1 Ability of peptide extracts obtained at different dry cured ham ripening
2 stages to bind aroma compounds

3 Isadora Martínez-Arellano, Mónica Flores*, Fidel Toldrá.

4 Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC) Avda. Agustín
5 Escardino 7, 46980 Paterna, Valencia, Spain.

6 *Corresponding author

7 *Email address:* mflores@iata.csic.es (F. Toldrá)

8 Abstract

9 The ability of peptide extracts obtained at different dry-cured ham ripening-stages to
10 bind volatile compounds has been examined using solid-phase microextraction and gas-
11 chromatography. The peptide extracts from dry-cured ham were previously defatted and
12 deodorised in order to be able to study peptide-volatile interactions. The binding effect of
13 each peptide extracts to volatile compounds was analysed **at different concentrations**. In the
14 presence of peptide extracts a release was observed for ethyl butyrate, ethyl 2-
15 methylbutyrate, ethyl 3-methylbutyrate and 3-methylthiopropional. On the other hand,
16 retention of about 20 and 30% was observed for 2-methylpropanal, hexanal and ethyl acetate
17 while the highest interaction was observed for trimethylpyrazine. All peptide extracts did not
18 exert any binding effect on 2-methylbutanal. No significant differences in binding-ability
19 were detected for the peptides obtained at different ripening-stages; therefore, the binding-
20 ability of peptide extracts was mainly based on volatile chemical characteristics and not on
21 the type of peptide extract obtained.

22

23 Keywords: volatile compounds, peptide extracts, interaction, dry-cured ham.

1. Introduction

Nearly 39 million dry-cured hams per year are manufactured in Spain, being this country **the world's biggest** producer (Estévez, Ventanas, Morcuende & Ventanas, 2015). Currently, there are five Protected Designations of Origin (PDO) in Spain; one of them is called "**Teruel dry-cured ham**" where hams are produced from heavy white pigs (Latorre, Ripoll, García-Belenguer & Ariño, 2009). Colour, texture, appearance and flavour determine the dry-cured ham quality (Ruiz, García, Muriel, Andrés & Ventanas, 2002). A long process of manufacturing including the stages of salting, post salting, and ripening is necessary to obtain a high quality dry-cured ham. Different biochemical reactions like proteolysis, lypolysis, glycolysis and transformations of nucleotides take place during these stages (Toldrá & Flores, 1998). Within these reactions proteolysis and lypolysis integrate two of the most important enzymatic phenomena. They generate compounds with high influence on taste, aroma and texture (Toldrá, 2006). Proteolysis is responsible for tenderisation, because an intense degradation of the myofibrillar structure is detected during the drying-curing process. Also, a large number of peptides resulting from muscle proteins like myosin, actin, troponin T, glycolytic enzyme, titin, LIM-domain breakdown have recently been identified in dry-cured ham (Mora, Sentandreu, Koistinen, Fraser, Toldrá & Bramley, 2009; Mora, Sentandreu & Toldrá, 2010; Gallego, Mora, Fraser, Aristoy & Toldrá, 2014). These peptides were identified by proteomic strategy and can be useful as industrial biomarkers for quality optimisation. These peptides also show antihypertensive effect and antioxidant activity (Escudero, Mora & Toldrá, 2014). Regarding flavour, several amino acids such as glutamic acid, glycine, alanine, valine, proline, histidine and leucine have been found in savoury fractions of dry-cured ham. Some small peptides were

also selected with interesting characteristics like ham flavour, salty, bitter, umami tastes in Spanish dry-cured ham (Sentandreu, Stoeva, Aristoy, Laib, Voelter & Toldrá, 2003).

Flavour is related to non-volatiles compounds like free amino acids and small peptides while aroma is due to the formation of volatile compounds (Flores, Sanz, Spanier, Aristoy & Toldrá, 1998). Several researches identified and quantified more than 261 volatile compounds in dry-cured ham from different origins such as French, Italian, and Spanish (Toldrá, Aristoy & Flores, 2009). The identified volatile compounds belonged to the different chemical classes: alkanes, alkenes, aldehydes, ketones, alcohols, aromatic hydrocarbons, carboxylic acids, esters, terpenes, sulphur compounds, furans, pyrazines, amines and chloride compounds (Flores, Grimm, Toldrá & Spanier, 1997). The final dry-cured ham aroma is based on volatile compounds concentration, odour threshold and the binding with the food matrix. Polarity, pH, molecular weight among others characteristics of volatile compounds affect the binding; therefore, it is fundamental to consider them (Seuvre, Espinosa Diaz & Voilley, 2002). However, there are few studies dealing with protein-flavour binding, being milk (Andriot, Marin, Feron, Relkin & Guicharda, 2000; Kühn, Considine & Singh, 2006) and soy proteins (Solina, Baumgartner, Johnson & Whitfield, 2005) those more investigated. In addition, several binding studies on animal proteins and peptides like fish actomyosin (Damodaran & Kinsella, 1983), sarcoplasmic and myofibrillar proteins from dry-cured ham (Pérez-Juan, Flores & Toldrá, 2006) and myoglobin and carnosine and anserine peptides (Gianelli, Flores & Toldrá, 2003) were investigated. Likewise, the structure of amino acids and peptides is important in their binding capacity to volatile compounds (Gianelli, Flores & Toldrá, 2002). In the food matrix several volatile compounds are bound to proteins while others are solubilised in fat. However, in fat reformulated foods these compounds are lost because fat acts as a reservoir

of the majority of volatile compounds (Kühn et al. 2006). Therefore, the information about the physicochemical interactions between proteins and volatile compounds can help in the development of reformulated food products.

Nevertheless, there are no studies about the role of small peptides produced endogenously during the ripening stages of dry-cured ham on the binding of volatile compounds. Therefore, the objective of this work was to analyse the ability of peptides obtained at different ripening stages of dry-cured ham to bind volatile compounds. In addition, the influence of the chemical characteristics of the volatile compounds and the source of peptide extracts was analysed.

2. Material and methods

2.1. Samples and reagents

Dry-cured hams (P.D.O. Teruel, Spain, Large White x Duroc; Escudero et al. 2014) at different stages of processing (post-salting (90 days), 5, 10 and 14 months of drying) were used. The muscle *Biceps femoris* was removed and used for peptide extraction.

The volatiles compounds were obtained from Fluka Chemie GmbH (Buchs, Switzerland): 3-methyl 1-butanol (99%), 3-methyl 2-butanol (99 %), 1-octen 3-ol (98%), 3-methylbutanal (97%), 2-methylbutanal (95%), ethyl acetate (99%), ethyl butyrate (99%), ethyl 2-methylbutyrate (98%), 2,3-butanedione (97%), 2-heptanone (98%) and Sigma Aldrich (St. Louis, Mo., USA): hexanal (98%), 3-methylthiopropional (98%), ethyl 3-methylbutyrate (98%), 2,6-dimethylpyrazine (98%), 2,3-dimethylpyrazine (99%), 2,3,5-trimethylpyrazine (99%).

2.1.2. Preparation of peptide extracts

A 50 g sample of *Bicep femoris* obtained at each ripening stage was placed in a stomacher (IUL Instrument, Barcelona, Spain) with 100 mL 0.01N HCl for 8 min. The homogenate was centrifuged at 10000 rpm, 4°C for 20 min. Then, the supernatant was filtered through glass wool and deproteinised by adding 3 volumes of ethanol. The sample was stored for 24 h at 4°C. Afterwards, it was centrifuged at the same conditions. The ethanol in supernatant was distilled to dryness, and the sample was lyophilised (Escudero et al. 2014). This constituted the peptide extract (PE).

The presence of lipids in the peptide extract (4g of PE) was determined by extracting the total lipids using dichloromethane:methanol (2:1; Folch, Lees & Sloane Stanley, 1957). The residual peptides were diluted again in 0.01 N HCl and further lyophilised to obtain the defatted peptide extract (Def-PE) which was further subjected to a deodorisation process to remove the volatile compounds. The Def-PE (4g) was dissolved in 200 mL HCl 0.01N, and sonicated during 20 min. Then, it was deodorised with 40 g of Amberlite XAD-2 resin (Sigma Aldrich, St. Louis, Mo., USA) in gentle agitation during 1.5 h. The resin was eliminated by filtration through glass wool. The obtained defatted-deodorised peptide extract (Def-DO-PE) was lyophilised and kept at -20°C until use.

2.2. Chemical characterisation of dry-cured ham and peptide extract

Sodium chloride in dry-cured ham and peptide extracts was determined by ion chromatography (Corral, Salvador & Flores, 2013) through the analysis of the sodium ion. Dry-cured ham moisture was measured using a moisture analyser Mettler Toledo HB43 Halogen (Mettler-Toledo, Greifensee, Switzerland). As described above, total lipids were extracted from 4 g of PE according to the method of Folch et al. (1957) and the content of free and total fatty acids in the total lipids was determined by conversion of fatty acids into fatty acids methyl esters (FAME) and analysed by gas chromatography with flame

ionisation detector (GC-FID) as described by Corral, Salvador, Belloch and Flores (2014) using an Agilent HP 7890A gas chromatograph (Hewlett Packard, Palo Alto, CA).

2.3. Volatile compounds of dry-cured ham

Five grams of minced dry-cured ham with 0.75 mg of BHT were placed in 20 mL headspace (HS) vial sealed with PTFE faced silicone septum. Subsequently, the extraction of volatile compounds was done by using a solid phase microextraction (SPME) device with a CAR/PDMS fibre as described by Corral et al. (2013).

The analysis of the volatile compounds was performed in a gas chromatograph Agilent 7890 CG with a mass spectrometer 5975 MS system (Hewlett Packard, Palo Alto, CA, USA). The equipment was supplied with a Gerstel MPS2 autosampler (Gerstel, Mülheim and der Ruhr, Germany). The headspace vial was kept at 37 °C during 30 min to equilibrate its headspace. Afterwards, the SPME fibre was exposed to headspace while maintaining the sample during 60 min at 37 °C for volatile extraction. Before, each injection the fibre was baked at 250 °C for 15 min. The compounds adsorbed by the fibre were desorbed in the injection port for 5 min at 240 °C with purge valve off (splitless mode). The volatiles were separated on a DB-624 column (30 m, 0.25 mm i.d., film thickness 1.4 µm). The carrier gas was helium with a flow rate of 34.3 cm/s. The oven temperature program began at 38 °C for 13 min, ramped to 100 °C at 3 °C/min and maintained at 100 °C during 5 min, then ramped to 150 °C at 4 °C/min, ramped to 210 °C at 10 °C/min, and lastly held at 210 °C for 5 min, the total run time was 62.2 min. Furthermore, the compounds were identified by comparison with mass spectra from the library database (Nist'05), Kovats retention index (Kovats, 1965) and by comparison with authentic standards. The quantification of the volatile compounds present in the headspace was done in SCAN mode employing either the area of total or extracted ion chromatogram (TIC or EIC) on an arbitrary scale.

2.4. Volatile compounds of peptide extract

The analysis of the volatile compounds was done by using 2 g of PE introduced in 20 mL HS vials. The volatile analysis was done by SPME–GC–MS using the same conditions as described above for dry-cured ham. The relative quantification of the volatile compounds present in the headspace was done in SCAN mode employing either the area of total or extracted ion chromatogram (TIC or EIC) expressed as a percentage of the total area present initially in the dry-cured ham.

2.5. Study of volatile binding with peptide extract

The volatile compounds used for the binding studies were selected from the compounds identified in the dry-cured ham (see 2.3.). A stock solution, containing 30 µg/L of each volatile compound was prepared in methanol. The volatile compound stock solution was dissolved to a final concentration of 2.5, 5, 7.5, 10 and 15 µg/L and added to Def–DO–PE obtained at each stage of ripening and to control solution. All the binding studies were done in triplicate. For the assays, the volatile compounds in the concentration cited above were added to 100 milligrams of each Def–DO–PE dissolved in 20 mM phosphate buffer pH 7 and located in 20 mL HS vial. The NaCl concentration was adjusted to saturation in all the assays. A buffer solution with saturated NaCl content, volatile compound solution and without peptide extract was used as control.

The quantification of volatile compounds in the headspace of the Def–DO–PE and control vials was assessed using SPME–GC–MS at the conditions mentioned above (see 2.3.); however, in this case volatile compounds were extracted by exposing the SPME fibre to headspace during 20 min at 37 °C.

The regression lines at the five concentration ranks assayed were calculated for peptide extracts and control vials. The binding was determined by comparison of slopes between

controls (slope of control vials were considered as 100 %) and slopes of peptide extracts.

Values lower or higher than 100% were considered binding and release, respectively.

Percentage of slope variation was calculated from the difference between the slope of control and that of peptide extracts.

2.6. Statistical analysis

The differences in the NaCl and fat contents, and peptide yield obtained at the different ripening stages were evaluated by one-way ANOVA analysis. The binding effect of volatile compounds interacted with each peptide extract and control, was analysed by linear regression. A one-way ANOVA was done and the slope means were compared using the Fisher's test least significant difference (LSD; $p < 0.05$). In addition, principal component analysis (PCA) was performed to evaluate the relationships among the binding of volatile compounds (slopes) and peptide extracts. XLSTAT (v 2011.5.01, Addinsoft, Barcelona, Spain) was used for data analyses.

3. Results

3.1. Optimisation of defatted and deodorised process of peptide extracts

During the optimisation process to obtain a peptide extract without the presence of volatile compounds, the NaCl and fat content was determined in the peptide extract and after the defatted and deodorised process at the different stages in the processing of dry-cured ham (table 1). The NaCl content of dry-cured ham (*Biceps femoris* muscle) at the different ripening stages was significantly different and their values were between 6–8% as typical reported for dry-cured ham (Estévez, 2015). Likewise, NaCl content in PE decreased while ripening time increased. As expected, these NaCl values decrease, in the total weight of the peptide extract, due to an enrichment of the proteolytic content during

processing time (Larrea, Hernando, Quiles, Lluch & Pérez-Munera, 2006). The fat content in PE was the lowest for post salting stage ($p<0.05$). Furthermore, the increase in fat was due to the moisture decrease during ripening (Olivares, Navarro, Salvador & Flores, 2010) and the values obtained are usual fat contents for pig meat (Wood, Enser, Fisher, Nute, Sheard, Richardson, Hughes & Whittington, 2008).

In addition, the fat content present in the PE obtained from 14 months dry-cured ham was evaluated in terms of composition and resulted in a similar content in total and free fatty acids. The fatty acid profile of total and free fatty acids was determined and resulted in 53% unsaturated, 28% monounsaturated and 20% polyunsaturated fatty acids. This fact means that during peptide extraction, not only a proportion of fatty acids are extracted but also a proportion of free fatty acids. These free fatty acids are generated as a consequence of lipolysis and get solubilised in the peptide extract. These fatty acids can act solubilising volatile compounds and also as a reservoir for their production (Latorre et al. 2009).

For this reason, a defatted process was established to eliminate the fat retained in the peptide extracts followed by a deodorisation process to complete the volatile elimination process. As observed in table 1, the yields of defatted deodorised peptide extracts (Def-DO-PE) from 10 and 14 months were higher than those obtained from post salting and 5 months, because of the proteolysis that occurs during ripening (Toldrá, 2004) generating high peptide and low NaCl contents.

The volatile compounds presented in dry-cured ham (14 months) and retained by the peptide extracts were analysed by headspace SPME extraction to confirm their elimination during the defatted and deodorised process. It is well known that the development of aroma is influenced by peptides and amino acids produced during the long aging of dry-cured hams (Toldrá et al. 1998). In this sense, a total of 48 volatile compounds were identified in

the HS of the 14 months dry-cured ham (table 1 supplementary, figure 1A). The identified volatiles compounds were 9 acids, 6 alcohols, 13 aldehydes, 2 esters, 1 furan, 9 ketones, 5 nitrogen compounds and 3 sulphide compounds. These results agree with those reported by Flores et al. (1997) who recognised that aldehydes are the most abundant group in dry-cured products. Lipolytic and oxidative degradation of unsaturated fatty acids formed alcohols and aldehydes. Furthermore, the esters are generated because of the interaction of free fatty acids and alcohols produced by lipid oxidation (Flores et al. 1997). From them, only 17 volatile compounds were found in the peptide extract (PE) obtained from a 14 month dry-cured ham (table 2, figure 1B). Figure 1 shows the reduction of volatiles compounds in 14 months dry-cured ham and peptides extracts (PE, Def-PE and Def-DO-PE). In general, the percentage of volatile compounds relative to their abundance in dry-cured ham was reduced (%PE-DCH) indicating that only few volatile compounds are retained in the peptide extract. Nevertheless, the high percentages of pyrazines and sulphur compounds retained in the PE were characteristic. These pyrazines contribute to roasted notes (Benjakul, Lertittkul & Bauer, 2004) while sulphur compounds had unpleasant odours like dirty socks and rotten eggs (Flores et al. 1997); also, other retained compounds like nonanal and hexanal had green and fatty aroma, respectively (Flores et al. 1997; Jurado, Carrapiso, Ventanas & García, 2009).

After the defatting process, the peptide extract (Def-PE) (figure 1C, table 2) showed an important reduction in volatile compounds because volatiles are stored in fat and fat is a flavour-release modulator (Brauss, Linforth, Cayeux, Harvey & Taylor, 1999). Subsequently, the lipophilic volatile compounds (high Log K_{ow} , table 1 Supplementary) were highly reduced in Def-PE, indicating that these volatile compounds were mainly present in the fat fraction. However, compounds with a hydrophilic character such as

butyrolactone and methanethiol also showed a larger decrease due to their ability to solubilise in alcohol and ether. In addition to the defatted process, the deodorisation process with Amberlite XAD-2 resin was efficient as it produced a significant reduction and only few traces of volatile compounds were finally present in the %Def-DO-PE (figure 1D, table 2). Therefore, this process was applied to several dry-cured hams obtained at different ripening stages to get peptide extracts free of volatile compounds to be used for the binding studies.

3.2. Binding of volatile compounds by peptide extracts

The changes in proteins during ripening are crucial due to the interaction with volatiles compounds and therefore its influence on the final perceived aroma (Toldrá et al. 1998). Thus, a total of 17 volatile compounds were selected for the binding studies because they were present in the dry-cured ham and peptide extract as described previously (see 2.1). The defatted and deodorised peptide extracts obtained at different ripening stages were used to study their binding ability. All the peptide extracts contained saturated NaCl concentration, except peptide extract from 14 months (table 1) that was corrected to saturation. In this way, in all binding studies the NaCl concentration did not contribute for a “salting in/ out effect” (Pérez-Juan, Flores & Toldrá, 2007).

Table 3 shows the slope of the regression lines for each volatile compound to assess its binding to Def-DO-PE extracts and control (linear regressions are shown in figures 1-5 of supplementary material). The binding of volatiles compounds by the peptide extracts (Def-DO-PE) obtained at different ripening stages showed significant differences except for compounds: 2-methylpropanal, 2-methylbutanal, ethyl 3-methylbutyrate and 2-heptanone ($p>0.05$). On the other hand, significant differences were detected among slopes from control (without peptide) and peptide extracts for almost all volatiles compounds ($p<0.05$).

In general a reduction in the slopes from control to peptide extracts was obtained except for compounds; 3-methylthiopropional and esters (ethyl butyrate, ethyl 2-methylbutyrate and 3-methylbutyrate) which resulted in higher slopes than the control indicating a release of these compounds in the presence of the peptide extracts.

The percentage of slope variation between control and peptide extracts obtained at the different ripening stages was calculated to clarify the binding (table 4). Therefore, values above or below 100 % indicated release or retained, respectively. A release was seen for ethyl butyrate, ethyl 2-methylbutyrate, ethyl 3-methylbutyrate and 3-methylthiopropional as reported Rodríguez-Bencomo, Muñoz-González, Andújar-Ortiz, Martín-Álvarez, Moreno-Arribas and Pozo-Bayón (2011) when studied the binding ability of wine matrix. On the other hand, the defatted deodorised peptide extracts (Def-DO-PE) obtained at different ripening stages, produced retention of about 20 and 30% for 2-methylpropanal, hexanal and ethyl acetate as also reported Gianelli et al. (2003) for a muscle dipeptide anserine. Moreover, all the Def-DO-PE had null binding ability with 2-methylbutanal probably due to a steric effect as indicated by Gianelli et al. (2003) and Goodridge, Beaudry, Pestka and Smith (2003) for carnosine and chicken myofibrillar proteins, respectively. Table 4 shows that peptides obtained at the post salting stage had more binding ability for alcohols, 3-methylbutanal, 2,3-butanedione and pyrazines. This finding would suggest that the post salting peptides had the highest size, mainly in the range 2700 to 4500 Da (Rodríguez-Núñez, Aristoy & Toldrá, 1995; Gallego et al. 2014); so, they had more binding sites available for these volatiles compounds. Nevertheless, during the processing of dry-cured ham proteolysis remains and free amino acids are produced and increased at the end of ripening (Mora et al. 2009; Gallego et al. 2014). On the other hand, myofibrillar proteins binding to volatile compounds have been also studied at different ripening times (pork

muscle, 7 and 12 months dry-cured ham) (Pérez-Juan et al. 2006) but no significant differences were reported in the binding of volatiles compounds. This fact suggested that although the myofibrillar proteins ability to bind volatile compounds is not affected during ripening time, the peptide fragments produced by their degradation are a source of peptides with high binding ability.

Among all the volatile compounds studied, trimethylpyrazine had the largest interaction with 85 to 97% binding with Def-DO-PE obtained at the different ripening stages. Pyrazines have almost 80% of hydrophobic surface that depends on the quantity of methyl groups in the ring and thus, the binding site should be predominantly non-polar (Tinndelli, Keen, Cavaggioni, Ehopoulos & Findlay, 1989). Several studies have suggested that myofibillar proteins degradation (Mora et al. 2009; Mora et al. 2010) produced peptides with hydrophobic residues like proline, glycine, valine, phenylalanine, isoleucine; so, trimethylpyrazine could be bound to this residues due to its characteristics (Tirindelli et al. 1989).

The different binding capacity of volatile compounds to peptide extracts can be explained by their chemical structure. Commonly, protein binding ability increases from alcohols to ketones and aldehydes. There are two kinds of binding: reversible (hydrogen bonds, hydrophobic binding, ionic bonds) and irreversible (covalent linkages, condensation of aldehydes with amino and sulfhydryl bonds) (Kühn et al. 2006). Moreover, Reiners, Nicklaus and Guichard (2000) suggested that alkyl chain linked with hydroxyl group had weakly binding with β -lactoglobulin. Nevertheless, we observed a higher peptide binding capacity for alcohols than aldehydes and ketones, because there is not a universal mechanism for volatile compounds binding with foods (table 4). The alcohols form strong hydrogen bonds with peptides because of the hydroxyl group has character of electron donor

and acceptor. On the other hand, aldehydes have the carbonyl group to form hydrogen bonds with peptides; but, 3-methylthiopropional has a sulphur group that provides inductive effect, so, this affects its capacity to form hydrogen bonds with peptides (Zhou & Cadwallar, 2006). The carbonyl group of esters and 2-heptanone is less electrophilic; therefore, they have low capacity to form hydrogen bonds with peptides and probably this is the reason for the low interaction observed. However, 2,3-butanedione has 2 carbonyl groups to form hydrogen bonds with peptides (table 4).

In order to understand the relationship between the binding of volatile compounds and peptides generated at the different ripening stages a principal component analysis was done using the volatiles compounds slopes (table 3) at different ripening stages. Figure 2 shows that 94% of the variability was explained by the two first principal components. Principal component 1 is the most important variable as it explains 84 % of the variability. PC1 is related to the presence of peptide in the medium as it displays control sample on the positive part while samples containing peptides were on the negative part. Also the volatile compounds, located in the negative part, were those released from the Def-DO-PE matrix, while those positioned in the positive part were bonded to the peptides. Also, PC 2 distinguished samples according to ripening stages. While generally volatile compounds binding was poorly affected by ripening time, only 3-methylthiopropional was positively related to the release observed for peptides obtained after 14 m of ripening. Subsequently the binding ability of peptide extracts to volatile compounds at different stages of ripening was mainly based on their chemical characteristics and not in the type of peptide extract generated during ripening.

4. Conclusion

In summary, peptide extracts obtained from dry-cured ham at different ripening stages have shown ability to bind volatile compounds. However, the main factor affecting the binding is the chemical nature of the volatile compound as observed by a high ability to bind alcohols and pyrazines. Probably the binding was due to their ability to form hydrogen bonds and hydrophobic interactions, respectively. On the other hand, peptides obtained in the early stages of ripening (post salting) would have high number of binding sites due to its high size as observed by the high binding values. The physicochemical interactions between peptides and volatile compounds shown in this manuscript would help to understand their role in flavour perception.

5. Acknowledgments

Isadora Martínez-Arellano thanks the CONACYT for a postdoctoral scholarship No.232230. Also, the projects AGL 2012-38884-CO2-01 from MINECO (Madrid, Spain), FEDER funds and PROMETEO 2012-001 (Generalitat Valenciana, Valencia, Spain) are acknowledged for financial support.

6. References

- Andriot, I., Marin, I., Feron, G., Relkin, P., & Guichard, E. (1999). Binding of **benzaldehyde** by β -lactoglobulin, by static headspace and high performance liquid chromatography in different physico-chemical conditions. *Lait*, 79, 577-586.
- Benjakul, S., Lertittikul, W., & Bauer, F. (2004). Antioxidant activity of maillard reaction products from porcine plasma protein-sugar model system. *Food Chemistry*, 93, 189-196.

357 Brauss, M. S., Linforth, R. S., Cayeux, T., Harvey, B., & Taylor, A. J. (1999). Altering the
 358 fat content affects flavor release in a model yogurt system. *Journal of Agricultural and*
 359 *Food Chemistry*, 47(5), 2055–2059.

360 Corral, S., Salvador, A., & Flores, M. (2013). Salt reduction in dry cured sausages affects
 361 aroma generation. *Meat Science*, 93, 776–785.

362 Corral, S., Salvador, A., Belloch, C., & Flores, M. (2014). Effect of fat and salt reduction
 363 on the sensory quality of slow fermented sausages inoculated with *Debaryomyces*
 364 *hansenii* yeast. *Food Control*, 45, 1–7.

365 Damodaran, S., & Kinsella, J. E. (1983). Binding of carbonyls in fish acotmyosin. *Journal*
 366 *Agriculture Food Chemistry*, 31, 856–858.

367 Escudero, E., Mora, L., & Toldrá, F. (2014). Stability of ACE inhibitory ham peptides
 368 against heat treatment and in vitro digestion. *Food Chemistry*, 161, 305–311.

369 Estévez, M., Ventanas, S., Morcuende, D., & Ventanas, J. (2015). Mediterranean products,
 370 In F. Toldrá, Y. H. Hui, I. Astiasaran, J. Sebranek, & R. Talon (Eds.), *Handbook of*
 371 *Fermented Meat and Poultry* (pp. 362–365). Chnai: John Wiley & Sons Ltd.

372 Flores, M., Grimm, C. C., Toldrá, F., & Spanier, A. M. (1997). Correlations of sensory and
 373 **volatile compounds of Spanish “Serrano” dry-cured ham** as a function of two
 374 processing times. *Journal of Agricultural and Food Chemistry*, 45, 2178–2186.

375 Flores, M., Sanz, Y., Spanier, A. M., Aristoy, M. C., & Toldrá, F. (1998). Contribution of
 376 muscle and microbial aminopeptidases to flavor development in dry-cured meat
 377 products. In: E.T. Contis, C.-T. Ho, C.J. Mussinan, T.H. Parliment, F. Shahidi and A.M.
 378 Spanier (Eds). *Developments in Food Science*, V40, 547–557, Elsevier.

379 Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and
 380 purification of total lipids from animal tissues. *The Journal of Biological Chemistry*,
 381 226, 497–509.

382 Gallego, M., Mora, L., Fraser, P., Aristoy, M., & Toldrá, F. (2014). Degradation of LIM
 383 domain-binding protein three during processing of Spanish dry-cured ham. *Food*
 384 *Chemistry*, 149, 121–128.

385 Gianelli, M. P., Flores, M., & Toldrá, F. (2002). Importance of meat proteins on flavour
 386 release. In F. Toldrá (Ed.), *Research Advances in the Quality of Meat and Meat*
 387 *Products* (pp. 79–94). Trivandrum: Research Sign-post.

388 Gianelli, M. P., Flores, M., & Toldrá, F. (2003). Interactions of soluble peptides and
 389 proteins from skeletal muscle on the release of volatile compounds. *Journal Agriculture*
 390 *and Food Chemistry*, 51, 6828–6834.

391 Goodridge, C. F., Beaudry, R. M., Pestka, J. J., & Smith, D. M. (2003). Solid phase
 392 microextraction-gas chromatography for quantifying headspace hexanal above freeze-
 393 dried chicken myofibrils. *Journal of Agricultural and Food Chemistry*, 51, 4185–4190.

394 Jurado, A., Carrapiso, A. I., Ventanas, J., & García, C. (2009). Changes in SPME-extracted
 395 volatile compounds from Iberian ham during ripening. *Grasas Aceites*, 60, 262–272.

396 Kovats, E. S. (1965). Gas chromatographic characterization of organic substance in the
 397 retention index system. In J. C. Giddings, & R. A. Keller (Eds.), *Advances in*
 398 *chromatography* (pp. 229–247). New York: Marcel Dekker, Inc.

399 Kühn, J., Considine, T., & Singh, H. (2006). Interactions of milk proteins and volatile
 400 flavor compounds: implications in the development of protein foods. *Journal of Food*
 401 *Science*, 71(5), R72–R82.

402 Larrea, V., Hernando, I., Quiles, A., Lluch, I., & Pérez-Munera, I. (2006). Changes in
 403 proteins during Teruel dry-cured ham processing. *Meat Science*, 74, 586–593.
 404 Latorre, M. A., Ripoll, G., García-Belenguer, E., & Ariño, L. (2009). The effect of gender
 405 and slaughter weight on loin and fat characteristics of pigs intended for Teruel dry-cured
 406 ham production. *Spanish Journal of Agricultural Research*, 7(2), 407–416.
 407 Mora, L., Sentandreu, M. A., & Toldrá, F. (2010). Identification of small troponin T
 408 peptides generated in dry-cured ham. *Food Chemistry*, 123, 691–697.
 409 Mora, L., Sentandreu, M. A., Koistinen, P., Fraser, P., Toldrá, F., & Bramley, P. (2009).
 410 Naturally generated small peptides derived from myofibrillar proteins in serrano dry-
 411 cured ham. *Journal of Agricultural and Food Chemistry*, 57, 3238–3234.
 412 Olivares, A., Navarro, J. L., Salvador, A., & Flores, M. (2010). Sensory acceptability of
 413 slow fermented sausages based on fat content and ripening time. *Meat Science*, 86, 251–
 414 257.
 415 Pérez-Juan, M., Flores, M., & Toldrá, F. (2006). Simultaneous process to isolate
 416 actomyosin and actin from post-rigor porcine skeletal muscle. *Food Chemistry*, 101,
 417 1005–1011.
 418 Pérez-Juan, M., Flores, M., & Toldrá, F. (2007). Effect of ionic strength of different salts
 419 on the binding of volatile compounds to porcine soluble protein extracts in model
 420 systems. *Food Research International*, 40(6), 687–693.
 421 **Reiners, J., Nicklaus, S., & Guichard, E. (2000). Interactions between β -lactoglobulin and**
 422 **flavour compounds of different chemical classes. Impact of the protein on the odour**
 423 **perception of vanillin and eugenol. *Lait*, 80, 347–360.**
 424 Rodríguez-Bencomo, J., Muñoz-González, C., Andújar-Ortiz, I., Martín-Álvarez, P.,
 425 Moreno-Arribas, M., & Pozo-Bayón, M. (2011). Assessment of the effect of the non-

426 volatile wine matrix on the volatility of typical wine aroma compounds by headspace
 427 solid phase microextraction/gas chromatography analysis. *Journal of Science and Food*
 428 *Agriculture*, 91, 2484–2494.

429 Rodríguez-Núñez, E., Aristoy, M. C., & Toldrá, F. (1995). Peptide generation in the
 430 processing of dry-cured ham. *Food Chemistry*, 53(2), 187–190.

431 Ruiz, J., García, C., Muriel, E., Andrés, A. I., & Ventanas, J. (2002). Influence of sensory
 432 characteristics on the acceptability of dry-cured ham. *Meat Science*, 61, 347–354.

433 Sentandreu, M. A., Stoeva, S., Aristoy, M. C., Laib, K., Voelter, W., & Toldrá, F. (2003).
 434 Identification of small peptides generated in spanish dry-cured ham. *Journal of Food*
 435 *Science*, 68(1), 64–69.

436 Seuvre, A. M., Espinosa Diaz, M. A., & Voilley, A. (2002). Transfer of aroma compounds
 437 through the lipidic-aqueous interface in a complex system. *Journal of Agricultural and*
 438 *Food Chemistry*, 50(5), 1106–1110.

439 Solina, M., Baumgartner, P., Johnson, R. L., & Whitfield, F. B. (2005). Volatile aroma
 440 components of soy protein isolate and acid-hydrolysed vegetable protein. *Food*
 441 *Chemistry*, 90(4), 861–873.

442 Tinndelli, R., Keen, J. N., Cavaggioni, A., Ehopoulos, E. E., & Findlay, J. B. (1989).
 443 Complete amino acid sequence of pyrazine binding protein from cow nasal mucosa.
 444 *European Journal of Biochemistry*, 185, 569–572.

445 Toldrá, F. (2004). Curing; Dry. In W. Jenses, C. Devine & M. Dikemman (Eds.),
 446 *Encyclopedia of meat sciences* (pp. 360–365). London: Elsevier Science Ltd.

447 Toldrá, F. (2006). Biochemical proteolysis basis for improved processing of dry-cured
 448 meats. In L. M. L. Nollet & F. Toldrá (Eds.), *Advances Technologies for Meat*
 449 *Processing*. Boca Ratón: CRC Press.

450 Toldrá, F., & Flores, M. (1998). The role of muscle proteases and lipases in flavor
451 development during the processing of dry-cured ham. *Critical Reviews in Food Science*,
452 38(4), 331–352.

453 Toldrá, F., Aristoy, M. C., & Flores, M. (2009). Relevance of nitrate and nitrite in dry-
454 cured ham and their effects on aroma development. *Grasas y Aceites*, 60 (3), 291–296.

455 Wood, J., Enser, M., Fisher, A., Nute, G., Sheard, P., Richardson, R., Hughes, S., &
456 Whittington, F. (2008). Fat deposition, fatty acid composition and meat quality: A
457 review. *Meat Science*, 78, 343–358.

458 Zhou, Q., & Cadwallader, K. R. (2006). Effect of flavor compound chemical structure and
459 environmental relative humidity on the binding of volatile flavor compounds to
460 dehydrated soy protein isolates. *Journal of Agricultural and Food Chemistry*, 54,
461 1838–1843.

462 463 464 Figure Legends

465 Figure 1. Volatile compounds obtained by SPME–GC–MS in (A) Dry-cured ham (B)
466 Peptide extract (C) Defatted peptide extract (D) Deodorized–defatted peptide extract.

467 Number of peaks in figure corresponds to volatile compounds shown in table 2.

468
469 Figure 2. Loadings of the two principal components (PC1–PC2) of peptide extracts (◇) at
470 different ripening stages (PS: post salting, 5M: 5 months, 10M: 10 months, 14M: 14
471 months) and the binding of volatile compounds.

Table 1. Characterisation of dry-cured ham and peptide extract used in the different ripening stages.

	Dry-cured ham	Peptide extract		Defatted-deodorised peptide extract		
	NaCl (% dm)	Fat (%)	NaCl (%)	Yield (%)	Peptide (g/50 g ham)	NaCl (g/50g ham)
Post Salting	6.5±1.2 ^b	1.7±0.3 ^c	78.2±4.7 ^a	4.4±1.7 ^b	0.4±0.2 ^c	1.8±0.7
5 months	10.9±0.2 ^a	2.3±0.1 ^b	75.8±6.3 ^a	5.6±1.6 ^b	0.8±0.2 ^c	2.0±0.4
10 months	7.2±0.2 ^b	2.6±0.2 ^{ab}	34.7±4.6 ^b	6.6±0.9 ^{ab}	2.1±0.2 ^b	1.3±0.1
14 months	6.5±0.2 ^b	2.7±0.2 ^a	26.6±6.9 ^b	9.1±1.3 ^a	3.2±0.5 ^a	1.4±0.2
<i>p</i>	***	***	**	**	***	ns

^a*p*-value of ripening time effect ****p*<0.001, ***p*<0.01, ns: no significant. Means followed by different letters in the same column indicate significant differences among ripening stages.

Table 2. Reduction of volatile compounds present in the dry-cured ham through the defatted and deodorisation processes of peptide extract from 14 months dry cured ham.

		LRI ¹	Dry-cured ham	Peptide extract	
			%PE-DCH ²	%Def-PE ³	%Def-DO-PE ⁴
Acids					
1	Acetic acid	714 ^a	25.9	33.7	64.3
2	2-methylpropanoic acid	859 ^a	26.3	45.7	— ⁵
3	3-methylbutanoic acid	938 ^a	40.1	1.6	—
4	2-methylbutanoic acid	944 ^a	44.7	21.7	—
5	Butanoic acid	887 ^a	73.1	28.8	—
6	Pentanoic acid	974 ^a	61.7	19.0	—
7	Hexanoic acid	1074 ^a	112.8	24.3	—
Aldehydes					
8	Hexanal	840 ^a	8.0	76.4	14.9
9	3-methylthiopropanal	965 ^a	64.6	97.6	54.5
10	Nonanal	1149 ^a	44.6	44.9	—
Ketones					
11	Butyrolactone	1022 ^a	48.5	7.7	35.3
Nitrogen compounds					
12	2,6-dimethylpyrazine	943 ^a	180.9	36.3	—
13	2,3-dimethylpyrazine	951 ^b	72.9	84	—
14	2,3,5-trimethylpyrazine	1037 ^a	41.9	44.9	—
15	Tetramethylpirazine	1117 ^a	68.6	23.1	—
Sulphur compounds					
16	Methanethiol	472 ^a	61.6	0.4	—
17	Dimethyl disulfide	772 ^a	74.4	28.1	—

¹Linear retention index of the compounds eluted from de GC-MS using a DB-624 capillary column (J&W Scientific 30 m x 0.25 mm i.d. x 1.4 µm film thickness). ^a: identification by mass spectrum and by coincidence with the LRI of an authentic standard and ^b: tentatively identification by mass spectrum.

²%PE-DCH: ratio peptide extract area vs dry-cured ham area.

³%Def-PE: ratio defatted-peptide extract area vs peptide extract area.

⁴%Def-DO-PE: ratio defatted-deodorised-peptide extract area vs peptide extract area.

⁵ not detected.

Table 3. Binding of volatiles compounds (slope of linear regression) to peptide extracts obtained at different ripening stages.

Compounds	Control slope	R ^{2,1}	Post salting slope	R ^{2,1}	5 months slope	R ^{2,1}	10 months slope	R ^{2,1}	14 months slope	R ^{2,1}	p-value ²	RMSE ³
Alcohols												
3-methyl-1-butanol	2000.0 ^a	0.98	150.6 ^d	0.96	376.2 ^c	0.94	346.9 ^c	0.98	442.8 ^b	0.97	***	20.9
2-methyl-1-butanol	3000.0 ^a	0.99	293.1 ^d	0.98	464.7 ^c	0.99	662.0 ^b	0.91	626.3 ^b	0.99	***	27.4
1-octen 3-ol	2666.7 ^a	0.93	734.8 ^b	0.99	1000.0 ^b	0.98	1333.3 ^b	0.89	1000.0 ^b	0.96	**	365.7
Aldehydes												
2-methylpropanal	2333.0	0.85	2000.0	0.80	2000.0	0.81	2000.0	0.85	2000.0	0.84	ns	258.2
3-methylbutanal	5000.0 ^a	0.69	3000.0 ^c	0.82	3333.3 ^c	0.87	3333.3 ^b	0.88	4000.0 ^c	0.78	***	365.2
2-methylbutanal	3000	0.72	3000	0.78	3000	0.84	3000	0.81	3000	0.89	ns	
Hexanal	5000.0 ^a	0.92	4000.0 ^b	0.79	5333.4 ^a	0.88	4000.0 ^b	0.95	4000.0 ^b	0.88	***	258.2
3-methylthiopropional	32.7 ^b	0.92	32.6 ^b	0.89	31.0 ^b	0.92	35.0 ^b	0.80	60.6 ^a	0.75	***	3.1
Esters												
Ethyl acetate	5000.0 ^a	0.92	3000.0 ^c	0.97	3666.7 ^b	0.98	4000.0 ^b	0.99	4000.0 ^b	0.99	***	258.3
Ethyl butyrate	6666.7 ^b	0.97	9000.0 ^a	0.83	9000.0 ^a	0.88	9000.0 ^a	0.91	9333.3 ^a	0.93	***	577.4
Ethyl 2-methylbutyrate	8333.3 ^b	0.86	10000.0 ^a	0.78	10000.0 ^a	0.81	10000.0 ^a	0.89	10000.0 ^a	0.86	***	258.2
Ethyl 3-methylbutyrate	8666.7	0.90	10000.0	0.76	10000.0	0.81	10000.0	0.88	10000.0	0.86	ns	683.1
Ketones												
2,3-butanedione	942.6 ^a	0.99	361.0 ^d	0.98	396.4 ^c	0.99	409.9 ^c	0.99	441.5 ^b	0.99	***	11.0
2-heptanone	5333.3	0.90	6000.0	0.95	6000.0	0.98	6333.3	0.99	6333.3	0.99	ns	447.2
Nitrogen compounds												
2,6-dimethylpyrazine	4666.7 ^a	0.99	655.2 ^b	0.99	792.2 ^b	0.99	926.0 ^b	0.91	895.6 ^b	0.99	***	260.1
2,3-dimethylpyrazine	1000.0 ^a	0.99	159.1 ^d	0.99	193.3 ^{cd}	0.99	267.0 ^b	0.91	229.1 ^{bc}	0.99	***	34.9
2,3,5-trimethylpyrazine	860.2 ^a	0.99	23.9 ^c	0.99	31.7 ^c	0.95	125.7 ^b	0.95	63.7 ^c	0.6	***	25.9

¹R²: coefficient of determination.

²p-value of ripening time effect in volatile retention. ***p<0.001, **p<0.01, ns: p>0.05. Means followed by different letters in the same compound indicate significant differences among ripening stages.

³RMSE: root mean square error

Table 4. Percentage of slope variation of the volatile compounds at each ripening stage compared to control slope.

	Post salting	5 months	10 months	14 months
Alcohols				
3-methyl-1-butanol	8	19	17	22
2-methyl-1-butanol	10	15	22	21
1-octen 3-ol	28	39	50	39
Aldehydes				
2-methylpropanal	89	89	89	89
3-methylbutanal	60	67	67	80
2-methylbutanal	100	100	100	100
Hexanal	80	107	80	80
3-methylthiopropenal	101	97	109	188
Esters				
Ethyl acetate	60	73	80	80
Ethyl butyrate	138	138	138	143
Ethyl 2-methylbutyrate	120	120	120	120
Ethyl 3-methylbutyrate	118	118	118	118
Ketones				
2,3-butanedione	38	42	44	44
2-heptanone	113	113	120	119
Nitrogen compounds				
2,6-dimethylpyrazine	14	17	20	19
2,3-dimethylpyrazine	16	19	27	23
2,3,5-trimethylpyrazine	3	4	15	7

Figure(s)
[Click here to download high resolution image](#)

Figure(s)
[Click here to download high resolution image](#)

Supplementary Material Table

[Click here to download Supplementary Material: Table 1 Supplem.docx](#)

Supplementary Material Figures

[Click here to download Supplementary Material: Supplementary Figures.docx](#)