This document is a postprint version of an article published in Food Research International© Elsevier after peer review. To access the final edited and published work see https://doi.org/10.1016/j.foodres.2020.109156

Document downloaded from:

1 **Effects of *post mortem* pH and salting time on Zinc-protoporphyrin content in**

2 **nitrite-free Serrano dry-cured hams**

3

4

5 **Ricard Bou[1], Mar Llauger[1], Jacint Arnau[1], Alejandro Olmos[2], Elena Fulladosa[1]**

6

7

8 [1] Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Food Technology, Monells, Finca

9 Camps i Armet s/n, 17121, Spain.

10 [2] Monte Nevado, C. San Ignacio, 6 Carbonero el Mayor 40270, Segovia, Spain

11

12

13

14 Corresponding author: Ricard Bou, ricard.bou@irta.cat, Institut de Recerca i Tecnologia

15 Agroalimentàries (IRTA), Food Technology, Monells, Finca Camps i Armet s/n, 17121, Spain

Abstract:

There is a growing demand for clean label products and thus the elimination of curing additives in various dry-cured meats is of interest while maintaining colour characteristics. This study was aimed to examine the effect of pH at 24 h *post mortem* ($pH_{SM24h} \leq 5.4$; $5.4 > pH_{SM24h} < 5.9$; $pH_{SM24h} \geq 5.9$) and salting time (standard vs reduced) on zinc-protoporphyrin content, heme content and other physicochemical parameters of Serrano dry-cured hams manufactured without the addition of curing agents. Results showed that in those hams with higher *post mortem* pH heme content was increased whereas ZnPP content and proteolysis index were decreased. Reduced salting time decreased salt content whereas ZnPP and heme contents remained unaffected. Lower *post mortem* pH and reduced salting time led to a higher content in various free fatty acids which, in turn, were found to correlate positively with ZnPP formation. However, the observed changes in heme and ZnPP contents had no effect on the instrumental color of the final product.

Keywords: porphyrin content, clean label, color development, ham processing, salt reduction

1 INTRODUCTION

Consumer awareness of additives drives the industry to reformulate and change production processes to obtain clean label products. The addition of nitrate and/or nitrite during the production of dry-cured meat products, such as French Bayonne ham or Serrano ham, is common due to their effects on color, safety, and antioxidant properties (Sebranek & Bacus, 2007; Skibsted, 2011). Conversely, the omission of nitrifying agents is mandatory in the Italian Parma hams (Parolari, 1996; Toldrá, 2002) and it is becoming popular in Spain (Olmos personal communication). However, the elaboration procedures and raw materials for Italian Parma ham are different from the ones used during the production of Serrano ham. Thus, it is important that the elimination of the curing agents in Serrano hams does not affect the typical color characteristics.

The principal chromophore in nitrified dry-cured hams is nitrosylmyoglobin, whereas Zn-protoporphyrin IX (ZnPP) has been identified as the main stable pigment in non-nitrified (sometimes also referred as uncured) hams (Wakamatsu, Nishimura, & Hattori, 2004). The formation of ZnPP in meat products, involving the replacement of Fe by Zn in the heme moiety, is mainly believed to be of enzymatic origin (Wakamatsu, Okui, Ikeda, Nishimura, & Hattori, 2004). The endogenous enzyme, ferrochelatase (also known as Zn-chelatase), is suggested to play a crucial role in the formation of ZnPP (Benedini, Raja, & Parolari, 2008; Khozroughi, Kroh, Schluter, & Rawel, 2018). This enzyme is active throughout the processing of dry-cured hams (Adamsen, Moller, Parolari, Gabba, & Skibsted, 2006; Parolari, Aguzzoni, & Toscani, 2016; Parolari, Benedini, & Toscani, 2009). The enzyme residual activity can explain the high ZnPP content found at the end of the process. However, the formation pathway of ZnPP in meat and meat products has not been completely elucidated. Several alternative non-enzymatic mechanisms have been proposed in addition to different factors that can modulate

58 the activity of ferrochelatase (Becker, Westermann, Hansson, & Skibsted, 2012; Grossi, do
59 Nascimento, Cardoso, & Skibsted, 2014; Parolari et al., 2016).

60 To gain a better understanding of the factors that can influence the formation of this pigment,
61 the relationships between the ZnPP content and various physicochemical parameters of the
62 commercial Parma hams were previously examined (Bou, Llauger, Arnau, & Fulladosa, 2018).
63 In this prior study, a high amount of ZnPP was associated with high salt content and increased
64 proteolysis and marbling. These findings are in good agreement with various *in vitro* studies.
65 For instance, the enzymatic formation of ZnPP in meat extracts was found to increase with
66 high amounts of sodium chloride (Benedini et al., 2008). Paganelli et al. (2016) reported that
67 the formation of ZnPP was favored in the presence of partly proteolyzed myoglobin. With
68 regards to the relationship between ZnPP and marbling in hams, it was hypothesized that
69 elevated marbling values may lead to a higher amount of free fatty acids during ham's
70 processing. In *in vitro* studies, the formation of ZnPP from heme was markedly activated by
71 fatty acids and phospholipids (Chau, Ishigaki, Kataoka, & Taketani, 2010; Taketani, 1993). In
72 addition to these factors, there are others such as pH that may also have an effect on the
73 formation of ZnPP in hams. In this regard, the enzyme ferrochelatase is able to remove the Fe
74 ion from porphyrin with an optimal pH range between 5.5–6.0, whereas Zn insertion is
75 favored at a neutral or basic pH (7.5–8.0) (Chau et al., 2010; Ishikawa et al., 2006).

76 Therefore, the formation of the stable pigment ZnPP could be influenced by acting on some
77 intrinsic and extrinsic factors (Bou et al., 2018). However, some factors can affect different
78 characteristics in the final product. It is well known that hams with a low *post mortem* pH are
79 more prone to proteolysis (Tabilo, Flores, Fiszman, & Toldra, 1999). Alternately, lipolysis can
80 be enhanced with high salt contents (Andres, Cava, Martin, Ventanas, & Ruiz, 2005; Motilva,
81 Toldra, Nieto, & Flores, 1993) and temperatures (del Olmo, Calzada, & Nunez, 2016; Martin,
82 Cordoba, Ventanas, & Antequera, 1999). In general, the biochemical changes and effects of
83 processing parameters on the dry-cured meat products are reasonably well understood.

84 However, the information regarding the factors that can affect the content of heme and ZnPP
85 is limited, considering that the research on ZnPP content in dry-cured hams is relatively
86 recent. In addition, most research on this pigment has been carried out in Parma hams. It is
87 important to note that the elaboration procedures and raw materials for Italian Parma ham
88 are different from the ones used during the production of Serrano ham. In comparison to
89 Serrano hams, the elaboration of Parma hams uses legs from heavier pigs, which are exposed
90 to a longer salting period. After salting, hams are exposed to resting and washing and, finally,
91 dried and ripened to lower temperatures than Serrano ham (Parolari, 1996; Toldrá, 2002).

92 To the best of our knowledge, no studies on ZnPP formation following the non-nitrified
93 Serrano dry-cured ham elaboration procedures have been performed till date. Therefore, the
94 aim of this work is to evaluate the effects of *post mortem* pH, salt content, and, indirectly, the
95 free fatty acid (FFA) content on the ZnPP and heme contents in dry-cured hams after the
96 Serrano ham elaboration procedures without the addition of nitrifying agents.

97

98 ## 2 MATERIAL AND METHODS

99

100 ### *2.1 Reagents and standards*

101 Chlorohemin (hemin) from porcine was purchased from Paneac Química SLU (Barcelona,
102 Spain) and ZnPP was obtained from Sigma-Aldrich (St. Louis, Missouri, United States).
103 Methanol was of HPLC grade (Merck KGaA, Darmstadt, Germany). Other ACS grade reagents
104 were used.

105 ### *2.2 Dry-cured ham elaboration and sampling*

106 Twenty-eight raw ham samples from animals with crosses of Large White and Landrace
107 breeds were selected in a commercial slaughterhouse according to the pH measured on the

108 *semimembranosus* (SM) muscle at 24 h *post mortem* (pH_{SM24h}) with a penetration electrode
109 (Crison 52-32) and a portable pH-meter (Crison PH 25, Crison Instruments, SA, Alella, Spain).
110 A 24-h post-slaughter pH ranging between 5.7 and 6.0 has been used as a threshold for dark,
111 firm and dry (DFD) meat (Ponnampalam et al., 2017). On the other hand, low *post-mortem*
112 pH is correlated with pastiness and anomalous color. In order to minimize the appearance of
113 defective texture in the final product, a cut-off point of pH 5.55 was reported for the
114 classification of hams (Garcia-Rey, Garcia-Garrido, Quiles-Zafra, Tapiador, & de Castro, 2004).
115 Accordingly, 10 hams with a low pH ($pH_{SM24h} \leq 5.4$), nine hams with normal pH ($5.4 < pH_{SM24h}$
116 < 5.9), and nine hams with high pH ($pH_{SM24h} \geq 5.9$) were selected. All hams were weighed and
117 salted according to the traditional method, but without using the nitrifying salts (nitrite and
118 nitrate). Hams were manually rubbed with 10 g of salt per kilogram of raw ham and then
119 covered with salt and piled at 3 ± 2 °C and 85 ± 5% relative humidity (RH) for 1 d/kg of raw
120 ham (standard salting) or 0.5 d/kg of raw ham (reduced salting). After salting, the hams were
121 washed with cold water and stored at 3 ± 2 °C and 85 ± 5% RH for two months. The drying of
122 hams was performed at 8 ± 2 °C and 70 ± 5% RH until three months of processing. The
123 temperature was then increased to 14 °C and 60 ± 5% RH until nine months of processing and
124 then to 25 °C until the end of process (12 months). Weight losses were monitored during the
125 entire elaboration process.

126 At the end of the process, the ham was boned the cushion part of the hams, which contains
127 *Biceps femoris* (BF) and SM muscles, was sampled. Three two-centimeter thick slices were cut
128 with a slicer machine, and BF muscle was excised to evaluate the instrumental color
129 characteristics within 10 min and determine, at the end of the process, the pH in BF and SM
130 muscles (pH_{BF} and pH_{SM}, respectively). Thereafter, the BF muscles from all slices were
131 homogenized together and some aliquots were frozen at -80 °C for pigment and other
132 physicochemical analyses.

133 ***2.3 Determination of ZnPP***

134 ZnPP was quantitatively extracted in subdued light conditions with ethyl acetate/acetic
135 acid/dimethyl sulfoxide solvent mixture (10:2:1, v/v/v) in quadruplicate as described
136 elsewhere with minor changes (Bou et al., 2018). In brief, two grams of ground ham samples
137 were weighed into 50-mL capacity centrifuge tubes and homogenized using an UltraTurrax
138 T25 model disperser (IKA Werke GmbH & Co. KG, Staufen, Germany) for one minute at 9000
139 rpm with 10 mL of the solvent mixture while the tube was immersed in ice. The sample
140 residues were re-extracted (few second burst) with the same volume of solvent mixture and
141 added to the previous one. After extraction on ice for 20 min and centrifugation (1100 *g*, 14
142 min, 4 °C), the supernatant was filtered through a filter paper (grade 1) and collected into a
143 volumetric flask. The solvent extractions were performed until the final volume was attained
144 (typically 20 mL). Two hundred microliters of extracts were transferred to 96-microwell plates
145 and sealed with a polyolefin acrylate sealing tape. The samples were then incubated for two
146 minutes at 30 °C and shaken for 30 sec before measuring the fluorescence of ZnPP using a
147 Thermo Fisher Scientific Varioskan microplate reader (Waltham, Massachusetts, USA) with
148 excitation at 416 nm and emission at 588 nm. Ethyl acetate/acetic acid/dimethyl sulfoxide
149 solvent mixture (10:2:1, v/v/v) was used as a blank. Each sample was analyzed four times, and
150 the excitation and emission spectra of the standards and samples were compared. ZnPP
151 content was calculated using a calibration curve prepared with ZnPP standard solutions and
152 expressed on the fresh weight basis, dry matter (DM) basis (ZnPP content DM = ZnPP
153 (mg)/(sample (kg) – water (kg))), and desalted DM basis (ZnPP content DM = ZnPP (mg)
154 /(sample (kg) – water (kg) – NaCl (kg))).

155 ***2.4 Determination of heme content***

156 The total heme pigments were determined after the extraction of heme in subdued light
157 conditions with 90% (v/v) aqueous acetone containing HCl (0.24 M) in triplicate as described
158 in literature with some minor modifications (Hornsey, 1956). Briefly, 1.5 g of ground ham was
159 weighed in subdued light conditions into 50-mL capacity centrifuge tubes, and 200 µL of 0.5%

160 (w/v) aqueous cysteine HCl solution and 10 mL of acidified acetone solution were added. The
161 mixture was homogenized using an UltraTurrax T25 model disperser for one minute at 9000
162 rpm while the tube was immersed in ice. The sample was further macerated on ice for one
163 hour in the dark and thereafter centrifuged for 15 min (1100 *g* at 4 °C). The supernatant was
164 filtered through filter papers (grade 42) and collected in a volumetric flask with maximum
165 protection from light. An aliquot was filtered through a PTFE syringe filter (0.45 µm) before
166 injection (40 µL) into an Agilent HPLC 1100 series instrument (Agilent Technologies, Inc., Santa
167 Clara, California, United States) equipped with a Luna C18 column (150 x 4.6 mm, 5 µm, 100
168 Å; Phenomenex, Torrance, California, USA) and a UV/Vis detector set at 414 nm. Aqueous
169 acetic acid (2%) and methanol were used as mobile phases A and B, respectively. Heme was
170 eluted with a gradient in which phase B was increased from 60 to 100% in 5 min and then
171 maintained for 10 min at a constant flow rate of 1 mL/min. Each sample was analyzed three
172 times. The total heme content was calculated by means of a calibration curve prepared with
173 chlorohemin standard solutions and expressed on a fresh weight basis, DM basis (heme
174 content DM = heme (mg)/(sample (kg) – water (kg))), and desalted DM basis (heme content
175 DM = heme (mg)/(sample (kg) – water (kg) – NaCl (kg))). The ratio between ZnPP and heme
176 contents was also calculated.

177 ***2.5 Physicochemical determinations***

178 The analyses were conducted in duplicate unless specified. The color was measured in
179 triplicate on the surface of the BF muscle on two-centimeter thick slices immediately after
180 slicing. A colorimeter (Minolta CM 600d, Konica Minolta, Inc., Chiyoda, Tokyo, Japan) was
181 used to measure the color in the CIE-Lab space (lightness (L^*), redness (a^*) and yellowness
182 (b^*)). The illuminant used was D65 with 10° observer angle. The final pH_{BF} and pH_{SM} values
183 were determined in quadruplicate using a S40 SevenMulti pH meter (Mettler Toledo,
184 Columbus, Ohio, United States) and an Inlab Solids Pro (Mettler Toledo) probe. The chloride
185 content was determined according to the ISO protocol 1841-2 using a 785 DMP Titrino

186 potentiometric titrator (Metrohm AG, Herisau, Switzerland) and expressed as the NaCl
187 content. The NaCl content on fresh weight basis (Salt = NaCl (g)/(100 g sample)) was
188 calculated. Water activity (a_w) was measured at 25 °C ± 0.3 with an AquaLab model Series 3
189 TE water activity meter (Decagon Devices, Inc., Pullman, Washington, USA). The moisture was
190 determined by drying at 103 ± 2 °C until a constant weight was reached (AOAC, 2000). The
191 protein content was calculated by multiplying the total nitrogen content obtained via Kjeldahl
192 digestion by a factor of 6.25 (AOAC, 2000). The non-protein nitrogen content was determined
193 by the precipitation of proteins with trichloroacetic acid followed by the determination of
194 total nitrogen (Careri et al., 1993). The proteolysis index was determined as a percentage of
195 the ratio between non-protein nitrogen and total nitrogen.

196 ### *2.6 Lipid analysis*

197 The lipids were extracted according to the Folch extraction procedure by homogenizing 10 g
198 of minced dry-cured ham (subcutaneous fat was previously removed) in 250 mL of
199 $CHCl_3$:methanol (2:1, v/v). The lipid extract was fractionated by passing 30–60 mg of lipid
200 dissolved in 5 mL of $CHCl_3$:methanol (2:1, v/v) through an aminopropyl column. The neutral
201 lipids were eluted with 5 mL of $CHCl_3$:isopropanol (2:1, v/v), FFAs with 5 mL of diethylic
202 ether:acetic acid (2%), and phospholipids with 5 mL of methanol:HCl (9:1, v/v). The fatty acid
203 composition of the fractionated FFAs and that of the total lipid fraction were determined by
204 gas chromatography after methylation as described in a prior literature report (Garcia
205 Regueiro, Gibert, & Diaz, 1994). Pentadecanoic acid was used as an internal standard for the
206 quantification of FFAs and the extent of lipolysis (total amount FFA). The samples were
207 analyzed in duplicate.

208 ### *2.7 Visual estimation of intramuscular fat*

209 To visually estimate the intramuscular fat content, high quality images were acquired using a
210 photographic system that included a calibrated Canon EOS 50D digital camera (Canon Inc.,

211 Tokyo, Japan) with a picture resolution of 15.1 megapixels and an objective Canon EF-S 18–
212 200 mm f/ 3.5–5.6 IS. White balance was carried out with a white card (Lastolite Ltd.,
213 Leicestershire, United Kingdom) to electronically adjust the color reproduction without
214 showing color dominants. The camera was connected to a computer into which the images in
215 RAW format were uploaded. The dry-cured ham slices were positioned below the camera lens
216 and an image of the entire slice surface was acquired. Capture One Pro software (Phase One
217 A/S Inc., Frederiksberg, Denmark) was used to perform the white balance of the RAW images
218 and digitalize them to 667 × 1000 pixels to afford a TIF file with 16-bit color and size of 4 MB.
219 This was considered as sufficiently high quality for computer image analysis.

220 The visual intramuscular fat of the two different sections of BF muscles was segmented using
221 the previously described procedures (Muñoz, Rubio-Celorio, Garcia-Gil, Guardia, & Fulladosa,
222 2015; Santos-Garcés, Muñoz, Gou, Garcia-Gil, & Fulladosa, 2014). In brief, Matlab scripts
223 written in-house were used for the segmentation of visual intramuscular fat using edge
224 detection based on the discrete Fourier transform. The total area of the BF muscles and visual
225 intramuscular fat area were segmented, and the number of pixels for each was determined.
226 The percentage of intramuscular fat area related to the total area of the BF muscle was
227 calculated.

228 ***2.8 Statistical analysis***

229 The means of replicates were employed for the statistical analysis. To evaluate the effects of
230 pH_{SM24h}, salt content, and its interaction on weight loss, pigment content, and
231 physicochemical composition, a two-way ANOVA was performed. The differences between
232 the mean values were tested using Tukey's test. Pearson correlations were calculated
233 between the ZnPP content, heme content, and physicochemical parameters. Principal
234 component analysis (PCA) was used to examine the correlations between different variables.

235 Statistical significance was predetermined at $p < 0.05$. All analyses were performed using the

236 JMP13 statistical package (SAS Institute, Cary, North Carolina, USA).

237

238 **3 RESULTS**

239 The interaction between the factors *post mortem* pH_{SM24h} and salting time was found to be

240 not significant for all the studied parameters. Accordingly, this interaction is not considered

241 in the model and the main effects are discussed separately. The overall appearances of the

242 final dry-cured hams are similar, particularly, the color of BF muscles (Figure 1).

243

244 ***3.1 Effect of post mortem*** pH_{SM24h}

245 As intended, the *post mortem* pH_{SM24h} values are different between the groups, whereas the

246 initial weight and weight losses during processing are similar (Table 1). During elaboration,

247 the hams with high pH_{SM24h} values result in a lower content of ZnPP than those for the hams

248 with medium and low pH_{SM24h}, regardless of the mode of expression ($p < 0.05$). The opposite

249 trend is observed for the heme content. This observation explains the lower ratio between

250 ZnPP and heme in hams with high pH_{SM24h} in comparison to those with medium and low

251 pH_{SM24h}, which have similar ratios. The Instrumental color is not affected regardless of the

252 *post mortem* pH_{SM24h} (Table 1).

253 At the end of the process, no differences are observed in final pH_{BF} and pH_{SM} of the medium

254 and low *post mortem* pH_{SM24h} hams (Table 1). However, the final pH_{BF} and pH_{SM} values are

255 higher in hams from animals with high *post mortem* pH_{SM24h} compared to those with medium

256 and low pH_{SM24h}. The salt contents are higher in the high pH_{SM24h} hams than in medium pH_{SM24h}

257 hams. However, the salt contents in the hams with the lowest *post mortem* pH_{SM24h} values are

258 not different from those with medium and high pH_{SM24h}. The higher salt contents in high

259 pH_{SM24h} hams compared to medium pH_{SM24h} hams are consistent with the previous findings
260 (Ruiz-Ramirez, Arnau, Serra, & Gou, 2005). Moisture and water activity are unaffected by the
261 *post mortem* pH_{SM24h}. Similarly, the protein content is similar in all hams. However, the
262 proteolysis index increases when pH_{SM24h} decreases. No differences are observed in the fat
263 content, marbling, and the extent of lipolysis (total amount of FFAs), regardless of the pH_{SM24h}
264 values.

265 The fatty acid profile of the total lipid fraction of the hams is not affected by the *post mortem*
266 pH_{SM24h} (data not included). Accordingly, the average fatty acid profile of the total lipid
267 fraction is included in Table 2. This table also shows the variation in the amounts of different
268 FFAs with different pH_{SM24h} values. The contents of saturated free fatty acids (SFFAs) remain
269 unchanged. However, the content of free erucic acid (22:1 n-9) is low in hams with high *post*
270 *mortem* pH_{SM24h}. This is the only fatty acid affected among the fatty acids belonging to the n-
271 9 series. Free linoleic acid (18:2 n-6) is high in hams with low *post mortem* pH_{SM24h}. A similar
272 trend is observed for 20:3 n-6 and docosahexaenoic (22:6 n-3) FFA which are also affected by
273 the variation in pH_{SM24h} value. These results explain the increase in the total polyunsaturated
274 fatty acid (PUFFA) content, and also of the PUFFAs corresponding to n-6 and n-3 series, at low
275 pH_{SM24h}.

276

277 *3.2 Effect of salting time*

278 After the salting step, the weight losses are higher in hams subjected to standard salting
279 times. However, the weight losses are similar after 3, 9, and 12 months (end of the process).
280 In addition, the salting time does not affect the porphyrin contents (ZnPP and heme). This
281 observation explains that the ZnPP/heme ratio remains unaffected (Table 1). These results
282 are consistent with the lack of effect on the instrumental color.

283 A slight increase in final pH_{BF} and pH_{SM} is observed in hams treated with a reduced salting
284 time. As expected, the final salt content is lower in hams exposed to a reduced salting time
285 compared to those subjected to standard salting time. The moisture content remains
286 unchanged, whereas the water activity is high in hams with reduced salting time. In addition,
287 no changes are observed in the protein content, proteolysis index, fat content, and marbling
288 with reduced salting times (Table 1). However, the lipolysis extent is higher in hams with
289 reduced salting time compared to that in hams subjected to standard salting time.

290 The fatty acid profile of the total lipid fraction is not affected by the salting time (data not
291 included). For this reason, the average of the fatty acid composition is reported in Table 2.
292 The contents of free stearic acid (18:0) and SFFA are higher in hams exposed to reduced
293 salting times compared to those subjected to standard salting times. However, the
294 monounsaturated free fatty acids (MUFFAs) remain unaffected by the variation in salting
295 time. Free linoleic acid (18:2 n-6), free arachidonic acid (20:4 n-6), and total n-6 PUFFAs are
296 higher in hams with reduced salting time compared to those subjected to standard salting
297 time. However, the FFA contents of the n-3 series do not change with the salting time.

298

299 *3.3 Relationships between parameters*

300

301 Principal component analysis (PCA) and Pearson's correlation analysis were performed to
302 examine the relationships between the obtained data. The first two dimensions of the PCA
303 exhibit 59.3% of the variance. As shown in Figure 2, all of FFA, water activity, moisture, ZnPP
304 content regardless of its mode of expression, proteolysis index, and the ratio of ZnPP and
305 heme positively influence the first component. The NaCl content is strongly characterized by
306 the first component, although it is situated on the negative axis. Final pH_{BF}, *post mortem*
307 pH_{SM24h} and different ways of expression of the heme content are localized in similar

308 coordinates and affected by the 1st and 2nd dimensions. However, the coordinates of the latter
309 parameters are opposite to those of the proteolysis index, marbling, and the ratio of ZnPP
310 and heme. The MUFFA content, redness, and ZnPP/heme ratio are also negatively influenced
311 by the 2nd principal component. Overall, all PUFFAs are plotted in similar coordinates and n-3
312 PUFFAs are grouped together with the ZnPP content.

313 The similar localization of the different ways of expression of heme and ZnPP contents (Figure
314 2) explain the close relationships between both the contents and with other parameters. The
315 final pH_{BF} is highly correlated with final pH_{SM} ($r = 0.944$, $P < 0.001$) and not shown. Likewise,
316 *post mortem* pH_{SM24h} is positively correlated with final pH_{SM} ($r = 0.730$, $P < 0.001$) and with
317 pH_{BF} ($r = 0.708$, $P < 0.001$). For better clarity, Table 3 only shows the most relevant correlations
318 between the contents of ZnPP and heme expressed on the desalted DM basis and the
319 remaining physicochemical parameters. The ZnPP content is positively correlated with the
320 ZnPP/heme ratio ($r = 0.650$, $P < 0.001$), free stearic acid (18:0; $r = 0.379$, $P = 0.047$), free linoleic
321 acid (18:2 n-6; $r = 0.480$, $P = 0.010$), and free arachidonic acid (20:4 n-6; $r = 0.568$, $P = 0.002$),
322 as well as with n-6 ($r = 0.534$, $P = 0.003$), n-3 ($r = 0.446$, $P = 0.017$), and total ($r = 0.537$, $P =$
323 0.003) PUFFAs. Contrarily, the ZnPP content is negatively correlated with the pH_{BF} ($r = -0.516$,
324 $P = 0.005$) and salt content ($r = -0.391$, P = 0.040). The heme content is positively correlated
325 with redness ($r = 0.434$, $P = 0.021$), pH_{BF} ($r = 0.854$, $P < 0.001$), and n-3 PUFFAs ($r = 0.557$, $P =$
326 0.002), and negatively correlated with the ZnPP/heme ratio ($r = -0.839$, $P < 0.001$), proteolysis
327 index ($r = -0.521$, $P = 0.004$), and n-3 PUFFAs ($r = -0.557$, $P = 0.002$). The ZnPP/heme ratio is
328 positively correlated with the proteolysis index ($r = 0.492$, $P = 0.008$), free linoleic acid (r =
329 0.391, $P = 0.040$), and n-6 ($r = 0.397$, $P = 0.037$), n-3 ($r = 0.500$, $P = 0.007$), and total (r = 0.406.
330 $P = 0.032$) PUFFAs. However, this ratio is negatively correlated with the final pH_{BF} ($r = -0.891$,
331 $P < 0.001$).

332 The onset of lipolysis causes the formation of FFA and as expected, these are highly correlated
333 (Table 3). In addition to the existing correlations with porphyrins, the pH_{BF} is negatively

334 correlated with the proteolysis index (r = -0.568, P = 0.002) and n-3 PUFFAs (r = -0.530, P =
335 0.004). However, the proteolysis index is positively correlated to moisture (r = 0.391, P =
336 0.040), free arachidonic acid (r = 0.474, P = 0.011), and n-6 (r = 0.377, P = 0.048), n-3 (r = 0.433,
337 P = 0.021), and total (r = 0.383, P = 0.044) PUFFAs. The NaCl content is negatively correlated
338 to water activity (r = -0.955, P <0.001), moisture (r = -0.735, P < 0.001) and in general, lipolysis
339 (Table 3). The opposite trend is observed for water activity. This latter parameter is generally
340 positively correlated with various FFAs and moisture (r = 0.845, P < 0.001), and inversely
341 correlated to protein content (r = -0.407, P = 0.032). The fat content is positively correlated
342 with MUFFA content (r = 0.586, P < 0.001).

343

344 # 4 DISCUSSION

345

346 ## *4.1 Effect of post mortem* pH_{SM24h}

347

348 The proximal compositions (moisture, protein, and fat) of hams at the end of the process (12
349 months) remains unaffected by the *post mortem* pH_{SM24h} (Table 1), which explains their similar
350 appearance (Figure 1). This lack of effect on the major food components allows a better
351 comparison between different treatments in terms of the porphyrin content. Table 1 shows
352 the differences in the final pH_{BF} and pH_{SM} of hams. The ability of ferrochelatase to remove and
353 insert the ions is regulated by pH (Chau et al., 2010). The ability of porcine ferrochelatase to
354 form ZnPP is reported to be maximum at a pH of ≤ 6 in *in vitro* studies (Benedini et al., 2008;
355 Ishikawa et al., 2006; Wakamatsu et al., 2019; Wakamatsu, Murakami, & Nishimura, 2015). It
356 has recently been reported that the optimal pH values for ZnPP formation in porcine skeletal
357 muscles are 5.5 and 4.75 depending on muscle fiber type (Wakamatsu et al., 2019). These

358 findings explain that at the end of the processing, the animals with a pH_{SM24h} of ≥ 5.9 afford
359 hams with lower ZnPP contents than those from animals with low pH_{SM24h} (Table 1).

360 The replacement of Fe ions in the protoporphyrin ring by Zn ions in hams has been mainly
361 attributed to the demetallation of heme by ferrochelatase at low pH (Parolari et al., 2009;
362 Wakamatsu, Okui, et al., 2004). Therefore, this explains the decreased content of ZnPP in
363 hams from animals with high pH_{SM24h} values (Table 1). This is in agreement with the similar
364 coordinates of final pH_{BF} and heme in the PCA (Figure 2), and the high correlation between
365 the final pH_{BF} and heme, as well as between the final pH_{BF} and ZnPP/heme ratio. These results
366 support the pH dependence of this enzyme and in particular, its crucial role in the removal of
367 Fe from the porphyrin ring as a first step toward forming ZnPP.

368 The pH value typically increases during processing, which is mainly attributed to proteolysis
369 (Arnau, Guerrero, Casademont, & Gou, 1995; Morales, Serra, Guerrero, & Gou, 2007).
370 However, high proteolysis indexes are usually observed in hams with low *post mortem* pH
371 (Morales et al., 2007; Ruiz-Ramirez, Arnau, Serra, & Gou, 2006; Tabilo et al., 1999), which is
372 mainly attributed to an increased cathepsin activity (Arnau, Guerrero, & Sárraga, 1998). It is
373 worth noting that the pH at the end of the process in SM and BF muscles is very similar (Table
374 1), which is in agreement with other authors (Arnau et al., 1995; Parolari et al., 2016).
375 Accordingly, the increased proteolysis in hams with low pH_{SM24h} can explain the absence of
376 differences in pH_{BF} and pH_{SM} compared to those for hams with medium *post mortem* pH_{SM24h}.
377 This reasoning is also consistent with the observed correlation between the proteolysis index
378 and pH_{BF}. However, in a prior study on commercial Parma hams, the correlation between the
379 final pH and proteolysis index, between final pH and ZnPP, and between the proteolysis index
380 and heme were not found to be significant (Bou et al., 2018). It is possible that the pH values
381 at the initial stages are particularly relevant to the formation of ZnPP because the activity of
382 ferrochelatase decreases significantly after 3–8 months of production (Parolari et al., 2009).

383 Therefore, the formation of ZnPP may not only be affected by the *post mortem* pH, but also
384 by the evolution of the pH during the complete elaboration process of hams.

385 In general, the content of ZnPP in Serrano hams seems to be higher than that reported in
386 Parma hams (Bou et al., 2018; Parolari et al., 2016) probably due to the higher process
387 temperatures in Serrano hams. After 12 months of processing at 16 °C, Parolari et al. (2016)
388 reported that in BF muscles the content of ZnPP averaged 59 mg/kg dry matter while the final
389 pH was around 5.9. This pH is comparable to the recorded final pH_{BF} in those hams with low
390 *post mortem* pH_{SM24h}. The formation of ZnPP has not been completely elucidated, and in
391 addition to pH, other factors can be involved (Khozroughi, Braga, Wagner, & Rawel, 2019;
392 Paganelli et al., 2016; Parolari et al., 2016). Paganelli et al. (2016) reported that the partial
393 proteolysis of myoglobin with pepsin facilitated its interaction with ferrochelatase. According
394 to the authors, the mild hydrolysis of the myoglobin facilitated the enzymatic removal of Fe
395 and subsequent insertion of Zn in the porphyrin ring. In agreement with this finding, the
396 proteolysis index is concomitant with the ZnPP/heme ratio and loss of heme (Figure 2 and
397 Table 3). A similar relationship between the proteolysis index and ZnPP/heme ratio was
398 observed in commercial Parma hams (Bou et al., 2018). Interestingly, the sum of porphyrins
399 is higher at high *post mortem* pH than at low pH. This finding is difficult to explain but it could
400 be attributed to an increased myoglobin oxidation at low pH and its subsequent interaction
401 with proteins (Chaijan, Benjakul, Visessanguan, Lee, & Faustman, 2008; Richards, 2013;
402 Thongraung, Benjakul, & Hultin, 2006). Thus, the low ZnPP/heme ratios could be partly due
403 to a higher stability or better extractability of heme at high pH compared to those at low pH
404 values. The relationships between the pH, proteolysis, and formation of ZnPP, as well as the
405 stability of heme should be investigated in more depth in future studies.

406 The proteolytic and lipolytic processes occur simultaneously during the processing of dry-
407 cured meat products and contribute to the flavor characteristics of dry-cured hams (Toldrá,
408 1998). Muscle lipases and phospholipases are responsible for lipolysis in dry-cured hams

409 (Andres et al., 2005; Motilva et al., 1993). Considering that ham is rich in oleic acid, the activity
410 of lipases can explain the correlation between fat content and MUFFAs. In addition, the
411 concurrent lipolytic and proteolytic processes can clarify the correlation between the
412 proteolysis index and some FFAs such as arachidonic acid, n-6 PUFFAs, n-3 PUFFAs, and total
413 PUFFAs (Table 3). However, the presence of FFAs and phospholipids can also activate the
414 enzymatic formation of ZnPP from heme (Chau et al., 2010). The onset of lipolysis and its
415 effect on ferrochelatase could explain the relationship between marbling and ZnPP in Parma
416 hams in a prior study (Bou et al., 2018). However, in the present study, the levels of marbling
417 and lipolysis (total amount of FFA) in BF are similar at different pH_{SM24h} values (Tables 1 and
418 2). This can explain the lack of correlation between these parameters and ZnPP content as
419 well as the ZnPP/heme ratio (Table 3). Despite this, the contents of free linoleic acid and other
420 PUFFAs are higher in hams with medium and low pH_{SM24h} compared to those in hams with
421 high pH_{SM24h} (Table 2). The increase in content of these PUFFAs rather than free oleic acid is
422 likely due to the high extent of lipolysis in the phospholipid fraction (Andres et al., 2005;
423 Toldrá, 1998). However, pH_{SM24h} also affected salt content and this may in turn cause changes
424 in the content of FFA. Despite that, the free linoleic acid, n-6 PUFFAs, and total PUFFAs are
425 correlated with the ZnPP content and ZnPP/heme ratio (Table 3). The content in FFA will be
426 further discussed in the following section but, as suggested by other researchers, the release
427 of certain fatty acids and phospholipids can also modulate the formation of ZnPP by
428 ferrochelatase to some extent (Chau et al., 2010; Taketani, 1993).

429 With regards to color, the overall redness values of these non-nitrified Serrano hams is higher
430 than that previously found in Parma hams (Parolari et al., 2016; Parolari et al., 2009). The
431 characteristic bright red color of Parma ham has been mainly attributed to the ZnPP content
432 (Wakamatsu, Nishimura, et al., 2004). However, ZnPP and heme are present in relatively
433 similar percentages in hams elaborated without the addition of nitrites (Bou et al., 2018;
434 Wakamatsu, Nishimura, et al., 2004). The relationship between porphyrins and color is of

435 technological interest. However, regardless of its effect on the different contents of
436 porphyrins, the production of dry-cured hams with a relatively broad range of pH_{SM24h} has a
437 small impact on the overall appearance of hams produced without the addition of curing salts
438 at the end of the process (Figure 1 and Table 1).

439

440 ***4.2 Effect of salting time***

441

442 The addition of salt in meat homogenates results in an increase in the formation of ZnPP
443 (Becker et al., 2012; Benedini et al., 2008). This is in agreement with the results of a prior
444 study with commercial Parma hams in which the salt content is correlated with the ZnPP
445 content, when expressed on a dry weight basis (Bou et al., 2018). However, in the present
446 study with Serrano processed hams, the ZnPP and heme contents are unaffected by the
447 salting time (Table 1). Moreover, the salt content is inversely correlated with ZnPP, when
448 expressed on desalted dry weight basis (Table 3) or on a dry weight basis (data not included).
449 Although these results suggest that the role of salt content in the conversion of Fe porphyrin
450 into ZnPP is less relevant than the effect of pH_{SM24h}, further studies aimed at explaining these
451 controversial results in dry-cured hams are needed as these utilize a narrow range of salt
452 concentrations than those assayed in the *in vitro* studies. The lack of any significant effect of
453 the salting time on ZnPP and heme contents can explain the similar instrumental color values
454 obtained in this study (Table 1).

455 The reduced salting time leads to a relatively low salt content in hams (Table 1) in comparison
456 to typical content values ranging from 4.3% to 6.4% in the BF muscles of the commercial
457 Parma hams (Bou et al., 2018). Considering that the weight losses are similar at the end of
458 their production, the low salt content in hams subjected to reduced salting time is consistent
459 with the increased water activities as this parameter is affected by both moisture and salt

460 content. Morales et al. (2007) reported that the final pH of dry-cured BF muscles reduced with
461 an increase in the salting levels, when studying the effect of three salting levels (1%, 2% or 4%
462 of added NaCl) and three different pH_{24h} values ($pH < 5.66$; $5.66 \leq pH \leq 6.00$; $pH > 6.00$). This
463 result is in agreement with the slight increase in the final pH_{BF} values of the hams subjected
464 to reduced salting time in the present study (Table 1). These results can be explained by the
465 activity of cathepsins, which has been reported to increase with the addition of salt, resulting
466 in an increase in the final pH (Arnau et al., 1998; Gil, Hortos, & Sarraga, 1998). However, the
467 proteolysis index remains unchanged regardless of the salting time as this effect appears to
468 be more dependent on the *post mortem* pH.

469 In pork muscle, the activity of acid lipase is increased by the addition of salt at 6-8% (Motilva
470 & Toldra, 1993). This fact explained the decrease in the fatty acids in both neutral and polar
471 lipid fractions during the processing of dry-cured hams with high salt contents (Andres et al.,
472 2005; Motilva et al., 1993). In the present study, the percentages of total SFFAs, MUFFAs, and
473 PUFFAs are 32%, 26–24%, and 42–44%, respectively. This PUFFAs levels are higher than that
474 corresponding to the total lipid fraction (Table 2). The composition of the total lipid fraction
475 is supposed to be mainly affected by the level of marbling (i.e. neutral lipids) which was similar
476 between salting treatments. Therefore, the relative increase in PUFFAs with respect to total
477 polyunsaturated fatty acids (PUFAs) suggests that the lipolysis is more intense in the
478 phospholipid fraction than in the neutral lipid fraction, which is in agreement with other
479 authors (Andres et al., 2005; Martin et al., 1999). However, higher amounts of FFAs are
480 expected in hams subjected to a standard salting time in comparison to those with a reduced
481 salting time because of a decrease in fatty acids. As shown in Table 1, an opposite trend is
482 observed herein which also agrees with results reported in previous studies (Andres et al.,
483 2005). The decreased amounts of FFAs in dry-cured hams subjected to standard salting time
484 are attributed to the high susceptibility of PUFA to oxidation, the existence of an equilibrium
485 between lipolysis and oxidation phenomena (Andres et al., 2005; Martin et al., 1999), and

486 possibly to low microbial counts. Thus, the oxidation processes can be responsible for the
487 negative correlation between FFAs and salt content, and high contents of a number of FFAs
488 in hams subjected to reduced salting time (Tables 2 and 3).

489 The enzyme ferrochelatase is presumed to have an important role with regards to the
490 formation of ZnPP in cured hams without the addition of nitrite (Parolari et al., 2016; Parolari
491 et al., 2009). Therefore, the presence of fatty acids and phospholipids may enhance the
492 activity of the enzyme which is located in the inner mitochondrial membrane (Chau et al.,
493 2010; Taketani, 1993). This fact may help to explain the correlations between PUFFAs and
494 ZnPP (Table 3). However, the higher lipolysis extent in hams with a reduced salting time had
495 no effect on pigments content. The substitution of Fe by Zn in the heme group catalyzed by
496 the enzyme takes place in both native and partly proteolyzed myoglobin (Khozroughi et al.,
497 2017; Paganelli et al., 2016; Wakamatsu et al., 2019). Despite that, it seems that the formed
498 ZnPP bound to protein can be transitioned into free ZnPP during the process of incubation
499 (Khozroughi et al., 2019). Interestingly, the formation of ZnPP in meat systems and fermented
500 sausages has been reported without causing a decrease in heme iron content (De Maere et
501 al., 2016; Wakamatsu et al., 2019). Thus, the formation of ZnPP in meat products can be very
502 complex and may involve different mechanisms.

503

504 5 CONCLUSIONS

505

506 In the production of Serrano dry-cured hams without the addition of curing salts, low ZnPP
507 and high heme contents are observed in hams with high pH_{SM24h} values. The reduction of the
508 salting time results in a decrease in the salt content but does not affect the ZnPP content.
509 However, the proteolytic and lipolytic processes, which are affected by the *post mortem* pH
510 and salt content, can also affect the formation of ZnPP. This issue should be clarified in future

511 studies as in the production of dry-cured hams proteolysis and lipolysis processes are
512 concomitant and thus difficult to interpret. At the end of the process, instrumental redness
513 seemed to be unaffected by the initial pH and salting period. Despite that, a high ZnPP content
514 can offer technological advantages owing to its color stability compared to that of heme,
515 which can be beneficial in obtaining a final product with better color characteristics and
516 stability.

517

518 **Acknowledgments:**

519 The CERCA Programme from the *Generalitat de Catalunya* and the Spanish *Centro para el*
520 *Desarrollo Técnico Industrial* (project IDI-20150895) supported this work. R. B. also received
521 financial support from the Ramon y Cajal Program (RYC-2013-12745) of the *Ministerio de*
522 *Economía y Competitividad*.

REFERENCES

Adamsen, C. E., Moller, J. K. S., Parolari, G., Gabba, L., & Skibsted, L. H. (2006). Changes in Zn-porphyrin and proteinous pigments in italian dry-cured ham during processing and maturation. *Meat Science, 74*(2), 373-379. doi:10.1016/j.meatsci.2006.04.003

Andres, A. I., Cava, R., Martin, D., Ventanas, J., & Ruiz, J. (2005). Lipolysis in dry-cured ham: Influence of salt content and processing conditions. *Food Chemistry, 90*(4), 523-533. doi:10.1016/j.foodchem.2004.05.013

AOAC (Ed.) (2000). *Official Methods of Analysis of AOAC International* (17th ed.). Gaithersburg, MD.

Arnau, J., Guerrero, L., Casademont, G., & Gou, P. (1995). Physical and chemical changes in different zones of normal and PSE dry-cured ham during processing. *Food Chemistry, 52*(1), 63-69. doi:10.1016/0308-8146(94)p4182-f

Arnau, J., Guerrero, L., & Sárraga, C. (1998). The effect of green ham pH and NaCl concentration on cathepsin activities and the sensory characteristics of dry-cured hams. *Journal of the Science of Food and Agriculture, 77*(3), 387-392. doi:doi:10.1002/(SICI)1097-0010(199807)77:3<387::AID-JSFA57>3.0.CO;2-H

Becker, E. M., Westermann, S., Hansson, M., & Skibsted, L. H. (2012). Parallel enzymatic and non-enzymatic formation of zinc protoporphyrin IX in pork. *Food Chemistry, 130*(4), 832-840. doi:10.1016/j.foodchem.2011.07.090

Benedini, R., Raja, V., & Parolari, G. (2008). Zinc-protoporphyrin IX promoting activity in pork muscle. *Lwt-Food Science and Technology, 41*(7), 1160-1166. doi:10.1016/j.lwt.2007.08.005

Bou, R., Llauger, M., Arnau, J., & Fulladosa, E. (2018). Zinc-protoporphyrin content in commercial Parma hams is affected by proteolysis index and marbling. *Meat Science, 139*, 192-200. doi:https://doi.org/10.1016/j.meatsci.2018.01.027

Careri, M., Mangia, A., Barbieri, G., Bolzoni, L., Virgili, R., & Parolari, G. (1993). Sensory property relationships to chemical data of Italian-type dry-cured ham. *Journal of Food Science, 58*(5), 968-972. doi:10.1111/j.1365-2621.1993.tb06090.x

Chaijan, M., Benjakul, S., Visessanguan, W., Lee, S., & Faustman, C. (2008). Interaction of fish myoglobin and myofibrillar proteins. *Journal of Food Science, 73*(5), C292-C298. doi:doi:10.1111/j.1750-3841.2008.00749.x

Chau, T. T., Ishigaki, M., Kataoka, T., & Taketani, S. (2010). Porcine Ferrochelatase: The Relationship between Iron-Removal Reaction and the Conversion of Heme to Zn-Protoporphyrin. *Bioscience Biotechnology and Biochemistry, 74*(7), 1415-1420. doi:10.1271/bbb.100078

De Maere, H., Fraeye, I., De Mey, E., Dewulf, L., Michiels, C., Paelinck, H., & Chollet, S. (2016). Formation of naturally occurring pigments during the production of nitrite-free dry fermented sausages. *Meat Science, 114*, 1-7. doi:10.1016/j.meatsci.2015.11.024

del Olmo, A., Calzada, J., & Nunez, M. (2016). Lipolysis, lipid peroxidation and texture of Serrano ham processed under different ripening temperature conditions. *International Journal of Food Science and Technology, 51*(8), 1793-1800. doi:10.1111/ijfs.13148

Garcia-Rey, R. M., Garcia-Garrido, J. A., Quiles-Zafra, R., Tapiador, J., & de Castro, M. D. L. (2004). Relationship between pH before salting and dry-cured ham quality. *Meat Science, 67*(4), 625-632. doi:10.1016/j.meatsci.2003.12.013

Garcia Regueiro, J. A., Gibert, J., & Diaz, I. (1994). Determination of neutral lipids from subcutaneous fat of cured ham by capillary gas-chromatography and liquid-

571 chromatography. *Journal of Chromatography A, 667*(1-2), 225-233.
572 doi:10.1016/0021-9673(94)89071-4
573 Gil, M., Hortos, M., & Sarraga, C. (1998). Calpain and cathepsin activities, and protein
574 extractability during ageing of longissimus porcine muscle from normal and PSE meat.
575 *Food Chemistry, 63*(3), 385-390. doi:10.1016/s0308-8146(98)00003-x
576 Grossi, A. B., do Nascimento, E. S. P., Cardoso, D. R., & Skibsted, L. H. (2014). Proteolysis
577 involvement in zinc-protoporphyrin IX formation during Parma ham maturation. *Food*
578 *Research International, 56*, 252-259. doi:10.1016/j.foodres.2014.01.007
579 Hornsey, H. C. (1956). The colour of cooked cured pork. I.—Estimation of the Nitric oxide-
580 Haem Pigments. *Journal of the Science of Food and Agriculture, 7*, 534–540.
581 Ishikawa, H., Yoshihara, M., Baba, A., Kawabuchi, T., Sato, M., Numata, M., & Matsumoto, K.
582 (2006). Formation of zinc protoporphyrin IX from myoglobin in porcine heart extract.
583 *Food Science and Technology Research, 12*(2), 125-130. doi:10.3136/fstr.12.125
584 Khozroughi, A. G., Braga, T. W., Wagner, J., & Rawel, H. (2019). Investigation of the post
585 mortem zinc protoporphyrin IX fluorescence with respect to its protein-bound and
586 unbound occurrence in aqueous meat extracts. *Food Chemistry, 283*, 462-467.
587 doi:10.1016/j.foodchem.2019.01.080
588 Khozroughi, A. G., Jander, E., Schirrmann, M., Rawel, H., Kroh, L. W., & Schluter, O. (2017).
589 The role of myoglobin degradation in the formation of zinc protoporphyrin IX in the
590 longissimus lumborum of pork. *Lwt-Food Science and Technology, 85*, 22-27.
591 doi:10.1016/j.lwt.2017.06.047
592 Khozroughi, A. G., Kroh, L. W., Schluter, O., & Rawel, H. (2018). Assessment of the bacterial
593 impact on the post-mortem formation of zinc protoporphyrin IX in pork meat. *Food*
594 *Chemistry, 256*, 25-30. doi:10.1016/j.foodchem.2018.01.045
595 Martin, L., Cordoba, J. J., Ventanas, J., & Antequera, T. (1999). Changes in intramuscular lipids
596 during ripening of Iberian dry-cured ham. *Meat Science, 51*(2), 129-134.
597 doi:10.1016/s0309-1740(98)00109-0
598 Morales, R., Serra, X., Guerrero, L., & Gou, P. (2007). Softness in dry-cured porcine biceps
599 femoris muscles in relation to meat quality characteristics and processing conditions.
600 *Meat Science, 77*(4), 662-669. doi:https://doi.org/10.1016/j.meatsci.2007.05.020
601 Motilva, M. J., & Toldra, F. (1993). Effect of curing agents and water activity on pork muscle
602 and adipose subcutaneous tissue lipolytic activity. *Zeitschrift Fur Lebensmittel-*
603 *Untersuchung Und-Forschung, 196*(3), 228-232. doi:10.1007/bf01202737
604 Motilva, M. J., Toldra, F., Nieto, P., & Flores, J. (1993). Muscle lipolysis phenomena in the
605 processing of dry-cured ham. *Food Chemistry, 48*(2), 121-125. doi:10.1016/0308-
606 8146(93)90045-h
607 Muñoz, I., Rubio-Celorio, M., Garcia-Gil, N., Guardia, M. D., & Fulladosa, E. (2015). Computer
608 image analysis as a tool for classifying marbling: A case study in dry-cured ham.
609 *Journal of Food Engineering, 166*, 148-155. doi:10.1016/j.jfoodeng.2015.06.004
610 Paganelli, M. O., Grossi, A. B., Dores-Silva, P. R., Borges, J. C., Cardoso, D. R., & Skibsted, L. H.
611 (2016). Limited proteolysis of myoglobin opens channel in ferrochelatase-globin
612 complex for iron to zinc transmetallation. *Food Chemistry, 210*, 491-499.
613 doi:10.1016/j.foodchem.2016.04.109
614 Parolari, G. (1996). Achievements, needs and perspectives in dry-cured ham technology: The
615 example of Parma ham. *Food Science and Technology International, 2*(2), 69-78.
616 doi:10.1177/108201329600200203
617 Parolari, G., Aguzzoni, A., & Toscani, T. (2016). Effects of Processing Temperature on Color
618 Properties of Dry-Cured Hams Made without Nitrite. *Foods, 5*(2).
619 doi:10.3390/foods5020033
620 Parolari, G., Benedini, R., & Toscani, T. (2009). Color Formation in Nitrite-Free Dried Hams as
621 Related to Zn-Protoporphyrin IX and Zn-Chelatase Activity. *Journal of Food Science,*
622 *74*(6), C413-C418. doi:10.1111/j.1750-3841.2009.01193.x

623 Ponnampalam, E. N., Hopkins, D. L., Bruce, H., Li, D., Baldi, G., & El-din Bekhit, A. (2017).
624 Causes and Contributing Factors to "Dark Cutting" Meat: Current Trends and Future
625 Directions: A Review. *Comprehensive Reviews in Food Science and Food Safety, 16*(3),
626 400-430. doi:10.1111/1541-4337.12258
627 Richards, M. P. (2013). Redox Reactions of Myoglobin. *Antioxidants & Redox Signaling, 18*(17),
628 2342-2351. doi:10.1089/ars.2012.4887
629 Ruiz-Ramirez, J., Arnau, J., Serra, X., & Gou, P. (2005). Relationship between water content,
630 NaCl content, pH and texture parameters in dry-cured muscles. *Meat Science, 70*(4),
631 579-587. doi:10.1016/j.meatsci.2005.02.007
632 Ruiz-Ramirez, J., Arnau, J., Serra, X., & Gou, P. (2006). Effect of pH(24), NaCl content and
633 proteolysis index on the relationship between water content and texture parameters
634 in biceps femoris and semimembranosus muscles in dry-cured ham. *Meat Science,*
635 *72*(2), 185-194. doi:10.1016/j.meatsci.2005.06.016
636 Santos-Garcés, E., Muñoz, I., Gou, P., Garcia-Gil, N., & Fulladosa, E. (2014). Including estimated
637 intramuscular fat content from computed tomography images improves prediction
638 accuracy of dry-cured ham composition. *Meat Science, 96*(2), 943-947.
639 doi:10.1016/j.meatsci.2013.09.018
640 Sebranek, J. G., & Bacus, J. N. (2007). Cured meat products without direct addition of nitrate
641 or nitrite: what are the issues? *Meat Science, 77*(1), 136-147.
642 doi:10.1016/j.meatsci.2007.03.025
643 Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide-*
644 *Biology and Chemistry, 24*(4), 176-183. doi:10.1016/j.niox.2011.03.307
645 Tabilo, G., Flores, M., Fiszman, S. M., & Toldra, F. (1999). Postmortem meat quality and sex
646 affect textural properties and protein breakdown of dry-cured ham. *Meat Science,*
647 *51*(3), 255-260. doi:10.1016/s0309-1740(98)00125-9
648 Taketani, S. (1993). Molecular and genetic characterization of ferrochelatase. *Tohoku Journal*
649 *of Experimental Medicine, 171*(1), 1-20. doi:10.1620/tjem.171.1
650 Thongraung, C., Benjakul, S., & Hultin, H. O. (2006). Effect of pH, ADP and muscle soluble
651 components on cod hemoglobin characteristics and extractability. *Food Chemistry,*
652 *97*(4), 567-576. doi:https://doi.org/10.1016/j.foodchem.2005.05.006
653 Toldrá, F. (1998). Proteolysis and lipolysis in flavour development of dry-cured meat products.
654 *Meat Science, 49*, S101-S110. doi:10.1016/s0309-1740(98)90041-9
655 Toldrá, F. (2002). Manufacturing of Dry-Cured Ham. In *Dry-Cured Meat Products* (pp. 27-62).
656 Trumbull, CT: Food & Nutrition Press Inc.
657 Wakamatsu, J., Akter, M., Honma, F., Hayakawa, T., Kumura, H., & Nishimura, T. (2019).
658 Optimal pH of zinc protoporphyrin IX formation in porcine muscles: Effects of muscle
659 fiber type and myoglobin content. *Lwt-Food Science and Technology, 101*, 599-606.
660 doi:10.1016/j.lwt.2018.11.040
661 Wakamatsu, J., Murakami, N., & Nishimura, T. (2015). A comparative study of zinc
662 protoporphyrin IX-forming properties of animal by-products as sources for improving
663 the color of meat products. *Animal science journal = Nihon chikusan Gakkaiho, 86*(5),
664 547-552. doi:10.1111/asj.12326
665 Wakamatsu, J., Nishimura, T., & Hattori, A. (2004). A Zn-porphyrin complex contributes to
666 bright red color in Parma ham. *Meat Science, 67*(1), 95-100.
667 doi:10.1016/j.meatsci.2003.09.012
668 Wakamatsu, J., Okui, J., Ikeda, Y., Nishimura, T., & Hattori, A. (2004). Establishment of a model
669 experiment system to elucidate the mechanism by which Zn-protoporphyrin IX is
670 formed in nitrite-free dry-cured ham. *Meat Science, 68*(2), 313-317.
671 doi:10.1016/j.meatsci.2004.03.014

672

673 **FIGURE CAPTIONS**

674

675 **Figure 1.** Images of the dry-cured ham sections. The hams displayed on the left (A, C, E) were
676 exposed to standard periods of salting whereas those in the right (B, D, F) were exposed to
677 reduced salting periods. On the top (A, B), middle (C, D) and bottom (E, F) positions are displayed
678 those hams with low, medium and high *post mortem* pH_{SM24h}, respectively.

679

680 **Figure 2**. Principal components analysis of the content in zinc-protoporphyrin (ZnPP), heme and
681 free fatty acid and other physicochemical parameters in non-nitrified dry-cured hams. The
682 abbreviation aw stands for water activity. See Tables 1 and 2 for other abbreviations and units.

683

Table 1. Effect of *post mortem* pH_{SM24h} range and salting time on weight losses during the elaboration of dry-cured hams and physicochemical composition of muscle *Biceps femoris* at the end of the process (12 months).

	Post mortem pH range			Salting time	
	High	Medium	Low	Standard	Reduced
pH_{SM24h}	6.12 ± 0.13 a	5.59 ± 0.03 b	5.40 ± 0.01 c	5.69 ± 0.34	5.69 ± 0.31
Initial weight (kg)	13.1 ± 0.37	13.0 ± 0.40	12.6 ± 1.12	12.85 ± 0.78	12.85 ± 0.74
Weight loss after salting (%)	5.4 ± 1.3	5.8 ± 1.2	6.4 ± 1.4	6.7 ± 1.1 b	5.1 ± 1.0 a
Weight loss after 3 months (%)	21.2 ± 1.8	21.5 ± 1.0	22.4 ± 2.3	22.1 ± 2.0	21.4 ± 1.6
Weight loss after 9 months (%)	32.4 ± 2.8	32.4 ± 1.1	33.8 ± 3.2	33.0 ± 3.1	32.8 ± 2.1
Weight loss after 12 months (%)	36.1 ± 3.3	36.0 ± 1.1	37.5 ± 3.4	36.6 ± 3.4	36.5 ± 2.3
ZnPP (mg/kg)[1]	25 ± 9 a	34 ± 7 b	36 ± 4 b	31 ± 8	33 ± 8
ZnPP (mg/kg dm)[2]	67 ± 24 a	91 ± 17 b	95 ± 11 b	83 ± 22	86 ± 22
ZnPP (mg/kg ddm)[3]	78 ± 29 a	105 ± 22 b	106 ± 13 b	94 ± 25	99 ± 26
Heme (mg/kg)[1]	47 ± 10 b	27 ± 5 a	23 ± 10 a	31 ± 12	34 ± 15
Heme (mg/kg dm)[2]	128 ± 26 b	74 ± 14 a	61 ± 22 a	82 ± 34	93 ± 40
Heme (mg/kg ddm)[3]	145 ± 30 b	84 ± 15 a	67 ± 26 a	93± 37	103 ± 47
ZnPP/heme ratio	0.52 ± 0.14 a	1.28 ± 0.38 b	1.73 ± 0.58 b	1.27 ± 0.76	1.11 ± 0.54
L*	43 ± 2.0	42 ± 1.6	43 ± 1.4	43± 1.5	42 ± 1.8
a*	11 ± 1.3	11 ± 0.3	12 ± 0.9	11 ± 0.8	11 ± 1.2
b*	6 ± 0.5	6 ± 0.9	6 ± 0.6	6 ± 0.7	6 ± 0.7
Final pH_{SM}	6.15 ± 0.11 b	5.93 ± 0.07 a	5.86 ± 0.14 a	5.93 ± 0.15 a	6.02 ± 0.16 b
Final pH_{BF}	6.14 ± 0.09 b	5.93 ± 0.06 a	5.86 ± 0.16 a	5.93 ± 0.15 a	6.01 ± 0.17 b
Salt content (%)	5.8 ± 1.5 b	4.2 ± 0.9 a	4.7 ± 1.1 ab	5.7 ± 1.2 b	4.0 ± 0.7 a
Water activity	0.925 ± 0.019	0.940 ± 0.008	0.929 ± 0.015	0.921 ± 0.015 a	0.941 ± 0.007 b
Moisture (%)	62 ± 1.9	63 ± 0.9	61 ± 2.7	61 ± 2.3	63 ± 1.6
Protein (%)	28 ± 0.8	28 ± 1.0	29 ± 2.0	28 ± 1.4	28 ± 1.4

Proteolysis index (%)	27 ± 3.3 a	29 ± 1.4 ab	33 ± 4.7 b	30 ± 4.1	30 ± 4.4
Fat (%)	2.6 ± 0.49	2.3 ± 0.56	2.6 ± 0.39	2.6 ± 0.48	2.4 ± 0.48
Marbling (%)	1.4 ± 0.77	1.8 ± 0.49	2.1 ± 0.87	1.7 ± 0.82	1.8 ± 0.71
Lipolysis extent (mg/100g)[4]	1016 ± 96	1054 ± 102	1062 ± 87	1002 ± 88 a	1090 ± 78 b

Results expressed as means ± standard deviation. Different letters in the same row (a-b) indicate significant differences within a factor ($p \leq 0.05$).

[1] Expressed on a fresh weight basis

[2] Expressed on a dry weight basis

[3] Expressed on a desalted dry matter basis

[4] The extent of lipolysis is calculated as the amount of free fatty acids in 100 g dry matter sample

Table 2. Fatty acid profile (expressed as % of area normalization) of the total lipid fraction of muscle *Biceps femoris* in dry-cured hams and the content in free fatty acids (expressed as mg/100 g dry matter) as affected by *post mortem* pH_{SM24h} range and salting time.

Fatty acid[1]	Total lipid fraction	*Post mortem* pH range			Salting time	
		High	Medium	Low	Standard	Reduced
	(%)	(mg/100 g)				
14:0	0.97 ± 0.20	10 ± 1	10 ± 1	9 ± 1	9 ± 1	10 ± 1
16:0	20.84 ± 1.04	182 ± 18	188 ± 20	185 ± 18	178 ± 19	192 ± 15
16:1 n-7	2.51 ± 0.67	19 ± 3	18 ± 5	15 ± 3	17 ± 4	17 ± 4
18:0	11.89 ± 1.12	136 ± 25	140 ± 12	142 ± 12	132 ± 15 a	147 ± 15 b
18:1 n-9	34.21 ± 4.68	199 ± 37	191 ± 33	181 ± 25	187 ± 35	192 ± 28
18:1 n-7	4.00 ± 0.46	38 ± 3	35 ± 6	35 ± 4	35 ± 5	37 ± 5
18:2 n-6	18.81 ± 3.62	268 ± 41 a	287 ± 27 ab	315 ± 30 b	275 ± 35 a	307 ± 34 b
18:3 n-6	0.21 ± 0.07	8 ± 1 a	10 ± 1 b	9 ± 1 ab	9 ± 1	9 ± 1
18:3 n-3	0.53 ± 0.09	10 ± 1	11 ± 1	11 ± 1	10 ± 1	11 ± 1
20:1 n-9	0.59 ± 0.14	9 ± 1	9 ± 1	9 ± 1	9 ± 1	9 ± 1
20:3 n-6	0.57 ± 0.21	15 ± 2 a	16 ± 1 ab	17 ± 2 b	16 ± 2	17 ± 2
20:4 n-6	4.55 ± 1.78	106 ± 14	115 ± 9	116 ± 11	107 ± 10 a	118 ± 12 b
22:1 n-9	0.10 ± 0.08	8 ± 1 a	9 ± 1 b	9 ± 1 b	8 ± 1	9 ± 1
22:6 n-3	0.20 ± 0.14	8 ± 1 a	10 ± 1 b	11 ± 2 b	9 ± 2	10 ± 2
SFA	33.70 ± 1.32	328 ± 42	338 ± 30	336 ± 28	320 ± 33 a	349 ± 27 b
MUFA	41.42 ± 5.53	272 ± 41	262 ± 44	248 ± 31	257 ± 43	264 ± 35
PUFA n-3	0.73 ± 0.15	19 ± 2 a	21 ± 1 b	21 ± 3 b	20 ± 2	21 ± 2
PUFA n-6	24.14 ± 5.44	397 ± 54 a	429 ± 34 ab	457 ± 41 b	406 ± 44 a	451 ± 43 b
Total PUFA	24.87 ± 5.55	415 ± 54 a	449 ± 35 ab	478 ± 43 b	426 ± 46 a	472 ± 45 b

Results expressed as means ± standard deviation. Different letters in the same row (a-b) indicate significant differences within a factor ($p \leq 0.05$).

[1] SFA stands for saturated fatty acids of the total lipid fraction and the amount of saturated free fatty acids; MUFA stands for monounsaturated fatty acids of the total lipid fraction and the amount of monounsaturated free fatty acids; PUFA stands for polyunsaturated fatty acids of the total lipid fraction and the amount of polyunsaturated free fatty acids.

Table 3. Pearson's coefficients of dry-cured hams' physicochemical variables and their content in free fatty acids[1].

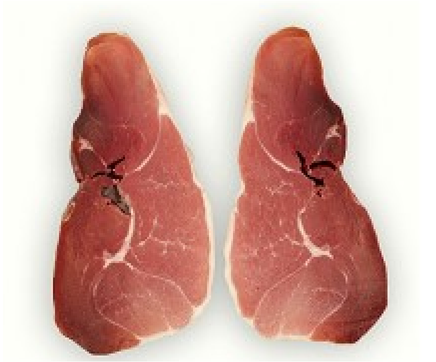
	ZnPP	Heme	ZnPP / heme	a*	pH_{BF}	Salt	aw	Moisture	Protein	PI	Fat	Marbling	16:0	18:0	18:2 n-6	20:4 n-6	SFFA	MUFFA	PUFFA n-6	PUFFA n-3	PUFFA	Total FFA
ZnPP	1.000	-0.357	0.650**	0.279	-0.516**	-0.391*	0.287	0.288	-0.069	0.219	-0.373	-0.027	0.276	0.379*	0.480*	0.568**	0.334	-0.247	0.534**	0.446*	0.537**	0.309
Heme		1.000	-0.839**	0.434*	0.854**	0.186	0.025	-0.021	0.191	-0.521**	0.054	-0.359	-0.053	0.017	-0.351	-0.192	-0.007	0.139	-0.348	-0.557**	-0.362	-0.142
ZnPP/heme			1.000	-0.154	-0.891**	-0.225	0.022	0.041	-0.032	0.492**	-0.181	0.358	0.065	0.146	0.391*	0.280	0.095	-0.287	0.397*	0.500**	0.406*	0.135
a*				1.000	0.337	-0.005	-0.004	-0.250	0.402*	-0.103	0.205	-0.258	0.169	0.225	0.248	0.242	0.209	0.002	0.233	-0.281	0.213	0.193
pH_{BF}					1.000	0.145	0.017	-0.084	0.226	-0.568**	0.109	-0.354	0.108	0.127	-0.257	-0.147	0.139	0.286	-0.261	-0.530**	-0.276	0.020
Salt						1.000	-0.955**	-0.735**	0.296	-0.291	0.140	-0.328	-0.462*	-0.503**	-0.636**	-0.741**	-0.512**	0.113	-0.701**	-0.534**	-0.702**	-0.521**
aw							1.000	0.845**	-0.407*	0.239	-0.181	0.291	0.415*	0.451*	0.503**	0.687**	0.463*	-0.103	0.581**	0.431*	0.581**	0.442*
Moisture								1.000	-0.705**	0.391*	-0.404*	0.249	0.301	0.313	0.277	0.631**	0.330	-0.152	0.395*	0.479*	0.404*	0.275
Protein									1.000	-0.452*	0.012	-0.337	-0.093	0.058	0.029	-0.275	-0.028	-0.043	-0.064	-0.355	-0.078	-0.071
PI										1.000	-0.122	0.228	-0.006	-0.068	0.310	0.474*	-0.048	-0.291	0.377*	0.433*	0.383*	0.070
Fat											1.000	0.314	0.122	-0.235	-0.050	-0.303	-0.036	0.586**	-0.125	-0.222	-0.131	0.165
Marbling												1.000	-0.015	-0.036	0.082	0.074	-0.023	-0.016	0.096	0.217	0.103	0.042
16:0													1.000	0.744**	0.663**	0.567**	0.945**	0.604**	0.671**	0.508**	0.672**	0.966**
18:0														1.000	0.680**	0.631**	0.921**	0.061	0.696**	0.476*	0.694**	0.738**
18:2 n-6															1.000	0.736**	0.706**	0.013	0.983**	0.715**	0.983**	0.799**
20:4 n-6																1.000	0.629**	-0.163	0.845**	0.565**	0.842**	0.620**
SFFA																	1.000	0.389*	0.719**	0.519**	0.718**	0.919**
MUFFA																		1.000	-0.033	0.050	-0.030	0.548**
PUFFA n-6																			1.000	0.726**	1.000**	0.794**
PUFFA n-3																				1.000	0.747**	0.617**
PUFFA																					1.000	0.795**
Total FFA																						1.000

[1] The content of zinc-protoporphyrin (ZnPP) and heme were expressed on a desalted dry matter basis. ZnPP, pH_{BF}, PI, SFFA, MUFFA, PUFFA and Total FFA stand for zinc-protoporphyrin, pH in *Biceps femoris* muscle at the end of the process, proteolysis index, saturated free fatty acids, monounsaturated free fatty acids, polyunsaturated free

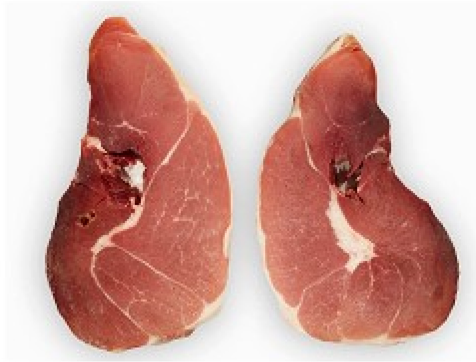
fatty acids and total amount of free fatty acids, respectively. The content of ZnPP and heme are expressed on a desalted dry matter basis. See table 1 for the units of the remaining parameters.

*$P < 0.05$, **$P < 0.01$

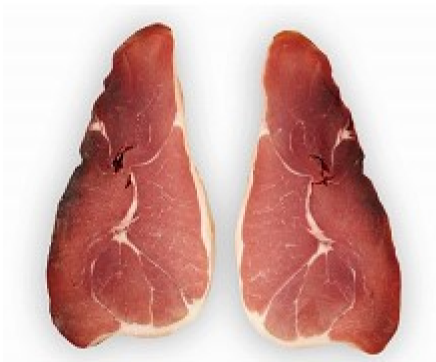
A



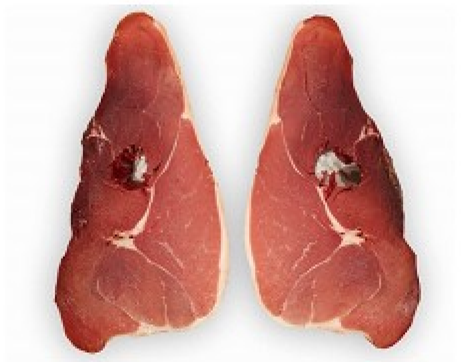
B



C

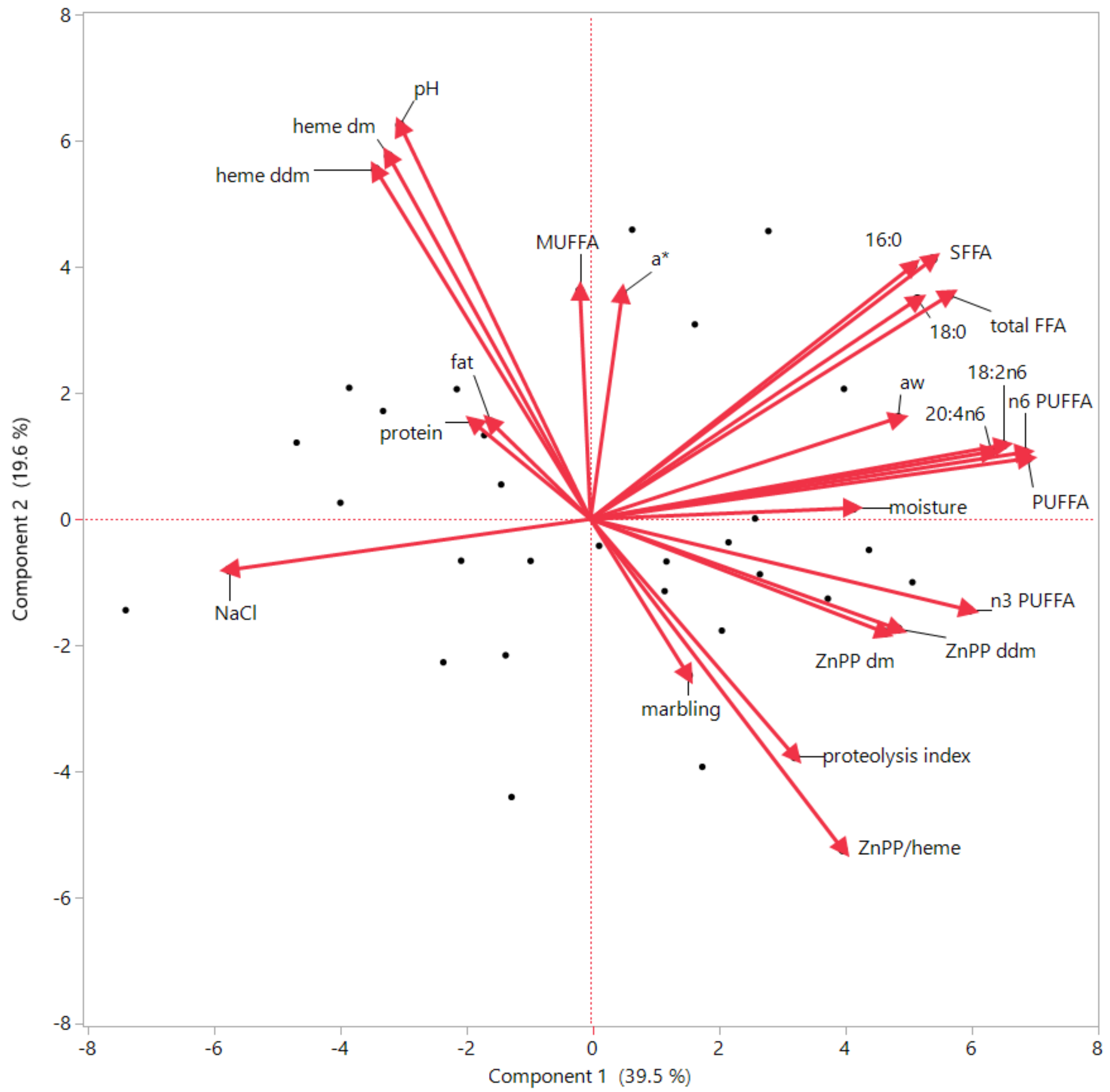


D



E

F


pH
heme dm
heme ddm
MUFFA
a*
16:0
SFFA
18:0
total FFA
fat
protein
aw
18:2n6
n6 PUFFA
20:4n6
PUFFA
moisture
NaCl
n3 PUFFA
ZnPP dm
ZnPP ddm
marbling
proteolysis index
ZnPP/heme
Component 1 (39.5 %)
Component 2 (19.6 %)
8
6
4
2
0
-2
-4
-6
-8