Valorization of The By-Products of Poultry Industry (Bones) By Enzymatic Hydrolysis and Glycation to Obtain Antioxidants Compounds

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Research Article

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VALORIZATION OF THE BY-PRODUCTS OF POULTRY INDUSTRY (BONES) BY ENZYMATIC HYDROLYSIS AND GLYCATION TO OBTAIN ANTIOXIDANTS COMPOUNDS

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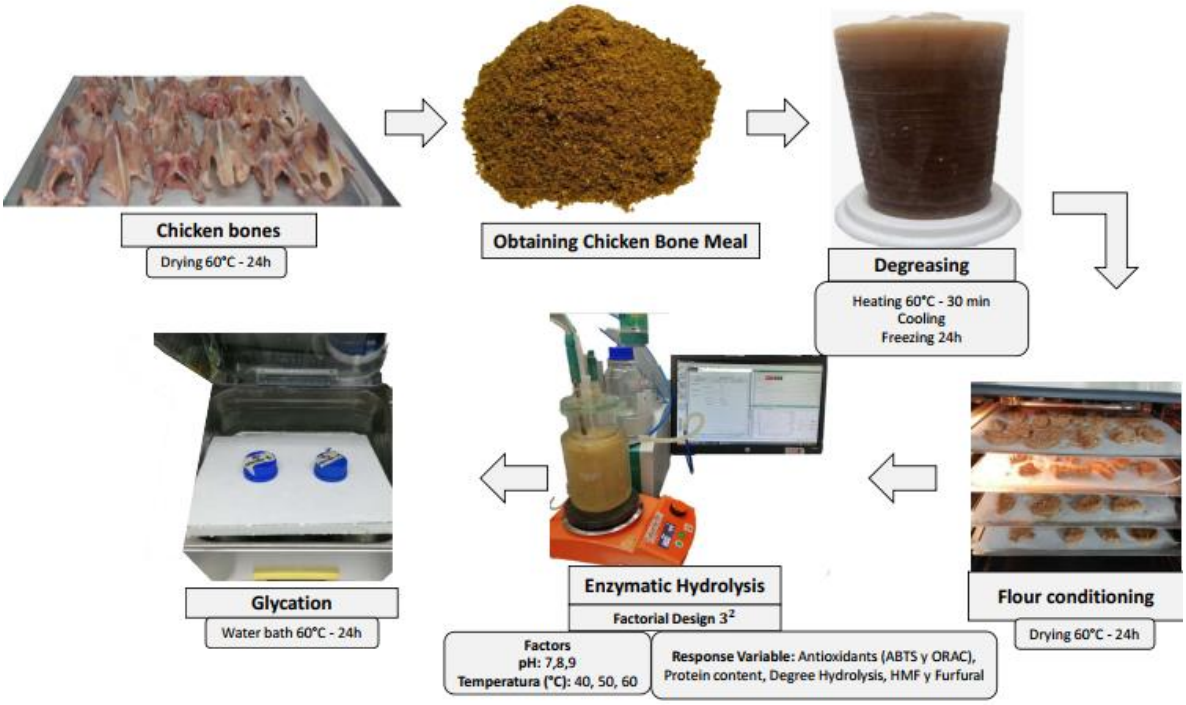
Graphical abstract



Abstract

Currently, one of the fastest growing industries in the world is the poultry industry; however, the increase in demand has generated the production of various byproducts, such as bones, and these byproducts have a negative impact on the environment. The aim of the present work was to evaluate the effect of glycation on the increase in antioxidant compounds and the formation of indicators of advanced glycation end products (AGE) in chicken bone hydrolysates; it also aimed to maximize the protein content, degree of hydrolysis and antioxidant content. Through analysis of variance (ANOVA), the content of AGE products (HMF and furfural) formed in the glycation process was analyzed. The chicken bone hydrolysate (CBH) had a protein content of 1.42 g/l, a degree of hydrolysis of 17.2% and an antioxidant capacity of 8334 and 10343 µmol ETrolox/l according to ABTS and ORAC evaluations, respectively. The glycation process increased the ORAC by 6.57%. The presence of hydroxymethylfurfural and furfural was determined in the glycated samples and detected at values between 0.05 and 0.22 and 0 and 0.26 ppm, respectively. In conclusion, hydrolysis and glycation are suitable alternatives that enable the use of chicken bones in producing food ingredients with higher added value.

Keywords. Chicken bones, enzymatic hydrolysis, glycation, antioxidant, HMF and furfural.

Statement of Novelty: The novelty of this study lies in obtaining compounds with antioxidant activity through enzymatic hydrolysis and glycation, evaluating the formation of indicators of advanced glycation end products in chicken bone hydrolysate to obtain a functional product with potential applications in the food industry, proposing an alternative that can reduce the environmental

impact of this waste product, which has a high content of nutritional protein that can be used to obtain bioactive compounds through enzymatic hydrolysis and glycation.

Introduction

Currently, the world consumption of chicken meat grows on average by 2.6% per year [1]. By 2030, the consumption per capita in the world will be 17.2 kg [2]. However, the increase in the consumption of chicken in its different forms and products generates a variety of byproducts, including viscera, legs, heads, bones, feathers and blood, which are approximately 37% of the live weight of the animal [3]. These byproducts have various impacts on the environment when the industry does not dispose of them properly [4], which leads to biological and biosafety problems [5]. The main poultry meat-producing countries in the world are the United States, Brazil, the European Union and China, which generate 20.3, 14.0, 12.8 and 12.3% world production, respectively; the remaining 40.6% of poultry products are produced in other countries [6]. On the American continent, Colombia ranks fifth with a production of 1.5 million tons of chicken meat per year, below the United States, Brazil, Mexico and Argentina [7].

The increase in the consumption of chicken meat worldwide is due to factors such as low cost, a lack of religious limitations, ease of accessibility, ease of transformation into processed foods [8], and its sensory and nutritional properties [9]. Chicken meat is recognized for its various health benefits, such as its low contents of cholesterol, calories and fat [10]; chicken meat also has a high content of proteins with high biological value [11], as well as essential amino acids and unsaturated fatty acids necessary in the human diet [12].

Given the quantity of production described above, several alternatives have been sought for the use and valorization of the byproducts of the poultry industry to mitigate its environmental impact. For example, the use of chicken feathers [13] and chicken hemoglobin powder [14] in animal food preparation has been reported. In addition, it has been determined that chicken feathers are an important source of amino acids for pigs [3]. Other studies have investigated the production of methane [15], biodiesel [16], flavorings and flavor enhancers from chicken blood, meat and bones [17].

However, one of the most interesting applications of the byproducts of the poultry industry is their use in the extraction of biomolecules, such as polyunsaturated fatty acids and proteins, by enzymatic hydrolysis [18]; chicken bones have specifically been used in this application due to their high protein content of approximately 19% [19]. These components have applications in the pharmaceutical, cosmetic, nutritional, food and microbiological industries [18] and have potential for application as functional ingredients [13]. In general, enzymatic hydrolysis has been used to obtain antioxidants from proteins of various sources, such as wheat germ [20], black beans [21], turkey byproducts [22], salmon gelatin [23] and chicken blood [24]. The extraction of antioxidant peptides derived from food proteins has provided natural ingredients for the formulation of functional foods and has improved their quality [25].

In addition, a prior study [19] determined that once antioxidants are obtained from proteins, they can be increased through a process known as glycation, the reaction between a reducing sugar and the amino acids that come from the peptides released during hydrolysis of the sample, from which the compounds derived from the last stages of the Maillard reaction are formed. [26]. Various authors

have reported an increase in antioxidant capacity by the glycation of different products, such as fish [27] and whey [28].

In addition, it has been reported that the antioxidant activity of compounds derived from glycation is due to different mechanisms, such as the chelation of metal ions, breaking of radical chains, decomposition of hydrogen peroxide and elimination of reactive oxygen species [29].

Recent studies report enzymatic hydrolysis as an alternative for the valorization of byproducts of the food industry [25], [30]; however, few studies have reported on the effect of glycation in these hydrolysates. This work aimed to evaluate the effect of glycation on the antioxidant capacity and the formation of indicators of thermal damage in CBH to obtain a product with potential applications in the food industry and generate an alternative to mitigate the environmental impact of this waste.

Materials and methods

Materials

Chicken bones (Carcasa) were purchased from a local market (Supermarkets EURO). The enzyme used was ALCALASE 2.4L® (Novozymes, Denmark), which contains subtilisin from Bacillus licheniformis. Its optimal temperature is between 55 °C and 70 °C, depending on the substrate, and the optimal pH is between 6.5 and 8.5 [31].

All reagents were of analytical grade and commercially available. Bovine serum albumin, fluorescein sodium, 2,2'-azino-bis(3-ethylbenzothiazolin)-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) were obtained from Sigma–Aldrich, St. Louis, MO, USA, and the phosphate buffer solution was prepared with reagents from MERCK®, Germany.

Sample treatment

For the treatment of chicken bones, the methodology described in a pervious study [32] was followed, with some modifications. The chicken carcass was conditioned by removing the adhered meat. The bones were dried in a convective dehydrator (Estructuras y Montajes SAS, Colombia) at 60 °C for 24 hours. Once the bones were dried, they were crushed in a Nutribullet food processor at maximum power for approximately 2 minutes (Nutribullet 600 W, USA).

Degreasing and drying of flour

Once the bone meal was obtained, it was mixed with distilled water at a ratio of 1:3; the solution was heated to 69 °C for 30 minutes with constant stirring; then, it was cooled to a temperature of 60 °C and refrigerated (Lassele, Mixed Refrigerator LRF-1382PC, Korea) until a temperature of 40 °C was achieved. Once this temperature was reached, the mixture was distributed in 250 mL polypropylene beakers, frozen for 24 hours, and stored in a freezer at a temperature of -18 °C (Lassele, LRF-1382PC Mixed Refrigerator, Korea). Subsequently, the sample was removed from the beakers, and the layer of suspended fat on top of the sample was cut. Once the sample had been degreased, it was cut by a chopper into blocks with an approximate thickness of 1.5 cm (Braher, Spain, Tajadora USA-250 Mono), and these were placed in a convective dehydrator (Estructuras y Montajes SAS, Colombia) at 60 °C for 24 hours.

Chemical characterization of flour

The proximal analysis of chicken bone meal was performed according to AOAC methods [33], and moisture, protein, fat, ash, and carbohydrates were determined by difference. All tests were done in triplicate. See Table 1.

Enzymatic hydrolysis

For the enzymatic hydrolysis process, the methodology provided in a published study [34] was followed with some modifications. Initially, chicken bone meal was diluted in distilled water in a 500 mL glass reactor at a protein concentration of 20 g/L. Hydrolysis was evaluated at 40, 50 and 60 °C and at pH 7.8 and 9. The apparatus was controlled with sensors connected to a Titrando 842 autotitrator (Metrohm, Switzerland) operated by a computer (Tiamo 1.2.1 software) that maintained the constant stirring of the reaction.

Once the pH and temperature conditions of the experimental design were reached (Table 2), Alcalase 2.4 L food grade was added to start the reaction. The hydrolysis process was carried out over 2 hours [35]. The base volume spent was recorded, and the degree of hydrolysis was calculated with the pH stat method, according to a prior study [36]; the released amino -NH groups were calculated according to Equation 1, and the average degree of dissociation was calculated according to Equations 2 and 3:

$$\% DH = \frac{V_B \times N_B \times 1 \times 1}{M_p \times \alpha \times h_{tot}} \times 100 \quad (1)$$

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \quad (2)$$

$$pK = 7.8 + \frac{298-T}{298 \times T} \times 2400 \quad (3)$$

where $V_B$is the volume of base consumed in L, N_B is the normality of the base (N), Mp is the mass of the protein in kg, h_{tot} is the total number of peptide bonds in the sample (eq/g protein) (for chicken proteins, its value is 7.6.), and α is the average degree of dissociation of the α-NH_2 groups released in the reaction, which depends on the pK, which in turn is a function of temperature, as indicated in Equations (2) and (3) [37]. Finally, the solution was heated at 85 °C for 10 min while stirring to inactivate the enzyme and stop the reaction.

Protein content

The protein content of the hydrolysate was determined by the Bradford method [38], [39]. The standard curve was made with bovine serum albumin. The absorbance was measured at 20 °C at a wavelength of 595 nm using a Thermo Scientific ™ Varioskan ™ LUX multimode microplate reader.

Glycation Process

The glycation methodology described in an earlier report[40] was followed; depending on the final protein content of the supernatant obtained from the hydrolysate by the Kjeldahl method, powdered lactose was added in a 1:1 protein mass ratio (Protein:Lactose). This solution was heated in a water bath (Lauda; Aqualine AL 5; Germany) at 60±2 °C for 24 hours. Once this process was finished, the product was frozen and stored (Lassele, LRF-1382PC Mixed Refrigerator, Korea) until further analysis.

Antioxidant activity

The antioxidant activity of the hydrolysate supernatant and glycated samples was determined by two methodologies: ORAC and ABTS, which are described below.

ORAC

First, 150 µL of fluorescein sodium working solution and 25 µL of the sample were added. For the blank, 25 µL of 75 mM phosphate buffer (pH 7.4) was added to the microplate. The samples were incubated for 30 minutes at 37 °C. Subsequently, 25 µL of AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) solution was added and shaken for 10 seconds at maximum intensity. The fluorescence intensity was measured every 5 minutes for 2 hours at excitation and emission wavelengths of 485 nm and 528 nm, respectively.

The ORAC values were calculated from the area under the curve of the sample data, and the results were expressed in micromoles of Trolox equivalents per L (µMol ETrolox/L) [39–41] according to Equation (4):

$$ORAC = [(AUC - AUC^{\circ})/(AUC_{Trolox} - AUC^{\circ})] \times f(Trolox) \quad (4)$$

where AUC is the area under the curve for the sample, AUC° is the area under the curve for the control, AUC_{Trolox} is the area under the curve for Trolox, and f is the dilution factor of the extracts.

ABTS

One hundred microliters of the sample or Trolox standard was taken for the blank, mixed with 1 ml of the ABTS solution, and incubated at 30 °C for 30 min. After that, absorbance measurements were performed at 730 nm. Aqueous solutions of Trolox with concentrations between 0 and 500 M were used for the calibration curve. The results are expressed as micromoles of Trolox equivalents per liter (µMol ETrolox/L) according to a prior study [27] with some modifications.

HMF Y Furfural

HMF and furfural compounds were determined as described in a previous study [44], with some modifications. Four milliliters of the homogenized sample was taken and diluted in 2 mL of water, clarified with 250 µL of Carrez I and II, and centrifuged for 10 minutes at 5000 rpm three times. Subsequently, it was filtered, and a volume of 10 mL was obtained. The supernatant was then passed through a filter with a pore size of 0.2 mm, and 50 µL of the filtered sample was analyzed on a C18 column at a rate of 1 mL/min with a mobile phase of water:acetonitrile (95:5) and a wavelength of 284 nm [45]. The analyses were performed in duplicate.

Experimental design

A factorial design of 3^2, with 3 center points, was carried out. The hydrolysis temperature and pH were the independent variables, and the protein content, degree of hydrolysis (DH), and antioxidant content by ABTS and ORAC were the dependent variables. This design was performed on hydrolyzed chicken bone meal samples. Each factor was studied at three levels (see Table 2). The design values were evaluated by analysis of variance (ANOVA) to determine the possible significance of the independent variables and their effects on the dependent variables. The experimental trials were randomized to reduce the results of unexpected variability in the observed responses. To find the optimal enzymatic hydrolysis conditions and to analyze how the independent variables affected the overall response, regression analyses and response surface plots were generated. The response variables that were found to be significant in the study were optimized.

The effect of glycation on antioxidant content and the formation of thermal damage indicators was evaluated by analysis of variance (ANOVA) as a function of the glycation process; the antioxidant, HMF and furfural contents were considered the response variables. The samples analyzed by this

design were those subjected to hydrolysis and subsequent glycation processes. The data were analyzed with Statgraphics Centurion XVI version 16.1.03 software.

Results

Characterization of chicken bone meal

Table 1 shows the results obtained from the proximal characterization of chicken bone meal. These values are similar to those reported by the Spanish Foundation for the Development of Animal Nutrition [44–47], which obtained protein content values of 49.3, 55.45, and 43%, respectively, for chicken bone meal. Several authors have also determined the protein contents in blood [50], offal [51], and chicken feet [52], and the values change depending on the part of the chicken used and can range from 20 to 60%. The contents of other components, such as ash, moisture, fat, and carbohydrates, are reported in Table 1.

Effect of hydrolysis conditions on the content of antioxidants, protein, and degree of hydrolysis of chicken bones.

Table 2 shows the experimental design, as well as the results of the variables analyzed, while Table 3 shows the significance levels for each of the factors and their interactions, as well as the coefficients of determination (R^2). Fig. 1 shows the response surface graphs for each variable.

Protein content

Table 2 presents the protein content in the CBH; the results varied between 0.84 and 1.65 mg/mL of protein; these results varied because the limited hydrolysis allows for alterations in the molecule size and the structure and strength of intermolecular and intramolecular interactions between proteins; the molecules are separated into peptides and free amino acids, decreasing the protein content and indicating structural changes [53] that confer functional properties, such as antioxidant activity, to the hydrolysates [54].

According to Table 3, temperature and pH had a significant effect ($p<0.05$) on the protein content. Temperatures close to 50 °C allowed a higher enzymatic activity for hydrolysis [53-54], which is evidenced by the decrease in protein content with increasing temperature.

Degree of hydrolysis

Table 2 shows that the degree of hydrolysis (DH) ranged from 13.59 to 17.98%, and similar values of DH have been reported in a previous study [35] on salmon byproducts.

According to Table 3, DH was significantly affected by pH ($p<0.05$); a higher DH was obtained with increasing pH because at a pH close to 9, the enzyme has higher performance [57]. Likewise, Table 3 shows that the quadratic effect of temperature on the degree of hydrolysis is also significant ($p<0.05$), showing that a point of maximum DH can be obtained for a temperature of 50 °C (see Fig. 1b). These results are in agreement with those described by [58], who, at pH 8.5 and 50 °C, achieved a higher degree of hydrolysis employing Alcalase 2,4*L*® in Catla visceral waste proteins.

Antioxidant content: ABTS and ORAC

Table 2 shows the results obtained for ABTS and ORAC found for chicken bones under the different processing conditions. These values range from 6531.52 to 8703.26 μmol ETrolox/L for the ABTS method and 2779.7 to 11565.04 μmol ETrolox/L for ORAC. These antioxidant capacity values are higher than those found for mango [59], orange [60], and beer [61].

According to Table 3, none of the factors analyzed significantly affected the ABTS content ($p>0.05$). However, the quadratic effect of temperature did significantly affect the ORAC ($p<0.05$). It was also observed that at higher temperatures and pH values, the antioxidant content increased according to both the ABTS and ORAC experiments (Fig. 1c and 1d). This behavior has been reported by other scholars [62],[63]. This behavior occurs because through enzymatic hydrolysis, low molecular weight peptides and amino acids are generated; these factors are related to an increase in antioxidant activity, decreasing the chain reactions of free radical formation through the compounds obtained [55–57].

Finally, Equations 5, 6, 7, and 8 show the models obtained by the experimental design for each of the response variables analyzed. In this sense, the coefficients of determination R2 for protein content and degree of hydrolysis were 87.91 and 83.83%, respectively, while for the antioxidant content by ABTS and ORAC, the R2 values were 52.43 and 60.75, respectively.

$$Protein = 6.8225 - 0.165917 * Temperature - 0.516667 * pH + 0.0011125 * Temperature^2 + 0.005 * Temperatura * pH + 0.03125 * pH^2 \quad (5)$$

$$DH = -22.6967 + 1.20783 * Temperature + 0.548333 * pH - 0.0125 * Temperature^2 + 0.011 * Temperature * pH + 0.01 * pH^2 \quad (6)$$

$$ABTS = -4689.31 + 640.308 * Temperature - 1282.07 * pH - 7.01902 * Temperature^2 + 9.61957 * Temperature * pH + 70.9239 * pH^2 \quad (7)$$

$$ORAC = 185915 + 1630.2 * Temperature - 54839.7 * pH - 35.6886 * Temperature^2 + 242.333 * Temperature * pH + 2670.19 * pH^2 \quad (8)$$

Optimization of the enzymatic hydrolysis process.

To optimize the process, a multiple-response optimization methodology was used, maximizing the protein content and degree of hydrolysis; the pH and temperature conditions were 9 and 50 °C, respectively, and these values were similar to those reported by Novozymes [31].

Table 4 shows the results of the experimental validation of the optimum process conditions, which were tested in triplicate. In general, the values obtained were very close to the values predicted by the model, with the experimental value for protein being 1,830.01 and the degree of hydrolysis being 17.49%.

Table 5 shows the content of nitrogen solubilized through hydrolysis, which was 62.42% of the initial content in chicken bone meal, for which it has been reported that, through hydrolysis, the protein can be solubilized up to 90% with Alcalase from treated fleshing meat [67]. It has also been reported that, with this enzyme, a greater number of free amino acids and small peptides is produced than those obtained with the enzyme papain [68].

Glycation process.

Table 6 shows the results obtained for the antioxidant content by ABTS and ORAC before and after glycation.

In general, and according to Table 6, the antioxidant content determined by ORAC increased by 6.57%, while the antioxidant content determined by ABTS decreased by 3.73%; this behavior can also be seen in Fig. 2a and 2b. Likewise, in both cases, the glycation process significantly affected the antioxidant content ($p<0.05$), as shown in Fig. 2a and 2b. The content of antioxidants decreased according to the ABTS tests , probably because they are thermosensitive; however, it can be said that after glycation, antioxidants can undergo further hydrogen transfer (HAT), which is the reason that the ORAC is higher [69].

This result is related to the fact that through the glycation of the amino groups of the proteins or peptides present in the matrix and a reducing sugar, in this case, lactose, antioxidant compounds can be generated; these antioxidant compounds have the capacity to eliminate free radicals. Antioxidants can delay oxidative deterioration in different matrices, which is why it has been suggested that compounds obtained through glycation could be used as functional ingredients in the food industry [70].

HMF and Furfural

Through the glycation process, compounds known as melanoidins are formed; these compounds have been reported to reduce peroxyl radicals [71]. In turn, this reaction also detects the formation of HMF and furfural, which are indicators of advanced glycation end products (AGE) of the intermediate stages of the Maillard reaction [45]. Both compounds have been reported to have toxic and carcinogenic effects [72]. The EFSA has already established an ADI (acceptable daily intake) value of 0.5 mg/kg bw/d for furfural [73]. Additionally, the Scientific Panel on Food Additives, Flavorings, Processing Aids and Food Contact Materials estimated a dietary intake of HMF of 1.6 mg/person per day based on a modified Theoretical Maximum Daily Added Intake (mTAMDI) approach [74]. According to the European Union Regulation of honey, maximum HMF limits have been established, and these limits vary according to the type of honey; in general, a maximum value of 40 mg/kg is accepted, except for honey for industrial use. A maximum of 80 mg/kg is accepted for honey of tropical origins [75].

Table 2 shows the HMF and furfural contents of the glycated samples, which ranged from 0.05 to 0.22 mg HMF/L and 0 to 0.26 mg furfural/L. The HMF values are lower than those reported in previous studies [76] of beer and balsamic vinegar [77]. The measured furfural contents are lower than those reported to be in fruit juices [76] and [78] in Marsala wine. It should be noted that the HMF and furfural contents are low, so glycation of CBH at 60 °C for 24 hours does not lead to a significant formation of the analyzed thermal damage indicator compounds because the Maillard reaction is promoted when food systems containing reducing sugars and amino acids are treated at high temperatures (> 120 °C) [79].

The furfural content presented in Table 2 is low because lactose mainly degrades to form HMF [80], because this disaccharide is composed of two hexoses, which generally promotes the formation of HMF; in contrast, furfural is derived from pentoses [81].

Fig. 3a shows that the highest HMF content was observed after treatments 1 and 2, which were significantly different from the other treatments ($p<0.05$); these results corresponded to temperature conditions of 50 °C and pH 8 and 9, respectively. Significant differences in the furfural content (Fig. 3b) were found mainly for treatment 4, which involved conditions of 50 °C at pH 7.

Conclusion

In general, it can be concluded that chicken bones are a raw material that can be used in enzymatic hydrolysis to obtain bioactive compounds such as antioxidants. In addition, by means of the factorial experimental design, it was possible to establish the best operating conditions of enzymatic hydrolysis to maximize the protein content and the degree of hydrolysis; it was evidenced that glycation has a significant effect on the content of antioxidant compounds and does not lead to the formation of advanced glycation end products (AGE) such as HMF and furfural. Finally, the compounds obtained here could be used in the development of new food matrices, thus serving as an alternative to the disposal of the byproducts generated by the chicken industry.

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Disclosure statement

The authors declare no conflict of interest

Declarations

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Availability of data and material: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability: The data were analyzed with Statgraphics Centurion XVI version 16.1.03 software.

Author's contributions: All authors had readen and agree with the published version of the manuscript. Conceptualization, methodology, formal analysis, experimental research, writing: preparation of the original draft, Luisa Londoño, Sara Franco and Sandra Restrepo; accompaniment of the experimental phase and resources, Lina Suárez, Fáver Gómez; writing: proofreading and editing, Óscar Vega, Pedro Valencia; visualization, Helena Núñez, Ricardo Simpson.

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List of figures

Fig. 1 Surface response showing the effect of temperature and pH on a) protein content, b) degree of hydrolysis (DH), c) antioxidant content by ABTS and d) ORAC.

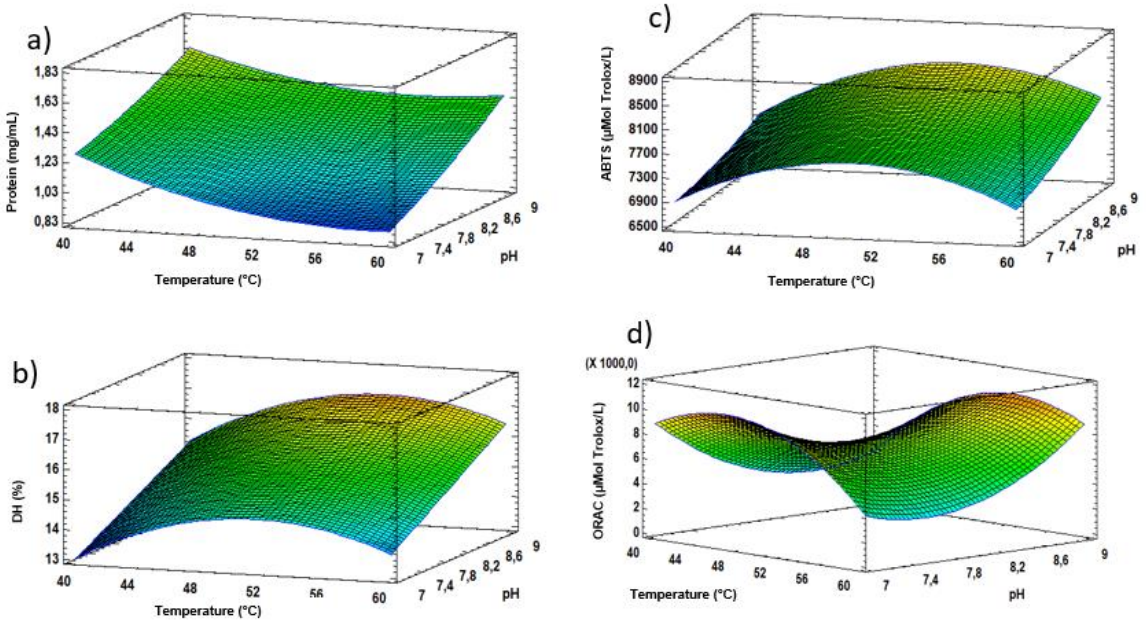
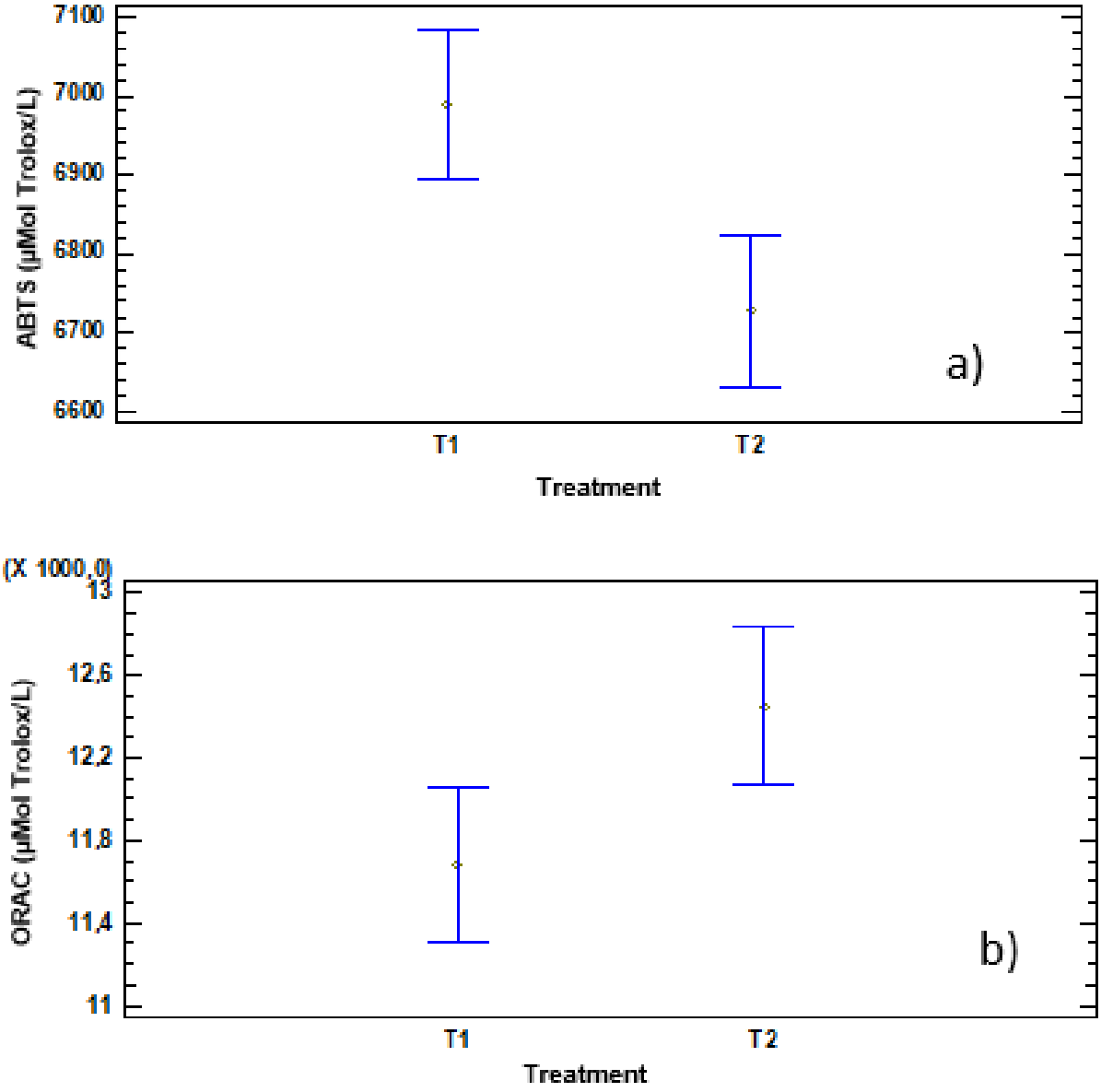
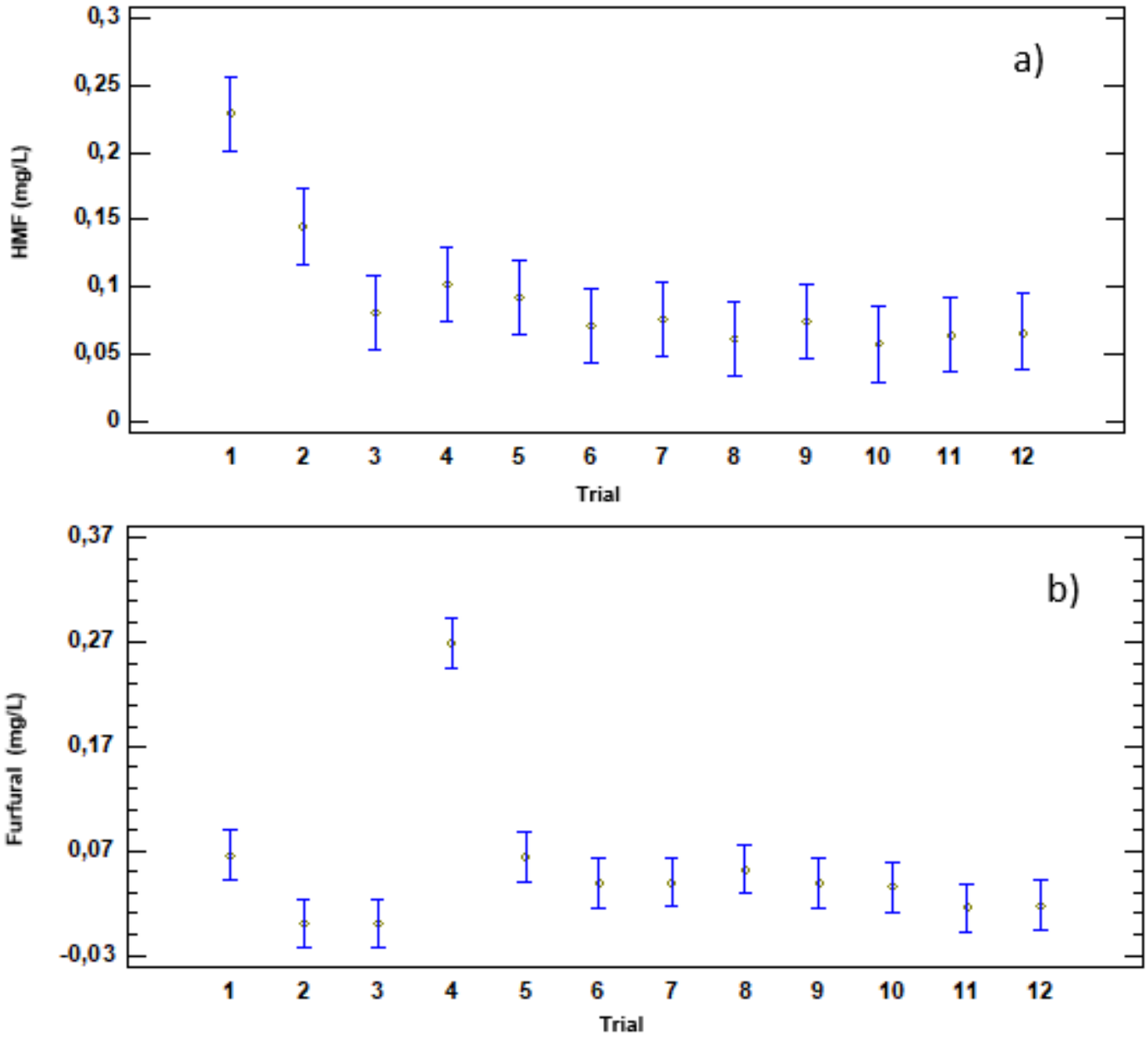


Fig. 2 Graph of average antioxidant content by a) ABTS and b) ORAC.



**T1: Hydrolyzed, T2: Glycated*

Fig. 3 Graph of average contents of a) HMF and b) furfural in the glycated samples.



List of tables

Table 1. Proximal characterization of chicken bone meal.

Component	Percentage (%)
Protein (N x 6,25)	47.17±3.93
Ash	24.61±0.76
Moisture	7.2±0.05
Total fat	19.04±0.17
Carbohydrates	2±3.16

Table 2. Experimental data for the different combinations of temperature and pH for the enzymatic hydrolysis of chicken bones.

Trial N°	Factor		Response variable				HMF	
	Temperature (°C)	pH	ORAC (µMol ETrolox/L)	ABTS (µMol ETrolox/L)	DH (%)	Protein (mg/mL)	(mg/L)	Furfural (mg/L)
1	50	8	9888.55	7968.48	15.83	1.04	0.23	0.17
2	50	9	11565.04	8233.7	17.98	1.38	0.14	ND
3	40	7	9616.69	6531.52	13.59	1.18	0.08	ND
4	50	7	10779.09	8077.17	13.85	1.12	0.10	0.27
5	50	8	7707.29	8703.26	15.27	1.16	0.09	0.06
6	60	8	6623.65	7256.52	15.47	1.13	0.07	0.04
7	60	9	6864.6	8041.3	15.86	1.51	0.08	0.04
8	50	8	5768.67	7516.3	16.4	1.21	0.06	0.05
9	60	7	4008.25	6926.09	13.93	0.84	0.07	0.04
10	50	8	3499.42	7096.74	15.92	1.2	0.06	0.04
11	40	9	2779.7	7261.96	15.08	1.65	0.06	0.02
12	40	8	3242.38	7508.7	13.84	1.53	0.07	0.02

Table 3. *p values* of the response variables in relation to the factors and their interactions.

Factors and interactions	Protein	Degree of hydrolysis	ABTS	ORAC
T	0.0161*	0.1625	0.5419	-
pH	0.0019	0.0047	0.2098	-
T^2	0.1449	0.0275	0.0965	0.0335
pH× T	0.392	0.7656	0.7522	0.0656
pH^2	0.6545	0.9823	0.8489	0.0915
R^2	87.9056	83.8294	52.4266	60.7536

* *p* value <0.05, indicate that there is a significant incidence.

Table 4. Experimental validation.

Response variable	Predicted value	Observed value
Protein (mg/mL)	1.44	1.83
Degree of hydrolysis (%)	17.14	17.49

Table 5. Nitrogen content in the chicken bone meal and hydrolysate phases.

Sample	Nitrogen content (g)
Chicken bone meal	1.58
Hydrolyzed	1.00

Table 6. Antioxidant content of the hydrolysate before and after glycation.

Sample	ABTS (µMol ETrolox/L)	ORAC (µMol ETrolox/L)
Hydrolyzed	6988.41[a]	11683.61[a]
Glycated	6727.54[b]	12450.92[b]

[a,b] Values with different letters are significantly different from each other ($P < 0.05$).