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HYDROLYZED COLLAGEN FROM MEAT BY-PRODUCT SOURCES: EXTRACTION AND CHARACTERIZATION

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Abstract: Hydrolyzed collagen, a biomaterial with numerous health and wellness applications, can be extracted from different animal sources. The increasing necessity for hydrolyzed collagen in the food and medical industries, as well as in household consumption, is prompting consideration of diversifying its natural sources. The valorization of waste by obtaining useful materials is a desire of modern society and one of the circular economy objectives. In light of these factors, we employed a straightforward and affordable method to extract collagen from by-products of the meat processing industry, highlighting the impact of extraction duration on the extract's properties and the structure of the collagen. The amount of hydroxyproline determined by a spectrometric method was used to estimate the collagen content. The protein content was quantified by the Kjeldahl method and the antioxidant capacity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The extracts were characterized by UV-visible spectroscopy, and some other properties, like *p*H, electrical conductivity, and phosphate content, were determined for a further characterization of extracts. FTIR spectroscopy was used to analyze the hydrolyzed collagen precipitated from extracts. The results demonstrated a relationship between extraction time and hydroxyproline concentration, as well as with collagen hydrolysis, also proving that waste and by-products from pork meat processing could be used as a sustainable source of hydrolyzed collagen in mild conditions in the absence of enzymes or other reagents.

Keywords: *animal collagen, antioxidant activity, biopolymers, bone soup, FTIR spectroscopy, residual raw materials*

INTRODUCTION

Collagen is one of the most abundant proteins in vertebrates which has attracted enormous interest in various practical and theoretical domains. It is a fibrillar protein found in bones, muscles, skin, blood vessels, and connective tissues, providing the body's strength, elasticity and mechanical stability, and conforming the conjunctive and connective tissues in the human body, essentially skin, joints, and bones [1, 2]. Animal tissue contains about 20 genetically unique collagens that vary in structure, function within the body, and amino acid composition and sequence. The collagens were divided into several groups according to their structure [1, 3]. The common sources from which collagen is extracted are the bovine (hides, bones, and tendons), porcine (skin and bones), marine (fish skin, scales, and bones), and avian (chicken cartilage and bones) [3 – 5]. As vital supplies of protein for the human diet, meat products also contain vitamins, minerals, and necessary amino acids. Large volumes of protein-rich leftover raw materials (such as heads, bones, carcasses, blood, skin, viscera, etc.) are produced during the processing of fish and animals. These materials have a great deal of potential for higher-value uses in food and feed. According to estimates, residuals make up between 40 and 60 percent of the total weight of fish and animals. The residual raw materials / by-products from the processing of fish and animals can be further processed, including to obtain proteins and amino acids [6 – 8].

The triple helix collagen structure consists of three α-chains Gly-X-Y, where Gly = glycine, X = proline (Pro), and Y is mainly hydroxyproline (Hyp), which is almost unique to collagen and crucial for stabilizing the triple helix. The hydrogen bonds between the N-H groups of glycine residues in one chain and the C=O groups of other amino acid residues in adjacent chains stabilize the triple helix. The repetition of $(Gly\text{-}Pro\text{-}Hyp)_n$, the collagen's most prevalent tripeptide unit, depends on the collagen type [2, 3]. In addition to the triple helical area, the collagen molecule has two nonhelical regions at either end of the helix structure. The average composition of collagen in amino acids consists of glycine (33 %), proline (10 - 15 %), and hydroxyproline (10 %) [9].

Collagen hydrolysate, also known as hydrolyzed collagen (HC), is a collection of small, low molecular weight (3 - 6 kDa) peptides that are produced when natural collagen is proteolyzed [3, 4]. In the first stage of collagen hydrolysis (proteolysis) by thermal treatment, above 40 ^{o}C, the denaturation of collagen occurs with the generation of three α-chains, with random coiled structure, which are further hydrolyzed. The hydrolysis can be acidic (by using CH_3COOH, HCl, or H_3PO_4), alkaline, or enzymatic (by means of proteolytic enzymes such as pepsin, papain, alcalase, etc.). Alternative methods include thermal treatment, at high temperature and pressure [9]. The properties of HC make it an important biomaterial; for example, it is soluble in cold water without gelling, is colorless and odorless, being used in emulsions as a stabilizer, with applications in pharmaceutical, cosmetic, and food industries [3]. Also, several benefits like quick digestion, high assimilation, and efficient intestine absorption, make it important in food systems [9]. The antioxidant activity of HC from different sources was evidenced in many studies [10 – 12], being dependent on the amino acid content and molecular mass. Namely, the peptides with 2 to 10 amino acid residues show high radical scavenging because of their accessibility to active radicals [3]. Other bioactivities of the collagen di- and tripeptides, especially those with C-terminal Pro or Hyp residues, like immunomodulatory and antibacterial, were evidenced by numerous researchers [4].

Among other sources, porcine by-products (e.g., pig skin) can be used to extract the native collagen of type I, with the advantage of high similarity to human collagen. Porcine collagen has been widely used in medical applications like skin and wound healing, hernia repair, and tendon reinforcement [9]. The extraction of HC porcine in industrial conditions involves treatments including high temperature (150 - 250 °C) and pressure (350 - 3900 kPa), conditions that result in the production of peptides having molecular weights less than 15 kDa [9].
In our study we involved a traditional method for obtaining hydrolyzed collagen from porcine by-products, in a similar manner to the one in which bone broth is obtained in households, having in view the recent increased interest in this drink [13]. The advantage is that the obtaining a functional product with potential applications in the food consumption / food industry is combined with the valorization of by-products, which might otherwise end up as waste. We studied the influence of time on collagen extraction, in correlation with Hyp content, antioxidant activity, and protein content.

MATERIALS AND METHODS

Materials and reagents

The porcine by-products (bones with marrow and meat) were purchased from the local market, being produced by a farm in Constanta County, Romania, and used as without further processing. The reagents (phosphate buffer solution *p*H = 6.8; chloramine T (*N*-chloro-*p*-toluene sulfonamide sodium salt) trihydrate, $CH_3C_6H_4SO_2NClNa \cdot 3H_2O$; 4-dimethylaminobenzaldehyde (DMBA), $(CH_3)_2NC_6H_4CHO$; hydroxyproline, $C_5H_9NO_3$; perchloric acid, $HClO_4$; 2-propanol, $(CH_3)_2CHOH$); DPPH, 2,2-diphenyl-1-picrylhydrazyl; ammonium molybdate, $(NH_4)_2MoO_4$; κ-carrageenan; glacial acetic acid, CH_3COOH; sodium chloride, NaCl) were produced by Sigma Aldrich and used as received, without further purification.

Extraction of collagen

The pork bones, also containing bone marrow and meat, were merged with distilled water (400 mL). The as-prepared samples were heated for different times (4 - 12 h) in similar conditions. During the uniform heating on a sand bath, the mixtures were manually stirred, the temperature was kept at about 85 ^oC, and the volume was maintained approximately constant by adding distilled water. The extraction conditions were chosen similar to the preparation of homemade bone broth. After completing the heating time, the hot samples were filtered through a cotton pad, and the cooled filtrates were brought to a volume of 500 mL, with H_2O, and analyzed. The extracts were denoted **1** - **5** (*i.e.*, **1** - 4 h, **2** - 6 h, **3** - 8 h, **4** - 10, **5** - 12 h).

Characterization of extracts

The extracts were characterized by UV-Vis spectroscopy, as well as by measuring *p*H, electrical conductivity, antioxidant activity, hydroxyproline, total protein content, and phosphate content.

UV-Vis spectroscopy. The electronic spectra of extracts were recorded in the 200 - 900 nm domain on a Jasco V 550 spectrophotometer.

Protein content. Protein content was determined by the Kjeldahl method [14], using a mineralization system DK 6 and distillation system UDK 127, Velp Scientific. Protein content was measured in order to determine the collagen / protein ratio (expressed as the percentage of collagen in meat protein), which must be at least 18 % for every meat product sold on the European market [15]. The nitrogen content was multiplied by 6.25 to determine the total protein amount.

Antioxidant activity - DPPH. The radical scavenging activity of extracts was determined by using DPPH assay according to the method of Sánchez-Moreno [16]. In brief, a Cecil CE2501 spectrophotometer was used to measure the decreasing absorption of the DPPH solution at 517 nm following the addition of the antioxidant. The results are expressed as IC_{50} at a fixed antioxidant concentration for all the samples.

Phosphates. Phosphates were determined by the colorimetric procedure, using ammonium molybdate as a reagent [17]. A Hach DR3900 VIS Spectral Photometer was used to measure the complex's absorbance at 680 nm, and a calibration curve originated from the measurements of a series of standard PO_4^{3-} solutions was used to determine the phosphate content ($y = 1.2029x + 0.008$; $R^2 = 0.9683$).

pH and electrical conductivity. The *p*H of extracts was determined by using a multiparameter dual-channel analyzer (Consort, C3030). The electrical conductivity (EC), expressed in $mS \cdot cm^{-1}$, was measured using a calibrated digital apparatus (*p*H/Cond 340i SET 1, WTW, Germany).

Hydroxyproline determination

A spectrometric method was used to determine hydroxyproline [18]. In brief, the hydroxyproline is oxidized with chloramine T to pyrrole, which further reacts with 4-dimethylaminobenzaldehyde (DMBA). The solution absorbance at 558 nm (Hach DR3900 VIS Spectral Photometer) is used to determine the resultant addition compound content.

Preparation of the measuring solutions. The preparation of the sample, blank, and standard solutions was made in test tubes, as follows: (*i*) 2.00 mL of solution is transferred into a test tube, and 5.00 mL of chloramine-T reagent is added. The solution is stirred and left at room temperature for 20 ± 1 min; (*ii*) 2.00 mL of color reagent is added, thoroughly mixed, and transferred into a water bath with 60 ± 0.5 °C, for 20 min. The samples were cooled down to ambient temperature, and they were let stand for 30 min; the absorbance was measured at 558 nm against the blank (Hach DR3900 VIS Spectral Photometer). The stock solution of the standard to obtain a calibration curve was 0.1, 0.5, 1.0, 1.5, and 2.0 $mg \cdot mL^{-1}$. All standard solutions were prepared directly before use.
The applied method was verified to show that it is suitable for the determination of hydroxyproline. The performance parameters evaluated were: limit of detection (LOD), limit of quantification (LOQ), linearity, working range, and accuracy. The following was

demonstrated by the studied method's performance parameters: (*i*) LOD was set at 0.1088 mg·mL^{-1}; (*ii*) LOQ was set at 0.3295 mg·mL^{-1}; (*iii*) the linearity range was between 0.06 - 1.07 mg·mL^{-1}, with a correlation coefficient (R^2) of 0.9748; (*iv*) the equation of the linear regression function is y = 0.9574·x. The degree of similarity between the actual value and the value found in the analyzed sample is referred to as accuracy. Ten replicate samples with a hydroxyproline concentration of 0.5 mg·mL^{-1} were prepared for the determination of accuracy, and the absorbance values (Yi) were measured at 558 nm. The experimentally determined concentration (Xi) values were calculated using the calibration curve equation. The obtained values (> 95 %) are within the range provided by the performance criterion established for accuracy [19].
The content of collagen was calculated by the following formula [15]:

$$Collagen\ (\%) = 8\ \times hydroxyproline\ content\ (\%) \qquad [1]$$

Precipitation and characterization of HC

HC was precipitated from aqueous solutions by using κ-carrageenan [20]. In brief, 100 mL of a cold solution of 0.5 % κ-carrageenan in 0.5 M CH_3COOH was slowly added, under continuous stirring, in 100 mL of cold collagen extract, simultaneously with the addition of 100 mL of a 3 % NaCl cold solution, and a white powder was obtained. After 30 min of stirring on an ice bath, the precipitate was separated by centrifugation (10,000 rpm, 10 min, Elektro-Mag 815M centrifuge), washed with distilled water and EtOH, and dried in an oven for 4 h at 85 °C. The powders were characterized by FTIR spectroscopy. The FTIR spectra were recorded in the range of 4000 - 650 cm^{-1} on an Agilent Cary 630 FTIR spectrometer, ZnSe ATR mode. The number and position of the bands that make up each spectral interval were determined using the second derivative spectrum. Using this method, the successive minima of the second derivative spectrum reveal the bands that comprise a certain spectral interval [21].

RESULTS AND DISCUSSION

The collagen extraction from porcine meat processing by-products was done as a simple extraction in water at a temperature of around 85 °C, without any initial pretreatment (like deproteinization, demineralization, and fat removal) as a general collagen extraction procedure would require. The goal was to reproduce the process used in home kitchens and in small or medium-sized production units, where the final product is the extract; the extract (as a "bone soup") should contain other nutrients besides hydrolyzed collagen, being an advantage for food. Therefore, in addition to collagen content, other characteristics were determined for the extracts obtained, which can be correlated with their use as such in food.

Characterization of extracts

The samples (**1** - **5**) were characterized mainly to estimate the amount of collagen extracted by determination of hydroxyproline, an amino acid specific to collagen, whose

measurement is used as an indicator for quantifying collagen levels [22]. In addition, other characteristics of the obtained extracts were determined.

Electronic spectra. The UV-Vis spectra of extracts (Figure 1) were recorded to obtain information about chemical composition. To obtain the electronic spectra, the successive dilution of the samples with distilled water was performed (1:10).

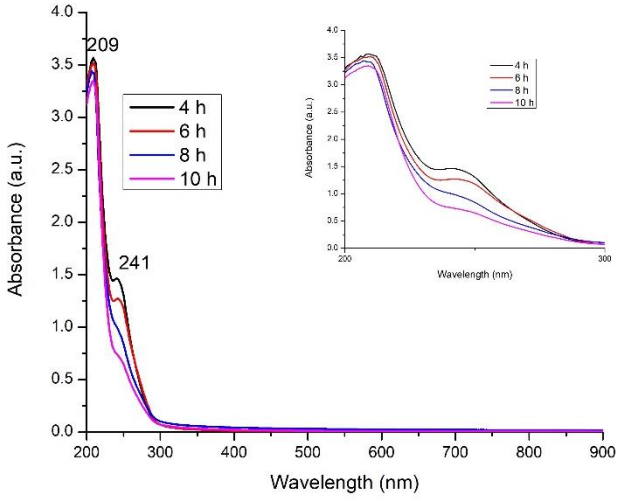

Figure 1. *UV-Vis spectra of **1**-**4** samples (in insert, the UV range of the spectra)*

A very intense band, at about 209 nm, can be observed in all spectra of extracts **1**-**4**. The bands in the far - UV domain (*i.e.*, 190 - 230 nm), such as those at 209 nm, can be assigned to the absorption of peptide bonds [23], therefore being evidence for the presence of proteins in studied samples. Furthermore, it was demonstrated the directly proportionality between the intensity of absorption bands and the number of peptide bonds [23]. For the studied extracts, the intensity of the band decreased as the extraction time increased, as evidence of the peptide bonds breakage by hydrolysis.

In the spectrum of sample **1**, with the smallest heating time (4 h), a band situated at 241 nm is partially overlapped with the intense band at 209 nm, but its shape is distinct. With the increasing heating time, the intensity of this band, reported to the band at 209 nm, is smaller and finally turns into a shoulder. Considering that absorption at 241 nm is not characteristic of a particular species, the band at 241 nm can be assigned to the phenylalanine residues (with a characteristic absorption at 257 nm), peptide bonds, or charged amino acids [24]. Since its intensity decreases with the extraction time, it is most likely assigned to peptide bonds, which are broken by hydrolysis. In the visible domain, no absorption was observed for any of the samples, which is also characteristic of collagen solutions [24].

As the present study aimed at collagen extraction without fat removal, it is expected that fatty components will also be present in the analyzed samples. Pork fat primarily consists of triglycerides, which have limited absorption in the UV region; therefore, no information can be obtained from the spectra regarding the presence of fats in the studied samples. The pork fat is typically pale or white, indicating minimal absorption in the visible region as well.

Chemical composition. The results in Table 1 indicate that with increasing extraction time, the amount of hydroxyproline (Hyp) also increases so that after 8 h of heat treatment, the Hyp content reached a maximum value of 0.184 %. This increase in Hyp content in the first part of the heat treatment might be explained by the polypeptide chain's hydrolysis [25]. After this interval, the Hyp level decreased, remaining constant at around 0.17 %, until the experiment's finalization (12 h). It can be observed that the Hyp content was affected by the extraction time; during the hydro extraction step, the proteins underwent degradation, leading to compounds with modified structures. Proteins with shorter chains and lower molecular weights are formed, called free peptides, which dissolve easily in water, leading to higher protein content, as observed in this study (Table 1).

Table 1. *Content of hydroxyproline (Hyp), hydrolyzed collagen (HC) and protein (P) (mean ± SD) %, and HC/P ratio in hydrolyzed collagen extracts as a function of hydrolysis time*

Sample	Time [h]	Hyp [%]	HC [%]	P [%]	HC/P [%]
1	4	0.069±0.01	0.55±0.07	8.23±1.78	6.68
2	6	0.161±0.09	1.32±0.21	10.29±2.01	12.8
3	8	0.184±0.11	1.47±0.30	8.44±1.20	17.45
4	10	0.172±0.08	1.38±0.40	8.87±2.41	15.5
5	12	0.170±0.10	1.36±0.15	8.73±1.09	15.68
					* < 18 %

* as per Reg. 1169/2011 [15]

This trend is in agreement with previous research performed on fish bone collagen [22]. The study's data proven that the amount of hydroxyproline increased significantly with the time of thermal treatment, the highest yield being determined after eight hours of heating.

Additionally, the hydrolyzed extract's total protein level was ascertained to estimate the collagen/protein ratio, which is a quality indicator for both meat and by-products. The results in Table 1 show that the extraction time (hydrolysis) led to variations in protein content. A slight increase in protein content was observed after the first 4 hours of thermal treatment, which may be a consequence of the gelatin formed during this extraction step. Continuing the hydrolysis process, a decrease in the protein percentage was observed; during the hydrolysis step, the hydrogen bonds are cleaved, which caused a change in the structure of the procollagen to become collagen, affecting the protein content, extraction yield, and water content. Also, macromolecular degradation during hydrolysis can lead to proteins with altered structures as well as an increase in protein hydrophilicity [26]. As regards the collagen/protein ratio (HC/P), the variation depends on the concentrations of the two components. The HC/P ratio, during the hydrolysis process, is below the maximum value accepted for meat by-products, which shows the nutritional quality of these extracts [15].

Antioxidant activity. The antioxidant activity of extracts was estimated by determining the half maximal inhibitory concentration (IC_{50}), meaning the concentration of an antioxidant required to scavenge 50 % of the free radicals (Table 2). The dependence between the antioxidant activity and IC_{50} is inverse, namely the lower the IC_{50}, the

stronger the antioxidant activity. The antioxidant activity of hydroxyproline peptides was demonstrated by several studies, but the Hyp's antioxidant abilities are affected by the size of molecule, *i.e.*, the smaller the peptide's molecular weight, the more readily it may donate an electron or hydrogen to stabilize radicals [27, 28]. The peptides hydrophobic amino acid content is primarily responsible for HC's antioxidant potential [29]. Additionally, the position and composition of amino acids in peptides influence collagen's antioxidant activity since the collagen peptides with -OH and -NH_2 groups can bind free radicals.

In this study, we observed that the antioxidant activity of extracts was high at the beginning of the hydrolysis process, reaching a maximum of around 6 h, after which it showed a gradual decrease until the end of hydrolysis (Table 2). Prolonging the hydrolysis time caused changes in the collagen protein structure, possibly generating less functional peptides or inactive or free amino acids [30]. It can be concluded that peptides exhibiting DPPH scavenging activity were released up to a maximum of 6 h and then hydrolyzed to inactive sequences, considering that both the size of the peptides and the number and sequence of amino acids can affect the antioxidant activity of the hydrolysates [31].

Table 2. *Antioxidant activity (IC_{50}), electrical conductivity (EC), and phosphates content (mean ± SD) in hydrolyzed extract as a function of hydrolysis time*

Sample	Time [h]	IC_{50}	EC [mS/cm]	PO_4^{3-} [%]
1	4	8.69±2.78	2.16±0.92	0.30±0.07
2	6	6.44±1.65	4.18±1.50	0.39±0.12
3	8	20.6±3.18	4.01±1.02	0.21±0.09
4	10	27±4.10	3.90±0.90	0.32±0.10
5	12	168±29.15	4.10±1.14	0.26±0.14

Electrical conductivity. The overall concentration of ions in a solution determines its electrical conductivity (EC). Measuring electrical conductivity is one of the non-destructive ways to assess the quality of meat. Intracellular and extracellular fluids are regarded as electrolytes, and meat is made up of many cells encased in insulating cell membranes [32]. Muscle experiences varying degrees of cell membrane injury following sacrifice due to the post-mortem metabolism. Meat's electrical characteristics vary as a result of increased cell membrane permeability and altered intracellular and extracellular fluid composition [33, 34]. Therefore, it is proposed that electrical conductivity can be used to forecast factors impacting the final quality of meat and by-products, as the change in electrical conductivity of meat reflects its qualitative features [35]. In this study, the hydrolysis process led to changes in the cell membranes of the by-products analyzed, resulting in cellular fluids with high ion content with electrical properties (Table 2).

*p*H is an important factor for collagen quality. During hydrolysis, *p*H showed values between 6.5 - 6.8, indicating that extraction time did not influence this parameter. In absence of a single standardized value for all collagen applications in Europe, the collagen's functional *p*H range in the majority of European applications is generally accepted to be between *p*H 4.0 and 7.0, with particular optimal ranges depending on the final product and its intended use. In some countries there are standards for collagen quality; for example, in Indonesia the *p*H value of crude collagen from fish scale must be between 6.5 and 8 [36]. Therefore, the *p*H levels of the collagen extracts used in this study are acceptable regardless of the standards referred to.

Phosphate content. Phosphates are not permitted in fresh meat but may be added to meat preparations and meat products (EC Regulation No. 1333/2008). The maximum permitted level of phosphates in meat and meat products under European legislation is 5 $g \cdot kg^{-1}$ expressed as phosphorus pentoxide (P_2O_5) individually or in combination in the finished product (Commission Regulation (EU) 2018/74). Meat products contain phosphates for a number of reasons, including adjusting and/or stabilizing the *p*H level, enhancing water retention, reducing cooking losses, enhancing texture and sensory qualities, and extending shelf life. Additionally, people can obtain phosphorus, a necessary nutrient for good health, from the phosphates found in meat products [37].
The phosphate content in the analyzed extracts showed small variations depending on the hydrolysis time (Table 2). The amount of phosphate is below the maximum permitted limit of 0.5 % in meat products [38] and it can be considered that the analyzed by-product extracts may represent mineral resources.
Pearson correlation was used to understand the relationship between the evaluated parameters (Table 3). The analysis showed a positive and strong correlation between collagen content and collagen/protein ratio (r = 0.96). This strong correlation may be related to the amino acid composition of the hydrolysates which may be similar to that of the initial proteins, *i.e.*, to be rich in glycine and proline. Also, a strong and significant correlation was observed between electrical conductivity and collagen content (r = 0.97) and HC/P ratio (r = 0.89), respectively. In this case, it can be said that along with the amino acids resulting during hydrolysis, by protein degradation, a quantity of ions is formed in the solution. In the present study, using a traditional extraction method (t = 85 °C), the electrical conductivity may be mainly given by ions released in extracellular fluids.

Table 3. *Correlation analysis*

	HC	**P**	**HC/P**	**PO_4^{3-}**	**IC_{50}**	**EC**	***p*H**
HC	1	0.31	**0.96**	-0.24	0.36	**0.97**	0.38
P		1	0.05	0.77	-0.26	0.45	-0.41
HC/P			1	-0.47	0.45	**0.89**	0.52
PO_4^{3-}				1	-0.56	-0.13	-0.41
IC_{50}					1	0.34	-0.21
EC						1	0.15
***p*H**							1

Correlation is significant at the 0.05 level and is displayed in bold.

Weak correlations were obtained between antioxidant activity and collagen, respectively, HC/P ratio, as well as between collagen and *p*H. The antioxidant properties of peptides are closely related to amino acid composition [28]. The hydrolysis time may have affected the amino acid composition by maintaining the level of collagen amino acids; possibly some amino acids as arginine, histidine, lysine, which are involved in DDPH radical scavenging activity, were altered by thermal treatment.

Characterization of precipitated collagen
In its native form, collagen is insoluble in water. The hydrolysis of collagen to smaller peptides increases the solubility, so the collagen hydrolysate is soluble in water (HC

solutions), which is also an advantage for its applications (for example, it is easy to incorporate into various products, etc.). So, the HC extracts can be used as obtained, without requiring the collagen separation. In our study, the precipitated HC was obtained to be analyzed by FTIR spectroscopy for information about its structure.

The IR spectra of collagen samples (Figure 2) contain the characteristic bands of proteins, assigned to the amide group. In addition to the characteristic bands of amides, bands assigned to other functional groups can also be found in the IR spectrum of collagen, but structural changes can only be correlated with the characteristic bands of amides. The characteristic amide bands which can be found in the IR spectra of the precipitated collagen samples are as follows [39, 40]:

1. The amide bands A and B, situated at around 3300 cm^{-1} (3500 - 3300 cm^{-1}), respectively 3070 cm^{-1}, are associated with N-H stretching vibrations (ν_{NH}). The amide band A is unaffected by the protein conformation, but its position depends on the strength of the hydrogen bond. The second component of the amide band A is the amide band B, a weak band between 3100 and 3030 cm^{-1}, which can be correlated with the stretching vibration of NH groups connected by intramolecular hydrogen bonds.
2. The amide I band, at around 1650 cm^{-1} (1700 - 1600 cm^{-1}), which is assigned mainly to the C=O stretching vibration of the peptide bond, is very sensitive to the secondary structure of collagen, particularly the triple helix. The structure of polypeptides can be related to the FTIR spectrum, mainly due to the amide I band, the nature of the side chain having very little effect on the amide I vibration. Furthermore, the hydrolysis, which can occur during the extraction of collagen by the breaking peptide bonds, can be related to the FTIR spectrum through changes in the amide bands.
3. The amide II band, at around 1550 cm^{-1} (1570 - 1470 cm^{-1}), is assigned to C-N stretching and N-H bending vibrations. Side-chain vibrations barely affect it, and the relationship between band position and protein secondary structure is more complicated than it is for the amide I vibration.
4. The amide III band, at 1400 - 1200 cm^{-1}, is mainly assigned to C-N stretching and N-H bending vibrations. Because it depends on side-chain structure and N-H bending contributes to many modes in the 1400-1200 cm^{-1} domain, the composition of this vibration mode in polypeptides is more complex. The amide III mode can be utilized for secondary structure prediction despite side chain contributions.

All four bands characteristic of amides were found in the FTIR spectra of HC precipitated from **1** - **4** samples (Figure 2).

The number and position of the bands that constitute each characteristic spectral interval were determined using the second derivative spectra (Table 4) [21].

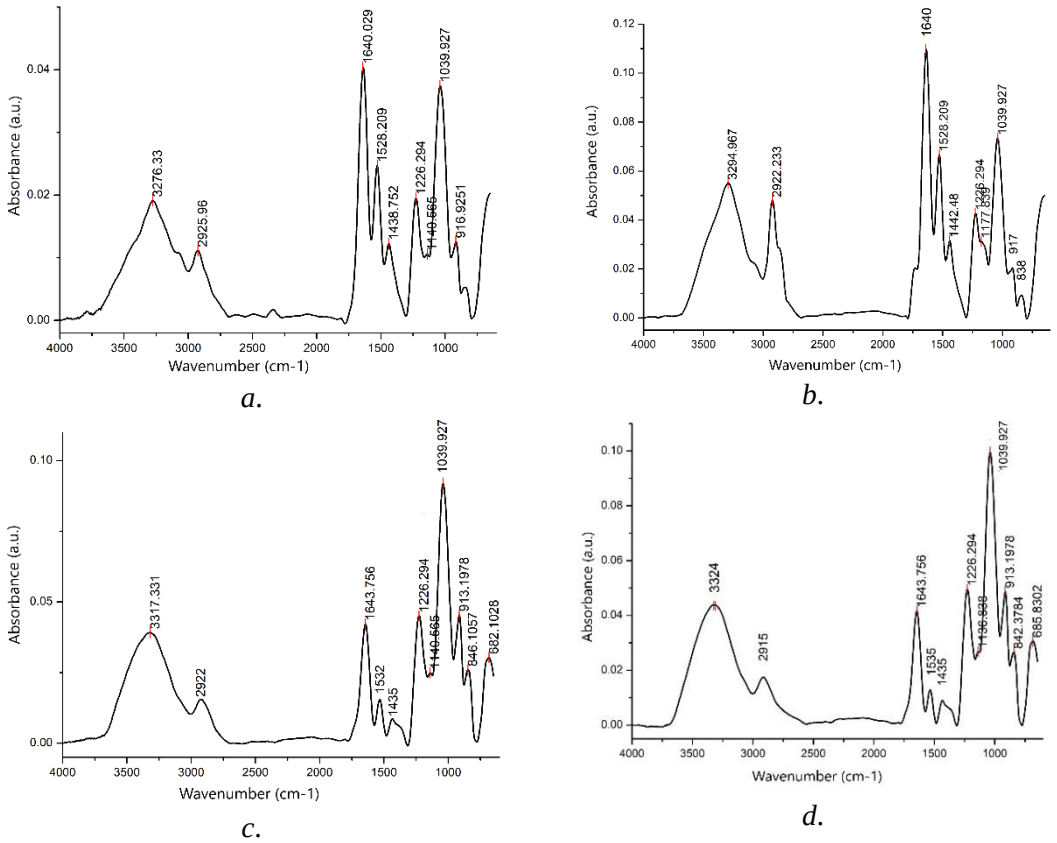

Figure 2. *FTIR spectrum of collagen precipitated from samples* ***1*** *(a),* ***2*** *(b),* ***3*** *(c) and* ***4*** *(d)*

Table 4. *The position of characteristic bands of precipitated HC obtained from the second derivative of FTIR spectra*

Sample	Amide I [cm^{-1}]	Amide II [cm^{-1}]	Amide III [cm^{-1}]
1 (4 h)	692, 1677, 1658, 1640, 1625	1546, 1528, 1509, 1494, 1480	1375, 1334, 1226
2 (6 h)	1658, 1640, 1625	1546, 1524, 1498, 1480	1375, 1334, 1278, 1234
3 (8 h)	1692, 1681, 1666, 1644	1554, 1535, 1502, 1480	1382, 1275, 1230
4 (10 h)	1658, 1640, 1625	1550, 1546, 1524, 1509, 1498, 1480	1375, 1263, 1219

The bands situated at 1450 - 1430 cm^{-1} (*i.e.*, 1438 (**1**), 1442 (**2**), 1435 (**3**), and 1435 cm^{-1} (**4**)), which can be assigned to pyrrolidine ring vibration of proline and hydroxyproline [41], are also found in the FTIR spectrum of collagen samples. All spectra exhibited absorptions at about 1040 cm^{-1}, assigned to ν(C-O) and ν(C-O-C) vibrations of the organic molecules, which were also identified in the spectra of other collagen samples [42]. By comparing the IR spectra, some correlations can be made with collagen hydrolysis. For example, it was evidenced that the higher intensity of the vibrations of ~ 1040 cm^{-1}, the longer hydrolysis time can be correlated. These results agree very well with the literature [3]. The bands assigned to the asymmetric (ν_{as}) and symmetric (ν_s) stretch of aliphatic C-H are situated in the range of 3000 - 2850 cm^{-1}, their intensity being proportional to the number of C-H bonds. The presence of bands in the FTIR spectra of

collagen samples can also be due to the retention of ethanol in HC, the precipitates being washed with ethanol. However, it is known that the bands assigned to the stretching vibrations of O-H group are found in the region of 3650 - 3200 cm^{-1}, the broad band at 3550 - 3200 cm^{-1} indicating the presence of hydrogen-bonded O-H groups. Therefore, the O-H stretching band can overlap with the N-H stretching band of amines and amides, specific with amide A band of collagen. The broader bands found in the FTIR spectra of HC samples may be due to the presence of the OH group, which could possibly originate from molecules of water and/or ethanol, considering the method used for HC separation from the extracts. The comparison between HC FTIR spectra is based on the fact that all samples were subjected to an identical treatment, except for the hydrolysis time.
The ratio between the intensity of collagen characteristic bands can provide some additional information about its structure. Thus, the hydrolysis degree can be correlated with the ratio of the band intensities $A_{\sim3300}/A_{\sim1630}$ [43]. Nevertheless, the value of $A_{\sim3300}$ can be affected by the amount of water present in the collagen sample, so the drying of the precipitated HC is a factor that can influence the ratio value; consequently, these quantitative results should be approached with carefulness.

Table 5. *The IR absorption ratios*

Sample	$\mathbf{A_{OH}/A_I}$ $\mathbf{[A_{\sim3300}/A_{\sim1630}]}$	$\mathbf{A_I/A_{OH}}$ $\mathbf{[A_{\sim1630}/A_{\sim3300}]}$	$\mathbf{\Delta\tilde{\nu}}$ $\mathbf{[cm^{-1}]}$
1 (4 h)	0.4737	2.1107	112
2 (6 h)	0.5025	1.9898	112
3 (8 h)	0.9284	1.0771	112
4 (10 h)	1.0522	0.9503	109

The ratio values calculated for collagen precipitated from samples **1-4** (Table 5) revealed an increase of the hydrolysis degree with the hydrolysis time. The cross-linking degree can be associated with the ratio of the band intensities $A_{\sim1630}/A_{\sim3300}$, a high degree of cross-linking being estimated for high values of this ratio [43]. A decrease in the ratio with the hydrolysis time revealed the diminution of the cross-linking degree during the hydrolysis, as expected. The difference between the wavenumbers for amide I and amide II bands can be correlated with the distortion process, which is considered to have occurred if the difference is greater than 100 cm^{-1} [43]. It can be seen from Table 5 that the values are similar or even identical and therefore cannot be correlated with the extraction time. Since all these values are greater than 100, it is assumed that a distortion of the peptide chain occurred during the heat treatment.

CONCLUSIONS

Hydrolyzed collagen, a soluble form of collagen with a smaller peptide chain and lower molecular mass, is a valuable source of essential amino acids. It is a biomaterial with wide applications in several industries but also present in the daily consumption of the population. We used porcine meat processing by-products as a source of collagen and obtained HC without using any chemical reagents, the collagen hydrolysis with HC obtaining being due only to temperature. Under these conditions, we varied the heating time, and the highest quantity of HC, estimated by spectrometric determination of hydroxyproline, was found after 8 h. All the obtained extracts had antioxidant activity,

mainly due to the hydroxyproline. In addition, it was observed that hydrolyzed collagen showed a significant correlation with some compounds, such as amino acids resulting from hydrolysis or ions released in extracellular fluids. Both the electronic spectra of the extracts and the FTIR spectra of precipitated HC demonstrated the increase in the degree of hydrolysis with heating time, but also the presence of amino acids in the obtained extracts. The raw materials used proved to be good sources for HC in facile and cheap conditions, in accordance with a circular economy.

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