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Effect of cooking and *in vitro* digestion on the antioxidant activity of dry-cured ham by-products

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Abstract

Dry-cured ham by-products have been traditionally used in Mediterranean household cooking of broths and stews. The aim of this work was to evaluate the effect of cooking treatments and *in vitro* gastrointestinal digestion on the antioxidant activity of natural peptides found in dry-cured ham by-products including bones. The antioxidant activity was tested using five different assays and results demonstrated that cooking using conventional household methods increased the antioxidant activity of ham by-products when assessed using different antioxidant assays with the exception of the ABTS radical scavenging measurement assay. Simulated gastrointestinal digestion showed no significant effect on the antioxidant activity of ham by-products and antioxidant activity decreased when assessed using the ORAC and β-carotene bleaching assays. Analysis by MALDI-TOF MS revealed a considerable breakdown of peptides due to the action of gastrointestinal enzymes, mainly in samples cooked at 100 ºC for 1 h. In addition, 459 peptides derived from 57 proteins were identified using mass spectrometry in tandem. These peptides were derived from collagen protein and were found to be responsible for the differences in antioxidant activities observed between uncooked and cooked samples after digestion. The results show the potential of dry-cured ham bones as a source of antioxidant peptides that retain their bioactivity after household cooking preparations and gastrointestinal digestion.

Keywords: Antioxidant, peptides, mass spectrometry, quantification, cooking, gastrointestinal digestion, by-products.

1. Introduction

Every year the meat industry produces tons of by-products including bones, skin, blood and horns resulting from both animal slaughtering and trimming or deboning during the processing of meat products (Lafarga & Hayes, 2014). This waste represents a high cost for the meat processing sector and serious environmental problems (Mora, Reig & Toldrá, 2014). As a result, meat processing industries are making a strong effort to convert wastes and by-products into useful products for animal feeds, human foods, pharmaceutical products, fertilisers and biodiesel generation (Arvanitoyannis & Ladas, 2008; Toldrá, Aristoy, Mora, & Reig, 2012). Regarding edible products, the production of protein hydrolysates from pork, beef or lamb by-products using commercial proteases is one of the most studied and promising markets (Di Bernardini, Harnedy, Bolton, Kerry, O'Neill, Mullen, & Hayes, 2011). These hydrolysates may be added to enhance the flavor, emulsion stability and water holding capacity of food products as well as for added nutrients to produce valuable products and functional ingredients like bioactive peptides (Zhang, Xiao, Samaraweera, Lee, & Ahn, 2010; Toldrá & Reig, 2011; Mora, Reig, & Toldrá, 2014). Residues derived from the slicing of hams such as rinds and bones are traditionally used in Mediterranean cooking as ingredients that add flavor to soups, broths, and stews.

A large number of bioactivities including antihypertensive, antimicrobial and antioxidant activities were reported for peptides generated though enzymatic hydrolysis of by-products to date (Di Bernardini et al., 2011, Mora et al., 2014, Lafarga & Hayes, 2014). These bioactive peptides need to be resistant to degradation by gastrointestinal proteases and must be absorbed through the intestinal epithelium and reach the bloodstream in an active form to exert physiologically effects (Vercruysse, Van Camp, & Smagghe, 2005). For this reason simulated digestion of meat proteins with gastrointestinal enzymes

including pepsin, trypsin and pancreatin is frequently used to determine the bioaccessibility and availability of bioactive peptides (Escudero, Sentandreu, Arihara, & Toldrá, 2010; Zhu, Zhang, Zhou, & Xu, 2016). Among bioactive peptides, those showing antioxidant capacity are interesting as their use in foods can provide natural protection against oxidative processes, which are associated with changes in sensory traits and nutritional value, quality deterioration and consequently, economic losses for food industries. Moreover, in terms of health effects in the human body, antioxidant peptides are thought to decrease the adverse effects of reactive oxygen species (ROS) on normal physiological functions and thus the risk for development of some degenerative diseases (Sarmadi & Ismail, 2010; Samaranayaka & Li-Chan, 2011).

The main purpose of the present study was to evaluate the effect of cooking treatments that simulated traditional Mediterranean household cooking of broths and *in vitro* gastrointestinal digestion on the antioxidant activity and peptide profile of water-soluble extracts obtained from dry-cured ham bones.

2. Materials and methods

2.1 Chemicals and reagents

Enzymes used for the *in vitro* gastrointestinal digestion: salivary α-amylase, porcine pepsine, porcine pancreatic α-amylase, porcine pancreatic lipase, and porcine bile extract were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Trypsin and chymotrypsin enzymes were from Fluka (Sigma-Aldrich, Co., St. Louis, MO, USA). Regarding chemicals used in the antioxidant assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferrycianide, ferric chloride, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), fluorescein, 2,2’-azobis(2-methylpropionamidine) dihydrochloride (AAPH), β-carotene, and linoleic acid were

from Sigma-Aldrich, Co. (St. Louis, MO, USA). Potassium persulfate, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were purchased from Panreac Quimica SAU (Barcelona, Spain). All used chemicals and reagents were of analytical grade.

2.2 Sample preparation and cooking procedure

Samples of femoral bones were obtained from six Spanish dry-cured hams with 18 months of processing, after the removal of muscles and fat. Bones were minced and subjected to different cooking conditions. A quanitity of 50 g of minced bones were cooked in water at 100 ºC for 20 min and 100 ºC for 1 h in order to simulate Mediterranean household cooking methods.

2.2 Extraction of peptides

Five different extraction procedures were tested. Two samples were cooked as described in the preceding section, followed by the extraction of peptides using water. The third sample was only submitted to extraction with water as a control, and the final two samples were subjected to acidic extraction using 0.5 N HCl and 0.01 N HCl in order to study the influence of different extraction solutions on peptides.

A total of 50 g of minced, dry-cured ham bones were homogenised with 200 mL of the corresponding extraction solvent and kept at 4 °C overnight with continuous stirring. The resulting homogenate was centrifuged at 12,000 *g* (20 min at 4 °C) and filtered through glass wool. Precipitation of proteins was done by the addition of 3 volumes of ethanol and maintaining the sample at 4 °C for 20 h. After centrifugation at 12,000 *g* for 10 min (4 ºC), the supernatant was dried in a rotatory evaporator and finally lyophilised.

2.3 *In vitro* gastrointestinal digestion

All samples were subjected to *in vitro* digestion according to the methodology described by Minekus et al. (2014) with some modifications. Briefly, 500 mg of sample were suspended in 2 mL of 0.2 M $NaHCO_3$ (pH 7.0). A total of 27 U/mL of salivary α-amylase solution and 37 μL of 50 mM $CaCl_2$ were added and the mixture was maintained for 3 min at 37 ºC to simulate the oral phase. Then, the pH was adjusted to 3.0 with 1 M HCl to expose samples to the gastric phase. For that, porcine pepsine was added to achieve 2000 U/mL in the final mixture followed by 4 μL of 50 mM $CaCl_2$. After 3 h of digestion at 37 ºC and constant stirring, the enzyme was inactivated by adjusting pH to 7.0 with 1 M NaOH. Digestive enzymes were added to the mixture to achieve the following activities in the final mixture: 100 U/mL of trypsin, 25 U/mL of chymotrypsin, 200 U/mL of porcine pancreatic α-amylase, 2000 U/mL of porcine pancreatic lipase and 10 mM of porcine bile extract. A total of 16 μL of 50 mM $CaCl_2$ was also added, and after 3 h at 37 ºC the intestinal digestion was finished by heating for 2 min at 95 ºC. The mixture was deproteinised by adding 3 volumes of ethanol maintaining the sample at 4 °C for 20 h, and centrifuged at 12,000 *g* and 4 °C for 10 min. Finally, the supernatant was dried in a rotatory evaporator and lyophilised.

2.4 Antioxidant activity

The antioxidant activity was determined in triplicate using five different methods. For that, stock solutions of 500 mg/mL in bidistilled water were prepared for all samples, before and after *in vitro* digestion.

2.4.1 DPPH radical scavenging activity

The DPPH activity of samples was determined as described by Bersuder, Hole, and Smith (1998). Briefly, 100 μL of each sample was mixed with 500 μL of ethanol and 125 μL of DPPH solution (0.02 % of DPPH in ethanol). The mixture was incubated in the dark for 60 min and the reduction of DPPH radicals was measured at 517 nm. A reduction in the

absorbance of the reaction mixture indicates higher free radical scavenging activity. Bidistilled water was used as the negative control and BHT as a positive control. The scavenging activity was calculated using the following equation: DPPH radical scavenging activity (%) = (Absorbance control – Absorbance sample) / Absorbance control x 100.

2.4.2 Ferric-reducing antioxidant power

The reducing power was measured based on the ability to reduce ferric iron to ferrous iron (Huang, Tsai, & Mau, 2006). Briefly, 70 µL of each sample 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 ºC for 20 min, and 70 µL of trichloroacetic acid (100 mg/mL) was added before a centrifugation step was carried out at 200 X *g* for 10 min. Then, 200 µL of the supernatant was mixed with 200 µL of bi-distilled water and 40 µL of ferric chloride (1 mg/mL). The absorbance was measured at 700 nm after 10 min of incubation. Higher absorbance values indicated higher ferric-reducing power. BHT was used as the positive control.

2.4.3 ABTS radical scavenging capacity

The ABTS assay was performed as described by Re et al. (1999) with slight modifications. Briefly, 7 mM of ABTS was dissolved in potassium persulfate (2.45 mM). The mixture was kept in the dark at room temperature for 12-16 h to produce $ABTS^{\cdot+}$. The $ABTS^{\cdot+}$ solution was diluted with phosphate buffer saline (PBS) (50 mM, pH 7.4) to obtain an absorbance of 0.70 ± 0.02 at 734 nm. An amount of 10 µL of sample was mixed with 990 µL of $ABTS^{\cdot+}$ solution and the absorbance was measured at 734 nm after 6 min of incubation. PBS was used as negative control and ascorbic acid as positive control. Trolox at a concentration between 0.05 and 2 mM was used to obtain a calibration curve. The ABTS radical scavenging activity was calculated and plotted against the concentration of

trolox and the results were expressed as nanomoles of TEAC (trolox equivalent antioxidant capacity) per mg of sample.

2.4.4 Oxygen radical absorbance capacity assay (ORAC)

ORAC assay using fluorescein (FL) was carried out according to the method described by Dávalos, Gómez-Cordovés, and Bartolomé (2004) with slight modifications. 140 μL of sample prepared in phosphate buffer (75 mM, pH 7.4) was mixed with 70 μL of fluorescein (200 nM) and incubated at 37 ºC for 15 min. Then, 70 μL of AAPH (80 mM) was added and the fluorescence was measured at 1 min intervals for 100 min using excitation and emission wavelengths of 485 and 538 nm, respectively. Trolox (2–16 μM) was used as standard and tryptophan as positive control. The area under curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The ORAC-FL values were calculated and plotted against the concentration of trolox to obtain a standard curve and the results were expressed as nanomoles of TE (Trolox equivalents) per mg of sample.

2.4.5. β-carotene bleaching assay

The ability of samples to prevent β-carotene bleaching was assayed as described by Koleva, Van Beek, Linssen, De Groot, and Evstatieva (2002) with some modifications. A total of 0.5 mg of β-carotene, 20 μL of linoleic acid and 200 μL of Tween 80 were dissolved in 1 mL of chloroform to obtain the β-carotene/linoleic acid solutionprepared by dissolving . Then, chloroform was totally evaporated in a rotatory evaporator at 40 °C, and 100 mL of bidistilled water was added and vigorously stirred. Finally, 50 μL of sample was mixed with 250 μL of the β-carotene solution and the absorbance was measured at 450 nm immediately (t_0) and after 180 min of incubation at 50 ºC (t_f). Bidistilled water was used as blank and BHA as positive control. The antioxidant activity

was calculated as: Antioxidant activity (%) = (1 - (Absorbance sample t_0 – Absorbance sample t_f) / (Absorbance blank t_0 – Absorbance blank t_f)) x 100.

2.5 MALDI-TOF MS analysis

In order to analyse the profile of peptides obtained after the different extractions, samples before and after gastrointestinal digestion were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) as described by Gallego, Mora, Aristoy, and Toldrá (2015a). Thus, an aliquot of 15 µL of sample from stock solutions was concentrated using Zip-Tip C18 with standard bed format (Millipore Corporation, Bedford, MA) and 1 µL of each sample was located into a MALDI plate for analysis.

2.6 nLC-MS/MS analysis

The identification of peptides was performed by nanoliquid chromatography-tandem mass spectrometry (nLC-MS/MS) according to the methodology used by Gallego, Mora, Aristoy, and Toldrá (2015b). In this regard, 5µL of control sample (peptides extracted with H_2O) and samples submitted to cooking (100 ºC during 20 min and 1 h, respectively) and all after the *in vitro* digestion at a concentration of 0.3 mg/mL in H_2O with 0.1% of TFA were injected into the nLC-MS/MS system.

2.7 Data analysis

Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA, USA; http://www.matrixscience.com) was used in the identification and quantification of the natural peptides extracted from broths. The identification of peptides and protein of origin was done using UniProt database with a significance threshold of $P < 0.05$ and a tolerance on the mass measurement of 100 ppm in MS mode and 0.3 Da for MS/MS ions. Mammalia taxonomy and none specific enzyme were selected.

The relative quantification was performed using the label-free approach described by Gallego et al. (2015b). This label-free approach is based on the measurement of the integrated areas of extracted ion chromatograms (XICs), whose combination allows the ratios for individual peptides to be determined. For that, during identification Mascot search engine assigns peptide matches to the origin protein, requiring robust search parameters as quantification is done at peptide level. Quantitative data was obtained using Peak View 1.1 software (AB Sciex, Framingham, MA, USA) and then analysed using Marker View 1.2 software (AB Sciex, Framingham, MA, USA). Principal Component Analysis (PCA) and loading plot analysis were performed using SIMCA-P+ 13.0 (Umetrics AB, Sweden) software. Finally, statistical analysis including one-way analysis of variance (ANOVA) and Fisher's multiple range test were carried out using XLSTAT program (2011).

3. Results and discussion

3.1 Effect of cooking treatment and simulated gastrointestinal digestion on the antioxidant activity

In order to optimise the extraction of peptides from dry-cured ham bones, the effect of acid concentrations (0.5 N and 0.01 N HCl, respectively) on the extracted peptides was also evaluated. Additionally, two different cooking treatments in boiling water (100 ºC) for 20 min and 1 h respectively were studied. All samples were also subjected to *in vitro* gastrointestinal digestion to assess the antioxidant capacity before and after the simulated digestion.

Different methodologies are currently used to assess the *in vitro* antioxidant activity of protein hydrolysates or peptides, which are classified into two groups depending on the basis of the chemical reactions involved: methods based on hydrogen atom transfer (HAT) and methods based on electron transfer (ET) (Huang, Ou, & Prior, 2005). The

253 HAT-based assays evaluate the ability of an antioxidant to quench free radicals by
254 hydrogen donation in a competitive reaction. These assays include ORAC, total radical
255 trapping antioxidant parameter (TRAP) and β-carotene bleaching assay. ET-based assays
256 measure the ability of a potential antioxidant to transfer one electron to reduce an oxidant,
257 so these reactions are pH dependent. The ABTS radical scavenging assay, ferric-reducing
258 antioxidant power, and DPPH radical scavenging activity are examples of ET-based
259 assays (Huang et al., 2005; McDonald-Wicks, Wood, & Garg, 2006). Thus, the
260 antioxidant activity was measured by two different HAT-based assays (ORAC and β-
261 carotene bleaching assay) and three ET-based assays (DPPH radical scavenging activity,
262 ferric-reducing antioxidant power and ABTS radical scavenging assay). The obtained
263 results, before and after digestion of samples, are shown in Figures 1 and 2.
264 In the DPPH radical scavenging activity assay, the highest antioxidant activities were
265 obtained for samples cooked for 20 min and 1 h, reaching percentage values of 53.05 ±
266 2.03 and 64.89 ± 1.51, respectively (Figure 1A), when assayed at a concentration of 2.5
267 mg/mL. After digestion, no significant effect was observed in any sample, except in the
268 case of the sample extracted with H_2O that showed a notable increase at all the tested
269 concentrations (Figure 1B). On the other hand, ferric-reducing antioxidant power assay
270 revealed that the sample cooked at 100 ºC for 20 min showed the highest absorbance
271 measured at 700 nm for a concentration of 2.5 mg/mL, indicating a significant higher
272 antioxidant activity. However, all samples at 5 mg/mL reached maximum values around
273 1.5, with the exception of the sample extracted with 0.5 N HCl (0.63 ± 0.04) (Figure 1C).
274 After samples were digested using gastrointestinal enzymes, the antioxidant activity at
275 2.5 mg/mL increased in samples extracted with 0.5 N HCl, 0.01 N HCl and H_2O, showing
276 no effect for cooked samples during 20 min or 1h. What is more, the sample 0.5 N HCl

277 displayed a marked increase on the antioxidant activity after the simulated digestion,
278 although it was the lowest value obtained (Figure 1D).
279 In contrast to the other methods, the β-carotene bleaching assay revealed that the sample
280 0.5 N HCl presented the highest antioxidant capacity (91%), followed by samples
281 subjected to cooking treatments that reached values around 85 % (Figure 1E).
282 Nevertheless, the antioxidant activity after digestion was decreased significantly for the
283 sample 0.5 N HCl (75%) and the cooked at 100 ºC for 1h (from 85% to 63%), whereas
284 the activity of the sample H_2O presented a sharp increase at the lowest concentrations
285 tested in the assay (Figure 1F).
286 Figure 2A shows no statistically differences between samples before and after *in vitro*
287 digestion in the ABTS radical scavenging assay, obtaining values between 187.47 ± 13.72
288 and 220.34 ± 17.81 nmol TEAC/mg sample. However, a significant increase in the
289 antioxidant activity was observed for the sample extracted with 0.5 N HCl after the
290 simulated digestion. In the ORAC assay (Figure 2B), the highest value (230.22 ± 6.59
291 nmol TE per mg) was obtained in the sample cooked at 100 ºC during 20 min, whereas
292 the antioxidant activity was considerably decreased after digestion with the exception of
293 the sample extracted with 0.5 N HCl.
294 Technological treatments such as mincing or cooking are particularly important in the
295 development of oxidation and denaturation processes as they affect the structural
296 properties and physic-chemical state of proteins and peptides (Liu & Xiong, 2000; Santé-
297 Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008). Moreover, at high temperatures,
298 oxidation and aggregation of proteins can lead to an increase of surface hydrophobicity
299 that modify the rate of digestion by gastrointestinal enzymes depending on the nature of
300 protease, temperature and time of cooking (Santé-Lhoutellier, Aubry, & Gatellier, 2007;
301 Bax et al., 2012). In fact, recent studies showed that the temperature of cooking affects

the digestion rate of proteins more than digestibility, showing that conformational changes due to protein denaturation favored the bioaccessibility of the digestive proteases to their cleavage sites (Sayd, Chambon, & Santé-Lhoutellier, 2016). In this regard, some studies have shown that cooking reduced the antioxidant capacity whereas simulated digestion could lead to the formation of novel antioxidant peptides (Jensen, Dort, & Eilertsen, 2014; Remanan & Wu, 2014). In the present study, samples subjected to cooking showed higher antioxidant activity than uncooked samples in all methods evaluated except for the ABTS radical scavenging assay. This fact evidences that there has been no reduction, but rather an increase of potentially bioactive peptides after household preparations using dry-cured ham bones. Nevertheless, the *in vitro* digestion of samples showed no significant effect on the antioxidant activity. On the other hand, regarding the evaluated effect of acid concentration on the extracted peptides, the low values of antioxidant activity obtained indicate that more acidic conditions did not increase the generation/extraction of a higher amount of peptides.

3.2 Peptide profile before and after digestion of samples

The gastrointestinal digestion has a key influence on the release of peptides from parent proteins as well as on the modification or breakdown of peptides that can exert antioxidant properties. In fact, enzymes specificity affects amount, size, composition of peptides and their amino acid sequence, influencing the antioxidant activity of the digested samples (Sarmadi & Ismail, 2010; Samaranayaka & Li-Chan, 2011). So, the analysis by MALDI-TOF MS was used to evaluate the stability of the peptides to the digestion method, comparing the peptide profile of samples before and after the *in vitro* digestion. As illustrated in Figure 3, there is a larger amount of peptides with smaller mass in the samples taken after the simulated digestion, suggesting the degradation of bigger sized peptides into smaller ones by the action of the added enzymes. In particular, samples

subjected to cooking at 100 ºC during 1 h were those whose digestion was more noticeable, showing a larger amount of peptides lower than 450 Da. In this regard, recent studies have shown that cooking treatments affect the digestion rate of proteins due to changes in their conformation that can modify the bioaccesibility of enzymes to their cleavage sites (Bax et al., 2012; Wen et al., 2015). However, this work revealed that the antioxidant activity of these cooked samples decreased for the ORAC and β-carotene bleaching assays, while the values obtained with the other antioxidant methods were not modified. These results could be explained by the fact that antioxidant activity is not only related to the size of the peptides but also to their amino acid composition, structure, and hydrophobicity (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). In this regard, a study done by Damgaard, Lametsch, and Otte (2015) showed that the antioxidant capacity of hydrolysed animal by-products was correlated with the amino acid composition, not with the amount of low molecular weight peptides. The aromatic amino acids Trp, Tyr, and Phe, as well as Cys, Met, and His have been reported to show high antioxidant activities through several mechanisms such as free radical scavengers, reducing agents, metal chelators and inhibitors of lipid peroxidation (Samaranayaka & Li-Chan, 2011; Power, Jakeman, & FitzGerald, 2013; Damgaard et al., 2015).

3.2 Peptide profile of the digested cooked samples

The peptide profile of samples subjected to cooking treatments and *in vitro* digestion was fully characterised by analysing the samples through nLC-MS/MS for the identification of peptides. The relative quantification of extracted peptides was performed using a label-free method by extracting peak intensities. So, a total of 459 peptides derived from 57 proteins were identified and quantified to establish differences between the control (extracted with H_20 and no cooking treatment) and cooked samples (100 ºC 20 min, and 100 ºC 1 h). To this end, a Principal Component Analysis (PCA) was carried out to assess

352 the possible differences between samples and to obtain information on the most
353 influential peptides (see Figure 4). In fact, the PCA showed two statistically different
354 groups, being sample cooked at 100 ºC for 1 h located in a different cluster of the other
355 two samples (Figure 4A). Component 1 is responsible for 52.9 % of the variability in the
356 dataset, and Component 2 explains 20 % of the variance within the dataset. Additionally,
357 the loading plot (Figure 4B) revealed all the peptides responsible for influencing the
358 clustering of data, being those derived from collagen protein the peptides showing the
359 greatest influence for the description of the two discriminant components. These peptides
360 were thus extracted for a new statistical analysis as shown in Figure 5. In this case, the
361 PCA showed three statistically different groups that match with the three different
362 treatments (Figure 5A). Component 1 explains 62.3 % of the variability in the dataset,
363 which allows differentiating between samples cooked for 1 h from the other two samples.
364 On the other hand, Component 2 is responsible for 14.3 % of the variance within the
365 dataset for these two discriminant components, discriminating between the control
366 sample and the sample cooked for 20 min. However, all the peptides do not show an
367 identical influence on the clustering so the loading plot (Figure 5B) revealed the main
368 responsible peptides for the observed differences, which are indicated with higher colour
369 density values. In this respect, Table 1 shows the sequences of those peptides mainly
370 responsible for the differences observed after digestion of sample cooked for 1h at 100ºC.
371 The complete list of sequences that have been identified and relatively quantified using
372 the label-free approach is shown in Table 1_supplementary material.
373 Even though the digestion process leads to the breakdown of proteins, proteolysis is
374 greatly responsible for the generation of thousands of small peptides and free amino acids
375 due to the action of endogenous enzymes on sarcoplasmic and myofibrillar proteins and
376 collagen (Toldrá & Flores, 1998). Figure 6 shows the percentage of peptides identified in

the three digested samples (control, 100 ºC 20 min, and 100 ºC 1 h) from main proteins of origin. As expected according to the observed PCA results, the largest percentage of peptides was from collagen protein (61 %), including collagen α-1(I) chain, collagen α-1(II) chainlike partial, collagen α-1(III) chain precursor, and collagen α-2(I) chain precursor. In fact, collagen is an insoluble fibrous protein and is the major component of some by-products such as bones, skin, cartilages and tendons (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). When collagen is heated, its structure in triple helix begins to unravel into single strands, shrinks and dissolves leading to a disruption of the myosin gel and loss of its functional properties such as gelling and water-binding capacity (Tornberg, 2005; Voutila, Mullen, Ruusunen, Troy, & Puolanne, 2007). Moreover, the enzymatic hydrolysis of collagen results in the release of many small peptides, some of them have been reported as bioactive peptides with ACE inhibitory and antioxidant activity. In this regard, some studies have identified antioxidant collagen peptides derived from hydrolysates of porcine skin (Li, Chen, Wang, Ji, & Wu, 2007) and bovine tendon (Ryder, Bekhit, McConnell, & Carne, 2016) as well as the naturally generated peptide GLAGA identified in dry-cured ham (Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013). What is more, collagen protein has repeated unique Gly-Pro-Hyp sequence on its structure, and observed antioxidant and antihypertensive activities have been associated with this unique peptide composition (Gómez-Guillén et al., 2011). So, due to the large amount of peptides identified from collagen in this work, they could be the main responsible for the antioxidant activity of samples. However, further research would be needed to characterise the specific sequences responsible for the antioxidant activity in dry-cured ham bones.

4. Conclusions

This study reports the presence of antioxidant peptides derived from dry-cured ham bones which have been traditionally used in the Mediterranean household cooking of broths and stews. In general, cooking increased the antioxidant activity whereas simulated gastrointestinal digestion did not shown a significant effect on the antioxidant activity of samples despite the analysis of peptide profile by MALDI-TOF MS revealed the generation of novel peptides, mainly in the samples cooked at 100 ºC for 1 h. Moreover, a total of 459 peptides derived from 57 proteins of origin were identified and relatively quantified by LC-MS/MS, being the peptides derived from collagen the most influential to establish differences between uncooked and cooked samples after digestion. In conclusion, dry-cured ham bones appear to be a good source of antioxidant peptides after household cooking preparations and considering gastrointestinal digestion, giving an added-value to these by-products that was not considered to date.

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FIGURE CAPTIONS

Figure 1. Antioxidant activities of the five samples (0.5 N HCl, 0.01 N HCl, H_2O, 100 ºC 20min, 100 ºC 1h) before and after the *in vitro* digestion determined using three different methods: DPPH radical scavenging activity, Ferric-reducing antioxidant power, and β-carotene bleaching assay. The values represent means of three replicates ± standard deviations.

Figure 2. Antioxidant activities of the five samples (0.5 N HCl, 0.01 N HCl, H_2O, 100 ºC 20min, 100 ºC 1h) before and after the *in vitro* digestion determined using A) ABTS radical scavenging capacity, and B) Oxygen radical absorbance capacity assay. Bars represent standard deviations from three replicates. The values represent means of three replicates ± standard deviations, and bar letters indicate significant differences among the values at $p < 0.05$.

Figure 3. MALDI-ToF mass spectra of the samples (0.5 N HCl, 0.01 N HCl, H_2O, 100 ºC 20min, 100 ºC 1h) before digestion (B.D.) and after digestion (A.F.).

Figure 4. A) Principal Component Analysis (PCA) score plot to assess the variance among all the peptides of samples H_2O, 100 ºC 20 min, and 100 ºC 1 h in three replicates (n=3). **B)** PCA loading plot showing the proteins of origin of those peptides more responsible for main differences between samples.

Figure 5. A) Principal Component Analysis (PCA) score plot to assess the variance among the peptides derived from collagen protein of samples H_2O, 100 ºC 20 min, and 100 ºC 1 h in three replicates (n=3). **B)** PCA loading plot showing peptides from collagen protein. Higher colour density values indicate those peptides more responsible for influencing the clustering of data.

Figure 6. Distribution of the 459 peptides identified by nLC-MS/MS according to their 57 protein of origin.

Table 1

Sequences of collagen peptides responsible for main differences between control and samples cooked for 20 min and 1 h at 100ºC, as is shown in the loading plot of the PCA analysis. All the reported sequences are located in the Q1 of the loading plot. Data of other sequences identified have been included in Supplementary material_Table 1.

		Location[a]	
Peptide sequence*	**Collagen chain**	**M1.p[1]**	**M1.p[2]**
GIP[Oxi]GPAGAAGATGA	α-2(I)	0.072	0.000
GLTGPIGPP[Oxi]GP[Oxi]AGAP[Oxi]GDKGETGPSGPAGPTGA	α-1(I)	0.072	0.001
GISVPGPMGPSGPR	α-1(I)	0.067	0.002
NGPVGPTGPVGA	α-2(I)	0.073	0.006
GDGGPP[Oxi]GATGFP[Oxi]GAAGR	α-2(I)	0.077	0.007
GNDGSVGPVGPAGPIGSAGPP[Oxi]GFP[Oxi]GAP[Oxi]GP[Oxi]K	α-2(I)	0.077	0.007
NGETGPQGPPGPTGPGGDK	α-1(III)	0.077	0.009
QGPP[Oxi]GPP[Oxi]GSP[Oxi]GEQGPSGA	α-1(I)	0.076	0.009
QGPAGEP[Oxi]GEP[Oxi]GQTGPAGA	α-2(I)	0.075	0.010
TGETGASGPP[Oxi]GFAGEK	α-2(I)	0.073	0.011

GETGPAGPAGPVGPVGAR	α-1(I)	0.076	0.012
GIPGP[Oxi]AGAAGATGA	α-2(I)	0.071	0.013
GEHGPP[Oxi]GPAGFP[Oxi]GAP[Oxi]GQNGEP[Oxi]GAK	α-1(III)	0.077	0.013
GELGPVGNP[Oxi]GPAGPAGPR	α-2(I)	0.077	0.015
P[Oxi]GPAGAAGAP[Oxi]GPQGAVGPAGK	α-2(I)	0.076	0.016
EKGSP[Oxi]GADGPAGAP[Oxi]GTPGPQ	α-1(I)	0.074	0.017
GEP[Oxi]GAAGPQGPP[Oxi]GPSGE	α-2(I)	0.069	0.017
GEP[Oxi]GSP[Oxi]GENGAPGQM[2Ox]GPR	α-1(I)	0.068	0.018
GESGPAGPP[Oxi]GAP[Oxi]GAP[Oxi]GAPGPVGPAGK	α-1(I)	0.074	0.018
GPP[Oxi]GAVGNP[Oxi]GVNGAP[Oxi]GEAGR	α-2(I)	0.077	0.019
GAP[Oxi]GTAGPSGPSGLPGER	α-2(I)	0.075	0.020
HGDQGAPGPVGPAGPR	α-2(I)	0.076	0.021
GEVGPAGPNGF	α-2(I)	0.061	0.027
GENGPVGPTGPVGA	α-2(I)	0.076	0.028
GAP[Oxi]GTAGPSGPSGLP[Oxi]GER	α-2(I)	0.076	0.029
PGQ.QGPAGEP[Oxi]GEP[Oxi]GQTGPAGA	α-2(I)	0.076	0.030
GSP[Oxi]GADGPAGAP[Oxi]GTPGPQ	α-1(I)	0.073	0.030
P[Oxi]GEQGVP[Oxi]GDLGAP[Oxi]GPSGA	α-1(I)	0.076	0.030
GPNGEVGSAGPP[Oxi]GPP[Oxi]GL	α-2(I)	0.075	0.032
GEPGP[Oxi]P[Oxi]GPAGAAGPAGNP[Oxi]GADGQP[Oxi]GGK	α-1(I)	0.076	0.032
GEP[Oxi]GPPGP[Oxi]AGAAGPAGNP[Oxi]GADGQPGGK	α-1(I)	0.076	0.032
GPTGPIGPP[Oxi]GPAGQP[Oxi]GDK	α-1(III)	0.075	0.033
GEP[Oxi]GAAGPQGPP[Oxi]GPSGEEGK	α-2(I)	0.074	0.034
GESGAP[Oxi]GLP[Oxi]GIAGPR	α-1(III)	0.076	0.034
NGETGPQGPP[Oxi]GPTGPGGDK	α-1(III)	0.075	0.036
GNDGSVGPVGPAGPIGS	α-2(I)	0.075	0.036
GLTGPIGPP[Oxi]GPAGAN[Dea]GEK	α-1(I)	0.076	0.036
GEQGPAGSP[Oxi]GFQ	α-1(I)	0.075	0.036
GESGPAGPP[Oxi]GAP[Oxi]GAP[Oxi]GAP[Oxi]GPVGPA	α-1(I)	0.071	0.036
PGQ.QGPP[Oxi]GPP[Oxi]GSP[Oxi]GEQGPSGASGPAGPR	α-1(I)	0.076	0.037
GP[Oxi]PGPM[Oxi]GPP[Oxi]GLAGPP[Oxi]GESG	α-1(I)	0.076	0.037
GETGPAGPAGAPGP[Oxi]AGSR	α-1(III)	0.075	0.038
PGQ.QGP[Oxi]PGEP[Oxi]GEP[Oxi]GASGPMGPR	α-1(I)	0.075	0.038
GENGSP[Oxi]GAP[Oxi]GAP[Oxi]GHPGPPGP[Oxi]VGPAGK	α-1(III)	0.076	0.038
DGPP[Oxi]GPP[Oxi]GSSGAP[Oxi]GSP[Oxi]GVSGPK	α-1(III)	0.074	0.038
GSPGP[Oxi]QGPP[Oxi]GAP[Oxi]GPGGISGITGA	α-1(III)	0.073	0.039
DGLNGLP[Oxi]GPIGP[Oxi]PGP[Oxi]R	α-1(I)	0.075	0.040
GEP[Oxi]GPPGP[Oxi]AGAAGPAGNP[Oxi]GADGQP[Oxi]GGK	α-1(I)	0.075	0.041
GEAGAQGPP[Oxi]GPAGPAGER	α-1(I)	0.075	0.041
GPAGPP[Oxi]GPP[Oxi]GAAGTP[Oxi]GLQGM[2Ox]PGER	α-1(III)	0.075	0.042
LGPVGNP[Oxi]GPAGPAGPR	α-2(I)	0.075	0.042
GEP[Oxi]GPP[Oxi]GPAGAAGPAGNP[Oxi]GADGQP[Oxi]GGK	α-1(I)	0.075	0.042
P[Oxi]GEAGPP[Oxi]GPP[Oxi]GPAGE	α-1(I)	0.072	0.043
TGDAGPVGPP[Oxi]GPP[Oxi]GPP[Oxi]GPP[Oxi]GPPSGGFD	α-1(I)	0.075	0.043
QGPP[Oxi]GEP[Oxi]GQAGPAGPPGP[Oxi]P[Oxi]GAIGPSGPAGK	α-1(III)	0.075	0.043
LQGPP[Oxi]GP[Oxi]PGSP[Oxi]GEQGPSGASGPAGPR	α-1(I)	0.063	0.044
GNDGSVGPVGPAGPIGSAGPPGFPGAPGPKGELGPVGNPGPAGPAGPRGEV	α-2(I)	0.072	0.044
EGPAGLP[Oxi]GIDGR	α-2(I)	0.071	0.044
GAAGLP[Oxi]GVAGAP[Oxi]GLP[Oxi]GPR	α-2(I)	0.075	0.044
GAAGLP[Oxi]GVAGAP[Oxi]GLPGP[Oxi]R	α-2(I)	0.075	0.044
GLTGPIGPPGP[Oxi]AGAN[Dea]GEK	α-1(I)	0.075	0.045
P[Oxi]GEAGPP[Oxi]GPPGP[Oxi]AGEK	α-1(I)	0.074	0.045
QGLP[Oxi]GPAGPP[Oxi]GEAGK	α-1(I)	0.075	0.046
QGPP[Oxi]GPP[Oxi]GSP[Oxi]GEQGPSGASGPAGPR	α-1(I)	0.074	0.046
GDAGPP[Oxi]GPAGPTGPP[Oxi]GPIGS	α-1(I)	0.074	0.047
GEP[Oxi]GAP[Oxi]GENGTPGQTGAR	α-2(I)	0.072	0.047
AGPAGPNGPP[Oxi]GPAGSR	α-2(I)	0.072	0.048
GSQGSQGPAGPP[Oxi]GPP[Oxi]GPP[Oxi]GPP[Oxi]GPSGGGY	α-2(I)	0.074	0.049
GDAGPP[Oxi]GPAGPTGPP[Oxi]GPIGSVGAP[Oxi]GP[Oxi]K	α-1(I)	0.074	0.049
GDAGPP[Oxi]GPAGPTGPP[Oxi]GP[Oxi]IGSVGAPGP[Oxi]K	α-1(I)	0.074	0.049
GEP[Oxi]GAP[Oxi]GENGTP[Oxi]GQTGAR	α-2(I)	0.071	0.049

GLP[Oxi]GPAGPP[Oxi]GEAGK	α-1(I)	0.074	0.049
SGLQGP[Oxi]P[Oxi]GPP[Oxi]GSP[Oxi]GEQGPSGASGPAGPR	α-1(I)	0.072	0.049
GLTGPIGPP[Oxi]GPAGAP[Oxi]GDK	α-1(I)	0.074	0.049
GEP[Oxi]GVLGAP[Oxi]GTAGPSGPSGLP[Oxi]GER	α-2(I)	0.073	0.050
SGLQGPP[Oxi]GPP[Oxi]GSP[Oxi]GEQGPSGA	α-1(I)	0.073	0.050
PGQ.QGPP[Oxi]GEP[Oxi]GEPGASGPM[2Ox]GPR	α-1(I)	0.074	0.051
GPP[Oxi]GAVGAP[Oxi]GPQGF	α-2(I)	0.074	0.051
QGPPGEP[Oxi]GEP[Oxi]GASGPM[Oxi]GPR	α-1(I)	0.074	0.051
QGPP[Oxi]GEP[Oxi]GEP[Oxi]GASGP[Oxi]MGPR	α-1(I)	0.074	0.052
GEVGLP[Oxi]GVSGPVGPP[Oxi]GNP[Oxi]GAN	α-2(I)	0.073	0.052
PGQ.QGPAGEP[Oxi]GEP[Oxi]GQTGPAGAR	α-2(I)	0.074	0.052
QGPAGEP[Oxi]GEP[Oxi]GQTGPAGAR	α-2(I)	0.073	0.053
SGLQGPP[Oxi]GPP[Oxi]GSP[Oxi]GEQGPSGASGPAGPR	α-1(I)	0.074	0.053
SGLQGP[Oxi]PGPP[Oxi]GSP[Oxi]GEQGPSGASGPAGPR	α-1(I)	0.074	0.053
GDAGPP[Oxi]GPAGPTGPP[Oxi]GPIGSVGAP[Oxi]GPK	α-1(I)	0.073	0.053
GEQGPAGPP[Oxi]GFQGLP[Oxi]GPAGT	α-2(I)	0.066	0.053
AGP[Oxi]PGPTGPAGPP[Oxi]GFP[Oxi]GAVGA	α-1(I)	0.073	0.053
GDAGPP[Oxi]GPAGPTGPP[Oxi]GPIGSV	α-1(I)	0.071	0.054
TGDAGPVGPP[Oxi]GPP[Oxi]GPP[Oxi]GPP[Oxi]GPPSGGF	α-1(I)	0.073	0.054
GSP[Oxi]GADGPAGAP[Oxi]GTP[Oxi]GPQ	α-1(I)	0.061	0.054
P[Oxi]GEQGVP[Oxi]GDLGAPGP[Oxi]SGAR	α-1(I)	0.073	0.055
GENGLP[Oxi]GENGAP[Oxi]GPM[Oxi]GPR	α-1(III)	0.072	0.055
GPP[Oxi]GPM[Oxi]GPP[Oxi]GLAGPP[Oxi]GESGR	α-1(I)	0.073	0.055
GETGPAGPAGPVGPV	α-1(I)	0.070	0.055
PGQ.QGPPGEP[Oxi]GEP[Oxi]GASGPM[Oxi]GPR	α-1(I)	0.070	0.056
GNDGSVGPVGPAGPIG	α-2(I)	0.070	0.056
GEPGPP[Oxi]GP[Oxi]AGAAGPAGNP[Oxi]GADGQP[Oxi]GGK	α-1(I)	0.071	0.056
AGP[Oxi]PGPTGPAGPP[Oxi]GFP[Oxi]GAVG	α-1(I)	0.070	0.057
GEQGPAGSP[Oxi]GFQGLP[Oxi]GPAGPP[Oxi]GEAGK	α-1(I)	0.073	0.058
GETGPAGPAGPVGPVGA	α-1(I)	0.073	0.058
AGPAGPNGPPGP[Oxi]AGSR	α-2(I)	0.071	0.059
GPP[Oxi]GPM[Oxi]GPPGLAGPP[Oxi]GESGR	α-1(I)	0.072	0.059
GNDGATGAAGPP[Oxi]GPTGPAGP[Oxi]P[Oxi]GFP[Oxi]GAVGAK	α-1(I)	0.072	0.060
GPP[Oxi]GESGAAGPAGPIGSR	α-2(I)	0.070	0.061
ISVPGPM[Oxi]GPSGPR	α-1(I)	0.071	0.064
GPP[Oxi]GPAGAP[Oxi]GPQGF	α-1(I)	0.069	0.065

*[Oxi] means that the previous residue has been oxidised. [Dea] means that the previous residue has been deamidated.

a- Location of the sequence in the Principal Component Analysis loading plot. All reported sequences are located in Q1.

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573 Figure 1

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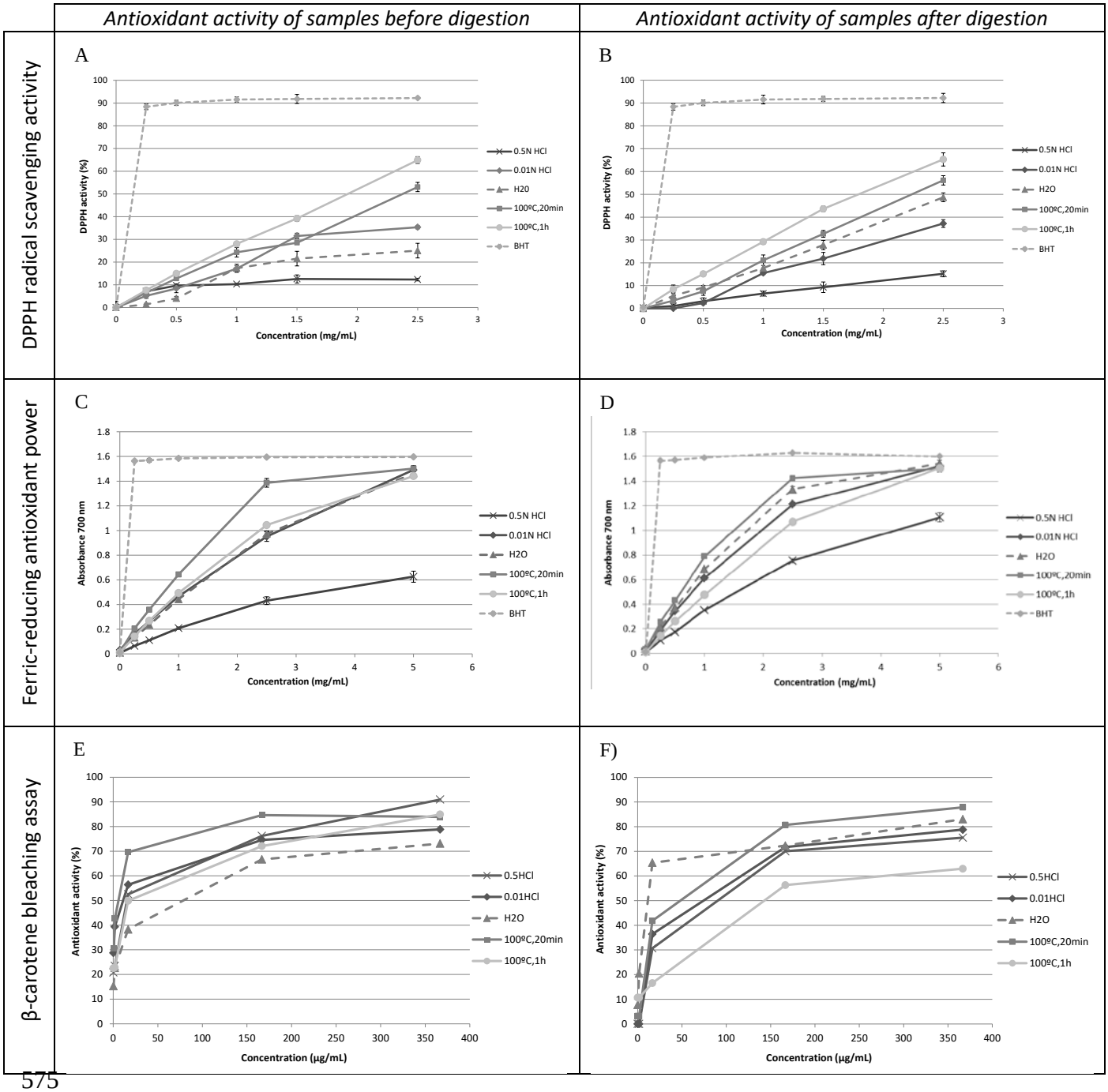

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579 Figure 2

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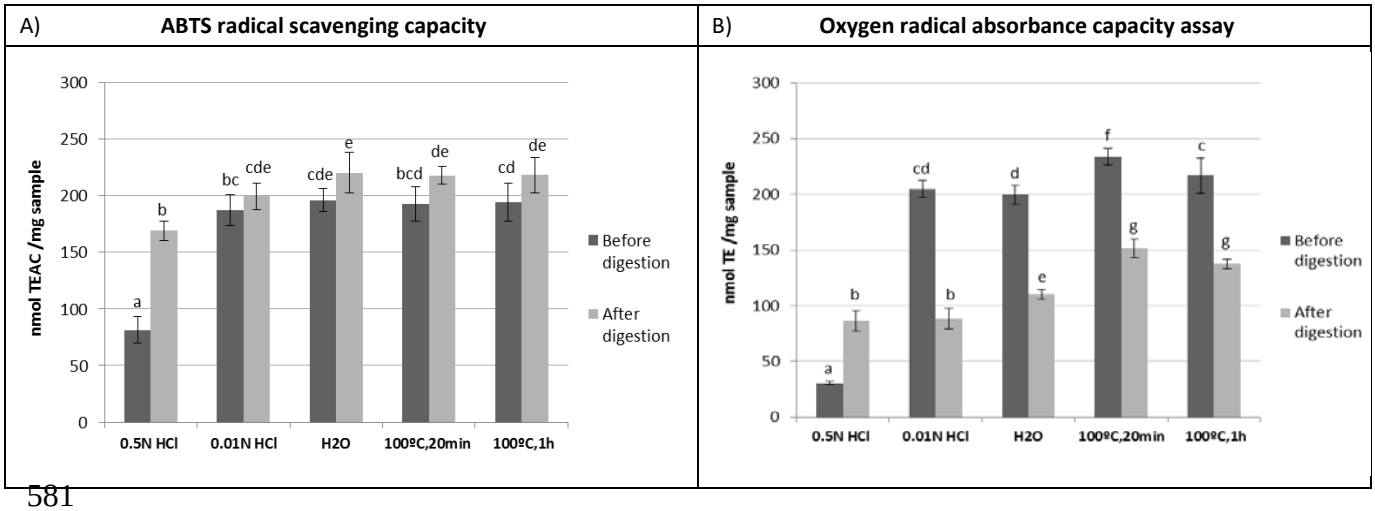

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585 Figure 3
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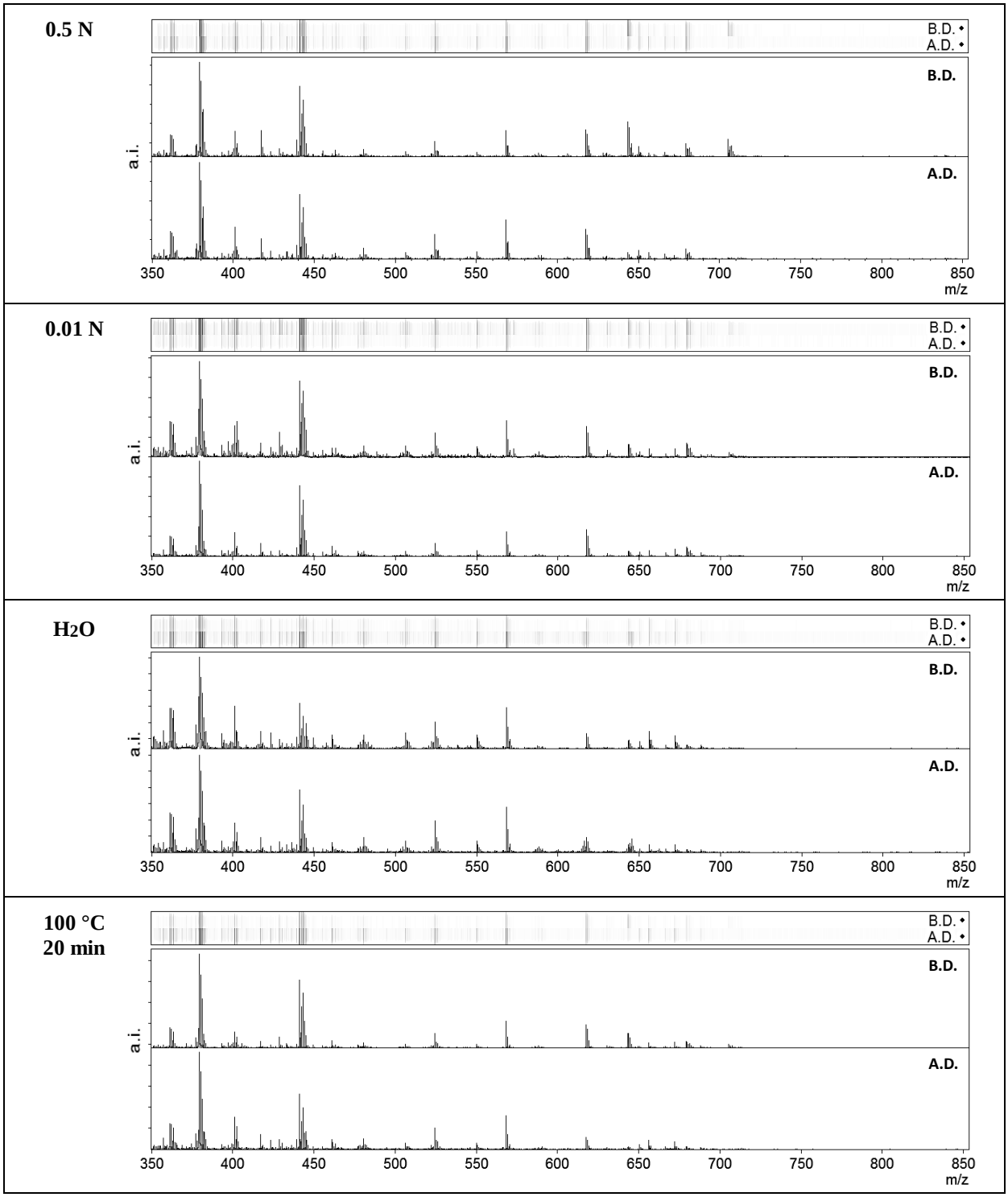

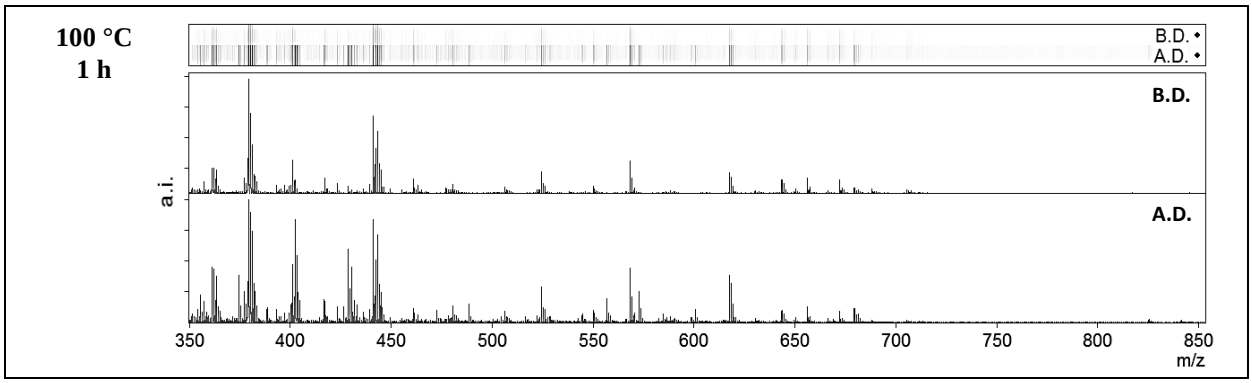
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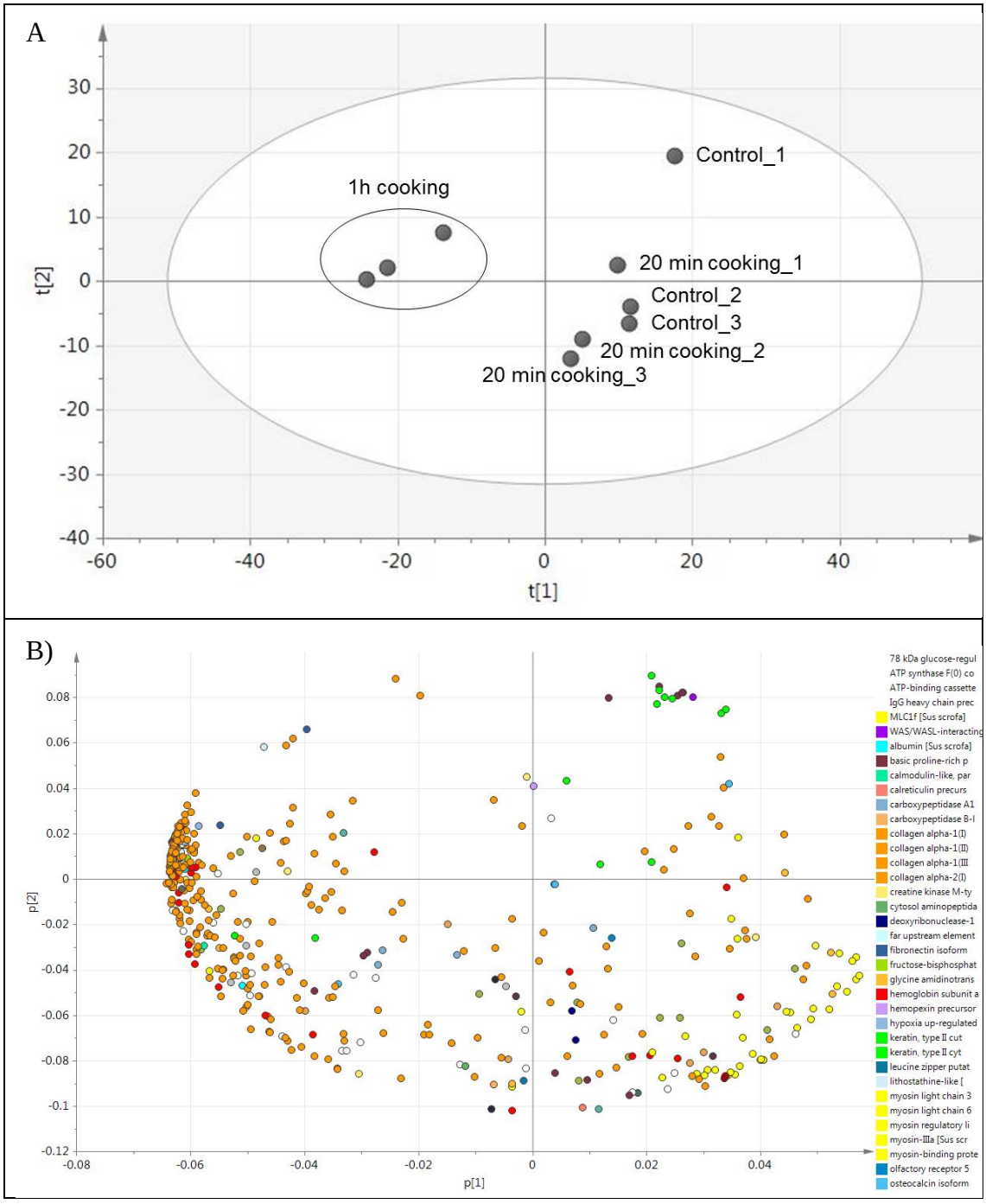
Figure 4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t[1]
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20 min cooking_1
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Control_3
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p[1]
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ATP-binding cassette
IgG heavy chain prec
MLC1f [Sus scrofa]
WAS/WASL-interacting
albumin [Sus scrofa]
basic proline-rich p
calmodulin-like, par
calreticulin precurs
carboxypeptidase A1
carboxypeptidase B-l
collagen alpha-1(I)
collagen alpha-1(II)
collagen alpha-1(III
collagen alpha-2(I)
creatine kinase M-ty
cytosol aminopeptida
deoxyribonuclease-1
far upstream element
fibronectin isoform
fructose-bisphosphat
glycine amidinotrans
hemoglobin subunit a
hemopexin precursor
hypoxia up-regulated
keratin, type II cut
keratin, type II cyt
leucine zipper putat
lithostathine-like [
myosin light chain 3
myosin light chain 6
myosin regulatory li
myosin-IIIa [Sus scr
myosin-binding prote
olfactory receptor 5
osteocalcin isoform

595 Figure 5

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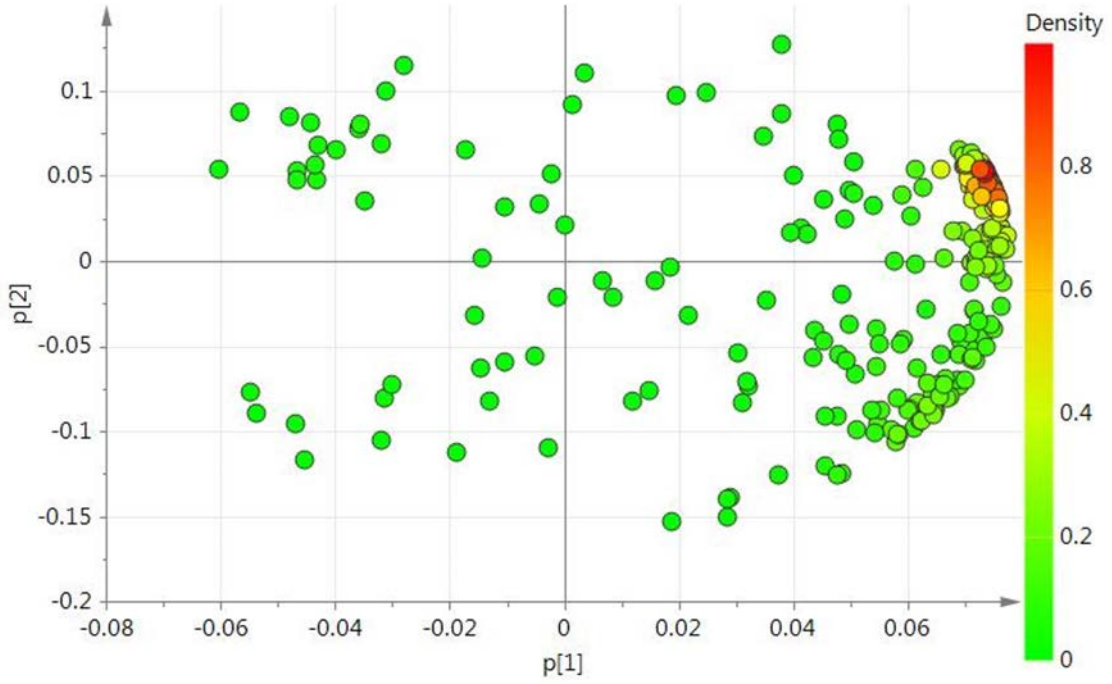

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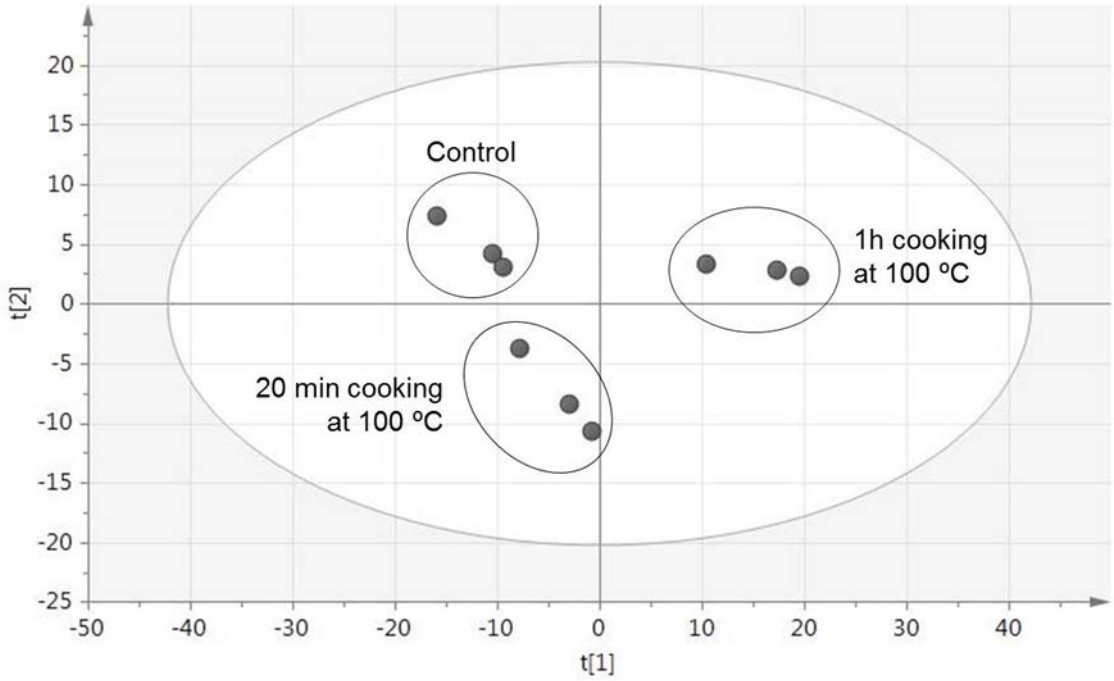

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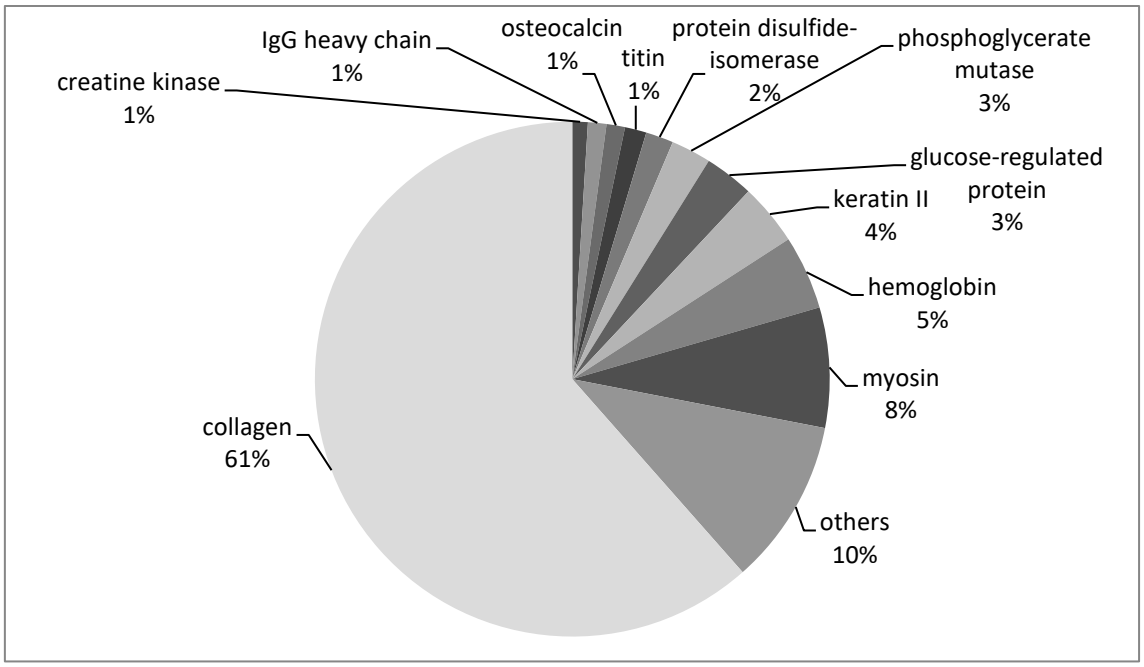
Figure 6


IgG heavy chain
1%
osteocalcin
1%
titin
1%
protein disulfide-
isomerase
2%
phosphoglycerate
mutase
3%
creatine kinase
1%
glucose-regulated
protein
3%
keratin II
4%
hemoglobin
5%
myosin
8%
collagen
61%
others
10%