Innovative Non-Destructive Measurements of Water Activity and the Content of Salts in Low-Salt Hake Minces

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Abstract

Impedance spectroscopy (IS), Low-field proton Nuclear Magnetic Resonance (LF ^{1}H NMR), chloride titration, ion chromatography and ion selective electrode were used to investigate physicochemical parameters and measure sodium and potassium contents in low salt brines and fish. Salt solutions (0-3 w/w,%) and model products of minced hake with added NaCl (0.5–3.0 w/w,%), or a mixture of NaCl and KCl (50/50 w/w,%) were analyzed. Good correlation was observed between the sodium content determined by ion selective electrode method and ion chromatography (R^2=0.97). In both salt solutions and fish minces, the impedance spectroscopy measurements could detect the difference in salt contents in mince with salt contents down to 0.5%. The NMR transversal relaxation time T_2 measurements clearly distinguished samples with 0, 0.5% and 1.0 - 3.0% salt, based on the principal component analysis (PCA). Therefore, LF ^{1}H NMR seems to be a suitable technique for studies of low-salt products.

Key words: Impedance spectroscopy, low sodium, low salt, low-field NMR, T_2 relaxation, hake

INTRODUCTION

A high consumption of sodium has been directly associated with a greater likelihood of increased blood pressure, which in turn has been directly related to the development of cardiovascular and renal diseases. [1] For these reasons, national and international bodies have set targets for a reduction in sodium consumption. [2] Salt is commonly employed in fish processing, because it helps increase shelf-life, reduce water activity (a_W) and it has an important effect on water holding capacity, fat binding, color, flavor, and texture. [3-5] Fish is used as a main ingredient in numerous seafood products, such as fish sausages, surimi and surimi-based products, like fish puddings.

The development of low-sodium fish products without affecting product quality and safety is of interest, especially considering the otherwise good nutritional characteristics of fish. The partial substitution of NaCl by KCl has shown to be one of the best alternatives for reducing sodium content. [6-8] Indeed, both salts have similar properties and the health effects of increased potassium intake are continuously evaluated by the international health authorities. [9-11] Replacement of NaCl by to high concentrations of KCl may have a negative influence on the flavor intensity and produce bitter tastes. [12]

Parameters such as a_w and salt content have important implications for product shelf-life and consumer safety. In this regard, the development of rapid, accurate, and non-destructive methods for monitoring, these parameters, independently of the sodium replacement, is of industrial interest as well as to determine the sodium content accurately in food. The increasing use of salt- replacers such as potassium chloride [8] makes it necessary to find new rapid techniques for determining the sodium content directly, since measuring the chloride content no longer represents the sodium content in the food.

Analytical methods for the determination of salt include flame atomic absorption spectrophotometry (FAAS), inductively coupled plasma/MS (ICP/MS), ionic chromatography, and sodium selective electrodes [13]. Other methods, such as Volhart method (AOAC method 971.27) [14] and potentiometric titration [15] measure the chloride contents and the sodium content is then calculated stoichiometrically.

To meet the objective of developing fast, non-destructive methods to monitor product quality as affected by sodium reduction, electronic sensors based on impedance spectroscopy (IS) may be an option. The relationship between sodium chloride content and impedance measurements has already been demonstrated. [16-19] In the IS technique, an electrical sinusoidal stimulus is applied to the electrodes to measure the impedance of the sample at different frequencies. The module and phase of the impedance can vary significantly according to the charges present (free ions), types of microstructure and electrolytes, as well as texture, geometry, and the electrodes used. [18] However, this technique has not yet been applied to food products in which sodium has been replaced by other cations.

The effect of salting can also be determined indirectly, for example by using low-field (LF) ^{1}H NMR to monitor changes in proton relaxation behavior as a result of salt addition. In foods, the NMR proton signals basically originate from small molecules like water and fat. Changes in tissue microstructure due to salting will affect proton exchange with the surrounding environment. For example, tissue swelling after the addition of salt leads to a more open microstructure causing higher water mobility. Several studies have been carried out where LF NMR has been used to monitor changes during fish salting processes. [20-26] However, since none of these studies have dealt with low-salt tissues, it would be of interest to explore the method further as a potential tool for low-salt applications.

The objectives of the present research are to (1) evaluate the application of impedance spectroscopy to monitor physicochemical parameters in salted fish products with, and without sodium replacement, (2) establish a fast and consistent method to measure sodium and potassium contents in fish products, and (3) assess the feasibility of employing LF NMR in low-salt tissues.

MATERIALS AND METHODS

Chemicals

Ammonium Chloride (NH_4Cl), Ammonium Hydroxide (NH_4OH), Ammonium Hydrogen Fluoride (NH_4FHF < 1%, LD_{50} mg/kg not found), Chloroform ($CHCl_3$), Ethanol (C_2H_5OH), Sulfuric acid (H_2SO_4), Potassium sulfate (K_2SO_4), Copper Sulfate ($CuSO_4$), Hydrogen Peroxide (H_2O_2) and Sodium hydroxide (NaOH) (Scharlau, S.A. or Thermo Fisher Scientific, USA). All the chemicals were of analytical-reagent grades.

Experimental protocol

Experiments using the impedance system were carried out in two phases. In the first phase, the system`s capability to distinguish between different types and quantities of salts was evaluated. The second phase evaluated the impedance system for discriminating between fish samples salted with different salt mixtures and quantities of salt.

Phase I: Salt solutions

Different brines were prepared by using NaCl, KCl, and a mixture of NaCl/KCl (50/50, w/w, %) at different contents. The total salt contents assessed were 0.0, 0.1, 0.5, 1.0 1.5, 2.0 2.5 and 3.0% (g salt/100 g distilled water). NaCl and KCl reagents (analytical-reagent grade) were obtained from Panreac Química S.A.U. (Barcelona, Spain). The brines were prepared the day before analysis to ensure that all of the salt was completely dissolved. The parameters measured in brines were a_w, pH, conductivity and sodium and chloride contents. Sodium content in brines was determined by a Na-selective electrode. All measurements were done in triplicate. Impedance spectroscopy measurements were also carried out on the same brine solutions.

Phase II: fish minces

Fresh hake (*Merluccius paradoxus/capensis*) were used as raw material. The fish were caught in June 13. 2012 by trawling of the coast of South Africa (FAO fishing area 47, Atlantic Southeast) and were obtained June 19th 2012 from a local supermarket in Valencia (Spain). The fish specimens were placed in styrofoam boxes with ice and transported immediately to the laboratory. Upon arrival to the laboratory, two fish were headed and gutted. Then, the fish were filleted, skinned, and the flesh was chopped with a standard food processor at low speed (Minirobot D81, Moulinex, Group SEB Iberica, Barcelona, Spain). Samples were prepared by mixing the fish mince (fish mince and salt, 200 g total) with an exact amount of salt before homogenising for 1 min. in the food processor. The amount of salt added to the fish mince had been pre-weighed to achieve an exact salt content (NaCl or NaCl/KCl) in the final sample 0.0, 0.5, 1.0, 2.0 and 3.0 % salt (g NaCl or NaCl/KCl/100 g salted fish mince). The homogenized fish minces were divided into five plastic containers (40 g. in each). Three of the plastic containers were used for the physicochemical analyses and impedance spectroscopy, whereas the remaining two containers were used for LF-NMR measurements. According to the results obtained by Sánchez-Alonso, [27] the mince composition does not suffer significant alterations during the frozen storage period. The samples were stored at -18°C and thawed to 4°C during 18 h before analysis. Moisture, lipid, protein, ash, a_w, pH, chloride, sodium and potassium contents, were determined in the same subsamples as were subjected to impedance spectroscopy. The minces assigned for LF NMR analysis were kept frozen for 86 days before the measurements were carried out.

ANALYTICAL METHODS

Physicochemical analyses

Moisture, lipid, protein and ash contents were assayed by AOAC Methods 950.46, 991.36, 928.08, and 920.153, respectively, [28] whereas pH and conductivity of brines were determined by using a multimeter MM 40 (Crison Instruments, S.A., Barcelona, Spain). The pH measurements of fish were carried out using a digital pH-meter micropH 2001 (Crison Instruments) with a puncture electrode (Crison 5231). Water activity was assessed in brines and fish minces with a fast water activity-meter (GBX FAst/lab, Romans sur Isère Cedex, France).

The chloride and sodium contents in brines were measured directly in the solutions, using a Chloride Analyzer (Sherwood mod. 926, Cambridge, UK) and a Dual Star^{TM} pH/ISE Meter (Thermo Fisher Scientific, Waltham, MA, USA) with a Na-selective electrode (Ross® Sodium Ion Selective Electrode, Thermo Fisher Scientific, USA), respectively. Chloride, sodium (by two different analytical methods) and potassium contents of fish minces were measured in an extract of the sample. For preparing the extract, 1.5 g of the mince was homogenised in ultra-pure water using an Ultra-turrax T-25 (IKA, Labortechnik, Staufen, Germany) at 9000 rpm for 1 min. Then, samples were warmed up to 90 ºC for 30 min, cooled down to room temperature, transferred to a volumetric flask and deluted up to 200 mL with ultra-pure water. Finally, samples were filtered through a cellulose filter paper (Whatman nº 1, Whatman International Ltd., Maidstone, UK). For chloride and sodium determinations, an aliquot of the extract was measured at room temperature by using the Chloride Analyzer and the Na-selective electrode as described above. The Na-selective electrode method was a modification of the Kivikari method.[29] In this study the direct calibration method was used, contrary to the method of Kivikari, where the known addition method was used. A calibration curve was made by using three standards of analytical-grade NaCl from Panreac Química S.A.U. (Barcelona, Spain). Sodium ion strength adjustor (Sodium ionic strength adjustor, Thermo Fisher Scientific, USA) was added to all solutions to ensure that samples and standards had similar ionic strength. Sodium and potassium contents of the samples were determined by ion chromatography (Compact IC 761, Metrohm® Ltd., Herisau, Switzerland) by using an ion exchange column (Metrosep C2, 250/4.0, Metrohm® Ltd., Herisau, Switzerland). The separation was monitored by using a regulated (20°C) conductivity detector and the IC Net 2.3 (Methrom® Ltd.) software was used for data collection and processing. Prior to analysis, samples were filtered through 0.45 μm nylon syringe filters. The isocratic elution was carried out using a solution of tartaric acid (4.0 mM)/dipicolinic acid (0.75 mM) at a flow rate of 1 mL/min. Samples were injected using a 20 μl loop injector. The content of each cation was determined by interpolation in the corresponding calibration curve. The calibration was established using a triplicate set of standard solutions of Na$^+$ (Fluka, Buchs, Switzerland) and K$^+$ (Sigma-Aldrich, St. Louis, MO, USA).

Impedance spectroscopy

The impedance spectroscopy measurement system was developed by the Instituto de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) at the Universidad Politécnica de València (UPV). [18] It consists of a software application that runs on a PC, electronic equipment and an electrode (for more information look in the supplementary information).

Using the software application the user chooses the frequencies and the amplitudes of the sinusoidal voltage signals. For each of the frequencies the electronic equipment generates the corresponding sinusoidal voltage waveform to the electrode. The current (I) and voltage (V) signals at the electrode are then sampled and the collected data are sent to the PC where a Discrete Fourier transform analysis (DFT) is performed to determine their amplitude and phase. The module |Z| and the phase (ϕ) of the *impedance* are then calculated using Eq. 1, where v(t) is the voltage signal, i(t) the current signal, f the frequency of the signals, and Δt is the time interval between the zero crossing of the voltage and current signals (**Figure 1**).

$$Z = |Z| e^{j\varphi} \quad \begin{cases} |Z| = \dfrac{|v(t)|}{|i(t)|} & \text{Module} \\ \varphi = 2\pi f \Delta t & \text{Phase} \end{cases} \tag{1}$$

The electronic equipment includes a digital processing block based on two CPLD's and three Random-access memories (RAM), one digital-to-analog converter, two analog-to-digital converters and some analog signal adaption circuits. [18]

The sensor employed in this study is a double electrode designed at IDM-UPV. The sensor consists of two steel needles 1.5 cm long and 1 mm in diameter, separated by a distance of 1 cm in a non-conductive frame. This design keeps the separation between both needles constant during measurements.

The impedance measurements were taken by inserting the sensors into the middle of the plastic containers (n=3) containing the solutions or the fish minces. Ten parallel measurements were performed in each plastic container. The penetration depth of the electrodes was constant in all the analyses (1.5 cm). All measurements were carried out at room temperature.

Preliminary Impedance Spectroscopy measurements showed that information given by low frequencies was not relevant for this study. Therefore all the measurements were carried out in the range of [10 kHz-1 MHz]. Seventeen frequencies were chosen in this range, thus a set of 34 values (17 module values and 17 phase values) were obtained for each sample.

Low field ^{1}H- NMR

LF ^{1}H NMR measurements were made on all fish minces. After thawing, approximately 2 grams samples were taken from each subsample of fish mince (n=2), and placed in NMR tubes (diameter 10 mm). There were analyzed three parallels from each subsample with fish mince in the LF ^{1}H NMR measurements. The tubes were immediately placed in ice and kept there until the NMR measurements were carried out. The measurements were performed using a Bruker minispec mq 20 (Bruker Optik GmbH, Ettlingen, Germany) with a magnetic field strength of 0.47 T corresponding to a proton resonance frequency of 20 MHz. The instrument was equipped with a 10 mm temperature-variable probe. A built-in heating element was connected to the temperature control unit (BVT3000, Bruker Optik GmbH). The temperature in the probe was regulated to 4°C by blowing compressed air through the sample holder. Transversal (T_2) relaxation was measured using the Carr-Purcell-Meiboom-Gill pulse sequence (CPMG). [30, 31] The T_2 measurements were performed with a time delay between the 90° and 180° pulses (τ) of 150 μs. Data from 4000 echoes were acquired from 16 scan repetitions. The repetition time between two succeeding scans was set to 3 s. All even echoes were sampled. The NMR transverse relaxation data were analyzed using two different calculation methods. (1) Biexponential analysis of T_2 relaxation data was performed by fitting of the following equation to the experimental CPMG curves, similar to that reported by Erikson et al. [21] and Lambelet et al. [32] :

$$Signal = A_{21} e^{-t/T_{21}} + A_{22} e^{-t/T_{22}} \qquad \text{(Eq. 2)}$$

where T_{21} and T_{22} are the relaxation time components, and A_{21} and A_{22} are the corresponding amplitudes, 4000 data points were used, and the calculations were made using MatLab (The Mathworks Inc., Natric, MA). Since the absolute relaxation amplitudes are proportional to the amount of water and fat in the sample, the relative amplitudes within samples were used. The T_{21} populations are calculated as: $A_{21}/(A_{21} + A_{22})$.

For the biexponential fitting, the populations sum up to 100%. Three parallel samples from each fish mince (n=2) were averaged. (2) Multivariate data analysis was performed for all raw relaxation (CPMG) curves. These curves were normalized by setting the first sampled echo to a value of 100, and thereafter scaling the rest of the echo-train. The first 600 data points were used for the principal component analysis (PCA).

Statistical analyses

Statistical treatment of the data was performed using the Statgraphics Centurion (Statpoint Technologies, Inc., Warrenton, VA, USA). A multifactor analysis of variance (ANOVA) was conducted for each evaluated parameter to test whether there were significant differences between the samples. These analyses were performed for the salt solutions and fish mince samples (phases I and II); in both cases, the physicochemical parameters were considered as dependent variables in these analyses. The type of cations and salt content, as well as its interaction were the factors. The Tukey test (least significant difference) was used to test for differences between averages at the 5% significance level.

In order to evaluate the measurement techniques used in this paper, different multivariate analyses [33] were carried out using the software SOLO PLS_Toolbox (Eigenvector Research, Inc., Wenatchee, WA).

Principal Component Analysis (PCA) was used to discriminate the salt content level for NaCl, KCl and mixtures. Typically, in PCA projects a multi-dimensional data set onto a new coordinate base formed by the orthogonal directions with data maximum variance. The eigenvectors of the data matrix are called principal components and they are uncorrelated between them. The principal components (PCs) are ordered so that PC1 displays the greatest amount of variance, followed by the next greatest PC2 and so forth. The main features of PCA are the coordinates of the data in the new base (scores plot) and the contribution to each component of the sensors (loads plot).

To create predictive models of physicochemical parameters, Partial Least Square (PLS) regressions were applied to both impedance spectroscopy and NMR measurements. The main objective of PLS is to predict one or more parameters (dependent variables Y) from a set of measured data (independent variables X). First, the set of independent variables is projected onto a new coordinate space by maximizing the covariance between Y and X. The axes of this new space are called latent variables (LV's). The important information that correlates Y and X is contained in the first LV's. Then a prediction model is built by applying a multiple regression to a reduced number of the LV's. PLS prediction models for a_w, Na, K, NaCl, and solute contents (g/100g) as well as solutes content in the water phase (g solutes/100g liquid phase) were created using a set of experimental data (calibration set). First, cross validation was used to select the number of LV's. The model was then validated with a new set of experimental data (validation set).

In the case of impedance measurements PCA's and PLS regressions were performed using impedance module and phase values obtained for the 17 frequencies in the range from [10 kHz to1 MHz]. In the case of NMR measurements, the relaxation times for each defined frequency were used.

RESULTS AND DISCUSSION

Phase I: Salt solutions

Physicochemical parameters

The results of the physicochemical analyses carried out for the salt solutions are shown in **Table 1**. As expected, the a_w of brines decreased with increasing brine content regardless of type of salt and the conductivity increased as salt content increased. Conductivity correlates with the total dissolved solids independently of the solute composition. In water, ions pass the electricity from one to another, therefore, the more Na^+, K^+, and Cl^- the solution contain the more electricity is carried and the higher the conductivity. This explained the fact that the conductivity was affected by the amount of salt but not by the sodium replacement. The initial conductivity of distilled water employed for preparing the salt solutions was 0.025 ± 0.003 mS/cm. The value increased with increasing salt content, from to 2 to 60 mS/cm for the lowest and the highest salt content, respectively. **Table 1** also shows the resulting contents of Na^+ and Cl^- after different salt additions to distilled water. In the solution prepared from KCl only, sodium was present in the range of 0.3 to 2.2 mg/L. The observed differences in chloride content depending on the type of salt are due to the different atomic mass of sodium and potassium (23 and 39 atomic mass units, respectively) owing to the fact that the salts were added equally by weight.

Impedance spectroscopy

Module and phase impedance spectra of KCl solutions are shown in **Figure 2a** and **2b**, respectively. Differences in both module and phase of impedance were observed to depend on salt content.

The module of the impedance decreased as the salt content increased, and the values were much higher for the lowest content (0.1% KCl) than for the other contents. Similar differences between salt contents were observed for NaCl and the mixture of NaCl:KCl (data not shown). These results are in agreement with those observed for the conductivity parameters for the brine (**Table 1**). The results are in accordance with previous studies on impedance spectroscopy. [16-18] The correlation can be explained by the conductance of an aqueous solution as a function of the ion content of the samples, and in fact impedance measurements are related with the ions capability of movement under the influence of an electrical field in this aqueous solution. In the present study, the behavior observed for NaCl solutions was similar to what was observed for KCl and NaCl:KCl solutions, which would indicate that impedance values were highly correlated with solute content. These results were confirmed by ANOVAs carried out for each impedance value (module and phase of impedance for each frequency), which established significant differences for solute content ($p<0.001$) but not for the type of salt ($p>0.05$) (ANOVA data not shown).

A PCA was performed with the data obtained in the impedance measurements (**Figure 3**). The statistical analysis was able to reduce the initial variables (34 variables, 17 values of module and 17 values of phase of impedance) into a set of values of linearly uncorrelated variables called principal components (PCs), being the number of principal components less than or equal to the number of original variables. Most of the variation in the sample was explained by PC 1 (68.75%) and PC 2 (27.82%). According to the results obtained, the impedance spectroscopy method could distinguish between salt contents; however, it was difficult to establish a correct classification of solutions according to the type of salt (**Figure 3**).

Phase II: Fish mince

Physicochemical analyses

The composition of the frozen/thawed raw material was determined. Moisture, protein, lipid and ash contents for unsalted hake were 80.2 ± 0.1 (**Table 2**), 15.6 ± 1.3, 0.5 ± 0.2 and 1.20 ± 0.02 g/100 g, respectively. These results are similar to those reported in other studies carried out with the same fish species. [27, 34]

The results of the physicochemical analyses for salted hake mince are summarized in **Table 2**. As expected, adding salt to the mince led to a reduction in moisture, from about 80.2 % (mince without additions) to 78.3 % and 77.8 % , for minces containing sodium- and potassium chloride (Na:K) and minces containing sodium chloride (Na), respectively. Due to the increase in mineral contents (up to 3.0g/100g mince) the a_w decreased from 0.992 (mince without additions) to 0.974 (Na:K) and 0.969 (Na). The moisture and a_w were significantly lower in minces containing 3.0% salt compared to minces containing less. Both the type of salt and their contents had a significant effect on the $a_{w,}$ compared to the brines where the a_w correlates only with the contents of salt. As expected, slightly higher water activities were found in the NaCl:KCl minces than in minces containing NaCl. Water activity decreases with increasing number of colligative units dissolved per volume. As K^+ is a larger ion than Na^+, replacing NaCl with an equal amount by weight of KCl will lead to a lower number of dissolved ions (colligative units) per volume and thus an increase in a_W of the product.

The pH of the unsalted mince (pH 6.97) was reduced after preparing the mince with different salts (Na) and (Na:K) and content (**Table 2**). The pH values of the raw material employed in this study is in accordance with the results obtained in other studies[34, 35] for fresh hake. A decrease in pH was observed when salt was added to our minces, a little more pronounced in case of Na than with most Na:K mixtures. Similar results have been observed in a study by Leroi & Joffraud, [36] indicating that pH decreases in fish flesh by the addition of salt due to the increase of the ionic strength of the solution inside the cells. Another explanation might be that an increased amount of chloride ions would open the myosin filament and the more dissociable acidic groups would be water-accesible. [37] Samples containing Na exhibited lower pH than the corresponding Na:K samples: pH 6.76 vs 6.81, respectively. Similar results with fish products subjected to partial sodium replacement have also been observed. [38]

The measured contents of sodium, potassium and chloride are shown in **Table 2**. The sodium (0.05-0.06 g/100 g) and potassium contents (0.35 g/100 g) of fresh fish mince (**Table 2**) agree with those reported in another study [39] for deboned hake. The chloride content in mince without additions was 0.21 g/100 g. When only NaCl was added to the minces, the potassium levels remained almost constant at 0.30 – 0.40 g/100g, resembling the level in mince without additions.

Table 2 shows a comparison between sodium contents in the different minces as determined by the ion selective electrode and by ion chromatography. Good correlation was observed between the sodium content determined by the ion selective electrode method and ion chromatography, which was confirmed by a simple regression carried out on the data obtained by both methodologies ($y=1.066x+7.961$, $R^2=0.967$).

Impedance Spectroscopy measurements

Impedance spectroscopy was used to detect changes in the fish mince adding different salt content and type of salt. A PCA was performed on the impedance spectroscopy measurements in fish mince samples with different type of salts.

The discrimination between the different salt contents observed in the PCA plot for fish minces was better than the one obtained for salt solutions (**Figure 4**). The percentage of variance explained by the first principal component in **Figure 4** is 90.17% while in **Figure 3**

PC1 only explains 68.75% of the total variance. This means that the correlation between impedance spectroscopy data and salt content is stronger in fish samples than in solutions. A possible explanation for this behavior is the salting-in effects on muscle proteins. [37] At salt contents lower than 0.5 M, the swelling of myofibrils starts and reaching a maximum at 0.8–1 M. [37] This usually causes a decrease myofibril volume, because the myofibril tends to disolve. However, in our study, the highest content in the minces corresponded to 0.65M and 0.55M for the minces with 3.0% NaCl and NaCl:KCl, respectively. The conformational changes, together with the increase in the conductivity, could be responsible for the different behavior in the IS observed among our hake minces and in the solutions. At some contents, the method also distinguished between types of cations (Na^+ or Na^+/K^+) in the fish mince, a behavior that can be explained by the different effects of sodium (kosmotrope, water-structure maker) and potassium (chaotrope, water-structure breaker) in actin and myosin. [40] Further work is needed to reveal significant differences between cations in the minces.

LF NMR

A LF-NMR T_2 relaxation method was used to study the relaxation behaviors in the mince when different types of salt were added to the mince in different amounts. The two transversal relaxation times with corresponding populations obtained from fitting of NMR data, are shown in **Table 3**. In fish muscle, typically two or three relaxation components are reported Erikson et al. and references therein [41]. The two major ones have relaxation times in the range of 40-60 ms (T_{21}) and 150-400 ms (T_{22}) , similar to those of the present research. The mean T_{21} and T_{22} relaxation times for the unsalted hake mince were 54 and 219 ms, respectively. The interpretation of such data have been controversial, but it is now becoming more accepted that the observed changes in relaxation behavior are due primarily to chemical and diffusive proton exchange between water molecules and biopolymers (e.g. proteins).[42, 43] A number of studies have nevertheless shown that these processes are linked to the morphology of the sample that in turn can be affected by, example.g., processing, such as salting and mincing. [23, 25, 41].

After addition of 0.5% NaCl or NaCl/KCl to the mince, the proton relaxation times, found by bioexponetial fitting, increased to 59-61 ms in case of T_{21} whereas the T_{22} value remained largely unchanged. Addition of more salt led to an increase in both T_{21} and T_{22} relaxation times, with mean values of 67-71 ms and 286-496 ms, respectively.

By comparison, when frozen/thawed Atlantic salmon fillets were salted to 2.7% NaCl in the head part of the fillet, T_{21} increased from 47 ms (unsaltet) to 48 ms (salted), whereas the tail part of the fillet had 2.9 % NaCl and T_{21} increased from 47 ms (unsalted) to 50 ms (salted), respectively. No significant changes were observed in T_{22} (140-150 ms (head part) and 140-169 ms (tail part)). [21] Similar values were obtained when fillets of the same species were salted in a 15 % NaCl brine. [20] Thus, it seems that the magnitude of change in T_{21} can be similar in highly concentrated brines (whole, lean fillets) as in our lean hake mince. Notably, the mincing of cod fillets does not alter the magnitude of the T_{21} values [44]. A stronger effect of salting of cod was, however, reported [24] where T_{21} values increased from 51 ms (raw material) to 86 to 94 ms after presalting by different methods (12 % salt).

A PCA score plot of the relaxation time curves is presented in **Figure 5**. Most of the variation in the sample was explained by PC 1 (76.60%) and PC 2 (20.54%) and it separates between minces with 0, 0.5% and 1.0-3.0% salt. Otherwise, the relaxation data did not reveal any clear trends, that is, between the magnitudes of the relaxation times at increasing salt contents above 0.5 %. The increase in relaxation times when 0.5 % salt was added, reflecting higher water proton mobility, suggests that a more open mince microstructure was formed. This was possibly caused by the binding of chloride ions to myosin filaments which would induce electrostatic repulsive forces causing an increase of filament spacing. [37]

In contrast to the increase in T_{21} as a result of the addition of 0.5 % salt, the corresponding population (T_{21} pop) did not change accordingly. The T_{21} pop values remained similar to those in the mince without additions (85-87 %). With further addition of salt, the values increased to 96-99 %, regardless of type and amunt of salt (1.0, 2.0 or 3.0 % salt) with a corresponding decrease in T_{22} pop. The latter population with high mobility decreased to 1-4 %. The changes in T_2 populations reflect a shift of the proton populations, increasing the amount of protons with higher mobility and decreasing the amount of protons with lower mobility. This may be explained by the changes in muscle structure due to the salting-in effect previously discussed.

Based on PCA analyses of the NMR T_2 relaxation data, a clear separation between samples with 0, 0.5 and 1.0 - 3.0% of salt was obtained. However, the LF NMR method was unable to distinguish between minces with different types of cations. To sum up, the fact that the most pronounced changes in relaxation behavior occurred at low contents of salt (0 – 0.5%) that LF ^{1}H NMR can be a suitable tool for indirectly studies of structural changes in low-salt systems. [41]

Partial Least Square (PLS) results

In order to create predictive models of physicochemical parameters PLS regression were applied to both impedance spectroscopy and LF NMR measurements. **Table 4** shows the values of the determination coefficient (R^2), the root-mean-square error of prediction (RMSEP) and the number of latent variables corresponding to the prediction models built for a_w, Na^+ (mg/100g), K^+ (mg/100g), NaCl (g/100g), gram salts (g/100g) and gram solutes/100g liquid phase using impedance spectroscopy data. Models for a_w, gram salts (g/100g) and gram solutes/100g liquid phase show very good behavior with R^2 values close to, or higher than 0.9. However, the results obtained for Na^+ (mg/100g), K^+ (mg/100g) and NaCl (g/100g) demonstrate that the proposed technique is not able to discriminate between the different types of salt. As shown in **Figure 3**, in the PCA plots most of the total variance corresponds to PC1. In the module and phase plots a similar discrimination between the different salt content levels could be obtained considering the module and phase values for all the frequencies or considering just the module value for one frequency (for example 1MHz). Based on this idea, new PLS models were built for a_w and gram salts (g/100g) using only one latent variable. The R^2 values for these new models are similar to those obtained using the number of latent variables established by cross-validation. This opens the the possibility to limit impedance measurements to the module at one single frequency so that the measurement process would be greatly shortened and the prediction could be made using a simple regression. There were no significant correlations between the LF NMR measurements and the physicochemical results. In conclusion, the PLS models of impedance spectroscopy measurements showed good correlations with R^2 values close to or higher than 0.9 for a_w, solute content and solute content in the liquid phase. However, the results obtained for Na^+ (mg/100g), K^+ (mg/100g) and NaCl (g/100g) demonstrate that the proposed technique is not able to discriminate between the different types of salt.

In conclusion, good correlations were observed between the sodium content determined by ion selective electrode method and ionic chromatography, which was confirmed by a simple regression, carried out using the data obtained by both methodologies. In both salt solutions and fish minces, the impedance spectroscopy measurements could separate between different salt contents down to 0.5%. However, the results obtained for cation determinations demonstrate that the proposed technique is not able to discriminate between the different types of salt. Furthermore, impedance spectroscopy measurements showed good correlations for a_w, solute content and solute content in the liquid phase. The NMR transversal relaxation time T_2, clearly distinguishes samples with 0, 0.5% and 1.0 - 3.0% salt, based on the principal component analysis (PCA). We conclude that LF ^{1}H NMR can be a suitable technique for studies of low-salt products. However, the LF NMR method was unable to distinguish between minces with different types of cations.

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SUPPORTING INFORMATION

Supporting Information Available: System Block Diagram. This material is available free of charge via the Internet at http://pubs.acs.org.

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Figure captions

Figure 1. Scheme of impedance measurement and registered signals. (Module |Z|, phase (ϕ) , v(t) is the voltage signal, i(t) the current signal, f the frequency of the signals, and Δt is the time interval between the zero crossing of the voltage and current signals)

Figure 2. Mean values of modulus (a) and phase (b) of impedance spectra for the KCl solutions with different salt contents (0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g salt/100 g distilled water, respectively)

Figure 3. PCA score plot of data obtained from the impedance spectroscopy measurements in solutions with different types of salt (NaCl, KCl and NaCl/KCl (NaK) , 50/50 w/w%) and contents 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g salt/ 100 g distilled water, respectively)

Figure 4. PCA score plot of data obtained from the impedance spectroscopy measurements in hake minces with different salt contents (NaCl (Na) or NaCl/KCl (Na:K) 0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 g/100 g salted mince, respectively)

Figure 5. PCA score plot of LF ^{1}H NMR T_2 relaxation data obtained from fish mince with salt content (NaCl (Na) or NaCl/KCl (Na:K) 0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 g/100 g salted mince, respectively)

Table 1

Physicochemical parameters of brine solutions prepared with different salts (S: KCl (K), NaCl:KCl (Na:K) and NaCl (Na)) and contents (C: g salt/100 g brine). Mean values ± SD (n=3). ANOVA F-ratio for each of the 2 factors (S and C) and its interaction in the physicochemical parameters.

S	C	a_w	Conductivity (mS/cm)	Na (g/L)	Cl (g/L)
K	**0.1**	0.999 ± 0.000^{aA}	2.10 ± 0.11^{A}	(0.34 ± 0.02)*10^{-3aA}	1.03 ±0.06^{aA}
	0.5	0.993 ± 0.001^{aB}	10.27 ± 1.15^{B}	(0.76 ± 0.00)*10^{-3aB}	3.50 ± 0.14^{aB}
	1.0	0.996 ± 0.003^{aB}	19.31 ± 1.24^{C}	(0.89 ± 0.01)*10^{-3aC}	5.78 ± 0. 13^{aC}
	1.5	0.990 ± 0.002^{aC}	31.20 ± 0.28^{D}	(1.26 ± 0.02)*10^{-3aD}	8.30 ± 0. 26^{aD}
	2.0	0.985 ± 0.001^{aC}	41.67 ±1.21^{E}	(1.37 ± 0.02)*10^{-3aE}	10.54 ± 0.09^{aE}
	2.5	0.980 ± 0.002^{aCD}	51.23 ± 3.61^{F}	(1.69 ± 0.04)*10^{-3aF}	12.64 ± 0.13^{aF}
	3.0	0.984 ± 0.002^{aD}	63.30 ± 5.30^{G}	(2.18 ± 0.16)*10^{-3aG}	14.68 ± 0.33^{aG}
K: Na	**0.1**	0.995 ± 0.002^{bA}	2.07 ± 0.04^{A}	0.26 ± 0.00^{bA}	1.08 ± 0.08^{bA}
	0.5	0.989 ± 0.002^{bB}	9.90 ± 0.42^{B}	0.77 ± 0.00^{bB}	3.74 ± 0.21^{bB}
	1.0	0.991 ± 0.001^{bB}	19.33 ± 1.26^{C}	1.67 ± 0.02^{bC}	6.44 ± 0.17 ^{bC}
	1.5	0.985 ± 0.004^{bC}	30.17 ± 3.06^{D}	2.60 ± 0.01^{bD}	9.12 ± 0.19^{bD}
	2.0	0.985 ± 0.001^{bC}	38.27 ± 0.81^{E}	3.45 ± 0.01^{bE}	11.86 ± 0.2 ^{bE}
	2.5	0.980 ± 0.002^{bCD}	50.63 ± 3.10^{F}	4.23 ± 0.07^{bF}	14.72 ± 0.3 ^{bF}
	3.0	0.981 ± 0.001^{bD}	63.10 ± 4.27^{G}	5.17 ± 0.02^{bG}	16.52 ± 0.15^{bG}
Na	**0.1**	0.998 ± 0.002^{bA}	2.57 ± 0.68^{A}	0.39 ± 0.00^{cA}	1.05± 0.08^{cA}
	0.5	0.989 ± 0.001^{bB}	10.44 ± 1.50^{B}	1.60 ± 0.01^{cB}	3.63 ± 0.21^{cB}
	1.0	0.984 ± 0.004^{bB}	19.37 ± 0.97^{C}	3.56 ± 0.02^{cC}	6.72 ± 0.28^{cC}
	1.5	0.980 ± 0.002^{bC}	28.37 ± 1.51^{D}	5.53 ± 0.03^{cD}	10.01 ± 0.19^{cD}
	2.0	0.981 ± 0.002^{bC}	39.87 ± 2.00^{E}	7.65 ± 0.00^{cE}	12.64 ± 0.05^{cE}
	2.5	0.983 ± 0.001^{bCD}	50.80 ± 3.10^{F}	9.45 ± 0.19^{cF}	16.20 ± 0.24^{cF}
	3.0	0.984 ± 0.002^{bCD}	59.10 ± 3.05^{G}	11.07 ± 0.06^{cG}	18.78 ± 0.26^{cG}
			F- ratio		
S		22.45***	0.61^{ns}	69815.08***	600.85***
C		80.43***	589.51***	14371.42***	10525.71***
S x C		7.90***	0.29^{ns}	5104.16***	69.82***

p-values : *** $p<0.001$; ** $p<0.01$; * $p<0.05$; ns: non significant

Different lower-case letters indicate significant differences ($p<0.05$) for factor S (salt composition).

Different capital letters indicate significant differences ($p<0.05$) for factor C (salt content).

Table 2

Physicochemical parameters of fish mince prepared with different salts (S: NaCl (Na) and NaCl:KCl (Na:K) and contents (C: g salt/100 g fish mince). Mean values ± SD (n=3).

S	C	Moisture (%)	a_w	pH	Chloride (g/100g)	Sodium (g/100g) (ISE)	Sodium(g/100g)(IC)	Potassium (g/100/g)	Ionic strength (mol/ kg solvent)
Fish mince	0	80.15 ± 0.08	0.992 ± 0.004	6.97 ± 0.13	0.21 ± 0.01	0.05 ± 0.00	0.06 ± 0.01	0.35± 0.00	0.11 ± 0.00
Na:K	0.5	80.37 ± 0.24^{aA}	0.987 ± 0.004^{aA}	6.81 ± 0.01^{aA}	0.43 ±0.01^{aA}	0.12 ± 0.01^{aA}	0.12 ± 0.04^{aA}	0.48 ±0.09^{aA}	0.18 ± 0.03^{aA}
Na:K	1.0	79.13 ± 0.41^{aA}	0.984 ± 0.003^{aAB}	6.71 ± 0.04^{aA}	0.69 ± 0.00^{aB}	0.19 ± 0.00^{aB}	0.19 ± 0.05^{aA}	0.56±0. 12^{aA}	0.26 ± 0.04^{aB}
Na:K	2.0	79.57 ± 0.09^{aA}	0.980 ± 0.005^{aB}	6.83 ± 0.02^{aA}	1. 29 ± 0.06^{aC}	0.43 ± 0.04^{aC}	0.42 ± 0.03^{aB}	0.79 ± 0.11^{aB}	0.47 ± 0.03^{aC}
Na:K	3.0	78.32 ± 0.41^{aB}	0.974 ± 0.001^{aC}	6.69 ± 0.02^{aB}	1.70 ± 0.03^{aD}	0.57 ± 0.02^{aD}	0.68 ± 0.08^{aC}	1.17 ± 0.14^{aC}	0.69 ± 0.05^{aD}
Na	0.5	79.09 ± 0.94^{aA}	0.985 ± 0.002^{bA}	6.76 ± 0.05^{bA}	0.50 ± 0.01^{bA}	0.19 ± 0.00^{bA}	0.26 ± 0.04^{bA}	0.40 ± 0.004^{bA}	0.22 ± 0.01^{bA}
Na	1.0	80.47 ± 0.88^{aA}	0.982 ± 0.001^{bAB}	6.78 ± 0.03^{bA}	0.82 ± 0.06^{bB}	0.35 ± 0.01^{bB}	0.38 ± 0.07^{bA}	0.30 ±0.0 6^{bA}	0.30 ± 0.03^{bB}
Na	2.0	78.67 ± 0.14^{aA}	0.978 ± 0.002^{bB}	6.67 ± 0.04^{bA}	1.34 ± 0.11^{bC}	0.76 ± 0.06^{bC}	0.78± 0.02^{bB}	0.40 ±0.04^{bB}	0.52 ± 0.03^{bC}
Na	3.0	77.77 ± 0.06^{aB}	0.969 ± 0.001^{bC}	6.67 ± 0.04^{bB}	1.90 ± 0.03^{bD}	1.01 ± 0.00^{bD}	1.09 ± 0.11^{bC}	0.39± 0.04^{bC}	0.71 ± 0. 03^{bD}
					F-ratio				
S		2.79^{ns}	6.82***	6.42***	28.40***	527.67***	116.86***	112.43***	8.26***
C		15.07***	31.63***	10.26***	819.47***	720.83***	153.16***	21.40***	300.80***
S x C		7.80***	0.67^{ns}	12.58***	2.71^{ns}	60.63***	6.65***	17.82***	0.17^{ns}

Different lower-case letters indicate significant differences ($p<0.05$) for factor S (salt composition). Different capital letters indicate significant differences ($p<0.05$) for factor C (salt content).

Table 3

Biexponential fitting of LF ^{1}H NMR T_2 relaxation data obtained in fish mince and fish mince prepared with different salts (S: NaCl and NaCl:KCl) and concentrations (C: g salt/100g fish mince). Mean values ± SD (n =2)

S	C (%)	T_{21} (ms)	T_{22} (ms)	T_{21} pop (%)	T_{22} pop (%)
Fish mince	0	54 ± 1	219 ± 7	86 ± 3	14 ± 3
NaCl/KCl	0.5	61 ± 1^{aA}	226 ± 10^{aA}	87 ± 2^{aA}	13 ± 2^{aD}
NaCl/ KCl	1.0	67 ± 1^{aB}	286 ± 46^{aB}	96 ± 1^{aBD}	4 ± 1^{aAC}
NaCl/ KCl	2.0	71 ± 1^{aD}	496 ± 42^{aD}	99 ± 0^{aBC}	1 ± 0^{aBC}
NaCl/ KCl	3.0	68 ± 2^{aBC}	342 ± 15^{aC}	98 ± 0^{aCD}	2 ± 0^{aAB}
NaCl	0.5	59 ± 1^{bA}	215 ± 10^{aA}	85 ± 1^{bA}	15 ± 1^{bD}
NaCl	1.0	69 ± 1^{bC}	366 ± 48^{bB}	98 ± 1^{bB}	2 ± 1^{bC}
NaCl	2.0	67 ± 0^{bB}	427 ± 34^{bD}	99 ± 0^{aCD}	1 ± 0^{aAB}
NaCl	3.0	68 ± 0^{aBCD}	423 ± 34^{bC}	99 ± 0^{aD}	1 ± 0^{aA}
			F - ratio		
S		9.20^{**}	4.99^{*}	0.30^{ns}	0.30^{ns}
C		214.59^{***}	132.32^{***}	609.87^{***}	609.87^{***}
S x C		22.49^{***}	17.43^{***}	12.14^{***}	12.14^{***}

p-values : *** $p<0.001$; ** $p<0.01$; * $p<0.05$; ns: non significant

Different lower-case letters indicate significant differences ($p<0.05$) for factor S (salt composition).
Different capital letters indicate significant differences ($p<0.05$) for factor C (salt concentration).

Table 4

Parameters of the PLS models of physicochemical parameters from the impedance measurements.

	LV	R^2	RMSEP
a_w	5	0.886	0.003
	1	0.812	0.003
Na (mg/100g)	4	0.480	276.154
K (mg/100g)	4	0.094	269.090
NaCl (g/100g)	4	0.425	0.730
KCl (g/100g)	3	0.393	0.628
Solute content (g/100g)	5	0.950	0.210
	1	0.953	0.203
Solute content in liquid phase (g/100g)	4	0.946	0.286

LV: number of Latent Variables; R^2: coefficient of determination; RMSEP: Root Mean Square Error of Prediction

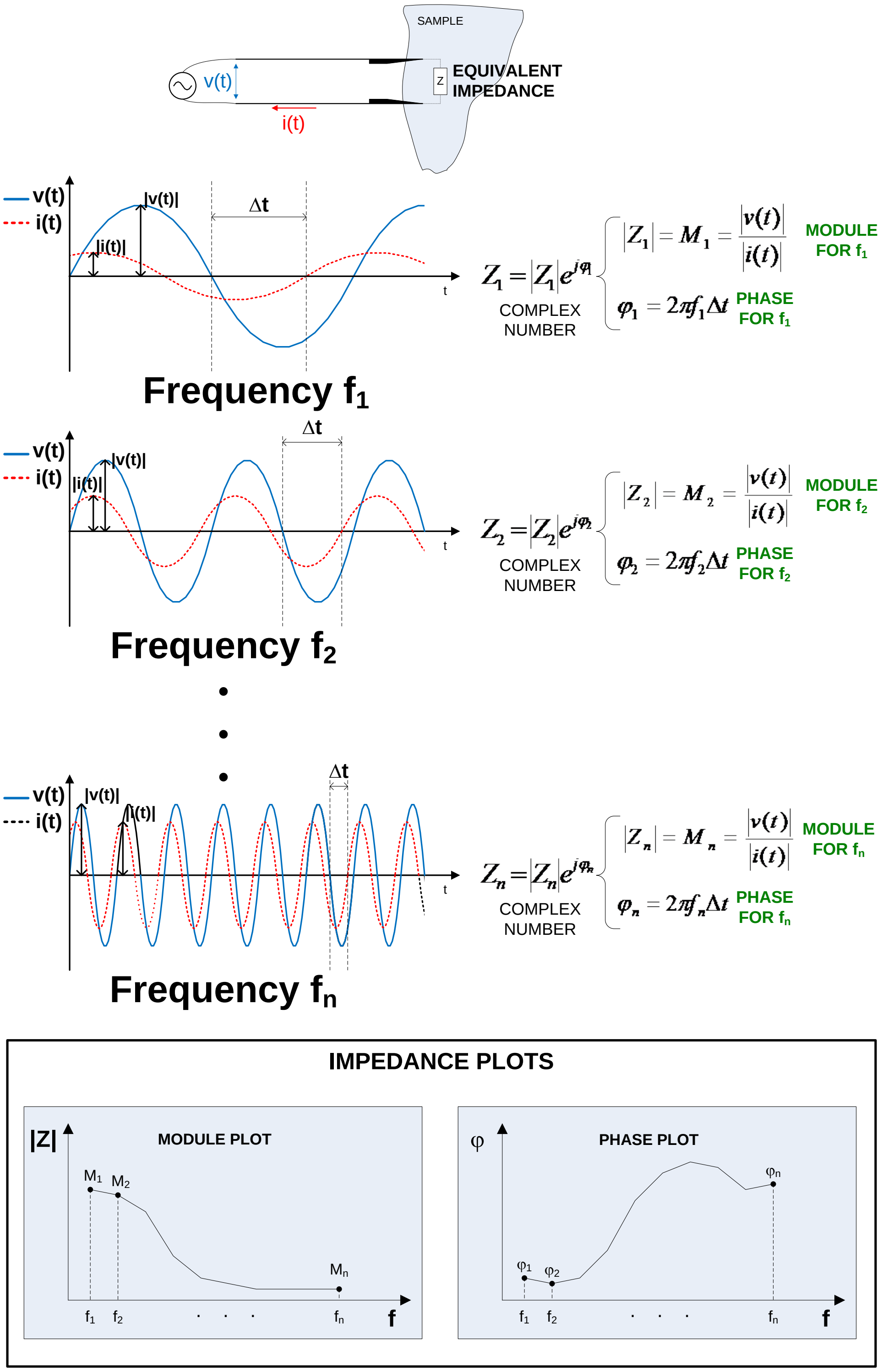

SAMPLE
v(t)
i(t)
Z
EQUIVALENT IMPEDANCE
v(t)
i(t)
|v(t)|
|i(t)|
Δt
t
$Z_1 = |Z_1| e^{j\varphi_1}$
COMPLEX NUMBER
$|Z_1| = M_1 = \frac{|v(t)|}{|i(t)|}$
MODULE FOR f1
$\varphi_1 = 2\pi f_1 \Delta t$
PHASE FOR f1
Frequency f1
$Z_2 = |Z_2| e^{j\varphi_2}$
COMPLEX NUMBER
$|Z_2| = M_2 = \frac{|v(t)|}{|i(t)|}$
MODULE FOR f2
$\varphi_2 = 2\pi f_2 \Delta t$
PHASE FOR f2
Frequency f2
$Z_n = |Z_n| e^{j\varphi_n}$
COMPLEX NUMBER
$|Z_n| = M_n = \frac{|v(t)|}{|i(t)|}$
MODULE FOR fn
$\varphi_n = 2\pi f_n \Delta t$
PHASE FOR fn
Frequency fn
IMPEDANCE PLOTS
MODULE PLOT
|Z|
M1
M2
Mn
f1
f2
fn
f
PHASE PLOT
φ
φ1
φ2
φn
f1
f2
fn
f

Figure 1

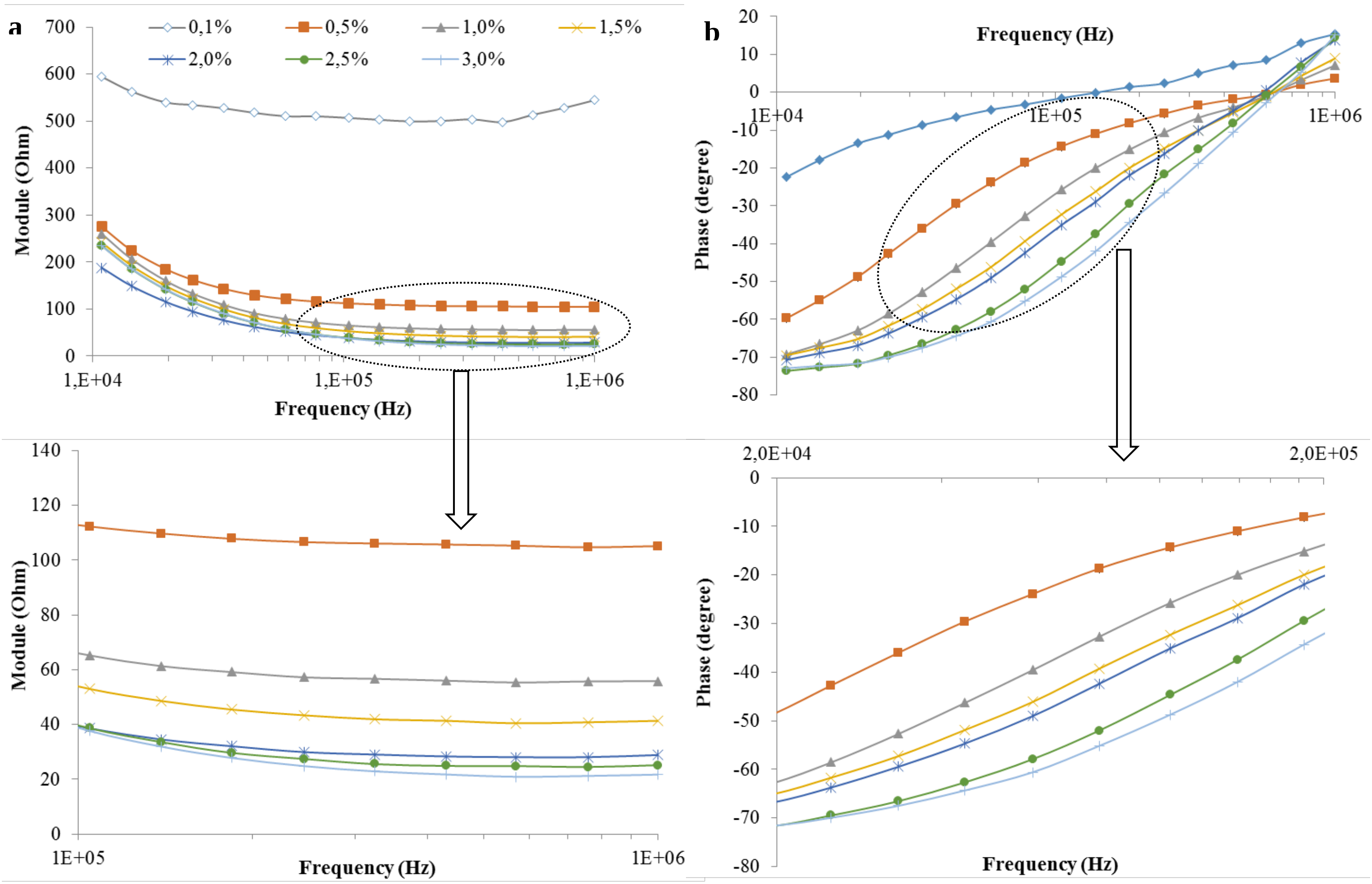

a
b
0,1%
0,5%
1,0%
1,5%
2,0%
2,5%
3,0%
Module (Ohm)
700
600
500
400
300
200
100
0
1,E+04
1,E+05
1,E+06
Frequency (Hz)
Phase (degree)
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
1E+04
1E+05
1E+06
Frequency (Hz)
Module (Ohm)
140
120
100
80
60
40
20
0
1E+05
1E+06
Frequency (Hz)
2,0E+04
2,0E+05
Phase (degree)
0
-10
-20
-30
-40
-50
-60
-70
-80
Frequency (Hz)

Figure 2

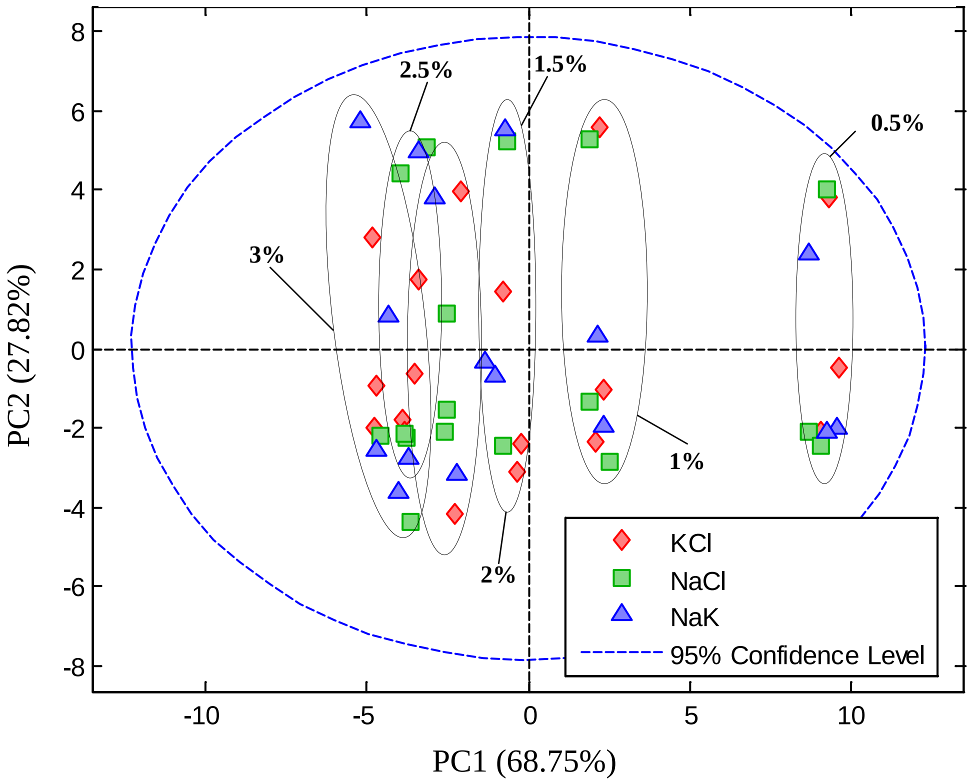

PC2 (27.82%)
PC1 (68.75%)
8
6
4
2
0
-2
-4
-6
-8
-10
-5
0
5
10
2.5%
1.5%
0.5%
3%
1%
2%
KCl
NaCl
NaK
95% Confidence Level

Figure 3

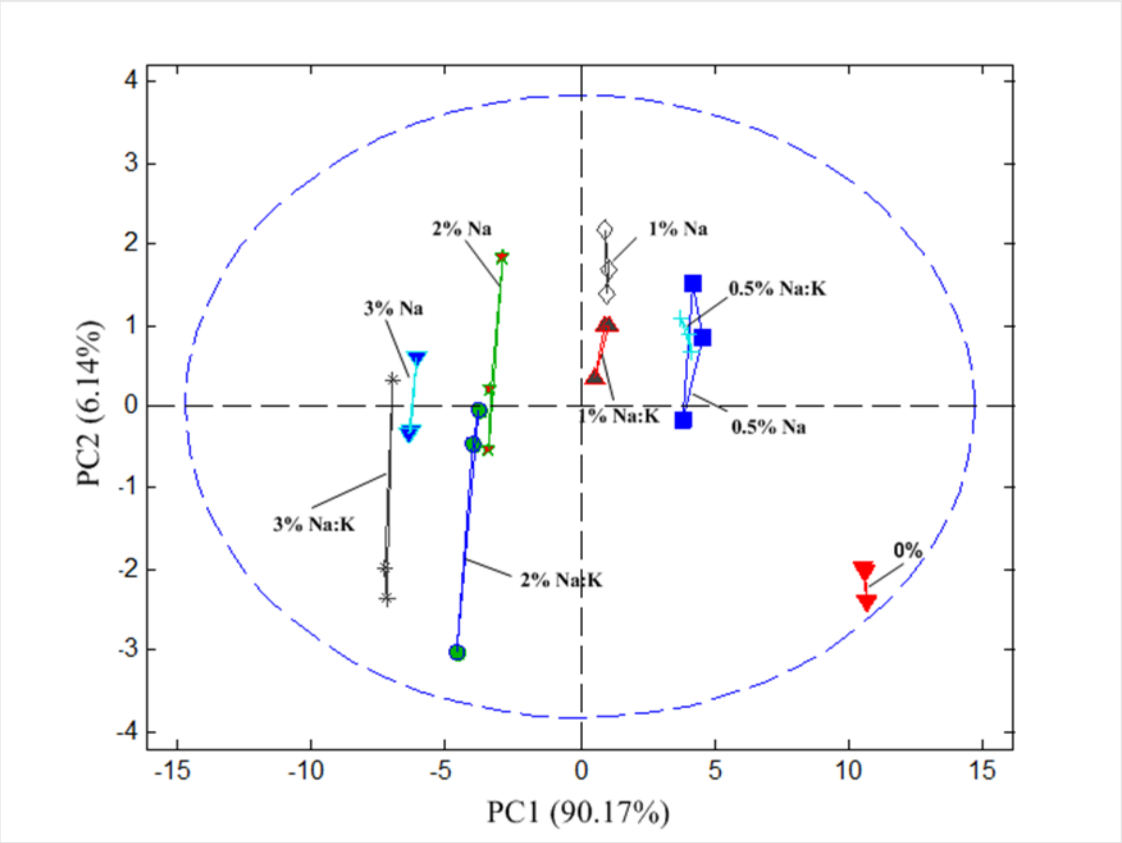

4
3
2
1
0
-1
-2
-3
-4
-15
-10
-5
0
5
10
15
PC1 (90.17%)
PC2 (6.14%)
2% Na
1% Na
0.5% Na:K
3% Na
1% Na:K
0.5% Na
3% Na:K
2% Na:K
0%

Figure 4

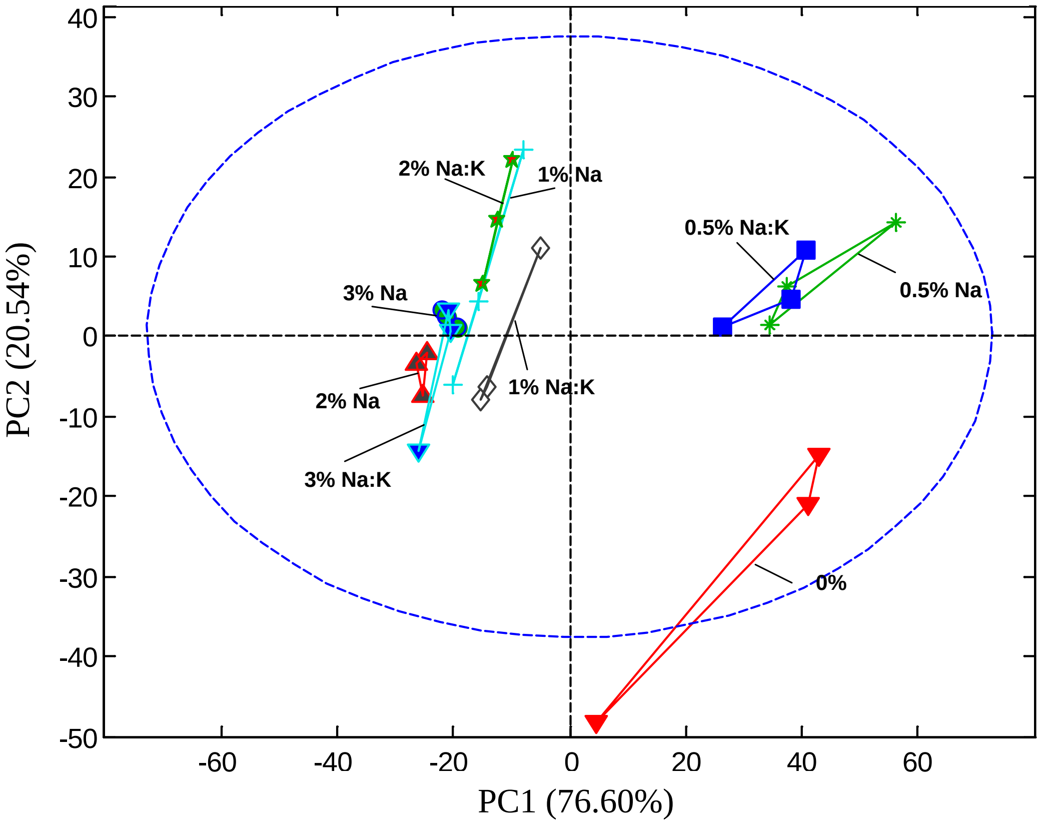
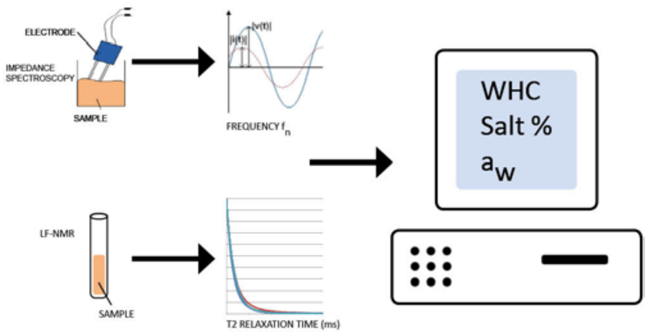

40
30
20
10
0
-10
-20
-30
-40
-50
-60
-40
-20
0
20
40
60
PC2 (20.54%)
PC1 (76.60%)
2% Na:K
1% Na
0.5% Na:K
0.5% Na
3% Na
2% Na
1% Na:K
3% Na:K
0%

Figure 5

TOC graphic


ELECTRODE
IMPEDANCE SPECTROSCOPY
SAMPLE
|v(t)|
|i(t)|
FREQUENCY f_n
LF-NMR
SAMPLE
T2 RELAXATION TIME (ms)
WHC
Salt %
a_w