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

Ricard Bou[a], Mar Llauger[a], Jacint Arnau[a], Elena Fulladosa[a]

[a] Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Food Technology, Monells, Finca Camps i Armet s/n, 17121, Spain.

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

Abstract:

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

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

Highlights:

- Typical contents of zinc-protoporphyrin (ZnPP) in commercial Parma ham were reported
- ZnPP content was positively correlated with redness and salt content
- Proteolysis and marbling were related with higher conversions of heme into ZnPP

1. Introduction

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

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

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

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

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

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

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

2. Material and Methods

2.1. Acquisition and processing of Parma ham samples

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

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

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

2.2 Reagents

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

2.3 Physicochemical determinations

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

Fat content was determined according to the ISO 1443:1973 protocol (ISO 2016). Water content (moisture) was determined by drying at 103 ± 2 °C until a constant weight was reached (AOAC 2000). The water content of the samples on defatted basis was also calculated from the chemical composition of the ham (Moisture = g H_2O/(100 g sample

– g fat)). Water activity (a_w) was measured at 25 °C ± 0.3 °C with a Novasina AW SPRINT – TH 500 instrument (Axair Ltd., Pfäffikon, Switzerland). Chloride content was determined according to ISO 1841-2:1996 protocol using a potentiometric titrator 785 DMP Titrino (Metrohm AG, Herisau, Switzerland) and expressed as NaCl content. The NaCl content on dry matter basis (NaCl DM = g NaCl/(100 g sample – g H_2O)) and on defatted dry mater basis (NaCl, defatted DM = g NaCl/(100 g sample – g H_2O – g fat)) was also calculated. Determination of pH was performed by means of a S40 SevenMulti (Mettler-Toledo S.A.E., Barcelona, Spain) and an Inlab Solids Pro (Mettler-Toledo S.A.E.) probe. All analyses were done in triplicate.

2.4 Visual estimation of intramuscular fat (marbling)

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

Visual intramuscular fat of the entire slice was segmented using the procedures previously described elsewhere (Santos-Garcés et al. 2014; Muñoz et al. 2015). In brief, Matlab scripts written in-house were used for segmentation of visual subcutaneous, intermuscular 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

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

2.8. Statistical analysis

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

3. Results and Discussion

3.1 Dry-cured Parma ham characterization

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

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

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

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

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

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

3.2 Relationships between physicochemical parameters and pigment contents

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

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

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

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

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

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

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

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

382 1999). In an *in vitro* study, Chau et al., (2010) reported that the presence of fatty acids
383 and phospholipids affect Zn-chelatase enzyme and promote iron removal from the
384 porphyrin ring. Therefore, elevated marbling values may contribute to a higher amount
385 of free fatty acids in the internal parts of ham during processing. These fatty acids may in
386 turn interact with the enzyme and explain the existing correlation between ZnPP and
387 marbling. The correlation between ZnPP/heme ratio and fat content was not significant (r
388 = 0.357; P = 0.087). In this regard, it should be noted that the fat content on the surface
389 of the sliced ham increased during slicing due to the spreading of subcutaneous fat by the
390 slicing blade. This increase would depend on subcutaneous fat content and slicing
391 conditions (personal data). In addition, the subcutaneous fat of the slice could be in
392 contact with other parts during their packaging and storage. In order to clarify the effect
393 of intramuscular fat on ZnPP/heme ratio it is necessary to design a specific experiment.

394 The observed range of ZnPP concentrations may obey to a number of intrinsic and
395 extrinsic factors. Thus, a Partial Least Squares regression analysis was conducted to
396 identify the compositional factors and production conditions (e.g. producer, processing
397 time) that were relevant (i.e. different from 0) with regard to ZnPP formation. In Figure
398 2a it can be observed that samples content of ZnPP was higher in those with higher salt
399 content. Despite the fact that this variable depends on ham production conditions (i.e.
400 salting), none of the producers showed an effect on ZnPP formation. Likewise, the
401 elaboration time, which ranged from between 12 to 20 months, had no effect on ZnPP
402 content (Figure 2a). Although ZnPP content has been shown to increase during hams'
403 processing, this has been shown to be very limited or nonsignificant when comparing ham
404 aged for 12 months with those having longer stages of maturation (18-20 months) which
405 can be related, among other factors, with the progressive decrease of enzyme activity
406 (Parolari et al. 2009; Parolari et al. 2016).

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

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

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

4. Conclusion

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

Acknowledgments:

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

Declaration of interest: none

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
Physicochemical characterization:					
Protein content	g/100g	30.6	3.0	25.3	36.1
Proteolysis index[1]	%	27.0	2.9	21.6	31.0
Fat content	g/100g	11.5	3.7	5.9	22.2
Marbling[2]	%	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
Sensory evaluation and pigment content:					
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

[1] The proteolysis index of the samples was determined as a percentage of the ratio between non-protein nitrogen and total nitrogen.

[2] Marbling is the estimation of the visual intramuscular fat expressed as the percentage of intramuscular fat area related to the total area of the slice.

465 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** (0.000)	0.994** (0.000)	-0.185 (0.386)	-0.159 (0.459)	-0.163 (0.448)	0.632** (0.001)	-0.228 (0.284)	0.444* (0.030)	0.243 (0.253)	0.145 (0.498)	0.440* (0.032)	-0.004 (0.984)	0.007 (0.975)	-0.458* (0.024)	0.176 (0.410)	-0.167 (0.434)	0.181 (0.396)	0.564** (0.006)
ZnPP (dm)		1	0.768** (0.000)	0.087 (0.684)	0.141 (0.510)	0.123 (0.566)	0.444* (0.030)	-0.042 (0.844)	0.268 (0.206)	0.502* (0.012)	0.503* (0.012)	0.500* (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** (0.008)
ZnPP (dfdm)			1	-0.119 (0.581)	-0.084 (0.698)	-0.088 (0.682)	0.586** (0.003)	-0.170 (0.426)	0.404 (0.051)	0.300 (0.154)	0.228 (0.284)	0.508* (0.011)	0.083 (0.700)	0.112 (0.601)	-0.420* (0.041)	0.147 (0.493)	-0.202 (0.344)	0.179 (0.402)	0.545** (0.009)
Heme (fw)				1	0.992** (0.000)	0.995** (0.000)	-0.751** (0.000)	0.482* (0.017)	-0.355 (0.089)	0.360 (0.084)	0.505* (0.012)	0.359 (0.085)	0.498* (0.013)	0.543** (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** (0.000)	-0.729** (0.000)	0.517** (0.010)	-0.375 (0.071)	0.414* (0.044)	0.582** (0.003)	0.425* (0.039)	0.585** (0.003)	0.616** (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** (0.000	0.509* (0.011)	-0.366 (0.079)	0.400 (0.053)	0.556** (0.005)	0.419* (0.041)	0.545** (0.006)	0.618** (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* (0.016)	0.029 (0.892)	-0.182 (0.396)	0.064 (0.766)	-0.421* (0.040)	-0.351 (0.093)	-0.591** (0.002)	0.357 (0.087)	-0.039 (0.855)	0.445* (0.029)	0.100 (0.658)
pH								1	-0.518** (0.010)	0.331 (0.115)	0.450* (0.027)	0.345 (0.099)	0.456* (0.025)	0.476* (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* (0.026)	0.300 (0.154)	-0.154 (0.472)	0.380 (0.067)	0.209 (0.351)
NaCl (fw)										1	0.928** (0.000)	0.893** (0.000)	0.487* (0.016)	0.525** (0.008)	-0.374 (0.072)	-0.204 (0.338)	-0.467* (0.021)	0.174 (0.415)	0.175 (0.436)
NaCl (dm)											1	0.861** (0.000)	0.765** (0.000)	0.728** (0.000)	-0.066 (0.759)	-0.435* (0.034)	-0.373 (0.073)	0.122 (0.571)	0.162 (0.472)
NaCl (dfdm)												1	0.489* (0.015)	0.658** (0.000)	-0.338 (0.107)	-0.018 (0.932)	-0.527** (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

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 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. The numbers within parenthesis indicate p-values; levels of significance are indicated as ** = P<0.01; * = P<0.05. n = 24.

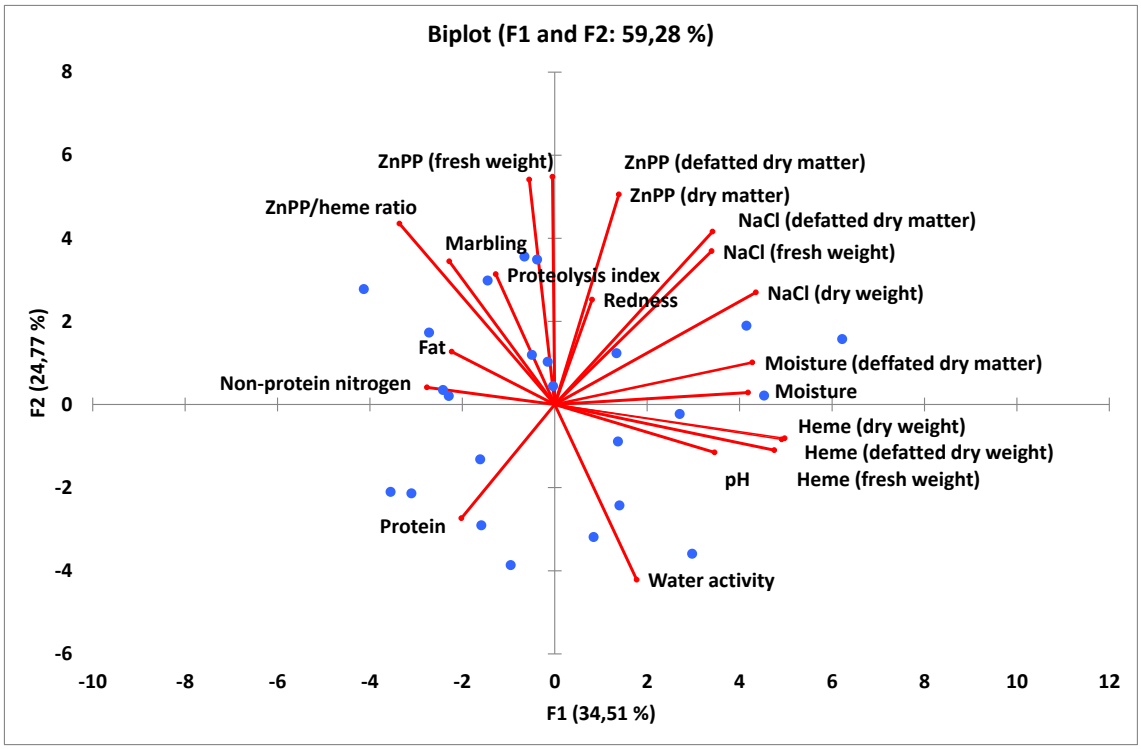
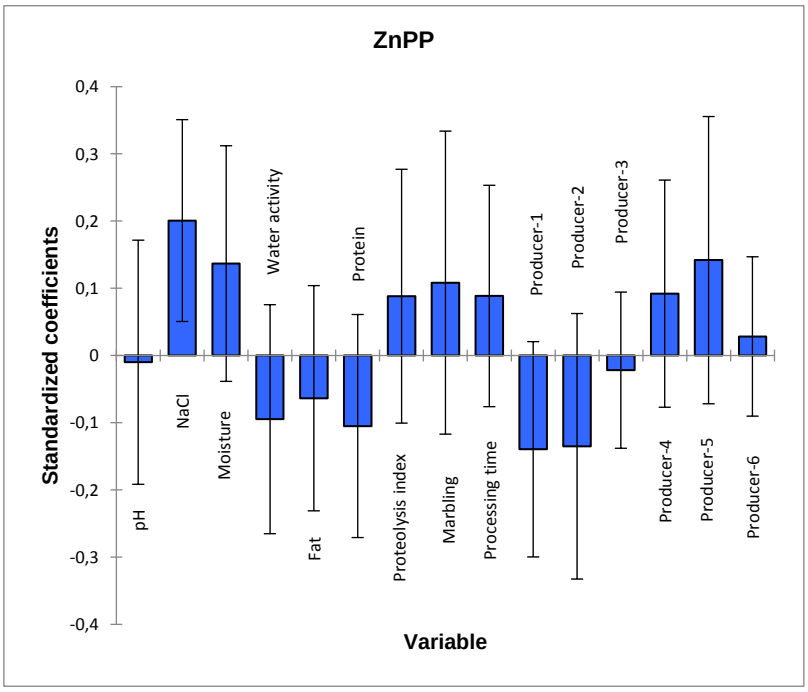


Figure 1. Principal component analysis. The abbreviation of ZnPP stands for zinc-protoporphyrin.

A)


ZnPP
Standardized coefficients
0,4
0,3
0,2
0,1
0
-0,1
-0,2
-0,3
-0,4
pH
NaCl
Moisture
Water activity
Fat
Protein
Proteolysis index
Marbling
Processing time
Producer-1
Producer-2
Producer-3
Producer-4
Producer-5
Producer-6
Variable

B)

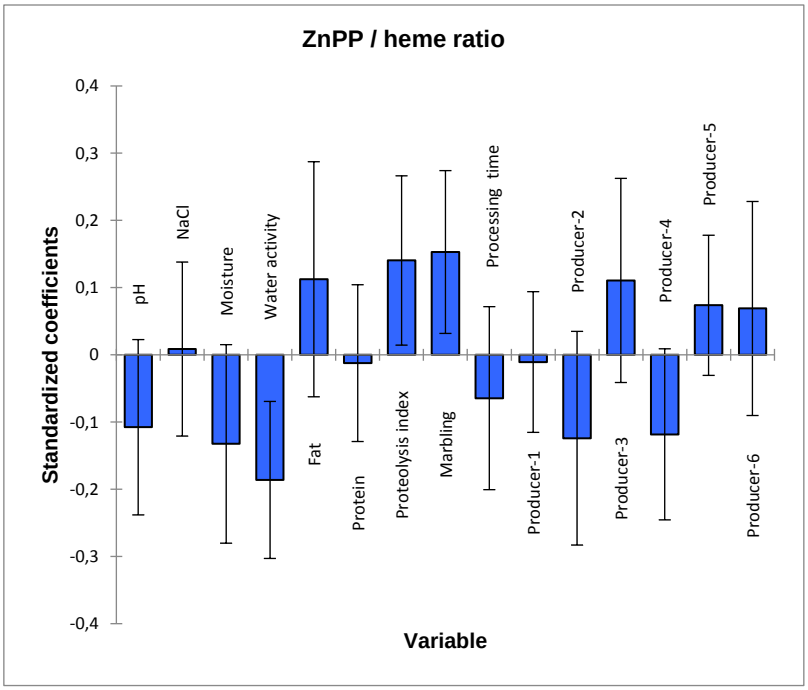


Figure 2. Standardized coefficients of Partial Least Squares regression analysis for zinc-protoporphyrin (ZnPP) and ZnPP/heme ratio both expressed on a dry matter basis. Error bars represent the 95% confidence interval.

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