Chapter

# Collagen: From Waste to Gold

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## Abstract

Industrial processing of bovine hides into leather results in many unusable hide off-cuttings, shavings and trimmings. This waste raw material is under-utilised and presents a waste valorisation opportunity to derive a high-value product such as collagen. Collagen is a highly sought-after protein which consists of three polypeptide chains, comprising 30% of the mammalian body's protein, being the main component of skin, connective tissue and cartilage. The demand for collagen is rising at approximately 20% annually and global collagen-based biomaterials market is predicted to reach US$5 billion by 2025. This chapter presents a waste valorisation opportunity to extract collagen from waste bovine hide off-cuttings. Further, it discusses collagen extraction method optimization and methods used to investigate physicochemical properties of collagen are reviewed.

**Keywords:** collagen extraction, bovine collagen, bovine hide, acid-enzyme, solubilisation, extraction, waste valorisation

## 1. Introduction

Increased environmental awareness has led researchers to find alternative solutions to replace petroleum-based materials in a sustainable manner. With an increase in world's population, one of the most concerning problems the planet is currently experiencing is the cumulative waste from various industries. The world's population produce an astounding 3.6 million tonnes of municipal solid waste each day. It is projected to rise to 6.1 million tonnes per day by the year 2025. It is adversely affecting health, contaminating our air, landscape, fresh water and ocean life. Waste valorisation is one method of managing waste in a sustainable manner and in return deriving a high-value product. The meat industry constitutes many by-products, which are under-exploited, from which a large number of valuable proteins, fats and chemicals can be derived from. Specifically related to the meat industry are tanneries and rendering plants, which process bovine and cattle hides for leather and fat production.

Hide off-cuttings, shavings and finished leather scrap are generated as waste in tanneries. These are currently disposed of in landfill sites and they have high landfilling costs per mass unit due to their low density and present low compaction ability. At best, the hide off-cuttings and shavings are converted to animal feed providing little or no economical or sustainable value, despite their content in valuable biopolymers. Bovine hides are rich in the valuable protein collagen, especially in the corium layer of the skin.

Considering the high cost of collagen and the vast number of applications and industries it can be of value, a more sustainable and a waste valorisation option would be to recover as much collagen as possible from hide off-cuttings.

Collagen is a structural protein, which provides strength, stability, and flexibility and is a major constituent of the skin tissue. Hence, bovine hides contain an abundance of collagen. The collagen molecule is a triple-helix comprised of three distinct alpha chains of repeating units of $(GLY\text{-}X\text{-}Y)_N$ amino acids, where X is often proline and Y is often hydroxyproline [1, 2].

Collagen is a highly sought-after protein, finding use in regenerative medicine, in cosmetics, used as casings, in supplements, films, pharmaceuticals, as a precursor to biodegradable materials, for use in tissue engineering and more recently in 3D printing [3–8]. The demand for collagen is rising at approximately 20% annually and global collagen-based biomaterials market is predicted to reach US$5 billion by 2025. Specifically extracting bovine collagen has many advantages over other potential sources, such as having a higher denaturation temperature in comparison to collagen from marine sources, extracting fish and porcine collagen present limitations; applications of fish collagen are limited because of its lower hydroxyproline content [9] and porcine products are prohibited by Muslim and Jewish communities [10].

This chapter aims to represent a background on waste generation in tanneries, to use the tannery waste bovine hide off-cuttings for extraction of high value collagen. Further, collagen extraction methodologies are discussed in detail and finally methods used to investigate physicochemical properties of collagen are reviewed.

## 2. Tannery processing: waste production

In recent years waste valorisation has attracted a significant amount of attention with the sole aim of managing waste in the most sustainable way. Waste from various industries is one of the most concerning problems the planet is currently experiencing and will increase with the increase in population and needs to be addressed. The meat industry constitutes many by-products that are underexploited, from which a large number of valuable proteins, fats and chemicals can be derived from.

Tanneries and rendering plants process bovine and cattle hide for leather and fat production. Casualty and cattle used for meat consumption result in a large quantity of waste and one of the most valuable by-products is the bovine and cattle skins or hides.

Industrial rendering separates animal by-products into value-added products such as animal protein meal and rendered animal fat and tanneries aim to process hides into leather, however, a substantial amount of waste is still produced from these processes that can be used to derive high-value products. Collagen is such a product that can be extracted from hide off-cuttings that is additional waste generated during leather preparation steps. Considering the high cost of collagen and its vast number of applications, extraction of such high-value product from bovine hide off-cutting is both sustainable and economical.

### 2.1 Impact of tannery waste on the environment

As much as this sector is considered to play a vital role because it recycles and reuses the by-products of the meat industry, the processes carried out in the different stages have a serious environmental impact. Environmental concerns that result from tanneries are due to resource consumption such as water, chemicals, energy and the generation of emissions such as volatile organic compounds, wastewater and solid waste. Moreover, hide off-cuttings, trimmings, hair and fleshings are removed from the hides during the tanning process. Only about 25% by weight of raw salted hides results in the finished leather [11]. Furthermore, other solid wastes are also produced from wastewater and sludge treatment.

**Figure 1** shows the stages carried out in a tannery and post tanning in order to convert hides into leather. These steps result in the release of corrosive gasses into the atmosphere and in large quantities contaminated wastewater. Though leather is used for many applications, from furniture to bags and shoes and is economical in many industries, some bovine hides such as bull-hides are often too thick to process and requires additional processing steps for thinning of hides.

During the conversion of bovine hides into leather (**Figure 2**) a vast number of chemicals are released into the environment and waste products are generated at each stage. **Table 1** is showing chemicals used and wasted generated at each stage of leather production.

### 2.2 Use of bovine hides for collagen production

As bovine hides are being converted to leather, additional waste is generated during the preparation steps. Collagen-rich hide off-cuttings, trimmings and defected parts end up in landfill or at best as animal protein feed which is of low value considering the processing costs.

Bovine hide off-cuttings, trimmings and potentially bull-hides that are too thick to process for leather production and calf-hides that have defects can be used for collagen extraction. Collagen is the most abundant protein found in the mammalian body, making up approximately 30% of the total body protein. This structural protein which provides strength, stability and flexibility is a major constituent of skin tissue [14] and hence bovine hides are rich in collagen, especially in the corium section of the hide [15].

Hide off-cuttings can come from various bovine sources, such as bull, cow, ox, calf and even bovine face-piece hides. Additional to bovine hide off-cuttings, bull-hides that are too thick to process and require additional thinning processes can also be used for collagen extraction. This reduces extra processing costs and can directly be used for collagen extraction.

Bovine hide off-cuttings can be processed for collagen extraction. This collagen can be used by various industries for many applications from biodegradable films, pharmaceuticals to cosmetics. Several methods and techniques can be applied to extract collagen from bovine hide off-cuttings and the most efficient, economical and environmentally favourable methods can be worked with in order to reduce chemical and solid waste. Further, the market value of collagen is a lot more than leather, ranging from $37 per gram to as high as $1000 per gram for native lab-grade collagen [16].

## 3. Collagen

Collagen is the most abundant structural protein found in the vertebrate body. Collagen is a rigid, inextensible, fibrous protein that is the principal component of connective tissue in animals, including tendons, cartilage, bones, teeth, skin and blood vessels. As a structural protein it is mainly used to give strength to structures in the body, however, it has different functions depending on the location of the body [17]. One-third of the total protein content in the mammalian body is collagen and accounts for three-quarters of the dry weight of the skin.

### 3.1 Collagen structure

The triple-helix of collagen consists of three distinct alpha chains coiled around each other and this is termed as tropocollagen. The tropocollagen units are arranged

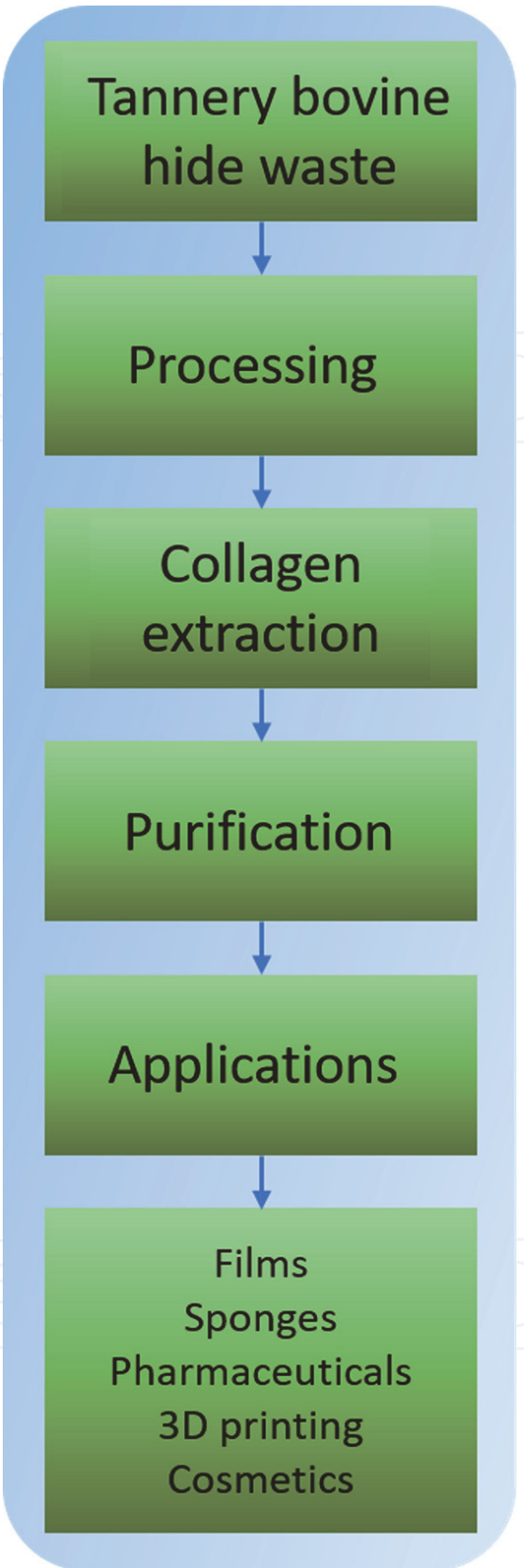


**Figure 1.**
*Process flow of waste valorisation from tanneries to collagen extraction and possible collagen-based applications.*

as fibres or sheets. A tropocollagen unit is about 285 kDa, 3000 Å in length and 15 Å in diameter. The triple helix is composed of repeating units of $(Gly\text{-}X\text{-}Y)_N$ amino acids, where X and Y are any amino acids, however, often X is proline and Y is hydroxyproline. The individual polypeptide chains of collagen each contain

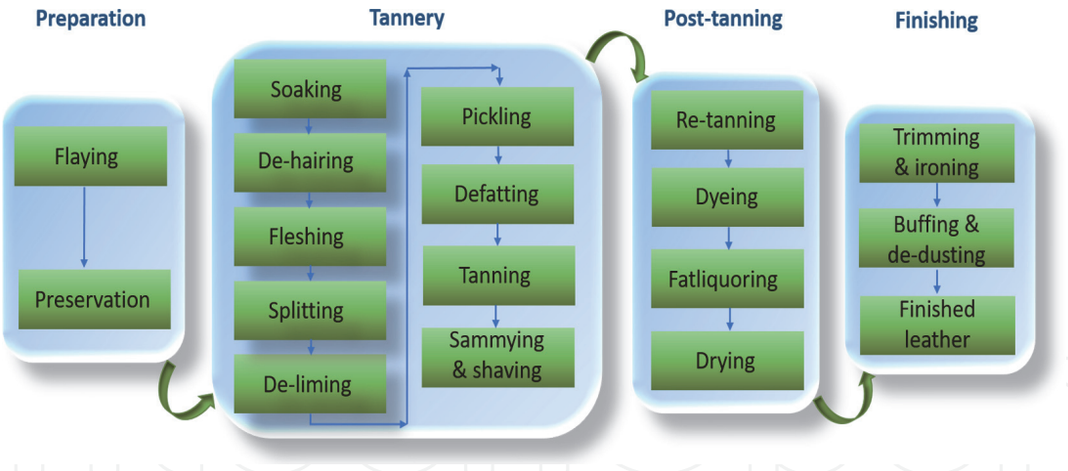


**Figure 2.**
*Process flow of transformation of hides into leather [12].*

| Tanning step | Chemicals | Wastes generated |
|---|---|---|
| Preservation | | |
| | Salt | Contaminated salt, raw hide trimmings |
| Soaking | | |
| | Water, surfactants, and enzymes | Salted and contaminated wastewater |
| De-hairing | | |
| | Water, sodium sulphide, and enzymes | Hair, alkaline water |
| Fleshing | | |
| | Water, mechanical processes | Flashings, alkaline water |
| Splitting | | |
| | Skin/hide | Limed hide |
| De-liming | | |
| | Water, ammonium sulphate and weak acids | Acidic wastewater |

**Table 1.**
*Chemicals used at each stage of hide to leather conversion and wastes generated [13].*

approximately 1000 amino acid residues. The accurate folding of these chains requires a glycine residue to be present in every third position of the polypeptide chain [1]. One-third of the amino acids in collagen in glycine and it always occupies the first position of the triplet. This is due to glycine being a small and an uncharged amino acid near the axis of the collagen triple helix. Glycine is a very crucial part of collagen molecule inherent characteristic as substitution of a single glycine for another amino acid disrupts the triple helix and results in skeletal deformities such as ontogenesis imperfect.

Imine acids make up approximately 25% of the residues in the collagen triple-helix. Imine acids – proline and hydroxyproline are typically found around the outside of the trip helix and the pentagon structure of these two amino acids includes the amine nitrogen and the α-carbon of the backbone chain. These limit the possible rotation in the amino acid (**Figure 1**) and hence forcing each collagen chain to form a left-handed helix. The high content of these imine acids makes the α-helix and β-sheet arrangements (generally found in proteins) unstable. Collagen triple-helix is held together by hydrogen bonding between chains. The NH group in

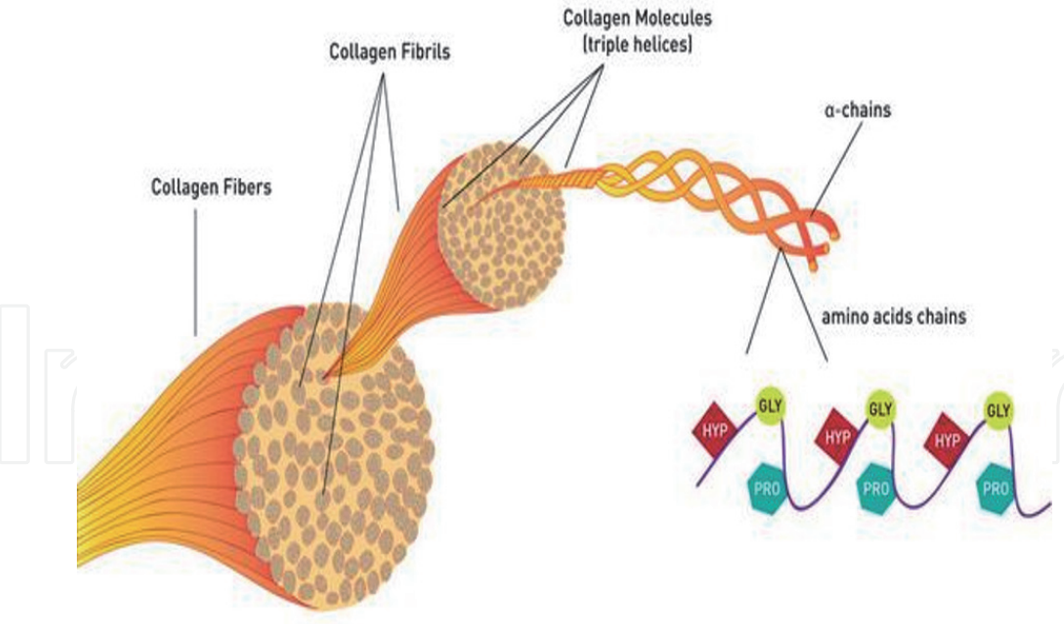


**Figure 3.**
*Collagen structure being broken down to fibre, fibril, triple helix and an alpha chain respectively [20] (used with permission).*

glycine in polypeptide chains forms H-bonds with adjacent peptide CO groups of the other chains.

After the formation of the collagen polypeptide chain, proline in the third position of the triplet in the amino acid sequence is hydroxylated by the enzyme propyl hydroxylase. The hydroxyl groups of the hydroxyproline and water molecules form hydrogen bonds that stabilise the triple-helix. Inhibition of hydroxylation causes diseases such as scurvy (caused by a lack of vitamin C in the diet) which is the inability of the triple-helix to form at body temperature (37°C) [18]. A decrease in imine acids (proline and hydroxyproline) content lowers the thermal stability of collagen as collagen loses its helical structure and shrinkage or denaturation occurs [18]. Avian and mammalian collagen have very similar amounts of hydroxyproline at 13.5% of the total amino acids. In comparison, aquatic animals have a lower level of hydroxyproline at approximately 10.3% [19].

The alpha-triple helix of collagen is shaped into a right-handed helix. The alpha chains each are shaped into a left-handed symmetry (the opposite direction), and then three of these alpha coiled strands get together to form a right-handed triple helix so when under strain, the chains twist into each other, giving strength and preventing unravelling. Each alpha helix is approximately 1.4 nanometres in diameter and 300 nanometres in length (approx. 1000 amino acids). The collagen molecule can be composed of either three identical alpha chains (homotrimers), or two or three different alpha chains (heterotrimers), however, the chain configuration depends on the collagen type being synthesised [2]. The hierarchical structure of collagen is zoomed-in starting from the alpha chains coiling together to form the triple helix is shown in **Figure 3**.

Cross-links that are covalent bonds occur between the ends tropocollagen before the formation of the collagen fibre. The triple helix and the cross-linking give rise to a collagen material that is very rigid, inextensible and stable. Since collagen on the primary level is composed of repeating units of Gly-X-Y amino acids, it is therefore rich in carboxylic acid groups, hydroxyl groups, amide and amine groups. The triple helix structure is stabilised by inter-chain hydrogen bonding and triple helix (tropocollagen) molecules parallel to each other are covalently cross-linked with each other through their aldehyde and amino groups, forming collagen fibrils. There are

multiple types of hydrogen bonding patterns found in the triple-helix. These include, i) direct hydrogen bonding among the peptides (i.e. the NH group in glycine in each polypeptide chain forms H-bonds with adjacent peptide CO groups of the other chains), ii) water-mediated hydrogen bonding linking carbonyl groups, and iii) water-mediated hydrogen bonding, which links hydroxyproline OH groups and carbonyl groups. Collagen self-organisation forms bundles or a meshwork that determines the tensile strength and the elasticity and geometry of the tissue.

The various collagen types are distinguished by the ability of their helical and non-helical regions to associate into fibrils and to form sheets or to cross-link different collagen types. For example, a two-dimensional network of type IV collagen is unique to the basal lamina. Most collagen is fibrillar and is composed of type I molecules (**Figure 4**) [2].

### 3.2 Collagen synthesis

Tropocollagen is produced by fibroblasts found in connective tissue in mammals and birds. The collagens α-chains are translated on the rough endoplasmic reticulum (RER). Inside the ER hydroxylation of the specific proline and lysine residues occurs, however lack of vitamin C will hinder this step. Inside the Golgi apparatus glycosylation of pro-α-chain lysine residues and formation of procollagen occurs. Procollagen molecules are exocytosed into extracellular space. The rest of the synthesis steps occur outside the fibroblasts. Procollagen peptidases cleave terminal regions of procollagen, transforming procollagen into insoluble tropocollagen. Many staggered tropocollagen molecules are reinforced by covalent lysine-hydroxylysine cross-linkage (by Lysyl oxidase) to make collagen fibrils. Lysyl oxidase requires copper (Cu++) for its activity [22].

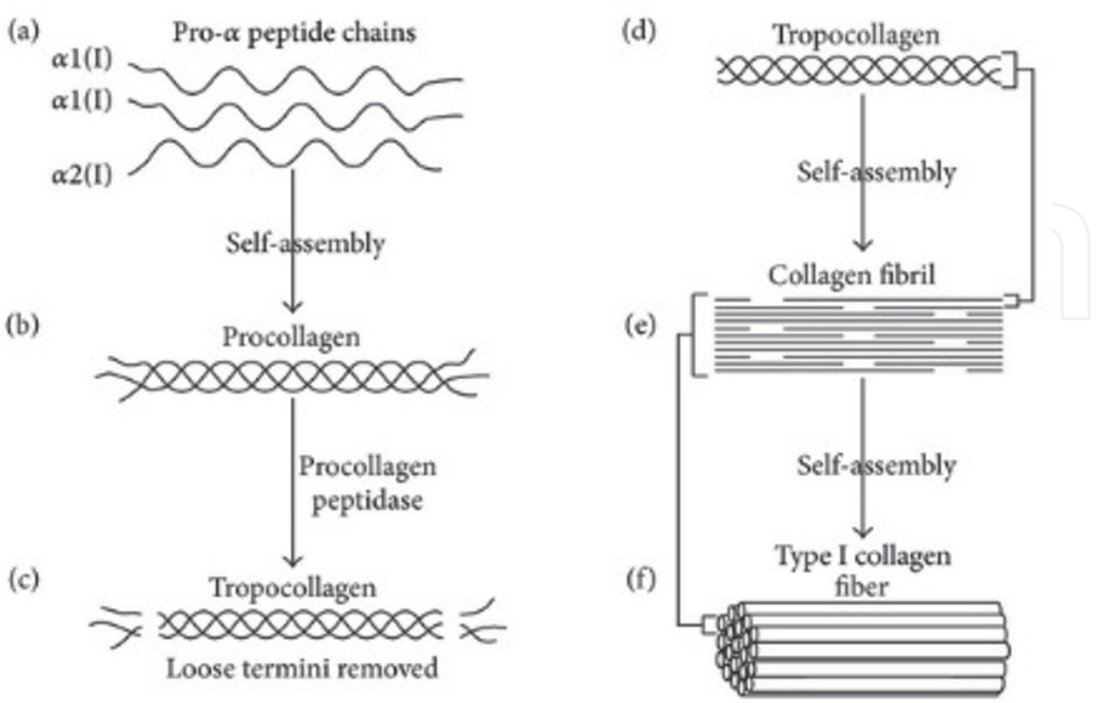


**Figure 4.**
*structure of collagen, with b) procollagen (loose ends), triple-helix wound together and c) collagen subunit tropocollagen (loose terminal removed) for final self-assemble of the collagen fibril and fibre (d-f) [21] (used with permission).*

### 3.3 Collagen fibres

The assembly of collagen fibrils into parallel bundles forms collagen fibres that have high strength and flexibility. When tropocollagen is assembled into collagen, it forms fibrous or sheet-like staggered structures. These fibrous structures have striations every 680 Å consisting of a dense-packed region where fibres overlap, and a loose-packed region is formed (**Figure 5**). In one single row, tropocollagen units are separated by 400 Å gaps, and these gaps are found in the loose-packed region. If the tropocollagen rows are aligned next to each other, each adjacent row is offset by 680 Å, forming a structure that repeats every five rows.

Hydrophobic and charged amino acid residues along the length of tropocollagen cause the staggered arrangement of tropocollagen. Tropocollagen units are aligned where the sum of the hydrophobic and charged region interaction between two units is strongest, hence the 680 Å staggering between units.

### 3.4 Collagen maturation

Inter-and intra-molecular covalent cross-links are formed between and within tropocollagen (collagen triple-helix) units giving strength to collagen fibres. Intra-molecular cross-links form between adjacent lysine groups and within individual triple-helix units and intermolecular cross-links occur between two triple-helix units comprising of two hydroxylysine groups and a lysine group.

The enzyme Lysyl oxidase converts the $NH_3^+$ group on the lysine and hydroxylysine sidechains to an aldehyde that then undergoes a condensation reaction forming an adol cross-link with other converted lysine sidechains. In each tropocollagen unit, four groups can contribute in the intermolecular cross-linking; lysines near the amino and carboxyl ends in the non-helical regions and hydroxylysines in the helical region. A hydroxyl-pyridinium cross-link is formed between one lysine and two hydroxylysine between residues near the amino-acid

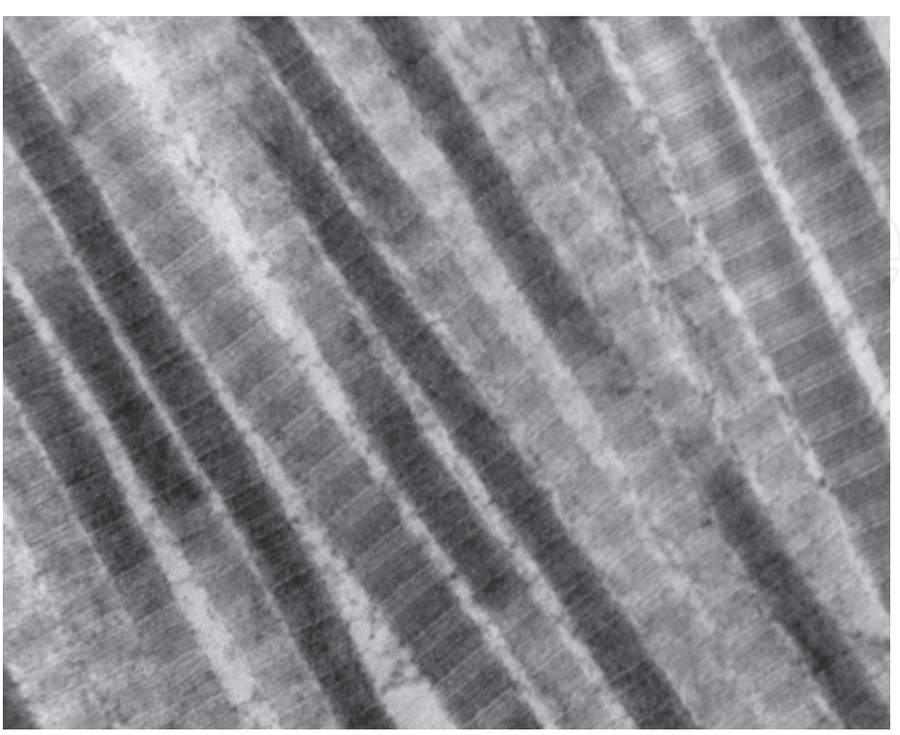

**Figure 5.**
*Collagen fibre showing the striations where tropocollagen is densely packed (light sections) [23] (used with permission).*

end of one tropocollagen unit and the residues near the carboxyl-end of an adjacent tropocollagen unit. The enzyme Lysyl oxidase is small enough to fit between the 400-Å gaps between the triple-helix molecules to initiate the intermolecular cross-linking.

Collagen maturity or the amount of cross-linking increases drastically with age of the tissue and depends on the type and function of the tissue where collagen is found.

### 3.5 Collagen types

Collagen has a wide range of structural roles in mammalian and aquatic tissue. It is the major constituent of skin, bone, tendon, cartilage, blood vessels and teeth. Collagen is found in almost every organ of the body, starting from skin to the cornea of the eye. To serve functions in such diverse tissues, there are different types of collagen that differ in how they interact with each other and with other tissue.

There are more than 28 types of collagen identified. Collagen types I, II, III are the most abundant and most investigated for various applications. However, over 90% of the collagen found in the body is type I. The variations are due to the differences in the assembly of basic polypeptide chains, different lengths of the helix, and differences in the terminations of the helical domains [24].

Each collagen molecule is composed of three different polypeptide chains (α1, α2, and α3). Each chain is identified by its amino acid composition (**Table 2**). Collagen type I, for example, is identified for its constitution of α1 (I) and/or α2 (I) chains. The most commonly occurring variant of type I collagen consists of two α1 (I) and one α2 (I) chain. The alpha symbol is used to indicate a single chain component seen after collagen denaturation and the letter β, γ, and δ have been used to indicate covalently linked dimers, trimers or tetramers of the alpha chain.

The most common types of collagen are:

- Collagen type I: found in skin, tendon, organs and bone tissues.
- Collagen type II: main component of cartilage.
- Collagen type III: the main component of reticular fibres, alongside type I.
- Collagen type IV: Forms the bases of the cell basement membrane.
- Collagen type V: the main component of cell surfaces, hair and placenta.

| Function | Description |
|---|---|
| Structural integrity | Collagens within the body serve largely for the maintenance and structural integrity of tissues and organs. |
| Entrapment and storage | The collagen within the body fulfils the role of entrapment, local storage, delivery of growth factors and cytokines and hence it plays an important role during organ development, wound healing and tissue repair. |
| Biodegradable | Collagen possesses the feature of being biodegradable and low immunogenicity. |
| Variety of applications | Collagen has been used in many industries, from the biomedical, cosmetic, pharmaceutical, leather, film industry to tissue engineering. |

**Table 2.**
*Collagen and its features [25].*

## 3.6 Collagen sources

As collagen is one of the most abundant proteins on earth, it can be extracted from various sources. Collagen can be extracted from almost every living animal, including alligators and kangaroos. However, common sources of collagen for the food industry and tissue engineering applications include bovine skin and tendons, porcine skin and rat-tail. Collagen can also be extracted from marine life; it can be extracted from sponges to fish and jellyfish. All collagen sources are worth investigating as each source differs in the collagen type in terms of characteristics.

### *3.6.1 Bovine collagen*

Collagen is extracted from many different sources; however, bovine collagen is seen to be the most used collagen type in a variety of different applications, such as the food industry, cosmetics, and medical applications. As the name implies, bovine collagen is a by-product of cows, mainly from the hides. It is a naturally occurring substance found in the skin, muscle, bones and tendons of cows. In the 1970s, the research on bovine collagen gained momentum, as researchers developed a system of extracting collagen and processing it in a liquid form [26].

The natural, unbleached skin and hair of cattle is the bovine hide (skin). Bovine hides are a by-product of the food industry from cattle. Bovine hides without complex processing can be manufactured into leather, which in turn can be used in the shoes and clothing industry. However further complex processing of the hides can be carried out to obtain the corium section of the hide for a variety of different medical and scientific applications [27]. One of the main applications of the corium is in the production of collagen.

Animal hide constitutes 60–65% water, 25–30% protein and 5–10% fats. The protein is mainly collagen [28]. Raw hides have four main parts; epidermis (6–10%), grain (less than 10%), corium (55–65%) and flesh and the thickness vary all over the animal (**Figure 6**) [29].

The epidermis and flesh layers are removed during tanning leaving the grain and corium layers. The grain is made up of collagen and elastin protein fibres. The corium is packed with collagen protein fibres. The thickness of corium also increases with age [30].

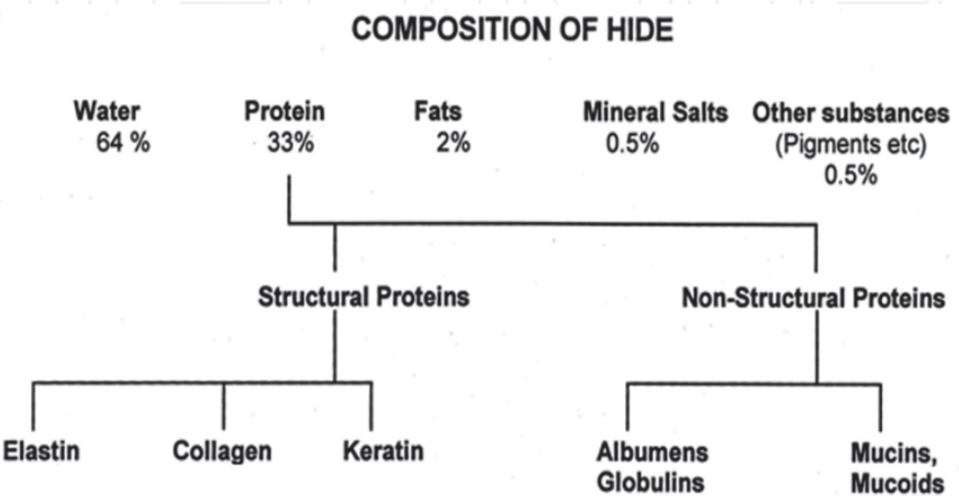


**Figure 6.**
*The approximate composition of bovine hide [28] (used with permission).*

*3.6.1.1 Properties of bovine hides*

Each section of the animal hide for its properties is discussed further [29] (**Figure 7**) [29]:

- *Epidermis:* There are two epidermis layers; one being the thin protective layer of cells during the life of the animal and the other being the flesh remain which is removed during tanning (leather production) by a process called liming.

- *Grain:* This layer is composed of elastin and collagen protein fibres. This layer is mainly used in the cosmetic industry for moisturisers and facial creams.

- *Corium:* The corium layer is made of collagen fibres, arranged in bundles and interwoven to give the structure strength, favourable elasticity and durability. Calf hides corium layer is thinner and smoother than the hides of mature animals; this is because the thickness of the corium increases with age.

*3.6.2 Collagen from fish*

Collagen from aquatic animals have been used as a safe substitute for bovine collagen, this is due to collagen from bovine sources have shown to be contaminated with some diseases. Fish solid wastes constitute 50–70% of the original raw material; however, this depends on the method of meat extraction [6].

Shark type I collagen forms fibrils under different conditions compared to bovine and porcine collagen [32]. For example, shark type I collagen gels and membranes have stronger rigidity and higher affinity to water vapour than those of porcine collagen, thus indicating the potential for utilising shark collagen as a new type I collagen material for various uses such as cell culture and medical technology [33].

*3.6.3 Porcine collagen*

Pigskin is a by-product of the pork production industry. Collagen extracted from pigskin or bone is not favourable to be a component of foods or pharmaceuticals

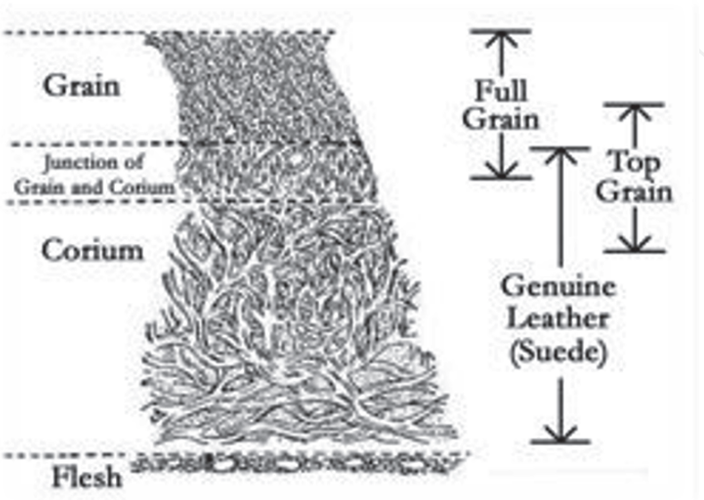


**Figure 7.**
*Structure of bovine hide [31] (used with permission).*

due to religious objections. Porcine collagen type I is extracted from pig hides, and in the medical field. Porcine collagen sheet material has proven to be useful as an implant for reconstructive surgery [34].

## 4. Collagen market and its applications

### 4.1 Collagen industries

There are many collagen-producing companies in around the world. However, not all of them produce 100% pure collagen but rather gelatine (hydrolysed collagen). These companies lack further innovation with the collagen, thus distributing the collagen in powder or liquid form to pharmaceutical and research industries. Therefore, extracting collagen from bovine hides and using this collagen to investigate high value applications would possibly generate huge economic potential for a product that is derived of waste materials.

Collagen plays an important role both in the mammalian and the non-mammalian body and in its extracted form. Due to collagen's high mechanical strength, it finds applications in several different industries, ranging from biomedical to the food industries.

#### *4.1.1 Gelita*

Gelita is the world's leading supplier of hydrolysed collagen proteins for the food, health and pharmaceutical industries. Gelita is based in numerous locations around the world with its headquarters in Germany [35]. However, the collagen Gelita produces is not 100% native collagen but hydrolysed collagen, in other terms it is gelatine.

#### *4.1.2 Southern lights biomaterials*

Based in Napier New Zealand, Southern Lights Biomaterials was founded in 2003. They provide high-quality processed and semi-processed biomaterials to medical device manufacturers across the globe. One of their flagships processed products is polymeric collagen, which is delivered to contracted customers [36].

The polymeric collagen produced by Southern Lights Biomaterials is type I collagen derived from bovine tendon and is naturally cross-linked [36]. They do not take advantage of using cattle hides or face-pieces. Their collagen is sold to independent contractors without further processing.

#### *4.1.3 Revolution fibres*

Revolution Fibres produce and market nano-fibre and nano-fibre products. Based in Auckland New Zealand, Revolution Fibres has developed its own technology for the industrial production of nano-fibre. This technology is called electrospinning [37]. Revolution Fibres manufacture biodegradable air filters from nano-particle sized fibres that are 'electro-spun' from collagen extracted from Hoki fish skins. They have launched a skincare range using collagen fibres to deliver plant extracts into the skin [38].

#### *4.1.4 Waitaki biosciences*

Waitaki Biosciences based in Christchurch New Zealand manufactures speciality nutritional supplement ingredients from natural, biological sources. Waitaki

Biosciences aims to target joint and bone health, immune and digestive support, along with skin and hair care. Marine collagen, natural collagen and chondroitin complex are some of their products [39]. The marine collagen produced by Waitaki Biosciences is in powder form, with a blend of ingredients selected from marine species. This marine collagen is designed for use as an oral supplement to support skin, nail and hair health [40].

Observing the collagen suppliers in New Zealand, there is a clear shortage in further innovation with the extracted collagen. Most of the above collagen suppliers distribute the collagen in a powder form or a liquid solution and export to external markets or distribute to local contractors. This collagen once supplied to contractors is usually blended in cosmetic products or encapsulated as pills in the pharmaceutical industry.

## 4.2 Collagen applications

Collagen has been widely used in a range of applications in cosmetic, biomedical, pharmaceutical, film industries, tissue engineering and recently in 3D/bio-printing.

### *4.2.1 Biomedical uses of collagen*

i. Collagen sponges

The collagen sponges act as a biological absorbance material. They have been useful in the treatment of severe burns and as a dressing for pressure sores, leg ulcers and donor sites. Collagen sponges can absorb large quantities of tissue exudate, smooth adherence to the wet wound bed with preservation of low moist climate as well as shielding against mechanical harm and bacterial infection [41].

Collagen sponges have also been found to be effective as drug delivery systems. For example, the collagen sponges were found to be suitable for short term delivery of antibiotics, such as gentamicin [42].

ii. Collagen shields

Originally, collagen shields were designed for bandage contact lenses. However, it's mostly used as a delivery device and has led to the development of drug delivery systems for ophthalmic applications [43]. For example, the collagen corneal shield is produced from porcine sclera tissue that closely resembles collagen molecules of the human eye. The collagen corneal shield promotes epithelial healing after corneal transplantation [44].

iii. Collagen mini pellets

A mini pellet made from collagen is usually a rod with a diameter and length of 1 mm and 1 cm respectively. These are very useful as a drug delivery device. This is because the mini pellet (rod) is small enough to be injected into the subcutaneous space through a syringe needle and still spacious enough to contain large molecular weight protein drugs, such as interferon [42].

iv. Skin replacement

Collagen has been widely used as vehicles for transportation of cultured skin cells or drug carrier for skin replacement and burn wounds [45]. Type

I collagen is suitable for skin replacement and burn wounds due to their mechanical strength and biocompatibility [7].

v. Bone substitutes

Collagen has been previously used as implantable carriers for bone inducing proteins [41]. Due to osteo-inductive activity; collagen itself has recently been used as bone substitutes [42]. Collagen combined with other polymers has been used for orthopaedic defects. Demineralised bone collagen in combination with hydroxyapatite was used as a bone graft material to treat acquired and congenital orthopaedic defects in rats [46].

vi. 3D printing and collagen

3D printing is the process of converting digital designs to three-dimensional solid objects. 3D printing works by initially designing a 3D image of the desired object, with computer-aided design (CAD) [47]. The object is divided into digital cross-sections by the program so that the printer can build the object layer-by-layer. Once the specified design is sent to the 3D printer, a specific material can be chosen. Depending on the printer type, this material can be rubber, plastics, paper, metals and more [48]. However, in the case of bio-printing; bio-ink (cells) and bio-paper (collagen, nutrients) are required [49].

*4.2.2 Collagen and rheumatoid arthritis and osteoarthritis*

Collagen has shown to have positive effects on rheumatoid arthritis and osteo-arthritis [50]. Published studies [51] have reported that ingestion of type II collagen relieves joint discomfort associated with osteoarthritis and rheumatoid arthritis. The authors also conducted a randomised trial involving 60 patients with severe active rheumatoid arthritis; a decrease in the number of swollen joints and tender joints occurred in subjects fed with type II collagen [51].

*4.2.3 Cosmetic applications of collagen*

Collagen has great tensile strength and being rich in proline and hydroxyproline, it is the main component of fascia, cartilage, ligaments, tendons, bone and skin. Having these properties, it is responsible for skin strength and elasticity. Its degradation leads to wrinkles that accompany ageing. Collagen has become a valuable ingredient of many cosmetic formulations. Cosmetic uses include skin and hair products. Collagen type III is predominant in young skin; it is referred to as "restructuring" collagen as it appears during the wound healing process [7]. With ageing collagen type III decreases leading to wrinkles and lines, thus moisturising creams and cosmetic injects containing collagen have become in high demand [52].

Bovine collagen has been the most widely used source for cosmetic applications. Recently, collagen from other sources such as fish skin, pigskin, and range of cattle skin has been used in the cosmetics industry. However, collagens from various sources differ in their physiochemical properties. For example, they all have different thermal stabilities, and this can affect the formulation or the shelf life of the products [3].

*4.2.4 Collagen films*

Thin films or biodegradable films are flexible, transparent and often strong materials derived from natural polymers such as whey protein, collagen, starch,

gelatine and many other natural renewable polymers [53, 54]. Due to rising environmental concerns, biodegradable films have attracted considerable attention especially from the food and drug packaging industries as they in constitution with other natural polymers can potentially replace plastic films which are derived from synthetic polymers [55].

Due to collagen being a biodegradable, biocompatible and a non-toxic polymer it has been used in the meat industry to form edible films and coatings through extrusion [56]. Collagen-based films in constitution with other biodegradable materials have been prepared in several studies to be used as packaging materials. Collagen's high tensile strength and the added advantage of biodegradability makes it an ideal agent for natural polymer films.

One of the main applications of collagen films in the biomedical industry is as a barrier membrane. These collagen films have been used for slow-release drug delivery and they have been used for the treatment of tissue infection, such as infected corneal tissue or liver cancer [42].

Edible films and coatings are a category of packaging materials. They differ from other bio-based packaging materials, and conventional packaging, by being formed from edible ingredients. These films and coatings may be used to reduce the amount of synthetic packaging used in a product or allow conversion from a multi-layer, multi-component packaging material to a single component material. The purpose of edible films and coatings may be to inhibit migration of moisture, oxygen, carbon dioxide and or to improve the mechanical integrity or handling characteristics of the food. Edible films may also be used to separate different components in multi-component foods, thereby improving the quality of the product. Edible films may also help to maintain food quality by preventing moisture and aroma uptake or loss after opening of the synthetic packaging.

The use of natural polymers such as collagen for film preparation has many advantages over synthetic and petroleum-based polymers. Biopolymer films for the purpose of packaging materials have the advantages of biodegradability, renewability, and environmental compatibility. Collagen also has good film-forming properties, high tensile strength, good thermal stability, and the fact that the collagen is derived from waste hide off-cuttings presents a sustainable solution. One drawback of collagen-based films is the inflexibility of films. However, this can be overcome by the addition of plasticizers to improve the flexibility and elongation (%) properties of the films. The use of plasticizers has been shown to provide improvement of films in terms of flexibility and elongation; however, this is generally at the expense of strength and stiffness. The effect of plasticiser concentration should, therefore, be investigated to identify best concentration results in the optimum mechanical, thermal and physical properties.

Biopolymer films made for the food industry as coatings or packaging needs to be transparent, have desirable tensile strength and elongation, it should be edible and possibly have a high resistance to transmission of liquids, gases and fats and oils. However, the above criteria will vary depending on the food industry application of the film.

Sionkowska et al. [57] prepared biopolymer films based on blends of collagen and silk fibroin. Films were prepared by method solution casting and characterised for their mechanical properties and structure. Film blends of collagen and silk fibroin showed better mechanical properties than for pure silk fibroin films. Sionkowska et al. [57] concluded that the better mechanical properties of the blend films were due to molecular interactions between collagen and silk fibroin. No plasticizing agent was added in the preparation of collagen and silk fibroin blend films. This would result in a very brittle and stiff film due to interactions between protein chains through hydrogen bonding, electrostatic forces and hydrophobic

interaction [58]. Hence the per cent elongation values of the film blends were very low (0.30–5.10%) [57].

Not all collagen extraction methods result in a collagen product that will be suitable for film preparation. Hence, to develop a collagen film with desired properties, it is necessary to investigate the various processes to prepare acid/alkaline/enzyme/acid-enzyme collagen that could easily be used as a raw material for extruded or casting of collagen-based films. O'Sullivan [6] reported that hydrochloric acid solubilisation extraction method of collagen is not favourable for the fabrication of edible films. However, acetic acid solubilisation with further processing gave a suitable collagen product as a raw material for the fabrication of edible film fabrication.

## 5. Methods used to extract collagen from bovine hides

### 5.1 General extraction procedure requirements

Every bovine collagen extraction procedure is restricted to the following four variable conditions:

- De-hairing, cleaning and storage of the hide section off-cutting.
- Cutting the de-haired hide section into approximately 1 cm x 1 cm pieces.
- Extraction temperature: For bovine tissue, the extraction procedure can be carried out at room temperature, as collagen denaturation temperature for bovine is ~39°C. However, it is preferable to extract collagen at a temperature of ~4°C to prevent contamination.
- Solubilisation: acid solubilisation, acid and enzyme solubilisation, or modified methods combining acids and enzymes.

Prior to collagen extraction, the sample is chopped to increase the extraction surface area and to speed up the extraction process. However, the temperature of the sample needs to be monitored, as high temperatures will unravel the tropocollagen making it soluble in solution, resulting in gelatine (denatured collagen). This greatly reduces the value of the protein, thus if native collagen is desired, any heating or denaturation of collagen should be avoided at every step of the process. Bovine collagen extraction is mostly carried out at temperatures of approximately 4°C to prevent bacterial contamination [9].

Collagen from juvenile sources (e.g. new-born calves or chicken embryos) will readily swell and dissolve in a low concentration of acetic acid solution and can be recovered by precipitating out the collagen by adding 1 to 5 M NaCl. However different types of collagen from different tissues will precipitate at different NaCl concentrations [59].

The older the animal/tissue sample, the greater the amount of lysine-hydroxylysine covalent cross-links that form between tropocollagen units. These cross-links typically form between the unwound part of a tropocollagen strand and another part of another tropocollagen unit, improving structural strength and chemical resistance of collagen, making the sample largely insoluble in acetic acid. The amount of cross-linking depends on the type of tissue (i.e. tendons are highly cross-linked to give strength) and age of the tissue (i.e. mature sources, such as bull-hides have high cross-linking in comparison to younger sources such as calf-hides) [60].

In order to dissolve mature collagen, pepsin enzyme can be added to the acetic acid solution, which attacks and cleaves the unwounded part of tropocollagen, allowing the tropocollagen units to separate and dissolve [59].

The following sub-sections discuss the main extraction steps/parameters or variables in more detail.

### 5.2 Temperature control

To prevent collagen denaturation and contamination, majority of the researchers carry out the collagen extraction process at approximately 4°C. Contamination occurs due to thermal denaturation or microbial degradation (**Table 3**).

### 5.3 Fat removal and demineralisation

Once the collagen source is de-haired, sized and cleaned it is then processed for defatting. Majority of collagen extraction processes defat the tissue of interest with an organic solvent or detergent prior to extraction (**Tables 4** and **5**).

### 5.4 Non-collagenous protein removal

Contaminating proteins need to be removed after defatting and demineralization. Most collagen extraction methods utilise salt or alkali solutions to solubilise the contaminants. Collagen is a lot more chemically resistant than most other proteins therefore, it is much less likely to be degraded or solubilised by a weak salt (**Table 6**).

| Collagen source | Temperature (°C) | Reference |
|---|---|---|
| Bovine | 4 °C | [14, 61–65] |
| Fish | 4–9°C | [61, 62, 66–72] |

**Table 3.**
*Processing temperatures used to extract collagen.*

| Source | Solvent | Reference |
|---|---|---|
| Bovine | Acetone | [19, 65] |
| Fish | 0.5% detergent | [69, 73] |
| | 10% butyl alcohol | [72, 74] |
| | 15% Butyl Alcohol | [75] |

**Table 4.**
*Solvents used for de-fatting of collagenous tissue in literature.*

| Collagen source | Chemical | Reference |
|---|---|---|
| Bovine | 0.5% HCl | [64] |
| | 0.5 M EDTA | [76] |
| Fish | 0.5 M EDTA | [74] |

**Table 5.**
*Chemicals used for demineralisation in literature.*

| Collagen source | Chemical | Reference |
|---|---|---|
| Bovine | 0.5 M NaCl | [77] |
| | 1 M NaCl | [78] |
| | $K_2HPO_4$ $^-$ | [79] |
| | 0.1 M NaOH | [65] |
| Fish | 0.1 M NaOH | [70–74] |

**Table 6.**
*Chemicals used for non-collagenous protein removal in literature.*

## 6. Possible collagen extraction methods

There are various methods to extract collagen from different animal tissues. The methods used to extract collagen from bovine or any other tissue such as fish skin; pigskin, rat tail, tendons etc. vary slightly, differing in enzyme concentration, acid concentration, salt concentration or pre-treatment period [6]. These variations can be studied and the most optimal method for bovine hide extraction can be obtained. However, acid extraction which results in acid-soluble collagen (ASC), pepsin extraction that gives pepsin solubilised collagen (PSC) and salt extractions. Some of the main extraction procedures found in literature are discussed in detail below [80].

### 6.1 The salting-out method

This method is seen as the least favourable method of collagen extraction. Collagen proteins, like general proteins have the property of being salt soluble. Different types of collagen proteins can be separated using the relationship between different collagen sources and salt concentrations. Neutral salt solutions are usually used, such as NaCl, Tris–HCl, phosphate, or citrate. In the salting-out method, the concentration of salt is the key factor to control, if for example, the concentration of NaCl is less than 1 mol/L in the neutral solution, its suitable for dissolution of type I collagen, however, if the concentration is bigger than 1 mol/L, it will precipitate the type I collagen. Since mature sources of collagen are less soluble because most collagen protein molecules have cross-linked, the salting-out method is not an efficient method alone to extract collagen [80].

### 6.2 The alkali method

The main chemicals used in the alkali method of collagen extraction are sodium hydroxide and monomethylamine [81]. This extraction method is not favoured as the main extraction method due to similar reasons as the salting-out method.

Hattori et al. [81] prepared collagen from bovine hides by alkaline solubilisation with 3.0% NaOH and 1.9% monomethylamine. The study also extracted bovine hide collagen by acid and enzymatic methods for comparison. These methods were carried out on animals of different ages. The amount of collagen extracted through this method was estimated by comparing the hydroxyproline content in the whole hide with that in the extracted collagen.

### 6.3 The alkali-enzyme method

The alkali-enzyme method is not as effective as the acid-enzyme method. This is because alkali is such as NaOH does not have the ability to fully solubilise collagen

and disrupt the cross-linking in a collagen molecule. This method is more preferred for gelatine production [80].

### 6.4 The acid-alkali oxidation method

A series of repetitive steps having acid then alkali soaking of samples for a long period can be used to extract collagen. However, this method requires a very long period and the reaction time is very slow. It does not work for mature tissues as it is near impossible for acid and alkali alone to disrupt the cross-linking developed in mature tissue, thus an enzyme is a must requirement. The collagen yield extracted decrease or increase for the same tissue type depending on the literature. These differences are due to denaturation of protein during the process of extraction, the difference in environmental temperature and the solubilisation method used to extract the collagen.

The yield of collagen by the different acid (HCl, citric acid, acetic acid) is dependent on the reaction time. The longer the period of solubilisation, the greater the yield of collagen being extracted. For example, Skierka [82], concluded that during a 24 hour of collagen extraction in acid, about 33% of collagen was solubilised, and after 72 hours, about twice as much collagen was solubilised.

The solubility of collagen in acids depends up the enzyme concentration. A low concentration of enzyme with an acid can completely solubilise collagen; however, it will also depend on the type of acid. For example, enzyme concentration on the solubility of collagen in citric acid and HCl gave a maximum yield of 75% for citric acid and 85% for HCl [82].

### 6.5 The acid method

Acids such as acetic acid, citric acid and hydrochloric acid (HCl) of low concentration can be added to collagen-containing samples. Acids at a pH of 2–3 and a concentration of approximately 0.5 mol/L can be used to solubilise collagen. In acid extraction of collagen, the acids swell collagen, disrupting the hydrophobic and electrostatic interactions between the tropocollagen units, and release the acid-soluble collagen (ASC). Yang et al. [80] concluded that citric acid has the best effect to extract collagen, second being acetic acid and last being hydrochloric acid. However, according to Skierka [82] and Higham [83], the most effective acid for collagen solubilisation was acetic acid and the least effective solvent was HCl. In order to achieve a sound conclusion, experiments need to be carried out to investigate the solubilisation efficiencies of each acid.

The acid molecules disrupt the collagen cross-linking in order to solubilise the collagen by allowing ligand substitution for each peptide side chain, causing disassociation of the cross-link. Thus, swelling the collagen and solubilising it out of the tissue and into solution [73].

The acid method is seen to be corrosive to the experimental equipment in terms of large-scale production. However, using a low concentration of acid in combination with an enzyme will avoid equipment corrosion and achieve a high yield product (**Table 7**).

### 6.6 The enzyme method

The enzyme method is seen to be as the ideal method of collagen extraction. The three commonly used enzymes for collagen extraction are pepsin, papain and tryptase [80]. The enzyme acts on the non-helical peptide chains of the collagen protein, having no effect on the helix peptide chains of the collagen protein. The enzyme has better reaction selectivity and it is less destructive to the collagen

| Collagen source | Acid type and concentration | Reference |
|---|---|---|
| Bovine | 0.5 M acetic acid | [14, 62, 81, 84–87] |
| | 10% acetic acid with 0.2% chlorhydric acid | [88] |
| Fish | 0.5 M acetic acid | [62, 66, 67, 69, 70] |
| | 0.15 M HCl | [73, 82] |
| | Citric acid | [73, 82] |

**Table 7.**
*Acids used for collagen extraction.*

| Collagen source | Enzyme type and conc. | Reference |
|---|---|---|
| Bovine | 1% Trypsin | [90] |
| | Pepsin | [62, 86–88] |
| Fish | 1% (w/w) pepsin | [62, 66, 69] |

**Table 8.**
*Enzyme extraction methods used for collagen extraction.*

protein, resulting in a protein whose triple helix structure is better preserved. Thus, the extracted collagen will have a better purity, and retain stable physical and chemical properties. The enzyme method also provides mild reaction conditions that avoid equipment corrosion and less energy consumption. However, reaction time may be long, depending on the type of enzyme used [89].

The enzyme solubilisation method works by disrupting the cross-linking that occurs in collagen. The chosen enzyme cleaves to the amino telopeptides from the tropocollagen molecule thus disrupting the cross-linking and allowing solubilisation of the collagen molecule. Enzyme solubilisation is mostly required when extracting collagen from mature tissue, this is due to the cross-links forming keto-imines which are increasingly difficult to disrupt as they contain strong intermolecular bonds. However, the enzyme method has the disadvantage of not only breaking the collagen molecule but also resulting in the scission of other proteins may occur too, hence, causing protein contamination as a result [83]. Enzymes have been used in collagen extraction, McClain et al. [78] used papain at 0.1% in buffers containing 0.02 M phosphate and 0.003 M EDTA as a solubilisation method for collagen.

The enzyme method is usually combined with the acid method to enhance the extraction process (**Table 8**).

### 6.7 The acid-enzyme method

The enzyme-acid solubilisation method is seen to be the most effective way to extract collagen. Both acids (citric acid, hydrochloric acid, acetic acid) and enzymes have the capability to disrupt the cross-links in a collagen molecule and make collagen soluble in solution. Addition of both an acid and an enzyme speed up the reaction time and results in a collagen protein well-kept in its triple helix structure [80]. Concentration and acid/enzyme type greatly depend on the collagen tissue and method optimization.

## 7. Collagen purification via dialysis and filtration

### 7.1 Collagen purification via dialysis

Dialysis is a preferred method of purification for collagen extraction, however, scaling up this technique for commercialisation has proven to be difficult. Dialysis

tubes are utilised with different cut off molecular weights to separate pure collagen from other solvents, salts and enzymes and other impurities.

### 7.2 Collagen purification via filtration

Ultrafiltration can be applied to remove the non-collagenous material prior to lyophilisation of collagen. Ultrafiltration utilises positive pressure to force a liquid through a semi-permeable membrane to separate species in an aqueous solution by molecular size, shape or charge. Ultrafiltration has the advantages of having a high throughput, cost-effective and large-scale purification is possible without being limited to lab-scale purification by dialysis.

Ultrafiltration enables the removal of solvents and salts of lower molecular weight from a solution (permeate). Thus, this results in the enrichment of the retained molecule (pure collagen). Ultrafiltration membranes can retain molecules in the range of 10 kDa to 1 MDa, thus concentration and purification of collagen (300 kDa) can be successfully achieved through this process.

- Crossflow/tangential flow filtration: the incoming feed passes parallel across the surface of a semi-permeable membrane. A permeate and a retentate stream are generated, where the permeate is the portion of the fluid that passes through the membrane and the remainder of the feed stream, which does not pass through the cross-flow membrane, is known as the retentate stream.

- Dead-end filtration: The feed moves towards the filter membrane. The particles that can be filtered are settled on the filter surface, however, this type of filtration is not sustainable as the accumulated solids need to be removed periodically or the filter needs to be replaced.

## 8. Collagen preservation

In order to preserve extracted collagen, it is usually freeze-dried and stored at conditions not exceeding −4°C. However, some researchers use hydrogen peroxide (0.3–3%) to disinfect collagen after extraction especially from fish sources.

## 9. Collagen extraction research and its applications

The popularity of collagen extraction continues to increase due to many reasons. It is a high strength protein, bio-derived, has excellent biocompatibility, biodegradability, and has weak antigenicity. Another main reason that relates to waste valorisation and sustainability is the fact that collagen can be extracted from almost any mammalian skin, bones, cartilage, fish skin and even chicken feet. Most often, the meat industry results in these by-products that can end up in landfill. These advantageous characteristics have made collagen one of the most useful biomaterials.

Research to this day is being carried out to improve extraction methods in terms of efficiency and economics. In addition to improving extraction methodologies, research is being carried out on collagen to enhance its use in several industries (**Table 9**).

| Period | Collagen extraction research |
|---|---|
| **1960–1969** | • Bakerman [91] extracted human skin collagen with age via the acid-solubilisation method. No defatting or demineralization steps were carried out, citric acid was used as the solubilisation agent. There was no mention of methods of collagen content analysis, only extracted yield was reported. |

| Period | Collagen extraction research |
|---|---|
| | • In 1968 Rigby [92] analysed the amino acid composition and thermal stability of ice-fish skin. The fish skin was swollen in 0.1 M HCl for extraction purposes. Td was found to be in the range 5.5–6°C.<br>• Young et al. [93] extracted cod skin collagen with mild solvents in the pH range of 3.4–8.7 at 3–90°C.<br>• Grant et al. [94] studied the carbohydrate content of bovine collagen. It was shown that crude preparations of collagen were contaminated with mannose, fucose and hexosamine.<br>• Bronstein et al. [95] studied human collagen and the relation between intra and intermolecular cross-linking.<br>• Miller et al. [96] extracted and characterised chick bone collagen with acetic acid. Specific methodology is not given, and the focus of this study was to understand collagen compositional changes with ageing via chromatography.<br>During this period, research was mainly carried out to understand collagen as a protein. There was no research on method optimization or investigation of different extraction methodologies. |
| **1970–1979** | • Anderson et al. [97] extracted bovine nasal collagen with 4 M guanidinium chloride or 1.9 M $CaCl_2$ and examined the structure by studying their scanning electron microscopy images.<br>• Pierson et al. [98] studied the effect of post-mortem ageing, time and temperature on pH, tenderness and soluble collagen fractions in bovine Longissimus muscle. Salt and acid-soluble collagen were not affected by temperature nor length of post-mortem ageing.<br>• Maekawa et al. [99] extracted collagen from the skin of mice. Extraction was carried out with 0.5 M acetic acid at 40°C for different times.<br>• Francis et al. [100] extracted collagen from biopsies of human skin. The study concluded to show that polymeric collagen of normal and diseased human skin from biopsies was feasible.<br>• Uitto et al. [101] analysed the solubility of skin collagen in normal human subjects and in patients with generalised scleroderma. Extraction was carried out with 0.14 M NaCl and number of extractions were varied.<br>• In 1976, Trelstad et al. [102] applied differential separation to separate native collagen types I, II, and III. The main precipitants were ammonium sulfate, sodium chloride and ethanol.<br>• Riemschneider et al. [103] extracted collagen from cow placenta via pepsin solubilisation.<br>The focus of collagen extraction in this period remained to be for medical purposes. The main sources of collagen were of human skin and rat skin. |
| **1980–1989** | • Merkel et al. [104] studied the content of type I and III collagen of healing wounds in fatal and adult rats. Collagen was extracted with 0.5 M acetic acid with pepsin. Collagen content ratio was estimated from densitometer scans of electrophoretically separated α-chains.<br>• Graham et al. [105] extracted and quantified types of collagen both in control intestine and as well as in both in inflamed and structured intestine resected from patients with Crohn's disease. This study mainly focused on differences in collagen types between the controlled and both inflamed and structured intestines.<br>• Murata et al. [106] studied the changes in collagen types in various layers of the human aorta and their changes with the atherosclerotic process. Collagen from human aortas was extracted by repeated pepsin digestion and the collagen types were identified by SDS-PAGE analysis.<br>• Elstow et al. [107] extracted and characterised type V foetal calf skin collagen. Neutral salt solutions (pH 9.2) with phosphate-buffered saline (PBS) were used to extract collagen and SDS-PAGE analysis was used to characterise and identify type V collagen.<br>• Laurent et al. [108] showed a simplified method for quantification of the relative amounts of type I and type III collagen in rabbit lung samples. This extracted involved repetitive homogenisation of the collagenous tissue in 2% sodium dodecyl sulfate and dried acetone powder.<br>• Van Amerongen et al. [109] analysed the concentration and extractability of collagen in human dental pulp. Premolar and third molar dental pulps were studied for their |

| Period | Collagen extraction research |
|---|---|
| | collagen content and acetic acid or neutral salts were used to extract collagen. Using SDS-PAGE analysis, 42.6% of extracted collagen to be type III.<br>• Kurita et al. [110] analysed the changes in collagen types during the healing of rabbit tooth extraction wounds. Collagen type was identified by use of SDS-PAGE analysis and hydroxyproline analysis was applied to observe collagen content.<br>Collagen quantification and understanding types of collagen present in diseased tissue vs. normal human tissue was the focus of collagen research in this period. |
| **1990–1999** | • Montero et al. [111] extracted collagen from Plaice skin and analysed its functional properties. Acetic acid was the main solubilising agent in this study and homogenisation was carried out with 0.4 M NaCl.<br>• Ambrose et al. [112] extracted and characterised collagen from bone and teeth for isotopic analysis. Carbon to nitrogen ratios of bone and teeth collagen was analysed the use of purification procedures that removed acid and alkaline-soluble contaminants were recommended.<br>• Nomura et al. [113] extracted and analysed properties of type I collagen from fish scales. Collagen was extracted with 0.5 M acetic acid and it was concluded that a large portion (80% of collagen remained insoluble which was further denatured to gelatine to be used for food purposes.<br>• Ciarlo et al. [114] extracted collagen from hake skin. Acetic acid was used for solubilisation and collagen was characterised for its viscosity and collagen type (SDS-PAGE).<br>• Bishop et al. [115] extracted and characterised collagen types II and IX from bovine vitreous. Centrifugation and precipitation with 4.5 M NaCl were applied and the collagen types were identified.<br>In this era, extraction of collagen from waste materials such as fish skin began, however, the methodologies were mainly focused on salt extractions, which is not very efficient, and the study of collagen in human tissue was still predominant. |
| **2000–2009** | • In 2007, Nalinanon et al. [59] extracted collagen from the skin of bigeye snapper using pepsin. Acid-extracted collagen resulted in lower collagen yields in comparison to pepsin-solubilised collagen.<br>• Woo et al. [116] extracted collagen from yellowfin tuna skin. Methodology was optimised by varying NaOH concentration, treatment time and pepsin concentration. The objective of this study was to determine the optimum conditions for extracting collagen from yellowfin tuna skin and characterisation was carried out by SDS-PAGE, FTIR, and solubility analysis.<br>• In 2003, Sadowska et al. [73] isolated collagen from the skin of Baltic cod. The aim of this investigation was also to determine optimum conditions for the extraction of collagen from cod skin. Acetic acid and citric acid were used as the main collagen solvents and within the two solvent extractions, time of treatment and digestion were varied.<br>• Jongjareonrak et al. [117] extracted and characterised collagen from bigeye snapper. Acid and pepsin solubilised collagen were isolated and characterised for their properties. The Td of the acid-solubilised and pepsin solubilised collagen varied slightly with, pepsin solubilised collagen having a higher Td (30.87°C).<br>• Zhang et al. [69] extracted and characterised collagen from the skin of grass carp via the method of pepsin-solubilisation. A collagen yield of 46.6% (dry-basis) was obtained and SDS-PAGE showed that the extracted collagen was type I and collagen Td was found to be 24.6°C.<br>• Nalinanon et al. [118] used pepsin from the stomach of tuna fish to use it for collagen extraction of threadfin bream skin. Pepsin from different tuna species were obtained to use for collagen extraction and to determine the differences in collagen extraction efficiency.<br>• Cao et al. [119] extracted and characterised type II collagen from chick sternal cartilage. Pepsin was the main solubilising agent in this study and NaCl was used for collagen precipitation. SDS-PAGE confirmed the presence of collagen type II and the amino acid composition of the type II collagen extracted was very close to the reference collagen type II obtained from Sigma Aldrich.<br>In this period, a vast number of different fish species were analysed for their collagen extractability. The main reason for this was due to adding value to waste fish skin and due to a large acceptance of fish collagen by a diverse group of people (Jewish, Hindu and Muslim religions not accepting certain meat products). |

| Period | Collagen extraction research |
|---|---|
| | Method optimization and comparison of different acids and enzymes for extraction efficiency had started in this period, due to a high demand for collagen from various markets. |
| **2010–2015** | • In 2010, Uriarte-Montoya et al. [120] extracted and characterised collagen from Jumbo squid and analysed its potential to be formed into a composite film with chitosan. Acid-solubilised collagen was extracted, and film blends of chitosan-collagen were prepared by casting. The films were characterised for their thermal and mechanical properties. The purpose of these films was to be used as bio-friendly packaging materials.<br>• Muralidharan et al. [71] extracted collagen from skin, bone and muscle of both trash fish and leather jacket fish. The collagen was characterised for their properties. Three methods of extraction were applied, and each method resulted in different collagen yields with the highest collagen yield being 71%. It was concluded that collagen from both trash fish and leather jacket fish could be used to extract collagen use it for potential pharmaceutical and biomedical applications.<br>• In 2012, Liu et al. [121] extracted collagen from fins, scales, skin, bones, and swim bladders of bighead carp. The aim of this study was to characterise pepsin-solubilised collagen from the five sources for simultaneous comparison purposes. It was concluded that all five tissues could be used as a potential substitute for mammalian collagen.<br>• Matmaroh et al. [122] extracted collagen from the scale of spotted golden goatfish via acid and pepsin solubilisation. SDS-PAGE showed both methods had revealed type I collagen and FTIR confirmed the presence of collagen triple helical structure. The main purpose of this study was to study collagen from the scale of spotted golden goatfish.<br>• Kittiphattanabawon et al. [123] extracted and characterised collagen from the skin of brown-banded bamboo shark. Both acid soluble and pepsin soluble collagen were extracted and the collagen yield with pepsin solubilisation was slightly lower than with acid solubilisation. It was concluded that collagen from skin of brownbanded bamboo shark could serve as an alternative source of collagen.<br>• Singh et al. [124] isolated collagen from skin of striped catfish. Once again, both methods of acid and pepsin solubilisation were applied to extract collagen. Collagen yield with pepsin-solubilisation was slightly higher (7.7%) in comparison to acid solubilised collagen (5.1%).<br>In this period and currently, most research being carried out on collagen extraction is based on method improvement and investigating novel tissues for possibility of collagen extraction. Sources that were not investigated and sources that potentially be environmentally friendly are being analysed for collagen extraction in order to find cheaper and efficient means of extraction. |

**Table 9.**
*Timeline of advancements in collagen extraction (1960s – 2015).*

## 10. Methods used to investigate the physiochemical properties of collagen and collagen-based films

There are at least 27 collagen types with 42 distinct polypeptide chains identified. Types I to XXVII collagen are fibril-forming collagens, containing triple-helix structures that can bundle into fibrils. Some collagen types are only present in certain tissues, for example, collagen types II, IX and XI are mostly found in cartilage tissues. Collagen types I to III are the ones mostly present in all collagen-containing tissues, type I being mainly present in skin tissue. Collagen characterisation is carried out to acquire information on structure, denaturation temperature, quantity, quality, thermal stability and fibril arrangement. Understanding the properties of each type of collagen will result in a better picture of what applications it can further be applied in.

The properties of the extracted collagen can be characterised by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Fourier transform infrared spectroscopy (FTIR), thermal stability (thermogravimetric analysis (TGA), differential scanning calorimetry (DSC)), morphology analysis, such as scanning

electron microscopy (SEM) and transmission electron microscopy (TEM); collagen moisture content, and hydroxyproline analysis.

The results from these analyses can be compared to standard collagen found in the market to compare yields and quality. The investigation of physiochemical properties of collagen through these characterisation methods is a way of optimising future collagen extraction methods.

## 10.1 Collagen molecular stability

### *10.1.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE analysis can be used to differentiate between the different collagen types and their individual chains. SDS-PAGE patterns of the extracted collagen can be obtained through any electrophoresis device such as the Mini-Protean or a PhastGel system. The collagen sample is boiled in SDS, resulting in collagen to break down into its polypeptide chains so that the α and β components of the collagen molecule can be analysed.

Though SDS electrophoresis has been utilised for preparative separation of collagen [125], it has been mainly used to compare collagen from different tissue types and to identify collagen types and polypeptide chains. Wu et al. [126] extracted bovine collagen and applied SDS-PAGE to identify the different collagen types present.

### *10.1.2 Fourier transform infrared spectroscopy (FTIR)*

In order to assess the collagen for abnormal formation and organisation and changes in its secondary structure, Fourier Transform Infrared Spectroscopy (FTIR) can be applied to reveal the collagen bio-distribution. FTIR has been used to study collagen denaturation [127], collagen cross-linking [128], and thermal self-assembly [129].

The vibrational bands characteristic of peptide groups and side chains provide information on protein structures. Spectral changes in amide A, amide I (1636–1661 $cm^{-1}$), amide II (1549–1558 $cm^{-1}$), and amide III (1200–1300 $cm^{-1}$) regions are indicative of changes in collagen secondary structure [127]. An increase in the intensity of amide III and broadening of amide I are related with increased intermolecular interactions via hydrogen bonding in collagen. Among these, the amide I band (peptide bond C=O stretch) is especially sensitive to secondary structures. A reduction in the intensity of amide A, I, II and III peaks and narrowing of amide I band are associated with collagen denaturation (Td) [127].

An FT-IR spectrophotometer can be used to obtain a spectrum for collagen. Approximately 2–4 mg of collagen in 100 mg potassium bromide (kBr) can be used to obtain spectra from 4000 to 1000 $cm^{-1}$.

## 10.2 Collagen content: hydroxyproline analysis

Hydroxyproline is an amino acid found in collagen, comprising about 13% of the collagen molecule, this amino acid is not found in any other proteins apart from elastin. Thus, determining the hydroxyproline content in a specified tissue enables the calculation of the total amount of collagen present. Experimentally, the amount of hydroxyproline content in a sample for mammals [130] is multiplied by 7.46 to give the amount of collagen in the sample.

Many studies on collagen extraction have applied hydroxyproline analysis to calculate collagen content [81, 87, 131, 132]. Researchers have developed methods to effectively measure hydroxyproline concentration of collagen using calorimetric

assays [19, 133], high-performance liquid chromatography, and enzymatic methods [133]. Calorimetric methods usually require complete hydrolysis of collagen to its individual amino acids, oxidising hydroxyproline present to a pyrrole, and then reacting the pyrrole with a colour forming agent. This colour change is measured using a UV/Vis spectrophotometer and compared against calibration data to determine hydroxyproline concentration [19]. To obtain the amount of collagen in a sample for mammals, the amount of hydroxyproline in the sample (mg) is multiplied by a factor of 7.46 [67].

## 10.3 Collagen denaturation temperature (td) and thermal stability

### *10.3.1 Differential scanning calorimetry (DSC) and thermal denaturation temperature ($T_d$)*

Any DSC calorimeter brand can be used, such as a Perkin Elmer DSC7. The thermal behaviour; stability of the native molecular structure and denaturation of collagen can be determined by carrying out differential scanning calorimetry (DSC). Denaturation temperature is obtained from the transition in the baseline in the 30–80°C region by taking the inflexion point reading. Total denaturation enthalpy (ΔH) can be estimated by measuring the area in the DSC thermogram.

Collagen denaturation temperature (Td) depends on collagen water content, collagen extraction method, collagen source, degree of collagen cross-linking and hydroxyproline content. Thermal stability of the collagen triple helix depends on hydrogen bonds (inter- and intra-hydrogen bonding) which further influences the folding and unfolding process when hydrogen bonds are broken and connected [134, 135]. Hence, the thermal stability of collagen depends on the cross-linking of collagen molecules (inter and intra).

Due to the polymeric nature of collagen, the thermal-induced denaturation of collagen is usually complicated. Heating collagen in wet or dry state reveals a series of thermal transitions. Thermal denaturation of collagen occurs due to hydrogen bonds breaking and hence the unfolding of the triple helices forming random polypeptide coils [136]. Cross-linking among the collagen molecules increase and mature with age and provides further stability. The age-related accumulation of cross-links increases the thermodynamic stability of collagen by increasing the activation energy required for collagen denaturation. However, the maturity of collagen cross-linking is limited to the functionality of the tissue. Post-mortem cross-linking of collagen can increase to the point where the tissue may become brittle [137].

Within the collagen fibril, there are complex interactions within and between the packed molecules. In addition to inter, intramolecular cross-links, and different forms of cross-linkages, there are several additional hydrophobic and ionic interactions that must be accounted for regarding collagen denaturation. The presence of non-collagenous components in the extracted collagen sample can cause variations in thermal denaturation [138].

Due to the domain structure of the triple helix, not all parts of the collagen molecule may denature at the same rate and it is almost impossible to define a definite equilibrium Td. Studies have also shown an increase in Td with an increase in hydroxyproline content [61, 139].

### *10.3.2 Thermogravimetric analysis (TGA)*

Thermal stability of extracted collagen is investigated using a gravimetric analyser. Approximately 5–10 mg of the sample can be used. The mass loss is

recorded while the sample is heated from room temperature up to 800°C at a rate of 10°C per minute. The first derivative of percentage mass change versus temperature can also be calculated to investigate temperature regions where mass loss was occurring.

Ramanathan et al. [140] used TGA to assess the thermal stability of fish skin collagen which was extracted via acid-solubilisation. They report using samples of approximately 5 mg and heating samples at 10°C/min in the temperature range of 0–800°C. The acid-solubilised collagen showed two weight loss steps on the TGA thermogram, relating the first stage to the loss of structural and bound water and stage two to thermal degradation of the polypeptide chain. The study concluded to show that the two peaks observed on the TGA differential curve were of collagen denaturation and collagen degradation respectively.

## 10.4 Collagen morphology

### *10.4.1 Scanning electron microscopy (SEM)*

The protein morphology of the extracted collagen can be studied using SEM. The morphology of the extracted collagen can be compared to the standard bovine

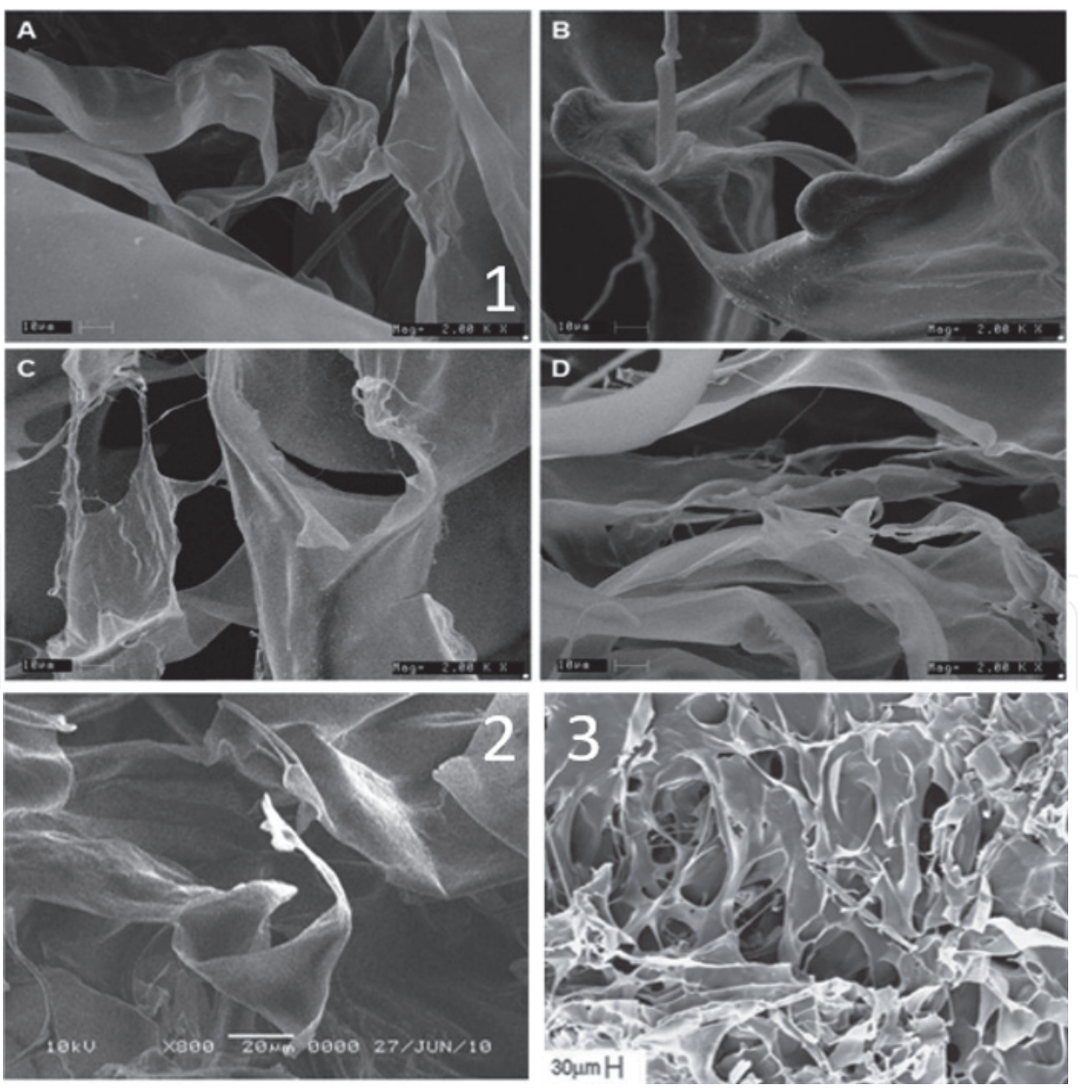


**Figure 8.**
*SEM images of extracted collagen, with 1) acid-soluble collagen of Catla fish (a), pepsin-soluble collagen of Catla fish (B), acid-soluble collagen of Rohu fish (C) and pepsin-soluble collagen of Rohu fish (D) [139], (2) being from buffalo skin [14] and 3) being SEM image of porcine skin collagen [142] (used with permission).*

collagen available in the market. The expected microstructure of collagen from SEM images would be to observe collagen sheets which would be a combination of collagen fibrils and fibres that are bundled together to form a fibril network and dense sheet-like structure.

Ramanathan et al. [140] used SEM to observe the surface morphologies of freeze-dried acid-solubilised fish skin collagen. The images showed a smooth surface texture, in two of the images, a layer-by-layer structure was observed (no definite fibres), and this was related to the intertwining of collagen fibres. Similarly, Rizk et al. [14], Tziveleka et al. [141], Rodrigues et al. [142], Pal et al. [139] all carried out SEM to assess the surface morphology of extracted collagen and all showed SEM images to have smooth or slightly wrinkled surfaces or sheet-like structures (**Figure 8**).

*10.4.2 Transmission electron microscopy (TEM)*

Transmission electron microscopy is usually carried out to observe collagen fibril structure and it is uniformity in a much deeper level. SEM only provides limited information on collagen morphology. **Figure 8** is showing an electron transmission image of mammalian lung tissue collagen at a magnification of 50 nm, while is showing a TEM image of collagen fibrils and fibres.

The preparative steps of collagen TEM are very specific and usually requires a technician to carry out each step carefully in order to observe the fibrillar structure of collagen. The Karnovsky fixative is mostly used as a preparative method prior to taking TEM images.

## 11. Conclusion

Collagen has risen its rank to be an integral material and an element of importance both in biomedical and non-biomedical sectors. In conclusion, research has shown that collagen can be effectively extracted from bovine and cattle hides. Using waste valorization concepts, collagen containing waste materials can be utilized to derive high-value products.

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