



**This document is a postprint version of an article published in Science of the Total Environment © Elsevier after peer review. To access the final edited and published work see <https://doi.org/10.1016/j.meatsci.2018.01.027>**

1 **Zinc-protoporphyrin content in commercial Parma hams is affected by proteolysis**  
2 **index and marbling**

3

4

5

6 Ricard Bou<sup>a</sup>, Mar Llauger<sup>a</sup>, Jacint Arnau<sup>a</sup>, Elena Fulladosa<sup>a</sup>

7

8 <sup>a</sup> Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Food Technology, Monells,  
9 Finca Camps i Armet s/n, 17121, Spain.

10

11 Corresponding author: Ricard Bou, [ricard.bou@irta.cat](mailto:ricard.bou@irta.cat), Institut de Recerca i Tecnologia  
12 Agroalimentàries (IRTA), Food Technology, Monells, Finca Camps i Armet s/n, 17121,  
13 Spain

14 **Abstract:**

15 The contents of zinc-protoporphyrin (ZnPP) and heme in twenty-four sliced Parma hams  
16 made without the addition of curing agents were determined. Expressed on a dry weight  
17 basis, ZnPP averaged 45 mg/kg on a dry matter basis and ranged from 23 to 85 mg/kg.  
18 The heme content averaged 37 mg/kg on a dry matter basis and ranged from 17 to 73  
19 mg/kg. A Principal Component Analysis (PCA) and Partial Least Squares (PLS)  
20 regression analyses were carried out to examine the existing correlations between these  
21 pigments and various physicochemical parameters in the final product. PCA showed the  
22 existence of associations between ZnPP, sensory redness and salt content. PLS suggests  
23 that the conversion of ZnPP from heme is facilitated in those hams with a higher  
24 proteolysis index and higher marbling.

25

26

27 **Keywords:** dry-cured ham, colour, pigments, proteolysis, marbling

28

29

30 **Highlights:**

31 - Typical contents of zinc-protoporphyrin (ZnPP) in commercial Parma ham were  
32 reported

33 - ZnPP content was positively correlated with redness and salt content

34 - Proteolysis and marbling were related with higher conversions of heme into ZnPP

35

## 36 1. Introduction

37

38 Dry-cured ham with its characteristic red colour, is a popular meat product in the  
39 Mediterranean region of Europe. This colour is typically obtained by reaction of nitric  
40 oxide, coming from curing agents (i.e. nitrate or nitrite), with muscle myoglobin to form  
41 nitrosylmyoglobin (Haldane 1901; Hornsey 1956). However, the main pigments in dry-  
42 cured hams produced without the addition of curing agents (e.g. *Prosciutto di Parma*) are  
43 heme and zinc-protoporphyrin IX (ZnPP) chromophores (Wakamatsu et al. 2004a;  
44 Adamsen et al. 2006a; Wakamatsu et al. 2009a). In ZnPP, the porphyrin moiety  
45 coordinates with Zn(II) instead of Fe(II) and is responsible for the distinctive bright,  
46 stable colour of non-nitrified Parma hams (Wakamatsu et al. 2004a; Adamsen et al. 2004).

47 However, the formation pathway of ZnPP in meat and meat products is complex and not  
48 yet completely elucidated. Non-enzymatic reactions, endogenous enzymatic reactions  
49 and enzymatic reactions caused by typical ham bacteria may be involved (Morita et al.  
50 1996; Becker et al. 2012; Wakamatsu et al. 2004b). Despite this, the formation of ZnPP  
51 is widely believed to be of enzymatic origin mainly given that this chromophore is not  
52 formed after treating the meat thermally (Wakamatsu et al. 2004b). Ferrochelatase (Zn-  
53 chelatase, heme synthase, E.C. 4.99.1.1) is the endogenous enzyme suggested as being  
54 responsible for the formation of ZnPP in meat (Benedini et al. 2008). This enzyme is  
55 active throughout the processing time of dry-cured hams and is considered to some extent,  
56 responsible for the gradual formation of ZnPP (Parolari et al. 2016; Parolari et al. 2009;  
57 Adamsen et al. 2006b). The residual activity of this enzyme, even in hams with a low  
58 water activity and a high salt concentration, would explain why many authors find a  
59 higher ZnPP content at the end of the process (Adamsen et al. 2006b; Parolari et al. 2009;  
60 Parolari et al. 2016). However, in addition to meat endogenous enzymes, various authors

61 have indicated the existence of other alternative and complex mechanisms that could be  
62 involved in this pigment formation and occur simultaneously (Becker et al. 2012; Grossi  
63 et al. 2014; Parolari et al. 2016).

64 As shown in various meat models, the enzymatic formation of ZnPP can be affected by  
65 several factors. For instance, Wakamatsu et al. (2007) reported that the presence of  
66 oxygen decreases the formation of ZnPP in meat solutions. In fresh meat extracts,  
67 Benedini et al. (2008) reported that the enzyme is temperature-dependent with an increase  
68 in activity from 10 °C to 37 °C. The same authors indicated that the formation of ZnPP is  
69 favoured by the presence of high amounts of sodium chloride. However, elevated sodium  
70 chloride contents have been also reported to limit the formation of ZnPP in meat systems  
71 (Adamsen et al. 2006b; Becker et al. 2012). In porcine heart extracts, the formation of  
72 ZnPP is favoured at pH between 5.5 and 6.0 (Ishikawa et al. 2006; Ishikawa et al. 2007).  
73 According to Chau et al. (2010) the enzyme is able to remove the iron atom from  
74 porphyrin in the latter pH range whereas the zinc insertion is favoured at neutral or basic  
75 pH (7.5-8.0). The latter authors also reported that the presence of fatty acids and  
76 phospholipids affect ferrochelatase activity. It is also worth mentioning that myoglobin  
77 suffers significant modifications during ham maturation which have been reported to  
78 facilitate the substitution of the heme iron by zinc (Adamsen et al. 2006b). Furthermore,  
79 Paganelli et al. (2016) reported that heme moiety transmetallation is enhanced by a partial  
80 proteolysis of the globin which facilitates Zn-chelatase activity.

81 However, in dry-cured hams, it has been found that the formation of ZnPP occurs even  
82 when they are manufactured at 4 °C, thus suggesting that non-enzymatic processes could  
83 also be involved given that at this temperature the activity of the enzyme is limited  
84 (Parolari et al. 2016). Therefore, a number of endogenous and exogenous factors that have  
85 not yet been completely elucidated could have an effect on the formation of ZnPP in

86 Parma hams (Grossi et al. 2014; Paganelli et al. 2016). Although several factors affecting  
87 ZnPP have been studied in *in vitro* models, data is scarce on typical ZnPP contents in dry-  
88 cured Parma ham and studies dealing with the factors involved in the formation of this  
89 pigment in hams are still necessary (Wakamatsu et al. 2009b; Parolari et al. 2009; De  
90 Maere et al. 2014). Therefore, it is important to gain a better understanding of the factors  
91 that contribute to this pigment content because they determine the characteristic colour  
92 of non-nitrified dry-cured hams. The formation of ZnPP is also interesting because of its  
93 increased stability in comparison with the pigments obtained from nitrified hams (Durek  
94 et al. 2012; Adamsen et al. 2004).

95 Besides, a better knowledge about elements that could influence the formation of ZnPP  
96 may help to develop strategies for obtaining a more intense and homogeneous colour in  
97 non-nitrified dry-cured meat products. The aim of this work was to report typical ZnPP  
98 and heme contents in commercial sliced non-nitrified Parma ham. In addition, the  
99 relationships between ZnPP and heme, and various physicochemical characteristics  
100 (proximate composition, proteolysis index, water activity, NaCl content and pH) plus  
101 sensory redness were studied.

102

## 103 **2. Material and Methods**

104

### 105 ***2.1. Acquisition and processing of Parma ham samples***

106 Twenty-four packages of PDO Parma sliced dry-cured ham obtained from six different  
107 producers were used and each package of ham came from a different batch. The hams  
108 were manufactured in compliance with the Parma ham production guidelines (European

109 Commission 2013) with an elaboration time ranging between 12 and 20 months. It must  
110 be mentioned that during Parma ham elaboration no nitrite or nitrate are added.

111 High quality images from the first slice of each package were acquired as described in  
112 section 2.4 in order to evaluate intramuscular fat. Subsequently, subcutaneous and  
113 intermuscular fat was discarded from all the slices. The lean part of the slice, which  
114 included *Semimembranosus*, *Semitendinosus* and *Biceps femoris* muscles, was minced  
115 and homogenized for further physicochemical analysis. Homogenized samples were  
116 aliquoted and kept vacuum packed in aluminium bags at -80 °C to further determine ZnPP  
117 and heme content.

## 118 **2.2 Reagents**

119 Chlorohemin (hemin) from porcine was from Panreac (Barcelona, Spain) whereas  
120 protoporphyrin IX and ZnPP were from Sigma-Aldrich (Madrid, Spain). Ethyl acetate,  
121 acetic acid and methanol were of a suitable grade for instrumental analysis. Other reagents  
122 were of ACS grade.

## 123 **2.3 Physicochemical determinations**

124 Protein content was calculated by multiplying by a factor of 6.25 total nitrogen which  
125 was determined via Kjeldahl digestion (AOAC 2000). Non-protein nitrogen content was  
126 determined by precipitation of proteins with trichloroacetic acid followed by  
127 determination of the total nitrogen (Careri et al. 1993). Proteolysis index was determined  
128 as a percentage of the ratio between non-protein nitrogen and total nitrogen.

129 Fat content was determined according to the ISO 1443:1973 protocol (ISO 2016). Water  
130 content (moisture) was determined by drying at  $103 \pm 2$  °C until a constant weight was  
131 reached (AOAC 2000). The water content of the samples on defatted basis was also  
132 calculated from the chemical composition of the ham (Moisture = g H<sub>2</sub>O/(100 g sample

133 – g fat)). Water activity ( $a_w$ ) was measured at  $25\text{ }^\circ\text{C} \pm 0.3\text{ }^\circ\text{C}$  with a Novasina AW  
134 SPRINT – TH 500 instrument (Axair Ltd., Pfäffikon, Switzerland). Chloride content was  
135 determined according to ISO 1841-2:1996 protocol using a potentiometric titrator 785  
136 DMP Titrino (Metrohm AG, Herisau, Switzerland) and expressed as NaCl content. The  
137 NaCl content on dry matter basis ( $\text{NaCl DM} = \text{g NaCl}/(100\text{ g sample} - \text{g H}_2\text{O})$ ) and on  
138 defatted dry matter basis ( $\text{NaCl, defatted DM} = \text{g NaCl}/(100\text{ g sample} - \text{g H}_2\text{O} - \text{g fat})$ )  
139 was also calculated. Determination of pH was performed by means of a S40 SevenMulti  
140 (Mettler-Toledo S.A.E., Barcelona, Spain) and an Inlab Solids Pro (Mettler-Toledo  
141 S.A.E.) probe. All analyses were done in triplicate.

#### 142 ***2.4 Visual estimation of intramuscular fat (marbling)***

143 In order to estimate the visual intramuscular fat content, high quality images were  
144 acquired using a photographic System that included a calibrated digital camera Canon  
145 EOS 50D with a picture resolution of 15.1 megapixels and an objective Canon EF-S 18–  
146 200 mm f/3.5–5.6 IS. White balance was carried out with a white card (Lastolite) in order  
147 to electronically adjust the colour reproduction without showing colour dominants. The  
148 camera was connected to a PC into which the images with RAW format were uploaded.  
149 Dry-cured ham slices were positioned below the camera lens and an image of the entire  
150 slice surface was taken. Capture One Pro software (Phase One A/S Inc., Frederiksberg,  
151 Denmark) was used to carry out the white balance of the RAW images and digitalize them  
152 to 667 x 1000 pixels resulting in a .tif file with 16 bits colour and 4 MB. This was  
153 considered to be high enough in quality for computer image analysis.

154 Visual intramuscular fat of the entire slice was segmented using the procedures previously  
155 described elsewhere (Santos-Garcés et al. 2014; Muñoz et al. 2015). In brief, Matlab  
156 scripts written in-house were used for segmentation of visual subcutaneous, intermuscular  
157 and intramuscular fat using edge detection based on the discrete Fourier transform. The



158 total area of the slice, the subcutaneous fat area of the slice, the intermuscular fat area and  
159 visual intramuscular fat area were segmented and the number of pixels for each one was  
160 determined. The percentage of intramuscular area related to the total area of the slice was  
161 calculated.

## 162 ***2.5. Sensory analysis: redness***

163 Sensory analysis of the samples was carried out by eight trained panelists according to  
164 the ISO 8586-2:2012 protocol (ISO 2016). Visual appearance of red colour (redness) was  
165 evaluated in the entire slice using an unstructured scale from 0 (very low) to 10 (very  
166 high).

## 167 ***2.6. Determination of zinc-protoporphyrin***

168 ZnPP was quantitatively extracted in subdued light conditions with ethyl acetate/acetic  
169 acid solvent mixture (4:1, v/v) as described elsewhere with minor changes (Wakamatsu  
170 et al. 2009a). The chromatographic elution of this extract as described in the  
171 determination of heme iron showed traces or the absence of demetallated protoporphyrin  
172 in our samples (data not shown). Hence, the determination of ZnPP content was achieved  
173 by measuring the fluorescence of samples directly. No differences were observed when  
174 comparing the excitation and emission spectra of samples from those obtained from the  
175 ZnPP standard. Therefore, this method was preferred because of its sensitivity and faster  
176 analysis compared with alternative HPLC methods. In brief, 2 g of ground Parma ham  
177 was weighed into 50 mL capacity centrifuge tubes and homogenized using an UltraTurrax  
178 T25 model for 1 min at 9000 rpm with 10 mL of the solvent mixture while the tube was  
179 immersed in ice. Sample residues were re-extracted (few sec burst) with the same volume  
180 of solvent mixture and added to the previous one. After extraction on ice for 20 min and  
181 centrifugation (1100 g, 14 min, 4 °C), the supernatant was filtered through filter paper

182 (grade 1) and collected into a volumetric flask. Extractions using the solvent were  
183 performed as thoroughly as possible till reaching final volume. Two hundred microL of  
184 the extract were transferred to 96-microwell plates and thereafter sealed with a polyolefin  
185 acrylate sealing tape. Samples were then incubated for 2 min at 30 °C and shaken for 30  
186 sec before measuring fluorescence of ZnPP using a Thermo Scientific Varioskan  
187 microplate reader (Waltham, USA) with excitation set at 415 nm and emission at 590 nm.  
188 Ethyl acetate/acetic acid (4:1, v/v) was used as a blank. A stock ZnPP standard was  
189 prepared by dissolving 20 mg of ZnPP in 20 mL of dimethyl sulfoxide and thereafter  
190 conveniently diluted with the ethyl acetate/acetic acid solution. Coefficients of  
191 determination ( $R^2$ ) higher than 0.99 were obtained from linear regression analysis made  
192 with calibration curves ranging from 0.01 to 8 mg/L using ZnPP standard solutions  
193 prepared in the ethyl acetate-acetic acid mixture (4:1, v/v). Excitation and emission  
194 spectra of standards and samples were compared. Each sample was analysed four times.  
195 ZnPP content was also expressed as dry matter basis (ZnPP content DM = mg ZnPP/(kg  
196 sample – kg water)) and defatted dry matter basis (ZnPP content defatted DM = mg  
197 ZnPP/(kg sample – kg water – kg fat)).

## 198 ***2.7. Determination of heme content***

199 Total heme pigments were determined after extraction of hemin in subdued light  
200 conditions with 90% (v/v) aqueous acetone containing HCl (0.24 M) as described  
201 elsewhere with some minor modifications (Hornsey 1956). Briefly, 1.5 g of ground ham  
202 were weighed in subdued light conditions into 50 mL capacity centrifuge tubes and 200  
203 microL of 0.5% (w/v) aqueous cysteine HCl solution plus 10 mL of the acidified acetone  
204 solution were added. Using an UltraTurrax T25 model, the mixture was homogenized for  
205 1 min at 9000 rpm while the tube was immersed in ice. The sample was further macerated  
206 on ice for 1 h in the dark and thereafter centrifuged for 15 min (1100 g at 4 °C). The

207 supernatant was filtered through filter papers (grade 42) and collected in a volumetric  
208 flask and was protected from light as much as possible during the process. An aliquot was  
209 filtered through a PTFE syringe filter, 0.45  $\mu\text{m}$ , before its injection (40  $\mu\text{L}$ ) into an  
210 Agilent HPLC 1100 series (Waldbronn, Germany) provided with a Luna C18 column  
211 (150x4.6 mm, 5  $\mu\text{m}$ , 100  $\text{\AA}$ ) from Phenomenex (Torrance, USA) and a UV/Vis detector  
212 set at 414 nm. A 2% aqueous acetic acid and 100% methanol were used as mobile phase  
213 A and B, respectively. Hemin was eluted with a gradient in which phase B increased from  
214 60 to 100% in 5 min and then maintained at a constant flow rate of 1 mL/min for 10 min.  
215 Coefficients of determination ( $R^2$ ) higher than 0.99 were obtained from linear regression  
216 analysis performed with calibration curves ranging from 0.1 to 10 mg/L using hemin  
217 standard solutions prepared in acidified acetone. Each sample was analysed three times.  
218 Total heme content was also expressed as dry matter basis (heme content DM = mg heme  
219 / (kg sample – kg water)) and defatted dry matter basis (heme content defatted DM = mg  
220 heme / (kg sample – kg fat))

## 221 **2.8. Statistical analysis**

222 For each sample the average of the replicates was treated as a single measurement before  
223 Principal component analysis (PCA). Pearson's correlation was used to examine linear  
224 correlations between different determinations (ZnPP and heme content expressed on dry  
225 matter basis, the ratio between ZnPP and heme, pH, visual intramuscular fat, moisture,  
226 water activity, fat content, protein content, proteolysis index and redness). Partial least  
227 squares (PLS) modelling was used to identify the factors (all considered quantitative with  
228 the exception of the type of producer) underlying the formation of ZnPP and the  
229 ZnPP/heme ratio. These analyses were performed using the XLStat 2015 for Microsoft  
230 Excel (Addinsoft, Paris) and considering significant a P value lower than 0.05.

231

## 232 **3. Results and Discussion**

233

### 234 **3.1 Dry-cured Parma ham characterization**

235 Table 1 shows the proximate composition and pigment contents of the Parma hams  
236 analysed in this study. The salt content ranged between 4.3% and 6.4% whereas moisture  
237 ranged between 42.6% and 56.5% and water activity was below 0.92. These results are  
238 in agreement with the existing literature and the expected standards of dry-cured Parma  
239 hams (Consorzio del Prosciutto di Parma 2016; Parolari et al. 2016; Adamsen et al.  
240 2006a). Fat content and visual intramuscular fat estimate (marbling) were relatively  
241 variable as was the proteolysis index.

242 Sensory analysis averaged a redness intensity of 4.4. The average ZnPP content in the  
243 sliced dry-cured Parma hams is reported in Table 1. These values are in agreement with  
244 those reported by other authors. For instance, Wakamatsu et al. (2009a) reported ZnPP  
245 contents that ranged between 27-40 mg/kg fresh weight in three different muscles  
246 (*Semimembranosus*, *Semitendinosus* and *Biceps femoris*) and sections of a Parma ham.  
247 Expressed on a dry weight basis, Parolari et al. (2016) reported ZnPP concentrations that  
248 ranged from 40 to 54 mg/kg in *Biceps femoris* and *Semimembranosus* muscles. In a  
249 previous study, the same authors reported that on a dry weight basis, the content of ZnPP  
250 in different muscles of finished Parma hams was similar (Parolari et al. 2009). It is worth  
251 noting that the obtained results in this study are comparable to those from other authors  
252 using similar solvent mixtures (Wakamatsu et al. 2009a; Parolari et al. 2016). Differences  
253 in moisture and fat content may contribute to an increased variability when studying ZnPP  
254 content. Therefore, it would be of interest to express results on defatted dry matter basis  
255 for a better comparison (Table 1). In this regard, it can be observed that, depending on  
256 the samples, the amount of this chromophore can be about 4-fold higher regardless of the

257 considered basis. These results indicate the existence of a broad range of ZnPP  
258 concentrations in Parma hams which could be attributed to a number of intrinsic factors  
259 such as the myoglobin concentration and different Zn-chelatase activity in muscles  
260 (Parolari et al. 2016; Wakamatsu et al. 2009a; Parolari et al. 2009) as well as extrinsic  
261 factors such as the amount of added salt and porphyrin photooxidation (Adamsen et al.  
262 2004; Ericson et al. 2003; Benedini et al. 2008).

263 Regarding heme content, it can be observed that this pigment was present in lower  
264 amounts when compared with that of the ZnPP (Table 1). The reported amounts are also  
265 in line with those reported by other authors ranging from 15-29 mg/kg expressed on fresh  
266 weight basis (Wakamatsu et al. 2009a; De Maere et al. 2014; Wakamatsu et al. 2009b).  
267 Expressed on a dry weight basis, Parolari et al. (2016) reported heme contents of  
268 approximately 50 mg/kg in Parma hams after 12 months of maturation whereas the sum  
269 of pigments, i.e. including protoporphyrin, accounted for 95-106 mg/kg of dry matter.  
270 This total porphyrin content is close to the observed averaged sum of approximately 80  
271 mg/kg dry matter without including demetallated protoporphyrin because, as explained  
272 in the material and methods section, this was found to be present at trace levels.

273 Various authors have suggested that the direct interchange of zinc with iron in the heme  
274 moiety does not occur (Wakamatsu et al. 2009b; Becker et al. 2012). Therefore, it is  
275 important to know the existing relationships between these two main pigments and look  
276 for those elements that may influence the ratio between them. In this study, it can be  
277 observed that ZnPP was about 1.5 times the heme content (Table 1), showing a broad  
278 variability between samples. Similarly to our results, Wakamatsu et al. (2009a) reported  
279 that generally in Parma hams, the ZnPP and heme content accounted for two-thirds and  
280 one-third of all porphyrins, respectively. Similarly, de Maere et al. (2014) reported  
281 concentrations of 15.9 and 19.9 mg/kg ham for heme and ZnPP, respectively. Parolari et

282 al. (2016) studied the effect of processing temperature on pigments content and reported  
283 ratios slightly higher than 1 in hams processed under typical temperatures for Parma ham.  
284 Overall, the ZnPP content in *Biceps femoris* and *Semimembranosus* muscles expressed  
285 on dry weight basis (54 and 40 mg/kg, respectively) seems to be in line with the present  
286 study. Conversely, the heme content seems to be slightly lower in commercial samples  
287 and thus explains the higher ZnPP/heme ratio. A number of factors such as the processing  
288 time and loss of heme pigments may have contributed to these changes. Therefore, further  
289 studies are required to clarify those factors responsible for the different ZnPP/heme ratios  
290 during production and retail conditions.

### 291 **3.2 Relationships between physicochemical parameters and pigment contents**

292 Table 2 shows the Pearson's correlation coefficients between the studied variables. As it  
293 can be observed there are several significant correlations between physicochemical  
294 variables and ZnPP content or ZnPP/heme ratio. Some of these correlations depend on  
295 the weight basis and thus making the interpretation more difficult. For this reason, the  
296 analysis of principal components was aimed at focusing on the most relevant  
297 physicochemical variables that may contribute to Parma ham colour. Results are  
298 displayed in Figure 1 where it can be observed that the first two principal components  
299 explained 59.28% of the total variance. The content of either ZnPP, heme or moisture  
300 expressed by different means are relatively close to each other and provide similar  
301 information regardless of the form of expression. It can also be observed that heme is  
302 plotted in the first component direction as for moisture and pH, and opposed to non-  
303 protein nitrogen and fat. In the second component, ZnPP, proteolysis index and redness  
304 are plotted in similar co-ordinates and relatively opposed to water activity. Salt content is  
305 localized between ZnPP and heme content. Therefore, the variables that are distributed  
306 along with the second component seem to be related with the formation of ZnPP. On the

307 other hand, factors such as the proteolysis index, marbling and water activity may have  
308 had an influence on the ZnPP/heme ratio (Figure 1).

309 Based on these results, a number of variables were selected to examine the existing  
310 correlations between physicochemical parameters to gain a better knowledge of the  
311 factors that may be involved in ZnPP formation (dry matter basis). In this regard,  
312 Pearson's correlation analysis (Table 2) showed that ZnPP, expressed on a dry matter  
313 basis, was positively correlated with salt content ( $r = 0.502$ ;  $P = 0.012$ ). This result is in  
314 agreement with various studies performed in different meat model systems which state  
315 that the formation of ZnPP is enhanced by the addition of NaCl (Benedini et al. 2008;  
316 Becker et al. 2012; Adamsen et al. 2006a). This is due to the fact that the activity of Zn-  
317 chelatase in pork meat homogenates was higher with increased amounts of sodium  
318 chloride ranging from 0 to 80 g/L (Benedini et al. 2008). The authors stated that this latter  
319 concentration may be considered as an upper level for salt content in hams, however, it is  
320 not clear whether the enzyme activity in hams is determined by the chloride content  
321 expressed as a percentage or alternatively by the NaCl/moisture ratio which can be higher  
322 and vary with processing time and muscle type (Arnau et al., 1995). Therefore, this limit  
323 should be considered with caution. In this connection, Becker et al., (2012) reported that  
324 the formation of ZnPP in meat homogenates was inhibited when NaCl was added at 70  
325 g/L. Similarly, Adamsen et al. (2006a) reported a decreased formation of ZnPP in meats  
326 treated with 25% (w/w) brines in comparison to those with 15% (Adamsen et al. 2006a).  
327 In the present study, the distribution plot of ZnPP vs salt concentration regardless of the  
328 way of expression seems to be linear. In cured products, Adamsen et al (2006a) did not  
329 find significant correlations between fluorescence intensity and salt content but it should  
330 be noted that in this study, hams and other meat products produced with and without  
331 nitrites were considered. Therefore, these controversial results could be explained by the

332 addition of curing agents (i.e. nitrates and nitrites) which have been shown to inhibit the  
333 formation of ZnPP (Benedini et al. 2008; Wakamatsu et al. 2010). Overall, these findings  
334 suggest that the formation of ZnPP in hams without curing agents is promoted by the  
335 addition of salt. It is also relevant to note that the range of salt concentrations found in  
336 real samples is narrower than those assessed in *in vitro* studies. Further studies are  
337 necessary to determine whether this pigment formation is affected by the amount of salt  
338 uptake and its distribution. The content in ZnPP was also correlated with redness ( $r =$   
339  $0.550$ ;  $P = 0.008$ ) thus suggesting its relevance on the formation of Parma ham's  
340 characteristic colour (Wakamatsu et al. 2004a).

341

342 Regarding heme, this content expressed on a dry weight basis has been found to be  
343 positively correlated with moisture ( $r = 0.585$ ;  $P = 0.003$ ). As shown in Table 2, heme is  
344 also correlated with pH ( $r = 0.517$ ;  $P = 0.010$ ). The Zn-chelatase enzyme has been  
345 reported to be affected by pH and takes part in the heme iron demetallation (Chau et al.  
346 2011; Wakamatsu et al. 2015; Ishikawa et al. 2006; Benedini et al. 2008). Indeed, Chau  
347 et al., (2010) reported that insertion and removal of metal ions by Zn-chelatase is affected  
348 by various factors, but the optimal pH for the iron removal from the porphyrin can be set  
349 between 5.5-6.0 whereas that for the zinc-ion insertion can be set at pH 7.5-8.0. As it can  
350 be observed in Table 1, the pH values at the end of the process were optimal for heme  
351 iron removal. Providing that heme moiety demetallation is less likely to occur at the  
352 higher pH range this may likely explain the recorded correlation between pH and heme  
353 content in the studied samples. Here it is worth noting that in real samples, the pH range  
354 is narrower than that assayed in various *in vitro* conditions (Benedini et al. 2008; Chau et  
355 al. 2010). It should also be kept in mind that pH differs slightly depending on the muscle  
356 and can also vary during the dry-curing of ham as it is affected by various factors



357 including proteolysis (Toldrá 2010). Nevertheless, the optimal pH for zinc ion insertion,  
358 i.e. reverse reaction, is far from the pH range in these samples and therefore it may explain  
359 the lack of correlation between pH and ZnPP.

360 Regarding the formation of ZnPP, different complex mechanisms have been proposed by  
361 various authors (Becker et al. 2012; Parolari et al. 2016; Paganelli et al. 2016; Grossi et  
362 al. 2014). Therefore, it may be interesting to consider the conversion of heme into ZnPP  
363 by examining the ratio between ZnPP and heme contents, calculated on a dry matter basis,  
364 as it could help to identify other factors that would maximize the formation of ZnPP  
365 during the production of dry-cured hams without nitrites. As expected, the ZnPP/heme  
366 ratio is negatively correlated with heme ( $r = -0.729$ ;  $p < 0.001$ ) and positively with ZnPP  
367 ( $r = 0.444$ ;  $P = 0.030$ ). In agreement with the previously reported pigment correlation,  
368 this ratio is also correlated with moisture ( $r = -0.421$ ;  $P = 0.040$ ) and water activity ( $r = -$   
369  $0.591$ ;  $P = 0.002$ ). As discussed previously, hams with reduced water activities  
370 corresponded to those with longer ripening periods and thus recorded a higher conversion  
371 into ZnPP. Despite its previously discussed relationship with ZnPP, the salt content was  
372 not correlated with the ZnPP/heme ratio (data not shown). However, this ratio was  
373 positively correlated with proteolysis index ( $r = 0.445$ ;  $P = 0.029$ ). In this connection,  
374 myoglobin partial proteolysis has been reported to affect the iron/zinc transmetallation  
375 mediated by Zn-chelatase in *in vitro* studies (Paganelli et al. 2016; Grossi et al. 2014).  
376 The existence of such a mechanism in Parma ham not only contributes to explaining the  
377 modifications in the protein part of ZnPP (Adamsen et al. 2006b) but also to the observed  
378 positive effect of proteolysis on the ZnPP/heme ratio.

379 This ratio between ZnPP and heme also correlated with marbling ( $r = 0.487$ ;  $P = 0.016$ ).  
380 The intramuscular fat of hams is hydrolyzed during their processing and thus free fatty  
381 acids are released from either neutral or polar lipids (Andres et al. 2005; Martin et al.

1999). In an *in vitro* study, Chau et al., (2010) reported that the presence of fatty acids and phospholipids affect Zn-chelatase enzyme and promote iron removal from the porphyrin ring. Therefore, elevated marbling values may contribute to a higher amount of free fatty acids in the internal parts of ham during processing. These fatty acids may in turn interact with the enzyme and explain the existing correlation between ZnPP and marbling. The correlation between ZnPP/heme ratio and fat content was not significant ( $r = 0.357$ ;  $P = 0.087$ ). In this regard, it should be noted that the fat content on the surface of the sliced ham increased during slicing due to the spreading of subcutaneous fat by the slicing blade. This increase would depend on subcutaneous fat content and slicing conditions (personal data). In addition, the subcutaneous fat of the slice could be in contact with other parts during their packaging and storage. In order to clarify the effect of intramuscular fat on ZnPP/heme ratio it is necessary to design a specific experiment.

The observed range of ZnPP concentrations may obey to a number of intrinsic and extrinsic factors. Thus, a Partial Least Squares regression analysis was conducted to identify the compositional factors and production conditions (e.g. producer, processing time) that were relevant (i.e. different from 0) with regard to ZnPP formation. In Figure 2a it can be observed that samples content of ZnPP was higher in those with higher salt content. Despite the fact that this variable depends on ham production conditions (i.e. salting), none of the producers showed an effect on ZnPP formation. Likewise, the elaboration time, which ranged from between 12 to 20 months, had no effect on ZnPP content (Figure 2a). Although ZnPP content has been shown to increase during hams' processing, this has been shown to be very limited or nonsignificant when comparing ham aged for 12 months with those having longer stages of maturation (18-20 months) which can be related, among other factors, with the progressive decrease of enzyme activity (Parolari et al. 2009; Parolari et al. 2016).

407 In Figure 2b, it can be observed that hams with higher values of proteolysis index and  
408 marbling values favoured the conversion of heme into ZnPP. It is well known that during  
409 the production of dry-cured hams both proteolytic and lipolytic processes occur (Toldrá  
410 2010; Martin et al. 1999). As discussed previously, a partial proteolysis of the globin  
411 facilitates the interaction between myoglobin and Zn-chelatase. Besides, the released fatty  
412 acids and phospholipids could affect the activity of Zn-chelatase in hams as described in  
413 *in vitro* studies (Chau et al. 2010). The interaction between the enzyme and hydrolyzed  
414 acylglycerides is expected to be higher in hams with increased percentages of  
415 intramuscular fat. Therefore, proteolysis and intramuscular fat content can be crucial for  
416 the elevated conversion of heme into ZnPP. In addition, an increased water activity seems  
417 to have a negative effect on the conversion ratio. Indeed, water activity is affected by  
418 moisture and salt content, therefore, it is possible that this parameter better explains the  
419 ZnPP/heme ratio than salt content as observed previously with ZnPP content (Figure 2a).  
420 Given that water activity is affected by extrinsic factors, it can be presumed that these  
421 factors can affect ZnPP formation. Despite this, neither the maturation time (over 12  
422 months) nor the producers were found to affect this ratio.

423 Additional studies addressing the effect of these factors on the formation of ZnPP would  
424 be of interest to manufacturers. Despite the fact that the insertion of Zn in the heme moiety  
425 still needs to be elucidated, these results suggested that the formation of ZnPP may be  
426 affected by various factors and complex interactions. In this regard, it is reasonable to  
427 think that processing conditions such as salting and drying may have an important effect  
428 on ZnPP formation as salt content and water activity seem to play a role. In addition, these  
429 results also reinforced the hypothesis that proteolysis and marbling (and perhaps  
430 lipolysis), among other possible mechanisms, can enhance iron/zinc transmetallation and  
431 thus modulate the formation of ZnPP. The production of hams with a higher or maximized

432 content in ZnPP is of technological interest as it may contribute to non-nitrified dry-cured  
433 hams with an improved and more stable colour.

434

#### 435 **4. Conclusion**

436

437 ZnPP and ZnPP/heme ratio showed a high variability in sliced Parma hams aged for 12  
438 to 20 months. In addition, the observations of this study are in agreement with various  
439 proposed mechanisms in different *in vitro* studies thus highlighting the importance of  
440 such effects in ham samples. The complexity involving the formation of ZnPP is thus not  
441 only supported by previous findings in various meat models but also seems to be  
442 confirmed in commercial Parma ham. In this regard, not only proteolysis but also  
443 marbling may influence ZnPP formation. However, further studies are required to  
444 confirm these relationships. Moreover, the effect on ZnPP of salt content and water  
445 activity are of great interest when dealing with the manufacture of hams without the  
446 addition of curing agents.

447

448

449 **Acknowledgments:**

450 This work was supported in part by INIA (RTA2013-00030-CO3-01) and CDTI (IDI-  
451 20150895) from the Spanish *Ministerio de Economía y Competitividad* and by the  
452 CERCA Programme from the *Generalitat de Catalunya*. R. B. received financial support  
453 from the *Ramon y Cajal* Program (RYC-2013-12745) of the Spanish *Ministerio de*  
454 *Economía y Competitividad*. The authors would like to thank Luis Guerrero for his help  
455 with data analysis.

456 **Declaration of interest:** none

457

458

459 Table 1. Physicochemical composition, pigment content and sensory evaluation of  
 460 redness in sliced commercial Parma dry-cured hams (n = 24).

Parameter	Units	Mean	Standard deviation	Minimum	Maximum
<b>Physicochemical characterization:</b>					
Protein content	g/100g	30.6	3.0	25.3	36.1
Proteolysis index <sup>1</sup>	%	27.0	2.9	21.6	31.0
Fat content	g/100g	11.5	3.7	5.9	22.2
Marbling <sup>2</sup>	%	1.35	0.37	0.48	1.92
Moisture	g/100g	50.8	3.4	42.6	56.5
Moisture, defatted basis	g/100g	57.2	2.6	50.3	61.2
Water activity	dimensionless	0.899	0.008	0.883	0.916
NaCl content	g/100g	5.3	0.58	4.3	6.4
NaCl content, dry matter basis	g/100g	10.9	1.7	8.1	14.4
NaCl content, defatted dry matter basis	g/100g	12.4	1.7	9.3	15.8
pH	dimensionless	5.84	0.09	5.66	6.00
<b>Sensory evaluation and pigment content:</b>					
Overall redness	10-point scale	4.3	1.2	2.0	7.0
Zn-protoporphyrin content, fresh weight	mg/kg	21.3	6.4	11.0	37.2
Zn-protoporphyrin content, dry matter basis	mg/kg	43.8	14	23.0	73.6
Zn-protoporphyrin content, defatted dry matter basis	mg/kg	50.3	16	25.5	84.6
Heme content, fresh weight	mg/kg	17.2	7.0	8.2	33.1
Heme content, dry matter basis	mg/kg	35.6	16	14.3	72.8

Heme content, defatted dry matter basis	mg/kg	40.0	17	19.5	79.3
Zn-protoporphyrin /heme ratio (dry matter)	dimensionless	1.5	0.69	0.49	2.71

---

461 <sup>1</sup> The proteolysis index of the samples was determined as a percentage of the ratio  
462 between non-protein nitrogen and total nitrogen.

463 <sup>2</sup> Marbling is the estimation of the visual intramuscular fat expressed as the percentage of  
464 intramuscular fat area related to the total area of the slice.

Table 2. Pearson's correlation coefficients between physicochemical composition, pigment contents and sensory evaluation of redness in Parma hams

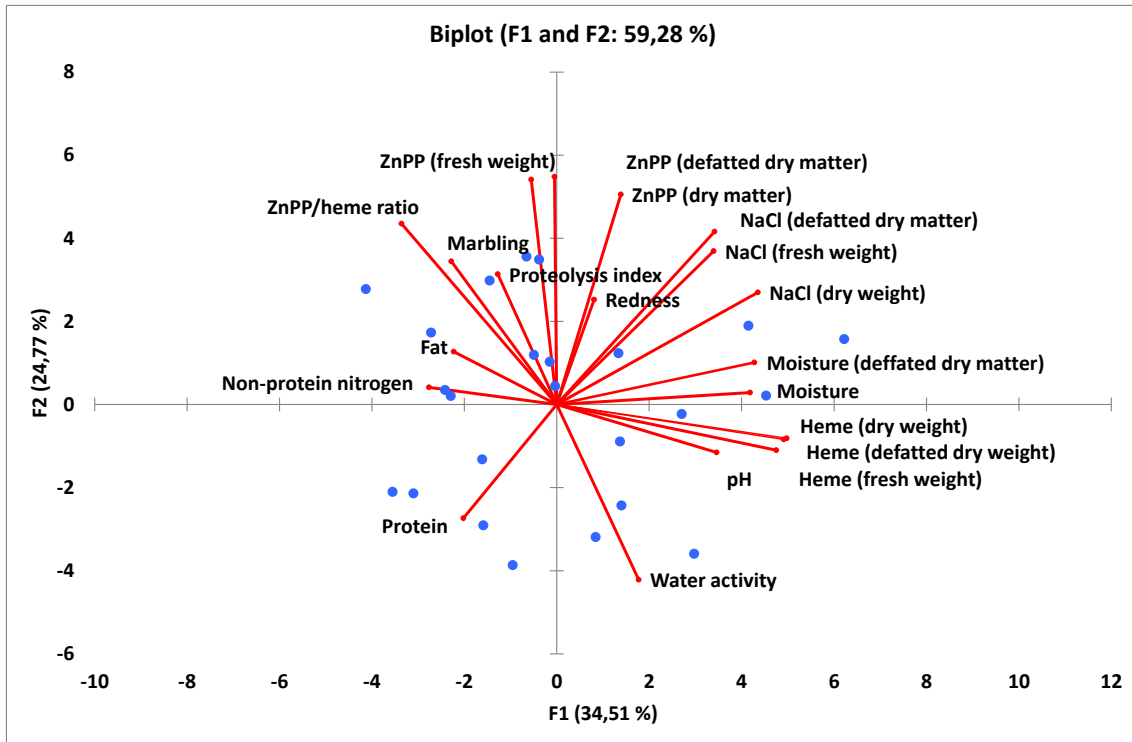
	ZnPP (fw)	ZnPP (dm)	ZnPP (dfdm)	Heme (fw)	Heme (dm)	Heme (dfdm)	ZnPP / heme	pH	Marbling (%)	NaCl (fw)	NaCl (dm)	NaCl (dfdm)	Moisture (%)	Moisture (df)	Water activity	Fat (%)	Protein (%)	PI (%)	redness
ZnPP (fw)	1	0.730 <sup>**</sup> (0.000)	0.994 <sup>**</sup> (0.000)	-0.185 (0.386)	-0.159 (0.459)	-0.163 (0.448)	0.632 <sup>**</sup> (0.001)	-0.228 (0.284)	0.444 <sup>*</sup> (0.030)	0.243 (0.253)	0.145 (0.498)	0.440 <sup>*</sup> (0.032)	-0.004 (0.984)	0.007 (0.975)	-0.458 <sup>*</sup> (0.024)	0.176 (0.410)	-0.167 (0.434)	0.181 (0.396)	0.564 <sup>**</sup> (0.006)
ZnPP (dm)		1	0.768 <sup>**</sup> (0.000)	0.087 (0.684)	0.141 (0.510)	0.123 (0.566)	0.444 <sup>*</sup> (0.030)	-0.042 (0.844)	0.268 (0.206)	0.502 <sup>*</sup> (0.012)	0.503 <sup>*</sup> (0.012)	0.500 <sup>*</sup> (0.013)	0.341 (0.103)	0.314 (0.135)	-0.236 (0.267)	-0.158 (0.460)	-0.261 (0.217)	0.220 (0.302)	0.550 <sup>**</sup> (0.008)
ZnPP (dfdm)			1	-0.119 (0.581)	-0.084 (0.698)	-0.088 (0.682)	0.586 <sup>**</sup> (0.003)	-0.170 (0.426)	0.404 (0.051)	0.300 (0.154)	0.228 (0.284)	0.508 <sup>*</sup> (0.011)	0.083 (0.700)	0.112 (0.601)	-0.420 <sup>*</sup> (0.041)	0.147 (0.493)	-0.202 (0.344)	0.179 (0.402)	0.545 <sup>**</sup> (0.009)
Heme (fw)				1	0.992 <sup>**</sup> (0.000)	0.995 <sup>**</sup> (0.000)	-0.751 <sup>**</sup> (0.000)	0.482 <sup>*</sup> (0.017)	-0.355 (0.089)	0.360 (0.084)	0.505 <sup>*</sup> (0.012)	0.359 (0.085)	0.498 <sup>*</sup> (0.013)	0.543 <sup>**</sup> (0.006)	0.287 (0.174)	-0.247 (0.244)	-0.327 (0.119)	-0.345 (0.098)	0.257 (0.249)
Heme (dm)					1	0.997 <sup>**</sup> (0.000)	-0.729 <sup>**</sup> (0.000)	0.517 <sup>**</sup> (0.010)	-0.375 (0.071)	0.414 <sup>*</sup> (0.044)	0.582 <sup>**</sup> (0.003)	0.425 <sup>*</sup> (0.039)	0.585 <sup>**</sup> (0.003)	0.616 <sup>**</sup> (0.001)	0.301 (0.152)	-0.305 (0.148)	-0.325 (0.121)	-0.300 (0.154)	0.266 (0.231)
Heme (dfdm)						1	-0.729 <sup>**</sup> (0.000)	0.509 <sup>*</sup> (0.011)	-0.366 (0.079)	0.400 (0.053)	0.556 <sup>**</sup> (0.005)	0.419 <sup>*</sup> (0.041)	0.545 <sup>**</sup> (0.006)	0.618 <sup>**</sup> (0.001)	0.287 (0.174)	-0.240 (0.259)	-0.353 (0.091)	-0.307 (0.144)	0.242 (0.279)
ZnPP / heme							1	-0.355 (0.088)	0.487 <sup>*</sup> (0.016)	0.029 (0.892)	-0.182 (0.396)	0.064 (0.766)	-0.421 <sup>*</sup> (0.040)	-0.351 (0.093)	-0.591 <sup>**</sup> (0.002)	0.357 (0.087)	-0.039 (0.855)	0.445 <sup>*</sup> (0.029)	0.100 (0.658)
pH								1	-0.518 <sup>**</sup> (0.010)	0.331 (0.115)	0.450 <sup>*</sup> (0.027)	0.345 (0.099)	0.456 <sup>*</sup> (0.025)	0.476 <sup>*</sup> (0.019)	0.304 (0.149)	-0.251 (0.237)	-0.132 (0.537)	-0.255 (0.229)	-0.181 (0.419)
Marbling (%)									1	0.037 (0.863)	-0.145 (0.499)	0.006 (0.978)	-0.301 (0.152)	-0.254 (0.232)	-0.453 <sup>*</sup> (0.026)	0.300 (0.154)	-0.154 (0.472)	0.380 (0.067)	0.209 (0.351)
NaCl (fw)										1	0.928 <sup>**</sup> (0.000)	0.893 <sup>**</sup> (0.000)	0.487 <sup>*</sup> (0.016)	0.525 <sup>**</sup> (0.008)	-0.374 (0.072)	-0.204 (0.338)	-0.467 <sup>*</sup> (0.021)	0.174 (0.415)	0.175 (0.436)
NaCl (dm)											1	0.861 <sup>**</sup> (0.000)	0.765 <sup>**</sup> (0.000)	0.728 <sup>**</sup> (0.000)	-0.066 (0.759)	-0.435 <sup>*</sup> (0.034)	-0.373 (0.073)	0.122 (0.571)	0.162 (0.472)
NaCl (dfdm)												1	0.489 <sup>*</sup> (0.015)	0.658 <sup>**</sup> (0.000)	-0.338 (0.107)	-0.018 (0.932)	-0.527 <sup>**</sup> (0.008)	0.169 (0.430)	0.129 (0.568)



Moisture (%)												1	0.778** (0.000)	0.472' (0.020)	-0.731** (0.000)	-0.022 (0.920)	-0.007 (0.976)	0.033 (0.8849)
Moisture (df)													1	0.321 (0.127)	-0.166 (0.4389)	-0.402 (0.051)	0.071 (0.741)	-0.086 (0.703)
Water activity														1	-0.465' (0.022)	0.201 (0.347)	-0.410' (0.047)	-0.181 (0.419)
Fat (%)															1	-0.371 (0.074)	0.101 (0.640)	-0.176 (0.434)
Protein (%)																1	-0.347 (0.097)	-0.137 (0.542)
PI (%)																	1	0.046 (0.838)
redness																		1

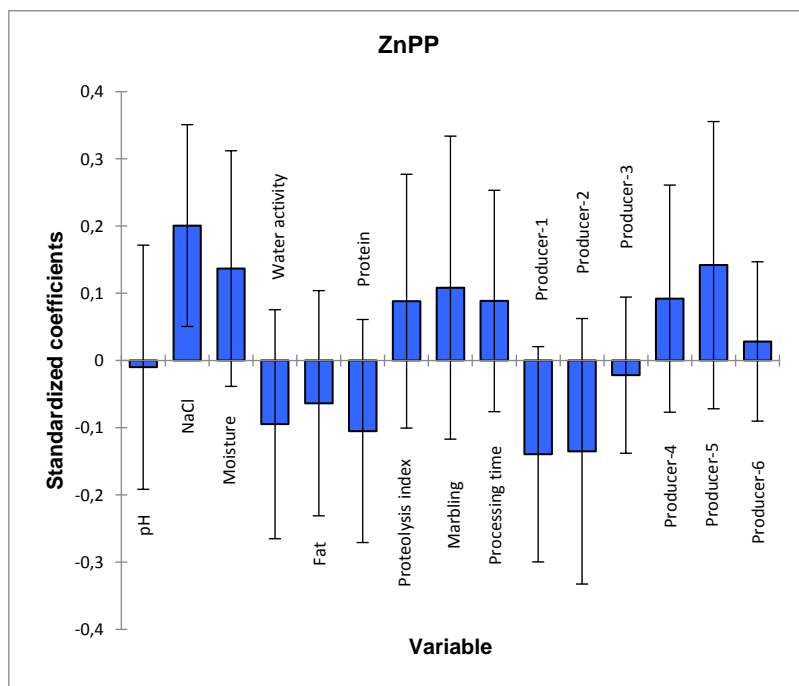
466 ZnPP stands for the content in zinc-protoporphyrin expressed on fresh weight (fw), dry matter (dm) and defatted dry matter (dfdm) basis; the same applies to the heme iron and NaCl content. Moisture is expressed  
467 as a percentage of the fresh weight and as a percentage of the defatted fresh weight (df). PI stands for proteolysis index and redness for the sensory evaluation of red colour using an unstructured scale from 0 to 10.  
468 The numbers within parenthesis indicate p-values; levels of significance are indicated as \*\* = P<0.01; \* = P<0.05. n = 24.

469

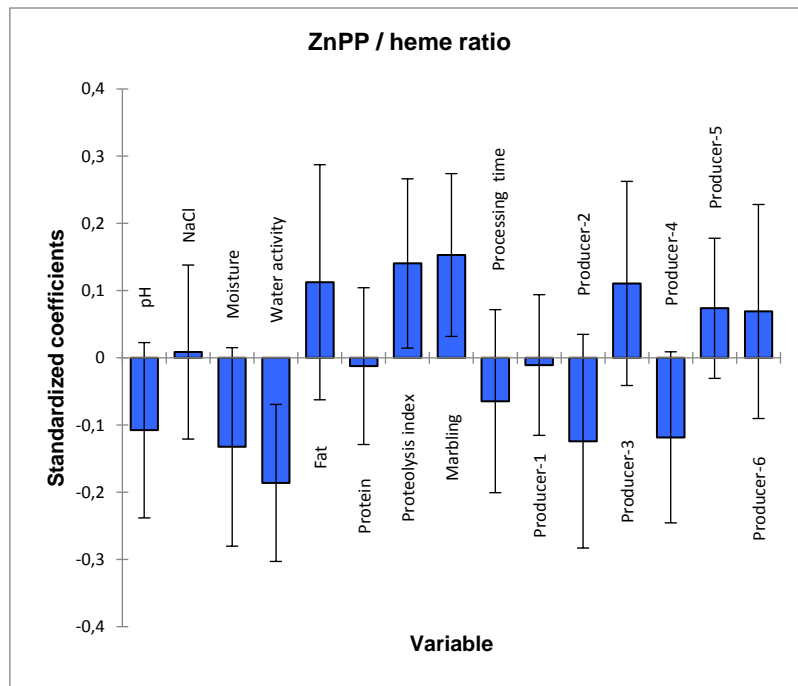


**Figure 1.** Principal component analysis. The abbreviation of ZnPP stands for zinc-protoporphyrin.

A)



B)



**Figure 2.** Standardized coefficients of Partial Least Squares regression analysis for zinc-protoporphyrin (ZnPP) and ZnPP/heme ratio both expressed on a dry matter basis. Error bars represent the 95% confidence interval.

## References

- Adamsen, C. E., Moller, J. K. S., Hismani, R., & Skibsted, L. H. (2004). Thermal and photochemical degradation of myoglobin pigments in relation to colour stability of sliced dry-cured Parma ham and sliced dry-cured ham produced with nitrite salt. *European Food Research and Technology*, 218(5), 403-409, doi:10.1007/s00217-004-0891-8.
- Adamsen, C. E., Moller, J. K. S., Laursen, K., Olsen, K., & Skibsted, L. H. (2006a). Zn-porphyrin formation in cured meat products: Effect of added salt and nitrite. *Meat Science*, 72(4), 672-679, doi:10.1016/j.meatsci.2005.09.017.
- Adamsen, C. E., Moller, J. K. S., Parolari, G., Gabba, L., & Skibsted, L. H. (2006b). 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.
- 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, 63-69.
- AOAC (Ed.). (2000). *Official Methods of Analysis of AOAC International* (17th ed.). Gaithersburg, MD.
- 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.
- Careri, M., Mangia, A., Barbieri, A., 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.
- 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.
- Chau, T. T., Ishigaki, M., Kataoka, T., & Taketani, S. (2011). Ferrochelatase Catalyzes the Formation of Zn-protoporphyrin of Dry-Cured Ham via the Conversion Reaction from Heme in Meat. [Article]. *Journal of Agricultural and Food Chemistry*, *59*(22), 12238-12245, doi:10.1021/jf203145p.
- Consorzio del Prosciutto di Parma (2016). [http://www.prosciuttodiparma.com/en\\_UK/consortium/specifications](http://www.prosciuttodiparma.com/en_UK/consortium/specifications).
- De Maere, H., De Mey, E., Baca, M., Sajewicz, M., Paelinck, H., Fraeye, I., et al. (2014). Determination of Selected Protoporphyrins in Parma Ham With use of 5,10,15,20-tetra(4-hydroxyphenyl) porphyrin as a Surrogate Standard in the Recovery Study. *Acta Chimica Slovenica*, *61*(4), 771-777.
- Durek, J., Bolling, J. S., Knorr, D., Schwagele, F., & Schluter, O. (2012). Effects of different storage conditions on quality related porphyrin fluorescence signatures of pork slices. *Meat Science*, *90*(1), 252-258, doi:10.1016/j.meatsci.2011.07.010.
- Ericson, M. B., Grapengiesser, S., Gudmundson, F., Wennberg, A. M., Larko, O., Moan, J., et al. (2003). A spectroscopic study of the photobleaching of protoporphyrin

IX in solution. *Lasers in Medical Science*, 18(1), 56-62, doi:10.1007/s10103-002-0254-2.

European Commission (2013). Commission implementing regulation (EU) approving minor amendments to the specification for a name entered in the register of protected designations of origin and protected geographical indications (Prosciutto di Parma (PDO)). In E. Commission (Ed.), (Vol. No 1208/2013 of 25 November 2013 ).

Grossi, A. B., do Nascimento, E. S. P., Cardoso, D. R., & Skibsted, L. H. (2014). Proteolysis involvement in zinc-protoporphyrin IX formation during Parma ham maturation. *Food Research International*, 56, 252-259, doi:10.1016/j.foodres.2014.01.007.

Haldane, J. (1901). The red colour of salted meat. *The Journal of Hygiene*, 1(1), 115-122.

Hornsey, H. C. (1956). The colour of cooked cured pork. I.—Estimation of the Nitric oxide-Haem Pigments. *Journal of the Science of Food and Agriculture*, 7, 534–540.

Ishikawa, H., Kawabuchi, T., Kawakami, Y., Sato, M., Numata, M., & Matsumoto, K. (2007). Formation of zinc protoporphyrin IX and protoporphyrin IX from oxymyoglobin in porcine heart mitochondria. *Food Science and Technology Research*, 13(1), 85-88, doi:10.3136/fstr.13.85.

Ishikawa, H., Yoshihara, M., Baba, A., Kawabuchi, T., Sato, M., Numata, M., et al. (2006). Formation of zinc protoporphyrin IX from myoglobin in porcine heart extract. *Food Science and Technology Research*, 12(2), 125-130, doi:10.3136/fstr.12.125.

ISO (2016). <http://www.iso.org/iso/home/standards.htm>. Accessed 19th December 2016.

- Martin, L., Cordoba, J. J., Ventanas, J., & Antequera, T. (1999). Changes in intramuscular lipids during ripening of Iberian dry-cured ham. *Meat Science*, *51*(2), 129-134, doi:10.1016/s0309-1740(98)00109-0.
- Morita, H., Niu, J., Sakata, R., & Nagata, Y. (1996). Red pigment of Parma ham and bacterial influence on its formation. *Journal of Food Science*, *61*(5), 1021-1023, doi:10.1111/j.1365-2621.1996.tb10924.x.
- Muñoz, I., Rubio-Celorio, M., Garcia-Gil, N., Guardia, M. D., & Fulladosa, E. (2015). Computer image analysis as a tool for classifying marbling: A case study in dry-cured ham. *Journal of Food Engineering*, *166*, 148-155, doi:10.1016/j.jfoodeng.2015.06.004.
- Paganelli, M. O., Grossi, A. B., Dores-Silva, P. R., Borges, J. C., Cardoso, D. R., & Skibsted, L. H. (2016). Limited proteolysis of myoglobin opens channel in ferrocyclase-globin complex for iron to zinc transmetallation. *Food Chemistry*, *210*, 491-499, doi:10.1016/j.foodchem.2016.04.109.
- Parolari, G., Aguzzoni, A., & Toscani, T. (2016). Effects of Processing Temperature on Color Properties of Dry-Cured Hams Made without Nitrite. *Foods*, *5*(2), doi:10.3390/foods5020033.
- Parolari, G., Benedini, R., & Toscani, T. (2009). Color Formation in Nitrite-Free Dried Hams as Related to Zn-Protoporphyrin IX and Zn-Chelatase Activity. *Journal of Food Science*, *74*(6), C413-C418, doi:10.1111/j.1750-3841.2009.01193.x.
- Santos-Garcés, E., Muñoz, I., Gou, P., Garcia-Gil, N., & Fulladosa, E. (2014). Including estimated intramuscular fat content from computed tomography images improves prediction accuracy of dry-cured ham composition. *Meat Science*, *96*(2), 943-947, doi:10.1016/j.meatsci.2013.09.018.
- Toldrá, F. (Ed.). (2010). *Handbook of Meat Processing*. Ames, Iowa: Wiley-Blackwell.



- Wakamatsu, J., Hayashi, N., Nishimura, T., & Hattori, A. (2010). Nitric oxide inhibits the formation of zinc protoporphyrin IX and protoporphyrin IX. *Meat Science*, 84(1), 125-128, doi:10.1016/j.meatsci.2009.08.036.
- Wakamatsu, J., Murakami, N., & Nishimura, T. (2015). A comparative study of zinc protoporphyrin IX-forming properties of animal by-products as sources for improving the color of meat products. *Animal science journal = Nihon chikusan Gakkaiho*, 86(5), 547-552, doi:10.1111/asj.12326.
- Wakamatsu, J., Nishimura, T., & Hattori, A. (2004a). A Zn-porphyrin complex contributes to bright red color in Parma ham. *Meat Science*, 67(1), 95-100, doi:10.1016/j.meatsci.2003.09.012.
- Wakamatsu, J., Odagiri, H., Nishimura, T., & Hattori, A. (2009a). Quantitative determination of Zn protoporphyrin IX, heme and protoporphyrin IX in Parma ham by HPLC. *Meat Science*, 82(1), 139-142, doi:10.1016/j.meatsci.2008.12.011.
- Wakamatsu, J., Okui, J., Hayashi, N., Nishimura, T., & Hattori, A. (2007). Zn protoporphyrin IX is formed not from heme but from protoporphyrin IX. *Meat Science*, 77(4), 580-586, doi:10.1016/j.meatsci.2007.05.008.
- Wakamatsu, J., Okui, J., Ikeda, Y., Nishimura, T., & Hattori, A. (2004b). Establishment of a model experiment system to elucidate the mechanism by which Zn-protoporphyrin IX is formed in nitrite-free dry-cured ham. *Meat Science*, 68(2), 313-317, doi:10.1016/j.meatsci.2004.03.014.
- Wakamatsu, J., Uemura, J., Odagiri, H., Okui, J., Hayashi, N., Hioki, S., et al. (2009b). Formation of zinc protoporphyrin IX in Parma-like ham without nitrate or nitrite. *Animal Science Journal*, 80(2), 198-205, doi:10.1111/j.1740-0929.2008.00619.x.