Proton Nuclear Magnetic Resonance Rapid and Structure-Specific Determination of ω-3 Polyunsaturated Fatty Acids in Fish Lipids

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Based on proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, a rapid and structure-specific method for the determination of ω-3 polyunsaturated fatty acids (PUFAs) in fish lipids is presented. The different chemical shift observed for the methyl resonance of ω-3 PUFAs ($\delta = 0.95$ ppm) with respect to the methyl resonance of all other fatty acids ($\delta = 0.86$ ppm) has provided the possibility of proposing a new and rapid method for the determination of ω-3 PUFA content. Twenty-four fish lipid samples (raw, cooked and canned albacore tuna) produced results that showed good agreement between ^{1}H-NMR analysis and gas chromatographic determination. Raw and cooked samples showed significantly higher levels of ω-3 PUFA than canned tuna.

KEY WORDS: 1D-TOCSY, CG, ^{1}H-NMR, ω-3 PUFA determination, fresh, cooked and canned tuna, fish oils, *Thunnus alalunga* lipids.

The role of ω-3 polyunsaturated fatty acids (PUFAs) of fish oils on human health is now well recognized. Several studies have suggested that diets rich in ω-3 PUFAs promote positive changes in blood serum lipids, such as lower levels of cholesterol and triglycerides and higher levels of high-density lipoproteins (HDLs) (1,2), and that this may reduce the risk of cardiovascular disease (3–6).

The content of ω-3 PUFAs in fish may depend on the fish species and its age but also on storage and processing, due to the oxidative deterioration of the highly unsaturated lipid substrate (7,8).

ω-3 PUFAs have been analyzed widely by gas chromatography (GC) together with the other kinds of fatty acids. The most commonly used method converts lipid extracts of biological samples into methyl esters, which then are analyzed by GC (9,10). However, marine lipids provide complicated mixtures of fatty acids (11,12), which have to be separated under optimum chromatographic conditions to avoid overlapping. Another problem arises from formation of artefacts during the transmethylation step (13); artefacts have to be detected by GC-mass spectrometry (14,15).

This work presents an improved, rapid and structure-specific method for the global quantitation of ω-3 PUFAs in fish lipids by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. NMR quantitative results obtained from raw, cooked and canned albacore tuna (*Thunnus alalunga*) were compared to the ω-3 PUFA content as determined by GC of fatty acid methyl-esters.

EXPERIMENTAL PROCEDURES

Raw material and processing. Fresh, cooked and canned tuna (*Thunnus alalunga*) were used in this research. After arrival at the laboratory, the fish were frozen at −40°C and stored at −18°C. Processing was performed in the pilot plant of the Instituto de Investigaciones Marinas of CSIC (Vigo, Spain). Steam cooking was carried out (102–103°C) until a final backbone temperature of 65°C was reached (90 min); the fish were then cooled at room temperature (14°C) for about 5 h. The fish were cleaned and 90-g portions of cooked muscle were placed in RO-100 cans (6.52 cm diameter, 3 cm height), and soybean oil (20 mL) and salts (2 g) were added. The cans were vacuum-sealed and sterilized in a retort at 110°C (55 min). The cans were stored at room temperature until required for analysis.

Lipid extraction. Lipids were extracted directly from raw and cooked muscles by the Bligh and Dyer method (16). For the canned samples, the liquid part was drained off carefully, and the muscle part was minced and wrapped up with filter paper; lipids were extracted from the resulting minced muscle also according to the Bligh and Dyer method (16). Lipid extracts were stored at −20°C in chloroform until analysis; propyl gallate was used as antioxidant.

GC. Lipid extracts were transesterified with CH_3COCl/CH_3OH according to Christie (17). Fatty acid methyl esters (FAME) were analyzed on a Perkin-Elmer 8700 gas chromatograph (Norwalk, CT). Conditions: SP-2330 fused-silica capillary column (0.25 mm i.d., 30 m length) (Supelco, Inc, Bellefonte, PA), 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. The carrier gas used was N_2 (10.0 psig). The injector (split ratio 150:1) was warmed to 225°C at 15°C/s. The individual esters were identified by comparison with the retention times of standard mixtures (Supelco: PUFAs No. 1 and No. 2; Larodan, Qualmix Fish).

NMR spectroscopy. ^{1}H-NMR spectra were recorded on two AM-400 and AC-270 Bruker high-resolution spectrometers (Bruker, Karlsruhe, Germany), located at the Centro Interdipartimentale di Metodologie Chimico-Fisiche of the University of Naples (Naples, Italy), operating at a proton frequency of 400 and 270 MHz, respectively. The following acquisition parameters were used: temperature 25°C, 16K of data points, spectral width of 10 ppm, acquisition time 2.7 s, pulse width 45°. Sixteen to thirty-two transients were accumulated for each spectrum, and therefore, running times varied between 1 and 2 min. Spectra were recorded at concentrations of 2% (wt/vol) by dissolving 10 mg oil in 0.5 mL chloroform-d (Aldrich Chemicals, Milwaukee, WI) with 0.03% tetramethylsilane (TMS) as an internal reference.

The structure-specific identification of ω-3 PUFA resonances was carried out on a Varian UNITY 400 high-resolution spectrometer (Varian Associates, Palo Alto, CA) operating at a proton frequency of 400 MHz for 1-D selective excitation TOCSY experiments (18,19). TOCSY 1-D spectra were acquired by the standard sequence with a pulse width of 90° and a spin lock duration of 70 ms.

Data obtained from NMR-spectra were subjected to the

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analysis of variance (ANOVA) one-way method according to Sokal and Rohlf (20).

RESULTS AND DISCUSSION

Figure 1a shows the ^{1}H-NMR spectrum recorded at 400 MHz on a fresh tuna oil sample. As recently reported by Gunstone (21,22), some signals can be specifically related to PUFAs in addition to the usual methylenic ($\delta = 1.3$ ppm), allylic ($\delta = 2.1$ ppm) and doubly allylic protons ($\delta = 2.8$ ppm). Attention should be drawn in particular to the low-field methyl signals ($\delta = 0.95$ ppm) which can be assigned to the ω-3 PUFA.

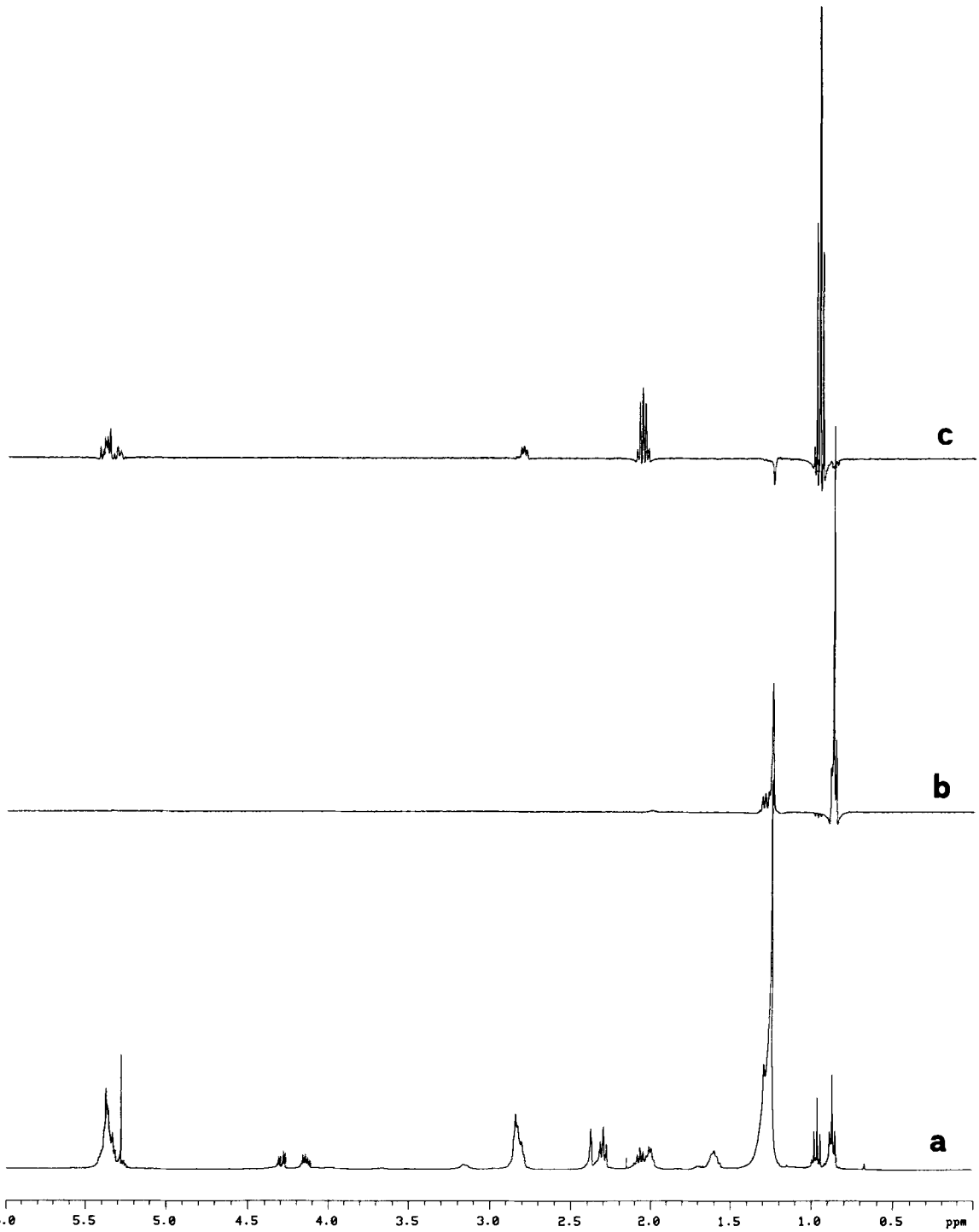

FIG. 1. Structural identification of ω-3 polyunsaturated fatty acids (PUFA) methyl resonances by proton nuclear magnetic resonance 400-MHz 1-D TOCSY experiments: (a) full spectrum, (b) selective excitation pulse on methyl triplet at 0.86 ppm, (c) selective excitation pulse on methyl triplet at 0.95 ppm. Assignments are as follows: 0.86 ppm (*t*), methyl of saturated, monounsaturated and ω-6 PUFA (3H); 0.95 ppm (*t*), methyl protons of ω-3 PUFA (3H); 1.31 ppm (*b s*), bulk methylenes; 1.65 ppm (*m*), 2H on C-3; 2.05 ppm (*m*), allylic protons (2H); 2.31 ppm (*t*), 2H on C-2; 2.76 ppm (*m*), doubly allylic protons (2H); 3.37 ppm (*b s*), phospholipid methyl (9H); 4.2 ppm (*m*), glyceryl protons (4H); 5.39 ppm (*m*), olefinic (1H) and glyceryl protons (1H).

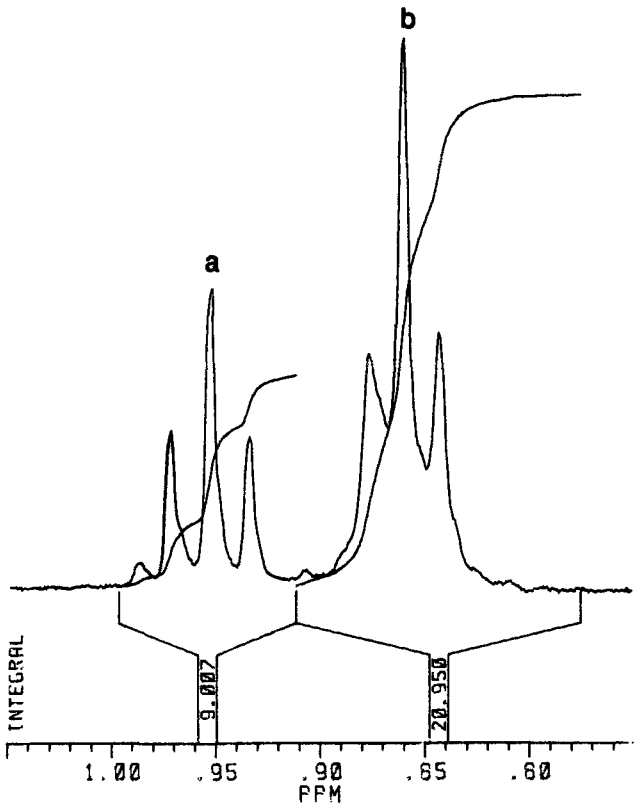

FIG. 2. Expansion of the 270-MHz, proton nuclear magnetic resonance methyl spectral region of a fish lipid sample from canned albacore tuna muscle with integration of the ω-3 polyunsaturated fatty acids methyl triplet (a, $\delta = 0.95$ ppm) and of the methyl resonance of other fatty acids (b, $\delta = 0.86$ ppm).

To confirm these assignments, two 1-D TOCSY experiments were carried out on a fish oil sample (Figs. 1b and 1c). With a selective excitation pulse in the first experiment, only the methyl signal at 0.95 ppm was selectively excited (Fig. 1c). The pattern observed, arising from the magnetization transfer from the excited methyl group to the coupled protons, shows a decreasing effect on allylic (proton bonded to the ω-2 carbon), then olefinic (ω-3 and ω-4 double-bond carbon) and doubly allylic (ω-5 carbon) bonds. In the second experiment (Fig. 1b), the methyl groups at 0.86 ppm were excited, and magnetization is only transferred to methylene groups, thus demonstrating that the methyl resonance at 0.86 ppm is essentially due to the saturated, monounsaturated and ω-6 PUFAs. On this basis the methyl signal intensity at 0.95 ppm can be taken as a global measure of ω-3 PUFA present in the fish lipid sample.

With high-field spectrometers (270–400 MHz), two methyl triplets are fully resolved, so that relative quantitation can be obtained by direct integration of the two envelopes (Fig. 2). The total ω-3 PUFA content can be simply calculated as indicated by the following expression:

$$\omega\text{-3 PUFA (\% molar fraction)} = 100\ A/A + B \qquad [1]$$

where A = integral value of methyl signal at.0.95 ppm and B = integral value of methyl signal at.0.86 ppm.

Twenty-four fresh, cooked and canned fish samples were analyzed. Each NMR intensity measurement was repeated at least four times to get statistically averaged values, and a good reproducibility was found (relative standard deviation less than 4%).

The NMR results were compared with those obtained by gas chromatography of the fatty acid methyl esters (Fig. 3). Total ω-3 PUFA content was determined by GC

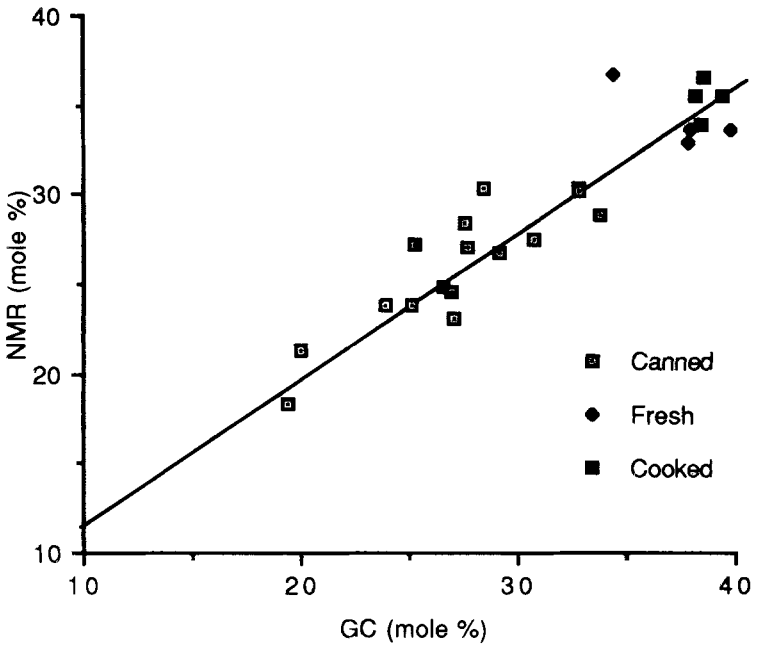

FIG. 3. Relationship between proton nuclear magnetic resonance (^{1}H-NMR) and gas chromatography (GC) determination of ω-3 polyunsaturated fatty acid content (%, mole fraction) in raw, cooked and canned fish samples. Linear regression: $Y = 5.579 + 0.753\ X$, $R^2 = 0.900$.

TABLE 1

Fatty Acid Composition and Total ω-3 Polyunsaturated Fatty Acid (PUFA) Content of Three Lipid Samples from Fresh, Cooked and Canned Albacore (*Thunnus alalunga*) as Determined by GC as Well as Total ω-3 PUFA Content Determined by Proton Nuclear Magnetic Resonance Spectroscopy[a]

Fatty acid	Fish lipid sample		
	Fresh	Cooked	Canned
14:0	3.37	3.41	1.30
15:0	0.77	0.77	0.25
16:0	22.18	23.60	16.24
16:1ω7	3.25	2.82	1.52
17:0	1.26	1.39	0.78
17:1ω7	1.04	0.93	0.78
18:0	5.52	5.55	5.33
18:1ω9	16.10	14.51	45.60
18:1ω7	2.48	2.08	2.42
18:2ω6	1.32	1.55	3.39
18:3ω6	0.43	0.43	0.53
18:3ω3	0.43	0.43	0.53
20:1ω4	1.59	0.99	0.86
18:4ω3	1.32	1.58	0.54
20:4ω6	1.20	1.12	0.69
22:1ω11	0.37	0.74	0.35
20:4ω3	0.80	0.93	0.49
20:5ω3	7.21	6.36	3.04
24:1ω9	0.77	0.93	0.57
22:4ω6	0.64	0.65	0.45
22:5ω3	1.38	1.24	0.63
22:6ω3	26.56	27.97	13.94
Total ω3 (GC)	37.70	38.51	19.00
Total ω3 (NMR)	32.81	33.93	18.29

[a]Data expressed as percentage of total fatty acids (mole fraction); Gas chromatographic (GC) values represent the mean of three determinations (relative standard deviation less than 5%), nuclear magnetic resonance (NMR) values represent the mean of four determinations (relative standard deviation less than 4%).

TABLE 2

ANOVA Analysis of the Content of ω-3 PUFA in Fresh Cooked and Canned Samples, as Determined by GC and NMR ($P < 0.01$)[a]

	NMR		GC	
	Average	St. Error	Average	St. Error
Fresh cooked	34.428	0.439	38.598	0.248
Canned	25.959	0.915	27.257	1.063

[a]St., standard; ANOVA, analysis of variance. Abbreviations as in Table 1.

by adding the percentage of all chromatographic peaks corresponding to ω-3 PUFA, as exemplified in Table 1. The regression between the NMR and GC data expressed as mole % (Fig. 3), shows a satisfactory agreement ($R^2 = 0.900$) between NMR and GC, although the Y intercept (5.579) and the line slope (0.753) indicates an error of 5% of NMR with respect to GC, with an underestimate of ω-3 PUFA determined by the NMR method.

For the ω-3 PUFA content, as determined by GC and NMR in raw, cooked and canned fish samples, two statistically different clusters were obtained from an ANOVA analysis ($P < 0.05$) (Table 2). The first corresponded to the raw and cooked samples, the second to the canned ones. No significant difference was observed between raw and cooked samples, while canned samples showed a lower content of ω-3 PUFA. These results are consistent with a previous experience where an interaction between fish lipids and soybean oil (fill oil) was observed (23). The flesh lipids showed an increase of fatty acids abundant in the soybean oil (*i.e.,* 18:2 and 18:3), and at the same time the characteristic ω-3 PUFA of the flesh lipids (20:5, 22:6 and others) showed a decrease with a corresponding increase in fill oil (23).

On the basis of experimental results, ^{1}H-NMR spectroscopy seems to be a rapid and satisfactory accurate tool for the global evaluation of ω-3 PUFA content in fish oils. Being based on a structure-specific spectroscopic measurement, the method does not require identification of diagnostic signals from standard compounds. On the contrary, gas-chromatographic procedures require sample derivatization (FAME) and the calculation of ω-3 PUFA level as a sum of different chromatographic peaks. The NMR quantitative analysis is nondestructive and rapid (1–2 min), and it can be carried out directly on the lipids extracted from the fish muscle or fish oils and applied to the nutritional evaluation and quality monitoring of fresh and processed fish products.

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