LF ^{1}H NMR T_2 relaxation rate as affected by water addition, NaCl and pH in fresh, frozen and cooked minced hake

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Abstract

Low-Field Nuclear Magnetic Resonance of proton transverse relaxation signal (T_2) was monitored in hake (*Merluccius merluccius*) mince with different thermal histories (fresh, frozen, cooked) as affected by pH, water and NaCl addition, and it was related to water holding capacity (WHC). Modifications of T_2 signals were found in terms of changes in relaxation times and relative abundance of the relaxation components. The relaxation rate of the major component ($1/T_{21}$) increased significantly upon frozen storage or pH increase, whereas water or NaCl addition had the opposite effect. WHC decreased with freezing or water addition and increased with NaCl or basic pH; thus, T_2 and WHC best correlated when each factor was analysed separately. Linearity found for pH and protein concentration with $1/T_{21}$ was consistent with chemical exchange being responsible for these changes. The significance of these results for technological situations where compositional and biochemical changes are occurring is discussed.

Keywords

Low-Field ^{1}H NMR; T_2 transverse relaxation signal; quality; fish; muscle; water addition; NaCl addition; freezing; cooking

1. Introduction

Fishing is one of the major commercial activities in the world and the diversity of seafood species that are traded, processing types and ways of storage or presentation bring about a large amount of products with different nutritional, sensory and commercial values. In turn this leads to many potential points where the intrinsic characteristics of a given product need to be measured to have an estimation of its quality (Bremner, 2000). Therefore it is necessary to develop standardized methods to estimate various properties of seafood, ideally obtained in a rapid, non-destructive or non-invasive way. Spectroscopic methods offer these advantages in many situations of interest, and one of these methods is low-field nuclear magnetic resonance proton relaxometry (LF ^{1}H NMR) which refers to the study of the relaxation of protons in a static magnetic field after being exposed to a radiofrequency pulse (Belton 2011).

LF ^{1}H NMR has been used widely in the study of meat and meat products, and its applications to seafood are constantly increasing, in particular for the transverse relaxation times (T_2). In muscle, three components can usually be detected. The first, named T_{2b}, is characterized by short relaxation times of 1–10 ms, but the components that have received the highest attention are the intermediate relaxation time T_{21}, which accounts for most of the signal, and T_{22}, a more slowly relaxing component (Belton & Ratcliffe, 1985; Bertram, Karlsson, Rasmussen, Pedersen, Donstrup & Andersen, 2001; Erikson, Standal, Aursand, Veliyulin & Aursand, 2012). T_{2b} has been attributed to water tightly associated with macromolecules (Erikson et al., 2012), but also to non-exchangeable protein protons (Hills, Takacs & Belton, 1989a), and/or to matrix protons located in muscle structures that are plasticized by the addition of water (Venturi, Rocculi, Cavani, Placucci, Dalla Rosa & Cremonini, 2007). Most authors consider that T_{21} represents water located within highly organized protein structures, and T_{22} is ascribed to water that can be lost as drip (Bertram et al., 2001; Erikson et al., 2012). In food systems, the interpretation of changes in T_2 signals is not always straightforward (Erikson et al., 2012). On the basis of studies with protein systems where there is an excess of water, some authors (Hills et al., 1989a; Hills, Takacs & Belton, 1989b;

Belton, 2011) proposed that a) the modifications in the signal are governed by chemical exchange between exchangeable protons of protein and water protons which leads to increasing apparent relaxation rates (i.e. the inverse of the relaxation time) of water, and b) the appearance of multiple relaxation times is due to the effects of diffusion and exchange.

LF ^{1}H NMR in seafood has been used to evaluate raw material characteristics, including anatomical location (Andersen & Rinan, 2002; Løje, Green-Petersen, Nielsen, Jørgensen & Jensen, 2007; Aursand, Erikson & Veliyulin, 2010), fat, water content and seasonal variation (Jensen, Jørgensen, Nielsen & Nielsen, 2005), wild vs. farmed (Gudjónsdóttir et al., 2010), the effect of post-mortem events such as pre-slaughter handling stress (Aursand et al., 2010), rigor mortis development (Aursand, Veliyulin, Bocker, Ofstad, Rustad & Erikson, 2009, 2010) or different processing and storage factors on product characteristics such as salting, desalting, dry salting and rehydration (Aursand et al., 2009; Gudjonsdottir, Arason & Rustad, 2011; Greiff et al., 2014; Carneiro et al., 2016), smoking (Løje et al., 2007), superchilling (Erikson et al., 2012), pressure treatment (Shang, Liu, Zheng, Wang & Yin, 2015), freezing rates and freezing methods (Sánchez-Alonso, Moreno & Careche, 2014; Erikson et al., 2016), time and temperature of storage (Lambelet, Renevey, Kaabi & Raemy, 1995; Steen & Lambelet, 1997; Jepsen, Pedersen & Engelsen, 1999; Yano, Tanaka, Suzuki & Kanzaki, 2002; Erikson, Veliyulin, Singstad & Aursand, 2004; Sánchez-Alonso, Martínez, Sánchez-Valencia & Careche, 2012, 2014; Sánchez-Valencia, Sánchez-Alonso, Martinez & Careche, 2015; Erikson et al., 2016), or presence of additives (Carneiro, Marsico, Ribeiro, Conte, Alvares & De Jesus, 2013a,b; Gudjónsdóttir, Karlsdóttir, Arason & Rustad, 2013; Nikoo, Regenstein, Ghomi, Benjakul, Yang & Xu, 2015; Asli, Ofstad, Ulrike, Jessen, Einen & Morkore, 2016).

Attempts have been made to correlate T_2 relaxation signals with other indicators known to give some information about the technological and sensory properties of fish muscle (Erikson et al., 2012; Careche, Sánchez-Alonso & Martinez, 2017). Special attention has been devoted to WHC,

and in some cases (i.e. cod muscle) T_2 relaxation data were able to predict WHC over a range of 30 to 90% (Jepsen et al., 1999). In protein/water systems Hills et al. (1989a; 1989b) showed that the relaxation rates increased both with protein concentration and with denaturation and aggregation. However, it is expected that WHC in muscle would have opposite trends in both situations, so it is hypothesized that this correlation would depend on the type of matrix and factors that affect changes in the matrix.

In order to gain a better understanding of the significance of the changes in the relaxation signal observed in some technological situations of interest in fish technology, there is a need for a systematic study of the changes of T_2 as affected by protein concentration, NaCl addition, pH or denaturation of muscle proteins, since many of the scenarios in which LF ^{1}H NMR has been applied as an indicator involve modification of one or more of the above factors. This would in turn make it possible to establish the relation of T_2 signals with other parameters known to give relevant information about the changes occurring in muscle, such as WHC.

The first objective of this work was to study the LF ^{1}H NMR relaxation signal as a function of moisture, protein concentration, addition of NaCl, and pH in hake minces with different thermal histories. The second objective was to relate this signal with WHC. For this purpose, hake (*Merluccius merluccius*) muscle that had been kept fresh, frozen stored for 11 days at –10 °C or at –30 °C for 833 days (hereafter referred to as fresh, frozen, and frozen stored) was used. Also, the effect of thermal treatment in formulations with different moisture and NaCl contents was studied.

2. Materials and methods

2.1. Sample preparation

Hake (*Merluccius merluccius*) captured in the Northeast Atlantic Ocean (FAO fishing area 27) were used. They were purchased refrigerated (i.e. unfrozen specimens stored in ice from catch to sale) from a local fishmonger (El Corte Inglés, Madrid) in April 2015, June 2017 and August

2017. On arrival, gutted and filleted fish were visually inspected and washed to remove any remains of blood, viscera, etc.

Three different batches of fish were used (Supplementary Table 1). Two fish from the first batch that was analysed (June 2017) were used for the studies on fresh muscle (Fresh), and 2 more fish from the same batch were kept frozen for up to 11 days in a home freezer (GGPV 5520, Liebherr GmbH, Korneuburg, Austria) with a final temperature set at –10 °C (Frozen). The second batch that was analysed had been frozen in April 2015 and stored for 833 days in a home freezer (CHS-441-30, Radiber S.A., Barcelona, Spain) at –30 °C (Frozen stored). The third batch of hake was bought in August 2017 for the studies on thermal processing (Cooked). The time elapsed between landing and purchase of the various batches was 4, 4 and 5 days, respectively.

In the first and second batches, fillets were wrapped in food-grade polyethylene film (thickness 12.5 µm, Playdesa Plásticos y Desarrollos, S.A., Pontevedra, Spain) and subjected to freezing. Time-temperature profiles were recorded in the thermal centre of the muscle with a model SE-520 data logger (TC Direct S.A., Madrid, Spain). When needed, frozen samples from either the first or the second batch were thawed in a water bath and treated as described below.

In all three batches, fish fillets were cut and minced in a meat mincer machine, with a hole diameter of 3 mm. The minced fish was used for the preparation of 5 different formulations: minced hake with water added to increase the final moisture to 1% or 2%, minced hake with NaCl added to reach 3% in the formulation, either with maintenance of the initial moisture content or without moisture correction, and minced hake, which served as the control. For the calculations, the initial moisture content of the mince was assumed to be 80%. The final temperature was kept below 6 °C in all cases. Each formulation was placed in food-grade plastic bags, labelled and stored at 4 °C until needed (within 24 hours).

In the batches that correspond to fresh, frozen and frozen stored samples, part of the control mince was stored at 4 °C for up to 8 days in order to study the effect of pH on the LF ^{1}H NMR signal and WHC (Supplementary Table 1).

The effect of water and NaCl addition was also studied in cooked samples. For this purpose, minces from the third batch of fresh muscle were used to make the different formulations, which were placed in metal cylinders (30mm height, 30 mm Ø) and subjected to thermal treatment (90 °C, 20 min) in a water bath (Bunsen, BTG thermostatic shaking water bath, Madrid, Spain) (Supplementary Table 1). In this case, an unheated, untreated sample (uncooked sample) was used as the control, thus making a total of 6 groups of samples.

2.2. Determination of pH, moisture, crude protein and WHC

pH was measured with a pHmeter (827pH lab, Metrohm, Switzerland) with 10 grams of sample mixed with 90 mL of distilled water at ambient temperature for 20 seconds. Two parallels were performed per formulation. Moisture content was determined according to AOAC (1995). Samples were placed in aluminium caps and heated in an oven at 100 °C for 24 hours. Results were expressed as g of water per 100 g of sample. Crude protein was determined by the Dumas combustion method in a LECO TruMac® N analyser (LECO Corporation, St. Joseph, MI, USA), using a nitrogen-to-protein conversion factor of 6.25 (AOAC, 1995). The results were expressed as grams of protein per 100 g of sample. WHC was determined according to Sánchez-Alonso, Careche, Moreno, González and Medina (2011). Three grams of muscle was wrapped in 3 filters (Whatman No. 1, 110 mm diameter), placed in 50 mL Falcon tubes and centrifuged (Sorvall RT6000B Refrigerated Centrifuge, A500 Rotor, Dupont) at 3,000 *g* for 15 minutes at 20 °C. The filter papers were weighed to determine the amount of absorbed water and the results expressed as a percentage of retained water per total water in the sample. Three parallels were performed for each formulation. The resulting pellets from the centrifuged samples were collected and subjected to LF ^{1}H NMR analysis as described below.

2.3. LF ^{1}H NMR relaxometry

The analyses were performed according to Sánchez-Alonso et al. (2014) with some modifications. Approximately 2 grams either of minced hake or of the pellets obtained from the WHC measurement was placed in NMR glass tubes (1.8 cm diameter and 18 cm height), weighed accurately and kept in an ice and water bath until analysis.

A minispec mq20 LF ^{1}H NMR analyser (Bruker Optik GmbH, Germany) with a magnetic field strength of 0.47 T corresponding to a proton resonance frequency of 20 MHz was used. During the measurements the samples were kept at ~4–8 °C by using a variable temperature probe head (model PA225) coupled to a Thermo Haake® C/DC class DC10-K10 refrigerated circulator (Fisher Scientific S.L., Madrid, Spain). Transverse relaxation data (T_2) were measured using the Carr–Purcell Meiboom–Gill pulse sequence (CPMG) (Carr & Purcell, 1954; Meiboom & Gill, 1958) with a τ-value of 150 μs. For each sample, 16 scans were acquired at 2 s intervals with a total of 3,000 echoes. Three parallels were performed per formulation.

In the case of the pellets, once analysed by LF ^{1}H NMR they were recovered for determination of moisture content. After the percentage of water had been obtained, the dried samples were subjected to crude protein analysis according to the Dumas method, as described above. These data (moisture and protein concentrations in the dried sample) were used to calculate the percentage of protein in the pellet.

The decay curves were analysed with the CONTIN algorithm, resulting in the corresponding distributions of the relaxation times; this is part of the software provided with the equipment (CONTIN – the minispec – v. 1.2).

2.4. Design and data analysis

In order to minimize the modifications made to muscle structure and to study the effect of these parameters in conditions relevant to the seafood industry, the effects of protein concentration

were studied by adding water to fish mince; salt addition was performed with or without moisture adjustment, and the possible effects of protein denaturation or aggregation were studied by means of the study of the above factors in three batches of fish with different thermal histories (i.e. fresh, frozen stored for short and long times, and cooked). For the study of the effect of pH the fish minces were stored at 4 °C for up to 8 days so that an increase in pH would occur naturally (Supplementary Table 1). Hake was chosen because of its very low fat content (i.e. less than 0.8%), so the signal due to lipid protons is negligible. Moreover, this species is highly appreciated in some European countries (i.e. Spain, Portugal), where it possesses a significant economic importance for the fish sector.

Two-way analysis of variance (ANOVA) was performed with type of sample (fresh, frozen, frozen stored, cooked) as covariate to see the effect of a) addition of water, or b) addition of salt, on the WHC and the relaxation rate ($1/T_{21}$). Then, for each type of sample a one-way analysis of variance was performed with addition of water or salt as the factor. The Levene test was used to check the equality of variances. Where variances were equal, the difference between means was analysed by the F test. Where equality of variances could not be assumed, Welch and Brown-Forsythe's robust test for equality of means was used. Once the difference between means had been assumed, multiple paired comparisons were used to determine differences between means. The Bonferroni test was used where variances were presumed to be equal, and the Tamhane T2 test was used where equality of variances could not be assumed. ANOVA as a function of the type of sample and pH as covariates was also performed. Then one way ANOVA with pH as covariate was analysed.

Linear regression analyses with some of the responses (i.e. $1/T_{21}$) as the Y-variable and the percentage moisture, protein or pH as X-variables, respectively, and correlation analyses were also calculated. As a first approach, the relaxation rate of the major component was calculated as the inverse of the relaxation time corresponding to the maximum of this component. In a second step,

since abundance of T_{22} component was high (i.e. higher than 10%) in many samples, the average relaxation rates of $1/T_{21}$ were adjusted for the relative abundance of this relaxation component. For this purpose, $1/T_{21}$ was multiplied by its relative abundance. SPSS software (IBM SPSS Statistics Version 24, IBM Corporation, 2016) was used.

2.5. UNE-EN ISO 9001 certification

The Institute of Food Science, Technology and Nutrition (ICTAN-CSIC) has been certified since 2008 under UNE-EN ISO 9001 for "Management and execution of research projects and contracts in the area of Food Science and Technology and Nutrition" (certificate number ER-0366/2008).

3. Results and discussion

3.1. Moisture, protein concentration, and pH

The values for percentages of moisture and protein in the different formulations are shown in Supplementary Table 2. Control minced samples (i.e. without water or NaCl addition or heating) had moisture contents and protein concentrations that ranged from 80.7 to 80.9% and from 18.5 to 18.9%, respectively, in all the fish batches (i.e. fresh, frozen, frozen stored and uncooked control), within the expected values for this species, and pH was between 6.9 and 7.3 for the various batches. The final water achieved in the formulations ranged between 81.3 and 83.3, not always reaching the +1 or +2% target, whereas the water and protein contents were as expected when salt was added, which is compatible with the higher ability of the muscle to retain water if some other ingredients (i.e. salts) are added to the formulations.

3.2. Effect of water and NaCl addition, pH and cooking on WHC

WHC values were significantly higher in fresh and frozen batches than in frozen stored controls. In these three batches water addition led to a decrease in WHC values, and addition of

NaCl led to an increase (Figure 1a). This increase with salt was higher when no water correction was made to maintain constant moisture. Cooking led to a significant decrease in WHC as compared to control muscle, with no further modification with added water, whereas there was a clear increase in WHC after cooking with addition of salt, especially in those samples where no moisture correction was performed. A pH shift towards basic values tended to significantly raise WHC values, but the increase was dependent on the type of sample (Figure 1b). These changes were within the expected behaviour of WHC in these conditions.

3.3. LF ^{1}H NMR relaxometry

3.3.1. Effect of addition of water and NaCl on CONTIN profiles

Figure 2a shows the LF ^{1}H NMR CONTIN profiles of fresh, frozen, frozen stored and cooked hake mince, with and without added water and NaCl. In the fresh muscle with no water addition, a major relaxation component, T_{21}, with a relaxation time of 59.1 ms, which represents 96.4% of the signal, was observed. This component has been reported to vary between 40 and 65 ms in post-rigor hake muscle (Careche et al., 2017), in agreement with the present results. The minor component T_{2b}, close to 1 ms, was also detected in these samples. In the frozen mince control (i.e. no water addition), the relative amplitude of T_{21} component decreased as compared to the fresh sample, and T_{22} was more evident. These results agree with previous findings about the effect of freezing for this species (Sánchez-Alonso et al., 2014). In frozen stored control samples, the amplitude of T_{22} component (i.e. A_{22}) increased significantly, and both relaxation time and the corresponding amplitude (i.e. A_{21}) of T_{21} component were lower than in the fresh or frozen controls, also in agreement with previous results for this species (Sánchez-Alonso et al., 2012; Sánchez-Valencia et al., 2015). In all three batches (i.e. fresh, frozen and frozen stored) the addition of water led to a decrease of the T_{21} relative amplitude and to an increase in T_{22} component which was greater in the samples with the highest addition of water. The addition of NaCl, either with maintenance of the initial moisture of the fish muscle or without any further adjustment to the

formulation, resulted in an increase of the T_{21} component, and at the same time T_{22} component was less evident and in some cases the signal disappeared. CONTIN profiles of cooked samples show that there was an increase in T_{22} component due to heating, and the changes due to water and NaCl addition were, in general, similar to the ones observed in unheated muscle, but there was also some residual T_{22} in NaCl-treated samples.

The presence of T_{22} component after freezing or cooking (Figure 2a) can be interpreted as an increase in spatial heterogeneity, which, as explained by other authors (Hills et al., 1989b; Lambelet et al., 1995), must exist on a large distance scale compared to that of the diffusion of water molecules, with the result that the rate of diffusion is not fast enough for the water molecules to experience all the environments on a short time scale compared to the chemical exchange time scale.

When muscle pellets obtained in the conditions used for determination of WHC were analysed, the T_{22} component disappeared in all the fresh, frozen, frozen stored and cooked samples (Figure 2b). These results support the idea that T_{22} represents water loosely bound (i.e. exuded by drip loss or by centrifugation).

3.3.2. Effect of addition of water and NaCl on relaxation rate of the major component ($1/T_{21}$)

There was a significant increase in $1/T_{21}$ between the fresh and frozen stored or cooked controls. In general, for each type of sample addition of water to muscle led to a slight but significant decrease in the relaxation rates (Figure 3a). Significant decreases in this parameter upon addition of NaCl occurred in all four types of samples, and they were more evident in those in which water was added in order to maintain the same moisture as in the initial muscle.

In the pellets the relaxation rate was in all cases higher than in intact, non-centrifuged mince (Figures 3b), and the values were highest in the cooked batch. No significant differences were

found as a consequence of addition of water per type of sample, whereas the decrease in relaxation rates in the pellets with NaCl followed a similar pattern to that of the non-centrifuged samples.

The observed decrease in relaxation rates with NaCl is in agreement with the effect of this salt at low concentrations, because it causes swelling of the myofibrils by an increase in electrostatic repulsion, expanding the protein network, which in turn leads to a lower protein-to-water ratio in the myofibril, and therefore longer relaxation times for T_{21} component would be expected (Erikson et al., 2012).

3.3.3. The effect of protein concentration on $1/T_{21}$

The addition of water or NaCl involved changes in composition, i.e. protein concentration, with the result that samples had less protein in both cases. In protein/water model systems the increase in relaxation rates with protein concentration has been observed to be linear, and attributed to chemical exchange, so in the presence of fast exchange the observed apparent relaxation rate of the water would be the sum of the fractional populations of protein and water multiplied by their corresponding relaxation rates; since the relaxation rates of protein are very high compared to that of bulk water, the average relaxation would be biased towards that of the protein, and therefore relaxation rates would increase with increasing protein concentration (Hills et al., 1989a; Belton, 2011). Another factor that could influence the relaxation rate is denaturation and/or aggregation. In model systems of denatured and aggregated proteins the apparent relaxation rates have been found to be significantly higher than in native protein systems because the former would have a more rigid structure and thus much higher relaxation rates (Hills et al., 1989b). Accordingly, the differences in relaxation rates found in section 3.3.2 could be a) the consequence of a different protein/water ratio or b) due to denaturation and aggregation of protein.

In order to see whether protein concentration could be the underlying factor in the changes observed in the relaxation rates, $1/T_{21}$ was plotted as a function of protein concentration. In the first step only centrifuged samples were used, because there was no T_{22} component and quantitative

analyses are easier with monoexponential signals (Hills et al., 1989a). Figure 4a shows a linear trend in the relaxation rate as a function of protein concentration after addition of water (R^2=0.975; y = 1,6967x - 16,442). As expected, the pellets corresponding to the fresh muscle retained more water (i.e. had a lower protein concentration) than those of the frozen samples, the cooked pellets being the ones with the highest protein content. This was also evident when these rates were plotted as a function of moisture (R^2=0.969, not shown).

When relaxation rates in the pellets of the formulations with added NaCl were plotted as a function of moisture, three distinct curves were observed, each with a slightly different linear trend (results not shown). However, when they were plotted as a function of protein concentration (Figure 4b), they merged into one (R^2=0.981; y = 1,6416x - 14,906). Therefore, also when salt is added the main factor affecting the T_2 signal is the decrease in the protein/water ratio.

The fact that all the samples (i.e. fresh, frozen, frozen stored, cooked) had a similar linear trend (R^2 = 0,987; y = 1,6382x - 14,794 when data were plotted together) suggests that the differences in the relaxation rate observed among them were due to an increased protein/water ratio in the pellets resulting from frozen storage or cooking, rather than to a significant modification of the protein due to denaturation or aggregation. This lack of effect could be due to the fact that the muscle structure is rigid enough for no further differences due to protein aggregation or denaturation to occur without disrupting the muscle structure.

This linearity was also observed when all the samples studied (i.e. pellets and mince resulting from water or salt addition) of all the types of muscle (fresh, frozen, frozen stored and cooked) were plotted together (R^2=0.952), but there was some lack of fit due to the samples with a high proportion of the T_{22} component (i.e. higher than 20%). When these were taken out, the coefficient of determination improved substantially but the improvement was slightly higher when adjustments to $1/T_{21}$ were performed to take into account the relative proportion of water in the T_{21} component (see Materials and methods), so all samples (Figure 4c), including those with a

relatively high proportion of T_{22} component, were linearly correlated with protein concentration (R^2=0.988; y = 1,575x - 13,158).

Therefore, any change in protein concentration, as a result of either addition of water or adjustments in formulation (i.e. by salt addition at a relatively low salt concentration) will affect the T_2 signal, and in many instances the protein concentration could be quantified. This result would open the possibility of using this technique for the analysis of undeclared addition of water on the basis of the information obtained about water retention (i.e. CONTIN profiles) and composition (i.e. information about protein concentration and moisture). However, other factors such as pH could affect the NMR signal, and they are analysed in the next section.

3.3.4. Effect of pH

The rise in pH resulting from storage at 4 °C produced a decrease in the proportion of T_{22} component only in frozen samples, whereas T_{21} shifted towards shorter relaxation times in fresh, frozen and frozen stored minced hake kept in the fridge for several days (Figure 5a), and, as in the previous samples, centrifugation led to a disappearance of T_{22} (Results not shown). The change in the apparent relaxation rates ($1/T_{21}$) of the muscle displayed a linear trend as the pH increased from 6.9 to 8.2 for all batches (y = 5,4533x - 21,932; R^2 = 0,816). However, there was some deviation in the samples obtained from frozen stored muscle (Figure 5b).

The effect of pH on proton transverse relaxation rates has been observed in muscle tissue (Fung & Puon, 1981; Erikson et al., 2004), and it has been attributed to an increase in the fraction of labile protein protons (Hills et al., 1989a) of NH and OH groups, so at a lower pH the exchange rates of the NH protons of lysine, arginine and histidine are slow, whereas serine and threonine, on the other hand, would be expected to have faster exchange rates. As a result of increasing the pH there would be an increase in fast-exchange NH proton (Hills et al., 1989a). Factors such as exchange of other amino acid residues (i.e. from aspartate and glutamate) or some conformational

changes in proteins owing to pH changes could also affect the signal according to those authors (Hills et al., 1989a).

3.4. Relationship between LF ^{1}H NMR and WHC

Variables extracted from biexponential analysis could not be used for this study since in some of the samples monoexponential curves were obtained (i.e. NaCl added minces). Thus, data extracted from CONTIN (i.e. T_{21}, T_{22} relaxation times and their corresponding maximum amplitudes A_{21}, A_{22}) were correlated with WHC. Results showed that A_{21} and A_{22} where correlated to WHC (R^2=0.774 and -0.760 respectively, significant at P=0.01 level. The relaxation time T_{22} was also significantly correlated to WHC (R^2=0.469, P=0.05) whereas T_{21} was not. This is consistent with the fact that T_{22} is water that it is loosely bound to the fish matrix. The high correlation of A_{21} with WHC can be explained in terms of amount of water present in this major component. However, there is a certain separation depending on the type of sample, so that frozen stored and cooked samples had a high linearity whereas fresh and recently frozen ones deviated from lineality in NaCl-added samples (Figure 6a).

On the other hand, as expected, the relation of the T_{21} signal and WHC did not run in parallel, since we had observed that $1/T_{21}$ decreased upon addition of water or NaCl (Figure 3) and increased with pH (Figure 5), whereas WHC decreased upon water addition and increased in the other two situations (i.e. NaCl addition and pH increase) (Figure 1) for a given batch of fish. Thus, correlations could exist in cases where a single factor is affecting the modifications of the fish matrix. For example, Figure 6b, shows a linearity between $1/T_{21}$ and WHC with increasing water which is different as a function of the type of sample, where fresh-frozen and frozen stored- cooked were grouped together respectively.

Therefore, the present paper shows good correlations between some parameters of T_2 signal and WHC. This is supported with results in the literature, where in general good correlations

between WHC and NMR signals have been obtained for cod (Jepsen et al., 1999; Andersen & Jorgensen, 2004; Erikson et al., 2004; Burgaard & Jorgensen, 2010), frozen stored hake muscle (Sánchez-Alonso et al., 2012) and frozen shrimps treated with different concentrations of sodium polyphosphate (Carneiro, Marsico, Ribeiro, Conte, Alvares & De Jesus, 2013b). However, whenever the signal is simultaneously affected by more than one factor these correlations may not necessarily occur which suggests that both WHC and LF ^{1}H NMR could be complementary indicators, rather than one being used as a substitute for the other.

4. Conclusions

LF ^{1}H NMR is sensitive to water and NaCl addition as well as pH, and the signal depends on the state of the fish muscle (i.e. fresh, recently frozen, frozen stored or cooked). Protein concentration was linearly related with the relaxation rate of the major component (i.e. $1/T_{21}$) and so it could be considered as the most important underlying factor for the changes upon addition of water or NaCl. This, together with the effect of pH on the NMR signal, is consistent with the effect of chemical exchange on the observed relaxation rates of this signal in the above situations.

The fact that LF ^{1}H NMR provides information related to the water that is retained in fish muscle or easily lost shows a relationship with WHC. On the other hand, correlations may only be found if each factor is analysed separately.

The water retention and composition characteristics obtained by LF ^{1}H NMR make this technique a useful tool as a quality indicator for several technological applications (i.e. freezing, frozen storage or addition of ingredients) that could be used in combination with other indicators. However, the interpretation should take into account that there may be counteracting effects in a given signal due to the protein concentration or pH of the samples.

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Conflict of interest

The authors declare that no competing interests exist.

References

Andersen, C. M., & Jorgensen B. M. (2004). On the Relation Between Water Pools and Water Holding Capacity in Cod Muscle. *Journal of Aquatic Food Products Technology*, *13*, 13–23.

Andersen, C. M., & Rinnan, A. (2002). Distribution of water in fresh cod. *Lebensmittel-Wissenschaft und -Technologie – Food Science and Technology*, *35*(8), 687–696.

AOAC. (1995). Official Methods of Analysis of the Association of Official Agricultural Chemists. Gaithersburg, Maryland, USA: AOAC International.

Asli, M., Ofstad, R., Ulrike, U., Jessen, F. Einen, O., & Morkore, T. (2016). Effect of Sodium Bicarbonate and Varying Concentrations of Sodium Chloride in Brine on the Liquid Retention of Fish (*Pollachius virens* L.) Muscle. *Journal of the Science of Food and Agriculture*, *96*(4), 1252–1259.

Aursand, I. G., Erikson, U., & Veliyulin, E. (2010). Water properties and salt uptake in Atlantic salmon fillets as affected by ante-mortem stress, rigor mortis, and brine salting: A low-field ^{1}H NMR and ^{1}H/^{23}Na MRI study. *Food Chemistry*, *120*, 482–489.

Aursand, I. G., Veliyulin, E., Bocker, U., Ofstad, R., Rustad, T., & Erikson, U. (2009). Water and salt distribution in Atlantic Salmon (*Salmo salar*) studied by Low-Field ^{1}H NMR, ^{1}H and

^{23}Na MRI and light microscopy: Effects of raw material quality and brine salting. *Journal of Agricultural and Food Chemistry*, *57*(1), 46–54.

Belton, P. S. (2011). Spectroscopic approaches to the understanding of water in foods. *Food Reviews International*, *27*(2), 170–191.

Belton, P., & Ratcliffe, R. G. (1985). NMR and compartmentation in biological tissues. *Progress in Nuclear Magnetic Resonance Spectroscopy*, *17*, 241–279.

Bertram, H. C., Karlsson, A. H., Rasmussen, M., Pedersen, O. D., Donstrup, S., & Andersen, H. J. (2001). Origin of multiexponential T_2 relaxation in muscle myowater. *Journal of Agricultural and Food Chemistry*, *49*(6), 3092–3100.

Bremner, H. A. (2000). Toward practical definitions of quality for food science. *Critical Reviews in Food Science and Nutrition*, *40*, 83–90.

Burgaard, M. G., & Jørgensen, B. M. (2010). Effect of temperature on quality-related changes in cod (*Gadus morhua*) during short- and long-term frozen storage. *Journal of Aquatic Food Products Technology*, *19*, 249–263.

Careche, M., Sánchez-Alonso, I., & Martinez, I. (2017). Estimation of Quality in Frozen Fish by Low Field NMR. In: G. A. Webb (Ed.), *Trends in Fish Processing Technologies* (pp. 1–16). Springer International Publishing.

Carneiro, C. D., Marsico, E. T., Ribeiro, R. D. R., Conte, C. A., Alvares, T. S., & De Jesus, E. F. O. (2013a). Quality Attributes in Shrimp Treated with Polyphosphate after Thawing and Cooking: A Study Using Physicochemical Analytical Methods and Low-Field H-1 NMR. *Journal of Food Process Engineering*, *36*, 492–499.

Carneiro, C. D., Marsico, E. T., Ribeiro, R. D. R., Conte, C. A., Alvares, T. S., & De Jesus, E. F. O. (2013b). Studies of the effect of sodium tripolyphosphate on frozen shrimp by physicochemical analytical methods and Low Field Nuclear Magnetic Resonance (LF H-1 NMR). *LWT-Food Science and Technology*, *50*, 401–407.

Carneiro, C. D., Marsico, E. T., Ribeiro, R. D. R., Conte, C. A., Mano, S. B., Augusto, C. J. C., & De Jesus, E. F. O. (2016). Low-Field Nuclear Magnetic Resonance (LF NMR 1H) to assess the mobility of water during storage of salted fish (*Sardinella Brasiliensis*). *Journal of Food Engineering*, *169*, 321–325.

Carr, H. Y., & Purcell, E. M. (1954). Effects of diffusion on free precession in nuclear magnetic resonance experiments. *Physical Review*, *94*(3), 630–638.

Erikson, U., Kjørsvik, E., Bardal, T., Digre, H., Schei, M., Søreide, T. S., & Aursand, I. G. (2016). Quality of Atlantic cod frozen in cell alive system, air-blast, and cold storage freezers. *Journal of Aquatic Food Product Technology*, *25*, 1001–1020.

Erikson, U., Standal, I. B., Aursand, I. G., Veliyulin, E., & Aursand, M. (2012). Use of NMR in fish processing optimization: a review of recent progress. *Magnetic Resonance in Chemistry*, *50*(7), 471–480.

Erikson, U., Veliyulin, E., Singstad, T. E., & Aursand, M. (2004). Salting and desalting of fresh and frozen-thawed cod (*Gadus morhua*) fillets: A comparative study using ^{23}Na NMR, ^{23}Na MRI, low-field ^{1}H NMR, and physicochemical analytical methods. *Journal of Food Science*, *69*(3), 107–114.

Fung, B. M., & Puon, P. S. (1981). Nuclear magnetic resonance transverse relaxation in muscle water. *Biophysical Society* 33, 27–38.

Greiff, K., Fuentes, A., Aursand, I. G., Erikson, U., Masot, R., Alcañiz, M., & Barat, J. M. (2014). Innovative Nondestructive Measurements of Water Activity and the Content of Salts in Low-Salt Hake Minces. *Journal of Agricultural and Food Chemistry*, *62*(12), 2496–2505.

Gudjonsdottir, M., Arason, S., & Rustad, T. (2011). The Effects of Pre-Salting Methods on Water Distribution and Protein Denaturation of Dry Salted and Rehydrated Cod – A Low-Field NMR Study. *Journal of Food Engineering 104*(1), 23–29.

Gudjónsdóttir, M., Gunnlaugsson, V. N., Finnbogadottir, G. A., Sveinsdottir, K., Magnusson, H., Arason, S., & Rustad, T. (2010). Process Control of Lightly Salted Wild and Farmed

Atlantic Cod (*Gadus morhua*) by Brine Injection, Brining, and Freezing – A Low Field NMR Study. *Journal of Food Science*, *75*(8), E527–E536.

Gudjónsdóttir, M., Karlsdóttir, M. G., Arason, S., & Rustad T. (2013). Injection of fish protein solutions of fresh saithe (*Pollachius virens*) fillets studied by low field nuclear magnetic resonance and physicochemical measurements. *Journal of Food Science and Technology-Mysore*, *50*, 228–238.

Hills, B. P., Takacs, S. F., & Belton, P. S. (1989a). The effects of proteins on the proton N.M.R. transverse relaxation time of water. II. Protein aggregation. *Molecular Physics*, *67*(4), 919–937.

Hills, B. P., Takacs, S. F. & Belton, P. S. (1989b). The effects of proteins on the proton NMR transverse relaxation time of water. II. Protein aggregation. *Molecular Physics*, *67*(4), 919–937.

Jensen, K. N., Jørgensen, B. M., Nielsen, H. H., & Nielsen, J. (2005). Water distribution and mobility in herring muscle in relation to lipid content, season, fishing ground and biological parameters. *Journal of the Science of Food and Agriculture*, *85*, 1259–1267.

Jepsen, S. M., Pedersen, H. T., & Engelsen, S. B. (1999). Application of chemometrics to low-field $^{(1)}$H NMR relaxation data of intact fish flesh. *Journal of the Science of Food and Agriculture*, *79*(13), 1793–1802.

Lambelet, P., Renevey, F., Kaabi, C., & Raemy, A. (1995). Low-Field nuclear-magnetic-resonance relaxation study of stored or processed cod. *Journal of Agricultural and Food Chemistry*, *43*(6), 1462–1466.

Løje, H., Green-Petersen, D., Nielsen, J., Jørgensen, B. M., & Jensen, K. N. (2007). Water distribution in smoked salmon. *Journal of the Science of Food and Agriculture*, *87*, 212–217.

Meiboom, S., & Gill, D. (1958). Modified Spin-Echo Method for Measuring Nuclear Relaxation Times. *Review of Scientific Instruments*, *29*(8), 688–691.

Nikoo, M, Regenstein, J. M., Ghomi, M. R., Benjakul, S., Yang, N., & Xu, X. (2015). Study of the combined effects of a gelatin-derived cryoprotective peptide and a non-peptide antioxidant in a fish mince model system. *LWT-Food Science and Technology*, *60*, 358–364.

Sánchez-Alonso, I., Careche, M., Moreno, P., González, M. J., & Medina, I. (2011). Testing caffeic acid as a natural antioxidant in functional fish-fibre restructured products. *LWT - Food Science and Technology*, *44*(4), 1149–1155.

Sánchez-Alonso, I., Martínez, I., Sánchez-Valencia, J., & Careche, M. (2012). Estimation of freezing storage time and quality changes in hake (*Merluccius merluccius*, L.) by low field NMR. *Food Chemistry*, *135*(3), 1626–1634.

Sánchez-Alonso, I., Moreno, P., & Careche, M. (2014). Low Field Nuclear Magnetic Resonance (LF-NMR) Relaxometry in Hake (*Merluccius merluccius*, L.) Muscle after Different Freezing and Storage Conditions. *Food Chemistry*, *153*, 250–257.

Sánchez-Valencia. J., Sánchez-Alonso, I., Martinez, I., & Careche, M. (2015). Low-field nuclear magnetic resonance of proton (1H LF NMR) relaxometry for monitoring the time and temperature history of frozen hake (*Merluccius merluccius* L.) muscle. *Food and Bioprocess Technology*, *8*, 2137–2145.

Shang, X., Liu, A., Zheng, J., Wang, P., & Yin, S. (2015). High Pressure Processing and Water Holding Capacity of Sea Bass Skeletal Muscle. *Journal of Aquatic Food Product Technology*, *24*(8), 740–751.

Steen, C., & Lambelet, P. (1997). Texture changes in frozen cod mince measured by low-field nuclear magnetic resonance spectroscopy. *Journal of the Science of Food and Agriculture*, *75*, 268–272.

Venturi, L., Rocculi, P., Cavani, C., Placucci, G., Dalla Rosa, M., & Cremonini, M. A. (2007). Water Absorption of Freeze-Dried Meat at Different Water Activities: A Multianalytical Approach Using Sorption Isotherm, Differential Scanning Calorimetry, and Nuclear Magnetic Resonance. *Journal of Agricultural and Food Chemistry*, *55*(26), 10572–10578.

Yano, S., Tanaka, M., Suzuki, N., & Kanzaki, Y. (2002). Texture change of beef and salmon meats caused by refrigeration and use of pulse NMR as an index of taste. *Food Science and Technology Research*, *8*, 137–143.

Figure legends

Figure 1. Water Holding Capacity (%) of **a)** fresh (white), frozen (dotted), frozen stored (rhomboid), and cooked (grey) hake mince without (control) or with added water to reach 1% or 2% moisture increase, and with 3% salt adjusting the water to get the initial moisture, and with 3% salt. Different letters in the same type of sample (i.e. fresh, frozen, frozen stored or cooked) show significant differences for the effect of water addition and different numbers show significant differences for the effect of NaCl addition. In the control column, different asterisks represent significant differences as a function of the type of sample. Significance established at $P<0.05$ level; **b)** minced hake stored at 4 ºC up to 8 days from fresh (diamond), frozen (square), and frozen stored (triangle) samples as a function of pH. Error bars, standard deviation.

Figure 2. LF ^{1}H NMR CONTIN profiles of **a)** minced samples and **b)** centrifuged pellets (3,000 *g* x 15 min). From top to bottom: fresh, frozen, frozen stored, and cooked hake mince. From left to right: control, with water added up to 1% or 2% moisture increase, with 3% NaCl adjusting the water to get the initial moisture and 3% NaCl without adjusting water. Curves are average of three replicates.

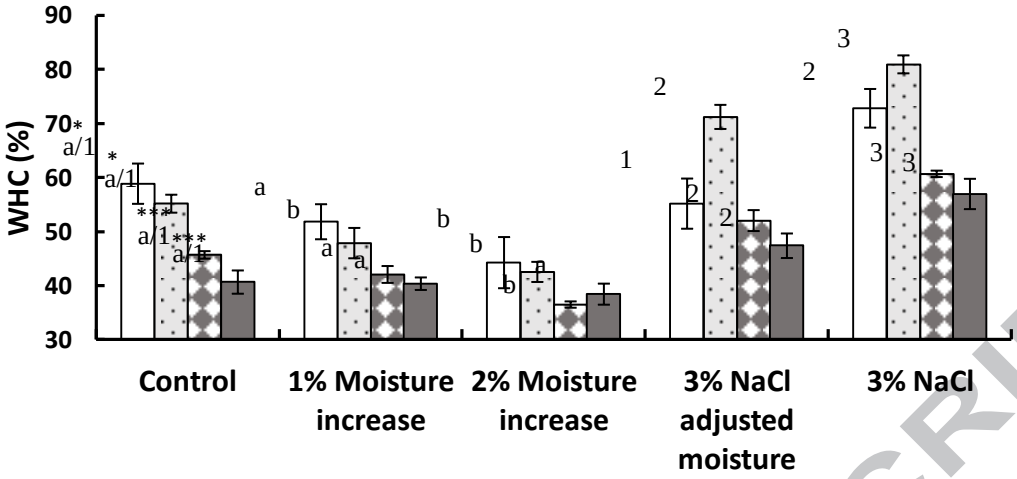
Figure 3. T_{21} relaxation rates ($1/T_{21}$) (s^{-1}) of **a)** minced samples and **b)** centrifuged pellets (3,000 *g* x 15 min). Fresh (white), frozen (dotted), frozen stored (rhomboid), and cooked (grey) hake mince without (control), or with added water to reach 1% or 2% moisture increase, and with 3% salt adjusting the water to get the initial moisture, and with 3% salt. Error bars, standard deviation. Different letters in the same type of sample (i.e. fresh, frozen, frozen stored or cooked) show significant differences for the effect of water addition and different numbers show significant differences for the effect of NaCl addition respectively. In the control column, different asterisks

represent significant differences as a function of the type of sample. Significance established at $P<0.05$ level.

Figure 4. T_{21} relaxation rates ($1/T_{21}$) (s^{-1}) as a function of protein concentration of centrifuged minced hake pellets (3,000 *g* x 15 min) for **a)** fresh (◆), frozen (■),frozen stored (▲), and cooked (●) samples without and with water addition (up to 2%) and **b)** without (◆), or with 3% NaCl adjusting the water to the initial moisture (■), and with 3% NaCl (▲). **c)** Adjusted $1/T_{21}$ as a function of protein concentration for all (i.e. fresh, frozen, frozen stored, and cooked) minced hake samples and pellets without and with NaCl or water addition. Adjustments were performed according to Materials and methods.

Figure 5. a) LF ^{1}H NMR CONTIN profiles of fresh (left), frozen (middle), and frozen stored (right) minced hake kept at 4 ºC up to 8 days. The arrow in the right axis shows the pH value for each sample. **b)** Adjusted T_{21} relaxation rates ($1/T_{21}$) (s^{-1}) as a function of pH for fresh (diamond), frozen (triangle), and frozen stored (open circle) of minced hake samples. Adjustments were performed according to Materials and methods.

Figure 6. Relation of T_2 signals and WHC in fresh (◆), frozen (■), frozen stored (▲), and cooked (●) samples: **a)** Maximum amplitude of T_{21} peak for all formulations (i.e. without and with NaCl or water addition, and effect of pH). Data points are averages per formulation. **b)** Adjusted T_{21} relaxation rates ($1/T_{21}$) (s^{-1}) in samples were water was added for up to 2%. Adjustments were performed according to Materials and methods.



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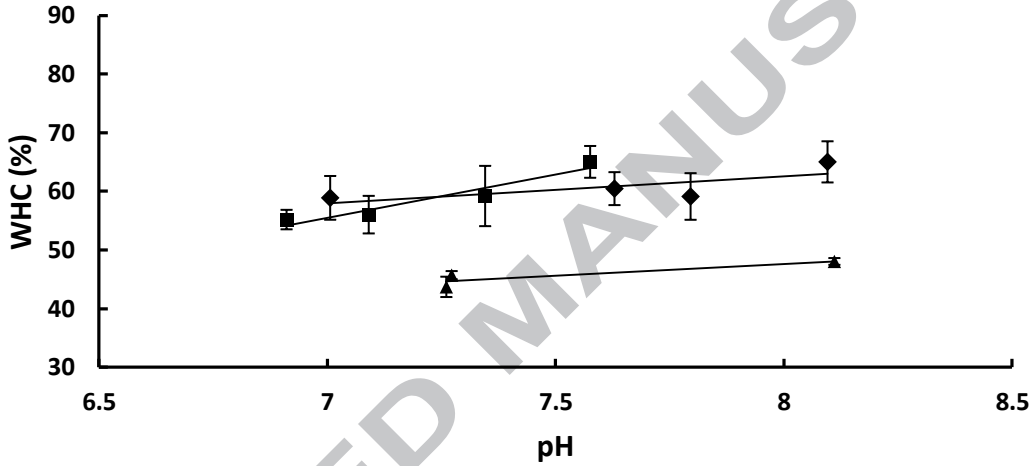
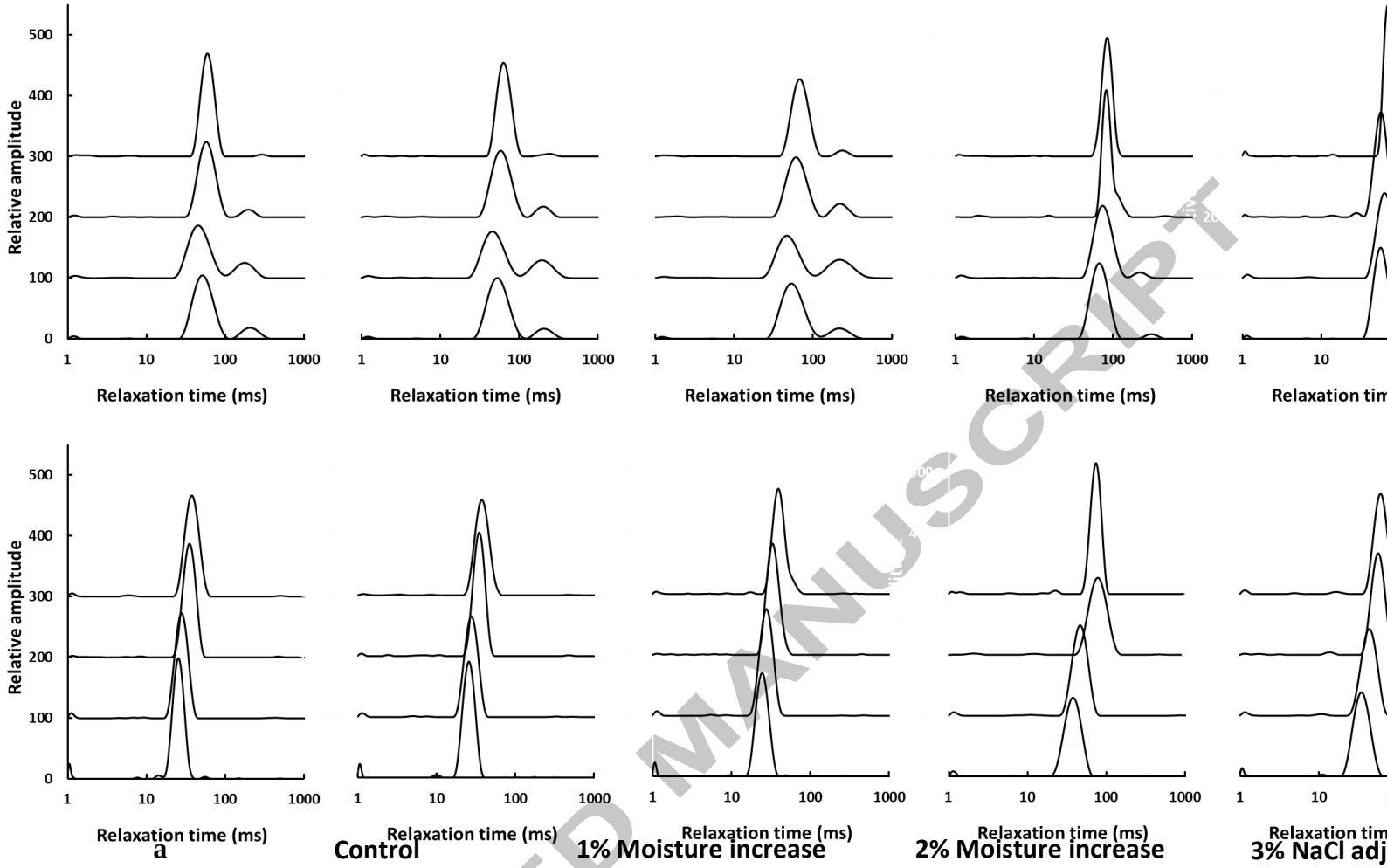
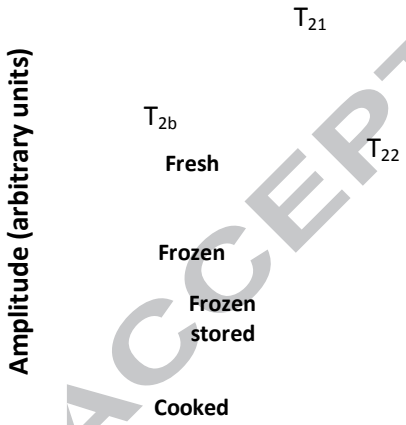


Figure 1



a **Control** **1% Moisture increase** **2% Moisture increase** **3% NaCl adj**



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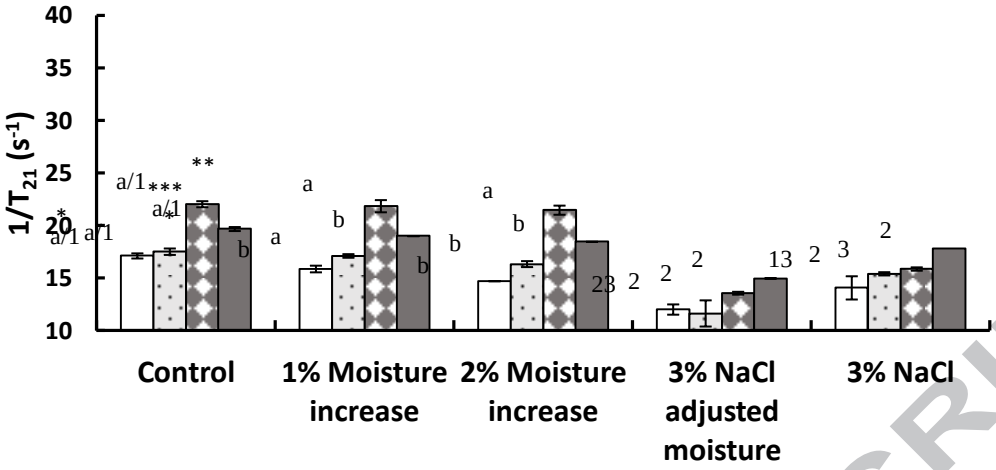
a



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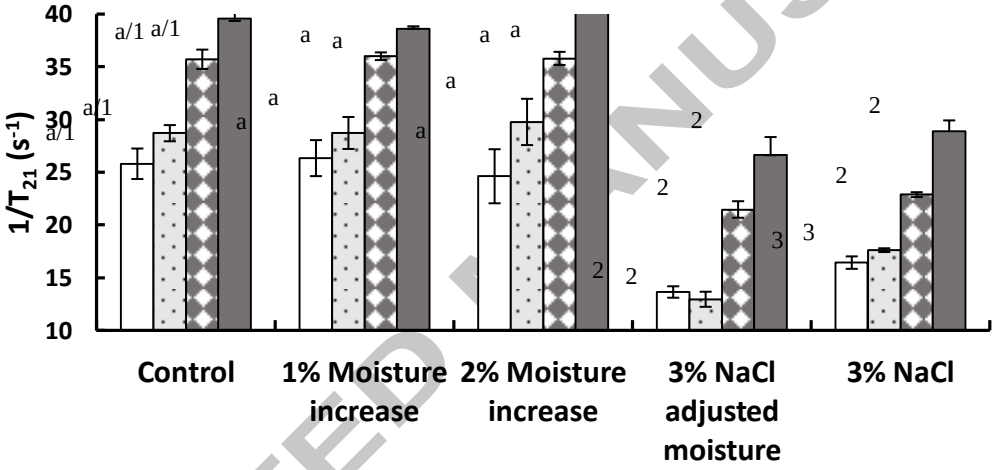
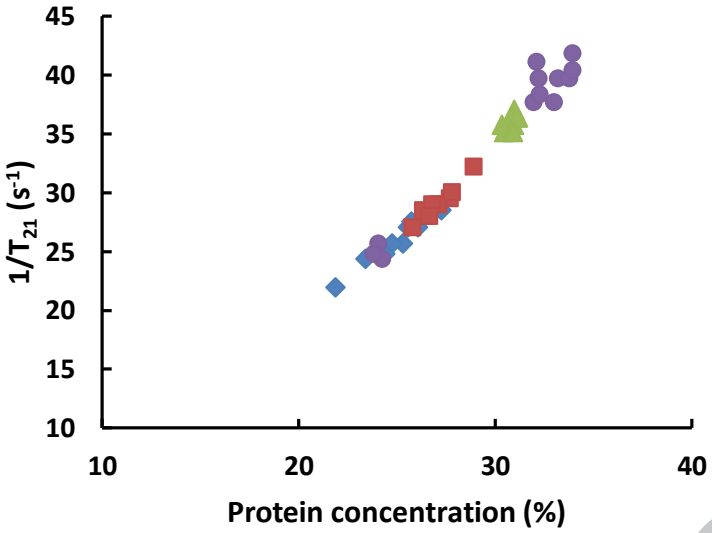


Figure 3

a



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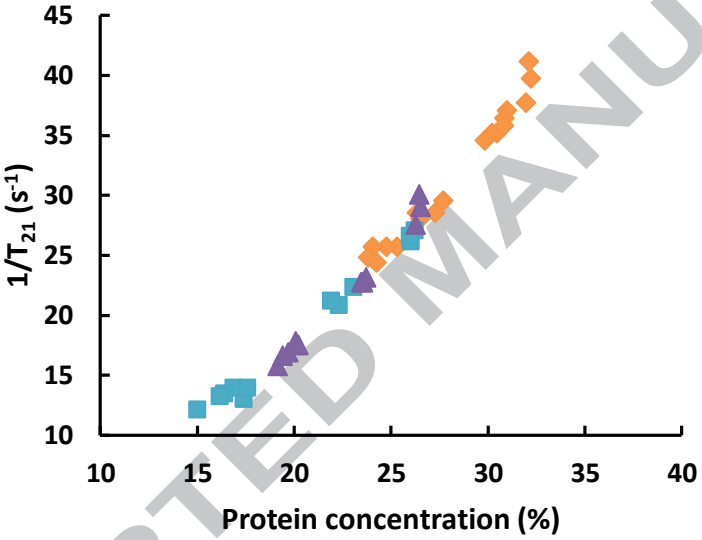

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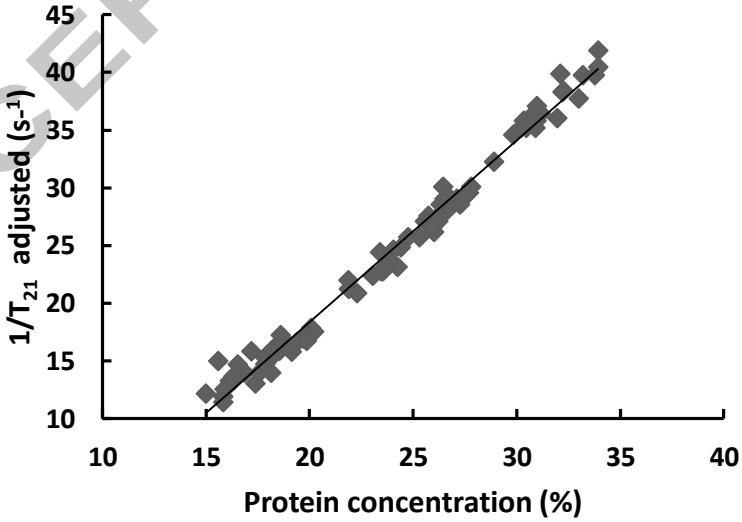
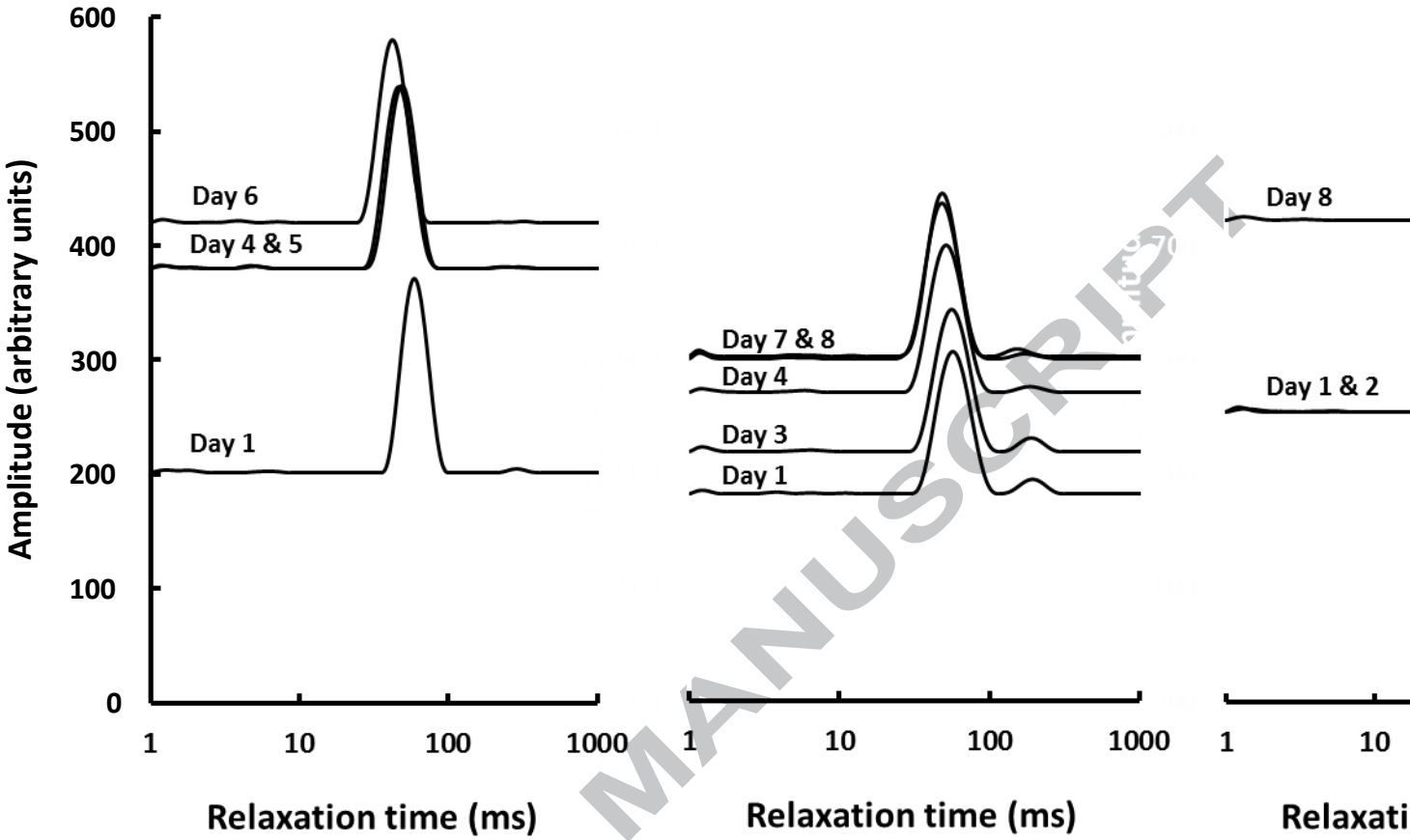


Figure 4

a



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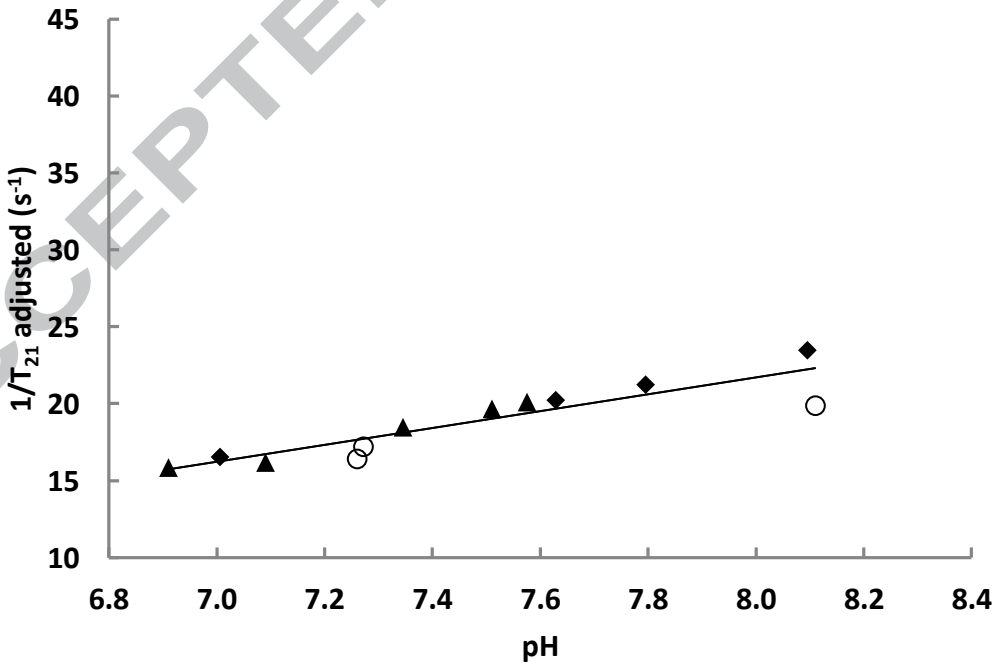

Figure 5

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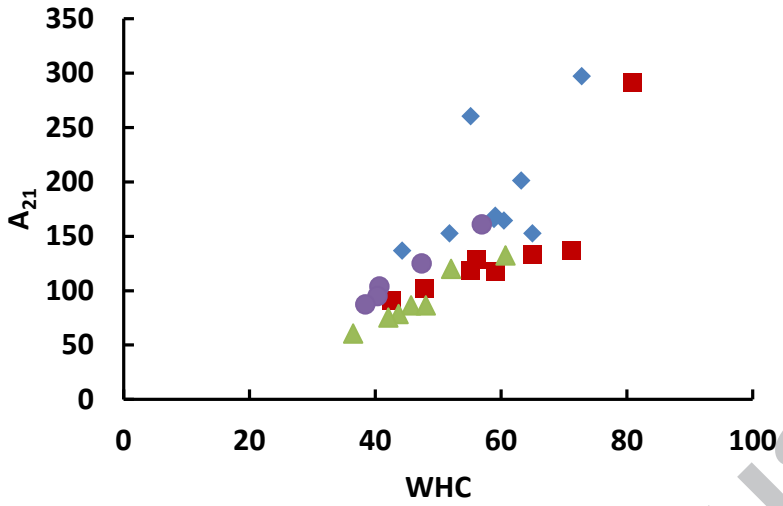

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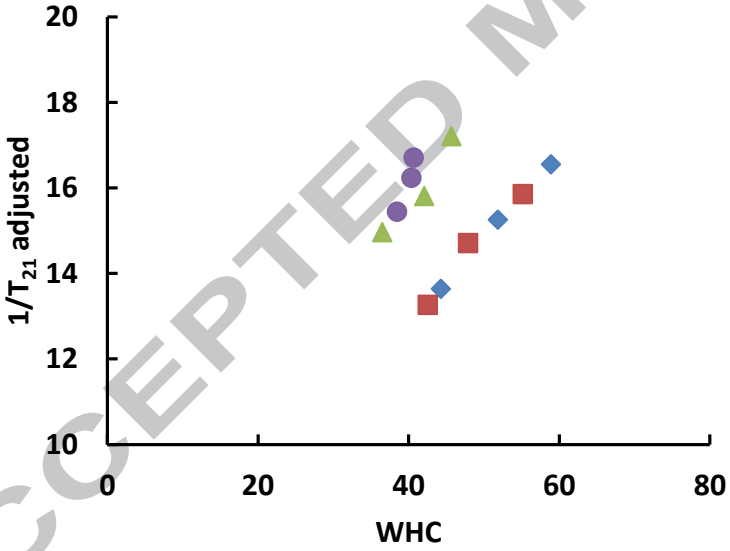

Figure 6

Highlights

- Relaxation rate of the major peak ($1/T_{21}$) increased upon freezing or pH increase
- $1/T_{21}$ is linearly related with protein concentration at constant pH
- $1/T_{21}$ is linearly related with pH at constant protein concentration
- Chemical exchange is responsible for changes with pH and protein concentration
- T_2 & water holding capacity correlated when each factor was analysed separately