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Additional Information

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

3

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21

22 **Abstract**

23 Impedance spectroscopy (IS), Low-field proton Nuclear Magnetic Resonance (LF ^1H NMR),
24 chloride titration, ion chromatography and ion selective electrode were used to investigate
25 physicochemical parameters and measure sodium and potassium contents in low salt brines and
26 fish. Salt solutions (0-3 w/w,%) and model products of minced hake with added NaCl (0.5–3.0
27 w/w,%), or a mixture of NaCl and KCl (50/50 w/w,%) were analyzed. Good correlation was
28 observed between the sodium content determined by ion selective electrode method and ion
29 chromatography ($R^2=0.97$). In both salt solutions and fish minces, the impedance spectroscopy
30 measurements could detect the difference in salt contents in mince with salt contents down to
31 0.5%. The NMR transversal relaxation time T_2 measurements clearly distinguished samples
32 with 0, 0.5% and 1.0 - 3.0% salt, based on the principal component analysis (PCA). Therefore,
33 LF ^1H NMR seems to be a suitable technique for studies of low-salt products.

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

36

37 INTRODUCTION

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

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

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

60 Analytical methods for the determination of salt include flame atomic absorption
61 spectrophotometry (FAAS), inductively coupled plasma/MS (ICP/MS), ionic chromatography,

62 and sodium selective electrodes ¹³. Other methods, such as Volhart method (AOAC method
63 971.27) ¹⁴ and potentiometric titration ¹⁵ measure the chloride contents and the sodium content
64 is then calculated stoichiometrically.

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

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

83 The objectives of the present research are to (1) evaluate the application of impedance
84 spectroscopy to monitor physicochemical parameters in salted fish products with, and without
85 sodium replacement, (2) establish a fast and consistent method to measure sodium and

86 potassium contents in fish products, and (3) assess the feasibility of employing LF NMR in
87 low-salt tissues.

88

89 **MATERIALS AND METHODS**

90 **Chemicals**

91 Ammonium Chloride (NH_4Cl), Ammonium Hydroxide (NH_4OH), Ammonium
92 Hydrogen Fluoride ($\text{NH}_4\text{FHF} < 1\%$, LD_{50} mg/kg not found), Chloroform (CHCl_3), Ethanol
93 ($\text{C}_2\text{H}_5\text{OH}$), Sulfuric acid (H_2SO_4), Potassium sulfate (K_2SO_4), Copper Sulfate (CuSO_4),
94 Hydrogen Peroxide (H_2O_2) and Sodium hydroxide (NaOH) (Scharlau, S.A. or Thermo Fisher
95 Scientific, USA). All the chemicals were of analytical-reagent grades.

96 **Experimental protocol**

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

101 **Phase I: Salt solutions**

102 Different brines were prepared by using NaCl, KCl, and a mixture of NaCl/KCl (50/50, w/w,
103 %) at different contents. The total salt contents assessed were 0.0, 0.1, 0.5, 1.0 1.5, 2.0 2.5 and
104 3.0% (g salt/100 g distilled water). NaCl and KCl reagents (analytical-reagent grade) were
105 obtained from Panreac Química S.A.U. (Barcelona, Spain). The brines were prepared the day
106 before analysis to ensure that all of the salt was completely dissolved. The parameters measured
107 in brines were a_w , pH, conductivity and sodium and chloride contents. Sodium content in brines
108 was determined by a Na-selective electrode. All measurements were done in triplicate.
109 Impedance spectroscopy measurements were also carried out on the same brine solutions.

110 **Phase II: fish minces**

111 Fresh hake (*Merluccius paradoxus/capensis*) were used as raw material. The fish were caught
112 in June 13. 2012 by trawling of the coast of South Africa (FAO fishing area 47, Atlantic
113 Southeast) and were obtained June 19th 2012 from a local supermarket in Valencia (Spain). The
114 fish specimens were placed in styrofoam boxes with ice and transported immediately to the
115 laboratory. Upon arrival to the laboratory, two fish were headed and gutted. Then, the fish were
116 filleted, skinned, and the flesh was chopped with a standard food processor at low speed
117 (Minirobot D81, Moulinex, Group SEB Iberica, Barcelona, Spain). Samples were prepared by
118 mixing the fish mince (fish mince and salt, 200 g total) with an exact amount of salt before
119 homogenising for 1 min. in the food processor. The amount of salt added to the fish mince had
120 been pre-weighed to achieve an exact salt content (NaCl or NaCl/KCl) in the final sample 0.0,
121 0.5, 1.0, 2.0 and 3.0 % salt (g NaCl or NaCl/KCl/100 g salted fish mince). The homogenized
122 fish minces were divided into five plastic containers (40 g. in each). Three of the plastic
123 containers were used for the physicochemical analyses and impedance spectroscopy, whereas
124 the remaining two containers were used for LF-NMR measurements. According to the results
125 obtained by Sánchez-Alonso,²⁷ the mince composition does not suffer significant alterations
126 during the frozen storage period. The samples were stored at -18°C and thawed to 4°C during
127 18 h before analysis. Moisture, lipid, protein, ash, a_w , pH, chloride, sodium and potassium
128 contents, were determined in the same subsamples as were subjected to impedance
129 spectroscopy. The minces assigned for LF NMR analysis were kept frozen for 86 days before
130 the measurements were carried out.

131 **ANALYTICAL METHODS**

132 **Physicochemical analyses**

133 Moisture, lipid, protein and ash contents were assayed by AOAC Methods 950.46, 991.36,
134 928.08, and 920.153, respectively,²⁸ whereas pH and conductivity of brines were determined

135 by using a multimeter MM 40 (Crison Instruments, S.A., Barcelona, Spain). The pH
136 measurements of fish were carried out using a digital pH-meter micropH 2001 (Crison
137 Instruments) with a puncture electrode (Crison 5231). Water activity was assessed in brines and
138 fish minces with a fast water activity-meter (GBX FAsT/lab, Romans sur Isère Cedex, France).

139 The chloride and sodium contents in brines were measured directly in the solutions,
140 using a Chloride Analyzer (Sherwood mod. 926, Cambridge, UK) and a Dual StarTM pH/ISE
141 Meter (Thermo Fisher Scientific, Waltham, MA, USA) with a Na-selective electrode (Ross®
142 Sodium Ion Selective Electrode, Thermo Fisher Scientific, USA), respectively. Chloride,
143 sodium (by two different analytical methods) and potassium contents of fish minces were
144 measured in an extract of the sample. For preparing the extract, 1.5 g of the mince was
145 homogenised in ultra-pure water using an Ultra-turrax T-25 (IKA, Labortechnik, Staufen,
146 Germany) at 9000 rpm for 1 min. Then, samples were warmed up to 90 °C for 30 min, cooled
147 down to room temperature, transferred to a volumetric flask and deluted up to 200 mL with
148 ultra-pure water. Finally, samples were filtered through a cellulose filter paper (Whatman n° 1,
149 Whatman International Ltd., Maidstone, UK). For chloride and sodium determinations, an
150 aliquot of the extract was measured at room temperature by using the Chloride Analyzer and
151 the Na-selective electrode as described above. The Na-selective electrode method was a
152 modification of the Kivikari method.²⁹ In this study the direct calibration method was used,
153 contrary to the method of Kivikari, where the known addition method was used. A calibration
154 curve was made by using three standards of analytical-grade NaCl from Panreac Química
155 S.A.U. (Barcelona, Spain). Sodium ion strength adjustor (Sodium ionic strength adjustor,
156 Thermo Fisher Scientific, USA) was added to all solutions to ensure that samples and standards
157 had similar ionic strength. Sodium and potassium contents of the samples were determined by
158 ion chromatography (Compact IC 761, Metrohm® Ltd., Herisau, Switzerland) by using an ion
159 exchange column (Metrosep C2, 250/4.0, Metrohm® Ltd., Herisau, Switzerland). The

160 separation was monitored by using a regulated (20°C) conductivity detector and the IC Net 2.3
161 (Methrom® Ltd.) software was used for data collection and processing. Prior to analysis,
162 samples were filtered through 0.45 µm nylon syringe filters. The isocratic elution was carried
163 out using a solution of tartaric acid (4.0 mM)/dipicolinic acid (0.75 mM) at a flow rate of 1
164 mL/min. Samples were injected using a 20 µl loop injector. The content of each cation was
165 determined by interpolation in the corresponding calibration curve. The calibration was
166 established using a triplicate set of standard solutions of Na⁺ (Fluka, Buchs, Switzerland) and
167 K⁺ (Sigma-Aldrich, St. Louis, MO, USA).

168 **Impedance spectroscopy**

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

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

$$181 \quad Z = |Z|e^{j\varphi} \quad \begin{cases} |Z| = \frac{|v(t)|}{|i(t)|} & \text{Module} \\ \varphi = 2\pi f\Delta t & \text{Phase} \end{cases} \quad (1)$$

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

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

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

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

197 **Low field ¹H- NMR**

198 LF ¹H NMR measurements were made on all fish minces. After thawing, approximately 2 grams
199 samples were taken from each subsample of fish mince (n=2), and placed in NMR tubes
200 (diameter 10 mm). There were analyzed three parallels from each subsample with fish mince
201 in the LF ¹H NMR measurements. The tubes were immediately placed in ice and kept there
202 until the NMR measurements were carried out. The measurements were performed using a
203 Bruker minispec mq 20 (Bruker Optik GmbH, Ettlingen, Germany) with a magnetic field
204 strength of 0.47 T corresponding to a proton resonance frequency of 20 MHz. The instrument
205 was equipped with a 10 mm temperature-variable probe. A built-in heating element was
206 connected to the temperature control unit (BVT3000, Bruker Optik GmbH). The temperature

207 in the probe was regulated to 4°C by blowing compressed air through the sample holder.
208 Transversal (T₂) relaxation was measured using the Carr-Purcell-Meiboom-Gill pulse sequence
209 (CPMG).^{30, 31} The T₂ measurements were performed with a time delay between the 90° and
210 180° pulses (τ) of 150 μs. Data from 4000 echoes were acquired from 16 scan repetitions. The
211 repetition time between two succeeding scans was set to 3 s. All even echoes were sampled.
212 The NMR transverse relaxation data were analyzed using two different calculation methods.
213 (1) Biexponential analysis of T₂ relaxation data was performed by fitting of the following
214 equation to the experimental CPMG curves, similar to that reported by Erikson et al.²¹ and
215 Lambelet et al.³² :

$$216 \quad S_i = A_1 e^{-t/T_{21}} + A_2 e^{-t/T_{22}} \quad (\text{Eq. 2})$$

217 where T₂₁ and T₂₂ are the relaxation time components, and A₂₁ and A₂₂ are the corresponding
218 amplitudes, 4000 data points were used, and the calculations were made using MatLab (The
219 Mathworks Inc., Natick, MA). Since the absolute relaxation amplitudes are proportional to the
220 amount of water and fat in the sample, the relative amplitudes within samples were used. The
221 T₂₁ populations are calculated as: A₂₁/(A₂₁ + A₂₂).

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

227 **Statistical analyses**

228 Statistical treatment of the data was performed using the Statgraphics Centurion (Statpoint
229 Technologies, Inc., Warrenton, VA, USA). A multifactor analysis of variance (ANOVA) was
230 conducted for each evaluated parameter to test whether there were significant differences
231 between the samples. These analyses were performed for the salt solutions and fish mince

232 samples (phases I and II); in both cases, the physicochemical parameters were considered as
233 dependent variables in these analyses. The type of cations and salt content, as well as its
234 interaction were the factors. The Tukey test (least significant difference) was used to test for
235 differences between averages at the 5% significance level.

236 In order to evaluate the measurement techniques used in this paper, different multivariate
237 analyses³³ were carried out using the software SOLO PLS_Toolbox (Eigenvector Research,
238 Inc., Wenatchee, WA).

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

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

257 used to select the number of LV's. The model was then validated with a new set of experimental
258 data (validation set).

259 In the case of impedance measurements PCA's and PLS regressions were performed
260 using impedance module and phase values obtained for the 17 frequencies in the range from
261 [10 kHz to 1 MHz]. In the case of NMR measurements, the relaxation times for each defined
262 frequency were used.

263

264 **RESULTS AND DISCUSSION**

265 **Phase I: Salt solutions**

266 **Physicochemical parameters**

267 The results of the physicochemical analyses carried out for the salt solutions are shown in **Table**
268 **1**. As expected, the a_w of brines decreased with increasing brine content regardless of type of
269 salt and the conductivity increased as salt content increased. Conductivity correlates with the
270 total dissolved solids independently of the solute composition. In water, ions pass the electricity
271 from one to another, therefore, the more Na^+ , K^+ , and Cl^- the solution contain the more
272 electricity is carried and the higher the conductivity. This explained the fact that the
273 conductivity was affected by the amount of salt but not by the sodium replacement. The initial
274 conductivity of distilled water employed for preparing the salt solutions was 0.025 ± 0.003
275 mS/cm. The value increased with increasing salt content, from 2 to 60 mS/cm for the lowest
276 and the highest salt content, respectively. **Table 1** also shows the resulting contents of Na^+ and
277 Cl^- after different salt additions to distilled water. In the solution prepared from KCl only,
278 sodium was present in the range of 0.3 to 2.2 mg/L. The observed differences in chloride content
279 depending on the type of salt are due to the different atomic mass of sodium and potassium (23
280 and 39 atomic mass units, respectively) owing to the fact that the salts were added equally by
281 weight.

282 **Impedance spectroscopy**

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

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

300 A PCA was performed with the data obtained in the impedance measurements (**Figure**
301 **3**). The statistical analysis was able to reduce the initial variables (34 variables, 17 values of
302 module and 17 values of phase of impedance) into a set of values of linearly uncorrelated
303 variables called principal components (PCs), being the number of principal components less
304 than or equal to the number of original variables. Most of the variation in the sample was
305 explained by PC 1 (68.75%) and PC 2 (27.82%). According to the results obtained, the
306 impedance spectroscopy method could distinguish between salt contents; however, it was

307 difficult to establish a correct classification of solutions according to the type of salt (**Figure**
308 **3**).

309 **Phase II: Fish mince**

310 **Physicochemical analyses**

311 The composition of the frozen/thawed raw material was determined. Moisture, protein, lipid
312 and ash contents for unsalted hake were 80.2 ± 0.1 (**Table 2**), 15.6 ± 1.3 , 0.5 ± 0.2 and $1.20 \pm$
313 0.02 g/100 g, respectively. These results are similar to those reported in other studies carried
314 out with the same fish species.^{27, 34}

315 The results of the physicochemical analyses for salted hake mince are summarized in
316 **Table 2**. As expected, adding salt to the mince led to a reduction in moisture, from about 80.2
317 % (mince without additions) to 78.3 % and 77.8 % , for minces containing sodium- and
318 potassium chloride (Na:K) and minces containing sodium chloride (Na), respectively. Due to
319 the increase in mineral contents (up to 3.0g/100g mince) the a_w decreased from 0.992 (mince
320 without additions) to 0.974 (Na:K) and 0.969 (Na). The moisture and a_w were significantly
321 lower in minces containing 3.0% salt compared to minces containing less. Both the type of salt
322 and their contents had a significant effect on the a_w , compared to the brines where the a_w
323 correlates only with the contents of salt. As expected, slightly higher water activities were found
324 in the NaCl:KCl minces than in minces containing NaCl. Water activity decreases with
325 increasing number of colligative units dissolved per volume. As K^+ is a larger ion than Na^+ ,
326 replacing NaCl with an equal amount by weight of KCl will lead to a lower number of dissolved
327 ions (colligative units) per volume and thus an increase in a_w of the product.

328 The pH of the unsalted mince (pH 6.97) was reduced after preparing the mince with
329 different salts (Na) and (Na:K) and content (**Table 2**). The pH values of the raw material
330 employed in this study is in accordance with the results obtained in other studies^{34, 35} for fresh
331 hake. A decrease in pH was observed when salt was added to our minces, a little more

332 pronounced in case of Na than with most Na:K mixtures. Similar results have been observed in
333 a study by Leroi & Joffraud,³⁶ indicating that pH decreases in fish flesh by the addition of salt
334 due to the increase of the ionic strength of the solution inside the cells. Another explanation
335 might be that an increased amount of chloride ions would open the myosin filament and the
336 more dissociable acidic groups would be water-accessible.³⁷ Samples containing Na exhibited
337 lower pH than the corresponding Na:K samples: pH 6.76 vs 6.81, respectively. Similar results
338 with fish products subjected to partial sodium replacement have also been observed.³⁸

339 The measured contents of sodium, potassium and chloride are shown in **Table 2**. The
340 sodium (0.05-0.06 g/100 g) and potassium contents (0.35 g/100 g) of fresh fish mince (**Table**
341 **2**) agree with those reported in another study³⁹ for deboned hake. The chloride content in mince
342 without additions was 0.21 g/100 g. When only NaCl was added to the minces, the potassium
343 levels remained almost constant at 0.30 – 0.40 g/100g, resembling the level in mince without
344 additions.

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

350 **Impedance Spectroscopy measurements**

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

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

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

370 **LF NMR**

371 A LF-NMR T_2 relaxation method was used to study the relaxation behaviors in the mince when
372 different types of salt were added to the mince in different amounts. The two transversal
373 relaxation times with corresponding populations obtained from fitting of NMR data, are shown
374 in **Table 3**. In fish muscle, typically two or three relaxation components are reported Erikson
375 et al. and references therein⁴¹. The two major ones have relaxation times in the range of 40-60
376 ms (T_{21}) and 150-400 ms (T_{22}), similar to those of the present research. The mean T_{21} and T_{22}
377 relaxation times for the unsalted hake mince were 54 and 219 ms, respectively. The
378 interpretation of such data have been controversial, but it is now becoming more accepted that
379 the observed changes in relaxation behavior are due primarily to chemical and diffusive proton
380 exchange between water molecules and biopolymers (e.g. proteins).^{42, 43} A number of studies

381 have nevertheless shown that these processes are linked to the morphology of the sample that
382 in turn can be affected by, example.g., processing, such as salting and mincing.^{23, 25, 41}

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

387 By comparison, when frozen/thawed Atlantic salmon fillets were salted to 2.7% NaCl
388 in the head part of the fillet, T_{21} increased from 47 ms (unsalted) to 48 ms (salted), whereas the
389 tail part of the fillet had 2.9 % NaCl and T_{21} increased from 47 ms (unsalted) to 50 ms (salted),
390 respectively. No significant changes were observed in T_{22} (140-150 ms (head part) and 140-169
391 ms (tail part)).²¹ Similar values were obtained when fillets of the same species were salted in a
392 15 % NaCl brine.²⁰ Thus, it seems that the magnitude of change in T_{21} can be similar in highly
393 concentrated brines (whole, lean fillets) as in our lean hake mince. Notably, the mincing of cod
394 fillets does not alter the magnitude of the T_{21} values⁴⁴. A stronger effect of salting of cod was,
395 however, reported²⁴ where T_{21} values increased from 51 ms (raw material) to 86 to 94 ms after
396 presalting by different methods (12 % salt).

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

405 In contrast to the increase in T_{21} as a result of the addition of 0.5 % salt, the
406 corresponding population (T_{21} pop) did not change accordingly. The T_{21} pop values remained
407 similar to those in the mince without additions (85-87 %). With further addition of salt, the
408 values increased to 96-99 %, regardless of type and amount of salt (1.0, 2.0 or 3.0 % salt) with a
409 corresponding decrease in T_{22} pop. The latter population with high mobility decreased to 1-4
410 %. The changes in T_2 populations reflect a shift of the proton populations, increasing the amount
411 of protons with higher mobility and decreasing the amount of protons with lower mobility. This
412 may be explained by the changes in muscle structure due to the salting-in effect previously
413 discussed.

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

420 **Partial Least Square (PLS) results**

421 In order to create predictive models of physicochemical parameters PLS regression were
422 applied to both impedance spectroscopy and LF NMR measurements. **Table 4** shows the values
423 of the determination coefficient (R^2), the root-mean-square error of prediction (RMSEP) and
424 the number of latent variables corresponding to the prediction models built for a_w , Na^+
425 (mg/100g), K^+ (mg/100g), NaCl (g/100g), gram salts (g/100g) and gram solutes/100g liquid
426 phase using impedance spectroscopy data. Models for a_w , gram salts (g/100g) and gram
427 solutes/100g liquid phase show very good behavior with R^2 values close to, or higher than 0.9.
428 However, the results obtained for Na^+ (mg/100g), K^+ (mg/100g) and NaCl (g/100g)
429 demonstrate that the proposed technique is not able to discriminate between the different types

430 of salt. As shown in **Figure 3**, in the PCA plots most of the total variance corresponds to PC1.
431 In the module and phase plots a similar discrimination between the different salt content levels
432 could be obtained considering the module and phase values for all the frequencies or
433 considering just the module value for one frequency (for example 1MHz). Based on this idea,
434 new PLS models were built for a_w and gram salts (g/100g) using only one latent variable. The
435 R^2 values for these new models are similar to those obtained using the number of latent variables
436 established by cross-validation. This opens the the possibility to limit impedance measurements
437 to the module at one single frequency so that the measurement process would be greatly
438 shortened and the prediction could be made using a simple regression. There were no significant
439 correlations between the LF NMR measurements and the physicochemical results. In
440 conclusion, the PLS models of impedance spectroscopy measurements showed good
441 correlations with R^2 values close to or higher than 0.9 for a_w , solute content and solute content
442 in the liquid phase. However, the results obtained for Na^+ (mg/100g), K^+ (mg/100g) and NaCl
443 (g/100g) demonstrate that the proposed technique is not able to discriminate between the
444 different types of salt.

445

446 In conclusion, good correlations were observed between the sodium content determined
447 by ion selective electrode method and ionic chromatography, which was confirmed by a simple
448 regression, carried out using the data obtained by both methodologies. In both salt solutions and
449 fish minces, the impedance spectroscopy measurements could separate between different salt
450 contents down to 0.5%. However, the results obtained for cation determinations demonstrate
451 that the proposed technique is not able to discriminate between the different types of salt.
452 Furthermore, impedance spectroscopy measurements showed good correlations for a_w , solute
453 content and solute content in the liquid phase. The NMR transversal relaxation time T_2 , clearly
454 distinguishes samples with 0, 0.5% and 1.0 - 3.0% salt, based on the principal component

455 analysis (PCA). We conclude that LF ^1H NMR can be a suitable technique for studies of low-
456 salt products. However, the LF NMR method was unable to distinguish between minces with
457 different types of cations.

458

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464

465 **SUPPORTING INFORMATION**

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

468

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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 ^1H 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 \pm 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 \pm 0.000 ^{aA}	2.10 \pm 0.11 ^A	(0.34 \pm 0.02)*10 ^{-3aA}	1.03 \pm 0.06 ^{aA}
	0.5	0.993 \pm 0.001 ^{aB}	10.27 \pm 1.15 ^B	(0.76 \pm 0.00)*10 ^{-3aB}	3.50 \pm 0.14 ^{aB}
	1.0	0.996 \pm 0.003 ^{aB}	19.31 \pm 1.24 ^C	(0.89 \pm 0.01)*10 ^{-3aC}	5.78 \pm 0.13 ^{aC}
	1.5	0.990 \pm 0.002 ^{aC}	31.20 \pm 0.28 ^D	(1.26 \pm 0.02)*10 ^{-3aD}	8.30 \pm 0.26 ^{aD}
	2.0	0.985 \pm 0.001 ^{aC}	41.67 \pm 1.21 ^E	(1.37 \pm 0.02)*10 ^{-3aE}	10.54 \pm 0.09 ^{aE}
	2.5	0.980 \pm 0.002 ^{aCD}	51.23 \pm 3.61 ^F	(1.69 \pm 0.04)*10 ^{-3aF}	12.64 \pm 0.13 ^{aF}
	3.0	0.984 \pm 0.002 ^{aD}	63.30 \pm 5.30 ^G	(2.18 \pm 0.16)*10 ^{-3aG}	14.68 \pm 0.33 ^{aG}
K: Na	0.1	0.995 \pm 0.002 ^{bA}	2.07 \pm 0.04 ^A	0.26 \pm 0.00 ^{bA}	1.08 \pm 0.08 ^{bA}
	0.5	0.989 \pm 0.002 ^{bB}	9.90 \pm 0.42 ^B	0.77 \pm 0.00 ^{bB}	3.74 \pm 0.21 ^{bB}
	1.0	0.991 \pm 0.001 ^{bB}	19.33 \pm 1.26 ^C	1.67 \pm 0.02 ^{bC}	6.44 \pm 0.17 ^{bC}
	1.5	0.985 \pm 0.004 ^{bC}	30.17 \pm 3.06 ^D	2.60 \pm 0.01 ^{bD}	9.12 \pm 0.19 ^{bD}
	2.0	0.985 \pm 0.001 ^{bC}	38.27 \pm 0.81 ^E	3.45 \pm 0.01 ^{bE}	11.86 \pm 0.2 ^{bE}
	2.5	0.980 \pm 0.002 ^{bCD}	50.63 \pm 3.10 ^F	4.23 \pm 0.07 ^{bF}	14.72 \pm 0.3 ^{bF}
	3.0	0.981 \pm 0.001 ^{bD}	63.10 \pm 4.27 ^G	5.17 \pm 0.02 ^{bG}	16.52 \pm 0.15 ^{bG}
Na	0.1	0.998 \pm 0.002 ^{bA}	2.57 \pm 0.68 ^A	0.39 \pm 0.00 ^{cA}	1.05 \pm 0.08 ^{cA}
	0.5	0.989 \pm 0.001 ^{bB}	10.44 \pm 1.50 ^B	1.60 \pm 0.01 ^{cB}	3.63 \pm 0.21 ^{cB}
	1.0	0.984 \pm 0.004 ^{bB}	19.37 \pm 0.97 ^C	3.56 \pm 0.02 ^{cC}	6.72 \pm 0.28 ^{cC}
	1.5	0.980 \pm 0.002 ^{bC}	28.37 \pm 1.51 ^D	5.53 \pm 0.03 ^{cD}	10.01 \pm 0.19 ^{cD}
	2.0	0.981 \pm 0.002 ^{bC}	39.87 \pm 2.00 ^E	7.65 \pm 0.00 ^{cE}	12.64 \pm 0.05 ^{cE}
	2.5	0.983 \pm 0.001 ^{bCD}	50.80 \pm 3.10 ^F	9.45 \pm 0.19 ^{cF}	16.20 \pm 0.24 ^{cF}
	3.0	0.984 \pm 0.002 ^{bCD}	59.10 \pm 3.05 ^G	11.07 \pm 0.06 ^{cG}	18.78 \pm 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 \pm 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 \pm 0.08	0.992 \pm 0.004	6.97 \pm 0.13	0.21 \pm 0.01	0.05 \pm 0.00	0.06 \pm 0.01	0.35 \pm 0.00	0.11 \pm 0.00
Na:K	0.5	80.37 \pm 0.24 ^{aA}	0.987 \pm 0.004 ^{aA}	6.81 \pm 0.01 ^{aA}	0.43 \pm 0.01 ^{aA}	0.12 \pm 0.01 ^{aA}	0.12 \pm 0.04 ^{aA}	0.48 \pm 0.09 ^{aA}	0.18 \pm 0.03 ^{aA}
Na:K	1.0	79.13 \pm 0.41 ^{aA}	0.984 \pm 0.003 ^{aAB}	6.71 \pm 0.04 ^{aA}	0.69 \pm 0.00 ^{aB}	0.19 \pm 0.00 ^{aB}	0.19 \pm 0.05 ^{aA}	0.56 \pm 0.12 ^{aA}	0.26 \pm 0.04 ^{aB}
Na:K	2.0	79.57 \pm 0.09 ^{aA}	0.980 \pm 0.005 ^{aB}	6.83 \pm 0.02 ^{aA}	1.29 \pm 0.06 ^{aC}	0.43 \pm 0.04 ^{aC}	0.42 \pm 0.03 ^{aB}	0.79 \pm 0.11 ^{bB}	0.47 \pm 0.03 ^{aC}
Na:K	3.0	78.32 \pm 0.41 ^{aB}	0.974 \pm 0.001 ^{aC}	6.69 \pm 0.02 ^{bB}	1.70 \pm 0.03 ^{aD}	0.57 \pm 0.02 ^{aD}	0.68 \pm 0.08 ^{aC}	1.17 \pm 0.14 ^{cC}	0.69 \pm 0.05 ^{aD}
Na	0.5	79.09 \pm 0.94 ^{aA}	0.985 \pm 0.002 ^{bA}	6.76 \pm 0.05 ^{bA}	0.50 \pm 0.01 ^{bA}	0.19 \pm 0.00 ^{bA}	0.26 \pm 0.04 ^{bA}	0.40 \pm 0.004 ^{bA}	0.22 \pm 0.01 ^{bA}
Na	1.0	80.47 \pm 0.88 ^{aA}	0.982 \pm 0.001 ^{bAB}	6.78 \pm 0.03 ^{bA}	0.82 \pm 0.06 ^{bB}	0.35 \pm 0.01 ^{bB}	0.38 \pm 0.07 ^{bA}	0.30 \pm 0.06 ^{bA}	0.30 \pm 0.03 ^{bB}
Na	2.0	78.67 \pm 0.14 ^{aA}	0.978 \pm 0.002 ^{bB}	6.67 \pm 0.04 ^{bA}	1.34 \pm 0.11 ^{bC}	0.76 \pm 0.06 ^{bC}	0.78 \pm 0.02 ^{bB}	0.40 \pm 0.04 ^{bB}	0.52 \pm 0.03 ^{bC}
Na	3.0	77.77 \pm 0.06 ^{aB}	0.969 \pm 0.001 ^{bC}	6.67 \pm 0.04 ^{bB}	1.90 \pm 0.03 ^{bD}	1.01 \pm 0.00 ^{bD}	1.09 \pm 0.11 ^{bC}	0.39 \pm 0.04 ^{bC}	0.71 \pm 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 ^1H 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 \pm 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	$61 \pm 1^{\text{aA}}$	$226 \pm 10^{\text{aA}}$	$87 \pm 2^{\text{aA}}$	$13 \pm 2^{\text{aD}}$
NaCl/ KCl	1.0	$67 \pm 1^{\text{aB}}$	$286 \pm 46^{\text{aB}}$	$96 \pm 1^{\text{aBD}}$	$4 \pm 1^{\text{aAC}}$
NaCl/ KCl	2.0	$71 \pm 1^{\text{aD}}$	$496 \pm 42^{\text{aD}}$	$99 \pm 0^{\text{aBC}}$	$1 \pm 0^{\text{aBC}}$
NaCl/ KCl	3.0	$68 \pm 2^{\text{aBC}}$	$342 \pm 15^{\text{aC}}$	$98 \pm 0^{\text{aCD}}$	$2 \pm 0^{\text{aAB}}$
NaCl	0.5	$59 \pm 1^{\text{bA}}$	$215 \pm 10^{\text{aA}}$	$85 \pm 1^{\text{bA}}$	$15 \pm 1^{\text{bD}}$
NaCl	1.0	$69 \pm 1^{\text{bC}}$	$366 \pm 48^{\text{bB}}$	$98 \pm 1^{\text{bB}}$	$2 \pm 1^{\text{bC}}$
NaCl	2.0	$67 \pm 0^{\text{bB}}$	$427 \pm 34^{\text{bD}}$	$99 \pm 0^{\text{aCD}}$	$1 \pm 0^{\text{aAB}}$
NaCl	3.0	$68 \pm 0^{\text{aBCD}}$	$423 \pm 34^{\text{bC}}$	$99 \pm 0^{\text{aD}}$	$1 \pm 0^{\text{aA}}$
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	R²	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; R²: coefficient of determination; RMSEP: Root Mean Square Error of Prediction

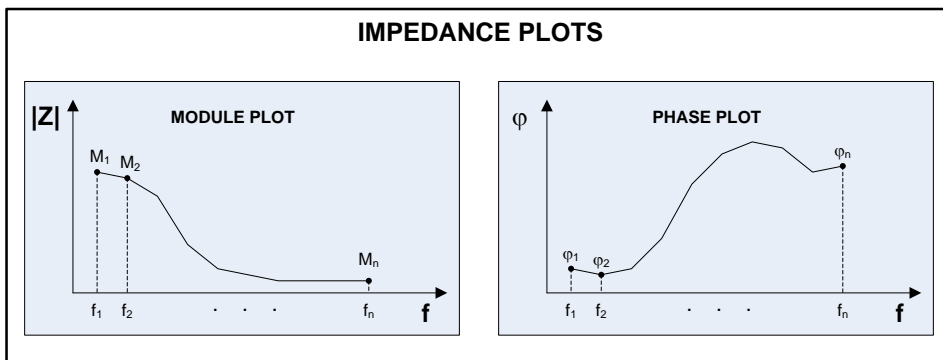
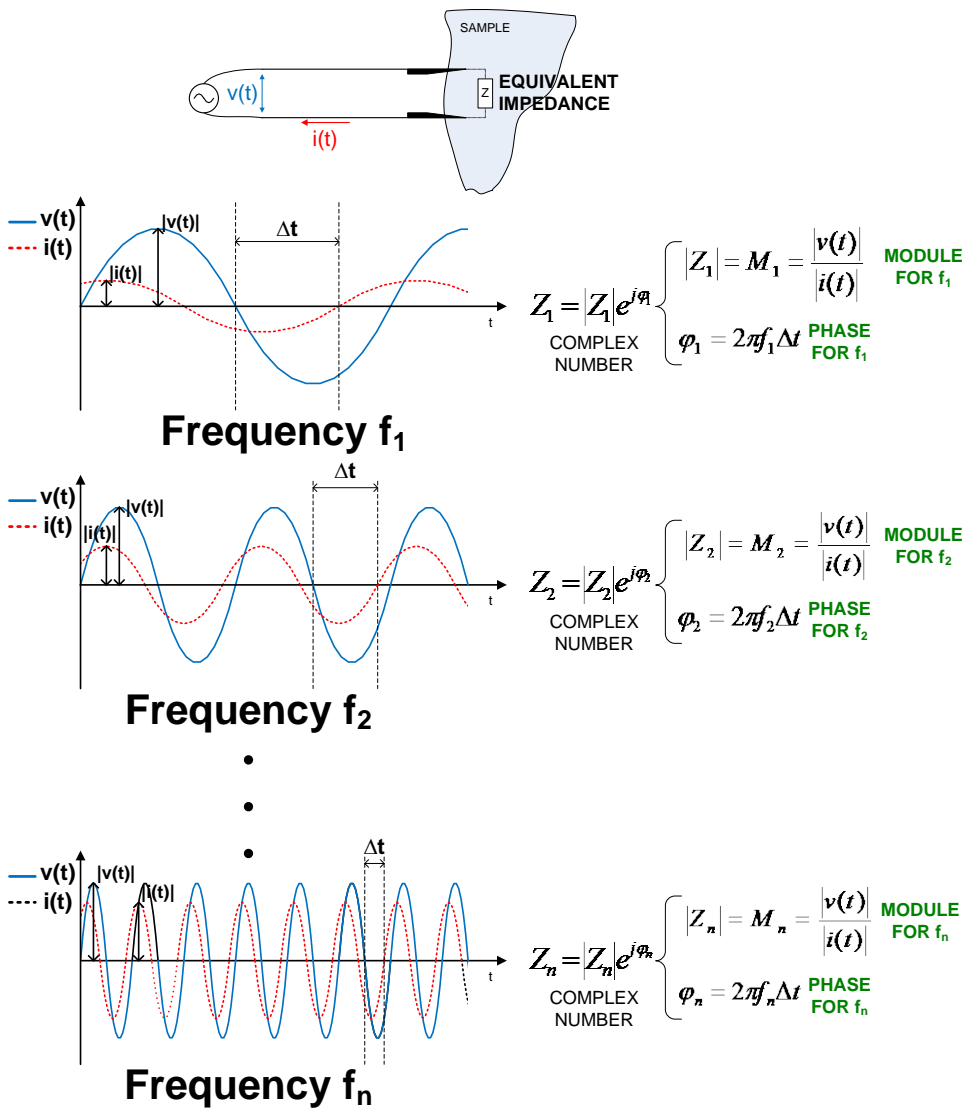


Figure 1

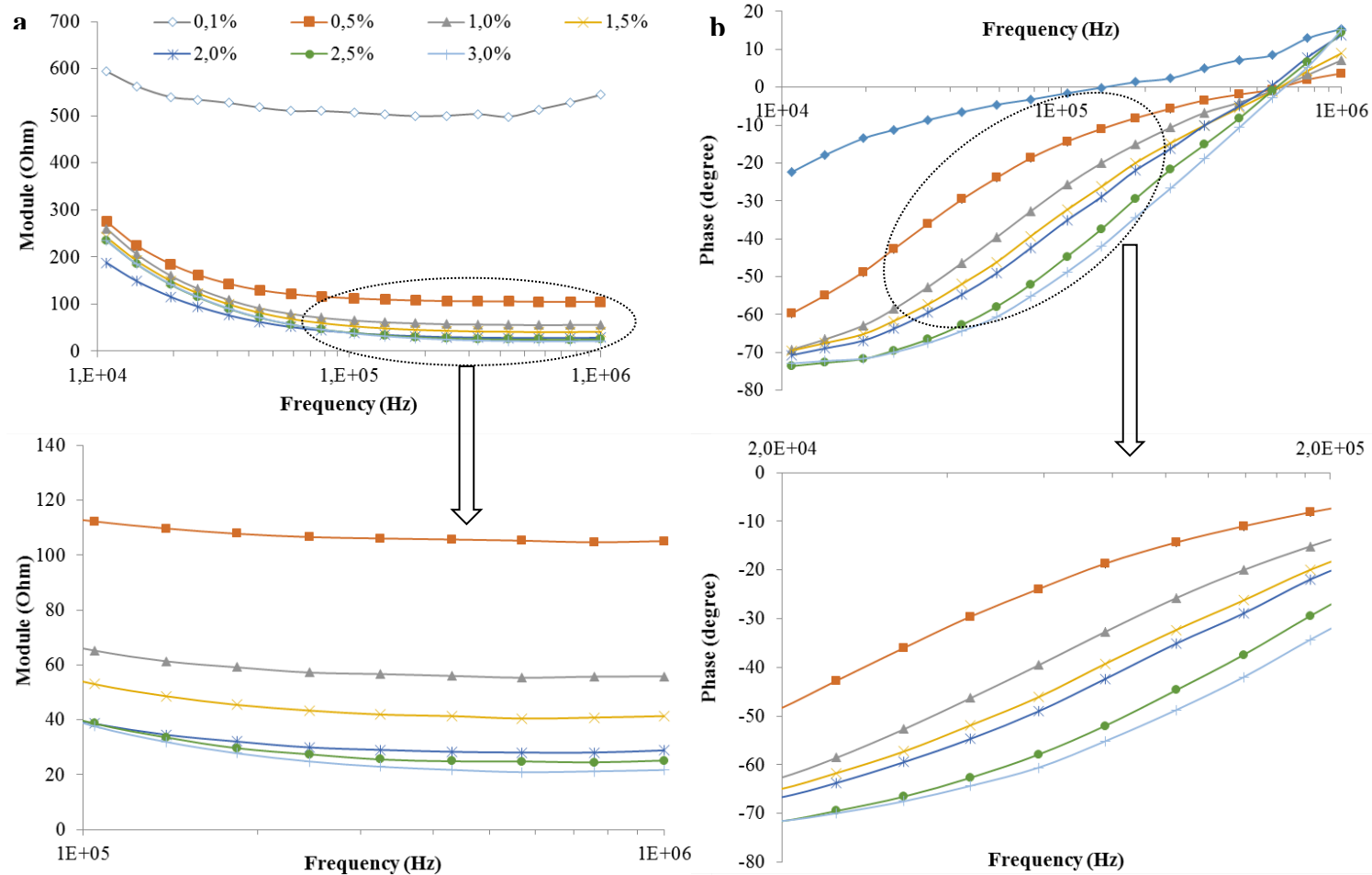


Figure 2

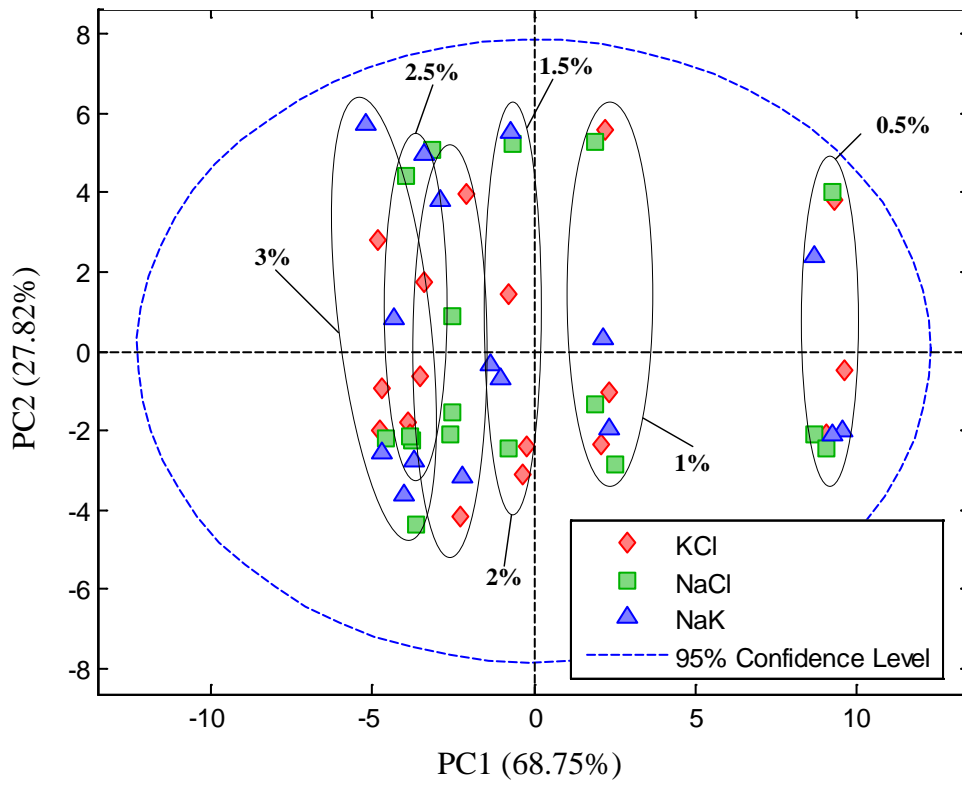


Figure 3

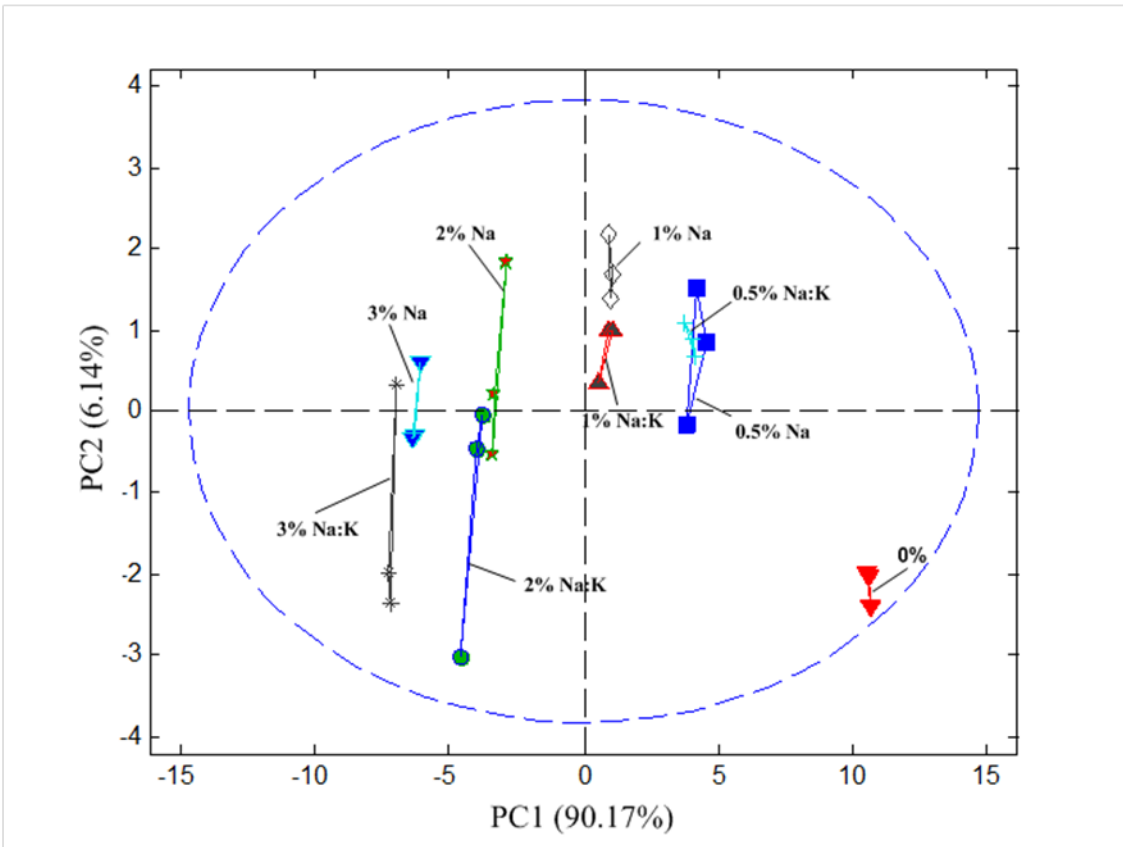


Figure 4

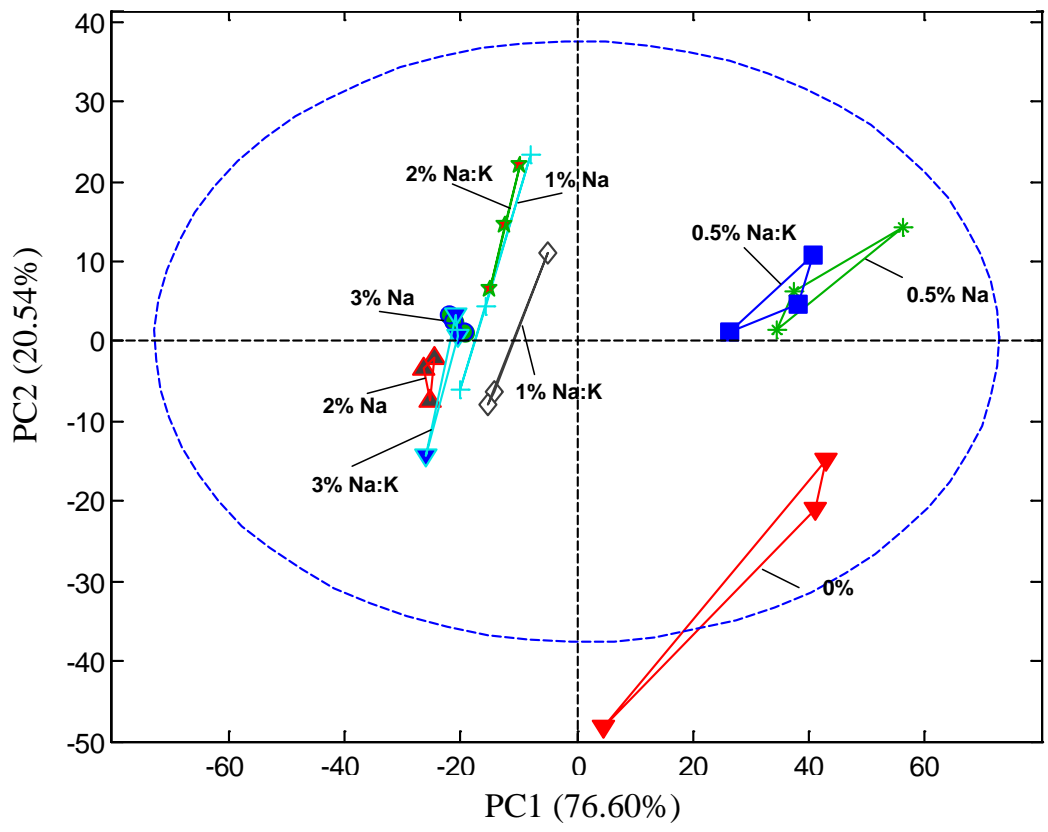


Figure 5

TOC graphic

