Extraction and characterization of collagen from bovine hides for preparation of biodegradable films

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Dedicated

To my parents,

Who are the epitome of wisdom, beauty and kindness,
who have influenced me and continue to inspire me every day.
At every obstacle and moment of doubt, you were there for me with endless support.
Thank you for your invaluable advice, encouragement and empathy.
I know I could not do this without you two and your duas. I wish I could show you how much I love and appreciate you. May Allah swt grant you both the highest of Jannah.
I love you so much.

Abstract

Industrial processing of bovine hides into leather results in a large number of 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 purpose of this study was to extract and characterize collagen from bovine hide off-cuttings in order to establish the potential of using these waste raw materials as sources of collagen. Secondly, to prepare and characterize biopolymer films from the extracted collagen.

Methods of acid-solubilisation (AS), acid-enzyme solubilisation (AES1) and a modified method of acid-enzyme solubilisation (AES2) were applied to extract collagen from bovine hide off-cuttings of bull, calf, cow, ox-hide and bovine face-pieces. The hide sources differed with respect to the animal's age, sex, diet and environment and influenced collagen yield, and therefore the economic benefit of extraction. The highest dry collagen content was obtained from cowhides using the AES2 method (75.13%), followed closely by bull hides at 74.45%. On the other hand, the lowest collagen content was also from cowhides (3.80%) but with the AS extraction method. The AS method proved to be inefficient for collagen extraction from bull, cow, face-piece and ox-hide sources. The analysis concluded that all the samples were of Type I collagen with α, β, and γ components.

Waste bovine hide off-cuttings proved to be a potential source of high-value product collagen. AES2 proved to be the most preferable method of extraction out of the three methods applied and considering leather to collagen revenue, these waste bovine hide off-cuttings could potentially result in substantial revenue.

Further work was carried out on BH and CH1 collagen to be investigated as potential raw materials for film preparation. Scanning electron microscopy (SEM) showed collagen sheets with a combination of threads and collagen strands, that were bundled together to form a fibril network and hence, a dense sheet-like

structure was observed for both BH and CH1 collagen. Differential scanning calorimetry (DSC) and Fourier transform spectroscopy (FTIR) indicated some age-related changes between calf hide collagen and bull hide collagen. DSC analysis resulted in thermal denaturation temperature of BH and CH1 collagen to be 51.90 ℃ and 45.36 ℃, respectively. FTIR spectra indicated BH collagen to possess a higher molecular order than CH1 collagen as indicated by a shift in the wavenumber of characteristic bands of amide A, amide I, amide II and amide III. Transmission electron microscopy (TEM) showed uniform, defined and highly ordered collagen fibrils for BH collagen.

BH and CH1 collagen-based films with varying glycerol concentration were successfully prepared. Films were characterized for their physical properties, secondary structure, thermal and mechanical properties. The films appeared to be uniform in thickness, transparent and showed good flexibility. Surface properties and thickness of both collagen sources were very similar. Film solubility (%) decreased with decreasing content of glycerol, which is a feature of glycerol. DSC and DTA curves of films indicated higher thermal stability for BH collagen-based films in comparison to CH1 collagen-based films. The FTIR spectra of films helped to understand the structural changes and interactions occurring with the collagen sources and glycerol. Films prepared with 40% glycerol were found to be optimum in terms of resulting in the most preferable film properties, especially resulting in superior mechanical properties for both BH and CH1 collagen sources. . Both BH and CH1 collagen-based films resulted in similar film properties. This is a positive finding as CH1 collagen is extractable without the use of enzymes, hence presenting a cheaper alternative.

Collagen-reinforced gelatine-based films were prepared in order to decrease the amount of collagen used with film preparation. The concentration of collagen reinforced had an influence on film moisture content, solubility (%), mechanical properties, thermal stability and the morphology of the gelatine-based films. Reinforcement of gelatine-based films with collagen improved both physical, thermal and mechanical properties of films. Increased addition of collagen resulted in a reduction of film moisture content, lower water solubility, high glass transition temperature (Tg) values and an increase in tensile strength of films. Tg

values of pure gelatine films (0C-100G) to reinforced films at 30% collagen increased from 46.80 ℃ to 52.76 ℃, respectively. The only downfall of adding increased amounts of collagen as a reinforcement was the reduction in elongation (%).

In general, the results showed that bovine waste hide off-cuttings have potential as sources of high-quality collagen. The modified acid-enzyme solubilisation proved to be an efficient method of extraction. Films were successfully prepared from extracted collagen and proved to have favourable properties. Further, collagen-reinforced gelatine-based films showed to have excellent film-forming properties and exhibited good thermal stability and mechanical strength. Blend films containing 30 wt % collagen displayed the best mechanical and physical properties. The blend film properties investigated by scanning electron microscopy, differential scanning calorimetry, and film surface properties showed a clear interaction between extracted collagen and gelatine - forming a new material with enhanced mechanical properties.

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Chapter 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 the 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 [1]. It is adversely affecting health, contaminating our air, landscape, freshwater 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 a large number of 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.

Wallace Group Ltd. (New Zealand) provide tannery and rendering services to casualty calves and cows collected from across the country. Wallace group in Waitoa, New Zealand processes over 120,000 casualty hides per year. These range from calves (the youngest source) to bull-hides being the thickest to process and the heaviest [2]. The rendering processes separate the animal by-products into value-added products such as animal protein meal and rendered animal fat, while the tannery aims to process hides into leather.

Hide off-cuttings, shavings and finished leather scrap are generated as waste in tanneries such as in Wallace Group. 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 economic or sustainable value, despite their high content of valuable biopolymers. Bovine hides are rich in the valuable protein collagen, especially in the corium layer of the skin [3]. Considering the high cost of collagen and the vast number of applications and industries it can be of value, a more sustainable and 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 [4; 5].

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 [6-11]. The demand for collagen is rising at approximately 20% annually and global collagen-based biomaterials market is predicted to reach US$4.6 billion by 2020 . 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 [12] and porcine products are prohibited by Muslim and Jewish communities [13].

One main application of the extracted collagen that is investigated in this thesis is the preparation of biopolymer films. 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 plasticizer concentration should, therefore, be investigated to identify which concentration results in the optimum mechanical, thermal and physical properties.

In section A of this study, collagen from various bovine hide sources was extracted.

Bull-hides being too thick to process for leather and often requiring multiple pre-thinning steps and calf-hides which are the youngest source requiring the least expensive method of extraction were chosen to carry forward to Part B of this study. It is also noteworthy to consider the age difference of bull and calf hides. The extent of cross-linking is a lot greater in bull-hide collagen in comparison to calf-hide collagen. In order to fully use this opportunity to utilize waste bovine hide off-cuttings in a sustainable manner, the second part of this thesis focused on collagen and gelatine-based films.

Section B starts with an extensive literature review on biopolymer films, followed by two experimental chapters on film preparation. The first film preparation chapter investigates the feasibility of preparing films from CH1 and BH collagen. Subsequently, investigating the effect of glycerol concentration on mechanical and thermal properties as well as the secondary structure of these films knowing that the degree of cross-linking among the collagen sources differ. Collagen is a high-value product and can be a very expensive raw material if extracted in its native form. Gelatine, the denatured form of collagen, on the other hand, is substantially cheaper in comparison to collagen and can be extracted requiring less time and chemicals. Gelatine has excellent film-forming properties; however, it has weak mechanical properties, whereas collagen compensates for that. Hence, the last film chapter investigates the preparation and properties of collagen-reinforced gelatine films.

In this work, research efforts to address the issues identified above were undertaken. The purpose of Part A of this study was to waste valorise bovine hide off-cuttings by extracting collagen from a number of different bovine hide sources. Further to characterize the extracted collagen and to develop a methodology to extract high purity collagen in the most efficient manner. Systematic approaches to extract collagen in a new efficient manner was introduced. Part B focused on the applications of collagen and the development of collagen-based biopolymer films.

More specific objectives of the thesis were:

Section A:

- Investigate the feasibility of extracting collagen from various bovine hide off-cuttings. This included hides from bull, calf, cow, ox-hides and bovine face-pieces. Three different extraction methodologies to be used in this investigation to identify which method is the most efficient in terms of extractables yield and collagen content. Further, characterizing the extracted collagen to better understand its physiochemical properties. The characterization techniques include proximate analysis of the hides, SDS-PAGE analysis, FTIR analysis, hydroxyproline analysis, extractables yield and estimated collagen production flow.
- To investigate and characterize the differences between the youngest (calf-hide) collagen source and the more mature and thickest source which would be the hardest to process into leather (bull-hide). Specifically, understanding the secondary structure, thermal properties and morphology of these two sources specifically in relation to the age-related increase in cross-linking. The purpose of this objective is to use known techniques to assess the structure and thermal properties of the two selected bovine collagen sources and compare their suitability for film preparation. This includes scanning electron microscopy (SEM), transmission electron microscopy (TEM), Fourier-transform spectroscopy (FTIR), thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). If both sources result in similar collagen characteristic then that is beneficial as there will be potential to use a large number of available hide off-cuttings, however, if there are slight differences in their characteristics then each can serve a different application.

Section B:

- Prepare biodegradable films from bovine calf-hide (CH1) and bull-hide (BH) collagen. These two sources were of interest as CH1 collagen is from a younger source and has a lot less inter- and intramolecular cross-linking than collagen from the older source of BH. To apply the

wet process of film casting to prepare films from these sources. The second part of this investigation will be to study the effect of plasticizer concentration on the physical and mechanical properties of films. The effect of glycerol on mature collagen derived from BH with extensive cross-linking and on younger CH1 collagen with a lot less cross-linking will be investigated. This way, we attempt to build a more complete picture of changes occurring in the system at the molecular level.

- To explore the feasibility of preparing composite films based on collagen-gelatine blends at different blend ratios via the casting method and using glycerol as a plasticizer. The amount of collagen to be varied between 0 and 30% to determine the optimal proportion of collagen required for the blend films. In addition, the thermal, morphological, mechanical and surface properties of the prepared films will be comprehensively studied as a function of the blend composition.

These specific objectives were addressed in nine chapters and divided into two parts, Section A making up the literature review of the first part of this thesis, followed by experimental Chapters 3 and 4. Section B starts with an extensive literature review on biopolymer film preparation and is followed by experimental chapters 6 and 7.

Chapter 1 is the thesis introduction gives a general overview of the study, including an introduction, research rationale, research goals and an outline of the thesis.

Section A, Chapter 2 presents an extensive literature review on the waste valorisation of bovine hide off-cuttings and collagen extraction. It further focuses on the importance and the demand for collagen and in-depth research on collagen extraction methodologies and the need for the development of more efficient methods of extraction. It concludes with a focus on methods used to investigate the physiochemical properties of collagen.

Chapter 3 investigates three different methodologies to extract collagen from bovine hide off-cuttings of five different sources. These include, bull-hides (BH),

cowhides (CH), calf-hides (CH1), ox-hides (OH), and bovine face-pieces (FP) and these sources differ with each other in terms of hide thickness, age, sex and environmental conditions.

Chapter 4 examines the thermal, morphological and secondary structure of BH collagen and CH1 collagen. Collagen from these two sources showed the most promising results in Chapter 3 in terms of collagen from calf hide being extractable efficiently without the use of enzymes and bull hides resulting in high collagen content with all three extraction methods. Collagen from these sources was carried forward for further characterizations to understand their properties for the preparation of biopolymer films.

Section B, Chapter 5 presents an introduction and an extensive literature review of biopolymer film preparation.

Chapter 6 examines film preparation from BH and CH1 collagen via solution casting. Effect of glycerol on the extent of collagen cross-linking is examined as the two collagen sources differ in collagen maturity and hence extent of inter- and intra-cross-linking. Subsequently, the effect of glycerol concentration on the mechanical, thermal, surface properties and secondary structure is investigated.

Chapter 7 aims to reduce the amount of collagen utilized in films prepared in experimental Chapter 6 in order to make them more economically viable. Collagen-reinforced, gelatine-based films are prepared at different blend ratios. The collagen-reinforced films are characterized for thermal stability, morphology, mechanical and surface properties.

Chapter 8 draws the main conclusions of the study and suggests some recommendations and future work based on the findings of the study.

Chapter 9 proposes some recommendations for future work to be investigated.

Section A

Extraction and characterization of collagen

Chapter 2

Literature review

2.1 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 a large number of by-products that are under-exploited, 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.

Wallace Group LP (New Zealand) was established to provide tannery and rendering services to casualty calves and cows collected across the country from North Island to Otago. Wallace Group in Waitoa, New Zealand processes over 120,000 (Figure 2.1) casualty hides per year ranging from calves being the youngest source to bull-hides being the thickest and heaviest [2]. Wallace Group LP process from the lightest skins up to heaviest bull and ox-hides and after tanning, hides are graded according to their weight and defects.

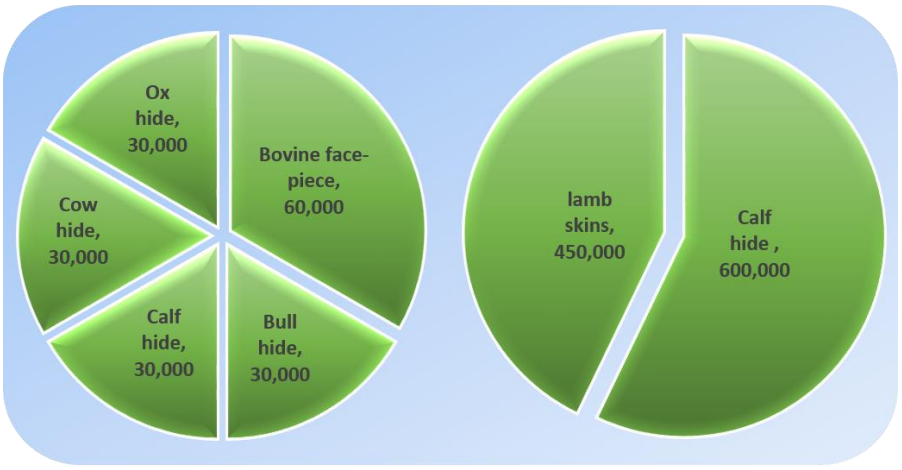

Figure 2.1: Number of aw hide/skin processed at Waitoa Wallace Corp yearly (left) and lambskin and calf-hides processed every season across its Waitoa and Fielding plants [2].

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.

2.2 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. In reality, only about 25% by weight of raw salted hides results in the finished leather [14]. Furthermore, other solid wastes are also produced from wastewater and sludge treatment.

Casualty cattle coming in Wallace Group tannery plants are processed (up to "Tannery" step of Figure 2.2), cleaned and prepared for "wet blue". Figure 2.2 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.

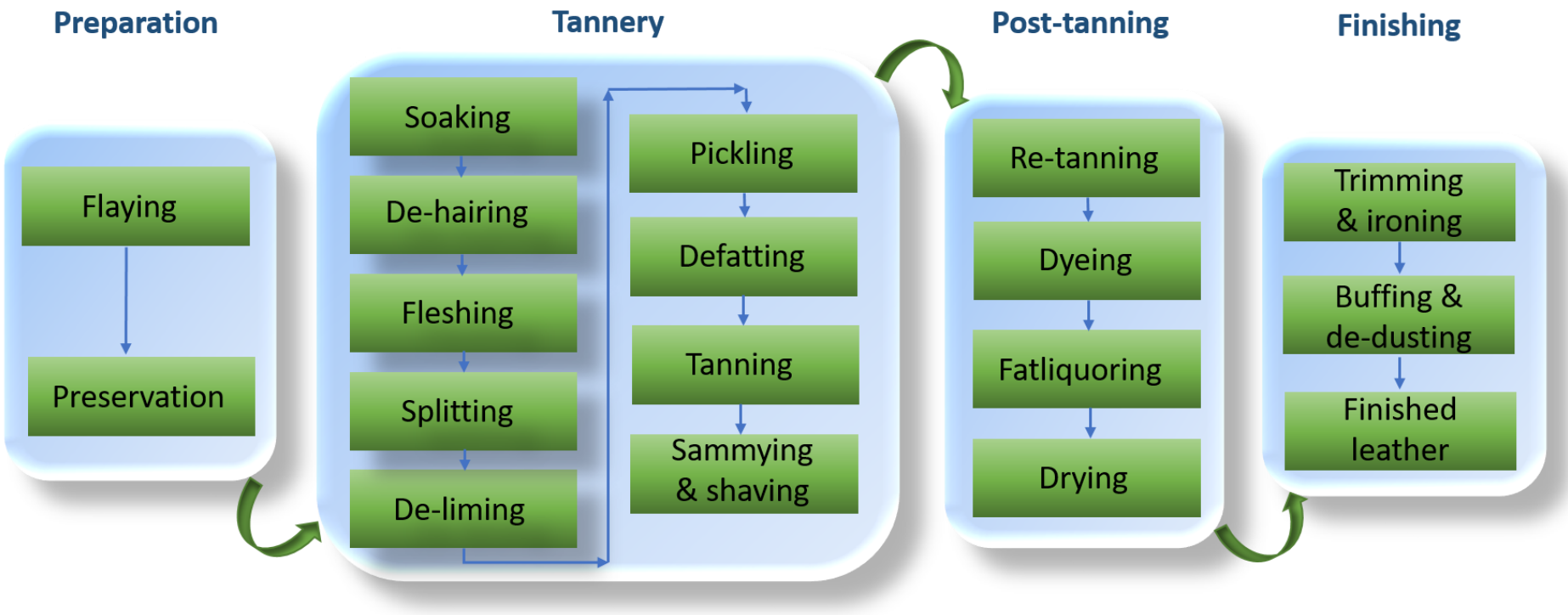

Figure 2.2: Process flow of transformation of hides into leather [2].

During the conversion of bovine hides into leather (Figure 2.2) a vast number of chemicals are released into the environment and waste products are generated at each stage. Table 2.1 is showing chemicals used and wasted generated at each stage of leather production

Table 2.1: Chemicals used at each stage of hide to leather conversion and wastes generated [15].

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

2.3 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 [16] and hence bovine hides are rich in collagen, especially in the corium section of the hide [17].

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 as a whole.

Wallace Group processes over 600,000 calf hides across its Waitoa and Fielding plants. Calf-hides are knowingly from a younger source and its collagen has a lot less cross-linking and a lot less mature cross-links than what is found in older sources of collagen such as from bull-hides. It will be even more cost-efficient and economically favourable to extract collagen from calf-hides than converting them into leather. The less mature cross-linking found in calf-hide collagen does not necessarily require costly enzymes or chemicals of high concentration.

Bovine hide off-cuttings can be processed for collagen extraction (Figure 2.3). This collagen can be used by various industries for a large number of applications from biodegradable films, pharmaceuticals to cosmetics. A number of 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 [18].

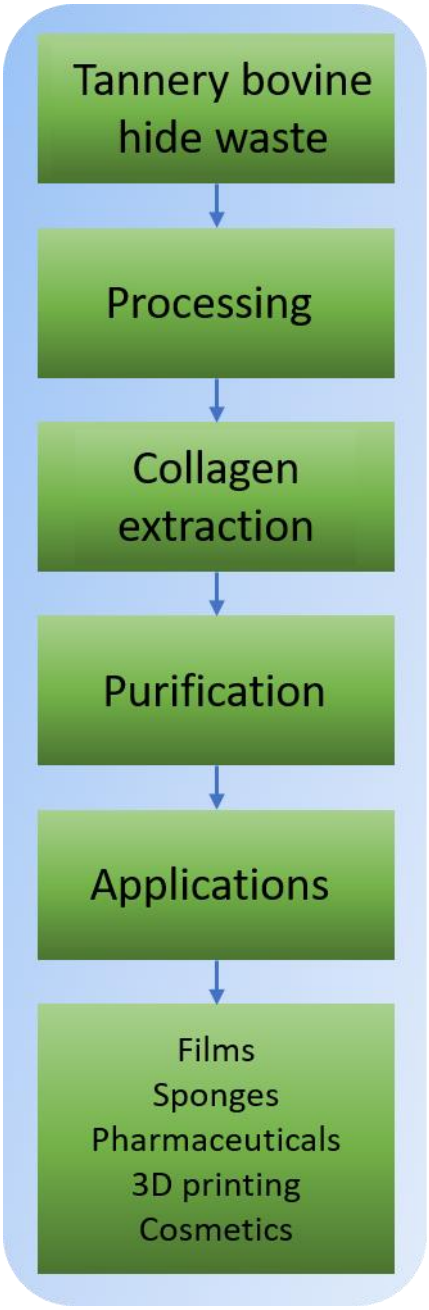

Figure 2.3: Process flow of waste valorisation from tanneries to collagen extraction and possible collagen-based applications.

2.4 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 [19]. 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 [4].

2.4.1 Collagen structure

The triple-helix of collagen consists of three distinct alpha chains coiled around each other and this is termed as tropocollagen [4]. The tropocollagen units are arranged 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 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 [4]. 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 2.4) 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 glycine in polypeptide chains forms H-bonds with adjacent peptide CO groups of the other chains.

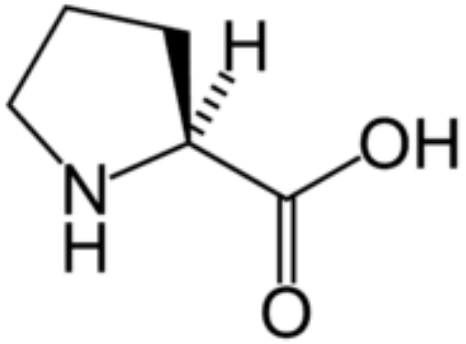

Figure 2.4: The structure of proline, alpha carbon covalently bonded to nitrogen as a part of a five-membered ring that limits the rotation of N relative to the alpha carbon [20] (used with permission).

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 stabilize 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) [21]. 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 [21]. 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% [22].

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 nanometers in diameter and 300 nanometers 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 [5]. The hierarchical structure of collagen is zoomed-in starting from the alpha chains coiling together to form the triple helix is shown in Figure 2.5.

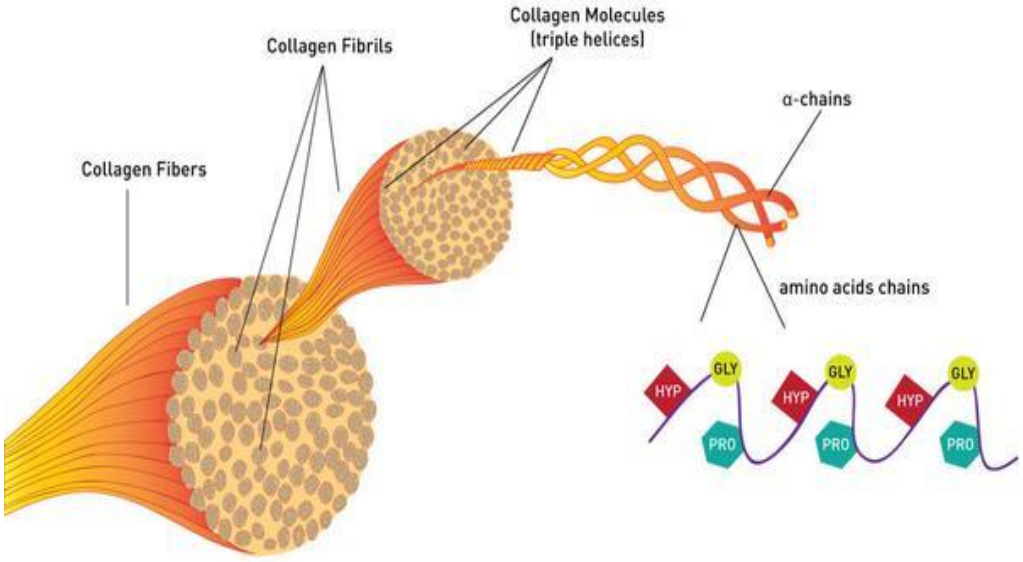

Figure 2.5: Collagen structure being broken down to fibre, fibril, triple helix and an alpha chain respectively [23] (used with permission).

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 stabilized 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-organization 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 [5].

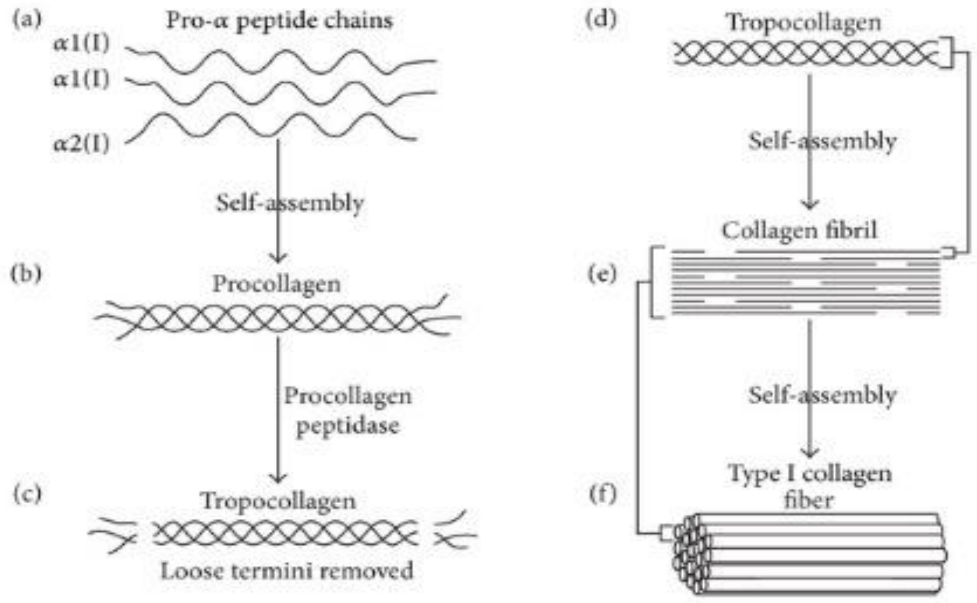

Figure 2.6: structure of collagen, with b) procollagen (loose ends), triple-helix wound together and c) collagen subunit tropocollagen (loose terminal removed) for final self-assemble of the collagen fibril and fibre (d-f) [24] (used with permission).

2.4.2 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 [25].

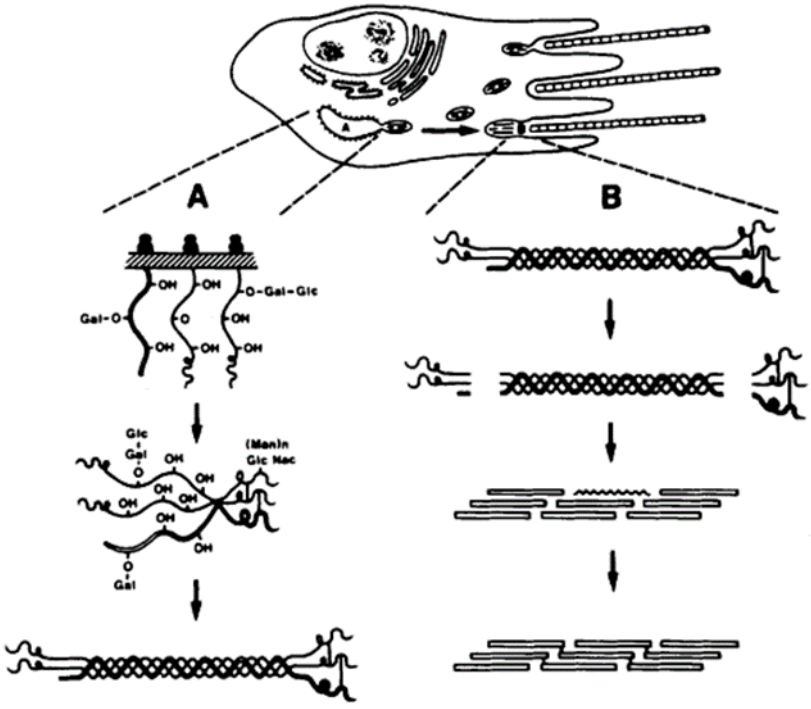

Figure 2.7: Collagen synthesis stages [26] (used with permission).

2.2.1. 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 2.8). 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.

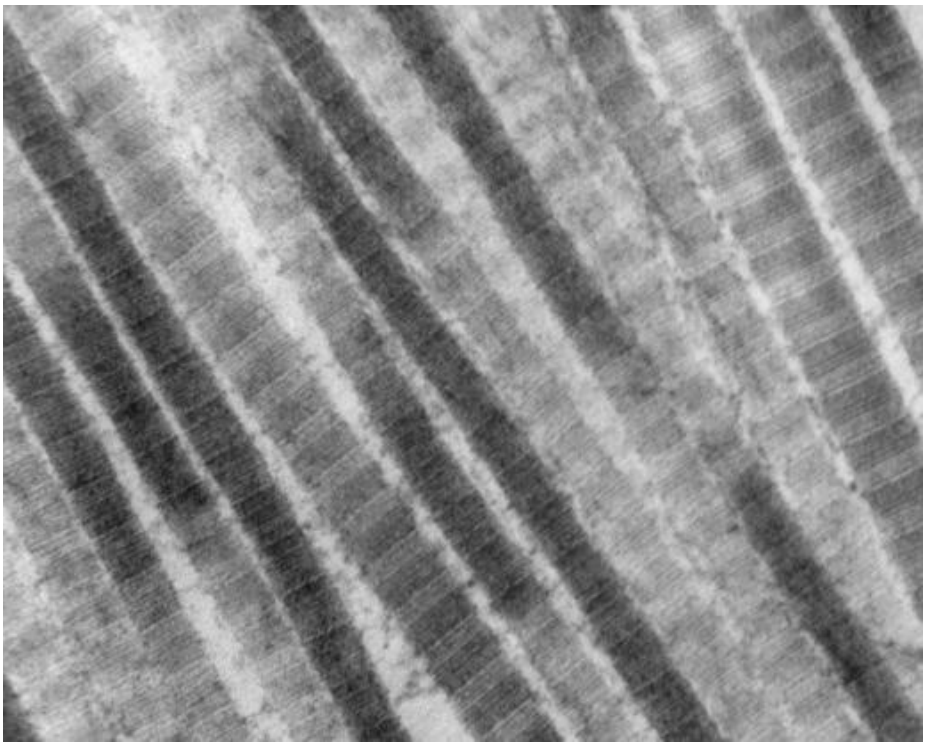

Figure 2.8: Collagen fibre showing the striations where tropocollagen is densely packed (light sections) [27] (used with permission).

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.

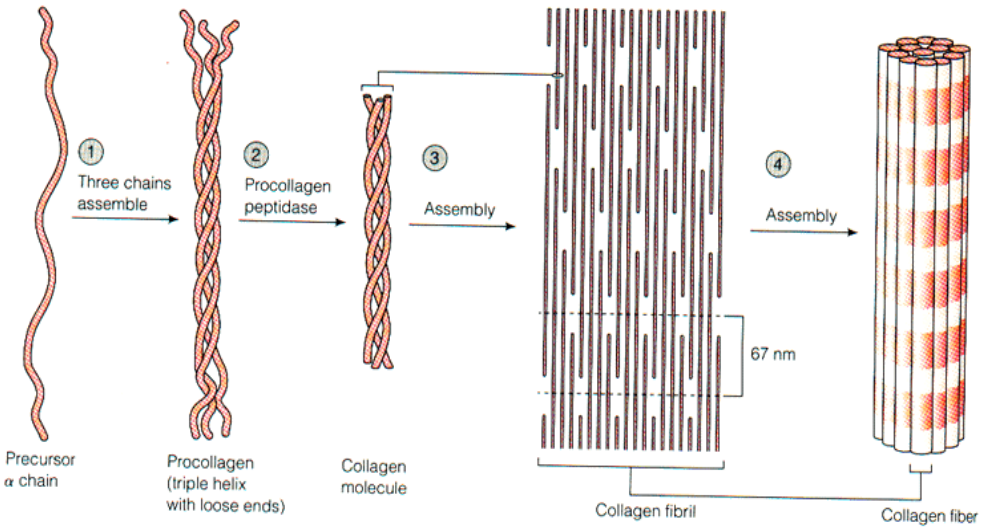

Figure 2.9: Assembly of a collagen fibre [28] (used with permission).

2.4.3 Collagen maturation

Inter-and intra-molecular covalent cross-links are formed between and within tropocollagen (collagen triple-helix) units giving strength to collagen fibres (Figure 2.9). Intramolecular 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 side-chains to an aldehyde that then undergoes a condensation reaction forming an adol cross-link with other converted lysine side-chains. 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 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.

2.4.4 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 also differences in the terminations of the helical domains [29].

Each collagen molecule is composed of three different polypeptide chains (α1, α2, and α3). Each chain is identified by its amino acid composition (Table 2.2). 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.

Table 2.2: Differing content of amino acid composition of human collagen chains [30].

Amino acid	a1(I)	a2(I)	a1(II)	a1(III)	a1(IV)	a2(IV)	a1(V)	a2(V)	a3(V)
3-Hydroxyproline	1	1	2	0	1	1	5	3	1
4-Hydroxylproline	108	93	97	125	122	110	110	106	91
Aspartic acid	42	44	43	42	45	49	49	50	42
Threonine	16	19	23	13	19	30	21	29	19
Serine	34	30	25	39	38	30	23	34	34
Glutamic acid	73	68	89	71	78	65	100	89	97
Proline	124	113	120	107	85	73	130	107	98
Glycine	333	338	333	350	334	324	332	331	330
Alanine	115	102	103	96	30	47	39	54	49
Half cystine	0	0	0	2	0	2	0	0	1
Valine	21	35	18	14	33	27	17	27	29
Methionine	7	5	10	8	15	14	9	11	8
Isoleucine	6	14	9	13	32	38	17	15	20
Leucine	19	30	26	22	52	56	36	37	56
Tyrosine	1	4	2	3	5	7	4	2	2
Phenylalanine	12	12	13	8	27	36	12	11	9
Hydroxylysine	9	12	20	5	50	36	36	23	43
Lysine	26	18	15	30	6	7	14	13	15
Histidine	3	12	2	6	6	6	6	10	14
Arginine	50	50	50	46	22	42	40	48	42
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000
Gal-hydroxylysine	1	1	4	-	2	2	5	3	7
Glc-Gal-hydroxylysine	1	2	12	-	44	29	29	5	17

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.

Table 2.3 describes the collagen types in more detail and its associated disorders. This is to highlight how much of an important role each collagen type plays in the tissue.

Table 2.3: Types of collagen and associated disorders [31].

Type	Description	Disorders
I	Type I collagen is the most abundant collagen type. It is found in tendons, skin, artery walls, cornea, cartilage and the organic part of bone and teeth.	Osteogenesis imperfecta, Ehlers–Danlos syndrome, Infantile cortical hyperostosis aka Caffey's disease.
II	Type II collagen makes up 50% of all cartilage protein. Also, found in the vitreous humour of the eye.	Collagenopathy, types II and X.
III	Type III collagen is found in artery walls, skin, intestines and the uterus. This collagen is mainly found in granulation tissue, it is produced by young fibroblasts before the tougher type I collagen is synthesized.	Ehlers–Danlos syndrome, Dupuytren's contracture.
IV	Serves as a part of the filtration system in capillaries and the glomeruli of the nephron in the kidney.	Alport syndrome, Goodpasture's syndrome
V	This collagen comprises most of the interstitial tissue.	Ehlers-Danlos Syndrome
VI	Comprises the majority of the interstitial tissue.	Ulrich myopathy, Bethlem myopathy, Atopic dermatitis
VII	Forms anchoring fibrils in dermoepidermal junctions	Epidermollysis bullosa dystrophica
VIII	Found in endothelial cells	Posterior polymorphous corneal dystrophy 2
IX	Comprises majority cartilage tissue	EDM2 and EDM3
X	Comprises most of the cartilage tissue	Schmid metaphyseal dysplasia
XI	Comprises most of the cartilage tissue	Collagenophathy, types I and XI

2.5 Gelatine

Gelatine is a colourless, translucent, foodstuff that is brittle when dry and gel-like after preparation. Gelatine is collagen that has been irreversibly hydrolysed. This happens with thermal denaturation. It is derived from animal skins and bones and can be extracted from fish skins. Gelatine is a low-quality product as the proteins are denatured and it is mainly used by the food industry as a gelling agent [32].

Gelatine is used for glues, by the pharmaceutical industry and cosmetic manufacturing.

2.5.1 Gelatine molecular structure

The chemical composition of gelatine is very close to that of collagen. The triple helix of the collagen molecule (which is composed of two α1 chains and one α2 chain) is broken down into a mixture of single or multi-stranded polypeptides, making the gelatine structure [32].

2.5.2 Amino acid composition of gelatine

Table 2.4: Approximate amino acid content of gelatine [33].

Amino acid	Content (%)	Amino acid	Content (%)
Glycine	21	Lysine	4
Proline	12	Serine	4
Hydroxyproline	12	Leucine	3
Glutamic acid	10	Valine	2
Alanine	9	Phenylalanine	2
Arginine	8	Threonine	2
Aspartic acid	6	Isoleucine	1

2.5.3 Isolation of gelatine

The annual global production of gelatine is approximately 375,000 metric tonnes [34]. More than 40% of gelatine production occurs in Europe, 20% in North America and the rest of production occurs in Latin America and "other" countries. Gelatine is usually derived from pork skins, cattle hides and bones, however recently fish by-products have also been considered due to obstacles surrounding religious restrictions.

There are a number of methods for converting collagen to gelatine; however, they all have several factors in common. One of these factors is that the intermolecular and the intermolecular bonds that stabilise insoluble collagen must be broken. The hydrogen bonds which stabilise the collagen helix must also be broken [35].

The gelatine isolation procedure is not seen to be complex or time-consuming. It does not require careful observation of temperatures as it is the case for collagen extraction. In order to isolate gelatine, collagen is heated past its melting point hence denaturing the collagen molecule and breaking the bonds that are holding collagen together[32]. Once collagen has been denatured, it has lost its triple helix tertiary structure. Hence collagen loses its chemical resistance and becomes soluble in water [36].

2.6 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.

2.6.1 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 [37].

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 [38]. 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 [3]. 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 [39].

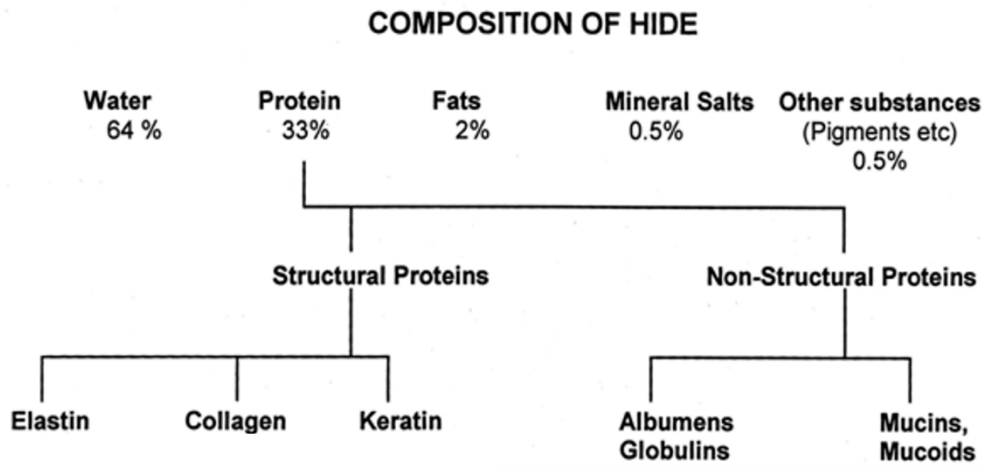

Figure 2.10: The approximate composition of bovine hide [3] (used with permission).

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 [40].

2.6.2 Properties of bovine hides

Each section of the animal hide for its properties is discussed further [39]:

- *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 moisturizers 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.

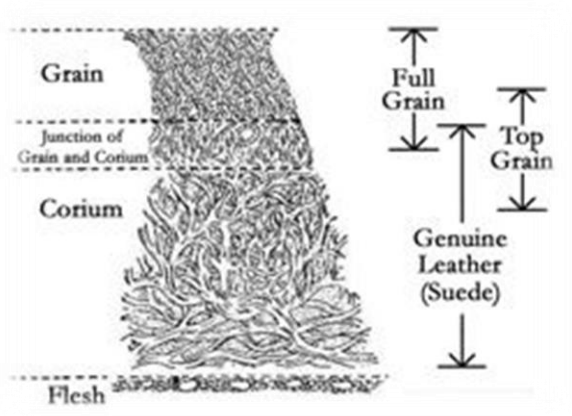

Figure 2.11: Structure of bovine hide [41] (used with permission).

2.6.3 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 [9].

Shark type I collagen forms fibrils under different conditions compared to bovine and porcine collagen [42]. 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 utilizing shark collagen as a new type I collagen material for various uses such as cell culture and medical technology [43].

2.6.4 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 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 [44].

2.7 Collagen market and its applications

2.7.1 New Zealand collagen industries

There are a number of collagen-producing companies in New Zealand. 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 film formability would possibly generate huge economic potential for New Zealand.

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 a number of different industries, ranging from biomedical to the food industries.

Table 2.5: Collagen and its features [45].

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.

2.7.2 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 flagship processed products is polymeric collagen, which is delivered to contracted customers [46].

The polymeric collagen produced by Southern Lights Biomaterials is type I collagen derived from bovine tendon and is naturally cross-linked [46]. They do not take advantage of using cattle hides or face-pieces. Their collagen is sold to independent contractors without further processing.

2.7.3 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 [47]. 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 [48].

2.7.4 Gelita NZ

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 and in Christchurch NZ with its headquarters in Germany [34]. However, the collagen Gelita produces is not 100% native collagen but hydrolysed collagen, in other terms it is gelatine.

2.7.5 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 [49].

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 [50].

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.

2.7.6 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.

2.7.6.1 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 have the ability to 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 [51].

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 [52].

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 [53]. 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 [54].

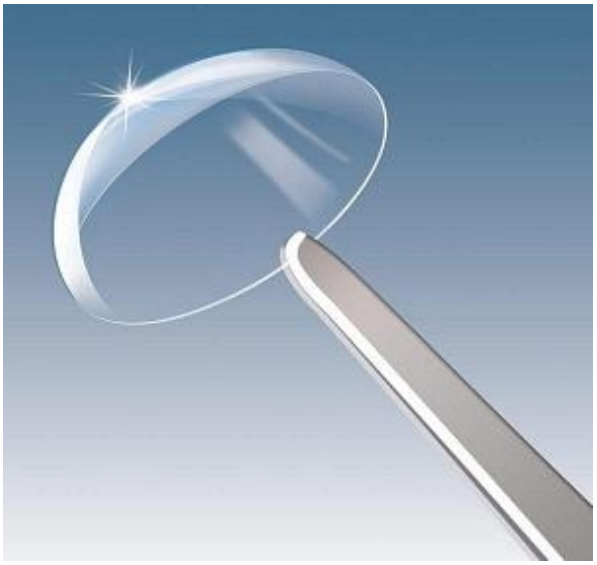

Figure 2.12: Collagen corneal shield used in ophthalmology [55] **(used with permission)**.

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 due to the fact that 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 [52].

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 [56]. Type I collagen is suitable for skin replacement and burn wounds due to their mechanical strength and biocompatibility[10].

v) Bone substitutes

Collagen has been previously used as implantable carriers for bone inducing proteins[51]. Due to osteoinductive activity; collagen itself has recently been used as bone substitutes[52]. 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[57].

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), using a 3D camera or animation modelling software[58]. 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 [59]. However in the case of bio-printing; bio-ink (cells) and bio-paper (collagen, nutrients) are required [60].

The process of 3D printing varies depending on the printer type; however, the material is usually sprayed, squeezed or otherwise transferred from the printer onto the platform. The 3D printer makes passes over the platform, depositing a layer on top of layer of material to create the finished product. The printing process of the product can take several hour or days depending on the size and complexity of the object. The layer thickness depends on the printer type also; the average layer thickness is approximately 100 microns. However, some printers can even deposit layers as thin as 16 microns [59].

The market for 3D printing is huge; $3.7 billion to date and the number is expected to reach $6.5 billion by 2019 [61].

There is an increasing lack of replacement organs throughout the world. In the United States alone on a daily basis, about 60 000 people are on the waiting list for kidney transplants, 3000 for heart transplants and 17 000 for liver transplants [62]. With the increasing life expectancy and lack of replacement organs, this presents a huge problem if no solutions are found. Recent developments give hope that three-dimensional bio-printing of living structures may offer the solution. The points below summarise the growing research in the development of 3D printed organs.

- *Ear:* Researchers at the University of Cornell started with a 3D camera that took a picture of the existing child's ear to match. This became the ear's

geometry into a computer. From the image derived from the camera, the 3D printer built a soft mold of the ear. Using a 3D printer they injected the mold with collagen derived from rat-tails, and then added 250 million cartilage cells from the ears of cows. The collagen served as a scaffold upon which cartilage grew [63].

- *Kidneys:* Researchers at Wake Forest Institute have 3D printed prototypes of kidneys. Layer-by-layer using a 3D/bio-printer to build the scaffold and deposition of kidney cells. Assembly is carried out when it is transplanted into the patient. The scaffold (collagen) degradation follows in-vivo [62].

- *Skin Grafts*: The 3D printing of skin tissue is simplified by representing it as a two-compartment tissue. The first of these two tissues is the multi-stratified epidermis that is composed of the basal, spinous, and granular layers in the live layer, all of which are represented by keratinocytes (KCs). The dead stratum corneum is represented by terminally differentiated KCs (corneocytes) in a lipid-rich bilayer matrix. The second compartment, dermis, is represented by synthetic substrates or acellular matrix protein scaffolds (e.g. collagen) or fibroblasts that are dispersed within protein scaffolds [64].

2.7.7 Collagen and rheumatoid arthritis and osteoarthritis

Collagen has shown to have positive effects on rheumatoid arthritis and osteoarthritis [65]. Published studies [66] have reported that ingestion of type II collagen relieves joint discomfort associated with osteoarthritis and rheumatoid arthritis. The authors also conducted a randomized 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 [66].

2.7.7.1 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 [10]. With ageing collagen type III decreases leading to wrinkles and lines, thus moisturizing creams and cosmetic injects containing collagen have become in high demand [67].

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 [6].

The Auckland Company, Revolution Fibres has developed their own technology for the industrial production of nano-fibre, being the only commercial producer of electrospun nano-fibre in Australasia. The nanofibers that are “electro-spun” from collagen extracted from Hoki fish skins have many applications. Revolution

Fibres have launched a skincare range using collagen fibres to deliver plant extracts into skin [48].

2.7.8 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 [68; 69]. 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 [70].

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 [71]. 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 [52].

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.

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.

A possible collagen-derived product is collagen films. Collagen films can have a variety of different applications depending on the innovative engineering applied. One of the commonly applied applications of the collagen films is as coatings or as a packaging material in the food industry.

Figure 2.13: Collagen film used as food coating [72] (used with permission).

Sionkowska et al. [73] prepared biopolymer films based on blends of collagen and silk fibroin. Films were prepared by method solution casting and characterized 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. [73] 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 [74]. Hence the per cent elongation values of the film blends were very low (0.30-5.10%) [73].

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 [9] 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.

2.8 Methods used to extract collagen from bovine hides

2.8.1 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 1cm 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.

The general method for the extraction of collagen from any tissue follows these main steps:

- Removal of non-collagenous protein using an organic salt
- Removal of fat using a detergent or an organic solvent and salt
- Dissolution of collagen using an organic acid

- Precipitation of collagen from solution using salt
- Purification of collagen through dialysis
- Lyophilisation of collagen for storage

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^{o}C to prevent bacterial contamination [12].

Collagen from juvenile sources (e.g. newborn 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 [75].

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) [76]. 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 [75].

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

2.8.2 Temperature control

To prevent collagen denaturation and contamination, majority of the researchers carry out the collagen extraction process at approximately 4℃. Contamination occurs due to thermal denaturation or microbial degradation.

Table 2.6: Processing temperatures used to extract collagen.

Collagen source	Temperature (℃)	Reference
Bovine	4℃	[16; 77-81]
Fish	4-9℃	[77; 78; 82-88]

2.8.3 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 (Table 2.7). Chemicals used for demineralisation is shown in Table 2.8.

Table 2.7: Solvents used for de-fatting of collagenous tissue in literature.

Source	Solvent	Reference
Bovine	Acetone	[22; 81]
Fish	0.5% detergent 10% butyl alcohol 15% Butyl Alcohol	[85; 89] [88; 90] [91]

Table 2.8: Chemicals used for demineralisation in literature.

Collagen source	Chemical	Reference
Bovine	0.5% HCl 0.5 M EDTA	[80] [92]
Fish	0.5 M EDTA	[90]

2.8.4 Non-collagenous protein removal

Contaminating proteins need to be removed after defatting and demineralization. Most collagen extraction methods utilize salt or alkali solutions to solubilise the contaminants (Table 2.9). 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 2.9: Chemicals used for non-collagenous protein removal in literature.

Collagen source	Chemical	Reference
Bovine	0.5 M NaCl	[93]
	1 M NaCl	[94]
	$K_2HPO_4^-$	[95]
	0.1 M NaOH	[81]
Fish	0.1 M NaOH	[86-90]

2.8.5 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 [9]. 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 [96].

2.8.5.1 The salting-out method

This method is seen as the least favourable method of collagen extraction. Collagen proteins, similar to 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 [96].

2.8.5.2 The alkali method

The main chemicals used in the alkali method of collagen extraction are sodium hydroxide and monomethylamine [97]. This extraction method is not favoured as the main extraction method due to similar reasons as the salting-out method.

Hattori et al [97] 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.

2.8.5.3 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 [96].

2.8.5.4 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 also 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 [98] 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 [98].

2.8.5.5 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 [96] concluded that citric acid has the best effect to extract collagen, second being acetic acid and last being hydrochloric acid. However, according to Skierka [98] and Higham [99], 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 [89].

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 2.10: Acids used for collagen extraction.

Collagen source	Acid type and concentration	Reference
Bovine	0.5 M acetic acid 10% acetic acid with 0.2 % chlorhydric acid	[16; 78; 97; 100-103] [104]
Fish	0.5 M acetic acid 0.15 M HCl Citric acid	[78; 82; 83; 85; 86] [89; 98] [89; 98]

2.8.5.6 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 [96]. 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 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 [105].

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 [99]. Enzymes have been used in collagen extraction, McClain et al. [94] 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 2.11: Enzyme extraction methods used for collagen extraction.

Collagen source	Enzyme type and conc.	Reference
Bovine	1% Trypsin Pepsin	[106] [78; 102-104]
Fish	1% (w/w) pepsin	[78; 82; 85]

2.8.5.7 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 speeds up the reaction time and results in a collagen protein well-kept in its triple helix structure [96]. Concentration and acid/enzyme type greatly depend on the collagen tissue and method optimization.

2.8.5.8 Collagen preservation

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

2.9 Collagen extraction work 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 a number of industries.

Table 2.12: Timeline of advancements in collagen extraction.

Period	Collagen extraction research
1960-1969	- Bakerman [107] extracted human skin collagen with age via the acid-solubilization 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. - .In 1968 Rigby [108] 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 ℃. - Young et al. [109] extracted cod skin collagen with mild solvents in the pH range of 3.4-8.7 at 3-90 ℃. - Grant et al. [110] studied the carbohydrate content of bovine collagen. It was shown that crude preparations of collagen were contaminated with mannose, fucose and hexosamine. - Bronstein et al. [111] studied human collagen and the relation between intra and intermolecular cross-linking. - Miller et al. [112] extracted and characterized 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.

	During this period, research was mainly carried out to understand collagen as a protein. There were no research on method optimization or investigation of different extraction methodologies.
1970-1979	- Anderson et al. [113] 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. - Pierson et al. [114] 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. - Maekawa et al. [115] extracted collagen from the skin of mice. Extraction was carried out with 0.5 M acetic acid at 40 ℃ for different times. - Francis et al. [116] 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. - Uitto et al. [117] analysed the solubility of skin collagen in normal human subjects and in patients with generalized scleroderma. Extraction was carried out with 0.14 M NaCl and number of extractions were varied. - In 1976, Trelstad et al. [118] applied differential separation to separate native collagen types I, II, and III. The main precipitants were ammonium sulfate, sodium chloride and ethanol. - Riemschneider et al. [119] extracted collagen from cow placenta via pepsin solubilisation. 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. [120] 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. - Graham et al. [121] extracted and quantified types of collagen both in control intestine and as well as in both in inflamed and strictured intestine resected from patients with Crohn's disease. This study mainly focused on differences in collagen types between the controlled and both inflamed and strictured intestines. - Murata et al. [122] 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. - Elstow et al. [123] extracted and characterized 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 characterize and identify type V collagen. - Laurent et al. [124] 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 homogenization of the collagenous tissue in 2% sodium dodecyl sulfate and dried acetone powder. - Van Amerongen et al. [125] analysed the concentration and extractability of collagen in human dental pulp. Premolar and third molar dental pulps were studied for their collagen content and acetic acid or neutral salts were used to extract collagen. By the use of SDS-PAGE analysis, 42.6 % of extracted collagen to be type III. - Kurita et al. [126] 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. 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. [127] extracted collagen from Plaice skin and analysed its functional properties. Acetic acid was the main solubilizing agent in this study and homogenisation was carried out with 0.4 M NaCl. - Ambrose et al. [128] extracted and characterized 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. - Nomura et al. [129] 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.

	- Ciarlo et al. [130] extracted collagen from hake skin. Acetic acid was used for solubilisation and collagen was characterized for its viscosity and collagen type (SDS-PAGE). - Bishop et al. [131] extracted and characterized collagen types II and IX from bovine vitreous. Centrifugation and precipitation with 4.5 M NaCl were applied and the collagen types were identified. 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. [75] extracted collagen from the skin of bigeye snapper by the use of pepsin. Acid-extracted collagen resulted in lower collagen yields in comparison to pepsin-solubilized collagen. - Woo et al. [132] extracted collagen from yellowfin tuna skin. Methodology was optimized 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 characterization was carried out by SDS-PAGE, FTIR, and solubility analysis. - In 2003, Sadowska et al. [89] 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. - Jongjareonrak et al. [133] extracted and characterized collagen from bigeye snapper. Acid and pepsin solubilized collagen were isolated and characterized for their properties. The Td of the acid-solubilized and pepsin solubilized collagen varied slightly with, pepsin solubilized collagen having a higher Td (30.87 °C). - Zhang et al. [85] extracted and characterized 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. - Nalinanon et al. [134] 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.

	- Cao et al. [135] extracted and characterized type II collagen from chick sternal cartilage. Pepsin was the main solubilizing 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. 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). 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. [136] extracted and characterized collagen from Jumbo squid and analysed its potential to be formed into a composite film with chitosan. Acid-solubilized 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. - Muralidharan et al. [87] extracted collagen from skin, bone and muscle of both trash fish and leather jacket fish. The collagen was characterized 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. - In 2012, Liu et al. [137] extracted collagen from fins, scales, skin, bones, and swim bladders of bighead carp. The aim of this study was to characterize pepsin-solubilized 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. - Matmaroh et al. [138] 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.

- Kittiphattanabawon et al. [139] extracted and characterized 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.
- Singh et al. [140] 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%).

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.

2.10 Collagen purification via dialysis and filtration

2.10.1 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 utilized with different cut off molecular weights to separate pure collagen from other solvents, salts and enzymes and other impurities.

2.10.2 Collagen purification via filtration

Ultrafiltration can be applied to remove the non-collagenous material prior to lyophilisation of collagen. Ultrafiltration utilizes 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 are able to 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.

- Cross-flow/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.

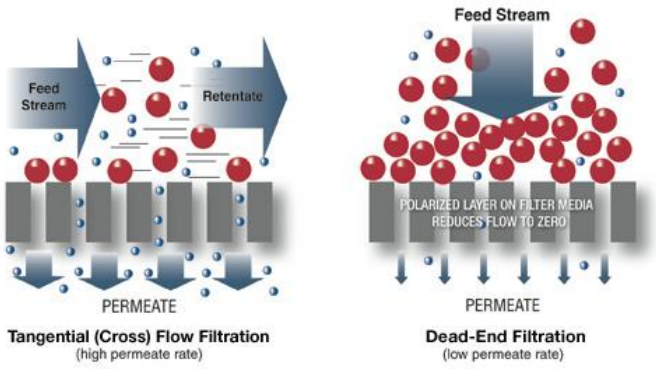

Figure 2.14: Diagram of cross-flow filtration (left) and dead-end filtration (right) [141] (used with permission).

2.11 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 are able to 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 characterization 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 characterized 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.

2.11.1 Collagen molecular stability

2.11.1.1 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 utilized for preparative separation of collagen [142], it has been mainly used to compare collagen from different tissue types and to identify collagen types and polypeptide chains. Wu et al. [143] extracted bovine collagen and applied SDS-PAGE to identify the different collagen types present.

2.11.1.2 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 [144], collagen cross-linking [145], and thermal self-assembly. [146].

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 [144]. 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) [144].

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

2.11.1.3 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 [147] is multiplied by 7.46 to give the amount of collagen in the sample.

A large number of studies on collagen extraction have applied hydroxyproline analysis to calculate collagen content [97; 103; 148; 149]. Researchers have developed methods to effectively measure hydroxyproline concentration of collagen using calorimetric assays [22; 150], high-performance liquid chromatography, and enzymatic methods [150]. 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 [22].

When carrying out hydroxyproline assays for collagen content analysis, due to potential variations such as in temperature, humidity and air pressure in the hydroxyproline assays, calibration standards should be prepared on a daily basis. Due to changes in water content, moisture content analysis should be carried out prior to hydroxyproline analysis. For the preparation of the calibration curve, calibration dilutions of hydroxyproline must be prepared and analysed within an hour before carrying out the hydroxyproline assays for collagen samples. According to the method of Neuman et al. [22], collagen samples should be hydrolysed in 6 M HCl in a tube block heater for 24 hours at 105 ℃. The hydroxyproline assays were prepared in 6% hydrogen peroxide in distilled water, 5% p-dimethylaminobenzaldehyde in n-propanol, 0.01 M $CuSO_4$ and 0.5 M NaOH. From each acid hydrolysed collagen samples, six sub-samples were added to 20 ml Pyrex test tubes. To each test tube, 0.5 ml of 0.01 M CuSO4, 0.5 M NaOH and 6% H_2O_2 were added, resulting in a colour change from clear to blue, to green to brown and to dark blue or black. The capped test tubes were mixed well. The test tubes were further placed in an 80°C water bath for 10 minutes and mixed twice to release the hydrogen peroxide. The release of hydrogen peroxide is crucial as any remaining H_2O_2 would react with p-dimethylaminobenzaldehyde in the following step affecting colour formation. The test tubes are removed from the water bath and placed in ice. 1.5 M H_2SO_4 was added to each test tube, 5% p-

dimethylaminobenzaldehyde in n-propanol was also added to each test tube. The test tubes were mixed well and placed in a 70°C water bath for 15 minutes to develop the pink/red colour. Lastly, the test tubes were cooled and mixed again. The solution absorbance was measured utilizing a UV spectrophotometer at 540 nm. 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 [83].

2.11.2 Collagen denaturation temperature (Td) and thermal stability

2.11.2.1 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℃ 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 has an effect on the folding and unfolding process when hydrogen bonds are broken and connected [151; 152]. 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 [153]. 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 [154].

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 a number of additional hydrophobic and ionic interactions that must be accounted for in regards to collagen denaturation. The presence of non-collagenous components in the extracted collagen sample can cause variations in thermal denaturation [155].

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 [77; 156].

2.11.2.2 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. [157] 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-solubilized 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. On the other hand, Nakano et al. [158] showed a two-stage degradation profile, indicating the first stage (150-200 °C) to be related to elimination reaction such as dehydration

and decarboxylation at side chains of collagen and relating the second stage (300-400 ℃) to collagen degradation.

2.11.3 Collagen morphology

2.11.3.1 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 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. [157] used SEM to observe the surface morphologies of freeze-dried acid-solubilized fish skin collagen. The fish skin collagen was mounted on brass studs and coated with gold using an ion coater. The Accelerating voltage was 5-20 kV and images were taken with a scanning electron microscope (VEGA3 SBH TESCAN). 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. [16], Tziveleka et al. [159], Rodrigues et al. [160], Pal et al. [156] 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 2.15).

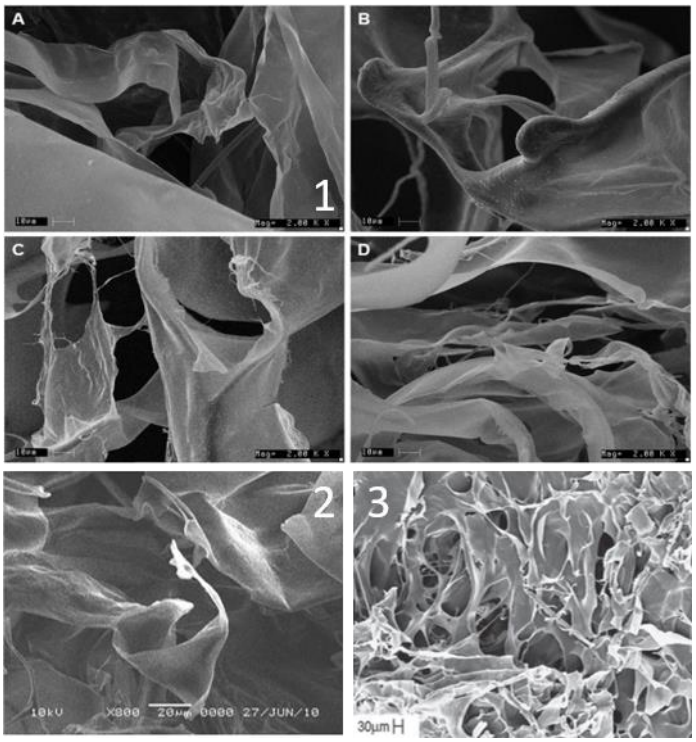

Figure 2.15: SEM images of extracted collagen, with 1) acid-soluble collagen of Catla fish (A), pepsin-soluble collagen of Catla fish (B), acid-soluble collagen of Rohu fish (C) and pepsin-soluble collagen of Rohu fish (D) [156], (2) being from buffalo skin [16] and 3) being SEM image of porcine skin collagen [160] (used with permission).

2.11.3.2 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 2.16 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.

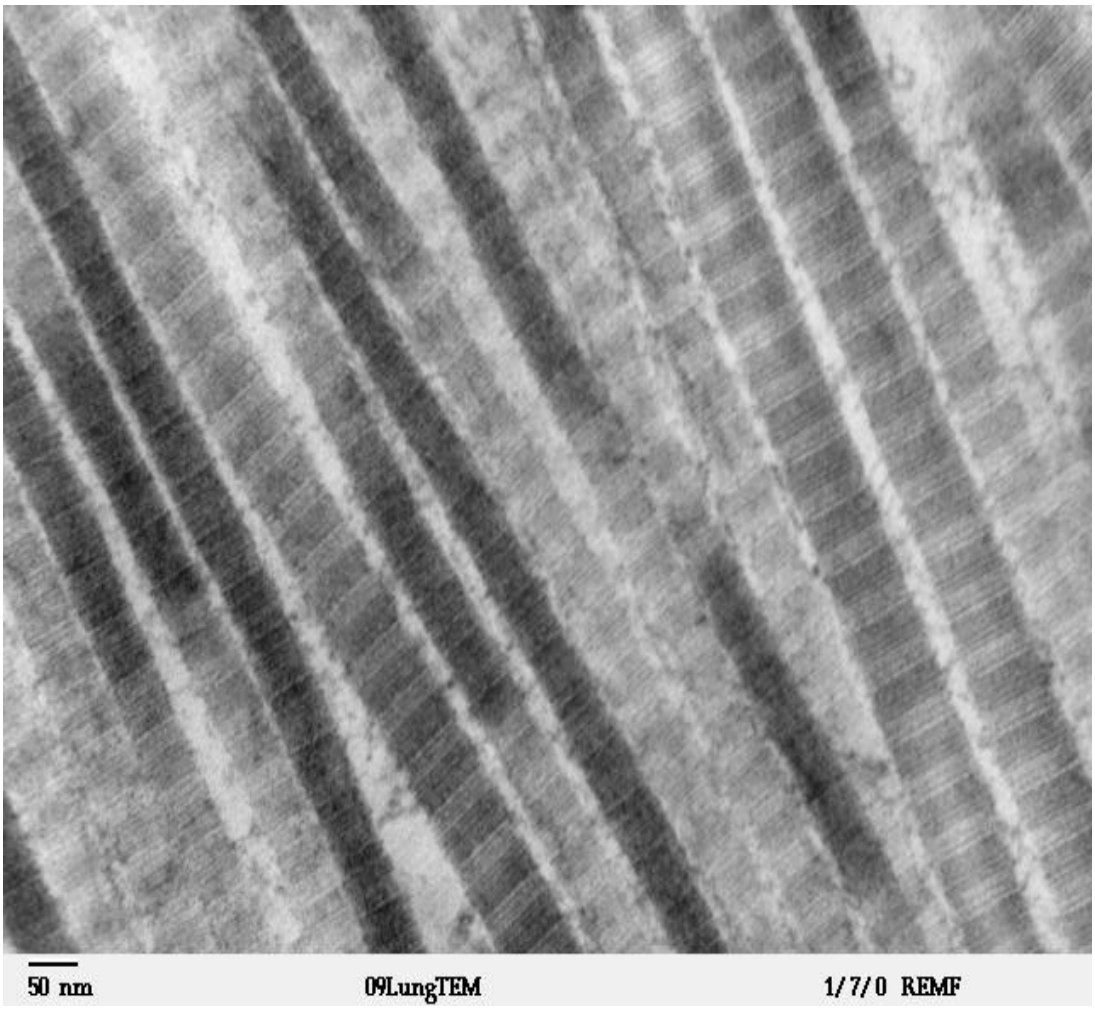

Figure 2.16: Transition electron microscope image of lung tissue collagen showing fibres of collagen [27] (used with permission).

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. The Karnovsky fixative is explained in more detail in Chapter 4.

Chapter 3

Collagen extraction from various waste bovine hide sources

A paper

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By

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[1]As first author of this paper, I prepared the initial draft manuscript, which was refined and edited with consultation with my supervisor, who has been credited as co-author. This Chapter is presented as a journal paper in the submitted format.

Collagen Extraction from Various Waste Bovine Hide Sources

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3.1 Abstract

Purpose: Hide is a by-product of meat production and is mostly used for leather production. Collagen is the main protein in mammalian skin, connective tissue, and cartilage and presents an opportunity for value addition to waste hide off-cuttings by extracting collagen. Three different extraction methods were applied to five different hide sources. The hide sources differed with respect to the animal's age, sex, diet and environment and influenced collagen extractables yield, and therefore the economic benefit of extraction.

Methods: Acid-solubilisation (AS), acid-enzyme solubilisation (AES1) and modified acid-enzyme solubilisation (AES2) were used to extract collagen from bull, calf, cow, face-pieces and ox-hides.

Results: The highest dry collagen content was from cow hides using the AES2 method (75.13 %), followed closely by bull hides at 74.45 %. On the other hand, the lowest collagen content was from cow hides (3.80 %) with the AS extraction method and the AS method proved to be inefficient for collagen extraction from bull, cow, face-piece and ox-hide sources. Analysis concluded that all the samples were of Type I collagen with α, β, and γ components.

Conclusions: Waste bovine hide off-cuttings can be used to extract high value product of collagen. AES2 proved to be the most preferable method of extraction out of the three

methods applied and considering leather to collagen revenue, these waste bovine hide off-cuttings could potentially result in substantial revenue.

Keywords: waste hide, collagen, collagen content, acid-soluble collagen, pepsin soluble collagen

3.2 Introduction

Collagen is the most abundant protein in mammals, making up to 30 % of whole-body protein content [52]. The collagen molecule is a triple-helix of three distinct alpha chains of repeating units of $(Gly\text{-}X\text{-}Y)_N$ amino acids, where X and Y are any amino acids. However, X is often proline and Y is often hydroxyproline [4; 5].

Purified collagen can be used for regenerative medicine and cosmetics, such as collagen injections for improving appearance, in body lotions and mascaras [67; 161]. Collagen is also used in casings [7], supplements [8], films [9], pharmaceuticals [10], as a precursor to biodegradable materials, for tissue engineering and more recently in 3D printing [10; 11; 25; 46; 51-54; 57; 60; 62; 162-174]. Demand for collagen is rising at approximately 20% annually and global collagen and hyaluronic acid (HA) based biomaterials market predicted to reach US$4.6 billion by 2020 [147; 167].

Hides, a by-product of meat production, are mostly used for leather production [175]. The bovine hide is approximately 30% protein and the inner corium layer of the hide is rich in collagen. This collagen has a high denaturation temperature in comparison to collagen from marine sources. Collagen can be extracted from fish and porcine sources but present limitations. Applications of fish collagen are limited because of its lower hydroxyproline content [12; 85] giving the collagen a low denaturation temperature while porcine products are prohibited by Muslim and Jewish communities [13].

Hide off-cuttings, not used in the leather industry; mostly end up in landfill or at most as animal feed. Converting this waste material into a high-value end-product, such as collagen, will benefit both the environment as well as the leather processor. The structure of bovine hide is influenced by age, sex, diet and environment [176] and may affect collagen structure and product yield. The research examines the effect of extraction method on collagen yield and collagen characteristics from different bovine sources with the aim of identifying a cost-competitive process for applications such as biopolymer films or bio-scaffolds. Methods of acid-solubilization (AS), acid-enzyme solubilization (AES1) and a modified acid-enzyme solubilization (AES2) method were applied. The AS method works due to collagen polypeptides in a solution having a positive charge and addition of an acid, it becomes dominant. Hence, solubilization is enhanced by repulsion among tropocollagen. In acid-

enzyme collagen extraction, the enzyme (pepsin) removes the non-helical ends of the collagen, allowing solubilisation [98] which is dependent on the acid concentration [177].

Proximate composition (ash, fat, and moisture), collagen content and average hide thickness of raw hides were determined. Materials were pre-treated then three different extraction methods (acid-solubilization, acid enzyme solubilisation and a modified acid-enzyme solubilisation) were used to solubilize the collagen. The extracts were purified by dialysis and characterized using SDS-PAGE and FITR. Extractables dry yield was calculated and hydroxyproline assays were done to quantify collagen content.

3.3 Experimental

3.3.1 Materials

Hide sections from different, unknown cattle breeds, of bull (BH), calf (CH1), cow (CH2), ox (OH) and bovine face-pieces (FP) were collected from a local tannery (Wallace Corporation Ltd, Waitoa, New Zealand) and stored at -20 $^{\circ}$C until used. Normally, the hide samples would not need to be stored at -20 $^{\circ}$C if extraction is to proceed immediately after sample collection. These represent animals slaughtered at 1-24 weeks (CH1), 16-18 months (BH), 2-4 years (OH) or four years (CH2) [45, 46]. The face-pieces came from animals of all ages.

Dialysis membranes (MWCO, 14 kDa), standard commercial collagen, pepsin enzyme from porcine gastric mucosa, L-hydroxyproline, high-molecular-weight protein markers, sodium acetate, citric acid, hydrochloric acid, acetic acid and SDS gel buffer strips were purchased from Sigma Aldrich. All other chemicals such as mercaptoethanol, sodium hydroxide, sodium chloride and methanol were analytical grade.

3.4 Extraction methods

3.4.1 Pre-treating the hide sections

Hide off-cuttings were de-haired and bleached in 0.5 M NaOH for 24 hours prior to size reduction. Samples were washed with distilled water and cut into 1 X 1 cm squares. The pre-treated hides were stored at 4°C until processed using one of three methods (Figure 3.1).

Extraction temperature, acetic acid concentration and pH used in each method are summarised in Table 3.1.

3.4.2 Acid-soluble (AS) collagen extraction

Non-collagenous material was removed by soaking pre-treated hide sections in 1:10 w/v of 0.1 M NaOH for 6 hours. The tissue was washed with distilled water until a neutral pH was achieved. The pieces were defatted by soaking in 1:10 w/v of 10% butyl alcohol for 18 hours and acid solubilized in 30 volumes of 0.5 M acetic acid for 24 hours. Collagen was precipitated using 2.5 M NaCl and the precipitate collected by centrifugation at 4000 rpm for 30 minutes. The pellet was dissolved in 0.5 M acetic acid (1:9 w/v) for 24 hours before being placed in dialysis tubing and dialysed against distilled water.

3.4.3 Acid-enzyme soluble (AES1) collagen extraction

All steps were done with continuous stirring. Hide samples were defatted in 20 volumes of 0.1 M NaOH and 0.1M NaCl for 24 hours washed with distilled water until a neutral pH was obtained. Samples were demineralized in 0.1 M HCl and 0.1 M NaCl (1:20 w/v) for 24 hours and washed with distilled water to a neutral pH. The defatted samples were suspended in 20 volumes of 0.5 M acetic acid and 1% (w/w) pepsin for 24 hours and filtered through a 4-mm and 250-µm filter. The collagen in the filtrate was precipitated using 2.5 M NaCl and left for 4°C for 24 hours before being centrifuged at 4000 rpm for 60 minutes. The pellet containing collagen was rinsed with distilled water, frozen overnight and freeze-dried for 48 hours.

3.4.4 Modified acid-enzyme soluble (AES2) collagen extraction

By combining all of the useful extraction procedures described in the literature, a more comprehensive method was developed (AES2). The AES2 method was modified in terms of temperature, time of extraction steps, repetition of extraction steps, concentration of chemicals and purification steps.

All steps were done with continuous stirring. Pre-treated hide sections were defatted by being mixed for six hours in 1:20 w/v of 0.1 M NaOH and 0.1 M NaCl. This was repeated a further three times before the samples were washed with distilled water until a neutral pH was obtained. The samples were demineralized in 1:20 w/v 0.1 M HCl and 0.1 M NaCl for 2 hours. The tissue was soaked in 20 volumes of 0.7 M acetic acid and 1% (w/w) pepsin for 48 hours

to solubilize the collagen. The supernatant was filtered through a 4-mm and then a 250-µm filter. The collagen in the filtrate was precipitated with 2.5 M NaCl then left at 4°C for 24 hours and centrifuged at 4000 rpm for 15 minutes. The pallet was dissolved in 1:9 w/v 0.7 M acetic acid at for 24 hours being dialyzed (Membra-Cel MD44-14) in 20 volumes of acetic acid, followed by distilled water for 24 hours. The dialysate was renewed every 2 hours. The purified extractables were frozen overnight and freeze-dried for 24-72 hours.

3.4.5 Lyophilisation

A known amount of purified collagen was placed in 250 ml LabServ plastic containers and frozen overnight. The samples were then placed in a Freezone 2.5 Labcono freeze drier under a vacuum of 42.0 Pa and -52°C for 48-72 hours.

Temperature control is an important factor to consider with collagen extraction. Table 3.1 outlines the temperatures used for each extraction method in this study. For collagen extraction, the majority of researchers use low temperatures of around 4 ℃ to preserve collagen from possible contamination and spoilage. Contamination might occur due to enzyme hydrolysis or microbial degradation. However, some literature has carried out extraction at room temperature. In order to observe differences attained in the collagen properties with the influence of temperature, extractions were carried out both at 4 ℃ and 21 ℃.

Table 3.1: Details of temperature and acetic acid concentration used for the extractions.

Method	Temperature (°C)	Acetic acid concentration (M)	pH
AS	21	0.5	2.60
AES1	4	0.5	2.60
AES2	4	0.7	2.35

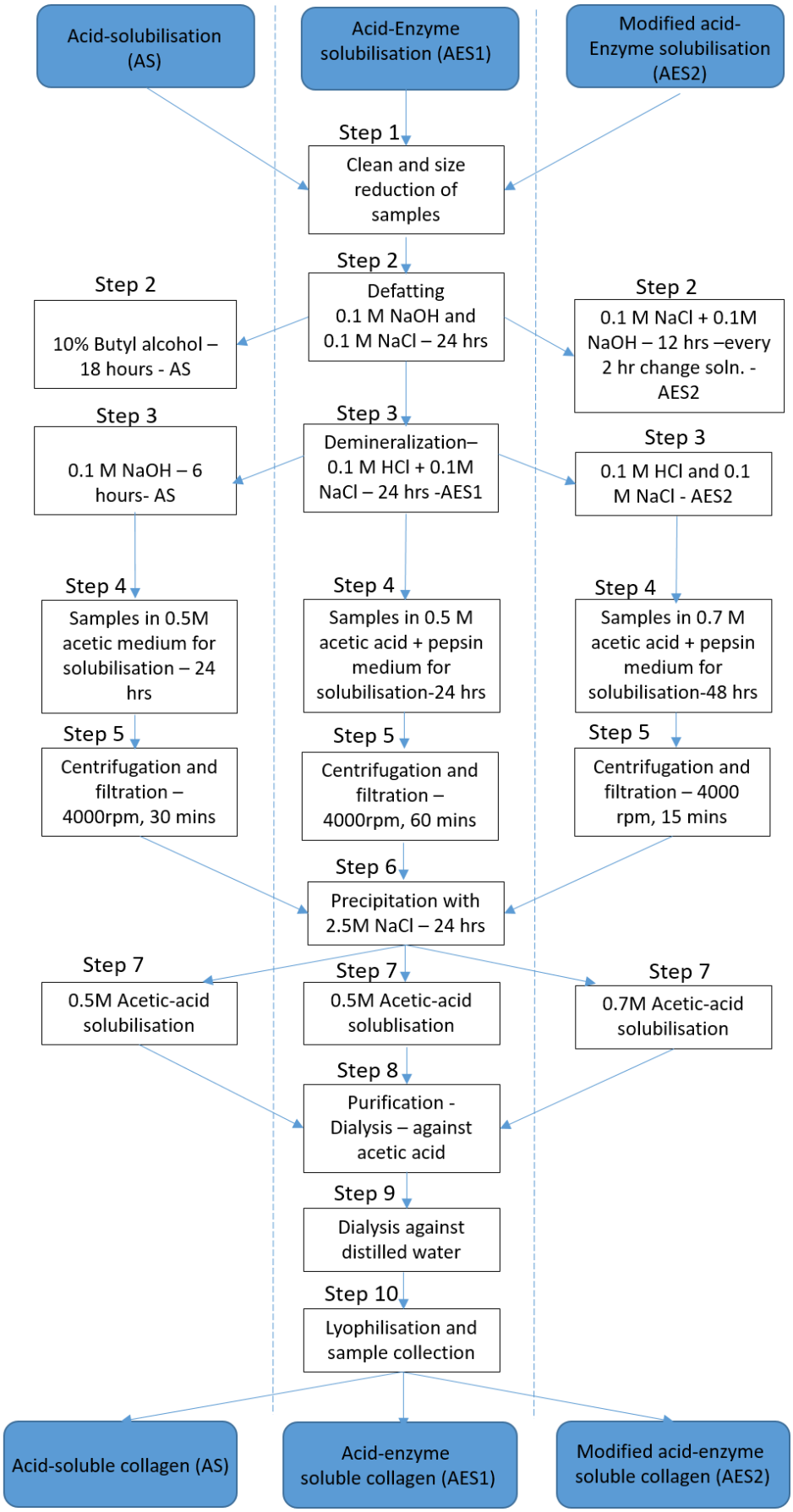


Figure 3.1: Flow chart of the three extraction methods.

3.5 Proximate analysis of raw material

Moisture content (MC) was measured by drying samples at 105°C for 24 hours in a Contherm Thermotech 2000 oven (Equation 1-1). Fat content was determined by Soxhlet extraction with petroleum ether (60-80°C) for 24 hours (Equation 1-2). Ash content was determined by heating approximately 5 g of defatted sample in a K2F/4 B&L-TetFlow furnace at 600°C for 24 hours (Equation 1-3). Hydroxyproline content was determined by digesting the raw hides in 6 M HCl for 24 hours at 105°C then using the calorimetric method of Carlson and Neuman [22; 178] at 540 nm. Total collagen content was obtained by multiplying the hydroxyproline content by 7.46 (Equation 1-4). Raw material density was determined by recording the weight and dimensions of rectangular hide samples and averaging the data.

$$\%\ Moisture\ content = \frac{M_{initial,wet} - M_{dry}}{M_{initial,wet}} * 100 \quad (1\text{-}1)$$

$$\%\ Fat = \frac{M_{Fat}}{M_{initial,dry}} * 100 \quad (1\text{-}2)$$

$$\%\ Ash\ (dry\ basis) = \frac{M_{Ash}}{M_{initial,dry}} * 100 \quad (1\text{-}3)$$

$$\%\ Collagen\ content = \frac{\mu g\ hyp\ in\ 1mg\ hydrolsate}{\mu g\ tissue\ represented\ in\ 1mg\ hydrolysate} * 7.46 * 100 \quad (1\text{-}4)$$

where:

- $M_{initial,wet}$ = initial wet mass of hide sample
- $M_{initial,dry}$ The = initial mass of hide after oven drying
- M_{Fat} = mass of fat collected from hide sample
- M_{Ash}= mass of ash from hide sample

3.6 Extractables and collagen characterization

Collagen content was determined using a modified hydroxyproline assay [22; 178]. Type I commercial standard collagen was used as a control. Because temperature, humidity and air pressure can affect the assay, calibration standards were prepared daily. As water content can change during storage and preparation, moisture content was analysed immediately before the hydroxyproline assay. Samples were hydrolysed in 6 M hydrochloric acid at 105°C for 24 hours. The hydroxyproline assays were done using 6% hydrogen peroxide in distilled water, 5% p-dimethylaminobenzaldehyde in n-propanol, 0.01 M $CuSO_4$ and 0.5 M NaOH. Samples of approximately 5 mg of acid hydrolysed collagen was added to six test tubes and then 0.5 ml of 0.01 M CuSO4, 0.5 M NaOH and 6% H_2O_2 were added. The test tubes were placed in an 80°C water bath for 10 minutes and mixed twice to release the hydrogen peroxide. 2 ml of 1.5M H_2SO_4 and 5% p-dimethylaminobenzaldehyde in n-propanol was then added to each test tube, the test tubes were mixed well and placed in a 70°C water bath for 15 minutes. UV absorbance was measured at 540 nm. 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 (Equation 1-2)[83].

Extractables yield (Equations 1-5) and collagen content (Equation 1-4) were calculated and moisture content of extracted solids determined.

$$\%\ Extractables\ yield\ \ (dry\ basis) = \frac{M_{dry\ extract}}{M_{initial,dry}} * 100 \tag{1-5}$$

Fourier-transform infrared spectroscopy (FTIR) was performed on the extracted purified collagen. Discs with 2 mg of collagen and approximately 100 mg potassium bromide (KBr) were prepared and spectra from 4000 to 500 cm^{-1} measured in an infrared spectrophotometer (FTIR Digilab FTS-40).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a modified method of Wu et al. [143]. Before analysis, extracted collagen samples were denatured in SDS-buffer solution at 100°C. The sample (10 mg) was added to micro test tubes containing 0.5 M Tris-HCl buffer, 50% glycerol, 10% SDS, 0.02% β-bromophenol blue, 5% β-

mercaptoethanol and water then loaded onto a PhastGel gradient and run at 200 V on the PhastSystem. A control containing commercial standard collagen and a protein marker was also run. After 10 minutes, the PhastGels were collected and soaked overnight in Coomassie staining solution containing 0.2% (w/v) Coomassie blue, 45% (v/v) methanol, 45% (v/v) distilled water and 10% (v/v) acetic acid. The gels were further de-stained by soaking them in 5% (v/v) methanol, 7% (v/v) acetic acid and 88% (v/v) distilled water until only the stained bands remained. The gels were removed from the solution and dried at room temperature.

3.7 Results and discussion

The five types of hide sources had observable structural and physical differences and it was hypothesised hide type would affect extractable yield and collagen content. Collagen cross-linking between typically increases with animal age [179] and the main difference between calf hides (CH1) and the rest was the age at which the animals were slaughtered (Table 3.2). Calves are typically slaughtered at a very young age compared to the other sources [180; 181]. The main difference between bull and cows is their sex while for cows and oxen is that cows are mostly kept for meat/milk production and breeding purposes while oxen are working animals [182].

Table 3.2: Thickness and density of hides from different bovine sources.

Material	Thickness (mm)	Density (g/cm^3)
Bull hide (BH)	12-15	1.53 ± 0.10
Calf hide (CH1)	3-5	1.32 ± 0.12
Cow hide (CH2)	5-10	1.58 ± 0.11
Face-piece (FP)	3-5	1.34 ± 0.19
Ox hide (OH)	5-10	1.60 ± 0.16

BH was the thickest (Table 3.2) and hide from young animals (CH1) and face pieces were the thinnest. Hides from older animals (BH, OH and CH) were denser than that from young animals (CH1) or face pieces (FP) (Table 3.2).

3.7.1 Proximate composition of the raw material

Table 3.3 illustrates the proximate composition of the hides prior to collagen extraction. Collagen content (dry basis) of all hide sources were high, however, BH resulted in the highest collagen content at 65.40% followed by OH at 54.82% (Table 3.3). FP resulted in the lowest collagen content of 36.00%, followed by CH1 at 41.1%. Ash content (%) ranged from 1.34-3.16%. Fat content (%) ranged from 12.12%-22.17% for the five hide sources and water content (%) of hides ranged from 62.91-66.48%. which correlates to literature values for cattle hides (63-65%) [51]. The raw samples all had similar dry matter contents of about 35%.

Table 3.3: Effect of source on proximate composition of raw bovine hide.

	Raw material				
	Calf hide (CH1)	Cowhide (CH)	Bovine face-piece (FP)	Bull hide (BH)	Oxhide (OH)
Sample mass (g)	100	100	100	100	100
-Water (g)	62.91	63.99	66.48	65.32	63.58
-Solids (g)	37.09	36.01	33.52	34.68	36.42
-Fat (dry basis)(g)	5.60	4.38	7.43	5.17	5.67
-Ash (dry basis) (g)	1.13	1.09	0.89	1.10	0.49
-Tissue (dry basis) (g)	30.37	30.55	25.20	28.42	30.26
-Collagen (dry basis) (g)	15.21	19.06	12.07	22.68	19.97
-Other material (dry basis) (g)	15.16	11.49	13.13	5.74	10.30
Raw hide collagen content (Dry basis, %)	40.87	52.93	36.01	65.40	54.83

Based on the results presented here, it would be logical to focus on recovering collagen from bull hides, especially considering that processing leather from bull hides are more difficult for some tanneries. However, the values presented in Table 3.3 are the total collagen content and does not necessarily mean it is easily recoverable, nor does it guarantee good quality. A tannery processing all the animal sources listed may, in fact, wish to process only parts of each of the sources, possibly those that would alternatively yield the lowest quality leather.

3.7.2 Proximate composition of extracted dry solids

3.7.2.1 Extractables yield

Figure 3.2 illustrates the total extractable yield, which is a mixture of salts, acids, small fragments of tissue and collagen. Wet and dry yields are often considered as "collagen yield" in literature with no specification of purity, however, this is clearly inaccurate and the product should be further analysed for its actual collagen content.

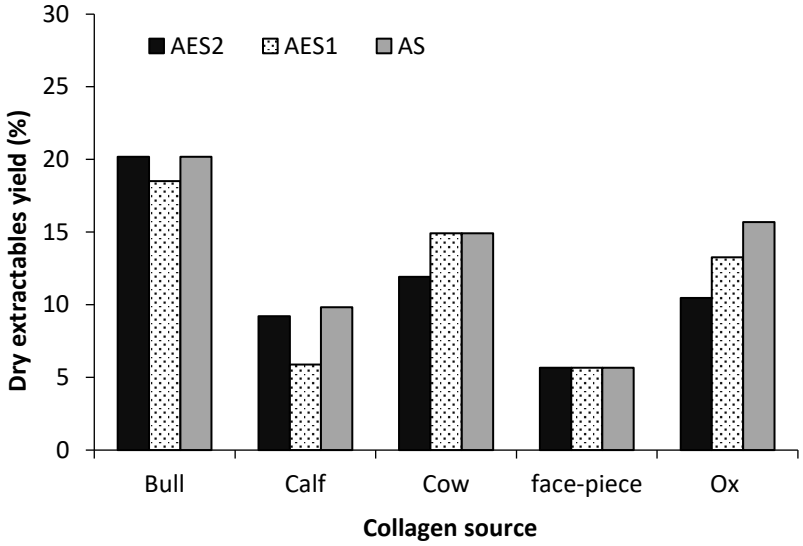


Figure 3.2: Effect of hide source and extraction method on the amount of material extracted: extractables (%) dry product yield.

Dry product yields (%) for each method were similar within each source (Figure 3.2). The AS extraction method resulted in the highest extractables dry yield amongst all cattle hide types. Both enzyme extraction methods produced lower yields. However, these are not true "collagen yields" as it does not consider purity. Pre-treatment parameters such as physical handling, pH, temperature and time of extraction could result in variances of collagen yield during the extraction processes.

3.7.3 Collagen content

The collagen content of the extracted collagen material was determined by hydroxyproline analysis and UV spectrometry.

The AS extraction method resulted in low collagen content (Table 3.4); significantly lower than the other methods used. The method of AS works due to collagen polypeptides in solution having a positive charge and addition of an acid, such as in acid-solubilisation, it becomes dominant. Hence solubilisation is enhanced by repulsion among tropocollagen. Thus, the sole use of the AS method is unable to break the crosslinks holding the non-helical ends of tropocollagen molecules to one another. Collagen fibres are stabilized by this extensive network of intermolecular cross-links. The degree of cross-linking in collagen is a big determinant of how much collagen can possibly be extracted. The proportion and the degree of intramuscular collagen crosslinking depend on muscle type, species, genotype, age, sex, and level of physical exercise [183]. Incomplete solubility of collagen suggests that inter-molecular cross-links are still present in collagen molecules as this is the case with AS extraction of older sources such as BH, CH2 and OH [88; 98].

The number of collagen cross-links increases with age [179]. The AS extraction method resulted in the highest collagen content for CH1 (Table 3.4). Taking into consideration the fact that younger sources such as calf hide have a very small amount of cross-linking as compared to older sources, it would be cost-efficient to extract collagen via the AS method. Reduction of pepsin enzyme and the sole use of AS extraction could reduce production costs significantly. Hides maintained at elevated temperatures prior to collagen extraction result in the denaturation of collagen leading to relaxation of the forces holding the collagen molecule together. The relaxation of tension is an important characteristic of hides since it is different for animals of different ages. In particular, hides from younger animals such as calves are generally weaker and exhibit a great degree of relaxation. This could possibly be another reason for the low extracted collagen content with the AS method as the extraction temperature was the highest (21℃) in comparison to AES1 and AES2 methods (4℃).

Table 3.4: Collagen content of extractables as obtained through hydroxyproline analysis, via extraction methods of AS, AES1 and AES2 and statistical summary of extraction method relevance.

Source	Collagen content (%)		
	AS	**AES1**	**AES2**
Bull hide (BH)	5.30 ± 4.7	30.20 ± 0.87	74.45 ± 0.36
Calf hide (CH1)	25.70 ± 1.3	19.50 ± 0.78	65.72 ± 0.31
Cow hide (CH2)	3.80 ± 3.3	26.90 ± 0.32	75.13 ± 0.16
Face-piece (FP)	4.90 ± 3.0	15.40 ± 1.16	48.86 ± 0.20
Ox hide (OH)	5.20 ± 2.6	30.10 ± 0.26	64.52 ± 0.45
Mean (%)	8.98	24.42	65.74
St.dev.S	9.37	6.66	10.61
Sum	44.90	122.10	328.68
Variance.S	87.72	44.35	112.61
T-Test	**AS-AES1**	**AS-AES2**	**AES1-AES2**
P-value	0.017	1.904E-5	7.811E-5

Alongside with collagen, structural proteins such as elastin and keratin are also present in the bovine hide. There is a high possibility that with collagen extraction, elastin and keratin are also solubilised to a certain extent. This means that with the acid solubilisation method, a high amount of extractables was recovered. However, as collagen is not solubilised with acid, the collagen content was low suggesting it was mostly hydrolysed elastin and keratin and would explain why the dry extractable yield was similar between the methods. As collagen content is determined by the hydroxyproline content of the extractables, it should be noted that elastin also contains hydroxyproline [184], which may lead to some error.

Both enzymatic methods of AES1 and AES2 resulted in an increased collagen content for all bovine hides compared to AS extraction. The main reason for this is the cleaving action of the enzyme to the cross-links which hold the tropocollagen molecule which is formed into collagen fibres with the aid of cross-links. This result supports previous acid extraction work on fish skin where significant lower collagen yields were obtained in comparison to enzymatic methods [75; 85; 89; 98; 185].

The AES1 and AES2 also resulted in a higher collagen content due to the fact that the initial stage of collagen solubilisation is the hydration of fibrous collagen which proceeds by exposure to acids. Acid solubilisation (AS) aids in removal of acidic proteins due to weakening of interactions between the acidic proteins and collagen fibrils thus acid-enzyme solubilisation (AES1 and AES2) maximizes the extraction process by further removing the cross-links that are stable in acid-solubilization are also removed, hence this method is a lot more effective than sole acid-solubilization [186]. However, the efficiency of enzyme-solubilisation is limited by location and type of intermolecular cross-links present in each source. Hence, there is a collagen content difference among sources tested [88; 98].

Heu et al [187] reported that one source can result in different collagen yields depending on what extraction method is used. He concluded that collagen from the skin of flatfish resulted in different yields, where, acid-solubilized collagen resulted in a yield of 57.3% and pepsin-solubilized collagen was 85.5%. Singh [140] also observed this increase with pepsin-solubilisation.

Kim et al. [186] reported that collagen content is influenced by a number of factors, such as acid concentration, the ratio of raw material to the acid solution, incubation time and temperature. Collagen content isn't solely dependent on extraction conditions but also on the raw material source and tissue type. Time of incubation has a huge influence on collagen content as the longer the incubation time, the greater the collagen content. AES2 had the longest incubation time, followed by AES1 and AS. Hence it was further observed that the AES2 method showed higher values in comparison to the other two extraction methods.

Kiew [88] reported the influence of acetic acid concentration on the extractability of collagen from the skin of fish. Kiew [88] concluded that the maximum yield of pepsin-acid solubilised collagen was achieved when 0.7 M acetic acid was used. A substantial drop in collagen yield was seen if the concentration of acetic acid was increased beyond 0.7 M. This was the case for the method of AES2 where 0.7 M (Figure 3.1) acetic acid was used and an increase both in collagen yield and collagen content was seen compared to the AES1 and AS methods where 0.5 M acetic acid was used (Figure 3.1). The difference in collagen content obtained with different acid concentrations could also be due to different solubility of collagen in the extracting medium. Kiew [88] showed 0.1 M acetic acid concentration to be the least effective

solvent for acid-pepsin collagen extraction, however, the amount of dissolved collagen increased as the concentration of acetic acid was increased. Wang et al. [188] also reported increased yields of collagen from the skin of grass carp as acetic acid concentration was increased. It is also reported by Wang et al [188] that collagen is denatured at extremely low pH, such as pH 2.0 or below collagenous fibres start to shrink and thus making protein hydration impossible.

Bowes et al [189] observed different hydroxyproline contents with acid-soluble and alkaline soluble collagen. Their study also concluded that only about two-thirds of the total protein extracted under acidic conditions are precipitated by salt and is of a collagenous type. Concluding that the remaining third part consists of albumins and globulins.

T-Test analysis determined if there is a significant difference between collagen yields from the different sources as well as between the extraction methods (Table 3.4).

P-values (for all combinations AS- AES1, AS-AES2, and AES1-AES2) confirmed that on a 95% confidence interval, that there was a significant difference between the extraction methods and collagen content from each source.

3.7.4 Fourier-transform infrared spectroscopy (FTIR)

AES2 extracted collagen from all hide sources were analysed for their secondary structure. The spectrum showed similar trends to literature by showing similar bands of type I collagen. This includes the main absorption bands of amide A, amide B, amide I, amide II, amide III. Amide A band is associated with N-H stretching vibration which occurs in the range 3400-3440 cm^{-1} and indicates the presence of hydrogen bonds [83]. Drastic differences in Amide peak position among the collagen sources were not observed.

Amide B bands for BH, CH2, CH1 and OH-collagen were observed at 2928, 2923, 2921, and 2923 cm^{-1} respectively. Amide B band is related to the asymmetrical stretch of CH_2 stretching vibration. The wavenumber and amplitude differences of the Amide A and Amide B found in the collagen sources indicated that the secondary structure of collagen could possibly be different, especially between BH and FP-collagen (Figure 3.3).

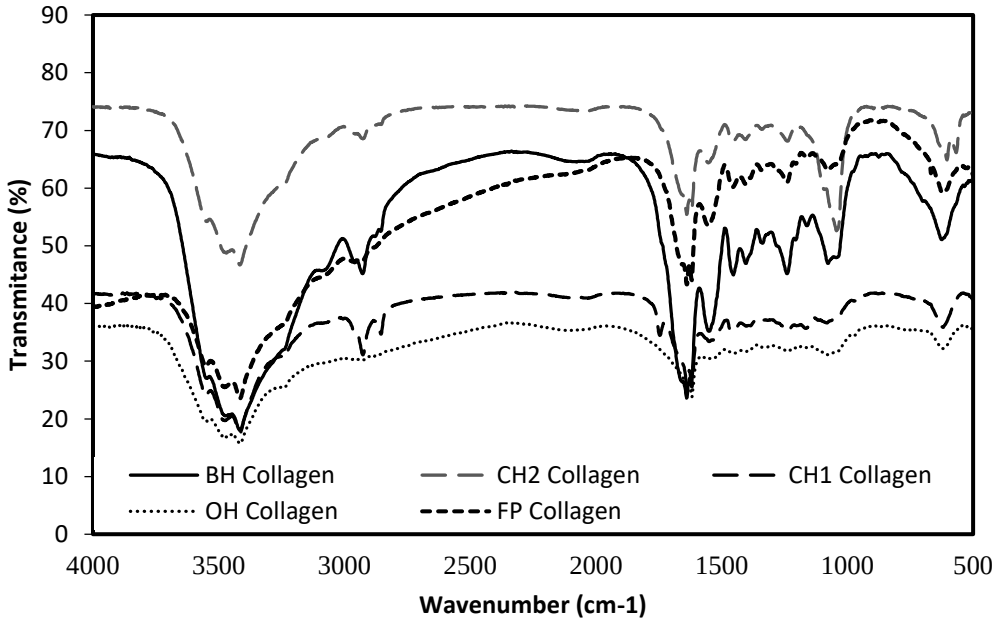


Figure 3.3: FTIR spectra for BH, CH1, CH2, OH, and, FP collagen.

Table 3.5: Main FTIR peak locations of BH, CH2, CH1 and OH-collagen.

	Component peak location (cm^{-1})				
Collagen source	Peak 1 (Amide A)	Peak 2 (Amide B)	Peak 3 (Amide I)	Peak 4 (Amide II)	Peak 5 (Amide III)
BH	3416	2928	1635	1548	1246
CH2	3418	2923	1620	1548	1229
CH1	3410	2921	1640	1477	1236
OH	3418	2923	1620	1524	1225
FP	3415	2935	1617	1546	1236

The Amide I peak is associated with C=O stretching vibration or stretching or possibly hydrogen bonding coupled with COO- (1600-1700 cm^{-1}). The Amide I-III peak locations for all collagen sources are shown in (Table 3.5) where the Amide I peak is thought to be a sensitive marker for the secondary structure of proteins. BH-collagen Amide I peak is very prominent and sharp in comparison to the other collagen sources (Figure 3.3). The Amide II band is associated with N-H bending vibration coupled with C-N stretch (1540-1560 cm^{-1}), indicative of the N-H group being involved in hydrogen bonding. The intensity of OH, CH1, FP and CH2 collagen was less than BH collagen for Amide II peak, which could possibly indicate less involvement of N-H groups involved in hydrogen bonding and thus a lower stability of triple

helix. Amide III is associated with C-N stretching vibration with N-H bend and C-O stretching. Amide III peak and intensity is an important characteristic of collagen. Generally, for collagen, the absorption intensity ratio between Amide III band and the 1450 cm^{-1} band (CH_2 bend) should be 1 for triple helix conformation and 0.5 is usually observed for gelatine [139; 190]. The absorption intensity ratio between Amide III band for collagen sources BH, CH2, CH1, OH and FP collagen were 0.86, 0.85, 0.85, 0.84, and 0.85 respectively. All absorption intensity ratios were way above 0.5, this indicates that the triple helix of all collagen sources was preserved.

3.7.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

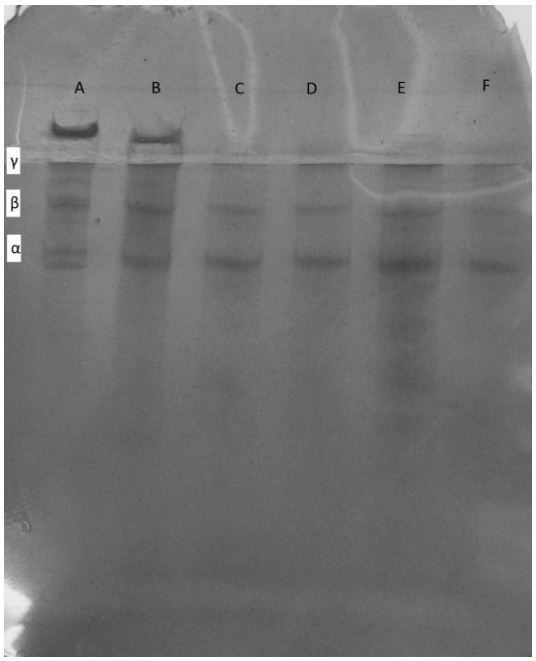


Figure 3.4: SDS-PAGE bands for BH collagen (B), CH1 collagen (C), CH2 collagen (D), OH collagen (E), and FP collagen (F), along with a protein marker (A) to identify the molecular weights.

During collagen preparation for SDS-PAGE, the boiling process denatures the collagen and the native triple-helix structure of collagen is lost. Thus, the individual polypeptide chains assume a random coil configuration. Covalent bonds occur between polypeptide chains in collagen and these can link either two or all three chains together.

Collagen from all sources tested displayed one γ-band (290-340kDa), one β-band (≈170-200kDa) and α-band (≈95-100kD) and confirmed the presence of unfolded polypeptide chains from the triple helix of collagen. This pattern also indicates that the extracted collagen from bovine hide is type I.

Extracted collagen showed the three polypeptide chain molecular distribution as the pepsin only attacks the non-triple helical domain of native collagen and the preparative procedures are not severe thus the peptide bonds are not broken [80]. Wide distributions would only be observed if the extracted collagen was destroyed during the extraction procedure. Thus, it can be stated that acid-pepsin solubilized purified collagen has not been destroyed (denatured) during the extraction procedure and resembles the standard bovine collagen both visually and in terms of its molecular distribution. Cheng et al [149], Lin et al [77], and Skierka and Sadowska [98] observed similar molecular weight distributions for extracted collagen with different species.

3.7.6 Estimated collagen production flow

Table 3.6 illustrates the likely collagen production based on raw material proximate composition, extracted collagen content and dry extractables yield. Based on collagen extraction recovery for each source and mass of collagen per gram of wet sample, collagen produced per year was calculated. BH would result in the largest product production rate (135.30 tonnes per year) followed by collagen from cow and OH.

Considering the high cost of collagen, bovine hide off-cuttings can be used to produce a very attractive high value-end product. Collagen markets include cosmetics industries, biomedicine, collagen films and tissue engineering. Majority of collagen that is sold in the market for food industries and cosmetics is not pure native collagen but rather broken down collagen amino acids (gelatine) or hydrolysed forms. These latter forms are sold at more reasonable prices due to the lack of specific processing steps and chemicals used.

At a wholesale price of $6.17 per gram of collagen, a revenue of $834,801,000 could be generated per year just from BH-collagen. Hence at a low selling price of $6.17 per gram of collagen can still result in substantial revenue. Collagen costing $6.17 or less per gram in most cases is collagen peptides or in its hydrolysed form. If this is the market then any bovine hide

off-cuttings and shavings could be processed into collagen peptides (gelatine) without the requirement of costly enzymes or chemicals. However, for biomedical and lab grade purposes, collagen must be in its stable insoluble triple-helical form and this requires careful extraction steps and use of both chemicals and enzymes which add up the processing costs. Native high purity collagen which is usually extracted for medicinal purposes is in its stable triple-helix form costs as high as $26,666.67 per gram (Sigma Aldrich [191]). Some chemicals companies do provide cheaper alternatives, such as "Collagen Solutions" which is still insoluble in water and can be used for lab-grade purposes. Hence, if the reference cost of collagen from "Collagen Solutions" at $76 NZD/g [192] is used to approximate a revenue based on the estimated collagen production from bull-hide (135.30 tonnes/year) and calf-hide (30.9 tonnes/year), a revenue of $1.026E10 and $234,840,0000 per year could be generated respectively. However, the collagen production (tonnes/year) (Table 3.6) values are based on the hypothetical ideal assumption that the whole bovine hide will be processed for collagen rather than just hide off-cuttings and shavings. On the other hand, looking at the cost of finished tanned skins and its revenue. Hides are sold on a per skin basis with an estimated average weight when it is packed and calf skins are graded from Premium to light. Some tanneries sell bovine skin in the range of $500-$800 per skin [193]. Not considering the processing costs, if ideally according to Table 3.6, 900 bull hides are sold at a maximum price of $800 per skin, a revenue of $720,000 could be generated. This value is still significantly lower in comparison to the lowest cost that collagen can be sold at $6.17 per gram.

However, environmental concerns, which 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. In reality, only about 25% by weight of raw salted hides results in the finished leather [14]. Furthermore, other solid wastes are also produced from wastewater and sludge treatment. Bovine waste off-cuttings can result in a favourable revenue reducing landfill waste and adding value to the economy.

Table 3.6: Mass balance of collagen production per year for each source.

	Source				
	Bovine face-piece	**Bull hide**	**Calf hide**	**Cow hide**	**Ox hide**
Mass processed per year (tonnes)*	360	900	510	810	900
Collagen (g/g wet sample)	0.12	0.23	0.15	0.19	0.20
Processing					
Collagen Recovery (%)**	22.9	66.2	39.8	46.0	34.3
Collagen produced (tonne/year)	9.9	135.3	30.9	71.0	61.7

*= based on an approximate number of hides processed by Wallace Corporation Ltd (NZ) annually and the average weight of each hide.

**= based on mass inflow and outflow of collagen, considering collagen content/purity.

3.8 Conclusions

It can be concluded that collagen of high yield and content can be extracted from bovine hides of bull, calf, cow, ox and face-pieces by acid-solubilisation, acid-enzyme solubilisation and a modified acid-enzyme solubilisation. Modification of the acid-enzyme soluble collagen extraction method resulted in significantly higher collagen content. Purification of collagen was a necessary step and it resulted in a clean white agglomerated powder, which resembled the standard collagen from Sigma Aldrich. Hydroxyproline assays indicated a sufficient content of hydroxyproline in the extracted collagenous tissue, thus it can be confirmed that the extracted material is collagen. SDS-PAGE analysis and FTIR confirmed the secondary structure and the triple helix and the chains that comprise the collagen molecule. This study has resulted in a new method to extract collagen of high extractables yield and content in an efficient manner. Thus, the extracted collagen from cow, calf, bull, face-piece and ox-hide, via the method of AES2 are suitable for a variety of different collagen-derived applications such as collagen biopolymer films is to be investigated in the near future.

3.9 Acknowledgements

We would like to acknowledge Wallace Corporation Ltd (New Zealand) for providing the raw materials.

Chapter 4

Microscopy and thermal analysis

4.1 Introduction

This chapter investigates the morphology, thermal stability and secondary structure of collagen recovered from calf (CH1) and bull hide (BH). In chapter 3, extraction from CH1 and BH resulted in the highest yield, using the least expensive method (addition of pepsin was not necessary for CH1).

Generally, in literature it is agreed that the thermal denaturing temperature (Td) of collagen is dependent on the source [156], extraction method [156], hydroxyproline content [77; 155; 156; 194], degree of cross-linking [195-197], water content [195-198], and presence of other compounds [198]. Td is an important property of collagen because film casting requires heating the film-forming solution (FFS) to temperatures as high as 50 ℃. For optimal film properties, the temperature during film preparation should be kept below Td. Kittiphattanabawon et al. [148] studied collagen from the skin of splendid squid and found a denaturation temperature of 34.1 ℃ using of acid-enzyme solubilisation, while Yan et al. [84] used the method of acid-solubilization and showed that the denaturing temperature was as low as 24.6 ℃. On the other hand, Lin et al. [77] extracted bovine skin collagen using the method of acid-solubilization and showed the Td to be 82.71 ℃, while Hickman et al. [194] extracted bovine hide collagen via the method of acid-enzyme solubilization and shown Td to be 42.0 ℃.

Fourier transform spectroscopy is a useful technique to assess collagen denaturation [137; 138; 144; 156]. Kumar et al. [156] and Muyonga et al. [144] have shown a reduction in the intensity of the amide A, I, II and III to be indicators of collagen denaturation.

The morphology of extracted collagen is of interest to observe fibre uniformity. Hayes et al. [199] used transmission electron microscopy (TEM) to observe uniform, regularly spaced collagen fibres as well as aggregates of smaller collagen bundles. Rizk et al. [16] and Tziveleka et al. [159] examined the microstructure of collagen with scanning electron microscopy (SEM) and observed smooth, wrinkled

and folded sheets, while Rodrigues et al. [160] revealed images of dense porous structures.

The purpose of this chapter is to use known techniques to assess the structure and thermal properties of the two selected bovine collagen sources and compare their suitability for film preparation. This includes scanning electron microscopy (SEM), transmission electron microscopy (TEM), Fourier-transform spectroscopy (FTIR), thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC).

4.2 Materials and methods

4.2.1 Materials

Calf and bull-hide off-cuts were obtained from Wallace Corporation Ltd (New Zealand). Pepsin, sodium hydroxide, acetic acid, hydrochloric acid, sodium chloride, dialysis tubes (Membra-Cel MD44-14) and all other chemicals and enzymes used were of analytical grade and obtained from Sigma Aldrich.

4.2.2 Collagen extraction

A modified acid-enzyme solubilisation method (described in detail in chapter 3) was used for extraction. The skin samples were cut in 2 x 2 cm^2 sections, weighed and placed in 0.5 M NaOH at 1:10 (w/v) for de-hairing purposes for 24 hours at 4°C. Pre-treated hide sections were stirred in 20 volumes of 0.1 M NaOH and 0.1 M NaCl for 12 hours with a change of solution every 6 hours (de-fatting). This step was repeated with a new batch of 0.1 M NaOH and 0.1 M NaCl every 6 hours for another 12 hours and then further washed with distilled water until a neutral pH was achieved. Demineralization was carried out in a solution of 0.1M HCl and 0.1M NaCl (1:20 w/v) for 12 hours with a change of solution every 2 hours. The defatted, demineralised tissue was washed with distilled water until a neutral pH was achieved.

The tissue was soaked in of 0.7 M acetic acid and 1% (w/w) pepsin at a 1:20 w/v for 48 hours to solubilize the collagen. The solubilized hide tissue was then filtered through a coarse 4 mm filter, followed by a fine 250 µm filter. The filtrate solution was collected and precipitated by addition of 2.5 M NaCl. The filtrate samples

were inverted gently to precipitate the collagen and left at 4 °C for 24 hours. The filtrate samples were centrifuged at 4000 rpm for 15 minutes. The pellet containing the precipitated collagen was collected, dissolved in 0.5M acetic acid at 1:9 (w/v) for 24 hours.

Dialysis was used for purification and samples were placed in dialysis tubing (Membra-Cel MD44-14) using 0.1M acetic acid and final dialysis against distilled water for 24 hours with dialysate being renewed every 2 hours. The purified collagen was frozen overnight prior to lyophilization for 24-72 hours.

4.2.3 Scanning electron microscopy

Collagen morphology was examined a Hitachi S-4700 scanning electron microscope (SEM). Before imaging, the freeze-dried and oven-dried collagen samples were platinum-coated for (80 seconds) under 3 kV current utilizing Ion Sputter Coater (Hitachi E-1030, Japan). Type I native collagen from bovine Achilles tendon (Sigma Aldrich) was used as a reference. Subsequently, the collagen samples were observed at an acceleration of 20 kV.

4.2.4 Transmission electron microscope

Collagen samples were fixed for 8 hours at room temperature in a modified Karnovsky's fixative for two hours at room temperature. The fixative contained 3% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer (pH 7.2). The samples were washed, then post-fixed three times for 10–15 min each in phosphate buffer (0.1 M, pH 7.2). The samples were dehydrated in a graded series of ethanol washes (25%, 50%, 75%, 95%, and 100%) for 10-15 minutes. This was followed by a final 100% ethanol wash for 1 h. Samples were critical-point (CP) dried using a Polaron E3000 series II critical-point drying apparatus in which liquid CO_2 was used as the CP fluid and 100% ethanol was used as the intermediary fluid. A Tecnai (FEI Tecnai G2 BioTwin) Transmission Electron Microscope was used for imaging.

4.2.5 Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) was performed on the extracted collagen. Discs containing 2 mg collagen in approximately 100 mg potassium

bromide (KBr) were prepared. Spectra were obtained by using an infrared spectrophotometer (FTIR Digilab FTS-40) from 4000 to 500 cm^{-1} with each sample being subjected to an average of 16 scans.

4.2.6 Thermogravimetric analysis

Thermal stability of extracted collagen was investigated using a gravimetric analyser (SDT 2960, TA Instruments). Approximately 5 mg of sample was used. The mass loss was recorded while the sample was heated from room temperature to 700 °C at a rate of 10 °C per minute in air purged at 150 ml/min with an empty pan as a reference. Differential thermal analysis (DTA) and thermal analysis (TGA) curves were obtained at the end of the operation.

4.2.7 Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to determine the thermal denaturation temperature (Td) of collagen samples using a Perkin Elmer Differential Scanning Calorimeter (DSC-8500). Approximately 2-8 mg collagen samples were weighed into aluminium pans and sealed. The samples were scanned at 50 ℃/min over the range of -50 ℃-150 ℃ using liquid nitrogen as the cooling medium. An empty pan was used as a reference. Denaturation temperature was obtained from the transition in the baseline in the 30-80 ℃ region by taking the inflexion point.

4.3 Results and discussion

4.3.1 Fourier-transform infrared spectroscopy

FTIR spectra of BH and CH1 are presented in Figure 4.1 alongside with spectra of standard Sigma collagen and gelatine for comparison purposes. Spectral changes, which are indicative of changes in collagen secondary structure, have been shown to include changes in the amide-A, amide B, amide I, amide II and amide III [144; 200; 201]. Generally, for collagen spectra, a slight shift to a lower wavenumber in the characteristic bands (amide A, amide B, amide I, amide II and amide III) are associated with increased molecular interactions via hydrogen bonding in collagen [146; 202; 203]. Increased hydrogen bonding indicates a sign of a stable triple helix, as the formation of collagen alpha chains into a triple helix requires the presence of hydrogen bonding both at inter and at the intra-molecular level.

FTIR spectra (Figure 4.1) showed the amide A peak position at 3414 cm^{-1} for both extracted collagen sources and standard Sigma collagen (Table 4.1). This band is associated with N-H stretching and indicates hydrogen bonding of N-H group with a carbonyl group of the peptide chain when the position of this peak is shifted to a lower frequency as compared to gelatine, suggesting the presence of intact triple-helical structures.

Asymmetrical stretching of CH_2 was shown by the presence of amide B band positions (Figure 4.1) at 2928 cm^{-1} for both BH and CH1 collagen (Table 4.1). This peak was very weak for the gelatine spectra (Figure 4.1, d), indicating loss of structure. CH1 also had relatively weak absorbance in this region, suggesting that some denaturing could have taken place.

The amide I peak positions for both BH and CH1 collagen was observed at 1638 cm^{-1}. This band represents hydrogen bonding between N-H and C=O along the polypeptide backbone. According to Muyonga et al. [144], the presence of this absorption confirms the formation of hydrogen bonding between N-H and C=O and hence, an indication of increased molecular interactions and helical stability.

The amide II band, which is due to N-H bending vibration coupled with a C-N stretching vibration, was observed at 1540 cm^{-1} and 1541 cm^{-1} for BH and CH1

collagen respectively. The shift of this wavenumber to a lower value indicates the presence of extensive hydrogen bonding in collagen and this was observed with both BH and CH1 collagen in comparison to gelatine (Table 4.1). A stable triple-helix of collagen requires the presence of hydrogen bonding both at inter and intra-molecular level. Hence, the greater the presence of hydrogen bonding at each characteristic band, the more stable the collagen triple helix.

The amide III band is associated with intermolecular interactions in collagen, comprising of C-N stretching vibration with N-H bend and C-O stretching was observed at 1239 cm^{-1} and 1240 cm^{-1} for BH and CH1 collagen respectively. The FTIR spectra for BH and CH1 collagen were consistent with the results of the SDS-PAGE in Chapter 3, where, gel electrophoresis did not show band smearing for CH1 collagen.

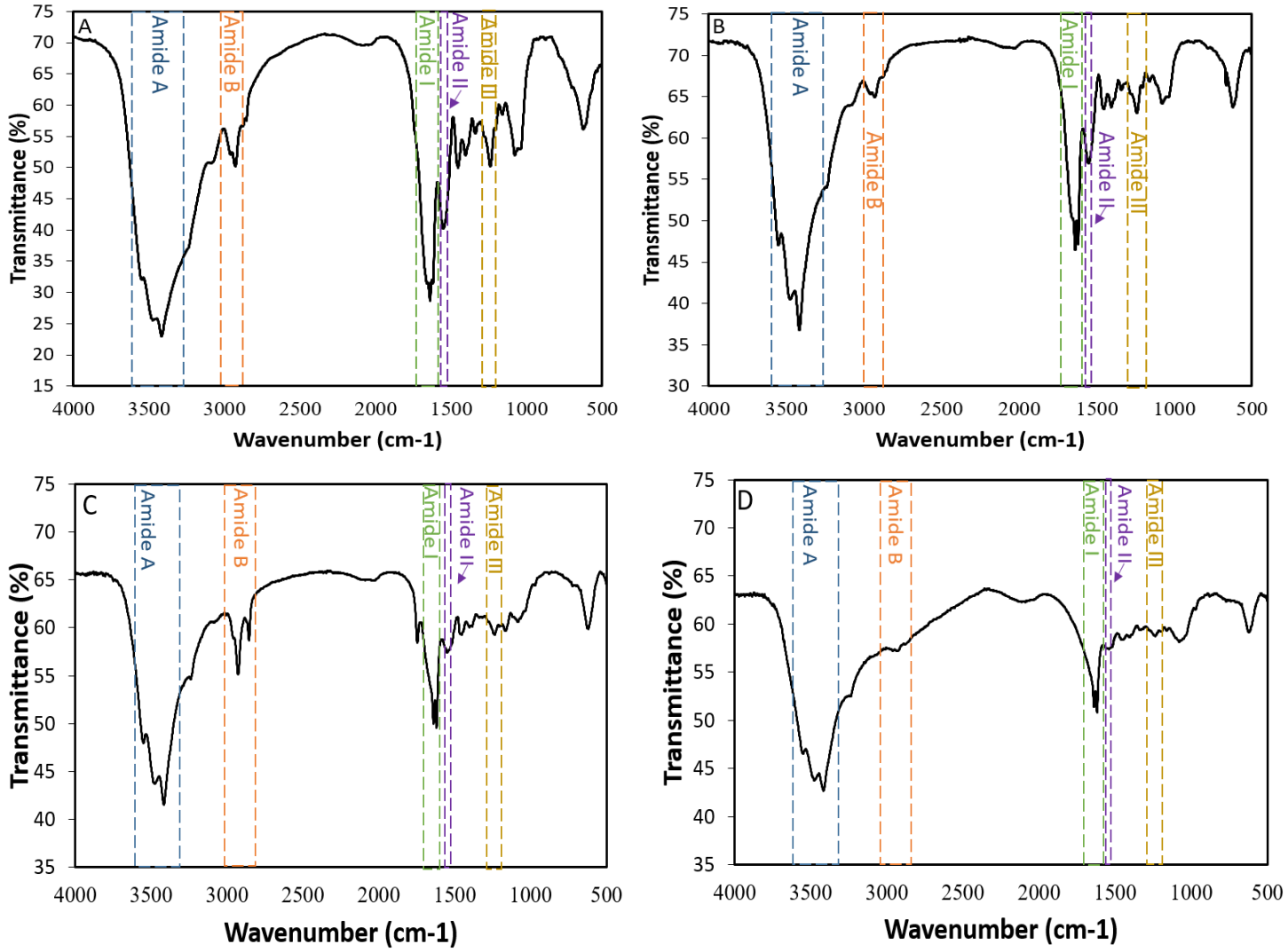


Figure 4.1: FTIR curves for BH collagen (A), CH1 collagen (B), standard sigma collagen (C) and gelatine (D).

Table 4.1: Peak location (cm-1) for BH and CH1 collagen.

	Component peak location (cm^{-1})				
Collagen source	Peak 1 (Amide A)	Peak 2 (Amide B)	Peak 3 (Amide I)	Peak 4 (Amide II)	Peak 5 (Amide III)
BH	3414	2928	1638	1540	1239
CH1	3414	2928	1638	1541	1240
Standard Sigma collagen	3414	2925	1638	1535	1237
Gelatine	3417	2930	1641	1557	1241

Amide I is the most crucial band for the secondary structure of proteins. This band is observed because of the stretching vibration of the peptide carbonyl group (-C=O). For a better understanding of the secondary structure of collagen and its main difference to gelatine, amide I band is deconvoluted. The deconvoluted spectrum of this band for collagen should show three components [204]. Deconvoluted amide I band peak wavenumbers of collagen has been associated to represent the presence of β-sheets in the range 1616-1637 cm^{-1}, α-helices in the range 1656-1662 cm^{-1} and β-turns when the peak falls in the range 1663-1696 cm^{-1} [205]. The deconvoluted spectrum of amide I band shows three components positioned at in the range 1620-1690 cm^{-1} for BH, CH1, and Sigma collagen (Figure 4.2). These associate with the presence of β-sheets, α-helices and β-turns and thus, an indication of a stable triple helix. However, the deconvoluted amide I band for gelatine shows presence of a peak at 1626 cm^{-1} indicating only presence of β-sheets.

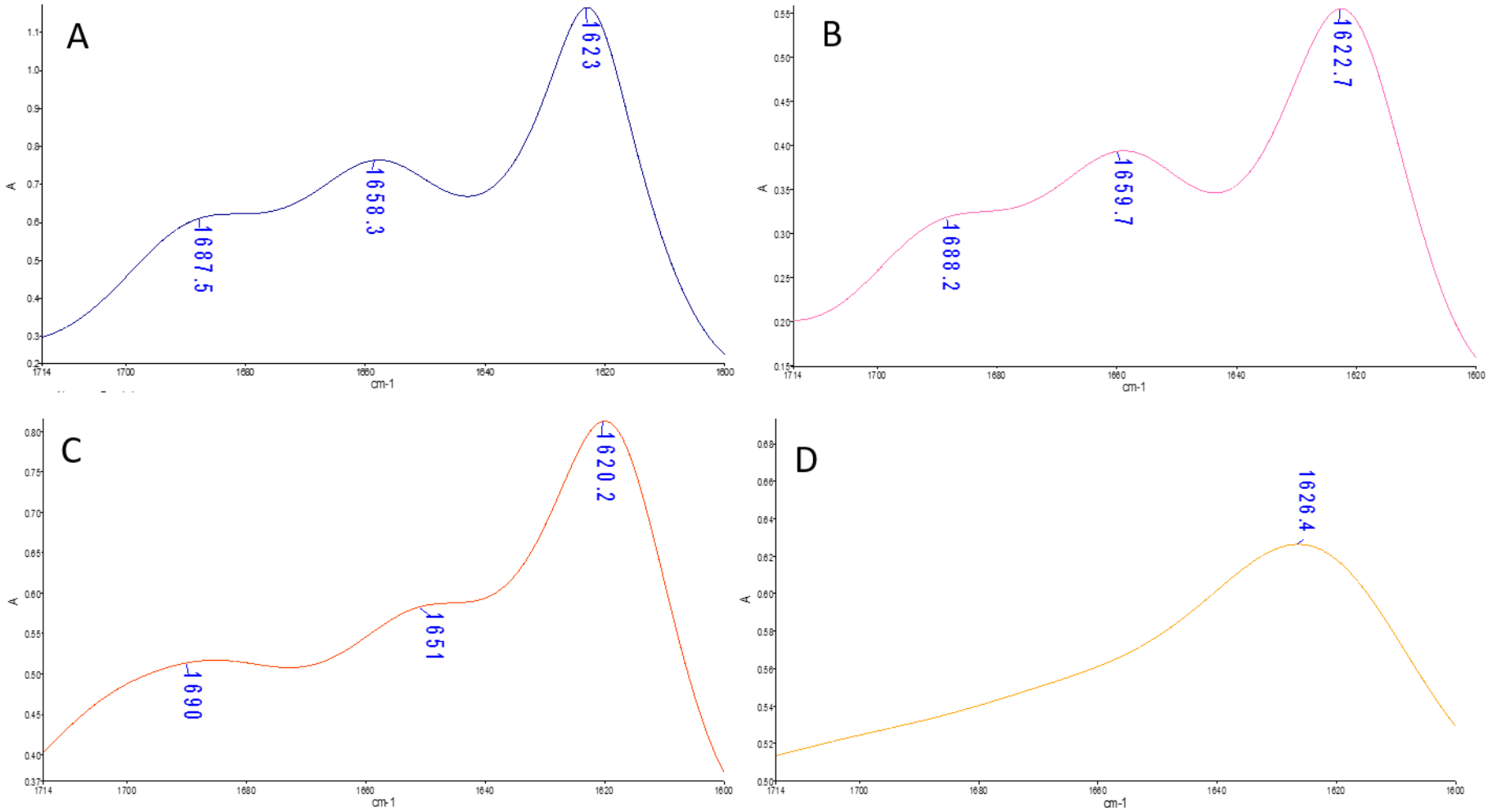


Figure 4.2: Deconvolution of amide I band for BH collagen (A), CH1 collagen (B), Sigma collagen (C), and gelatine (D).

4.3.2 Thermogravimetric analysis

Thermal degradation curves of BH and CH collagen are presented in Figure 4.3. Both samples showed a three-step process. The first notable weight loss occurring at approximately 30 ℃-100 ℃ was related to the removal of physically absorbed and bound water. The second transition corresponding to thermal decomposition and the last transition, corresponding to carbonization. The constant mass past 600 ℃ represents char. The most important transition in terms of collagen thermal stability is the second phase– both collagen sources showed this transition at approximately the same temperature. Rodrigues et al. [160] similarly observed a three-stage weight loss profile for porcine skin collagen with full degradation at 650 ℃. It is important to note that the degradation stage of collagen can alternatively increase or decrease depending on the extraction method, pre-conditioning treatments and possible absorbed moisture [158; 206]. Collagen from both sources showed to have similar thermal stability and hence, both sources can be utilized as the primary material for film preparation.

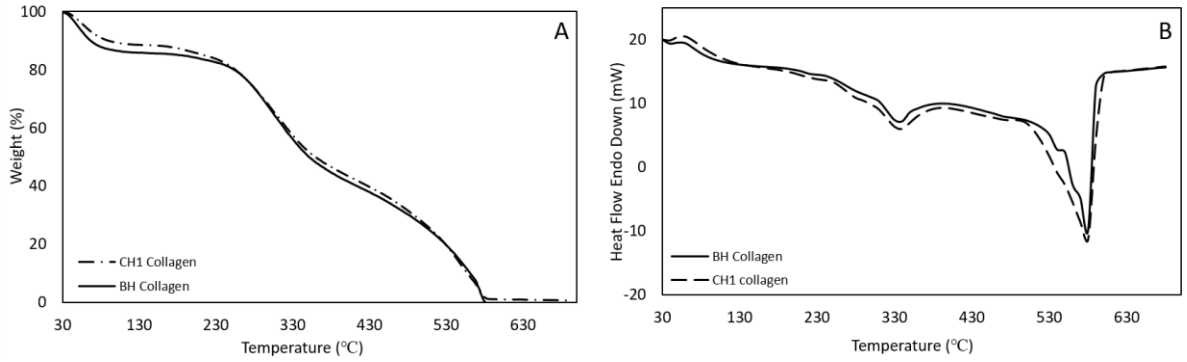


Figure 4.3: TGA weight loss (A) and DTA (B) curves of BH and CH1 collagen.

4.4 Differential scanning calorimetry

The denaturation of collagen (Td) was determined using differential scanning calorimetry (DSC) (Figure 4.4). The first endothermic transition, which has been magnified (smaller graph), is due to the helix-coil transition of collagen, which is the indication of denaturation. Denaturation temperatures of 51.90 ℃ and 45.36 ℃

were observed for BH and CH1-collagen respectively (Figure 4.4, a, b and Table 4.2).

The second transition on the DSC thermographs (Figure 4.4) corresponded to the release of water and BH and CH1 collagen showed this transition at 112.77 ℃ and 107.57 ℃, respectively.

DSC analysis of standard Sigma collagen and commercial gelatine were carried out to compare them as a reference to BH and CH1 collagen. Gelatine is fully denatured; hence, a Td is not detected. According to Mukherjee et al. [207] the first endothermic transition observed with gelatine (Figure 4.4, d), Table 4.2) in the range 70-80 ℃ is the glass transition temperature of gelatine. On the other hand, the standard Sigma collagen indicated a Td at 58.64 ℃ (Table 4.2)

The approximate 6 ℃ increase in denaturation temperature with BH collagen could be due to the higher content of hydroxyproline in the older tissue of BH. The hydroxyl groups of hydroxyproline may possibly aid as hydrogen donors for binding between the α-chains of the triple helix through hydrogen bonding. Benjakul et al. [208] associated a higher content of hydroxyproline with increasing thermal stability of collagen and higher values of Td – this was related to pyrrolidine rings of proline and hydroxyproline and hydrogen bonding via the hydroxyl group of hydroxyproline. On the other hand, Sinthusamran et al. [209] related variance of Td to confirmation and amino acid sequence of collagen. Method of collagen extraction and collagen source also results in fluctuation of Td [139; 144; 209]. Duan et al. [210] and Muyonga et al. [144] reported differences in thermal denaturation of collagen to be dependent on habitat temperature, age of tissue and season.

Drying results in a much higher denaturation temperature [195]. Hence, Td of collagen depends and varies directly with water content. Self-cross-linking of collagen molecules, while heat is being applied, is another factor, which contributes to the higher denaturation temperature.

The thermal properties of collagen via TGA and DSC indicated to show both sources could potentially be used for film preparation. FTIR confirmed the high Td values of BH and CH1 collagen by the presence of α-helices and β-sheets.

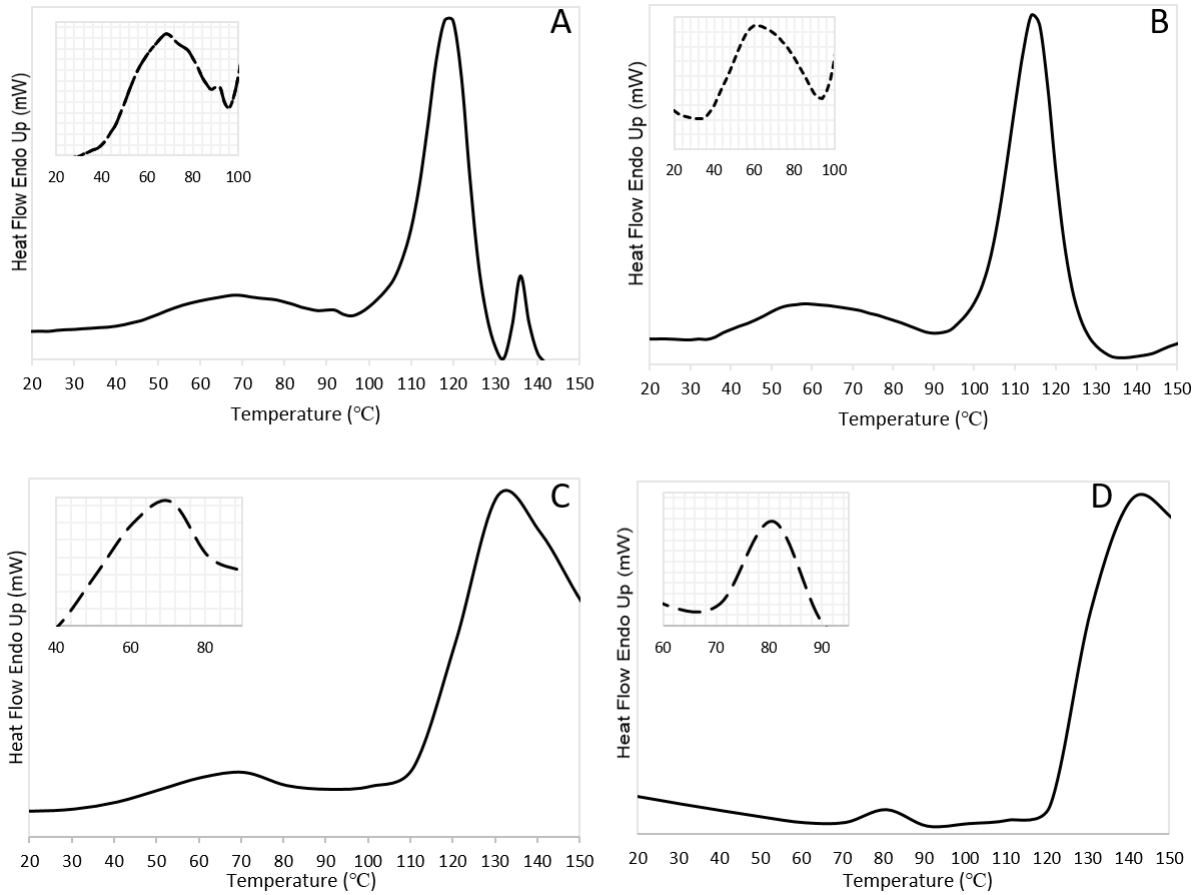


Figure 4.4: DSC thermogram of BH collagen (A), CH1 collagen (B), standard Sigma collagen (C) and gelatine (D).

Table 4.2: Denaturation temperatures and transitions BH, CH1 collagen, standard sigma collagen and gelatine from the DSC thermogram.

Sample	BH collagen	CH1 collagen	Standard sigma collagen	Gelatine
Endo. Transition 1	51.90 ℃	45.36 ℃	58.64 ℃	76.29 ℃
Endo. Transition 2	112.77 ℃	107.57 ℃	121.64 ℃	117.77 ℃

4.4.1 Scanning electron microscopy

SEM images (Figure 4.5), showed smooth flat sheet surfaces, with protruding strands observed occasionally for both BH and CH1 collagen. Standard Sigma collagen similarly showed the same microstructure as BH and CH1 collagen. This was the expected observation, to detect collagen sheets with a combination of threads and collagen strands that are bundled together to form a fibril network and a dense sheet-like structure [211].

Rizk et al. [16] and Tziveleka et al. [159] examined the microstructure of insoluble collagen and observed smooth wrinkled and folded sheets similar to BH and CH1 (Figure 4.5). Rodrigues et al [160] examined the morphology of pigskin collagen and found structures very similar to what was observed here. Barzideh et al. [211] extracted collagen from ribbon jellyfish via the method of pepsin-solubilization and examined the microstructure of collagen by SEM analysis and observed collagen sheets with occasional collagen strands as observed with both BH and CH1 collagen (Figure 4.5). Pal et al. [156] did not observe any drastic changes between collagen from different fish sources and both sources showed irregular dense sheet-like surfaces, similar to what was seen in Figure 4.5.

At SEM level, the structure of BH and CH1 collagen were collagen sheets with a combination of threads and strands, which was also seen with Sigma collagen. For a better understanding of differences in collagen, morphology between collagen extracted from BH and CH1 transition electron microscope (TEM) is used. TEM enables images to be taken to a resolution of nano-meters to observe the characteristic banding pattern formed by native collagen when precipitated into fibrils.

Small white spots were observed on both extracted BH and CH1 collagen types (as circled on Figure 4.5) indicating possible impurities, possibly salts. The small white spots were not observed with the standard Sigma collagen.

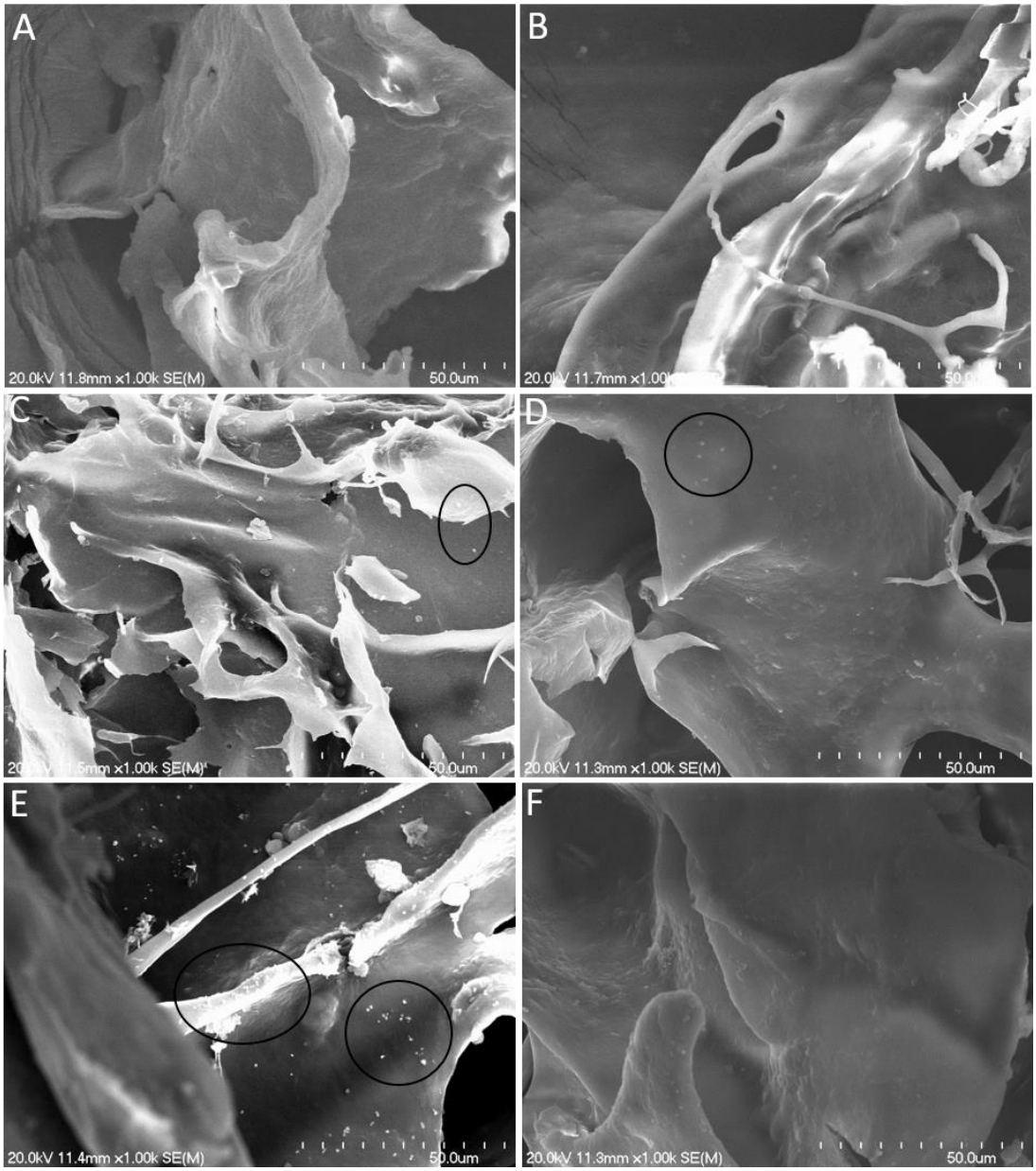


Figure 4.5: SEM images of commercial bovine collagen (Sigma Aldrich) (a-b), bull-hide collagen (c-d), and calf-hide collagen (e-f).

4.4.2 Transmission electron microscope

Figure 4.6 and Figure 4.7 are showing Transition Electron Microscopy (TEM) images of acid-enzyme extracted BH and CH1 collagen at different magnifications. Uniform, defined and highly ordered collagen fibrils can be observed in all magnifications (1µm, 100nm, 200nm) for BH collagen (Figure 4.6). Fibres (fibril bundles) are seen in images of A-F (Figure 4.6) indicating native collagen is extracted without any damage to the fibril structure. The banding pattern of individual fibrils (Figure 4.6, c and f) is a result of tropocollagen (collagen molecules) associating into collagen fibres.

On the other hand, CH1 showed no sign of an organized structure (Figure 4.7). It was hypothesized that CH1 collagen would also show uniform and defined collagen fibrils under TEM. Thermal stability and the secondary structure of both sources were very similar as observed with DSC, TGA and FTIR analysis. This cannot be due to collagen denaturation as FTIR analysis indicated a stable triple-helical structure and both TGA and DSC indicated the thermal stability of CH1 collagen to be very similar to the BH collagen. One possibility for the loss of order in TEM images of CH1 could be due to sections or a specific batch of CH1 collagen (being from a younger source and therefore having a lot less cross-linking) to have been digested by the acids used in the extraction. A very small amount of collagen is used for TEM analysis and the digested part of CH1 collagen could possibly have been used for analysis.

It can be concluded from this section that potentially bits of CH1 collagen to have been digested by acids during extraction. Furthermore, a specific batch of CH1 collagen, which was sent for TEM analysis, could have possibly been denatured. This point is emphasized on as all other characterizations showed CH1 collagen to be stable and very similar to BH and Sigma collagen.

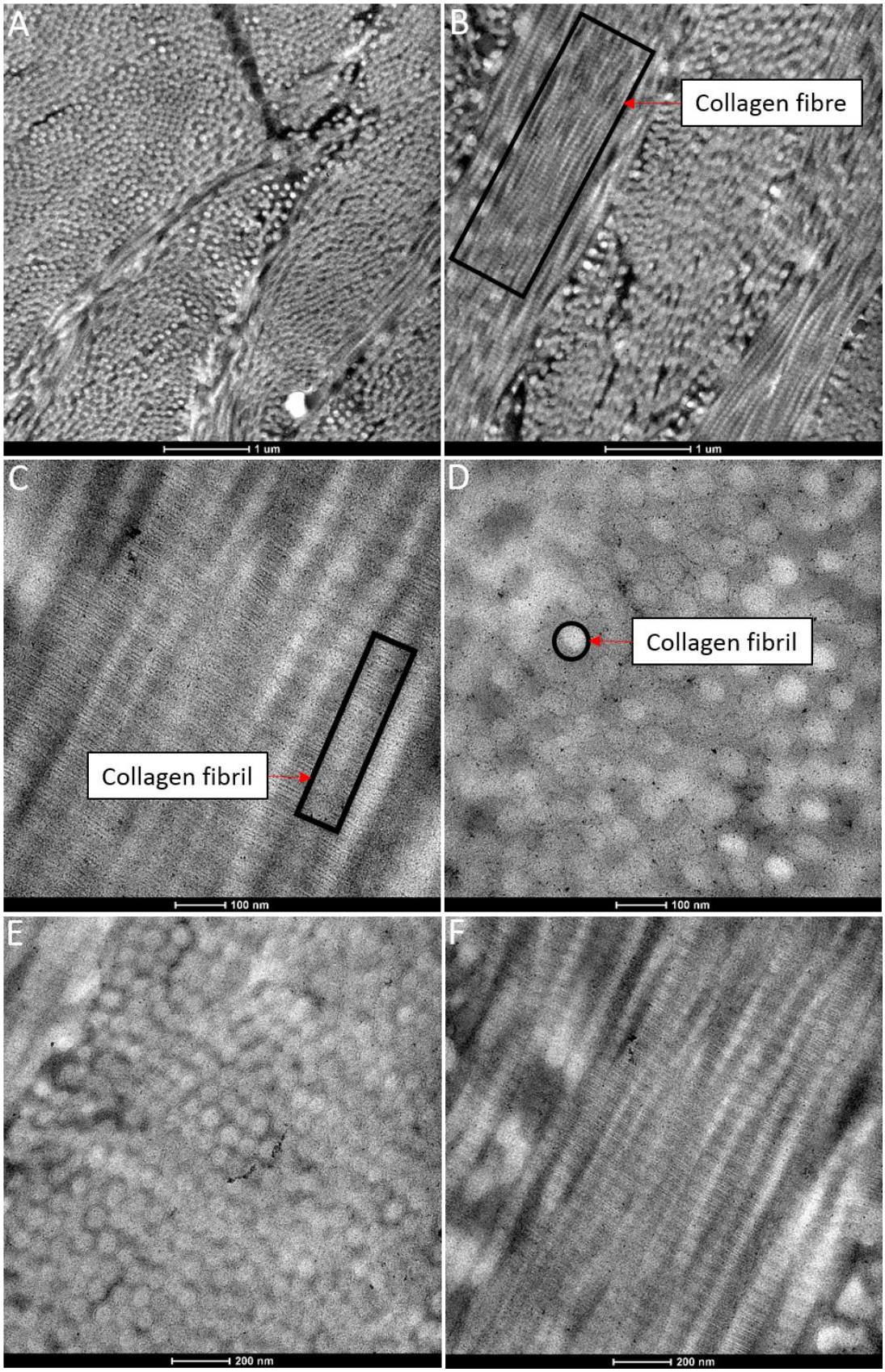


Figure 4.6: Transmission electron microscopy images of Bull-hide collagen at 1um (a-b), 100nm (c-d) and 200nm (e-f) shown both cross-sectional and longitudinal views.

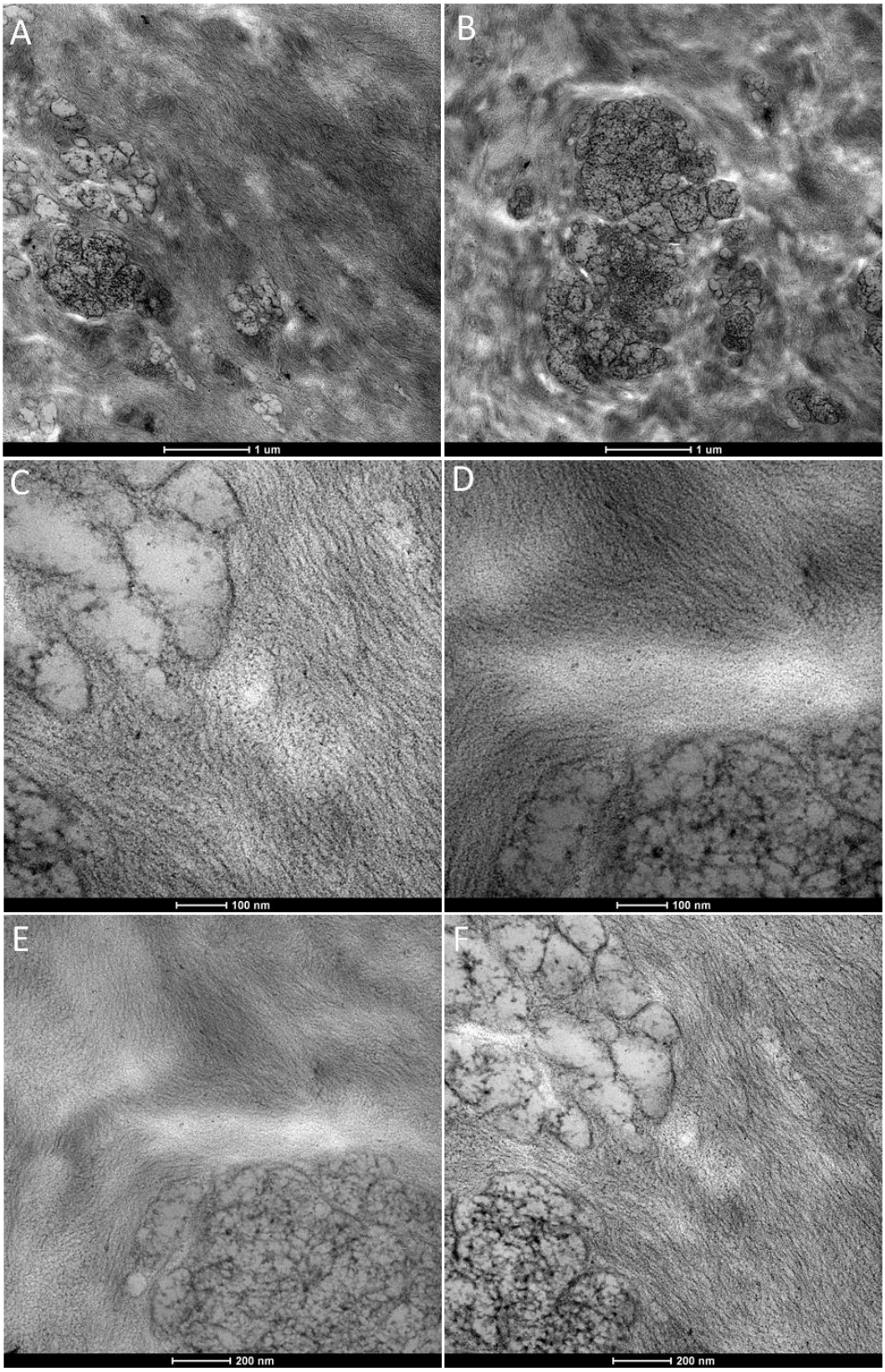


Figure 4.7: Transmission electron microscopy images of calf-hide collagen at 1um (a-b), 100nm (c-d) and 200nm (e-f).

4.5 Conclusion

FTIR analysis indicated a stable triple helix presence for both BH and CH1 collagen. The thermal stability of collagen was investigated and the denaturation temperatures of BH and CH1-collagen were determined by DSC studies as 51.90 ℃ and 45.36 ℃ respectively and TGA analysis showed a three-stage weight loss transition for both BH and CH1 collagen. SEM showed aggregated collagen sheets for all collagen including Sigma collagen. TEM images of BH collagen showed a uniform, organized structure in the form of fibres and fibrils. However, under TEM, CH1 collagen did not show any sign of organized structure. The thermal properties, secondary structure of CH1 collagen did show large similarities to BH and Sigma collagen, hence, it was concluded that parts of CH1 collagen might have gelatinized or digested during the extraction process and those parts have been possibly investigated under TEM. It can be concluded that collagen from both sources of BH and CH1 can, therefore, be possible sources of collagen from waste hide off-cuttings to be produced into high-value-added products such as collagen films which full be investigated in part B of this thesis.

4.6 Acknowledgements

Immense thanks to Professor Richard Haverkamp (Processor of nanotechnology at the School of Engineering and Advanced Technology – Massey University) for his invaluable help with the TEM images.

Section B

Biopolymer film preparation

Chapter 5

Literature review

5.1 Introduction

Due to rising environmental concerns, natural polymers are seen as an attractive solution for replacing petroleum-based plastics. Biodegradable food packaging is seen to be favourable as it aims to solve the problem of plastic waste and offer an alternative solution which does not contribute to environmental pollution. This review considers biodegradable polymers in application of films for packaging purposes with a focus on relevant polysaccharides and proteins. Special emphasis on mechanical properties of biopolymer films is placed as it is a crucial factor in terms of food packaging. Here we discuss the main results in recent developments in preparation of biopolymer films for packaging applications with a focus on their mechanical properties during the last decade.

The overuse of synthetic non-biodegradable plastics for single-use packaging has caused serious environmental pollution problems. Biopolymers produced from renewable natural sources can be used to overcome the problem of synthetic plastics. Biopolymers as an alternative are regarded as favourable as they are renewable, biodegradable, readily available, cheaper and environmentally friendly [212-215].

There is a range of derived biopolymers found in nature from proteins such as collagen, soy protein, and silk to polysaccharides such as cellulose, starch, chitosan and pectin (Figure 5.1). Biopolymers have found application in many industries, such as in packaging, agriculture, cosmetics, medical and pharmaceutical industries. This review will focus on the application of a few selective biopolymers used in preparation of films.

Biodegradable films for food packaging purposes aims to increase shelf-life, provide an alternative solution to traditional food packaging, act as food coatings and as a means of adding functional ingredients directly to the films. The biopolymer films act as a barrier against the transfer of oxygen, water, aromas and flavours between the food and the environment. These films also aim to protect the food by improving food integrity during physical handling and decrease mechanical damage while operating as a vehicle for active compounds such as

antioxidants and antimicrobials. Ultimately the use of some biopolymers as packaging films enables the use of natural resources that are often food waste by-products, hence reducing solid waste pollution, among many other benefits.

Natural polymer-based packaging won't necessarily replace synthetic plastics overnight. The downside of natural polymer-based packaging is the weak mechanical properties, however, research has and is being carried out to improve the properties and to obtain environmentally friendly, biodegradable, and cheap biopolymer-based packaging.

The high cost of processing and performance limitations are the major barriers to the lack of general acceptance of biopolymers as a substitute for petroleum-based non-biodegradable polymers. For example, very small amounts of moisture lead to hydrolytic degradation of PLA under melt processing conditions. Therefore the hygroscopic characteristic of biopolymers has an adverse effect on the adhesion mechanism as well as the biodegradability of the bio-based composites [216]. Films made from natural polymers also exhibit the disadvantages of brittleness, weak barrier and physiochemical properties but mostly weak mechanical properties. The disadvantage of brittleness can be dealt with by the addition of suitable plasticizers however an increase in plasticizer content will decrease mechanical properties. Thus plasticizers, reinforcements, fillers, nanoparticles, and antimicrobial agents are added to improve certain properties while sacrificing others.

Polysaccharides and proteins are most commonly used in the preparation of biopolymer films. Polysaccharides (chitosan, starch, cellulose, pectin etc.) and proteins (collagen, gelatine, soy protein etc.) are also referred to as agro-polymers as they are extracted from animal or plant sources. Agro-polymers are believed to reduce dependence on fossil resources, as agricultural resources are mostly sustainable [217]

One main disadvantage of biopolymer films especially those derived from agro-polymers is that they generally have lower physiochemical properties in comparison to synthetic films. This results in films having high water vapour permeability, poor mechanical and barrier properties. For protein films, their

performance is dictated by their amino acid profile and structure. However, both polysaccharides and proteins are hydrophilic hence they have the ability to absorb large quantities of moisture and hence this limits their uses in packaging films. Additives such as lipids which are hydrophobic are added to films to reduce film hygroscopicity and these include lipids such as paraffin, hydrocarbon-based waves, acetyl-oligosaccharides, fatty acids, minerals, and vegetable oils can enhance barrier properties of films.

This review will focus on the mechanical properties of biopolymer films for packaging applications. Mechanical properties aim to determine the tensile strength of the films, per cent elongation and elastic modulus. The biopolymer molecular makeup and structure play an important role on the mechanical properties and hence on the preparation process of the final product. Understanding the mechanical properties of biopolymer films will give valuable information on the ability of the film to perform as a packaging material for foodstuffs. Mechanical property information of films is crucial as they give an understanding of film durability and also as a means of comparison to commercial plastic packaging [218].

For the purpose of this review on recent developments in preparation of biopolymer films for packaging applications only a selected number of film-forming polysaccharides and proteins have been chosen to extensively review and update on their improvements in regards to mechanical properties.

5.2 Film-forming biopolymers

A number of biopolymers found in nature which are renewable, biodegradable, cheap, and some biocompatible have been used to prepare films for packaging purposes. This review will only focus on the main ones used by the majority of researchers (most literature in the past decade) for the purpose of film preparation and packaging purposes. Figure 5.1 shows a classification of biopolymers, under naturally derived biopolymers there a division between proteins and polysaccharides, for the purpose of this paper only natural biopolymers will be looked into specifically a few selective polysaccharides (chitosan, starch, cellulose, and pectin) and proteins (collagen, gelatine and soy protein).

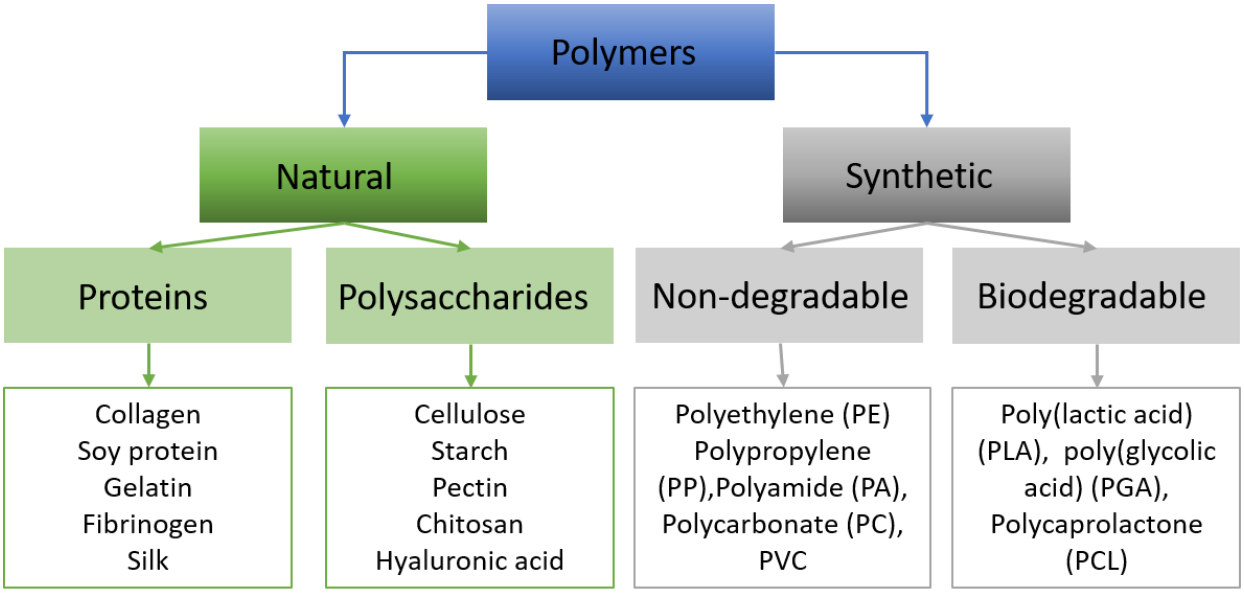


Figure 5.1: Some examples of natural and synthetic polymers [217; 219].

5.2.1 Polysaccharides

5.2.1.1 Chitosan

Chitosan is a natural polymer derived from chitin. Chitin is a polysaccharide made up of repeating units of (1→4) linked 2-deoxy-2-acetamido-b-D-glucose and when deacetylated chitosan is formed. After cellulose, chitosan is the second most abundant polysaccharide found in nature.

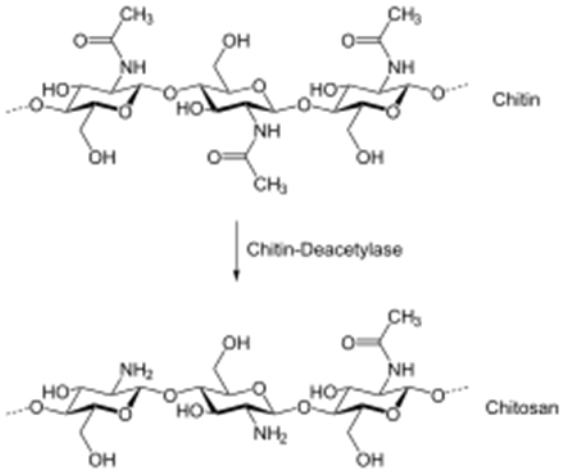


Figure 5.2: Formation of chitosan by deacetylation of chitin [220] **(used with permission).**

Chitosan is seen as a favourable natural polymer to be used in the preparation of biomaterials due to its biodegradability, biocompatibility, non-toxicity, bio-functionality and favourable antimicrobial characteristics [221-223]. Minerals, vitamins and other functional substances can be incorporated in chitosan-based films and the advantage of antibacterial activity makes chitosan-based films favourable packaging materials. This results in biodegradable chitosan films which are used as packaging for the preservation of foods [224; 225].

The antimicrobial characteristic of chitosan has led to many studies investigating film preparation from chitosan [226-228]. Due to chitosan being biodegradable, biocompatible and non-toxic it has received significant attention as it is also antimicrobial, and this factor makes it ideal for food preservation. The preparation of chitosan films is a simple procedure, involving evaporation of its solvent or dilute acid solutions [229].

Recently Dominguez-Martinez et al [230] prepared chitosan-based composite films containing mucilage, polyvinyl alcohol (PVA) and chitosan at different concentrations. Glycerol at 14% was used as a plasticizer. Films were cast on glass plates and dried with a convective dehydrator. Mechanical and barrier properties of the films were investigated and SEM was used to study the film uniformity. The prepared films water vapour permeability (WVP) was affected by glycerol and mucilage content as higher WVP values were observed than in films composed of

pure PVA or chitosan. High tensile strength chitosan-based films with a homogenous structure were obtained.

The preparative method of chitosan-based films has not changed over the years. Solution casting is still the most widely used preparative method for chitosan-based films (2016-2018 literature) [230-236].

5.2.1.2 Starch

Starch is a natural polymer found mainly in rice, potatoes, corn and beans. It is one of the most abundant natural polysaccharides, it is renewable and cheap [237]. Depending on the origin of the starch, the chemical composition, size and the structure of the starch granules will vary. However, native starch is composed of two polymer macromolecules of amylose and amylopectin [238]. The film-forming ability of starch is from amylose as it is a linear part of starch which is known to result in clear, robust and strong films in comparison to amylopectin films which tend to be brittle and uneven in surface.

The film-forming ability of starch for packaging purposes specifically has been investigated for the past decade by a number of researchers [239-245]. Films based on starch generally have lower mechanical properties in comparison to synthetic polymer films. With the addition of a plasticizer such as water, a thermoplastic behaviour is exhibited by starch. Either casting or extrusion can be applied to prepare starch films. In order to reduce starch film brittleness, plasticizers such as glycerol, xylitol or sorbitol are typically incorporated in the film-forming solution. Studies have shown that at low plasticizer concentration such as glycerol, both strain and strength of starch film are decreased. However above 20% glycerol concentration elongation increases and resulting in lower tensile strength. Talja et al [242] investigated the effects of glycerol, xylitol, sorbitol, and water on the physical and mechanical properties of starch films. Both young's modulus and tensile strength decreased with an increase in plasticizer content and at the same time resulted in an increase in film elongation. An increase in both water and plasticizer content increased elongation and a reduction in tensile strength. Due to phase separation and crystallization, high plasticizer contents resulted in

changes in mechanical properties of films. Glycerol had a bigger effect on the mechanical properties of starch films and sorbitol having the smallest effect.

Jiménez et al. [238] have written an extensive review on edible starch films from raw material to film formation mechanism. Starch has attractive characteristics for packaging applications such as biodegradability, transparency, and being environmentally friendly. However, it has weak mechanical properties which are unfavourable for food packaging purposes. In order to improve this disadvantageous characteristic of starch films, reinforcing them with stronger natural polymers such as cellulose fibres has enhanced mechanical properties greatly [246-251]. Muller et al [244] reinforced starch-based films with cellulose fibres and concluded that films reinforced with cellulose fibres had higher tensile strength and elasticity modulus than the films with no reinforcement. Savadekar et al [252] and Tongdeesoontorn et al [253] also reported an increase in tensile strength of starch-based films when reinforced with cellulose.

5.2.1.3 Cellulose

Cellulose is an insoluble polysaccharide found in the cell wall of plants. This biopolymer is composed of repeating units of β(1→4) linked D-glucose monomers [254].

Cellulose is biodegradable, inexpensive and one of the most readily available renewable resource. The film-forming ability of cellulose derivatives has been investigated by a number of researchers. Cellulose derivatives of methylcellulose (MC), hydroxypropyl methylcellulose (HPMC) and carboxymethyl cellulose (CMC) have the ability to form continuous matrices and are water-soluble hence they are of great interest to researchers. Films formed from low water content cellulose derivatives have shown to have very efficient barrier properties [255].

The film-forming ability of CMC, MC and HPMC cellulose has been reported by several studies [255-262]. Commercial films of regenerated cellulose such as cellophane have been used for packaging properties, however, it has weak barrier properties in comparison to synthetic films used for packaging purposes. Preparation of cellophane also generates hazardous by-products (CS_2, H_2S)

resulting in pollution. However, if cellulose films are kept in controlled dry conditions then their barrier properties are better or equivalent to dense polyethylene and low-density polyethylene [263-266].

5.2.1.4 Pectin

Pectin is a plant-based complex macromolecule found in the cell wall of plants giving the tissue firmness and integrity [267]. Industrially pectin is extracted mostly from apple pomace and citrus peels [268]. Pectin has attracted more attention recently due to its low cost and environmentally friendly properties as its biodegradable and obtained from renewable resources [269].

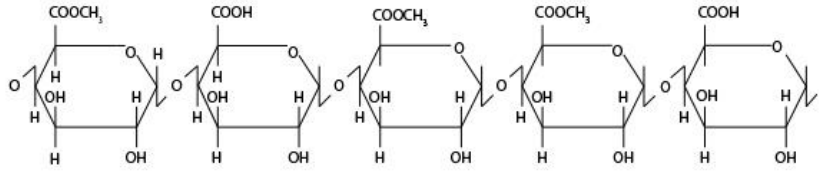


Figure 5.3: Pectinic acid monomers joined to make the biopolymer pectin [270] (used with permission).

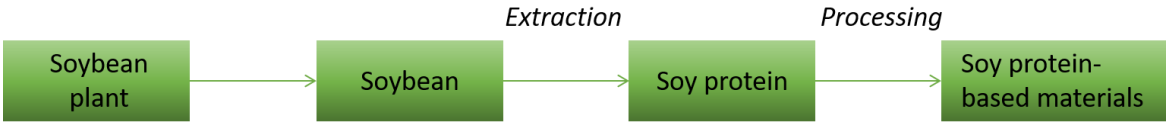
Pectin has numerous applications and its use has been found in medical products, food industry, cosmetics and the textiles industries [267; 271]. Edible pectin-based films with good mechanical and barrier properties have also been produced by several studies [267; 269; 272-279]. Cabello et al [269] prepared pectin-based films plasticized with glycerol and polyethylene glycol which resulted in favorable mechanical and barrier properties. Jo et al [279] prepared pectin-gelatine-based composite films in combination with irradiation to increase the mechanical properties of the films for packaging materials.

5.2.2 Proteins

5.2.2.1 Soy protein

Soy protein is plant-based biopolymer extracted from the soybean plant. Soybean consists of protein (42%), oil (20%), carbohydrates (33%) and approximately 5%

ash on a dry basis. Soybean is mainly used in in the food industry as a consumable, however, it has attracted more attention to be used for extraction of soy protein



to be used as a biopolymer in non-food applications [280].

Figure 5.4: Process of soy protein extraction to processing.

Due to soy protein being renewable and biocompatible, it has been shown to be favourable as a raw material for the preparation of films as packaging [281]. Soy protein is readily available, biodegradable, cheap and nutritious. Films have been prepared from soy protein by several studies and shown to have good film-forming capacity, resulting in consistent film thickness and with good barrier and mechanical properties [282]. Soy protein-based films are edible, biodegradable and non-toxic.

Soy protein as a substitute for non-biodegradable films and plastics has been researched by several studies and looks to be a promising potential to be used in packaging applications [280; 283-286]. The application of soy protein-based films for packaging is limited due to its poor mechanical properties and hygroscopic nature [287]. However, research has been carried out to improve soy protein-based films mechanical properties and moisture sensitivity. Chemical, physical and enzymatic treatments have been applied to improve these properties. For instance, Yildirim et al. [288] introduced enzymatic cross-linking to improve the mechanical properties of soy protein-based films. Heat curing [289], UV radiation [290] and alkali treatments [291] have also been introduced in an effort to improve the mechanical properties and moisture sensitivity of soy protein-based films.

5.2.2.2 Collagen

Collagen is the most abundant structural protein found in the vertebrate body. Collagen is a rigid, inextensible, fibrous protein that the main component of connective tissue in animals, including tendons, cartilage, bones, teeth, skin and blood vessels. As a structural protein, it is used to give strength to structures in the body [19].

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 [71]. 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.

Sionkowska et al [73] prepared biopolymer films based on blends of collagen and silk fibroin. Films were prepared by method solution casting and characterized for their mechanical properties and structure. Films blends of collagen and silk fibroin showed better mechanical properties than for pure silk fibroin films. Sionkowska et al 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 [74]. Hence the percent elongation values of the film blends were very low (0.30-5.10%) [73].

The casting method of film preparation requires the presence of a biopolymer, a solvent, a compatible plasticizer and/or other blends and additives. Collagen is soluble in acid, precipitates in alkaline solutions and is insoluble in water. Hence for collagen film preparation, the solvent needs to be an acid. There seem to be some misconceptions or blurred definitions of "collagen"-based films. Some literature has used water as a solvent for preparation of "collagen"-based films.

Collagen does not dissolve in water unless it's in its denatured form (gelatine). Some literature have used the terms collagen and gelatine interchangeably which is inaccurate as they are not the same thing. They are both derived from the same sources and have the same amino acid profiles but they differ in their molecular structure arrangement. Hence collagen-based films must involve an acidic solvent to be miscible with the other film-forming components. Yang et al. [292] prepared collagen-based packaging films from extracted pig-skin collagen. However, it can be argued that the "collagen" used in preparation of these films were not collagen but gelatine as in the method Yang et al. [292] states "0.2g of collagen powder was dissolved in 1ml of water at 40℃." The prepared composite films were characterized for their tensile strength and structure.

Collagen-based films as packaging in non-food applications have also been investigated. Sommer et al. [74] prepared collagen-based packaging films for the automotive industry and compared them with commercial polypropylene block copolymer films. Bovine collagen solubilized in lactic acid was prepared into films by casting. Other additives such as stabilizing and cross-linking agents were added. Glycerol and lecithin were used as plasticizers for the preparation of the films. The resultant films were of high tensile strength (20-27 MPa) and elongation at break (%) ranged between 19-34% (based on UV and temperature controls). Sommer et al. [74] concluded that due to brittleness and limited resistance of collagen-based films to polar substances their use in the automotive industry as body paint protection is restricted.

5.2.2.3 Gelatine

Gelatine is a colourless, translucent, foodstuff which is brittle when dry and gel-like after preparation. Gelatine is collagen that has been irreversibly hydrolysed. This happens by thermal denaturation of collagen. It is derived from animal skins and bones and can also be extracted from fish skins. Gelatine has been used in many applications due to its cheap processing cost and favourable biodegradability characteristics. It is mainly used in the food industry as a gelling agent, however, it is also used by the pharmaceutical and cosmetics industries.

Gelatine films that are transparent, water-resistant and flexible are successfully prepared by several studies [70; 293-305]. These films were usually prepared by a method called film-casting where the films are cooled, followed by drying the aqueous film-forming solution. Microencapsulation of aromas, vitamins and sweeteners has involved gelatine films and as a raw material for photographic films [306].

The preparation of gelatine films date as back as 1952, Bradbury et al. [293] studied the effect of preparation temperature on the mechanical properties of gelatine films using the method of film casting. It was concluded that gelatine films prepared under high temperature resulted in low strength and high recoverable extraction.

The preparation method of edible biopolymer films has remained the same over the years. In literature researchers still use the method of casting for preparation of biopolymer films. Sobral et al. [294] investigated the mechanical, water vapour and thermal properties of gelatine-based edible films. Gelatine from bovine and porcine sources were used with water as the solvent and sorbitol as a plasticizing agent at different concentrations. The film solutions were cast on Plexiglas plates. Mechanical properties of the gelatine-based films in this study were analysed in a different way in comparison to conventional method of using a tensile tester. Puncture tests were performed to determine the force and the deformation of the films at breaking points. The results focusing on the mechanical properties of the films indicated that the higher the plasticizer (15-60 g sorbitol/100g gelatine) content the lower the puncture force was required.

5.3 Additives

Additives are an important component of film preparation. Additives such as plasticizers, reinforcements and fillers are added to the film-forming solution to enhance its properties such as improving flexibility and mechanical properties. Films used for food coating purposes include additives such as antimicrobials, antioxidants, flavours and colours and flavours to achieve ideal desired packaging and to increase the shelf life, improve barrier properties and enhance taste. In this section, only plasticizers, reinforcements and fillers will be the focal point as this review focuses on films for packaging purposes and not on film-coatings for foodstuffs hence. The role of plasticizers, reinforcements and fillers in relation to film mechanical properties is reviewed.

5.4 Plasticizers

Plasticizers are non-volatile, small molecular weight (M_w) compounds applied in the film and plastic-forming industries to enhance flexibility and processability of polymers by lowering the glass transition temperature (Tg) [307; 308]. Scientifically a plasticizer is defined by International Union of Pure and Applied Chemistry as “a substance or material incorporated in a material (usually plastic or elastomer) to increase its flexibility, workability, or dispensability” [309]. Plasticisers position themselves between polymer molecules and interfere with polymer-polymer chain interactions to increase flexibility and processability, hence they increase the free volume of the polymer structure.

Plasticisers play a huge role in film preparation formulation, they increase film stretchability and flexibility as without it the films would be brittle and break upon film cast peeling. Low molecular weight compounds are generally used as plasticizers as they need to be miscible in the biopolymer. Sorbitol, glycerol, polyethylene glycol and mannitol are the most commonly used food-grade plasticizers [310]. There is a growing interest in the use of natural-based plasticizers especially with the preparation of biopolymer films. Natural-based plasticizers are preferred as they are characterized by low toxicity, lower production risk in terms of health and safety and low migration. Fatty acid esters,

epoxidized triglyceride vegetable oils from soybean oil, sunflower oil and caster-oil etc. are some natural plasticizers applied mostly for edible films. With natural plasticizers being an important factor of biopolymer films, most traditional plasticizers used in synthetic polymer processing are not suitable for biodegradable thermoplastics [308]. The total replacement of synthetic plasticizers by natural plasticizers is far out of reach, however, replacement of them in certain applications is a good positive start.

During biopolymer film preparation procedure usually, the protein or the polysaccharide is heated to denaturation point where intermolecular interactions such as hydrogen bonding and hydrophobic interactions among the chains can occur resulting in brittle films. Therefore addition of a compatible plasticizer is necessary in order to reduce brittleness and make the film more flexible by reducing the interactions protein-protein chains [307]. Water can also act as a plasticizer and it can influence the plasticizing ability of another added plasticizer. Polyols such as glycerol (Gly), polyethylene glycol (PEG), sorbitol (Sor); and water are hydrophilic plasticizers which have the ability to improve the mechanical properties of biopolymer films due to their interference with protein chain-chain hydrogen bonding [311].

Incorporation of plasticizers in biopolymer films is essential as they improve mechanical properties [312]. However, plasticizers can also negatively affect edible films for example if plasticization is with water or polyols where an increase of gas and vapour transfer can occur [261]. There are a number of plasticizers used for preparation of biopolymer-based films for packaging purposes. Table 5.1 shows the plasticizer types used in literature for biopolymer-based films.

Table 5.1: Plasticizers used in the preparation of biopolymer-based films.

Biopolymer film	Plasticizer	Reference
Chitosan-based		
	Glycerol (GLY)	[221; 251; 313-315]
	GLY, lactic acid	[274]
	GLY, ethylene glycol (EG), poly(ethylene glycol) (PEG), propylene glycol (PG)	[225]
Collagen-based		
	PG	[316]
	PEG 400	[317]
	PEG 1500	[318]
	GLY	[319-321]
	GLY and Lecithin	[74]
Gelatine-based		
	GLY, Sorbitol	[322]
	PEG (M_w = 300, 400, 600, 800, 1500, 4000, 10000, 20000), mannitol (Man), sorbitol, EG, diethylene glycol (DEG), triethylene glycol (TEG), ethanolamine (EA), diethanolamine (DEA), triethanolamine (TEA), malic acid (MA), sucrose, oleic acid, citric acid, tartaric acid.	[70]
	Fatty acids	[323]
	Sorbitol	[294]
	Glycerol	[298]
PLA-based		
	Tributyl citrate (TbC), diethyl bishydroxymethyl malonate (DBM), DEG, TEG	[324]

Biopolymer film	Plasticizer	Reference
	Monomeric and polymeric commercial plasticizers (di-2-etylhexyladipate, polymeric adipates (G206/2 and G206/7)	[325]
	Chloroform	[326]
	Ascorbyl palmitate, α-tocopherol	[327]
	Diisodecyl adipate, diethyl adipate, acetyl triethyl citrate, acetyl tributyl citrate, tributyl citrate	[328]
Soy protein-based		
	GLY, sorbitol	[329]
	GLY	[215; 290; 330-332]
Pectin-based		
	GLY, PEG	[333]
	GLY	[273; 275; 278]
	GLY, lactic acid	[274]
Starch-based		
	GLY, sorbitol	[241]
	GLY	[240; 244]
	GLY, xylitol, sorbitol	[242]
	GLY, citric acid	[334]
Cellulose-based		
	PEG, acetyl(tributyl citrate) (ATBC)	[335]
	PEG	[260; 336; 337]
	PEG, stearic acid (SA), palmitic acid (PA) and lauric acid (LA)	[338]

5.5 Reinforcements and fillers

The use of biopolymers has been limited because of their generally low mechanical and barrier properties. Reinforcements and fillers are used in film preparation to enhance mechanical properties resulting in composites. There are a number of fillers that are used to modify and enhance resin properties that become a part of the polymer matrix. Nanoparticle fillers are added to biopolymer film forming formulation to improve their mechanical and barrier properties. Advancement of nanotechnology has enabled the production of various types and sizes of fillers such as cellulosic nanofibers, nano-clays and nano-metals and many more. The resultant composite material potentially can be used in a wide variety of applications due to its large surface area and a greater aspect ratio [339]. These are added to the biopolymer matrix for the main goal of improving mechanical properties of films but also additionally leading to better barrier properties. Many studies have shown the benefits of addition of fillers in comparison to without [340].

Organic fillers have gained a lot of attention due to their biodegradability, low cost, fewer health hazards and lower specific weight in comparison to mineral fillers. Several studies have incorporated these natural fillers with synthetic polymers in the hope of further improving their properties. Balasuriya et al [341] prepared composites of polyethylene with wood flakes acting as a filler. The mechanical properties of the composite was studied and significant improvements in tensile strength were obtained.

Biopolymers themselves, which exhibit higher mechanical properties, can also be used as reinforcements. Kunanopparat et al [342] used cellulose fibres to reinforce wheat gluten films and hence improved their mechanical properties. Ghanbarzadeh et al [334] also used cellulose (carboxymethyl cellulose) at varying concentrations as a filler with citric acid to improve the mechanical properties of starch films. Starch films were prepared with the aid of glycerol as a plasticizer and cellulose and citric acid acting as fillers, the film solutions were cast on Teflon trays and characterized. A significant increase in tensile strength was seen with the addition of cellulose, with tensile strength increasing from 6.57 to 16.11 MPa for

the films that contained 20% cellulose. The citric acid added in this particular paper was used as a cross-linking agent. Plasticizers and cross-linking agents have opposite effects on the tensile strength of materials. With an increase in the cross-linking agent (citric acid) the tensile strength increased but strain at break decreased, however, the opposite effect was seen with an increase in plasticizer content. In the case of these starch films, the citric acid at different concentrations both acted as a cross-linking agent and a plasticizer. Citric acid acts as both a cross-linking agent and a plasticizer were observed in this study as at different concentrations different favourable properties were achieved. For example at 10% (w/w) citric acid the mechanical properties of starch were increased and at 15-20% citric acid stress-strain curves showed a transition from ductile to plastic material behaviour. Hence optimizing experimentations and trial and error can potentially result in finding of a favourable formulation which can increase both mechanical and other properties.

However, cellulose possesses poor solubility in organic solvents, has low thermal stability and polarity of cellulose results in some difficulties when used as fillers in biopolymer films. It further results in poor dispersion in melted polymer, weak interaction between cellulose fibre and matrix thus leading to challenges in thermal processing. However, these challenges can be overcome by chemical modification of cellulose, using a compatibilizer, and or by dissolving cellulose in a suitable solvent prior to dispersing in the polymer matrix. Some natural polymers used as natural fillers and reinforcements in films and their influence on increased tensile strength are summarized in Table 5.2.

Table 5.2: Reinforcement/fillers used in biopolymer films and their influence on tensile strength.

Reinforcement/filler	Polymer	Tensile strength (MPa) increase	Reference
Starch nanocrystals	Pullulan	2 – 25 MPa	[343]
Nanocellulose	Chitosan		
		47.68 – 57.45 MPa	[344]
		79 – 98 MPa	[345]
		85 – 120 MPa	[346]
Halloysite nanotubes	Pectin	2370 – 2991 MPa	[347]
Nanoclay	Whey protein	3.4 – 3.29 MPa	[348]
Nanocellulose	Sodium caseinate	3.4 – 5.5 MPa	[349]
Montmorillonite	Soy protein	2.87 – 8.73 MPa	[350]
Nanoclay	Chitosan	10 – 78 MPa	[351]
Microcrystalline cellulose fillers	Hydroxyl propyl methyl cellulose		
		28.5 – 56.5 MPa	[352]
		35.6 – 54 MPa	[353]
Starch microparticles	Starch	12.8 – 25.3 MPa	[354]
Chitin nanofibrils	Carrageenan	30.2 – 44.7 MPa	[355]
Cellulose nanocrystals	Alginate	18.03 – 25 MPa	[356]
Lignin	Starch	22.4 – 16.9 MPa	[357]
Chitosan nanoparticles	Starch	2.84 – 10.80 MPa	[358]
Cellulose nanoparticles	Hydroxyl propyl methyl cellulose	49.3 – 60.1 MPa	[359]
Nanoclay	Agar	28.06 – 36.87 MPa	[360]
Carboxymethyl cellulose and citric acid	Starch	6.57 – 16.1 MPa	[334]

5.6 Biopolymer film preparation

Biopolymer films are prepared by two main methods; wet and dry process. Essentially all preparation methods requires a natural polymer, solvent, plasticizing agent(s), and or other additives. The film solution or matrix will be an interacting polymer network 3D structure [361]. Film preparation is affected by a number of factors which in turn affect the mechanical properties of the film. Film solution preparation temperature, plasticizer concentration, water content, casting temperature, biopolymer content and concentration are some of the major key factors that need to be accounted for which ultimately control the characteristics of the end product film. The film preparation method plays an important role in the end product characteristics such as its mechanical strength. Thus it is very important to understand what and how literature has addressed biopolymer film preparation over the last decade and how it has or hasn't affected mechanical properties. This section will focus on the two main biopolymer film preparative methods and how it plays a role in the mechanical properties of the film. Figure 5.5 has summarized both wet and dry processing of biopolymer films.

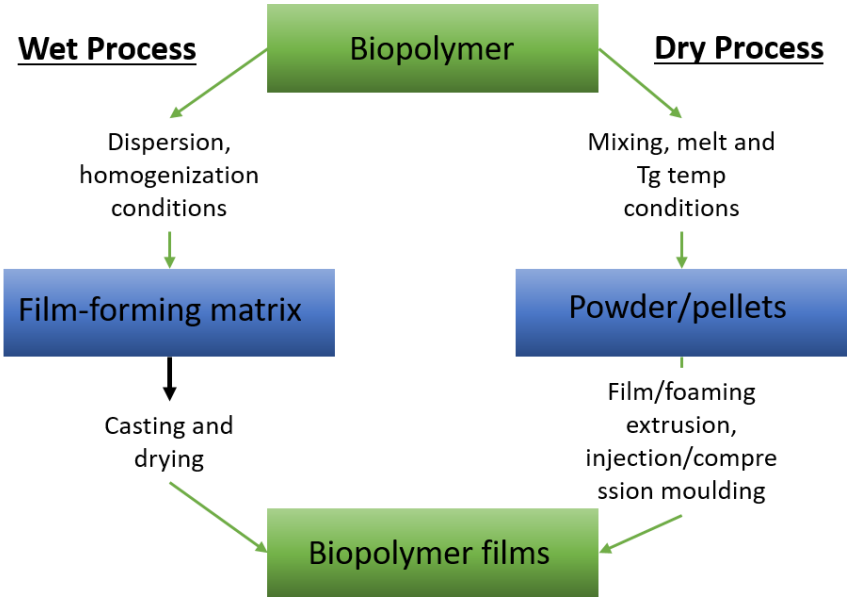


Figure 5.5: Schematic summary of the two process of biopolymer film preparation.

5.6.1 Dry process

Preparation of biopolymer films by the wet process at an industrial level can be inefficient, energy and time consuming. This is also one of the major limitations when considering replacing synthetic polymers with natural polymers. Thermoplastic treatment can be applied to some biopolymers in order to produce large scale production of biopolymer films.

Dry process of biopolymer film preparation is carried out by film and foaming extrusion, injection and compression moulding and reactive extrusion. The required biopolymer with or without other blends is mixed with plasticizer(s) and extruded with a film-blowing die or injection/compression moulded [238]. Majority of biopolymer films cannot generally be extrusion blown, like synthetic polymers, as they do not have defined melting points and undergo decomposition upon heating.

Film-blowing of thermoplastic starch was prepared by Thunwall et al. [362]. Starch as a biopolymer with glycerol as a plasticizer and water were used to identify the best possible way of preparing films by film-blowing extrusion. The resulting product was a sticky double-walled film which was contributed to the presence of glycerol or concentration of glycerol. The tensile strength ranged from approximately 7.5-10.9 MPa.

5.6.2 Wet process

Wet process or generally referred as to as solution casting is the main method of biopolymer film preparation. Wet casting of biopolymer films has five main steps: coagulation, dispersion, homogenization, casting and drying. The film matrix is prepared by mixing the biopolymer with a solvent, a plasticizing agent added and/or other additives. It is then homogenised and casted on a tray (covered with Teflon sheets or another non-adhesive surface), finally it is left to dry in a vacuum oven or in an open air environment. This method of biopolymer film preparation is the most widely used literature and has not been changed over the years [214; 245; 292; 322; 325; 328; 363; 364]. Film uniformity is a key characteristic that needs to be achieved by film casting. Film uniformity is also crucial with casting of

films as when characterizing for tensile strength, films of irregular uniformity will have a huge impact on mechanical properties. Film porosity is another problem which arises from dissolved air in the film forming solution. Porous films will lead to cracks and result in premature failure of films. Prior to casting films on a preferred surface, degassing of the film solution can be carried out to remove as much dissolved air as possible. Vacuum degassing by using a vacuum pump for approximately an hour at a specified pressure can remove all if not most dissolved air in the film forming solution.

However, there are always continuous efforts made to modify biopolymer film preparation methods in order to enhance their properties. As mentioned in the additives section, natural reinforcements/fillers such as nanoclays, Nanocellulose and chitin nanofibrils etc. are used to enhance film properties and provide better barrier and mechanical properties. Similarly over the years researchers have used various additives and additional steps to biopolymer film preparation methodologies in order to gain optimum end product results. In addition to the above mentioned reinforcements and fillers, the barrier and mechanical properties of biopolymer films can be enhanced by chemical and physical cross-linking and or with surface treatments such as grafting and coating. For example, Lahtinen et al [365] investigated the effect of heat treatment on barrier and functionality of polyolefin-coated papers. The results showed significant improvement in barrier characteristic of the films by a drastic reduction in both water vapour and oxygen transmission rates of polyolefin coatings. Heating films and coatings over their melting point, then further slowly cooling them back to ambient room temperature causes changes in film density, secondary structure, crystallinity and spherulite size and can potentially result in better barrier properties. Heating above some biopolymer's melting point can result in irreversible hydrogen bonding which can improve both barrier and mechanical properties.

5.7 Film characterization

5.7.1 Film surface properties

5.7.1.1 Film thickness

Film thickness is an important factor, which affects the migration rate of the liquid film-forming dispersion and causes differences in film structure. Hence, monitoring this feature is vital for mechanical properties. Moreover, the thickness of films may perhaps increase with the concentration of the poured solution and the final thickness can be governed by on the retraction of the film when solution evaporates and the rate of solution evaporation strongly affects the polymer network arrangements.

Film thickness is usually measured with hand-held micrometres or digital calipers. Five to 10 measurements at random positions on the film are taken and an average thickness value is obtained. The components of the film-forming solution affect the alignment, sorting and compacting of the molecules during film drying process, thereby causing the differences in thickness.

Visually, films prepared from collagen and gelatine should be transparent, colourless and flexible with no apparent voids or cracks. Addition of plasticizers such as glycerol can give films a hint of yellow in color. Reinforcements and additives can lead to increased film thickness. Wang et al. [366] prepared collagen-based films with starch and observed an increase in film thickness with an increase in starch concentration.

5.7.1.2 Moisture content

Moisture content of films are carried out by a series of weighing and drying. Film samples are cut into approximate 2 * 2 cm squares and weighed (W_1). Samples are placed in an oven at 105 ℃ and weighed again after 24 hours (W_2). Subsequently, water content or moisture content is determined as the percentage of initial film weight lost after drying, and reported as:

$$MC\ (\%) = [(W_1 - W_2)/W_1] * 100$$

Nor et al. [367] prepared gelatine-based films and reported MC values to range between 7.86 % to 24.4 % depending on the film formulation and the content of plasticizer added. Moisture content influences other properties of films such as tensile strength, film solubility, and thermal stability. Therefore, it is important to observe and investigate moisture content of biopolymer films in order to understand changes in tensile strength and glass transition temperatures.

5.7.1.3 Film solubility (%)

Film solubility or water solubility is an important factor of biopolymer films, especially since most potential applications require low water solubility to enhance product integrity and water resistance. Water solubility of films can be viewed as a measure of the water resistance and integrity of a film.

Addition of plasticizers can have a large effect on the percent solubility of films [367], as plasticizers such as glycerol are hydrophilic and highly soluble. Hence, it is important to research what concentrations of plasticizers will result in optimum water solubility (%) without the drawback of reducing other properties such as tensile strength and thermal properties. Water solubility is determined by using the following equation:

$$Solubility\ (\%) = [(W_i - W_f)/W_i] * 100$$

-*where:* = W_i = initial weight of the film, W_f = weight of the un-dissolved dried film residue.

The use of reinforcements and additives can increase or decrease film solubility depending on the functional properties of the additive. For example, Wang et al. [366] prepared collagen reinforced starch films and upon increasing amounts of collagen, the films exhibited lower solubility values, owing to insoluble nature of collagen fibres.

5.7.2 Differential scanning calorimetry (DSC)

A differential scanning calorimeter (DSC) is used to determine the glass transition temperature (Tg) and helix coil transition of the polymer films. A transition in the range 30-70 ℃ indicates the glass transition temperature of collagen and gelatine

films, characterized by a discontinuity at baseline. The inflection point of this discontinuity provides the glass transition of films.

5.7.3 Film morphology – Scanning electron microscopy (SEM)

Surfaces and cross-sectional areas of composite films are examined by a scanning electron microscope (SEM). Interfacial properties of composite films are studied from the SEM images and the effect of additives or reinforcements is analysed.

5.7.4 Fourier transform spectroscopy (FTIR)

Fourier transform spectroscopy (FTIR) analysis is a crucial characterization to carry out in film production. It helps to determine the potential functional groups that perform new interactions formed in collagen films with plasticizers and other additives if added. This further helps to understand the changes observed in other film properties such as tensile strength. The most important functional groups that correlated with collagen films plasticized with polyols such as glycerol include; amide A (3000-3500 cm^{-1}), amide I (1630-1650 cm^{-1}), amide II (1539-1550 cm^{-1}) and amide III (1033-1135 cm^{-1}) [368]. For raw collagen, the FTIR spectra should show a shift to a lower wavenumber for each characteristic amide band, the lower the shift in the wavenumber, the great presence of hydrogen bonding. Collagen triple helix requires hydrogen bonding for its stability, hence, a shift to higher wavenumbers in the characteristic bands would indicate a decrease in the amount of hydrogen bonding and hence a less stable triple-helix molecule. FTIR spectra for collagen films with glycerol as the plasticizer should still show the characteristic amide bands of amide A to amide III. In this case a shift to a higher wavenumber in the characteristic bands indicates changes in structural arrangement of collagen, hence, representing interactions with glycerol.

5.7.5 Mechanical performance of biopolymer films

Understanding and investigating the mechanical properties of biopolymer films for packaging purposes is crucial as it gives information on the strength and durability of the films.

There are a number of ways that can be carried out in the film preparation method to increase the mechanical strength of biopolymer films. The reinforcements and

fillers section of this chapter has focused on that. This section has summarized the tensile strength and elongation of the biopolymers discussed in this paper found in literature (mostly focused on literature from the last two decades (2000-2018)) versus commercially utilized plastics such as polyethylene, poly(lactic) acid and Mater-bi.

Table 5.3: Summary of mechanical properties of some biopolymer films discussed in this review.

Biopolymer film	Tensile Strength (MPa)	Elongation (%)	Reference
Chitosan-based	12.7-82.4	2.1-65.0	[363; 369-372]
Starch-based	2.4-66.8	4.86-80	[245; 334; 364; 373]
Soy protein-based	0.91-40.6	3.95-1226	[215; 280; 281; 374-376]
Gelatine-based	15.12-76.8	5.9-62.86	[370; 377-380]
Collagen-based	1.42-44.5	1.76-42	[9; 74; 381-383]
Pectin-based	4.48-69.33	1.98-71.64	[269; 277; 384-386]
Cellulose-based	2.23-17.77	1.42-89.93	[387-391]

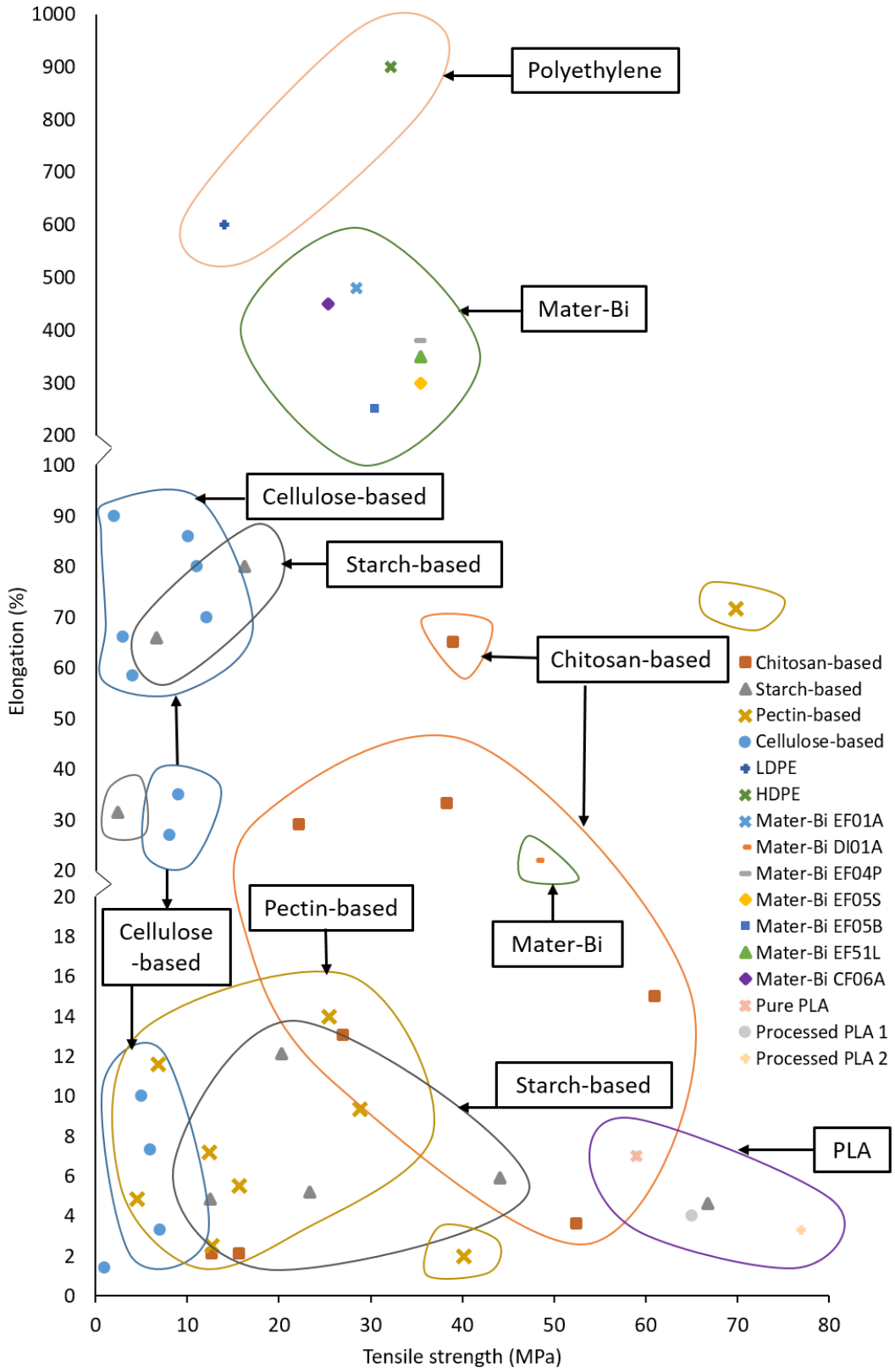


Figure 5.6: Mechanical properties of polysaccharide-based films (discussed in this review) vs. mater-bi [392], polyethylene [393] (LDPE and HDPE), and poly(lactic) acid (PLA) [394].

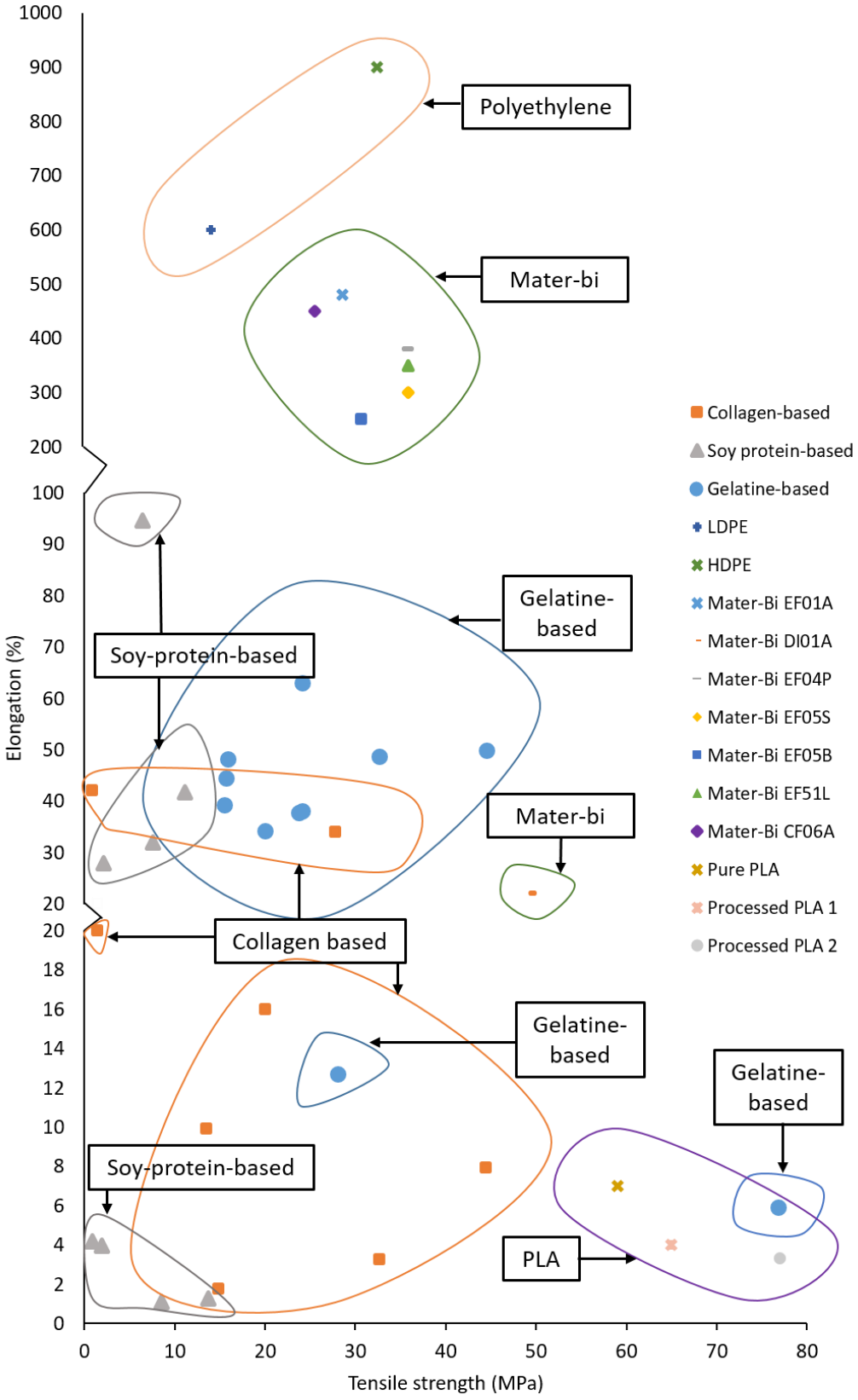


Figure 5.7: Mechanical properties of protein-based films (discussed in this review) vs. mater-bi [392], polyethylene [393] (LDPE and HDPE) and poly(lactic) acid (PLA) [394].

Figure 5.6 and Figure 5.7 are showing the tensile strength (MPa) versus elongation (%) for polysaccharide-based and protein based films respectively; based on literature and data in Table 5.3. The graphs further in comparison show the tensile strength (MPa) and elongation (%) of polyethylene which is the most common plastic used in the world (low density polyethylene (LDPE), and high density polyethylene (HDPE)), Mater-bi and poly(lactic) acid. Unlike petroleum based plastics, both Mater-bi and poly (lactic) acid are derived from renewable resources such as starch and commercially utilized worldwide for packaging purposes.

Chitosan-based films show a wide range of mechanical properties, with tensile strength as high as 82 MPa (Table 5.3) and elongation as low as 2.1% to as high as 65% (Figure 5.6). In some literature, chitosan-based films' mechanical properties overlap with pectin-based, starch-based films and in some cases even with PLA and Mater-Bi DI10A.

Cellulose, pectin and starch-based films overlap greatly in their mechanical properties. However, starch and pectin-based exhibit higher tensile strength (MPa) in comparison to cellulose-based films. It is interesting to observe cellulose and starch-based films overlap in two different regions – both in high elongation (%) and low tensile strength and in region of low elongation (%) and high tensile strength. In the case of high elongation (%) and low strength, starch-based composites with citric acid and carboxymethyl cellulose (CMC) blends were prepared into films by casting method. It was concluded that at 10% citric acid and 15% CMC, the starch films showed the highest tensile strength. Interestingly, in both regions that cellulose-based and starch-based films share similar mechanical properties, literature shows that in both cases cellulose-starch blends were utilized to prepare the biopolymer films.

Observing the mechanical properties of commercial plastics, PLA displays the highest tensile strength in comparison to polyethylene and Mater-bi. However, PLA exhibits much lower (%) elongation in comparison to Mater-bi and polyethylene. Pure PLA shares similar mechanical properties with chitosan-based films in the region of high tensile strength and low elongation (%). In this particular case, the chitosan-based films were prepared with blends of PLA. Interestingly, in

this paper, with the increase of PLA to chitosan ratio, both tensile strength and elongation (%) decreased.

Further observing the mechanical properties of protein-based films (Figure 5.7), gelatine-based films exhibit an ideal ratio of elongation (%) to tensile strength. In most cases, gelatine-based films were blends of gelatine, cross-linking agents, antioxidants, nanoclay, and or other biopolymers in order to improve tensile strength and other properties [293; 295; 297; 314; 370; 377; 378].

Collagen being the un-denatured form of gelatine, when used to prepare films shares similar tensile strength but a lot lower elongation (%). The decrease in elongation (%) could be attributed to collagen in its denatured form is heavily cross-linked and has limited mobility. Upon denaturation, collagen cross-links are broken and with addition of plasticizers, collagen-based films become flexible and hence an increase in elongation (%) at a cost of lower tensile strength. However, reinforcements and fillers (5.5) can be added to increase the mechanical properties of collagen-based films.

Over all, there are a few outliers in terms of really high tensile strength or elongation for protein-based and polysaccharide-based films which coincide with mechanical properties of commercially produced plastics. Further investigation and optimization needs to be carried out in order to efficiently improve the mechanical properties of biopolymer films.

5.8 Conclusions

Rising interest in sustainable materials to be used in food packaging applications combined with great mechanical strength will continue to grow. Biopolymers can offer this alternative to synthetic petroleum based plastics as a starting point as they are renewable, biodegradable and inexpensive. Researchers have developed high strength biopolymer films in combination with bio-based fillers and reinforcements and plasticizers over the past decade. Future work will need to focus on bettering mechanical strength of biopolymer films at the same time not sacrificing other characteristics such as barrier properties.

Chapter 6

The effect of glycerol concentration on mechanical, thermal and structural properties of collagen-based films

6.1 Introduction

Generally, thin films that find application in packaging industries are flexible, transparent and often strong materials which can be derived from natural polymers such as whey protein, collagen, starch, gelatine and potentially other natural renewable polymers [68; 69]. Due to rising environmental concerns, biodegradable films have attracted considerable attention especially from the food and drug packaging industries as natural polymer-based films can potentially replace plastic films which are derived from synthetic polymers [70].

Preparation of films from renewable resources will in most cases degrade more readily and contribute to the reduction of environmental waste. Specifically, in case of collagen-based films considered here, it will subsequently contribute to waste valorisation as the collagen used to prepare films in this chapter is derived from waste bovine hide off-cuttings. In the US alone, edible films total annual revenue exceeding $100 million US dollars.

Films must provide physical protection to food products from mechanical damage; and to guard food from chemical and microbiological activities. Films that are to be used in the food industry should also prevent moisture losses, while selectively allowing controlled exchange of important gases such as oxygen, carbon dioxide and ethylene. However, if the films are to be used in the medical industry, characteristics such as biocompatibility, controllable biodegradability, mechanical strength and the ability to absorb body fluids for delivery of nutrients are more important. Hence, depending on the application of the films certain functionalities need to enhanced over others

The main steps required to prepare films from proteins are as follows: 1) Breaking of low-energy intermolecular bonds that stabilize the polymers. 2) Changes in the arrangement and shaping of the polymer chains. 3) Rearrangement and formation of the film by stabilization of new interactions and bonds [395]. There are two processes of preparing films from proteins such as collagen: the wet process that involves the homogenization and solubilisation of the proteins and the dry process which is based on the thermoplastic properties of proteins under low moisture

content [395]. Preparation of films from proteins with the wet process has been studied extensively [335; 395; 396], however modifications and improvements to the general method is continuously being applied depending on the raw material. These modifications include the use of reinforcements to improve mechanical strength, addition of antibacterials to increase film shelf life and incorporation of appropriate plasticizers to increase flexibility and at the same time not sacrifice film tensile strength. The wet process requires the formation of a protein solution under controlled conditions and thus this process is often described as a film casting method [397].

Plasticizers are incorporated in film preparation methodologies to achieve flexibility and workability. Interactions of a plasticizer and the polymer depends on a number of factors such as molecular size, configuration and the total number of functional hydroxide groups of the plasticizer, as well as the polymer compatibility with the particular plasticizer [398]. Glycerol was used as the only plasticizer for this study due to its low molecular weight and the absence of a ring molecular conformation that sterically hinder insertion between the protein chains which is the case with sorbitol (higher molecular weight) [398]. Glycerol helps to overcome film brittleness caused by extensive intermolecular forces found in collagen. Addition of plasticizer changes some properties of films, such as increasing flexibility, moisture sensitivity, as well mechanical properties. The effect of glycerol concentration as a plasticizer on mechanical properties, secondary structure (FTIR), solubility, thickness, thermal properties, and mechanical properties of the films were investigated in this chapter.

For this investigation, collagen extracted from bovine calf-hide (CH1) and bull-hide (BH) were used to prepare biodegradable films. These two sources were of interest as CH1 collagen is from a younger source and has a lot less inter- and intramolecular cross-linking than collagen from the older source of BH. Extraction of CH1 collagen can also be carried out without the use of expensive enzymes as discussed in Chapter 3. In this chapter, the wet process was applied to prepare films from these sources. The second part of this investigation was to study the effect of plasticizer concentration on the physical and mechanical properties of films. The effect of glycerol as a plasticizer on biodegradable films has been studied

[70; 225; 298; 302; 311; 399-405] , however the effect of glycerol at these specific concentrations used in this chapter on collagen from sources of CH1 and BH has not been considered yet. Hence, the effect of glycerol on mature collagen derived from BH with extensive cross-linking and on younger CH1 collagen with a lot less cross-linking will be investigated. This way, we attempt to build a more complete picture of changes occurring in the system at the molecular level. It will be beneficial to observe if there are major differences in properties of these films as it will utilize collagen from off-cuttings of bovine hides and hence help in terms of waste valorisation.

6.2 Materials and methods

6.2.1 Materials

Bovine hide off-cuttings from bull and calf were obtained from Wallace Corporation, Waikato, New Zealand. Glycerol, pepsin, silica gel, acetic acid, hydrochloric acid, sodium chloride, and dialysis tubing were obtained from Sigma Aldrich, PTFE coated glass fabrics were obtained from Dotmar Universal plastics. All remaining chemicals used for collagen extraction were analytical grade.

6.2.2 Collagen extraction

Hides were washed with cold tap water. They were de-haired and cut into pieces (approximately 1 cm x 1 cm). The de-haired, cut hide pieces were either stored at -20℃ (long-term storage) or at 4℃ to be used for collagen extraction. BH and CH1 collagen were extracted by acid-enzyme solubilisation as described in detail in Chapter 3.

6.2.3 Film preparation

Purified, freeze-dried collagen was suspended in 50 ml 0.5 M acetic acid and left for 24 hours at 4 ℃. Following this step, distilled water was added to the collagen solution and for a homogenous suspension; it was stirred continuously for 10 minutes at room temperature. Finally, glycerol was added to the film forming solution and placed on a water bath at 60℃ for 45 minutes with continuous stirring. Film forming solution was poured on 150 mm diameter glass petri dishes

that were PTFE coated and placed in oven at 55 ℃ for 48 hours. The dried films were manually peeled off and stored in a desiccator prior to analysis. Figure 6.1 shows a simplified schematic diagram of the film preparation method.

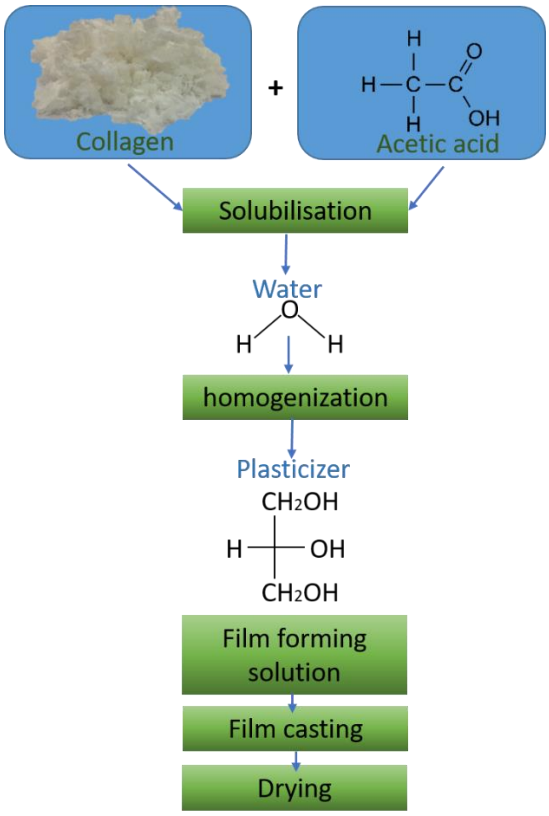


Figure 6.1: Schematic diagram of film preparation carried out in this investigation.

6.2.3.1 Experimental design

BH and CH1 collagen-based films were prepared by solution casting. For each collagen source three formulations containing varying amounts of plasticizer were used (Table 6.1).

Table 6.1: Composition of each film formulation.

Formulation and collagen source	Composition	Remaining materials added to each film formulation
	Glycerol concentration	Collagen solubilized in 0.5 M acetic acid. 10 Parts dH2O relative to weight of collagen.
F1(BH) **F1(CH1)**	80% glycerol relative to weight of collagen	
F2(BH) **F2(CH1)**	40% glycerol relative to weight of collagen	
F3(BH) **F3(CH1)**	20% glycerol relative to weight of collagen	

Where:

- F1 = formulation 1 , F2 = formulation 2, F3 = formulation 3
- BH collagen = bull hide collagen
- CH1 collagen = calf hide collagen
- dH_2O = distilled water

These formulations were selected based on of scoping experiments that gave an indication of the amount of plasticizer and water to use to prepare collagen-based films. The scoping experiments included using glycerol concentrations of 0%, 5%, 10%, 20%, 40%, and 80%. Films with 0-10% glycerol were extremely brittle and would break upon peeling off the cast. 20%, 40% and 80% glycerol concentrations were therefore chosen to further work with.

6.2.4 Film characterization

6.2.4.1 Film thickness

Thickness of the collagen-based films were determined using a digital caliper (Limit Triple Read Digital Caliper) to the nearest 0.01mm. Thickness measurements (mm) were taken in five random locations for each film and an average was calculated.

6.2.4.2 Moisture content (MC)

All films were weighed (W_1) and dried at 105°C for 24 hours in a Contherm Thermotech 2000 oven. The dried film samples were weighed again (W_2) and moisture content was determined as the percentage of initial film weight lost during drying. Triplicate measurements of moisture content for each film sample was taken and an average was taken to account for MC according to the Equation 1.

$$MC\ (\%) = [(W_1 - W_2)/W_1] * 100 \qquad [1]$$

6.2.4.3 Solubility

Film samples were cut into 2 cm*2 cm samples, wrapped in filter paper and dried in a Contherm Thermotech 2000 oven at 105°C for 24 hours. Film samples in filter paper were weighed (W_i). The film with the filter paper were soaked in water for 24 hours at 28°C with occasional stirring. The film samples with the filter paper were oven dried one last time at 105°C for 24 hours to determine the weight of the film which is not soluble in water (W_f).

$$Solubility\ (\%) = [(W_i - W_f)/W_i] * 100 \qquad [2]$$

-where: = W_i = initial weight of the film, W_f = weight of the un-dissolved dried film residue.

6.2.4.4 Microstructure characterization – Fourier transform infrared spectroscopy (FTIR)

The secondary structure of the films were analysed by FTIR. FTIR Digilab FTS-40 was used to record the spectrum from with the KBr method. Films were cut in approximately 1 mm strips and further cut into smaller pieces to be mixed with KBr. Discs containing 2 mg of film sample in approximately 100 mg potassium bromide (KBr) were prepared. Spectra were taken in the wavenumber region 500-4000 cm^{-1} with each sample being subjected to an average of 16 scans.

6.2.4.5 Thermogravimetric analysis (TGA)

The thermal properties of the collagen-based films were characterized using the PerkinElmer STA 8000 analyser. The analysis was operated in a dynamic mode, film samples (4-7 mg) were heated from 30°C to 800°C at 10°C/min in air purged at 150 ml/min with an empty pan used as a reference. Differential thermal analysis (DTA) and thermal gravimetric analysis (TGA) curves were obtained at the end of the operation.

6.2.4.6 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) was used to determine the glass transition temperature (Tg) or the denaturation temperature (Td) of film samples using a Perkin Elmer Differential Scanning Calorimeter (DSC-8500). Approximately 4-7 mg

film samples were weighed into aluminium pans and sealed. The samples were scanned at 50 ℃/min over the range of -50 ℃-300 ℃ using liquid nitrogen as the cooling medium. An empty pan was used as a reference. Td or Tg was obtained from the transition in the baseline in the 30-80 ℃ region by taking the inflection point.

6.2.4.7 Mechanical properties

Film tensile strength properties were measured in accordance to ASTM D882-12. The test was performed using Instron-4204 universal testing machine fitted with a 5kN load cell and the cut films were conditioned at 23 ± 2 ℃ and 50 % RH for 48 hours before analysis. Initial grip separation distance was set to 50 mm and mechanical crosshead speed to 50 mm/min. In order to prevent slippage and premature failure occur near the grips, the specimen ends were cushioned with abrasive paper. Stress versus strain graphs were obtained from which the ultimate tensile strength, Young's modules and elongation (%) could be determined. Tensile strength measurements of each type of film were repeated 5 times.

6.3 Results and discussion

6.3.1 General observations

Table 6.2 summaries the resulting surface properties of each film formulation. In the absence of sufficient amounts of plasticizer, a very brittle inflexible film was formed which broke into pieces upon peeling off the casting surface (observed while carrying out scoping experiments). F1 for both collagen sources were very flexible and this is due to the high plasticizer content. The higher plasticizer content changes some of the functional and physical properties of films and this includes the flexibility of the films as the plasticizer reduces the interactions between the adjacent chains in the biopolymer and thus affecting the flexibility of the material [294].

Prepared collagen-based films from all formulations and collagen sources were very similar in their physical surface properties. The films were flexible, very light yellow in colour – which is due to addition of glycerol, and there were not any visible bubbles, cracks or pores. Film thickness ranged from 551 μm to 588 μm

(Table 6.2) with no significant variance in thickness amongst the films based on glycerol content.

Table 6.2: Observed film surface properties of BH and CH1-based collagen films with formulation 1,2 and 3.

Film source	Formulation	Observed visual properties	Film thickness (μm)
BH collagen films	**F1**	Easy removal, smooth, transparent, sticky, very flexible, no bubbles	558.3 ± 1.6
	F2	Easy removal, smooth, transparent, not sticky, flexible, no bubbles	565.7 ± 1.4
	F3	Hard to peel off, smooth, transparent, limited flexibility, no bubbles	551.7 ± 2.6
CH1 collagen films	**F1**	Easy removal, smooth, transparent, slightly sticky, very flexible, no bubbles	570.3 ± 5.6
	F2	Easy removal, smooth, transparent, not sticky, flexible, no bubbles	559.3 ± 0.6
	F3	Hard to peel off, smooth, transparent, limited flexibility, no bubbles	588.3 ± 2.5

6.3.2 Moisture content (MC) and solubility

Table 6.3 shows the moisture content (%) and solubility (%) of films. A small increase in moisture content was observed with increasing glycerol concentration. The highest moisture content was observed with F1 films. . This could be due to the hydrophilic character of glycerol, and resulting in films with high glycerol content becoming more susceptible to moisture. Within the two sources of collagen, the CH1 collagen-based films had slightly higher moisture contents for all formulations. This was also reflected in Tg values of films with DSC analysis (Figure 6.5, Table 6.6). Lower Tg values were obtained upon an increase of MC due to the plasticizing effect of water.

Moisture content (MC) of films is regarded as an important characteristic as it greatly affects the mechanical properties of the film [406]. Water can also act as a plasticizer in protein films, thus monitoring the water content of films helps to

understand its plasticizing effects on the resulting films. High MC can adversely affect the mechanical properties of films resulting in very weak films [406]. Moisture content also has a huge effect on the glass transition temperature of biopolymer films. Cuq et al. [395] stated, the higher the water content of films the lower the glass transition temperature and the lower the water content the higher the glass transition temperature of films [395; 407]. This was similarly observed with Tg values of films obtained in this chapter.

Table 6.3: Moisture content (%) and solubility (%) of BH and CH1 collagen-based films with 80% glycerol (F1), 40% glycerol (F2) and 20% glycerol (F3).

Film source	Formulation	Moisture content (%)	Solubility (%)
BH- collagen based films			
	F1(BH)	9.1 ± 1.4	36.14 ± 2.3
	F2(BH)	8.3 ± 0.9	33.80 ± 0.9
	F3(BH)	8.1 ± 1.2	13.4 ± 2.6
CH1-collagen based films			
	F1(CH1)	13.4 ± 2.1	33.7 ± 0.82
	F2(CH1)	12.6 ± 1.6	29.2 ± 1.5
	F3(CH1)	12.4 ± 1.3	14.2 ± 4.6

Films must have great water resistance to preserve the integrity of the product [408] and should easily degrade naturally once deposited in the environment [409] thus film solubility to a certain extent is favored. However, high solubility is not favored as the films will not be able protect the product from humidity and moisture loss [399].

Table 6.3 shows solubility of BH and CH1-based collagen films with F1, F2 and F3 formulations. A significant increase or decrease in film solubility (%) was not seen between the two film sources. However, approximately 3% reduction in solubility with F1 and F2 was observed with CH1 films (Table 6.3). This could possibly indicate that BH films contained portions of gelatinized collagen, which is water-soluble. However, this is not a significant amount as in the DSC section of this chapter Tg values of BH collagen films were slightly higher than the CH1 collagen films.

Within the formulations, F1 having a greater glycerol content (80%) showed a slight increase in solubility in comparison to F2. An increase in the addition of

glycerol as a plasticizer in the film matrix is understood to reduce hydrogen bonding and increase the mobility of the polymer chains [410] and hence causes an increase in the free volume and the network becomes more soluble [408]. This explains the huge reduction in solubility with F3 as it had the lowest content of glycerol at 20%. Both BH and CH1-collagen based films with formulation 3 had a drastic decrease in solubility (%) at 13.43% and 14.17% respectively (Table 6.3). A reduction in film solubility is favored, especially if it has to be used for high-moisture foods. As much as this low solubility (%) of formulation 3 for both BH and CH1-collagen based films are favored, the brittleness of the F3 films is not favored. Brittle films have little or no applications.

6.3.3 Fourier transform infrared spectroscopy (FTIR)

Interactions between glycerol and collagen sources could play an important role in determining characteristics of the subsequent films, especially their mechanical properties. The most important functional groups related to interactions of glycerol with collagen are hydroxyl groups occurring at amide A (3000-3500 cm^{-1}), amide I (1630-1650 cm^{-1}), amide II (1539-1550 cm^{-1}) and amide III (1033-1135 cm^{-1}) [411]. A shift in any of these amide peaks to a lower wavenumber indicate presence of hydrogen bonding and the lower it is within the band region the greater the presence of hydrogen bonding and collagen molecular stability. On the other hand, a shift to higher wavenumbers within these characteristics bands indicate structural rearrangements occurring in the film structure [406] .

The Amide A peak position for BH and CH1 collagen-based films was found in the range 3455-3438 cm^{-1} (Figure 6.2 and Figure 6.3, Table 6.4) which is due to hydrogen bonding of N-H group with a carbonyl group of the peptide chain. A shift of Amide A group to lower frequencies indicates presence of hydrogen bonding between collagen molecules as it is required for the formation of the triple helix. As the glycerol content increased, the position of this peak shifted to higher wavenumbers (Table 6.4) This indicates loss of structure within the collagen molecules as glycerol is introduced into the film matrix. Amide A peak for F3 films of both sources was more intense in comparison to F1 and F2 films. Siripatrawan et al.[412] also observed this with low plasticized chitosan-based films and related

it to a stronger presence of free hydroxyl groups and N-H bonds contributing to hydrogen bonding. With the addition of increasing amounts of plasticizer, this band became wider and less intense.

Amide I band position for BH and CH1 collagen-based films ranged between 1635-1645 cm^{-1}. The main attribute of this band is hydrogen bonding between N-H stretch and C=O stretching vibration along the collagen polypeptide backbone. Again, as glycerol content was increased for either film source, the wavenumber shifted to higher values. Similarly, amide II and amide III band peak positions shifted to higher wavenumbers with increasing glycerol concentration (Table 6.4) for both BH and CH1 collagen-based films.

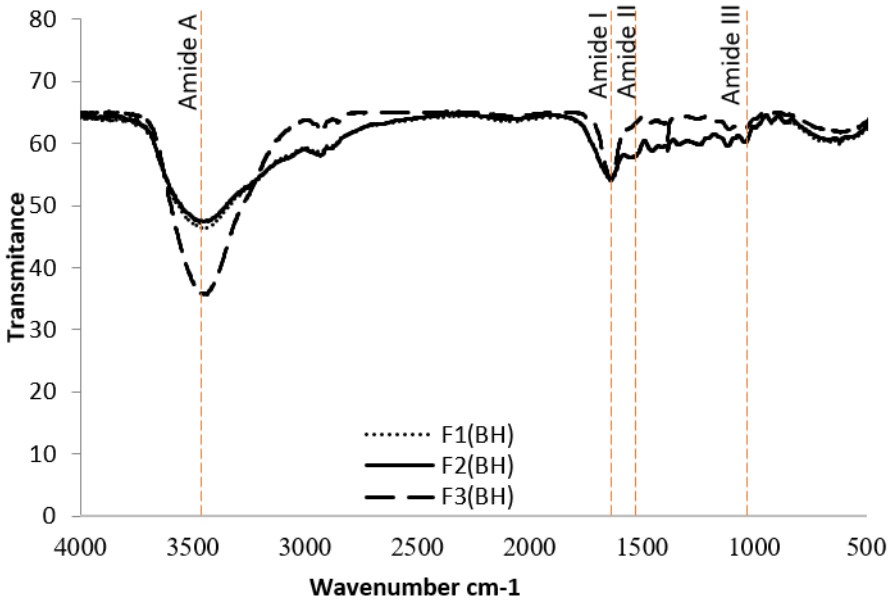


Figure 6.2: FTIR spectra of BH collagen-based films with with 80% (F1) glycerol, 40% (F2) glycerol, and 20% (F3) glycerol.

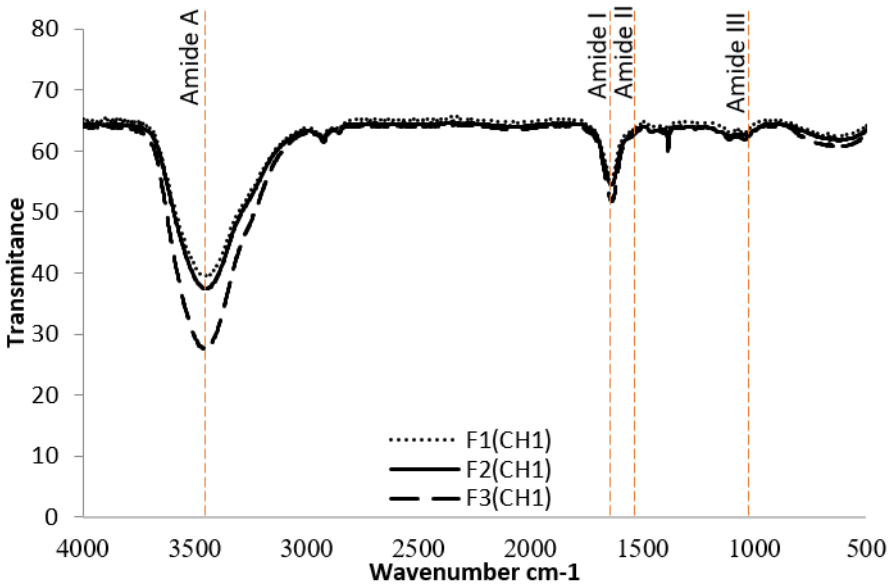


Figure 6.3: FTIR spectra of CH1-collagen-based films with 80% (F1) glycerol, 40% (F2) glycerol, and 20% (F3) glycerol.

Table 6.4: Main FTIR spectra peaks for BH and CH1-collagen based films and their respective absorbance ratios.

	Component peak location (cm^{-1})			
Collagen film formulation	Peak 1 (Amide A)	Peak 2 (Amide I)	Peak 3 (Amide II)	Peak 4 (Amide III)
F1(BH)	3455	1645	1554	1043
F2(BH)	3452	1639	1554	1040
F3(BH)	3438	1638	-	1037
F1(CH1)	3453	1639	-	1042
F2(CH1)	3452	1638	-	1040
F3(CH1)	3449	1635	-	1038

6.3.4 Mechanical properties

Table 6.5 is showing the mechanical properties of BH and CH1-collagen films. Tensile strength and elongation (%) are particularly important mechanical measurements of biopolymer films as tensile strength accounts for the film's mechanical resistance due to interconnected forces between the chains and elongation (%) correlates to the plasticity of the film.

Tensile strength (MPa) increased with decreasing plasticizer concentration (80% to 40%) for both BH and CH1-collagen based films (Table 6.5), but then decreased at 20% glycerol. F1 films resulted in very similar tensile strengths at 1.18 MPa and 1.33 MPa for BH and CH1-collagen based films respectively. The highest tensile strength was attained at 40% glycerol for both BH and CH1 collagen-based films. BH-collagen films with 40% glycerol resulted in an increase of approximately 81% in tensile strength in comparison to CH1 collagen-based films with an increase of approximately 83% in tensile strength from 80% to 40% glycerol reduction.

F2 films with 40% glycerol exhibited superior tensile strength in comparison to F3 films with 20% glycerol. This phenomenon can also be related to "anti-plasticization" effect of glycerol at 40% with collagen. Suderman et al [368] associated this with stronger interaction between the plasticizer and the polymer molecules that hinder macromolecular mobility. Zhang et al. [413] described anti-plasticization with starch-based films and related the anti-plasticization of films to occur when the amount of plasticizer molecules increased above the critical value. Sanyang et al. [414] observed a decrease in elongation (%) of starch-based films with an increase in glycerol content and associated this to formation of glycerol-rich and starch-rich areas due to migration of glycerol from the film matrix. Further, Sanyang et al. [414] related this migration of glycerol from the film matrix to enable stronger starch intermolecular bonding interactions and thus a reduction in elongation (%) of the films.

Chang et al. [415] similarly observed both the plasticization and anti-plasticization of water with starch-based films. Water acted as a plasticizer in the case of thermal stability and tensile modulus; however, it acted as an anti-plasticizer with tensile strength of the starch films. This was the exact observation in the case of this chapter, at 40% plasticizer, glycerol acted as plasticizing agent in regards to DSC measurements resulting in lower Tg values in comparison to 20% glycerol and acted as an anti-plasticizer with tensile strength measurements. Chang et al. [415] related this contradictory plasticizing effects of water on mechanical properties of films to lower and higher deformation of films being resolved by allocating water different roles but primarily operating via the opposite of free volume effects.

Additionally, the mechanical properties of biopolymer films are greatly associated with distribution of intra-and intermolecular interactions, depending on the orientation and arrangement of polymer chains in the network [321]. Due to this, it was hypothesised that the younger collagen source (CH1-collagen) would result in lower or similar tensile properties but not higher than the older source collagen (BH-collagen). In terms of waste valorisation collagen extraction from hide off-cuttings of both BH and CH1 are favourable. However, since collagen from CH1 can be extracted without the use of expensive enzymes (such as pepsin) it is advantageous that CH1-collagen based films resulted in similar mechanical properties as BH collagen films. This way collagen from CH1-collagen could be used for biopolymer film preparation and collagen from BH off-cuttings for more expensive end use applications as extraction of pure native BH collagen requires use of high cost enzymes. In this chapter, both collagen types resulted in films with high enough tensile strength to be used as packaging purposes for food related applications such as coatings and wrappings.

Table 6.5: Mechanical properties of BH and CH1-collagen based films from stress-strain analysis with varying glycerol concentrations.

Film Formulation	Test conditions	Thickness (μm)	Tensile strength (MPa)	Elongation at break (%)
F1(BH)	23±2℃, 50% RH, 48 hrs	558.33 ± 1.6	1.18 ± 5.3	249.18 ± 15.5
F2(BH)	23±2℃, 50% RH, 48 hrs	565.67 ± 1.4	6.11 ± 5.1	184.92 ± 12.3
F3(BH)	23±2℃, 50% RH, 48 hrs	551.67 ± 2.6	5.19 ± 4.2	179.47 ± 16.4
F1(CH1)	23±2℃, 50% RH, 48 hrs	570.33 ± 5.6	1.33 ± 5.5	254.21 ± 16.1
F2(CH1)	23±2℃, 50% RH, 48 hrs	559.33 ± 0.6	7.79 ± 5.7	102.27 ± 11.4
F3(CH1)	23±2℃, 50% RH, 48 hrs	588.33 ± 2.5	5.94 ± 5.1	50.14 ± 17.5

Elongation at break (%) for both film types increased only up to 40% glycerol then decreased again with 20% glycerol. BH-collagen based films exhibited highest elongation (%) with 80% glycerol concentration and lowest elongation (%) with 20% glycerol concentration at 249.18% and 179.47% respectively. On the other hand, CH1-collagen based films resulted in elongation (%) in the range of 50.14 % to

254.21%. An increase in elongation (%) with increasing glycerol concentration was also reported by Jongjareonrak et al. [296] and Hoque et al. [416]. At large, the mechanical properties of collagen-based films were affected by glycerol content, with the most optimum glycerol content being 40% for this case.

In terms biopolymer film mechanical properties, it can be concluded from the above observations that in order to choose a film with high strength, elongation will need to be sacrificed and to choose a formulation that results in high elongation (%) then tensile strength will need to be overlooked. This observation of biopolymer films with higher tensile strength values showing lower elongation (%) value is correlated to the structural nature of those attributes. Inherently, collagen has a vast network of cross-linking which makes it mechanically stable and strong. The introduction of heat during film preparation does denature the collagen to a certain extent and hence it will have a lower number of cross-linking and become weaker.

6.3.5 Thermal properties - Thermogravimetric analysis (TGA)

TGA weight loss thermographs of BH and CH1 collagen-based films are presented in Figure 6.4. It can be observed that the films from both CH1 and BH-collagen have similar properties and the multiple stages relate to evaporation and decomposition of different components of films.

The film thermograph regarding weight loss is divided in four phases for F1 and F2 of both CH1 and BH-collagen films. F3 thermographs for both CH1 and BH-collagen films shows three phases of weight loss (%) with increasing temperature. Formulations F1 and F2 show dehydration or evaporation of moisture from the films, presented in phase one which ranges from 50-100℃. The second stage of thermal degradation for F1 and F2 films observed at approximately 100-270 ℃ corresponded to the evaporation of glycerol. The degradation of glycerol at this stage has been reported by Sanyang et al. [414] with glycerol plasticized starch films and by Zhong et al. [417] . Finally, decomposition of F1 and F2 films is observed in two phases of the thermograph, as rapid weight loss, observed at the approximate temperature range of 270 to 360℃ in phase three and in the fourth

phase ranging from 360-600℃ indicating full collagen degradation. Thermal stability of the films increase with decreasing glycerol content (F1→F3).

F3 film thermographs look similar to the raw collagen thermographs observed in chapter 4 with a three-phase weight loss profile. DTA curves (Table 6.6, b and d) of both film sources with F3 show a more thermally stable curve in comparison to F1 and F2 films. The results of the DTA curves agree to the Tg values obtained with DSC analysis (Table 6.6). BH collagen-based films DTA curve shows to be slightly more thermostable than the CH1 collagen-based films and this is confirmed with the DSC Tg values. Addition of glycerol results in a drop in the thermal stability of the biopolymer film. Films plasticized with higher contents of glycerol at 80% (F1) and 40% (F2) degrade at slight lower temperatures and this indicates that the presence of glycerol reduces the intermolecular hydrogen bonding and thus increasing the free volume within the polymer network. Therefore, there is not a drastic difference in thermal stability of films within collagen sources, however, it BH collagen films were slightly more thermostable, especially F3 films (Figure 6.4, a, b). Similarly, this difference in thermal stability between BH and CH1 collagen films were observed in Tg values with DSC analysis. Within formulations and has direct correlation to plasticizer content.

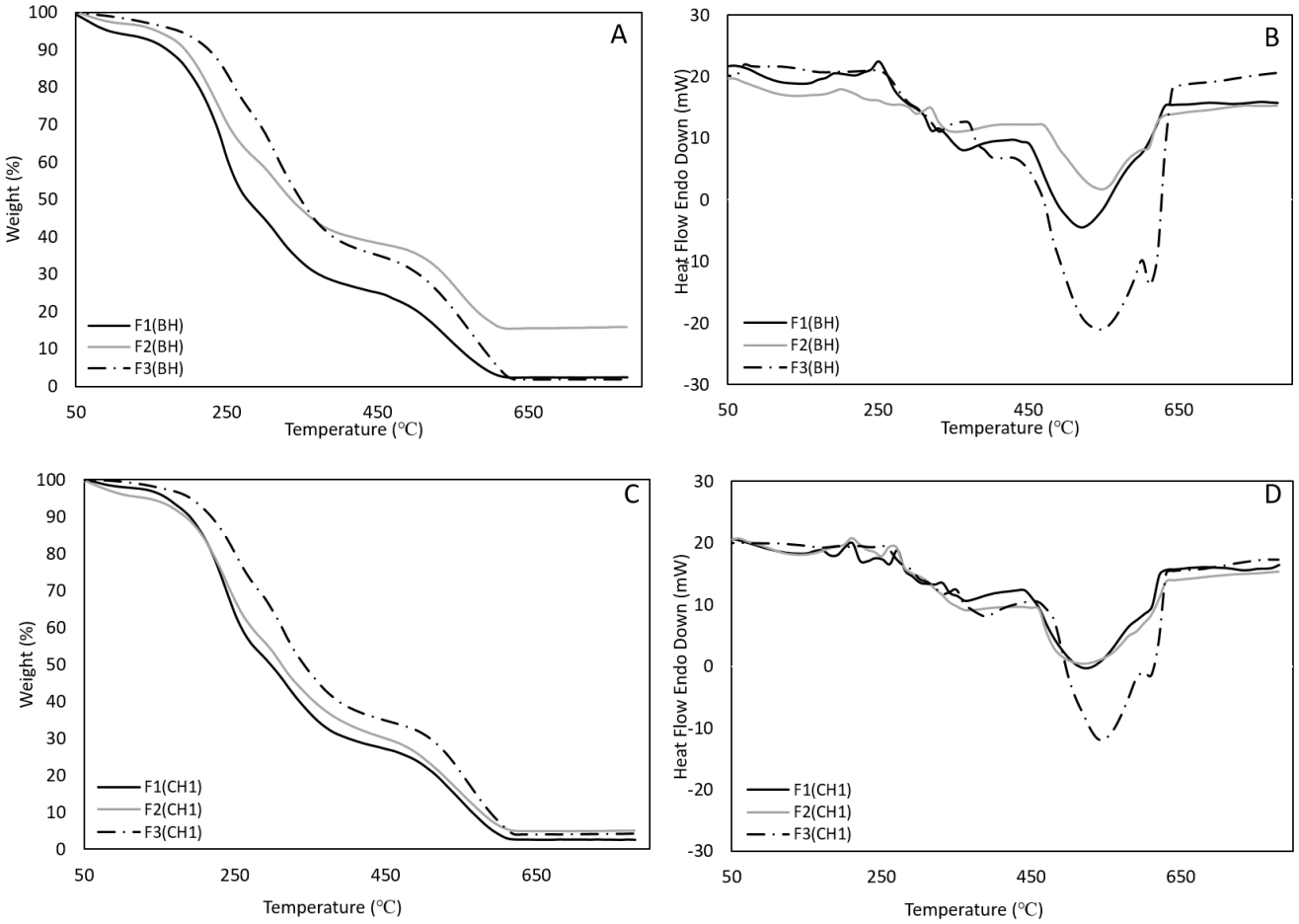


Figure 6.4: TGA weight loss and DTA curves for BH collagen films (A-B) and for CH1 collagen films (C-D).

6.3.6 Differential scanning calorimetry

DSC thermograms showed four endothermic transitions for both BH and CH1 collagen-based films (Figure 6.5). The first and most important endothermic transition being related to film the glass transition temperature (Tg) of films. The second transition being associated to water evaporation, the third endothermic transition to loss of glycerol and the fourth transition to collagen degradation.

The glass transition temperature (Tg) of both BH and CH1 collagen based films increased with decreasing glycerol concentration (Table 6.6, Figure 6.5). The highest Tg for both BH and CH1 collagen-based films were observed with F3 at 62.46 ℃ and 60.67 ℃, respectively. As the glycerol content decreased, film Tg values increased linearly. Patil et al. [418] related a decrease in Tg of biopolymer films upon water uptake, which explains why F1 films had the lowest Tg values due to glycerol being a hygroscopic substance it absorbs water. The addition of glycerol in collagen-based films results in more hydrophilic hydroxyl groups as active sites, which can be occupied by water molecules. Hence, films with the higher glycerol concentration demonstrated a higher moisture content (as presented in section 6.3.2) and therefore resulted in lower Tg values.

Horn et al. [419] prepared collagen and gelatine-based films with pequi oil as the plasticizer and all resultant films exhibited Tg values of approximately 51 ℃, concluding that the triple helix structure of collagen was preserved with addition of pequi oil due observance of such a high Tg value. Wang et al. [321] prepared collagen-based films plasticized with glycerol and attained a Tg value of approximately 55 ℃. Hence, the Tg values obtained in this chapter are like other studies where biopolymer films were plasticized with glycerol – the fact that the films in this chapter were prepared via hot solution casting did not have a drastic negative effect on the thermal properties of the films.

Considering the collagen sources, BH collagen-based films resulted in higher Tg values with all formulations in comparison to CH1 collagen-based films. The observation of higher Tg values being exhibited for BH collagen-based films is understandable as in chapter four; CH1 collagen indicated lower denaturation temperatures than BH collagen. This phenomenon was explained by the concept

that CH1 collagen has a lot less cross-linking in comparison to BH collagen and its lower thermal stability, hence it is more readily affected by heat and harsh conditions such as high acid concentrations. DTA curves (Figure 6.4, b) also showed higher thermal stability for all formulations of BH collagen-based films in comparison to CH1 collagen-based films. Another factor for the higher Tg values of BH collagen films could be attributed to the lower moisture content of BH films (Table 6.3). Water does act as a plasticizer and CH1 collagen films did exhibit higher moisture content values in comparison to BH collagen films. This is hence reflected in film Tg values.

Table 6.6: DSC thermogram transitions of BH and CH1 collagen-based films.

Film source	Formulation	Transition 1 (Tg)	Transition 2 (Water evaporation)	Transition 3 (Loss of glycerol)	Transition 4 (collagen degradation)
BH collagen -based films	F1(BH)	53.06 ℃	107.45 ℃	134.21 ℃	274.24 ℃
	F2(BH)	57.33 ℃	96.11 ℃	141.64 ℃	250.90 ℃
	F3(BH)	62.46 ℃	109.57 ℃	134.58 ℃	255.78 ℃
CH1 collagen -based films	F1(CH1)	51.80 ℃	99.68 ℃	135.64 ℃	202.37 ℃
	F2(CH1)	52.59 ℃	97.87 ℃	135.24 ℃	258.05 ℃
	F3(CH1)	60.67 ℃	100.41 ℃	143.34 ℃	288.31 ℃

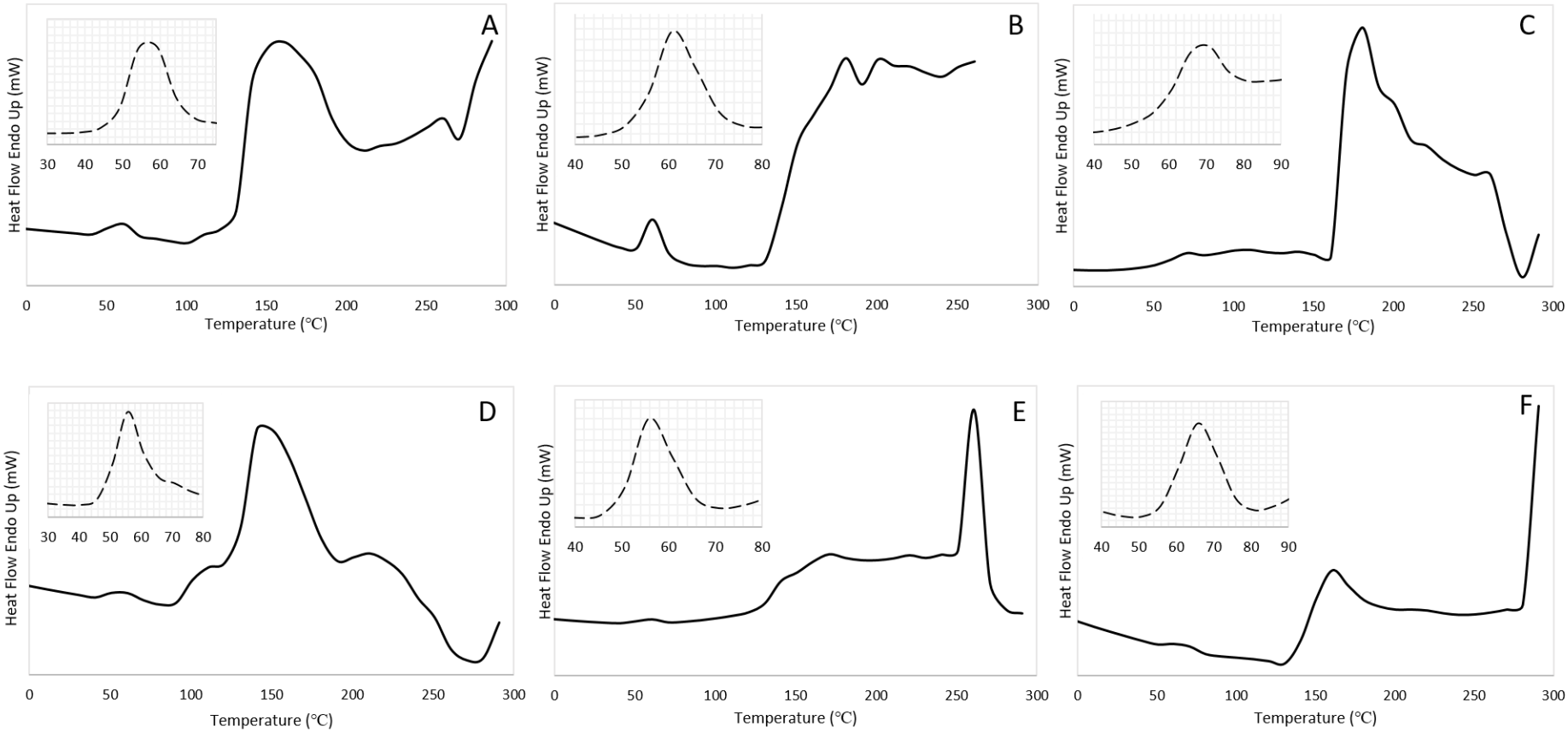


Figure 6.5: DSC thermograms of BH and CH1 collagen films with A = F1(BH), B= F2(BH), C = F3(BH), D = F1(CH1), E = F2(CH1), and F = F3(CH1).

Considering all the characterizations carried out on the films in this chapter, it can stated that transparent, flexible, and without presence of pores or cracks films can be prepared both from BH and CH1 collagen. Moisture content of BH collagen films being lower than CH1 collagen films were also reflected in film thermal stability with TGA analysis and also in Tg values with DSC analysis. F1 films for both collagen sources exhibited the highest moisture content values and solubility (%) and this was correlated to the hydrophilic nature of glycerol and resulting in films with high glycerol content to become more susceptible to moisture. The moisture content variance amongst the formulations also affected the mechanical properties of films. High MC can adversely affect the mechanical properties of films, resulting in weaker films. However, F2 films for both collagen sources resulted in superior tensile strengths in comparison to F3 films (lowest glycerol content) – this factor was correlated to the anti-plasticization effect of glycerol. FTIR analysis showed the interactions between glycerol and collagen at each formulation. As the glycerol content was increased for both collagen sources, a shift to a higher wavenumber within each characteristic band (amide A-amide III) was found, indicating structural rearrangements occurring in the film structure. These results were reflected both in Tg values and with mechanical properties of films – with an increase in glycerol resulting in lower Tg values and resulting in lower tensile strength values. Overall, both sources resulted in films sturdy enough to be used for packaging purposes, especially F2 films.

6.4 Conclusion

BH and CH1-based collagen films with three formulations varying in the plasticizer content were successfully prepared. The films were characterized for their physical properties, secondary structure, thermal and mechanical properties. The films appeared to be uniform in thickness, transparent and showed good flexibility. Surface properties and thickness of both collagen sources were very similar, however film solubility (%) decreased with decreasing content of glycerol, which is a feature of glycerol. DSC and DTA curves of films indicated higher thermal stability for BH collagen-based films in comparison to CH1 collagen-based films. The FTIR spectra of films helped to understand the structural changes and

interactions occurring with the collagen sources and glycerol. The structural changes observed with FTIR spectra helped to understand the trend in tensile strength increase with a decrease in glycerol content. Interestingly, both collagen sources resulted in similar mechanical properties. This is a positive finding as extraction of CH1 collagen is inexpensive in comparison to extraction of BH collagen, which is from an older source. F2 films glycerol content was found to be most optimum as it resulted in the most preferable film properties, especially resulting in superior mechanical properties for both collagen sources in comparison to F1 and F3 films. Hence, in chapter 6 a constant concentration of glycerol at 40% will be used to prepare collagen-reinforced gelatine films.

Chapter 7

Films from collagen and gelatine blends

7.1 Introduction

Reduction of petroleum resources, increase of environmental pollution and the demand for sustainable materials has driven researchers to obtain alternative, bio-friendly materials. The production of sustainable films and coating materials from renewable or waste resources has received considerable attention. Proteins, lipids and carbohydrates that are obtained from renewable resources such as plant and animal sources are a great alternative for production of bio-friendly materials and subsequently reducing waste produced from these sectors.

Amongst the proteins, collagen is such a biomaterial, which can be utilized for film preparation. This is due to its unique amino acid residues being variable in proportions and distributions along the macromolecular super helix structure, which combine much functionality applicable for film forming applications. Collagen also has great tensile strength and excellent thermal stability [420].

Gelatine is readily available, inexpensive and a biodegradable polymer that has favourable film forming properties. Moreover, gelatine-based films are flexible, translucent and colourless. Research has been carried out on gelatine-based films as a potential packaging material to preserve food [322; 421-424]. Due to the non-toxic nature of gelatine, ease of processability, low –cost and film-forming ability, gelatine attracts a great interest as an alternative for synthetic polymers used in the packaging industry. However, the major disadvantage of gelatine as a food packaging material is its poor mechanical properties and low thermal stability, which limit their application in the packaging industry. To overcome such limitations, the blending of gelatine with other compatible and miscible biopolymers with different structures could contribute in desired packaging material properties in the resulting composite films.

The raw material for collagen is extracted from waste bovine hide off-cuttings throughout this project. The processing steps of collagen extraction require the use of acids, enzymes and salts, hence, the costs can eventually add up. Collagen to be used as the sole raw material for the production of packaging materials can be costly and ineffective in terms of using such a sought after biomaterial,

especially in the medical industries. Gelatine, on the other hand is derived from the same waste sources of bovine hide off-cuttings but at a substantially lower cost. Extraction of gelatine does not require the use of enzymes, careful control processing steps nor the need of systematic purification. Combining the great mechanical and thermal properties of collagen with the great flexibility and film forming ability of gelatine, a unique, cost efficient film could possibly be prepared.

Collagen is insoluble in water, but soluble in weak acid concentrations; however, gelatine is highly soluble in water and forms transparent elastic thermoreversible gels upon cooling below approximately 25 ℃. The amphiphilic nature of gelatine molecules allows useful emulsification and foam stabilizing properties. Upon dehydration of gelatine, irreversible conformational changes take place that are useful in the formation of films [420]. The sol-gel transition of gelatine solutions is a reversible reaction unique to gelatine and is used for various applications such as film formation. The sol-gel transition is temperature responsive, where, it can form a gel upon cooling and gelatine can be liquefied upon heating. Compatibility and miscibility of these two polymer blends are an important feature to consider. Collagen-gelatine blends will need to be miscible or compatible in order for a favourable composite film to be prepared. Gelatine being the thermally denatured form of collagen (Figure 7.1) has the same amino acid profile of collagen but broken down into smaller units of protein rather than comprising of a stable triple helix found in collagen; they also differ in their structure and properties. Hypothetically, a blend of collagen and gelatine should be miscible in solution upon collagen solubliztion in a weak acid solution. On the other hand, one of the main differences between collagen and gelatine in terms of miscibility of the two polymers is the hydrophobic characteristic of collagen and the hydrophilic nature of gelatine. Considering the hydrophobic characteristic of collagen separately, it can be speculated that the blend with gelatine could lead to distinctive films having enhanced solubility characteristics. Due to structural differences and amino acid composition between collagen and gelatine, the blend of both proteins could yield films with unique properties.

Addition of collagen to gelatine-based films as a reinforcement could potentially increase the mechanical and thermal properties of the composite films. Hence,

possibly resulting in films that have similar properties to pure collagen films at the prevention of utilizing just collagen as the raw material and resulting in more cost effective films. To date, no information regarding the effect of collagen on the material properties of gelatine-based films has been reported. In this context, the aim of this chapter, therefore, was to explore the feasibility of preparing composite films based on collagen-gelatine blends at different blend ratios via the casting method and using glycerol as plasticizer. The amount of collagen varied between 0 and 30% to determine the optimal proportion of collagen required for gelatine-based films. In addition, the thermal, morphological, mechanical and surface properties of the prepared films were comprehensively studied as a function of blend composition.

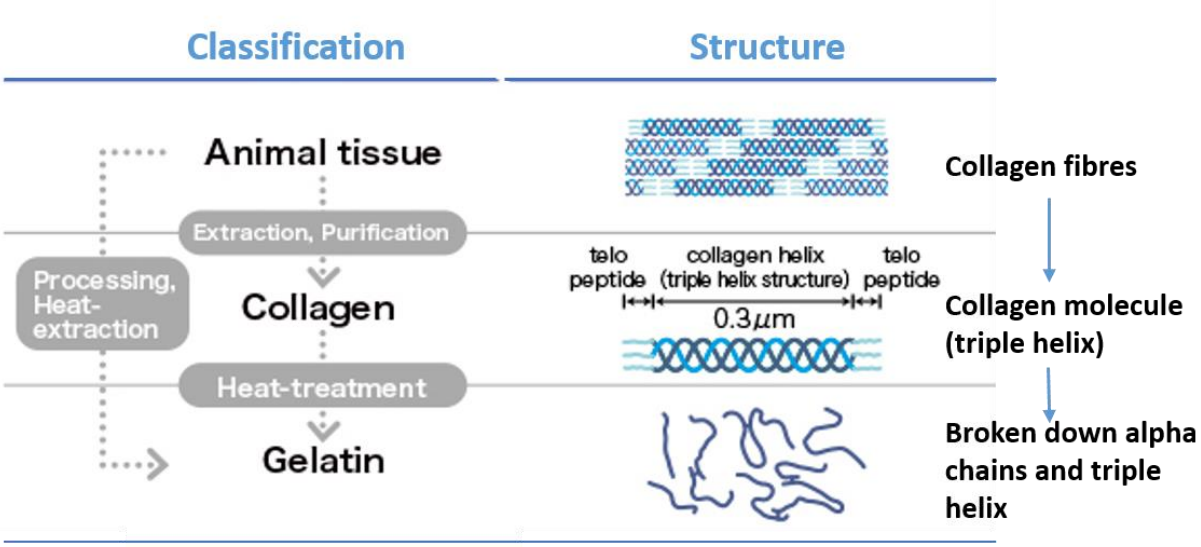


Figure 7.1: Structural and extraction differences between collagen and gelatine (used with permission).

7.2 Materials and methods

7.2.1 Chemicals

Bovine hide (bull-hides) off-cuttings were obtained from Wallace Corporation Ltd. (New Zealand). Acetic acid, glycerol, hydrochloric acid, sodium hydroxide, sodium chloride, and gelatine (225 g Bloom) were obtained from Sigma-Aldrich.

7.2.2 Collagen extraction

Hide off-cuttings were prepared for collagen extraction via the modified method of acid-enzyme extraction as described in Chapter 3.

7.2.3 Film preparation

Film preparation was carried out in four main steps. Weighed samples of collagen were dissolved in 0.5 M acetic acid for 24 hours at 4 ℃ to obtain the collagen film-forming solution (CFFS). Gelatine was dissolved in 60 ml distilled water to form the gelatine film-forming solution (GFFS). GFFS was placed on a water bath while being stirred continuously with a magnetic stirrer for 20 minutes at 50 ℃ to obtain a homogenous solution. GFFS was removed from heat, once it reached approximately 30 ℃ CFFS was added to it, and the total film-forming solution was stirred for 30 minutes at room temperature. Glycerol as a plasticizer was added to the total film-forming solution at a 40% concentration based on the dry weight of the protein content, and stirred for another 30 minutes. Subsequently, the total film-forming solution was poured on glass petri dishes coated with Teflon sheets and left to dry for 48 hours in fume hood. Films were peeled off manually and conditioned at 25 ± 0.5 ℃ and 50 ± 5% RH for 48 h prior to analyses. Table 7.1 is showing the detailed concentration of each component added to each formulation.

Table 7.1:Film forming formulations that were prepared in this study to the proportion (w/w) of gelatine (G) and collagen (C). .

Formulation	Collagen (%)	Gelatine (%)
0C-100G*	0	100
10C-90G	10	99
20C-80G	20	80
30C-70G	30	70
100C-0G**	100	0

*Where: * = control gelatine film, ** = control collagen film*

7.2.4 Film thickness

Film thickness was measured using a digital caliper (Limit Triple Read Digital Caliper). The thickness value reported was the mean of 10 random measurements.

7.2.5 Moisture content (%)

All films were weighed (W_1) and dried at 105°C for 24 hours in a Contherm Thermotech 2000 oven. The dried film samples were weighed again (W_2) and moisture content was determined as the percentage of initial film weight lost during drying. Triplicate measurements of moisture content for each film sample was taken and an average was taken to account for MC according to the equation (1).

$$MC\ (\%) = [(W_1 - W_2)/W_1] * 100 \qquad [1]$$

7.2.6 Solubility (%)

Film samples were cut into approximately 2 cm X 2 cm and wrapped in filter paper to be dried in an oven (Contherm Thermotech 2000) at 105°C for 24 hours. After a constant weight was achieved, the initial dry weight (W_i) of samples were recorded. The films were then immersed in distilled water (50 ml) for 24 hours at room temperature with occasional stirring. Films were oven dried one last time at 105°C for 24 hours to determine the weight of the film which is not soluble in water (W_f). Five replicates were carried out for each film formulation and percent solubility of the collagen-reinforced gelatine films were calculated according to equation (2).

$$Solubility\ (\%) = [(W_i - W_f)/W_i] * 100 \qquad (2)$$

-*where:* = W_i = initial weight of the film, W_f = weight of the un-dissolved dried film residue.

7.2.7 Mechanical properties

Film tensile strength properties were measured in accordance to ASTM D882-12. The test was performed using Instron-4204 universal testing machine fitted with a 5kN load cell and the cut films were conditioned at 23 ± 2 ℃ and 50 % RH for 48 hours before analysis. Initial grip separation distance was set to 50 mm and mechanical crosshead speed to 50 mm/min. In order to prevent slippage and premature failure occur near the grips, the specimen ends were cushioned with abrasive paper. Stress versus strain graphs were obtained from which the ultimate tensile strength and elongation (%) could be determined. Tensile strength measurements of each type of film were repeated 5 times and the averages were taken as the result.

7.2.8 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) was used to determine the thermal properties of films using a Perkin Elmer Differential Scanning Calorimeter (DSC-8500). Film samples were conditioned at 25 ℃ and 53 % relative humidity for 48 hours prior to testing. Approximately 5-8 mg film samples were weighed into aluminium pans and sealed. The samples were scanned at 50 ℃/min over the range of -50 ℃-150 ℃ using liquid nitrogen as the cooling medium. An empty pan was used as a reference. The glass transition temperature (T_g) was obtained from the transition in the baseline in the 30-80 ℃ region by taking the inflection point.

7.2.9 Film morphology – scanning electron microscopy (SEM)

Film morphology of collagen-gelatine composite films were studied using a scanning electron microscope (SEM) (Hitachi S-4700). Dried film samples cut transversally and surface pieces and were platinum coated for 80 seconds under 3kV utilizing Ion Sputter Coater (Hitachi E-1030, Japan), subsequently the films were observed at an acceleration of 20 kV.

7.3 Results and discussion

7.3.1 Surface properties

All films were translucent, flexible and showed no sign of cracks or bubbles (Figure 7.2). Thickness and observed surface properties of films are presented in Table 7.2. Film thickness ranged from 0.521 mm to 0.552 mm. A significant difference in thickness amongst the films as the collagen content increased was not observed, in fact there was no correlation between an increase in collagen content and film thickness. Thickness and physical properties of collagen-reinforced films were very similar to pure collagen films with 40% plasticizer attained in chapter 5. This also indicates a sign of good miscibility between collagen and gelatine as bumps or uneven thickness was not observed with any of the composite films. This included similar flexibility, transparency and no sign of visible cracks or bubbles.

Generally, film thickness is governed by preparation methods, drying conditions, composition of the film-forming solution and the nature of the film components. Increases in composite film thickness is related to polymer immiscibility, low degree of compactness of polymer chains within the film matrix, and aggregation of peptide chains [423; 425]. Ahmad et al. [425] prepared collagen-soy protein composite films and observed an increase in composite film thickness with increasing addition of soy protein and attributed this to low degree of compactness of the protein chains within the film matrix. The thinner films were noted to have a higher degree of compactness in which the peptide chains align themselves with less protrusion in the film matrix. Therefore, relating literature findings to this chapter, it can be suggested that collagen and gelatine had some type of compatibility, as film thickness was not affected by an increase in addition of collagen.

Table 7.2: Film thickness and observed properties of all formulations.

Film formulation	Film thickness (mm)	Observed surface properties
0C-100G	0.552 ± 0.3	Transparent, flexible, no bubbles, no sign of cracks, easy removal off cast
10C-90G	0.532 ± 2.8	Transparent, flexible, no bubbles, no sign of cracks, easy removal off cast
20C-80G	0.540 ± 1.7	Transparent, flexible, no bubbles, no sign of cracks, easy removal off cast
30C-70G	0.521 ± 3.6	Transparent, flexible, no bubbles, no sign of cracks, easy removal off cast
100C-0G	0.526 ± 2.9	Transparent, limited flexibility, no bubbles, no sign of cracks, easy removal off cast

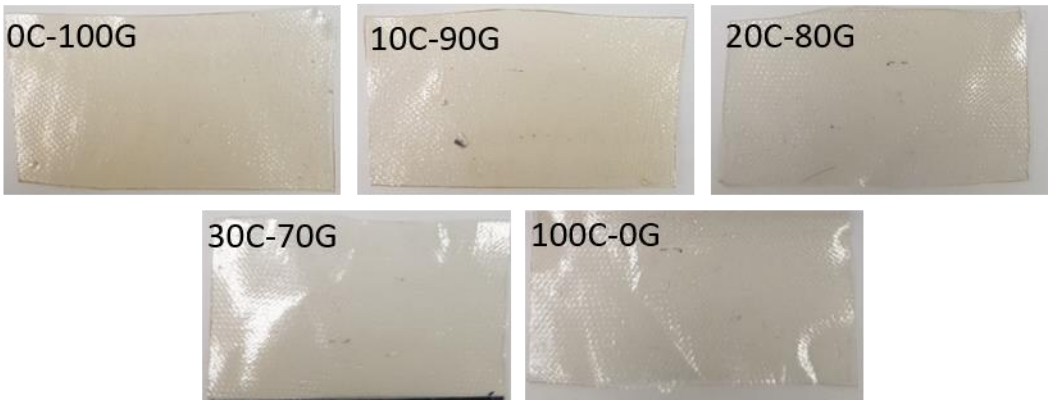


Figure 7.2: Images of films at different concentrations of collagen reinforcement with control gelatine and collagen films.

7.3.2 Moisture content (MC) and film solubility (%)

MC of films are important as it has an influence on mechanical properties due to its plasticizing effects. The highest MC was exhibited by the control gelatine film (0C-100G) at 22.85 %, followed closely by 10C-90G at 22.28 % (Table 7.3). The high MC content of the pure gelatine film and the 10C-90G films could be attributed to the hydrophilic nature of gelatine and with addition of glycerol further reducing the interaction between polymer chains. Hence, an increased availability of hydroxyl groups leading to more absorption of water. As the collagen content was increased, MC decreased. The lowest MC content resulting from the control collagen (100C-0G) film at 14.30 %.

Film formulations of 0C-100G and 10C-90G resulted in MC values slightly higher than 20%. Moisture content of films can have a plasticizing effect on the resultant films. Aguirre-Loredo et al. [426] for example reported plasticization of chitosan-based films when their moisture content was higher than 20 %, which subsequently affected the film's thermal, mechanical and barrier properties. Hence, film blends at exhibiting MC values higher than 20% can adversely affect mechanical properties of films.

Both water and glycerol are capable of plasticizing gelatine and collagen. This results in higher in greater film flexibility and extensibility; however, those properties are preferred only to a certain extent in order to prevent loss of high tensile strength.

Table 7.3: Moisture content (%) and water solubility (%) of of films.

Film formulation	Film moisture content (%)	Film water solubility (%)
0C-100G	22.85 ± 3.7	18.65 ± 4.8
10C-90G	22.28 ± 6.3	17.65 ± 3.6
20C-80G	19.26 ± 5.5	16.81 ± 4.5
30C-70G	18.27 ± 4.1	14.02 ± 5.4
100C-0G	14.30 ± 3.0	6.17 ± 8.7

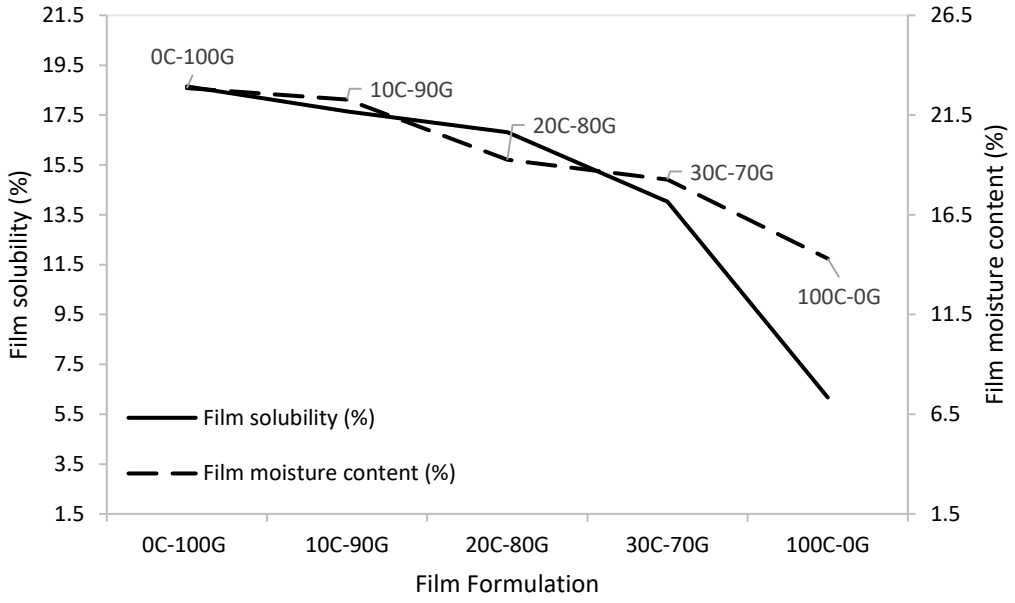


Figure 7.3: Film solubility (%) vs. film moisture content (%) for all formulations.

Water solubility is an important property of biodegradable films. The extent of preferred film solubility typically depends on its potential application [427]. Film water solubility (%) are presented in Table 7.3: Moisture content (%) and water solubility (%) of of films.. Highest solubility (%) being exhibited by 0C-100G at 18.65 %, followed closely by composites films of 10C-90G at 17.65 %. Further with increased addition of collagen film solubility decreased linearly, with the lowest solubility (%) being exhibited by 100C-0G at 14.30 % (Table 7.3). 100C-0G exhibiting the lowest solubility (%) could possibly be due to the large extent of cross-linking both at inter- and intramolecular level. Liu et al. [427] related a decrease in solubility (%) of gelatine-based films with an increase in content of cross-linking, and as glycerol content was increased, a decrease in content of cross-linking and subsequently lower film solubility values were obtained.

The lower solubility of composite films may be due to the low solubility of collagen molecules in aqueous solutions and the reduction of gelatine and subsequent increase of collagen in the composite films. Keeping in mind that gelatine is highly hydrophilic, but collagen being soluble in acidic and some alkaline solutions. Additionally, the decrease in the solubility of the composite films could be due to

strong interaction between gelatine and collagen in the film matrix, which also correlated with the highest tensile strength amongst the composite films (30C-70G films, Table 7.4), due to lower hydrophilic sites available for water absorption which can have an inverse effect on tensile strength. Furthermore, the non-polar components of gelatine has potentially interacted with the hydrophobic domains of collagen, resulting to increased hydrophobicity of the resultant composite films. Therefore, the water solubility of the composite films decreased.

Essentially, during polymer film preparation, water is eliminated during drying, with protein conformational changes, and with the degree of protein unfolding determining the type and the proportion of covalent (Disulfide bonds) or non-covalent interactions established between protein chains. Alongside hydrogen bonding between collagen and gelatine, Disulfide bond formation might have also taken place in the composite films. Hence, the interaction of peptide chains would be a lot stronger, especially via Disulfide bonds. Subsequently, the cohesion of the composite films final structure would be a function of these bonds and a hence having an effect on the solubility of the films.

7.3.3 Mechanical properties

Film mechanical properties were measured as reported in Table 7.4 and Figure 7.4. The control gelatine film (0C-100 G) at 2.02 MPa exhibited the lowest tensile strength, while the control collagen film (100C-0 G) at 7.19 MPa exhibited the highest tensile strength. The tensile strength of composite films increased with an increased addition of collagen. In comparison to the control gelatine film, the tensile strength of the highest collagen content blend film (30C-70G) increased by 55.4 %, while at only 10% collagen, the tensile strength increased by 29.71 %.

The increase of tensile strength in composite films in comparison to the control gelatine film indicated that the tensile strength values were affected substantially by the addition of collagen in the film formulation. This could be due to the interactions formed between collagen and gelatine especially via hydrogen bonding and promoting development of new bonds formed in the composite films. As a result, the tensile strength of the composite films increased. The higher

tensile strength values of the composite films with respect to the control gelatine films suggested also reinforcement of the film matrix, which was potentially induced by a certain degree of interaction between the collagen and gelatine molecules. This phenomenon could be attributed to the reticulation of the composite film network structure caused by higher protein-protein interactions via hydrogen bonding, hydrophobic interaction and or as well as disulphide bonding [428]. Overall, it can be stated that collagen and gelatine blends in the composite films underwent molecular interactions in a way where strong bonds were formed – especially with the increase in collagen content. This in turn allows higher chain entanglement, which results in the reduction of molecular slippage upon tensile deformation. Subsequently, this was coincidental with the lowered elongation (%) values with the increased incorporation of collagen at all blend ratios of the composite films.

Wang et al. [321] prepared collagen-based films with sodium alginate and attributed an increase in tensile strength with addition of sodium alginate to formation of cross-linking between the collagen matrix and sodium alginate. Therefore, with the case of 30C-70G a high tensile strength could be attributed to a higher degree of hydrogen bonding between collagen and gelatine. According to SEM analysis (Figure 7.6), a better uniformity (cross-sectional image) for the 30C-70G blend films was also obtained, leading to higher tensile strength.

Elongation (%) of the films decreased with increased addition of collagen. The highest elongation (%) was exhibited by the control gelatine film at 128.23 %, while the lowest elongation (%) being exhibited by the composite film 30C-70G at 36.70 %. Although an increase in tensile strength of composite films are observed with increased addition of collagen, there is the drawback of decreased elongation. Observing Figure 7.4 and taking both tensile strength and elongation (%) into consideration, the composite films of 20C-80G shows ideal mechanical properties. Considering solely, tensile strength, then 30C-70G films exhibit higher tensile strength at the expense of low elongation (%).

The higher elongation (%) values reflected the increased extensibility of the composite films at 10 % and 20 % collagen. The higher elongation (%) values were coincidental with decreased tensile strength values.

Table 7.4: Tensile strength and elongation at break of all films.

Film formulation	Test condition	Tensile strength (MPa)	Elongation (%)
0C-100G	23±2℃, 50% RH, 48 hrs	2.02 ± 4.6	128.23 ± 12.8
10C-90G	23±2℃, 50% RH, 48 hrs	2.62 ± 5.4	90.44 ± 11.5
20C-80G	23±2℃, 50% RH, 48 hrs	2.90 ± 11.5	73.69 ± 16.6
30C-70G	23±2℃, 50% RH, 48 hrs	3.14 ± 1.5	36.70 ± 13.8
100C-0G	23±2℃, 50% RH, 48 hrs	7.19 ± 9.9	112.82 ± 8.2

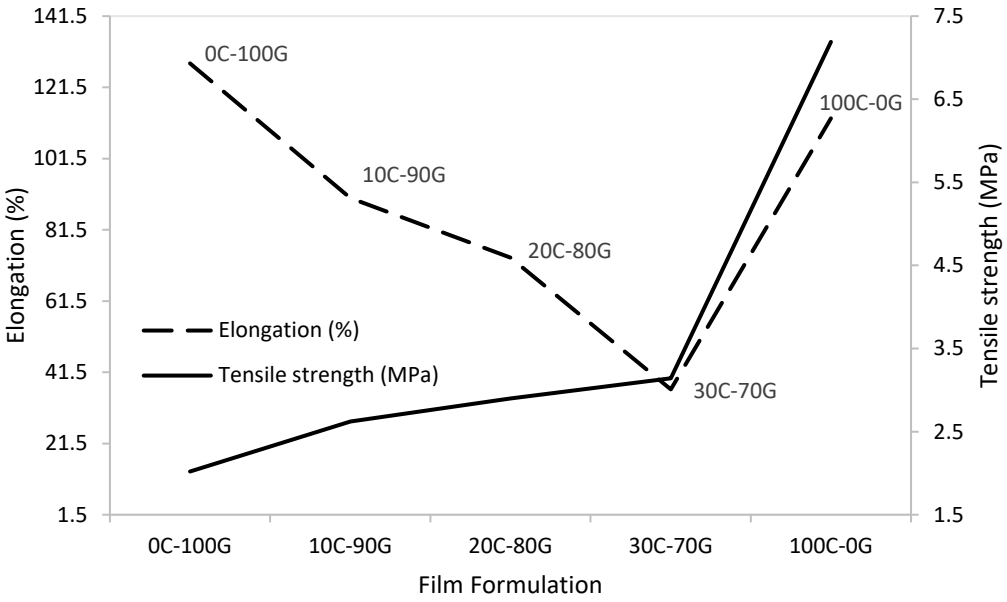


Figure 7.4: Film formulation and its relationship with tensile strength (MPa) and elongation (%).

In regards to mechanical properties of blend films attained in this chapter, a few factors needs to be addressed in the future. Ideally, composite films at 10% collagen would have been preferred to have the desired mechanical properties. This preference is due to utilizing the lowest concentration of collagen as collagen is of high cost and requires careful processing steps. Acknowledging the cost and processing limitations of collagen, increasing the gelatine content of the blend films and concurrently acquiring high tensile strength and good thermal stability

is preferred. In order to develop such composite films derived of collagen and gelatine, a few factors such as addition of cross-linking agents, addition of different plasticizer concentration, different types of plasticizers, novel preparation methods need to be studied and optimized. Though Chapter 5 did focus on studying the effect of plasticizer concentration on film mechanical, structural and thermal properties, it did not however address different types of plasticizers. The mere fact that this chapter involves polymer blends in preparation of films and the previous chapter focused on just one type of polymer, suggests that plasticizer concentration and type could affect these polymer blends to a different unique manner. For future works, these factors should be investigated. Therefore, it can be concluded that the mechanical properties of composite films prepared in this chapter from biopolymers of collagen and gelatine were largely affected type of molecular interactions and the ratio of biopolymers used.

7.3.4 Differential scanning calorimetry (DSC)

DSC thermograms presented in (Figure 7.5) revealed two endothermic processes for all films. The first endothermic transition (Figure 7.5) was attributed to glass transition temperature (T_g). The second endothermic transition being related to evaporation of water. The control gelatine film exhibited T_g at 46.80 ℃, and the control collagen film exhibited a T_g value of 60.85 ℃. As collagen was incorporated into the film matrix, T_g of films increased linearly. At 20% and 30% collagen, the composite films showed the highest T_g values at 51.11 ℃ and 52.76 respectively ℃ (Table 7.5).

All composite films demonstrated a higher T_g value than the pure gelatine films. This could be due to interactions between collagen and gelatine and formation of new hydrogen bonds, thereby restricting chain movement and resulting in slightly higher Tg values with blend films. Another possibility for the raised T_g values of the blend films could be associated with the collagen rich phases due to the increased addition of collagen, leading to higher T_g values.

As polymer T_g is governed by structural features of polymeric materials such as molecular weight, chain branching, crystallinity and extent of cross-linking – it is

important to take into account these factors when analysing the T_g of composite films. The first endothermic transition in films involves the disruption of crystalline or aggregated structure, which is stabilized by various intermolecular interactions. The composite films in this chapter exhibited higher Tg values in comparison to the control gelatine films. This indicates that interactions between collagen and gelatine had taken place resulting in formation of hydrogen bonding between the two polymers, which had stabilized the composite films. The higher T_g values amongst the composite films was in agreement with stronger film structure, as accompanied with increased tensile strength, compared to the control gelatine film.

In case of the composite films, the thermograms (Figure 7.5) clearly show one single endothermic peak for the T_g. This suggests that the two polymers were miscible in the composite film matrix or that they had interfacial interaction and stability. Hence, this was correlated to the higher T_g values attained with the blend formulations. However, if the T_g endothermic transitions were well separated in the composite films then it can be suggested that the polymers involved are immiscible due to their distinct chemical structures.

The increase in T_g of collagen reinforced films can be also correlated to the moisture content of films (Table 7.3) as water can act as a plasticizer and hence result in a reduced T_g value. The reduced T_g is resulted by the ability of water just like plasticizers to penetrate between the polymer chains and hence weaken the interaction between gelatine and collagen. A reduction in moisture content of films was observed with addition of collagen and hence higher T_g values were exhibited with all blend films.

Similarly, Hosseini et al. [299] observed an increase in the T_g of gelatine-based films with an increase in addition of chitosan. This increase in T_g was related to good miscibility between gelatine and chitosan and formation of new hydrogen bonding networks.

In chapter 5, where pure collagen films were analysed, T_g values of films at 40% plasticizer ranged from 52.59 ℃ to 57.33 ℃. The T_g values of the composite films in this chapter reached as high as 52.76 ℃. Therefore, utilizing a smaller content

of collagen in gelatine-based films rather than preparing pure collagen films can result in favourable properties. Considering the overall concept of waste valorisation of this project, it will be beneficial, efficient and cost effective to prepare gelatine-based films and reinforcing them with collagen to gain the favourable properties of pure collagen films. Not only does preparation of gelatine require substantially lower processing costs, it also does not need the use of careful processing steps, which is required for collagen extraction.

Overall, with the composite films, it can be concluded that hydrophobic interactions were predominant between the two polymers, compared to the control gelatine films. Additionally, presence of disulphide bonds in the composite films resulted in higher thermal resistance. The presence of a single endothermic transition for composite film T_g value calculation indicated miscibility of the two polymers – which was accompanied with higher T_g values. The results suggested that a single ordered structure existed in the composite film matrix due to molecular re-orientation in the film network induced by the addition of collagen. This results in a certain degree of interaction between collagen and gelatine via hydrogen bonding. This conclusion was consistent with the increase in tensile strength. The greater interaction amongst the protein molecules, restricted chain mobility in the composite films and subsequently resulting in higher tensile in comparison to the control gelatine films. Hence, the addition of collagen exhibited a prominent impact on the thermal properties (T_g) of the resultant composite films due to the intermolecular interaction and molecular organisation inn in the film matrix.

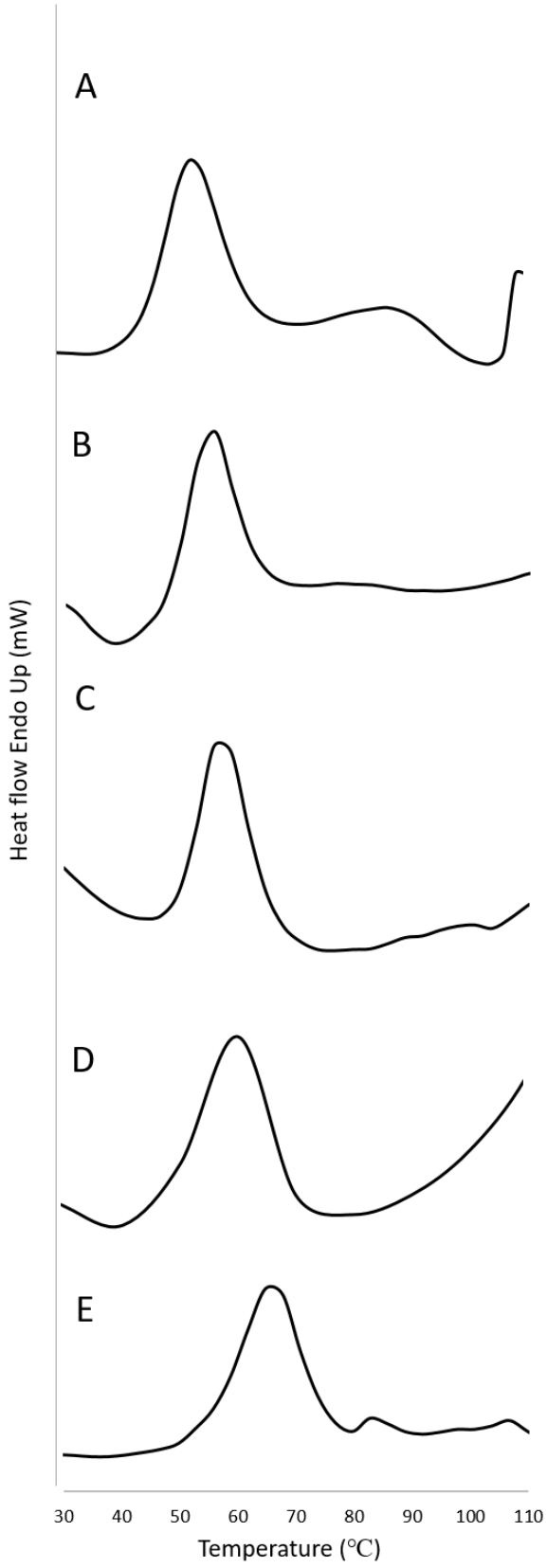


Figure 7.5: DSC thermographs of films 0C-100G (a), 10C-90G (b), 20C-80G (c), 30C-70G (d), and 100C-0G (e).

Table 7.5: 1st and 2nd transition values of films obtained from DSC thermograms.

Film formulation	1st Temp. transition	2nd Temp. transition
(A) 0C-100G	46.80 ℃	108.05 ℃
(B) 10C-90G	50.26 ℃	109.35 ℃
(C) 20C-80G	51.11 ℃	95.07 ℃
(D) 30C-70G	52.76 ℃	99.27 ℃
(E) 100C-0G	60.85 ℃	104.21 ℃

7.3.5 Film morphology – scanning electron microscopy (SEM)

The SEM micrographs of the cross-section films are illustrated in Figure 7.6 at 50 μm and 100 μm. The control gelatine films along with the composite films showed irregular and non-uniform surface. With increased addition of collagen, the composite films showed a more homogenous surface. The composite films of 10C-90G and 20C-80G had similar protruded surfaces similar to the control gelatine films. The presence of slightly uneven and protruded microstructure of the composite films of 10C-90G and 20C-80G could be due to the small content of collagen present and lack of adhesion at small concentrations. This was accompanied with reduction in tensile strength, which was observed with mechanical analysis and reduced T_g values as observed with DSC analysis.

A more compact structure was observed with 30C-70G composite films. This indicated that with increased addition of collagen, molecules were dispersed uniformly throughout the 30C-70G composite films, with the formation of hydrogen bonds forming strong adhesion between the two polymers, which was responsible for good structural integrity in 30C-70G composite films.

Wang et al. [429] prepared collagen-hydroxyapatite blend films and with increased addition of hydroxyapatite, the blend films became compact and a single phase was observed. This was associated with hydroxyapatite facilitating the enhancement of interfacial bonding with collagen, and hence resulting in a more morphological compact film blend. The changes in microstructure of the blend films induced by addition of hydroxyapatite were consistent with mechanical properties of films as similarly observed in this chapter.

Overall, from SEM of composite films it can be concluded that collagen was uniformly distributed within the gelatin matrix, indicating good compatibility of collagen and gelatine blends. This observation was even more evident with increased amounts of collagen as with each increased formulation of collagen, the composite film microstructure became more compact. Nevertheless, minor heterogeneities were still present in the highest collagen content (30C-70G) composite film, which is typical form of brittle fracture. In general, the microstructure of films are governed by molecular organization in the film network. The presence of strong intermolecular interactions in the 30C-70G composite films led to improved mechanical and thermal (T_g) properties. The microstructure of 10C-90G and 20C-80G composite films were due to the low content of collagen leading to dispersion inefficiency and poorer alignment and distribution amongst the two polymers.

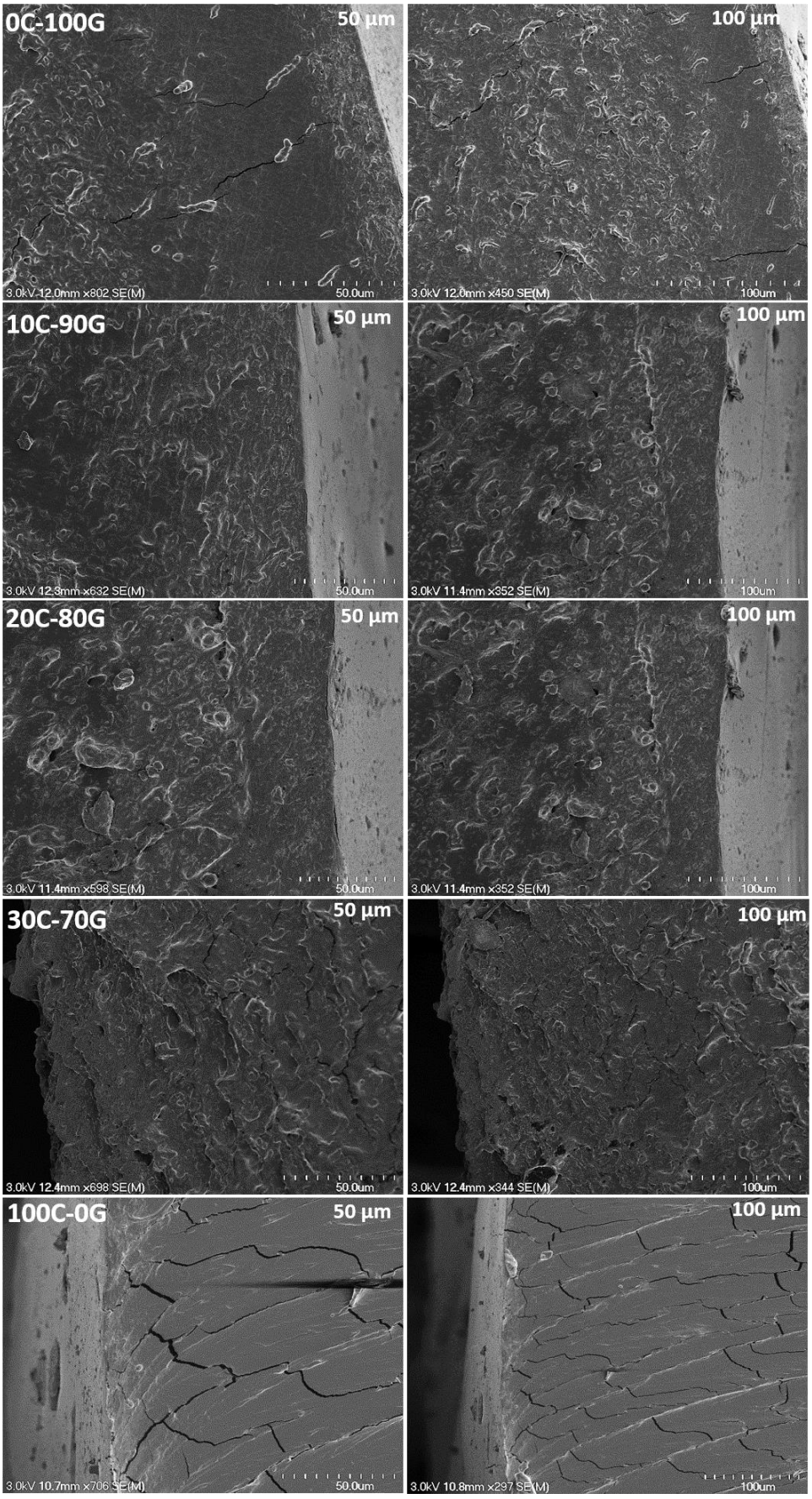


Figure 7.6: Cross-section SEM images of films at 50 and 100 µm.

7.4 Conclusions

Gelatine-collagen blends were utilized to prepare composite films with collagen acting as reinforcement. The properties of the composite films were significantly affected by the different blend ratios. The concentration of collagen had an influence on film moisture content, solubility (%), mechanical properties, thermal stability and the morphology of the composite films. DSC analysis and mechanical properties demonstrated good miscibility of the two polymers. Increased addition of collagen lead to a reduction in the film moisture content, lower water solubility, high T_g values and an increase in tensile strength of composite films.

Composite films containing 30 wt % collagen displayed the best mechanical and physical properties. Furthermore, the composite films of 30C-70G formed a compatible and miscible blend compared to 20C-80G and 10C-90G composite films as evidenced by SEM micrographs. This indicated that with subsequent increase of collagen there was enhancement of intermolecular interactions between collagen and gelatine, specifically via hydrogen bonding. The only downfall of adding increased amounts of collagen as a to the composite films was the reduction in elongation (%). The results suggested that the prepared composite films have potential to be used as packaging materials, however further studies need to be carried out to determine improve the mechanical properties of these films in terms of elongation (%) and tensile strength for these films to be used as active packaging in commercial food systems.

This chapter therefore showed that it is possible to prepare films that mimic pure collagen films in terms of thermal, physical and mechanical properties. This will not only benefit the environment in terms of waste valorisation and sustainability but also reduce material costs, as gelatine is substantially cheaper than collagen. In general, in order to prepare cost effective films, the most ideal composite film properties should be attained at 10% or at an even lower collagen concentration. Hence, further work will need to focus on utilizing lower contents of collagen in the blend formulations and simultaneously improving the films physical, thermal and mechanical properties. A few factors that could be addressed are, the use of

different glycerol concentrations, combination different plasticizers, addition of cross-linking agents such as glutaraldehyde, different conditioning systems prior to film characterization, moisture content control and use of different film preparative methods.

Chapter 8

Conclusions

Collagen has risen its rank to be an integral material and element of importance both in biomedical and non-biomedical sectors. Using concepts of waste valorisation, collagen from bovine hide off-cuttings was successfully extracted.

It can be concluded from the first experimental chapter that collagen of high yield and content can be extracted from bovine hides of bull, calf, cow, ox and face-pieces by acid-solubilisation, acid-enzyme solubilisation and a modified acid-enzyme solubilisation. Modification of the acid-enzyme soluble collagen extraction method resulted in significantly higher collagen content. Purification of collagen was a necessary step and it resulted in a clean white agglomerated powder, which resembled the standard collagen from Sigma Aldrich. Hydroxyproline assays indicated a sufficient content of hydroxyproline in the extracted collagenous tissue, thus it can be confirmed that the extracted material is collagen. SDS-PAGE analysis and FTIR confirmed secondary structure, the triple helix, and the chains that comprise the collagen molecule. The first experimental chapter resulted in a new method to extract collagen of high extractables yield and content in an efficient manner. Thus, the extracted collagen from cow, calf, bull, face-piece and ox-hide, via the method of AES2 are suitable for a variety of different collagen-derived applications such as collagen biopolymer films.

Collagen from calf hide was extractable without the use of enzymes. Hence, the method of acid-solubilisation without enzymes could be applied to extract collagen from calf hides and hence reducing excess cost of enzymes. Due to age difference and increase of age-related cross-linking content, further characterizations of calf hide collagen and bull hide collagen were carried out in order to determine differences in morphology, thermal stability and secondary structure. FTIR analysis indicated stable triple helix presence for both BH and CH1 collagen. The thermal stability of collagen was investigated and the denaturation temperatures of BH and CH1-collagen were determined by DSC studies as 51.90 ℃ and 45.36 ℃ respectively and TGA analysis showed a three-stage weight loss transition for both BH and CH1 collagen. SEM showed aggregated collagen sheets for all collagen including Sigma collagen. TEM images of BH collagen showed uniform, organized structure in the form of fibres and fibrils. However, under TEM, CH1 collagen did not show any sign of organized structure. The thermal properties,

secondary structure of CH1 collagen did show large similarities to BH and Sigma collagen, hence, it was concluded that parts of CH1 collagen might have gelatinized or digested during the extraction process and those parts have been possibly investigated under TEM. It can be concluded that collagen from both sources of BH and CH1 can therefore be possible sources of collagen from waste hide off-cuttings to be produced into high-value added products such as collagen films.

BH and CH1-based collagen films with three formulations varying in the plasticizer content were successfully prepared. The films were characterized for their physical properties, secondary structure, thermal and mechanical properties. The films appeared to be uniform in thickness, transparent and showed good flexibility. Surface properties and thickness of both collagen sources were very similar, however film solubility (%) decreased with decreasing content of glycerol, which is a feature of glycerol. DSC and DTA curves of films indicated higher thermal stability for BH collagen-based films in comparison to CH1 collagen-based films. The FTIR spectra of films helped to understand the structural changes and interactions occurring with the collagen sources and glycerol. The structural changes observed with FTIR spectra helped to understand the trend in tensile strength increase with a decrease in glycerol content. Interestingly, both collagen sources resulted in similar mechanical properties. This is a positive finding as extraction of CH1 collagen is inexpensive in comparison to extraction of BH collagen, which is from an older source. F2 films glycerol content was found to be most optimum as it resulted in the most preferable film properties, especially resulting in superior mechanical properties for both collagen sources in comparison to F1 and F3 films. Hence, in Chapter 6 a constant concentration of glycerol at 40% will be used to prepare collagen-reinforced gelatine films.

Gelatine-collagen blends were utilized to prepare composite films with collagen acting as reinforcement. The properties of the composite films were significantly affected by the different blend ratios. The concentration of collagen had an influence on film moisture content, solubility (%), mechanical properties, thermal stability and the morphology of the composite films. DSC analysis and mechanical properties demonstrated good miscibility of the two polymers. Increased addition

of collagen lead to a reduction in the film moisture content, lower water solubility, high T_g values and an increase in tensile strength of composite films.

Composite films containing 30 wt % collagen displayed the best mechanical and physical properties. Furthermore, the composite films of 30C-70G formed a compatible and miscible blend compared to 20C-80G and 10C-90G composite films as evidenced by SEM micrographs. This indicated that with subsequent increase of collagen there was enhancement of intermolecular interactions between collagen and gelatine, specifically via hydrogen bonding. The only downfall of adding increased amounts of collagen as a to the composite films was the reduction in elongation (%). The results suggested that the prepared composite films have potential to be used as packaging materials, however further studies need to be carried out to determine improve the mechanical properties of these films in terms of elongation (%) and tensile strength for these films to be used as active packaging in commercial food systems.

It was possible to prepare films that mimic pure collagen films in terms of thermal, physical and mechanical properties. This will not only benefit the environment in terms of waste valorisation and sustainability but also reduce material costs, as gelatine is substantially cheaper than collagen. In general, in order to prepare cost effective films, the most ideal composite film properties should be attained at 10% or at an even lower collagen concentration. Hence, further work will need to focus on utilizing lower contents of collagen in the blend formulations and simultaneously improving the films physical, thermal and mechanical properties. A few factors that could be addressed are, the use of different glycerol concentrations, combination different plasticizers, addition of cross-linking agents such as glutaraldehyde, different conditioning systems prior to film characterization, moisture content control and use of different film preparative methods.

In conclusion, these results prove that collagen from waste bovine hide off-cuttings have the capability to be an alternative source of collagen for many uses in numerous fields, especially in the field of biopolymer films as applied in this study.

Chapter 9

Recommendations

Results obtained during the course of this research project have laid an important platform to further advance the extraction of collagen from bovine hide off-cuttings and enhancement of film preparation from collagen and gelatine. Some recommendations for future work have been proposed:

- In this work, collagen was extracted and bovine hide off-cuttings were only cut to 2 * 2 cm pieces during pre-treatment steps. Mincing of hide samples, prior to collagen extraction could be further investigated, in order to observe if the greater surface area would result in higher collagen content.
- Further work on optimisation of the extraction of collagen is recommended. The method of alkali extraction can be attempted and its efficiency on the five sources of collagen could be explored.
- The variables of acid concentration, pH, temperature and step duration were applied with collagen extraction via the three methods applied in chapter 3. The influence of different variables and parameters such as enzyme type, re-extraction of collagen from used hide off-cuttings on the efficiency of collagen extraction could be investigated.
- Studies on management of waste generated such as hair during the extraction process of collagen from bovine hide off-cuttings can be explored. To possibly extract keratin from the waste hair and use it as a reinforcement agent in collagen-based films.
- In chapter 5, the effect of glycerol concentration on mechanical and thermal properties of BH and CH1 collagen-based films were investigated. The effect of multiple different plasticizers such as sorbitol, fatty acids and propylene glycol at different concentrations on mechanical and thermal properties of films could be explored.
- The collagen-reinforced gelatine-based films showed improved tensile strength with increased addition of collagen, at the expense of reduced elongation (%). The effect of additives such as citric acid (which both acts as a crosslinking agent and as a plasticizer) at different concentrations on mechanical properties could be added to the film-forming solution.
- In order to further improve the mechanical properties and thermal stability of collagen-reinforced gelatine-based films, the incorporation of cross-linking agents such as glutaraldehyde could be investigated.

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Appendices

A1 Collagen extraction – pictorial library

Figure 1: Raw hides prior to de-hairing and size reduction.

Figure 2: De-haired, sized reduced hides.

Figure 3: collagen extraction performed prior to precipitation and centrifugation.

Figure 4: Salt precipitated collagen solution.

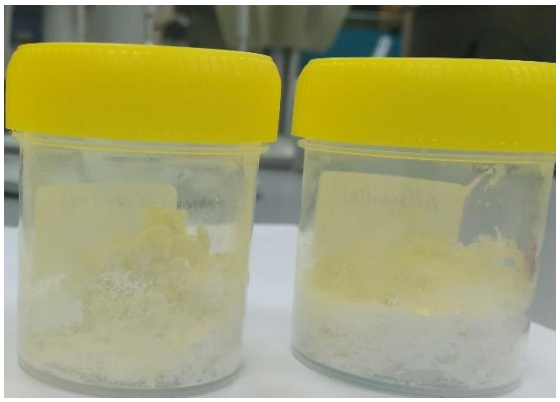

Figure 5: Extractables prior to purification via dialysis.

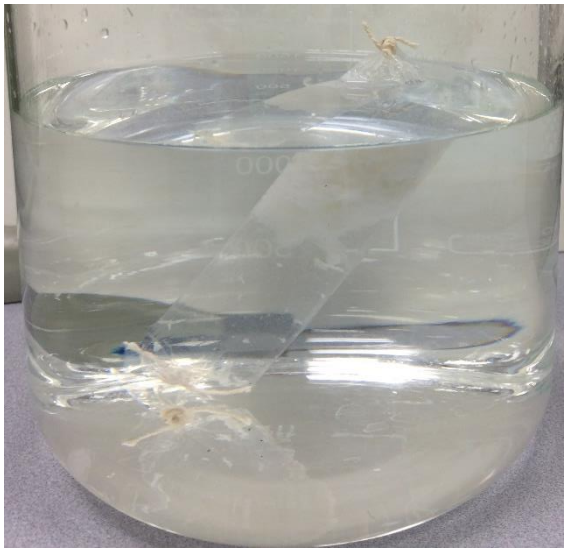

Figure 6: collagen purification by dialysis.

Figure 7: Freeze-dried semi-purified collagen - dialysis had to be carried out once more to remove salts and impurities.

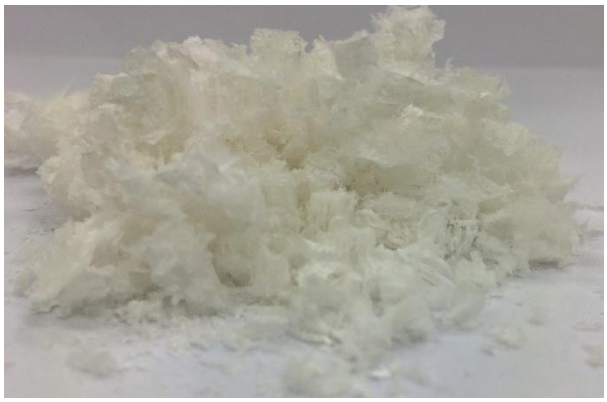

Figure 8: Freeze-dried purified collagen.

A2 Raw hide ash content measurement

Table 1: Ash content of bovine hides measured with the Tetflow furnace at 600 ℃.

Ash content - Tetflow furnace 600°C									
	Before				After				
Sample	*Repeats*	*Sample + container (g)*	*container (g)*	*Sample mass before (g)*	*Sample + container (g)*	*container (g)*	*Sample mass after (g)*	*Ash content (%)*	*Average Ash content (%)*
Calf hide	1	20.7877	15.2946	5.4931	15.4718	15.2946	0.1772	3.225865176	3.03863153
	2	21.3607	16.6858	4.6749	16.8191	16.6858	0.1333	2.851397891	
Cow hide	1	20.3538	15.6542	4.6996	15.9025	15.6542	0.2483	5.283428377	3.02169519
	2	19.0400	15.0398	4.0002	15.0702	15.0398	0.0304	0.759962002	
bull hide	1	20.7049	16.0974	4.6075	16.3123	16.0974	0.2149	4.664134563	3.61523953
	2	19.3390	15.1073	4.2317	15.2159	15.1073	0.1086	2.566344495	
Ox hide	1	20.7282	16.0330	4.6952	16.1335	16.0330	0.1005	2.140483898	1.33858279
	2	19.3120	15.3618	3.9502	15.3830	15.3618	0.0212	0.536681687	
Bovine face-piece	1	20.9125	16.0230	4.8895	16.2410	16.0230	0.2180	4.458533592	2.65178281
	2	20.3640	15.9618	4.4022	15.9990	15.9618	0.0372	0.845032029	

Raw hide density

Table 2: density of bovine hides.

	Density (g/cm^3)						
Repeats	1	2	3	4	5	Average	% error
Bull hide	1.526	1.523	1.524	1.527	1.526	1.5252	0.096361
Calf hide	1.323	1.325	1.328	1.326	1.321	1.3246	0.182441
Cow hide	1.584	1.582	1.586	1.581	1.584	1.5834	0.110115
Bovine face-piece	1.338	1.335	1.336	1.332	1.331	1.3344	0.193107
Ox hide	1.592	1.596	1.593	1.598	1.591	1.594	0.163594

Extractables dry and wet yield.

Table 3: Extractables dry and wet yields with the extraction methods of AS, AES1 and AES2.

				AES2		
Source	Collagen yield (g collagen/g wet skin in)	Collagen yield (g collagen/g wet skin in) (%)	Product yield (g dry product out/ g wet skin in)	Product yield (g dry product out/ g wet skin in) (%)	Freeze dried product yield (g wet product out/g wet skin in)	Freeze dried product yield (g wet product out/g wet skin in) (%)
BH	0.150330767	15.03307667	0.201921782	20.19217821	0.239520958	23.95209581
CH1	0.060608519	6.060851856	0.092222335	9.222233499	0.115768463	11.57684631
CH2	0.089616144	8.961614371	0.119281437	11.92814371	0.143712575	14.37125749
FP	0.027732549	2.773254945	0.056759209	5.675920886	0.067864271	6.786427146
OH	0.068582434	6.858243398	0.104674045	10.46740445	0.119760479	11.9760479

AES1						
Source	Collagen yield (g collagen/g wet skin in)	Collagen yield (g collagen/g wet skin in) (%)	Product yield (g dry product out/ g wet skin in)	Product yield (g dry product out/ g wet skin in) (%)	Freeze dried product yield (g wet product out/g wet skin in)	Freeze dried product yield (g wet product out/g wet skin in) (%)
Bull	0.05589868	5.589868	0.185094967	18.50949669	0.219560878	21.95608782
Calf	0.01148982	1.148982036	0.058922156	5.892215569	0.071856287	7.185628743
Cow	0.04011145	4.011145014	0.149113198	14.91131975	0.183632735	18.36327345
face-piece	0.008740918	0.874091816	0.056759209	5.675920886	0.067864271	6.786427146
Ox	0.039908724	3.990872405	0.132587123	13.25871231	0.151696607	15.16966068

AS						
Source	Collagen yield (g collagen/g wet skin in)	Collagen yield (g collagen/g wet skin in) (%)	Product yield (g dry product out/ g wet skin in)	Product yield (g dry product out/ g wet skin in) (%)	Freeze dried product yield (g wet product out/g wet skin in)	Freeze dried product yield (g wet product out/g wet skin in) (%)
Bull	0.010701854	1.070185445	0.201921782	20.19217821	0.239520958	23.95209581
Calf	0.025238323	2.523832335	0.098203593	9.820359281	0.119760479	11.9760479
Cow	0.005665868	0.566586826	0.149101796	14.91017964	0.179640719	17.96407186
face-piece	0.002781201	0.278120123	0.056759209	5.675920886	0.067864271	6.786427146
Ox	0.008164575	0.816457547	0.157011067	15.70110668	0.179640719	17.96407186

Hydroxyproline analysis

Table 4: Hydroxyproline standard curve data 1 a), b) and c).

Hydroxyproline assay calibration curve data (540 nm) a)								
Absorbance reading (540 nm)	**Hydroxyproline concentration (µg/ml)**	**0**	**2.5**	**5**	**7.5**	**10**	**12.5**	**15**	**17.5**
	Sample 1	0.000	0.021	0.034	0.056	0.078	0.091	0.108	0.136
	Sample 2	0.000	0.020	0.033	0.055	0.079	0.093	0.112	0.135
	Sample 3	0.000	0.020	0.035	0.057	0.077	0.092	0.109	0.136
	Average	0.000	0.020	0.034	0.056	0.078	0.092	0.110	0.136
	%St.dev.S	0	2.83943	2.94118	1.78571	1.28205	1.08696	1.89818	0.42557

Hydroxyproline assay calibration curve data (540 nm) b)								
Absorbance reading (540 nm)	**Hydroxyproline concentration (µg/ml)**	**0**	**2.5**	**5**	**7.5**	**10**	**12.5**	**15**	**17.5**
	Sample 1	0.000	0.023	0.033	0.061	0.078	0.095	0.109	0.137
	Sample 2	0.000	0.022	0.036	0.060	0.077	0.097	0.111	0.135
	Sample 3	0.000	0.022	0.034	0.063	0.079	0.098	0.113	0.138
	Average	0.000	0.022	0.034	0.061	0.078	0.097	0.111	0.137
	%St.dev.S	0.000	2.585	4.449	2.491	1.282	1.580	1.802	1.118

Hydroxyproline assay calibration curve data (540 nm) c)									
Absorbance reading (540 nm)	**Hydroxyproline concentration (µg/ml)**	**0**	**2.5**	**5**	**7.5**	**10**	**12.5**	**15**	**17.5**	**20**
	Sample 1	0.000	0.023	0.034	0.066	0.079	0.096	0.114	0.138	0.164
	Sample 2	0.000	0.024	0.035	0.067	0.078	0.095	0.112	0.137	0.163
	Sample 3	0.000	0.023	0.033	0.067	0.078	0.097	0.113	0.138	0.167
	Average	0.000	0.023	0.034	0.067	0.078	0.096	0.113	0.138	0.165
	%St.dev.S	0.000	2.474	2.941	0.866	0.737	1.042	0.885	0.419	1.264

Table 5: Hydroxyproline standard curve data for AS method extracted collagen..

AS extraction calibration curve									
Hydroxyproline assay calibration curve data (540 nm)									
Absorbance reading (540 nm)	**Hydroxyproline concentration (µg/ml)**	**0**	**2.5**	**5**	**7.5**	**10**	**12.5**	**15**	**17.5**
	Sample 1	0.000	0.021	0.034	0.056	0.078	0.091	0.108	0.136
	Sample 2	0.000	0.020	0.033	0.055	0.079	0.093	0.112	0.135
	Sample 3	0.000	0.020	0.035	0.057	0.077	0.092	0.109	0.136
	Average	0.000	0.020	0.034	0.056	0.078	0.092	0.110	0.136
	%St.dev.S	0	2.839428	2.94118	1.78571	1.28205	1.08696	1.89818	0.42557

Table 6: Hydroxyproline standard curve data for AES1 method extracted collagen.

AES1 extraction calibration curve												
Hydroxyproline assay calibration curve data (540 nm)												
Absorbance reading (540 nm)	Hydroxyproline concentration (µg/ml)	0	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25	27.5
	Sample 1	0.000	0.023	0.033	0.061	0.078	0.094	0.109	0.137	0.165	0.196	0.235	0.298
	Sample 2	0.000	0.022	0.036	0.060	0.077	0.095	0.111	0.135	0.168	0.198	0.234	0.314
	Sample 3	0.000	0.022	0.034	0.062	0.079	0.096	0.113	0.138	0.164	0.197	0.230	0.316
	Average	0.000	0.022	0.034	0.061	0.078	0.095	0.111	0.137	0.166	0.197	0.233	0.309
	%St.dev.S	0.000	2.585	4.449	1.639	1.282	1.053	1.802	1.118	1.257	0.508	1.136	3.189

Table 7: Hydroxyproline standard curve data for AES2 method extracted collagen.

AES2 extraction calibration curve															
Hydroxyproline assay calibration curve data (540 nm)															
Absorbance reading (540 nm)	Hydroxyproline concentration (µg/ml)	0	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25	27.5	30	32.5	35
	Sample 1	0.000	0.023	0.034	0.066	0.079	0.096	0.114	0.138	0.164	0.196	0.233	0.311	0.389	0.468	0.517
	Sample 2	0.000	0.024	0.035	0.067	0.078	0.095	0.112	0.137	0.163	0.198	0.238	0.321	0.388	0.462	0.519
	Sample 3	0.000	0.023	0.033	0.067	0.078	0.097	0.113	0.138	0.167	0.199	0.235	0.310	0.387	0.465	0.513
	Average	0.000	0.023	0.034	0.067	0.078	0.096	0.113	0.138	0.165	0.198	0.235	0.314	0.388	0.465	0.516
	%St.dev.S	0.000	2.474	2.941	0.866	0.737	1.042	0.885	0.419	1.264	0.773	1.069	1.937	0.258	0.645	0.592

Hydroxyproline analysis – calibration curves

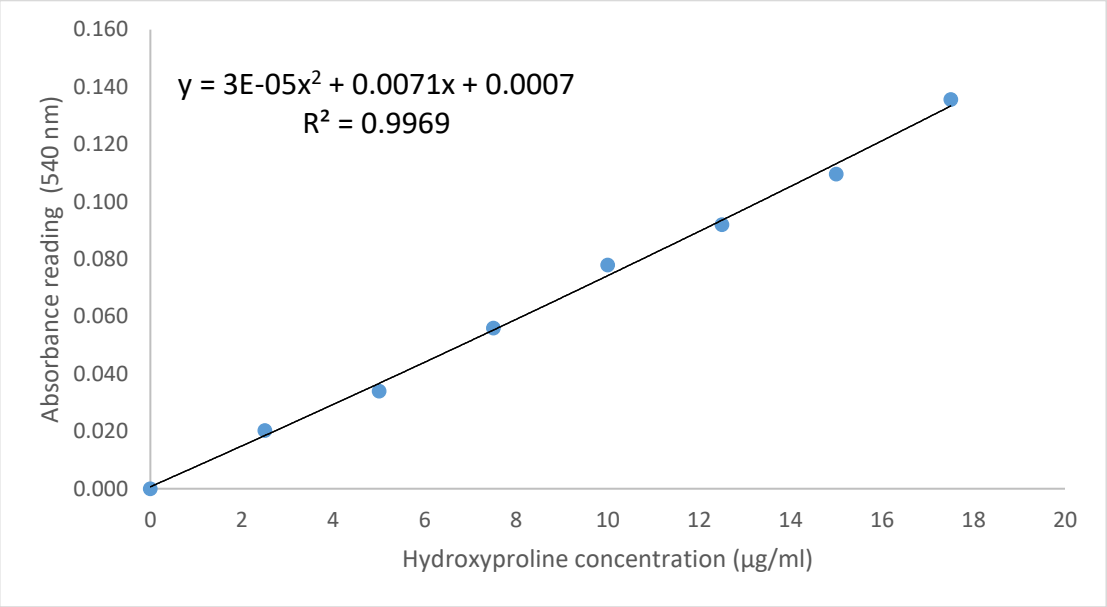

Figure 9: Hydroxyproline standard curve 1.

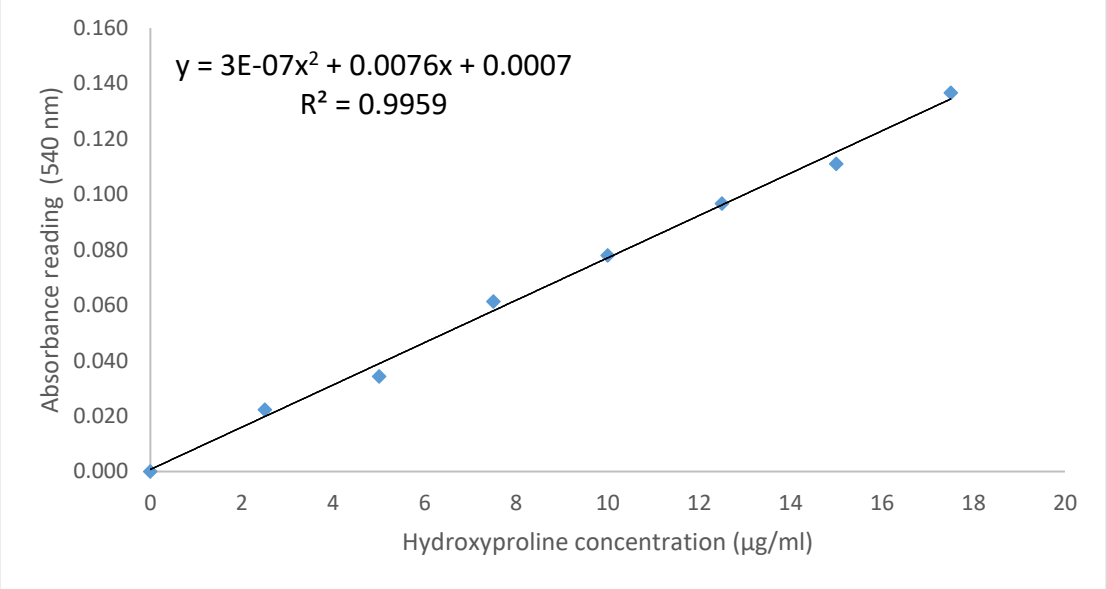

Figure 10: Hydroxyproline standard curve 2.

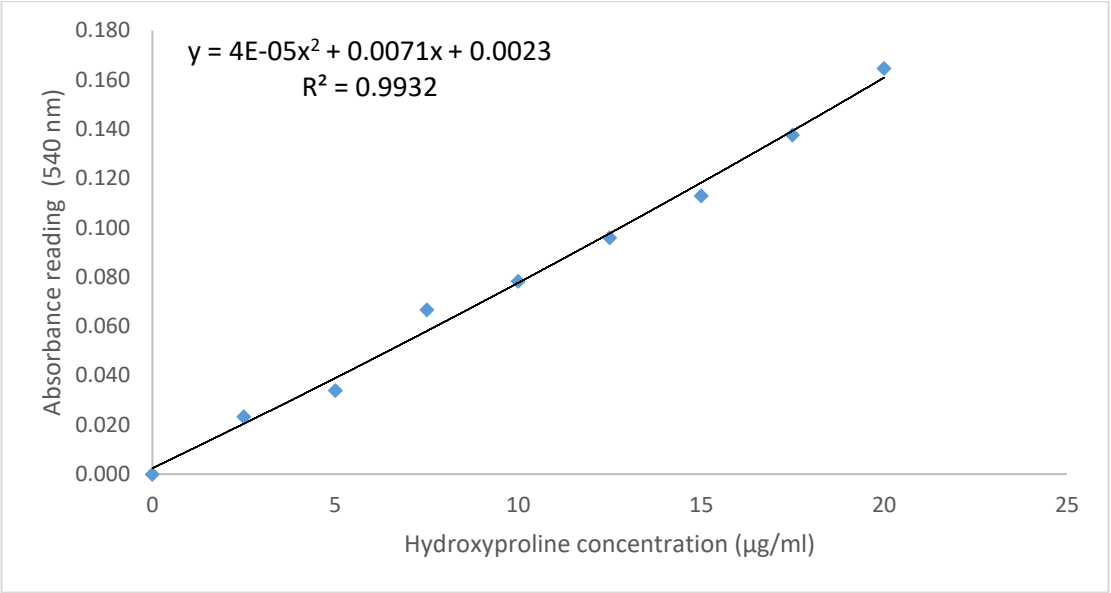

Figure 11: Hydroxyproline standard curve 3.

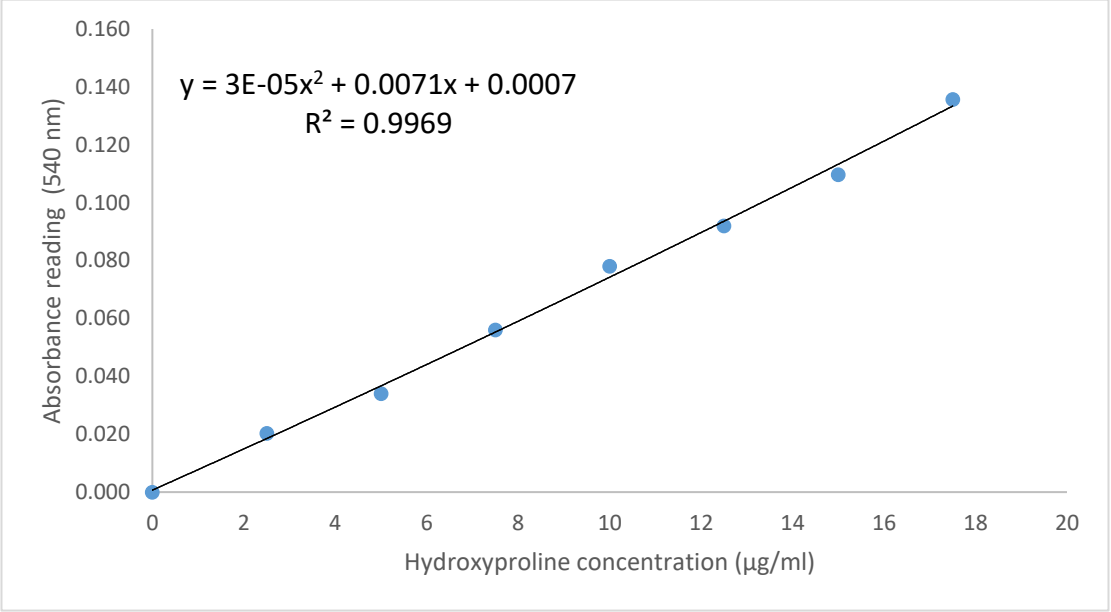

Figure 12: Hydroxyproline standard curve 4.

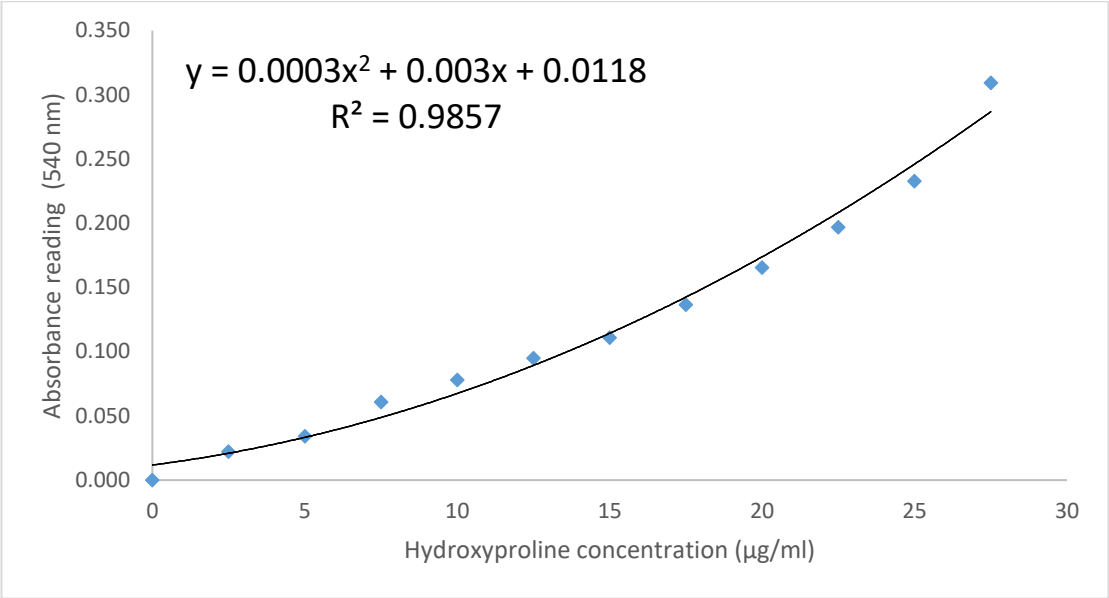

Figure 13: Hydroxyproline standard curve 5.

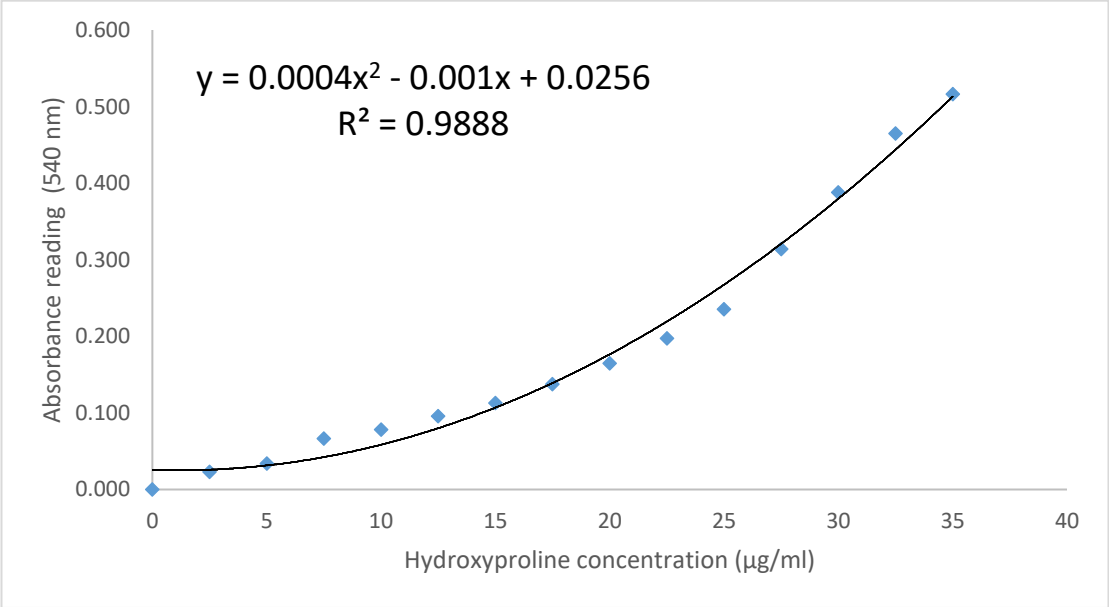

Figure 14: Hydroxyproline standard curve 6.

Hydroxyproline analysis – data analysis

Table 8: Hydroxyproline data analysis for AS extraction of collagen from the 5 bovine hide types.

Hydroxyproline assay data																						
Sample	Vol 1.5M H2SO4 (ml)	Vol p-dimethyl. (ml)	Sum volume in absorbance solution (ml)	Assay absorbance values (540 nm) - Six trials for each sample								Conc. Hydroxyproline (µg/ml)	Conc. Hydroxyproline (mg/ml)	Initial dry mass (mg)	Actual dry mass conc. In assay (mg collagen/ml)	Conc. HP in collagen sample [(mg HP)/(mg collagen sample)]	% HP in collagen sample	Bovine multiplication factor	mg collagen / mg sample	Raw % collagen in sample	Average % collagen in sample	% error
				1	2	3	4	5	6	Average	% stdev											
BH Collagen (bull hide)	2	1	5	0.021	0.020	0.022	0.021	0.024	0.023	0.0218	6.7418	2.943336815	0.002943337	43.2	0.432	0.00681328	0.681327966	7.46	0.0508271	5.0827066		
	2	1	5	0.022	0.024	0.023	0.022	0.023	0.022	0.0227	3.6022	3.057808284	0.003057808	41.2	0.412	0.007421865	0.742186477	7.46	0.0553671	5.5367111		
	2	1	5	0.025	0.021	0.024	0.022	0.023	0.021	0.0227	7.2044	3.057808284	0.003057808	45.2	0.452	0.006765063	0.676506258	7.46	0.0504674	5.0467367	5.2761	
	2	1	5	0.024	0.025	0.023	0.024	0.022	0.021	0.0232	6.3538	3.126439379	0.003126439	41.5	0.415	0.007533589	0.753358887	7.46	0.0562006	5.6200573		
	2	1	5	0.022	0.021	0.019	0.022	0.023	0.024	0.0218	7.8889	2.943336815	0.002943337	43.1	0.431	0.006829088	0.682908774	7.46	0.050945	5.0944995		4.713
CH1 Collagen (calf hide)	2	1	5	0.112	0.109	0.110	0.106	0.112	0.108	0.1095	2.1417	14.44591816	0.014445918	42.5	0.425	0.033990396	3.399039568	7.46	0.2535684	25.356835		
	2	1	5	0.111	0.110	0.109	0.112	0.110	0.109	0.1102	1.0612	14.52957499	0.014529575	41.7	0.417	0.034843105	3.484310549	7.46	0.2599296	25.992957		
	2	1	5	0.109	0.109	0.107	0.107	0.108	0.110	0.1083	1.1179	14.29939175	0.014299392	41.1	0.411	0.034791707	3.479170742	7.46	0.2595461	25.954614	25.708	
	2	1	5	0.109	0.112	0.110	0.108	0.111	0.112	0.1103	1.4801	14.55048097	0.014550481	41.8	0.418	0.034809763	3.480976308	7.46	0.2596808	25.968083		
	2	1	5	0.108	0.109	0.108	0.110	0.105	0.113	0.1088	2.4252	14.3622086	0.014362209	42.4	0.424	0.033873133	3.387313348	7.46	0.2526936	25.269358		1.261
CH2 Collagen (cow hide)	2	1	5	0.018	0.015	0.016	0.015	0.017	0.014	0.0158	9.2966	2.1159307	0.002115931	41.9	0.419	0.005049954	0.504995394	7.46	0.0376727	3.7672656		
	2	1	5	0.015	0.016	0.013	0.014	0.015	0.017	0.0150	9.4281	2.000563218	0.002000563	41.3	0.413	0.004843979	0.484397874	7.46	0.0361361	3.6136081		
	2	1	5	0.018	0.015	0.014	0.017	0.016	0.019	0.0165	11.3384	2.208145184	0.002208145	41.2	0.412	0.005359576	0.535957569	7.46	0.0399824	3.9982435	3.7957	
	2	1	5	0.019	0.017	0.019	0.014	0.012	0.015	0.0160	17.6777	2.138990939	0.002138991	41.5	0.415	0.005154195	0.515419503	7.46	0.0384503	3.8450295		
	2	1	5	0.011	0.019	0.015	0.018	0.015	0.018	0.0160	18.5405	2.138990939	0.002138991	42.5	0.425	0.00503292	0.503291986	7.46	0.0375456	3.7545582		3.314
FP Collagen (face-piece)	2	1	5	0.019	0.021	0.020	0.017	0.020	0.021	0.0197	7.6553	2.645204071	0.002645204	40.1	0.401	0.006596519	0.659651888	7.46	0.04921	4.9210031		
	2	1	5	0.020	0.017	0.020	0.022	0.021	0.019	0.0198	8.6844	2.668163439	0.002668163	40.3	0.403	0.006620753	0.662075295	7.46	0.0493908	4.9390817		
	2	1	5	0.016	0.020	0.021	0.019	0.022	0.020	0.0197	10.5030	2.645204071	0.002645204	42.1	0.421	0.006283145	0.628314506	7.46	0.0468723	4.6872262	4.9322	
	2	1	5	0.021	0.018	0.020	0.023	0.019	0.022	0.0205	9.1260	2.759957378	0.002759957	41.6	0.416	0.006634513	0.663451293	7.46	0.0494935	4.9493466		
	2	1	5	0.022	0.020	0.022	0.021	0.021	0.018	0.0207	7.2849	2.782894992	0.002782895	40.2	0.402	0.006922624	0.692262436	7.46	0.0516428	5.1642778		3.066
OH Collagen (ox hide)	2	1	5	0.021	0.022	0.023	0.023	0.021	0.020	0.0217	5.5895	2.920429553	0.00292043	40.3	0.403	0.007246723	0.724672346	7.46	0.0540606	5.4060557		
	2	1	5	0.022	0.023	0.020	0.021	0.018	0.021	0.0208	8.2675	2.805828263	0.002805828	40.5	0.405	0.006927971	0.692797102	7.46	0.0516827	5.1682664		
	2	1	5	0.025	0.022	0.021	0.020	0.022	0.022	0.0220	7.6060	2.966239751	0.00296624	40.8	0.408	0.007270195	0.727019547	7.46	0.0542357	5.4235658	5.2972	
	2	1	5	0.020	0.021	0.023	0.021	0.025	0.023	0.0222	8.2775	2.989138364	0.002989138	41.2	0.412	0.00725519	0.72551902	7.46	0.0541237	5.4123719		
	2	1	5	0.018	0.020	0.021	0.025	0.019	0.021	0.0207	11.7199	2.782894992	0.002782895	40.9	0.409	0.006804144	0.680414423	7.46	0.0507589	5.0758916		2.757

Table 9: Hydroxyproline data analysis for AES1 extraction of collagen from the 5 bovine hide types

Hydroxyproline assay data																						
Sample	Vol 1.5M H2SO4 (ml)	Vol p-dimethyl . (ml)	Sum volume in absorbance solution (ml)	Assay absorbance values (540 nm) - Six trials for each sample								Conc. Hydroxyproline (µg/ml)	Conc. Hydroxyproline (mg/ml)	Initial dry mass (mg)	Actual dry mass conc. In assay (mg collagen/ml)	Conc. HP in collagen sample [(mg HP)/(mg collagen sample)]	% HP in collagen sample	Bovine multiplication factor	mg collagen / mg sample	Raw % collagen in sample	Average % collagen in sample	% error
				1	2	3	4	5	6	Average	stdev											
BH Collagen (bull hide)	2	1	5	0.140	0.145	0.140	0.143	0.139	0.144	0.141833	1.598293	16.4113158	0.016411316	41.0	0.410	0.0400276	4.002759952	7.46	0.298606	29.86059	30.2142217	
	2	1	5	0.142	0.139	0.136	0.145	0.141	0.146	0.142	2.405251	16.38535324	0.016385353	40.2	0.402	0.040759585	4.075958518	7.46	0.304067	30.40665		
	2	1	5	0.140	0.145	0.142	0.147	0.141	0.141	0.143	1.748438	16.47608489	0.016476085	40.4	0.404	0.040782388	4.078238834	7.46	0.304237	30.42366		
	2	1	5	0.144	0.143	0.142	0.141	0.148	0.139	0.143	1.956016	16.48901528	0.016489015	41.1	0.411	0.040119259	4.011925859	7.46	0.29929	29.92897		
	2	1	5	0.143	0.145	0.140	0.142	0.141	0.143	0.142333	1.123146	16.45020072	0.016450201	40.3	0.403	0.040819357	4.081935664	7.46	0.304512	30.45124		0.867
CH1 Collagen (calf hide)	2	1	5	0.083	0.084	0.081	0.079	0.080	0.083	0.082	2.198026	11.05891929	0.011058919	42.1	0.421	0.026268217	2.626821685	7.46	0.195961	19.59609	19.5020532	
	2	1	5	0.079	0.081	0.083	0.080	0.084	0.078	0.080833	2.616202	10.97219807	0.010972198	42.5	0.425	0.025816937	2.581693663	7.46	0.192594	19.25943		
	2	1	5	0.082	0.078	0.084	0.080	0.083	0.080	0.081167	2.50648	11.00694294	0.011006943	42.3	0.423	0.026021142	2.60211417	7.46	0.194118	19.41177		
	2	1	5	0.081	0.083	0.085	0.089	0.080	0.081	0.083167	3.700638	11.21384868	0.011213849	42.8	0.428	0.026200581	2.620058102	7.46	0.195456	19.54563		
	2	1	5	0.080	0.085	0.083	0.084	0.087	0.082	0.0835	2.655516	11.24807681	0.011248077	42.6	0.426	0.026403936	2.640393617	7.46	0.196973	19.69734		0.781
CH2 Collagen (cow hide)	2	1	5	0.127	0.128	0.124	0.125	0.124	0.121	0.124833	1.815951	15.04439517	0.015044395	41.6	0.416	0.036164411	3.616441147	7.46	0.269787	26.97865	26.9021103	
	2	1	5	0.126	0.120	0.125	0.123	0.126	0.128	0.124667	2.053782	15.03053225	0.015030532	41.9	0.419	0.035872392	3.587239201	7.46	0.267608	26.7608		
	2	1	5	0.121	0.124	0.126	0.126	0.125	0.127	0.124833	1.56271	15.04439517	0.015044395	41.6	0.416	0.036164411	3.616441147	7.46	0.269787	26.97865		
	2	1	5	0.123	0.126	0.124	0.122	0.121	0.124	0.123333	1.296171	14.91928156	0.014919282	41.3	0.413	0.036124168	3.612416842	7.46	0.269486	26.94863		
	2	1	5	0.120	0.124	0.122	0.124	0.125	0.126	0.1235	1.602475	14.93322185	0.014933222	41.5	0.415	0.035983667	3.598366711	7.46	0.268438	26.84382		0.321
FP Collagen (face-piece)	2	1	5	0.063	0.059	0.058	0.062	0.059	0.061	0.060333	2.975229	8.666666667	0.008666667	42.1	0.421	0.020585907	2.058590657	7.46	0.153571	15.35709	15.3925105	
	2	1	5	0.061	0.062	0.060	0.063	0.060	0.062	0.061333	1.802513	8.788078587	0.008788079	42.1	0.421	0.020874296	2.087429593	7.46	0.155722	15.57222		
	2	1	5	0.058	0.060	0.063	0.061	0.062	0.059	0.0605	2.822851	8.686976778	0.008686977	42.0	0.420	0.020683278	2.068327804	7.46	0.154297	15.42973		
	2	1	5	0.060	0.057	0.054	0.059	0.065	0.063	0.059667	6.094253	8.585122582	0.008585123	42.5	0.425	0.020200288	2.020028843	7.46	0.150694	15.06942		
	2	1	5	0.062	0.064	0.058	0.060	0.064	0.061	0.0615	3.481093	8.808210118	0.00880821	42.3	0.423	0.020823192	2.082319177	7.46	0.155341	15.5341		1.16
OH Collagen (ox hide)	2	1	5	0.148	0.149	0.143	0.145	0.147	0.141	0.1455	1.933785	16.69485346	0.016694853	41.2	0.412	0.040521489	4.052148898	7.46	0.30229	30.22903	30.1054939	
	2	1	5	0.145	0.144	0.148	0.145	0.143	0.148	0.1455	1.30101	16.69485346	0.016694853	41.5	0.415	0.040228563	4.022856256	7.46	0.300105	30.01051		
	2	1	5	0.147	0.145	0.149	0.142	0.143	0.144	0.145	1.641708	16.65640783	0.016656408	41.3	0.413	0.040330285	4.03302853	7.46	0.300864	30.08639		
	2	1	5	0.144	0.149	0.145	0.149	0.148	0.146	0.146833	1.32857	16.79704363	0.016797044	41.7	0.417	0.04028068	4.028068017	7.46	0.300494	30.04939		
	2	1	5	0.145	0.147	0.146	0.149	0.145	0.144	0.146	1.118488	16.73323108	0.016733231	41.4	0.414	0.040418433	4.041843257	7.46	0.301522	30.15215		0.257

Table 10: Hydroxyproline data analysis for AES2 extraction of collagen from the 5 bovine hide types

Sample	Vol 1.5M H2SO4 (ml)	Vol p-dimethyl. (ml)	Sum volume in absorbance solution (ml)	Assay absorbance values (540 nm) - Six trials for each sample								Conc. Hydroxyproline (µg/ml)	Conc. Hydroxyproline (mg/ml)	Initial dry mass (mg)	Actual dry mass conc. In assay (mg collagen/ml)	Conc. HP in collagen sample [(mg HP)/(mg collagen sample)]	% HP in collagen sample	Bovine multiplication factor	mg collagen / mg sample	Raw % collagen in sample	Average % collagen in sample	% error
				1	2	3	4	5	6	Average	stdev											
BH Collagen (bull hide)	2	1	5	0.380	0.383	0.374	0.372	0.366	0.381	0.3760	1.5734	42.47142546	0.042471425	42.7	0.427	0.099464697	9.94647	7.46	0.742007	74.20066		
	2	1	5	0.375	0.373	0.381	0.371	0.372	0.370	0.3737	0.9731	42.24896628	0.042248966	42.3	0.423	0.099879353	9.987935	7.46	0.7451	74.51		
	2	1	5	0.370	0.372	0.375	0.363	0.373	0.376	0.3715	1.1499	42.04205825	0.042042058	42.0	0.420	0.100100139	10.01001	7.46	0.746747	74.6747	74.452423	
	2	1	5	0.367	0.375	0.371	0.373	0.369	0.372	0.3712	0.7029	42.01019718	0.042010197	42.3	0.423	0.099314887	9.931489	7.46	0.740889	74.08891		
	2	1	5	0.368	0.369	0.378	0.369	0.381	0.368	0.3722	1.4169	42.1057571	0.042105757	42.0	0.420	0.100251803	10.02518	7.46	0.747878	74.78784		0.361
CH1 Collagen (calf hide)	2	1	5	0.316	0.313	0.321	0.319	0.307	0.310	0.3143	1.5549	36.45940793	0.036459408	41.3	0.413	0.088279438	8.827944	7.46	0.658565	65.85646		
	2	1	5	0.305	0.322	0.310	0.312	0.315	0.315	0.3132	1.6635	36.34288216	0.036342882	41.5	0.415	0.08757321	8.757321	7.46	0.653296	65.32961		
	2	1	5	0.315	0.318	0.314	0.317	0.320	0.320	0.3173	0.7201	36.75854885	0.036758549	41.7	0.417	0.088149997	8.815	7.46	0.657599	65.7599	65.7246237	
	2	1	5	0.318	0.312	0.321	0.318	0.314	0.312	0.3158	1.0724	36.60906762	0.036609068	41.5	0.415	0.088214621	8.821462	7.46	0.658081	65.80811		
	2	1	5	0.316	0.320	0.318	0.312	0.322	0.325	0.3188	1.3110	36.90785225	0.036907852	41.8	0.418	0.088296297	8.82963	7.46	0.65869	65.86904		0.306
CH2 Collagen (cow hide)	2	1	5	0.375	0.373	0.371	0.372	0.368	0.369	0.3713	0.6347	42.02612868	0.042026129	41.6	0.416	0.101024348	10.10243	7.46	0.753642	75.36416		
	2	1	5	0.372	0.375	0.365	0.370	0.373	0.375	0.3717	0.9277	42.05798587	0.042057986	41.8	0.418	0.100617191	10.06172	7.46	0.750604	75.06042		
	2	1	5	0.366	0.371	0.378	0.368	0.375	0.373	0.3718	1.0915	42.07391155	0.042073912	41.8	0.418	0.100655291	10.06553	7.46	0.750888	75.08885	75.1345825	
	2	1	5	0.369	0.376	0.371	0.379	0.373	0.374	0.3737	0.8695	42.24896628	0.042248966	42.0	0.420	0.100592777	10.05928	7.46	0.750422	75.04221		
	2	1	5	0.372	0.370	0.375	0.371	0.375	0.369	0.3720	0.6208	42.08983529	0.042089835	41.8	0.418	0.100693386	10.06934	7.46	0.751173	75.11727		0.156
FP Collagen (face-piece)	2	1	5	0.219	0.225	0.227	0.226	0.229	0.218	0.2240	1.8225	27.09067723	0.027090677	41.3	0.413	0.06559486	6.559486	7.46	0.489338	48.93377		
	2	1	5	0.220	0.224	0.229	0.223	0.227	0.224	0.2245	1.2794	27.14461808	0.027144618	41.4	0.414	0.06556671	6.556671	7.46	0.489128	48.91277		
	2	1	5	0.225	0.223	0.230	0.224	0.227	0.226	0.2258	1.0038	27.28833777	0.027288338	41.8	0.418	0.065283105	6.52831	7.46	0.487012	48.7012	48.8618124	
	2	1	5	0.227	0.223	0.228	0.226	0.222	0.225	0.2252	0.9392	27.21650019	0.0272165	41.6	0.416	0.065424279	6.542428	7.46	0.488065	48.80651		
	2	1	5	0.231	0.216	0.221	0.230	0.228	0.215	0.2235	2.9083	27.03671124	0.027036711	41.2	0.412	0.065623086	6.562309	7.46	0.489548	48.95482		0.195
OH Collagen (ox hide)	2	1	5	0.311	0.309	0.314	0.317	0.312	0.315	0.3130	0.8453	36.32622676	0.036326227	42.3	0.423	0.085877605	8.58776	7.46	0.640647	64.06469		
	2	1	5	0.315	0.310	0.318	0.313	0.316	0.312	0.3140	0.8426	36.42612592	0.036426126	42.1	0.421	0.086522864	8.652286	7.46	0.645461	64.54606		
	2	1	5	0.312	0.318	0.310	0.311	0.317	0.314	0.3137	0.9505	36.39283506	0.036392835	41.9	0.419	0.086856408	8.685641	7.46	0.647949	64.79488	64.5233615	
	2	1	5	0.318	0.314	0.312	0.310	0.316	0.319	0.3148	1.0114	36.50931436	0.036509314	42.0	0.420	0.086926939	8.692694	7.46	0.648475	64.8475		
	2	1	5	0.310	0.315	0.312	0.319	0.313	0.314	0.3138	0.8902	36.4094816	0.036409482	42.2	0.422	0.086278392	8.627839	7.46	0.643637	64.36368		0.447

Mass balance – collagen production

Table 11: Mass balance for AS extraction method.

AS extraction					
			Raw material		
	Calf hide	Cow hide	Bovine face-piece	Bull hide	Ox hide
Fat content					
Sample mass (dry) (g)	4.57133	5.37438	6.37711	5.61726	4.90179
Fat content (g)	0.68991	0.65325	1.41393	0.837	0.76336
% fat (on dry basis)	15.09211	12.15489	22.17195564	14.9005	15.57309
Water content (% wet basis)					
Wet weight (g)	2.219	2.0519	2.1673	2.3861	2.0198
Dry weight (g)	0.8231	0.7389	0.7264	0.8275	0.7357
Water content (%)	62.90671	63.98947	66.48364324	65.31998	63.5756
Solids content (%)	37.09329	36.01053	33.51635676	34.68002	36.4244
Ash content					
Ash dry solids (%)	3.038632	3.021695	2.65178281	3.161524	1.338583
Collagen content of tissue (dry basis) (%)	41	52.93	36	65.4	54.82
Extracted collagen content aka purity (%)	25.7	3.8	4.9	5.3	5.2
Mass balance of collagen production per year					
Sample mass (g)	100	100	100	100	100
Water (g)	62.90671	63.98947	66.48364324	65.31998	63.5756
Solids (g)	37.09329	36.01053	33.51635676	34.68002	36.4244
Fat (dry basis)(g)	5.598158	4.37704	7.431231751	5.167498	5.672403
Ash (dry basis) (g)	1.127128	1.088128	0.888780987	1.096417	0.487571
Tissue (dry basis) (g)	30.368	30.54536	25.19634402	28.41611	30.26442
-Collagen (dry basis) (g)	15.20825	19.06037	12.06588843	22.68073	19.96786
-Other material (dry basis) (g)	15.15975	11.48499	13.13045559	5.735373	10.29657
Collagen (g)/(g) wet sample	0.152082	0.190604	0.120658884	0.226807	0.199679
grams of collagen in skin wet basis (g)	15.20825	19.06037	12.06588843	22.68073	19.96786
grams of collagen extracted (g)	3.908519	0.724294	0.591228533	1.202079	1.038328
Recovery (%)	16.59516	2.972591	2.305012	4.718478	4.08886
Hides processed per year*	30000	30000	60000	30000	30000
Weight per hide (kg)**	17	27	6	30	30
Mass processed (kg)	510000	810000	360000	900000	900000
Collagen produced per year (kg)	**12871.55**	**4589.354**	**1001.232635**	**9631.669**	**7348.119**

Table 12: Mass balance for AES1 extraction method.

AES1 extraction					
			Raw material		
Fat content	**Calf hide**	**Cow hide**	**Bovine face-piece**	**Bull hide**	**Ox hide**
Sample mass (dry) (g)	4.57133	5.37438	6.37711	5.61726	4.90179
Fat content (g)	0.68991	0.65325	1.41393	0.837	0.76336
% fat (on dry basis)	15.09211	12.15489	22.17196	14.9005	15.57309
Water content (% wet basis)					
Wet weight (g)	2.219	2.0519	2.1673	2.3861	2.0198
Dry weight (g)	0.8231	0.7389	0.7264	0.8275	0.7357
Water content (%)	62.90671	63.98947	66.48364	65.31998	63.5756
Solids content (%)	37.09329	36.01053	33.51636	34.68002	36.4244
Ash content					
Ash dry solids (%)	3.038632	3.021695	2.651783	3.161524	1.338583
Collagen content of tissue (dry basis) (%)	41	52.93	36	65.4	54.82
Extracted collagen content aka purity (%)	19.5	26.9	15.4	30.2	30.1
Mass balance of collagen production per year					
Sample mass (g)	100	100	100	100	100
Water (g)	62.90671	63.98947	66.48364	65.31998	63.5756
Solids (g)	37.09329	36.01053	33.51636	34.68002	36.4244
-Fat (dry basis)(g)	5.598158	4.37704	7.431232	5.167498	5.672403
-Ash (dry basis) (g)	1.127128	1.088128	0.888781	1.096417	0.487571
-Tissue (dry basis) (g)	30.368	30.54536	25.19634	28.41611	30.26442
-Collagen (dry basis) (g)	15.20825	19.06037	12.06589	22.68073	19.96786
-Other material (dry basis) (g)	15.15975	11.48499	13.13046	5.735373	10.29657
Collagen (g)/(g) wet sample	0.152082	0.190604	0.120659	0.226807	0.199679
grams of collagen in skin wet basis (g)	15.20825	19.06037	12.06589	22.68073	19.96786
grams of collagen extracted (g)	2.965608	5.12724	1.858147	6.849582	6.010324
Recovery (%)	7.339493	21.04442	7.244322	24.64589	19.98649
Hides processed per year*	30000	30000	60000	30000	30000
Weight per hide (kg)**	17	27	6	30	30
Mass processed (kg)	510000	810000	360000	900000	900000
Collagen produced per year (kg)	**5692.662**	**32490.27**	**3146.731**	**50308.82**	**35917.86**

Table 13: Mass balance for AES2 extraction method.

AES2 extraction					
	Raw material				
	Bovine face-piece	Bull hide	Calf hide	Cow hide	Ox hide
Fat content					
Sample mass (dry) (g)	6.38	5.62	4.57	5.37	4.90
Fat content (g)	1.41	0.84	0.69	0.65	0.76
% fat (on dry basis)	22.17	14.90	15.09	12.15	15.57
Water content (% wet basis)					
Wet weight (g)	2.17	2.39	2.22	2.05	2.02
Dry weight (g)	0.73	0.83	0.82	0.74	0.74
Water content (%)	66.48	65.32	62.91	63.99	63.58
Solids content (%)	33.52	34.68	37.09	36.01	36.42
Ash content					
Ash dry solids (%)	2.65	3.16	3.04	3.02	1.34
Collagen content of tissue (dry basis) (%)	36.00	65.40	41.00	52.93	54.82
Extracted collagen content aka purity (%)	48.86	74.45	65.72	75.13	65.52
Mass balance of collagen production per year					
Sample mass (g)	100.00	100.00	100.00	100.00	100.00
-Water (g)	66.48	65.32	62.91	63.99	63.58
-Solids (g)	33.52	34.68	37.09	36.01	36.42
-Fat (dry basis)(g)	7.43	5.17	5.60	4.38	5.67
-Ash (dry basis) (g)	0.89	1.10	1.13	1.09	0.49
-Tissue (dry basis) (g)	25.20	28.42	30.37	30.55	30.26
-Collagen (dry basis) (g)	12.07	22.68	15.21	19.06	19.97
-Other material (dry basis) (g)	13.13	5.74	15.16	11.48	10.30
Collagen (g)/(g) wet sample	0.12	0.23	0.15	0.19	0.20
grams of collagen in skin wet basis (g)	12.07	22.68	15.21	19.06	19.97
grams of collagen extracted (g)	5.90	16.89	9.99	14.32	13.08
Recovery (%)	22.98	66.28	39.85	46.00	34.35
Hides processed per year*	60000.00	30000.00	30000.00	30000.00	30000.00
Weight per hide (kg)**	6.00	30.00	17.00	27.00	30.00
Mass processed (kg)	360000.00	900000.00	510000.00	810000.00	900000.00
Collagen produced per year (kg)	**9983.72**	**135297.69**	**30910.34**	**71016.48**	**61724.19**

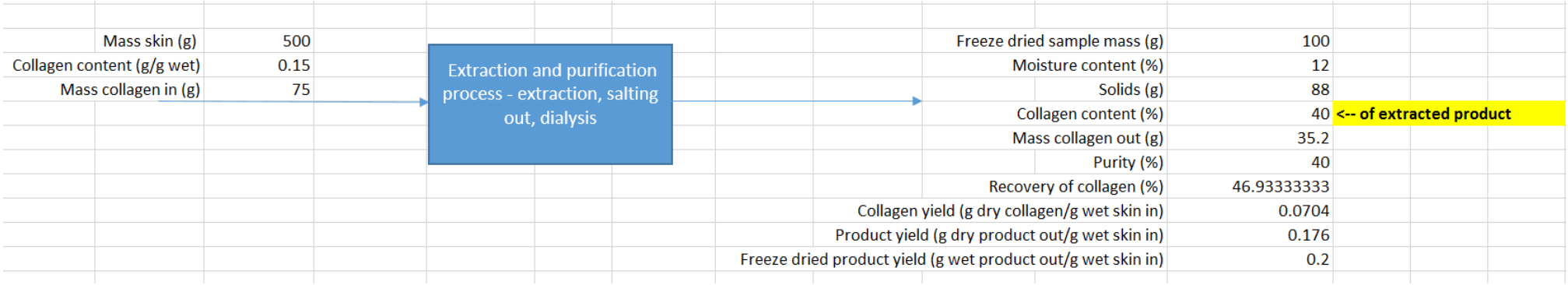

Figure 15: Example of collagen extraction mass balance.

Table 14: Extractables recovery calculations via AES2 extraction method.

	AES2											
	IN				OUT					RECOVERY		
Sample	Mass of skin (g)	Collagen (g/g wet)	Mass collagen in (g)	Freeze dried sample mass (g)	Moisture content (%)	Solids (g)	Collagen content - purity (%)	Mass collagen out (g)	Recovery of collagen (%)	Collagen yield (g collagen/g wet skin in)	Product yield (g dry product out/ g wet skin in)	Freeze dried product yield (g wet product out/g wet skin in)
Bull	250.5	0.226807343	56.8152393	60	15.69765599	50.58141	74.45	37.6578571	66.28126103	0.150330767	0.201921782	0.239520958
Calf	250.5	0.15208247	38.09665863	29	20.33898305	23.10169	65.72	15.1824339	39.85240293	0.060608519	0.092222335	0.115768463
Cow	250.5	0.190603719	47.74623149	36	17	29.88	75.13	22.448844	47.01699653	0.089616144	0.119281437	0.143712575
face-piece	250.5	0.120658884	30.22505052	17	16.36363636	14.21818	48.86	6.94700364	22.98425814	0.027732549	0.056759209	0.067864271
Ox	250.5	0.199678552	50.01947736	30	12.59717281	26.22085	65.52	17.1798997	34.34641987	0.068582434	0.104674045	0.119760479

Table 15: Extractables recovery calculations via AES1 extraction method.

AES1												
	IN				OUT				RECOVERY			
Sample	Mass of skin (g)	Collagen (g/g wet)	Mass collagen in (g)	Freeze dried sample mass (g)	Moisture content (%)	Solids (g)	Collagen content - purity (%)	Mass collagen out (g)	Recovery of collagen (%)	Collagen yield (g collagen/g wet skin in)	Product yield (g dry product out/ g wet skin in)	Freeze dried product yield (g wet product out/g wet skin in)
Bull	250.5	0.226807343	56.8152393	55	15.69765599	46.36629	30.2	14.0026193	24.64588641	0.05589868	0.185094967	0.219560878
Calf	250.5	0.15208247	38.09665863	18	18	14.76	19.5	2.8782	7.554993282	0.01148982	0.058922156	0.071856287
Cow	250.5	0.190603719	47.74623149	46	18.79813917	37.35286	26.9	10.0479183	21.04442162	0.04011145	0.149113198	0.183632735
face-piece	250.5	0.120658884	30.22505052	17	16.36363636	14.21818	15.4	2.1896	7.244322051	0.008740918	0.056759209	0.067864271
Ox	250.5	0.199678552	50.01947736	38	12.59717281	33.21307	30.1	9.99713537	19.98648507	0.039908724	0.132587123	0.151696607

Table 16: Extractables recovery calculations via AS extraction method.

AS												
	IN			OUT					RECOVERY			
Sample	Mass of skin (g)	Collagen (g/g wet)	Mass collagen in (g)	Freeze dried sample mass (g)	Moisture content (%)	Solids (g)	Collagen content - purity (%)	Mass collagen out (g)	Recovery of collagen (%)	Collagen yield (g collagen/g wet skin in)	Product yield (g dry product out/ g wet skin in)	Freeze dried product yeild (g wet product out/g wet skin in)
Bull	250.5	0.226807343	56.8152393	60	15.69765599	50.58141	5.3	2.68081454	4.718477951	0.010701854	0.201921782	0.239520958
Calf	250.5	0.15208247	38.09665863	30	18	24.6	25.7	6.3222	16.59515618	0.025238323	0.098203593	0.119760479
Cow	250.5	0.190603719	47.74623149	45	17	37.35	3.8	1.4193	2.972590623	0.005665868	0.149101796	0.179640719
face-piece	250.5	0.120658884	30.22505052	17	16.36363636	14.21818	4.9	0.69669091	2.305011562	0.002781201	0.056759209	0.067864271
Ox	250.5	0.199678552	50.01947736	45	12.59717281	39.33127	5.2	2.04522616	4.088859509	0.008164575	0.157011067	0.179640719

Table 17: Collagen production flow per year via AS extraction method calculations.

AS					
Raw material	**Bovine face-piece**	**Bull hide**	**Calf hide**	**Cow hide**	**Ox hide**
Hides processed per year*	60000	30000	30000	30000	30000
Weight per hide (kg)**	6	30	17	27	30
Mass processed per year (kg)	360000	900000	510000	810000	900000
Collagen content of tissue (dry basis) (%)	36.00	65.40	41.00	52.93	54.82
Composition					
Sample mass (g)	100	100	100	100	100
-Water (g)	66.48364324	65.31998	62.90671	63.98947	63.5756
-Solids (g)	33.51635676	34.68002	37.09329	36.01053	36.4244
-Fat (dry basis)(g)	7.431231751	5.167498	5.598158	4.37704	5.672403
-Ash (dry basis) (g)	0.888780987	1.096417	1.127128	1.088128	0.487571
-Tissue (dry basis) (g)	25.19634402	28.41611	30.368	30.54536	30.26442
-Collagen (dry basis) (g)	12.06588843	22.68073	15.20825	19.06037	19.96786
-Other material (dry basis) (g)	13.13045559	5.735373	15.15975	11.48499	10.29657
Collagen (g/g wet sample)	0.120658884	0.226807	0.152082	0.190604	0.199679
Processing					
Total collagen in (kg/year)	15843.06122	17901.26	28678.58	33500.01	33754.17
Collagen Recovery (%)	2.305012	4.718478	16.59516	2.972591	4.08886
Collagen produced (kg/year)	**1001.232635**	**9631.669**	**12871.55**	**4589.354**	**7348.119**
Collagen purity (%)	4.9	5.3	25.7	3.8	5.2
Product flow (kg/year)	**20433.31907**	**181729.6**	**50083.84**	**120772.5**	**141310**

Table 18: Collagen production flow per year via AES1 extraction method calculations.

	AES1				
Raw material	**Bovine face-piece**	**Bull hide**	**Calf hide**	**Cow hide**	**Ox hide**
Hides processed per year*	60000	30000	30000	30000	30000
Weight per hide (kg)**	6	30	17	27	30
Mass processed per year (kg)	360000	900000	510000	810000	900000
Collagen content of tissue (dry basis) (%)	36.00	65.40	41.00	52.93	54.82
Composition					
Sample mass (g)	100	100	100	100	100
-Water (g)	66.48364324	65.31998	62.90671	63.9894732	63.5756
-Solids (g)	33.51635676	34.68002	37.09329	36.0105268	36.4244
-Fat (dry basis)(g)	7.431231751	5.167498	5.598158	4.37704008	5.672403
-Ash (dry basis) (g)	0.888780987	1.096417	1.127128	1.08812836	0.487571
-Tissue (dry basis) (g)	25.19634402	28.41611	30.368	30.5453584	30.26442
-Collagen (dry basis) (g)	12.06588843	22.68073	15.20825	19.0603719	19.96786
-Other material (dry basis) (g)	13.13045559	5.735373	15.15975	11.4849865	10.29657
Collagen (g/g wet sample)	0.120658884	0.226807	0.152082	0.19060372	0.199679
Processing					
Total collagen in (kg/year)	15843.06122	17901.26	28678.58	33500.0137	33754.17
Collagen Recovery (%)	7.244322	24.64589	7.339493	21.04442	19.98649
Collagen produced (kg/year)	**3146.730517**	**50308.82**	**5692.662**	**32490.2721**	**35917.86**
Collagen purity (%)	15.4	30.2	19.5	26.9	30.1
Product flow (kg/year)	**20433.31504**	**166585.5**	**29193.14**	**120781.681**	**119328.4**

Table 19: Collagen production flow per year via AES2 extraction method calculations.

AES2					
Raw material	**Bovine face-piece**	**Bull hide**	**Calf hide**	**Cow hide**	**Ox hide**
Hides processed per year*	60000	30000	30000	30000	30000
Weight per hide (kg)**	6	30	17	27	30
Mass processed per year (kg)	360000	900000	510000	810000	900000
Collagen content of tissue (dry basis) (%)	36.00	65.40	41.00	52.93	54.82
Composition					
Sample mass (g)	100	100	100	100	100
-Water (g)	66.48	65.32	62.91	63.99	63.58
-Solids (g)	33.52	34.68	37.09	36.01	36.42
-Fat (dry basis)(g)	7.43	5.17	5.60	4.38	5.67
-Ash (dry basis) (g)	0.89	1.10	1.13	1.09	0.49
-Tissue (dry basis) (g)	25.20	28.42	30.37	30.55	30.26
-Collagen (dry basis) (g)	12.07	22.68	15.21	19.06	19.97
-Other material (dry basis) (g)	13.13	5.74	15.16	11.48	10.30
Collagen (g/g wet sample)	0.12	0.23	0.15	0.19	0.20
Processing					
Total collagen in (kg/year)	15843.06	17901.26	28678.58	33500.01	33754.17
Collagen Recovery (%)	22.98	66.28	39.85	46.00	34.35
Collagen produced (kg/year)	**9983.72**	**135297.69**	**30910.34**	**71016.48**	**61724.19**
Collagen purity (%)	48.86	74.45	65.72	75.13	65.52
Product flow (kg/year)	**20433.32**	**181729.60**	**47033.39**	**94524.80**	**94206.64**

Table 20: Collagen production flow per year via AES2 extraction method calculations.

	Raw material				
Property	**Calf hide**	**Cow hide**	**Bovine face-piece**	**Bull hide**	**Ox hide**
Fat content					
Sample mass (dry) (g)	4.57	5.37	6.38	5.62	4.90
Fat content (g)	0.69	0.65	1.41	0.84	0.76
% fat (on dry basis)	15.09	12.15	22.17	14.90	15.57
Water content (% wet basis)					
Wet weight (g)	2.22	2.05	2.17	2.39	2.02
Dry weight (g)	0.82	0.74	0.73	0.83	0.74
Water content (%)	62.91	63.99	66.48	65.32	63.58
Solids content (%)	37.09	36.01	33.52	34.68	36.42
Ash content					
Ash dry solids (%)	3.04	3.02	2.65	3.16	1.34
Collagen content of tissue (%)	41.00	52.93	36.00	65.40	54.82
Mass balance of collagen production per year					
Sample mass (g)	100.00	100.00	100.00	100.00	100.00
Water (g)	62.91	63.99	66.48	65.32	63.58
Solids (g)	37.09	36.01	33.52	34.68	36.42
Fat (g)	5.60	4.38	7.43	5.17	5.67
Ash (g)	1.13	1.09	0.89	1.10	0.49
Tissue (g)	30.37	30.55	25.20	28.42	30.26
Collagen (g)	12.45	16.17	9.07	18.58	16.59
Collagen (g)/(g) wet sample	0.12	0.16	0.09	0.19	0.17
Recovery	65.72	75.13	48.86	74.45	65.52
Hides processed per year*	30000.00	30000.00	60000.00	30000.00	30000.00
Weight per hide (kg)**	17.00	27.00	6.00	30.00	30.00
Mass processed (kg)	510000.00	810000.00	360000.00	900000.00	900000.00
Collagen produced per year (kg)	**4,173,186.19**	**9,838,876.90**	**1,595,497.01**	**12,452,298.92**	**9,783,355.86**

Soxhlet extraction data – raw hides

Table 21: Raw hide fat content determination calculations.

Soxhlet extraction data							
	Bull collagen (T1)	**Bull collagen (T2)**	**Calf hide (T3)**	**Cow hide (T4)**	**Bovine face-piece (T5)**	**Bull hide (T6)**	**Ox hide (T7)**
Initial thimble mass (g)	3.95086	3.60670	4.00260	3.89161	3.87611	4.09517	3.77626
Mass of thimble + sample (g)	8.65100	6.01340	8.57393	9.26599	10.25322	9.71243	8.67805
Mass of tray (g)	5.39396	5.43160	5.43377	5.43205	5.34565	5.33980	5.36386
Mass of dried sample + thimble + tray (g)	9.67341	9.24270	13.31779	14.04479	14.18494	14.21523	13.27855
Oil recovered (g)	4.37155	2.20230	0.68991	0.65325	1.41393	0.83700	0.76336
Sample mass (dry) (g)	4.70014	2.40670	4.57133	5.37438	6.37711	5.61726	4.90179
Fat content (g)	0.69106	0.11370	0.68991	0.65325	1.41393	0.83700	0.76336
% fat (on dry basis)	**14.70297**	**4.72431**	**15.09211**	**12.15489**	**22.17196**	**14.90050**	**15.57309**

Commercial prices of standard collagen

Table 22: Cost of lab grade standard collagen.

Tissue type	Price (NZD)/g	Form	Reference
Calf skin	1,725.00	Powder	http://www.sigmaaldrich.com/catalog/product/sigma/c3511?lang=en&region=NZ
Bovine skin (general)	26,666.67	Powder	http://www.sigmaaldrich.com/catalog/product/sigma/5006?lang=en&region=NZ
Bovine Achilles tendon	90.30	Powder	http://www.sigmaaldrich.com/catalog/product/sigma/c9879?lang=en&region=NZ
Kangaroo tail	20,850.00	Powder	http://www.sigmaaldrich.com/catalog/product/sial/c3929?lang=en&region=NZ
Human skin	483,500.00	Powder	http://www.sigmaaldrich.com/catalog/product/sigma/c5483?lang=en&region=NZ
Rabbit skin	8,010.00	Powder	http://www.sigmaaldrich.com/catalog/product/sigma/c5608?lang=en&region=NZ

Number of hides processed per year across Waitoa and Fielding plants of Wallace Corp Group.

Table 23: Hides processed at Wallace Corp Group per year.

	Bovine face-piece	Bull hide	Calf hide	Cow hide	Ox hide
Hides processed per year*	60000	30000	30000	30000	30000
Weight per hide (kg)**	6	30	17	27	30
Mass processed per year (kg)	360000	900000	510000	810000	900000

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ORIGINAL PAPER

Collagen Extraction from Various Waste Bovine Hide Sources

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Abstract

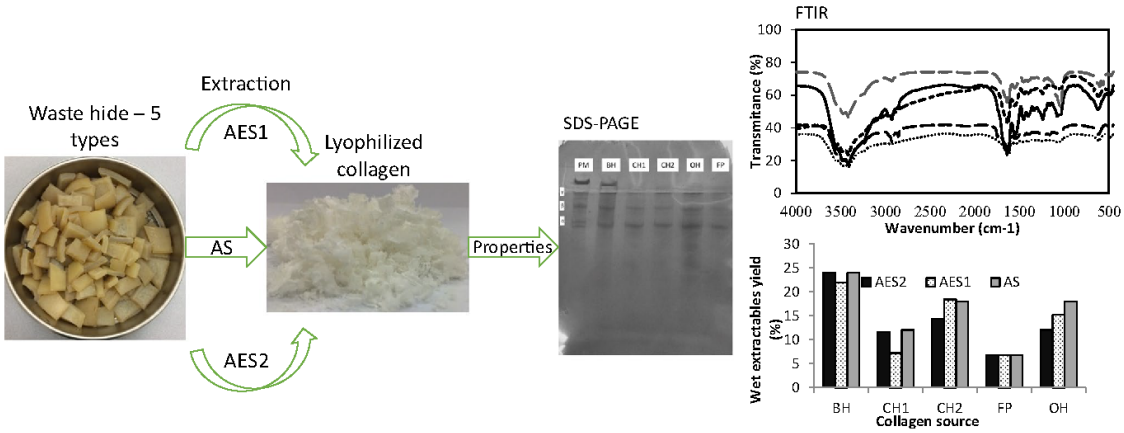
Purpose Hide is a by-product of meat production and is mostly used for leather production. Collagen is the main protein in mammalian skin, connective tissue and cartilage and presents an opportunity for value addition to waste hide off-cuttings by extracting collagen. Three different extraction methods were applied to five different hide sources. The hide sources differed with respect to the animal's age, sex, diet and environment and influenced collagen extractables yield, and therefore the economic benefit of extraction.

Methods Acid-solubilisation (AS), acid-enzyme solubilisation (AES1) and modified acid-enzyme solubilisation (AES2) were used to extract collagen from bull, calf, cow, face-pieces and ox-hides.

Results The highest dry collagen content was from cow hides using the AES2 method (75.13%), followed closely by bull hides at 74.45%. On the other hand, the lowest collagen content was from cow hides (3.80%) with the AS extraction method and the AS method proved to be inefficient for collagen extraction from bull, cow, face-piece and ox-hide sources. Analysis concluded that all the samples were of Type I collagen with α, β, and γ chains.

Conclusions Waste bovine hide off-cuttings can be used to extract high value product of collagen. AES2 proved to be the most preferable method of extraction out of the three methods applied and considering leather to collagen revenue, these waste bovine hide off-cuttings could potentially result in substantial revenue.

Graphic Abstract



Keywords Waste hide · Collagen · Collagen content · Acid soluble collagen · Pepsin soluble collagen

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Statement of Novelty

To our knowledge, no work has been carried out on extraction of collagen from waste bovine hide off-cuttings from the five sources used in this paper. The novelty further lies in the application of three extraction methods and understanding which extraction method works best with each different bovine hide source, hence providing efficient methods of extraction rather than just using one method for any source. In terms of waste valorization and economics, there is benefit in terms of using waste bovine hide off-cuttings and producing high value product collagen.

Introduction

Collagen is the most abundant protein in mammals, making up to 30% of whole-body protein content [1]. The collagen molecule is a triple-helix of three distinct alpha chains of repeating units of $(Gly\text{-}X\text{–}Y)_N$ amino acids, where X and Y are any amino acids. However, X is often proline and Y is often hydroxyproline [2, 3].

Purified collagen can be used for regenerative medicine and cosmetics, such as collagen injections for improving appearance, in body lotions and mascaras [4, 5]. Collagen is also used in casings [6], supplements [7], films [8], pharmaceuticals [9], as a precursor to biodegradable materials, for tissue engineering and more recently in 3D printing [1, 9–34]. Demand for collagen is rising at approximately 20% annually and global collagen and hyaluronic acid (HA) based biomaterials market predicted to reach US$4.6 billion by 2020 [21, 35].

Hides, a by-product of meat production, are mostly used for leather production [36]. The bovine hide is approximately 30% protein and the inner corium layer of the hide is rich in collagen. This collagen has a high denaturation temperature in comparison to collagen from marine sources. Collagen can be extracted from fish and porcine sources but present limitations. Applications of fish collagen are limited because its lower hydroxyproline content [37, 38] giving the collagen a low denaturation temperature while porcine products are prohibited by Muslim and Jewish communities [39].

Hide off-cuttings, not used in the leather industry; mostly end up in landfill or at most as animal feed. Converting this waste material into a high-value end-product, such as collagen, will benefit both the environment as well as the leather processor. The structure of bovine hide is influenced by age, sex, diet and environment [40] and may affect collagen structure and product yield. The research examines the effect of extraction method on collagen yield and collagen characteristics from different bovine sources with the aim of identifying a cost-competitive process for applications such as biopolymer films or bio-scaffolds. Methods of acid-solubliztion (AS), acid-enzyme solubilisation (AES1) and a modified acid-enzyme solubliztion (AES2) method were applied. The AS method works due to collagen polypeptides in a solution having a positive charge and addition of an acid, it becomes dominant. Hence, solubilisation is enhanced by repulsion among tropocollagen. In acid-enzyme collagen extraction, the enzyme (pepsin) removes the non-helical ends of the collagen, allowing solubilisation [41] which is dependent on the acid concentration [42].

Proximate composition (ash, fat, and moisture), collagen content and average hide thickness of raw hides were determined. Materials were pre-treated then three different extraction methods (acid-solubilisation, acid enzyme solubilisation and a modified acid-enzyme solubilisation) were used to solubilise the collagen. The extracts were purified by dialysis and characterized using SDS-PAGE and FITR. Extractables dry yield was calculated and hydroxyproline assays were done to quantify collagen content.

Experimental

Materials

Hide sections from different, unknown cattle breeds, of bull (BH), calf (CH1), cow (CH2), ox (OH) and bovine face-pieces (FP) were collected from a local tannery (Wallace Corporation Ltd, Waitoa, New Zealand) and stored at – 20 °C until used. Normally, the hide samples would not need to be stored at – 20 °C if extraction is to proceed immediately after sample collection. These represent animals slaughtered at 1–24 weeks (CH_1), 16–18 months (BH), 2–4 years (OH) or 4 years (CH_2) [43, 44]. The face-pieces came from animals of all ages.

Dialysis membranes (MWCO, 14 kDa), standard commercial collagen, pepsin enzyme from porcine gastric mucosa, L-hydroxyproline, high-molecular-weight protein markers, sodium acetate, citric acid, hydrochloric acid, acetic acid and SDS gel buffer strips were purchased from Sigma Aldrich. All other chemicals such as mercaptoethanol, sodium hydroxide, sodium chloride and methanol were analytical grade.

Extraction Methods

Pre-treating the Hide Sections

Hide off-cuttings were de-haired and bleached in 0.5 M NaOH for 24 h prior to size reduction. Samples were washed

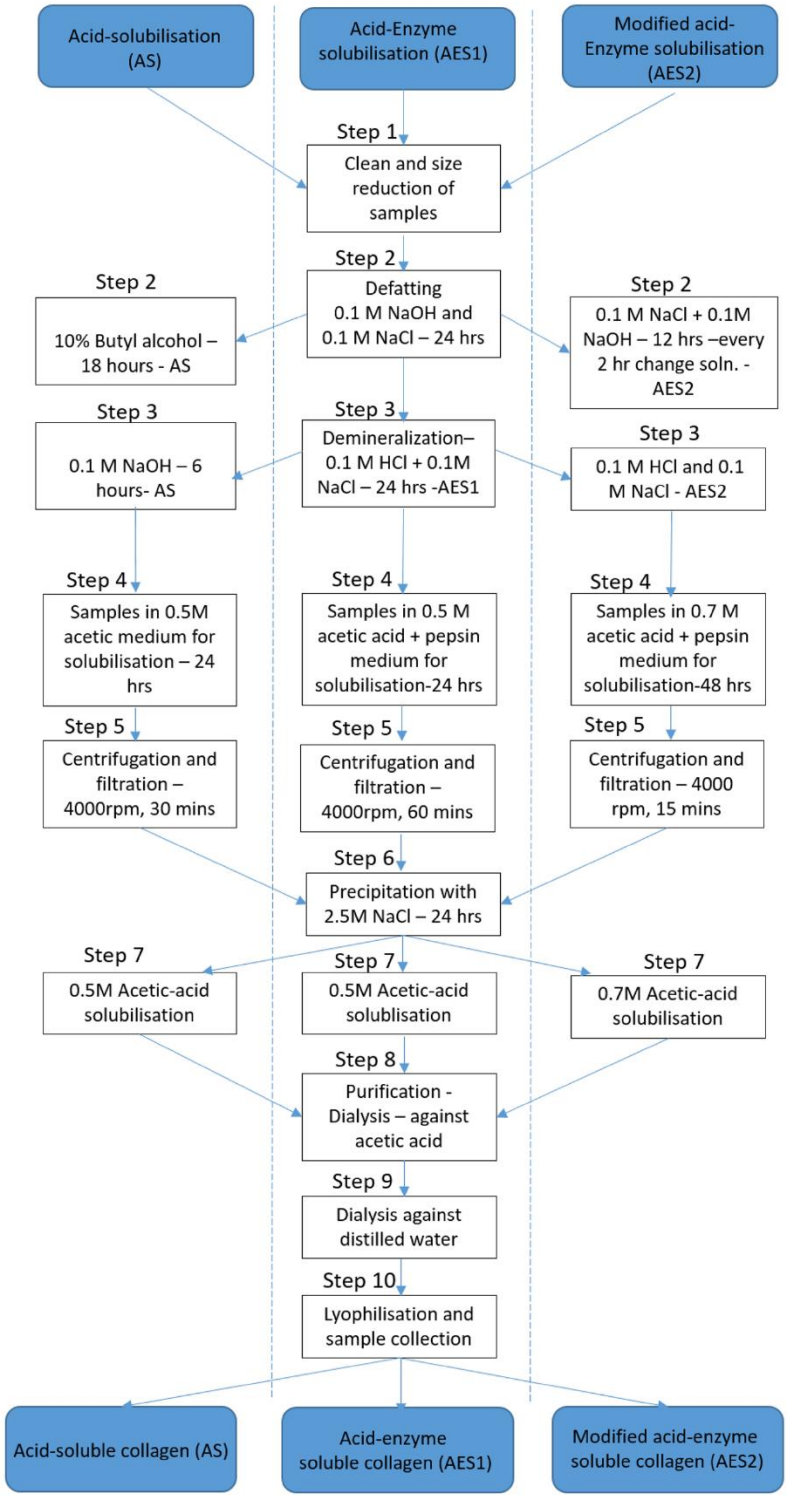

Fig. 1 Flow chart of the three extraction methods. *Details* created on PowerPoint 2016, grouped, and saved as a PNG image

with distilled water and cut into 1 × 1 cm^2. The pre-treated hides were stored at 4 °C until processed using one of three methods (Fig. 1). Extraction temperature, acetic acid concentration and pH used in each method are summarised in Table 1.

Acid-Soluble (AS) Collagen Extraction

Non-collagenous material was removed by soaking pre-treated hide sections in 1:10 w/v of 0.1 M NaOH for 6 h. The tissue was washed with distilled water until a neutral pH was achieved. The pieces were defatted by soaking in 1:10 w/v of 10% butyl alcohol for 18 h and acid solubilized in 30 volumes of 0.5 M acetic acid for 24 h. Collagen was precipitated using 2.5 M NaCl and the precipitate collected by centrifugation at 4000 rpm for 30 min. The pellet was dissolved in 0.5 M acetic acid (1:9 w/v) for 24 h before being placed in dialysis tubing and dialysed against distilled water.

Acid-Enzyme Soluble (AES1) Collagen Extraction

All steps were done with continuous stirring. Hide samples were defatted in 20 volumes of 0.1 M NaOH and 0.1M NaCl for 24 h washed with distilled water until a neutral pH was obtained. Samples were demineralized in 0.1 M HCl and 0.1 M NaCl (1:20 w/v) for 24 h and washed with distilled water to a neutral pH. The defatted samples were suspended in 20 volumes of 0.5 M acetic acid and 1% (w/w) pepsin for 24 h and filtered through a 4-mm and 250-µm filter. The collagen in the filtrate was precipitated using 2.5 M NaCl and left for 4°C for 24 h before being centrifuged at 4000 rpm for 60 min. The pellet containing collagen was rinsed with distilled water, frozen overnight and freeze-dried for 48 h.

Modified Acid-Enzyme Soluble (AES2) Collagen Extraction

By combining all of the useful extraction procedures described in the literature, a more comprehensive method was developed (AES2). The AES2 method was modified in terms of temperature, time of extraction steps, repetition of extraction steps, concentration of chemicals and purification steps.

Table 1 Details of temperature and acetic acid concentration used for the extractions

Method	Temperature (°C)	Acetic acid concentration (M)	pH
AS	21	0.5	2.60
AES1	4	0.5	2.60
AES2	4	0.7	2.35

Details created on Microsoft word

All steps were done with continuous stirring. Pre-treated hide sections were defatted by being mixed for six hours in 1:20 w/v of 0.1 M NaOH and 0.1 M NaCl. This was repeated a further three times before the samples were washed with distilled water until a neutral pH was obtained. The samples were demineralized in 1:20 w/v 0.1 M HCl and 0.1 M NaCl for 2 h. The tissue was soaked in 20 volumes of 0.7 M acetic acid and 1% (w/w) pepsin for 48 h to solubilize the collagen. The supernatant was filtered through a 4-mm and then a 250-µm filter. The collagen in the filtrate was precipitated with 2.5 M NaCl then left at 4°C for 24 h and centrifuged at 4000 rpm for 15 min. The pallet was dissolved in 1:9 w/v 0.7 M acetic acid at for 24 h being dialyzed (Membra-Cel MD44-14) in 20 volumes of acetic acid, followed by distilled water for 24 h. The dialysate was renewed every 2 h. The purified extractables were frozen overnight and freeze-dried for 24–72 h.

Lyophilisation

A known amount of purified collagen was placed in 250-ml LabServ plastic containers and frozen overnight. The samples were then placed in a Freezone 2.5 Labcono freeze drier under a vacuum of 42.0 Pa and − 52 °C for 48–72 h.

Temperature control is an important factor to consider with collagen extraction. Table 1 outlines the temperatures used for each extraction method in this study. For collagen extraction, the majority of researchers use low temperatures of around 4 °C to preserve collagen from possible contamination and spoilage. Contamination might occur due to enzyme hydrolysis or microbial degradation. However, some literature has carried out extraction at room temperature. In order to observe differences attained in the collagen properties with the influence of temperature, extractions were carried out both at 4 ℃ and 21 ℃.

Proximate Analysis of Raw Material

Moisture content (MC) was measured by drying samples at 105 °C for 24 h in a Contherm Thermotech 2000 oven (Eq. 1). Fat content was determined by Soxhlet extraction with petroleum ether (60–80 °C) for 24 h (Eq. 2). Ash content was determined by heating approximately 5 g of defatted sample in a K2F/4 B&L-TetFlow furnace at 600°C for 24 h (Eq. 3). Hydroxyproline content was determined by digesting the raw hides in 6 M HCl for 24 h at 105 °C then using the calorimetric method of Carlson and Neuman [45, 46] at 540 nm. Total collagen content was obtained by multiplying the hydroxyproline content by 7.46 (Eq. 4). Raw material density was determined by recording the weight and dimensions of rectangular hide samples and averaging the data.

$$\%Moisture\ content = \frac{M_{initial,wet} - M_{dry}}{M_{initial,wet}} * 100 \quad (1)$$

$$\%\,Fat = \frac{M_{Fat}}{M_{initial,dry}} * 100 \quad (2)$$

$$\%\,Ash(dry\,basis) = \frac{M_{Ash}}{M_{initial,dry}} * 100 \quad (3)$$

$$\%\,Collagen\,content = \frac{\mu g\,hyp\,in\,1\,mg\,hydrolsate}{\mu g\,tissue\,represented\,in\,1\,mg\,hydrolysate} * 7.46 * 100 \quad (4)$$

where $M_{initial,wet}$initial wet mass of hide sample, $M_{initial,dry}$ initial mass of hide after oven drying, M_{Fat}mass of fat collected from hide sample, M_{Ash}mass of ash from hide sample.

Extractables and Collagen Characterization

Collagen content was determined using a modified hydroxyproline assay [45, 46]. Type I commercial standard collagen was used as a control. Because temperature, humidity and air pressure can affect the assay, calibration standards were prepared daily. As water content can change during storage and preparation, moisture content was analysed immediately before the hydroxyproline assay. Samples were hydrolysed in 6 M hydrochloric acid at 105 °C for 24 h. The hydroxyproline assays were done using 6% hydrogen peroxide in distilled water, 5% *p*-dimethylaminobenzaldehyde in *n*-propanol, 0.01 M $CuSO_4$ and 0.5 M NaOH. Samples of approximately 5 mg of acid hydrolysed collagen was added to six test tubes and then 0.5 ml of 0.01 M CuSO4, 0.5 M NaOH and 6% H_2O_2 were added. The test tubes were placed in an 80 °C water bath for 10 min and mixed twice to release the hydrogen peroxide. 2 ml of 1.5M H_2SO_4 and 5% p-dimethylaminobenzaldehyde in n-propanol was then added to each test tube, the test tubes were mixed well and placed in a 70 °C water bath for 15 min. UV absorbance was measured at 540 nm. 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 (Eq. 2) [43].

Extractables yield (Eq. 5) and collagen content (Eq. 4) were calculated and moisture content of extracted solids determined.

$$\%\,Extractables\,yield(dry\,basis) = \frac{M_{dry\,extract}}{M_{initial,dry}} * 100 \quad (5)$$

Fourier-transform infrared spectroscopy (FTIR) was performed on the extracted purified collagen. Discs with 2 mg of collagen and approximately 100 mg potassium bromide (KBr) were prepared and spectra from 4000 to 500 cm^{-1} measured in an infrared spectrophotometer (FTIR Digilab FTS-40).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a modified method of Wu et al. [44]. Before analysis, extracted collagen samples were denatured in SDS-buffer solution at 100 °C. The sample (10 mg) was added to micro test tubes containing 0.5 M Tris–HCl buffer, 50% glycerol, 10% SDS, 0.02% β-bromophenol blue, 5% β-mercaptoethanol and water then loaded onto a PhastGel gradient and run at 200 V on the PhastSystem. A control containing commercial standard collagen and a protein marker was also run. After 10 min, the PhastGels were collected and soaked overnight in Coomassie staining solution containing 0.2% (w/v) Coomassie blue, 45% (v/v) methanol, 45% (v/v) distilled water and 10% (v/v) acetic acid. The gels were further de-stained by soaking them in 5% (v/v) methanol, 7% (v/v) acetic acid and 88% (v/v) distilled water until only the stained bands remained. The gels were removed from solution and dried at room temperature.

Results and Discussion

The five types of hide sources had observable structural and physical differences and it was hypothesised hide type would affect extractable yield and collagen content. Collagen cross-linking between typically increases with animal age [47] and the main difference between calf hides (CH1) and the rest was the age at which the animals were slaughtered (Table 2). Calves are typically slaughtered at a very young age compared to the other sources [48–50]. The main difference between bull and cows is their sex while for cows and oxen is that cows are mostly kept for meat/milk production and breeding purposes while oxen are working animals [51].

BH was the thickest (Table 2) and hide from young animals (CH1) and face pieces were the thinnest (Table 2). Hides from older animals (BH, OH and CH) were denser than that from young animals (CH1) or facepieces (FP) (Table 2).

Table 2 Thickness and density of hides from different bovine sources

Material	Thickness (mm)	Density (g/cm^3)
Bull hide (BH)	12–15	1.53 ± 0.10
Calf hide (CH1)	3–5	1.32 ± 0.12
Cow hide (CH2)	5–10	1.58 ± 0.11
Face-piece (FP)	3–5	1.34 ± 0.19
Ox hide (OH)	5–10	1.60 ± 0.16

Details: Created on Microsoft word 2016

Proximate Composition of the Raw Material

Table 3 illustrates the proximate composition of the hides prior to collagen extraction. Collagen content (dry basis) of all hide sources were high, however, BH resulted in the highest collagen content at 65.40% followed by OH at 54.82% (Table 3). FP resulted in lowest collagen content of 36.00%, followed by CH1 at 41.1%. Ash content (%) ranged from 1.34 to 3.16%. Fat content (%) ranged from 12.12 to 22.17% for the five hide sources and water content (%) of hides ranged from 62.91–66.48%. which correlates to literature values for cattle hides (63–65%) [51]. The raw samples all had similar dry matter contents of about 35%.

Based on the results presented here, it would be logical to focus on recovering collagen from bull hides, especially considering that processing leather from bull hides are more difficult for some tanneries. However, the values presented in Table 3 are the total collagen content and does not necessarily mean it is easily recoverable, nor does it guarantee good quality. A tannery processing all the animal sources listed may, in fact, wish to process only parts of each of the sources, possibly those that would alternatively yield the lowest quality leather.

Proximate Composition of Extracted Dry Solids

Extractables Yield

Figure 2 illustrates the total extractable yield, which is a mixture of salts, acids, small fragments of tissue and collagen. Wet and dry yields are often considered as "collagen yield" in literature with no specification of purity, however, this is clearly inaccurate and the product should be further analysed for its actual collagen content.

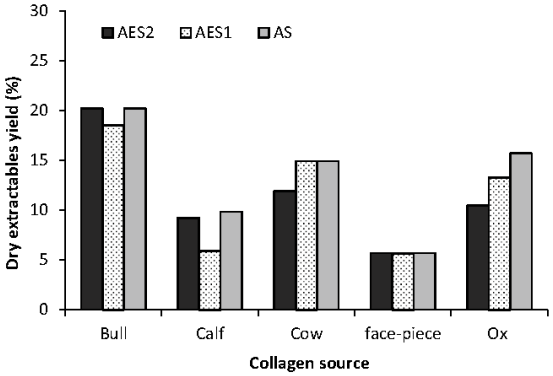

Fig. 2 Effect of hide source and extraction method on amount of material extracted: extractables (%) dry product yield. *Details* This graph was created on Excel 2016

Dry product yields (%) for each method were similar within each source (Fig. 2). The AS extraction method resulted in the highest extractables dry yield amongst all cattle hide types. Both enzyme extraction methods produced lower yields. However, these are not true "collagen yields" as it does not consider purity. Pre-treatment parameters such as physical handling, pH, temperature and time of extraction could result in variances of collagen yield during the extraction processes.

Collagen Content

The collagen content of the extracted collagen material was determined by hydroxyproline analysis and UV spectrometry.

The AS extraction method resulted in low collagen content (Table 4); significantly lower than the other methods

Table 3 Effect of source on proximate composition of raw bovine hide

	Raw material				
	Calf hide (CH1)	Cow hide (CH)	Bovine face-piece (FP)	Bull hide (BH)	Ox hide (OH)
Sample mass (g)	100	100	100	100	100
Water (g)	62.91	63.99	66.48	65.32	63.58
Solids (g)	37.09	36.01	33.52	34.68	36.42
Fat (dry basis)(g)	5.60	4.38	7.43	5.17	5.67
Ash (dry basis) (g)	1.13	1.09	0.89	1.10	0.49
Tissue (dry basis) (g)	30.37	30.55	25.20	28.42	30.26
Collagen (dry basis) (g)	15.21	19.06	12.07	22.68	19.97
Other material (dry basis) (g)	15.16	11.49	13.13	5.74	10.30
Raw hide collagen content (Dry basis, %)	40.87	52.93	36.01	65.40	54.83

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Table 4 Collagen content of extractables as obtained through hydroxyproline analysis, via extraction methods of AS, AES1 and AES2 and statistical summary of extraction method relevance

Source	Collagen content (%)		
	AS	AES1	AES2
Bull hide (BH)	5.30 ± 4.7	30.20 ± 0.87	74.45 ± 0.36
Calf hide (CH1)	25.70 ± 1.3	19.50 ± 0.78	65.72 ± 0.31
Cow hide (CH2)	3.80 ± 3.3	26.90 ± 0.32	75.13 ± 0.16
Face-piece (FP)	4.90 ± 3.0	15.40 ± 1.16	48.86 ± 0.20
Ox hide (OH)	5.20 ± 2.6	30.10 ± 0.26	64.52 ± 0.45
Mean (%)	8.98	24.42	65.74
St.dev.S	9.37	6.66	10.61
Sum	44.90	122.10	328.68
Variance.S	87.72	44.35	112.61
T-Test	AS-AES1	AS-AES2	AES1-AES2
P-value	0.017	1.904E-5	7.811E-5

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used. The method of AS works due to collagen polypeptides in a solution having a positive charge and addition of an acid, such as in acid-solubilisation, it becomes dominant. Hence solubilisation is enhanced by repulsion among tropocollagen. Thus, the sole use of AS method is unable to break the crosslinks holding the non-helical ends of tropocollagen molecules to one another. Collagen fibres are stabilized by this extensive network of intermolecular cross-links. The degree of cross-linking in collagen is a big determinant of how much collagen can possibly be extracted. The proportion and the degree of intramuscular collagen crosslinking depend on muscle type, species, genotype, age, sex, and level of physical exercise [52]. Incomplete solubility of collagen suggests that inter-molecular cross-links are still present in collagen molecules as this is the case with AS extraction of older sources such as BH, CH_2 and OH [41, 53].

The number of collagen cross-links increases with age [47]. The AS extraction method resulted in the highest collagen content for CH1 (Table 4). Taking into consideration the fact that younger sources such as calf hide have a very small amount of cross-linking as compared to older sources, it would be cost-efficient to extract collagen via the AS method. Reduction of pepsin enzyme and the sole use of AS extraction could reduce production costs significantly. Hides maintained at elevated temperatures prior to collagen extraction result in the denaturation of collagen leading to relaxation of the forces holding the collagen molecule together. The relaxation of tension is an important characteristic of hides since it is different for animals of different ages. In particular, hides from younger animals such as calves are generally weaker and exhibit a great degree of relaxation [54]. This could possibly be another reason for the low extracted collagen content with the AS method as the extraction temperature was the highest (21 °C) in comparison to AES1 and AES2 methods (4 °C).

Alongside with collagen, structural proteins such as elastin and keratin are also present in the bovine hide. There is a high possibility that with collagen extraction, elastin and keratin are also solubilised to a certain extent. This means that with the acid solubilisation method, a high amount of extractables was recovered. However, as collagen is not solubilised with acid, the collagen content was low suggesting it was mostly hydrolysed elastin and keratin and would explain why the dry extractable yield was similar between the methods. As collagen content is determined by the hydroxyproline content of the extractables, it should be noted that elastin also contains hydroxyproline [55], which may lead to some error.

Both enzymatic methods of AES1 and AES2 resulted in an increased collagen content for all bovine hides compared to AS extraction. The main reason for this is the cleaving action of the enzyme to the cross-links which hold the tropocollagen molecule which are formed into collagen fibres with the aid of cross-links. This result supports previous acid extraction work on fish skin where significant lower collagen yields were obtained in comparison to enzymatic methods [38, 41, 56–58].

The AES1 and AES2 also resulted in a higher collagen content due to the fact that the initial stage of collagen solubilisation is the hydration of fibrous collagen which proceeds by exposure to acids. Acid solubilisation (AS) aids in removal of acidic proteins due to weakening of interactions between the acidic proteins and collagen fibrils thus acid-enzyme solubilisation (AES1 and AES2) maximizes the extraction process by further removing the cross-links that are stable in acid-solubilisation are also removed, hence this method is a lot more effective than sole acid-solubilisation [59]. However, the efficiency of enzyme-solubilisation is limited by location and type of intermolecular cross-links present in each source. Hence, there is a collagen content difference among sources tested [41, 53].

Heu et al. [60] reported that one source can result in different collagen yields depending on what extraction method is used. He concluded that collagen from the skin of flat-fish resulted in different yields, where, acid-solubilized collagen resulted in a yield of 57.3% and pepsin-solubilized collagen was 85.5%. Singh [61] also observed this increase with pepsin-solubilisation.

Kim [59] reported that collagen content is influenced by a number of factors, such as acid concentration, the ratio of raw material to the acid solution, incubation time and temperature. Collagen content isn't solely dependent on extraction conditions but also on the raw material source and tissue type. Time of incubation has a huge influence on collagen

content as the longer the incubation time, the greater the collagen content. AES2 had the longest incubation time, followed by AES1 and AS. Hence it was further observed that AES2 method showed higher values in comparison to the other two extraction methods.

Kiew [53, 62] reported the influence of acetic acid concentration on the extractability of collagen from the skin of fish. Kiew [53] concluded that maximum yield of pepsin-acid solubilised collagen was achieved when 0.7 M acetic acid was used. A substantial drop in collagen yield was seen if the concentration of acetic acid was increased beyond 0.7 M. This was the case for the method of AES2 where 0.7 M (Fig. 1) acetic acid was used and an increase both in collagen yield and collagen content was seen compared to the AES1 and AS methods where 0.5 M acetic acid was used (Fig. 1). The difference in collagen content obtained with different acid concentrations could also be due to different solubility of collagen in the extracting medium. Kiew [62] showed 0.1 M acetic acid concentration to be the least effective solvent for acid-pepsin collagen extraction, however the amount of dissolved collagen increased as the concentration of acetic acid was increased. Wang et al. [63–65], also reported increasing yields of collagen from skin of grass carp as acetic acid concentration was increased. It is also reported by Wang et al. [64] that collagen is denatured at extremely low pH, such as pH 2.0 or below collagenous fibres start to shrink and thus making protein hydration impossible.

Bowes et al. [66] observed different hydroxyproline contents with acid-soluble and alkaline soluble collagen. Their study also concluded that only about two-thirds of the total protein extracted under acidic conditions are precipitated by salt and is of a collagenous type. Concluding that the remaining third part consists of albumins and globulins.

T-Test analysis determined if there is a significant difference between collagen yields from the different sources as well as between the extraction methods (Table 4).

P-values (for all combinations AS- AES1, AS-AES2, and AES1-AES2) confirmed that on a 95% confidence interval, that there was a significant difference between the extraction methods and collagen content from each source.

Fourier-Transform Infrared Spectroscopy (FTIR)

AES1 extracted collagen from all hide sources were analysed for their secondary structure. The spectrum showed similar trends to literature by showing similar bands of type I collagen. This includes the main absorption bands of amide A, amide B, amide I, amide II, amide III. Amide A band is associated with N–H stretching vibration which occurs in the range 3400–3440 cm^{-1} and indicates the presence of hydrogen bonds [43]. Drastic differences in Amide peak position among the collagen sources were not observed.

Amide B bands for BH, CH2, CH1 and OH-collagen were observed at 2928, 2923, 2921, and 2923 cm^{-1} respectively. Amide B band is related to the asymmetrical stretch of CH_2 stretching vibration. The wavenumber and amplitude differences of the Amide A and Amide B found in the collagen sources indicated that the secondary structure of collagen could possibly be different, especially between BH and FP-collagen (Fig. 3).

The Amide I peak is associated with C=O stretching vibration or stretching or possibly hydrogen bonding coupled with COO– (1600–1700 cm^{-1}). The Amide I–III peak locations for all collagen sources are shown in (Table 5) where the Amide I peak is thought to be a sensitive marker for the secondary structure of proteins. BH-collagen Amide I peak is very prominent and sharp in comparison to the other collagen sources (Fig. 3). The Amide II band is associated with N–H bending vibration coupled with C–N stretch (1540–1560 cm^{-1}), indicative of the N–H group being involved in hydrogen bonding. The intensity of OH, CH1, FP and CH2 collagen was less than BH collagen for Amide II peak, which could possibly indicate less involvement of N–H groups involved in hydrogen bonding and thus a lower stability of triple helix. Amide III is associated with C–N stretching vibration with N–H bend and C–O stretching. Amide III peak and intensity is an important characteristic of collagen. Generally, for collagen, the absorption intensity ratio between Amide III band and the 1450 cm^{-1} band (CH_2 bend) should be 1 for triple helix conformation and 0.5 is usually observed for gelatine [67, 68]. The absorption intensity ratio between Amide III band for collagen sources BH, CH2, CH1, OH and FP collagen were 0.86, 0.85, 0.85, 0.84, and 0.85 respectively. All absorption intensity ratios were way above 0.5, this indicates that the triple helix of all collagen sources was preserved.

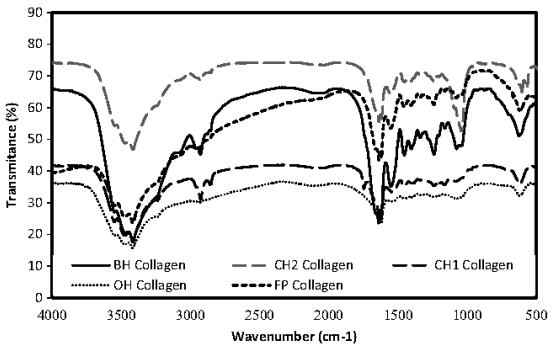

Fig. 3 FTIR spectra for BH, CH1, CH2, OH, and, FP collagen. *Details* This graph was created on Excel 2016

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

During collagen preparation for SDS-PAGE, the boiling process denatures the collagen and the native triple-helix structure of collagen is lost. Thus, the individual polypeptide chains assume a random coil configuration. Covalent bonds occur between polypeptide chains in collagen and these can link either two or all three chains together (Fig. 4).

Collagen from all sources tested displayed one γ-band (290–340 kDa), one β-band (≈ 170–200 kDa) and α-band (≈ 95–100 kD) and confirmed the presence of unfolded polypeptide chains from the triple helix of collagen. This pattern also indicates that the extracted collagen from bovine hide is type I.

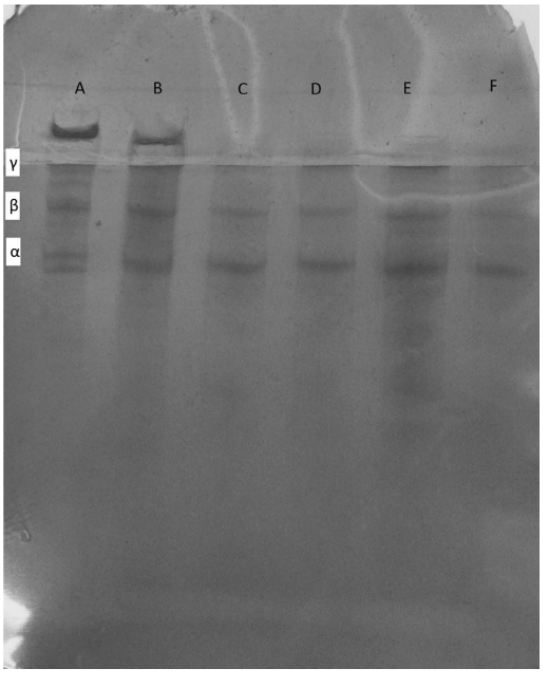

Fig. 4 SDS-PAGE bands for BH collagen (B), CH1 collagen (C), CH2 collagen (D), OH collagen (E), and FP collagen (F), along with a protein marker (A) to identify the molecular weights. *Details* This image was labelled and grouped on PowerPoint, then saved as a PNG image

Extracted collagen showed the three polypeptide chain molecular distribution as the pepsin only attacks the non-triple helical domain of native collagen and the preparative procedures are not severe thus the peptide bonds are not broken [69]. Wide distributions would only be observed if the extracted collagen was destroyed during the extraction procedure. Thus, it can be stated that acid-pepsin solubilized purified collagen has not been destroyed (denatured) during the extraction procedure and resembles the standard bovine collagen both visually and in terms of its molecular distribution. Cheng et al. [70], Lin et al. [71], and Skierka and Sadowska [41] observed similar molecular weight distributions for extracted collagen with different species.

Estimated Collagen Production Flow

Table 6 illustrates the likely collagen production based on raw material proximate composition, extracted collagen content and dry extractables yield. Based on collagen extraction recovery for each source and mass of collagen per gram of wet sample, collagen produced per year was calculated. BH would result in the largest product production rate (135.30 tonnes per year) followed by collagen from cow and OH.

Considering the high cost of collagen, bovine hide off-cuttings can be used to produce a very attractive high value-end product. Collagen markets include cosmetics industries, biomedicine, collagen films and tissue engineering. Majority of collagen that is sold in the market for food industries and cosmetics is not pure native collagen but rather broken down collagen amino acids (gelatine) or hydrolysed forms. These latter forms are sold at more reasonable prices due to lack of specific processing steps and chemicals used.

At a wholesale price of $6.17 per gram of collagen, a revenue of $834,801,000 could be generated per year just from BH-collagen. Hence, at a low selling price of $6.17 per gram of collagen can still result in a substantial revenue.

Table 5 Main FTIR peak locations of BH, CH2, CH1 and OH-collagen

Collagen source	Component peak location (cm^{-1})				
	Peak 1 (Amide A)	Peak 2 (Amide B)	Peak 3 (Amide I)	Peak 4 (Amide II)	Peak 5 (Amide III)
BH	3416	2928	1635	1548	1246
CH2	3418	2923	1620	1548	1229
CH1	3410	2921	1640	1477	1236
OH	3418	2923	1620	1524	1225
FP	3415	2935	1617	1546	1236

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Table 6 Mass balance of collagen production per year for each source

Source	Bovine face-piece	Bull hide	Calf hide	Cow hide	Ox hide
Mass processed per year (tonnes)[a]	360	900	510	810	900
Collagen (g/g wet sample)	0.12	0.23	0.15	0.19	0.20
Processing					
Collagen Recovery[b] (%)	22.9	66.2	39.8	46.0	34.3
Collagen produced (tonne/year)	9.9	135.3	30.9	71.0	61.7

Details This table was created on Microsoft Word 2016. (This table will need to be placed vertically when journal paper is structured into two columns OR placed across two columns)

[a]Based on approximate number of hides processed by Wallace Corporation Ltd (NZ) annually and average weight of each hide

[b]Based on mass inflow and outflow of collagen, considering collagen content/purity

Native high purity collagen which is usually extracted for medicinal purposes is in its stable triple-helix form costs as high as $26,666.67 per gram (Sigma Aldrich). Therefore, native collagen is significantly more valuable and could generate more revenue than stated in this paper. Collagen costing $6.17 or less per gram in most cases is collagen peptides or in its hydrolysed form. If this is the market then any bovine hide off-cuttings and shavings could be processed into collagen peptides (gelatine) without the requirement of costly enzymes or chemicals. However, for biomedical and lab grade purposes, collagen must be in its stable insoluble triple-helical form and this requires careful extraction steps and use of both chemicals and enzymes which add up the processing costs.

Environmental concerns, which 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. In reality, only about 25% by weight of raw salted hides results in the finished leather [72]. Furthermore, other solid wastes are also produced from wastewater and sludge treatment. Bovine waste off-cuttings can result in a favourable revenue reducing landfill waste and adding value to the economy.

Conclusion

It can be concluded that collagen of high yield and content can be extracted from bovine hides of bull, calf, cow, ox and face-pieces by acid-solubilisation, acid-enzyme solubilisation and a modified acid-enzyme solubilisation. Modification of the acid-enzyme soluble collagen extraction method resulted in significantly higher collagen content. Purification of collagen was a necessary step and it resulted in a clean white agglomerated powder, which resembled the standard collagen from Sigma Aldrich. Hydroxyproline assays indicated a sufficient content of hydroxyproline in the extracted collagenous tissue, thus it can be confirmed that the extracted material is collagen. SDS-PAGE analysis and FTIR confirmed secondary structure and the triple helix and the chains that comprise the collagen molecule. This study has resulted in a new method to extract collagen of high extractables yield and content in an efficient manner. Thus, the extracted collagen from cow, calf, bull, face-piece and ox-hide, via the method of AES2 are suitable for a variety of different collagen-derived applications such as collagen biopolymer films is to be investigated in the near future.

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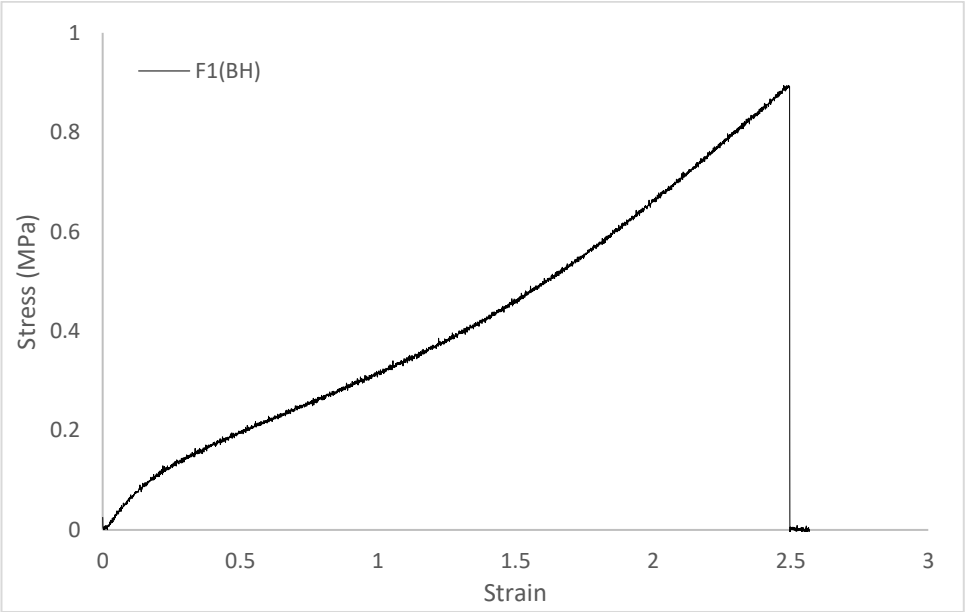
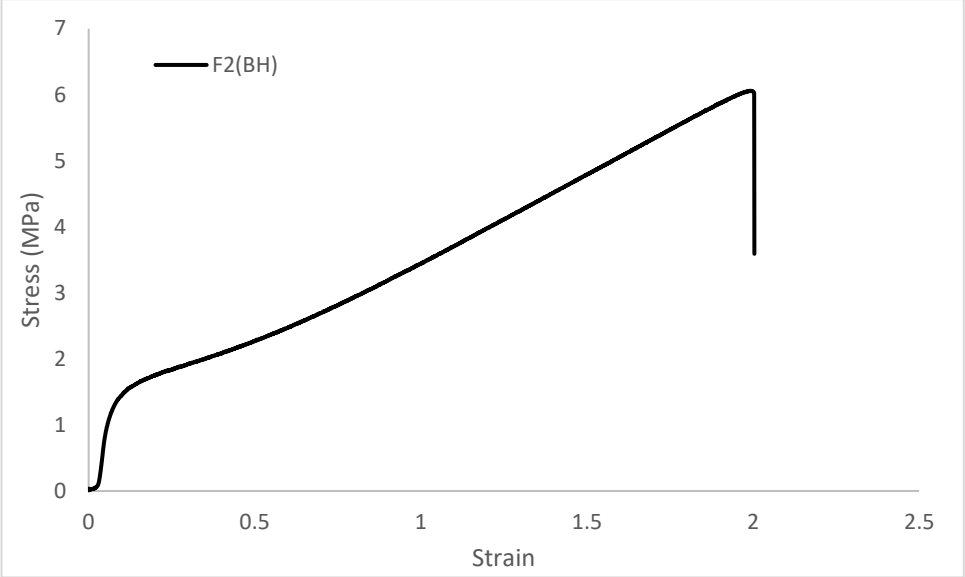
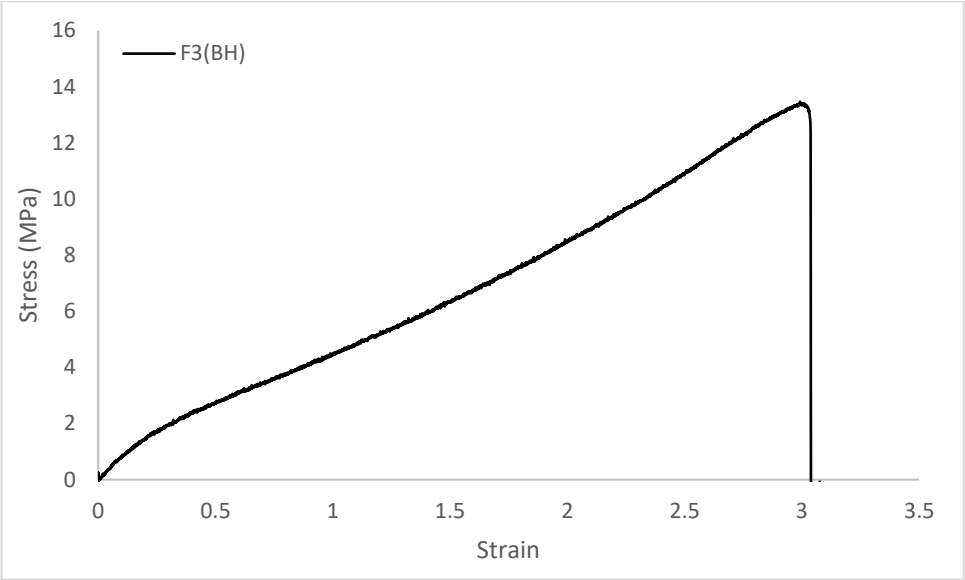
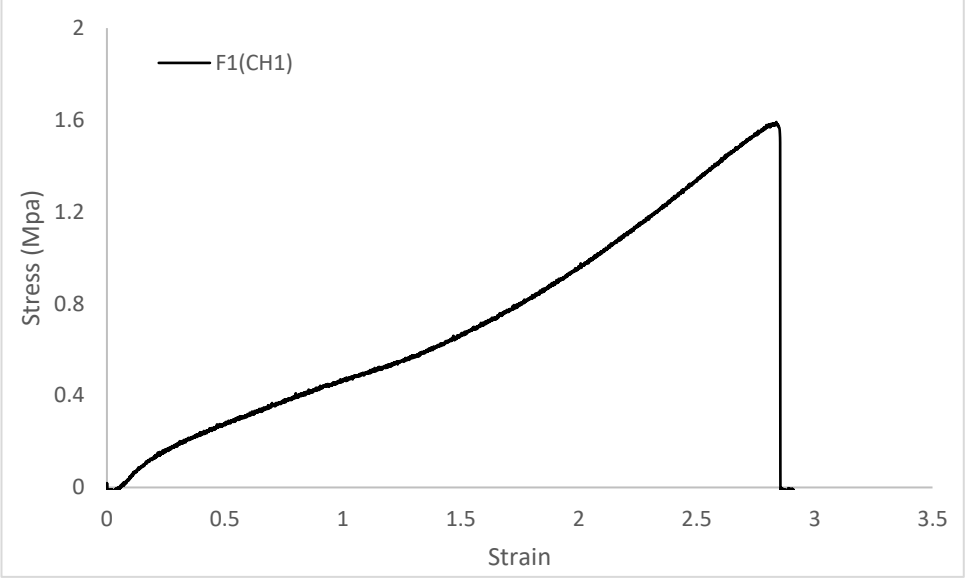
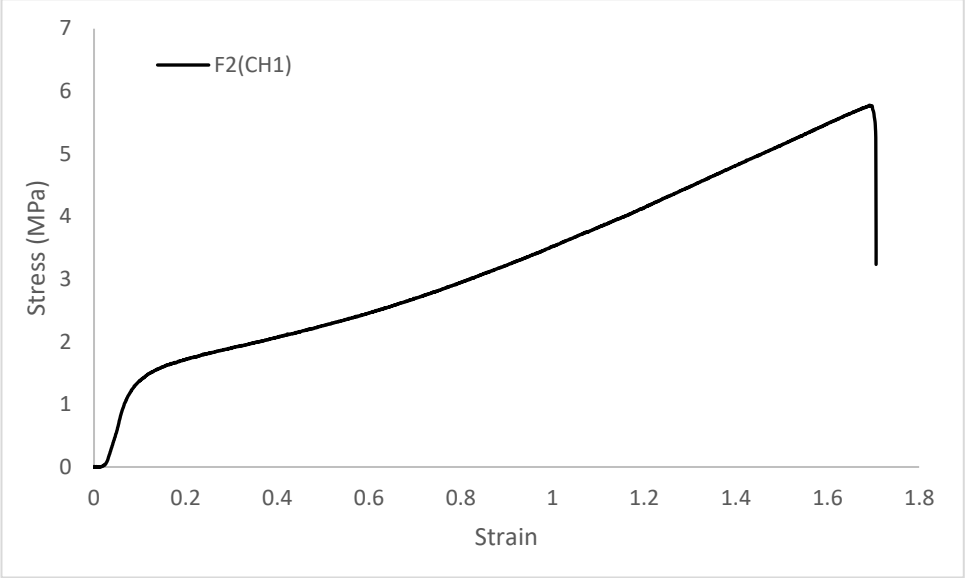
A4 Stress – strain representation graphs for Chapter 6

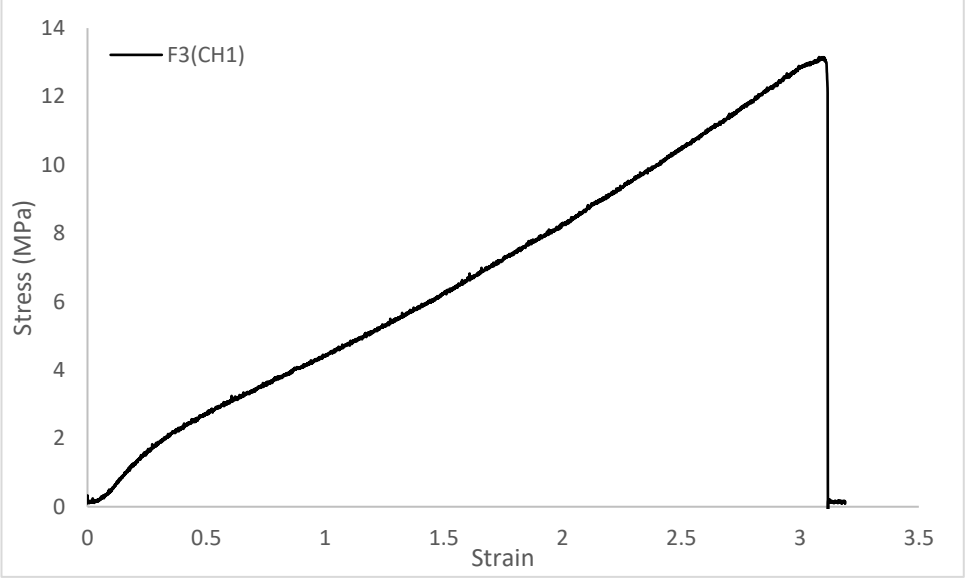





F3(BH)
Stress (MPa)
Strain
16
14
12
10
8
6
4
2
0
0
0.5
1
1.5
2
2.5
3
3.5


F1(CH1)
Stress (Mpa)
Strain
2
1.6
1.2
0.8
0.4
0
0
0.5
1
1.5
2
2.5
3
3.5


F2(CH1)
Stress (MPa)
Strain
0
1
2
3
4
5
6
7
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8


F3(CH1)
Stress (MPa)
Strain
0
2
4
6
8
10
12
14
0
0.5
1
1.5
2
2.5
3
3.5

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  - Friedrichs, J. (2009). Analyzing interactions between cells and extracellular matrix by atomic force microscopy. PhD thesis, Technical University of Dresden, Dresden, Germany.

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  - URL:https://www.google.com/search?q=Soft+Shield+Collagen+Corneal+Shields+by+Oasis+Medical.&rlz=1C1GCEA_enNZ803NZ804&sxsrf=ACYBGNSeAdYkzc6ifq8TkjyaSMPdYf8UAQ:1581312914545&source=lnms&tbm=isch&sa=X&ved=2ahUKEwjfjLPUocbnAhU3zzgGHZZOC_0Q_AUoAXoECAsQAw&biw=1280&bih=881#imgrc=TQ5TtIsIjSAZkM
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