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Mass spectrometric evidence for a zinc–porphyrin complex as the red pigment in dry-cured Iberian and Parma ham

Jens K.S. Møller [a,*], Christina E. Adamsen [a], Rodrigo R. Catharino [b], Leif H. Skibsted [a], Marcos N. Eberlin [b]

[a] Royal Veterinary and Agricultural University, Department of Food Science, Food Chemistry, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark
[b] Thomson Mass Spectrometry Laboratory, Institute of Chemistry, State University of Campinas, UNICAMP, Campinas, SP 13083-970, Brazil

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Abstract

Extracts containing red pigment complexes from the two types of dry-cured hams, Italian Parma and Spanish Iberian ham, were obtained using water and acetone as extraction solvents followed by a crude purification with C18 column filtration. The purified extracts were then analyzed spectroscopically by recording absorption and fluorescence spectra ($\lambda_{ex} = 420$ nm), which both indicate the presence of chemically identical red chromophores with properties similar to a complex of transition metals and protoporphyrin IX. Electrospray ionization mass spectrometry (ESI-MS) in the positive ion mode confirms the presence of identical chemical compounds. ESI-MS in the negative ion mode detects a cluster of seven isotopologue ions (that of m/z 623.2 as the most intense) with a pattern matching that of a Zn protoporphyrin IX complex. Based on mass spectral data it is concluded that a Zn–porphyrin complex constitutes a major chromophore in dry-cured Iberian ham as well as in Parma ham.

Keywords: Parma and Iberian dry-cured hams; Colour; Zinc–porphyrin complexes; Nitrite-free meat products; Structural elucidation; Electrospray ionization mass spectrometry

1. Introduction

The Mediterranean region is generally renowned for producing high quality food products, and this wide recognition is often related to the unique taste and aroma attributes of the final products (Flores, 1997). An important aspect of these traditional foods is the application of long-established manufacturing technologies, which include prolonged maturation and ripening periods, and these foods are most often made without the use of artificial additives (Gonzalez & Ockerman, 2000). Certain types or qualities of dry-cured hams originating from either Italy or Spain can particularly be differentiated from other cured meat products with respect to their flavour (Dirinck, Van Opstaele, & Vandedriessche, 1997), while other quality parameters such as texture and colour also will differ between these. Although the production methods related to such traditional foods rely on long experience, diverse cultures, gastronomic heritage and different climates, considerable research efforts have recently been devoted to the understanding of the fundamental aspects of production of these products (Toldra & Navarro, 2000), thereby allowing improvement in process technologies with emphasis on product safety, quality and nutritional value.

The products locally known as ''Prosciutto di Parma'' and ''Jamón Ibérico'' (Parma ham and Iberian ham will be used herein, respectively) are unique in the sense that

* Corresponding author. Tel.: +45 3528 3290; fax: +45 3528 3344.
E-mail address: jemo@kvl.dk (J.K.S. Møller).

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the only additive used is sodium chloride, which is added as coarse salt during the salting with no other additives added subsequently during the prolonged drying and maturation period. This means that both nitrate and nitrite are absent in these cured meats products, but still their colour develops into an intense, dark red colour of the lean part of the ham. In the manufacturing of Parma ham the additives are regulated through the Parma Consortium, which since 1992 only allows NaCl as additive (Parolari, 1996), while for dry-cured Iberian ham by tradition only coarse salt from the Mediterranean sea is used although no official regulations exist to the best of our knowledge.

A limited number of studies dealing with the pigment formed in especially Parma ham have been published in support of various theories concerning the chemical nature of the primary pigment formed in Parma ham. The main hypotheses include suggestions such as coordination of basic ligands, formed during the degradation processes, to the central heme iron in myoglobin (Parolari, Chizzolini, Bellatti, & Dazzi, 1983), and microbial metabolism generating NO, thereby enabling the formation of nitrosylmyoglobin ($MbFe^{II}NO$) (Morita, Niu, Sakata, & Nagata, 1996). However, the possible presence of $MbFe^{II}NO$ as the primary pigment in dry-cured Parma ham has been ruled out by a comparison between electron spin resonance (ESR) spectra of nitrite-cured dried ham and Parma ham. The Parma ham pigment was found to be ESR silent, while a characteristic ESR signal for $MbFe^{II}NO$ was observed in nitrite-cured ham samples (Møller, Adamsen, & Skibsted, 2003). Further studies of the chemical properties and extractabilities of the pigment in Parma ham showed that extraction yields for aqueous solvents decreased during the numerous stages of the dry-curing process, while the yield of a red pigment extracted with polar solvents increased until the final matured Parma ham indicating that the association between globin and the heme chromophore becomes weaker with time (Møller et al., 2003; Parolari, Gabba, & Saccani, 2003).

More recently, a Zn–porphyrin complex has been reported to contribute to the red colour of Parma ham (Wakamatsu, Nishimura, & Hattori, 2004), and formation of this hydrophobic pigment during maturation is in agreement with the varying extractabilities found for Parma ham using either water or polar solvents.

The main objective of the present study was to further elucidate structural details of red-coloured chromophores in particular dry-cured hams from Spain and Italy, respectively, with the application of electrospray ionization mass spectrometric (ESI-MS). Herein we report conclusive proof for the presence of an identical Zn–porphyrin complex as the primary pigment in Spanish Iberian dry-cured ham using ESI-MS. We also present spectroscopic and ESI-MS evidence for the presence of a Zn–porphyrin complex in dry-cured Parma ham originating from Italy, thereby confirming the previous findings by Wakamatsu, Nishimura, et al. (2004).

2. Materials and methods

2.1. Chemicals

Acetone used for extractions was purchased from Lab-Scan (Dublin, Ireland) and of analytical HPLC grade. Formic acid and ammonium hydroxide analytical grade were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Water for extraction was prior to use purified using a Millipore Q-Plus purification train (Millipore, Bedford, MA, USA). Hemin, zinc protoporphyrin IX and protoporphyrin IX used for spectroscopic analysis were all obtained from Sigma Aldrich Chemical Co.

2.2. Raw materials

Fully matured Parma ham (12 months manufacturing time) was obtained from a local producer in Parma through Stazione Sperimentale per I'Industria delle Conserve Alimentari, Parma, Italy. Fully matured Iberian ham (30 months manufacturing time) was obtained from the manufacturer; Señorio de Montanera S.L. Criadores de Cerdo Ibérico Asociados, Badajoz, situated in the region of Extremadura, Spain.

2.3. Extraction of dry-cured ham pigments

Whole slices of each of the two types of dry-cured ham were trimmed for visible fat and connective tissue, and the extraction method described by Wakamatsu et al. was applied with minor adjustments (Wakamatsu, Nishimura, et al., 2004; Wakamatsu, Okui, Ikeda, Nishimura, & Hattori, 2004). The resulting lean meat (5 g) originating from muscles in a whole slice was chopped finely and homogenized for 1 min in 20 ml of distilled water using an Ultra Turrex. Subsequently, the homogenate was centrifuged at 3000 rpm for 10 min at 4 °C and the supernatant was filtered through S&S 589[2] filter paper (Schleicher & Schuell GmbH, Dassel, Germany). The filtrate was added 3 vol. of cold acetone while being kept on ice for 15 min and then centrifuged at 3000 rpm for 5 min at 4 °C.

2.3.1. Crude purification

The isolated pigment of the dry-cured hams lean section in 75% acetone was further submitted to a crude purification carried out by diluting 1:1 v/v with distilled water and subsequent transferring to a disposable C18 column, Sep-Pak® Vac C18 Cartridge (12 cm^3/2 g; Waters Co., Manchester, UK), which was pre-washed with 15 ml methanol and 15 ml distilled water as previously described by Wakamatsu, Nishimura, et al. (2004) and Wakamatsu, Okui, et al. (2004). The column was washed with 25 ml distilled water followed by elution of the red pigmented fraction with 10 ml of 75% acetone resulting in purified extracts.

2.4. Absorption and fluorescence spectroscopy

The purified fractions of ham pigments in 75% acetone were analyzed by measurement of electronic absorption spectrum using a Cintra 40 spectrophotometer (GBC Scientific Equipment, Dandenong, Vic., Australia). The absorbance spectra of the purified extracts recovered from two types of dry-cured hams were recorded at room temperature in the range $350 < \lambda < 700$ nm with a spectral resolution of 1 nm. Fluorescence emission spectra were recorded for the two different purified extracts of dry-cured ham at room temperature using an Aminco Bowman Series 2 Luminescence spectrometer (SLM-Aminco, Urbana, IL, USA). The spectrometer was set with excitation wavelength at $\lambda_{ex} = 420$ nm and the fluorescence emission intensities were recorded from 500 to 700 nm with 1 nm intervals. Standard solutions of Zn-pp were prepared and analyzed with absorbance spectroscopy in order to compare spectral patterns and characteristic observed for the purified extracts of dry-cured ham.

2.5. Determination of zinc and iron in purified extracts

In order to establish the zinc and iron content in the purified extracts (75% acetone), these were submitted to destruction and subsequently re-dissolved in 0.1 M HCl as described by Møller et al. (2003). The solutions were analyzed for zinc and iron using AAS (Atomic Absorption Spectrometer 3300, Perkin–Elmer with a zinc-lamp or an iron-lamp, Intensitron lamp, Perkin–Elmer, Wellesley, MA 02481-4078, USA). The zinc or iron concentrations were estimated from a standard curve prepared from a zinc/iron standard (1.000 g/l, Titrisol, Merck) diluted with 0.1 M HCl to yield: 0.10, 0.30, 0.50, 1.00, 2.50 and 5.00 ppm zinc/iron.

2.6. Electrospray ionization mass spectrometry

A Q-TOF mass spectrometer (Micromass, Manchester, UK) was used for ESI-MS analysis of the dry-cured ham 75% acetone purified extracts to which was added 15% methanol to facilitate ESI. The general conditions were: source temperature of 100 °C, capillary voltage of 2.1 kV and cone voltage of 40 V. For analysis in the negative ion mode (ESI(−)-MS) 10.0 μl of an aqueous solution of ammonium hydroxide 0.1% was added to the purified extracts of dry-cured ham. For analysis in the positive ion mode (ESI(+)-MS), 10.0 μl of an aqueous solution of formic acid 0.1% was introduced to the dry-cured ham purified extracts. ESI-MS was preformed by direct infusion with a flow rate of 10 μl min^{-1} using a syringe pump (Harvard Apparatus, Holliston, MA, USA). Mass spectra were acquired and accumulated over 60 s and spectra were scanned over m/z 200–1000 range.

2.6.1. Tandem mass spectrometry

Ions of interest detected by ESI-MS from the purified extracts of the dry-cured hams were further analyzed by ESI-MS/MS. The ion was mass-selected and subjected to 15–55 eV collisions with argon in the collision quadrupole. The collision gas pressure was optimized to produce extensive fragmentation.

2.7. Data handling

All data obtained from ESI-MS of the purified extracts of the two dry-cured hams were treated and presented using MassLynx v4.0 (Waters, Manchester, UK). Mass spectral data were accumulated over approximately 20 s. and the relevant mass range was selected and enlarged, usually being from m/z 350 to 950 (a range that contained all ions of interest as judged by visual inspection).

3. Results

After homogenization, centrifugation and filtration the initial raw aqueous extracts of both types of dry-cured ham have a reddish colour. However, adding 75% of acetone results in immediate precipitation of water-soluble proteinous components and the resulting 75% acetone/water solution of the extract had a clear bright red colour. The formed precipitate presumably of protein nature having a white/grey colour was discharged and not included in further analysis. Subsequently, purification of the crude mixture using a C18 column yields a final extract with an intense bright red colour, which is the sample used for all following analysis and hereafter is refereed to as the purified extracts of the dry-cured hams.

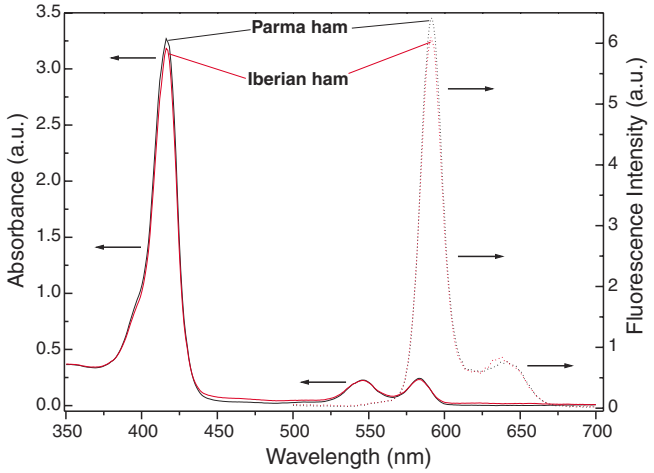

Fig. 1. Representative spectra for electronic absorption (full lines) and fluorescence emission (dashed lines) in the UV–vis wavelength range from 75% acetone/water extracts of dry-cured Parma ham (black lines) and Iberian ham (red lines). Fluorescence emission intensity for dry-cured Parma ham and Iberian ham extracts in 75% acetone/water solution was obtained with excitation wavelength $\lambda_{ex} = 420$ nm and emission spectra recorded between 500 and 700 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 1 shows the absorption and fluorescence spectra of the purified extracts from Parma ham and Iberian ham, respectively. It is evident from the electronic absorption spectra that one or more red coloured compounds are present in both extracts in quite equal amounts. The observed spectral patterns for electronic absorption show a high degree of resemblance to complexes of transition metal and protoporphyrins, such as heme or Zn–porphyrin, or the bright red myoglobin derivates oxy- and nitrosylmyoglobin, $MbFe^{II}O_2$ and $MbFe^{II}NO$. The two electronic absorption spectra both have a characteristic high energy transition at 418 nm, known as the Soret band, as well as two low intensity bands at 546 nm and 584 nm. These electronic absorption spectra indicate after comparisons to spectra of authentic heme compounds and zinc protoporphyrin in 75% acetone (results not shown), that similar chemical compounds are present in the two dry-cured ham extracts.

Fluorescence spectra are also shown in Fig. 1 and emission is monitored upon excitation at $\lambda_{ex} = 420$ nm, which results in a very high intensity emission peak at 590 nm and a lower emission peak at 642 nm. This corresponds very well with $\lambda_{ex,max} = 417$ nm and $\lambda_{em,max} = 589$ nm previously reported for Zn–porphyrin complexes (Masuda et al., 1999), and is further in agreement with the well-documented absence of fluorescence from Fe-heme compounds (Leonard, Yonetani, & Callis, 1974), in contrast to other metal complexes (Mg and Co), all exhibiting fluorescence with a distinctly different pattern compared to Zn complex (Castelfranco, Weinstein, Schwarcz, Pardo, & Wezelman, 1979). The total contents of Zn and Fe in the purified extracts were determined by atomic absorption spectroscopy (AAS). The results for Zn contents were 0.21 ± 0.03 and 0.23 ± 0.05 ppm in purified extract of Iberian ham and Parma ham, respectively. The total Fe content in purified extracts of Iberian ham and Parma ham were found to be 0.46 ± 0.10 and 0.41 ± 0.09 ppm, respectively.

Fig. 2 shows the ESI(+)-MS of both Parma ham and Iberian ham purified extracts. The spectra display only two noticeable positive ions of $m/z > 350$. The most intense ion is that of m/z 579.3 with a simple isotopic pattern: that is, with m/z 579/580/581 ratio for the three isotopologues of approximately 100:40:10. The positive ion of protoporphyrin IX as shown in Fig. 3 has an exact mass 563.3 Da, which indicates that the observed positive ions at higher m/z values are derivatives of protoporphyrin IX, as the addition of formic acid to the extracts is likely to cause dissociation between the central metal and the protoporphyrin molecule due to protonization of the pyrrole N-atoms (Duprat et al., 1995). The observed positive ions in Parma ham and Iberian ham exhibit m/z shifts relative to the unchanged protoporphyrin in steps of 16 units, e.g. m/z 579.3 and m/z 595.3. This chemical modification could be due to formation of amine adducts of one or both of the propanoic acids present in protoporphyrin IX which is possible as this could correspond to the observed stepwise addition of 16 mass units. These structural similarities

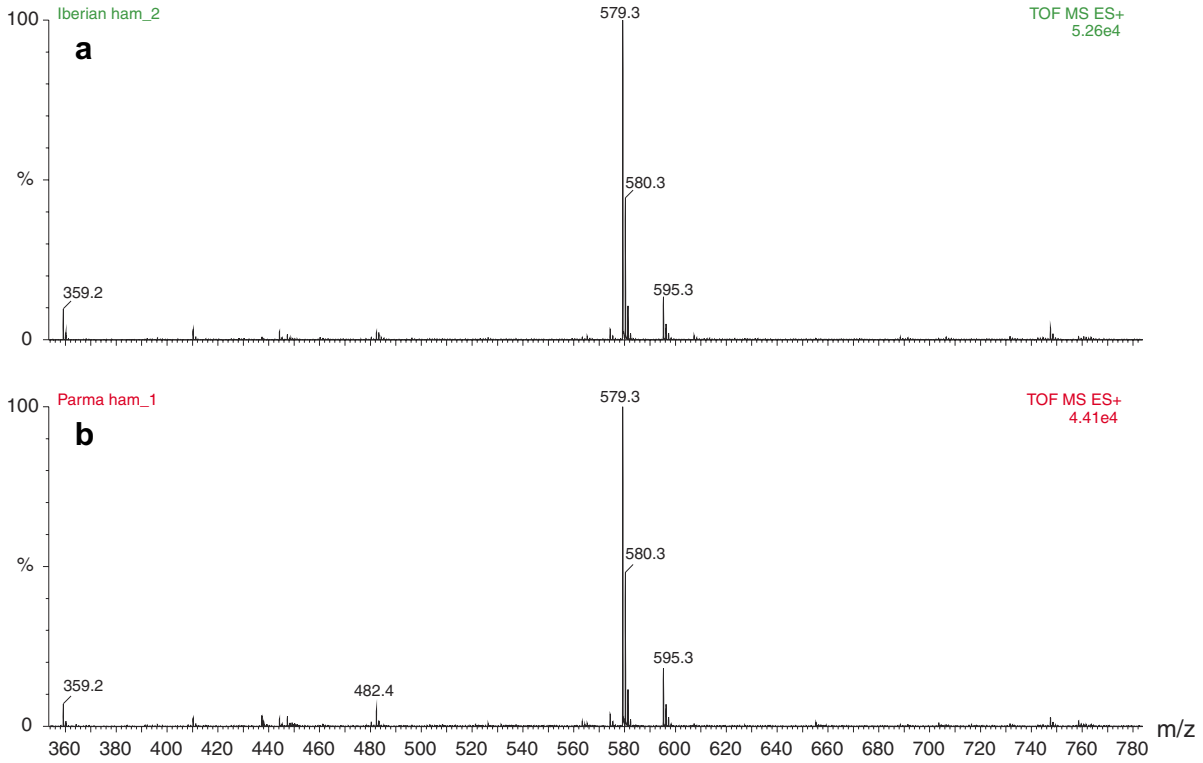

Fig. 2. Electrospray ionization mass spectra in the positive ion mode of dry-cured Iberian ham (a) and Parma ham (b) extracts in 75% acetone/water solution.

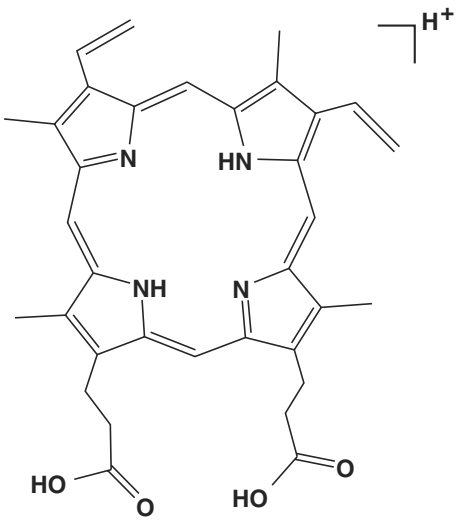

Fig. 3. Structure of positive ion formed from protoporphyrin IX. The ion has m/z of 563.26.

are further supported by performing ESI(+)-MS/MS on the observed positive ions (results not shown), which results in identical fragmentation and loss of 278 mass units for both ions with m/z 579.3 and m/z 595.3, respectively.

Fig. 4 displays the ESI-MS in the negative ion mode for Iberian ham and Parma ham purified extracts. These spectra contain several negative ions for the purified extracts of the two dry-cured hams, e.g. ions of m/z 455.2, 524.3 and 533.3, which are detected in relative high abundances. The most interesting observation for the ESI(−)-MS data is the cluster of isotopologue ions of m/z 623.2 (the most intense). Zinc is naturally present as 5 isotopes; ^{64}Zn: 45.89%, ^{66}Zn: 27.81%, ^{67}Zn: 4.11%, ^{68}Zn: 18.57% and ^{70}Zn: 0.62% (Aylett, 1975), and in Fig. 5 the magnification of the range of interest clearly confirms the presence of a Zn complex of protoporphyrin IX in dry-cured Iberian ham with the detection of six negative isotopologue ions of m/z 623.196 (100%), 625.204 (57%), 627.186 (39%), 624.200 (37%), 626.210 (30%) and 628.193 (15%). Furthermore, isotope modelling of a compound corresponding to Zn protoporphyrin IX (molecular formula: $C_{34}H_{31}N_4O_4$-Zn) proves that for the deprotonated molecule of the complex as illustrated in Fig. 6 yields a distinct pattern of 7 isotopologue ions with the same distribution and abundances as those observed in Fig. 5. ESI(−)-MS of the purified extract of Parma ham reveals that Zn–porphyrin is the most abundant ion present, while in comparison the purified extract of Iberian ham yields a more complex mass spectrum with numerous negatively charged ions.

Fig. 7 shows the ESI(−)-MS/MS of the ion of m/z 623.2 in the purified extract of Parma ham. The fragmentation of this anion is not very pronounced and relatively high collision energy needs to be applied in order to induce dissociation that yields mainly the fragment of m/z 535.2. This ion is formed by the loss of 88 Da that is likely to be by the loss of two CO_2 molecules from cleavages at the two carboxyl groups of the intact protoporphyrin ring, while the fragment of m/z 520.1 appears from a loss of 103 Da, possible due to additional loss of one of several methyl substituents.

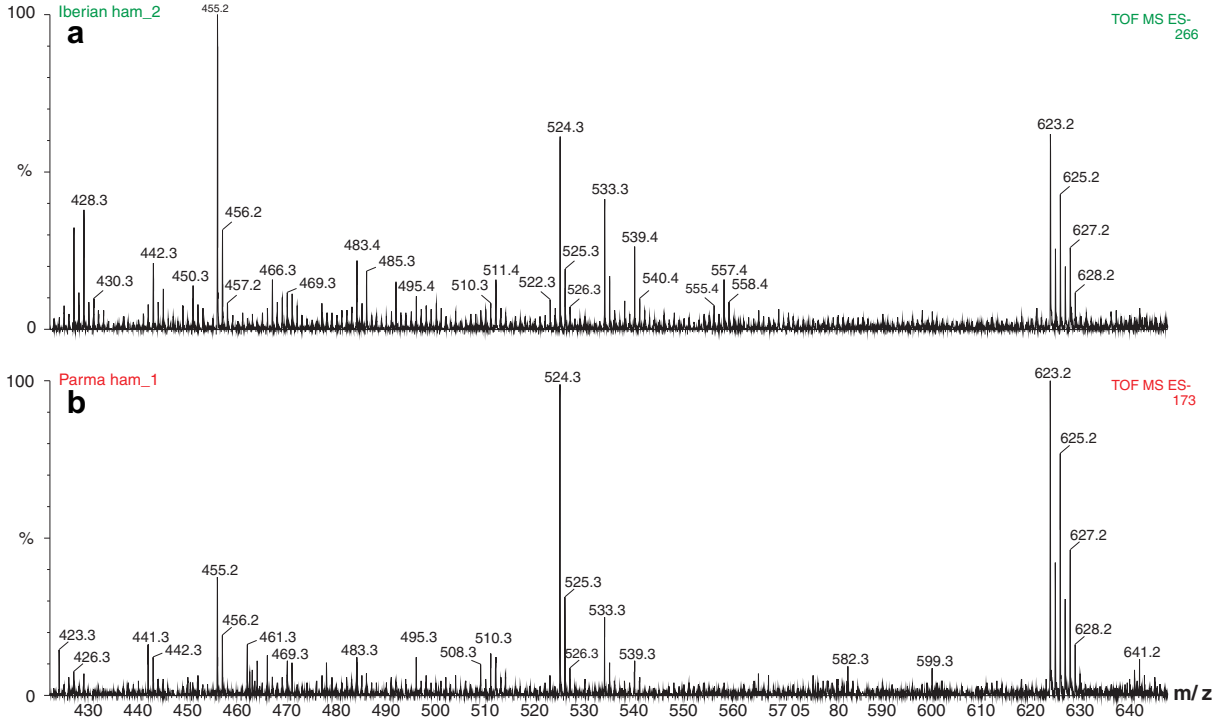

Fig. 4. Electrospray ionization mass spectra in the negative ion mode of dry-cured Iberian ham (a) and Parma ham (b) extracts in 75% acetone/water solution.

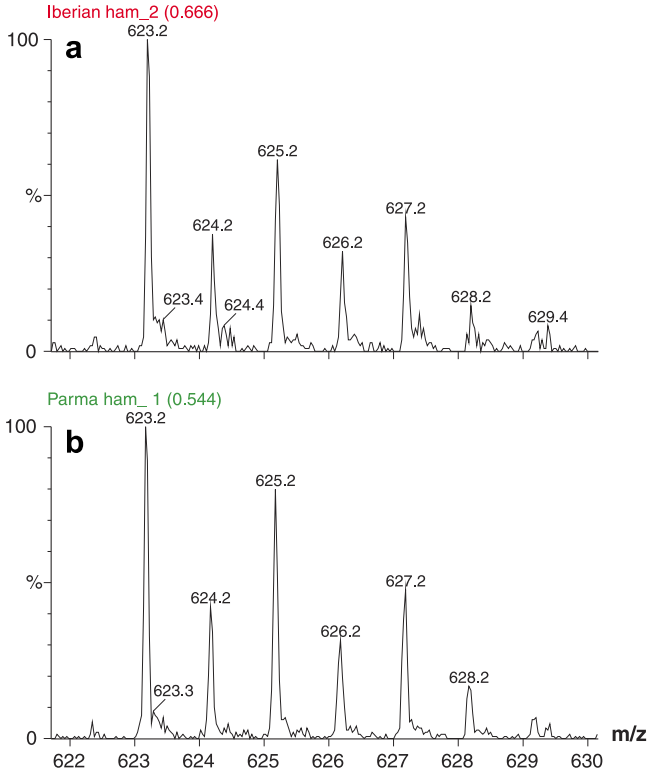

Fig. 5. Electrospray ionization mass spectra in the negative ion mode of dry-cured extracts Iberian ham (a) and Parma ham (b) in 75% acetone/water solution magnifying the range for *m*/*z* 622–630 showing a characteristic isotopologue distribution of Zn–porphyrin complex with the ion of *m*/*z* 623.2 as the major isotopologue.

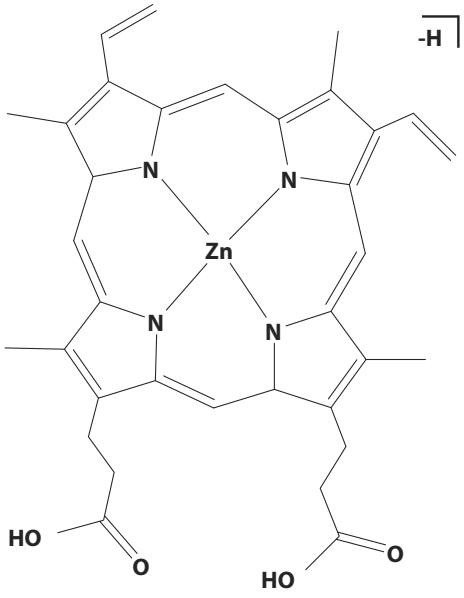

Fig. 6. Structure of negative ion of zinc–porphyrin complex with *m*/*z* of 623.26 for the most abundant ion.

A MS study identifying porphyrin derivatives in the urine of patients with deficiencies in the heme biosynthesis enzymes also found that ESI-MS/MS results mainly in cleavage of side-chains on methyl esters of various porphyrin structures (Danton & Lim, 2004). Likewise, free base alkyl derivatives of porphyrin have been shown mainly to generate methyl and ethyl fragments during atmospheric pressure chemical ionization (APCI) MS/MS investigations (Rosell-Mele, Carter, & Maxwell, 1999).

4. Discussion

The various instrumental analyses of the 75% acetone/water extracts of Parma ham and Iberian ham all point towards the presence of a Zn–porphyrin complex in relatively high amounts in the two types of dry-cured hams. This Zn–porphyrin complex is expected to be able to establish the unique red colour characteristic of these meat products. The patterns of the absorption spectra were similar to previous observations of Zn–porphyrin in polar solvents (ethanol:dimethylfuran) (Zaitoun, 2005), and also findings made during assays of the enzyme ferrochelatase capable of incorporating Zn into protoporphyrin confirm the spectral nature (Camadro & Labbe, 1982). The fluorescence emission peaks also correspond precisely with results reported several years ago in a study of Zn–porphyrin complexes in aqueous solution (Leonard et al., 1974).

Physiological and nutritional studies have investigated the interrelated metabolism of minerals such as Fe and Zn, and high Zn intake has been shown to affect Fe level in liver tissue of chicken (Pimentel, Greger, Cook, & Stahl, 1992). The formation and accumulation of Zn–porphyrin have been explored in human biology as an indicator of anaemia and iron status (Labbe & Dewanji, 2004; Cook, 2005). In context, the enzyme, ferrochelatase, has been found to compete for the substrates Fe and Zn in yeast cells, thus, the enzyme can also incorporate Zn into porphyrin (Camadro & Labbe, 1982). This activity of the enzyme may be operating during the prolonged production period of the dry-cured hams under investigation, and results obtained in model systems indicate that the Zn complex appears as a result of an endogenous enzyme-linked reaction (Wakamatsu, Okui, et al., 2004). Despite the unfavourable physicochemical conditions of reduced water activity and elevated concentrations of NaCl in dry-cured hams, numerous endogenous meat enzymes maintain activity during processing resulting in optimal flavour and texture development (Sentandreu & Toldra, 2001; Toldra, Flores, & Sanz, 1997). In effect, such harsh conditions may alter the specificity of some enzymes as known from industrial utilization (Balcao, Paiva, & Malcata, 1996), e.g. as immobilized enzymes combined with the use of organic solvents that can shift the equilibrium of certain enzyme-catalyzed reactions.

The mechanism by which the iron originally present as central iron atom in Mb is exchanged with Zn is still unknown. Previous studies using ESR spectroscopy performed directly on various dry-cured hams have shown a characteristic signal for $MbFe^{II}NO$, slightly modified in the protein conformation, indicating the presence of

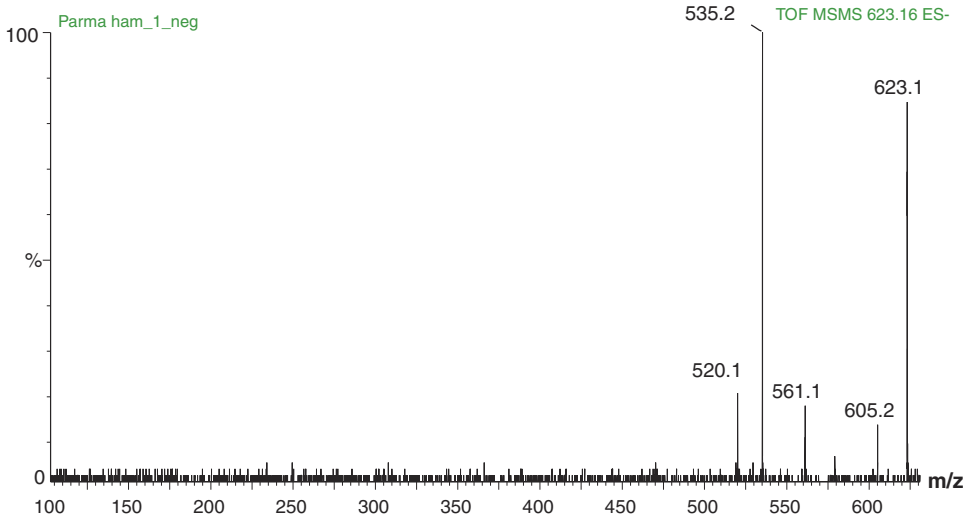

Fig. 7. Electrospray ionization tandem mass spectra of negative ions of m/z 623.2 from dry-cured Parma ham extracts in 75% acetone/water solution.

nitrosyl complexes in Spanish Serrano ham known to be produced with nitrite/nitrate, while ESR spectra of Parma ham showed that paramagnetic complexes were absent (Møller et al., 2003). The Zn–porphyrin content in different kinds of meat products including dry-cured and brine-cured meat products has now been shown to depend on the absence of added nitrate and nitrite, which apparently totally inhibit formation of Zn–porphyrin (Adamsen, Møller, Laursen, Olsen, & Skibsted, 2006). The present measurements of total content of Zn and Fe surprisingly find approximately 2 times more Fe than Zn in the purified extracts from the two types of dry-cured ham, which means that some degree of heme contribution to the observed UV–vis spectra cannot be ruled out. However, mass spectrometric analysis completely fails to detect either positive or negative ions indicating presence of heme having a M_w of 616.2 Da, thereby signifying that heme structures either are subject to CID during ionisation process or that Fe is present as free metal ions in the extracts.

In this context, it is noteworthy that red meats are a major source of both Fe and Zn in human nutrition (Higgs, 2000), and the content as an average of three different pork muscles of Zn relative to total Fe (heme-Fe and free Fe) in pork muscle tissue is as high as 3:1 (Hazell, 1982; Lombardi-Boccia, Lanzi, & Aguzzi, 2005). The daily intake of these minerals monitored in Italy gave an estimate of a 2:1 ratio for Zn:Fe originating from meat and meat products (Lombardi-Boccia, Aguzzi, Cappelloni, Di Lullo, & Lucarini, 2003). This remarkable high Zn:Fe ratio explains how an enzyme like ferrochelatase under the right conditions can be involved in the formation of Zn–porphyrin in dry-cured hams, although further knowledge is necessary with respect to enzyme activity at high salt concentration and low water activity in muscle tissue. The presently measured ratio of Zn:Fe in the purified ham extracts, which are surprisingly high in Fe relative to Zn could be due to uneven fractions of these metals being lost during the extraction process, e.g. in the precipitate.

The current findings could form the basis for production meat products without the use of additives like nitrite, which then allows a classification as organic processed meat providing that the breeding, rearing and slaughtering methods all meet the standards and regulations defined for such products. In conclusion, our results suggest that production of cured meat products without the use of additives such as nitrate or nitrite significantly alters the chemistry leading to colour formation. The herein presented ESI-MS results are supported by other spectroscopic techniques further confirming the presence of a Zn–porphyrin complex in the two types of dry-cured hams. These findings suggest a mutual mechanism of colour formation in various traditional dry-cured meat products for which only NaCl is used as additive. The absence of strong field ligands that normally bind and apparently block the central Fe atom in heme permits the central metal atom to be exchanged in porphyrin, which is the main chromophore in red-pigmented muscles. Zn–porphyrin is rather inert as the major red pigment in Iberian and Parma ham, and the association between this complex and the globin part of Mb is weakened throughout the process as indicated by extractions performed with either polar or apolar solvents (Møller et al., 2003), but currently the fate of the protein part of Mb is uncertain and calls for further investigation.

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References

Adamsen, C. E., Møller, J. K. S., Laursen, K., Olsen, K., & Skibsted, L. H. (2006). Zn–porphyrin formation in cured meat products. Effect of added salt and nitrite. *Meat Science, 72*(4), 672–679.

Aylett, B. J. (1975). The chemistry of zinc, cadmium and mercury. In: *Comprehensive Inorganic Chemistry* (pp. 187–308). Oxford: Pergamon Press Ltd.

Balcao, V. M., Paiva, A. L., & Malcata, F. X. (1996). Bioreactors with immobilized lipases: State of the art. *Enzyme and Microbial Technology, 18*(6), 392–416.

Camadro, J. M., & Labbe, P. (1982). Kinetic studies of ferrochelatase in yeast – zinc or iron as competing substrates. *Biochimica et Biophysica Acta, 707*(2), 280–288.

Castelfranco, P. A., Weinstein, J. D., Schwarcz, S., Pardo, A. D., & Wezelman, B. E. (1979). Mg insertion step in chlorophyll biosynthesis. *Archives of Biochemistry and Biophysics, 192*(2), 592–598.

Cook, J. D. (2005). Diagnosis and management of iron-deficiency anaemia. *Best Practice & Research Clinical Haematology, 18*(2), 319–332.

Danton, M., & Lim, C. K. (2004). Identification of monovinyl tripropionic acid porphyrins and metabolites from faeces of patients with hereditary coproporphyria by high-performance liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry. *Rapid Communications in Mass Spectrometry, 18*(22), 2779.

Dirinck, P., Van Opstaele, F., & Vandedriessche, F. (1997). Flavour differences between northern and southern European cured hams. *Food Chemistry, 59*(4), 511–521.

Duprat, A. F., Traylor, T. G., Wu, G. Z., Coletta, M., Sharma, V. S., Walda, K. N., et al. (1995). Myoglobin NO at low pH – free 4-coordinated heme in the protein pocket. *Biochemistry, 34*(8), 2634–2644.

Flores, J. (1997). Mediterranean vs northern European meat products. Processing technologies and main differences. *Food Chemistry, 59*(4), 505–510.

Gonzalez, C. B., & Ockerman, H. W. (2000). Dry-cured Mediterranean hams: long process, slow changes and high quality: a review. *Journal of Muscle Foods, 11*(1), 1–17.

Hazell, T. (1982). Iron and zinc compounds in the muscle meats of beef, lamb, pork and chicken. *Journal of the Science of Food and Agriculture, 33*(10), 1049–1056.

Higgs, J. D. (2000). The changing nature of red meat: 20 years of improving nutritional quality. *Trends in Food Science & Technology, 11*(3), 85–95.

Labbe, R. F., & Dewanji, A. (2004). Iron assessment tests: Transferrin receptor vis-a-vis zinc protoporphyrin. *Clinical Biochemistry, 37*(3), 165–174.

Leonard, J. J., Yonetani, T., & Callis, J. B. (1974). Fluorescence study of hybrid hemoglobins containing free base and zinc protoporphyrin-IX. *Biochemistry, 13*(7), 1460–1464.

Lombardi-Boccia, G., Aguzzi, A., Cappelloni, M., Di Lullo, G., & Lucarini, M. (2003). Total-diet study: dietary intakes of macro elements and trace elements in Italy. *British Journal of Nutrition, 90*(6), 1117–1121.

Lombardi-Boccia, G., Lanzi, S., & Aguzzi, A. (2005). Aspects of meat quality: trace elements and B vitamins in raw and cooked meats. *Journal of Food Composition and Analysis, 18*(1), 39–46.

Masuda, T., Inoue, K., Masuda, M., Nagayama, M., Tamaki, A., Ohta, H., et al. (1999). Magnesium insertion by magnesium chelatase in the biosynthesis of zinc bacteriochlorophyll a in an aerobic acidophilic bacterium *Acidiphilium rubrum*. *Journal of Biological Chemistry, 274*(47), 33594–33600.

Møller, J. K. S., Adamsen, C. E., & Skibsted, L. H. (2003). Spectral characterisation of red pigment in Italian-type dry-cured ham. Increasing lipophilicity during processing and maturation. *European Food Research and Technology, 216*(4), 290–296.

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.

Parolari, G. (1996). Achievements, needs and perspectives in dry-cured ham technology: the example of Parma ham. *Food Science and Technology International, 2*(2), 69–78.

Parolari, G., Chizzolini, R., Bellatti, M., & Dazzi, G. (1983). Physical and chemical changes occurring in proteins during the maturation of Parma ham. II. – Colour. *Industria Conserve, 58*, 147–149.

Parolari, G., Gabba, L., & Saccani, G. (2003). Extraction properties and absorption spectra of dry cured hams made with and without nitrate. *Meat Science, 64*(4), 483–490.

Pimentel, J. L., Greger, J. L., Cook, M. E., & Stahl, J. L. (1992). Iron-metabolism in chicks fed various levels of zinc and copper. *Journal of Nutritional Biochemistry, 3*(3), 140–145.

Rosell-Mele, A., Carter, J. F., & Maxwell, J. R. (1999). Liquid chromatography tandem mass spectrometry of free base alkyl porphyrins for the characterization of the macrocyclic substituents in components of complex mixtures. *Rapid Communications in Mass Spectrometry, 13*(7), 568–573.

Sentandreu, M. A., & Toldra, F. (2001). Dipeptidyl peptidase activities along the processing of Serrano dry-cured ham. *European Food Research and Technology, 213*(2), 83–87.

Toldra, F., Flores, M., & Sanz, Y. (1997). Dry-cured ham flavour: enzymatic generation and process influence. *Food Chemistry, 59*(4), 523–530.

Toldra, F., & Navarro, J. L. (2000). Improved traditional foods for the next century - Foreword and highlights. *Food Research International, 33*(3–4), 145.

Wakamatsu, J., Nishimura, T., & Hattori, A. (2004). A Zn–porphyrin complex contributes to bright red color in Parma ham. *Meat Science, 67*(1), 95–100.

Wakamatsu, J., Okui, J., Ikeda, Y., Nishimura, T., & Hattori, A. (2004). 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.

Zaitoun, M. A. (2005). Spectroscopic study of protoporphyrin IX zinc(II) encapsulated in sol–gel glass. *Spectrochimica Acta Part A-Molecular and Biomolecular Spectroscopy, 61*(8), 1715–1719.