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3 # Evolution of proteolytic and physico-chemical characteristics of
4 # Norwegian dry-cured ham during its processing
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Abstract

Proteolytic activity and physico-chemical characteristics were studied for Norwegian dry-cured ham at four different times of processing: raw hams, post-salted hams (3 months of processing), hams selected in the middle of the production (12 months of processing) and hams at the end of the processing (24 months). Cathepsin H activity decreased until negligible values after 3 months of processing, whereas cathepsins B and B+L were inactive at 12 months. AAP was the most active aminopeptidase whereas RAP and MAP were active just during the first 12 months of processing. Proteolysis index reached a value of 4.56±1.03 % with non-significant differences between 12 and 24 months of ripening. Peptide identification by LC-MS/MS was done and two peptides (GVEEPPKGHKGNKK and QAISNNKDQGSY) showing a linear response with the time of processing were found. Unfreezable water content and glass transition temperature were investigated using differential scanning calorimetry (DSC) technique with non-significant differences in the temperature of glass transition for 12 and 24 months of processing.

Keywords: dry-cured ham, proteolysis, muscle enzymes, DSC, peptides

58 **1 Introduction**

59 The production of Norwegian ham is energy and time consuming (up to two years). It is the
60 reason why the product is considered to be in the high price food segment. The demands of
61 Norwegian consumers are growing and the competition with European brands of dry-cured
62 ham is consequently high. Due to this, an urgent need of better understanding of the processes
63 taking place during the manufacture has arisen.

64 The published information regarding Norwegian dry-cured ham process is limited by a few
65 studies mainly dealing with sensory analysis (Hersleth et al., 2011; Hersleth et al., 2013) or
66 salt distribution processes in hams (Håseth et al., 2012; Vestergaard et al., 2005). Biochemical
67 changes occurring during the processing are mainly responsible for the final quality of dry-
68 cured ham and therefore processes taking part in the biochemical activity should be
69 investigated. Enzymatic activity is known to contribute to the breakdown of proteins and their
70 degradation products – peptides – mainly by the action of cathepsins and aminopeptidases,
71 respectively (Toldrá, 2002). The knowledge about their activity can be linked to the potential
72 of protein degradation and therefore, it is essential for the processing evaluation.

73 Some works have focused on the study of peptides as marker compounds of the processing
74 (Gallego et al., 2015; Gallego et al., 2014). Thus, peptide identification can be used as a
75 measure to prove the curing time as well as in the modelling of the proteolytic process.
76 Previous attempts investigated certain stages of the processing (salting, post-salting, final etc.)
77 but did not focus on the evolution of peptides during the whole production. As far as it has not
78 been done before, main objective of the present research was to identify and relatively
79 quantify peptides that can be linked to the protein degradation in order to be implemented into
80 the modelling in a further research.

81 The other aim of the work is to estimate physico-chemical and thermal properties of the ham
82 which can be linked to the completeness of the processing and quality of the final ham. Thus,

83 in the present study the biochemical, physico-chemical and thermal characteristics were used
84 in the characterization of Norwegian dry-cured ham for the first time. Some of the obtained
85 values would result necessary to implement the modelling of the proteolytic process as well as
86 the identification processing time marker peptides.

87 ## 2 Materials and methods.

88 ### 2.1 Material and reagents

89 Cathepsin fluorescent substrates n-alpha-CBZ-Arg-Arg 7-amido-4-methylcoumarin
90 hydrochloride (N-CBZ-Arg-Arg-AMC), n-CBZ-Phe-Arg 7-amido-4-methylcoumarin
91 hydrochloride (N-CBZ-Phe-Arg-AMC) and Arg 7-amido-4-methylcoumarin hydrochloride
92 (Arg-AMC) were purchased from R&D Systems (R&D Systems, Inc., MN, US) as well as the
93 aminopeptidase fluorescent substrates Ala-AMC and Arg-AMC. Reagents for aminopeptidase
94 assays: ethylene glycol tetraacetic acid (EGTA), 2-mercaptoethanol, dithiothreitol (DTT),
95 bestatin, and puromycin as well as sodium citrate, ethylenediaminetetraacetic acid (EDTA),
96 Triton X-100, and cysteine used for cathepsins assays were from Sigma (Sigma-Aldrich CO.,
97 US). For peptide identification assays, HCl at 37%, ethanol, trifluoroacetic acid (TFA),
98 acetonitrile (ACN), and formic acid (FA) from Scharlab (Scharlau Chemie, Barcelona, Spain)
99 were used.

100 ### 2.2 Sampling

101 A total of twenty-four hams from a cross-breed of 50% Norwegian Duroc, 25% Norwegian
102 Landrace and 25% Yorkshire pork of same age were provided by a local dry-cured ham
103 producer. The raw hams were randomly selected the same day of slaughtering according to
104 their meat content. The values of meat content in the range of 55 – 65 % were considered
105 suitable.

106 A total of four groups of six hams representing the four stages of production were studied:
107 raw hams (RH), post-salted hams (PSH, 3 months of processing), hams selected in the middle

108 of the production (MH, 12 months of processing) and final ripened dry-cured hams (FH, 24
109 months of processing). *Biceps femoris* muscle was used in all the analytical determinations
110 which were done each in triplicate.
111 The processing of the hams was done under same identical conditions and consisted on two
112 stages of salting, one week each, accomplished by sprinkling of hams' cuts. Between the two
113 salting steps, hams were washed and kneaded. The salting was done at 4 °C and high air
114 relative humidity (ARH) (≥ 95%). Salting was followed by a post-salting stage at 8-10 °C
115 with high ARH (≥ 95%) during 10 weeks. After that, hams were dried during 4 months at 13
116 °C and 74% ARH and finally ripened during at least 17 months at the same temperature and
117 ARH as during the drying step but covered by fat grease.
118 **2.3 pH measurment**
119 The raw hams were controlled at 24 hours postmortem in muscle *Semimembranosus* to check
120 the pH (691 pH Meter, Metrohm, Switzerland).
121 **2.4 Moisture content and water activity determination**
122 For moisture determination the samples were minced and dried at 105 ºC (drying oven
123 DryLine, VWR, Oslo Norway). The bone dry mass (BD) of the muscle was determined by
124 measuring the weight decrease until it reached equilibrium, according to the standard
125 methodology (AOAC, 2012).Water activity measurements were done using an AquaLab CX-
126 2 instrument (Decagon Devices, Pullman, WA, USA).
127 **2.5 Salt content measurement**
128 Salt content was measured using the conductivity method with a digital salt meter (PAL-
129 SALT Salt Meter, ATAGO CO. LTD., Tokyo, Japan). All samples were minced, mixed with
130 double-distilled water until 250 mL at 60 °C, and left for two hours with intensive shaking to
131 obtain aqueous extracts. Then, samples were filtered through glass wool and analyzed by
132 triplicate.

133 **2.6 Enzymatic activity measurements**

134 The enzymatic extraction buffer for cathepsin assays was prepared as described in (Toldrá and
135 Etherington, 1988). For the cathepsin assays, 50 mM of sodium citrate extraction buffer (pH
136 5) was mixed with 1 mM EDTA and Triton X-100 0.2 % (v/v). The fluorometric assay was
137 done according to (Toldrá and Etherington, 1988). Cathepsin B (EC 3.4.22.1) and cathepsin L
138 (EC 3.4.22.15) were tested in 40 mM phosphate reaction buffer (pH 6) containing 0.4 mM
139 EDTA and 10 mM cysteine with the addition of fluorescent substrates, namely N-CBZ-Arg-
140 Arg-AMC 0.05 mM for cathepsin B and N-CBZ-Phe-Arg-AMC 0.05 mM for cathepsins B +
141 L. Cathepsin H (EC 3.4.22.16) was assayed in 40 mM phosphate reaction buffer (pH 6.8)
142 consisted of 0.4 mM EDTA, 10 mM cysteine and 0.05 mM Arg-AMC as a fluorescent
143 substrate.

144 The enzymatic extraction buffer for aminopeptidase assays was described by (Toldrá et al.,
145 2000). Sodium phosphate extraction buffer (50 mM, pH 7.5) with 5 mM EGTA was used for
146 the aminopeptidase assays. Reaction buffers were 100 mM phosphate extraction buffer (pH
147 6.5) containing 2 mM 2-mercaptoethanol and 0.1 mM Ala-AMC for alanyl aminopeptidase
148 (AAP; EC 3.4.11.14) assay; 50 mM phosphate extraction buffer (pH 7.5) containing 0.2 M
149 NaCl, 0.1 mM Arg-AMC, and 0.25 mM puromycin for arginylaminopeptidase (RAP; EC
150 3.4.11.6) assay; 100 mM phosphate extraction buffer (pH 6.5) containing 10 mM DTT, 0,15
151 mM Ala-AMC and 0.05 mM bestatin for methionylaminopeptidase (MAP; EC 3.4.11.18)
152 assay.

153 For cathepsin assays, 2.5 g of muscle were homogenized in 25 mL of sodium citrate
154 extraction buffer (50 mM, pH 5). For aminopeptidase assays, 4 g of muscle were
155 homogenized in 20 mL of the sodium phosphate extraction buffer (50 mM, pH 7.5). The
156 extracts for cathepsin and aminopeptidase assays were homogenized using a Polytron
157 homogenizer (PT-MR 2100, Kinematica AG, Luzenerstrasse, Switzerland) three times during

158 10 s at 26.000 rpm and 4 °C. The resulting homogenates were centrifuged at 10.000 *g* for 20
159 minutes at 4 °C (Evolution Sorvall® RC, Thermo Fisher Scientific Inc., Waltham, TX, USA).
160 The supernatants were collected, filtered through glass wool, and stored at 4 °C until use. The
161 extracts for cathepsin B, cathepsins B + L and cathepsin H assays were used without dilution
162 in all cases. The extracts used in the measurement of aminopeptidase activity were diluted
163 using sodium phosphate extraction buffer 1:5 for AAP, and 1:2 for RAP, and used without
164 dilution for MAP when the RH and PSH were analyzed. During the analysis of MH and FH
165 samples, the extract for AAP, RAP and MAP was used without dilution.
166 The measurement of cathepsin enzymes and aminopeptidases activity was performed using a
167 fluorescence reader (Fluoroskan Ascent FL, Thermo Electron Corporation Labsystems,
168 Helsinki, Finland). Thus, 250 µL of each of the corresponding reaction buffers and substrate
169 and 50 µL of the corresponding extract was added to the wells (n=4), and incubated at 37 °C
170 during 15 minutes. Fluorescence reading was carried out at 0 and 15 minutes using
171 wavelengths of excitation and emission of 355 nm and 460 nm, respectively. One unit of
172 enzymatic activity was defined as the amount of enzyme able to hydrolyze 1 µmol of
173 substrate per minute at 37 °C. Results were expressed as units (U) per gram of muscle.

174 **2.7 Proteolysis index measurement**

175 Proteolysis index was measured as the percentage of mg of leucine divided by the total
176 nitrogen. The amount (mg) of leucine was estimated using the reaction of derivatization with
177 Cd-ninhydrin described by (Doi et al., 1981). The total nitrogen was obtained by Kjeldakhl
178 method using freeze-dried samples by CHN-S/N elemental analyser 1106 (Carlo Erba
179 Instruments s.p.a., Milan, Italy).
180 Peptide extraction was done according with (Gallego et al., 2015). Briefly, a total of 20 g of
181 muscle were minced and mixed with 80 mL of 0.01 N HCl. The mixtures were homogenized
182 for 8 minutes and centrifuged at 10.000 *g* and 4 °C for 20 minutes. A volume of 250 µL was

183 taken from the supernatant and three volumes of ethanol (750 µL) were added. Finally, the
184 solutions were stored at 4 °C for 20 hours to precipitate the proteins and centrifuged again at
185 10.000 *g* and 4 °C for 10 minutes. The supernatants were dried in a rotary evaporator
186 RC10.10 (Jouan, Thermo Fisher Scientific, MA) and in a vacuum-freeze drier. The remained
187 dried extracts were resuspended in 100 µL of H_2O with 0.1 % of TFA for the nLC-MS/MS
188 analysis.

189 **2.8 Peptide identification by nanoLC-MS/MS**

190 The analysis of nLC-MS/MS was done according to (Mora et al., 2015) with minor changes. 5
191 µL of each sample (diluted 1:2 with 0.1 % of trifluoroacetic acid (TFA)) were loaded onto a
192 nanoLC C18-CL trap column (3µm, 75 µm x 15cm; Eksigent, AB Sciex, CA, USA) and
193 desalted with 0.1% TFA as a mobile phase A at flow rate of 3µL/min during 5 min. The trap
194 column was automatically switched in-line onto a nano-HPLC C18-CL capillary analytical
195 column (3µ, 75um x 12cm; Nikkyo Technos Co, Ltd. Japan) equilibrated in 5% acetonitrile
196 (ACN) and 0.1% formic acid (FA). Peptide elution was carried out with a linear gradient of 5
197 to 35% over 120 min of solvent B, containing 0.1% FA in 100% ACN at a flow rate of 300
198 nL/min. The outlet of the capillary column was directly coupled to a nano-electrospray
199 ionisation system (nano-ESI) and a quadrupole/time-of-flight (Q/ToF) mass spectrometer
200 (5600 TripleTOF, ABSCIEX). The mass spectrometer was operated in positive polarity and
201 data-dependent acquisition mode, in which a 250-ms TOF MS scan from 350–1250 *m/z*, was
202 performed, followed by 50-ms product ion scans from 350–1250 *m/z* on the 25 most intense
203 2-5 charged ions.

204 ProteinPilot v4.5. search engine (ABSciex) was used to identify the peptide sequences.
205 ProteinPilot default parameters were used to generate a peak list and Paragon algorithm was
206 used to search in *Sus scrofa* (pig) Expasy protein database using the parameters of no
207 digestion and no cys-alkylation.

2.9 Differential Scanning Calorimertry (DSC) procedures

The DSC analysis was done using a DSC Q2000 (TA Instruments, USA) instrument equipped with a Liquid Nitrogen Cooling System (TA Instruments, USA). The temperature and cell constant calibration was done using indium. The heat capacity was calibrated with a sapphire in the range between –150.0 and 150.0 °C. Helium was chosen as a purge gas at 25 mL x min^{-1} according to manufacturer recommendations. An empty hermetically sealed aluminum pan was used as reference.

Sample (7 - 10 mg) was placed into aluminum pans with hermetic lids. The pans were sealed with a Tzero® DSC Sample Encapsulation Press (TA Instruments, USA) and placed using an autosampler into the DSC cell. Samples were cooled (10 °Cxmin^{-1}) and equilibrated at –40.0 °C for 30 minutes to achieve the complete crystallization of ice in tissues. Then, they were cooled down to –150.0 °C (10 °Cxmin^{-1}) and equilibrated for 60 minutes. The DSC scanning to +40.0 °C was performed at the rate of 5 °C x min^{-1}. The analysis of data was done using the Universal Analysis 2000 v. 4.5A software (TA instruments, USA). The glass transition was characterized with the following parameters of the endothermic baseline shift: onset, end and inflection points. The onset and end points were measured extrapolating the baselines to the intersection with the glass transition line.

The amount of unfreezable water was calculated as the difference between the total water content and the ice fraction. The ice fraction was detected by the integration of DSC melting curve (with the help of sigmoidal tangent baseline function) in the range between the onset of ice melting and the freezing point. The onset of ice melting was detected by analyzing the first derivative of the heating curve by temperature (Tolstorebrov et al., 2014b). The freezing point was estimated as a minimum value of the ice melting endothermic peak on the DSC heat flow curve. Straight baselines were drawn from each limit, and S-shaped curve was drawn to connect the two baselines before and after the transition. The shape of the curve was obtained

233 considering the slope of the heating curve before and after the ice melting peak. The
234 estimation of the mass of ice was calculated with respect to melting enthalpy of ice at
235 different temperatures according to (Tolstorebrov et al., 2014b).

236 **2.10 Statistical analysis**

237 The ANOVA (single test) procedure was used to determine significant differences in physico-
238 chemical characteristics, enzymatic activities and DSC analysis (glass transition temperature
239 and unfreezable water content) during the different times of processing. The difference was
240 considered significant at $P < 0.05$. The homogeneity of variance of the data was tested before
241 applying the ANOVA procedure by employing of Levene's test for all the data. Each
242 statistical analysis was done using the software Microsoft Excel 2010 (Microsoft Office
243 Proffessional Plus 2010) by analyzing six hams per time in triplicate. SigmaPlot 13.0 was
244 used for statistical analysis of the data on peptide identification and drawing all the figures.
245

246 **3 Results and discussion**

247 **3.1 *Physicochemical parameters of hams***

248 The mean of pH in the raw meat at 24 hours post-mortem ranged between 5.6 and 5.8. The
249 values of moisture content, water activity and salt content were measured for the RH, PSH,
250 MH and FH samples (**Table 1**). The values of salt content for the pair of RH and PSH and the
251 values of water activity for the pairs of RH and PSH, PSH and MH and MH and FH could not
252 be compared by ANOVA because the Levene's test was failed. According to the table,
253 moisture content and water activity values decreased with time. Salt content increased in PSH
254 samples compared with RH as expected. **Table 1** shows a significant decrease in moisture
255 content values for the analyzed hams between the MH and FH groups ($P < 0.05$) with
256 corresponding differences in water activity values. The salt content rose gradually with time
257 due to the moisture loses occurring in hams during the dry-ripening period with non-
258 significant differences between MH and FH samples.

259 **3.2 Effect of processing time on proteolytic activity**

260 The activity of endogenous muscle proteolytic enzymes was analyzed for RH, PSH, MH, and
261 FH samples to investigate the evolution of cathepsin and aminopeptidase activity during the
262 processing of Norwegian dry-cured ham. The cathepsin activity (cathepsins B, B + L and H)
263 is shown in **Figure 1**. Standard deviation is shown as error bars; means near one line with
264 different letters differed significantly ($p < 0.05$; ANOVA single test). The values of cathepsin
265 B + L for the pair of MH and FH and the values of cathepsin H for the pairs of PSH and MH
266 and MH and FH could not be compared by ANOVA because the Levene's test was failed.
267 Cathepsin activity is almost totally suppressed in MH samples (reduced by 97 and 96 % for
268 cathepsins B and B + L, respectively, compared with RH samples), indicating almost no
269 activity after one-year of processing. In fact, other previous studies reported a residual activity
270 of cathepsins B, B + L at the end of ripening for both *Biceps femoris* and *Semimembranosus*
271 muscles in Spanish-style dry-cured hams (Armenteros et al., 2012; Parreño et al., 1994;
272 Toldrá and Etherington, 1988). In addition, the activity of cathepsin H resulted almost totally
273 suppressed in PSH samples (a decrease of 86 % compared with the RH samples), which is in
274 contrast to previously described by (Flores et al., 2009) and (Toldrá and Etherington, 1988)
275 for Iberian (*Biceps femoris* muscle) and Serrano (*Semimembranosus* muscle) hams
276 respectively, where cathepsin H remained active after the post-salting stage, but in agreement
277 with other previously published reports (Armenteros et al., 2012; Parreño et al., 1994) which
278 also showed the reduction of cathepsin H activity after the post-salting stage. These
279 differences could be due to differences in the processing as well as the used raw material
280 which have been described to highly influence the enzymatic activity during the dry-cured
281 ham processing (Mora et al., 2015). In fact, the fast decrease in the activity of endogenous
282 enzymes observed in Norwegian dry-cured ham can be related to the high pH of the raw
283 matter, low temperature used, low air relative humidity of the drying air, or even a result of

284 several factors (Petrova et al., 2015). The salt content, even though an inhibitor of cathepsins
285 (Rico et al., 1991) and aminopeptidases (Toldrá et al., 1993) is unlikely to be the reason of the
286 activity fall due to the relatively low salt content in the PSH, MH and FH samples.

287 The activity of aminopeptidases (AAP, MAP and RAP) is shown on **Figure 2**. Standard
288 deviation is shown as error bars; means near one line with different letters differed
289 significantly ($p < 0.05$; ANOVA single test). The values of AAP for the pair of RH and PSH
290 and the values of MAP for the pair of PSH and MH could not be compared by ANOVA
291 because the Levene's test was failed. The three types of assayed aminopeptidases showed
292 non-significant activity after one year of processing. The activity of RAP and MAP followed
293 a similar trend to cathepsins B and L: they were almost completely inactive in MH samples
294 (the activity was reduced by 95 % for both RAP and MAP). AAP was active during the whole
295 processing, but its activity was significantly reduced from MH and FH samples (reduced by
296 75 and 78 % correspondingly). In this respect, aminopeptidase activity was detected in
297 Spanish dry-cured ham even after 12 months of processing, suggesting that AAP and RAP
298 may be involved in the latter stages of protein degradation (Toldrá et al., 2000). (Toldrá et al.
299 2000) showed a good stability of AAP and RAP during dry-curing and both enzymes were
300 suggested to be main contributors to the generation of free amino acids during the processing
301 of dry-cured ham. MAP, whose activity is enhanced in the presence of low amounts of NaCl,
302 can also generate free amino acids in cured meat products. This enzyme has broad substrate
303 specificity, exhibiting maximal activity in the hydrolysis of N-terminal Met (100%) and Lys
304 (81%) amino acids (Flores et al., 2000). In this sense, AAP was predictively more active and
305 has been described to be responsible for an important part of porcine muscle aminopeptidase
306 activity during its processing (Petrova et al., 2015; Zhao et al., 2005).

307 The proteolysis index (PI) during dry-cured ham processing is directly related to the
308 enzymatic activity. In fact, **Table 2** shows the PI values of RH, PSH, MH and FH with

309 significant differences between RH, PSH and MH samples (P < 0.05), while MH and FH did
310 not differ statistically (P> 0.05) (n=6). Low activity values of proteolytic enzymes would
311 result in low protein degradation and a smaller amount of non-protein nitrogen in the samples.

312 **3.3 Peptide identification**

313 The analysis of mass spectrometry resulted in the identification of 6129 peptides. The origin
314 of the peptides was also identified and a total of 229 proteins were determined with 95% of
315 confidence. The main purpose of the peptide identification was to detect those peptides that
316 could be used as markers of the time of processing. The selection of those peptides showing
317 the potential to be process biomarkers was done as follows: peptide ought to be stable and
318 homogeneous during the manufacture and certain kinetics (negative or positive) of
319 quantitative changes ought to be clearly observed; the dependence of quantitative parameter
320 and time should follow a simple geometrical behavior (a straight line dependence was chosen
321 as the best and simplest alternative) with R > 0.90 for further modelling. As a quantitative
322 parameter, the intensity of absorbance of the peptide ion was used.

323 From the total number of identified peptides, two potential candidates to be a biomarker of the
324 manufacturing process of Norwegian dry-cured ham were detected (**Table 3**):
325 QAISNNKDQGSY peptide is a myosin light chain fragment from *Sus scrofa* and
326 GVEEPPKGHKGNKK is a fragment of myomesin-1 protein from *Sus scrofa*, both showing
327 100% homology with the origin sequences. Statistically, the values between the time groups
328 of hams differed significantly (P < 0.05) probably due to differences in the proteolysis
329 occurred during the processing period.

330 In order to evaluate the possibility to follow the ripening process through the relative
331 quantification of the generated peptides the PSH, MH and FH samples were taken. The
332 average intensity of those peptides identified using nLS-MS/MS and the time of processing is
333 shown in **Figure 3**. Standard deviation is shown as error bars; means near one line with

334 different letters differed significantly (P < 0.05; ANOVA single test). Two linear curves of
335 the form $f = y_0 + a * x$ corresponding with the two graphs of intensity are also shown on the
336 figure together with their equations in order to visualize the linear behavior of the intensity-
337 time dependence.

338 Several attempts to find potential peptide biomarkers have been previously done in 9 months
339 Spanish dry-cured ham. These peptides resulted to be generated from titin (Gallego et al.,
340 2015) and from protein and LIM-domain protein (Gallego et al., 2014). However, this is the
341 first time that data of intensities of the peptide absorbance for potential biomarkers in relation
342 to the time of processing are showed. The presence of these peptides at PSH, MH and FH
343 make possible to utilize them in the modelling of proteolytic changes. Despite both peptides
344 seems to be adequate, the peptide QAISNNKDQGSY is considered as a prefered candidate to
345 be implemented in the modelling, since the relation between the intensity and time is more
346 pronounced.

347 **3.4 Differential Scanning Calorimetry results**

348 Glass transition is a second-order transition from viscous-rubber to brittle-solid state, when
349 cooling down the system. The viscosity of the system reaches a value of 10^{12} Pa x s in a glassy
350 state (Champion et al., 2000). Glass transition in protein food products with high moisture
351 content (usually higher than 25.0% d.b.) occurs at low and ultra-low temperatures, when a
352 significant amount of water is transited into ice. The temperature of the glass transition differs
353 from product to product and it depends (among other factors) on the average molecular
354 weight of the system. Low average molecular weight will cause a decrease in the glass
355 transition temperature (Roos, 1995). At the same time, the glass transition temperature does
356 not depend on the initial water content of a product (Tolstorebrov et al., 2014b). The so-called
357 unfreezable water, which forms maximal freeze concentrated solution at freezing

358 temperatures, influences that phenomenon (Tolstorebrov et al., 2016). The occurrence of the
359 glass transition can be used as an indicator of biochemical changes in foods.
360 Glass transition of RH samples was detected in the temperature range between -81.78 and -
361 61.73 ºC. This data can be referred to relatively high average molecular weight of the
362 maximal freeze concentrated solution in the samples at the beginning of the manufacture.
363 Similar values of glass transition temperatures were previously observed for fresh ham by
364 (Tolstorebrov et al., 2014a). PSH samples showed glass transition in the temperature range
365 between -81.56 and -65.97 °C with non-significant statistically differences with RH samples
366 ($P > 0.05$). The relation between the inflection temperatures of glass transition and the time of
367 production is shown in **Figure 4**. Standard deviation is shown as error bars; means near one
368 line with different letters differed significantly ($P < 0.05$; ANOVA single test).
369 MH and FH samples showed a significant decrease in the glass transition temperature in
370 comparison with RH and PSH samples. The values for MH and FH samples decreased to a
371 temperature range between -88.68 and -74.95 ºC for the onset of glass transition and the end
372 point respectively. This can be explained by the fact that some meat compounds such as
373 proteins are hydrolyzed into small peptides decreasing the average molecular weight of the
374 sample. The glass transition temperatures of MH and FH did not differ statistically ($P > 0.05$),
375 probably due to the low enzymatic activity detected after one year of processing. In fact, a
376 strong negative correlation (R>-0.97) between glass transition temperatures and proteolysis
377 indeces was observed. Thus, the decrease in glass transition temperature was probably due to
378 the decrease in the average molecular weight of the unfrozen (maximal freeze concentration)
379 solution. On the other hand, the influence of salt could also be important since salt molecules
380 (see salt concentration in **Table 1**) could take part in the formation of the unfreezable
381 solution, as salt crystallization was not reached in the samples (crystallization peak of salt was
382 not detected on the DSC heat flow curves in all the studied samples). According to this, the

383 presence of salt could also decrease the average molecular mass of maximal freeze
384 concentrated solution and the temperature of glass transition.
385 The amount of unfreezable water was calculated at 26.78±3.71 % d.b. for RH samples and
386 28.17±4.29 for PSH samples ($P > 0.05$), which is in agreement with earlier reports (Pham,
387 1987; Reid and Fennema, 2007). The evolution of unfreezable water content with time is
388 shown on **Figure 5**. Standard deviation is shown as error bars; means near one line with
389 different letters differed significantly ($p < 0.05$; ANOVA single test). The curing process
390 resulted in a sharp increase of the unfreezable water content in MH samples (38.34±3.41%
391 d.b.). A significant increase in the amount of unfreezable water was also observed in FH
392 samples (45.41 ±5.0 % d.b). The presence of an additional amount of unfreezable water in FH
393 samples should have decreased the average molecular weight of the maximal freeze
394 concentrated solution and, as a consequence, decreased the glass transition temperature. At
395 the same time, non-statistically significant differences in glass transition temperature were
396 detected between the MH and FH groups ($P > 0.05$). Probably some processes involving the
397 aggregation of peptides, lipids etc., are occurring during the curing process. However, such
398 processes were out of the scope of this study. The amount of unfreezable water showed
399 moderate correlation with proteolysis index ($R > 0.91$).
400 **Conclusions**.
401 Proteolytic, physico-chemical and thermal characteristics of Norwegian ham were
402 investigated at different times of dry-cured processing (RH, PSH, MH and FH samples).
403 Cathepsin H activity fell down until negligible values after 3 months of the processing,
404 whereas cathepsins B and B + L were almost totally inactivated after 12 months. AAP was the
405 most active aminopeptidase during the whole processing whereas RAP and MAP were only
406 active during 12 months. Proteolysis index values increased up to 4.56±1.03 % at 24 months
407 of ripening (FH samples) with non-significant differences with the obtained values at 12

408 months of ripening (MH samples). Thermal properties showed non-significant differences in
409 the temperature of glass transition for MH and FH samples, probably due to similarities in the
410 generated peptides. Two possible marker peptides (GVEEPPKGHKGNKK and
411 QAISNNKDQGSY) showing a linear response with the time of processing were found and
412 their values of absorbance intensity were reported. They will be implemented into the
413 attempts with the modelling of proteolysis index for the processing of Norwegian dry-cured
414 ham.

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512 **Figure captions**

513 **Figure 1** – Cathepsin-like activity along the processing of Norwegian dry-cured ham.

514 **Figure 2** – Aminopeptidase activity along the processing of Norwegian dry-cured ham..

515 **Figure 3** – Relative intensity values versus time of ripening of the potential biomarkers

516 identified.

517 **Figure 4** – Relation between the inflection temperature of glass transition and the time of
518 processing.
519 **Figure 5** – Relation between unfreezable water content and the time of processing.
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537 **Tables**.
538 **Table 1.** Physico-chemical parameters for the different production time

Sample	Moisture content*(w.b.)	Water activity*	Salt concentration*, % (w. b.)
RH	74.1±1.11[a]	0.99±0.00	0.20±0.00
PSH	73.4±1.71[a]	0.97±0.02	0.99±0.68[a]
MH	60.9±3.37[b]	0.93±0.01	4.87±0.67[b]
FH	47.7±2.58[c]	0.88±0.02	5.42±0.10[b]

539 *The data is indicated as average mean ± SD;

540 [a-c] Means in the same column with different letters differ significantly ($P < 0.05$; ANOVA

541 single test).

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559 **Table 2.** Proteolysis index for the different production time

Sample	Proteolysis index, %*
RH	0.48±0.15[a]
PSH	0.28±1.19[b]
MH	4.99±0.57[c,d]
FH	4.56±1.03[d]

560 *The data is indicated as average mean ± SD;

561 [a-c] Means in the same column with different letters differ significantly ($P < 0.05$; ANOVA

562 single test).

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582 **Table 3.** Potential biomarkers identified using nLC-MS/MS and relatively quantified with a
583 label-free approach.

Peptide	Molecular mass	m/z	Theoretical molecular mass	Theoretical m/z	Theoretical z
GVEEPPKGHKGNKK	1504.81	502.61	1504.80	502.61	3
QAISNNKDQGSY	1324.59	663.30	1324.59	663.30	2

584 [*]The data is indicated as average mean ± SD;
585 [a-c] Means in the same column with different letters differ significantly ($P < 0.05$; ANOVA
586 single test).

Highlights

- Proteolytic and physico-chemical characteristics for Norwegian ham were studied
- Thermal properties were studied by DSC teqnique
- Peptide identification by LC-MS/MS was done
- Two marker peptides of the processing were found

Figure 1

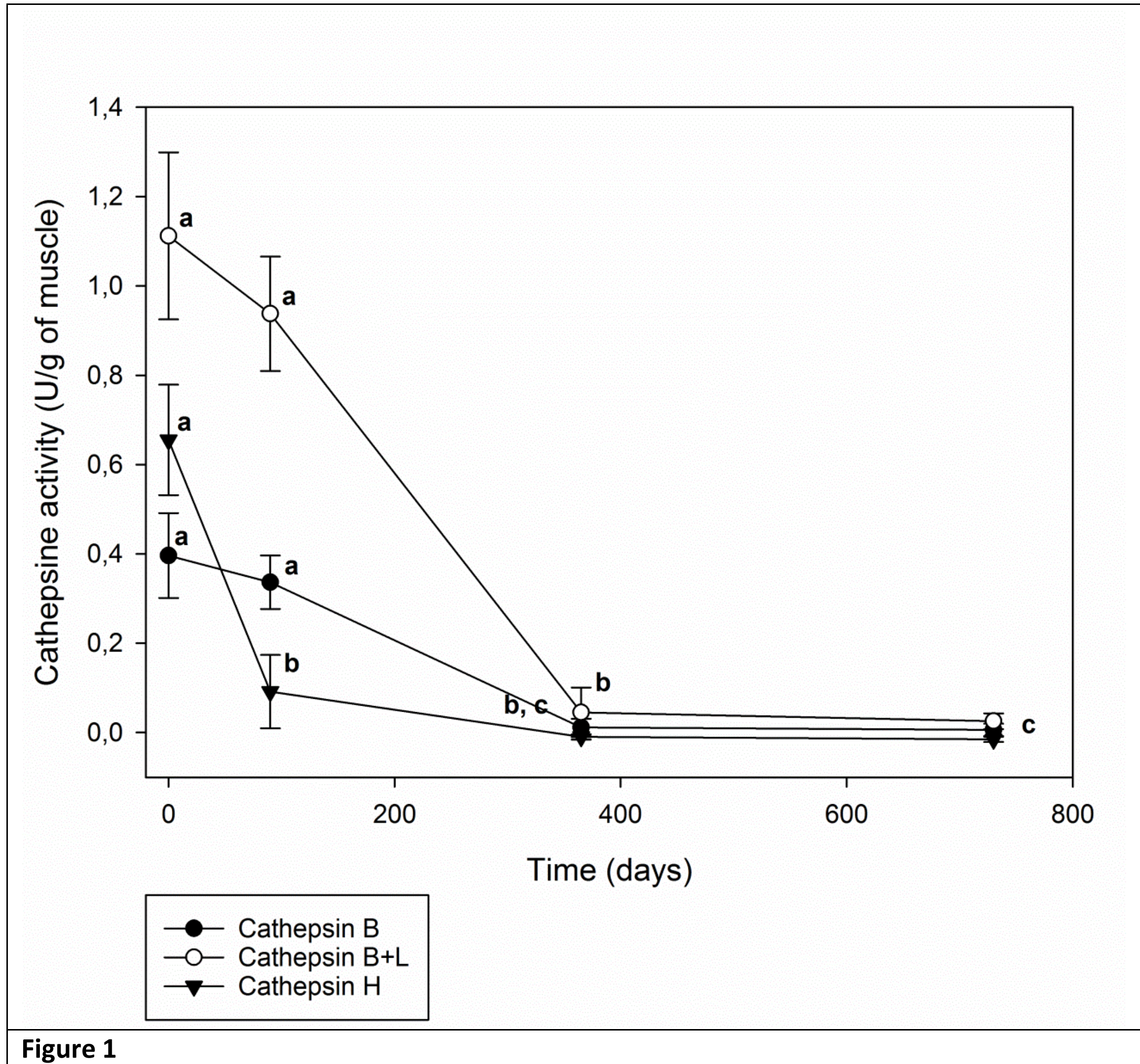
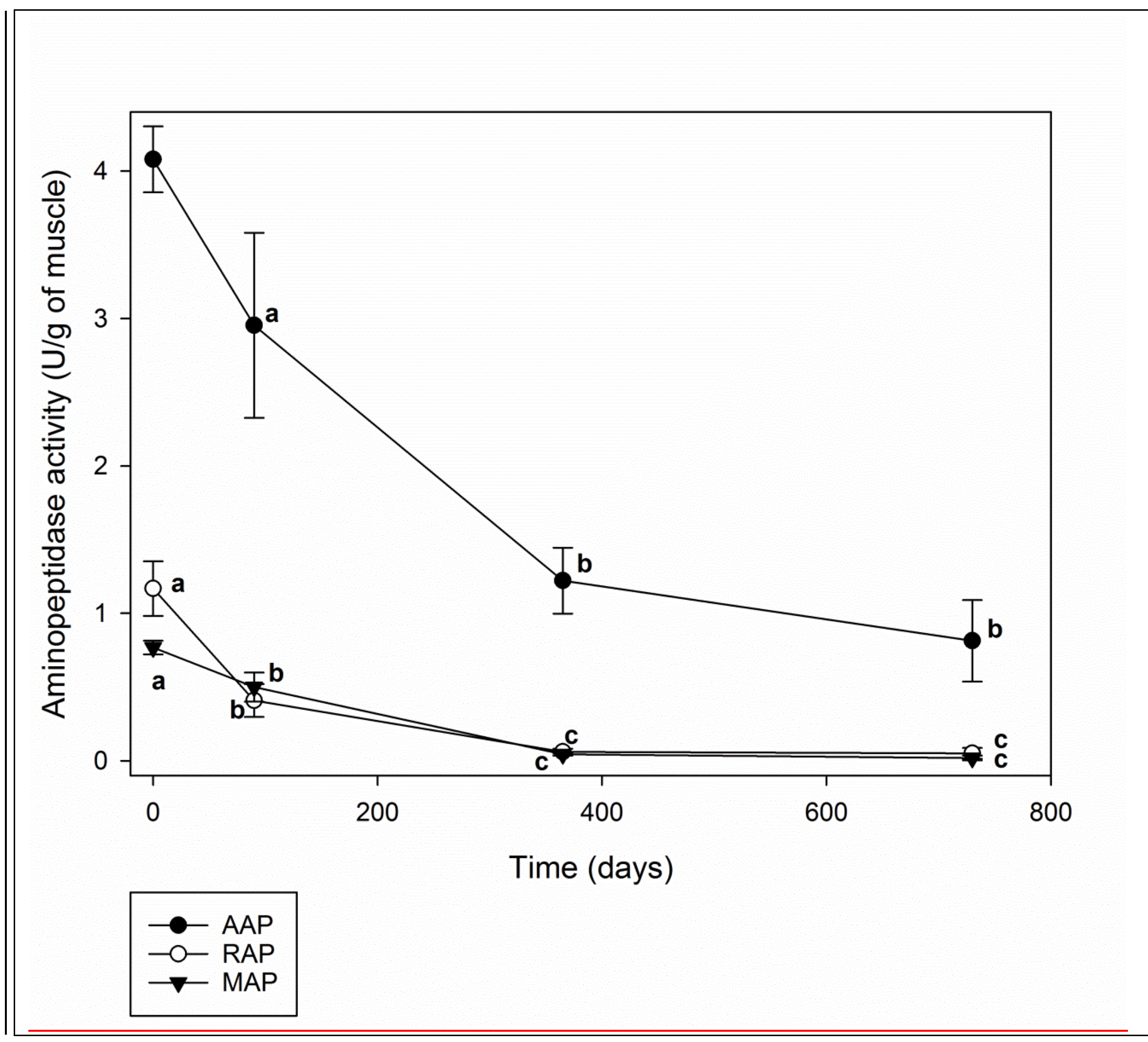


Figure 1

Figure 2



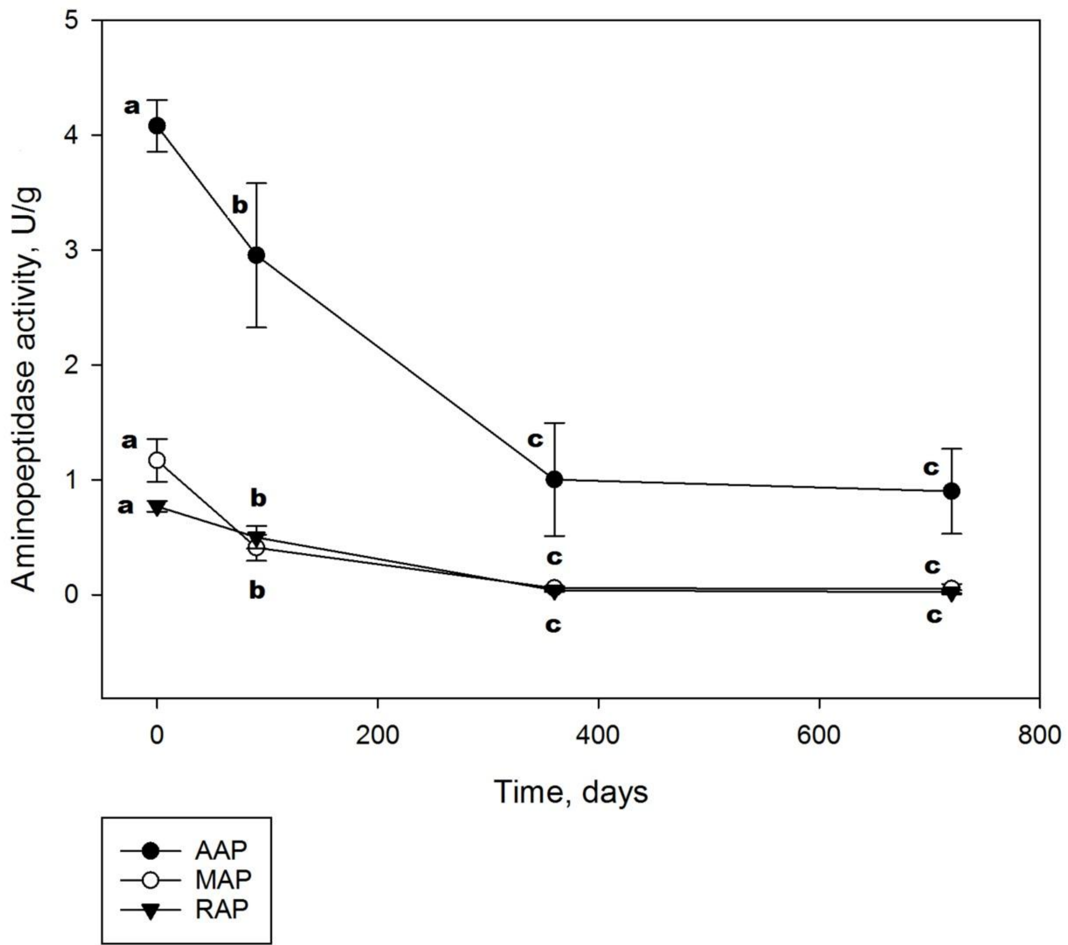
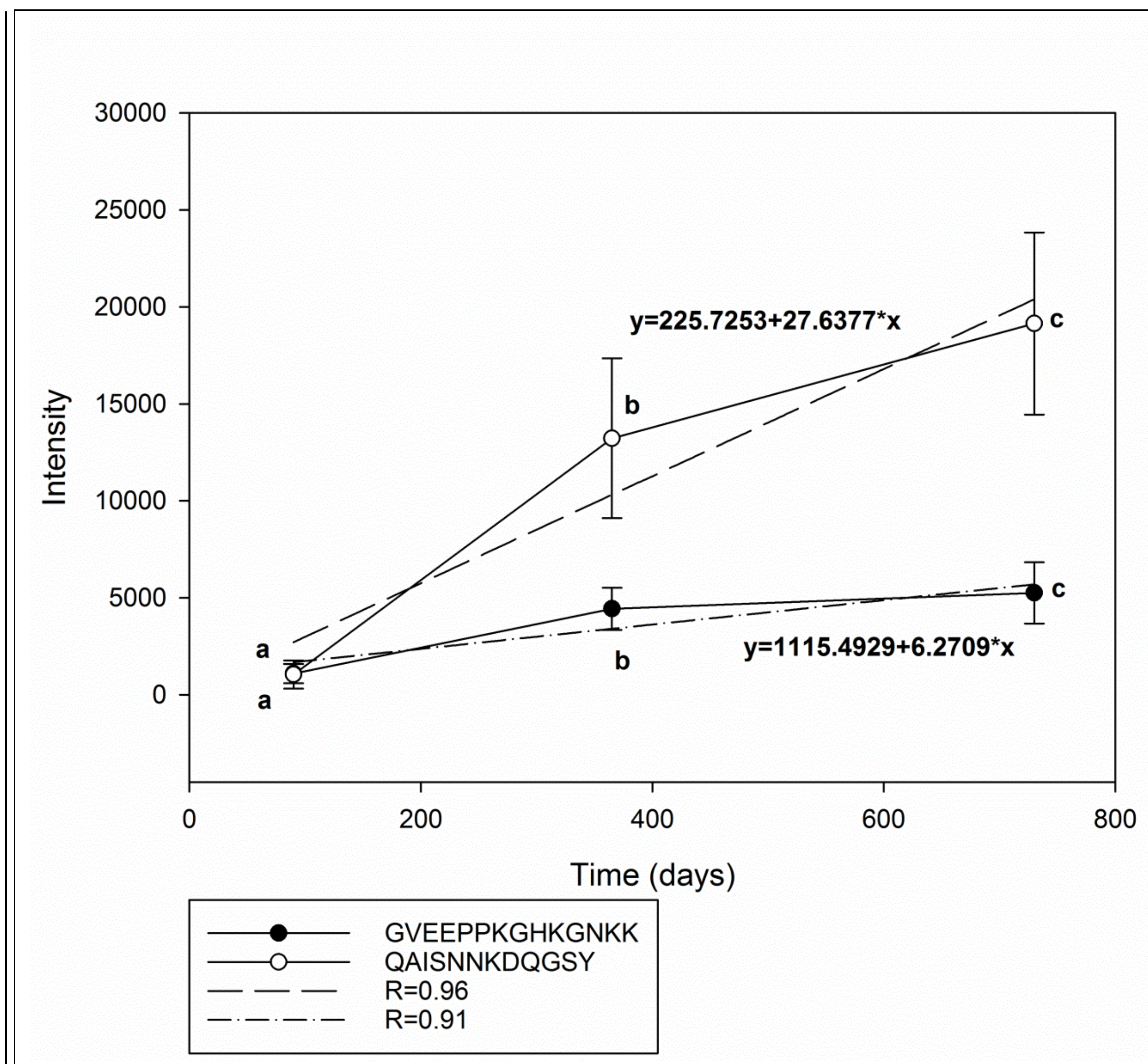


Figure 2 –

Figure 3



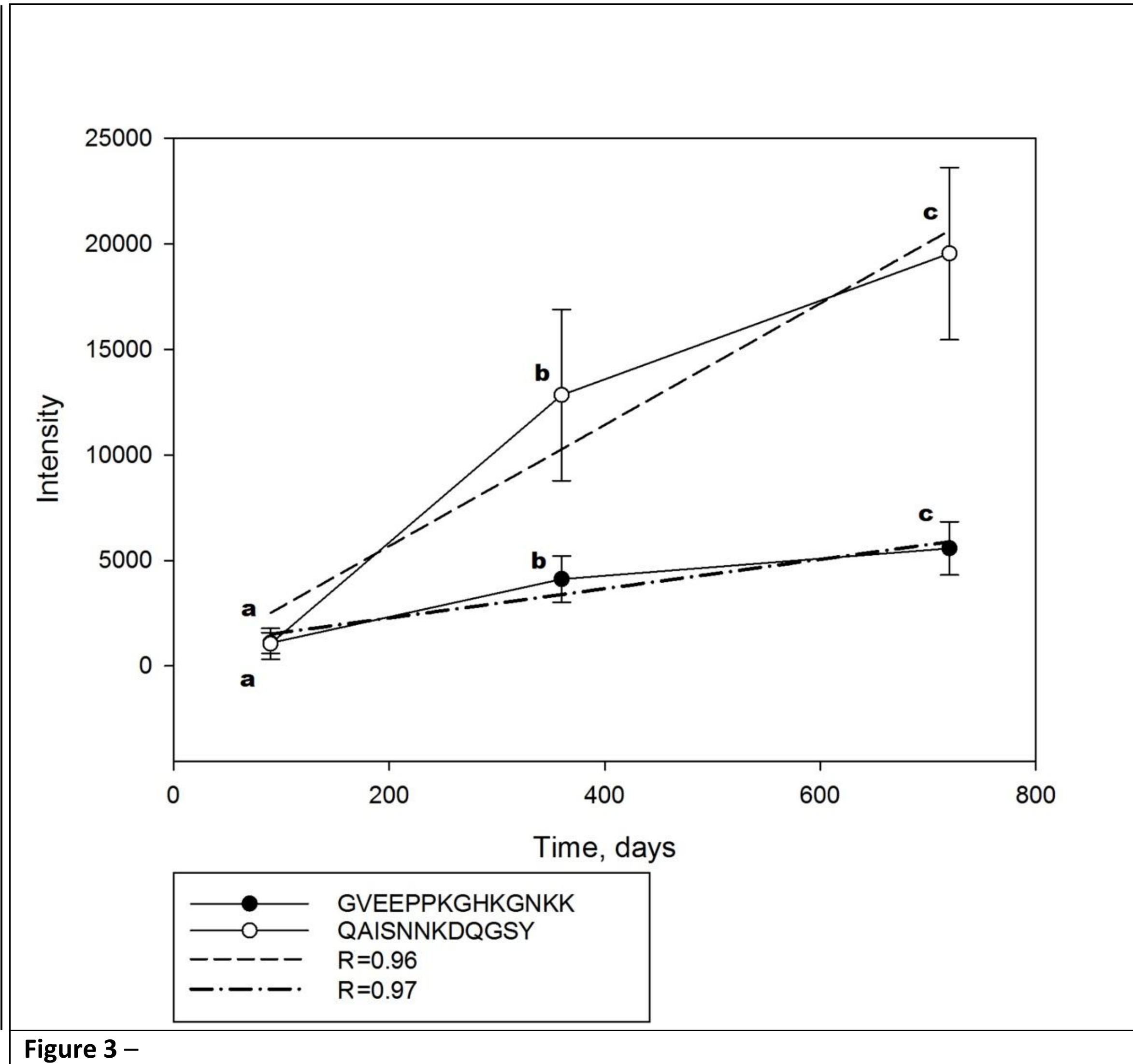


Figure 3 –

Figure 4

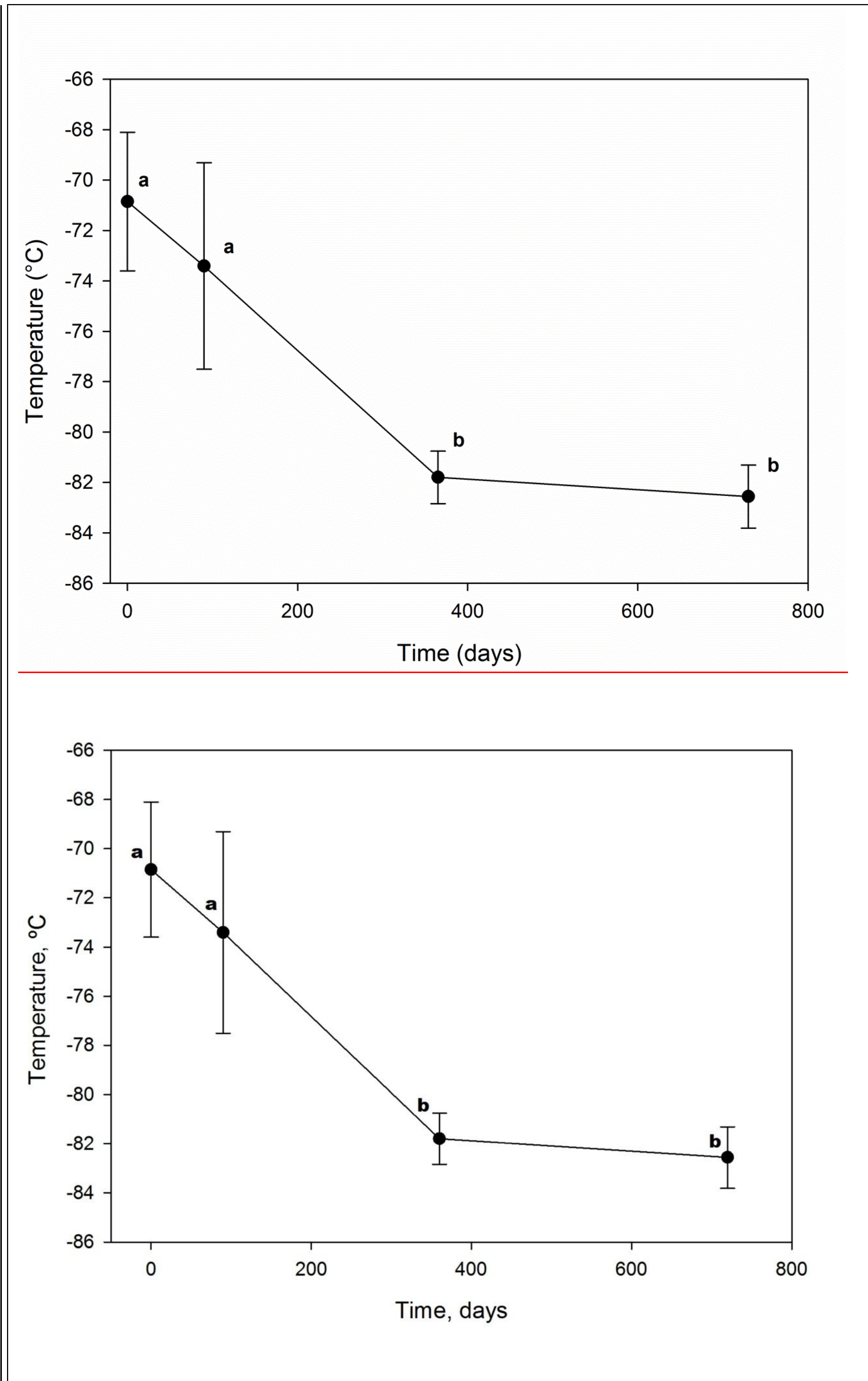


Figure 4 –

Figure 5

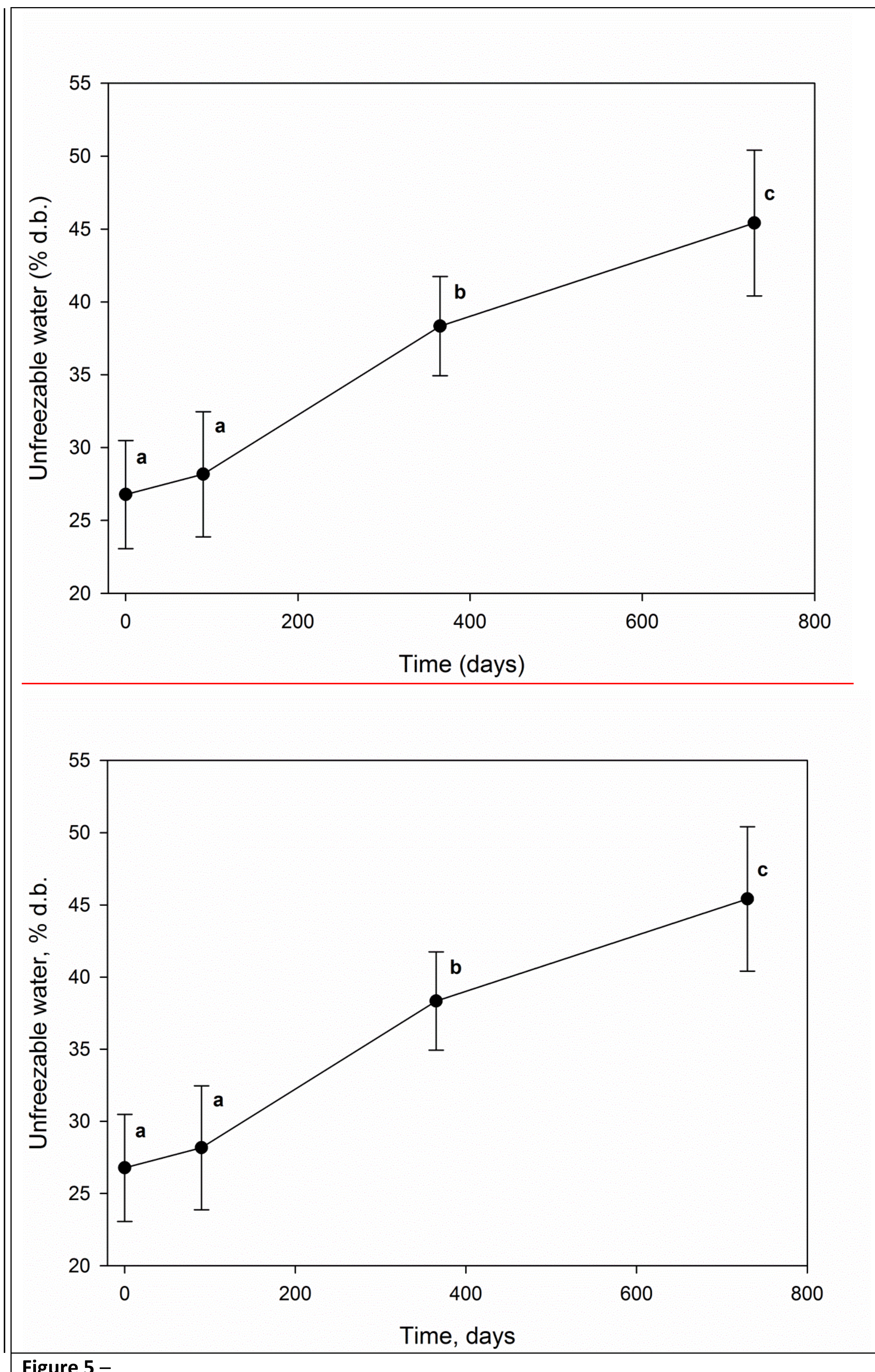


Figure 5 –