

## Optimization of a Multi-Step Procedure for Isolation of Chicken Bone Collagen

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### Abstract

Chicken bone is not adequately utilized despite its high nutritional value and protein content. Although not a common raw material, chicken bone can be used in many different ways besides manufacturing of collagen products. In this study, a multi-step procedure was optimized to isolate chicken bone collagen for higher yield and quality for manufacture of collagen products. The chemical composition of chicken bone was 2.9% nitrogen corresponding to about 15.6% protein, 9.5% fat, 14.7% mineral and 57.5% moisture. The lowest amount of protein loss was aimed along with the separation of the highest amount of visible impurities, non-collagen proteins, minerals and fats. Treatments under optimum conditions removed 57.1% of fats and 87.5% of minerals with respect to their initial concentrations. Meanwhile, 18.6% of protein and 14.9% of hydroxyproline were lost, suggesting that a selective separation of non-collagen components and isolation of collagen were achieved. A significant part of impurities were selectively removed and over 80% of the original collagen was preserved during the treatments.

**Keywords:** chicken bone, collagen, isolation, optimization

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### Introduction

Collagen is the main fibrous protein in skins, tendons, and bones of animals (Brinckmann, 2005). It is a heavy weighted protein and not water soluble due to its hydrophobic nature (Ergel and Bachinger, 2005). Insoluble collagen must be pretreated before converted into a soluble form such as gelatin. This is usually done by heating in water at temperatures higher than 40°C, which is known as shrinking temperature of collagen (Schrieber and Gareis, 2007). Collagen products are manufactured from skins and bones of animals. The most common raw materials used in collagen products are pork skin and bones. Due to objections against to use of pork in collagen products, alternative resources gained tremendous attention from the researchers.

Poultry processing by-products might be used as alternative raw materials in collagen products. Poultry processing industry showed enormous development in the last decades. Chicken, as one of the poultry species, is currently the number one meat animal in the US because of its

amount of consumption (USDA, 2011). Chicken processing creates a very large amount of waste, some of which have high amount of nutritive components. Chicken bone is one of these wastes, which is mostly not utilized or is limitedly used in manufacturing of animal feeds, pet foods and fertilizers. Some countries prohibit use of animal wastes in feed production and apply legal limitations (Lefferts *et al.*, 2007). Therefore, alternative ways in utilization of chicken bone are highly desirable. One of these alternatives might be use in manufacturing of collagen products. However, bone is a very complex composite material including fibrous collagen surrounded by an extremely dense material, calcium apatite crystals (Meyers *et al.*, 2008). Not only the minerals but also the fat, as two major components of the bone dry matter beside the protein, need to be removed prior to the manufacturing of collagen products to obtain the highest yield and quality. Organic part of the bone, which is mostly protein, may account up to 35% of the bone. About 70% of the organic part is protein and about 90% of this protein is collagen, which may account up to 20% of the bone tissue (Hawkins, 2001; Ockerman and Hansen, 1988; Sealy *et al.*, 2014).

The objective of this study was to design a multi-step procedure for isolation of chicken bone collagen and to

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optimize the treatment conditions keeping the highest amount of collagen isolated. For this purpose, immersion of bone samples in hot water (Cleaning, CL) was utilized to help in removing impurities including blood and non-protein components. Acid treatment (Demineralization, DM) was used to weaken the mineral part of the bone and to remove calcium phosphates (Hosseini-Parvar *et al.*, 2009). And, solvent extraction (Degreasing, DG) was applied for removing fats and grease (Zhang *et al.*, 2010). These three successive treatments were studied at varying temperatures and for varying treatment times to remove non-collagen components selectively. There were 2 independent factors at 5 levels for each treatment. And, 2 dependent variables were selected for evaluation of the corresponding treatment. These dependent variables were the protein loss (%) for all treatments; the weight loss (%), the mineral loss (%), and the fat loss (%) for the treatments of CL, DM, and DG, respectively.

## Materials and Methods

Whole tibia bones of broiler chickens (5-6 wk old) were used as the starting material. This was the bone in the middle part of a chicken leg called 'drumstick' (Fig. 1). Bone samples were obtained from a local meat market and brought to the laboratory for further processing. After remaining muscles and ligaments were removed using a knife, bone samples were stored at  $-18^{\circ}\text{C}$  until use (within 30 d) in treatments. All treatments were duplicated and measurements were done at least in triplicate. All reagents used were of analytical grade and obtained from Sigma (USA) and Merck (USA).

### Chemical composition of chicken bone

Thawed bone samples were ground using a heavy-duty blender (Waring 7011HS, USA) to increase the surface area before the analyses of chemical composition (Fig. 1). Moisture, protein, fat, and mineral content of ground bone

samples were determined according to AOAC (2000) methods. Protein content was calculated based on the total nitrogen estimated by the Kjeldahl method (AOAC method 984.13), in where a factor of 5.4 was used for nitrogen-protein conversion as the most abundant protein in bone is collagen and the nitrogen content of collagen is about 18.5% (Muyonga *et al.*, 2004). Fat content was determined by using a solvent extraction unit (Ankom XT15, USA), where n-hexane was the solvent (Bligh and Dyer, 1959). Moisture content was determined based on the weight difference after drying the samples in an air circulating oven (Mikrotest MKD420, Turkey) at  $105^{\circ}\text{C}$  until constant weight was obtained (AOAC method 927.05). Mineral content was determined by incineration of the samples in a muffle furnace (Nüve MF106, Turkey) at  $550^{\circ}\text{C}$  for 7 h, which was the typical incineration time determined in preliminary studies (AOAC method 942.05).

### Treatments of cleaning, demineralization, and degreasing

Frozen bone samples were thawed at  $4^{\circ}\text{C}$  overnight, and then the treatments of CL, DM, and DG were carried out successively at different conditions according to the study design. The level of independent variables was determined based on the trials and preliminary studies. Independent variables and their levels are given in Table 1. For CL treatment, about 50 g (3 pieces) of whole 'tibia' bone was placed into a 500 mL erlenmeyer flask. Then, 250 mL (1:5, w/v) of distilled water previously heated to the set temperature was added. After immersion of bone samples in hot water at varying temperatures for different times, the content of the flask was filtrated through 4 layers of cheesecloth. Immersion solution was examined for its protein content by the Biuret method for protein loss calculations. The bone residue was washed with tap water (1:5, w/v) for 3 times, filtrated through 4 layers of cheesecloth, dried by paper towel to remove excessive water, and weighted for weight loss calculations.

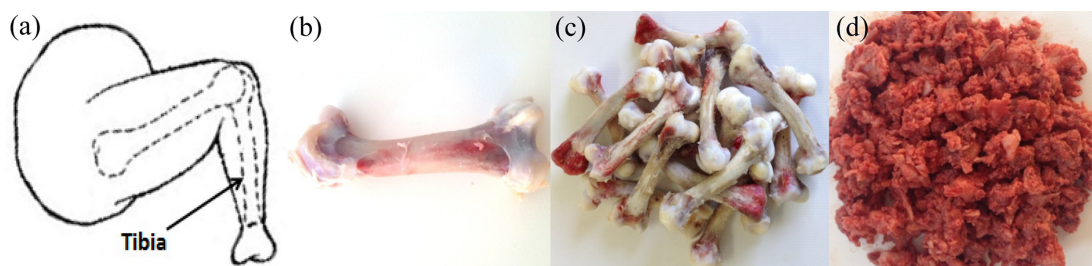


Fig. 1. Schematically represented location of the drumstick bone 'tibia' (a), the actual photo of the bone (b), a bulk of the bones used in the treatments (c), and the ground bone tissue used for chemical composition analyses (d).

**Table 1. Independent variables and their levels for the treatments of CL, DM, and DG**

|    | Independent variables         | Levels |    |     |     |     |
|----|-------------------------------|--------|----|-----|-----|-----|
|    |                               | -2     | -1 | 0   | +1  | +2  |
| CL | Temperature (°C)              | 50     | 55 | 60  | 65  | 70  |
|    | Duration (min)                | 45     | 90 | 135 | 180 | 225 |
| DM | HCl Concentration (%)         | 1      | 2  | 3   | 4   | 5   |
|    | Duration of HCl Treatment (h) | 6      | 12 | 18  | 24  | 30  |
| DG | Temperature (°C)              | 20     | 25 | 30  | 35  | 40  |
|    | Duration (h)                  | 6      | 12 | 18  | 24  | 30  |

CL, Cleaning; DM, Demineralization; DG, Degreasing.

Bone residue obtained in the CL treatment was subsequently used in the DM treatment by placing into a 500 mL erlenmeyer flask. Then, a proper amount of HCl (about 240 mL, 1:5, w/v) at varying concentrations was added. DM treatment was carried out at ambient temperature (at  $24\pm 2^\circ\text{C}$ ) for different treatment times according to the study design (Table 1). Upon completion of the DM treatment, flask content was filtrated through 4 layers of cheesecloth. The solution obtained was examined for its protein content for protein loss calculations. Meanwhile, the bone residue was processed as given for CL treatment and tested for mineral content to determine the mineral loss.

Bone residue obtained from the DM treatment was used in the DG treatment. The bone residue placed into a 500 mL flat bottom volumetric flask was mixed with about 150 mL (1:4, w/v) of n-hexane. The bone residues were degreased under continuous shaking at 150 rpm using a shaking incubator (Heidolph, Germany) according to the conditions given in Table 1. Upon completion of the DG treatment, flask content was filtrated through 4 layers of cheese cloth and a small part of the extract was used for the analysis of protein content to determine the protein loss. For determination of the fat separated, the solvent was collected from the extract by using a rotary evaporator (IKA, Germany). Then, excessive solvent was removed by holding the extract at  $60^\circ\text{C}$  for an hour. Solvent used in DG treatment was changed every 6 h and the fat loss (%) was calculated accordingly based on the initial amount of bone fat. Throughout the treatments of CL, DM, and DG; hydroxyproline (HYP) concentration was followed in the extracts along with the protein concentration of the bone residues, to follow the HYP loss and to verify the protein loss, respectively.

#### Calculation of weight, protein, mineral and fat loss values

The weight loss (%) was calculated based on the difference between the initial weight of the original bone and the bone residues after the corresponding treatments. Pro-

tein loss (%) was calculated based on the difference between the initial protein content of the bone determined by the Kjeldahl method and the protein content determined in the extracts after each treatment, which was measured by the Biuret method. Protein loss was also verified by measuring the protein concentration of the bone residues by the Kjeldahl method. The verification was not for all the samples but just for the samples from experimental runs at center points, to keep the number of experiments feasible. Mineral loss (%) was determined in a similar way, based on the difference between the initial mineral content of the original bone and the mineral content of the bone residue after the DM treatment. Mineral loss was also verified by the mineral analysis of the extracts after the DM treatment only for those experimental runs at center points. Fat loss (%) was similarly calculated based on the difference between the fat content of the original bone and the amount of fat separated in the extraction solutions after the DG treatment. After removing solvent as described, the fat amount separated was gravimetrically measured. Fat content of the bone residues after the DG treatment were also measured by the Soxhlet method at center points to verify the amount of the fat loss.

#### Determination of protein and hydroxyproline content

The protein concentration of the extracts was determined using the Biuret method as described by Gornall *et al.* (1949). Bovine serum albumin (BSA) was used as the standard in the range of 0 to 1 mg/mL (Zhou and Regenstein, 2006). The dry BSA powder was corrected for its salt and water content by calibration based on the absorbance of BSA at 280 nm (absorbance of BSA at 280 nm is 6.66 for a 1% BSA solution) with the absorbance at 320 nm subtracted as a background scattering correction (Regenstein and Regenstein, 1984).

Hydroxyproline (HYP) content was followed in both the bone residues and the extracts after the treatments of CL, DM, and DG at the center points thus, the HYP loss (%)

was determined and verified throughout the treatments. HYP content of the bone residues and the extracts was determined based on the method given by Woessner (1961), in which L-hydroxyproline was used as the standard. 2 g of bone sample was used for hydrolysis while the sample was 2 mL of the extract in case of liquid samples. An appropriate amount of HCl was used for samples to obtain a final concentration of 6 N HCl during hydrolysis. Proper dilutions were prepared after hydrolysis and HYP concentration of each sample was calculated based on the absorbance obtained at 557 nm (Boran and Regenstein, 2009; Woessner, 1961).

### Statistical analysis

Response surface methodology was used for the design and data analysis. Response surface methodology is a mathematical modeling technique that relates independent and dependent variables, and establishes regression models that describe the interrelations between input parameters and output responses (Yang *et al.*, 2007). The JMP 8.0 statistics software (SAS, USA) was used to analyze the data obtained to produce analysis of variance (ANOVA) tables, to determine significant regression terms for regression models, and to draw the surface plots for all dependent variables. Sample order was completely randomized as given in Table 2. A 2 factor 5 level 2 center point central composite design was duplicated separately for all

3 treatments. This requires 20 experimental runs for each treatment, which makes 60 runs in total. The desirability function was utilized for a multi-objective optimization task to determine optimum levels of independent variables.

## Results

Chemical composition of the tibia bone and the bone residues obtained after the treatments are given in Table 3. According to the results, dry matter of chicken bone is composed of 3 major components including protein (36.7%), minerals (34.6%) and fats (22.4%), suggesting that there are 2 major components to be removed for collagen isolation, namely fats and minerals. HYP concentration of the original bone samples was calculated to be 1.35% on wet weight basis.

Effects of the treatments on chemical composition and HYP concentration are summarized in Table 4. The results showed that minerals were effectively removed while fat removal was not quite complete considering the cumulative amount of fat separated. While mineral and fat were removed, about 19% of the protein and 15% of the HYP were lost, suggesting that the fat and the minerals were selectively separated (Table 4). Although each treatment removed some fat, the CL step was the most effective. The cumulative amount of fat separated was over 57% of the total present. Mineral separation was more successful

**Table 2. Randomized order of experimental runs and the coded levels of independent variables**

| Order | Cleaning |     |      | Demineralization |     |         | Degreasing |     |      |
|-------|----------|-----|------|------------------|-----|---------|------------|-----|------|
|       | Pattern  | Dur | Temp | Pattern          | Dur | HCl Con | Pattern    | Dur | Temp |
| 1     | 00       | 0   | 0    | a0               | -2  | 0       | +          | 1   | -1   |
| 2     | 0a       | 0   | -2   | A0               | 2   | 0       | 0a         | 0   | -2   |
| 3     | 00       | 0   | 0    | 0A               | 0   | 2       | +          | -1  | 1    |
| 4     | a0       | -2  | 0    |                  | -1  | -1      | +          | -1  | 1    |
| 5     |          | -1  | -1   | a0               | -2  | 0       | +          | 1   | -1   |
| 6     | +        | 1   | -1   |                  | -1  | -1      |            | -1  | -1   |
| 7     | 0A       | 0   | 2    | +                | 1   | -1      | 0A         | 0   | 2    |
| 8     | 0a       | 0   | -2   | 00               | 0   | 0       | A0         | 2   | 0    |
| 9     | +        | -1  | 1    | 0A               | 0   | 2       | 0A         | 0   | 2    |
| 10    |          | -1  | -1   | ++               | 1   | 1       | a0         | -2  | 0    |
| 11    | 00       | 0   | 0    | 00               | 0   | 0       | A0         | 2   | 0    |
| 12    | A0       | 2   | 0    | 00               | 0   | 0       |            | -1  | -1   |
| 13    | +        | 1   | -1   | +                | 1   | -1      | 00         | 0   | 0    |
| 14    | a0       | -2  | 0    | +                | -1  | 1       | 0a         | 0   | -2   |
| 15    | 00       | 0   | 0    | A0               | 2   | 0       | 00         | 0   | 0    |
| 16    | 0A       | 0   | 2    | ++               | 1   | 1       | 00         | 0   | 0    |
| 17    | ++       | 1   | 1    | 00               | 0   | 0       | a0         | -2  | 0    |
| 18    | A0       | 2   | 0    | 0a               | 0   | -2      | ++         | 1   | 1    |
| 19    | ++       | 1   | 1    | +                | -1  | 1       | ++         | 1   | 1    |
| 20    | +        | -1  | 1    | 0a               | 0   | -2      | 00         | 0   | 0    |

a, -, 0, +, A represent the levels of the variables in the ascending order (Dur: Duration, Temp: Temperature, Con: Concentration).

**Table 3. Composition of bone and bone residues after treatments of CL, DM, and DG (%)**

| Sample       | Moisture                | Crude fat | Crude mineral | Crude nitrogen | Protein (N×5.4) | Total organic matter | Total dry matter |
|--------------|-------------------------|-----------|---------------|----------------|-----------------|----------------------|------------------|
| Chicken Bone | 57.5 <sup>1)</sup> ±0.2 | 9.5±0.1   | 14.7±0.4      | 2.89±0.10      | 15.6±0.5        | 27.8                 | 42.5             |
| CL Residue   | 57.4±0.2                | 7.2±0.2   | 15.0±0.3      | 2.88±0.05      | 15.5±0.2        | 27.6                 | 42.6             |
| DM Residue   | 67.0±0.1                | 7.5±0.2   | 2.8±0.2       | 3.04±0.04      | 16.4±0.2        | 30.2                 | 33.0             |
| DG Residue   | 70.1±0.3                | 5.1±0.1   | 2.3±0.1       | 2.94±0.03      | 15.9±0.2        | 27.6                 | 29.9             |

<sup>1)</sup>Values were given based on wet weight of fresh bone and bone residues of each treatment. Values were average values and standard deviation of triplicate measurements. Values given were for CL, DM and DG residues obtained at optimum treatment conditions.

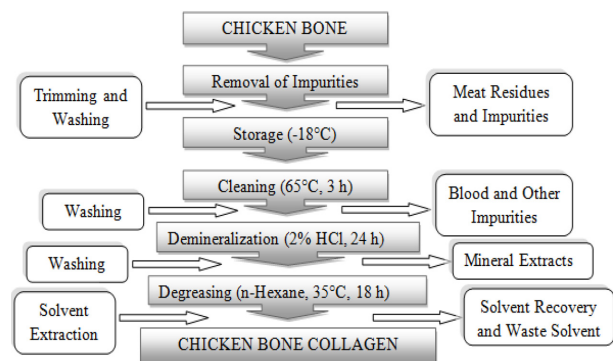
**Table 4. Cumulative loss in macro-nutrients and HYP content during CL, DM, and DG**

| Sample       | Weight loss (%)   | Fat loss (%) | Mineral loss (%) | Protein loss (%) | HYP loss (%) | HYP con (%) | HYP/Pro ratio (%) |
|--------------|-------------------|--------------|------------------|------------------|--------------|-------------|-------------------|
| Chicken Bone | NA                | NA           | NA               | NA               | NA           | 1.35 ±0.02  | 8.66              |
| CL Residue   | 6.1 <sup>1)</sup> | 28.9         | 4.0              | 6.4              | 13.8         | 1.24 ±0.03  | 7.98              |
| DM Residue   | 17.9              | 35.2         | 84.2             | 13.4             | 14.3         | 1.41 ±0.07  | 8.58              |
| DG Residue   | 20.2              | 57.1         | 87.5             | 18.6             | 14.9         | 1.44 ±0.06  | 9.06              |

<sup>1)</sup>Values were average of triplicate measurements at optimum conditions. Loss values were all given based on initial amount of corresponding component and their concentration in bone residues obtained after each treatment. HYP concentration was given based on wet weight of initial bone or bone residue. HYP/Pro ratio was given based on HYP and protein content of fresh bone and bone residues of each treatment. NA, Not applicable.

as the cumulative amount of mineral separated was almost 88% of the total present. The DM treatment seemed to be very effective in removing minerals from the bone. During the treatments, a gradual increase in the relative amount of HYP and HYP/Pro ratio was observed in the bone residues, suggesting that non-collagen protein was slightly removed. Optimization studies on the overall results showed that optimum treatment conditions were as follows: immersion of the bone samples in distilled water at 65°C for 90 min in the CL process, followed by a demineralization step using 2% HCl solution for 24 h in the DM process, and finally fat removal using n-hexane at 35°C for 18 h in the DG process. Schematic process flow of this isolation procedure is given in Fig. 2.

Experimental results were analyzed using JMP 8.0 software to obtain the regression models for each dependent variable. Predicted results based on these regression models are given in Table 5 along with the experimental data obtained. Experimental results and response surface plots (Fig. 3) showed that increasing temperature in the CL step increased the weight loss in the bone residues while also increasing the amount of protein loss. The effect of the duration was limited compared to that of the temperature in CL treatment (Fig. 3(a) and 3(b)). As minimum protein and maximum weight loss were aimed, the higher temperatures seemed to be more effective in removing impurities but also responsible for higher protein loss. Therefore, mild treatment temperatures for relatively short duration seemed to be preferable.

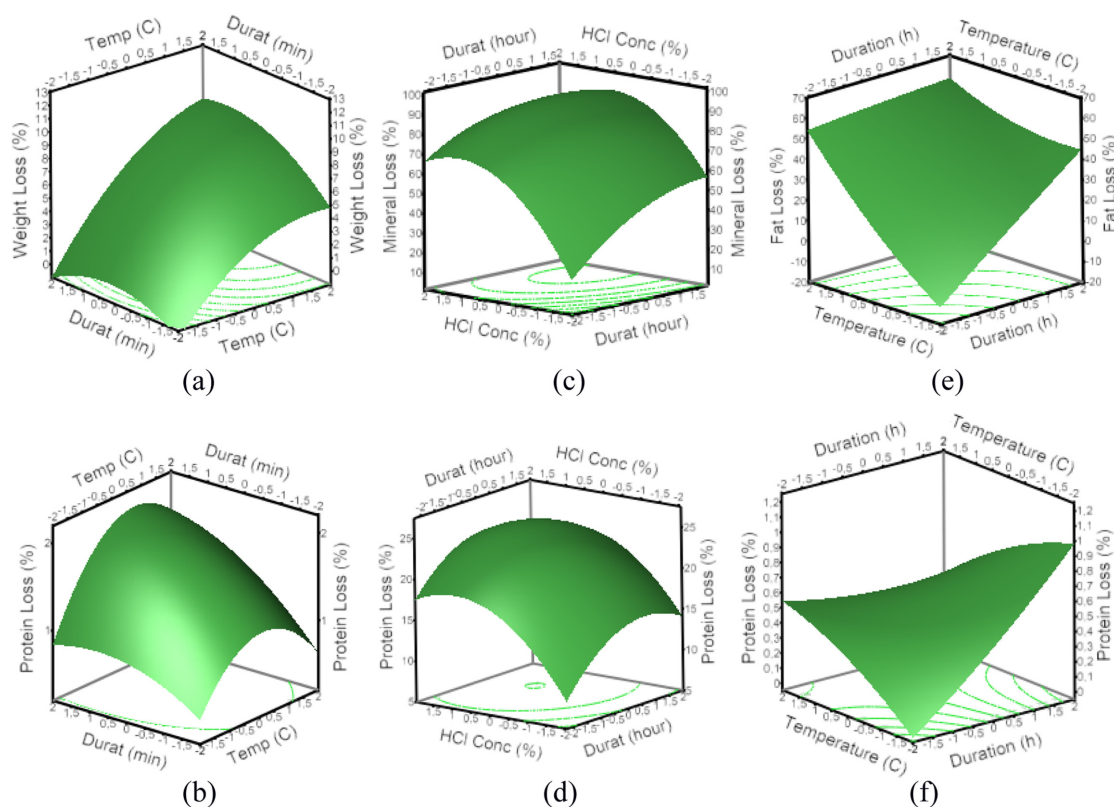
**Fig. 2. Optimized process flow for isolation of chicken bone collagen.**

The weight loss (%) was a dependent variable only in the CL treatment and not in successive treatments but it was followed throughout the treatments as the loss in protein, HYP, mineral and fat was only possible to be calculated if the weight of the corresponding bone residue is known after each treatment. With respect to the DM process, the levels of both HCl concentration and the treatment duration had significant effects on dependent variables. The highest amount of mineral was removed around at the middle levels of independent variables. These middle levels also caused the highest amount of protein loss (Fig. 3(c) and 3(d)). Therefore, low HCl concentration and middle level treatment duration was preferable for effective separation of minerals along with limited loss in the protein.

**Table 5. Experimental and predicted results for dependent variables of CL, DM, and DG**

|    | Cleaning           |      |                  |      | Demineralization |      |                  |      | Degreasing   |      |                  |      |
|----|--------------------|------|------------------|------|------------------|------|------------------|------|--------------|------|------------------|------|
|    | Weight loss (%)    |      | Protein loss (%) |      | Mineral loss (%) |      | Protein loss (%) |      | Fat loss (%) |      | Protein loss (%) |      |
|    | Exp                | Pre  | Exp              | Pre  | Exp              | Pre  | Exp              | Pre  | Exp          | Pre  | Exp              | Pre  |
| 1  | 6.75 <sup>1)</sup> | 6.37 | 1.89             | 1.75 | 64.9             | 67.3 | 19.7             | 19.0 | 30.4         | 34.3 | 0.60             | 0.69 |
| 2  | 3.56               | 3.50 | 1.27             | 1.15 | 90.8             | 90.3 | 22.0             | 22.4 | 16.8         | 17.4 | 0.42             | 0.44 |
| 3  | 6.92               | 6.37 | 1.83             | 1.75 | 87.6             | 82.9 | 24.2             | 23.0 | 36.4         | 38.9 | 0.63             | 0.47 |
| 4  | 1.33               | 0.36 | 1.07             | 0.87 | 71.6             | 64.1 | 18.9             | 19.3 | 37.7         | 38.9 | 0.59             | 0.47 |
| 5  | 1.89               | 3.05 | 0.99             | 1.27 | 66.1             | 67.3 | 19.9             | 19.0 | 31.9         | 34.3 | 0.70             | 0.69 |
| 6  | 7.26               | 6.71 | 1.45             | 1.44 | 71.2             | 64.1 | 19.5             | 19.3 | 15.0         | 12.7 | 0.45             | 0.32 |
| 7  | 6.19               | 5.86 | 1.81             | 1.91 | 83.3             | 78.4 | 22.2             | 21.2 | 60.6         | 57.0 | 0.48             | 0.45 |
| 8  | 3.66               | 3.50 | 1.21             | 1.15 | 86.8             | 87.6 | 25.2             | 24.2 | 50.1         | 47.1 | 0.76             | 0.79 |
| 9  | 2.65               | 3.78 | 1.34             | 1.55 | 85.8             | 82.9 | 24.5             | 23.0 | 60.5         | 57.0 | 0.33             | 0.45 |
| 10 | 1.97               | 3.05 | 0.97             | 1.27 | 88.5             | 92.8 | 23.9             | 24.7 | 15.9         | 16.7 | 0.20             | 0.33 |
| 11 | 6.83               | 6.37 | 1.76             | 1.75 | 85.0             | 87.6 | 24.8             | 24.2 | 52.4         | 47.1 | 0.90             | 0.79 |
| 12 | 7.24               | 8.58 | 1.33             | 1.41 | 85.9             | 87.6 | 24.5             | 24.2 | 15.6         | 12.7 | 0.40             | 0.32 |
| 13 | 7.05               | 6.71 | 1.49             | 1.44 | 84.1             | 78.4 | 22.0             | 21.2 | 34.1         | 32.3 | 0.47             | 0.52 |
| 14 | 1.42               | 0.36 | 1.08             | 0.87 | 82.2             | 84.2 | 21.0             | 23.3 | 16.1         | 17.4 | 0.44             | 0.44 |
| 15 | 6.79               | 6.37 | 1.83             | 1.75 | 90.1             | 90.3 | 22.5             | 22.4 | 34.9         | 32.3 | 0.53             | 0.52 |
| 16 | 6.23               | 5.86 | 1.98             | 1.91 | 89.2             | 92.8 | 23.2             | 24.7 | 35.6         | 32.3 | 0.43             | 0.52 |
| 17 | 8.89               | 8.35 | 2.03             | 1.91 | 85.9             | 87.6 | 24.7             | 24.2 | 14.4         | 16.7 | 0.28             | 0.33 |
| 18 | 8.81               | 8.58 | 1.26             | 1.41 | 46.2             | 48.3 | 14.8             | 15.6 | 39.7         | 47.7 | 0.63             | 0.56 |
| 19 | 8.23               | 8.35 | 2.06             | 1.91 | 82.2             | 84.2 | 21.4             | 23.3 | 40.6         | 47.7 | 0.50             | 0.56 |
| 20 | 2.18               | 3.78 | 1.37             | 1.55 | 39.3             | 48.3 | 15.1             | 15.6 | 34.6         | 32.3 | 0.42             | 0.52 |

<sup>1)</sup>All values of protein, mineral and fat loss were given based on the difference between their concentrations in initial bone sample and in extraction solutions obtained after each treatment.



**Fig. 3. Response surface plots for weight (a) and protein (b) loss of CL, mineral (c) and protein (d) loss of DM, fat (e) and protein (f) loss of DG treatments.**

Considering the results of the DG process, the treatment duration was effective at high temperatures for both fat and protein removal; and longer treatment durations caused higher loss in both fat and protein content (Fig. 3(e) and 3(f)). Temperature was very effective as increasing temperature caused higher protein loss. Increasing temperature was also effective in fat removal. Thus, relatively high temperature and short treatment duration were preferable for effective separation of fat along with limited loss in the protein.

The isolation procedure determined for chicken bone collagen was verified by running the procedure at optimum conditions given. After the isolation procedure carried out at optimized conditions, about 82% of the original protein and 85% of the initial HYP were preserved in the final bone residue while over 88% of the initial mineral content and about 57% of fats were selectively removed. The final bone residue was about 80% of the original bone in weight with a higher percentage of protein and HYP as given in Table 3 and 4. The highest HYP concentration found in treated bone residues was 1.44% and the highest HYP/Pro ratio was 9.06 as given in Table 4.

## Discussion

Chemical composition of the bone tissue changes greatly depending on species, age, diet, nourishment and from bone to bone of the same animal. Field *et al.* (1974) reported that the age and the species were important factors significantly affecting the composition of animal bone, i.e., the older the age the higher the fat and the dry matter of the bone. They reported similar fat content for chicken bone but a bit higher dry matter (49.9%) compared to the value (42.5%) reported in this study, probably because the bone samples used in this study were from younger animals. In another study, chemical composition of the chicken bone was reported 53.2% moisture, 8.4% fats, 20.5% protein (conversion factor for Kjeldahl nitrogen was 6.25) and 15.9% minerals (Kettawan *et al.*, 2002). Although moisture and fat content reported in our study was similar, protein content was lower most probably due to the conversion factor used in this study. As previous studies reported that over 90% of the bone protein is collagen, 5.4 was the factor used for nitrogen-protein conversion in this study to estimate the protein concentration of the chicken bone (Brinckmann, 2005; Fratzl, 2008; Schrieber and Gareis, 2007). However, this might slightly underestimate the protein content of the bone as there are obviously other proteins beside collagen in bone tissue.

Hydroxyproline is used for estimation of collagen content as it is a unique iminoacid for collagen (Fratzl, 2008). Other than collagen, elastin has some HYP but at lower levels compared to collagen (Eastoe and Leach, 1977). It is reported that HYP ratio in proteinaceous material of bone samples from different species was close to 10% (Herpandi *et al.*, 2011; Li *et al.*, 2009). Therefore, when an arbitrary factor of 10 was used for conversion of HYP to collagen, this would conclude that about 90% of chicken bone protein was collagen, which is consistent with previous reports for bone tissues from various animals. Thus, removing fats and minerals would predominantly leave the collagen isolated in the remaining bone residue as intended.

Demineralization is a key process when bone protein is intended to be isolated. In the literature, acid treatment looks very common and HCl is one of the most common inorganic acids used for this purpose (Castro-Ceseña *et al.*, 2011; Figueiredo *et al.*, 2011). In this study, HCl was used for demineralization of the bone samples at several concentrations for varying treatment durations. HCl was very effective in demineralization of the bone, removing about 88% of the initial bone minerals. The amount of mineral separated was consistent with previously reported values (Castro-Ceseña *et al.*, 2011; Figueiredo *et al.*, 2011).

High pressure or hot water treatment is common in the literature for fat removal from various bones (Hosseini-Parvar *et al.*, 2009; Nawrocki, 1997). Our preliminary studies, on the other hand, showed that immersion of the bone samples as whole in hot water had very limited success in degreasing. In addition, initial immersion in hot water increases the porosity, which may harm the collagen. Boiling, on the other hand, is more effective in degreasing of the bones, but may cause extensive and irreversible damage on the collagen, which should be avoided for high yield and quality. Especially when cleaning is insufficient, bones may be degreased in organic solvents (Guilminot *et al.*, 2014). There are some chemicals used for bone degreasing as reported in the literature. For example, Lander *et al.* (2014) used trichloroethylene at 82°C for 3 d for degreasing human bones after boiling the samples in distilled water. Guilminot *et al.* (2014) used hexane, methyl alcohol, and other organic solvents along with other methods including enzymatic treatments and supercritical CO<sub>2</sub> extraction to remove fat from whale bones. They concluded that enzymatic methods and supercritical CO<sub>2</sub> extraction were not much effective in fat removal from the bones. On the other hand, organic solvents gave the best performance in removing fats from



the bones with a reasonable harm on the bone tissue (Guilminot *et al.*, 2014).

Therefore, in this study, the initial hot water immersion was applied for relatively short durations especially for removing color pigments and visible impurities. This treatment was followed by the immersion of the resultant bone residues in HCl solution for removal of minerals, which was very successful. During these two successive treatments, some fat was separated, but still the larger part of the fats was in the bone residue. Therefore, a final step of solvent extraction was applied for removal of the remaining fats. The amount of fats separated was actually calculated based on the amount of fats collected in DG treatment, which was about 57% of the initial fat, suggesting that the greater part of the fats was separated in DG treatment. This was also confirmed by the fat content of the bone residue after DG treatment.

The experimental data was used for optimization of the level of independent variables for each treatment. In general, the desirability function is used as an indicator of how closely the goal (that is, minimizing or maximizing the response or matching a target value) is achieved by the model. The desirability level for each response is set manually and this affects the overall desirability of the results. The prediction profiler of the JMP software was used to obtain the highest desirability for each response, the highest overall desirability, the highest values for maximized responses and the lowest values for minimized

responses, based on the settings given in Table 6.  $R^2$  values obtained for each dependent variable were high in general, indicating that the regression models were successful in describing the treatments. Regression coefficients for all models are given in Table 7. According to the regression models, the dependent variables of each treatment can be estimated using the following equations:

$$\text{Wgh L in CL} = 6.37 + (2.06 \times \text{Temp}) + (0.59 \times \text{Dur}) - (0.47 \times \text{Temp}^2) - (0.42 \times \text{Dur}^2)$$

$$\text{Pro L in CL} = 1.75 + (0.13 \times \text{Temp}) + (0.19 \times \text{Dur}) - (0.15 \times \text{Temp}^2)$$

$$\text{Min L in DM} = 87.56 + (8.66 \times \text{Con}) + (5.73 \times \text{Dur}) - (5.50 \times \text{Con}^2) - (2.19 \times \text{Dur}^2)$$

$$\text{Pro L in DM} = 24.19 + (1.85 \times \text{Con}) + (0.85 \times \text{Dur}) - (1.22 \times \text{Con}^2) - (0.87 \times \text{Dur}^2)$$

$$\text{Fat L in DG} = 32.28 + (9.91 \times \text{Temp}) + (7.60 \times \text{Dur})$$

$$\text{Pro L in DG} = 0.52 + (0.11 \times \text{Dur})$$

According to the models, the linear terms of all independent variables were significant except the temperature for protein loss in the DG treatment. Besides the linear terms, all quadratic terms were significant for both dependent variables in the DM process. The quadratic terms were also significant in the CL treatment only except the duration for protein loss in the CL treatment. On the other hand, the interaction terms were found insignificant in all regression models.

**Table 6. Settings of optimization, predicted results and corresponding  $R^2$  values of the models**

|    | Dependent Variables  | Low | Middle | High | Predicted results | $R^2$ | Desirability <sup>1)</sup> |
|----|----------------------|-----|--------|------|-------------------|-------|----------------------------|
| CL | Weight loss (% max)  | 1.3 | 5.1    | 8.9  | 6.71±1.02         | 0.91  | 0.592                      |
|    | Protein loss (% min) | 0.9 | 1.5    | 2.1  | 1.4±0.2           | 0.82  |                            |
| DM | Mineral loss (% max) | 38  | 78     | 92   | 78.4±5.4          | 0.91  | 0.442                      |
|    | Protein loss (% min) | 10  | 20     | 26   | 21.2±1.3          | 0.88  |                            |
| DG | Fat loss (% max)     | 10  | 40     | 65   | 42.5±3.5          | 0.94  | 0.513                      |
|    | Protein loss (% min) | 0.1 | 0.5    | 0.9  | 0.5±0.1           | 0.72  |                            |

<sup>1)</sup>Desirability of low, middle, and high levels was set to 0.01, 0.60, and 0.99, respectively, for variables maximized (max: maximized) and 0.99, 0.40, and 0.01, respectively, for variables minimized (min: minimized).

**Table 7. Regression coefficients of the models for each treatment**

| Cleaning  |        |        | Demineralization |        |        | Degreasing |        |       |
|-----------|--------|--------|------------------|--------|--------|------------|--------|-------|
| Term      | Wgh L  | Pro L  | Term             | Min L  | Pro L  | Term       | Fat L  | Pro L |
| Intercept | 6.37*  | 1.75*  | Intercept        | 87.56* | 24.19* | Intercept  | 32.28* | 0.52* |
| Temp      | 2.06*  | 0.13*  | Con              | 8.66*  | 1.85*  | Temp       | 9.91*  | 0.01  |
| Dur       | 0.59*  | 0.19*  | Dur              | 5.73*  | 0.85*  | Dur        | 7.60*  | 0.11* |
| Temp×Temp | -0.47* | -0.15* | Con×Con          | -5.50* | -1.22* | Temp×Temp  | 1.23   | -0.02 |
| Dur×Dur   | -0.42* | 0.05   | Dur×Dur          | -2.19* | -0.87* | Dur×Dur    | -0.09  | 0.01  |
| Temp×Dur  | 0.23   | 0.05   | Con×Dur          | -1.41  | -0.14  | Temp×Dur   | -3.18  | -0.07 |

\* denotes significant difference from zero at  $p < 0.05$ . Temp, Temperature; Dur, Duration; Con, Concentration (HCl); Pro, Protein; L, Loss; Min, Mineral; Wgh, Weight.



The optimum isolation procedure was cleaning at 65°C for 90 min, the demineralization in 2% HCl solution for 24 h, and the degreasing by n-hexane extraction at 35°C for 18 h. Under optimum conditions, the multi-step procedure for isolation of chicken bone collagen resulted in 87.5% mineral and 57.1% fat removal along with 18.6% protein and 14.9% HYP loss, which suggests that the chicken bone collagen was selectively separated and isolated to some extent. Verification of the optimized procedure also confirmed the predicted results. The bone samples used in the isolation treatments were whole tibia bones. Therefore, optimum conditions reported in this study would not be valid if the bone samples were ground to increase the surface area. In that case, milder treatments might be required for preservation of collagen in the treated residue. Dry matter of the final bone residue was composed of about 53% protein, which might be slightly underestimated due to the factor used for nitrogen-protein conversion. Thus, a reasonable success was achieved with respect to the isolation of collagen in chicken bone tissue.

### Conclusion

A multi-step procedure was defined and optimized for isolation of chicken bone collagen. Under optimum conditions, this procedure resulted in 87.5% mineral and 57.1% fat removal along with 18.6% protein and 14.9% HYP loss, which suggests that the chicken bone collagen was effectively isolated. Almost 85% of the initial collagen was preserved and isolated in the final bone residue, while other two major components, namely minerals and fats, were selectively removed at reasonable levels. Further studies are needed to characterize the resultant collagen in terms of its quality and functional properties.

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