^{13}C Nuclear Magnetic Resonance Monitoring of Free Fatty Acid Release After Fish Thermal Processing

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^{13}C Nuclear magnetic resonance spectroscopy was applied to the study of lipid hydrolysis occurring during industrial canning of tuna (*Thunnus alalunga*). An increase in the free fatty acid (FFA) level was observed after cooking and sterilization, and a different FFA pattern was found when storage of the frozen raw material and thermal steps (cooking and can sterilization) were compared. Lipolysis in raw muscle occurs preferentially in the *sn*-1 and *sn*-3 acyl positions of triacylglycerols, with a consequent cleavage of saturated and monounsaturated fatty acids. After thermal processing, an increase of docosahexaenoic acid (DHA) was found in the FFA fraction, as well as a relative decrease of the peak intensity of DHA in the *sn*-2 position of triacylglycerols. This finding indicates a different mechanism of FFA release during the frozen storage and thermal processing of raw fish.

KEY WORDS: Cooked and canned tuna, DHA, free fatty acids, hydrolysis, ^{13}C-NMR, raw, *Thunnus alalunga* lipids.

For some years, canneries have used frozen fish as the raw material for later processing. The storage life of fish is limited due to biochemical modifications of the natural components. Some alterations can affect lipids [oxidation, accumulation of free fatty acids (FFA)], which are susceptible to the development of rancidity and other off-flavors. Many works have described lipid changes in fish muscle during storage at low temperatures (1-3), and the effect of postmortem lipolysis on the production of FFA and glycerol (4). As for processed fish, some information is available on the effects of cooking and canning on lipid composition, as well as on the amounts of FFA formed (5,6). The mechanism of FFA release and the extent of lipid hydrolysis due to thermal treatment of fish muscles, however, have not been studied in detail.

^{13}C Nuclear magnetic resonance (NMR) spectroscopy is a useful tool in the study of various problems related to lipid technology. As described previously, complementary information about the lipid class composition and the total acyl profile can be inferred simultaneously from the same ^{13}C NMR spectrum (7,8). It was also suggested that ^{13}C NMR spectroscopy may be used for the study of lipid hydrolysis in fish samples to determine the amount and composition of FFA formed when the carbonyl region of the spectrum is studied (8).

The aim of this work was the application of ^{13}C NMR to the study of FFA formation during the three steps of industrial fish canning: raw material, cooked and canned fish muscles. Particular attention was given to the major polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA), to obtain information on the stereoselectivity and specificity of the hydrolysis taking place during the thermal process.

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EXPERIMENTAL PROCEDURES

Raw material. Seven Atlantic tuna samples (*Thunnus alalunga*) were obtained from commercial sources. After arrival at the laboratory, the fish were frozen at −40°C and stored at −20°C for 18-24 mon.

Cooking and canning. Processing was performed in the pilot plant of the Instituto de Investigaciones Marinas of CSIC (Vigo, Spain). Whole eviscerated and beheaded fish were steamed (102-103°C) until a final backbone temperature of 65°C was achieved (90 min); they were then cooled and maintained 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 salt (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 (three months).

Lipid extraction. Lipids were extracted from raw and cooked muscles by the Bligh and Dyer method (9). The liquid part was carefully drained off from canned samples, and the muscle was minced and wrapped up in filter paper. The lipids were then extracted from the resulting minced muscle according to the Bligh and Dyer method (9). Lipid extracts were stored at −20°C in chloroform until analysis; propyl gallate was used as antioxidant.

^{13}C NMR. ^{13}C NMR spectra were recorded on a Bruker (Karlsruhe, Germany) spectrometer operating at a ^{13}C frequency of 67.88 MHz. Spectra were recorded at concentrations of 10-20% wt/vol by dissolving 50-100 mg lipid in 0.5 mL chloroform-*d* (Aldrich Chemical, Milwaukee, WI) and using controlled temperatures of 30 ± 0.1°C to obtain the best reproducibilities in chemical shifts and relaxation rates. The following acquisition parameters were used: 200 ppm spectral width, 16 K data points (with resulting digital resolution of 2.7 Hz/pt and 0.37 s acquisition time), relaxation delay 2 s and 45° pulse width. All flame-ionization detectors, prior to Fourier transformation, were filtered by using an exponential multiplication (line broadening of 2 Hz) for sensitivity enhancement. Chemical shifts were indirectly referred to tetramethylsilane (δ = 0 ppm) by using the central resonance of chloroform-*d* (δ = 77.00 ppm). ^{13}C spin-lattice relaxation times (T_1) were measured by using the inversion-recovery (180-τ-90) pulse sequence (10). Quantitative analysis of FFA was performed on the basis of FFA carbonyl resonance intensities according to the procedure previously described (8). To observe low levels of FFA (0.5-1% mole fractions), a good signal-to-noise ratio is required. This condition was obtained with concentrated lipid solutions (10-20% wt/vol) at 3,000-5,000 scans (2-3 h total time of accumulation). Integrations of NMR intensity were repeated three times for each spectrum, and a relative standard deviation of less than 10% was found. Data obtained from NMR spectra were subjected to the analysis of variance (ANOVA) one-way method, according to Sokal and Rohlf (11).

RESULTS AND DISCUSSION

In a previous study, it was observed that FFA carbonyl resonances are well resolved from those corresponding to esterified carbons in the ^{13}C NMR spectrum of fish lipids. We also verified that the quantitative response of ^{13}C NMR compares to that of a classical method (8). Table 1 summarizes some chemical shift data from this earlier paper (8), which will be used in the subsequent discussion.

As discovered for other lipid classes, carbonyl resonances of FFA with a double bond close to the carbonyl end exhibit an up-field-induced shift with respect to saturated (SFA) or monounsaturated (MUFA) fatty acids, that have double bonds far from the acyl end. Therefore, different FFA species can be singled out in the carbonyl region of the ^{13}C NMR spectrum (178–176 ppm). Major acyl components of fish lipids, such as SFA–MUFA, 20:5ω3 eicosapentaenoic acid (EPA) and 22:6ω3 (DHA) can be distinguished in the ^{13}C NMR spectra of lipids extracted from raw and processed fish muscles. Figure 1 shows the ^{13}C NMR carbonyl patterns of a tuna sample for the three stages studied (raw, cooked, and canned) with the assignments of FFA and glyceride carbonyl resonances (Table 1).

The total FFA content found by ^{13}C NMR in 21 samples is shown in Figure 2. A variable level of hydrolysis was observed in individual raw starting samples. This is due to different storage times and conditions of fish prior to arrival at the laboratory.

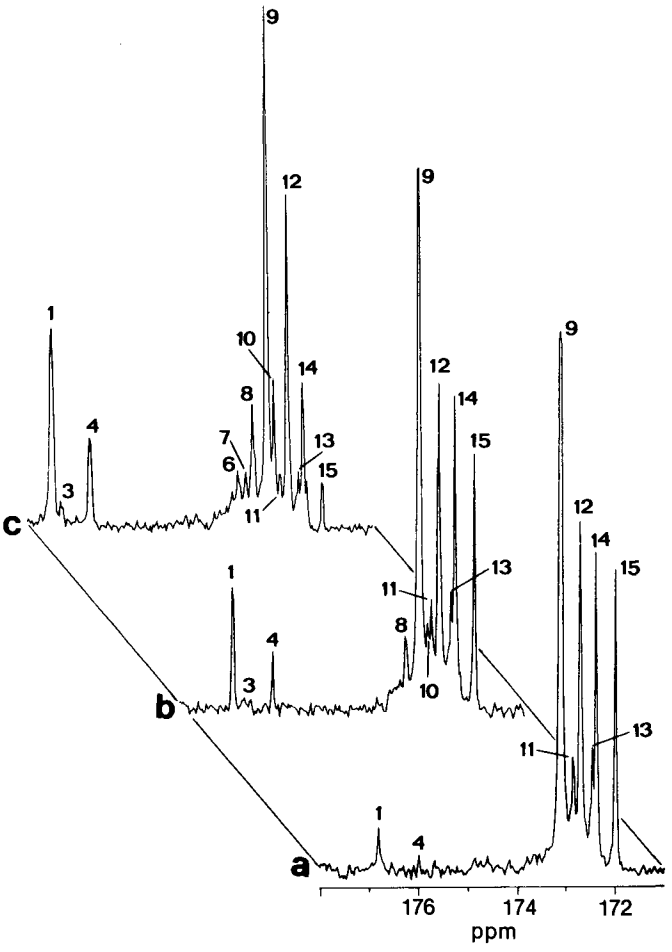

FIG. 1. Expansion of the carbonyl regions of the ^{13}C nuclear magnetic resonance spectra of lipids extracted from raw (a), cooked (b) and canned (c) tuna muscles. Labelled peaks are assigned as shown in Table 1.

Compared to each raw starting sample, cooked samples showed a significant increase in FFA, and a further increase was found after canning for all samples examined (Figs. 1 and 2).

As for FFA composition, a pattern, in which mainly SFA and MUFA and only low levels of EPA and DHA are

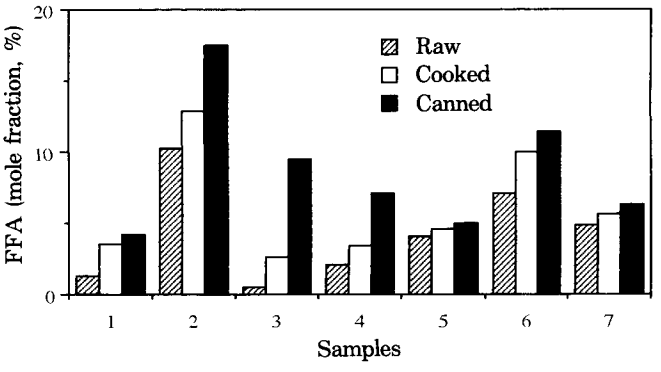

FIG. 2. Free fatty acid (FFA) content (%, molar fraction) found in seven samples of raw tuna muscles and in the corresponding processed (cooked, canned) samples.

TABLE 1

^{13}C NMR Chemical Shift (ppm/$CDCl_3$) Assignment of Carbonyl Resonances of Tuna (*Thunnus alalunga*) Lipids

Peak[a]	ppm[b]	Assignment[c]	
		Lipid class	Acyl position-chain
1	177.33	FFA	SFA, MUFA
2	177.29	FFA	Linoleyl
3	177.05	FFA	EPA
4	176.51	FFA	DHA
5	174.2–174.6	PC/PE	(broad envelope)
6	173.83	1,2-DG	*sn*-1-SFA
7	173.70	1,2-DG	*sn*-2-SFA
8	175.40	1,3-DG	*sn*-1,3-SFA
9	173.21	TG	*sn*-1,3-SFA, MUFA, PUFA
10	173.06	1,3-DG	*sn*-1,3-DHA
11	172.95	TG	*sn*-1,3-EPA
12	172.82	TG	*sn*-2-SFA, MUFA, PUFA
13	172.55	TG	*sn*-2-EPA
14	172.48	TG	*sn*-1,3-DHA
15	172.08	TG	*sn*-2-DHA

[a]See Figure 1 for peak assignment.
[b]At a digital resolution of 2.7 Hz/pt, the accuracy of the shift values recorded was ± 0.01 ppm. Peaks are referenced to internal (tetramethylsilyl (δ = 0 ppm).
[c]Abbreviations: NMR, nuclear magnetic resonance; FFA, free fatty acid; TG, triacylglycerols; 1,2-DG, *sn*-1,2-diacylglycerols; 1,3-DG, *sn*-1,3-diacylglycerols; PE, phosphatidylethanolamine; PC, phosphatidylcholine; FFA, DG and TG carbons are split into different signals in relation to the fatty acid chain: Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), linoleyl, polyunsaturated (PUFA), DHA and EPA.

present, is characteristic for raw fish (Fig. 1a). Resonances corresponding to free PUFAs (DHA) increase in cooked and in canned samples (Fig. 1b and 1c), thus suggesting major hydrolysis of DHA after the thermal steps. To confirm this preliminary observation, an ANOVA analysis was performed on the complete set of results, with the molar ratio DHA + EPA/total FFA as variable. The results of this ANOVA (Table 2) showed significant statistical differences between the three steps involved in the process ($P < 0.05$). Canned samples showed the highest level of free DHA and EPA and raw samples the lowest level.

These results seem to confirm a preferential hydrolysis of SFA and MUFA during frozen storage of raw muscle, whereas a more intense hydrolysis (with a higher release of DHA and EPA) takes place during the thermal steps (cooking and canning).

Several conclusions on the lipolytic mechanism and stereoselectivity in raw and heat-treated fish muscles can be drawn. It is well known that lipolytic enzymes are active during frozen storage of fish samples (12,13). NMR has also demonstrated that PUFAs in tuna triacylglycerols and phospholipids are preferentially esterified in the *sn*-2 acyl position of the glycerol moiety (8, 14). The results of the present study indicate that there is a predominant enzyme action on the *sn*-1 and *sn*-3 positions at low temperatures. This finding is also confirmed by the study of the glycerol region (15), in which only *sn*-1,2-diacylglycerols resonances were observed (data not shown). This agrees with results presented by authors who have theorized that the preferential positional distribution of PUFAs in the *sn*-2 acyl position (16) can be related to *in vivo* biological protection against hydrolytic cleavage and oxidative damage (17,18).

On the other hand, the thermal breakdown of glycerides must be considered in relation to hydrolysis after processing. The observed acyl pattern, with a major concentration of free DHA after cooking and sterilizing, suggests a different (physical or enzymatic) lipolysis mechanism at high temperatures than that found during frozen storage. The origin of PUFAs (DHA), released during thermal processes, can then be studied indirectly from the triacylglycerol carbonyl resonances in which DHA peaks are upfield shifted and clearly resolved from the complex envelope of esterified carbons (Fig. 1, peaks no. 14 and 15). In fact, from the comparison between the relative intensity of the *sn*-1,3- and *sn*-2-DHA peaks in raw, cooked and canned samples (Fig. 1), a more intense decrease of the *sn*-2 DHA resonance (peak no. 15) can be seen, resulting in an increase of the *sn*-1,3/*sn*-2 DHA ratio (Table 3).

TABLE 2

ANOVA Analysis of the Content (% mole fraction) of Free ω3 PUFA (DHA and EPA) in the FFA of Lipid Extracted from Raw, Cooked and Canned Samples, as Determined by ^{13}C NMR ($P < 0.01$)[a]

Samples	Average	Standard error
Raw	4.975	0.051
Cooked	33.450	0.071
Canned	46.375	0.065

[a]ANOVA, analysis of variance. Abbreviations as in Table 1. Data are expressed as % of FFA (mole fraction).

TABLE 3

Ratio Between the Docosahexaenoic Acid Content (% mole fraction) in the *sn*-1,3 and *sn*-2 Acyl Position of Triacylglycerols in Three Samples of Raw and Processed (cooked and canned) Tuna Samples[a]

Sample no.	Raw	Cooked	Canned
1	0.90	1.23	1.43
2	1.22	1.23	2.17
3	0.94	0.91	1.78

[a]Mean of three determinations. Standard deviations were less than 4%.

This observation and the highest level of free DHA in thermally treated samples confirmed the preferential cleavage of DHA in the *sn*-2 triacylglycerol position. As for the mechanism of hydrolysis, enzyme activation associated with temperature may be excluded due to the rapid heating of the fish muscle, while a physical breakdown of acyl chains esterified to the secondary hydroxyl group may be due to a heat effect. The study of this aspect is now in progress, and results obtained from heating tests made on model compounds will be discussed elsewhere.

On the basis of results shown here, ^{13}C NMR spectroscopy appears to be a useful, modern method for the quantitation and characterization of the acyl composition of the FFA fraction. These data can be inferred directly from the lipid sample. This appears to be interesting for the study of FFA compositions in highly unsaturated lipids, due to the fact that conventional methods may be susceptible to losses in PUFA content (thin-layer chromatographic purification, oxidation, etc).

The possibility of obtaining simultaneous information on diacylglycerols (15) and the acyl positional distribution on triacylglycerols (8) and phospholipids (14) suggests that NMR could be a clean and complete technique for the study of enzyme specificity and stereoselectivity, as well as for lipolysis monitoring in other oils, fats and fatty foods.

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REFERENCES

1. Maclean, J., and C.H. Castell, *J. Fish. Res. Board Can. 21*:1345 (1964).
2. Castell, C.H., B.A. Moore, P.M. Jangaard and W.E. Neal, *Ibid. 23*:1385 (1966).
3. Dyer, W.J., and M.L. Morton, *Ibid. 13*:129 (1956).
4. Hardy, R., and J.G.M. Smith, *J. Sci. Food Agric. 27*:595 (1976).
5. Aubourg, S., C. Sotelo and J.M. Gallardo, *J. Agri. Food Chem. 38*:809 (1990).
6. Hale, M.B., and T. Brown, *Marine Fisheries Review 45*:4 (1983).
7. Aursand, M., and H. Grasdalen, *Chem. Phys. Lipids 62*:239 (1992).
8. Sacchi, R., I. Medina, S. Aubourg, I. Giudicianni, L. Paolillo and F. Addeo, *J. Agri. Food Chem. 41*:1247 (1993).
9. Bligh, E. and W. Dyer, *Can. J. Biochem. Physiol. 37*:911 (1959).

10. Freeman, R., *A Handbook of Nuclear Magnetic Resonance*, Longman Scientific and Technical, Oxford, 1988, p. 228.
11. Sokal, R., and F. Rohlf, *Biometry*, W. Freeman and Co., San Francisco, 1981.
12. De Koning, A.J., S. Milkovitch and T.H. Mol, *J. Sci. Food Agric. 39*:79 (1987).
13. De Koning, A.J., and T.H. Mol, *Ibid. 39*:79 (1987).
14. Medina, I., and R. Sacchi, *Chem. Phys. Lipids*, in press (1994).
15. Sacchi, R., F. Addeo, I. Giudicianni and L.Paolillo, *Riv. Ital. Sostanze Grasse 67*:245 (1990).
16. Christie, W.W., in *Analysis of Oils and Fats*, edited by Elsevier Applied Science Publishers, London and New York, 1986, pp. 313–348.
17. Brockerhoff H., R.G. Ackman, and R. Hoyle, *Arch. Biochem. Biophys. 100*:9 (1963).
18. Brockerhoff, H., M. Yurkowski, R.G. Ackman and R. Hoyle, *J. Fish. Res. Board. Can. 21*:1379 (1964).

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