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Molecular mobility of fish flesh measured by low-field nuclear magnetic resonance (LF-NMR) relaxation: effects of freeze–thaw cycles

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Abstract In this study, the molecular mobility of fish flesh was measured by low field nuclear magnetic resonance (LF-NMR) relaxation. Sardine, tuna and mackerel were frozen at −40 °C and stored for 1 day (24 h); and then these samples were thawed at room temperature (20 °C). The relaxation of water protons in fish flesh was measured for fresh (i.e., before freezing) and multi-cycle freeze–thaw samples (i.e., up to 12 times). Three domains from different pools of protons (i.e., low-mobile, medium-mobile and high-mobile) were identified from the relaxation curve. The T_{2b} (low-mobile), T_{21} (medium-mobile) and T_{22} (high-mobile) indicated the proton populations in the protein molecules, strongly bound water molecules, and weakly bound water molecules, respectively. In all cases, the relaxation time (T_{2b}: sardine $r = 0.736$ and $p < 0.01$, tuna $r = 0.857$ and $p < 0.001$, mackerel $r = 0.904$ and $p < 0.001$; and T_{22}: sardine $r = 0.956$ and $p < 0.0001$, tuna $r = 0.927$ and $p < 0.0001$, mackerel $r = 0.890$ and $p < 0.0001$) increased with the freeze–thaw cycles and it reached a nearly constant value after 6 freeze–thaw cycles. The increased relaxation time (i.e., higher mobility) up to 6 freeze–thaw cycles could be due to the increase in proton mobility. However, relaxation time (T_{21}: sardine $r = -0.510$ and $p > 0.05$, tuna $r = 0.162$ and $p > 0.5$, mackerel $r = 0.513$ and $p > 0.01$) showed insignificant change with the increase of freeze–thaw cycles, which indicated minimal change in the medium-mobile protons. The results in this study revealed that the changes in proton mobility in the fish flesh during freeze–thaw cycles could be identified using T_{2b} and T_{22} relaxation of LF-NMR.

Keywords Freezing · Freeze–thaw cycle · NMR relaxation · Mackerel · Fish quality

Introduction

Freezing is one of the conventional food preservation techniques for extending shelf life. Thawing of frozen fish causes structural changes, thus affecting quality of fish. Temperature abuse can cause multiple freeze–thaw cycles in restaurants and retail shops as well as during transport. In cuttlefish, non-heme iron decreased significantly after 5 freeze–thaw cycles and the damage affected hemeproteins, and released pro-oxidants [1]. The physicochemical and enzymatic changes of cod muscle protein have been observed during freeze–thaw cycles [2].

Low field nuclear magnetic resonance (LF-NMR) is gaining interest for quality assessment of foods. Another name for this is time domain nuclear magnetic resonance (TD-NMR). Most of these NMR applications depend on time-domain spectra and frequency-domain spectra with moderate resolution. LF-NMR uses longitudinal T_1 (spin–lattice or T_1, perpendicular to the magnetic field) relaxation and transversal T_2 (spin–spin or T_2, parallel to the magnetic field) relaxation [3] and it is commonly used to explore the information on the binding nature of water in the matrix (i.e., proton mobility). The relaxation time of bulk water varies between 2 and 2.5 s. In the muscle tissue the relaxation time is reduced due to interactions or binding of water molecules. In the case of fish muscle, usually two relaxation times are referred to: T_{21} (in the range of 40–60 ms) and T_{22} (in the range of 150–400 ms) [3].

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However, another, lower, relaxation time (in the range of 1–10 ms) has also been reported as T_{2b} [4, 5]. Erickson et al. [3] reviewed different explanations of T_{2b}, T_{21} and T_{22} as reported in the literature. The first simple model explained that T_{21} and T_{22} represented intracellular and extracellular water, while T_{2b} was ascribed to the water tightly associated with macromolecules [5, 6]. Recent studies have moved relaxation times beyond the concept of "water phase," "bound water" or "water mobility." Instead, relaxation times can be used as an indicator of morphology and the state of polymers (i.e., physiochemical and structural variations) with exchangeable protons as dominated by chemical and diffusive proton exchange of water-biopolymer. Thus, the applications of LF-NMR to fish and fish products extends the possibility of using relaxation times as affected by various postharvest, storage and processing conditions (i.e., quality of fish and fish products) [3].

Four relaxation times were determined for the freeze–thaw cod muscle, which were related to different water pools [6]. Gudjonsdottir et al. [7] also reported that the relaxation times correlated with physicochemical properties of fish fillet. The effect of salts on the raw (pre-rigor and post-rigor) and freeze–thaw salmon flesh was studied by LF-NMR [8]. The pre-rigor muscle showed a very dense structure (i.e., difficult to differentiate individual myofibrils), whereas post-rigor muscle opened up (i.e., the endomysium was easier to discern, and individual myofibrils appeared more distinct). The freeze–thaw salmon showed extracellular space in the sarcoplasm due to the damage by ice crystals. In the case of raw flesh, added salt decreased T_{21}, whereas T_{21} was increased for freeze–thaw salted flesh. This indicated that salt formed compact (i.e., low mobile) myofibrils (i.e., lower T_{21}) in the case of raw flesh, whereas salt formed swollen (i.e., higher mobility) myofibrils (i.e., higher T_{21}). In the literature, there is negligible information available on the use of low frequency LF-NMR to determine the effects of multi-freeze–thaw cycles on fish muscle structure. The objective of this study was to apply LF-NMR relaxation for sardine, tuna and mackerel flesh in order to assess proton mobility in fish flesh as a function of freeze–thaw cycles. Increased proton mobility is commonly correlated with reduced quality due to more deteriorative reactions during storage and distribution.

Materials and methods

Materials

Sardine *Sardinella longiceps*, longtail tuna *Thunnus tonggol* and Indian mackerel *Rastrelliger kanagurta* were all purchased from one fisherman's boat before the catch was brought onto the dock (i.e., around 6 h after catching), and transported to the lab. The postmortem age of the fish was considered similar since the fish were collected from the same boat and the same batch. Crushed ice was used to keep the fish cool during transport. Whole fish were cleaned with tap water and the skin was peeled off using a sharp knife. A 2-g sample of fish flesh (sardine, tuna and mackerel) was cut from a dorsal location of each fish separately and was placed inside 10-mm LF-NMR tubes (3 cm height). The tubes with the fish flesh were placed inside the NMR sample hole one at a time, and their initial (i.e., before freezing) LF-NMR relaxation was measured. The flesh inside the tubes was then frozen at −40 °C and kept for 24 h. The frozen samples were then taken out and thawed at room temperature (20 °C) for 1 h (an initial experiment had shown that this was adequate time to reach 20 °C) and their TD-NMR relaxation was measured. This procedure provided one freeze–thaw cycle, and the same procedure was used for the multi-freeze–thaw cycle samples. The magnet was always maintained at 40 °C and an initial experiment with an inserted thermocouple showed that the increase in temperature during each experiment was less than 1 °C. Each measurement was replicated 4 times for fish flesh taken from four different fish (i.e., samples from the same location of different fish).

Low field nuclear magnetic resonance

Low field nuclear magnetic resonance (LF-NMR) measurements were performed using a Bruker Minispec 20 MHz RE (mq one with modular or pulse generator, 0.47T, p20, Karlsruhe, Germany) equipped with NMR data acquisition and analysis software. The equipment was calibrated with 3 standards provided by the manufacturer. The protocol was first optimized with gain (65), number of scans (32), and duration of the relaxation. Proton spin–spin T_2 relaxation was measured with a Carr-Purcell pulse sequence procedure and signal after the first 90° pulse was acquired (i.e., fid-cp-mb procedure). The sequence had a pulse separation of 0.04 ms and 2000 data points were collected with a recycle delay of 1.5 s and attenuation 4. The free induction decay (FID) signal was recorded automatically and analyzed by tri-exponential function as [3, 9]:

$$S_2 = I_{2b} \exp\left(-\frac{t}{T_{2b}}\right) + I_{21} \exp\left(\frac{t}{T_{21}}\right) + I_{22} \exp\left(\frac{t}{T_{22}}\right), \quad (1)$$

where S_2 is the intensity at any time for T_2 relaxation, I_{2b}, I_{21}, and I_{22} are the first, second and third onset intensities for first, second and third linear regions of the T_2 relaxation curve (i.e., pre-exponential factors), and T_{2b}, T_{21}, and T_{22}

are the relaxation times for strongly bound protons within the protein molecule, medium-bound water pool (i.e., on the surface of the protein molecule) and weak-bound water domains, respectively. The pre-exponent factors and relaxation times were plotted as a function of freeze–thaw cycles in order to identify different pools of protons in the fish flesh. A graphical segmentation method was used to determine the number of segments clearly (i.e., numbers of linear portions of the plot ln S_2 versus time) rather presuming 3 relaxation components. The linear parts were first selected by plotting ln ($S_2/S_{2,o}$) versus time and the segments were identified visually. The linearity of the segments was then assessed by considering the regression coefficient (r^2) and p value of each segment using MS-Excel. The statistical variables or factors are relaxation times (i.e., T_{2b}, T_{21} and T_{22}) and numbers of freeze–thaw cycles. The variations of T_{2b}, T_{21} and T_{22} as a function of freeze–thaw cycles were statistically determined from the correlations (linear and non-linear Spearman's) using PAST software, and p values are reported [10].

Results

Figure 1 shows 3 linear segments when the relaxation curve of mackerel flesh was plotted according to Eq. 1. Figure 1a shows the first portion of the ln ($S_2/S_{2,o}$) versus time plot, and a clear change in slope was observed after 0.04 ms, thus the time from 0.01 to 0.04 ms was considered to be the first linear segment. The second segment was identified in a similar way (Fig. 1c), but this segment may be biased to some extent due to the non-linear curvature of the data points. However, this bias was reduced by maintaining a high regression coefficient (higher than 0.90) when selecting this segment. In the case of the third segment, the selection of the linear section was similar to that of the first segment. In most of the cases in the literature, LF-NMR identified two to four linear segments (i.e., types of relaxing domains). The relaxation times and intensities were estimated from the slopes and intercepts of the linear regression lines (Fig. 1, b: $r^2 = 0.973$ and $p < 0.001$; c: $r^2 = 0.925$ and $p < 0.001$; and d: $r^2 = 0.998$ and $p < 0.001$). The segments' regression

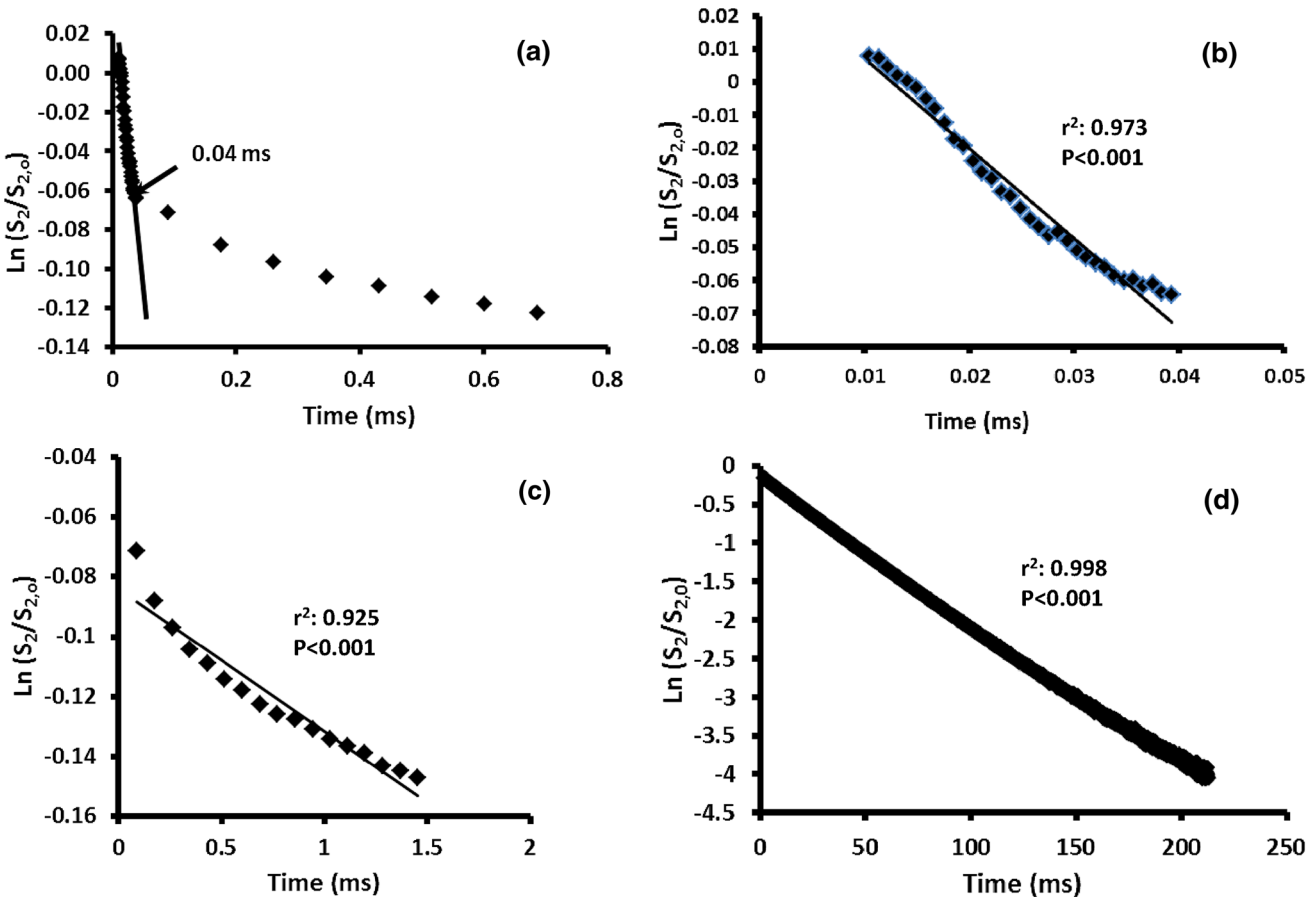

Fig. 1 Plot of ln ($S_2/S_{2,o}$) versus relaxation time, **a** first portion of the relaxation curve, **b** low-mobile protons (first linear part), **c** medium-mobile protons (second linear part), **d** high-mobile protons (third linear part)

coefficients (r^2) were always greater than 0.90 and the p values were less than 0.001, indicating highly significant regression lines. The percent intensity decay indicated that T_{2b}, T_{21} and T_{22} were 6.1, 8.1, and 85.8%, respectively. Table 1 shows the initial relaxation times of low-mobile (T_{2b}), medium-mobile (T_{21}) and high-mobile (T_{22}) protons of the sardine, tuna and mackerel flesh. The values of T_{2b} were observed as 0.365, 0.367, and 0.404 ms for sardine, tuna and mackerel flesh, respectively. Similarly, T_{21} were observed as 11.65, 28.57 and 22.20 ms, while T_{22} were observed as 58.8, 64.6 and 55.2 ms, respectively.

Figure 2 shows the relaxation time (T_{2b}) of sardine, tuna and mackerel increased significantly as a function of freeze–thaw cycles (linear: sardine $r = 0.736$ and $p < 0.01$, tuna $r = 0.857$ and $p < 0.001$, mackerel $r = 0.904$ and $p < 0.001$; and non-linear Spearman: sardine $r = 0.549$ and $p < 0.05$, tuna $r = 0.846$ and $p < 0.001$, mackerel $r = 0.934$ and $p < 0.0001$). In all cases, T_{22} increased until 6 freeze–thaw cycles and then it remained nearly constant up to 12 freeze–thaw cycles. Similar increasing trends were also observed in the case of the high-mobile domain (T_{22}) (linear correlation: sardine $r = 0.956$ and $p < 0.0001$, tuna $r = 0.927$ and $p < 0.0001$, mackerel $r = 0.890$ and $p < 0.0001$; and non-linear Spearman: sardine $r = 0.978$ and $p < 0.0001$, tuna $r = 0.989$ and $p < 0.0001$, mackerel $r = 0.901$ and $p < 0.0001$) (Fig. 3). However, T_{21} did not show significant change with the freeze–thaw cycles (linear correlation: sardine $r = -0.510$ and $p > 0.05$, tuna $r = 0.162$ and $p > 0.5$, mackerel $r = 0.513$ and $p > 0.01$; and non-linear Spearman: sardine $r = -610$ and $p > 0.01$, tuna $r = 0.159$ and $p < 0.5$, mackerel $r = 0.142$ and $p > 0.1$) (Fig. 4).

Table 1 Initial relaxation times and intensities (i.e., before freezing) of sardine, longtail tuna an Indian mackerel

Fish	T_{2b} (ms)	T_{21} (ms)	T_{22} (ms)	I_{21}	I_{22}	I_{23}
Sardine	0.365 ± 0.003	11.65 ± 0.41	58.8 ± 1.4	92.7 ± 2.2	81.8 ± 1.7	65.9 ± 1.4
Tuna	0.367 ± 0.004	28.57 ± 0.21	64.6 ± 1.4	96.5 ± 1.1	84.5 ± 1.0	67.6 ± 1.3
Mackerel	0.404 ± 0.007	22.20 ± 0.84	55.2 ± 0.8	83.2 ± 1.9	75.6 ± 0.3	66.4 ± 1.8

Values are mean ± standard deviation

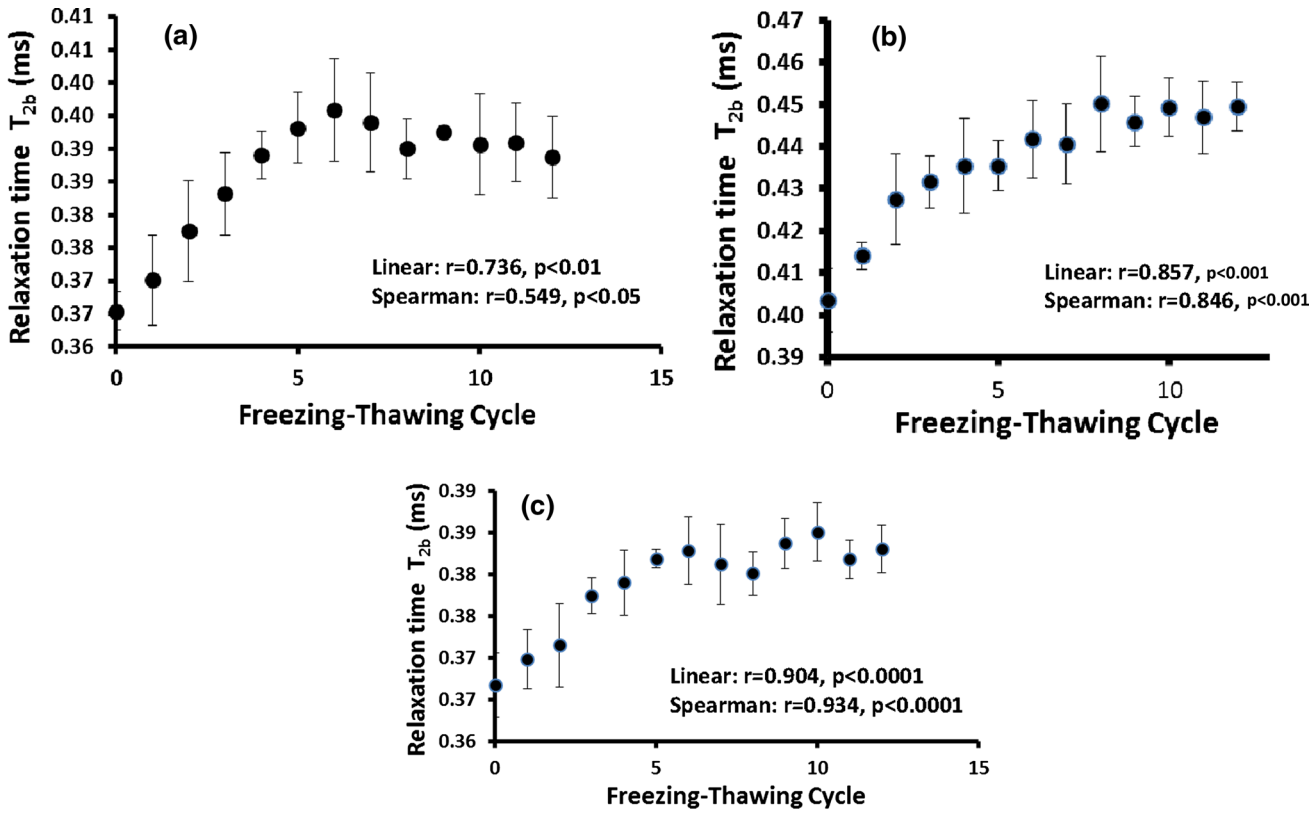

Fig. 2 Relaxation time (T_{2b}) (i.e., low-mobile protons) as a function of freeze–thaw cycles. **a** Sardine, **b** tuna, **c** mackerel

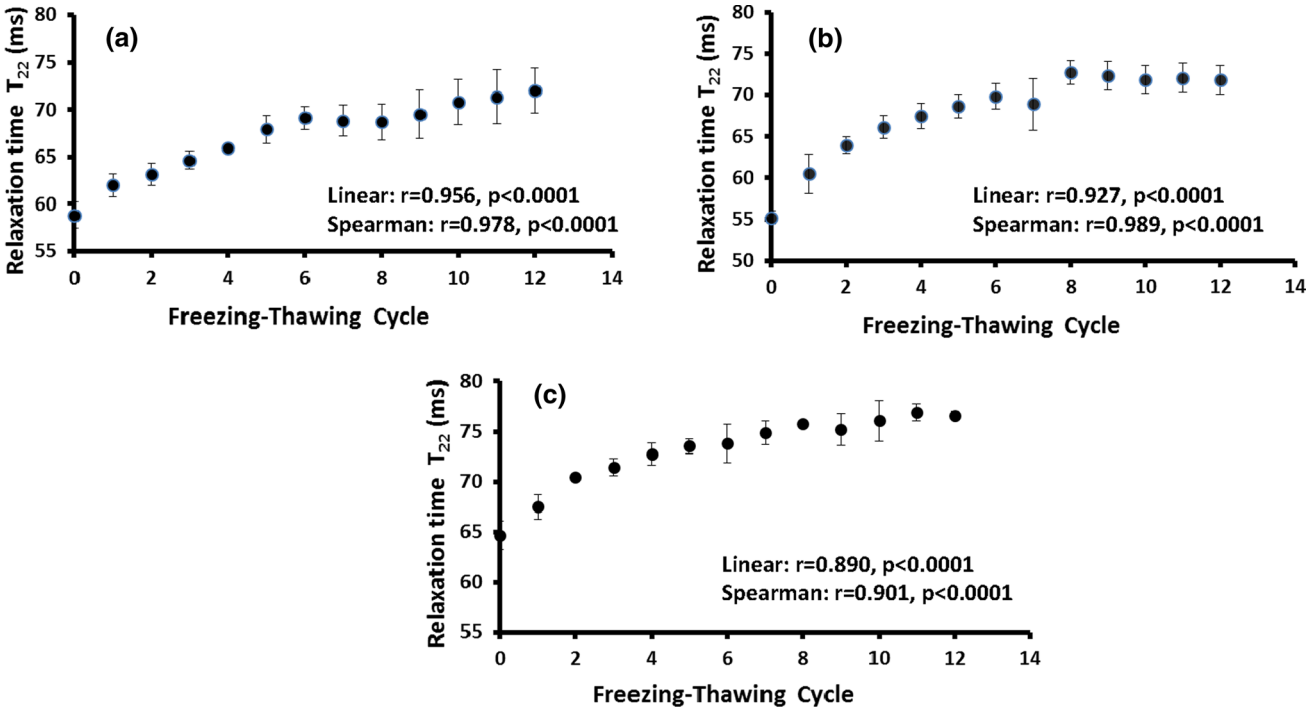

Fig. 3 Relaxation time (T_{22}) (i.e., high-mobile protons) as a function of freeze–thaw cycles. **a** Sardine, **b** tuna, **c** mackerel

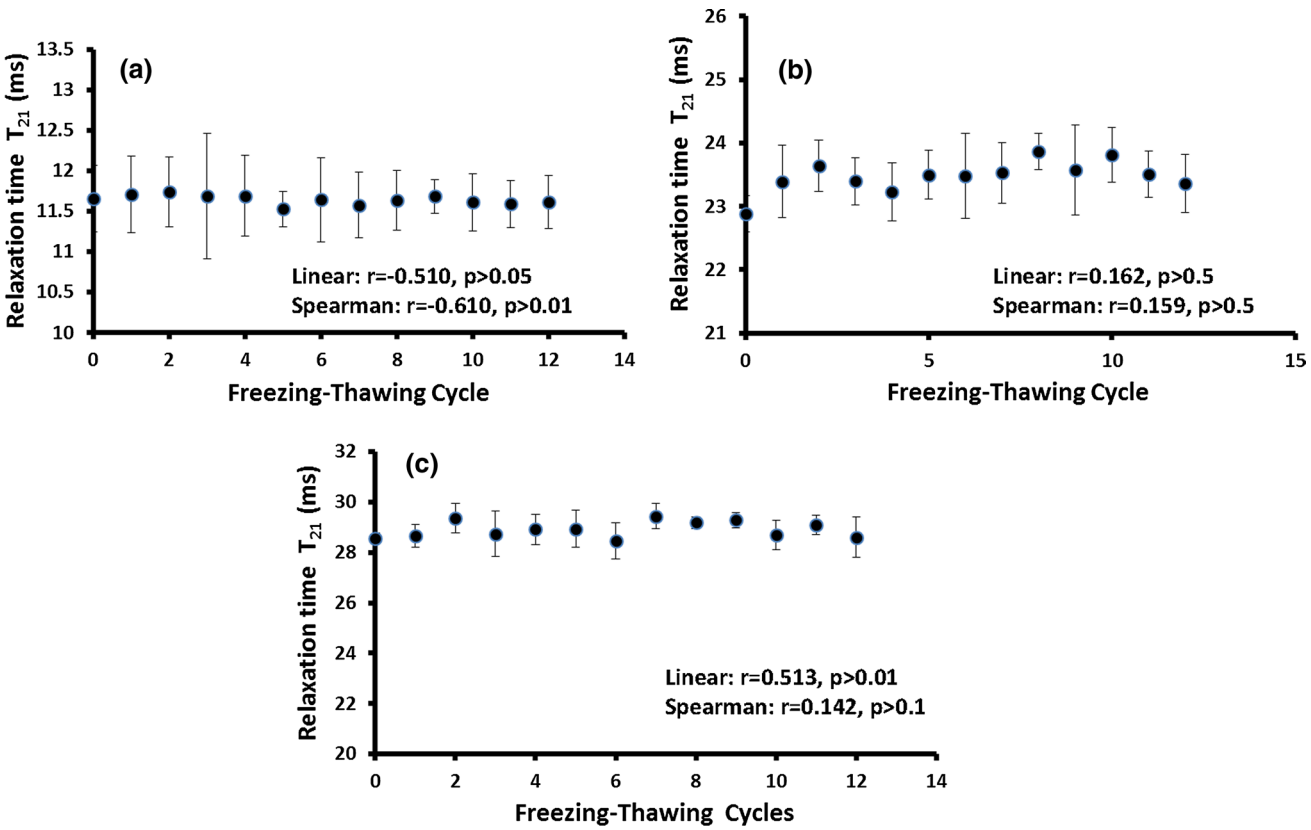

Fig. 4 Relaxation time (T_{21}) (i.e., medium-mobile protons) as a function of freeze–thaw cycles. **a** Sardine, **b** tuna, **c** mackerel

Discussion

Microscopy of fish muscle indicated that myofibrils were the highest fraction, followed by sarcolemma and then sarcoplasm [11]. Protons could be distributed in the polymer structures (i.e., proteins) and different water molecules with varied degrees of binding. Therefore, it was possible to assign T_{2b} (low-mobile) to the proton population in the protein matrix of sarcoplasm, sarcolemma and myofibril, and T_{21} (medium-mobile) to the proton population in the strongly bound water attached to the protein matrix. Similarly, T_{22} (high-mobile) could be assigned to the proton population in the weakly bound water or capillary water. However, protons in bound water were less mobile compared with those in unbound or relatively free water.

A higher relaxation time indicated a higher mobility of the domain [9]. Considering different fish species used in this study, low variation was observed in the case of low-mobile protons (i.e., for T_{2b}) as compared to the medium-domain and high-mobile domains. In the case of fresh cod flesh, T_{2b} and T_{21} were observed as 1.22 and 52.5 ms, respectively; while mackerel fresh flesh showed T_{2b} and T_{21} as 0.74 and 47.3 ms, respectively [12]. Similarly, trout flesh showed T_{2b} and T_{21} as 1.37 and 41.7 ms, respectively [13]. In the case of raw cod muscle, T_{21} and T_{22} relaxation times varied within 20–100 and 100–300 ms, respectively [8, 14]. The results of this study showed lower values (i.e., T_{21} varied 11.65–22.20 ms and T_{22} varied from 55.2 to 64.6 ms) as compared to other fish reported in the literature. These variations of relaxation in different species could be due to the compositional and structural variations of different fish species. The T_{21} relaxation was observed to be lower (i.e., nearly half) in the case of sardine as compared to tuna and mackerel (Table 1). The protons of fat could be observed near to T_{21}, thus protons in the fat of fatty fish (i.e., sardine) could interfere the protons' mobility in the medium bound water and reduced the relaxation time, T_{21}. The literature showed that the fat content of sardine was 6.30 g/100 g sample as compared to the 0.70 g and 2.71 g/100 g sample for tuna and mackerel, respectively [15].

The increased relaxation time indicated higher mobility of the protons in different domains. This increase in the case of T_{2b} could be explained by the cross-linking of protein [12], when ice was formed by exclusion of water from the protein matrix. The cross-linking of the protein matrix could increase the mobility of the proton population in the protein matrix. In this case, more weakly bound water was relocated outside the protein structure due to freeze–thaw, thus it increased the population of mobile protons (i.e., T_{22}). It is important to mention that protein denaturation and cross-linking depended on the fish species [2, 16, 17]. However, negligible change was observed in the medium-mobile bound water attached to the protein matrix. In the literature, the types of structural changes were commonly explained by the increasing or decreasing trend of relaxation time. In the case of raw salmon flesh, salting decreased the mobility (i.e., decreased T_{21}) while in the case of freeze–thaw flesh, salting increased the structural mobility (i.e., increased T_{21}) [14]. In the case of single slow and fast freeze–thaw cycles of cod and mackerel flesh, Nott et al. [12] observed that T_{21} increased while T_{22} decreased. The increase or decrease in relaxation time was more pronounced as duration of frozen storage increased. However, in the case of trout, Nott et al. [14] observed that both T_{21} and T_{22} increased with a fast freeze–thaw cycle, while a decreasing trend was observed in the case of a slow freeze–thaw cycle. This indicated that even methods of processing (i.e., fast or slow freeze–thaw cycles) could be differentiated from the LF-NMR relaxation times (i.e., T_{21} and T_{22}).

LF-NMR identified 3 types of protons (low-mobile, medium-mobile and high-mobile) in fish flesh. The relaxation times (i.e., T_{2b} and T_{22}) of low-mobile and high-mobile protons increased up to 6 freeze–thaw cycles and then remained nearly constant in value, while an insignificant difference was observed for the medium-mobile domain. The increased structural mobility could be explained by protein cross-linking, which caused relocation of more mobile water outside the protein molecules. Fresh water prawn remained stable (i.e., physicochemical properties) for up to 3 freeze–thaw cycles; however, a detrimental effect was observed with further freeze–thaw cycles [18].

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