish species, which means that even if fish oils to be used for interesterification were to contain relatively large amounts of these fatty acids, the lipase would substitute them primarily with EPA and DHA from an n-3 concentrate.

The degrees of conversion for the two n-3 fatty acids show a clear preference for EPA over DHA, not only when present alone but also in the different mixtures examined. The reason for this great difference in the lipase specificity can probably be explained by the fact that DHA, in contrast to EPA, has a double bond nearer its carboxylic end. A similar investigation by Haraldsson (13) has shown that a double bond close to the carboxylic end of the fatty acid has a negative influence on the suitability of the fatty acid as a substrate for the lipase from *R. miehei*.

Yamane *et al.* (14) recently studied *R. miehei* lipase in an attempt to increase the n-3 PUFA content of fish oil by acidolysis in a bioreactor system. They obtained an equal incorporation of EPA and DHA in the triacylglycerols, but this might be explained by the high concentration of 20:1 in both the original cod liver oil and in the enriched FFA fraction used.

Lipases from other microorganisms have also been studied. Sonnet *et al.* (15) examined the selectivity of *Geotrichum candidum* lipase and reported a high preference for oleic acid compared to two longer-chain monoenoic fatty acids. Studies with lipase from *Candida cylindracea* by Tanaka *et al.* (16) showed a possible interference of specific triglyceride structures, particularly in those that contain DHA. These results

TABLE 2
Competitive Factors (α) Calculated After Two Hours of Incubation

Experiment no.	EPA	20:1n-9	DHA	22:1n-9
1,3,5 and 7[a]	5.05	1	24.27	1.38
2,4,6 and 8[b]	4.55	1	46.01	1.46
9	4.22	1	—	—
10	—	—	32.86	1
11	4.24	1	51.75	2.05[c]
12	4.50	1	42.55	1.35
13	4.22	1	52.92	1.31

[a]Pure FFA. See Table 1 for abbreviations; EPA, eicosapentaenioc acid; DHA, docosahexaenoic acid.
[b]Pure FFAE.

emphasized the importance of not only the overall fatty composition of the fish oils used for PUFA enrichment reactions, but also the triglyceride structure and the selection of microorganism for the isolation of appropriate enzymes. The results obtained in the present experiments suggest that, when using the lipase from *R. miehei,* it may be possible to diminish the content of 20:1 and 22:1 present in the outer positions in the triacylglycerols of fish oils and also to incorporate relative more 20:5n-3 than 22:6n-3 into the oil. The degree of incorporation will greatly depend on the composition of the n-3 concentrate and the state of the fatty acids because FFA are much more reactive than the corresponding ethyl esters.

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REFERENCES

1. Bang, H.O., and J. Dyerberg, *Acta. Med. Scand. 192*:85 (1972).
2. Carrol, K.K., and C.J. Woodward, *Marine Biogenic Lipids, Fats and Oils*, Vol. II, edited by R.G. Ackman, CRC Press, Boston, 1989, p. 435.
3. Haagsma, N., C.M. van Gent, J.B. Luten, R.W. de Jong and E. van Doorn, *J. Am. Oil Chem. Soc. 59*:117 (1982).
4. Haraldsson, G.G., P.A. Höskuldsson, S.T. Sigurdsson, F. Thorsteinsson and S. Gudbjarnason, *Tetrahedron Lett. 30*:1671 (1989).
5. Pedersen, S., Thesis, Oparbejdning af langkædede n-3 fedtsyrer fra fiskeolier, the Technical University of Denmark, 1992.
6. Verger, R., in *Lipases,* edited by B. Borgstrom, and H.L. Brockman, Elsevier, Amsterdam, 1984, p. 83.
7. Deleuze, H., G. Langrand, H. Millet, J. Baratti, G. Buono and C. Triantaphylides, *Biochim. Biophys. Acta 911*:117 (1987).
8. Rangheard, M.-S., G. Langrand, C. Triantaphylides and J. Baratti, *Ibid. 1004*:20 (1989).
9. Hansen, T.T., and P. Eigtved, in *Conference on Emerging Technologies in the Fats and Oils Industry*, edited by A.R. Baldwin, American Oil Chemists' Society, Champaign, 1986.
10. Christie, W.W., *Gas Chromatography and Lipids, A Practical Guide*, The Oily Press, Ayr, 1989, p. 65.
11. Duncan, A.J., *Quality Control and Industrial Statistics*, 4th edn., Richard D. Irwin Inc., Homewood, 1974, pp. 60–62.
12. Verger, R., and G.H. de Haas, *Ann. Rev. Biophys. Bioenerg. 5*:77 (1976).
13. Haraldsson, G.G., *INFORM 1*:305 (1990).
14. Yamane, T., T. Suzuki and T. Hoshino, *J. Am. Oil Chem. Soc. 70*:1285 (1993).
15. Sonnet, P.E., T.A. Foglia and M.W. Baillargeon, *Ibid. 70*:1043 (1993).
16. Tanaka, Y., T. Funada, J. Hirano and R. Hashizume, *Ibid. 70*:1031 (1993).

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