Mechanisms of Transverse Relaxation of Water in Muscle Tissue

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ABSTRACT:

Nuclear magnetic resonance (NMR), and in particular transverse relaxation (T_2), has been used to characterize meat and seafood products for decades. Despite many years of research, it is still not possible to reproducibly correlate the transverse relaxation of muscle foods to attributes that determine their quality and value. Instead of directly trying to interpret the T_2 spectrum itself, typically chemometrics is used to try to relate the relaxation distributions to other measured properties on the sample. As muscle tissue is a porous medium, it is tempting to use equations developed to analyze other porous systems to provide a more direct, quantitative description of the tissue. However, the standard equations used to characterize porous materials have been developed for predominantly geological systems. This article discusses the foundations of transverse relaxation theory in porous media and the challenges that arise when attempting to adapt the equations to a biological system like tissue.

One of the biggest issues that needs to be overcome before porous media theory can be reliably applied to characterize meat and seafood is to determine the source of relaxivity in the tissue. In order to better understand how the NMR signal originates, T_2, diffusion, T_1-T_2 correlation and T_2-T_2 exchange experiments were performed on Atlantic cod (*Gadus morhua*) tissue in a variety of states (e.g. fresh, thawed, homogenized, etc.). In the literature, typically four T_2 peaks are reported for meat and seafood samples. Results of this study

indicate that the fastest relaxation peak is attributable to hydrogen within the protein itself and therefore arises from dipolar coupling. The T_{2B} peak appears to belong to a type of bound water in protein called “buried water”, and its relaxation stems from a combination of restricted motion and interaction with the hydrogen in the protein. For the T_{21} peak, attributed to fluid in myofibrils, the main relaxation mechanism is the interaction between water molecules and the hydrogen in myosin/actin matrix. The T_{22} peak arises predominantly from the interaction of water with dissolved protein in the sarcoplasm. An important finding from the study is the need to include both surface sinks and volume sinks in the interpretation of T_2 relaxation results. Given these sources of the transverse relaxation in tissue, it is highly likely that changes to the T_2 distribution that have been attributed to microstructural changes in the tissue are in reality due to a combination of changes in microstructure, surface relaxation and fluid properties. These findings aid in better interpreting T_2 measurements in meat and seafood products and present a step towards a systematic approach for using transverse relaxation to quantitatively describe changes in tissue, with the ultimate aim of eventually predicting product quality and value from NMR relaxometry.

1. INTRODUCTION

1.1. NMR of Seafood and Meat

Nuclear magnetic resonance has been used for decades to characterize tissue food products such as meat and fish. The method has the advantages of being non-invasive, functions on opaque samples and can be performed on relatively large samples. While some work has been performed using magnetic resonance imaging, the majority of the research has been done using transverse (T_2) relaxation. This stems from the fact the Carr-Purcell-Meiboom-Gill (CPMG) (Carr and Purcell, 1954; Meiboom and Gill, 1958) method typically

used to measure T_2 relaxation is quick, stable, and can be performed reliably with relatively inexpensive low-field NMR systems like benchtop or one-sided magnets. This makes the method well suited for industrial applications, where equipment cost and throughput are of paramount importance.

T_2 relaxation has been used by numerous researchers to observe changes in tissue with various types of processing: freezing (Jepsen et al., 1999; Jensen et al., 2002; Sanchez-Alonso et al., 2014; Xu et al., 2018), salting (Wu et al., 2006; Aursand et al., 2008; Gudjonsdottir et al., 2011; McDonnell et al., 2013, Gudjonsdottir et al., 2015), smoking (Hullberg and Bertram, 2005; Løje et al., 2007), etc. When peak shifts are seen in the T_2 distributions, this is typically attributed to structural changes, such as swelling of tissue or cellular damage. The analysis of the T_2 distributions usually involves qualitative descriptions of shifts in peak areas, locations, and shapes. In order to gain useful information from the NMR measurements, researchers have relied on using chemometric methods (Bechmann et al., 1999; Jepsen et al., 1999; Jensen et al., 2002; Bertram et al., 2003; Gudjonsdottir et al., 2011; McDonnell et al., 2013; Gudjonsdottir et al., 2019) like principal component analysis (PCA), multiple linear regression (MLR) or partial least squares (PLS) to relate the T_2 results to other laboratory measurements.

However, using chemometrics to correlate T_2 relaxation to sample quality attributes suffers from reproducibility problems (Zhu et al., 2017). For example, using the various multivariate analysis methods, researchers are frequently able to obtain good correlations between T_2 results and water holding capacity (WHC), an attribute related to product yield and sensory properties of the sample. Unfortunately, the models appear to develop local calibrations that are not globally applicable. Although good results are obtained for a

particular set of samples, the developed models fail to work well on a new set of samples, limiting the usefulness of chemometric-derived T_2 relationships in an industrial setting.

While chemometrics is often criticized as being a black box, direct interpretation of the T_2 relaxation itself in tissue is not straightforward either. Meat and seafood are porous materials, where fluid interacts with a solid matrix. The interaction of the fluid with the porous structure serves to enhance the transverse relaxation rate, such that the T_2 distribution is frequently treated as reflecting the underlying porous structure (Song, 2013). However, much of the developed porous media analysis theory was developed for geological samples. Relaxation mechanisms in stone are generally well understood. For sandstones and carbonate rocks, relaxation occurs due to interaction of fluid molecules with paramagnetic impurities. The geological systems are assumed to be in the fast diffusion limit, where fluid molecules rapidly explore the pore space but exchange between domains is slow. Even on first glance, it is clear that many of the underlying principles relevant to rocks may not hold for tissue samples. Furthermore, several problems and ambiguities arise with the current interpretation of transverse relaxation in meat and seafood when the systems are interpreted as porous media. We seek in this paper to better understand the relaxation mechanisms in tissue food products like seafood and meat and the physical meaning of the peaks in the T_2 distribution.

1.2. Tissue Structure

Meat and seafood are composed of the skeletal muscle (Hill and Olson, 2012) of mammals, poultry, and fish, which is a multiscale porous medium. At the smallest scale, the tissue is made up of repeating elements called sarcomeres. These are composed of thick filaments, the protein myosin, and thin filaments, the protein actin. These filaments are arranged in a lattice pattern with liquid filling the space between them. This liquid,

sarcoplasm, is predominantly water (80-85%), dissolved protein (10-15%) and small amounts of various salts and biomolecules. At the next scale, myofibrils are made up of the sarcomeres. Each muscle cell, or fiber, is made up of numerous myofibrils. This package of myofibrils is surrounded by the sarcolemma, which is the plasma membrane of the cell, a lipid bilayer, and a thin layer of polysaccharide, gycocalyx. A bundle of muscle fibers is called a fascicle, which is surrounded by the perimysium, a connective tissue made up of several types of collagen and elastic fibers. Within the fascicle, the muscle fibers are surrounded by endomysium, a thin layer of connective tissue. The fascicle itself is surrounded by a connective tissue called the perimysium. Finally, at the largest scale, the whole muscle itself is composed of multiple fascicles and is surrounded by the epimysium, another type of connective tissue. Blood vessels run between the fascicles to supply oxygen and nutrients to the cells.

1.3. Porous Media Theory Background

When a transverse relaxation measurement is made, the signal is a summation of all the exponential decays resulting from different T_2 relaxation times present:

$$M(t) = \sum_i A_i e^{-t/T_{2i}} \quad [1]$$

where M is the measured signal at time t, and A_i is the amplitude of the i^{th} T_2 time. The ubiquitous equation relating transverse relaxation time to porous media structure is:

$$\frac{1}{T_2} = \rho_2 \frac{S}{V} \quad [2]$$

Where S is the surface area, V is the fluid volume, and ρ_2 is the surface relaxivity, or how efficient the surface is in enhancing relaxation of the saturating fluid. Then assuming a constant ρ_2 and a homogenous fluid saturation, the distribution of pore sizes in the system can be obtained from the distribution of T_2 times present.

Underneath these tidy equations lie numerous simplifications. Equation 2 originates from the work of Korringa, Seevers and Torrey (KST) (Korringa et al., 1962), who first investigated relaxation of fluid at surfaces in a porous medium. Three relaxation processes were identified. One, T_{1B}, was the longitudinal relaxation rate of the saturating fluid itself. The other two were surface processes: T_{1S}, which is the surface relaxation that occurs at all sites of the surface, and T_{1M}, which is the surface relaxation that occurs due to interaction with small numbers of paramagnetic impurities present on the pore surface. The equations laid out by KST provided a very general equation framework for longitudinal relaxation in a porous media. Based on experimental observations on water in rocks, the equations could be simplified to disregard the contributions of T_{1B} and T_{1S}, producing the equation for longitudinal relaxation:

$$\frac{1}{T_1} = \left(\frac{S \times h}{V}\right) \frac{n_M}{T_{1M} + \tau_M} \quad [3]$$

Where h is the thickness of the surface layer of fluid, n_M is the number of relaxation sites, T_{1M} is the longitudinal relaxation rate at the relaxation site and τ_M is the residence time of the fluid molecule at the relaxation site.

Brownstein and Tarr later built upon this theory to include transverse relaxation (Brownstein and Tarr, 1979). However, they only looked at the effect of surface relaxation, stating its source was beyond the scope of the paper. Brownstein and Tarr combined the effects of number of relaxation sites, correlation time and relaxation rate into a single constant *M* (now commonly μ) to produce the general equation for both T_1 and T_2:

$$\frac{1}{T_i} = M_i \frac{S}{V} \quad [4]$$

and solved for three simple geometries: the sphere, the cylinder and the plane. From this, they were able to accurately estimate the diameter of a rat muscle cell. Importantly,

Brownstein and Tarr also coined the terminology of the “fast diffusion regime” and the “slow diffusion regime”, based on the work of Zimmerman and Brittin (Zimmerman and Brittin, 1957). In the fast diffusion regime, relaxation is slow compared to diffusion throughout the pore space, such that a single pore produces a single relaxation time. In the slow diffusion regime, relaxation is fast compared to diffusion throughout the pore space, such that multiple relaxation times may arise in a single pore.

Kleinberg and Horsfield continued to investigate the source surface relaxivity of transverse relaxation in geological materials (Kleinberg and Horsfield, 1990). One of the additional challenges with transverse relaxation is that diffusion of spin-bearing molecules through magnetic gradients will lead to additional dephasing of the transverse relaxation, enhancing the relaxation rate. Kleinberg and Horsfield found for short echo spacings and low magnetic field strengths, the influence of internal gradients could be neglected. In Kleinberg, Kenyon and Mitra, they returned to the KST theory (Kleinberg et al., 1994). As relaxation rate in rocks was found to be independent of temperature, this indicated the source of relaxivity was paramagnetic. As the exchange rate of fluid at the surface is fast compared to the surface relaxation, the residence time could be neglected, and the equation simplified to:

$$\frac{1}{T_2} = \left(\frac{S \times h}{V}\right)\left(\frac{n_M}{T_P}\right) \quad [5]$$

Where T_p is the relaxation rate due to dilute paramagnetic impurities on the pore surfaces.

To investigate relaxometry in seafood and meat, we begin with no assumptions about the sources of relaxivity or the terms that can be discarded and start by combining Brownstein and Tarr with KST theory. While Brownstein and Tarr did not consider them in analysis of their rat cells, they formulated the theory of volume sinks in their paper. As the sarcoplasm contains dissolved proteins that enhance the relaxation rate of water, we

anticipate this to a be a relevant term, such that we include it in our investigation. This gives the equation

$$\frac{1}{T_2} = \frac{\rho_2 S + \Gamma_2 \upsilon}{V} \quad [6]$$

Where Γ_2 is average value of the volume strength density over the active volume, υ. Adding in KST to expand the surface relaxivity term, the full equation for consideration is:

$$\frac{1}{T_2} = \sum_i \left(\frac{S \times h}{V}\right) \frac{n_m}{T_{2m} + \tau_m} + \frac{\Gamma_2 \upsilon}{V} \quad [7]$$

where T_{2m} is the longitudinal relaxation rate at the relaxation site. As multiple relaxation mechanisms may be present in a system, the effect on T_2 is summed over each of the *i* relaxation mechanisms relevant for the sample. Therefore, in order to accurately interpret T_2 measurements in seafood and meat, information regarding the source of relaxivity in the tissue is needed in order to determine values for the terms n_m, T_{2m}, τ_m for each surface relaxation mechanism and Γ_2 for the volume sinks.

1.4. Relaxation Mechanisms of Tissue Background

Although research into relaxivity mechanisms of meat and seafood in food science has been limited (Bertram et al., 2007; Wu et al., 2007; Gudjonsdottir et al., 2015), researchers in other areas have done extensive research into sources of relaxation in tissue. As early as the 1960's, researchers started to look at the effect of proteins on the relaxation rate of water. Brey and colleagues (Brey et al., 1968) studied water in lysozyme and bovine serum albumin solutions and speculated that the relaxation behaviour may stem from the movement of water molecules relaxing at the surface of the protein to the bulk fluid. Through the years, many other researchers have performed studies that support the theory that the interaction between the water and proteins is the source of relaxation in muscle tissue (Koenig et al., 1975; Fung and Puon, 1981). However, the exact interaction mechanism

for relaxation has been hotly debated. Three possible mechanisms exist. One is an increased relaxation rate due to restricted molecular tumbling of the water molecules due to their interaction with surfaces. Another is through dipolar coupling between the adsorbed water molecule and protons in the protein matrix. The last is through chemical exchange of hydrogen between the protein and water molecules. These mechanisms would serve to increase the relaxation rate of the hydrogen in water molecules at the protein surface, and then mixing of the water molecules with the bulk fluid would lead to an overall decrease in the relaxation rate of the saturating fluid. Reviewing the literature, one finds often contradictory studies on which mechanism seems to be responsible. Fung and Puon found that pH had a significant effect on transverse relaxation time (Fung and Puon, 1981). As the hydrogen exchange rate between protein and water molecules is pH dependent, they concluded that chemical exchange between the two constituents was responsible for the enhanced relaxation time of the fluid. On the other hand, Koenig, Hallenga and Shporer found that the spin-lattice dispersion behaviour of ^{1}H, ^{2}H and ^{17}O in protein solutions to be virtually identical (Koenig et al., 1975), suggesting that relaxation was due to the interaction of the entire water molecule, not just exchanging of protons. In reality, one would expect a combination of both effects to contribute to the relaxation and the relative strengths of the two to depend on the structure and chemistry of the protein system under investigation.

Another possible source of relaxation in tissue is interaction of the fluid molecules with paramagnetic molecules. Reviewing the literature, no studies were found where metallic paramagnetic ions were indicated as a possible source of relaxation in muscle tissue. For foods classed as “white meat”, it is not expected that paramagnetic ions would have a large influence on the relaxation rate. However, it is possible that for a poorly bled animal after slaughter, significant remaining blood in the tissue could affect relaxation rates, particularly

as the hemoglobin transitions from oxyhemoglobin, a diamagnetic molecule, to deoxyhemoglobin, a paramagnetic molecule. For “red meats”, although they are rich in the iron-bearing protein myoglobin, it is a diamagnetic molecule. Another scenario of where paramagnetic relaxation would be a potential relaxation mechanism is dissolved O^{17}, which would act as a volume sink for the water molecules in the tissue (Swift and Connick, 1962, Fung and McGaughy, 1979). After slaughter, the oxygen level in the tissue begins to fall, as it is no longer being replenished by the blood. It is the falling level of oxygen in the tissue that sets about the rigor mortis process in the muscle and studies of pre- and post-rigor fish have shown a shift to longer T_2 relaxation times (Aursand et al., 2009). However, it is not possible from these studies to isolate the effect of the change in tissue oxygenation from other structural or chemical changes that occur during rigor. Reviewing the literature did not produce any results on the influence of oxygen levels on relaxation time in food science, but we anticipate that the influence of tissue oxygenation on transverse relaxation to be relatively small.

1.5. Correlation time of Water in Tissue

In addition to relaxation mechanisms, significant research into the correlation times of water with macromolecules, both in model systems of protein and in muscle tissue itself has also been performed. Knipsel, Thompson and Pintar used the spin-lattice dispersion measurements in mouse tissue (Knipsel et al., 1974) to find that the correlation time for rotation of water molecules and exchange of protons to be approximately 20 nanoseconds and 10 microseconds respectively. Packer performed similar measurements using systems of hydrated protein powders (Packer, 1977) and found that the protein caused anisotropic tumbling of nearby water molecules with a correlation time on the order of a nanosecond, with residence time on the order of a microsecond. He also found the influence of the

protein on water only seemed to extend one or two water molecules away, such that the majority of fluid in the tissue would experience bulk behaviour. Research by Koenig and Brown had a similar conclusion, that only water in direct contact with solids are affected by their presence (Koenig and Brown, 1985). As they describe it, “solvent molecules only learn about the presence of stationary surfaces when they bump into them”. Similarly, a study by Cleveland and colleagues (Cleveland et al., 1976) found that obstruction by the myosin/actin network could only account for approximately 15% of the decrease of the measured diffusion coefficient of water in rat skeletal muscle compared to bulk water, such that the majority of the restriction must arise from another mechanism.

1.6. Transverse Relaxation Mechanisms and Food Science

The turn of the century ushered in a new era for NMR research in food science. Two important developments occurred during the 90’s. One was the widespread availability of benchtop NMR spectrometers (Blumich, 2019). Previously, NMR systems had been either limited to homebuilt systems or large, expensive superconducting magnets which required significant capital, space and upkeep. With the development of permanent magnet-based equipment, NMR was now cheaper, easier to maintain, and possible to fit in a regular lab, making it accessible to a wider range of researchers. Similarly, industry began to seriously consider NMR for quality control once benchtop systems became commonly available, as high-field systems were not seen as viable option due to their cost and upkeep requirements. The second was improvements in computing power. Before, analysis of transverse relaxation was limited to fitting a limited number of decaying exponentials to the relaxation decay curves. As computers became more powerful, numerical inverse Laplace transforms could be routinely performed on the data (Kroeker and Henkelman, 1986; Menon and Allen, 1991). This enabled many decaying exponentials to be fitted to the data,

creating smooth T_2 curves. Now, not only could the average time of a T_2 peak be determined, but information about the width and shape of the peak could be obtained as well.

These advancements enabled food scientists to use NMR to study an unprecedented number of systems, studying how different processing and handling effected the T_2 distributions of seafood and meat. For T_2 relaxation of fresh meat and fish, the transverse relaxation spectrum of fish and meat typically consists of three characteristic peaks (T_{2B}, T_{21}, T_{22}). A fourth peak at very fast relaxation times is seen if short enough echo spacings are used. There has been some dispute as to the source of the different relaxation populations, but in recent years, a general consensus has arisen in food science as to the constituents believed to be associated with each peak (Bertram et al., 2001). The current interpretation of the different peaks is as follows: the first peak (T_{2b}) is generally on the order of a few milliseconds and is ascribed to bound water. The second peak (T_{21}) is in the range of 30-70ms and is ascribed to intra-myofibrillar water. The third peak (T_{22}) is on the order of a 100-300 ms and is ascribed to extra-myofibrillar water. As previously mentioned, if a short enough echo spacing is used for the CPMG, an additional fourth peak is seen on the order of a few hundred microseconds. This has been attributed to the protons in the protein molecules itself (Venturi et al., 2007).

Although much effort has been put into understanding the origin of the different peaks in the T_2 spectrum of water in meat and seafood, the relaxation mechanisms have not been comprehensively investigated in depth. Typically, the observed relaxivity associated with each peak is simply ascribed to the mobility of the water, the T_{2B} peak being described as water that is most restricted and the T_{22} peak the least restricted. Given the limited influence of tissue on the mobility of water found by previous researchers, this description

seems inadequate to explain the T_2 results found by food science researchers in meat and seafood. In this study, we seek to better understand the different relaxation mechanisms present in tissue-based foods. We achieve this by applying a range of different NMR measurements (T_2, Diffusion, T_1-T_2, T_2-T_2 exchange) to cod that has been treated in a variety of ways. From these results, we are able to infer the underlying mechanisms of relaxivity.

2. EXPERIMENTAL

2.1. NMR System

NMR measurements were performed on a SpinSolve Benchtop Spectrometer (Magritek, Aachen, Germany). The system operates at 43 MHz and is equipped with 160 mT/m diffusion gradients. Samples are placed in 5mm tubes for measurement. The system operates at approximately 25°C, though temperature control of the sample is not maintained.

2.2. NMR Measurements

2.2.1. Transverse Relaxation Measurement

T_2 measurements were performed using the standard CPMG pulse sequence. Pulse length was 30 μs and, unless noted otherwise, echo spacing was 80 ?s. A total of 20000 echoes were used with a relaxation delay of 10 seconds after each measurement. Measurement time was approximately 45 seconds.

2.2.2. Diffusion measurements

Diffusion measurements were performed using a standard pulsed field gradients spin echo (PGSE) measurement (Stejskal and Tanner, 1965). Small delta was 3 ms and large delta was 25ms. Gradients strength was 160 mT/m and were ramped in 10 steps with a 0.1ms stabilization delay.

2.2.3. T_1-T_2 correlation experiments

The T_1-T_2 correlation experiment (Song et al., 2002) can be used to identify liquid-like and solid-like components in a sample. This ability comes from Bloemberg-Pound-Purcell (BPP) theory (Bloembergen et al., 1948). Liquids tend to have similar T_1 and T_2 values, whereas solids tend to have long T_1 and short T_2. T_1-T_2 correlations were run using a combined Inversion Recovery-CPMG sequence. T_1 wait values ranged from 0.1 ms to 3 seconds in 32 steps. CPMG parameters were the same as for the one-dimensional T_2 measurement.

2.2.4. T_2-T_2 exchange experiments

The T_2-T_2 exchange measurement, or REXSY, is used to observe exchange in a system (Washburn and Callaghan, 2006; Monteilhet et al., 2006). The measurement begins with an initial encode for T_2, followed by a mixing period where the magnetization is stored along the z-axis. During the mixing period, the system does not experience T_2 relaxation, only T_1. At the end of the mixing period, the magnetization is returned to the transverse plane and a second T_2 encode is performed. The data is then inverted using a 2D inverse Laplace transform. Signal that appears along the diagonal is from spins that have remained in their original T_2 environment between the first and second T_2 encodes. Signal that occurs on the off-diagonal arises from spins that have changed T_2 environment during the mixing period. Off-diagonal peaks can indicate both molecular exchange and exchange of magnetization through spin diffusion. The T_2-T_2 exchange experiment was performed using the pulse sequence presented in Washburn and Callaghan. A total of 50 initial encode values were used. A long relaxation delay of 10s was used in between each measurement in order to minimize heating of the sample. Other parameters were the same as the 1D CPMG.

While the T_2-T_2 exchange experiment is a powerful tool for observing exchange of magnetization in a system, caution must be taken in interpretation of the results. Multisite

exchange can lead to asymmetries and peak shifts in the plot (Van Landeghen et al., 2010; Gao and Blumich, 2020). For long mixing times, T_1 weighting of the signal can cause the signal along the axis of the first encode to be shifted to longer T_2 relaxation times. Furthermore, as exchange peaks evolve, the off-diagonal peak frequently "buds" off the diagonal peak. This can lead to a diagonal peak shifting away from the diagonal before the off-diagonal signal is distinct enough to be resolved by the inversion at a longer mixing time.

2.3. Data Analysis

Analysis of the NMR data was performed using the accompanying system software, Prospa. Both one and two-dimensional inverse Laplace transforms were performed using a Non-Negative Least Squares algorithm (Lawson and Hanson, 1987). Regularisation of the inversions was determined by the l-curve method (Hansen, 2000), where the regularization term is selected to minimize the sum of the residuals and no further.

2.4. Samples

Atlantic cod fish (*Gadus morhua*) were received from the Tromsø Aquaculture Research Station, Norway. The fish were killed by a blow to the head and immediately gutted. They were bled for 30 mins, iced and transported to Nofima, where they were kept on ice for 4 days to ensure that the fish were out of rigor prior to filleting.

2.4.1. Fresh state samples

For fresh state measurements, small subsamples were taken from fillets once they were out of rigor and placed inside 5mm tubes for measurement. For chilled measurements, samples were placed in a 4-degree cold room and allowed to equilibrate overnight, then immediately placed in the NMR machine for measurement. Otherwise, samples were allowed to reach ambient temperature before measurement.

2.4.2. Frozen samples

In order to induce tissue damage in the samples, several subsections of tissue were taken, placed in 5mm tubes and placed into a -5 °C freezer. Freezing at this warm of temperature has been shown to cause severe tissue damage due to the formation of large ice crystals that tear the tissue (Powrie, 1984). In order to observe sample changes upon thawing, samples were placed immediately into the NMR equipment for T_2 measurement. All other measurements on the samples were performed after they had warmed up to ambient temperature. Measurements were performed in triplicate.

2.4.3. Homogenized samples

Homogenization of samples serves to disrupt the muscle cell structure. The process tears apart the cell membranes and disrupts the myofibrillar network. Samples of fresh state fillet were macerated with a chilled mortar and pestle and immediately transferred to 5mm test tubes for T_2 measurement to observe the evolution of the T_2. All other measurements in the homogenized state were performed after the samples had come to equilibrium overnight. Measurements were performed in triplicate.

2.4.4. Freeze-dried samples

Thin slices of fillet were placed in a -80 °C freezer. Once frozen, the samples were freeze-dried using Freezone 12Plus freeze dryer (LabConco, Kansas City, USA) at 0.04 mBar. Samples remained in the freeze drier for 60 hours and were then removed and stored in a tightly sealed container until use.

2.4.5. Drip loss

Four different drip loss samples were taken. One was sampled from the container holding the fresh fillets. A second sampling was taken from the container after 14 days of storage. In addition, drip loss was taken from samples that had been stored at -20 °C and -40 °C and then thawed.

3. RESULTS

3.1. T2 relaxation of fresh state tissue

Figure 1 shows an example T_2 relaxation distribution for a sample of the fresh state cod. We see the standard four distinct peaks typically seen in the T_2 distribution of tissue. The terms T_{2B}, T_{21} and T_{22} are commonly used in literature. The fastest-relaxing peak does not have a standard nomenclature. For ease of discussion, we refer to it as T_{2S}, as it is assumed to arise from hydrogen in the solid protein. A simple experiment to test for internal gradients or exchange is repeating the CPMG measurement with different echo spacings. Hills and colleagues (Hill et al., 1989; Hill et al., 1990; Hills et al., 1991) recorded dispersion in the T_2 relaxation with changing echo spacing in a variety of different foods, indicating exchange on the time scale of the CPMG measurement. Changing the echo spacing produced no appreciable difference in the T_{21} and T_{22} peaks.

For the T_{2S} and T_{2B} peaks, notable differences were observed. The two peaks began to merge towards one another with increased echo spacing. T_{2B} also decreased in intensity, suggesting it may also be exchanging with the other two peaks, but its influence is too weak on the stronger peaks to be resolved by the inverse Laplace transform. The T_{2S} and T_{2B} peaks finally merging into a single peak when the echo spacing was increased to 400 us, indicating significant exchange between the two peaks on the time scale of the CPMG measurement. While the shift in peaks with echo spacing is similar to the effect seen when spin-bearing molecules diffuse through internal gradients, given the two constituents are believed to be associated with immobilized hydrogen, we do not expect this is the situation here.

3.2. T_2 relaxation of homogenized tissue

The relaxation distribution of homogenized tissue changed as a function of time, shown in Figure 3. These changes are summarized in Figure 4.

Upon initial measurement, the T_{22} peak had a very low intensity, approximately 1% of the total signal. As the sample was allowed to sit, signal intensity shifted from the T_{21} peak into the T_{22}. At the same time, the maximum amplitude of the T_{22} peak shifted toward the T_{21} peak while the center of T_{21} peak slowly shifted to shorter relaxation times. Over time, as much as 15% of the signal intensity shifted from the T_{21} to T_{22} peak. Though the behaviour was consistent between samples, the exact amount varied appreciably between samples. This suggests that upon damage to the cell membranes and myofibrils, liquid leaks from the myofibrils into the surrounding tissue.

3.3. T2 relaxation of thawed tissue

As with the homogenized tissue, the T_2 distribution of the thawed samples changed with time, shown in Figure 5. Initial measurement directly from the freezer showed a distribution that looked similar to the fresh state. Curiously, in contrast to the simply chilled sample, shifts were seen to shorter relaxation times for the T_{2S} peak as well. As the sample warmed up, the location of T_{2S} returned to its location in the fresh state samples and the T_{21} peak broadened and intensity transferred over to the T_{22} peak. This initially produced two separate peaks. As the sample was allowed to equilibrate, the two peaks appeared to merge together, producing a single peak with a shoulder. We believe that upon thawing, because of damage to the cell membrane, internal cell pressure causes sarcoplasm to flood from the myofibrils into the surrounding tissue. Given time, the system comes to equilibrium.

3.4. T2 relaxation of chilled tissue

Cooling the fresh state sample did not produce a shift in the T_{2S} peak (Figure 6). This suggests that for this peak, residence time τ_m is not a factor in relaxivity. For T_{2B}, a slight shift to shorter relaxation times is seen. This is in line with increased relaxation due to slower

molecular rotation. In contrast, there was a slight shift to a longer relaxation time at low temperature in the T_{21} and T_{22} peaks, indicating that residence time is a factor. Cooling down the sample slows the exchange between bulk water and bound water, such that there is less interaction with the surface, leading to longer relaxation times. Note, because temperature affects the NMR signal intensity, it is not possible to make definitive statements regarding the changes in signal intensity between measurements.

T_2 results for chilled thawed and homogenized tissue are shown in Figures 7 and 8 respectively. Both treatments produced different results compared with the fresh state. As with the fresh state, the T_{2S}, T_{2B} and T_{21} peaks remained relatively unchanged. However, the T_{22} peak shifted to much shorter relaxation times, in some cases practically merging with the T_{21} peak.

3.5. T_2 relaxation of freeze-dried tissue

The T_2 relaxation of the freeze-dried tissue shows signal predominantly in the T_{2S} peak, though weak peaks are seen at longer relaxation times (Figure 9). This is expected, as the freeze-drying process will not completely remove liquid water from the sample. We believe that for the freeze-dried sample, the T_{2B} signal overlaps with T_{2S} signal. The freeze-drying process does not remove all the bound water in the protein (Takano et al., 2005), but the motion of the water will be hampered by the drying process. The restriction of motion will serve to speed up the relaxation rate of the remaining water molecules.

3.6. T2 relaxation of drip loss

Figure 10 shows the T_2 distribution for different drip loss samples. We note a range of different relaxation times, depending on the sample. Visually, the drip loss from the sample stored 14 days and the -40 °C sample were more opaque, indicating either a higher protein concentration or larger proteins and both samples showed shorter T_2 relaxation times. This

is in line with established theory (Koenig et al., 1978), where cross relaxation between the water and dissolved protein leads to a single, averaged relaxation rate dependent on protein size and concentration (Hallenga and Koenig, 1976). For all the drip loss samples, the values obtained are in the time range typically observed for the T_{22} peak in tissue. Measurement of drip loss in the chilled state (Figure 11) produced a shift from in the peak towards a shorter relaxation time. We believe the shift arises from the slower rotational tumbling of the protein molecules, such that when water molecules interact with the protein, this leads to a faster relaxation rate.

3.7. T_1-T_2 Correlation

Figure 12 shows the T_1-T_2 results. The T_1-T_2 correlation plot of the fresh state samples showed very little variation in the T_1 values, all clustered between 300 and 500 ms. The T_{2S} and T_{2B} had very high T_1/T_2 ratios, 5623 and 416 respectively, indicating restricted rotation and suggesting that they stem from hydrogen in solids. In contrast, T_{21} and T_{22} had T_1/T_2 ratios of 8 and 2.5, indicating relatively free rotation of the molecules and suggesting liquid components. For the freeze-dried sample, similar ratios are observed, indicating the long T_1 times for T_{2S} and T_{2B} are not simply the product of efficient exchange with the liquid components during the long T_1 wait time. The majority of signal appears to come from solid constituents. A weak peak with a low T_1/T_2 ratio (~4) at a T_2 of 100 us indicates there is still a small amount surface water present in the system.

3.8. T_2-T_2 exchange of fresh state tissue

For fresh state samples, at the shortest mixing time, exchange was already seen between the T_{2S} and the T_{21} peaks. There is also an exchange peak between T_{2S} and T_{2B} that is skewed towards the location of the diagonal T_{2S} peak, suggesting there is some residual influence of the T_{2S} diagonal peak that cannot be resolved by the inverse Laplace transform. No exchange

peaks with T_{2S} are seen in the first encode axis, only the second encode axis. This is due to the T_1 and T_2 relaxation that occurs during the encode process, such that this very short component is lost by the time the signal is acquired. Exchange between the T_{2B} and the T_{21} peaks starts to arise on the order of 1 ms. This is first seen as a shift in the diagonal T_{2B} peak, followed by the emergence of a separate exchange peak between T_{2B} and T_{21} at around 5 ms. No exchange was seen between the T_{21} and T_{22} peaks at short exchange times. At longer mixing times, from approximately 50 ms and onward, the beginnings of exchange is seen between the two peaks. This is in line with results of Sobel and colleagues (Sobel et al., 1986) who estimated a 29 s^{-1} exchange rate between intra and extracellular fluid. Interestingly, even at long mixing times, no exchange is seen between the T_{2S} and T_{22} peaks. This suggests that interaction with the myosin/actin protein matrix is not the source of relaxation for that T_2 component.

3.9. T_2-T_2 exchange of homogenized tissue

Subtle changes in the exchange plots occur in the homogenized samples. At the shortest mixing times, the exchange peaks with the T_{2S} peak have shifted from clearly associated to T_{21} to halfway between T_{21} and T_{22}. This typically arises when both cross-peaks are present, but the two populations do not have enough intensity to be resolved separately by the inverse Laplace transform. Interestingly, exchange appears to be more rapid between the T_{2B} and T_{21} peaks in the homogenized samples even though the peak location of T_{2B} is not altered from the intact samples. Weak exchange is already evident between T_{2B} and T_{21} at 100 us. At 500 us, two distinct exchange peaks with T_{2S} form for both T_{21} and T_{22}. This indicates that the T_{22} component is now interacting with hydrogen in solid proteins when the cell membrane has been damaged. However, still no exchange at short mixing times is seen between the T_{21} and T_{22} peaks, but cross peaks begin to form at shorter mixing times

499 (5-10 ms) than in the fresh state samples. Although it might seem surprising that there is not
500 more rapid exchange between the two environments when the cell membrane is disrupted,
501 the myofibrillar matrix is still tortuous, such that it takes some time for a significant amount
502 of water molecules to diffuse between it and the surrounding fluid. Another very curious
503 result is the appearance of the exchange peak between T_{2S} and T_{22} before there appears to
504 be significant exchange between the intra and extramyofibrillar water. Several possible
505 explanations exist. This simply could be an artefact of the inversion, where the weaker
506 exchange peaks between T_{21} and T_{22} are not able to be resolved from the nearby, much
507 stronger diagonal peaks. It may also be that the water in the extramyofibrillar spaces is able
508 to interact more with other sources of hydrogen in solids than myosin and actin lattice when
509 the structure is damaged.

510 **3.10. Diffusion measurements in tissue**

511 Diffusion measurements were made on the fresh state, the thawed state, and
512 homogenized tissue samples. Due to equipment limits, only the diffusion of T_{21} and T_{22} were
513 possible to observe. Single exponential decay was observed for all samples and little
514 variation was observed in the measured diffusion coefficients.

515 Frequently, the changes in the T_2 distribution of seafood and meat with processing are
516 referred to as changes in water mobility. However, this is not supported by the diffusion
517 results, which do not show a significant change in values between the fresh, frozen-thawed
518 or homogenized states. For the thawed samples, where the T_2 distributions sometimes
519 showed significant differences from each other, the diffusion coefficient remained close to
520 1.6×10^{-9} m^2/s. In particular, even when the T_2 spectrum showed strongly bimodal
521 behaviour (e.g. thawed tissue), the diffusion attenuation curve only showed a single
522 diffusion value. As the self-diffusion coefficient of pure water at 25°C is 2.299×10^{-9} m^2/s, only a

minor restriction of movement in the water molecules is observed. Therefore, it is unlikely that the changes in the T_2 distribution can be attributed to changes in mobility. This is supported by the results of Koenig and Brown (Koenig and Brown, 1985), who found that water molecules could diffuse almost uninhibited through the myofibrils when they were more than a few angstroms away from the surfaces, and Cleveland and colleagues (Cleveland et al., 1976) who found only a minor reduction in water mobility due to the myosin/actin lattice.

4. DISCUSSION

In order to use the T_2 distribution as a proxy for the microstructure in the system, the assumptions of a constant surface relaxivity and a homogeneous fluid saturation need to hold true. Based on the results of the experiments, we believe these conditions are only potentially met in the fresh state, and in particular shortly after slaughter. In the case of processing or storage of the tissue, the T_2 distribution is influenced by the interplay of microstructure, surface chemistry and fluid composition changes.

We identify multiple different sources of relaxation within the tissue. The T_{2S} peak appears to arise from hydrogen in the structural proteins. Therefore, its relaxation time will be controlled by the dipolar coupling that the spins experience due to their restricted motion. The T_{2B} peak shows more complicated behaviour. It has a high T_1/T_2 ratio, indicating solid like behaviour, but has an exchange rate with liquid like water on the order of a few hundred microseconds to a few milliseconds depending on the sample state. We believe that the T_{2B} peaks comes from protein bound water trapped within cavities of the protein, dubbed “buried water” (Levitt and Park, 1993). As buried water is considered structurally part of the protein, the source of relaxation comes from the restricted rotational motion of the molecule when it is integrated into the protein structure (Martini et al., 2013). Unlike

normal bound water, which has an exchange rate with the bulk of picoseconds, buried water is known to have a much slower exchange rate with the surrounding bulk, ranging from a few nano seconds up to tens of milliseconds (Levitt and Park, 1993).

An important point we seek to emphasize is that, despite the name, bound water is not stuck to the protein surface, but in constant exchange with the bulk fluid. Perusing the food science literature, many authors tend to describe "bound water" as a static entity that stays bound during the course of a measurement. This is not an accurate description because it is well established that bound water on proteins exchanges with the surrounding fluid on the order of pico- to nanoseconds (Otting et al., 1991; Otting et al., 1992). Therefore, the water molecules that are bound to the protein will change tens to hundreds of thousands of times during the space of the first echo of a CPMG measurement. This is why an average decay rate is seen for the bound protons exchanging with the bulk water.

The T_{21} peak appears to arise from water inside the muscle cells interacting with the myosin/actin lattice. One question in interpreting the results is whether it is the structure of the muscle cell or the underlying lattice of myosin and actin that influences the T_{21} peak. One possibility is that the myofibril lattice acts as numerous, small nanopores. The other is that the larger muscle cell is the structural unit and the myosin/actin lattice acts instead as surface sinks. Based on a review of the literature and the results of our measurements, we believe the second scenario is more likely. The diffusion coefficients measured here and reported in the literature are in line with a microporous structure, not a nanoporous one. The exchange rate between the T_{21} and T_{22} peaks is consistent with exchange between intracellular and extracellular water. Therefore for Equation 7, we believe it is not so much the cell surface to volume ratio that influences the transfer relaxation rate, as the volume of water relative to the amount of myosin and actin in the region. This would also explain why

571 disruption of the cell membranes and myofibrillar structure during homogenization and
572 freezing does not seem to have a dramatic impact on the T_{21} peak, as it is interaction of
573 water with the hydrogen in the myofibrillar proteins, not the lipids in the cell membrane nor
574 the structure of the myofibrils, that drives the relaxation process. This presents challenges
575 going forward for definitive interpretation of transverse relaxation, as many of the
576 processing steps that will alter the tissue microstructure will also affect the interaction of
577 water with the proteins. In fact, in many situations, the processing may influence the
578 myofibrils more than the sarcolemma. Changes to pH will affect the residence time, τ_m, of
579 water on the protein surfaces. Loss of the myofibrillar protein into drip loss will affect the
580 number of relaxation sites, n_m. Denaturation of the protein structure from storage, salting or
581 heating will affect the binding sites and therefore $T_{2m,}$ the relaxation rate of water at the
582 protein surface.
583 While it appears interaction of water molecules with the protein is the source of
584 relaxation, we are not able to say at this time the relative influence of chemical exchange
585 versus intermolecular dipolar coupling. More detailed study is needed to determine this and
586 we expect that the relative importance of the two interactions may vary depending on tissue
587 state. However, both types of interactions will affect the relaxation rate through $\tau_{m.}$
588 Therefore, changes that affect the chemical exchange rate or correlation time of water
589 molecules, such as pH or temperature, will influence the T_2 results. This is seen in the
590 lowered T_2 value of the T_{21} peak in the chilled and thawing samples. While investigated in
591 this study (data not shown), no anisotropy in the T_2 signal was observed depending on
592 orientation of the fibers with the applied magnetic field and no reported cases of T_2
593 anisotropy with muscle fiber direction were found in the literature. This suggests that
594 restricted motion of the water itself is not a significant relaxation mechanism in the tissue,

595 as the highly ordered structure of the tissue would be expected to show anisotropy if it
596 were.
597 Two different constituents appear to contribute to the T_{22} peak. For fresh samples, the
598 peak appears to be associated with extracellular water. The peak shifts to longer relaxation
599 times at lowered temperature indicates a surface relaxivity process. This would be consistent
600 with a weak interaction of water with the lipids of the sarcolemma, similar to what is seen in
601 water in oil emulsions. However, the leakage of sarcoplasm out of the muscle cells will also
602 produce a T_2 peak in this range of relaxation times. Cross-relaxation between the dissolved
603 protein and water molecules will lead to a relaxation value for the sarcoplasm that is lower
604 than that of bulk water (Koenig et al., 1978) Therefore, once damage starts to occur in the
605 tissue, it appears that the relaxation time of the sarcoplasm itself becomes the dominant
606 influence of T_{22}. We expect that the relaxation time of the sarcoplasm also influences the
607 relaxation time of the T_{21} peak, but its effect appears to be weaker than the presence of the
608 myofibrillar matrix.
609 We believe that this helps explain some of the discrepancies seen in the literature. In a
610 work by Cole and colleagues (Cole et al., 1993), it was observed that homogenization of the
611 frog muscle tissue with a mortar and pestle produced a single exponential decay. This led to
612 the conclusion that the relaxation must stem from intracellular and extracellular water. In
613 contrast, when Bertram et al. (Bertram et al., 2001) performed a similar experiment using
614 pork, they observed that the T_{22} peak remained relatively unchanged, indicating that the T_{21}
615 and T_{22} peaks belonged to intramyofibrillar and extramyofibrillar water. Based on our
616 results, it appears that both situations are possible and depend on sample handling. In the
617 fresh state, the T_{22} peak appears to stem from fluid in the extracellular space. However,
618 upon damage to the cell membranes, sarcoplasm begins to leak out of the cells into the

surrounding space. As the dissolved protein in the sarcoplasm acts as volume sinks, the relaxation time is on the order of a few hundreds of milliseconds, even without any surface interaction, and the apparent relaxation time of water is lowered from bulk.

Often in porous media research, the T_{2bulk} term is neglected as it is much longer than the other relaxation processes in the system of study and therefore does not have any appreciable effect on the measured signal. Furthermore, T_{2bulk} is frequently treated interchangeably with volume sinks. For example, in water that has been doped with copper sulfate, the lowered relaxation time is referred to as the "bulk" value for the water. Technically, the bulk relaxation time of the water is still approximately 3 seconds. However, given the uniform system and rapid, efficient interaction between the water molecules and paramagnetic ions, for all practical purposes, the water phase can be treated as a uniform fluid with a lower relaxation time. For geological systems, even for porous media with multiphase saturation, each fluid type is typically considered to be homogeneous. On the contrary, in a system like tissue, particularly once it begins to undergo changes and damage, a homogeneous saturating fluid cannot be assumed. When tissue is damaged either through processing or aging, biomolecules that can potentially act as relaxation agents will be lost into the sarcoplasm. At the same time, water is able to exchange more effectively from intramyofibrillar and extramyofibrillar spaces. For some types of processing, like brining, additional water may be taken into the tissue. Furthermore, damage and changes to tissue is non-uniform throughout the samples with processing. All this serves to produce compositional changes of the sarcoplasm throughout the tissue, allowing for complex mixing of the local environments.

Pulling these results together, a better understanding of how the transverse relaxation distribution changes with handling in tissue is revealed. For freshly slaughtered fish and

643 meat, narrow, distinct peaks indicate a uniform structure and fluid composition. The
644 broadening and shifts of peaks with processing indicate increased heterogeneity in the
645 system. The T_{21} peak can extend to lower relaxation times if fluid is lost from the myofibrillar
646 matrix, lowering the relative fluid volume compared to the surface sinks present. Similarly,
647 an increase in the dissolved protein in the sarcoplasm will also serve to lower the relaxation
648 time. Correspondingly, loss of myofibrillar protein through drip loss may shift the T_{21} peak to
649 longer relaxation times, as the number of relaxation sites are decreased. Damage to the
650 sarcolemma may increase the amount of fluid in contact with the myofibrils, also shifting the
651 T_{21} peak to longer times. Location of the T_{22} peak appears to be dominated by the protein
652 dissolved in the fluid. The exact relaxation time will be combination of both protein
653 concentration and average protein size. The shift of the T_{21} and T_{22} peaks towards each other
654 indicates increased interaction between fluid molecules in intra-myofibrillar and extra-
655 myofibrillar spaces. Understanding how these different mechanisms of relaxation influence
656 the measured T_2 enables a much more thorough description of the changes experienced by
657 the tissue than simply interpreting the changes as differences in water mobility. Frequently
658 in the food science literature, in order to simplify the analysis, the T_2 relaxation data analysis
659 of tissue is performed using a limited number of exponentials, which does not take
660 advantage of the full information present in the data. These results also underscore the
661 importance of using a full T_2 distribution from an inverse Laplace transform to characterize
662 samples, and not simply a bi or tri-exponential fitting to the data. Brown and colleagues
663 (Brown et al., 2000) had a similar finding, where T_2 results analyzed using an inverse Laplace
664 transform correlated better with wet lab properties than when the results were analyzed by
665 bi or tri-exponential fitting.

At the same time, the findings of this study indicate that, while not impossible, using the transverse relaxation alone to accurately characterize meat and seafood will be a significant challenge. Because so many aspects of the tissue structure and composition can influence the relaxation rate, determining the physical meaning behind a given T_2 distribution requires controlling many variables. This potentially could be done by calibration to other laboratory measurements, as is sometimes done with geological samples. The drawback here is that while relaxivity in geological samples is controlled by a single, stable variable, the paramagnetic impurity content, tissue has multiple variables that evolve with time. It was observed that even short storage times could influence the T_2 results as the tissue aged. The developed calibration would likely only be applicable for a short period of time and would need to be repeated after any sort of processing or aging.

Another possibility is to characterize the systems with more advanced NMR measurements. More complete information about sample state could be obtained from other two-dimensional types of inverse Laplace measurements, such as T_2-D (Hurlimann and Venkataramanan, 2002) which correlates transverse relaxation and diffusion or DDCOSY (Callaghan et al., 2003), which can give information regarding anisotropy in diffusion. Researchers have worked to develop rapid T_2-T_2 exchange methods (d'Eurydice et al., 2016), which may be useful for estimating tissue damage. Combined inverse Laplace and Fourier measurements, such as DOSY or spectrally resolved T_2, may also yield information on protein content in solution, helping to control for the effect of volume sinks on the relaxation. Advanced NMR measurements also become necessary to use T_2 relaxation in a broader range of food products. White fish and lean cuts of pork and poultry have low enough fat content that its contribution can be considered negligible. However, if fatty fish, such as salmon or mackerel, or fattier cuts of meat are examined, this can no longer be considered

690 true and fat will have an appreciable contribution to the measured T_2 signal. Although the
691 theories tested here should be applicable to both meat and seafood, measurements in this
692 paper were only tested on fish. Therefore, further testing is necessary to experimentally
693 confirm similar results in meat and shellfish. There is still much work to be done to fully
694 understand NMR relaxation in muscle tissue and the results presented here are only the
695 start in developing a framework to improve repeatability in these complex materials that are
696 highly sensitive to variations of sample preparation and heterogeneity.

697 **5. CONCLUSIONS**

698 The results of the study demonstrate the inherent complexity of using transverse
699 relaxation measurements in food science. The transverse relaxation is sensitive to a variety
700 of tissue properties that are related to quality attributes, such as tissue damage and protein
701 denaturation. The challenge is to disentangle the effects of the different relaxation
702 mechanisms from one another when interpreting a relaxation time distribution. The
703 mechanisms that control transverse surface and volume relaxivity arise from properties that
704 can vary greatly between samples and can easily be altered during processing or storage.
705 The plethora of effects that can influence the transverse relaxation rate indicates that one
706 dimensional T_2 measurements alone are likely insufficient to adequately characterize
707 attributes that affect quality of seafood and meat products. However, through careful
708 experimental design and application of more advanced NMR tools, we anticipate that
709 information about tissue changes can be obtained. Results highlight the influence of
710 dissolved protein in the sarcoplasm on relaxation time, underscoring the need for including
711 the effect of volume sinks in addition to surface relaxivity when interpreting results. The full
712 T_2 distribution should be used for data analysis, as information on the tissue state is
713 contained in not only the peak location and amplitude, but the shape of the curves as well.

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