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Influence of process conditions used to obtain protein isolates from chicken feather meal

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Abstract

Poultry processing industries generate huge amount of by-products throughout the world, including feather meal. The objective of the present work was to study the influence of different concentrations of sodium sulphite, urea and extraction temperatures on the yield and protein content of protein isolates from feather meal. Central rotational experimental design consisting of 17 trials was used. The proximal composition of the raw material and the sulfhydryl groups of the isolates that presented the best results in the experimental design was then evaluated. The response surface obtained for the protein isolate yield indicated that a decrease in the concentration of sodium sulphite and increase in the urea concentration enhanced the yield values, while temperature did not have an influence on any of the variable-responses. It was also found that the use of a higher concentration of sodium sulphite increased the content of sulfhydryl groups, which indicated greater breakage of the disulphide bonds which in turn is a desirable factor in protein extraction.

Introduction

By-products from different animal sources can be used as raw materials for the processing of different products. Poultry processing industries around the world generate huge amounts of solid by-products such as heads, feet, bones, viscera, blood and feathers. These wastes are often transformed into animal feed, fertilisers or are completely discarded. The inadequate disposal of these wastes causes environmental pollution, diseases and loss of useful biological resources such as proteins, enzymes and lipids. The use of methods that make use of these biological components for the processing of products with greater added values, instead of the direct use of the residual materials, could be a viable option (Lasekan *et al.*, 2013).

Chicken feathers have very versatile applications, ranging from composite materials, fibres, skeletal tissue engineering, and nano- and micro-particles, among others. Despite their low cost, abundant availability, wide applicability and peculiar properties, non-food industrial applications of feather keratin are very limited. Poor thermoplasticity, difficulty in dissolving keratin, and limited knowledge on the processability and properties of the developed products are some of the limitations for a large-scale use of feather keratin. However, the growing interest in the use of renewable and sustainable raw materials, and the need to reduce dependence on non-renewable petroleum resources, make feathers an attractive raw material for the generation of bioproducts (Reddy, 2015).

Keratin is characterised by high content in the amino acid cysteine, which ranges from 2 to 18% by mass. The presence of cysteine results in high resistance, mainly against the action of proteolytic enzymes, due to the disulphide bonds between the cysteine molecules. Keratin is also characterised by its high hydroxyl content of the amino acid serine, which corresponds to 15% by mass. Reducing agents, such as urea and sodium sulphite, can be used for the extraction of protein compounds that have large amounts of disulphide bonds and hydrogen bonds, which are interactions that occur in proteins and promote the stability of their native structure such as in keratin (Barone *et al.*, 2006; Du *et al.*, 2016). Protein extraction or solubilisation of keratinous products, such as feather meal, should be done to promote the change in protein conformation

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to primary structure. Without this step, due to its resistance, it would not be possible to carry out the protein isolation (Colembergue *et al.*, 2016).

Response surface methodology is an important tool that aims to optimise processes, and consists of a group of mathematical and statistical procedures to study the influence of factors (independent variables) on one or more responses (dependent variables or response variables). This methodology gives rise to a mathematical correlation that relates product properties and process parameters, with the objective of developing, optimising and analysing processes, products and formulations (Thakur and Saxena, 2000; Rodrigues and Iemma, 2014).

The objective of the present work was therefore to evaluate the influence of different concentrations of sodium sulphite and urea, and different temperatures on the production of protein isolates from feather meal, in the effort to obtain higher yield and protein content from the isolate.

Materials and methods

Proximate composition and pre-treatment of raw material

The raw material (chicken feather meal) was obtained from a poultry processing industry located in the State of Rio Grande do Sul, Brazil, and taken to the Laboratory of Food Technology at the School of Chemistry and Food, Federal University of Rio Grande. The proximate composition of the feather meal was performed by obtaining the values of moisture in the De Leo A1SE oven at 105°C (method 950.46), crude protein by the micro-Kjeldahl method in the Gerhardt Kjeldatherm VA digester with nitrogen conversion by 6.25 (Method 928.08), ether extract in Sohxlet Quimis extractor Q-308-26B using petroleum ether solvent (method 960.39), and mineral content, charring and then incinerating the sample in a Quimis Q318M24 muffle at 550°C (method 920.153), according to AOAC (2000).

The sample was then degreased according to Wang *et al.* (1999) with some changes by using hexane at a ratio of 1:3 (w/v) at 50°C for 30 min in a Cientec shaker CT-712RNT under 125 rpm agitation. This procedure was repeated four times, and the meal was dried at room temperature for 24 h.

Extraction of protein isolates

To extract the protein isolates from the defatted sample, different solutions of urea and sodium sulphite were used as described by Arruda (2010) with slight modifications. Protein precipitation was performed according to Gupta *et al.* (2012) with slight modifications.

The dried sample was subjected to protein solubilisation by agitation in a Cientec Shaker CT-712RNT at 125 rpm for 5 h at pH 9.0 using a mixture of urea and sodium sulphite at different concentrations and temperatures. Following solubilisation, the slurry was filtered through nylon filter to remove insolubilised particles and centrifuged at 14,308 g in a Hanil 1544-6906 centrifuge for 15 min to collect the liquid fraction. From this fraction, the volume was measured, and ammonium sulphate slurry 7:10 (w/v) (ammonium sulphate/water deionized slurry) was added in the same amount of liquid fraction collected (1:1 v/v) to precipitate the proteins by salting out under agitation using Fisatom 712 agitator at 700 rpm. The slurry was then subjected to centrifugation using the same conditions previously mentioned to collect the precipitated fraction (solid), and this was washed with deionised water for 25 min under stirring with Fisatom 712 propeller shaft stirrer at 700 rpm for the removal of possible reagents present in the solution. Centrifugation was carried out again under the same conditions previously mentioned, and the solid fraction was collected and subjected to drying in a Quimis Q314D242 forced circulation oven at 50°C for 18 h. Each dry protein isolate was then weighed to obtain yield values and milled in a knife mill by Tecnal TE-633 Tec Mill for storage in closed flasks at room temperature.

Experimental design

Experiments were performed according to a central composite rotatable design (CCRD) with six axial points and three repetitions at the central point, totalling 17 trials. The process parameters (independent variables) used were sodium sulphite concentration (x_1), urea concentration (x_2) and extraction temperature (x_3), and the response variables (dependent variables) were the yield at the end of the processing (y_1) and the protein content (y_2) of the isolates. The initial mass of the sample was 30 g for each test, and the yield was calculated considering 100% initial mass. The levels of the studied variables were selected according to the preliminary tests and based on the literature (Schrooyen *et al.*, 2000; Moore *et al.*, 2006; Arruda, 2010). The parameters used in the experimental design were: 0.032, 0.1, 0.2, 0.3 and 0.368 M for sodium sulphite, 1.65, 2.5, 3.75, 5.0 and 5.85 M for urea, and 33.2, 40, 50, 60 and 66.8°C for temperature (coded levels -1.682, -1, 0, +1 and +1.682, respectively).

The software Statistica® for Windows, version 5.0 was used for the treatment of the data. Statistical analysis, or theoretical validation of the proposed mathematical model, was performed by applying

Analysis of Variance (ANOVA) to test the lack of fit, determine the regression coefficients and generate the response surface.

The yield of the process (y_1), was determined as the ratio between the final mass of dry protein isolate (g) and the initial mass of dry feather meal (g), expressed as a percentage. The protein content (y_2) was evaluated by the micro-Kjeldahl method (N × 6.25) according to AOAC (2000).

Sulfhydryl group analysis

The isolates that gave the best yields and protein contents were submitted to sulfhydryl group analysis. The total sulfhydryl group content was determined using Ellman reagent (5,5 dithiobis-2-nitrobenzoic acid), 10 mM) following the procedure described by Shimada and Cheftel (1988), with slight modifications. Samples (100 mg) of each dried and crushed protein isolate were homogenised for 3 min with 50 mL 0.1 M phosphate buffer, pH 8.0, containing 1 mM ethylenediamine tetraacetic acid, 6 M urea and 0.5% sodium dodecyl sulphate. The mixture was then centrifuged at 8,667 g in Biosystems centrifuge, model MPW-350/350R for 20 min. Next, 3 mL supernatant was collected and 30 μL Ellman reagent was added and the mixture was incubated for 15 min at room temperature (25 ± 1°C). After the reaction, the absorbance of the mixture was measured at 412 nm in Biospectro UV spectrophotometer, model SP-22, and the sulfhydryl groups were determined using a molar extinction coefficient of 13600 M^{-1} cm^{-1}.

Statistical analysis

For the analysis of the proximal composition and the sulfhydryl groups, the data were obtained in triplicate. The average values were compared through analysis of variance and significant statistical differences ($p < 0.05$) using the Tukey test.

Results and discussion

Proximate composition

The proximate composition of the raw material resulted in the following values: moisture content of 8.4 ± 0.1% (91.6 ± 0.1% dry matter), 79.9 ± 0.2% crude protein, 8.7 ± 1.9% ether extract and 3.4 ± 0.2% minerals on a wet basis. Studying the composition of feather meal, Nunes *et al.* (2005) found similar values for dry matter (89.5%); and lower values for crude protein (64.5%), ether extract (4.2%) and ash (2.1%). Eyng *et al.* (2012) also found similar values for proteins (71.2 to 82.4%) and minerals (1.8 to 3.6%) and lower value for ether extract (4.1 to 6.2%). Industries can modify the percentage of components used to produce feather meals, justifying the reasons of the differences found in meals, especially ether extract.

Nascimento *et al.* (2002) studied the composition of six feather and blood meals of different origins and found values of 88.9 to 90.9% for dry matter which were lower than those obtained in the present work; 72.3 to 81.9% crude protein, 1.3 to 10.1% ether extract, and 1.4 to 3.4% minerals which were almost similar to those obtained in the present work.

Table 1. Results of the experimental design trials for the yield variable in the processing and protein content of protein isolates from feather meal.

Trial	[ ] Na_2SO_3	[ ] CH_4N_2O	Temperature (°C)	Yield (%)	Protein (%)
1	-1	-1	-1	7.97	96.75
2	+1	-1	-1	0.93	91.36
3	-1	+1	-1	18.79	97.47
4	+1	+1	-1	11.5	96.86
5	-1	-1	+1	9.76	95.06
6	+1	-1	+1	0.78	95.24
7	-1	+1	+1	19.12	97.39
8	+1	+1	+1	11.49	95.98
9	-1,682	0	0	17.11	96.88
10	+1,682	0	0	0.55	94.09
11	0	-1,682	0	0.17	91.32
12	0	+1,682	0	17.08	99.11
13	0	0	-1,682	10.01	93.99
14	0	0	+1,682	12.49	92.68
15	0	0	0	10.95	96.37
16	0	0	0	11.93	95.35
17	0	0	0	11.22	95.61

Experimental design

The yields and the protein contents of the trials are presented in Table 1. Yield values ranged from 0.17 to 19.12%, from the start of the solubilisation process to the post-drying step. The statistical analysis showed a significant negative effect on the average for the linear and quadratic sodium sulphite concentration, and a significant positive effect on the average for the linear and quadratic urea concentration, as can be seen in Table 2. It was observed that when lower concentrations of sodium sulphite and higher urea were used, higher yield was obtained.

Du *et al.* (2016) reported that urea acts on the breakdown of the hydrogen bonds, while Barone *et al.* (2006) commented that the sodium sulphite acts in the reduction of the disulphide bridges. The keratin found in the feather meal is β-keratin (Arai *et al.*, 1983), which contains approximately 7 to 20% of the

Table 2. Estimated effects by the regression model for the yield variable in the processing of protein isolates from feather meal.

Independent variable	Estimated effect	Pure error	*t*-value	*p*-value
Average	11.32926	0.554799	20.4205	<0.000001
Sodium sulphite (L)	-8.60929	0.521042	-16.5232	0.000001
Sodium sulphite (Q)	-1.53464	0.573420	-2.6763	0.031709
Urea (L)	10.23596	0.521042	19.6452	<0.000001
Urea (Q)	-1.67956	0.573420	-2.9290	0.022055
Temperature (L)	0.89783	0.521042	1.7231	0.128529
Temperature (Q)	0.17614	0.573420	0.3072	0.767653
Sulphite - urea	0.27500	0.680809	0.4039	0.698319
Sulphite - temperature	-0.57000	0.680809	-0.8372	0.430111
Urea - temperature	-0.33000	0.680809	-0.4847	0.642676

L: linear, Q: quadratic. The bold *p*-value showed significant effect on the experimental design to the 95% significance level.

amino acid cysteine (Yamauchi *et al.*, 1996), and can form intra- and intermolecular disulphide bonds with other cysteine molecules, forming cystine (Barone *et al.*, 2006). Feather keratin has a large amount of disulphide bonds (due to high cysteine content) and hydrogen bonds, which is the main reason for protein insolubility conferring strength, stability and proteolytic resistance (Onifade *et al.*, 1998). These bonds must be disrupted (by urea and sodium sulphite) and their secondary structure destroyed so that protein extraction could occur.

The analysis of variance makes it possible to observe whether the significance of the regression equation follows an F distribution based on the degrees of freedom numbers of the quadratic average, due to the regression and residual quadratic average, where the calculated F-test values are compared to tabulated values in the same interval. In addition, the correlation coefficient R between observed responses and predicted values explains the percentage of variability of the mean (Barros Neto *et al.*, 1995).

For the yield variable in the processing, the residue did not present evidence of lack of fit, because the F_{calc} (181.1) higher than the F_{tab} (3.26) proves an equation with the Fcalc value 55.6 times superior to the F_{tab}, leading to a valid and statistically significant equation. The coefficient of determination found, $R^2 = 0.98371$, was significant ($p < 0.05$), and the model could be considered as valid. This coefficient measures the proportion of the total response variance that is explained by the model. Thus, the larger the R^2, the smaller the error, hence the better the model.

The obtained model was significant and also predictive because according to Barros Neto *et al.* (1995), for a regression to be statistically significant and valid for predictive purposes, the value of F_{calc} must be at least four to five times the value of F_{tab}, and R^2 should be greater than or equal to 60%. The model adjusted using only the significant variables, obtained for the processing yield in function of the studied variables in the codified form, is described in Equation 1:

$$y_1 = 11{,}44183 - 8{,}60929x_1 - 1{,}5867x_1^2 + 10{,}23596x_2 - 1{,}73162x_2^2 \quad \text{(Eq. 1)}$$

From Figure 1, it was possible to verify that the increase in the urea concentration (5 M or more) and the decrease in the sodium sulphite concentration (0.2 M or less) resulted in a higher yield during the protein extraction. Of the 17 trials, four of them presented percentage of yield greater than 15% and were selected for the analysis of sulfhydryl groups.

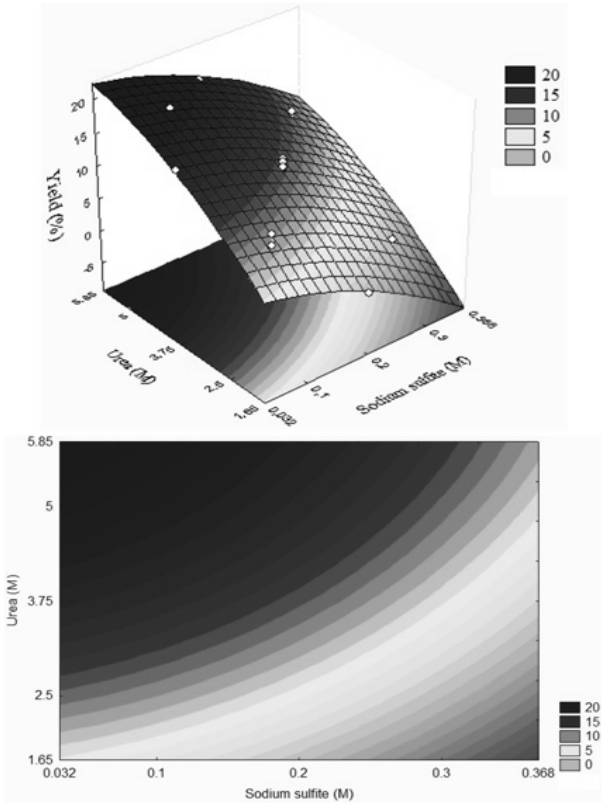

Figure 1. Response surface (above) and contour lines (below) showing the effects of reagent concentrations on the percentage of processing yield in obtaining protein isolates from feather meal.

Low concentrations of urea are inefficient for a satisfactory protein extraction process, mainly keratin. Values above 3.75 M urea in the extraction process are sufficient to obtain high concentrations of keratin, based on the results found in the present work which also agree with Arruda (2010).

Table 3. Estimated effects by the regression model for the independent variable protein content in the processing of protein isolates from feather meal.

Variable	Estimated effect	Pure error	t-value	p-value
Average	95.70273	0.305420	313.3480	0.000010
Sodium sulphite (L)	-1.74587	0.286836	-6.0867	0.025947
Sodium sulphite (Q)	0.30490	0.315671	0.9659	0.436003
Urea (L)	3.27901	0.286836	11.4316	0.007565
Urea (Q)	0.11403	0.315671	0.3612	0.752511
Temperature (L)	-0.14254	0.286836	-0.4969	0.668484
Temperature (Q)	-1.21500	0.315671	-3.8489	0.061355
Sulphite - urea	0.79750	0.374789	2.1279	0.167162
Sulphite - temperature	1.19250	0.374789	3.1818	0.086197
Urea - temperature	-0.78750	0.374789	-2.1012	0.170404

L: linear, Q: quadratic. The bold *p*-value showed significant effect on the experimental design to the 95% significance level.

Protein content

The protein content of the isolates varied from 91.32 to 99.11%, showing that there was an increase in protein content between 3.88 and 11.63% as compared to the value initially found in the raw material (79.9%). The variable "linear sodium sulphite concentration" had a significant and negative effect, and the variable "linear urea concentration" had a significant positive effect on the average of the response "protein content" (Table 3). This fact indicated that, when low concentrations of sodium sulphite and high concentrations of urea were used, high protein content was obtained. The results indicated that 64% of the variability in the protein content response could be explained by the model ($R^2 = 0.6395$). Barros Neto *et al.* (1995) report that the R^2 should be greater than or equal to 60%, with R^2 being the variable y_2 according to these authors. By the analysis of variance for regression at 5% significance, the value of F_{calc} was 3.3 times higher than that of F_{tab}. In addition, the lack of fit of the model was not significant, because the F_{calc} was superior to the F_{tab}, indicating that there was no evidence of lack of fit of the model and agreeing with Pighinelli *et al.* (2008). Since R^2 was slightly above 60%, we opted not to obtain the response surface for the variable protein content. The adjusted model with the significant variables present, as a function of the variables studied in the codified form is shown in Equation 2:

$$y_2 = 95{,}38294 - 1{,}74587x_1 + 3{,}27901x_2 \quad \text{(Eq. 2)}$$

Cortez-Vega *et al.* (2013) prepared a protein isolate of Whitemouth croaker and obtained 97.37 ± 0.31% of proteins on dry basis, which were close to those obtained in the present work. Martins *et al.* (2009), working with Whitemouth croaker, obtained 86.94 and 72.34% protein isolate through acid and alkaline extraction, respectively, and for protein isolate from fillet 77.98% by acid extraction and 88.28% by alkaline extraction. These results were lower than those found in the present work.

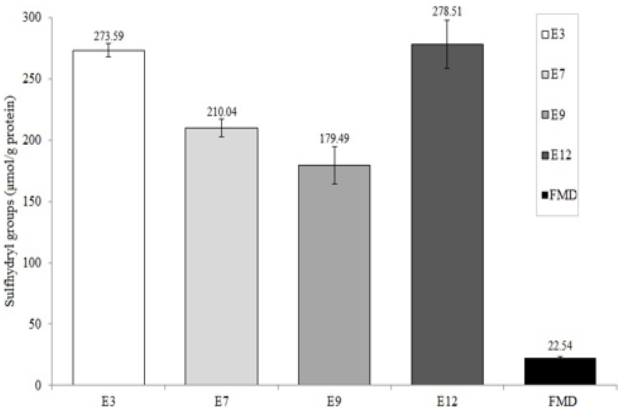

Figure 2. Sulfhydryl groups of the protein isolates from feather meal that presented the highest yields in the experimental design. E3: isolate produced with 0.1 M sodium sulphite and 5 M urea at 40°C; E7: isolate produced with 0.1 M sodium sulphite and 5 M urea at 60°C; E9: isolate produced with 0.032 M sodium sulphite and 3.75 M urea at 50°C; and E12: isolate produced with 0.2 M sodium sulphite and 5.85 M urea at 50°C.

Figure 2 shows the results obtained in the analysis of sulfhydryl groups of the isolates that presented the highest yields in the experimental design. It was possible to verify that the E3 and E12 assays presented higher amounts of sulfhydryl groups, indicating the reduction of the disulphide bonds that were present in the sample before the isolates were obtained. In addition, the averages obtained from the E3 - E12 trials did not show significant differences between them ($p > 0.05$), as well as between assays E7 - E9, using the Tukey's Test. From these results, it

was possible to consider a higher solubility in E3 (0.1 M sodium sulphite and 5.0 M urea solution) and E12 (0.2 M sodium sulphite and 5.85 M urea solution), comparing with E7 and E9 assays.

Another finding of the present work was that the tests that presented higher content of sulfhydryl groups in the analysis were those that used higher concentrations of sodium sulphite. In the E12 assay, the concentration at the central point (0.2 M) resulted in 278.51 μmol/g protein, however, in the E9 assay, the concentration used was the lowest sodium sulphite at the axial point (0.032 M), resulting in 179.49 μmol/g protein. According to Barone *et al.* (2006), sodium sulphite acts on the breakdown of cystine disulphide bonds, and this breakage results in the appearance of cysteine-free sulfhydryl groups.

Keratin from chicken feathers is a water-insoluble protein because it has a high content of disulphide bonds and a high amount of hydrophobic amino acids. The extraction of feather keratin without the cleavage of the peptide bonds is only possible by breaking the disulphide bonds. A mild procedure involves the use of thiol groups to reduce the disulphide bonds under moderately alkaline conditions in the presence of urea to provide the breakdown of the hydrogen bonds (Jones and Mecham, 1943; Schrooyen *et al.*, 2000). Schrooyen *et al.* (2000) also mentioned that pH and urea concentration are some of the factors that improve the extraction of keratin by about 75%. The pH of 7 to 9.0 and 5 M or more of urea increased keratin extraction in a study carried out by these authors.

Lin *et al.* (1992) evaluated the reduction of disulphide bonds in SH groups through the Ellman reaction to study the enzymatic keratinolytic mechanism, and did not detect an increase in the production of the free SH groups during a hydrolysis process for 1 h. The present work indicated the need for the use of reducing agents, such as urea and sodium sulphite to break the disulphide bonds to obtain protein isolates and make the proteins suitable for the extrusion process to obtain new materials.

Conclusion

Low concentrations of sodium sulphite and high concentrations of urea influenced the increase in yield and protein content of protein isolates. Temperature had no significant effect on any of the response variables. Treatments with 0.1 M sodium sulphite and 5.0 M urea, and with 0.2 M of sodium sulphite and 5.85 M urea gave the highest yield and protein. Finally, the best treatments previously mentioned resulted in a greater amount of sulfhydryl groups, indicating a greater rupture in the disulphide bonds, necessary to unfold the native structure and fundamental in obtaining the primary structure of the keratin for better extraction and isolation of the proteins.

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