Optimization of a Sustainable Keratin Extraction Process from Waste Slaughterhouse Feathers: A Practice and Business Model Innovation

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ABSTRACT: *The extraction of keratin from natural feathers has been studied for its use in various cosmetics and drug delivery applications. There are various reducing agents to dissolve the hard keratin such as sodium dodecyl sulfate and 2-mercaptoethanol, in the present work, a novel extraction method has been developed using sodium sulphite, sodium bisulphite, and sodium dodecyl sulfate in the presence of urea, 2-mercaptoethanol, Ethylenediaminetetraacetic acid (EDTA), and thiourea. To increase extraction yield, the weight of feathers, time of incubation, pH, and temperature were investigated using a Central Composite Design and Mixture plan for Optimization. With the present process, we evaluated the apport of keratin treatment and extraction techniques utilizing sodium sulphite, sodium bisulphite, and sodium*

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dodecyl sulfate in the presence of urea, 2-mercaptoethanol, Ethylenediaminetetraacetic acid (EDTA), and thiourea. The percentage yield and keratin concentration were measured using UV-Vis absorbance, Bradford, and Biuret assays. Then, the protein profile and their functional groups were characterized using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Fourier Transform Infrared Spectroscopy (FTIR). The purpose was to compare the different procedures in terms of keratin protein quality and quantity, as well as their cost-effectiveness, and to determine the optimum conditions for the keratin extraction process. The results proved that the yield of white chicken feathers keratin (81.2 %) increased using sodium sulphite (1M), sodium bisulphite (0.1 M), and Sodium Dodecyl Sulfate (0.1 M). The highest protein production was measured at 80°C in 10 h with 5 g of feathers at pH 10. This process of keratin extraction can be used from the laboratory to industrial production with high recoverability and stable properties.

KEYWORDS: *Optimization; Mixture plan; Waste poultry feathers; Keratin extraction; Business model.*

INTRODUCTION

Every year, millions of tons of feathers biomass are produced by poultry farms throughout the world. This generates huge waste management issues, as well as human diseases such as chlorosis and fowl cholera [1]. As a result, timely disposal of this waste remained critical, while incineration is the most common technique for dealing with feather debris, its high energy consumption, and considerable carbon pollution make it unsuitable [2,3]. Composting them with manure is another option, but the composting process is time-consuming and subject to a lot of veterinary inspection requirements [4]. The chemical composition of a feather reveals that it includes 91 % ~ keratin protein, a very valuable protein; hence, it is advantageous to extract this protein for use in disciplines like health care and cosmetics [5, 6]. Previous studies reported that keratin can be extracted from feathers using a variety of methods, including chemical hydrolysis (acid and alkali). Sodium sulfide and sodium metabisulphite can dissolve feathers with good conversion percentages [7-9]. It has been reported that enzymatic and microbiological treatment seems to be another way to extract feather keratin. Many microorganisms aid in the extraction of keratin by secreting keratinolytic and proteolytic enzymes known as keratinases. Such microorganisms include mesophilic fungi, actinomycetes, and several bacillus species [10, 11]. Microwave irradiation was utilized in other investigations to obtain keratin from feathers. Due to the rapid homogenous heating, this process is considered another tool to extract keratin from feathers [12,13]. Furthermore, keratin from feathers can be extracted using a steam explosion approach, ionic liquid dissolution, thermal hydrolysis, or a superheated process [11, 14].

Keratin has been claimed to be capable of producing materials such as biofilms, nanofibers, hydrogels, sponges, membranes, and cosmetics [3, 11, 13]. If pure keratin is recovered from feathers, hydrolysis is undoubtedly easier since there is no protective film and the structure is compact, and eco-friendly enzymatic hydrolysis may be used in industrial applications [15]. It has been reported that feathers are a great source of keratin that has numerous applications. Recently, a novel study highlighted the importance of keratin extracted from chicken feathers as an additive for electrospinning preparation, which enhances polymeric nanofiber mats. Keratin extracted from chicken feathers acts as a bioactive membrane created for wounds such as burns and diabetes-related ulcers [16]. Another study described the extraction of feather keratin serving as a biocompatible and inexpensive polymer, a kind of dual-sensitive keratin-based polymer hydrogel with interpenetrating network structure was prepared following two-step polymerization of N-isopropyl acrylamide and itaconic acid in the presence of crosslinker. Thus, in the biomedical and clinical nursing domains, these keratin-based biopolymer hydrogels with an interpenetrating network structure, pH sensitivity, and temperature sensitivity might be used to maintain drug carriers and humid medicinal materials [17]. As a result, developing effective processes for extracting keratin from poultry feathers is highly coveted from both an environmental and economic perspective. Despite its environmental and economic advantages, as well as its smooth conduct, this method has not been optimized;

the concentrations of sodium bisulphite, sodium dodecyl sulfate, and β-mercaptoethanol, as well as the featherweight, temperature, and the time required to run this extraction, vary from one researcher to another [18]. To achieve a high yield of keratin protein, sodium sulphide, β-mercaptoethanol, and sodium dodecyl sulfate were employed [19]. In a prior investigation, urea and sodium dodecyl sulfate was added to a solution containing sodium bisulphite and the results show that the addition of urea increased both the process rate and the product yield [20]. It was demonstrated, also, that after 2 h of extraction using β-mercaptoethanol and sodium bisulphite as the reducing agents, good yields of soluble keratin were obtained, respectively. It was also noted that the use of sodium bisulphite reduced the extraction time to approximately 1 h with the same result [7]. Further, the author demonstrates that chemically treating the feathers with 2.5 percent of concentrated sodium hydroxide improves the extraction efficiency and increases the yield of keratin [7].

The current study aims to optimize the process of extracting keratin from waste chicken feathers by studying chicken feather weight, incubation time, pH, and temperature using a Central Composite Design. To the best of our knowledge, this work is the first to evaluate the cumulative effect using Minitab 19 software of sodium sulphite, sodium bisulphite, and sodium dodecyl sulphate on poultry feathers keratin extraction. Thus, our work offers optimal avenues for future product development and paves the way for the recovery of poultry feather waste.

EXPERIMENTAL SECTION

Material

This study focused on waste poultry feathers (white chicken feathers), which were collected from a chicken meat processing plant in Sousse, Tunisia. These white poultry feathers are scoured, combed, and analyzed for moisture, ash, and residual lipid as described by *Taylor et al.* [21]. Reagent-grade chemicals were used except where otherwise noted. For feathers keratin extraction, 2-Mercaptoethanol and urea (Sigma-Aldrich, USA), DTT (Fluka, USA), SDS (Merck), sodium sulphite, sodium bisulphite (40 % solution), and EDTA were used.

Pre-treatment of the feathers and fats removal

Fresh and wet white body feathers from 35 to 65 days old were washed and cleaned with detergent in hot water at 65 ±2 °C, then dried for 30 hours at 75± 2 °C. Poultry feathers were cut and chopped into tiny pieces (0.05 to 1.5 mm) before being cleaned of residual lipids. The removal of residual lipids took place in two stages. First, the feathers were washed for 2 h in a 0.05 % solution of Tween 20, rinsed with MilliQ plus sterilized water, then vacuum filtered on Whatman #2 paper. The cleaned small feather pieces were then immersed in a new combination of chloroform/methanol/ethanol (2:1:1, v/v/v) for 24 h, and filtered as above. The small feather fibers were then air-dried for two days to evaporate the solvents before being packed and stored at room temperature (20-25 °C).

Extraction and solubilization process

For the final comparison, the defatted poultry feathers (0.5 to 5 g) were immersed in 100 ml of hydrogen chloride solution (20 mM) with a pH range of 8 to 10 containing 2-mercaptoethanol, EDTA, Na_2SO_3 (sodium sulphite), sodium dodecyl sulfate (SDS) or sodium bisulphite ($NaHSO_3$) as described on the DOE experiment on Table 1. To speed up the dissolution of keratin, Na_2SO_3 can unfold the disulfide bonds as seen in Eq.1. Since Na_2SO_3 is a weak alkaline salt, it cannot destroy keratin, but can be used as an accelerator for keratin extraction.

$$RSSR' + {SO_3}^{2-} \rightarrow RSSO_3^{-} + R'S^{-} \quad (1)$$

Whereas, sodium bisulphite was used to cross covalent disulfide bonds according to Eq.2 [18,22].

$$\text{Feather keratin-S-S-keratin} + SO3^{2-} \rightarrow \text{Keratin-S}^{-} + \text{Keratin-S-S}_3^{2-} \quad (2)$$

Feather keratin Sodium bisulphite Cysteine thiol Cysteine –s-sulphonate

The mixtures were treated for 10 to 48 h at temperatures ranging from 40 to 80 °C. The components employed in these reaction mixtures are shown in Table 1 and Table 2. To avoid feather aggregation during the reaction, the defatted feathers were mixed with a magnetic bar during the extraction procedure. The obtained mixture was centrifuged at 15000 rpm for 30 min at room temperature to separate insoluble material, and the supernatant was filtered through a folded filter. The filtrates obtained with various reducing agents were then dialyzed in double-distilled water (ddH_2O) for 72 h using Spectra / Por dialysis membranes in regenerated cellulose with an MWCO 6000-8000 (molecular weight cut off) changing the external ddH_2O three times a day. The supernatant was centrifuged at 15000 rpm for 12 min. All procedures were performed in triplicate.

Yield calculation of keratin

The yield of soluble keratin was determined by different extraction methods. Tests were performed varying the extraction solution, temperature, and pH. The chicken feathers' keratin yield could be calculated according to Eq. (3):

$$\text{Keratin yield (\%)} = (\text{Wfk}) / \text{Wik} \times 10 \quad (3)$$

Where W_{ik} is the initial keratin weight (before keratin extraction) and W_{fk} is the final weight of keratin (after keratin extraction) respectively [22, 23]. The amount of keratin in feathers was measured using three different dosing techniques, and all tests were performed in triplicate. The initial assay measured UV-Vis absorbance at 280 nm with a Bio Photometer plus spectrophotometer (Hamburg, Germany). For the second analysis, keratin content was determined by the Bradford colorimetric method with a commercial protein assay kit (Bio-Rad, Hercules, CA [24, 25]. Briefly, the amounts of the total protein were quantified by detecting the optical density at the wavelength 595 nm and comparing them to a standard curve of Bovine Serum Albumin (BSA) using a spectrophotometer (HP Agilent 8453, Palo Alto, CA). In the third analysis of the keratin content, an assay according to the Biuret protocol was carried out. The Biuret reagent was added to the feather keratin extract in the 2:1 v/v and incubated in the dark for 15 min. The BSA was used as a reference standard and the plates were read at 550 nm.

Electrophoresis using sodium dodecyl sulfate-polyacrylamide gel

Molecular weight and the profile of the feather proteins were evaluated by Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate (SDS-PAGE). The Acetone precipitation led to a better resolution of cleaned chicken feathers proteins. To determine the feather molecular weight, pellets were dissolved and mixed with 4X electrophoresis buffer (containing 6 M Urea, 10 % SDS, 250 mM Tris - HCl buffer, 5% 2-mercaptoethanol, glycerol 30% (v/v) and 0.01% (w/v) bromophenol blue) at ratio 4:1 for analysis by 12% SDS- PAGE. Then, the samples were heated in a water bath at 100 °C for 5 min. The protein standard was used for calibration (Bio-RAD, USA). The stacking and running gels were 4 and 12% polyacrylamide, respectively. The applied electrophoresis voltage was 80 V for 30 min followed by 140 V for 120 min. Protein separation was performed on a Mini-PROTEAN® Precast Gels system (Bio-RAD, USA). Protein patterns were visualized by Coomassie brilliant blue G250 and silver staining methods [26,27].

Gel staining

The gels were washed with ddH_2O after separation, and the feather proteins were visualized using a sensitive colloidal Coomassie G250 stain [28]. The dye solution containing 17% (w/v) ammonium sulfate, 3% (v/v) phosphoric acid, 0.1% (w/v) Coomassie G250, and 34% (v/v) methanol were changed after 12 h staining and the gel slabs were subjected to a 24-hour cycle for increasing dye deposition on low-abundance proteins. The detection was then increased by placing the gel into 1% v/v acetic acid to produce a better contrast between spots and gel. Silver staining was done according to *Oakley et al*. [29]. All Coomassie and silver-stained gels were treated using PDQuest 7.1 software (Bio-rad). The individual spot volumes were normalized by dividing their Optical Density (OD) values by the total OD values of all the spots present in the gel and expressed as % vol. The significance of expression differences of protein spots was estimated by a student's t-test, $p \leq 0.05$.

Fourier-Transform Infrared (FT-IR) spectroscopy

Fourier-Transform InfraRred (FT-IR) spectroscopy was performed to examine the chemical structures of keratin powders using a PerkinElmer spectrum 100/Universal Diamond Attenuated Total Reflectance (ATR) (Beaconsfield, Buckinghamshire, England). Each obtained spectrum was an average of 4 scans with a resolution of 4 cm^{-1} and a wavenumber range of 550-4000 cm^{-1}.

Statistical and experimental design

The first optimization is based on an experimental design, and the second optimization is based on a mixed design experiment, with each experiment identifying three measurements or responses such as protein concentration in absorbance at (280 nm) protein concentration using the Bradford technique and protein concentration using the Biuret method.

Proteins extraction optimization using an experimental design

Minitab (Ver. 19.0, U.S. Federal Government Commonwealth of Pennsylvania, USA) was used to set up

the Design of the experiment, optimizing the extraction process by setting up a small number of targeted experiments, controlling multiple parameters (or variables), and emphasizing the interactions between these variables. A complete factorial design was developed to reduce the number of experiments and define the ranges of variation for each factor. The collected data were analyzed to compute statistical values such as the mean and Standard Deviation (SD). The normality and constant variance assumptions are confirmed.

Design of experiment (DOE) and variance analysis (ANOVA) were used to determine the regression coefficients, and the statistical significance of the model components, and to fit the mathematical models of the experimental data, aiming to optimize the overall region for all response variables. The response variables were predicted using a second-order polynomial model, as illustrated in Eq. (4):

$$Y = \beta 0 + \sum_1^3 \beta i\, Xi + \sum_1^3 \beta ij\, Xi\, Xj + \sum_1^3 \beta ijk\, Xi\, Xj\, Xk \quad (4)$$

Y is the predicted dependent variable; $\beta 0$ is a constant regression coefficient, βi are the regression coefficients for the linear effect terms, and βij and βijk are the regression coefficients of two, three, and four-factor interaction effect terms, respectively. X_1, X_2, and X_3 are the factors (independent variables).

The impact of the various independent variables on all measurements was investigated using one factor at a time to identify significant ones and define their preliminary range. Based on our previous experimental findings and other prior studies, intervals are established based on semi-industrial or even industrial feasibility [30]. For this feather keratin extraction procedure, the following minimum/maximum values were chosen for the DOE design experiment: feather sample mass (g) X_1 [min value = 0.5, max value = 5], temperature (°C) X_2 [40; 80], and incubation time X_3 (h) [10; 48].

The adequacy of the model was predicted *via* regression analysis (R^2) and ANOVA. Thus, the combined effect of these three components on the obtained Response (Y_i) is as follows for four response values: yield proteins, protein concentration (UV-Vis 280nm), protein concentration (Bradford), and protein concentration (Biuret). Full factorial Design (FFD) with two levels (-1 as min value and +1 as max value) with 2^3 reflecting 8 experiences. The experimental points in this design are created on the edges of a cubic shape, indicating the values for the different comment's responses. To reduce the effect of unforeseen variation in observed responses, the experimental trials were randomized.

Proteins extraction optimization using a mixture design

Formulating components' mixture approach for optimum protein extraction is performed by the determination of the protein amount in each sample using the three methods indicated above. The effect of modifying variables and component doses on the responses may be seen on the ternary contour map in the mixture design. In this paper, the mixture design was employed in the experimental design to define the ideal formula. The relationship between the different combinations was analyzed by Minitab 19.0.

Formulation of an optimized mixture

The mixture design is commonly used to investigate the relationships between the proportion of various factors and responses. A Simplex Lattice Design was developed and the P-value was the probability that the magnitude of a contrast coefficient was due to random process variability. In this study, the factors were sodium sulphite, sodium bisulphite, and sodium dodecyl sulfate (SDS). They were utilized as combination starters in this investigation, with concentrations ranging from 0 to 100%. To optimize the formulation of the aforesaid component concentrations, the Mixture design approach was supplied by the program Minitab.

RESULTS AND DISCUSSION

Results of the different responses

To optimize the formulation, the concept of a mixing plan was chosen using Minitab 19 Software, which allowed the implementation of the matrix of experiments. This software is widely used in the modeling and optimization of industrial processes [31,32]. Choosing "Simplex Lattice Design" (Fig. 1) for the construction of the mixing plan ensures a perfect distribution of the uniformity of the ten mixtures in the experimental field by following the different yield protein extraction sought for this ecological process.

The efficiency of the protein extraction process was evaluated by examining the results and searching for the optimum of each of the four different responses, one by one:

Table 1: Yield for the different experiments according to the three different parameters (Weight Temperature, Incubation (Design of experiment)

Sample	Weight (g)	Temperature (°C)	Incubation (h)	SDS (M)	Sodium sulphite (M)	Sodium bisulphite (M)	Yield (%)
1	5	40	10	0.1	1	0.1	69.0
2	0.5	40	48	0.1	1	0.1	43.2
3	0.5	80	48	0.1	1	0.1	51.9
4	0.5	40	10	0.1	1	0.1	49.01
5	5	80	48	0.1	1	0.1	68
6	5	80	10	0.1	1	0.1	81.2
7	0.5	80	10	0.1	1	0.1	50.9
8	5	40	48	0.1	1	0.1	62.2

Table 2: Yield for the different experiments according to the three different parameters (Sodium sulphite, SDS, Sodium bisulphite

Sample	Weight (g)	Temperature (°C)	Incubation (h)	Sodium sulphite (%)	SDS (%)	Sodium bisulphite (%)	Yield (%)
1	5	80	10	0.00	0.50	0.50	61.0
2	5	80	10	0.00	0.00	1.00	55.9
3	5	80	10	0.33	0.33	0.33	76
4	5	80	10	0.66	0.17	0.17	79.6
5	5	80	10	1.00	0.00	0.00	81.2
6	5	80	10	0.50	0.50	0.00	70.5
7	5	80	10	0.50	0.00	0.50	77
8	5	80	10	0.17	0.66	0.17	53.9
9	5	80	10	0.00	1.00	0.00	49.8
10	5	80	10	0.17	0.17	0.66	58.9

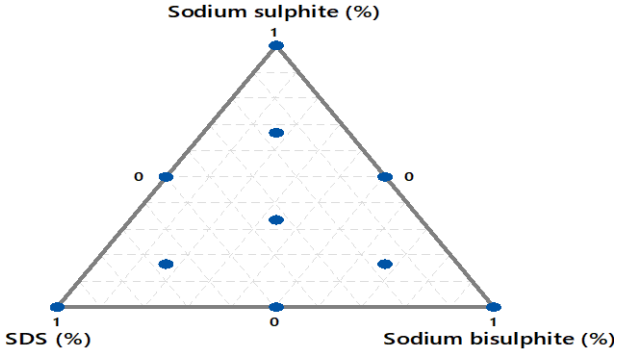


Fig. 1: Simplex lattice design graph in quantities of the mixture of the three compounds Sodium sulphite, SDS, and Sodium bisulphite varying from 0 to 1

yield, protein concentration (UV-vis 280 nm), protein concentration (Bradford), and protein concentration (Biuret). The greater the number of responses, the it is easier to converge towards the optimum of the optima. All measurements of the different experiments were carried out using an experimental design based on the three variables (Feather mass, Temperature, and incubation time; Table 1) for variables optimization using the full factorial design experiment.

Table 1 contains a full description of the mathematical expressions used to determine the design distribution and to decode the ranges of the tested variables. Once the optimal conditions (Weight (g) X_1, Temperature (°C) X_2, and Incubation (h) X_3), SDS (Molar concentration) as well as, Sodium sulphite (M), Sodium bisulphite (M), have been found for a better protein concentration determined by yield, protein concentration at (UV-vis 280 nm), by Bradford method and by Biuret method. Table 1 provides the different experiments matrix offered by the Minitab software as a full factorial experimental design. All the results of Yield, protein concentration at (UV-vis 280nm), (Bradford), and (Biuret) were expressed as a percentage and the response format (Yi) was specifically ascertained in the mathematical form.

Concentrations were varied within a range of 0 to 1 for each of the chemical components to be examined, and yield was measured, protein concentration at (UV-vis 280 nm), (Bradford) and (Biuret) for each experiment shown in Table 2, a study conducted differently than that of *Kamarudin et al*. [19].

The novelty of this study is the simultaneous adjustment of the parameters required for excellent protein extraction as well as the dosages of the chemical compounds required for this protein extraction and, among other things, the extraction of keratin desired.

Table 3: Comparison of the 280nm, bradford, and biuret methods according to the three different parameters (Design of experiment)

Sample	Weight (g)	Temperature(°C)	Incubation (h)	Protein yield (UVvis 280nm) %	Protein yield (BRADFORD) %	Protein yield (BIURET) %
1	5	40	8	77.2	86.8	84.3
2	0.5	40	10	54.6	55.6	60.8
3	0.5	80	10	69.8	77.7	78.6
4	0.5	40	8	60.2	62.4	70.2
5	5	80	10	75.6	81.3	83.2
6	5	80	8	81.0	88.9	87.2
7	0.5	80	8	65.7	67.8	75.2
8	5	40	10	73.3	81.3	81.9

Table 4: Comparison of 280 nm, bradford, and biuret methods according to the three different parameters (mixture design plan)

Sample	Sodium sulphite (%)	NaOH (%)	Sodium bisulphite (%)	Protein yield (UV-vis 280nm) %	Protein yield (BRADFORD) %	Protein yield (BIURET) %
1	0.00	0.50	0.50	75.8	69.5	60.2
2	0.00	0.00	1.00	62.8	56.2	55.2
3	0.33	0.33	0.33	82.5	80.1	74.2
4	0.66	0.17	0.17	85.7	82.4	79.1
5	1.00	0.00	0.00	88.2	87.6	80.2
6	0.50	0.50	0.00	78.5	74.5	70.8
7	0.50	0.00	0.50	83.2	83.0	75.4
8	0.17	0.66	0.17	67.8	62.9	55.9
9	0.00	1.00	0.00	64.9	55.3	55.5
10	0.17	0.17	0.66	70.2	64.8	57.8

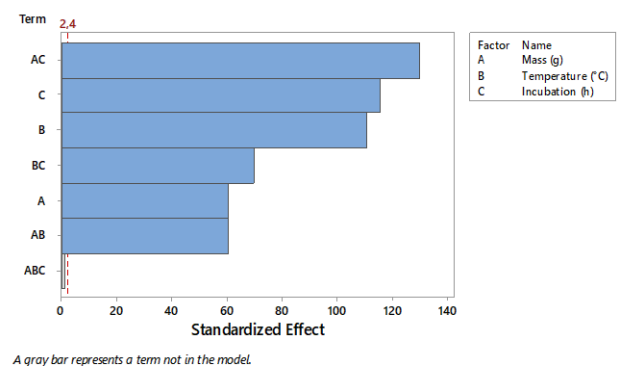


Fig. 2: Pareto Diagram of the feather proteins yield response

Other measurements were taken for the various experiments by the Mixture design plan based on the three variables (Sodium sulphite, SDS, and Sodium bisulphite) shown in Table 2 for variable optimization by the mixture design plan. The results of protein extraction are summarized in Tables 3 and 4.

Pareto diagram representation

The Pareto chart was used to determine the size and magnitude of the effects on which the bars intersecting the baseline are statistically significant. In Fig. 2, for example, the horizontal bars representing factors X_i and X_iX_j connect the red reference line at 2.4.

With the present model's terms, these factors were statistically significant at the 0.05 level. Since the Pareto chart showed the absolute value of the effects. This allowed the elements to be evaluated and compared with each other and their interactions two-by-two.

The analysis of the Pareto diagram (Fig. 2) showed that the interaction between the mass of the feather and the incubation period had the greatest effect on the response of the yield of the protein extraction, followed by the incubation time, temperature, and mass, which was previously proven [19,33]. This demonstrated that the interactions between the components were sometimes more influential than the components themselves [19]. The other interactions (AB, ABC) illustrated the slightest effect on the evaluated yield response. These observations could never be verified and highlighted without the DOE strategy of optimizing experiments and highlighting the effect of the interactions on a well-determined phenomenon, something that was not raised or treated by subsequent publications [19,31,33].

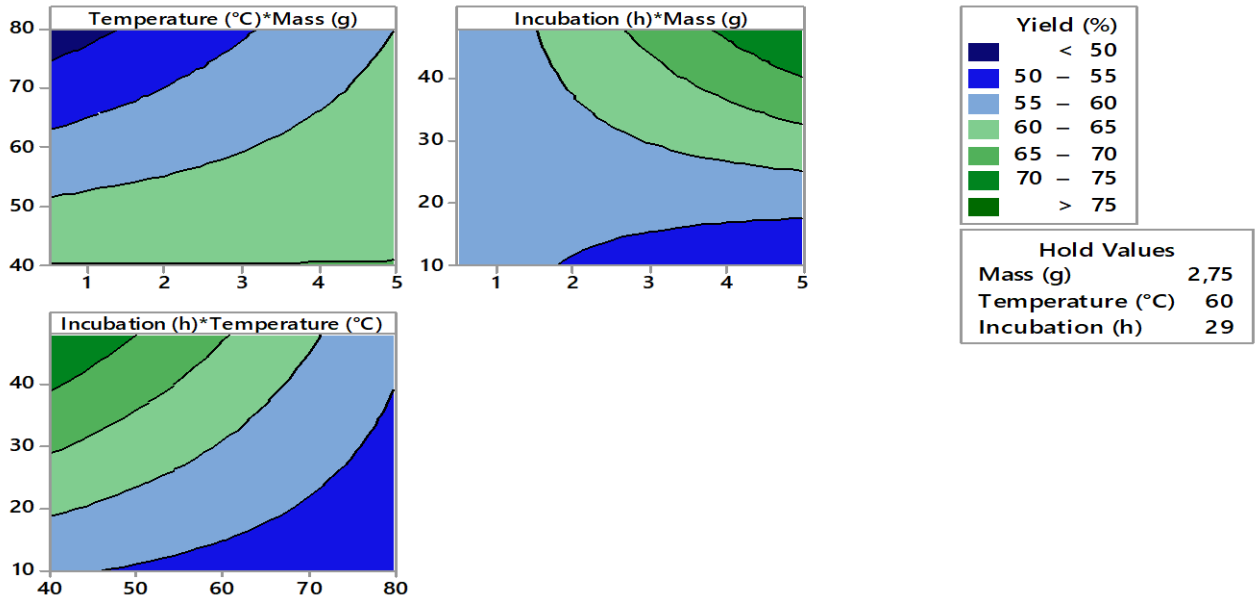


Fig. 3: Contour Plots of protein yield for each two-by-two interactions parameters (on maximum value)

Discussion for each response

Each response was represented by colorful bands in the Minitab software 19: Interval values were represented by a different color. The 2-dimensional representation called contour plot was chosen to give simple and understandable results. Then, the contour plot was easily interpreted with other graphs: The main factor controlling the maximum value of the response was pointed out by the Pareto chart.

The interpretation of the four responses: two-by-two interactions contour plot, the main effect plot, the interactions blot; and the equation of the proposed mathematical model are expressed in these graphs.

Proteins yield response

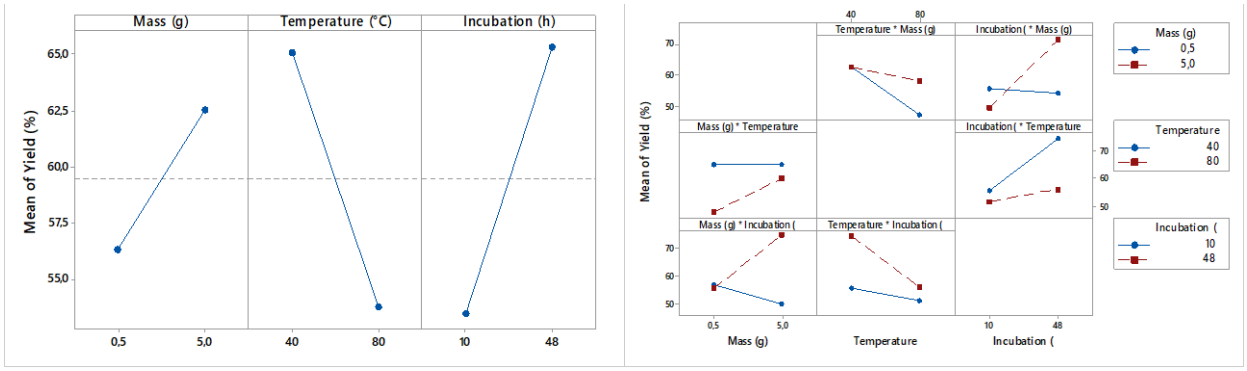
The contour graph (Fig. 3) showed that protein feathers' yield can be greater than 75 % (dark green band) when the values of the controlled parameters are maximal. The protein extraction increased when the temperature (40-50 °C), feather weight (4-5g), and incubation time (40-48 h) were elevated. Previous studies have proven these results. They explain that the chemical hydrolysis of keratins is often assisted by good heating to ensure high yield, however, elevated temperature following good incubation may increase also amino acid separation [7, 34, 35].

The main effects graph (Fig. 4a) depicts the specific influence of each of the three parameters on the protein Yield process. The maximum yield values using temperature value (T=40°C) and incubation time equal to 48h gave up to 65% for protein Yield extraction [19, 34]. The featherweight (equivalent to 5g) provided the highest protein production (62.5 %). The main effects graph shows the effects of each parameter, such as featherweight, temperature, and incubation period [19]. The intersecting effects in diagrams (Fig. 4b) demonstrate the relevance of weight and temperature in achieving a high protein yield (70%) for the same parameters discussed.

Protein yield was obtained at a high level, just when all the parameters were optimized to their maximum. Fig.4 b presents the diagram of interaction and highlights the varied responses according to the two-by-two interactions. Protein yield was depicted on the ordinate line, while parameter variations were depicted on the abscissa. For each parameter, two limit values (Maximum and Minimum) were determined [36].

Eq.5 illustrates all parameters including the main factor, the two-by-two, and the three interaction parameters in the mathematical model equation with a regression coefficient R^2= 99%. According to the Pareto diagram shown in Fig. 2. This depends on the affinity of the statistical calculations and their compliance with the statistical laws taken into account with a probability P <0.05.

$$\text{Yield (\%)} = 71.024 - 7.2636\ \text{Mass (g)} - 0.20045\ \text{Temperature (°C)} + 0.44836\ \text{Incubation (h)} + 0.06886\ \text{Mass (g)} * \text{Temperature(°C)} + 0.15553\ \text{Mass (g)} * \text{Incubation (h)} - 0.009405\ \text{Temperature (°C)} * \text{Incubation (h)}\ R^2=99\% \quad (5)$$



(a) (b)

Fig. 4: Diagrams of the interaction effects for protein concentration (280nm) (a: Main effects plot; b: Interaction plot)

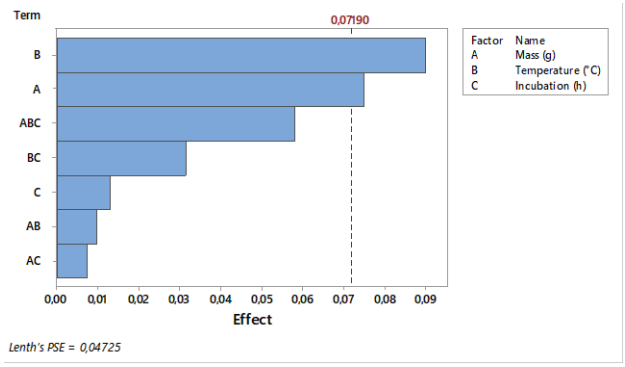


Fig. 5: Pareto diagram of the protein's concentration (280 nm) response

Protein concentration (UV-Vis 280nm) response

The Pareto chart helped to determine the size and magnitude of the effects. Data in Fig. 5 show that bars intersecting with the baseline were statistically significant and the horizontal bars representing Xi and XiXj intersect with the red reference line at 0.0719 value as indicated in Fig. 5. With the current model, factors are statistically significant at the 0.05 level [37].

From the Pareto diagram (Fig. 5), the rank of these parameters can be inferred from starting temperature, featherweight, and the interaction between the three factors impacting the protein concentration's response at 280 nm. Thus, the interaction between factors might be, sometimes, more important than the incubation time. Whereas, when the parameter values varied to their maximum (feather weight equal to 5g, T=80°C, and incubation time=48h), the contour graph (Fig. 6) showed a higher protein concentration (UV-Vis 280 nm) than 0.350 (dark green band).

The main effects graph (Fig. 7a) illustrated the impact of the incubation time of 48 hours on the keratin extraction. The incubation time of 48 hours, of 5 g at 80°C, can reach the protein yield to 0.31 than 0.275 value. This conclusion has been well validated by *Shavandi et al*. [38]. He showed that keratin yield is proportional to incubation, high temperature, and biomass.

The intersecting effect in diagrams (Fig.7b) showed the impact of feather mass and temperature in predicting maximum protein concentration up to 0.32 and 0.36 when the parameters are set to their maximum values.

The main graph illustrates the impact of the variation of each parameter like feather mass, temperature, and incubation time. According to the Pareto diagram, shown in Fig. 5, all parameters were set in the mathematical model equation (Eq. 6). This depended on the affinity of the statistical calculations and their compliance with the statistical laws taken into account with a probability P <0.05.

$$\text{Proteins Concentration (UV} - \text{vis)} = 0.1921 - 0.05123\ \text{Mass (g)} + 0.000457\ \text{Temperature (°C)}$$

R^2=100 % (6)

Protein concentration (bradford)

The intersection between the horizontal bars presenting Xi and XiXj factors and the red reference line value was shown by the diagram (Fig. 8). At the 0.05 level, these factors are statistically significant. Based on this Pareto diagram, feather mass, and temperature seem to be the most important factors influencing protein extraction. In addition, the results of the different two-by-two interactions (AC, BC, and AB) respectively, showed a minimum effect. The intersection of the red reference line at 0.0576 with the feather mass, temperature, and the three intersections parameters mentioned is considered a decisive factor for this response.

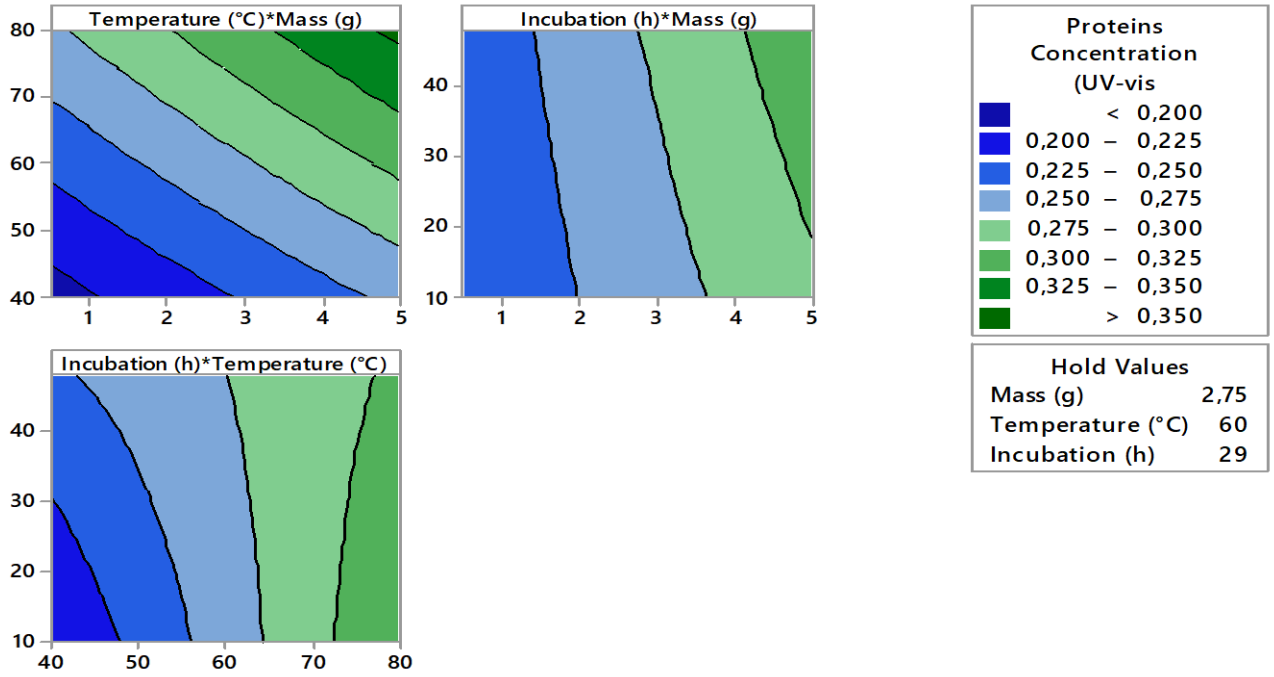
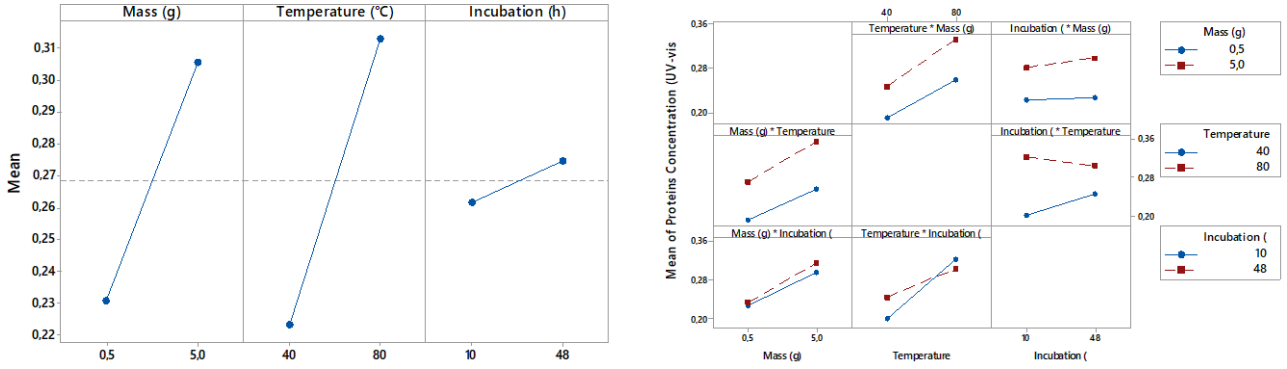


Fig. 6: Contour Plots of protein concentration (UV-vis 280nm) for each two-by-two interaction parameter (on maximum value)



(a) (b)

Fig. 7: Diagrams of the interaction's effect on protein concentration (280nm) response (a: Main effects plot; b: interaction plot

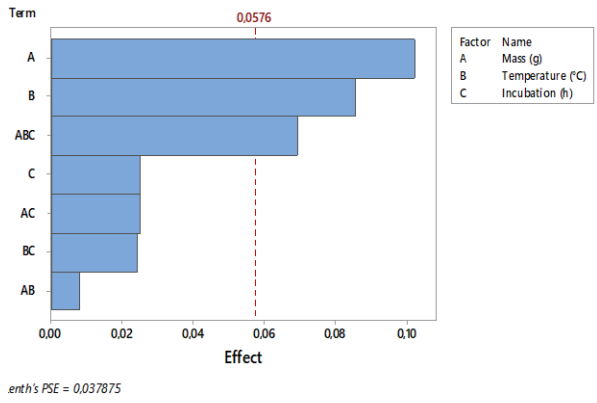


Fig. 8: Pareto chart for the response proteins concentration (bradford)

The contour graph (Fig. 9) shows the impact of the incubation time of 40 hours on the keratin extraction. The incubation time of 40 hours, of 5 g of a feather at 80°C, can reach the protein yield of 0.40 mentioned as the maximum protein concentration referred to in dark green color.

The effect of each factor on the protein concentration (Bradford) response is illustrated in Fig. 10 a and 10 b, indicating that the responses increased using the feather mass, the temperature, and the incubation time. The ideal keratin extraction was obtained with 5g of feather mass, 80 °C, and an incubation time equal to 48h (Fig. 10 a). Despite the studies that have been carried out, the process of

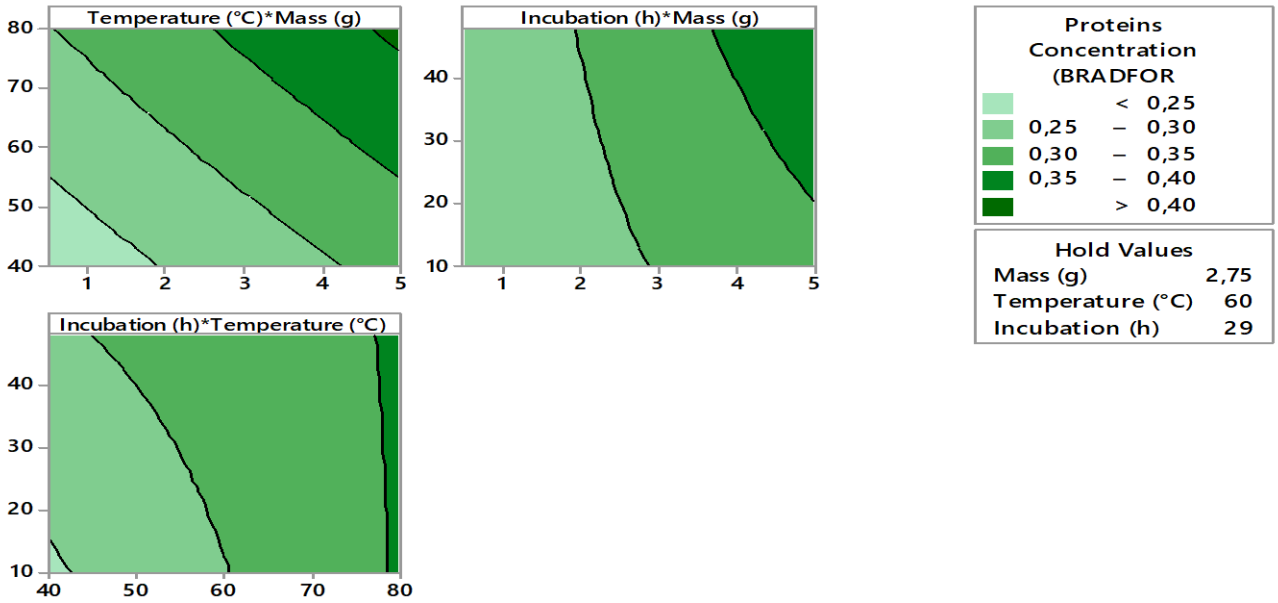
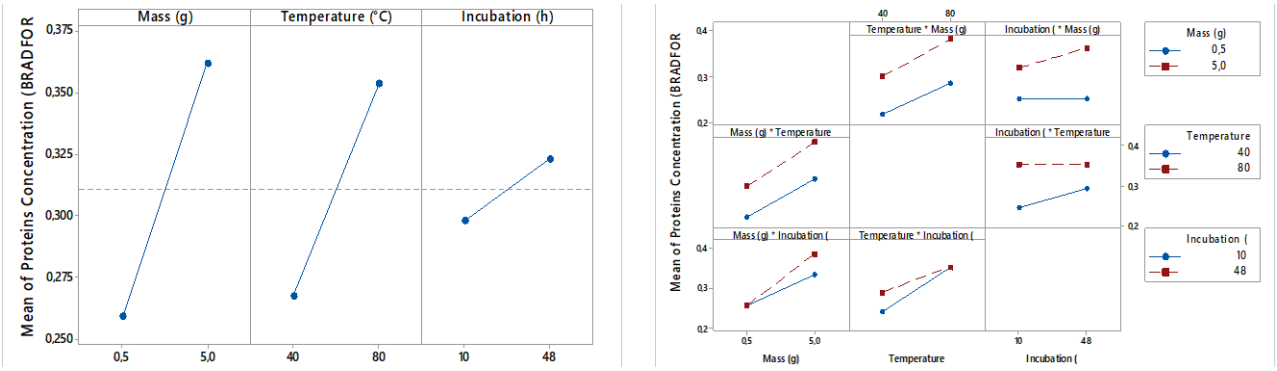


Fig. 9: Contour Plots of protein concentration (bradford) for each two-by-two interaction parameter (on maximum value)



(a) (b)

Fig. 10: Effects of interactions diagram for factorial Plots for Protein Concentration (Bradford) response (a: Main effects plot; b: Interaction plot)

extracting keratins from poultry feathers is not yet optimized. The concentrations of sodium bisulphite, sodium dodecyl sulfate, and β-mercaptoethanol, as well as the weight, temperature, and number of hours always, vary from one researcher to another. This is reflected in the work of Tonin and his colleagues [39], who used this mixture of chemicals with a final yield of keratin of 33%, while that of *Kamarudin* et *al.* [19] who used the same extraction technique under different conditions and obtained only 18.3% of keratin biomass. The main graph (Fig. 10 a) reveals the impact of the variation of each parameter like feather mass, temperature, and incubation time. Feather mass and temperature were chosen as the main factors in the mathematical model equation depending on the Pareto diagram, the affinity of the statistical calculations, and their compliance with the statistical laws were considered with a probability at $P < 0.05$.

In this case, the mathematical model considered only the feather mass, temperature, and interactions as shown in Eq. (7).

$$\text{Proteins Concentration (BRADFORD)} = 0.2763 - 0.06147\ \text{Mass (g)} - 0.000397\ \text{Temperature (°C)} - 0.004915\ \text{Incubation (h)} + 0.001261\ \text{Mass (g)} * \text{Temperature (°C)} + 0.002725\ \text{Mass (g)} * \text{Incubation (h)} + 0.000079\ \text{Temperature (°C)} * \text{Incubation (h)} - 0.000040\ \text{Mass (g)} * \text{Temperature (°C)} * \text{Incubation (h)} \quad R=100\% \qquad (7)$$

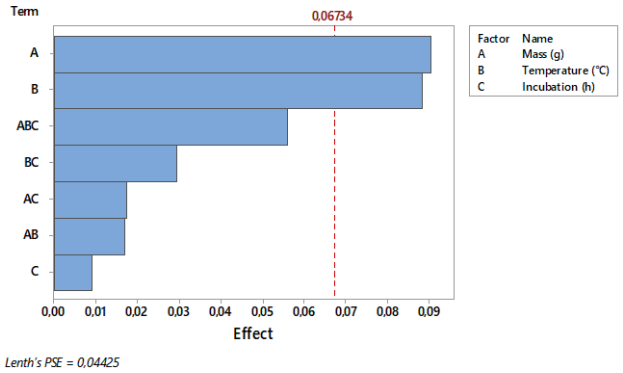


Fig. 11: Pareto Diagram of Proteins Concentration (BIURET)

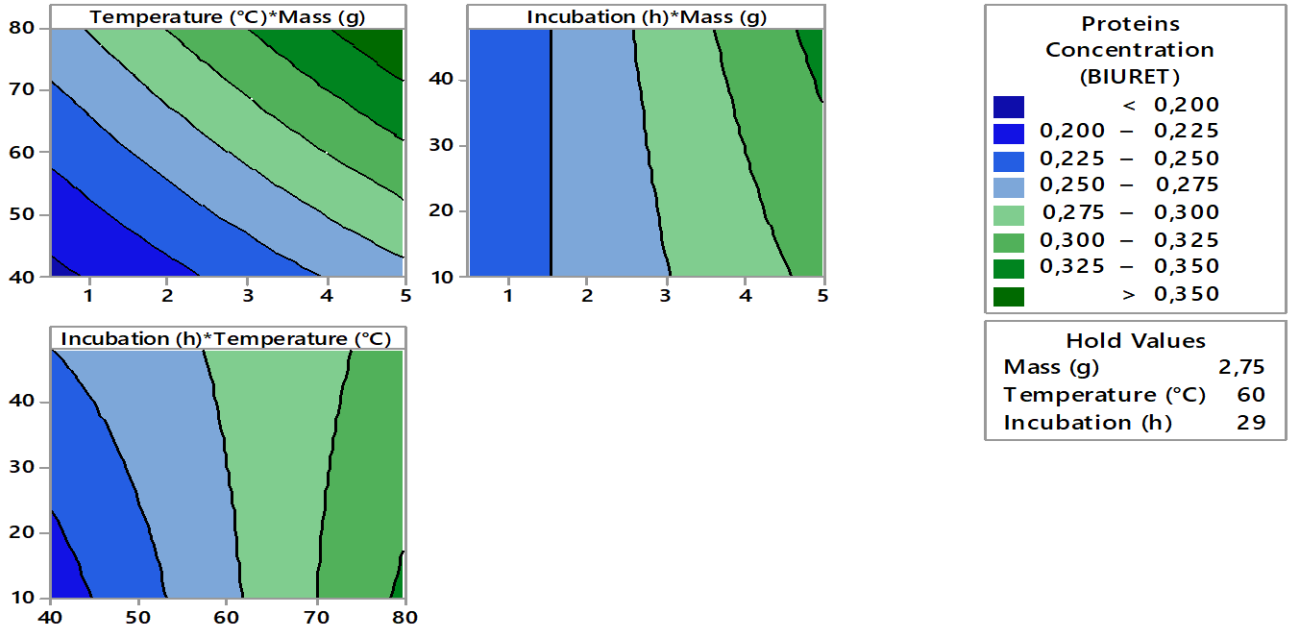


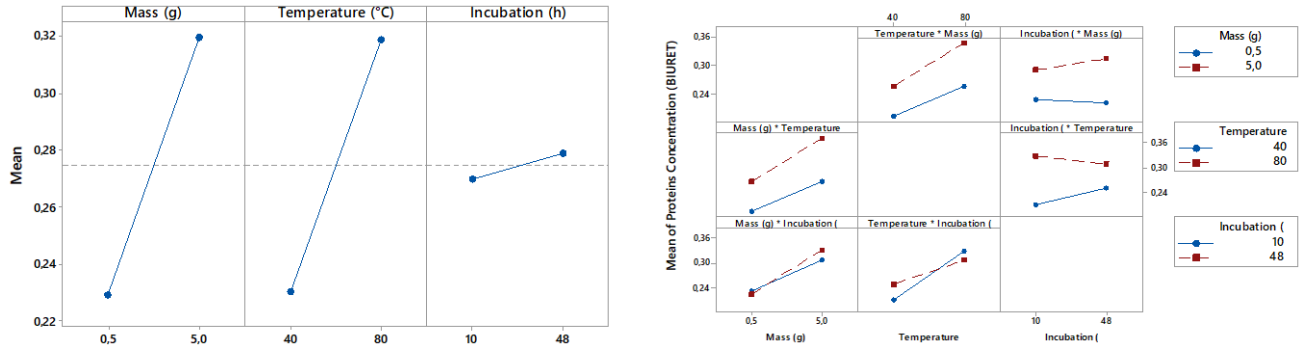
Fig. 12: Contour Plots of protein concentration (BIURET) contour graphs for each two by the two interaction parameters

Proteins concentration (biuret)

The Pareto diagram (Fig.11) shows that the horizontal bars representing Xi and XiXj factors intersect the red reference line value. These factors are statistically significant at the 0.05 level with the terms of the current model. Data from the Pareto diagram reveal that feather mass and temperature were the main factors impacting protein extraction. Moreover, the analysis of results indicated that different two-by-two interactions (BC, AC, and AB) respectively have the minimum effect. The red reference line at 0.0673 intersected only with the feather mass, temperature parameters are mentioned as a decisive factor for this response.

Analysis of the contour graph (Fig. 12) indicates the impact of the incubation time of 40 hours on the keratin extraction. The incubation time of 40 hours, of 5 g of a feather at 80°C, can reach a protein yield of 0.35 (dark green band).

The effect of each factor on the protein concentration (Biuret) response is shown in Fig. 13a and 13b with the increase of the feather mass and temperature and decrease of the incubation step. According to Fig 13a, the ideal protein extraction was obtained using 5g of feather mass at 90 °C and 48 h of incubation time. The main graph (Fig. 13a) reveals the impact of the variation of each parameter like feather mass, temperature, and incubation time. This graph is closely related to the results of *Kamarudin et al*. [19]. He proves that good keratin yield is linked to prolonged exposure to well-studied temperatures. Feather mass and temperature were chosen as the main factors in the mathematical model



(a) (b)

Fig. 13: Diagrams of the effects of interactions for proteins concentration (biuret) response (a: main effects plot; b: interaction plot)

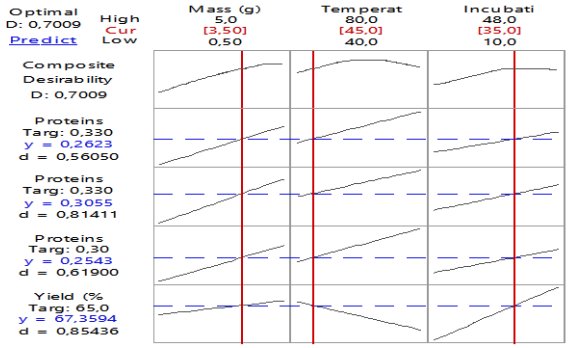


Fig. 14: Optimization graph with higher desirability coefficient.

equation depending on the Pareto diagram, the affinity of the statistical calculations, and their compliance with the statistical laws were taken into account with a probability of $P < 0.05$.

In this case, the mathematical model takes into account only the feather mass, temperature, and time of incubation as shown in Eq.8. The three factors and their interaction were significant and mentioned in the mathematical model (Eq. (8)).

$$\begin{aligned}\text{Proteins Concentration (BIURET)} &= 0.2162 - 0.05414\ \text{Mass (g)} \\ &+ 0.000207\ \text{Temperature (°C)} \\ &- 0.003401\ \text{Incubation (h)} + 0.001139\ \text{Mass (g)} \\ &* \text{Temperature (°C)} + 0.002170\ \text{Mass (g)} \\ &* \text{Incubation (h)} + 0.000051\ \text{Temperature (°C)} \\ &* \text{Incubation (h)} - 0.000033\ \text{Mass (g)} \\ &* \text{Temperature (°C)} * \text{Incubation (h)}\end{aligned}$$

$R^2 = 100\ \%$ (8)

Optimization of optima

This method of superimposing and optimizing the optima found, aimed to determine the values of the parameters giving us the optimum of the protein's extraction process of the three methods of measuring responses: proteins concentration (280 nm), Bradford, and Biuret. These optima were reached by fixing the satisfaction limits for the optimum sought for the different responses studied for the protein extraction process parameters that should be used. Our objective is to find the conditions to have the three responses at maximum optimum. In Fig. 14, the values in red correspond to the optimal values of each factor, and in blue the value of each response under these optimal conditions. The confirmed result after processing the data gives the following optimal values: feathers weight is 3.5 g, Temperature = 45 °C, and incubation time equal to 35 h with desirability.

Thus, the theoretical results given by the data processing can be compared to the practical result after the process of the extraction of the protein. Table 5 confirms the theoretical model's accord with the experimental values. This demonstrated that implementing the experimental plan can provide a promising result in terms of the extraction process with the fewest experiments and cost treatment.

Optimization of the components of the extraction mixture

According to the results obtained during the evaluation of the proteins extraction method of all experiments, the maximum protein concentration was 83.92 % corresponding to 0.7525 % of sodium sulphite, 0.014 % of SDS, and 0.2335 % of sodium bisulphite. Eventually, the spots are a dark green color that indicates the amount of protein extracted (Fig. 15).

For proteins concentrations (280 nm), Bradford and Biuret responses and according to the results obtained during the evaluations of the keratins extractions methods

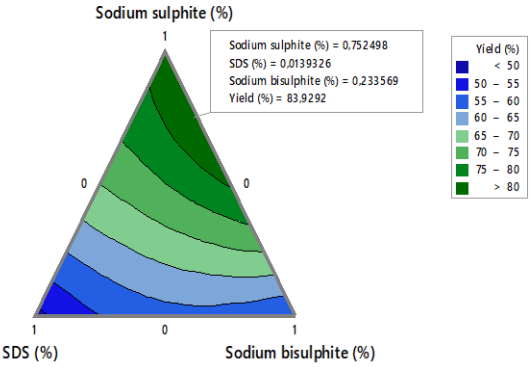


Fig. 15: Optimum optimization graph with higher desirability coefficient

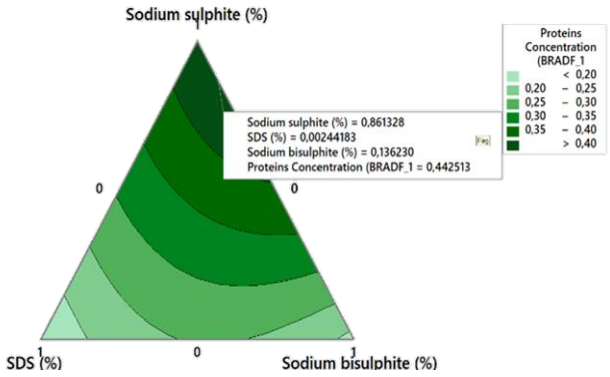


Fig. 17: Contour graph for proteins concentration (bradford)

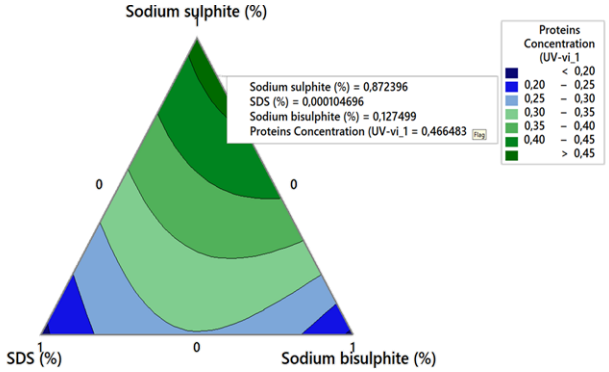


Fig. 16: Contour graph for proteins concentration (280 nm)

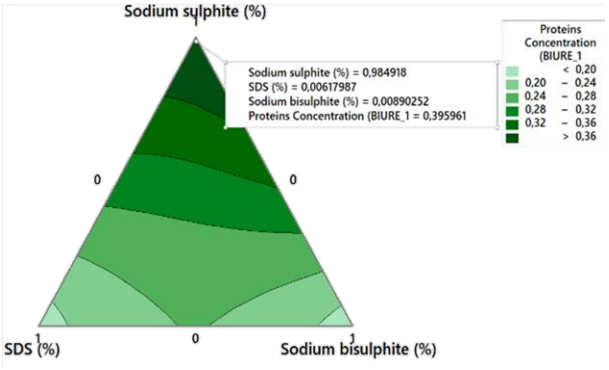


Fig. 18: Contour graph for proteins concentration (biuret)

of all experiments, the maximum concentrations were obtained with an OD greater than 0.45 and 88.2 % of yield; 0.40 and 82.4 % of yield; 0.388 and 80.2 % of yield, respectively, reflecting the amounts of proteins detected. In conclusion, the optimal areas are those with green and dark green colors. These zones expressed the quantity of the extracted protein (Fig. 16, Fig. 17 and Fig.18) whose optimum for these responses proteins concentrations (280), Bradford and Biuret correspond to combinations as follows (0.872 % of sodium sulphite, 0.0001 % of SDS and 0.127 % of sodium bisulphite); (0.861 % of sodium sulphite, 0.0024 % of SDS and 0.1362 % of sodium bisulphite); (0.985 % of sodium sulphite, 0.0062 % of SDS, and 0.009 % of sodium bisulphite) respectively. Our work follows the same reasoning as that of *Khumalo et al.* [18] with slightly different results.

The contour graphs (Fig. 15, Fig. 16, Fig. 17, and Fig. 18) show the evolution of the four responses for each parameter as sodium sulphite, SDS, and Sodium bisulphite components and their interactions. All parameters and their interactions were chosen as the main factors in the mathematical model equation (Eqs. (9-11 depending on the affinity of the statistical calculations and their compliance with the statistical laws taken into account with a probability P <0.05 with a significant regression coefficients R^2.

$$\begin{aligned} &Yield\ (\%) \\ &= 54.32 + 27.78\ Sodium\ sulphite\ (\%) \\ &- 5.94\ SDS\ (\%) + 19.0\ Sodium\ sulphite\ (\%) \\ &* SDS\ (\%) + 49.3\ Sodium\ sulphite\ (\%) \\ &* Sodium\ bisulphite\ (\%) + 26.6\ SDS\ (\%) \\ &* Sodium\ bisulphite\ (\%) \\ &- 65\ Sodium\ sulphite\ (\%) * SDS\ (\%) \\ &* Sodium\ bisulphite\ (\%) \end{aligned}$$

R^2= 89.53 % (9)

$$\begin{aligned} &\text{Proteins Concentration (UV} - \text{vis 280 nm)} = \\ &0.1890 + 0.2745\ \text{Sodium sulphite (\%)} \\ &- 0.0018\ \text{SDS (\%)} + 0.034\ \text{Sodium sulphite (\%)} \\ &* \text{SDS (\%)} + 0.341\ \text{Sodium sulphite (\%)} \\ &* \text{Sodium bisulphite (\%)} + 0.440\ \text{SDS (\%)} \\ &* \text{Sodium bisulphite (\%)} \\ &- 0.27\ \text{Sodium sulphite (\%)} * \text{SDS (\%)} \\ &* \text{Sodium bisulphite (\%)} \end{aligned}$$

R^2 = 90.80 % (10)

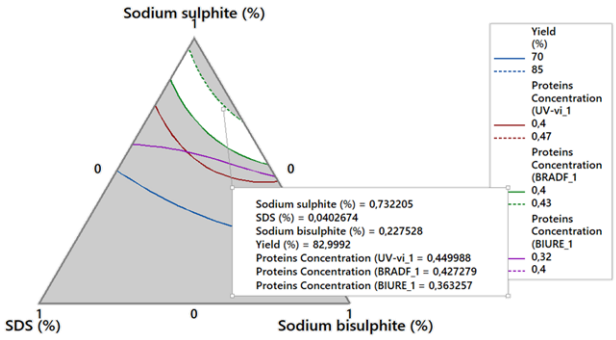


Fig. 19: Superimposed optimization graph with target limits

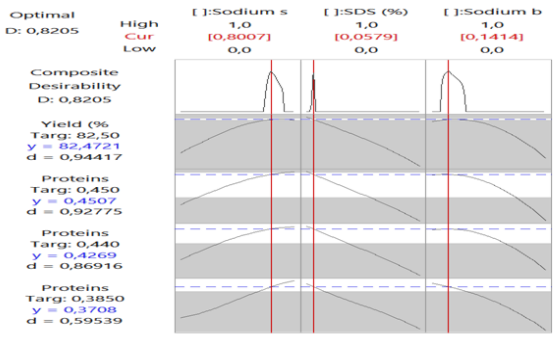


Fig. 20: Superimposed optimization graph with target limits

$$\text{Proteins Concentration (BRADFORD)} = 0.1842 + 0.2548\,\text{Sodium sulphite (\%)} - 0.0325\,\text{SDS (\%)} + 0.041\,\text{Sodium sulphite (\%)} * \text{SDS (\%)} + 0.330\,\text{Sodium sulphite (\%)} * \text{Sodium bisulphite (\%)} + 0.340\,\text{SDS (\%)} * \text{Sodium bisulphite (\%)} - 0.24\,\text{Sodium sulphite (\%)} * \text{SDS (\%)} * \text{Sodium bisulphite (\%)}$$

$R^2 = 90.02\ \%$ (11)

$$\text{Proteins Concentration (BIURET)} = 0.1734 + 0.2243\,\text{Sodium sulphite (\%)} + 0.0047\,\text{SDS (\%)} + 0.044\,\text{Sodium sulphite (\%)} * \text{SDS (\%)} + 0.158\,\text{Sodium sulphite (\%)} * \text{Sodium bisulphite (\%)} + 0.259\,\text{SDS (\%)} * \text{Sodium bisulphite (\%)} - 0.68\,\text{Sodium sulphite (\%)} * \text{SDS (\%)} * \text{Sodium bisulphite (\%)}$$

$R^2 = 82.92\ \%$ (12)

Optimization analysis

The optimum was sought for the four responses shown in Fig. 19, including the extraction yield of 83%, protein concentration (280nm (OD = 0.449), Bradford (OD = 0.427), and Biuret (OD = 0.363), which corresponded to the following component combinations: 0.732% sodium sulphite, 0.040% SDS, and 0.227% sodium bisulphite. The maximum number of proteins detected by the extraction yield and the three methods are provided by this composition mixture. The solid and dotted lines represent the target value's upper and lower limits. As shown in Fig. 19, the white area represents the optimum area for protein extraction, which is supported by the results of the four methods.

In Fig. 20, the red and blue values correspond to the optimal results values of each factor under these optimal conditions. After processing the data, the following optimal values were obtained: 0.8% of sodium sulphite, 0.058% of SDS, and sodium bisulphite equal to 0.14% giving a yield of extraction of 82.47%. The OD of 280 nm was equal to 0.451, the OD of Bradford was 0.427, and finally, the OD value of Biuret was equal to 0.371 with 82.05% of desirability.

This methodology demonstrates that using the Mixture design plan provides encouraging results in terms of having an extraction process with the fewest experiments and low-cost treatment [40].

SDS-PAGE analysis

Fig. 21a depicts the electrophoretic SDS-PAGE gel patterns and average molecular mass values of the extracted keratin from chicken feathers under various conditions. This figure summarizes the SDS-PAGE results for the optimum experiments conditions and parameters (Tables 1 and 2) according to the three different parameters (Design of experiment): weight feather, temperature, and incubation time with the three other different parameters (Mixture design plus): sodium sulfite, sodium bisulphite, and SDS to have the maximum of protein extraction. The result showed the presence of two hyper-focused tracks with upper and lower bands of 24-45 kDa and 12-18 kDa and which were marked with black and blue dotted lines. Fig. 21a, proved also that the two upper and lower bands were obtained when feather keratin was extracted using 1 M of sodium sulphite, 0.1 M SDS, and 0.2 sodium bisulphite M and it was higher than used 1M of only sodium hydroxide according to samples (8 and 9) in Table 2. This higher yield may be due to the dissociation of disulphide bonds by the sodium bisulphite (sulphitolysis reaction)

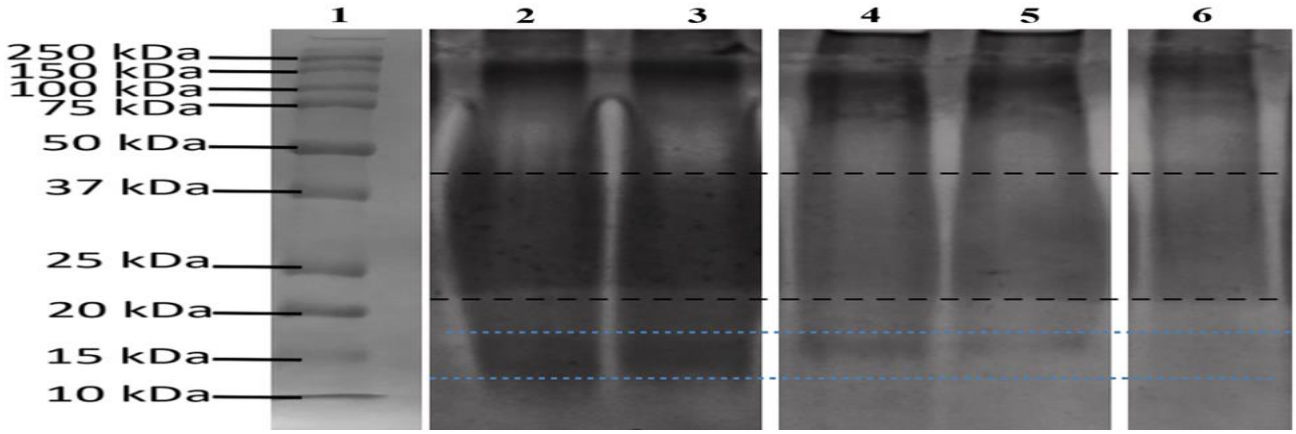


Fig. 21a: The SDS-PAGE of keratin analysis. 10 µg of protein extracts from feathers using acetone precipitation and different extracted protocols. Column 1: protein marker; Columns (2, 3, 4, 5, and 6) represents samples (6, 1, 8, 3, and 8) respectively. Stain made with colloidal coomassie blue G-250

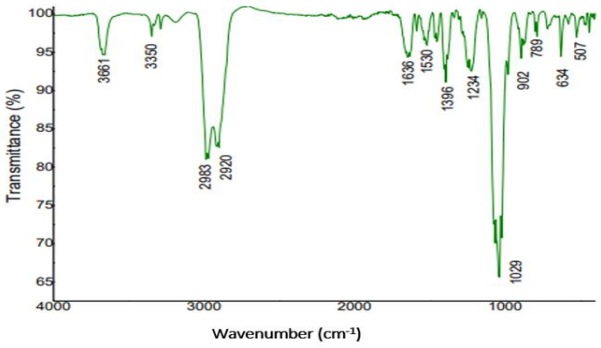


Fig. 21b: Mid-infrared spectra of keratin feather from the 81.2 % sample

and SDS. The profile was three times higher than that of the extracted keratin by *Oakley et al*. [28] and almost similar to those of *Khumalo et al*. [18]. Fig. 21a recapitulates the SDS-PAGE data according to the mixing plan described in (Table 2). This approach follows sodium sulphite, SDS, and sodium bisulphite components are needed for maximum protein extraction. These results proved that the feather keratin depends mainly on the extraction conditions (Fig. 21a and 21b).

CONCLUSIONS

The molecular weights fractions of feather keratin were obtained using a novel and simple method with sodium sulphite, sodium bisulphite, and SDS reagents that produced mostly two bands of 24-45 kDa and 12-18 kDa. The natural feather keratin yield was remarkably increased to 81.2% by the addition of sodium sulphite/sodium bisulphite to the SDS. This step demonstrated that reduction processes are the quickest and most efficient method. These findings aid researchers in determining which method is most likely to generate the optimal component of a feather keratin product. Furthermore, statistical optimization of feather keratin extraction using a central composite design and a mixture plan for optimization increased protein yield. This process of keratin extraction can be used from the laboratory to industrial production with high recoverability and stable properties.

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