Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 1, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

KERATIN EXTRACTION FROM CHICKEN FEATHERS IN AQUEOUS SOLUTIONS

Victor Alexandru FARAON[1#], Eliza Gabriela MIHĂILĂ[1, 2#], Naomi TRITEAN[1, 3], Bogdan TRICĂ[1, 2], Luiza CAPRĂ[1], Mihail-Bogdan ROMAN[4], Diana CONSTANTINESCU-ARUXANDEI[1], Florin OANCEA[1, 5]

[1]National Institute for R & D in Chemistry and Petrochemistry - ICECHIM, 202 Splaiul Independenței, 060021, Bucharest, Romania
[2]POLITEHNICA University of Bucharest, 313 Splaiul Independenței, 060042, Bucharest, Romania
[3]Faculty of Biology, University of Bucharest, 91-95 Splaiul Independentei, R-050095, Bucharest, Romania
[4]FIA Crop Control, 3 Interioară Street, 900330, Constanța, Romania
[5]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnologies, Bucharest, Romania
[#]These authors contributed equally

Corresponding author emails: diana.constantinescu@icechim.ro, florin.oancea@icechim.ro

Abstract

Biowastes have emerged as a promising source for the production of value-added products, reducing the burden on landfills and promoting the concept of a circular economy. Chicken feathers, constituting a significant fraction of the poultry industry's waste stream, possess a robust protein structure composed mainly of keratin. Keratin is a biopolymer with unique properties, including high nitrogen content and slow degradation, making it an attractive candidate for various applications in agriculture and other fields. One of the main problems is the development of more eco-friendly methods for the efficient extraction of this biopolymer. The aim of this study was to compare the yield of keratin extraction from chicken feathers by three methods, alkaline, acidic and subcritical water and to characterize the keratin obtained by the most eco-friendly method, i.e., subcritical water. The subcritical water extraction is a promising alternative to the alkaline and acidic extractions, if proper optimization is carried out. We show that SDS-PAGE electrophoresis combined with FTIR analysis can offer valuable Information in this respect.

Key words*: poultry industry, feather keratin, subcritical water extraction, alkaline extraction, acidic extraction.*

INTRODUCTION

Keratin is one of the most abundant biopolymers, being produced by the epithelial cells of vertebrates (Meyers, Chen, Lin, & Seki, 2008). Together with collagen, keratin assures the structural rigidity of the different vertebrate coverages – skin, nails, hair, horns (McKittrick et al., 2012).

Keratin is a complex mixture of filament-forming proteins (Tinoco et al., 2020; Tomlinson, Mülling, & Fakler, 2004), it is characterised by a high content of sulfur-containing amino acids and is organized in two types of secondary structures, α-helix (e.g., wool) and β-sheet (e.g., feathers) (Calvaresi, Eckhart, & Alibardi, 2016; Chilakamarry et al., 2021).

The keratin chains are cross-linked by di-sulfuric bridges, which stabilize and rigidize the secondary and tertiary structures (Wang, Yang, McKittrick, & Meyers, 2016). Due to its complex and highly organized structures, keratin is insoluble in most of the known protein solvents and is resistant to the hydrolysis of the usual proteases, such as subtilisin, pepsin, papain or trypsin (Chaitanya Reddy et al., 2021; Jaouadi et al., 2013).

The bioeconomy, i.e., the economic sectors producing and using bioresources, generates large amounts of keratin- rich side streams – wool from sheep rearing or feathers, nails, hairs, skins from slaughterhouses, hair and wool from leather industries (Gaidau et al., 2019; Sharma & Gupta, 2016). Overall, such keratin-rich side streams are estimated to exceed yearly the

threshold of 40 million tons (Hussain, Memon, Khatri, & Memon, 2020; Suarato et al., 2020).
Disposal of keratin-rich material is difficult. Landfilling generates high epidemiological risks, due to the proliferation of the keratinolytic (human) pathogens (Călin et al., 2017; Duan et al., 2020). Incineration raises technical difficulties due to low flammability of keratin and produces hazardous substances like sulfur oxides (Ossai, Hamid, & Hassan, 2022; Tesfaye, Sithole, Ramjugernath, & Chunilall, 2017).
The ideal solution for keratin is to use the high protein content of keratin- rich side streams for various applications - in food and feed industries or as agricultural inputs (Alvarez-Castillo, Felix, Bengoechea, & Guerrero, 2021; Giteru et al., 2023; Perța-Crișan, Ursachi, Gavrilaș, Oancea, & Munteanu, 2021). Keratin protein is a source of plant biostimulants (Kaur, Bhari, & Singh, 2021; Popko et al., 2018), increasing plant tolerance to abiotic stress, improving crop quality traits and enhancing nutrients uptake and nutrients utilization. Due to their high resistance to biodegradation, keratin proteins are good candidates for sustainable fertilizer coating (Chen, Li, & Zhang, 2021; Choi & Nelson, 1996; Mihăilă et al., 2020; Yang, Tong, Geng, Li, & Zhang, 2013).
For such utilizations it is necessary to solubilize keratin from keratin-rich material. The aim of this work was to compare the yield of different methods for protein solubilization from chicken feathers and to characterize the protein extracted by the most eco-friendly method, subcritical water extraction.

MATERIALS AND METHODS

Materials

The chicken feathers were acquired from a local Romanian poultry farm. Cetrimonium bromide (CTAB) and Hydrochloric (HCl) acid 37% were from Sigma-Aldrich (Missouri, USA) and sodium hydroxide (NaOH) was from Chimreactiv (Bucharest, Romania). The chemicals for SDS-PAGE, Bradford and Biuret were: Precision Plus Protein™ Dual Xtra Standards, Mini-PROTEAN TGX Stain-Free Gels 4-20%, 2X Laemmli sample buffer, 2-mercaptoethanol, (Bio-Rad, California, USA), Bradford Reagent (Sigma-Aldrich, Missouri, USA), Brilliant Blue G ultrapure powder, Sodium n-dodecyl sulfate 99% (dry wt.) (Alfa Aesar, Massachusetts, USA), Glycine, Tris-(hydroxymethyl)-aminomethane, Sodium hydroxide, Hydrated copper(II) sulfate, Potassium sodium tartrate, Potassium iodide (Scharlau, Barcelona, Spain), Glacial acetic acid (Chimreactiv, Bucharest, Romania), Methanol (Honeywell, Indiana, USA), Albumin bovine fraction V, pH 7.0 (Janssen Chimica, Beerse, Belgium).
The feathers were initially washed with double distilled water (ddH_2O) + 1% CTAB in a ratio of 1/20 (w/v) at 50°C and magnetic stirring at 400 rpm for 2h, followed by rinsing with ddH_2O to remove CTAB. The rinsing process involved gently agitating the feathers in clean water until no more foam was observed. After rinsing, the feathers were air dried to remove excess moisture. They were spread out in a well-ventilated area and allowed to dry overnight at room temperature, ensuring complete drying. After the washing and drying steps, the feathers were further processed through milling to obtain a fine and homogeneous powder. Feather milling was conducted using a centrifugal ball mill (Retsch Type S 100, Haan, Germany) equipped with a ceramic grinding jar and ceramic balls. The centrifugal ball mill was operated at 380 rpm. The milling process lasted for a total of 4 hours to ensure thorough grinding and particle size reduction. To promote uniform milling and prevent sample aggregation, the direction of rotation was changed alternately between clockwise and counterclockwise every 2 minutes.

Alkaline hydrolysis of keratin from chicken feathers

For this extraction of keratin 6M solution of HCl and 1N solution of NaOH were used. The prewashed and grinded feathers were immersed in a mixture of NaOH 1N solution, with a ratio of 1:10 (w/v) for 1 hour at 90-95°C, under stirring (20 g feathers + 200 ml NaOH 1N). Samples were taken as to neutralize the hydrolysate with HCl 6M to a neutral pH (Mettler Toledo Seven Compact S210 pH meter) and further to acidify it down to the isoelectric point of feather keratin previously determined for the extraction in subcritical water, pH = 4.4 (Škerget et al., 2023). When the pH reached 4.4 the total sample was centrifuged at 15°C, 8500 rpm, 20 minutes (Hettich Universal Centrifuge

320 R, Tuttlingen, Germany). After that the precipitate was washed with ddH_2O and lyophilized (SCANVAC CoolSafe, Labogene, Lillerød, Denmark). The N content was determined in the supernatant after each reaction using the Kjeldahl method and reported to 100 g of feathers.

Acidic hydrolysis of keratin from feathers

For this extraction of keratin, 4 M solution of HCl and 1 M solution of NaOH were used. Pre-washed and grinded feathers (30 g) were mixed with 200 ml HCl 4 M. The mixture was boiled at 95°C until the feathers were dispersed. The resulted solution was filtered twice. The pH of filtrate was adjusted with NaOH 1M to reach the isoelectric point of keratin (pH = 4.4), at which the keratin precipitated. After that the precipitate was centrifuged (15°C, 8500 rpm, 20 minutes), washed with ddH_2O and lyophilized.

Keratin hydrolysis using super-heated (subcritical) water

The pre-washed and grinded feathers were mixed with ddH_2O at a substrate/water ratio of 1/20 (w/v), i.e., 6 g feathers with 120 mL ddH_2O. The experiments were done at two temperatures, 150°C and 200°C for 2 h, using a Parr Series 4523 Bench Top Reactor (Moline, USA), with 200 rpm agitation and pressure of 9 bar at 150°C and 11 bar at 200°C.

Characterization of keratin extracts

Infrared spectroscopy (FTIR) analyses were performed using a Shimadzu FTIR IRTracer-100 and FTIR automated microscope AIM-9000 spectrometer. A resolution of 4 cm^{-1} and a wavelength interval of 4000-400 cm^{-1} were used. For cryo - TEM analyses a Tecnai ™ G2 F20 TWIN Cryo-TEM (FEI Company ™) apparatus was used. The sample was prepared by embedding it in an epoxy resin (Agar 100 Resin Kit) from Agar Scientific (Stansted, UK). First, the fine powder was kept overnight at room temperature in a 1.5 mL volume from a mixture of epoxy resin (without accelerator) and acetone (1: 1 ratio by volume). The next day, the whole mixture was separated by centrifugation at 3000 g for 10 minutes at room temperature. The supernatant was discarded and the wet pellets containing the sample were left in a well-ventilated space at room temperature to remove all acetone by evaporation. The acetone-free wet pellets were then mixed with another 1.5 mL of epoxy resin with added accelerator and added to a silicon mold. After 48 h of crosslinking at 60°C, the embedded sample was cut to 80 nm slices using a Leica EM UC7 ultramicrotome (Wetzlar, Germany) at a cutting rate of 2 mm/s which were then collected on 200 mesh lacey carbon film/formvar grids from Ted Pella, Inc. (USA). The sample was analyzed at an accelerating voltage of 200 kV.

Biuret and Bradford assays

The protein concentration in the two keratin hydrolysate samples obtained by subcritical water was determined using Coomassie Brilliant Blue G-250 dye binding-assay – Bradford, and a cooper-based assay – Biuret. The Biuret assay was performed by mixing 40 µL of 1:4 diluted samples with 200 µL of Biuret reagent in 96-well plates. After 30 minutes of incubation at room temperature, the absorbance of the samples was read at 550 nm using a microplate reader (CLARIOstar Plus microplate reader, BMG LABTECH, Ortenberg, Germany) against a bovine serum albumin (BSA) standard curve prepared from a 10 mg/mL BSA stock solution and which underwent the same steps (Janairo, Linley, Yap, Llanos-Lazaro, & Robles, 2011). The Bradford assay was performed by mixing 200 µL of 1: 50 diluted samples with 50 µL of Bradford reagent in 96-well plates. After 5 minutes of incubation at room temperature, the absorbance of the samples was read at 595 nm. The BSA standard curve was prepared from a stock solution of 246 µg/mL BSA which was subjected to the same treatment (Bradford).

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Keratin hydrolysate samples obtained by subcritical water were analysed by SDS-PAGE using protein electrophoresis Mini-PROTEAN Tetra Cell (Bio-Rad, California, USA). After mixing 10 µL of sample with 5 µL of loading buffer, the samples were thermally denatured for 5 minutes at 95°C. 15 µL of sample and 5 µL of molecular mass marker were loaded into the electrophoresis gel well according to the manufacturer's instructions. The migration time up to the bottom of the gel was about 45 minutes at a constant voltage of 30 mA. Subsequently, the electrophoresis gel was washed 3 times with double distilled water and immersed in the staining solution based on Coomassie Blue R-250, where it was left for a few hours under stirring (ROCKER 3D digital, IKA, Staufenim

Breisgau, Germany). The gel was then washed with double distilled water, heated in the microwave and left under stirring for 24 hours, changing the water several times. A gel documentation system was used for the image acquisition (G:BOX Chemi XRQ, Syngene, Cambridge, United Kingdom).

RESULTS AND DISCUSSIONS

The alkaline and acidic hydrolysis gave the yield of 72.5% (14.5 g from 20 g feathers) and 64.6% (19.38 g from 30 g) extracted keratin, respectively, based on the total N measurements of extracted keratin. In fact, considering the previously reported keratin percent of 91% in feather, the yields are 79.7% and 71%, respectively. The precipitation of keratin at the isoelectric point resulted in approx. 50% precipitation of keratin in the case of the alkaline extraction and approx. 60% precipitation in the case of acidic extraction. This suggests higher molecular weight keratin fragments in the acidic extract than in the alkaline extract.

The yield in the case of the extractions with the subcritical water was 26.4% (29% of the total feather keratin) at 150°C (resulting approx. 13.75 mg/ml keratin concentration) and 90.5% (99.45% of the total feather keratin) at 200°C (resulting approx. 42 mg/ml keratin concentration). This is consistent with previous studies that reported, during the finalization of our studies, yield below 30% at 150°C and almost 80% yield at 200°C after 1h (Škerget et al., 2023). In the case of 200°C, although the yield was almost 100%, there were signs of compound degradation after the 2 h extraction, the extract color suffering a radical change from dark yellow to dark brownish (Figure 1).

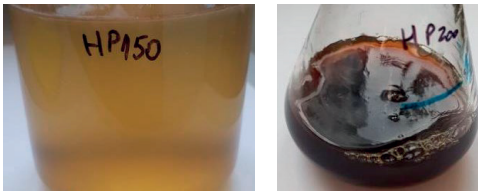

Figure 1. Reaction mixtures after hydrolysis with super-heated water: at 150°C (HP150) and at 200°C (HP200)

Signs of compounds degradation have been also reported previously at 250°C after 10 min of extraction, leading to a decrease in the yield probably caused by some volatile formation (Škerget et al., 2023). It is possible that in our case, after breaking down in small peptides and amino-acids at 200°C, some compounds suffered oxidation and possibly also Maillard-type reactions. Our study shows in addition that the extraction at 150°C does not improve after an additional hour compared to the previous study (Škerget et al., 2023), which indicates, together with the previous study, that temperatures up to 150°C are not efficient for extracting keratin in subcritical water even when using longer time periods.

Cryo-TEM analysis indicated the presence of high aggregates of colloidal-like structures formed from keratin proteins (Figure 2). Keratin was previously shown to have the tendency to form colloids. The aggregation is higher as the pH approaches the isoelectric point of the hydrolysate. The striations that can be observed at the 100 nm scale are an indication of the presence of keratin fragments obtained by the hydrolysis process.

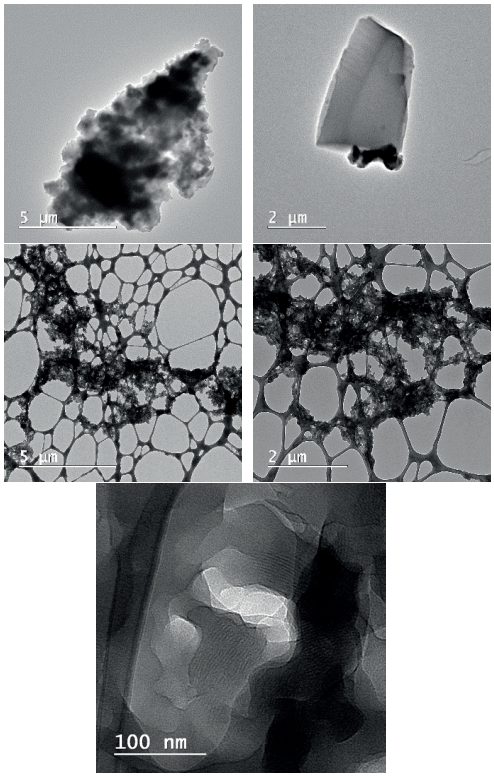

Figure 2. Cryo-TEM of keratin hydrolysate (HP200)

Bradford gave a protein concentration more than 50 times lower than Biuret i.e., 0.25 mg/mL compared to 14.81 mg/mL for HP150, and 0.45 mg/mL compared to 21.89 mg/mL for HP200 (Figure 3A). It is well known that the results obtained by the Bradford method depend on the amino acid composition, the Coomassie Blue R-250 i.e., dye binding readily to arginine and lysine residues (Compton & Jones, 1985). Lysine has not been found previously in significant amounts in keratin extracted by subcritical water, while arginine gave less clear results, one study reporting moderate to relatively high amounts and one study no mention about arginine (Di Domenico Ziero et al., 2022; Škerget et al., 2023). The difference between the two studies was that the first was performed in a semi-continuous flow. Moreover, the Bradford method is more suitable to pure proteins and other compounds could interfere with this method, including eventual CTAB traces remained after washing and rinsing (Aminian, Nabatchian, Vaisi-Raygani, & Torabi, 2013). The Biuret method does not depend on the amino-acid composition, but it is a direct evaluation of peptide bonds. Some differences between Bradford and Biuret have been reported in other cases as well, for example in the quantification of the protein from rapeseed meal (Kalaydzhiev, Ivanova, Uzunova, Manolov, & Chalova, 2018). Therefore, the differences between the two methods were not completely unexpected, although the differences are higher than estimated. The values obtained by Biuret are closer to the previously reported values (Di Domenico Ziero et al., 2022). Compared to the extracted keratin estimated based on the total N content, the keratin determined by Biuret was similar at 150°C (14.81 versus 13.74 mg/ml) but lower at 200°C (21.89 mg/ml versus 42 mg/ml). This indicates that at 200°C almost half of the peptide bonds got hydrolysed to amino-acids and other compounds that do not react with Biuret. The higher difference between the protein concentration of HP200 and HP150 measured with Bradford (ratio HP200/HP150 = 1.80) than with Biuret (HP200/HP150 = 1.5) show the differences between the two methods. The contribution to Bradford comes also from the free amino-acids and the results confirm that the amino-acids are probably more abundant in the HP200 sample than in the HP150 sample.

In the case of the SDS-PAGE the HP150 and HP200 samples were diluted 4-fold before mixing with loading buffer, and the 4-fold diluted HP200 sample (that had higher protein concentration than HP150) was additionally diluted to the concentration of the 4x diluted HP150 sample (HP200d).

In the case of the hydrolysate obtained at 150°C (HP150) the SDS gel shows a relatively narrow profile, with most protein fragments between 3 and 10 kDa (Figure 1B). This is in agreement with the previous study which reported molecular weights of extracted peptides in the range 4-12 kDa (Škerget et al., 2023).

Although the protein concentration shown in Figure 3A is higher in the case of sample HP200, apparently hydrolysis at a temperature of 200°C induces further hydrolysis of peptides the loss of high temperature sensitive amino acids (Bhavsar et al., 2016; Rajabinejad et al., 2017), as can be seen in Figure 1 B by the absence of bands for HP200 sample, and it also leads to an oxidation process, given by the brown colour of the hydrolysate, so these oxidation products may interfere with the methods used to determine protein content.

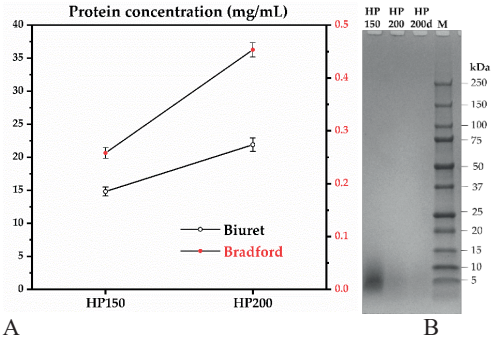

Figure 3. Protein analysis of keratin hydrolysates: (A) Protein concentration (mg/mL) determined by the Biuret and Bradford methods (error bars ± standard deviation (SD)); (B) Molecular weight analysis by SDS-PAGE; HP150=keratin hydrolysate obtained at 150°C, 4-fold dilution; HP200=keratin hydrolysate obtained at 200°C, 4-fold dilution; HP200d=keratin hydrolysate obtained at 200°C diluted to the protein concentration of sample H150; M=molecular weight marker

The FTIR spectra showed that the keratin extracted at 150°C maintained the structural features found in the feathers (Figure 4). The

main peaks the untreated feathers, known as the fingerprint for proteins, are present at approx. 1629 cm^{-1} (amide I), 1535 cm^{-1} (amide II), and 1240 cm^{-1} (amide III). Additionally, there is an additional band at 3285 cm^{-1}, characteristic for inter- and intra-molecular hydrogen (H)-bonds. The peaks characteristic for aliphatic C-H bonds around 2900 cm^{-1} are present as well. All these bands are present in the HP150 as well, with only small shifts in the case of certain peaks, indicating that the extraction at 150°C generates a significant amount of peptides. In the case of the extraction at 200°C, there are significant changes in the FTIR spectra. The amide and H-bond bands are almost completely reduced, and a large peak appears at 997 cm^{-1}. The peaks around 2900 cm^{-1} are maintained. This indicates that at this temperature the peptide structure is significantly hydrolyzed to amino acids/very small peptides together with the disruption of the H-bonds that stabilized the secondary and higher order structures. These data correlate with the observations from the SDS-PAGE, where the peptides are almost absent at 200°C.

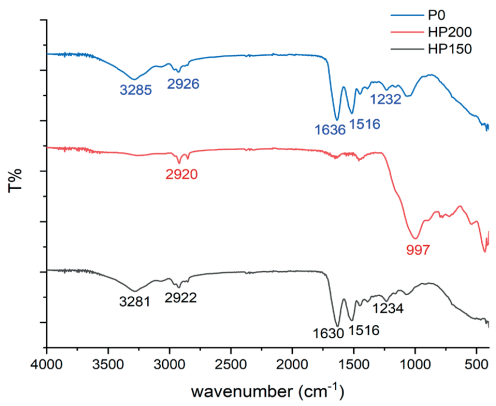

Figure 4. FTIR spectra of chicken feather (P0), keratin extraction by subcritical water at 200°C (HP200) and at 150°C (HP150)

CONCLUSIONS

In this study we compared three different methods of keratin extraction from chicken feathers in aqueous solutions: alkaline, acidic and subcritical water. Our data are in agreement with previous studies and show that the subcritical water extraction can be a viable alternative to the alkaline and acidic extractions, but that proper optimization, product characterization and selection of parameters need to be performed in order to meet the quality criteria depending on the application. SDS-PAGE electrophoresis combined with FTIR analysis can offer valuable Information in this respect.

ACKNOWLEDGEMENTS

The research leading to these results has received funding from European Regional Development Fund (ERDF), the Competitiveness Operational Programme (POC), Axis 1, project POC-A1-A1.2.3-G-2015-P_40_352, My_SMIS 105684, "Sequential processes of closing the side streams from bioeconomy and innovative (bio)products resulting from it - SECVENT", subsidiary project 2040/2017. We thank Dr. Gabriel Vasilievici for the help with the subcritical water extraction.

REFERENCES

Alvarez-Castillo, E., Felix, M., Bengoechea, C., & Guerrero, A. (2021). Proteins from Agri-Food Industrial Biowastes or Co-Products and Their Applications as Green Materials. *Foods, 10*(5). doi:10.3390/foods10050981

Aminian, M., Nabatchian, F., Vaisi-Raygani, A., & Torabi, M. (2013). Mechanism of Coomassie Brilliant Blue G-250 binding to cetyltrimethylammonium bromide: an interference with the Bradford assay. *Anal Biochem, 434*(2), 287-291. doi:10.1016/j.ab.2012.11.014

Bhavsar, P., Zoccola, M., Patrucco, A., Montarsolo, A., Rovero, G., & Tonin, C. (2016). Comparative study on the effects of superheated water and high temperature alkaline hydrolysis on wool keratin. *Textile Research Journal, 87*(14), 1696-1705. doi:10.1177/0040517516658512

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. (0003-2697 (Print)).

Călin, M., Constantinescu-Aruxandei, D., Alexandrescu, E., Răut, I., Doni, M. B., Arsene, M.-L., Oancea, F., Jecu, L., & Lazăr, V. (2017). Degradation of keratin substrates by keratinolytic fungi. *Electronic Journal of Biotechnology, 28*, 101-112.

Calvaresi, M., Eckhart, L., & Alibardi, L. (2016). The molecular organization of the beta-sheet region in Corneous beta-proteins (beta-keratins) of sauropsids explains its stability and polymerization into filaments. *J Struct Biol, 194*(3), 282-291. doi:10.1016/j.jsb.2016.03.004

Chaitanya Reddy, C., Khilji, I. A., Gupta, A., Bhuyar, P., Mahmood, S., Saeed Al-Japairai, K. A., & Chua, G. K.

(2021). Valorization of keratin waste biomass and its potential applications. *Journal of Water Process Engineering, 40*, 101707. doi:10.1016/j.jwpe.2020.101707

Chen, Y., Li, W., & Zhang, S. (2021). A multifunctional eco-friendly fertilizer used keratin-based superabsorbent as coatings for slow-release urea and remediation of contaminated soil. *Progress in Organic Coatings, 154*, 106158. doi:10.1016/j.porgcoat.2021.106158

Chilakamarry, C. R., Mahmood, S., Saffe, S., Bin Arifin, M. A., Gupta, A., Sikkandar, M. Y., Begum, S. S., & Narasaiah, B. (2021). Extraction and application of keratin from natural resources: a review. *3 Biotech, 11*(5). doi:10.1007/s13205-021-02734-7

Choi, J.-M., & Nelson, P. V. (1996). Developing a slow-release nitrogen fertilizer from organic sources: II. Using poultry feathers. *Journal of the American Society for Horticultural Science, 121*(4), 634-638.

Compton, S. J., & Jones, C. G. (1985). Mechanism of dye response and interference in the Bradford protein assay. *Analytical Biochemistry, 151*(2), 369-374. doi:https://doi.org/10.1016/0003-2697(85)90190-3

Di Domenico Ziero, H., Ampese, L. C., Sganzerla, W. G., Torres-Mayanga, P. C., Timko, M. T., Mussatto, S. I., & Forster-Carneiro, T. (2022). Subcritical water hydrolysis of poultry feathers for amino acids production. *The Journal of Supercritical Fluids, 181*, 105492. doi:https://doi.org/10.1016/j.supflu.2021.105492

Duan, Y., Awasthi, S. K., Liu, T., Pandey, A., Zhang, Z., Kumar, S., & Awasthi, M. K. (2020). Succession of keratin-degrading bacteria and associated health risks during pig manure composting. *Journal of Cleaner Production, 258*, 120624.

Gaidau, C., Epure, D.-G., Enascuta, C. E., Carsote, C., Sendrea, C., Proietti, N., Chen, W., & Gu, H. (2019). Wool keratin total solubilisation for recovery and reintegration - An ecological approach. *Journal of Cleaner Production, 236*, 117586. doi:10.1016/j.jclepro.2019.07.061

Giteru, S. G., Ramsey, D. H., Hou, Y. K., Cong, L., Mohan, A., & Bekhit, A. (2023). Wool keratin as a novel alternative protein: A comprehensive review of extraction, purification, nutrition, safety, and food applications. *Comprehensive Reviews in Food Science and Food Safety, 22*(1), 643-687. doi:10.1111/1541-4337.13087

Hussain, F. S., Memon, N., Khatri, Z., & Memon, S. (2020). Solid waste-derived biodegradable keratin sponges for removal of chromium: A circular approach for waste management in leather industry. *Environmental Technology & Innovation, 20*, 101120. doi:10.1016/j.eti.2020.101120

Janairo, G., Linley, M., Yap, L., Llanos-Lazaro, N., & Robles, J. (2011). Determination of the Sensitivity Range of Biuret Test for Undergraduate Biochemistry Experiments. *e-J. Sci. Technol, 5*.

Jaouadi, N. Z., Rekik, H., Badis, A., Trabelsi, S., Belhoul, M., Yahiaoui, A. B., Ben Aicha, H., Toumi, A., Bejar, S., & Jaouadi, B. (2013). Biochemical and molecular characterization of a serine keratinase from Brevibacillus brevis US575 with promising keratin-biodegradation and hide-dehairing activities. *PLoS One, 8*(10), e76722. doi:10.1371/journal.pone.0076722

Kalaydzhiev, H., Ivanova, P., Uzunova, G., Manolov, I., & Chalova, V. (2018). Biuret and Bradford Methods Suitability for Protein Quantification in Rapeseed Meal. *Contemporary Agriculture, 67*, 87-92. doi:10.2478/contagri-2018-0013

Kaur, M., Bhari, R., & Singh, R. S. (2021). Chicken feather waste-derived protein hydrolysate as a potential biostimulant for cultivation of mung beans. *Biologia*. doi:10.1007/s11756-021-00724-x

McKittrick, J., Chen, P. Y., Bodde, S. G., Yang, W., Novitskaya, E. E., & Meyers, M. A. (2012). The Structure, Functions, and Mechanical Properties of Keratin. *JOM, 64*(4), 449-468. doi:10.1007/s11837-012-0302-8

Meyers, M. A., Chen, P.-Y., Lin, A. Y.-M., & Seki, Y. (2008). Biological materials: Structure and mechanical properties. *Progress in Materials Science, 53*(1), 1-206. doi:10.1016/j.pmatsci.2007.05.002

Mihăilă, E.-G., Faraon, V. A., Deșliu-Avram, M., Neamțu, C., Constantinescu-Aruxandei, D., & Oancea, F. (2020). New Controlled-Release Fertilizers with Keratin-Based Coating from Chicken Waste Feathers. *Proceedings, 57*(1), 20.

Ossai, I. C., Hamid, F. S., & Hassan, A. (2022). Valorisation of keratinous wastes: A sustainable approach towards a circular economy. *Waste Management, 151*, 81-104. doi:10.1016/j.wasman.2022.07.021

Perța-Crișan, S., Ursachi, C., Gavrilaș, S., Oancea, F., & Munteanu, F. D. (2021). Closing the Loop with Keratin-Rich Fibrous Materials. *Polymers (Basel), 13*(11). doi:10.3390/polym13111896

Popko, M., Michalak, I., Wilk, R., Gramza, M., Chojnacka, K., & Górecki, H. (2018). Effect of the New Plant Growth Biostimulants Based on Amino Acids on Yield and Grain Quality of Winter Wheat. *Molecules, 23*(2), 470. Retrieved from https://www.mdpi.com/1420-3049/23/2/470

Rajabinejad, H., Zoccola, M., Patrucco, A., Montarsolo, A., Rovero, G., & Tonin, C. (2017). Physicochemical properties of keratin extracted from wool by various methods. *Textile Research Journal, 88*(21), 2415-2424. doi:10.1177/0040517517723028

Sharma, S., & Gupta, A. (2016). Sustainable Management of Keratin Waste Biomass: Applications and Future Perspectives. *Brazilian Archives of Biology and Technology, 59*(0). doi:10.1590/1678-4324-2016150684

Škerget, M., Čolnik, M., Zemljič, L. F., Gradišnik, L., Semren, T. Ž., Lovaković, B. T., & Maver, U. (2023). Efficient and Green Isolation of Keratin from Poultry Feathers by Subcritical Water. *Polymers, 15*(12), 2658. Retrieved from https://www.mdpi.com/2073-4360/15/12/2658

Suarato, G., Contardi, M., Perotto, G., Heredia-Guerrero, J. A., Fiorentini, F., Ceseracciu, L., Pignatelli, C., Debellis, D., Bertorelli, R., & Athanassiou, A. (2020). From fabric to tissue: Recovered wool keratin/polyvinylpyrrolidone biocomposite fibers as artificial scaffold platform. *Mater Sci Eng C Mater*

Biol Appl, 116, 111151. doi:10.1016/j.msec.2020.111151

Tesfaye, T., Sithole, B., Ramjugernath, D., & Chunilall, V. (2017). Valorisation of chicken feathers: Characterisation of chemical properties. *Waste Manag, 68*, 626-635. doi:10.1016/j.wasman.2017.06.050

Tinoco, A., Rodrigues, R. M., Machado, R., Pereira, R. N., Cavaco-Paulo, A., & Ribeiro, A. (2020). Ohmic heating as an innovative approach for the production of keratin films. *Int J Biol Macromol, 150*, 671-680. doi:10.1016/j.ijbiomac.2020.02.122

Tomlinson, D. J., Mülling, C. H., & Fakler, T. M. (2004). Invited Review: Formation of Keratins in the Bovine Claw: Roles of Hormones, Minerals, and Vitamins in Functional Claw Integrity. *Journal of Dairy Science, 87*(4), 797-809. doi:https://doi.org/10.3168/jds.S0022-0302(04)73223-3

Wang, B., Yang, W., McKittrick, J., & Meyers, M. A. (2016). Keratin: Structure, mechanical properties, occurrence in biological organisms, and efforts at bioinspiration. *Progress in Materials Science, 76*, 229-318. doi:10.1016/j.pmatsci.2015.06.001

Yang, Y. C., Tong, Z. H., Geng, Y. Q., Li, Y. C., & Zhang, M. (2013). Biobased Polymer Composites Derived from Corn Stover and Feather Meals as Double-Coating Materials for Controlled-Release and Water-Retention Urea Fertilizers. *Journal of Agricultural and Food Chemistry, 61*(34), 8166-8174. doi:10.1021/jf402519t