Evaluation and Characterization of Protein Isolates from Poultry Processing Industries by-Product

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Abstract

Brazil is one of the largest global producers of poultry meat and can thus be considered a large producer of by-products from poultry industries. The objective of this study was to obtain a protein isolate from feather and blood meal and to evaluate the extraction process at different times. The proximal composition of three different feather and blood meals resulted between 4.69-13.59% moisture, 60.17-79.97% protein, 12.76-17.06% fat, and 2.95-10.14% ash on a wet basis. We observed that the extraction for 6 h showed the highest breakage of disulfide bonds. The spectroscopy Fourier transform infrared analysis revealed characteristic bands of protein, and characteristic bands of cysteine and cysteic acid, indicating breakage of disulfide bonds. Differential scanning calorimetry analysis of protein isolate showed that there were two endothermic transitions. With this study, it was possible to obtain protein isolates from feather and blood meal with potential application in processing of proteinous bioplastics.

Keywords: Poultry by-product, protein extraction, keratin, sulfhydryl group.

1. Introduction

According to the Brazilian Poultry Association, the 2014 chicken production in Brazil was 12.7 million tons and the third largest in the world, behind only the US (the world's largest producer) and China. Brazil is the largest exporter of chicken meat (4.1 million tons), with 32.3% of its production destined for export (UBABEF, 2015). With this high production, there is a large amount of by-products and waste from poultry processing industries. One of these by-products are feathers, which are used to produce meal along with blood for the production of animal feed (Eyng *et al*., 2012). The poultry industry produces many waste feathers every year, creating an environmental problem that is difficult to eliminate. Therefore, both economically and environmentally, it is necessary to develop an efficient and profitable process to use these by-products. However, feathers have been mainly used as a low-nutritional-value animal feed (Wang & Cao, 2012). Poultry slaughterhouses produce large amount of feathers, which approximately five percent of the body weight of chicken is feathers. The slaughterhouse with a capacity of fifty thousand poultries can produce 2-3 tons of dry feathers per day (Gupta *et al*., 2012).

Feather meal contains high crude protein content, but 85% to 90% of the protein is keratin, which by virtue of its structure and the large amount of sulfur amino acids has low solubility and high resistance to the action of enzymes, thus necessitating hydrolysis in order to be digested by animals (Scapim *et al*., 2003). Feather meal is obtained by cooking under pressure, cleaning the feathers, and not allowing decomposition, which allows the presence of blood, provided that the percentage included does not significantly alter the composition of meal, according to Brazilian Compendium of Animal Feeding (2005). Reducing agents help decrease the stability of keratin fibers in the solid form found in feathers. These reagents will break down the disulfide bonds, hydrogen bonds, and salt linkages of the keratin fibers and allow dissolution into protein solution.

Currently, there is increasing interest in the development of materials that are environmental friendly and obtained from renewable resources. The main renewable materials are obtained from polysaccharides, lipids, and proteins. Proteins are polymers formed by various amino acids capable of promoting intra and inter-molecular bonds, allowing the resultant materials to have a large variation in their functional properties (Gupta *et al*., 2012). Feather is cheap, renewable, biodegradable, and widely available, but it is underexplored by the industries. Except for applications in animal feed, duvet and down coat, feather is mainly disposed in the landfills as the solid waste, occasioning environmental and economic issues (Jin *et al*., 2011). Several researchers have worked on developing potential materials from feather to resolve the solid waste pollution (Dou *et al*., 2016). Development of chicken feather and blood meal is dedicated to manufacturing animal feed and has not truly been explored for other purposes. As this is a highly protein product, it can be used in the production of various products, such as in the elaboration of biomaterials as biodegradable plastics. The main objective of this study was to evaluate a process for isolation and extraction of proteins from feather and blood meal at different times evaluating the sulfhydryl groups production and to characterize both the raw materials and the final product.

2. Material and Methods

2.1 Raw material

Feather and blood meal produced in chicken processing industries of Southern Brazil. The samples were collected and then maintained under refrigeration until use.

2.2 Proximate composition of feather and blood meals

We performed proximate composition on chicken feather and blood meals from three different poultry industries: moisture was determined in an oven De Leo A1SE (Brazil) to 105°C until constant weight, protein concentration was determined by the micro-Kjeldahl method in a digester Gerhardt Kjeldatherm VA (Germany) with conversion of nitrogen by 6.25, fat concentration was determined in a Soxhlet extractor Quimis Q-308-26B (Brazil) using petroleum ether as solvent, and ash was determined beforehand by charring samples and then incinerating in a muffle Quimis Q318 M24 (Brazil) at 550°C until constant weight according to Association of Official Analytical Chemists (AOAC, 2000).

2.3 Pretreatment: defatting of feather and blood meal

Two methods for the extraction off at from meal samples were tested: first, degreasing was performed according to Schrooyen *et al*. (2001) in a Quimis Q-308-26B Soxhlet extractor (Brazil) at a temperature between 40 and 60°C for 12 h using petroleum ether as a solvent; the second method was carried out according to Wang *et al*. (1999) with some modifications using hexane in a Cientec CT-712RNT shaker (Brazil) and shaking at 125 rpm at 50°C for 30 min. Then, the solvent was exchanged, and the same process was performed three or four times at a ratio of 1:3 (w/v). For all tests, after removal of the solvent at room temperature (25ºC) for 24 h, the defatted meal was sieved into a set of sieves with a maximum particle size of 355 µm, and yields were calculated by considering 100% of the weight of the original sample.

2.4 Obtaining a protein isolate from feather and blood meal

Initially, aliquots of 30 g of defatted feather and blood meal were weighed to perform the method of protein isolation. Obtaining the protein isolate from the meal was conducted according to Gupta *et al*. (2012) with some modifications. First, the defatted feather meal was dissolved using 0.5 M sodium sulfide (1:9 w/v) in a Cientec CT-712RNT shaker (Brazil) at 30°C under stirring at 125 rpm with testing extraction times of 4, 5, 6, 7 and 9 h. The pH of the solution during the process remained alkaline (10 - 13). After dissolving, the solution was filtered through a nylon filter and centrifuged at14308 x *g* in a Hanil 1544-6906 centrifuge (South Korea) for 15 min. The supernatant was collected, and the solid fraction was discarded. The supernatant was measured and submitted to protein precipitation using ammonium sulfate solution (7:10 w/v) in a 1:1 ratio (v/v) by adding it drop wise under stirring with a Fisatom712 propeller shaft stirrer (Brazil) at 700 rpm. Then, the solution was centrifuged in a Hanil1544-6906 centrifuge (South Korea) at 14308 x *g* for 15 min, and the solid particles were collected. The liquid fraction was discarded. The solid content that was collected previously was submitted to washing under stirring with a Fisatom 712 propeller shaft stirrer at 700 rpm with 100 ml of deionized water to obtain the purified protein; it was then centrifuged at 14308 x *g* for 15 min to collect the solid fraction again. The solid fraction was submitted to drying in a Quimis Q314D242 oven (Brazil) with air circulation at 50°C for 18 h. The solids were then weighed and ground in a Tecnal TE-633 Tec Mill knife mill (Brazil) for subsequent storage in closed flasks at room temperature (25ºC).

2.5 Sulfhydryl group's analysis

Sulfhydryl groups were determined by using Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid, 10 mmol/L) according to the procedure described by Shimada & Cheftel (1988) with some modifications. Samples (100 mg) of defatted meal and protein isolates from 4, 5, 6, 7 and 9 h of extraction were homogenized for 3 min with 50 ml of phosphate buffer 0.1mol/L, pH 8.0, containing 1mmol/L EDTA, 6mol/L urea and 0.5% SDS. Then, the mixture was centrifuged at 8667 x *g* in a High Speed Brushless Centrifuge MPW-350 (Poland) for 20 min. To 3 mL of the supernatant, we added 30 µL of Ellman's reagent, and the mixture was then incubated for 15 min at room temperature (25ºC). After the reaction, the absorbance of the mixture was measured at 412 nm in a Biospectro UVSP-22 (Brazil) spectrophotometer, and SH groups were determined using a molar extinction coefficient of 13600 $M^{-1}cm^{-1}$ (Beveridge *et al*., 1986).

2.6 Fourier transforms infrared spectroscopy analysis (FTIR)

The defatted meal samples and protein isolates from different extraction times (5, 6 and 7 h) were submitted to spectroscopy in the infrared region (FTIR) using a Fourier transform infrared spectrometer Prestige-21, Shimadzu (Japan), 4000-400 cm^{-1} region, using the diffuse reflectance technique in potassium bromide (KBr) (Zavareze *et al*., 2014).

2.7 Thermal properties

The thermal properties of the protein isolate extracted to 6 h using differential scanning calorimetry (DSC) were assessed in a Shimadzu DSC-60 calorimeter (Japan). Sample sizes of 2–3 mg were used, and only one heating cycle from 30°C to 300°C at a rate of 10°C/min in a N_2 atmosphere (Barone *et al*., 2006), verifying the peak temperature and the enthalpy (ΔH) of each transition.

2.8 Statistical analysis

The averages were obtained from triplicate experiments and were submitted to analysis of variance (ANOVA) and Tukey's test with at a 95% significance level.

3. Results and Discussion

3.1 Proximate composition

Table 1 shows the values of the proximal composition of three samples of feather and blood meal collected from different chicken processing industries.

Table 1: Proximate composition of chicken feather and blood meal collected from different chicken processing industries.

Components	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)
Dry matter	95.31 ± 0.14^a	86.41 ± 0.15^b	91.41 ± 0.06^c
Protein	63.13 ± 2.07^a	81.39 ± 2.68^b	87.48 ± 0.21^c
Fat	17.53 ± 0.54^a	7.14 ± 0.24^b	9.60 ± 2.04^b
Ash	10.64 ± 0.66^a	3.41 ± 0.01^b	3.78 ± 0.25^b

Identical letters on the same line mean that there was no significant difference between samples (p>0.05).

In the proximate composition analysis, sample 3 showed 8.59 ± 0.6% moisture and 79.97 ± 0.21% protein on a wet basis and it was selected for the degreasing tests and to obtain protein isolates, mainly for the higher content of protein. Almost all samples showed a significant difference, except in fat and ash content for samples 2 and 3.Scapim *et al*. (2003) evaluated feather meal samples and showed moisture values between 12.2 to 12.6% and protein contents between 80.6 to 81%. These results were higher than sample 3 on a wet basis (8.59 and 79.97%, respectively), but only the protein values are presented as the values stipulated by legislation described below. Ash content was between 2.1 to 2.4%, lower than sample 3 (3.78 ± 0.25%).

The Ministry of Agriculture, Livestock and Supply (MAPA) limits the minimum protein content to 80%, the maximum moisture to 8% and the ash content to approximately 3%, in order to characterize the by-product as "feathers meal" (Brazil, 1988). Feather is commonly used for animal feed because practically all feather meal production is designed for the elaboration of animal feed. Although the meal contains not just chicken feathers but also chicken blood, and the legislation allows the addition of blood in the feather meal, the sample that was closest to the Brazilian standards and selected for the protein extraction was sample 3.

Considering that the composition of the animal by-products may vary by processing, type and proportion of its original components, the determination of chemical composition is extremely important for application (Nunes *et al*., 2006).

3.2 Meal yield after defatting

The defatting of meal was realized with two purposes: to remove the fat content of the sample and to allow less interference during the protein isolation process. Furthermore, defatting increases the shelf-life of the product because the presence of fat allows the sample to be susceptible to lipid oxidation. Table 2 shows the yield percentage of defatted samples (Yield 1) and the yield of defatted samples that were then sieved (Yield 2), relative to the initial weight of sample.

Table 2: Yield in percentage obtained from feather and blood meal samples defatted, and defatted and sieved.

Samples	Yield 1 (%)	Yield 2 (%)
1E	86.0 ± 4.3[a]	72.9 ± 2.2[a]
2H	91.3 ± 0.9[a]	73.5 ± 0.1[a]

Yield 1: Yield corresponding to the weight after defatting.
Yield 2: Yield corresponding to the weight after scouring and sifting.
E: Samples defatted with petroleum ether.
H: Samples defatted with hexane.

Table 2 notes that the samples that had a lower weight variation during the process of defatting, evidenced by the low standard deviation, were the sample submitted to defatting with hexane in a 4-stage solvent exchange (2H). It was verified through the standard deviation values on the step before sieving (0.9%) and after sieving (0.1%), which were less in the samples defatted with hexane. There was no statistical difference between the methods of defatting at the 95% significance level. Possible explanations for this result may be related to a smaller loss of raw material during the steps of solvent removal and sieving. It was verified that the mass of the sample to be defatted no affects the yield of defatted meal after the sieving, and it also presents lower weight variation at the end of the process. However, there was less mass variation in two stages at the end of the process for 2H sample found through of standard deviations: 86.0 ± 4.3% (1E) and 91.3 ± 0.9% (2H) for defatting without sieving, and 72.9 ± 2.2% (1E) and 73.5 ± 0.1% (2H) for defatting and sieving samples, respectively. From these results, the defatting method with hexane was faster than with petroleum ether, so defatting can be performed in shorter process time using hexane in shaker.

3.3 Obtaining evaluations of protein isolates

The method of solubilization with 0.5 M sodium sulfite made it possible to obtain the isolated protein from feather and blood meal. Other protein isolation methods were tested (pH shifting according to Freitas *et al*., 2011), but could not yield protein in a satisfactory amount because the proteins that compose the feather meal are distinct from the proteins extracted by this method – myofibrillar proteins – which are obtained from muscle or waste fish. Feather meal is primarily composed of keratin, which is a structural protein that contains disulfide bonds between two protein chains. Disulfide bonds lend stability and resistance to keratin and should be broken using a reducing agent. This study used sodium sulfide as a reducing agent. The proximal composition of wet protein isolates at different solubilization times with sodium sulfide was similar, with values of 7% moisture, 92% protein, 0.4% ash and less than 0.1% fat, leading to the ability to accurately characterize them as "protein isolates" from feather and blood meal. However, the isolate yields at different extraction times were distinct, which the extraction for 6 hours resulted in a highest yield of isolated approximately 20.6%. Reducing agents, such as sodium sulfide, thioglycolic acid and potassium cyanide, act very quickly and do not cause any chemical alterations or damage to the protein yield from chicken feathers. Products prepared from the solutions behave as true proteins and not as products of hydrolysis. Their solutions are precipitated by ordinary protein precipitants such as sulfosalicylic acid and ammonium sulfate (Gupta *et al*., 2012), the latter of which was used in this study.

3.4 Sulfhydryl group's analysis

The analysis of sulfhydryl groups in the protein isolates aimed to view the breaking of disulfide bonds contained in the raw materials through the isolated protein processing and to identify and quantify the free sulfhydryl groups by spectrophotometry.

Figure 1 shows the graph obtained from the analysis results of the protein extraction times of 4, 5, 6, 7 and 9 h. From Figure 1, it can be observed that there were more sulfhydryl groups at an extraction time of 6 h in a shaker at 30°C.

The highest solubilization of feather meal proteins may be related to the appearance of sulfhydryl groups in the samples because initiating the breakdown of inter-chains disulfide bonds leads to cysteine being converted to cysteic acid (Cardamone *et al*., 2009). The analysis of the identified sulfhydryl groups of this compound can be related to the FTIR analysis described below. The breaking of the disulfide bonds resulted in decreased stability of keratin because these bonds along with hydrogen bonds cause stability and make the protein insoluble in aqueous solution, thus requiring reducing agents to modify these properties of keratin. Reducers act very quickly and do not cause any chemical change in the protein (Gupta *et al*., 2012). However, the pure keratin solution without additive is instable and the keratin has poor mechanical property so that it is difficult to satisfy the demand for produce and application of biomaterials, such as keratin films. It was suggested that natural or synthetic polymers can be added to enhance the mechanical properties of keratin (Xing *et al*., 2016).

3.5 Fourier transform infrared spectroscopy analysis (FTIR)

FTIR analysis were performed on defatted feather meal samples with hexane and isolated from 5, 6 and 7 h of extraction; these samples were selected because they showed a higher content of sulfhydryl groups (Figure 2).

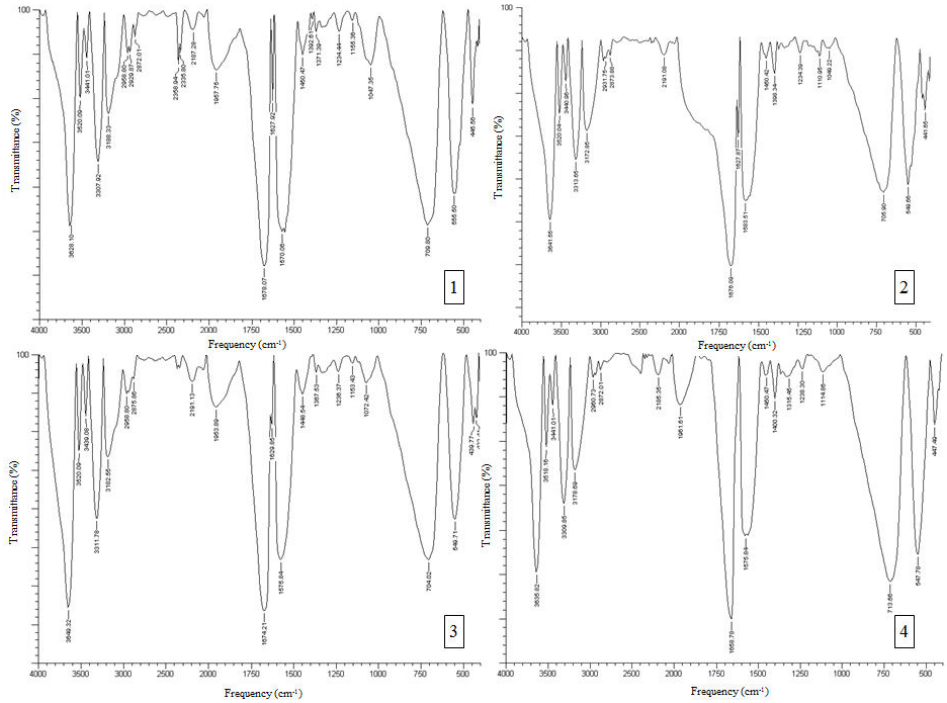

Figure 2: Fourier transform infrared spectra of samples of defatted feather meal (1) and protein isolated from 5 h (2), 6 h (3), and 7 h (4) of extraction.

Figure 2 shows that the infrared spectrometry graphics did not show any practical differences in the bands between samples. However, the FTIR spectrum of the defatted meal sample feather (1) yielded bands in the region 2358 and 2335 cm^{-1}, which were not observed at any other spectral intensity. This region showed weak bands characterized by alkyne compounds or spurious bands due to atmospheric CO_2 absorption (Barbosa, 2011); these were revealed only in the defatted meal sample. Most of the FTIR information on protein secondary structure (α-helix, β-sheet, β turns and irregulars) is obtained from the analysis of the amide I band, which occurs in the region of 1700-1600 cm^{-1}. This band is mainly due to the stretching of C=O bonds; the peptide bond is sensitive to different conformations of protein secondary structures (Forato *et al*., 1997). There was bands visualized within this range in all samples (1: 1678 cm^{-1}, 2: 1676 cm^{-1}, 3: 1674 cm^{-1}, and 4: 1659 cm^{-1}) can be a contribution from segments of disordered peptides (Xing *et al*., 2016). A wide spectrum and moderate intensity band in the region of 3188-3172 cm^{-1} was observed, indicating the presence of the NH_3^+ group. According to Barbosa (2011), when the spectrum is obtained in a KBr tablet (solid phase), a wide spectrum of moderate intensity between 3200-3000 cm^{-1} is observed due to the asymmetric stretching of the NH_3^+ group.

Another intense and broad band in the spectra is seen between 713-704 cm^{-1}, which is generally observed in the spectrum of primary and secondary amides resulting from symmetrical angular deformation of the N-H located out of bond plane at approximately 900-650 cm^{-1} (Silverstein *et al*., 2005).

Bands between 1034-1078 cm^{-1} can be attributed to cysteine (Xing *et al*., 2016), formed from the break of disulfide bonds when keratin is dissolved. The cysteine may have been oxidized again from 7 h of extraction, because in this time was not observed characteristic bands of this amino acid. Bands were observed very close to the spectrum of cysteic acid (R-SH) in the range of 1445 cm^{-1}, indicating that there was interchain cysteine oxidation and the breaking of disulfide bonds (Cardamone *et al*., 2009).

Other spectrum structures at 445-422 cm^{-1} may be associated with the presence of disulfide linkages, which can be observed with weak peaks between 500 and 400 cm^{-1} (Silverstein *et al*., 2005), probably indicating that not all of these bonds were broken in the protein structure, or disulfide bonds were formed again between cysteine residues.

3.6 Thermal properties

Figure 3 shows the DSC thermogram of the protein isolate extracted for 6 h.

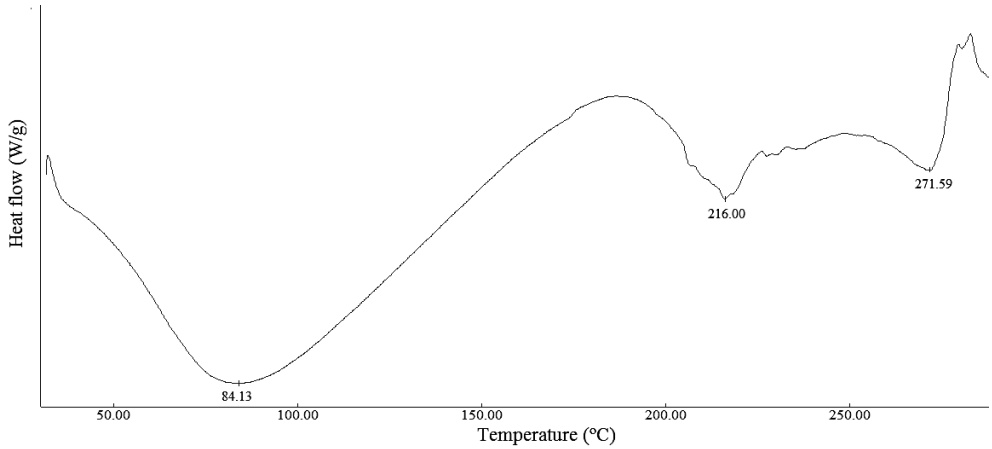

Figure 3: Differential scanning calorimetry of protein isolate extracted for 6 h.

DSC thermogram (Figure 3) shows two main endothermic transitions for the protein isolate extracted to 6 h: the first endothermic transition was a low temperature peak (temperature 84.13ºC, ΔH 417.74 mJ) related to the moisture loss. According to Barone *et al*. (2005), the low temperature peak corresponds to the amount of hydrogen-bound water in the keratin and, in some cases, protein denaturation. In a higher temperature transition was found two peaks (first temperature 216.00ºC, last temperature 271.59ºC, ΔH 180.65 mJ), related to the protein degradation, which the first temperature can correspond to loss of keratin cristallinity, and the last temperature corresponded to the protein melting. DSC analysis is essential to know the temperatures that the proteins undergo some type of transition related to the temperature increase. The processing at high temperatures can lead to loss of intrinsic properties of the proteins, affecting the structure of the material formed.

4. Conclusion

Sample 3 of feather and blood meal presented the most parameters within the standards of the Brazilian legislation (moisture and proteins). In addition, it also had higher protein content and was thus used for the defatting test and subsequently for protein extraction. The yield on the different methods of defatting showed no significant difference between them, but the extraction that used hexane as solvent reduced the process time, with less loss of sample during the process, observed by the standard deviation values. This work showed that it is possible to obtain protein isolates from chicken feather and blood meal and that the protein extraction time with the highest content of sulfhydryl groups was from isolates extracted for 6 h. The appearance of these groups is directly related to the breaking of the disulfide bonds. Cysteine characteristic bands were not detected in the isolated extracted for 7 h, indicating that disulfide bonds can have been formed again from this extraction time. FTIR analysis showed typical bands of proteins and breaking products of disulfide bonds, indicating the keratin solubilization. DSC analysis showed that the protein isolate had two main endothermic transitions, which the second endothermic transition was related to the protein isolate degradation.

According to the performed study, it was possible to obtain protein isolate from feather and blood meal and it could aggregate value to the isolate with potential application in processing of proteinous bioplastics.

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Tables

Table 1: Proximate composition of chicken feather and blood meal collected from different chicken processing industries.

Components	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)
Dry matter	95.31 ± 0.14[a]	86.41 ± 0.15[b]	91.41 ± 0.06[c]
Protein	63.13 ± 2.07[a]	81.39 ± 2.68[b]	87.48 ± 0.21[c]
Fat	17.53 ± 0.54[a]	7.14 ± 0.24[b]	9.60 ± 2.04[b]
Ash	10.64 ± 0.66[a]	3.41 ± 0.01[b]	3.78 ± 0.25[b]

Identical letters on the same line mean that there was no significant difference between samples ($p>0.05$).

Table 2: Yield in percentage obtained from feather and blood meal samples defatted, and defatted and sieved.

Samples	Yield 1 (%)	Yield 2 (%)
1E	86.0 ± 4.3[a]	72.9 ± 2.2[a]
2H	91.3 ± 0.9[a]	73.5 ± 0.1[a]

Yield 1: Yield corresponding to the weight after defatting.
Yield 2: Yield corresponding to the weight after scouring and sifting.
E: Samples defatted with petroleum ether.
H: Samples defatted with hexane.

Figures

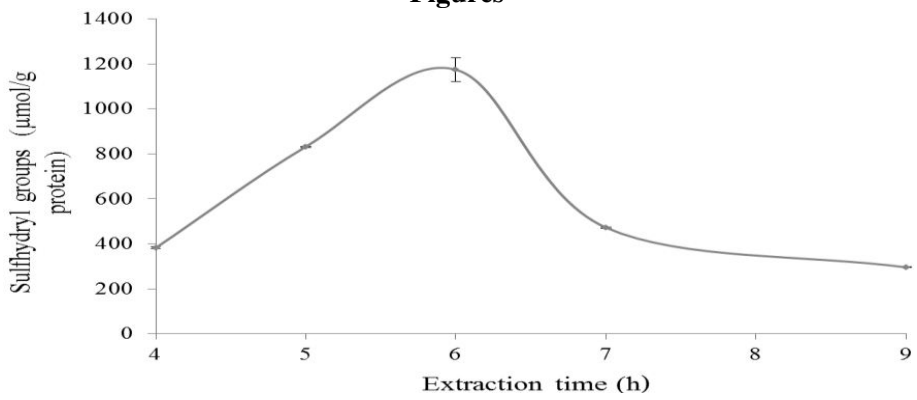
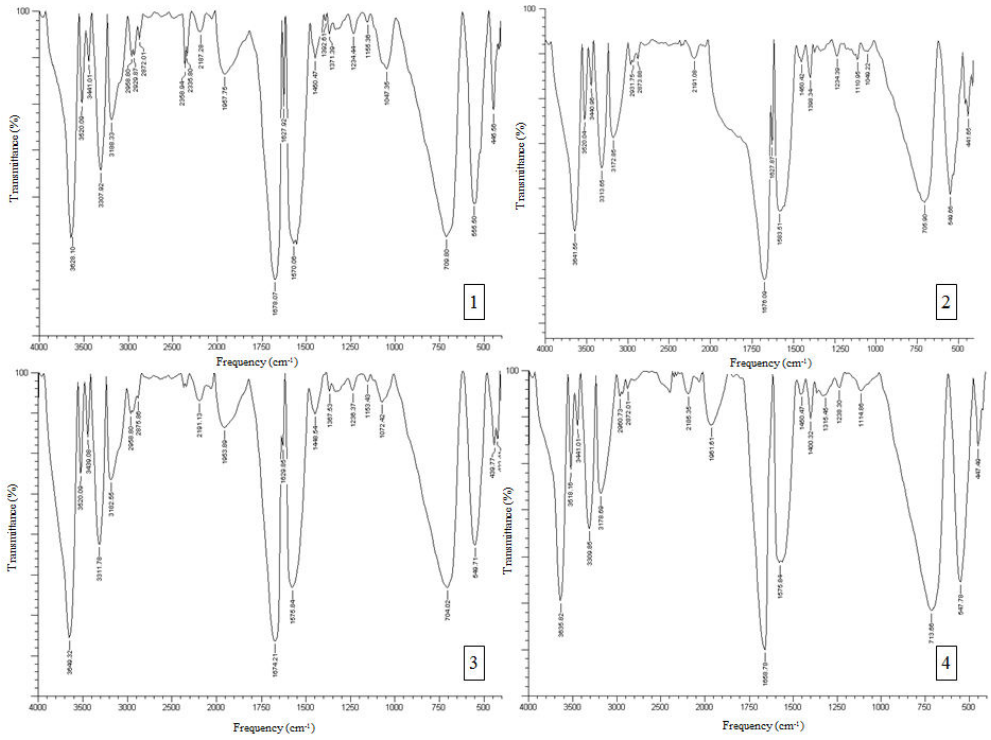


Figure 1: Sulfhydryl groups obtained at different times of protein extraction from feather and blood meal.



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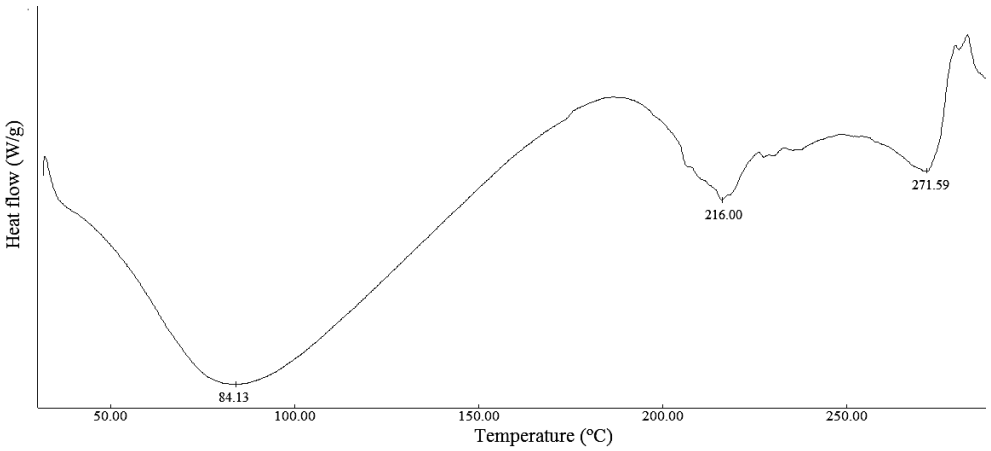

Figure 3: Differential scanning calorimetry of protein isolate extracted for 6 h.