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Meat Science 59 (2001) 363–368

MEAT SCIENCE

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Free amino acids and other non-volatile compounds formed during processing of Iberian ham

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Received 18 October 2000; received in revised form 2 January 2001; accepted 16 March 2001

Abstract

Fifty-five legs from Iberian pigs were traditionally processed into dry cured hams. Free amino acids and other non-volatile compounds in the water-soluble fraction from the *biceps femoris* muscle were analyzed by HPLC. At the drying stage and in the last months in the cellar the largest increases in these water-soluble compounds took place. There was a clear influence on free amino acid formation of salt content and on the formation of peptides of the temperature at each processing stage. As the amount of non-volatile compounds in the water-soluble fraction increases with processing time, their determination could provide a maturation index for Iberian ham. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Iberian ham; Non-volatile compounds; Processing; Proteolysis

1. Introduction

Dry-cured hams from Iberian pigs are considered the most valuable meat products in Spain due to their high sensorial quality. The characteristic flavour develops in the highly marbled legs of Iberian pigs raised during the period prior to slaughter exclusively on acorns and pasture and processed following a traditional method over at least 18 months.

The chemical and biochemical changes occurring during the successive stages of processing have been investigated as well as the volatile and non-volatile compounds responsible for the desirable flavour (Antequera et al., 1992; Córdoba, Antequera, García, Ventanas, López-Bote, & Asensio, 1994; García, Berdagué, Antequera, López-Bote, Córdoba, & Ventanas, 1991; Ruiz, García, Díaz, Cava, Tejeda, & Ventanas, 1999). The proteolysis that takes place during ripening is an important source of flavour compounds (free amino acids, small peptides and Maillard-reaction products) in dry-cured hams such as Serrano (Aristoy & Toldrá, 1995; Flores, Aristoy, Spanier, & Toldrá, 1997; Toldrá, 1998), Parma (Careri, Mangia, Barbieri, Bolzoni, Virgili, & Parolari, 1993; Hansen-Moller, Hinrichsen & Jacobsen, 1997) and French hams (Buscailhon et al., 1994). These flavours are more marked in Iberian ham (Virgili, Parolari, Soresi-Bordini, Schivazappa, Cornet, & Monin, 1999) probably due to the long ageing time and the high temperatures involved during some periods of the maturation. Thus, large increases in amino acid nitrogen are found during the processing of traditional Iberian ham; the largest increases (73%) are found in the non-protein nitrogen fractions (Córdoba et al., 1994; Ventanas, Córdoba, Antequera, García, López-Bote, & Asensio, 1992). Martín, Antequera, Ruiz, Cava, Tejeda, and Córdoba (1998) and Martín, Córdoba, Antequera, Timón, & Ventanas (1998) observed that the processing conditions (temperature and salt content) determine the level and the kind of compounds released from protein breakdown during the dry-curing of Iberian hams. Due to the trend in consumption of low-salted products, many manufacturers processing Iberian hams have reduced the salting period to 1 day per kg of raw ham. A lower salt concentration seems to contribute to a difference in the quantity of the non-protein nitrogen fraction in dry-cured Iberian hams. Thus, Martín, Córdoba et al. (1998) found a lower rate of total amino acid nitrogen formation (48% of the overall

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PII: S0309-1740(01)00088-2

non-protein nitrogen) and an increase in peptide nitrogen in lower salted Iberian hams. The non-volatile compounds generated from proteins may be of importance in the sensory quality in dry-cured hams. Therefore, information regarding the nature and proportions of non-volatile taste-active compounds is of interest to help to understand the mechanisms of flavour development. Such information could also provide a means of enhancing the sensory quality of Iberian cured hams.

The aim of our work was to investigate the changes in the composition of free amino acids during the ripening of Iberian hams cured under two different processing conditions, one according to the traditional method (high-salted hams), and the other one based on a more modern process (low-salted hams). Also, other non-volatile water soluble compounds were analyzed during processing.

2. Materials and methods

2.1. Processing and sampling of the hams

A total of 55 homogeneous legs (about 10 kg) were obtained from Iberian pigs (160 kg live weight). Pigs were grown extensively on pasture with acorns from *Quercus ilex* and *Quercus suber*. The hams were processed under two different regimes. Thirty-one hams were submitted to a traditional ripening process (T) consisting of salting for 1.5 days kg^{-1}, post-salting at low temperature and drying-ageing steps under environmental conditions. Twenty-four hams was ripened by a modern technique (M) in which the hams experienced a shorter salting period (1 day kg^{-1}). This processing (M) was carried out under controlled conditions of temperature and relative humidity.

Temperatures for each processing are shown in Fig. 1. Stages and number of hams removed at each stage were as follows:

	Traditional	Modern
Green State	0 days ($n=5$)	0 days ($n=5$)
Salting–postsalting	75 days ($n=5$)	76 days ($n=5$)
Drying	168 days ($n=4$)	197 days ($n=6$)
Four month cellar		314 days ($n=6$)
Six months cellar	360 days ($n=5$)	
Eight months cellar		456 days ($n=5$)
Fully matured hams	588 days ($n=5$)	665 days ($n=4$)

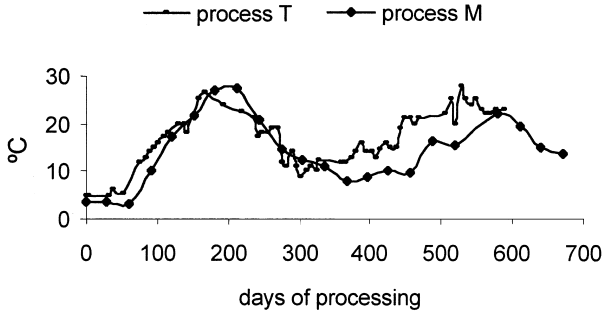

Fig. 1. Environmental temperature during the T and M processes.

Samples of *biceps femoris* muscles of each ham were removed and kept at −80°C until analyzed.

2.2. Chemical analysis

Moisture was determined following the ISO recommended method (ISO/1442).

Salt content was estimated as chloride, extracted with water-ethanol (60:40 v/v) and quantified by the Carpentier Volhard method (AOAC, 1984).

Free amino acids (FAA) were determined by homogenizing 10 g of sample and 25 mg of norleucine (added as an internal standard) with sulphosalicylic acid (10%). After 17 h the homogenates were centrifuged at 15 300 ***g*** for 10 min at low temperature (2°C) and filtered through Whatman No. 54 paper. After the pH of the filtrates was adjusted to 6 with 30% potassium hydroxide, they were filtered again through Whatman No. 54 paper. Amino acid derivatization was carried out with phenyl isothiocyanate (PITC) according to the modified method of Yang and Sepúlveda (1985). Filtrate (100 μl) was mixed with 200 μl of PITC solution (ethanol-trietylamine-PITC 7:2:1) for 10 min, and dried in a Speedvac (Savant Instruments) with no heating for 20 min. The residue was dissolved in 200 μl of 0.5 M phosphate buffer, pH 7.4.

Twenty microlitres of filtrate were used for chromatographic separation. Samples were analyzed in a Beckman HPLC equipped with a UV detector (254 nm). The column was a reverse-phase Supelcosil LC18 column, 25 cm×4.6 mm (5 μm particle size) protected with a Bio Rad guard column. The temperature was controlled at 35°C. Chromatographic separation used a solvent system consisting of two eluents: (A) 0.03M sodium acetate containing 0.05% of triethylamine, adjusted to pH 6.8 with acetic acid, and (B) 90:10 of acetonitrile-water. At a flow rate of 1 ml/min using the following solvent composition gradient: Initial to 3.2% B ; 0.5-min linear change to 4.5%; 5-min linear change to 8.5% B; 10-min linear change to 11.5% B; 9-min linear change to 26.5% B; 22-min linear change to 99% B; wash for 12 min at 99% B and re-equilibrate at 3.2%. Identification was based on the retention times of reference compounds (Sigma) including L-Ala (alanine), L-Arg (arginine), L-Asn (asparagine), L-Asp (aspartic acid), L-Glu (glutamic acid), L-Gln (glutamine), L-Gly (glycine), L-His (histidine), L-Pro (proline), L-Ile (isoleucine), L-Leu (leucine), L-Lys (lysine), L-Met (methionine), L-Phe (phenylalanine), L-Pro (proline), L-Ser (serine), L-Thr

(threonine), L-Trp (tryptophan), L-Tyr (tyrosine) and L-Val (valine). The concentration of the different amino acids was calculated from the standard curves of the pure amino acids prepared and derivatized simultaneously with the samples and run under identical conditions.

Non-volatile compounds were determined by homogenizing 10 g of sample with 0.6 N perchloric acid. The homogenates were centrifuged at 15 300 ***g*** for 10 min and filtered through Whatman No. 54 paper. After the pH of the filtrates was adjusted to 6 with 30% potassium hydroxide, they were filtered again through Whatman No. 54 paper. Twenty microlitres of filtrate were used for the HPLC analysis, using a reverse-phase C18 column, 25 cm×4.6 mm (5 μm particle size) and UV detector (214 nm). The eluents used were (A) water HPLC grade and (B) acetonitrile containing 0.1% of trifluoroacetic acid.

An atmospheric pressure chemical ionization (APCI) interface mass spectrometer detector was used for some of the extracts, coupled to the HPLC, using the same chromatographic conditions as before. Positive ions were detected under a vaporizer temperature of 450°C and a discharge current of 5 μA. The temperature of the capillary was 150°C at 0 V of voltage. Full scan data (range 50–2000 m/z), were acquired and processed by Navigator 1.1 software.

2.2.1. Statistical analysis

The comparison of means between the T (traditional) and M (modern) processes was made using Student t-test. Differences between stages were performed by analysis of variance, using the Bonferroni test of the StatGraphics 5.0 software package from Statistical Graphics Corp. (Rockville, MD).

3. Results and discussion

Table 1 shows the moisture and salt contents in the hams throughout ripening. The hams from M had lower salt contents than those from T because of the shorter salting period. The free amino acids detected at the green stage increased significantly ($P<0.05$) during ripening in both T and M (Fig. 2); the increases being greatest during the drying stage, agreeing with results obtained previously (Martín, Córdoba, et al., 1998). The temperature reached during the drying of Iberian ham seems to stimulate proteolytic activity of catepsin D and exopeptidases of both muscle and microbial origin leading the release of amino acids; some exopeptidases present in raw meat remain active during the whole production process (Toldrá et al., 1992). During the cellar stage there were also big increases in free amino acids, but the increase was always less than observed at the drying stage in both T and M (Fig. 2). The high quantity of free amino acids accumulated in the drying stage could explain the small increases during the last processing stage, as inhibition due to the presence of released hydrophobic free amino acids of some aminopeptidases (Flores, Aristoy, & Toldrá, 1998). In T, the generation of free amino acids was progressive. However, in M there was a sudden increase during drying which then remained almost constant. This, could be due to the higher temperatures during the cellar stage in T than in M.

Glutamic acid, alanine, lysine, leucine and arginine were the most abundant free amino acids in the final product. These amino acids have also been reported as the most abundant in Iberian ham (Ruiz et al,. 1999), serrano (Toldrá et al., 1992) and Parma ham (Careri et al., 1993).

Martín, Córdoba et al. (1998) found that the fraction of amino acid nitrogen in hams from T was higher than in hams from M, agreeing with this study. A number of free amino acids such as Gly, Thr, Pro, Tyr, Val, Met, Ile, Leu, Phe, Trp and Lys, were found in significantly larger quantities ($P<0.05$) in the final products from T than from M (Fig. 3). Also, the sum of all the free amino acids in T was significantly greater ($P<0.05$) than in M (4808 mg/100 g DM and 3591 mg/100 g DM, respectively). These results do not agree with those obtained by Virgili et al. (1999), who found that the free amino acid content was negatively correlated with salt content. This could be due to the different levels of salt in the hams in the two studies.

Although at the end of processing, hams from T had higher contents of free amino acids than hams from M, there was a slight difference in the profiles of free amino acids in the two groups. The relative quantity of desirable amino acid which possess sweet (alanine, glycine and serine) and umami taste (aspartic and glutamic acids) was calculated (Mau & Tseng, 1998). The percentage representing the sum of these desirable amino acids, with respect to the total free amino acids in each

Table 1
Variation in moisture and NaCl in *biceps femoris* muscle during the ripening of Iberian hams

Stage	Moisture (%)		NaCl (%)[a]	
	Process T	Process M	Process T	Process M
GR	^{1}71.43±0.78	^{1}72.21±0.48	^{1}0.00±0.00	^{1}0.00±0.00
PS	^{2}64.92±1.47	^{12}63.63±1.54	^{12}3.57a±0.35	^{1}1.36b±0.13
DR	^{2}63.74±2.78	^{23}57.39±1.51	^{23}4.76a±0.35	^{2}2.77b±0.16
4C		^{34}54.73±0.78		^{23}3.29±0.19
6C	^{3}54.66b±3.61		^{3}5.35±0.50	
8C		^{4}49.39±2.02		^{23}3.51±0.37
FM	^{3}48.67±0.53	^{4}47.28±0.28	^{3}5.85a±0.74	^{3}4.09b±0.16

[a] Percentage in fresh matter.

Means with different letters show significant differences between muscles ($P<0.05$). Means with different numbers show significant differences between stages ($P<0.05$).

group of the final product was 47% in M, compared with 38% in T. This difference in relative proportions is remarkable, considering both groups of hams came from a homogeneous group of Iberian legs. The difference in relative proportions of the amino acids, could be attributed to the different proteolytic phenomena that takes place at the two salt contents, and could partially explain the larger consumer acceptance of hams made under the shorter salting periods (Ruiz, Ventanas, Cava, Timón, & García, 1998). These results agree with those of Virgili, Parolari, Schivazappa, Parolari, Bordini, and Degni (1998), who found that the free amino acid contents were greater in the more bitter samples, as also were some dipeptidase activities.

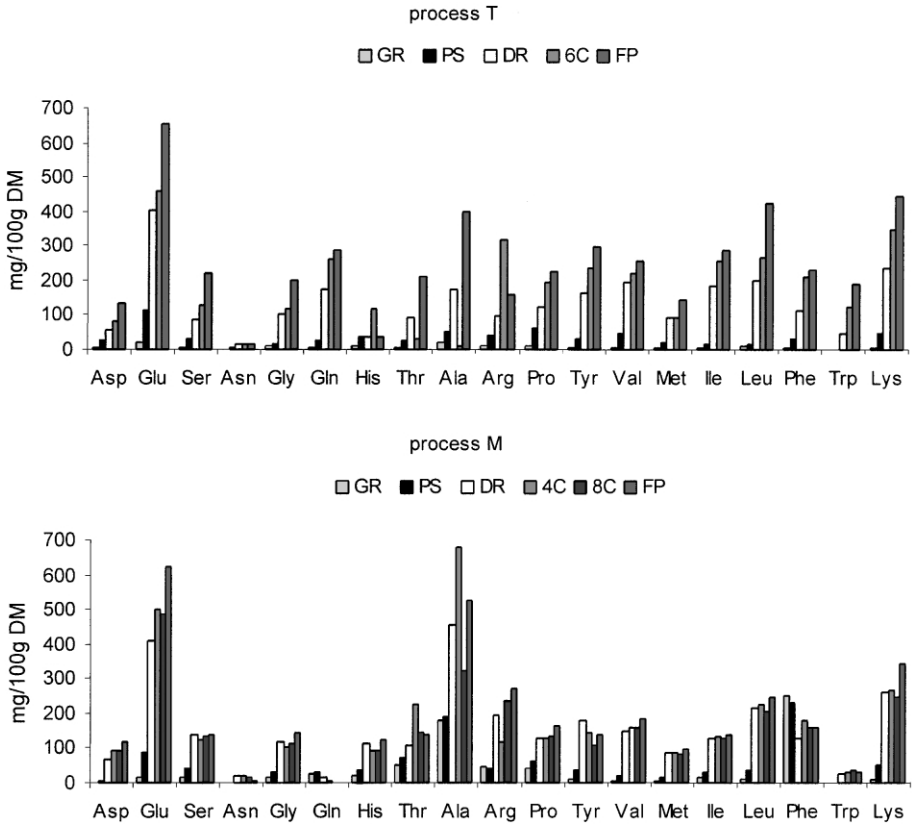

Fig. 2. Free amino acid contents at the different stages of the T and M processes (green ham, GR; postsalting PS; drying DR; months in cellar 4C, 6C, 8C; final product FP).

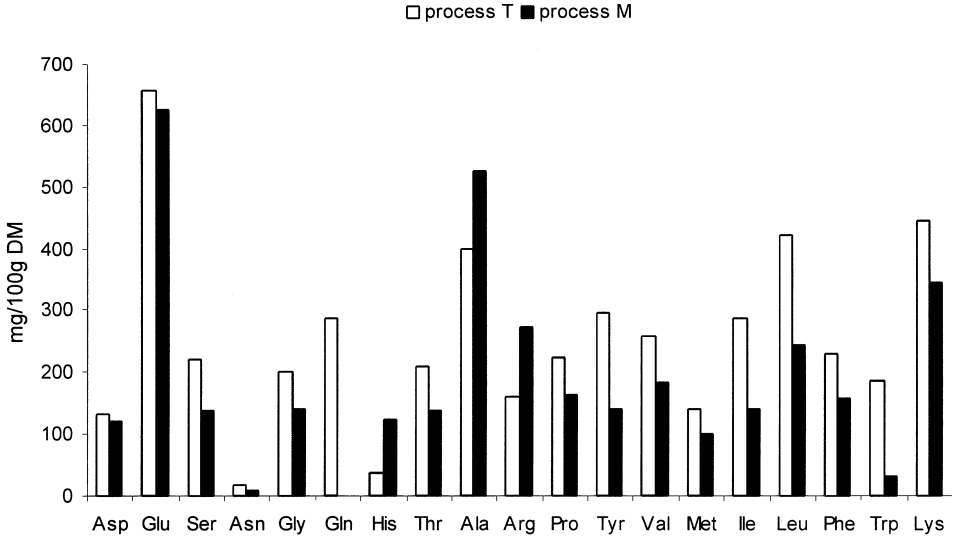

Fig. 3. Free amino acid contents in dry-cured hams from the T and M processes.

Less salted hams were subjected to further analysis for several non-volatile substances using reverse HPLC separation of the perchloric acid extract of the dry-cured hams from M. In this analysis fourteen peaks which exhibited absorbance at 214 nm were detected. The chromatography procedure was set up so as to allow the early elution (5.5 min) of the free amino acids in the extract, thus avoiding their interference.

Their presence in this fraction and the polarity exhibited in the chromatography analysis indicated that they were probably peptides since their retention behaviour indicated larger molecular weights, than the free amino acids. Also, the evolution of these compounds was similar to the peptide nitrogen analyzed in these hams (Martín, Córdoba et al., 1998).

Further analysis of these 14 characteristic peaks by LC-MS analysis supported this conclusion since the results indicated the compounds had molecular weights between 185 and 320.

These results indicate massive proteolytic breakdown took place in hams from M, leading to the formation of low molecular weight peptides. Fig. 4 shows the increase in the amount of these compounds throughout maturation.

There was a relationship between the successive curing steps and the formation of these low molecular weight peptides (Fig. 5). The largest increase in the area of the peaks took place during drying, the increase being significant for most of the peaks ($P<0.05$). Also, during the latter stages of processing, in the cellar, the rate of formation showed a significant increase ($P<0.05$). The temperature in the drying stage was highest during ripening, and the salt concentration was lower than in subsequent stages. Therefore, enzymatic activity was probably greater during drying compared to the later stages leading to more marked peptide release. This two-step increase was in good agreement with the growth of free amino acids in low-salted Iberian hams reported previously, and is consistent with the relatively high temperatures (20–25°C) reached during these two phases (Fig. 5).

Other authors have investigated the peptides present in dry-cured ham (Flores et al., 1998; Hansen-Moller et al., 1997; Rodríguez-Nuñez, Aristoy, & Toldrá, 1995;

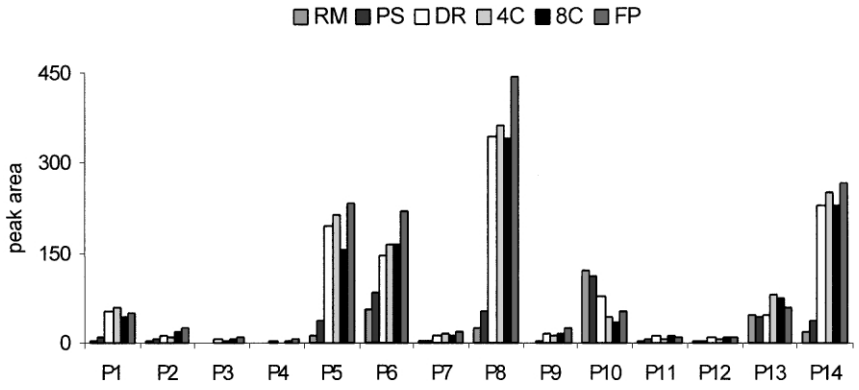

Fig. 4. Development of low molecular weight compounds during process M.

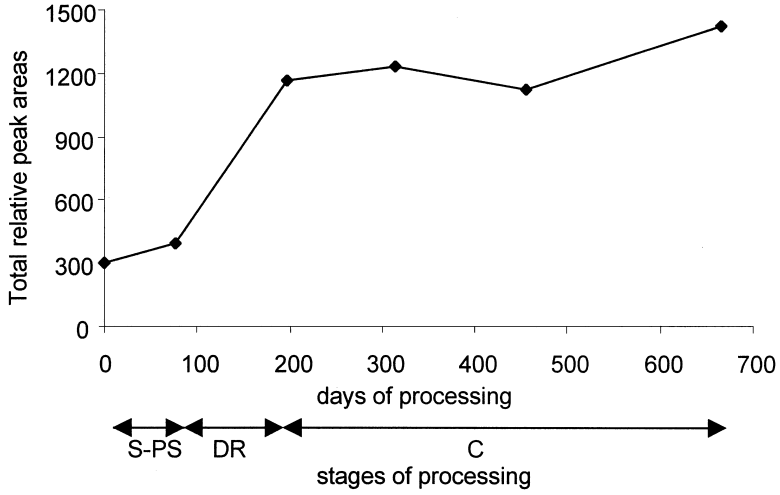

Fig. 5. Evolution of the total low molecular weight peptides in hams from process M during the different stages of ripening: salting-postsalting (S-PS), drying (DR), cellar (C).

Ruiz et al., 1999) and found a marked increase in the concentration of peptides as dry-curing progressed, although in some studies the peptides were larger than those reported here.

As free amino acids are the ultimate products of proteolysis, the small peptides present would represent a dynamic state in which both formation and breakdown occurred. However, the pattern of liberation of free amino acids during ripening of Iberian hams is supposedly not selective, reflecting their proportion in porcine muscle (Cordoba et al., 1994). This is not consistent with the results of the small peptides analysis. A detailed examination of the 14 individual peaks during processing showed that the individual peaks behaved differently. Some which were absent in the raw meat (P3, P9) increased during ripening ($P<0.05$), indicating an accumulation of proteolysis products during processing. P10 decreased ($P<0.05$) to virtually nothing and P13 showed a slight reduction, which could be due to inhibition of the proteases that produced them during further proteolysis. On the other hand, P5, P6, P8 and P14 which were low in the green state gave a marked increase during drying. Thus, the proteolytic system responsible for their generation (oligopeptidases) seems to be active during the whole dry-curing process, being the most abundant peaks in the final product. Overall, an increase in the small peptides in the processed hams is found (Fig. 5).

As they increase during maturation, these small peptides could be used as a maturation index for Iberian ham, since their determination is easier than that for amino acids. It is important to continue to investigate the generation of these small peptides as they contribute to the specific flavour of Iberian dry-cured ham.

References

Antequera, T., López-Bote, C., Córdoba, J. J., García, C., Asensio, M. A., Ventanas, J., García-Regueiro, J. A., & Díaz, I. (1992). Lipid oxidative changes in the processing of Iberian pig hams. *Food Chemistry*, *45*, 105–110.

Aristoy, M. C., & Toldrá, F. (1995). Isolation of flavor peptides from raw pork meat and dry-cured ham. In G. Charalambous (Ed.), *Food flavors: generation analysis and process influence* (pp. 1323–1344). Amsterdam: Elsevier Science BV.

AOAC (1984). *Official methods of analysis* (3rd ed.) 24010: 432. Association of Official Analytical Chemistry, Washington D.C.

Buscailhon, S., Berdagué, J. L., Bousset, J., Cornet, M., Gandemer, G., Touraille, C., & Monin, G. (1994). Relations between compositional traits and sensory qualities of French dry-cured ham. *Meat Science*, *37*, 229–243.

Careri, M., Mangia, A., Barbieri, G., Bolzoni, L., Virgili, R., & Parolari, G. (1993). Sensory property relationships to chemical data of Italian-type dry-cured ham. *Journal of Food Science*, *58*, 968–972.

Córdoba, J. J., Antequera, T., García, C., Ventanas, J., López-Bote, C., & Asensio, M. A. (1994). Evolution of free amino acids and amines during ripening of Iberian cured ham. *Journal of Agricultural and Food Chemistry*, *42*, 2296–2301.

Flores, M., Aristoy, M. C., Spanier, A. M., & Toldrá, F. (1997). Non-volatile components effects on quality of 'Serrano' dry-cured ham as related to processing time. *Journal of Food Science*, *62*, 1235–1239.

García, C., Berdagué, J. J., Antequera, T., López-Bote, C., Córdoba, J. J., & Ventanas, J. (1991). Volatile components of dry cured Iberian ham. *Food Chemistry*, *41*, 23–32.

Flores, M., Aristoy, M. C., & Toldrá, F. (1998). Feedback inhibition of porcine muscle alanyl and arginyl aminopeptidases in cured meat products. *Journal of Agricultural and Food Chemistry*, *46*, 4982–4986.

Hansen-Moller, J., Hinrichsen, L., & Jacobsen, T. (1997). Evaluation of peptides generated in Italian-style dry-cured ham during processing. *Journal of Agricultural and Food Chemistry*, *45*, 3123–3128.

Martín, L., Antequera, T., Ruiz, J., Cava, R., Tejeda, J. F., & Córdoba, J. J. (1998). Influence of the processing conditions on the proteolysis during the ripening of Iberian ham. *Food Science and Technology International*, *4*, 17–22.

Martín, L., Córdoba, J. J., Antequera, T., Timón, M. L., & Ventanas, J. (1998). Effects of salt and temperature on proteolysis during ripening of Iberian ham. *Meat Science*, *49*, 145–153.

Mau, J. L., & Tseng, Y. H. (1998). Nonvolatile taste components of three strains of Agrocybe cylindracea. *Journal of the Agricultural and Food Chemistry*, *46*, 2071–2074.

Ruiz, J., Ventanas, J., Cava, R., Timón, M. L., & García, C. (1998). Sensory characteristics of Iberian ham: influence of processing time and slice location. *Food Research International*, *31*, 53–58.

Ruiz, J., García, C., Díaz, M. C., Cava, R., Tejeda, J., & Ventanas, J. (1999). Dry cured Iberian ham non-volatile components as affected by the length of the curing process. *Food Research International*, *32*, 643–651.

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

Toldrá, F., Aristoy, M. C., Part, C., Cerveró, C., Rico, E., Motilva, M. J., & Flores, J. (1992). Muscle and adipose tissue aminopeptidase activities in raw and dry-cured ham. *Journal of Food Science*, *57*, 816–833.

Toldrá, F. (1998). Proteolysis and lipolysis in flavour development of dry-cured meat products. *Meat Science*, *49*, 101–110.

Ventanas, J., Córdoba, J. J., Antequera, T., García, C., López-Bote, C., & Asensio, M. A. (1992). Hydrolysis and Maillard reactions during ripening of Iberian ham. *Journal of Food Science*, *57*, 813–815.

Virgili, R., Schivazappa, C., Parolari, G., Bordini, C. S., & Degni, M. (1998). Proteases in fresh pork muscle and their influence on bitter taste formation in dry-cured ham. *Journal of Food Biochemistry*, *22*, 53–63.

Virgili, R., Parolari, G., Soresi-Bordini, C., Schivazappa, C., Cornet, M., & Monin, G. (1999). Free amino acids and dipeptides in dry cured ham. *Journal of Muscle Foods*, *10*, 119–130.

Yang, C., & Sepúlveda, F. (1985). Separation of phenylthiocarbamyl amino acids by high-performance liquid chromatography on Spherisorb octadecylsilane columns. *Journal of Chromatography*, *346*, 413–416.