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ORIGINAL PAPER

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Spectral characterisation of red pigment in Italian-type dry-cured ham. Increasing lipophilicity during processing and maturation

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Abstract Spectroscopic studies of Parma ham during processing revealed a gradual transformation of muscle myoglobin, initiated by salting and continuing during ageing. Electron spin resonance spectra did, however, conclusively show that the pigment in dry-cured Parma ham at no stage is a nitrosyl complex of ferrous myoglobin as found in brine-cured ham and Spanish Serrano hams. Both near-infra red reflectance spectra of sliced ham and UV/visible absorption spectra of extract of hams, obtained with aqueous buffer or acetone, showed the presence of different red pigments at varying processing stages for both solvents. Especially, the pigment extracted with aqueous buffer exhibited unique spectral features different from those of well-known myoglobin derivatives. At the end of processing, the pigment(s) becomes less water extractable, while the fraction of red pigment(s) extractable with acetone/water (75%/25%) increases throughout the processing time up to full maturation at 18 months. The chemical identity of the 6th ligand of myoglobin could not be conclusively established, but possible candidates are discussed. The partition of the pigment(s) between pentane and acetone/water showed a strong preference for pentane, suggesting that only the heme moiety is present in the acetone/water extract, and that Parma ham pigment is gradually transformed from a myoglobin derivative into a non-protein heme complex, which was found to be thermally stable in acetone/water solution

Keywords Colour · Red myoglobin derivatives · Dry-curing · Parma ham, · ESR spectroscopy

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Introduction

Raw, dry-cured ham is a meat product with high added value originating from the Mediterranean region of Europe [1]. One Italian variant, known as Parma ham, is produced using salt as the only additive, and is renowned throughout the world for its flavour, aroma and superior palatability. Other variants of raw, dry-cured ham have added nitrite or nitrate during processing, while for Parma ham nitrate was banned in 1993 [2]. The added sodium chloride facilitates the high degree of dehydration occurring during the prolonged period of processing and provides the salty taste to the matured meat product.

The colour of Parma ham changes during processing from the initial bright red of raw muscle to a darker red colour characteristic for the matured meat product after approximately 18 months of processing. Due to the high degree of dehydration, myoglobin will, like other components, be more concentrated in the muscle, thereby yielding higher colour intensity. A study of the changes in the red pigment(s) occurring during processing of Parma ham showed distinct spectral changes in the UV/visible range for pigments extracted with aqueous buffer [3]. A more recent study has shown that a fraction of the pigments in Parma ham extractable with aqueous buffer is stable toward oxidation by the strong oxidising agent ferricyanide and also is rather stable toward (thermal) autoxidation [4]. Previously, the myoglobin derivative present in matured Parma ham has been suggested to be nitrosylated myoglobin, which has been speculated to be formed by microbial activity in the ham during the prolonged process [5]. Several bacterial strains of relevance to meat have been found to modify the chemical structure of myoglobin, either due to their general reducing capacity [6] or by the specific formation of nitric oxide [7, 8], which is a key intermediate formed during curing with nitrite and ascorbate as additives. Additional aspects of meat curing and microbial metabolism involving NO have recently been covered in a review of the interactions between myoglobin and NO [9]. Microbial activity is most likely to play a role in the

flavour development during processing of Parma ham [10], but whether this microbial action also contributes to the development of a uniform, stable dark red colour in the ham during processing is doubtful, since microorganisms are mainly situated at the surface of the meat during the process.

In general, studies dealing with colour development in Parma ham are limited, but attempts have been made to correlate the colour of " green" hams to the final colour of fully matured Parma hams by combining sensory and instrumental colour evaluation [11]. The specific muscle investigated greatly affects the colour attributes, and *semi membranous* have been found to differ from both *biceps femoris* and *semi tendinosus* in both Parma ham [11], and in Spanish dry-cured ham [12]. Furthermore, no differences with respect to colour intensity or stability could be observed for Parma ham manufactured from pork supplemented with vitamin E in comparison to pork from a normal feeding regime [13].

It is well established that both lipolysis [14, 15] and proteolysis [16, 17, 18] occur during processing of dry-cured ham, which results in increasing amounts of free fatty acids [19], peptides and free amino acids [18, 20]. Lipid oxidation during processing has been shown to increase to a certain level at the intermediate stages of processing, but the markers for rancidity and lipid oxidation decreases significantly in the final product [21]. This may signify either that lipid oxidation is halted at a certain point of processing due to formation of antioxidative compounds or alternatively that lipid oxidation products disappear by further reaction at the end of processing. Small peptides and certain free amino acids will directly affect the taste of dry-cured ham [22], and are furthermore precursors for volatile compounds that establish the flavour of the final product [10]. In light of the chemical changes observed in the protein and lipid fraction of Parma ham during processing, it is surprising how the red colour develops and becomes rather stable in the matured product.

In the present study, various spectroscopic studies of pigments isolated from Parma ham were undertaken in order to investigate and further elucidate the chemical nature and transformations of the red pigments formed in Italian dry-cured Parma ham during processing.

Methods and materials

Chemicals

$Na_2HPO_4{\cdot}2H_2O$ and $NaH_2PO_4{\cdot}H_2O$, hydrogen peroxide (30%), nitric acid (65%), sulphuric acid (95–97%), sodium ascorbate, sodium nitrite and an iron standard (Tritisol, 1.000 g/L) were all obtained from Merck (Darmstadt, Germany), and acetone and pentane were obtained from Lab-Scan (Dublin, Ireland). All chemicals were of analytical grade. Water was purified through a Millipore Q-Plus purification train (Millipore, Bedford, MA, USA). Metmyoglobin (MbFe(III), horse heart, Type III) was from Sigma (St. Louis, MO, USA).

Meat samples

Parma ham was obtained from Stazione Sperimentale per ÍIndustria delle Conserve Alimentari, (Parma, Italy) and included samples from 5 processing stages: post-salting (3 months old) and 3 samples at intermediate maturation stages (11, 12 and 15 months of processing), and finally Parma ham at the end of process (18 months old). In addition, raw pork leg was purchased locally together with commercial samples of Spanish Serrano ham and Danish cooked, brine-cured ham. Either a 0.20 M phosphate buffer (pH adjusted to 6.0 with 1.0 M NaOH) or a 75% acetone/water solution was used to extract myoglobin derivatives from raw pork ham and the five Parma ham samples.

ESR spectroscopy

Samples of whole pieces of meat (0.25 g) or of 0.30 mL of liquid extract were transferred to an ESR capillary tube (Wilmad Glass, Bueno, NJ, USA) and frozen by submerging the tube in liquid nitrogen. Other meat products, e.g. dry-cured Spanish Serrano ham and cooked, brine-cured ham, and solutions of MbFe(II)NO in buffer, which all posses a characteristic ESR signal for MbFe(II)-NO, were included in the experiment as references. MbFe(II)NO was synthesised from MbFe(III), sodium ascorbate and sodium nitrite and purified as previously described [23].

ESR capillary tubes were placed in a Bruker ECS 106 spectrometer equipped with a Bruker 4103 TM/9216 rectangular cavity (Bruker, Rheinstetten, Germany). The temperature inside the cavity was held at 150 K by a Bruker ER 4112 HV continuous-flow liquid nitrogen cryostat. The microwave frequency was measured with a HP 5350B microwave frequency counter (Hewlett-Packard, Palo Alto, CA, USA). The magnetic field was modulated with a frequency of 100 kHz using 5 Gauss field modulation amplitude, 2 mW microwave power and microwave frequencies were in all experiments between 9.418–9.426 GHz. ESR spectra were recorded as a single scan with a conversion time of 40.96 msec and a time constant of 372.68 msec. In order to both accommodate signals from high spin ferric MbFe(III) and nitrosyl complexes of MbFe(II) two scan ranges were employed with a centre field of 2600 G and a sweep width of 3400 G for high spin ferric myoglobin, while details of MbFe(II)NO was probed using a centre field of 3325 G and a sweep width of 225 G. A standard, strong pitch, with a known g-value, was measured each day as a standard in order to calculate g-values for the experimentally observed signals.

Visible/near-infra red (VIS/NIR) reflectance spectroscopy

Prior to pigment extraction samples were subjected to VIS/NIR reflectance spectroscopy (model 6500, NIRSystems, Silver Springs, MD) using a " spinning cup" measuring cell. The spectrophotometer was equipped with a split detector system applying a silicon detector between 400 and 1100 nm and a lead sulphide detector between 1100 and 2500 nm. The angle of incident light was 90° with reflectance measured at a 45° angle. A meat sample of approximately 3.5 cm in diameter and 1 cm in thickness was fitted into the cup and the surface reflectance (R) measured, which was converted by log 1/R.

Extraction with buffer

Lean meat (10 g), taken either from *biceps femoris* or *semi membranosus*, was trimmed for any visible fat, chopped very fine and homogenised with 90 g of phosphate buffer using a Ultra Turrex homogeniser (Janke & Kunkel, Kika Labortechnik, Staufen, Germany) at 15 000 rpm for 1 min. Subsequently, the mixture was separated by centrifugation (2 °C, 15 000 rpm for 15 min). For supernatants still turbid after the first centrifugation, filtration through S&S 589[2] filter paper (Dassel, Germany) was employed followed by centrifugation (2 °C, 15 000 rpm for 15 min). Finally,

the extract was filtered through MiniSart filter with φ=0.45 μm (Sartorius, Goettingen, Germany) and subsequently purified on a Sephadex PD10 column (Pharmacia Biotech, Uppsala, Sweden), which had been equilibrated with cold (5 °C) phosphate buffer. The extract was used for measurements within 2 h during which it was kept in the dark and on ice.

Extraction with acetone/water and pentane

The method described by Hornsey [24] for isolation of nitrosylmyoglobin in nitrite brine-cured meat products was used slightly modified. Lean meat (10 g) was homogenised together with 75% acetone/water solution (90 g) for 1 min at 15 000 rpm. Subsequently, the slurry was filtered through S&S 589[2] filter paper, after which the filtrate was kept on ice in the dark and used for measurements within 2 h. Acetone/water fraction was washed with pentane (2:1 v/v) for separation as described below and the pentane phase was used for further measurements.

Pentane-acetone partition coefficient

The partition coefficient between pentane and aqueous acetone (K_{pen}) for the fraction of Parma ham pigment extracted with acetone/water was determined spectrophotometrically (Soret band) and from the iron concentration in the two phases. Equal volumes of the acetone/water fraction and pentane were mixed in a separatory funnel. For a better separation of the two fractions, 4% (v/v) of saturated aqueous sodium chloride was added to the separatory funnel.

Following separation, absorption spectra were recorded for the pentane and the acetone/water phase as described below. Subsequently, the iron concentrations in the pentane phase and in the acetone/water phase were determined by atomic adsorption spectroscopy (AAS) as described below.

Partition coefficient (K_{pen}) was estimated both as the ratio of the absorbance at the maximum of the Soret band (λ=418 nm) for the two phases and as the ratio between the iron concentration in the two phases.

UV-VIS absorption spectrophotometry

A Hewlett-Packard 8452A UV-VIS diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA) equipped with a thermostatted cell compartment with magnetic stirring was used to record spectra in the range 350<λ<650 nm with a spectral resolution of 2 nm of the various kinds of extracts recovered from the meat samples.

Iron determination in extracts

In order to establish the molar extinction coefficients for the various pigments, iron was determined following extraction or partition by AAS using a Spectra AA-200 instrument (Varian, NSW, Australia). Parma ham pigment extracted with buffer was determined by direct injection to the AAS.

Extracts of Parma ham pigment in acetone/water or pentane underwent destruction in nitric acid before measurement. Nitric acid (7.5 mL) was mixed with 5.0 mL of the extract or solution from partition in a 500 mL Erlenmeyer flask. Each flask was left at room temperature overnight for digestion. The flasks were subsequently placed on a hot plate and evaporated until dryness. Hydrogen peroxide–sulphuric acid reagent, prepared according to [25], was added in 1.0 mL aliquots to each sample until the solution became clear. The flaks were left on the hot plate until white vapours of sulphuric acid appeared (approximately 10 min). After cooling, the digest was quantitatively transferred to a 10.0 mL volumetric flask and diluted with 0.100 M HCl. The solutions were analysed for iron using AAS. The iron concentration was estimated from a standard curve prepared from an iron standard (1.000 g/L, Titrisol, Merck) diluted with 0.100 M HCl to yield: 0.100, 0.300, 0.500, 1.00, 1.50 and 2.50 ppm iron.

Results and discussion

ESR spectra were recorded under experimental conditions that enabled the detection and identification of low amounts of either nitrosylmyoglobin, MbFe(II)NO ($NO^{\cdot}$, s=1/2), or the low-field portion of high-spin aquametmyoglobin, $MbFe(III)OH_2$ (Fe(III), s=1/2) [26]. No ESR signal for high-spin ferric myoglobin could be detected in the low-field region in any of the investigated samples (results not shown), and as may be seen from the ESR spectra shown in Fig. 1, no signal for a NO complex was detected in Parma ham (spectrum d), indicating that both MbFe(II)NO and high spin MbFe(III) are absent in matured Parma ham. Signals originating from the paramagnetic ligand NO were present in the ESR spectra of phosphate solution of pure 0.20 mM MbFe(II)NO frozen in liquid nitrogen to form a glass (spectrum a). Serrano dry-cured ham (spectrum b) or cooked, brine-cured ham (spectrum c) also showed the characteristic ESR signal of the NO complex of myoglobin. ESR spectra of cooked, brine-cured ham, dry-cured Serrano ham and pure MbFe(II)NO solution all exhibit hyperfine splitting into a triplet yielding three corresponding g-values in the g_z region originating from NO ligation and indicating the

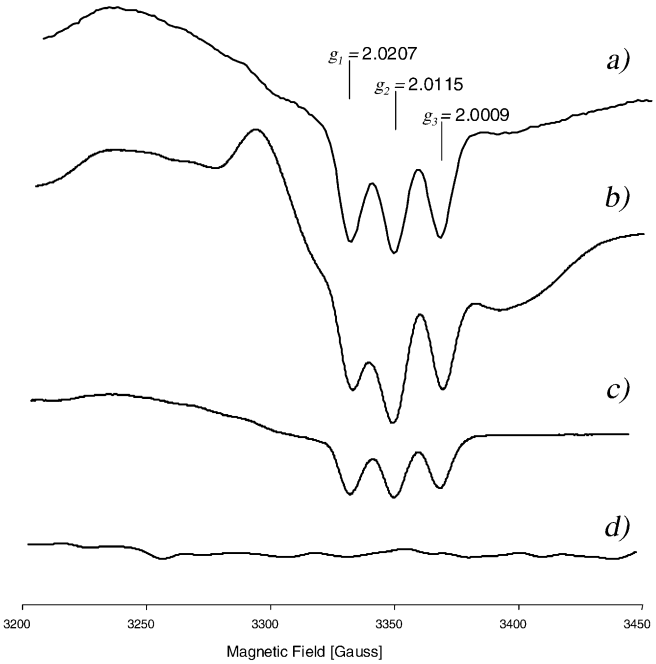

Fig. 1 Electron spin resonance spectra of dry-cured meat products (Parma and Serrano ham), cooked brine-cured ham and pure nitrosylmyoglobin recorded at 150 K. Spectrum *a* Nitrosylmyoglobin (0.20 mM), *b* Serrano ham (nitrate added during curing), *c* Cooked, brine-cured ham (60 ppm nitrite added during curing) and *d* Parma ham (only NaCl added)

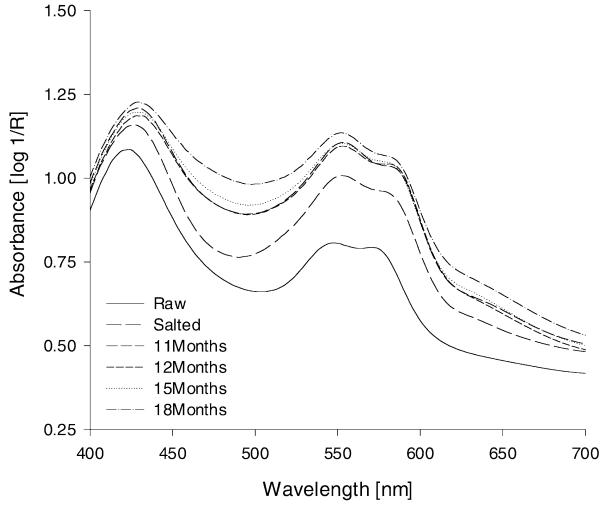

Fig. 2 Visible region of visible/near-infra red reflectance spectra of raw pork leg, salted ham and dry-cured Parma ham at 4 different processing stages recorded at 25 °C using a "spinning cup" cell (sampling area=12.5 cm^2)

presence of a penta-coordinated heme NO complex. The minor variations in these spectra can be attributed to differences in protein conformation or pigment concentration, and to a different degree of hydration, which is higher for cooked, brine-cured ham compared to dry-cured Serrano ham. Likewise, substantial differences in low-temperature ESR spectra of MbFe(II)NO in either raw or heat-treated bacon have previously been reported [27]. It is accordingly safe to conclude from the ESR spectra that the myoglobin derivative present in fully matured Parma ham is not a nitrosylated form of myoglobin, as has been suggested to be formed as a result of microbial activity [5]. It should also be noted that if bacteria were responsible for colour formation, the colour hue and intensity in the centre of the ham most likely would be different from the hue and intensity at the surface zone, in contrast to what is observed for Parma ham. Given the increasing amounts of salt and reduced moisture content during processing of dry-cured hams, the formation of hemichrome (a denatured low-spin form of ferric myoglobin) could occur, in which the 6th ligand most likely is histidine or another amino acid from globin, e.g. methionine. These inactivated complexes of myoglobin are paramagnetic and thus detectable by ESR spectroscopy. The applied temperature is especially critical and in order to obtain a signal it should be at least 77 K or below [28]. Therefore, based on the present ESR experiments, it cannot be ruled out that hemichrome is absent in matured Parma ham. However, as will be observed later, the absorption spectral characteristics of pigments isolated from Parma ham show no resemblance to purified hemichromes [29].

Reflectance spectra of raw meat and Parma ham at various stages of processing were measured in the wavelength range 400–2500 nm. Figure 2 shows absorbance (log 1/R) spectra between 400–700 nm, the spectral region in which myoglobin and heme absorb light with the three distinct bands, i.e. the high intensity Soret band and, at higher wavelengths, the so called β– and α–bands. The spectral changes occurring as the ham undergoes maturation are characterised by an increase in intensity of the bands between 500 and 600 nm, corresponding to an increase in the concentration of red pigment and evident as an increase in sample redness. More interestingly, the peak of the Soret band in the near-UV region is shifted from 422 nm in raw meat to 428 nm in salted Parma ham and finally to 430 nm for mature Parma ham. Similar changes were observed for the most intense absorption (the β–band), which shifts from 546 nm in raw meat to 552 nm in both salted and mature Parma ham. These spectral changes indicate a change from a predominance of oxymyoglobin ($MbFe(II)O_2$) in raw meat towards a predominance of a pigment having some spectral properties similar to those of deoxymyoglobin (MbFe(II)) in mature Parma ham. Absorption maxima for the Soret band and the β–band are at 434 and 555 nm for MbFe(II), respectively, and at 418 and 546 nm for $MbFe(II)O_2$, respectively [30]. The Soret band, as measured by VIS/NIR reflectance spectroscopy, confirms that the predominant myoglobin derivative changes during maturation of Parma ham. However, based on these measurements, we were not able to identify the myoglobin species present in mature Parma ham, but the measurements do confirm that the pigment is not MbFe(II)NO, since the Soret band has a maximum of 421 nm and the β–band absorbs at 548 for MbFe(II)NO.

Determination of iron in ham extracts (obtained using aqueous buffer or acetone/water, and in pentane used for partition of pigment between acetone/water and pentane in each case), in combination with absorption spectra of the solution, allowed the determination of the molar extinction coefficient for the various fractions of the pigment. Table 1 summarises spectral properties and molar extinction coefficients for various myoglobin derivatives in relation to the values found in the present study for aqueous buffer and acetone/water extracts, or for pentane used for partition of pigment between acetone/water and pentane. It should be noted that aqueous extract of Parma ham pigment was purified by gel filtration through a column prior to spectral measurements of iron, while the acetone/water and pentane extracts were analysed without further treatment. This means that extinction coefficients for the aqueous extract are exact, whereas the values obtained in apolar solvent are qualitative as small amounts of free iron may contribute, although this type of extract is used as a standard method for determination of heme iron.

Figure 3 shows absorption spectra of pigments from raw pork and Parma ham at various processing stages using aqueous buffer for extraction. As observed for VIS/NIR reflectance spectroscopy, the visible adsorption spectrum of pigment extracted with aqueous buffer from raw pork is similar to the aqueous spectrum of $MbFe(II)O_2$, whereas the spectra of extract from Parma ham are red-shifted both with respect to the Soret band

Table 1 Spectral characteristics of myoglobin derivatives and red pigments isolated from matured Parma ham by aqueous phosphate buffer or acetone/water extraction

Myoglobin derivative	Absorption maxima		
	Soret: ε (M^{-1} cm^{-1})	Visible bands	
		β: ε (M^{-1} cm^{-1})	α: ε (M^{-1} cm^{-1})
Deoxymyoglobin[a]	434 nm–113 200	555 nm–11 900	
Oxymyoglobin[a]	418 nm–136 000	544 nm–14 400	582 nm–15 100
Metmyoglobin[a]	410 nm–163 900	505 nm–10 200	630 nm–3 900
Nitrosylmyoglobin[a]	421 nm–146 800	548 nm–12 800	580 nm–11 600
Carboxymyoglobin[b]	419 nm–207 000	540 nm–15 400	579 nm–13 900
Mature Parma ham extracted with aqueous buffer[c, d]	424 nm–152 100	550 nm–19 300	586 nm–10 800
Mature Parma ham extracted with acetone/water[d]	416 nm–199 200	546 nm–23 300	584 nm–22 200
Mature Parma ham pentane separated phase[d]	418 nm–135 300	548 nm–9 900	582 nm–10 100

[a] From Millar et al. [30]
[b] From Antonini and Brunori [34]
[c] Spectrum from buffer-extracted Parma ham was baseline corrected by subtracting background $A_{650\ nm}$=0.19
[d] Dielectric constants of solvent at 20 °C from Weast [39]: water: 80.1; acetone: 21.2; pentane: 1.84

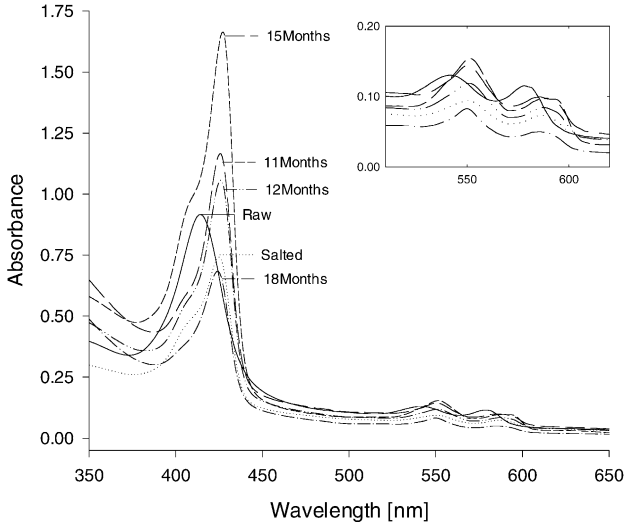

Fig. 3 UV/visible spectra of pigments extracted from raw pork leg, salted ham and dry-cured Parma ham at 4 different processing stages by 0.20 M aqueous phosphate buffer obtained within 2 h of extraction and recorded at 25 °C. *Inset* shows β– and α–bands in detail

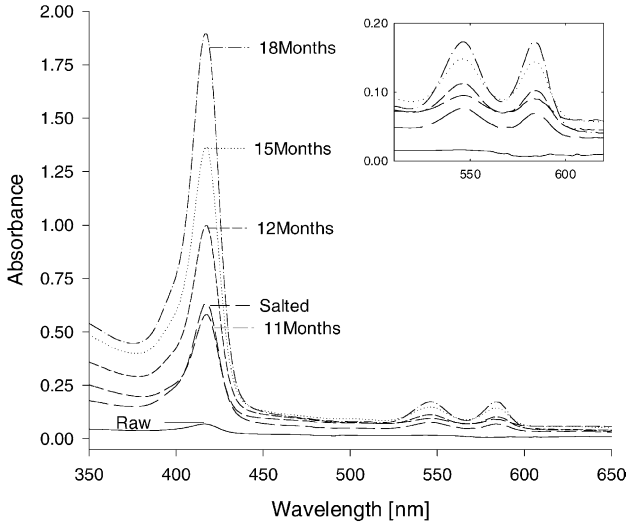

Fig. 4 UV/visible spectra of acetone/water (75%/25%) extracts of pigments from raw pork leg, salted ham and dry-cured Parma ham at 4 different processing stages obtained within 2 h of extraction and recorded at 25 °C temperature. *Inset* shows β– and α–bands in detail

and the visible bands. All Parma ham samples between 3 and 15 months exhibit a shoulder on the high-energy side of the Soret band, which indicates that at least two myoglobin derivates are found in the mixture. Furthermore, spectra of Parma ham pigment show a deformation of the β– and α–bands, compared to the spectrum of $MbFe(II)O_2$, resulting in shift towards red at 542 nm and a decrease in the intensity at 580 nm. Both findings are in agreement with previous observations for this wavelength region [3, 4]. The spectral changes observed both for Parma ham samples using VIS/NIR reflectance and for pigment extracted by aqueous buffer using UV/visible spectroscopy are thus in mutual agreement, and the small variation in the positions of the Soret and visible bands can be accounted for by differences in the environment in the meat matrix compared to aqueous buffer and the acetone/water solvent.

The absorption spectra of meat extracts using acetone/water in Fig. 4 show adsorption maxima corresponding to a bright red pigment. In contrast to the changes seen for spectra originating from samples extracted with aqueous buffer, no spectral changes relating to peak position can be observed in the pigments extracted with acetone/water from Parma ham at various stages of processing. The spectral differences between the red pigment in the two types of extracts obtained during processing of Parma ham, suggest that the concentration of the water-soluble pigment increases to a maximal level around 11–12 months of processing, and thereafter diminishes. The pigment extractable with acetone/water, on the other hand, is practically absent in the raw pork, but increases

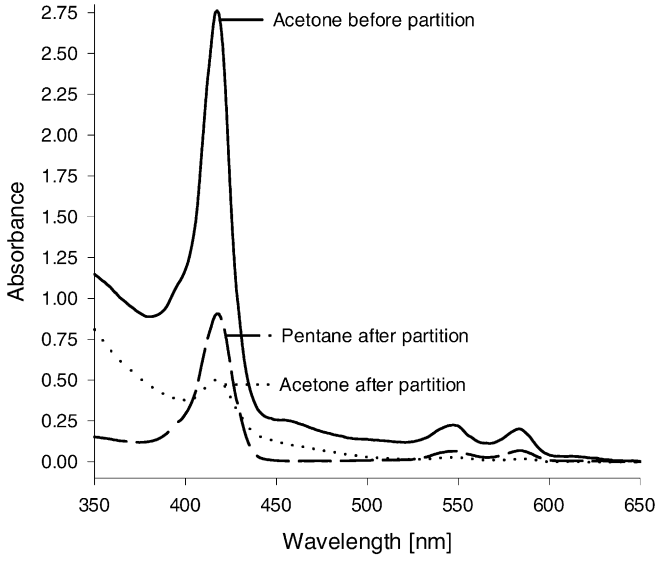

Fig. 5 UV/visible spectra of acetone/water (75%/25%) extracts of pigments from mature, dry-cured Parma (18 months) followed by separation into 2:1 (v/v) pentane added aqueous 4% NaCl

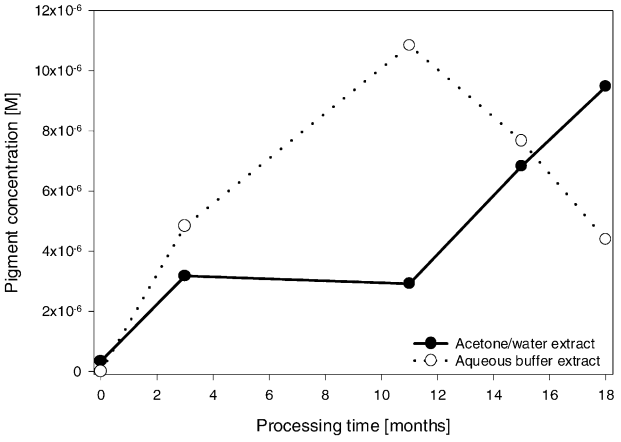

Fig. 6 Changes in concentration of red pigments present in aqueous buffer and acetone/water extract of dry-cured Parma ham during the course of processing. Samples for 12 months maturation are omitted from the plot for purpose of clarity. Estimation of concentration is based on Soret band absorbance and extinction coefficients (see Table 1) for the two respective Parma ham extracts

throughout the processing of Parma ham. Based on these observations for the two types of extracts, it is suggested that the extract obtained using aqueous buffer contains a red myoglobin derivative with a so-far-unidentified ligand bound to the central iron atom.

The pigment extractable with acetone/water is different from the pigment extracted with aqueous buffer and, based on the pigment distribution between pentane and acetone/water observed for the acetone/water extract, it seems likely that the pigment extracted with acetone/water is the heme moiety from which the globin has been dissociated. Heme is poorly soluble in polar solvent, but will be extracted by more apolar solvents such as acetone/water and will subsequently be transferred to pentane (see Fig. 5). Correspondingly, Möhler noted that only the heme–NO moiety was extracted by acetone from cooked, brine-cured ham, and accordingly the globin has been detached [31]. It is further known that acetone and other methylketones cause cleavage of the heme-protein linkage [32, 33]. The small amount extractable with acetone/water from raw pork leg and the increasing concentration found in salted Parma ham indicates that the level of free heme or the extractability from myoglobin increases significantly during salting and maturing of Parma ham. The affinity for heme to apomyoglobin is normally very high with an equilibrium constant of 10^{12}–10^{15} M^{-1} [34]. This constant will be greatly affected by conformational changes in the protein [35], which is likely to take place as myoglobin is exposed to high salt concentration and dehydration during processing of Parma ham. This is also in accordance with the present finding of decreasing amounts of pigment in aqueous buffer and an increasing amount of pigment in acetone/water extracts, indicative of a change toward a less water-soluble, more hydrophobic pigment during processing. The time course of the transformation of the pigment in Parma ham during processing from a water-extractable to an acetone-extractable has been visualised in Fig. 6, in which pigment in aqueous extracts from raw meat is estimated to be absent, based on the fact that spectra for extracts from raw meat and salted or matured Parma ham show no resemblance.

The partition coefficient between acetone/water and pentane (Eq. 1) determined for the pigments extracted with acetone/water was found to have the value K_{pen}=1.76±0.11 (n=4) based on the visible absorption spectra. Based on the iron measurements for the two phases, the value is 0.58±0.07 (n=4). One possible explanation for this difference is that more than one pigment is present in the extracts, and that these pigments have different lipophilicity and absorption spectra. The pigment may also undergo some degradation during extraction and the subsequent partition experiment, resulting in iron dissociation from the pigment. The iron ions will stay in the acetone/water phase, in effect decreasing the value of the apparent K_{pen}.

$$K_{pen} = \frac{[\text{Mb/heme}]_{\text{pentane}}}{[\text{Mb/heme}]_{\text{acetone/water}}}$$

The reactivity of the Parma ham pigments, extracted with aqueous buffer or acetone/water, towards oxygen was found to be very low. In a kinetic experiment, the reaction of the pigment with atmospheric oxygen was measured spectrophotometrically at room temperature. The pigment extracted with aqueous buffer precipitated within 1 h and hampered evaluation of spectral changes. The pigment in extracts made with acetone/water was found to be stable for more than 24 h at room temperature. In a study using aqueous extracts of Parma ham, the pigment was found to be stable towards autoxidation for 7 days and was not oxidised by ferricyanide [4]. Ferricyanide readily causes

oxidation of both MbFe(II)O_2 and MbFe(II)NO to MbFe(III) [36], whereas MbFe(II) also is readily oxidised [37].

In summary, VIS/NIR spectra of sliced Parma ham together with absorption spectra of extracted pigment suggested that a low-spin ferrous iron complex of myoglobin or heme is formed in Parma ham during processing. However, the oxidative stability of pigment fractions makes it more likely that the red colour of Parma ham originates from a ferric complex with an unidentified ligand coordinated to Fe(III), as also previous suggested by Parolari et al. [3]. High-field ligands such as CN^- or N_3^- have been shown to form complexes with ferric myoglobin having Soret band peaks at approximately 422 nm [38], which corresponds well to the observed Soret band for the pigment extracted from Parma ham with aqueous buffer. In addition, the alkaline form and the azide complex of ferric myoglobin exhibit absorption maxima (at 540 and 570/585 nm, respectively) in the visible region, which will yield a reddish colour [34]. In Parma ham, a myriad of low molecular weight chemical components of either lipid or protein origin will gradually be formed, and some of these are potential ligands for myoglobin. Certain free amino acids are likely candidates as the 6th ligand of myoglobin in Parma ham, as either sulphur-containing or basic amino acids have chemical groups capable of donating electrons in the bond formation with the central Fe atom. Furthermore, the relatively high salt concentration together with the high degree of dehydration may denature the protein moiety, which may in turn facilitate the movement and binding of more bulky ligands from the distal side in the heme pocket. We are currently investigating such complex formation.

Conclusion

ESR spectroscopy rules out the possibility that a nitrosylated ferrous iron complex of myoglobin is the major pigment in mature Parma ham. Other spectral evidence further shows that the oxygenated form of myoglobin present in raw meat is gradually transformed into a red pigment possessing unique spectral properties. Aqueous or acetone/water extraction of the pigment of raw meat and of Parma ham proves that the initial oxymyoglobin, which is quite unstable upon isolation, is slowly altered to a red, thermally stable heme complex, which is preferentially distributed in organic solvent.

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