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4 **Characterisation of the antioxidant peptide AEEEYPDL and its**

5 **quantification in Spanish dry-cured ham**

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Abstract

Bioactive peptides derived from food matrices are object of intensive research due to multiple health benefits they can exert in humans. However, the small size and low abundance of these peptides in complex matrices as well as the effect of food processing and gastrointestinal digestion on their structure, sequence, and functionality, are important challenges. Spanish dry-cured ham has been reported as a good and natural source of bioactive peptides mainly showing antihypertensive and antioxidant activities. In this work, the antioxidant peptide AEEEYPDL, identified from 9 months of curing dry-cured ham, showed good resistance to different heat treatments and salt contents. On the other hand, the bioactivity of the peptide significantly decreased after simulated gastrointestinal digestion. Finally, an optimised multiple reaction monitoring methodology was able to quantify the peptide AEEEYPDL as 0.148 fg per g of dry-cured ham despite the existing challenges in the quantification of bioactive peptides from complex matrices.

Keywords: Antioxidant peptides, stability, mass spectrometry, peptide quantification, MRM, dry-cured ham.

1. Introduction

The identification and characterisation of protein-derived bioactive peptides has been an important area of interest due to their high specificity, small size, low toxicity, high activity and frequent multifunctional properties make them suitable for their use as therapeutic agents (Agyei, Ongkudon, Wei, Chan & Danquah, 2016).

Antioxidant peptides are interesting as they are able to delay or prevent lipid and protein oxidation in food systems that could lead to quality deterioration, as well as protect the body against oxidative damages related to diseases such as inflammation, cancer and aging (Mittler, 2002; Liu, Xing, Fu, Zhou & Zhang, 2016). Some studies have reported the identification of antioxidant peptides or protein hydrolysates derived from meat and meat products, including Spanish dry-cured ham (Escudero, Aristoy, Nishimura, Arihara & Toldrá, 2012; Escudero, Mora, Fraser, Aristoy & Toldrá, 2013; Mora, Escudero, Fraser, Aristoy & Toldrá, 2014). In this product, the naturally generated peptides SNAAC and AEEEYPDL were the most active antioxidants by using DPPH radical scavenging assay and reducing power analysis (Mora et al., 2014). Additionally, AEEEYPDL was previously identified in water soluble fractions of dry-cured ham showing angiotensin I-converting enzyme (ACE) inhibitory activity (Escudero, Mora, Fraser, Aristoy, Arihara & Toldrá, 2013).

The use of peptidomic approaches, advanced mass spectrometry methodologies, and bioinformatics tools is fundamental for the discovery and identification of bioactive peptides in food. However, there are several difficulties to be considered due to the small size and low abundance of the molecules in complex samples as well as the modification of the structure and activity of the bioactive peptides during food processing and/or gastrointestinal digestion. Moreover, the quantitative analysis of bioactive peptides in complex matrices, which is crucial for further studies of bioavailability and potential

effect of the peptide in the organism, is still a challenge (Li-Chan, 2015; Mora, Gallego, Reig & Toldrá, 2017). In dry-cured meat products, the mixture of peptides showing a wide range of sizes and unspecific cleavage sites that were naturally generated during the dry-curing process requires a peptidomics approach including different pretreatments and chromatographic steps to avoid interferences and to concentrate peptides for the separation and identification of bioactive peptides (Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles & Recio, 2014; Mora et al., 2017). Despite the simplest method to determine peptide abundance by LC-MS is the measurement of peak areas or peak heights of extracted ion chromatograms (EIC) using label-free quantitation methodology, Multiple Reaction monitoring (MRM) is the most used method for the quantification of bioactive peptides in complex mixtures and it has been applied for studying ACE-inhibitory peptides derived from enzymatic hydrolysis of food samples (Hu, Stromeck, Loponen, Lopes-Lutz, Schieber & Gänzle, 2011; Nakashima, Kudo, Iwaihara, Tanaka, Matsumoto & Matsui, 2011; Rawendra et al., 2014; Priyanto et al., 2015). MRM is a greatly sensitive method of targeted MS that can selectively detect and quantify small and low abundant peptides based on the screening of specific transitions from precursor peptides to product ions (Lange, Picotti, Domon & Aebersold, 2008; Picotti & Aebersold, 2012). However, there are several difficulties in the analysis due to the complexity of the samples especially when peptides have been naturally generated by unspecific enzymes or during processing conditions. Also the optimisation of the MRM parameters for an accurate and sensitive quantification without interferences from other peptides or signal suppression is an important challenge for low abundant natural peptides (Capriotti, Cavaliere, Piovesana, Samperi & Laganà, 2016; Mora et al., 2017).

The goal of the present study was to characterise the antioxidant peptide AEEEEYDDL evaluating its stability against different processing conditions and *in vitro* gastrointestinal

digestion. Moreover, the quantification of this peptide was carried out using MRM methodology in Spanish dry-cured ham samples processed for 9 months.

2. Materials and methods

2.1 Chemicals and reagents

Peptide AEEEYPDL was synthesised by GenScript Corporation (Piscataway, NJ, USA).

All the enzymes for *in vitro* digestion, fluorescein, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), tryptophan, and ascorbic acid were from Sigma-Aldrich, Co. (St. Louis, MO, USA). Potassium persulfate was from Panreac Quimica S.A.U. (Barcelona, Spain). Regarding reagents for QQQ analysis, water and acetonitrile were of LC-MS grade and were purchased from Sharlab, S.L. (Barcelona, Spain), whereas formic acid for LC-MS was from Fluka Analytical of Sigma-Aldrich, Co. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

2.2 Stability of the antioxidant peptide AEEEYPDL

2.2.1 Effect of heat treatments and salt concentrations

The stability of the antioxidant peptide AEEEYPDL against different heat treatments and salt concentrations was assessed. Peptide solutions (1 mg/mL) were kept during 10 min at different temperatures: 37 °C, 50 °C, 72 °C, and 90 °C, as well as exposed to different salt concentrations: 0 %, 2%, 4%, and 8% NaCl at 100 °C for 10 min (n=3). After the treatments, samples were immediately cooled in ice to room temperature and then stored at -20°C for following antioxidant assays.

2.2.2 Effect of *in vitro* gastrointestinal digestion

The peptide AEEEEYPDL was also subjected to a simulated digestion according to the method described by Minekus et al. (2014), with some modifications. Briefly, 2 mg of the peptide was dissolved in 1 mL of NaHCO₃ (200 mM, pH 7.0). The oral phase was simulated by adding 21 U/mL of salivary α -amylase and 0.75 mM of CaCl₂, maintaining the mixture at 37 °C for 3 min. After adjusting the pH to 3.0 for the gastric phase, pepsin enzyme and CaCl₂ were added to achieve 2000 U/mL and 0.075 mM, respectively. The mixture was continuously stirred for 3 h at 37 °C, and then the pH was adjusted to 7.0 for the intestinal phase. For that, 100 U/mL of trypsin, 25 U/mL of chymotrypsin, 200 U/mL of pancreatic α -amylase, 2000 U/mL of pancreatic lipase, 10 mM of bile extract, and 0.3 mM CaCl₂ were added to the mixture, which was maintained for 3 h at 37 °C. The digestion was finished by heating at 95 °C for 2 min, and the sample was subsequently deproteinised using 3 volumes of ethanol (4 °C, 20 h), and centrifuged (12,000 g, 4 °C, 10 min). The supernatant was dried in a rotatory evaporator and lyophilised for the following assays. The assay was performed in triplicate.

2.3 Antioxidant activity

2.3.1 Oxygen radical absorbance capacity assay (ORAC)

ORAC assay was carried out following the procedure described by Dávalos, Gómez-Cordovés and Bartolomé (2004) with some modifications. Briefly, 140 μ L of samples at concentrations of 1, 2.5, and 5 μ g/mL in phosphate buffer (75 mM, pH 7.4) was mixed with 70 μ L of fluorescein (200 nM) and incubated for 15 min at 37 °C in the dark. After the addition of 70 μ L of AAPH (80 mM), the fluorescence was measured every min for 100 min using excitation and emission wavelengths of 485 and 538 nm, respectively. Several concentrations of trolox (2–16 μ M) were used to obtain a calibration curve, and tryptophan was used as positive control. The area under curve (AUC) was calculated for each sample by integrating the relative fluorescence curve, and the values were plotted

against the concentration of trolox. Results were expressed as nmol of TE (trolox equivalents) per mg of peptide.

2.3.2. ABTS radical scavenging capacity

The ABTS assay was carried out according to the methodology described by [Re et al. \(1999\)](#), with some modifications. Thus, 3.8 mg of ABTS were dissolved in 1 mL of potassium persulfate (2.45 mM), mixed, and kept in the dark at room temperature for approximately 15h to produce ABTS^{•+}. Phosphate buffer saline (PBS, 50 mM, pH 7.4) was used to dilute the ABTS^{•+} solution until an absorbance of 0.70 ± 0.02 at 734 nm was reached. A total of 10 μ L of samples at different concentrations (250, 500, and 750 μ g/mL) was mixed with 990 μ L of ABTS^{•+} solution, measuring the absorbance at 734 nm after 6 min of incubation in the dark. Different concentrations of trolox (0.05–2 mM) were used to obtain a standard curve, and ascorbic acid was used as positive control. The ABTS radical scavenging activity of samples was calculated and plotted against the concentration of trolox, showing the results as nmol of TEAC (trolox equivalent antioxidant capacity) per mg of peptide.

2.4 Preparation of dry-cured ham samples: extraction, deproteinisation and fractionation of peptides

Samples from Spanish dry-cured hams at 9 months of curing were prepared according to the methodology described by [Gallego, Mora and Toldrá \(2016\)](#). So, 50 g of *Biceps femoris* muscle was used for the extraction of peptides with 0.01 N HCl and deproteinisation with ethanol, followed by a size-exclusion chromatography in a Sephadex G25 column in order to fractionate peptides according to their molecular mass. Fractions of 5mL were automatically collected and monitored by ultraviolet absorption at 214 nm (see [Figure 1_supplementary material](#)). Fractions including elution volumes from 180 to 280 mL were selected and pooled in five groups named samples s1 (180-200

mL), s2 (205-220 mL), s3 (225-240 mL), s4 (245-260 mL) and s5 (265-280 mL), and lyophilised. Finally, samples were resuspended in 30 μ L of water for the quantification of the peptide AEEEYPDL through MS analysis using MRM methodology.

2.5 Liquid chromatography – tandem mass spectrometry 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).

Peptide solutions (1000 fmol/ μ L) were subjected to *in vitro* digestion and heat treatments as described previously, and then analysed by LC-MS/MS in order to evaluate the stability of the peptide. A total of 5 μ L of each sample was injected and concentrated on a ZORBAX StableBond guard column (5 μ m, 1 μ m $\times$ 17 mm; Agilent Technologies, Denmark) at a flow rate of 0.1 mL/min for 5 min and using 5% (v/v) acetonitrile (ACN) in water as mobile phase. The trap column was automatically switched in-line onto a ZORBAX 300SB-C18 capillary column (5 μ m, 250 μ m $\times$ 0.3 mm; Agilent Technologies, Denmark). Mobile phases were 0.1% (v/v) formic acid (FA) in water as solvent A, and 100 % ACN as solvent B. Gradient elution for LC was 0-2 min, 5 % B; 2-20 min, linear from 5 to 45% B; 20-25 min, linear from 45 to 65 % B; 25-28.5 min, linear from 65 to 98 % B; and 28.5-30 min, 98 % B; at a flow rate of 6 μ L/min at 30 °C. The column outlet was directly coupled to a ESI, and the QQQ (MS/MS) was operated in positive polarity to acquire full scan mass spectra from 80 to 1200 *m/z*. MS parameters for the specific peptide were: nitrogen gas temperature, 350 °C; gas flow, 6 L/min; nebulizer pressure, 15 psi; capillary, 3500 V; fragmentor, 100 V; scan time, 500 ms; cell accelerator, 4 V.

The standard AEEEYPDL (1000 fmol/ μ L) was analysed by LC-MS/MS in order to obtain information on *m/z* and retention time of the peptide from the MS profiling. Then, in order

to evaluate the degradation of the peptide samples under different temperatures, a calibration curve from the standard peptide at concentrations from 100 to 3000 fmol/ μ L was prepared using peak areas obtained from EIC. For that, the EICs were extracted from the total ion chromatograms (TIC) by indicating the m/z of the double charged ion (483.2), and then peaks were integrated. Samples were processed and data 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 quantitative analysis of the peptide AEEEEYPDL in dry-cured ham samples was carried out in the QQQ-MS under MRM mode after optimisation of the acquisition parameters using Skyline software (version 3.5.0.9319; <https://skyline.gs.washington.edu>). Standard peptide solutions for the establishment of calibration curves were prepared at concentrations from 50 to 1000 fmol/ μ L, and analysed by MRM to calculate peak areas. Then, dry-cured ham samples were analysed using the same optimised parameters. Data analysis was done using MassHunter Quantitative Analysis software (version B.07.01; Agilent Technologies, Inc.).

2.6 Bioinformatics and statistical analysis

Statistical analysis including one-way analysis of variance (ANOVA) and Fisher's multiple range tests were performed using XLSTAT 2011 v5.01 software (Addinsoft, Barcelona, Spain). Results were expressed as the mean of 3 replicates $\pm$ standard deviations. Differences were considered significantly at $p < 0.05$.

The PEPstrMOD software (<http://osddlinux.osdd.net/raghava/pepstrmod/>) was used to predict the tertiary structure of the peptide AEEEEYPDL. The prediction strategy uses both the regular secondary structure information predicted from PSIPRED and β -turns information predicted from BetaTurns (Kaur, Garg & Raghava, 2007; Singh et al., 2015). PeptideCutter tool of ExPASy (https://web.expasy.org/peptide_cutter) was used for *in*

silico analysis in order to predict the stability of the peptide during gastrointestinal digestion.

3. Results and discussion

3.1 Characterisation of the antioxidant peptide AEEEYPDL

AEEEYPDL has been previously reported as a potent antioxidant peptide derived from the hydrolysis of creatine kinase protein during the dry-cured ham processing (Mora et al., 2014). The effectiveness of the peptide to act as antioxidant and the mechanism underlying this activity are related to its molecular mass, composition and location of amino acids in the sequence as well as its structure and hydrophobicity (Elias, Kellerby, & Decker, 2008; Sarmadi & Ismail, 2010). In this regard, the aromatic amino acid Tyr has been described as a strong radical scavenger. Furthermore, the carboxyl group of Glu next to the Tyr residue could induce the donation of the hydrogen atom from the phenolic hydroxyl in Tyr, improving the antioxidant activity of the peptide. The hydrophobic residues Ala, Pro and Leu, and the acidic amino acids Glu and Asp contained in the AEEEYPDL sequence could also display free radical quenching activity (Liu et al., 2016; Zou, He, Li, Tang & Xia, 2016). As an example, Fig. 1 shows the predicted structure of AEEEYPDL in hydrophilic environment using the PEPstrMOD software, as the characteristic peptide chemical structure could be the main factor influencing its antioxidant activity. In fact, electronic, steric, hydrophobic, and hydrogen bonding properties of amino acids at the C- and N-terminal regions, which are given by the peptide structure, seem to be closely related to the antioxidant activity (Li & Li, 2013; Zou et al., 2016).

The antioxidant activity of the peptide can be modified due to processing conditions and gastrointestinal digestion as they can influence its structure and susceptibility to hydrolysis, and thus its bioactivity. So, the stability of AEEEYPDL against different heat

treatments (37 °C, 50 °C, 72 °C, 90 °C for 10 min), salt concentrations (0 %, 2 %, 4 %, 8 % NaCl at 100 °C for 10 min) and *in vitro* digestion (t0: before digestion, G: after the gastric phase, GI: after the gastrointestinal digestion) was evaluated. For a complete characterisation of the bioactivity, two methodologies were used: ORAC assay, which is a method based on hydrogen atom transfer (HAT), and ABTS radical scavenging assay that is based on electron transfer (ET). Both methods measure the radical scavenging ability, but use different model compounds with various side reactions and other disturbing factors that can lead to different results (Huang, Ou & Prior, 2005; MacDonald-Wicks, Wood & Garg, 2006).

ORAC antioxidant values for AEEEYPDL after the different treatments are shown in Fig. 2a, evidencing that the highest antioxidant activity of the peptide was obtained after exposure to 37 °C and 90 °C for 10 min as well as before digestion (t0), reaching values close to 900 nmol TE/mg peptide in all these cases. The activity decreased 30-40 % when the temperature was increased to 50 °C, 72 °C, and after exposure to 100 °C for 10 min at the different salt concentrations, as high temperatures could affect the secondary structure of the peptide and consequently its antioxidant activity (Zhu, Zhang, Kang, Zhou & Xu, 2014; Wang, Yu, Xing, Chen, Liu & Li, 2017). The exposure of the peptide to different salt concentrations had a minimal influence on the antioxidant activity. After the G and GI digestion, the antioxidant activity was significantly decreased to 250 nmol TE/mg, probably due to the modification of the peptide characteristics or its degradation into smaller fragments by the enzymatic action.

The results obtained from ABTS radical scavenging capacity are displayed in Fig. 2b, obtaining values between 1300 and 1500 nmol TEAC/mg of peptide after all the tested temperatures, salt treatments, and before digestion. Similar to ORAC results, the

antioxidant activity decreased significantly when the peptide was subjected to G and GI digestion, showing a 90 % reduction in both cases.

In order to evaluate the stability of the peptide AEEEYPDL after *in vitro* digestion, samples were analysed by LC-MS/MS. Figure 3 shows the TICs obtained after G and GI digestion. Additionally, a computer simulation of the gastrointestinal digestion including pepsin, trypsin and chymotrypsin enzymes was carried out using the PeptideCutter tool of ExPASy. This *in silico* analysis showed the cleavage by pepsin of the peptide AEEEYPDL into the fragments AEEEY and PDL, which have not been previously described as functional peptides in BIOPEP database (<http://www.uwm.edu.pl/biochemia/index.php/en/biopep>). So, the degradation of the peptide during the *in vitro* digestion would explain the marked decrease in its antioxidant activity.

Gastrointestinal digestion has a major role in the release of peptides from parent proteins as well as in the modification or degradation of peptides, which determine their chemical composition and physical properties and thus their bioactivity (Elias et al., 2008). In this regard, several studies have reported an increase in the antioxidant activity of cooked pork and beef meats after *in vitro* digestion (Simonetti, Gambacorta, & Perna, 2016; Mora, Bolumar, Heres, & Toldrá, 2017), whereas other works have evidenced the generation of novel peptides by gastrointestinal enzymes with no significant effect or decrease on the antioxidant activity (Gallego, Mora, Hayes, Reig, & Toldrá, 2017; Gallego, Mora, Reig, & Toldrá, 2018). Simulation of digestion is very useful to evaluate the stability of the bioactive peptides against digestive enzymes, but it could also contribute to a greater knowledge about the active form, post-translational modifications, bioavailability, and involved mechanisms during digestion and transport processes to reach their target sites. Nevertheless, *in vivo* studies are needed to demonstrate the physiological effects of

bioactive peptides as there is no a direct relationship between *in vitro* and *in vivo* assays (Vermeirssen, Van Camp & Verstraete, 2004; Segura-Campos, Chel-Guerrero, Betancur-Ancone & Hernandez-Escalante, 2011).

3.2 Quantification of the peptide AEEEYPDL after heat treatments

The degradation of peptide after the different heat treatments (37 °C, 50 °C, 72 °C, 90 °C, and 100 °C for 10 min) was evaluated by MS analysis on the basis of peak intensity measurements, quantifying peptide samples (1000 fmol/μl) using the EICs. In a mass spectrum, the peak area or height at a certain *m/z* measures the number of ions detected by the mass spectrometer. The integration of the ion intensity over time results in an EIC that represents the ion abundance for the peptide (Wong, Sullivan & Cagney, 2007; Lange et al., 2008).

The TIC, mass spectrum and EIC obtained are shown in Fig. 4. Scan spectra indicated that the ion was double charged (*m/z* of 483.2) with a retention time of 16.13 min. The degradation of the peptide AEEEYPDL was evaluated by analysing changes in the EIC peak areas of the samples plotted against the concentration of AEEEYPDL peptide and the obtained calibration curve was $y = 3400.7x - 247716$. A good linearity was established in the range 100-3000 fmol/μL with a coefficient of determination (R^2) of 0.99. Peptide samples subjected to different temperatures were analysed, showing a progressive decrease in the initial concentration (1000 fmol/μL) as the temperature was increased (Fig. 5). So, values of 873.41 ± 15.9 and 808.14 ± 3.5 fmol/μL after treatments at 90 °C and 100 °C for 10 min, respectively, were obtained for AEEEYPDL, probably as a result of the degradation of the peptide into smaller fragments due to the high temperatures. Peptide hydrolysis due to either processing conditions or digestion can maintain, decrease, or increase the bioactivity depending on the characteristics of the generated peptide fragments (Sánchez-Rivera et al., 2014). In this regard, peptide

degradation can lead to the exposure of internal groups and release of free amino acids, which can modify the size, amount, structure, amino acid composition and hydrophobicity of peptides, and thus affect their antioxidant capacity (Samaranayaka & Li-Chan, 2011; Zhu et al., 2014). This fact could explain previous ORAC antioxidant results showing a certain increase of the peptide activity after exposure to 90 °C but a significant decrease after the treatment at 100 °C (see Fig. 2).

3.3 MRM quantification of the peptide AEEEYPDL in dry-cured ham samples

In order to quantify the antioxidant peptide AEEEYPDL in dry-cured ham samples, a LC-MS/MS analysis in MRM mode was carried out. Parent and product ions, collision energy, resolution, voltage, and dwell time are key parameters for optimisation of the methodology, whereas the selectivity is achieved by the MRM characteristic of triple quadrupole MS instrument due to the two stages of mass selection (precursor ion and product ion) allows greater signal-to-noise ratio needed for quantification (Prasad & Unadkat, 2014). The intensity of the MRM signal depends on the MS response of parent and product ions, so fragmentation pattern with relative intensities of product ions should be predicted by MRM transition design tools (Prasad & Unadkat, 2014). In this work, transition list detailing the optimal collision energy for each precursor ion and product ion was obtained by the Skyline software, selecting 3 transitions to measure for each product ion and a voltage interval of 3 V. Based on theoretical calculations, each transition was examined to select the collision energy that increased fragment ion intensity and thus generated the greatest peak area. The dwell time for each transition was 5 ms and the fragmentor voltage was established as 130 V. The optimised MRM transition was set as m/z 483.2 $\rightarrow$ 622.2 and the limit of quantification was determined as 50 fmol/ μ L after testing the peptide at lower concentrations. The peptide amounts were calculated on the basis on peak areas in the MRM chromatogram using the standard curve

of the peptide AEEEYPDL (Fig. 6). In dry-cured ham samples, the MRM analysis indicated that the content of the peptide in the sample s1 was 0.074 fg/mL whereas in the remaining four samples it was not able to be quantified, thus the amount of AEEEYPDL was calculated to be 0.148 fg per g of dry-cured ham. As far as we know, this is the first time that an antioxidant peptide has been quantified by MRM in dry-cured ham despite the complexity of this food matrix. Quantitative analysis of bioactive peptides is fundamental as the effect and impact in the organism depend on the amount ingested and able to reach the target site (Mora et al., 2017). Moreover, peptide-food matrix interactions could significantly affect the bioavailability and bioactivity of peptides (Udenigwe & Fogliano, 2017), which should be also considered in future studies.

4. Conclusions

The results obtained in this study evidenced a good stability of the antioxidant peptide AEEEYPDL derived from Spanish dry-cured ham against different temperatures and salt concentrations, but its activity was significantly reduced after a simulated gastrointestinal digestion due to its degradation by protease enzymes. The measurement of peak area intensities allowed the quantification of the peptide after different heat treatments, evidencing the degradation of AEEEYPDL with temperature. Finally, an optimised MRM-based methodology was able to quantify the peptide AEEEYPDL in the complex dry-cured ham sample, being a decisive step in further studies for the monitoring and characterisation of bioactive peptides in this product.

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502 **Figure 1.** Structure of the AEEEEYDDL peptide in hydrophilic environment using
503 PEPstrMOD software.

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Figure 2. Antioxidant activity of the peptide AEEEEYPDL after exposure to different heat treatments (37 °C, 50 °C, 72 °C, and 90 °C for 10 min), salt contents (0%, 2%, 4%, and 8% NaCl at 100 °C for 10 min), and *in vitro* gastrointestinal digestion (t0: before digestion, G: after gastric phase, GI: after gastrointestinal digestion) measured by A) ORAC assay and B) ABTS radical scavenging capacity.

a)

b)

Figure 3. Total ion chromatogram (TIC) of the peptide AEEEEYPDL subjected to *in vitro* digestion: **a)** after the gastric phase (G) and **b)** after the gastrointestinal (GI) digestion.

A)

B)

Figure 4. a) Total ion chromatogram (TIC), **b)** mass spectrum, and **c)** extracted ion chromatogram (EIC) of the peptide AEEEEYPDL after MS analysis.

a)

b)

c)

Figure 5. Degradation of the peptide AEEEYPDL after heat treatments by analysing changes in the EIC peak areas.

Figure 6. Calibration curve of the standard peptide AEEEYPDL obtained from peak areas in MRM chromatograms.