Quantitative High-Resolution ^{13}C NMR Analysis of Lipids Extracted from the White Muscle of Atlantic Tuna (*Thunnus alalunga*)

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Lipids extracted from albacore tuna (*Thunnus alalunga*) were studied using high-resolution ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Simultaneous quantitative determination of free fatty acids, lipid classes, and fatty acids α–β positional distribution in triacylglycerols was obtained. Glyceride and phospholipid composition was studied using glyceryl resonances. Lipids extracted from fresh white muscles of Atlantic tuna had a different triacylglycerol and phospholipid content, related to fish weight. Free fatty acid carbonyl resonances were detected at the lower field of the ^{13}C NMR carbonyl region and used for its quantitative determination. NMR data agreed with those obtained using UV spectrophotometry, showing that NMR is a suitable method to follow lipolytic alterations. The careful study of glyceryl and carbonyl resonances also leads to the direct determination of fatty acid α–β distribution in triacylglycerols. The quantitative analysis of fatty acid composition and distribution was then achieved, and results obtained using high-resolution NMR were in good agreement with those obtained by gas chromatography. Polyunsaturated fatty acids showed a preferential location in the β-acyl position, whereas monounsaturated fatty acids were found preferentially esterified in the α-position. The method may be used to monitor the composition and changes in fish lipids and oils during processing or storage.

High-resolution NMR is increasingly applied to the analysis of food and food components. In the field of edible oils and fats, NMR provides useful information on the composition of palm, seed, and olive oils (Ng, 1985; Wollenberg, 1990; Sacchi et al., 1992) and some terrestrial animal fats (Bonnet et al., 1990). However, its application to more complicated lipid substrates, such as those extracted from marine sources, has proved to be more difficult (Gunstone, 1991; Aursand and Grasdalen, 1992).

The analysis of fish lipid composition, however, is particularly interesting due to the well-known benefits of some components to human health, particularly the omega-3 (or n–3) polyunsaturated fatty acids (PUFA) in preventing cardiovascular disease (Kinsella, 1986). The amounts of n–3 PUFAs, such as DHA (22:6, n–3) and EPA (20:5, n–3), vary from species to species and may also change depending on fish processing and storge (Aubourg et al., 1989, 1990; Yamamoto and Imose, 1989; Cronin and O'Sullivan, 1990).

Lipid class composition (the relative amount of triacylglycerols, diacylglycerols, free fatty acids, sterols, phospholipids, and other minor components) is another important aspect, from which lipolytic alterations can be studied and used to determine the freshness of fish stored in ice (Barassi et al., 1987).

A further important characteristic of fish lipids and oils, extremely complex from an analytical point of view, is the distribution of fatty acids on the glycerol backbone of triacylglycerols. For many, years, the analysis of triglyceride acyl distribution has implied the use of enzymatic and chemical hydrolysis. Wide use has been found for lipase hydrolysis, although it turned out not to be accurate enough for marine lipids since the presence of double bonds in the proximity of the carbonyl group of fish PUFAs reduces the rate of deacylation of glycerides (Walker, 1975; Christie, 1986). The Grignard reagent is useful for marine substrates (Aubourg et al., 1990), and new high-performance liquid chromatography and gas–liquid chromatography combined with mass spectrometry methods have provided quick, useful procedures (Holmer, 1989; Nikolova-Damuyanova et al., 1990), as has chiral chromatography (Christie, 1992).

The aim of this work was to apply high-resolution ^{13}C NMR spectroscopy as an improved, nondestructive, direct, and quantitative method for the study of fish lipid composition and structure. For this purpose, lipids extracted from the white muscle of albacore tuna (*Thunnus alalunga*) were analyzed on high-field NMR spectrometers (67.88–100.64 MHz), and quantitative NMR-derived data were compared with those obtained using the classical methods (gas–liquid chromatography and UV spectrophotometry). The instrumental conditions for the simultaneous quantitative determination of lipid classes and free fatty acids and quantities and positional distribution of PUFAs in triacylglycerols are described, and the general applicability of the method is discussed.

MATERIALS AND METHODS

Standards and Reagents. Standard triacylglycerols, phospholipids, cholesterol, fatty acid methyl esters, and free fatty acids were obtained from Fluka (Buchs, Switzerland) and Nu-Chek-Prep Inc. (Elysian, MN). Chloroform-*d* [with 0.03% v/v internal tetramethylsilane (TMS)] was an Aldrich Chemical Co. (Milwaukee, WI) product. All solvents were of reagent grade and were purchased from Fluka.

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Fish Samples and Lipid Extraction. The albacore tuna (*T. alalunga*) used in this work were caught by a commercial tuna vessel on the Atlantic Ocean (around 43° N and 27° W). After arrival at the laboratory, the fish were frozen and kept at −20 °C. Lipids were extracted from the white muscle of 8–10-kg tuna samples using the Bligh and Dyer (1959) method and then stored at −20 °C in dichloromethane (CH_2Cl_2) until analyzed; propyl gallate was added as an antioxidant. The composition of lipid extracted was checked by means of preliminary NMR spectroscopy (Sacchi et al., 1990, 1991), and triacylglycerol acyl distribution was studied on lipid samples containing no appreciable quantities of material other than triacylglycerols.

Free Fatty Acid Determination. Free fatty acids were measured using NMR spectroscopy (Sacchi et al., 1990) and using the Lowry and Tinsley (1976) method based on the formation of a complex with $(AcO)_2Cu$–pyridine and UV spectrophotometric measurements.

Gas Chromatography. Lipid extracts were transesterified with CH_3COCl/CH_3OH as described by Christie (1982). Fatty acid methyl esters (FAME) were analyzed on a Perkin-Elmer 8700 gas–liquid chromatograph. Conditions: SP-2330 fused silica capillary column (0.25 mm i.d., 30 m length) (Supelco, Inc., Bellefonte, PA), with temperature programmed from 145 to 190 °C at 1.0 °C/min, from 190 to 210 °C at 5.0 °C/min, 13.5 min at 210 °C, and from 210 to 230 °C at 5.0 °C/min; detector temperature, 250 °C; carrier gas, N_2 (10.0 psig); injector (split ratio 150:1), heated to 225 °C at 15 °C/s. The individual FAMEs were identified by comparison with the retention times of standard mixtures (Supelco: PUFAs 1 and 2; Larodan, Qualmix Fish).

Carbon-13 Nuclear Magnetic Resonance. Full ^{13}C NMR spectra and high-resolution spectra of the carbonyl region were recorded on two Bruker (Karlsruhe, Germany) spectrometers operating at ^{13}C frequencies of 67.88 and 100.64 MHz, respectively. Spectra were recorded at concentrations of 10–20% w/v (50–100 mg of lipid in 0.5 mL of chloroform-*d*) using 5-mm NMR tubes. Spectra were recorded at controlled temperatures of 30 ± 0.1 °C to obtain the best chemical shift and relaxation rate reproducibility. Two different sets of acquisition parameters were used. The full ^{13}C NMR spectrum was recorded with 256-3000 scans using a 200 ppm spectral width, 16K data points, a 0.37-s acquisition time, a relaxation delay of 5 s, and a 45° pulse width. High-resolution carbonyl spectrum was recorded with the following acquisition parameters: data points, 16K; spectra width, 800 Hz; acquisition time, 12–20 s; pulse width, 45–90°. The free induction decay (FID) was transformed with zero filling up to 32K data points to yield a digital resolution of 0.05–0.08 Hz/point. All FIDs, prior to Fourier transformation (FT), were filtered using an exponential multiplication (line broadening of 0.2 Hz) for sensitivity enhancement.

Carbon-13 spin–lattice relaxation times (T_1) were measured using the inversion–recovery (180–τ–90) pulse sequence (Freeman, 1988). Nuclear Overhauser effects were calculated from the difference between the broad band and the inverse-gated decoupled spectra obtained with the same acquisition parameters and number of scans (Freeman, 1988).

Quantitative full spectra were obtained using the inverse-gated decoupling mode (without NOE). Carbonyl high-resolution spectra were obtained in broad band noise decoupling mode, considering the similarity of the NOE enhancements. ^{13}C NMR peak intensities were accurately quantified using a computer curve resolution program (Bruker, Karlsruhe, Germany).

RESULTS AND DISCUSSION

The ^{13}C NMR spectra of fish oils show very complex patterns of resonances if compared to vegetable oils, due to the higher complexity of fatty acids and the presence of phospholipids (Figure 1). The olefinic and methylene regions appear to be very complex due to the molecular complexity of fish PUFAs involving a high number of NMR signals. The present study focuses on glyceryl resonances, to quantify lipid classes, and on carbonyl (acyl) resonances, to define the free fatty acid content and composition and the acyl positional distribution on triacylglycerols. By

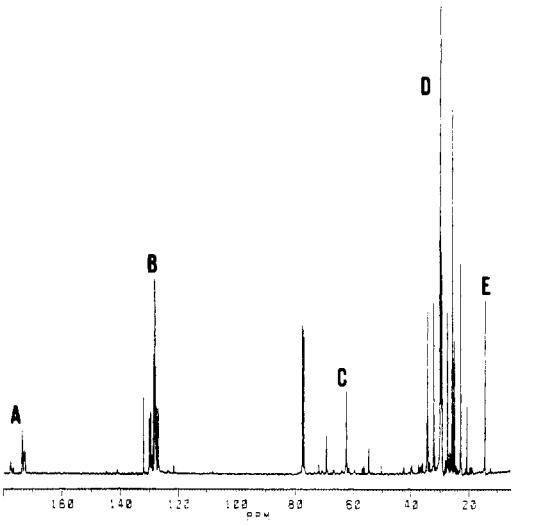

Figure 1. ^{13}C NMR spectrum of lipid extracted from the white muscle of albacore tuna (*T. alalunga*). Spectral regions are indicated as follows: A. carbonyls; B, olefinic carbons; C, glyceryl carbons; D, methylene carbons; E, methyl carbons. See Materials and Methods for spectral recording conditions.

recording ^{13}C NMR spectra on high-resolution spectrometers, at the two observed frequencies of 67.88 and 100.64 MHz, in spite of the high magnetic field applied, the resolution of esterified carbonyl signals requires appropriate instrumental conditions (high numbers of data point) during spectral acquisition (Wollenberg, 1990). For this reason, the spectra were recorded using two different acquisition conditions: a full spectrum with low resolution obtained with few data points, rapid recycle delay (thus allowing about 50 scans/min), and then a good signal to noise ratio in a short time (5000 scans for a total accumulation time of less than 2 h) from which the lipid classes and free fatty acid content can be determined; and a carbonyl high-resolution spectrum acquired with a longer acquisition time (12–20 s) and then longer accumulation for the specific study of acyl positional distribution in triacylglycerols. To obtain an NMR quantitative response for the different lipid components in the mixture, spectra were acquired under experimental conditions ensuring the complete recovery of each NMR resonance used in quantitative measurements. In fact, the linearity between the NMR signal intensity and the concentration of the component can be distorted for two reasons: a different relaxation rate and/or a different nuclear Overhauser enhancement (NOE) of carbons considered in the relative calculation (Freeman, 1988). In routine ^{13}C NMR qualitative analysis, most spectra are recorded using broad band proton decoupling (irradiation of all protons at the same time with the result of a completely proton decoupled spectrum with a single line for each carbon) and using a delay time (D_1) between two subsequent pulses. When carbons have different relaxation behaviors, the longitudinal relaxation time (T_1) has to be known for all carbons to ensure that the D_1 used between pulses is compatible with the longer T_1. Thus, all carbons are fully relaxed before the next pulse (Gillet and Delpuech, 1980; Wollenberg, 1990). As for the NOE factor, NOE enhancement may not be the same for all resonances, which means the evaluation of the peak areas would not correspond to the true population of the signal species.

On the basis of these considerations, both T_1 and NOE values were determined for all glyceryl carbons before the acquisition parameters were set for quantitative experiments. T_1 values, measured at 100.64 MHz using the inversion–recovery method, ranged between 4 and 5.8 s

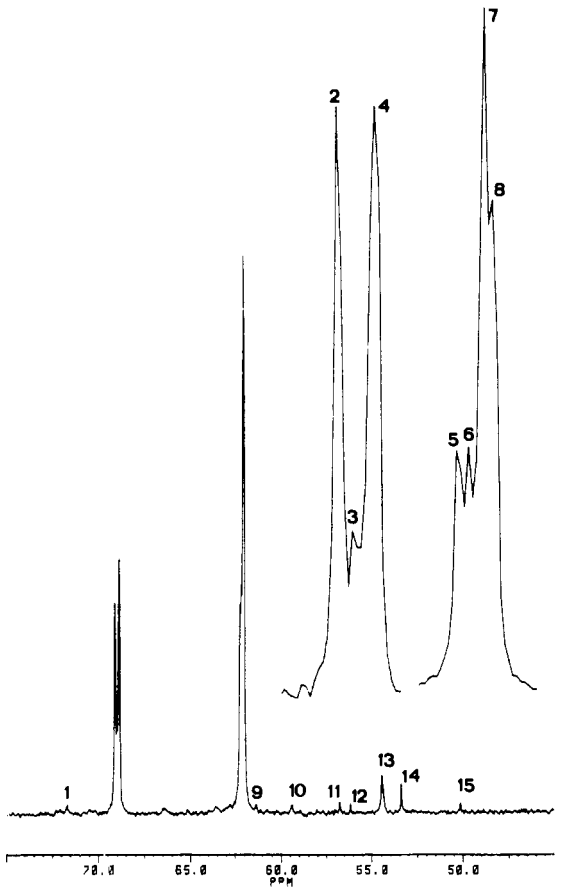

Figure 2. Expansion of the glyceryl region of the ^{13}C NMR spectrum of lipids extracted from the white muscle of albacore tuna (*T. alalunga*). Labeled peaks are assigned in Table I.

for carbonyls and were less than 1 s for all glyceryl carbons. NOE factors ranged between 3.1 and 4.1 for different glyceryl carbons and were very low and almost identical for carbonyls, according to data reported in the literature (Ng, 1983). This suggests that the spectra for accurate quantification of glycerides should be acquired in inverse-gated decoupling mode for complete NOE suppression, while the broad band mode can still be used for rapid quantitative analysis of free fatty acids and semiquantitative evaluation of glycerides. Considering the small NOE effect on carbonyl signal intensities, the α–β acyl distribution on triacylglycerols was finally studied from the high-resolution (0.04 Hz/point) spectra of the carbonyl region using the broad band mode.

Lipid Class Analysis. Lipid class composition of Bligh and Dyer extracts can be directly monitored from the full broad band decoupled ^{13}C NMR spectra (Figure 1). As reported previously for olive oils (Sacchi et al., 1989, 1990), glyceryl regions provide an easy characterization of the carbon nuclei related to different glyceride species, i.e., triglycerides, diglycerides, and monoglycerides. Figure 2 shows the expansion of the 50–75 ppm region in a raw fish lipid ^{13}C NMR spectrum. In addition to glycerides, phospholipid resonances were observed and assigned by comparison with the chemical shifts recorded on model compounds (Table I).

Lipid composition of raw albacore tuna lipids was calculated on the basis of the signal intensity of diagnostic carbons for each glyceride and phospholipid species (Table II). Lipids from freshly caught raw samples showed a very high content of TG (96–98%), a corresponding low level of phospholipids (2–4%), and traces of other glycerides. No cholesterol resonances were detected, thus

Table I. ^{13}C NMR Chemical Shift Assignment of Glyceryl Resonances of Lipid Extracted from the White Muscle of Atlantic Tuna (*T. alalunga*) (See Figure 2)

	chemical shift	
peak	ppm[a] ($CDCl_3$)	assignment[b]
1	71.593	β-1,2-DG
2	69.008	β-TG (DHA)
3	68.903	β-TG (EPA)
4	68.777	β-TG (MU, PUFA)
5	62.151	α-TG (DHA)
6	62.072	α-TG (EPA)
7	61.966	α-TG (MU, PUFA)
8	61.913	α-TG (ST)
9	61.306	α'-1,2-DG
10	59.249	PC, lyso-PC
11	56.664	α-PE
12	56.057	α'-PE
13	54.475	PC, lyso-PC
14	53.201	solvent peak (CH_2Cl_2)
15	50.044	PE

[a] At a digital resolution of 2.6 Hz/point, the accuracy of the shift values recorded was ±0.006 ppm. Peaks are referenced to internal TMS (0 ppm). [b] Abbreviations: the carbons of glyceryl backbone are designated α,β,α'; TG, triacylglycerols; 1,2-DG, *sn*-1,2-diacylglycerols, PE, phosphatidylethanolamine; PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine. Triacylglycerol (TG) α and β carbons are split into different signals in relation to different attached fatty acids: saturated (ST), monounsaturated (MUFA), polyunsaturated (PUFA), DHA, and EPA.

Table II. Lipid Composition of Fresh Albacore Atlantic Tuna (*T. alalunga*) White Muscle As Determined by ^{13}C NMR Spectroscopy

lipid class[a]	% mole fractions[b]	lipid class[a]	% mole fractions[b]
TG	96.3 ● 2.1	PE	1.5 ± 0.3
1,2-DG	0.2 ± 0.1	PC	2.4 ± 0.4
1,3-DG		lyso-PC	
MG			

[a] TG, *sn*-triacylglycerols; 1,2-DG, *sn*-1,2-diacylglycerols; 1,3-DG, *sn*-1,3-diacylglycerols; MG, *sn*-1- and *sn*-2-monoacylglycerols; PE, phosphatidylethanolamine; PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine. [b] Mean of three determinations with standard deviations.

indicating that cholesterol levels in the extracted lipid were less than 0.4% mole fraction (limit of detection from the spectral noise in our experimental conditions). The lower level of phospholipids as compared to the values found in the same fish species (Gallardo et al., 1989) can be explained by the high weight (8–10 kg) of fatty fish samples used here. In fact, it is indicated that the increase in fat content is almost entirely due to a large increase in triacylglycerols (Pearson et al., 1977), and the use of large samples explains the relative lower amount of phospholipids in the lipids extracted.

Free Fatty Acid Determination. The carbonyl region of all ^{13}C NMR spectra showed two distinct signal patterns (Figure 3). Two intense signals were observed at 177.329 and 176.515 ppm in the ^{13}C NMR spectra of lipid samples from albacore muscles (Figure 3b) and assigned to free fatty acids (FFA) by comparison with the chemical shifts observed in a known mixture of standard FFAs (Figure 3a). The lower field signal (177.329 ppm) was assigned to saturated and monounsaturated fatty acids, while the peak at 176.515 ppm was assigned to DHA. The 172–174 ppm envelope corresponds to the chemical shift range of triacylglycerol acyl carbons and other esterified carbonyls as summarized in Table III (Sacchi et al., 1989). Therefore, a quantitative determination of FFAs can be obtained by

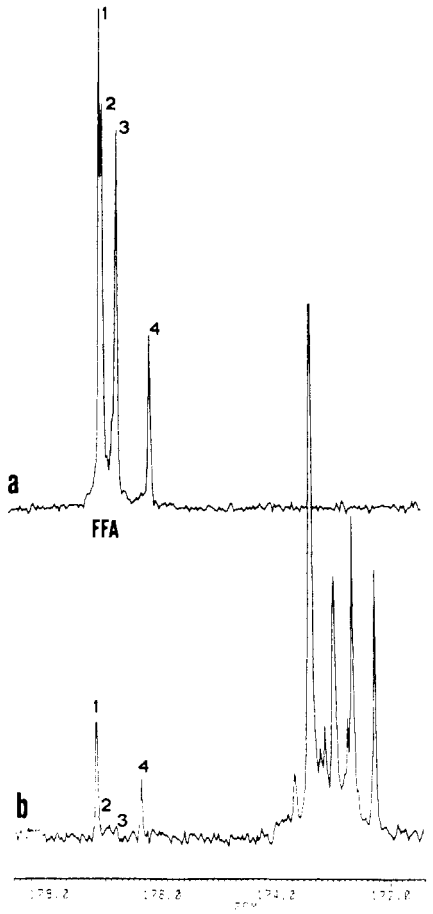

Figure 3. ^{13}C NMR carbonyl spectral region of a standard mixture of free fatty acids (a) and of a sample of albacore lipids (b). Labeled peaks are assigned in Table III.

integrating the FFA carbonyl spectral region using the expression

$$\text{free fatty acids (mole fraction \%)} = \text{FFA} \times 100/(\text{FFA} + \text{EC})$$

where FFA is the integral (average value of three experiments) of the free fatty acids envelope (177.500–176.500 ppm) and EC is the integral (average value of three experiments) of the esterified carbonyl envelope (174.500–171.000 ppm).

The total free fatty acid content of seven lipid samples determined using NMR was compared to the free fatty acid values obtained using the Lowry and Tinsley (1976) UV method. As shown in Figure 4, an excellent linearity was found between the NMR and UV data ($R^2 = 0.990$).

Each NMR determination was repeated three times, and a good reproducibility was found (relative standard deviation less than 10%).

In addition to the total level of free fatty acids, it must be stressed that high-resolution NMR also provides useful information on the composition of free fatty acids, particularly on the most abundant components, such as saturated, monounsaturated, and DHA (Figure 3).

Positional Distribution. Fatty acid distribution in animal and vegetal triacylglycerols has been widely studied (Litchfield, 1972; Breckenridge, 1978; Christie, 1982, 1992), and a structural pattern was obtained for most fats in which polyunsaturated fatty acids are preferentially located in the β-position on the glycerol molecule (Brockerhoff et al., 1963, 1964). It was suggested that this special location in PUFAs may protect them from *in vivo* metabolic oxidation processes.

Table III. ^{13}C NMR Chemical Shift Assignment of Carbonyl Resonances of Lipid Extracted from the White Muscle of Atlantic Tuna (*T. alalunga*)

peak[a]	ppm[b] ($CDCl_3$)	assignment[c]
1	177.329	FFA (ST, MUFA)
2	177.291	FFA (linoleyl)
3	177.055	FFA (EPA)
4	176.512	FFA (DHA)
	174.2–174.6	α,β-PC+PE (broad envelope)
	173.831	α-1,2-DG (ST)
	173.701	β-1,2-DG (ST)
	175.398	α-1,3-DG (ST)
	173.214	α-TG (ST, MU, PUFA)
	172.950	α-TG (EPA)
	172.819	β-TG (ST, MU, PUFA)
	172.555	β-TG (EPA)
	172.476	α-TG (DHA)
	172.080	β-TG (DHA)

[a] See Figure 3. [b] At a digital resolution of 2.6 Hz/point, the accuracy of the shift values recorded was ±0.006 ppm. Peaks are referenced to internal TMS (0 ppm). [c] Abbreviations: the carbons of glyceryl backbone are designated α,β,α'; FFA, free fatty acids; TG, triacylglycerols; 1,2-DG, *sn*-1,2-diacylglycerols; PE, phosphatidylethanolamine; PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine. FFA and TG α and β carbons are split into different signals in relation to different attached fatty acids: saturated (ST), monounsaturated (MUFA), linoleyl, polyunsaturated (PUFA), DHA, and EPA.

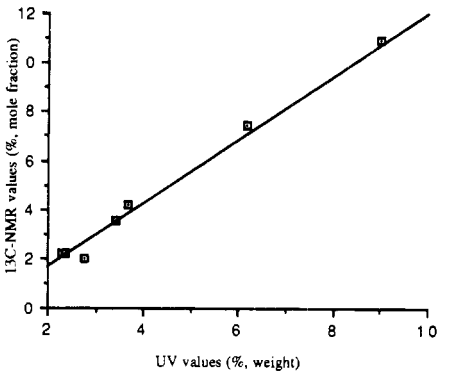

Figure 4. Relationship between ^{13}C NMR and UV values of free fatty acid content as detected in seven different samples of tuna lipids. Linear regression: $Y = -1.08 + 1.35X$, $R^2 = 0.990$.

In the present work, fatty acid positional distribution was examined in three samples of fresh albacore white muscle by studying the glyceryl and high-resolution carbonyl spectra (digital resolution of 0.04 Hz/point). Preliminary full spectra were recorded on standard triacylglycerols, *sn*-1,2- and *sn*-1,3-diacylglycerols, *sn*-1- and *sn*-2-monoacylglycerols, phospholipids, and their mixtures (Table III). Due to the possibility that *sn*-1,2-diacylglycerol carbonyl peaks (upfield-shifted in PUFA by the double bond near carbonyl groups) might overlap with the triacylglycerol carbonyl zone, three tuna samples were selected on the basis of their low diacylglycerol content. This was verified, as specified above, by the preliminary observation of the spectral region of glyceryl carbons (50–75 ppm) which did not show resonances corresponding to *sn*-1,2- and *sn*-1,3-diacylglycerols. Phospholipid acyl resonances do not overlap with triacylglycerols due to the lower field chemical shift of phospholipid carbonyls (Table III).

^{13}C NMR glyceryl (Figure 2) and acyl (Figure 5) triacylglycerol resonances were then used to define the stereospecific distribution of fatty acids, in particular of DHA and EPA. In the glyceryl region of the full spectra acquired with low digital resolution (2.6 Hz/point), the

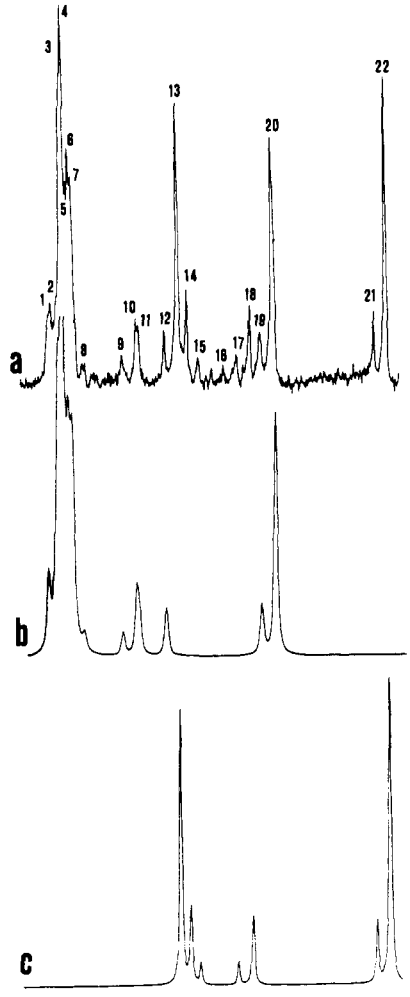

Figure 5. ^{13}C NMR triacylglycerol carbonyl spectral region of lipids extracted from the white muscle of albacore tuna. High-resolution spectrum was recorded with a digital resolution of 0.08 Hz/point (a), simulated triacylglycerols α-position (b) and β-position (c). Labeled peaks are assigned in Table IV.

splitting of α-CH_2 into four peaks (62.151, 62.072, 61.966, and 61.913 ppm) and of β-CH into three peaks (69.008, 68.903, and 68.777 ppm) was observed (Figure 2). The assignment of partially resolved glyceryl carbons (Table I) was made on the basis of the chemical shifts recorded on pure compounds, indicating a downfield induced shift occurring with increasing unsaturation of the acyl chain and when the first double bond is closer to the acyl end (Sacchi et al., 1989; Gunstone, 1991). The α-CH_2 glyceryl pattern was assigned to DHA (62.151 ppm), EPA (62.072 ppm), monounsaturated fatty acids (MUFA), and other PUFAs (61.966 ppm) and saturated fatty acids (61.913 ppm). β-CH resonances were also assigned to DHA (69.008 ppm), EPA (68.903 ppm), and bulk fatty acids (saturated, MUFAs, and other PUFAs) (68.777 ppm). From the relative signal intensities, and in spite of a partial overlapping of signals, a larger concentration of DHA in the β-position was singled out.

The carbonyl region was then carefully investigated, by recording the spectra with a restricted spectral width (800 Hz), an acquisition time of 12–20 s, and increased digital resolution to 0.05–0.08 Hz/point. Twenty-two triacylglycerol peaks were resolved using this procedure (Figure 5). Several combined observations were made to assign the carbonyl peaks to different fatty acids in both the α- and β-acyl positions (Table IV). The first was that the carbonyl resonances of fatty acids attached to the α- and β-positions of glycerol exhibit a systematic chemical shift

Table IV. ^{13}C NMR Chemical Shift Assignment of Carbonyl Resonances of Traicylglycerols Extracted from the White Muscle of Albacore Tuna (*T. alalunga*) (See Figures 5 and 6)

		assignment[b]	
peak	ppm[a] ($CDCl_3$)	position-acyl chain	current name
1	173.285	α-ST	
2	173.262	α-ST	
3	173.243	α-18:0	stearyl
4	173.236	α-16:0	palmityl
5	173.225	α-20:1Δ11	eicosenoyl
6	173.215	α-18:1Δ9	oleyl
7	173.207	α-18:2Δ9, 18:3Δ9	linoleyl, lineolenyl
8	173.172	α-20:4Δ8	arachidonyl
9	172.036	α-22:5Δ7	DPA
10	172.986	α-20:4Δ5	arachidonyl
11	172.977	α-20:5Δ5	EPA
12	172.889	unassigned	
13	172.842	β-ST, 18:0	stearyl
14	172.811	β-18:1Δ9	oleyl
15	172.773	β-20:4Δ8	arachidonyl
16	172.742	β-22:5Δ7	DPA
17	172.620	β-20:4Δ8	arachidonyl
18	172.596	β-20:5Δ5	EPA
19	172.560	α-PUFA[c]	
20	172.517	α-22:6Δ4	DHA
21	172.168	β-PUFA[c]	
22	172.126	β-22:6Δ4	DHA

[a] At a digital resolution of 0.08 Hz/point, the accuracy of the shift values recorded was ±0.002 ppm. Peaks are referenced to the DHA β-acyl resonance = 171.126 ppm, which agrees with TMS = 0 ppm. [b] Fatty acids are defined with their carbon number and unsaturation; Δn indicates the first double bond on the acyl chain with respect to the carbonyl ($\Delta 1$ carbon). Abbreviations are specified in Table I. [c] These assignments are only tentative.

difference of about 0.40 ppm (Sacchi et al., 1989, 1990; Gunstone, 1991). On the basis of this experimental evidence, several pairs of resonances were singled out, corresponding to as many fatty acids attached in the α- and β-glycerol positions (Figure 5b,c). To assign each peak pair, the relative order of the chemical shifts for saturated, monounsaturated, and polyunsaturated fatty acids was studied, considering particularly the numbers of double bonds and their positions with respect to the carbonyl end. It is well-known that the chemical shift of fatty acid carbonyls decreases (high field induced shift) (i) when the first double bond is close to the carbonyl end, (ii) with an increasing number of double bonds in the chain, and (iii) the shorter the chain (methyl group close to the carbonyl end) (Ng, 1985; Wollenberg, 1990; Gunstone, 1991; Lie Ken Jie et al., 1992). The first point (double bond position with respect to the carbonyl end) appears to be the most important in determining the chemical shift order of the triglyceride carbonyls (Table IV). Two signals were found at 172.560 and 172.168 ppm ($\Delta\delta$ = 0.4), shifted slightly downfield from the DHA resonances, and only tentatively assigned to another unidentified PUFA in the α- and β-positions. Combined NMR and chromatographic experiments are currently in progress to identify these signals.

The assignments made were indirectly confirmed by comparing the quantitative values found using NMR with the total fatty acid composition as determined by gas chromatography (Table V). Good agreement was found between the NMR- and GC-derived data, with the saturated component slightly overestimated in the NMR results. This may be attributed to the partial overlapping between saturated and monounsaturated α-acyl resonances (Figure 6). A 2:1 ratio between the NMR signal intensities of the α- and β-peaks was also found, thus confirming the quantitative response given by NMR under

Table V. Total Fatty Acid Composition of Lipid Extracted from the White Muscle of Tuna Lipids As Determined by NMR and Gas Chromatography[a]

fatty acid[b]	NMR	GC
ST	34.42 ± 1.70	29.73 ± 0.43
MUFA, linoleyl	23.05 ± 2.16	25.47 ± 1.06
arachidonyl	2.02 ± 0.41	1.02 ± 0.08
EPA	7.89 ± 0.17	8.16 ± 0.66
DPA	2.27 ± 0.37	2.02 ± 0.66
DHA	30.36 ± 0.46	31.04 ± 1.13

[a] NMR and GC data are expressed as percent weight (means of three determinations with standard deviations). [b] Abbreviations: see footnote to Tables III and IV.

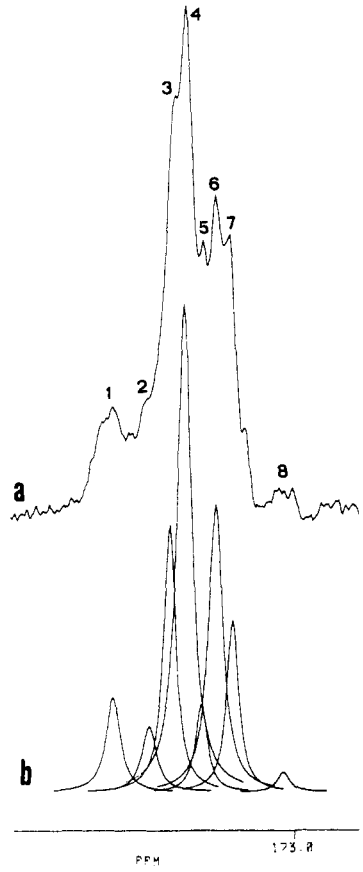

Figure 6. Expansion of the ^{13}C NMR carbonyl spectral region corresponding to the saturated (ST), monounsaturated (MU), and linoleyl and linolenyl (L) fatty acids esterified in the triacylglycerol α-position. Spectrum conditions were as in Figure 5. (a) Experimental envelope; (b) individual simulated peaks. Labeled peaks are assigned in Table IV.

Table VI. Fatty Acid Positional Distribution of Tuna Lipids As Determined Using High-Resolution ^{13}C NMR[a]

fatty acid[b]	α-position	β-position
ST	40.86 ± 1.75	35.15 ± 3.18
MUFA	30.09 ± 2.92	10.87 ± 0.85
arachidonyl	1.65 ± 0.57	2.23 ± 0.49
EPA	6.33 ± 0.09	9.57 ± 0.36
DPA	1.28 ± 0.36	3.25 ± 0.43
DHA	19.80 ± 1.47	38.90 ± 2.17

[a] NMR data are expressed as mole fraction percent of each glycerol position. Means of three determinations with standard deviations. [b] Abbreviations: see footnote to Tables III and IV.

the experimental conditions used to record the high-resolution carbonyl spectra.

Table VI shows the positional acyl distribution found using NMR in albacore tuna triacylglycerols. Saturated fatty acids appear to be randomly distributed on triacylglycerol α- and β-acyl positions. Monounsaturated fatty acids such as palmitoyl, oleyl, erucyl, and eicosenoyl appear to be preferentially esterified to the α-acyl position, as well as linoleyl and linolenyl. PUFAs such as arachidonyl, docosapentanoyl, EPA, and mainly DHA were present in larger concentrations in the β-position. The acyl distribution determined by NMR appears to be mostly in agreement with data in the literature (Aubourg et al., 1990).

CONCLUSIONS

Carbon-13 NMR spectroscopy seems to be a useful, direct, and nondestructive method for the study of fish lipid and oil composition. The major advantage of NMR over the classical methods is essentially the absence of enzymatic or chemical manipulation of lipid samples. This seems to be very important for the positional analysis of marine lipids, considering the possibility that fatty acids are transposed onto glycerol (Litchfield, 1972) and of unsaturated damage during enzymatic or chemical hydrolysis (Chang, 1977). ^{13}C NMR offers the simultaneous possibility of monitoring the composition of lipid classes, free fatty acid content, and composition and acyl positional distribution.

This suggests that NMR can be widely applied (i) to follow lipid changes occurring in fish during processing or storage, (ii) to the on-line monitoring of the purity of fish triacylglycerols extracted or fractionated using preparative chromatographic procedures, and (iii) to direct acyl distribution analysis in different fish species.

Further improvement of NMR sensitivity and resolution, as recently demonstrated (Maple and Allerhand, 1988) on modified ultra-high-resolution spectrometers (with new decoupling conditions and elimination of temperature gradients in the samples), will abbreviate the analytical times, now in the range of 1–2 h to study lipid composition and in the range of 3–4 h for accurate α–β acyl distribution analysis.

As illustrated, NMR determination of α–β distribution of fatty acids from fish triacylglycerols does not present problems for lipid samples with a low level of hydrolysis. Samples containing higher amounts of diacylglycerols, due to the overlapping of carbonyl peaks, may require a preliminary purification of triacylglycerols.

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