1 **Effect of cooking and *in vitro* digestion on the peptide profile of**
2 **chicken breast muscle and antioxidant and alcohol dehydrogenase**
3 **stabilizing activity**

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Abstract:

The present study aimed to investigate the effect of cooking and simulated gastrointestinal digestion on the antioxidant and alcohol dehydrogenase (ADH) stabilizing activity of peptides extracted from chicken breast muscle. Results showed that cooking would not affect peptide bioactivity, whereas further digestion using gastrointestinal enzymes could lead to significant changes, producing an increase in ORAC (112.5 to 682.0 $\mu\text{M TE/g}$) and ABTS radical scavenging activities (164.0 to 848.9 $\mu\text{M TE/g}$), whereas a decrease in DPPH radical scavenging (from 36.1% to 4.4%), ferric-reducing power (OD 700 from 0.50 to 0.15) and ADH stabilizing activities (from 44.1% to 20.5%) was observed. The peptidomic analysis resulted in the identification and relative quantitation of 777 peptides from 76 different parent proteins and evidenced that peptides derived from titin and collagen were mainly responsible for the differences detected in the peptide profile. The decrease of DPPH radical scavenging, ferric reducing power, and ADH stabilizing activity may result from release of inactive peptides containing oxidized residues, mainly from collagen, leading to the dilution of active sequences. Results obtained indicated the negative effect of protein and peptides oxidation on the bioactivity of chicken peptides. Nevertheless, further work would be needed to confirm the peptide sequences responsible for the bioactivity.

Keywords: bioactive peptides, heat treatment, gastrointestinal digestion, mass spectrometry, meat protein

1. Introduction

Bioactive peptides have attracted extensive attention worldwide due to not only their health promoting potential, but also their natural and safe properties (Mora, Gallego & Toldrá, 2018; Sultan, Huma, Butt, Aleem & Abbas, 2018; Toldrá, Reig, Aristoy & Mora, 2018). In recent years, peptides derived from food protein sources have been massively studied as functional food ingredients driving by the increasing demand for improving human health and wellness through daily diet supplement (Chalamaiah, Yu & Wu, 2018; Kim & Wijesekara, 2010; Zhang et al., 2018). To date, peptides derived from various food proteins have been reported to exhibit positive effect on the prevention of diseases and modulation of physiological functions, including antioxidant, anti-hypertension, anti-cancer, anti-gout, anti-diabetes, anti-inflammation, alcohol hydrogenase (ADH) activating and/or stabilizing, hepatoprotection, cardioprotection, osteoprotection, immunomodulation, antimicrobial and antiviral activity etc. (Chalamaiah, Yu & Wu, 2018; Mora, Gallego & Toldrá, 2018; Pan et al., 2018; Todorov, Wachsman, Knoetze, Meincken & Dicks, 2005; Yu, Li, He, Huang & Zhang, 2013). Bioactive peptides are inactive within protein sequence but once released through fermentation, enzymatic hydrolysis, or gastrointestinal digestion, they can exert their activity in the target organ. Enzymatic hydrolysis is still the most commonly used method for peptide preparation due to the controllable and reproducible process in addition to high production efficiency, regardless the introduction of salt when adjusting pH, which may be difficult to remove (Wang, Yu, Xing & Li, 2017). Also, peptides are more absorbable and

bioavailable than proteins (Chalamaiah, Yu & Wu, 2018).

Chicken meat is well accepted worldwide as an important source of high quality dietary protein. Chicken breast contains over 90% of crude proteins (dry basis), which are rich in essential amino acids, offering a well-balanced amino acids composition and are considered a good source of bioactive peptides (Cui, Zhou, Zhao & Yang, 2009; Murphy, Marks & Marcy, 1998; Sun, Pan, Guo & Li, 2012). Previous studies indicated that Alcalase digestion of chicken breast muscle could generate great amounts of bioactive peptides (alcohol dehydrogenase stabilizing and antioxidant activity) that benefit to the prevention and amelioration of alcohol-induced liver injury (Xiao et al., 2019; Xiao, Zhou, Zhao, Su & Sun, 2018). In addition, chicken liver hydrolysate could also enhance the catalytic activity of hepatic alcohol metabolic enzyme (ADH and ALDH) and antioxidative enzymes (SOD, CAT and GPx) (Lin et al., 2017). However, bioactive peptides could also be generated during the transportation and storage of chicken breast under the action of endogenous peptidases (Mora, Bolumar, Heres & Toldrá, 2017; Toldrá, 2006). Moreover, pre-treatment before meat consumption such as cooking, fermentation, and curing, may also affect the action of endogenous enzyme and subsequently result in the generation of bioactive peptides (Sayd, Chambon & Santé-Lhoutellier, 2016). For instance, protein and peptides oxidation could happen during the cooking of chicken breast muscle, resulting in the modification of structural properties and their bioactivity (Serpen, Gökmen & Fogliano, 2012).

On the other hand, ingested peptides should be able to circulate intact through the

gastrointestinal tract in order to be absorbed, reach the blood stream, and exert their biological function in target organs. Simulated digestion using gastrointestinal enzymes is generally applied to confirm the biological accessibility and availability of bioactive peptides after ingestion. However, its effect on bioactivity must also be considered because peptides are easily degraded due to the action of endogenous gastrointestinal enzymes (Escudero, Sentandreu, Arihara & Toldrá, 2010; Mora, Bolumar, Heres & Toldrá, 2017).

The main purposes of this study were to evaluate the changes occurred in chicken breast extracted peptides as a consequence of cooking and simulated gastrointestinal digestion, and the effect of these factors on alcohol dehydrogenase stabilizing capacity and antioxidant activity of peptides. Peptides were identified using in tandem mass spectrometry and analyzed using a label-free quantitation approach.

2. Materials and methods

2.1 Materials

Chicken breast was obtained from a local market 24 h after slathering (Valencia, Spain). Enzymes for *in vitro* digestion: salivary α -amylase, porcine pepsin, porcine pancreatic α -amylase, porcine pancreatic lipase, and porcine bile extract were obtained from Sigma Aldrich, Co. Ltd (St. Louis, MO, USA), whereas trypsin and chymotrypsin were from Fluka (Sigma-Aldrich, Co., St. Louis, MO, USA). 2,2'-Diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide, ferric chloride, trichloroacetic acid (TCA), 2,6-di-tertbutyl-4-methylphenol (BHT, B1378),

2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), fluorescein, 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), alcohol dehydrogenase (from *Saccharomyces cerevisiae*), and β-nicotinamide adenine dinucleotide (NAD⁺) were purchased from Sigma Aldrich, Co. Ltd (St. Louis, MO, USA). Acetonitrile, hydrochloric acid 37%, and trifluoroacetic acid (TFA) were of HPLC grade from Scharlab (Barcelona, Spain). All other chemicals and reagents were of analytical grade and obtained from Scharlab (Barcelona, Spain).

2.2 Preparation of chicken breast muscle bioactive peptides

2.2.1 Cooking

Cooking was carried out according to the method described by Mora et al. (2017). As indicated in Fig. 1, one third of the chicken breast was preserved as control, while the rest was placed in a vacuum bag and cooked for 30 min in a water bath until the internal temperature of the chicken breast reached 70 °C. This temperature is used in traditional cooked ham production to assure the quality and safety characteristics of the product. The temperature was monitored using a hand held digital thermometer. After cooking, the chicken breast was cooled to 25 °C in an ice bath (0 °C) and stored at -18 °C for further peptide extraction and hydrolysis.

2.2.2 Simulated gastrointestinal digestion

The digestion was carried out using a two-stage assay in the digestor (Carousel 6 Plus Reaction Station, Radleys, UK) adapted from the standard consensus protocol for static simulated digestion released by Infogest (Minekus et al., 2014). In this assay,

oral phase digestion was not performed because chicken breast contains over 90% of crude protein (in dry basis) and the duration of chicken meat in oral cavity during chewing is short. The effect of salivary amylase is limited but can influence further identification of peptide sequences. Chicken breast was cut into small pieces and minced before used. Firstly, 5 g of chicken breast sample was weighed and suspended in 10 mL of 0.01 N HCl at pH 3. The gastric phase was started by adding porcine pepsin (2000 U mL⁻¹), followed by 15 µL of 50 mM CaCl₂. After 2 h of digestion at 37 °C and constant stirring, the enzyme was inactivated by adjusting pH to 7.0 with 1 M NaOH. Then the intestinal phase was initiated by adding an enzyme mixture of 100 U mL⁻¹ of trypsin, 25 U mL⁻¹ of chymotrypsin, 200 U mL⁻¹ of porcine pancreatic α-amylase, 2000 U mL⁻¹ of porcine pancreatic lipase and 10 mM of porcine bile extract, followed by 70 µL of 50 mM CaCl₂. Samples were incubated for 3 h at 37 °C and the digestion was completed by heating in a water bath at 95 °C for 2 min.

2.2.3 Peptide extraction and deproteinization

Control and cooked chicken breast samples were minced and 30 g of each sample was homogenized with 120 mL of 0.01 N HCl (IUL Instrument homogenizer, Barcelona, Spain) for 8 min under cold conditions to extract peptides.

After peptide extraction or simulated digestion, the mixtures were centrifuged at 12 000 g and 4 °C for 20 min. The supernatants were collected and 3 volumes of ethanol (1 : 3) were added and the mixtures were store at 4 °C for 20 h to remove the proteins. Subsequently, the samples were centrifuged at 12 000 g and 4 °C for 20 min. The supernatant was filtered with Nylon filters of 0.45 µm and ethanol was

evaporated using a rotatory evaporator. Finally, the samples were lyophilised and stored at -18 °C for further analysis.

2.3 Determination of antioxidant activity

The antioxidant activity was determined in triplicate for all the assays. For that, stock solutions of 500 mg/mL (on basis of lyophilized powder) in bidistilled water were prepared for all the samples.

2.3.1 Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was performed as described by Dávalos et al. (2004) with some modifications. Briefly, 140 µL of sample was mixed with 70 µL of fluorescein (200 nM) in a microplate and pre-incubated at 37 °C for 15 min. Then, 70 µL of AAPH (80 mM) was added and the fluorescence was measured exactly every min for 100 min using a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, MA, USA). The excitation and emission wavelengths were 485 and 538 nm, respectively. Trolox was used as a positive control and the ORAC values were expressed as µM TE (Trolox equivalents)/mg of sample.

2.3.2. ABTS radical scavenging capacity

The ABTS assay was carried out according to Gallego et al. (2017). Briefly, 7 mM ABTS stock was prepared in 2.45 mM potassium persulfate and stored in the dark at room temperature for 12–16 h to produce ABTS⁺•. The ABTS⁺• 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. Then, 10 µL of sample was mixed with 990 µL of ABTS⁺• solution, measuring the absorbance at 734 nm after 6 min of incubation in dark.

Trolox was used as positive control, while PBS as negative control. Results were expressed as $\mu\text{M TE/mg}$ of sample.

2.3.3. DPPH radical scavenging activity

DPPH scavenging activity analysis was carried out according to Gallego et al. (2017). Briefly, 100 μL of sample was mixed with 500 μL of ethanol and 125 μL DPPH solution (2%, w/v in ethanol). The mixture was kept for 60 min in the dark before the measuring of absorbance at 517 nm. Distilled water and BHT were used as negative and positive control, respectively. Results were expressed as DPPH scavenging activity, calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = (\text{Absorbance control} - \text{Absorbance sample}) \times 100 / \text{Absorbance control}.$$

2.3.4. Ferric-reducing antioxidant power

The ferric-reducing antioxidant power (FRAP) was measured conducted according to Mora et al. (2017). Briefly, 100 μL of sample was mixed with 250 μL of potassium phosphate buffer (0.2 M, pH 6.6) and 250 μL of potassium ferricyanide (1% w/v) and incubated at 50 °C for 30 min. Then, 250 μL of 10% TCA was added, mixed, and centrifuged at 12 000 g for 10 min. After that, 250 μL of the supernatant were taken and 250 μL of distilled water and 50 μL of 0.1% (w/v) ferrous chloride were added. Absorbance was measured at 700 nm after 10 min. Results were expressed as absorbance values at 700 nm.

2.4. ADH stabilizing activity assay

The ADH stabilizing activity assay was carried according to our previous study

(Xiao, Zhao, Zhou, Gallego, Gao, Toldrá & Mora, 2019). In brief, peptide samples (0.1 mg/mL in bidistilled water, 50 μ L) were pre-incubated with the same volume of ADH (0.2 U/mL dissolved in 10 mM PBS, pH 7.4) at 37 °C for 60 min before the detection of enzyme activity. ADH activity was determined by adding 150 μ L of preincubated reaction reagent (containing 22.4 mM sodium pyrophosphate buffer, 3.3% ethanol and 7.8 mM NAD⁺). The resultant β -nicotinamide adenine dinucleotide hydrate (NADH) was recorded at 340 nm every 10 s for 10 min under 37 °C incubation using a CLARIOstar microplate reader (BMG LABTECH, Ortenberg, Germany). The reaction curve was fitted and the first derivative of the fitting curve at 0 min was calculated as the initial reaction rate. The original ADH activity was considered 100%, bi-distilled water instead of peptides was used as a negative control. Relative activity was calculated according to the following formula:

$$\text{Relative activity (\%)} = V_s / V_0 \times 100\% \quad (1)$$

Here, V_0 was the original ADH activity, while V_s was that of the negative control or tested samples. All the determinations were carried out in triplicate.

2.5. Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis

An Agilent 1100 HPLC system (Agilent Tech., California, USA) with a Symmetry C18 column (250 $\times$ 4.6 mm, 5 μ m; Waters Co. Milford, MA, USA) were used to analyze the differences in peptide profiles after different treatments. The mobile phases consisted of solvent A: 0.1% TFA in milli Q water (v/v) and solvent B: 0.085% TFA in 60% acetonitrile (milli Q water:ACN; v/v). Samples (50 mg/mL $\times$ 20

μL) were injected and separated under a flow rate of 1 mL/min using the following gradient: 0% solvent B for the first 5 min, and linearly increase to 50% solvent B in 25 min. Peptide fractions were monitored at 214 nm.

2.6. Identification of peptides by nLC–MS/MS

Peptides were identified using a nano-liquid chromatography tandem mass spectrometry system (nLC-MS/MS, AB Sciex Instruments, MA, USA), according to Gallego et al. (Gallego, Mora, Aristoy & Toldrá, 2015a). Briefly, samples (5 μL, 0.3 mg/mL) was loaded onto an Eksigen trap column (3μm C18-CL, 350 μm × 0.5 mm) for 5 min before injected into the analytical column (3μm C18-CL, 75 μm × 123 mm; Nikkyo Technos Co, Ltd. Japan). The mobile phases consisted of solvent A: 0.1% v/v formic acid (FA) in water and solvent B: 0.1% FA in acetonitrile (ACN). Peptides were separated linearly from 5% to 35% solvent B over the first 90 min, and then from 35% to 65% solvent B for 10 min under a flow rate of 0.3 μL/min at 30 °C. The flow was ionized applying 2.8 kV. Q-ToF was operated in positive polarity and information-dependent acquisition mode. MS1 scan was acquired from 100 to 1200 m/z for 250 ms, while MS2 scan was required from 100 to 1500 m/z for 50 ms on 50 of the most intense ions charging from 1 to 5.

2.7. Label free quantitation of the identified peptides

Relative quantitation was performed using a label-free method according to Gallego et al. (2015a). The quantitation of peptide is based on the relative integrate area of extracted ion chromatogram (XIC) in each sample. For that, quantitative data was obtained using Peak View 1.1 software (AB Sciex, Framingham, MA, USA) and

then analysed using Marker View 1.3 software (AB Sciex, Framingham, MA, USA). No multiple corrections are performed. Only peptides with confidence of 95% or greater were quantified. The area of extracted ion chromatograms (XICs) for each peptide was obtained from the median according to the predefined algorithm in Peak View 1.1 software. Each sample was quantified in triplicate.

2.8. Statistical analysis

Principal Component Analysis (PCA) and loading plot analysis were performed using SIMCA P+ 13.0 (Umetrics AB, Sweden) software. Statistical analysis was performed using an SPSS package (SPSS 13.0 for Windows, SPSS Inc, Chicago, IL). The homogeneity of the data was tested before mean comparison applying the one-way analysis of variance (ANOVA) following Duncan's test. Difference of $p < 0.05$ was considered statistically significant. Standard deviation (SD) was shown as error bars on the figures.

3. Results and discussion

3.1. Effect of cooking and simulated gastrointestinal digestion on the antioxidant activity of chicken breast

It is recommended to use different assays to evaluate the antioxidant activity of a sample, since no single standard method can characterize its overall antioxidant capacity (Gallego, Mora, Reig & Toldrá, 2018). Among the most commonly used assays, some are based on hydrogen atom transfer mechanism (HAT, i.e. oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and β -carotene bleaching assay), while the others are based on electron

transfer (ET, i.e. DPPH radical scavenging, FRAP, and ABTS radical scavenging assays) (MacDonald-Wicks, Wood & Garg, 2006).

In this article, four different antioxidant assays were applied, including ORAC, ABTS, DPPH, FRAP assays. As can be seen in Fig. 2, non-significant differences were observed between control and the cooked samples on all the assays except the FRAP assay, in which OD 700 increased from 0.43 to 0.50 after cooking (Fig 2B). These results are in agreement with a previous study describing that cooking does not significantly influence the antioxidant and antihypertensive activity of beef extract (Mora, Bolumar, Heres & Toldrá, 2017). The obtained results could be due to the stability of chicken peptides towards heat treatment. However, the increase in ferric reducing power assay indicated that some positive changes occurred on peptides during cooking. Previous studies indicated that cooking can trigger the generation of reactive oxygen species (ROS), resulting in the oxidative modifications in proteins and peptides, and masking the antioxidant capacity of peptides (Mora, Bolumar, Heres & Toldrá, 2017; Soladoye, Juárez, Aalhus, Shand & Estévez, 2015). As an example, active free thiol group in Cys and Met are very sensitive to ROS and may be oxidized to disulfide, sulfenic acid, sulfinic acid, methionine sulfoxide, and methionine sulfone, etc. during cooking under the action of ROS, resulting in the loss of antioxidant activity (Simonetti, Gambacorta & Perna, 2016). However, some researches described an increase in antioxidant activity after thermal process because the denaturation of protein or peptide and generation of peptides with antioxidant activity (Gallego, Mora, Hayes, Reig & Toldrá, 2017; Sayd, Chambon &

Santé-Lhoutellier, 2016). In the present study, results suggested the stability of the obtained peptides during cooking, and the maintenance of antioxidant activity possibly due to the generation of peptides that compensate the loss of activity induced by thermal treatment.

However, after further simulated gastrointestinal digestion, a significant decrease of DPPH radical scavenging rate (from 36.1% to 4.4%) and FRAP (OD 700 from 0.50 to 0.15), and a sharp increase of ORAC (from 112.5 to 682.0 $\mu\text{M TE/g}$) and ABTS radical scavenging activity (from 164.0 to 848.9 $\mu\text{M TE/g}$) were observed. These results further indicated the importance to characterize the antioxidant capacity using different assays. The apparently contradictory results could be due to the fact that the antioxidant activity of peptides is related not only to the peptide size and amino acid composition, but also amino acid sequence, peptide structure and hydrophobicity (or polarity), etc., which also determine the antioxidant mechanism (HAT or ET) (Gallego, Mora, Hayes, Reig & Toldrá, 2017; Zheng, Zhao, Dong, Su & Zhao, 2016). In present study, HAT based assay (ORAC), whereas ET based assays (DPPH radical scavenging and FRAP) decrease except ABTS radical scavenging. Though ABTS radical scavenging was classified as an ET-based assay, the reaction mechanism between $\text{ABTS}^+\bullet$ and peptide is initiated by a labile hydrogen atom, as a result, ABTS radical scavenging assay could also considered a HAT-based method (Gallego, Mauri, Aristoy, Toldrá & Mora, 2020). Furthermore, previous studies reported that Cys, Met, and related peptides were found to display noticeable ferric reducing power and DPPH radical scavenging activity due to the strong reducing functional group of $-\text{SH}$

in Cys, whereas Trp, Tyr and related peptides exhibit strong ORAC and ABTS radical scavenging activity (Zheng, Lin, Su, Zhao & Zhao, 2015; Zheng, Zhao, Dong, Su & Zhao, 2016). In the present study, gastrointestinal digestion simulation was performed using pepsin, chymotrypsin, and trypsin. The primary targets of these enzymes were Phe, Leu, Trp, Tyr, Lys, and Pro cleavage sites (Altinelataman et al., 2019). In this regard, the simulated digestion resulted in the release of Trp and Trp-containing peptides, which subsequently contributed to the increase of ORAC and ABTS radical scavenging activity. The obtained results also indicated that the gastrointestinal digestion is not influencing ferric reducing power and DPPH radical scavenging activities. This fact could be due to that antioxidant peptides are degraded and novel antioxidant sequences are generated to compensate the observed activity or that there is a release of non-antioxidant peptide sequences that results in the dilution of active sequences and the decrease of ferric reducing power and DPPH radical scavenging activities. Similar results were also obtained during the study of antioxidant peptides derived from dry-cured ham (Gallego, Mauri, Aristoy, Toldrá & Mora, 2020).

3.2. Effect of cooking and simulated gastrointestinal digestion on ADH stabilizing activity

Regarding ADH stabilizing activity, non-significant difference was observed after cooking, although simulated gastrointestinal digestion resulted in a significant decrease from 44.1% to 20.5% relative ADH activity (Fig. 3). This result was in line with that of DPPH and ferric-reducing power assay as aforementioned (Fig. 2A and B). Similar result was observed when bovine peptides were subjected to

gastrointestinal digestion (Fu, Young, Dalsgaard & Therkildsen, 2015). It was demonstrated that longer peptide tended to form a more favorable spatial conformation to interact with an enzyme, inducing a conformation change in the enzyme and modifying enzyme activity (Ma, Wu & Li, 2018; Yu, Li, He, Huang & Zhang, 2013). In this research, gastrointestinal enzymes resulted in the degradation of relatively longer peptides, leading to the loss of favorable conformation for the interaction between peptide and ADH. Moreover, a recent study reported that tripeptide KPC stabilizes ADH possibly through binding to the active site of ADH and protecting ADH against oxidative inactivation (Xiao, Zhao, Zhou, Gallego, Gao, Toldrá & Mora, 2019). In this regard, the decrease in ADH stabilizing activity may also related to the release of oxidized sequences during simulated digestion, sharing the similar mechanism as that of the DPPH scavenging and ferric reducing power activity.

3.3. Effect of cooking and gastrointestinal digestion on the peptide fractions

To further understand how cooking and gastrointestinal digestion could influence peptide bioactivity, the samples were analyzed by RP-HPLC, which is one of the most common methods used for peptide separation and analysis (Moayedi et al., 2018). Samples were prepared using lyophilized peptide powder and normalized to the same peptide powder concentration by subtracting the induction of digestive enzymes and salt. As shown in Fig. 4, the profile of the cooked sample was almost overlapped with that of control, indicating that cooking did not lead to significant changes in peptide fractions. This result was in accordance with the maintenance of antioxidant (Fig. 2)

and ADH stabilizing activities (Fig. 3) after cooking. However, further gastrointestinal digestion led to significant changes in fraction intensity. After gastrointestinal digestion, a higher amount of peptides were observed due to newly released sequences from chicken proteins, as well as the degradation of peptides into shorter fragments or amino acids, resulting in a markedly decrease in intensity was found in the fractions eluted at about 3.5-6 min and 10.5-11.5 min, whereas an increase in intensity was found at about 8.5-10.5 min and 11.5-30 min. Similarly, Gallego et al. found that a large amount of small antioxidant peptides (< 450 Da) were generated after the simulated gastrointestinal digestion of dry-cured ham (Gallego, Mora, Hayes, Reig & Toldrá, 2017). As aforementioned, digestive enzymes used in present study tend to firstly release peptides contained Trp, Tyr, and Phe etc. residues. On one hand, such aromatic amino acids residues exhibit strong absorption at around 220 nm, resulting in significant increase in HPLC profile (Fig. 4). On the other hand, Trp, Tyr and related peptides can significantly increase ABTS and ORAC activity (Fig. 2C and D) (Zheng, Dong, Su, Zhao & Zhao, 2016; Zheng et al., 2012).

3.4. Effect of cooking and gastrointestinal digestion on peptide profile

Samples were fully characterized and quantified by nLC-MS/MS to determine the effect of cooking and gastrointestinal digestion on the peptide profile. The relative quantitation of peptides was performed using a label-free peptidomic approach by extracting peak intensity. A total of 777 peptides from 76 parent proteins were identified in control, cooked, and cooked plus digested samples. A Principal Component Analysis (PCA) was applied to evaluate the differences between the

groups of samples, obtaining the information of the most influencing peptides. In Fig. 5A, the PCA showed three statistically different groups, showing that cooking and further GI digestion resulted in significant differences regarding peptide concentration. Principal component 1 (PC 1) explained the 61.1 % of the variability in the dataset, while PC 2 was responsible for the 22.4 % within the dataset, allowing the differentiation between the three groups of samples. Thus, peptides identified in the ‘Cooked and Digested’ group (Fig.5) might be the most important influencing bioactivity. Fig. 5B revealed all the peptides influencing the clustering of data. According to these results, the most abundant peptides responsible for the differences in the peptide profiles were derived from titin (green) and collagen (red) proteins. A similar study demonstrated that peptides from collagen were responsible for the differences in antioxidant activities between uncooked and cooked peptides (Gallego, Mora, Hayes, Reig & Toldrá, 2017). Interestingly, collagen and titin peptides could easily be oxidized during cooking and digestion, especially Met and Cys residues which are the most susceptible to oxidation by all forms of ROS due to the existence of sulfur atoms (Gallego, Mora, Aristoy & Toldrá, 2015b; Zhang, Xiao & Ahn, 2013). Another research found that a large number of peptides from collagen showed the oxidation of Pro residue after cooking and *in vitro* GI digestion of Spanish dry-cured ham (Gallego, Mora, Hayes, Reig & Toldrá, 2017). More recently, Pro residues from collagen were reported to be the main targets during *in vitro* oxidation of chicken breast (Márquez-Lázaro, Mora, Méndez-Cuadro, Rodríguez-Cavallo & Toldrá, 2020). In this regard, the accumulation of oxidized peptides can reduce the antioxidant

capacity in meat peptides (Serpen, Gökmen & Fogliano, 2012).

Hence, all the 46 detected oxidized peptides were subjected to further analysis, and results are shown in Fig. 6. The PCA separated two statistically different groups, with the 'Cooked and Digested' group located in a different cluster (Fig. 6A). PC 1 explained the 94.4 % of the variability in the dataset, which allowed the differentiation between the 'Cooked and Digested' samples from the other. On the other hand, PC 2 was responsible for 2.7 % of the variance within the dataset for the two discriminant components. The loading plot (Fig. 6B) revealed the main peptides responsible for the observed differences, indicated by the higher color density value. As can be seen, the highest color density were represented by 9 peptides, including

MIEM[Oxi]DGTENK	(1167.316	Da),
NGDRGETGPAGPAGPP[Oxi]GPAGA	(1802.85	Da),
GETGPAGP[Oxi]AGPP[Oxi]GPAGA	(1360.424	Da),
GSP[Oxi]GADGPIGAP[Oxi]GTP[Oxi]GPQ	(1532.618	Da),
AGPAGNP[Oxi]GADGQP[Oxi]GAK	(1364.42	Da),
GP[Oxi]SGPQGPPSGAP[Oxi]GPK	(1290.386	Da),
GAP[Oxi]GPHGQVGPSGKP[Oxi]GN	(1513.634	Da),
HGDQGPP[Oxi]GNNGPAGPR	(1527.57	Da), and

GLP[Oxi]GAIGAP[Oxi]GPAGGAGDR (1490.618 Da), indicating the most influencing effect of these peptides on the difference between samples. The complete list of the 46 oxidized peptides and their relative quantitation is shown in Fig 7. A total of 40 among the 46 peptides were originated from chicken collagen, containing

at least one oxidized Pro residue in each sequence. Three peptides were derived from chicken myosin with oxidized Met residue in the sequences, including NLTEEM[Oxi]AVLDETI AKLT (1891.134 Da), VFDKEGNGTV M[Oxi] (1196.340 Da), and KMEGDLNEM[Oxi] (1066.202 Da). Furthermore, almost all the oxidized peptides were observed with increase concentration after digestion, indicating further released of such peptides during digestion. In this regard, cooking leads to protein oxidation in amino acid residues like Met and Pro, and further simulated gastrointestinal digestion resulted in the release of oxidized peptides that are inactive, diluting the concentration of active sequences, and subsequently decreasing activity in ferric reducing power, DPPH radical scavenging and ADH stabilizing activity, while the release of Trp and Tyr containing peptides contribute to the increase in ORAC and ABTS radical scavenging assays.

4. Conclusion

This study reports the effect of cooking and simulated gastrointestinal digestion on the generation of bioactive peptides in chicken breast and the influence on peptide bioactivity. The obtained results showed that cooking did not result in significant changes in the bioactivity detected, whereas gastrointestinal digestion could lead to an increase in ABTS and ORAC activity, but a decrease of DPPH, ferric reducing power, and ADH stabilizing activity. The change in bioactivity may result mainly from peptides identified from titin and collagen, as well as from the newly generated peptides during gastrointestinal digestion. Protein oxidation occurred during cooking and the oxidized sequences were released during further digestion, resulting in the

change of bioactivity. Results obtained further illustrated the promising potential to use bioactive chicken peptides as functional food ingredient, however, more work would be needed to synthesize and validate the antioxidant and ADH stabilizing activity of these specific peptide sequences that may be responsible for the bioactivity of chicken breast.

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FIGURE CAPTIONS

Fig. 1 Scheme of chicken breast samples indicating different processing and analysis methodologies.

Fig. 2 Antioxidant activity of chicken breast peptides using different processing treatments. A: DPPH radical scavenging, B: ferric reducing power, C: ORAC and D: ABTS radical scavenging assays. The values represent means of triplicates and bars represent standard deviations. Bars with different letters are significantly different at $p < 0.05$.

Fig. 3 ADH stabilizing activity of chicken breast peptides after different processing treatments. The values represent means of triplicates and bars represent standard deviations. Bars with different letters are significantly different at $p < 0.05$.

Fig. 4 HPLC profile of chicken breast peptides after different processing treatments: after cooking and after cooking plus simulated GI digestion.

Fig. 5 A: Principal Component Analysis (PCA) score plot of main identified proteins to assess the variance among all the peptides generated after cooking and simulated gastrointestinal digestion of chicken breast in triplicates ($n = 3$). Principal component 1 (PC 1) explained the 61.1 % of the variability in the dataset while PC 2 was responsible for the 22.4 % of variance within the dataset, allowing the differentiation between the three groups of samples. B: Loading plot showing the parent protein of peptides affecting score plot distribution obtained in the Score plot after cooking and simulated gastrointestinal digestion of chicken breast.

Fig. 6 A: Principal Component Analysis (PCA) score plot of oxidized protein to

630 assess the variance among all the oxidized peptides generated after cooking and
631 simulated gastrointestinal digestion of chicken breast. B: Loading plot showing the
632 oxidized peptides affecting the score plot distribution. Principal component 1 (PC 1)
633 explained 94.4 % of the variability in the dataset, while PC 2 was responsible for 2.7 %
634 of variance, allowing the differentiation between cooked and digested samples.
635 Fig. 7 Heat map showing the relative intensity of the oxidized peptides under different
636 processing treatments.

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