Evaluation of main post-translational modifications occurred in naturally generated peptides during the ripening of Spanish dry-cured ham

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Abstract

Peptidyl post-translational modifications (PTMs) could influence the final quality of processed meat. In this study, the peptide oxidative phenomena in Spanish dry-cured ham (*Biceps femoris* muscle) was evaluated at different ripening times (9, 12, 15, 18 and 24 months of processing) in evidence of interactions amongst the lipid and protein oxidation, major peptidyl PTMs and the release of free amino acids (FAAs). Results showed that 12 months of processing enabled the most abundant protein-bound carbonyls, while TBARS value was significantly favored ($P < 0.001$) by ripening process. However, the FAAs were still intensively generated during overall ripening. Peptidomics and chemometrics further revealed that proteolysis was mostly hampered for the oxidized peptides rather than the deamidated ones during ripening. Myosin light chain (MYL1 and MYL3) showed high oxidative susceptibility owing to peptidyl methionine and proline oxidation as well as acetaldehyde adduct formation on lysine or histidine residues.

Keywords: Peptides, post-translational modifications, dry-cured ham, oxidation, deamidation, peptidome, mass spectrometry

1. Introduction

Traditional Spanish dry-cured ham, a well-known food product in the Mediterranean area, is manufactured by conventional curing with salt, nitrite, and nitrate followed by a long period of maturation (Flores, Aristoy, Spanier and Toldrá, 1997). Since low counts of microorganisms have been identified in dry-cured ham (Molina and Toldrá, 1992), the majority of proteolytic changes were due to the action of endo- and exopeptidases (Toldrá, Aristoy and Flores, 2000). As a result of proteolysis, free amino acids and peptides have been extensively generated which are considered as crucial parameters for obtaining good sensory characteristics and nutritional value (Flores et al., 1997). Proteolysis activity in dry-cured ham can be affected by many factors such as salt content, moisture content, temperature, pH and anatomic location of muscles inside the ham (i.e. external muscle *Semimembranosus* or internal muscle *Biceps femoris*) (Flores et al., 1997; Toldrá, 1998; Ruiz-Ramírez, Arnau, Serra and Gou, 2005; Toldrá, 2006). Thus, a better understanding about the relationship between the proteolysis and these factors during prolonged processing would favour an optimised industrial manufacturing, product preservation and health implication.

Due to the proteolytic process and processing conditions such as the time and temperature, the muscle matrix (i.e. lipid, protein and peptides) can be affected by oxidative processes that would influence the ultimate quality traits of the product, causing relevant economic loss for meat industries (Mora, Gallego, Aristoy and Toldrá, 2018). During the production of dry-cured meat, both reactive oxygen species (including such free radicals as $\bullet$ OH, $\text{O}_2\bullet^-$, $\text{RS}\bullet$ or $\text{ROO}\bullet$ and non-radical species as H_2O_2 or ROO) and reactive nitrogen species (i.e. nitric oxide and peroxynitrite) can be generated in muscle, resulting in an increased susceptibility to oxidation *via* a free radical chain reaction (Lund, Heinonen, Baron and Estévez, 2011). Moreover, the pro-oxidative effect of salt has been reported to facilitate

the disruption of the structural integrity of membranes, allowing a closer interaction of lipids or protein reactants with metal-mediated catalysts, owing to the transition of redox status from ferrous (Fe^{2+}) of oxymyoglobin to ferric (Fe^{3+}) of metmyoglobin *via* the Fenton reaction (Soladoye, Juárez, Aalhus, Shand and Estévez, 2015). Thus, the addition of salt has been related to promote the oxidation of myofibrillar proteins, leading to the formation of protein cross-linkages, such as intra/inter-molecular disulfide bonds under high salt conditions (Li, Xiong and Chen, 2013) or cross-linked Schiff-adduct mediated by secondary lipid oxidation products (Veberg, Vogt and Wold, 2006; Gürbüz and Heinonen, 2015). Due to the complex matrix of dry-cured ham, major peptidyl post-translational modifications (i.e. deamidation, oxidation, etc.) have been reported (Gallego, Mora, Aristoy and Toldrá, 2015a; Cañete, Mora and Toldrá, 2017; Gallego, Mora and Toldrá, 2018a), but their contribution to the oxidative susceptibility of ham muscle during processing has not been fully understood yet.

In this study, two major hypotheses were set: i) if hydrolysis of peptides might be affected by different post-translational modifications with ripening times and ii) if there is a key peptidyl oxidative modification that might potentially hamper the protein hydrolysis during ripening process. To achieve that, the evolution of lipid/protein oxidation events and major peptidyl post-translational modifications occurring in Spanish dry-cured ham were profiled with different ripening times (9, 12, 15, 18 and 24 months of processing), thereafter the main drivers behind these events were evaluated by chemometrics and bioinformatics tools. The results of present study provide valuable information on the quality control and technical advance in the production of dry-cured ham.

2. Materials and methods

2.1 Dry-cured ham processing and sampling

Spanish dry-cured ham was manufactured according to the traditional procedure (Escudero, Mora, Fraser, Aristoy, Arihara and Toldrá, 2013), from the same batch of white pigs (6-months-old pigs, Landrace x Large White breeds) by ripening-drying up to 24 months. Briefly, the production of dry-cured ham had the following stages: 1) the pre-salting period using a mixture of sodium chloride, with nitrate and nitrite for 30 min; 2) a salting stage where hams were covered with salt and maintained at 2–4 °C to reach a 90–95% relative humidity for 12 days; 3) the removal of residual salt from the external surface followed by a post-salting/resting stage at 4–5 °C and 75–85% relative humidity for 60 days; 4) and finally, a ripening period in a drying chamber at an increasing temperature to 13–20 °C and relative humidity being dropped to 70%. Six ham slices were sampled from different hams (n=6) at different processing times (9, 12, 15, 18 and 24 months, respectively). *Biceps femoris* (BF) muscle was separated from each ham slice, vacuum-packaged, and frozen to –80 °C until further analysis.

2.2 Determination of moisture and salt contents

Moisture content was measured according to the methodology of Gallego, Mora and Toldrá (2018b), with a slight modification. Briefly, two grams of minced BF muscle were dried to constant weight at 150 °C using a halogen moisture analyzer HB43 (Mettler Toledo, Switzerland). Final results were expressed as mg moisture/g BF sample (wet weight).

Salt content was measured following the methodology described by Armenteros, Aristoy, and Toldrá (2012). Sodium (Na⁺) content was determined by ion chromatograph (Metrohm Advanced compact IC-861, Metrohm® Ltd., Herisau, Switzerland) equipped with a Metrosep C3 column (250 × 0.4 mm; Metrohm® Ltd.). 3 mM HNO₃ with 5% acetone was used as the mobile phase at a flow rate of 1.2 mL/min. Data collection and processing were performed using IC Net 2.3 software (Metrohm® Ltd.) using a calibration curve prepared with standard

solutions of Na⁺ (0.5–100 ppm) (Fluka, Switzerland, Sigma, St. Louis, MO, USA). Final results were expressed as mg Na⁺/g BF sample (wet and dry weight basis).

2.3 Measurement of lipid oxidation (TBARS) and protein oxidation (DNPH-assay)

Lipid oxidation was measured using the thiobarbituric acid reactive substances (TBARS) method (Salih, Smith, Price and Dawson, 1987), with a slight modification. Five grams of minced BF sample were homogenized with 20 mL trichloroacetic acid (TCA) (5%, m/v) and 500 µL butylated hydroxytoluene (BHT) (4% in ethanol). The mixture was then centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant (4 mL) was vortex-mixed thoroughly with 4 mL 0.02 M thiobarbituric acid (TBA). The mixture was incubated in a boiling-water bath (JP Selecta, Barcelona, Spain) for 60 min to develop color, then cooled in the ice-water for 15 min prior to analysis. The absorbance of the resulting supernatant solution was measured at λ 532 nm using a ultraviolet–visible (UV–Vis) spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, CA, USA) against a blank prepared with 4 mL TCA and 4 mL TBA solution. The amount of TBARS was calculated from standard calibration curve using 1,1,3,3-tetra ethoxy propane (TEP) (0.1–2 µg/mL malondialdehyde (MDA)) as standard, presented as mg MDA/kg BF muscle sample (wet weight basis).

Protein oxidation was evaluated by carbonyl index of BF samples using the 2,4-dinitrophenylhydrazine (DNPH) derivatization method as described by Armenteros, Heinonen, Ollilainen, Toldrá and Estévez (2009). The amount of protein carbonyls was measured at λ 370 nm by UV–Vis spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, CA, USA). Meanwhile, the final pellets were washed and dissolved in 20 mM sodium phosphate buffer pH 6.5 containing 6 M guanidine hydrochloride, and their absorption at λ 280 nm were collected to calculate the protein amount by plotting absorption value against a calibration curve prepared with bovine serum albumin (BSA) as standard (0.2–2 mg/mL).

Final result of carbonyl index was expressed as nM hydrazones/mg protein using the absorption coefficient for the protein hydrazones ($22.0 \text{ mM}^{-1}\text{cm}^{-1}$).

2.4 Determination of free amino acids (FAAs) and dipeptides (DPs)

For the preparation of muscle extract, 30 g of BF sample were homogenized with 0.01 M HCl (1:4; w/v) in a stomacher (IUL Instruments, Barcelona, Spain) for 8 min at 4 °C, and centrifuged ($10,000 \times g$, 20 min, 4 °C). The supernatant was filtered through glass wool and immediately frozen at -80 °C until use. For analysis of FAAs and DPs, sample pretreatment including chemical deproteinization and derivatization were carried out following the procedure described by Aristoy and Toldrá (1991). An internal standard solution (10 mM norleucine in 0.01 M HCl) was used. The resulting supernatants were filtered through a $0.45 \mu\text{m}$ membrane filter (Millipore), and sealed in an HPLC injection vial (Agilent Technologies, USA) prior to instrumental analysis. Chromatographic separation for derivatized molecules was done by using the methodology of Flores et al. (1997). Reversed-phase HPLC system (Series 1100 Agilent chromatograph, Agilent, Palo Alto, CA, USA) was used and equipped with a Waters Nova Pack[®] C18 column ($3.9 \times 300 \text{ mm}$) (Waters Corporation, Milford, MA, USA) and a photodiode array detector. The temperature was controlled at 52 °C , and the separated amino acids were detected at $\lambda 254 \text{ nm}$. Quantification was accomplished by considering the response factors calculated for each amino acid/dipeptide in the series of mixed standard, varying the relative proportion of added internal standard (norleucine). The final results were expressed as mg FAAs (or DPs)/100 g dry matter of BF muscle.

2.5 Qualitative and quantitative peptidomic analysis by nLC-MS/MS

The supernatant that was stored at -80 °C during the preparation of muscle extract (according to section 2.4) was deproteinized using three volumes of ethanol, and kept at 4 °C

overnight. Then, the samples were centrifuged to eliminate the precipitated proteins, the solvent was evaporated using a rotavapor, and the remaining peptide sample was lyophilized.

The identification of peptides was performed by nano-liquid chromatography-tandem mass spectrometry (nLC-MS/MS) using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to the quadrupole/time-of-flight (Q-ToF) TripleTOF[®] 5600 system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionization source (nESI). Chromatography separation and MS analytical strategy were performed according to the methodology of Gallego et al. (2018b). Information Dependent Acquisition (IDA) mode was performed to measure individual peptide samples in random groups. Afterwards, ProteinPilot v5.0 (AB Sciex, MA, USA) was used to generate the peptide library where the Paragon algorithm (Shilov et al., 2007) of ProteinPilot assisted in searching the UniProt database (Mammalian species) with the following parameters: no enzyme specificity, no taxonomy restriction, including all possible peptidyl post-translational modifications (PTMs). All the peptides were identified at 1% FDR. A label-free methodology was carried out for the relative quantification of peptides according to Gallego, Mora, Aristoy and Toldrá (2015b). In this label-free approach, quantification of peptides was done based on the measurement of the integrated areas of extracted ion chromatograms (XICs). Peak View v1.1 (AB Sciex, MA, USA) was used to quantify the areas for all peptides assigned in the library. Only those peptides with confidence of $\geq 95\%$ were quantitated. Finally, the areas were exported to Marker View v1.3 (AB Sciex, MA, USA) to normalize the obtained peak areas by total area summary.

2.6 Statistics, chemometrics and bioinformatics

All analytical experiments for each sample (n=6) were made in triplicate. The data were presented as mean $\pm$ standard deviation (SD) where indicated. A one-way analysis of variance

(ANOVA) was used to determine the statistical difference by SAS software (v.9.4) (SAS Institute, Inc., Cary, North Carolina, USA). Significant differences between means were detected by Tukey's HSD test ($P < 0.05$). Principal component analysis (PCA) was performed by SAS software to assess covariance structure and identify the differences between BF muscles. Pearson's coefficients amongst different technical parameters were determined by SAS program and visualized in a heat map using Heatmap Illustrator (v.1.0) (HemI, CUCKOO Workgroup, Wuhan, Hubei, P. R. China) (Deng, Wang, Liu, Cheng and Xue, 2014). To better understand the main drivers behind those peptidyl PTMs of interest, the correlations between the identified peptides and technical parameters were analyzed by partial least squares regression (PLSR) (following Kernel Algorithm and cross-validation assisted by Jack-knife uncertainty test) using the Unscrambler X (v.10.4) (CAMO ASA, Oslo, Norway), where the resulting data of peak area (as mentioned in Section 2.5) worked as X-matrix and the technical parameters (i.e. moisture and salt content, carbonyl index and TBARS value) as Y-variables. Hierarchically cluster analysis (HCA) was performed by HemI software to discriminate different PTMs of interest in peptidomic data. For a better understanding of PTMs, protein-protein interaction analysis was performed using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (v.11.0) (<https://string-db.org/>) to classify the reactome pathways, molecular locations, domains and features of the related proteins of origin, depending on the strength from the database. The resulting data was visualized as an interactive map in an edge confidence of 0.4 for the associated nodes. K-means clustering was performed by STRING for inspected nodes at 3 sets of clusters.

3. Results and discussion

3.1 Moisture and salt content

The moisture and salt content can be considered as two critical factors affecting the kinetics of chemical, enzymatic and microbial reactions occurring in dry-cured ham (Toldrá, 1998). The water migration showed a clear gradient between SM muscle and BF muscle with a trend to equilibration towards the end of the ripening-drying process (Toldrá, 1998; Toldrá, 2006). As shown in **Figure S1A**, an intense dehydration was observed during the overall ripening-drying stage ($P < 0.05$), reaching a moisture content around 50% in the inner part of the ham (BF muscle). Accordingly, the salt content significantly increased in wet weight basis of BF muscle during processing from 9 months to 24 months ($P < 0.05$) (**Figure S1B**), probably owing to the salt migration from the surface to inner location (Toldrá, 1998; Gallego et al., 2018b). Indeed, the dehydration and salt diffusion during ripening may greatly depend on many intrinsic characteristics of muscle, i.e. the weight/size of ham, adiposity, pH, fibril size and morphology (Toldrá, 1998). In general, as a result of salt penetration, the myofibrillar and sarcoplasmic proteins are likely to denature and/or dissociate between different muscles, further introducing oxidative modifications at the side chains of amino acid residues, including thiol oxidation, aromatic hydroxylation, and formation of carbonyl groups (Mora et al., 2018). Meanwhile, the action and stability of proteolytic enzymes during dry-cured ham manufacturing are closely related to the length of processing time, which definitively affected the textural and sensory traits of final products (Toldrá, 1998; Toldrá, 2006).

3.2 Lipid and protein oxidation

In our TBA test conditions, BHT was added to the homogenization solutions to prevent lipid auto-oxidation during heat incubation. Lipid oxidation, expressed as TBARS value, was observed to be significantly increased during the prolonged processing times ($P < 0.001$) (**Figure 1A**), probably owing to the pro-oxidant action of metallic ions present as impurities in the use of salt during the curing process (Soladoye et al., 2015). Particularly, there was a

drastic increase at the end of ripening stage (24 months of processing) (Figure 1A). Similar behavior of TBARS was found in Celta dry-cured ham by Bermúdez, Franco, Carballo and Lorenzo (2014) who observed an increase in MDA contents during the ripening-drying period in the internal (BF) muscle. Apart from the effect of salt, many other factors can be involved in lipid oxidation in dry-cured ham, such as temperature, length of ripening/drying time, fatty acid composition, oxygen atmosphere, initial pH, feeding, etc. (Toldrá, 1998; Jiménez-Colmenero, Ventanas & Toldrá, 2010). Furthermore, Figure 1B showed that the amount of carbonyls reached the maximum level at 12 months of processing ($P < 0.05$), with non-significant differences between the samples of 15, 18 and 24 months ($P > 0.05$).

3.3 FAAs and DPs

3.3.1 Hydrolysis profile

Despite of oxidative stress, the generation of free amino acids such as aspartic acid, glutamic acid, serine, glycine, alanine, valine, leucine and lysine remained intense along the whole ripening process (Table S1), resulting from the action of muscle carboxy- and aminopeptidases (Toldrá et al., 2000). Flores et al. (1997) reported that all amino acids exhibited an increased concentration at 12 months of processing when compared with 7 months of curing of dry-cured ham. Moreover, the amount of total hydrophilic FAAs, total hydrophobic FAAs and total FAAs were observed to be elevated after 12 months of processing, but their amount remained constant when the processing times subsequently increased up to 24 months ($P > 0.05$) (Table S1).

Carnosine and anserine are considered as the two major histidine-containing DPs in ham. The present study showed non-significant changes in the histidine abundance during the processing times ($P > 0.05$), while the amount of total DPs significantly decreased at 24

months of processing with depletion of carnosine compared to 9 months of processing probably owing to oxidative stress (Table S1).

3.3.2 Multivariate analysis by PCA

PCA was subsequently performed to interpret the correlations among FAAs, DPs and the studied technical parameters. A total 81.74% of variance was successfully explained, where 62.81% and 18.93% of variance were contributed by PC1 and PC2, respectively (Figure 2A). From PCA bi-plot, data set 9 months was located at PC1(-)PC2(+) quadrant and interacted with the loading of moisture content. Moreover, data set 12 months positioned in PC1(-)PC2(-) quadrant clustered with the loading of carbonyl index, implying a variation of possible carbonyls precursors over ripening process. As ripening proceeded, the remaining data sets (15-24 months) were distributed in PC1(+) quadrant, which allowed a strong interaction with the loadings of major FAAs (Figure 2A). So, our results revealed a discriminated pattern owing to the ripening time-dependent release of FAAs and DPs.

To move forward, TBARS showed a strong positive correlation to the salt concentration (Figure 2 and Table S2), implying the role of salt as a pro-oxidant in the development of lipid oxidation. In addition, carnosine had strong negative correlation to TBARS (Figure 2 and Table S2). In the present study, the products of lipid peroxidation (i.e. aliphatic aldehydes or α,β -Unsaturated aldehydes) expressed by TBARS were observed to be increased over ripening times particularly in the 24 months of sample (Figure 1A). Thus, histidine-containing DPs such as carnosine can be oxidized by lipid-derived aldehydes particularly MDA-like products, further involving enzyme-assisted secondary reactions (Xie, Baba, Sweeney and Barski, 2013). It is worthwhile to note that TBARS showed strong positive correlation to both lysine and glycine (Figure 2 and Table S2). Despite that glycine and lysine could be susceptible to react with lipid peroxidation products such as MDA owing to their high

reactivity of side chains (Veberg et al., 2006), both of them were intensively generated by the enzymatic action over ripening-drying process (see Table S1). Thus, a hydrolysis-induced compensation for the oxidative loss of glycine and lysine might be responsible for their positive correlations to TBARS.

3.5 Chemometrics and bioinformatics in the peptidomic analysis

3.5.1 Identification of peptidyl PTMs

Peptide extracts from BF muscle at different processing times (9, 12, 15, 18 and 24 months) were analyzed by nLC-MS/MS to identify and quantify the peptides showing PTMs of amino acid residues. Following label-free quantification approach, more than 7,800 peptides have been identified, including more than 1,400 peptide fragments which contain PTMs evidences (i.e. carbamylation, formylation, oxidation, amide-type adduct formation, deamidation, dehydration, or pyro-glutamic acid formation of susceptible amino acid residues, etc.). The key PTMs of identified peptides fall into four major categories: i) deamidation of glutamine (Q) or asparagine (N); ii) proline (P) oxidation; iii) lysine (K) or histidine (H) modification for acetaldehyde adduct formation; and iv) methionine (M) oxidation. These peptidyl PTMs were distributed in 315 modified peptides within a total of 2151 peptides (Table S3), mainly resulting from the degradation of proteins in myofibril within four structural units. These structural units included: i) the thick filaments (i.e. MYHC2, MYH1, MYH2, MYH7, MYL1, MYL3, MLC2B, MLC2, MYBPC2); ii) the thin filaments (i.e. ACTA1, TNNT3, TNNT2); iii) the cytoskeletal filaments (i.e. TTN); and iv) the Z-line (i.e. LDB3). Peptidyl PTMs were also identified from sarcoplasmic proteins due to the degradation of hemoglobin subunit beta (HBB) and a series of endogenous enzymes (i.e. ENO3, PFMK, APOBEC2, GAPDH, PYGM, LDHA and PKM). Furthermore, the percentage

of these peptidyl PTMs in sequence summary was reported in Table 1, which provided a quantitative point of view about the susceptibility of peptides being modified during ripening process. Among them, protein/peptide deamidation can be achieved in both enzymatic and nonenzymatic/chemical process. Enzymes involved in the deamidation include transglutaminase, protease, peptide-glutaminase and protein-glutaminase (PG) (Hamada and Swanson, 1994). In many cases, the released peptides enabled a higher deamidation rate than the intact protein or protein isolate owing to the flexibility of the polypeptide, thereby giving more variables to food functionalities (Robinson and Rudd, 1974; Hamada and Swanson, 1994). During dry-cured ham processing, peptide deamidation was less influenced by the muscle type but probably closely related to characteristic conditions (i.e. temperature, pH, salting) (Cañete et al., 2017). As a result, Q or N can be deamidated in a “selective” manner during ripening according to four main mechanisms:

i) As indicated by the percentage data, N exhibited a higher general susceptibility to deamidation (7.22%) than Q (5.10%) (Table 1), which may be a result of N being closer to the side chain amide group from the peptide nitrogen (Robinson and Rudd, 1974).

ii) Deamidation susceptibility of Q and N was also greatly dependent on their molecular localization on sequences. For instance, the Q and N residues present at the extreme end of peptide chain were preferred to be deamidated rather than being situated in central positions (Table 1) (Cañete et al., 2017).

iii) Bioavailability of Q and N might also affect their deamidation efficiency, varying from the protein origin or even the functional domain. Compared to Q, N residues on myosin light chain were observed to be more accessible to deamidation regardless of the family isoforms (Table 1) probably as a result of a higher amount of N than Q (Weeds and Frank, 1974). In contrast, Q residues on troponin T protein were more easily deamidated than N (Table 1), resulting from the higher abundance of Q on sequences (Pearlstone, Carpenter,

340 Johnson and Smillie, 1976). Similarly, according to Strehler, Strehler-Page, Perriard,
341 Periasamy and Nadal-Ginard (1986), the content of Q in the rod segment of myosin heavy
342 chain (8.2%) was higher than that in the head segment (4.2%), which partly explained the
343 relatively high deamidation ratio of Q residues on the sequences derived from myosin heavy
344 chain, particularly for MYHC2, MYH2 and MYH7 (Table 1).

345 iv) The adjacent amino acid residues also favored deamidation efficiency of Q and N,
346 especially being those surrounded by small residues (e.g., Valine (V), Alanine (A) or Glycine
347 (G)). In present study, the percentage summary of peptides containing deamidated N nearby V,
348 A or G and that of peptides containing deamidated Q nearby V, A or G accounted for 31.56%
349 and 19.56% of total studied sequences, respectively (Table S3 and Table 1), probably owing
350 to the less steric hindrance of these small residues to inhibit the deamidation process
351 (Kempkes, Martens, Grzetic, Berden and Oomens, 2016).

352 Apart from deamidation modification, peptidyl PTMs mediated by oxidizing conditions
353 also occurred. According to the literature, secondary lipid oxidation product such as
354 malondialdehyde (MDA) has been shown to form stable Schiff base adducts (m/z +54 amu)
355 on N-termini of peptides, which predominantly include lysine residues (Gürbüz and Heinonen,
356 2015). Interestingly, 50 of 56 (89.29%) peptide fragments containing oxidized proline
357 residues were identified to be a result of peptidyl acetaldehyde adduct formation (m/z +26
358 amu) on either lysine (K) or histidine (H) (Table S3 and Table 1). Thus it can be expected that
359 salt diffusion during ripening allowed for in-depth formation of reactive carbonyl derivatives
360 (Gallego et al., 2018a), especially on myosin light chain fragments (MYL1 and MYL3) (Table
361 1). Similar mass spectrometry evidence has been reported, showing that acrolein could enable
362 site-specific, rather than diffuse, protein modification *via* Michael additions. These additions
363 may occur on either on components of the sarcomere/cytoskeleton (cardiac α -actin, desmin,
364 myosin light polypeptide 3), or involved in mitochondrial energy metabolism (mitochondrial

creatine kinase (CK)-2, ATP synthase) in murine myocardium (Luo et al., 2007). It is worthwhile to mention that unlike troponin T, troponin I is a basic protein with an isoelectric point of 9.3. As a result, troponin I conceivably exhibited higher oxidative susceptibility than troponin T protein (Table S3 and Table 1), due to the abundance of major basic amino acids (i.e. arginine (R), lysine (K) and histidine (H)) in troponin I sequences (Pearson and Young, 2012).

On the other hand, peptidyl methionine residue can be a vulnerable target of oxidative damage. as described in Table S3 and Table 1, 19 of 43 (44.19%) methionine residues were detected in myosin-derived peptide sequences (MYHC2, MYL1, MYL3, MLC2B and MYH2). Among them, the highest percentages of methionine residues were present in myosin heavy chain (MYHC2) (3.92%) and myosin regulatory light chain 2 (MLC2B) (4.08%) (Table 1). Methionine residues in other sequences derived from thin filaments and Z-line (e.g., alpha-actin (ACTA1), troponin I (TNNI2) and LIM domain binding 3 (LDB3)) also showed susceptibility to oxidation (Table S3 and Table 1), which possibly depended on their location within myofibril and their accessibility to oxidants (Gallego et al., 2015a). It was reported that the neighboring basic amino acid residues favored methionine oxidation (Levine, Mosoni, Berlett and Stadtman, 1996). So, in identified actin fragments (from M1 to M8), all oxidized methionine residues were surrounded by lysine residues, which coincided with this previous research (Table S3). Meanwhile, the oxidized methionine residues were co-identified with the deamidation of N or the acetaldehyde adduct formation in peptide fragments, including those from ENO3, LDB3, MYL1, MYL3, MLC2B and TNNI2 (Table S3).

3.5.2 PLSR analysis and Pearson's correlation analysis

To better understand the main factors to those peptidyl PTMs, a quantitative structure-activity relationship (QSAR) study has been performed by PLSR analysis.

Regardless of deamidation or oxidative modification, regression analysis between X and Y, basically reflected their interaction within the PLS components that can be shown in the correlation loading plots (Figure 3A-C), where the inner and outer ellipse referred to 50 and 100% explained variance, respectively. From Figure 3A, the deamidated peptides (X) explained the variance of 74% and 7% for factor component 1 (FC-1) and factor component 2 (FC-2), respectively, while the key technical parameters (Y) spanned the FC-1 and FC-2 with an explained variance of 46% and 15%, respectively. It was clear that 225 loadings of deamidated fragments (Table S3) dynamically escaped from outer ellipse onto the inner one in such a manner as to correspond to the moisture loss and salt diffusion over times. Indeed, temperature, pH and salting process were considered as the main drivers behind deamidation, which further caused protein hydrolysis and protein unfolding (Cañete et al., 2017) as represented by FC-1 and FC-2, respectively. Thus, it was not surprising that the majority of significant cross-validated X-variances of deamidated pattern showed negative correlation to FC-1 but had positive weights on FC-2 (Figure 3A, Figure S2A) (Kempkes et al., 2016), particularly the peptide D16, D118, D142 and D176 (Figure S2A). Deamidated peptides from myosin heavy chain (MYH2 and MYH7) showed relatively lower negative weights on FC-1 (D179, D180, D190, D191, D192, D206, D207 and D208) than those pieces from other myosin family proteins (Figure S2A, Table S3). Thus, the deamidated residues in myosin rod seemed difficult to cleavage probably due to inaccessibility of endopeptidases within the rigid helical structure (Xiong, 1997).

In addition, predictive X-matrix from P oxidation, K or H modification and M oxidation data were inspected *via* responding to Y-variation as a fixed effect (Figure 3B and Figure 3C). Interestingly, the X-matrix represented in Figure 3B and Figure 3C both explained a much higher variance (17% and 15%, respectively) for FC-2 than the deamidation effect on FC-2 (7%) (Figure 3A), indicating that the solvation of hydrophobic residues (i.e. P, K and M)

played a critical role in the unfolding process (Karplus and Šali, 1995). In addition, the distributed variance of X-matrix on FC-1 was observed to be lower in such oxidation pattern (Figure 3B and Figure 3C) (61% and 53%, respectively) than that in deamidation pattern (Figure 3A). This result provided strength to the hypothesis that these oxidized peptides were closely related to an impaired proteolytical behavior. From Figure S2B, all the significant X-variables computed from P oxidation, K or H modification data were negatively correlated to FC1 while showing positive correlation to FC2 (particularly peptides P22, P34, P35 and P38), proving an intense proteolysis experienced by myosin light chain isoforms following enhanced accessibility to oxidants through unfolding process (Table S3) (Mora, Sentandreu and Toldrá, 2011). Indeed, after PLSR analysis, these significantly varied acetaldehyde-modified peptides derived from MYL1 and MYL3 (Figure 3B, Figure S2B and Table S3) were identified to be negatively correlated to both salt content and TBARS value ($P < 0.05$) while showing non-significant correlation to carbonyl index ($P > 0.05$) (Table S4), indicating their high oxidative susceptibility to lipid peroxidation.

However, nine significant peptide fragments showing M oxidation in PLSR were positively correlated to FC1 and all the computed myosin-derived fragments (M18, M26, M27 and M35) showed relatively higher negative correlation to FC2 (Figure 3C, Figure S2C and Table S3). Such methionine residues in myosin-derived sequences were highly susceptible to neighboring oxidants, but unlike peptidyl P oxidation pattern, their unfolding process tended to be compromised during ripening (Figure 3B and Figure 3C). Additionally, protein/peptide breakdown can be largely interfered by oxygen atmosphere, since the intra/inter-molecular cross-links formed in highly oxidative conditions may enable a hampered pattern to enzymatic proteolysis (Lund et al., 2011). Meanwhile, the positive weights on FC-1 implied that peptidyl methionine might be reduced from methionine sulfoxides by the action of methionine sulfoxide reductases (Gallego et al., 2018a).

3.5.3 HCA of peptidyl PTMs of interest and protein-protein interaction networks

The profile of significant X-loadings in all PLSR analysis (Figure 3A-C and Figure S2) was mapped by a hierarchical clustering analysis (Figure 3D). Ham samples from different processing times (9, 12, 15, 18 and 24 months) displayed different color distributions depending on their relative abundance, indicating the characteristic changes in the peptidome in respect to ham processing times. The studied deamidated sequences, basically separated by the oxidized peptides (mainly distributed in the middle section of heatmap), were enriched into the clusters showing rapid degradation pattern over time, particularly the peptides D38 (from GLOBIN domain-containing protein) and D63, D96, D112, D118, D161, D162 (from myosin light chain family) (Figure 3D). Thus, during ripening process, myosin light chain family peptides might have undergone extensive degradation in which the deamidation was implicated as a “molecular clock” signaling the sites for proteolytic cleavage (White, Cordwell, McCarron, Tchen, Hambly and Jeremy, 2003). From Figure 3D, it is apparent that the significant degradation of oxidized sequences occurred at 12 months of processing. However, the prolonged ripening process (> 12 months of processing) implied less variance in their degradation, particularly for those oxidized peptides derived from MYL1 and MYL3 family (P10, P21, P22, P31, P32, P34, P35, P36). These peptides showed both peptidyl P oxidation and acetaldehyde adduct formation on peptidyl K residues (Table 2), supporting the hypothesis that such characterized multi-PTMs may in turn hindered the hydrolysis of myosin light chain sequence during prolonged ripening.

Finally, the protein family of those peptides showing PTMs during ripening times and their protein-protein interacting networks were visualized in Figure S3. These proteins of origin (nodes) were connected mainly based on the known interactions (edges) from the experimental and curated database evidence (STRING database). The p-value of

protein-protein interactions enrichment was $< 1.0 \times 10^{-16}$, and the K-means clustering coefficient was 0.719. Proteins in myofibril (MYHC2, MLC2B, MYL1, MYL3, MLC2, MYH1, MYH2, MYH7, MYBPC2) and alpha-1,4 glucan phosphorylase (PYGM) were enriched into a cluster, whereas proteins participating in glycolysis and energy metabolism (PFKM, PKM, GAPDH, ENO3, LDHA) showed close association in another separated cluster (Figure S3). Myosin heavy chain (MYH1, MYH2, MYH7) completely covered the rod and motor segments, where MYH2 and MYH7 had more unique domains in their N-terminus (Figure S3). These increased domains likely enabled their high susceptibility to deamidation, as the number of deamidated MYH2 and MYH7 sequences was 4 times higher (compared to deamidated MYH1 peptides) and 77.5 % occurred at their N-terminus (Table S3).

4. Conclusions

Salting diffusion and moisture loss during ripening-drying process of Spanish dry-cured ham (*Biceps femoris* muscle) not only played a vital role in proteolysis, but also favored the peptide modification following protein unfolding. Methionine and proline residues in myosin light chain (MYL1 and MYL3) sequences were the most susceptible to oxidation, indicating their potential being good biomarkers of oxidative damage during the ripening of dry-cured ham. Following ripening process, lipid peroxidation might enable the formation of stable acetaldehyde adducts to lysine or histidine residues, mostly on myosin light chain, thereby introducing oxidative damage and carbonyl functionalities. Continued investigation on structure-function of such adducts should provide greater insight into the relationships among generation of peptides, oxidative stress, and antioxidant activity of dry-cured ham.

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Conflict of interest

The authors declare that ‘NO Conflict of interest’ exist in this research article.

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LEGENDS FOR THE FIGURES

Figure 1 Dynamic changes of TBARS (**A**) and carbonyl index (nM hydrazones/mg protein) (**B**) determined by DNPH assay for dry-cured ham (*Biceps femoris* muscle) during different processing times (9–24 months). Values were expressed as means $\pm$ SD (n=6). Different letters on the same bar denote statistical differences ($P < 0.05$).

Figure 2 (**A**) Bi-plot from principal component analysis considering the data of free amino acids and dipeptides, moisture and salt content, lipid and protein oxidation for dry-cured ham (*Biceps femoris* muscle) during different processing times (9–24 months). (**B**) Pearson's coefficients amongst variables of interest visualized by HemI Illustrator.

Figure 3 Correlation loading plot of PLSR modeling for identified peptides with PTMs categorized as deamidation of N or Q (**A**), P oxidation, and K or H modification for acetaldehyde adduct formation (**B**) and M oxidation (**C**), as a function of technical parameters. The PTMs data were determined as X-matrix (predictor, loaded in blue) and the technical parameters as Y-variables (response, loaded in red). Those modified peptides (from X-matrix) showing significant regression coefficients to Y-variables were code-labeled with black circles (●). (**D**) Hierarchically clustering analysis was performed on those modified peptides showing significant X-loadings in PLSR at processing times (9–24 months). Numerical quantitative values of peak intensity were log-transformed (base 2) for improving normality and expressed as a color gradation of square by HemI Illustrator.

Figure 1

639 Figure 2

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648 **Table 1.** List of the key post-translational modifications (PTMs) on peptide sequences and their distribution corresponding to the proteins identified by nLC-MS/MS
649 in Spanish dry-cured ham (*Biceps femoris* muscle) under the ripening process*.

PTMs ¹	Formula	Mass Shift (Da)	Amino acid residue	Protein name	Gene origin ²	Number of modified peptides	Number of non-modified peptides	Percentage of modified peptides in relation to total number of peptides (%) ³	General percentage of modified peptides in relation to total number of peptides (%) ⁴
Deamidation	H(-1) N(-1) O(1)	0.98	Glutamine (Q)	Actin, alpha 1, skeletal muscle	ACTA1	5	175	2.56	5.10
				Beta-enolase	ENO3	2	217	0.85	
				GLOBIN domain-containing protein	LOC110259958	2	29	5.41	
				Muscle 6-phosphofructokinase	PFMK	1	9	10.00	
				Myosin binding protein C, fast type	MYBPC2	1	80	1.23	
				Myosin heavy chain isoform fast type	MYHC2	4	43	7.84	
				Myosin light chain 1/3, skeletal muscle isoform 1f	MYL1	25	278	7.18	
				Myosin light chain 3	MYL3	4	115	2.61	
				Myosin regulatory light chain 2	MLC2B	1	200	0.41	
				Myosin-1	MYH1	5	47	10.64	
				Myosin-2	MYH2	24	115	16.44	
				Myosin-7	MYH7	10	131	6.71	
				Troponin T fast skeletal muscle type	TNNT3	6	114	4.96	
				Uncharacterized protein	TMPO	3	3	50.00	
			Asparagine (N)	Actin, alpha 1, skeletal muscle	ACTA1	7	175	3.59	7.22
				Beta-enolase	ENO3	11	217	4.66	
				C->U-editing enzyme APOBEC-2	APOBEC2	8	9	47.06	
				GLOBIN domain-containing protein	LOC110259958	6	29	16.22	
				Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	3	91	3.19	
				Hemoglobin subunit beta	HBB	2	15	11.76	
				LIM domain binding 3	LDB3	1	8	11.11	
				L-lactate dehydrogenase	LDHA	1	4	20.00	
				Myc box-dependent-interacting protein 1 isoform 8	BIN1	2	0	100.00	
				Myosin heavy chain isoform fast type	MYHC2	2	43	3.92	
				Myosin light chain 1/3, skeletal muscle isoform 1f	MYL1	35	278	10.06	
				Myosin light chain 2V	MLC2	4	42	8.70	
				Myosin light chain 3	MYL3	5	115	3.27	
				Myosin regulatory light chain 2	MLC2B	38	200	15.51	
				Myosin-1	MYH1	5	47	10.64	
				Myosin-2	MYH2	5	115	3.42	
				Myosin-7	MYH7	1	131	0.67	
				Troponin T fast skeletal muscle type	TNNT3	1	114	0.83	
				Uncharacterized protein	TMPO	2	3	33.33	
Schiff base formation via acetaldehyde	H(2) C(2)	26.02	Lysine (K)	Beta-enolase	ENO3	4	217	1.69	5.55

Category	Residue	Weight (g/mol)	Residue	Weight (g/mol)	Weight (g/mol)	Weight (g/mol)	Weight (g/mol)			
Oxidation	O(1)	15.99	Histidine (H)	Myosin light chain 1/3, skeletal muscle isoform 1f	MYL1	13	278	3.74	6.78	
				Myosin light chain 3	MYL3	28	115	18.30		
				Myosin-7	MYH7	8	131	5.37		
				Titin isoform X1	TTN	1	86	1.15		
			Proline (P)	Troponin I, fast skeletal muscle	TNNI2	1	32	3.03		2.65
				Myosin light chain 1/3, skeletal muscle isoform 1f	MYL1	13	278	3.74		
			Myosin light chain 3	MYL3	28	115	18.30			
			Myosin-7	MYH7	8	131	5.37			
			Titin isoform X1	TTN	1	86	1.15			
			Methionine (M)	Beta-enolase	ENO3	6	217	2.54		2.65
				Troponin I, fast skeletal muscle	TNNI2	1	32	3.03		
				Actin, alpha 1, skeletal muscle	ACTA1	8	175	4.10		
				Alpha-1,4 glucan phosphorylase	PYGM	1	11	8.33		
			LIM domain binding 3	LDB3	1	8	11.11			
			Myosin heavy chain isoform fast type	MYHC2	2	43	3.92			
			Myosin light chain 1/3, skeletal muscle isoform 1f	MYL1	3	278	0.86			
			Myosin light chain 3	MYL3	2	115	1.31			
			Myosin regulatory light chain 2	MLC2B	10	200	4.08			
			Myosin-2	MYH2	2	115	1.37			
			Pyruvate kinase	PKM	2	2	50.00			

¹ PTMs of interest were listed as deamidation modification (Q & N), acetaldehyde adduct formation on peptidyl amino acid residues (K & H) as well as oxidation of P & M.

³ Percentage of modified peptides in relation to total number of peptides (%) = (the number of modified peptides per protein) × 100/ (total number of peptides per protein regardless of modification). Overview of total number of peptides (per protein) regardless of the PTMs of interest refers to **Table S5**.

number of peptides regardless of modification for all the corresponding proteins). Overview of total number of peptides (per protein) regardless of the PTMs of