Evaluation of Ethanolysis with Immobilized *Candida antarctica* Lipase for Regiospecific Analysis of Triacylglycerols Containing Highly Unsaturated Fatty Acids

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ABSTRACT: The suitability of a recently proposed method based on ethanolysis with immobilized *Candida antarctica* lipase for regiospecific analysis of oils containing long-chain PUFA such as EPA and DHA has been evaluated using selected marine oils and regio-isomerically enriched synthetic TAG substrates. 1,3-Regioselectivity of the lipase was enhanced when the ethanolysis was conducted in a high excess of ethanol, typically 10–50 times by weight of the oil. This enabled the reaction to be conducted on a milligram scale. However, irrespective of the ethanol-to-oil ratio, *C. antarctica* lipase released FA from TAG at different rates depending on the degree of unsaturation and/or chain length of the FA. Differences in lipolysis rates were particularly significant for EPA and DHA, with EPA released faster than DHA. Although DHA can be measured with reasonable accuracy by ethanolysis with *C. antarctica,* the method requires further optimization before it can be adopted for reliable regiospecific analyses that are as accurate as those obtainable by ^{13}C NMR analysis for all major FA occurring in oils rich in long-chain PUFA.

Paper no. J11359 in *JAOCS 83,* 923–927 (November 2006).

KEY WORDS: *Candida antarctica*, docosahexaenoic acid, eicosapentaenoic acid, FA composition, lipase, MAG, marine oils, ^{13}C NMR, regiospecific analysis, TAG.

Methods used for regiospecific analysis of FA can be divided into three broad categories: enzymatic, chemical, and spectroscopic. The enzymatic method is based on release of the FA attached to the *sn*-1 and *sn*-3 positions of glycerol by partial or complete hydrolysis in the presence of a 1,3-specific lipase followed by determination of the FA profile of the residual 2-MAG (1,2). The chemical method involves partial deacylation of the TAG with a Grignard reagent such as ethyl magnesium bromide or allyl magnesium bromide (2–4) and determination of the FA profile of the resulting 1,3- or 1,2-(2,3)-DAG. In both cases, the FA profile at the *sn*-1(3) positions is subsequently calculated by difference from the total FA profile. In both enzymatic and chemical methods, it is assumed that partial deacylation occurs randomly to yield representative mixtures of MAG and DAG. NMR, especially ^{13}C NMR, is the most useful spectroscopic technique for regiospecific FA analysis. Characteristic signals in the olefinic and carbonyl regions of the ^{13}C NMR spectrum allow unambiguous determination of the positional distribution of FA between the *sn*-2 and *sn*-1(3) positions with the exception of a few. ^{13}C NMR carbonyl signals are particularly useful for PUFA in which the polyunsaturated system is located close to the carboxyl carbon, such as in DHA (5–7).

The enzymatic method is considered unsuitable for regiospecific analysis of long-chain (LC) PUFA such EPA and DHA, as they have been shown to resist hydrolysis by lipase (8). This leaves ^{13}C NMR and deacylation using Grignard reagent as the methods deemed suitable for analyzing the positional distribution of these nutritionally important *n*-3 LC-PUFA. Although ^{13}C NMR is an excellent technique for determining the positional distribution of DHA, it is unable to distinguish EPA from arachidonic acid and other PUFA with Δ5 unsaturation and can give erroneous results for EPA. The Grignard method also has limitations in that the DAG on which the analysis is performed can be contaminated by acyl migration. Furthermore, separation of DAG from the rest of the reaction products involves laborious processes.

Unlike most commercial lipases, which inefficiently release EPA and DHA from TAG, the immobilized yeast lipase from *Candida antarctica* (CAL) has been shown to exhibit significant activity toward these FA. For example, Haraldsson *et al.* (9) used this lipase for the synthesis of homogeneous trieicosapentaenoin and tridocosahexaenoin from glycerol. CAL not only displayed significant activity toward LC-PUFA but also exhibited strict 1,3-regiospecificity for ethanolysis of TAG in the presence of a high excess of ethanol (10,11). Recently, Shimada and co-workers (12,13) claimed that, in contrast to pancreatic lipase, CAL acts on saturated and unsaturated C_{14}–C_{24} acids to a similar degree and suggested that ethanolysis in the presence of this lipase may be used for regiospecific analysis of TAG containing LC-PUFA. We have further investigated the CAL ethanolysis reaction using both marine oils and synthetic TAG as substrates. Here we report on the development of a semimicro enzymatic method based on lipolysis with CAL for regiospecific analysis of TAG containing LC-PUFA.

EXPERIMENTAL PROCEDURES

Materials. Tuna oil (HiDHA 25S5) and cod liver oil were purchased from Nu-Mega Lipids Pty. Ltd. (Brisbane, Queensland,

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Australia) and Melrose Laboratories P/L (Mitcham, Australia), respectively. Refined seal blubber oils (harp seal oil) were obtained from two sources: Atlantic Marine Products (Catalina, Newfoundland, Canada) and Canomega Industries Inc. (Nepean, Onario, Canada). Randomized seal blubber oil was prepared at BioCentrum-DTU (Lyngby, Denmark) by chemical randomization of a sample of the seal blubber oil obtained from Atlantic Marine Products. 1,2-Distearoyl-3-oleoyl-glycerol, 1,3-dipalmitoyl-2-oleyol-glycerol and 1,3-distearoyl-2-oleoyl-glycerol (all at least 99% pure) were purchased from Sigma-Aldrich (Sydney, New South Wales, Australia). Immobilized CAL (Novozym 435) was a gift from Novozyms Australia Pty. Ltd. (Sydney, Australia). The homogeneous TAG triolein (OOO), trilinolein (LLL), trilinolenin (LnLnLn), trieicosapentaenoin (EEE), and tridocosahexaenoin (DDD), all of 99% or greater purity, were purchased from Nu-Chek-Prep, Inc (Elysian, MN). The symmetrical TAG 1,3-dipalmitoyl-2-docosahexaenoyl-glycerol (PDP), 1,3-dipalmitoyl-2-linoleoyl-glycerol (PLP), and 1,3-dipalmitoyl-2-linolenoyl-glycerol (PLnP) were synthesized from glycerol by CAL-mediated reaction with vinyl palmitate, followed by coupling with the corresponding PUFA in the presence of 4-dimethylaminopyridine (DMAP) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) using a modification of the method of Halldorsson *et al*. (14). The unsymmetrical TAG 1,2-dipalmitoyl-3-docosahexaenoyl-glycerol (PPD), 1,2-dipalmitoyl-3-linoleoyl-glycerol (PPL), and 1,2-dipalmitoyl-3-linolenoyl-glycerol (PPLn) were synthesized from glycerol-1,2-acetonide by reaction with the corresponding PUFA in the presence of DMAP and EDCI, removal of the protecting group, followed by coupling with palmitic acid using DMAP and EDCI as before.

Lipolysis. Lipase-catalyzed ethanolysis was performed on a milligram scale using a modification of the method of Shimada *et al*. (12). Typically, 10–60 mg of oil was dissolved in 125–760 mL of absolute ethanol (oil/ethanol 1:10 w/w) and mixed in a culture tube with 4–26 mg of the lipase (4%, w/w of the solution of oil in ethanol). The tube was flushed with nitrogen, capped, and agitated (200 rpm) for 4 h in a water bath maintained at 30°C. Triplicate enzyme reactions were performed for each sample. In preliminary experiments designed to verify or optimize reaction conditions, the ethanolysis was also performed at an oil/ethanol ratio of 1:3 w/w, and products were analyzed at hourly intervals up to 4 h.

Analysis of reaction products. The reaction mixture (0.5 mL) containing the products of ethanolysis was filtered using a 0.45 μm polytetrafluoroethylene membrane syringe filter (Vivascience AG, Hanover, Germany), and the retentate washed with distilled dichloromethane (DCM, 8 mL). The washings and the retenate were combined, and an aliquot of 0.5 mL was analyzed by GC without further purification to obtain the *sn*-1(3) FA composition. The rest of the solution (*ca*. 8 mL) was chromatographed on a single Isoelute Diol SPE cartridge (500 mg; International Sorbent Technology Ltd., Hengoed, Mid Glamorgan, United Kingdom). The cartridge was washed with DCM (3 mL) to remove the ethyl esters and any DAG and unreacted TAG. The MAG were recovered by elution with methanol (8 mL). FAME were prepared from the isolated MAG according to the method of Bannon *et al*. (15) to obtain the *sn*-2 FA composition.

Analysis of FA. FA composition was determined by GC on a BPX70 fused-silica column (25 m, 0.25 mm i.d., and 0.25 μm film thickness; SGE, Ringwood, Australia). Helium was used as the carrier gas at a constant head pressure of 120 kPa. The GC oven temperature was increased from 170 to 205°C at a rate of 1°C /min, then to 230°C at 10°C/min, and then held at the final temperature for a further 20 min. Temperatures of the injector and the FID detector were maintained at 220 and 250°C, respectively. The peak area counts were integrated using the Ezchrom™ Chromatography Data System (Scientific Software, Palo Alto, CA). FA composition at *sn*-1(3) was calculated from the measured FA composition at *sn*-2 [alternatively, FA composition at *sn*-2 from the measured FA compostion at *sn*-1(3)] using the following equation:

$$\text{FA at } sn\text{-2 (mol\%)} = 3 \times \text{total FA (mol\%)} - 2 \times \text{FA at } sn\text{-1(3) (mol\%)} \quad [1]$$

Postitional distribution of FA by ^{13}C NMR. Spectra were recorded at 200 MHz on a Bruker Avance 800 spectrometer using $CDCl_3$ as solvent. The spectra were referenced using the solvent carbon signal ($CDCl_3$ = 77.16 ppm). 2D NMR techniques such as Homonuclear Correlation Spectroscopy, Heteronuclear Multiple Quantum Coherence, Heteronuclear Multiple Bond Coherence, and Nuclear Overhauser Effect Spectroscopy were used to aid assignment of the spectra.

RESULTS AND DISCUSSION

Regiospecific analyses with CAL have been performed on 2–30 g of oil using an oil-to-ethanol ratio of 3:1 (w/w) (12,16). However, there are practical difficulties with this low ethanol/oil ratio when conducting the reaction on a milligram scale. Analysis on a milligram scale is required in situations where the amount of oil is limited such as in the case of novel or experimental oils. Conducting the reaction at a higher ethanol/oil ratio can potentially overcome this problem. It has been shown that CAL exhibits strict 1,3-regiospecificity and furnishes a higher yield of 2-MAG when the reaction is conducted in excess ethanol using ethanol/oil ratios in the range of 3:1 to 10:1 w/w (10,11). The reaction was also reported to be faster in excess ethanol, and constant 2-MAG yields were obtained at ethanol/oil ratios of 3:1 and above. It has been suggested that the enhanced 1,3-regiospecificity of CAL in excess ethanol may be due to the ability of ethanol to fix the tertiary structure of the lipase, hindering the *sn*-2 acyl group from accessing the catalytic pocket (11). There was no significant difference in the FA composition when ethanol-to-oil ratios below 10:1 (w/w) were used. However, precision of the measurement worsened as reflected in the high % CV values (data not shown). Too low ethanol/oil ratios must be avoided because these conditions can lead to loss of regiospecificity of the lipase. Ethanolysis of fish

TABLE 1
Effect of Ethanol-to-Oil Ratio on Ethanolysis of PLP, PLnP, and PDP with *Candida antarctica* Lipase[a] (4% by weight of oil/ethanol mixture)

	Ethanol/oil ratio, w/w		
TAG	10:1	25:1	50:1
PLP	82.0 (1.3)	89.6 (0.7)	93.1 (0.3)
PLnP	80.6 (0.9)	89.9 (2.0)	89.2 (0.7)
PDP	89.3 (1.2)	96.1 (0.9)	96.6 (0.4)

[a]P, L, Ln, and D refer to palmitic acid, linoleic acid, linolenic acid, and DHA, respectively. Values shown are *sn*-1(3) mole percentages for P from direct GC analysis of ethyl esters formed by ethanolysis, averaged for quadruplicate measurements. % CV is shown in parentheses.

oil with CAL at an ethanol/oil molar ratio of 1.7:1 resulted in complete conversion of the oil to ethyl esters, suggesting that the lipase acted nonspecifically under these conditions (17).

Table 1 shows the 1(3)-palmitic acid mol% (determined by direct analysis of ethyl esters after ethanolysis with *ca*. 10 mg oils) when the lipolysis was performed on three synthetic TAG, namely, PLP, PLnP, and PDP at various ethanol-to-oil ratios. The highest accuracy (>90%) was obtained when the highest ethanol-to-oil ratio (50:1) was used. This implies stricter 1,3-regiospecificity for the lipase and reduced acyl migration at higher ethanol-to-oil ratios. Thus, a higher ethanol-to-oil ratio is not only permissible but indeed preferable when performing the reaction with a small amount of oil.

In regiospecific analysis using hydrolysis of TAG with a 1,3-specific lipase, it is customary to measure the FA composition of the resultant 2-MAG and subsequently calculate the *sn*-1(3) FA by difference from the total FA. This procedure is followed because FA liberated by lipolysis (ethyl esters in the case of ethanolysis) may not adequately represent the FA originally present in positions 1 and 3 of the TAG, owing to preferential release by lipase of some FA over others, and contamination with FA released from positions 1 and 3 after they have migrated from position (2). However, Luddy *et al.* (18), who analyzed lard for positional distribution of FA, found little difference between results from analysis of 2-MAG and direct analysis of FA liberated by lipolysis. Indeed, direct analysis of FA ester formed by alcoholysis of TAG has been recommended for regiospecific analysis (19) because it is quick and does not require chromatographic separation of 2-MAG and esterification prior to GC.

Table 2 shows the positional distribution of FA in PLP, PLnP, and PDP determined by both methods. The synthetic TAG used for this comparison were highly regio-isomerically enriched (97–98% purity for the symmetrical TAG and 88–92% for the unsymmetrical TAG) on the basis of high-field ^{13}C NMR spectroscopy. Thus, in the case of the symmetrical TAG, the *sn*-2 position should be almost entirely esterified to one of the PUFA and contain little or no palmitic acid. Good results were obtained when regiospecific analysis was performed through determination of the FA composition of 2-MAG. For the symmetrical TAG, PUFA contents ranging from 94.8 to 98.0% were obtained by this method for the *sn*-2 composition. This amounted to 97.4–99.0% palmitic acid at the *sn*-1(3) position. The corresponding figures obtained by direct analysis of the ethyl esters resulting from ethanolysis were 78.4–96.6 mol% and 89.2–96.6 mol%, respectively. Table 2 also shows the regiospecific analysis results for three unsymmetrical TAG, namely, PPL, PPLn, and PPD. The regiopurities of the unsymmetrical TAG (88–92%) were not as high as that of the corresponding symmetrical compounds (97–98%), but absolute regiopurity was not required for the comparison of methods. As previously observed for the symmetrical TAG, the regiopurity of the unsymmetrical TAG measured by the lipase method *via* 2-MAG was more accurate compared with that determined by direct analysis of the ethyl esters produced by ethanolysis. From these results, it can be concluded that while more accurate results are obtained when the analysis is performed through 2-MAG, reasonable results can also be obtained by direct analysis of the ethyl esters formed. The latter method may be used when quick analysis is required for comparative purposes.

The ability of CAL to release *n*-3 LC-PUFA and common C_{18} unsaturated acids from TAG without discrimination was tested by conducting the ethanolysis reaction on an equimolar

TABLE 2
Regiospecific Analysis of Symmetrical and Unsymmetrical Synthetic TAG by Different Methods[a]

TAG (purity by	*sn*-1(3) measured[b]		*sn* -2 calculated[c]		*sn*-2 measured[d]		*sn*-1(3) calculated[e]	
^{13}C NMR)	P	L, Ln, or D	P	L, Ln, or D	P	L, Ln, or D	P	L, Ln, or D
Symmetrical								
PLP (97% pure)	93.1 (0.3)	6.9 (4.3)	13.8	86.2	5.2 (18.9)	94.8 (1.0)	97.4	2.6
PLnP (98% pure)	89.2 (0.7)	10.8 (5.4)	21.6	78.4	2.0 (2.6)	98.0 (0.2)	99.0	1.0
PDP(97% pure)	96.6 (0.4)	3.4 (9.6)	6.8	96.6	3.4 (26.1)	96.6 (0.8)	98.3	1.7
Unsymmetrical								
PPL (92% pure)	58.1 (0.2)	41.9 (0.3)	83.8	16.2	86.7 (1.7)	13.3 (10.8)	56.6	43.4
PPLn (88% pure)	59.5 (0)	40.5 (0)	81.0	19.0	84.1 (1.3)	15.9 (0.4)	57.9	42.1
PPD (91% pure)	61.9 (0.5)	38.1 (0.8)	76.2	23.8	82.2 (4.3)	17.8 (18.0)	58.9	41.1

[a]FA composition is expressed as mol% averaged for quadruplicate measurements. % CV is shown in parentheses. For abbreviations see Table 1.
[b]*sn*-1(3) FA composition measured by GC analysis of ethyl esters generated by ethanolysis by *Candida antarctica.*
[c]*sn*-2 FA composition calculated from measured total and *sn*-1(3) FA compositions.
[d]*sn*-2 FA composition determined by analysis of the FA composition of separated 2-MAG.
[e]*sn*-1(3) FA composition calculated from the measured total and *sn*-2 FA composition.

TABLE 3
Effect of FA Chain Length and Degree of Unsatuation on Ethanolysis by *Candida antarctica* Lipase[a]

Reaction time (h)	18:1	18:2	18:3	20:5	22:6
1	20.9 (0.7)	20.8 (0.6)	21.4 (0.2)	23.9 (0.9)	13.1 (3.6)
2	20.6 (6.6)	20.6 (6.7)	21.0 (6.3)	23.2 (6.1)	14.7 (10.9)
3	20.7 (3.5)	20.5 (3.6)	20.9 (3.1)	23.2 (3.2)	14.7 (10.0)
4	20.3 (1.9)	20.1 (2.0)	20.3 (1.9)	22.6 (2.0)	16.7 (3.6)
Theoretical *sn*-1(3) composition	20.0	20.0	20.0	20.0	20.0

[a]The reaction was performed on an approximately equimolar mixture of trioctadecenoin, trioctadecadienoin, trioctadecatrinoin, trieicosapentaenoin, and tridocosahexaenoin at 30°C using an ethanol to TAG ratio of 10:1 w/w. Values shown are averaged (n = 4) composition of *sn*-1(3) FA (mol%) at different time points. % CV is shown in parentheses.

mixture of the homogeneous TAG OOO, LLL, LnLnLn, EEE, and DDD using conditions recommended by Shimada *et al*. (12,13) except that an ethanol-to-oil ratio of 10:1 (w/w) was used. The ethyl esters formed were analyzed after 1, 2, 3, and 4 h of reaction (Table 3). It was found that the C_{18} acids were released at approximately the same rate irrespective of the degree of unsaturation. DHA was released more slowly, forming only 16.7 mol% out of the theoretical 20.0 mol% after 4 h reaction, whereas EPA was released the fastest. Faster ethanolysis of EEE compared with DDD has been reported before (10), where, under similar conditions of reaction the 2-MAG content in the resulting acylglycerol mixture after 1 h reaction was 91.7 mol% for EEE and only 14.7 mol% for DDD. Approximately 4 h were required for DDD to undergo 90% conversion to 2-MAG, which is similar to the rate observed in the present study. The faster hydrolysis of EEE compared with DDD may be caused not only by a FA specificity but also by a TAG specificity as suggested by Tanaka *et al*. (20). Shimada *et al.* (12), who used randomized TGA40 oil (a single-cell oil containing a high degree of PUFA) as substrate to investigate FA selectivity of CAL lipase found that the longer-chain acids $C_{22:0}$ and $C_{24:0}$ were released more slowly than C_{16}, C_{18}, and C_{20} acids. Unfortunately, they did not report on the relative reaction rates of EPA and DHA although the TGA-40 substrate they used contained significant amounts of these acids. Based on the findings of the present study and those of others, a minimum of 4 h reaction time (at a lipase loading of 4% by weight) appears to be necessary for regiospecific analysis of oils containing significant amounts of EPA and DHA in combination with relatively more saturated FA. Our results concur with those of Shimada *et al.* (12) who recommended a reaction time of 4 h with 4% (w/w) lipase loading.

Table 4 shows results for regiospecific analysis of a sample of chemically randomized seal blubber oil by ethanolysis with CAL for 4 h at 30°C. Because chemical randomization rearranges the FA in a random fashion, the FA composition at each glycerol carbon should be the same as the total FA composition, and each FA should be equally distributed (33.3%) among the three glycerol positions. FA compositions determined by direct analysis of FA ethyl ester resulting from ethanolysis [*sn*-1(3) composition] and *via* 2-MAG (*sn*-2 composition) were both in reasonably good agreement with the theoretical values, with the former method giving particularly good accuracy (less than 5% error for FA occurring at >5% mol%). In regard to PUFA, direct *sn*-1(3) analysis somewhat underestimated DHA and overestimated EPA. In contrast, the *sn*-2 composition determined through 2-MAG significantly underestimated EPA. These differences can be attributed to the different rates of release of EPA and DHA during CAL-mediated ethanolysis.

Table 5 shows the *sn*-2 positional distribution of EPA and DHA in selected marine oils measured by both ^{13}C NMR and CAL methods. In the present study, the two methods gave similar results for DHA, which were also in agreement with literature values obtained by various methods including reaction with lipase followed by analysis of 2-MAG (12,21), reaction with Grignard reagent followed by analysis of 1,2(2,3)-DAG (22,23), and ^{13}C NMR (7,24). In the present study as well as in the literature, values for EPA by the CAL method generally tended to be lower than those measured by ^{13}C NMR.

TABLE 4
Regiospecific Analysis of Randomized Seal Blubber Oil by Ethanolysis with *Candida antarctica* Lipase[a]

FA compostion (mol%)	14:0	16:0	16:1 n-7	18:0	18:1 n-9	18:1 n-7	18:2 n-6	18:4 n-3	20:1 n-9	20:5 n-3	22:1 n-11	22:5 n-3	22:6 n-3
Total	6.0	8.8	20.2	1.0	22.6	4.9	1.9	1.4	10.7	7.1	1.6	3.8	7.9
sn-1(3) by direct analysis of ethyl esters formed by ethanolysis	6.4	9.2	19.7	1.2	22.0	5.2	1.8	1.5	11.2	7.7	1.6	3.6	6.8
sn-2 through 2-MAG analysis	7.5	9.9	25.6	1.2	21.2	4.6	2.3	1.4	7.3	5.2	1.0	3.0	7.6

[a]FA composition is expressed as mol% and is averaged for quadruplicate measurements.

TABLE 5
Comparison of *sn*-2 Positional Distribution of EPA (20:5) and DHA (22:6) in Selected Marine Oils Determined by Ethanolysis Using *Candida antarctica* Lipase with ^{13}C NMR Spectroscopy and Literature Data

	DHA			EPA		
	Lipase method	^{13}C NMR	Literature values	Lipase method	^{13}C NMR	Literature values
Tuna oil	53.1	52.0	54.0[a], 47.4[b], 49.5[c], 49.5[d]	25.8	37.2	28.6[a], 31.2[b], 27.5[c], 43.0[d]
Cod liver oil	69.4	72.5	74.4[e]	27.7	33.3	37.8[e]
Seal blubber oil (source 1)	8.7	ND	3.2[e], 7.4[f]	8.6	ND	4.6[e], 7.6[f]
Seal blubber oil (source 2)	6.3	ND		6.2	ND	
Randomized seal blubber oil (source 2)	32.3	30.9	33.3[g]	24.5	31.8	33.3[g]

[a]Shimada *et al.* (12).
[b]Calculated from Myher *et al.* (22).
[c]Amate *et al.* (21).
[d]Calculated from Sacchi *et al.* (24).
[e]Aursand *et al.* (7).
[f]Calculated from Wanasundera and Shahidi (23).
[g]Theoretical values. ND, not detected.

ACKNOWLEDGMENTS

The work was conducted as part of the Food Futures Flagship, a CSIRO Australia's National Research Flagship. We thank Frances Separovic (University of Melbourne) and Neeranat Thienthong (Monash University) for ^{13}C NMR spectra of the synthetic TAG and marine oils, respectively.

REFERENCES

1. Brockerhoff, H., Stereospecific Analysis of Triglycerides, *Lipids 6*:942–956 (1971).
2. Christie, W.W., The Positional Distribution of Fatty Acids in Triglycerides, in *Analysis of Fats and Oils*, Elsevier, New York, 1986, pp. 313–339.
3. Yurkowski, M., and H. Brockerhoff, Fatty Acid Distribution of Triglycerides Determined by Deacylation with Methyl Magnisium Bromide, *Biochim. Biophys. Acta. 125*:55–59 (1966).
4. Becker, C.C., A. Rosenquist, and G. Holmer, Regiospecific Analysis of Triacylglyerols Using Allyl Magnesium Bromide, *Lipids 28*:147–149 (1993).
5. Gunstone, F.D., High Resolution NMR Studies of Fish Oils, *Chem. Phys. Lipids 59*:83–89 (1991).
6. Bergana, M.M., and T.W. Lee, Structure Determination of Long-chain Polyunsaturated Triacylglycerols by High Resolution ^{13}C Nuclear Magnetic Resonance, *J. Am. Oil Chem. Soc. 73*:551–556 (1996).
7. Aursand, M., L. Jorgensen, and H. Grasdalen, Positional Distribution of Omega-3 Fatty Acids in Marine Lipid Triacylglycerols by High Resolution ^{13}C Nuclear Magnetic Resonance Spectroscopy, *Ibid. 72*:293–297 (1995).
8. Bottino, N.R., G.A. Vandenburg, and R. Reiser, Resistance of Certain Long-Chain Polyunsatuated Fatty Acids of Marine Oils to Pancreatic Lipase Hydrolysis, *Lipids 2*:489–493 (1967).
9. Haraldsson, G.G., B.O. Gudmundsson, and O. Almarsson, The Synthesis of Homogeneous Triglycerides of Eicosapentaenoic Acid and Docosahexaenoic Acid by Lipase, *Tetrahedron 51*:941–952 (1995).
10. Irimescu, R., K. Furihata, K. Hata, Y. Iwasaki, and T. Yamane, Utilization of Reaction Medium-Dependent Regiospecificity of *Candida antarctica* Lipase (Novozym 435) for the Synthesis of 1,3-Dicapryloyl-2-docosahexaenoyl (or eicosapentaenoyl) Glycerol, *J. Am. Oil Chem. Soc. 78*:285–289 (2001).
11. Irimescu, R., Y. Iwasaki, and C.T. Hou, Study of TAG Ethanolysis to 2-MAG by Immobilized *Candida antarctica* Lipase and Synthesis of Symmetrically Structured TAG, *Ibid. 79*:879–883 (2002).
12. Shimada, Y., J. Ogawa, Y. Watanabe, T. Nagao, A. Kawashima, T. Kobayashi, and S. Shimizu, Regiospecific Analysis by Ethanolysis of Oil with Immobilized *Candida antarctica* Lipase, *Lipids 38*:1281–1286 (2003).
13. Shimada, Y., and Y.Watanabe, Regiospecific Analysis of Triacylglycerols by an Enzymatic Method, *Lipid Technol. 17*:39–41 (2005).
14. Halldorsson, A., C.D. Magnusson, and G.G. Haraldsson, Chemoenzymatic Synthesis of Structured Triacylglycerols by Highly Regioselective Acylation, *Tetrahedron 59*:9101–9109 (2003).
15. Bannon, C.D., J.D. Craske, and A.E. Hilliker, Analysis of Fatty Acid Methyl Esters with High Accuracy and Reliability. IV Fats with Fatty Acids Containing Four or More Carbon Atoms, *J. Am. Oil Chem. Soc. 62*:1501–1507 (1985).
16. Kawashima, A., T. Nagao, Y. Watanabe, T. Kobayashi, I. Ikeda, Y. Tominaga, and Y. Shimada, Preparation of Regioisomers of Structured TAG Consisting of One Mole of CLA and Two Moles of Caprylic Acid, *Ibid. 81*:1013–1020 (2003).
17. Breivik, H., G.G. Haraldsson, and B. Kristinsson, Preparation of Highly Purified Concentrates of Eicosapentaenoic Acid and Docosahexaenoic Acid, *Ibid. 74*:1425–1429 (1997).
18. Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman, and R.W. Riemenschneider, Pancreatic Lipase Hydrolysis of Triglycerides by a Semimicro Technique, *Ibid. 41*:693–696 (1964).
19. Dourtoglou, T., E. Stefanou, S. Lalas, V. Dourtoglou, and C. Poulos, Quick Regiospecific Analysis of Fatty Acids in Triacylglycerols with GC Using 1,3-Specific Lipase in Butanol, *Analyst 126*:1032–1036 (2001).
20. Tanaka, Y., T. Funada, J. Hirano, and R. Hashizume, Triglyceride Specificity of *Candida cylindracea* Lipase: Effect of Docosahexaenoic Acid on Resistance of Triglyceride to Lipase, *J. Am. Oil Chem. Soc. 70*:1031–1034 (1993).
21. Amate, L., M. Ramirez, and A. Gil, Positional Analysis of Triglycerides and Phospholipids Rich in Long-chain Polyunsaturated Fatty Acids, *Lipids 34*:865–871 (1999).
22. Myher, J.J., A. Kuksis, K. Geher, P.W. Park, and D.A. Diersen-Schade, Stereospecific Analysis of Triacylglycerols Rich in Long-chain Polyunsaturated Fatty Acids, *Ibid. 31*:207–215 (1996).
23. Wanasundara, U.N., and F. Shahidi, Positional Distribution of Fatty Acids in Triacylglycerols of Seal Blubber Oil, *J. Food Lipids 4*:51–64 (1997).
24. Sacchi, R., I. Medina, S.P. Aubourg, I. Giudicianni, L. Paolillo, and F. Addeo, Quantitative High-Resolution ^{13}C NMR Analysis of Lipids Extracted from the White Muscle of Atlantic Tuna (*Thunnus alalunga*), *J. Agric. Food Chem. 41*:1247–1253 (1993).

[Received March 8, 2006; accepted August 8, 2006]