Quantitative High-Resolution ^{13}C and ^{1}H Nuclear Magnetic Resonance of $\omega 3$ Fatty Acids from White Muscle of Atlantic Salmon (*Salmo salar*)

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High-resolution ^{13}C nuclear magnetic resonance (NMR) spectra have been obtained and used to define the $\omega 3$ (n-3) fatty acid distribution in lipid extract and white muscle from Atlantic salmon (*Salmo salar*). The ^{13}C spectrum of lipid extracted from muscle gives quantitative information about the individual n-3 fatty acids, 18:2n-6, 20:1/22:1 and groups of fatty acids. The quantitative data compare favorably with those obtained by gas–liquid chromatography. The ^{1}H NMR spectrum of the lipid extract gives information about the amount of 22:6n-3 and the total content of n-3 fatty acids. The ^{13}C NMR technique also revealed the positional distribution (1,3- and 2-acyl) of the important 20:5n-3 and 22:6n-3 acids in the triacylglycerol molecules. In the quantitative ^{13}C NMR spectrum of white muscle, the methyl region of the acyl chains of triacylglycerols gave rise to sufficiently resolved signals to permit estimation of the total concentration of lipids and the n-3 fatty acid content. The NMR data are in good agreement with corresponding data obtained by traditional methods.

KEY WORDS: Fish muscle, lipid extract, $\omega 3$ fatty acid distribution, quantitative ^{13}C and ^{1}H NMR.

With the increasing availability of modern instrumentation in scientific institutions, high-resolution nuclear magnetic resonance (NMR) spectroscopy has become popular in the study of lipids. NMR offers the opportunity to study heterogeneous lipid mixtures, oils and depot fat noninvasively and nondestructively. Analytical NMR measurements can be carried out with a high degree of automation.

^{13}C NMR has proven useful in determining the distribution of triacylglycerols and the position of the fatty acids on the glycerol backbone (1–6). Early quantitative analysis of triacylglycerols by NMR were performed by Shoolery (7). More recently, ^{13}C NMR has been used to obtain quantitative information about the fatty acid composition of vegetable oils (2,3), vegetable seeds (8) and other intact tissues (9).

Quantitative and qualitative information can also be obtained from ^{1}H spectra of lipids. Interpretation of the ^{1}H spectra of different cells, tissue and lipid extracts has been given (3,10–12).

In earlier papers (1,13) we reported the lipid composition of depot fat in white muscle of Atlantic salmon (*Salmo salar*) derived from the ^{13}C NMR technique.

In this paper, we present further evidence about the usefulness of the NMR technique (^{13}C and ^{1}H) for the quantitation of fatty acids in lipid extracted from white muscle of Atlantic salmon and in the corresponding muscle.

EXPERIMENTAL PROCEDURES

Hexamethyldisiloxane and 18:2,Δ*cis*-9,12 (linoleic acid), 20:5,Δ*cis*-5,8,11,14,17 [eicosapentaenoic acid (EPA)] and 22:6,Δ*cis*-4,7,10,13,16,19 [docosahexaenoic acid (DHA)] were purchased from Sigma Chemical Co. (St. Louis, MO). $CDCl_3$ (99.5% purity) was purchased from E. Merck (Darmstadt, Germany). D_2O (99.9% purity) was obtained from Cambridge isotope laboratories (Cambridge, England). Chrom(III)-acetyl-acetonate [$Cr(acac)_3$] was supplied by Merck-Schuchardt (München, Germany).

Lipid extraction. Lipids were extracted from white muscle of farmed Atlantic salmon (*Salmo salar*) according to the method of Bligh and Dyer (14). Before analyzing the lipid extracts by NMR, parts of the chloroform phase were removed by evaporation and replaced by $CDCl_3$.

Gas chromatography (GC). An internal standard, 21:0 methyl ester, was added to the extracted sample prior to methylation. Fatty acid methyl esters (FAMEs) were prepared as described by Metcalfe *et al.* (15). The FAMEs were determined quantitatively by capillary GC [Carlo Erba HRGC 5160 series (Milano, Italy) equipped with a SP2330 glass capillary column, on-column injection and flame-ionization detector] fitted with a Shimadzu-Cromatopac C-3rA computing integrator (Tokyo, Japan). The GC oven was programmed from an initial 60 to 150°C at a rate of 25°C/min and maintained 3 min at the latter temperature. Thereafter, the temperature increase was 2°C/min to a final temperature of 190°C. Hydrogen was used as the carrier gas. Identification of FAMEs and quantitation was based on the comparison to standard reference samples (Nu-Chek-Prep., Inc., Elysian, MN).

NMR spectrometry. NMR was performed on a Jeol EX-400 spectrometer (Tokyo, Japan). Lipid extracts and mixtures of free fatty acids (approximately 50–100 mg in 0.6 mL) were examined in $CDCl_3$ in 5-mm NMR tubes. Muscle was minced and mixed with 2–3 drops of D_2O before it was placed in a 10-mm NMR tube. The chemical shifts were referenced indirectly to tetramethylsilane (TMS) by using the central peak of $CDCl_3$ (δ = 77.08). All spectra, except those obtained for T_1 measurements, were acquired at ambient temperature (≈20°C).

The quantitative ^{13}C spectra of lipid extracts and mixtures of free fatty acids were obtained at a frequency of 100.40 MHz with the NOE-suppressed, inverse-gated, proton-decoupled technique. The free induction decay (FID) was acquired with a pulse delay of 40 s for the lipid extract and 60 s for the mixture of free fatty acids, a sweep width of 25 KHz, and 131 K data points. Scans (1000) were collected at a 90° excitation pulse. The quantitative ^{13}C spectra of lipid extract containing relaxation reagent [0.025M or $Cr(acac)_3$] were obtained with the same spectrometer parameters as for the spectra of lipid extract without relaxation reagents. The only difference was the pulse delay, which was changed to 8 s.

The quantitative ^{1}H spectra were obtained at a frequency of 399.65 MHz. The FID was acquired with a pulse delay of 6 s, a sweep width of 5 KHz and 32 K data points, and 16 scans were collected at a 45° excitation pulse. The quantitative ^{13}C spectrum of minced muscle was obtained with the NOE-suppressed, inverse-gated, proton-de-

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coupled technique, with a pulse delay of 25 s, a sweep width of 25 KHz and 131 K data points. Scans (2700) were collected at a 90° excitation pulse. A coaxial two-compartment 10-mm (V_s = 2.6 mL, V_{ref} = 0.41 mL) NMR tube was used, and in the central tube, hexamethyldisiloxane in $CDCl_3$ (C_{ref} = 0.059 M/L) served as an external standard when analyzing the total amount of lipid and n-3 fatty acids in muscle. The chemical shifts of the muscle spectrum were referenced indirectly to TMS by using the central peak of $CDCl_3$ placed in the central tube (δ = 77.08). The relative intensities of ^{13}C resonances were determined by a computer program developed by O.E. Bakøy in our laboratory.

Spin-lattice relaxation times (T_1). T_1 values were measured at 24°C by fast inversion recovery (16). The lipid extracts in $CDCl_3$ were used directly without degassing, and the T_1 values were calculated after examination of the height of the peaks.

RESULTS AND DISCUSSION

Muscle lipids of Atlantic salmon (*Salmo salar*) are composed mainly of triacylglycerols (≈93%) (17). The chainlength of the fatty acids ranges from C14 to C22, which are either fully saturated or contain one to six double bonds. In salmon, lipid reserves (cellular) are mainly incorporated into connective tissue of muscle surrounding single and bundles of muscle cells (18).

Figures 1-3 show expanded ^{13}C spectra of white muscle from Atlantic salmon and corresponding extracted

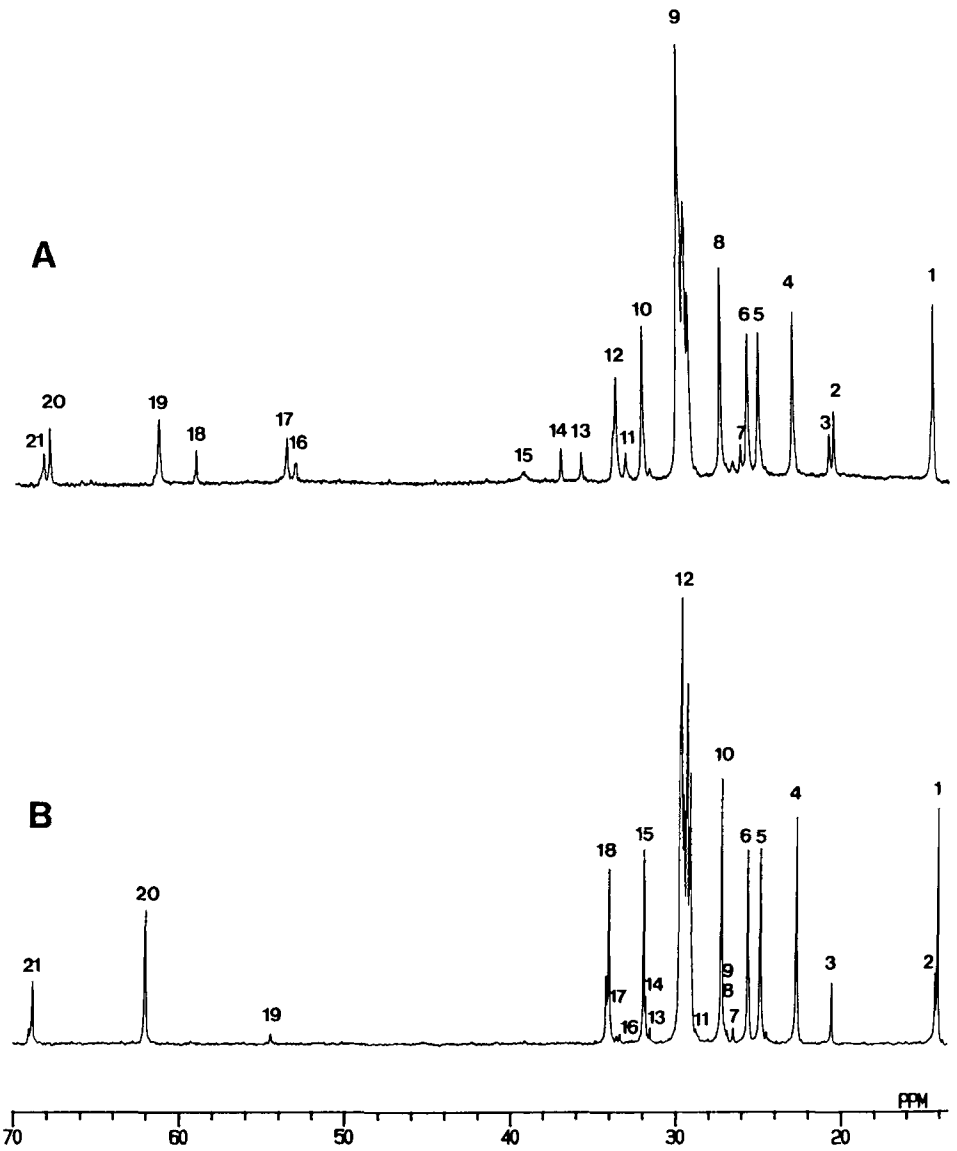


FIG. 1. An expansion of the aliphatic region of the ^{13}C nuclear magnetic resonance spectrum of: (A) white muscle from Atlantic salmon (*Salmo salar*) in D_2O. The proton noise-decoupled spectrum was taken from 32 K data points at a frequency of 100.40 MHz at ambient temperature, with 59,000 scans, pulse delay 0.755 s and a 33° pulse. Assignments for the numbered resonances are given in Table 1; (B) spectrum of the extracted lipids from the corresponding muscle shown in A, dissolved in $CDCl_3$. The spectrum was accumulated with inverse-gated decoupling by using 131 K data points at a frequency of 100.40 MHz at ambient temperature, with 1062 scans, pulse delay 60 s and a 90° pulse. Assignments for the numbered resonances are given in Table 2.

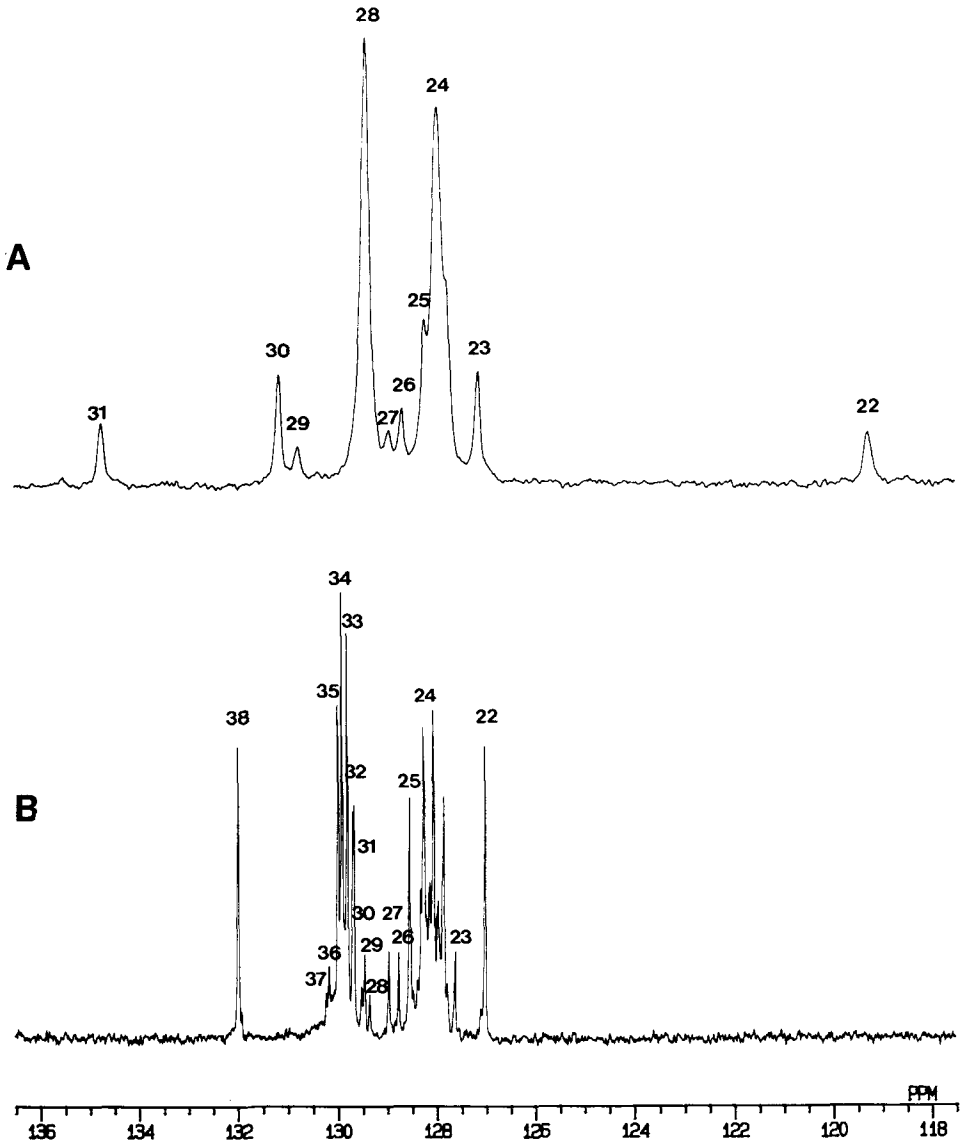


FIG. 2. An expansion of the olefinic region of the ^{13}C nuclear magnetic resonance spectrum of (A) white muscle from Atlantic salmon and (B) lipid extract of corresponding muscle. Assignments for the numbered resonances are given in Tables 1 and 2, respectively. Spectrometer parameters: see Figure 1.

triacylglycerols from muscle. The interpretation of the lipid-extract spectrum (Figs. 1B, 2B and 3B) is based on our previous studies of defined free fatty acids, triacylglycerols, lipid extract of muscle from Atlantic salmon (13) and data on the muscle lipids.

Further evaluation of the ^{13}C NMR signals (Figs. 1A, 2A and 3A) from white muscle was made on the basis of ^{13}C NMR spectra of intact bovine muscle (12), liver (19,20) and other adipose tissues (9,19,21). Interpretation of the observed resonances in the spectra of muscle and in the lipid extract (Figs. 1, 2 and 3) is presented in Tables 1 and 2, respectively.

In the carbonyl region (Figs. 3A and 3B), we observed a 1.12–1.50 ppm shift of the carbonyl carbon resonances of the triacylglycerols when they are extracted from muscle and dissolved in $CDCl_3$. The nuclear resonances from carbon near the glycerol moiety are influenced by the dielectric constant of the medium. A similar effect of the solvent on the resonance frequencies of carbonyl carbon of phospholipid has been interpreted as a result of solvent competition for intermolecular hydrogen bonds (22).

Quantitative NMR parameters. For quantitative measurements, the T_1 values for carbons in the different fatty acids have to be taken into account. T_1 values for the lipids in white muscle of Atlantic salmon and the corresponding extracted lipids in $CDCl_3$ are given in Table 3. The carbon nuclear spin relaxation mechanism is dominated by direct dipolar interaction with the covalently attached protons for all triacylglycerol ^{13}C-nuclei except the fatty acyl carboxyl. As shown in Table 3, the shortest T_1 values are found at the glyceride junction of the three fatty acyl side chains. There is a mobility gradient down the chain, with the shortest correlation time (longest T_1) appearing at the $\omega 2$ carbon (T_1 = 6.15 s) in the n-3 fatty

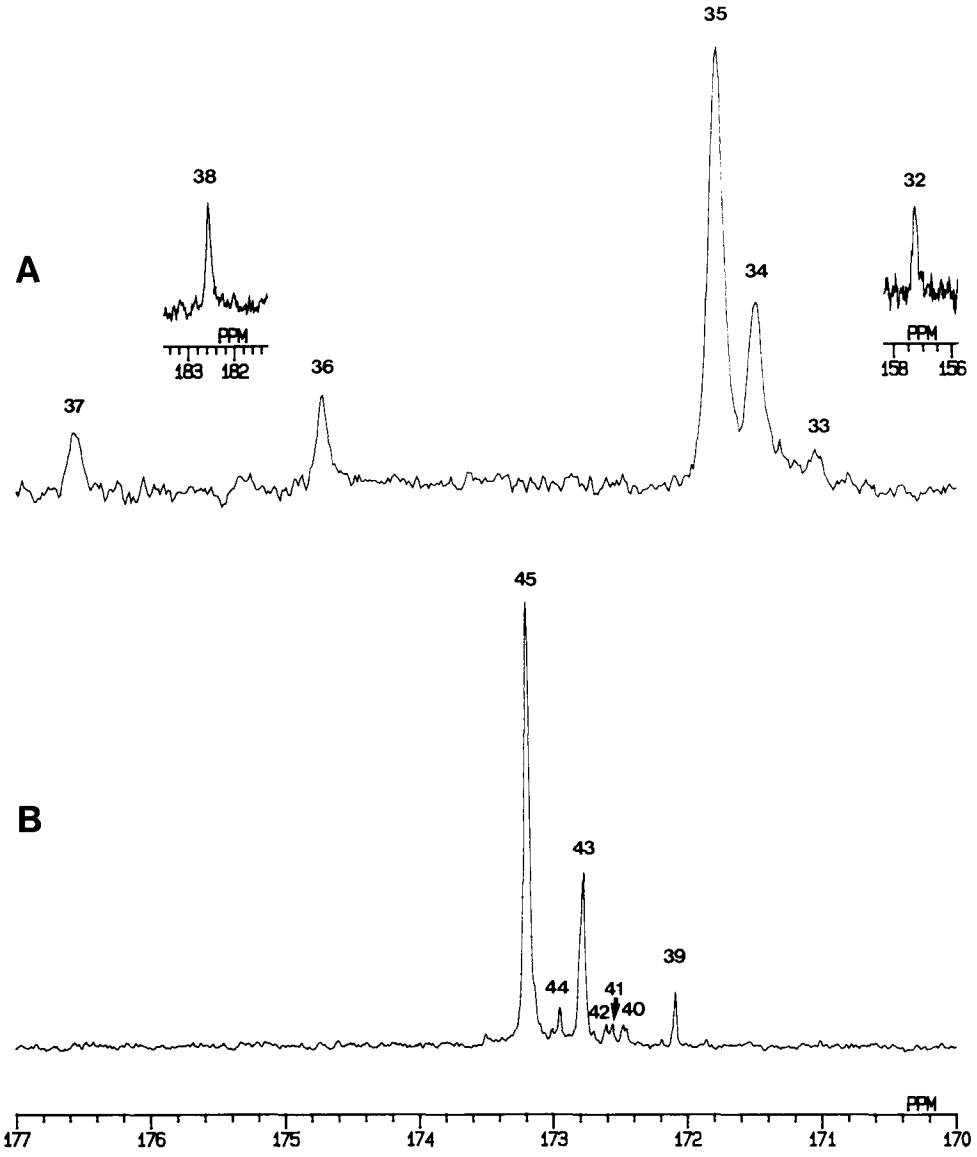


FIG. 3. An expansion of the carbonyl region of the ^{13}C nuclear magnetic resonance spectrum of (A) white muscle from Atlantic salmon and (B) lipid extract of corresponding muscle. Assignments for the numbered resonances are given in Tables 1 and 2, respectively. Spectrometer parameters; see Figure 1.

acids. This is in agreement with recently obtained T_1 data for EPA and DHA in $CDCl_3$ (13). *In situ*, there is a mobility gradient down the chain, with the longest T_1 appearing at the methyl end of the chains, as reported by Sillerud *et al.* (23). Furthermore, the T_1s were found to be two- to threefold longer for the extracted triacylglycerols dissolved in $CDCl_3$ than for the molecules *in situ*. This is understood from the known dependence of rotational correlation time on viscosity (24). The interior of the fat cells in muscle is pure lipid with a viscosity much higher than that of $CDCl_3$.

The quantitation of the total amount of n-3 fatty acids in the ^{13}C spectrum of lipid extract is based on the unique signals from $\omega 1$, $\omega 2$, $\omega 3$ and $\omega 4$ carbons in these fatty acids, with T_1 values 5.29, 6.15, 5.85 and 5.85 s, respectively. To get satisfactory quantitative results, the recycle time has to be ≥ 6.5 T_1 (25). Because of the limited sensitivity of the ^{13}C nucleus, nearly 1000 scans must be collected, and an experimental time of nearly 10 h would be needed, which is not practical for most applications. To decrease the experimental time for performing quantitative measurements, addition of paramagnetic compounds, which decrease the relaxation time, is provided (7,26). $Cr(acac)_3$ (conc. 0.025M) was added to the sample. Addition of the relaxation reagent in this concentration resulted in minimal line broadening. The effect on the T_1 values for the triacylglycerol carbons is shown in Table 3.

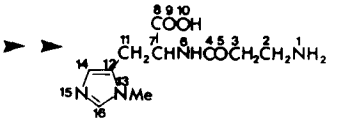
The $\omega 4$ carbon of the n-3 fatty acids has the longest T_1 value (T_1 = 1.6 s). Addition of relaxation reagent reduced the experimental time fivefold when analyzing the lipid extract in $CDCl_3$ solution. Mooney (26) reported that the normal concentration of paramagnetic reagent used is 0.1M $Cr(acac)_3$. This reduces the relaxation time to less than 1 s for all types of carbon nuclei. However, we have

TABLE 1

Peak Assignments[a] for ^{13}C Nuclear Magnetic Resonance Spectrum of White Muscle of Atlantic Salmon (*Salmo salar*)

Peak	Compound	Carbon	Chemical shift (ppm)
1	All fatty acids (f.a.)	$-CH_3$	14.06
2	Lactate	C3	20.23
3	All n-3 f.a.	$\omega 2$[b]	20.51
4	All f.a. except 18:3, 18:4, 20:5, 22:5	$\omega 2$/C3 in DHA	22.80
5	All f.a. except 22:6	C3	24.88
6	Unsaturated f.a.	$=CH-\underline{C}H_2-CH=$	25.56
7	Anserine	11[c]	25.96
8	Mono, di and 18:3	$-\underline{C}H_2-CH=CH$	27.23
9	All f.a. except 20:5, 22:6	$-(CH_2)n-$	29.89
10	All f.a. except n-3 f.a.	$\omega 3$	32.09
11	Anserine	$-N(-CH_3)$	33.15
12	All f.a.	C2	33.67
13	Anserine	2 $(-CH_2-)$[c]	35.78
14	Not identified		35.78
15	Not identified		37.05
16	Anserine	7 (-CH-)[c]	53.43
17	Creatine/phosphocreatine	$-CH_2$	53.93
	Phosphatidylcholine	$-N^+(CH_3)_3$	
18	Not identified		59.50
19	Glyceryl	C1,C3	61.84
20	Lactate	C2	68.59
21	Glyceryl	C2	68.94
22	Anserine	14 (=CH-N)[c]	118.24
23	All n-3 f.a.	$\omega 4$	127.09
24	Polyunsaturated f.a.	$-\underline{C}=\underline{C}$	127.99
			128.28
25	Highly unsaturated f.a.[d]	$-\underline{C}=\underline{C}-$	128.79
26	Eicosapentaenoic acid (20:5)	C5,C6	129.07
27	DHA (22:6)	C4,C5	129.42
28	Monounsaturated and 18:2, 18:3 f.a.	$-\underline{C}=\underline{C}-$	129.60
29	Anserine	12 (=C-N)[c]	131.12
30	All n-3 f.a.	$\omega 3$	131.53
31	Anserine	16 (N-C=N)[c]	135.47
32	Creatine/phosphocreatine	$N-C(NH_2)_2$	157.33
33	DHA in β-position in glyceryl	$-\underline{C}OOCH_2H$	170.99
34	f.a. in β-position[e]	$-\underline{C}OOCH_2H$	171.45
35	f.a. in α-position	$-\underline{C}OOCH_2H$	171.73
36	Not identified	$-\underline{C}OOCH_2H$	174.73
37	Anserine	$-\underline{C}OOH$	176.50
38	Lactate	$-\underline{C}OOH$	182.53

[a]The chemical shifts are referenced indirectly to trimethylsilane by using the central peak of $CDCl_3$ (δ = 77.08 ppm).
[b]Carbon number two from the methyl end.
[c]IUPAC (*International Union of Pure and Applied Chemistry*, Oxford, 1982) numbering of the atoms in anserine;



[d]18:4, 20:4n-6, 20:4n-3, 20:5, 22:5 and 22:6.
[e]All fatty acids except docosahexaenoic acid (DHA) in β-position of the triacylglycerols and phospholipid.

used only 0.025M, and because of the linewidth increase at higher levels of $Cr(acac)_3$, 0.025M was a compromise. Shoolery (7) has also studied the effect of different concentrations of $Cr(acac)_3$ on relaxation times.

Furthermore, to obtain quantitative ^{13}C spectra with minimal recycle time, a correction factor dependent on the Nuclear Overhouser Effect (NOE) and T_1 *vs.* repetition rate can be found (9). This will be a subject for further research, especially when studying intact muscle.

Quantitation of lipid extract by ^{13}C NMR. A standard mixture of 18:2n-6, EPA and DHA was prepared to evaluate the accuracy of the NMR-derived fatty acid content values (Table 4). The quantitative examination was carried out on the fatty acid mixture with and without relaxation reagent. The relative concentrations are within $\pm 1.5\%$ and $\pm 0.7\%$ of the actual values with and without relaxation reagent, respectively. These error margins are within an acceptable range for NMR as a quantitative technique.

From the interpretation of the ^{13}C spectrum of the

TABLE 2

Peak Assignments[a] for ^{13}C Nuclear Magnetic Resonance Spectrum of Lipid Extracted from White Muscle of Atlantic Salmon (*Salmo salar*)

Peak	Compound	Carbon	Chemical shift (ppm)
1*[b]	n-6 fatty acids (f.a.)	$-CH_3$	14.09
	All f.a. except n-3/n-6 f.a.	$-CH_3$	14.13
2*	n-3 f.a.	$-CH_3$	14.29
3*	n-3 f.a.	$\omega 2$[c]	20.56
4*	All f.a. except 18:3, 18:4, 20:5, 22:5	$\omega 2$/C3 in	22.63
		22:6	22.68
			22.71
5*	All f.a. except 22:6	C3	24.67
			24.73
			24.86
6	Unsaturated f.a.	$=CH-\underline{C}H_2-CH=$	25.53
			25.63
7*	EPA and 20:4n-6	C4	26.49
8*	18:4	C5	26.84
9*	22:5	C6	27.00
10	Mono-, diunsaturated and 18:3 f.a.	$\underline{C}H_2-CH=CH$	27.03
			27.17
		$\underline{C}H_2-CH=CH$	27.22
11*	22:5	C4	28.75
12*	All f.a. except 20:5 and 22:6	$-(CH_2)n-$	29.00
			29.78
13*	n-6 f.a.	$\omega 3$	31.80
14*	n-7 f.a.	$\omega 3$	31.92
15*	Saturated and n-9/n-11 f.a.	$\omega 3$	31.94
16*	20:4, 20:5 in α-position[d]	C2	33.36
17*	20:4, 20:5 in β-position	C2	33.55
18*	All f.a. except 20:5	C2	33.88
			34.03
			34.18
19	Phosphatidylcholine	$-N^+(CH_3)_3$	54.16
20	Glyceryl	C1,C3	62.01
			62.07
21	Glyceryl	C2	68.86
			68.93
			68.98
			69.08
22*	All n-3 f.a.	$\omega 4$	127.00
23*	22:6	C4	127.61
24*	Polyunsaturated 2-3 double bonds	-C=C-	127.78
		-C=C-	128.38
25*	Polyunsaturated 4-6 double bonds[e]	-C=C-	128.46
		-C=C-	128.54
26*	20:4, 20:5	C6	128.76
27*	20:4, 20:5	C5	128.95
28	Not identified		129.35
29*	22:6	C5	129.44
30	Not identified		129.51
31*	18:1 + 16:1	C9	129.66
32*	16:1	C9	129.69
33*	20:1/22:1	C11/C13	129.80
34*	20:1/22:1	C12/C14	129.91
35	18:1/16:1	C9/C10	129.99
36*	18:2	C13	130.18
37*	18:3	C9	130.22
38*	All n-3 f.a.	$\omega 3$	131.99
39*	22:6 in β-position in triacylgl.	$-\underline{C}OOCH_2H$	172.11
40*	22:6 in α-position in triacylgl.	$-\underline{C}OOCH_2H$	172.51
41	20:4, 20:5 in β-position in triacylglcerol	$-\underline{C}OOCH_2H$	172.59
		$-\underline{C}OOCH_2H$	172.63
42	Not identified	$-\underline{C}OOCH_2H$	172.77
43	All f.a. except 20:4, 20:5 and 22:6 in β-position of triacylglycerol		172.81

(continued)

TABLE 2 (continued)

Peak	Compound	Carbon	Chemical shift (ppm)
44	20:4, 20.5 in α-position in triacylglycerol	-$\underline{C}$OOCH$_2$H	172.98 173.04
45	All f.a. except 20:4, 20:5 and 22:6 in α-position	-$\underline{C}$OOCH$_2$H	173.23

[a]The chemical shifts are referenced indirectly to trimethylsilane by using the central peak of $CDCl_3$ (δ = 77.08).
[b]The area of the peaks designated with an asterisk (*) have been used to calculate the distribution of fatty acids with the least-square method.
[c]Carbon number two from the methyl end.
[d]20:4n-6 and 20:5 in α-position of the triacylglycerol molecule.
[e]18:4, 20:4n:6, 20:4n-3, 20:5, 22:5 and 22:6.

TABLE 3

^{13}C Spin-Lattice Relaxation Times Values (s) for Lipid Extract with and without Relaxation Reagent [Cr(acac)$_3$][a] and White Muscle of Atlantic Salmon (*Salmo salar*)

Carbon	Lipid extract		*In situ*
		with Cr(acac)$_3$	
Glyceryl, α	0.29	0.22	0.14
β	0.36(0.43)		0.17
C1	3.08	0.87	0.47
C2	0.58	0.50–0.58	0.22
C3	0.72	0.22	0.29
C4 in 20:5	1.00		
C4 in n-6 fatty acids (f.a.)	0.70		
=CH-$\underline{C}$H$_2$-CH=	2.14	0.87	0.39
	3.46[b]	1.15	
-$\underline{C}$H$_2$-CH=CH-	1.01	0.79	0.43
ω3[c] carbon in;			
n-6 f.a.	3.08		1.01–1.15
n-7 f.a.	2.72		1.01–1.15
n-9 and sat. f.a.	2.72		1.01–1.15
ω2 carbon in;			
All except n-3 f.a.	2.72	1.15	1.01
n-3 f.a.	6.15	1.15	1.47
Methyl end in;			
All except n-3 f.a.	3.36	1.15	2.31
n-3 f.a.	5.29		2.31
Olefinic carbon;			
ω3 carbon in n-3 f.a.	5.85	1.45	1.47
ω4 carbon in n-3 f.a.	5.85	1.60	1.78
20:5 (C5/C6)	1.60	1.01–1.15	0.87[d]
22:6 (C4/C5)	1.60		
Not identified	1.75	1.01–1.15	
ω6 carbon in polyunsaturated f.a.	4.08	1.01–1.15	
ω7 in 20:5, 22:6	3.66	1.01–1.15	
$\geqslant$ ω7 carbon in unsaturated	2.22–2.72	1.01–1.15	
20:1/22:1; (C11, C13/C12, C14)	1.37	1.01–1.15	0.50[e]
18:1/16:1 (C9/C10)	1.45	1.01–1.15	
18:2 (C13)	2.55	1.01–1.15	
18:3 (C9)	1.45	1.01–1.45	

[a]Concentration of Cr(acac)$_3$ is 0.025M.
[b]Allylic carbon near the methyl end in polyunsaturated fatty acids.
[c]Carbon number three from the methyl end.
[d]Olefinic carbon mainly from the polyunsaturated fatty acids.
[e]Olefinic carbon mainly from the monounsaturated fatty acids.

TABLE 4

Quantitation of Known Fatty Acid Solution in $CDCl_3$ with and without Relaxation Reagent [$Cr(acac)_3$ 0.025M] by ^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy

	Known solution			
			Examined by ^{13}C NMR	
Fatty acids	wt%	mol%	mol%	mol% with $Cr(acac)_3$
18:2n-6	50.42	53.83	54.5	55.3
20:5n-3	12.43	12.29	12.2	11.6
22:6n-3	37.15	33.86	33.3	33.1

TABLE 5

Comparison of the Determination of Fatty Acid Composition[a] of Lipid Extracted from White Muscle of Atlantic Salmon Examined by ^{13}C NMR, ^1H NMR Spectroscopy and GC[b]

Fatty acid	^{13}C NMR (mol%)	^1H NMR (mol%)	GC (mol%)
18:1n-9	12.4 (13.6)[c]		14.7
20:1/22:1	26.5 (25.0)		23.2
16:1/18:1n-7	11.5 (10.3)		12.5
18:2n-6	3.4 (3.1)		2.8
18:3n-3	0.9 (0.7)		1.0
18:4n-3	1.9 (2.3)		2.0
20:4n-3	3.1 (1.5)		1.4
20:5n-3	3.9 (5.2)		5.2
22:5n-3	2.0 (2.1)		1.4
22:6n-3	7.1 (7.1)	6.1	6.7
Saturated	27.0 (28.9)		27.0
Monounsaturated	50.5 (48.9)		50.5
Polyunsaturated	22.4 (22.1)		20.5
HUFA[d]	16.1 (16.0)		16.7
ω3	19.0 (19.0)	18.9	17.7
ω6	3.4 (3.1)		2.8

[a]Quantitative data obtained by nuclear magnetic resonance (NMR) are given directly in terms of molar fractions. Gas chromatography (GC) data were converted into mol% from wt%.
[b]The NMR analysis were done on $CDCl_3$ solutions of extracted lipid, while the GC analysis were done on derived fatty acid methyl esters.
[c]Composition of lipid extract with relaxation reagent $Cr(acac)_3$ (0.025M).
[d]18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3. HUFA, high unsaturated fatty acids.

lipid extracted from white muscle of Atlantic salmon (Table 2), lines from both the methylene (Fig. 1B), the olefinic (Fig. 2B) and the carbonyl (Fig. 3B) region in the ^{13}C spectrum must be considered to obtain quantitative information about the fatty acid distribution and the position of the fatty acids in the triacylglycerols of EPA and DHA. Obviously, in the ^{13}C spectrum of the lipid extract, there are many well-separated resonances that can be assigned to specific carbon atoms from fatty acids and groups of fatty acids (1,13). In spectra obtained under quantitative conditions, the peak areas of these unique signals are directly proportional to the amount of the fatty acids.

Wollenberg (2) has examined the extracts of different vegetable seeds. He concluded that a combination of the olefinic and carbonyl spectral regions provides the necessary information to quantify the position of the fatty acids in the triacylglycerols of complex oils.

We have collected the ^{13}C spectrum of lipid extract of white muscle from Atlantic salmon. The carbon peaks used for the calculations are designated with an asterisk (*), (Table 2). The data obtained with the least square method (7,27) in the calculations are listed in Table 5. The GC-derived distribution of the corresponding extract is included for comparison. The data show a satisfactory correlation between the NMR-derived triacylglycerol distribution and GC-derived results.

The NMR determination (1–2 h) is as rapid as the GC method, which involves hydrolysis of the esters and their subsequent methylation. The NMR method has the advantage of being gentle to temperature-sensitive samples. This is particularly important for n-3 fatty acids. The content of EPA in the α-(*sn*-1,3) and the β-(*sn*-2) positions can be calculated from the area under peaks 16 and 17, respectively, in Figure 1B, and for DHA from the areas under peaks 39 (β) and 40 (α) in the carbonyl region of the ^{13}C spectrum (Fig. 3B and Table 2). The data in Table 6 indicate that DHA preferentially is in the β-position, and EPA has a slight preference for the β-position of the triacylglycerol and phospholipid moieties (28). Brockerhoff *et al.* (29) and Litchfield (30,31) have pointed out the general tendency of 20:5, 22:5 and 22:6 to be preferentially esterified at β-position in fish and invertebrate triacylglycerols. Ando *et al.* (32) has shown that the positional distribution of DHA has some relation to the amount of 20:1/22:1 fatty acids in fish triacylglycerols. In fish oils with high contents of 20:1/22:1, nearly 70–80% of the DHA is in the β-position of the glycerol moiety. The data (Table 6) derived by ^{13}C NMR analysis are in good agreement with the results presented by Ando *et al.* (32). If we want to examine simultaneously the distribution of other fatty acids in addition to the positional distribution of EPA and DHA, the NMR parameters have to be set as described under Experimental Procedures. However, if only the positional distribution of EPA and DHA is to be examined, the ^{13}C spectrum of the lipid extract with relaxation reagent can be collected with 20–30 min. The T_1 values for the carbons in EPA and DHA that are involved in these measurements are in the range of 0.50–0.87 s (Table 3).

Quantitation of lipid extract by ^1H NMR. The resonance data given in Table 7, for the ^1H NMR spectrum

TABLE 6

1,3(α)-,2(β)-Acyl Distribution for 20:5n-3 and 22:6n-3 Fatty Acid in Lipid Extracted from White Muscle of Atlantic Salmon Obtained by ^{13}C Nuclear Magnetic Resonance Spectroscopy

Fatty acid	1,3-Position (%)	2-Position (%)
20:5[a]	57.0	43.0
22:6[b]	18.4	81.6

[a]The area at 33.36 and 33.55 ppm, respectively, indicates the amount of 20:5 in 1,3- and 2-position of the glyceryl moiety (Table 2, Fig. 1).
[b]The area at 172.51 and 172.11 ppm, respectively, indicates the amount of 22:6 in 1,3- and 2-position of the glyceryl moiety (Table 2, Fig. 3).

TABLE 7

Peak Assignments[a] for ^{1}H NMR Spectrum of Lipid Extracted from White Muscle of Atlantic Salmon (*Salmo salar*)

Peak	Compound	Carbon	Chemical shifts (ppm)
1	All fatty acids (f.a.) except n-3 f.a.	$-CH_3$	0.85–0.89
2	n-3 f.a.	$-CH_3$	0.95–0.98
3	All f.a. except 20:5 and 22:6	$-(CH_2)_n-$	1.25–1.29
4	All f.a. except 22:6	$-C\underline{H}_2-CH_2-COOH$	1.60
5	Unsaturated f.a.	$-C\underline{H}_2-CH=CH$	1.99–2.07
6	All f.a. except 22:6	$-C\underline{H}_2-COOH$	2.28
7	22:6	$=CH-C\underline{H}_2-C\underline{H}_2-COOH$	2.38
8	Polyunsaturated f.a.	$=CH-C\underline{H}_2-CH=$	2.81–2.84
9	PC[b]	$-N(C\underline{H}_3)_3$	3.35
10	Phospholipid	—	3.64
11	Phospholipid	—	3.80
12	PC/PE[b]	Glyceryl moiety	4.00
13	Glyceryl	C1,3 protons	4.11
14	Glyceryl	C1,3 protons	4.31
15	Glyceryl	C2	5.26
16	Unsaturated f.a.	$-C\underline{H}=C\underline{H}-$	5.34–5.36

[a]The chemical shifts are referenced indirectly to trimethylsilane using the peak of $CHCl_3$ (δ = 7.26 ppm).
[b]PC, phosphatidylcholine; PE, phosphatidylethanolamine; NMR, nuclear magnetic resonance.

(Fig. 4) of lipid extracted from the white muscle of Atlantic salmon are partly based on data reported in the literature (3,10,12,33–36).

^{1}H NMR has two significant advantages. The ^{1}H nucleus has the highest NMR sensitivity of any stable nucleus, and it has nearly 100% natural abundance. However, compared to ^{13}C spectra, the ^{1}H NMR spectra show small chemical shift dispersion and extensive multiplicity due to homonuclear j-coupling, which often result in severe spectral overlap, makes quantitation difficult. In addition, ^{1}H spectra may contain broad resonances from phospholipid. The second advantage is the much shorter experimental time when collecting quantitative spectra, compared to ^{13}C NMR.

The lipid extract shows the expected range of signals in the ^{1}H NMR spectrum (Fig. 4). In addition to the usual $-CH_3$ signals at 0.85–0.89 ppm, a triplet at 0.95 ppm arises from the $-CH_3$ carbon of the n-3 fatty acids. Additional signals also appear on the side of signals at 2.28, 2.0 (allylic) and 1.6 ppm (Fig. 4, Table 7). The first of these (2.38 ppm) is a signal assigned to C2 and C3 hydrogens (four) of DHA (10), the others arise from allylic or C3 hydrogens under the additional influence of a nearby double bond in polyene acids (33).

Consequently, we can estimate from the ^{1}H spectrum of the lipid extracted from muscle the total amount of n-3 fatty acids and the content of DHA. As can be seen in Table 5, values compared well with thoe obtained from the ^{13}C spectrum and the GC method.

Quantitation of lipid in muscle by ^{13}C NMR. A semiquantitative ^{13}C NMR spectrum of white muscle from Atlantic salmon is presented in Figures 1A–3A. ^{13}C NMR determination of the total concentration of lipids and groups of fatty acids within white muscle from Atlantic salmon is possible due to the liquid-like nature of the tissue lipids, with high contents of polyunsaturated fatty acids. As mentioned before, the fat reserves are incorporated into muscle as cellular lipids in connective tissue (18). Consequently, the lipids dispersed in white muscle produce an inhomogeneous sample, and there is considerable line broadening, thus decreasing resolution and sensitivity compared to the ^{13}C spectrum of lipid extract of muscle (Figs. 1–3). The line broadening originates from the heterogeneous nature of the muscle and ultimately from induced dipolar fields produced by differences of bulk magnetic susceptibility between the lipid cells and muscle cells (37,38,39). However, the resolution obtainable in the quantitative NMR measurement was adequate to quantitate the total concentration (wt%, from external reference) of lipids and the total amount of n-3 fatty acids, in addition to the muscle metabolites lactate and anserine. ^{13}C NMR can also be used to estimate the intracellular pH *via* the C14 (peak designated 22, see Fig. 2A, Table 1) carbon in the histidine ring of anserine (12). The data for quantitation of lipids are given in Table 8. Peaks used in the present analysis are designated 1 ($-CH_3$ carbons), 30 (ω3 carbons of n-3 fatty acids) and 31 (C16 in anserine) (Fig. 1A and 2A, Table 1). To verify the quantitative NMR results obtained for the muscle, the lipids in the muscle examined by NMR were extracted and analyzed by ^{13}C, ^{1}H NMR spectroscopy and GC (Table 5). The total lipid content was examined gravimetrically (Table 8).

T_1 values of triacylglycerol carbons in lipids of muscle show that the $-CH_3$ carbon resonances have the longest T_1 time (T_1 = 2.31 s), and the quantitative ^{13}C NMR spectrum must be collected with a recycle time $\geq$ 6.5 T_1 and nearly 1000 scans. ^{13}C spectra of white muscle collected in this way required long measuring times. To lower the recycle time, correction factors dependent on NOE and T_1 can be used (9).

The measured values for the total concentration of fatty acids are in good agreement with the total lipid content

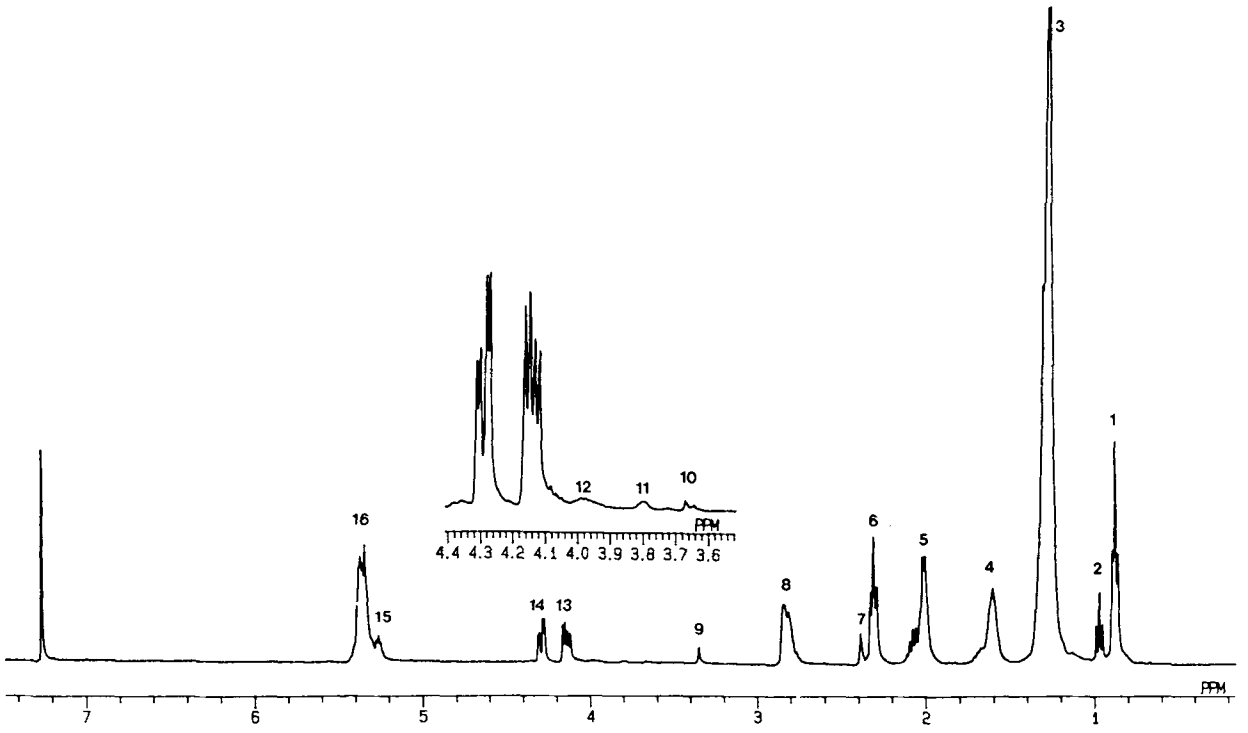


FIG. 4. ^{1}H nuclear magnetic resonance of lipid extracted from white muscle of Atlantic salmon (*Salmo salar*). The spectrum was accumulated from 32 K data points at a frequency of 399.65 MHz at ambient temperature, with 16 scans and pulse delay of 6 s and 45° pulse. Assignments for the numbered resonances are given in Table 7.

TABLE 8

Quantitation of Total Lipid and ω3 Fatty Acids in White Muscle from Atlantic Salmon (*Salmo salar*) by Traditional Methods (lipid extraction/gravimetrical examination and GC, respectively) and by ^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy of Muscle and the Corresponding Lipid Extract

	Lipid extract			
	GC[a]	Gravimetrical method	^{13}C NMR	Muscle ^{13}C NMR[b]
Total lipid (wt%)	—	11.1	—	10.9
n-3 Fatty acids (mol%)	17.7	—	19.0	20.6

[a]Examined by gas chromatography (GC), see Table 5.
[b]$C_s = (I_s/I_r)\cdot(V_r/V_s)\cdot C_r, C_s$ = conc. sample, I_s = intensity sample signal, V_s = volume sample, C_r = conc. external reference (ref), I_r = intensity ref. signal, V_f = volume ref.

obtained gravimetrically (Table 8). Consequently, the methyl end region (ω1 to ω3 carbon) of the acyl chains of the triacylglycerols has sufficient mobility at ambient temperature to give rise to resolved signals in the ^{13}C NMR spectrum of the minced white muscle. This is not in accordance with that reported by Lundberg *et al.* (12) for intact bovine muscle. The main reason for this is probably the negligible content of polyunsaturated fatty acids in bovine muscle and the much higher amount of shorter-chain saturated and mono- and diunsaturated fatty acids with higher melting points.

The n-3 fatty acid content obtained by analyzing ^{13}C spectrum of white muscle (Table 8) also compares well with data obtained by ^{13}C, ^{1}H NMR and GC when analyzing the corresponding lipid extract.

However, Wollenberg (8) has used the magic-angle sample spinning (MASS) NMR technique to obtain quantitative data in whole vegetable seeds. MASS reduces line broadening, which arises from differences in magnetic susceptibility (38–40). Because any reduction of the linewidth makes signals higher, the signal-to-noise ratio is also improved. Therefore, this method can be applied to intact muscle from Atlantic salmon and might result in additional quantitative and qualitative information for the muscle.

The results from this study show that ^{13}C NMR is a useful method to obtain information about the distribution of individual n-3 and groups of fatty acids in lipid extracted from muscle, within 1–2 h experimental time. The positional distribution of EPA and DHA in the glycerol moiety can be determined in 20–30 min. ^{1}H NMR gives information about the total amount of n-3 fatty acids and DHA within 2–5 min.

When analyzing the ^{13}C spectrum of white muscle, satisfactory quantitative and qualitative data can be obtained. We hope, however, to examine intact muscle by MASS ^{13}C and ^{1}H NMR to obtain still higher sensitivity and better resolution of the quantitative spectra.

NMR analysis of muscle and of lipid extracted from muscle appears to be an interesting approach to study changes occurring during storage of fish.

ACKNOWLEDGMENTS

Research grants from University of Trondheim and the Norwegian Fisheries Research Council (to M.A.) are gratefully acknowledged.

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[Received November 30, 1992; accepted July 7, 1993]