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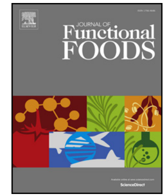

Antioxidant peptides profile in dry-cured ham as affected by gastrointestinal digestion

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ABSTRACT

Dry-cured hams are a good source of bioactive peptides, whose stability to gastrointestinal (GI) digestion determines their bioaccessibility and thus their bioavailability to exert beneficial effects. The aim of this study was to evaluate the effect of *in vitro* GI digestion of dry-cured hams on the peptide profiles and their antioxidant activity. Results showed that the antioxidant activity decreased in the digested samples when measured by DPPH radical scavenging activity and ferric-reducing antioxidant power methods, but increased when assayed by ABTS radical scavenging assay. Chromatographic and mass spectrometry analysis revealed changes in the peptide profiles and evidenced the degradation of proteins, mainly titin, by the digestive enzymes. The study of the peptide profiles before and after digestion of dry-cured hams would help to extend the knowledge about the influence of peptide characteristics on their antioxidant activity and resistance to hydrolysis by digestive enzymes, allowing a better understanding of the bioaccessibility of the bioactive peptides generated in dry-cured hams.

1. Introduction

Spanish dry-cured hams constitute widely appreciated products by consumers due to their organoleptic characteristics resulting from the proteolysis and lipolysis phenomena that occur during its processing. Proteolysis is produced by the action of endogenous muscle enzymes (endopeptidases and exopeptidases), which breaks down proteins and generates high amount of small peptides and free amino acids that contribute to the final organoleptic and textural characteristics of dry-cured hams (Toldrá & Flores, 1998; Toldrá, Aristoy, & Flores, 2000). As a result of proteolysis, dry-cured hams are also a good source of bioactive peptides that can exert functional properties in human health. Bioactive peptides are generally 2–20 amino acids in length that remain inactive within the sequence of their parent proteins and they have to be released by endogenous or exogenous enzymes during processing in order to exert a physiological effect in the organism (Lafarga & Hayes, 2014; Udenigwe & Aluko, 2012). Several studies have reported the identification of bioactive peptides in water soluble extracts obtained from dry-cured hams, mainly showing *in vitro* angiotensin I-converting enzyme (ACE-I) inhibition, but also *in vivo* antihypertensive activity on spontaneously hypertensive rats (Escudero et al., 2013; Escudero, Mora, & Toldrá, 2014; Mora, Escudero, Arihara, & Toldrá, 2015). Moreover, dry-cured ham peptide extracts have shown an intense antioxidant activity (Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013; Xing et al., 2016; Zhu et al., 2013), and SNAAC has been the strongest antioxidant peptide identified to date in ham with IC_{50} values of 75.2 μM in DPPH radical scavenging activity and 205 μM in ferric-reducing power assay (Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014). Antioxidant peptides can exert a beneficial role in the organism by providing protection from oxidative stress caused by free radicals and/or reactive oxygen species (ROS), which are implicated in several human disorders. The effectiveness of a peptide to act as antioxidant and the mechanism underlying this activity are majorly determined by the size, composition and location of the amino acids in the sequence, structure, and hydrophobicity of the peptide (Nwachukwu & Aluko, 2019; Sarmadi & Ismail, 2010).

Nevertheless, some peptides showing bioactivity *in vitro* fail to exert effects *in vivo* (and vice versa) due to possible modifications o degradation of the peptides by the enzymes participating in the gastrointestinal (GI) digestion or intestinal absorption processes (Iwaniak, Minkiewicz, & Darewicz, 2014). In this regard, simulated *in vitro* GI digestion systems are commonly applied for studying the release of potentially bioactive peptides as well as determining their bioaccessibility. Several studies have used static GI digestion models in order to evaluate the generation of antioxidant peptides in water soluble extracts derived from dry-cured hams and by-products (Gallego, Mora, Hayes, Reig, & Toldrá, 2017; Zhu, Zhang, Zhou, & Xu, 2016) as well as to study the stability of specific antioxidant peptides against simulated

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digestion (Gallego, Mora, & Toldrá, 2018a; Gallego, Mora, Reig, & Toldrá, 2018). Also *in silico* approaches have been used for predicting the release of antioxidant peptides from meat digestion (Kęska & Stadnik, 2016; Sayd et al., 2018).

The aim of this study was to evaluate the effect of *in vitro* GI digestion on the antioxidant activity of the peptides naturally generated in Spanish dry-cured hams with 12 months of processing. Additionally, chromatographic and mass spectrometry techniques have been used for the characterisation of the peptide profiles in the undigested and digested dry-cured ham samples.

2. Materials and methods

2.1. Chemicals and reagents

Enzymes porcine pepsin, pancreatic α-amylase, pancreatic lipase, and bile extract as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, potassium ferricyanide, 2,2′-aAzino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Trypsin and chymotrypsin enzymes were from Fluka (Sigma-Aldrich, Co., St. Louis, MO, USA). Potassium persulfate and butylated hydroxytoluene (BHT) were purchased from Panreac Química, S.A.U. (Barcelona, Spain), whereas formic acid (FA), acetonitrile (ACN), ethanol, and trichloroacetic acid (TCA) was from Sharlab, S.L. (Barcelona, Spain). All used chemicals and reagents were of analytical grade.

2.2. Dry-cured ham samples

Spanish dry-cured hams of 12 months of processing were from white-breed pigs (Landrace × Large White and Duroc). Three dry-cured ham samples were prepared by grinding and mixing *Biceps femoris* muscle after removing extramuscular fat.

2.3. In vitro gastrointestinal digestion

A total of 5 g of sample, in duplicate, was dissolved in 10 mL of 0.01 N HCl (pH 3.0). Samples were subjected to *in vitro* digestion in a digestor (Carousel 6 Plus Reaction Station, Radleys, UK) following the methodology described by Minekus et al. (2014) with some modifications. The gastric phase was simulated by adding 2000 U/mL pepsin and 0.075 mM $CaCl_2$, maintaining the mixture 2 h at 37 °C with continuous stirring. Then, the enzyme was inactivated by adjusting pH to 7.0. In the intestinal phase, 100 U/mL of trypsin, 25 U/mL of chymotrypsin, 200 U/mL of pancreatic α-amylase, and 2000 U/mL of pancreatic lipase were added. Also bile extract and $CaCl_2$ were added to give a final concentration of 10 mM and 0.3 mM, respectively, and the mixture was kept at 37 °C for 2 h. Finally, the digestion was finished by heating for 2 min at 95 °C. The study was also done in control samples (undigested), in which the procedure was carried out in the same way but without the addition of enzymes. One control including all enzymes (without sample) was also considered, and final antioxidant values obtained from this control were used to recalculate the antioxidant activity of the dry-cured ham digested samples. Finally, all samples were deproteinised by adding 3 volumes of ethanol (4 °C, 20 h), centrifuged (12,000*g*, 4 °C, 10 min), and the resultant supernatants were dried in a rotatory evaporator and lyophilised for subsequent assays.

2.4. Antioxidant activity

2.4.1. DPPH radical scavenging activity

The DPPH activity of the undigested and digested samples was carried out according to the method described by Bersuder, Hole, and Smith (1998). For that, 100 µL of each sample (10 mg/mL) was mixed with 500 µL of ethanol and 125 µL of DPPH solution (0.02%). The mixture was incubated for 60 min in the dark, measuring the reduction of DPPH radicals at 517 nm. Lower absorbance values indicate higher free radical scavenging activity. BHT (20 mg/mL) was used as positive control. The scavenging activity was calculated as: DPPH activity (%) = (Absorbance control − Absorbance sample) × 100/Absorbance control.

2.4.2. Ferric-reducing antioxidant power

The ferric reducing power activity was evaluated following the methodology of Huang, Tsai, and Mau (2006). A total of 70 µL of each sample (10 mg/mL) was mixed with 70 µL of phosphate buffer (200 mM, pH 6.6) and 70 µL of potassium ferricyanide (10 mg/mL). The mixture was incubated at 50 °C for 20 min, and 70 µL of TCA (100 mg/mL) was added before centrifugation (200*g*, 10 min). Then, 140 µL of the supernatant was mixed with 140 µL of bidistilled water and 28 µL of ferric chloride (1 mg/mL), measuring the absorbance at 690 nm. Higher absorbance values indicate higher ferric-reducing antioxidant power. BHT (20 mg/mL) was used as positive control.

2.4.3. ABTS radical scavenging capacity

The ABTS assay was performed following the method described by Re et al. (1999) with some modifications. Briefly, 7 mM ABTS was dissolved in 2.45 mM potassium persulfate, and the mixture was kept for 15 h in the dark at room temperature to produce the ABTS radical cation ($ABTS^{\cdot+}$). Then, this solution was diluted with 50 mM phosphate buffer saline (PBS; pH 7.4) to obtain an absorbance of 0.70 ± 0.02 at 734 nm. An amount of 10 µL of each sample at different concentrations (2, 2.5, and 5 mg/mL) was mixed with 990 µL of $ABTS^{+}$ solution, and the absorbance was measured at 734 nm after 6 min of incubation in dark. Ascorbic acid (4 mM) was used as positive control, and Trolox at different concentrations (0.05–2 mM) were used to obtain a calibration curve. Results were expressed as nmol of trolox equivalent antioxidant capacity (TEAC) per mg of sample.

2.5. Reversed-phase high-performance liquid chromatography

The peptide profiles of the samples were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA). The column was a Symmetry C18 (4.6 × 250 mm, 5 µm; Walters Co., Milford, MA, USA), using 0.1% trifluoroacetic acid (TFA) in water as solvent A, and 0.085% TFA in ACN:water (60:40, v/v) as solvent B. Peptides were eluted by a gradient consisting of 100% solvent A for 2 min, followed by a linear gradient from 0 to 50% of solvent B during 50 min at a flow rate of 1 mL/min. The elution profile was monitored by UV absorbance at 214 nm.

2.6. Tandem mass spectrometry

2.6.1. Peptide identification

The identification and relative quantification of peptides was performed by nanoliquid chromatography-tandem mass spectrometry (nLC-MS/MS) using an Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to the quadrupole/time-of-flight (Q/ToF) TripleTOF® 5600+ system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionisation source (nESI).

A total of 5 µL of each sample (1 mg/mL in 0.1% of TFA with 2% ACN) was loaded onto a trap column (NanoLC Column, 3 µm C18-CL, 350 µm × 0.5 mm, Eksigent of AB Sciex, CA, USA) using 0.1% TFA as mobile phase at a flow rate of 3 µL/min for 5 min. Then, the peptides were loaded onto an analytical column (LC Column, 3 µm C18-CL, 75 µm × 12.3 cm, Nikkyo Technos Co., Ltd., Japan) equilibrated in 0.1% FA with 5% ACN. Solvent A was 0.1% FA and solvent B was 0.1% FA in ACN. Peptide elution was carried out with a linear gradient from 5% to 35% solvent B over 60 min, at a flow rate of 0.3 µL/min and

30 °C. The column outlet was directly coupled to a nESI, and the Q/ToF (MS/MS) was operated in positive polarity and data-dependent mode. Survey MS1 scans was acquired from 350 to 1250 *m/z* for 250 ms, and from 100 to 1500 *m/z* for 50 ms in 'high sensitivity' mode for MS2 experiments.

Data were processed using ProteinPilot™ v5.0 software (AB Sciex, MA, USA) for the identification and quantification of peptides. The Paragon algorithm was used to search in Uniprot database with no enzyme specificity and no taxonomy restriction.

2.6.2. Peptide quantification

The quantification of peptides was performed by using the label-free methodology described by Gallego, Mora, Aristoy, and Toldrá (2015a). This approach is based on the measurement of relative ion intensities of extracted ion chromatograms (XICs) to determine the ratios for individual peptides, using 3 replicates per sample. Peptides were quantified using PeakView v1.1 software (AB Sciex, Framingham, MA, USA) and analysed with Marker View v1.3 software (AB Sciex, Framingham, MA, USA).

2.7. Statistical analysis

Statistical analysis was carried out using XLSTAT 2011 v5.01 (Addinsoft, Barcelona, Spain). One-way analysis of variance (ANOVA) was performed for antioxidant analyses. Fisher's multiple range tests were used to evaluate significant differences among mean values at $P < 0.05$.

Data obtained from the relative quantification of peptides was statistically analysed with SIMCA-P + 13.0 (Umetrics AB, Sweden) software, performing Principal Component Analysis (PCA) and Student's *t*-test analysis ($P < 0.001$) for testing differences between samples.

3. Results and discussion

3.1. Effect of in vitro GI digestion on the antioxidant activity of samples

The antioxidant activity of the samples was evaluated by DPPH radical scavenging activity, ferric-reducing antioxidant power, and ABTS radical scavenging assay, as there is not a standardised assay to accurately characterise the antioxidant capacity of a sample. These assays are usually categorised as electron transfer (ET)-based methods, although DPPH and ABTS assays can also act in hydrogen atom transfer (HAT) reactions (Prior, Wu, & Schaich, 2005).

Results obtained from the DPPH radical scavenging activity assay are shown in Fig. 1A. Undigested samples showed 23.4% of antioxidant activity, whereas the *in vitro* GI digestion of the samples led to a reduction of around 10% in the activity. Similar results were obtained in the ferric-reducing power antioxidant assay (Fig. 1B), showing a decrease in the absorbance values measured at 690 nm after the simulated digestion. In this study, DPPH was dissolved in ethanol, which could form strong hydrogen bonds with antioxidants and then, the predominant reaction mechanism could be based on ET as in the reducing power assay (Boudier et al., 2012). DPPH and reducing power assays correlated well with the hydrophobicity of the peptides (Liao et al., 2016).

Conversely, ABTS radical-scavenging assay showed higher antioxidant values in digested than undigested samples (Fig. 1C), reaching a value of 482.76 nmol TEAC/mg in the digested sample. The ABTS radical is not correlated with the hydrophobicity of the peptides because it is both a water- and lipid-soluble compound (Liao et al., 2016). ABTS reacts rapidly with antioxidants, and the reaction mechanism between $ABTS^{\cdot +}$ and amino acid residues seems to be initiated by a labile hydrogen atom (Aliaga & Lissi, 2000), therefore ABTS radical-scavenging assay could be considered in this study as a HAT method.

Differences in reactive species, mechanisms, reaction conditions, reaction kinetics, and quantitation methods make it difficult to compare

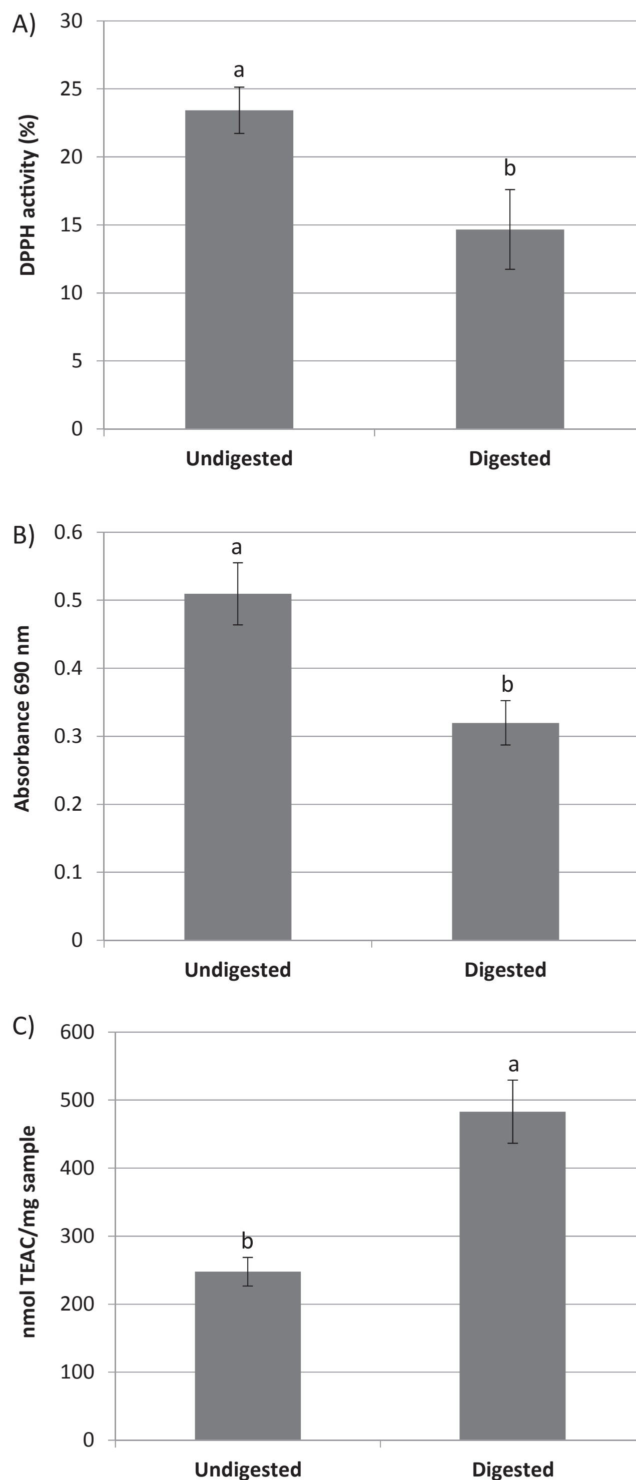

Fig. 1. Antioxidant activity of the undigested and digested dry-cured ham samples determined by (A) DPPH radical scavenging activity, (B) Ferric-reducing antioxidant power, and (C) ABTS radical scavenging assay. Bar letters indicate significant differences among mean values ($P < 0.05$).

results between the different antioxidant assays (Huang, Ou, & Prior, 2005). For example, similar compounds react in both ferric-reducing antioxidant power and ABTS assays but the pH of reaction differs, which could lead to a significant difference in the structure of the same peptide, especially for those containing non-neutral amino acids (Zou, He, Li, Tang, & Xia, 2016).

In agreement with the obtained results, a previous study carried out

by Wang et al. (2018) with Xuanwei ham before and after cooking followed by *in vitro* digestion revealed an increment of about four-fold in the antioxidant activity measured by ABTS assay, and significant decrease in the DPPH activity after digestion of samples. Also pepsin-trypsin simulated digestion of peptide extracts from Jinhua hams led to a significant reduction in the DPPH radical scavenging activity, probably due to the accumulation of smaller peptides and free amino acids that could make the medium more hydrophilic and then more difficult to react with lipid-soluble DPPH radicals (Zhu et al., 2016; Zhu, Zhang, Kang, Zhou, & Xu, 2014). Conversely, a significant increment of the antioxidant activity measured by DPPH and ferric-reducing power assays was observed in beef peptide extracts after simulated GI digestion (Mora, Bolumar, Heres, & Toldrá, 2017), as well as an increased antioxidant activity determined as thiols content of digested pork meat (Simonetti, Gambacorta, & Perna, 2016).

The action of GI enzymes leads to the breakdown of peptides, release of amino acids and/or exposition of internal groups, affecting the size, amount and physico-chemical characteristics of the peptides, thus affecting their antioxidant capacity. In fact, the amino acid composition and structure of peptides as well as their solubility in the reaction media have the greatest influence on the antioxidant activity (Samaranayaka & Li-Chan, 2011; Sarmadi & Ismail, 2010). Also the interactions between amino acids in the peptide sequence as well as the steric and electronic properties of the residues, mainly of those located at the C-terminus, could also play an important role in the antioxidant activity (Acquah, Di Stefano, & Udenigwe, 2018; Zou et al., 2016).

Sometimes peptides showing *in vitro* bioactivity are inactive in the human body after ingestion mainly due to the action of salivary, gastric and intestinal enzymes, that hydrolyse them into smaller size peptides that lose their bioactivity. What is more, small bioactive peptides can be hydrolysed in the intestine by peptidases of the microbial flora, by brush border peptidases in the epithelium of the intestinal membrane, or even by peptidases in the blood stream. Thus, *in vivo* assays are always necessary in order to confirm the physiological effects of bioactive peptides in the organism.

3.2. Characterisation by RP-HPLC of the peptide profiles in the undigested and digested samples

The composition and levels of peptides released during *in vitro* GI digestion can provide further information about the antioxidant activity of the samples. The analysis by RP-HPLC revealed the peptide pattern of the samples in order to evaluate the stability of the peptides to simulated GI digestion. As can be seen in Fig. 2, a higher amount of peptides was observed in the profile of the digested sample in comparison with the control sample due to digestive enzymes could have released peptides encrypted in proteins as well as degraded peptides into shorter sequences and free amino acids. It has been reported that peptides with high antioxidant activity usually contain a high proportion of hydrophobic amino acids (Ala, Leu, Ile, Val, Pro,…) or aromatic residues (Trp, Tyr and Phe), and also, the imidazole, pyrrolidine, and indole ring of His, Pro, and Trp, respectively, can act as radical scavengers (Acquah et al., 2018; Zou et al., 2016). Thus, peptides containing acidic amino acids, those negatively charged or less hydrophobic would be more resistant to enzymatic hydrolysis (Wang, Xie, & Li, 2019).

3.3. Identification and quantification by nLC-MS/MS of the peptides generated in the undigested and digested samples

Samples were also analysed by nLC-MS/MS for a complete characterisation of the peptide profiles in the undigested and digested samples, identifying thousands of peptides derived from more than 50 different proteins. The analysis revealed differences in terms of molecular weight (MW) of the identified peptides in the dry-cured ham samples. In the undigested sample, 90.5% of the peptides had MW between 1000 and 5000 Da and only 9.5% of the peptides were lower than 1000 Da, whereas most of the peptides after digestion were from 500 to 3300 Da, with 22.6% of the peptides having MW $<$ 1000 Da. Smaller peptides, with MW ranging from 200 to 1700 Da, and high amount of di- and tripeptides were identified by Paolella et al. (2015) in 18 and 24 months aged Parma hams after *in vitro* GI digestion. That study underlined the high influence of the proteolysis phenomena occurring during ham ageing on the peptide pattern of digested samples, as proteolysis allows a higher digestibility and bioaccessibility of muscle proteins. Moreover, this size of peptides is the most interesting to study of bioactive peptides and those able to cross the intestinal barrier to exert *in vivo* effects. So, although there is no a strict relationship between the antioxidant activity and MW of the peptides, most of the reported antioxidant peptides from food proteins are usually between 3 and 6 amino acids showing MWs lower than 1000 Da (Zou et al., 2016).

Additionally, Fig. 3 shows the distribution of the identified peptides in each sample according to their protein of origin. The largest percentage of peptides identified in the undigested sample was from myosin protein, reaching a value of 37% (Fig. 3A). Myosin is a major structural muscle protein and its intense degradation during dry-cured ham processing has been widely described (Di Luccia et al., 2005; Fabbro et al., 2016; Toldrá, Rico, & Flores, 1993). Identified peptides were also derived from other structural myofibrillar proteins such as actin, troponin, and tropomyosin as well as from sarcoplasmic proteins such as creatine kinase and glycolytic enzymes. Several studies have reported the degradation of these muscle proteins in Spanish dry-cured hams by the action of endogenous enzymes, and numerous derived peptides have been identified by MS techniques (Gallego, Mora, & Toldrá, 2018b). Moreover, some studies have reported that antioxidant peptides from different dry-cured hams were mainly lower than 1000 Da and originated from myosin, actin, creatine kinase and beta-enolase proteins (Mora et al., 2014; Xing et al., 2018). Regarding the digested sample, most of the identified peptides were from titin (28%), myosin (18%), and collagen (11%) (Fig. 3B). These results evidence the intense action of the digestive enzymes on titin, whose percentage of identified peptides increased by 25% in the digested sample compared to the control. Titin is a giant protein and the third most abundant of the striated muscle, which is intensively degraded throughout the dry-cured ham processing (Gallego, Mora, Aristoy, & Toldrá, 2015b). Titin protein and the existing derived peptides would be further hydrolysed by the enzymes added for the *in vitro* GI digestion, generating large amounts of peptides and free amino acids. Probably titin protein is degraded during the curing process in big fragments, many of them protein fragments or oligopeptides, and later GI digestion liberates novel peptides from these fragments through the action of GI enzymes. Besides, peptides from collagen protein represented 11% of the identified peptides in the digested sample, and those from major sarcoplasmic proteins reached a similar percentage (12%) (Fig. 3B). Collagen protein is an insoluble fibrous protein which has been described as a good source of bioactive peptides showing antihypertensive and antioxidant activities, probably associated with the unique repeating pattern Gly-Pro-Hyp in its sequence (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011; Minkiewicz, Dziuba, & Michalska, 2011). In this regard, a previous study showed that collagen-derived peptides could be responsible for the antioxidant activity obtained after household cooking preparations and simulated GI digestion of dry-cured ham bones (Gallego et al., 2017). On the other hand, Paolella et al. (2015) identified several peptides in the digestion of *Biceps femoris* muscle from Parma dry-cured hams at 18 and 24 months of ageing, which were mainly originated from the myofibrillar proteins actin and myosin, and sarcoplasmic proteins such as pyruvate kinase, beta-enolase and fructose-biphosphate aldolase. Differences in the amino acid composition among proteins could determine the extent of digestion because residues such as Phe, Tyr, Trp, Lys, and Arg are the target cleavage sites of the GI enzymes pepsin and trypsin (Savoie, Agudelo, Gauthier, Marin, & Pouliot, 2005).

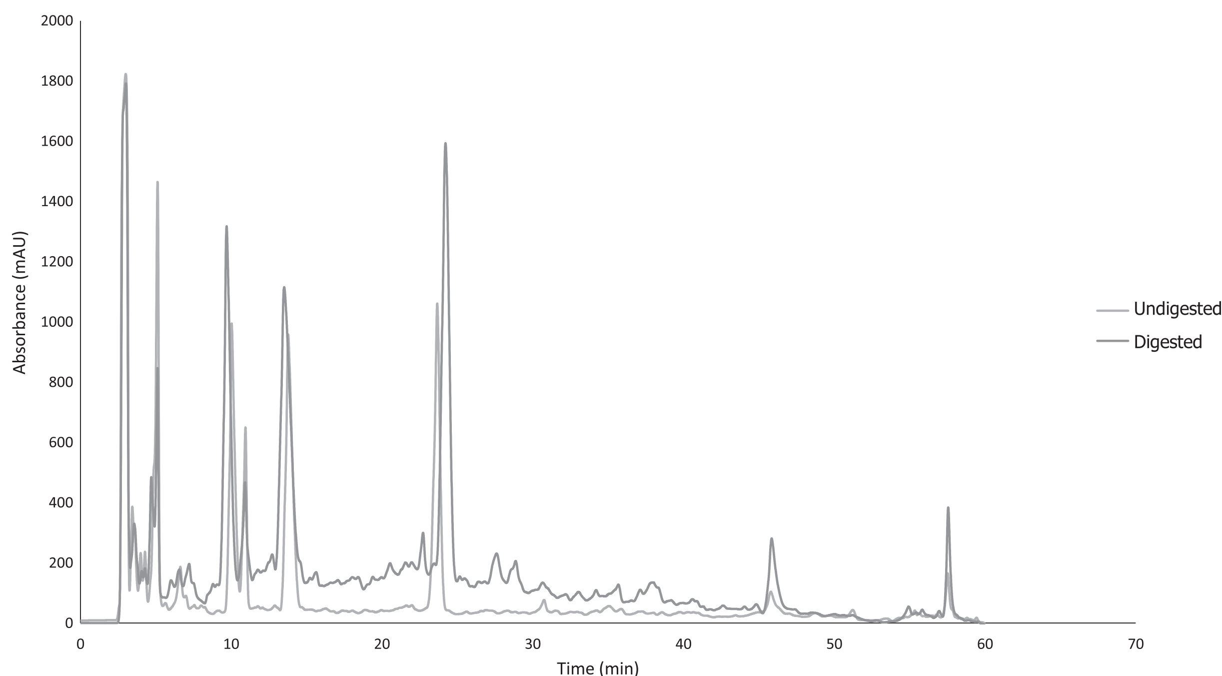

Fig. 2. Reversed-phase chromatographic separation of the undigested sample compared to the digested dry-cured ham sample.

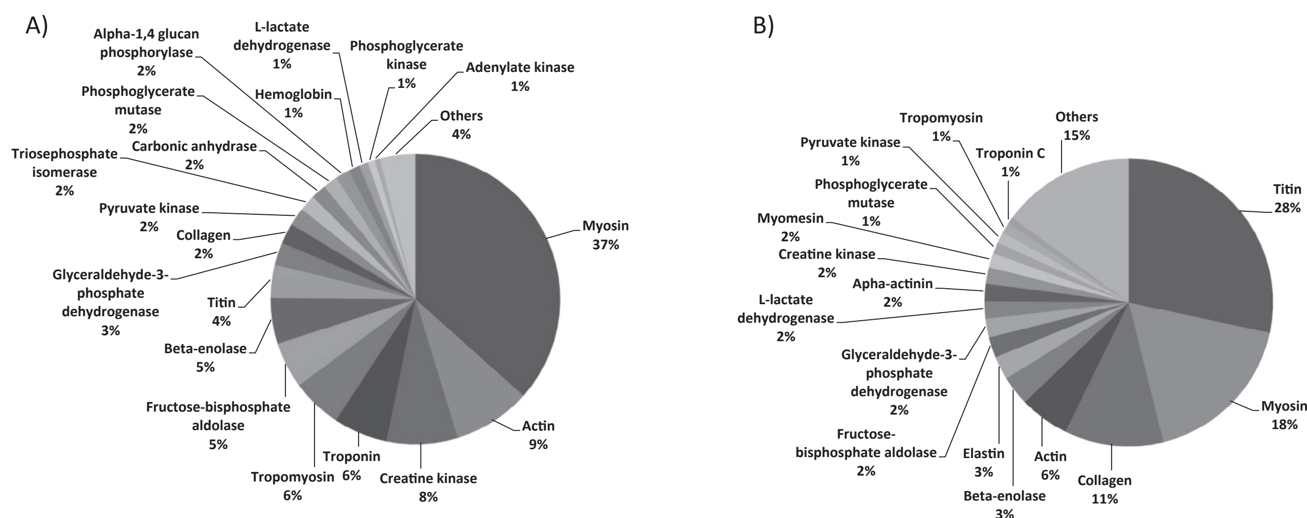

Fig. 3. Distribution of the peptides identified by nLC-MS/MS according to their origin proteins in (A) undigested and (B) digested dry-cured ham samples.

On the other hand, the relative quantification of peptides was done by using a label-free methodology. A PCA was performed to assess differences between the undigested and digested samples as well as to obtain information about the most influential peptides (Fig. 4). The PCA score plot of main identified proteins showed the variance among the peptides present before and after the simulated GI digestion (Fig. 4A). The loading plot showed the peptides responsible for influencing the clustering of data (Fig. 4B). Principal component 1 (PC 1) explained the 51.5% of the variability in the dataset while PC 2 was responsible for the 22.2% of variance within the dataset, allowing the differentiation between undigested and digested samples. Peptides derived from titin (TTN) would affect primarily the score plot distribution, which would be expected given the large number of titin-derived peptides identified after the simulated GI digestion (Fig. 3). Also peptides from troponin (TNN) and myosin heavy chain (MYH) proteins could have considerable influence for the description of the two discriminant components.

Conformational changes of proteins and peptides during food processing such as cooking or aging determine the accessibility of the digestive peptidases to their cleavage sites. Therefore, the limiting factor for the dynamics of protein hydrolysis is the bioaccessibility, which would be primarily determined by the processing and complexity of the food matrix (Bax et al., 2012; Sayd, Chambon, & Santé-Lhoutellier, 2016).

4. Conclusions

The simulated GI digestion of dry-cured ham led to a decrease in the antioxidant activity when measured by DPPH radical scavenging activity and ferric-reducing antioxidant power methods, but an increase when determined by ABTS radical scavenging assay. Structural properties of peptides such as amino acid composition, peptide size or hydrophobicity determine not only their reactivity, but also their stability and bioavailability. The characterisation of the peptide profiles before and after the simulated digestion of dry-cured hams evidenced the degradation of proteins, mainly titin, by the digestive enzymes, and

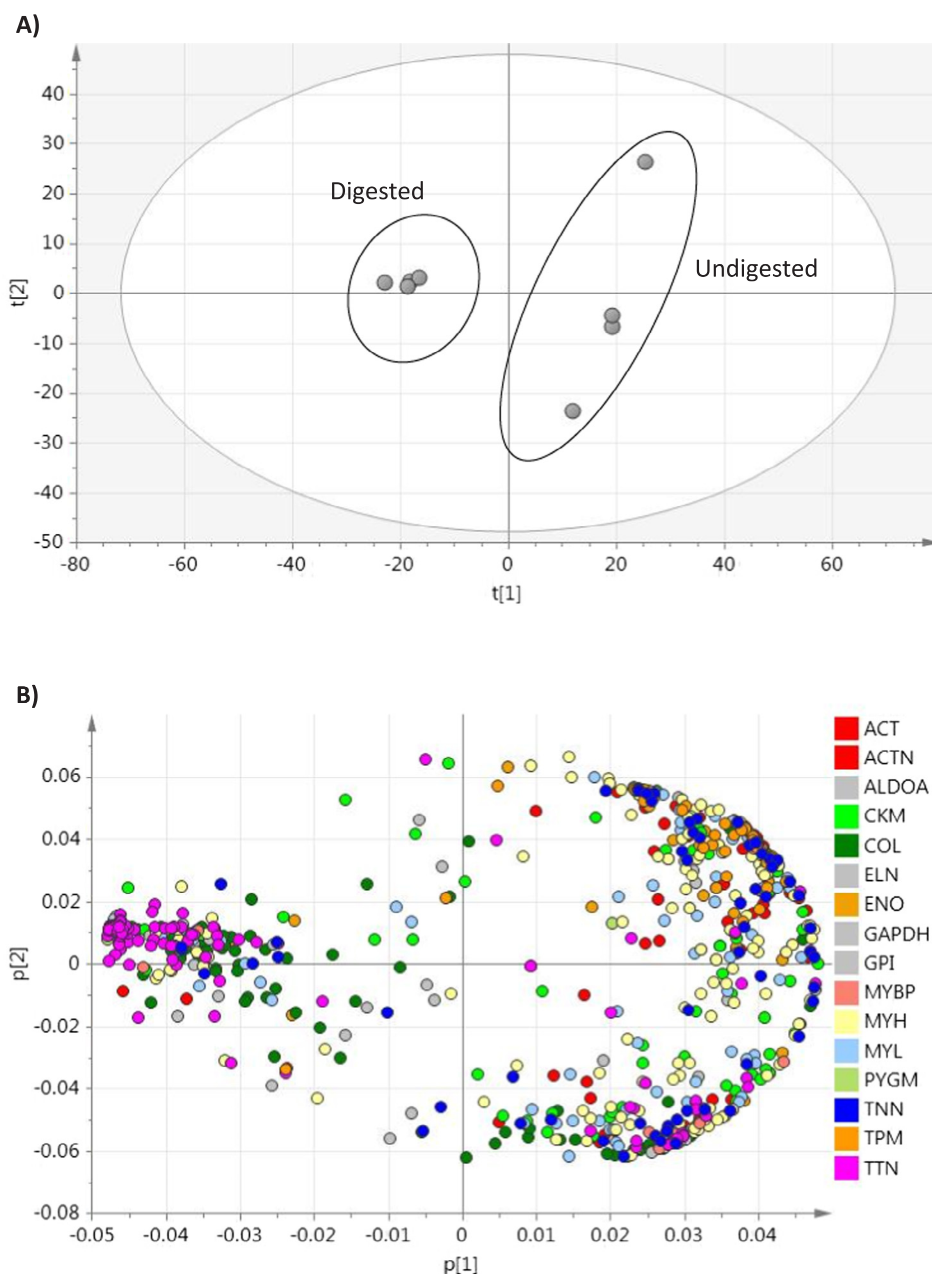

Fig. 4. (A) Principal Component Analysis (PCA) score plot of main identified proteins to assess the variance among all the peptides generated in the undigested and digested dry-cured ham samples (n = 4). (B) Loading plot showing the peptides affecting the score plot distribution and coloured by protein of origin.

would help to improve the knowledge about the influence of peptide characteristics on the antioxidant activity and their resistance to digestive enzymes. Nevertheless, differences between *in vitro* and *in vivo* environments make necessary further studies to confirm the antioxidant behavior under physiological conditions. Peptide-food matrix interactions should also be considered as they can lead to chemical modifications affecting the bioaccessibility and bioavailability of bioactive peptides.

5. Ethics Statement

This research did not include any human subjects and animal experiments.

CRediT authorship contribution statement

Marta Gallego: Methodology, Supervision, Writing - original draft, Writing - review & editing. **Lodovica Mauri:** Investigation. **M. Concepción Aristoy:** Methodology, Supervision. **Fidel Toldrá:** Conceptualization, Methodology, Resources, Writing - review & editing. **Leticia Mora:** Conceptualization, Methodology, Resources, Supervision, 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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