Evolution of oxidised peptides during the short processing of Spanish dry-cured ham

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Abstract

Sensory and nutritional properties of dry-cured ham can be negatively affected due to oxidative modifications of muscle proteins during its processing. In this study, a peptidomic approach has been used in order to study the evolution of oxidised peptides generated throughout a short dry-curing process (0, 2, 3.5, 5, 6.5 and 9 months), focusing on those derived from major myofibrillar proteins. A total of 67 peptides showing methionine, proline, and tryptophan oxidations were identified by nano liquid chromatography coupled to tandem mass spectrometry and then relatively quantified using a label-free methodology, showing the hydrolysis of some of them during the process. So, the peptidomics strategy used in this work has resulted to be very useful as a complementary tool to the methods currently used to study protein oxidation, allowing a better understanding of the oxidation at peptide level and the influence of ham processing conditions.

Keywords: Peptidomics, peptides, oxidation, mass spectrometry, quantification, label-free, dry-cured ham.

1. Introduction

Oxidative processes play an important role in quality and sensory perception of meat products during their processing and storage (Lund, Heinonen, Baron, & Estévez, 2011; Zhang, Xiao, & Ahn, 2013). Numerous studies are focused on lipid oxidation as it is fundamental in the maturation of meat and the desired final flavour of dry-cured ham (Ladikos, & Lougovois, 1990; Toldrá & Flores, 1998; Gandemer, 2002), whereas protein oxidation has been less studied to date. Oxidation of proteins has been defined as a covalent modification occurring directly by reactive species or indirectly by reaction with secondary products of oxidative stress. The most important oxidative modifications of proteins involve the modification of amino acid side chains, cleavage of polypeptide backbones and protein cross-linking. As a result, proteins can be subjected to changes in their physical and chemical properties such as conformation, functionality, susceptibility to enzymatic hydrolysis, solubility, hydrophobicity, and stability, as well as in their nutritional values due to the loss of essential amino acids and lower digestibility of proteins (Stadtman & Levine, 2003; Xiong, 2000; Lund et al., 2011). Regarding oxidation of amino acid residues, methionine and cysteine are those more susceptible due to their reactive sulfur atoms, whereas aromatic amino acids (phenylalanine, tyrosine, tryptophan, and histidine) or other residues (arginine, lysine, leucine, proline, and threonine) need more severe conditions to be oxidised (Stadtman, 1990; Shacter, 2000; Zhang et al., 2013).

Several works have reported protein carbonylation as a way of protein oxidation in meat during maturation or chilled storage (Martinaud et al., 1997; Santé-Lhoutellier, Engel, Aubry, & Gatellier, 2008), during meat processing like cooking, irradiation, frozen storage or packaging (Rababah et al., 2004; Xia, Kong, Liu, & Liu, 2009; Estévez, Ventanas, Heinonen, & Puolanne, 2011; Utrera, Parra, & Estévez, 2014), and during

fermentation or dry-curing processes (Ventanas, Estévez, Tejeda, & Ruiz, 2006; Ventanas, Ventanas, Tovar, García, & Estévez, 2007; Armenteros, Heinonen, Ollilainen, Toldrá, & Estévez, 2009; Wang et al., 2011; Koutina, Jongberg, & Skibsted, 2012). Nevertheless, peptide oxidation has been little reported and only a few studies have been described based on mass spectrometry (MS) in tandem for the identification of peptides showing amino acid modifications such as methionine oxidation in dry-cured hams (Gallego, Mora, Fraser, Aristoy, & Toldrá, 2014; Gallego, Mora, Aristoy, & Toldrá, 2015b).

Despite the analysis of post-translational modifications such as oxidation continues to be highly challenging due to the difficulty in detecting the high variety of oxidation products, the low abundance and lability of some of them under MS analysis, and the complexity of the mechanisms (Silva et al., 2013), the novel advances in MS during the last decades have allowed a notorious progress in the field. Thus, peptidomics approaches are a valuable tool to study oxidative mechanisms as they allow to determine the specific site and nature of such modification as well as to identify and quantify the oxidised peptides (Schey & Finley, 2000; Silva, Vitorino, Domingues, Spickett, & Domingues, 2013, Verrastro, Pasha, Jensen, Pitt, & Spickett, 2015).

The present work was focused on studying the evolution of peptide oxidation during the short processing of dry-cured ham. For this purpose, a peptidomics strategy has been used for the identification and relative quantification of oxidised peptides using a label-free method on the basis of ion intensity measurements.

2. Materials and methods

98 **2.1 Dry-cured ham processing**

99 Spanish dry-cured hams (6-months-old pigs, Landrace × Large White) were prepared
100 according to the traditional procedure consisting on pre-salting for 30 min (with a
101 mixture of salt, nitrate and nitrite), salting for 10 days (2-4 ºC, 90-95% relative humidity
102 (RH), post-salting for 2 months (4-5 ºC, 75-85% RH), and ripening-drying up to 9
103 months (14-20 ºC, 70% RH). The study was done in triplicate, taking samples at six
104 different processing times: 0 months (raw ham), 2 months (post-salting stage), 3.5, 5,
105 and 6.5 months (ripening-drying stage), and 9 months (dry-cured ham).

106 **2.2 Extraction of peptides**

107 For the extraction of peptides at each processing time (0, 2, 3.5, 5, 6.5, and 9 months),
108 50 g sample of *Biceps femoris* muscle were minced and homogenised with 200 mL of
109 0.01 N HCl for 8 min in a stomacher (IUL Instrument, Barcelona, Spain). The
110 homogenate was centrifuged at 12,000 *g* for 20 min in cold, filtered through glass wool,
111 and then deproteinised by adding 3 volumes of ethanol and kept at 4 °C for 20 h. The
112 sample was centrifuged again at 12,000 *g* for 10 min and the supernatant was
113 lyophilised. The resulting extract was dissolved in 25 mL of 0.01 N HCl, filtered
114 through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA, USA), and stored at
115 -20 °C until use.

116 **2.3 Size-exclusion chromatography**

117 Extracts were fractionated according to the molecular mass of peptides using size-
118 exclusion chromatography on a Sephadex G25 column (2.5 x 65 cm; Amersham
119 Biosciences, Uppsala, Sweden). For that, 5 mL of each extract was injected on the
120 column and the separation was performed using 0.01 N HCl as mobile phase at a flow
121 rate of 15 mL/h in a cool room. Fractions of 5 mL were collected using an automatic
122 fraction collector and then monitored at 214 nm using an UV spectrophotometer

(Agilent 8453, Agilent Technologies, Palo Alto, CA, USA). Finally, fractions including elution volumes from 125 to 160 mL, corresponding to the highest molecular weight peptides, were pooled and divided in aliquots of 100 µL that were lyophilised for the following analysis.

2.4 nLC-MS/MS analysis

Peptide identification was performed using nano liquid chromatography-tandem mass spectrometry with an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to a quadrupole/time-of-flight (Q/ToF) TripleTOF® 5600+ system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionisation source (nano-ESI), according to the methodology described by Gallego, Mora, Aristoy, and Toldrá (2015a).

Lyophilised extracts at different processing times were resuspended in 100 µL of 0.1 % (v/v) trifluoroacetic acid (TFA) in H_2O. A total of 10 µL of each sample was cleaned and concentrated using Zip-Tip C18 with standard bed format (Millipore Corporation, Bedford, MA). Then 4 µL of the supernatant was injected and concentrated on an Eksigent C18 trap column (3 µm, 350 µm × 0.5 mm; Eksigent of AB Sciex, CA, USA), at a flow rate of 3 µL/min for 5 min and 0.1% TFA as mobile phase. The trap column was automatically switched in-line onto a nano-HPLC capillary column (3 µm, 75 µm × 12.3 cm, C18; Nikkyo Technos Co., Ltd., Japan). Mobile phases were 0.1% (v/v) formic acid (FA) in H_2O as solvent A, and 0.1% (v/v) FA in acetonitrile as solvent B. HPLC conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of 0.30 µL/min at 30 °C. The column outlet was directly coupled to a nano-ESI, and the Q/ToF (MS/MS) was operated in positive polarity and information-dependent acquisition mode, in which a 250 ms ToF

147 MS scan from 300 to 1250 *m/z* was performed, followed by 50 ms product ion scans
148 from 100 to 1500 *m/z* on the 50 most intense 1 to 5 charged ions.

149 **2.5 Data analysis**

150 Data were processed using ProteinPilot™ v4.5 software (AB Sciex, MA, USA) for the
151 identification and quantification of the peptides. The Paragon algorithm of ProteinPilot
152 was used to search in ExPASy database (http://www.expasy.org) with no enzyme
153 specificity and no cysteine alkylation. A novel database from Uniprot including all
154 proteins from *Sus scrofa* species, and specifically titin protein from *Mus musculus*
155 species and LIM domain binding protein 3 (LDB3) from *Homo sapiens* species
156 (accession numbers A2ASS6 and O75112, respectively) was generated for the search.
157 A label-free methodology was used for the relative quantification of peptides, following
158 the methodology described by Gallego et al. (2015a). In this label-free approach,
159 quantification was done at peptide level based on the measurement of the integrated
160 areas of extracted ion chromatograms (XICs). Thus, the combination of XICs allows the
161 determination of the ratios for individual peptides, using 3 replicates per sample and
162 normalising by total areas summary. Peptides were quantified using Peak View 1.1
163 software (AB Sciex, Framingham, MA, USA) and then data were statistically analysed
164 using Marker View 1.3 software (AB Sciex, Framingham, MA, USA). Principal
165 Component Analysis (PCA) and loading plot analysis were performed using SIMCA-P+
166 13.0 software (Umetrics AB, Sweden).

167

168 **3. Results and discussion**

169 Proteolysis is the main biochemical reaction that takes place during the long-time
170 processing of dry-cured hams, generating large amounts of peptides and free amino
171 acids through the enzymatic hydrolysis of proteins that contribute to the valuable

172 flavour, texture and quality of the final product (Toldrá & Flores, 1998; Lametsch et al.,
173 2003). However, oxidative modifications of muscle proteins can negatively modify the
174 physico-chemical, sensory, and nutritional properties of dry-cured hams (Lund et al.,
175 2011; Zhang et al., 2013; Soladoye, Juárez, Aalhus, Shand, & Estévez, 2015).
176 In this study, peptide extracts from dry-cured hams sampled at different processing
177 times (0, 2, 3.5, 5, 6.5, and 9 months) were analysed by nLC-MS/MS to identify and
178 quantify oxidised peptides. More than 19,500 peptides from 189 different proteins were
179 identified in the peptide extracts, showing around 6,500 oxidative modifications. The
180 study was focused on main myofibrillar proteins, and a total of 67 oxidised peptides
181 were identified in common in all samples and then relatively quantified using a label-
182 free methodology based on peak intensity measurements. Oxidative modifications were
183 observed in methionine (M), proline (P), and tryptophan (W) amino acid residues,
184 identifying 34, 27, and 6 oxidised peptides, respectively, derived from the proteins
185 myosin heavy chain (MYH), myosin light chain (MYL), titin (TITIN), nebulin (NEBU),
186 LIM domain-binding 3 (LDB3), and PDZ and LIM domain protein 3 (PDLIM3). As an
187 example, Figure 1 shows the MS/MS spectra of the peptide IDGVNTDTMTHL derived
188 from LDB3 protein and the peptide VSPGTAIGKTPEM from nebulin protein, both
189 showing methionine oxidations.
190 Tables 1, 2, and 3 show the sequences of the identified oxidised peptides, their protein
191 of origin, and their relative quantitation at different times of dry-cured ham processing
192 presented as a heat map where numerical quantitative values were expressed as
193 percentage and converted into a color gradation. Specifically, Table 1 shows those
194 peptides with methionine oxidation, which is the most susceptible amino acid to
195 oxidative modifications due to the reactive sulfur atom contained in its side chain.
196 Oxidation of methionine is involved in cross-linking and leads to the formation of

197 sulfur-containing derivatives such as methionine sulfoxide and methionine sulfone,
198 although this process can be reversible (Shacter, 2000; Zhang et al., 2013). In this work,
199 all oxidised methionines were identified in the sulfoxide form (+ 16 Da shift), whereas
200 methionine sulfone was not observed (+ 32 Da shift). The identified peptides showing
201 methionine oxidation (Table 1) were mainly derived from three proteins, LDB3, NEBU,
202 and PDLIM3. A previous study performed by Gallego et al. (2015b) identified 120
203 peptides showing methionine oxidation from major myofibrillar proteins in 14 months
204 Teruel dry-cured ham, evidencing that amino acids surrounding methionine residue
205 influence its susceptibility to be oxidised. In the present study, most of the identified
206 oxidised methionines were adjacent to threonine (T), aspartic acid (D), and glutamic
207 acid (E) residues. Moreover, methionine oxidation has been detected in peptides
208 generated during the in-solution digestion of different isoforms of myosin protein from
209 porcine muscles (Jeong, Jung, Jeong, Yang, & Kim, 2016).
210 On the other hand, the oxidation of proline (Table 2) was mainly identified in peptides
211 derived from myosin and titin proteins. Proline is one of the most likely amino acid
212 residues to yield carbonyl derivatives after its oxidation and it is also implicated in
213 peptide bond cleavage through the formation of 2-pyrrolidone that is later hydrolysed to
214 4-aminobutyric acid (Stadtman, 1993; Berlett & Stadtman, 1997). Finally, the oxidation
215 of tryptophan residues was identified in peptides derived from LDB3, all derived from
216 the same region of the sequence (Table 3). Tryptophan residue has been described to be
217 easily oxidised due to the indole ring that contains in its structure, forming N-
218 formylkynurenine, kynurenine and various hydroxy derivatives (Simat & Steinhart,
219 1998; Schey & Finley, 2000).
220 Moreover, this study evidenced that some oxidised peptides were further hydrolysed
221 during the process into shorter peptides, which is observed in the case of LDB3 protein.

222 So, Table 1 shows the degradation of FNM[Oxi]PLTISRITPGSKAAQSQL that
223 generates the peptides FNM[Oxi]PLTISRITPGSKA, FNM[Oxi]PLTISRITPGSK,
224 FNM[Oxi]PLTISRITPG, and FNM[Oxi]PLTIS. Relative quantification indicated that
225 FNM[Oxi]PLTISRITPGSKAAQSQL at 3.5 months of processing was the peptide
226 showing the highest percentage between all the identified peptides with methionine
227 oxidation, whereas its derived peptide FNM[Oxi]PLTISRITPGSK showed the highest
228 amount during the initial steps of the process (2 and 3.5 months). However, the amount
229 of the shortest peptide resulting from this hydrolysis, FNM[Oxi]PLTIS, increased
230 during the processing time probably as a consequence of the hydrolysis of oxidised
231 peptides at initial stages, leading to its accumulation at the end of the process.
232 Furthermore, Table 3 shows the hydrolysis of the peptide
233 LTGPGPW[Oxi]GFRLQGGKD into LTGPGPW[Oxi]GFRL, LTGPGPW[Oxi]GFR,
234 and LTGPGPW[Oxi]GF. In this case, the amount of the peptide LTGPGPW[Oxi]GFR
235 at 6.5 months of the processing was the highest of all peptides showing tryptophan
236 oxidation as it could be generated from longer oxidised peptides. However, the quantity
237 of this peptide decreased at 9 months of processing probably due to its further
238 hydrolysis into shorter peptides. These results are in agreement with a previous work
239 reported by Gallego et al. (2014) in which most of the identified peptides from LDB3
240 protein showing methionine oxidation could derive from the hydrolysis of peptides
241 oxidised at earlier stages of processing.
242 A peptidomics approach was used in order to establish statistical differences between
243 the different stages of the dry-cured ham processing according to the influence of the
244 oxidised peptides and variance among them. Thus, a Principal Component Analysis
245 (PCA) score plot with two components was carried out, showing four statistically
246 different groups corresponding to 0 months (raw ham), 2 and 3.5 months (post-salting

247 and beginning of the ripening period), 5 and 6.5 months (ripening-drying stage), and 9
248 months (final dry-cured ham) (Figure 2A). Discriminant components 1 and 2 explain
249 20.3 and 17.8 % of the variability in the dataset, respectively, allowing the
250 differentiation between the earlier stages (from 0 to 3.5 months) from the latter stages of
251 the dry-cured ham processing (from 5 to 9 months). Although little is known about the
252 influence of each step of processing on oxidation mechanisms, the results of the present
253 work show that oxidative reactions are extended as the process advances due to the
254 effect of curing salts and conditions used for dry-curing.
255 The salt (NaCl) used during the salting stage of hams could exert a pro-oxidative effect
256 similar to that on lipids due to the link between the two processes as lipid oxidative
257 products could promote protein oxidation (Souza et al., 2013; Xiong, 2000; Soladoye et
258 al., 2015; Hęś, 2017). Moreover, salt can lead to changes on the conformation,
259 functionality, and solubility of myofibrillar proteins which favour their susceptibility to
260 radicals and pro-oxidant factors (Liu, Xiong, & Chen, 2011) as well as could alter the
261 iron state of myoglobin from ferrous (Fe^{2+}) to ferric (Fe^{3+}) increasing its pro-oxidant
262 potential (Souza et al., 2013). In this regard, Wang et al. (2011) reported that the salting
263 process of Xuanwei dry-cured ham boosted the hydrolysis of proteins, and increased
264 their oxidation although at a slower rate.
265 On the other hand, the nitrite added as curing agent in the salting process could offer
266 certain protection against both lipid and protein oxidation in meat products due to its
267 ability to chelate the free Fe^{3+} and react with meat pigments forming nitrosylmyoglobin-
268 Fe^{2+} (Honikel, 2008; Souza et al., 2013). However, other studies hypothesised that
269 nitrite could promote the formation of protein carbonyls in fermented sausages
270 (Villaverde, Ventanas, & Estévez, 2014) as well as convert reactive oxygen species
271 (ROS) into reactive nitrogen species (RNS) initiating oxidative and nitrosative reactions

(Skibsted, 2011). Regarding the ripening-drying process of hams, studies by Ventanas et al. (2006, 2007) showed the relationship between protein and lipid oxidation, reporting higher protein carbonylation as drying conditions of meat products were longer and more severe. Furthermore, Koutina et al. (2012) reported that the oxidation rate of proteins increased throughout the processing of Parma dry-cured ham but tended to stabilise towards the final stages of maturation.

Furthermore, the loading plot shown in Figure 2B revealed those proteins having the highest influence on the clustering of data. Thus, oxidised peptides generated from LDB3 and PDLIM3 proteins are the main responsible for the differences between raw hams (0 months) and the other stages of processing, whereas those derived from myosin differentiate the final product from those hams at initial stages. In this regard, peptides generated from the hydrolysis of myosin light chain protein were previously reported as the most influential at the end of the ham dry-curing process (Gallego et al., 2016).

The use of tandem mass spectrometry allows a fast, sensitive and accurate determination of the site and nature of peptide oxidation as modified amino acids are mass-shifted and thus identifiable in the fragmentation pattern (Schey & Finley, 2000). However, the difficulty in the analysis of oxidative modifications depends on i) the variety of differences in the molecular mass of peptides, showing increments of 16 Da for the consecutive addition of oxygen atoms or other mass shifts when oxidation is followed by other reactions, ii) the low amount and instability of modified peptides when analysed under MS analysis which complicate their identification and quantification, and iii) the matrix complexity and potential to be spontaneously oxidised during sample preparation, HPLC, or ionization that can make the interpretation of the results rather difficult (Parker, Mocanu, Mocanu, Dicheva, & Warren, 2010; Silva et al., 2013; Verrastro et al., 2015).

297 Only a few number of studies focused on peptide oxidation have been reported to date,
298 and most of the current knowledge about oxidative processes has been obtained from
299 evaluating protein oxidation through the quantification of carbonyl compounds.
300 However, peptidomics can be used as a complementary tool to the currently existing
301 methodologies, allowing to obtain more information about oxidation at peptide level as
302 well as to study the influence of the ham processing conditions on post-translational
303 modifications of the generated peptides.
304
305 **4. Conclusions**
306 A peptidomics strategy has proved to be useful to study the evolution of peptide
307 oxidation during the short processing of Spanish dry-cured hams, identifying and
308 quantifying a total of 67 peptides derived from main myofibrillar proteins showing
309 methionine, proline, and tryptophan oxidations. A label-free approach based on the
310 measurement of peak intensities was used for the relative quantification of oxidised
311 peptides, being possible to establish oxidation differences at different processing times
312 as a consequence of specific processing conditions. Thus, this study allows a better
313 knowledge of oxidative mechanisms and their effects at peptide level in the short
314 processing of Spanish dry-cured ham.
315
316 **Acknowledgements**
317 Grant AGL2014-57367-R and FEDER funds from the Spanish Ministry of Economy,
318 Industry and Competitiveness and postdoctoral contract to MG are acknowledged. Juan
319 de la Cierva de Incorporación postdoctoral contract to LM is also acknowledged. The
320 peptidomic analysis was performed in the proteomics facility of SCSIE University of
321 Valencia that belongs to ProteoRed, PRB2-ISCIII (PT13/0001-ISCIII-SGEFI/FEDER).

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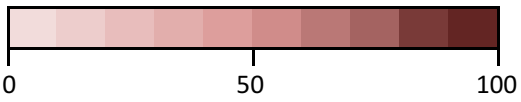
FIGURE CAPTIONS

Figure 1. MS/MS spectra of the peptides IDGVNTDTMTHL derived from LDB3 protein and VSPGTAIGKTPEM from nebulin protein, showing the oxidised methionines in blue.

Figure 2. A) Principal Component Analysis score plot to assess the variance among oxidised peptides at different times of curing; **B)** Loading plot showing the oxidised protein fragments affecting the score plot distribution. Discriminant component 1 (t[1]) and Discriminant component 2 (t[2]) explained a 20.3 and 17.8 % of variability in the dataset, respectively.

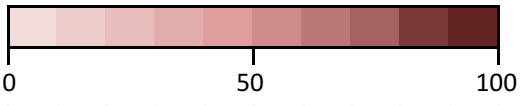
Table 1

Table 1. Peptides showing methionine oxidation identified by nLC-MS/MS and relatively quantified using a label-free approach based on peak intensity. Numerical quantitative values at different times of the dry-cured ham processing were expressed as percentage and converted into a color gradation: 

Protein*	Peptide sequence	Processing times (months) 0	2	3.5	5	6.5	9
LDB3	VAIDGVNTDTM[Oxi]THL						
	IDGVNTDTM[Oxi]THL						
	FNM[Oxi]PLTISRITPGSKAAQSQL						
	FNM[Oxi]PLTISRITPGSKA						
	FNM[Oxi]PLTISRITPGSK						
	FNM[Oxi]PLTISRITPG						
	FNM[Oxi]PLTIS						
	M[Oxi]PLTISRITPGSKA						
	VVAIDGVNTDTM[Oxi]THL						
	DAIM[Oxi]DAIAGQAQAQGSDFSGSLPIKD						
	M[Oxi]DAIAGQAQAQGSDFSGSLPIKD						
	KPPDIPDSRVPIPTM[Oxi]PIR						
MYH1	M[Oxi]AIFGEAAPYLRKSEK						
MYL1	EVKKVLGNPSNEEM[Oxi]						
NEBU	TM[Oxi]DPDVPQFIQA						
	DVSPGTAIGKTPEMM[Oxi]						
	VSPGTAIGKTPEMM[Oxi]						
	VSPGTAIGKTPEM[Oxi]						
	YKENVGKGTPTPVTPEM[Oxi]						
	VGKGTPTPVTPEM[Oxi]						
	MGKGTPLPVTPEM[Oxi]						
PDLIM3	APNIPLEM[Oxi]ELPGVKIVH						
	M[Oxi]E[Dhy]LPGVKIVHAQF						
	M[Oxi]ELPGVKIVHAQF						
	M[Oxi]ELPGVKIVHA						
	M[Oxi]ELPGVKI						
TITIN	KPPDIPDSRVPIPTM[Oxi]PIRAVPP						

*Protein name according to Uniprot database. LDB3: LIM domain-binding protein 3; MYH1: myosin heavy chain 1; MYL1: myosin light chain 1; NEBU: nebulin; PDLIM3: PDZ and LIM domain protein 3; TITIN: titin.

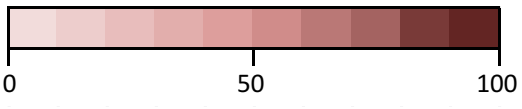
Table 2. Peptides showing proline oxidation identified by nLC-MS/MS and relatively quantified using a label-free approach based on peak intensity. Numerical quantitative values at different times of the dry-cured ham processing were expressed as percentage and converted into a color gradation:



Protein*	Peptide sequence	Processing times (months) 0	2	3.5	5	6.5	9
LDB3	FNMP[Oxi]LTISRITPGSKA						
	LTVDSASP[Oxi]VYQAVIK						
	TVDSASP[Oxi]VYQAVIK						
MYL1	VKKPAAAAAPAPAPAP[Oxi]AP[Oxi]APAPAPPKEEK						
	VKKPAAAAAPAPAP[Oxi]APAPAPAPAPPKEE						
	VKKPAAAAAPAPAPAPAP[Oxi]AP[Oxi]APAPPKE						
	VKKPAAAAAPAPAPAP[Oxi]APAPAPAPPKE						
	APAPAPAPAPAPPP[Oxi]EPAKEP						
	APAPAPAPAPAPAPP[Oxi]KEE						
	APAPAPAPAPAPAP[Oxi]APPKEEKI						
	APAPAPAPAPAPAP[Oxi]AP[Oxi]						
	PAPAP[Oxi]AP[Oxi]APAPAPAPPKEE						
	PAPAPAPAP[Oxi]APAPAPPKEEKID						
MYL3	APKKP[Oxi]EPKKDDAKAA						
	AP[Oxi]KKPEPKKDDAKA						
	AP[Oxi]KKPEPKKDDAK						
	APKKP[Oxi]EPKKDDAKAAAKAAPAPAPAPAPAPEPPKEPEFD						
	APKKP[Oxi]EPKKDDAKAAAKAAPAPAPAPAPAPEPPKEPEF						
	KAAPAPAPAPAPAP[Oxi]EP[Oxi]PKEPEF						
	APAPAPAPAPAP[Oxi]EPPKEPEF						
	APAPAPAPAPAPEPP[Oxi]KEPE						
	APAPAPAPAPAPEP[Oxi]PKEP						
	PAPAPAPAPAP[Oxi]EPPKEPEF						
	PAPAPAPAPAPEPP[Oxi]KE						
MYL4	APAPAPAPAPPP[Oxi]EP[Oxi]AKEP[Oxi]						
	APAPAPAPAPAPEP[Oxi]P[Oxi]KEP[Oxi]						
	PAPAPAPAPAPAPPP[Oxi]EPAKEP						
	APAPAPAPAPAPPPEPAKEP[Oxi]TFDP						
TITIN	IPAKVP[Oxi]EKKVPPPKVVKKPVVE						
	KIEEPPPTKVPEP[Oxi]P[Oxi]KKIVPE						
	LAP[Oxi]PQEPEAPPAKVPEAPKEVVPEK						
	KVPEVPKKPVPEKKVPAP[Oxi]TPK						
	PEKKVPVPVPKKVEPPPPP[Oxi]K						
	MKPP[Oxi]DIPDSRVPIPTMPIR						

*Protein name according to Uniprot database. LDB3: LIM domain-binding protein 3; MYL1: myosin light chain 1; MYL3: myosin light chain 3; MYL4: myosin light chain 4; TITIN: titin.

Table 3. Peptides showing tryptophan oxidation identified by nLC-MS/MS and relatively quantified using a label-free approach based on peak intensity. Numerical quantitative values at different times of the dry-cured ham processing were were expressed as percentage and converted into a color gradation:



Protein*	Peptide sequence	Processing times (months) 0	2	3.5	5	6.5	9
LDB3	SVTLTGPGPW[Oxi]GFRLQGGKD						
	SVTLTGPGPW[Oxi]GFR						
	LTGPGPW[Oxi]GFRLQGGKD						
	LTGPGPW[Oxi]GFRL						
	LTGPGPW[Oxi]GFR						
	LTGPGPW[Oxi]GF						

*Protein name according to Uniprot database. LDB3: LIM domain-binding protein 3.

Figure 1

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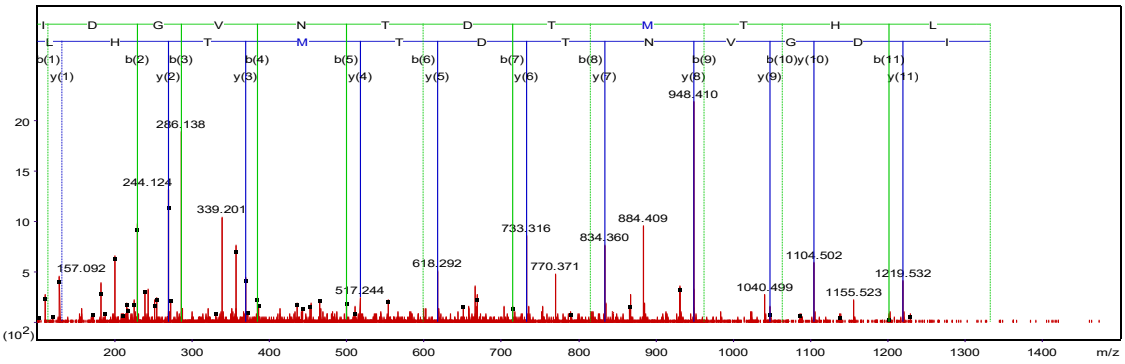
A) IDGVNTDTM[Oxi]THL (666.81, 2+)



B) VSPGTAIGKTPEM[Oxi] (652.33, 2+)

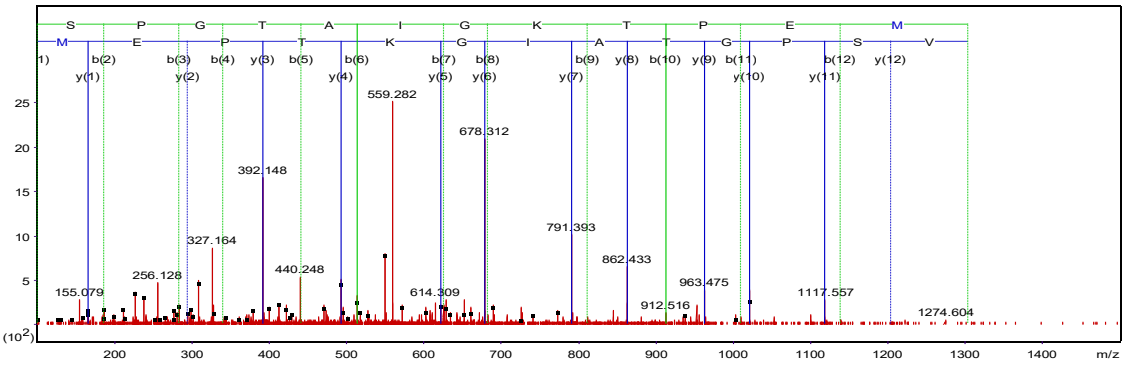


Figure 1.

Figure 2
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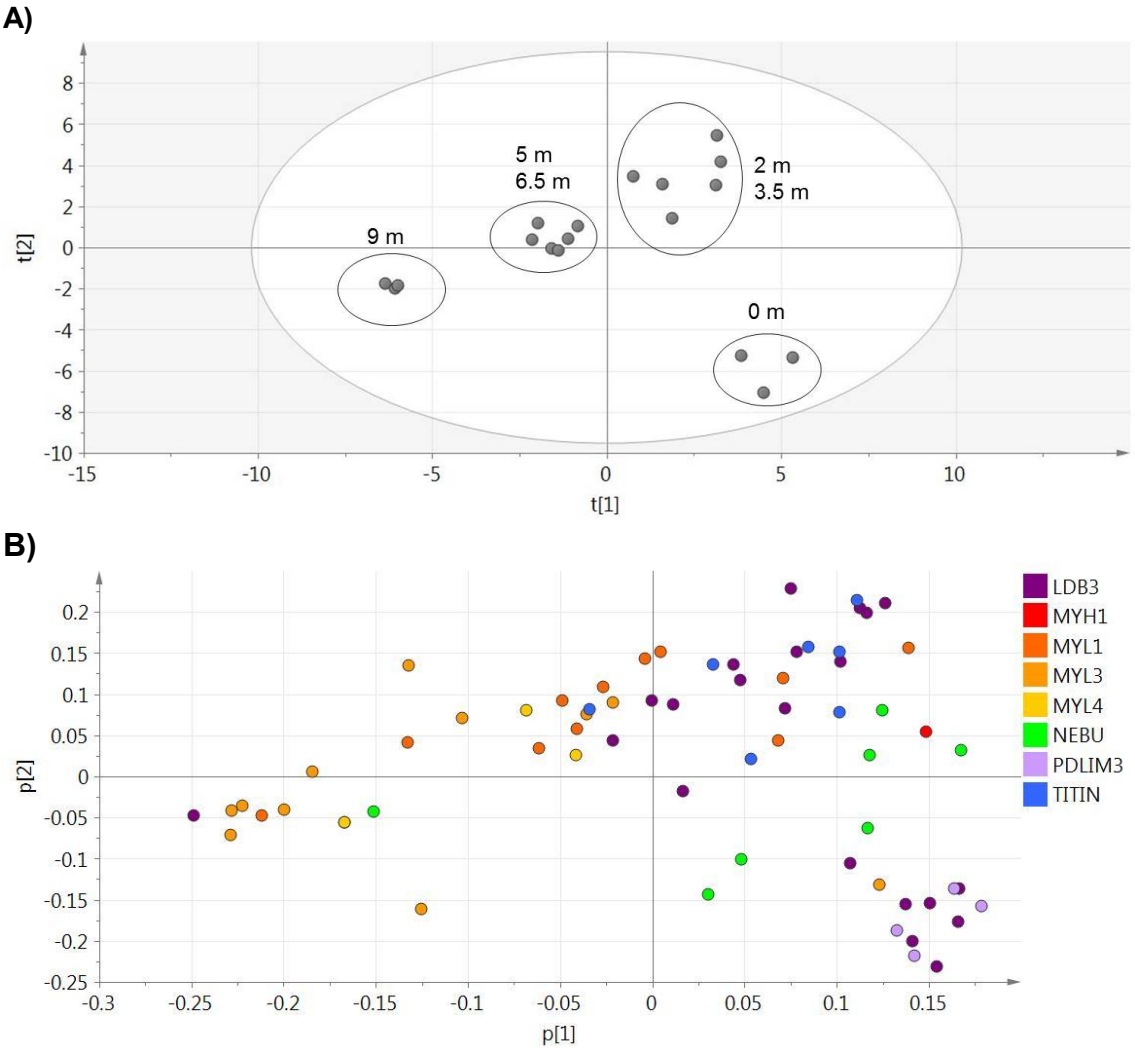

Figure 2.