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Quantification and *in silico* analysis of taste dipeptides generated during dry-cured ham processing

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ABSTRACT

Small peptides such as dipeptides contribute to a great extent to the characteristic taste of dry-cured hams. In this study, hydrophilic interaction liquid chromatography (HILIC) combined to tandem mass spectrometry was used to separate, identify, and quantify seven dipeptides in dry-cured hams sampled at different processing times (6, 12, 18, and 24 months). Results showed an increased concentration of dipeptides DA, DG, EE, ES, and EV with the length of processing, obtaining values up to 23 μg/g of dry-cured ham, which suggests an intense action of muscle enzymes dipeptidyl peptidases during the process. The dipeptide VG significantly decreased from 7 to 4 μg/g of dry-cured ham as the processing increased from 6 to 24 months, whereas the dipeptide PA showed low values between 380 and 550 ng/g of dry-cured ham at all the sampling times. Additionally, *in silico* analyses reported the sensory characteristics of the studied dipeptides, mostly giving bitter and umami taste, and predicted their allergenicity, toxicity, and physicochemical properties. These results could be useful for further studies related to the pleasant taste of dry-cured hams.

1. Introduction

The use of liquid chromatography (LC) coupled to mass spectrometry (MS) is an effective and accurate technique for the identification and quantification of peptides. In the case of dipeptides and tripeptides, their identification is principally done by *de novo* sequencing or matching the accurate mass and retention time to those of standards. However, these analyses involve several difficulties related to the complexity of the matrix sample, small size of dipeptides, their poor separation on common reversed phase (RP) chromatographic columns, the wide range of polarity and retention times during LC analysis due to matrix interferences, as well as the limited type and number of generated fragment ions that avoid an unambiguous sequence identification (Tang, Li, Lin, & Li, 2014; Piovesana et al., 2019). The use of an efficient LC separation, accurate MS instruments, and optimisation of MS parameters are all crucial to achieve an efficient analysis of small peptides in complex matrices such as dry-cured ham.

Dry-cured ham is a traditional and high-valuable product with characteristic texture, flavour and quality that are directly related to the raw material and processing conditions. Proteolysis is the main biochemical reaction that takes place during the processing of hams, generating high amounts of small peptides and free amino acids. These compounds contribute, together with the volatile compounds generated from lipolysis, lipid oxidation and Maillard reactions, to the organoleptic characteristics of dry-cured hams (Toldrá, Flores, & Sanz, 1997; Toldrá & Flores, 1998). In fact, several studies have reported the influence of muscle enzymes, processing conditions and processing time on the sensory characteristics of dry-cured hams, noting that the pleasant taste of the final product is the result of an appropriate balance of enzymatic activities during processing (Flores, Aristoy, Spanier & Toldrá, 1997; Toldrá et al., 1997; Ruiz et al., 1998; Sforza et al., 2006; Del Olmo, Calzada, Gaya, & Nuñez 2015).

Bitter, salty, sweet, sour, and umami constitute the five basic taste sensations that are mainly produced by non-volatile or water-soluble compounds such as salts, sugars, nucleotides, amino acids, and peptides (Fisher & Scott, 1997; Farmer, 1999). Food-derived peptides can exhibit all these tastes. Bitter, umami and sweet tastes result from electronic and/or conformational features of peptides, whereas salty and sour tastes are related to their zwitterionic and nature of amino acid side chains (Temussi, 2012). Numerous dipeptides and tripeptides have been reported to influence meat sensory traits both directly or indirectly through interaction with other compounds such as volatiles, amino acids, or nucleotides (Dashdorj et al., 2015; Kęska & Stadnik, 2017). So, empirical approaches have reported taste characteristics of several dry-

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cured ham dipeptides such as the bitterness given by GF (Gly-Phe), GL (Gly-Leu), LE (Leu-Glu), and PL (Pro-Leu), or the sour taste of dipeptides DV (Asp-Val), GE (Gly-Glu), and VE (Val-Glu), among others (Sforza et al., 2001; Sentandreu et al., 2003). Nevertheless, *in silico* approaches are gaining ground as a fast and cheap tool to predict the generation, characteristics, and structure-taste relationships of sensory peptides in foods prior to complex *in vitro* and *in vivo* studies using instrumental and sensory analyses.

The aim of this study was the identification and quantification of a certain number of taste dipeptides generated throughout the dry-cured ham processing by combining hydrophilic interaction liquid chromatography (HILIC) and non-targeted analysis by MS/MS. Moreover, *in silico* analyses regarding sensory characteristics, allergenicity, toxicity, and physicochemical properties of the studied dipeptides were performed.

2. Materials and methods

2.1. Chemicals and reagents

Peptides are named all through the manuscript using the standard IUPAC single-letter codes for amino acid sequences. Standard dipeptides DG, EE, ES, EV, PA, and VG were purchased from Bachem AG. (Bubendorf, Switzerland), and the dipeptide DA was from Sigma-Aldrich, Co. (St. Louis, MO, USA). Water and acetonitrile of LC-MS grade were obtained from Sharlab, S.L. (Barcelona, Spain), and ammonium acetate for LC-MS was from Sigma-Aldrich, Co. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

2.2. Peptide extraction and deproteinisation

Spanish dry-cured hams from pigs of industrial genotypes Landrace × Large White were processed in a local factory in Spain following the traditional procedure up to different lengths of processing: 6, 12, 18, and 24 months. Hams were obtained from different pigs. Then, *Biceps femoris* muscles of hams were subjected to peptide extraction and deproteinisation following the methodology used and described by Gallego et al. (2015). The study was done with 6 hams per curing time.

2.3. Ultrafiltration

A total of 50 mg of each peptide extract, in duplicate, was mixed with 1.5 mL of water and fractionated by ultrafiltration using Amicon® ultra 0.5 mL centrifugal filters (Merk Millipore Ltd., Cork, Ireland) with a membrane having molecular mass cut-off of 3 kDa. The collected fractions containing peptides lower than 3 kDa were freeze-dried and then resuspended in water to reach a concentration of 10 mg/mL. After centrifugation at 4 °C and 10,000 *g* for 10 min, the supernatants were analysed by mass spectrometry.

2.4. HILIC LC-MS/MS analysis

The LC-MS/MS analysis was performed using an Agilent 1260 Infinity LC system (Agilent, Palo Alto, CA, USA) coupled to a triple quadrupole mass spectrometer (QQQ) 6420 Triple Quad LC/MS (Agilent, CA, USA) with an electrospray ionisation source (ESI).

A total of 5 μL of each sample was injected and concentrated on a SeQuant ZIC®–HILIC guard fitting PEEK column (5 μm, 1 mm × 14 mm; Merk KGaA, Darmstadt, Germany) at a flow rate of 0.02 mL/min for 5 min and using 90% (v/v) acetonitrile (ACN) in 10 mM ammonium acetate as mobile phase. The trap column was automatically switched in-line onto a SeQuant ZIC®–HILIC capillary column (5 μm, 150 mm × 0.3 mm; Merk KGaA, Darmstadt, Germany). Mobile phases were 10 mM ammonium acetate as solvent A, and ACN as solvent B. Gradient elution for LC was 0–8 min, 80% B; 8–25 min, linear from 80 to 30% B; 25–28 min 30% B; and 28–35 min, linear from 30 to 80% B; at a flow rate of 6 μL/min at 30 °C. The column outlet was directly coupled to an ESI, and the QQQ (MS/MS) was operated in positive polarity to acquire full scan mass spectra from 70 to 500 *m/z*. Other MS parameters were: nitrogen gas flow, 6 L/min; gas temperature, 350 °C; nebulizer pressure, 15 psi; capillary, 3500 V; fragmentor, 100 V; scan time, 500 ms; cell accelerator, 4 V.

The standard dipeptides (1 nmol/μL) were analysed by LC-MS/MS in order to obtain information on *m/z* and retention time of each peptide from the MS profiling. In order to quantify the dipeptides in the dry-cured ham samples, calibration curves from the standard peptides were prepared using peak areas obtained from extracted ion chromatograms (XICs). Interference studies were performed by spiking the dry-cured ham samples with standard dipeptides in order to confirm retention times through co-elution. Samples were processed and data were evaluated using MassHunter LC/MS Data Acquisition (version B.08.00) and MassHunter Qualitative Analysis software (version B.07.00) (Agilent Technologies, Inc.), respectively. The analyses of the standard dipeptides were done in triplicate (n = 3), whereas dry-cured ham samples were done in six samples (n = 6).

2.5. In silico analysis

The BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) was used in the search of similar sequences previously identified as sensory peptides (Minkiewicz, Iwaniak, & Darewicz, 2019).

The potential allergenicity of dipeptides was predicted using the AllerTOP v.2.0 software (http://www.ddg-pharmfac.net/AllerTOP/index.html), in which peptides are classified as probable allergen or non-allergen based on their physicochemical properties (Dimitrov, Bangov, Flower, & Doytchinova, 2014).

Peptide toxicity and physicochemical properties such as hydrophobicity, steric hindrance, side bulk, hydropathicity, amphipathicity, hydrophilicity, and charge were evaluated using the ToxinPred tool (http://crdd.osdd.net/raghava/toxinpred/). Peptide toxicity was predicted according to the amino acid composition using the SVM (support vector machine) based method, and a threshold value of 0.0 was used to classify toxic and non-toxic peptides (Gupta et al., 2013). In addition, primary structures of the dipeptides were drawn using the PepDraw tool (http://pepdraw.com/).

2.6. Statistical analysis

Statistical analysis including one-way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) tests were performed using Statgraphics Centurion XVI software (Statgraphics Technologies, Inc., The Plains, VA, USA). Results were expressed as the mean of 6 replicates ± standard deviations, and differences were considered significantly at $p < 0.05$.

3. Results and discussion

3.1. Quantification of dipeptides in dry-cured hams

Dipeptides can be generated in large amounts during the dry-cured ham processing by the action of muscle enzymes dipeptidyl peptidases (DPP), which release dipeptides from the *N*-terminal position of polypeptides. In particular, DPP I could be the main enzyme involved in the generation of dipeptides during dry-curing, as it would remain strongly active during processing. The action of DPP II, III and IV could be limited depending on processing conditions such as salt, pH and temperature (Sentandreu & Toldrá, 2001; Zhao et al., 2005).

Quantitative changes in the profiles of dipeptides in dry-cured hams at different times of processing (6, 12, 18, and 24 months) were evaluated using LC-MS/MS. In order to fractionate the dry-cured ham samples, the obtained peptide extracts were ultrafiltered and only those

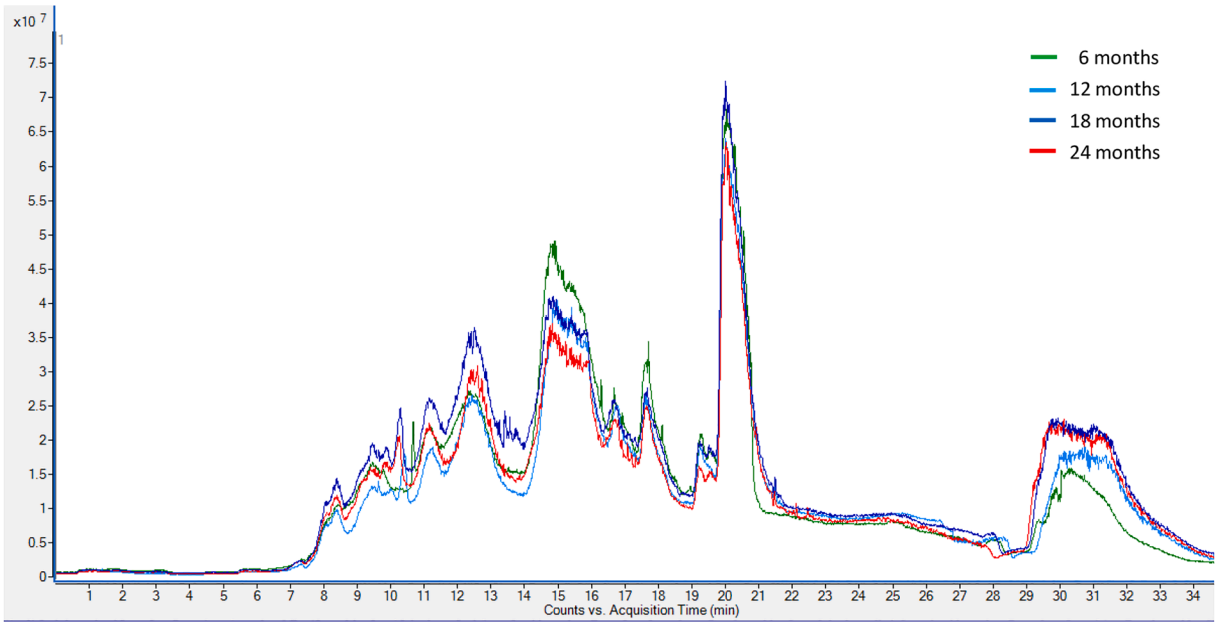

Fig. 1. Total ion chromatograms (TIC) of dry-cured ham samples at 6, 12, 18, and 24 months of processing obtained from HILIC LC-MS/MS analysis.

peptide fractions lower than 3 kDa were analysed. However, the study of dipeptides in a complex matrix such as dry-cured ham involved additional difficulties including the poor retention and separation on RP-LC columns commonly used for MS analysis (data not shown). To overcome this problem, small peptides are usually chemically derivatised, but this labelling sometimes results in the loss of sequence and structure information due to the reduced MS/MS fragmentation of the dipeptide backbone (Tang et al., 2014). For this reason, the strategy employed in the present study was the use of HILIC, which effectively improves retention and separation of dipeptides without the need for derivatisation with the additional advantage of compatibility with further MS analysis (Nguyen & Schug, 2008; Guo & Gaiki, 2011; Mora, Hernández-Cázares, Aristoy, Toldrá, & Reig, 2011).

Dry-cured ham samples at 6, 12, 18, and 24 months of processing were analysed using HILIC LC-MS/MS, showing in Fig. 1 the resulting total ion chromatograms (TIC). The identification of dipeptides in the dry-cured ham samples was done by matching the accurate mass and retention time of those of standards, whereas the quantification was done based on peak intensity areas of XIC using calibration curves from the standard dipeptides. In a mass spectrum, the area or height of a peak at certain m/z measures the number of detected ions, and the integration of the ion intensity over time results in a XIC that represents the ion abundance for the peptide (Wong, Sullivan, & Cagney, 2007). However, some dipeptides presented retention time drifts in comparison to standards due to matrix interferences, being mandatory the spiking of the dry-cured ham samples for a precise identification and quantification of the dipeptides.

Seven dipeptides including DA, DG, EE, ES, EV, PA, and VG were accurately identified and quantified by HILIC LC-MS/MS analysis in the dry-cured ham samples. As example, Fig. 2 shows the XIC of the standard dipeptide DG at different concentrations (1–100 pmol/μL) as well as the calibration curve obtained from the integration of peak areas in XICs. Quantitative results of the seven dipeptides are presented in Fig. 3, evidencing their evolution in dry-cured hams at different processing times (6, 12, 18, and 24 months). Results showed that the concentration of dipeptides DA and DG increased over processing time, ranging values of about 5 to 15 μg/g of dry-cured ham. The dipeptide EE increased almost 10 times from 6 months to 12 months of processing and reached values around 18 μg/g in dry-cured hams at 18 and 24 months. This dipeptide may be derived from the degradation of the titin protein, which has been described to be intensively hydrolysed during dry-cured ham processing (Gallego et al., 2015). Meanwhile, the concentration of the dipeptide ES was below the detection limit of the method when analysed in hams with a short processing time (6 months), but it increased gradually at longer times up to a value of 7.5 μg/g at 24 months (Fig. 3). Similarly, dipeptide EV was only quantified in long processing times, showing a concentration of 7.7 μg/g in 18 months dry-cured hams and greatly increasing up to 23.4 μg/g in 24 months hams. These results evidence the action of DPP during dry-cured ham processing, being probably DPP I the key enzyme responsible for the generation of dipeptides (Sentandreu & Toldrá, 2001; Zhao et al., 2005). On the other hand, the dipeptide PA showed the lowest concentration among the studied ones, with values below 0.56 μg/g in all the dry-cured ham samples. Several myofibrillar proteins such as myosin light chain, titin and LIM domain-binding protein 3 have been reported to be potential sources for PA release during dry-cured ham processing (Gallego, Mora, & Toldrá, 2019). However, the low concentration of PA could be due to its further hydrolysis into free amino acids. Finally, the dipeptide VG was the unique whose concentration decreased over time, decreasing from 7.4 to 4.3 μg/g as dry-cured ham processing time increased (Fig. 3). The action of dipeptidases, which hydrolyse dipeptides into their constituent amino acid residues, could be responsible for these results (Mora, Fraser, & Toldrá, 2013).

Zhu et al. (2017) employed MS/MS analysis for the identification and relative quantification of 63 peptides (from 2 to 13 amino acid residues) in Jinhua dry-cured hams. The study reported that dipeptides, tripeptides, and tetrapeptides were the most abundant peptides in the post-ageing stage of hams (8 months of processing), showing total relative peak areas of 23.59%, 48.28%, and 21.08%, respectively. Among the ten quantified dipeptides, VE showed the highest relative peak area with a value of 5.62%, whereas PL, AR, and AH reached values higher than 3%. On the other hand, an absolute quantitative targeted analysis was used by Degnes et al. (2017) to evaluate changes in the profiles of metabolites during dry-cured hams ripening. In that study, the final concentration of the dipeptide PG was 1.3 mg/100 g dry weight ham, whereas AP, AV and GV reached values between 0.5 and 1 mg/100 g dry weight ham. In a recent study, a peptide profile was established in different types of dry-cured ham (from Japan, Spain and Italy) using a metabolomics approach, resulting in a gradual increase of the studied dipeptides AK, AA, GL, EE, and GG during ripening (Sugimoto et al., 2020).

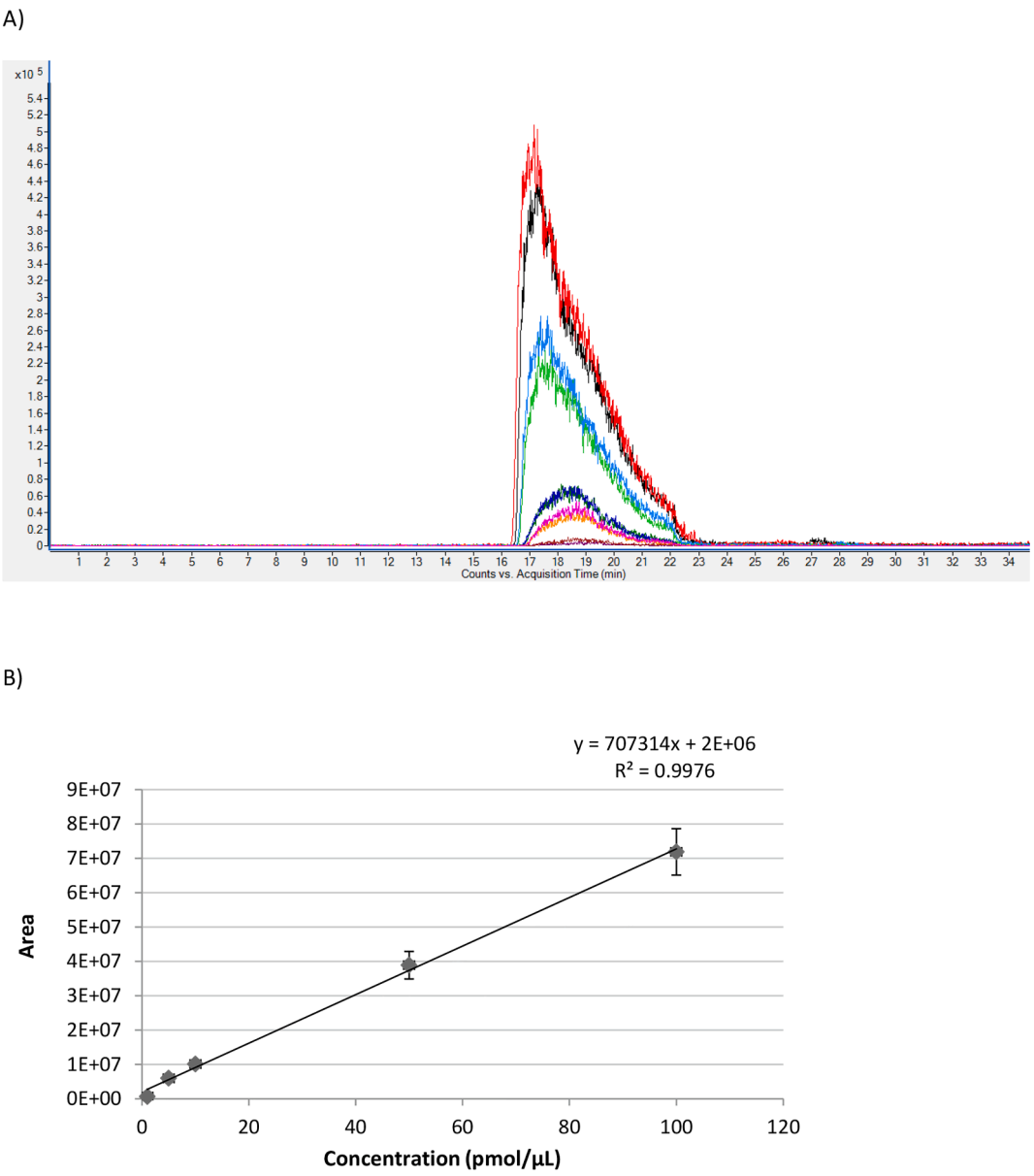

Fig. 2. A) Extracted ion chromatogram (XIC) of the standard dipeptide DG at different concentrations and B) calibrated curve obtained from integration of peak areas in XICs.

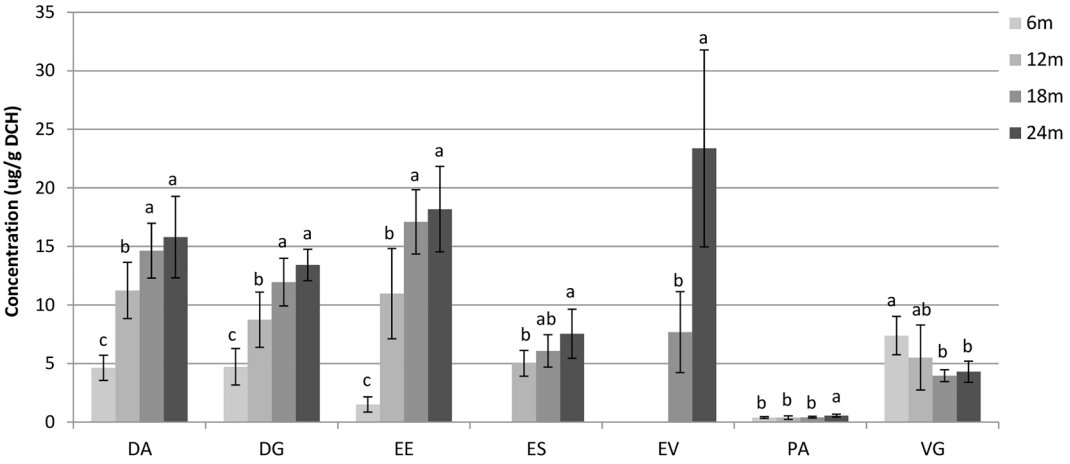

Fig. 3. Quantitative results obtained from peak areas in XICs of the studied dipeptides in dry-cured hams at 6, 12, 18, and 24 months of processing.

Table 1
In silico prediction of sensory characteristics, allergenicity, toxicity and physicochemical characteristics of the dry-cured ham dipeptides.

	Dipeptide sequence	DA	DG	EE	ES	EV	PA	VG
	Mass (Da)	204.18	190.15	276.24	234.21	246.26	186.21	174.2
Sensory characteristics[a]	Bitter	x	x	x		x	x	x
	Umami	x	x	x	x	x		x
	Salty			x				
	Sour					x		
	Sweet					x		
	Bitterness suppressing			x	x			
	Sweetness suppressing			x				
Allergenicity prediction[b]		Non-allergen	Probable allergen	Non-allergen	Probable allergen	Probable allergen	Non-allergen	Probable allergen
Toxicity prediction[c]		Non-toxic	Non-toxic	Non-toxic	Non-toxic	Non-toxic	Non-toxic	Non-toxic
Physicochemical characteristics[d]	Hydrophobicity	−0.23	−0.28	−0.62	−0.44	−0.04	0.09	0.35
	Steric hindrance	0.64	0.72	0.68	0.60	0.69	0.44	0.69
	Side bulk	0.64	0.72	0.68	0.60	0.69	0.44	0.69
	Hydropathicity	−0.85	−1.95	−3.50	−2.15	0.35	0.10	1.90
	Amphipathicity	0.00	0.00	1.27	0.64	0.64	0.00	0.00
	Hydrophilicity	1.25	1.50	3.00	1.65	0.75	−0.25	−0.75
	Charge	−1.00	−1.00	−2.00	−1.00	−1.00	0.00	0.00

According to ToxinPred tool.
[a] Sensory characteristics of the dipeptides according to BIOPEP database.
[b] Allergenicity prediction according to AllerTOP tool.
[c] Toxicity prediction according to ToxinPred tool.
[d] Physicochemical characteristics of the dipeptides.

3.2. *In silico analysis of dipeptides*

Empirical approaches have reported that the combination of small peptides, mainly dipeptides, and free amino acids may contribute to the characteristic taste of dry-cured hams (Sentandreu et al., 2003). However, bioinformatic tools and databases can be used as a fast and cheap alternative to empirical methods for the prediction of the generation of sensory peptides and amino acids in foods as well as of their characteristics. A recent *in silico* study described the sequences of numerous dipeptides to be generated in dry-cured hams with predicted taste characteristics (Gallego et al., 2019). In addition, several tools available in the BIOPEP database were used to predict that myofibrillar proteins would yield the greatest number of taste-active dipeptides and free amino acids in dry-cured hams, mainly giving bitter and umami sensations (Kęska & Stadnik, 2017).

The potential taste characteristics of the studied dipeptides were predicted using BIOPEP database (Table 1). So, dipeptides DA, DG, EE, EV and VG were previously described to impart both bitter and umami tastes, PA would give bitter taste, and ES was described to be umami. Bitter taste is mainly associated with peptides that contain hydrophobic amino acids (A, I, L, P, V), aromatic (F, W, Y) or basic residues (H, K, R) (Kęska & Stadnik, 2017). Bitterness is the most frequent sensation associated with protein hydrolysis (Maehashi & Huang, 2009), and several dipeptides such as ID, IV, LE, and PL have been described to impart this taste in dry-cured hams (Sforza et al., 2001; Sentandreu et al., 2003). On the other hand, acidic amino acids (D, E) are related to umami taste, which is a good trait in dry-cured hams for its association with an aged product (Nishimura & Kato, 1988). Umami dipeptides such as ER, EY, PE, and VE have been reported to be generated during the aging of different types of hams including Spanish dry-cured hams (Sforza et al., 2001; Sentandreu et al., 2003; Dang, Gao, Ma & Wu, 2015). Additionally, dipeptide EV would also provide sour taste (Table 1), which is associated with peptides having D, E and K residues, as well as it would impart sweet taste, which is related with the presence of hydrophobic amino acids or hydrophilic residues such as G and K (Kęska & Stadnik, 2017). Dipeptide EE was further described to impart saltiness, which is a taste associated with the acidic amino acid D, as well as to exert both bitter and sweet suppression. Also ES was reported as bitterness suppressing dipeptide (Table 1). Bitterness suppressors mainly contain E, K and R residues, and their generation during dry-cured ham processing would play an important role to prevent undesired tastes in hams resulting from the accumulation of bitter peptides (Sforza et al., 2006; Kęska & Stadnik, 2017).

It should be noted that sensory peptides do not always encode the residues responsible for taste sensation. For example, dipeptides DG and EE described as bitter dipeptides do not present hydrophobic, aromatic or basic residues in their sequences, and dipeptide VG was associated with umami taste despite that it does not contain acidic amino acids (Table 1). Furthermore, the same amino acid residue could impart different tastes as its properties may depend on the spatial structure and position in the peptide sequence (Kęska & Stadnik, 2017). Most studies on peptide structure and sensory attributes have been focused on bitter peptides. Chemometric studies showed that the presence of hydrophobic amino acids located at the C-terminus and bulky residues adjacent to this position would determine the intensity of bitter peptides (Wu & Aluko, 2007; Iwaniak, Hrynkiewicz, Bucholska, Darewicz, & Minkiewicz, 2018). What is more, there may be taste-taste interactions of peptides with synergistic or suppressive effects between tastants. Umami taste would have the greatest synergistic effect by enhancing sweet and salty tastes as well as suppressing bitterness and sourness (Dang, Gao, Xie, Wu, & Ma, 2014). Salty peptides would also suppress bitterness, and salty-sour and sour-bitter interactions would enhance each taste (Kim, Son, Kim, Misaka, & Rhyu, 2015).

Taste peptides are important to determine the quality of foods, but also they may have potential as valuable and natural ingredients for modulation of taste and improving sensory characteristics. In this regard, some studies have reported that the addition of the *kokumi* peptide γ-Glu-Val-Gly could enhance umami, mouthfulness, thickness or continuity of food in which is added (Miyaki, Kawasaki, Kuroda, Miyamura, & Kouda, 2015; Miyamura, Jo, Kuroda, & Kouda, 2015). Furthermore, most taste dipeptides and tripeptides are also associated with biological activities, especially inhibition of proteolytic enzymes such as angiotensin converting enzyme (ACE), DPP IV, and DPP III, among others (Iwaniak, Minkiewicz, Darewicz, & Hrynkiewicz, 2016). It is therefore

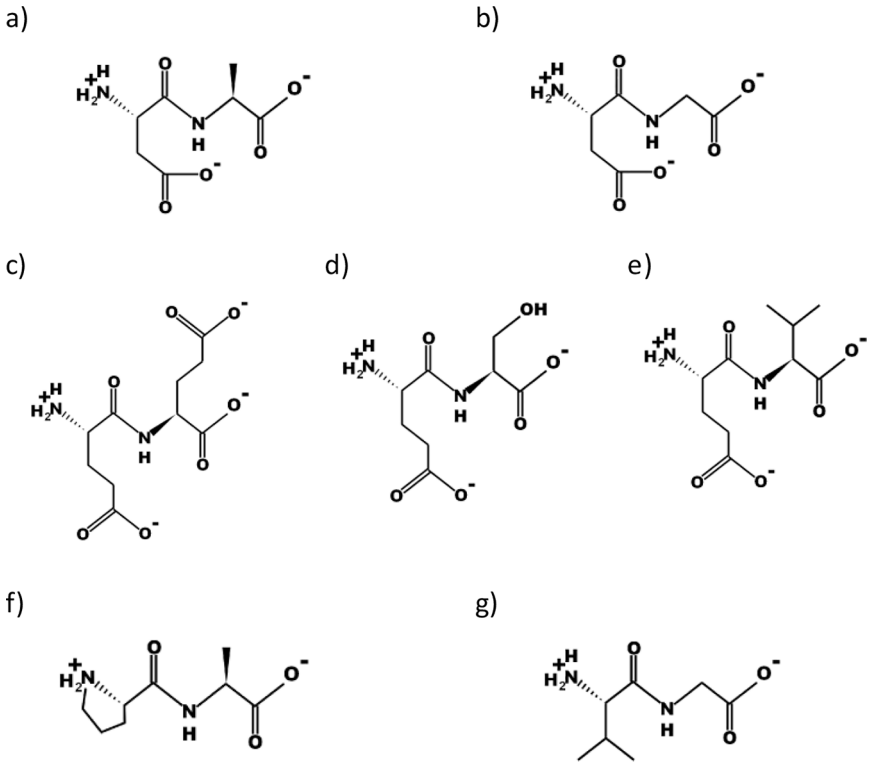

Fig. 4. Primary structure of dipeptides a) DA, b) DG, c) EE, d) ES, e) EV, f) PA, and g) VG using PepDraw tool.

important to consider the safety of peptides, especially when the aim is developing nutraceuticals or functional food ingredients. In the present study, the allergenicity and toxicity of the studied dipeptides were assessed *in silico* using AllerTop and ToxinPred tools, respectively. As shown in Table 1, dipeptides DG, ES, EV and VG were predicted to be allergen, whereas none of the studied dipeptides would be toxic (SVM scores < 0). Gupta et al. (2013) reported that certain amino acids such as C, H, N, and P were abundant and preferred at various positions (C- or *N*-terminus) in toxic peptides.

Moreover, the structure and spatial conformation of peptides result in specific physicochemical properties which are determinants of peptide properties. Some physicochemical characteristics of the studied dipeptides predicted using ToxinPred tool are indicated in Table 1, whereas their primary structures are shown in Fig. 4. Chemical information obtained from databases and *in silico* tools are very useful to understand the relationship between the role of molecular or physicochemical properties (size, electronic attributes, hydrophobicity, polarity, steric properties, etc.) of a peptide and its activities or properties, as a function of the chemical structures. In this regard, structure-bitterness relationship of peptides is the most extensively studied to date. Quantitative structure–activity relationship (QSAR) prediction models have reported significant correlations between ACE inhibition and bitterness of dipeptides, attributing this relationship to hydrophobic properties (Pripp & Ardö, 2007). Moreover, multivariate analyses using principal component analysis (PCA) or multiple linear regression (MLR) models stated the importance of molecular weight, bulkiness, number of carbon and hydrogen atoms of amino acids forming the sequences, and hydrophobicity of amino acids in the bitterness of dipeptides and tripeptides (Iwaniak et al., 2018; Iwaniak, Hrynkiewicz, Bucholska, Minkiewicz, & Darewicz, 2019). Findings achieved to date reflect the complexity between molecular structure and biological or functional properties of peptides, and therefore further studies are needed for broadening the knowledge about these relationships.

4. Conclusions

The use of HILIC LC-MS/MS allowed an efficient chromatographic separation as well as the identification and quantification of seven dipeptides in dry-cured hams at different processing times (6, 12, 18, and 24 months). Quantitative results evidence the intense action of DPP enzymes throughout dry-cured ham processing, generating high amount of dipeptides DA, DG, EE, ES, and EV with concentrations up to 23 μg/g of dry-cured ham. Dipeptide PA showed low values between 380 and 550 ng/g at all the dry-cured ham sampling times, whereas dipeptide VG significantly decreased from 7 to 4 μg/g of dry-cured ham as the processing time increased from 6 to 24 months. This suggests that both PA and VG might be hydrolysed into free amino acids during the dry-curing process. *In silico* analyses reported the sensory characteristics of the studied dipeptides, mostly giving bitter and umami taste to dry-cured hams, as well as predicted their allergenicity, toxicity, and physicochemical properties. These results could be useful for further studies using instrumental and sensory analyses in order to know those peptides with the highest contribution to the pleasant taste of dry-cured hams.

CRediT authorship contribution statement

Marta Gallego: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Visualization. **Fidel Toldrá:** Supervision, Funding acquisition, Project administration, Resources, Writing - review & editing. **Leticia Mora:** Methodology, Supervision, Funding acquisition, Project administration, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Dang, Y., Gao, X., Xie, A., Wu, X., & Ma, F. (2014). Interaction between umami peptide and taste receptor T1R1/T1R3. *Cell Biochemistry and Biophysics, 70*(3), 1841–1848.

Dang, Y., Gao, X., Ma, F., & Wu, X. (2015). Comparison of umami taste peptides in water-soluble extractions of Jinhua and Parma hams. *LWT-Food Science Technology, 60*(2), 1179–1186.

Dashdorj, D., Amna, T., & Hwang, I. (2015). Influence of specific taste-active components on meat flavor as affected by intrinsic and extrinsic factors: An overview. *European Food Research and Technology, 241*(2), 157–171.

Degnes, K. F., Kvitvang, H. F. N., Haslene-Hox, H., & Aasen, I. M. (2017). Changes in the profiles of metabolites originating from protein degradation during ripening of dry cured ham. *Food and Bioprocess Technology, 10*(6), 1122–1130.

Del Olmo, A., Calzada, J., Gaya, P., & Nuñez, M. (2015). Proteolysis and flavor characteristics of Serrano ham processed under different ripening temperature conditions. *Journal of Food Science, 80*(11), C2404–C2412.

Dimitrov, I., Bangov, I., Flower, D. R., & Doytchinova, I. (2014). AllerTOP v. 2 - a server for *in silico* prediction of allergens. *Journal of Molecular Modeling, 20*(6), 2278.

Farmer, L. J. (1999). The role of nutrients in meat flavor formation. *Proceedings of the Nutrition Society, 53*, 327–333.

Fisher, C., & Scott, T. R. (1997). The chemical senses. In Fisher, C. & Scott, T. R. (Eds.). Food flavours: Biology and chemistry (pp. 56–99). UK, RSC. Cambridge.

Flores, M., Aristoy, M. C., Spanier, A. M., & Toldra, F. (1997). Non-volatile components effects on quality of "Serrano" dry-cured ham as related to processing time. *Journal of Food Science, 62*(6), 1235–1239.

Gallego, M., Mora, L., Aristoy, M. C., & Toldrá, F. (2015). Titin-derived peptides as processing time markers in dry-cured ham. *Food Chemistry, 167*, 326–339.

Gallego, M., Mora, L., & Toldrá, F. (2019). The relevance of dipeptides and tripeptides in the bioactivity and taste of dry-cured ham. *Food Production, Processing and Nutrition, 1*(1), 2.

Guo, Y., & Gaiki, S. (2011). Retention and selectivity of stationary phases for hydrophilic interaction chromatography. *Journal of Chromatography A, 1218*(35), 5920–5938.

Gupta, S., Kapoor, P., Chaudhary, K., Gautam, A., Kumar, R., Raghava, G. P., & Open Source Drug Discovery Consortium. (2013). In silico approach for predicting toxicity of peptides and proteins. *PloS One, 8*(9).

Iwaniak, A., Minkiewicz, P., Darewicz, M., & Hrynkiewicz, M. (2016). Food protein-originating peptides as tastants - Physiological, technological, sensory, and bioinformatic approaches. *Food Research International, 89*, 27–38.

Iwaniak, A., Hrynkiewicz, M., Bucholska, J., Darewicz, M., & Minkiewicz, P. (2018). Structural characteristics of food protein-originating di-and tripeptides using principal component analysis. *European Food Research and Technology, 244*(10), 1751–1758.

Iwaniak, A., Hrynkiewicz, M., Bucholska, J., Minkiewicz, P., & Darewicz, M. (2019). Understanding the nature of bitter-taste di-and tripeptides derived from food proteins based on chemometric analysis. *Journal of Food Biochemistry, 43*(1), e12500. https://doi.org/10.1111/jfbc.12500.

Kęska, P., & Stadnik, J. (2017). Taste-active peptides and amino acids of pork meat as components of dry-cured meat products: An *in-silico* study. *Journal of Sensory Studies, 32*(6), e12301.

Kim, M. J., Son, H. J., Kim, Y., Misaka, T., & Rhyu, M. R. (2015). Umami–bitter interactions: The suppression of bitterness by umami peptides via human bitter taste receptor. *Biochemical and Biophysical Research Communications, 456*(2), 586–590.

Maehashi, K., & Huang, L. (2009). Bitter peptides and bitter taste receptors. *Cellular and Molecular Life Sciences, 66*(10), 1661–1671.

Miyaki, T., Kawasaki, H., Kuroda, M., Miyamura, N., & Kouda, T. (2015). Effect of a kokumi peptide, γ-glutamyl-valyl-glycine, on the sensory characteristics of chicken consommé. *Flavour, 4*(17), 99–106.

Miyamura, N., Jo, S., Kuroda, M., & Kouda, T. (2015). Flavour improvement of reduced-fat peanut butter by addition of a kokumi peptide, γ-glutamyl-valyl-glycine. *Flavour, 4*(16), 95–98.

Mora, L., Hernández-Cázares, A. S., Aristoy, M.-C., Toldrá, F., & Reig, M. (2011). Hydrophilic interaction chromatography (HILIC) in the analysis of relevant quality and safety biochemical compounds in meat, poultry and processed meats. *Food Analytical Methods, 4*(1), 121–129.

Mora, L., Fraser, P. D., & Toldrá, F. (2013). Proteolysis follow-up in dry-cured meat products through proteomic approaches. *Food Research International, 54*(1), 1292–1297.

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM database of bioactive peptides: Current opportunities. *International Journal of Molecular Sciences, 20*(23), 5978.

Nguyen, H. P., & Schug, K. A. (2008). The advantages of ESI-MS detection in conjunction with HILIC mode separations: Fundamentals and applications. *Journal of Separation Science, 31*(9), 1465–1480.

Nishimura, T., & Kato, H. (1988). Taste of free amino acids and peptides. *Food Reviews International, 4*(2), 175–194.

Piovesana, S., Montone, C. M., Cavaliere, C., Crescenzi, C., La Barbera, G., Laganà, A., & Capriotti, A. L. (2019). Sensitive untargeted identification of short hydrophilic peptides by high performance liquid chromatography on porous graphitic carbon coupled to high resolution mass spectrometry. *Journal of Chromatography A, 1590*, 73–79.

Pripp, A., & Ardö, Y. (2007). Modelling relationship between angiotensin-(I)-converting enzyme inhibition and the bitter taste of peptides. *Food Chemistry, 102*(3), 880–888.

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*(1), 53–58.

Sentandreu, M., & Toldrá, F. (2001). Dipeptidyl peptidase activities along the processing of Serrano dry-cured ham. *European Food Research and Technology, 213*(2), 83–87.

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

Sforza, S., Pigazzani, A., Motti, M., Porta, C., Virgili, R., Galaverna, G., … Marchelli, R. (2001). Oligopeptides and free amino acids in Parma hams of known cathepsin B activity. *Food Chemistry, 75*(3), 267–273.

Sforza, S., Galaverna, G., Schivazappa, C., Marchelli, R., Dossena, A., & Virgili, R. (2006). Effect of extended aging of Parma dry-cured ham on the content of oligopeptides and free amino acids. *Journal of Agricultural and Food Chemistry, 54*(25), 9422–9429.

Sugimoto, M., Sugawara, T., Obiya, S., Enomoto, A., Kaneko, M., Ota, S., & Tomita, M. (2020). Sensory properties and metabolomic profiles of dry-cured ham during the ripening process. *Food Research International, 129*, 108850.

Tang, Y., Li, R., Lin, G., & Li, L. (2014). PEP search in MyCompoundID: Detection and identification of dipeptides and tripeptides using dimethyl labeling and hydrophilic interaction liquid chromatography tandem mass spectrometry. *Analytical Chemistry, 86*(7), 3568–3574.

Temussi, P. A. (2012). The good taste of peptides. *Journal of Peptide Science, 18*(2), 73–82.

Toldrá, F., Flores, M., & Sanz, Y. (1997). Dry-cured ham flavour: Enzymatic generation and process influence. *Food Chemistry, 59*(4), 523–530.

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

Wong, J. W., Sullivan, M. J., & Cagney, G. (2007). Computational methods for the comparative quantification of proteins in label-free LCn-MS experiments. *Briefings in Bioinformatics, 9*(2), 156–165.

Wu, J., & Aluko, R. E. (2007). Quantitative structure-activity relationship study of bitter di- and tri-peptides including relationship with angiotensin I-converting enzyme inhibitory activity. *Journal of Peptide Science, 13*(1), 63–69.

Zhao, G. M., Zhou, G. H., Xu, X. L., Peng, Z. Q., Huan, Y. J., Jing, Z. M., & Chen, M. W. (2005). Studies on time-related changes of dipeptidyl peptidase during processing of Jinhua ham using response surface methodology. *Meat Science, 69*(1), 165–174.

Zhu, C. Z., Tian, W., Li, M. Y., Liu, Y. X., & Zhao, G. M. (2017). Separation and identification of peptides from dry-cured Jinhua ham. *International Journal of Food Properties, 20*(S3), S2980–S2989.