Positional Distribution of ω3 Fatty Acids in Marine Lipid Triacylglycerols by High-Resolution ^{13}C Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT: The positional distribution [α(1,3)-acyl and ß(2)-acyl] of ω3 fatty acids [18:4(n-3), 20:4(n-3), 20:5(n-3), 22:5(n-3) and 22:6(n-3)] in depot fat of Atlantic salmon (*Salmo salar*), harp seal oil and cod liver oil triacylglycerols has been examined by ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The positional distribution data can be defined from the spectrum of the carbonyl (C1 carbon) and the methylene (C2 and glyceryl carbon) regions. In depot fat of Atlantic salmon and cod liver oil, docosahexaenoic acid (DHA) was concentrated in the ß-position of the triacylglycerides with 72.6 and 74.4%, respectively. Only 3.2% of DHA and 4.6% of eicosapentaenoic acid (EPA) were esterified to the ß-position of the triacylglycerides in harp seal oil. EPA is nearly randomly distributed in cod liver oil and muscle lipids of Atlantic salmon, with 37.8 and 39.7%, respectively, in the ß-position. In general, the ^{13}C NMR-derived data were in accordance with corresponding data reported in the literature obtained by conventional techniques.
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KEY WORDS: ^{13}C NMR spectroscopy, ω3 fatty acids, positional distribution (1,3-acyl and 2-acyl).

High-resolution ^{13}C nuclear magnetic resonance (NMR) spectroscopy is an adequate method in examining the positional distribution of fatty acids of triacylglycerols of vegetable oils (1–4), whole vegetable seeds (5) and meat products (6).

Compared to conventional chemical (7) and enzymatic (8,9) procedures used to obtain structural information, the NMR technique offers the opportunity to study heterogeneous lipid mixtures, oils and depot fat noninvasively and nondestructively.

Recently we reported the interpretation of the ^{13}C NMR spectra of ω3 fatty acids and lipid extracted from the white muscle of Atlantic salmon (*Salmo salar*) (10,11) and quantitative NMR measurements of lipid composition of depot fat in muscle of Atlantic salmon (12).

In the present work, ^{13}C NMR has been used to explore the positional distribution of ω3 fatty acids on the glycerol backbone of triacylglycerols from depot fat of Atlantic salmon, cod liver oil and seal oil. Detailed information about the distribution of other fatty acids in triacylglycerols from marine lipids can also be obtained *via* high-resolution ^{13}C NMR and is under further investigation.

EXPERIMENTAL PROCEDURES

The cod liver oil was a product from Peter Møller A/S (Oslo, Norway). Harp seal oil was provided by The Norwegian Institute of Fisheries and Aquaculture Ltd. (Tromsø, Norway).

Lipid extraction. Lipids were extracted from white muscle of farmed Atlantic salmon (*S. salar*) according to the method of Bligh and Dyer (13). Parts of the chloroform phase were removed by evaporation and replaced by CDCl$_3$ before analyzing the lipid extracts by NMR.

NMR spectroscopy. NMR was performed on a Jeol EX-400 spectrometer (Tokyo, Japan). Lipid extracts (approximately 50–100 mg in 0.6 mL) were examined in CDCl$_3$ in 5-mm NMR tubes. The chemical shifts were referenced indirectly to tetramethylsilane (TMS) by using the central peak of the CDCl$_3$ (δ = 77.08). The ^{13}C broad-band proton-decoupled spectra of the lipid extracts were obtained at ambient temperature (~20°C) at a frequency of 100.40 MHz, a spectral width of 23 KHz, 131 K data points and a pulse repetition time of 2.94 s. Scans (2000–3000) were collected at a 63° excitation pulse. A Gaussian filter of 0.15 Hz Lorentzian narrowing and 0.18 Hz Gaussian broadening was applied to the FID (free induction decay) before Fourier transformation. The relative intensities of ^{13}C resonances were determined by a computer program developed in our laboratory.

RESULTS AND DISCUSSION

The ^{13}C chemical shifts and assignments for glycerol, C1 and C2 carbon signals of depot fat of Atlantic salmon, cod liver oil and harp seal oil are given in Table 1. The interpretation is

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TABLE 1
[13]C Chemical Shift (ppm) and Assignments for Glycerol, C1 and C2 Carbon Atoms

	Atlantic salmon	Cod liver oil	Harp seal oil	Assignment
Glycerol	69.11	69.08	69.11	22:6 ß
	69.02	68.99	69.03	20:5 ß
	68.96	68.94	—	] ß
	68.90	68.86	68.89	]
	62.26	62.20	62.26	22:6 α
	62.18	62.12	62.19	] α
	62.09	62.03	62.10	]
	62.03	61.96	—	
C1	173.52	—	—	Unknown
	—	—	173.25	] α
	173.23	173.15	173.23	]
	173.16	173.06	173.19	20:4n-3 α
	173.12	173.00	173.15	22:5 α
	173.07	172.88	173.06	18:4 α
	172.98	172.83	173.00	20:5 α
	172.95	—	—	20:5 α[i]
	172.84	172.69	172.86	] ß
	172.81	172.67	172.82	]
	—	172.66	172.78	20:4n-3 ß
	172.68	—	172.76	22:5 ß
	172.63	172.50	172.66	18:4 ß
	172.59	172.45	172.60	20:5 ß
	172.51	172.36	172.53	22:6 α
	172.48	—	—	22:6 α[i]
	172.11	171.98	172.13	22:6 ß
C2	34.83	—	—	Unknown
	34.20	34.14	34.21	ß
	—	—	34.20	
	34.11	33.96	34.05	α
	34.04	—	34.01	
	—	—	33.97	
	33.90	33.83	33.91	22:6 α
	33.73	—	—	Unknown
	33.57	33.49	33.58	20:5 ß
	—	—	33.44	Unknown
	33.39	33.31	33.40	20:5 α

FIG. 1. The carbonyl region of the ^{13}C nuclear magnetic resonance spectrum of (A) harp seal oil and (B) lipids from white muscle of Atlantic salmon, with assignments of the unique lines that allow examination of the positional distribution of the ω3 fatty acids. EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

based on our study of ω3 fatty acids (10,11) and lipid extracted from muscle of Atlantic salmon (12). The carbonyl region (Fig. 1) and the methylene region (Figs. 2 and 3) can be used to explore the positional distribution of ω3 fatty acids in marine lipid triacylglycerols. In general, the ^{13}C resonance of the carbon close to the carbonyl end in triacylglycerols and phospholipids is influenced by esterification of the glycerol moiety, with two separate signals for the C1 and C2 carbon in the acyl chain, depending on whether the chain is present in the α- or ß-position (3,4,11,12).

C1 carbon. The carbonyl signals in triacylglycerides appear at about 173.2 and 172.8 ppm (Fig. 1) of which the higher shift is associated with the α chains. However, the carbonyl chemical shifts of unsaturated chains in triacylglycerides indicate a progressive decreasing shift as the fatty acid double bond is positioned closer to the carbonyl carbon (Fig. 1 and Table 1). The carbonyl chemical shift order (both for α and ß carbon) going from low field to high field, for the ω3 fatty acids is shown here; 20:4δ8,11,14,17 > 22:5δ7,10,13,16,19 [docosapentaenoic acid (DPA)] > 18:4δ6,9,12,15 >
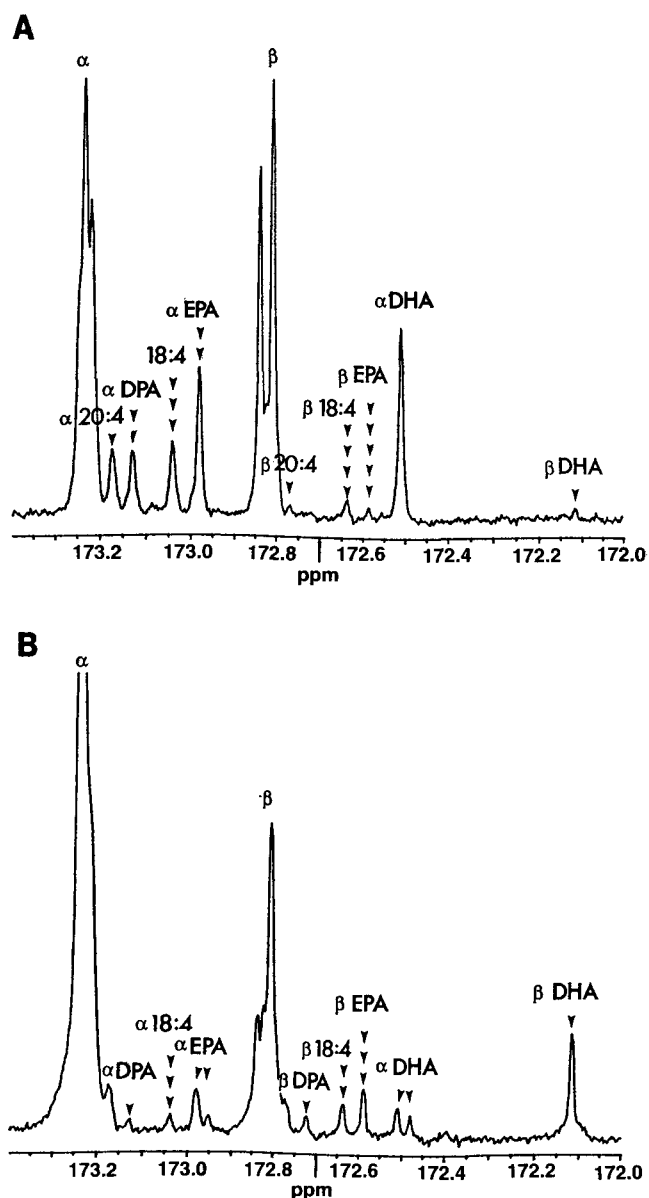
20:5δ5,8,11,14,17 [eicosapentaenoic acid (EPA)] > 22:6δ4,7,10,13,16,19 [docosahexaenoic acid (DHA)]. The same trend has been found by Wollenberg (2) by ^{13}C NMR examination of the acyl positional distribution of triacylglycerols derived from edible vegetable oils. Consequently, the carbonyl region alone can be used to define the ω3 fatty acid positional distribution (except 18:3n-3) for marine lipids (depot fat of Atlantic salmon, cod liver oil and seal oil) examined in this study (Fig. 1 and Table 2).

In general, the difference in chemical shift for C1 resulting from α and ß chains is about 0.4 ppm (3). Gunstone (3)
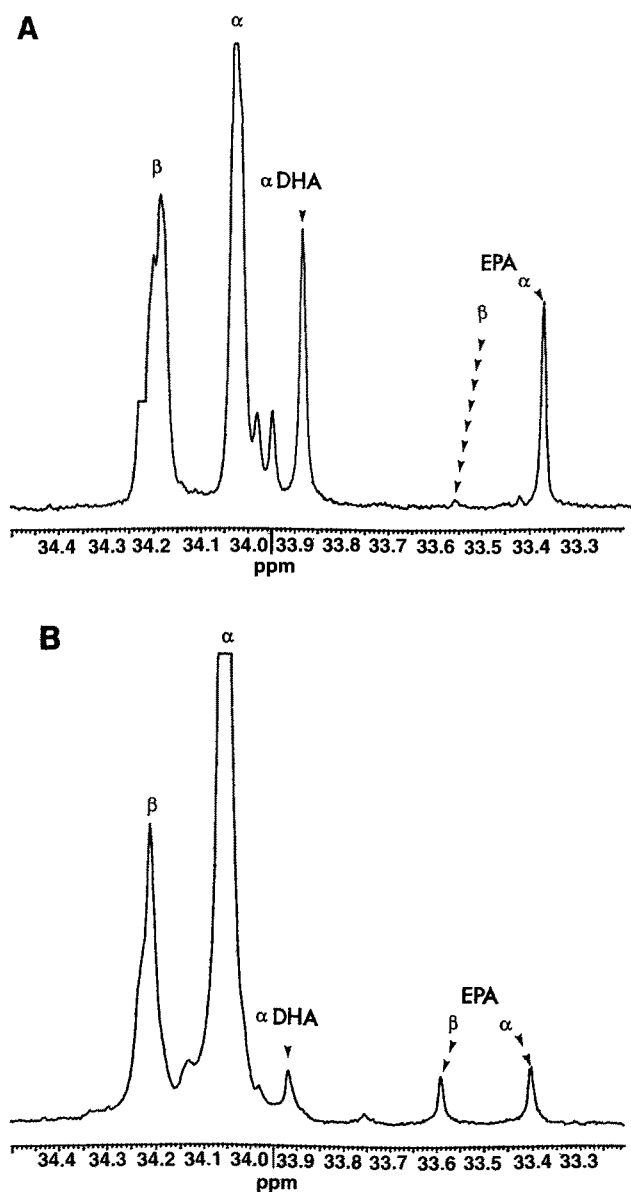
A

B

FIG. 2. An expansion of the methylene region of the ^{13}C nuclear magnetic resonance spectrum of (A) harp seal oil and (B) lipids from white muscle of Atlantic salmon where the C2 carbon gives signals. Abbreviations as in Figure 1.

FIG. 3. An expansion of the ^{13}C nuclear magnetic resonance spectrum of (A) harp seal oil and (B) lipids from white muscle of Atlantic salmon where the glycerol carbons give signals. Abbreviations as in Figure 1.

has shown, by studying synthetic triacylglycerols, that the differences between the α and ß signals depend on the nature of the acyl chains. Triacylglycerols with saturated fatty acids in the α-position and unsaturated in the ß-position show differences of about 0.43 ppm, and 0.38 ppm for the opposite situation. The observed differences in chemical shifts for carbonyl carbon of the ω3 fatty acids in cod liver oil, harp seal oil and depot fat of Atlantic salmon, resulting from α and ß chains, are given in Table 2.

In the ^{13}C spectrum of depot fat of Atlantic salmon (Fig. 1B), both 20:5n-3 and 22:6n-3 give rise to two C1 α signals with α chemical shift difference of about 0.03 ppm. The C1 α shift of EPA and DHA is presumably influenced by the na-
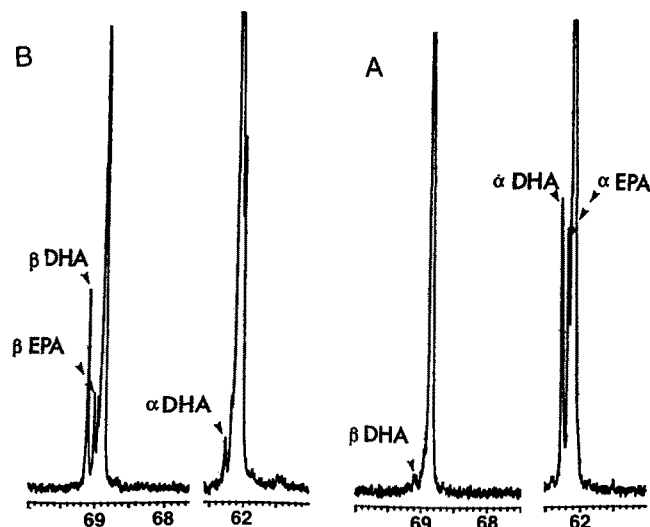
ture of the acyl chain (unsaturated or saturated acyl chains) esterified to the ß-position of the triacylglycerol molecule and the symmetry of the molecule. Muscle lipids of Atlantic salmon may contain triacylglycerol molecules with EPA or DHA both in α- and ß-position of the molecule. To confirm this theory it is necessary to study synthetic glycerides with ω3 fatty acids both in α- and in ß-positions of triacylglycerol molecules.

C2 carbon. The C2 carbon atom in the acyl chain also shows two signals at about 34.2 and 34.0 ppm for the ß and α acyl chains, respectively (Fig. 2). Further, the C2 chemical shift is also influenced by the position of the double bond, related to the carbonyl end.

The C2 carbon of EPA gives rise to two well-separated resonances in the ^{13}C spectrum at nearly 33.40 ppm (a-position) and 33.58 ppm (ß-position), both for depot fat of Atlantic salmon (Fig. 2B) and harp seal oil (Fig. 2A). The positional distribution data for EPA in depot fat of Atlantic salmon, cod liver oil and harp seal oil, defined from this region of the ^{13}C spectrum, are presented in Table 2. In lipid mixtures where both EPA and 20:4n-6 fatty acid exist in abundance, C2 signals (α- and ß-position) will be overlapped by corresponding carbon signals from 20:4n-6 fatty acid due to identical chemical structure in the region near the carbonyl end of these fatty acids. However, in general, the 20:4n-6 fatty acid exists in minimal amounts in marine lipids (≤1%).

For DHA, only the C2 α resonance gives rise to resolved signals; the C2 ß resonance will overlap with α signals from other fatty acids (Fig. 2).

Glycerol carbon. The glyceryl carbon resonances will also be influenced by the nature of the esterified fatty acid and shift to a lower field when the double bond in the fatty acids is positioned closer to the carbonyl carbon (Fig. 3). Esterification of DHA to the α- and ß-position of the triacylglycerols

TABLE 2
Positional [1,3(α)- and 2(ß)-acyl] Distribution (%) for 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3 Fatty Acids in Muscle Lipids of Atlantic Salmon, Cod Liver Oil and Harp Seal Oil Obtained by ^{13}C Nuclear Magnetic Resonance Spectroscopy

	18:4n-3		20:4n-3		20:5n-3		22:5n-3		22:6n-3	
	α	ß	α	ß	α	ß	α	ß	α	ß
Lipids from white muscle of										
Atlantic salmon (*Salmo salar*)	26.6[a]	73.4	—		60.3(57.4)[b]	39.7(42.6)	31.3	68.7	27.4(26.0)[c]	72.6(74.0)
Cod liver oil										
(*Gadus morhua*)	66.8	33.2	—		62.2(61.9)	37.8(38.1)	—	—	25.6(24.1)	74.4(75.9)
Seal oil										
(*Paghophilus groenlandica*)	81.9	18.1	83.8	16.2	95.4(95.4).	4.6(4.6)	100	—	96.8(96.9)	3.2(4.1)

[a]The positional distribution is defined from the carbonyl carbon resonances.
[b]The C2 carbon resonances (the value in parentheses).
[c]The glycerol carbon resonances (the value in parentheses).

and phospholipids results in downfield shifts of the glyceryl carbon resonances to 62.07 and 69.08 ppm, respectively. Consequently, this region of the ^{13}C spectrum also provides the necessary information to quantify the positional distribution of DHA (Table 2). Glyceryl signals due to esterification of EPA are not fully resolved, and, consequently, each of these signals (Fig. 3) cannot be accurately integrated in this ^{13}C spectrum.

Quantitative results. In general, for quantitative measurements, both the nuclear Overhauser effect (NOE) and T_1 values for the carbons have to be taken into account (12). The carbons of current interest for measurements of the positional distribution of the ω3 fatty acids have similar NOE. In addition, the T_1 values for these carbons are not particularly influenced by the position of the fatty acids in the glycerol molecule (2,12). Consequently, to explore the positional distribution of ω3 fatty acids, ^{13}C NMR measurements can be done semi-quantitatively by pulsing relatively fast, resulting in short experimental time (maximum 1–2 h). Wollenberg (2), in connection with NMR studies of vegetable oils, has examined a standard triglyceride mixture to check the reliability of the NMR-derived positional distribution data. The error margins were within an acceptable range, and NMR was found to be a superior technique for obtaining positional distribution data.

The NMR-derived positional distribution data, obtained by examining the carbonyl region of the ^{13}C spectrum for the ω3 fatty acids of depot fat of Atlantic salmon, cod liver oil and harp seal oil, are presented in Table 2. We have also included the positional distribution data for EPA and DHA, de-

fined from the spectrum of the glycerol carbon and the C2 carbon region, respectively. Cod liver oil and depot fat of Atlantic salmon have nearly identical positional distributions of EPA, DPA and DHA.

According to expectation, DHA was not randomly distributed (random is defined as 67% α and 33% ß), but preferentially esterified at ß-position of the triacylglycerols, with 74.4 and 72.6%, respectively, for cod liver oil and depot fat of Atlantic salmon. Corresponding data, obtained by traditional techniques, were in the range of 68–74% for cod liver oil (8,14).

NMR data showed that 39.7 and 37.8% of EPA was located at the ß-position in lipids from Atlantic salmon and cod liver oil, respectively. Corresponding literature data for cod liver oil indicated 44% in the ß-position. It is important to notice that EPA is nearly randomly distributed in the triacylglycerol molecule as compared to DHA.

DPA was preferentially esterified at ß-position (68.7%) in muscle lipids from Atlantic salmon. The DPA content of cod liver oil was too low for analyzing the positional distribution of this fatty acid. The distribution of the polyunsaturated fatty acids in triacylglycerols of muscle from Atlantic salmon has not been, as far as we know, previously reported. However, Brockerhoff *et al.* (8) and Litchfield (15) have pointed out the general tendency of EPA, DPA and DHA to be preferentially esterified at the 2-position of fish and invertebrate triacylglycerols. Furthermore, Ando *et al.* (7) have shown that the positional distribution of both DPA and DHA is related to the amount of 20:1 and 22:1 fatty acids in fish triacylglycerols. They found that in fish lipids with high contents of 20:1 and 22:1 nearly 70–80% of the DHA was in the ß-position of the glycerol moiety.

18:4n:3 was randomly distributed in cod liver oil with 33.2% in the ß-position, whereas in the muscle lipids of Atlantic salmon, 18:4 has nearly the same distribution as DHA (73.4% in ß-position).

However, the distribution ω3 fatty acids is quite different in fish oils vs. oils from marine mammals. In harp seal oil, nearly 100, 96.8 and 95.4% of DPA, DHA and EPA, respectively, was esterified to the 1,3-positions of the glycerol moiety (Table 2). This is in accordance with positional data for triacylglycerols of harp seal blubber, presented previously

TABLE 3
Differences in Chemical Shifts Between α and ß Chains

Fatty acid	Atlantic salmon diff.[a] shift α and ß	Cod liver oil diff. shift α and ß	Harp seal oil diff. shift α and ß
18:4n-3	0.44	0.38	0.40
20:4n-3	—	—	0.41
20:5n-3	0.39 0.36	0.38	0.40
22:5n-3	0.44	—	0.39
22:6n-3	0.40 0.37	0.38	0.40

[a]Differential.

(8,14,15,16) as examined by conventional techniques, where 93.7, 87–88 and 70–90% DPA, DHA and EPA, respectively, were located in the 1,3-positions of the glycerol molecule.

The distribution of 20:4n-3 followed the same pattern as the other ω3 fatty acids, with esterification preferentially at the α-position (81.9%). Further, the positional distribution data for EPA and DHA, obtained by examining the carbonyl region, are in good agreement with data obtained by examining the C2 and the glycerol resonances.

In conclusion, our results clearly demonstrate that the NMR method is a noninvasive and nondestructive technique for obtaining information about the positional distribution of ω3 fatty acids in marine lipid triacylglycerols without affecting their chemical structure by preparative treatment. This aspect is under further investigation to examine the positional distribution of monounsaturated fatty acids that exist in abundance in marine lipids.

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REFERENCES

1. Ng, S., *Lipids 20*:778 (1985).
2. Wollenberg, K.F., *J. Am. Oil Chem. Soc. 67*:487 (1990).
3. Gunstone, F.D., *Chem. Phys. Lipids 56*:195 (1990).
4. Gunstone, F.D., *Ibid. 56*:201 (1990).
5. Wollenberg, K., *J. Am. Oil Chem. Soc. 68*:391 (1991).
6. Bonnet, M., C. Denoyer and J.P. Renou, *Int. J. Food Sci. Technol. 25*:399 (1990).
7. Ando, T., K. Nishimura, N. Aoyanagi and T. Takagi, *J. Am. Oil Chem. Soc. 69*:417 (1992).
8. Brockerhoff, H., R.J. Hoyle, P.C. Hwang and C. Litchfield, *Lipids 3*:24 (1968).
9. Myher, J.J., A. Kuksis and L.-Y. Yang, *Biochem. Cell Biol. 68*:336 (1990).
10. Aursand, M., J.R. Rainuzzo and H. Grasdalen, in *Quality Assurance in the Fish Industry, Vol. 30*, edited by H.H. Huss, M. Jakobsen and J. Liston, Elsevier, 1992, pp. 407–416.
11. Aursand, M., and H. Grasdalen, *Chem. Phys. Lipids 62*:239 (1992).
12. Aursand, M., J.R. Rainuzzo and H. Grasdalen, *J. Am. Oil Chem. Soc. 70*:971 (1993).
13. Bligh, E.G., and W.J. Dyer, *Can. J. Biochem. Physiol. 37*:911 (1959).
14. Ackman, R.G., *Adv. Seafood Biochem. 269*:289 (1992).
15. Litchfield, C., *Lipids 3*:417 (1969).
16. Ackman, R.G., and W.M.N. Ratnayake, in *Health Effects of Fish and Fish Oils*, edited by R.K. Chandra, ARTS Biomedical Publishers & Distributors, Newfoundland, 1989.