ADDIS ABABA UNIVERSITY

INSTITUTE OF TECHNOLOGY

SCHOOL OF BIO & CHEMICAL ENGINEERING

EXTRACTION OF GLYCOSAMINOGLYCANS FROM RAWHIDE TRIMMING WASTE BY USING PAPAIN ENZYME

BY
WOLDEAMANUEL WONDAFERASH

Jan 2023
ETHIOPIA, ADISS ABABA

ADDIS ABABA UNIVERSITY
INSTITUTE OF TECHNOLOGY
SCHOOL OF BIO & CHEMICAL ENGINEERING

Extraction of Glycosaminoglycan's from raw hides trimming waste by using papain enzyme

This thesis paper submitted to Addis Ababa University, institute of technology, School of Chemical and Bio Engineering in Partial Fulfillment of the Requirements the accomplishment of the Degree of Master of Science in Chemical Engineering (Leather stream).

BY

Woldeamanuel Wondaferash

Advisor: - Shegaw Ahmed (Dr)

Jan 2023
ADDIS ABABA, ETHIOPIA

ADDIS ABABA UNIVERSITY
INSTITUTE OF TECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING

Board of examining committee

Shegaw Ahmed (Dr) Advisor	Signature_____	Date_____
Shimelis Kebede (Dr) Internal Examiner	Signature_____	Date_____
Anteneh Marelign (Dr) External Examiner	Signature_____	Date_____

DECLARATION

This is to verify that this thesis under topic “Extraction of Glycosaminoglycan’s from Raw hide trimming waste by using papain enzyme” given in partial compilation of the requirements for the award of the degree of Master of Science in “chemical Engineering” Leather Stream in Addis Ababa institute of technology (AAiT) School of Chemical & and Bio Engineering for Degree of Masters of Science in chemical Engineering by Mr.Woldeamanuel Wondaferash; (Id Number: GSR/7927/11) is carried out by him under our guidance. The paper work has not been submitted before for any reward of degree or diploma to the best of our knowledge and belief.

Signature & date _____

Acknowledgements

First of all i would like to thanks my almighty GOD for giving me courage to cope up my research even in the complicated situations and I express my regards to my external advisor Dr. Jakuamer (India) and internal advisor Shegaw Ahimed (Dr), for their invaluable supervision enabled me to complete my research work effectively. My appreciation also goes to Dr. Madan for his supportive contribution by giving all the necessary materials need for the completion of my research work and also I thank my granted organization (LIDI) as part of the CLRI-LIDI twinning program, which has sponsored the study.

Grateful acknowledgements will be extended to the workers of (CLRI), laboratory experts for their willingness; in supplying relevant equipment and materials in addition to their hospitality during the period of laboratory work will thanks. Finally, but most significantly, I would like to convey my truly regards to my family for their help and advice without which I cannot complete the study on time.

Abbreviations and Acronyms

AAT	Addis Ababa Tannery
HP	Hydroxy proline
GAGs	Glycosaminoglycan's
CD	Circular Dichroism Spectra
CLRI	Central leather research institute
FTIR	Fourier Transform Infrared Spectroscopy
LIDI	Leather industry development institute
M	Protein marker
GC	Green collagen
CS	Chondroitin Sulphate
E	Experiment
SEM	Scanning Electronic Microscope

Table of Contents

Contents **Page**

DECLARATION i
Acknowledgements ii
Abbreviations and Acronyms iii
Table of Contents vii
List of table ix
List of Figure x
List of Annex xi
Abstract x
CHAPTER ONE 1
INTRODUCTION 1
1.1 Background of the study 1
1.2. Problem Statement 3
1.3 Objective 4
1.3.1 General objective *4*
1.3.2 Specific Objective *4*
1.3.3 Scope *4*
1.4 Significance of the Studies 4
1.5 Framework of the Studies 5
CHAPTER TWO 6
LITERATURE REVIEW 6
2.1 Introduction 6
2.2 Glycosaminoglycan's and proteoglycans 6
2.2.1 Glycosaminoglycan *7*
2.3 Extraction and structural analysis of Glycosaminoglycan's 10
CHAPTER THREE 14
METHODOLOGY 14
3.1 Experimental Methods 14
3.2 Materials 15
3.2.1 Raw materials collection *15*

3.2.2 Chemicals and reagents used for analysis *15*
3.2.3 Equipment, Instruments and Apparatuses *16*
3.3 Methods 18
3.3.1 Determination of the basic components of raw hide *18*
3.3.1.1 Moisture value *18*
3.3.1.2 Percentage of Fat *18*
3.3.1.3 Protein content analysis *18*
3.3.1.4 Determination of total ash content *19*
3.3.1.5 Glayconsaminoglaycans content *19*
CHAPTER FOUR 21
RESULTS AND DISCUSSION 21
4.1 The Composition of Hide Trimming 21
4.1.1 The percentage of moisture content *21*
4.1.1.1 The percentage of fat content *21*
4.1.1.2 The percentage of protein content *22*
4.1.1.3 The percentage of ash *23*
4.1.1.4 The percentage of Glayconsaminoglaycans content *24*
4.2 Factors affecting Glayconsaminoglaycans analysis 24
4.2.1 The effect of pH Glayconsaminoglaycans content *24*
4.2.2 The Effects of pH on Glayconsaminoglaycans *25*
4.2.3 Temperature effect on GAGs content *26*
4.2.4 The effect of enzymes concentration on glycosaminoglycan's *26*
4.2.5 Time duration factors of glycosaminoglycan's production *27*
4.3 Chemical composition of extracted Glycosaminoglycan's 28
4.4 FTIR result of Glayconsaminoglaycans 29
4.5 NMR Result of NMR Glayconsaminoglaycans 31
CHAPTER FIVE 33
CONCLUSION AND RECOMMENDATION 33
5.1 Conclusion 33
5.2 Recommendation 34
REFERENCES 35
APPENDIX 39

List of table

Table3.1 Chemicals used on optimization 16

Table3.2 List of apparatuses 17

Table4.1 Moisture content value 21

Table4.2 Fat content percentage 22

Table4.3 Protein content percentage 23

Table4.4 Ash content percentage 23

Table4.5 The effect of pH on gags 25

Table4.6 Effect of temperature on GAGs 26

Table4.7 Effect of enzyme on GAGs 27

Table4.8 Effect of time on gags 28

Table 4.9 Composition of glycosaminoglycan's preparation 28

Table4.10 Glayconsaminoglaycans FTIR result 30

List of Figure

Figure 2.1 Structure of the saccharide 7

Figure 2.2 Extraction of GAGs from disaccharide 13

Figure 3.1 Extractions of Glayconsaminoglaycans 14

Figure 4.1 Glayconsaminoglaycans FTIR results 30

Figure 4.2 NMR Result 31

List of Annex

Annex 1 Enzyme selection based on the Analysis 39

Annex 2 Dilution factor selection based on the Analysis 40

Annex 3 List of chemicals types 41

Annex 4 List of Laboratory equipment 42

Annex 5 List of Laboratory apparatus and Range of Operations 43

Annex 6 UV spectroscopy reading at 525nm 44

Annex 7 Raw material collection and Sample preparation 45

Abstract

Glycosaminoglycans are the most plentiful type of organic active polysaccharides mainly found in animals. Chondroitin sulfate (CS) are one of the classes of glayconsaminoglaycans that can commonly use in pharmaceutical, cosmetics and beverage industries as well as in treatment of arthritis and eye diseases. Therefore, taking out of Glayconsaminoglaycans from cow hide trimming waste by papain enzyme is necessary. In these process temperature, time, enzyme and PH are variables that can affect the result of Glayconsaminoglaycs. The best result of data was taken at pH of 6.7, enzymes concentration 0.6 %, temperature of 68^{O}C and hydrolysis time 3 hours. The extracted Glayconsaminoglaycans were 0.5 % and its composition were 62.7% carbohydrates and16.4 % proteins, moisture content 13.1%, and ash content 7.8 %. The experiment analysis was analyzed by using UV spectroscopy and characterize by FTIR and NMR analysis.

Keywords:-GAGs, rawhide, trimming waste

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Our country Ethiopia has much population of animals. From this huge livestock population, extensive amount of hides and skin production is collected from a slaughterhouse. These hide and skin sectors helps to attain income for our industry and give as jobs opportunities for many peoples. To change wastes to high value products various researches are necessary. So we have to focus on the leather sector challenge and solution for the development of leather manufacturing. The extraction of glycosaminoglycan's by papain enzyme from raw hides trimming waste research is important to have new products and to utilize wastes (Tahir et al., 2018).

Glycosaminoglycan's plays important biological roles and that can be classified based on the groups of sulfated complex and the variance of repeating disaccharide units. Glycosaminoglycan's based on sulfated complex can be grouped into four main classes: heparin sulfate, Chondroitin sulfate, Derma tan sulfate, Keratin sulfate, and Hyaluronic. Glycosaminoglycan's are extremely plentiful type of organic active polysaccharide mainly found in animals. Glycosaminoglycan is referred to as mucopolysaccharides. These muco polysaccharides are consisting of repeating disaccharide units. The main groups of glycosaminoglycan's are based on the difference of sugar content and glycoside linkage (Tahir et al., 2018).

The groups of Glycosaminoglycan are classified based on their central disaccharide units and include heparin, Chondroitin, Derma tan, Keratin sulfate, and Hyaluronic. The amine group can be attached with and without the monomer of carbon ring. A glycoside bond has connection with the monomers together.

In all kinds of life carbohydrates are the main building block. The chemical nature of these molecules is self- explanatory name already found in the nature of compounds. These compounds are the composed of carbon, hydrogen, oxygen. Also all carbohydrates are the

consist of all these exclusively composed of elementary atoms. The composition of the constituent of monosaccharide's are goes to the linear or branched polysaccharides. (Piepkorn et al., 1989).

In the Glycosaminoglycan the additional result of these sulfates is the highly negative charge with the carboxyl blocks. These give Glycosaminoglycan heparin being the most negatively charged biological macro molecule. In another differentiating property glycosaminoglycan are often sulfated at different positions and resulting from differences in disaccharide composition. The fact that this sulfation pattern can vary in any given type of glayconsaminoglaycans, implies that the heterogeneity of GAGs is enormous (Acott et al., 1988).

The extraction of gags by using papain enzyme with different percentage conditions and by considering enzyme factors like (pH, and temperature and extraction time, dilution factor) are needs to know the right steps to collect high percentages of gags. The extracted gags from trimming row hide waste and gain raw materials are useful for medicine, beauty products and food production. The research also focuses on the main activities such as extraction of Glayconsaminoglaycans solution preparation, characterization of Glayconsaminoglaycans and collagen, assessing the quality gabs of extracted gags and finally assessing the chemical composition of extracted Glayconsaminoglaycans.

1.2. Problem Statement

The presence of gags in various invertebrates as well as vertebrates has been nicely documented. Many researchers have reported extracting glycosaminoglycan's from fish, pigs, chicken, sea snakes, marine and Bacteria in different years, but still, The demand of glycosaminoglycan's for the market supply is high because glycosaminoglycan's are very important product for medicine, beauty products and food production and widely used in (Raghuraman, 2013).

The production of glycosaminoglycan's is needed in different industries. Extraction of gags from rawhide trimming waste is another part of gaining high products from byproducts. Even in the composition of rawhide and skin, the percentage of gags is in small amounts, but it has high values and is very important in the industry.

Research projects like glue production and leather boarding were done to change products of hide trimming waste to high-value products. But glycosaminoglycans are a very much higher-value product than the others. Because of the extraction method's high cost, still extracting the glycosaminoglycan content is difficult to solve the shortage of glycosaminoglycan's production, and to benefit the company, we have done our research, study on extraction of glycosaminoglycan's by using papain enzyme from rawhide trimming waste so in these projectam designing a short and easy method to extract high value product glycosaminoglycan from cow hide trimming waste and to change wastes in to useful materials from byproducts of cow hide.

About 60% of the total non-chrome solid wastes are generated from tanneries solid waste. The major disadvantages of conventional beam raw hide trimming are the causing of fleshing waste with lime that can pollute the environment. If these wastes were without lime, it was easy to use the constituents. So, green fleshing was the best option for the regaining of valuable collage nous constituent. In Ethiopian tanning industries around 26 tons/day fleshing wastes was produced.

But still now a small amount of fleshing wastes are try to use for glue production. The large amount fleshing wastes are damped to the surrounding environment. To extracting high valuable byproduct from fleshing wastes is achievable with acetic acid solubilisation (Tsegaw, 2018).

1.3 Objective

1.3.1 General objective

The main idea of this research is to extract glycosaminoglycan's from raw hide trimming waste by using papain enzyme and to gain an income for the leather industry and our country.

1.3.2 Specific Objective

- To extract optimum amount of Glycosaminoglycan's from raw cow hide trimming waste
- To Study the effect of enzyme hydrolysis time (1, 3, 5, 7 and 9 hours) and papain concentration (0.2 to 1.2 %), pH (5.5 to 8) and temperature (50 to 75 °C).
- To gain high value product glycosaminoglycan's by using short and appropriate method for extraction from cow hides trimming waste
- To characterize the extract glycosaminoglycan's from raw hide trimming waste.

1.3.3 Scope

The scope of the paper focuses on extraction of glycosaminoglycan's by using papain Enzyme analysis from the waste to gain very important product glycosaminoglycan's and using commercially available and few chemicals, by designing new process to collect high amount of Gags from raw hide trimming waste.

1.4 Significance of the Studies

In the leather sector manufacturing process different byproducts are dumped to environment. Even in first level of process contributes about more than 60% wastes of the wastes are discharged in the form of solid, liquid and gases. From this process raw hide trimming wastes are one of the solid byproducts wastes that must change to useful and valuable products. Reducing the wastes that are discharged to the environment is very critical point to see new technology that friendly to environment.

The study gives a way for rawhide trimming waste management and income for the sector by recovering useful products from the wastes, which otherwise is dump to the surrounding. Extraction of Glycosaminoglycan from rawhide trimming waste is one of the ways of gaining high value products which is useful for pharmaceutical and cosmetic industries. Introducing and creating such types of research is very important to use byproducts and change into useful product and income generation.

1.5 Framework of the Studies

The paper is grouped into five sections. In the initial step: enzyme assists Extraction process based on the result of research and hydrothermal process of rawhide wastes will discuss. Second section: preparation and quantification of glycosaminoglycan by different temperature, time, and pH and enzyme concentration from raw hide trimming wastes. The third steps enzymatic unhairing activity with each parameter. Fourth; extract glycosaminoglycan from rawhide trimming wastes. Finally the detail chemical properties of the glayconsaminoglaycans are analyze in the laboratory.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Leather making process is the transforming of putrefying raw hide/skin in to non-putrefying. In this process, from beam house to finishing different chemical and materials are used in any steps. Beam house stages are the waste removal operation section and ready for the second steps of the process. It includes several operations, waste removal stages, removal of hair and adding liming deliming, and pickling procedure (Tsegaw, 2018).

Glycosaminoglycan's can be classified based on the groups of sulfated complex and the variance of repeating disaccharide units. Glycosaminoglycan's can be grouped into four main classes: heparin sulfate, Chondroitin sulfate, Derma tan sulfate, Keratin sulfate, and Hyaluronic. Glycosaminoglycans are extremely plentiful type of organic active polysaccharide mainly found in animals. Glycosaminoglycan is referred to as mucopolysaccharides. These muco polysaccharides are consisting of repeatingdisaccharide units. (Van Wijk et al., 2012).

For the creativity of new technology we have to focus on the undiscovered things need to be study are the main wide study area and so much focus on applications of this high-molecular-weight polysaccharide to tissue engineering have been investigated. Because of these still the market supply needs high production of glycosaminoglycan's (Tahir et al., 2018).

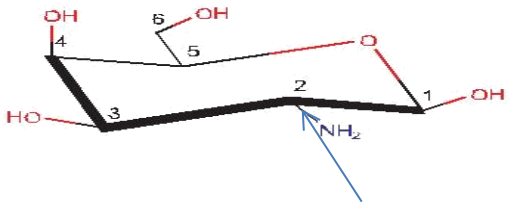
2.2 Glycosaminoglycan's and proteoglycans

Glycosaminoglycan, which are long linear polysaccharides that are sulfated and have a negative charge, have a molecular weight ranging from 10 to 100 kDa. In the Glycosaminoglycan the additional consequence of these sulfates is the highly negative charge with the carboxyl blocks of the hexuronic acids, these gives Glycosaminoglycan heparin being the most negatively charged biological macro molecule known. (AMS Biotechnology, n.d.).

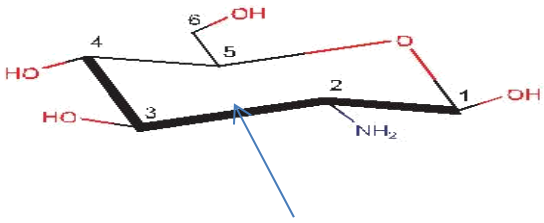
2.2.1 Glycosaminoglycan

Heparin sulfate is plays a significant role control in the interaction between cells and in the interaction of extracellular matrix.. Heparin sulfate is motivating the adhesion of cells to extracellular matrix by binding themselves to matrix macro molecules. Structurally, most glycosaminoglycan's are among other things either a glucuronic or iduronic acid, linked to either a glucosamine or a galactosamine to form a repeating disaccharide (Raghuraman, 2013).

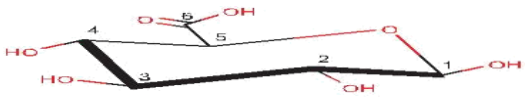
Iduronic acid



Glucuronic acid



Galactosamin



Glucosamine

Figure 2.1 Structure of the saccharide

Chondroitin sulfate is containing a monosulfated replicate disaccharide collected of D glucuronic acid as well as N-acetyl-galactosamine. Chondroitin sulfate is a group of chain of alternating sugars. Mostly chondroitin sulfate is found linked to proteins as part of glycosaminoglycans. The link can have over 100 individual sugars. (Henrotin et al., 2010).

In virtually all connective tissues of the body Chondroitin sulfate are attached to proteoglycans. Salvation patterns are determine specific biological activities of

Chondroitin sulfate and its core protein play an important role in the collagen. The saccharide of chondroitin is shown to ease collagen cross linking. When it exposure to UV light chondroitin sulfate content are shown. (Henrotin et al., 2010).

Dermatan sulfate are consists of repeating units like L-iduronic acid and N-acetyl-galactosamine. Both Dermatan and chondroitin sulfate are reflecting structural isomers. Epimerization of the carboxyl group around of glucuronic acid can creates the iduronic acid of dermatan sulfate. The existence of iduronic acid plays a vital role in determining the specificity of glycosaminoglycan with protein interactions. Dermatan are the major glycosaminoglycan found in skin and when associated with decorin and it is directly involved in collagen fibril formation (Fransson & Rodén, 1967).

Heparin sulfate are a type of glycosaminoglycan consisting of uronic acid with linear polysaccharides Variable styles of substitution of the disaccharide subunits give upward push to an wide of verities of complex sequences. Structurally among the glycosaminoglycan (GAG) family of polysaccharides Heparin is the most complex members. In contrast to heparin, Heparin sulfate are occurs only in most cells. HS proteoglycans are mostly located on cell surfaces and in the extracellular matrix. With a verities core protein there are many families of proteoglycans. (Yan, 2017).

In different studies have reported heparin sulfate are exists more in skin meanwhile in vitro functions in skin are not yet been fully discovered. Study suggested that heparin sulfate hold back collagen fibrillogenesis. (Yan, 2017).

Keratin sulfate are consists of repeating disaccharides unit. Unlike other glycosaminoglycan's, protein core keratin sulfate can be O-linked to threonine or N linked to aspargine. keratan sulfate are linked to lumican covalently in the side chain and associated with fibrillar collagen(Habuchi et al., 2002).

Hyaluronic acid is non-covalently linked to any protein and synthesized as a non-sulfated polymer. Because of its linear poly anion character it has a very high molecular weight. It holds water with high concentration and found in synovial fluid, articular cartilage, skin, vitreoushumor and ECM of loose connective tissue (Panelewen et al., 2015).

In the extra molecular matrix of skin hyaluronic acid is the major component and it maintains the normal hydration of skin and distributes salts and nutrients. Generally, skin hyaluronic acid is about 50% of total body hyaluronic acid. It shown that hyaluronic acid enhances the enzymatic resistance and the mechanical properties of collagen (Panelewen et al., 2015).

In skin duration of age the concentration of hyaluronic acid does not change but its length and its saccharide unit's change. The concentration of hyaluronic acid in dermises significantly higher than in the epidermis but it is found in both the dermis and epidermis associated with both collagen fibrils and elastin fibers. (Panelewen et al., 2015).

The extraction of Glayconsaminoglaycans is in particular executed by means of the enzymatic deployment of exogenous proteases or sodium hydroxide Palmy. So for extractions of gags enzymes are very important but its price is so expensive papain is the best for glycosaminoglycans extraction and to give high yields of Glayconsaminoglaycans and the price also better (Le Vien et al., 2017).

2.3 Extraction and structural analysis of Glycosaminoglycan's

The viability of glayconsaminoglaycans extraction from FFPE tissues were used rat liver and pores and skin. The tissues were both with formalin- constant and paraffin embedded. Initially paraffine embedded tissues have been removed ear. (Figure 2.1A). The system with papain digestion are need (TCA) for precipitation and followed by alternate chromatography and methanol precipitation. The special styles of glycosaminoglycan's should certainly be extracted and isolated from FFPE tissues (Figure2.1B). The liver contained roughly similar level of both CS and HS compared to the skin. The Glayconsaminoglaycans chains size could be affected as in the case with RNA contained from FFPE tissues. Glycosaminoglycan's from frozen and FFPE samples were study using poly acryl amide gel electrophoresis (Figure2.1C). The GAGs domain structure was compromised by FFPE treatment. (Figure 2.1D) (Van Wijk et al., 2012).

Glayconsaminoglaycans separated from FFPE (with pre-deparaffination and without) or frozen tissues, for the skin and the liver two enormous contrast between the GAG composition of the skin and liver was the huge innovation of the skin with the anti-D S anti body. Most cells contain heparin, a largely sulfated which is recognized by the anti-body. The structural composition of GAGs was further analyzed on the disaccharide level by using heparinases. (Figure2.1E), (Figure 2.1F) (Van Wijk et al., 2012).

The main disaccharide constituents of the liver and their relation were relatively related between FFPE and frozen samples shown in the below (Figure 2.1E). Again, the composition was not changed by FFPE treatment (Van Wijk et al., 2012).

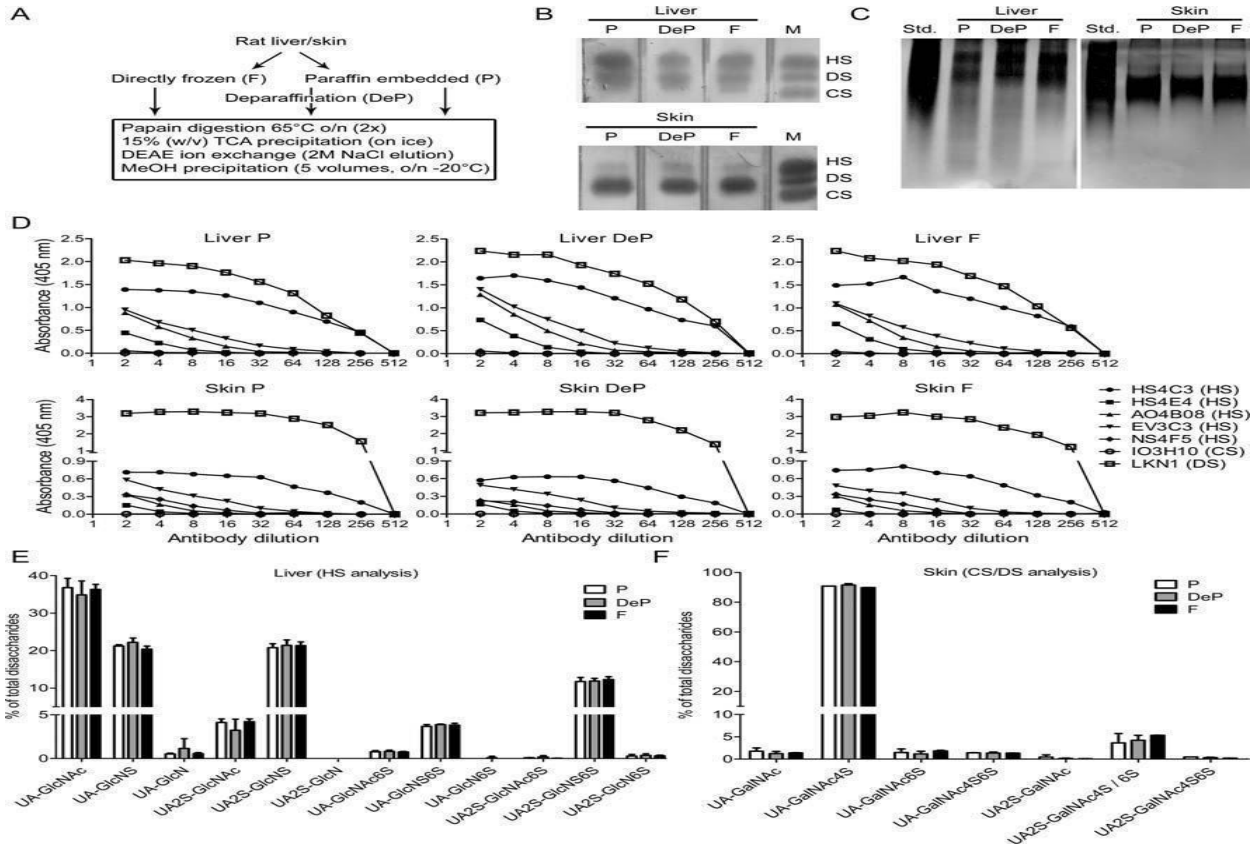
The concentrations of extracted Glayconsaminoglaycans using the meta chromatic dye dimethyl methylene blue. The amount isolated from FFPE skin tissues was less than that the amount significantly com- pared with frozen tissue. For the liver, a direct relation could not be made since frozen material was homogenized, for the preparation of FFPE material was cut into pieces both frozen and FFPE materials Combined. These results indicate the skin is sufficient to amount to analyze Glayconsaminoglaycans related with frozen tissue, but further research may be needed for other tissues (Van Wijk et al., 2012).

Glayconsaminoglaycans amount and categorize have been identified in high performance liquid chromatography. The analysis was extracted to know the disaccharide composition. The relation in composition may be seen by the relative increase in chondroitin sulfate relate with the light sensitivity and the differences were noted as well (Raghuraman, 2013).

To identify the researcher methodology and to extract high-quality Glayconsaminoglaycans from FFPE tissues and show that properties and application related with those evaluated from frozen tissues are Glayconsaminoglaycans can be analyzed from as little amount. In different section and laser- dissected structures can be tasted as shown for different layers of the skin. Associated with differences in disaccharide structure were performed using the steps of tissue blocks from the diseased skin. The use of archival FFPE material and opens new avenues enables to determine Glayconsaminoglaycans and relate this with patient man related data (Raghuraman, 2013).

Glycosaminoglycan extraction from different tissues in Fig.2.1 analysis and isolation of the structural and extraction of GAGs from different tissues work flow. The types of analysis by agarose gel electrophoresis are obtained by taking some amount of sample for gags determination. The Size analysis of Glayconsaminoglaycans link by poly acryl amide

electrophoresis some amount of gags was added. The Standard was added from the bovine (Le Vien et al., 2017).



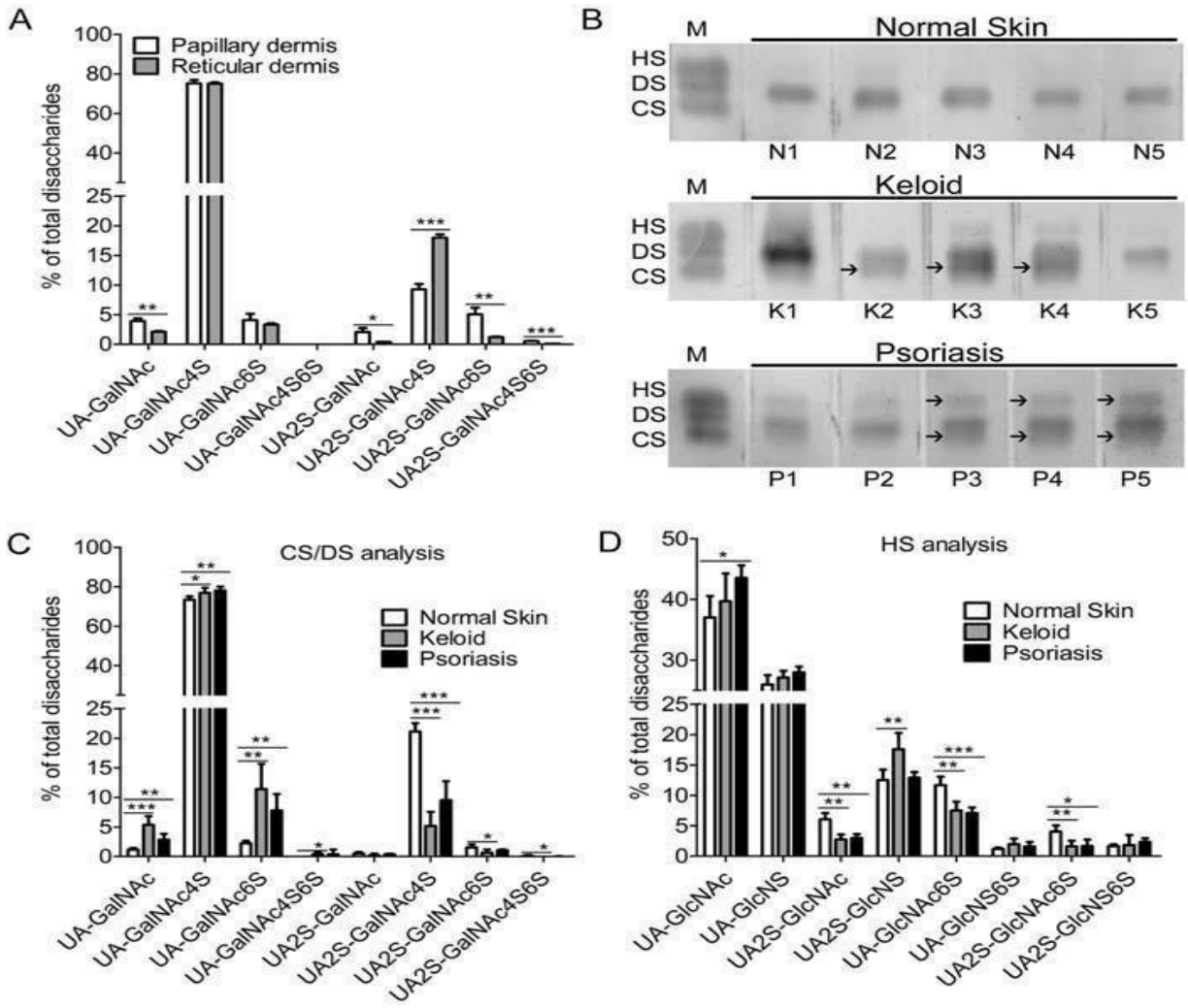


Figure 2.2 Extraction of GAGs from disaccharide

The compositional disaccharide analysis by high performance liquid chromatography Glayconsaminoglaycans was enzymatically obtained under good condition in the disaccharides extraction and the data are presented from the three different isolations for disaccharide extraction.

Glycosaminoglycan's from skin layer can take by laser micro dissection. Among those three multiple enzyme digestions and Composition can be gather in the layer different sections of skin.

CHAPTER THREE

METHODOLOGY

3.1 Experimental Methods

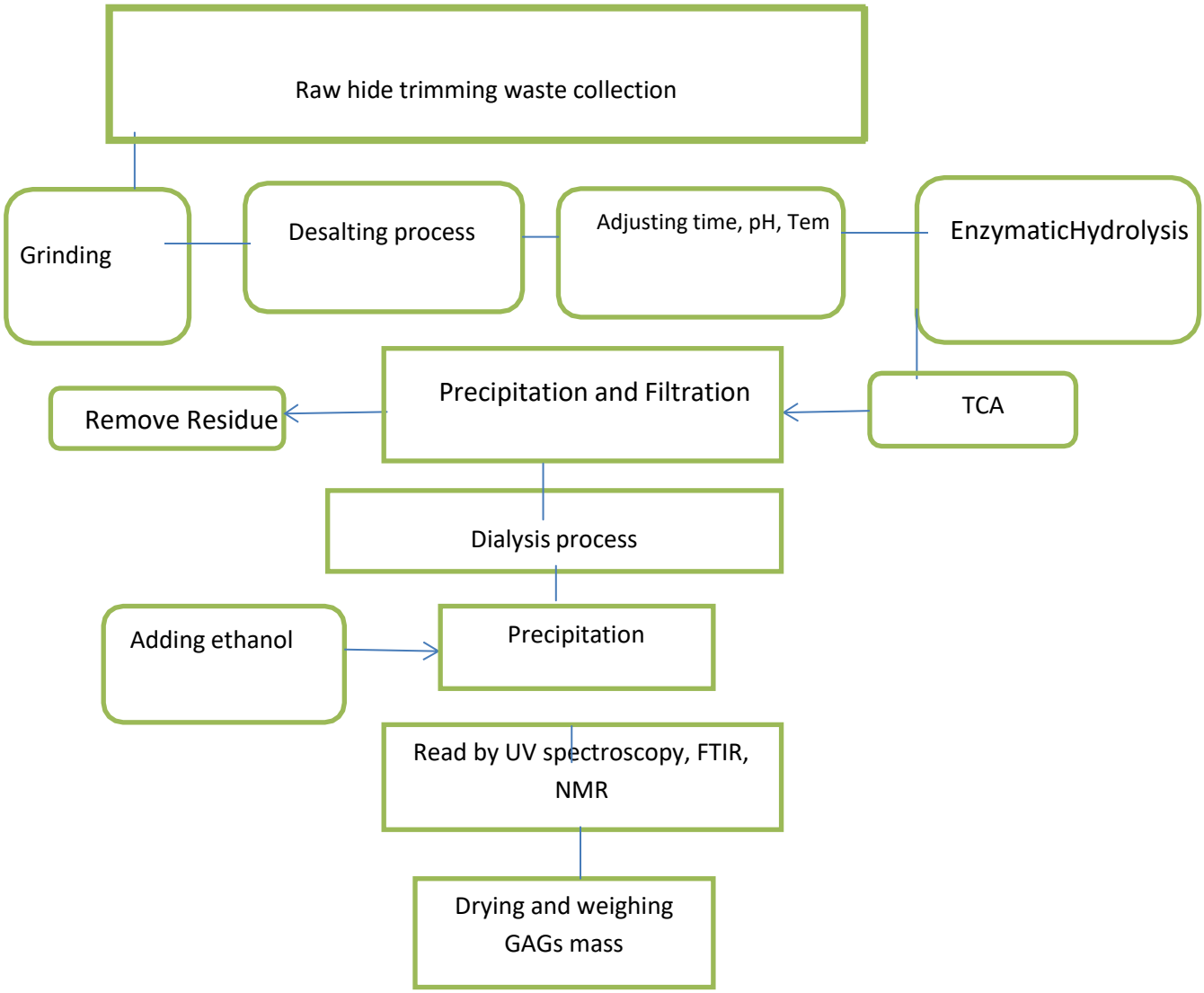


Figure 3.1 Extractions of Glayconsaminoglaycans

The steps of laboratory process describe the Optimization of GAGs by Enzymatic Hydrothermal analysis from raw hide trimming waste. Trimming cow hide waste was used asraw materials for optimization of glycosaminoglycan's. The sample was washed and cleaned well for lab processing to avoid any salt, dung and any contamination. The sample size was reduced by scissor cut followed by mince grinding machine to grained and ready for experiment. The hydrolysis was done by adjusting the pH in the range of 5.5 to 8 and heat range was in the limit of 50 to 75 ^{O}C.

The percentage of enzyme was in the limit of 0.2 to 1.2 (w/w) and time was in the range of 60 to 540 minutes. After hydrolysis the papain was deactivated at 90 oc for 10 minutes.The solution was spark by TCA of 6% was added for precipitation and filtration and precipitate over night at 10 ^{O}C, the mixture was separated by vacuum filtration through filter paper. The solution was dialyzed by the membrane with the size of 14KDa for four hours changed periodically and the water was changed every 30 minutes to remove wastes and any un necessarily things, and then finally the solution was precipitated glycosaminoglycans over night at 10oc by 99% ethanol with the ratio of the mixture per solvent ethanol of one to one ratio. The solution was dried at 60 ^{O}C to collect power. The gags were determined by chondroitin sulfate by UV spectroscopy and characterized by FTIR and NMR.

3.2 Materials

3.2.1 Raw materials collection

For the purpose of this study the raw material which is raw hide trimming waste was collected from (CLRI) institute of model tannery in Chennai, India. The raw material was wet salted cow hide so it was washed to remove the salt, dung and any dirt's from sample.

3.2.2 Chemicals and reagents used for analysis

The chemical used for laboratory analysis was all lab grades and the reagent and chemical used for the laboratory analysis was listed below in the Table 3.1.

Table3.1 Chemicals used on optimization

Chemicals	Use
Chondroitin sulfate	Used as standard to determine glycosaminoglycan's
Papain	Used to extract GAGs
Sulphuric acid	Used for digestion of raw hide trimming waste to determine Protein
Acetic acid	To adjust pH in the analysis
Ethanol	Used for the separation of gages as solvent
Trichloroacetic acid (TCA)	Used for the precipitation of sample mixture in gages analysis
Copper sulphate anhydrous	Used as catalyst
Potassium sulphate Anhydrous	Used as catalyst
1.9-dimethylmethylenblue (DMMB)	Used to Determine the chondroitin sulfate form skin .
Dichloro methane (DCM)	Used as solvent fort the analysis of fat content

3.2.3 Equipment, Instruments and Apparatuses

The gags were determined by chondroitin sulfate by UV spectroscopy and characterized by FTIR and NMR. For the laboratory analysis we used different equipment and apparatus. The list is mentioned name of instrument below with its model and efficiency. Instruments used in the analysis are listed in Table 3.2.

Table3.2 List of apparatuses

Equipment	Model	Use
Uv spectroscopy	V-730UV	ıe content of glycosaminoglycan's by 525nmwavelength
Heating oven		To determine the moisture content of raw hide Trimming waste
Muffle furnaces		To analyze the percentage of ash content
Kjeldahle distillation Unit	IK-194	To determine the hide substance of raw hide trimming Waste
Soxlet apparatus	LTSW-5	the percentage of fat content in the Raw hidetrimming waste
Simple distillation		To separate dichloromethane solvent in the analysis of Fat
Rotary shaker		For extraction of gags
pH		To read the pH value of sample
FTIR	Jasco FTIR/4200 Ty pe A	To analyze the functional Group of the sample
NMR		To know the functional group
Weighing balance		To weighing the sample Amount of taken in the analysis
Mince grinding machine		To reduce the sample size

3.3 Methods

3.3.1 Determination of the basic components of raw hide

3.3.1.1 Moisture value

Moisture value is one of the compositions of hide and skin that varies based on the heat and humidity of the surrounding. To calculate the Moisture value we can take some amount of sample and small amounts of solvents. The percentage of moisture can be determined by taking the amount of sample both sides before and after drying and subtracting the weight of crucible. Sample was taken from the raw hide trimming waste. The apparatuses used for this analysis were crucible, heating oven and weighing balance. SLC-3 method was used to determine moisture content. A 3-gram trimming waste weighed, then dried for five hours at 105^{O}C in heating oven, and then cooled to room temperature in desiccators and weighed. Then drying, and weighing process up to gain the some weight. Moisture value is expressed as a percentage of its dry weight.

$$moisture\ contetn\ , \% = m1 - m2/m1 * 100$$

Where, m1 = wet sample m2 = dry sample

3.3.1.2 Percentage of Fat

Soxhlet extraction method was used to determine fat percentage. By taking samples (3gm) from the hide trimming waste, the sample was weighed on balance and loaded to fat extraction apparatuses, In Soxhlet fats are extracted for five hours by using Dichloro methane. This method is the official extraction method. The fat was obtained in the Soxhlet, and the solvent isolated and obtained in the tank for recovery and dried at 103 ^{O}C.

$$(fat\ contetn,\ \% = (\ wf/ws)) * 100\ )$$

Where, Wf = extracted fat weight,Ws=sample weight

3.3.1.3 Protein content analysis

The reagent was 0.5N sulphuric acid, copper sulphate anhydrous, potassium sulphate anhydrous. The hide trimming waste sample was collected and 3g weight was used for analysis in 250 ml flask with 20ml sulfuric acid and the flask was heated. The sample was digested with 5g

potassium sulphate anhydrous and 5g copper sulphate anhydrous catalyst, the process was heated until the solution shows color changed. This process could take about 30 minutes to finish the steps. The solution was collected and put in to an ammonia distillery. Very clean funnel was used to add sodium hydroxide solution (0.05m) until the solution turned to colors of black. Methyl orange was an indicator and the ammonia was distilled in to100 ml of 0.05 molar of sulfuric acid. But ammonia has been distilled after obtaining roughly 150 ml of distillate, and these steps could takes around 40 minutes. 0.05molar NaOH was used to return titrate the 0.5N sulfuric acid. (SLC-7).

$$protien\ contetn\ ,\% = \left({}^{a}/_{b}\right) * 100$$

Where, a = hide substance wt in gram,

b = hide trimming waste wt in gram

3.3.1.4 Determination of total ash content

Three-gram hide trimming waste was placed in a ceramic crucible. The trimming waste sample was burn on a hotplate fume before 800 ^{O}C furnaces. The concentrated ammonium nitrate solution was added to burn all of the carbon. After adding of hot water to the residue the solution was filtered. To complete all ashes the residue was washed again and again. SLC-6 method was used for the analysis. Thepercentage was calculated.

$$\text{Ash contet ,\%} = \left(\frac{T_1 - T_2}{T_1}\right) * 100$$

Where, T1= wet sample (g)

T2= dry sample (g)

3.3.1.5 Glayconsaminoglaycans content

The sample was washed and cleaned well for lab processing to avoid any salt, dung and any contamination. The sample size was reduced by scissor cut followed by mince grinding machine

to grained and ready for experiment. The hydrolysis was done by the pH in the range of 6.5 and temperature was 65 ^{O}C. The percentage of papain enzyme was 0.6 % (w/w) and the time was 180 minutes. After hydrolysis at 90 ^{O}C the papain was deactivated for ten minutes. The solution was settled by TCA acid with percentage of 6% was added for precipitation and filtration and precipitate over night at 10 ^{O}C, the solution was separated by air sucking filtration method by filter paper and then dialyzed by the membrane with the 14KDa for four hours steps changed the water each 30 minutes to separate impurities. Finally the solution was precipitated GAGs over night at 10oc by 99% ethanol solvent at the related solution per ethanol of one to one ratio.. The solution was dried at 60 ^{O}C to collect power. The gags were determined by CS content by UV spectroscopy (Le Vien et al., 2017).

$$\%\ \text{of GAGs contents} = \left(\frac{OD - 0.040KV}{0.004m(100-h)1000}\right)100$$

Where, OD= absorbance at 525nm

V: volume of mixture after final process (ML)

m: trimming waste (g),

K: dilution ratio, H: moisture value]

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 The Composition of Hide Trimming

4.1.1 The percentage of moisture content

Hide has different composition. The main components of hide are moisture, protein, fat and ash. Moisture is the highest composition in hide which contributes 67.6 % of the total composition. The second components proteins were 23 % and the third other 5.2% the fourth one fat was 2.35% and fifth composition of hide ash was 1.9%. The result of experiment listed below in the table.

Table4.1 Moisture content value

S, No	B1(gram)	B2(gram)	B1andB2(gram) after drying= B3(gram)	B1-B2= B3 (gram)	Percentage (%)
1	5.06	0.486	2.16	1.7	66.7
2	5.10	0.550	2.15	1.6	68.6
3	5.02	0.409	2.009	1.6	68.1
					Average=67.7

Where; M1; sample (g) M3 = dried sample and aluminum foil (g)

M2; aluminum foil (g) M4= mass of dried sample

As shown in the table, the experiment was repeated three times with slightly similar sample weight and aluminum weight. From the average the percentage of moisture content was calculated. The moisture content of the sample nature has great role in the value of glycosaminoglycan's result calculation. So moisture value analysis is important for the analysis of glycosaminoglycan.

4.1.1.1 The percentage of fat content

Fat is one of the compositions of raw hide trimming waste. Soxhlet apparatus is very common for analysis of percentage of fat value. The amount of fat was 2.3% and the experiment was repeated three times. Determination of fat is important to know the percentage of fat available in

the sample and to understand the sample nature for the gags experiment. The lab data listedbelow in the table.

Table4.2 Fat content percentage

SN.	B1	B2	B1+B2=M3 after extraction	B3-B2=B3 After room temperature	% of fat content
1	3.0124	30.3450	33.3574	0.0658	2.18
2	3.006	31.023	34.029	0.068	2.26
3	3.1325	30.230	33.3625	0.0768	2.45
					Average=2.29

Where;

B1= sample (g) B3 = fat and crucible (G)

B2 = crucible (g) B4 = fat (g)

As shown in the table 4.2, the analysis was done 3 times and the average fat content was 2.296 %. The weight of crucible was subtracted before calculation to know the extracted fatcontent in gram. The crucible weight measured before and after the extraction to know the net dry weight of fat content.

4.1.1.2 The percentage of protein content

To determine the protein content 3g of hide trimming waste was taken and digest by 30ml sulphuric acid with 5g of catalyst mixture of 50g of copper sulphate anhydrous and 500g of potassium sulphate anhydrous for one hour up to all carbon oxidized. Then digested sample was cool to room temperature and dilute with 50ml of distilled water. After digestion 100ml saturated boring acid was added and distillated by kjeldahl distillation unit for 3 minutes. The receiver was settled properly to collect the solution. Finally, I used 0.5N sulphuric acid for titration of the solution and 23% of protein was recorded from the test result. The experiment was done three times. The result recorded below in the table.

Table4.3 Protein content percentage

S,NO	Sample	30ml (H_2SO_4) and 50g catalyst digest (1hour)	100 ml boric acid digest 3 minutes	Amount of 0.5N H_2SO_4) taken	Percentage of protein
Blank	-	1 hour	3 minutes	0.5 ml	-
1	3.0012	1 hour	3 minutes	11.3ml	25
2	3.0134	1 hour	3 minutes	12.4ml	21
3	3.0120	1 hour	3 minutes	11.5ml	24
					Average=23.3

As shown in the table 4.3, the analysis of protein was proceeding by acidic digestion followed by kjeldahl digestion. After digestion 0.5N sulphuric acid was used for titration. Protein analysis is important to know the sample nature and in the recovery of collagen.

4.1.1.3 The percentage of ash

For the analysis of ash value, 3gram of grinding hide trimming waste was taken and burns by flame and ignited at the furnace at 750 ^{O}C up to all changed to ash, the cool in the desiccators at room temperature the result of ash was 1.9%. The experiment was done three times.

Table4.4 Ash content percentage

S.NO	Sample weight in gram	Crucible weigh in gram	Sample and Crucible wt. after ignition	Net weightof ash	% of ash Content
1	3.0012	20.456	23.3694	2.95	1.7
2	3.0	22.134	25.0252	2.94	2.0
3	3.04	20.657	23.5535	2.98	1.97
					Average=1.89

As shown in the table 4.4, the percentage of ash content nearly the same amount from the three analyses, so the result taken from the average of three repeatedly laboratory result.

4.1.1.4 The percentage of Glayconsaminoglaycans content

The total Glayconsaminoglaycans content in the samples were measured using a quantitative dye-binding method. The dye binding occurs at both sulfate and carboxyl groups on the Glayconsaminoglaycans molecules. Chondroitin 4-sulfate was used as standard to determine total Glayconsaminoglaycans content in the samples. 525nm UV spectroscopy was used to read and the analysis was done 4 times.UV spectroscopy analysis result recorded on Annex 5.

The blank was read by 100 ml distilled water and2.5ml1.9-dimethylmethylen blue reagent but thestandard start from 10ul to 100mul. 50ul sample was taken for the analysis and dilute by 50ul distilled water. All samples one to seven are the same sample of glycosaminoglycan's analysis. 2.5 ml reagent wastaken to read by 525nm UV spectroscopy. The analysis was done four times.

4.2 Factors affecting Glayconsaminoglaycans analysis

The amount of Glayconsaminoglaycans contents was fixed and the dilute amount was varied it showed different result was gained. Maximum amount of Glayconsaminoglaycans was gained in a ratio of 1:4. The amount of Glayconsaminoglaycans was decrease as the amount of dilution rate increased. We can conclude that the amount of Glayconsaminoglaycans was high at1:4 level of dilution rate, Annexes 2. Time, temperature, pH and amount of enzyme content are the main factor of Glayconsaminoglaycans extraction.

4.2.1 The effect of pH Glayconsaminoglaycans content

From the data of analysis at a faster rate at pH 6.5 Sulfated proteoglycans were released. From the analysis the data shows that pH value can affect the analysis and the steps of distribution of active analyzed sulfated Glycosaminoglycan's.

Table4.5 The effect of pH on gags

PH	Glayconsaminoglaycans percentage	Unit
5.5	0.28	%
6.0	0.36	%
6.5	0.48	%
7.0	0.37	%
7.5	0.35	%
8.0	0.34	%

4.2.2 The Effects of pH on Glayconsaminoglaycans

The colony-stimulating can affect by Granulocyte-macrophage and controls progress and steps of the hematopoietic cells. According to the earlier reports have indicate that the mitogenic activity maybe documented by the glycoside moiety of proteoglycans and that can related with the membrane of stromal cells in different condition. In this work, we have carry out different studies of the relation between those two GM-CSF and glycosaminoglycan.

The presence of heparin assist a marked blue shift in the fluorescence emission spectrum of GM-CSF and also a 30-fold increase drastically in the intensity of light scattering, But this shows that the origination of huge molecular weight complexes between the two molecules. Heparin-induced big changes in the different spectral properties were only observed at acidic ph. The relation of acidic pH glycosaminoglycan sulfation are depends one another. The fact that high ionic strength are disturb the reaction. It indicates that the relation between GM-CSF and glycosaminoglycan is regulated by electro static interactions in the process.

Probably these interactions require sulfate groups in the glycosaminoglycan and positively charged histidine residues in GM-CSF. These negatively charged glycol lipids present on the plasma membrane could regulate an acidic micro environment ability of activate interaction with GM-CSF and membrane-bound proteoglycans.

As the result indicated above table 4.6 the high activity of papain at pH 6.5 washigh because here the velocity was the highest and the percentage of Glayconsaminoglaycans was high. The amount of Glayconsaminoglaycans was decreased when the PH increased. In the table below the least amount of Glayconsaminoglaycans content was found at pH of 8.

4.2.3 Temperature effect on GAGs content

The papain analysis of hide trimming waste at temperature of 60°C and 65°C it was observed that more protein hydrolyzed drastically but after 65°C it decrease

Table4.6 Effect of temperature on GAGs

Temperature	GAGs content in (%)	UNIT
50°C	0.22	%
55°C	0.36	%
60°C	0.38	%
65°C	0.49	%
70°C	0.36	%
75°C	0.29	%

As table 4.7 showed; initially as temperature increased; the content of glycosaminoglycan's was increased and at 65°C it yields high glycosaminoglycan's content and after these it decline. In these experiment 65°C is the optimum temperature for papain enzyme and hide substrate reaction.

4.2.4 The effect of enzymes concentration on glycosaminoglycan's

In this project analysis we use papain enzyme for hydrolysis. Papain enzyme contains in the active center a highly reactive cysteine group that is essential for its function. This enzyme hydrolyzes big molecular proteins into easily absorbable small peptides and amino acids. Papain is an enzyme mainly that can extract and collected from fruit of papaya plant. These enzymes can break proteins in to peptides and amino acids. Basically papain is a main known component in meat tenderizer. The result showed in the table 4.8 below.

Table4.7 Effect of enzyme on GAGs

Enzyme (%)	Glycosaminoglycans content (%)	UNIT
0.2	0.19	%
0.4	0.4	%
0.6	0.5	%
0.8	0.47	%
1	0.48	%
1.2	0.49	%

As the concentration of papain enzyme increased; the glycosaminoglycan's content also proportionally increased; but after 0.6% of papain enzyme concentration the glycosaminoglycan content is almost similar. So, 0.6 % is enough to hydrolyze hide trimming waste for extraction of glycosaminoglycans. Based on the result indicated above by using 0.6% enzyme concentrations, it's possible to produce high amount of glycosaminoglycan's content on limited enzyme percentage.

4.2.5 Time duration factors of glycosaminoglycan's production

There are also visible changes in the effect of reaction time between one, three, and five hours. But after five hours there is no result varies on the hydrolysis gags with papain. This shows that papain had higher efficiency at the hydrolysis of three hours.

Table4.8 Effect of time on gags

Time(Hr.)	glycosaminoglycans content	UNIT
1	0.2	%
3	0.5	%
5	0.49	%
7	0.46	%
9	0.5	%

Time was one of the factors for enzyme hydrolysis in this experiment 3 hours was enough for glycosaminoglycan's extraction from hide because there was no significant change after 3 hours. Almost all the content of glycosaminoglycan's was similar; so no need of more time for hydrolysis.

4.3 Chemical composition of extracted Glycosaminoglycan's

After extraction of glycosaminoglycan's from raw hide trimming the powered was dried and the chemical composition of extracted glycosaminoglycans was carbohydrate 62.7%, protein 16.7%, and moisture 13.1% and ash was 7.8%.

Table 4.9 Composition of glycosaminoglycan's preparation

Composition	Amount	Unit	Method
Protein	16.4	%(w/w)	SLC7
Carbohydrate	62.7	%(w/w)	AOAC 974.06
Moisture	13.1	%(w/w)	SLC-3
Ash	7.8	%(w/w)	SLC-6

As shown in the table 5 above, the major proportion of glayconsaminoglaycans was carbohydrates that hold 62.7% of the total composition. The second major component was protein others were put under the third ratio in the composition. From the analysis the results showed that papain can active around the pH 6.5. We can get high Gags at the PH of 6.5. So if we consume more enzymes for the analysis we couldn't get extra gags so from this experience we can choose that 0.8%.(%w/wpro).is.much.enough.

4.4 FTIR result of Glayconsaminoglaycans

The infrared spectroscopes work on the concept that radiation passes over a sample, some of the light radiation is captivated. The radiation that passes over the sample is listed and put as a result. Many materials with their various light sensibilities produce different spectra. The light spectra canbe used to isolate and know molecules Sensitive and precise. FTIR is used in the synthesis of organic, science of polymer, engineering of petrochemical, food analysis and pharmaceutical industry. Fourier Transform Infrared spectroscopy benefits to run from the use of an interferometer, which is the infrared "source" and which gives for the higher speed, and the Fourier transform. To read the spectrum is a matter of analyzing which molecules and bonds related to which peaks.(Lettow et al., 2020)

To isolate and determine various materials Fourier Transform Infrared spectrophotometers (FTIR) are used to know light sensitivity in the range of 4,000 to 400 $cm^{-1.}$ FTIR characterization helps to understand secondary structure and functional group found in sample. The spectra were pointed in the region between 4000 and 400 cm^{-1} and from the spectrum amide II (mainly C–N stretching) FTIR band is shown in figure 4.3 in the light spectra range between 1180cm-1and 1360 cm-1 wave numbers. The amide III (N–H in plane deformation) and amide A (N–H stretching) FTIR bands are shown in figure 4.2 bands Fourier Transform Infrared spectroscopy are shown in the light spectra range between 3000cm-1to 3500cm-1 wave numbers.

Table4.10 Glayconsaminoglaycans FTIR result

S No,	Frequency expected Between	Frequency from the Result
(C–N stretching) amide II	1180cm^{-1} and 1360 cm^{-1}	1237 cm^{-1}
(N–H stretching)amide A	3000 cm^{-1} to 3500 cm^{-1}	3269 cm^{-1}
Amide I' band	1600-1700 cm $^{-1}$	1639 cm^{-1}

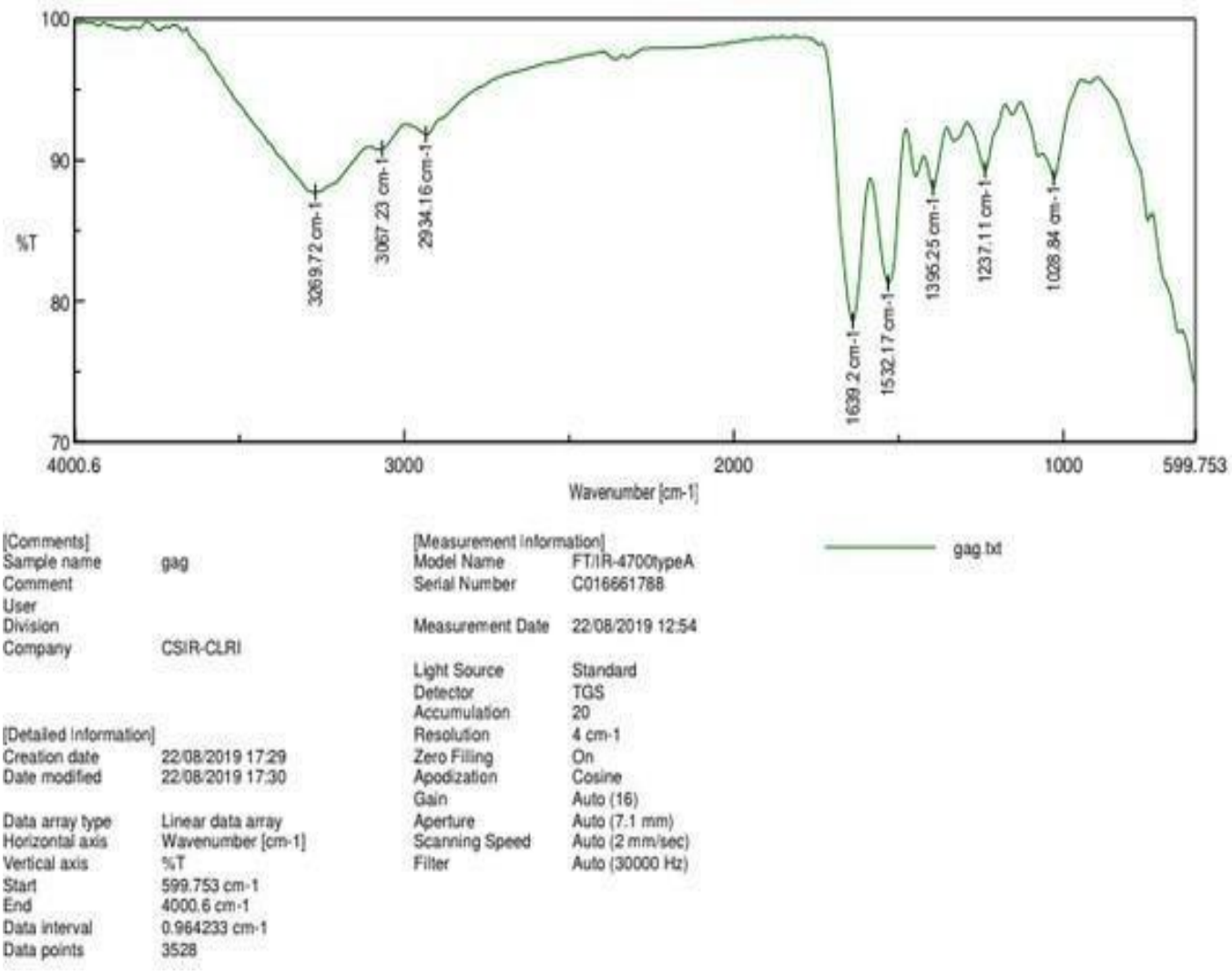
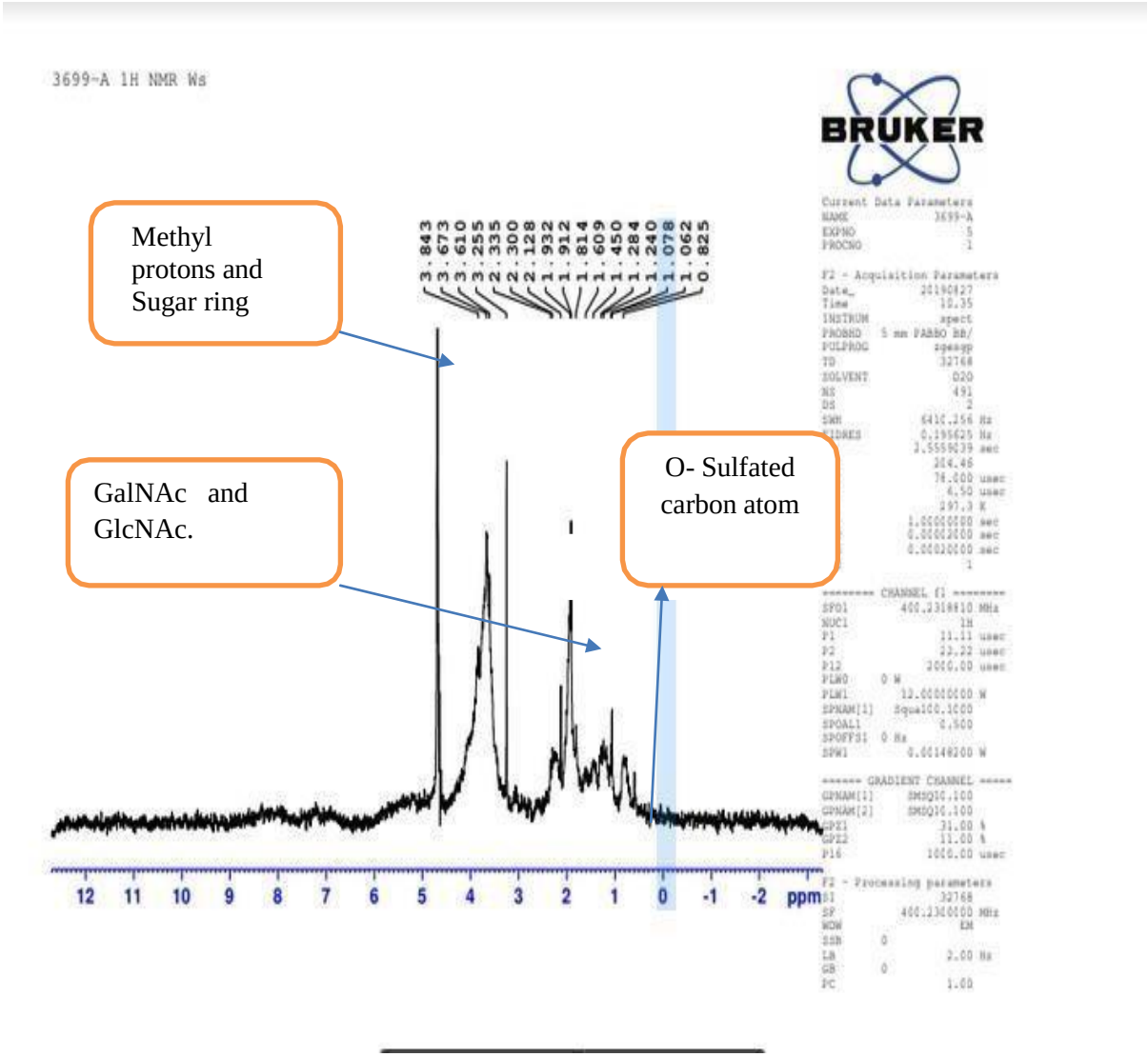


Figure 4.1 Glayconsaminoglaycans FTIR results

4.5 NMR Result of NMR **Glayconsaminoglaycans**

NMR is the best technique for the analysis of glayconsaminoglaycans because it a straight quiz a sample without derivatization procedure. NMR is nondestructive; it gives a sample to be extra used for other analytical methods. Moreover, NMR is very sensitive to compliance changes and can allow information on the secondary structure of Glayconsaminoglaycans. But their complexes sill containing some other compound with proteins.(Rudd et al., 2009) So in this analysis we used NMR spectroscopy the result record as show below.

Figure 4.2 NMR Result



From the spectra we can be understood that from 0.825-2.335 ppm and 3.255-3.843 ppm,shows that the existence of sugar ring protons and methyl protons very useful in Glayconsaminoglaycans. O-sulfated carbon atoms can be existing in the range of 0.825-2.335 ppm. The value between 1.91-2.19 ppm shows the existence of methyl group in GalNAc and GlcNAc. The existence of O-sulfated group and methyl group of GalNAc and GlcNAc in NMR spectra shows the indication of chondroitin sulfate.

CHAPTER FIVE
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this paper the researcher had used papain Enzyme to hydrolyze raw hide trimming by considering time, PH, temperature difference, enzyme percentage are highly determined and affect the glayconsaminoglaycans value that hydrolyzed from raw hide trimming. In this analysis few chemicals and recovered it again like solvents and collagen. The extraction rate of trimming waste with that of collagen recovery and Glayconsaminoglaycans was 18 % and 0.5 % respectively from 25gm trimming waste. This indicates that producing in more potential can decrease foreign exchange/used as import substation/ for and country, could create job opportunity, and also producing with least cost raw material but produce too high valuable product. Also used as, environmental pollution since it uses the residue materials that can pollute the environment.

However, it's a trial case, but producing much more Glayconsaminoglaycans and collagen could lead to exporting the product that gain foreign exchange rate for a nation. The raw hide trimming can be a potential source of Glayconsaminoglaycans which is useful for the treatment many biological medicine,especially with function of joint improvement.

Hide wastes were collected largely from hide trimming when the hide and skin ready for loading in wetback process which use for extraction of glayconsaminoglaycans that gives income instead of wastes. In this research, only glayconsaminoglaycans were synthesized from hide trimming. Hide trimming can be used as raw material resource of Glayconsaminoglaycans whichis used for pharmacitual and cosmetic industries. That different product provides different of application like. Beauty, agriculture, syntan utilize for post tanning, tissue, packing material, food and medicinal application.

This study shows the way forward for decreasing of wastes by using chemicals that produce valuable product by new technology. An enzymatic process in the leather sector need further work sin the advantages of cost minimization, reaction life time and weather condition.

5.2 Recommendation

So based on the result of the analysis the following direction is forwarded;

- The process of enzymatic analysis should be design at minimum cost and short time interval.
- The skins that collected from surrounding were employed for traditional soaking operations, wet finishing processes so here using enzyme for the process is recommended.
- In our country, the application of new technology like enzyme application in leather sectors was not adapted. So introducing the way of processing is much recommended.
- The enzyme and its application should be considering the weather condition with consideration of cost and time.
- The project was extracting only Glayconsaminoglaycans from caw hide trimming. We can further prepare other new type gelatin and collagen relates products, for medicine packing materials, gelatin film and pharmacitual raw materials for different application.

REFERENCES

Acott, T. S., Kingsley, P. D., Samples, J. R., & Van Buskirk, E. M. (1988). Human trabecular meshwork organ culture: Morphology and glycosaminoglycan synthesis. *Investigative Ophthalmology and Visual Science*, *29*(1), 90–100.

AMS Biotechnology. (n.d.). Proteoglycans & Glycosaminoglycans. *Glycobiology Research*, 1– 36.

Fransson, L.-Å., & Rodén, L. (1967). Structure of Dermatan Sulfate. *Journal of Biological Chemistry*, *242*(18), 4161–4169. https://doi.org/10.1016/s0021-9258(18)95792-3

Habuchi, H., Nogami, K., & Kimata, K. (2002). Heparan sulfate: Structure, biosynthesis, and functions. *Connective Tissue*, *34*(3), 249–259.

Henrotin, Y., Mathy, M., Sanchez, C., & Lambert, C. (2010). Chondroitin sulfate in the treatment of osteoarthritis: From in vitro studies to clinical recommendations. In *Therapeutic Advances in Musculoskeletal Disease* (Vol. 2, Issue 6, pp. 335–348). https://doi.org/10.1177/1759720X10383076

Le Vien, N. T., Nguyen, P. B., Cuong, L. D., An, T. T. T., & Dao, D. T. A. (2017). Optimization of papain hydrolysis conditions for release of glycosaminoglycans from the chicken keel cartilage. *AIP Conference Proceedings*, *1878*. https://doi.org/10.1063/1.5000177

Lettow, M., Grabarics, M., Mucha, E., Thomas, D. A., Polewski, Ł., Freyse, J., Rademann, J., Meijer, G., von Helden, G., & Pagel, K. (2020). IR action spectroscopy of glycosaminoglycan oligosaccharides. *Analytical and Bioanalytical Chemistry*, *412*(3), 533–537. https://doi.org/10.1007/s00216-019-02327-7

Panelewen, J., Pusponegoro, A. D., Labeda, I., Islam, A. A., & Hatta, M. (2015). Analysis of expression of mRNA Matrix Metalloproteinase (MMP)-8 gene and Tissue Inhibitors of Metalloproteinase (MMP)-1 in Intraperitoneal Adhesion after Usage of Hyaluronate acid-Carboxymethylcellulose (HA-CMC) or Virgin Coconut Oil (VCO). *American Journal of Medical and Biological Research*, *3*(4), 113–116. https://doi.org/10.12691/ajmbr-3-4-6

Piepkorn, M., Hovingh, P., & Linker, A. (1989). Glycosaminoglycan Free Chains. *Journal of Biological Chemistry*, *264*(15), 8662–8669. https://doi.org/10.1016/s0021-9258(18)81843-9

Raghuraman, H. (2013). *EXTRACTION OF SULFATED GLYCOSAMINOGLYCANS DEDICATION TO : My parents*. *July*.

Rudd, T. R., Skidmore, M. A., Guimond, S. E., Cosentino, C., Torri, G., Fernig, D. G., Lauder, R. M., Guerrini, M., & Yates, E. A. (2009). Glycosaminoglycan origin and structure revealed by multivariate analysis of NMR and CD spectra. *Glycobiology*, *19*(1), 52–67. https://doi.org/10.1093/glycob/cwn103

Tahir, M. B., Wossen, A. M., & Mersso, B. T. (2018). Evaluation of livestock feed balance undermixed crop-livestock production system in the central highlands of Ethiopia. *Agriculture and Food Security*, *7*(1). https://doi.org/10.1186/s40066-018-0170-8

TSEGAW, D. (2018). *"Studies on Development of Cleaner Beamhouse Process, Extraction and Characterization of Collagen From Fleshing Wastes." June*.

Van Wijk, X. M. R., Vallen, M. J., Van De Westerlo, E. M., Oosterhof, A., Hao, W., Versteeg, E. M., Raben, J., Wismans, R. G., Smetsers, T. F. C. M., Dijkman, H. B. P. M., Schalkwijk, J., & Van Kuppevelt, T. H. (2012). Extraction and structural analysis of glycosaminoglycans from formalin-fixed, paraffin-embedded tissues. *Glycobiology*, *22*(12), 1666–1672. https://doi.org/10.1093/glycob/cws119

Yan, X. (2017). 2018_Pnas_Si_Spe. *Proceedings of the National Academy of Sciences*, 2017. https://doi.org/10.1073/pnas

Acott, T. S., Kingsley, P. D., Samples, J. R., & Van Buskirk, E. M. (1988). Human trabecular meshwork organ culture: Morphology and glycosaminoglycan synthesis. *Investigative Ophthalmology and Visual Science*, *29*(1), 90–100.

AMS Biotechnology. (n.d.). Proteoglycans & Glycosaminoglycans. *Glycobiology Research*, 1– 36.

Fransson, L.-Å., & Rodén, L. (1967). Structure of Dermatan Sulfate. *Journal of Biological Chemistry*, *242*(18), 4161–4169. https://doi.org/10.1016/s0021-9258(18)95792-3

Habuchi, H., Nogami, K., & Kimata, K. (2002). Heparan sulfate: Structure, biosynthesis, and functions. *Connective Tissue*, *34*(3), 249–259.

Henrotin, Y., Mathy, M., Sanchez, C., & Lambert, C. (2010). Chondroitin sulfate in the treatment of osteoarthritis: From in vitro studies to clinical recommendations. In *Therapeutic Advances in Musculoskeletal Disease* (Vol. 2, Issue 6, pp. 335–348).

https://doi.org/10.1177/1759720X10383076

Le Vien, N. T., Nguyen, P. B., Cuong, L. D., An, T. T. T., & Dao, D. T. A. (2017). Optimization of papain hydrolysis conditions for release of glycosaminoglycans from the chicken keel cartilage. *AIP Conference Proceedings*, *1878*. https://doi.org/10.1063/1.5000177

Lettow, M., Grabarics, M., Mucha, E., Thomas, D. A., Polewski, Ł., Freyse, J., Rademann, J., Meijer, G., von Helden, G., & Pagel, K. (2020). IR action spectroscopy of glycosaminoglycan oligosaccharides. *Analytical and Bioanalytical Chemistry*, *412*(3), 533–537. https://doi.org/10.1007/s00216-019-02327-7

Panelewen, J., Pusponegoro, A. D., Labeda, I., Islam, A. A., & Hatta, M. (2015). Analysis of expression of mRNA Matrix Metalloproteinase (MMP)-8 gene and Tissue Inhibitors of Metalloproteinase (MMP)-1 in Intraperitoneal Adhesion after Usage of Hyaluronate acid-Carboxymethylcellulose (HA-CMC) or Virgin Coconut Oil (VCO). *American Journal of Medical and Biological Research*, *3*(4), 113–116. https://doi.org/10.12691/ajmbr-3-4-6

Piepkorn, M., Hovingh, P., & Linker, A. (1989). Glycosaminoglycan Free Chains. *Journal of Biological Chemistry*, *264*(15), 8662–8669. https://doi.org/10.1016/s0021-9258(18)81843-9

Raghuraman, H. (2013). *EXTRACTION OF SULFATED GLYCOSAMINOGLYCANS DEDICATION TO : My parents*. *July*.

Rudd, T. R., Skidmore, M. A., Guimond, S. E., Cosentino, C., Torri, G., Fernig, D. G., Lauder, R. M., Guerrini, M., & Yates, E. A. (2009). Glycosaminoglycan origin and structure revealed by multivariate analysis of NMR and CD spectra. *Glycobiology*, *19*(1), 52–67. https://doi.org/10.1093/glycob/cwn103

Tahir, M. B., Wossen, A. M., & Mersso, B. T. (2018). Evaluation of livestock feed balance undermixed crop-livestock production system in the central highlands of Ethiopia. *Agriculture and Food Security*, *7*(1). https://doi.org/10.1186/s40066-018-0170-8

TSEGAW, D. (2018). *"Studies on Development of Cleaner Beamhouse Process, Extraction and Characterization of Collagen From Fleshing Wastes." June*.

Van Wijk, X. M. R., Vallen, M. J., Van De Westerlo, E. M., Oosterhof, A., Hao, W., Versteeg, E. M., Raben, J., Wismans, R. G., Smetsers, T. F. C. M., Dijkman, H. B. P. M., Schalkwijk, J., & Van Kuppevelt, T. H. (2012). Extraction and structural analysis of

glycosaminoglycans from formalin-fixed, paraffin-embedded tissues. *Glycobiology*, *22*(12), 1666–1672. https://doi.org/10.1093/glycob/cws119

Yan, X. (2017). 2018_Pnas_Si_Spe. *Proceedings of the National Academy of Sciences*, 2017. https://doi.org/10.1073/pnas

APPENDIX

Annex 1 Enzyme selection based on the Analysis

Sample(g)	Papain enzyme (%)	Tripson enzyme (%)	Extracted GAG s Glycosaminoglycans(gram) by papain	Extracted Glycosaminoglycans (gram)by tripson
25	0.5	0.5	65	25
25	0.5	0.5	60	28
25	0.5	0.5	55	31
25	1	1	81	50
25	1	1	79	45
25	1	1	75	47

Note: - Based on the result of analysis the above scientific parameters papain enzyme givesas high yields Glycosaminoglycans.

Annex 2 Dilution factor selection based on the Analysis

Sample(g)	Dilution (1:4) GAGs content(mg)	Dilution(1:6)GAGs content(mg)	Dilution (1:8) GAGs content(mg)	Dilution (1:10)GAGs content (mg)
25	64	40	39	30
25	70	55	30	35
25	60	45	40	31
25	71	40	41	28
25	65	57	38	35
25	59	50	29	27

Annex 3 List of chemicals types

No.	Chemicals	Brands	Amount	Concentration
1	Chondroitin sulfate	Sigma	100g	
2	Papain	Sigma	150g	
3	Sulphuric acid	India	0.5	98%
4	Acetic acid	Sigma	0.5	99-100%
5	Ethanol	CDH	2.8	100%
6	Methanol	CDH	2.6	100%
7	copper sulphate anhydrous	India	0.025g	98%
8	potassium sulphate anhydrous	India	0.025g	99.5%
9	1.9-dimethylmethylen blue (DMMB)	Sigma	100g	
10	Dichloromethane (DCM)	India	2.5ml	99.5%

Annex 4 List of Laboratory equipment

No.	Glassware type	Volume
1	Conical flask	250ml
2	Measuring cylinder	5-100ml
3	Beaker	50-500ml
4	Round bottom flask	250ml
5	Test tubes	10ml-150ml
6	Micropipettes	0.5-10 ul, 2-20ul, 20-200ul, 200-1000ul
7	Burettes	10 and 100ul
8	Crucible	10-30ml
9	Filter paper	10-50mm

Annex 5 List of Laboratory apparatus and Range of Operations

No.	Laboratory Equipment name	Brand	Range of operation
1	NMR		
2	Uv spectroscopy	SHIMADZU Corp Sr no. A11635202825	UV-1800 240V
3	FTIR	Jasco FTIR/4200 Type A	
4	Kjeldahl distillation unit		

Annex 6 UV spectroscopy reading at 525nm

No.	Standards	standard chondroitin sulfate (ul)	Distilled water (ul)	DMMB reagent (ml)	Concentration read from uv at 525nm(ug)
1	Blank	0	100	2.5	0.0006
2	Standard 1	10	90	2.5	0.0860
3	standard 2	25	75	2.5	0.2453
4	standard 3	50	50	2.5	0.3703
5	standard 4	75	25	2.5	0.4935
6	standard 5	100	0	2.5	0.5565
7	Sample 1	50	50	2/5	0.0730
8	Sample 2	50	50	2.5	0.0654
8	Sample 3	50	50	2.5	0.0667
10	Sample 4	50	50	2.5	0.0687
11	Sample 5	50	50	2.5	0.0675
12	Sample 6	50	50	2.5	0.0679
13	Sample 7	50	50	2.5	0.0683

Annex 7 Raw material collection and Sample preparation