VALORISATION OF WASTE CHICKEN FEATHERS: REGENERATION OF KERATIN FIBRE INTO TUBULAR NANOFIBRES VIA ELECTROSPINNING

By

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PREFACE

The research work contained in this thesis about the valorisation of waste chicken feathers: regeneration of keratin fibre into tubular nanofibres via electrospinning was carried out by the candidate for a PhD degree while based in the Discipline of Chemical Engineering, School of Engineering of the College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal, Howard Campus, Durban, South Africa.

This work represents the original work of the author and has not been submitted in any form to another university. Where the work of others was used, it was accordingly acknowledged in the text.

Signed: _____ Date: _____
Prof. Bruce Sithole (Supervisor)

DECLARATION 1: PLAGIARISM

I, Mduduzi Blessing Khumalo, declare that:

i. the research reported in this thesis, except where stated otherwise, is my original work.
ii. this dissertation has not been submitted for any degree or examination to any other university.
iii. this thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;
iv. this thesis does not contain other persons' writing, unless specifically acknowledged and referenced as being sourced from other researchers. Where other written sources have been quoted, then:
    a) their words have been re-written but the general information attributed to them has been referenced;
    b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;
v. where I have used material for publications, I have indicated my role in such work;

Signed: Mduduzi Blessing Khumalo

Date: _____

DECLARATION 2: PUBLICATIONS

LIST OF JOURNAL PUBLICATIONS

- Khumalo, M., Sithole, B., Tesfaye, T. Valorisation of waste chicken feathers: Optimisation of keratin extraction from waste chicken feathers by sodium bisulphite, sodium dodecyl sulphate and urea. Published in the Journal of Environmental Management, 2020, volume 262, 110329 .

- Khumalo, M., Sithole, B., Tesfaye, T. Beneficiation of keratinous biomass: conversion into biomaterials. Under revision for the Journal of Biomass Conversion and Biorefinery.

- Khumalo, M., Sithole, B. Valorisation of waste chicken feathers: fabrication and characterisation of novel keratin nanofibres conduits for potential application in peripheral nerve regeneration. Submitted to the Journal of Biomaterials.

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- Khumalo, M., Sithole, B. and Tesfaye, T. Oral presentation, titled Conversion of waste chicken feathers into regenerated keratin nanofibres; at the Postgraduate Research and Innovation Symposium, at Westville Campus, University of KwaZulu-Natal; 25 October 2018.

- Khumalo, M., Sithole, B. and Tesfaye, T. Oral presentation, titled Conversion of waste chicken feathers into regenerated keratin nanofibres at The 7th International Conference on Biorefinery, at Johannesburg; 18-21 August 2019.

- Khumalo, M., Sithole, B. and Tesfaye, T. Oral presentation, titled Conversion of waste chicken feathers into regenerated keratin nanofibres; at the Postgraduate Research and Innovation Symposium, at Westville Campus, University of KwaZulu-Natal; 17 October 2019.

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Regards

Mduduzi kaMandla kaHlabekisa Khumalo

GENERAL SUMMARY

The poultry industry generates billions of kilograms of chicken feathers as a by-product during chicken meat processing. A large proportion of the produced feathers is disposed of by landfilling, burial or incineration, but this causes environmental concerns due to greenhouse gas emissions and land pollution. Thus beneficiation of feathers into high-value materials is desirable. In the past few decades, there has been an influx of research on fabrication of nanofibres for applications in the health care sector. This is due to nanofibrous materials' unique properties, including high surface area to volume ratio, porosity and flexibility. These properties are vitally important in the clinical health care sector as they allow nanofibres to mimic the native extracellular matrix of most human tissues and organs, including peripheral nerves. This work aimed to valorise waste chicken feathers by extracting keratin and converting it into keratin nanofibres tubes for potential application as nerve regeneration conduits. The first step was the development of a process for extraction of keratin from chicken feathers. The method was optimised by Response Surface Methodology to obtain the best extraction conditions that included sodium bisulphite, sodium dodecyl sulphate, urea, temperature and time as independent factors that affect the yield of keratin. The process was statistically analysed to evaluate the effects of each factor and the factors' interactions on the extraction. Through scientific evidence, this work discovered that temperature is the most significant factor in the extraction process, followed by reaction time, the concentration of sodium bisulphite, and concentration of sodium dodecyl sulphate. The optimisation analysis produced a new model that predicts keratin yield (up to 67.23%) from the keratin extraction process. The second step was studying electrospinnability of the extracted keratin to generate tubular keratin nanofibres for possible use as nerve conduits in nerve regeneration. The results showed that pure keratin could not be electrospun to keratin nanofibres; however, keratin blended with polyvinyl alcohol (PVA) was spun to novel keratin nanofibres tubes. As far as the author's knowledge, this is the first time that this has been demonstrated. Analysis of the nanofibres' thermal stability, chemical properties, and morphological properties showed that the increase in keratin content in keratin/PVA mixtures increases the thermal stability of keratin/PVA nanofibres conduits, and causes a decrease in nanofibres diameters and porosity. The distribution of the nanofibers diameters also narrows as keratin content increase. Nano dimensions of the fibres were confirmed by scanning electron microscopy. These results imply a possible breakthrough in plausible applications of chicken feathers keratin into the nerve regeneration space as nerve repair conduits. This innovation will benefit the clinical health care sector in saving patients' livelihood and promoting a rapid utilisation of waste chicken feathers, resulting in a cleaner and safer environment.

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CHAPTER 1
INTRODUCTION

1.1 Background

The poultry industry generates billions of kilograms of chicken feathers as a by-product during chicken meat processing. Chicken meat has become a global necessity for most households due to its nutritional benefits and cost-efficiency. South Africa slaughter 19 million chicken per week to account for a demand of 28 million chickens per week (Business Insider South Africa 2018). Worldwide, 50 billion chickens are slaughtered per year (Compassion in World Farming). In contrast, the United States of America used to slaughters 8 billion chickens per year, increasing from 580 million chickens per year in 1950 (Gupta et al. 2004). Therefore, chicken meat production is directly proportional to the growth of the global population. Hence, the generation of chicken feathers will continue to grow, resulting in the growth of waste feathers. The world produce approximately 5 billion kg of chicken feathers as by-product, South Africa contributes about 100 million kg of chicken feathers. Considering that South Africa doesn't meet the production demand of 26 million broilers per week, mitigations are underway to increase production, further driving up feathers production. The produced feathers are dumped in landfills, buried or incinerated. Landfills have been identified as significant contributors to the emission of greenhouse gases.

On the other hand, incineration produces a large amount of carbon dioxide and is energy-intensive, whereas buried feathers have a lengthy degradation period, meanwhile polluting the land. Thus, all these waste management methods are environmentally unfriendly, and attract levies and transport costs. These 90%-keratin waste fibres have good mechanical and thermal properties that, when appropriately utilised, can be a valuable side stream of the poultry industry, and contribute significantly to the development of circular economy and zero-waste aspects.

1.2 Study Rational

The inevitable growth of chicken meat production due to the growing demand for this meat generates an enormous amount of chicken feathers as waste. The common tactics to deal with this waste has been incineration and burial in landfills. However, waste management regulations and by-laws exert stringent measures on these practices due to their environmental unfriendliness. Therefore, sustainable solutions to minimise or possibly eliminate poultry waste are needed. Chicken feathers have exceptional properties such as comfort, thermal stability, chemical stability and tensile strength (Bessa et al. 2017, Okonkwo et al. 2019). In addition to physical and mechanical properties, feathers are composed of 91% keratin, 8% and 1% lipid (Saravanan et al.

2013). These properties have drawn researchers' attention to exploring potential applications of industrial waste chicken feathers from the poultry farms.

Chicken feathers have been used as pillow fillers and have shown good performance in accumulating less asthma-triggering pets allergens than synthetic fillers (Custovic et al. 2000). A cotton-covered pillow filled with chicken feathers was also the 2nd best fire-resistant in the burning rate test after polyester fibrefill covered with fibreglass, outperforming polyurethane foam and latex foam fillers. The feathers are rich in amino acids, and they can nourish the soil with macronutrients nitrogen, phosphorus and potassium for good plant growth (Zul et al. 2020). Chicken feathers can be blended with other agricultural wastes like cow dung to make compost with a high content of iron, zinc, sulphur, potassium, nitrogen, carbon and boron. This compost enriches the quality of fruits (Arthanari and Dhanapalan 2019). The chicken feathers can be hydrolysed to make liquid fertilisers, which enriches leafy plants with nitrogen and phosphorus to enhance plant growth (Zul et al. 2020). The nutritional value of feathers has motivated the conversion of chicken feathers to feather meal for animal feed. This is due to high protein content and a number of amino acids, of which some are essential. Chicken feather protein hydrolysate can be used as an antioxidant in animal feed formulation, encouraging using this waste material in making feather meals (Oluba et al. 2020). Feather meal has become an essential ingredient for ruminant animals as it is an exceptional source of amino acids, especially sulphur-containing amino acid cystine (Ockerman and Basu 2014). Among the properties of chicken feather keratin are biocompatibility and biodegradation. Hence, research haver intensified in the field of applying this keratin in biomedicine. The widely investigate approach involves the extraction of keratin from biomass and conversion into biomaterials such as tissue scaffolds, drug delivery carriers and wound dressing materials. The resultant materials are in the form of biofilms, sponges, hydrogels and nanofibres. The wide range of nanofibres applications has further drawn research interest to explore the viability of using chicken feathers keratin to make nanofibres biomaterials. When successfully established, this field can commercially feed the health care sector, pharmaceutical industry, beauty, and cosmetic industry with biomedical products.

1.3 Problem Statement

The worldwide processing of chicken meat from the poultry industry is a significant aspect of humanity as it is both a food security matter and an employment opportunity. Its by-products, including feathers, are a critical issue that contradicts the global population's meat contribution as they pollute the environment during disposal. This negative impact is a health risk hazard as it pollutes the air and groundwater, and emits greenhouse gases, the contributors to global warming. Research has shown that chicken feathers can produce high-value products that can economically boost the poultry industry and benefits sectors of interest, such as the health care sector. This

dissertation critically and scientifically analysed possible beneficiations of waste chicken feathers, and explored the production of a biomaterial from chicken feathers keratin through the electrospinning technique. The final keratin nanofibres tubes advance the process of valorising waste chicken feathers.

1.4 Research Questions

This project aims to answer the following questions which, when answered correctly, can be a major contribution to the valorisation of waste chicken feathers and the biomedicinal field:

How to optimise an environmentally friendly process for extraction of keratin from chicken feathers?

The extraction of keratin from chicken feathers is a critical step to valorise waste chicken feathers. The extraction method affects the desired keratin properties, consequentially predetermining applications of the extracted keratin. Extraction of keratin from chicken feathers using an effective and environmentally friendly method, a combination of sodium bisulphite, urea and sodium dodecyl sulphate, has not been optimised. Hence, extraction yield and keratin properties can not be easily controlled. Defining the keratin extraction process's optimal parameters will advance the valorisation of waste chicken feathers and benefits processes that utilise keratin, such as electrospinning.

Can waste chicken feathers be converted to keratin nanofibre tubes through electrospinning?

Keratin nanofibres have been previously shown to be biodegradable, biocompatible and suitable cell culture properties; these properties minimise immunological reactions and create a cell-friendly microenvironment. Fabricating keratin nanofibres tubes from polymer solution to tubes, without folding mat to the tube, will provide a firm and seamless conduit structure that offers an even lateral strength. This fabrication technique of keratin nanofibres tube eliminates the unevenness of the lateral strength.

1.5 Thesis Objectives

This project aims to produce keratin nanofibres tubes through electrospinning by using poultry industry's waste chicken feathers. It also aims to develop an optimised environmentally friendly keratin extraction method.

The objectives of this research are:

- The development of an optimised green extraction method to extract keratin from waste chicken feathers and formulate a mathematical model that can predict keratin yield from the extraction.
- Characterisation of the extracted chicken feathers keratin to link keratin properties to the optimised extraction process.
- Fabrication, by electrospinning, of new tubular keratin nanofibres.
- Characterisation of the electrospun tubular keratin nanofibres to profile their chemical composition, thermal behaviour and morphology.

1.6 Thesis Contributions

The main contributions of this research are the following:

- Development of an environmentally friendly keratin extraction method that yields predictable results. The prediction is done through a novel mathematical model that was experimentally confirmed. This process improved the extraction method to high efficiency and yield while some extraction parameters remained low to moderated levels.

- Detailed information of chicken feather keratin, showing keratin's elemental composition, functional groups, molecular weight distribution and crude protein content.

- Development of electrospinning method for the production of novel tubular keratin nanofibres. And a report on the effect of keratin on the properties of nanofibers.

1.7 Thesis Outline

The structure of this thesis is in publications format. Each chapter represents a free-standing manuscript published under peer-review or submitted for publication in an accredited peer-reviewed journal. The manuscripts are written in a general manuscript format, i.e., abstract, introduction, methodology, results, discussion and conclusion.

Chapter 1 introduces the research by highlighting the background of the problem, study significance, aims and objectives.

Chapter 2 consists of a literature review that briefly explains the world demand for an environmentally friendly biopolymer such as keratin. The chapter also analyses the structures and classifications of keratin according to their sources, methods of keratin extraction and analysis of possible biomedical applications of keratin materials.

Chapter 3 covers the optimisation of keratin extraction from waste chicken feathers using an aqueous solution of sodium bisulphite, urea and sodium dodecyl sulphate.

Chapter 4 deals with the electrospinning and characterisation of keratin nanofibres tubes as potential nerve regeneration conduits.

Chapter 5 is an overall discussion of the project which sums the outcomes of chapter two to four and concludes the project.

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CHAPTER 2
LITERATURE REVIEW

Chapter Overview

Chicken feathers consist of more than 90% keratin protein, which is also found in other sources such as wool, hair, nails, claws, hoofs, horns and beaks. Therefore, it is vital to review and analyse keratin's variations critically and classifications, alpha and beta keratin, chemical structures and composition, soft and hard keratin according to their sources to expose beneficiation technologies applicable to waste chicken feathers.

The review critiqued keratin extraction methods that have been used on chicken feathers and other keratin sources, including limitations encountered during extraction processes. The review deals with the frequently utilised methods to dissolve and extract keratin from keratinous materials, including chemical, electromagnetic waves (microwave), supercritical water and steam explosion extractions. The study aimed to identify efficient, less harmful and less environmentally harsh extraction methods to eliminate further burden to the environment with harmful chemicals with the current project.

This chapter also reviews different forms of keratinous biomaterials that are potentially applicable in the biomedical field. These biomaterials can be in the form of hydrogels, biofilms and scaffold composites which include nanofibres. Their applications are based on their excellent bioproperties such as biocompatibility, biodegradation and cell proliferation. Keratinous biomaterials can be used in tissue engineering, drug delivery systems and as wound dressing materials.

BENEFICIATION OF KERATINOUS BIOMASS: CONVERSION INTO BIOMATERIALS

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2.1 Abstract

The consumptions of large quantities of wool in the textile industry, and chickens in the poultry industry produce enormous waste keratinous biomass. The textile industry produces unspinnable wool short fibres as by-products, whereas the poultry industry generates feathers as by-products during meat production. As these by-products are underutilised, they end up as wastes that pollute the environment after disposal by either incineration or burial in landfill sites. This problem can be solved via the beneficiation of the wastes. Wool and chicken feathers are biomass that is comprised of more than 90 percent keratin protein. Keratin is a valuable protein with good properties that can be exploited in the production of biomaterials such as nanofibres, films and hydrogels. This report is a critical review of keratin and its beneficiation into biomaterials. It thoroughly classifies different types of keratin in relation to their sources. Furthermore, it looks at the often-used keratin extraction techniques, aligning them to the extracted keratin properties.

Keywords: Keratin, waste chicken feathers, wool keratin, keratinous waste biomass, beneficiation.

2.2 Introduction

The world is in desperate need of sustainable and environmentally friendly non-petroleum-based resources for applications in different industries. Therefore, biopolymers can be suitable substitutes for unsustainable petroleum-based polymers. Biopolymers are attractive to researchers due to their superior properties such as biocompatibility, biodegradability and biochemically activeness. Keratin is a biopolymer that can be utilised in many sectors, especially clinical health care (Idris et al. 2014). Its desirable properties include superior thermal, mechanical and chemical

stability characteristics (Selmin et al. 2012). Keratin can also serve as an extracellular matrix, facilitating cell attachment and cell proliferation. It is a fibrous structural protein that consists of a significantly high amount of sulphur content. These properties enable keratin to be utilised in diverse applications such as in the beauty industry, agriculture and healthcare (Karthikeyan et al. 2007, Kumaran et al. 2016). From literature reports covering research in broad areas concerning deriving value from keratinous biomass, it is evident that most efforts focus on the utilisation of keratin protein in the health care sector. The extracted keratin can make various biomaterials such as nanofibres, biofilms, powders and hydrogels. In some applications though, the keratin materials must be mixed with other polymers to supplement or change its properties. Keratin can be combined with polyvinyl alcohol (PVA), polyethene terephthalate (PET) polyethene oxide (PEO) and others to improve processability and mechanical properties (Aluigi et al. 2008, Li and Yang 2014). Nanoparticles such as silver nanoparticles can be incorporated into keratin biomaterials to advance keratin antibacterial effects (He et al. 2020). This review aims to critically study different methods to solubilise and extract keratin from keratinous biomass for biomaterials production.

2.3 Structural biology and classifications of keratin

Keratins belong to the family of scleroproteins, i.e., fibrous structural proteins found in epithelial cells of vertebrates (Yeo et al. 2018). Keratins in different vertebrates exist in two forms called alpha and beta keratin (Modesto et al. 2004, Greenwold and Sawyer 2010, Cilurzo et al. 2013, Wang 2016).

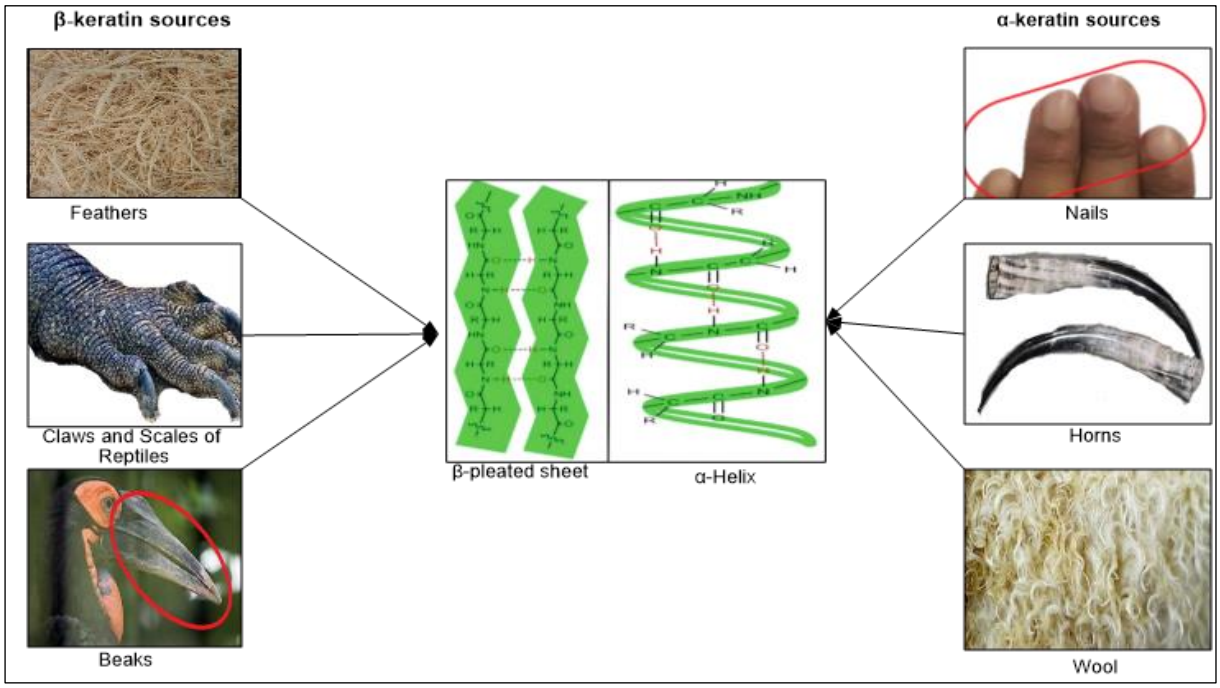

Figure 2.1: Examples of sources of alpha and beta keratins. (Saha et al. 2019).

As illustrated in Figure 2.1, the only difference between alpha and beta keratin is the arrangement of polypeptide chains which are either folded into coils, α-helix structures or zigzag β-sheets that

form α-keratin and β-keratin. The stability of coil and zigzag shapes of keratin is attributed to hydrogen bonds between carboxyl groups and amino groups of amino acids (Shah 2019). The polypeptide chains of keratin protein consist of repeating sequences of amino acids, including amino acids with nonpolar side chains that provide keratin water insolubility. Both the structures and properties of keratin are determined by the primary amino acids forming polypeptides. Each keratin source contains different amounts, types, and varying proportions of alpha and beta keratins. For example, human hair consists of 90% keratin (Kajiura et al. 2006, Villa et al. 2013), of which 50-60% of it is alpha keratin. Stratum corneum of the human skin is 70% keratin (Selmin et al. 2012), and nails keratin has properties of both human hair and skin keratins (Kitahara and Ogawa 1991, Inoue et al. 2001).

The primary keratin structure consists of varying, yet, high amounts of cysteine, an amino acid that contains sulphur atoms in its side chains. The sulphur atoms bond to form a strong disulphide bridge between polypeptides, a bridge that gives the keratin molecule its hardness and strength properties (Sahoo et al. 2012). Keratins are also classified according to their sulphur content; whereby those with 1,5 to 2% sulphur content, as found in skin, are soft keratins, and those with 4 to 8% sulphur content, e.g., from feathers, wool, hooves, nails and horns, are hard keratins (Karthikeyan et al. 2007, Cilurzo et al. 2013).

Keratins from feathers and wool are plentiful, globally, due to the generation of feathers and wool from food and textile industries, respectively (McGovern 2000, Xie et al. 2005, Li and Wang 2013). Wool keratin, accounting for 95 wt% of wool, consists of both soft and hard keratin in a proportion of 60% to 26%, respectively (Idris et al. 2014, Li and Yang 2014). The textile industry generates large quantities of wool waste during yarn spinning. Like wool, feathers are made of about 90% beta keratin and produced in large quantities during poultry processing (Stiborova et al. 2016).

2.4 Chicken feathers as a source of keratin

Around the world, chicken slaughterhouses and farms process an average of 58 billion chickens per annum for chicken meat production (Compassion in World Farming. 2013). There is an increasing demand for chicken meat (Conway 2016). Considering that a 2kg slaughter-mass chicken has 5-7% of its weight in the form of feathers per chicken, chicken meat production produces large amounts of feathers as waste. Inconsequential amounts of waste chicken feathers are beneficiated into low-value products such as pillow fillers, decoration products, fertilisers and animal feed. The rest are disposed of in landfill sites or incinerated (Veerabadran et al. 2012, Bhattacharyya et al. 2015). These disposal methods pollute the environment and are expensive for producers (Sahoo et al. 2012, Yang et al. 2013). Chicken feathers are composed of 91% keratin, 8% water and 1% lipids (Saravanan et al. 2013): this makes them a good source of keratin,

a high-value product sold for about $280 per kg. The biocompatibility and biodegradation properties of keratin have attracted research interests for keratin applications in biomedicine (Swetlana and Jain 2010, Sigma-Aldrich Pty. Ltd. 2017). However, these biomaterials have not passed the research stage, which means more studies are still required to overcome the challenges that delay their healthcare sector applications.

2.5 Methods for extraction of keratin

The extraction of keratin entails the unbinding of polypeptides that are covalently bonded by strong sulphur-sulphur bridges. This process's results vary significantly, leading to the formation of a mixture of polypeptide-derived compounds that can be as short as individual amino acids, depending on the extraction method (Wang and Cao 2012, Ayutthaya et al. 2015). Keratin can be extracted using various chemical matrices, microwave radiation, microbial or enzyme treatment, supercritical water extraction, and steam explosion pretreatment (Shavandi et al. 2017). Extractions of keratin using chemical matrices are often used due to their noticeable yield of up to 94% (Sinkiewicz et al. 2017).

Table 2.1: Advantages and disadvantages of the major keratin extraction techniques, namely chemical, enzymatic, microbial, supercritical steam and water explosion

Extraction Technique	Advantages	Disadvantages	References (Ref.)
Chemical	Effectively solubilises raw material Extraction yield can be as high as 91%, and a high dissolution rate of 94% Soluble and chemically reactive keratin can be obtained. Water-soluble keratin can be extracted under low pH, in the presence of reducing or oxidising agent. Easily modifiable by changing pH, concentration, reaction time and temperature to extract keratin with specific properties	Requires a significant amount of chemicals May require harmful and toxic chemicals like 2-mercaptoethanol which may be difficult to handle The processes are often environmentally unfriendly due to the presence of chemicals	(Earland and Knight 1955, Zahn et al. 1960, Shavandi et al. 2017, Donato and Mija 2019)

Microbial and Enzymatic	Consumes less energy and has mild reaction conditions, hence, environmentally safe green technique. The microbial used are green and cost-efficient. Actinomycetes, keratinophilic fungi and other bacteria can fully break keratin	The extraction cycle is longer than others; 50% solubilisation after 21 days was reported several times.	(Korniłłowicz-Kowalska and Bohacz 2011, Shavandi et al. 2017)
Supercritical water and steam explosion	Reduced energy consumption due to shortening explosion time, which can be as short as 0.0875 s No or fewer chemicals are used; hence, it is environmentally friendly and green hydrolysis. Highly digestible and soluble products. 80% of raw material can be converted to pepsin digestible material.	Most of the raw material is not converted into soluble keratin. At one instance, 62.4% remained insoluble solid product, and only 18.7% was soluble in water. Low cysteine content. Only 50% of cysteine retained Low-quality extract.	(Tonin et al. 2006, Zhao et al. 2012, Xu et al. 2014)
Microwave Irradiation	Homogeneous distribution of heat in the reactor as compared to conventional heating. All reaction molecules are capable of absorbing microwave radiation, thus rapidly increasing the temperature of reactants and solvents Shortens reaction time, hence, minimising or eliminating side reaction, resulting in purer extractives. Reduces the activation energy of keratin extraction. It is faster than conventional steam methods.	Significant loss of some amino acids such as cysteine; a reduction of cysteine from 9.4 mol% to 0.5 mol% was reported.	(Zoccola et al. 2012, Chen et al. 2015, Feroz et al. 2020)

Each extraction method, as can be seen in Table 2.1, has its advantages and disadvantages. Thus specific benefits and challenges must be further evaluated based on the intended application, considering the extracted keratin properties from each extraction technique.

Chemical extraction of keratin takes place under reduction, oxidation or hydrolysis processes or solubilisation in ionic liquids (Sinkiewicz et al. 2017). Hydrolysis of keratin source with alkalis disrupts the keratin polypeptides and modifies the keratin structure by the disrepair of cysteine to form thioether bond (Li and Wang 2013). However, cysteine sensitivity to alkalis leads to rapid decomposition of keratin, resulting in lower cysteine yields than other chemical extraction methods. Nonetheless, the resulting keratin remains intact during the hydrolysis process (Xie et al. 2005, Idris et al. 2014, Tsuda and Nomura 2014). A discussion of the predominantly used methods of extraction keratin from different sources follows below.

2.5.1 Reduction extraction

The extraction of keratin by reduction process utilises reducing agents to disrupt disulphide bridges to form cysteine. Thioglycolic acid, thioglycolate salts, 2-mercaptoethanol, sodium sulphite, sodium bisulphite and sodium metabisulphite are frequently used as reducing agents to extract keratin (Aluigi et al. 2008, Sinkiewicz et al. 2017). Surfactants and denaturing agents are used in advancing the extraction of this protein (Shavandi et al. 2017).

2.5.1.1 Reduction extraction via thiolysis

Thiol groups such as 2-mercaptoethanol (Yin et al. 2013, Sinkiewicz et al. 2017), thioglycolic acid and thioglycolate (Gupta et al. 2012) are the most used substances in the extraction of keratin from keratinous sources. This reduction reaction, illustrated in Equations 1-3, reaches dynamic equilibrium, where the reduced keratin residues ($\mathrm{Cys-S^-}$) can react to form an aggregated keratin, eq.3, unless they are chemically modified, eq.5 (Cardamone 2010).

$$\mathrm{Cys-S-S-Cys} + \mathrm{S^- - R} \rightleftharpoons \mathrm{Cys-S-S-R} + \mathrm{Cys-S^-} \quad (1)$$

$$\mathrm{Cys-S-S-R} + \mathrm{S^- - R} \rightleftharpoons \mathrm{R-S-S-R} + \mathrm{Cys-S^-} \quad (2)$$

$$\mathrm{Cys-S-S-R} + \mathrm{Cys-S^-} \rightleftharpoons \mathrm{Cys-S-S-Cys} + \mathrm{S^- - R} \quad (3)$$

Where $\mathrm{Cys-S-S-Cys}$, is untreated keratin, $\mathrm{S^- - R}$ a deprotonated thiol (mecarptide / thioglycol ion), $\mathrm{Cys-S-S-R}$ the half-reduced keratin, $\mathrm{Cys-S^-}$ is the reduced keratin (cysteine thiol), and R is the hydroxyl or carboxyl group.

Therefore, thiol-containing chemicals are often used in combination with surfactants and denaturing agents. Surfactants, like sodium dodecyl sulphate (SDS), are used to increase the keratin extraction rate and yield and stabilise the extracted keratin in aqueous solutions. The surfactant prevents aggregation of polypeptide chains and oxidation of cysteine residues. It forms complexes with the reduced keratin (Yamauchi et al. 1996). SDS sulphate anions bind with positively charged side chains, whereas the dodecyl side-chain bind with nonpolar regions of the protein surface adjacent to cationic sites (Schrooyen et al. 2001). In reducing disulphide bonds in

proteins with 2-mercaptoethanol, one molecule of surfactant binds with seven amino acid residues.

Furthermore, cooperative binding before the critical micelle concentration (CMC), takes place. The protein unfolds to expose the hydrophobic interior and reveal more potential binding sites for free SDS ions. Hence, an increase in the average number of surfactant ions per amino acids is observed as the surfactant concentration approaches CMC. After the cooperative binding, one SDS molecule is bound to two amino acid residues (Reynolds and Tanford 1970). Moreover, SDS prohibits the aggregation of the extracted keratin after removing the reducing and denaturing agents (Schrooyen et al. 2001). Urea and thiourea are the most utilised protein denaturing agents to increase keratin solubility in water and prevent reversal of the reaction by carbamylation of cysteine residues (Cardamone 2010). When the reaction temperature reaches 70 ^{o}C, urea is converted to cyanic acid, and carbamylate free thiol groups of proteins, cysteines and cystine, eq. 4 and 5 (Fearon 1923, Lippincott and Apostol 1999).

$$\mathrm{CON_2H_4} \xrightarrow{\Delta} \mathrm{NH_3 + HCNO} \quad (4)$$

$$\mathrm{NCNO + SH - Cys} \rightarrow \mathrm{H_2NCO - S - Cys} \quad (5)$$

The swelling of keratin structure is a desirable feature as it facilitates the disruption of hydrophobic interactions within the protein chains. Urea effectively does this in conjunction with 2-mercaptoethanol or thioglycol compounds (Xu et al. 2014). Examples of keratin extraction using thiols are summarised in Tables 2.2 and 2.3.

Table 2.2: Summaries of some keratin extraction using 2-mercaptoethanol.

Source of keratin	**Extraction solution**	**Temp (^{o}C)**	**Time (hours)**	**Result(s)**	**Ref.**
Chicken feathers	1.66 M 2-mercaptoethanol 8 M urea 0.2 M Tris-HCl, at 8.0 pH	70	2	84% yield	(Sinkiewicz et al. 2017)
Wool	3 ml 2-mercaptoethanol 7 M urea 1.2 g SDS, at 7.0 pH	50	24	-	(Alemdar et al. 2005)
Wool	1.4 M 2-mercaptoethanol 6 M urea 3 mM EDTA, at 9.1 pH	62-65	4	Mw: 40-60 kDa	(Cardamone 2010)
Chicken feathers	1.66 M 2-mercaptoethanol 8 M urea 0.26 M SDS	50	16	Mw:15 –60 kDa	(Sionkowska et al. 2011)

Chicken Feathers	0.095 M 2-mercaptoethanol 0.33 M urea 0.05 M SDS 0.016 M Tris-HCl at 8.0 pH	70	2	93% keratin yield, Mw:20 kDa and 14-15% cysteine residues	(Yin et al. 2013)
Chicken Feathers	1.1168 ml 2-mercaptoethanol 31.8 g urea 117g EDTA 3.29 g THAH	70	48	Mw bands at 10 and 25 kDa	(Alahyaribeik and Ullah 2019)
Bovine Hair	125 mM 2-mercaptoethanol	55	3	30% yield	(De Souza et al. 2020)
Hair	1.66 M 2-mercaptoethanol 0.26 M SDS	50	12	96% keratin yield, Mw: 40-64 kDa	(Fernández-d'Arlas 2018)

In general, keratin extracted by mild 2-mercaptoethanol is left intact because only disulphide bonds are cleaved (Cardamone 2010). Moreover, the yields of extraction of keratin from this method are significantly high, ranging from 27% (Nakamura et al. 2002) to 93% (Yin et al. 2013). The variation in the extraction yield of keratin and keratin properties can be attributed to the multiple alterations of this extraction method from solution components and their concentrations to solution pH, temperature and reaction time. The variations in extraction conditions result in the extracted keratin's diversified properties, as illustrated in Table 2.2. The variations in keratin properties include a wide range of molecular weight, from 15 kDa to 60 kDa. Nakamura et al. (2002) introduced thiourea as a denaturing agent in this method and reported a yield increase from 50% to 65% compared to Yamauchi et al. (1996). The results also showed keratin with a molecular weight from 40-60 kDa and disappearance of high (110-135kDa) and low (10-20 kDa) molecular weight keratins (Yamauchi et al. 1996, Nakamura et al. 2002). Although keratin extraction via 2-mercaptoethanol is the point of reference for high yield and undamaged keratin, its high cost and environmental harm limit its industrial applications (Ramya et al. 2020).

Table 2.3: Summaries of some keratin extraction using thioglycol (-ic acid/ -ate).

Keratin source	Extraction solution	Temp (ºC)	Time (hours)	Reported Result(s)	Ref.
Wool	0.2 M thioglycolic acid at a pH range of 11-12	25	6-24	%Yield: 35-75%. Molecular weight (Mw): 48-62.8, 19.4, 17.7 and 12.6 kDa	(Hatakeyama et al. 2009)
Hair	1 M thioglycolic acid 100 mM Tris-base at 10.2 pH	-	12	Mw: 43 kDa and 64 kDa	(Hill et al. 2010)
Wool	0.4 M thioglycolic acid 8 M urea with EDTA under nitrogen gas	50	24	Keratin type I; Mw: 48 kDa Keratin type II: Mw:61 kDa	(Ikkai and Naito 2002)
Bovine hair	0.1 M thioglycolate 6 M urea at 7.0 pH	35	-	Mw: 45 and 55 kDa	(Kamal et al. 1998)
Chicken feathers	0.5 M thioglycolate at a pH of about 10-13	30	6	Only 30% of feathers dissolved, yielding 8.8% of protein	(Gupta et al. 2012)
Bovine Hair	125 mM thioglycolic acid	55	3	50% keratin yield, Mw: 20 kDa	(De Souza et al. 2020)
Chicken Feathers	0.5 M thioglycolic acid	50-55	2	76.9 % keratin yield	(Fernández-d'Arlas 2018)

The earliest reports of keratin extraction by thioglycol (-ic acid/-ate) suggested keratin can only be extracted at a pH greater or equal to 10.5. In contrast, an increase in solubility was observed at a pH greater than 11 (Goddard and Michaelis 1935, Patterson et al. 1941). A study by Thompson and O'donnell (1962) revealed that the reducing abilities of thioglycolic acid and mercaptoethanol are similar at low concentrations. However, recent studies show that keratin can be extracted by thioglycolic acid from a neutral pH to 13, while thiols concentrations range from 0.1-1 M, see Table 2.3. Even though there has been no optimisation of this TGA extraction method, the resulting keratin has a molecular weight that spreads from low to 64 kDa, with 43-64 kDa being the most reported molecular range from this extraction method (Agrawal and Ray 2001, Hatakeyama et al. 2009, Hill et al. 2010).

Thioglycolic acid and 2-mercaptoethanol are toxic when ingested or in contact with the skin. They cause severe eye damage. When skin contact occurs, 2-mercaptoethanol causes skin irritation, whereas thioglycolic acid causes skin-burn. Prolonged exposure to 2-mercaptoethanol results in organ damage; hence, precautional measures must be taken when working with it. Both of these chemicals endanger aquatic life due to their toxicity. They are combustible; 2-MEC must be stored below 30 °C, whereas TGA should be kept between 2 and 8 °C (Hodge and Sterner 1949, The State of New Jersey Department of Helth and senior services. 2005, United Nations Economic Commission for Europe. 2017).

2.5.1.2 Reduction Extraction via Sulphitolysis

The extraction of keratin via sulphitolysis method involves the reduction of the disulphide bridge of cystine by either sodium sulphite, sodium bisulphite or any other sulphite ion (SO_3^{2-}) producing chemical substances. This reduction process results in the formation of either two cysteine S-sulphonate molecules as per eq.6, cysteine thiol, and cysteine S-sulphonate (eq.7) or cysteine thiol and perthiocysteine in eq.8.

$$\mathrm{Cys-S-S-Cys} + 2SO_3^{2-} \rightleftharpoons 2Cys-S-SO_3^{2-} \quad (6)$$

$$\mathrm{Cys-S-S-Cys} + HSO_3^{-} \rightleftharpoons Cys-S-SO_3^{2-} + \mathrm{Cys-SH} \quad (7)$$

$$\mathrm{Cys-S-S-Cys} + Na_2S \rightleftharpoons Cys-S-S^{-} + \mathrm{Cys-SH} \quad (8)$$

Similar to the reduction of cystine through thiolysis, sulphitolysis reduction is a reversible reaction at pH above 9, which decreases the rate of sulphitolysis of cystine (Shavandi et al. 2017). While sulphite, bisulphite and disulphite ions co-exist in equilibrium in an aqueous solution, the equilibrium position depends on the extraction solution's pH. Bisulphite ions have the strongest presence in an acidic medium, whereas sulphite ions are in high concentration at pH above 7. Reduction of cystine by sodium bisulphite may result in high concentrations of cysteine-S-sulphonate as more cysteine thiol molecules may react with sulphite ions depending on the pH of the reaction solution (Betts and Voss 1970).

The reduction reaction by sodium sulphide is a two steps process. First, sodium sulphide reacts with water to form hydrogen sulphide ion as per eq.9.

$$Na_2S + H_2O \rightarrow 2Na^{+} + SH^{-} + OH^{-} \quad (9)$$

Second, the hydrogen sulphide ion reduces the cystine's disulphide bridge as in eq.10 to reach eq.8.

$$\mathrm{Cys-S-S-Cys} + SH^{-} \rightleftharpoons Cys-S-S^{-} + \mathrm{Cys-SH} \quad (10)$$

Table 2.4: Summaries of some keratin extraction through sulphitolysis.

Keratin source	Extraction solution	Temp (ºC)	Time (hours)	Reported Result(s)	Ref.
Chicken feathers	0.5 M sodium sulphide at a pH of 10-13	30	6	Keratin yield of 53%	(Gupta et al. 2012)
Chicken feathers	0.2 M sodium disulphite 8 M urea 0.6 g SDS at a pH of 6.5	65	5	Keratin yield of 87.6% Mw: 11–20, 32, 37, 50, 75 kDa	(Ayutthaya et al. 2015)
Chicken feathers	0.5 M sodium disulphide 8 M urea at a pH of 6.5	65	2	Keratin decreases the viscosity of the polymer solution and increases its conductivity	(Zoccola et al. 2008)
Chicken feathers	0.13 M sodium sulphide	30	1 & 24	Keratin yield of 62% Mw: 10 KDa for 68-70% of extracted keratin Mw: 20 kDa for 15-19% of extracted keratin	(Poole et al. 2011, Poole and Church 2015)
Wool	0.2 M sodium disulphite 8 M urea SDS at a pH of 6.5	65	2	Multiple bands at a Mw: 4-18 kDa and big bands between 38-62 kDa	(Cilurzo et al. 2013)
Chicken feathers	0.5 M sodium bisulphite 8 M urea 0.08 M SDS	70	4	Keratin Yield of 84%	(Sinkiewicz et al. 2017)
Chicken feathers	0.92 M sodium sulphide	80.9	9.5	Keratin yield of 91%	(Kamarudin et al. 2017)
Bovine Hair	125 mM sodium sulphide	55	3	Keratin yield of 90%	(De Souza et al. 2020)
Chicken feathers	10 g sodium sulphide	70	48	Keratin with Mw of 10 kDa	(Alahyaribeik and Ullah 2019)

Reducing agents like sodium sulphide cleave cystine cross-links and preserve the primary structure's long chains (Wormell and Happey 1949). However, the sulphitolysis process does not normally proceed to completion. The extent of the solubilisation can be increased by the inclusion of urea and dodecyl sulphate. Further, increasing temperature, the concentration of the reducing

agent, or adding highly concentrated urea also increase the reaction rate. The reaction conditions also affect the extraction yield and properties of the extracted keratin like molecular weight.

One study investigated the effect of the concentration of sodium metabisulphite on the extraction yield and molecular weight. The results showed that as the concentration was increased from 0.0 to 0.2 M, the extraction yield increased from 36.2 to 87%; the low concentration could not break the macromolecules of keratin. The yield decreased when the concentration of sodium metabisulphite was increased above 0.2 M. Higher concentration of the reducing agent break most of the disulphide bridges, resulting in short peptide chains that are lost during dialysis, leading to low yields. The lower ranges of molecular weight of the extracted keratin at 0.2 and 0.3 M were 10-20 kDa and 10-19 kDa, respectively; whereas at 0.4 and 0.5 M, the lower ranges of 10-15 kDa and 11-13 kDa, respectively. The highest molecular weight at a concentration of 0.0 to 0.3 M was 75 kDa. It decreased to 30 kDa and 18 kDa at 0.4 M and 0.5 M, respectively. Similar to the effect of concentration on yield, a low shift of low and high molecular weight can be attributed to a higher concentration of the reducing agent that breaks most of the disulphide bridges (Ayutthaya et al. 2015).

Precautional measures must accompany keratin extraction through sulphitolysis method because most of the sulphitolysis reagents are both harmful and toxic to some degree. Even though sodium sulphite's overall toxicity is moderate, it is slightly hazardous on skin and eyes if it is in contact. Like sodium sulphide, sodium disulphide and sodium bisulphite, the sodium sulphite liberates toxic gases when in contact with acids. When heated, it may also decompose to form a chocking toxic sulphur dioxide and a strong corrosive sodium oxide, hence, precaution must be considered when handling it. Sodium sulphide, on the other hand, is dangerous to skin and eyes and can cause blindness. Over-exposure to it results in lung damage, choking, unconscious or death; therefore, extreme care is required when working with it. Likewise, sodium bisulphite and sodium disulphite are harmful when ingested. Sulphitolysis reagents endanger aquatic life; hence, they must be controlled during disposal (Hodge and Sterner 1949, German Federal Water Management Act. 2005, Merck 2015, United Nations Economic Commission for Europe. 2017).

2.5.2 Oxidation Extraction

Oxidising agents cleave cystine disulphide bonds to form cysteine amino acids, containing a sulphonic group. Protein groups such as thiol, disulphide, thioether, imidazole, phenolic and indole are susceptible to attack by oxidising agents. Oxidation reactions take different mechanisms due to the diversity of oxidising agents and reaction conditions. Organic peroxy acids such as performic acid and peracetic acid offer good solubility and reaction irreversibility. Hence, they are the preferred oxidising agents for protein. Peracetic acid and performic acid attack and

convert cystine to two molecules of cysteic acid without or with minimal hydrolysis of peptide bridge, as in eq.11.

$$\mathrm{Cys-S-S-Cys + 5RCO_3H + H_2O \rightarrow 2Cys-SO_3H + 5RCO_2H} \quad (11)$$

The yield of performic acid-soluble keratin can be increased by increasing reaction temperature or oxidation time. The frequently adopted oxidation extraction of keratin was performed by Earland and Knight (Earland and Knight 1955). They used 2% peracetic acid at 30 ºC for 20 hours to solubilise wool and hair, followed by 0.2 N ammonia and hydrochloric acid to precipitate the extract, yielding 6.6 % keratin. During this extraction, insoluble beta-keratin residues were observed, while most of the extracted keratin was a much soluble alpha-keratin. Alpha, beta and gamma keratin are separated according to their solubilities. Alpha keratin can be separated by solubilisation in ammonia followed by precipitation in acid. Beta keratin is insoluble in ammonia, while gamma keratin is soluble in ammonia, but it does not precipitate in acids. Additionally, alpha keratin can be precipitated by adjusting the pH to 4, while beta keratin can be separated by adjusting the oxidising solution to alkaline (Alexander and Earland 1950)

The use of inorganic peroxy acids like peroxydisulphuric acid at 90 ºC and pH of 1.6, then 4.6, have been reported. Peroxydisulphate ($S_2O_8^{2-}$) significantly attacks cystine, methionine, arginine, histidine, proline, tyrosine and possible phenylalanine. This oxidation reaction consists of multiple intermediate reactions which generally end with the formation of either cystine monoxide, dioxide or sulphonate groups, with the sulphonate as the main product.

$$\mathrm{Cys-S-S-Cys + S_2O_8^{2-} \rightarrow 2Cys-S-O-SO_3^{2-} \xrightarrow{OH^-} 2Cys-S-OH + 2SO_4^{2-}} \quad (12)$$

Cysteinesulphanic acid ($Cys-S-OH$) is followed by immediate deprotonation to give $Cys-SO^-$ ion, followed by other possible reactions.

Oxidation by peracetic acid and performic acid has noticeable disadvantages such as incomplete oxidation of cystine to cysteic acid and loss of threonine, serine, tyrosine, histidine and phenylalanine (Simmonds 1954). However, tyrosine and phenylalanine are slowly recoverable using hydrogen peroxide and performic acid (Smith and Stockell 1954). Carbon dioxide and ammonia gases are liberated during this reaction, reducing the total nitrogen content. Table 2.4 contains other examples of where keratin was extracted via oxidation reaction.

Table 2.5: Examples of extraction of keratin through an oxidation reaction.

Keratin source	Extraction solution	Temp (°C)	Time (hours)	Reported Result(s)	Ref.
Human hair	2% Peracetic acid	37	15	Soluble keratose with the main Mw at 40-60 kDa	(Wang et al. 2017)
Human hair	2% peracetic acid	37	10	Keratin Type I and Type II, Mw: 10.5, 21.6, 38.7,69.2, 83.6 kDa, with big band at 40-60 kDa	(de Guzman et al. 2011)
Horn	20% and 30% hydrogen peroxide	25	12	Keratose: High Mw: 40-160 kDa, Low Mw: 15-25 kDa	(Zhang et al. 2015)
Chicken feathers	2 M hydrogen peroxide	50	2	87.1% keratin yield	(Fernández-d'Arlas 2018)
Bvine hair	125 mM hydrogen peroxide	55	3	50% keratin yield with a Mw of 20 kDa	(De Souza et al. 2020)

Some researchers have adopted a 2% peracetic acid concentration for oxidation extraction of keratin. Others have modified the process to increase the extraction yield by increasing the peracetic acid concentration. Shavandi et al. (2017) optimised this process by setting peracetic acid concentration (6, 12, 24 and 36%) and reaction time (1, 2 and 3 days) as extraction factors. They showed an increase in extraction yield as the concentration and reaction time increased, except day three yield of 24 and 36% acid concentrations. Extraction with 24% peracetic acid recorded 54.93% yield after two days; hence, it was selected as the optimum condition. Nevertheless, 36% of peracetic acid showed the fastest extraction, yielding 52.86% in one day. The extracts contained water-soluble and insoluble keratin fractions. Both fractions showed strong molecular weight bands at 100-130 kDa and 45-58 kDa. The distinct bands at 45 kDa and 58 kDa could be assigned to alpha-keratin fractions (Hill et al. 2010).

The oxidation reaction by peroxide (O_2^{2-}) is a slow irreversible reaction that forms two molecules of cysteic acid per oxidation of a cystine molecule, as in eq.13 (Poole et al. 2009).

$$\mathrm{Cys-S-S-Cys} + \mathrm{O_2^{2-}} \xrightarrow{[O]} 2\mathrm{Cys-SO_3^-} \qquad (13)$$

Hydrogen peroxide attacks sulphur-containing amino acids like cystine and methionine. However, it may also attack cystine, tryptophan and tyrosine residues in the presence of certain metal ion or organic acids. Cystine is reported to be less susceptible to hydrogen peroxide than

cysteine and methionine. The pH of the extraction medium affects the oxidation rate of cystine and methionine differently; the oxidation rate of methionine increases slightly as the pH is reduced from 5 to 1, while cysteine's oxidation rate decreases with a reduction in pH (Neumann 1972).

Peracetic acid is a flammable substance (in both liquid and vapour form). It is a dangerous self-reactive substance that may cause a fire if heated. Its vapour is irritating to the eye, nose and throat, whereas, the liquid is an irritant to skin and eyes. The other oxidiser, hydrogen peroxide, may cause fire or explosion; it is a robust corrosive oxidiser that may cause severe skin burns and eye damage. Hydrogen peroxide is acutely toxic when inhaled or swallowed (National Center for Biotechnology Information. 2020). Therefore, proper protective gear must be used when working with these oxidisers.

2.5.3 Extraction via ionic liquids

In search of green chemistry requirements that seek to protect the environment, ionic liquids have attracted considerable attention due to their nature (Li and Wang 2013, Holkar et al. 2017). Ionic liquids are salts that exist as liquids in low temperatures. These are eco-friendly solvents that have good solubility. The properties of ionic liquids have been beneficial in the quest to extract keratin from keratinous sources such as wool and feathers because they can disrupt hydrogen bonds (Holkar et al. 2017). Even though keratin can be extracted through oxidation and reduction processes, ionic liquids' extraction results in different high-value-non-degraded keratin (Ji et al. 2014). Properties of ionic liquids make them favourites for keratin regeneration. These liquids have low vapour pressure that allows ease containment, product recovery and recyclability. Recoverability is also attributed to its hydrophobicity and miscibility in an organic solvent (Wang and Cao 2012). Furthermore, ionic liquids have high thermal stability, i.e., they do not decompose at relatively high temperatures, allowing high-temperature reactions to proceed conveniently (Mallakpour and Dinari 2012).

Imidazole based ionic liquids are the most utilised ionic liquids to extract keratin (Xie et al. 2005, Wang and Cao 2012, Li and Wang 2013, Chen et al. 2014, Ghosh et al. 2014, Ji et al. 2014, Wang et al. 2014).

Table 2.6: Examples of some of the reported ionic extraction of keratin.

Keratin source	Ionic liquid	Temp (ºC)	Time (hours)	Solubility (wt%)	Ref.
Wool	1-allyl-3-methylimidazolium chloride [Amim]Cl	130	10.5	8	(Li and Wang 2013)
Wool	1-butyl-3-methylimidazolium chloride [Bmim]Cl	130	10	11	(Xie et al. 2005)
Duck feathers	1-butyl-3-methylimidazolium bromide [Bmim]Br	120	1	4	(Ji et al. 2014)
Human hair	1-ethyl-3-methylimidazolium diethyl phosphate [EMIM]DEP	120	0.5	70.2	(Seghir et al. 2020)
Turkey feather	choline thioglycolate	130	10	45	(Idris et al. 2013)
Human Hair	40% tetrabutylammonium hydroxide	25	9	38	(Singh and Prasad, 2019)
Wool	[AMIM][dicyanamide]	130	10	47.5	(Idris et al. 2014)
Wool	[BMIM][Acetate]	120	0.5	92.92	(Zhang et al. 2017)
Chicken feathers	[HOEMIM][NTf_2]	80	4	21	(Wang and Cao 2012)
Human hair	50% choline hydroxide	25	12	10	(Singh and Prasad, 2019)

Xie et al. (2005) showed that wool solubility is higher in [BMIM]Cl than in other ionic liquids, while Li and Wang (2013)'s investigation revealed that there is higher solubility rate of wool in

[AMIM]Cl than in [BMIM]Cl. For imidazolium cations and chloride anion-based ionic liquids, the joint effects of cations and anions account for breaking the covalent and non-covalent interactions (Wang et al. 2014).

In small-cation imidazolium ionic liquids derivatives, the anion is responsible for the quality of regenerated keratin. In contrast, chloride's nucleophile strength was proposed as the cause of disulphide bond degradation, leading to the cysteic acid formation (Ghosh et al. 2014). The ionic liquid reactivity can be manipulated by choosing different combinations of cations and anions for different reaction requirements (Wang and Cao 2012). The regenerated wool keratin from ionic liquids shows a beta-sheet structure and the disappearance of the alpha-helix structure owing to coagulation solvents (Li and Wang 2013). The thermal stability of regenerated wool keratin is slightly higher than that of natural wool keratin fibres (Xie et al. 2005). Ionic liquids reactivities can be enhanced by introducing additives such as sodium hydroxide and sodium bisulphite (Wang and Cao 2012, Ji et al. 2014). However, Li and Wang (2013) found that the thermal stability of films of the regenerated wool keratin decreased slightly to some extent because of the partial molecular chain breakage. While the extracted keratin was with less damage, it was extracted without the formation of the pollutant by-products. Choline based ionic liquids have also been reported in the dissolution of keratinous sources. These ionic liquids showed the highest regeneration of keratin as compared to imidazole based ionic liquids (Chen et al. 2014).

The disadvantages of using ionic liquids are that they can cause skin, an acute eye, and respiratory irritations (Merck 2015, United Nations Economic Commission for Europe. 2017). They may be dangerous if ingested because of their mild toxicity level (Hodge and Sterner 1949). 1-butyl-3-methylimidazolium chloride is very hazardous to marine life with chronic effects (German Federal Water Management Act. 2005). Another disadvantage is that ionic liquids are expensive and, currently, are difficult to recover for reuse.

2.5.4 Enzymatic hydrolysis

Keratin extraction by chemicals may break both disulphide and peptide bonds in proteins, and alter the extracted keratin structure. Extraction in extreme pH levels for prolonged periods at high temperatures results in peptide fragments with low molecular weight. The breakage of these bonds limits the keratin's numerous biomedical applications (Jones 1975, Smith et al. 1994, Eslahi et al. 2013). Enzymatic hydrolysis of keratin is an alternative process that uses biological substances to catalyse chemical reactions during keratin extractions. Enzymes are frequently used, commercially, for hydrolysis of keratin due to their environmental friendliness. Enzymatic hydrolysis does not require harsh conditions, hence preserves functional properties of extracted keratin (Eslahi et al. 2013).

2.5.5 Microwave-assisted extraction

The extraction of keratin using microwaves is one of the fasted keratin extraction methods as it lasts for short periods as low as 5 minutes (Shin et al. 2010). This method uses microwave radiation that facilitates the extraction of keratin from keratinous biomass by direct heating of the keratin molecules using microwave energy. Electromagnetic energy is transferred to thermal energy when dipole rotation and ionic induction mechanisms occur concurrently (Destandau et al. 2013). Zoccola et al. (2012) extracted keratin from wool with subcritical water exposed to temperatures of 150 to 180 ^{o}C for 1 hour. The microwave radiation had a power that ranged from 150-570 Watts, and it took 5-7 minutes to reach the set temperatures. The extracts contained free amino acids, peptides, and low molecular weight proteins, with small cystine and lanthionine amounts. The disappearance of high molecular weight keratin (43-67 kDa) suggested a significant change in the structure of the extracted keratin; hence, this keratin's secondary structure differed from that of reduced and oxidised keratin. The extraction yield changed significantly with change in temperature, time, and wool-water mass ratio; For example, changing temperature from 150 ^{o}C to 180 ^{o}C increased the extraction yield from 10% to 60% whereas halving the reaction time reduced the extraction yield from 50% to 40% (Zoccola et al. 2012). Shin et al. (2010) extracted keratin from human hair by exposing it to microwave radiation (600 W) for specified incubation periods between 5–120 min. The microwave-assisted extraction also resulted in a significant loss of some amino acids, such as cysteine (Shin et al. 2010).

2.5.6 Supercritical water and steam explosion extraction

In the extraction of keratin with supercritical water and steam explosion, steam is exerted to the tissues and cells of keratinous biomass. Scorching water vapour enters cells and tissues, applying pressure and quickly releasing it. The phenomenon results in a millisecond explosion reaction, exposing the protein. Zhao et al. (2012) applied high-density steam flash (HDSF) explosion, a modification of the conventional steam explosion, but effects high-intensity conditions that are different from other physicochemical methods. An HDSF system consisting of a cylinder and a piston enables catapult explosions within 0.0875 seconds. The release of high-density energy at a very short time exerts a force that disrupts and unfolds the structure of fibrous proteins. Beta-sheet crystals and intermolecular disulphide bonds are successfully destroyed or destabilised without causing significant damage to the keratin protein chain. This process increases enzymatic accessibility, extraction, and dissolution of feather keratins in polar solvents like water, salt solutions, and weak bases. However, the process results in keratins with reduced molecular weight and loss of mechanical properties (Zhao et al. 2012, Shavandi et al. 2017).

2.6 Optimisation of extraction procedures

Many of the afore-mentioned reports on extraction procedures do not entail optimisation of the processes. Hence, it is difficult to ascertain which procedures are ideal for large scale extractions of keratin. Recently, researchers in South Africa have reported optimising a number of chemical procedures for the extraction of keratin from waste chicken feathers. Extraction of keratin from chicken feathers using sodium bisulphite, SDS and urea were optimised. The concentration of sodium bisulphite, SDS, reaction time and temperature were set as independent variables. Response Surface Methodology (RSM) joined with Box-Behnken Design (BBD) was used to design the experiments, analyse the effects of the combinations of independent variables of the response (protein yield). The study showed that the reaction temperature is the most significant factor, followed by reaction time, sodium bisulphite concentration, and sodium dodecyl sulphate concentration. SDS PAGE results of molecular weight analysis showed broad, strong bands between 10 kDa and 15 kDa, and weak broad bands at 20-25 kDa, 30-40 kDa and at 55 kDa suggesting that the extracted keratin has multiple monomers of low to high molecular weight (Khumalo et al. 2020).

A similar optimisation study was also carried to extract keratin from chicken feathers using sodium bisulphite and sodium hydroxide. The concentration of sodium bisulphite, sodium hydroxide, reaction temperature and time were chosen as independent variables. RSM was used to design the experiment and analyse the results. The optimised conditions were 87 ^{o}C, 111 minutes, 0.5% NaHSO3 and 1.78% NaOH. This keratin had a molecular weight between 3 kDa to 15 kDa (Fagbemi et al. 2020). These optimisation studies allow the prediction of the response variables, namely the yield of hydrolysate and protein.

2.7 The utilisation of keratin in biomedical applications

Keratins have unique properties that can be exploited to fabricate materials that could be beneficial to human health. The properties contribute to various chemical, physical, and biological behaviour of keratin materials. For example, keratin's biocompatibility, biodegradability, and natural ability to facilitate cell proliferation are some of the material's main attractive properties (Rouse and Van Dyke 2010). As a result, keratin materials can function as extracellular cell matrices to provide structural and biochemical support to surrounding cells (Shavandi et al. 2017). Keratin properties are modifiable in different stages of either extraction or material fabrication, allowing keratin biomaterials to have characteristics tailor-made for specific applications. Keratin can self-assemble and polymerise to form solid biomaterials (Rouse and Van Dyke 2010). It can be converted into porous nanofibres, composites, scaffolds, films, sponges and hydrogels that can be used in various biomedical applications. Regenerated keratin biological activity could be

beneficial in controlling specific applications. The following subsections describe some of the more popularly reported keratin biomaterials, i.e., films, nanofibres and hydrogels.

2.7.1 Keratin films

Tanabe et al. (2001) produced keratin films by casting the reduced keratin solution after mixing with chemical diepoxy cross-linkers and evaluated its mechanical, waterproof, and cell proliferation properties in comparison with keratin–chitosan composite films. Keratin without the addition of a plasticiser resulted in a fragile film, whereas keratin-treated with cross-linkers yielded tenacious and flexible films similar to keratin-chitosan films. The study showed that chemically cross-linked keratin films are highly stretchable than keratin-chitosan composite films and had significantly advanced waterproof features, i.e., no swelling was observed in acidic and neutral aqueous conditions, and small swelling occurred in alkaline condition. When mouse fibroblast cells were cultured on a cross-linked keratin-coated surface, cells' attachment to keratin, keratin-chitosan composite, and chitosan film was slightly delayed. However, once attached to the cross-linked keratin surface, the cells proliferated excellently, implying the biocompatibility of chemically cross-linked keratin films (Tanabe et al. 2001). Li and Wang (2013) produced keratin film by evenly distributing keratin / ionic liquid solution to glass and immersing it in water, methanol and ethanol as coagulating solvents for 12 h at room temperature. The regenerated films were then cleaned with deionised water and dried at room temperature. The thermal stability of the regenerated wool keratin film was reduced slightly due to molecular chain breakage (Li and Wang 2013). Fujii (2012) prepared human hair keratin film to be used as a model to simulate average human hair. For this study, the focus was on whether keratin film can survive physical and chemical stimulation, such as ultraviolet (UV), bleaching, perm, and thermal treatments. The results demonstrated that keratin films responded to hair damage causes in the same way as hair, whereas the keratin film responses were significantly higher than those of hair. Keratin film can be used as a human hair replacement to effectively examine hair damage in the development of hair care products (Fujii 2012). Other examples of keratin-based films are shown in Table 7.

Table 2.7: Keratin films, sources and biomedical applications.

Keratin source	**Application and Observations on Films**	**Ref.**
Chicken feathers	Drug delivery carrier: Drugs were delivered effectively. The films had excellent mechanical properties. They had a discharging period of 12 hours.	(Yin et al. 2013)

Merino wool	Drug delivery carrier: The film supported fibroblast cell attachment and growth.	(Posati et al. 2018)
Wool	Drug delivery carrier: Films had a lower drug release ratio, which showed they could be used to release drugs over a longer period. The films also showed improved mechanical properties.	(Cui et al. 2013)
Human Hair	Corneal implantation: Evaluated corneal biocompatibility of keratin film in rats. The results showed good corneal biocompatibility with minor tissue reaction. Corneal transparency was also conserved. The administration of steroids slowed down the implant degradation, which might benefit applications like transplantation of epithelial cell sheets.	(Borrelli et al. 2015)
Human hair	Ocular surface reconstruction: The films showed enhanced light transmission and biomechanical strength compared to the human amniotic membrane (AM). In addition, the cell behaviour of the films was similar to that found on AM. It was deduced that keratin films could offer a new and promising substitute for reconstructing the ocular surface.	(Reichl et al. 2011)
Human hair	Artificial human skin: the skin distinguished between the touch sensation of a bare hand and a glove-protected finger. This artificial thermoplastic polyurethane-keratin skin could also differentiate the tactile feel of other objects like aluminium foil, cotton gloves and wood.	(Li et al. 2018)

Keratin films have also been reported in other biomedical applications such as the human nail model (Lusiana et al. 2011), tissue engineering, a substrate for cell culture (Reichl 2009), contact lens material (Pannebaker et al. 2010), bone implantation (Dias et al. 2010) and wound dressing (Ganesan 2017).

2.7.2 Keratin Hydrogels

Wang et al. (2012) assessed the effectiveness of keratin hydrogels as substrates for cell cultures. They produced the hydrogels using human hair keratin extracted by inducing Ca^{2+} polymerization; The hydrogels were highly branched and porous micro-architectures. In a preliminary cell culture study, L929 murine fibroblasts were used to compare in vitro biocompatibility of keratin hydrogels with collagen type 1 hydrogels with similar viscoelastic

properties. The results indicated that keratin hydrogels are comparable to collagen hydrogels in cell adhesion promotion, proliferation and cell viability preservation. The study showed that cells remained agglomerated in proliferative colonies inside keratin hydrogels and were homogeneously distributed as single cells within collagen hydrogels. The findings indicate that keratin hydrogels can be used as substrates for cell cultures and may be used as frameworks for soft tissue regeneration (Wang et al. 2012). In another study, Wang et al. (2015) developed and optimised a method to produce hydrogels for a 3D cell culture system from human hair. Concentration, pH and temperature were chosen as critical factors that affect keratin gelation and encapsulation of living cells. SEM analysis of the hydrogels showed a highly interconnected porous micro-architecture in the interior.

The keratin hydrogels had a stiffness comparable to brain tissue. Further results, from L929 culture studies, showed the preservation of cell viability during encapsulation; however, the cells tended to spread towards peripheral regions of the hydrogel because of diffusion limitations. Cell proliferation measurements of keratin hydrogels implied that they were comparable to collagen hydrogels, without an acute host response. The conclusive evidence from this investigation suggested that keratin hydrogels can be used as effective 3D cell culture systems (Wang et al. 2015). Table 2.7 summarises other advancements on keratin-based hydrogels.

Table 2.8: Keratin hydrogels, sources and applications in biomedicine.

Keratin source	**Application and Observations**	**Ref.**
Chicken feathers	Drug delivery carrier: the cumulative release of an anticancer drug (Dox·HCl) reached 93.3% within 16 h, and that of a macromolecular drug (BSA) was 75.9% in 24 h. Hence, the hydrogels were pH and temperature sensitivity, making them appropriate for drug delivery.	(Sun et al. 2016)
Chicken feathers	Drug delivery carrier: pH-sensitive keratin hydrogels were developed. For small molecular drugs, 97% of drugs were delivered within 24 hours at pH 8.4, whereas for macromolecular drugs, the release rate was 89% in 24 hours at pH 7.4.	(Guo et al. 2015)
Wool	Effect of chemical modification on biomedical applications: Keratin hydrogels (KH) and chemically modified keratin hydrogels (AAK, CMK, and AEK). All hydrogels slightly shrank in acidic medium. KH, AAK and AEK supported cell proliferation, though cell elongation was slightly suppressed on AAK and AEK hydrogel. KH and AAK hydrogels showed more than three days of drug release rate. AEK and CMK hydrogels completed drug release in one day.	(Nakata et al. 2015)

Human hair	Peripheral nerve conduit: Neuromuscular recovery with keratin was higher than in other conduits without keratin. Nerves in keratin hydrogels had lower conduction delays, greater amplitudes, more myelinated axons, and larger axons than nerves in other conduits without keratin. Sensory nerve autografts and keratin hydrogels were statistically equivalent in compound motor action potential measurements at six months. Moreover, keratin-filled conduits demonstrated greater axon density and larger average axon diameter than other conduits and autograft at six months.	(Apel et al. 2008)
Human Hair	Filler material: an investigation on the feasibility of keratin hydrogels as filler materials. Keratin hydrogels could be controlled by adjusting some preparation parameters. With water absorption of 850%, these hydrogels had excellent biocompatibility and could promote angiogenesis formation and cell proliferation. The conclusion was that the hydrogels could be suitable filler materials.	(Xu et al. 2016)
Wool	Wound repair: keratin increased cell viability by over 1.5 fold. It also eliminated free radicals within 10 minutes, with an elimination rate of 20% for 0.01% keratin, 50% for 0.1% keratin and 80% for 1% keratin. These hydrogels exhibited complete wound healing after 12 days, while positive control chitosan hydrogels only resulted in 90% wound closure.	(Su et al. 2020)
Human Hair	Hydrogels as cell culture: cell seeded on keratin hydrogels were viable up to 7 days with a viability of approximately 90%. They showed slow kinetics indicating their suitability for long-term application in cell therapy.	(Yue et al. 2018)

Other applications of keratin hydrogels include haemostatic dressing gel (Aboushwareb et al. 2009), nanocarriers for chemotherapeutic agents (Curcio et al. 2015), wound healing (Wang et al. 2017), skin regeneration (Li et al. 2019) and pulp-tissue engineering (Sharma et al. 2017).

2.7.3 Keratin nanofibres

Keratin nanofibres are mostly produced through electrospinning methods. In this process, polymeric fluid is charged by a high voltage and then erupts, forming a Taylor cone. The polymer jet is drawn to the grounded collector while drying to microfibres and/ nanofibres before reaching the collector (Hyun et al. 2011, Alazab et al. 2017). Nanofibre properties are driving forces for such a vast interest in nanofibres (Huang et al. 2003). Nanofibres can be applied in various healthcare sectors, electronics, wastewater treatment and polluted-air cleaning (Subbiah et al. 2005, Jirsák and Dao 2009). Nanofibres and their production have been advanced by blending keratin with other polymers such as polyethene and polyvinyl alcohol (Aluigi et al. 2008, Li and Yang 2014). Keratin-based nanofibres can substitute petroleum-based polymer nanofibres since they have an advanced cell to material interactions (Rouse and Van Dyke 2010).

Ye et al. (2020) prepared high molecular keratin (HMK) from wool through a multi-enzyme pathway. The 120 kDa soluble keratin was extracted from wool by keratinase, and its molecular weight was upgraded by transglutaminase. Keratin, mixed with PHBV at 30% to 70% ratio, was converted to nanofibres by electrospinning. The resulting nanofibrous mat had improved mechanical properties. Silver (Ag) particles were then incorporated into the mat to improve the mat's potential biomedical applications. The results showed that HMK/PHBV/Ag mat had suitable antibacterial property and excellent biocompatibility. The mat also had good wound healing properties (Ye et al. 2020). He et al. (2020), in a separate study, also prepared keratin/PVA/PEO nanofibres incorporated with silver nanoparticles and investigated their antimicrobial effects. Silver nanoparticles improved crystallinity, thermal stability and antibacterial activity of Keratin/PVA/PEO nanofibres. The antibacterial activity was effective against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. Furthermore, both elongation at break and tensile strength of nanofibres were highest when silver content was 1.2 percent (He et al. 2020).

Another study reported on the manufacture of keratin/PET composite nanofibres using electrospinning to absorb Cr (VI) in an acidic medium. Wool was solubilised by immersion in tris(hydroxypropyl)phosphine (THP) solution at 90 ^{o}C for 12 hours. The purified keratin was blended, in different ratios, with polyethene terephthalate (PET) before electrospinning into nanofibres. The absorption results showed maximum absorption when the keratin-PET blending ratio was 50/50 at a pH value of 3. The blend showed a maximum of 75.86 mg/g compared to 27.27 mg/g of pure PET nanofibres. The results showed that the redox reaction of disulphide bonds and electrostatic adsorption of amino was responsible for the absorption process (Jin et al. 2020). Other recently reported developments about keratin nanofibres are summarised in Table 8.

Table 2.9: Keratin nanofibres, blends, sources and applications.

Keratin nanofibres	Keratin source	Observations and application	Ref.
Keratin/polyethene oxide/hydroxyapatites	Human hair	Wound dressing: Tensile strength of K/PEO/15%HA membrane was twice high, 2.23 MPa, as that without HA. The K/PEO/HA membrane enhanced cell proliferation, reduced inflammatory response, enhanced burnt-skin recovery. However, the keratin/PEO/HA nanofibre membrane's thermostability decreases with an increase of HA content.	(Fan et al. 2020)

Keratin/polyacrylonitrile	Wool	Antibacterial agent: composite nanofibres had sound antibacterial effects and high moisture permeability. 89.21% inhibiting ability against E. coli and 60.70% against S. aureus. The composite had excellent wetting performance and hydrophilic properties.	(Zhong et al. 2020)
Keratin/poly (L-Lactide) nanofibres	Wool	Drug delivery carrier: 5-fluorouracil (5-FU) was loaded and distributed evenly in nanofibres. Nanofibres were smooth and uniform and had a pH-stimuli responsive character in acidic conditions. Keratin increased the inhabitation of tumour cells after 120 hours. Interaction of keratin and 5-FU increase the release period of 5-FU in acidic conditions. 83% of the drug was released in acidic medium, while only 42% of 5-FU was released in 120 hours in neutral medium. K/FU/PLLA had an enhanced antitumor efficiency.	(Zhang et al. 2020)
Keratin/polycaprolactone	Wool	Cell instructive scaffolds for inter-face tissue engineering: hMSC cell well adhered and proliferated on the nanofibres scaffold. However, the nanofibres had a negligible antibacterial effect on E. coli and S. aureus.	(Cruz-Maya et al. 2019)
Keratin/hydrotalcite loaded with diclofenac (HTD)	Wool	Drug delivery carrier: the scaffold was loaded with diclofenac. Nanosized HTD showed a reduced swelling ratio, a slower degradation. The drug's toxicity decreased when loaded into nanofibres and confirmed the biocompatibility of keratin/HTD nanofibres mat. The controlled release of diclofenac within 24 hours did not significantly compromise the cell growth.	(Giuri et al. 2019)

The research reports show promising results that demonstrate the potential of keratin nanofibres in biomedical applications. However, more in-depth investigations are required to confirm if the *in-vitro* and *in-vivo* results can be transferred to clinical practices and improve their properties (Mehta and Pawar 2018).

2.8 Conclusions

This work discussed different types of keratin structures, namely alpha- and beta-keratins. It further discussed keratin composition, i.e., amino acids, amount of sulphur in different keratin concerning soft and hard ones. This work also discussed various techniques available to extract keratins from their sources; keratin extraction techniques such as chemical extractions and non-chemical extractions. Properties of extracted keratin concerning extraction methods were also highlighted. One must anticipate the properties of keratin required before the actual extraction so that its properties will be aligned to intended biomaterials such as nanofibres, hydrogels and films. These keratin biomaterials are examined for their potential biomedical applications such as drug delivery carrier, tissue engineering, wound dressing and wound healing. This work demonstrated that though techniques to utilise keratinous wastes are available, it is challenging for them to reach the commercial breakthrough to the respective sectors. Hence, more innovative ways to overcome the existing challenges are still required.

Future work may include:

- Finding the commercialisable keratin extraction methods that are effective, environmentally friendly and economically viable.
- Simple and quick separation of the components of the extract.
- Improving the properties of keratin biomaterials so that they can be commercialised.

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CHAPTER 3
OPTIMISATION OF KERATIN EXTRACTION PROCESS

Chapter Overview

This chapter, based on the published research paper, describes the extraction process and its optimisation. Keratin must be extracted from waste chicken feathers before it can be electrospun to keratin nanofibre tubes. Different extraction methods, highlighted in chapter 2, result in the variation of keratin properties such as molecular weight. For example, harsh extraction conditions can denature keratin and over-digest it; hence, the extraction process is vital for keratinous waste valorisation. Therefore, the research work in this chapter is based on the optimised keratin extraction process undertaken in this chapter, chapter 3, because a different extraction method would result in keratin with different nanofibre properties, such as electrospinnability.

The chapter highlights the advantages of using reduction extraction techniques, specifically sodium bisulphite, compared to the harmful 2-mercaptoethanol. Even though the sodium bisulphite extraction method was effective, its non-optimisation status allowed wide variation on the recovered keratin properties.

The chapter further describes designing the experiments using the Design Of Experiment (DOE) technique of Design-Expert software, creating 29 runs as optimisation runs, before accurately analyzing the results through Response Surface Methodology (RSM). The study ends with the optimized conditions of the extraction process and the mathematical model that is used to determine the extraction yield. The optimized parameters allow detailed insight into the extraction process.

VALORISATION OF WASTE CHICKEN FEATHERS: OPTIMISATION OF KERATIN EXTRACTION FROM WASTE CHICKEN FEATHERS BY SODIUM BISULPHITE, SODIUM DODECYL SULPHATE AND UREA

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3.1 Abstract

Extraction of keratin from keratinous waste materials, such as chicken feathers, has been identified as the favourable approach in beneficiation of this biomass. The chemical extractions of keratin by reducing agents are usually preferred because they are much faster than its counterpart, oxidation extraction. One such reduction extraction is a mixture of sodium bisulphite, sodium dodecyl sulphate and urea. At least five factors may affect the keratin extraction process and its final properties when using this extraction. Even though this extraction method is often used, the effects of its independent variables have not been studied; thus, the effects of independent variables cannot be fully linked to the extraction process and final keratin properties. Therefore, this study aimed to optimise keratin extraction from waste chicken feathers using sodium bisulphite, sodium dodecyl sulphate, and urea. The optimisation was statistically performed using Response Surface Methodology (RSM) linked with Box-Behnken Design. After screening the independent variable using one factor at a time method, the sodium bisulphite concentration, concentration of sodium dodecyl sulphate, reaction temperature and reaction time were chosen for the study. Twenty-nine experiments were statistically designed and executed, and their results were used to analyse the effects of all the independent variables to optimise the extraction process. The reaction temperature was the most significant factor, while the concentration of sodium dodecyl sulphate was the most insignificant factor of this extraction process. Independent variables significance order was reaction temperature > reaction time > concentration of $NaHSO_3$ > concentration of $NaC_{12}H_{25}SO_4$. The designed reduced cubic model was significant and was used to predict the protein yield from the keratin extraction using sodium bisulphite.

Keywords: keratin, extraction, optimisation of extraction, waste chicken feathers.

3.2 Introduction

Extraction of keratin from chicken feathers is an important aspect when valorising the abundant waste chicken feathers from the poultry industry. This is because the industry produces an enormous amount of waste chicken feathers during meat production. An insignificant amount is used to make low-value products such as feather meal for livestock and fertilisers (Tesfaye et al. 2017). The larger portion of feathers is considered waste and is either disposed of in landfill sites or incinerated. Both disposal techniques, landfills and incineration, are environmentally unfriendly due to slow decomposition of feathers in landfills and air pollution from the latter. The solid waste chicken feathers require a large area for landfill, and contaminate groundwater. The chemical composition of a feather shows that it contains 91% of β-keratin protein, a precious protein. Therefore, it is beneficial to extract this protein for the applications such as in health care and cosmetic industries (Swetlana and Jain 2010). It has been reported that materials such as biofilms, nanofibres, hydrogels, sponges, membranes and cosmetics can be produced from keratin (Karthikeyan et al. 2007, Kumaran et al. 2016).

Extraction of keratin protein from chicken feathers using reducing agents is often preferred because it is generally faster than the extraction of keratin from feathers using oxidising agents (Holkar et al. 2017). Even though keratin extraction by 2-mercaptoethanol produces the highest yield, its price and toxicity cause reluctance on its application (Holkar et al. 2017, Sinkiewicz et al. 2017). Hence, keratin extraction using sodium bisulphite is an excellent alternative technique due to its simplicity, effectiveness, and economically and environmentally friendliness. During the keratin extraction process, sodium bisulphite ($NaHSO_3$) cleaves and further prevents disulphide bonds forming (Holkar et al. 2017). Moreover, this extraction technique includes the addition of urea as a denaturing agent, and a surfactant, sodium dodecyl sulphate (SDS); urea increases the solubility of keratin in water and facilitates the effect of reducing agent on the polypeptide chain (Katoh et al. 2004, Xu et al. 2014). Sodium dodecyl sulphate prevents the aggregation of polypeptide chains by blocking the formation of new cross-linkages, thus, increasing extraction rate and improving the stability of the extracted keratin (Schrooyen et al. 2001, Tonin et al. 2007).

Despite the economic and environmental friendliness and smooth execution, this extraction technic has not been optimised, i.e., the concentration of sodium bisulphate, sodium dodecyl sulphate and urea, as well as temperature and the number of hours to run this extraction vary from one researcher to another. Sinkiewicz et al. (2017) extracted keratin from chicken feathers using this mixture of chemicals. They obtained 82% of keratin, while Tonin et al. (2007) and Kamarudin et al. (2017) used the same extraction technique under different conditions and obtained the maximum yields of 33% and 18,3% respectively. This means the yield, physical and chemical properties of the extracted keratin cannot be easily predicted. For instance, the molecular weight

and molecular weight distribution of keratin are essential properties for keratin applications. Severe extraction conditions such as very high pH/low pH at prolonged exposure to high-temperature breaks both disulphide bonds and peptide bonds producing low-molecular-weight keratins which are mostly used to make animal feeds, with limitations to biomedical applications (Jones 1975, Smith et al. 1994). Whereas extraction of keratin under mild conditions breaks disulphide bonds of proteins fibres without significant cleavage of polypeptide bonds, resulting in an unaffected macromolecular structure of keratin, this yields high-molecular-weight keratin that can be used for biomedical applications (Eslahi et al. 2013). Thus, the current work aims to optimise the process of extracting keratin from waste chicken feathers using sodium bisulphite, sodium dodecyl sulphate and urea to obtain experimental conditions that result in a maximum yield of keratin.

3.3 Methods and materials

White chicken feathers were collected from chicken meat processing plant RCL Foods at Hammarsdale, South Africa. Sodium bisulphite, sodium hydroxide, sodium dodecyl sulphate and urea were purchased from Sigma-Aldrich South Africa. The main instruments for the keratin's characterisation were Fourier transform infrared spectroscopy (FTIR) and a Carbon Hydrogen Nitrogen Sulphur and Oxygen (CHNS/O) Analyser.

3.3.1 Pre-treatment of chicken feathers

Wet chicken feathers were rinsed with water, at 60 ^{o}C, to remove excess blood while manually removing other meat by-products. Feathers were then autoclaved at a temperature of 121 ^{o}C and pressure 120 kPa for 0.5 hours. Furthermore, they were soaked for 24 hours in 0.5% v/v sodium hypochlorite to disinfect and remove stains, afterwards, rinsed with water and dried at 25 ^{o}C.

3.3.2 Extraction procedure

Cleaned-disinfected chicken feathers were comminuted in a milling machine to increase the feathers' surface area, thus enhancing their dissolution rate during the extraction process. The milled feathers were soaked in 99% ethanol for 24 hours to remove fatty materials, rinsed with water, filtered, and dried at 50 ^{o}C for three days. Dry, cleaned and degreased 15 g of chicken feathers were immersed in various solutions of sodium bisulphite, sodium dodecyl sulphate and 1.5 M urea as shown in Tables 3.1 and 3.2, of sodium bisulphite and sodium dodecyl sulphate. The reaction mixtures were mechanically shaken on a desktop shaker for homogeneous distribution of all mixture components in a container and then heated in a heated oil bath. After heating and then cooling, the mixtures were centrifuged at 9000 rpm for 15 min and thereafter filtered to separate the insoluble materials and supernatants. The filtrates obtained were dialysed

in distilled water using cellulose membrane dialysis tubes (MWCO 14 kDa) for five days. The retained keratin solutions were freeze-dried to obtain keratin powder and then stored in a cold room at 4 ^{o}C.

3.3.3 Experimental design and statistical analysis

On single factor analysis, independent variables, viz, the concentration of sodium bisulphite ($NaHSO_3$), concentration of sodium dodecyl sulphate (SDS), extraction temperature (T) and extraction time (t), and their ranges were selected for the investigation of their effects on the response variable, the protein yield (%). A three-level (-1, 0, +1) Box-Behnken design (BBD) joint with response surface methodology (RSM) was employed to design the experiments of the project. Table 3.1 shows the independent variables and their three levels, low, middle, and high, as per the selected ranges.

Table 3.1: Independent variables and levels as per Box-Behnken Design.

Variable (units)	Variable symbol	Low level (-1)	Middle level (0)	High level (+1)
$NaHSO_3$ (M)	A	0.2	0.3	0.4
SDS (M)	B	0	0.035	0.07
Temperature (^{o}C)	C	50	70	90
Time (h)	D	2	3	4

BBD, according to the number of experiments (N) equation (eq).1, developed twenty-five experiments and four replicates as per the parameters defined in Table 3.1.

$$N = 2k(k-1) + C_0 \quad (1)$$

where k is the number of factors, and C_0 is the number of central points. Designed experiments were carried out according to Table 3.2. The experimental data were statistically analysed and fitted into a second-order polynomial equation, eq.2.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i Y_j \quad (2)$$

where *Y* is the predicted response, *Xi* and *Xj* are the independent variables, β_o is the offset term, β_i is the *i*th linear coefficient, β_{ii} is the *i*th quadratic coefficient, and β_{ij} is the *ij*th interaction coefficient. The variables possess a statistically significant effect on the response, protein yield, were analysed through analysis of variance (ANOVA). The analysis included the effect of variables and their interactions on protein yield.

Table 3.2: Box-Behnken design matrix of uncoded values along with experimental protein yield (%).

Run	$NaHSO_3$ (M)	$NaC_{12}H_{25}SO_4$ (M)	Temp (°C)	Time (h)	Protein (%)
1	0,3	0	50	3	0,17
2	0,3	0,07	50	3	7,01
3	0,3	0,035	50	2	4,34
4	0,3	0,035	50	4	6,89
5	0,2	0,035	50	3	5,60
6	0,4	0,035	50	3	9,17
7	0,3	0,07	70	4	51,38
8	0,2	0,07	70	3	33,80
9	0,3	0,035	70	3	26,21
10	0,3	0,035	70	3	30,65
11	0,3	0	70	4	11,51
12	0,3	0,035	70	3	29,60
13	0,4	0,035	70	4	3,95
14	0,4	0	70	3	7,74
15	0,2	0,035	70	4	18,99
16	0,2	0	70	3	6,08
17	0,3	0,035	70	3	16,84
18	0,4	0,07	70	3	38,45
19	0,2	0,035	70	2	12,69
20	0,3	0,07	70	2	32,98
21	0,3	0,035	70	3	5,08
22	0,4	0,035	70	2	1,81
23	0,3	0	70	2	12,86
24	0,3	0,035	90	4	43,41
25	0,3	0,07	90	3	17,63
26	0,2	0,035	90	3	20,01
27	0,3	0,035	90	2	9,66
28	0,3	0	90	3	9,72
29	0,4	0,035	90	3	14,10

3.3.4 Characterisations of feather keratin extract

3.3.4.1 Fourier transform infrared spectroscopy (FTIR)

Functional groups of the extracted keratin were analysed using Fourier Transform Infrared spectroscopy (Frontier Universal model, from PerkinElmer), in an attenuated total reflection mode (ATR). Each spectrum obtained was an average of 4 scans at a wavenumber range of 550 – 4000 cm-1 with a resolution of 4 cm^{-1}.

3.3.4.2 CHNS Analysis for crude protein content in extracted keratin (done)

Elemental composition of extracted keratin was performed, where carbon, hydrogen, nitrogen and sulphur were analysed using a CHNS/O analyser. This was mainly to determine the crude protein content, which is calculated from nitrogen content in a sample by multiplying it by a crude protein conversion factor of 6.25.

3.3.4.3 Bradford Assay for protein concertation

To further analyse the protein content in the extracted sample, a Bradford assay was performed using a spectrophotometer (Promega GloMax Microplate Multimode Reader). Bovine serum albumin (BSA) was used as the standard to generate a calibration curve from absorption measurements at 595 nm. Keratin solutions were prepared by dissolving 35 mg into 1 ml of deionised water before the absorption measurements.

3.3.4.4 SDS PAGE analysis for molecular weight determination

The molecular weight of extracted keratin was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Keratin samples 35 mg were dissolved in 1 ml distilled water and boiled for 5 min. Subsequently, 15 uL of 35 mg keratin sample was added into a solution of 5 uL of NuPAGE LDS sample buffer (4X) containing 5% β-mercaptoethanol, and boiled for 7 min. The denatured samples were then loaded onto 16% and 12% polyacrylamide gels for low and mid-high molecular weight determination, respectively. The gels were subjected to 80V for 30 min, followed by 120V for 2 hours. The 12% gel was then stained, for 30 minutes, with Coomassie Brilliant Blue (CBB) G-250 followed by an overnight destaining with an ethanol-acetic acid solution, while the 16% gel was first fixed with 5% glutaraldehyde for 25 minutes before a 20 minutes staining with CBB G-250. The 16% polyacrylamide gel was destained with 10% acetic acid solution for 20 minutes. GENE imaging software was used to take images of both gels for analysis.

3.4 Results and discussions

3.4.1 Effect of pre-treatment of waste chicken feathers

The originally golden-brown waste chicken feathers showed significant improvement of whiteness index and a decrease in yellowness due to bleaching ability of sodium hypochlorite. Moreover, the treatment of feathers with ethanol further increased their whiteness index due to the recovered golden-yellow oil responsible for low whiteness index. This effect of pre-treatment on waste chicken feathers was in line with the findings of Tesfaye et al. (2018), which showed the inverse relationship between whiteness index and yellowness index.

3.4.2 Extraction of keratin from waste chicken feathers

Cooking feathers resulted in a heterogeneous mixture of solution and chicken feather particles of different sizes. The variation of feather particles' sizes was due to different combinations of the concentration of sodium bisulphite, concentration of sodium dodecyl sulphate, reaction temperature and time. The hydrolysate formed was due to the sulphitolysis of disulphide bonds between keratin molecules by the reducing agent, sodium bisulphite, according to eq.3 (Yin et al. 2013, Pilehvar et al. 2019).

$$Keratin - S - S - Keratin + SO_3^{2-} \rightarrow Keratin - S^- + Keratin - S - S_3^{2-} \quad (3)$$

Light brown supernatant followed by light brown glittering powder was obtained after purification and freeze-drying, respectively.

3.4.3 Statistical analysis of the keratin extraction from feathers

Twenty-nine experimental runs were conducted, and the results are tabulated in

Table **3.2**. Independent variables and dependent variable allowed the use of statistical data processing to determine the dependency of the response on the independent variable. Table 3**.3** showed the analysis of variance (ANOVA) based on the statistically processed data.

Table 3.3: Analysis Of Variance (ANOVA) of the reduced cubic model for the optimisation of extraction.

Source	**Sum of Squares**	**Mean Square**	**F-value**	**p-value**	
Model	19,72	2,47	2,84	0,0277	Significant
A-$NaHSO_3$	1,63	1,63	1,88	0,9810	
C-Temperature	7,27	7,27	8,39	0,0089	
D-Time	0,0204	0,0204	0,0236	0,8795	
AC	4,51	4,51	5,20	0,0337	
AD	0,5677	0,5677	0,6546	0,4280	
C^2	2,47	2,47	2,85	0,1068	
D^2	0,8418	0,8418	0,9707	0,3363	
AD^2	3,52	3,52	4,05	0,0577	

The Table showed that the reduced cubic model of the experiment had a p-value of 0.0277 (less than 0.05), which indicated that the model was significant. The model also had an F-value of 2.84, which implied that there was only 2.77% chance that an F-value this large could be due to noise; hence, this F-value emphasised the model significance. The adequate precision measure was 8.234 (greater than 4), meaning the model had a strong enough signal to be used for optimisation. The reaction temperature and the product of the concentration of sodium bisulphite and temperature were significantly affecting the response since they had p-values of 0.0089 and 0.0337, respectively. In addition, the ANOVA Table showed that the concentration of sodium bisulphite was marginally significant, whereas reaction time and the concentration of sodium dodecyl sulphate were insignificant.

Table 3.4: Coefficients estimates for the model of optimisation.

Factor	Coefficient Estimate	Standard Error	VIF
Intercept	2.51	0.2808	—
A-$NaHSO_3$	-0.4520	0.3292	1.50
C-Temperature	0.7785	0.2688	1.0000
D-Time	0.0413	0.2688	1.0000
AC	1.06	0.4656	1.0000
AD	-0.3767	0.4656	1.0000
C^2	-0.5987	0.3545	1.02
D^2	0.3492	0.3545	1.02
AD^2	1.15	0.5703	1.50

The coefficients Table, Table 3.4, contained the coefficients estimates that represented the expected change in response per each unit change of each independent variable when others are unchanged, whereas, each coefficient magnitude was directly proportional to its influence on the response in relation to other coefficients, i.e. the factor with bigger coefficient is more significant than others and has the least p-value. Table 3.4 also showed small and similar standard errors as this must be the case for a balanced design. The Variation Inflation Factors (VIF) for each coefficient were within 1-1.5 range. VIF value of 1 is an ideal value for measuring orthogonality of a factor in an orthogonal design. In contrast, a value of 10 is an acceptable upper limit. The coefficient estimates were used to formulate the model equation, equation 4.

$$\ln(Protein\ Recovered\ \%) = +2{,}51 - 0{,}4520\,A + 0{,}7785C + 0{,}0413D + 1{,}06AC - 0{,}376AD - 0{,}5987C^2 + 0{,}3492D^2 + 1{,}15AD^2 \quad (4)$$

where A, C, D is sodium bisulphite, temperature and time, respectively. The effects of sodium sulphite concentration, the concentration of sodium dodecyl sulphate, reaction temperature and reaction time on the recovered protein are shown on the 3D-graphs in Figure 3.1. The standard error for each model term is 0,2887; similar and low standard errors are encouraged for a balanced design. The Variance Inflation Factor (VIF) is 1, which is an ideal VIF value is 1.0. VIF that is greater than 10 is cause for concern. The model has a Predicted R^2 of 0,2250 in agreement with the Adjusted R^2 of 0,3811; i.e. the difference is less than 0.2. The Adeq Precision (noise ratio) is 8,121, which is greater than the desirable 4.

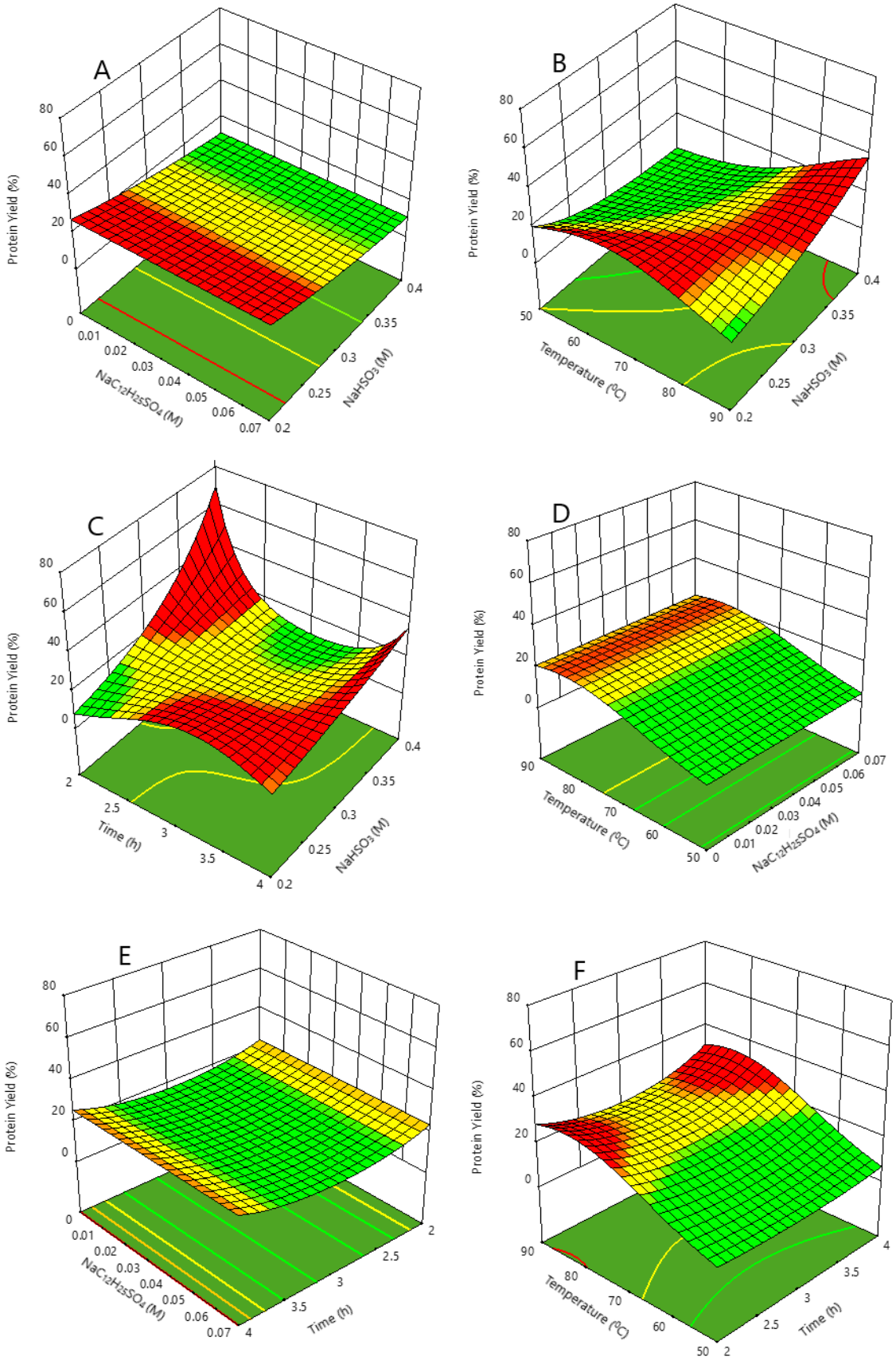

Figure 3.1: Three dimensional graphs are showing the effects of temperature, time, the concentration of $NaC_{12}H_{25}SO_4$ and $NaHSO_3$(M) on the protein yield.

The graphs A, B, C and D in Figure 3.**1** represent each independent variable's effects and their interactions on the protein yield. Graph A shows the effect of sodium bisulphite and sodium dodecyl sulphate concentrations on the amount of the extracted keratin. Graph B represents the effect of sodium bisulphite concentration and reaction temperature on the response. Graph C shows the effect of sodium bisulphite concentration and reaction time, and D is the effect of sodium concentration dodecyl sulphate and reaction temperature on keratin yield. Furthermore, E and F are the effects of sodium dodecyl sulphate and reaction time and the temperature-time effect on the yield. Graphs A, D and E show that protein yield had no dependency on the variation of the concentration of sodium dodecyl sulphate. This is in line with ANOVA and coefficients estimate Tables that excluded it from the significance descriptions due to the lack of it. The effects of other independent variables were interaction dependent; for example, the reaction temperature may be observed as being directly proportional to the protein yield (on graph C). However, its effect highly depends on other variables (graph B and F)

3.5 Characterisation of the extracted keratin

3.5.1 Structural analysis of the extract by FTIR

The analysis of extracted keratin and raw chicken feathers by FTIR showed absorbance peaks corresponding to multiple amide groups. The spectra of these analyses are contained in Figure 3.2. Literature shows that the presence and positions of amides, from amide I, are used to confirm the presence of the protein material (Dabalos et al. 2019).

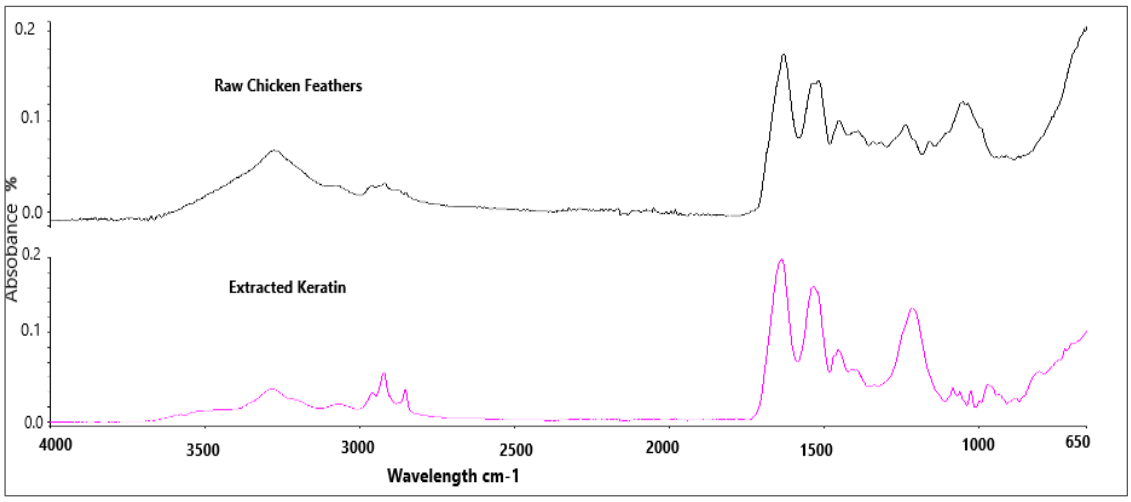

Figure 3.2: FTIR spectra of extracted keratin, raw feathers and keratin standard.

The peak at 3280.79 mc^{-1} represents amide A while peak 2919.88 mc^{-1} is amide B, 1633.74 mc^{-1} amide I, 1532.24 mc^{-1} amide II, 1212.05 mc^{-1} amide III, 623 mc^{-1} amide IV, 1000 mc^{-1} thiosulphate ion and 550 mc^{-1} is for disulphide bonds of keratins (Kakkar et al. 2014). Peaks positions of the extracted keratin show no significant shift compared to raw feathers, indicating

the conservation of the protein's secondary structure during the extraction process (Cardamone 2010). Furthermore, thiosulphate ion's presence was due to the attachment of sulphite ion to one of the sulphur atoms of the disulphide bond, indicating the breakage of this bond during the extraction process. However, the peak at 550 mc^{-1} for extracted keratin suggests that there are still traces of unbroken disulphide bonds after the keratin extraction process.

3.5.2 Structural analysis of the extracted keratin using MNR

The ^{13}C NMR spectra of the extracted keratin, in Figure 3**.3**, showed peaks at 25 ppm, which correspond to alkyl side chains. The cleavage of the disulphide bonds may reduce the β-carbon signals from 40 ppm to 25-29 ppm to give thiol signals, hence, these alkyl group peaks overlapped with cystine groups signals that show up between 25 ppm and 29 ppm.

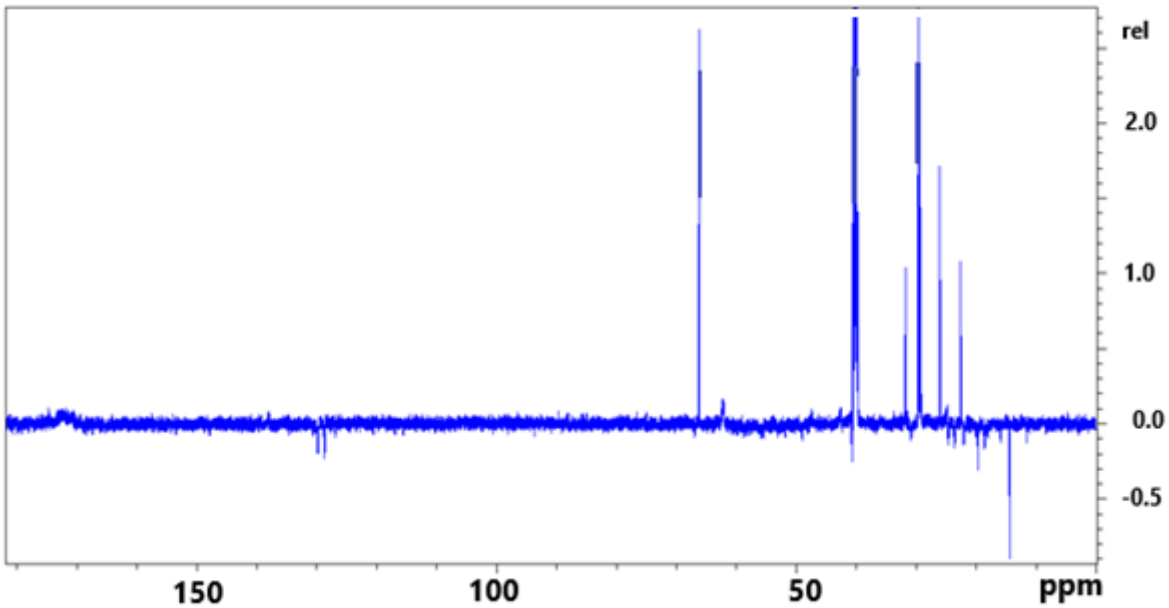

Figure 3.3: The ^{13}C NMR spectrum of keratin extracted from chicken feathers.

Beta carbon peaks present in leucine and cross-linked cysteine residues were also observed at 40 ppm, while disulphide bonds signals may be represented at the same 40 ppm (Baias et al. 2009). Alpha carbon signals were observed at 60 ppm. There were peaks at 130 ppm, indicating the presence of aromatic carbon, confirming the presence of the amino acids in the extracted keratin. Low carbonyl signal at 170 ppm represented the keratin protein's amide carbonyl (Idris et al. 2014).

3.5.3 Analysis of the molecular weight of the extract using SDS-PAGE

The analysis of the extracted keratin's molecular weight was analysed using DSD PAGE technique, the resulting gels A and B are shown in Figure 3.4.

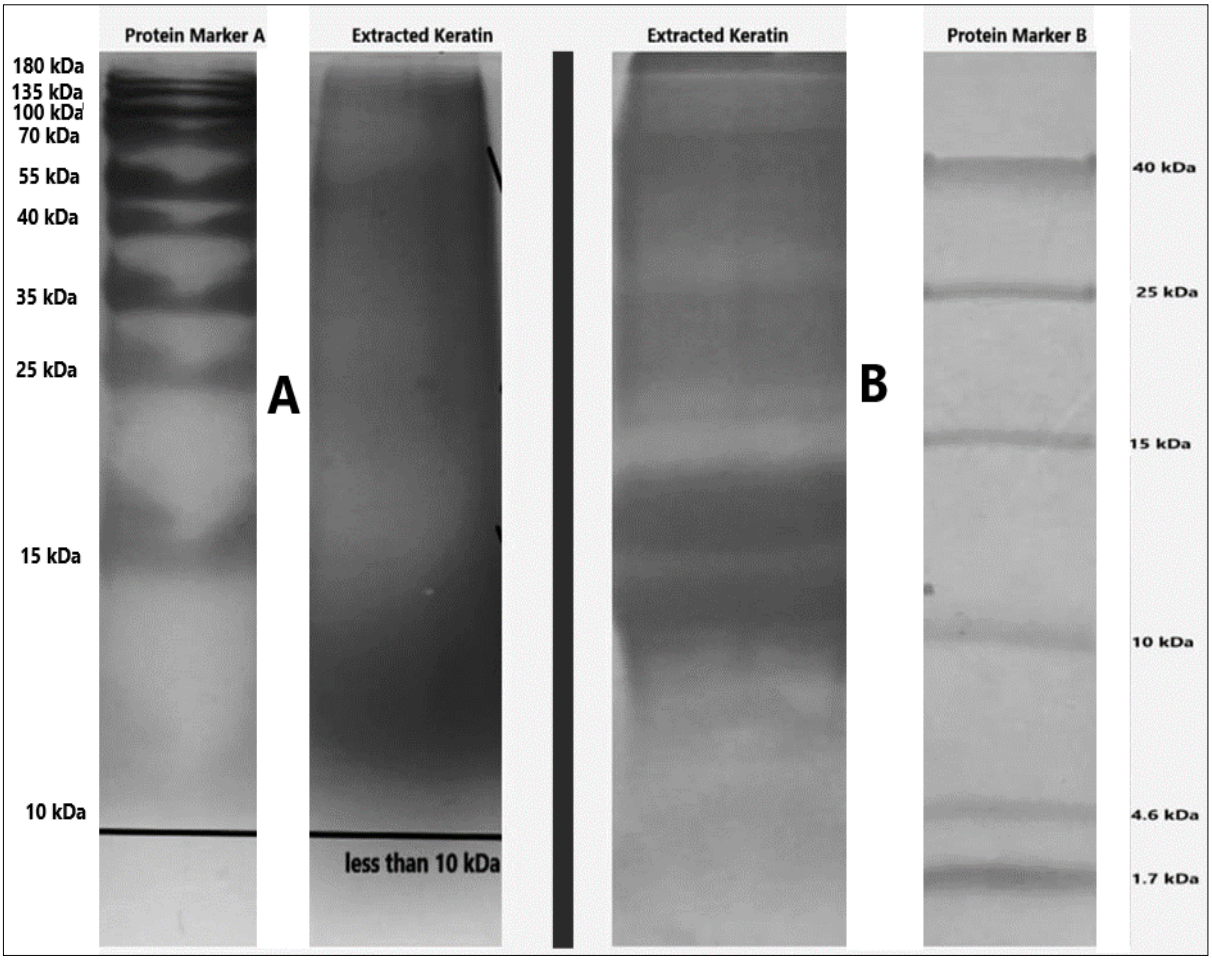

Figure 3.4: SDS-PAGE gels of extracted keratin protein, glycine gel (A), tricine gel (B).

Gel A was a glycine gel whose bands were analysed in reference to medium to high molecular weight markers that can determine molecular weight from 10 to 180 kDa. Whereas, gel B was a tricine gel used to analyse low to medium molecular weights of the sample using a marker with a range of 1.7 to 40 kDa. The Coomassie Brilliant Blue (staining agent) appeared all over the lane in each gel, with weak bands at about 20-25 kDa, 30-40 kDa and 55 kDa, meaning that the extracted keratin consisted of multiple monomers that have low to high molecular weights. These molecular weights were in line with Kamarudin et al. (2017) results that showed keratin proteins with low molecular weight (3 kDa) and high molecular weight (62 kDa). Therefore, molecular weights of extracted keratins depend mainly on the extraction conditions.

3.5.4 Crude protein analysis using CHNS analyser

The qualitative and quantitative analysis of chemical elements in extracted keratin showed, in Figure 3.5, that there was 46.64% carbon, 7.72% hydrogen, 10.82% nitrogen, 5.02% sulphur and 29.74% of other elements, including oxygen. This composition was comparable to that of raw chicken feathers shown in Figure 3.6 (Tesfaye et al. 2017).

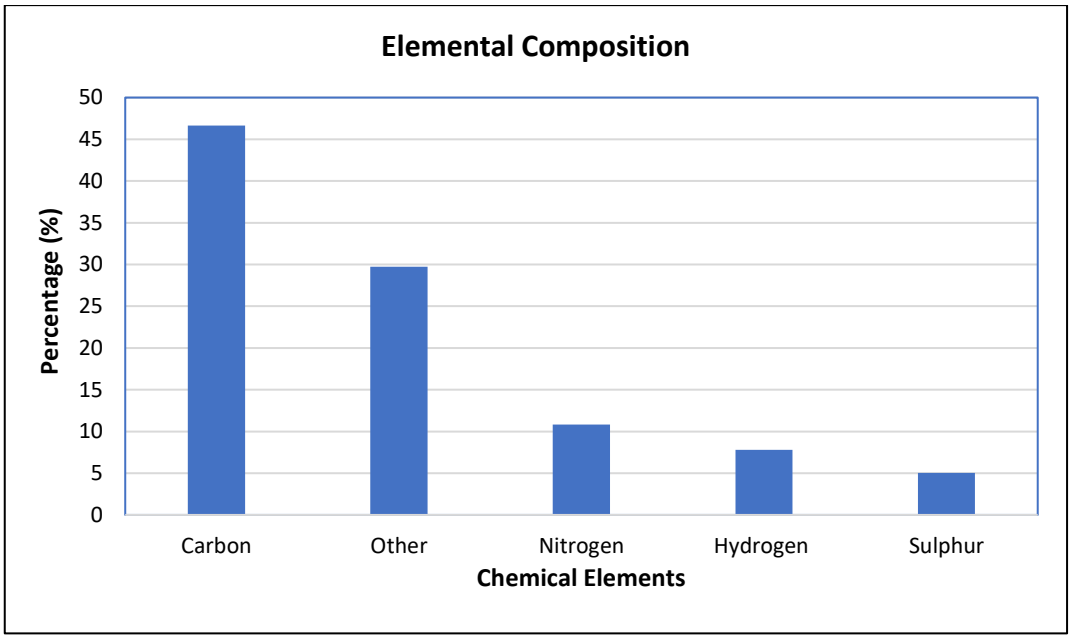

Figure 3.5: Graph of the elemental composition of the extracted chicken feather keratin.

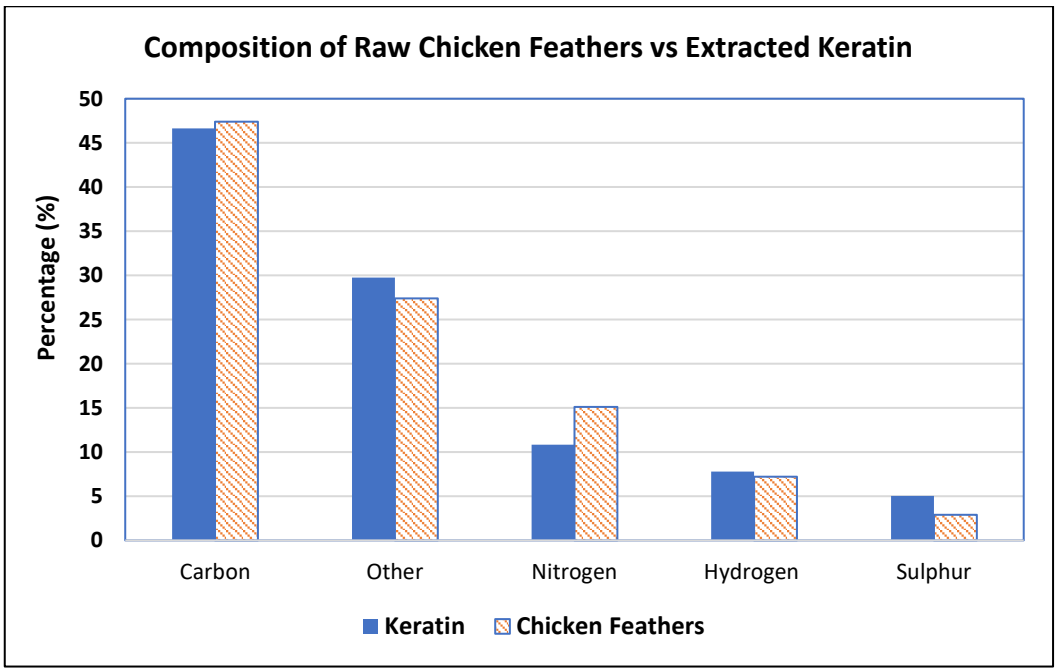

Figure 3.6: Graph of the elemental composition of extracted keratin and raw chicken feathers.

The noticeable difference in element content was that of sulphur, i.e. the average sulphur content in extracted keratin was almost double to that of raw chicken feathers. This was due to sulphite ion attached to one of the sulphur atoms from disulphide bond, i.e. one additional sulphur atom for every molecule of keratin. This significant change in sulphur content can be used to confirm the cleavage of disulphide bonds during keratin extraction. The average nitrogen content in the sample was then used to determine average crude protein in the extracted keratin by multiplying it by a nitrogen-to-protein conversion factor of 6.25. Therefore, the 10.82% nitrogen content in

the extracted keratin gave 67.63% of crude protein. The protein presence was further confirmed by Bradford assay, which showed a protein concentration of 0.021 $\mu g.ml^{-1}$.

3.6 Conclusion

This study emphasised that keratin can be extracted from wasted chicken feathers using a solution of sodium bisulphite, sodium dodecyl sulphate and urea. The application of response surface methodology (RSM) was useful in studying the effects of the four independent variables on keratin extraction from waste chicken feathers. The effect of reaction temperature was the most significant, and variable significance in descending order is reaction temperature > reaction time > concentration of $NaHSO_3$ > concentration of $NaC_{12}H_{25}SO_4$. The developed model will help in predicting the amount of keratin that can be extracted from feathers. This work will enable utilising this cheaper and environmentally friendly extraction technique to recover keratin from keratinous waste materials for their beneficiation.

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CHAPTER 4
ELECTROSPINNING OF KERATIN NANOFIBRES TUBES

Chapter overview

The demand for biodegradable polymers for biomedical applications is high due to the shortage of grafts for the injured person. Hence, polymers are utilized to make tissue scaffolds. Synthetic polymers have been favourites for this because of the modifiable properties. Their applications, though, is limited by the high level of toxicity. Hence, the research focus has turned to natural biocompatible polymers such as keratin.

Valorisation of waste chicken feathers requires conversion of this waste to high-value products that are in demand in various fields such as clinical health care sector. Research on keratin nanofibres' biomedical applications have been reported, appreciative of the excellent keratin bioproperties such as biodegradability, biocompatibility, and cell growth promotion. Previous studies of applications of keratin nanofibres mats on nerve regeneration showed some success. However, folding nanofibres mats to conduits, since tubes are gold standard nerve regeneration scaffolds, disadvantages the regeneration process.

Chapter 4 focuses on converting waste chicken feathers to high-value product, keratin nanofibres tubes through electrospinning. The fabricated biomaterial has a potential application as a nerve regeneration conduit to treat severe nerve injuries.
This chapter also presents chemical properties, composition, thermal behaviour, and morphological properties of the produced keratin nanofibres tubes as functions of keratin content.

VALORISATION OF WASTE CHICKEN FEATHERS: FABRICATION AND CHARACTERIZATION OF NOVEL KERATIN NANOFIBRES CONDUITS FOR POTENTIAL APPLICATION IN PERIPHERAL NERVE REGENERATION

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4.1 Abstract

The treatment of peripheral nerve injuries continues to be problematic for the patient. Health practitioners have to repair nerve defects or gaps while the distal and proximal stumps are under tension, complicating the process. Whereas end-to-end autografts and allografts work for nerve repair, they are accompanied by limitations such as gap-size dependency, immunological rejections, and reduced functionality of the donor/recipient tissue or organ. Alternatives to grafts include synthetic conduits. However, although they are commercially available, they are rigid, non-flexible structures with biocompatibility and biodegradability limits.

Keratin protein from chicken feathers can be used as a biopolymer for production of nerve regeneration conduits. This work aimed to fabricate flexible, porous, biocompatible and biodegradable nerve regeneration conduits from keratin protein. Keratin was extracted from waste chicken feathers, blended with polyvinyl alcohol and then transformed into nanofibres conduits using electrospinning. The nanofibres conduits had average diameters that ranged from 170 nm to 234 nm. The average diameter of nanofibres decreased when keratin content was increased. In contrast, the range of nanofibre diameter distribution narrowed, suggesting that their numbers increased as nanofibres became thin, thus reducing the interfacial spaces between them. The analysis confirmed the presence of keratin protein in nanofibres, guaranteeing biocompatibility and biodegradation. Characterization by TGA showed that keratin improved the thermal stability and hydrophilicity of the nanofibres.

Keywords: keratin/PVA nanofibre tubes, keratin nerve conduits, nerve regeneration nanofibres conduits, waste chicken feathers, keratin nanofibres.

4.2 Introduction

The peripheral nervous system (PNS) is an integral part of the nervous system that enables the body to move muscles and feel normal sensations. Its primary function is to connect the central nervous system (CNS) to tissues and organs (Dodla et al. 2019). The role of PNS is made possible by the central nervous system basic units, called neurons, which are composed of, among others, bundles of axons that form electric-cable-like peripheral nerves. Unlike CNS that is protected by the skull and vertebrae, the PNS is exposed to injuries that can either be neuropraxia, axonotmesis, or neurotmesis depending on the severity of the injury; where neuropraxia is the least severe whereas neurotmesis is the most severe nerve injury (Moskow et al. 2019). The complicated anatomy of the nerve bundle makes nerves repair difficult, leading to the most unsuccessful treatments. Peripheral nerve injuries remain the challenge for both clinical and basic research despite the advancements that have been made in this field (Zhan et al. 2013). Current repair methods include end-to-end repair, grafts and synthetic conduits. While end-to-end suturing, extending and reconnecting distal stump to proximal stump is the simplest and preferred nerve repair method, its success is limited to the gap size and the proximal nerve's availability stump. Furthermore, it is also limited to the amount of tension induced during stumps extension over a nerve gap; if it exceeds the optimal level, the repair process results in partial nerve recovery. The treatment may be done by donating a nerve from another part of the body, autograft. The autograft is advantageous in eliminating or minimising immunological rejection, and is considered the reference standard for closing the nerve gap (Sinis et al. 2011). However, the balance of functionality between the donor and recipient tissue or organ has to be established. This balance limits the treatment if there is a substantial loss of nervous tissue that may require multiple small grafts. Another form of nerve donation treatment is an allograft, whereby the nerve tissue is harvested from the donor of the same species. Even though this process increases nerve tissue availability, it also introduces the chances of disease transmissions and immunological response (Panseri et al. 2008, Dodla et al. 2019).

Therefore, synthetic nerve conduits are favoured for the nerve regeneration process due to their flexibility in mimicking the natural nervous tissue. However, the current commercially available conduits are rigid and inflexible, and may cause cell loss due to its lack of physiological properties required during nervous tissue movement (Murphy et al. 2019). The conduit must be biocompatible, biodegradable, porose, bioresorbable and mechanically strong (Tevlin et al. 2014). Biocompatible synthetic polymers suit most of the fundamental requirements for nerve conduits because of their adequate mechanical strength, ductility, and physiochemical compatibility, especially electrospun nanofibres scaffolds. Due to softness, flexibility, high porosity, high surface area to volume ratio and nanoscale diameter, nanofibres have attracted attention for their application as nerve conduits. Research has shown that nanofibres scaffolds support cellular

ingrowth due to their ability to mimic the extracellular matrix of the native tissue or organ (Yoshimoto et al. 2003, Kim and Fisher 2007). Nanofibres can be electrospun into tubular forms that are flexible and soft to allow easy suturing of distal and proximal stum during nerve repair. Furthermore, their porous nanostructure acts as a substrate for cell attachment and allows nutrients exchange (Panseri et al. 2008).

While biocompatible polymers have been used to fabricate electrospun nanofibres conduits (Agrawal and Ray 2001), there is still a need for biodegradable polymers derived from natural resources in order to increase biocompatibility and lower immunological rejection. Among other synthetic polymers, polyvinyl alcohol (PVA) has been used in biomedical applications, including nerve repair treatment, due to its physical and chemical properties. Polyvinyl alcohol is FDA approved for clinical use in humans, and it has good electrospinnability to form nontoxic nanofibres with large pore sizes. However, cell adhesion for cell growth, and biocompatibility need improvement (Wang et al. 2010, Guo et al. 2018). Consequently, keratin is to be used to blend with PVA to increase scaffold biocompatibility. Keratin is a fibrous protein that acts as an extracellular matrix and promotes biocompatibility to minimise immunological rejection. It, therefore, enhances cell adhesion and cell proliferation during biomedical applications. Guo et al. (2018) used oxidative hair keratin nanoparticles to coat PVA nanofibres for nerve repair, whereby large pore size enhanced neural cell viability and proliferation. Sierpinski et al. (2008) produced a biomedical hair-keratin gel and concluded that it promotes vigorous nerve regeneration response by activating Schwann cells. Keratin inclusion facilitates the treatment result that is comparable to the gold standard conduit, the autograft. Similar results were observed from the application of hydrogel conduit filled with hair keratin for nerve regeneration, in which results were also comparable to the gold standard for nerve repair (Pace et al. 2013). Meanwhile, chicken feathers are produced in large quantities during poultry processing. Only a small amount is used to make valuable products, and the majority of chicken feathers are disposed of as solid waste that leads to contamination of the environment. Fortunately, this waste is a good source of keratin as feathers are comprised of 90% keratin protein. Therefore, the current study aims to fabricate and characterise the seamless nanofibres conduit of polyvinyl alcohol and chicken feather keratin to apply in nerve regeneration.

4.3 Methodology and Materials

White chicken feathers were collected from chicken meat processing plant RCL Foods at Hammarsdale, South Africa. Analytical grade sodium bisulphite, sodium hydroxide, sodium dodecyl sulphate, urea and polyvinyl alcohol were purchased from Sigma-Aldrich South Africa. The fabrication of seamless nanofibres conduits was done using a Nano Spinner NE200 from Inovenso (in Durban, South Africa). The nanofibres were characterised by Fourier transform

infrared (FTIR), Thermal gravimetric Analyser, DTG-60AH model, Carbon Hydrogen Nitrogen Sulphur and Oxygen (CHNS/O) Analysis, and Scanning Electron Microscopy (SEM).

4.3.1 Pre-treatment of chicken feathers

Wet chicken feathers were rinsed with water at 60 ^{o}C to remove excess blood while manually removing other meat by-products, and then autoclaved at a temperature of 121 ^{o}C and pressure 120 kPa for 30 minutes. After that, soaked in 0.5% v/v sodium hypochlorite for 24 hours before rinsed with water and dried at 25 ^{o}C.

4.3.2 Extraction and analysis of keratin

Cleaned-disinfected chicken feathers were grounded in a milling machine to increase the dissolution rate during the extraction process. They were then soaked in 99% ethanol for 24 hours to remove fatty materials, rinsed with water before dried at 50 ^{o}C for three days. Fifteen grams of dry and degreased chicken feathers were put in a solution of 0.23 M sodium bisulphite, 0.07 M sodium dodecyl sulphate, and 1.5 M urea. The reaction mixture was shaken on a linear platform shaker for homogeneous distribution of all mixture components in a container and then heated in a 90 ^{o}C oil bath. After cooking, the mixture was centrifuged at 9000 rpm for 15 min and filtered to separate the insoluble materials and supernatants. The filtrate was dialysed in distilled water using cellulose membrane dialysis tubes (MWCO 3.5 kDa) for five days. The keratin solution in the dialysis tubes was freeze-dried to obtain keratin powder, sealed, and stored in a cold room at 4 ^{o}C.

4.3.3 Preparation for electrospinning solutions

Solutions of various ratios of 0/100 to 30/70 of keratin/polyvinyl alcohol (K/PVA), at a constant concentration of 12 wt%, were prepared by initially dissolving chicken feather keratin in deionized water, stirred at 50 ^{o}C while adding about two drops of 1 M NaOH, and then cooled to room temperature. Polyvinyl alcohol was slowly added into the solution while stirring, and then further stirred for 30 minutes. The temperature was then increased to 80 ^{o}C for 2 hours before cooling to room temperature. The solutions were then ready for electrospinning.

4.3.4 Fabrication of seamless nanofibres conduits

Each electrospinning solution was drawn into a 10 ml polyurethane (PU) syringe that was then connected by a plastic tube to a Nano-Spinner nozzle of 0.8 mm inside diameter. The electrospinning parameters were set to 30 kV, 20 cm nozzle-collector distance, 3 ml/hour feed rate and a collector rotation speed of 52 cm/s.

4.3.5 Characterization of keratin and nanofibres conduits

4.3.5.1 SDS PAGE analysis

The molecular weight of the extracted keratin was analysed using two SDS-PAGE methods for low-medium and medium-high molecular weights. Fifteen ul of 35 mg/ml sample was dissolved in a buffer solution of 5% beta-mercaptoethanol, 5 ul of molecular weight marker, before boiling the solution for 7 minutes. The sample was then loaded into 12% and 16% SDS-PAGE gels, before applying 80V-120V for 0.5 hours to 2 hours. The gels were stained, distained and imaged using GENE imaging software.

4.3.5.2 Fourier transform infrared spectroscopy (FTIR)

Functional groups of the keratin, polyvinyl alcohol and nanofibres were analysed using Fourier Transform Infrared spectroscopy (Frontier Universal model, from PerkinElmer) in an attenuated total reflection mode (ATR). Each sample was scanned four times at a wavenumber range of 550 – 4000 cm^{-1} with a resolution of 4 cm^{-1}.

4.3.5.3 Protein content

The protein content on the nanofibres was determined using a CHNS/O analyser, which determines the content of carbon, hydrogen, nitrogen and sulphur in the sample. Thereafter, the protein content is calculated by multiplying the nitrogen content by a crude protein conversion factor of 6.25 (Salo-väänänen and Koivistoinen 1996).

4.3.5.4 Scanning electron microscopy (SEM)

Scanning electron microscopy was used to determine morphological properties of the nanofibers such as shape, diameter, porosity, smoothness and beads. Samples were set up on a metal stub using a sticky carbon disc; they were then gold-coated using a sputter coater before analysis in a ZEISS LEO 1450 Scanning Electron Microscopy

4.3.5.5 Thermal Gravimetric Analysis

Thermophysical properties were investigated using Thermal Gravimetric Analyser at a heating rate of 30 ^{o}C/min from 30 to 600 ^{o}C.

4.4 Results and Discussions

4.4.1 Extraction and Characterization of keratin

White chicken feathers were obtained after pre-treatment. After that, a mixture of an aqueous solution and chicken feathers particles were recovered after 4 hours of cooking. The final

purification steps resulted in light brown supernatant and keratin powder after filtration and freeze-drying, respectively. The SDS-PAGE analysis of the extracted keratin molecular weight showed that the extracted keratin has molecular weights that range from about 3 kDa to approximately 60 kDa, whereas the broad, intense band between 10 to 15 kDa indicates that multiple monomers have a molecular weight of 10-15 kDa range. FTIR analysis confirmed the presence of polypeptide amides, namely amide A, amide B, amide I, II and III. Elemental analysis showed that the keratin had 5.02% sulphur, 46.64% carbon, 10.82% nitrogen, 7.72% hydrogen and 29.74% of other elements, including oxygen. The keratin extract was composed of 67.63% protein, as calculated from the nitrogen content in the powder.

4.4.2 Preparation and characterization of keratin/PVA nanofibres

The prepared electrospinning solutions of various ratios of keratin and PVA, from 00/100 to 30/70, were homogeneous and had a pH of 8 at 24,1 ^{o}C. The graph in Figure 4.1 shows the effect of increasing keratin content on the conductivity of the electrospinning solution.

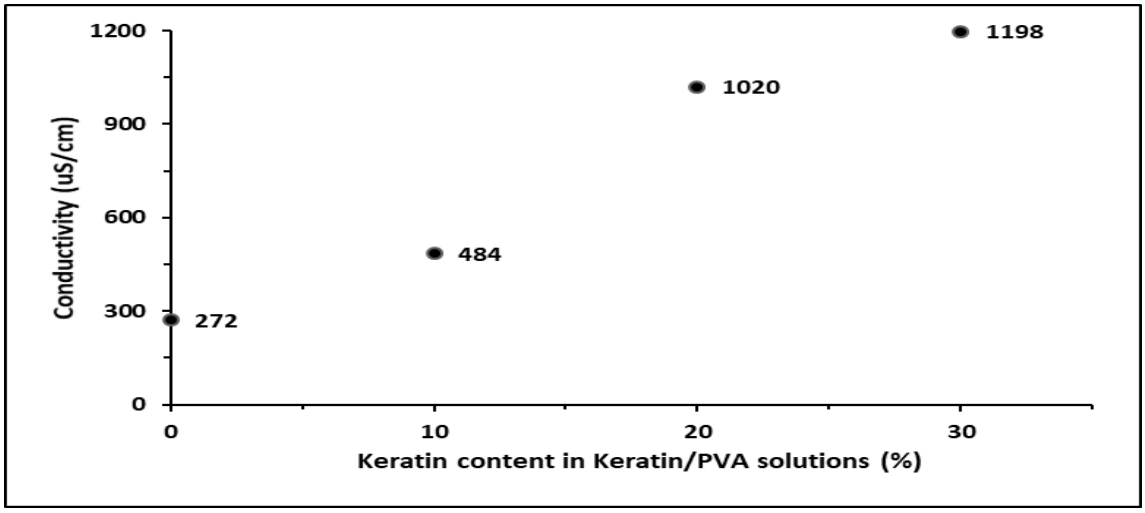

Figure 4.1: Effect of keratin content on the conductivity of keratin/PVA solutions.

The conductivity of electrospinning solutions increased with an increase in keratin content. The increase in conductivity may be due to the existence of polar amino acids in keratin. The morphology of keratin/PVA nanofibres with different keratin content from 0% to 30% is shown in Figure 4.2. All nanofibres were cylindrical and became thinner as keratin content was increased. That is, as keratin content increases, the conductivity of the spinning solution increases.

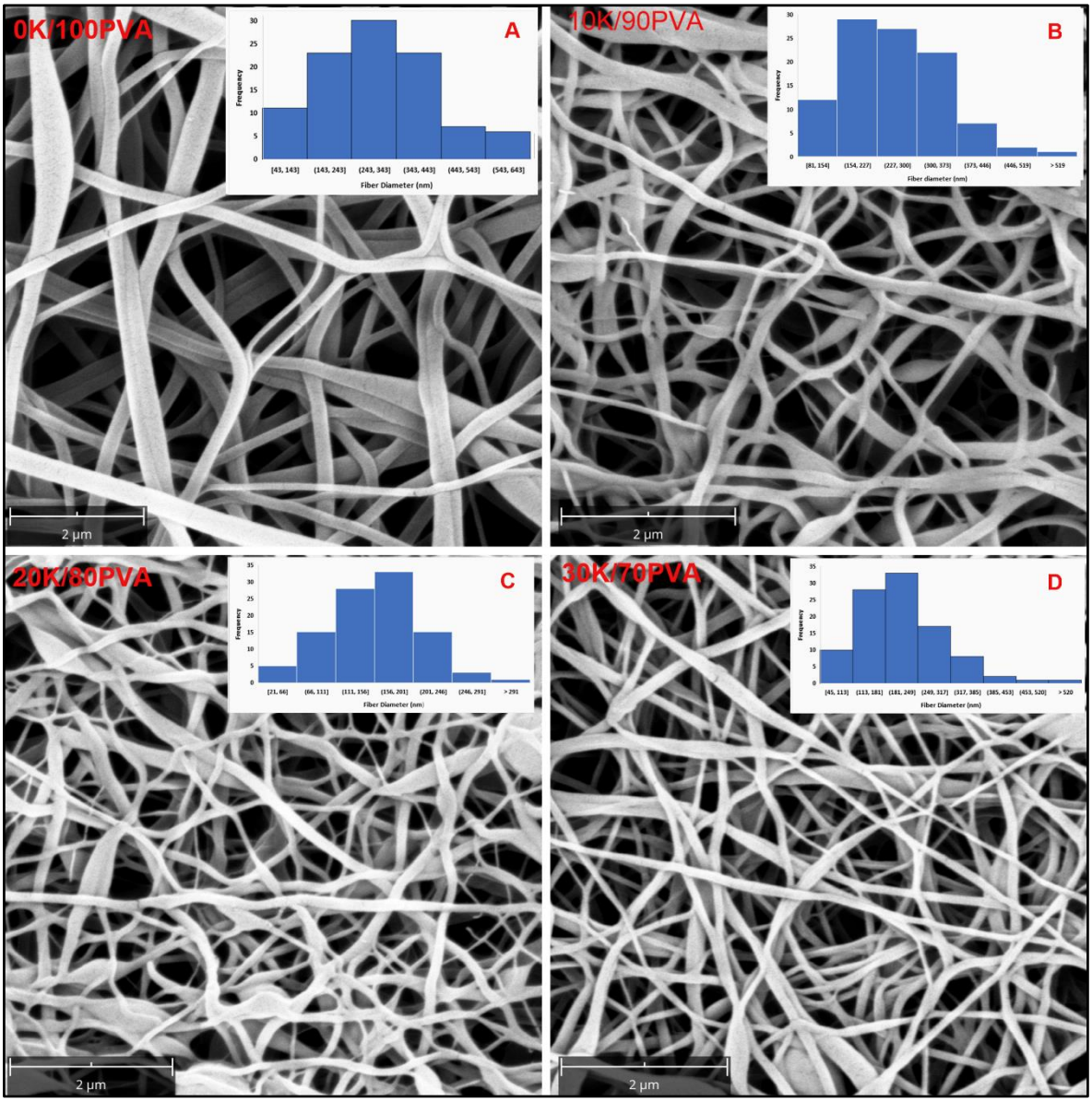

Figure 4.2: SEM images and distributions of nanofibres diameters of the keratin (k)/polyvinyl alcohol (PVA) system. A) 0% keratin/100%PVA, B) 10%K/90%PVA, C) 20%K/80%PVA and D) 30%K/70%PVA.

The increase in conductivity increases the polymer jet's electrical charge, increasing the electric field force, and resulting in thinner jets and nanofibres, that is, smaller fibre diameters. In addition to greater electric field strength, increasing conductivity decreases the solution's viscosity, resulting in smaller nanofibre's average diameters. However, there was no noticeable change on the individual nanofibres' cross-sectional shape from 100%PVA to 90%PVA nanofibres, as shown in Figure 4.3.

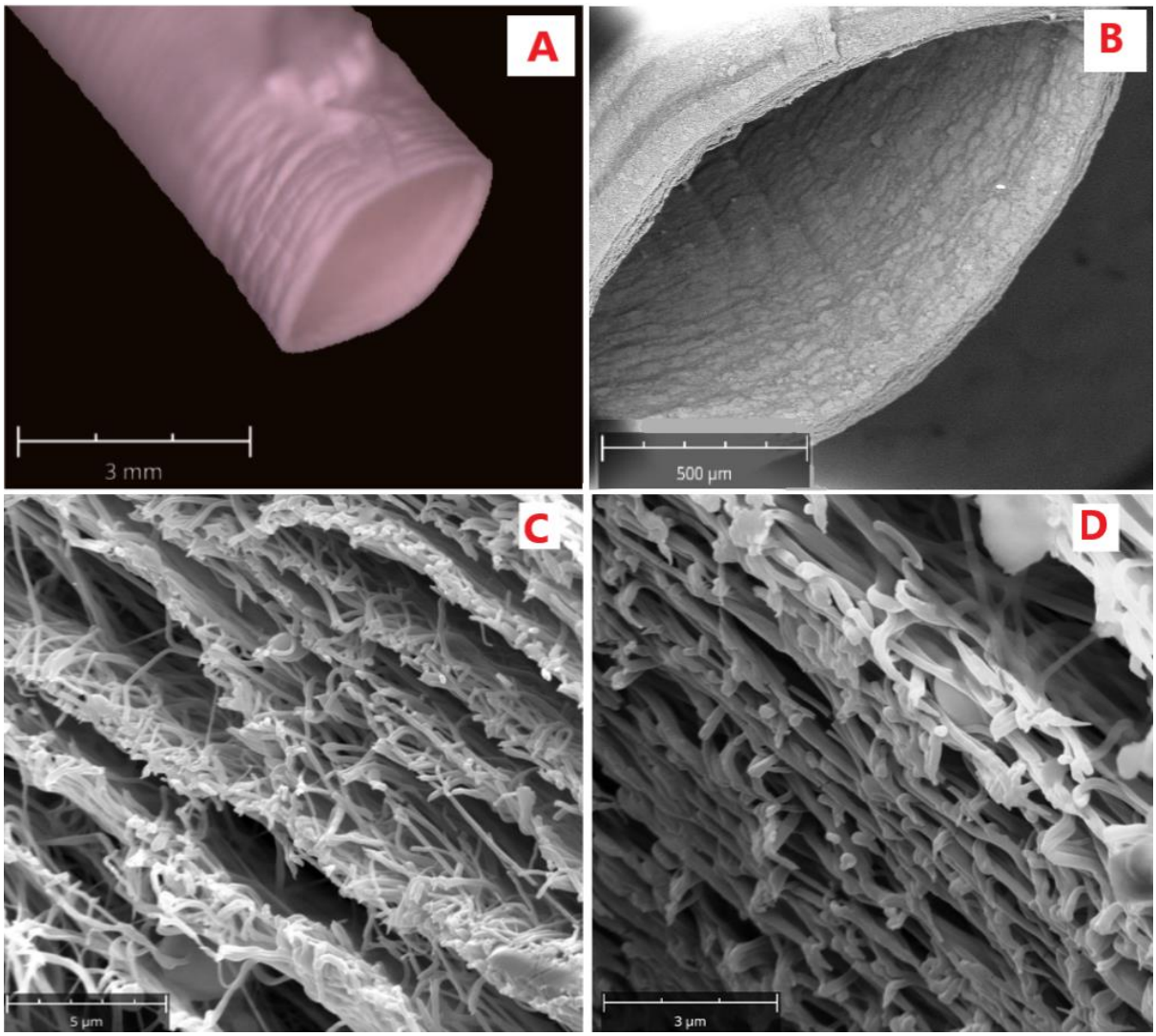

Figure 4.3: SEM images of keratin nanofibres; A) tubular structure and B) the outer and inner surfaces of the tube, C) and D) cross-sections of individual nanofibres, 0%K/100%PVA and 10%K/90%PVA nanofibres, respectively.

Figure 4.2 also shows histograms of nanofibre diameters to indicate the diameter distributions that narrow as keratin content increases. Energy dispersive x-ray (EDX) results, shown in Figure 4.4, indicate that keratin/PVA nanofibres are mainly composed of carbon, oxygen, nitrogen and Sulphur.

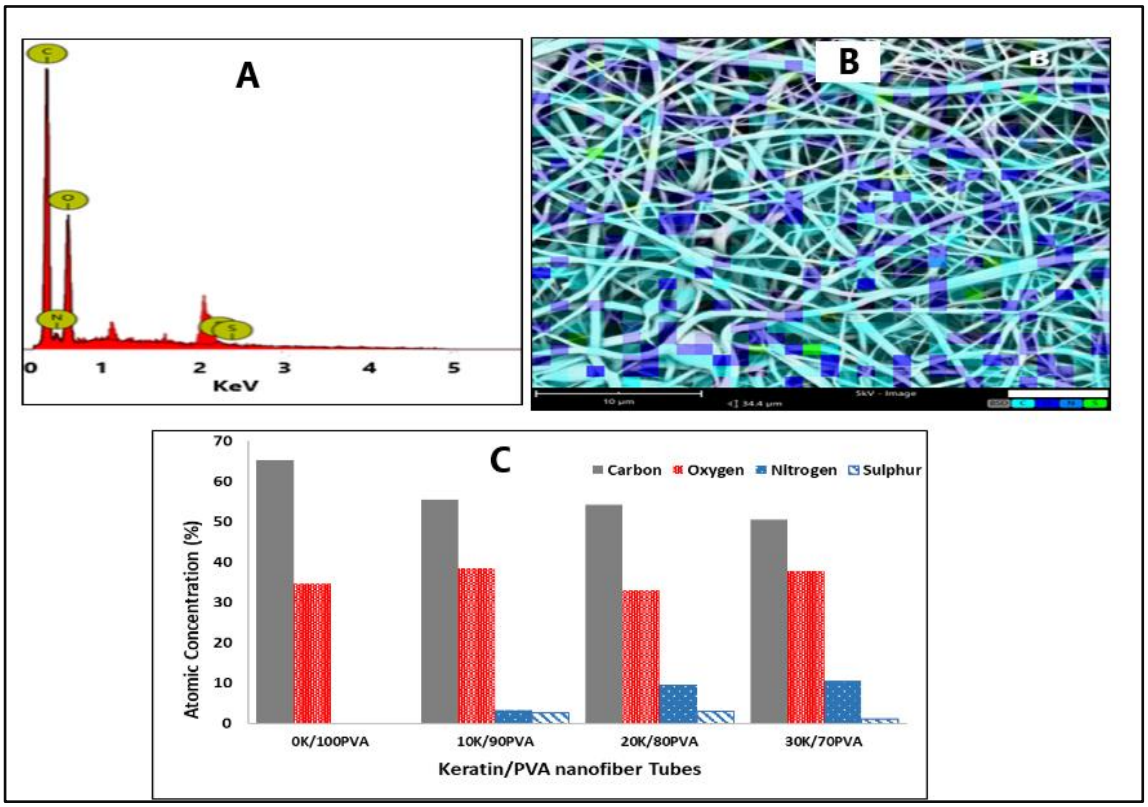

Figure 4.4: Energy-Dispersive X-ray (EDX) results of keratin/PVA nanofibres tubes; A) EDX spectrum of elements, B) elements mapping, C) atomic concentration of nanofibres.

Results of elemental mapping by EDX showed that all the atoms were evenly distributed throughout the nanofibres materials. The significant amounts of nitrogen and sulphur indicated the presence of keratin proteins. There was a noticeable increase in nitrogen percentage as keratin content increased in nanofibres. Conductivity and average diameters of nanofibres are tabulated in Table 3.1 and Figure 4.4.

Table 4.1: Conductivity of different keratin/PVA solutions and average diameters of relevant nanofibres.

Keratin/PVA	Conductivity (mS/cm)	Average Diameter ± Std. dev (nm)
0%/100%	272	234 ±87
10%/90%	484	212 ±63
20%/80%	1020	196 ±37
30%/70%	1198	170 ±50

Furthermore, SEM image analysis shows pores that are evenly distributed throughout the nanofibres tubes. The average pore sizes decreased from 0.09 to 0.07 pm, when keratin content was increased from 0% to 30%. This effect can also be attributed to the keratin conductivity; high conductivity within the polymer jet causes the jet to split into thinner jets due to the greater repulsive force, resulting in a higher number of fibres per unit area, thus, reducing pore size (He et al. 2020).

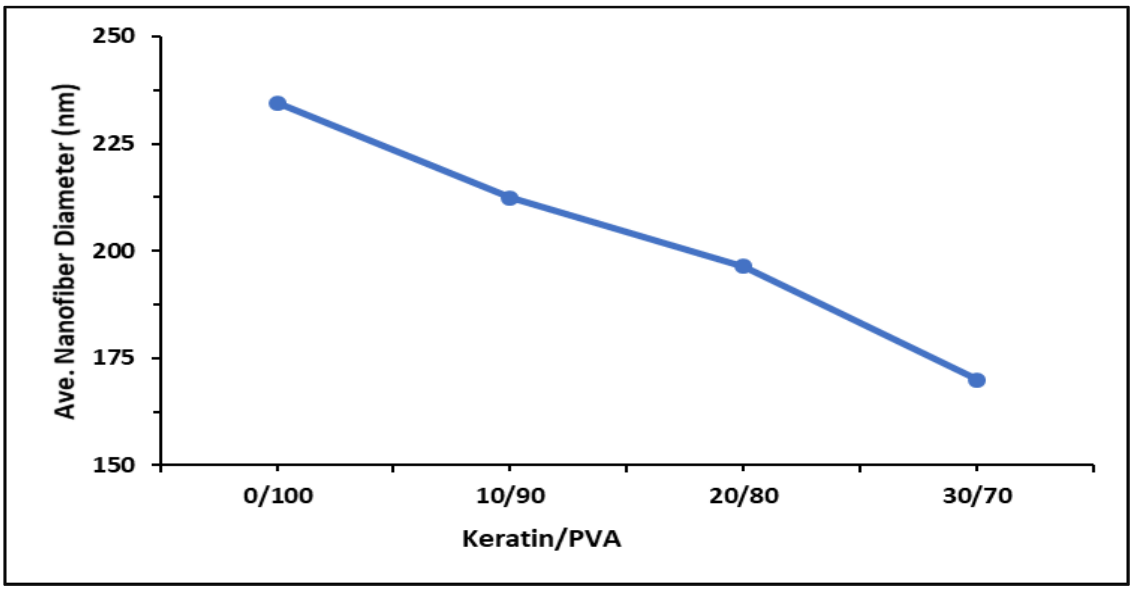

Figure 4.5: Graph of the effect of keratin content on average diameters of keratin/PVA nanofibres.

The FTIR spectra of PVA nanofibres, keratin powder and keratin/PVA nanofibres are compared in Figure 4.4. The keratin spectrum exhibited peaks indicative of different amides of keratin protein. An N-H stretching of amide A at 3282,56 cm^{-1}; a C-N bending amide Bat (2919.88 cm^{-1}; a, CO stretching of amide I at 1633.74 cm^{-1};a C-N and N-H bending amide II at1532.24 cm^{-1}; an amide III CO stretching at 1200 cm^{-1}; a C-H bending of amide IV at 623 c0m^{-1}; a S-O stretching of cysteine-sulphonate at 1212.05 cm^{-1}; and disulphide bonds at 550 cm^{-1} (Guo et al. 2018). Some of the peaks that are exhibited in the PVA spectrum includes -OH stretching peak at 3100 cm^{-1}, -CHO stretching at 2950 cm^{-1} and CO stretching at 2900 cm^{-1}. The addition of keratin into PVA increased the intensity of amide A and B peaks; this can be attributed to the interaction of -NH_2 group of keratin and -OH group of PVA, resulting in hydrogen bond formation.

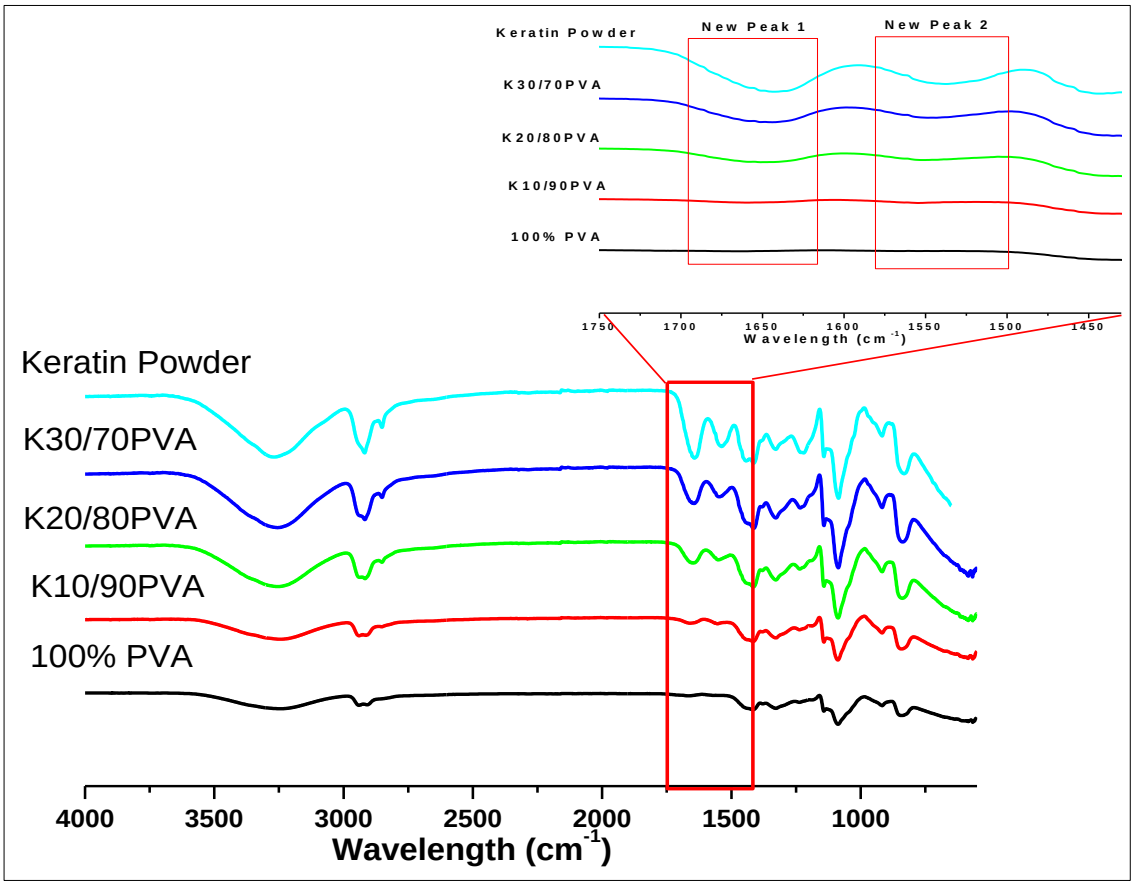

Figure 4.6: FTIR spectra of chicken feathers keratin, PVA nanofibres, keratin/PVA nanofibres and a zoomed-in sub-graph of new peaks.

The increased amount of keratin corresponds with the formation of two new peaks, in relation to PVA, at 1642.26 cm^{-1} and 1547.21 cm^{-1}. These peaks shift to shorter wavelengths as keratin content increases. The changes in the intensities of peaks, forming new ones and shifting to shorter wavelengths, confirm keratin in the nanofibres and suggest the chemical interactions between keratin and PVA. This chemical reaction between PVA and keratin functional group minimises the interaction between keratin macromolecules and increases the keratin's electrospinnability; the amide-carbonyl interaction further prevents phase separation of keratin and PVA in blended spinning solution (Cruz-Maya et al. 2019).

TGA results in Figure 4.5 show the mass-loss curves and their derivatives, reflecting the degradation of polyvinyl alcohol, keratin, and keratin-PVA nanofibres. These graphs show three major mass-loss events for nanofibres and keratin. The first event at just below 100 ^{o}C was attributed to water evaporation. The mass loss, shown in Table 4.2, resulted in a mass reduction of 2.59% for 100% PVA nanofibres, and 2.49% to 3.98% for 10% to 30% keratin nanofibres and keratin.

Table 4.2: Quantity of mass loss of keratin/PVA nanofibres at 100 °C and 400 °C.

Sample	Mass loss (%) T_{100} °C	T_{400} °C
100% PVA	2,59	80,39
10%K/ 90%PVA	2,49	70,11
20%K/ 80%PVA	3,27	71,51
30%K/ 70%PVA	3,77	70,91
Keratin Powder	3,98	68,11

The increasing trend of water mass with the increase in keratin content in PVA indicates keratin nanofibres' hydrophilicity. The second and significant mass loss occurred between 190 °C and 400 °C. This was attributed to the degradation of alpha-helix and peptide bonds of amino acid residues (He et al. 2020).

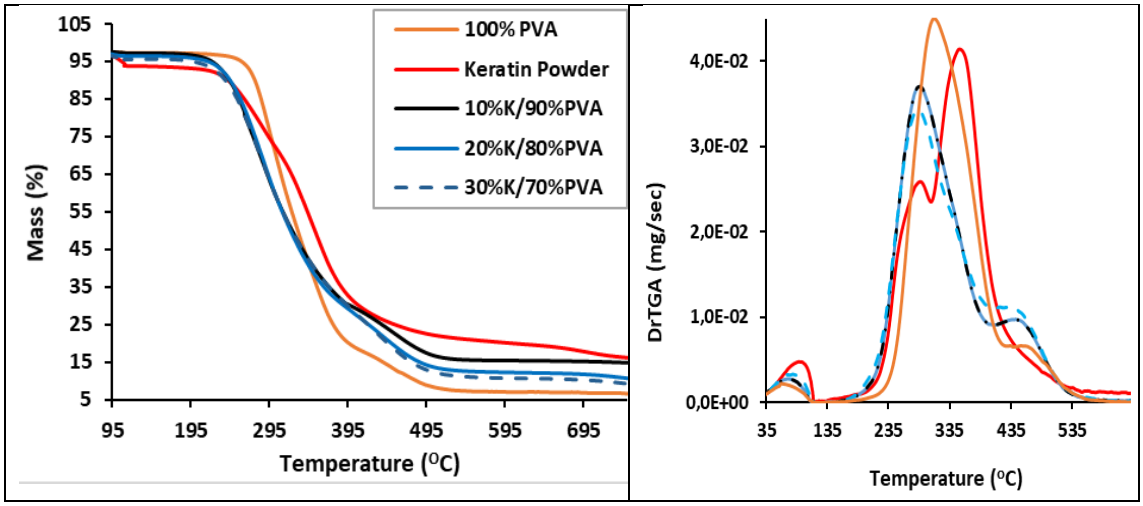

Figure 4.7: Thermogravimetric analysis and TGA derivative of keratin/PVA nanofibres.

The onset temperature of PVA nanofibres, 190 °C, was higher than that of keratin and keratin/PVA nanofibres at 160 °C. However, increasing keratin content showed no further effect on the onset temperature of keratin/PVA nanofibres. Nevertheless, PVA nanofibres and keratin showed a high degradation rate, with PVA nanofibres having the highest rate. Both PVA nanofibres and keratin exhibited a high degradation rate than their blend nanofibres. The 3rd mass loss event took place between 405 °C and 490 °C; this loss was due to the degradation of the previous mass loss event's by-products. Keratin had a high amount of residuals than PVA, indicating better stability than PVA. Ash contents of keratin/PVA nanofibres fell in between keratin and PVA while showing an increasing trend with an increase in keratin content.

4.5 Conclusions

Keratin/PVA nanofibres tubes were successfully fabricated without longitudinal seams. The addition of polyvinyl alcohol improved the electrospinnability of keratin by interrupting the interaction of keratin macromolecules and forming hydrogen bonds. On the other hand, 30% keratin advanced the thermal stability of PVA nanofibres as it reduced the nanofibers mass loss by 10% at 400 ^{o}C. Keratin nanofibre tubes have a smaller diameter than keratin free PVA nanofibres, increasing their surface area. Increasing keratin concentration in nanofibers from 10% to 30% decreased diameters of nanofibers from 234 nm to 170 nm. The decrease in diameters was accompanied by an increase in pore size from 0.07 pm to 0.09 pm. These permeable tubes can provide transportation of nutrients and metabolic waste (Xie et al. 2010) and increase cell proliferation, cell spreading and differentiation of neural stem cells while decreasing the level of cell aggregation (Christopherson et al. 2009). These keratin-based nanofibres tubes are potential nerve regeneration frames; they may enhance electrophysiological recovery and axon density (Apel et al. 2008). Hence, the future investigation on the keratin/PVA nanofibre tubes must include the in vitro and in vivo experiments.

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CHAPTER 5
SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

5.1 Summary and conclusions

This work aimed to develop a solution to produce a highly valuable product from the problematic waste chicken feathers for the South African poultry industry. There is currently a lack of value-adding processing to utilize the enormous waste of chicken feathers produced as by-products during meat processing. The industrial waste chicken feathers are disposed off in landfills, buried or incinerated, polluting groundwater, land and fresh air. Hence, value-added products from chicken feathers are in demand. Therefore, this project covered optimisation of keratin extraction from waste chicken feathers by developing a novel mathematical model for a predictable, high-efficient and environmentally friendly keratin extraction technique. The end-product of the feather valorising process developed a potentially high-value keratin material, keratin nanofibres tubes, that can be used as nerve regeneration conduits. Characterization and analysis of properties of the products, such as molecular weight, chemical functional groups, protein content, thermal behavior, and elemental composition, were performed. The results illustrated that a high quality keratin product was obtained from waste chicken feathers.

The 3rd chapter of this thesis, optimisation of keratin extraction, showed that the combination of sodium bisulphite, sodium dodecyl sulphate, and urea exposed to 90 ^{o}C for 4 hours is an effective extraction method of keratin. Extraction medium temperature was the most significant factor in the yield on keratin, followed by reaction time and sodium bisulfite concentration. In contrast, the concentration of SDS had the least significance on the keratin yield. Under these extraction conditions, the developed statistical model gives the 70% keratin yield. The molecular weight of 10 kDa and above indicates that the extraction process preserved most of the keratin protein. The well-defined extraction process can help the South African poultry industry produce this high-value protein and receive lucrative income instead of wasting money on feathers waste disposal activities. The availability of characterization results allows in-depth understanding and enables informed decisions on possible valorisation measures of waste chicken feathers by benefiting the extracted keratin.

The keratin was then studied for possible applications in the biomedical field, specifically for nerve regeneration purposes. Severe nerve injuries include breakage of the tubular axon; hence, the ideal nerve regeneration conduits are in tubular form. Moreover, nanofibres conduits have attracted attention due to their unique biological friendly properties. However, there has been no reported production of keratin nanofibres. Thus this work has described successful fabrication

and characterisation of the novel keratin nanofibres tubes from chicken feathers for potential application as nerve regeneration conduits. 100% keratin nanofibers conduits could not be achieved; increasing keratin content from 0% to 30% reduced the diameter of nanofibers, increased their surface area and reduced their pore size. Also, addition of keratin improved thermal stability of the conduits. However, the keratin addition limit was 30% keratin beyond which there is no improvement in stability of the nanofibers. Unlike other nanofibres tubes electro-sprayed with keratin particles, the keratin nanofibres tubes have keratin incorporated on the nanofibres at the electrospinning process. This method offered an even distribution of keratin protein throughout the nanofibres, as proven by the analytical data.

5.2 Recommendations for future work

The successful fabrication of keratin nanofibres seamless tubes suggests a potential tube application in the nerve regeneration process. Hence, the author recommends the following studies to enable keratin nanofibres tubes utilization as nerve conduits:

- Optimisation of fabrication of the keratin nanofibres conduits.
    - Investigation of the effect of process parameters of polymer solution preparation such as type of solvent, blending method for keratin and supporting polymer, e.g. PVA, concentration, blending and dissolving temperature and dissolving time on the electrospinning process parameters and throughput.
    - The optimisation of keratin nanofibres properties such as diameter, porosity, wettability, and mechanical strength according to their effects on nerve repair biological activities.
- The in vitro and in vivo studies of the tubes on nerve regeneration to determine the degree of effectiveness.
- Effect of cellulose nanocrystal on physiochemical and mechanical properties of keratin nanofibres conduits.
- Techno-economic studies of keratin extraction and nanofibres production in order to evaluate the commercialisation of the developed technologies.
- Analysis of the environmental impact of keratin nanofibres from production to service to evaluate the circular economy concept.

This work can also be extended to other sectors such as automotive, electronics and water treatment processes.

APPENDICES

A. Published journals articles

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Research article

Valorisation of waste chicken feathers: Optimisation of keratin extraction from waste chicken feathers by sodium bisulphite, sodium dodecyl sulphate and urea

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ABSTRACT

Extraction of keratin from keratinous waste materials, such as chicken feathers, has been identified as the favourable approach in beneficiation of this biomass. The chemical extractions of keratin by reducing agents are usually preferred because the process is much faster than its counterpart, oxidation extraction. One such reduction extraction is the use of a mixture of sodium bisulphite, sodium dodecyl sulphate and urea. There are at least five factors that may affect the keratin extraction process and its final properties when using this extraction. Even though this extraction method is often used, the effects of its independent variables have not been studied; as a result, the effects of independent variables cannot be fully linked to the extraction process and final keratin properties. Therefore, this study aimed to optimise the extraction of keratin from waste chicken feathers using sodium bisulphite, sodium dodecyl sulphate and urea. The optimisation was statistically performed using Response Surface Methodology (RSM) linked with Box-Behnken Design. After screening the independent variable using one factor at a time method, the concentration of sodium bisulphite, concentration of sodium dodecyl sulphate, reaction temperature and reaction time were chosen for the study. Twenty-nine experiments were statistically designed and executed, and their results were used to analyse the effects of all the independent variables in order to optimise the extraction process. The reaction temperature was found to be the most significant factor, while the concentration of sodium dodecyl sulphate was the most insignificant factor of this extraction process. Independent variables significance order was reaction temperature > reaction time > concentration of $NaHSO_3$ > concentration of $NaC_{12}H_{25}SO_4$. The designed reduced cubic model was significant and was used to predict the protein yield from the keratin extraction using sodium bisulphite.

1. Introduction

Extraction of keratin from chicken feathers is an important aspect when valorising the abundant waste chicken feathers from the poultry industry. This is because the industry produces enormous amount waste chicken feathers during meat production, of which insignificant amount is used to make low-value products such feather meal for livestock and fertilisers (Tesfaye et al., 2017a, b). The larger portion of feathers is considered as waste and is either disposed of in landfill sites or incinerated. Both disposal techniques, landfills and incineration, are environmentally unfriendly due to slow decomposition of feathers in landfills and air pollution from the latter. The solid waste chicken feathers require a large area for landfill and contaminate groundwater. The chemical composition of a feather shows that it contains 91% of β-keratin protein, a highly valuable protein, therefore, it is beneficial to extract this protein for the applications in the fields such health care and cosmetic industries (Swetlana and Jain, 2010). It has been reported that materials such as biofilms, nanofibres, hydrogels, sponges, membranes and cosmetics can be produced from keratin (Karthikeyan et al., 2007; Kumaran et al., 2016).

Extraction of keratin protein from chicken feathers using reducing agents is often preferred because it is generally faster than the extraction of keratin from feathers using oxidising agents (Holkar et al., 2017). Even though the extraction of keratin by 2-mercaptoethanol produces the highest yield of keratin, its price and toxicity cause reluctance on its application (Holkar et al., 2017; Sinkiewicz et al., 2017). Hence, the

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extraction of keratin using sodium bisulphite is an excellent alternative technique due to its simplicity, effectiveness, and economically and environmentally friendliness. During the keratin extraction process, sodium bisulphite ($NaHSO_3$) cleaves and further prevents the formation of disulphide bonds (Holkar et al., 2017). Moreover, this extraction technique includes the addition of urea as a denaturing agent, and a surfactant, sodium dodecyl sulphate (SDS); urea increases the solubility of keratin in water and facilitates the effect of reducing agent on the polypeptide chain (Katoh et al., 2004; Xu et al., 2014). Whilst sodium dodecyl sulphate prevents the aggregation of polypeptide chains by blocking the formation of new cross-linkages, thus, increasing extraction rate and improves the stability of the extracted keratin (Schrooyen et al., 2001; Tonin et al., 2007).

Despite the economic and environmental friendliness and smooth execution, this extraction technique has not been optimised, i.e., the concentration of sodium bisulphate, sodium dodecyl sulphate and urea, as well as temperature and the number of hours to run this extraction vary from one researcher to another. Sinkiewicz et al. (2017) extracted keratin from chicken feathers using this mixture of chemicals and obtained 82% of keratin, while Tonin et al. (2007) and Kamarudin et al. (2017) used the same extraction technique under different conditions and obtained the maximum yields of 33% and 18,3% respectively. This means the yield, physical and chemical properties of the extracted keratin cannot be easily predicted. For instance, the molecular weight and molecular weight distribution of keratin are essential properties for keratin applications; severe extraction conditions such as very high pH/low pH at prolonged exposure to high-temperature breaks both disulphide bonds and peptide bonds producing low-molecular-weight keratins which are mostly used to make animal feeds, with limitations to biomedical applications (Jones, 1975; Smith et al., 1994). Whereas extraction of keratin under mild conditions breaks disulphide bonds of proteins fibres without significant cleavage of polypeptide bonds, thus resulting to an unchanged micromolecular structure of keratin, this yields high-molecular-weight keratin that can be used for biomedical applications (Eslahi et al., 2013). Thus, the current work aims to optimise the process of extracting keratin from waste chicken feathers using sodium bisulphite, sodium dodecyl sulphate and urea to obtain experimental conditions that result in a maximum yield of keratin.

2. Methods and materials

White chicken feathers were collected from chicken meat processing plant RCL Foods at Hammarsdale, South Africa. Sodium bisulphite, sodium hydroxide, sodium dodecyl sulphate and urea were purchased from Sigma-Aldrich South Africa. The main instruments for the characterisation of the keratin were Fourier transform infrared spectroscopy (FTIR) and a Carbon Hydrogen Nitrogen Sulphur and Oxygen (CHNS/O) Analyser.

2.1. Pre-treatment of chicken feathers

Wet chicken feathers were rinsed with water, at 60 °C, to remove excess blood while manually removing other meat by-products. Feathers were then autoclaved at a temperature of 121 °C and pressure 120 kPa for 0.5 h. Furthermore, they were soaked for 24 h in 0.5% v/v sodium hypochlorite to disinfect and remove stains, afterwards, rinsed with water and dried at 25 °C.

2.2. Extraction procedure

Cleaned-disinfected chicken feathers were comminuted in a milling machine to increase the surface area of the feathers, thus enhancing their dissolution rate during the extraction process. The milled feathers were soaked in 99% ethanol for 24 h to remove fatty materials, rinsed with water, filtered and then dried at 50 °C for three days. Dry cleaned and degreased 15 g of chicken feathers were immersed in various concentrations of sodium bisulphite, sodium dodecyl sulphate and 1.5 M urea as shown in Tables 1 and 2, of sodium bisulphite and sodium dodecyl sulphate. The reaction mixtures were mechanically shaken on a desk top shaker for homogeneous distribution of all mixture components in a container and then heated in a heated oil bath. After heating and then cooling, the mixtures were centrifuged at 9000 rpm for 15 min and thereafter filtered to separate the insoluble materials and supernatants. The filtrates obtained were dialysed in distilled water using cellulose membrane dialysis tubes (MWCO 14 kDa) for five days. The retained keratin solutions were freeze-dried to obtain keratin powder and then stored in a cold room at 4 °C.

Table 1
Independent variables and levels as per Box-Behnken Design.

Variable (units)	Variable symbol	Low level (-1)	Middle level (0)	High level (+ 1)
$NaHSO_3$ (M)	A	0.2	0.3	0.4
SDS (M)	B	0	0.035	0.07
Temperature (°C)	C	50	70	90
Time (h)	D	2	3	4

Table 2
Box-Behnken design matrix of uncoded values along with experimental protein yield (%).

Run	$NaHSO_3$ (M)	$NaC_{12}H_{25}SO_4$ (M)	Temp (°C)	Time (h)	Protein (%)
1	0,3	0	50	3	0,17
2	0,3	0,07	50	3	7,01
3	0,3	0,035	50	2	4,34
4	0,3	0,035	50	4	6,89
5	0,2	0,035	50	3	5,60
6	0,4	0,035	50	3	9,17
7	0,3	0,07	70	4	51,38
8	0,2	0,07	70	3	33,80
9	0,3	0,035	70	3	26,21
10	0,3	0,035	70	3	30,65
11	0,3	0	70	4	11,51
12	0,3	0,035	70	3	29,60
13	0,4	0,035	70	4	3,95
14	0,4	0	70	3	7,74
15	0,2	0,035	70	4	18,99
16	0,2	0	70	3	6,08
17	0,3	0,035	70	3	16,84
18	0,4	0,07	70	3	38,45
19	0,2	0,035	70	2	12,69
20	0,3	0,07	70	2	32,98
21	0,3	0,035	70	3	5,08
22	0,4	0,035	70	2	1,81
23	0,3	0	70	2	12,86
24	0,3	0,035	90	4	43,41
25	0,3	0,07	90	3	17,63
26	0,2	0,035	90	3	20,01
27	0,3	0,035	90	2	9,66
28	0,3	0	90	3	9,72
29	0,4	0,035	90	3	14,10

2.3. Experimental design and statistical analysis

On single factor analysis, independent variables, viz, concentration of sodium bisulphite ($NaHSO_3$), concentration of sodium dodecyl sulphate (SDS), extraction temperature (T) and extraction time (t), and their ranges were selected for the investigation of their effects on the response variable, the protein yield (%). A three-level (−1, 0, +1) Box-Behnken design (BBD) joint with response surface methodology (RSM) was employed to design the experiments of the project. Table 1 shows the independent variables and their three levels, low, middle and high level as per the selected ranges.

BBD, according to the number of experiments (N) equation (eq).1, developed twenty-five experiments and four replicates as per the

Table 3
Analysis Of Variance (ANOVA) of the reduced cubic model for the optimisation of extraction.

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	19,72	2,47	2,84	0,0277	Significant
A-$NaHSO_3$	1,63	1,63	1,88	0,9810	
C-Temperature	7,27	7,27	8,39	0,0089	
D-Time	0,0204	0,0204	0,0236	0,8795	
AC	4,51	4,51	5,20	0,0337	
AD	0,5677	0,5677	0,6546	0,4280	
C^2	2,47	2,47	2,85	0,1068	
D^2	0,8418	0,8418	0,9707	0,3363	
AD^2	3,52	3,52	4,05	0,0577	

parameters defined in Table 1.

$$N = 2k(k-1) + C_0 \quad (1)$$

where k is the number of factors, and C_0 is the number of central points. Designed experiments were carried out according to Table 2. The experimental data was statistically analysed and fitted into a second-order polynomial equation, eq. (2).

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i Y_j \quad (2)$$

where *Y* is the predicted response, *Xi* and *Xj* are the independent variables, β_o is the offset term, β_i is the *i*th linear coefficient, β_{ii} is the *i*th quadratic coefficient, and β_{ij} is the *ij*th interaction coefficient. The variables possess a statistically significant effect on the response, protein yield, were analysed through analysis of variance (ANOVA). The analysis included the effect of variables and their interactions on protein yield.

2.4. Characterisations of feather keratin extract

2.4.1. Fourier transform infrared spectroscopy (FTIR)

Functional groups of the extracted keratin were analysed using Fourier Transform Infrared spectroscopy (Frontier Universal model, from PerkinElmer), in an attenuated total reflection mode (ATR). Each spectrum obtained was an average of 4 scans at wavenumber range of 550–4000 cm^{-1} with a resolution of 4 cm^{-1}.

2.4.2. CHNS analysis for crude protein content in extracted keratin (done)

Elemental composition of extracted keratin was performed, where carbon, hydrogen, nitrogen and sulphur were analysed using a CHNS/O analyser. This was mainly to determine the crude protein content, which is calculated from nitrogen content in a sample by multiplying it by a crude protein conversion factor of 6.25.

2.4.3. Bradford assay for protein concertation

To further analyse the protein content in the extracted sample, a Bradford assay was performed using a spectrophotometer (Promega GloMax Microplate Multimode Reader). Bovine serum albumin (BSA) was used as the standard to generate a calibration curve from absorption measurements at 595 nm. Keratin solutions were prepared by dissolving 35 mg into 1 ml of deionised water prior to the absorption measurements.

2.4.4. SDS PAGE analysis for molecular weight determination

The molecular weight of extracted keratin was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Keratin samples 35 mg were dissolved in 1 ml distilled water and boiled for 5 min. Subsequently, 15 μL of 35 mg keratin sample was added into a solution of 5 μL of NuPAGE LDS sample buffer (4X) containing 5% β-mercaptoethanol, and boiled for 7 min. The denatured samples were then loaded onto 16% and 12% polyacrylamide gels for low and mid-high molecular weight determination, respectively. The gels were subjected to 80 V for 30 min, followed by 120 V for 2 h. The 12% gel was then stained, for 30 min, with Coomassie Brilliant Blue (CBB) G-250 followed by an overnight destaining with an ethanol-acetic acid solution, while the 16% gel was first fixed with 5% glutaraldehyde for 25 min before a 20 min staining with CBB G-250. The 16% polyacrylamide gel was destained with 10% acetic acid solution for 20 min. GENE imaging software was used to take images of both gels for analysis.

3. Results and discussions

3.1. Effect of pre-treatment of waste chicken feathers

The originally golden-brown waste chicken feathers showed significant improvement of whiteness index and a decrease in yellowness due to bleaching ability of sodium hypochlorite. Moreover, the treatment of feathers with ethanol further increased their whiteness index owing to the recovered golden-yellow oil which was also responsible for low whiteness index. This effect of pre-treatment on waste chicken feathers was inline with the findings of Tesfaye et al. (2018) which showed the inverse relationship between whiteness index and yellowness index.

3.2. Extraction of keratin from waste chicken feathers

Cooking feathers resulted in a heterogeneous mixture of solution and chicken feather particles of different sizes. The variation of sizes of feather particles was due to different combinations of the concentration of sodium bisulphite, concentration of sodium dodecyl sulphate, reaction temperature and time. The hydrolysate formed was due to the sulphitolysis of disulphide bonds between keratin molecules by the reducing agent, sodium bisulphite, according to eq. (3) (Yin et al., 2013; Pilehvar et al., 2019).

$$Keratin - S - S - Keratin + SO_3^{2-} \rightarrow Keratin - S^- + Keratin - S - S_3^{2-} \quad (3)$$

Light brown supernatant followed by light brown glittering powder was obtained after purification and freeze-drying, respectively.

3.3. Statistical analysis of the keratin extraction from feathers

Twenty-nine experimental runs were conducted, and the results are tabulated in Table 2. Independent variables and dependent variable allowed the use of statistical data processing to determine the dependency of the response on the independent variable. Table 3 showed the analysis of variance (ANOVA) based on the statistically processed data.

The table showed that the reduced cubic model of the experiment had a p-value of 0.0277 (less than 0.05) which indicated that the model was significant. The model also had an F-value of 2.84 which implied that there was only 2.77% chance that an F-value this large could be due to noise; hence, this F-value emphasised the model significance. The adequate precision measure was 8.234 (greater than 4), meaning the model had a strong enough signal to be used for optimisation. The reaction temperature and the product of the concentration of sodium bisulphite and temperature were significantly affecting the response since they had p-values of 0.0089 and 0.0337, respectively. In addition,

Table 4
Coefficients estimates for the model of optimisation.

Factor	Coefficient Estimate	Standard Error	VIF
Intercept	2.51	0.2808	–
A-$NaHSO_3$	−0.4520	0.3292	1.50
C-Temperature	0.7785	0.2688	1.0000
D-Time	0.0413	0.2688	1.0000
AC	1.06	0.4656	1.0000
AD	−0.3767	0.4656	1.0000
C^2	−0.5987	0.3545	1.02
D^2	0.3492	0.3545	1.02
AD^2	1.15	0.5703	1.50

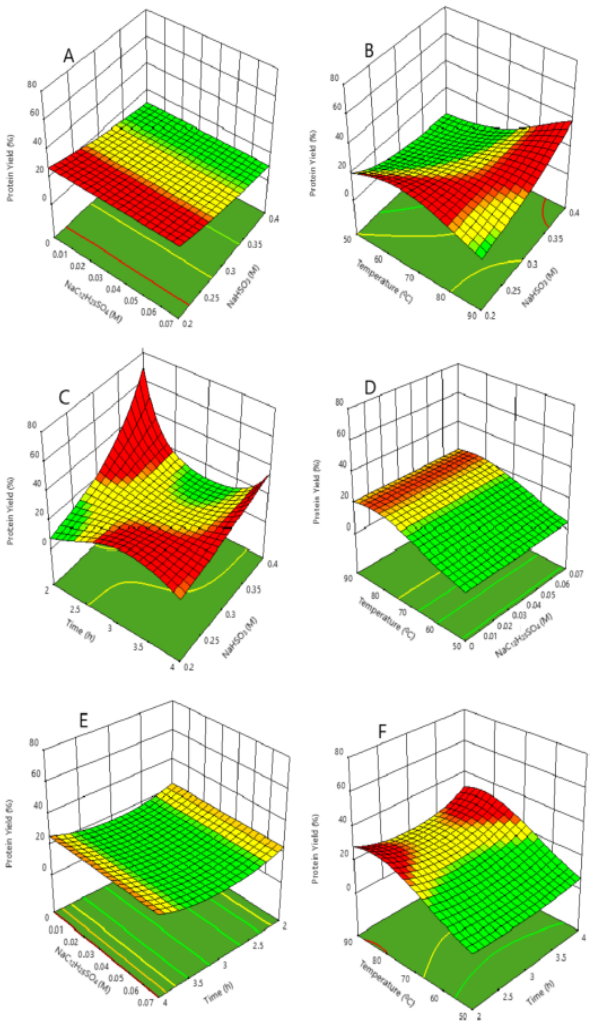

Fig. 1. Three dimensional graphs are showing the effects of temperature, time, the concentration of $NaC_{12}H_{25}SO_4$ and $NaHSO_3$(M) on the protein yield.

the ANOVA table showed that the concentration of sodium bisulphite was marginally significant, whereas reaction time and the concentration of sodium dodecyl sulphate were insignificant.

The coefficients table, Table 4, contained the coefficients estimates that represented the expected change in response per each unit change of each independent variable when others are unchanged, whereas, each coefficient magnitude was directly proportional to its influence on the response in relation to other coefficients, i.e. the factor with bigger coefficient is more significant than others and has the least p-value. Table 4 also showed small and similar standard errors as this must be the case for a balanced design. The Variation Inflation Factors (VIF) for each coefficient was within 1–1.5 range, as VIF value of 1 is an ideal value for a measure of orthogonality of a factor in an orthogonal design while a value of 10 is an acceptable upper limit. The coefficient estimates were used to formulate the model equation, equation (4).

$$\ln(\text{Protein Recovered }\%) = +2{,}51 - 0{,}4520A + 0{,}7785C + 0{,}0413D + 1{,}06AC - 0{,}376AD - 0{,}5987C^2 + 0{,}3492D^2 + 1{,}15AD^2 \quad (4)$$

where A, C, D is sodium bisulphite, temperature and time, respectively. The effect of the concentration of sodium sulphite, concentration of sodium dodecyl sulphate, reaction temperature and reaction time on the recovered protein are shown on the 3D-graphs in Fig. 1.

The graphs A, B, C and D in Fig. 1 represent the effects of each independent variable and their interactions on the protein yield, where graph A shows the effect of the concentrations of sodium bisulphite and sodium dodecyl sulphate on amount of the extracted keratin. Graph B represents the effect of sodium bisulphite concentration and reaction temperature on the response, graph C shows the effect of sodium bisulphite concentration and reaction time, and D is the effect of the concentration of sodium dodecyl sulphate and reaction temperature on keratin yield. Furthermore, E and F are the effects of sodium dodecyl sulphate and reaction time, and temperature-time effect on the yield, respectively. Graphs A, D and E show that protein yield had no dependency on the variation of the concentration of sodium dodecyl sulphate, this is inline with ANOVA and coefficients estimate tables which excluded it from the significance descriptions due to the lack of it. The effects of other independent variables were interaction dependent, for example, the reaction temperature may be observed as being directly proportional to the protein yield (on graph C), however, its effect highly depends on other variables (graph B and F).

3.4. Characterisation of the extracted keratin

3.4.1. Structural analysis of the extract by FTIR

The analysis of extracted keratin and raw chicken feathers by FTIR showed absorbance peaks that corresponded to multiple amide groups. The spectra of these analyses are contained in Fig. 2. Literature shows that the presence and positions of amides, from amide I, are used to confirm the presence of the protein material (Dabalos et al., 2019).

The peak at 3280.79 mc^{-1} represents amide A while peak 2919.88 mc^{-1} is amide B, 1633.74 mc^{-1} amide I, 1532.24 mc^{-1} amide II, 1212.05 mc^{-1} amide III, 623 mc^{-1} amide IV, 1000 mc^{-1} thiosulphate ion and 550 mc^{-1} is for disulphide bonds of keratins (Kakkar et al., 2014). Peaks positions of the extracted keratin show no significant shift as compare to raw feathers which indicate the conservation of the secondary structure of the protein during the extraction process (Cardamone, 2010). Furthermore, the presence of thiosulphate ion was due to the attachment of sulphite ion to one of the sulphur atoms of the disulphide bond, which also indicate the breakage of this bond during the extraction process. However, the peak at 550 mc^{-1} for extracted keratin suggests that there are still traces of unbroken disulphide bonds after the keratin extraction process.

3.4.2. Structural analysis of the extracted keratin using MNR

The ^{13}C NMR spectra of the extracted keratin, in Fig. 3, showed peaks at 25 ppm, which correspond to alkyl side chains. The cleavage of the disulphide bonds may reduce the β-carbon signals from 40 ppm to 25–29 ppm to give thiol signals, hence, these alkyl group peaks overlapped with cystine groups signals that show up between 25 ppm and 29 ppm.

Beta carbon peaks present in leucine and cross-linked cysteine residues were also observed at 40 ppm, while disulphide bonds signals may be represented at the same 40 ppm (Baias et al., 2009). Alpha carbon signals were observed at 60 ppm. There were peaks at 130 ppm, indicating the presence of aromatic carbon, confirming the presence of the amino acids in the extracted keratin. Low carbonyl signal at 170 ppm represented the amide carbonyl of the keratin protein (Idris et al., 2014).

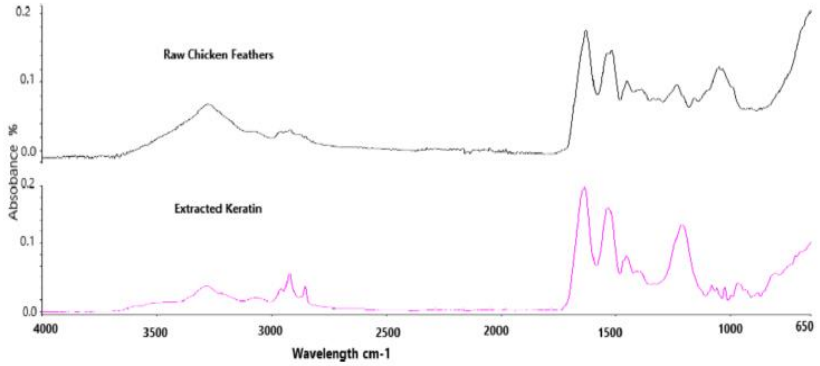

Fig. 2. FTIR spectra of extracted keratin, raw feathers and keratin standard.

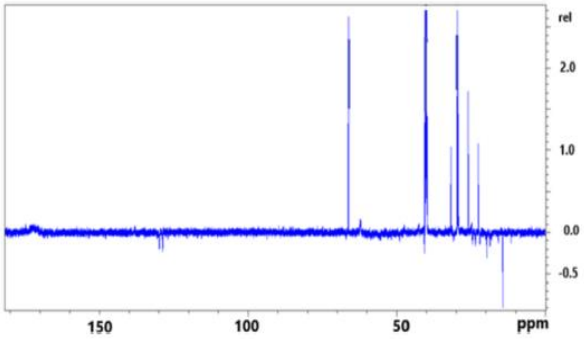

Fig. 3. The ^{13}C NMR spectrum of keratin extracted from chicken feathers.

3.4.3. Analysis of the molecular weight of the extract using SDS-PAGE

The analysis of the molecular weight of the extracted keratin was analysed using DSD PAGE technique, the resulting gels A and B are shown in Fig. 4.

Gel A was a glycine gel of which its bands were analysed in reference to medium to high molecular weight marker that can determine molecular weight from 10 to 180 kDa. Whereas, gel B was a tricine gel that was used to analysed low to medium molecular weights of the sample using a marker with a range of 1.7–40 kDa. The Coomassie Brilliant Blue (staining agent) appeared all over lane in each gel, with weak bands at about 20–25 kDa, 30–40 kDa and 55 kDa, meaning that the extracted keratin consisted of multiple monomers that have low to high molecular weights. These molecular weights were in line with Kamarudin et al. (2017) results that showed keratin proteins with low molecular weight (3 kDa) and high molecular weight (62 kDa). Therefore, molecular weights of extracted keratins depend mainly on the extraction conditions.

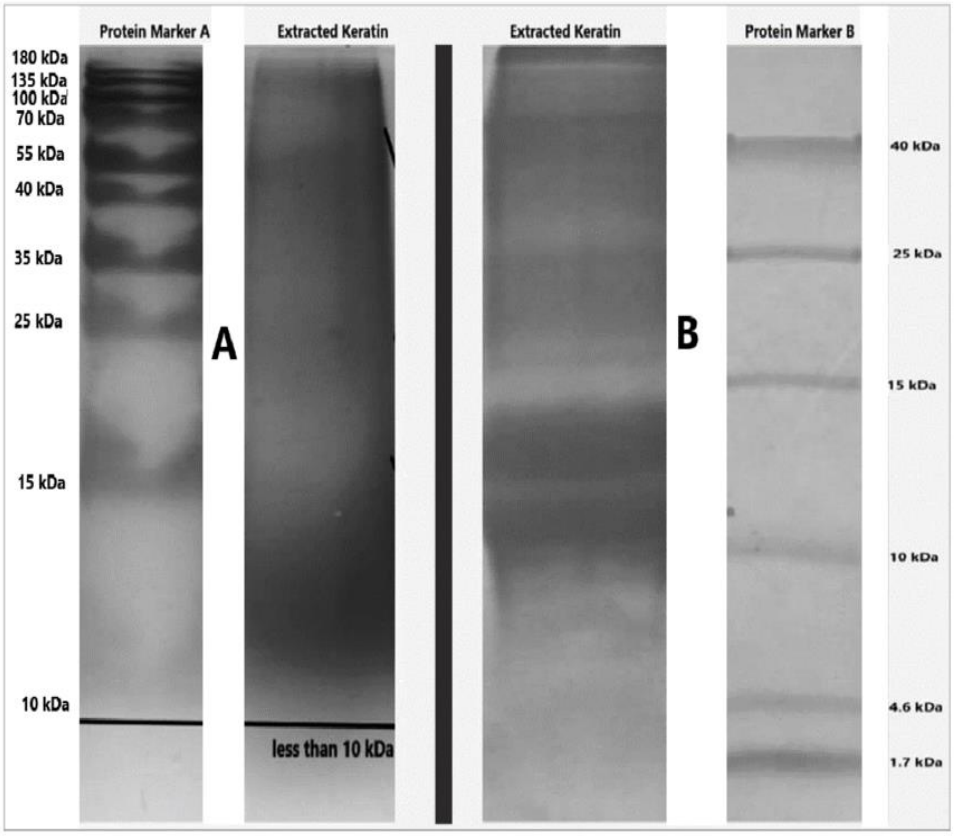

Fig. 4. SDS-PAGE gels of extracted keratin protein, glycine gel (A), tricine gel (B).

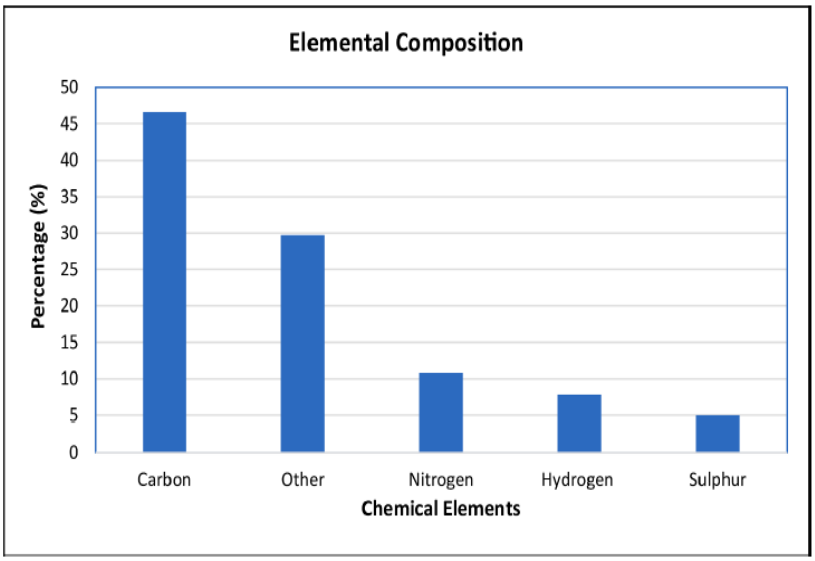

Fig. 5. Graph of the elemental composition of the extracted chicken feather keratin.

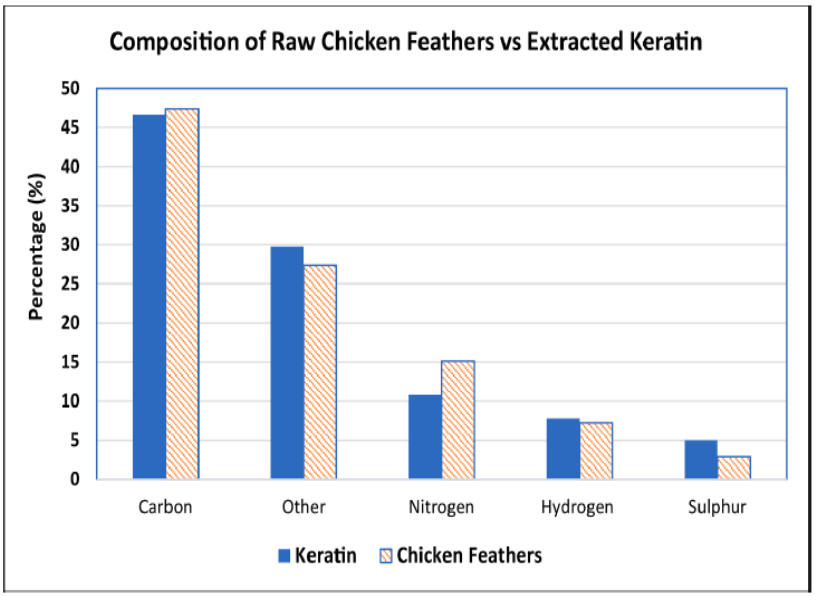

Fig. 6. Graph of the elemental composition of extracted keratin and raw chicken feathers.

3.4.4. Crude protein analysis using CHNS/O analyser

The qualitative and quantitative analysis of chemical elements in extracted keratin showed, in Fig. 5, that there was 46.64% carbon, 7.72% hydrogen, 10.82% nitrogen, 5.02% sulphur and 29.74% of other elements, including oxygen. This composition was comparable to that of raw chicken feathers shown in Fig. 6 (Tesfaye et al., 2017a, b).

The noticeable difference in element content was that of sulphur, i.e. the average sulphur content in extracted keratin was almost double to that of raw chicken feathers. This was due to sulphite ion that attached to one of the sulphur atoms from disulphide bond, i.e. one additional sulphur atom for every molecule of keratin. This significant change in sulphur content can be used to confirm the cleavage of disulphide bonds during keratin extraction. The average nitrogen content in the sample was then used to determine average crude protein in the extracted keratin by multiplying it by a nitrogen-to-protein conversion factor of 6.25. Therefore, the 10.82% nitrogen content in the extracted keratin gave 67.63% of crude protein. The protein presence was further confirmed by Bradford assay which showed a protein concentration of 0.021 μg ml^{-1}.

4. Conclusion

This study emphasised that keratin can be extracted from wasted chicken feathers using a solution of sodium bisulphite, sodium dodecyl sulphate and urea. The application of response surface methodology (RSM) was effective in studying the effects of the four independent variables on the extraction of keratin from waste chicken feathers, where, the effect of reaction temperature was found to be most significant one, and variables significance in a descending order is reaction temperature > reaction time > concentration of $NaHSO_3$ > concentration of $NaC_{12}H_{25}SO_4$. The developed model will help in predicting the amount of keratin that can be extracted from feathers. This work will enable the utilisation of this cheaper and environmentally friendly extraction technique to recover keratin from keratinous waste materials for their beneficiation.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mduduzi Khumalo: Conceptualization, Methodology, Investigation, Data curation, Validation, Formal analysis, Visualization, Writing - original draft. **Bruce Sithole:** Supervision, Project administration, Funding acquisition, Conceptualization, Resources, Writing - review & editing. **Tamrat Tesfaye:** Supervision, Project administration, Conceptualization.

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Possible Beneficiation of Waste Chicken Feathers Via Conversion into Biomedical Applications

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Abstract

Keratin-based waste materials such as wool and waste chicken feathers are driving investigations to beneficiate them. The poultry industry in South Africa generates about 230 million kg of waste chicken feathers per annum, which makes them the abundant keratin source. most of which is disposed of by landfilling or combustion. The current disposal techniques through landfilling or combustion are not environmentally friendly. Thus, methods for beneficiation of the waste are needed. Considering that chicken feathers are comprised of mainly keratin, it is plausible that the keratin can be exploited for application in biomedical applications. However, keratin biomaterials have not found a breakthrough in clinical applications. The keratin can be recovered in the form of fibres or dissolved from feathers in suitable solvents. Regenerated keratin biomaterials can take the form of hydrogels, membranes, films, sponges, scaffolds, and nanofibres. These materials possess excellent properties that can be applied to different fields, including the health sector. Currently, there is no review paper that puts together all possible beneficiations of waste chicken feathers keratin in biomedicine. Therefore, this work exposes the chemistry and characteristics of keratin from different sources including chicken feather keratin in relation to their potential use in the biomedical applications. This review also highlights the properties of regenerated keratin and corresponding biomaterials, including electrospun regenerated keratin fibres for biomedical applications. Keratin nanofibres, also possess advanced properties for biomedical applications due to nanofibres reception in medical applications. Keratin is one biopolymer that can function as an acceptable biopolymer. The review indicates that there is a need for biopolymers as many fields rely on petroleum-based polymers which tend to have biocompatibility limits and are unsustainably resourced.

Keywords: Regenerated keratin; Keratin; Waste chicken feathers; Keratin-based waste material

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Introduction

In various fields, there is a need for biopolymers that can be used as alternative sources to petroleum-based polymers that have biocompatibility problems and/or environmentally unfriendly production. Keratin is one biopolymer that can replace some of these materials [1]. Keratin is fibrous protein that has high concentrations of sulphur in its amino acids which are responsible for mechanical, thermal and chemical stability properties [2]. Protein-based biomaterials can facilitate cell-to-cell and cell-to-material interactions which makes them more biocompatible than their counterparts [3]. Previous research work has shown that regenerated keratin can be used for different applications such as in cosmetics, animal feedstocks, fertilisers and pharmaceutical engineering applications [4,5].

Potential biomedical applications of regenerated keratin also include materials for drug delivery, tissue engineering and wound healing [2,6]. These materials are made in forms that are relevant to their applications; the regenerated keratin materials can be membranes, films, powders, sponges and fibres [2,7-9]. The fact that keratin can blend with other polymers such as polyethene oxide, polylactic acid and so forth, to supplement each other widens it fields of applications [10,11]. It also plays a vital role in dissolving drugs in the body; hence, keratin protein supports dissolution of drugs to body cells. Therefore, pure and or blended regenerated keratin membranes can be used for drug delivery because of their properties match the human stratum corneum [2]; pores in keratin film provide excellent drug delivery property for the film, even though the keratin as a biopolymer has not been characterised in terms extracellular polymeric substance (EPS) and soluble microbial product (SMP), the fact that it is a protein and has a high molecular weight may suggest has a high ratio of EPS than SMP [8]. Regenerated keratin partnered with glycerol to make clear films can be used as wound dressing materials [7]. The aim of this report is to review the possible economical way of keratin extraction and possibilities of valorisation of chicken feathers keratin into regenerated keratin nanofibres.

Sources and related classifications of keratin

There are different types of keratins depending on the classification that is used. One way to classify keratin is based on the sulphur content, which is proportional to the amount of cysteine, the main amino acid of keratin. Keratins with 1,5 to 2% and 4 to 8% sulphur content are referred to as soft and hard keratins, respectively [5,6]. This sulphur content is proportional to structural stability and some degree of resistance to forces before fracture [7]. Both soft and hard keratin contain protein mixture that is set in a filament matrix structure which is supported by sulphide bond [8]. The other method of keratin classification is based on the source of keratin concerned; this filament protein, keratin is mainly found in vertebrates and reptiles, including chickens. The keratin proteins from these two classes are distinguishable by the way in which polypeptide chains are laid, which may either form alpha helices or beta pleated sheets that result in alpha-keratin and beta keratin, respectively. Alpha and beta keratins of mammals and reptiles are found in stratum corneum, horns, nails, hooves, claws, hagfish slime threads, scales, shells such as that of tortoise and beaks, claws and feathers of birds [9-11].

The earlier mentioned soft keratin is typically found in the skin while hard keratin is found in nails, hooves, horns, hair including wool and feathers [6]. Human skin consists of stratum corneum which is 70% keratin [2] while a human hair is 90% keratin [12,13]. The hair keratin consists of 50-60% alpha keratin while nail keratin, contrariwise, has characteristics of both hair and skin keratins in varying proportions [14,15], with 21.9% of amino acids being cysteine and serine [16]. Wool and feathers are the most abundant sources of keratin worldwide due to their utilisation in the textile and food industry, respectively [17,18]. The usage of wool in textile industry results in masses of un-spinnable short wool waste [19]. Wool is

95% keratin, of which 60% wt is soft keratin, and 26% is hard keratin [20]. Comparatively, feathers consist of 90% beta keratin [21]. The poultry industry produces the bulk of chicken feathers as waste during meat production [22]. Valuable materials produced from regenerated keratin include electrospun nanofibres [20]. Regeneration of keratin via electrospinning demands its extraction from its source. Equally, keratin must be extracted from chicken feathers for the production of nanofibres [23].

Chicken feathers as the most abundant source of keratin

Compassion in World Farming stated, in 2013, that 58 billion chickens are slaughtered per year [24]. Considering a 2 kg slaughter-weight of a chicken with 5-7% of feathers per chicken, a minimum of 5,8 billion kg of chicken feathers are produced per year as a by-product [25]. USA and India produce approximately 1.044 billion kg and 140 million kg chicken feathers, as waste, per year, respectively [26,27]. Two to three tons of chicken feathers can be effortlessly produced by a slaughterhouse that processes 50 000 chickens per day [28]. According to Tesfaye South Africa produces 258 million kg of chicken feathers as a by-product while producing meat [29]. Insignificant amounts of these feathers are used as useful products, for low-value applications like animal feed which cost about 13 Rand, and fertilizer; the other significant portion is considered as a waste product [30,31]. The trend of poultry production indicates an increase in chicken production.

Consequently, chicken feathers amount will increase [32]. Currently, the poultry industry disposes of the waste chicken feathers using landfilling and incineration techniques [7,33]. These disposal procedures are restricted, generate greenhouse gases such as carbon monoxide or pose a danger to the environment; chicken feathers are solid waste which pollutes land due to their degradation resistance, increase cost of production due to levy paid by poultry meat producers, covers large area and can contaminate groundwater [34,35].

Conversely, chicken feathers contain about 90% of keratin protein [25], a highly valued protein; the price of one-milligram keratin from a human cell sells for R2840 per gram [36]. Hence, keratin can be extracted from chicken feathers and be converted into essential and valuable products which can add extra value and revenue to the poultry industry. Biomedical applications are amongst the applications of keratin [3,37-39]. However, keratin extraction for these applications has not yet optimised. The succeeding section of this paper describes some of the extraction methods that are or can be used to extract keratin from waste chicken feathers.

Keratin extraction techniques

Keratin extraction involves breaking strong disulphide bonds that crosslink keratin molecules, this allows extraction of keratin protein. Depending on extraction technique, keratin chains may also be shortened during the process [40,41]. This section describes extraction techniques that can be used to extract keratin from different sources. These extracting techniques can be categorised into chemical techniques, microwave radiation technique, microbial, enzymatic extraction supercritical water and steam explosion [42]. The subsections below give a brief description of the mentioned techniques.

Chicken feather keratin can be extracted via chemical solutions or mixtures of sodium metabisulphite, sodium bisulphite, sodium sulphide, sodium hydroxide, 2-mercaptoethanol, thioglycolic acid and ionic liquids [28,40,41,43,44]. These keratin extraction methods may produce keratin proteins with different physical properties such as viscosity, molecular weight and

others which later affect the properties of the final products that will be produced from keratin. Therefore, this section aims to review the different methodologies of keratin extraction technologies for waste chicken feathers.

Chemical extraction of keratin

Chemical extraction uses chemical substances to extract keratin from keratinous fibres. The most common classes of chemicals used to extract keratin from keratinous materials are reducing agents, oxidising agents, ionic liquids and alkalines [43]. Hydrolysis extraction method requires a significant amount of alkaline chemicals such as sodium hydroxide. When the keratinous fibre is treated with an alkaline solution, keratin chain is damaged, and keratin structure is disrupted and altered through degradation of cysteine which forms thioether linkage [17]. However, cysteine is very sensitive to alkalis, hence, it quickly decomposes in their presence, therefore, the amount of cysteine decreases rapidly when compared to the reduction method. Nevertheless, the hydrolysed keratin remains undamaged during the process [19,45].

Reducing agents: On the other hand, keratin can be extracted by reduction process, where reducing agents break cystine disulphide bonds and produce cysteine. The often used reducing agents are thiol containing chemicals such as thioglycolic acid or thioglycolate salts and 2-mercaptobisulphite, sulphitolysis agents like sodium sulphite, sodium bisulphite and sodium metabisulphite [43,46]. The reducing agents are often used with denaturing agents and surfactants to enhance the extractability of keratin [42].

Extraction of keratin using sodium metabisulphite

Ayutthaya investigated keratin extraction from chicken feathers using various concentrations of sodium metabisulphite [40]. In this method, short cleaned feathers are dissolved by immersing in solutions of sodium-metabisulphite (varying from 0 to 0.5 M) with 8 M urea, 0.6 M sodium dodecyl sulphate and 5 N sodium hydroxide and stirred at 65°C for 5 hours. The next step is to filter solution and dialyse it with distilled water for three days, changing water three times a day. To further purify the extracts, the solution is then concentrated by a rotary evaporator at 40°C and 45 mbar. The concentrated keratin is then freeze-dried before determining the percentage yield of extracted keratin and store it in a closed container. The results, according to Ayutthaya paper, showed the increased in keratin percentage yield from 36.2% to 87.6% as the concentration of sodium metabisulphite increased from 0.0 M to 0.2 M respectively. Sodium metabisulphite solution of 0.2 M concentration yielded keratin with a molecular weight range of 12-20 kDa. The sodium metabisulphite concentration above 0.2 M resulted in a decrease in keratin percentage yield because most disulphide bonds of keratin are broken, leading to keratin short chains which escape dialysis tube during dialysis. Sinkiewicz and Ayutthaya separately recorded 62.9% and 60.2% from 0.5 M sodium metabisulphite extraction [40,43]. Precaution must be considered when working with sodium metabisulphite as it is slightly toxic in both LD50 oral and LD50 dermal exposure according to Hodge and Sterner acute toxicity scale. Sodium metabisulphite is harmful if swallowed, causes severe eye blindness and liberates toxic gases if it reacts with acids, hence, eye protection must be considered when working with this substance [47-49]. According to the German Federal Water Management Act, sodium metabisulphite poses slightly danger to aquatic life, therefore it must be neutralized before discharging, like treating using sodium hypochlorite solution [47].

Extraction of keratin using sodium bisulphite

This section reviews keratin extraction from chicken feathers using sodium bisulphite. In Sinkiewiczinvestigation, one gram of pre-treated feathers was immersed in 25 ml of aqueous solution of 0.5 M sodium bisulphite, 8 M urea and 0.08 M SDS.

The mixture was then stirred at 70°C for 4 hours. For purification purpose, the mixture was centrifuged at 9000 rpm for 15 minutes before filtered through a folded filter paper. The filtrate was then dialysed using regenerated cellulose (MWCO 3500-500 Da) in distilled water for 72 hours, changing the outer water every day. The keratin sediments were then washed with distilled water and centrifuged at 9000 rpm for 15 minutes. The insoluble residue was collected, dried at 105°C and weighed. The maximum percentage yield of keratin from Sinkiewicz investigation was 84%; however, the addition of 2.5% sodium hydroxide increases the percentage yield of keratin by approximately 10%.

Sodium bisulphite is non-combustible, however, precautions must be taken when handling it because it is harmful if swallowed, it liberates toxic gases when it reacts with acids. It slightly endangers aquatic life; hence, its disposal must be controlled by stirring into sodium hypochlorite [47-50].

Extraction of keratin using sodium sulphide

Sodium sulphide can be used to extract keratin from chicken feathers; In Gupta investigation 50 g of blended clean chicken feathers was put into 2 L of 0.5 M sodium sulphide solution. The mixture was heated to the temperature of 30°C and pH was maintained between 10-13 while the mixture was continuously stirred for 6 hours. For purification purpose, the mixture was then filtered and centrifuged at 10 000 rpm for 5 minutes. The liquid was filtered again to make it particle free. 5.3 M ammonium sulphate was added dropwise to feather solution at 1:1 ratio to precipitate protein. The solution is then centrifuged at 10 000 rpm for 5 minutes, and the particles are collected. The collected solid particles are washed with 1000 ml of deionised water and centrifuged at 10 000 rpm for 5 minutes. The solid particles are collected, dissolved in 2 M sodium hydroxide, centrifuged at 10 000 for 5 minutes. The liquid is collected and stored. The percentage yield of keratin from Gupta procedure was recorded to be 53% relative to 50 g of starting chicken feathers [28]. Despite the simplicity of this method, sodium sulphide is very dangerous; it is a permeator that also causes skin irritation that may result in inflammation and eye irritation which can injure or cause blindness in case of contact. The substance is also dangerous when ingested and inhaled. Precautions must be considered when handling sodium sulphide because severe over-exposure can produce lung damage, choking and unconsciousness or death; moreover, sodium sulphide is slightly water endangering.

Extraction of keratin using Shindai method

In this method, Sinkiewicz investigated keratin extraction using 2-mercaptoethanol. To execute this method, one gram of pre-treated feathers is immersed in 25 ml of aqueous solution of 1.66M 2-mercaptoethanol with 8 M urea and 0.2 M Tris-HCl at pH 8.0. The mixture is then stirred at 70°C for 2 hours. To purify the extract, the mixture is centrifuged at 9000 rpm for 15 minutes and then filtered through a folded filter paper before dialysed using regenerated cellulose (MWCO 3500-5000 Da) in distilled water for 72 hours, changing the outer water every day. The keratin sediments are then washed with distilled water and centrifuged at 9000 rpm for 15 minutes. The insoluble keratin residues are then dried at 105°C and weighed to determine percentage yield of keratin. The percentage yield of keratin from Sinkiewicz investigation was 90% [43]. Yin used the similar method of keratin extraction from chicken feather and obtained 93% keratin yield; hence, this method can be replicated to obtain this high percentage yield of keratin in just 2 hours of dissolving feathers [51].

Nevertheless, 2-mercaptoethanol is moderately toxic in both oral and dermal exposure; it causes skin irritation, severe eye damage, and allergic skin reaction. It causes damage to organs through continued or frequent exposure if consumed. 2-

mercaptoethanol is also toxic to marine life with enduring effect. Moreover, it is a high-water endangering substance. 2-mercaptoethanol must be kept below 30°C due to its combustibility.

Extraction of keratin using thioglycolic acid

The other keratin extraction method is by use of thioglycolic acid; Gupta extracted keratin from 50 g of chicken feathers using 2 L of 0.5 M thioglycolate solution with 0.1 N sodium hydroxide. The mixture is then heated to 30°C while the solution is being stirred and pH maintained between 10-13 for 6 hours. To purify the extract, the mixture is then centrifuged at 10 000 rpm for 5 minutes, and the collected liquid is filtered to remove insoluble residues further. To refine the extracted keratin, it is precipitated by adding 5.3 M of ammonium sulphate solution dropwise, while stirring the solution. Keratin solid particles are then collected after 5 minutes centrifugation at 10 000 rpm. The percentage yield of keratin obtained from Gupta investigation was 8.8% [28]. However, Hatakeyama obtained percentage yield of 75% when they used thioglycolic acid and sodium hydroxide to extract keratin from wool, a method that can be adapted for keratin extraction from chicken feathers, while allowed to dissolving time of 16 hours for a solution pH value of 13 [52].

Thioglycolic acid is toxic if swallowed, in contact with skin or if inhaled. The liquid causes severe skin burn and eye damage, hence, protective gear must be worn to avoid skin contact, inhalation and eye irritation. This substance should be stored in a temperature range of 2°C to 8°C due to its combustibility [47-49,53]. According to the German Federal Water Management Act, thioglycolic acid slightly endangers aquatic life, thus, precautional measures must be taken [50].

Extraction using ionic liquids: Idris (2014) and Xie (2005) reported the use of imidazole ionic liquids such as 1-allyl-3-methylimidazolium chloride, 1-butyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium bromide to extract keratin. The reports show that chlorine-containing ionic liquids are more effective than other ionic liquids, this might be due to nucleophilic strength of chlorine ions as compared to other halogen ions. The extracted keratin by these liquids is mainly composed of beta-sheet structure than alpha helix structure which seems to be destroyed during extraction process [1,19]. Moreover, the extract from wool showed greater thermal stability than the original wool. Ionic liquids are said to be environmentally friendly and cost-effective because of their recyclable ability which minimises their disposal to the environment and reduces the consumption of fresh raw ionic liquids [44].

Extraction of keratin using imidazole ionic liquids

Imidazole ionic liquids can also be used to extract keratin from chicken feathers; Ji used imidazole ionic liquids 1-allyl-3-methylimidazolium chloride [Amim]Cl, 1-butyl-3-methylimidazolium chloride [Bmim]Cl and 1-butyl-3-methylimidazolium bromide [Bmim]Br. These liquids were used in proportion to different weights of Na_2SO_3 and water to dissolve chicken feathers. The mixture of liquids with chicken feathers is then heated, to advance disulphide bond cleavage, under magnetic stirring condition. The sample is filtered by suction filtration before the protein is precipitated by adding water to the mixture. Keratin solid particles are then collected using suction filtration; at this stage, the sample is ready for percentage yield determination. Ji investigation obtained reported a maximum yield of 75.1% using [Bmim]Cl ionic liquid under extraction time of 60 minutes at 90°C [44].

Precautional measured must be taken when one works with imidazole ionic liquids because they cause skin irritation, acute eye irritation and may cause respiratory irritation [47,49]. The liquids also pose a danger if swallowed due to its moderate

toxicity level on Hodge and Sterner acute toxicity scale [48]. According to the German Federal Water Management Act, [Bmim]Cl is highly dangerous to water life with chronic effect [50]. Wang and Cao extracted keratin from chicken feathers using hydrophobic ionic liquid, IL,1-hydroxyethyl-3-methylimidazolium bis(trifluoromethanesulfonic)amide ([HOEMIm][NTf_2]) and sodium hydrogen sulphate. The maximum percentage yield of keratin obtained from the investigation of Wang and Cao was recorded as low as 21.5%. The addition of the ionic liquid increased the percentage yield of keratin up to some extent depending on the mass ratio of ionic liquid, feathers, and $NaHSO_3$ [41]. The primary objective of keratin extraction from chicken feathers is to valorise chicken feathers. Hence, the percentage yield of keratin is prioritised during chicken feather dissolving; therefore, keratin extraction by the [HOEMIm][NTf_2] liquid is not efficient on keratin recovery. This substance, 1-hydroxyethyl-3-methylimidazolium bis(trifluoromethanesulfonic) amide is moderately toxic if swallowed according to Hodge and Sterner acute toxicity scale, a precaution against swallowing is vital; it is also hazardous to aquatic life [47,50].

Oxidising agents: Keratin can also be extracted by an oxidation method, where oxidising agents like peracetic acid, performic acid, potassium permanganate, sodium hypochlorite and hydrogen peroxide are used to break cystine disulphide bonds and release cysteine amino acids with sulfonic groups as side chains. The extracted keratin may contain alpha helix structures and beta sheet structure, in which oxidation by peracetic acid or performic acid allows their separation due to their solubility at different pH values [42].

Costs of the reviewed methods of chemical extraction of keratin: Cost of implementing the keratin extraction procedure is one of the vital factors for choosing a keratin extraction technique. This section is, therefore, describing them, excluding purification after cooking the mixture. The Table 1 below illustrates the unit costs of the materials and equipment, as per Sigma-Aldrich Pty. Ltd catalogue, that is used in the keratin extraction procedures, described above, at laboratory scale [36].

TABLE 1. **Material for keratin extraction, for selected methods, and cost per, in rand, per gram.**

Materials	**Unit cost (Rand per gram)**
Sodium metabisulphite	0.86
Urea	2.04
Sodium dodecyl sulphite	1.59
Sodium hydroxide	1.69
Sodium bisulphite	0.32
Sodium sulphide	0.85
2-Mercaptoethanol	55.4
Tris-HCl	3.09
Thioglycolic acid	6.4

The tabulated unit costs in Table 1, with concentrations of chemicals that are required to prepare 100 ml keratin extraction mixtures and electrical energy consumed, as per Eskom tariffs, during extraction are the bases of the cost calculations. Table 2 shows the costs of keratin extraction and keratin percentage yields of various extraction techniques [54].

TABLE 2. **Keratin extracting methods, cost of extraction and percentage yield of keratin.**

Keratin extraction method	Cost (rands)	%Yield of Keratin
Thioglycolic acid	33.24	75
Sodium sulphide	50.28	53
Sodium bisulphite	111.08	84
Sodium metabisulphite	176.02	87
2-Mercaptoethanol (Shindai)	758.80	90-93

As it is clearly seen in Table 2, an increase in extraction cost from thioglycolic acid to 2-mercaptoethanol and cost dependent percentage yield with an exception of thioglycolic acid which seems not to correlate with others. Shindai, 2-mercaptoethanol, method is the expensive extraction technique (Table 2), however, the keratin percentage yield may attract the interests of the researchers.

Enzymatic hydrolysis

Keratin extraction in a very high or low pH for extended time at high temperatures produces low molecular weight peptide fragments because chemicals break both disulphide and peptide bonds, and this limits several biomedical applications [23,55,56]. The enzymatic hydrolysis of keratin is the use of biological catalysts to catalyze chemical reaction during keratin extractions. Enzymatic hydrolysis requires mild treatment conditions hence conserves the functional properties of extracted keratin [23].

Microwave-assisted keratin extraction

Microwave keratin extraction uses microwave radiation to extract keratin from fibres. The extraction is done by using the principle of direct heating the molecules of the material using microwave energy. The transformation of electromagnetic energy to thermal energy occurs when the mechanisms, ionic induction and dipole rotation take place simultaneously [57]. Keratinous fibres can also be put into superheated water at the temperature ranging from 150-180°C in a microwave reactor [58]. Shavandi used microwave radiation varying power in a range of 150-570 Watts [42]. The power was applied for up to 7 minutes at a temperature of 180°C. Microwave-assisted keratin extraction is advantageous in a way that it may take place in low temperatures over a short period of time.

Supercritical water and steam explosive

In supercritical water and steam explosive keratin extraction, steam is forced into keratinous tissue and cells of biomass. The high-temperature steam penetrates tissues and cells, pressurising and then rapidly depressurises them. This causes an explosion in millisecond reaction which releases keratin. The process results in reduced molecular weight of keratin and loss of mechanical properties [42,59].

Applications of keratin biopolymer in biomedicine

Keratin biomaterials have been produced and experimentally used in several biomedical applications, this is due to their biocompatibility, biodegradability and the capability of keratin to act as an extracellular matrix to facilitate cell adhesion. The

following subsections highlight areas of focus in applications of feather keratin-based biomaterials to emphasize the potential biomedical applications of keratin extracted from waste chicken feathers.

Drug delivery carriers

Drug delivery systems are technologies that are engineered for the targeted delivery and or controlled release of therapeutic substances to improve health and extend lives [60]. Several synthetic and natural polymers have been used as drug carriers. Even though synthetic polymers are often used compared to natural polymers due to modifiable properties to cover a wide range of application, they pose side effects. Therefore, the focused has turned to natural polymers for safe use as drug delivery carriers. Multiple proteins, including keratin, have been investigated in the development of biomaterials, and keratin-based materials showed positive outcomes. Researches are underway to improve this system to make it more effective and precise; one way to do this is to incorporate nanotechnology and biomaterials in development of drug delivery carrier [61]. Keratin biocompatibility property, that is due to the presence of amino acids, allows keratin to be modified to meet drug delivery requirement [62] (Table 3).

TABLE 3. **Drug delivery keratin-based carriers, keratin sources and results of investigations.**

Type of Keratin biomaterials	**Keratin source**	**Drug delivery ability**	**References**
Keratin film	Chicken feathers	Drugs were loaded and released successfully. Good mechanical properties films provided a continuous release of loaded drug for up to 12 hours.	[51]
Keratin-hydrotalcite hybrid films	Merino wool	support fibroblast cells adhesion and growth suggesting their potential use as drug delivery systems	[63]
Keratin graft polyethylene glycol (Keratin-g-PEG)	Wool	The drug released from the loaded keratin-g-PEG nanoparticles showed that it can be internalized into the cells efficiently, and the loaded drug indicated a faster release into the nuclei of the cells	[64,65]
keratin/doxorubicin nanoparticles	Human hair	Keratin-based drug carrier is potential for cancer therapy. Keratin/doxorubicin nanoparticles were able to catalyse nitric oxide release from blood endogenous donor	[66]
Unextracted	Cancer cell plasma membrane in human	The study establishes keratin 1 as a new marker for breast cancer targeting	[67]
Keratin Hydrogel	Chicken feathers	The cumulative release of the anticancer drug (Dox·HCl) reached 93.3% within 16 h, and the cumulative release rate of macromolecular drug (BSA) got to 75.9% in 24 h. Therefore, the keratin-based biopolymer hydrogel with	[68]

		interpenetrating network structure, pH-sensitivity and temperature sensitivity are potentially applied to sustain drug carrier	
Keratin films with catalysed crosslinking	Wool	Films showed a lower drug release ratio in which drugs can be loaded and released over a longer period for prolonged healing. These films also showed an increase in tensile strength and decrease in elongation at break.	[69]
Keratin hydrogel	Chicken feathers	The hydrogels were able to release 97% drugs for 24 hours	[70]
Keratin hydrogel	Wool	Keratin hydrogel and chemically modified keratin hydrogels showed good drug delivery carrier properties, with a delivery rate that ranges from 1-3 days.	[71]

Wound dressing

Wound management is a necessity for wound healing process. To archive this wound dressing and wound treatment play vital roles. The wound dressing material may include multiple layers to maintain moist environment at the wound, be able to control excess exudates, the material must be able to protect wound from the outside environment, adhere well to the skin and be comfortable to the body part [72]. Wound dressing materials are in various forms including, but not limited to, nanofibrous mats, hydrogel, films, sponges or foams. Keratin has a potential of being incorporated in the development of wound dressing materials due to its biocompatibility property and more. Investigations are ongoing to introduce keratin wound dressing biomaterials to clinical application. Table 4 highlights some keratin wound dressing materials which have been developed from across the world.

TABLE 4. **Wound dressing keratin-based biomaterials, keratin sources and results of investigations.**

Type of Keratin biomaterials	**Keratin source**	**Observations**	**References**
Keratin/gelatin nanofibres with polyurethane as an outer layer	Human hair	*In vivo* study, keratin nanofibres mat gave reduction in wound area at 4 days, and better wound repair at 14 days with a thicker epidermis and larger number of newly formed hair follicles, thus, this material could be a good candidate for wound dressing applications.	[73]

Keratin nanofibres	Chicken feathers	Three keratin blended wound dressing materials were prepared, namely keratin nonwoven (KN), keratin-sodium alginate (KSAN) and keratin-chitosan (KCN). *In vivo* observed at 15, 17, 21 and 23 days showed better wound dressing effects of KSAN and KCN than KN.	[74]
Keratin powder	Mouse fur	Fur keratin-derived protein dressings significantly accelerated wound healing in the mouse mode, which is a good outcome.	[75]

Tissue engineering

Damaged body tissues or cells may require the assemble of functional construct that restores, maintains, improves them or the whole organ. Generally, cells are the building blocks of tissues which are the basic unit of body functioning. This is tissue engineering, it evolved from the field of biomaterials development to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues, however, this field is now extending using tissues as biosensors and chips that detect threads agents and toxicity test, respectively. The tissue engineering process begins with building a scaffold from synthetic and or natural sources. Keratin can be used in development of scaffolds for tissue engineering [76-80].

Electrospinnability of keratin

Keratin nanofibres could be used in wide range of keratin biomedical applications including drug delivery carrier, wound dressing and tissue engineering, therefore, it is important to gather the evidence of chicken feather electrosinnability in order to valorise them. To verify the electrospinnability of keratin, it is important to recall the properties of keratin, that might affect its electrospinnability, and relate them to the basic requirements of electrospinning. Examples of how the keratin properties influences the electrospinnability of this biopolymer are described below.

Keratin properties in relation to its electrospinnability

Viscosity is one of the most important parameters on electrospinning. The viscosity of the solution and its electrical properties determine the extent of elongation of the solution. These influence the diameter and other morphological properties of the resultant electrospun fibres [81]. However, this property has limits, at low viscosity; it is common to find beads along the fibres deposited on the collector. When the viscosity increases, there is a gradual change in the shape of the beads from spherical to spindle-like until a smooth fibre is obtained. High viscosity discourages the bending instability to set in for a longer distance as it emerges from the roller surface. As a result, the jet path is reduced and the bending instability spreads over a smaller area [82,83]. This reduced jet path also means that there is less stretching of the solution resulting in a larger fibre diameter. However, when the viscosity is high enough, it may discourage secondary jets from breaking off from the main jet which may contribute to the increased fibre diameter [84]. Therefore, determining the viscosity range of each polymer to be spun is required. One of the factors that affect the viscosity of the solution is its molecular weight. The molecular weight of the spannable polymers ranges from 13 kDa to about 200 kDa [85] (Table 5).

TABLE 5. **Keratin-based scaffolds for tissue engineering, keratin sources and results of investigations.**

Type of Keratin biomaterials	Keratin source	Tissue engineering ability	References
Keratin/Gelatin/Chitosan	Hooves	The scaffold exhibited good porosity and interconnectivity of pores, and cells demonstrated good cell viability of keratin scaffold.	[76]
Keratin/poly (ε-caprolactone) nanofibres mat (keratin/PLC)	Human hair	Test showed that fibroblast cells adhered more to keratin/PLC mat than PLC. And blood clothing time test indicated that the mats are blood compatible which makes them potential scaffolds for vascular tissue engineering	[77]
Powdered scaffolds	Chicken feathers	*In vitro* cell viability test indicated that the scaffolds are biocompatible and support cell growth, this were positive results for tissue engineering application	[78]
Keratin based hydrogels	Human hair	*In vitro* study showed positive results for peripheral nerve regeneration over 6 months period.	[79]
Keratin/chitosan mats	Human hair	The cells that were cultured in nanofibers showed growth, forming the layer on the scaffold, mimicking the epidermis tissue.	[80]

The Aluigi investigation of the structure and properties of keratin/polyethylene oxide nanofibres shows extracted keratin molecular weight ranges from 11kDa to 60kDa in which a large distribution of it falls within the molecular weight range of spannable polymers [46]. The molecular weight of the polymer may also influence electrical conductivity and surface tension of the solution; these properties have significant effect on electrospinnability of a polymeric material [86].

Electrical conductivity of the polymer solution is one of the requirements for electrospinning because the polymer solution must allow charge induction to form Taylor cones and then polymer jets. Aluigi investigation also reported that increasing the amount of keratin in electrospinning solution results in significant increase of solution electrical conductivity which might be due to the polarity of its amino acids [46]. Nevertheless, the polymer solution electrical conductivity can always be improved by adding an electrical conductive substance like salts or conductive solvents even though this compromises the diameter of the nanofibres [87,88].

Surface tension is also a critical factor in electrospinning. The formation of droplets, beads and fibres depends on the surface tension of solution. Generally, high surface tension of a solution inhibits the electrospinning process because of instability of the jets and the generation of sprayed droplets [89]. Low surface tension of the spinning solution helps electrospinning to occur at a lower electric field [90]. However, not necessarily a lower surface tension of a solvent will always be more suitable for electrospinning, surface tension determines the upper and lower boundaries of the electrospinning window if all other variables are held constant [91-93]. The surface tension mostly depends on the composition of the solvent, hence, extracted

keratin may insignificantly influence surface tension of the electrospinning solution. The following section reveals the work that have been done on electrospinning of keratin to keratin nanofibres.

Electrospinability of keratin

Literature shows the existence of biopolymer keratin nanofibres [58], therefore, the co-existence of keratin from the worldwide abundant chicken feathers [32], and applications of large surface area-to-volume ratio nanofibres [94] is a phenomenon that shall be embraced. Nanofibrous materials have been investigated for their extravagant applications such as gas-sensor ability [95]. Biocomposite nanofibres in urea biosensing have also been investigated and showed improvements, over existing technology, in properties such as response time and sensitivity to lower concentrations of urea [96]. Moreover, nanofibrous materials in biomedical applications have drawn much attention due to their abilities in biomedicine field [3]. Polyvinyl alcohol nanofibres can controllably release ketoprofen when it is used as a drug carrier [97]. Antimicrobial wound dressing nanofibres materials loaded with silver nanoparticles for aerobic bacteria reduction showed excellent properties and antibacterial effects [98]. Also, researchers revealed potential nanofibres applications in tissue engineering, including tissue scaffolding [99,100]. Nanofibres biomedical applications are advanceable by petrochemical polymers replacement or blending with biopolymers, this enhances nanofibres matrix-to-cell interactions. Thus, keratin is one of the biopolymers that can supplement petrochemical polymers lacking properties for biomedical applications. Therefore, electrospinnability of keratins, including chicken feathers keratin is vital to nanofibres materials biomedical applications [3]. Although pure keratin nanofibres are non-existence, researchers have managed to produce keratin blended nanofibres [40].

Wool alpha keratin can blend, with good interaction with, with polyvinyl alcohol to form nanofibrous materials which have improved thermal and mechanical properties as compared to keratin properties. The wool alpha keratin-polyethene blend nanofibres, with various ratios, can be spun which suggests that alpha keratin blends with a range of synthetic polymers [20,46]. In addition, human hair alpha keratin blends with, at least one synthetic polymer, poly(ε-caprolactone) and electro-spun to nanofibres that can act as a composite base of uniform fibre morphology and suitable mechanical properties for biomedical applications [101]. The other type of keratin, beta keratin from chicken feathers, has not yet been electrospun to 100% keratin nanofibres rather blended with other electrospinnable synthetic polymers such as polylactic acid (PLA) even though the mats reflect poor mechanical properties and instability in water [102]. This keratin/PLA composite nanofibrous material can be used as scaffolds for tissue engineering in biomedicine [103].

Keratin-based materials have not made it to any commercial industry across the spectrum despite their potential applications and a demand for such products. All the work that has been done on these keratin-based materials proves to be, somehow, inadequate to commercialise them, therefore, more investigations must be done to eliminate all the limitations that prohibit commercialisation of these materials. Table 6 summarises some of the work that has been done regarding converting keratin to keratin nanofibres. This table does not include work that has not been discussed in this section; a lot more work has been done on the electrospinnability of keratin.

For each investigation, Table 6 shows the source of keratin, extraction technique of keratin, type of polymer that was used to blend with keratin to improve its properties and reference.

TABLE 6. **Advancements of the properties of keratin nanofibres by blending keratin with synthetic polymers.**

Source of Keratin	Extraction Technique	Type of Polymer used	Aim	Reference
Chicken feathers	Sodium metabisulphite	Polylactic acid	To advance processibility of keratin	[40]
Wool	Sodium metabisulphite	Polyvinyl alcohol	To enhance wool-keratin properties	[20]
Wool	Sodium metabisulphite	Polyethylene oxide	Biomedical application	[46]
Human hair	Trizma base	Poly(ε-caprolactone)	Biomedical application	[101-103]
Human Epidermis	2-mercaptoethanol	Polylactic acid	Biomedical application	[104,105]

Discussion

Regenerated keratin can also be used in nanofibres form that is produced via electrospinning [106]. In electrospinning, a high voltage is applied to a polymer fluid to charge it; when charges within the fluid reached a critical amount, a fluid jet will erupt from the droplet resulting in the formation of a Taylor cone. The electrospinning jet travels towards the region of lower potential, which in most cases is a grounded collector [106-108]. This process results in the formation of microfibres and nanofibres. The high surface area to volume ratio, flexibility, and some mechanical properties are some of the properties that draw attention to nanofibres applications [94].

Applications of nanofibre products in various fields such as tissue engineering, drug delivery carriers, cancer diagnosis, optical sensors, oil-water separation, air filtration and lithium-air battery are driving forces for improvement in production of nanofibres for such applications. Thus, keratin nanofibres can be used for these nanofibres applications [109,110]. Like other forms of keratin materials, keratin nanofibres have thus far been produced by blending keratin with another polymer like polyethene and polyvinyl alcohol [46,20]. Keratin-based nanofibres are an alternative to nanofibres of petroleum polymers as they possess an improved cell to material interaction since they are protein based [3].

Conclusion

Persistence of a problem of keratin-based waste, including waste chicken feathers, indicates the need for further investigation of their valorisation process. Pure keratin is worth paying attention to due to its high price per kilograms. Variety of keratin-based products across different fields is sufficient to valorise keratin-based waste by-product, especially waste chicken feathers from the poultry industry. There are adequate keratin extraction techniques that can assist in the valorisation of keratin-based waste by-products. Electrospinnability of keratin blend enhances its potential for biomedical applications especially as nanofibres receive an overwhelming welcome to the same field. Nonexistence of breakthrough of keratin-based biomaterial in the clinical applications shows innovative opportunities for further investigation of keratin biomaterials, including chicken feather keratin-based biomaterials. Extensive research and development work is required to develop appropriate technologies for utilisation of waste chicken feathers as a source of some of the proposed biomedical applications mentioned in this review.

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B. Abstracts of selected oral presentations

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POSSIBLE BENEFICIATION OF WASTE CHICKEN FEATHERS VIA CONVERSION INTO BIOMEDICAL APPLICATIONS

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Keratin-based waste materials such as wool and waste chicken feathers are driving investigations to benefitiate them. The poultry industry in South Africa generates about 230 million kg of waste chicken feathers per annum, which makes them the abundant keratin source, most of which is disposed of by landfilling or combustion. Such disposal methods are not environmentally friendly. Thus, methods for beneficiation of the waste are needed. Considering that chicken feathers are comprised of mainly keratin, it is plausible that the keratin can be exploited for application in biomedical applications. However, keratin biomaterials have not found a breakthrough in clinical applications. The keratin can be recovered in the form of fibres or dissolved from feathers in suitable solvents. Regenerated keratin biomaterials can take the form of hydrogels, membranes, films, sponges, scaffolds, and nanofibres. These materials possess excellent properties that can be applied to different fields, including the health sector. This review summarises the chemistry and characteristics of keratin in relation to their potential for use in the biomedical applications. This review also highlights the properties of regenerated keratin and corresponding biomaterials, including electrospun regenerated keratin fibres for biomedical applications. It also looked at keratin nanofibres, which possess advanced properties for biomedical applications due to nanofibres reception in medical applications. These includes extraction of keratin protein, keratin fibres, and regenerated keratin nanofibers and description of characteristics suitable for biomedical applications. The review indicates that there is a need for biopolymers as many fields rely on petroleum-based polymers which tend to have biocompatibility limits and are unsustainably resourced. Keratin is one biopolymer that can function as an acceptable biopolymer. Research is ongoing to demonstrate potential applications of regenerated keratin based biomaterials in the biomedical field.

KEYWORDS: Regenerated keratin, keratin, waste chicken feathers, keratin-based waste material

Conversion of waste chicken feathers into regenerated keratin nanofibers

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Key words: Regenerated keratin; keratin; waste chicken feathers; keratin nanofibres.

The poultry industry in South Africa generates about 230 million kg of waste chicken feathers per annum, which makes them the abundant keratin source, most of which is disposed of by landfilling or combustion. Such disposal methods are not environmentally friendly. Thus, methods for beneficiation of this organic waste rich in keratin are needed. Considering that waste chicken feathers are comprised of 91% keratin, it is plausible that the feathers keratin can be exploited for application in several fields. Keratin possesses excellent properties, can be obtained from biomass, doesn't contain any harmful chemicals, more compatible to apply or use on hair and human skin and can be used in various application areas, including cosmetics, shampoos and conditioners and the health sector. Research indicates that there is a need for biopolymers as many fields rely on petroleum-based polymers which tend to have biocompatibility limits, negative environmental impact and are unsustainably resourced. This work aims to extract keratin from waste chicken feathers via the chemical method and convert it into keratin nanofibers via electrospinning. Keratin nanofibers possess advanced properties for biomedical applications due to nanofibres reception in medical applications. The final product from this work will be used in biomedical application such as wound dressing material. Fourier-transform infrared spectroscopy and CHNS characterisations qualitatively and quantitatively confirmed the successful extraction of keratin from waste chicken feathers. The ongoing experimental work is about conversion of extracted keratin to keratin nanofibres.