
**ADDIS ABABA UNIVERSITY**

**ADDIS ABABA INSTITUTE OF TECHNOLOGY (AAiT)**

**SCHOOL OF CHEMICAL AND BIO-ENGINEERING**

# Extraction and Characterization of Elastin from Raw Hide Trimmings using Autoclave Extraction Technology

**By**

**Zerihun Yoseph**

**June, 2018**

**Addis Ababa**

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***A Thesis submitted to Addis Ababa University, Addis Ababa Institute of Technology, School of Chemical and Bio Engineering in Partial Fulfilment of the Requirements the Attainment of the Degree of Master of Science in Chemical Engineering (Leather Technology stream).***

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**GSR /3476/08**

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Approved by Board of Examines:

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Chairman, Department's Graduate Committee

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## Declaration

I, the undersigned declare that this thesis is my original work and that all sources of materials used for the thesis have been dully acknowledged.

Zerihun Yoseph Signature: - _____ Date:-_____

The thesis has been approved for submission by:

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|---|---|---|
| Dr. Ing. Berhanu Assefa (Advisor) | _____ | _____ |
| Dr. Gladstone Christopher Jayakumar (Advisor) | _____ | _____ |

## *Abstract*

*During leather processing, raw skins/hides are trimmed and thrown as wastes and accounting for pollution load in leather industry. Elastin is a fibrous protein with multifaceted applications in biomedical field due to its intrinsic biological annotations viz elasticity and cell interactions, the extraction process is challenging task due to their cell interactive properties, the application is versatile and the price is in current international market is highly expensive. In this context, leather solid wastes specifically raw hide trimmings have been found out in the research as the potential source of raw material for elastin extraction. In this research, the raw Hide Trimming elastin content was estimated between in the range of 122 mAUmin and 97 mAUmin using the combination of FPLC, UV-Spectroscopy, SDS-PAGE protein scientific characterization techniques. The Elastin was extracted from leather waste through autoclaving technology, the method yield was found to be 90%. In addition, the biocompatibility study confirms that there is no toxic nature of extracted elastin for biological application. This implies that the selected extraction process was effective and efficient. The physical characterization using DSC result indicates that the melting peak of elastin was -0.5420 watt/g at 103.23 $^{o}C$, elastin showed very high denaturation temperature of 275.82$^{o}C$ and TGA studies confirm that elastin have six mass loss degradation steps. The chemical characterization result using $^{13}C$ Solid state NMR and FTIR that elastin shows random coil, α-helix and β-strand secondary structure. Moreover, the FTIR confirm that amide A, I, II, III, IV, V, VI, and VII functional group existed in the elastin fiber. The biological characterization using amino acid analysis result show that the amino acid composition chain of the product have high amount of glycine, non-polar amino acid, low amount of acidic amino acids, low amount of hydroxyl amino acid and there is no hydroxylsine, tryptophan and Cysteine amino acids in the amino acid composition of the protein fiber. The little variations were the existence of high amount of basic amino acids in the protein amino acid composition due to the presence of collagen residues. Finally, the biocompatibility study shows that there is no toxicity nature of the extracted elastin fiber for human keratinocyte cells culture. There result is scientifically justified and confirmed that the raw hide trimmings extracted elastin makes feasible for biological and biomedical applications.*

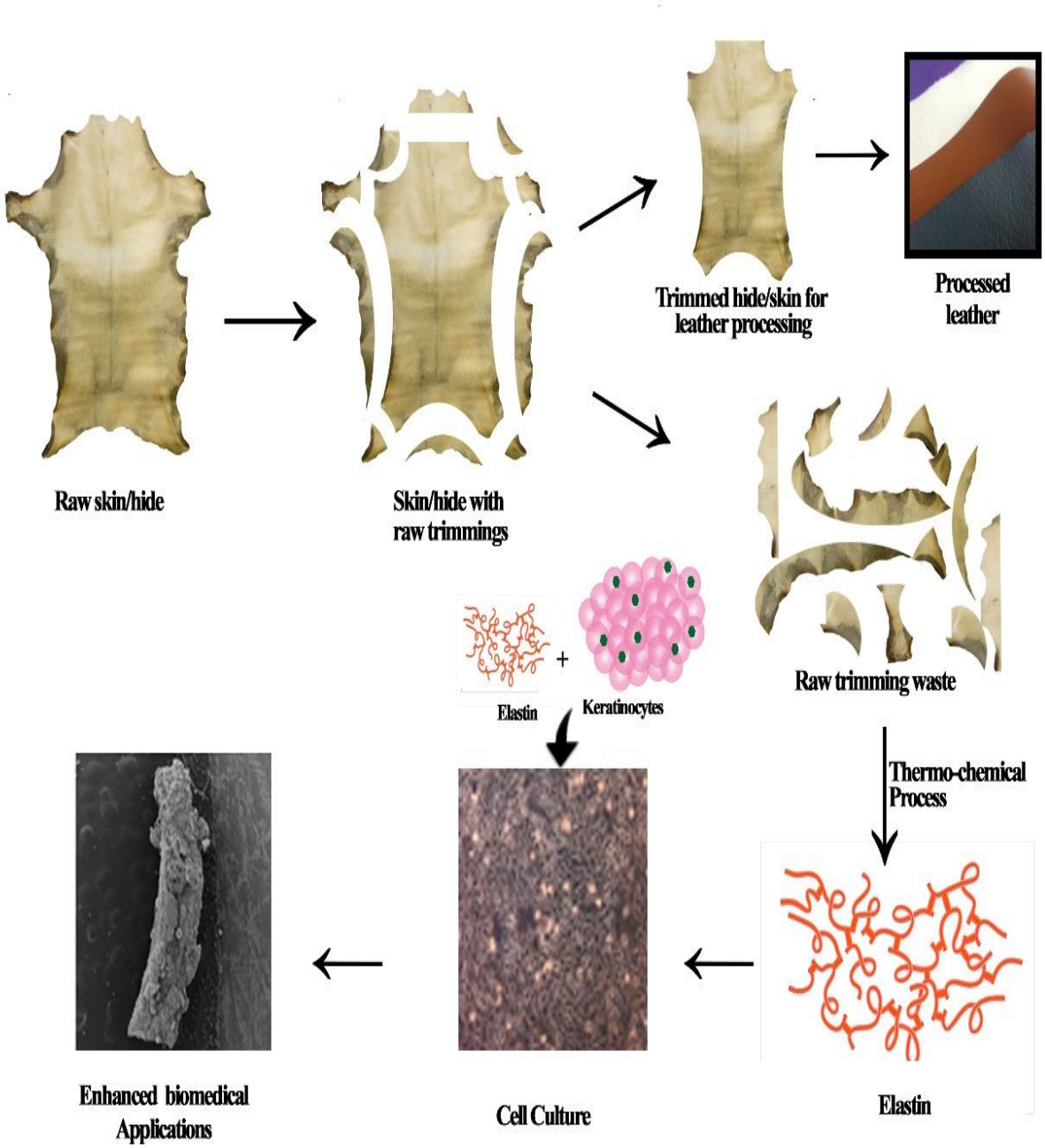


**Fig. 1 Graphical Abstract**

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## List of Abbreviations

| | |
|---|---|
| **CD** | Circular Dichorism |
| **CPMAS** | Cross-Polarization Magic Angle Spinning |
| **DSC** | Differential Scanning Calorimetry |
| **EVG** | Verhoeff-Van Gieson (EVG) |
| **FPLC** | Fast Protein Liquid Chromatography/ |
| | Fast Performance Liquid Chromatography |
| **FRET** | Fluorescence Resonance Energy Transfer |
| **FTIR** | Fourier transform Infrared Spectroscopy |
| **FID** | Free Induction Decay |
| **H/D** | Hydrogen/Deuterium |
| **HP** | Hydroxyl Proline |
| **HPLC** | High Performance Liquid Chromatography |
| **KD** | Kilo Dalton |
| **MTT** | Methylthiazolyldiphenyl-tetrazolium |
| **NMR** | Nuclear Magnetic Resonance |
| **OD** | Absorbance Values |
| **PBS** | Phosphate-buffered Saline |
| **pH** | Potential of hydrogen |

| | |
|---|---|
| **RPM** | Revolution per Minute |
| **SDS-PAGE** | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| **SEM** | Scanning Electron Microscope |
| **SS** | Suspended Solids |
| **TDS** | Total Dissolved Solids |
| **TGA** | Thermogravimetry |
| **UV** | Ultra Violet Spectroscopy |
| **mAU** | mile absorbance Unit |
| **WBS** | Work Breakdown Structure |
| **WB** | Wide Bore |

# Chapter One

# Introduction

## 1.1Background

Tanning consists of a series of successive operations converting raw hides and skins into leather. The raw material in the production of leather is a by-product of the meat industry. Tanners recover the hides and skins from slaughterhouses and transform them into a stable material that can be used in the manufacture of a wide range of products [1]. In line with that, Leather or tanning industry has played an important role in the development of civilization.

The tanning operation consisting of converting the raw hide or skin, a highly putrescible materials, in to leather, a stable material, which can be used in the manufacture of a wide range of products. The whole process involves a sequence of complex chemical reactions and mechanical processes. The production processes in a tannery can be split into seven main categories: Hide and skin curing, preservation, trimming and sorting, beam house operations, tanning operations, post-tanning operations and finishing [2] & [3].

Trimming and sorting is one of the processes in beamhouse. Trimming is done to remove unwanted long Shank, horn, hooves, ears, tails etc, and give a proper shape to hide/ skin. Then sorting is carried out according to size, weight, thickness, grade etc and formed into batches to maintain the quality of leather [4].

During tanning process, there is a significant generation of high pollution to the environment. The main pollutants are categorized in to air pollution, wastewater and solid wastes. Among these pollutants, the solid waste is one of the pollutant that adverse impact on the environment, ecological system and human health. The term solid waste in the tannery includes raw and lime trimmings, raw hide and skin rejects, limed pelt splits, fleshing wastes, hairs, chrome shavings, chrome splits and buffing dusts [5].

The solid waste generated by the tanning operation is one of the vital and focus area of research due to its hidden and unexploited wealth. Now days, thanks for the researchers' the solid wastes are utilized for various purpose that is environmentally friendly products.

Several measures have been taken for effectively managing the solid wastes generated during leather processing. Protein hydrolysate, biodiesel, glue, gelatin, fat liquors and reactive proteins are some of the by-products from fleshing wastes. From chrome shaving wastes, splits and trimmings by-products like glue, gelatin, protein flavor and reconstituted collagen are also prepared. The other major solid waste is hair which contains keratin; rich in cysteine are also well exploited for the by-product utilization [5], [6] & [7].

Though, several proteins based materials are derived from leather wastes but the extraction of elastin from tannery wastes have not much explored. Elastin is the principal constitutes of the connective tissue, particularly those tissues that contains elastomeric properties such as walls of arteries, the alveoli of the lungs, skins, tendons and elastic ligaments [8]. It is estimated that the elastin content of tendons and the bovine ligamentum are 90%. Elastin has significant applications in the biomedical fields. One of the applications is for tissue engineering and bio sensing. The other area of valuable and vital application of elastin is the cosmetics industry as lotions, creams, skin care products, jells, moisturizers and anti-ageing products [8]. Therefore, extracting high end biomedical product from leather wastes would create a new avenue for effective and profitable solid waste management to the leather research fraternity.

### 1.2 Problem Statement

Elastin is a fibrous protein that histologically found in the grain and dermis layer of the skin structure [2] & [3]. Elastin gives elasticity property to skin. Moreover, elastin have multidimensional and vital application namely in Biomedical, Pharmaceuticals and Cosmetics industries. The key and multipurpose biomedical and biological applications are tissue engineering, bio sensing, pathology of lung disease, pathology of the arterial wall, modification with ageing, human breast tumors, neosythesis in response to vascular injury, the temporal arteritis/Polymyalgia Rheumatic Syndrome, it has been used in studies of Chronic obstructive pulmonary disease and others[8]. Furthermore, the elastin product price in the international market infers that the product is very costly due to useful application in various industries. For example, according to sigmaaldrich online market the price of elastin from bovine neck ligament

powder of five gram in a polybottle is 251.68(USD). In this context, viewing the technological perspectives problems in general and extraction process specifically elastin is a challenging task due to their cell interactive properties and less percentage of availability in skin histological structure. Thus, the primary problem is allied with the product.

The extraction methodology of elastin was broadly categorized as physical, alkaline/acid and enzymatic methods/procedures [8] & [9]. In depth review and analysis in the context of technical feasibility by compare and contrast each procedure using various scientific parameters shown in Annex1. The physical extraction procedure mainly focuses on primarily treating with alkaline in the case of hide/skin only and repeating autoclaving method [8] & [9]. Autoclaving is the heart of elastin extraction process. The problem associated with this technology is the microfibrillar components are not completely removed [8] & [9] and the energy cost is relatively high. The secondary methodology is alkaline/acid extraction methodology/protocol mainly use NaCl extraction of elastin and defatted with acetone and dried with NaOH and lyphollized [8] & [9]. This technology have a lot of problems such as existence of non- elastin material and microfibrillar components are not completely removed [9].There is a possibility of toxic substance generation due to presence of chemicals. Hence, this method extracted elastin is not applicable as bio material. The third enzymatic extraction protocol mainly uses NaCl extraction, organic solvent extraction, treatment with guanidium-HCl extraction or CNBr treatment, Collagenase digestion, trypsin digestion and NaCl extraction. This technology have a high potential for bio applicability but problem that not avoiding alkaline and acid hydrolytic treatment, the time of the process is very long, it involves complex chemical and biological process, there is also a possibility of microbial contamination of the product and the technology substrate compatibility is low and not yet tried on hides and skin. Hence, comparing based on the technology compatibility with the basic raw material of the research, many merits regarding the technology selection parameter, less process time and reactions complexity, and the most important parameter that there no possibility of the product contamination with chemical and microbial than enzymatic process. In addition, the extraction technology is designed based on less process and reactions complexity and free of microbial contamination. Therefore, the selected feasible technology for elastin extraction for the research is autoclaving method with alkaline with pretreatment.

The other problem is related with the environmental, human health and knowledge gap viewpoints. The problems are solid waste generated during the trimming and sorting and along with other solid wastes have a high amount of pollutants that have magnified impact to the environment and human health [5], [6] & [10], the leather making process of 1000 kg of raw hide process there is 850 kg of solid waste generated during the process only 150 kg of raw material is converted in to leather and 120 kg of raw trimming waste generated which accounts almost 85% of by weigh of cow hide have generated solid waste in which 14% are takes raw trimming [5], [11] & [12], among the total solid waste generated the raw skin and hides trimmings accounts 5-7% of the total share of solid wastes[5], comparing the solid waste generated tanning sub process the beam house operations takes 80% of share of the total solid waste of the tanning process [5]. Furthermore, the solid waste management system in Ethiopian tanning sub sector context indicated that disposal of tannery solid waste along with municipal solid wastes in open dumping area called "Koshe" or "Rapi"[13]. These concrete problems collectively implies that the solid waste generation of tanning process is significantly high proposition and load to the environment and human health, the tannery solid waste management have given less focus and knowledge and technology gap along with low research in exploiting and utilizing the tannery solid waste management in Ethiopia circumstance.

In conclusion, to overwhelm the problem's the research was developed for high value and marketable bio product from a raw hide trimming waste through filling and transferring the knowledge and technology gap.

## 1.3 Objectives

### 1.3.1 General Objective

- The main aim of the study is to recover high value protein bio material for biological application by the extraction of elastin from hide raw trimming.

### 1.3.2 Specific Objectives

The specific objectives of this study were:

- To characterize the raw material for protein and elastin content
- To select elastin extraction protocol from trimming wastes base material

- To characterize the physical, chemical and biological properties of the product
- To study the secondary structure and functional group analysis
- To evaluate biocompatibility of the extracted elastin and forward its applications

## 1.4. Scope of the Study

The study was primarily focus on extensive literature survey in the title area; secondly collection of raw trimmings of cow hides. Thirdly, characterization of the raw Hide Trimming for protein and elastin content using selected scientific methods. Fourthly, conduct extraction of elastin using combination of chemical and heat treatment methods. Fifthly, scientific characterization of extracted elastin using different protein characterization techniques collectively names as physical, chemical and biological methods. Finally, forwarded its application of the extracted elastin based on the biocompatibility characterization result.

## 1.5 Significance of Study

Every research should have something to contribute sine lots of time, money, and human power is exerted to do it. So this study is believed to consider the following;

- It can help to utilize the solid wastes generated during tanning process
- The research helps to minimize the solid waste environmental pollution problems associated with tanneries
- The research helps to produce eco-friendly product from solid tannery waste
- The research helps the tanneries to produce value addition products from the waste and to generate extra income
- The research provide a new innovation of producing bio material from waste
- It can help in the product integration of tanneries with other industries
- The, outcome of this research would provide a new scope to researchers to work further.

## 1.6 Framework of the Study

The frameworks of this research have three distinct phases. The first phase of the research is determination, estimation and characterization of raw material for elastin content. In this phase, protein is extracted based on designed method and scientifically characterize using protein characterization techniques and estimate the protein and elastin of the sample. In addition, the characterization methods were used in this phase was FPLC, UV-Spectroscopy and SDS-PAGE. The second phase of the research was extraction of elastin from raw hide trimmings waste by using selected autoclaving technology with pre-treatment with alkaline mixture. Finally, the most vital and innovative phase three of research was a multidimensional protein characterization techniques to have a holistic view and scientific assessment of extracted elastin. This phase characterizations majorly categorized as physical, chemical and biological techniques. Moreover, the protein characterization techniques were provided a concrete scientific justification, assessment and explanation about the physical, chemical, and biological properties of extracted Elastin. Furthermore, it can able to determine the functional group analysis, protein secondary structure determination and gave a scientific justification about the application of the product. The general conceptual framework of the study was shown in the Fig.1.1.

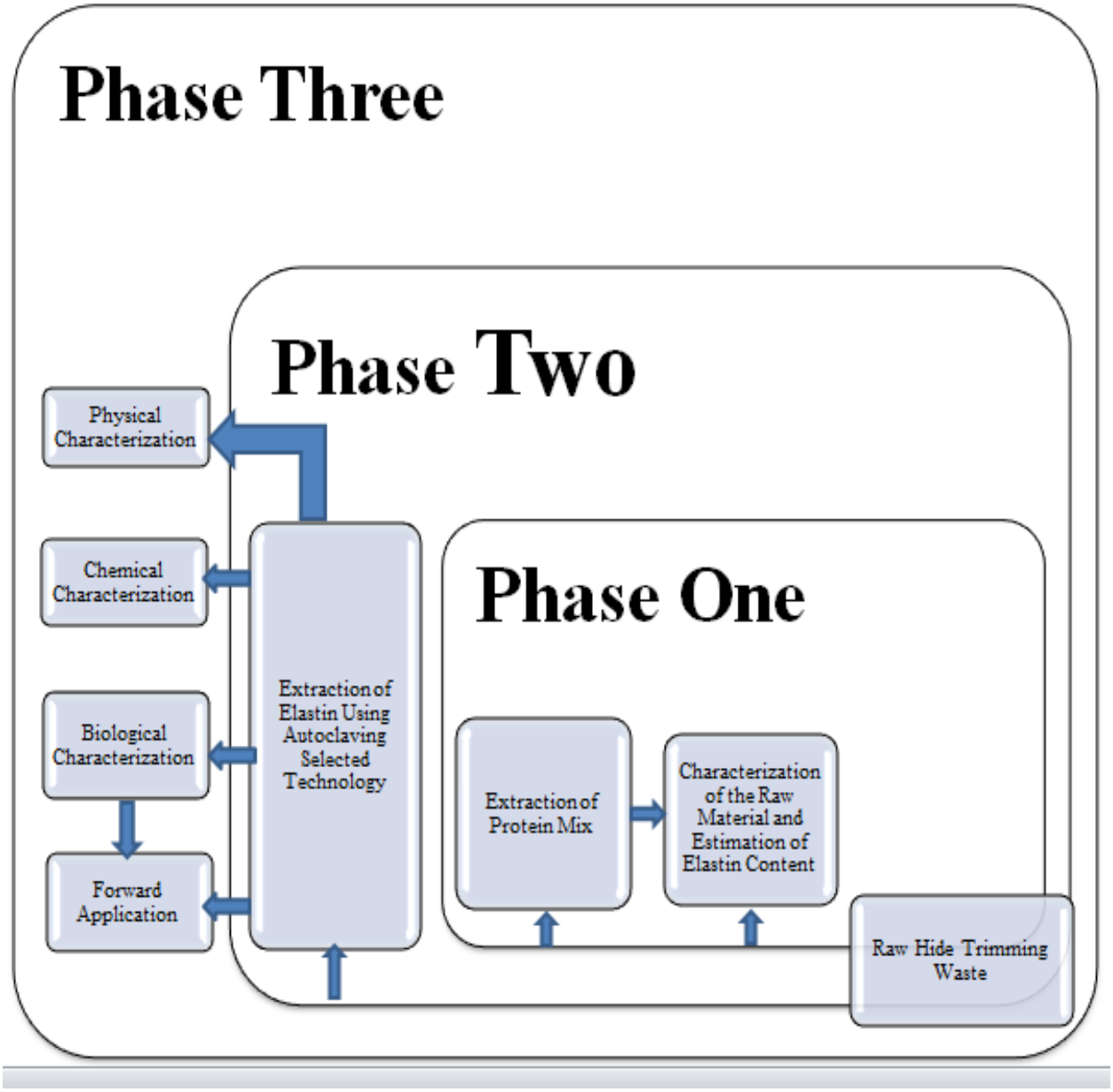


**Fig. 1.1 General Framework of the Research**

### 1.7 Organization of the Study

The thesis was organized in to five chapters. Each chapter presentation and their respective content overviews briefly were listed and discussed as follows:-

**Chapter One: -** Presents the introduction part of the research. These are background of the study, problem statement of the research, general and specific objectives of the research were

shown in detail. In addition, the research scope, significance, and framework and organization were presented in the chapter.

**Chapter Two**: - Presents review of related literatures regarding the thesis title. Briefly, the skin structure, the components of the skin and Histological structure of skin were discussed. In addition, the overview of leather process, hide and skin storage, explanation of leather solid wastes and its proportion and detail overview of leather waste utilization. Moreover, it briefly explains and discusses the meaning of proteins, classification of proteins, the protein structure/configuration, the techniques of proteins characterization and purification, the meaning of amino acids and its type. Furthermore, a detail review of Elastin, the structure and formation of elastin, the property of elastin, the amino acid composition of elastin and the scientific difference of elastin and collagen were discussed.

**Chapter Three**: -This chapter was showed the detail material and methods of the research. Basic raw materials, name of chemicals, name of laboratory Glassware's and equipment's type and amount of each were listed. Moreover, it was briefly explained the method of the raw material characterization and estimation of elastin content. Furthermore, it explained in detail the selected extraction method of elastin from the basic raw materials. Further, it showed all the methods of physical, chemical and biological protein characterization of the sample.

**Chapter Four**: - Presented all the results and discussions of the research. These are the raw material elastin content characterization and determination using FPLC, UV-Spectroscopy and SDS- PAGE. Moreover, it briefly explained and computed the quantitative and qualitative analysis of FPLC Chromatogram result of the sample. In addition, brief explanations of the physical, chemical and biological characterization results of the research by correlating with previously reported journals. Furthermore, it briefly explains the major findings of each research phase results were shown respectively.

**Chapter Five**: - This chapter presents the Conclusion and Recommendations of the research. In brief the research work.

# Chapter Two

# Literature Review

## 2.1 Introduction

### 2.1.1 The Skin Structure

It is important to understand the nature of hide and skin, in order to rationalize the structure–function, structure–reactivity and structure–property relationships.

The skin and its structure system are divided into five distinct parts as shown in Fig.2.1. These are skin, hair, glands, nails and nerve endings. The skin is an organ because it consists of different tissues that are joined to perform a specific function. In addition, the skin is largest organ of the body in surface area and weight [2] & [3].

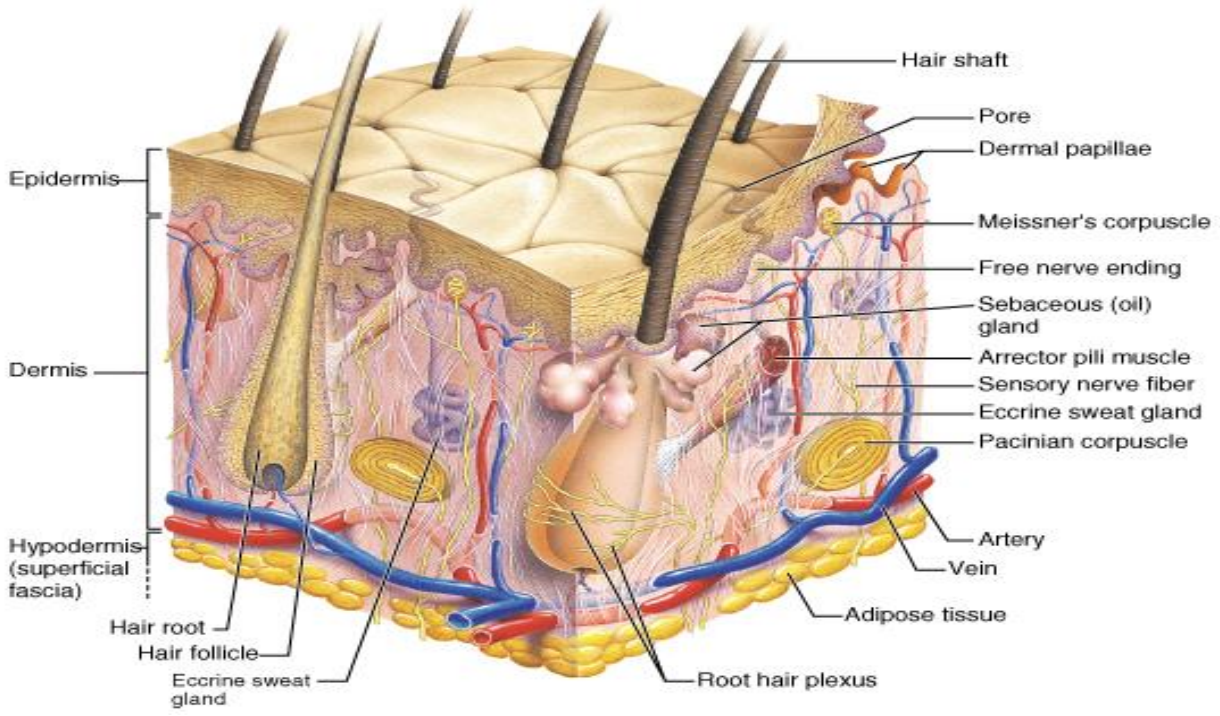


**Fig. 2.1 Skin Structure**

### 2.1.2 Components of Skin

The skin components are Epidermis, Grain, Junction, Corium, Flesh layer and Flesh. Each layers were discussed as follows:-

**Epidermis:-**The outermost layer of the skin, the barrier between the animal and its environment: it is composed of so called 'soft keratin', characterized by a relatively low content of cystine compared to cysteine, i.e. less oxidation of the thiol groups to the crosslinking disulfide group[2] & [3].

**Grain:-**The upper most layers in unhaired or de-wooled pelt. The the corium minor is also referred to in the jargon as the grain layer. The structure is fibrous, but the fibres are so fine and the appearance is more like a solid. The lack of fiber interaction, in comparison with lower layer of the skin, makes the grain weak. The macro-structure is a convoluted sheet, because the grain layer is larger in area than the lower layers, so it has to be folded. This conformation is held in place by the presence of elastin [2] & [3].

**Junction:-**The grain-corium junction is the transition zone between the very fine fibers of the grain and the much larger fibers of the corium. It is an open structure, consisting of relatively small fibres and carrying other structural components of the skin: these include the veinous system and, in the case of sheep skins, lipocytes, which are cells that contain triglyceride fat.

**Corium:-**The main part of the skin is the obviously fibrous structure called the corium or the corium major. The fibres structure varies through the cross section of hide or skin: the fibres increase in size, reaching the maximum fiber diameter in the center of the corium and then decreasing a little they approach the next lower layer [2] & [3].

**Flesh Layer:-**The so called flash layer is the layer of the skin closest to the flesh of the animal: although it has a distinct fiber structure, it is still part of the corium [2] & [3].

**Flesh:-**Hides and skins are inevitably presented to the tanner with adhering flesh (muscle) and fat. This must be removed at the earliest stage possible in the processing programme, because it creates a barrier to the uniform penetration of chemicals, which would cause non-uniformity of leather properties [2] & [3].

## 2.2 Histological Structure of Cowhide

The Cowhide is the natural, unbleached skin and hair of a cow. It retains the original coloring of the animal. In addition, cowhides are a natural product/by-product of the food industry from cattle. The compositions are of hide (free, bonded and unbounded) water, protein, fats, mineral salts and other substances. Each composition parts estimated percentage share of the total are shown in detail Fig. 2.2.

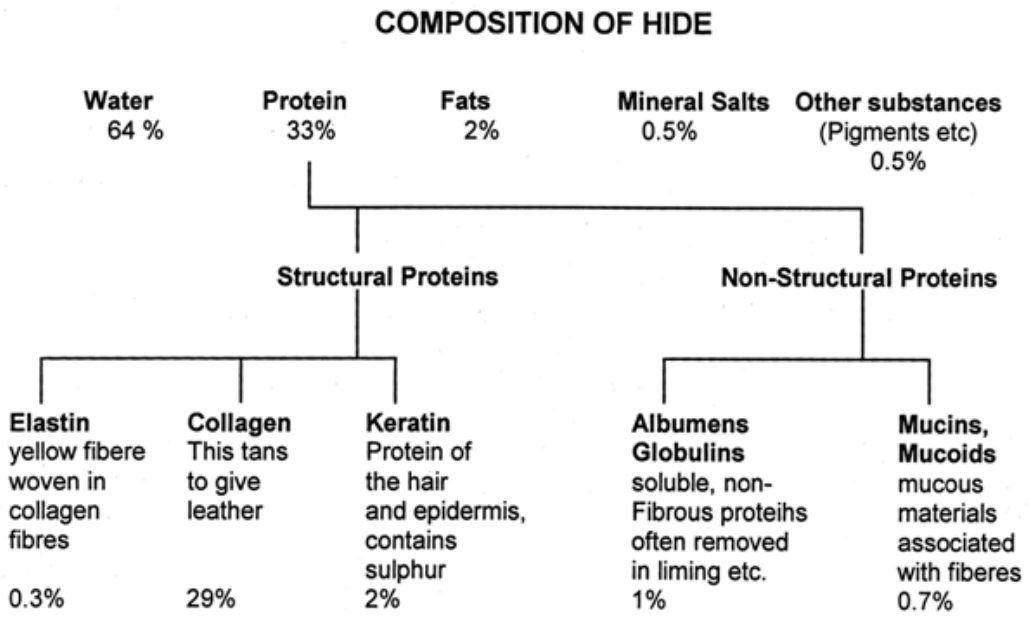


**Fig. 2.2 Composition of Hide**

## 2.3 Processes in Leather Making

Tanning, the process of transforming the animal hides to leather, involves the conversion of skin, a natural renewable resource, which is putrescible and inflexible when dried, to a chemically and structurally modified form with long shelf life and high flexibility. The leather production process is made up of several operations and typically includes hide and skin storage and beam house operations, tanning operations, post-tanning operations and finishing operations [2] & [3].

## 2.4 Hide and Skin Storage and Beam House Operations

The main objective of the storage and beam house operations is to prepare hides and skins for the tanning operations. The detail process under taking in storage and beam house are sorting, trimming, curing and sorting, soaking, unhearing and liming, painting and liming, fleshing and splitting [2], [3] & [4].

**Trimming:-**Trimming is generally carried out during the sorting process. Some of the edges (legs, tails, face, udders, etc.) of the raw hides and skins can be cut off. Usually this process step can be carried out in tanneries before soaking process. [4].

## 2.5 Solid Waste Generated by Leather Industry

Solid wastes generated by the leather industry were found to be huge impact to the environment and the society as well. The solid wastes generated in each distinct tanning sub process were classified as follows:

i. Wastes from untanned hides/skins (trimmings, fleshing wastes)
ii. Wastes from tanned leather (shaving wastes, buffing dust)
iii. Wastes from dyed and finished leather (trimmings from leather).

When comparing the tanning sub-process solid waste generation 80% of solid wastes are generated during pre-tanning processes, while 20% of the wastes are caused by post-tanning processes. Due to the bad smell they produce during their putrefaction and their harmful chemical content such as sodium sulphade, untanned hide/skin wastes collectively have negative effects on the soil, socity water resources of the environment [6] & [7].

## 2.6 The Proportion of Solid Waste Generated Tanning Process

The proportion of solid waste is generated during in tanning processing are the first is fleshing waste accounts 50-60% of the total solid waste. Secondly, the Chrome shaving, chrome split and buffing and buffing dusts this waste takes 5-7% share of the total. Thirdly, raw skin and hides trimmings accounts 5-7% of the total solid waste. The proportion of solid waste generated in tanning sub unit operations are beam house 80% of the total, Tanning accounts 19% of the total and finishing unit operation generate 1% of the total waste [5] & [6].

Therefore, the research was focus on reducing beam house solid waste and converting the raw trimming wastes to high valued product by extraction elastin. This research helps and plays a vital role to reduced environmental load generated by trimming wastes and makes feasible the waste contains a high value product. Moreover, the research shows a new venture of converting the trimming waste to environmental friendly product.

## 2.7 Tannery Solid Waste Utilization Overview

The tannery solid waste was found to be a hidden wealth that should be used for the production of different environmentally friendly products. Now days, the researchers tried to exploit this wealth for the design of differed environmentally friendly product. These products will discuss in detail as follows:-

**Fleshing wastes**: Fleshing has been explored for the possible utilization as Fleshing hydro lysate that improved uptake of chromium in chromium tanning and rechroming, for the preparation of Biodiesel by using transesterification process, Fleshing hydro lysate contains proteins was used for preparation of Sytan chemicals which used in post tanning process of leather, Glue, Gelatin, Lipids in fleshing waste used for the preparation of fat liquors, Lime flashings have been utilized for stabilizing the delimited pelt, which has been named as Reactive Protein(RP) [5], [6] & [7].

**Chrome Shaving Wastes**: Chrome tanned leather, splits and trimmings have been useful in obtaining glue, gelatin, protein flavor and reconstituted collagen [5], [6] & [7].

**Hair**: The hair is Keratin is rich in cystine has been hydrolyzed using concentrated NaOH or HCl. The hydrolsate prepared from keratinous material has been employed in chrome tanning and rechroming. Moreover, the study showed that hydrolysate helps to improve the chrome exhaustion of tanning bath and rechroming bath. In addition, the keratin also used in natural compost preparation [5], [6] & [7].

**Raw trimmings and Wet blue trimmings:** this solid waste was utilized for the preparation of gelatin and glue [5], [6] & [7].

**Chrome and buffing dust:** are useful in developing retanning agent, poultry feed, fertilizer and landfill sites [5], [6] & [7].

To sum up, this research by utilizing the raw trimming was able to produce high value product called elastin that have versatile application in in the area of biomedical and tissue engineering , Pharmaceuticals and cosmetics industries.

## 2.8 Proteins

Proteins are the most abundant biological macromolecules, occurring in all cells and all parts of cells. Proteins also occur in great variety; thousands of different kinds, ranging in size from relatively small peptides to huge polymers with molecular weights in the millions, may be found in a single cell. Moreover, proteins exhibit enormous diversity of biological function and are the most important final products of the information pathways. Proteins are the molecular instruments through which genetic information is expressed [14].

Relatively simple monomeric subunits provide the key to the structure of the thousands of different proteins. All proteins, whether from the most ancient lines of bacteria or from the most complex forms of life, are constructed from the same set of constituent parts of 20 amino acids, covalently linked in characteristic linear sequences [14].

Proteins are the most versatile macromolecules were found in living systems and serve vital functions in all biological processes. They function are variety and important role to living organisms. These functions are as catalysts, they transport and store other molecules such as oxygen, they provide mechanical support and immune protection, they generate movement, they transmit nerve impulses, and they control growth and differentiation [15].

Proteins are built from 20 simpler constituent compounds called amino acids. The amino acids contain a basic amino (NH2) group at one end and an acid (COOH) group at the other end. The amino acids in the protein molecule are linked together through peptide (-CO NH-) bonds. The peptide bond formation was carried out using two amino acids by releasing water during the bond formation. The detail process was shown in Fig.2.3.

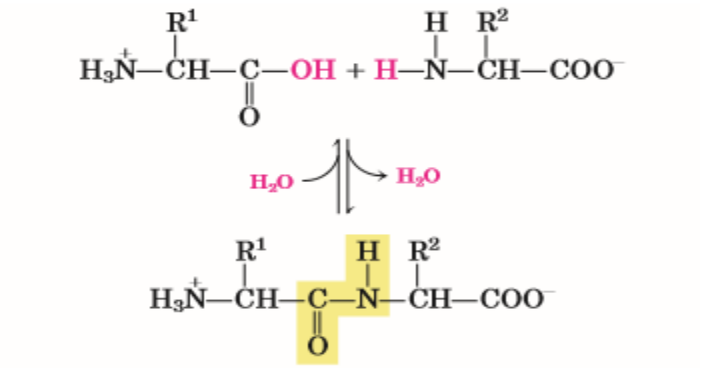

**Fig. 2.3 Formation of a peptide bond by condensation**

The amino acid unit in a peptide is often called a residue (the part left over after losing a hydrogen atom from its amino group and the hydroxyl moiety from its carboxyl group). In a peptide, the amino acid residue at the end with a free α-amino group is the amino-terminal (or N-terminal) residue; the residue at the other end, which has a free carboxyl group, is the carboxyl-terminal (C-terminal) residue [14].

## 2.9 Classification of Proteins

Since proteins have different amino acid composition chains (the type and number of each amino acid present in the protein), sequence of amino acids (the order in which amino acids are linked), size, conformation in space (three dimensional folding pattern) and function, their classification becomes a rather difficult task[16]. Although all schemes have some overlapping features, depending on their physicochemical properties and functions proteins can be classified in different ways in the following:-

### Classification Based on the Source of Protein Molecule

Proteins have been traditionally divided into two well-defined groups. These are animal proteins and plant proteins. Animal proteins are the proteins derivative from animal sources such as eggs, milk, meat and fish. They are usually called higher-quality proteins because they contain adequate amounts of all the essential amino acids. On the other hand, plant proteins are called lower-quality proteins since they have a low content (limiting amount) of one or more of the essential amino acids. The four most common limiting amino acids are methionine, lysine, threonine and tryptophan [16].

**Classification Based on Shape**

Based on the shape of protein molecule the proteins have been grouped under two categories. These are globular and fibrous.

***i. Globular or Corpuscular Proteins.***

These proteins have an axial ratio (length: width) of less than 10 (usually not over 3 or 4) and possess a relatively spherical or ovoid shape in space structure. These are usually soluble in water or in aqueous media containing acids, bases, salts or alcohol, and diffuse readily. The globular proteins are more complex in conformation than fibrous proteins, have a far greater variety of biological functions and are dynamic rather than static in their activities. Tertiary and quaternary protein structures are usually associated with this class of proteins. Nearly all enzymes are globular proteins, as are protein hormones, blood transport proteins, antibodies and nutrient storage proteins [16].

***ii. Fibrous or Fibrillar Proteins.***

These proteins have axial ratios greater than 10 and resemble long ribbons or fibres in shape. These are mainly of animal origin and are insoluble in all common solvents such as water, dilute acids, alkalies and salts and also in organic solvents. Most fibrous proteins serve in a structural or protective role in the animal body. The fibrous proteins are extremely strong and possess two important properties which are characteristic of the elastomers. These are: (a) they can stretch and later recoil to their original length. (b) They have a tendency to creep, i.e., if stretched for a long time, their basic length increases and equals the stretched length but, if the tension on the two ends of the fibril is relaxed, they creep to their shorter and shorter length [16].

It is a heterogeneous group and includes the proteins of connective tissues, bones, blood vessels, skin, hair, nails, horns, hoofs, wool and silk. The important examples are:

I. Collagens: - These are of mesenchymal origin and form the major proteins of white connective tissues (tendons, cartilage) and of bone. More than half the total protein in mammalian body is collagen[16] & [17].

II. Elastin: - Also of mesenchymal origin; form the major constituents of yellow elastic tissues (ligaments, blood vessels); differ from collagens in not being converted to soluble gelatins[16] & [17].

III. Keratins:-These are of ectodermal origin; form the major constituents of epithelial tissues (skin, hair, feathers, horns, hoofs, nails); usually contain large amounts of sulfur in the form of cystine– human hair has about 14% cystine. It is the principal constituent of the fibres of silk; composed mainly of glycine, alanine and serine units [16] & [17].

**Classification Based on Solubility**

Proteins differ in their aqueous solubility from being highly soluble to totally insoluble in water. Water-soluble proteins are generally smaller in size and contain proportionally higher amount of polar amino acids. They usually have compact and globular structures and can be coagulated by exposure to heat. Some common examples of water soluble proteins include plasma albumin and the well-known egg proteins, namely ovomucoid and ovalbumin. Water-insoluble proteins are usually larger is size, fibrous in nature and contain relatively larger amount of non-polar amino acids. They are more common among plants and are soluble in alcohol and acidic or alkaline solvents. Gliadin from wheat and zein of maize are well known examples of this class of proteins. In addition, there are many proteins that share the properties of both of these classes to varying extent. They are sparingly soluble in water and have variable sizes and diverse functions. Collagen and fibrinogen are well known examples of this class of protein [16] & [17].

**Classification Based on Composition**

Composition of proteins is the most common criteria for their classification. Proteins devoid of structural constituents other than amino acids are called simple proteins. Examples of simple proteins are Albumins, Globulins, Glutenins etc. If a protein is associated with a non-amino acid component it is named as conjugated protein. Examples of conjugated proteins are Glycoprotein, Lipoproteins, Nucleoproteins, Metalloproteins and Flavoproteins [16] & [17].

**Classification Based on Biological Function**

Depending upon their physical and chemical structure and location inside the cell, different proteins perform various functions. As such diverse proteins may be grouped under following categories, based on the metabolic functions they perform [16].

**Table 2.1 Classification based on biological function of proteins**

| Class of protein | Function | Examples |
|---|---|---|
| **Enzymic proteins** | Biological catalysts | Urease, Amylase, Catalase, Cytochrome C, Alcohol dehydrogenase |
| **Structural proteins** | Strengthening or protecting Biological structures | Collagen, Elastin, Keratin, Fibroin |
| **Transport or carrier proteins** | Transport of ions or molecules in the body | Myoglobin, Hemoglobin, Ceruloplasmin, Lipoproteins |
| **Nutrient and storage proteins** | Provide nutrition to growing embryos and store ions | Ovalbumin, Casein, Ferritin |
| **Contractile or motile proteins** | Function in the contractile system | Actin, Myosin, Tubulin |
| **Defense proteins** | Defend against other organisms | Antibodies, Fibrinogen, Thrombin |
| **Regulatory proteins** | Regulate cellular or metabolic activities | Insulin, G Proteins, Growth hormone |
| **Toxic proteins** | Hydrolze (or degrade) enzymes | Snake venom, Ricin |

## 2.10 Proteins Structure/Configuration

According Linderstom-Lang proposed four levels of structural organization for proteins based on the degree of complexity of their molecules. They are:

i) Primary structure ii) Secondary structure iii) Tertiary structure iv) Quaternary structure

Three of these structural levels (primary, secondary and tertiary) can exist in molecules composed of a single polypeptide chain, whereas the fourth (i.e., quaternary) involves interactions of polypeptides within a multichained protein molecule. In mathematical term, these are also depicted as $1^o$, $2^o$, $3^o$ and $4^o$ respectively [16].

Fibrous proteins are relatively simple molecules with primary and secondary levels of structure. On the other hand, globular proteins have high complexity due to all the four levels of structural organization [18] & [19].

**Primary structure: Amino Acid Sequence**

The primary structure of a protein refers to the number and sequence of amino acids, the constituent units of the polypeptide chain. The main mode of linkage of the amino acids in proteins is the peptide bond which links the α-carboxyl group of one amino acid residue to the α-amino group of the other. The proteins may consist either of one or more peptide chains [14].

The chain formed by polymerization of amino acid molecules provides the primary structure of a protein. Together with any covalent cross linkages and other modifications, this may also be called the covalent structure of the protein. Each monomer unit in the chain is known as an amino acid residue. This term acknowledges the fact that each amino acid has lost one molecule of $H_2O$ during polymerization [19].

**Secondary Structure of Proteins: Helix Formation or Local Folding**

The folding and hydrogen bonding between neighboring amino acids results in the formation of a rigid and tubular structure called a helix. This constitutes the secondary structure of proteins, which refers to the steric or spatial relationship of amino acids that are near to each other in the amino acid sequence. Based on the nature of hydrogen bonding (whether intramolecular or

intermolecular), Pauling and Corey (1951) identified two regular types of secondary structure in proteins: alpha helix (α-helix) and beta pleated sheet (β-pleated sheet) [16].

**Alpha helix (α-helix):** The polypeptide chain with planar peptide bonds would form a right-handed helical structure by simple twists about the α-carbon-to-nitrogen and the α-carbon-to-carboxyl carbon bonds. They called this helical structure as α-helix. The helix is so named because of the mobility of α-carbon atoms [16].

**Beta pleated sheet (β-pleated sheet):** The second type of repetitive, minimum-energy or stable conformation, which they named β-pleated sheet (β because it was the second structure they elucidated, the α-heilx having been the first). The formation of β-pleated sheets depends on intermolecular (inter chain) hydrogen bonding, although intra molecular hydrogen bonds are also present. The pleated sheet structure is formed by the parallel alignment of a number of polypeptide chains in a plane, with hydrogen bonds between the >C = O and –N-H- groups of adjacent chains. The R groups of the constituent amino acids in one polypeptide chain alternately project above and below the plane of the sheet, leading to a two-residue repeat unit. The β sheet structures are quite common in nature and are favoured by the presence of amino acids, glycine and alanine [16].

**Other Secondary Structures**: The γ-helix, is an example of a highly hydrogen-bonded structure that is insufficiently stable to be used in proteins because of the lack of interatomic contacts. In addition to the α-helix and β-pleated sheet structures, other repetitive structures also exist in one or a few specialized proteins. Two such was structures (β-turn and collagen triple helix) [16].

**Elastin**

Elastin is found in elastic connective tissues such as ligaments and blood vessels and skin. It resembles collagen in some of its properties, but differs in other. The polypeptide subunit of elastin fibrils is tropoelastin (MW 72,000), containing about 800 amino acid residues. Tropoelastin differs from tropocollagen in having many lysine but few proline residues. Also, the helix which it forms is quite different from a helix and the collagen helix[16].

Tropoelastin consists of α helical portions of polypeptides rich in Gly residues, separated by short regions containing Lys and Ala residues. The helical regions stretch on applying tension

but regain their original length when tension is released. The regions containing Lys residues form covalent cross-links. Four lysine side chains come together and are enzymatically converted into desmosine and a related compound, isodesmosine ; these amino acids are found exclusively in elastin. Like collagen, elastin also contains lysinonorleucine. These nonstandard amino acids are capable of joining tropoelastin chains into arrays that can be stretched reversibly in all directions (Fig. 2.4) [16].

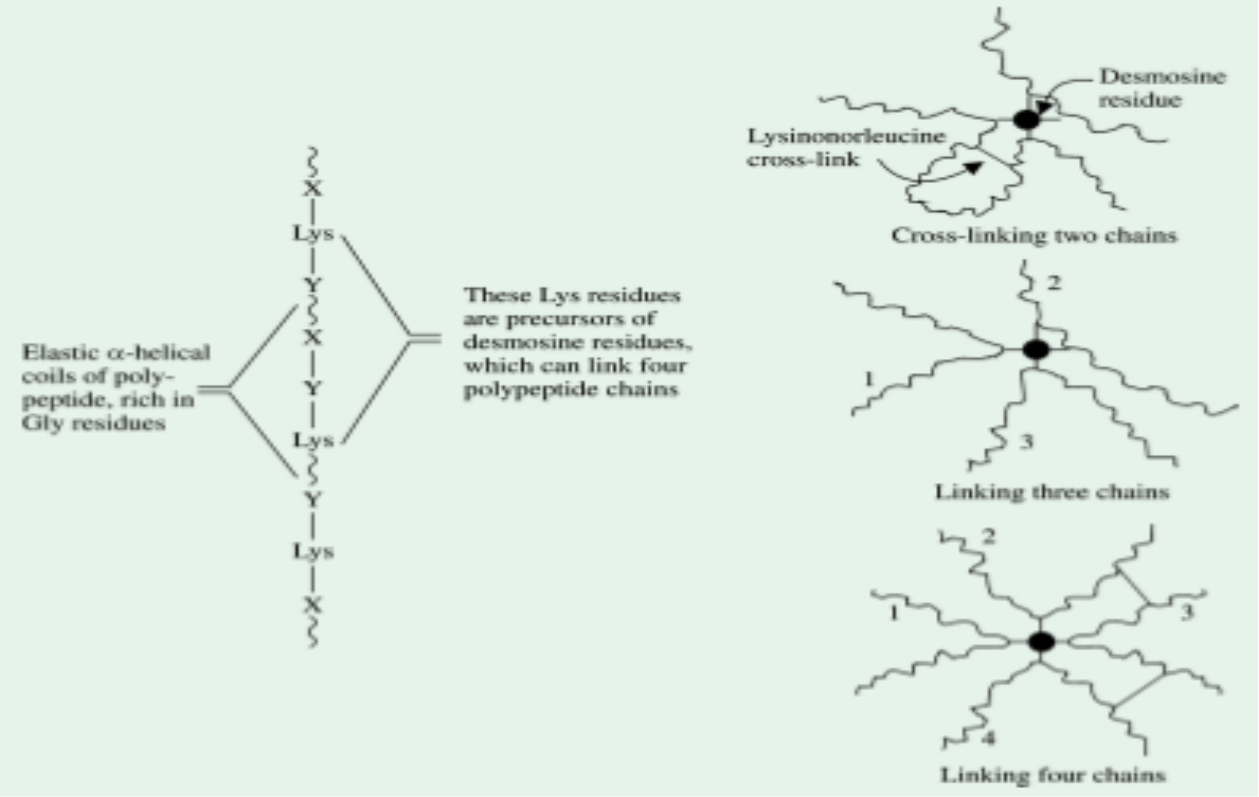


**Fig 2.4 Tropoelastin molecules and their linkage to form a network of polypeptide chains in elastin**

**Tertiary structure: Folding of the Chain or Overall Folding**

Globular proteins, the helix must, therefore, possess many other types of bonds placed at regular intervals. These additional bonds include disulfide, hydrogen, hydrophobic and ionic. In such globular proteins (including enzymes, transport proteins, some peptide hormones and immunoglobulins), polar groups because of their hydrophobicity are most often located on the

molecule's exterior and nonpolar R groups in the interior, where their interactions create a hydrophobic environment. The tertiary structure, thus, involves the folding of the helices of globular proteins. It refers to the spatial arrangement of amino acids that are far apart in linear sequence and to the pattern of disulfide bonds. The dividing line between secondary and tertiary structure is, hence, a matter of taste. Xray crystallographic studies have revealed the detailed 3-'D' structures of more than 300 proteins [16].

**Quaternary Structure of Proteins: Protein-Protein Interactions**

A fourth degree of complexity in protein structure has recently been recognized to be of great value in many proteins. Some globular proteins consist of 2 or more interacting peptide chains. Each peptide chain in such a protein is called a subunit. These chains may be identical or different in their primary structure. This specific association of a number of subunits into complex large-sized molecules is referred to as the quaternary structure. In other words, quaternary structure refers to the spatial arrangement of subunits and the nature of their contact. The same forces (disulfide, hydrogen, hydrophobic and ionic bonds) involved in the formation of tertiary structure of proteins are also involved here to link the various polypeptide chains [16].

## 2.11 Techniques of Protein Purification and Characterization

Proteins are purified by fractionation procedures. In a series of independent steps, the various physicochemical properties of the protein of interest are utilized to separate it progressively from other substances. The idea here is not necessarily to minimize the loss of the desired protein, but to eliminate selectively the other components of the mixture so that only the required substance remains [18].

It may not be philosophically possible to prove that a substance is pure. However, the operational criterion for establishing purity takes the form of the method of exhaustion: the demonstration, by all available methods that the sample of interest consists of only one component. Therefore, as new separation techniques are devised, standards of purity may have to be revised. Experience has shown that when a sample of material previously thought to be a pure substance is subjected to a new separation technique, it occasionally proves to be a mixture of several components[18].

The characteristics of proteins and other biomolecules that are utilized in the various separation procedures are solubility, ionic charge, polarity, molecular size, and binding specificity for other

biological molecules. Some of the procedures of protein purification that depend on protein characteristics are summarized in table 2.2 [18].

**Table 2.2 Different Protein Purification and characterization Techniques**

| Characteristic | Procedure |
|---|---|
| **Solubility** | 1. Salting in<br>2.Salting out |
| **Ionic Charge** | 1. Ion exchange chromatography<br>2. Electrophoresis<br>3.Isoelectric focusing |
| **Polarity** | 1.Adsorption chromatography<br>2.Paper chromatography<br>3.Reverse-phase chromatography<br>4.Hydrophobic interaction chromatography |
| **Molecular Size** | 1. Dialysis and ultrafilteration<br>2.Gel electrophoresis<br>3.Gel filtration chromatography<br>4.Ultracentrifugation |
| **Binding Specificity** | 1.Affinity chromatography |

**Protein Folding and Structure Characterization:-**The study of protein folding and characterization requires rapid mixing and observational techniques such as stopped-flow devices, circular dichroism (CD), pulsed H/D exchange followed by NMR, and fluorescence resonance energy transfer (FRET) [18].

**Protein Dynamics Characterization: -** Proteins are flexible and fluctuating molecules whose group motions have characteristic periods ranging from $10^{-15}$ to over $10^{-3}$ s. X-ray analysis, which reveals the average atomic mobility's in a protein, indicates that proteins tend to be more mobile at their peripheries than in their interiors. Molecular dynamics simulations indicate that native protein structures each consist of a large number of closely related and rapidly interconverting conformational sub states of nearly equal stabilities. The rates of aromatic ring

flipping, as revealed by NMR measurements, indicate that internal group mobilities within proteins vary both with the protein and with the position within the protein [18].

## 2.12 Amino Acids

Proteins are polymers of amino acids, with each amino acid residue joined to its neighbor by a specific type of covalent bond. Proteins can be broken down (hydrolyzed) to their constituent amino acids by a variety of methods, and the earliest studies of proteins naturally focused on the free amino acids derived from them. Twenty different amino acids are commonly found in proteins[14].

**Amino Acids Common Structural Features**

All twenty of the common amino acids are α-amino acids. They have a carboxyl group and an amino group bonded to the same carbon atom (the αcarbon) (Fig.2.4). They differ from each other in their side chains, or R groups, which vary in structure, size, and electric charge, and which influence the solubility of the amino acids in water. In addition to these 20 amino acids there are many less common ones. Some are residues modified after a protein has been synthesized; others are amino acids present in living organisms but not as constituents of proteins [14].

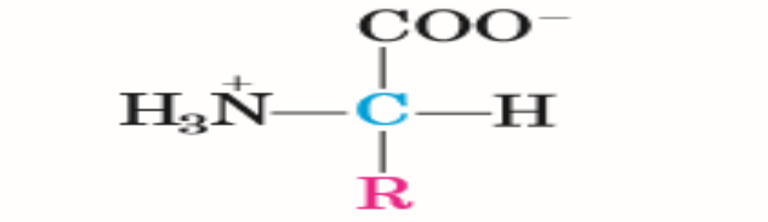


**Fig. 2.5 General Structure of an Amino Acid**

This structure is common to all but one of the α-amino acids. (Proline, a cyclic amino acid, is the exception.) The R group or side chain (red) attached to the α carbon (blue) is different in each amino acid.

**Classification of Amino Acids**

The various amino acids are usually classified according to the polarities of their side chains, R, which is substituent to the Cα atom[18].

Glycine, Alanine, Valine, Leucine, Isoleucine, Methionine, Proline (which is really a secondary amino acid), phenylalanine, and tryptophan are nonpolar amino acids [18].

Serine, Threonine, Asparagine, Glutamine, Tyrosine, and Cysteine are uncharged and polar amino acids [18].

Lysine, Arginine, Histidine, Aspartic acid, and Glutamic acid are charged and polar amino acids. The side chains of many of these amino acids bear acid–base groups, and hence the properties of the proteins containing them are pH dependent [18].

## 2.13 Elastin

Tissues like ligaments and arterial blood vessels need highly elastic fibers a property not shared by collagens. Such tissues therefore, contain large quantities of an elastic protein known as elastin. These proteins are very flexible and extendable. They contain large amount of glycine, alanine and valine amino acid residues. They have very little conventional types of secondary structures and generally occur as random coils. The primary structure of these proteins contains significant amount of lysine residues that are probably involved in inter fiber cross-linking. The nature of cross-linking is not similar to what we have seen in the case of collagen. Instead, four lysine residues are interconnected. There are fewer cross-links because four polypeptide chains are cross-linked at the same time making the structure highly interconnected rubbery network [3] & [8].

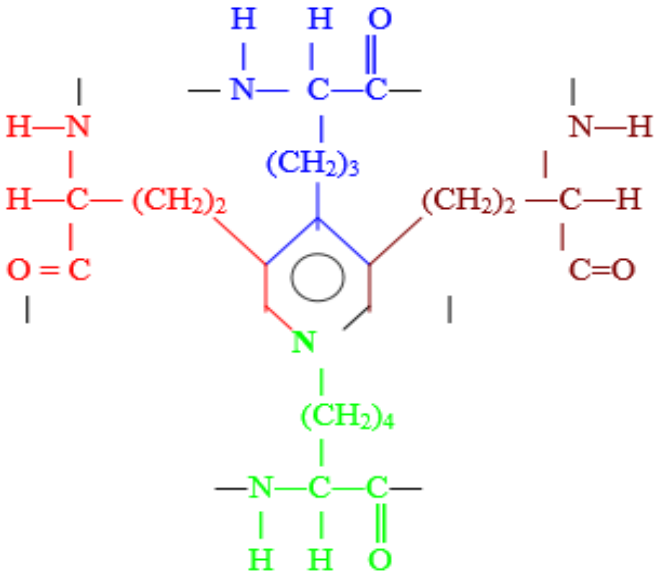

**Fig. 2.6 Interconnection of Four Lysine Residues in Elastin**

### Elastin Functions and Structure in the Skin Matrix

Elastin contributes greatly to the physical properties of skin and leather, because it controls the elasticity of the grain layer. The material of the grain is weak, so it cannot stretch to accommodate stresses in the skin when, for example, a joint is flexed. Hence it is adopts a convoluted, rippled form, that can flatten as the corium stretches. The mechanism by which it returns to its convoluted state when a stress is removed is through the action of elastin fibres, which extend when the skin is stretched, then contract to the resting position when the stress is removed. The elastin fibres are centered on the follicles, with coarse fibres running parallel to the skin surface and finer fibres running at right angles to the skin surface [3] & [8].

### The Difference between Elastin and Collagen

The several differences of elastin and collagen were summarized below in table 2.3.The difference mainly on the amino acid composition of each group indicated.

**Table 2.3 Comparison of elastin and collagen in Amino Acid composition per 1000**

| Amino acid type | Elastin | Collagen |
|---|---|---|
| **Glycine** | 355 | 330 |
| **Apolar** | 431 | 170 |
| **Acidic** | 14 | 120 |
| **Basic** | 10 | 96 |
| **Hydroxy** | 20 | 57 |
| **Proline** | 125 | 126 |
| **Hydroxyproline** | 23 | 93 |

The amino acid composition deference was:-

- The proteins have similar glycine content – by comparison with collagen, this indicates that the structure of elastin could be helical [3].
- Elastin has more apolar amino acids – therefore, the protein is more hydrophobic than collagen [3].
- There are more acid and basic amino acids in collagen than in elastin – making collagen relatively hydrophilic [3].
- The proteins have similar proline contents, which supports the suggestion of helical structure in elastin [3].
- There is less hydroxyproline in elastin – so the structure is less reliant on hydrogen bonding than collagen [3].
- The lack of basic residues in elastin means there is little lysine and very little histidine: covalent crosslinking of the type found in collagen is not possible.[3]

**In addition, the following major difference's observed Collagen and Elastin:**

- Although we know that collagen structure depends on covalent bonding, to hold the triple helix units together, there is not enough information within the amino acid composition alone to comment on the presence of covalent bonding in elastin [3].
- Collagen structure depends on electrostatic, salt links from the charged side chains, but they are not important in elastin [3].
- Collagen structure depends on hydrogen bonding, based on the high HP content – since the HP content is low in elastin, its structure relies less on H-bonding. This, too, supports the notion of the hydrophobic character of elastin [3].
- Bonding in elastin is dependent on hydrophobic interactions, due to the high content of apolar side chains. Such bonding is relatively unimportant in collagen [3].

### 2.14 The Price value of Elastin in the Market Expensive

According sigmmaldrich marketing website the price of elastin from bovine neck ligament powder is five gram in a polybottle is about 251.68(USD) and 10g in polybottle is about 410.54(USD).Elastin, soluble from bovine neck ligament salt-free lyophilized powder of one gram is about marketed 646.37(USD).

# Chapter Three

# Materials and Methods

## 3.1 Materials and Equipment's

The basic raw material were used for elastin extraction was the bovine raw hide trimmings. The bovine wet salted raw hide trimmings from shank, neck, ear and head regions were used to study for the research.

The chemicals and reagents were used for the research during characterization raw materials, extraction of elastin, physical, chemical and biological characterization are of laboratory grade chemicals. The detail chemical and reagent types, their concentration and brand were shown in Annex-2.

The laboratory equipment's that used during the experimental laboratory work are Weighing balance laboratory scale, pH paper and meter, measuring cylinder, conical flask, sample collecting bottles, burettes, micropipettes, different sized standard measuring flasks, round bottom flask, centrifuge, different size centrifuge tubes, test tubes, different volume beakers, crucible ,separation funnel , electrical stirrer, filter paper, scissors, Cutting knives. These laboratory Glassware's types and their respective volumes were shown in detail Annex-3.

The description of Major Laboratory machineries of the research work were listed as follows:-

- **High resolution light microscope-** for identification of staining image and structure of elastin**.**
- **Scanning Electron Microscope:** To study the topography, morphology, composition and crystallographic information of elastin.
- **DSC Analysis:** to study the behavior elastin during heat treatment .In addition, to identify the melting peak, endothermic and exothermic thermal shifts and the denaturation temperature.
- **TGA Analysis:** to identify the decomposition stage temperature and the percent of weight loss of the elastin.

- **UV-Spectroscopy Analysis:** to detect protein in the extracted sample by the analysis of the absorbance peaks.
- **Solid-State NMR Analysis:** to study and characterize the secondary structure of elastin using the spectrum amide carboxyl, aromatic and aliphatic region. Moreover, to identify the constituent amino acids that will be responsible for the chemical shifts.
- **FPLC analysis:** to detect soluble elastin, to chromatographic separations of proteins and to detect desmosine and isodesmosine aromatic amino acid of elastin cross link using absorbance in the chromatogram.
- **HPLC analysis:** to detect proteins, amino acids crosslinks of elastin and to perform amino acid analysis of elastin.
- **FTIR analysis:** to study the secondary structure and functional group analysis of elastin.

In addition, the laboratory equipment's and instruments that were used during the experimental work of the research are autoclave, sample preserving refrigerator, Heating bath, UV-spectrophotometer, scanning electron microscope (SEM), ultrasonic bath, soxhlet apparatus ,Shaker, sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis(SDS-PAGE) apparatus, reflective index measuring machine, light microscope, Differential Scanning Calorimetry (DSC) machine, Thermogravimetry (TGA) machine, Fast Protein Liquid Chromatography (FPLC), High Performance Liquid Chromatography (HPLC), Fourier-transform infrared spectroscopy (FTIR) and Solid State Nuclear Magnetic Resonance (NMR ) Spectroscopy. The laboratory equipments their brands and range of operation were shown in detail in Annex-4.

## 3.2 Raw Material Preparation and Characterization

### 3.2.1 Sample Preparation for Protein and Elastin Estimation

The 74.15gm raw trimming of hide sample was taken from ear area trimming. The sample cut in to small pieces and washed three times by 300% water then mixed with 300% water, 10% lime, 5% NaOH and 5% NaCl. The hide, water and alkaline mixture were mixed using shaker for 8hrs per day for seven days. Then, supernatant separated by decantation.

The supernatant (protein soup and lipid mixture) undergo micro particle separation process using mechanical separation, solvent extraction, separation and evaporation of solvent. First the

supernatant and residues forms clear two phase by using gravity sedimentation for three days. The supernatant was decanted and separated from residue. Secondly, the supernatant purified from solid particles using vacuum filtration. The vacuum filtrate further purified the sample from micro solid particles using centrifuge using 4500 rpm for one hour and taking supernatant and discard the residue.

The protein soup and lipid mixture were under go solvent extraction process using ethyl acetate with stoichiometric amount ratio1:2.The reaction under taken using batch reaction with the mixer speed was 1360 rpm for eight hours at the 35ºC.Then the protein, lipid and solvent mixture were kept in the shaker with 100 rpm and 20 ºC for two days. The protein soup solution, lipid and solvent mixture were separated using separation funnel. Then the excess solvent in the protein solution was extracted using rotary evaporator at 80 ºC for one hour. The protein soup free from lipids was ready for elastin content estimation and characterization.

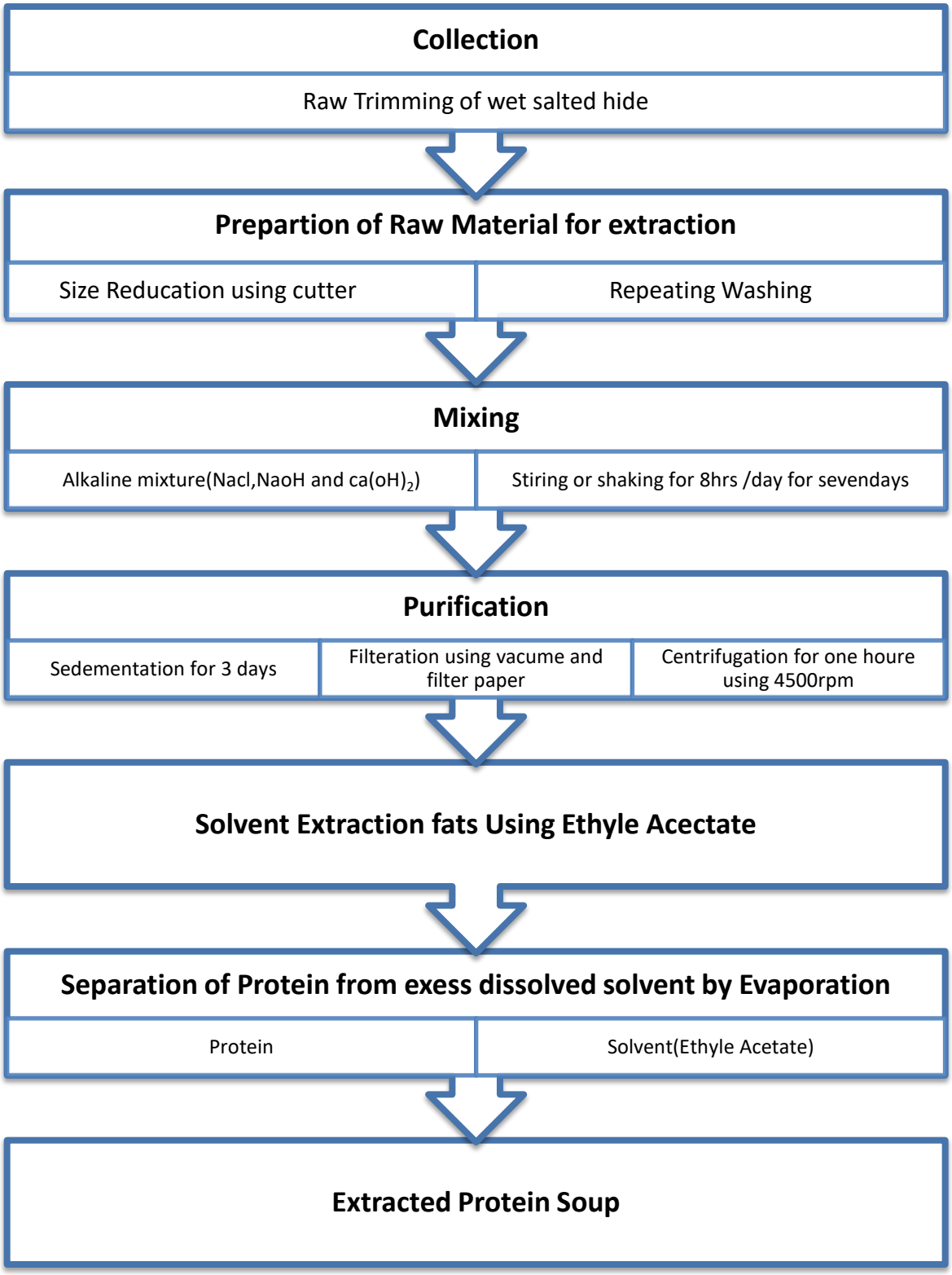


**Fig.3.1 Process Flow sheet of Protein Soup extraction**

### 3.2.2 Detection of Aromatic Cross links of Elastin Using UV- Spectroscopy

The Sample (10 ml protein soup) were taken and transferred in the volumetric flask .Then, the deionized water taken as a base and read in the UV spectroscopy. Then the protein soup diluted with deionized water and read the absorbance of peptide bond in the UV-Spectroscopy.

### 3.2.3 Fast Performance Chromatograph (FPLC) Detection & Estimation of Elastin

FPLC analysis of protein soup was using Sephadex G100 Column with the maximum pre column pressure of 80psi and maximum delta column pressure of 3650 psi. The flow rate was 1 ml/min and the maximum flow rate of 2 ml/min. The fraction collection rack type is F1 (12-13mm x 100mm) and the collection pattern was Serpentine. The start tube was A1 and fraction size of 1.0ml.The UV detector wavelengths were adjusted to at 275 nm, 280 nm, 285nm and 290 nm. Finally, the protein detection, analysis or purify mixtures of proteins, separation of protein were carried out by injecting the sample in the FPLC.

### 3.2.4 Electrophoretic Detection of Elastin content

The Elastin content and protein types were detected using SDS-PAGE Electrophoretic characterization Techniques. This characterization technique was used in the extracted protein soup (mixture) to separate the proteins according to their respective molecular weight. Therefore, the SDS-PAGE solution preparation, Gel preparation and the standard Electrophoretic Detection of Elastin content Procedure were used in the experiment show in Annex-5.

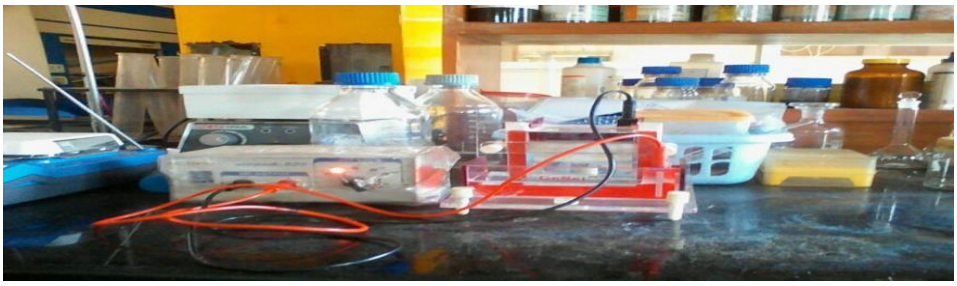

**Fig.3.2 SDS-PAGE Setup**

### 3.3 Preparation and Extraction of insoluble elastin sample

#### 3.3.1 Insoluble Elastin Preparation and Extraction Method

The insoluble elastin from was prepared according to U.S. patent [20] protocol with little modification. Briefly, the hide raw trimming 87.26gm was taken and cut in to small pieces and washed three times by 300% of water. The sample added in the vessel and mixed with 300% water, 10% $Ca(OH)_2$, 5% NaOH and 5% NaCl. Then, the alkaline and sample mixture held kept for five days under ambient temperature.

The supernatant impurities were drained and fibrous proteins free from hair and fat were three times washed by water. The fibrous protein added in the beaker and mixed with 30% of water, 1.5% ammonium chloride and 1.5% of hydrochloric acid and kept for 30 minutes. After the completion of the reaction time the PH were adjusted to be 7.The supernatant were drained and the fibrous protein washed 300% of water. Then, 600% of distilled water was added in the sample and kept for 12 hours. After 12 hours the water drained and added 10% of water in the fibrous protein sample.

The sample was autoclaved for 16 hours at a pressure of 1bar (15psi) at the temperature of 120 ºC. The treatment of fibrous protein under heat and pressure hydrolyzed the polypeptide bonds within the collagen while leaving the elastin in a cross-linked condition.

The oligopeptide solution were cooled at the temperature of 4°C.This temperature cooling separates a small amount of fat rose to the surface of the solution(supernatant) and leave elastin along with other minor impurities precipitated. Then, the collagen oligopeptide solution and precipitated elastin along the minor impurities heated and filtered by using filter paper.

The filter cake was autoclaved for 4hrs at the temperature of 120 ºC. The dry matters have 25.56 percent dry matter, 0.7 percent ash, and 14 percent cross-linked elastin based upon the weight of the dry matter.

The dry matter that contain elastin other impurities was washed by 300% of water by weight at 60° C for one hour. The elastin was free from salt and other soluble impurities. Then, the cross linked elastin was separated using filtration by water in the stainless steel sieve.

Finally, the filter cake was washed by 300% of water by weight at 60° C for one hour. Then, the insoluble elastin was extracted.

### 3.3.2 Insoluble Elastin Extraction Process Descriptions

The extraction processes were collectively and pictorially represented in the Fig. 3.3.


**Fig.3.3 Pictorial view of Insoluble Elastin Extraction Process**

### 3.3.3 Process Flow sheet of Extraction Process of Insoluble Elastin

The extraction of elastin fiber from raw hide trimming waste has eight distinct processes as shown Fig.3.4 after the collection of the raw material. The primary process, washing was carried out repeatedly to remove the impurities from the skin. Secondly, the size reduction was carried out to enhance the efficiency of extraction process.

The third step was pretreatment process, in which skin was treated with alkaline solutio ( sodium chloride, Sodium hydroxide and lime chemicals) in order to remove hairs, fats and soluble proteins from the skin matrix. The next process was separation of alkaline residue and fats using sedimentation, decantation and filtration using sieve. Afterwards, washing was carried out to remove impurities from the skin matrix.

The sixth step and the heart of the extraction process was autoclaving to dismantle and separate collagen and elastin from the skin histological structure. This process required 1 bar of steam pressure for 16 hrs of autoclaving. This process was effectively extracted 90% of elastin from the skin matrix while changing collagen to liquid gelatin and melting the fats.

The final process was filtration using filter paper and washing to clean the extracted elastin fibers.

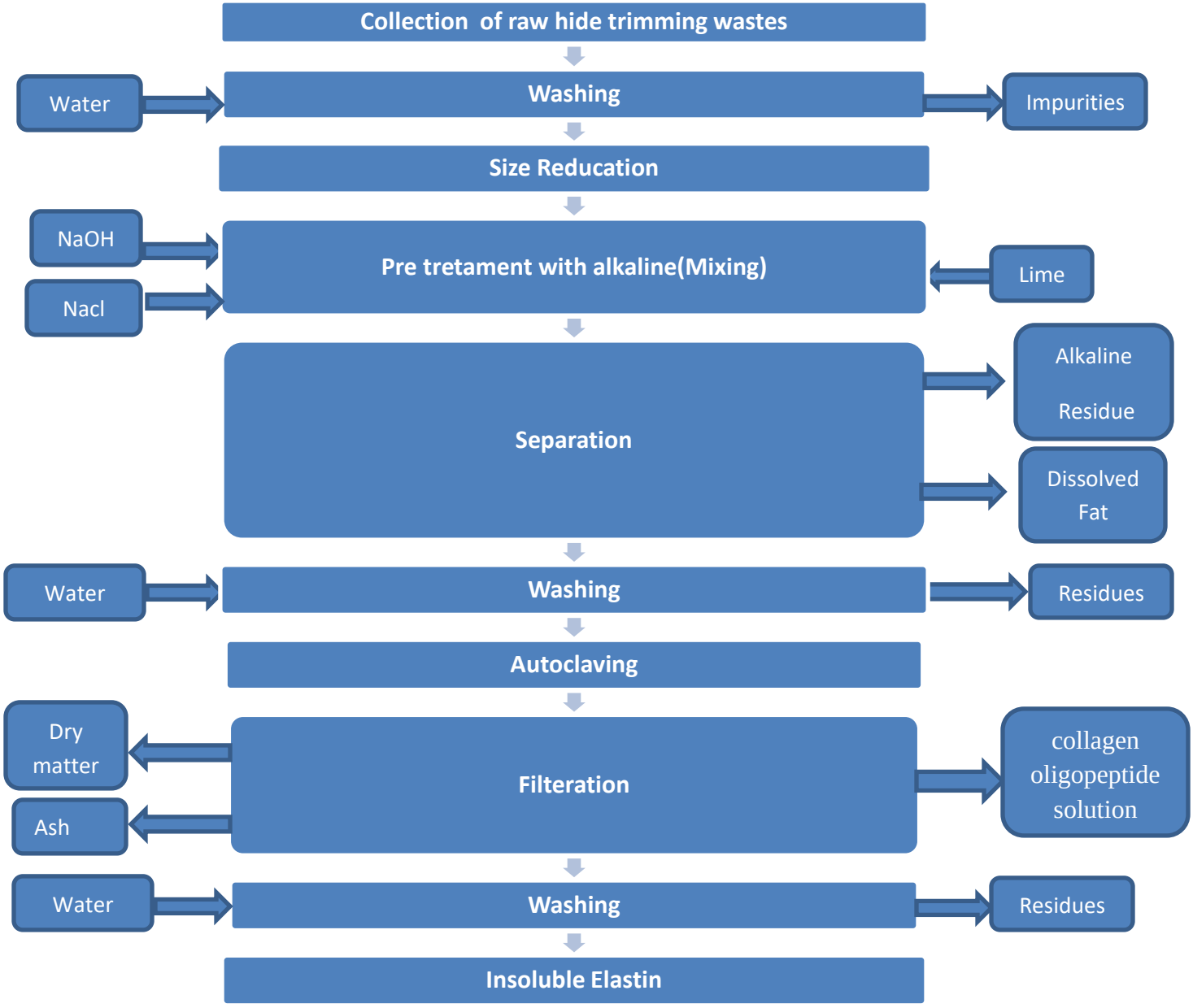


**Fig.3.4 Process Flow Diagram of Insoluble Elastin Extraction Process**

### 3.4 Elastin Characterization Techniques Performed

## 3.4.1 Physical Characterization of Elastin

The physical characterization of elastin was carried out using Differential Scanning Calorimetric (DSC), Thermal Gravimetric Analysis (TGA) and Scanning Electron Microscope (SEM) characterization techniques. These characterizations enable to study thermal property, the topography, morphology, composition and crystallographic information of elastin. The physical characterization each method will be summarized as follows:-

**Differential Scanning Calorimetric (DSC)**

The insoluble elastin of 4.1900 mg was taken Differential Scanning Calorimetric analysis. The analyses were performed with Tzero Aluminum Panandh Gas1: Nitrogen 50.0 ml/min and Gas2: Nitrogen 50.0 ml/min. The Temperature Range selected for the analysis was from-90.06 to 396.70 °C at 19.99 °C/min Heat Only. The heat flows (mW) against temperature °C were recorded for the sample.

**Thermal Gravimetric Analysis (TGA)**

The insoluble elastin of 9.29700 mg was taken to the Thermal Gravimetric Analysis (TGA) analysis. The analysis was performed using with the Platinum with Gas1 and Gas 2: was Nitrogen. The flow rate of balance was Gas: Nitrogen 40.0 ml/min and Sample Gas: Nitrogen 60.0 ml/min. The Ramp heat flow was adjusted between 20.00 °C/min to 800.00 °C. Then, the decomposition of sample weight (%) against temperature °C was recorded.

**Scanning Electron Microscope (SEM)**

The elastin fiber was dried in oven at 110ºC for ten minutes and grinded to make a power. Then, 50 µm elastin powder was coated with gold to enhance electron interaction with the specimen. Then, TESCAN VEGA 3 microscope with accelerating voltage10kv electron beams was projected on the specimen. The resolution range carefully chosen to scan the specimen was between 2µm to500 µm. Next, magnifications range selected to deeply scan the specimen is between 1.17kx to 694 kx. Then, the Different specimen SEM images were

taken to study topographical allied with surface features of specimen, Crystallographic information that tell the arrangement of atoms and morphological that is the shape and size of the elastin fibers were studied.

### 3.4.2 Chemical Characterization of Elastin

The chemical characterization of extracted elastin was performed using Solid State NMR experiments, HP content Determination, Verhoeff-Van Gieson (EVG) Staining, Physio-chemical test using Phosphate buffer and acetic acid and Fourier transforms infrared spectroscopy (FTIR).These characterization techniques were helps to study the secondary structure or protein dynamics property ,the HP content, the chemical property, reaction with staining chemicals and the functional group of the extracted elastin. The chemical characterization detail methods implemented during experimental work will be summarized here under.

**Solid State NMR experiments**

The solid-state NMR experiments were performed on a Bruker Avance-III HD 400 WB NMR spectrometer (9.4 T). The proton and carbon resonance frequencies were 400.07 and 100.61 MHz respectively. Sample was packed in a 4-mm-diameter zirconia rotor with a Kel-F cap. $^{13}C$ MAS spectrum of the sample was recorded at room temperature using a double resonance 4 mm MAS probe. $^{13}C$ cross polarization/total sideband suppression (CP/TOSS) experiment was performed at a spinning speed of 7 kHz to get sideband free spectrum. A contact time of 3 ms and a $^{1}H$ $90^{\circ}$ pulse length of 4 μs were used. Typically, 3072 scans were acquired with a relaxation delay of 4s. SPINAL-64 decoupling sequence was used to decouple protons during the carbon acquisition by employing radiofrequency field strength of 83 kHz. FID was subjected to an exponential multiplication function with a line broadening value of 150 Hz prior to Fourier transform. $^{13}C$ chemical shifts were referenced to the carbonyl signal of glycine at 176.03 ppm as an external reference standard.

**HP content Determination**

The Elastin hydroxylproline amino acid composition content was determined by using standard HP content protocol. This characterization technique helps to describe the property of elastin. Hence, the methods of solution and reagent reparation, standard preparation for the estimation of hydroxyproline, sample preparation for the estimation of hydroxyproline and HP Content Determination Procedure standard protocol were used in the laboratory work were shown in detail Annex-6.

**Verhoeff-Van Gieson (EVG) Staining Characterization**

The verhoeff-Van Gieson(EVG) staining characterization is one of the methods to determine the chemical properties of elastin. In addition, it can also to differentiate elastin from other fibrous proteins by generating different coloration on the surface of the protein fiber. Therefore, the methods of Verhoeff-Van Gieson Solution, different chemicals, Reagents preparations were Annex-6. Moreover, the Verhoeff-Van Gieson (EVG) Staining Procedure was used during the experimental works were shown in Annex-7.

**Phosphate buffer and acetic acid Test/Characterization (Physio-chemical test)**

The 1mg of the sample was taken and putted in closed test tubes. Then, using micro pipette 4ml of phosphate buffer was dropped on the sample surface in the test tube. The phosphate buffer detail preparation was shown in annex8. Then, the sample was heated in the ultrasonic apparatus at 60 ºC for one hour. After that, the sample was taken out from the ultrasonic apparatus and 4ml of acetic acid solution was dropped in the sample. Finally, the sample along with the mixed solution was heated in ultrasonic apparatuses at 60ºC for three days. Finally, the sample was taken out for the observation.

**Fourier transforms infrared spectroscopy (FTIR)**

The 6 mg of elastin fiber was taken using the crucible. Then, the sample was dried using water bath at 110º C for 10minutes.After that, the dried sample was grinded using mortar and made a powder. Then, the powder was mixed with 3 teaspoons of KBr and mixed thoroughly in a mortar while grinding with the pestle. Next, the sample and KBr mixture was pressed about 5000psi and formed pellet. Finally, pellet was placed in the FTIR sample holder and the generated FTIR spectrums of the sample were recorded in the resolution range between 349.0525$cm^{-1}$ and 7800.6488$cm^{-1}$.

### 3.4.3 Biological Characterizations

The biological characterizations on extracted elastin was enable us to determine the free amino acid composition and the compositional amino acid types of the elastin, the purity determination of elastin, to analyze the histological features, it also test and check that the biocompatibility of the product(extracted Elastin). Therefore, the biological characterization of the product was done using free amino acid analysis, histological study using Using Orcein and Methylene Blue method and biocompatibility test using immortalized human keratinocytes cells characterization techniques were used. The details of each characterization method will be shown in the following:-

**Free Amino Acid Analysis of Elastin using HPLC (High Performance Liquid Chromatography)**

**The Hydrolysis of Elastin and Preparation of sample for HPLC analysis:-**The 5mg purified elastin was dissolved in 6 ml of 6N HCl and was subjected to hydrolysis in boiling water bath for a period of 24 h. The tubes were cyclo-mixed for every 1 h for proper hydrolysis to take place. After 24 h of hydrolysis, the tubes were centrifuged at 3500 rpm for 15 min. The supernatant was filtered and was neutralized with 1N NaOH. Then the filtered solution was diluted to 1:100 of the volume (1 ml diluted to 100 ml) with milli-Q water and was proceed for estimation of protein amino acids in HPLC (High Performance Liquid Chromatography) and HP(Hydroxylproline) content determination [21], [22] & [23].

## Histology Using Orcein and Methylene Blue Method

### (Modified Taenzer-Unna orcein Method)

The histology characterization of the extracted elastin was done using Orcein and Methylene standard method. The method reagent solution preparations and the method sequential procedures were shown in detail in Annex-9.

## Toxicity Study (Bio compatibility study) using keratinocyte cells

Biocompatibility of fabricated scaffolds (sample) was assessed by MTT Assay. Briefly, fabricated scaffolds was cut into small pieces and placed it into the cell culture plate and kept it 2 hours under UV radiation for sterilization. After sterilization, HaCaT (immortalized human keratinocytes 20 K cell/well) cell were seeded onto cell culture plate and allowed it to grow for 24hours and 48 hrs at $37^{o}C$ in 5% $CO_2$ and 95% $O_2$ humidified incubator. After incubation the spent medium was removed and cells were examined under and observed the cells morphology using phase contrast microscope (Leical systems). Subsequently, 0.5mg/ml of MTT(Thiazolyl Blue Tetrazolium Bromide salt) in 1X PBS(500µL/well) was added and kept it for 4 hours in a dark/ $37^{o}C$.The MTT assay was carried out in triplicates. Following 4 hours incubation with MTT solution, the MTT solution was removed and formazan crystal was solubilized with 200µL DMSO and measured the absorbance at 570nm using BioRad ELISA Plate reader. The Percentage of cell viability was calculated by following formula.

Cell Viability (%) = (O.D of treated cells/O.D of untreated cells)*100

# Chapter Four

# Results and Discussion

## 4.1 Characterization of the Raw Material and Estimation of Elastin Content Results

### 4.1.1 Absorption Spectrum

The UV spectrum the extracted protein mix is shown in the Fig.4.1. The maximum absorbance found to be in 221nm wavelength. This result confirms that detection of protein at the region 215–220 nm. Moreover, this phenomenon is due to bonds of protein and detection of aromatic amino acids at the absorbance region of 280 nm [18].

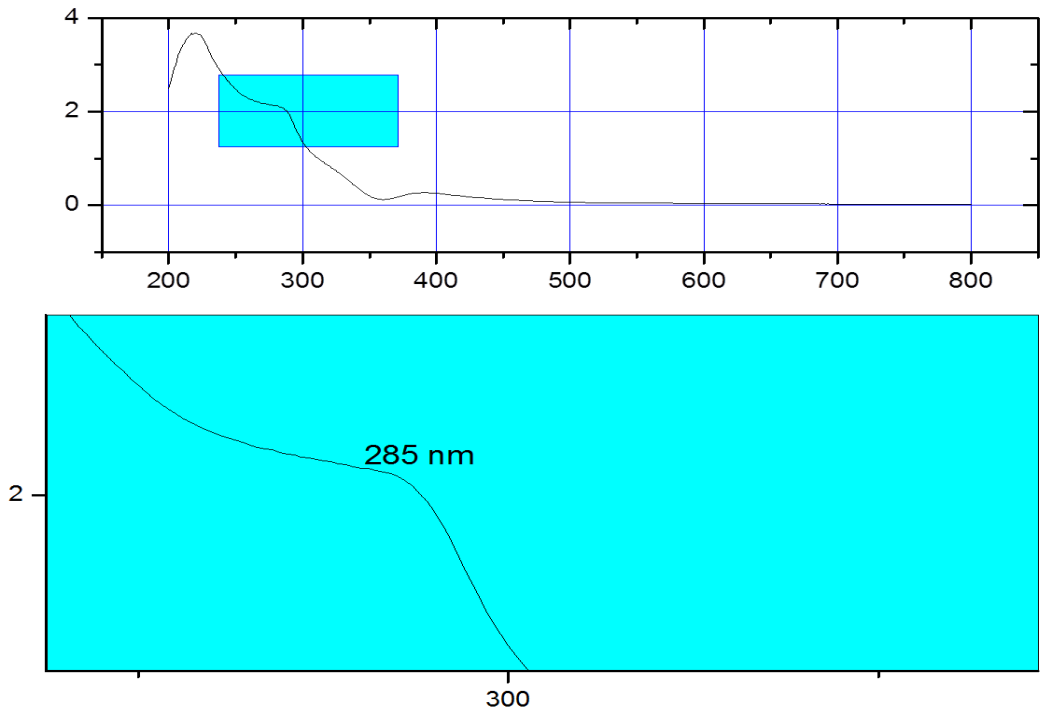


**Fig.4.1 Absorption Spectroscopy of Protein Mix**

Hence, the peptide UV absorbance results is shown in the Fig.4.1 at 221 nm which matches the previously reported literatures, which confirms the existence of aromatic amino acids in the protein mix[18]. The UV absorbance in 391nm region is due to the presence of pigment in the protein solution. Moreover, this region absorbance indicates that the protein mix contains aromatic amino acids namely Tyrosine, Tryptophan and Phenylalanine [18]. The isodesmosine aromatic cross link of elastin has been detected in the absorbance at 285nm region [24]. Furthermore, the UV absorption Spectrum result collectively specifies that the protein solution contains aromatic amino acids with high and significant level of protein concentration. To sum up, the detection of isodesmosine aromatic cross links of elastin indicates that alpha and beta elastin in the protein mix.

### 4.1.2 Electrophoretic Detection of Elastin in Protein Mix

The solvent purified protein mix was characterized using 12% SDS-PAGE and the result is shown in Fig.4.2A and B. This result indicates the molecular weight regions of proteins. The electrophoresis jel result indicates that there are two separate band regions. The bottom band a region extends from 5 to 8 KD and the upper regions ranges from 50 to 90 KD.

The first region (5-8kd) is tiny, scattered and non-uniform bands as shown in the Fig.4.2B.This band areas are characterized by β-elastin detection molecular weight regions. In addition, from electrophoretic lower bands result, this indicates that there is a high probability of detection of β-elastin [2] & [8]. Hence, the molecular weight of this type of elastin is between 5.5-6 KD [2] & [8]. The second region ranges from 50 to 90kd.This region has wide range of spectrum, uniform bands and clear concentrated bands as shown in the Fig.4.2 of SDS-PAGE Jel result. Moreover, this region possesses high possibility of detection of α-Elastin in the protein soup [2] & [8]. Therefore, the upper bands that are extends from 60-84 KD confirms that α-Elastin presence in the protein soup [2] & [8]. For further information, the upper and lower band regions are shown in the following Fig.4.2B.

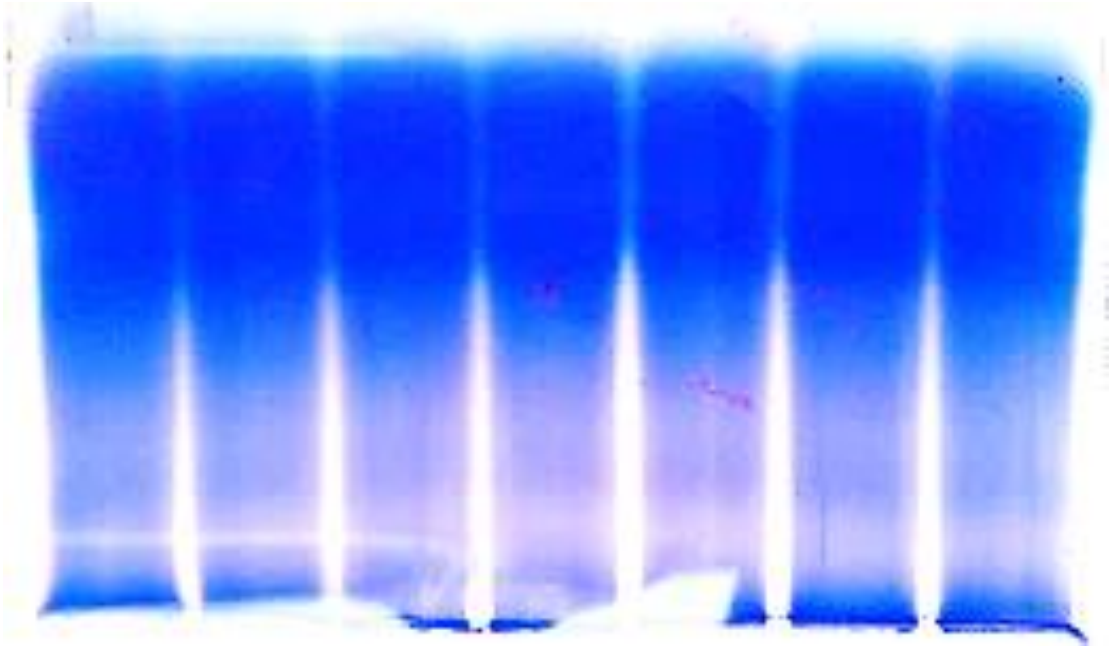

**Fig.4.2. A Original image of SDS-Gel Electrophoresis in 12% Jel of solvent purified extract with seven same samples.**

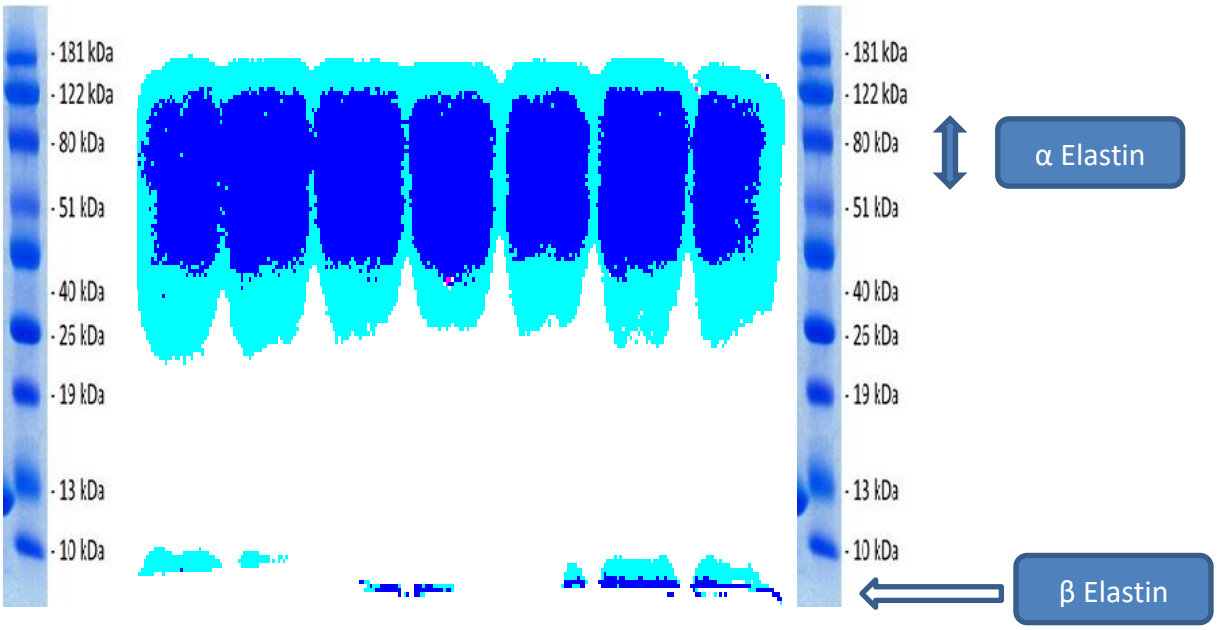


**Fig.4.2.B Contrasted Image of SDS-Gel Electrophoresis in 12% Jel of solvent purified extract with seven same samples. The figure showed two major bands in the extract.**

To sum up, SDS-PAGE result analysis shows that β-elastin and α-Elastin are detected in the extracted protein soup and the result confirms presence of elastin in the sample.

### 4.1.3 FPLC (Fast Protein Liquid Chromatography) Chromatogram Analysis

The FPLC chromatogram of the protein sample is shown in Fig.4.3A. From the chromatogram, it is shown that there are two peaks in the 280nm wavelength with retention time range has 50-80 min and 160-180 min. These two chromatogram peaks indicates that proteins exists in the extracted sample. Moreover, the small and narrow chromatographic band confirms that there is a better separation of proteins [25].

In Fig 4.3B, chromatograms with four different wavelengths (275,280,285 and 290 nm) are shown. The maximum chromatogram peak is obtained in the 275nm wavelength. This region of absorbance represents high concentration desmosine aromatic amino acid of elastin cross link presence in the extracted sample [2], [26] & [27]. In addition, it is indicated from FPLC chromatogram peak that there is also absorbance at 285 nm wavelength. This region of absorbance contains high concentration of isodesmosine aromatic amino acid of elastin [2], [26] & [27].

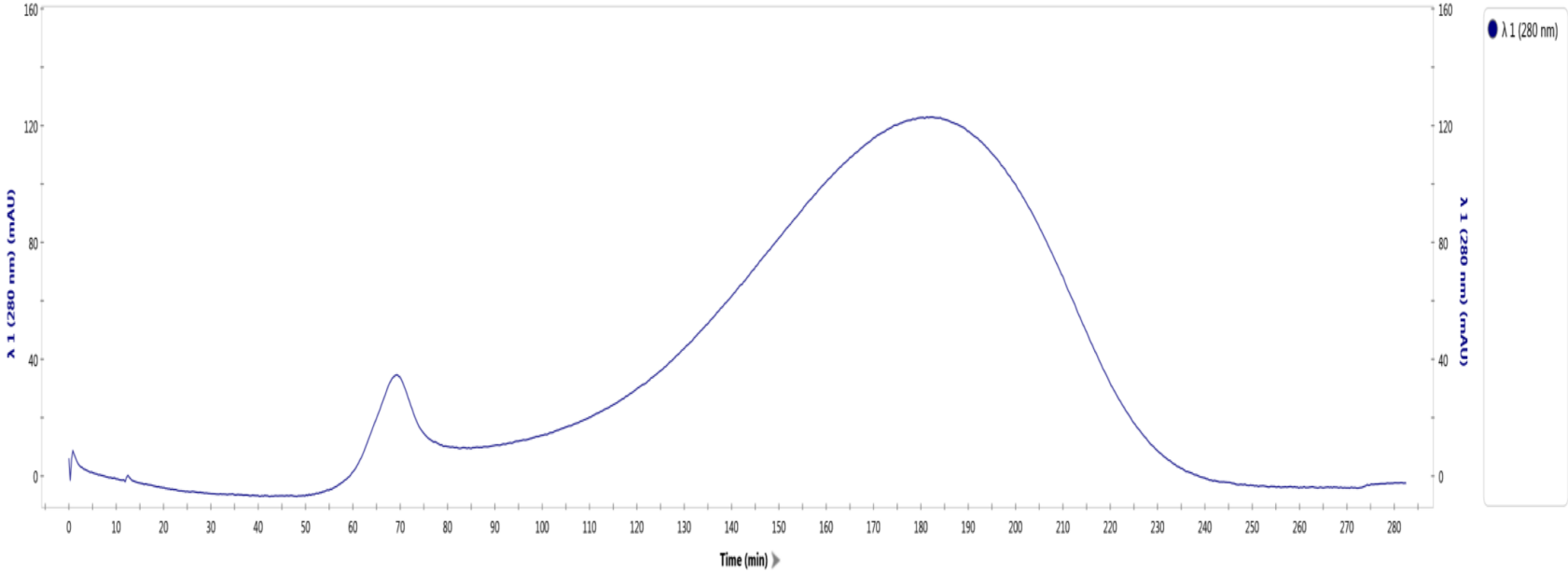


**Fig.4.3 A. FPLC Chromatogram at 280 nm Wavelength**

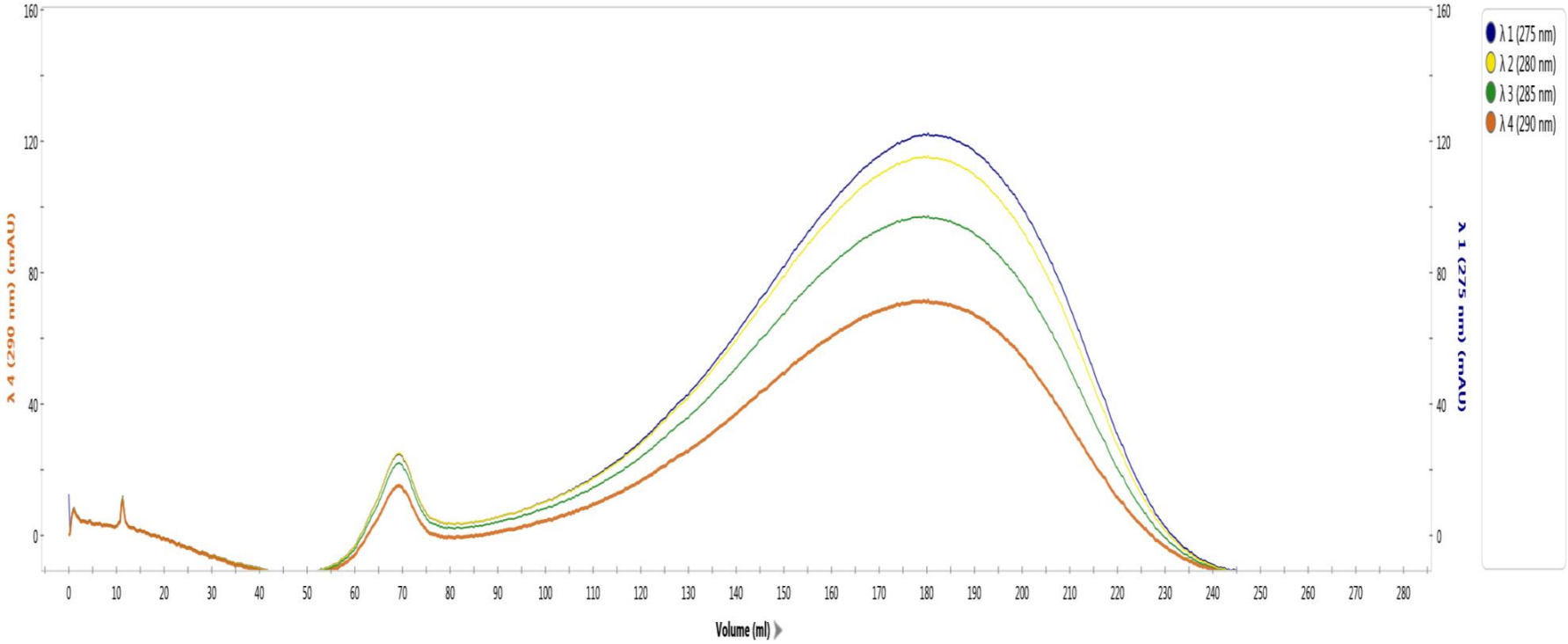


**Fig.4.3 B. FPLC chromatogram at 275, 280, 285 and 290 nm wavelengths**

From the chromatographic detections analysis, it confirms that desmosine and isodesmosine aromatic amino acids of elastin exist in the solution. This result confirms the presence of elastin in the extracted protein mixture.

### 4.1.4 Quantitative and Qualitative Analysis of FPLC Chromatogram

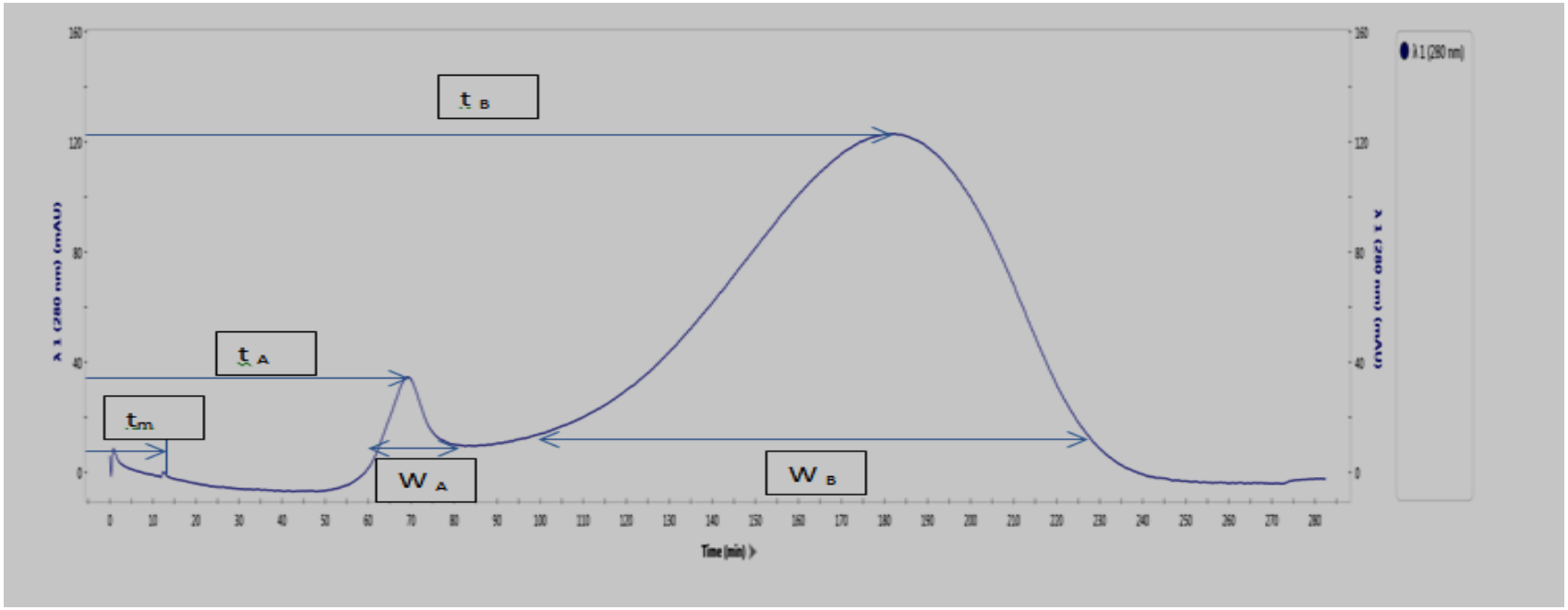


**Fig.4.3 C. FPLC chromatogram at 280nm that indicates mobile phase and Protein mix**

Quantitative and Qualitative Analysis and computation of FPLC Chromatogram was computed using Fig.4.3 C. It was found out that the resolution (RAB) equal to be 1.07. Hence, RAB >1 the separation was good and effective. Furthermore, the retention factor (K) and selectivity factor (α) for protein mix found to be $K_A = 5$ , $K_B = 14.22$ and $\alpha = 2.844$ respectively in which both of them values are greater one that in tells the separation of protein mix was good and effective[28]. Moreover, from quantitative and qualitative analysis of FPLC chromatogram, it was scientifically found out that the concentration of elastin in terms of peak area is estimated in the range of 122.515004 mAUmin and 97.19099849 mAUmin..Please refer Annex-11 for detail quantitative and qualitative computation of FPLC chromatogram

### 4.1.5 Findings of Raw Material Characterization and Elastin Content Estimation

- Tiny solid particles, suspended solids and lipids effectively have been purified from protein soup using developed mechanical separation, solvent extraction and evaporation methods.
- UV absorption Spectrum at 221nm wavelength specifies that protein solution contains aromatic amino acids with high protein concentration and also the detection of isodesmosine aromatic cross links of elastin at 285nm wavelength. It is also indicates presence of alpha and beta elastin.
- SDS-PAGE result analysis shows that β-elastin and α-Elastin detected in the extracted protein soup with higher level of concentration.
- From FPLC chromatographic detection, peaks at 275nm and 285nm wavelengths confirms that desmosine and isodesmosine aromatic amino acids are at a significant level in the solution. Moreover, the result confirms and scientifically justifies the elastin existence in the sample with high concentration.
- Quantitative and Qualitative Analysis of FPLC Chromatogram was enabled to compute the protein soup Retention time and volume, Chromatographic Resolution, Retention factor and Selectivity factor. The retention time, volume and peak width for the mobile phase, protein A and protein B were quantitatively determined by the analysis. In addition, the resolution ($R_{AB}$) was found to be 1.07. Hence, $R_{AB}$ >1 the separation was good and effective. Furthermore, the retention factor (K) and selectivity factor ($\alpha$) for protein mix was found to be $K_A = 5$ , $K_B = 14.22$ and $\alpha = 2.844$ respectively in which

both of values are greater than one. These results indicate the separation of protein mix was good and effective.

- The FPLC Chromatogram absorption peak scientifically determines Desmosine and Isodesmosine aromatic amino acids concentration of elastin crosslinks in the protein soup. Therefore, the concentration in terms of peak area estimate the elastin content of the skin matrix in the range of 122.515004 mAUmin and 97.19099849 mAUmin.

## 4.2 Extraction and Purification of Elastin Result

### 4.2.1 Extraction Process Results

The extraction process the extracted Elastin weight, weight of hide on dry basis and the Yield of process results were shown in Table 4.1.For Detail Extraction process result computation refer Annex-11.

**Table 4.1 Raw Material Input Mass, Extracted Elastin Mass and Yield Results**

| No. | Description | Measurement | Amount |
|---|---|---|---|
| 1. | **Raw Material (Input) Results** | | |
| **1.1** | The weight of the Hide sample in wet basis | Grams | 87.2627 |
| **1.2** | The moisture content of the sample | Grams | 56.72355 |
| **1.3** | The weight of the hide in dry basis | Grams | 30.54345 |
| 2. | **Extracted Elastin Products (output) Results** | | |
| **2.1** | The total weight of Filter cake | Grams | 1.98 |
| **2.2** | Dry Matter | Grams | 0.5061 |
| **2.3** | Ash | Grams | 0.1386 |
| **2.4** | Insoluble Elastin | Grams | 0.275 |
| 3. | **Extraction Process Yield Results** | | |
| **3.1** | Theoretical Yield of Elastin | Grams | 0.305435 |
| **3.2** | The actual Yield of the extraction process | Grams | 0.275 |
| **3.3** | Extraction Method Yield | Percentage | 90.04% |

### 4.2.2 Findings of the Elastin Extraction and Purification Process

- The Autoclaving process taken 1 bar of steam pressure and 16 h autoclaving time.
- The weight of the hide (Input) in dry basis found to be 30.54345 gram.
- The weight of filter cake was measured to be 1.98 gram.
- The mass of extracted insoluble elastin was found to be 0.275gram.
- The dry matter and ash impurities weight was found 0.5061gram and 0.1386 gram respectively.
- The yield the extraction process was computed to be 90%. This result indicates that the extraction was effective. In addition, the extraction process was optimized.

## 4.3 Physical Characterization of Elastin

### 4.3.1 Thermal Stability Studies of Extracted Elastin

#### 4.3.1.1 Differential Scanning Calorimetric (DSC) Analysis

The DCS thermogram of extracted elastin is shown in Fig.4.4.From the thermogram, it is observed that elastin has glass state in the temperature range of 0 ºC to 25 ºC at the heat flow of -0.03420318W/g. Then, it starts to change to rubbery state in the temperature range of 25 ºC to 50 ºC at the heat flow rate between -0.03420318W/g and-0.7672901W/g.

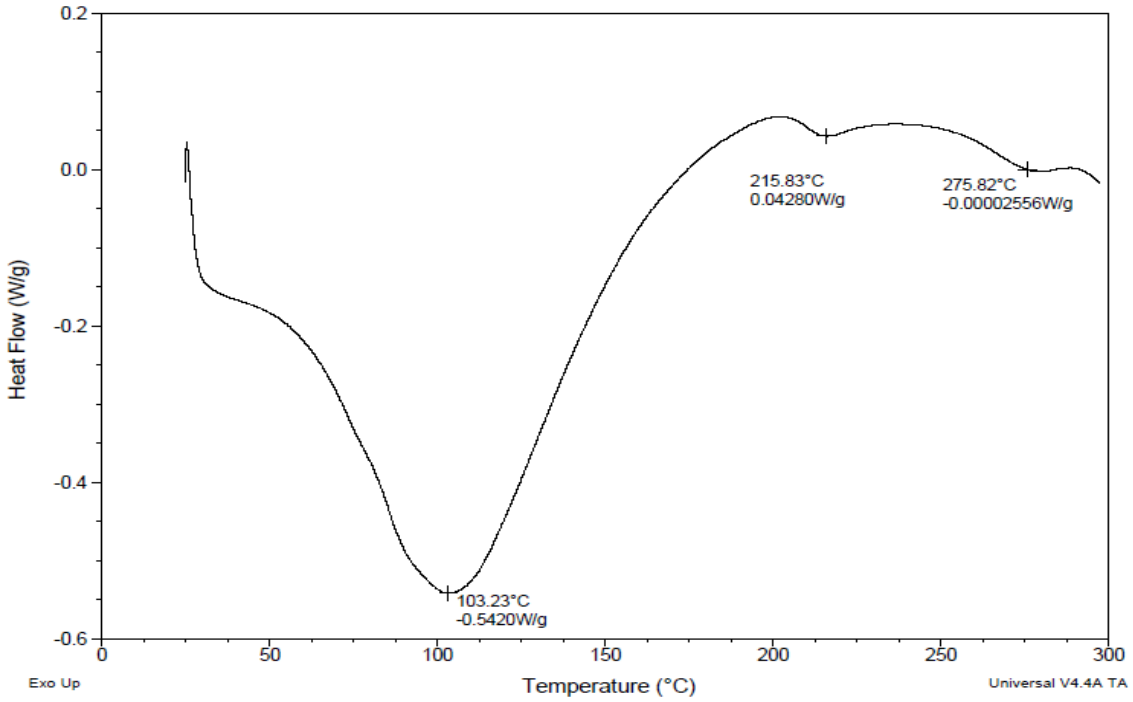


**Fig.4.4 DSC Curves of Elastin in the temperature range of -90.06°C to 396.70 °C at 19.99 °C/min Heat Only.**

The DSC thermogram study also indicates that the extracted elastin started to denature at the temperature of 215.83°C in the heat flow rate of 0.04280W/g. Further increase of temperature the elastin shows very high denaturation temperature at 275.82ºC in the heat flow rate of -0.00002556W/g.

### 4.3.1.2 Thermal Gravimetric Analysis (TGA) Analysis

The Thermal Gravimetric Analysis thermogram is shown briefly in the Fig.4.5 with the decomposition peaks along with temperature. Based on the TGA thermogram result of extracted elastin degradation peaks occur in six mass loss steps.

The first decomposition peak occurs by 12.73% weight loss in the temperature range of 0°C to 64.53°C.The second degradation peak indicate in the TGA thermogram by 11.14% weight loss in from 64.53°C to 253.25°C.The third decomposition peak displays thermogram by 4.33% weight loss in the temperature range of 253.24°C to 298.05 °C. The fourth degradation shown in

theromogram by 30.32% weight loss in the temperature range of 298.05°C to 367.04°C.The fifth degradation peak occur by 11.61% weight loss in the temperature range of 367.04°C to 626.33°C. The last decomposition peaks indicate in the TGA thermogram by 7.56% weight loss between 626.33°C and 800°C.All the percentage of mass lose step together with the temperature ranges are shown in the following Table 4.2 and Fig.4.5.

**Table 4.2 Six Mass loss steps percentage of Elastin along with temperature range**

| **Steps** | **% Mass loss** | **Temperature Range $^{0}$C** |
|---|---|---|
| 1 | 12.73 | 0-64.53 |
| 2 | 11.14 | 64.53-253.24 |
| 3 | 4.33 | 253.24-298.05 |
| 4 | 30.32 | 298.05-367.04 |
| 5 | 11.61 | 367.04-626.33 |
| 6 | 7.56 | 626.33-800 |

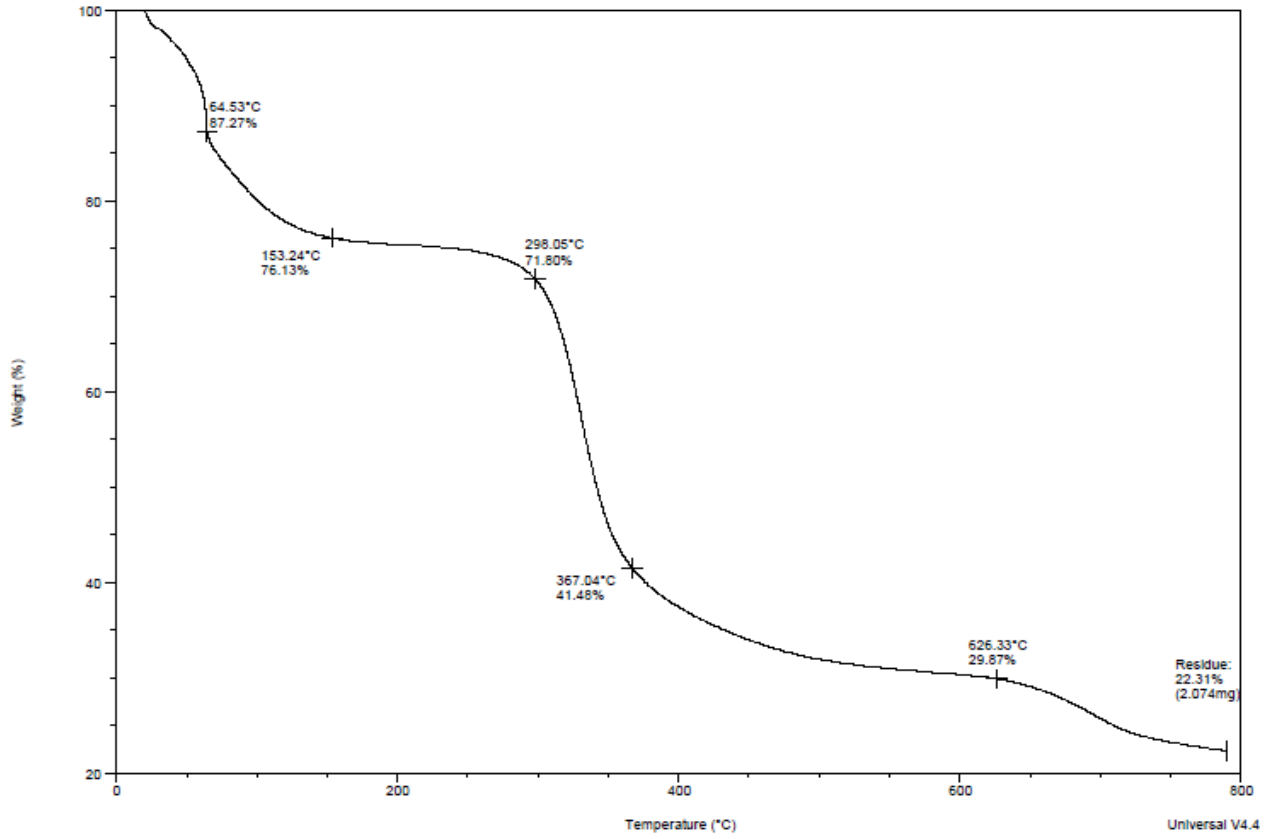


**Fig.4.5 TGA Curves of Elastin in the temperature calibration three points 25.00°C to 25.00°C, 362.48°Cto 358.00°C and 567.30°C to 554.00 °C and the ramp of range of 20°C/ min to 800 °C/ min.**

From the TGA Analysis, the result indicates that the maximum elastin decomposition or weight loss is 30.32%.It occurs in the temperature range of 298.05 ºC to 367.04 ºC. Moreover, the TGA analysis showed that the minimum elastin decomposition or weight loss is 4.33% in the temperature ranging from 76.13 ºC to 71.80 ºC.

### 4.3.2 Scanning Electron Microscope (SEM) Topography and Morphology studies of Elastin

The SEM image of the sample is shown in (Fig.4.6.A & B).It is observed that elastin has fiber like structure in an enlarge view with rough and non-uniformity surface topographic structure. In addition, shape also looks elongated tail structure.

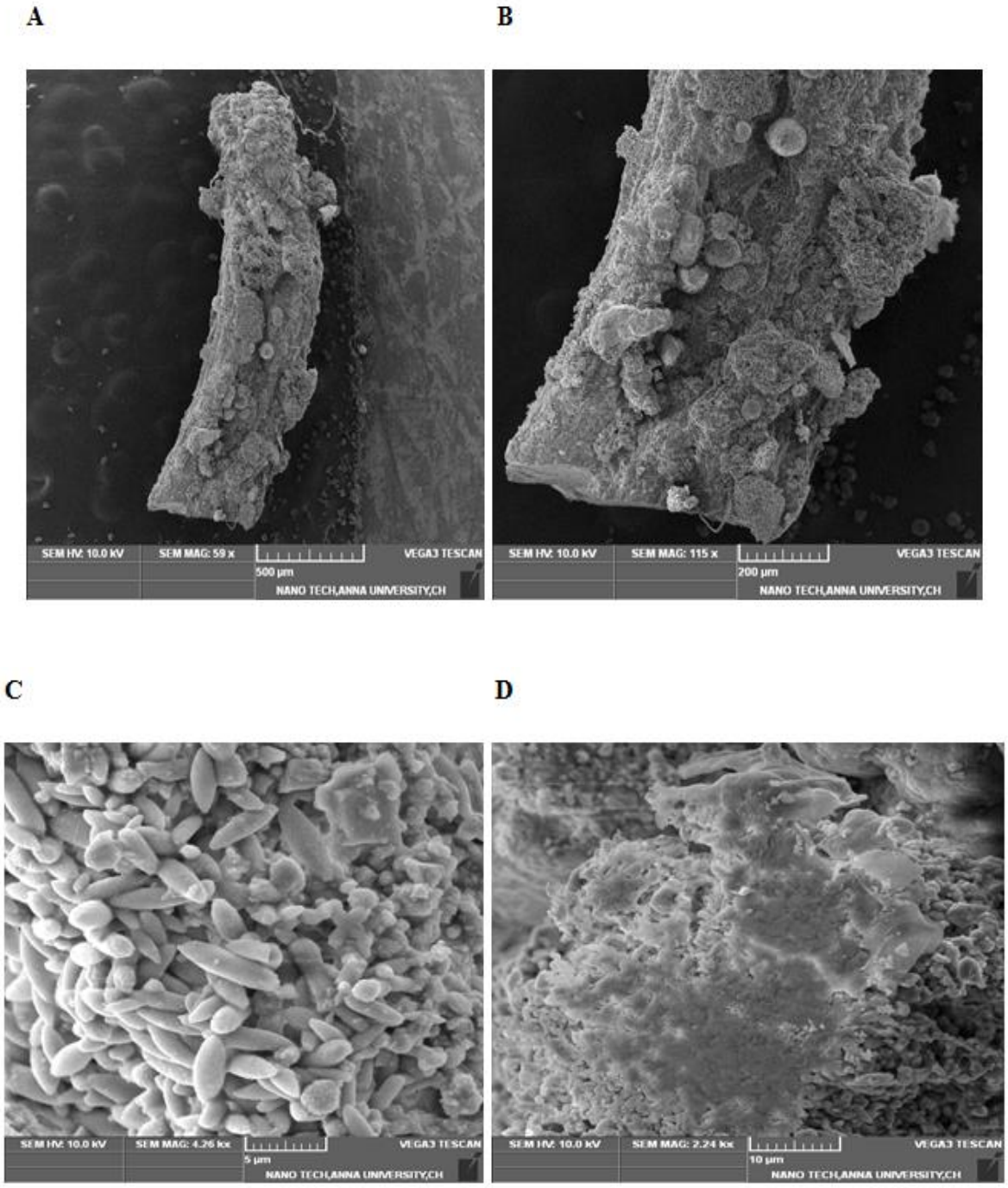


**Fig.4.6 SEM Image of Elastin sample taken with (A) Magnification of 59x and resolution-n 500µm (B) Magnification of 115x and resolution 200µm (C) Magnification of 4.26kx and resolution 5µm (D) Magnification of 2.24kx and resolution 10µm**

From Fig.6.C, the 5um oval structure form aggregation and stick together in bundles. Moreover, the oval shapes aggregation indicates some irregularity and lacks uniformity distribution on the fiber surface. The above topographical structure draws resemblance to the fibril structure of collagen previously reported [30].

The SEM image indicated in Fig4.6.D, the elastin fibers shows continuous pattern with which they form network. Furthermore, this network shows similarity to the bundles of micro fibrils structure of collagen. Moreover, this structural feature shows some uniformity on coving the surface of oval structure of the fiber surface [31].

## 4.4 Analysis of Chemical Characterization of Elastin

### 4.4.1 Analysis of $^{13}C$ Solid state NMR Spectrum and Chemical Shifts

The sample was subjected to $^{13}C$ Solid state NMR analysis in dry state at 37 ºC. The sample generates multiple spectrums as shown in the Fig.4.7 A and B. These multiple spectrums are detected in three distinct regions namely amide carboxyl, aromatic and aliphatic region of the elastin fiber [32].

The first spectrum group chemical shifts are shown in the amide carboxyl region of elastin. These are shown in the Fig.4.7 at 242.51, 186.15 and 174.34 ppm. This region of chemical shifts are found to be broad resonance in the backbone carbonyl region at 172.34 pm. In addition, the carbonyl region has characteristics splitting at 172.34 ppm is assigned to glycine residues in β-strand structure of elastin [33]. The peak at 172.34 ppm exactly matches from previous reported literatures of elastin Solid State NMR spectrum [34].

The second group spectrum of chemical shifts is shown in the aromatic region of elastin. This region totally generates four chemical shifts. The region of chemical shifts peaks are observed at 137.70, 129.59, 106.27 and 102.82 pm respectively. The dominant peak of aromatic region of chemical shifts is observed at 129.59 ppm which, indicates that aromatic resonance and the region of aromatic carbons do not have adjacent hetero-atoms [33]. Moreover, 129.59 ppm chemical shift of elastin in this region shows the existence of the side-chain carbons of the phenylalanine amino acids [33].

In Figures, the third set of NMR spectrums is shown by the elastin sample in the aliphatic region. This region generates maximum set of spectrums comparing the other two regions. The NMR peaks of this region are found to be nine. These chemical shifts peaks are observed at 67.94, 59.66, 51.93, 47.90, 42.32, 29.76, 24.05, 19.11 and 4.15 ppm. In addition, the chemical shifts at 59.66, 51.93, 47.90 and 42.32 ppm are assigned to C(α) of the amino acids[33].The name of the amino acids are Valine , Alanine, Phenylalanine and Glycine respectively [34].Moreover, the chemical shifts at 29.76 and 24.05ppm are assigned to Valine and Proline amino acids residues with aliphatic side chain carbons of C(β) and C(ϒ) respectivly [32].The detail chemical shifts along with assigned amino acids are briefly summarized in the following Table 4.3.

**Table 4.3 Solid State $^{13}$C NMR Spectrum of Chemical shifts, Regions and Amino acids assignations**

| NMR Chemical Peak (ppm) | Regions and Assigned Amino Acids |
|---|---|
| 242.51,186.15 and 174.34 | Amide carboxyl regions |
| 172.34 | glycine residues in β-strand structure |
| 129.59 | Aromatic Resonance<br>side-chain carbons of the phenylalanines |
| 59.66, 51.93, 47.90 and 42.32 | C(α) of the amino acids. |
| 42.32 | Glycine |
| 47.90 | Phenylalanine |
| 51.93 | Alanine |
| 59.66 | Analine |
| 29.76 ppm and 24.05ppm | aliphatic side chain carbons of C(β),C(ϒ) |
| 29.76 | Valine |
| 24.05 | Proline |

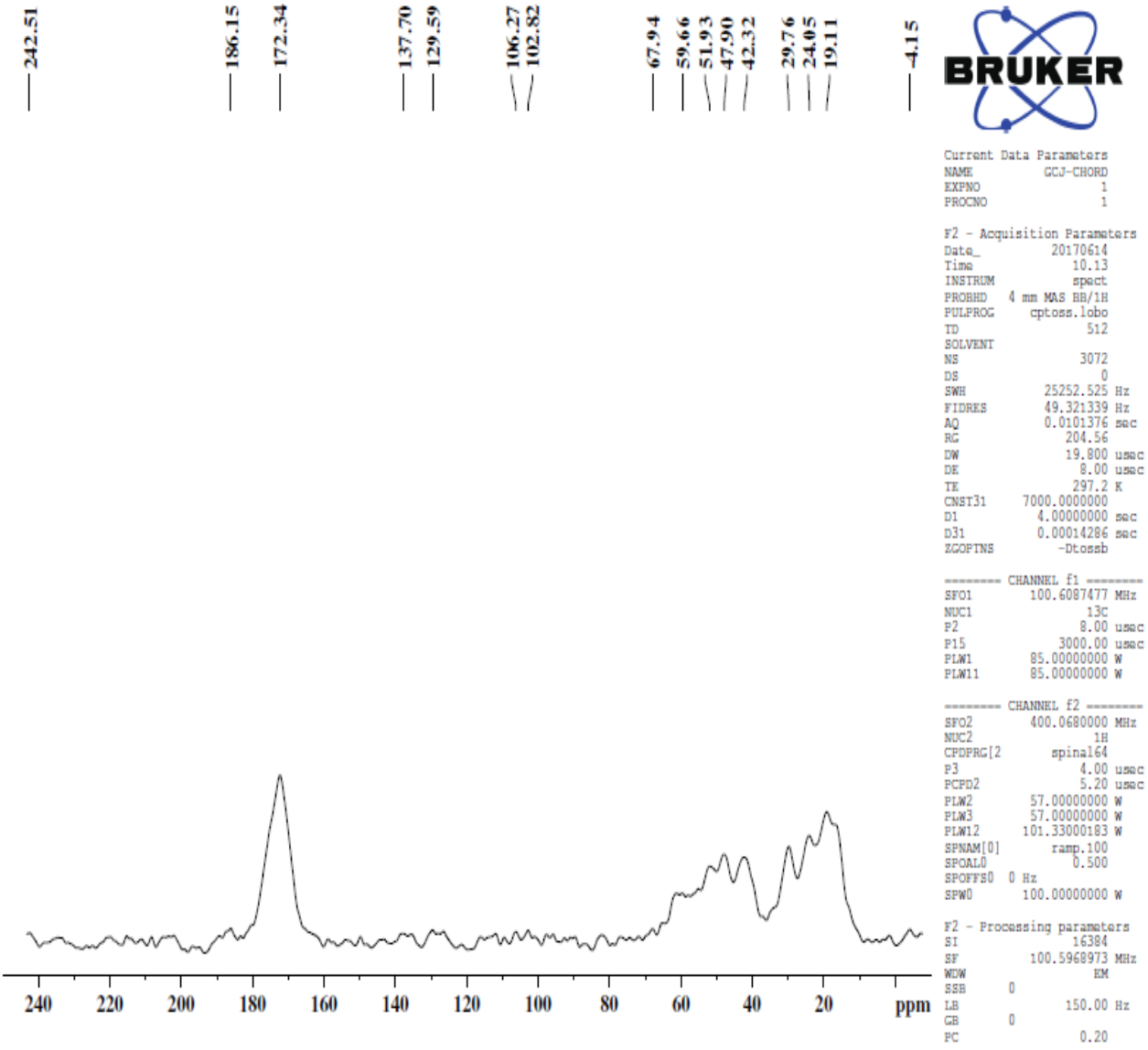


**Fig.4.7 A. Solid State $^{13}$C NMR CPMAS spectra of elastin sample with dry state. The data for the samples was taken at 37°C.The chemical shift values are indicated in ppm.**

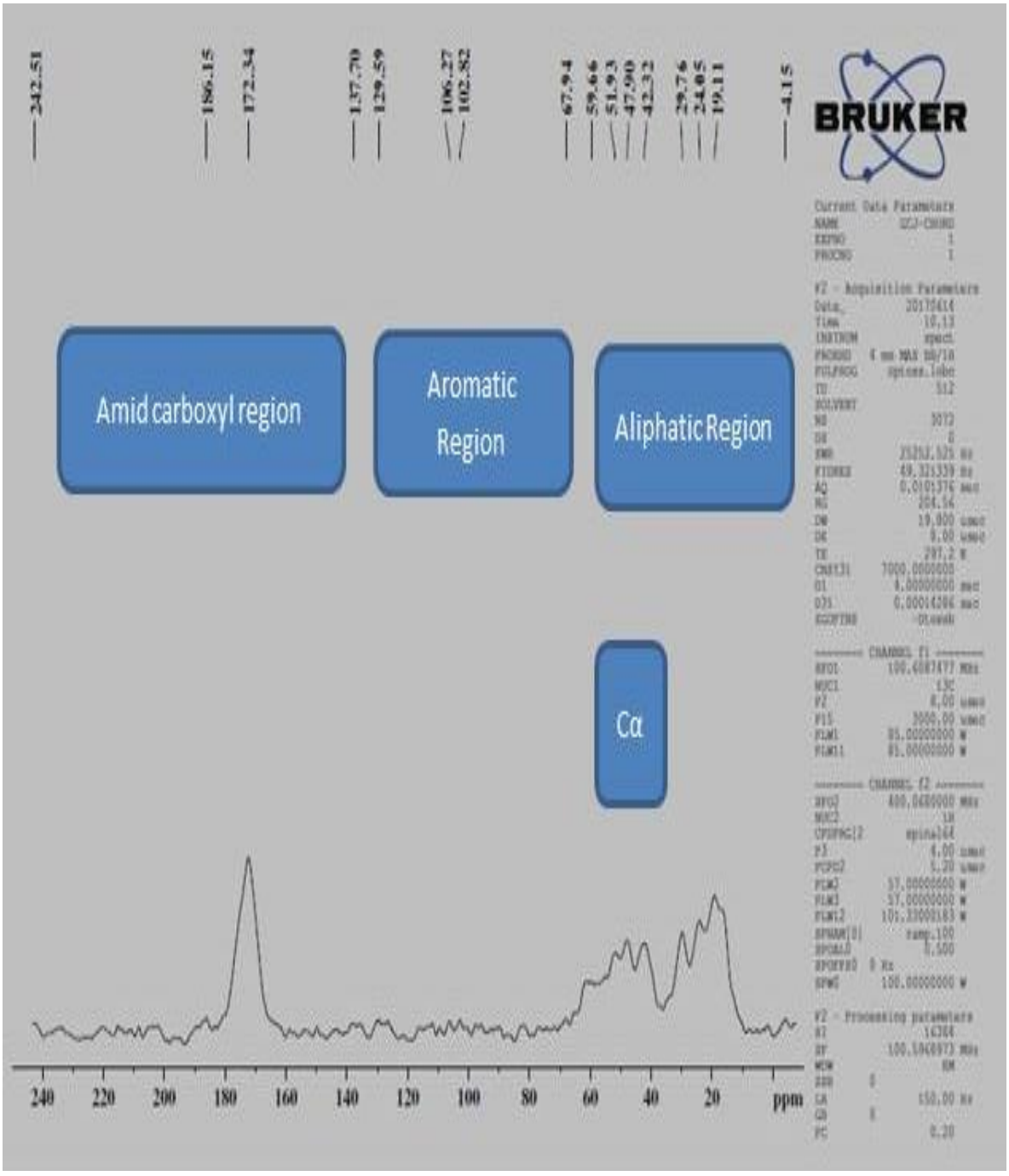


**Fig.4.7 B. Solid State $^{13}C$ NMR CPMAS spectra of elastin sample with dry state. The chemical shift values with respect to regions are indicated in ppm.**

In summary, the $^{13}C$ Solid state NMR spectrum peaks along with chemical shifts are correctly correlated and matched to the amino acids chains of the elastin of previous reported studies [33] & [34]. Furthermore, the NMR chemical shifts are confirmed by analyzing and correlating chemical shifts by cited previous literatures that extracted elastin has random coil,α-helix and β-strand secondary structure with high level of purity.

### 4.4.2 HP content Determination

One of the principal differences between elastin and collagen is the hydroxyproline (HP) content. The HP content of elastin is low, when comparing to the collagen [3]. In addition, the estimate value of HP content of Elastin ranges from 1.5 to 2.3 per cent depending on the source [35]. 0.6 mg of extracted sample was hydrolyzed with 6N of HCl for 24h [21], [22] & [23]. Then, the standard protocol of HP determination method was used to quantify the HP content [35].

The standard HP and the OD value were summarized and given in the table 4.4.

**Table 4.4 The UV Absorbance Values at 557nm with different HP standard Solutions**

| µg/µL | µg/1000 µL | OD Value |
|---|---|---|
| **20** | **2** | **0.057** |
| **40** | **4** | **0.177** |
| **60** | **6** | **0.256** |
| **80** | **8** | **0.369** |

From the Table 4.4, the data was plotted by assigning the HP concentration as independent variable and Absorbance (OD value) as dependent variables. The Design Expert 6.0 software was used in order to create empirical relation, the regression equation was computed relates the two variables and to perform detail statistical analysis. The software output results were summarized and given in the following Table 4.5, 4.6, 4.7 and Fig.4.10.

**I. Design Summary**

The experiment type, the design model, number of experiment, the maximum and minimum response variable and factor details are given in Table 4.5.

**Table 4.5 The Design Summary of the Experiment**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Study Type | Factorial | | Experiments | 4 | | | |
| Initial Design | Full Factorial | | Blocks | No Blocks | | | |
| Design Model | Linear | | | | | | |
| Response | Name | Units | Obs | Minimum | Maximum | Trans | Model |
| Y1 | OD value | | 4 | 0.057 | 0.369 | None | Linear |
| | | | | | | | |
| Factor | Name | Units | Type | Low Actual | High Actual | Low Coded | High Coded |
| A | Concentration | ug/1000 | Numeric | 2 | 8 | -1 | 1 |

From table 4.5, the experiment design model was linear. The response variable was OD value and the experiment was found to be one factorial. In addition, the number of observation of the experiment was found to be four, the minimum and maximum OD value was found to be 0.057 and 0.369. Moreover, the low and high HP concentrations were found to be 2 and 8 µg/1000 µL.

**II. Analysis of FIT Summary**

The model equation of the experiment as given in Table 4.6 is to be linear. In addition, the correlation coefficient ($R^2$) was 0.9943345, the adjusted correlation coefficient ($R^2$) of the experiment was 0.9915017 and the correlation coefficient ($R^2$) was found to be 0.98244105.All the above computed values were shown in the Table 4.6.

**Table 4.6 The Model Summary Experiment**

| Source | Std. Dev. | R-Squared | Adjusted R-Squared | Predicted R-Squared | PRESS | |
|---|---|---|---|---|---|---|
| Linear | 0.012114 | 0.9943345 | 0.9915017 | 0.98244105 | 0.00091 | Suggested |
| Quadratic | 0.016771 | 0.99457096 | 0.9837129 | 0.75870938 | 0.0125 | |
| Cubic | | | | | + | |
| + Case(s) with leverage of 1.0000: PRESS statistic not defined | | | | | | |
| | | | | | | |
| "Model Summary Statistics": Focus on the model maximizing the "Adjusted R-Squared" | | | | | | |
| and the "Predicted R-Squared". | | | | | | |

**III. The ANOVA (Analysis of Variance) of the Experiment**

The ANOVA results is given in Table 4.7.As per the results, the Model F-value of 351.01 implies the model is significant. There is only a 0.28% chance that a "Model F-Value" this large could occur due to noise. In addition, Values of "Prob > F" less than 0.0500 indicate model terms are significant. The ANOVA detail results are summarized in Table 4.7.

**Table 4.7 The ANOVA for Response Surface Linear Model Analysis**

| Source | Sum of Squares | DF | Mean Square | F Value | Prob > F | Comment |
|---|---|---|---|---|---|---|
| Model | 0.05151125 | 1 | 0.05151125 | 351.0136286 | 0.0028 | significant |
| A | 0.05151125 | 1 | 0.05151125 | 351.0136286 | 0.0028 | |
| Residual | 0.0002935 | 2 | 0.00014675 | | | |
| Cor Total | 0.05180475 | 3 | | | | |

## IV. Plotted Model Graph for Standard Solution

The Fig.4.8 shows the absorbance vs concentration of HP(µg). From the figure, it obtained that the relationship between the absorbance and concentration linear.

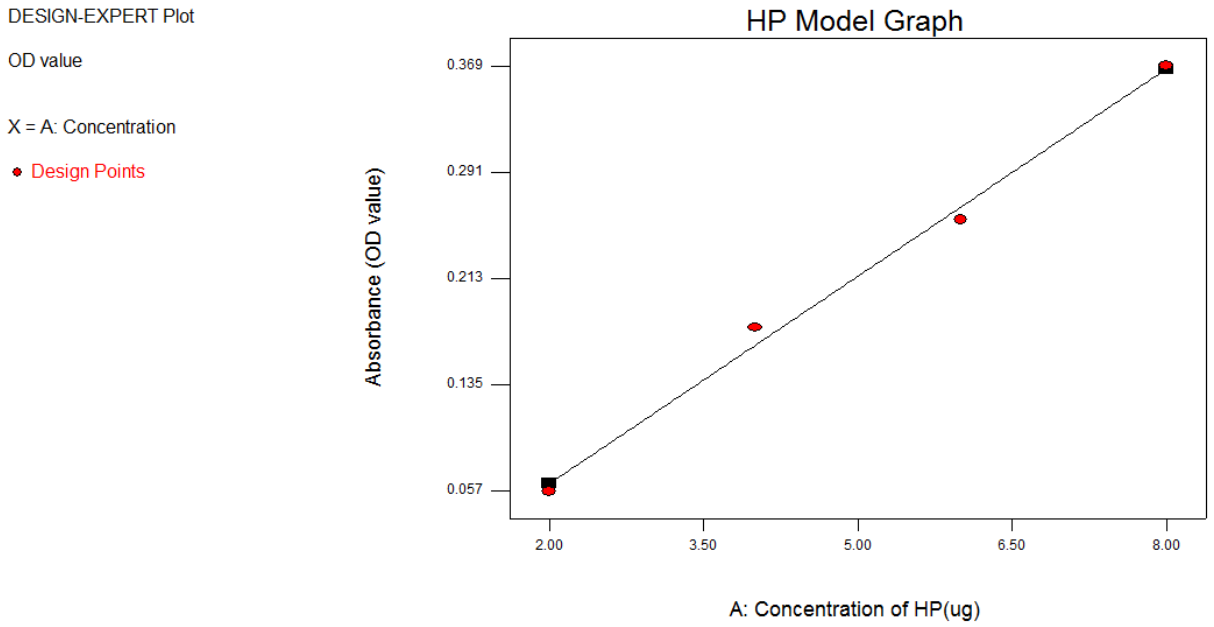


**Fig.4.8 Standard Graph for Hydroxyproline Estimation**

From Fig.4.8, it is clearly shown that the absorbance was linearly related to the HP concentration. Moreover, the plotted graph was helps to derive the regression equation that relates the OD value and concentration of HP variables. Finally, it helps to determine the HP content of the sample.

## V. The Regression Equation (Model Equation) and Determination of HP content

The design Expert software derives the model equation from the above I-IV analysis results. The final models Equation in terms of Actual factors were found to be:-

$$OD\ Value = -0.039 + 0.05075 * Concentration\ of\ HP$$ ---------Eq. 1

From the above equation 1 the Hp concentration was derived in terms of OD value

$$HP\ concentration(ug) = \frac{OD\ Value + 0.039}{0.05075}$$ ----------Eq.2

By using Eq.2 it was computed the unknown HP of the sample. The sample HP concentration was found to be 0.49ug/ml. It is approximated to be 0.5ug/ml. Hence, the HP content of the sample was computed to be 2.1%. Then, by correlating the Hp content result from previous reported literatures that the elastin hydroxyproline content ranges between 1.5 to 2.3% [35]. Therefore, the 2.1% HP content value confirms that it lies within the acceptable range of mammalian elastin.

### 4.4.3 The Verhoeff-Van Gieson (EVG) Staining Characterization Result

This staining was done according to Verhoeff-Van Gieson(EVG) standard staining Protocol. As per the protocol, if the sample is elastin it has the strongest affinity of the iron hematoxylin complex chemical by retain the dye longer time. Due to that the reaction gives the black color on the surface of the elastin.

Samples of different sizes were taken in the glass slides and stained as per Verhoeff-Van Gieson (EVG) staining method. The surface of stained samples was visualized using Light Microscope and results are shown in Fig.4.9 A - F.

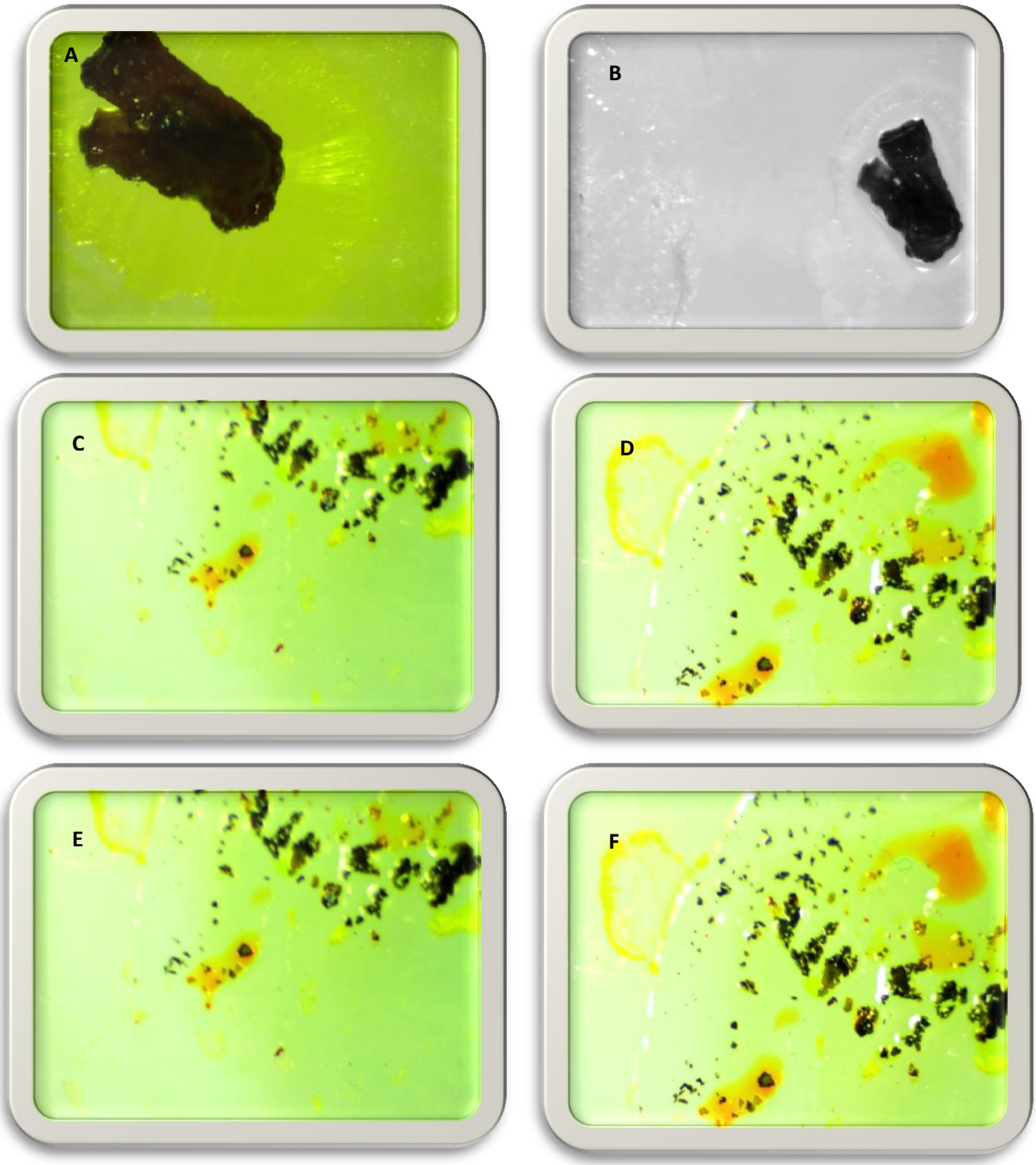


**Fig. 4.9 Microscopic Image of Stained Elastin sample (A) Stained Elastin Fiber with color image (B) Stained Elastin Fiber with black and white image (C), (D) (E) & (F) Stained Elastin Powder and Collagen residue of the sample.**

The stained sample image is shown in Fig.4.9 A that the surface of the sample becomes pure black. The generation of black coloration result indicates that the sample surface has a strong affinity to iron hematoxylin complex chemical and retain on the surface of the sample. The Fig.4.9 B confirms that the fiber surface generates black color. Moreover, the Microscopic stained images results from Fig.4.9 C to F shows that the samples changes to black color with aggregation and forms network. Moreover, the red color spots are shown in the microscopic image confirms the presence of collagen.

In conclusion, the Verhoeff-Van Gieson (EVG) staining coloration result of the sample ratifies that the black and red coloration is due to the presence of elastin and collagen residuals.

### 4.4.4 Microscopic Images of Elastin fiber Result

The physical property of elastin fiber was studied using the Light Microscopic image. The Microscopic image of the elastin fiber was shown in the Fig.4.10.

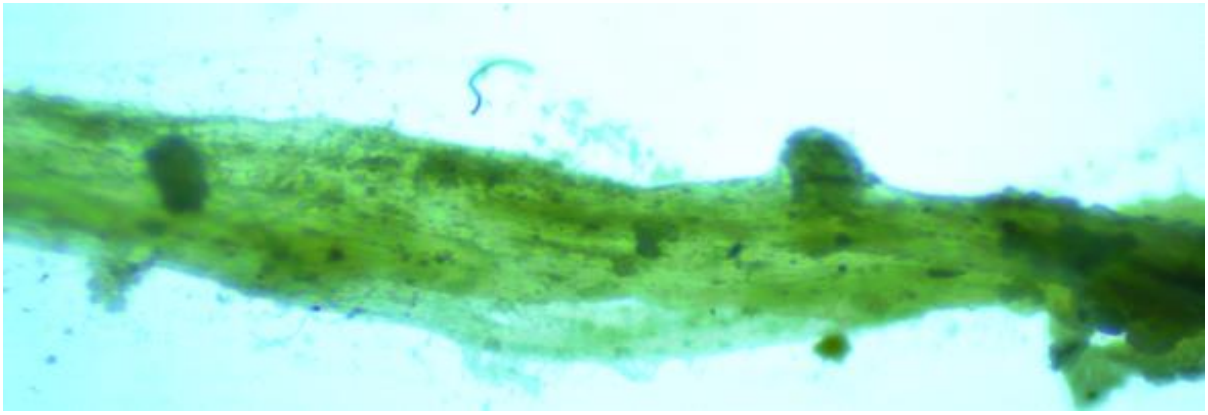

**Fig. 4.10 Microscopic Image of Elastin Fiber**

From Fig 4.10, it is inferred that the elastin fiber has a rope like structure that resembles longitudinal cylindrical shape. The fiber has amorphous structure that lacks uniformity along the longitudinal direction as shown in the figure. In addition, the fiber contains an oval shape of different size widely scattered bodies are existed on the fiber surface structure. Moreover, black spots images exist on the elastin surface which indicates the presence of microfibrile collagen [36].The above Microscopic image found to be similar in accordance with early reported literatures [36].

### 4.4.5 Physico-chemical Characterization

The physico-chemical test of the sample was performed using phosphate buffer and acetic acid. The sample showed strong resistant during treating with phosphate buffer. In addition, the sample also has showed a high resistance when it was mixed with acetic acid and heating at 60 $^{O}$C in the ultrasonic apparatus. The sample resistance property to physio-chemical tests confirms that the extracted protein has elastin character.

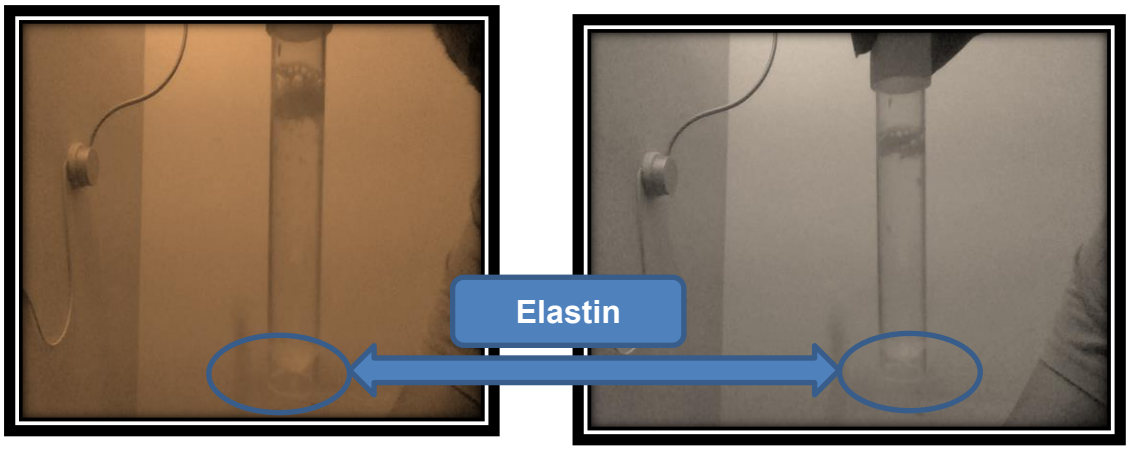


**Fig. 4.11 The Image of Physio-chemical test Result of Elastin Sample**

### 4.4.6 Functional group analysis using FTIR (Fourier Transform Infrared Spectroscopy)

FTIR characterization helps to determine secondary structure and functional group presence in sample. The FTIR spectrums result of the sample are shown in Fig.4.12. From the FTIR spectrum, the amide I region (C=O stretching) approximate frequency expected between 1650cm-$^{1}$ to 1780 cm-$^{1}$ wave number in which it is shown in Fig.4.12 at 1694cm$^{-1}$ wavenumber [35]&[36]. Moreover, the amide II (mainly C–N stretching) FTIR band is shown Fig4.12 in the frequency range between 1180cm$^{-1}$and 1360 cm$^{-1}$ wavenumbers [37] . Furthermore, the amide III (N–H in plane deformation) and amide A (N–H stretching) FTIR bands are shown in the Fig.4.12 in the frequency range between 3000cm$^{-1}$ to 3500cm$^{-1}$ wavenumbers [37] & [38].

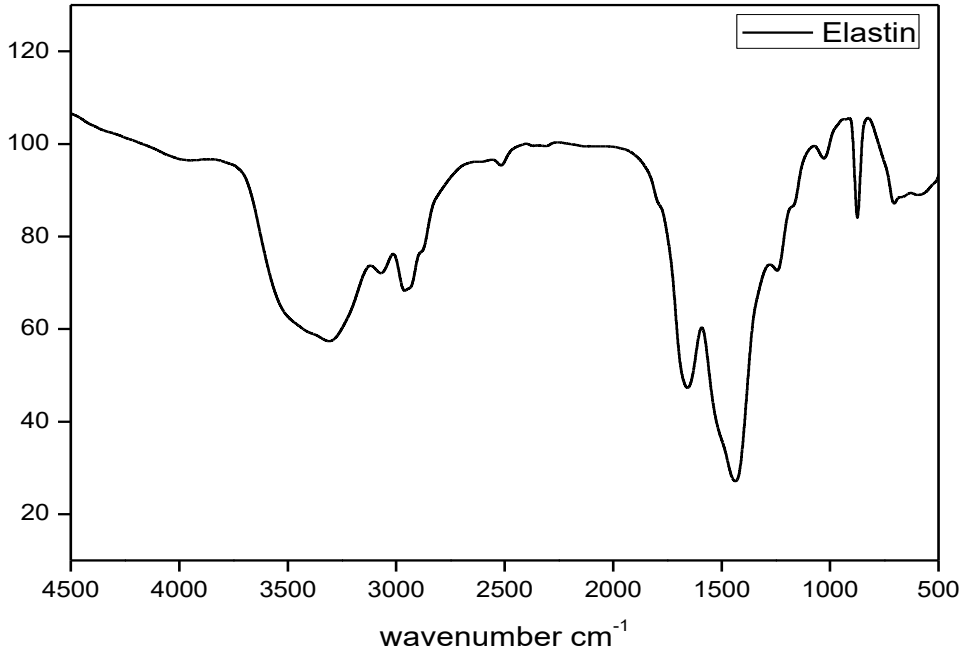


**Fig.4.12 FTIR Spectrums of Bovine Extracted Elastin**

As specified in literatures, the amide I region has α-helix, β-strand and undefined(turn + unordered) conformational structures shown and bands at 2000cm$^{-1}$ which assign to random coil, turns, and alpha -helix parts of elastin structure [37]&[38].Moreover, the region below 1000cm$^{-1}$ is shown in the Fig.4.14 has amide V,IV,VI, and VII[37].

The amine A, I, II and III has in plane rocking and in plane scissoring symmetric vibrations and amide V, IV, VI, and VII regions has out of plan wagging and out of plane twisting asymmetric vibrations [37]&[38].The eight modes can be describe as in five in-plane: C = O stretching, C-N stretching, N-H stretching, OCN bending, CNH bending; and three out-of-plane: C-N torsion, C = O and N-H out-of-plane bending [37].

## 4.5 Biological Characterizations Results and Analysis

### 4.5.1 Free Amino Acid Analysis Result of Elastin by HPLC

The free amino acid analysis was carried out for extracted elastin sample. This characterization technique helps to determine the free amino acid composition of the extracted elastin fiber. The

analysis was carried out using the High Performance Liquid Chromatography (HPLC). The Amino acid analysis result is shown in Table 4.8 and Fig.4.13.

**Table 4.8 The Amino Acid Compositions in nmoles/ml of Extracted Elastin**

| No. | Name of Amino Acids | Concentration(nmole/mL) |
|---|---|---|
| 1. | Aspartic acid(Asp) | 13 |
| 2. | Glutamic acid(Glu) | 8 |
| 3. | Serine(Ser) | 17 |
| 4. | Histidine(His) | 237 |
| 5. | Glycine(Gly) | 209 |
| 6. | Threonine(Thr) | 24 |
| 7. | Arginine(Arg) | 193 |
| 8. | Alanine(Ala) | 164 |
| 9. | Tyrosine(Tyr) | 11 |
| 10. | Methionine(Met) | 143 |
| 11. | Valine(Val) | 118 |
| 12. | Phenylalanine(Phe) | 41 |
| 13. | Isoleucine(lle) | 29 |
| 14. | Leucine(Leu) | 72 |
| 15. | Lysine(Lys) | 11 |

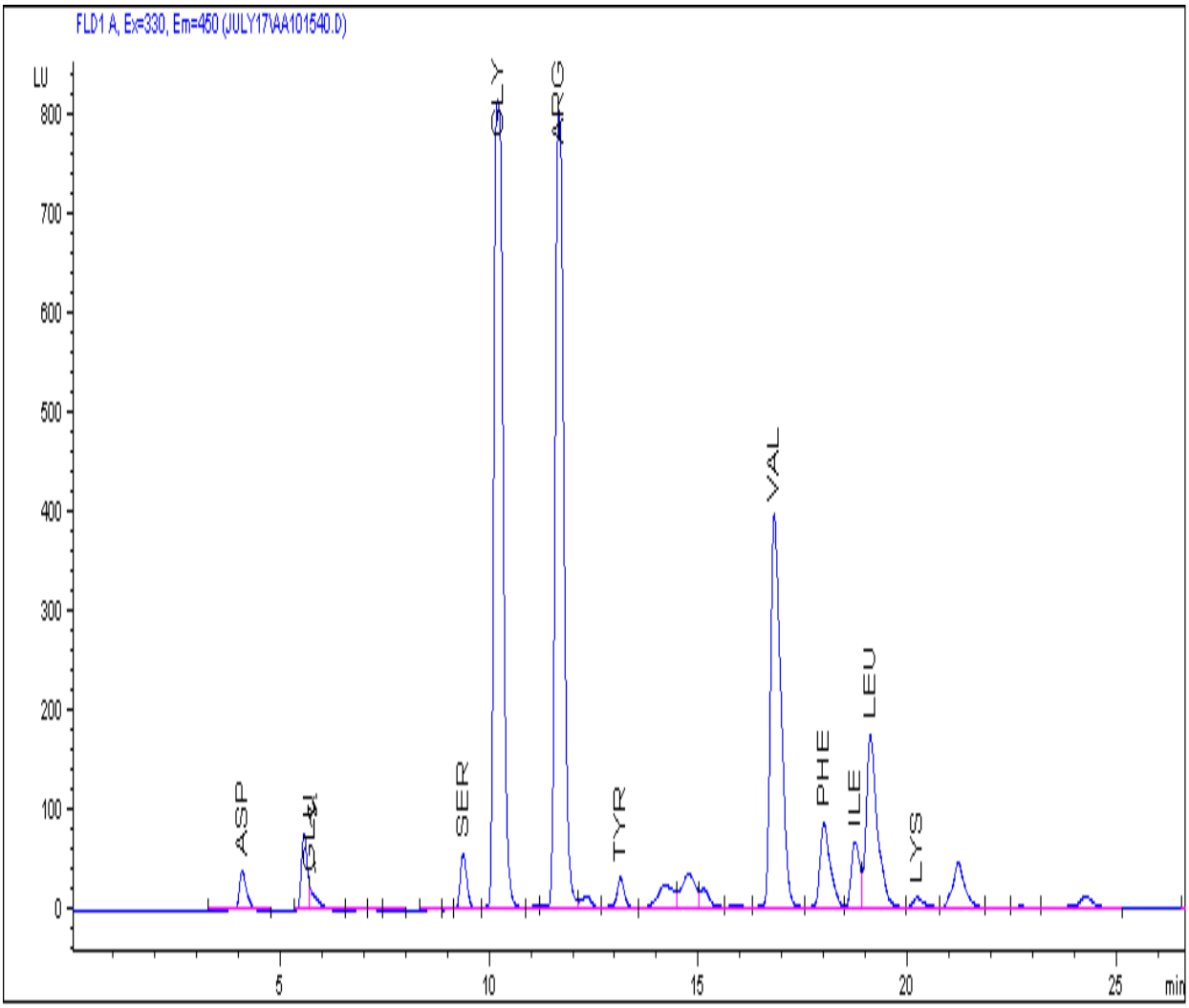


**Fig.4.13 Free Amino Acid Analysis of extracted Elastin using HPLC**

From the, Table 4.8 and Fig.4.13 free amino acids composition results indicates that the extracted elastin sample is composed of high amount of glycine and non-polar amino acids. Moreover, the sample has a low amount of acidic, hydroxyl and hydroxylproline amino acids. Furthermore, the amino acid analysis results are confirms that there was no hydroxylysine and tryptophan amino acid in the extracted sample.

### 4.5.1.1 Free Amino Acid Result Analysis

The Fig 4.14 is shows the percentage shares of amino acid from the total amino acid composition of elastin fiber. Therefore, the amino acids percentage share from the total is shown in the following Pie graph.

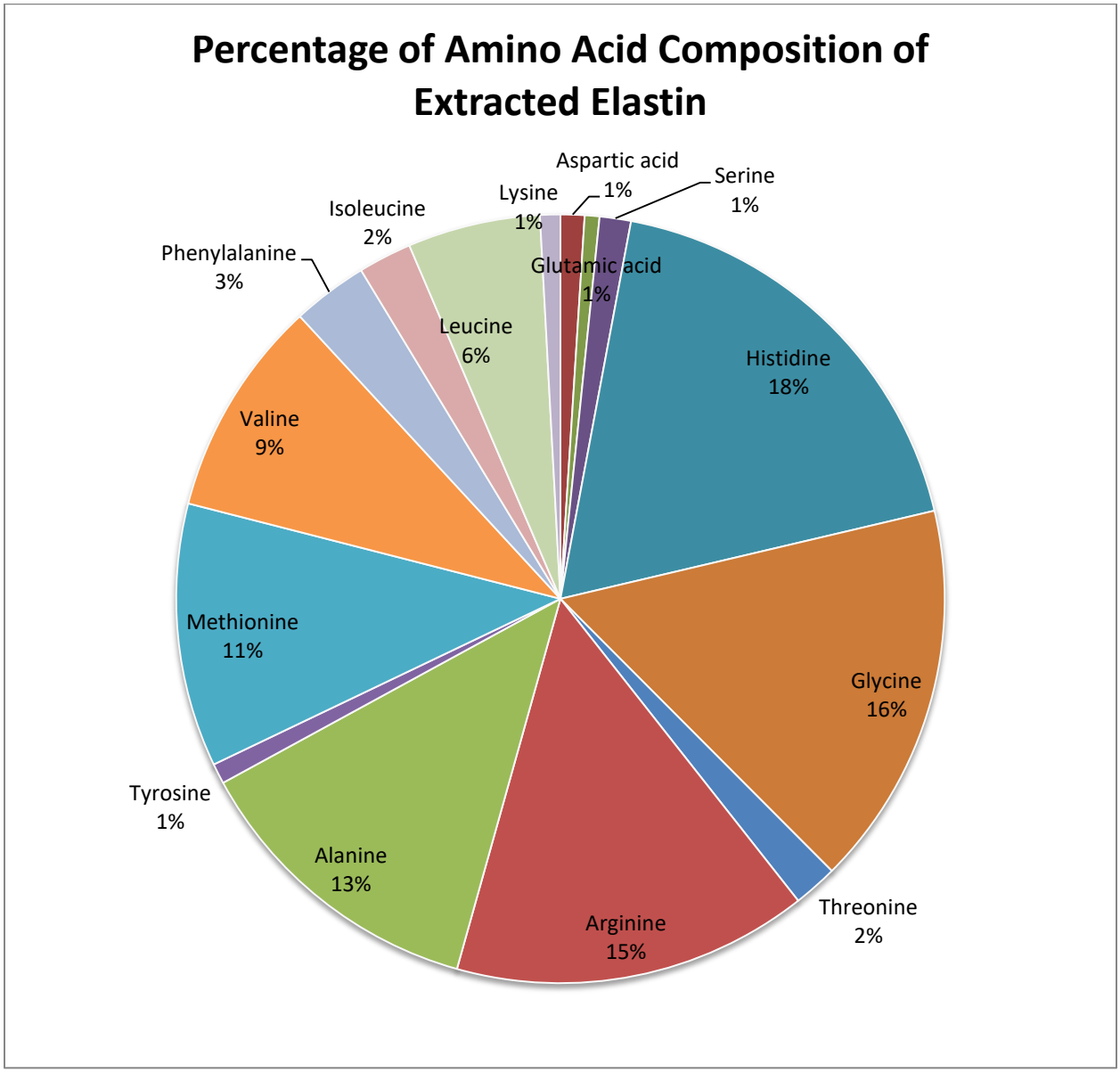


**Fig.4.14 Percentage shares of free amino acids of Extracted Elastin**

From Fig. 4.14 shows amino acid percentage shares of each amino acid from the total results of the extracted elastin purity was assessed.

The level of Glycine and Alanine concentration found to be 209 nmol/mL and 164 nmole/mL respectively. The Glycine takes 16% share and Alanine takes 13% share of the total amino acids. This result indicates that the two amino acids are present in high amount in the elastin fibers which deplicates that they are the major constituents of the elastin fiber. In addition, the concentration of Valine is found to be 118nmol/ml which takes the amino acid composition share of 9%.This high amount of Valine amino acid result is also one of the scientific parameter that confirms the extracted sample has elastin property [3] & [9].

The level of Aspartic acid and Glutamic acid is found to be 13nmol/mL and 8nmol/mL respectively. These amino acids are 1% shares of the elastin. Furthermore, the amount of Lysine is found to be very little it is almost about 1% of the total share of amino acids. The amounts of these types of amino acids are found to be low. Therefore, the above result confirms that the extracted elastin has highly hydrophobic property [3] & [8].

The amount of Alanine amino acid concentration is found to be 164nmol/ml that takes 13% share of the total amino acid composition of extracted elastin fiber [3] & [9].

There are no Hydroxylysine, Tryptophan and Cystine amino acids presence in the amino acid composition of the elastin fiber. These characters indicate that the extracted protein has elastin nature. Moreover, the detection and determination of the concentrations of Methionine and Histidine in the amino acid composition of the elastin fiber is due to the presence of fibrillies and microfibrial residual collagen [3], [8] & [9].

### 4.5.1.2 Free Amino Acid Types Analysis of Elastin fiber

The amino acid composition types of the extracted sample were analyzed using the following Fig. 4.15 and Fig. 4.16.

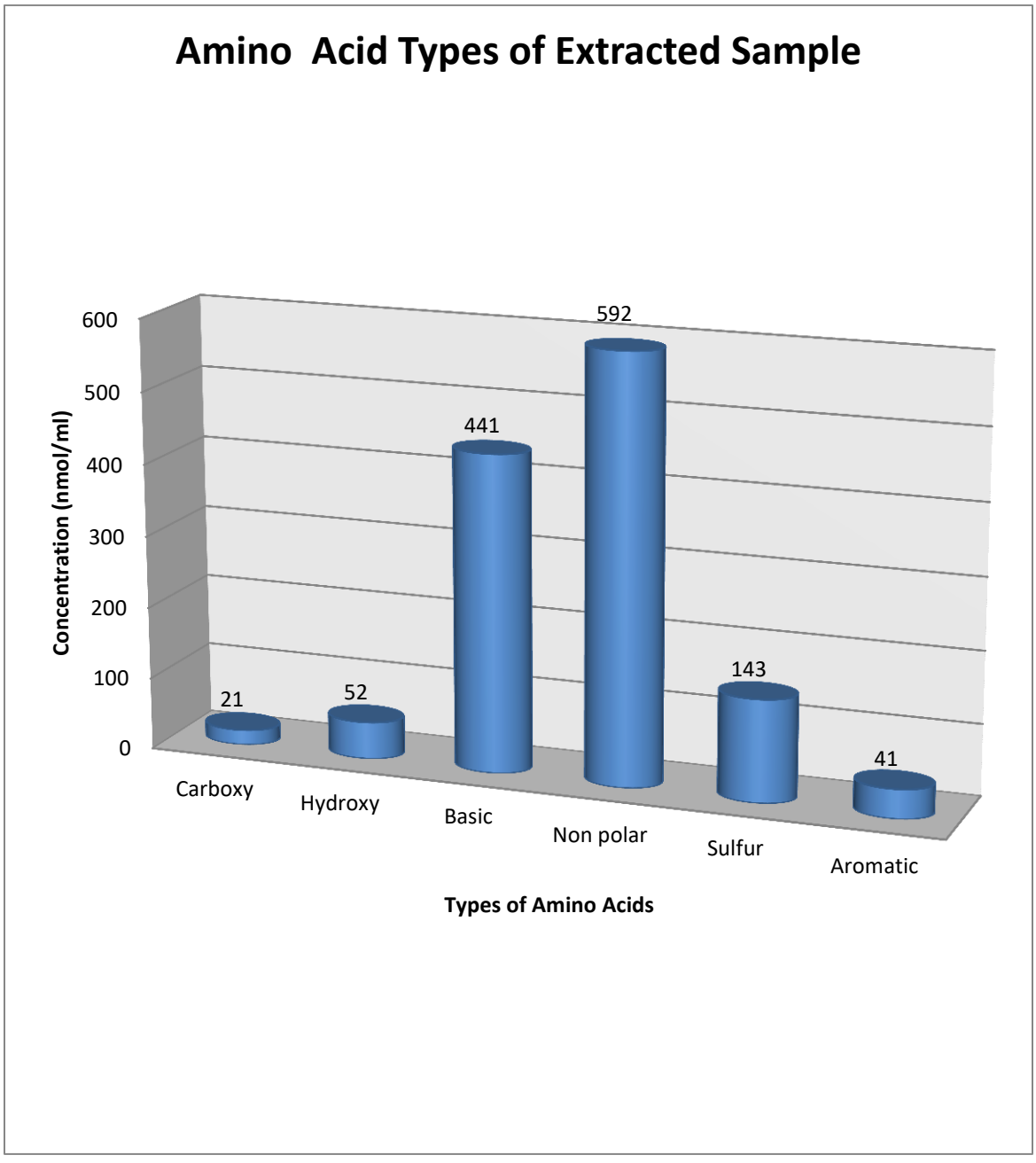


**Fig.4.15 The Amino Acid Types of Extracted Elastin Concentration**

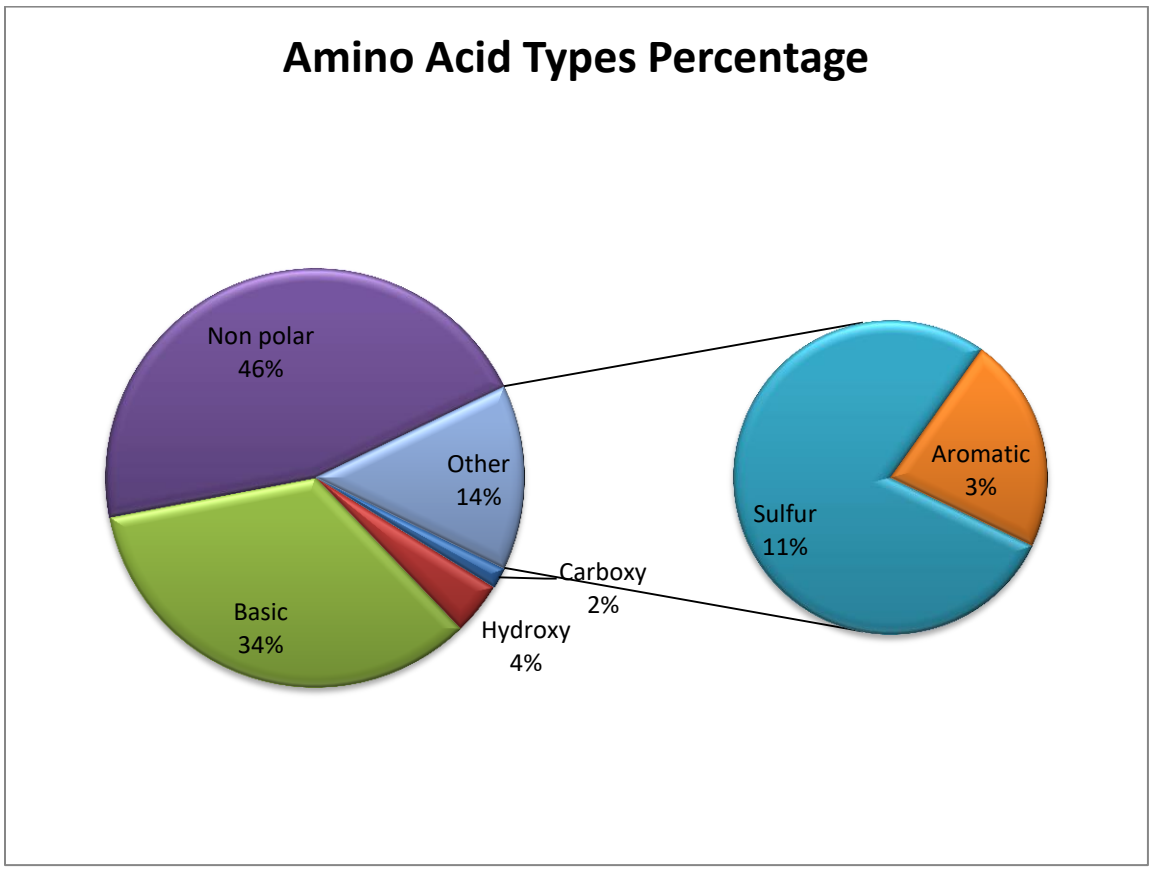


**Fig.4.16 Free Amino Acid Types and Percentage of Extracted Elastin**

From the amino acid types analysis, that there is a highest amount of non-polar amino acids. The total concentrations of these amino acid types are found to be 592nmol/mL which shares 46% of the total amino acid composition. This character of the extracted elastin has a highly hydrophobic property [3].

The second higher amount of amino acid type is found to be a basic amino acid category. This type of amino acid has total concentration of 441 nmol/mL and shares 34% of the total amino acid composition. The figures are magnified due to the presence of high amount of Histidine and Arginine amino acids that has 237 nmol/mL and 193nmol/mL concentrations respectively. These amino acids take 98% shares of the basic amino acids. Furthermore, the presence of sulfur type amino acid composition is found to be 143nmol/mL which takes 11% shares of the total amino

acids. The above analysis were indicates that existence of collagen type I and III in the microfibrial structure elastin fiber [3], [8] & [9].

The presence of acidic and hydroxyl types of amino acids are determined to be 21nmol/mL and 52nmol/mL concentrations respectively. The total shares of these types of amino acid are estimated to be 6%. The low amount of acidic and a hydroxyl type of amino acids confirm that the extracted protein shows more of elastin property [3] & [8].

The level of Aromatic amino acids in the elastin fiber is also one of the important factors for the identification of proteins. From the amino acid result analysis, the concentration of aromatic amino acid is found to be 41nmol/mL that has 4% share of total amino acids. The presence of aromatic amino acids confirms that the extracted sample has elastin character [3] & [9].

The above result analysis collectively confirms and concludes that the extracted elastin matches the previous reported literature of amino acid composition of elastin [3], [8], [9] & [35]. But, with little variations of has high amount of basic amino acid due presence of residual collagen.

### 4.5.2 Histology Result Using Orcein and Methylene Blue Staining Method

The modified Taenzer-Unna orcein method was used to demonstrate fine and delicate elastin fiber histological structure. As per the standard procedure, the staining was performed has extracted sample surface. The result of the stained image of the extracted sample is shown in the Fig.4.17.

The Microscopic image shown in Fig.4.17 A is the original Orcein and Methylene stained image of the sample. As it is shown in the image the light to dark brown color is the elastin fiber

The image shown in Fig. 4.17 C is the original image of the sample with Orcein and Methylene stained. The color range from light to dark brown image is the elastin fiber. The elastin fiber forms irregular pattern and shape on the sample surface.

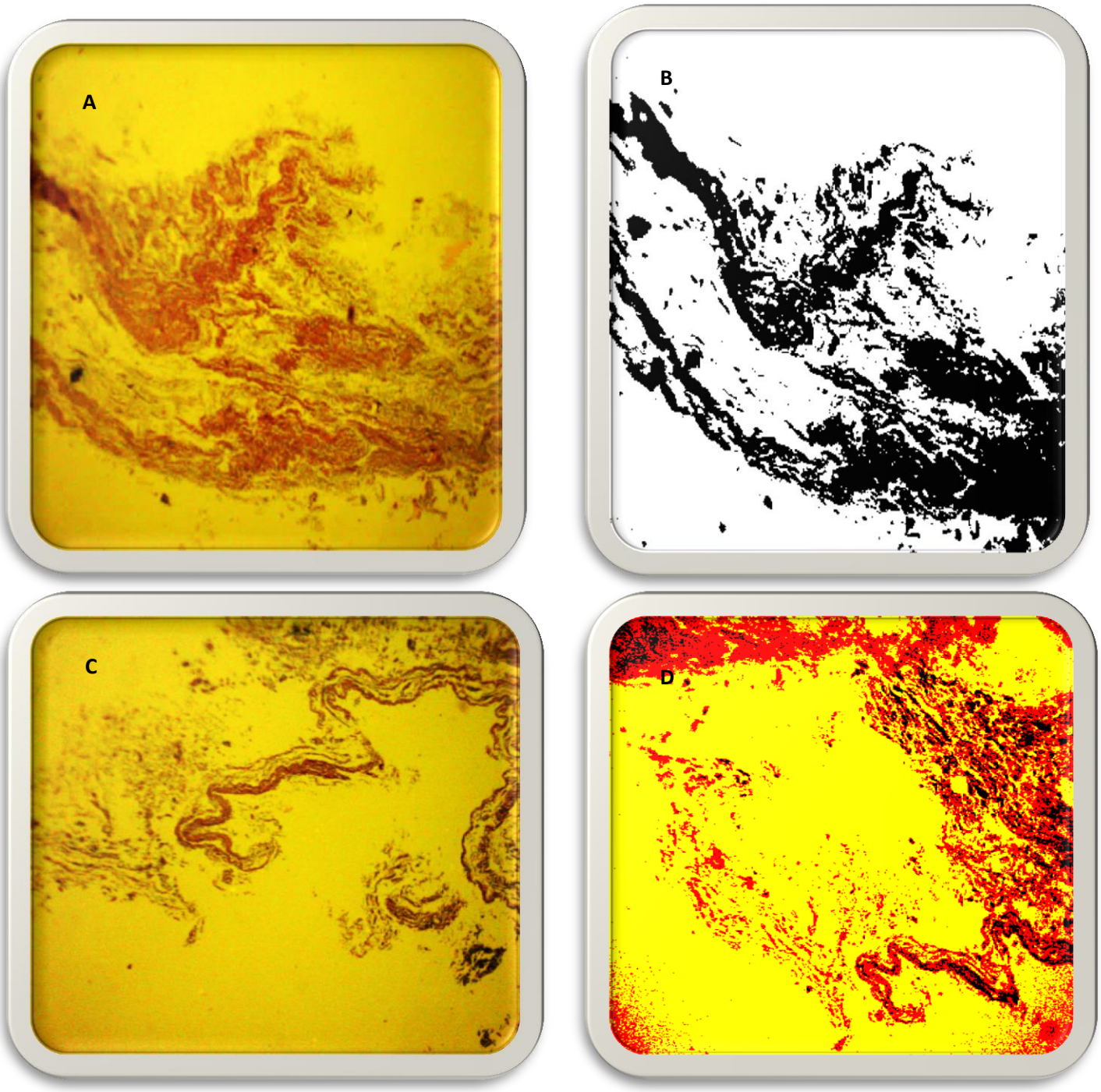


**Fig. 4.17 Microscopic Image of Orcein and Methylene Stained Elastin sample Histological structure taken (A) & (C) Stained Elastin Structure with color image (B) Stained Elastin structure recolor with black and white image (D) Stained Elastin structure with brightness 30% and contrast 100% of the sample.**

The modified the stained images are shown in Fig.4.17 B and D. The black and white image of shown Fig.4.17 B indicates that the black coloration is the elastin fiber distribution in the sample surface.

The modified high contrast and low brightness stained image of sample is shown in Fig.4.17 D. In the image, the light to dark brown coloration indicates that the elastin fiber structure distribution on the sample. In addition, the yellowish image is also shown in the skin matrix of the sample surface. Moreover, the fiber distribution has aggregation in some area of sample. Furthermore, the fiber networks had some irregular patterns and non-uniformity behavior shown on the surface of extracted sample.

### 4.5.3 Results of Toxicity Study (Bio compatibility study)

Toxicity study was carried out using human keratinocyte cells. The result of extracted elastin fiber is shown in the Fig.4.18 and Fig.4.19.

The cell proliferations are shown in both Fig.4.18 and Fig.4.19 indicates an increase in absorbance with higher concentration. This result scientifically confirms that the non-toxic nature of extracted elastin. In addition, the selected extraction process did not develop any toxic substances on the elastin fiber. Moreover, from toxicity study result analysis forwarded that extracted elastin has no impurities that can hinder cell proliferation.

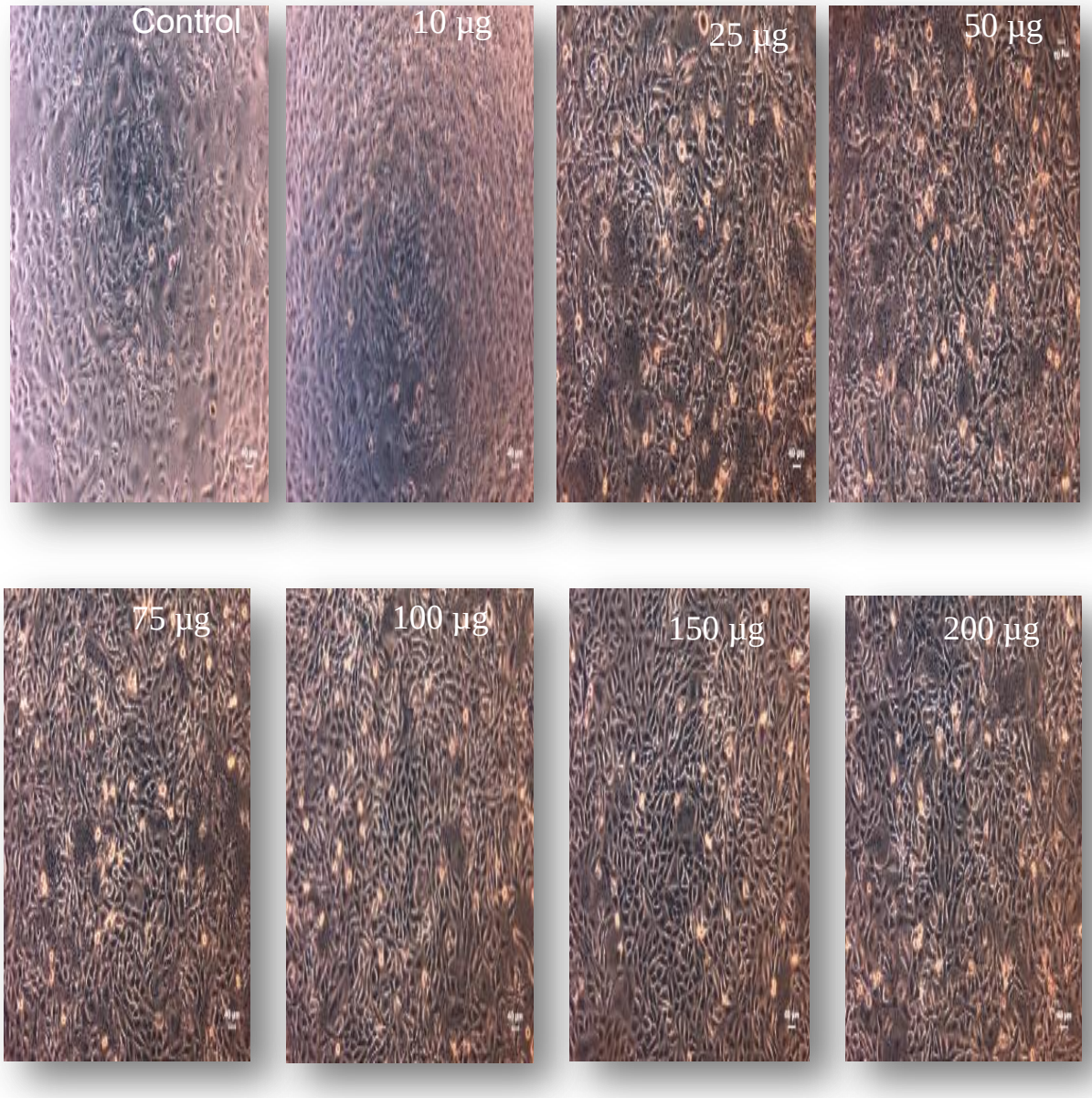


**Fig.4.18 Biocompatibility Studies of elastin using human keratinocyte cell (HaCaT)**

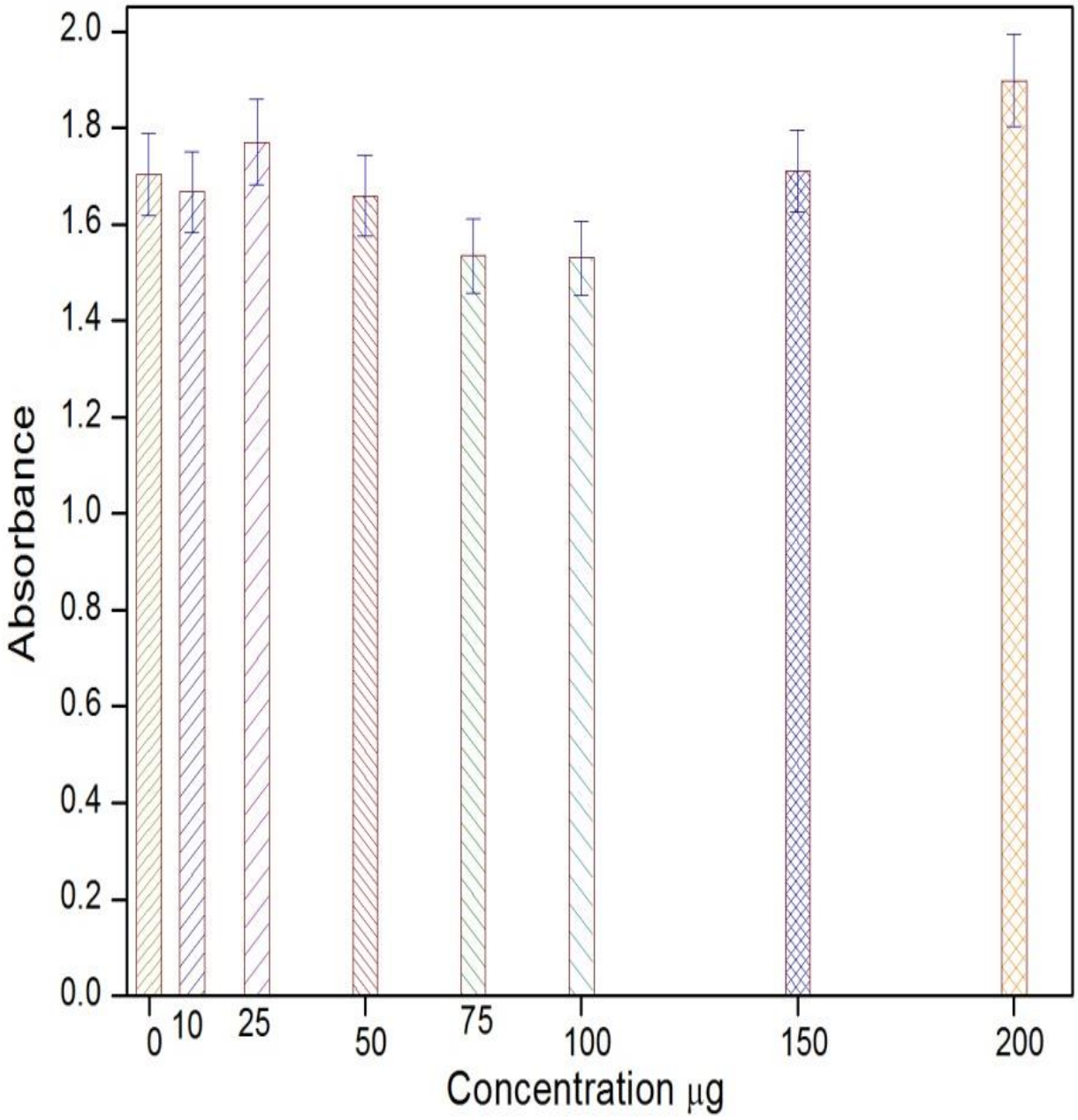


**Fig.4.19 The Cell Proliferation vs Absorbance**

To sum up, the toxicity study analysis result of the elastin shows that extracted elastin has high potential for biochemical and biomaterial applications. In addition, the biocompatibility studies scientifically justify and confirm that the extracted elastin from raw hide trimming solid waste can be used as a biological application.

### 4.6 Findings of the Physical, Chemical and Biological Characterization of Elastin

- Thermal Stability Studies of Elastin using DSC it is found out that the melting peak of extracted elastin is -0.5420 watt/gram and the heat flow rate at 103.23 $^{o}$C temperature. In addition, the elastin shows very high denaturation temperature of 275.82ºC.
- Thermal Stability Studies of Elastin using TGA result analysis has shown that elastin has a degradation peaks occur in six mass loss steps. Moreover, the maximum and minimum the elastin decomposition or weight loss is 30.32% and 4.33% occurs in the temperature ranges of 298.05 ºC to 367.04 ºC and 76.13 ºC to 71.80 ºC respectively.
- The SEM & Microscopic image results are shown that the elastin topographical structure has fiber like structure with surface roughness and non-uniformity surface observed. In addition, shape also looks elongated cylindrical structure and there is an oval structure with length of about 5µm that form aggregation and stick together.
- The $^{13}$C Solid state NMR of the sample generates multiple spectrums are detected in three distinct regions namely amide carboxyl, aromatic and aliphatic region of the elastin. These shifts due to the presence of glycine residues in β-strand structure, aromatic resonance side-chain carbons of the phenylalanines, C(α) of the amino acids, Glycine , Phenylalanine, Alanine, Analine, Aliphatic side chain carbons of C(β),C(ϒ) , Valine and Proline amino acid chains of elastin.
- Solid State NMR chemical shifts result analysis of the extracted elastin shows and confirms that elastin has the random coil, α-helix and β-strand secondary structure of protein.
- The sample HP concentration is found to be 2.1%.This value is in the acceptable ranges of mammalian elastin.
- Physico-chemical, Staining and Histology combination result color change was confirmed that the extracted protein was elastin.
- Functional Groups Analysis result are indicated that the amide I region has α-helix, β-strand and undefined (turn + unordered) conformations structure observes. In addition, the FTIR bands at 2000cm$^{-1}$are assigned to random coil turns and alpha -helix parts of elastin structure. Moreover, the FTIR region below 1000 cm$^{-1}$ has amide V,I V, VI, and VII.

- The free amino acid analysis show that the extracted elastin has high amount of glycine and non-polar amino acids and low amount of acidic and hydroxyl amino acids. Moreover, there are no Hydroxylysine, Tryptophan and Cysteine amino acids found in the amino acid composition of the protein fiber. The amino acid compostion of the extracted elastin correlating with previous reported literatures [3], [8], [9] & [35].
- The variations in the amino acid composition is due to the presence of fibrillies and microfibrial residual collagen which correspond to the presence of Methionine and Histidine.
- The biocompatibility study indicates that the extracted elastin has not accumulated any toxic compounds that hinder the human keratinocyte cells proliferation. Therefore, the biocompatibility study confirms that the extracted elastin can be used as bio-material for different biomedical applications.

# Chapter Five

# Conclusions and Recommendations

## 5.1 Conclusion

The research major findings during raw material characterization, elastin extraction and characterization of the developed product the following conclusions were drawn.

The raw material characterization and elastin content estimation using FPLC, SDS-PAGE and UV-Spectroscopy combination characterization techniques are confirms and effectively determined the elastin content of the skin matrix.

The holistic characterization techniques using physical, chemical and biological of extracted elastin are studied the physical, chemical and biological properties. The physical characterization results using DSC and TGA thermal stability studies it can conclude that the melting peak of elastin is -0.5420 watt/g at 103.23 ºC, elastin shows very high denaturation temperature of 275.82ºC and the product have six mass loss degradation steps. The chemical characterization using physico-chemical test, EVG staining and histology studies combined results show that the extracted protein has shown elastin chemical property. In addition, the $^{13}C$ Solid State NMR chemical shifts and FTIR spectrum combined results have show along with correlated literatures that extracted elastin has shown random coil, α-helix and β-strand secondary structure. Furthermore, the FTIR spectrum peaks demonstrated that elastin has amide A, I, II, III, IV, V, VI, and VII functional group. Moreover, the biological characterization using the free Amino Acid Analysis results shows and scientifically confirms that the extracted protein matches the amino acid composition of elastin and the only impurity is the presence of fibrillies and microfibrial residual collagen. Additionally, biocompatibility study using human keratinocyte cells study result is confirms that there no toxic nature of the product for biological applications.

In conclusion, from the aforementioned characterization and cellular biocompatibility studies results forwarded and concluded that the extracted elastin has a high potential in biochemical and biomaterial applications. Moreover, the biocompatibility studies are scientifically justified and confirm that the developed high value bio material product from raw hide trimming solid waste will be used as a different biological application.

## 5.2 Recommendations

Based on the research results the following recommendations were given;

- The extracted elastin from raw hide trimming solid waste biocompatibility result indicates that the product has not developed any toxic substance during human keratinocyte cell proliferation. Hence, it is recommended that the product(Extracted Elastin) should be used as biomaterial for different biological applications, tissue engineering and biomedical applications
- The cited market surveys indicate that the elastin products has now day very expensive price in the international market due to its versatile applications in many industries. Therefore, it is recommended that further feasibility study should be done in future to assess the profitability of the product. Based on the feasibility study extracted elastin from tannery solid waste have a potential product to export international market and will generated huge amount of foreign currency to the country.
- The tannery solid wastes have been unexploited resources due to less focus and research in the country context .Therefore, it is recommend that more similar research will be need in the future for exploitation of wealth from tannery solid waste.
- The tannery solid waste management system of Ethiopia should be improved in future. It is recommended that one of the improvement areas is more research needed in future for converting tannery solid waste to useful product.

## References

[1] Langmaier F., Kolozmik K., Sukop S., Mla Dek M. Products of Enzymatic Decomposition of Chrome-Tanned Leather Waste. JSLTC. 83 (4), 187, 1999.

[2] Bienkiewicz, Krzysztof J. *Physical chemistry of leather making*. Krieger Publishing Co. Inc., 1983

[3] Covington, Anthony D. Tanning chemistry: the science of leather. Royal Society of Chemistry, 2009.

[4] JRC, DIRECTORATE-GENERAL. "Integrated Pollution Prevention and Control Draft Reference Document on Tanning of Hides and Skin Draft 27 February 2009."

[5] Kanagaraj, J., K. C. Velappan, N. K. Babu, and S. Sadulla. "Solid wastes generation in the leather industry and its utilization for cleaner environment-A review." (2006).

[6] Puntener, Alois. "The ecological challenge of producing leather."The Journal of The American Leather Chemists Association(1995).

[7] Ozgunay, H., S. Colak, M. M. Mutlu, and F. Akyuz. "Characterization of Leather Industry Wastes." Polish Journal of Environmental Studies 16, no. 6 (2007).

[8] Robert, Ladislas and William Hornebeck.Elastin and elastases.Vol.1.CRC Press,1989

[9] W.F.Daamen,T.Hafmans,J.H. Veerkamp, T.H. van Kuppevelt “Comparison of five procedures for the purification of insoluble elastin” Biomaterial 22(2001) 1997-2005

[10] Thanikaivelan, Palanisamy, Jonnalagadda Raghava Rao, Balachandran Unni Nair, and Thirumalachari Ramasami. "Biointervention makes leather processing greener: an integrated cleansing and tanning system." Environmental science & technology 37, no. 11 (2003): 2609-2617.

[11] Jorgen P., Protrade: Foot wear and Leather Goods Division Deutsche Gesenschaft Esch born (1995).

[12] Buljan, J., Reich, G., and Ludvik, J. (1997). Mass Balance in Leather Processing. London: IULTCS Congress, p. 138

[13] Bench Marking (Technology Up-gradation) Program in Ethiopian Tanning Industry, Report – Consolidated EMS Report, Prepared by Central Leather Research Institute (CLRI), (Council of Scientific and Industrial Research) Chennai, India October, 2010.

[14] Nelson, David L., Albert L. Lehninger, and Michael M. Cox.Lehninger principles of biochemistry. 4-th Edition,Macmillan, 2008.

[15] Berg, J. M., J. L. Tymoczko, and L. Stryer. "Biochemistry Fifth Edition, 2002."

[16] Jain, J. L., Sunjay Jain and Intln Jain. Fundamentals of biochemistry, Sixth revised edition S. Chand & Compay Ltd. 2005.

[17] M. Y. Khan and Sangeeta Saxena Biomolecules: Introduction, Structure and Function Department of Biotechnology B.B. Ambedkar University Raibareli Road Lucknow (October 2006).

[18] Voet, Donald, and Judith G. Voet. "Biochemistry, 4$^{-th}$ Edition."New York: John Wiley& Sons Inc (2011).

[19] Metzler, David E. Biochemistry: the chemical reactions of living cells. Academic Press, 2003. Second edition.

[20] Cioca, Gheorghe. "Partially hydrolyzed elastin from limed hide trimmings." U.S. Patent 4,363,760, issued December 14, 1982.

[21] Vyavahare, Narendra, Matthew Ogle, Frederick J. Schoen, and Robert J. Levy. "Elastin calcification and its prevention with aluminum chloride pretreatment." *The American journal of pathology* 155, no. 3 (1999): 973-982.

[22] Soskel, Norman T. "High-performance liquid chromatographic quantitation of desmosine plus isodesmosine in elastin and whole tissue hydrolysates." *Analytical biochemistry* 160, no. 1 (1987): 98-104.

[23] Suyama, Kyozo, and Fumihiko Nakamura. "Isolation and characterization of new cross-linking amino acid “allodesmosine” from hydrolysate of elastin." *Biochemical and* biophysical research communications 170, no. 2 (1990): 713-718.

[24] Mu, Plummer, and David T. Plummer. Introduction to practical biochemistry. Tata McGraw-Hill Education, 1988.

[25] Hatti-Kaul, Rajni, and Bo Mattiasson, eds. Isolation and purification of proteins. CRC Press, 2003.

[26] Baurain, Roger, Jean François LAROCHELLE, and François LAMY. "Photolysis of desmosine and isodesmosine by ultraviolet light." The FEBS Journal 67, no. 1 (1976) : 155-164.

[27] Watanabe, Mika, and Takashi Sawai. "Alteration of cross-linking amino acids of elastin in human aorta in association with dissecting aneurysm: analysis using high performance liquid chromatography." The Tohoku journal of experimental medicine 187, no. 4 (1999): 291-303.

[28] Harvey, David. "Analytical Chemistry 2.0—an open-access digital textbook." Analytical and bioanalytical chemistry 399, no. 1 (2011): 149-152.

[29] Kakivaya, S. R., and C. A. Hoeve. "The glass point of elastin." Proceedings of the National Academy of Sciences 72, no. 9 (1975): 3505-3507.

[30] Yang, Jie, Hui-Lin Zhao, Bai-Lu Tang, Xiu-Lan Chen, Hai-Nan Su, Xi-Ying Zhang, Xiao-Yan Song et al. "Mechanistic insight into the elastin degradation process by the metalloprotease myroilysin from the deep-sea Bacterium Myroides profundi D25." Marine drugs 13, no. 3 (2015): 1481-1496.

[31] Ushiki, Tatsuo. "Collagen fibers, reticular fibers and elastic fibers, A comprehensive understanding from a morphological viewpoint." Archives of histology and cytology 65, no. 2 (2002): 109-126.

[32] Jain, Dharamdeep, Alyssa Y. Stark, Peter H. Niewiarowski, Toshikazu Miyoshi, and Ali Dhinojwala. "NMR spectroscopy reveals the presence and association of lipids and keratin in adhesive gecko setae." Scientific reports 5 (2015).

[33] Perry, Ashlee, Michael P. Stypa, Brandon K. Tenn, and Kristin K. Kumashiro. "Solid-State $^{13}$C NMR Reveals Effects of Temperature and Hydrationon Elastin." Biophysical journal 82, no. 2 (2002): 1086-1095.

[34] Papaioannou, A., M. Louis, B. Dhital, H. P. Ho, E. J. Chang, and G. S. Boutis. "Quantitative comparison of structure and dynamics of elastin following three isolation schemes by 13 C solid state NMR and MALDI mass spectrometry." Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1854, no. 5 (2015): 391-401.

[35] Neuman, Robert E., and Milan A. Logan. "The determination of collagen and elastin in tissues." Journal of Biological Chemistry 186, no. 2 (1950): 549-556.

[36] Muiznieks, Lisa D., and Fred W. Keeley. "Molecular assembly and mechanical properties of the extracellular matrix: a fibrous protein perspective." Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1832, no. 7 (2013): 866-875.

[37] Arrondo, Jose Luis R., Arturo Muga, Jose Castresana, and Felix M. Goñi. "Quantitative studies of the structure of proteins in solution by Fourier-transform infrared spectroscopy." Progress in biophysics and molecular biology 59, no. 1 (1993): 23-56.

[38] Debelle, Laurent, Alain JP Alix, Marie-Paule Jacob, Jean-Pierre Huvenne, Maurice Berjot, Bernard Sombret, and Pierre Legrand. "Bovine elastin and κ-elastin secondary structure determination by optical spectroscopies." Journal of Biological Chemistry 270, no. 44 (1995): 26099-26103.

# Appendix

**Annex 1:** Methodology/Technology Selection based on Scientific Parameters

| No. | Selection Parameter | Extraction Technologies | | | Reference |
|---|---|---|---|---|---|
| | | Alkaline/Acid Method | Physical/Autoclaving Method | Enzymatic Method | |
| 1. | Soluble Protein contamination | No Present | No Present | No Present | [9] |
| 2. | HP content | Compatible with literature | Compatible with literature | Compatible with literature | [9] |
| 3. | % presence of Glycine, Alanine, Valnine & Proline | High | High | High | [9] |
| 4. | Concentration of Desmosine and Isodesmosine | Compatible with literature | Compatible with literature | Compatible with literature | [9] |
| 5. | Presence of sulphydryl group | Moderate content | Minor amount because oxidized | Between high and minor based on the chemical treatment | [9] |
| 6. | Van Gieson's staining Result | Positive Result | Positive Result | Positive Result | [9] |
| 7. | Type I and IV collagen Presence | Absent or not present on the product | Absent or not present on the product | Absent or not present on the product | [9] |
| 8. | Impurities in collagen fibrillies and microfibrilles | Non-elastin materials present | Microfibriall residual collagen present(Moderately pure) | Pure | [2],[9] |
| 9. | Process Time | Short | Very short | Very long | Own Analysis |
| 10. | Chemical and Microbial Contamination | High | Low | High | Own Analysis |
| 11. | Substrate Compatibility | Low | High | Low because Not yet tried | Own Analysis |
| 12. | Complexity of the process | Low | Low | High | Own Analysis |

**Note: -** Based on comparing and contrasting the above mentioned scientific parameters it's found out that the feasible technology for the research is autoclaving method

**Annex 2:** List of chemicals types, concentration and brands were used in the Experiment

| No. | List of Chemical | Concentration | Brand | Amount |
|---|---|---|---|---|
| 1 | HCl | 35% | Himedia | 2.5L |
| 2 | CaOH | 98% | Himedia | 500g |
| 3 | NaOH | 98% | SRL | 500g |
| 4 | NaCl | 99.5% | MERCK | 500g |
| 5 | Ammonium Chloride | 99.5% | SD fine | 500g |
| 6 | Ammonium Sulphate | 98% | SD fine | 500g |
| 7 | Ammonium Per sulphate | 98% | SD fine | 500g |
| 8 | H2O2(Hydrogen Peroxide) | 30% | Emplura | 500 ml |
| 9 | Filter Paper(dia125) | 125mm | whatmann | 100 no. |
| 10 | Sorbic Acid | 99.0% | Sigma | 100g |
| 11 | Sodium Dodecyl Sulphate | 98.5% | Sigma | 25g |
| 12 | Chloroform | 99% | Emplura | 2.5L |
| 13 | Metanol | 100% | CDH | 2.5L |
| 14 | Ethanol | 100% | CDH | 2.5L |
| 15 | Ether | 99.5% | SRL | 2.5L |
| 16 | Na2HPO4 | 99% | SRL | 500g |
| 17 | EDTA | 98% | SD fine | 100g |
| 18 | PH | 2-14; 5-7.5;7.5-14 | RANKEM | 2 Pkts |
| 19 | Phenonaphtaline | 0.1% | Himedia | 125ml |
| 20 | Metly Organe | 1% | SRL | 100g |
| 21 | Tris Buffer | 99.8% | Merck | 100g |
| 22 | $CaCl_2$ | 99.5% | SD fine | 500g |
| 23 | $MgCl_2$ | 99.5% | SD fine | 500g |
| 24 | KBr | 99% | Sigma | 100g |
| 25 | Acrylamide | 99% | SRL | 500g |
| 26 | N'N'-bis-methylene-acrylamide | 99% | Sigma | 100g |
| 27 | Tris base | 99% | Sigma | 100g |
| 28 | Glycine | 99% | Sigma | 500g |
| 29 | Glycerol | 99.5% | Himedia | 1L |
| 30 | 2-mercaptoethanol | 99% | Sigma | 100ml |
| 31 | 1% bromophenol | 99% | Sigma | 100g |
| 32 | APS | 98% | Himedia | 500g |
| 33 | TEMED | 99-101% | Himedia | 100ml |

| No. | List of Chemical | Concentration | Brand | Amount |
|---|---|---|---|---|
| 34 | Citric Acid monohydrate | 99% | Sigma | 25g |
| 35 | Glacial acetic | 99-100% | Sigma | 1.5ml |
| 36 | Sodium acetate trihydrate | 99% | Himedia | 500g |
| 37 | Hydroxyl proline | 98.5% | Sigma | |
| 38 | Chloramine-T | 99% | Himedia | 250g |
| 39 | Methyl Cellulose | | Sigma | 100g |
| 40 | Perchloric Acid | 70% | Merck | 500ml |
| 41 | PDAB (para dimethyl amino bezaldehyde) | 99% | Himedia | 100g |
| 42 | Hematoxylin | | Himedia | 25g |
| 43 | Ferric chloride | 10% | Sigma | 100g |
| 44 | Lugol's iodine | | Sigma | 50ml |
| 45 | 1% Acid fuchsin | 1% | Sigma | 25g |
| 46 | Picric Acid | 98% | | 100g |
| 47 | KCl | 99.5% | MERCK | 500g |
| 48 | Synthetic orcein | | Sigma | 25g |
| 49 | Methylene blue | 1% | SRL | 100g |
| 50 | Acetic Acid | 99.9% | SRL | 500ml |
| 51 | Acetone | 99% | RANKEM | 2.5L |
| 52 | Potassium bromide | 99.5% | SRL | 500g |
| 53 | $KH_2PO_4$ | 98% | Sigma | 100g |

**Annex 3:** List of Laboratory Glassware's Types and Volumes

| No. | Glass wares Types | Volume |
|---|---|---|
| 1 | Round bottam flask | 250ml |
| 2 | Beaker | 50-1000ml |
| 3 | Measuring cylinder | 5-100ml |
| 4 | Conical flask | 250ml |
| 5 | Sample collection bottles | 10-50ml |
| 6 | Micropipettes | 0.5-10 ul, 2-20ul, 20-200ul, 200-1000ul |
| 7 | Burettes | 2,10 and 100ul |
| 8 | Standard measuring flask | 50-500ml |
| 9 | Centrifuges tubes | 15-50ml |
| 10 | Test tubes | 9ml and 22ml |
| 11 | Crucible | 10-30ml |
| 12 | Separation Funnel | 250ml |
| 13 | Filter paper | 10-50mm |

**Annex 4:** List of Laboratory Equipment's Types, Brand and Range of Operations

| No. | Laboratory Machinery Types | Brand | Range of Operations/Capacity |
|---|---|---|---|
| 1 | SDS-PAGE Apparatus | ORANGE, cat NO. VMR0007; Serial No. OG/2018-02/21 | |
| 2 | HPLC | Schimadzu UFLC | |
| 3 | FTIR | Jasco FTIR/4200 Type A | |
| 4 | FPLC | GE AKTa purifier | |
| 5 | DSC | Q200 V23.10 Build 79 | |
| 6 | TGA | Q50 V 20.5 Build 30 | |
| 7 | SEM | PHENOMProx | 300-3µm |
| 8 | Centrifuge | Eppendorf 5810R | 1000-14000RPM |
| 9 | Vacuum pump/Filter | Type 350.350.400 Sr no. 3692 Rays scientific | 1500W 220volt |
| 10 | Autoclave | Equitron Sr no. 7421 STWLAAE.053 | 1800 Watts, 230VAC 50Hz,setted to one bar |
| 11 | Ultrasonic Bath | LABMAN Model no LMUC-4 | Frequency 40 KHz Power 100 |
| 12 | Heater/Water bath | Equitron sr.no 7514.AFI.318 | 1000W 230VAC 50Hz |
| 13 | UV-Spectrophotometer | SHIMADZU Corp Sr no. A11635202825 | UV-1800 240V |
| 14 | Electrical Stirrer | REMI model-1RML | RPM-1200, 220W, 50Hz. |
| 15 | Shaker | Labnet | Ambient-60C, 200rpm |
| 16 | Refractive index Measuring Machine | LABMAN-make LMAR-1317 | nD 1.300 -1.7003 |
| 17 | Light Microscope | Novex | 4-100X |
| 18 | Solid State NMR | | |

**Annex 5:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Method Protocol

## I. SDS-PAGE Solutions Preparation

**30% Acrylamide Solution:-**The solution was prepared using 30 g acrylamide mixed with 0.8 g N'N'-bis-methylene-acrylamide in the beaker. Then, diluted to 100ml with deionized water and stored in refrigerator at 4° C temperature.

**10% SDS Solution Preparation:-**The solution was prepared by dissolving 50 g SDS in 450 ml deionized water with gentle stirring .After that, deionized water added $^{in}$ the mixture up the total volume to be 500 ml.

**Lower Tris Buffer (4x):-** The lower Tris Buffer was prepared by dissolving 18.17gm Tris base in to 4 ml of 10% SDS. Then, the mixture pH was adjusted to 8.8 -9by adding conc. HCl.

**Upper Tris Buffer (4x):-**The upper Tris Buffer was prepared by dissolving 6.06gm Tris base in to 4 ml of 10% SDS. Then, the mixture pH was adjusted to 6.8-7 by adding conc.HCl. The solution stored in refrigerator at 4° C.

**Tris-glycine reservoir buffer (4x):-** The solution was prepared by mixing 12 g Tris base with 57.6 g glycine in 1000 ml deionized water. Then, the solution stored in refrigerator at 4° C.

**Sample buffer (5x):-**First, the mixture of 3.9 ml deionized water and 1.0 ml 0.5 M Tris, was prepared. Secondly, the mixture pH 6.8 was adjusted. Thirdly, the prepared mixture was mixed 0.8 ml Glycerol, 1.6 ml 10% SDS, 0.4 ml 2-mercaptoethanol and 0.4 ml 1% bromophenol blue respectively. Finally, the solution sored in the refrigerator.

**Ammonium Persulfate Solution (APS make fresh daily):-**This solution is freshly prepared during the experiment. The solution was prepared by dissolving 0.1 g APS (electrophoresis grade) in 1 ml deionized water.

**II. Gel preparation**

**Resolving gel (lower gel) Preparation:-**The resolving gel was prepared by selection 12% Acrylamide solution formulation. Firstly, 3.45 ml of deionized water mixed with 2.5ml Lower Tris Buffer. Secondly, in the mixture 4ml of 30% Acrylamide was added. Finally, 50µl of 10% APS and 5.0µl of TEMED simultaneously added in the solution.

**Stacking gel (upper gel) Preparation:-**The stacking gel was prepared initially by mixing 3.05ml deionized water with 1.25ml of Upper Tris Buffer. Next, in the solution 0.67ml of 30% Acrylamide was added. Lastly, 25µl of 10% APS and 5.0µl of TEMED simultaneously added in the solution.

**Electrode Buffer:-**The electrode buffer was prepared by mixing 370 ml deionized water with 125ml of Reservoir buffer .Then, in the solution 5ml of 10% of SDS was added.

**III. Electrophoretic Detection of Elastin content Procedure**

Electrophoresis studies were done using 10ml of 12% acrylamide separating gels. The solution was swirled gently but thoroughly. Then,500 µl separating gel solution in inserted using micropipette in the gap between glass plates. The plate with separating jell leave for 30 minutes. After wards, put the comb and the prepared 5ml stacking jel transferred in the compartment leave for 30 minutes to form gelation. The protein sample of 15 µl and 10 µl sample solubilizing buffer and 10 µl water are mixed and heated for ten minutes in boiling water. The prepared sample loaded into SDS-PAGE. Then covered and connect the anodes. The voltage adjusted to 50v the during the sample running in the stacking gel area and adjusted to 100V once entered in the separating gel portion of SDS-PAGE Compartment. The jell staining one day and destaining in the Single Platform Laboratory Shaker for three days.

**Annex 6:** HP content Determination Standard Method Protocol

## I. Solution and Reagent Preparation

**Buffer Preparation (100ml):-**The buffer initially was prepared by mixing 5g of Citric acid monohydrate with 1.2ml of Glacial acetic. Then, in the mixture 12 g of Sodium acetate trihydrate and 3.4gram of Sodium hydroxide was added and 100 ml of distilled water poured until the pH of the buffer to be 6.

**Standard HP:-**The standard HP solution was prepared by mixing in the beaker 10mg of hydroxyline with 100 ml of 0.001N HCl with gentle stirring.

**Chloramine-T (0.05M):-**The Chloramine-T (0.05M) solution initially was prepared by mixing 0.281gm of Chloramine-T and 4ml of Water .Then, in the solution 10 ml of Buffer and 6ml Methyl Cellusolve was added with gentle stirring.

**Perchloric acid [70%] (3.15M):-**The 70% of Perchloric acid was prepared by careful mixing 2.7ml of Perchloric acid with water to be the total volume of 10ml.

**PDAB (20%):-**The PDAB (20%) solution was prepared by mixing 2g of PDAB in 10 ml of Methyl cellulose by stirring.

## II. Standard Preparation for the estimation of hydroxyproline

Initially, five standard hydroxyproline solutions were dropped in five labeled test tubes. The added volume of standard hydroxyproline reagent in five test tubes are 20 µl, 40 µl, 60 µl, 100 µl and 120 µl respectively. Then, in each test tubes 1980 µl, 1960 µl, 1940 µl, 1900 µl and 1880 µl distilled water were added separately using micropipettes. Afterwards, in each five test tubes 1000 µl of Chloramine-T chemical added and incubated the solution for 20 minutes at room temperature.

In the primary prepared solutions in each test tubes 1000 µl of Perchloric acid were mixed subsequently and incubated for 5 minutes at room temperature. After that, 1000 µl of PDAB

(para dimethyl amino bezaldehyde) chemical was added in each test tube consecutively. Then, the prepared standard solution were incubated in water bath for 20 minutes at 60ºC temperature.

### III. Sample Preparation for the estimation of hydroxyproline

Initially, the blank solution was prepared without sample. This was done by mixing 2000µl of deionized water and 1000µl of Chloramine-T in test tube. The mixture was kept for 20 minutes at room temperature. Then, 1000 µl Perchloric Acid was added in the prepared mixture and kept the solution at room temperature for 5 minutes.

Secondly, five purified and lyophilized sample solutions were dropped in five labeled test tubes. The added sample volumes in five test tubes are 2000 µl each. Afterwards, in each five test tubes 1000 µl of Chloramine-T chemical added and incubated the solution for 20 minutes at room temperature. Then, the prepared solutions in each test tubes 1000 µl of Perchloric acid were mixed subsequently and incubated for 5 minutes at room temperature. After that, 1000 µl of PDAB (para dimethyl amino bezaldehyde) chemical was added in each test tube consecutively. Then, the prepared sample solution was incubated in water bath for 20 minutes at 60ºC temperature.

### IV. HP Content Determination Procedure

The Standard HP solutions, the blank solution and the five Sample solutions were taken for measurement of absorbance at 557nm in UV-Spectrophotometer respectively. Then, the Hydroxyproline content of the sample was determined directly from the drawn standard curve.

**Annex 7:** Verhoeff-Van Gieson (EVG) Staining Characterization Method Protocol

## I. Solution, chemicals and Regent preparation of Verhoeff-Van Gieson

**Alcoholic Hematoxylin:-**The solution was prepared by dissolving 5gram of Hematoxylin in 100 ml of Ethanol with gently heating and mixing in the beaker. Then, the prepared solution was labeled.

**10% Ferric Chloride:-**The solution was prepared by dissolving 10 gram of Ferric chloride in 100ml of deionized water by mixing in the beaker and the solution transferred to small round bottom flask .Next, the prepared solution was labeled.

**Verhoeff's Hematoxylin:-**The reagent was prepared initially by mixing 20 ml Alcoholic hematoxylin and 8ml of 10% ferric chloride in the beaker with gentle stirring. Then, in the solution 8 ml of Lugol's iodine was mixed. Lastly, the prepared solution was transferred in to round bottom flask and labeled it.

**Differentiating Solution (2% Ferric Chloride):-**The 2% Ferric Chloride solution was prepared by mixing 10ml of 10% ferric chloride with 40ml deionized water in beaker with gently stirring. The solution was prepared freshly at the time of the experiment.

**Van Gieson's Solution:-**The solution was prepared by mixing 1ml of 1%Acid fuchsin and 45 ml of saturated Picric acid. Then, the solution is allowed to stand overnight and labeled.

## II. Verhoeff-Van Gieson (EVG) Staining Procedure

Primarily, the elastin sample taken and washed with distilled water three times. Then, Verhoeff's hematoxylin was dropped in the sample using micro pipettes and leave for 30 minutes in room temperature. After that, the sample was washed using water and five drops of 2% ferric chloride solution added on the sample. The sample washed with water and five drops of Hypo solution added on the sample. The sample leaved for one minute in order to effectively remove iodine from the sample surface. Afterwards, the sample surface was washed with water. Finally, the sample surface was counter stained in Van Gieson's solution and dried at the room temperature. The stained sample surface was taken to microscope using glass film and different images of the sample stained surface were taken by adjusting the magnification.

**Annex 8:** 0.1 M Phosphate Buffer Preparation Standard Protocol

**1. Preparation of Stock Solutions**

A. 0.2 M solution preparation of monobasic sodium phosphate (NaH2PO4.H2O)

The 0.2M solution of monobasic sodium phosphate was prepared by dissolving 27.8g of monobasic sodium phosphate (NaH2PO4.H2O) in 1000 ml of distilled water.

B. 0.2 M solution preparation of dibasic sodium phosphate (NaH2PO4.H2O)

The 0.2M solution of dibasic sodium phosphate was prepared by mixing 52.65 g of Na2HPO4.7H2O ) in 1000 ml of distilled water.

**2. Preparation of 0.1 M Phosphate Buffer**

The 0.1M of Phosphate buffer was prepared by mixing 33ml of 0.2M solution of monobasic sodium phosphate with 67ml of 0.2M solution of dibasic sodium phosphate. Then, the mixture was diluted to a total volume of 200ml with distilled water. Finally, the PH of the prepared phosphate buffer was 7.1.

**Annex 9:** Histology Using Orcein and Methylene Blue Method Protocol

(Modified Taenzer-Unna orcein Method)

## I. Reagent solutions Preparations

**Solution 1 Preparation:-**The solution 1 was initially prepared by dissolving 1.0 grm of synthetic orcein in 100ml of 70% alcohol with gentle stirring. Then, in the mixture 1.0 ml of hydrochloric acid was carefully added using micropipettes. Finally, the solution was kept in closed cylinder.

**Solution 2 Preparation:-**The solution 2 was prepared by mixing 0.5% of methylene blue in 1.0% acetic acid in the beaker with gentle stirring. Then, the solution was kept in closed beaker.

## II. Orcein and Methylene Blue Method Procedure

Initially, 10 gram of sample was taken and five drops of formalin chemical added on the surface of the sample (elastin fiber) to stabilize the protein. Then, the sample was sectioned in to five distinct pieces. Next, each sample pieces was stained by a solution 1 in a covered jar for 30 minutes. The stained surface of each pieces of sample was rinse off excesses stain by washing the sample surface with 70% of alcohol. Afterwards, the sample surface was stained by solution 2 for 3minutes.The stained surface was rinse off excess stain with distilled water and blot gently with filter paper. The prepared sample was dehydrated, cleared and mounted in synthetic resin medium. Finally, the stained sample surface was taken to microscope using glass film and different images of the sample stained surface were taken by adjusting the magnification.

**Annex 10:** Quantitative and Qualitative Analysis of FPLC Chromatogram Computation

From FPLC data sheet the mobile phase, protein A and Protein B were collected and the analysis listed below.

**A. Computation of Retention time($t_r$)** [25]

I. Mobile Phase Data

$V_r$ (Retention Volume) = 10.64488312 ml

mAU=4.790999927

$V_r = t_r \times \mu$ ---------- Eq. (1)

Where $V_r$ is retention Volume, $t_r$ is retention time and $\mu$ is the mobile phase's velocity through the column

From Eq.1 tr is derived as

$$t_r = {}^{V_r}/_{\mu}$$

$\mu = 1.3\ ml/min$

$$t_r = {}^{10.64488312ml}/_{1.3ml/min}$$

$t_r = 8.188min \approx 9\ min$

$t_r = 9\ min$

II. Protein(Solute) A

$V_r$ (Retention Volume) = 70.0019455 ml

mAU= 24.57400039

$V_r = t_r \times \mu$ ---------- Eq. (1)

Where $V_r$ is retention Volume, $t_r$ is retention time and $\mu$ is the mobile phase's velocity through the column

From Eq.1 tr is derived as

$$t_r = {}^{V_r}/_{\mu}$$

$$\mu = 1.3\ ml/min$$

$$t_r = {}^{70.0019455ml}/_{1.3ml/min}$$

$$t_r = 53.847min \approx 54\ min$$

$$t_r = 54\ min$$

The width of Protein (solute) A was measured from Fig.4.3 C. The base width value was

$$W_A = 20\ min$$

III. Protein(Solute) B

$V_r$ (Retention Volume) = 178.1814575 ml

mAU= 115.0389984

$V_r = t_r \times \mu$ ---------- Eq. (1)

Where $V_r$ is retention Volume, $t_r$ is retention time and $\mu$ is the mobile phase's velocity through the column

From Eq.1 tr is derived as

$$t_r = {}^{V_r}/_{\mu}$$

$$\mu = 1.3\ ml/min$$

$$t_r = {}^{178.1814575ml}/_{1.3ml/min}$$

$$t_r = 137.062min \approx 137min$$

$$t_r = 137\ min$$

The width of Protein (solute) B was measured from Fig.4.3 C. The base width value was

$$W_B = 135\ min$$

**B. Computation of Chromatographic Resolution** [25]

The resolution between Protein (Solute) A and Protein (Solute) B Peaks computed in the following formula as

$$R_{AB} = \frac{2\Delta t_r}{W_A + W_B} = \frac{2(t_{r,B} - t_{r,A})}{W_B + W_A} \quad \text{---------- Eq.2}$$

$$R_{AB} = \frac{2(t_{r,B} - t_{r,A})}{W_B + W_A}$$

$$R_{AB} = \frac{2(137min - 54min)}{135min + 20min}$$

$$R_{AB} = 1.07$$

Hence, $R_{AB} > 1$ the separation was good and effective

**C. Computation of Retention factor [25]**

The retention factor for A and B of the chromatograph was calculated from the following formula

$$K_A = \frac{t_{r,A} - t_m}{t_m} \quad \text{----------Eq.3}$$

$$K_A = \frac{54min - 9min}{9min}$$

$$K_A = 5$$

$$K_B = \frac{t_{r,B} - t_m}{t_m}$$

$$K_A = \frac{137min - 9min}{9min}$$

$$K_B = 14.22$$

**Therefore, $K_A = 5$ and $K_B = 14.22$ respectively in which both of them values are greater one which indicates clearly that separation of protein was efficient.**

**D. Computation of selectivity factor [25]**

The selectivity factors of the two protein was calculated, as follows:-

$$\alpha = \frac{K_B}{K_A}$$----------Eq.4

$$\alpha = \frac{14.22}{5}$$

$$\alpha = 2.844$$

**E. The Peak area(mAU*min) of protein concentration[25]**

From the Fig.4.3 C the protein concentration using the peak area were found to be

Protein (A) = 24.57400039 mAU (mile absorbance Unit)

Protein (B) = 115.0389984 mAU

**F. The Peak area(mAU*min) of Desmosine Concentration**

The Peak Area(mAU*mim) of Desmosine aromatic amino acid Concentration at 275nm from FPLC data sheet was

Peak Area = 122.515004mAUmin $V_r$ = 180.2610321 ml

**G. The Peak area(mAU*min) of Isodesmosine Concentration**

The Peak Area (mAU*mim) of Isodesmosine aromatic amino Concentration at 275nm from FPLC data sheet is

Peak Area = 97.19099849 mAUmin $V_r$ = 179.6367798 ml

**Annex 11:** Extracted Elastin, Weight of hide on dry bases and Yield Computations

**1. The extracted Elastin Calculation**

The total weigh of the extracted (Filter cake) = 1.98 gram

According the selected autoclaving extraction protocol

- The dry matter weight is estimated to be 25.56 % of filter cake
- The Ash weight weight is estimated to be 0.7% of the filter cake
- The insoluble Elastin weight is estimated to be 13.8 to 14% of the filter cake

Based on the above assumption the parts of the filter cake computed as follows

$$\text{Dry matter} = 0.2556 * 1.98\text{gram}$$

$$\underline{\mathbf{Dry\ matter} = \mathbf{0.5061gram}}$$

$$Ash = 0.07 * 1.98gram$$

$$\underline{\boldsymbol{Ash} = \mathbf{0.1386}\boldsymbol{gram}}$$

Taking 13.9 % of the filter cake of the insoluble Elastin .Then, the weight of the extracted insoluble Elastin computed as follows

$$Insoluble\ Elastin = 0.139 * 1.98gram$$

$$\underline{\boldsymbol{Insoluble\ Elastin} = \mathbf{0.275}\boldsymbol{gram}}$$

Therefore, the weight of the extracted insoluble elastin is to be 0.275 grams.

**2. The weight of the hide on dry basis calculations**

The weight of the hide on dry basis detail computation is shown as follows:-

The weight of the hide in dry basis calculation

The weight of the skin sample in wet basis = 87.2627 grams

The moisture content of the hide is about 65%-70% weight of the fresh hide [2] & [3].

Taking 65% of moisture content, the moisture content computed as follows

The moisture content of the hide = 0.65*87.2627 gram

The moisture content was estimated to be = 56.72355gram

The weight of the hide in dry basis = 87.2627gram – 56.72355gram

**The weight of the hide in dry basis = <u>30.54345gram</u>**

**3. The Extraction process Yield Calculations**

The Yield the Extraction Process computes as the following formula

$$Yield = \frac{Actual\ Yield}{\text{Theoretical}\ Yield} * 100$$

The theoretical yield of Elastin computed using the following assumption.

The Elastin content of the skin matrix is in the range between 0.3-1% as per previous reported literatures [2] & [3].

Taking 1% of the elastin content of skin matrix

The Elastin content of the dry hide mass = 0.01*30.54345 gram

**The Elastin content of the dry hide mass = 0.305435 gram**

The theoretical yield of the hide sample was found to be 0.305435 gram

The actual yield of the extraction process was found to be 0.275 grams

$$Yield = \frac{Actual\ Yield}{\text{Theoretical}\ Yield} * 100$$

$$Yield = \frac{0.275}{0.305435} * 100$$

$$\underline{\boldsymbol{Yield = 90.04\%}}$$

Therefore, the Autoclaving (Chemo-Thermal) Extraction method yield was found to be 90%. This yield indicates that the selected extraction process was efficient.