Functional aptitude of hake minces with added TMAO-demethylase inhibitors during frozen storage

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ABSTRACT

The ability of compounds of natural origin (black, white, red, and green tea extracts, phytic acid) to inhibit TMAO-demethylase enzyme was assayed. Black tea and phytic acid exerted the highest inhibiting activities, similar to the already known inhibitor sodium citrate. Hake minces incorporating these three compounds were prepared and stored frozen (150 days, –12 °C). TMAO-demethylase enzyme was partially inhibited (lower enzyme activity, reduction of formaldehyde accumulation). The study of physicochemical properties of the minces (salt-soluble proteins, water holding capacity, structural water associated with myofibrils) pointed to evident protein aggregation and loss of functionality when phytic acid was added, whereas black tea and sodium citrate did not have a negative effect. Consequently, the salt-ground mince with phytic acid showed worse viscoelastic properties than the others. In conclusion, black tea polyphenols and sodium citrate can be used as additives to inhibit TMAO-demethylase enzyme during frozen storage of fish minces.

Keywords

Protein aggregation; formaldehyde; tea extract; gelation; fish muscle

1. INTRODUCTION

Freezing is extensively used to preserve fish from microbial and enzymatic spoilage during long periods. However, it is well known that fish muscle spoils during frozen storage because of muscle hardening derived from protein aggregation and, depending on the fish species, because of the development of rancid flavors derived from lipid oxidation (Gómez-Estaca, Giménez, Gómez-Guillén, & Montero, 2010; Tejada, Huidobro, & Mohamed, 2003). The main cause of muscle freeze-induced protein aggregation is the progressive dehydration of proteins as ice crystals form and grow, along with the consequent increase in the salt concentration in the liquid phase (Bigelow & Lee, 2007; Cheung, Liceaga, & Li-Chan, 2009), both resulting in the formation of intermolecular cross-links (Bigelow & Lee, 2007). Other factors influencing the loss of muscle protein quality during frozen storage are the accumulation of dimethylamine and formaldehyde derived from the demethylation of trimethylamine oxide (TMAO) catalyzed by the trimethylamine oxide demethylase enzyme, as well as the accumulation of lipid oxidation products (aldehydes), depending on the fish species. The accumulation of formaldehyde is especially important in gadoids, whereas that of lipid oxidation products is of importance in fatty fish (Leelapongwattana, Benjakul, Visessanguan, & Howell, 2005; Saeed & Howell, 2002; Sikorski & Kostuch, 1982; Sotelo, Gallardo, Piñeiro, & Pérez-Martin, 1995).

The quality of frozen fish depends on intrinsic factors such as species and season, as well as on technological factors such as handling practices prior to freezing, freezing rate, storage temperature, etc. (Careche, Herrero, Rodríguez-Casado, Del Mazo, & Carmona, 1999). Apart from this, in order to improve quality and shelf life of frozen fish, novel treatments have also been applied. Some of these strategies are based on the addition of antioxidants that retard lipid oxidation; cryoprotective agents such as sorbitol, sucrose, or protein hydrolysates that prevent ice crystal growth and protein dehydration; polyphosphates that improve water-binding capacity and protein solubility; sodium alginate that chelates calcium ions responsible for cross-

53 linking; or soy protein isolate that reduces free water available for ice crystallization (Badii &
54 Howell, 2002; Bigelow & Lee, 2007; Cheung, et al., 2009). Another alternative to improve frozen
55 fish quality could be the inhibition of TMAO demethylase enzyme, resulting in the reduction or
56 inhibition of formaldehyde accumulation, consequently retarding protein aggregation and loss
57 of functionality (Leelapongwattana, Benjakul, Visessanguan, & Howell, 2008, 2010). In this
58 regard, several compounds have been assayed in order to inhibit the activity of TMAO
59 demethylase, such as sodium citrate, pyrophosphate, H_2O_2, or hydrocolloids such as sodium
60 alginate, xanthan gum, or carrageenan (Da Ponte, Roozen, & Pilnik, 1986; Leelapongwattana, et
61 al., 2008, 2010; Parkin & Hultin, 1982). The mechanism of action of many of these additives
62 seems to be related to the chelating effect on ferrous ion, which is located in the active site of
63 the enzyme and is also required for TMAOase activity (Leelapongwattana, et al., 2008). Despite
64 this, the body of literature in this regard is quite scarce, and research on new compounds,
65 especially those of natural origin, could be of great interest. Polyphenols are known to be good
66 metal chelators (Bravo, 1998), so we hypothesize that they could be potential candidates to
67 inhibit TMAO demethylase enzyme, improving fish quality and gelation ability during frozen
68 storage. Tea (*Camellia sinensis*), a common beverage consumed worldwide, is well known for
69 inducing health benefits because it contains phenolic compounds and derivatives with biological
70 properties against a number of chronic diseases (Sanlier, Gokcen, & Altuğ, 2018). Catechins
71 predominate in the phenolic composition of unfermented tea (green tea) or slightly fermented
72 tea (white tea), while theaflavins and thearubigins (oxidized phenolic species) accumulate during
73 fermentation, reaching a maximum in fully fermented black tea (Wang & Ho, 2009). Besides
74 their radical scavenging capacity, green tea catechins and black tea theaflavins are also
75 recognized for possessing well-established iron-chelating properties (Hatcher, Singh, Torti, &
76 Torti, 2009). Similarly, phytic acid (inositol hexaphosphate), which is ubiquitous in eukaryotic
77 cells, could also be a possible natural candidate to inhibit TMAO demethylase enzyme, owing to
78 its strong iron affinity (Nielsen, Tetens, & Meyer, 2013). To the best of our knowledge, the

79 inhibiting activity of polyphenols and phytic acid on TMAO demethylase enzyme has not yet
80 been reported. Therefore the objective of the present work was to study the TMAO demethylase
81 inhibiting activity of several chelating agents, with special emphasis on those of natural origin
82 (tea polyphenolic extracts and phytic acid), and their impact on hake mince protein functional
83 aptitude, including gelation ability, during frozen storage.

84 **2. MATERIALS AND METHODS**

85 **2.1. Materials and reagents**

86 Four hakes (*Merluccius merluccius*), each weighing 3–4 kg, were acquired at a local market in
87 Madrid 36 h after fishing and immediately transported to ICTAN. TMAO was from Sigma-Aldrich
88 (Madrid, Spain). Phytic acid and sodium citrate were from Sigma-Aldrich (Madrid, Spain). White,
89 green, red, and black teas were acquired in a local market.

90 **2.2. Preparation and partial purification of TMAOase**

91 The partially purified enzyme extract was prepared following the method described by Benjakul,
92 Visessanguan, and Tanaka (2004). For this purpose, finely chopped viscera or minced muscle of
93 hake (*Merluccius merluccius*) were extracted with 3 volumes of chilled 20 mM Tris-acetate buffer
94 (pH 7), containing 0.1 M NaCl and 0.1% Triton X-100, in an Omnimixer-Homogenizer (model
95 17106, OMNI International, Waterbury, USA). The homogenates were centrifuged at 38,500×*g*
96 at 4 °C for 30 min (Sorvall Combiplus, Dupont, Wilmington, DE, USA), and the supernatants were
97 partially purified by acidification. For this purpose, they were mixed at a 1:1 ratio (v:v) with 0.1
98 M Na-acetate buffer, pH 4.5, centrifuged at 38,500×*g* at 4 °C for 30 min, and the supernatants
99 were neutralized to pH 7 with NaOH. The neutralized supernatants were centrifuged again
100 (38,500×*g*, 4 °C, 30 min) to remove undissolved debris. The supernatants obtained were used as
101 crude enzyme extract (partially purified TMAOase) for: (i) enzyme inhibiting experiments, (ii)

addition to the minces (extracts obtained from viscera), and (iii) determination of residual TMAOase activity in the minces during frozen storage (extracts obtained from minced muscle).

2.3. Determination of TMAO demethylase activity

The method described by Benjakul, et al. (2004) was used, with slight modifications. To 2.5 mL of assay buffer (24 mM Tris-acetate, 24 mM TMAO, 2.4 mM ascorbate, 0.24 mM $FeCl_2$, and 0.12 M NaCl, pH 7), 0.5 mL of properly diluted partially purified enzyme extract was added. The reaction was performed at 25 °C for 20 min, after which 1 mL of 10% trichloroacetic acid was added to terminate the reaction. The pH of the enzyme reaction was checked (6.8 ± 0.15) and no significant differences ($p \leq 0.05$) were observed among samples. The reaction mixture was centrifuged at 8,000×*g* for 30 min and the supernatant was subjected to formaldehyde determination. One unit of TMAOase was defined as the activity that released 1 μmol of formaldehyde per minute.

2.4. Extraction and determination of formaldehyde

Formaldehyde was extracted by steam distillation in the presence of phosphoric acid as described by Rehbein (1987). Formaldehyde determination was performed by means of the Hantzsch reaction as described by Nash (1953).

2.5. *In vitro* enzyme inhibiting assay

The TMAO-demethylase inhibiting activity of sodium citrate, phytic acid, and various types of tea extracts (white, green, red, and black) was evaluated. Sodium citrate and phytic acid were dissolved in distilled water (5 g/100 mL) by magnetic stirring for 1 h at room temperature. Tea extracts (5 g/100 mL) were prepared by infusion at 90 °C for 15 min in a thermostatic bath and filtration through Whatman No. 1 filter paper. For enzyme inhibiting assays, to 2.5 mL of assay buffer (24 mM Tris-acetate, 24 mM TMAO, 2.4 mM ascorbate, 0.24 mM $FeCl_2$, and 0.12 M NaCl,

125 pH 7), 0.5 mL of properly diluted partially purified enzyme extract from viscera and 0.12 mL of
126 various dilutions of enzyme inhibiting solutions or extracts were added. The reaction was
127 performed at 25 °C for 20 min, after which 1 mL of 10% trichloroacetic acid was added to
128 terminate the reaction. The reaction mixture was centrifuged at 8,000×*g* for 30 min and the
129 supernatant was subjected to formaldehyde determination. As positive control, a sample was
130 prepared following the same procedure but with the addition of 0.12 mL of distilled water
131 instead of enzyme inhibiting solution. Results have been expressed as IC_{50}, which is the
132 concentration of inhibiting agent needed to reduce the initial enzyme activity by 50%. In the
133 case of the tea extracts, IC50 is calculated based on the amount of leaves (w/v) used to prepare
134 the extracts.

135 **2.6. Mince preparation**

136 Hakes were headed, gutted, skinned, filleted, and washed with cold tap water.Chopped muscle
137 was ground with 1.5% NaCl and divided into 1250 g aliquots, which were mixed with partially
138 purified enzyme extract from viscera (1160 units/Kg mince) in a Stephan blender at 2 °C for 1
139 min, in order to magnify the subsequent TMAO activity. Afterwards, the various enzyme
140 inhibitors, cooled to 3 °C, were added to achieve a final concentration of 3.7 g/Kg mince and
141 mixed for 2 min. According to the results of the *in vitro* inhibiting assay, four different batches
142 were produced: (i) control without enzyme inhibitor (C), (ii) with phytic acid (PA), (iii) with
143 sodium citrate (SC), and (iv) with black tea extract (BT). All batches were vacuum-packed in 250
144 g aliquots in flexible bags (type BB4L, Cryovac, Barcelona, Spain), placed in stainless steel trays,
145 and frozen in a horizontal plate freezer (Sabroe, Aarhus, Denmark) at –40 °C for 2 h. All batches
146 were subsequently stored at –12 °C in order to simulate temperature abuse conditions and allow
147 magnification of the changes in formaldehyde accumulation (Sotelo, Aubourg, Perezmartin, &
148 Gallardo, 1994).

149 **2.7. Color determination**

150 For mince color determination, the minces were allowed to thaw and then placed in a glass
151 sample container, and the color coordinates L* [black (0) to white (100)], a* [green (–) to red
152 (+)], and b* [blue (–) to yellow (+)] were obtained with a Konica Minolta CM-3500d
153 spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan). Simple transformations were
154 used to convert a* and b* coordinates to C* and h° chromatic parameters. Total color
155 differences (ΔE) from control mince and whiteness index (WI) were calculated as shown below
156 in Eqs. 2 and 3, respectively:

157 Eq. 2

158 Eq. 3

159 where L^*, a^*, and b^* are the values of these parameters for each sample, and L^*_c, a^*_c, and b^*_c are
160 those of the control sample.

161 **2.8. Protein solubility and water holding capacity**

162 Protein solubility was determined as previously described by Gómez-Estaca, et al. (2010) and
163 expressed as g soluble protein/100 g protein present in the muscle. Water holding capacity was
164 determined as described by Gómez-Guillén, Montero, Hurtado, and Borderías (2000) and results
165 expressed as g water retained/100 g water present in the muscle.

166 **2.9. Protein aggregate size measurement and ζ-potential**

167 An amount of 1 g of muscle was homogenized with cold 0.8 M NaCl for 1 min in an Omnimixer-
168 Homogenizer (model 17106, OMNI International, Waterbury, USA) immersed in an ice/water
169 bath. The homogenates were centrifuged (6,000×*g*, 2 °C, 15 min) in a Sorvall Evolution RC
170 Centrifuge (Thermo Fisher Scientific Inc., Landsmeer, The Netherlands) and the supernatants
171 were used for analyses. Particle size and ζ-potential of the soluble protein fraction were
172 determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) at ≤5 °C.

Z-average was measured by dynamic light scattering (DLS), and ζ-potential by laser Doppler velocimetry through the electrophoretic mobility, provided by the Hückel approximation. All samples were diluted with 0.2 M phosphate buffer (pH 7.0) to a final concentration of 0.01 mg/mL, and the results were the means of at least 10 replicates.

2.10. Low-Field Proton Nuclear Magnetic Resonance (LF-NMR H^1)

Relaxometry analysis was carried out according to Sánchez-Alonso, Moreno, and Careche (2014). Portions of mince measuring 1 × 1 × 2 cm and weighing 2 g were placed in NMR tubes (1.8 cm diameter and 18 cm height) and sample temperature was kept at 4 °C using a Thermo Haake C/DC class DC10-K10 refrigerated circulator (Fisher Scientific S.L., Madrid, Spain). Transverse relaxation data (T2) were determined in a Low-Field Nuclear Magnetic Resonance Minispec mq20 analyzer (Bruker Optik GmbH, Germany) with a magnetic field strength of 0.47 T (proton resonance frequency of 20 MHz). Relaxation time distribution was analyzed using the CONTIN regularization algorithm employing the Carr–Purcell–Meiboom–Gill pulse sequence with a τ-value of 150 μs, and 16 scans at 2 s intervals with a total of 3,000 echoes were obtained per sample. At least three replicates were measured per sample.

2.11. Viscoelastic properties and gelling capacity

The salt-ground muscles were obtained by homogenizing the minces with NaCl for 2 min to a final concentration of 1.5 g/100 g mince, using a domestic homogenizer (Braun, Germany) immersed in an ice/water bath. Batter moisture was adjusted to 80% with the required amount of ice. Viscoelastic properties of the batters (elastic modulus G' and viscous modulus G") were determined using a Bohlin rheometer (Bohlin Instruments Ltd., model CVO, Worcestershire, UK) with a cone-plate geometry (cone angle 4°, gap=0.15 mm). A dynamic frequency sweep was carried out at 10 °C over the frequency range 0.1–10 Hz (γ=0.005). A dynamic temperature

196 sweep was done at 1 Hz by heating from 10 °C to 85 °C at a scan rate of 1 °C/min (γ=0.005). At
197 least two replicates per sample were measured.

198 **3. RESULTS AND DISCUSSION**

199 **3.1. *In vitro* TMAO demethylase inhibiting assay**

200 The compound showing the highest enzyme inhibiting activity was phytic acid (95.2 ± 4.8)
201 ($p \leq 0.05$), followed by black tea extract (116.9 ± 5.8) and sodium citrate (114.7 ± 5.7), which
202 showed similar values ($p > 0.05$). The red, white, and green tea extracts were significantly less
203 active than the other compounds studied ($p \leq 0.05$), showing IC_{50} values of 234.1 ± 11.7, 268.1 ±
204 13.4, and 268.9 ± 13.3, respectively. The inhibition exerted by sodium citrate in the present work
205 agrees with previous results obtained by Leelapongwattana, et al. (2008). The authors cited
206 studied the TMAO demethylase inhibiting activity of a number of potentially inhibiting
207 compounds, finding that sodium pyrophosphate, sodium citrate, and sodium alginate showed
208 the highest activities, in decreasing order. The proposed mechanism of action of the three
209 compounds was a chelating or complexing effect on ferrous or ferric ions. To the best of our
210 knowledge, there are no previous reports on the inhibiting activity of phytic acid or polyphenolic
211 extracts on TMAO demethylase enzyme. The mechanism of action of the natural inhibitors used
212 in the present work (tea polyphenols and phytic acid) would most probably also be iron ion
213 chelation (Hatcher et al., 2009; Gupta et al., 2013). Interestingly, among the various tea types
214 tested, there seems to be a positive relationship between the presumably higher oxidation level
215 of polyphenols in black tea and its higher TMAOase inhibiting activity, probably favored by a
216 higher Fe chelating activity (Wang & Ho, 2009).

217 **3.2. Physicochemical changes of hake minces with enzyme inhibitors during frozen storage**

218 Among the various inhibitors studied, black tea, phytic acid, and sodium citrate were selected
219 to be included in the hake minces. The optical properties of the minces are shown in Table 1.

220 Sodium citrate did not produce any change in optical properties (p>0.05) as compared to the
221 control batch. In contrast, significant changes (p≤0.05) were observed with the other enzyme
222 inhibitors, which were especially evident in the batch with black tea, resulting in decreased
223 lightness, higher chromaticity, and a change in hue angle toward the blue region. These
224 variations are clearly reflected in the total color difference and also resulted in a decrease in the
225 whiteness index (Table 1).

226 The results of TMAO demethylase activity of hake minces with enzyme inhibitors during frozen
227 storage are shown in Figure 1A. All samples showed a similar trend during storage, i.e., an
228 increase in activity during the first month followed by a decrease as storage continued.
229 Leelapongwattana, et al. (2008) also reported this trend when studying the TMAO demethylase
230 activity of minced lizardfish muscle with the addition of enzyme inhibitors during frozen storage.
231 According to those authors, this effect may be the result of the disruption of cell membranes at
232 the beginning of frozen storage induced by ice crystals, followed by a decrease in activities,
233 possibly because of denaturation of TMAO demethylase and lower extraction efficacy resulting
234 from cold-induced protein aggregation during frozen storage. The batches with phytic acid and
235 black tea generally showed lower values than the control batch during storage (p≤0.05), pointing
236 to a small degree of enzyme inhibition. In contrast, the batch with sodium citrate showed higher
237 enzyme activity than the control (p≤0.05). This result was unexpected, as it does not agree with
238 the *in vitro* inhibiting results. A possible explanation may be an improvement in enzyme
239 extraction due to cell disruption and muscle protein denaturation in the presence of this salt.

240 The accumulation of formaldehyde, which could be an indirect measure of the TMAO
241 demethylase activity, is shown in Figure 1B. From the first month of storage onwards, the
242 formaldehyde content in the samples with enzyme inhibitors showed a tendency towards lower
243 values than in the control batch, although differences were not always significant (p≤0.05),
244 suggesting a reduction in enzyme activity. The batch in which the lowest formaldehyde

245 accumulation was observed was the one with phytic acid (PA), followed by black tea (BT) and
246 sodium citrate (SC). These results seem to confirm that the apparent increase in enzyme activity
247 observed in the SC batch (Figure 2) might be due to an improvement in enzyme extraction, as
248 discussed, rather than a real increase in enzymatic activity in the mince. Leelapongwattana, et
249 al. (2008) studied the TMAO demethylase inhibitory effect of sodium alginate, pyrophosphate,
250 and mixtures of them added to minced lizardfish, finding a reduction both in enzyme activity
251 and in formaldehyde accumulation. However, the inhibitory effect observed by those authors
252 was higher than that found in the present work. The differences must be attributed to the
253 different fish species, freshness, enzyme inhibitors assayed, storage temperature, sample
254 preparation, etc.

255 The results of salt-soluble protein and water holding capacity (WHC) of hake minces containing
256 the various TMAOase inhibitors during frozen storage are depicted in Figures 1C and 1D,
257 respectively. Initially, muscle protein solubility was around 74% in the control batch. It
258 decreased sharply to 43% during the first 15 days of storage, and then decreased further to 33%
259 by the end of the storage period. A similar biphasic pattern of fish myofibrillar protein
260 denaturation during frozen storage has been reported previously (Jiang & Wu, 2018). The early
261 sharp decrease was attributed to a pronounced change in muscle protein conformation and
262 aggregation due to strong water recrystallization at the relatively high storage temperature used
263 (–12°C). This temperature of abuse was selected in order to maximize the accumulation of
264 formaldehyde, as its formation is directly related to frozen storage temperature (Sotelo, et al.,
265 1994). Sodium citrate and, more intensely, phytic acid reduced protein solubility significantly
266 ($p \leq 0.05$) at day 15 as compared to the control batch and the batch with black tea extract. No
267 significant differences were observed during the subsequent storage period, in which all batches
268 exhibited rather low salt-soluble protein. The water holding capacity decreased with storage
269 time in all batches ($p \leq 0.05$). Unlike the protein solubility, a pronounced drop in WHC during the
270 first 15 days was not observed, probably because the initial protein quality was not ideal, and

271 the muscle might have exuded weakly bonded water before and during the mince preparation.
272 Phytic acid reduced WHC greatly from day 15 onwards, in contrast to sodium citrate and black
273 tea extract, which showed values close to the control batch.

274 In order to obtain deeper knowledge about the quality of the myofibrillar proteins during frozen
275 storage, the evolution of the size and net charge of soluble aggregates was monitored (Bao,
276 Boeren, & Ertbjerg, 2018; Chihi, Mession, Sok, & Saurel, 2016) and shown in Figure 2. Figure 2A
277 shows the changes that occurred in the C batch during frozen storage, whereas Figures 2B, 2C,
278 2D, and 2E show the changes at days 15, 30, 90, and 150, respectively, depending on the enzyme
279 inhibitor added. At day 0 of storage, the soluble fraction of the C batch consisted mainly of
280 protein aggregates peaking around 700 nm, although fractions of lower (≈130 nm) and higher
281 (≈5.5 μm) average size were also observed, the latter coinciding with the detection limit of the
282 equipment. After 15 days of storage, a noticeable decrease in intensity of the main population
283 (≈700 nm) was concomitant with an increase in 5.5 μm particles, both events denoting protein
284 rearrangement into larger microaggregates that were still present in the soluble protein fraction.
285 As storage continued, the mean particle size of the main soluble aggregates showed a
286 progressive downward tendency, reaching a value near 250 nm at day 150 of storage; at the
287 same time, the intensity of the largest aggregates also tended to decrease with time (Figure 2A).
288 Although there is no specific literature on the effect of frozen storage of fish on the size of
289 soluble aggregates, the results obtained here are consistent with progressive protein
290 aggregation, with disruption of the original protein aggregates and formation of larger particles
291 that tended to leave the soluble fraction as frozen storage continued. Vate and Benjakul (2016)
292 reported an average particle size of 513 nm in heated natural actomyosin solution from sardine,
293 which upon addition of protein cross-linkers increased to 645 nm as a result of protein
294 aggregation. However, comparisons with the present work are difficult because the above-
295 mentioned natural actomyosin was previously heated and changes in particle size distribution
296 were not shown.

297 With regard to the effect of the various enzyme inhibitors, phytic acid was the one that induced
298 the greatest changes, at day 15 causing a marked reduction in the average size of soluble
299 aggregates as compared to the other samples. This was due not only to a shift of the most
300 abundant protein aggregates to a smaller size, but also to a reduction in the amount of larger
301 soluble microaggregates, which presumably went into the insoluble protein fraction (Figure 2B).
302 At the same day of storage, the most abundant protein fraction in SC also shifted toward a lower
303 particle size and the abundance of the 5.5 μm population decreased, as compared to the control
304 and BT batches (Figure 2B). All these events suggested considerable protein insolubilization at
305 the early stage of storage in both PA and SC batches, in agreement with the protein solubility
306 results (Figure 1C). During storage, the average size of the aggregates in the three batches
307 treated with enzyme inhibitors showed a downward tendency, as described for the control, and
308 at day 150 of storage only minor differences were observed between them and the control batch,
309 as observed before for protein solubility (Figure 1C).

310 The effect of frozen storage on the net charge of the soluble protein aggregates from the various
311 minces is shown in Figure 2F. All samples presented an electronegative ζ-potential, attributed
312 to the abundance of acidic amino acids in the fish muscle, which are largely deprotonated at
313 neutral pH. The ζ-potential of the control batch at day 0 was slightly lower than that reported
314 previously in salt-ground hake muscle (–20.8 mV) (Marín-Peñalver, Alemán, Montero, & Gómez-
315 Guillén, 2018), but slightly higher than in heated natural actomyosin from sardine (–13.0 mV)
316 (Vate & Benjakul, 2016). These differences could be related to different protein aggregation
317 status. Furthermore, coinciding with increasing protein aggregation, the protein net charge
318 tended to decrease ($p \leq 0.05$) in all batches during storage, and PA was the batch that exhibited
319 the lowest ζ-potential. According to Vate and Benjakul (2016), aggregation of protein molecules
320 had an impact on the surface charge of the aggregates by masking the charged amino acids,
321 which remain inside the protein complexes.

322 LF-NMR H^1 has been used as a tool to evaluate deeper changes in structural water associated
323 with morphological changes in protein (Sánchez-Alonso, et al., 2014). Figure 3 shows the
324 transversal proton relaxation time curves in the 10–1000 ms range of the various hake minces
325 during frozen storage. At day 0 of storage, the control sample showed a main T_{21} relaxation band,
326 indicative of water located predominantly in the intra-myofibrillar space (Figure 3A). This finding
327 would be compatible with muscle that has been frozen in adequate conditions (fast freezing)
328 and stored during a short period of time (Sánchez-Alonso, Martinez, Sánchez-Valencia, &
329 Careche, 2012; Sánchez-Alonso, et al., 2014). During storage, the T_{21} band gradually lost
330 amplitude and shifted toward lower relaxation times, which indicates a decrease in the spacing
331 between the myofibrils; at the same time, a slower relaxation component (T_{22}) appeared,
332 corresponding to extra-myofibrillar water, resulting from freeze-induced morphological changes
333 in protein (Sánchez-Alonso, et al., 2012). These changes were indicative of protein denaturation
334 and were consistent with the loss of protein solubility and water holding capacity during frozen
335 storage. In the samples with enzyme inhibitors (Figure 3 B–E), again, from day 15 onward, phytic
336 acid induced the greatest changes in structural water, showing an intense migration of water
337 protons the intra- to extra-myofibrillar space, in agreement with the strong protein aggregation.
338 As with the control batch, the T_{22} component showed a noticeable tendency to increase in all
339 batches during the storage period. After 90 days, the BT batch was apparently the least affected,
340 but at the end of storage no great differences were found among the various batches.

341 **3.3. Gel forming capacity of hake minces with added enzyme inhibitors during frozen storage**

342 In order to determine the effect of the various additives on the viscoelastic properties of the
343 salt-ground muscle, as a previous and necessary step for protein gelation a frequency sweep
344 test was carried out initially (in the control mince without additives at day 0) and at days 15 and
345 150 of frozen storage (Figure 4). The elastic modulus G' was greater than the viscous modulus
346 G" throughout the whole frequency range in all the samples tested, denoting a typically

predominant solid-like behavior, regardless of the additive or the storage period. All mechanical spectra in terms of G' fitted the power law model very well (R^2≥0.99). The n' exponent values of the corresponding equations are also shown in Fig. 4. According to Campo and Tovar (2008), the smaller the value of n', the greater the matrix stability. Within the first 15 days of storage, the n' value in the control batch (without inhibitors) increased from 0.134 to 0.149, but it registered the lowest value as compared to the minces with added inhibitors, indicating that at the early stage of storage this was the most stable batter. In contrast, the PA batch presented the highest degree of matrix instability (n' = 0.233), which coincided with the considerable early protein aggregation described in section 3.2. At the end of storage, G' and G" values increased considerably in all batches, but much more intensely in PA, in agreement with its more intense protein aggregation and lower matrix stability. No great differences in the viscoelastic behavior of the respective salt-ground batters were observed in SC and BT batches at the end of the storage period, and it was very similar to that of the control batch (without additives).

The thermal gelation profile of the salt-ground muscle without and with inhibitors during frozen storage is shown in Figure 5. Figure 5A shows the changes in G' as a function of the heating temperature of the control batch (without inhibitors) during the whole storage period. During the first 15 days of storage the control batch presented a sharp increase in G' between 30 and 39 °C, indicating a strong setting phenomenon (Figure 5A). This peak is largely attributed to the result of endogenous muscle transglutaminase activity that leads to the formation of ε-(γ-glutamyl)-lysine covalent bonds (Lanier, Yongsawatdigul, & Carvajal-Rondanelli, 2013). After that, the pronounced drop in G' to ≈47 °C could be ascribed to the activity of indigenous proteolytic enzymes causing a breakdown in the preformed protein network, constituting the so-called *modori* phenomenon (Ueki, Wan, & Watabe, 2016). This phenomenon has been also attributed to the destruction of alpha-helix and unwinding of coiled-coil structure of myosin rod (Fukushima, Satoh, Nakaya, Ishizaki, & Watabe, 2003). From 47 °C upwards the progressive rise in G' indicated continuous thermal aggregation of muscle proteins. Similar viscoelastic behavior

of salt-ground hake mince upon heating from 5 to 80 °C has been reported previously (Marín-Peñalver, et al., 2018). Interestingly, at days 0 and 15 the maximum values of G' registered at the end of the heating ramp did not exceed those of the setting peak, probably because of strong residual proteolytic activity. In contrast, after the first month of storage both setting and *modori* phenomena clearly tended to be less pronounced or even disappeared at the end of 150 days. This effect could be related to the loss of enzyme activities during frozen storage. Furthermore, values of G' in the thermal profiles tended to be higher with storage time, and the onset of thermal aggregation also showed a slight down-shift, both effects being compatible with progressive freeze-induced muscle protein aggregation and protease inactivation. After 150 days of storage, the pronounced increase in the G' values from the onset of the heating ramp indicated that the protein was highly aggregated, but it did not lose its thermal aggregation ability.

This pronounced effect of initial protein aggregation on rheological behavior during heating was also observed in the PA batch in the early stage of frozen storage (Figure 5B), where setting and *modori* were not clearly evidenced. In contrast to the SC and BT batches, the initially high degree of protein aggregation induced by phytic acid at day 15 also resulted in a greater increase in G' as a result of thermal aggregation. However, this effect was considerably reduced after 30 days (Figure 5C), so by the end of the storage period this batch had almost completely lost its thermal gelation ability. The early reduction in protein solubility and WHC induced by the interaction of phytic acid with the muscle proteins and the noticeable decrease in water binding properties of this batch during the entire storage period led to a final collapse of the protein network upon heating. In contrast, the evolution of the gelation profile in the presence of sodium citrate or black tea was quite similar to that of the control batch during 150 days of storage.

4. CONCLUSION

The ability of sodium citrate, tea polyphenolic extracts, and phytic acid to inhibit the activity of TMAO-demethylase enzyme was evidenced *in vitro* and subsequent evaluated *in vivo* during frozen storage of hake mince (–12 °C/150 days). Results from enzyme activity and formaldehyde accumulation during frozen storage evidenced a discrete inhibition, which was more intense for phytic acid and black tea polyphenolic extract. The study of the salt-soluble proteins (total amount, net charge, and size of aggregates), water holding capacity, and structural water associated with myofibrils pointed to evident protein aggregation and loss of functionality when phytic acid was added to the hake minces, whereas black tea polyphenols and sodium citrate did not have a negative effect. This resulted in a worsening of the viscoelastic properties of the salt-ground muscle for the mince with added phytic acid, whereas the other additives had a negligible effect. All the salt-ground minces were able to form thermally induced gels, but by the end of the storage period the mince with added phytic acid had almost lost this property, owing to extensive protein aggregation. Sodium citrate and black tea polyphenols can be used to inhibit TMAO-demethylase enzyme during frozen storage of fish minces, but they showed a limited capacity to protect the functional aptitude of the hake mince protein. However, as black tea polyphenols could provide fish restructured products with interesting health properties, the use of black tea as a potential bioactive agent should not be disregarded. Under the experimental conditions used, a clear relationship between formaldehyde-mediated protein aggregation inhibition during frozen storage and protein thermal aggregation ability could not be stablished. This could be due to a combined effect of (i) the boosting of protein aggregation at the storage temperature selected (–12 °C) and the muscle processing type (mincing), and (ii) the discrete formaldehyde inhibition observed *in vitro.* Further studies at lower storage temperatures would be necessary to fully understand the effect of TMAO-demethylase inhibitors on protein quality of fish muscle.

Acknowledgements

The authors wish to thank the European Commission for financial support under the FEDER-INTERCONNECTA program (project reference PROPESCA-ITC-20151199).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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548 Figure captions

549 Figure 1. TMAO-demethylase activity (A, enzyme units/g), formaldehyde accumulation (B,
550 µg/kg), salt-soluble protein (C, %), and water holding capacity (D, g/100 g water), determined in
551 various hake minces without and with addition of inhibitors during frozen storage. C: control
552 without inhibitors; SC: with sodium citrate; PA: with phytic acid; BT: with black tea. Different
553 letters (a, b, c, d) indicate significant differences ($p \leq 0.05$) as function of frozen storage. Different
554 letters (x, y, z, w) indicate significant differences ($p \leq 0.05$) as function of the enzyme inhibitor
555 added.

556 Figure 2. Particle size distribution of salt-soluble protein aggregates from various hake minces
557 without and with addition of inhibitors during frozen storage. A) control batch at days 0, 15, 30,
558 90, and 150; B) all batches at day 15; C) all batches at day 30; D) all batches at day 90, and E) all
559 batches at day 150. F) net charge (ζ-potential, mV). C: control without inhibitors; SC: with sodium
560 citrate; PA: with phytic acid; BT: with black tea.

561 Figure 3. LF-NMR relaxation time distribution of various hake minces without and with addition
562 of inhibitors during frozen storage. A) control batch at days 0, 15, 30, 90, and 150; B) all batches
563 at day 15; C) all batches at day 30; D) all batches at day 90, and E) all batches at day 150. C:
564 control without inhibitors; SC: with sodium citrate; PA: with phytic acid; BT: with black tea.

565 Figure 4. Mechanical spectra in terms of elastic modulus (G') and viscous modulus (G") of various
566 salt-ground hake minces without and with addition of inhibitors during frozen storage. a) elastic
567 modulus and c) viscous modulus, determined after 15 days of storage; b) elastic modulus and d)
568 viscous modulus, determined after 150 days of storage. C0d: control without inhibitors at day 0
569 of storage; C: control without inhibitors; SC: with sodium citrate; PA: with phytic acid; BT: with
570 black tea.

571 Figure 5. Thermal gelation profile in terms of elastic modulus (G') of various salt-ground hake
572 minces without and with addition of inhibitors during frozen storage. a) control batch at days 0,
573 15, 30, 90, and 150; b) all batches at day 15; C) all batches at day 30; D) all batches at day 150.
574 C: control without inhibitors; SC: with sodium citrate; PA: with phytic acid; BT: with black tea.

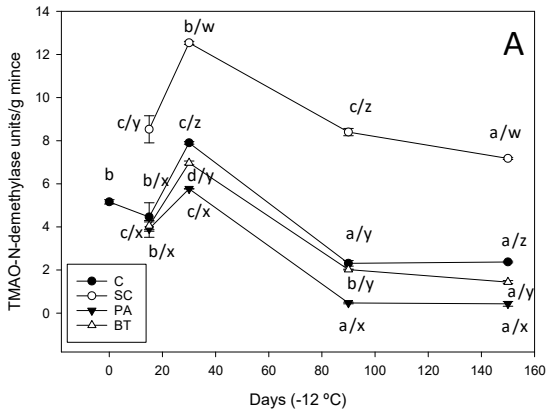
Highlights

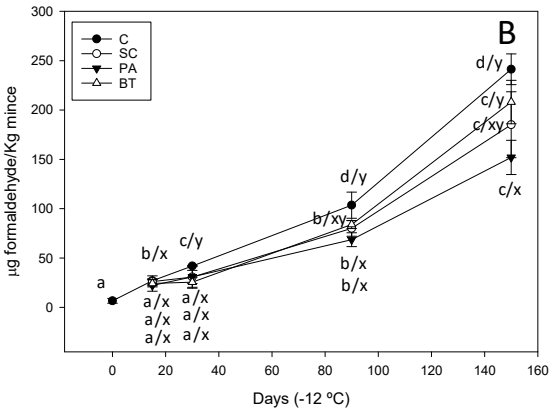
1. Tea extracts, phytic acid *PA*, sodium citrate *SC* inhibit TMAO-demethylase *in vitro*
2. Black tea extract *BTE* inhibits TMAO-d more intensely than red, white and green ones
3. *BTE*, *PA* and *SC* partially inhibit TMAO-d in hake mince during frozen storage
4. *PA* aggregates hake proteins and impairs protein functionality and gelation
5. *BTE* and *SC* do not affect hake protein functionality and gelation

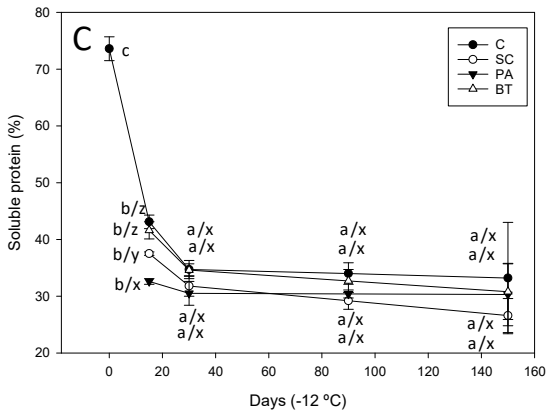
Table 1. Optical properties of the various minces developed: Lightness (L*), hue angle (h°), chromaticity (C*), total color difference (ΔE), and whiteness index (WI). C: control without inhibitors; SC: with sodium citrate; PA: with phytic acid; BT: with black tea.

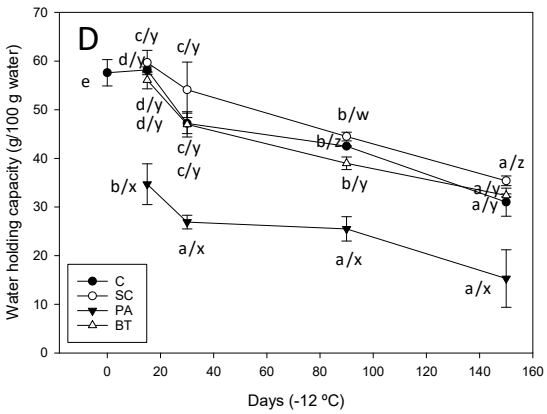
	C	**SC**	**PA**	**BT**
L*	63.5 ± 0.9c	63.3 ± 0.7c	61.2 ± 0.8b	57.7 ± 1.3a
h°	107.4 ± 2.3c	108.4 ± 2.0c	102.4 ± 2.0b	82.9 ± 1.2a
C*	7.2 ± 0.5a	7.2 ±0.5a	6.8 ± 0.8a	12.0 ± 0.9b
ΔE		1.17 ± 0.75a	2.63 ± 1.2a	8.65 ± 1.14b
WI	7.67 ± 0.08c	7.66 ± 0.07c	7.49 ± 0.08b	6.66 ± 0.11a

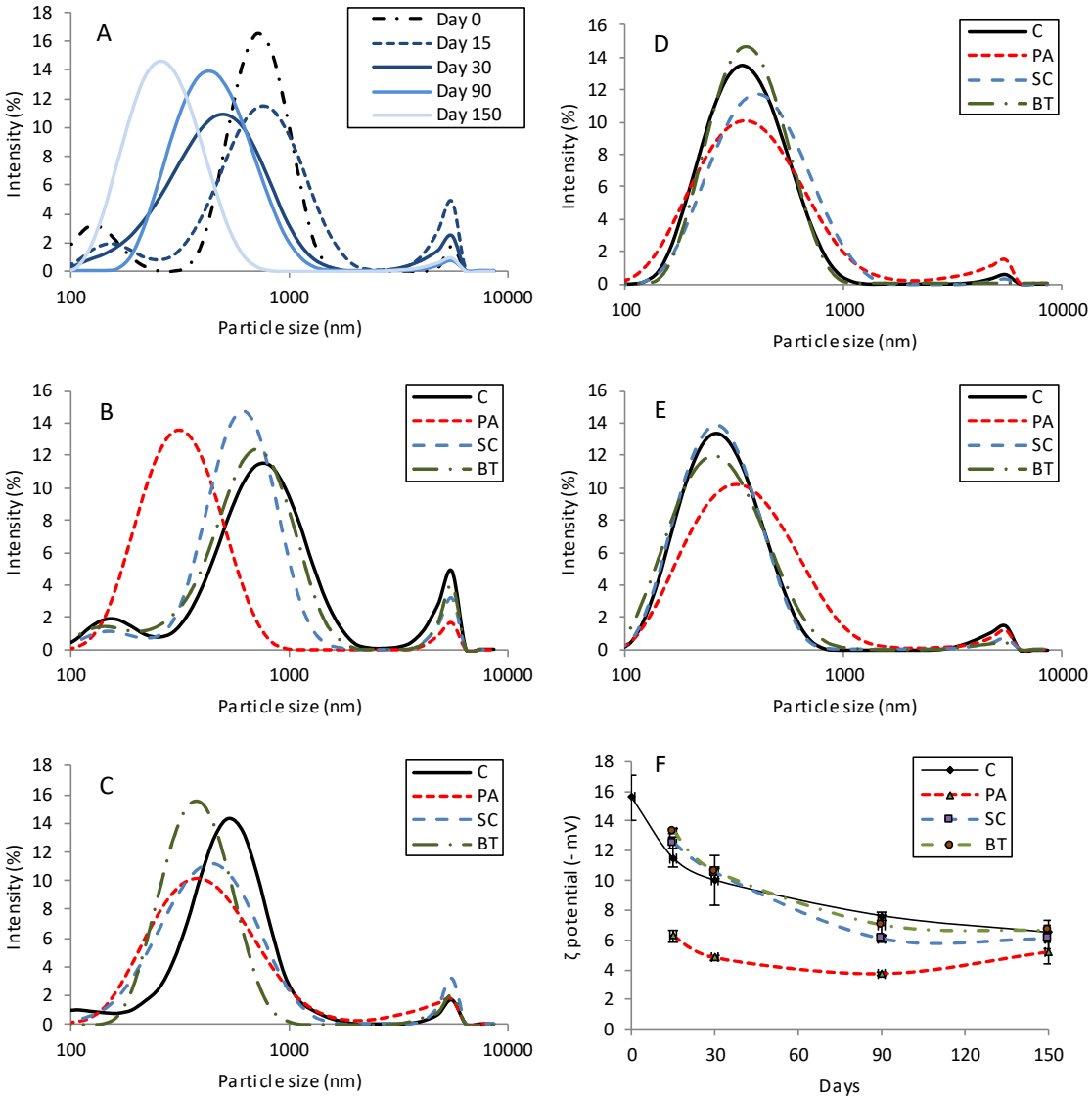
Different letters in the same row (a, b, c, d) indicate significant differences ($p \leq 0.05$) among samples.

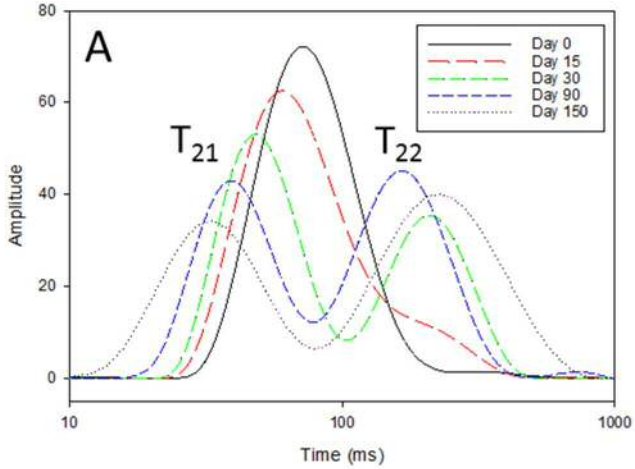

A
TMAO-N-demethylase units/g mince
Days (-12 ºC)
C
SC
PA
BT

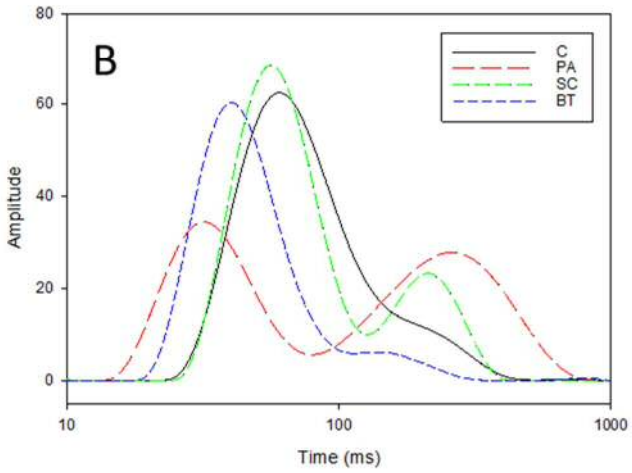

B
µg formaldehyde/Kg mince
Days (-12 ºC)
C
SC
PA
BT

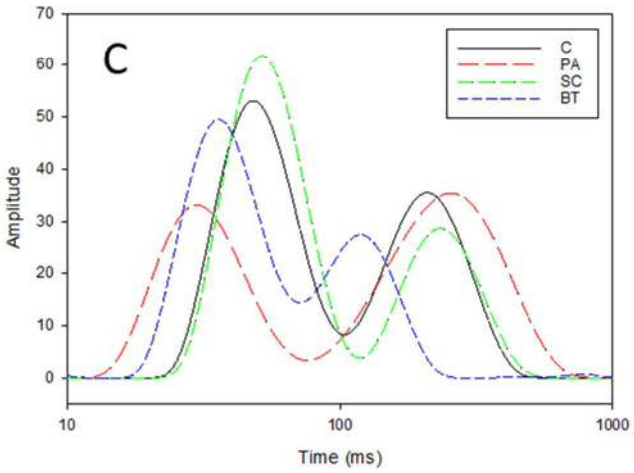

C
Soluble protein (%)
Days (-12 ºC)
C
SC
PA
BT

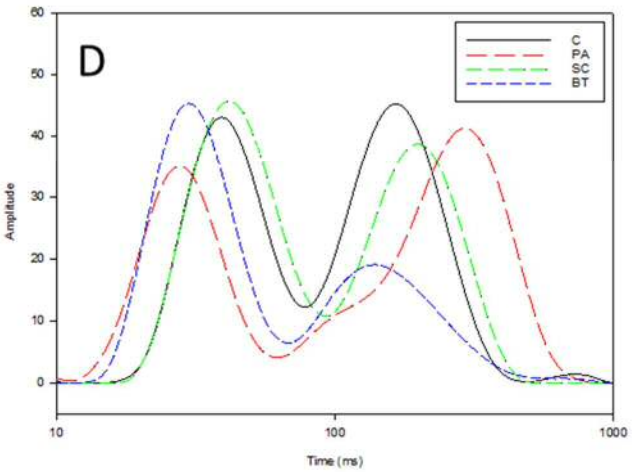

D
Water holding capacity (g/100 g water)
Days (-12 ºC)
C
SC
PA
BT

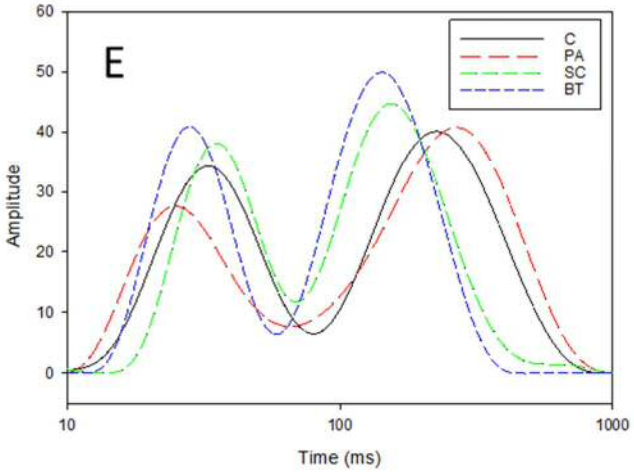

A
Day 0
Day 15
Day 30
Day 90
Day 150
Intensity (%)
Particle size (nm)
D
C
PA
SC
BT
B
E
C
F
ζ potential (- mV)
Days

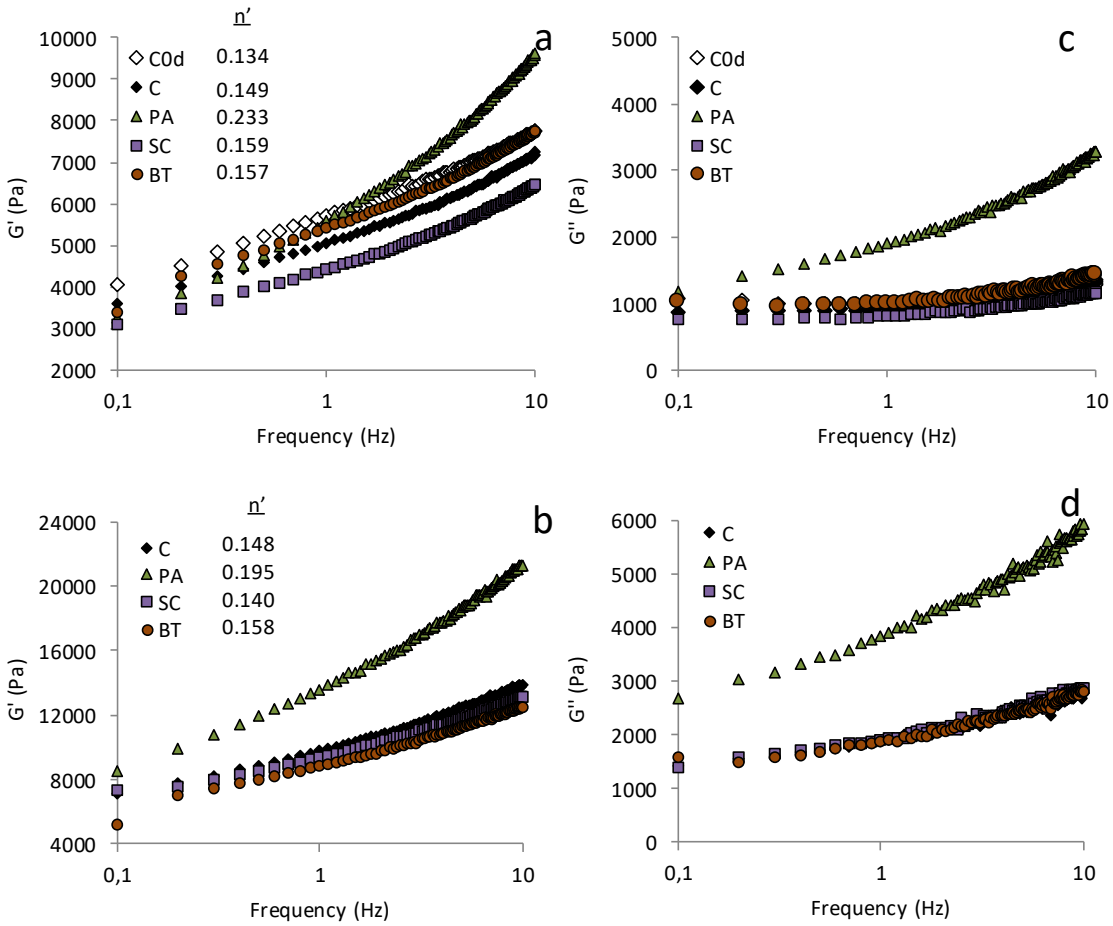

A
Day 0
Day 15
Day 30
Day 90
Day 150
T21
T22
Amplitude
Time (ms)

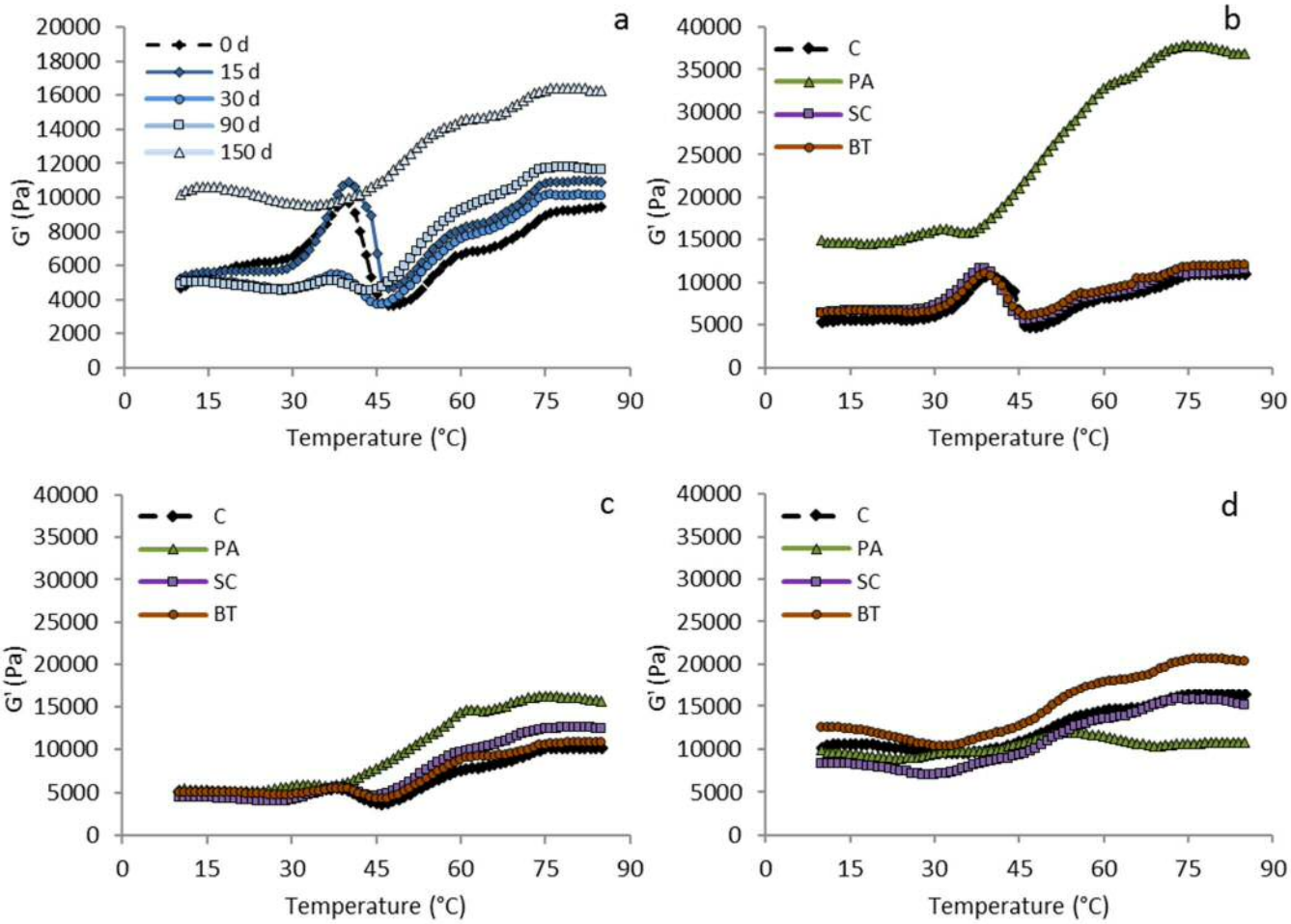

B
C
PA
SC
BT
Amplitude
Time (ms)


C
C
PA
SC
BT
Amplitude
Time (ms)


D
C
PA
SC
BT
Amplitude
Time (ms)


E
C
PA
SC
BT
Amplitude
Time (ms)


a
n'
C0d 0.134
C 0.149
PA 0.233
SC 0.159
BT 0.157
G' (Pa)
Frequency (Hz)
c
G'' (Pa)
Frequency (Hz)
b
n'
C 0.148
PA 0.195
SC 0.140
BT 0.158
G' (Pa)
Frequency (Hz)
d
G'' (Pa)
Frequency (Hz)


a
0 d
15 d
30 d
90 d
150 d
G' (Pa)
Temperature (°C)
b
C
PA
SC
BT
G' (Pa)
Temperature (°C)
c
C
PA
SC
BT
G' (Pa)
Temperature (°C)
d
C
PA
SC
BT
G' (Pa)
Temperature (°C)