Effect of proteolysis index level on instrumental adhesiveness, free amino acids content and volatile compounds profile of dry-cured ham

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Abstract

Defective textures in dry-cured ham are a common problem that causes important economic losses in the ham industry. An increase of proteolysis during the dry-cured ham processing may lead to high adhesiveness and consumer rejection of the product. Therefore, the influence of proteolysis index (PI) on instrumental adhesiveness, free amino acids and volatile profile of dry-cured ham was assessed. Two hundred Spanish dry-cured ham units were firstly classified according to their PI: low PI (<32%), medium PI (32-36%) and high PI (>36%). Instrumental adhesiveness was affected by PI, showing the lowest values in the batch with low PI. Significant differences (P<0.05) among groups were found in six amino acids: serine, taurine, cysteine, methionine, isoleucine and leucine. The content of leucine, serine, methionine, and isoleucine significantly (P<0.05) increased as the proteolysis index rose. However, taurine and cysteine content showed an opposite behaviour, reaching the highest values in the dry-cured hams with low PI.

Significant differences (P<0.001) in the total content of volatile compounds among ham groups were observed, with the highest concentration in the batch with low PI, and decreasing the concentration as the PI increased. Regarding the different chemical families of volatiles, the hydrocarbons (the main family), alcohols, aldehydes, ketones and acids were more abundant in the hams showing the lowest PI. Esters did not show significant differences among the three batches of hams studied. The present study demonstrated that, apart from the effect on the adhesiveness, an excessive proteolysis seems to be associated with negative effects on the taste and aroma of the dry-cured ham.

Keywords: Ham texture; Pastiness; Proteolysis; Texture defects; Nitrogen fraction; Aroma

1. Introduction

Texture is an important quality criterion for the certification of the Traditional Spanish dry-cured ham ‘‘Jamón Serrano’’ as a Guaranteed Traditional Speciality (Fundación Jamón Serrano, 1998). Dry-cured hams are usually classified into four texture types: very pasty, pasty, soft and normal, each identified by various properties (Harkouss *et al.*, 2015). Texture problems, such as pastiness, crusting and softness, frequently hinders slicing and provides a mouth-coating sensation, underlining the important role of texture for both retailer and consumer acceptability. Pastiness is a texture defect that appears in dry-cured ham when there is an excessive breakdown of the protein structure of the muscle due to the action of a series of autochthonous enzymes and therefore related to an excessive proteolysis.

Several authors reported that proteolysis activity in dry-cured ham is affected by many processing parameters such as water content, temperature, salt content, anatomic location and fresh ham pH (Ruiz-Ramirez, Arnau, Serra, & Gou, 2005; Ruiz-Ramirez, Arnau, Serra, & Gou, 2006; Bermudez, Franco, Carballo, Sentandreu, & Lorenzo, 2014). The intensity of proteolysis during dry-cured ham processing is often measured by the proteolysis index, that is defined as the non-protein nitrogen content expressed as percentage of the total nitrogen. In this regard, a relationship between proteolytic index (PI) and texture throughout the dry-cured ham manufacture process has been reported by several authors (García-Garrido, Quiles-Zafra, Tapiador, & Luque de Castro, 1999; Harkouss *et al.*, 2015; Ruiz-Ramírez *et al.*, 2006). In addition, Harkouss *et al.* (2015) showed that adhesiveness can be estimated as a function of PI values, water and salt content.

Proteolysis, one of the main biochemical reactions during the dry-cured ham processing, is considered to be the major contributor to texture changes (Jurado, García, Timón, & Carrapiso, 2007). It has been shown that textural defects are closely related to anomalous proteolysis (Virgili,

Parolari, Schivazappa, Soresi-Bordini, & Borri, 1995). In addition, lipolysis and proteolysis are the main biochemical reactions involved in the generation of a wide range of volatile compounds (Bermúdez, Franco, Carballo, & Lorenzo, 2015; Fulladosa, Garriga, Martín, Guàrdia, García-Regueiro, & Arnau, 2010). In this regard, the volatile compounds of dry-cured ham give an indication of the chemical and metabolic process that occurs during the ripening process. Several groups of volatile compounds have been reported in dry-cured ham, such as hydrocarbons, aldehydes, ketones, alcohols, esters, lactones, terpenes, nitrogen compounds, sulphur compounds, carboxylic acids and chloride compounds. These volatiles can be grouped according to their possible origin, into volatiles from lipid autooxidation (aldehydes, hydrocarbons, alcohols and ketones), microbial esterification (e.g. propyl acetate and ethyl propanoate), carbohydrate fermentation (e.g. 1,3-butanediol and phenyl acetaldehyde), amino acid catabolism (e.g. 2-methylbutanal, 2-methyl-1-butanol and 2,3-butanediol) and other origins (Narváez-Rivas, Gallardo, León-Camacho, 2012; Lorenzo, Montes, Purriños, & Franco, 2012; Lorenzo, Franco, & Carballo, 2014; Purriños, Carballo, & Lorenzo, 2013), although several of these volatiles may have more than one origin (Lorenzo, Bedia, & Banón, 2013). In order to deepen the knowledge of the correlation between proteolysis and the different sensory quality parameters of ham, the objective of this study was to assess the effect of the proteolysis index on the free amino acid and volatile profiles and the adhesiveness of Spanish dry-cured ham.

2. Materials and methods

2.1. Samples

Two hundred raw hams with a pH value<5.5, which were more prone to develop defective textures, were obtained from a commercial slaughterhouse. Hams were coming from pigs belonging to crosses of Large white and Landrace breeds (medium fat content). All animals (castrated male) were reared in the conditions. The pigs were allowed *ad libitum* access to water

and feed. The basal diet contained: barley (81.08%), lard (4.0%), soya (12.05%), methionine (0.08%), lysine (0.30%), threonine (0.11%), calcium carbonate (0.96%), dicalcium phosphate (0.66%), salt (0.33%) and minerals and vitamins (0.4%). All hams (n = 200) were weighted (11.9 kg ± 1.1 kg) and salted according to the traditional system. Hams were manually rubbed with the following mixture: 0.15 g of KNO_3, 0.15 g of $NaNO_2$, 1.0 g of dextrose, 0.5 g of sodium ascorbate and 10 g of NaCl per kilogram of raw ham. The hams were next pile salted at 3 ± 2 ºC and 85 ± 5% RH during 4 (n=50), 6 (n=50), 8 (n=50) or 11 days (n =50) according to their corresponding raw weight. After salting, hams were washed with cold water and post-salted at 3 ± 2 ºC and 85 ± 5% RH during 45 days. Drying of hams were performed at 12 ± 2 ºC and 70 ± 5% RH until reaching a weight loss of 29%, later they were vacuum packaged and kept at 30ºC during 30 days to induce proteolysis. After this time, hams continued the drying process at 12 ± 2 ºC and 65 ± 5% RH until reaching a weight loss of 34%, later they were vacuum packaged again and kept at 30ºC during 30 days more. After this period, hams were dried again until the end of the drying process (weight loss of 36%). At the end of the process, hams were cut and boned and the cushion part containing the *biceps femoris* muscle was excised and sampled. Ten slices from each dry-cured ham were vacuum packed and stored at room temperature (20 ºC) for no longer than 4 weeks, for texture and chemical analysis.

2.2. Instrumental adhesiveness

Textural analysis was performed using a texture analyzer (Stable Micro Systems, TA-XT Plus, London, UK) by carrying out a separation test using different load cells with a specific probe. Instrumental adhesiveness was measured in sliced ham samples (1 mm) by applying probe tests and calculating the negative area of a force-time curve in tension tests with a single-cycle. The texturometer was equipped with a probe connected to a special device that enables horizontal probe displacement. After the separation of the slices, the probe returned to the initial position.

117 The conditions for the measurement of adhesiveness of dry cured ham slices were reported by
118 Lopez-Pedrouso *et al.* (2018). From the obtained graph force vs. distance, the adhesiveness was
119 calculated. All the measurements were made in triplicate, at room temperature.

120 *2.3. Chemical analysis*

121 After instrumental adhesiveness determination, the *biceps femoris* samples were minced and
122 subjected to chemical analysis in triplicate. Water content was analysed by drying at 103 ± 2 °C
123 until reaching a constant weight (AOAC, 1990), whereas the chloride content was analysed
124 according to the ISO 1841-2 (ISO, 1996) standard using a potentiometric titrator 785 DMP Titrino
125 (Metrohm, Herisau, Switzerland), and results were expressed as percentage of NaCl.

126 *2.4. Nitrogen fraction analysis*

127 Total nitrogen content (NT) was determined according to the Kjeldahl method (ISO, 1978)
128 using the Vapodest 50S analyser (Gerhardt, Königswinter, Germany). It concerns a semi-micro
129 rapid routine method using block-digestion, copper catalyst and steam distillation into boric acid.
130 A known quantity of the sample (1 ± 0.1 g) was analysed.

131 The content of non-protein nitrogen was assessed as described by Lorenzo, García Fontán,
132 Franco, & Carballo (2008). Two and half g of sample were homogenised in 25 mL of deionized
133 water and centrifuged. Afterwards, 10 mL of 20% trichloroacetic acid (99.5% purity, Merck,
134 Darmstadt, Germany) were added, and the mix was stirred well and let to stabilize for 60 min at
135 room temperature. Next, it was centrifuged at 1734g during 10 min. After centrifugation, the
136 supernatant was filtered, and 15 mL of filtrate were used for determination of the nitrogen content,
137 following the same method used for the total nitrogen (NT) determination (ISO, 1978). The
138 proteolytic index (PI) was calculated as the ratio (non-protein nitrogen / total nitrogen) × 100
139 according to Ruiz-Ramírez *et al.* (2006).

Total volatile basic nitrogen (TVB-N) content was assessed according to the Commission Regulation (EC) No 2074/2005 (Commission Regulation, 2011). A 10 g sample of muscle was homogenized with 90 mL of perchloric acid, and the resulting suspension was centrifuged at 10000 g for 10 min using an Allegra X-22 centrifuge (Beckman Coulter, California, EEUU). Fifty mL of the supernatant were analysed for the nitrogen content following the Kjeldahl method using a Vapodest 50S analyzer (Gerhardt, Königswinter, Germany). The TVB-N values were expressed as mg nitrogen/100 g of dry matter.

Finally, dry-cured hams were categorized in different proteolysis index level groups according to their proteolysis index: low proteolysis level (IP <32%) (LP), medium proteolysis level (32%< IP >36%) (MP) and high proteolysis level (IP >36%) (HP).

2.5. Free Amino acid analysis

The amino acids were extracted following the procedure described by Lorenzo, Cittadini, Bermúdez, Munekata, & Domínguez (2015). Amino acids were derivatizated with 6-aminoquinolyl-Nhydroxysuccinimidyl carbamate (Waters AccQ-Fluor reagent kit) and analyzed by RP-HPLC using a Waters 2695 Separations Module with a Waters 2475 Multi Fluorescence Detector, equipped with a Waters AccQ-Tag amino acid analysis column. The results were expressed as mg of free amino acid/100 g of dry matter.

2.6. Volatile compound analysis

The extraction of the volatile compounds was performed using solid-phase microextraction (SPME). A SPME device (Supelco, Bellefonte, USA) containing a fused silica fibre (10 mm length) coated with a 50/30 layer of divinylbenzene/ carboxen/ polydimethylsiloxane was used. Headspace SPME extraction (from 1 g of sample) and chromatographic analyses were carried out under the conditions described by Domínguez, Gómez, Fonseca, and Lorenzo (2014). Volatile compounds were identified by comparing their mass spectra with those contained in the NIST14

(National Institute of Standards and Technology, Gaithersburg) library, and/or by comparing their mass spectra and retention time with authentic standards (Supelco, Bellefonte, PA, USA), and/or by calculation of retention index relative to a series of standard alkanes (C_5–C_{14}) (for calculating Kovats indexes, Supelco 44585-U, Bellefonte, PA, USA) and matching them with data reported in literature. The results are expressed as area units (AU) × 10^6 /g of dry matter.

2.7. Statistical analysis

The effect of proteolysis index group/level was examined using a one-way ANOVA, where this parameter was set as factor. The values were given in terms of mean values and standard error of the means (SEM). When a significant effect (P<0.05) was detected, means were compared using the Tukey´s test. Correlations between variables were determined by correlation analyses using the Pearson´s linear correlation coefficient. All analyses were conducted using the IBM SPSS Statistics 24.0 program (2016) software package.

3. Results and discussion

3.1. Instrumental adhesiveness, chemical parameters and nitrogen fractions

Table 1 shows the instrumental adhesiveness, chemical composition, nitrogen fractions and proteolysis index of dry-cured hams for the different proteolysis levels (low, medium and high). Several authors (Bermúdez *et al.*, 2014; Ruiz-Ramírez *et al.*, 2006; Virgili, Parolari, Schivazappa, Bordini, & Borri, 1995) noticed that proteolytic activity in ham is highly correlated to salt content. The negative relationship between salt content and proteolysis index has been extensively reported (Flores *et al.*, 2006; Armenteros, Aristoy, Barat, & Toldrá, 2009a; dos Santos *et al.*, 2015). In this regard, in the present study, it was found that there is a negative correlation (r=-0.218, P<0.01, data not shown) between the proteolysis index and the salt concentration. However, García-Garrido *et al.* (1999) showed hams of both normal and defective texture may contain salt contents from 6.2 to 8.1% in wet weight in agreement with the results of the present study (salt contents

188 ranging from 4.48 to 4.96%). Statistical analysis did not show significant differences on salt
189 content among the three PI levels studied, presenting mean values of 11.63 g/100 g dry matter.
190 No works related to instrumental adhesiveness of dry-cured ham slices were found in
191 literature. Results from this study showed that significant (P<0.001) differences between PI levels
192 groups were found. Thereby, the higher the PI, the higher the adhesiveness (71.43, 77.20 and
193 90.15 g, for LP, MP and HP groups, respectively). According to García-Garrido *et al.* (1999),
194 hams with a defective texture exhibited high moisture/protein ratios as a result of both increased
195 moisture and decreased protein contents related to hams with a normal texture.
196 The PI is defined as the percentage of non-protein nitrogen accounting for total nitrogen and
197 it is often used to describe the intensity of proteolysis during dry-cured ham processing. In Spanish
198 dry-cured ham, the PI reflecting a good quality could be considered between 33 and 36, whereas
199 in Italian dry-cured ham is between 22 and 30 (Careri, Mangia, Barbieri, Bouoni, Virgili, &
200 Parolari, 1993). Result in the present study are in agreement with data reported by other authors
201 (García-Garrido *et al.,* 1999; Pugliese *et al.*, 2015; Zhao, Tian, Liu, Zhou, Xu, & Li, 2008) who
202 observed values between 17.23 and 35.2 in dry-cured hams. These differences in PI among hams
203 could be due to differences in raw materials, salting procedure, ripening process, duration of steps,
204 and temperature and relative humidity used in the processing of dry-cured hams. In addition, Ruíz-
205 Ramírez *et al.* (2006) observed that the anatomic location of the muscle, the fresh ham pH, and
206 the amount of added NaCl affected the proteolysis index at the end of the dry-cured ham process.
207 Another noted relation is that stablished between the content of the nitrogen compounds and
208 the proteolysis reactions, because proteolytic processes break down the proteins giving rise to
209 smaller peptides and free amino acids (Armenteros, Aristoy, & Toldrá, 2009b). In addition,
210 Petrova, Tolstorebrov, Mora, Toldrá, and Eikevik (2016) noticed that the PI during dry-cured ham
211 processing is directly related to the enzymatic activity. In this regard, in the present study the non-

protein nitrogen content also showed significant (P<0.001) differences among ham groups, since the lowest values were observed in the LP batch (3.76 *vs*. 4.02 *vs*. 4.42 g/100 g of dry matter, for LP, MP and HP groups, respectively). This an expected result since the hams have been classified according to their IP. Low activity values of proteolytic enzymes would result in low protein degradation and in a smaller amount of non-protein nitrogen in samples (Petrova *et al.*, 2016). This finding is in agreement with data reported by García-Garrido *et al.* (1999) who observed that the non-protein nitrogen levels were 30% higher in hams of defective texture than in normal pieces. In addition, Martín, Córdoba, Antequera, Timón, and Ventanas (1998) noticed that the high temperatures during the drying stage stimulate the formation of non-nitrogenous compounds as the enzymatic activity increases.

Finally, the basic volatile nitrogen content was not affected by proteolysis index, showing this nitrogen fraction mean values of 389.88 mg/100 g of dry matter (Table 1). These values were higher than those reported by other authors in dry-cured ham (values ranging from 50 to 240 mg/100 g of dry matter) (Martín *et al.*, 1998; Ventanas, Córdoba, Antequera, García, López-Bote, & Asensio, 1992), and also higher than data reported by Lorenzo *et al.* (2008) in dry-cured lacón (85.6-109.7 mg/100 g of dry matter).

3.2. Free amino acids

The effect of proteolysis index on free amino acid content (expressed as mg/100 g dry matter) of dry-cured ham is shown in Table 2. No significant differences in the total amount of free amino acids among the three different groups (mean values of 5370 mg/100 g of dry matter) were observed. The total free amino acid content observed in the present study was higher than those in previous studies on dry-cured ham (about 4000 mg/100 g dry matter; Córdoba *et al.*, 1994; Martín *et al.*, 2001; Ruiz *et al.*, 1999). However, other studies showed higher total

concentrations in dry-cured ham (about 12,500 g/100 g dry matter, Jurado *et al.*, 2007; Zhao *et al.*, 2005).

In general, the free amino acid profile observed in the present study basically coincides with those reported in different types of dry cured ham (Jurado *et al.*, 2007; Martín *et al.*, 2001; Virgili, Saccani, Gabba, Tanzi, & Bordini, 2007; Zhao *et al.*, 2005, Bermúdez *et al.*, 2014). The individual free amino acids showed higher values in dry-cured hams with high PI, except for taurine, arginine, cysteine and lysine that presented higher concentrations in dry-cured ham with low PI than in medium and high PI groups. As discussed previously, an excess of proteolysis causes a texture defect which translates into a non-acceptance by the consumers; this excess of proteolysis also entails an increase in the concentration of nitrogen compounds of low molecular weight, such as peptides and free amino acids (Toldrá, 1998). The factors associated with animals (genotype, age, sex of animals, *pre* and *post-mortem* treatments) and the processing conditions and technological processes used (pH, humidity, water activity, time, temperature, salt concentration, etc.) have great important in the activity of the enzymes that cause proteolysis reactions (Sanz & Toldrá, 2002).

On the other hand, six of the 18 free amino acids quantified in this study showed significant differences among PI levels ($P<0.05$): serine, taurine, cysteine, methionine, isoleucine and leucine (Table 2). Leucine was the major amino acid in all groups, showing a significant increase ($P<0.01$) when proteolysis index increased (566.83, 586.15 and 623.75 mg/100 g of dry mater for LP, MP and HP batches, respectively). A similar trend was observed for serine, methionine and isoleucine, showing the highest levels in dry-cured hams with high PI. However, taurine and cysteine content presented an opposite behaviour, reaching the highest values in dry-cured hams with low PI (Table 2). According to Bermúdez *et al.* (2014), the free amino acid content variations depend on the ratio between free amino acid formation and degradation. In addition, during ripening process the

enzymes continue the protein degradation producing mainly small peptides and free amino acids (Toldrá, 2006). Some of these free amino acids contribute directly to taste (Jurado *et al.*, 2007), whereas other ones participate indirectly in flavor development because they are precursors of many odorants (Hidalgo & Zamora, 2004) important for dry-cured meat products.

These differences in the individual free amino acid content among the three ham groups studied could induce differences in flavour. In this regard, Henriksen and Stahnke (1997) noticed that specific amino acid groups might have an impact exceeding the individual effects on sensorial properties. In this sense, the concentration of alanine, serine, proline, threonine and glycine is related with sweet taste; bitter taste is mainly associated with aromatic amino acids such as leucine, valine, isoleucine, methionine, while phenylalanine, histidine, glutamic and aspartic acids impart an acid taste, and a characteristic aged flavour have been linked to lysine, tyrosine and aspartic acid (Table 2). According to the free amino acid profile and to the differences observed in the present study, our results seem to indicate that only bitter taste could be significantly (P<0.05) affected by PI, presenting the highest values in dry-cured hams with high PI. This result is in agreement with data reported by other authors (Careri *et al.*, 1993; Parolari, Virgili, & Schivazappa, 1994) who noticed that an excess of proteolysis is undesirable because it may give a bitter or metallic aftertaste in dry-cured hams.

3.3. Volatile compounds

Table 3 shows the effect of proteolysis index on the volatile compounds profile of dry-cured ham. An increase in the relative abundance of total volatiles in headspace of ham might suppose a more intense odor or flavour, or not, or it might have a negative or positive effect; this will depend on the type of volatile compounds that are formed. Thirty-nine volatile compounds were identified and quantified and they were classified into the following chemical families: hydrocarbons (14), alcohols (5), aldehydes (4), esters (2) ketones (4) acid (1), sulfur compounds (1) and other

283 compounds (2) according to Lorenzo & Carballo (2015). Most of the volatile compounds come
284 from chemical or enzymatic oxidation of unsaturated fatty acids and further interactions with
285 proteins, peptides and free amino acids. Other volatile compounds result from Strecker degradation
286 of free amino acids and Maillard reactions (Toldrá & Flores, 1998). Statistical analysis showed
287 significant differences (P<0.001) in the total volatiles content between groups, with the highest
288 concentration observed in the batch with low PI, and decreasing as the proteolysis index increased
289 (1575.24 *vs*. 133781 *vs*. 997.49 AU × 10^6 /g of dry matter for LP, MP and HP batches, respectively).
290 As shown in Table 3, the main family of volatile compounds were the hydrocarbons. These
291 compounds derived from the oxidative decomposition of lipids, which may be catalyzed by
292 hemocompounds such as hemoglobin and myoglobin (Ramírez, & Cava, 2007). In addition,
293 Martín, Córdoba, Aranda, Córdoba, & Asensio (2006) suggested that methyl hydrocarbons could
294 be synthesized by molds as a product of secondary degradation of triglycerides. It was observed
295 a higher content of hydrocarbons in the batch with lower PI compared to the other ones (759 *vs*.
296 605 *vs*. 416 AU × 10^6 /g of dry matter for LP, MP and HP groups, respectively). These outcomes
297 could be due to the greater lipid oxidation in the low PI ham group compared to the other two
298 groups. However, at the sensory level, these differences do not have a great impact on the quality
299 of the final product since the hydrocarbons are compounds that have little contribution to aroma
300 because of their high odour threshold values (Wu *et al.*, 2015). Among hydrocarbons, undecane
301 was the most abundant in the three ham categories studied and this compound could be used to
302 discriminate dry-cured hams according to their PI.
303 Regarding alcohols, significant differences were observed in the total content (P<0.001)
304 among groups, as well as in all of individual compounds. In all cases, the highest values
305 corresponded to the hams with lower PI (Table 3). Alcohols follow the same mechanism of
306 generation as acids; straight-chain aliphatic alcohols can be generated by the oxidation of lipids,

whereas branched alcohols are most likely derived from the Strecker degradation of amino acids through the reduction of their respective aldehydes (Pérez-Palacios, Ruiz, Martín, Grau, & Antequera, 2010). Alcohols, because of their low odour threshold, contribute to the aroma of ham, with fatty, woody and herbaceous notes (Garcia, & Timón, 2001). Among the alcohols, in the three ham groups, ethyl alcohol was the most abundant and represented about 72% of the total alcohols. On the other hand, high 1-octen-3-ol content was also found in the three groups (60.28 *vs*. 47.67 *vs*. 30.22 AU × 10^6 /g of dry matter for LP, MP and HP groups, respectively). Mainly in hams with low PI, this alcohol has low odor threshold and is associated with mushroom-like, earth, dust, fatty, sharp and rancid odors (García-González, Tena, Aparicio-Ruiz, & Morales, 2008; Théron, Tournayre, Kondjoyan, Abouelkaram, Santé-Lhoutellier, & Berdagué, 2010). In addition, it was found a positive correlation between 1-octen-3-ol and cysteine (r=0.766; P<0.01).

Aldehydes are known as the major contributors to the unique flavour of dry-cured ham due to their rapid formation during lipid oxidation and their low odour thresholds (Ramírez, & Cava, 2007). Linear aldehydes come mainly from an oxidative degradation of the unsaturated fatty acids: oleic, linoleic, linolenic and arachidonic (Sabio, Vidal-Aragon, Bernalte, & Gata, 1998; Chan, & Coxon, 1987). On the other hand, the major formation pathway of the branched chain aldehydes seems to be the oxidative deamination-decarboxylation, probably via Strecker-degradation (Narváez-Rivas *et al.*, 2012). Statistical analysis showed that the total aldehyde content was significantly affected (P<0.001) by PI, reaching the highest values in dry-cured hams with low PI (232.10 *vs*. 195.75 *vs*. 140.52 AU × 10^6 /g of dry matter for LP, MP and HP groups, respectively). Within aldehydes, hexanal was the most abundant, showing significant differences (P<0.001) among batches (104.42 *vs*. 79.42 *vs*. 43.87 AU × 10^6 /g of dry matter for LP, MP and HP groups, respectively). Hexanal at low concentrations has a pleasant and grassy aroma (Aparicio, & Morales, 1998), which turns fatty at medium concentration and extremely rancid and tallowy at high

331 concentrations (Morales, Rios, & Aparicio, 1997). At the concentrations determined in the analyzed
332 hams, hexanal contributes to grassy odour in hams with high PI, and, perhaps, to a fatty perception
333 in the case of hams with low PI. It was found a positive correlation between cysteine and hexanal
334 (r=0.599; $P<0.01$), heptanal (r=0.516; $P<0.01$) and benzeneacetaldehyde (r=0.561; $P<0.01$).
335 The low odour thresholds of ketones indicate that they have a great impact on ham aroma. In
336 dry-cured ham, their origin can be diverse. Ramírez, & Cava (2007) found that the majority of
337 ketones originated from lipid oxidation, whereas a few others, such as 3-hydroxybutan-2-one, are
338 formed through Maillard reactions; the methyl ketones are generated by microorganism
339 esterification. Hams with low PI presented higher total ketones content than those in the two other
340 groups (48.85 *vs*. 43.22 *vs*. 42.06 AU × 10^6 /g of dry matter for LP, MP and HP groups,
341 respectively). Although, the concentration of 2-heptanone did not show significant differences
342 among groups, this compound contributes to ham aroma with spicy/blue cheese/acorn sensory notes
343 due to low odour thresholds. Esters did not show significant differences among the three batches
344 studied (40.92 *vs*. 35.45 *vs*. 38.11 AU × 10^6 /g of dry matter for LP, MP and HP groups,
345 respectively). Esters are formed through the enzymatic esterification of fatty acids and alcohols
346 during curing, mostly by the action of microorganisms such as lactic acid bacteria and
347 *Micrococcaceae* (Purriños, Bermúdez, Franco, Carballo, & Lorenzo, 2011). Esters have low
348 olfaction threshold values; however, taking into account that the analysed samples presented very
349 low values of these compounds, it can be considered that they do not contribute to the aroma of dry-
350 cured ham.
351 Sulfur compounds mainly originate from the catabolism of amino acids that contain sulfur
352 (Sabio *et al.*, 1998; Ramírez, & Cava, 2007) from ribonucleotides (Dumont, & Ada, 1972), or they
353 are generated by the microbial population (Martín *et al.*, 2006). Only a sulfur compound, dimethyl
354 disulphide, was found at very low concentrations in the three batches studied, and its presence could

355 come from the degradation of sulfur amino acids through a microbial deamination (Belitz, &
356 Grosch, 1999). However, a significant correlation between dimethyl disulphide and cysteine,
357 taurine and methionine was not found.

358 Finally, acetic acid was the only acid identified in the headspace of the dry-cured ham
359 samples, showing the highest content in hams with low PI (55.16 vs. 40.46 vs. 35.72 AU × 106 /g
360 of dry matter for LP, MP and HP groups, respectively). This outcome is in agreement with data
361 reported by Pérez-Juan, Flores, and Toldrá. (2006) who observed that acetic acid was the most
362 abundant acid detected in dry-cured ham. The main straight-chain carboxylic acids are derived from
363 the hydrolysis of triglycerides and phospholipids and mainly from the oxidation of unsaturated fatty
364 acids (Pugliese *et al.*, 2015). In addition, some branched acids could be also originated from the
365 oxidation of their respective Strecker aldehydes, for example, 2-methyl butanal would come from
366 the degradation of isoleucine amino acid and 2-methyl butanoic acid would be formed from later
367 oxidation (Ramírez, & Cava, 2007). The origin of acetic acid in ham is not clear. According to some
368 authors, this is originated from carbohydrate fermentation by microorganisms (Kandler, 1983) and
369 from the Maillard reaction according to others (Martín *et al.*, 2006).

370 Most of the volatile compounds detected in the present study come from the oxidation of
371 lipids. Usually the processing conditions that favour the lipid oxidation (e.g. increased salt content)
372 inhibit the action of proteolytic enzymes. This is probably the reason by which hams having the low
373 PI showed the highest amounts of most of the volatile compounds determined. Apart from the
374 volatiles formed directly from the lipid oxidation, oxidized lipids formed during ripening could
375 react with the free amino acids converting them into Strecker aldehydes, α-keto acids and amines.
376 The lipid oxidation products (free radicals and reactive carbonyls) can also influence the subsequent
377 reactions suffered by these compounds: the formation of Strecker aldehydes and other aldehydes
378 from α-keto acids, the formation of Strecker aldehydes and olefins from amines, the formation of

shorter aldehydes from Strecker aldehydes, and the addition reactions suffered by the olefins produced from the amines (Hidalgo and Zamora, 2016). This could be the most probable origin of the butanal, 3-methyl (from leucine) and the benzeneacetaldehyde (from phenylalanine) detected in the present study; the 1-butanol, 3-methyl also probably comes from reduction of the butanal, 3-methyl having this same origin. The formation of these Strecker aldehydes from Maillard reactions is unlikely in hams, given the very low amounts of reducing sugars present in such food matrix. On the other hand, these compounds could also be formed by reactions between protein carbonyls and amino acids (Estévez, Ventanas, & Heinonen, 2011), but this origin in the present study is also unlikely, given that hams with a more intense proteolysis were those that presented the lowest values of these two compounds.

Due to the non-polar character of most of the volatiles determined, the special structure of the hams with abundant fat infiltrated in muscle tissue could favor the retention of these compounds. Fat solubilizes and traps these compounds avoiding its loss in the prevailing environmental conditions during maturation.

4. Conclusions

Proteolysis significantly increased the adhesiveness of dry-cured ham. The basic volatile nitrogen content and total free amino acid content was not significantly affected by the proteolysis index. Individual free amino acids content was higher in dry-cured hams with high PI level, except for taurine, arginine, cysteine and lysine that showed higher concentrations in the dry-cured hams with low PI levels. The bitter amino acids were significantly (P<0.05) affected by PI, showing the highest values in high PI level. Total content of volatile compound were significantly different among PI level groups, showing a decrease with the increase of the proteolysis index. Regarding the different chemical families of volatiles, the hydrocarbons (the main family), alcohols, aldehydes, ketones and acids were more abundant in the hams showing the lowest PI. However,

esters did not show significant differences among the three batches of hams studied. Most of the volatile compounds detected in the present study come from the oxidation of lipids. Usually the processing conditions that favour the lipid oxidation inhibit the action of proteolytic enzymes. This is probably the reason by which hams having the low PI showed the highest amounts of most of the volatile compounds determined. Apart from the effect on the adhesiveness, an excessive proteolysis seems to be associated with negative effects on the taste and aroma of the dry-cured ham.

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608

Highlights:

► An excessive proteolysis influenced negatively the aroma of dry-cured hams

► The total free amino acid content was not affected by the proteolysis index (PI)

► The content of leucine, serine, methionine, and isoleucine significantly increased as the PI rose.

► The highest concentration of volatile compounds were observed in hams with low PI

Table 1

Effect of proteolysis index on instrumental adhesiveness, chemical parameters and nitrogen fractions of dry-cured ham

Parameters	Groups			SEM	P-value
	LP	MP	HP		
Instrumental adhesiveness (g)	71.43[a]	77.20[a]	90.15[b]	1.580	0.005
Moisture (%)	58.98	58.83	58.86	0.071	0.065
Salt (% dry matter)	11.88	11.86	11.16	0.135	0.067
TN (% dry matter)	11.85	11.76	11.70	0.027	0.062
NPN (% dry matter)	3.76[a]	4.02[b]	4.42[c]	0.025	<0.001
TBVN (mg/100 g dry matter)	385.79	389.21	394.65	2.612	0.112
Proteolysis Index (%)	31.10[a]	34.50[b]	38.59[c]	0.249	<0.001

[a-c]Mean values in the same row (corresponding to the same parameter) not followed by a common letter differ significantly ($P<0.05$; Tukey´s Test)

SEM: standard error of mean

Groups: LP = low proteolysis (PI < 32%); MP= medium proteolysis (32%< PI > 36%) and HP = high proteolysis (PI>36%).

TN: Total Nitrogen; NPN: Non-protein nitrogen; TBVN: Total basic volatile nitrogen

Table 2

Effect of proteolysis index on free amino acids content (expressed as mg/100 g dry matter) in dry-cured ham

Amino acids	Groups			SEM	P-value
	LP	MP	HP		
Aspartic acid	183.62	181.84	182.64	2.53	0.961
Serine	176.95[a]	193.86[b]	203.05[b]	2.92	0.001
Glutamic acid	451.20	447.19	462.25	5.70	0.538
Glycine	198.66	194.48	195.69	2.54	0.788
Histidine	98.08	102.24	100.76	1.44	0.497
Taurine	97.45[b]	91.18[ab]	85.97[a]	1.27	0.001
Arginine	397.50	386.43	379.00	5.62	0.398
Threonine	209.87	220.07	223.57	2.83	0.117
Alanine	414.96	406.56	416.48	5.11	0.706
Proline	275.58	279.87	290.94	3.43	0.163
Cysteine	443.09[b]	286.77[a]	269.44[a]	9.93	<0.001
Tyrosine	189.49	194.15	197.59	2.78	0.485
Valine	383.68	389.34	400.21	4.42	0.291
Methionine	194.50[a]	206.57[ab]	216.58[b]	2.61	0.002
Lysine	266.70	251.38	248.40	3.70	0.094
Isoleucine	338.43[a]	349.81[ab]	371.49[b]	4.20	0.004
Leucine	566.83[a]	586.15[ab]	623.74[b]	6.89	0.002
Phenylalanine	374.93	392.15	400.76	4.62	0.061
Total free amino acids	5399.04	5333.27	5406.31	62.77	0.878
Flavors					
Sweet[1]	1235.86	1267.92	1299.09	12.348	0.096
Bitter[2]	1860.39[a]	1924.03[ab]	2003.98[b]	21.417	0.018
Acid[3]	718.79	729.23	737.25	7.775	0.601
Aged[4]	632.46	621.49	623.19	5.852	0.703

[a-b] Mean values in the same row (corresponding to the same parameter) not followed by a common letter differ significantly ($P<0.05$; Tukey´s Test)

SEM: standard error of mean.

Groups: LP = low proteolysis (PI < 32%); MP= medium proteolysis (32%< PI > 36%) and HP = high proteolysis (PI>36%)

[1]Sweet flavor = ∑ of alanine, glycine, threonine, serine and proline; [2] Bitter flavor = ∑ of leucine, valine, isoleucine, methionine and phenylalanine; [3]Acid flavor = ∑ of glutamic acid, aspartic acid and histidine; [4]Aged flavor = ∑ of lysine, tyrosine and aspartic acid

Table 3

Effect of proteolysis index on volatile compounds content (expressed as Area Units (AU) x10^6 / g dry matter) of dry-cured ham

Volatile compounds	LRI	R	Groups			SEM	P-value
			LP	MP	HP		
Octane	800	*ms,lri,s*	57.49^{c}	36.59^{b}	26.07^{a}	1.759	<0.001
Decane	1000	*ms,lri,s*	61.95^{c}	47.44^{b}	30.13^{a}	1.753	<0.001
Undecane	1100	*ms,lri,s*	143.42^{c}	123.26^{b}	71.39^{a}	4.014	<0.001
6-Tridecene	1223	*ms*	10.66^{b}	10.11^{b}	6.05^{a}	0.442	<0.001
Dodecane	1200	*ms,lri,s*	84.90^{b}	80.25^{b}	46.84^{a}	2.366	<0.001
Tridecane	1300	*ms,lri,s*	27.44^{b}	26.79^{b}	17.07^{a}	0.729	<0.001
Total lineal hydrocarbons			377.69^{c}	323.96^{b}	194.36^{a}	9.309	<0.001
Pentane, 2,3,4-trimethyl-	666	*ms*	13.92^{ab}	15.54^{b}	11.50^{a}	0.53	0.006
Pentane, 2,3,3-trimethyl-	675	*ms*	33.08^{b}	18.68^{a}	24.44^{a}	1.239	<0.001
Heptane, 3-methylene-	743	*ms*	30.73^{b}	22.33^{a}	19.37^{a}	0.905	<0.001
Heptane, 3-ethyl-	866	*ms,lri*	24.88^{c}	15.49^{b}	9.48^{a}	0.761	<0.001
2,3-Dimethyl-3-heptene, (Z)-	898	*ms*	8.69^{b}	6.49^{a}	5.83^{a}	0.254	<0.001
Octane, 3-ethyl-	996	*ms*	23.39^{b}	19.23^{a}	16.15^{a}	0.611	<0.001
Nonane, 3-methyl-	999	*ms*	16.97^{c}	12.85^{b}	8.94^{a}	0.422	<0.001
Cyclohexane, 1,2-diethyl-1-methyl-	1041	*ms*	13.85^{c}	11.27^{b}	5.69^{a}	0.449	<0.001
Cyclopentane, pentyl-	1082	*ms*	66.26^{c}	50.50^{b}	33.98^{a}	2.151	<0.001
5-Undecene, 9-methyl-, (Z)-	1169	*ms*	78.70^{c}	64.56^{b}	34.22^{a}	2.05	<0.001
Undecane, 3-methyl-	1215	*ms*	31.98^{c}	27.08^{b}	19.68^{a}	0.844	<0.001
Undecane, 3-methylene-	1233	*ms*	12.58^{b}	13.04^{b}	8.69^{a}	0.405	<0.001
5-Undecene, 3-methyl-, (E)-	1235	*ms*	12.46^{c}	9.92^{b}	5.77^{a}	0.527	<0.001
10-Methylnonadecane	1293	*ms*	2.92^{b}	2.68^{b}	1.92^{a}	0.117	<0.001
Total branched hydrocarbons			347.95^{c}	283.77^{b}	213.44^{a}	9.29	<0.001
Total hydrocarbons			759.93^{c}	605.28^{b}	416.99^{a}	21.711	<0.001
2-Pentanone	620	*ms,lri*	10.82^{b}	7.94^{a}	10.66^{b}	0.307	<0.001
2-Butanone, 3-hydroxy-	711	*ms,lri*	25.60^{b}	21.52^{a}	19.56^{a}	0.531	<0.001
3-Heptanone	940	*ms*	4.24^{c}	2.97^{b}	2.20^{a}	0.147	<0.001
2-Heptanone	950	*ms,lri*	11.08	11.13	9.93	0.264	0.089
Total ketones			48.85^{b}	43.22^{a}	42.06^{a}	0.654	<0.001
Ethylalcohol	307	*ms*	256.05^{b}	255.00^{b}	223.95^{a}	5.257	0.018
1-Butanol, 3-methyl-	737	*ms*	23.73^{c}	17.61^{b}	12.99^{a}	0.92	<0.001
1-Hexanol	932	*ms,lri*	20.43^{b}	17.67^{b}	11.70^{a}	0.812	<0.001
1-Octen-3-ol	1062	*ms,lri*	60.28^{c}	47.67^{b}	30.22^{a}	2.208	<0.001
Benzyl Alcohol	1157	*ms,lri*	24.78^{c}	21.97^{b}	17.53^{a}	0.444	<0.001
Total Alcohols			364.49^{b}	357.68^{b}	299.65^{a}	6.092	<0.001
Butanal, 3-methyl-	537	*ms,lri*	82.17^{b}	82.72^{b}	68.65^{a}	1.985	0.005
Hexanal	814	*ms,lri,s*	104.42^{c}	79.42^{b}	43.87^{a}	3.592	<0.001

Heptanal	959	*ms,lri,s*	21.64^{c}	17.02^{b}	11.60^{a}	0.554	<0.001
Benzeneacetaldehyde	1154	*ms*	22.85^{c}	17.34^{b}	14.55^{a}	0.572	<0.001
Total Aldehydes			232.10^{c}	195.75^{b}	140.52^{a}	5.969	<0.001
Acetic acid, ethylester	437	*ms*	35.57	31.22	34.97	0.909	0.128
Decanoic acid, ethylester	1442	*ms*	4.92^{c}	4.13^{b}	3.23^{a}	0.123	<0.001
Total Esters			40.92	35.45	38.11	0.937	0.072
Acetic acid	571	*ms*	55.16^{b}	40.46^{a}	35.72^{a}	1.519	<0.001
Total Acids			55.16^{b}	40.46^{a}	35.72^{a}	1.519	<0.001
Disulfide, dimethyl	702	*ms,lri*	4.86^{a}	6.06^{b}	4.30^{a}	0.179	<0.001
Total Sulfur Compounds			4.86^{a}	6.06^{b}	4.30^{a}	0.179	<0.001
Pyrazine, 2,6-dimethyl-	964	*ms,lri*	15.44^{b}	13.74^{a}	14.21^{ab}	0.259	0.029
Ethanol, 2-butoxy-	974	*ms*	41.42^{b}	31.17^{a}	25.95^{a}	1.187	<0.001
Total Other Compounds			56.86^{b}	44.91^{a}	40.16^{a}	1.328	<0.001
Total Compounds			1575.24^{c}	1337.81^{b}	997.49^{a}	37.224	<0.001

$^{a-c}$ Mean values in the same row (corresponding to the same parameter) not followed by a common letter differ significantly (P<0.05; Tukey´s Test)

SEM: standard error of mean; LRI: Lineal Retention Index calculated for DB-624 capillary column (J&W scientific: 30 m × 0.25 mm id, 1.4 µm film thickness) installed on a gas chromatograph equipped with a mass selective detector; R: Reliability of identification; lri: linear retention index in agreement with literature (Dominguez *et al.*, 2014; Flores *et al.*, 2005; Pateiro *et al.*, 2015); ms: mass spectrum agreed with mass database (NIST14); s: mass spectrum and retention time identical with an authentic standard

Groups: LP = low proteolysis (PI < 32%); MP= medium proteolysis (32%< PI > 36%) and HP = high proteolysis (PI>36%)