***In vitro* oxidation promoted by chlorpyrifos residues on myosin and chicken breast proteins**

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Abstract

Organophosphate pesticides are frequently used to eliminate or prevent insects in poultry. However, their residues may continue in meat after slaughtering. In this study, proteomics and peptidomics approaches were used to evaluate their oxidative impact on myosin and chicken breast proteins under *in vitro* conditions. Myosin protein was exposed to diazinon and chlorpyrifos showing an increase in its oxidation by increasing times, especially with chlorpyrifos.

Then, chicken breast was contaminated with chlorpyrifos to evaluate carbonylation and the effect of simulated gastrointestinal digestion. Proteins were isolated using size-exclusion-chromatography and identified by mass spectrometry in tandem. Myosin, β -enolase, CK-M-type and actin were identified as main proteins susceptible to oxidation. Also, oxidised peptides obtained before and after simulated gastrointestinal digestion were identified. Collagen peptides the most susceptible to oxidation. These results suggest that the presence of chlorpyrifos residues on meat could have a negative effect on its final quality and nutritional value.

Keywords: *Chicken breast, chlorpyrifos, carbonylation, peptidomics*

Chemical compounds studied in this article

Chlorpyrifos (PubChem CID: 2730); Diazinon (PubChem CID: 3017).

1. Introduction

In recent years, the global consumption of chicken meat has been increasing (FAO, 2018). The high demand is associated to its low cost, healthier profile and non-interference with religious or cultural beliefs as occurs with beef and pork meat (Özünlü, Ergezer, & Gökçe, 2018). Moreover, chicken meat is a rich source of proteins, with a high content in essential amino acids, necessary for the human metabolic processes, as well as vitamins and minerals (Estévez, 2015; Pereira & Vicente, 2013).

However, chicken meat proteins are more susceptible to undergo irreversible oxidation such as carbonylation. This fact can occur directly by incorporation of a carbonyl group on lysine, threonine, arginine or proline residues; or indirectly by reaction with end products of sugar and lipid oxidation (Márquez-Lázaro, 2020; Estévez, 2015; Estévez, 2011). The impacts of carbonylation in meat have been associated to a reduction of its quality traits such as texture, flavor, tenderness, color and nutritional value (Estévez, 2011).

External factors related to animal husbandry, slaughter, processing and storage conditions have shown to have an important role in the promotion of oxidative stress in meat (Ali et al., 2015; Li et al., 2016; Silva et al., 2018; Soyer, Özalp, Dalmış, & Bilgin, 2010). In this context, the use of organophosphate pesticides (POPs) such as diazinon and chlorpyrifos in poultry (to prevent or eliminate insects as well as preserving agents in poultry feed) could be considered a potential oxidative stress factor on meat proteins as these pesticides can be accumulated in the animal tissue when they are used at high concentrations or during a

prolonged time (Chawla, Kaushik, Shiva Swaraj, & Kumar, 2018; Han, Sapozhnikova, & Lehotay, 2016; Mahugija, Chibura, & Lugwisha, 2018). Several studies using different biological models show that pesticides can promote oxidative stress at trace concentrations (Aly, EL-Gendy, Mahmoud, & El-Sebae, 2010; Ojha & Srivastava, 2014; Shah & Iqbal, 2010; Zhang et al., 2019). In addition, these substances have been related to toxicological effects as endocrine disruption, carcinogenicity, genotoxicity, and neurological disorders (Chawla et al., 2018).

In order to prevent the toxicological effects associated to the presence of pesticide residues on consumer, international authorities such as European Union (EU) have established maximum residue levels (MRLs) in several products of animal origin. The MRLs values indicate the maximum concentration that can be present in food and not cause a toxic effect on the consumer (Villaverde, Sevilla-Morán, López-Goti, Alonso-Prados, & Sandín-España, 2016). However, it is still unknown if these MRLs concentrations might promote oxidative stress in muscle proteins from chicken and affect its meat quality properties. This is a matter of concern for scientists and the focus of several researches nowadays as developed and developing countries are using increasing amounts of pesticides for agricultural purpose, which can potentially increase the presence of their residues in products of animal origin (Chawla et al., 2018).

Thus, the objective of this study was to evaluate the *in vitro* oxidative effect of two organophosphate pesticides in myosin standard protein and the effect of chlorpyrifos on chicken muscle proteins and peptides after a simulated gastrointestinal (GI) digestion. For this, myosin protein standard and chicken breast proteins were exposed to pesticides at concentrations around MRLs and their oxidative effects were determined using the

carbonylation as a biological marker. Next, the identification of oxidized peptides after simulated gastrointestinal (GI) digestion was performed by mass spectrometry in tandem.

2. Materials and methods

A scheme including the developed experimental design and the analysis carried out in this study is described in Supplementary material Figure 1.

2.1 Chemicals and reagents

Chlorpyrifos (CPF), diazinon (DZN), and trifluoroacetic acid were purchased from Sigma-Aldrich, Co (St. Louis, MO, USA). Methanol, ethanol, and phosphoric acid were purchased from Scharlab, S.L (Barcelona, Spain). Sodium hydroxide, Tris, urea, thiourea, calcium chloride anhydrous, and chloride acid were from Panreac Quimica S.A (Barcelona, Spain) and iron sulphate was from BDH laboratory (Kuwait, Kuwait). Myosin, aldolase, bovine serum albumin, ovalbumin, myoglobin, cytidine, salivary α -amylase, porcine pepsin, porcine pancreatic α -amylase, porcine pancreatic lipase, and porcine bile extract used in the simulation of GI digestion were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Trypsin and chymotrypsin enzymes were from Fluka (Sigma-Aldrich, Co., St. Louis, MO, USA). The trypsin used in mass spectrometry assays was obtained from Promega, Co (Madrid, Spain).

2.2 Preparation of pesticides solutions

Pesticides individual stock solutions were prepared at a concentration of $100\text{ }\mu\text{g.mL}^{-1}$ in methanol. These solutions were stored at $4\text{ }^{\circ}\text{C}$ in the dark for not longer than one month. To avoid protein precipitation, serial dilutions were prepared with milli-Q water to obtain a working solutions of $10\text{ }\mu\text{g.mL}^{-1}$. Different dilutions were prepared from the working

solutions to obtain final concentrations of 100 and 20 $\mu\text{g.Kg}^{-1}$ for CPF and DZN, respectively, in meat samples (w/w). The final content of solvents in meat sample were less than 1%.

2.3 Myosin exposition to organophosphate pesticide residues

POPs residues are usually associated with acetylcholinesterase inhibition, but these have shown to produce oxidative stress on different biological models even at trace concentrations (Shah & Iqbal, 2010; Wang, Shen, Zhou, & Jin, 2019). Nevertheless, their oxidative effect in chicken meat is still unknown.

Thus, the oxidative capacity of CPF and DZN was initially evaluated on myosin protein standard assaying different exposition times and using carbonylation as control parameter. Myosin is a muscle protein susceptible to suffer oxidation (Ooizumi & Xiong, 2004), moreover is one of the major proteins in the muscle tissue and thus can act as a potential model before testing chicken muscle.

For this, a solution of myosin protein standard prepared at 200 $\mu\text{g.Kg}^{-1}$ in milli-Q water was contaminated individually with CPF and DZN at 100 and 20 $\mu\text{g.Kg}^{-1}$, respectively. Then, samples were incubated for 60, 90, 120, 180, 240 and 300 minutes at room temperature and darkness. At the end of each exposure time, carbonylation was measured using the 2,4 DNPH assay. Each experiment was performed in duplicate. At the end of the experiment, the organophosphate pesticide and time of incubation that induced the highest carbonylation in myosin were selected for the *in vitro* contamination of chicken breast. Myosin with FeSO_4 (100 $\mu\text{g.Kg}^{-1}$) and myosin in milli-Q water were used as positive and negative control, respectively.

2.4 Sample preparation and contamination

Chicken breast (*Pectoralis muscle*) was purchased at a local market in Valencia, Spain. In the laboratory, chicken breast was cleaned to remove visible adipose and connective tissue. Then, it was cut into small pieces and homogenized using a blender. Two and five grams of homogenized were weighed for the extraction of total proteins and the *in vitro* GI digestion assay, respectively. Then, samples were contaminated individually with CPF at 100 $\mu\text{g.Kg}^{-1}$ final concentration, followed by vortex for 30 seconds and incubated for 1 hour at room temperature and darkness. Samples without CPF were used as negative control. In order to minimize oxidation promoted by oxygen, nitrogen gas (N_2) was placed on each sample tube for the incubation.

2.5 Extraction of total proteins

10 mL of Tris-HCl 50 mM with urea 6 M, and thiourea 1M at pH 8 were added to each sample (2 grams), mixing with a vortex during five minutes. The homogenate was centrifuged at 4 °C and 10,000 rpm for 20 minutes and supernatants containing sarcoplasmic and myofibrillar proteins was collected. Protein concentration was determined using Bradford assay from Bio-Rad protein assay according to manufacturer's instructions (Bio-Rad Laboratories, USA), using bovine serum albumin (BSA) as protein standard. Protein concentration was expressed as mg protein mL^{-1} . Each experiment was performed in triplicate.

2.6 Protein carbonyl content

Protein carbonyls were measured by estimation of total carbonyl groups according to Mesquita et al., (2014) with some modifications. Briefly, 80 μL of DNPH (10mM in H_3PO_4

0.5M) was added to 80 μ L of protein solution and incubated during 10 minutes in the dark at 25°C. Then, 40 μ L of NaOH 6M were added. After incubation during 10 minutes at 25°C in dark, absorbance was read at 450 nm. Carbonyl content was calculated using molar extinction coefficient of DNPH corrected for microplates ($\epsilon=11154 \mu\text{M}^{-1} \text{cm}^{-1}$). Results were expressed as carbonyl index (nmol per mg protein). Due to the instability of dinitrophenylhydrazones in alkaline medium, incubation time after NaOH addition was rigorously controlled.

2.7 Size-exclusion chromatography and trypsin digestion

For the fractionation of total proteins according to molecular mass, an ÄKTA Start system (GE Healthcare GmbH, Germany) was used. The column employed was a HiPrep 16/60 Sephacryl S-100 High Resolution (GE Healthcare GmbH, Germany). The molecular mass range for this column is 1–100 KDa. As mobile phase, 50mM phosphate buffer 0.15M NaCl, at pH 7.0 was used. Chromatographic conditions were at a flowrate of $0.5 \text{ mL} \cdot \text{min}^{-1}$ and injection sample volume was 2.5 mL. Fractions of 5 mL were collected each 10 minutes using an automatic fraction collector (Model 2110, Biorad, USA). Before separation, the samples and mobile phases were filtered using $0.45 \mu\text{m}$ nylon membrane filters (Teknokroma, Spain). The fractions collected were lyophilized and pooled each six continuous fractions (30 mL). Protein content of the pooled fractions was measured by BCA assay and their carbonyl content by 2,4-DNPH alkaline method. Previous to the separation of proteins, a calibration curve using aldolase (158 KDa), bovine albumin serum (66.5 KDa), ovalbumin (44 KDa), myoglobin (16.7 KDa) and cytidine (≤ 1 KDa) standards was done.

The identification of the proteins collected during the chromatographic separation was done by mass spectrometry in tandem after a trypsin digestion. For this, between 18 to 60 µg of protein were reduced with 1.2 µL of 45 mM DTT at 50°C for 15 minutes and alkylated with 1.2 µL of 50mM IAA at room temperature and darkness. The IAA excess was eliminated by adding 1.2µL of 45mM DTT. Then, proteins were digested using trypsin to ratio 1:50 %w/w (trypsin/protein) and incubated overnight at 37°C. The reaction was stopped with TFA 10% decreasing the pH to 3.0.

2.8 Simulated GI digestion

Contaminated samples and negative control were subjected to *in vitro* digestion according to the methodology described by Minekus et al (2014) with some modifications (Gallego, Mora, Hayes, Reig, & Toldrá, 2017). All process was done in a digester (Carousel 6 Plus Reaction Station, Radleys, UK). For the gastric phase, samples were resuspended in 10 mL of HCl 0.01 N, pH 3.0. Then, porcine pepsin was added to achieve a final concentration of 2,000 U.mL⁻¹ followed by 15 µL of CaCl₂ 50 mM. After 2 hours of digestion at 37 °C and constant stirring, the enzyme was inactivated by adjusting pH to 7.0 with NaOH 1 M. The intestinal phase was simulated by adding to the mixture 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. A total of 70 µL of CaCl₂ 50 mM was also added and the sample was incubated during 2 hours at 37 °C. The intestinal digestion was finished by heating during 2 minutes at 95 °C. The mixture was deproteinised by adding 3 volumes of ethanol and keeping the sample at 4 °C for 20 hours. Then, the sample was centrifuged at 12,000 rpm and 4 °C for 10 minutes. Finally, the supernatant was dried

in a rotatory evaporator and lyophilized for mass spectrometry in tandem assay. Each experiment was performed by duplicate.

The pellet (undigested proteins) was dissolved in NH_4HCO_3 50 mM with Tris buffer 10mM pH 8.55 and protein content was determined by BCA assay that is based on the traditional Lowry assay (Smith et al., 1985). The undigested protein was subjected to trypsin digestion. For this, 100 μg of undigested protein were reduced with 2 μL of DTT 45 mM at 50°C for 15 minutes and alkylated with 2 μL of IAA 50 mM at room temperature and darkness. The IAA excess was eliminated by adding of 2 μL of DTT 45mM. Finally, the proteins were digested using trypsin in relation 1:50 %w/w (protein/trypsin) and incubated overnight at 37 °C. The reaction was stopped with TFA 10 %until obtained pH 3.0 in proteins digested. The resulting peptides were analyzed by mass spectrometry in tandem.

2.9 Identification by mass spectrometry in tandem

The tryptic proteins and the peptides obtained after simulated GI digestion were analyzed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX). In order to achieve this, samples were resuspended in 50 μL of TFA 0.1% in ACN 2%. 5 μL of every sample was loaded onto a trap column (NanoLC Column, 3 μm , C18-CL, 350 μm x 0.5 mm; Eksigent) and desalted with TFA 0.1% at 3 $\mu\text{L}.\text{min}^{-1}$ during 5 minutes. The peptides were then loaded onto an analytical column (LC Column, 3 μm , C18-CL, 75 μm x 12 cm, Nikkyo) equilibrated in ACN 5% FA 0.1% (formic acid). Elution was carried out with a linear gradient of 5 to 35% B in A for 60 minutes. (A: FA 0.1%; B: ACN, 0.1% FA) at a flow rate of 300 $\text{nL}.\text{min}^{-1}$. Sample was ionized applying 2.8 kV to the spray emitter. Analysis was carried out in DDA mode. Survey MS1 scans were acquired from 350–1250 m/z for 250 ms. The quadrupole resolution was set to ‘UNIT’ for MS2 experiments, which

were acquired 100–1500 m/z for 50 ms in ‘high sensitivity’ mode. The switch criteria that were used in the mass spectrometry analysis were charge from 1+ to 5+, and a minimum intensity of 70 counts per second (cps). Up to 25 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 seconds. The system sensitivity was controlled with 2 fmol of 6 protein standards (LC Packings).

2.10 Data analysis

2.10.1 Statistical analysis

Values are reported as mean $\pm$ SEM of independent determinations. To evaluate the effect of pesticides on carbonylation, data was analyzed by t-test unpaired using the statistical software Prism v.5.01 (GraphPad Software, San Diego, CA, USA).

2.10.2 Data analysis of proteomics and peptidomics data

2.10.2.1. Identification of proteins obtained from the SEC fractions and the non-digested protein fragments obtained after simulated GI digestion: a proteomic approach

ProteinPilot v4.5 search engine (AB Sciex, Framingham, MA, USA) default parameters were used to generate a peak list directly from the 5600 TripleTof instrument wiff files. The Paragon algorithm (Shilov, Seymour, et al., 2007) of Protein Pilot v 4.5 was used to search the Uniprot_Aves (Nov 2018) database with the following parameters: trypsin enzyme specificity, no taxonomy restriction, and the search effort set to through. The posttranslational modifications such as oxidation of methionine and proline amino acids were determined automatically by Paragon algorithm.

2.10.2.2 Identification of generated peptides after simulated GI digestion and relative quantitation using label-free mass spectrometry: a peptidomics approach

The analysis of the obtained spectra was done using ProteinPilot v 4.5. Search engine (ABSciex). ProteinPilot default parameters were used to generate a peak list directly from the 5600 TripleTof wiff files. The Paragon algorithm (Shilov et al., 2007) of ProteinPilot v 4.5 was used to search the Uniprot_Aves (Nov 2018) database using none enzyme as parameter. The posttranslational modifications such as oxidation of methionine and proline amino acids were determined automatically by Paragon algorithm. Samples were grouped by type for the search (CN and CPF). Additionally, all samples were combined in a single search to build the library for quantitation. The protein grouping was done by Pro group algorithm. Peak View 1.1 software (AB Sciex, Framingham, MA, USA) was used to quantify the areas for all the peptides assigned in the library. Only peptides with confidence of 95% or greater were quantified. The areas obtained previously were loaded on Marker View 1.3 software (AB Sciex, Framingham, MA, USA) and data were normalized by total areas sum. Normalized areas were used to extract the information of oxidized peptides, which were used for statistics analysis. Principal Component Analysis (PCA) and loading plot analysis were performed using SIMCA-P+ 13.0 software (Umetrics AB, Sweden).

3. Results and discussion

3.1 Myosin carbonylation induced by organophosphate pesticides

The oxidative power of CPF (100 $\mu\text{g.Kg}^{-1}$), and DZN (20 $\mu\text{g.Kg}^{-1}$) pesticides on standard myosin protein was determined *in vitro* and the results obtained are summarized in Fig. 1. In all assayed times the carbonylation induced by CPF was significantly higher in comparison to negative control ($P<0.05$), showing the maximum oxidative damage at 60 minutes (1.6 fold greater than negative control). Between 90 to 300 minutes the carbonylation promoted by CPF was similar, being these among 1.2 and 1.3 fold higher

than negative control (Fig. 1a). In contrast, the carbonylation produced by DZN was only significantly higher than control at 60 minutes of exposure ($P<0.05$) as observed in Fig. 1b.

Comparing carbonylation induced by both pesticides, CPF was the one that induced the most oxidative damage on myosin at the exposure time evaluated. According literature, this result is not related to chlorpyrifos concentration, because according to the results reported by Giordano et al, equal concentrations of diazinon and chlorpyrifos did not generate the same amount of oxygen reactive species (chlorpyrifos > diazinon) on neural cell (Giordano et al.,2007). In addition, toxicological studies in fish and frogs have showed the higher toxic power of chlorpyrifos compared to diazinon, even at lower concentrations (Sparling & Fellers, 2007; Cao et al.,2018).

Thus, chlorpyrifos was selected to study its oxidative effect in chicken breast proteins and 60 minutes was fixed as maximum exposure time.

3.2 Carbonylation induced by chlorpyrifos on chicken breast proteins

The carbonylation promoted by CPF on chicken breast proteins was quantified by a spectrophotometry assay and its results are showed in Fig. 2. The oxidative damage on exposed and control samples were significantly different ($P<0.05$); being the carbonyl index 2.7-fold higher in samples exposed to CPF than those used as control. These results showed the capacity of CPF to promote oxidative damage on muscle proteins, which are in accordance with the results observed on myosin protein (section 3.1) and other assays performed in biological models such as rat and fish (Altun, Özdemir, & Arslan, 2017; Owumi & Dim, 2019). Although the mechanism that promotes protein carbonylation induced by CPF is still unknown, it could be related to its high lipophilicity (Kim et al.,

2016). This can help its transference through the cell membrane and facilitate its interaction with intracellular components.

On the other hand, the carbonylation on meat proteins has been widely related to external or internal factors such as breeding and slaughter of animals as well as processing, preservation, curing, and storage of meat and meat products (Estévez, 2011) and, more recently, with antibiotic residues (Márquez-Lázaro, 2020). Thus, in this context, the animal exposure to chlorpyrifos can be considered an external factor included in animal breeding, which is capable to induce oxidative damage even under *in vitro* conditions.

3.2.1 Isolation and identification of carbonylated proteins

Total protein extracts from exposed samples and control were fractionated according to their molecular mass using size-exclusion chromatography. Groups of five consecutive fractions were pooled together, as it is shown in Fig. 3a. Then, their carbonyl index was determined (Fig. 3b). The molecular weight (MW) of mixed fractions were determined with the calibration curve obtained from protein standards ($y = -0,7351x + 1,5137$; $R^2 = 0,9819$), Table 1.

The carbonylation promoted by CPF on F2, F4 and F5 was significantly higher in comparison to control ($P < 0.05$). The calculated molecular weight of these fractions was $>100 - 49.9$ kDa for F2, and < 49.9 kDa for F4 and F5. In contrast, the carbonylation on F3 (< 49.9 kDa) did not show significant differences with the control ($P > 0.05$); while in F1 (>100 kDa), carbonylation in the control was significantly higher than exposed sample ($P < 0.05$).

Further study of the oxidative damage caused by CPF, mass spectrometry in tandem was used to identify the oxidized proteins in the different groups of fractions. For this, each fraction (F1 to F5) was matched with chromatographic peaks obtained at 280 nm (Fig. 3a and Table 1). The identified proteins in each fraction and their molecular weights are shown in Table 2. As it was described above, CPF specifically promoted carbonylation on F2, F4 and F5, which were matched with peaks 2, 3-4 and 4, respectively (Table 1). In F2, myosin heavy chain (MHC) protein was identified; in F4, β -enolase, creatine kinase M-type and actin proteins were identified, while kinase M-type and actin were identified in F5. In this regards, MHC and actin are myofibrillar proteins, whereas, β -enolase and creatine kinase are sarcoplasmic proteins (della Malva et al., 2017; Gallego, Mora, Aristoy, & Toldrá, 2015)

Actin and MHC are important proteins involved in muscle contraction and cell movement (energy dependent process) (The UniProt Consortium, 2018). β -enolase is an enzyme that participates in glycolysis and the gluconeogenesis pathway, where acts as 2-phospho-D-glycerate hydrolyase and phosphoenolpyruvate hydratase, respectively (Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014; The UniProt Consortium, 2018). Creatine kinase M-type is an enzyme involved in energetic metabolism of tissues. It can act as an antioxidant by scavenging free radicals in *postmortem* muscle (Malheiros et al., 2019; Mora et al., 2014).

Sarcoplasmic and myofibrillar proteins oxidation has been related to color and texture changes, formation of cross-links, increased toughness and hardness, impaired water holding capacity (WHC), loss of essential amino acids and digestibility (Estévez, 2015); important aspects involved in sensory, technological and nutritional properties of meat (Estévez, 2011). In this sense, the presence of CPF residues at trace concentrations could be

considered a potential factor for the decrease of meat quality, as well as occurs with other chemical substances such as certain food additives (nitrites, etc.) (Feng et al., 2016; Villaverde, Ventanas, & Estévez, 2014).

On the other hand, when comparing the molecular weight of identified proteins (by MS/MS) with the theoretical molecular weight calculated with the calibration curve, the results are similar (Table 1 and 2). In general, this is related to the fact that size-exclusion chromatography (SEC) is frequently used as an initial step of protein purification and gives a good estimation of the molecular weight of the obtained fractions (Burgess, 2018; Lecchi, Gupte, Perez, Stockert, & Abramson, 2003; Mora et al., 2014).

3.3 Identification of oxidized peptides obtained after simulated GI digestion

In this section, the simulated digestion of control and exposed chicken breast samples was carried out and a peptidomics approach based on label-free quantitation of the identified peptides was used to establish statistical differences between peptide profiles, according to the influence of the oxidized peptides and variance among them.

Thus, a Principal Component Analysis (PCA) score plot with two components was carried out (Fig. 4a). Discriminant components 1 and 2 explain the 74.0 and 9.5% of variability in the dataset, respectively, allowing the differentiation between control and exposed samples. These results showed that exposure to CPF could have an impact on peptides oxidation, which would probably be captured by the cells once the digestion process is completed. This effect is concerning, since the oxidized proteins intake have been related to alteration of cellular signalling pathways, intestinal flora disturbance, alteration of redox state of intestinal tissue and the formation of adducts with DNA that lead to gene expression

modification (Estévez et al. 2017). Also this effect could influence the bioavailability of essential amino acids in meat proteins (Gallego et al., 2015; Estévez, Li, Soladoye, & Van-Hecke, 2017).

The loading plot (Fig. 4b) showed that oxidized peptides generated from collagen are main responsible for the differences observed between exposed and control samples. The fact that collagen (300 kDa) was not identified in the peaks obtained from size-exclusion chromatography (section 3.2.1) could be due it was not retained in the column because of its high molecular weight (The UniProt Consortium, 2018), and would elute in the solvent front of the chromatography.

The oxidized peptides obtained from exposed samples could have been generated due to a higher susceptibility of collagen to suffer oxidation reactions, because its abundance in muscle is lower (10%) than sarcoplasmic and myofibrillar proteins (Mohammadkhah, Murphy, & Simms, 2018). This fact is in agreement with the distribution of total peptides obtained after digestion in exposed and control samples (Fig. S2), where most of the peptides were from titin (25.0 and 23.4%, respectively) and myosin (7.8 and 8.0%, respectively) proteins. Moreover, when comparing the percentage of peptides identified in collagen, it can be observed that it was higher in exposed samples compared to control samples (5.8 and 3.8%, respectively); a change that could be associated to oxidation promoted by CPF, which could affect the digestion process and thus, increase the number of oxidized peptides (Estévez et al., 2017).

The observed oxidation in collagen protein from exposed and control samples mainly occurred in proline residues probably due to proline is the most abundant amino acid in collagen (Hong, Fan, Chalamaiah, & Wu, 2019). Proline amino acid is usually susceptible

to suffer carbonylation mediated by direct attack of free radicals (Estévez, 2011), which could suggest that CPF oxidation mechanism might be linked to this phenomenon.

Additionally, collagen is approximately 10% of skeletal muscle volume and its function is associated with extracellular matrix (ECM), where it is the main protein. In muscle, the ECM organizes the fibers and participates as a retaining mechanism during deformation (Mohammadkhah et al., 2018). In this sense, collagen oxidation promoted by chlorpyrifos residues in *in vivo* conditions could affect the animal welfare, since carbonylation is associated to the loss of protein functionality (Estévez, 2015; Estévez, 2011). Also, the intramuscular collagen oxidation could affect meat quality favoring its tenderness (Archile-Contreras & Purslow, 2011).

3.4 Identification of undigested proteins after simulated GI digestion and evaluation of its oxidation

Digestibility is a technological property of meat related to its nutritive value, which can be affected by protein oxidation. In this context, the undigested proteins were identified by MS/MS after trypsin digestion. The oxidative impact of chlorpyrifos on protein digestibility was evaluated from the obtained peptides. The total number of oxidized tryptic peptides derived from undigested proteins is shown in Fig. S3, and it was very similar. However, the influence of oxidation in exposed sample was higher in collagen protein than in control samples. Fact that shows, once again, the susceptibility of collagen to the oxidative effect of chlorpyrifos as described in section 3.3.

The intake of collagen hydrolysates has been related to favorable effects in human health such as improving muscle strength, bone density, and skin health, as well as reduce obesity,

joint pain, and blood pressure, and prevent atherosclerosis and aging. Main sources of collagen are bones, meat, tendons and skin from animals (chicken, beef, pork, etc.) (Hong et al., 2019). However, collagen oxidation promoted by chlorpyrifos could affect its beneficial effects and contribute to metabolism disturbance (Estévez et al., 2017). Also, animal welfare could be affected as tissues with high collagen content as skin, bones, tendons could be targets of oxidation promoted by chlorpyrifos, being necessary to increase the attention especially when exposure is considered.

4. Conclusions

This study evidences that the presence of chlorpyrifos residues at trace concentrations promoted *in vitro* carbonylation on myosin and chicken breast proteins, while the oxidative power of diazinon was lower than chlorpyrifos and it was only observed on myosin at one of the studied times of exposure. The simulated GI digestion of chicken breast exposed to chlorpyrifos showed that collagen was the main protein affected by oxidation especially in its proline residues. The proteomic analysis of undigested proteins after simulated GI digestion, also showed collagen as main protein affected by oxidation. In addition, our results provide important insights about the impact of organophosphate pesticides residues on quality and nutritional value of meat. Also, this study can be the basis for an investigation line focused in the impact of veterinary drugs on the quality of animal origin products.

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582 **FIGURE CAPTIONS**

Fig1. In vitro carbonylation of myosin exposed to: a) Chlorpyrifos (CPF) at 100 µg.Kg-1 and b) Diazinon (DZN) at 20 µg.Kg-1, monitored during 300 minutes (5 hours). The carbonylation was measurement each hour and data are expressed as mean ± SEM (n=2). The positive control was myosin exposed to FeSO4 at 100 µg.Kg-1

Fig 2. Carbonyl content in total chicken breast proteins exposed to chlorpyrifos (CPF) at 100 µg.Kg-1 and control. Mean ± SEM were graphed (n = 3). a,bMeans having different superscripts differ between control and exposed samples (P < 0.05).

Fig 3. a) Chromatograms obtained from size-exclusion chromatography at three wavelengths (214, 254 and 280 nm) and b) Carbonyl content in mixed fractions of chicken breast proteins exposed to chlorpyrifos (100 µg.Kg-1) and control. Mean ± SEM were graphed (n = 2). a,bMeans having different superscripts differ between control and exposed samples (P < 0.05). F: fraction.

Fig 4. a) Principal Component Analysis (PCA) score plot to assess the variance among all the oxidized peptides generated after the simulated GI digestion of exposed and control samples. B) Loading plot showing the oxidized peptides affecting the score plot distribution. Principal component 1 (PC 1) explained the 74.0% of the variability in the dataset while PC 2 was responsible for the 9.5% of variance within the dataset, allowing the differentiation between control and exposed samples.

Table 1. Fractions matched with peaks obtained from size-exclusion chromatography

Fractions	Peak number	Molecular weight* (KDa)
1	1	>100
2	2	>100-49.9
3	2 and 3	<49.9
4	3 and 4	<49.9
5	4	<49.9

*Molecular weight calculated from calibration curve ($y = -0,7351x + 1,5137$; $R^2 = 0,9819$)

Table 2. Proteins identified in the peaks collected from size-exclusion chromatography using mass spectrometry in tandem (QToF).

<i>Peak N° 1</i>		
Accession number*	Protein	Molecular weight (kDa)
A0A1V4K6N2_PATFA	Titin	3906.5
A0A1D5NVW6_CHICK	Myosin	520.0
MYSS_CHICK	Myosin heavy chain	230.0
G1MYI4_MELGA	Tropomyosin	32.8
MLE3_CHICK	Myosin light chain 1	20.0
<i>Peak N° 2</i>		
Accession number*	Protein	Molecular weight (kDa)
Q9PTY2_CHICK	Myosin heavy chain	230.0
<i>Peak N° 3</i>		
Accession number*	Protein	Molecular weight (kDa)
KCRM_CHICK	β-enolase	47.2
ENOB_CHICK	Creatine kinase M-type	43.3
A0A0Q3Q7N5_AMAAE	Actin	42.0
<i>Peak N° 4</i>		
Accession number*	Protein	Molecular weight (kDa)
KCRM_CHICK	Creatine kinase M-type	43.3
A0A0Q3Q7N5_AMAAE	Actin	42.0

* Protein accession number according to UniProt data bases

Fig1. *In vitro* carbonylation of myosin exposed to: a) Chlorpyrifos (CPF) at 100 µg.Kg⁻¹ and b) Diazinon (DZN) at 20 µg.Kg⁻¹, monitored during 300 minutes (5 hours). The carbonylation was measurement each hour and data are expressed as mean ± SEM (n=2). The positive control was myosin exposed to FeSO₄ at 100 µg.Kg⁻¹. * Indicate significance respect to control (P<0.05).

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Fig 3. a) Chromatograms obtained from size-exclusion chromatography at three wavelengths (214, 254 and 280 nm) and b) Carbonyl content in mixed fractions of chicken breast proteins exposed to chlorpyrifos ($100 \mu\text{g.Kg}^{-1}$) and control. Mean $\pm$ SEM were graphed ($n = 2$). ^{a,b}Means having different superscripts differ between control and exposed samples ($P < 0.05$). F: fraction.

Figure 4

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Fig 4. a) Principal Component Analysis (PCA) score plot to assess the variance among all the oxidized peptides generated after the simulated gastrointestinal digestion of exposed and control samples. B) Loading plot showing the oxidized peptides affecting the score plot distribution. Principal component 1 (PC 1) explained the 74.0% of the variability in the dataset while PC 2 was responsible for the 9.5% of variance within the dataset, allowing the differentiation between control and exposed samples.

Fig.S1. Experimental design and analyzes carried out in this study.

a)

b)

Fig. S2. Distribution of the total peptides identified by nLC-MS/MS after gastrointestinal digestion according to their protein of origin ($\geq 88\%$ confidence) in: a) control and b) exposed samples. These results were obtained using peptidomics for the identification of the generated peptides.

Figure S3. Distribution of the oxidized peptides obtained after the trypsin digestion of proteins non-digested in the simulated GI digestion. a) Control and b) exposed samples. These results were obtained using a traditional proteomic approach for the identification of proteins.