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Sustainable Collagen Composites from New Zealand Sheepskin: From Extraction to Application

A thesis presented in partial fulfilment of the requirements for the degree of

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Abstract

Sheepskin, a low-value by-product of the New Zealand meat industry, is largely discarded despite its potential as a source of collagen. This thesis investigates the extraction, characterisation, and application of sheepskin collagen in sustainable fibre-based composites. Collagen was extracted using acid, acid-enzyme, and alkali methods, with pre-treatment and extraction parameters systematically evaluated to optimise yield and investigate its properties. Characterisation by FTIR, SDS-PAGE, SEM, and small-angle X-ray scattering confirmed preservation of fibrillar collagen under suboptimal and optimal conditions, while highlighting variability in molecular weight and purity across extraction methods.

Further investigation of the acid-enzyme extraction was conducted to establish the process variables determining the optimum yield and collagen content. The extracted collagen was applied as a coating on self-assembled cellulose fibre sheets, and performance was compared with gelatin coatings. Collagen improved surface wettability, barrier performance, tensile properties, and thermal behaviour, with effects dependent on coating concentration. Gelatin coatings provided some functional enhancements but were less effective at fibre network integration. Advanced synchrotron micro-computed tomography (micro-CT) revealed three-dimensional coating penetration, fibre-matrix interactions, and structural differences between collagen and gelatin composites, directly linking internal microstructure to observed macroscopic properties.

This work demonstrates that sheepskin, an underutilised agricultural by-product, can be transformed into functional collagen suitable for bio-based composites. The findings provide both a sustainable route for waste valorisation and foundational knowledge for developing collagen-based materials with tailored mechanical, barrier, and thermal properties. Future work should focus on scaling extraction processes, optimising coating integration, and extending micro-CT analysis to dynamic or environmental testing to further enhance the utility of sheepskin-derived collagen in sustainable materials applications.

Table of Contents

Acknowledgements.....	iii
Abstract	v
List of Tables	xi
List of Figures.....	xiv
List of Abbreviations.....	xxi
Publications.....	xxiii
Conferences	xxiii
Chapter 1. Introduction	1
1.1 Background.....	1
1.2 Aims and objectives.....	3
1.3 Thesis overview.....	3
Chapter 2. Literature Review 1: Collagen	5
2.1 Skin and sheepskin characteristics.....	5
2.2 Collagen.....	7
2.3 Collagen Extraction Process	11
2.4 Specific extraction methods	19
2.5 Future Considerations.....	28
2.6 Conclusions	29
Chapter 3. Literature Review 2: Collagen matrix composites.....	30
3.1 Introduction.....	30
3.2 Collagen sources	30

3.3	Crosslinkers	32
3.4	Additives and coatings	35
3.5	Composite forming methods	38
3.6	Conclusions	44
Chapter 4.	Gelatin and collagen from sheepskin.....	45
4.1	Abstract.....	45
4.2	Introduction.....	45
4.3	Materials and Methods.....	46
4.4	Results and Discussion	50
4.5	Commercialisation.....	59
4.6	Conclusions	59
Chapter 5.	Modified collagen extraction from raw and pickled sheepskin	60
5.1	Introduction.....	60
5.2	Materials and methods	61
5.3	Results and discussion.....	66
5.4	Conclusion.....	73
Chapter 6.	Protein coatings on cellulose sheets.....	75
6.1	Introduction.....	75
6.2	Experimental method.....	75
6.3	Results and discussion.....	83
6.4	Conclusion	110
Chapter 7.	Micro-computed tomography to characterise collagen- and gelatin-coated fibrous composite sheets.....	111

7.1	Introduction.....	111
7.2	Experimental method.....	115
7.3	Results	118
7.4	Discussion.....	140
7.5	Conclusion	143
Chapter 8.	Conclusions and recommendations for future work.....	147
	References	149
	Appendices	187
	Appendix A: Chapter 5 modified and large batch extraction.....	187
A1.	Yield T-test	187
A2.	Hydroxyproline raw data	188
A3.	Mass balance for large batch extraction	190
A4.	Yield extract for the modified methods	192
A5.	FTIR wavenumber legends	193
A6.	SAXS Images: Detection of collagen fibrils extracted from acid, acid-enzyme, and alkali.....	194
	Appendix B: Chapter 6 Collagen and cellulose sheet fabrication.....	196
B1.	Tensile plots.....	196
B2.	Coated and uncoated sheets properties before and after coating.	200
B3.	Properties of the permeability samples, initial and final oxygen levels, and oxygen transmission rate and oxygen permeability repeats	202
B4.	Tear sample properties and tear data repeats	206
B5.	Front and back of sheet FTIR plots and wavenumbers	208

B6.	Contact angle measurements.....	212
B7.	TGA supplementary data	217
Appendix C: Chapter 7 Micro-computed tomography to characterize coated and uncoated fibrous composite		218
C1.	ROI segmentation viewed in different axes and the pore space ROI considered for the aspect ratio/volume contribution.....	218
C1.	Anisotropy calculation results using SVD and MIL: property plots 226	
C2.	SVD and MIL iterations	232
Appendix D: Research output		244
D1.	Publication 1 in Biology: Collagen Extraction from Animal Skin	244
D2.	Publication 2 in Polymers: Gelatin and Collagen from Sheepskins 259	
D3.	Poster presented at Canadian Society for Chemistry, 2-6th June 2024, Winnipeg, Canada.....	271
Appendix E: Statement of Contribution Forms		272
E1.	Publication 1 in Biology: Collagen Extraction from Animal Skin	272
E2.	Publication 2 in Polymers: Gelatin and Collagen from Sheepskin	273

List of Tables

Table 1 Cattle, sheep, pig, and goat skin histology, skin weight percentage, fat and protein content, skin thickness, and grain layer.	6
Table 2 Properties and characteristics of different types of sheep.	7
Table 3 Skin as percentage of total body mass.	9
Table 4 Collagen types, compositions and sources (based on [103, 104])	11
Table 5 Pre-treatment, extraction and isolation methods for collagen production from skin.	19
Table 6 Chemical and physical crosslinkers.	32
Table 7 Freeze-drying in cylindrical conduit of collagen composite: parameters and resulting effect on the scaffold.	40
Table 8 Hydroxyproline and gelatin-collagen content of the material recovered using the different extraction methods (acid, alkali), and raw sheepskin.	52
Table 9 FTIR absorption bands of amide vibration modes from the literature [342] and measured in this work and keratin bands identified in these samples assigned from literature [343].	55
Table 10 The <i>D</i> -spacing of collagen extracted by acid or alkali.	59
Table 11 Hydroxyproline and collagen content of acid step, acid-enzyme step, acid-enzyme and large batch samples.	68
Table 12 SEM images of the freeze-dried collagen from the two-step acid and acid-enzyme, one-step acid-enzyme, and extraction from pickled pelts.	69
Table 13 Amide peaks for modified collagen extraction samples.	73
Table 14 FQA results on fibre length and width.	83
Table 15 Viscosity measurements of the different coatings.	83
Table 16 Sheet properties: coating percentage, before (initial) and after (final) coating sheet thickness, and grammage.	84
Table 17 Ultimate tensile strength of coated and uncoated sheets.	86

Table 18 Thickness and weight coating percentage of the permeability samples with resulting oxygen permeability and transmission rate of the different collagen and gelatin coated sheets.....	92
Table 19 FTIR Spectra peak detection for wood pulp components and coating.	99
Table 20 DTG Maximum degradation rate. Units in °C. The temperatures in brackets are slight indication of peaks that can be seen in the close-up in Figure 33.	106
Table 21 SEM images for the uncoated and collagen coated sheets at 1 mm and 100 um scale and both sides of the sheet, front (1) and back (2).	108
Table 22 SEM images for the high and low bloom gelatin coated sheets at 1 mm and 100 um scale and both sides of the sheet, front (1) and back (2).	109
Table 23 Sheet properties: coating percentage by weight, thickness, and grammage	118
Table 24 Region of interest (ROI) range parameters of fibre, coating and total sheet on Dragonfly	118
Table 25 Volume based aspect ratio (%) approximation of fibre, coating and total sheet on Dragonfly	119
Table 26 Fibre and coating ROIs for collagen 3% and 5%. Fibre ROI are shown in orange, collagen at 3% in purple and collagen at 5% in yellow.	120
Table 27 -X and -Y axes views of coating and fibre ROIs. Fibre ROI are shown in orange, collagen at 3% in purple, collagen at 5% in yellow, high bloom gelatin at 3% in pink and high bloom gelatin 5% in green.	124
Table 28 Converged degree of anisotropy result. Minimum values for parameters.	137
Table 29 Key findings correlating volume ratios and porosity analysis on composite properties. Abbreviations used: C- collagen, HBG- high bloom gelatin, LBG- Low bloom gelatin, and UC- uncoated.	145
Table 30 Yield T-test summary for all extraction methods	187
Table 31 Calculation data for HYP and collagen content.....	188
Table 32 Collagen extraction mass balance	190
Table 33 Extraction yield for the modified extraction methods	192
Table 34 Tensile data (and paper properties).....	200

Table 35 Permeance sample properties.....	202
Table 36 Data point consideration for oxygen diffusion during the permeability test...	203
Table 37 Oxygen transmission rate results.....	204
Table 38 Conversion from oxygen transmission rate results to oxygen permeability	205
Table 39 Tear sample properties.....	206
Table 40 Tear raw data	206
Table 41 Front side of the coated and uncoated sheet contact angle measurements	212
Table 42 Back side of the coated and uncoated sheet contact angle measurements.....	214
Table 43 Contact angle summary.....	216
Table 44 -X and -Y axes views of coating and fibre ROIs	218
Table 45 Pore space x and y axis view	220
Table 46 Pore space x- and y- axis view	222
Table 47 Pore space z and z- axis view	224
Table 48 SVD and MIL trial results	232
Table 49 SVD and MIL values with their respective frequency during calculation	236

List of Figures

Figure 1 World meat production (1961-2020). Data from Ritchie and Roser [78, 80]	9
Figure 2 Skin-collagen-molecular structure. Adapted with permissions from: (1) Tang et al., Journal of Voice, published by Elsevier Inc., 2017; (2) Reilly and Lozano, Plastic and Aesthetic Research, published by OAE Publishing Inc., 2021 (©2021 MINERVA Research Labs Ltd. (106 New Bond St. London UK) all rights reserved) [93, 94].....	10
Figure 3 Collagen content of different body parts of animals. Reproduced with permission from Jafari et al., Polymers, 2020; 12(10):2230. ©2020 MDPI [19].....	11
Figure 4 Overview of processing options for extraction of collagen from skin (based on Table 5).	28
Figure 5 Flowchart of the three extraction methods.	48
Figure 6 Dried gelatin-collagen (top, optical images; bottom, SEM images) from the different extraction methods: (a) acid hydrolysis, (b) acid-enzyme, (c) alkali hydrolysis.	51
Figure 7 FTIR spectra of collagen recovered by acid (blue line), acid-enzyme (green line) and alkali (red line) extraction. a) full spectrum; b) expanded region that assists in distinguishing between gelatin and collagen [341]. See Table 9 for details of peak assignments.....	54
Figure 8 SDS PAGE of collagen from acid, acid-enzyme and the alkali extraction processes. The lanes on extreme left and right show the globular protein molecular weight markers (2–250 kDa). There are three replicate lanes for each treatment. The figure has been modified to remove some lanes on the right-hand side not discussed in this work (full original SDS PAGE available).	57
Figure 9 a) SAXS integrated scattering intensity for extract obtained by acid (blue line), acid-enzyme (green line), and alkali (red line) extraction showing sharp peaks for diffraction from the collagen fibril D-banding; b) example of the SAXS pattern (acid extracted material) with the faint rings visible due to the collagen diffraction.	58
Figure 10 Process flow diagram for the one-step acid-enzyme and two-step collagen extraction methods.	64
Figure 11 Process flow diagram of the large batch collagen extraction from pickled pelts	65

Figure 12 Rope-like structure observed in the collagen samples extracted in the modified method. Scale bar is 10 μ m. SEM images are as follows: (a) A2, (b) AE2, (c) AE1, and (d) LBPP.....	70
Figure 13 SDS PAGE results for the modified extraction methods. Lanes are as follows: (1) protein marker, (2-4) A2, (5-7) AE2, (8-10) AE1, (11-13) LBPP, (14) gelatin standard, (15) collagen standard, (16) protein marker. Molecular weights are labelled to the left of the protein marker.	71
Figure 14 FTIR results for the modified extraction methods: acid step (A2), acid-enzyme step (AE2), one step acid-enzyme (AE1), and large batch from pickled pelts (LBPP).....	72
Figure 15 Collagen and gelatin coated sheet fabrication method.	78
Figure 16 Instron tensile testing setup	79
Figure 17 Oxygen permeability setup (a) parts of the setup and (b) assembled setup.....	80
Figure 18 Tear test sample setup (left), and samples after and before tear test (right).	81
Figure 19 Fibre coated with gelatin and collagen solutions (from left to right) after the coating was applied on the sheet, when drying with the excess water evaporated, and after the coated sheets had been drying for 12 hours showing the densification of the collagen coated sheet and the maintained pore space in the dried gelatin coated sheet. Legends are listed on the top left corner for peptide chains, fibres, and water.....	85
Figure 20 Stress-strain curves for the coated and uncoated cellulose sheets	86
Figure 21 (a) Elongation at break, (b) tensile strength, (c) elastic modulus of coated and uncoated cellulose sheets.....	87
Figure 22 Total elongation as a function of ultimate tensile strength	88
Figure 23 Force-elongation curve from tensile testing to determine the elastic modulus	89
Figure 24 Tensile energy absorption (TEA) as a function of tensile strength	90
Figure 25 Tear strength for different coated cellulose sheets.....	93
Figure 26 Tear index for different coated cellulose sheets.....	94
Figure 27 Tear strength as a function of grammage	95

Figure 28 FTIR stacked plot collagen used for coating (solid line) and uncoated wood pulp sheet (dashed line).	96
Figure 29 Solid line is the side facing up referred to as front and the dashed plot is the side in contact with the tray referred to as back during the drying process. (A) collagen 5%, (B) collagen 3%, (C) high bloom gelatin 5%, (D) high bloom gelatin 3%, (E) low bloom gelatin 5%, (F) low bloom gelatin 3%.	98
Figure 30 Surface contact angle (θ_c).....	100
Figure 31 Front and back contact angle measurements for side 1 and side 2, respectively, of the coated and uncoated sheets at 0.1 s, 1 s, and 10 s.....	101
Figure 32 Weight change over a temperature gradient from 30°C – 600°C	104
Figure 33 DTG plots for the different coated hand sheets.	105
Figure 34 DTG in the 400 – 600°C temperature range.....	106
Figure 35 Composite sheet mounted on the sample platform in the MCT beamline (Australian Synchrotron).	115
Figure 36 2D MROI OpenPNM segmentation for the different coated and uncoated sheets; Y to –Y slice view. Coloured regions are the segmented pores, colour indicates the pore size indicating yellow hues as larger pores, green hues as medium pores and purple hues as the small pores.....	126
Figure 37 X+Z+ axis view of MROI and porosity. On the right, the 3D MROI segmentation is shown alongside the corresponding 3D plots of pore equivalent diameter. Pores are represented as spheres (vertex), with the x-axis colour scale indicating pore size. Branch connections between pores are represented as edges (lines), with the y-axis colour scale indicating the measured distance between pore centres. (Figure continues on the next page.).....	128
Figure 38 X–Z– MROI and Porosity view of MROI and Porosity. (Figure continue on next page).....	130
Figure 39 Pore size (as equivalent diameter) histograms.....	133
Figure 40 X+Z+ axis view displaying the connectivity of the pore segments with edge branching colours correspond to the length between centres of connected pores.	134
Figure 41 X–Z– axis view of connectivity and inter-pore length.	135
Figure 42 Histograms for connectivity between pores.....	136

Figure 43 Fibre ROI for anisotropy analysis.....	138
Figure 44 Projection- and eigen- based anisotropy evaluation at different axis views....	139
Figure 45 FTIR plot results for the modified extraction methods (A) Step 1: Acid extraction step (B) Step 2 Acid-enzyme (C) One-step acid enzyme (D) Large batch acid-enzyme extraction	193
Figure 46 SAXS imaging: collagen sample extracted via acid hydrolysis	194
Figure 47 AXS imaging: collagen sample extracted via acid-enzyme hydrolysis	194
Figure 48 SAXS imaging: collagen sample extracted via alkali hydrolysis	195
Figure 49 Tensile plot: Low bloom gelatin 3%.....	196
Figure 50 Tensile plot: Low bloom gelatin 5%.....	196
Figure 51 Tensile plot: High bloom gelatin 3%.....	197
Figure 52 Tensile plot: High bloom gelatin 5%.....	197
Figure 53 Tensile plot: Collagen 3%.....	198
Figure 54 Tensile plot: Collagen 5%.....	198
Figure 55 Tensile plot: Uncoated.....	199
Figure 56 FTIR: Collagen and cellulose fibre	208
Figure 57 FTIR: Collagen 5%	208
Figure 58 FTIR: Collagen 3%	209
Figure 59 FTIR: High bloom gelatin 5%.....	209
Figure 60 FTIR: High bloom gelatin 3%.....	210
Figure 61 FTIR: Low bloom gelatin 5%.....	210
Figure 62 FTIR: Low bloom gelatin 3%.....	211
Figure 63 (a) Collagen 3% (b) Collagen 5% (c) High bloom gelatin 3%(d) High bloom gelatin 5% (e) Low bloom gelatin 3% (f) Low bloom gelatin 5% (g) no coating (h)	

collagen coatin. Weight – dash line, Heatflow – solid line, DTG dash double dot	217
Figure 64 SVD degree of anisotropy vs maximum iteration	226
Figure 65 SVD degree of anisotropy vs orientation	226
Figure 66 SVD degree of anisotropy vs radius	227
Figure 67 SVD degree of anisotropy vs sample	227
Figure 68 SVD degree of anisotropy vs minimum iteration	228
Figure 69 SVD degree of anisotropy vs tolerance	228
Figure 70 MIL degree of anisotropy vs maximum iteration	229
Figure 71 MIL degree of anisotropy vs orientation	229
Figure 72 MIL degree of anisotropy vs radius	230
Figure 73 MIL degree of anisotropy vs sampling	230
Figure 74 MIL degree of anisotropy vs minimum iteration	231
Figure 75 MIL degree of anisotropy vs tolerance	231
Figure 76 Spacing (um) = 15, Radius of influence (um) = 30, Evaluation: Projection, Mesh smoothing (repetitions) = 1	238
Figure 77 Spacing (um) = 15, Radius of influence (um) = 35, Evaluation: Projection, Mesh smoothing (repetitions) = 2	238
Figure 78 Spacing (um) = 15, Radius of influence (um) = 25, Evaluation: Projection, Mesh smoothing (repetitions) = 2	239
Figure 79 Spacing (um) = 20, Radius of influence (um) = 30, Evaluation: Projection, Mesh smoothing (repetitions) = 2	239
Figure 80 Spacing (um) = 10, Radius of influence (um) = 30, Evaluation: Projection, Mesh smoothing (repetitions) = 2	240
Figure 81 Spacing (um) = 15, Radius of influence (um) = 30, Evaluation: Projection, Mesh smoothing (repetitions) = 2	240

Figure 82 Spacing (um) = 15, Radius of influence (um) = 30, Evaluation: Eigen, Mesh smoothing (repetitions) = 2	241
Figure 83 Spacing (um) = 10, Radius of influence (um) = 30, Evaluation: Eigen, Mesh smoothing (repetitions) = 2	241
Figure 84 Spacing (um) = 20, Radius of influence (um) = 30, Evaluation: Eigen, Mesh smoothing (repetitions) = 2	242
Figure 85 Spacing (um) = 15, Radius of influence (um) = 35, Evaluation: Eigen, Mesh smoothing (repetitions) = 2	242
Figure 86 Spacing (um) = 15, Radius of influence (um) = 25, Evaluation: Eigen, Mesh smoothing (repetitions) = 2	243
Figure 87 Spacing (um) = 15, Radius of influence (um) = 30, Evaluation: Eigen, Mesh smoothing (repetitions) = 1	243

List of Abbreviations

ABS	Acrylonitrile butadiene styrene
BHT	Butylated hydroxytoluene
DA	Degree of anisotropy
DHT	Dehydrothermal temperature
ECM	Extracellular matrix
EDC/NHS	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide
FACIT	Fibril associated collagens with interrupted triple helices
FQA	Fibre quality analyser
FTIR	Fourier-transform infrared spectroscopy
GMP	Good manufacturing practices
HA	Hyaluronic acid
HAp	Hydroxyapatite
HYPQ	Hydroxyproline quantification
LOX	Lysyl oxidase
MCT	Micro-computed tomography
MIL	Mean intercept length
MROI	Multi-region of interest
MTG	Microbial transglutaminase
OTR	Oxygen transmission rate
PDMS	Polydimethylsiloxane
PET	Polyethylene terephthalate
PLA	Poly-lactic acid
PLGA	Poly(lactic-co-glycolic acid)
PU	Polyurethane
ROI	Region of interest
SAXS	Small angle X-ray scattering
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SVD	Star volume distribution
TEA	Tensile energy absorption
TGA	Thermogravimetric analysis
TIE	Transport of intensity
UV	Ultraviolet

Publications

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Chapter 1. Introduction

1.1 Background

New Zealand is known for its agricultural commodities such as dairy products, meat, forest products, fruit and vegetables, and wine [1]. One industry that has been a significant contributor to the New Zealand trade is the meat industry. This industry generates NZD 12 billion in income annually for the country [2, 3]. It is also the second largest goods export in New Zealand, where according to the Beef + Lamb NZ 2024/2025 forecast, 95% of the sheep meat produced is exported [3-5]. The total value export from beef and sheep meat generated NZD 10.6 billion for New Zealand in 2024/2025 production year [6]. These animals are processed before being exported and only a third of a whole animal is meat and the remainder are considered by-products and waste [7, 8].

The environmental impact of raising animals for meat and the production process are widely studied [9-11]. Raising animals requires natural resources such as water, energy, and land and consequently impacts the air, water, and soil. From emissions released to the environment to the consumption of resources in the production process [9]. According to Roos et al [12], 75-90% of the energy consumed by livestock animals is spent on body maintenance or lost in manure and by-products such as bones and skin. Moreover, disposing the skin and other by-products not only a loss in potential source of revenue but also the regulatory disposal process is an additional expense [13]. Therefore, it is imperative to optimize the use of the majority if not the whole animal that was raised for meat production.

By-products like head, liver, lungs, tongue, tail and blood are consumed in certain countries and have been used in various applications such as an additive, emulsifier and nutritional enrichment in food [13]. One of the by-products in meat production is animal skin [14]. A significant portion of the weight of the animal is skin, contributing between 4% to 15% of its weight, which is consequently one of the most valuable by-products [13, 15]. Although some skins are considered edible and have a small niche in the meat market, such as chicken and pig skin, skins from cows, sheep and goat, however edible, are not widely consumed. Fortunately, they are used in the leather industry. The majority of leather is made from cow, sheep, goat and pig [16]. However, as the fashion industry

shifts to the fast fashion trend where consumers have acquired a disposable mentality, the rise of aesthetically similar synthetic leather is now preferred, which is also significantly cheaper. New Zealand meat production has been dominated by both beef and sheep meat. Zooming into the by-product specifically the hide/skin, RNZ reported in 2019 the cattle hide market was oversupplied that significantly lowered the market value of leather [17]. Similarly, Meat and Livestock Australia has reported in 2019 that sheepskin is lower in value due to poor demand, and small or damaged sheepskin have no value in the market and are disposed [18].

Alternatives in utilising by-products from meat production such as skin and hide have been of research interest. One of the most abundant protein components in animals is collagen. It is a structural protein that forms extracellular matrix of various connective tissues such as the skin, bones, cartilage and tendons [19]. Recent rise of collagen study has led to utilising animal production by-products from cow [20-26], pig [27-36], goat [37-41], sheep [42, 43], chicken [28, 44-47] as well as marine animals [19, 48-53], covering not only skin, hides and scales, but also other parts of these animals [54-56].

In a recent review on collagen extraction from animal skin that includes all five most consumed animals mentioned, sheep skin is the least studied [57]. Very few studies have been done on collagen extraction from sheep skin as a direct by-product of the meat industry. Furthermore, disclosed yields have also been low compared to collagen extracted from other livestock animals. Previous studies have shown that different animals have different skin chemical composition and conformation [15, 58, 59]. However, it has also been observed in previous study that similar methods are applied for different species. Despite effectively extracting collagen, the efficiencies vary widely and is particularly low in sheepskin, and collagen content of their respective extracts have not been reported.

Extracted collagen is then further processed and used in a spectrum of applications. Collagen has been used in cosmetics [60, 61], yarn production [62], medicine [63] and many more. Collagen-based composite materials have been of research interest particularly in producing alternative materials for packaging [31, 64] and biomedical applications [65]. This variety of application of collagen is due to its ability to form fibres that can provide extra strength and stability by self-aggregation and cross-linking [66].

1.2 Aims and objectives

In this research, there are two research areas that will be explored (1) is to explore and shed light on collagen extraction from New Zealand sheepskin and (2) to develop novel collagen-based composite materials that could potentially further broaden the applications of collagen-based composites. The aim is to extract, characterise and apply collagen and gelatin from waste sheepskin as a sustainable coating for wood pulp fibre sheets.

The objectives for this project were:

Collagen extraction and characterisation

- (1) Develop extraction methods for collagen from sheepskin and evaluate its yield and composition.
- (2) Characterise the structural and functional properties of extracted collagen from different extraction methods.
- (3) Assess the influence of extraction parameters on the collagen yield and quality.

Composite fabrication and analysis

- (4) Fabricate plain fibre sheets and establish their baseline mechanical, thermal, and surface properties.
- (5) Apply collagen coatings and investigate effects on the fibre sheet performance.
- (6) Assess the influence of coating concentration on composite properties.
- (7) Compare collagen-coated sheets with gelatin-coated sheets, examining structural, mechanical, and barrier differences.
- (8) Critically evaluate the potential of sheepskin-derived collagen as a sustainable coating material for fibre-based composites.

1.3 Thesis overview

The thesis proceeds as follows:

- **Chapter 2** presents a literature review on collagen and its extraction from livestock skins, highlighting species-specific properties, pre-treatment requirements, and existing gaps in knowledge.

- **Chapter 3** extends the review to composite materials, including other collagen sources, crosslinkers, additives, and fabrication techniques, and applications in packaging and biomedical fields.
- **Chapter 4** compares extraction methods (acid, acid-enzyme, and alkali) for sheepskin collagen, assessing yield, purity, and structural integrity through FTIR, SDS-PAGE, SEM and SAXS.
- **Chapter 5** explores optimisation of acid-enzyme extraction. Including raw versus pickled sheepskins, extraction schedules, and parameter adjustment, revealing strategies to maximise yield and molecular weight retention.
- **Chapter 6** investigates the application of extracted collagen as a coating on cellulose fibre sheets, evaluating mechanical, thermal, surface, and barrier properties relative to different grades of gelatin.
- **Chapter 7** employs synchrotron micro-computed tomography (micro-CT) and advanced image analysis to characterise internal structure, porosity, fibre alignment, and coating distribution, linking structural insights to its functional performance.

These chapters provide a comprehensive framework demonstrating how low-value sheepskin can be upcycled into functional, sustainable collagen-based materials, addressing both resource efficiency and material innovation in New Zealand.

Chapter 2. Literature Review 1: Collagen

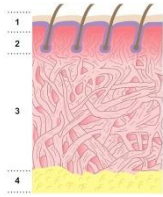
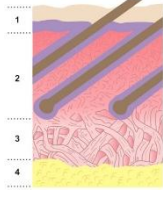
This chapter reviews collagen and its extraction from animal skin, with particular emphasis on livestock-derived sources and sheepskin characteristics. The chapter discusses skin structure, collagen composition and organisation, extraction methodologies, and factors influencing collagen recovery and quality. It also highlights current challenges and gaps in knowledge relevant to the extraction and utilisation of collagen from sheepskin.

2.1 Skin and sheepskin characteristics

Skins serve as a physical protective barrier [58]. The skin thickness depends on the species, age, sex and the area of the body. As illustrated in figure 1, it has three major layers: epidermis, dermis, and hypodermis. Furthermore, hide chemical composition differs depending on the animal type, age, sex, fat content and if the hide has undergone treatment. Hides are typically low in fat and high in protein, which is mostly collagen. In addition, hair follicles are in the dermis layer [58]. Collagen fibres form close to the surface and tend to be coarser towards the centre of the skin [15]. The upper part of the dermis composed of sebaceous glands, erectile follicle smooth muscles, elastic reticulum, and collagenous fibres. This part of the dermis is the grain layer and constitutes to 10% of the skin, which largely contributes to the elasticity of the skin and less on the strength. After this part of the dermis are bundles of interwoven collagen, which largely contributes to its strength. On the other hand, hair mainly contains keratin that accounts for 6-10% of the hide protein [15]. The third layer of the skin is the hypodermis, which stores energy, protects the skin, and enables the skin and the structure underneath to slide to facilitate movement [67].

As previously mentioned, skin differs depending on the animal species. Therefore, sheepskin is different from other animal skin. Table 1 compares cattle, sheep, pig, and goat skins.

Table 1 Cattle, sheep, pig, and goat skin histology, skin weight percentage, fat and protein content, skin thickness, and grain layer.

Animal and skin histology [68]	Skin (%)	Fat (%)	Protein (%) (collagen, keratin, elastin, reticulin)	Thickness (mm)	Grain layer thickness (ratio relative to thickness)
Cattle 	5.1 – 8.5 [13]	3.1 [15]	36 [15]	4 – 8 [59]	1/6 [59]
Sheep 	11.0 – 11.7 [13]	30 – 50 [15]	-	2 – 3 [59]	≈1/2 [59]
Pig 	3.0 – 8.0 [13]	30 – 50 [15]	14[15]	≈2 [69]	No distinct grain layer [59]
Goat 	~9.0 [70]	3 – 10[15]	31 [37]	1 – 3 [59]	1/3 [59]

There are 28 sheep breeds in New Zealand [71]. In table 2, the skin structure differs depending on the type of sheep [59]. In addition, sheep skin structure is affected by the density and texture of wool fibres. It restricts dermis fibre in the grain layer to interweave, which can result to loose contact between the grain layer and deeper dermis layer. Furthermore, fat is stored between the grain and the dermis specifically in sheep skin. Dermis fibres in sheepskins are fine and loosely interwoven compared to goat and calf skin [59].

Table 2 Properties and characteristics of different types of sheep.

Sheep type	Thickness (mm)	Area (m ²)	Characteristics	Use
Hair sheep	0.8	0.4 – 0.5	Fine dermis fibres, fairly compact. Close contact between grain and dermis layers	Gloving
Wool-bearing sheep			Grain layer is thick relative to the total thickness of skin to accommodate wool.	-
Coarse-woolled domestic hill sheep	2 – 3	0.5 – 0.6	Lower wool density. Unlikely to have loose grain and dermis layers contact.	Grain or nappa clothing leather
Fine-woolled sheep			-	Woolskin clothing

2.2 Collagen

One of the most abundant sources of collagen is the skin. There are 29 different types of collagen [72]. There are three types found in the skin, type I, III and IV. Type I is the most abundant one present. It contributes to 90% of the collagen in the skin [72].

The following section of the literature review has been published as an academic journal article in the MDPI Biology:

Matinong, A.M.E., et al. Collagen Extraction from Animal Skin. Biology, 2022. 11, DOI: 10.3390/biology11060905.

Collagen extraction from animal skin

2.2.1 Abstract

Collagen is the most abundant structural protein in animals. It is the major component of skin. It finds uses in cosmetics, medicine, yarn production and packaging. This paper reviews the extraction of collagen from hides of most consumed animals for meat with

the focus on literature published since 2000. The different pre-treatment and extraction techniques that have been investigated for producing collagen from animal skins are reviewed. Pre-treatment by enzymatic, acid or alkaline methods have been used. Extraction by chemical hydrolysis, salt solubilization, enzymatic hydrolysis, ultrasound assisted extraction and other methods are described. Post-extraction purification methods are also explained. This compilation will be useful for anyone wishing to use collagen as a resource and wanting to further improve the extraction and purification methods.

2.2.2 Introduction

Animal hides have been used in diverse applications since prehistoric times. Hide or skin contributes between 3% and 12% (Table 1) to the weight of a live mammal and may reach up to 20% in poultry (Table 1) [13]. Hides are mostly a byproduct of meat production [73-75]. As meat for human consumption is mostly bovine (beef), porcine (pork), ovine (lamb), hircine (goat) and galline (chicken) [76-78], hides of these animals are most readily available. Hides are generally a low value byproduct of meat production, and for some animals, for example sheep, typically go to landfill. Hides have the potential to be used for adding value to meat production operations [79]. Increasing global consumption of meat (Figure 1) inevitably means an increasing supply of hides [78]. These hides need to be better utilised also to minimize environmental impact [78, 80].

Among other applications, hides are used: as a source of the protein keratin for extraction and incorporation in animal feed [81], fertilizers [82], cosmetics [83] and food packaging [84]. In addition, gelatine, a food additive and packaging material [85, 86], is produced from hides. Further, hides are a major source of collagen for use in cosmetics [60], medicine [61, 63], yarn production [62] and packaging [31, 60].

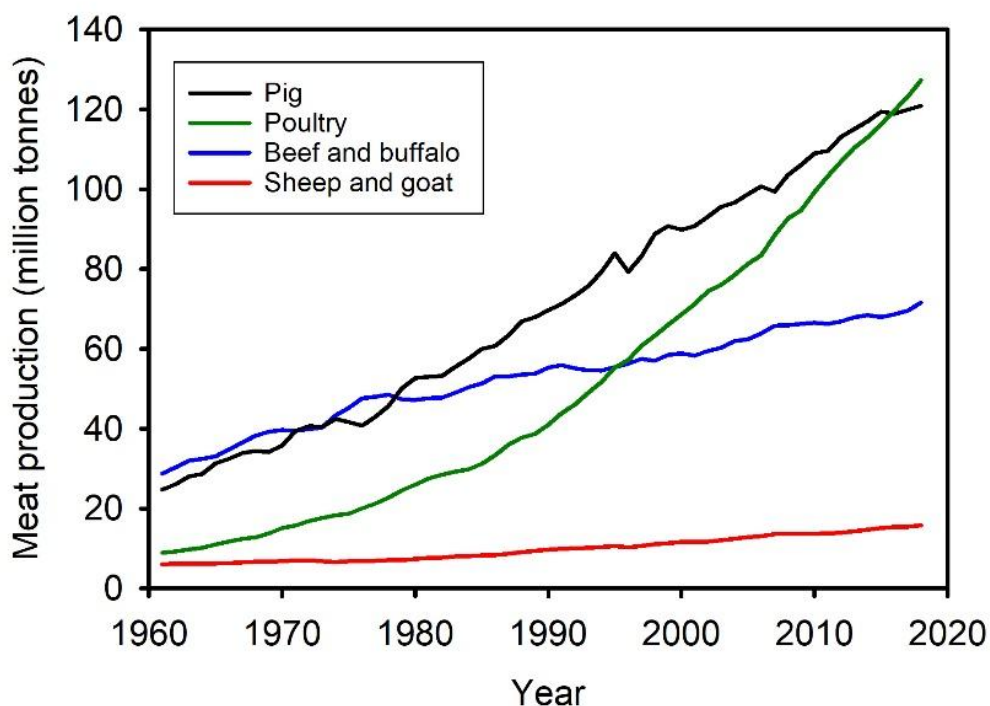


Figure 1 World meat production (1961-2020). Data from Ritchie and Roser [78, 80]

This paper reviews the extraction of collagen from hides of the most commonly consumed animals as listed in Table 3. The focus is on the literature published since 2000.

Table 3 Skin as percentage of total body mass.

Animal	Skin (% wt/wt)	Reference
Cattle	5.1-8.5	
Sheep	11.0-11.7	[13]
Pig	3.0-8.0	
Goat	~9.0	[70]
Chicken	8.0-20.0	[87]

2.2.3 Collagen

Collagen is the most abundant structural protein in all animals [88]. It is a component of the extracellular matrix (ECM) of various connective tissues such as skin, bones, cartilage and tendons [19]. Naturally synthesized collagen molecules consist of three long helicoidal chains of amino acid residues with nonhelical terminals at both ends [19]

(Figure 2). At least 46 unique polypeptide chains have been found in collagens of various animals [88].

Collagen chains most commonly consist of the repeating motif Gly-X-Y where Gly is the amino acid glycine while X and Y are generally the amino acids proline and 4-hydroxyproline, respectively [88]. This motif is distinct from the other ECM components [88-90]. The α -chains of different types of collagens vary in composition, depending on the frequency of repetition and the length of the segment containing the Gly-X-Y motif, with or without interruptions, and the amino acid residues that occur in positions X and Y [91, 92].

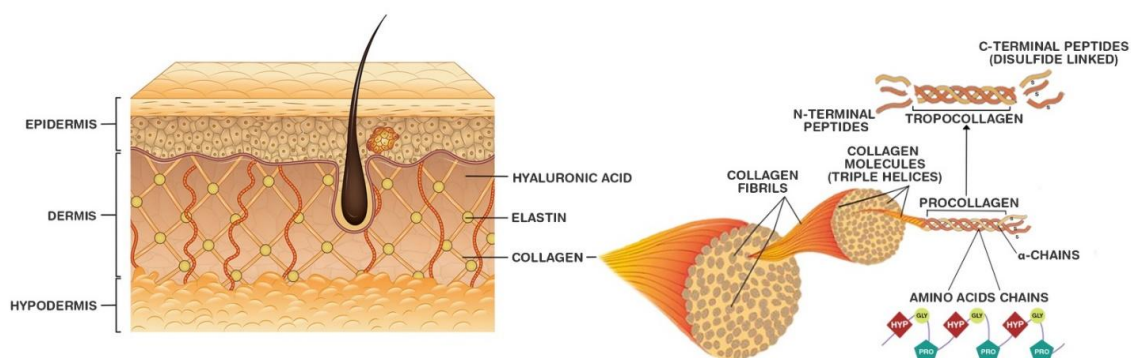


Figure 2 Skin-collagen-molecular structure. Adapted with permissions from: (1) Tang et al., Journal of Voice, published by Elsevier Inc., 2017; (2) Reilly and Lozano, Plastic and Aesthetic Research, published by OAE Publishing Inc., 2021 (©2021 MINERVA Research Labs Ltd. (106 New Bond St. London UK) all rights reserved) [93, 94].

The arrangement of the polypeptide chains and the variation of the terminals gives distinction to the types of collagens, both fibrillar and non-fibrillar. Collagen types vary in conformations resulting in different lengths of helices and distributions of non-helical segments. These criteria are used to group collagens into several groups. General groups include fibrillar collagens, FACIT (fibril associated collagens with interrupted triple helices), FACIT-like collagen, basement membrane collagen, beaded filament collagen, transmembrane collagen, short-chain collagen, and unclassified collagen [95, 96]. At least 29 types of collagens are currently recognized [91, 95]. Fibrillar collagens are the most abundant ECM proteins in vertebrates, providing stability, connectivity and form to tissues and organs [96, 97]. The most abundant fibrillar collagen in most tissues is type I collagen [98]. It is primarily present in fibril surfaces and connective tissues of the skin

and bones [99]. Collagen type I has a rod like structure made of three helically coiled chains. It has a molecular weight of approximately 300 kDa, with a length of 280 nm, a diameter of 1.4 nm and contains about 1020 amino acid residues per chain [91, 100, 101].

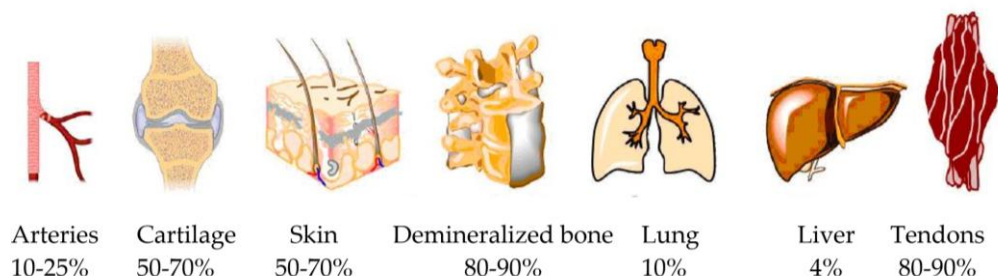


Figure 3 Collagen content of different body parts of animals. Reproduced with permission from Jafari et al., *Polymers*, 2020; 12(10):2230. ©2020 MDPI [19].

Collagen is widespread in mammal tissues (Figure 3) [19, 102] and therefore can be sourced from various abattoir by-products including hides [102]. The type of collagen depends on the tissue type. Fibrillar collagens associated with different tissue types are shown in Table 2 [96, 103]. Biocompatibility, biodegradability, and low antigenicity of collagen make it an attractive material for various applications [60].

Table 4 Collagen types, compositions and sources (based on [103, 104])

Type	Molecule	Source
I	$[\alpha 1(I)]_2[\alpha 2(I)]$	Skin, tendon, bone, ligaments, interstitial tissues.
II	$[\alpha 1(II)]_3$	Intervertebral disc, cartilage, vitreous humor
III	$[\alpha 1(III)]_3$	Cardiovascular vessel, uterus, skin, muscle.
V	$[\alpha 1(V)][\alpha 2(V)][\alpha 3(V)]$	Similar to type I, also cell cultures, fetal tissues; associates with type I.
XI	$[\alpha 1(XI)][\alpha 2(XI)][\alpha 3(XI)]$	Cartilage, in vertebral cartilage and bone enamel.

2.3 Collagen Extraction Process

The collagen extraction process depends on the source material. The aim is to remove all the non-collagenous matter and recover collagen as the final product. The recovery process typically involves pre-treatment of the source tissue, collagen extraction, and

further purification [105]. For recovery from skin, the pre-treatment steps are generally preceded by washing the skin by soaking in cold water for a few days while replacing the water every few hours. This is followed by cutting the skin into more manageable pieces. Typically, skin is cut into small 1 cm² pieces [106].

2.3.1 Pre-treatment

Pre-treatment is intended to break the covalent intermolecular crosslinks between collagen molecules. In vivo, these crosslinks facilitate collagen's structural and mechanical functions in tissues and organs [97]. These crosslinks breakdown very slowly even in boiling water. Various mild chemical treatments are used for breaking the covalent links [101]. Dilute acids and alkalis are typically used for partial hydrolysis of the collagen. This cleaves the crosslinks, while leaving the collagen chains intact [107]. Certain enzymes may also be used for the pre-treatment step [101].

Acid pre-treatment

For acid pre-treatment, washed and chopped skin pieces are immersed in dilute acid at a controlled temperature. The acid permeates the skin causing it to swell to between two- to three-times its initial volume and hydrolyses the crosslinks [102, 108, 109]. Acid pre-treatment is suitable for relatively fragile skins [109] that have a lower degree of fibre intertwinement, such as porcine and fish skins [102].

Alkaline pre-treatment

Dilute alkalis such as sodium hydroxide (NaOH) and calcium hydroxide (Ca(OH)₂) are used for pretreatment. The duration of pre-treatment depends on the thickness of the material being treated [108]. Alkalis are particularly effective in extracting collagen from thick and hard materials [79, 102]. Pretreatment with NaOH may take from a few days to weeks to complete [107]. The lengthy treatment notwithstanding, alkaline treatment with NaOH is often preferred as it swells the skin substantially ensuring diffusion of the alkali deep into the tissue matrix [110]. Alkalis hydrolyse the unwanted non-collagenous proteins, lipids, pigments, and other organic material [111]. The treatment conditions such as the temperature, duration and the concentration of alkali significantly impact the efficacy of removal of the unwanted non-collagen material [112]. A NaOH concentration ranging from 0.05 to 0.10 kmol m⁻³ is sufficient for the pre-treatment [102]. Within this

concentration range, much of the acid soluble collagen and its native structure are retained in the tissue so long as the treatment temperature is between 4 and 20 °C. A high concentration of NaOH (e.g. $\geq 0.2 \text{ kmol m}^{-3}$) can lead to a substantial loss of acid soluble collagen. A 0.5 M NaOH concentration may degrade the native structure of acid soluble collagen [102].

Skin-specific pretreatments

The source, or the nature of the skin, may necessitate skin-specific pretreatments including soaking, fleshing, dehairing, and cutting [105]. For example, skins with fur and feathers may require different pretreatments.

Mechanical slicing may be used to remove some of the fat adhering to the hide [27, 31, 35, 37, 113]. Alcohols such as butyl alcohol may be used to solubilize fat and pigments from chicken [55,56] and bovine skins [22, 23, 111, 112]. Other pre-treatment steps may use heating [44, 47], detergents [28, 47], solvents such as petroleum ether [30, 114], hexane [29], and hot water rinses [36]. A demineralization step may involve the use of the chelating agents such as ethylene diamine tetra acetic acid (EDTA) [42, 115].

2.3.2 Extraction

Conventional extraction methods have typically relied on chemical hydrolysis using acid, alkali or salt solubilization. In some cases, the chemical extraction may be aided by ultrasound or microwaves, and enzymes [49]. Extraction temperature is controlled at a relatively low value (4 °C) to minimize degradation of collagen [111].

Extraction methods can be tailored depending on the desired yield and the properties of the final product. Collagen properties such as the average length of the polypeptide chains, the solubility, the solution viscosity, thermal stability, emulsifying capacity, and water retention are affected by the specifics of the extraction method [49, 113]. In addition, the specific processing conditions, including the pre-treatment used, the storage conditions for the hides, and their specifics (animal type, age and gender) influence the quality of the extracted collagen [91]. For example, breed and age of poultry affects the characteristics of the collagen extracted [56,61].

Acid hydrolysis

Collagen is most commonly extracted from skins by a hydrolysis treatment involving either acids or alkalis. Both inorganic and organic acids can effectively cleave bonds in collagen to enable extraction of the fibrils [116]. Under acidic conditions the collagen molecules have a net positive charge and the resulting electrostatic repulsive force between them facilitates molecular separation [111]. The organic acids commonly used are acetic, chloroacetic, citric and lactic acids. Acetic acid has been widely reported for collagen extraction [16,48,50,54,55,56,59,71,73]. Among inorganic acids, the commonly used ones include hydrochloric, sulfuric and nitric acids [51, 79, 102, 117]. Organic acids are apparently more effective for cleaving collagen crosslinks, and result in higher extraction yield [51, 60, 118] compared to mineral acids. Organic acids also solubilize non-crosslinked collagens [117]. For extraction with acetic acid, a typical concentration is 0.5 kmol m^{-3} with contact time of 24–72 h and continuous stirring [119].

Alkali hydrolysis

Alkaline hydrolysis of pretreated skin may also be used to extract collagen. Aqueous sodium hydroxide and potassium hydroxide are the commonly used alkalis [111]. Aqueous calcium oxide, calcium hydroxide, and sodium carbonate are also used as extractants [120]. Alkalis have a strong tendency to hydrolyse collagen fibrils [116]. This offers an extreme extraction condition and for this reason amino acids like cysteine, histidine, serine, and threonine may be destroyed in the process [111, 120]. The use of alkali hydrolysis appears to be mostly confined to extracting collagen from leather processing waste [116, 121], however, a collagen-based flame retardant has been produced from collagen obtained by alkaline extraction of otherwise untreated cattle skin [20].

Salt solubilization

Solubilization with salts is used less commonly [102, 111]. Solutions of neutral salts are effective in solubilizing collagen and are commonly used in extraction. Examples of the salts used are citrates, phosphates, sodium chloride, and Tris-HCl [102]. Salt solubilization has been used in collagen being extracted. Collagen type I dissolves at salt concentrations of $<1.0 \text{ kmol m}^{-3}$ but precipitates at concentrations exceeding 1.0 kmol m^{-3} .

m^{-3} [116]. This limitation on salt concentration requires careful control when extracting with salts compared to alkaline and acid hydrolysis [49].

Enzyme hydrolysis

Enzyme hydrolysis has been developed to address some of the shortcomings of the more traditional methods [49]. Enzyme hydrolysis may be used in combination with some of the traditional chemical methods [116]. Enzyme hydrolysis offers better reaction selectivity and is less damaging to collagen. Therefore, it has the potential to maximize collagen yield and purity of the extracted product [116]. Enzymes tend to be much more expensive than acids, alkalis and salts, but they can be used under mild reaction conditions. Compared to chemicals, enzymatic treatment is less corrosive to processing equipment, consumes less energy, produces less waste, allows better control of degree of hydrolysis, and the final hydrolysate has a lower salt content [49, 116]. Hybrid methods involving chemicals as well as enzymes have been reported [116].

Proteolytic enzymes are used in collagen extraction. These enzymes may be of animal origin (e.g. trypsin, pepsin), plant origin (e.g. bromelain, papain, ficin), or microbially produced single or mixed enzymes (e.g. collagenase, proteinase K, Alcalase®, Nutrase®, Flavourzyme®, and Protamex®). Pepsin from animal sources is most widely used in collagen extraction [60, 79, 111]. Pepsin, trypsin and papain only act on the non-helix portion of the peptide chain of collagen (the ends) and leave the structurally important helical portion intact [116]. Also, the extracted pepsin soluble collagen generally has a higher purity as the non-collagenous proteins are efficiently hydrolysed by the enzyme. Pepsin treatment also increases acid solubility of collagen, which increases the extraction efficiency if used in combination with acid extraction. The yield of pepsin soluble collagen is significantly affected by the degree of crosslinking in the telopeptide region of the peptides [49]. Plant enzymes such as papain also allows good control of the extent of hydrolysis of the protein substrate [102].

A two-step extraction process without a preceding treatment has been reported for recovery of collagen from sheepskin; an enzymatic step was used first and this was followed by acid hydrolysis [43]. Another hybrid extraction procedure using acetic acid in combination with pepsin has been described by several authors [22, 23, 25-27, 33, 37, 40, 42, 46, 47].

Ultrasound-assisted extraction

Application of ultrasound (frequency of ≥ 20 kHz) during collagen extraction improves yield and reduces the time required for extraction [122]. Ultrasound generates intense turbulence in a liquid resulting in enhancement of mass transfer and rates of chemical reactions [102, 123]. In ultrasound-assisted acid extraction of collagen from sea bass skin the structure of the extracted collagen remained undamaged [124], although sufficiently intense ultrasonic treatment has the potential to damage protein structure. The collagen yield is influenced by the amplitude of ultrasound and the duration of the treatment. A higher amplitude has been found to shorten extraction time and enhance yield [49]. Ultrasound assistance has been found to damage collagen structure during alkali treatment but improve yield and collagen purity (and reduce extraction time) with enzyme treatment. In ultrasound-assisted alkali treatment for extraction of collagen from pigskin, damage to the triple helix structure of collagen was reported [114]. In ultrasound-mediated pepsin-based extraction of collagen from cattle tendon, extraction efficiency and the collagen quality were enhanced compared to a process involving only the enzyme [119]. Use of sonication in combination with enzymes increases collagen yield and shortens extraction time [125]. Ultrasound treatment is generally simple, reduces the need for corrosive chemicals, and can be carried out economically and safely. Damage to the structure of collagen has been observed in some cases [114]. Damage may be minimized by optimization of the treatment (ultrasound amplitude, duration, concentration of enzyme or other chemical, temperature) for the specific collagen source [102, 111, 125].

Other extraction methods

Microwave-assisted. Microwave-assisted extraction has been extensively described. Microwaves, a form of electromagnetic radiation used in microwave cooking, disrupt cell and tissue structure [126, 127], facilitating extraction. Microwaves penetrate deeply in tissue [127]. Microwave assistance has been found to speed up acid and enzyme action compared to equivalent treatments without the microwaves [126].

Mechanical agitation. Tissue solubilization processes involving acids, alkalis and enzymes are generally enhanced by mechanical agitation. Agitation improves hydrolysis by improving mass transfer of enzymes and chemicals into tissue. Thus, it shortens

extraction time or enhances collagen yield in a given extraction time [128]. Agitation-assisted hydrolysis for collagen recovery from jellyfish tissue was shown to enhance yield by at least 5-fold compared to hydrolysis under static conditions using acid, or enzymes alone [129]. Similar observations were reported by others [42].

Precipitation of solubilized collagen

Once the collagen has been solubilized by the methods described above, it needs to be precipitated from solution. Salts are most often used to achieve this, with a salt concentration exceeding 1.0 kmol m^{-3} . This treatment has been used for collagen from chicken skin [28, 46, 47, 53], sheep and goatskin [37, 38, 40, 42], pigskin [33, 130], and cattle skin [21, 22].

2.3.3 Post extraction: Purification

In addition to collagen, a crude collagen extract typically contains neutral salts and non-collagen proteins [105]. Collagen can be purified from this extract to obtain collagen fractions with different molecular weights. Purification processes use multiple steps [131] which may include filtration and centrifugation [85]. Target protein, or unwanted proteins, may be precipitated from solution by “salting out” (adding a high concentration of salt to extract).

Salting-out of collagen, or other proteins, in a crude extract is influenced by several factors, including temperature, pH, the ionic strength, the salt used, and the specifics of the proteins involved. Generally, collagen is less soluble than the contaminating proteins and may be precipitated by salting out. Salting out conditions must be selected to minimize coprecipitation of the other proteins.

Lyotropic series can be used as a guide in selecting salts for salting-out [105, 132]. Both anions and cations in a salt are ranked in the order of their salting-out ability [105].

Anions are more efficient in salting out than cations. For anions, the salting out efficiency decreases in the following order: $\text{F}^- \approx \text{SO}_4^{2-} > \text{HPO}_4^{2-} > \text{CHCOO}^- > \text{Cl}^- > \text{NO}_3^- > \text{Br}^- > \text{ClO}_3^- > \text{I}^- > \text{ClO}_4^- > \text{SCN}^-$. For cations, the salting out performance decreases in the following order: $\text{NH}_4^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+ > \text{Mg}^{2+} > \text{Ca}^{2+}$. Neutral salts produced by combining a strong anion and a cation from the above series, have a strong ability to precipitate collagen from a solution. Sodium chloride is a neutral salt

with a strong precipitating effect. Although ammonium sulfate is generally the best for salting out proteins, it is not a neutral salt and its constituent ions are often not acceptable in products intended for biomedical applications [105]. Neutral salts have the advantage of not significantly affecting the pH of a solution, as pH is also a factor in salting out.

For precipitation of collagen, a suitable mass of a neutral salt is added to a specific volume of the collagen containing solution [105]. The pH is adjusted to 7 by adding sodium hydroxide. The resulting solution is allowed to stand for 4-12 hours. The collagen precipitate is collected by centrifugation. The recovered precipitate may be dissolved and subjected to a second round of salting-out. This procedure may be repeated twice or more for further purification [105]. Refrigerated centrifuges (4 °C) are typically used in recovering the precipitate.

The precipitated collagen contains a large amount of salt. This is removed by dialysis. Precipitated collagen is placed in a dialysis bag and dialyzed against an acidic solution or deionised water. During dialysis, the dialysate is periodically changed to increase the rate of ion migration [131]. Various dialysis schemes have been reported [22, 102]. A single or multiple dialysis stages may be used. Dialysis may take 4 to 10 days to sufficiently remove the salts [105]. This slow step contributes nearly 50% to the total time required for the entire collagen extraction process [105].

Purification methods may be customized to recover collagen of a certain molecular weight range [49]. Various types of membrane filtrations and chromatographic separations can be used to fine-tune the molecular weight of the collagen product [131, 133].

2.4 Specific extraction methods

The collagen extraction methods that have been described are summarized in Table 5.

Table 5 Pre-treatment, extraction and isolation methods for collagen production from skin.

Pre-treatment	Extraction and isolation	Reference
Chicken		
Hot-water bath (40 and 60 °C, 1 h)	Multi-step extraction (4 °C): (1) Protease inhibitors solution: 1 kmol m ⁻³ NaCl and 50×10 ⁻³ kmol m ⁻³ Tris-HCl with PhCH ₂ SO ₂ F (1×10 ⁻³ kmol m ⁻³), MalNEt (10×10 ⁻³ kmol m ⁻³) and EDTA (20×10 ⁻³ kmol m ⁻³) for 24 h; (2) Ethylene diamine dihydrochloride, 24 h; (3) 0.5 kmol m ⁻³ acetic acid; (4) 0.5 kmol m ⁻³ acetic acid with pepsin (1 mg/mL). Followed by precipitation steps: Ammonium sulfate (25% saturation) for precipitation in between extraction rounds. NaCl (crystals) for collagen type-specific precipitation.	[47]
Non-collagen removal (0.1 kmol m⁻³ NaOH, 6 h); fat removal (10% v/v butyl alcohol, 24 h, 4 °C)	Acid hydrolysis (0.5 kmol m ⁻³ acetic acid, 42 h, 4 °C); precipitation (2 kmol m ⁻³ NaCl); dialysis (water); centrifugation	[53]
Non-collagen removal (0.1 kmol m⁻³ NaOH, 6 h); fat removal (4% detergent,	Acid hydrolysis (0.5 kmol m ⁻³ acetic acid, 3 days at <10 °C); precipitation (2.5 kmol m ⁻³ NaCl and 0.05 kmol	[28]

Triton X-100 and 5% KCl, 12 h)	m^{-3} Tris-(hydroxymethyl)-aminomethane); centrifugation	
Fat and pigment removal (centrifugation)	Acid hydrolysis (0.5 kmol m^{-3} acetic acid, 72 h at 4°C); centrifugation; dialysis (distilled water)	[44]
Non-collagen removal (0.1 kmol m^{-3} NaOH, 24 h at 4°C); fat removal (10% butyl alcohol, 24 h at 4°C)	Acid hydrolysis (0.5 kmol m^{-3} acetic acid, 24 h at 4°C); vacuum (varies depending on breed)	[45]
Fat and pigment removal (centrifugation); non-collagen removal (2 kmol m^{-3} NaOH, 12 h)	Acid hydrolysis (0.5 kmol m^{-3} acetic acid, 24 h at 4°C); precipitation (0.9 kmol m^{-3} NaCl); for acid insoluble collagen: (1) heat soluble collagen (95°C), (2) enzyme hydrolysis (1% w/w pepsin; centrifugation; dialysis (deionized water)	[46]
Sheep		
Conducted at 4°C. Non-collagen removal (0.1 kmol m^{-3} NaOH, 6 h); demineralize (0.5 kmol m^{-3} EDTA-2Na, 48 h)	Acid hydrolysis (0.5 kmol m^{-3} acetic acid, 3 h at 20°C); enzyme addition (pepsin 1 g L^{-1} , 48 h); precipitation (2.6 kmol m^{-3} NaCl); centrifugation; second hydrolysis (1 kmol m^{-3} NaCO_3 , trypsin 1:50 w/v at 60°C from 10 min to 4 h)	[42]
Wash; dehair (deionized water)	Acid-enzyme hybrid (0.5 kmol m^{-3} acetic acid, 0.01 and 0.001 g g^{-1} trypsin, at 20 and 35°C , pH 7 and 9 for 30–360 min); filtration and centrifugation	[43]

Goat		
Non-collagen removal (0.1 kmol m⁻³ NaOH, 0-48 h at 4 °C)	Conducted at 4 °C. Acid-enzyme hybrid (0.5 kmol m ⁻³ acetic acid with 0.1% w/v pepsin, 24 h); precipitation (2.6 kmol m ⁻³ NaCl, 12 h); centrifugation (4500 g, 30 min)	[37]
Non-collagen removal (0.1 kmol m⁻³ NaOH, 24 h at 4 °C)	Acid hydrolysis (0.5 kmol m ⁻³ acetic acid 24-72 h); precipitation (2.6 kmol m ⁻³ NaCl); centrifugation (7000 g); redissolution and dialysis (acetic acid)	[38]
Non-collagen removal (0.1 kmol m⁻³ NaOH, 0-48 h at 4 °C)	Acid-enzyme hybrid (0.5 kmol m ⁻³ acetic acid with 0.1% pepsin, 24-72h at 38 °C); precipitation (2.6 kmol m ⁻³ NaCl); centrifugation (7000 g, 30 min at 4 °C); redissolution and dialysis (acetic acid)	[40]
No pre-treatment detailed.	Enzyme hydrolysis (1 g pepsin in 100 mL buffer (pH 2.0) at 37 °C for 15 min); second enzyme addition (0.1 U pepsin, 1-120 min); neutralization (1 kmol m ⁻³ NaOH); centrifugation (1000 g, 15 min)	[39]
Pig		
Degrease in ultrasonic bath (75% sodium dodecylbenzene (SDBS)), skin to SDBS 1:2.5 volume ratio at 25 °C, 120 W); non-collagen removal (1% NaCl for 6 h)	Conducted at 4 °C. Acid-enzyme hybrid (2000 U g ⁻¹ pepsin in 0.5 kmol m ⁻³ acetic acid, 18 h); precipitation (NaCl, 8-12 h); centrifugation; redissolution and dialysis (both acetic acid, and water for second dialysis)	[27]

No pre-treatment detailed.	Alkaline treatment (3-7% NaOH in 6% NaCl solution, 24 h); neutral salt wash (NaCl); neutralization (7% acetic acid); vacuum freeze dry (75-90% moisture removal)	[130]
Fat removal (1:3 w/v petroleum ether at 30 °C, 1 h)	Enzyme hydrolysis (microfluidizer) (2,400 U g ⁻¹ pepsin (pH 7, 50 °C) and 3,000 U g ⁻¹ Alcalase (pH 8.5, 60 °C)); centrifugation (10,000 g, 4 °C, 20 min); dialysis and freeze dry	[30]
Fat removal (hexane, 60 g skin per 400 mL); drying (24 h at 60 °C, -76 mm Hg)	Hydrolysis with rotary evaporator (125 g L ⁻¹ of acid with pH 3 or alkali with pH 12 at 60°C for 1 h at rotary speed scale 6); centrifuge (8,000 g, 10 min); freeze dry	[29]
Fat removal (petroleum ether); non-collagen removal (1% w/v NaCl, 6 h); alkali pre-treatment (2% g g⁻¹ NaOH with 2:5 g mL⁻¹ ratio, 1 h); ultrasound (in alkali solution, at 25 kHz at 290 W, 40 min)	Maintain basic pH (0.1 kmol m ⁻³ phosphate buffer with pH 8.0); enzyme hydrolysis (Alcalase 1:100 w/v at 55 °C, varying hydrolysis times); centrifugation	[114]
Fat removal (10% Na₂CO₃; hot water bath 45 °C)	pH adjustment (pH 8); enzyme hydrolysis (2 h at 40 °C, enzyme not mentioned); filtration and centrifugation; freeze dry (supernatant)	[31]
In a rotating drum: Wash (0.3% peregral at 30 °C for 3 h); fat removal (manual	Alkali-enzyme hydrolysis (not detailed); freeze dry (-5 °C for 5 h)	[33] Based on [134] and

defleshing; 300% float with 2.5% Na₂CO₃ and 0.5% peregat at 30 °C for 3 h); dehair (2.5% trypsin (250 μ mg⁻¹) coated on flesh side at 25 °C overnight)	(stirred at 4 °C); acid hydrolysis (2 kmol m ⁻³ acetic acid with 1:50 w/v, 6 h) centrifugation (20,000 g, 30 min); neutralization (NaOH addition until pH 7.5); precipitation (1.5 kmol m ⁻³ NaCl, refrigerated desiccator for 12 h); centrifugation; dialysis(water until neutral pH, 30 kDa molecular weight cutoff membrane; freeze dry ; acid-enzyme hybrid (0.5 kmol m ⁻³ acetic acid solution containing 1.5% pepsin with 1:20 w/v ratio for 3 days); centrifugation and precipitation; dialysis (2×); freeze dry	patent CN1569260
Fat and flesh removal (mechanical removal); wash (phosphate-buffered saline)	Decellularized collagen: Supercritical CO ₂ vessel system (75% ethanol, 30-50 °C and 200-350 bar for 40min); neutralization (0.1-1 kmol m ⁻³ NaOH); drying and sterilization (γ-irradiation 25 kGy). Atelocollagen: Milling (freeze milled with liquid nitrogen, 50-200 μm); acid and enzyme digestion (0.01 kmol m ⁻³ HCl containing 1 g L ⁻¹ pepsin, stirred for 16-18 h at 25 °C); filtration (1) 0.1-0.4 μm, (2) >150 kDa, (3) 0.2 μm; fibrillogenesis (3 mg mL ⁻¹ acidic atelocollagen solution with 0.2 kmol m ⁻³ phosphate buffer at a ratio of 9:1 v/v); centrifugation (7,000 g, 30 min at 4 °C); freeze dry (precipitate)	[32]

Hot-water bath (1:9 w/v at 60 °C, 30 min)	Acid/alkali-enzyme hydrolysis ((1) 762 U g ⁻¹ pepsin, (2) trypsin and (3) Alcalase with 1 kmol m ⁻³ HCl or NaOH at their respective optimal temperature and pH, 4 h); inactivate enzyme, neutralization, filtration, dialysis and freeze dry	[34]
Alkali treatment (0.1 kmol m⁻³ NaOH at 1:5 volume ratio, 3 days)	Acid hydrolysis and filtration (0.5 kmol m ⁻³ acetic acid for 3 days, 2x. Filtrate was collected separately); freeze and precipitated (frozen 24 h; added in 0.9 kmol m ⁻³ NaCl for 12h). Centrifugation; dissolution and dialysis (acetic acid; dialysis at 1:10 for 2 days); freeze dry	[35]
Hot-water bath (90 °C, 1 min)	Control mixture (5:100 volume ratio skin to solution (water)); enzyme hydrolysis (1) Alcalase, (2) Flavorzyme, (3) Neutrase, (4) bromeline, (5) Protamex, (6) papain - at 1:100 enzyme substrate ratio with hydrolysis times 1-24 h at optimal temperature; inactivation and cooling; centrifugation (4,000 g, 15 min); filtration (3 kDa, 60 psi nitrogen gas at 20 °C); freeze dry	[36]
Cattle		
No pre-treatment detailed.	Alkali hydrolysis (3-5% NaOH, stirred at 60 °C); filtration (filtrate collected); neutralization (acetic acid); dialysis	[20]

Neutral salt wash (10 volumes (v/w) 0.5 kmol m^{-3} NaCl for 2 h); lime treatment (dehair) (40 g L^{-1} lime suspension at 1:10 w/v for 4 days); non-collagen removal (2% NaOH at 1:10 w/v for 5 days)	Conducted at 4°C , stirred. Acid-enzyme hybrid (0.5 kmol m^{-3} acetic acid at 1:100 w/v with pepsin addition at 1:20 w/w, 2 days); centrifugation (20,000 g, 20 min), [26] precipitation (2 kmol m^{-3} NaCl, 6 h); centrifugation (15 min); dialysis (14 kDa MW, deionized water for 4 days); freeze dry
Hair removal (1) sulfide solution [135]; (2) oxidative process [136]; relime [135]; acid pre-treatment (0.5% acetic acid first at pH 7.1 for 3 h and then at pH 5.4 for 4 h). Neutral salt wash (400% water containing 4.35% NaCl for 2 h, twice); lime treatment (400% water containing 0.3% lime for 2 h, thrice); acetone treatment (400% acetone, 12 h repeated 5-7 times); air dry and frozen; (pulverized)	Conducted at 4°C . Acid hydrolysis (200-250 mg hide powder in 100 mL 0.5 kmol m^{-3} acetic acid, 3 days); [41] centrifugation and freeze dry
No pre-treatment detailed.	Skin-buffer mixture (100 g L^{-1} skin to solution, 0.1 kmol m^{-3} phosphate buffer and 0.15 kmol m^{-3} NaCl with pH 7.5); alkali addition ($0.001 \text{ kmol m}^{-3}$ NaOH and 3.5g NaBH ₄ , [21] incubated for 24 h at 25°C); acid addition (glacial acetic acid, adjusting

	pH to 3); centrifugation (23,000 g for 30 min, pellet collected); freeze dry	
Hair removal (0.5 kmol m⁻³ NaOH, 24 h); non-collagen removal (3 different samples prepared: (1) 1:10 w/v 0.1 kmol m⁻³ NaOH , 6 h; (2) 20 vol 0.1 kmol m⁻³ NaOH and 0.1 kmol m⁻³ NaCl, 24 h; (3) 1:20 w/v 0.1 kmol m⁻³ NaOH and 0.1 kmol m⁻³ NaCl, 6h); fat removal/demineralization ((1) 1:10 w/v 10% butyl alcohol, 10 h; (2) 0.1 kmol m⁻³ HCl and 0.1 kmol m⁻³ NaCl 1:20 w/v, 24 h; (MAES) 0.1 kmol m⁻³ HCl and 0.1 kmol m⁻³ NaCl 1:20 w/v, 24 h)	Acid/acid-enzyme hydrolysis: (1) 30 vol. 0.5 kmol m ⁻³ acetic acid, 24 h, (2) 20 vol 0.5 kmol m ⁻³ acetic acid with 1% w/w pepsin, 24 h, (3) 20 vol 0.7 M acetic acid with 1% w/w pepsin, 48 h; filtration (4 mm and 250 µm filter); precipitation: (1) 2.5 kmol m ⁻³ NaCl, (2) 2.5 kmol m ⁻³ NaCl, (3) 2.5 kmol m ⁻³ NaCl; centrifugation (various times); dialysis (14 kDa MW, acetic acid and water); freeze dry (-52°C, 48-72 h)	[22]
Lime treatment (100 g hide in 1.5 g Na₂S and 5 g CaO solution, soaked for 2 days); de-lime treatment (2 g NH₄Cl and 4 mL concentrated HCl); wash (rinsed with water until neutral pH); fat removal (10 volumes 10% butanol for 24 h)	Acid hydrolysis (30 volumes 0.5 kmol m ⁻³ acetic acid solution containing 1% pepsin, 2 days stirred periodically); filtration and centrifugation (2-layer cheesecloth; 10,000 g for 20 min). Precipitation (3 kmol m ⁻³ NaCl); centrifugation and dissolution (acetic acid); dialysis (tris buffer)	[23]

Hot-water bath (70 °C for 15 min, dehair)	Solutions (500 g hide each sample, calcium hydroxide and acetic acid solutions were added to each beaker). Samples and concentrations (1) control not pretreated; (2) 5% w/v $\text{Ca}(\text{OH})_2$; (3) 15% w/v $\text{Ca}(\text{OH})_2$; (4) 5% w/v $\text{Ca}(\text{OH})_2$ and 5% v/v CH_3COOH ; (5) 15% w/v $\text{Ca}(\text{OH})_2$ and 5% v/v CH_3COOH . Hydrolysed for 4 days); hot water bath (60-70 °C for 24 h); filtration and dehydration (filtrate collected, 60 °C oven for 24 h)	[24]
De-lime treatment (2% NH_4Cl and 0.5% HCl, 60 min); neutralization (0.5% HCl until pH 6-7; rinsed with distilled water)	Acid-enzyme hybrid (30 volumes 0.5 kmol m^{-3} acetic acid containing 1% pepsin, 48 h, 4 °C); centrifugation (10,000 g at 4 °C, 15 min); precipitation (3 kmol m^{-3} NaCl); centrifugation; dissolution and precipitation (0.5 kmol m^{-3} acetic acid and 0.7 kmol m^{-3} NaCl); dissolution and dialysis (0.5 kmol m^{-3} and 0.1 kmol m^{-3} acetic acid, respectively)	[25]

The pre-treatments listed in Table 5 usually have the purpose of removing some of the non-collagenous material but also serve the important function of “opening up” the skin to enable easier penetration of the reagents that are used to solubilize collagen. In some cases, the pre-treatment might include similar chemistry to the subsequent treatment, but generally with lower concentrations of reagents. The main options described in Table 5 may be summarized pictorially, as in Figure 4.

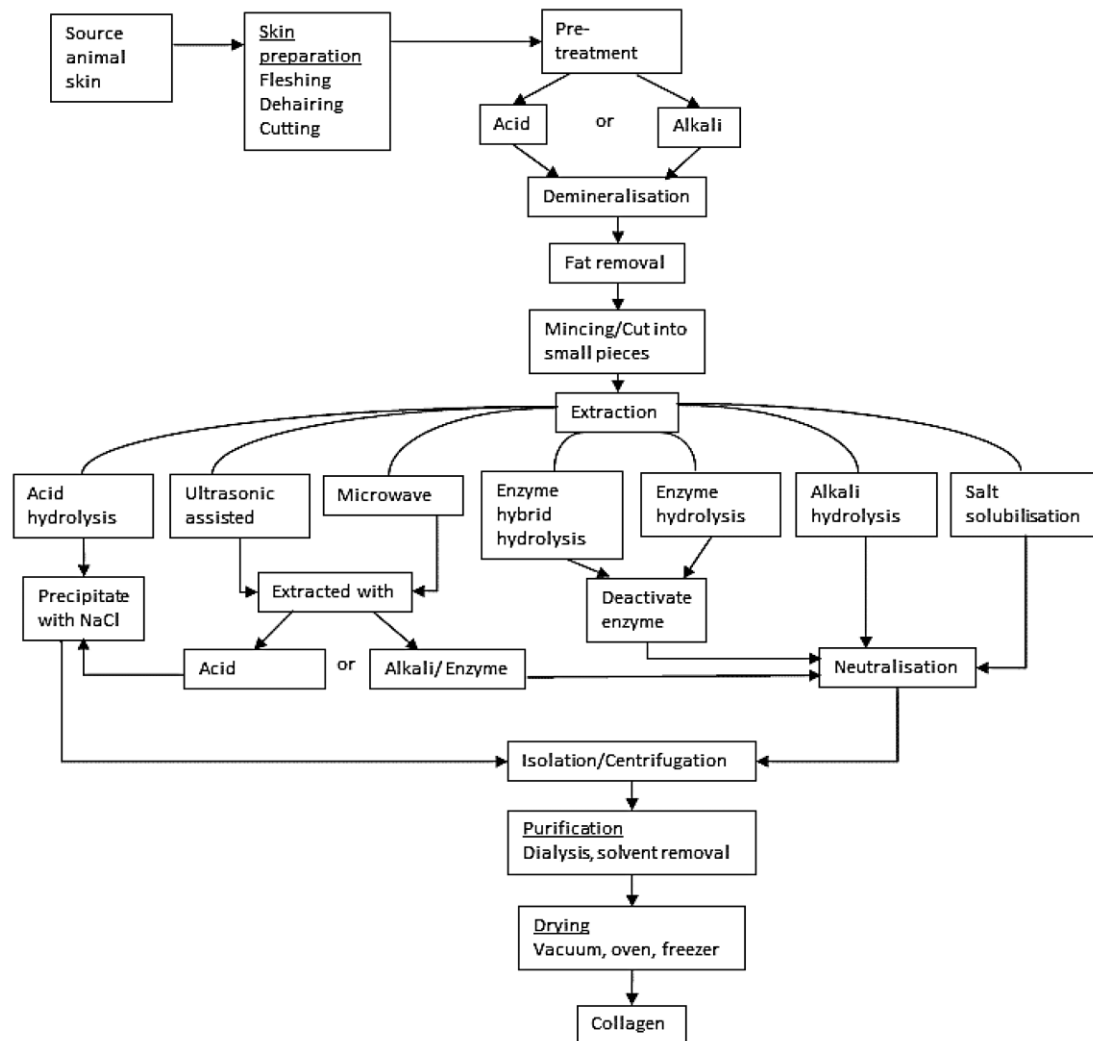


Figure 4 Overview of processing options for extraction of collagen from skin (based on Table 5).

2.5 Future Considerations

While a range of methods for extracting collagen from animal skins has been described, these do not cover the skin from all commercially reared meat animals. Skin from some animal species have not been investigated in detail, notably sheep and goats, which represents an opportunity for further study and exploitation. The nature of the collagen resulting from the extraction processes has often not been well characterised. For example, do the collagen fibrils remain intact, or do they reform spontaneously, or are they degraded partially? Also, what is the impact of these factors on the mechanical properties of materials manufactured using the extracted collagen? This is an area where further knowledge would be useful.

2.6 Conclusions

A wide variety of collagen extraction and separation methods have been described for animal skins from a number of meat-producing species. These have been categorized and summarized here showing the options available to produce a collagen extract. Multiple choices are clearly available for the commercial production of collagen from various animal skins.

Chapter 3. Literature Review 2: Collagen matrix composites

3.1 Introduction

The previous chapter reviewed collagen as a biomaterial, with particular emphasis on skin structure, collagen characteristics, and extraction methods from animal-derived sources. Building on this foundation, this chapter focuses on collagen matrix composites and the strategies used to modify and process collagen for functional applications. Alternative collagen sources, crosslinking methods, additives and coatings, and fabrication techniques are reviewed in relation to their influence on the structural, mechanical, and biological properties of collagen-based composites. Particular attention is given to approaches relevant to biomedical materials and sustainable fibre-based composite applications.

3.2 Collagen sources

Collagen has been sourced from a wide range of animal species using established extraction and purification techniques. The most common sources include the skin of cattle (bovine), pigs (porcine), sheep (ovine), goats (caprine), and chickens (galline). In addition to skin, collagen-rich tissues such as the dermis, tendon, and bone have been widely utilised. Other species, including rats, horses, ducks, birds, and frogs, have also been studied. More recently, marine organisms have gained attention as alternative sources, with collagen isolated from tissues such as sea cucumbers [137], sea pineapples [137], squid [138], mussel byssus [137], fish bones, scales, and skin [139].

Collagen has been used in a number of applications in medicine, cosmetics and the food industry [140]. Although animal-derived collagen remains widely used, several limitations restrict its broader application. These include the risk of allergenicity, infectious agents in vivo, religious and cultural restrictions, and broader ethical concerns [141, 142]. Marine-derived collagen offers some advantages in this regard, as it avoids mammalian proteins such as alpha-gal, which may trigger allergic reactions [143-145]. However, marine collagen exhibits lower thermal stability compared to mammalian-derived collagen, with a reduced denaturation temperature that limits its stability across temperatures ranges [141]. Additionally, animal-derived collagens often display batch-to-batch variation, making Good Manufacturing Practices (GMP) critical not only for preventing

contamination and ensuring quality and purity, but also for ensuring consistency and reproducibility [146-148].

In response to these challenges, recombinant systems are being investigated as alternative collagen production platforms [146, 148-151]. Recombinant human collagen has been expressed in various hosts, including mammalian and insect cells, yeast, bacteria, plants such as tobacco, maize, rice, barley, alfalfa, soybean, and thale cress, and algae such as *Volvox* and *Chlamydomonas reinhardtii*. The enzyme prolyl-4-hydroxylase (P4H) plays a key role in stabilizing collagen by converting proline residues into hydroxyproline, which is essential for triple-helix stability and fibril formation [152]. In plant systems, however, P4H exhibits different substrate preferences, which may reduce hydroxylation efficiency. Co-expression approaches involving human P4H have shown potential in mitigating this limitation. Yeast (*Pichia pastoris*) and *E. coli* have also been investigated as hosts, although they also require P4H activity. The hydroxylation process further depends on cofactors including ferrous iron (Fe^{2+}), α -ketoglutarate, oxygen (O_2), and ascorbate (vitamin C), the latter of which is often supplemented to recombinant systems to facilitate collagen synthesis [153].

Recombinant expression methods allow greater control over collagen properties compared to animal-derived sources [141]. This enables the production of collagen with a defined triple-helical structure, as well as the ability to tailor properties such as concentration, gelling behaviour, viscosity, and shear-thinning characteristics, which are relevant for applications such as composite fabrication and 3D bioprinting. Despite these advantages, challenges remain, including high production costs, relatively low yield, and limited stability under some conditions. Furthermore, while recombinant collagen closely resembles native collagen in structure, its mechanical performance often falls short, requiring additional modifications such as amino acid sequence engineering or crosslinking to enhance functionality [141].

3.3 Crosslinkers

Collagen composites have shown low durability to external factors like enzymes and high temperature, and integration of crosslinkers facilitates both stabilisation and added functionality in collagen composites. Crosslinkers are categorized as chemical and physical. Table 6 summarises the common crosslinkers used for collagen composites.

Table 6 Chemical and physical crosslinkers.

Chemical crosslinkers and key features	Crosslinking reaction mechanism	Function and requirement	Applications
Glutaraldehyde (GA) • Low cost [154] • High solubility in water [154]	Aldehyde groups react with ϵ -amino groups of lysine/hydroxylysine; may also react with histidine/arginine residues [155-158]	Stabilizes collagen against thermal/enzymatic degradation. Requires optimal collagen concentration (too low = weak, too high = inhomogeneous) [159].	Heart valves, vascular grafts, cartilage, tendons, artificial skin [160, 161]
Genipin • Natural, water soluble and less toxic than GA [162] • Antimicrobial, anti-tumour, anti-inflammatory [163] • Resistant to enzymatic degradation [164]	Covalently reacts with primary amines (lysine/hydroxylysine/arginine) via nucleophilic attack on dihydropyran ring $\rightarrow$ stable heterocyclic crosslinks [165]	Stabilizes collagen and increases acid/pepsin resistance. Mechanical properties depend on concentration & incubation time. Cytotoxic if ≥ 5 mM.[166]	Acellular tissues, injectable gels (e.g., spinal cord injury) [164], scleral reinforcement (myopia treatment) [167], delivery systems [162]
Dialdehyde starch (DAS)	Aldehyde groups crosslink free amino groups in collagen $\rightarrow$ DAS	Improves mechanical/thermal properties. Concentration-dependent:	Wound dressings [170], tissue engineering scaffolds

• Biodegradable, low toxicity [168] • Antiviral activity [169], blood compatibility [168]	bridges (intra/intermolecular) [168]	higher content = more rigid, less water uptake. Low content prevents calcification [168].	[171], cardiovascular [172], bone tissue engineering (porous structures) [173]
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (EDC-NHS) • “Zero-length” crosslinker [174] • Biocompatible, non-cytotoxic [175]	Activates –COOH (Asp/Glu) → unstable O-acylisourea intermediate → stabilized by NHS → reacts with –NH ₂ to form amide bonds [175]	Improves thermal/enzymatic stability, enhances fibril alignment, facilitates cell attachment & proliferation [176]. Optimal ratio EDC:NHS is ~1:1 [177].	Collagen–gelatin composites [178], decellularized arteries [179], neuronal, bone & cartilage in tissue engineering (piezoelectric signalling) [180-182]
Chitosan • Natural polysaccharide • Biocompatible, biodegradable [183] • Antibacterial, antifungal, haemostatic [184]	Forms hydrogen bonds + covalent interactions with collagen (via thermal, UV, GA, epoxy, tannic acid) [185-188]. Can bind metal ions via amino groups [183].	Enhances structural/mechanical properties, increases resistance to degradation, modulates pore size & swelling [189-191]. Enhances cell attachment & proliferation (esp. with light treatment) [192].	Biomedical: blood vessels, cartilage, scaffolds [184, 193, 194] Environmental: metal ion removal, beverage clarification [183, 195]
Physical crosslinkers and key features	Crosslinking reaction mechanism	Function and requirement	Applications
Temperature Dehydrothermal or DHT • Non-toxic • Sterilization effect	Heat (>90 °C) + vacuum removes H ₂ O → induces amide crosslinks (intramolecular & between Lys/Ala) [196-198]. Can cause partial denaturation [199].	Increases mechanical strength [200], reduces enzymatic solubility [200]. Exposure time/temp affects denaturation & modulus [196]. More efficient than UV [201].	Fibroblast attachment & proliferation, tissue repair/regeneration, stabilization of marine collagen [202, 203]

Ultra-Violet light (UV) • Non-toxic • Sterilization effect • Fast but shallow penetration [204]	Generates free radicals on tyrosine/phenylalanine → dityrosine crosslinks [205]. Can combine with photoactive reagents or EDC/NHS [206].	Produces moderate crosslink density [207]. Risk of denaturation at high dose [208]. Thin/transparent scaffolds preferred [204].	Ocular treatments (e.g., riboflavin–UVA) [208], surface modification of biomaterials [209]
Enzymatic crosslinking • Biocompatible, preserves bioactivity [210]	LOX: converts lysine/hydroxylysine → aldehydes → react with ϵ -amino groups [210]. MTG: catalyses bond between γ -carboxamide of glutamine & ϵ -amino of lysine → ϵ -(γ -glutamyl) lysine bond [211].	Improves collagen stability, elasticity, and mechanical strength without altering fibril morphology [210, 212, 213].	Food industry (MTG) [214]; Tissue Engineering: bone [215], vascular grafts [216], fibroblasts [217], endothelial cells [218], smooth muscle cells [219]

3.4 Additives and coatings

Other than crosslinkers, collagen can also be combined with other components to make a composite. These additives can be either in the form of additives incorporated in the matrix or a coating on the composite. These are integrated to form the majority of the composite and/or provide properties for the intended applications of the composite materials.

3.4.1 Natural additives and coatings

a) Biopolymers

Biopolymers can be plant-, animal-, and fungal-derived polysaccharides and proteins, and are commonly used in collagen composites. One of the most studied biopolymers is hyaluronic acid (HA). The addition of hyaluronic acid improves the biocompatibility and non-cytotoxic properties of collagen composites [220]. It also has the ability to bind with proteins and cells [221], and allows control of porosity and pore size [222]. The effect of HA is strongly dependent on the molecular weight employed, where low molecular weight HA has been shown to exhibit inflammatory responses that stimulate healing, whereas high molecular weight HA is anti-oedematous and protective against chronic tissue damage [220]. These features make it a desirable additive for composites used in regenerative medicine, supporting tissue regeneration through enhanced cell proliferation [223], wound healing [224], cartilage-like regeneration [225] and bone regeneration [226].

Another biopolymer commonly used in collagen scaffolds is alginate. It offers good bioactivity [227], biocompatibility [228] and haemostatic properties [229]. It also offers matrix stabilisation, which is possible through electrostatic interactions and hydrogen bonding [230]. It has been used in tissue engineering such as artificial organ and tissue production as well as drug delivery systems both in dry delivery system and as cell carriers [231].

Gelatin is a biopolymer derived from partial hydrolysis of collagen. It is a cheaper alternative for collagen and can be extracted in a relatively straightforward process [232-234]. It exhibits biocompatibility [235], antioxidant activity and promotes high alkaline phosphatase expression in dental cell models [236], indicating potential for mineralizing tissues. It also showed reduced swelling capacity of the composite, which is supportive of

applications in wound healing and cell proliferation in dental interfaces [235, 237]. It also showed potential in food packaging applications, which has proven shelf-life extension, barrier properties with regard to gas permeability and contamination [238].

Other organic components that have been combined with collagen composites are cellulose [239], starch derivatives [240], chitosan [241], silk fibroin [242], and elastin [243, 244].

b) Minerals and inorganics

Hydroxyapatite (HAp) is one of the most studied mineral components, which is a natural mineral component of bones. HAp is bioactive, biocompatible, osteoconductive, non-toxic and high histocompatibility [191, 245]. In situ integration of hydroxyapatite include microsphere formation (HAp microspheres dispersion) and precipitation ($\text{Ca}^{2+} + \text{PO}_4^{3-}$) methods [246-251]. Hydroxyapatite can chemically bond with surrounding tissues, making it ideal in certain repair strategies like bone substitute production, overall bone tissue regeneration and bone-marrow stem-cell proliferation and differentiation [245, 252].

Titanium dioxide is another inorganic component that has been studied in collagen composites. It is sought after in scaffolds used in biomedical applications due to its antibacterial activity against *Staphylococcus aureus* reducing infection in wound healing [253]. In addition, it can produce reactive oxygen species that can disrupt bacterial membranes and influence protein expression and gene activity contributing to its anti-microbial efficiency [254, 255]. It is a non-toxic additive that enhances mechanical strength and is resistant to degradation [253, 256]. At the nanoscale, titanium dioxide can provide high surface activity due to large specific surface area, which offers an abundance of reactive sites and stronger interface adhesion [254].

Silica-based bioglass is an inorganic filler used for its bioactivity, biocompatibility and Osteoconductivity [257]. It improves mechanical stability and facilitates cell growth on implants, which underpins its use in bone repair and other regenerative strategies [258].

Another filler is zinc oxide, used for its antibacterial effects that support treatment of burns and wound healing both chronic and diabetic infection wounds [259].

Magnetic particles, particularly iron oxides such as Fe_3O_4 and Fe_2O_3 are another inorganic additive that have been incorporated in collagen composites [260]. It improves cell viability for blended collagen composites and can be used to control alignment and conformation when subjected to a magnetic field. This enhances the directional properties of the composite that supports cell organization and tissue regeneration in bone and nerve engineering applications [260].

Other emerging mineral components integrated in collagen composites are gypsum in building construction specifically building restoration [261], zeolite in bone regeneration in rabbit femurs [262], beta-tricalcium phosphate and calcium carbonate in bone grafting [263, 264].

3.4.2 Synthetic additives and coatings

On the synthetic side, polylactic acid (PLA) is added to collagen composites for its structural stiffness while still maintaining porosity with interconnected pores [265]. Studies have shown that it improves mechanical stability in blended composites [266]. PLA supports functional activities such as cellular attachment and tissue ingrowth. These functionalities have been studied in wound healing, neo-tissue formation, full-thickness wound dressing and vascular grafts [229, 267-269]. Furthermore, it has also been explored for cartilage and bone marrow regeneration due to its strong interactions with chondrocytes for cartilage-tissue applications and low cytotoxicity in bone marrow-derived stem cells, respectively [267-269].

Polyurethane (PU) is another synthetic polymer that demonstrates potential in biocompatibility, stabilizing effect and ability to enhance mechanical (flexibility and high tensile strength) and surface properties of scaffolds [251, 270]. It was found to improve interaction between cells and materials (i.e. between components) [271]. PU addition to collagen composites allows tuneable slow degradation and resists enzymatic breakdown which supports structure during ingrowth required for regeneration. It has been studied for vessel repair nerve regeneration, such as blood vessel repair and peripheral nerve regeneration [247, 251, 272].

3.4.3 Functional additives and coatings

A wide range of functional additives have also been explored to provide antioxidant or antimicrobial activity in collagen/chitosan composites. Antioxidants such as butylated hydroxytoluene (BHT) and α tocopherol have been incorporated to scavenge free radicals and improve oxidative stability [273]. Essential oils, including oregano, rosemary and clove [274, 275], as well as spices such as turmeric [276] and ginger [277, 278], exhibit both antioxidant and antimicrobial activity, and are considered natural functional options. Plant and herb extracts like those from oregano, rosemary or other aromatic sources contribute similar benefits [278-282].

Antimicrobial additives are particularly important. Plant extracts in general [278, 279] and photoactivated chlorophyllins [279] have been reported to provide strong antimicrobial effects through photodynamic activity. Lysozyme, as a natural antimicrobial enzyme, offers protection through bacterial cell wall disruption [283]. Other options include benzoic acid, an aromatic acid preservative with broad antimicrobial activity [284], gelatin with additional antimicrobial contributions [275], and chitosan itself, which has been thoroughly studied for antibacterial efficacy [278, 285]. Collectively, these additives not only extend material stability but also transform collagen–chitosan and other polymeric matrices into multifunctional biomaterials with antimicrobial and antioxidant activities critical for wound healing and tissue engineering applications.

Functional additives have also been used in collagen composites used in packaging applications for food quality preservation, extending shelf life and preventing risks of foodborne illnesses [286]. Inorganic and metallic components zinc oxide and titanium dioxide, discussed earlier, alongside silver nanoparticles, have also been used for their antimicrobial properties in collagen composites [287].

3.5 Composite forming methods

3.5.1 Self-assembly

Collagen fibrillogenesis can occur spontaneously under physiological conditions, enabling the fabrication of composites with minimal external intervention. This mechanism is used in mineralized collagen composites and hydrogels, which mimic the native extracellular matrix and provide a biologically relevant nanostructure [288]. The process

leverages the inherent tendency of collagen molecules to align and form fibrillar structures, making it a useful strategy for scaffolds requiring natural-like organization without extensive processing.

3.5.2 Solvent Casting / Evaporative Methods

In this approach, collagen is dissolved in acidic conditions, combined with fillers or polymers, and crosslinked before drying [289]. Evaporation at controlled temperature and humidity consolidates the scaffold and removes the solvent. For example, a collagen–cellulose composite was prepared by drying at 37 °C and 50% humidity. The method is straightforward and scalable, but final structural and mechanical properties depend heavily on precise drying conditions and the need for post-treatment crosslinking. The resulting film was investigated for its potential for biomedical applications and was further processed by undergoing a secondary equilibrium process by submerging the films in CuCl and FeCl₃ solutions to induce a cation-specific effect. A similar method referred to as casting and rehydrating was used to fabricate collagen-hydroxyapatite films on glass plate casts at 30°C. The resulting films showed improved water vapour and oxygen permeability and exhibited potential for food sealing applications [290].

3.5.3 Freeze-drying (including Template- and Alignment-based Variants)

Freeze-drying (lyophilization) involves freezing a collagen solution, followed by sublimation of ice under low pressure and temperature, creating an interconnected porous structure [291]. Template-assisted freeze-drying further controls scaffold architecture. For example, a poly(lactic-co-glycolic acid) (PLGA) mesh coated with collagen in acidic solution was used to produce composites with either small- or large-aperture pores. Small-aperture scaffolds achieved uniform pore filling, strong fibroblast adhesion, and enhanced dermal regeneration, while large-aperture scaffolds retained open pores supporting adhesion with less homogeneity [292]. Freeze-drying can also promote collagen fibril alignment if performed in cylindrical conduits. Scaffold diameter, tensile properties, and fibril organization were influenced by conduit aspect ratio, diameter, collagen concentration, and freezing temperature [293, 294]. In addition to alignment, freeze-drying offers tuneable control over matrix density, porosity, and texture. A higher freezing temperature produces larger ice crystals and thus larger pores, resulting in scaffolds with faster rehydration but weaker mechanical strength. Conversely, lower

freezing temperatures generate smaller ice crystals and correspondingly smaller pores, leading to denser matrices with slower rehydration but superior mechanical properties [291]. Furthermore, Table 7 summarises the different parameters affecting collagen matrix in freeze drying in cylindrical conduits. This setup has been used to make porous scaffolds for bone regeneration, particularly for cranial defects [295].

Table 7 Freeze-drying in cylindrical conduit of collagen composite: parameters and resulting effect on the scaffold.

Factors	Result
Aspect ratio	Low aspect ratio, lower tensile strength
	High aspect ratio results in a more pronounced preferential orientation of topographical feature
Cylinder diameter	Smaller diameter results to a smaller scaffold diameter
Fibrillar network (Collagen concentration)	The lower the concentration of the collagen hydrogel, the smaller the scaffold diameter
Freezing temperature	The lower the freezing temperature, the smaller the scaffold diameter

3.5.4 Electrospinning

Electrospinning uses a high-voltage field to arrange collagen fibres in nano- and micro-size scales. A needle deposits a collagen solution onto a grounded collector. Both elements generate an electric field that stretches the fibres as it settles onto the collector and the solvent evaporates, creating an assembled collagen matrix [296, 297]. A study used electrospun collagen with polyethylene terephthalate (PET) in hexafluoro isopropanol solvent using controlled flow rates, voltages, and distances. The electrospun mats were subsequently stabilized with glutaraldehyde [298]. While electrospinning yields high-surface-area, fibrous composites favourable for cell adhesion, the method risks collagen denaturation from harsh solvents and therefore requires stabilization or blending with synthetic polymers [298]. A study reported that electrospun collagen fibres lack the characteristic axial periodicity of native fibrils, indicating a disruption of the hierarchical structure during fibre formation [299]. These nanoscale alterations are

expected to influence the functional and biological performance of the electrospun materials, particularly their capacity for cell attachment and recognition [299].

The electrospun collagen-PET composites exhibited good cell viability and adhesion potential for dermal tissue engineering applications [298]. In addition, there are studies combining collagen and a complex carbohydrate – pullulan – using this technique to explore it as an alternative for edible food packaging and wound healing treatment [300, 301].

3.5.5 Co-precipitation and Suspension (Mixing) / Immersion (No Mixing)

This straightforward method fabricates composites by directly mixing collagen with inorganic fillers (e.g., hydroxyapatite, bioactive glass) or polymers in suspension, followed by gelation and crosslinking. The process allows homogeneous filler distribution within the collagen matrix but requires stabilization to maintain structural integrity. Co-precipitation can also be used in combination with immersion, immersing the collagen scaffold in a mineral suspension for a few days [302]. Both integration methods are usually followed by freeze-drying [303]. This technique is commonly used for mineralized composites for bone applications [304].

3.5.6 Electro-compaction

Electro-compaction or electrochemical alignment is a technique that enables collagen fibrils to be concentrated and aligned by passing a weak electric field across a collagen suspension confined between electrodes. Applying a current repels the ampholytes in the collagen fibrils from both sides of the anode and cathode terminals, respectively, according to their local charge. This leads to concentrating the collagen fibres at the central region creating closely packed and organised collagen fibrils [305]. The electrode shape can be utilised in order to achieve a matrix with improved fibre orientation and mechanical properties. In one study, bovine collagen solution placed in an electro-compaction cell formed a thin film on a nylon filter at 5 V for 5 min, while excess solution was removed [306]. Subsequent incubation in PBS stabilized the compacted fibrils, and the resulting films supported adhesion of corneal stromal cells. This method produces dense, anisotropic scaffolds with improved strength and uniformity compared to conventional gelation, making it particularly suited for tissue engineering of load-bearing or aligned tissues.

3.5.7 Microfluidics

Microfluidic devices fabricated from PDMS or ABS allow fine control over collagen flow through microscale channels. Two main strategies exist: (a) droplet microfluidics, which generates uniform microspheres for drug delivery and cell encapsulation; and (b) microfluidic spinning, which forms continuous aligned collagen fibres [307-309]. Crosslinkers and buffers can be introduced via additional inlets, and channel topographies can be modified to define composite structures. This high-resolution technique enables the fabrication of tailored collagen microstructures [307]. In a study where the composite was fabricated by directing concentrated collagen solution through a microfluidic system, which enabled precise fibrillar alignment and hierarchical organization. The resulting fibres exhibited enhanced tensile properties, underscoring potential application in drug delivery and tissue engineering [310].

3.5.8 3D printing / Extrusion

3D printing via extrusion allows for site-specific deposition of collagen-based inks into scaffolds/moulds with precise architecture. Collagen is commonly combined with hydroxyapatite, a stabilizer like pectin and a crosslinker like genipin [141, 311-314]. This significantly enhances mechanical properties particularly the storage modulus. It also shifts the denaturation to a higher temperature. This demonstrates the composite's potential for bone tissue engineering, where both biomimetic structure and mechanical stability are necessary [311]. In addition to its bone regeneration applications, this technique has also been used for dentoalveolar and cartilage tissue regeneration [312]. The application of 3D printing by extrusion is limited due to the lack of resolution due to the aperture used as well as the resulting lower viable cell density. Such drawbacks may not be ideal for soft tissue applications but useful for hard tissue printing. Other techniques for 3D printing can be used if high precision and resolution is required for certain applications. Technologies such as (but not limited to) laser- and acoustic-based printing as well as stereolithographic can be used [312]. For brevity, these will not be discussed in detail.

3.5.9 Mechanical fabrication techniques

Stretching, compression or shearing are the mechanical forces used for collagen fibre alignment. Applying periodic mechanical deformation on a collagen scaffold causes the

fibrils to reorganise and align in the direction of the applied strain[315]. Potential limitations of the technique are nonuniform alignment and structural degradation or mechanical damage [316, 317].

3.5.10 Gravity-based fabrication

Gravity-based fabrication can be as simple as aggregating collagen fibrils by combining them with Eagle's medium and sodium hydroxide (adjusting pH to 7.6), and letting the aggregates settle, creating a hydrated collagen lattice [318]. Another example is from a study using microchambers, using glass sheets and a constant flowing collagen solution [319]. It utilises evaporation to increase the concentration of the collagen in the chamber and then inducing fibrillogenesis using ammonia vapour at room temperature to increase pH and prevent dilution and therefore stabilizing the collagen [319, 320].

3.5.11 Magnetic alignment

Magnetically aligned collagen scaffolds can be fabricated using either high-strength electromagnetic fields (4–12 T) or magnetic particle-assisted approaches with low-level fields (<0.25 T) [321]. The electromagnetic method exploits the diamagnetic properties of collagen's peptide bonds to orient fibrils perpendicular to the field, though it requires high field strength, low temperatures, and extended exposure times to yield effective alignment [322]. In contrast, the magnetic particle approach uses nanoparticles (e.g., iron oxide) that interact with collagen fibres and align parallel to the applied field, enabling scaffold fabrication under more practical conditions [323, 324]. Both strategies allow control over scaffold architecture and have shown promise across neural, vascular, corneal, cartilage, and musculoskeletal tissue engineering, with ongoing efforts to integrate them with advanced fabrication methods such as 3D printing and electrochemical deposition to improve mechanical and biological performance [325, 326].

3.5.12 Dispersion and vacuum filtration for film formation

Dispersion and vacuum filtration are essential steps for obtaining uniform collagen suspensions and fabricating smooth collagen films. In this study, collagen from sea pineapple species was extracted using NaCl pre-treatment followed by urea dissolution. The collagen was then dispersed through homogenization and sonication, creating a stable suspension, which was subsequently filtered through a 0.45 µm polycarbonate

membrane under vacuum, rinsed with water, and air-dried to obtain collagen films. Chitosan was deposited on the collagen film surface, and the composite was evaluated as an eco-friendly food packaging alternative. Its performance was tested on meat and fruit samples, assessing properties such as mechanical strength, water vapor permeability, surface wettability (contact angle), preservation efficacy, and biodegradability [137].

3.6 Conclusions

This chapter reviewed the major components and fabrication approaches used in collagen matrix composites. Collagen can be obtained from a range of sources, including animal-derived, marine-derived, and recombinant systems, each with different advantages and limitations. The incorporation of crosslinkers, additives, and coatings, together with fabrication approaches such as self-assembly, solvent casting, freeze-drying, electrospinning, 3D printing, and alignment-based techniques, enables the structural, mechanical, biological, and functional properties of collagen-based materials to be tailored for specific applications. Collectively, the literature demonstrates that collagen is a highly adaptable matrix material with considerable potential for the development of biomedical, packaging, and other sustainable composite applications.

Chapter 4. Gelatin and collagen from sheepskin

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4.1 Abstract

Abattoirs dispose of sheepskins as solid waste because of the low price and poor demand for sheepskin leather. In principle, instead of being disposed of in landfills, sheepskins can serve as a source of the protein collagen or the hydrolysis product gelatin. In this research, sheepskins collected from abattoirs were used as source of collagen. Three extraction methods were compared: acid extraction, acid with enzyme and alkali extraction. The extracted material was characterized using scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR), small angle X-ray scattering (SAXS) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The collagen and gelatin extraction yield ranged from 3.1% to 4.8% with the product purity determined by hydroxyproline ranging from 7.8% for the alkali process to 59% and 68% for the acid and acid-enzyme processes. SDS PAGE showed that the acid process produced fragments with molecular weight in the range 100 to >250 kDa while acid-enzyme resulted in smaller fragments, below 30 kDa. The FTIR region of the amide I band at 1800-1550 cm⁻¹, used as an indicator of the collagen and gelatin content, showed the gelatin dominated in the acid extracts, and the alkaline extract contained a large portion of keratin. SAXS was found to be a sensitive method to show the presence of intact collagen fibrils for material from all extraction methods, although at low concentration. Sheepskin is shown to be a useful source for collagen-gelatin material of varying molecular weight.

4.2 Introduction

Collagen is the most abundant structural protein in animals [88]. Collagen can be extracted from animal tissue such as skins from animals slaughtered for meat and further processed for diverse applications. Gelatin is a hydrolysis product of collagen, and maybe readily obtained for skins, sometimes in combination with collagen, where the two may grade into each other with a range of molecular weights.

Collagen and gelatin are used in cosmetics [327], medical and pharmaceutical products [328], and food formulation and packaging [329]. Methods used to extract collagen from tissue [57] include hydrolysis with acids [35] and alkalis [20], solubilization with salts [330], and enzymatic hydrolysis [30]. Often these chemical treatments are assisted with ultrasound [331] and microwaves [332].

This work compares gelatin and collagen extraction from sheepskins using acid hydrolysis, acid-enzyme hydrolysis and alkali hydrolysis. The aim was to identify a suitable process for collagen recovery from sheepskins in the form of gelatin or collagen for possible commercial systems.

4.3 Materials and Methods

Whole sheepskins from unknown sheep breeds were collected from a local abattoir (ANZCO Foods, Bulls, New Zealand). Sheepskins were stored at $-20\text{ }^{\circ}\text{C}$ until processed for extraction. Other materials and chemicals used were acetic acid (99.7%, LabServ), sodium chloride (99%, Summit AG Salt fine), EDTA-4Na (Hamilton Chemicals), butanol (99.5%, RP Normapur), sodium hydroxide (Scharlau), sodium acetate (Scharlau), pepsin (Sigma Aldrich), dialysis bags (8-14 kDa molecular weight cutoff), molecular weight markers (Bio Rad Precision Plus Protein Kaleidoscope Prestained Protein Standards and Dual Xtra Standards), Protein Gel Criterion Tris-HCl Precast Gels (Bio Rad), Laemmli sample buffer (Bio Rad), tris/glycine/SDS buffer (Bio Rad), 2-mercaptoethanol (Bio Rad), sodium acetate trihydrate (Sigma Aldrich), citric acid (AnalaR), chloramine-T (Sigma Aldrich), n-propanol (Unilab), p-dimethylaminobenzaldehyde (Sigma Aldrich), 70% perchloric acid (AnalaR), and L-hydroxyproline (Sigma Aldrich).

4.3.1 Pretreatment

The sheepskins were thawed overnight in a fridge ($4\text{ }^{\circ}\text{C}$). The skins were checked for adhering flesh, fat, and wool and, if present, these were trimmed. The skins were washed and cut into $120 \pm 5\text{ g}$ (fresh weight) samples. The pretreatment was conducted at $4\text{ }^{\circ}\text{C}$ with continuous stirring. The various pretreatment steps are shown in Figure 1. All skin samples underwent the same pretreatment with a skin to solution ratio of 1.2:10 kg skin:L solution.

Non-collagenous protein was removed by soaking the skins in 0.1 M NaOH for 6 h, the NaOH solution was changed every 3 h. The skins were then rinsed until the pH of the wash was neutral. The swollen skins were then manually dehaired with a tweezer and washed with water. The washed skin was cut into 1 cm² pieces and demineralized by soaking in EDTA-4Na solution (pH 7.4) for 48 h, changing the solution every 16 h. The demineralized skins were washed and defatted by soaking in 10% v/v butanol for 16 h. The pretreated skin pieces were processed using one of the two extraction methods identified below.

4.3.2 Acid extraction

The pretreated skin sample underwent acid hydrolysis to extract the acid-soluble collagen. The skins were immersed in 0.5 M acetic acid (a skin-to-acid ratio of 1.2:10 w/v) at 30 °C for 4-7 days in sealed Erlenmeyer flasks held on a shaker platform (150 rpm agitation speed).

4.3.3 Acid-enzyme extraction

For the acid-enzyme extraction, the skin pieces (Section 4.3.1) were suspended in 0.5 M acetic acid and 1 g L⁻¹ pepsin, with a skin-to-solution ratio of 1.2:10 w/v. This suspension was agitated at 150 rpm at 30 °C for 4-7 days until the skins dissolved then heated at 90 °C for 10 minutes to deactivate the enzyme.

4.3.4 Alkali extraction

Alkali soluble gelatin and collagen was extracted by suspending the skins in 0.5 M NaOH with a skin-to-alkali ratio of 1.2:10 w/v. The suspension was maintained at 60 °C (hotplate), with continuous stirring (150 rpm), for 10-12 h until the skins dissolved.

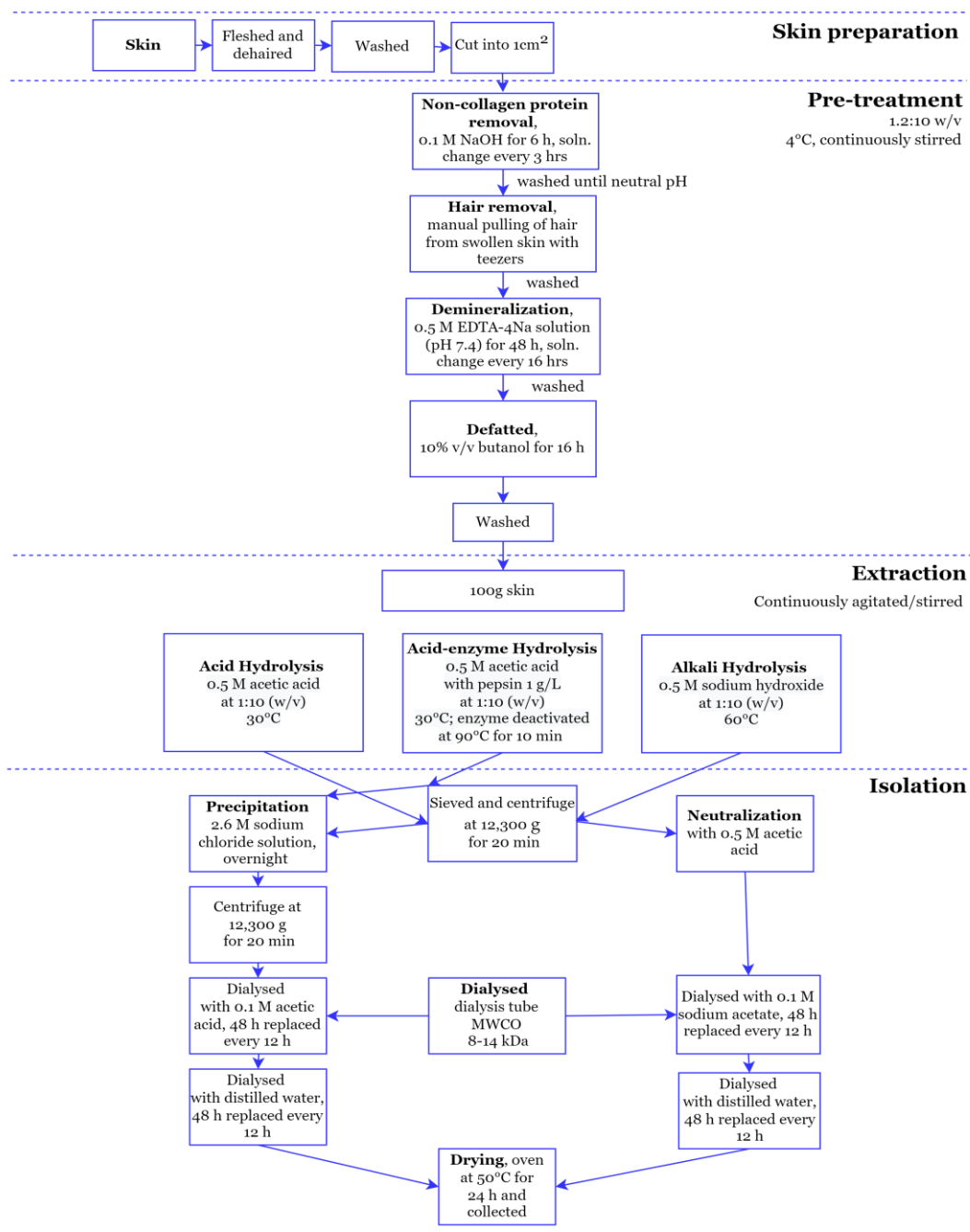


Figure 5 Flowchart of the three extraction methods.

4.3.5 Isolation of collagen and gelatin

The collagen and gelatin were isolated from the extracts of the acid (Figure 5) first by centrifugation (12,300 g, 20 min) with the supernatant subjected to salt precipitation by adding sodium chloride to attain a final concentration of 2.6 M and allowed to stand overnight at room temperature (~23 °C). The precipitate formed was recovered by centrifugation (12,300 g, 20 min). The recovered precipitate was dissolved in 0.5 M acetic

acid at a ratio of 1:9 v/v, transferred to a dialysis tube, and dialyzed against 0.1 M acetic acid for 48 h at room temperature. The 0.1 M acetic acid dialysate was replaced with distilled water and dialyzed for 48 h. Both dialysate solutions were replaced every 12 h.

For the alkali extraction, the sample was sieved and centrifuged (12,300 g, 20 min) (Figure 1). The supernatant was collected and neutralized using 0.5 M acetic acid. The neutralized sample is dialyzed with 0.1 M sodium acetate (48 h), and distilled water (48 h), with the dialysate solution replaced every 12 h. Sodium acetate rather than acetic acid was used in order to have a similar pH on either side of the dialysis membrane.

The concentrated solution from inside the dialysis bags was placed in trays and dried at 50 °C for 24 h.

4.3.6 Extractable yield

Extractable yield was calculated as the mass of the dry solids ($M_{\text{recovered solids}}$) recovered from the extracts divided by the mass of the dry skin (M_{skin}) used in extraction [22] with weighing taking place immediately after drying.

4.3.7 Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of the dried and ground collagen samples were recorded (Thermo Scientific Nicolet iS5 with iD7 ATR spectrometer; Thermo Fisher Scientific. MA, USA) over the wavenumber range of 400 to 4000 cm^{-1} with a resolution of 2 cm^{-1} and data spacing of 0.241 cm^{-1} .

4.3.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE followed Laemmli's methodology [333] to determine the molecular weights of the collagen and gelatin extracted by the different methods. The collagen or gelatin was dissolved in the buffer to obtain a concentration of 1 mg mL^{-1} . Afterwards, 10 μL of the denatured sample and 5 μL of a protein standard (molecular weight markers) were loaded into wells at the top of a precast gel (4–20% Criterion Tris-HCl Protein Gel, 18 well, 30 μL). Coomassie Blue R250 stain was used.

4.3.9 Hydroxyproline quantification

Due to consistent hydroxyproline:collagen ratios in sheepskin, hydroxyproline quantification can be used to provide assessment of the total collagen and gelatin content [334]. The hydroxyproline in collagen samples was quantified using an adaptation of a published method [335]. Briefly, 0.1 g of a sample (either the extracted collagen, or raw skin) in a 50 mL capped vial were digested with 10 mL of 6 M HCl for 24 h in an oven at 110 °C. After hydrolysis, the samples were cooled to room temperature, and 6 M NaOH was added until the pH was 6.0. The resulting solution was filtered, and the volume was adjusted to 50 mL with deionized water. A 50 μ L portion of this solution was mixed with 450 μ L of chloramine-T (0.056 M chloramine-T in acetate-citrate buffer, pH 6.5), and allowed to stand for 25 min at room temperature in a capped vial. A 500 μ L portion of Ehrlich's reagent was added to the vial and it was held in a 65 °C water bath for 20 min to allow the colour to develop. The absorbance values of the sample were measured against a blank (distilled water) in a UV spectrophotometer at 550 nm. The readings were converted to concentration of hydroxyproline using a calibration plot that had been prepared using standard solutions of hydroxyproline.

4.3.10 Small-angle X-ray scattering (SAXS)

SAXS diffraction patterns were recorded on the Australian Synchrotron SAXS/WAXS beamline using a high intensity undulator source. The energy resolution was 10–4 using a cryo-cooled Si(111) double crystal monochromator. The beam size (FWHM focused on the sample) was 250×80 μ m with total flux of approximately 2×10^{12} photons s^{-1} . A Pilatus 1 M detector with an active area of 170 × 170 mm, a sample detector distance of 3371 mm, and X-ray of 8 or 11 keV, was used to record all the diffraction patterns. Exposure time for diffraction patterns was in the range of 1–5 s. Data was processed using SAXS15ID software [336].

4.4 Results and Discussion

4.4.1 Extracted collagen

The samples extracted using the different methods differed in appearance and texture (Figure 6). The acid-extracted samples had a lighter off-white appearance (Figure 6a, b)

compared to the alkali-extracted sample (Figure 6c). The acid processed samples are comprised of larger aggregates with smoother surfaces and sharp edges, typical of gelatin (Figure 2, SEM images) whereas the alkali processed sample had solid particles with rougher surfaces.

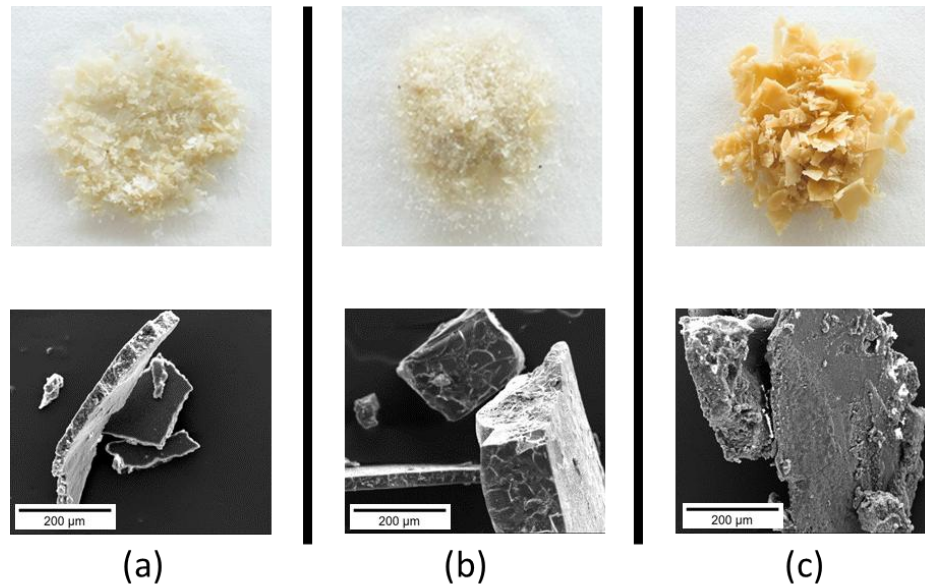


Figure 6 Dried gelatin-collagen (top, optical images; bottom, SEM images) from the different extraction methods: (a) acid hydrolysis, (b) acid-enzyme, (c) alkali hydrolysis.

4.4.2 Extractable yield

The extraction yield of gelatin-collagen using the different methods are as follows (average values $\pm$ standard deviation (% w/w); three replicates): 3.1 ± 1.6 for acid extraction; 4.8 ± 1.9 for acid-enzyme extraction; 4.1 ± 7.7 for alkali extraction (Table 8). Based on the Student's t-test, the yields from the acid and acid-enzyme extractions were not significantly different ($p > 0.05$). Including enzyme had no positive benefit on extraction yield, in contrast to other studies that have shown improved extraction of collagen from cattle and fish skin in the presence of enzymes [22, 337].

Table 8 Hydroxyproline and gelatin-collagen content of the material recovered using the different extraction methods (acid, alkali), and raw sheepskin.

Sample	Hydroxyproline content (mg g ⁻¹ dry product)	Gelatin-collagen in dry product (%)	Gelatin-collagen extracted (mg g ⁻¹ dry raw skin)	Extracted collagen-gelatin relative to the total amount of collagen in the raw skin (%)
Acid	74.2 ± 8.1	59.3 ± 6.5	10.7	3.1
Acid-enzyme	85.1 ± 25.3	68.1 ± 20.2	16.8	4.8
Alkali	9.7 ± 2.1	7.8 ± 1.7	14.3	4.1
Raw sheepskin	19.4 ± 7.3	15.5 ± 5.8	-	-

4.4.3 Hydroxyproline quantification

Collagen molecules have a specific characteristic motif of Gly-X-Y (Gly = glycine, X = proline, Y = 4-hydroxyproline) that stabilizes their triple-helix structure. The hydroxyproline component in the motif is also uniquely abundant in collagen compared to other proteins. The abundance of hydroxyproline in an extract is used in estimating its collagen content. The measured data (Table 8) was used in the following equation to calculate the hydroxyproline content:

$$\text{Hydroxyproline } (\mu\text{g g}^{-1} \text{ sample}) = \frac{\text{Hydroxyproline } (\mu\text{g mL}^{-1}) \times \text{Dilution volume (mL)}}{\text{Sample weight (g)}} \quad (4-1)$$

The collagen content was then estimated using the following equation:

$$\text{Collagen content } (\mu\text{g g}^{-1} \text{ sample}) = \frac{\text{Hydroxyproline } (\mu\text{g g}^{-1} \text{ sample}) \times \text{Conversion factor}}{1000} \quad (4-2)$$

For sheepskin collagen extracts and the raw sheepskin samples, it is known that ca. 12.5% of the collagen mass is hydroxyproline [43]. This is slightly less than in the hides of other animals such as cattle, deer, and goat. Using the percentage of hydroxyproline in sheepskin, the collagen content of the samples was calculated using a conversion factor of 8 (100/12.5) in Eq. (4-2). The hydroxyproline and collagen content of the samples are shown in Table 1. The material extracted by acid extracted material contained the most gelatin-collagen. The acid extract had 7.6-fold more gelatin-collagen than the gelatin-collagen content of the alkali-processed sample (Table 8). Although alkaline solution had a high extraction of material, it is also known that the alkali treatment with readily

hydrolyse collagen into smaller peptides [338]. The relatively high extraction temperature (60 °C) for the alkali method may also have been a factor in hydrolysing the collagen to small molecular weight components. These small molecular weight components would have been mostly removed during dialysis using the membrane with a molecular weight cutoff of 8–14 kDa and therefore are not included in the apparent yield. Nevertheless, such small molecular weight components are not useful for most gelatin applications and therefore it is useful not to include this component in the apparent yield calculation.

The hydroxyproline content in the materials produced by acid and acid-enzyme extraction here are similar or greater than that reported for cattle skin [22, 28] and fish skin [339] subjected to similar treatments.

The extraction yield from sheepskin is a little lower than that obtained from cattle hide [22]. Sheepskin has a higher fat content (30-50% in the dermis alone), compared to bovine hide (2-3% fat) and goatskin (3-10% fat) [15]. The extra fat, which was not effectively removed in the defatting stage, may have contributed to the lower extraction yield of collagen by providing the collagen some protection (by hydrophobicity) from the digestion chemicals.

The extraction yield of gelatin-collagen from skin could perhaps be improved by modifying the pretreatment conditions to better “open up” the skin matrix enabling better penetration of the hydrolysis chemicals. In the leather industry an initial liming stage of the skins and hides is performed to remove the hair, solubilize the fat and swell the skin which opens up the fibres for better chemical penetration at subsequent processing stages [340].

4.4.4 FTIR

The FTIR spectra of the material extracted using the different extraction protocols had some major differences (Figure 7). A summary of the positions of the main absorption bands and their assignment is provided in Table 9. Absorption band that can be assigned to collagen or gelatin and bands due to keratin are observed in all samples, but the proportions of each of these differs between samples. The material from the alkali extraction contained more intense peaks that are due to keratin.

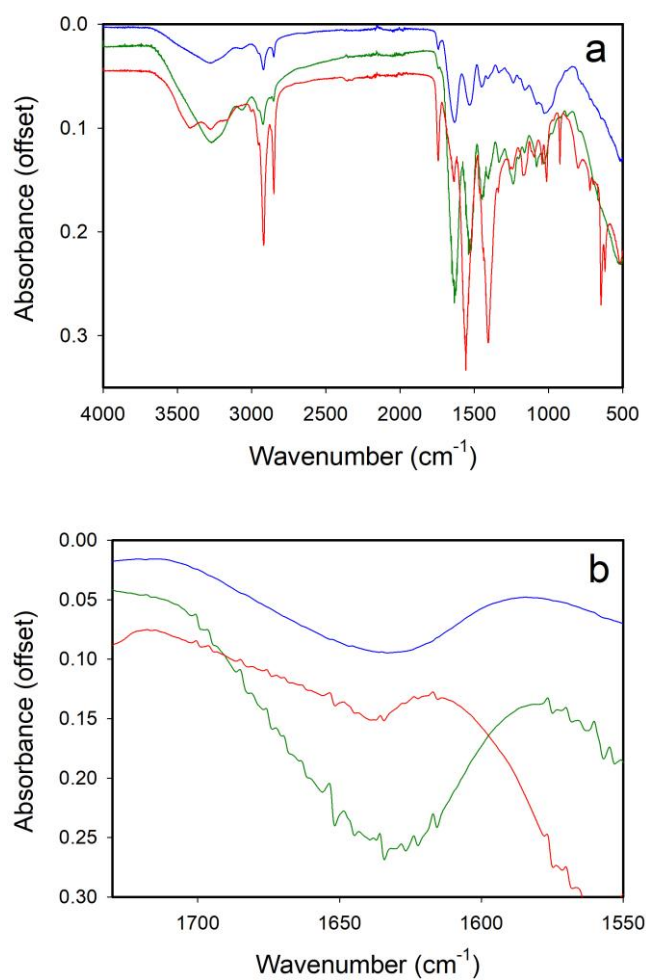


Figure 7 FTIR spectra of collagen recovered by acid (blue line), acid-enzyme (green line) and alkali (red line) extraction. a) full spectrum; b) expanded region that assists in distinguishing between gelatin and collagen [341]. See Table 9 for details of peak assignments.

Table 9 FTIR absorption bands of amide vibration modes from the literature [342] and measured in this work and keratin bands identified in these samples assigned from literature [343].

Band	Literature band detection (wavenumber, cm ⁻¹)	Absorption band positions from this work for different extraction methods (wavenumber, cm ⁻¹)			Functional group and vibration modes
		Acid	Acid Enzyme	Alkali	
Collagen and gelatin					
Amide A	3300-3500	-	-	-	NH stretching
Amide B	~3100	3065	3065	3163	NH stretching
Amide I	1600-1800	1630	1635	1638	C=O stretching
Amide II	1470-1570	1530	1528		CN stretching
Amide III	1250-1350	1334	1334	1339	CN stretching, NH bending
Keratin specific (keratin also displays amide bands above)					
	2931	2921	2923	2919	(CH ₃) symmetric stretch
	2875	2851	2852	2850	(CH ₂) symmetric stretch
		1743	1743	1743	unassigned
	1551			1559 (overlaps with collagen band)	(NH) bend; (CN) stretch
	1420	1409	1407	1408	(CH ₃) deformation
Water					
		3275	3275	3275	OH stretching (water)

That the material contains collagen or gelatin can be observed from the amide I bands, which are from C=O stretching in hydrogen-bonded carbonyls of proteins. These bands are sensitive to changes in the secondary structure of proteins. Amide II bands from C–N stretching are also affected, to a lesser extent, by changes in the secondary structure of proteins [344]. Amide III bands result from C–N and N–H planar deformation vibrations and other effects associated with carbon-nitrogen stretching and the bending of the nitrogen-hydrogen. These bands are sensitive to changes in the trip helix structure and the polarity of the protein.

To distinguish between collagen and the hydrolysis product gelatin it has been shown elsewhere that the amide I absorption bands between 1700 and 1600 cm⁻¹ can be used to [341]. Collagen has been shown to have a peak at 1659-1651 cm⁻¹ whereas in gelatin the

peak is at 1643-1633 cm^{-1} , depending on the temperature during the measurement (lower wavenumber at higher temperature). This region of the FTIR recorded here is displayed in Fig 3b. In these samples there is just one broad peak centred at 1630 cm^{-1} for the acid and acid-enzyme extracted material and a peak at 1638 cm^{-1} for the alkali extracted. These may indicate that gelatin dominates over collagen in all of these extracted materials and, because of the shift in band position, could suggest that the alkali extracted material is hydrolysed further than the acid extracted material, although the poor resolution of this region limits the conclusions that can be drawn.

The FTIR makes it clear that these materials are not pure collagen or pure hydrolysis products of collagen. There are intense peaks associated with keratin, and these are more prominent in the alkali extracted material. The low hydroxyproline content of the alkali material showing that it is less than 8% gelatin and the intense FTIR peaks for keratin show that the alkaline extracted material is likely largely keratin.

4.4.5 SDS PAGE

The developed SDS PAGE gel shows the molecular weights of the extracted proteins (Figure 8). The globular protein molecular weight standards are known to be not good molecular weight standards for the fibrous collagen components, possible due to different SDS binding, so are presented only as an approximate range standard. Collagen extraction by acid hydrolysis resulted in products with molecular weights in the range 100 to greater than 250 kDa. When enzyme was used in conjunction with the acid hydrolysis, much smaller molecular weight components resulted, mostly 13-30 kDa. For the alkali extraction process indistinct bands were observed in the SDS PAGE.

The simple acid hydrolysis therefore results in material that contains more of the longer collagen chains and less material that has been broken into smaller fragments of gelatin. The $\alpha 1$ -chains and $\alpha 2$ -chains, specific to collagen-1, produce bands at around 116 kDa [332], as observed here. The higher molecular weight bands observed here are result from either two crosslinked α -chains (β -band), or three crosslinked α -chains (γ -band).

The acid hydrolysis with enzymes results in the collagen being hydrolysed into small fragments, in other words, gelatin. The dialysis membrane used for separation after extraction had a cutoff of 8-15 kDa so the range observed here in the SDS PAGE of 13-30 kDa is limited by this separation and it is probable that smaller polypeptide fragments

were also produced by the acid-enzyme hydrolysis. Although pepsin is considered to digest only the non-helical parts of the procollagen [116], acid hydrolysis (as used here) is nonspecific.

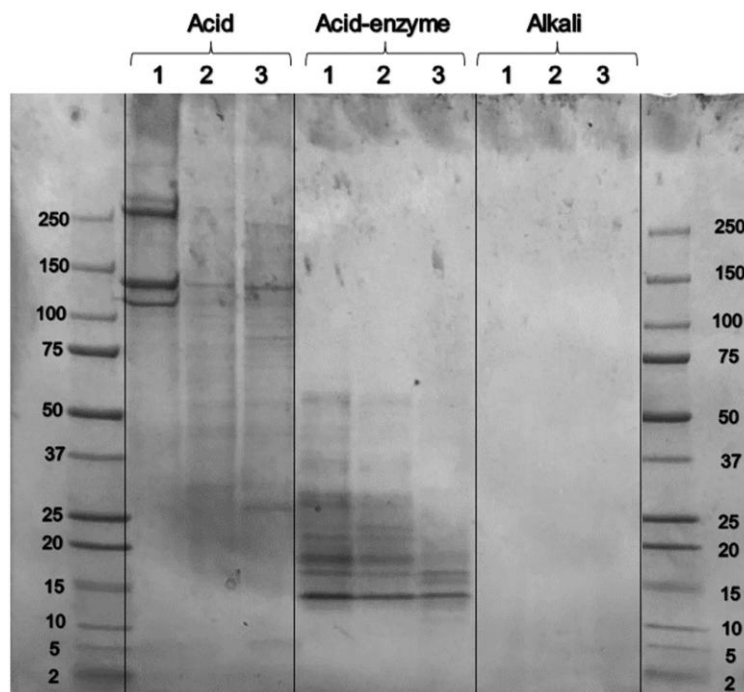


Figure 8 SDS PAGE of collagen from acid, acid-enzyme and the alkali extraction processes. The lanes on extreme left and right show the globular protein molecular weight markers (2–250 kDa). There are three replicate lanes for each treatment. The figure has been modified to remove some lanes on the right-hand side not discussed in this work (full original SDS PAGE available).

The samples extracted with alkali, did not have distinct bands in the SDS PAGE. However, from the hydroxyproline analysis and the FTIR, it was clear that this material was largely keratin and contained only a small proportion (7.8%) of collagen or collagen hydrolysis products. In other studies, alkali-based treatments have been found effective for extracting collagen under milder conditions than in the present work [345].

Current market conditions suggest that “collagen peptides” or “collagen hydrolysate” may represent an increasing market sector where the smaller components have distinct value. If these components could be efficiently recovered by methods such as those studied here, high-quality peptides may be more produced with more valuable than low bloom strength gelatin.

4.4.6 SAXS

The SAXS diffraction patterns of the gelatin-collagen material recovered by three extraction methods are shown in Figure 9. The diffraction peaks (Bragg peaks) are due to banding in the fibrillar structure of collagen [346]. The presence of this diffraction feature is definitive evidence for the presence of collagen fibrils in the samples. The distance between the bands (i.e., the D -spacing) in the collagen molecules is calculated from the diffraction data. Diffraction also occurs from the triple-helix, at around 0.287 nm[347]. Triple-helix structures exist in gelatin or collagen-like peptide fragments[348]. We did not measure diffraction at this scale, only the diffraction angle where we can see fibrillar banding at around 63 nm.

The presence of these D -spacing diffraction peaks in the acid-enzyme and the alkaline samples is a little surprising considering the SDS PAGE results showing little material of higher molecular weight in these samples (although high molecular weight material in the acid extracted material). This perhaps is an indication of the sensitivity and specificity of SAXS for detecting fibrillar collagen.

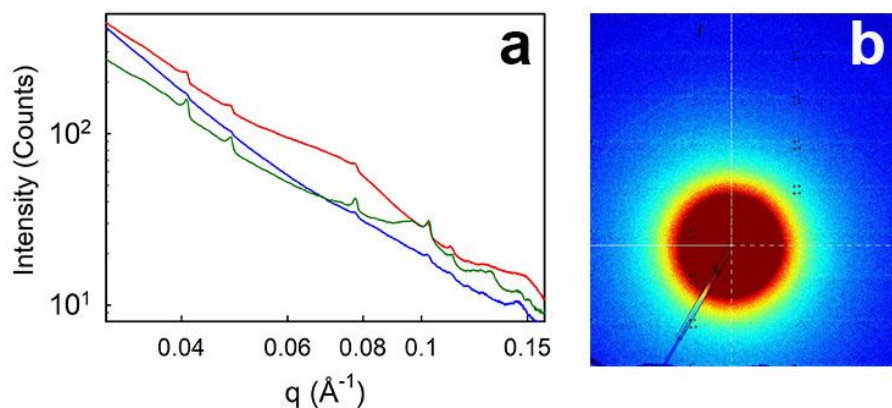


Figure 9 a) SAXS integrated scattering intensity for extract obtained by acid (blue line), acid-enzyme (green line), and alkali (red line) extraction showing sharp peaks for diffraction from the collagen fibril D -banding; b) example of the SAXS pattern (acid extracted material) with the faint rings visible due to the collagen diffraction.

The D -spacing is barely affected by the method of collagen extraction (Table 10) and is similar to that present in native tissues, of various types, for example [349].

Table 10 The *D*-spacing of collagen extracted by acid or alkali.

Extraction method <i>D</i> -spacing (nm)	
Acid	63.0
Acid-enzyme	63.0
Alkali	62.9

4.5 Commercialisation

The adaptation of this process for commercialisation would require some changes from what is described here in this laboratory study. For example, demineralization with EDTA is not required; the initial processing of the skins could be a conventional beamhouse operation where the skins are dehaired and defatted to produce pickled pelts.

4.6 Conclusions

This study trialled three different methods for extracting collagen from sheepskin. The key difference with sheepskin from other skins is the high fat content that must be removed at the start of the process. A gelatin-collagen mix was successfully extracted using both an acid process and an acid-enzyme process. All three approaches give distinct and different products with potential to adapt and give three different commercially relevant materials. Longer chain polypeptides were obtained from the acid process, while the acid-enzyme process broke the collagen into small fragments considered gelatin. Some fibrillar collagen was observed in all extracts. The alkaline extraction was dominated by keratin which may find markets in haircare. Small angle X-ray scattering was shown to be sensitive in detecting small quantities of intact fibrillar collagen in the extracts. The range of collagen derived molecules of varied molecular weight in these extracts, and the ability to select this range, informs the use of these materials in varied applications.

Chapter 5. Modified collagen extraction from raw and pickled sheepskin

5.1 Introduction

By the end of the breeding year, which concludes in September 2025, the number of processed lambs in New Zealand is expected to reach 16.8 million [4]. New Zealand, one of the world's largest exporters of sheep meat, exports approximately 90% of its lamb production [350, 351]. However, the meat is exported primarily in cuts, rather than as whole carcasses, leaving behind significant quantities of by-products that require management [352]. One such by-product is skin, which is $\sim 11\%$ of the sheep weight [353]. In recent years, the accumulation of sheepskins in landfills has raised environmental concerns, given their negative impact on both the ecosystem and local communities [354].

Conversely, increasing demand for natural materials has prompted interest in upcycling these by-products into a range of low-value products, such as clothing [355], home décor [356], medical accessories [357], and lanolin [353]. Among the various potential uses of sheepskin, collagen has emerged as a versatile product that has numerous applications from various medical [358] and dental implants [359] to food packaging [137]. Collagen extraction from bovine and porcine sources has been widely studied, along with marine sources as an alternative due to religious, cultural and health concerns associated with the former. However, ovine sources present another potential alternative that remains largely unexplored [57].

In addition to the meat industry, the leather industry is a potential source of sheepskin. After pre-tanning, skin trimmings are by-products of the process [360]. Pickling is the last step of the pre-tanning stage where pelts are produced [361]. The skins have undergone unhairing, liming, deliming and bating, and then pickled. Pickled pelts are free from wool (unhairing), fat (liming) and non-collagenous proteins (liming, deliming and bating) [361]. In addition to the raw sheepskin, pickled pelts were also explored as an alternative to raw sheepskin for collagen extraction. Given the treatments pickled pelts undergo, they may serve as a more time-efficient option for collagen recovery. The pickling process eliminates the need for unhairing and enables more effective fat removal, streamlining the extraction process. Additionally, the structural modifications

induced by pickling may facilitate the diffusion of collagen from skin matrix into the extraction solution, potentially enhancing extraction efficiency.

This study aims to fill this gap by further investigating the extraction methods of collagen from sheepskins sourced from abattoirs and leather factories, building upon previous research in this area, exploring other extraction parameters [362].

5.2 Materials and methods

Whole sheepskins from unknown sheep breeds were collected from a local abattoir (ANZCO Foods, Bulls, New Zealand). Pickled sheepskins were provided by the NZ Leather & Shoe Research Association (LASRA, Palmerston North, New Zealand). The sheepskins were stored at -20 °C until processed for extraction. The following materials and chemicals were used in the study: acetic acid (99.7%, LabServ), sodium chloride (99%, Summit AG Salt Fine), sodium hydroxide (Scharlau), sodium acetate (Scharlau), pepsin (Sigma Aldrich), molecular weight markers (Bio-Rad Precision Plus Protein Kaleidoscope Prestained Protein Standards and Dual Xtra Standards), Protein Gel Criterion Tris-HCl Precast Gels (Bio-Rad), Laemmli sample buffer (Bio-Rad), tris/glycine/SDS buffer (Bio-Rad), 2-mercaptoethanol (Bio-Rad), sodium acetate trihydrate (Sigma Aldrich), citric acid (AnalaR), chloramine-T (Sigma Aldrich), n-propanol (Unilab), p-dimethylaminobenzaldehyde (Sigma Aldrich), 70% perchloric acid (AnalaR), and L-hydroxyproline (Sigma Aldrich). For the isolation process in both the one-step and two-step acid-enzyme extractions, dialysis bags (8-14 kDa molecular weight cutoff) were used, while a diafiltration unit with a Pellican cassette (10 kDa Ultracel) (DeLand, U.S.A) was used for the large batch process.

5.2.1 One- and two-step acid enzyme extraction

Sheepskins were thawed before extraction. The process flow diagram, summarized in Figure 10, is described below. Pre-treatment procedures were conducted at 4°C. The skins were washed, drained, and soaked in a 0.5 M NaOH solution for 24 hours. After soaking, the wool was removed from the skin. The skins were washed again until the water reached a neutral pH, and then mechanically minced.

The minced sheepskin was soaked in a 1:1 solution of 0.1 M NaOH and 0.1 M NaCl for 12 hours, with a skin-to-solution ratio of 1:25 (w/v), stirred, and the solution was changed every 2 hours. The skins were then washed with water until the pH of the water was neutral.

The skins underwent demineralization by soaking in a 1:1 ratio of 0.1 M HCl and 0.1 M NaCl (pH 7.5), with a skin-to-solution ratio of 1:25 (w/v), for 2 hours. The skins were washed again with water until the pH of the water became neutral.

5.2.1.1 Two-step Acid Enzyme

The washed skins were soaked in 0.7 M acetic acid at a 1:25 (w/v) ratio at 4°C and stirred for 48 hours. After extraction, the skins and the extracting solution were separated using a sieve. The extract liquid was processed for isolation. The sample from this method is referred to as acid extraction step 1 (A2).

The residual sheepskin was soaked again in 0.7 M acetic acid at a 1:25 (w/v) ratio, with the addition of pepsin at a concentration of 1 g·L⁻¹ of acid solution, stirred at 4°C for 48 hours. Afterward, the extraction liquid was separated from the skin, and the liquid was heated at 90°C for 10 minutes to deactivate the pepsin. The extract liquid for the second part of the two-step acid-enzyme is the acid-enzyme step 2 sample (AE2).

5.2.1.2 One-step acid-enzyme

The washed skins were soaked in 0.7 M acetic acid at a 1:25 (w/v) ratio, with pepsin at a concentration of 1 g·L⁻¹ of acid solution, stirred at 4°C for 48 hours. After extraction, the liquid was separated from the skins, and the liquid was heated at 90°C for 10 minutes to deactivate the pepsin. The sample from this method is referred to as one-step acid-enzyme extraction (AE1).

5.2.2 Isolation

The three extract liquids underwent a series of steps for collagen isolation. Samples were centrifuged at $12,300 \times g$ for 20 minutes, separating the pellet from the supernatant. The supernatant was then precipitated by adding 2.6 M sodium chloride to the solution, stirred, and left overnight. The precipitated solution was centrifuged again at $12,300 \times g$ for 20 minutes. Protein pellets were then separated from the supernatant and dissolved in 0.5 M acetic acid at a ratio of 1:9 (v/v). The collected pellets were placed in dialysis bags with an 8-14 kDa molecular weight cutoff, and dialyzed against 0.1 M acetic acid for 48 hours, and the dialysate solution was replaced with distilled water and dialyzed for an additional 48 hours. Both dialysates were replaced every 12 hours. The protein extracts that were in the dialysis bags were freeze-dried at -20°C for 24 hours and collected. Four samples for each extraction method were collected and analysed.

5.2.3 Large batch collagen extraction

A large batch collagen extraction was conducted using a method similar to the one-step acid-enzyme extraction, with slight modifications to accommodate a larger amount of feedstock. The process flow diagram for the large batch process is illustrated in Figure 11. Pickled pelts weighing 2.4 kg were used for the extraction following the method described above, with modifications to the isolation process. The dialysis step of the isolation phase was replaced by diafiltration using a filtration unit with a 10 kDa Ultracel cassette. The liquid extract was diafiltered for 7 days, with the liquid in the stirred tank being replenished with 3 L of 0.1 M acetic acid for the first 3 days and 3 L of water for the final 3 days. The concentrated collagen solution was neutralized with 1 M sodium hydroxide until the pH reached 6.0 and then freeze-dried at -60°C . The sample from this method is referred to as large batch from pickled pelts (LBPP).

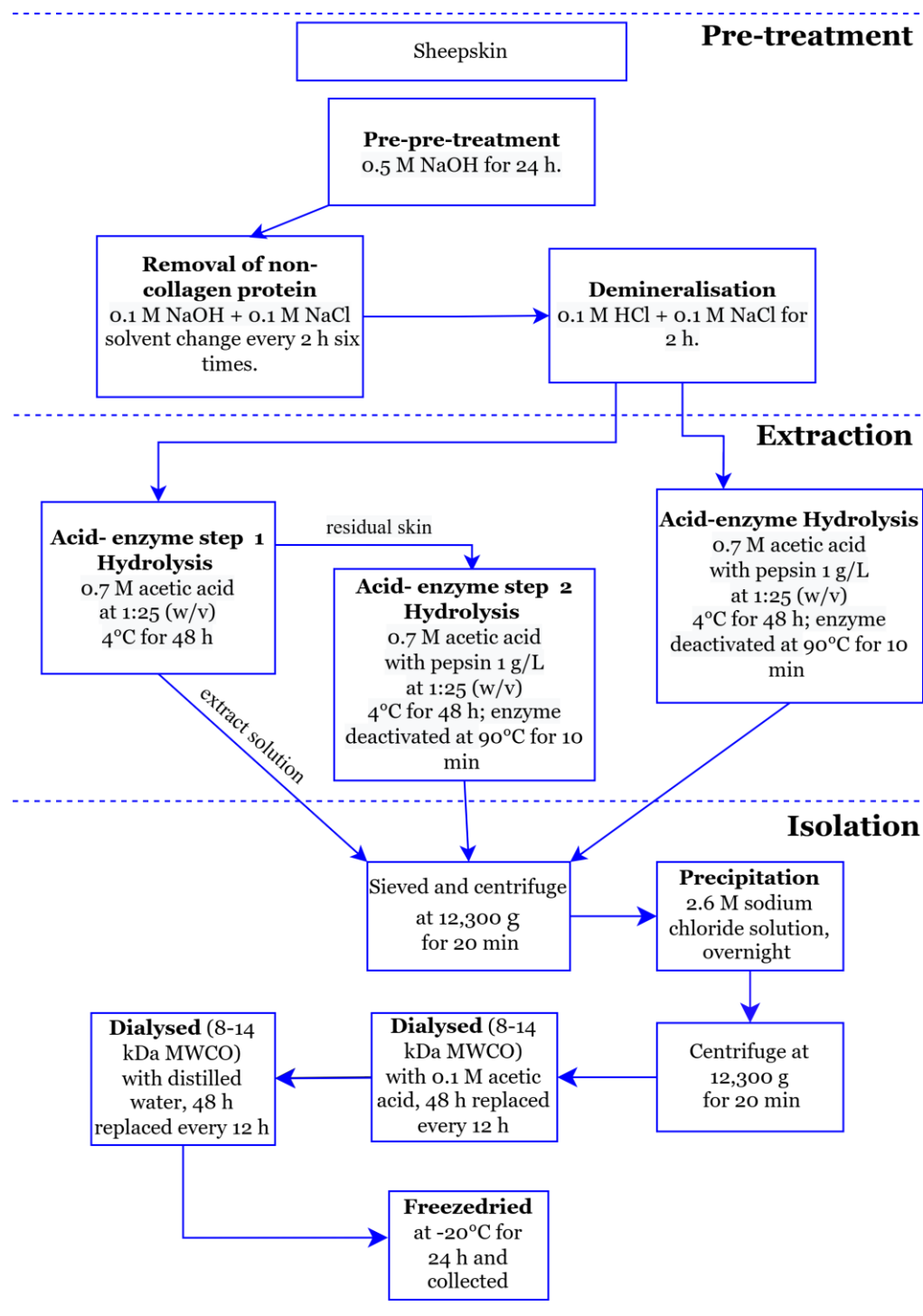


Figure 10 Process flow diagram for the one-step acid-enzyme and two-step collagen extraction methods.

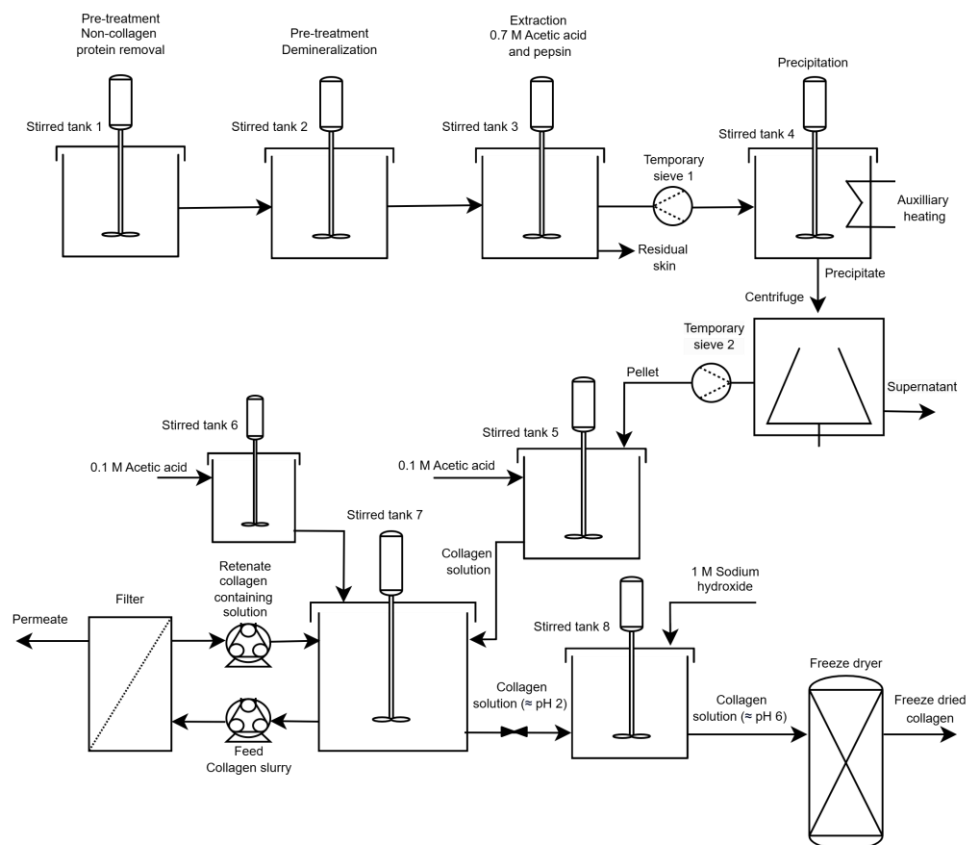


Figure 11 Process flow diagram of the large batch collagen extraction from pickled pelts

5.2.4 Yield

The extractable yield was calculated as the mass of the dry solids from freeze drying, divided by the dry skin used in extraction.

5.2.5 Hydroxyproline Quantification (HYPQ)

The Reddy and Enwemeka [335] method was used to determine the hydroxyproline content of the samples. From the hydroxyproline content, collagen content in the samples was quantified based on a published method by McNulty [334]. Samples were hydrolysed in 6 M HCl at a concentration of 0.001 g sample/mL of HCl solution. The samples were digested in a 110°C oven for 24 hours, then cooled, and the pH was adjusted to 6.0 using 6 M NaOH. The samples were filtered, and the volume was adjusted to 50 mL with deionized water. Aliquots of 50 μ L from each solution were mixed with chloramine-T reagent (pH 6.5) and allowed to react for 25 minutes in capped vials. Afterward, 500 μ L of Ehrlich's reagent was added to the samples, which were then placed in a water bath at 65°C for 20 minutes. The absorbance of the samples was

measured at 550 nm using a UV spectrophotometer, with distilled water used as a blank. A hydroxyproline standard curve was generated to determine the hydroxyproline content in the samples.

5.2.6 Scanning electron microscopy (SEM)

Thinly sliced freeze-dried collagen samples were mounted on SEM stubs and were platinum coated (~ 5 nm). Samples were observed under SEM (Hitachi Regulus SU8230) at an acceleration voltage of 3 kV and with varying magnifications: 1 mm, 500 μm , 100 μm .

5.2.7 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The molecular weights of the collagen samples were determined using SDS-PAGE, following Laemmli's method [333]. The collagen was dissolved and stained in a buffer to a concentration of 1 mg/mL using Coomassie Blue R250 stain. From this sample-buffer solution, 10 μL of the denatured sample and 5 μL of the protein standard were loaded into an 18-well precast gel.

5.2.8 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of each dried collagen sample were recorded using a JASCO FT/IR-6X FTIR spectrometer equipped with an ATR PRO ONE X attachment. The spectra were recorded over a wavenumber range of 400 to 4000 cm^{-1} , with a resolution of 4 cm^{-1} , based on the accumulation of 50 scans.

5.3 Results and discussion

5.3.1 Extract yield

The extraction yield of collagen using modified acid-enzyme methods was determined, with average values and standard deviations (% w/w) as follows: 2.81% $\pm$ 0.40 for one-step acid-enzyme (AE1S), 0.23% $\pm$ 0.03 for acid extraction step 1 (A2), 2.84% $\pm$ 0.60 for acid-enzyme step 2 (AE2), 3.07% $\pm$ 0.63 for the total two-step acid-enzyme (AE2S), and 5.09% for the large-batch acid-enzyme extraction from pickled pelts. Extraction yields were calculated based on the yield and mass balance method detailed in Appendix A. A Student's t-test was performed to compare AE1 and A2, as well as AE1 and AE2S, with

no significant differences observed in either comparison ($p > 0.05$). Incorporating pepsin in the extraction resulted in higher yields. However, an initial acid extraction before an acid-enzyme extraction had minimal effect on the total yield when the two-step and the one-step acid-enzyme extractions were compared. The extraction from pickled pelts resulted in the highest yield compared to both the one-step and two-step acid-enzyme extractions from raw skins. This may be due to the pre-tanning (soaking, fleshing, unhairing, liming, deliming, bating and pickling) process that removes fat, hair, flesh and non-collagenous proteins leaving the collagen in the skin and creating a more open matrix (due to the removal of other components of the skin) that facilitated a more efficient penetration of extraction solutions.

5.3.2 HYPQ

Hydroxyproline content was quantified using the calculation method detailed in a previous study [362]. Calculated hydroxyproline and collagen content for each sample are presented in Table 11. Collagen extracted at 4°C for 48 hours showed that, despite its low yield, A2 extraction contained the highest hydroxyproline content. The AE1S and AE2 extractions produced similar results, with hydroxyproline content slightly lower than A2. The hydroxyproline content in the large-batch extraction was marginally lower than in the acid-enzyme extraction samples.

When collagen yield was expressed relative to dry skin weight, results indicated that the two-step acid-enzyme extraction method did not provide a significant advantage in terms of either overall yield or collagen content. Studies optimizing collagen extraction suggest that acetic acid concentrations above 0.5 M but below 0.8 M improve extraction yields [51, 363-365]. However, despite the higher acetic acid concentration, extraction time remained a key factor influencing both yield and collagen content. In a previous study [362] [232], where collagen was extracted over seven days at 0.5 M acetic acid with and without pepsin, the **showed more collagen content** yields and collagen content were higher, highlighting the importance of extraction time in maximizing collagen recovery.

For shorter extraction periods, increasing acetic acid concentration and incorporating pepsin produced similar yields. However, differences in collagen content were more pronounced. Although the collagen extract in this study contained a substantial amount of collagen, these findings emphasize that extraction time is a critical parameter for maximizing collagen content. The large-batch extraction from pickled pelts exhibited

similar effects, though this was offset by a higher overall yield, despite relatively similar collagen content. The extract yield from pickled pelts was 5.09%, containing 40.39% $\pm$ 0.17 collagen.

Table 11 Hydroxyproline and collagen content of acid step, acid-enzyme step, acid-enzyme and large batch samples.

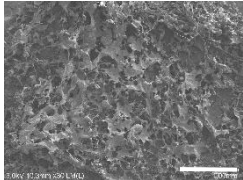
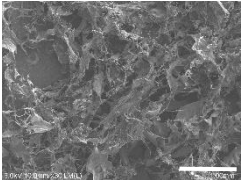
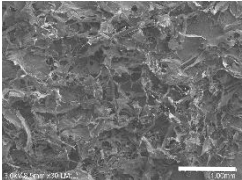
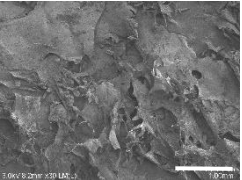
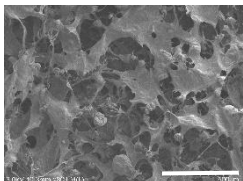
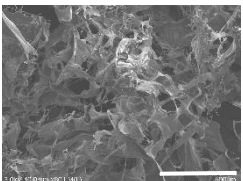
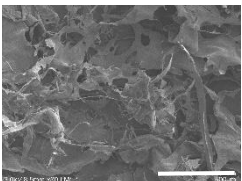
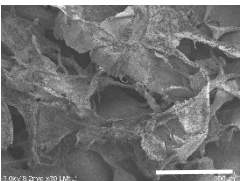
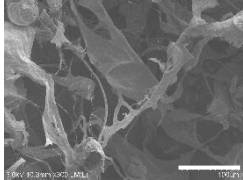
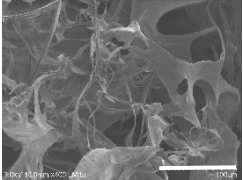
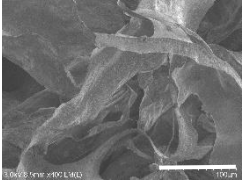
Sample	Extracted yield (g extract / g skin)	Hydroxyproline content (mg g ⁻¹ dry product)	Collagen in dry product (%)	Collagen extracted (mg g ⁻¹ dry raw skin)	Extracted collagen relative to the total amount of collagen in the raw skin (%)
A2	0.23 $\pm$ 0.03	57.81 $\pm$ 0.85	46.25 $\pm$ 0.68	1.01 $\pm$ 0.08	0.29 $\pm$ 0.03
AE2	2.84 $\pm$ 0.60	51.10 $\pm$ 0.30	40.88 $\pm$ 0.24	10.75 $\pm$ 2.14	3.07 $\pm$ 0.63
AE1S	2.81 $\pm$ 0.40	51.54 $\pm$ 0.03	41.23 $\pm$ 0.02	11.58 $\pm$ 1.66	3.31 $\pm$ 0.40
LBPP	5.09	50.50 $\pm$ 0.22	40.39 $\pm$ 0.17	20.55 $\pm$ 0.09	5.87 $\pm$ 0.025

A separate study optimized collagen extraction from delimed sheepskins using response surface methodology, employing acid hydrolysis with parameters such as an acetic acid solution mixing ratio of 0.04 (1/40 g/L), an extraction time of three days, and collagen precipitation using NaCl at 5% concentration after centrifugation [365]. This method resulted in a significantly higher extraction yield of 25.75%, though the collagen content of the extract was not reported. Another study using a less concentrated acetic acid both with and without pepsin but with significantly longer extraction time of 7 days, showed higher collagen content (68.1% and 59.3%, respectively). Moreover, other than collagen approximation, hydroxyproline content provides information thermal stability of the collagen extracted. Hydroxyproline provides thermal stability to the collagen by forming -O-H $\cdots$ O=C=C hydrogen bonds and stereochemical characteristics of the pyrrolidine ring [366].

5.3.3 SEM

Freeze-dried collagen samples were examined under SEM at various magnifications, as shown in Table 12. After lyophilization, differences in sponge-like texture were observed between samples extracted using only acetic acid and those extracted with acetic acid and pepsin. In acetic acid only extraction, collagen samples exhibited smaller pores. Additionally, large pores were present in the acetic acid and pepsin-extracted samples, creating a more open network of pores.

Table 12 SEM images of the freeze-dried collagen from the two-step acid and acid-enzyme, one-step acid-enzyme, and extraction from pickled pelts.

Scale	A2	AE2	AE1S	LBPP
1 mm				
500 um				
100 um				

The large-batch extraction resulted in larger pores but displayed a more closed structure with fewer pores than the other extraction methods. The differences in porosity between enzyme-assisted and acid-only extractions were attributed to disaggregation due to autolysis [367]. Enzymatic digestion leads to shorter collagen fibrils, creating larger pores with less connectivity and an overall more open collagen structure, indicative of the presence of shorter, lower molecular weight fibrils.

The rope-like texture observed in two-step acid and acid-enzyme extractions in Figure 12 was more pronounced than in one-step acid-enzyme extraction and was even less apparent in the large-batch extraction from pickled pelts. This may be due to the effect of the acid extraction in the first step opening the matrix by cleaving bonds and helping with molecular separation due to the low pH, and then the residual skin is exposed to the acetic acid and pepsin solution for the step two extraction creating more rope-like structures [111, 116]. Furthermore, the structural differences suggest greater disintegration of fibril bundles in the enzyme-assisted methods [368].

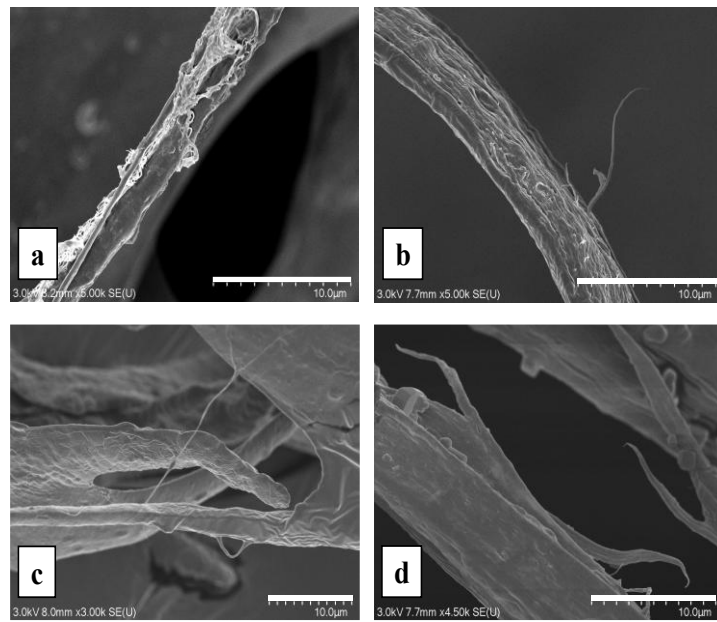


Figure 12 Rope-like structure observed in the collagen samples extracted in the modified method. Scale bar is 10 μ m. SEM images are as follows: (a) A2, (b) AE2, (c) AE1, and (d) LBPP.

5.3.4 SDS-PAGE

SDS-PAGE was performed using an 18-well precast gel, with the first and last wells left blank for band clarity. The second well was labelled as lane 1, continuing sequentially (Figure 13). The SDS-PAGE results corroborate SEM findings, showing that extractions involving acetic acid and pepsin produced bands around the ~ 20 kDa mark, indicating the presence of short fibrils. This band is more evident in the acetic acid pepsin extractions from raw sheep skin compared to the sample from pickled pelts.

Enzyme-assisted extractions also exhibited a higher proportion of high molecular weight fibrils compared to acid-only extractions, suggesting a greater presence of γ -chains (intra-collagen cross-links between three α -chains). Conversely, acid-extracted samples contained more β -chains (cross-links between two α -chains). The $\alpha 1$ and $\alpha 2$ collagen chains were present at approximately ~ 100 kDa for all samples [369]. Acid-extracted samples appeared to have slightly higher proportions of α -chains than those extracted with pepsin. The molecular weight distribution of collagen samples is an indicator of various properties, including antioxidant activity, solubility, and emulsifying and foaming

capacities [367].

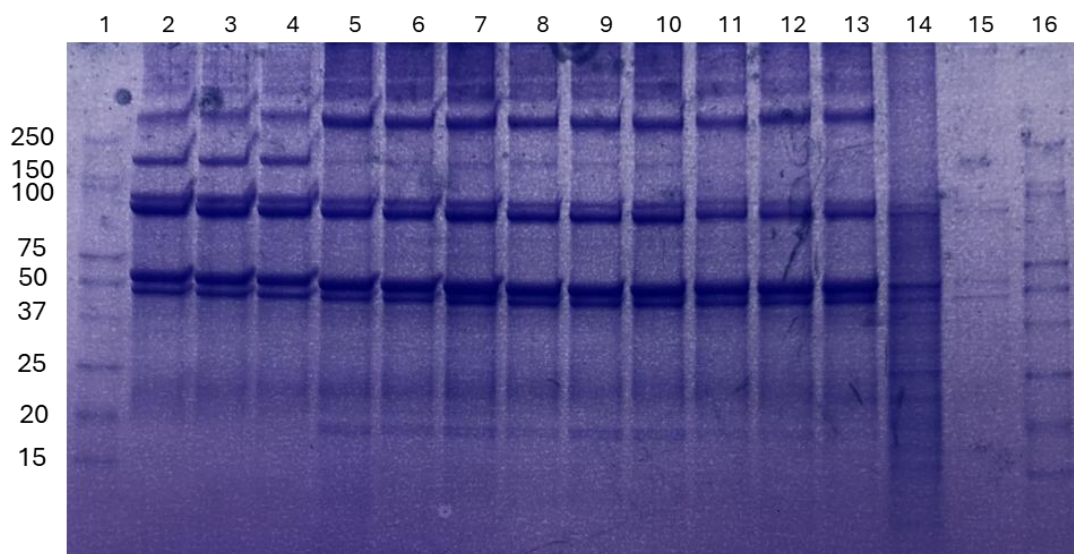


Figure 13 SDS PAGE results for the modified extraction methods. Lanes are as follows: (1) protein marker, (2-4) A2, (5-7) AE2, (8-10) AE1, (11-13) LBPP, (14) gelatin standard, (15) collagen standard, (16) protein marker. Molecular weights are labelled to the left of the protein marker.

5.3.5 FTIR

FTIR spectra of collagen extracted using different modified methods were consistent with characteristic collagen profiles as well as collagen sourced from sheep (Figure 14) [42, 362, 365, 370]. The positions of five key amide bands for type I collagen are summarized in Table 13 [371]. Variations in FTIR bands were influenced by extraction method, amino acid composition, and peptide sequence differences among samples [372]. The Amide A band, associated with NH stretching vibrations, was observed between 3300 and 3500 cm^{-1} . A peak near 3300 cm^{-1} indicates hydrogen bonding of the NH group, while a peak between 3400 and 3440 cm^{-1} suggests the presence of free NH stretching. The pickled pelts extract exhibited a shift to a lower frequency (3279 cm^{-1}), while other samples displayed peaks around 3301 – 3302 cm^{-1} . Moreover, symmetry of amide A peaks can also be an indication of the low water content of the collagen samples [373].

The Amide B band, related to NH and asymmetric CH_2 stretching, was observed at approximately $\sim 3100\text{ cm}^{-1}$. The acid-extracted sample showed a peak at 3090 cm^{-1} ,

while acid-enzyme samples had peaks at $2922\text{--}2932\text{ cm}^{-1}$. The difference in wavenumber and amplitude between these samples suggests differences in secondary structure of the extracted collagen [372].

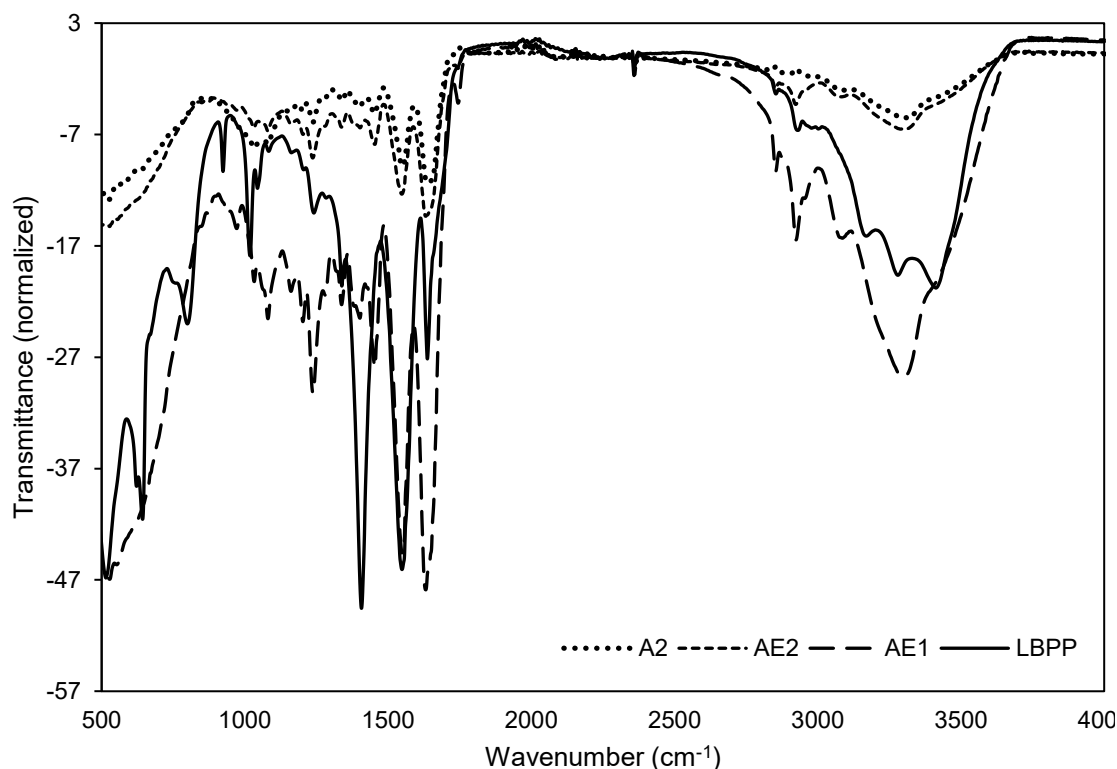


Figure 14 FTIR results for the modified extraction methods: acid step (A2), acid-enzyme step (AE2), one step acid-enzyme (AE1), and large batch from pickled pelts (LBPP).

The Amide I band provides information on the secondary structure and is a particularly sensitive indicator of peptide linkage, which corresponds to C=O stretching and hydrogen bonding with COO^- , was detected between 1600 and 1800 cm^{-1} [341, 374]. Acid-extracted collagen exhibited a peak at 1650 cm^{-1} , while enzyme-assisted extractions showed peaks between 1632 and 1636 cm^{-1} . The lower wavenumber in enzyme-assisted extractions suggests a partial loss of native collagen structure [371].

The Amide II band is associated with C–N stretching and NH bending vibrations, appeared between 1470 and 1570 cm^{-1} , with peaks near $1548\text{--}1552\text{ cm}^{-1}$. The resulting positions indicate the presence of water in the samples as well as the presence of tyrosine, asparagine and glutamine [325, 375, 376].

The Amide III band, which results from CN stretching, NH and CH bending, and CC stretching, was detected between 1250 and 1350 cm^{-1} [374]. Peaks were found at 1238–1242 cm^{-1} . Amide III peaks are usually about 5 times lower in amplitude compared to amide I [377].

Specific band ranges for amide I, II and III indicating peptide linkage are present in all samples [378]. The ratio of IR absorption intensity between Amide III and Amide II was close to 1.0 in all samples, confirming the presence of triple-helix structure [373, 379].

Table 13 Amide peaks for modified collagen extraction samples.

Amides	Functional group and vibration modes	Band from literature	A2	AE2	AE1	LBPP
A	NH stretching	3300-3500	3302	3301	3301	3279
B	NH, CH ₂ stretching	~3100	3090	2922	2922	2932
I	C=O stretching, H bond with COO ⁻	1600-1800	1650	1632	1636	1636
II	C–N stretching, NH bending	1470-1570	1549	1552	1548	1548
III	CN stretching, NH and CH bending, CC stretching	1250-1350	1238	1240	1238	1242

5.4 Conclusion

This study investigated collagen extraction from raw and pickled sheepskins using modified acid-enzyme methods. The findings demonstrated that while the one-step and two-step acid-enzyme extractions from raw sheepskins yielded similar amounts of collagen, the use of pickled pelts improved extraction efficiency. The large-batch acid-enzyme extraction from pickled pelts yielded the highest collagen recovery (5.09%), suggesting that pre-treated sheepskins offer a more efficient alternative to raw sheepskins for collagen extraction.

Hydroxyproline quantification revealed that despite the higher overall yield in pickled

pelts, the collagen content was comparable to raw sheepskin extractions, reinforcing the idea that optimizing extraction parameters is crucial for maximizing collagen recovery. SEM analysis indicated that pepsin-assisted extractions resulted in a more open collagen network, potentially influencing its functional properties. Additionally, increasing the acetic acid concentration to 0.7 M improved collagen yield compared to previous studies using lower acid concentrations. Furthermore, the use of pepsin improved the extractability of collagen.

Overall, these findings highlight the importance of pre-treatment processes, extraction conditions, and enzyme-assisted methods in improving collagen yield from sheepskin. Further research is needed to optimize large-scale collagen extraction.

Chapter 6. Protein coatings on cellulose sheets

6.1 Introduction

Collagen is the most abundant protein in the extracellular matrix of living organisms [380]. The term collagen originated from two Greek words *kolla* meaning glue and *gene* meaning generation [381]. Collagen can be extracted from different sources. Most commonly from bovine (beef), porcine (pork), ovine (lamb), hircine (goat) as well as various marine animals [57, 137]. It is a fibrous protein composed of polypeptide chains conformed into a characteristic triple helix shape. Its inherent mechanical stability and biological functionality make it a highly sought-after component in the development of bio-based composites [382].

Partial denaturation of collagen produces gelatin [383]. It has water-soluble molecules that form shorter chains and randomly oriented coils [384]. It is commonly sourced from porcine and bovine [385]. It is notable for its film-forming ability, adhesiveness and biodegradability [386].

Studies have shown collagen and gelatin as promising versatile natural polymers that are used in a vast array of applications. Literature has extensively covered the application of both in medicinal and surgical treatment [142, 387], cosmetics [60], smart and edible food packaging [137, 386], etc. Majority of the collagen-based and gelatin-based composites include other added components as a filler, additive, preservative, and/or functional components to improve mechanical and functional properties. However, the fundamental interaction between collagen and fibrous substrates like wood pulp fibres without such additives remain less explored. In this study, collagen and gelatin interactions with self-assembled wood pulp fibres are investigated for their mechanical, thermal and surface properties.

6.2 Experimental method

6.2.1 Extraction of large batch collagen

6.2.2 Materials

For the extraction of collagen used for the composite, whole pickled sheepskins were provided by the NZ Leather & Shoe Research Association (LASRA, Turitea, New Zealand). The sheepskins were stored at -20 °C until processed for extraction. The

following materials and chemicals were used in the study: acetic acid (99.7%, LabServ), sodium chloride (99%, Summit AG Salt Fine), sodium hydroxide (Scharlau), sodium acetate (Scharlau), pepsin (Sigma Aldrich). For isolation, a diafiltration unit with a Pellican cassette (10 kDa Ultracel) was used for the large batch process. For the hand sheets, unbleached cellulose nano fibres (made from *Pinus radiata*) (Oji Fibre Solutions, Kawerau, New Zealand), gelatine 150 bloom (Davis Gelatine Co., Beaudesert, Australia), gelatine 250 bloom (Gelita, Beaudesert, Australia).

6.2.3 Extraction

Before extraction, sheepskins were thawed and pre-treated at 4°C. The skins were washed, drained, and soaked in a 0.5 M NaOH solution for 24 hours to facilitate wool removal. Afterward, they were rinsed thoroughly until the water reached a neutral pH and then mechanically minced.

The minced sheepskin was then treated in a 1:1 solution of 0.1 M NaOH and 0.1 M NaCl (1:25 w/v) for 12 hours, with continuous stirring and solution replacement every 2 hours. Following this, the skins were rinsed with water until a neutral pH was achieved. To remove minerals, they were soaked in a 1:1 solution of 0.1 M HCl and 0.1 M NaCl (pH 7.5) for 2 hours, maintaining the same skin-to-solution ratio, and then washed again until the pH was neutral.

The pelts were soaked in 0.7 M acetic acid at a 1:25 (w/v) ratio, with pepsin at a concentration of 1 g·L⁻¹ of acid solution, stirred at 4°C for 48 hours. After extraction, the liquid was separated from the skins, and the liquid was heated at 90°C for 10 minutes to deactivate the pepsin.

Diafiltration was performed using a filtration unit equipped with a 10 kDa Ultracel cassette. The liquid extract underwent diafiltration for seven days, with 3 L of 0.1 M acetic acid replenishing the stirred tank for the first three days, followed by 3 L of water for the remaining three days. The resulting collagen solution adjusting to pH 6.0 using 1 M sodium hydroxide and then freeze-dried at -60°C.

6.2.4 Composite fabrication

The bulk fibres were dispersed in water for 24 hours with fibre-to-water ratio of 3:100 (w/v). A supermass colloid machine (SMC) (Masuko Sangyo Version IV ultrafine

friction grinder, Saitama, Japan) was used to defibrillate the fibres. The fibre slurry was processed four times at a set distance between the grinding disks, twice at 200 μm and twice at 100 μm . After processing, excess water was squeezed out and the fibres were stored damp at 4°C until further processing.

Damp fibres were dispersed once again in water (1:15 v/v) to homogenise for 1 min to ensure dispersion and prevent aggregation. The slurry was then transferred to a shallow basin and added more water 1:6 (v/v) slurry to water ratio. The sheets were fabricated by using a framed sieve, pictured in Figure 15, into the loose slurry. Making a gentle swaying motion, allowing the fibres to self-assemble, level and distribute throughout the perimeter of the sieve. The sheets were then transferred to a calico fabric using a sponge to release the pulp sheet from the sieve. The sheets are dried on the fabric at 45°C overnight. After drying, the sheets were separated from the fabric sheet. The dried sheets thickness were between 0.2 – 0.3 mm. The sheets were stored at 23°C and 50% relative humidity until further use.

Gelatin with 150 and 250 bloom were used for low and high bloom gelatin coating, respectively. Gelatin solution with concentrations of 3% and 5% (w/w) were hydrated for 30 minutes and then heated at 60°C for 30 minutes, occasionally stirring. The collagen coating solutions with concentrations of 3% and 5% (w/w), were hydrated for 30 minutes and then homogenised for 5 minutes. The coating solutions were cooled down to 30°C before being used for coating. The sheets were cut into 100 mm by 200 mm, immersed into the coating for 10 seconds and transferred onto a tray, and dried at 37°C overnight. Once dried, the coated sheets were stored in a 23°C and 50% relative humidity chamber until ready for analysis. Coated and uncoated sheet samples will be referred to according to the coating and are as follows: collagen 3% (C3), collagen 5% (C5), high bloom gelatin 3% (HG3), high bloom gelatin 5% (HG5), low bloom gelatin 3% (LG3), low bloom gelatin 5% (LG5), no coating (NC). At least 7 samples for each coating were fabricated and analysed.

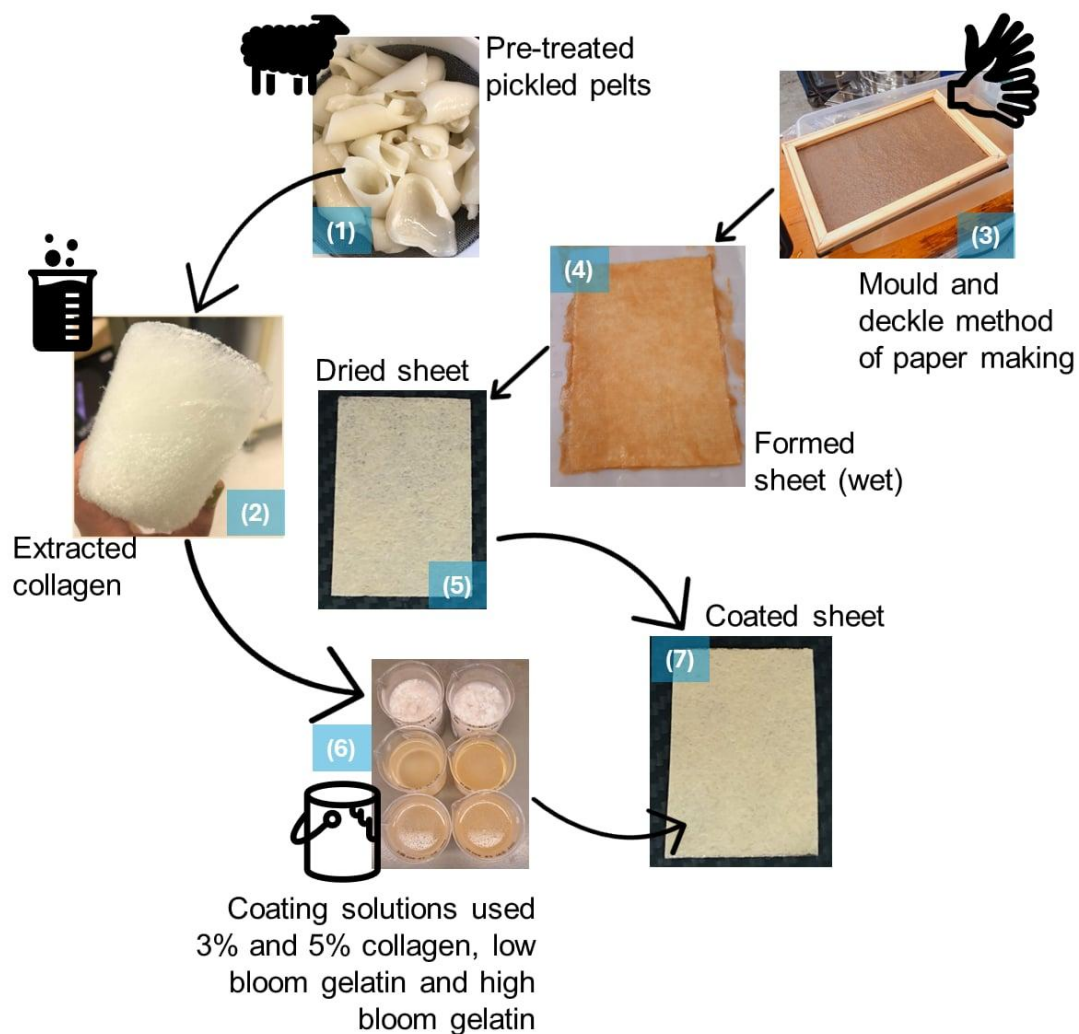


Figure 15 Collagen and gelatin coated sheet fabrication method.

6.2.5 Fibre length analysis

Fibre length was analysed using an OpTest Fibre Quality Analyser (FQA). A small amount of damp fibre was squeezed free of excess water and sourced from the same bulk material used in composite fabrication and suspended in 200 mL of water for each analysis. Measurements were conducted in triplicate using three independent subsamples to ensure reproducibility.

6.2.6 Tensile test

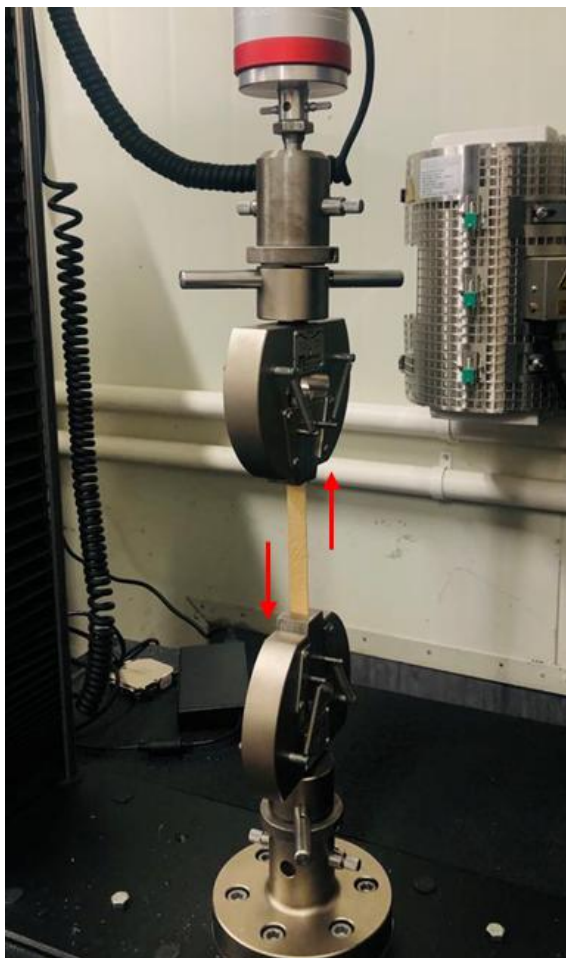


Figure 16 Instron tensile testing setup

A uniaxial tensile test was conducted in accordance with ASTM D828-22. An Instron 5982 universal testing machine (Instron, Massachusetts, US) equipped with a 500 N load cell (for coated) (pictured in Figure 16) and 10 N (for uncoated) was used to carry out tensile testing. Clamp span distance of 100 mm and crosshead speed 25 mm min^{-1} . Sample width of 15 mm was used and were conditioned at 23°C and 50% relative humidity for at least 24 hours prior to testing. At least seven samples for each sample type were tested for tensile testing.

6.2.7 Oxygen Permeability

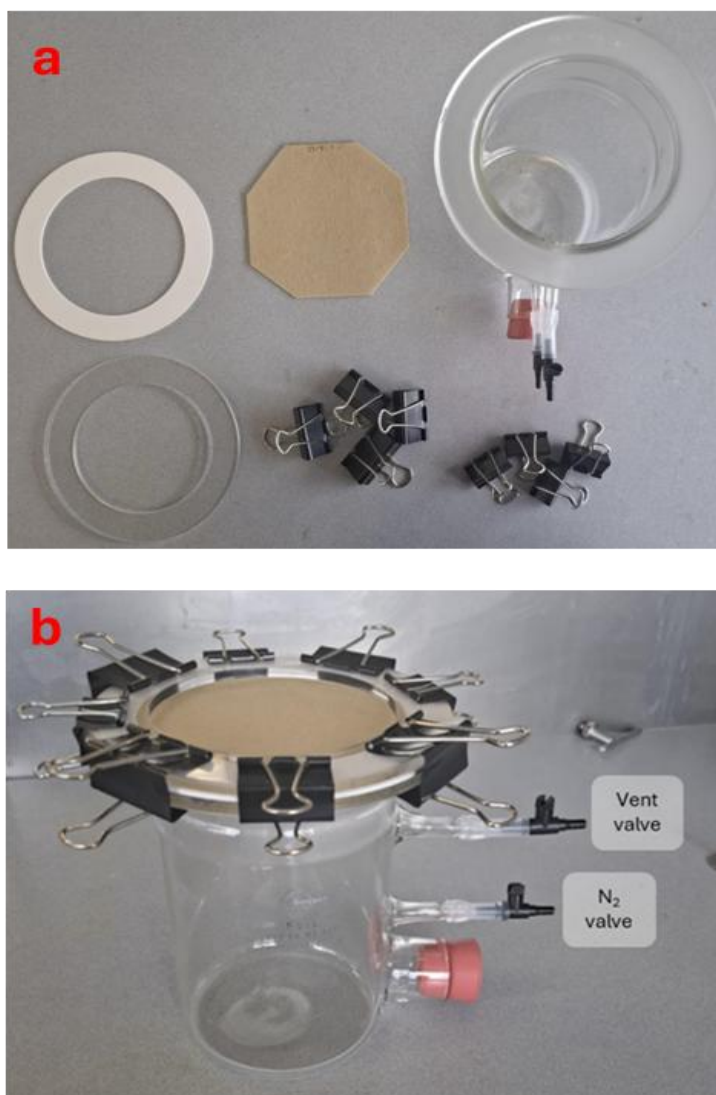


Figure 17 Oxygen permeability setup (a) parts of the setup and (b) assembled setup.

Oxygen permeability was carried out with a modified beaker setup, shown in Figure 17. Samples were 100 x 100 mm squares with corners cut to fit the lip of the beaker and lip band. An oxygen meter, WatchGas SST1 O₂ Gas detector (WatchGas detection, Capelle aan den IJssel, Netherlands), was placed inside the beaker to measure oxygen levels (%). The sample is placed on the beaker opening and secured with a rubber gasket, lip band and clips to secure in place. There are two apertures on the side of the beaker with valves attached. One was for the nitrogen supply and the other serving as a vent. Once the sample is in place, the interior of the chamber is purged using a nitrogen source for 15 minutes, while the vent valve remained open. An additional 5 minutes of purging to allow oxygen level to stabilize inside the beaker (reaching Oxygen level range 4–9%).

The purging was stopped by closing the nitrogen source, and the nitrogen valve and vent valve of the beaker. The initial oxygen levels were recorded and assigned as time = 0 s. The oxygen levels were monitored over time until it reached a stable oxygen levels or when equilibrium is achieved with the atmospheric oxygen level ($\sim 20.9\%$). The oxygen level and time elapsed were recorded.

6.2.8 Tear test

A tear test was conducted based on ASTM D2261-13(2024). The test was carried out using the Instron 5982 universal testing machine setup similar to the tensile analysis with the configuration and tested samples (Figure 18). Sample dimensions were set to 26 mm by 200 mm to accommodate the width of the existing clamp with a longitudinal cut of 75 mm. The clamp distance was set to 75 mm with a testing speed of 300 mm min^{-1} . The clamp tore along the longitudinal cut and was stopped when the tear was at least 75 mm in length. Samples with breakage within 5 mm of the edge of the clamp were disregarded. Repeats of at least 5 samples were used for the tear test.

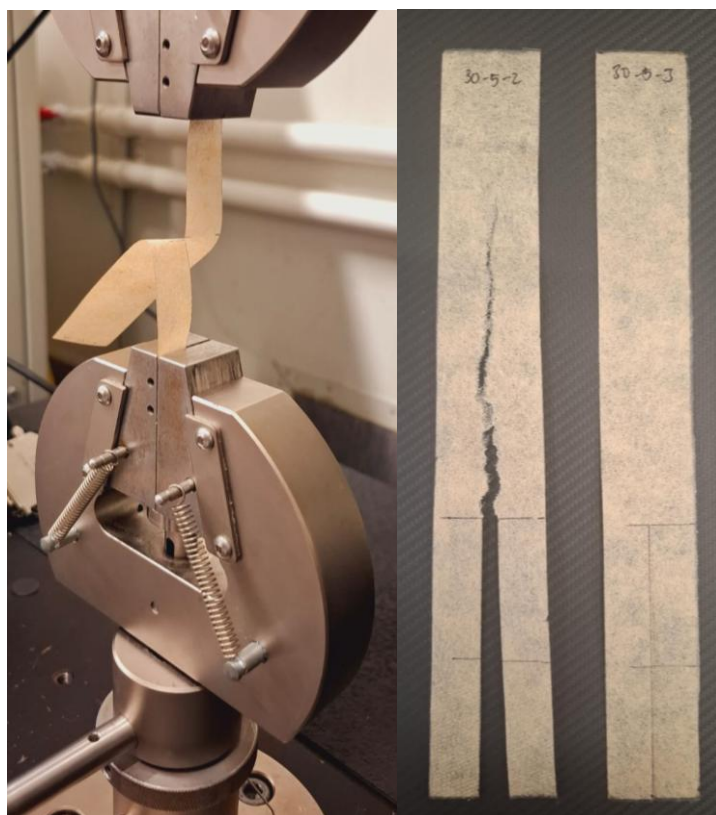


Figure 18 Tear test sample setup (left), and samples after and before tear test (right).

6.2.9 FTIR

The FTIR spectra of each dried collagen sample were recorded using a JASCO FT/IR-6X FTIR spectrometer equipped with an ATR PRO ONE X attachment. The spectra were recorded over a wavenumber range of 400 to 4000 cm^{-1} , with a resolution of 4 cm^{-1} , based on the accumulation of 50 scans.

6.2.10 TGA

Thermogravimetric analysis of the coated and uncoated sheets was conducted using Perkin Elmer STA 8000 thermal analyser. The sample weighing ~ 10 mg was placed in a crucible. The analysis was performed under argon atmosphere with a flowrate of 40 ml min^{-1} , heating rate of $10^\circ\text{C min}^{-1}$, and heating temperature range from 30 to 600°C .

6.2.11 SEM

Sheet samples showing both sides of the sheets (side facing up from the tray denoted as Side 1 and the side adjacent to the tray when drying denoted as Side 2) were mounted on SEM stubs and were platinum coated (~ 5 nm). Samples were observed under SEM (Hitachi Regulus SU8230) at an acceleration voltage of 3 kV and with magnifications of 1 mm and 100 μm .

6.2.12 Contact angle measurement

Contact angle measurement is based on EN ISO/TS 14778 Paper and board – measurement of water contact angle by optical methods. The samples were placed on a flat levelled platform. A 4 μL water droplet was delivered to the surface of the sample. Angles were measured at three different contact times: 0.1 s, 1.0 s, and 10.0 s. At least 5 samples for each coating type were taken for measurements.

6.3 Results and discussion

6.3.1 Fibre length analysis and coating solution viscosity

The components of the sheets were observed, fibre length and width, and collagen and gelatin coating viscosities were measured. Table 14 shows the length and width measurements of the processed wood pulp fibre used in fabricating the hand sheets.

Table 14 FQA results on fibre length and width.

Subsample	Length (mm)	Width (um)
1	2.67 ± 0.03	36.2
2	2.67 ± 0.03	36.4
3	2.75 ± 0.03	36.5

The viscosity of the coating solutions was measured (AMETEK Brookfield RVDV2T Viscometer, Massachusetts, U.S.A.) at 20°C and 30°C for collagen and gelatin coating solutions, respectively, shown in Table 15. Consequentially, the grammage of sheets coated in collagen was higher compared to the gelatin coated sheets. The grammage was also higher for the 5% coated sheets than the 3% coated sheets.

Table 15 Viscosity measurements of the different coatings.

Sample	Viscosity (Pa·s)	Temperature (°C)	Spindle number	Rotation speed (rpm)
Collagen 3%	0.14 ± 0.05	20.0	3	20
Collagen 5%	1.715 ± 0.02	19.8	3	20
High bloom gelatin 3%	0.008 ± 0.02	31.3	2	20
High bloom gelatin 5%	0.014 ± 0.02	29.2	2	20
Low bloom gelatin 3%	0.008 ± 0.02	28.2	2	20
Low bloom gelatin 5%	0.01 ± 0.02	29.6	2	20

6.3.2 Sheet properties

Table 16 Sheet properties: coating percentage, before (initial) and after (final) coating sheet thickness, and grammage

Sample	Initial thickness (mm)	Coating (wt.%)	Final thickness (mm)	Grammage (g·m ⁻²)
Collagen 3%	0.24 ± 0.02	43.29 ± 3.78	0.19 ± 0.03	74.24 ± 7.73
Collagen 5%	0.23 ± 0.03	55.57 ± 5.44	0.19 ± 0.02	92.78 ± 9.49
High bloom gelatin 3%	0.28 ± 0.01	28.66 ± 1.96	0.31 ± 0.04	58.37 ± 6.30
High bloom gelatin 5%	0.20 ± 0.02	49.99 ± 4.64	0.27 ± 0.02	66.96 ± 14.82
Low bloom gelatin 3%	0.22 ± 0.02	34.51 ± 2.72	0.28 ± 0.04	60.14 ± 4.87
Low bloom gelatin 5%	0.20 ± 0.03	47.17 ± 4.28	0.24 ± 0.03	68.19 ± 5.50
No coating	0.24 ± 0.02	-	-	41.90 ± 4.44

The coated sheets are measured before and after coating, detailed measurements are in Appendix B, the thickness, coating (wt%), and grammage are shown in Table 16. An observation from measuring dimensions of the coated sheets is that the collagen-coated sheets retained more coating by weight (wt%) than their gelatin coated counterparts. High bloom gelatin coated sheets contained more coating than low bloom gelatin. Higher molecular weight chains which are present in a descending order from collagen, high bloom gelatin (250 bloom) and low bloom gelatin (150 bloom).

Figure 19 shows a simplified illustration of the coated sheet before and after drying. High molecular weight promotes a more compact film network, resulting in thinner sheets [388]. In combination with high molecular weight, long drying time of collagen composites also increases this compaction effect by condensing the collagen [389]. Collagen fibrils, even when self-assembled, can form tighter bundles that result in a compact and dense structure [390]. This in turn pulls the fibres closer together. As a result, the sheets are thinner after coating with collagen (3% and 5%) compared to before coating. The overall longer peptide chains in collagen occupied the pore space of the fibre sheets effectively, while gelatin with its shorter chains was less effective in covering the pore space. Furthermore, when gelatin is combined with cellulose have been shown

to effectively absorb and retain water [391]. In addition, the self-assembled short amino acid chains enabled the retention of water, as the composite dries the gaps filled by the water remained leaving an open matrix. Thus, resulting to thicker sheets compared to both the uncoated and the collagen coated sheets. Another factor to consider is that cellulose fibres have the tendency to swell when heat dried [392]. However, with the low temperature used in the drying process of the fabrication ($<60^{\circ}\text{C}$), it had no negative effect on swelling properties of the fibre [393]. The high bloom gelatin coated sheets are thinner than when coated with low bloom gelatin. The sheets are slightly thicker when comparing the thickness before coating and after coating with gelatin.

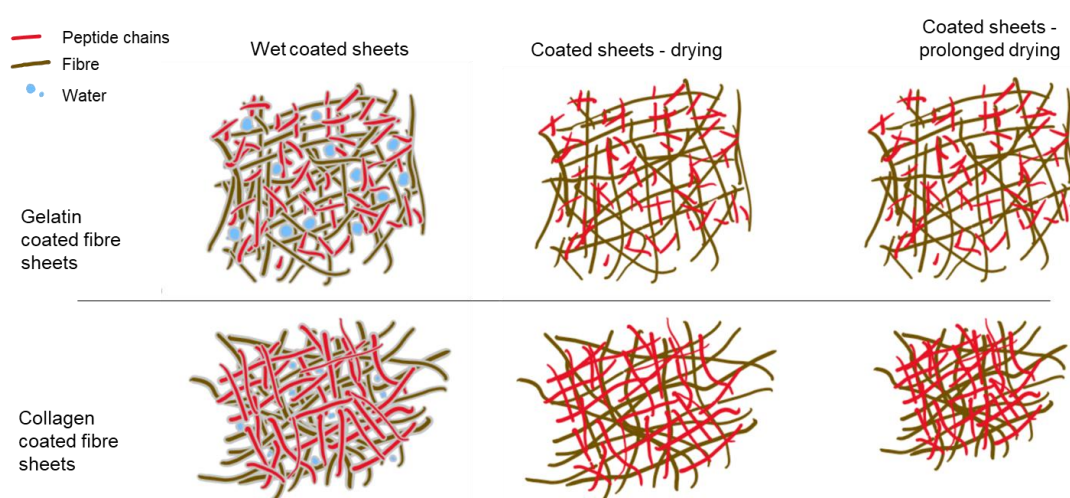


Figure 19 Fibre coated with gelatin and collagen solutions (from left to right) after the coating was applied on the sheet, when drying with the excess water evaporated, and after the coated sheets had been drying for 12 hours showing the densification of the collagen coated sheet and the maintained pore space in the dried gelatin coated sheet. Legends are listed on the top left corner for peptide chains, fibres, and water.

6.3.3 Tensile test

Tensile testing was performed on the samples. From the tensile strength values obtained, gelatin coated sheets showed higher tensile strength values, particularly high concentration low and high bloom gelatin as well as low concentration high bloom gelatin (Figure 20). However, looking at the representative plots for different coated and non-coated sheets of stress-strain curves provided more insight into the tensile properties of the sheets. Table 17 lists the average tensile strength for the coated and uncoated sheets. The high concentration gelatin coated sheets, both low and high bloom,

showed the highest tensile strength but with lower tolerance to strain. Collagen coated sheets at high concentration, on the other hand, provided both high tensile strength and generally better tolerance to strain. Overall, coating the sheets showed significant improvement in tensile properties compared to uncoated sheets.

Table 17 Ultimate tensile strength of coated and uncoated sheets

Sample	Tensile strength (kN/m)
Collagen 3%	1.99 ± 0.42
Collagen 5%	2.97 ± 0.78
High bloom gelatin 3%	3.18 ± 0.27
High bloom gelatin 5%	3.03 ± 0.51
Low bloom gelatin 3%	2.40 ± 0.27
Low bloom gelatin 5%	3.41 ± 0.64
Uncoated	0.20 ± 0.02

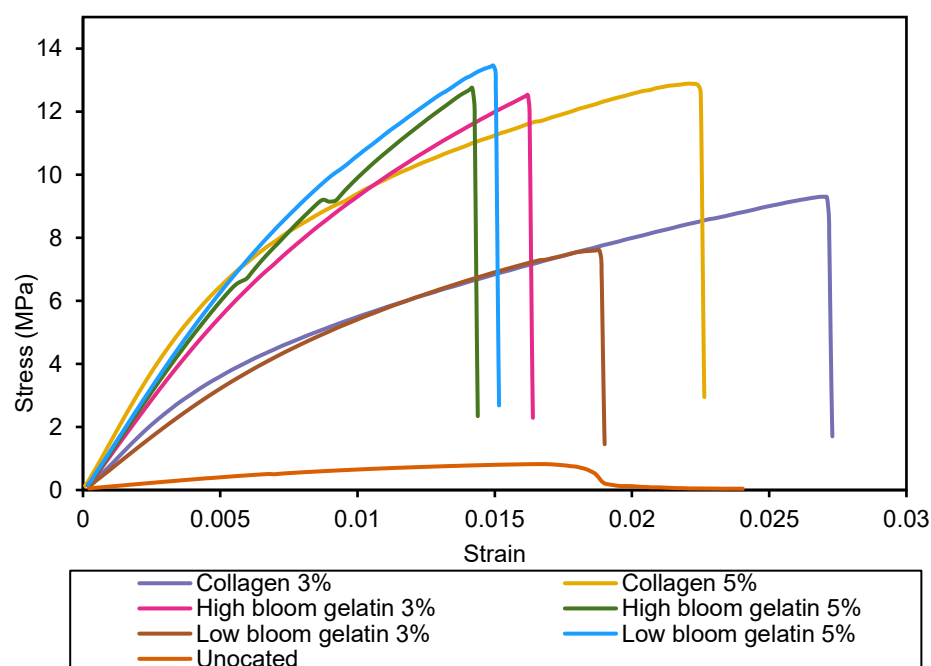


Figure 20 Stress-strain curves for the coated and uncoated cellulose sheets

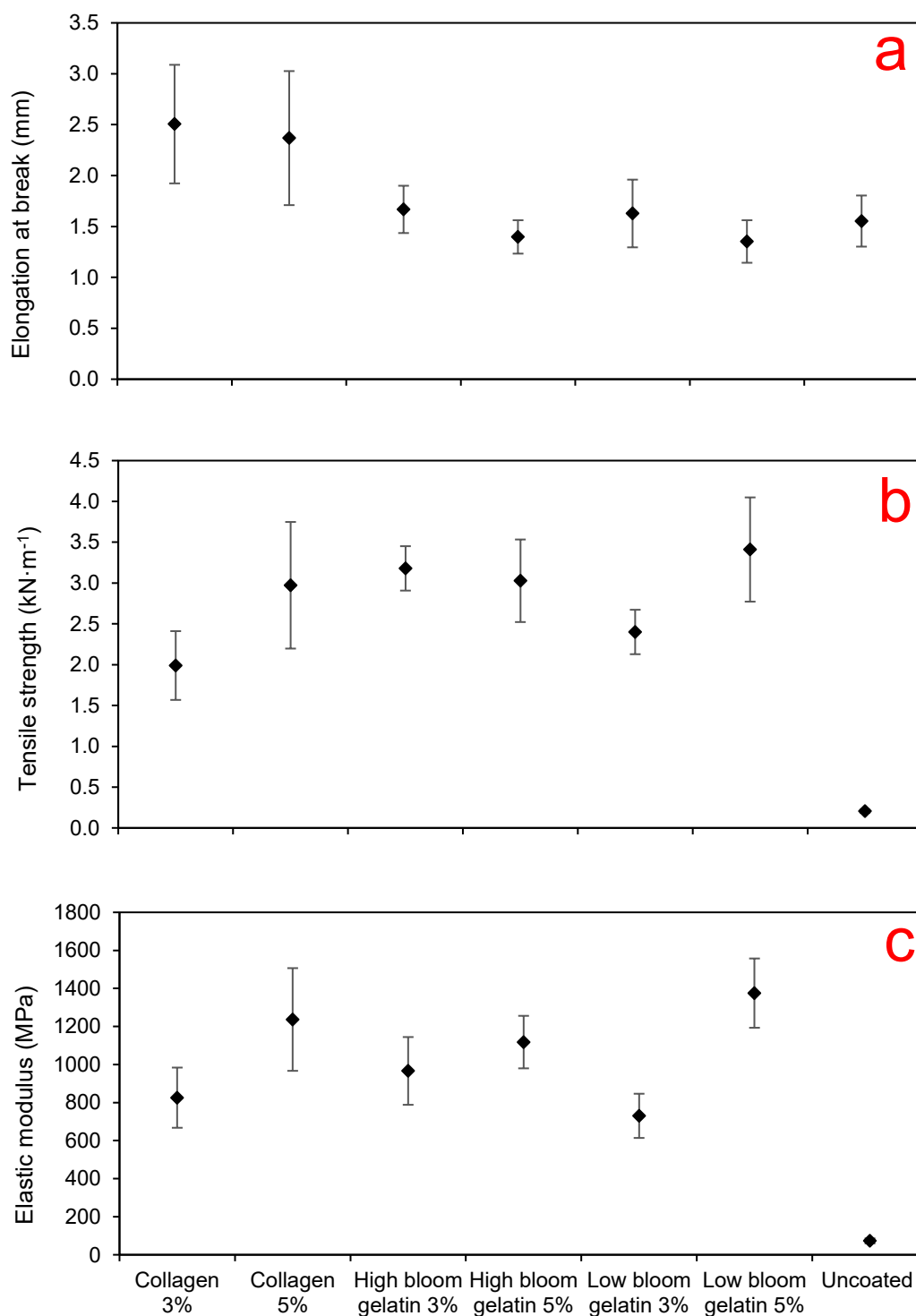


Figure 21 (a) Elongation at break, (b) tensile strength, (c) elastic modulus of coated and uncoated cellulose sheets.

In Figure 21, elongation at break, tensile strength, and elastic modulus are plotted comparing the various coated sheet samples. Elongation at break further shows the ductility and tensile strength of the collagen coated sheets. This is also shown when the ultimate tensile strength was plotted against total elongation in Figure 22. Gelatin

enhances the sheet properties in terms of tensile strength but falls short in tolerating strain and therefore elongation. Nonetheless, coating the sheets in collagen and gelatin improved tensile strength of the sheets by at least ten-fold compared to the uncoated samples.

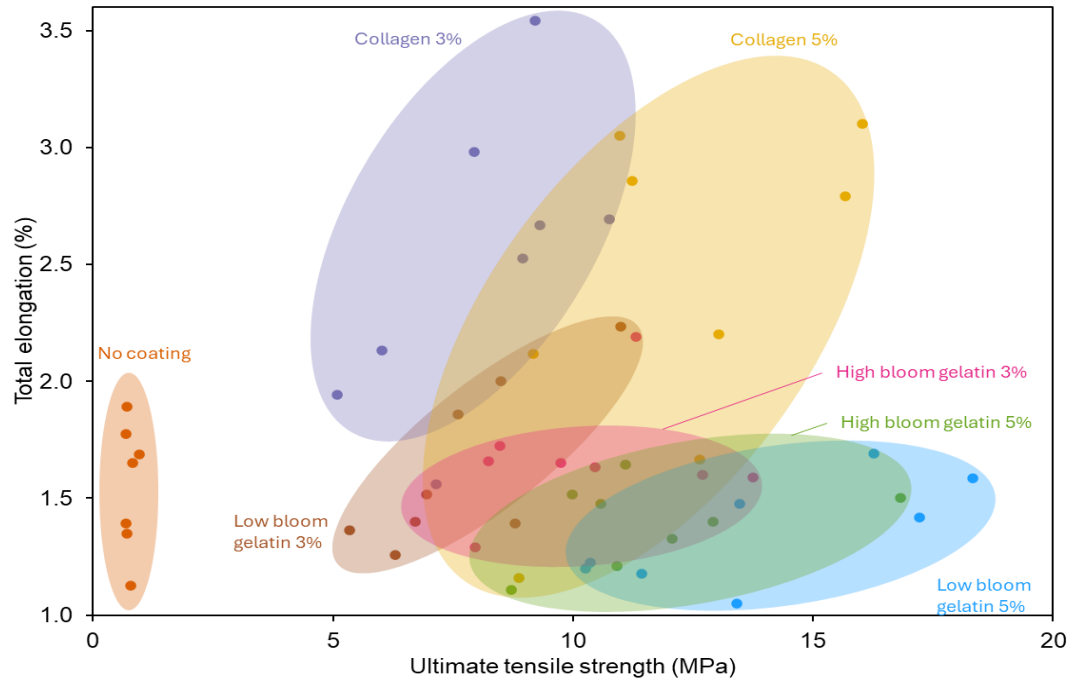


Figure 22 Total elongation as a function of ultimate tensile strength

Unlike other materials like metals and plastics, paper composite materials use force and elongation data to determine elastic modulus. The maximum slope, $\bar{S}_{max}$, from the force-elongation curve, shown in Figure 23, was used to calculate the elastic modulus in Equation 6-1.

$$E = \frac{\bar{S}_{max} \cdot l}{b \cdot t} \quad (6-1)$$

Results of the elastic modulus of the coated and uncoated sheets are shown in Figure 21c shows collagen 5% provided enhanced elastic properties. Low bloom gelatin at 5% also showed high elastic modulus. This may be due to lower molecular weight and lower viscosity contributing to a more evenly distributed sheet as well as the amount of gelatin present in the composite. The latter can also be said for high bloom gelatin at 5%, which showed moderate values for elastic modulus compared to the other coated sheets. The

3% concentrations of all coating types showed relatively similar values in terms of elastic modulus particularly for collagen at 3% and high bloom gelatin at 3%.

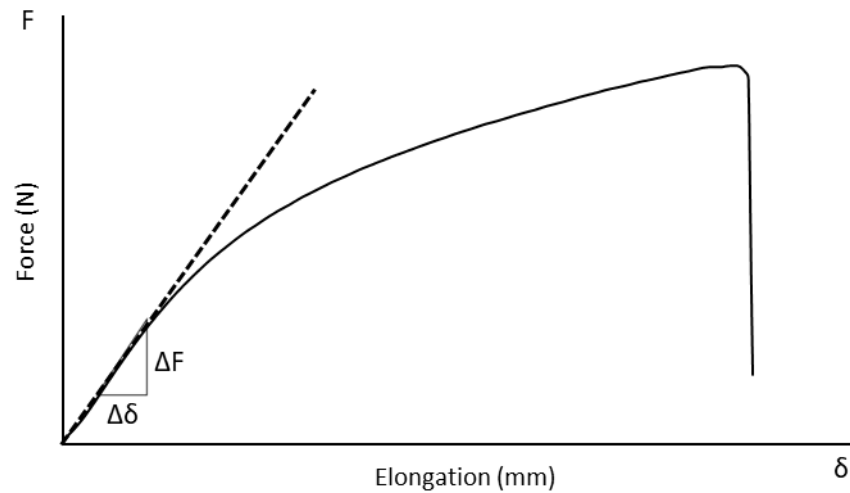


Figure 23 Force-elongation curve from tensile testing to determine the elastic modulus

Tensile energy absorption (TEA) was also determined by calculating the area under the force-elongation curve. Resulting plots are shown in Figure 24. Tensile energy absorption of a material gives information on the durability and toughness, which can be useful in determining whether the material is suitable for certain applications. From the plots in Figure 21, TEA and tensile strength of the collagen- and gelatin-coated sheets have a proportional relationship. The uncoated sheets, on the other hand, had been relatively constant TEA values with the range of tensile strength covered in this study.

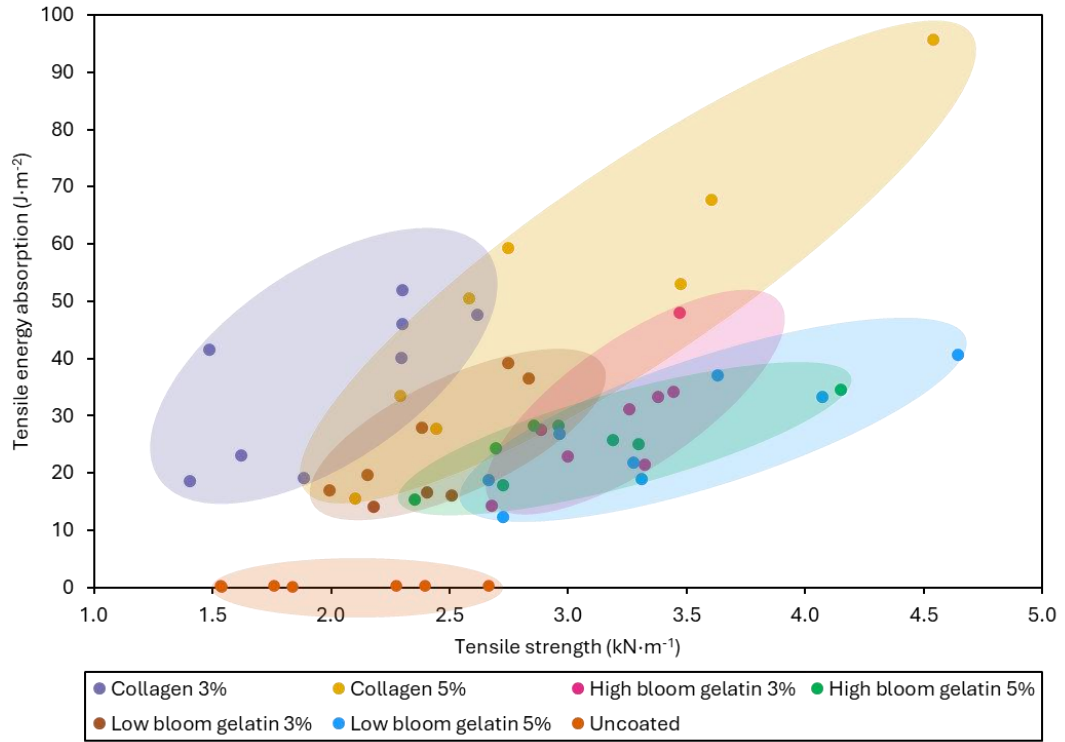


Figure 24 Tensile energy absorption (TEA) as a function of tensile strength

6.3.4 Oxygen transmission rate (OTR)

The oxygen level and time elapsed were recorded and was used to calculate oxygen permeability of the sheets. First-order reaction (equation 1) was used to calculate the oxygen transmission rate (OTR), which covers the volume transfer of oxygen from the atmosphere into the beaker. The first-order reaction is defined as r , the rate of reaction is equal to reaction rate constant, k , directly proportional to concentration of air C_A at time t , inner diameter of the beaker A and beaker volume V . The step-by-step calculation from the rate of reaction to oxygen transmission rate and oxygen permeability is shown below.

Rate of reaction expression (rate per unit volume)

$$-r = kC_A \cdot A \cdot V \quad (6-2)$$

Considering the derivation of eq 6-2 and integration gives:

$$kt \times \frac{A}{V} = \ln \frac{C_{A0}}{C_A} \quad (6-3)$$

Plotting $\ln C_{A0}/C_A$ vs time, slope $m = k \times \frac{A}{V}$. Isolating rate constant k gives

$$k = \frac{m}{A/V} = OTR, \quad \text{units: } \left(\frac{m^3}{m^2 \cdot s \cdot \%O_2} \right) \quad (6-4)$$

Expressing the equation in terms of the pressure by multiplying the equation with final percentage of O_2 which is expressed in atmospheric pressure.

$$OTR \times P_{O_2} = \left(\frac{m^3}{m^2 \cdot s \cdot atm} \right) \quad (6-5)$$

To calculate permeability by multiplying OTR with sheet thickness d

$$Permeability \left(\frac{m^3}{m \cdot s \cdot atm} \right) = OTR \left(\frac{m^3}{m^2 \cdot s \cdot atm} \right) \cdot \frac{d}{10^6} \quad (6-6)$$

The calculation for the OTR and oxygen permeability followed first order kinetics of the material assuming a steady state flux of oxygen. The first linear section of the oxygen concentration log plot was considered with the assumption that the dominant mechanism is diffusion of oxygen into the glass beaker. The data range considered, and supplementary data are detailed in Appendix B. Table 18 shows the OTR and permeability results for the different coated and uncoated sheets. OTR is a material specification that is measured by quantifying the O_2 passing through a unit area of material per unit time under test conditions [394].

A study reported on oxygen permeability results for polyethylene coated sulphate and sulphite paper values ranging from 1000 – 2000 and 100 – 400 $cm^3 \cdot m^{-1} \cdot d^{-1} \cdot atm^{-1}$, respectively [395]. Comparing this with the values obtained from the experimental setup for measuring oxygen transmission rate of the coated and uncoated sheets, showed significantly higher values for oxygen transmission in the setup used in this study. A commercially available baking paper was used in the OTR analysis. Comparing the values of the different coated and uncoated sheets and the baking paper. The collagen 5% coated sheet as well as the baking paper resulted in similar permeability values.

Table 18 Thickness and weight coating percentage of the permeability samples with resulting oxygen permeability and transmission rate of the different collagen and gelatin coated sheets.

Sample	Thickness (mm)	Coating (wt.%)	OTR ($\text{cm}^3 \cdot \text{m}^{-2} \cdot \text{d}^{-1} \cdot \text{atm}^{-1}$)	O ₂ permeability ($\text{cm}^3 \cdot \text{m}^{-1} \cdot \text{d}^{-1} \cdot \text{atm}^{-1}$)
Collagen 3%	0.17 ± 0.02	35.19 ± 6.01	$6.10 \times 10^8 \pm 3.93 \times 10^8$	104.68 ± 69.45
Collagen 5%	0.17 ± 0.02	55.26 ± 8.77	$6.08 \times 10^6 \pm 3.82 \times 10^6$	1.044 ± 0.64
High bloom gelatin 3%	0.28 ± 0.05	32.16 ± 3.67	$1.38 \times 10^9 \pm 1.01 \times 10^8$	386.48 ± 81.12
High bloom gelatin 5%	0.28 ± 0.05	44.46 ± 4.16	$1.00 \times 10^9 \pm 4.65 \times 10^8$	299.86 ± 169.69
Low bloom gelatin 3%	0.24 ± 0.04	29.82 ± 2.55	$1.43 \times 10^9 \pm 8.47 \times 10^8$	339.95 ± 40.69
Low bloom gelatin 5%	0.31 ± 0.02	47.31 ± 5.23	$1.38 \times 10^9 \pm 1.95 \times 10^8$	424.37 ± 48.39
No coating	0.24 ± 0.01	-	$1.62 \times 10^9 \pm 8.81 \times 10^7$	383.83 ± 41.29
Baking paper	0.05	-	$1.98 \times 10^7 \pm 1.96 \times 10^6$	0.99 ± 0.09

6.3.5 Tear test

The tear test provides more information about the coated and uncoated sheets particularly on the adhesion the coating offers between fibres in the sheets and its resistance to tear. Tear test data were recorded as the Instron equipment tears the composite sheets, the force applied (N) and displacement (mm) of the clamps were recorded and plotted. The tear strength was determined by the first five highest peaks in the force-displacement curve and averaged. Figure 25 shows the plots for tear strengths of the different coated and uncoated wood pulp sheets. Collagen 5% and 3% showed good tear strength compared to most of the gelatin coated samples. Low bloom gelatin 3% showed the highest values for tear strength with the average being comparable to that collagen 5%, and significantly higher than at the higher concentration (5%), and high bloom counterpart. Moreover, gelatin coated sheets at 3% for both the low and high bloom gelatin showed better results. This may be due to the low viscosity of the coating solution allowing it to effectively penetrate the internal fibre matrix (viscosity measurements in Table 15). Furthermore, coating the wood pulp sheets with collagen and gelatin significantly increased tear strength.

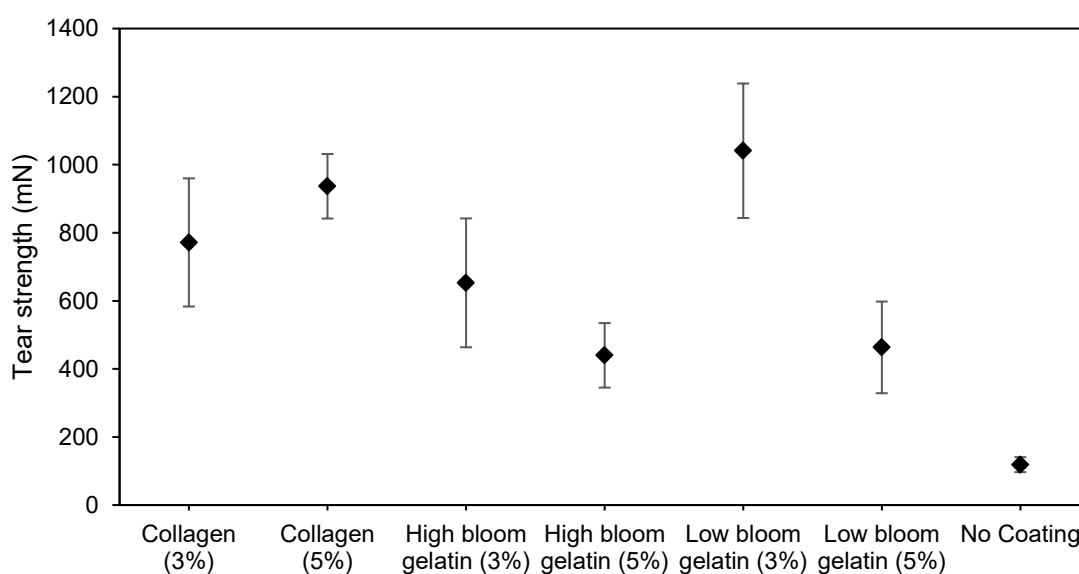


Figure 25 Tear strength for different coated cellulose sheets

The tear index values for the sheets were compared where the resulting motif is similar to the tear strength (Figure 26). However, both collagen coatings have lower tear index compared to 5% of both high and low bloom gelatin. Tear index corrects the tear strength,

taking into account the grammage of the sheets. Thus, higher grammage of the collagen coated sheets (Table 16) resulted in a lower tear index, since high grammage can contribute to a higher tear strength observed in the tear strength plot. To explore this further, the grammage of the samples as a function of tear strength was plotted in Figure 27 to highlight the impact of the coating itself rather than reflecting the grammage of the sheets. The sheets coated with 3% low bloom gelatin showed a good balance between grammage as well as good tear strength.

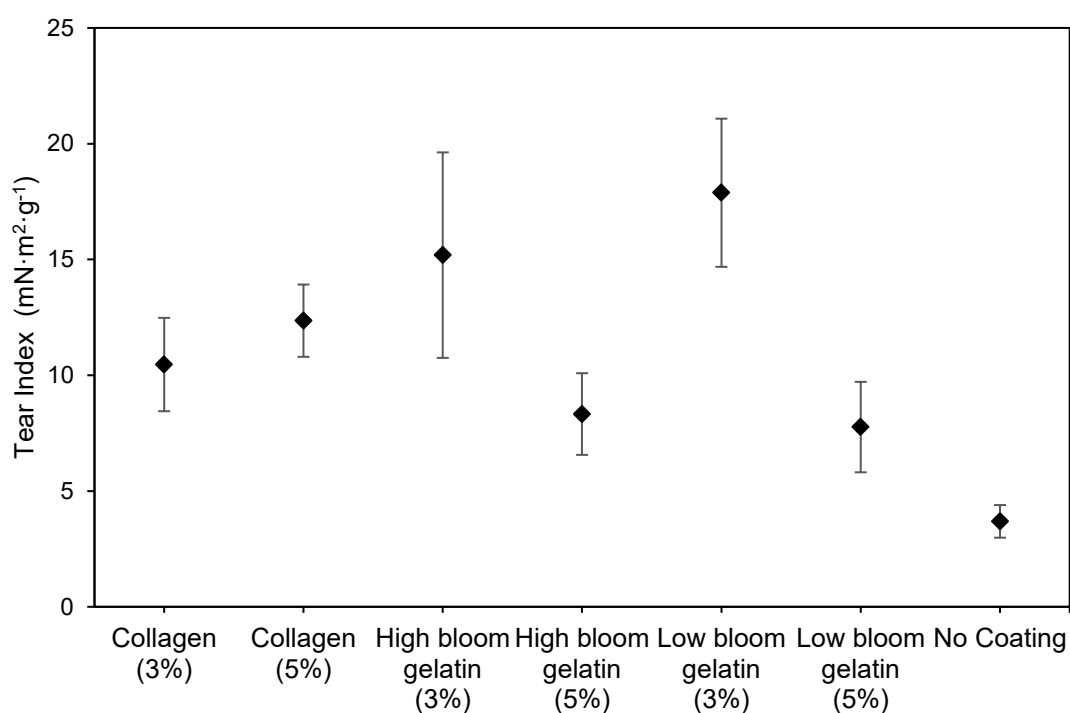


Figure 26 Tear index for different coated cellulose sheets

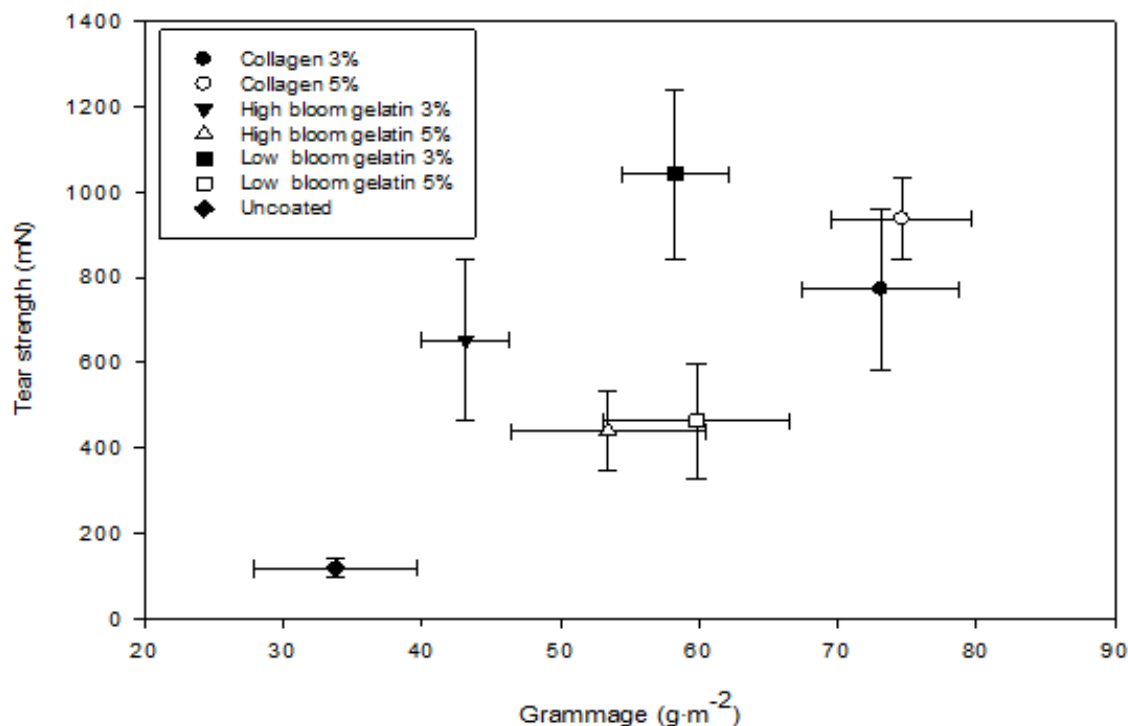


Figure 27 Tear strength as a function of grammage

6.3.6 FTIR

The FTIR spectra for collagen, gelatin and uncoated sheets are shown in Figure 28, and gelatin and collagen coated wood pulp sheets are plotted in Figure 29. Wood pulp consists of cellulose, lignin and hemicellulose. The peaks for these components were investigated in the spectra plots of the coated and the uncoated sheets. It was found that the signature peaks for lignin were not detected while one of the hemicellulose peaks that overlaps with a cellulose peak was present. Cellulose peaks, on the other hand, were all detected. The detected peaks and wavenumber values of the functional groups and vibration modes are detected are listed in Table 19. Some vibration and functional group have two values; the first value is for the 'front' spectra of the sheet and the second value is listed if the reading is different for the 'back' side.

A pure wood pulp FTIR peak indicating C-H stretching can be observed around 2800 – 3000 cm⁻¹ [396-398]. Intensity of C-H stretching of pure wood pulp is influenced by the composite structure. The C-H stretching peak intensified with the collagen coated sheets even when compared to uncoated fibre sheets, while the gelatin coated sheets showed a decreased in peak intensity. The latter shift indicates more hydrogen bonding generated in

the composites [399]. The former on the other hand could indicate changes in absorption peak of free -OH groups [400]. The absence of the RCOOR' linkage peaks associated with lignin and hemicellulose in the uncoated sheets is an indication of the removal of lignin and hemicellulose from the wood pulp fibre which is common in fibre processing [401, 402]. Nonetheless, significant spectral changes from base material with the addition of gelatin and collagen were observed. Thus, coating the wood pulp fibre sheets with either gelatin or collagen can change the mechanical properties of the sheets, and varying the content of collagen or gelatin allows further control of properties.

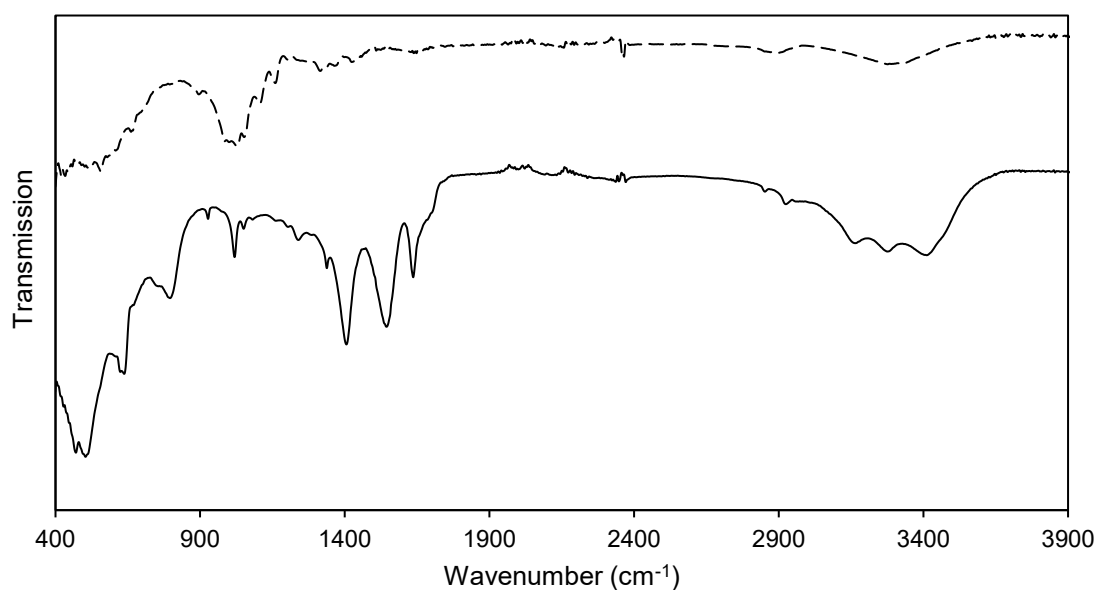


Figure 28 FTIR stacked plot collagen used for coating (solid line) and uncoated wood pulp sheet (dashed line).

Notable changes from the collagen, gelatin and wood pulp sheet only spectra to the coated sheets are that all the coated sheets displayed a magnitude increase and peak shape (instead of a clean peak, it is composed of three peaks forming a large peak characteristic of the wood pulp peak range for -OH stretch around $1030\text{--}1160\text{ cm}^{-1}$). However, the sheet coated with collagen 5% showed a significantly less intense peak compared to other coated sheets, the peak formed a clean sharp peak – characteristic of the collagen only spectra. Coating the sheets with collagen and gelatin resulted in an increased amplitude of the peak around $2800\text{--}3100\text{ cm}^{-1}$, which is contributed by the CH stretch from wood pulp fibre and cellulose, and amide B denotes vibrations such as NH , CH_2 , and C=O stretching from the collagen and gelatin coating. Similarly, the peak around $3300\text{--}3500\text{ cm}^{-1}$, from amide A denoting NH

stretching from the collagen and gelatin coating. This peak for the collagen 5% coated sheet is characteristic of a collagen-dominated peak particularly the front side spectra of the sheet indicating more coating deposited on one side. The gelatin coated sheet showed similar spectra for both the front and the back sides of the sheets, while the collagen coated sheets showed overall higher intensity peaks, notably on 3% collagen coated sheets.

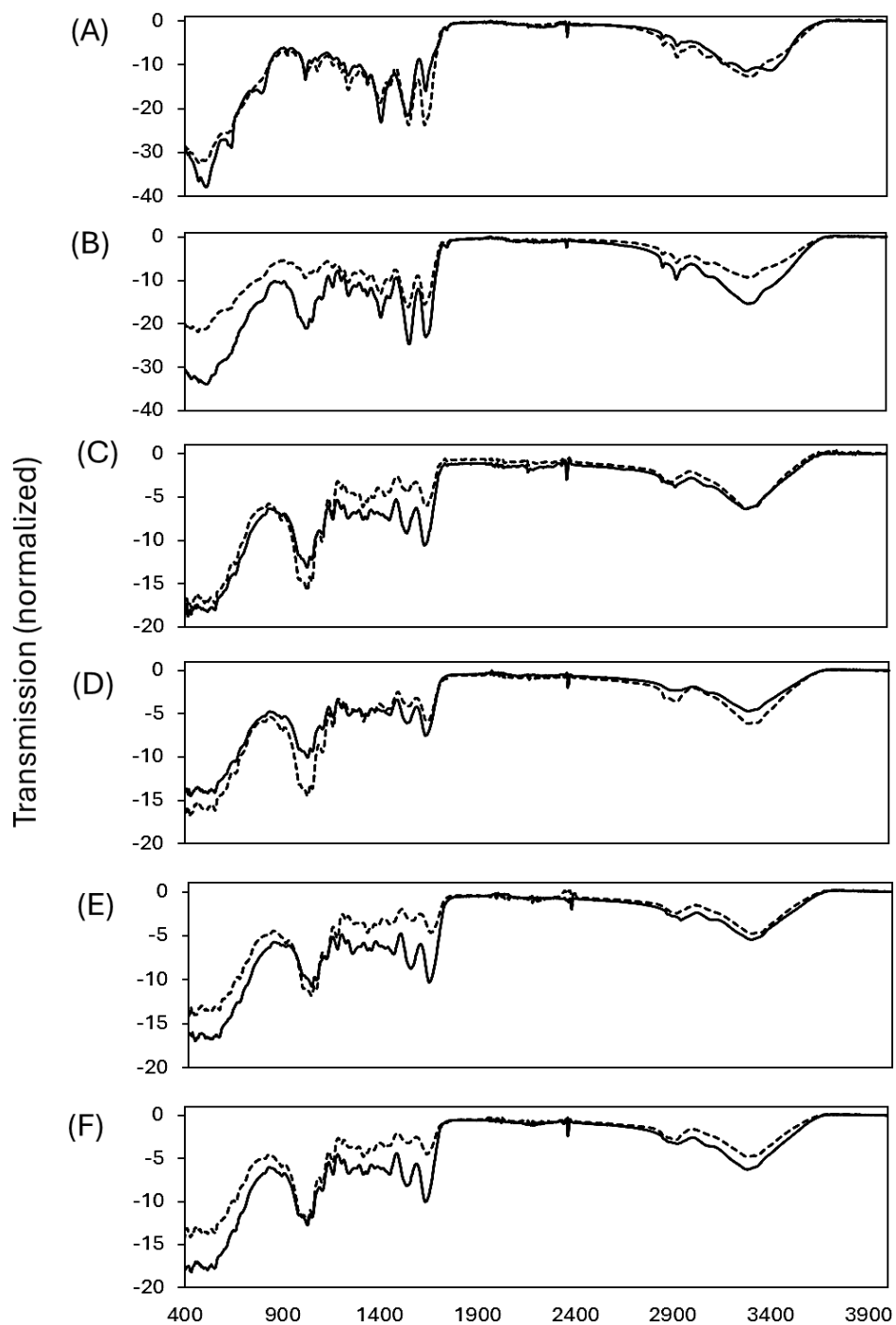


Figure 29 Solid line is the side facing up referred to as front and the dashed plot is the side in contact with the tray referred to as back during the drying process. (A) collagen 5%, (B) collagen 3%, (C) high bloom gelatin 5%, (D) high bloom gelatin 3%, (E) low bloom gelatin 5%, (F) low bloom gelatin 3%.

Table 19 FTIR Spectra peak detection for wood pulp components and coating.

Vibration/ functional group	C 5%	C 3%	HBG 5%	HBG 3%	LBG 5%	LBG 3%	UC
Detected peaks							
Cellulose							
O–H Stretch (~3300)	3302, 3275	3298	3280	3292	3282, 3276	3275	3309
C–H Stretch (2900)	2924	2922	2917, 2896	2919, 2907	2920, 2900	2921	2897
C–O/ C-O-C Stretch (1030-1160)	1047, 1083	1051, 1102	1052, 1104	1052, 1105	1061, 1104	1029, 1103	1027
Lignin							
aromatic skeletal (1510-1600) -- Not detected							
C=O stretch (1715-1740) -- Not detected							
Hemicellulose							
C=O acetyl stretch (1730-1740) -- Not detected							
C-O-C Stretch (1030- 1070) cellulose overlap	1047	1051	1052	1052	1061	1029	1027
Wood pulp fibre							
C–H (2800-3000)	2924	2922	2917, 2896	2919, 2907	2920, 2900	2921	2897
–OH (3324)	3302, 3275	3298	3280	3292	3282, 3276	3275	3309
RCOOR' linkages 1740 -- Not detected							
Collagen / Gelatin							
NH stretching 3300- 3500	3393, 3302	3298	3280, 3335	3292	3282, 3276	3275	–
NH CH ₂ stretching, C=O stretching ~3100 (not in gelatin)	2924	2922	2917, 2896	2907, 2919	2920, 2900	2921	–
H bond with COO [–] 1600-1800	1631	1637	1632, 1647	1637	1628, 1638	1632	–
C–N stretching, NH bending, CN stretching 1470-1570	1549	1551	1541, 1542	1537	1536, 1541	1538	–
NH & CH bending, CC stretching 1250- 1350	1336	1336	1314	1314	1314	1316	–

6.3.7 Water contact angle measurements

Contact angles were measured for the coated and uncoated sheets to determine the effect of coating on the sheet surface. A droplet of water was pipetted on a levelled surface of the sheet and the angle of the droplet in contact with the surface of the sheet is then measured. Depending on the measured angle, the surface can be characterised from three different wettability properties as shown in Figure 30.

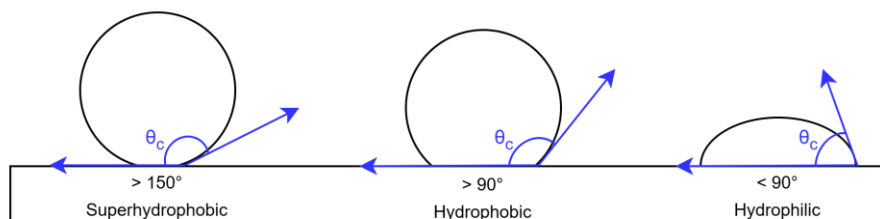


Figure 30 Surface contact angle (θ_c)

The surface interactions between the composite materials such as cellulose-water, collagen-water and gelatin-water as well as cellulose-collagen and cellulose-gelatin were considered to investigate the water contact angle. An illustration of the water droplet progression on the composite surface, and the coated and uncoated sheets contact angles for 0.1 to 10 seconds timestamp is plotted in Figure 31.

The contact angle of the uncoated sheets at 0.1 s for the front and the back of the sheet were 32.48° and 36.06° , respectively. However, the angles rapidly decreased to 0° , indicating complete wetting. The hydrophilic surface is due to the hydroxyl groups in the cellulose structure with a strong affinity to water molecules [403]. β (1-4) polysaccharides linked with D-glucose units in cellulose provide an abundance of $-\text{OH}$ groups that readily bond with water molecules [404, 405]. Figure 30 shows both uncoated and coated sheets average contact angle (repeats of front and back side of sheets) measured at 0.1 s, 1.0 s, and 10 s.

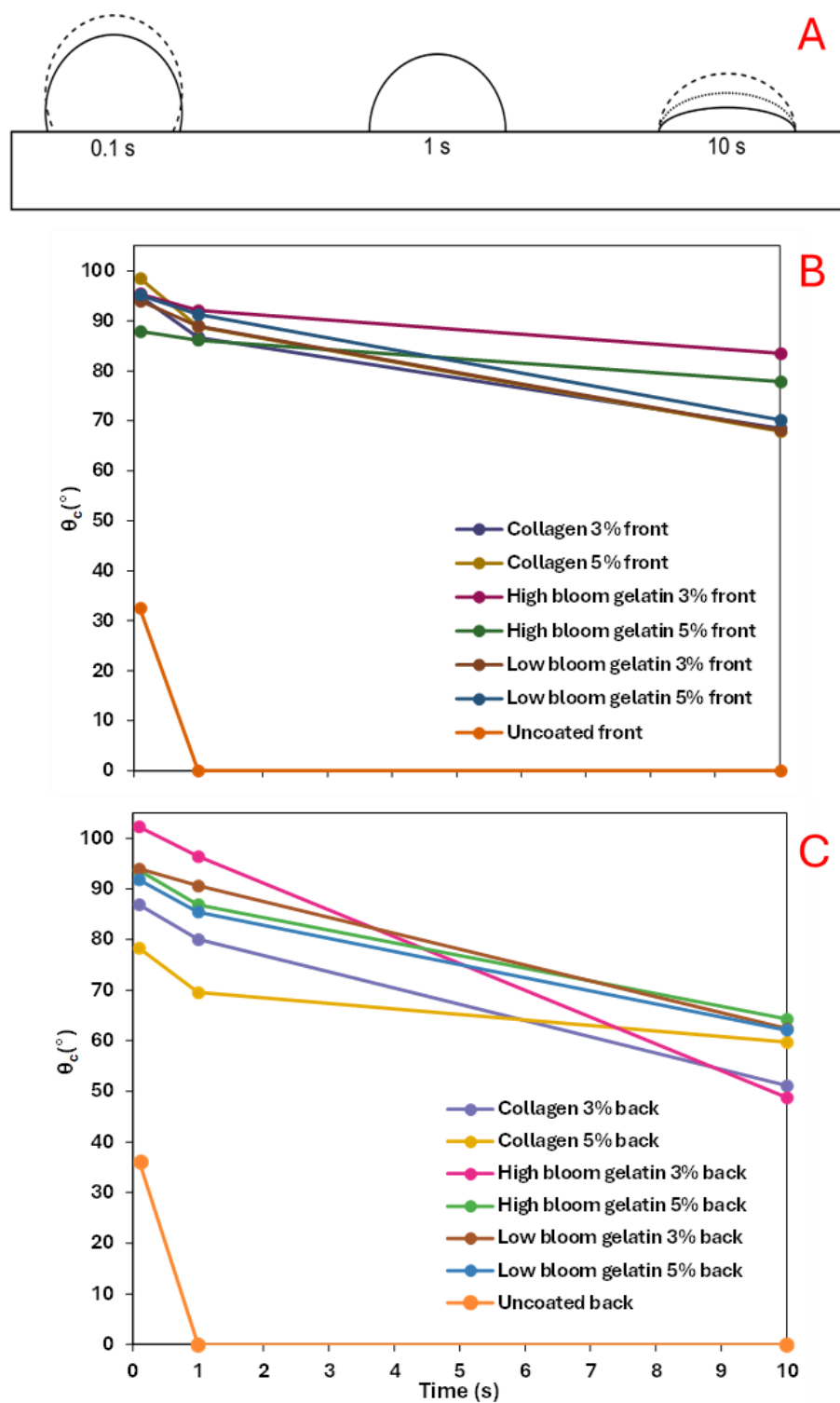


Figure 31 Front and back contact angle measurements for side 1 and side 2, respectively, of the coated and uncoated sheets at 0.1 s, 1 s, and 10 s.

Beyond the intrinsic properties of the raw materials, the fabrication process plays a critical role in determining the surface characteristics of the composite sheets. Key factors include

the drying method [406, 407], pH [408], and coating concentration and thickness [409, 410], all of which influence how the material interacts with water.

The amphipathic nature of collagen, which contains both hydrophilic and hydrophobic amino acids, means that the drying method can affect which functional groups are exposed at the surface [411]. During drying, hydrophobic tails tend to orient toward the air while hydrophilic heads are buried within the matrix. Drying the coatings at low temperatures, as was done in this study, promotes aggregation and partial unfolding of collagen chains, which exposes more hydrophobic regions and results in a more compact surface structure [406].

According to literature, the pH of collagen and gelatin solutions significantly affects molecular conformation and, consequently, surface wetting behaviour. Increasing the pH of collagen to around 7.83 results in higher β -sheet and random coil content, indicative of a looser molecular structure and increased exposure of negatively charged hydrophobic groups [412, 413]. At pH around 10, further conformational changes reduce α -helical content and lead to a disorganized fibrillar network, promoting rehydration and more hydrophilic surface behaviour. In this study, collagen coatings were applied at pH $\approx$ 6, which is below the isoelectric point of collagen type I (pI $\approx$ 7.2) [121]. This maintained good solubility and produced contact angles of 90.9° at 3% and 88.4° at 5% concentration. These values compare favourably to reported literature values for collagen surfaces, such as 24° [414], 18.6° [415], and 68° [416]. Another study reported a similar contact angle of approximately 87° for collagen at pH 6 [408]. The gelatin coating solutions had pH values of approximately 5.2 and 4.9 for the high and low bloom, respectively. Lin et al. [417] studied the effect of gelatin pH on contact angle where it was found that gelatin formed at a pH close to the isoelectric point (depending on the source and treatment for extraction) yielded the highest contact angle [418]. This is due to the charged group being more balanced and forming internal pair resulting in fewer free ionic groups on the surface. Contact angle measured at an average approximate of 73° for a type B alkali treated gelatin (pI $\approx$ pH= 5) [417]. This resulted in high contact angle values for the gelatin coated sheets compared to the collagen coated.

Coating concentration also affects the formation and surface characteristics of the films. Higher concentrations tend to form denser films, which limit the exposure of hydrophilic groups at the surface [419]. While collagen is known to hold more water than gelatin at the

same relative humidity and temperature, thicker collagen coatings can trap more water internally yet may exhibit lower hydration at the surface [420, 421]. In this study, direct comparisons of coating thickness were complicated by the penetration of the coating into the fibre sheet, particularly in the collagen-coated samples, which led to a thinner overall sheet profile as observed in Table 16.

At 3% concentration, collagen coatings penetrated more effectively and were observed on both the front and back surfaces of the sheet. In contrast, 5% collagen deposited primarily on the front and did not penetrate fully. Both high and low bloom gelatin at 3% demonstrated even distribution across the sheet and achieved favourable contact angles on both sides. Gelatin coatings exhibited even higher contact angles than collagen. High bloom gelatin produced values of 98.9° at 3% and 90.8° at 5%, while low bloom gelatin reached 94.0° at 3% and 93.5° at 5%. These values surpass literature reports of 34.3° and 71.3° . However, some samples, particularly high bloom gelatin at both concentrations and low bloom gelatin at 3%, showed contact angles of 0° at 10 seconds in some replicates. This suggests uneven distribution of the coating or localized differences in surface coverage, possibly due to rapid water spreading or thin spots on the sheet.

The bloom value of gelatin plays a substantial role in determining surface behaviour, influencing properties such as chain length, molecular interactions, and film uniformity. Low bloom gelatin, with shorter polymer chains, can either enhance hydrophilicity by increasing chain mobility and exposing polar groups or reduce it if the chains rearrange and conceal these groups. In contrast, high bloom gelatin, with longer chains and typically lower impurity levels, is considered higher quality and may form smoother and more hydrophilic films through uniform surface coverage, or alternatively promote hydrophobic aggregation through dense chain packing [422, 423]. In general, gelatin contains more hydrophilic than hydrophobic amino acids, suggesting an inherent tendency toward hydrophilicity unless modified by processing conditions [422]. Therefore, both the bloom value and fabrication parameters—especially drying conditions—can be strategically adjusted to modulate the surface wettability of the composite sheets [407]. A study by Bialopiotrowicz and Janczuk observed contact angles for 75 bloom gelatin sheets at approximately 5% and 3% concentrations and found values of 122° and 82° , respectively [424].

6.3.8 Thermogravimetric analysis (TGA)

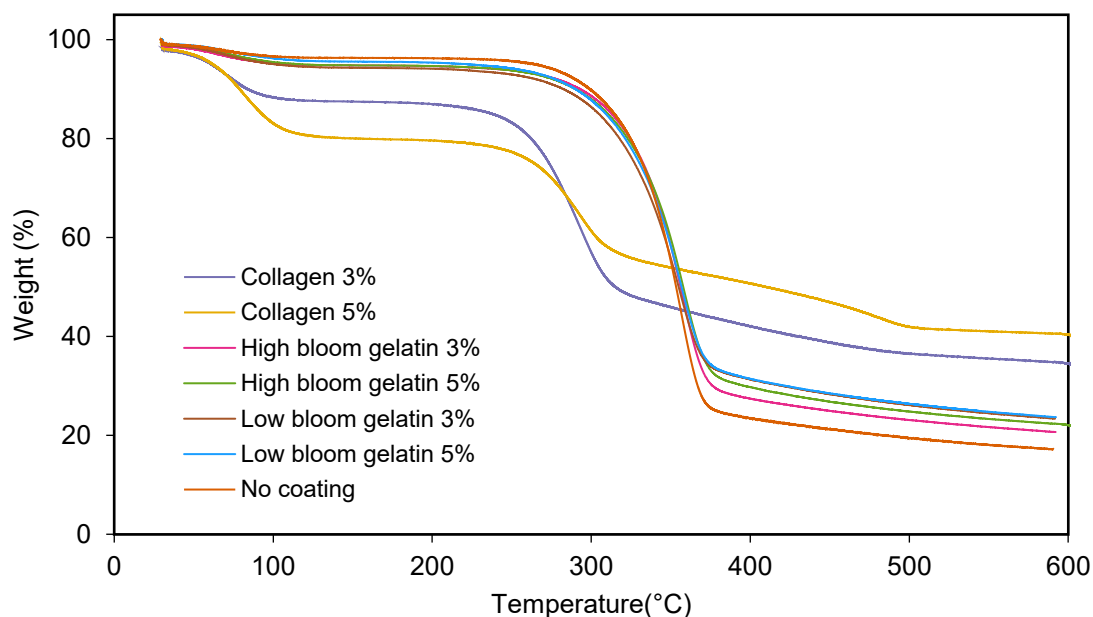


Figure 32 Weight change over a temperature gradient from 30°C – 600°C

The thermal performance of the coated and uncoated sheets was investigated using TGA. The weight loss as a function of temperature and differential TGA (DTG) curves are shown in Figure 32 and 33, respectively. Weight loss in the first decline was observed approximately from 35°C to 94°C for uncoated and gelatin sheets, and for 3% collagen and 5% collagen it continued until 103°C and 116°C, respectively. Maximum degradation in temperature ranges, listed in Table 20, from 63°C to 83°C was due to the moisture content and evaporation of physically absorbed water [425]. The uncoated sheet and the gelatin coated sheet showed similar pattern for weight loss, while the collagen coated sheets showed a faster and sharper loss.

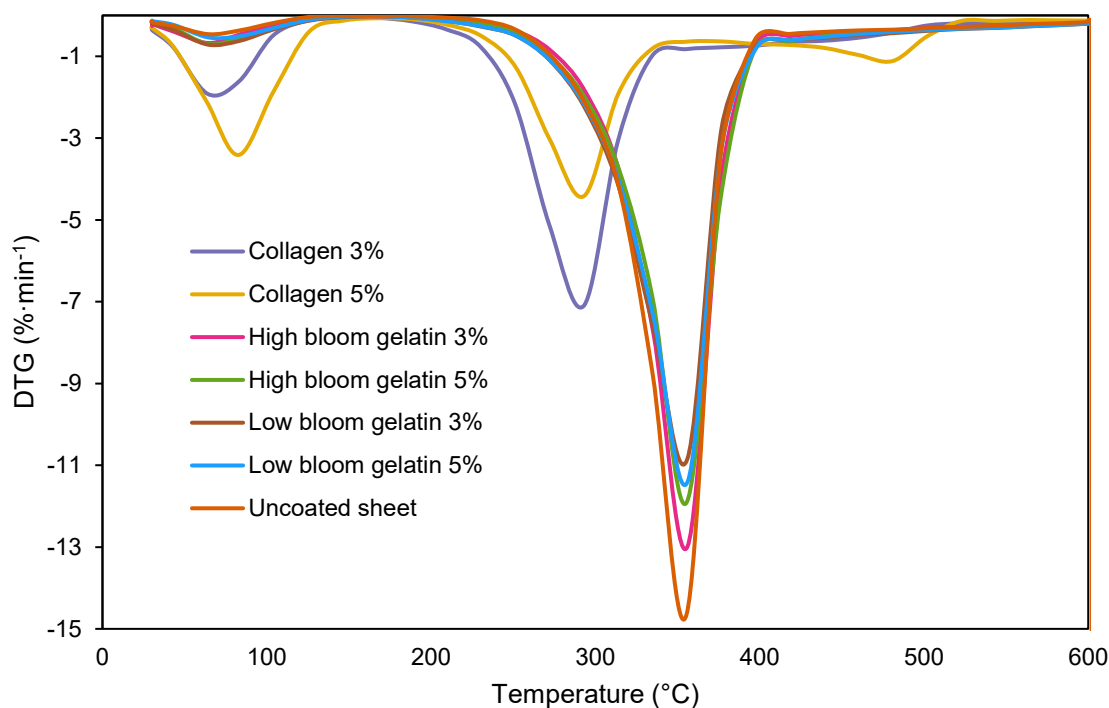


Figure 33 DTG plots for the different coated hand sheets.

In the second decomposition region, early onset was observed for the collagen coated sheets at 240°C and 249°C for 3% collagen and 5% collagen, respectively. The gelatin-coated and uncoated sheets were observed around 269°C. The second weight loss is from the decomposition of collagen, gelatin and cellulose molecules [426]. The second region of decomposition for the gelatin and uncoated sheet showed different behaviour, showing a steeper decrease in weight loss, which may be due to a rapid pyrolysis of cellulose in the uncoated sheet [427]. The gelatin coated sheets showed quick decomposition due to it not being crosslinked with the wood pulp fibre components and poor integration of gelatin and wood pulp fibres [428, 429]. However, gelatin coated sheets have a higher onset temperature in this region. The collagen coated sheets, particularly the 5%, showed the slowest weight loss progression, seconded by the 3% collagen. The sheets displayed better weight retention in the presence of collagen, although an earlier onset was observed in this region. Better retention of weight during TGA could indicate homogeneous mixing, which formed effective bonding of the components.

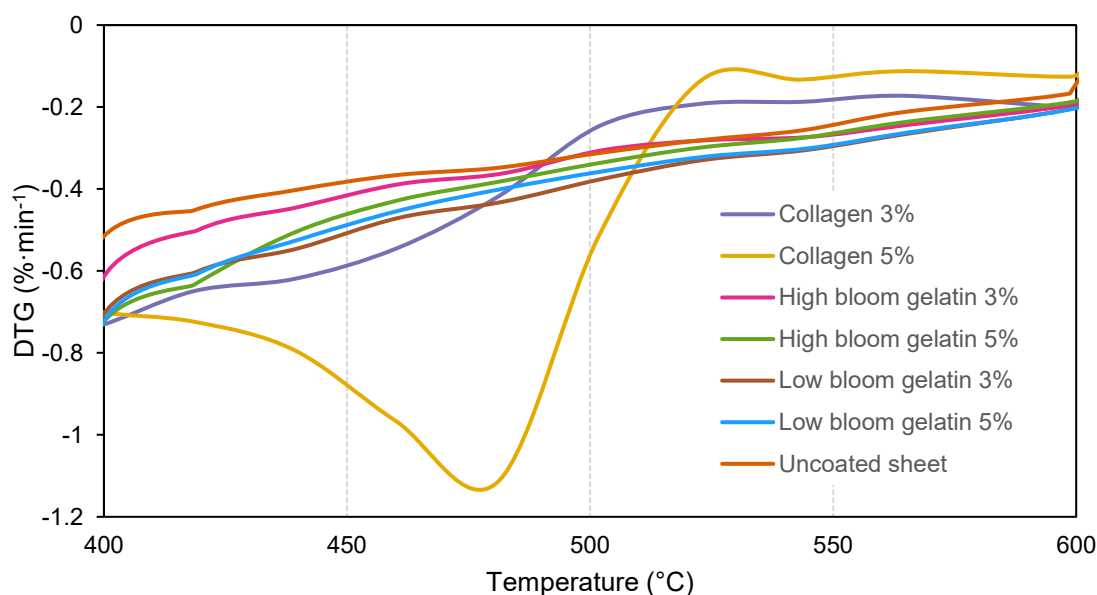


Figure 34 DTG in the 400 – 600°C temperature range.

The collagen coated sheets displayed better weight retention consequentially generated a third step in the weight loss plot as well as a third peak in its DTG as shown in Figure 34, which was not observed with the other coated and uncoated sheets. The third region of thermal degradation likely corresponds to secondary pyrolysis of collagen, including carbonization of residues from thermally stable triple-helical structures or crosslinked domains. Table 20 lists the maximum degradation rates for the three peaks observed in the DTG plots for each degradation region. For the gelatin-coated and uncoated sheets, values in the third region are shown in brackets, as the corresponding peaks are barely visible.

Table 20 DTG Maximum degradation rate. Units in °C. The temperatures in brackets are slight indication of peaks that can be seen in the close-up in Figure 33.

Step/Sample	First	Second	Third
Collagen 3%	63.97	293.08	334.67
Collagen 5%	82.86	293.08	480.96
High bloom gelatin 3%	64.36	356.07	(419.14)
High bloom gelatin 5%	64.37	355.34	(418.42)
Low bloom gelatin 3%	64.38	356.01	(418.92)
Low bloom gelatin 5%	64.45	356.25	(419.34)
Uncoated	64.58	355.34	(418.33)

6.3.9 SEM

Topographic images of the coated and uncoated sheets were obtained using SEM. Table 21 and Table 22 shows the images for the uncoated, collagen, high bloom gelatin, and low bloom gelatin coated sheets.

The uncoated sheets appear porous, with overlapping but loosely packed fibres. In the 100 μm scale, signs of defibrillation were visible, especially with the loose fibre strands at the edges.

The collagen coatings showed an effective filling of the gaps in the fibre matrix. The 3% collagen showed impressions of the fibres after coating, although some hollow spots were observed on the back side. In contrast, the 5% collagen coated sheets no longer showed visible fibre impressions, and black spots on the images indicate fibre tips protruding, which was particularly noticeable on the back side. Furthermore, the 5% collagen coated sheet eliminated the visible hollow spots on the reverse side.

For the 3% gelatin coated sheets, both on the high and low bloom, loose single-strand fibres at the edges are still present, similar to uncoated sheets. However, closer packing of overlapping fibres is evident, and gelatin accumulation at fibre overlaps appears to reinforce the structure. In the 5% gelatin coated sheets, loose strands are absent, as the coating holds stray fibres tightly within the fibre matrix. Overlapping fibres appeared denser such that the fibres were visible through the mesh apertures.

The high bloom coatings displayed a more interconnected fibre network, with the coating enveloping the overlaps more completely. This is observed in regions where the gelatin had pooled during drying, particularly on the back side of the sheet. A key observation for all the gelatin-coated sheets is that neither the fibre matrix nor the voids were not fully coated.

Table 21 SEM images for the uncoated and collagen coated sheets at 1 mm and 100 um scale and both sides of the sheet, front (1) and back (2).

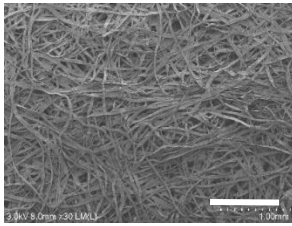
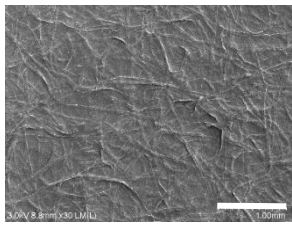
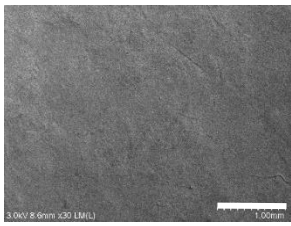
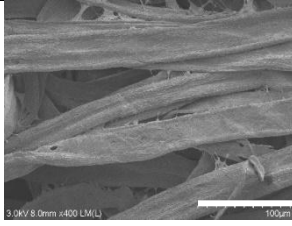
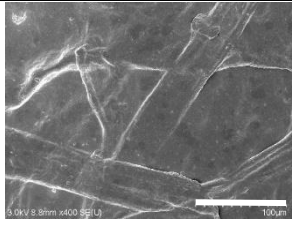
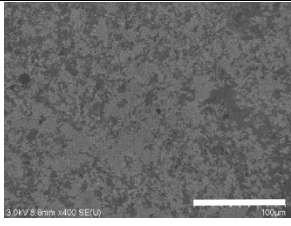
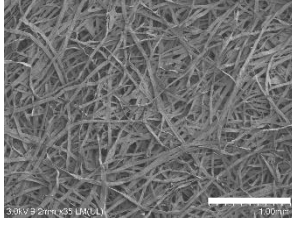
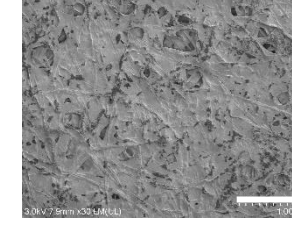
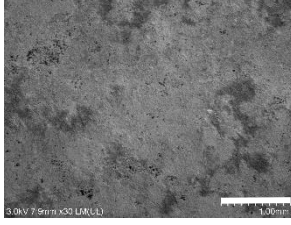
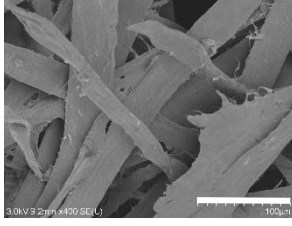
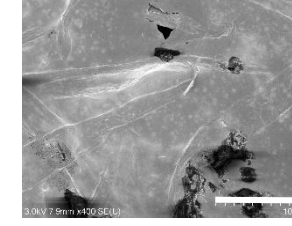
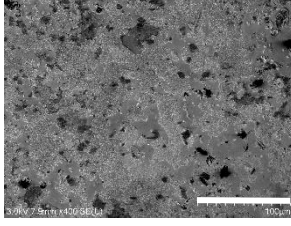
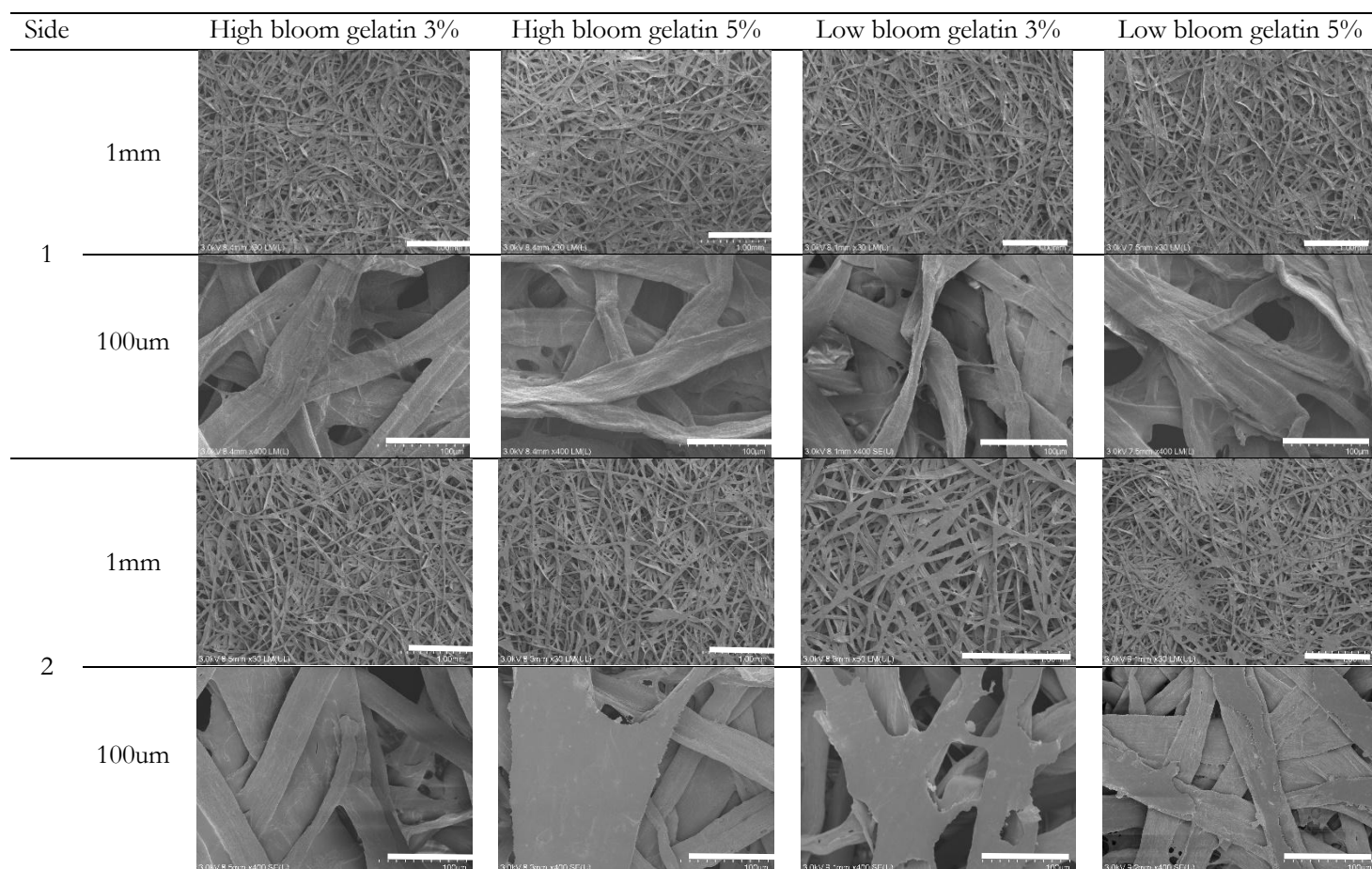
Side		Uncoated sheet	Collagen 3%	Collagen 5%
1	1mm			
	100um			
2	1mm			
	100um			

Table 22 SEM images for the high and low bloom gelatin coated sheets at 1 mm and 100 μ m scale and both sides of the sheet, front (1) and back (2).

6.4 Conclusion

This study investigated the fundamental interactions between protein coatings, collagen and gelatin, and self-assembled cellulose fibre sheets derived from wood pulp. Collagen, with its ordered triple-helical structure, and gelatin, as its partially denatured counterpart with random coil configurations, were applied to cellulose fibre networks to examine how their structural and molecular differences influence composite performance.

Collagen coatings, especially at 5%, provided the most balanced enhancement, improved tensile strength, elasticity, tear resistance, and oxygen barrier properties. The collagen-coated sheets also exhibited more gradual thermal degradation and a third decomposition region not observed in gelatin or uncoated samples, suggesting deeper integration within the cellulose sheets. SEM analysis further showed a more compact and cohesive fibre network with improved surface coverage.

Gelatin coatings, particularly high bloom at 3% and 5%, improved tensile and tear strength while exhibiting lower elongation and thermal stability compared to collagen. Although gelatin coatings remained visible on the fibre surface and produced more hydrophobic surfaces, they did not fully cover the fibre matrix or voids and resulted in a more expanded porous structure.

Overall, both proteins adhered effectively to cellulose fibre through hydrogen bonding and physical entanglement without the use of synthetic additives. The results demonstrate that naturally derived proteins can function as effective biopolymeric coatings for cellulose-based materials, with collagen favouring structural reinforcement and gelatin contributing hydrophobicity and flexibility. Further evaluation under application-relevant environmental conditions, including varying temperature and humidity, would provide additional insight into the performance of these protein-based coatings in sustainable packaging and related composite applications.

Chapter 7. Micro-computed tomography to characterise collagen- and gelatin- coated fibrous composite sheets

7.1 Introduction

Composites can be characterised and analysed for their surface, mechanical and thermal properties. The methods of fabrication and the addition of materials into the composite are designed to optimise functionality for the intended purpose or application. However, a limiting factor to obtaining the functional properties in spite of the optimised methods and materials are the possible defects and flaws that may occur during synthesis [430]. Such defects and flaws may not be visible due to size and their nature – being formed internally.

7.1.1 Micro-computed tomography

One method to analyse the internal macrostructure of composites undamaged is to observe it under microcomputed tomography (MCT).

MCT is a non-invasive technique used to capture 3D images using X-ray based on the principles of clinical computed tomography. Micro-CT scanners usually consist of an X-ray source, an area detector, and a means of producing images at regularly spaced intervals around the object being imaged [431]. Micro-CT at the Australian Synchrotron works similarly but instead of using an X-ray tube with an actual and effective focal spot to produce an X-ray field, it uses a bending magnet source [432]. As X-rays from the source interact with the sample, transmitted X-rays are converted into visible light through the scintillator (a thin plate dosed with Cerium or Europium/Terbium), which is transmitted onto a beam detector (monochromatic or white beam) to capture spectral characteristics by measuring the light after passing through the sample, and the magnifying lens focuses the visible light to achieve the desired spatial resolution, and then an image sensor (direct or indirect) collects and transforms the light into an electrical signal as data to generate a high resolution image [432-435].

7.1.2 Component contrast

Gathering data to generate images and constructing the volume of the sample, requires good phase and component contrast imaging to analyse the samples. Component contrast can be expressed using the Beer-Lambert law (equation 7-1), where I_0 is the unattenuated

x-ray beam, I is the intensity after passing through material with thickness, x , and linear attenuation coefficient of the material, μ (cm^{-1}) [436].

$$I = I_o \exp (-\mu x) \quad (7-1)$$

Introducing a density parameter allows recognition of the fundamental basis that attenuation amount is the number of atoms interacted with the x-ray beam.

$$I = I_o \exp \left[\left(-\frac{\mu}{\rho} \right) \rho x \right] \quad (7-2)$$

In MCT, I/I_o is measured at many angles in one rotational plane, calculating μ in the x , y and z direction for each voxel.

These equations do not take into account the refraction of the x-ray as it passes through a solid, and this refraction is used for phase contrast applications of MCT, but for component contrast absorption and density differences are covered in equation 7-2 [437]. However, analysing a porous medium with different components requires adequate phase contrast recognising boundaries throughout the composite particularly when materials used in the composites have similar resulting absorption intensities and/or similar absorption intensities to air when captured using MCT.

7.1.3 Phase contrast

Phase contrast utilises x-ray refraction as x-ray slightly bends, where the refractive index reading changes with a difference from one by parts per million, when it passes through solids. The shape and the characteristics of the x-ray changes as it passes through a region with different electron density. The method used at the synchrotron is parallel-beam propagation-based phase contrast [434]. In this method, the sample is placed farther away from the detector than normal x-ray imaging. After the x-ray from the source interacts with the sample, the refracted x-ray, displaying Fresnel diffraction effects, interferes with other x-rays at the detector plate. From these interferences, detectable fringes are captured as internal and external boundaries between different material components with varying electron densities [437]. The contrast is determined by the difference in the second

derivative of the x-ray phase [438]. Since phase retrieval methods applied for propagation-based phase contrast data utilises the transport of intensity equation 7-3 (TIE) for near-field approximation [439].

$$\nabla_{\perp} \cdot (I(\mathbf{r}_{\perp}, z) \nabla_{\perp} \phi(\mathbf{r}_{\perp}, z)) = -\frac{2\pi}{\lambda} \frac{\partial}{\partial z} I(\mathbf{r}_{\perp}, z) \quad (7-3)$$

Assuming a non-absorbing sample in its pure phase, $\kappa = 0$ for this equation and the image has no absorption contrast, resulting in a zero gradient. At position z , $I(\mathbf{r}_{\perp}, z)$, the near-field intensity can be approximated using the equation by Bronnikov [440] in equation 7-4. It is closely related to the Laplacian second derivative that expresses the phase shift imposed on the x-ray (wavefront) right after traversing through the sample.

$$I(\mathbf{r}_{\perp}, z) = I(\mathbf{r}_{\perp}, 0) \left(1 - \frac{\lambda_z}{2\pi} \nabla_{\perp}^2 \phi(\mathbf{r}_{\perp}, 0) \right) \quad (7-4)$$

Equation 7-4 representing the wavefront intensities, however, falls short at increased phase-induced intensity variations. Due to the increase in intensity variations when phase gradient increase from sample changes in thickness, density, sample composition or large sample to detector distance. In addition, x-ray absorption occurs in real experimental conditions. Introducing a Fourier domain filter can be solved to recover from the intensity variations but with assumptions such as weakly absorbing object or constant ratio between the refractive index, δ , and an arbitrary number related to the linear attenuation coefficient, β . The image algorithm described by Paganin et al [441] and other close revision is the widely applied method of correction due to the provision of computational speed and straightforward experimental requirements. The calculation assumes a single material and constant δ/β ratio and solves for the sample thickness, t , presented in equation 7-5.

$$t(r_{\perp}) = -\frac{1}{\mu} \ln \left(\mathcal{F}^{-1} \left\{ \frac{\mathcal{F} \left(\frac{I(r_{\perp}, z)}{I_0} \right)}{\alpha |k|^2 + 1} \right\} \right), \text{ where } \alpha = z \left(\frac{\delta}{\mu} \right) \quad (7-5)$$

$\mathcal{F}$ and $\mathcal{F}^{-1}$ are the Fourier transform and inverse Fourier transform, k is the spatial frequency, z is the propagation distance, $\left(\frac{I(r_{\perp}, z)}{I_0}\right)$ is the normalised image intensity and μ the linear attenuation coefficient.

7.1.4 Applications

These technologies and iterative algorithm allow the data collection, calculation and generation of images and reconstruction of the 3D structure of the sample. The imaging technique was initially used for biomedical imaging for research and treatment [442]. The non-invasive nature of X-ray micro-computed tomography, together with the growing availability of synchrotron radiation facilities worldwide, has expanded its use as an advanced imaging technique in materials science. Applications have extended to medical implants [443], and various composites used in energy storage and catalysis [444], building materials [445, 446], packaging particularly active and smart packaging materials detecting defects and flaws [430] and more.

In this chapter, cellulose fibre sheets are coated with gelatin and collagen and were observed under MCT for data collection and reconstructed into 2D and 3D images using ORS Dragonfly imaging software. The coated and uncoated sheets were investigated with respect to coating-to-fibre ratio, coating distribution, pore structure, pore connectivity and anisotropy using phase contrast, Open Pore Network modelling, and anisotropy analysis methods available in the imaging software. By linking three-dimensional structural features with the mechanical and barrier properties discussed in Chapter 6, this chapter provides insight into how protein coatings modify the internal architecture and functional performance of fibre-based composites.

7.2 Experimental method

7.2.1 Imaging



Figure 35 Composite sheet mounted on the sample platform in the MCT beamline (Australian Synchrotron).

Microcomputed tomography was performed at the micro-computed tomography beamline (MCT) at the ANSTO Australian Synchrotron. The sample was mounted on stage-Y for 3D imaging, aligned with the lasers, and positioned 155 mm from the detector to achieve good phase contrast (Figure 35). Imaging was performed using a monochromatic beam with an X-ray energy of 18 keV and a white beam detector equipped with an 18× objective lens, a field of view of $0.9 \times 0.8 \text{ mm}^2$, and an effective pixel size of $0.361 \text{ }\mu\text{m}$ [434]. A total rotational angle of 180° was used to capture 1800 projections, with an exposure time of 0.1 s per projection, resulting in a total acquisition time of 15 minutes per sample. The captured images were stored as H5 files and reconstructed using in-house software. For computational efficiency, the samples were cropped into smaller objects, a size determined through trial and error by incrementally reducing object dimensions to match the processing power of the computer used in this study. The analysis was performed using a 12th Generation Intel Core i7 with 1 TB hard drive storage, an 8 GB graphics card, and 32 GB RAM.

7.2.2 Phase volume percentages

In the volume aspect analysis, the objects were approximately 400-megabyte objects. For the uncoated sheets, components were divided into pore space and fibre regions of interest (ROIs) [447]. In the coated sheets, an additional ROI was included for the coating. These ROIs were used to quantify the volume of fibre, coating, and pore space. Dragonfly software can differentiate the components of the composite sheets using upper and lower Otsu thresholds, which exploit differences in pixel intensities (from X-ray absorption) between the materials [448]. The segmentation was further refined by visual inspection and additional software features, such as the Process Island tool and image operations applied to the Otsu-separated regions. Data for ROI ranges and percentage by volume were recorded manually.

7.2.3 Porosity analysis

The Open Pore Network (OPN) modelling feature of Dragonfly software was used to investigate the porosity of the coated and uncoated sheets [449]. The objects used for this part of the study are approximately 800 MB each. The pore space ROI was divided into its connected components, limited to 6-connected connectivity. The largest connected component was then extracted as an ROI and used for OPN analysis, with the phase index set to 1, sigma set to 1, $R_{\max}$ at 10, isolated pores trimmed and edge tolerance to 0.5%. To adjust the representation of arrows and colour coding, parameters were set under the ROI table as follows for pore size analysis:

- a) *Vertex = equivalent diameter*
- b) *Edge radius measurement = Cross sectional area*
- c) *Edge LUT measurement = volume*
- d) *Vertex LUT measurement = equivalent diameter*

To analyse pore connectivity, connectivity measurements were enabled and vertex LUT measurement was set to connectivity.

7.2.4 Anisotropy methods

The uncoated sheet used to perform anisotropy analysis. An ROI for the fibre was defined using the intensity range listed in Table 25. The object was cropped to a 2.45 MB volume,

resulting in dimensions of $243.73 \times 355.20 \times 341.58 \mu\text{m}^3$ ($680 \times 991 \times 953$ pixels). There are four methods in analysing anisotropy using the ORS Dragonfly software (1) star volume distribution (SVD) [450, 451], (2) mean length intercept (MIL), (3) surface normal eigen-vector based [452] and (4) surface normal projection-based [452]. The first two methods quantify the geometry, resulting in a value for degree of anisotropy (DA), while the latter generates a visual representation of anisotropy of the geometry with a reference scale bar for the DA that shows variation in the different regions of the geometry.

The ROI range used for the fibre component in the anisotropy analysis was 3.52–29.93. This ROI was then selected to compute anisotropy, and either a star volume distribution or mean intercept length-based DA value could be calculated. Parameters include maximum and minimum iteration limits, sampling, orientation, radius, and tolerance values were defined prior to calculation. The goal for both methods was to reach a stable DA value despite slight variations in parameters, essentially achieving convergence. The corresponding DA value for each run was recorded manually. In this study, convergence was first achieved using SVD, after which MIL was applied with the same parameters to confirm the results.

For the surface normal methods—(a) eigen vector-based and (b) projection-based—the sample geometry was confined within a box shape, and similar parameters, such as spacing, radius of influence, and mesh repetition, were defined. Either projection- or eigen-based evaluation was then applied, producing a colour-coded directional vector map of the fibre sheet geometry. The colour of the arrows corresponds to the DA values they represent. Averaging and visual analysis of the results, rather than relying on a single value, were used to interpret the data. For all anisotropy methods, DA follows the same scale, where a value of zero indicates perfectly isotropic orientation, and values approaching 1 indicate increasing anisotropy of the geometry.

For the anisotropy vector field mapping, the parameters set for both evaluation methods are as follows: spacing at 20 μm , radius of influence to 30 μm , mesh smoothing at 2 and the evaluation was set either as Eigen- or Projection-base.

7.3 Results

The composite sheets were measured to determine thickness, grammage and coating percentage by weight. These measurements were measured in Chapter 6 and are reproduced in Table 23.

Table 23 Sheet properties: coating percentage by weight, thickness, and grammage

Sample	Coating (wt.%)	Thickness (mm)	Grammage (g·m ⁻²)
Collagen 3%	43.3 ± 3.8	0.19 ± 0.03	74.2 ± 7.73
Collagen 5%	55.6 ± 5.4	0.19 ± 0.02	92.8 ± 9.49
High bloom gelatin 3%	28.7 ± 1.9	0.31 ± 0.04	58.4 ± 6.3
High bloom gelatin 5%	50 ± 4.6	0.27 ± 0.02	66.96 ± 14.8
Low bloom gelatin 3%	34.5 ± 2.7	0.28 ± 0.04	60.1 ± 4.9
Low bloom gelatin 5%	47.2 ± 4.3	0.24 ± 0.03	68.2 ± 5.5
No coating	-	0.24 ± 0.02	41.9 ± 4.4

7.3.1 Phase volume percentages

Table 24 Region of interest (ROI) range parameters of fibre, coating and total sheet on Dragonfly

Sample / ROI Range	Sheet		Fibre		Coating	
	Min	Max	Min	Max	Min	Max
Collagen 3%	1.07	39.98	4.92	39.98	0.8	6.42
Collagen 5%	0.91	36.27	5.68	36.27	0.91	4.71
High bloom gelatin 3%	1.6	33.02	4.25	33.02	1.6	4.25
High bloom gelatin 5%	1.17	70.15	4.11	70.15	1.17	4.11
Low bloom gelatin 3%	2.5	36.27	6.47	36.27	2.5	7.36
Low bloom gelatin 5%	2.67	29.12	6.29	29.12	2.67	6.29
No coating	-	-	3.17	29.93	-	-

The results for the volume percentage of each sample are listed in Table 25. One set of results considers only the fibre and coating as a solid sheet volume, while the other

considers the total sheet volume, including air gaps within the geometry as ‘pore space’ along with the fibre and coating.

The volume-based aspect ratio derived from the ROIs can be visualised in both 2D and 3D. In Table 26, the 3D views along the X- and Y-axes and the central 2D slice of each sample are presented. Table 27 shows negative X and Y axis biases for each sample.

Table 25 Volume based aspect ratio (%) approximation of fibre, coating and total sheet on Dragonfly

Sample / Volume percentage	Solid sheet volume		Total sheet volume		
	Fibre	Coating	Pore space	Fibre	Coating
Collagen 3%	67.7	32.3	54.4	33.3	15.9
Collagen 5%	50.5	50.7	43.4	26.9	27.1
High bloom gelatin 3%	78.5	21.5	82.4	13.8	3.8
High bloom gelatin 5%	76.6	23.5	86.9	18.4	5.6
Low bloom gelatin 3%	67.2	40.6	83.2	11.4	6.9
Low bloom gelatin 5%	60.9	39.3	78.7	12.9	8.3
No coating	-	-	86.3	13.9	-

Table 26 Fibre and coating ROIs for collagen 3% and 5%. Fibre ROI are shown in orange, collagen at 3% in purple and collagen at 5% in yellow.

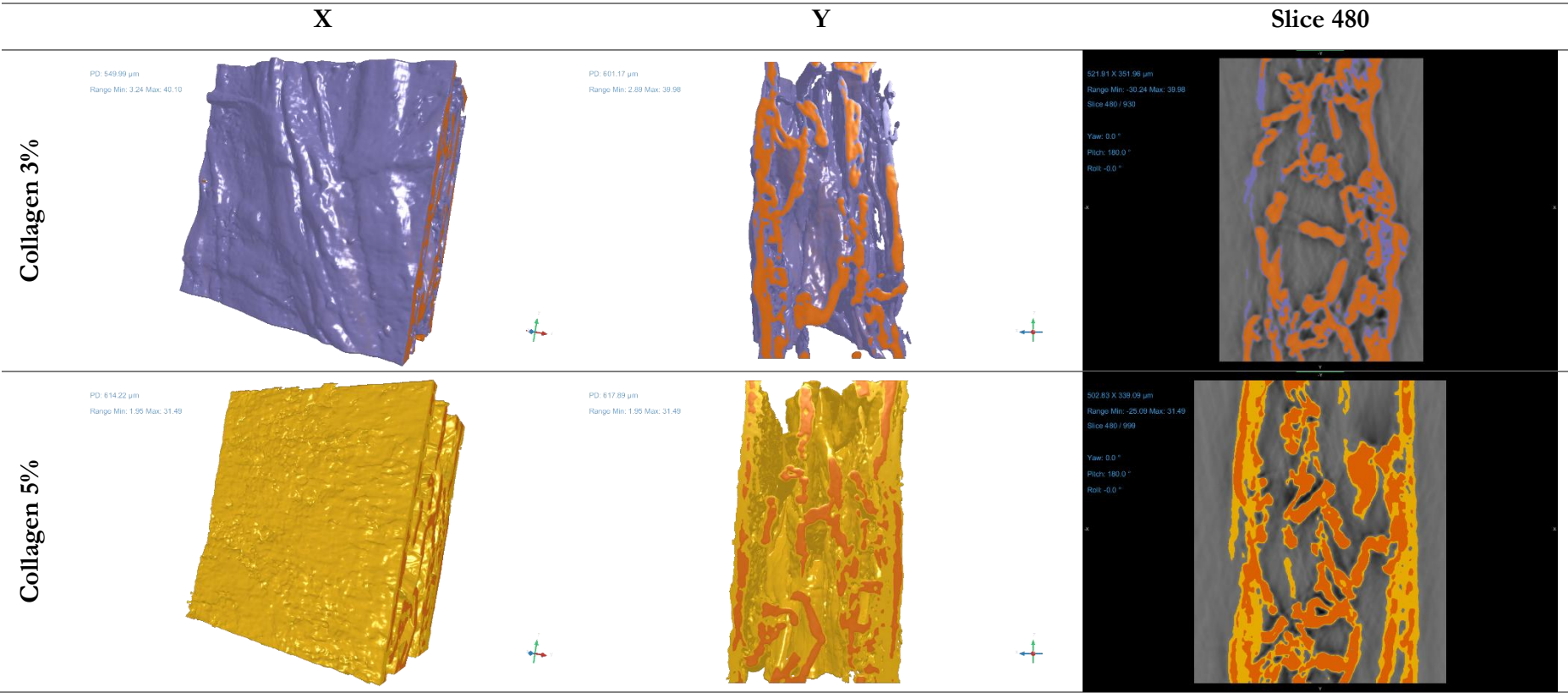


Table 26 (continued). Fibre and coating ROIs for high bloom gelatin 3% and 5% Fibre ROI are shown in orange, high bloom gelatin at 3% in pink and high bloom gelatin 5% in green.

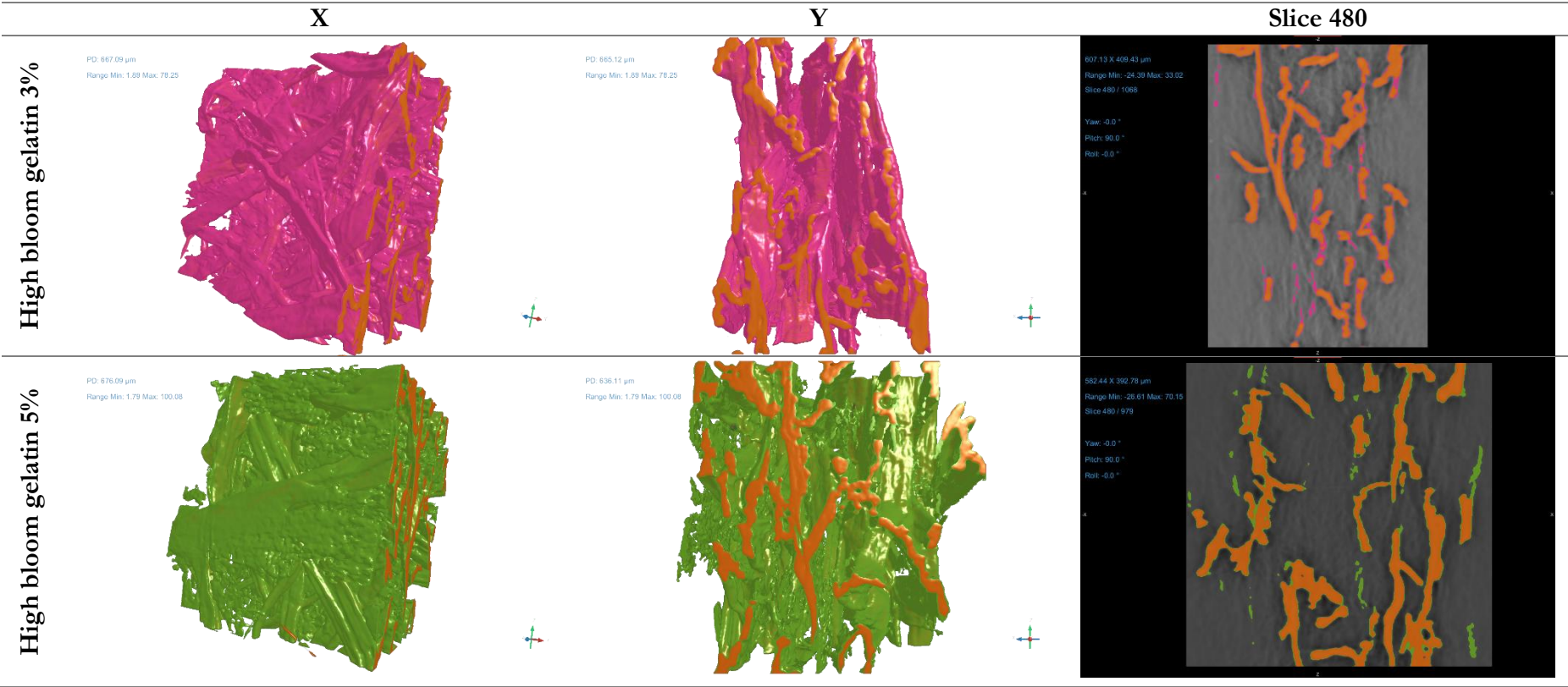


Table 26 (Continued). Fibre and coating ROIs for low bloom gelatin 3% and 5% Fibre ROI are shown in orange, low bloom gelatin at 3% brown, and low bloom gelatin at 5% in blue.

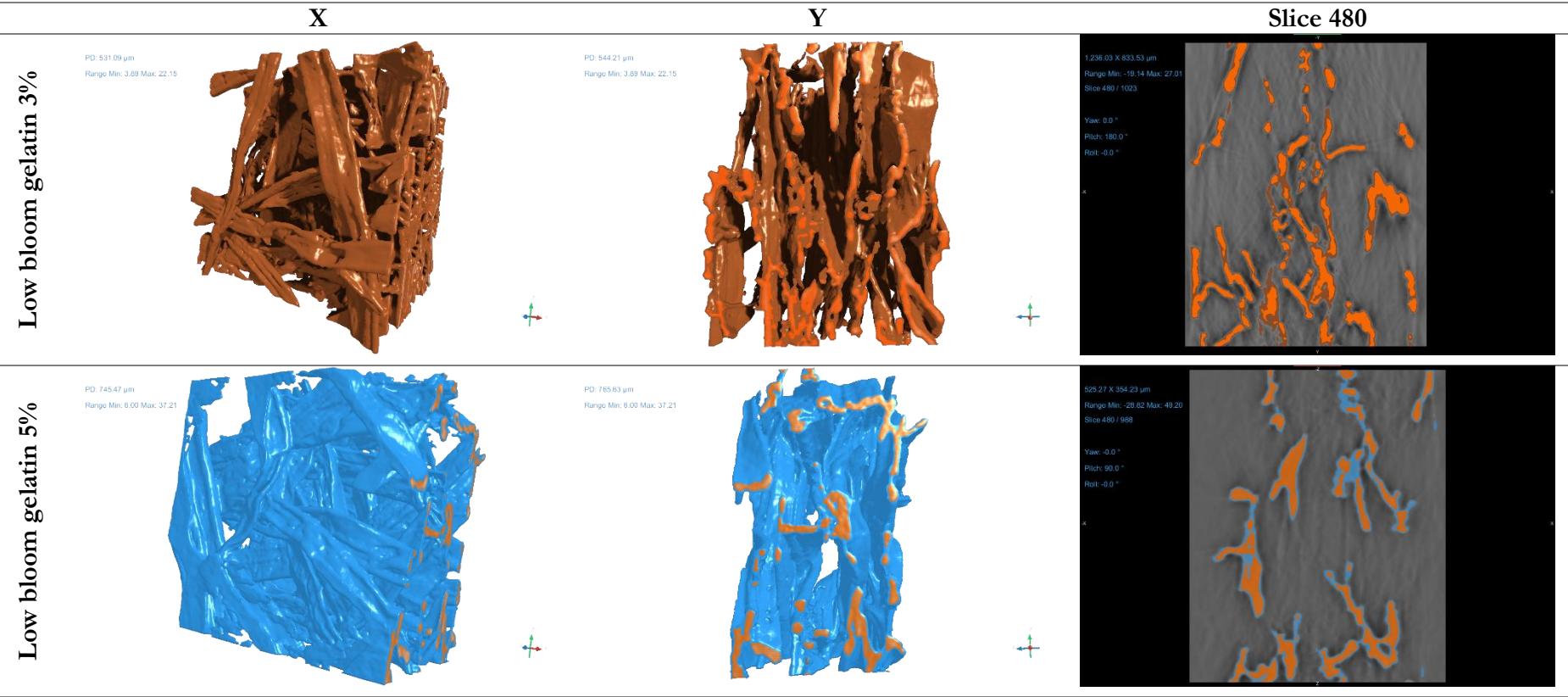


Table 26 (Continued). Fibre ROI for the uncoated sheet shown in orange.

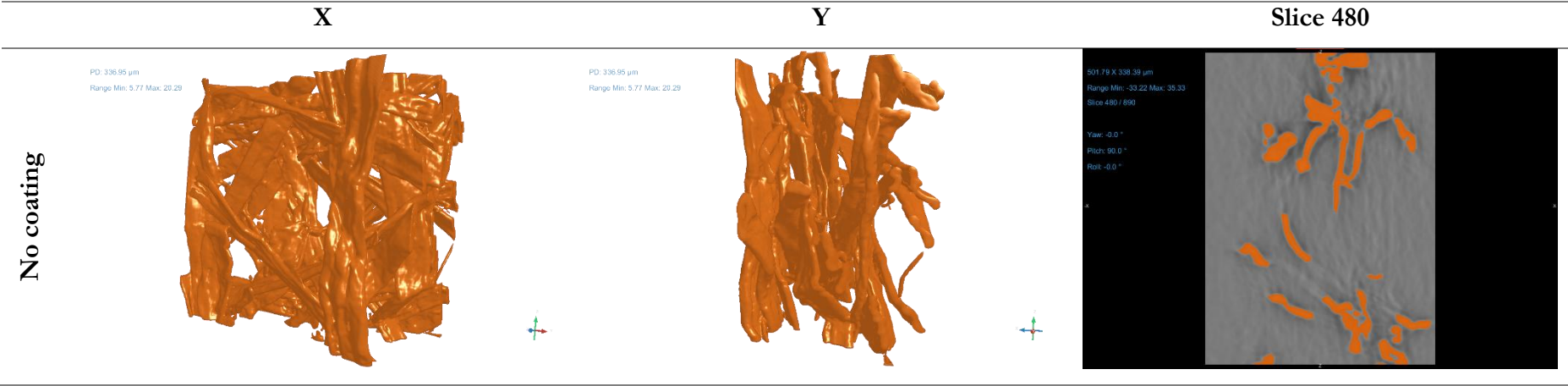


Table 27 -X and -Y axes views of coating and fibre ROIs. Fibre ROI are shown in orange, collagen at 3% in purple, collagen at 5% in yellow, high bloom gelatin at 3% in pink and high bloom gelatin 5% in green.

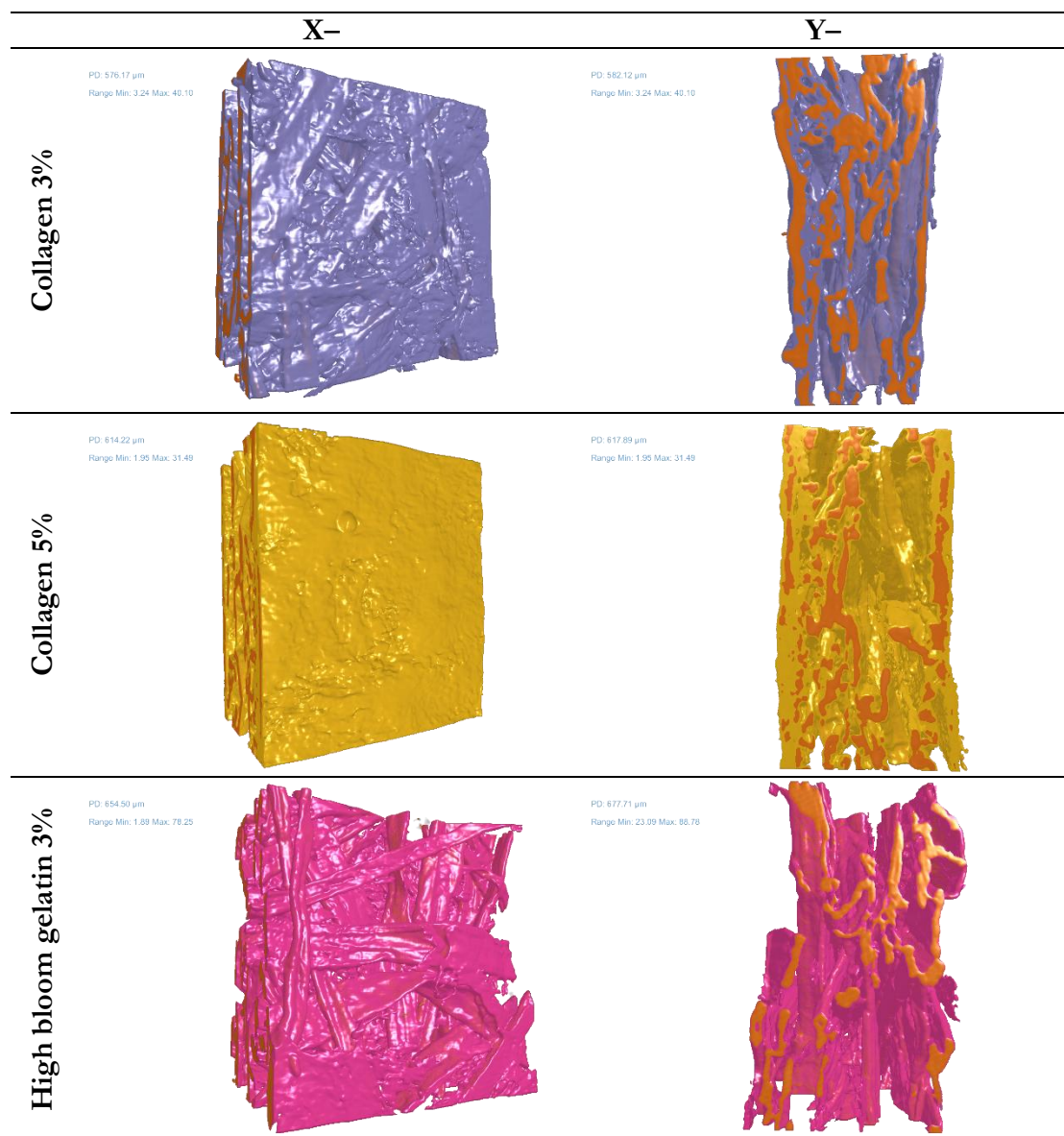
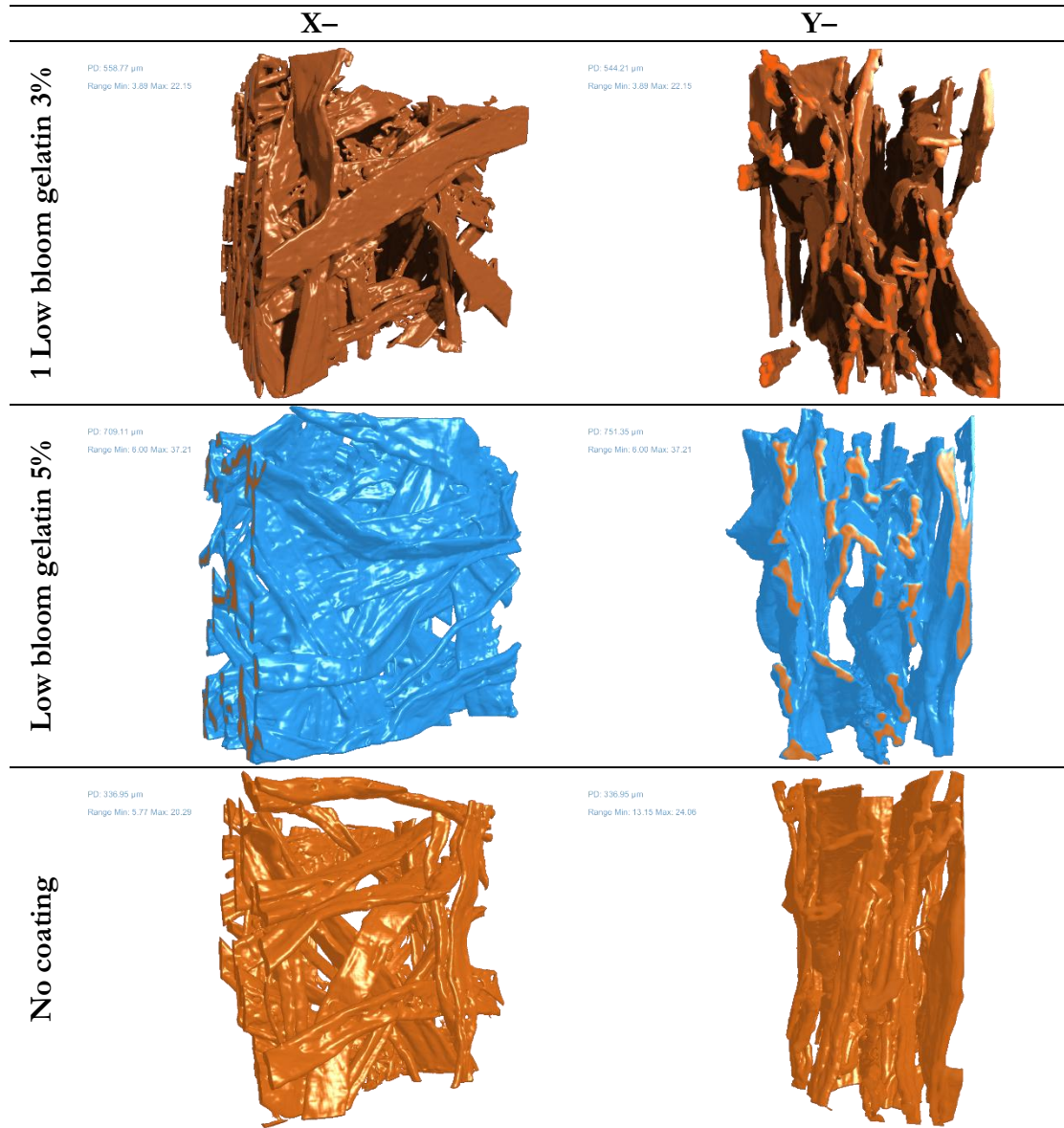


Table 27 (Continued) Fibre ROI are shown in orange, low bloom gelatin at 3% brown, and low bloom gelatin at 5% in blue.



7.3.2 Porosity analysis using Open Pore Network Modelling (OpenPNM)

The calculation produced a multi-region of interest (MROI) of the segmented pore space in both 3D and 2D slice views. The computed data can be expressed in the ROI segments of the pore space and the middle-slice 2D view of the MROI is shown in Figure 36. Multi-ROI 3D screenshots, along with plots of pore volume as equivalent diameter and pore connectivity, are presented from Figure 37 and Figure 40.

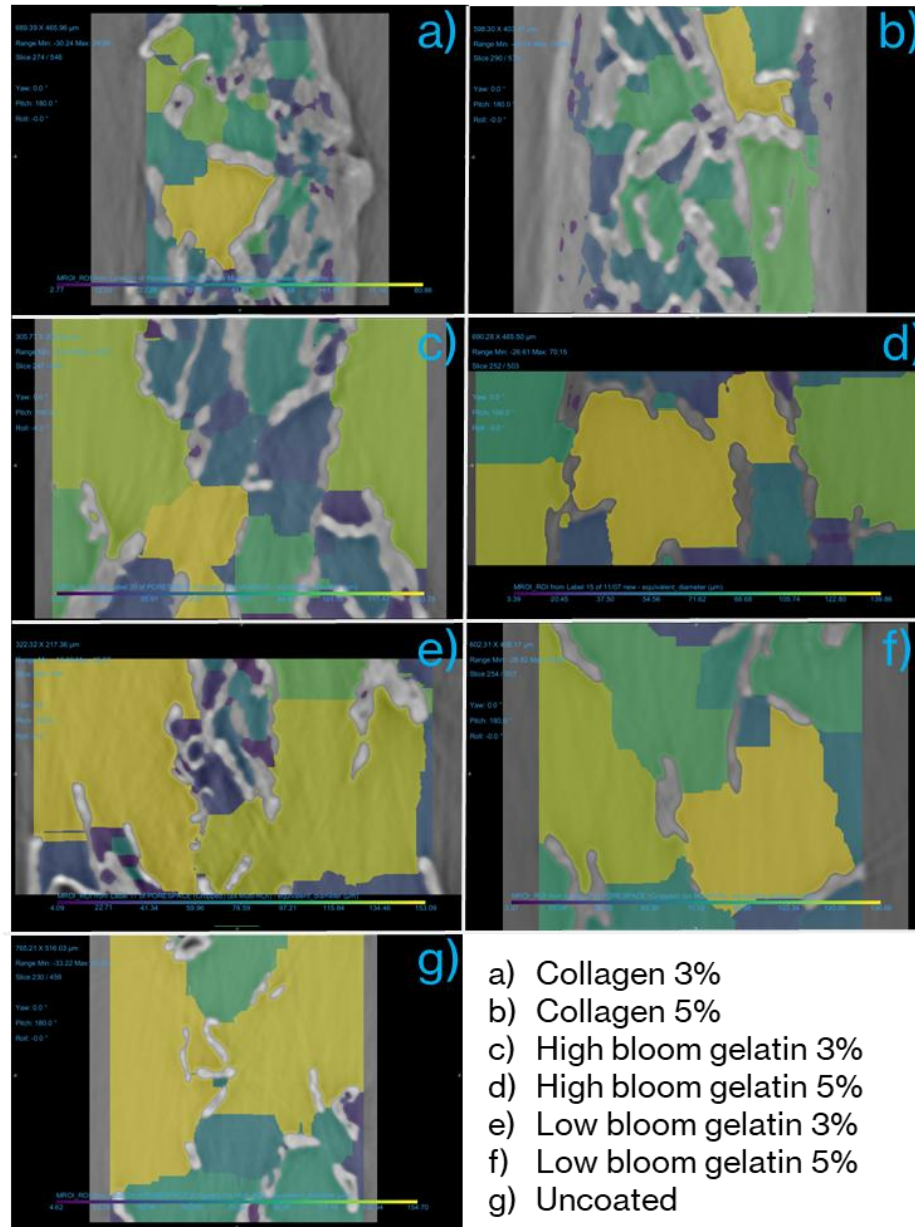


Figure 36 2D MROI OpenPNM segmentation for the different coated and uncoated sheets; Y to $-Y$ slice view. Coloured regions are the segmented pores, colour indicates the pore size indicating yellow hues as larger pores, green hues as medium pores and purple hues as the small pores.

The 2D slices of the MROI pore space (blue–green–yellow colour scale) and fibres (grey) in Figure 36 illustrate how segmentation is carried out in the OpenPNM module within Dragonfly. Although, there were a few occurrences where the pore is more elongated relative to the other pores within the geometry and due to the nature of the self-assembled fibre in which some mimic a corner from fibre ends, these are recognised as

separate pores. This approach was overall effective in regions with closely packed fibres, as observed in the collagen-coated and high-bloom gelatin-coated fibre samples.

In the 3D plots, spheres were shown representing the measured parameters mentioned in the methods section (vertex and edge parameters). The same colour scale was applied to both the MROI and the spheres. This representation allows the pore size to be expressed through pore volume converted into equivalent diameter. The pore size and colour are indicated by the equivalent diameter measured. Figure 37 shows the samples oriented toward the positive X and Z axes, while Figure 38 presents the negative X and Z axes view; in both cases, the axis indicator was set in the same orientation.

After segmentation, the changes in the matrices upon coating became more evident. Collagen-coated samples showed slight compaction, whereas gelatin-coated samples expanded, resulting in larger pore spaces. This effect was most pronounced in the 5% high-bloom gelatin samples, followed by 3% high-bloom gelatin, 3% low-bloom gelatin, 5% low-bloom gelatin, and finally the uncoated sample.

In the right column of Figures 37 and 38, the 3D plots show pore size (spheres) in terms of equivalent diameter (horizontal colour scale) and the connecting branches (edges) linking pore centres (vertical colour scale). The size (vertex radius) and colour of the spheres correlates with pore size. Larger and lighter-coloured spheres, indicating larger pore sizes, were observed more frequently in the gelatin-coated and uncoated samples. These were often connected by longer edges, reflecting greater distances between larger pores.

Another observation was that pores located on the surfaces of the collagen-coated samples were obscured beneath substantial fibre-coating layers: on one side for the 3% collagen sample (X+Z+ view), and more extensively on both sides for the 5% collagen sample.

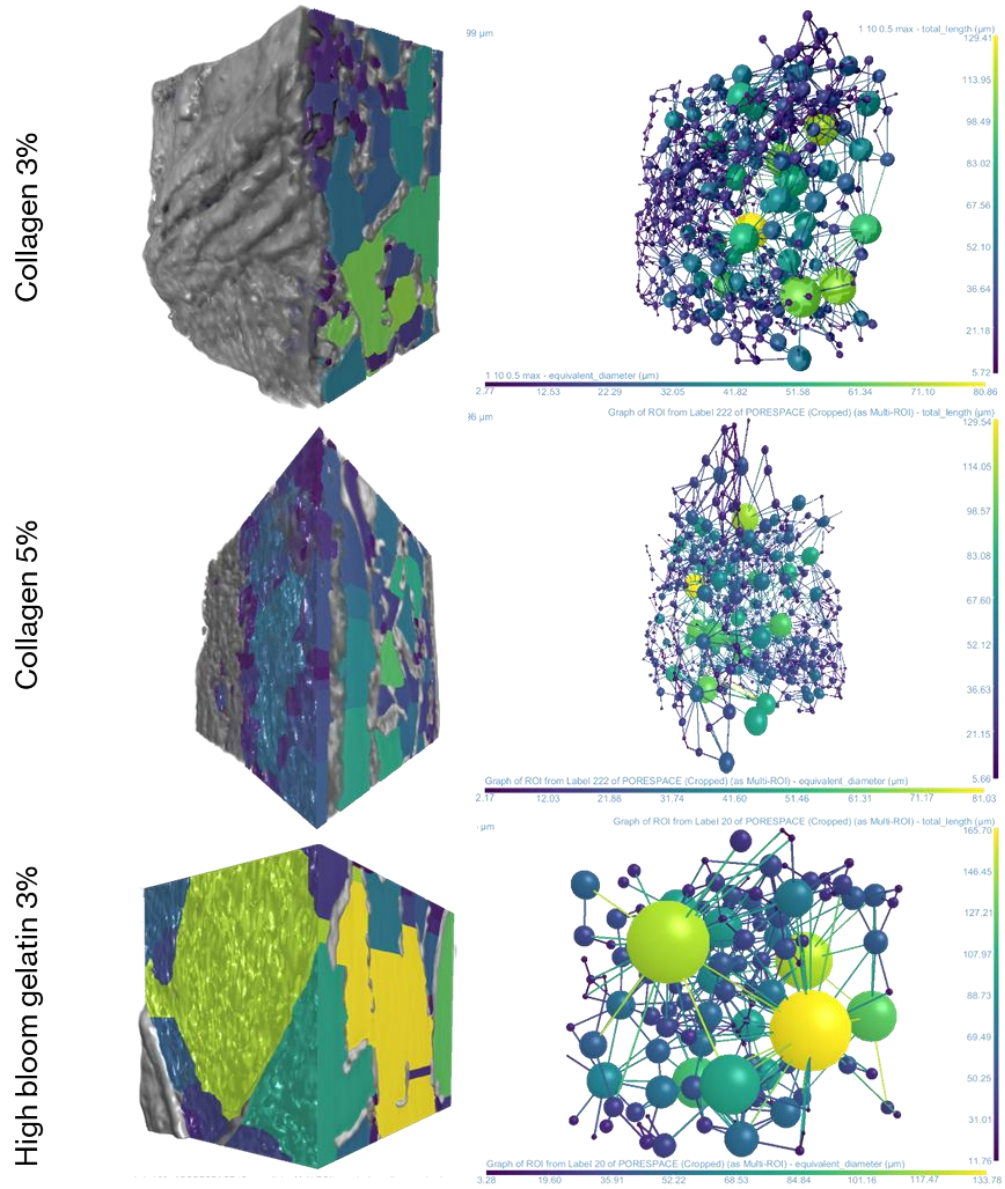


Figure 37 X+Z+ axis view of MROI and porosity. On the right, the 3D MROI segmentation is shown alongside the corresponding 3D plots of pore equivalent diameter. Pores are represented as spheres (vertex), with the x-axis colour scale indicating pore size. Branch connections between pores are represented as edges (lines), with the y-axis colour scale indicating the measured distance between pore centres. (Figure continues on the next page.)

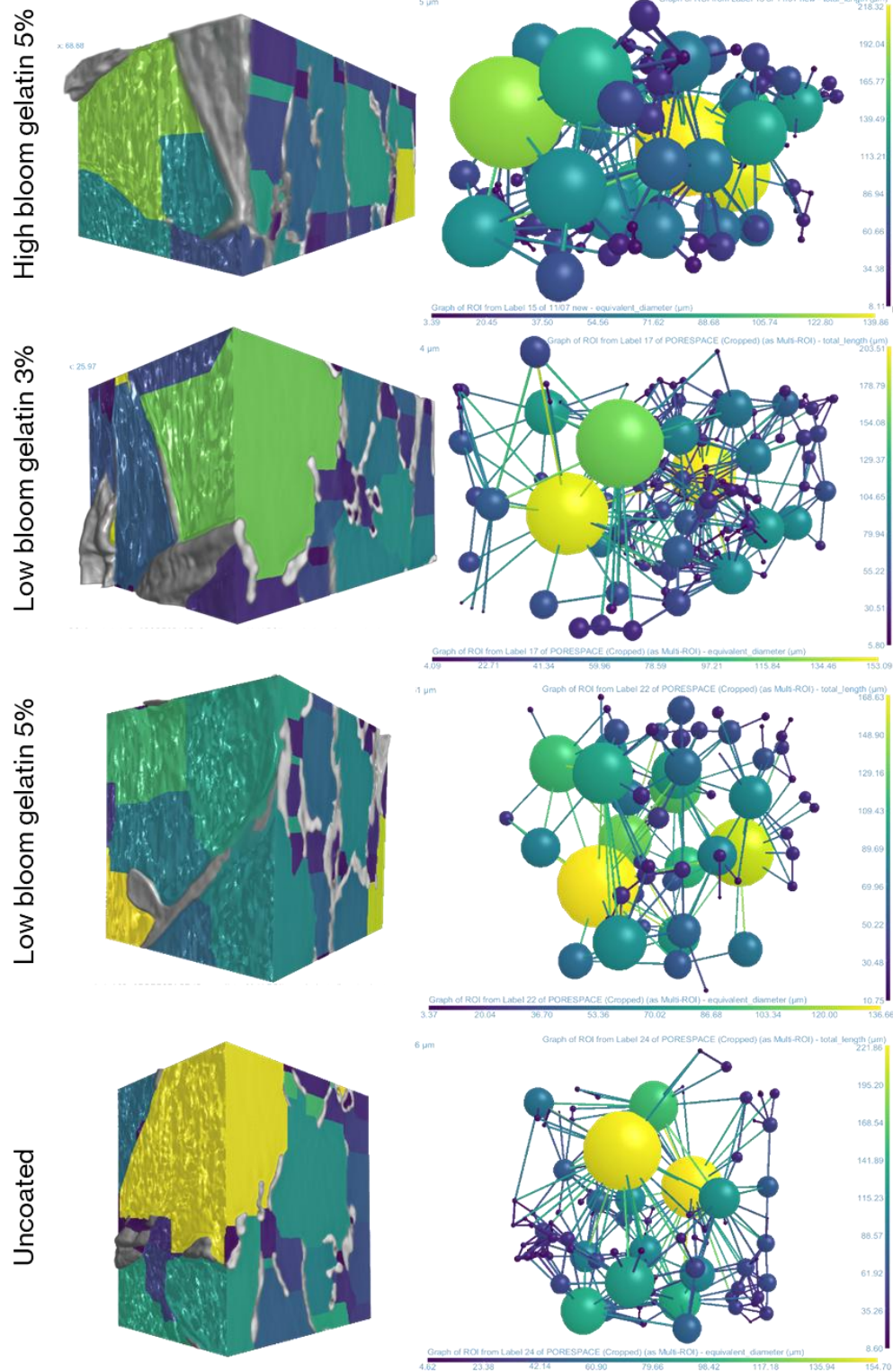


Figure 37 (continued).

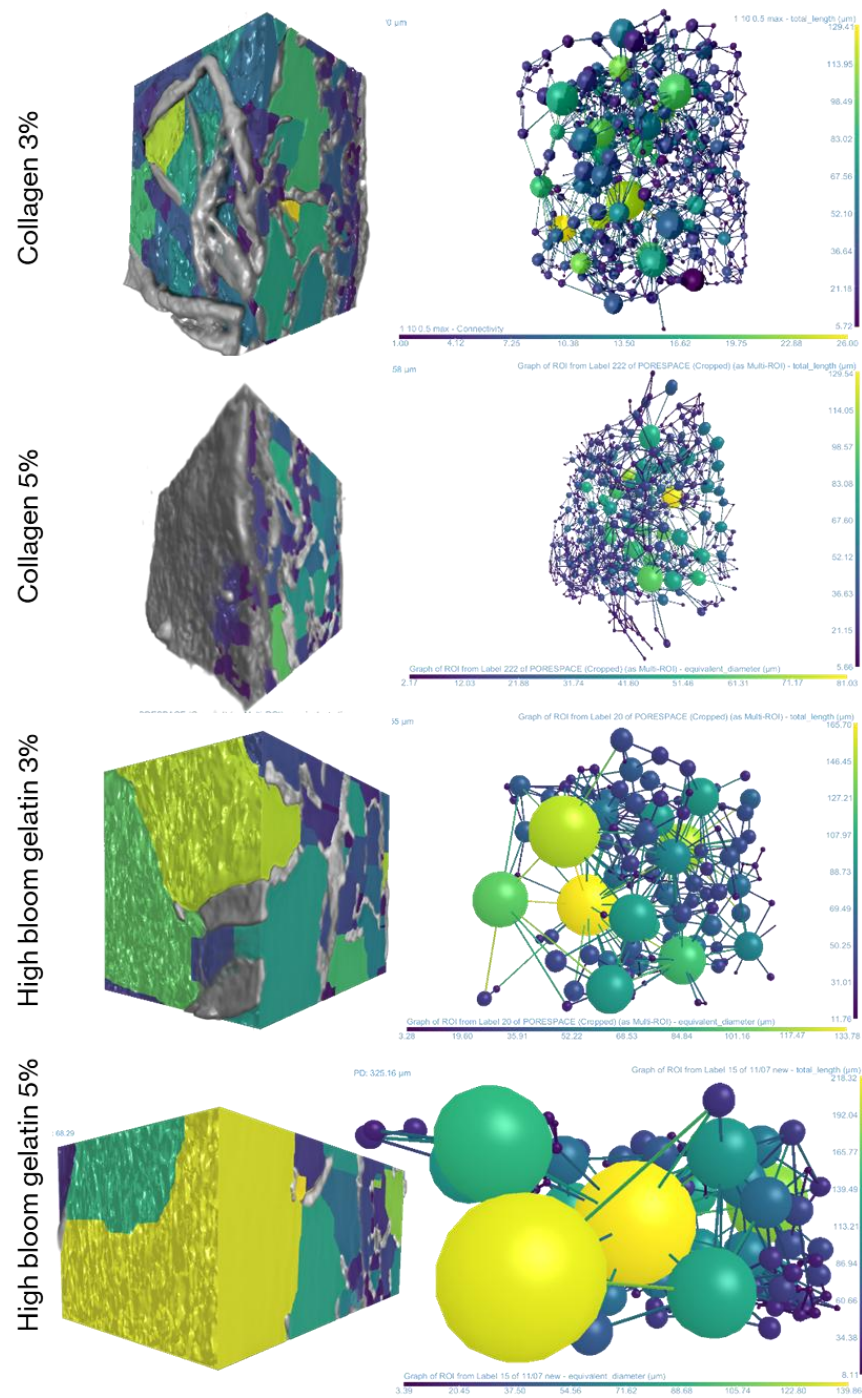


Figure 38 X-Z– MROI and Porosity view of MROI and Porosity. (Figure continue on next page).

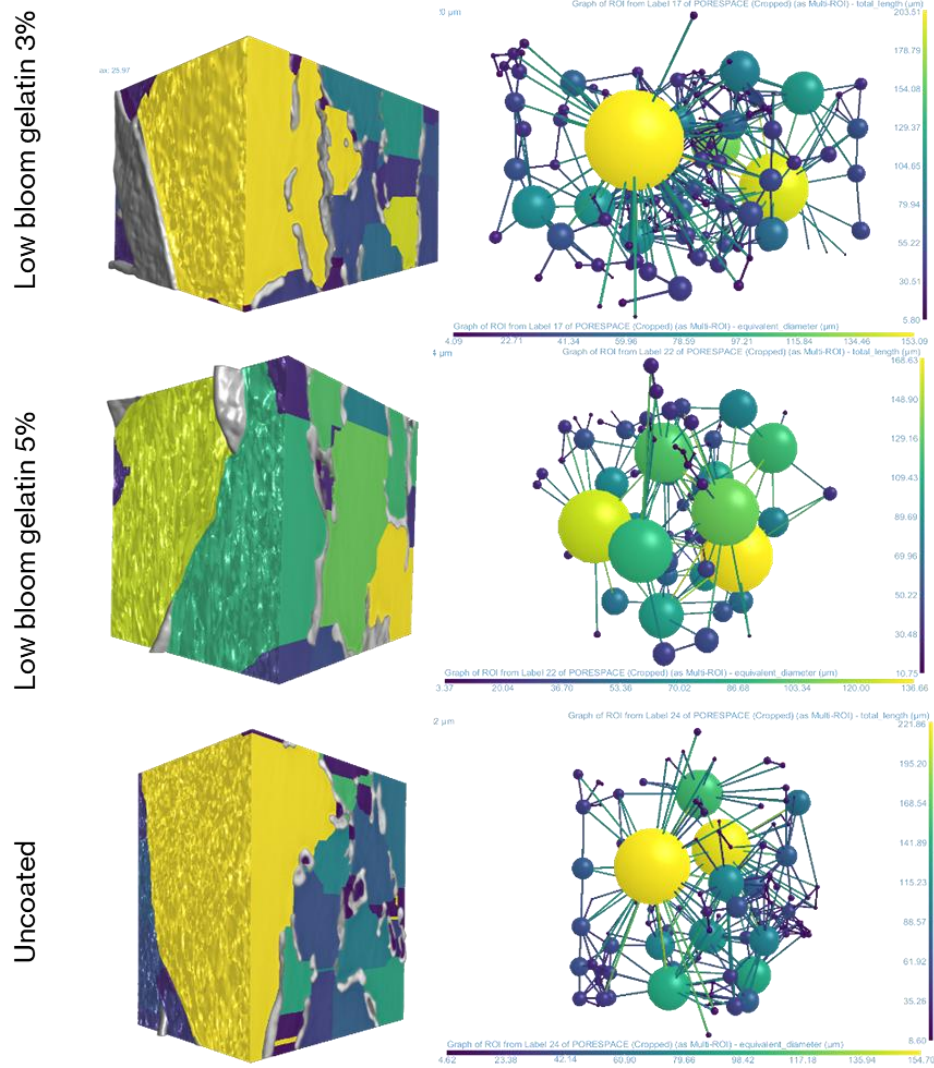


Figure 38 (continued).

The OpenPNM module allowed extraction of data as frequency versus pore equivalent diameter. Figure 39 presents the histograms of pore equivalent diameter derived from Figures 37 and 38 for each sample. All samples exhibited right-skewed pore distributions in which the majority of the population is composed of small-sized pores. This shows as high frequency on the left side of the scale (small pore size measured, μm) and the frequency tapers as the scale toward larger pores in decreasing numbers. Collagen at 5% (b) and high bloom gelatin at 5% (d) showed a distinct peak near the smaller pore sizes. Similar peaks were also present in collagen at 3% (a), high bloom gelatin at 3% (c), low bloom gelatin at 3% (e) and 5% (f), and the uncoated sheet (g), although these also displayed a minor peak between 20–40 μm . The most irregular distributions were observed in low bloom gelatin at 5% (f) and high bloom gelatin at 3% (c). In addition, the x-axis range for pore equivalent diameter varied across the samples. In ascending

order of maximum range: $\approx 80\text{ }\mu\text{m}$ for collagen at 3% and 5%; $\approx 130\text{ }\mu\text{m}$ for high bloom gelatin at 3% and 5%, and low bloom gelatin at 5%; and $\approx 150\text{ }\mu\text{m}$ for low bloom gelatin at 3% and the uncoated sheet.

An additional analysis was carried out using OpenPNM. The model segments pores into ROIs, but in fibrous sheets the pores are free-form and interconnected. This regenerates the plot into a representation of the connectivity of the pores. The spheres in the graphs represent the number of connections each pore has in the matrix, colour-coded with a legend indicating the connection count. Figures 40 and 41 show the 3D plots at X+Z+ and X-Z- orientations, respectively. Edge colour was kept as the measured distance between pore centres, while sphere size and colour represent the numbers of connections for each pore, thus the larger the pore and its colour -on the green to yellow - the more connectivity.

Within the same MROI, pore connectivity for collagen-coated samples was largely located beneath a layer of fibres and coating. Specifically, the 3% collagen sample exhibited a coating layer on the X+ axis, while the 5% collagen sample showed coating on the X- axis and partially on the X+ axis. The 3% collagen sample displayed more connectivity within the matrix, with larger and lighter-coloured spheres distributed across the cross-section. In contrast, 5% collagen exhibited smaller spheres distributed throughout the matrix, indicating reduced connectivity. Shorter connections between pores were generally observed in collagen-coated samples compared to both gelatin-coated and uncoated samples.

The gelatin-coated samples not only developed larger pores but also formed a more interconnected pore network across the cross-section. High bloom gelatin at 5% showed the greatest connectivity due to its large pores, resulting in an open and expansive matrix compared with the other coatings. Low bloom gelatin at 3% also promoted expansion, but rather than forming numerous connections, it generated elongated pore channels along the cross-section that still facilitated connectivity. From the 3D plots, the 3% high bloom gelatin, 5% low bloom gelatin, and uncoated samples displayed similar connectivity patterns.

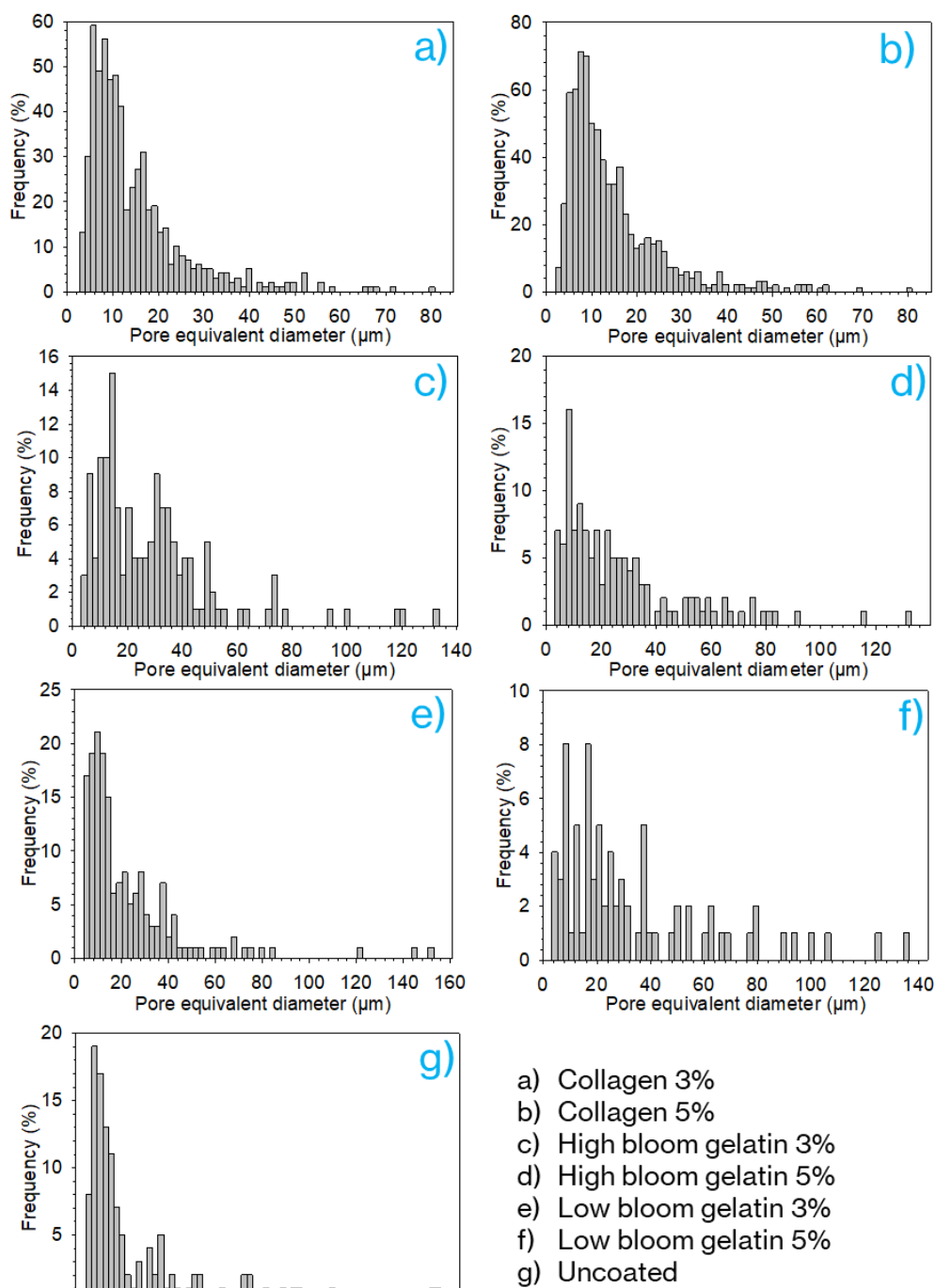


Figure 39 Pore size (as equivalent diameter) histograms.

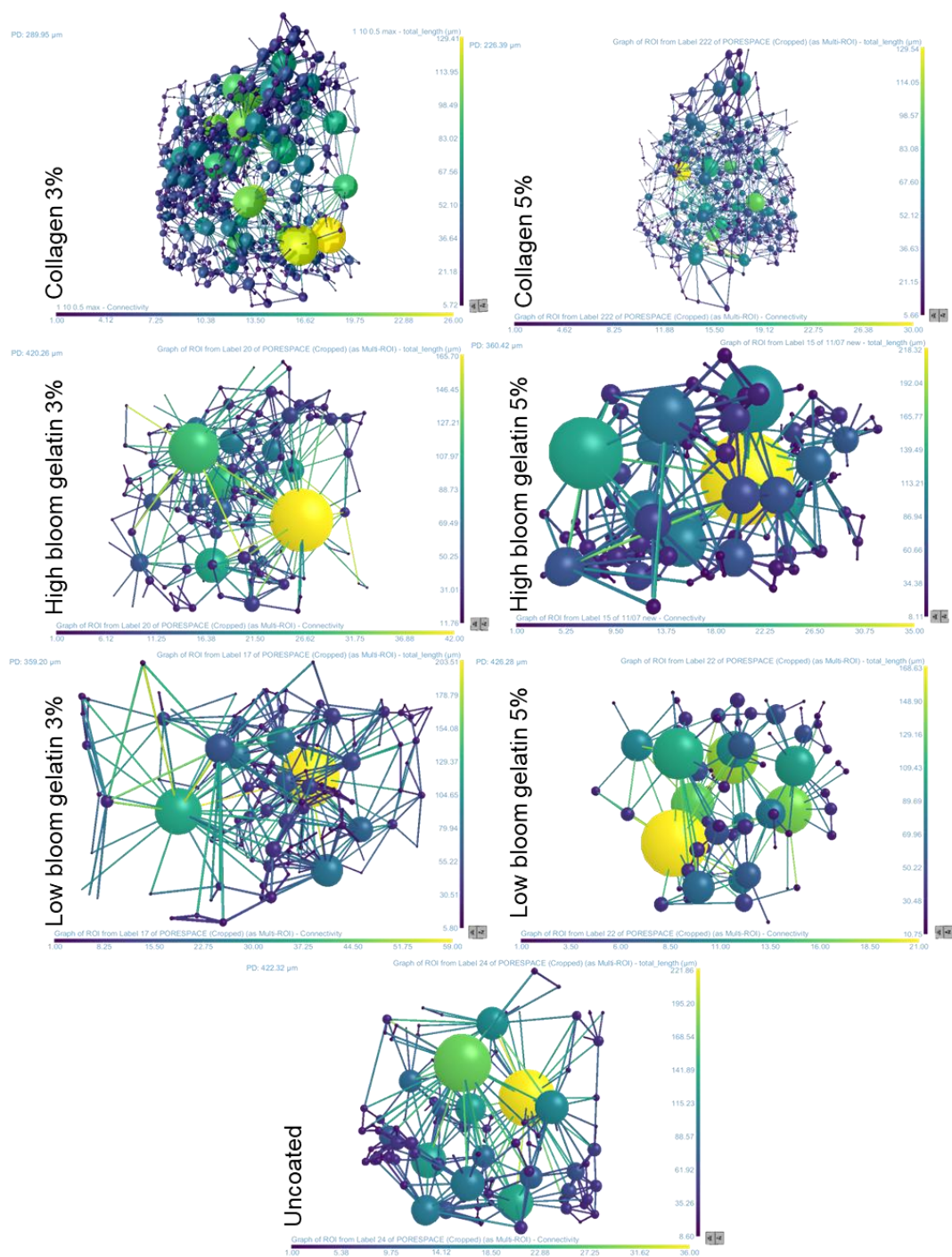


Figure 40 X+Z+ axis view displaying the connectivity of the pore segments with edge branching colours correspond to the length between centres of connected pores.

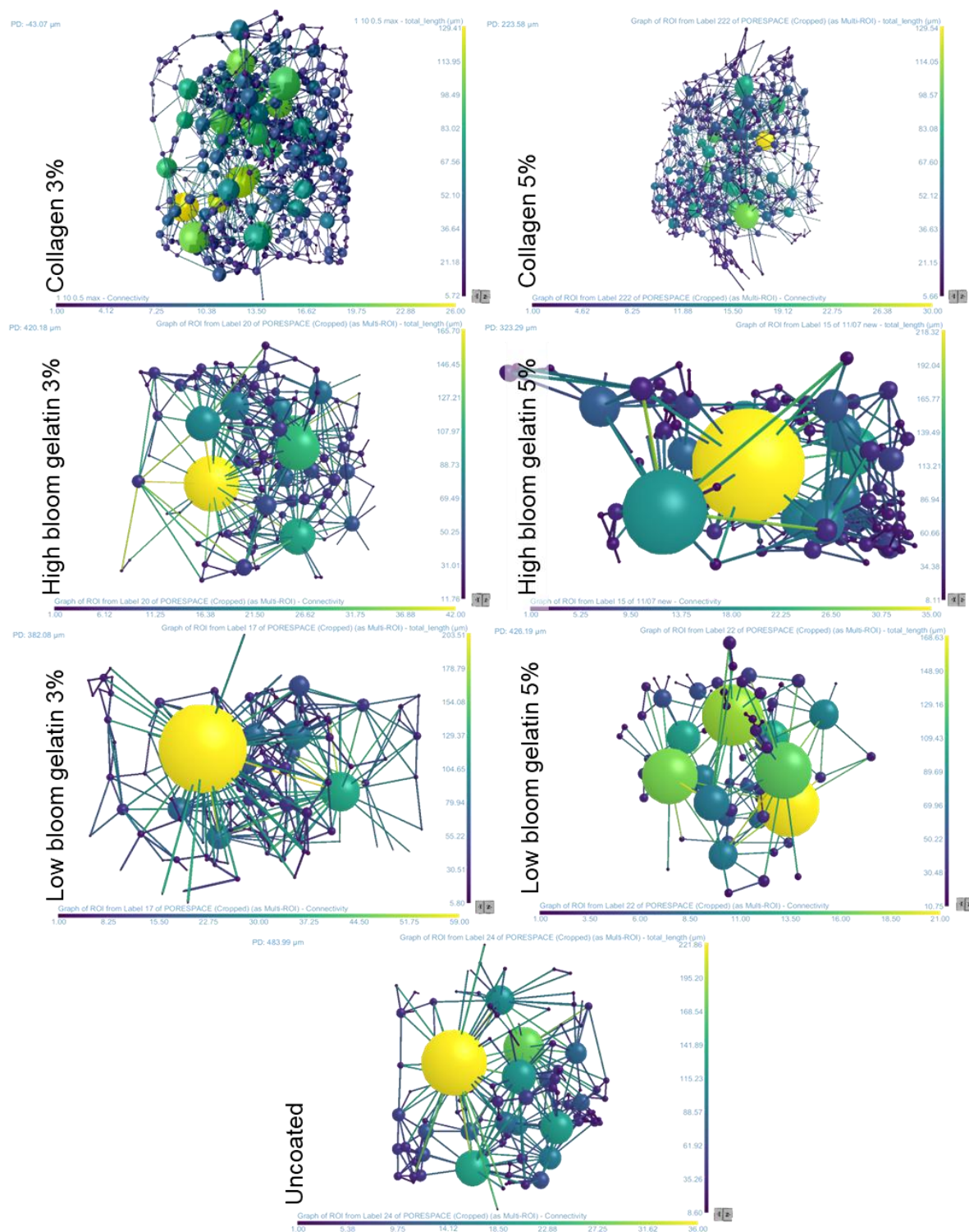


Figure 41 X–Z– axis view of connectivity and inter-pore length.

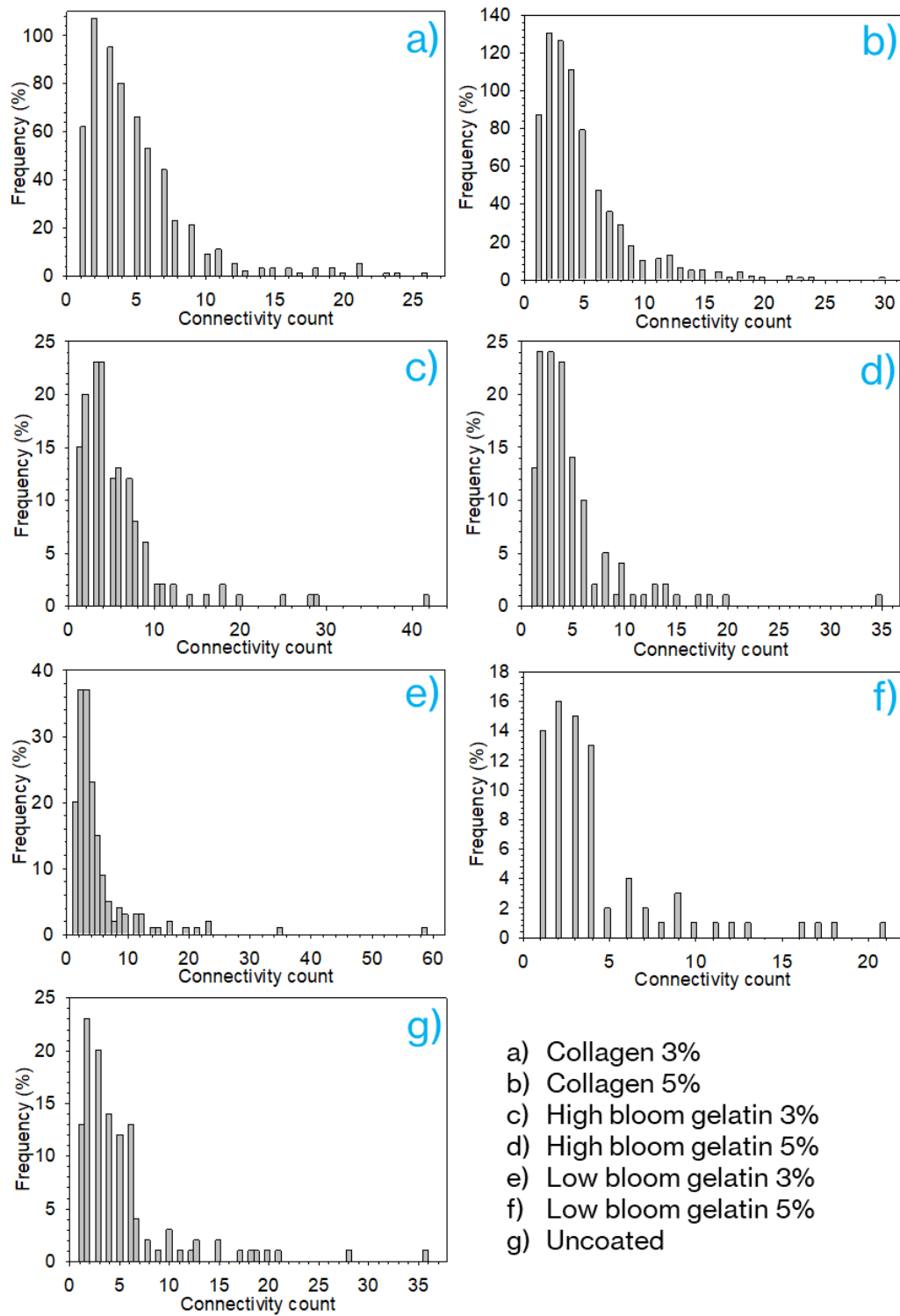


Figure 42 Histograms for connectivity between pores.

Histogram data presented in Figure 42 further analyse pore connectivity. All samples exhibited a right-skewed distribution, with most pores showing low connectivity. The 3% collagen (a), 5% collagen (b), 5% high bloom gelatin (d), and 3% low bloom gelatin (e)

displayed sharp peaks, suggesting a more uniform connectivity network dominated by pores with few connections. Among these, 5% collagen showed the highest overall pore count, despite its tighter matrix, as indicated by the higher frequency range within the lower connectivity counts. In contrast, 3% high bloom gelatin (c), 5% low bloom gelatin (f), and the uncoated sample (g) showed broader and more irregular distributions. Notably, 3% high bloom gelatin and 3% low bloom gelatin exhibited the widest distributions, with pores reaching approximately 40 and 60 connections, respectively. The 5% low bloom gelatin showed a more scattered connectivity distribution, consistent with the irregularity observed in its pore equivalent diameter histogram (Figure 42f).

7.3.3 Anisotropy analysis

Anisotropy analysis was performed on the uncoated sample. There were two anisotropy computation methods used: (1) mean intercept length (MIL) [453] and (2) star volume distribution (SVD) [454]. Parameter input value needed for the calculation include maximum iterations, sampling, orientations, minimum iterations, radius, and tolerance. These parameters were used to for the iteration runs solving for convergence (Table 28).

Table 28 Converged degree of anisotropy result. Minimum values for parameters.

Parameters	Value	
Maximum iteration	800	
Minimum iteration	400	
Sampling	15	
Orientations	16	
Radius	8	
Tolerance	0.05	
Method	SVD	MIL
DA value	0.44	0.43

Different parameters were tested to determine values representing the anisotropy of the matrix, with detailed results presented in Appendix C. Anisotropy vector field was also generated. The parameters input for the mapping are as follows: spacing at 20 μm , radius of influence to 30 μm , mesh smoothing at 2 and the evaluation was set either as Eigen- or Projection-base. The fibre ROI used is pictured in Figure 43.

The mapping parameters selected minimise overestimation of vector arrows and reduce crowding, with sampling, radius of influence, and mesh smoothing set to 20 μm , 30 μm , and 2, respectively. Projection- and eigen -based mapping is shown in Figure 44, with both methods displayed in XZ and Y-positive and Y-negative biased views.

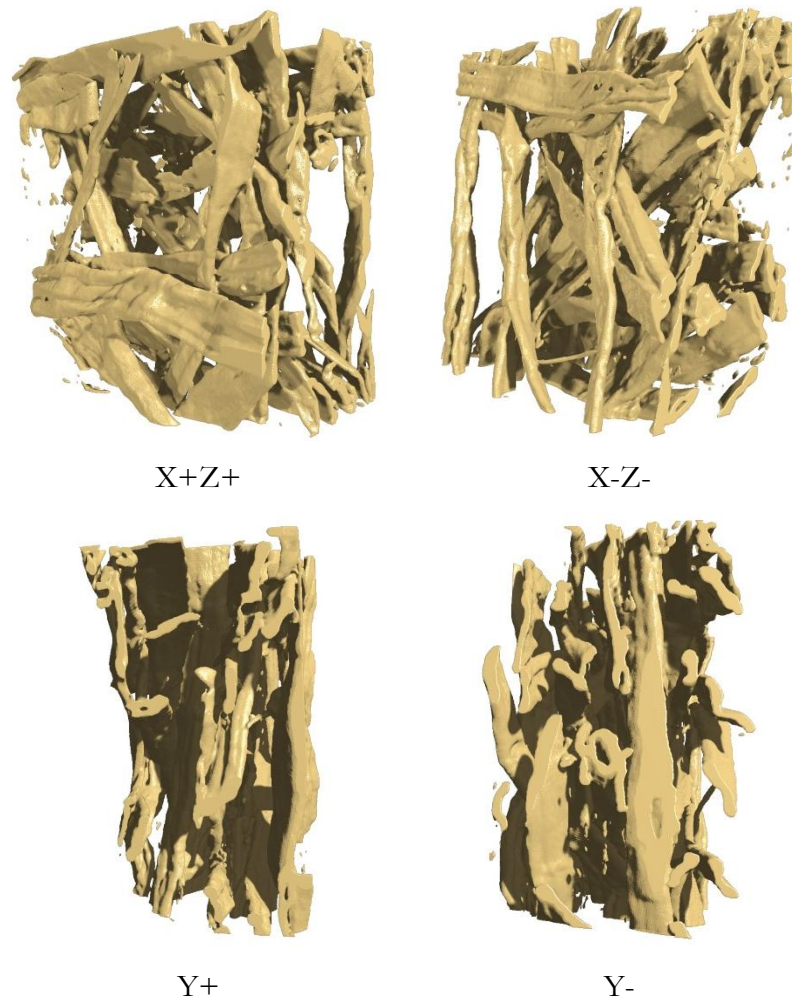


Figure 43 Fibre ROI for anisotropy analysis.

An immediate observation is that projection-based mapping suggests a more anisotropic matrix, while eigen-based mapping displays a wider variety of distributions, with colours corresponding to the degree of anisotropy (DA). The eigen-based method appears to display a scattered vector anisotropy compared to the projection-based method, which either indicates overestimation by the projection approach or greater sensitivity of that method to directional alignment (DA = 1, red colour). The anisotropic vectors for both methods showed concentration towards the surface of the fibre sheet.

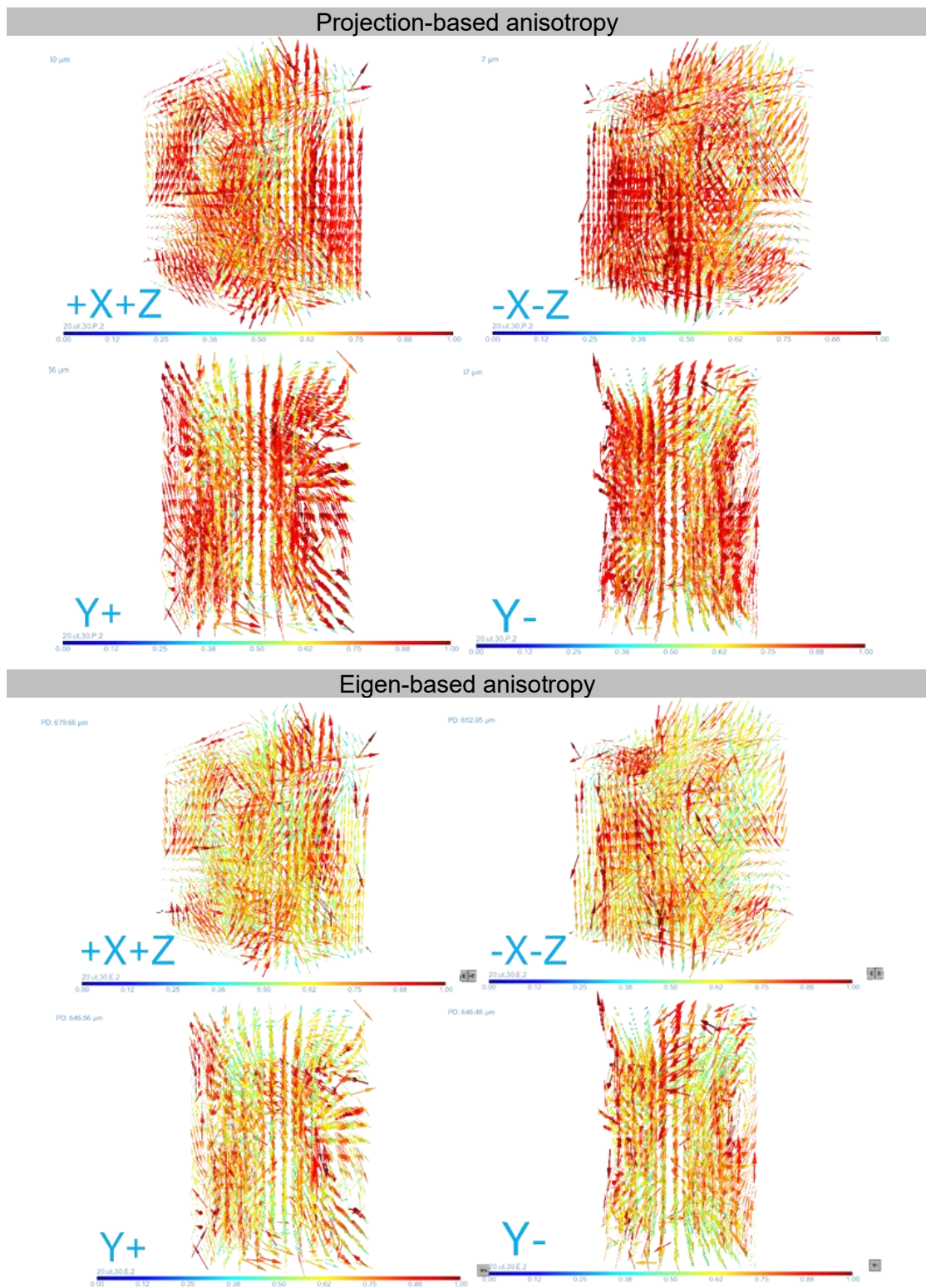


Figure 44 Projection- and eigen- based anisotropy evaluation at different axis views.

7.4 Discussion

7.4.1 Phase volume percentages

The composite sheet properties were presented in Chapter 6. This chapter rationalises these properties using the MCT technique and Dragonfly software.

The volume fractions utilises phase contrast within the matrix and allows observation of the openness and expansion of the structure. The expansion of the composite was measured in the cross-section thickness before and after coating the cellulose sheets.

Furthermore, the volume fraction contribution determined from the volume fractions (coating, fibre, and pore space of the coating and fibre) coincides with the weight of coating and cellulose sheet as shown in Table 23 for the fibre and coating weight contribution and Table 25 for the resulting volume contribution using the ROIs.

The summed percentage values for the aspect ratio can exceed 100%, as certain phases identified in the software analysis of MCT data overlap, while others remain distinct. The 3D and 2D visualization in Table 26 and 27 correspond to their SEM images shown in Table 21 and 22 (Chapter 6). Moreover, they show the distribution of the coating throughout the matrix. This is shown effectively in the cross section and the 2D slice. The deposit of coating on the surface of the sheet particularly for the 3% and 5% collagen, made substantial surface coverage despite the open structure, effectively forming a good barrier during permeability analysis (Table 18).

Moreover, on the 2D slices in Table 26, low bloom gelatin at 3% shows a concentration of fibres in the middle of the sheet and accumulation of the gelatin coating. Both 3% collagen and 3% high bloom gelatin show good aggregation of coating within the cross section. The former displayed it more on the outer matrix improving barrier properties, and the latter more in the centre effectively reinforcing the matrix improving tear strength. This is also observed with 5% collagen and 5% high bloom gelatin but with the addition of surface coating. Good tear strength performance was observed for 5% collagen but not for the 5% high bloom gelatin (Figure 25). This is due to its more open matrix (more pore space), which weakened the structure and required less force for tear to propagate.

The samples that display coating at the centre of the sheet such as 3% low bloom gelatin, 3% high bloom gelatin, 5% high bloom gelatin and 5% collagen showed good aggregation of coating or coating and fibre and performed well in the tensile testing (refer to Figure 21b). This may be due to deposition during coating and drying. Since the

sheets were fabricated via wet forming, heavier components may settle slightly, with the top portion sinking toward the middle and the bottom half draining toward the back-side surface. The resulting matrix developed a dense centre in the cross-section, affecting mechanical properties. This enabled the sheets to handle high stress and strain before failure and increasing total elongation, which was observed in 5% collagen. The 3% low bloom gelatin performed best during tear test possibly due to this aggregation. The uncoated sheets exhibited an open matrix. The absence of coating that could potentially hold fibres together showed the lack of structural integrity of the uncoated sheets which was evident during mechanical testing.

7.4.2 Porosity

The MROI segmentation was analysed and converted to pore equivalent diameter, presented in 3D graphs (Figure 37 and 38). This allowed visualization of the distribution of pore size as well as the spacing between pore centres (edges/branch). This further confirmed the compactness of the collagen coated samples and the resulting extent of openness of matrix once the sheets after gelatin coating. Coating the cellulose fibre sheets with gelatin aqueous solution, resulted to hydrogel absorption into the fibre, which caused the swollen gelatin to expand the fibre matrix upon penetration, consequentially increasing the cross-section thickness and formation of larger pores, increasing the total length between pores (presence of lighter edges). As for the collagen, it bridged fibres in the matrix and formed coating by further shrinking of the collagen fibrils upon drying pulls the fibres (purple edge indicating decreased length between pore centres). Moreover, the small purple spheres close to the surfaces of the matrix, particularly for the collagen coated samples could be false small pores (especially near edges or surfaces with a single connection) due to the sigma value used, introducing possible inaccuracy.

The data from the 3D plots were extracted and plotted as histograms (Figure 39). The low noise in the higher diameter bins indicated a more uniform structure. While a smoother right skewed distributions found in the collagen coated sample suggested a more homogenous porous network. Another indication of this was the narrower pore size distribution. The settling effect of the gelatin on the fibres during the initial stage of drying created agglomeration of both fibre and coating in the centre of the cross section, consequentially generated a substantial number of large pores, as reflected by the higher frequency of larger pores. This was particularly evident with 5% the low bloom gelatin,

further amplifying heterogeneity and creating a multi-modal distribution. Defects during fabrication could also have contributed to this distribution. These contributed to the low tear strength of 5% low bloom gelatin from Figure 25 (Chapter 6).

Pores with large equivalent diameter were more likely to form a connected pore network when located in the middle of the matrix. The large pores close to the threshold (pore space—fibre/coating interface) or on the surface of the sheet exhibited lower connectivity compared to pores near the centre. This was evident in the 3D connectivity plots (Figure 40 and 41). The dominant observation was that matrix expansion created connectivity paths, as observed in gelatin-coated and uncoated sheets. However, further analysis of histograms (Figure 39f and 42f) revealed that the irregularity of 5% low bloom gelatin in pore equivalent diameter resulted in lower connectivity counts, which did not fully represent the structure's open matrix, similar to other gelatin-coated samples. This emphasises the importance of visualization of the 3D images and plots for the samples. The false small pores were also observed, indicated by small spheres near surfaces and the cross-section thresholds showing single connections. Available data on both the pore size and connectivity allowed visualization and analysis of pore network throughout the volume of the composite. This information is valuable for mass transfer, such oxygen permeation and transmission rate, useful in food packaging applications [455]. Collagen samples displayed low mean connectivity, indicating more isolated pores that could restrict flow or transport. Also considering the thick fibre-coating layer on the surface of the sheets that were observed. While the gelatin coated and uncoated samples predominantly showed higher connectivity and a broad distribution, providing more and larger pathways for flow. Samples with occasional highly connected pores (Figure 42 c, e) might exhibit anisotropic transport or stress concentration zones [456]. This could have an effect both thermal and mechanical properties of the gelatin coated sheets.

7.4.3 Anisotropy

The convergence analysis produced stable degree of anisotropy (DA) values of 0.44 and 0.43 for the star volume distribution (SVD) and mean intercept length (MIL) methods, respectively. As DA values approach 1 with increasing anisotropy and orientational alignment, whereas values near 0 indicate isotropy, the mid-range values obtained here suggest moderate fibre alignment within the self-assembled cellulose sheet. The restricted imaging volume, imposed by computational constraints, may have influenced this outcome by emphasising local fibre organisation rather than large-scale alignment.

Furthermore, the limited numerical precision of the DA values (two significant figures) introduces uncertainty in pinpointing subtle changes at convergence. Nevertheless, the slightly higher DA from SVD compared with MIL indicates that SVD captured orientation with greater sensitivity and is generally considered a more effective method for characterising fibrous networks.

These differences are explained by the underlying assumptions of each method. MIL quantifies anisotropy by measuring intercepts of parallel test lines across the structure and assumes a degree of symmetry, which can underestimate anisotropy in irregular or self-assembled fibre networks [457]. In contrast, in SVD, a point grid is distributed on the image and rays of vectors extending from different directions until boundaries of the ROI are reached, enabling both primary and secondary orientations to be detected. While this heightened sensitivity can also result in overestimation if secondary orientations are suppressed, careful selection of sampling and radius parameters helps to avoid this effect [457].

Complementary insights were provided by anisotropy vector mapping (Figure 44). The projection-based approach revealed a predominance of highly anisotropic vectors, suggesting stronger alignment within the fibre network. By comparison, the eigen-based approach yielded a broader range of DA values (approximately 0.25–1), with fewer vectors at the highest anisotropy levels. This divergence reflects the conceptual basis of the two approaches: projection methods estimate orientation directly from voxel intensity gradients, offering high sensitivity but a tendency to overstate anisotropy [458, 459], whereas eigen-based analyses rely on structure tensor calculations within a defined neighbourhood, which provide robustness against noise but can smooth over finer directional detail [460]. Together, these results suggest that projection-based methods emphasise dominant orientations, while eigen-based methods provide a more balanced assessment of anisotropy in fibrous networks.

7.5 Conclusion

Micro-CT imaging combined with Dragonfly analysis enabled non-destructive, quantitative characterization of cellulose fibre composites under different collagen and gelatin coatings. Anisotropy calculation on uncoated sheets using both SVD and MIL showed fibre alignment was moderate with consistent anisotropy values across projection- and eigen- based methods. In addition, Projection mapping gave higher DA

values than eigen-based mapping, but both showed similar mapping results. The key findings in volume fractions and porosity analysis are summarised in Table 29.

Table 29 Key findings correlating volume ratios and porosity analysis on composite properties. Abbreviations used: C- collagen, HBG- high bloom gelatin, LBG- Low bloom gelatin, and UC- uncoated.

	Volume fractions	Porosity	Effect on properties
C 3%	• Reduced pore fraction compared with uncoated, but compaction was modest. • Solid fraction increased slightly, reflecting early stages of densification. • Aggregation at cross-section centre.	• Pores were smaller than uncoated but still showed some connectivity. • Network was denser, though less isolated than at 5% collagen.	• Displayed improvement in barrier properties (good coverage of pores). • Good tear strength
C 5%	• Strongly reduced pore fraction, with clear compaction of the fibre network. • Highest solid fraction among all samples, reflecting significant densification. • Aggregation at cross-section centre and added surface coating.	• Produced the smallest pores and lowest connectivity. • Networks contained short, isolated pore to pore links with minimal continuity.	• Significant improvement in permeability (substantial coverage from coating). • Increased tensile strength • High tear strength
HBG 3%	• Expanded the fibre network more strongly than low bloom at the same concentration. • Aggregation at cross-section centre. • Higher pore fraction achieved even at 3%.	• Generated larger pores and higher connectivity than low-bloom 3%. • Networks showed early development of continuity across the fibre matrix.	• Increased tensile strength • High tear strength
HBG 5%	• Produced the highest pore fraction of all samples, maximising openness of the fibre network. • Solid fraction was reduced to its lowest relative level. • Aggregation at cross-section centre and added surface coating.	• Produced the largest and most interconnected pores across all conditions. • Networks were highly continuous, spanning the matrix with strong pore to pore connectivity.	• High tensile strength • Improved barrier properties but not as well as the collagen coated sheets.

LBG 3%	• Expanded the fibre network compared with uncoated, increasing pore fraction. • Expansion was present but less pronounced than at 5% concentration.	• Generated larger pores with some improvement in connectivity. • Networks were more open than uncoated or collagen-coated sheets, though less continuous than at 5%.	• High tensile strength • High tear strength (performed best tear test)
LBG 5%	• Further expanded the fibre network, increasing pore fraction more than the 3% counterpart. • Shifted balance towards greater openness, reducing the sheet-to-pore space ratio.	• Produced larger and irregular, more connected pores than low-bloom 3%. • Networks were moderately continuous, but less developed than in high-bloom gelatin.	• Expansion caused an increased in permeability • Possible defects (pore irregularities) displayed poor tear properties.
UC	• Showed an intermediate pore fraction, neither highly compacted nor expanded. • Fibre and air volumes were balanced, providing a reference state for comparison.	• Displayed moderate pore size and connectivity. • Pore networks resembled low-bloom gelatin at higher concentration, but without extensive expansion.	• Poor barrier properties: fluid/gas can move through it easily • Lack structural integrity

Collectively, these findings establish a clear relationship between coating type, microstructural modification, and resulting mechanical behaviour. This integrated approach provides a robust framework for evaluating and optimizing natural fibre composites, offering pathways to tailor porosity, anisotropy, and properties for advanced applications such as sustainable packaging.

Chapter 8. Conclusions and recommendations for future work

This thesis explored the potential of New Zealand sheepskin, a low-value by-product of the meat industry, as a source of collagen for sustainable material applications. By moving from extraction and characterisation through composite fabrication and advanced imaging, the research demonstrated how underutilised biological resources can be transformed into functional materials, contributing both to waste valorisation and to the advancement of bio-based composites.

The research produced three major contributions. First, it established baseline knowledge on the efficiency and properties of collagen extracted from sheepskin, a species largely neglected in prior studies. Second, it demonstrated the feasibility of applying collagen as a functional coating for fibre sheets, improving barrier and surface properties while providing an alternative to synthetic additives. Third, it showcased the novel use of synchrotron micro-CT to probe collagen-fibre interactions in three dimensions, revealing structural insights that conventional techniques could not capture. Together, these contributions highlight both the scientific novelty and practical significance of valorising sheepskin for circular bioeconomy applications.

Chapters 4 and 5 developed and compared collagen extraction methods (acid, acid-enzyme, and alkali), identifying the influence of pre-treatment and acid concentration on yield, composition, and structural integrity. While yields were lower than cattle hide counterparts, the studies confirmed that sheepskin can supply collagen of suitable quality with significantly higher collagen content even when only acid is used for extraction, with FTIR, SDS PAGE, SEM, and SAXS establishing collagen properties and confirming preservation of its fibrillar structure under both suboptimal and optimal conditions. This proved that sheepskin is a reliable source of collagen.

Chapters 6 and 7 applied this collagen in fibre sheet coatings, beginning with baseline fibre characterisation and extending to performance testing of collagen- and gelatin-coated composites. Coating application improved surface wettability, reduced air permeability, and enhanced barrier behaviour, with concentration-dependent effects. Direct comparisons with gelatin revealed similarities in function but also distinct advantages of collagen in fibre-matrix interactions, particularly in surface and barrier

properties. A key novelty was the integration of synchrotron micro-CT to assess composite structure in three dimensions. This advanced analysis revealed how collagen penetrated and distributed within the fibre network, providing evidence of fibre-matrix bonding that directly explained the observed changes in properties. By contrasting collagen and gelatin, micro-CT highlighted unique structural mechanisms underpinning collagen's performance, marking one of the first uses of this technique in protein-fibre composites.

The final chapter synthesised these insights, framing sheepskin collagen as both a waste valorisation strategy and a sustainable material feedstock. By showing that low-value sheepskins can yield functional collagen suitable for composite coatings, this research links agricultural by-products with bio-based materials innovation.

Overall, this thesis demonstrates that:

1. Sheepskin collagen can be extracted and structurally preserved under optimised conditions, even if yields are modest compared to other livestock.
2. Collagen-based coatings substantially improve the surface and barrier properties of fibre sheets compared to uncoated samples.
3. Synchrotron micro-CT provides unprecedented structural insights, linking coating distribution with functional outcomes in collagen-fibre composites.

These contributions advance understanding of both sheepskin-sourced collagen processing and its application in sustainable materials, bridging waste management challenges with circular economy solutions.

Future research could focus on further optimising extraction efficiency and scalability (pilot plant scale), tailoring collagen-fibre interactions through crosslinking or blending strategies, conducting life cycle analysis, and expanding the use of synchrotron imaging to in-situ studies of mechanical or environmental behaviour. Such work will accelerate the transition from laboratory demonstration to practical application, positioning sheepskin collagen as a contributor to sustainable materials development in New Zealand and beyond.

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[1/Default.htm#ROIs%20and%20Multi-](https://www.theobjects.com/dragonfly/dfhelp/2024-1/Default.htm#ROIs%20and%20Multi-ROIs/Regions%20of%20Interest/Computing%20Anisotropy.htm)

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Appendices

Appendix A: Chapter 5 modified and large batch extraction

A1. Yield T-test

Table 30 Yield T-test summary for all extraction methods

Sample no.	One-step acid enzyme (AE1)	Acid extraction step 1 (A2)	Acid-enzyme step 2 (AE2)	Two-step acid-enzyme total (AES2S)	Chp 4 Acid	Chp 4 Acid enzyme
1	2.335	2.780	0.198	2.138	0.470	4.840
2	2.788	3.225	0.240	2.548	4.168	3.580
3	3.428	2.420	0.220	3.208	0.996	3.450
4	3.723	-	0.275	3.448	1.557	2.527
5	-	-	-	-	-	4.060
6	-	-	-	-	-	4.826
7	-	-	-	-	-	0.191
8	-	-	-	-	-	0.509
9	-	-	-	-	-	0.191
Average	3.068	2.808	0.233	2.835	1.798	2.686
Standard deviation	0.625	0.403	0.033	0.601	1.641	1.927
Variance	9.413	7.887	0.054	8.037	0.821	7.215
Number of samples	4	3	4	4	4	9
Dataset comparison		t-test value	degree of freedom	two tail, 0.05 critical value		
One step, two step		0.071	5	2.571		
Acid two step, acid enzyme two step		0.750	6	2.441		
One step, acid enzyme two step		0.007	5	2.571		
One step, acid two step		0.780	5	2.571		
Two step total vs old		0.101352	11	2.201		
one step acid-enz vs old		0.035782	10	2.228		
acid two step vs old acid		0.758107	6	2.441		

A2. Hydroxyproline raw data

Table 31 Calculation data for HYP and collagen content

Sample	2 Acid Step			3 AEst			4 AcidE nz			5 Largebatch		
Volume (mL)	5	5	5	5	5	5	10	10	10	10	10	10
Weight (mg)	5.2122	5.365	5.2711	5.157	5.186	5.201	10.042 5	10.028 7	10.042 5	10.282 5	10.245 1	10.225 3
6 M HCl (mL)	5	5	5	5	5	5	10	10	10	10	10	10
Hydrolysis vol (mL)	10	10	10	10	10	10	20	20	20	20	20	20
Initial dilution (mL)	25	25	25	25	25	25	50	50	50	50	50	50
Absorbance	0.148	0.148	0.148	0.133	0.133	0.133	0.131	0.130	0.131	0.130	0.131	0.131
Hyp (ug/ml) from abs	12.210	12.210	12.222	10.611	10.580	10.580	10.357	10.332	10.351	10.339	10.351	10.369
Hyp (ug/g sample)	58562. 747	56894. 828	57969. 367	51439. 631	51002. 360	50855. 266	51566. 239	51514. 078	51535. 495	50272. 585	50516. 365	50704. 777
Hyp (g/g)	0.059	0.057	0.058	0.051	0.051	0.051	0.052	0.052	0.052	0.050	0.051	0.051
Conversion factor	8	8	8	8	8	8	8	8	8	8	8	8
Collagen (ug/g)	46850 1.979	45515 8.624	46375 4.932	41151 7.046	40801 8.877	40684 2.126	41252 9.908	41211 2.628	41228 3.961	40218 0.679	40413 0.919	40563 8.216
Collagen (mg/g)	468.50 2	455.15 9	463.75 5	411.51 7	408.01 9	406.84 2	412.53 0	412.11 3	412.28 4	402.18 1	404.13 1	405.63 8
Coll (g/g)	0.469	0.455	0.464	0.412	0.408	0.407	0.413	0.412	0.412	0.402	0.404	0.406
Hyp mg/g sample	58.563	56.895	57.969	51.440	51.002	50.855	51.566	51.514	51.535	50.273	50.516	50.705
Collagen % in extract	46.850	45.516	46.375	41.152	40.802	40.684	41.253	41.211	41.228	40.218	40.413	40.564
Collagen % in relation to 12.5% Hyp in Coll	4.685	4.552	4.638	4.115	4.080	4.068	4.125	4.121	4.123	4.022	4.041	4.056

Hyp mg/g extract	58.563	56.895	57.969	51.440	51.002	50.855	51.566	51.514	51.535	50.273	50.516	50.705
Collagen mg/g extract	468.50 2	455.15 9	463.75 5	411.51 7	408.01 9	406.84 2	412.53 0	412.11 3	412.28 4	402.18 1	404.13 1	405.63 8
Percentage of collagen in the extracted collagen (%)	46.850	45.516	46.375	41.152	40.802	40.684	41.253	41.211	41.228	40.218	40.413	40.564
Extraction efficiency	0.2	0.24	0.22	2.14	2.55	3.21	2.78	3.23	2.42	5.09	5.09	5.09
Raw skin weight g	40	40	40	40	40	40	40	40	40	2400	2400	2400
Extracted product g	0.079	0.096	0.088	0.855	1.019	1.283	1.112	1.290	0.968	122.10 0	122.10 0	122.10 0
From efficiencies confirmation sheet: extract g/g skin	0.002	0.002	0.002	0.021	0.025	0.032	0.028	0.032	0.024	0.051	0.051	0.051
Collagen mg/g skin	0.925	1.092	1.020	8.796	10.394	13.049	11.468	13.291	9.977	20.461	20.560	20.637
Collagen g/g skin	0.001	0.001	0.001	0.009	0.010	0.013	0.011	0.013	0.010	0.020	0.021	0.021
Collagen content (~35%)(mg/mg dry raw skin) from Rafea thesis)	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Raw skin sample weight mg	40000	40000	40000	40000	40000	40000	40000	40000	40000	24000 00	24000 00	24000 00
Collagen content mg (total collagen content in sheepskin)	14000	14000	14000	14000	14000	14000	14000	14000	14000	84000 0	84000 0	84000 0
Collagen content g	14	14	14	14	14	14	14	14	14	840	840	840
Collagen from extract g in the total raw skin weight (mg)	37.012	43.695	40.810	351.84 7	415.77 1	521.97 8	458.73 3	531.62 5	399.09 1	49106. 261	49344. 385	49528. 426
Collagen from extract g in the total raw skin weight (g)	0.037	0.044	0.041	0.352	0.416	0.522	0.459	0.532	0.399	49.106	49.344	49.528
Percentage of collagen extracted from total collagen content of raw skin sample (%)	0.264	0.312	0.292	2.513	2.970	3.728	3.277	3.797	2.851	5.846	5.874	5.896

A3. Mass balance for large batch extraction

Table 32 Collagen extraction mass balance

Pickled skin (g)	Extract solution (L)	Extracted skin weight (g)	NaCl weight to 2.6 M (kg)	Empty centrifuge bottles (g)	Bottle + extract solution (g)	Extract solution (g)	Liquid (g)	Precipitate (g)	Freezedried collagen (g)	Yield (%)
2400.00	14.98	919.08	2.28	468.58	3197.49	2728.91	-	-	7.50	-
-	14.35	898.11	2.18	445.46	3186.36	2740.90	-	-	4.50	-
-	14.60	850.85	2.22	420.21	3128.69	2708.48	-	-	12.00	-
-	14.50	930.38	2.20	452.46	3148.31	2695.85	-	-	20.00	-
-	-	-	-	467.65	3145.60	2677.95	-	-	10.00	-
-	-	-	-	454.79	3158.19	2703.40	-	-	6.00	-
-	-	-	-	454.81	3159.40	2704.59	-	-	3.00	-
-	-	-	-	421.36	3168.10	2746.74	-	-	1.80	-
-	-	-	-	468.68	3080.53	2611.85	-	-	6.00	-
-	-	-	-	444.59	3064.64	2620.05	-	-	10.00	-
-	-	-	-	463.04	3160.42	2697.38	-	-	-	-
-	-	-	-	424.08	3183.18	2759.10	-	-	-	-
-	-	-	-	467.42	3172.03	2704.61	-	-	-	-
-	-	-	-	447.04	3176.07	2729.03	-	-	-	-
-	-	-	-	440.42	3116.17	2675.75	-	-	-	-
-	-	-	-	454.32	3167.07	2712.75	-	-	-	-
-	-	-	-	430.99	3147.62	2716.63	-	-	-	-

-	-	-	-	409.15	3164.31	2755.16	-	-	-	-
-	-	-	-	450.96	3114.28	2663.32	-	-	-	-
-	-	-	-	471.36	3170.47	2699.11	-	-	-	-
-	-	-	-	423.29	3143.43	2720.14	-	-	-	-
-	-	-	-	472.05	3187.87	2715.82	-	-	-	-
-	-	-	-	445.27	3185.19	2739.92	-	-	-	-
-	-	-	-	460.38	3244.14	2783.76	-	-	41.30	-
2400.00	58.43	3598.42	8.88	10758.36	75769.56	65011.20	62876.30	2134.90	122.10	5.09

A4. Yield extract for the modified methods

Table 33 Extraction yield for the modified extraction methods

	Container (g)	Container + Freezedried collagen (g)	Freeze-dried (g)	Yield (%)
Acid-enzyme extraction				
ACE 1	20.56	21.668	1.112	2.780
ACE 2	15.89	17.176	1.290	3.225
ACE 3	15.87	16.838	0.968	2.420
			Avg	2.808
			stdev	0.403
Acid step extraction				
AC 1	20.56	20.634	0.08	0.20
AC 2	20.56	20.652	0.10	0.24
AC 3	20.55	20.641	0.04	0.22
AC 4	20.55	20.664	0.06	0.28
			Avg	0.23
			stdev	0.03
Acid-enzyme step extraction				
ACES 1	20.56	21.411	0.86	2.14
ACES 2	20.56	21.574	1.02	2.55
ACES 3	20.55	21.835	1.28	3.21
ACES 4	20.55	21.933	1.38	3.45
			Avg	2.835
			stdev	0.601
Total Acid+ Acid-enzyme step				3.07

A5. FTIR wavenumber legends

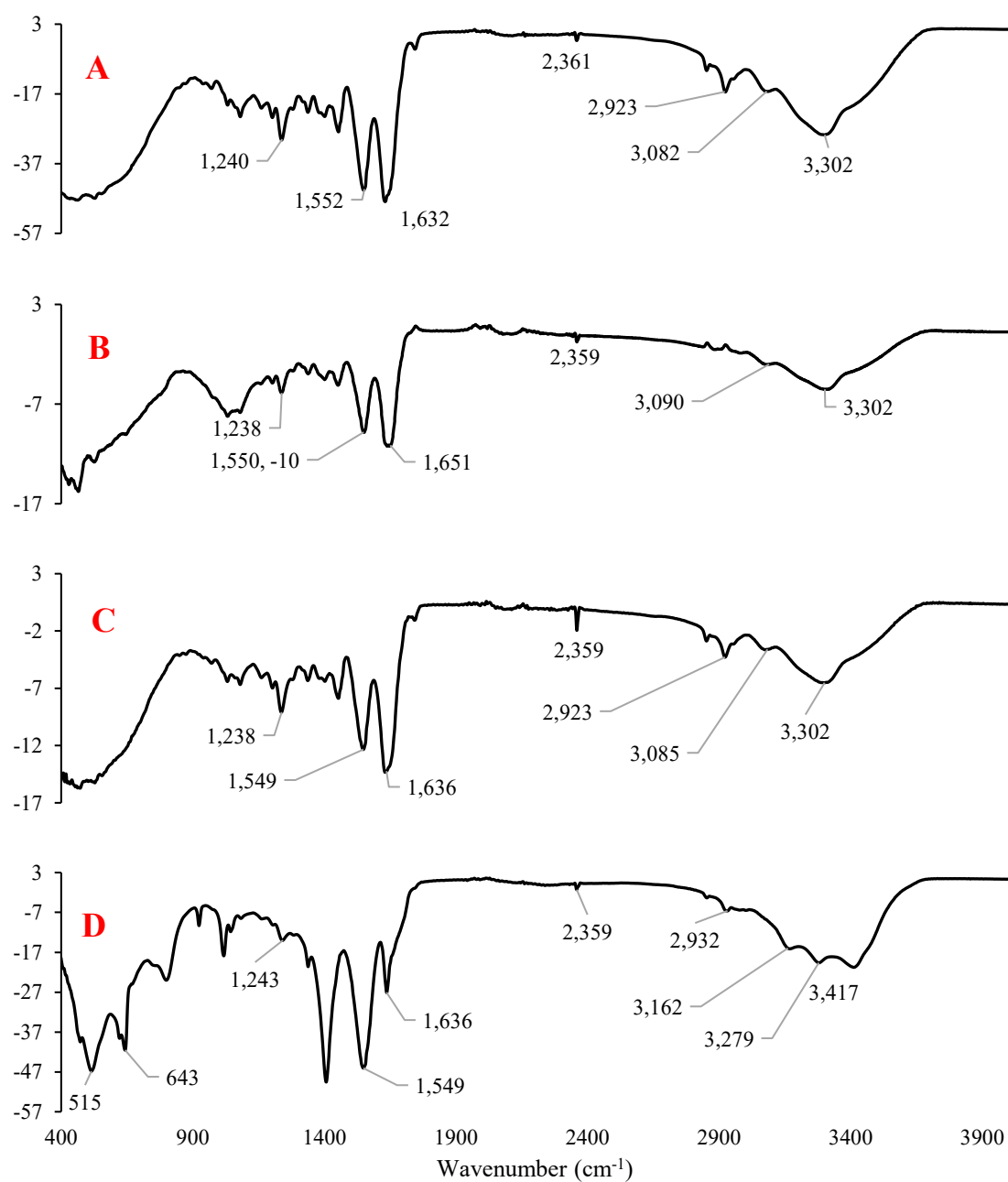


Figure 45 FTIR plot results for the modified extraction methods (A) Step 1: Acid extraction step (B) Step 2 Acid-enzyme (C) One-step acid enzyme (D) Large batch acid-enzyme extraction

A6.SAXS Images: Detection of collagen fibrils extracted from acid, acid-enzyme, and alkali

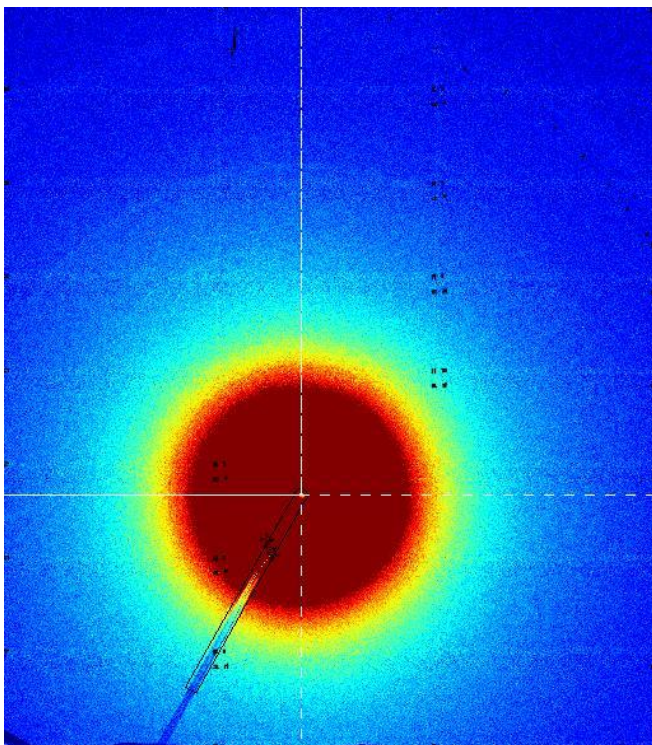


Figure 46 SAXS imaging:
collagen sample extracted via
acid hydrolysis

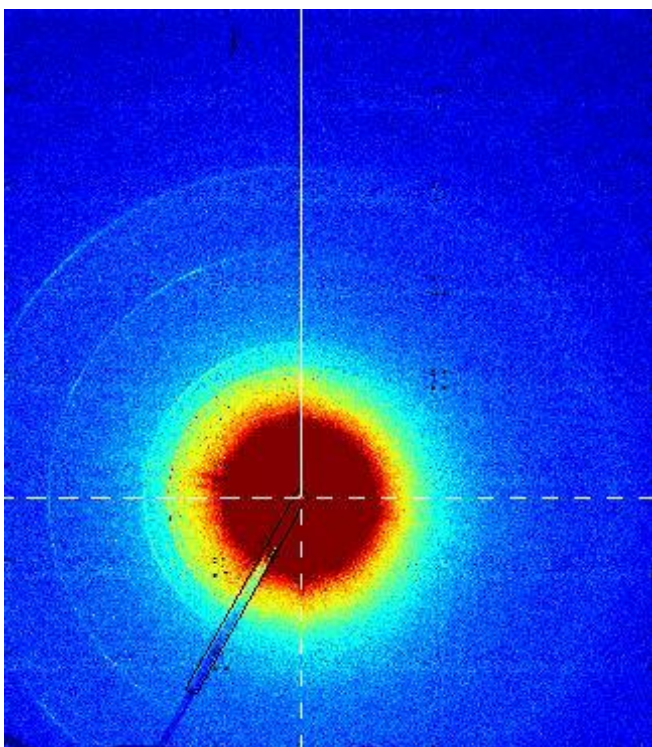


Figure 47 AXS imaging:
collagen sample extracted via
acid-enzyme hydrolysis

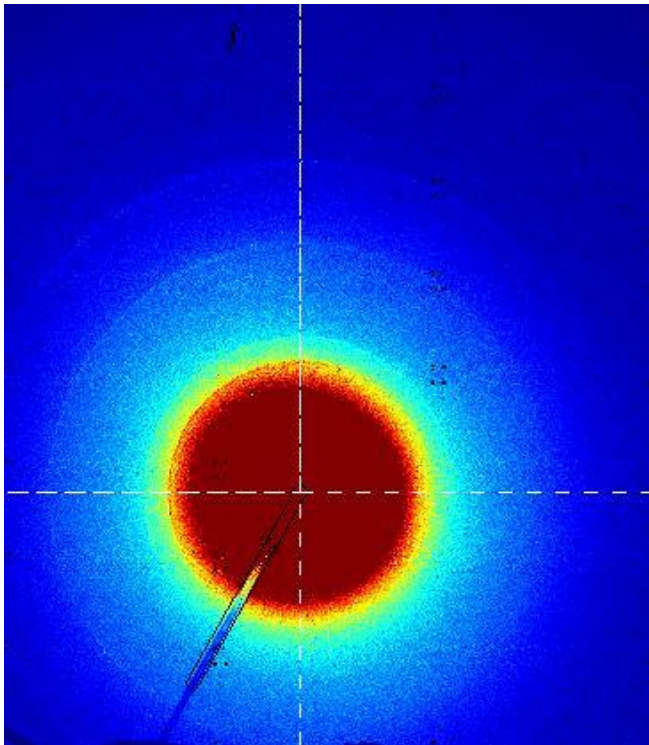


Figure 48 SAXS imaging:
collagen sample extracted via
alkali hydrolysis

Appendix B: Chapter 6 Collagen and cellulose sheet fabrication

B1. Tensile plots

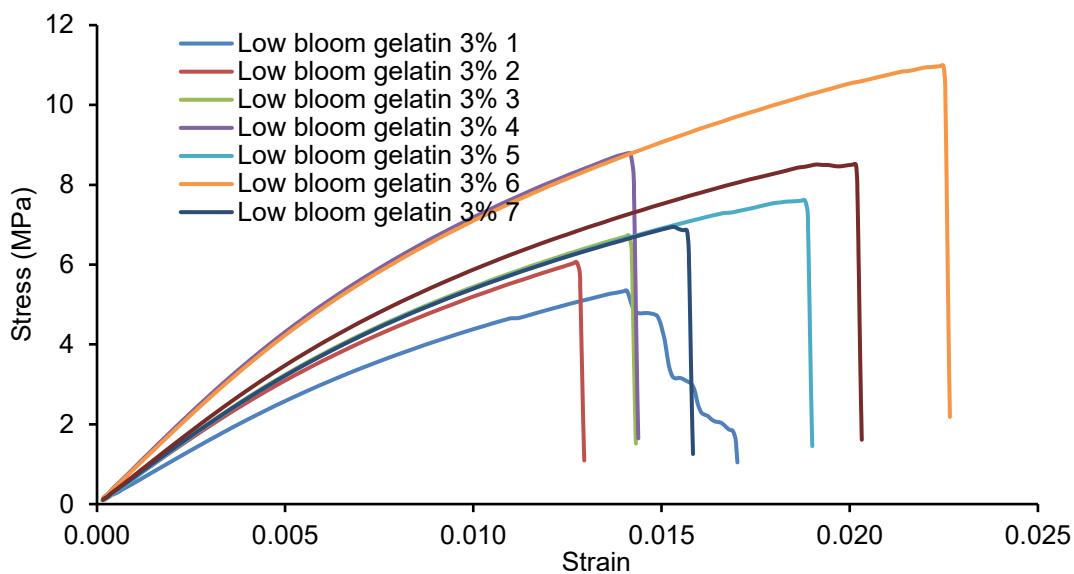


Figure 49 Tensile plot: Low bloom gelatin 3%

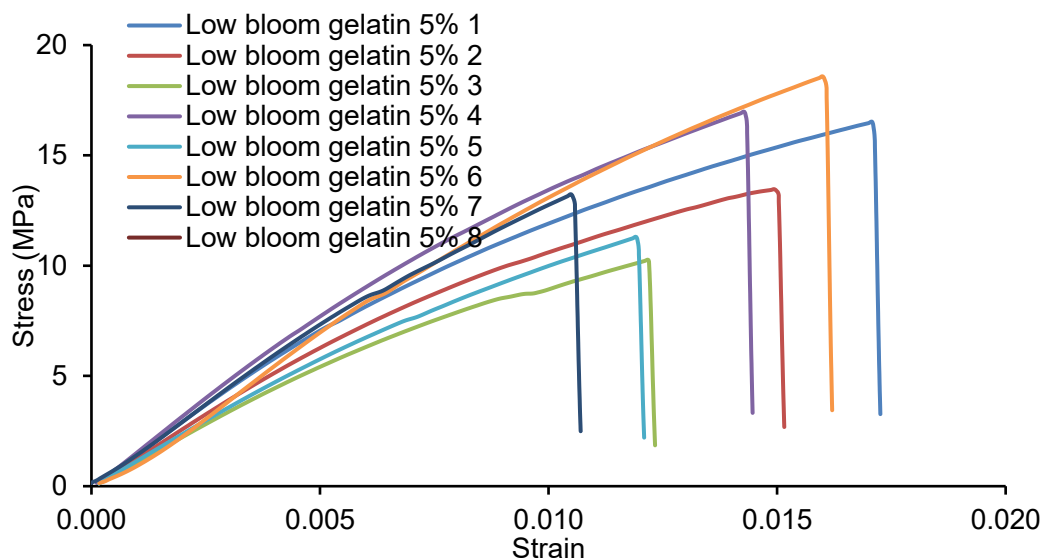


Figure 50 Tensile plot: Low bloom gelatin 5%

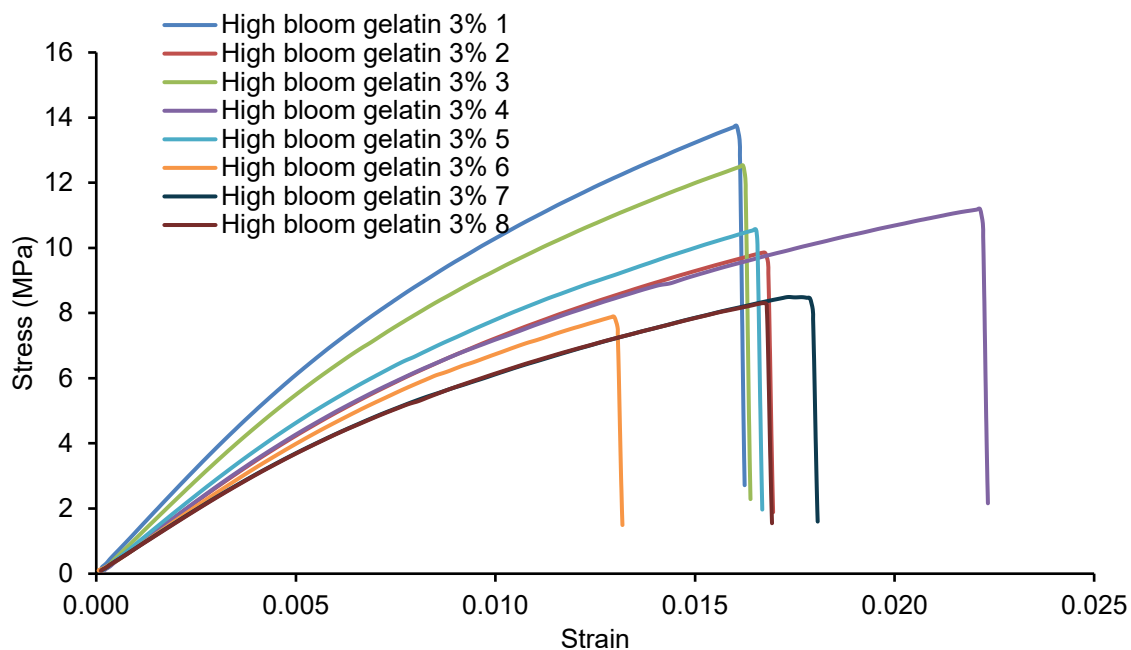


Figure 51 Tensile plot: High bloom gelatin 3%

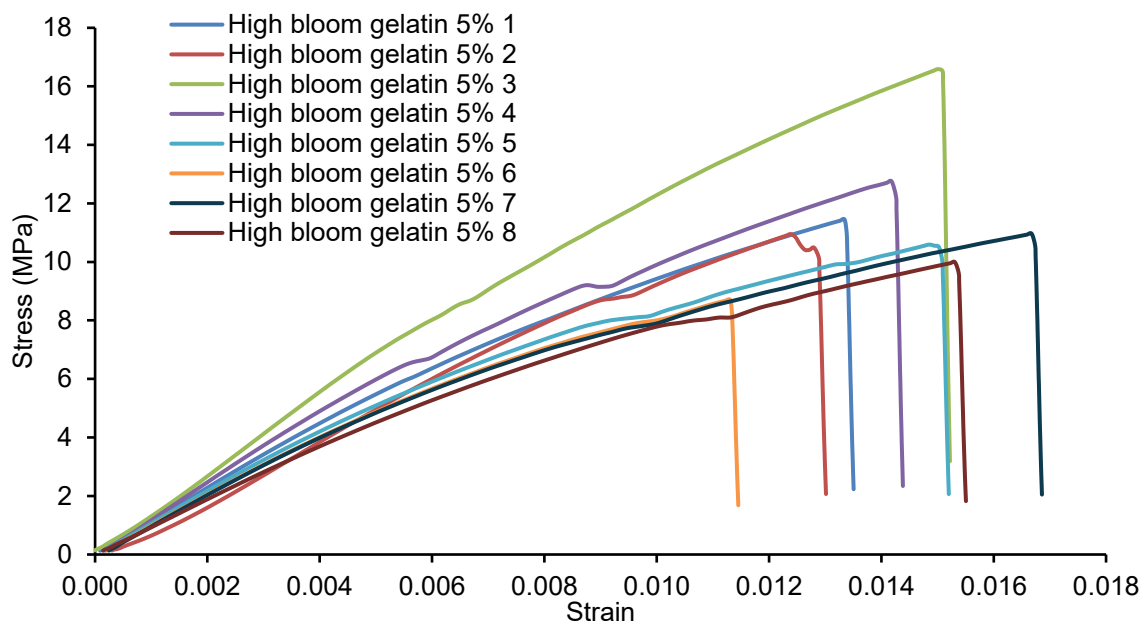


Figure 52 Tensile plot: High bloom gelatin 5%

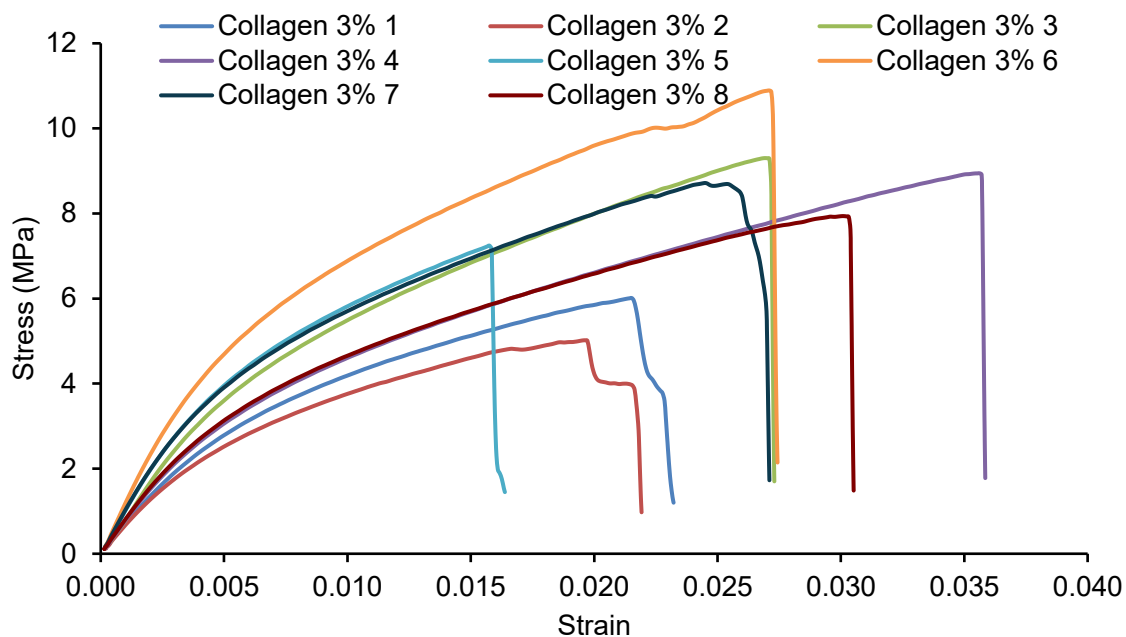


Figure 53 Tensile plot: Collagen 3%

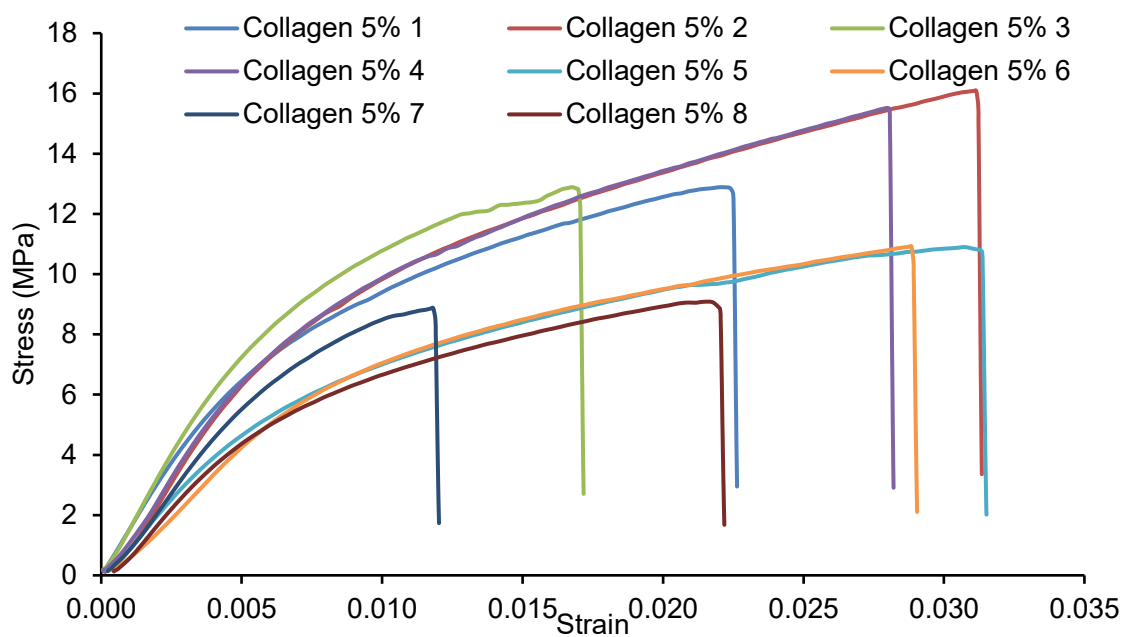


Figure 54 Tensile plot: Collagen 5%

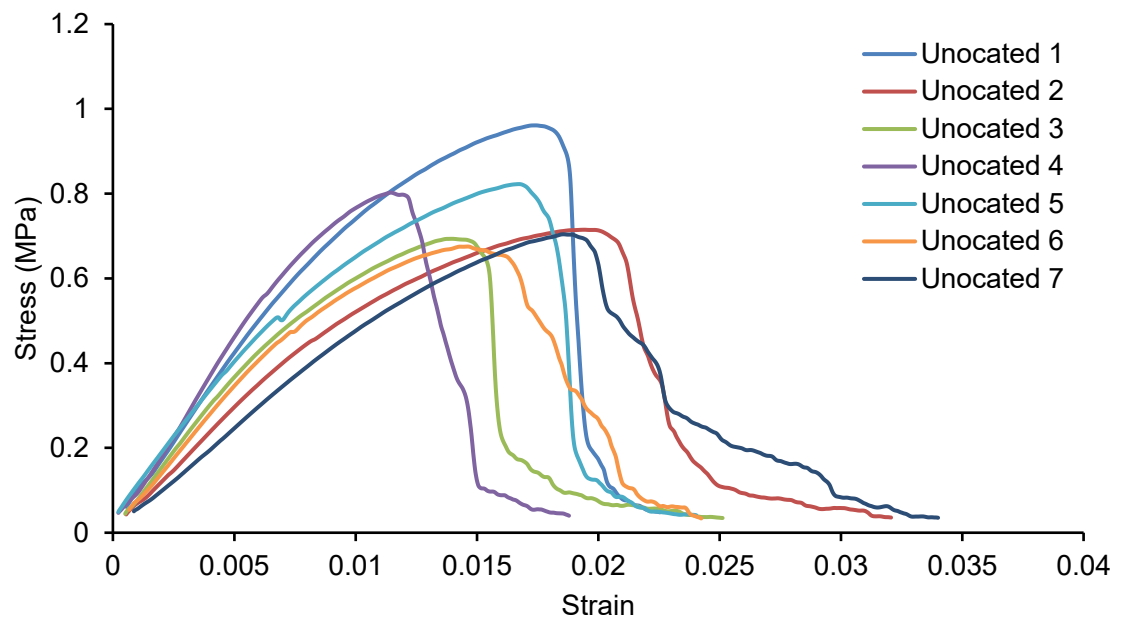


Figure 55 Tensile plot: Uncoated

B2. Coated and uncoated sheets properties before and after coating

Table 34 Tensile data (and paper properties)

Sample	LBG 3%		HBG 3%		LBG 5%		HBG 5%		C 3%		C 5%		UC	
	Avg.	St. dev.	A vg .	St. dev.	A vg .	St. dev.	A vg .	St. dev.	A vg .	St. dev.	A vg .	St. dev.	A vg .	St. dev.
Before coating														
Weight (g)	0.16	0.02	0.1 8	0.02	0.1 9	0.02	0.1 9	0.02	0.2 1	0.02	0.2 7	0.03	0.1 2	0.01
Length (mm)	150.51	0.79	15 0.0 6	0.91	14 9.9 6	0.87	14 9.9 1	1.01	14 8.0 8	1.43	14 8.2 6	1.27	14 6.9 6	0.88
Width (mm)	15.27	0.31	15. 29	0.36	15. 46	0.42	15. 05	0.33	14. 97	0.36	14. 95	0.45	14. 92	0.21
Thickness (mm)	0.33	0.07	0.3 2	0.06	0.2 5	0.02	0.2 6	0.01	0.2 5	0.04	0.2 4	0.03	0.2 7	0.03
After coating														
Weight (g)	0.05	0.01	0.0 6	0.01	0.1 0	0.02	0.1 0	0.02	0.1 0	0.01	0.1 6	0.03	-	-
Length (mm)	29.05	1.62	34. 81	1.66	50. 52	5.37	50. 81	4.82	45. 06	3.12	58. 86	4.81	-	-
Width (mm)	15.27	0.31	15. 29	0.36	15. 46	0.42	15. 05	0.33	14. 97	0.36	14. 95	0.45	-	-
Thickness (mm)	0.33	0.07	0.3 2	0.06	0.2 5	0.02	0.2 6	0.01	0.2 5	0.04	0.2 4	0.03	-	-
Coating wt.	0.05	0.01	0.0 6	0.01	0.1 0	0.02	0.1 0	0.02	0.1 0	0.01	0.1 6	0.03	-	-

Coating % w/w	29.05	1.62	34. 81	1.66	50. 52	5.37	50. 81	4.82	45. 06	3.12	58. 86	4.81	-	-
Max force (N)	36.73	4.73	48. 60	4.23	52. 94	11.00	45. 58	7.78	29. 73	6.09	44. 26	10.90	3.0 4	0.30
Tensile strength (kN/m)	2.40	0.27	3.1 8	0.27	3.4 1	0.64	3.0 3	0.51	1.9 9	0.42	2.9 7	0.78	0.2 0	0.02
Area (mm ²)	2298.7	41.9	22 93. 9	62.2	23 18. 9	64.98	22 56. 0	56.0	22 16. 6	59.5	22 17. 2	77.1	19 27. 8	700.1
w, grammage (g/m ²)	69.27	7.26	77. 77	5.09	82. 31	9.95	82. 22	6.46	96. 58	9.60	12 2.0 4	15.65	54. 54	3.66
Tensile Index (kNm/kg)	34.82	3.73	40. 95	3.27	41. 52	6.35	36. 70	4.14	20. 62	3.81	24. 10	3.70	3.7 4	0.27
Tensile displacement	1.63	0.33	1.6 7	0.23	1.3 5	0.21	1.4 0	0.16	2.5 1	0.58	2.3 7	0.66	1.5 5	0.25
Final length (mm)	101.63	0.33	10 1.6 7	0.23	10 1.3 5	0.21	10 1.4 0	0.16	10 2.5 1	0.58	10 2.3 7	0.66	10 1.5 5	0.25
Strain at break (%)	1.63	0.33	1.6 7	0.23	1.3 5	0.21	1.4 0	0.16	2.5 1	0.58	2.3 7	0.66	1.5 5	0.25
Tensile energy absorption (J/m ²)	23.38	9.23	29. 08	9.48	26. 18	9.33	24. 92	5.68	36. 00	12.68	50. 34	23.62	2.0 0	0.41
Tensile energy absorption index (J/kg)	339.9	135.13	37 5.2	124.2	32 1.6	117.0	30 2.8	62.9	38 0.9	149.2	40 5.3	158.2	36. 5	5.7
Force-elongation curve slope, Smax (N/mm)	35.26	3.16	45. 52	3.26	52. 71	7.04	43. 89	4.60	30. 76	6.33	44. 72	10.66	2.9 0	0.55
Modulus of elasticity (MPa)	730.3	116.2	96 6.4	178.0	13 75. 5	182.0	11 18. 0	138.0	82 5.8	158.2	12 36. 8	269.9	72. 8	13.2

B3. Properties of the permeability samples, initial and final oxygen levels, and oxygen transmission rate and oxygen permeability repeats

Table 35 Permeance sample properties

Permeance	Weight 1 (g)	Length (mm)	Width(mm)	Thickness 1 (mm)	Weight 2 (g)	Thickness 2 (mm)	Coating (wt%)	Grammage (g/m2)
Collagen 3%	0.508	122.107	122.107	0.210	0.790	0.166	35.187	53.013
	0.064	0.188	0.188	0.012	0.118	0.023	6.013	7.946
Collagen 5%	0.534	121.984	121.984	0.225	1.216	0.173	55.259	81.670
	0.055	0.290	0.290	0.026	0.119	0.016	8.770	7.750
High bloom gelatin 3%	0.602	121.834	121.834	0.184	0.883	0.277	32.155	59.508
	0.091	0.344	0.344	0.030	0.092	0.047	3.671	6.456
High bloom gelatin 5%	0.573	122.398	122.398	0.167	1.033	0.279	44.458	68.959
	0.039	0.397	0.397	0.028	0.044	0.050	4.155	3.039
Low bloom gelatin 3%	0.499	122.368	122.368	0.298	0.713	0.238	29.819	47.564
	0.071	0.546	0.546	0.018	0.111	0.042	2.548	7.118
Low bloom gelatin 5%	0.547	177.425	177.425	0.205	1.047	0.311	47.313	58.769
	0.035	110.226	110.226	0.023	0.116	0.016	5.226	27.259
No coating	0.559	122.215	122.215	0.236	-	-	-	37.410
	0.055	0.311	0.311	0.014	-	-	-	3.758

Table 36 Data point consideration for oxygen diffusion during the permeability test

No.		Collagen 3%	Collagen 5%	High bloom gelatin 3%	High bloom gelatin 5%	Low bloom gelatin 3%	Low bloom gelatin 5%	No coating	Baking paper
1	Initial point range	500	3980	200	200	200	200	200	3980
	Initial O ₂ %	4.2	5.5	6.1	3.8	5.9	4	6.6	4
	O ₂ % at end point	18.3	6	15.6	9.3	15.1	14.2	16.4	4.6
2	Initial point range	500	3980	200	200	200	200	200	3980
	Initial O ₂ %	5.1	4.9	5.3	3.9	4	3.9	5.8	7.2
	O ₂ % at end point	18	5.2	15.6	7.4	14.9	13.2	16.1	18.9
3	Initial point range	500	3980	200	200	200	200	200	3980
	Initial O ₂ %	5.5	5.6	4.8	3.6	6.3	7.6	3.9	5.6
	O ₂ % at end point	18.1	15	15.2	14.5	16.2	16.7	15.6	18.6
4	Initial point range	500	3980	200	200	200	200	200	-
	Initial O ₂ %	6.6	5.5	7.2	7	4.2	4.1	6.1	-
	O ₂ % at end point	12.3	9.7	15.8	15.4	15.2	15.5	16.3	-
5	Initial point range	500	3980	200	200	200	200	200	-
	Initial O ₂ %	3.6	3.6	4.5	4.2	3.9	4.9	6.4	-
	O ₂ % at end point	20.9	4.9	14.4	15.1	15.1	15.7	16.1	-

Table 37 Oxygen transmission rate results

OTR (cm ³ /m ² .d.atm)	Collagen 3%	Collagen 5%	High bloom gelatin 3%	High bloom gelatin 5%	Low bloom gelatin 3%	Low bloom gelatin 5%	No coating	Baking paper
1	9.72E+08	5.38E+06	1.32E+09	5.35E+08	1.31E+09	1.24E+09	1.71E+09	2.11E+07
2	9.01E+08	1.24E+06	1.52E+09	3.44E+08	1.38E+09	1.05E+09	1.62E+09	1.70E+07
3	9.01E+08	1.26E+07	1.49E+09	1.35E+09	1.56E+09	1.51E+09	1.66E+09	2.12E+07
4	2.54E+08	7.33E+06	1.31E+09	1.32E+09	1.43E+09	1.56E+09	1.67E+09	-
5	2.04E+07	3.84E+06	1.27E+09	1.46E+09	1.47E+09	1.51E+09	1.46E+09	-
Average	6.10E+08	6.08E+06	1.38E+09	1.00E+09	1.43E+09	1.38E+09	1.62E+09	1.98E+07
Standard deviation	3.93E+08	3.82E+06	1.01E+08	4.65E+08	8.47E+07	1.95E+08	8.81E+07	1.96E+06

Table 38 Conversion from oxygen transmission rate results to oxygen permeability

Permeability (cm ³ /m.d.atm)	Collagen 3%	Collagen 5%	High bloom gelatin 3%	High bloom gelatin 5%	Low bloom gelatin 3%	Low bloom gelatin 5%	No coating	Baking paper
1	194.40	0.97	463.50	128.40	393.31	410.79	392.52	1.05
2	135.08	0.25	470.51	72.31	385.63	337.51	355.43	0.85
3	144.08	2.14	417.78	445.75	312.53	468.00	415.97	1.06
4	45.77	1.25	275.52	357.55	300.34	437.54	434.75	-
5	4.09	0.61	305.11	495.28	307.93	468.00	320.47	-
Average	104.68	1.04	386.48	299.86	339.95	424.37	383.83	0.99
Standard deviation	69.45	0.64	81.12	169.69	40.69	48.39	41.28	0.10

B4. Tear sample properties and tear data repeats

Table 39 Tear sample properties

Tear	Weight 1 (g)	Length (mm)	Width(mm)	Thickness 1 (mm)	Weight 2 (g)	Thickness 2 (mm)	Coating (wt%)	Grammage (g/m2)
Collagen 3%	0.198	200.578	26.876	0.231	0.394	0.161	49.634	73.129
	0.012	0.592	0.293	0.019	0.032	0.021	2.226	5.653
Collagen 5%	0.189	202.388	26.400	0.219	0.399	0.150	52.576	74.632
	0.013	0.934	0.418	0.021	0.024	0.014	2.749	5.076
High bloom gelatin 3%	0.144	201.495	26.212	0.194	0.228	0.230	36.579	43.146
	0.007	1.066	0.391	0.022	0.017	0.014	2.817	3.065
High bloom gelatin 5%	0.155	203.133	26.415	0.173	0.286	0.188	46.231	53.414
	0.027	0.268	0.481	0.026	0.037	0.028	3.854	7.008
Low bloom gelatin 3%	0.226	201.021	26.449	0.255	0.310	0.359	27.094	58.285
	0.015	0.812	0.268	0.010	0.025	0.021	1.703	4.538
Low bloom gelatin 5%	0.151	202.408	26.224	0.190	0.318	0.248	52.157	59.805
	0.012	0.671	0.366	0.015	0.039	0.015	3.328	7.244
No coating	0.184	202.819	26.837	0.205				33.763
	0.033	0.329	0.394	0.025				5.904

Table 40 Tear raw data

	Max tear index	Average Tear Index	Min tear index	Standard dev Tear index	Average grammage	StdDev Grammage	Max tear strength	Average Tear Strength, mN	Min tear strength	Standard dev Tear strength
Collagen 3%	14.53	10.46	8.05	2.02	73.13	5.65	1132.44	771.61	510.24	188.31
Collagen 5%	14.48	12.36	10.15	1.56	74.63	5.08	1060.54	936.68	783.76	94.83
High bloom gelatin 3%	27.21	15.19	10.29	4.44	43.15	3.06	1157.76	652.96	481.38	189.32
High bloom gelatin 5%	10.32	8.32	4.96	1.76	53.41	7.01	597.38	439.86	280.58	94.79
Low bloom gelatin 3%	24.82	17.88	14.17	3.20	58.29	4.54	1471.26	1040.99	818.40	197.64
Low bloom gelatin 5%	10.69	7.76	5.10	1.95	59.81	7.24	748.52	463.28	313.36	134.71
No Coating	5.16	3.69	2.78	0.70	33.76	5.90	155.74	118.76	91.58	22.01

B5. Front and back of sheet FTIR plots and wavenumbers

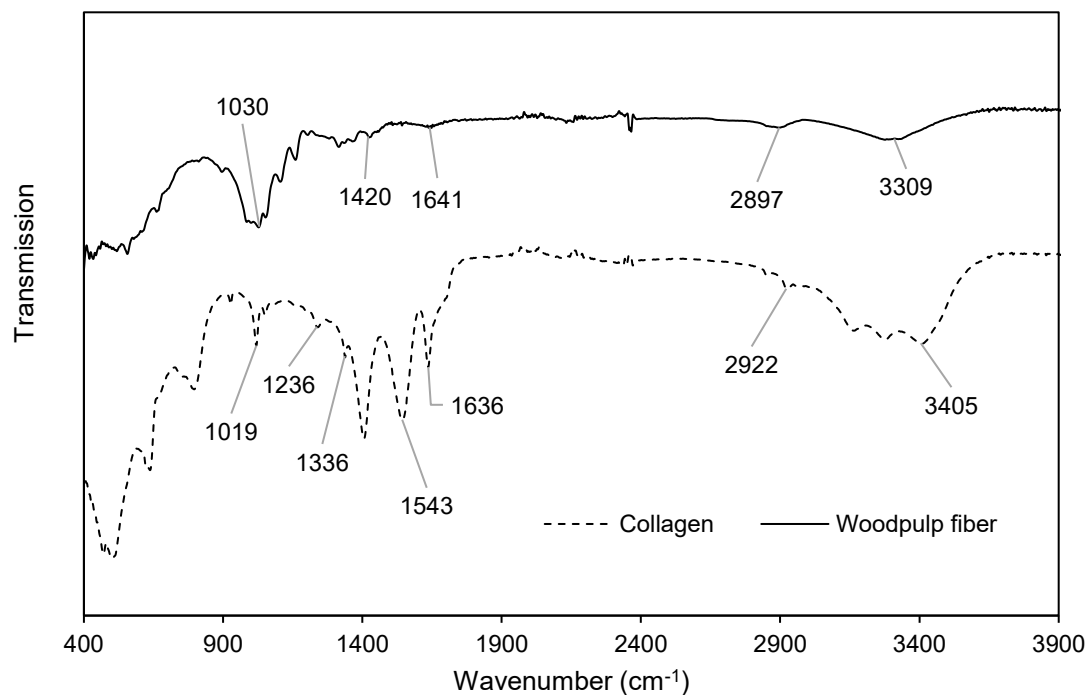


Figure 56 FTIR: Collagen and cellulose fibre

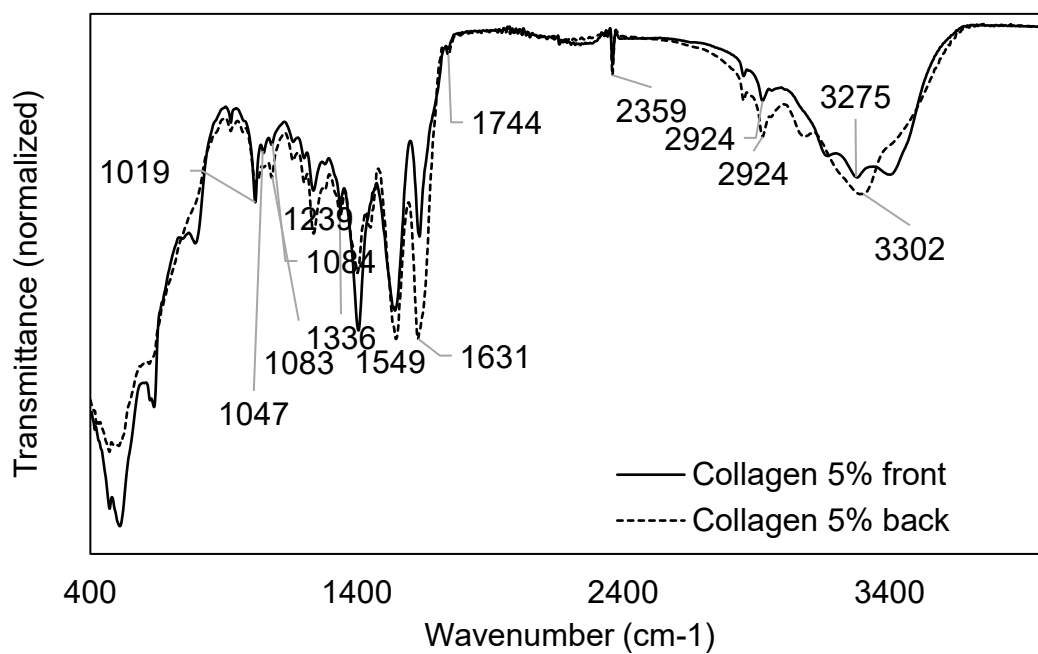


Figure 57 FTIR: Collagen 5%

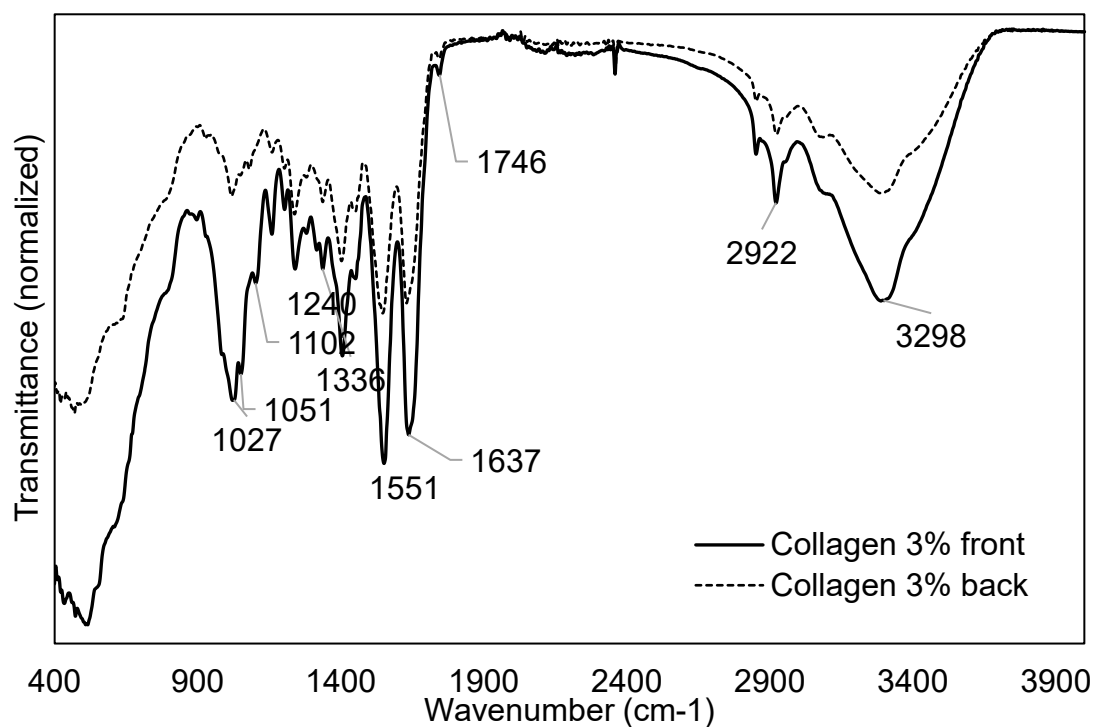


Figure 58 FTIR: Collagen 3%

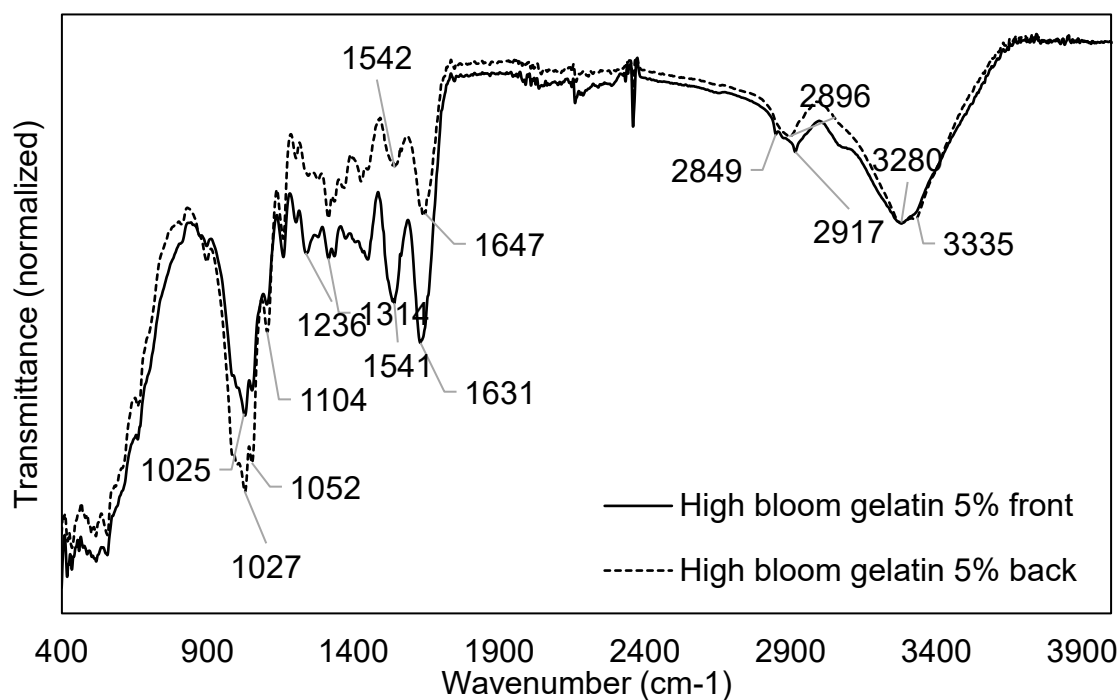


Figure 59 FTIR: High bloom gelatin 5%

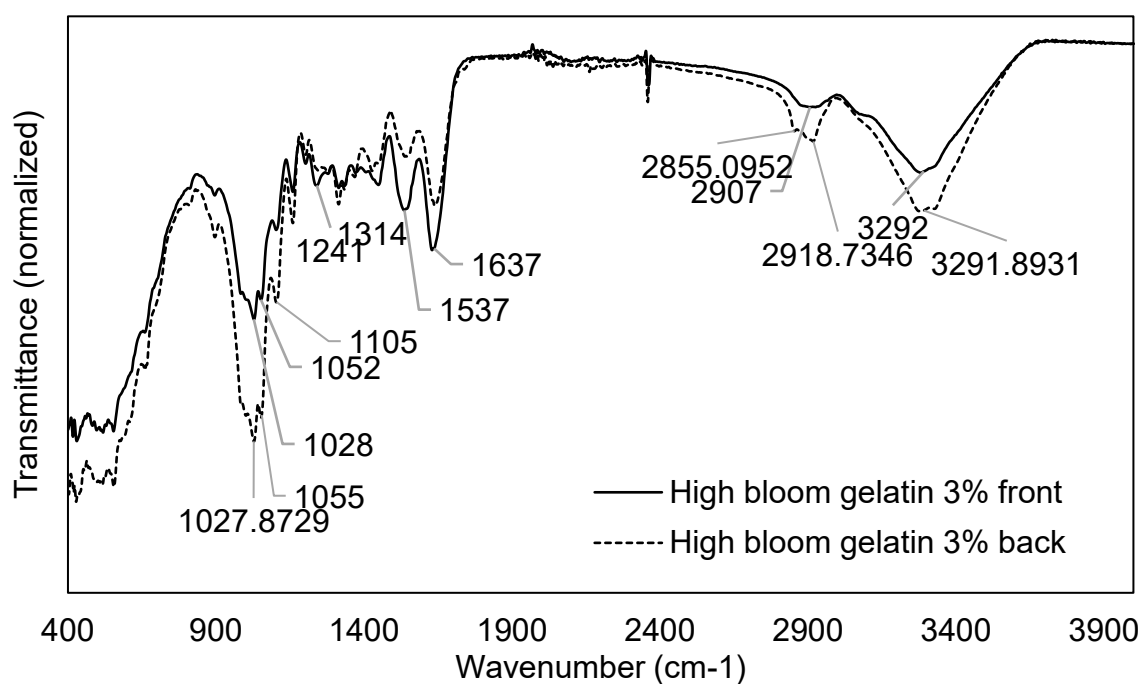


Figure 60 FTIR: High bloom gelatin 3%

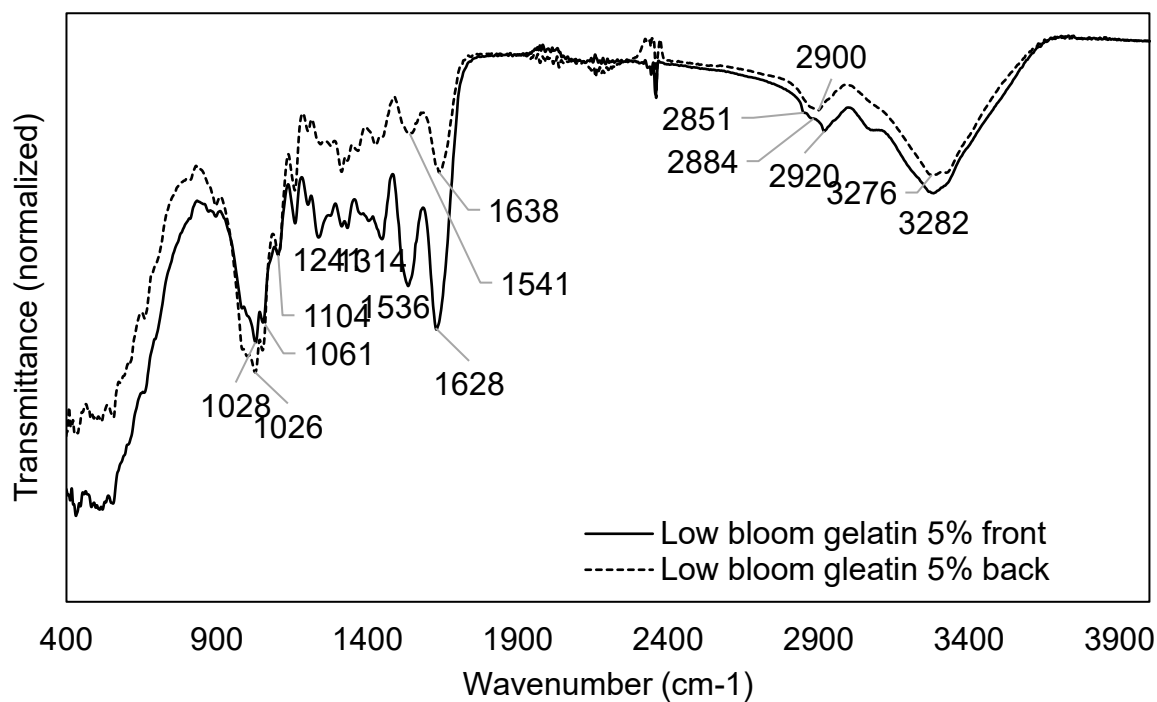


Figure 61 FTIR: Low bloom gelatin 5%

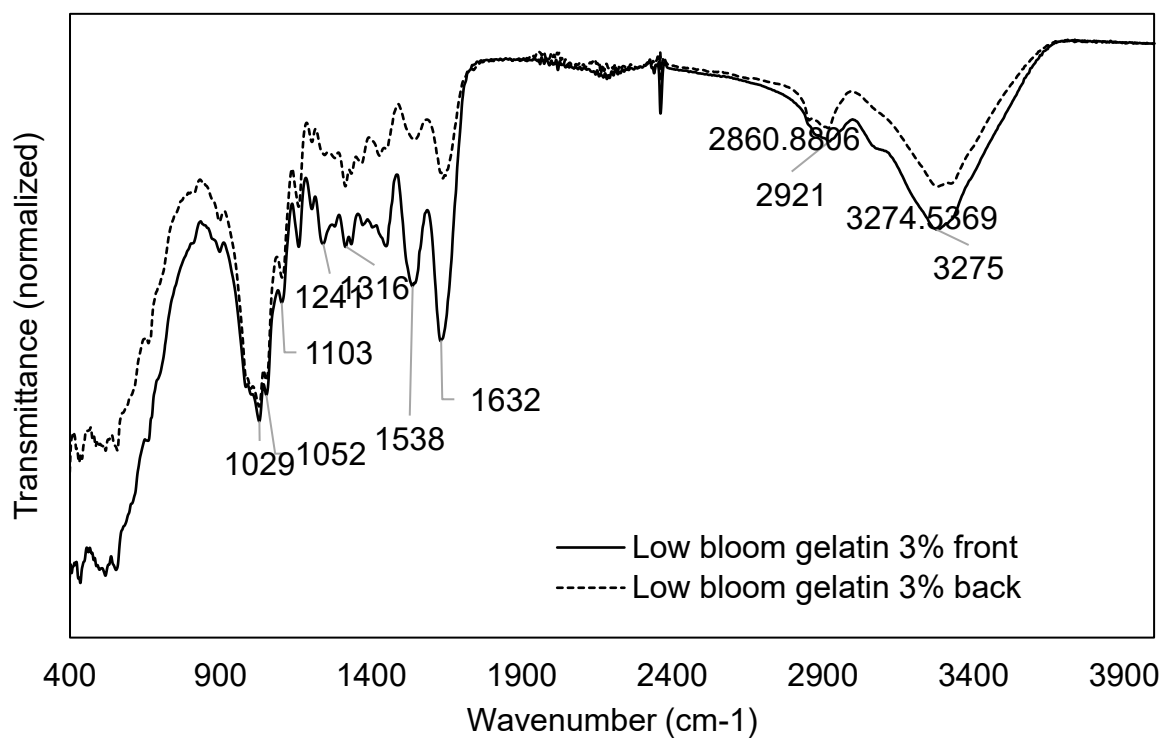


Figure 62 FTIR: Low bloom gelatin 3%

B6. Contact angle measurements

Table 41 Front side of the coated and uncoated sheet contact angle measurements

Sample		Timestamp (s)		
FRONT		0.1s	1s	10s
Collagen 3%	1	96.6	68.5	44.2
	2	95.9	88.9	78
	3	81.0	77.5	74.3
	4	113.5	109.5	79.5
	5	97.8	95.3	61.3
	6	84.9	80.5	73.5
Average		94.9	86.7	68.5
Std. Dev.		10.4	13.2	12.3
Collagen 5%	1	99.7	92.1	80.8
	2	65.9	64.9	52.2
	3	87.1	84.9	75.8
	4	111.6	87.9	65.6
	5	128.4	114.6	65.0
Average		98.5	88.9	67.9
Std. Dev.		21.3	15.9	9.9
High bloom gelatin 3%	1	97.7	96.0	92.5
	2	112.7	108.6	105.7
	3	99	94.6	90.0
	4	79.3	73.2	70.0
	5	88.5	88.1	59.4
Average		95.44	92.1	83.52
Std. Dev.		11.2	11.5	16.6
High bloom gelatin 5%	1	82.5	81.7	80.4
	2	86.5	84.4	82.9
	3	95.9	91.6	73.2
	4	77.2	77.0	72.7
	5	97.3	96.2	80.1
Average		87.9	86.2	77.9
Std. Dev.		7.7	6.9	4.1

Low bloom gelatin 3%	1	85.4	81.6	79.3
	2	98.1	93.4	14.8
	3	95.2	90.7	86.4
	4	90.6	84.5	66.8
	5	100.8	94.6	93.4
Average		94.0	88.9	68.1
Std. Dev.		5.5	5.1	28.1
Low bloom gelatin 5%	1	96.0	92.7	67.7
	2	102.9	90.7	76
	3	97.1	96.6	94.0
	4	77.6	76	51.7
	5	102.5	100.6	61.5
Average		95.2	91.3	70.2
Std. Dev.		9.2	8.4	14.3
Uncoated	1	26.2	0	0
	2	41.2	0	0
	3	18.3	0	0
	4	49.7	0	0
	5	27.0	0	0
Average		32.5	0	0
Std. Dev.		11.3	0	0

Table 42 Back side of the coated and uncoated sheet contact angle measurements

Sample		Time stamp (s)		
BACK		0.1	1	10
Collagen 3%	1	90.8	90.2	58.2
	2	88.7	75.9	56.3
	3	82.7	78.8	51.6
	4	88.7	88.5	68.9
	5	95.9	83.4	14.4
	6	74.5	63.5	57.4
Average		86.9	80.1	51.1
Std. Dev.		6.8	8.9	17.2
Collagen 5%	1	86.0	84.1	82.3
	2	81.0	77.5	71.3
	3	87.7	71	70.9
	4	66.8	54.9	34.0
	5	70.0	60.3	40.1
Average		78.3	69.56	59.72
Std. Dev.		8.4	10.7	19.1
High bloom gelatin 3%	1	113.7	104.7	95.6
	2	96.8	96.1	92
	3	103.9	95.3	0
	4	97.2	92.1	56.4
	5	99.9	93.8	0
Average		102.3	96.4	48.8
Std. Dev.		6.2	4.4	42.1
High bloom gelatin 3%	1	88.9	81.1	79.3
	2	79.2	73.6	72.5
	3	97.9	92.6	82.1
	4	105.2	93.8	0
	5	97.2	93.2	87.5
Average		93.68	86.86	64.28
Std. Dev.		8.9	8.1	32.5
Low bloom gelatin 3%	1	80.6	77.1	0
	2	90.9	89.4	83.3
	3	97.5	95.5	90.5
	4	97.5	95.3	84.9

	5	103.4	95.8	53.4
Average		93.98	90.62	62.42
Std. Dev.		7.8	7.2	33.8
Low bloom gelatin 5%	1	83.2	80.4	72.2
	2	100.6	98.6	31.7
	3	96.3	84.3	79.2
	4	73.9	64.3	60.9
	5	105	99.7	66.7
Average		91.8	85.46	62.14
Std. Dev.		11.5	13.0	16.4
Uncoated	1	46	0	0
	2	22.9	0	0
	3	48.5	0	0
	4	34.9	0	0
	5	28	0	0
Average		36.1	0	0
Std. Dev.		9.9	0	0

Table 43 Contact angle summary

Sample	Contact angle, θ_c at Time stamp (s)					
	Front			Back		
	0.1	1	10	0.1	1	10
C3	94.95 ± 10.42	86.7 ± 13.25	68.47 ± 12.33	86.88 ± 6.76	80.05 ± 8.93	51.13 ± 17.23
C5	98.54 ± 21.26	88.88 ± 15.9	67.88 ± 9.89	78.30 ± 8.44	69.56 ± 10.74	59.72 ± 19.05
HBG3	95.44 ± 11.17	92.1 ± 11.55	83.52 ± 16.61	102.30 ± 6.24	96.4 ± 4.37	$48.8 \pm 42.14^{**}$
HBG5	87.88 ± 7.72	86.18 ± 6.89	77.86 ± 4.13	93.68 ± 8.89	86.86 ± 8.13	$64.28 \pm 32.50^*$
LBG3	94.02 ± 5.48	88.96 ± 5.07	68.14 ± 28.08	93.98 ± 7.77	90.62 ± 7.17	$62.42 \pm 33.79^*$
LBG5	95.22 ± 9.25	91.32 ± 8.38	70.18 ± 14.31	91.80 ± 11.54	85.46 ± 13.04	62.14 ± 16.38
UC	32.48 ± 11.34	0.0 ± 0.0	0.0 ± 0.0	36.06 ± 9.93	0.0 ± 0.0	0.0 ± 0.0

*Resulted to one zero contact angle

**Resulted to two zero contact angle

B7. TGA supplementary data

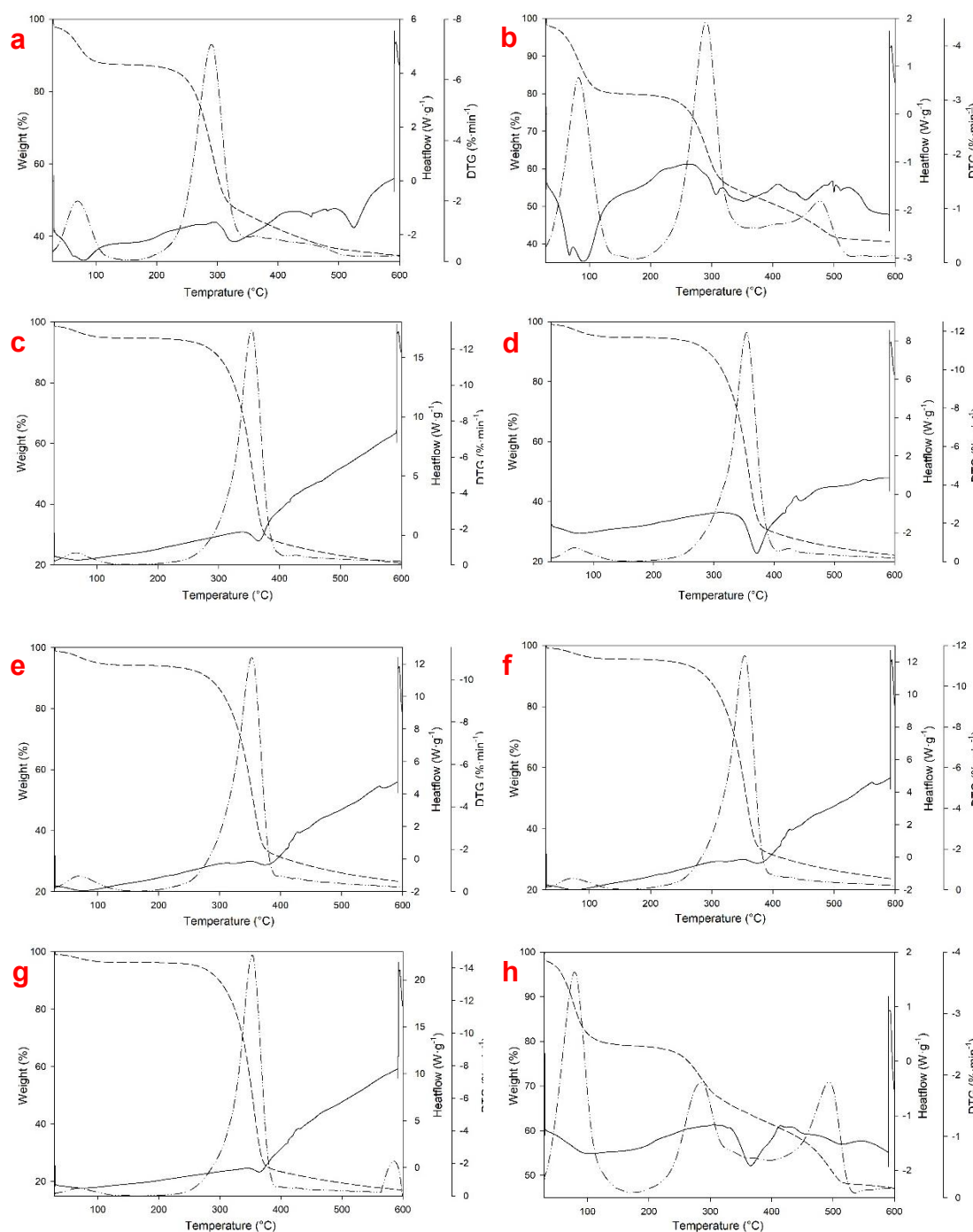
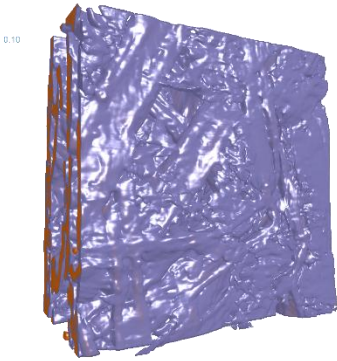
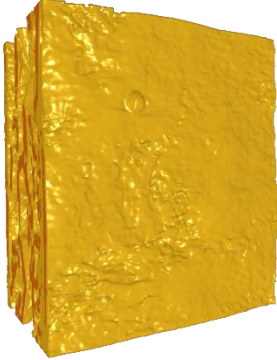
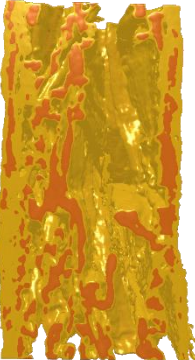


Figure 63 (a) Collagen 3% (b) Collagen 5% (c) High bloom gelatin 3% (d) High bloom gelatin 5% (e) Low bloom gelatin 3% (f) Low bloom gelatin 5% (g) no coating (h) collagen coating. Weight – dash line, Heatflow – solid line, DTG dash double dot

Appendix C: Chapter 7 Micro-computed tomography to characterize coated and uncoated fibrous composite

C1. ROI segmentation viewed in different axes and the pore space ROI considered for the aspect ratio/volume contribution

Table 44 -X and -Y axes views of coating and fibre ROIs

	X-	Y-
Collagen 3%		
Collagen 5%		
High bloom gelatin 3%		

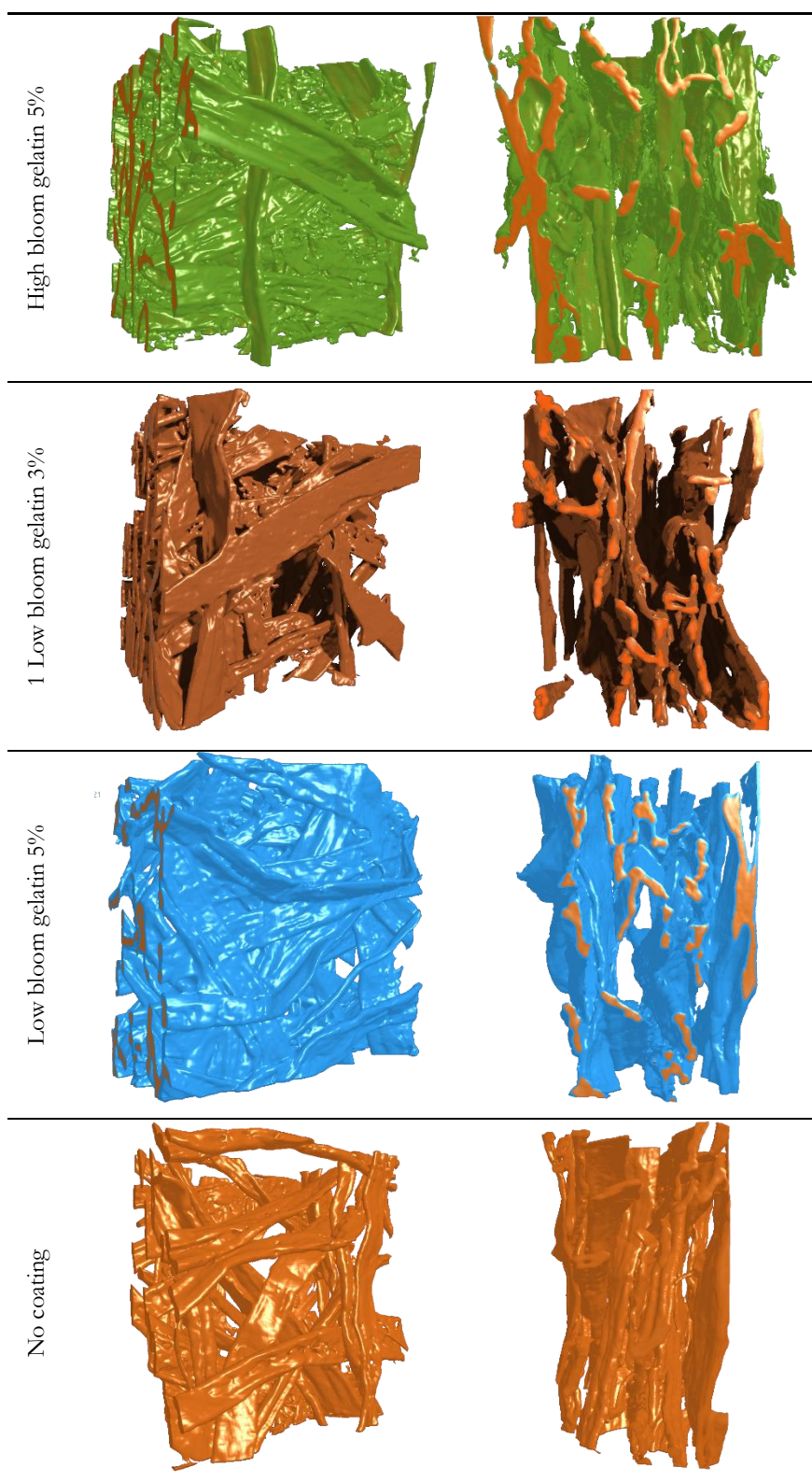
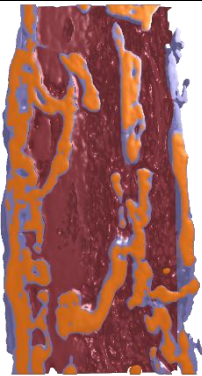
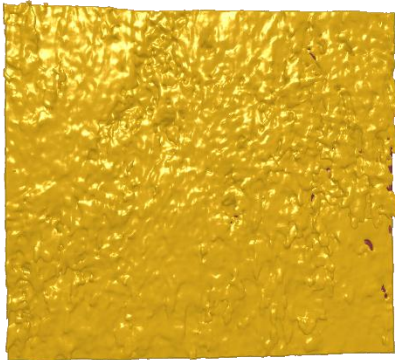
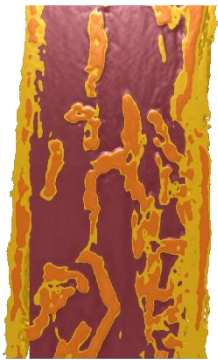
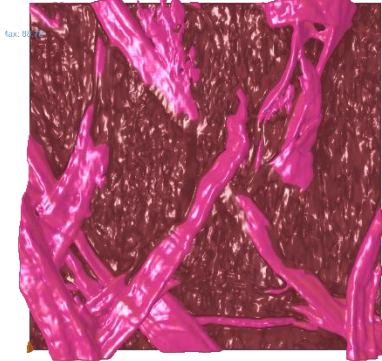
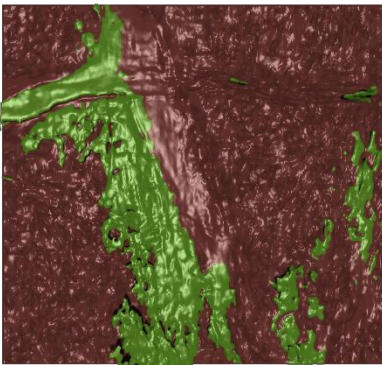


Table 45 Pore space x and y axis view

	X+	Y+
Collagen 3%		
Collagen 5%		
High bloom gelatin 3%		
High bloom gelatin 5%		

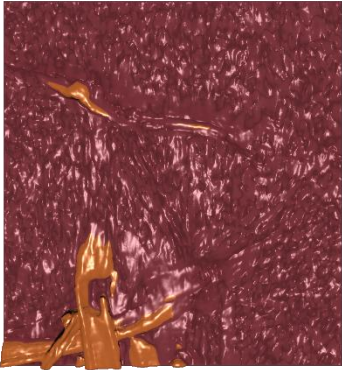
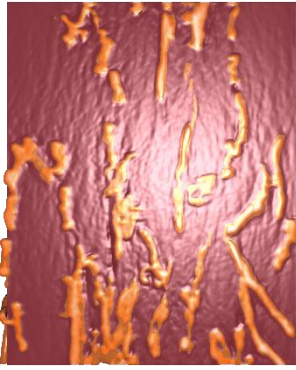
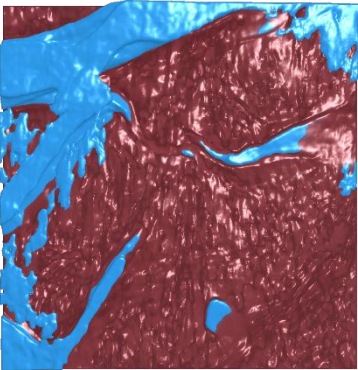
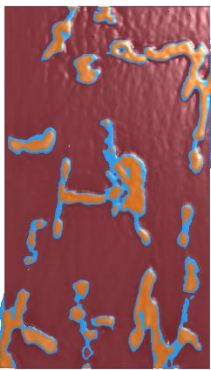
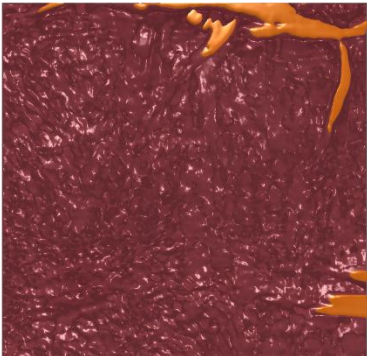
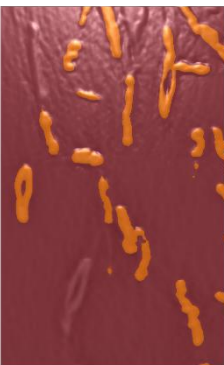
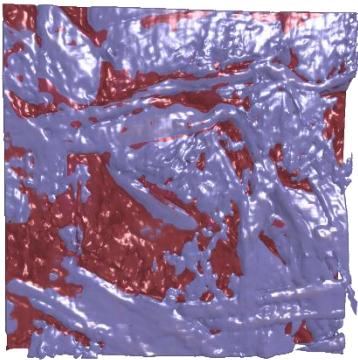
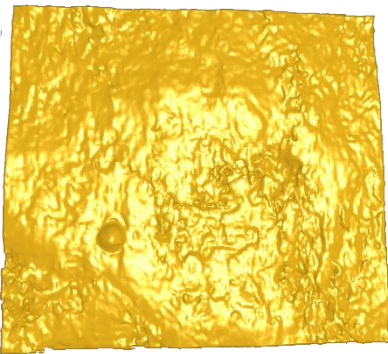
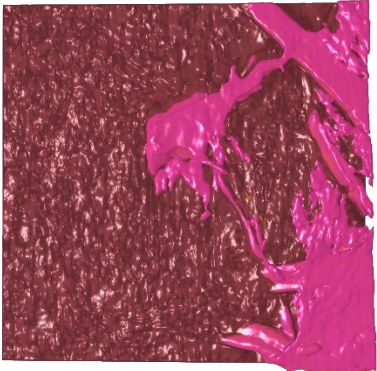
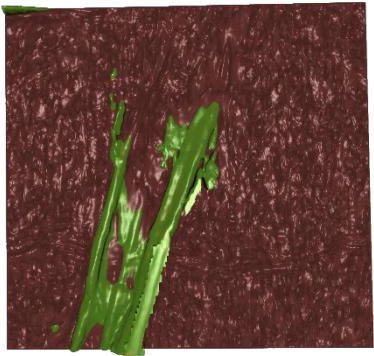
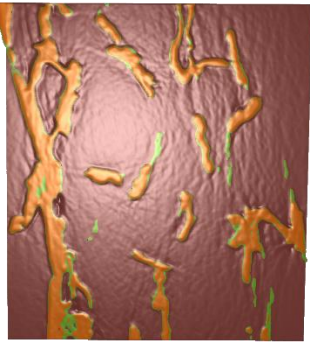
1 Low bloom gelatin 3%		
Low bloom gelatin 5%		
No coating		

Table 46 Pore space x- and y- axis view

	X-	Y-
Collagen 3%		
Collagen 5%		
High bloom gelatin 3%		
High bloom gelatin 5%		

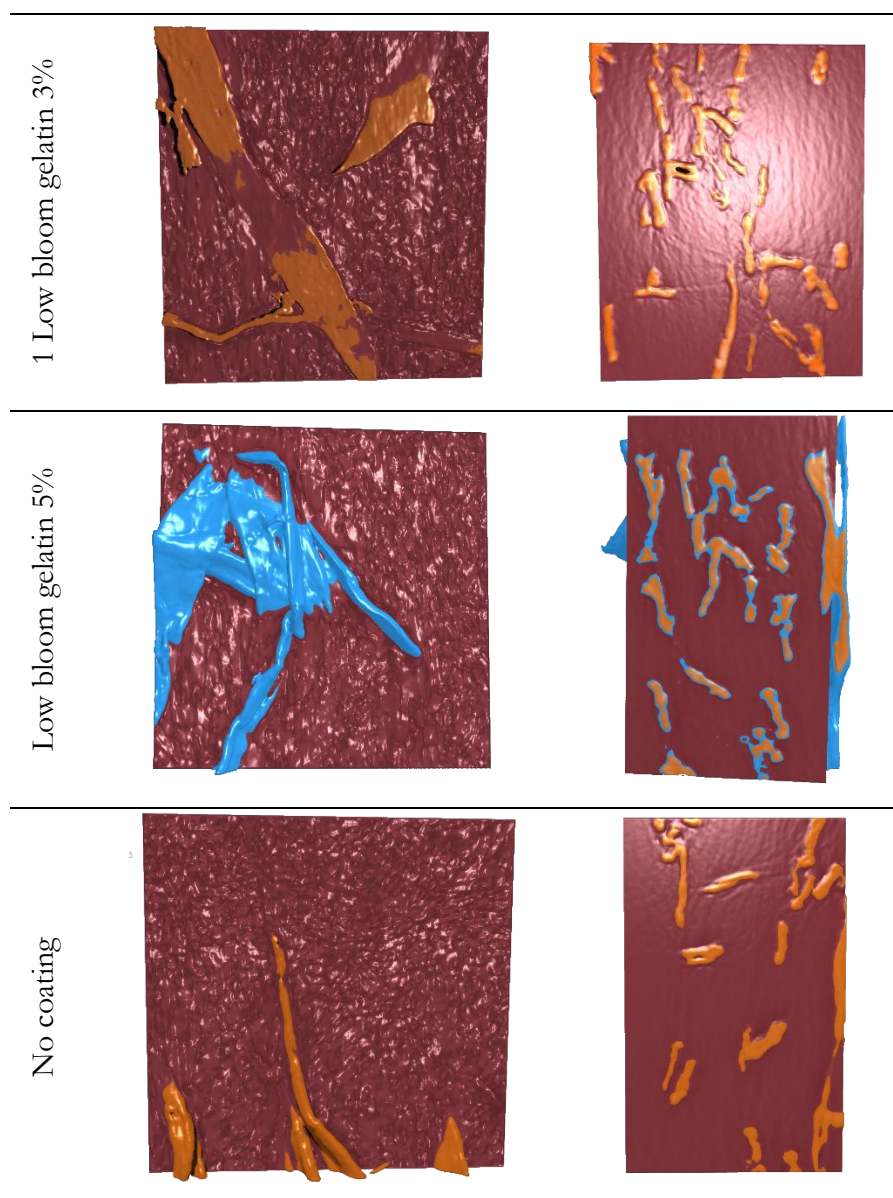
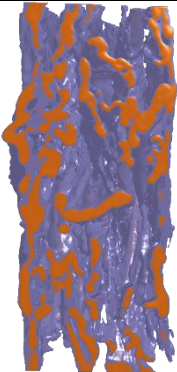
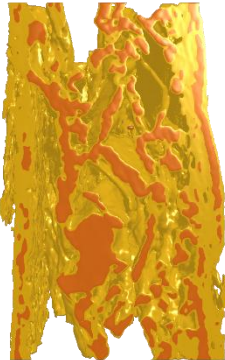
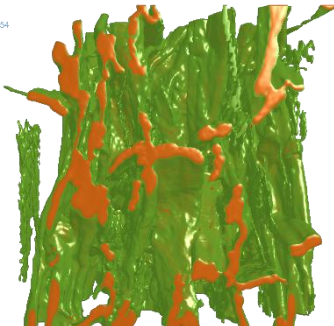
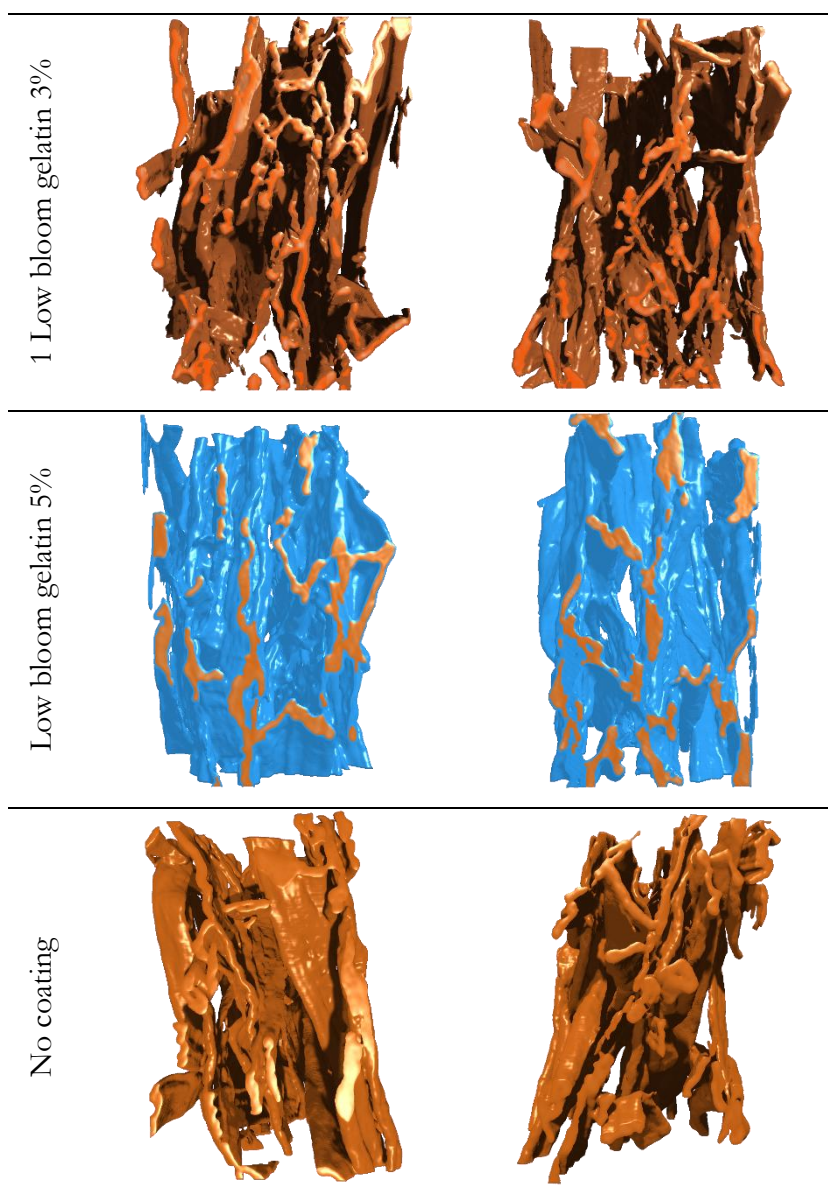


Table 47 Pore space z and z- axis view

	Z+	Z-
Collagen 3%		
Collagen 5%		
High bloom gelatin 3%		
High bloom gelatin 5%		



C1. Anisotropy calculation results using SVD and MIL: property plots

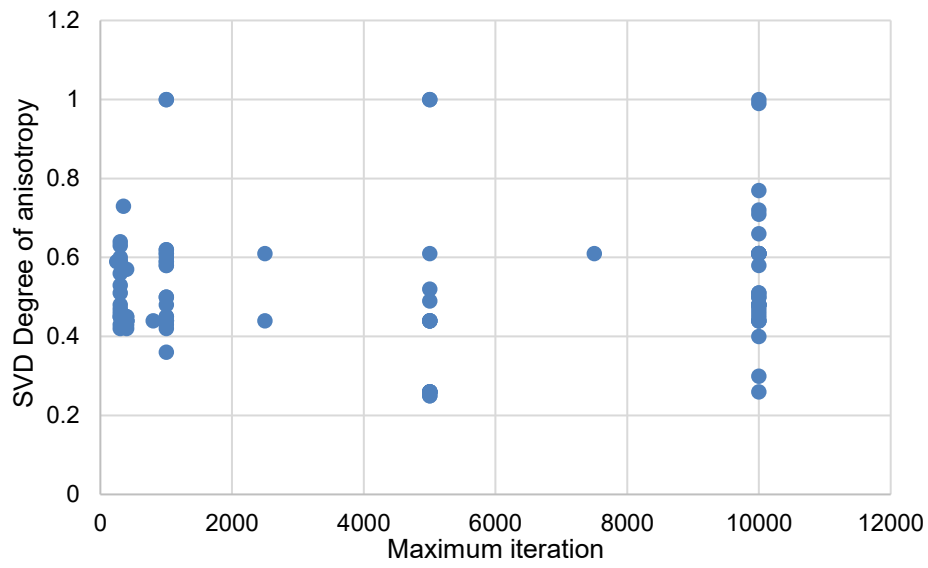


Figure 64 SVD degree of anisotropy vs maximum iteration

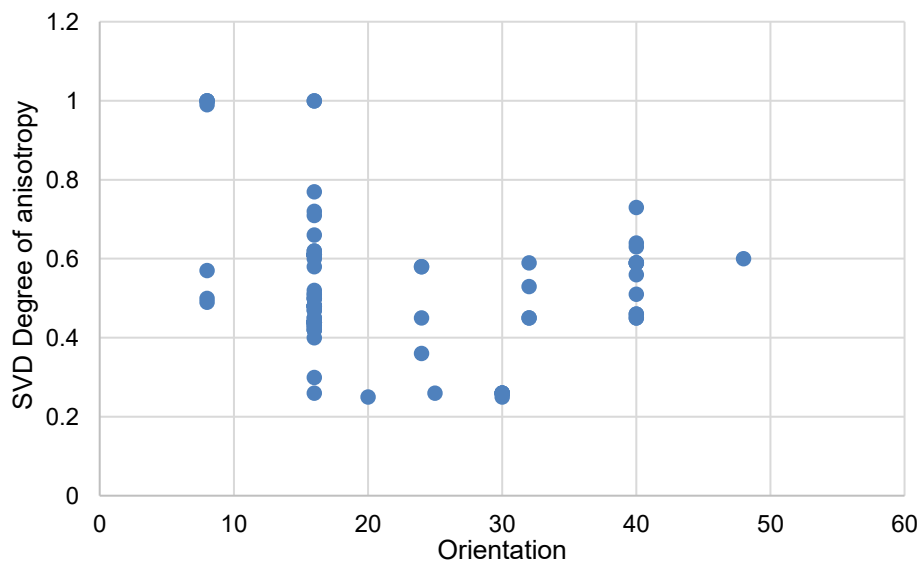


Figure 65 SVD degree of anisotropy vs orientation

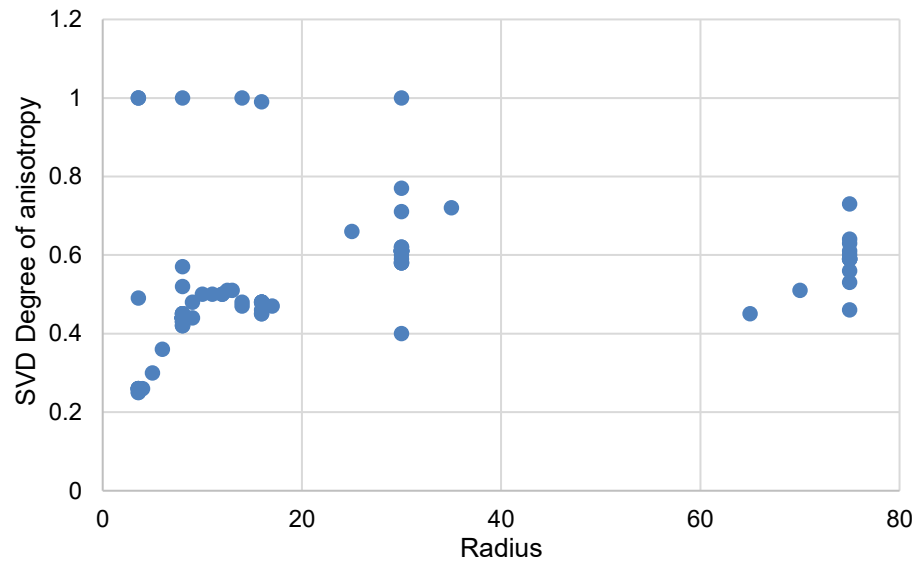


Figure 66 SVD degree of anisotropy vs radius

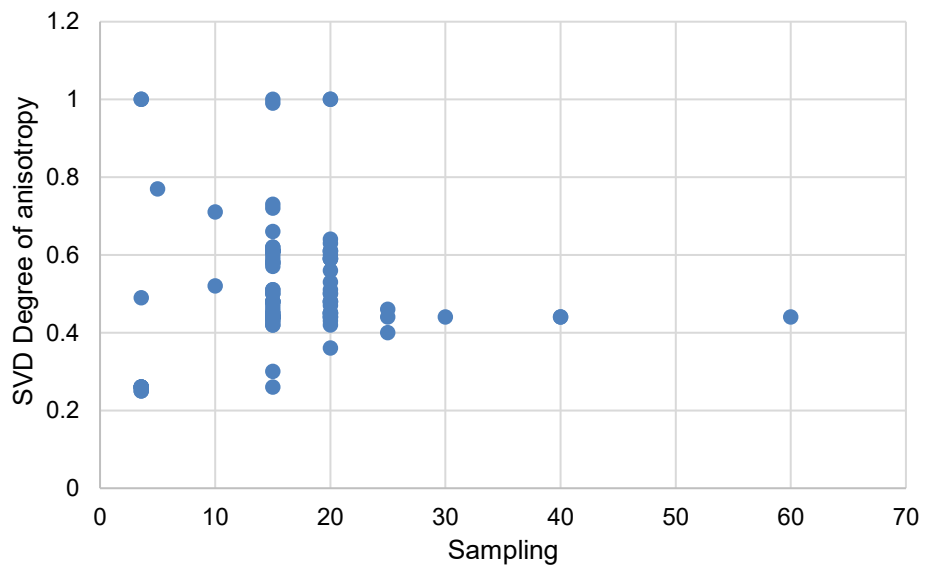


Figure 67 SVD degree of anisotropy vs sample

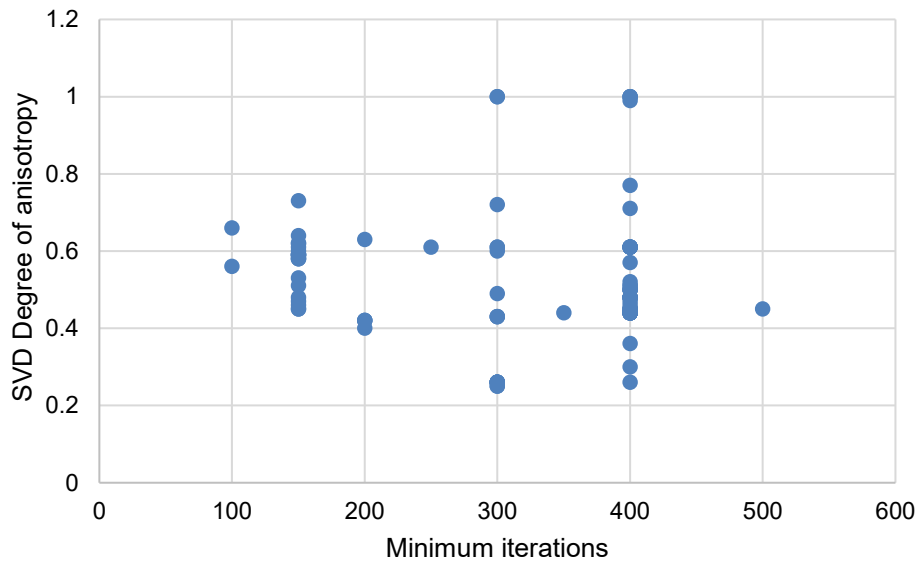


Figure 68 SVD degree of anisotropy vs minimum iteration

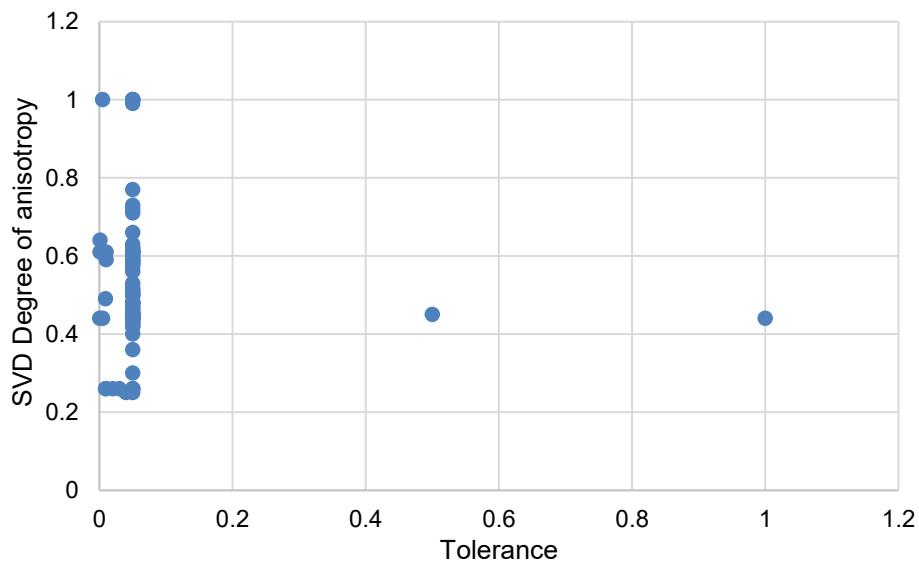


Figure 69 SVD degree of anisotropy vs tolerance

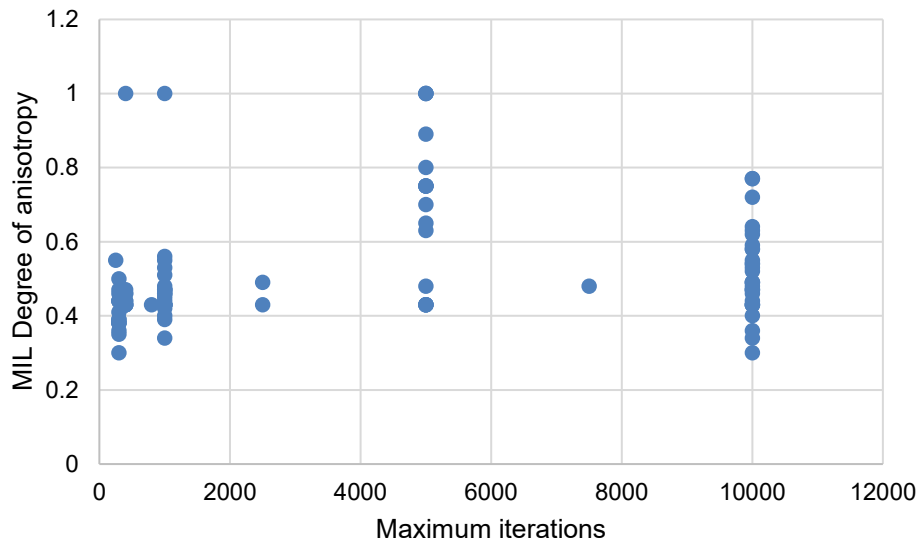


Figure 70 MIL degree of anisotropy vs maximum iteration

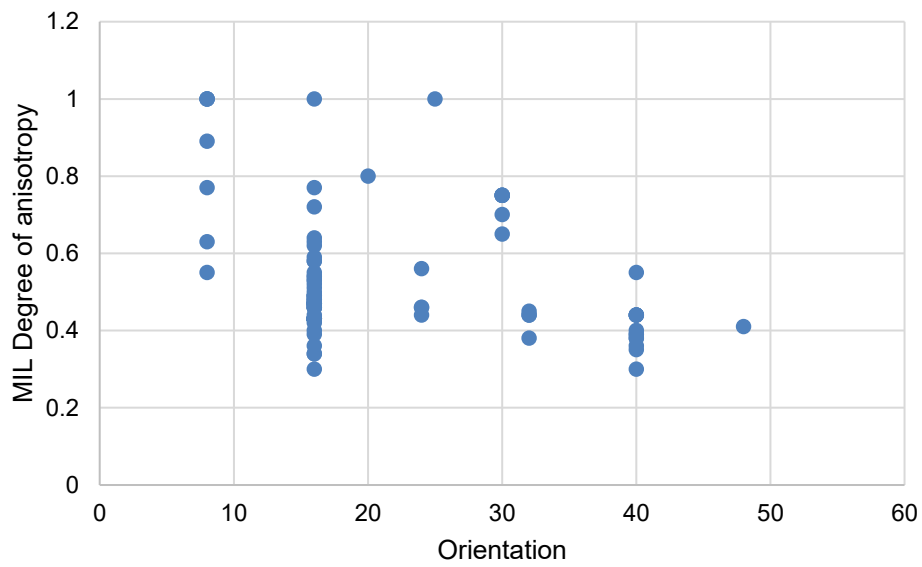


Figure 71 MIL degree of anisotropy vs orientation

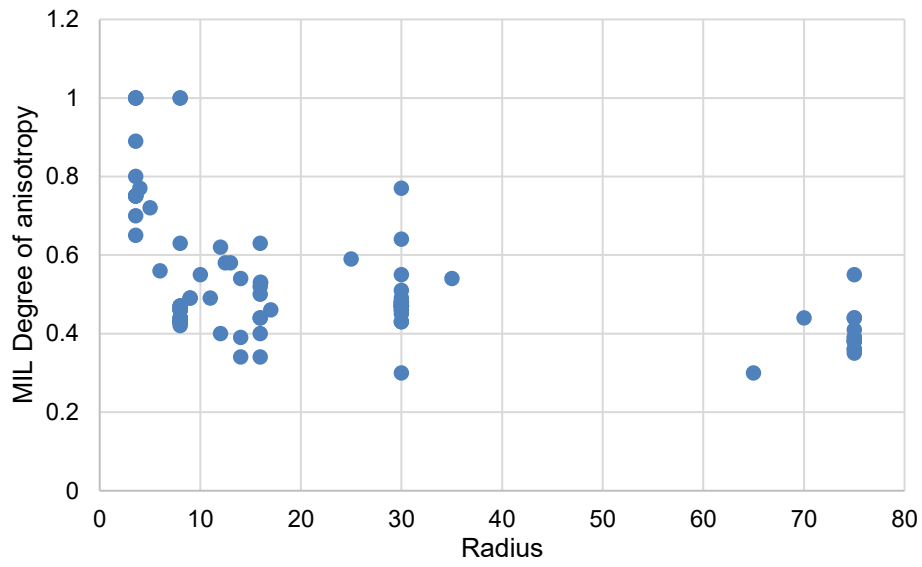


Figure 72 MIL degree of anisotropy vs radius

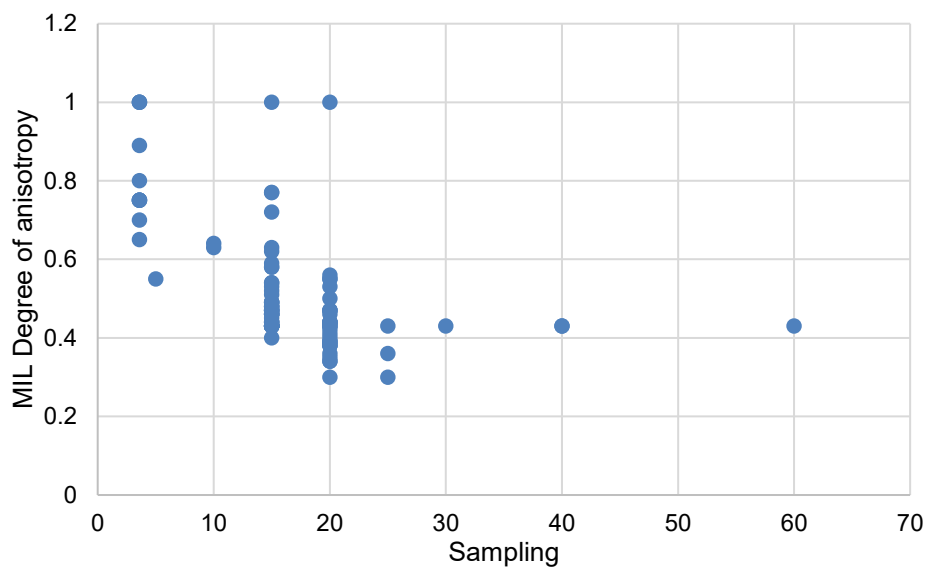


Figure 73 MIL degree of anisotropy vs sampling

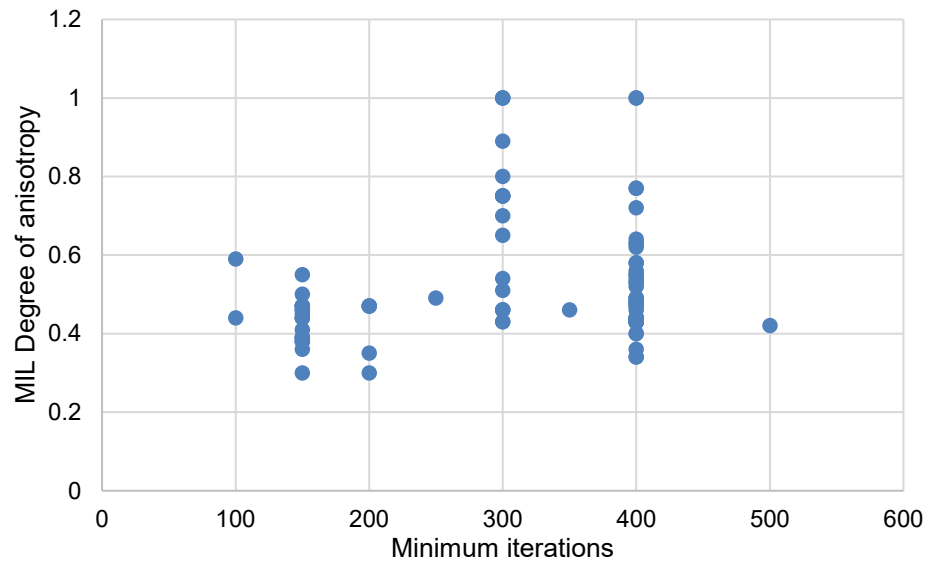


Figure 74 MIL degree of anisotropy vs minimum iteration

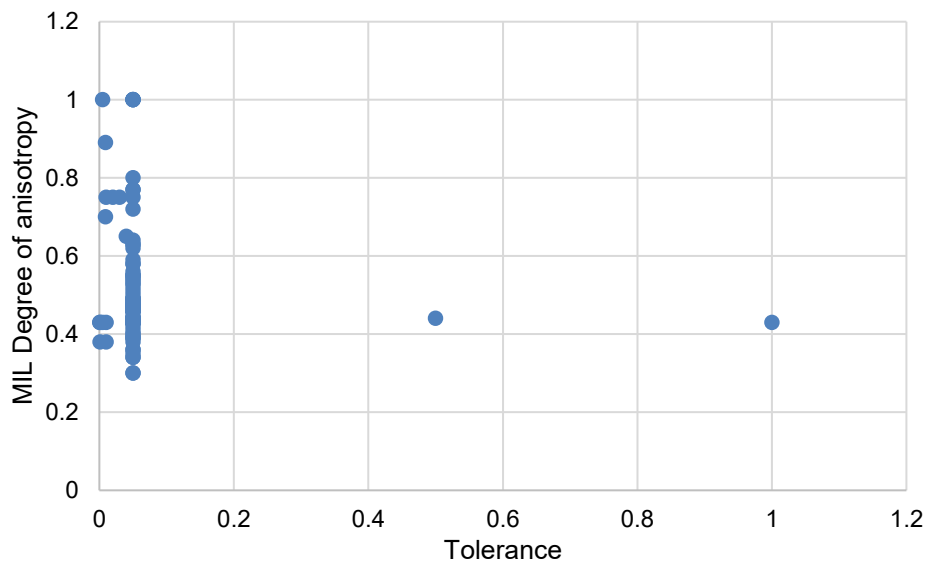


Figure 75 MIL degree of anisotropy vs tolerance

C2. SVD and MIL iterations

Table 48 SVD and MIL trial results

Max iteration	Sampling	Orientations	Min iteration	Radius	Tolerance	SVD value	MIL value
5000	3.59	20	300	3.59	0.05	0.25	0.8
5000	3.59	30	300	3.59	0.04	0.25	0.65
5000	3.59	30	300	3.59	0.05	0.26	0.75
5000	3.59	25	300	3.59	0.05	0.26	1
5000	3.59	30	300	3.59	0.03	0.26	0.75
5000	3.59	30	300	3.59	0.02	0.26	0.75
5000	3.59	30	300	3.59	0.01	0.26	0.75
5000	3.59	30	300	3.59	0.009	0.26	0.7
10000	15	16	400	3.99	0.05	0.26	0.77
10000	15	16	400	5	0.05	0.3	0.72
10000	25	16	200	30	0.05	0.4	0.3
10000	15	16	400	7.98	0.05	0.44	0.43
10000	15	16	400	9	0.05	0.44	0.49
300	20	40	150	65	0.05	0.45	0.3
10000	15	32	400	15.96	0.05	0.45	0.44
300	20	40	150	15.96	0.05	0.45	0.44
300	25	40	150	75	0.05	0.46	0.36
10000	15	40	400	15.96	0.05	0.46	0.4
10000	15	16	400	17	0.05	0.47	0.46
300	20	16	150	14	0.05	0.47	0.39
10000	15	16	400	9	0.05	0.48	0.49
10000	15	16	400	15.96	0.05	0.48	0.52
10000	15	16	400	16	0.05	0.48	0.53
10000	15	16	400	14	0.05	0.48	0.54
10000	20	16	400	15.96	0.05	0.48	0.34
300	20	16	150	15.96	0.05	0.48	0.5
5000	3.59	8	300	3.59	0.009	0.49	0.89
5000	3.59	8	300	3.59	0.005	1	1

10000	15	16	400	11	0.05	0.5	0.49
10000	15	16	400	12	0.05	0.5	0.62
300	20	40	150	70	0.05	0.51	0.44
10000	15	16	400	12.5	0.05	0.51	0.58
10000	15	16	400	13	0.05	0.51	0.58
300	20	32	150	75	0.05	0.53	0.38
300	20	40	100	75	0.05	0.56	0.44
10000	15	16	150	30	0.05	0.58	0.47
1000	15	24	150	30	0.05	0.58	0.46
1000	15	24	150	30	0.05	0.58	0.46
1000	15	32	150	30	0.05	0.59	0.45
1000	20	40	150	75	0.05	0.59	0.39
300	20	40	150	75	0.05	0.59	0.39
250	20	40	150	75	0.05	0.59	0.55
300	20	40	150	75	0.01	0.59	0.38
1000	15	16	300	30	0.05	0.6	0.51
300	20	48	150	75	0.05	0.6	0.41
10000	15	16	400	30	0.05	0.61	0.48
5000	15	16	400	30	0.05	0.61	0.48
7500	15	16	400	30	0.05	0.61	0.48
10000	20	16	400	30	0.05	0.61	0.47
2500	15	16	250	30	0.05	0.61	0.49
1000	15	16	400	30	0.05	0.61	0.48
10000	15	16	300	30	0.01	0.61	0.43
10000	15	16	300	30	0.001	0.61	0.43
1000	15	16	150	30	0.05	0.61	0.47
10000	20	16	400	30	0.05	0.61	0.47
10000	20	16	400	75	0.05	0.61	0.36
1000	15	16	150	30	0.05	0.62	0.47
1000	15	16	150	30	0.05	0.62	0.47
300	20	40	200	75	0.05	0.63	0.35
300	20	40	150	75	0.001	0.64	0.38
10000	15	16	100	25	0.05	0.66	0.59

10000	10	16	400	30	0.05	0.71	0.64
10000	15	16	300	35	0.05	0.72	0.54
350	15	40	150	75	0.05	0.73	0.44
10000	5	16	400	30	0.05	0.77	0.55
10000	15	8	400	15.96	0.05	0.99	0.63
5000	3.59	8	300	3.59	0.05	1	1
10000	15	8	400	30	0.05	1	0.77
5000	20	16	400	8	0.05	0.44	0.43
5000	30	16	400	8	0.05	0.44	0.43
5000	10	16	400	8	0.05	0.52	0.63
5000	40	16	400	8	0.05	0.44	0.43
5000	15	16	400	8	0.05	0.44	0.43
10000	15	16	400	8	0.05	0.44	0.43
2500	15	16	400	8	0.05	0.44	0.43
1000	15	16	400	8	0.05	0.44	0.43
800	15	16	400	8	0.05	0.44	0.43
400	15	16	400	8	0.05	0.44	0.43
400	15	16	200	8	0.05	0.42	0.47
400	15	16	300	8	0.05	0.43	0.46
300	15	16	200	8	0.05	0.42	0.47
300	15	16	300	8	0.05	0.43	0.46
400	15	16	350	8	0.05	0.44	0.46
400	15	8	400	8	0.05	0.57	1
400	15	24	400	8	0.05	0.45	0.44
400	15	32	400	8	0.5	0.45	0.44
400	15	16	400	8	0.005	0.44	0.43
400	15	16	400	8	0.0005	0.44	0.43
400	15	16	400	8	1	0.44	0.43
1000	20	16	400	8	0.05	0.44	0.43
1000	25	16	400	8	0.05	0.44	0.43
1000	20	16	400	8	0.05	0.44	0.43
1000	20	16	300	8	0.05	0.43	0.46
1000	20	16	200	8	0.05	0.42	0.47

1000	40	16	400	8	0.05	0.44	0.43
1000	60	16	400	8	0.05	0.44	0.43
1000	20	16	400	8	0.05	1	1
1000	20	16	400	8	0.05	0.45	0.44
1000	20	16	500	8	0.05	0.45	0.42
1000	20	8	400	10	0.05	0.5	0.55
1000	20	24	400	6	0.05	0.36	0.56
1000	20	16	400	12	0.05	0.5	0.4
1000	20	16	400	14	0.05	1	0.34
1000	20	16	400	16	0.05	0.48	0.53
1000	20	16	300	16	0.05	0.49	0.5
1000	20	16	200	16	0.05	0.47	0.46
1000	40	16	400	16	0.05	0.48	0.3
1000	60	16	400	16	0.05	0.48	0.3
1000	20	8	400	16	0.05	0.45	1
1000	20	24	400	16	0.05	0.45	0.42
10000	20	16	400	16	0.05	0.48	0.53
2500	20	16	400	16	0.05	0.48	0.53
800	20	16	400	16	0.05	0.48	0.53
400	20	16	400	16	0.05	0.48	0.53

Table 49 SVD and MIL values with their respective frequency during calculation

SVD	F-SVD	MIL	F-MIL
0	0	0	0
0.01	0	0.01	0
0.02	0	0.02	0
0.03	0	0.03	0
0.04	0	0.04	0
0.05	0	0.05	0
0.06	0	0.06	0
0.07	0	0.07	0
0.08	0	0.08	0
0.09	0	0.09	0
0.1	0	0.1	0
0.11	0	0.11	0
0.12	0	0.12	0
0.13	0	0.13	0
0.14	0	0.14	0
0.15	0	0.15	0
0.16	0	0.16	0
0.17	0	0.17	0
0.18	0	0.18	0
0.19	0	0.19	0
0.2	0	0.2	0
0.21	0	0.21	0
0.22	0	0.22	0
0.23	0	0.23	0
0.24	0	0.24	0
0.25	2	0.25	0
0.26	7	0.26	0
0.27	0	0.27	0
0.28	0	0.28	0
0.29	0	0.29	0

0.3	1	0.3	4
0.31	0	0.31	0
0.32	0	0.32	0
SVD	F-SVD	MIL	F-MIL
0.33	0	0.33	0
0.34	0	0.34	2
0.35	0	0.35	1
0.36	1	0.36	2
0.37	0	0.37	0
0.38	0	0.38	3
0.39	0	0.39	3
0.4	1	0.4	2
0.41	0	0.41	1
0.42	3	0.42	2
0.43	3	0.43	20
0.44	20	0.44	8
0.45	9	0.45	1
0.46	2	0.46	8
0.47	3	0.47	9
0.48	13	0.48	4
0.49	2	0.49	4
0.5	4	0.5	2
0.51	3	0.51	1
0.52	1	0.52	1
0.53	1	0.53	6
0.54	0	0.54	2
0.55	0	0.55	3
0.56	1	0.56	1
0.57	1	0.57	0
0.58	3	0.58	2
0.59	5	0.59	1
0.6	2	0.6	0
0.61	11	0.61	0
0.62	2	0.62	1

0.63	1	0.63	2
0.64	1	0.64	1
0.65	0	0.65	1
0.66	1	0.66	0
SVD	F-SVD	MIL	F-MIL
0.67	0	0.67	0
0.68	0	0.68	0
0.69	0	0.69	0
0.7	0	0.7	1
0.71	1	0.71	0
0.72	1	0.72	1
0.73	1	0.73	0
0.74	0	0.74	0
0.75	0	0.75	4
0.76	0	0.76	0
0.77	1	0.77	2
0.78	0	0.78	0
0.79	0	0.79	0
0.8	0	0.8	1
0.81	0	0.81	0

0.82	0	0.82	0
0.83	0	0.83	0
0.84	0	0.84	0
0.85	0	0.85	0
0.86	0	0.86	0
0.87	0	0.87	0
0.88	0	0.88	0
0.89	0	0.89	1
0.9	0	0.9	0
0.91	0	0.91	0
0.92	0	0.92	0
0.93	0	0.93	0
0.94	0	0.94	0
0.95	0	0.95	0
0.96	0	0.96	0
0.97	0	0.97	0
0.98	0	0.98	0
0.99	1	0.99	0
1	5	1	6

Vector field using surface normal projection- and eigen-based

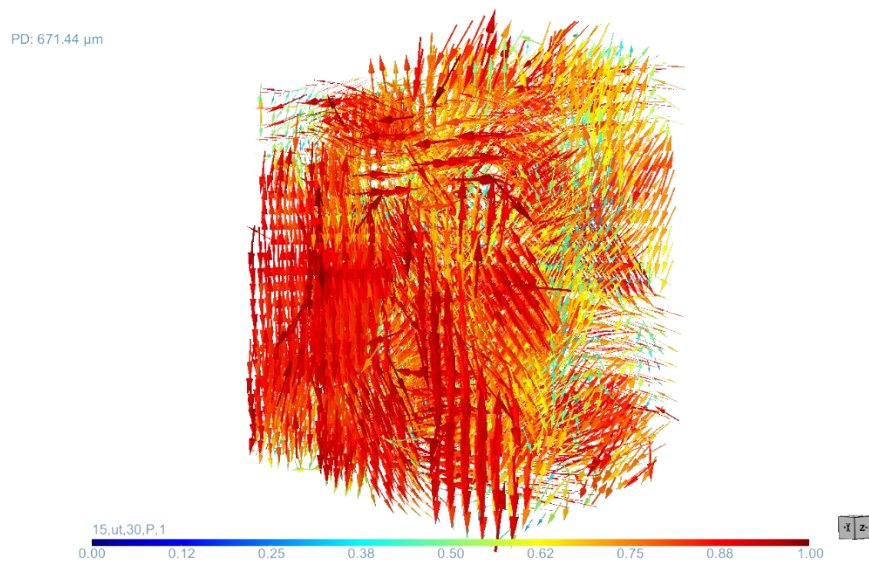


Figure 76 Spacing (μm) = 15, Radius of influence (μm) = 30, Evaluation: Projection, Mesh smoothing (repetitions) = 1

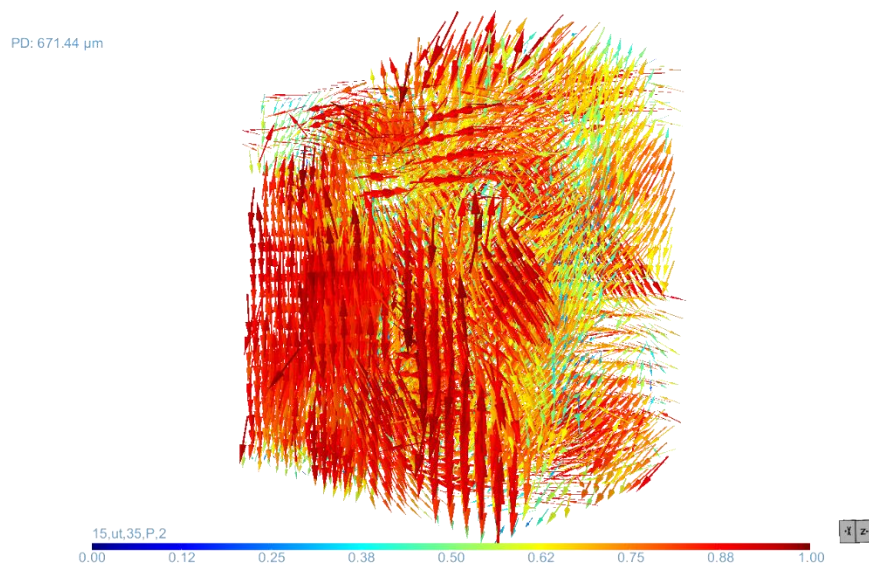


Figure 77 Spacing (μm) = 15, Radius of influence (μm) = 35, Evaluation: Projection, Mesh smoothing (repetitions) = 2

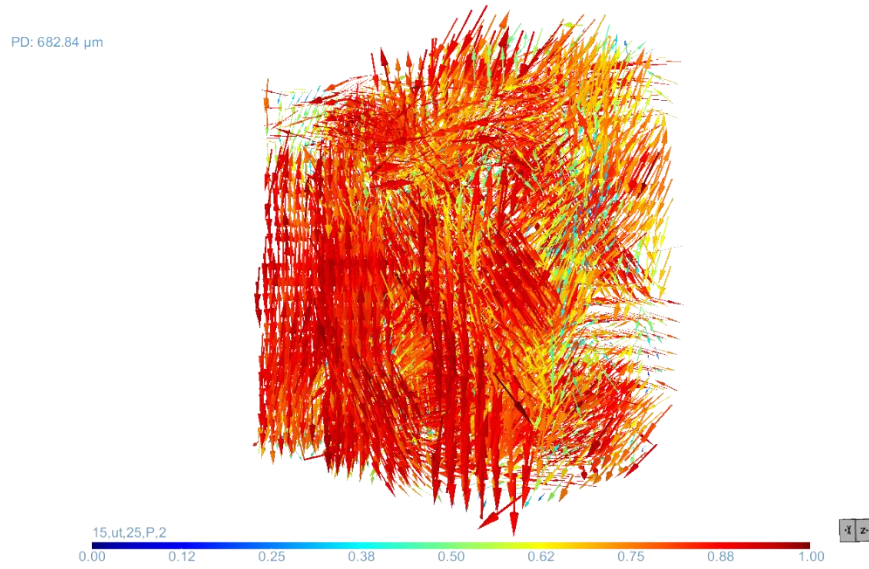


Figure 78 Spacing (μm) = 15, Radius of influence (μm) = 25, Evaluation: Projection, Mesh smoothing (repetitions) = 2

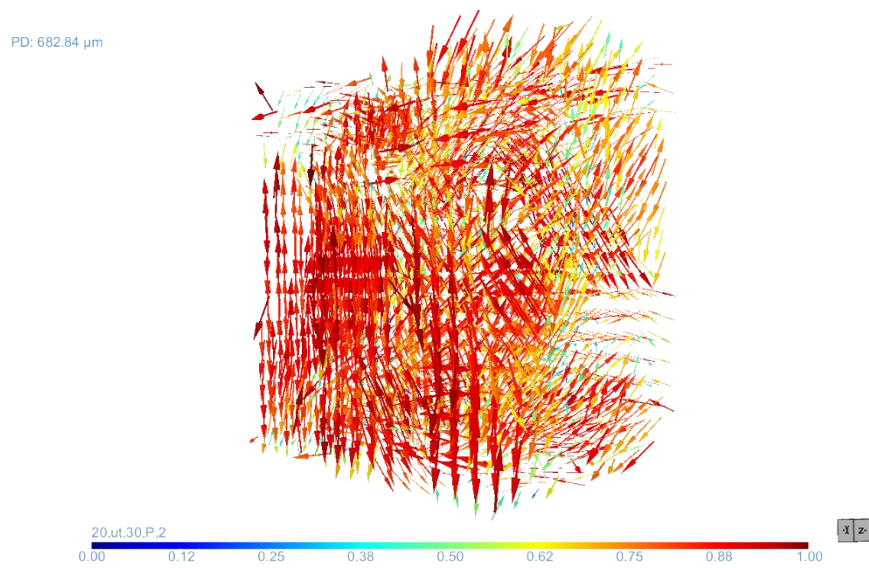


Figure 79 Spacing (μm) = 20, Radius of influence (μm) = 30, Evaluation: Projection, Mesh smoothing (repetitions) = 2

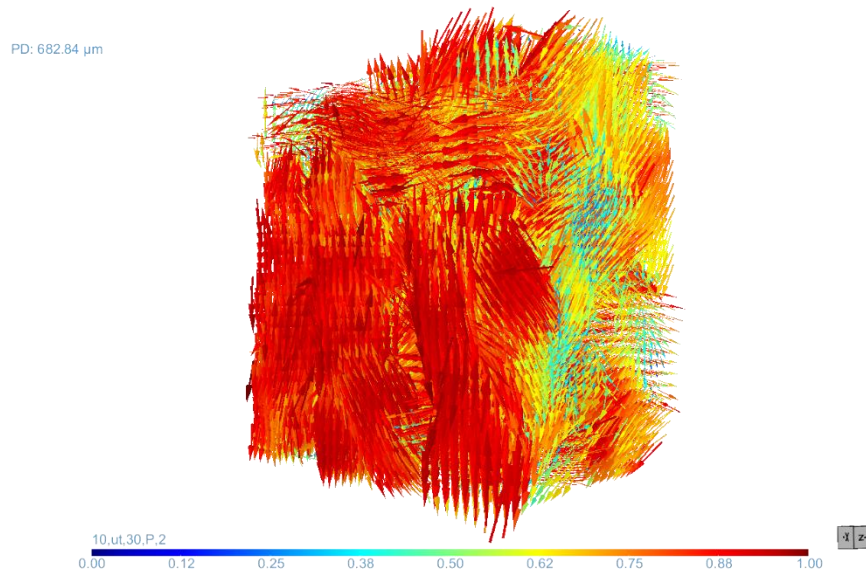


Figure 80 Spacing (μm) = 10, Radius of influence (μm) = 30, Evaluation: Projection, Mesh smoothing (repetitions) = 2

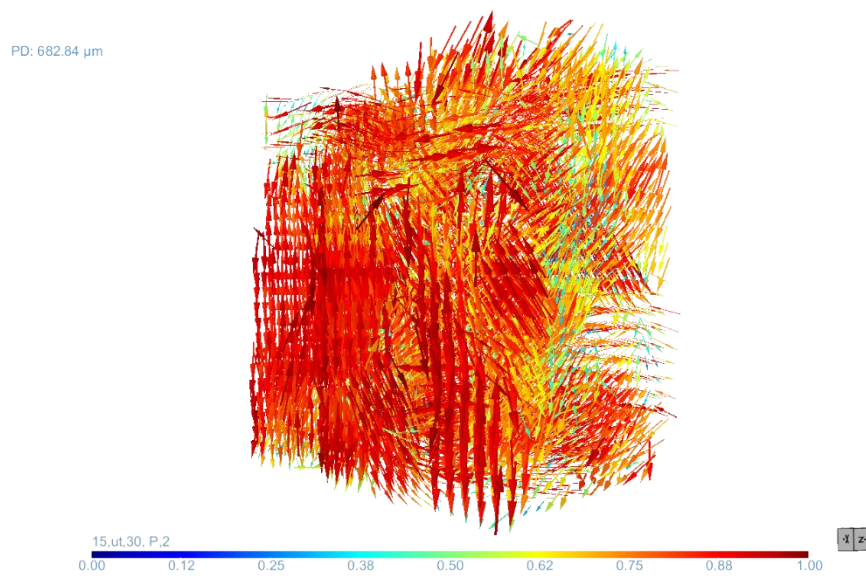


Figure 81 Spacing (μm) = 15, Radius of influence (μm) = 30, Evaluation: Projection, Mesh smoothing (repetitions) = 2

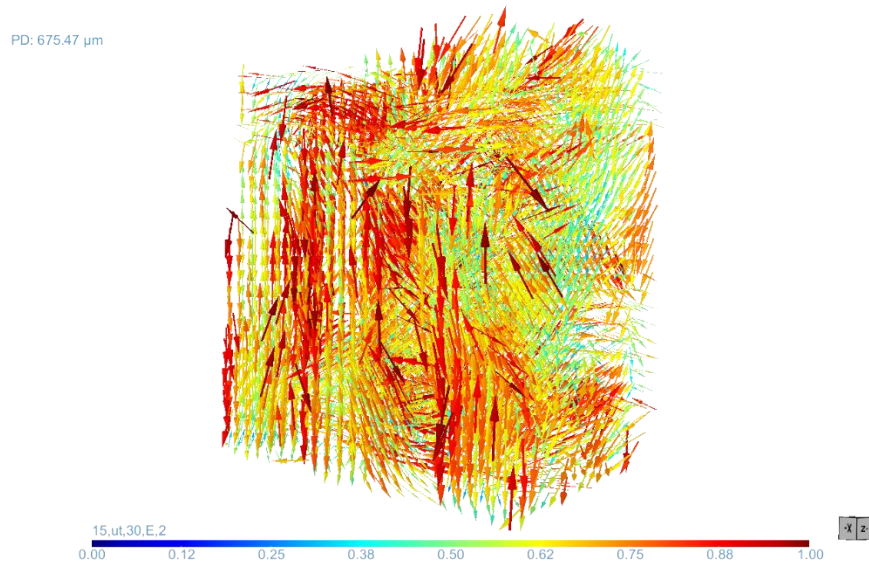


Figure 82 Spacing (μm) = 15, Radius of influence (μm) = 30, Evaluation: Eigen, Mesh smoothing (repetitions) = 2

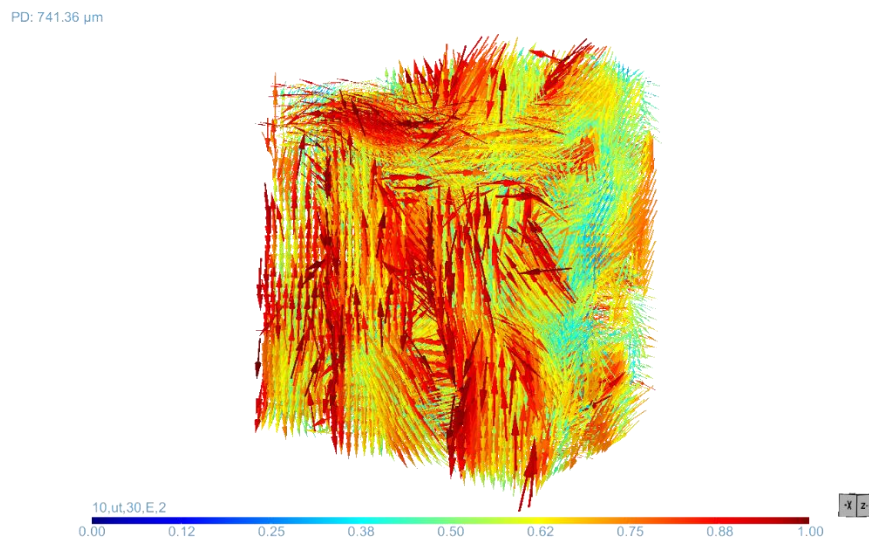


Figure 83 Spacing (μm) = 10, Radius of influence (μm) = 30, Evaluation: Eigen, Mesh smoothing (repetitions) = 2

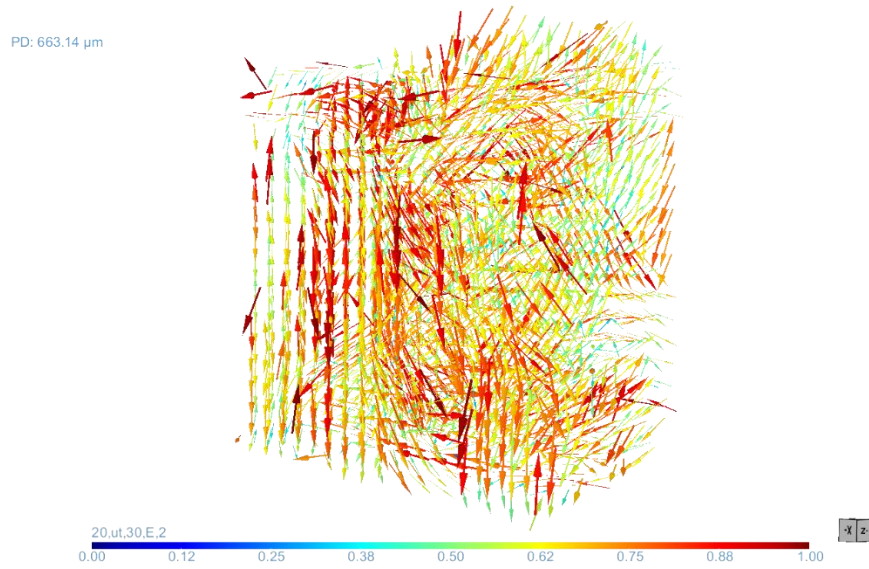


Figure 84 Spacing (μm) = 20, Radius of influence (μm) = 30, Evaluation: Eigen, Mesh smoothing (repetitions) = 2

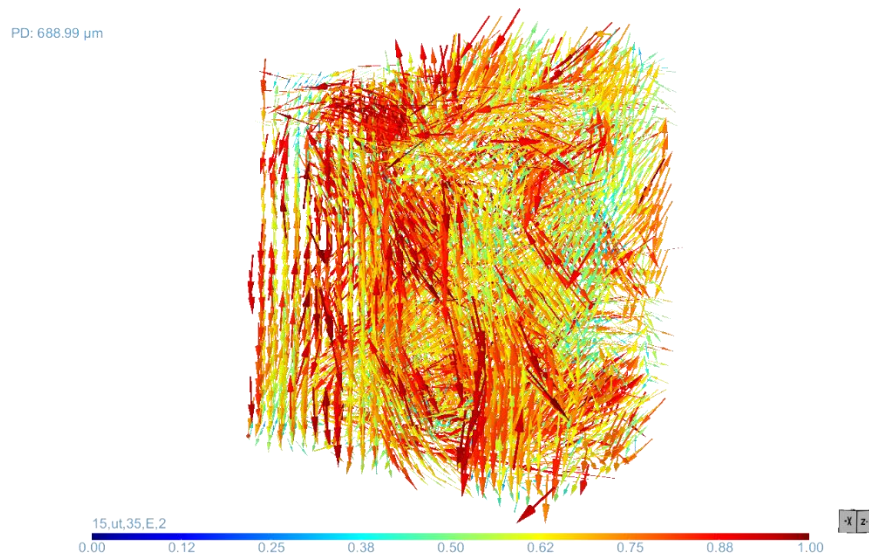


Figure 85 Spacing (μm) = 15, Radius of influence (μm) = 35, Evaluation: Eigen, Mesh smoothing (repetitions) = 2

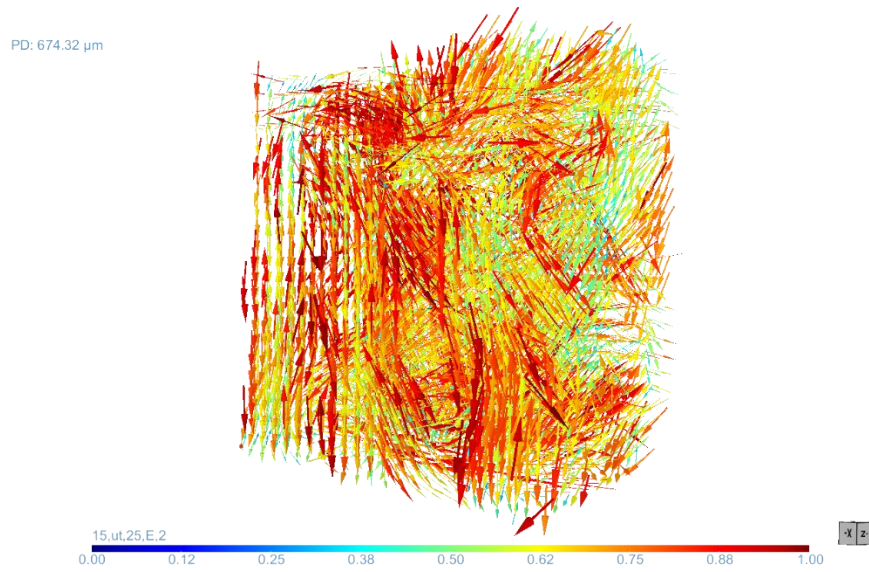


Figure 86 Spacing (μm) = 15, Radius of influence (μm) = 25, Evaluation: Eigen, Mesh smoothing (repetitions) = 2

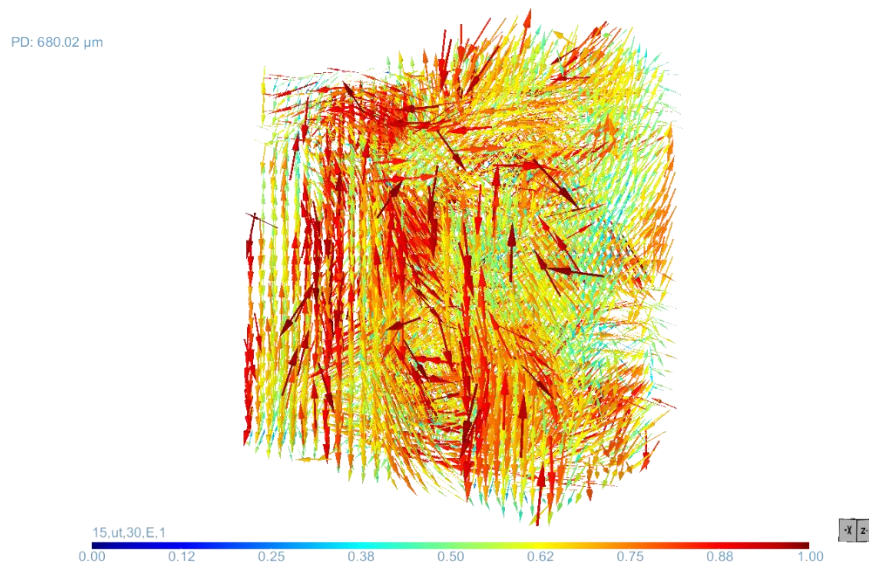


Figure 87 Spacing (μm) = 15, Radius of influence (μm) = 30, Evaluation: Eigen, Mesh smoothing (repetitions) = 1

Appendix D: Research output

D1.Publication 1 in Biology: Collagen Extraction from Animal Skin



Review

Collagen Extraction from Animal Skin

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Simple Summary: Collagen is useful in many applications including cosmetics, medicine, yarn production and packaging. Collagen can be recovered from skins of animals raised for meat. Here, we review methods for the extraction and purification of collagen from animal skins.

Abstract: Collagen is the most abundant structural protein in animals. It is the major component of skin. It finds uses in cosmetics, medicine, yarn production and packaging. This paper reviews the extraction of collagen from hides of most consumed animals for meat with the focus on literature published since 2000. The different pretreatment and extraction techniques that have been investigated for producing collagen from animal skins are reviewed. Pretreatment by enzymatic, acid or alkaline methods have been used. Extraction by chemical hydrolysis, salt solubilization, enzymatic hydrolysis, ultrasound assisted extraction and other methods are described. Post-extraction purification methods are also explained. This compilation will be useful for anyone wishing to use collagen as a resource and wanting to further improve the extraction and purification methods.

Keywords: collagen; extraction; skin



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1. Introduction

Animal hides have been used in diverse applications since prehistoric times. Hide or skin contributes between 3% and 12% (Table 1) to the weight of a live mammal and may reach up to 20% in poultry (Table 1) [1]. Hides are mostly a by-product of meat production [2–4]. As meat for human consumption is mostly bovine (beef), porcine (pork), ovine (lamb), hircine (goat) and galline (chicken) [5–7], hides of these animals are most readily available. Hides are generally a low value by-product of meat production, and for some animals, for example sheep, typically go to landfill. Hides have the potential to be used for adding value to meat production operations [8]. Increasing global consumption of meat (Figure 1) inevitably means an increasing supply of hides [7]. These hides need to be better utilized also to minimize environmental impact [7,9].

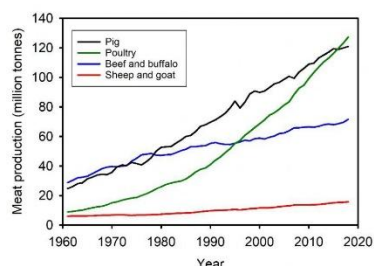


Figure 1. World meat production (1961–2020). Data from Ritchie and Roser [7,9].

Among other applications, hides are used: as a source of the protein keratin for extraction and incorporation in animal feed [10], fertilizers [11], cosmetics [12] and food packaging [13]. In addition, gelatine, a food additive and packaging material [14,15], is produced from hides. Furthermore, hides are a major source of collagen for use in cosmetics [16,17], medicine [18], yarn production [19] and packaging [16,20].

This paper reviews the extraction of collagen from hides of the most commonly consumed animals as listed in Table 1. The focus is on the literature published since 2000.

Table 1. Skin as percentage of total body mass.

Animal	Skin (% wt/wt)	Reference
Cattle	5.1–8.5	[1]
Sheep	11.0–11.7	[1]
Pig	3.0–8.0	[1]
Goat	~9.0	[21]
Chicken	8.0–20.0	[22]

2. Collagen

Collagen is the most abundant structural protein in all animals [23]. It is a component of the extracellular matrix (ECM) of various connective tissues such as skin, bones, cartilage and tendons [24]. Naturally synthesized collagen molecules consist of three long helicoidal chains of amino acid residues with nonhelical terminals at both ends [24] (Figure 2). At least 46 unique polypeptide chains have been found in collagens of various animals [23].

Collagen chains most commonly consist of the repeating motif Gly-X-Y where Gly is the amino acid glycine while X and Y are generally the amino acids proline and 4-hydroxyproline, respectively [23]. This motif is distinct from the other ECM components [23,25,26]. The α -chains of different types of collagens vary in composition, depending on the frequency of repetition and the length of the segment containing the Gly-X-Y motif, with or without interruptions, and the amino acid residues that occur in positions X and Y [27,28].

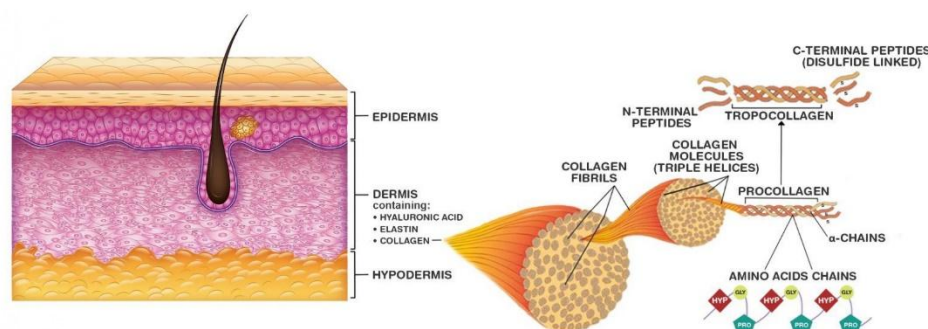


Figure 2. Skin-collagen-molecular structure. Adapted with permissions from: (1) Tang et al., Journal of Voice, published by Elsevier Inc. (Amsterdam, The Netherlands), 2017; (2) Reilly and Lozano, Plastic and Aesthetic Research, published by OAE Publishing Inc., 2021 (©2021 MINERVA Research Labs Ltd. (106 New Bond St. London, UK) all rights reserved) [29,30].

The arrangement of the polypeptide chains and the variation of the terminals gives distinction to the types of collagens, both fibrillar and non-fibrillar. Collagen types vary in conformations resulting in different lengths of helices and distributions of non-helical segments. These criteria are used to group collagens into several groups. General groups

include fibrillar collagens, FACIT (fibril associated collagens with interrupted triple helices), FACIT-like collagen, basement membrane collagen, beaded filament collagen, transmembrane collagen, short-chain collagen, and unclassified collagen [31,32]. At least 29 types of collagens are currently recognized [27,31]. Fibrillar collagens are the most abundant ECM proteins in vertebrates, providing stability, connectivity and form to tissues and organs [32,33]. The most abundant fibrillar collagen in most tissues is type I collagen [34]. It is primarily present in fibril surfaces and connective tissues of the skin and bones [35]. Collagen type I has a rod-like structure made of three helically coiled chains. It has a molecular weight of approximately 300 kDa, with a length of 280 nm, a diameter of 1.4 nm and contains about 1020 amino acid residues per chain [27,36,37].

Collagen is widespread in mammal tissues (Figure 3) [24,38] and therefore can be sourced from various abattoir by-products including hides [38]. The type of collagen depends on the tissue type. Fibrillar collagens associated with different tissue types are shown in Table 2 [32,39]. Biocompatibility, biodegradability, and low antigenicity of collagen make it an attractive material for various applications [16].

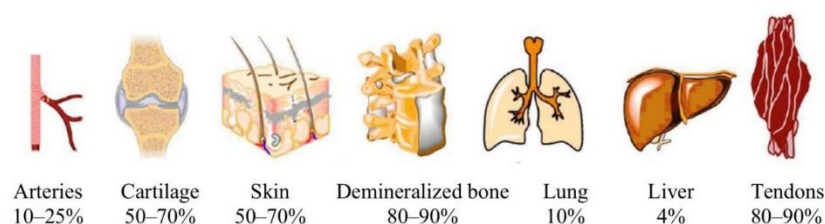


Figure 3. Collagen content of different body parts of animals. Reproduced with permission from Jafari et al., Polymers, 2020; 12(10):2230. ©2020 MDPI [24].

Table 2. Collagen types, compositions and sources (based on [39,40]).

Type	Molecule	Source
I	$[\alpha 1(I)]_2[\alpha 2(I)]$	Skin, tendon, bone, ligaments, interstitial tissues
II	$[\alpha 1(II)]_3$	Intervertebral disc, cartilage, vitreous humor
III	$[\alpha 1(III)]_3$	Cardiovascular vessel, uterus, skin, muscle
V	$[\alpha 1(V)][\alpha 2(V)][\alpha 3(V)]$	Similar to type I, also cell cultures, fetal tissues; associates with type I
XI	$[\alpha 1(XI)][\alpha 2(XI)][\alpha 3(XI)]$	Cartilage, in vertebral cartilage and bone enamel

Differences in Skin of Different Animals

There are significant differences in skins of different animals. These include both the composition and the structure of the skin. For example, skins of chicken [41] and sheep are high in fat whereas beef and goat have skins with a lower fat content [42]. There may be differences in the composition of the collagen, specifically the hydroxyproline content. The arrangement of the collagen fibrils also varies markedly among species, with a higher alignment of the fibrils in species such as goat, buffalo and cattle than in sheep and pig [43,44]. Within a species, different breeds may show differences in skin structure and composition. In cattle, ‘looseness’ is a known problem in leather processing which results from differences in collagen fibril alignment [45]. These compositional and collagen structural differences have to potential to affect ease of extraction.

3. Collagen Extraction Process

The collagen extraction process depends on the source material. The aim is to remove all the non-collagenous matter and recover collagen as the final product. The recovery process typically involves pretreatment of the source tissue, collagen extraction, and further

purification [46]. For recovery from skin, the pretreatment steps are generally preceded by washing the skin by soaking in cold water for a few days while replacing the water every few hours. This is followed by cutting the skin into more manageable pieces. Typically, skin is cut into small 1 cm² pieces [47].

3.1. Pretreatment

Pretreatment is intended to break the covalent intermolecular crosslinks between collagen molecules. In vivo, these crosslinks facilitate collagen's structural and mechanical functions in tissues and organs [33]. These crosslinks breakdown very slowly even in boiling water. Various mild chemical treatments are used for breaking the covalent links [37]. Dilute acids and alkalis are typically used for partial hydrolysis of the collagen. This cleaves the crosslinks, while leaving the collagen chains intact [48]. Certain enzymes may also be used for the pretreatment step [37].

3.1.1. Acid Pretreatment

For acid pretreatment, washed and chopped skin pieces are immersed in dilute acid at a controlled temperature. The acid permeates the skin causing it to swell to between two- to three-times its initial volume and hydrolyzes the crosslinks [38,49,50]. Acid pretreatment is suitable for relatively fragile skins [50] that have a lower degree of fibre intertwinement, such as porcine and fish skins [38].

3.1.2. Alkaline Pretreatment

Dilute alkalis such as sodium hydroxide (NaOH) and calcium hydroxide (Ca(OH)₂) are used for pretreatment. The duration of pretreatment depends on the thickness of the material being treated [49]. Alkalis are particularly effective in extracting collagen from thick and hard materials [8,38]. Pretreatment with NaOH may take from a few days to weeks to complete [48]. The lengthy treatment notwithstanding, alkaline treatment with NaOH is often preferred as it swells the skin substantially ensuring diffusion of the alkali deep into the tissue matrix [51]. Alkalis hydrolyze the unwanted non-collagenous proteins, lipids, pigments, and other organic material [52]. The treatment conditions such as the temperature, duration and the concentration of alkali significantly impact the efficacy of removal of the unwanted non-collagen material [53]. A NaOH concentration ranging from 0.05 to 0.10 kmol m⁻³ is sufficient for the pretreatment [38]. Within this concentration range, much of the acid soluble collagen and its native structure are retained in the tissue so long as the treatment temperature is between 4 and 20 °C. A high concentration of NaOH (e.g., ≥0.2 kmol m⁻³) can lead to a substantial loss of acid soluble collagen. A 0.5 kmol m⁻³ NaOH concentration may degrade the native structure of acid soluble collagen [38].

3.1.3. Skin-Specific Pretreatments

The source, or the nature of the skin, may necessitate skin-specific pretreatments including soaking, fleshing, dehairing, and cutting [46]. For example, skins with fur and feathers may require different pretreatments. Mechanical slicing may be used to remove some of the fat adhering to the hide [20,54–58]. Alcohols such as butyl alcohol may be used to solubilize fat and pigments from chicken [55,56] and bovine skins [52,53,59,60]. Other pretreatment steps may use heating [61,62], detergents [63], solvents such as petroleum ether [64,65], hexane [66], and hot water rinses [67]. A demineralization step may involve the use of the chelating agents such as ethylene diamine tetra acetic acid (EDTA) [68,69].

3.2. Extraction

Conventional extraction methods have typically relied on chemical hydrolysis using acid, alkali or salt solubilization. In some cases, the chemical extraction may be aided by ultrasound or microwaves, and enzymes [70]. Extraction temperature is controlled at a relatively low value (4 °C) to minimize degradation of collagen [52]. Extraction methods can be tailored depending on the desired yield and the properties of the final product. Collagen

properties such as the average length of the polypeptide chains, the solubility, the solution viscosity, thermal stability, emulsifying capacity, and water retention are affected by the specifics of the extraction method [57,70]. In addition, the specific processing conditions, including the pretreatment used, the storage conditions for the hides, and their specifics (animal type, age and gender) influence the quality of the extracted collagen [27]. For example, breed and age of poultry affects the characteristics of the collagen extracted [56,61]. Differences in collagen structure, for example the hydroxyproline content, can also affect the extraction process.

3.2.1. Acid Hydrolysis

Collagen is most commonly extracted from skins by a hydrolysis treatment involving either acids or alkalis. Both inorganic and organic acids can effectively cleave bonds in collagen to enable extraction of the fibrils [71]. Under acidic conditions the collagen molecules have a net positive charge and the resulting electrostatic repulsive force between them facilitates molecular separation [52]. The organic acids commonly used are acetic, chloroacetic, citric and lactic acids. Acetic acid has been widely reported for collagen extraction [16,48,50,54–56,59,71,72]. Among inorganic acids, the commonly used ones include hydrochloric, sulfuric and nitric acids [8,38,72,73]. Organic acids are apparently more effective for cleaving collagen crosslinks, and result in higher extraction yield [16,72,74] compared to mineral acids. Organic acids also solubilize non-crosslinked collagens [73]. For extraction with acetic acid, a typical concentration is 0.5 kmol m^{-3} with contact time of 2472 h and continuous stirring [75].

3.2.2. Alkali Hydrolysis

Alkaline hydrolysis may also be used to extract collagen most commonly with aqueous sodium hydroxide or potassium hydroxide [52] although calcium oxide, calcium hydroxide, and sodium carbonate can also be used as extractants [76]. Alkalis have a tendency to hydrolyze collagen fibrils [71] and the amino acids cysteine, histidine, serine, and threonine may be destroyed in the process [52,76]. The use of alkali hydrolysis appears to be mostly confined to extracting collagen from leather processing waste [71,77], however, a collagen-based flame retardant has been produced from collagen obtained by alkaline extraction of otherwise untreated cattle skin [78].

3.2.3. Salt Solubilization

Solubilization with salts is used less commonly [38,52]. Solutions of neutral salts are effective in solubilizing collagen and are commonly used in extraction. Examples of the salts used are citrates, phosphates, sodium chloride, and Tris-HCl [38]. Collagen type I dissolves at salt concentrations of $<1.0 \text{ kmol m}^{-3}$ but precipitates at concentrations exceeding 1.0 kmol m^{-3} [71]. This limitation on salt concentration requires careful control when extracting with salts compared to alkaline and acid hydrolysis [70].

3.2.4. Enzyme Hydrolysis

Enzyme hydrolysis has been developed to address some of the shortcomings of the more traditional methods [70]. Enzyme hydrolysis may be used in combination with some of the traditional chemical methods [71]. Enzyme hydrolysis offers better reaction selectivity and is less damaging to collagen. Therefore, it has the potential to maximize collagen yield and purity of the extracted product [71]. Enzymes tend to be much more expensive than acids, alkalis and salts, but they can be used under mild reaction conditions. Compared to chemicals, enzymatic treatment is less corrosive to processing equipment, consumes less energy, produces less waste, allows better control of degree of hydrolysis, and the final hydrolysate has a lower salt content [70,71]. Hybrid methods involving chemicals as well as enzymes have been reported [71].

Proteolytic enzymes are used in collagen extraction. These enzymes may be of animal origin (e.g., trypsin, pepsin), plant origin (e.g., bromelain, papain, ficin), or microbially

produced single or mixed enzymes (e.g., collagenase, proteinase K, Alcalase® (Novozymes, Bagsværd, Denmark), Nutrase® (Nutrex, Hoogbuul, Belgium), Flavourzyme® (Novozymes, Bagsværd, Denmark), and Protamex® (Novozymes, Bagsværd, Denmark)). Pepsin from animal sources is most widely used in collagen extraction [8,16,52]. Pepsin, trypsin and papain only act on the non-helix portion of the peptide chain of collagen (the ends) and leave the structurally important helical portion intact [71]. Additionally, the extracted pepsin-soluble collagen generally has a higher purity as the non-collagenous proteins are efficiently hydrolyzed by the enzyme. Pepsin treatment also increases acid solubility of collagen, which increases the extraction efficiency if used in combination with acid extraction. The yield of pepsin soluble collagen is significantly affected by the degree of crosslinking in the telopeptide region of the peptides [70]. The plant enzymes such as papain, also allow good control of the extent of hydrolysis of the protein substrate [38].

A two-step extraction process without a preceding treatment has been reported for recovery of collagen from sheepskin; an enzymatic step was used first and this was followed by acid hydrolysis [79]. Another hybrid extraction procedure using acetic acid in combination with pepsin has been described by several authors [55,56,59,60,62,68,80–84].

3.2.5. Ultrasound-Assisted Extraction

Application of ultrasound (frequency of ≥ 20 kHz) during collagen extraction improves yield and reduces the time required for extraction [85]. Ultrasound generates intense turbulence in a liquid resulting in enhancement of mass transfer and rates of chemical reactions [38,86]. In ultrasound-assisted acid extraction of collagen from sea bass skin the structure of the extracted collagen remained undamaged [87], although sufficiently intense ultrasonic treatment has the potential to damage protein structure. The collagen yield is influenced by the amplitude of ultrasound and the duration of the treatment. A higher amplitude has been found to shorten extraction time and enhance yield [70]. Ultrasound assistance has been found to damage collagen structure during alkali treatment because the alkali may weaken hydrogen bonds and break parts of covalent bonds in collagen which then facilitates damage by ultrasound [64]. However, with enzyme treatment, the yield and collagen purity are improved using ultrasound and the extraction time is reduced. In ultrasound-mediated pepsin-based extraction of collagen from cattle tendon, extraction efficiency and the collagen quality were enhanced compared to a process involving only the enzyme [75]. Use of sonication in combination with enzymes increases collagen yield and shortens extraction time [88]. Ultrasound treatment is generally simple, reduces the need for corrosive chemicals, and can be carried out economically and safely. Damage to the structure of collagen has been observed in some cases [64]. Damage may be minimized by optimization of the treatment (ultrasound amplitude, duration, concentration of enzyme or other chemical, temperature) for the specific collagen source [38,52,88].

3.2.6. Other Extraction Methods

Microwave-assisted. Microwave-assisted extraction has been extensively described. Microwaves, a form of electromagnetic radiation used in microwave cooking, disrupt cell and tissue structure [89,90], facilitating extraction. Microwaves penetrate deeply in tissue [90]. Microwave assistance has been found to speedup acid and enzyme action compared to equivalent treatments without the microwaves [89].

Mechanical agitation. Tissue solubilization processes involving acids, alkalis and enzymes are generally enhanced by mechanical agitation. Agitation improves hydrolysis by improving mass transfer of enzymes and chemicals into tissue. Thus, it shortens extraction time or enhances collagen yield in a given extraction time [91]. Agitation-assisted hydrolysis for collagen recovery from jellyfish tissue was shown to enhance yield by at least 5-fold compared to hydrolysis under static conditions using acid, or enzymes alone [92]. Similar observations were reported by others [68].

3.2.7. Precipitation of Solubilized Collagen

Once the collagen has been solubilized by the methods described above, it needs to be precipitated from solution. Salts are most often used to achieve this, with a salt concentration exceeding 1.0 kmol m^{-3} . This treatment has been used for collagen from chicken skin [62,63,80,93], sheep and goatskin [54,55,68,81], pigskin [82,94], and cattle skin [59,95].

3.3. Post Extraction: Purification

In addition to collagen, a crude collagen extract typically contains neutral salts and non-collagen proteins [46]. Collagen can be purified from this extract to obtain collagen fractions with different molecular weights. Purification processes use multiple steps [96] which may include filtration and centrifugation [85]. Target protein, or unwanted proteins, may be precipitated from solution by “salting out” (adding a high concentration of salt to extract).

The salting out of collagen, or other proteins, in a crude extract is influenced by several factors, including temperature, pH, the ionic strength, the salt used, and the specifics of the proteins involved. Generally, collagen is less soluble than the contaminating proteins and may be precipitated by salting out. Salting out conditions must be selected to minimize coprecipitation of the other proteins.

Lyotropic series can be used as a guide in selecting salts for salting-out [46,97]. Both anions and cations in a salt are ranked in the order of their salting-out ability [46]. Anions are more efficient in salting out than cations. For anions, the salting out efficiency decreases in the following order: $\text{F}^- \approx \text{SO}_4^{2-} > \text{HPO}_4^{2-} > \text{CHCOO}^- > \text{Cl}^- > \text{NO}_3^- > \text{Br}^- > \text{ClO}_3^- > \text{I}^- > \text{ClO}_4^- > \text{SCN}^-$. For cations, the salting out performance decreases in the following order: $\text{NH}_4^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+ > \text{Mg}^{2+} > \text{Ca}^{2+}$. Neutral salts produced by combining a strong anion and a cation from the above series, have a strong ability to precipitate collagen from a solution. Sodium chloride is a neutral salt with a strong precipitating effect. Although ammonium sulfate is generally the best for salting out proteins, it is not a neutral salt and its constituent ions are often not acceptable in products intended for biomedical applications [46]. Neutral salts have the advantage of not significantly affecting the pH of a solution, as pH is also a factor in salting out.

For precipitation of collagen, a suitable mass of a neutral salt is added to a specific volume of the collagen containing solution [46]. The pH is adjusted to 7 by adding sodium hydroxide. The resulting solution is allowed to stand for 4–12 h. The collagen precipitate is collected by centrifugation. The recovered precipitate may be dissolved and subjected to a second round of salting-out. This procedure may be repeated twice or more for further purification [46]. Refrigerated centrifuges (4°C) are typically used in recovering the precipitate.

The precipitated collagen contains a large amount of salt. This is removed by dialysis. Precipitated collagen is placed in dialysis bag and dialysed against an acidic solution or deionised water. During dialysis, the dialysate is periodically changed to increase the rate of ion migration [96]. Various dialysis schemes have been reported [38,59]. A single or multiple dialysis stages may be used. Dialysis may take 4 to 10 days to sufficiently remove the salts [46]. This slow step contributes nearly 50% to the total time required for the entire collagen extraction process [46].

Purification methods may be customized to recover collagen of a certain molecular weight range [70]. Various types of membrane filtrations and chromatographic separations can be used to finetune the molecular weight of the collagen product [96,98].

4. Specific Extraction Methods

The collagen extraction methods that have been described in the literature are summarized in Table 3.

Table 3. Pretreatment, extraction and isolation methods for collagen production from skin.

Pretreatment	Extraction and Isolation	Reference
Chicken		
Hot-water bath (40 and 60 °C, 1 h)	Multi-step extraction (4 °C): (1) Protease inhibitors solution: 1 kmol m ⁻³ NaCl and 50 × 10 ⁻³ kmol m ⁻³ Tris-HCl with PhCH ₂ SO ₂ F (1 × 10 ⁻³ kmol m ⁻³), MalNet (10 × 10 ⁻³ kmol m ⁻³) and EDTA (20 × 10 ⁻³ kmol m ⁻³) for 24 h; (2) Ethylene diamine dihydrochloride, 24 h; (3) 0.5 kmol m ⁻³ acetic acid; (4) 0.5 kmol m ⁻³ acetic acid with pepsin (1 mg/mL). Followed by precipitation steps: Ammonium sulfate (25% saturation) for precipitation in between extraction rounds. NaCl (crystals) for collagen type-specific precipitation.	[62]
Non-collagen removal (0.1 kmol m ⁻³ NaOH, 6 h); fat removal (10% v/v butyl alcohol, 24 h, 4 °C)	Acid hydrolysis (0.5 kmol m ⁻³ acetic acid, 42 h, 4 °C); precipitation (2 kmol m ⁻³ NaCl); dialysis (water); centrifugation	[93]
Non-collagen removal (0.1 kmol m ⁻³ NaOH, 6 h); fat removal (4% detergent, Triton X-100 and 5% KCl, 12 h)	Acid hydrolysis (0.5 kmol m ⁻³ acetic acid, 3 days at <10 °C); precipitation (2.5 kmol m ⁻³ NaCl and 0.05 kmol m ⁻³ Tris-(hydroxymethyl)-aminomethane); centrifugation	[63]
Fat and pigment removal (centrifugation)	Acid hydrolysis (0.5 kmol m ⁻³ acetic acid, 72 h at 4 °C); centrifugation; dialysis (distilled water)	[61]
Non-collagen removal (0.1 kmol m ⁻³ NaOH, 24 h at 4 °C); fat removal (10% butyl alcohol, 24 h at 4 °C)	Acid hydrolysis (0.5 kmol m ⁻³ acetic acid, 24 h at 4 °C); vacuum (varies depending on breed)	[99]
Fat and pigment removal (centrifugation); non-collagen removal (2 kmol m ⁻³ NaOH, 12 h)	Acid hydrolysis (0.5 kmol m ⁻³ acetic acid, 24 h at 4 °C); precipitation (0.9 kmol m ⁻³ NaCl); for acid insoluble collagen: (1) heat soluble collagen (95 °C), (2) enzyme hydrolysis (1% w/w pepsin; centrifugation; dialysis (deionized water)	[80]
Sheep		
Conducted at 4 °C. Non-collagen removal (0.1 kmol m ⁻³ NaOH, 6 h); demineralize (0.5 kmol m ⁻³ EDTA-2Na, 48 h)	Acid hydrolysis (0.5 kmol m ⁻³ acetic acid, 3 h at 20 °C); enzyme addition (pepsin 1 g L ⁻¹ , 48 h); precipitation (2.6 kmol m ⁻³ NaCl); centrifugation; second hydrolysis (1 kmol m ⁻³ NaCO ₃ , trypsin 1:50 w/v at 60 °C from 10 min to 4 h)	[68]
Wash; dehair (deionized water)	Acid-enzyme hybrid (0.5 kmol m ⁻³ acetic acid, 0.01 and 0.001 g g ⁻¹ trypsin, at 20 and 35 °C, pH 7 and 9 for 30–360 min); filtration and centrifugation	[79]
Goat		
Non-collagen removal (0.1 kmol m ⁻³ NaOH, 0–48 h at 4 °C)	Conducted at 4 °C. Acid-enzyme hybrid (0.5 kmol m ⁻³ acetic acid with 0.1% w/v pepsin, 24 h); precipitation (2.6 kmol m ⁻³ NaCl, 12 h); centrifugation (4500 × g, 30 min)	[55]
Non-collagen removal (0.1 kmol m ⁻³ NaOH, 24 h at 4 °C)	Acid hydrolysis (0.5 kmol m ⁻³ acetic acid 24–72 h); precipitation (2.6 kmol m ⁻³ NaCl); centrifugation (7000 × g); redissolution and dialysis (acetic acid)	[54]
Non-collagen removal (0.1 kmol m ⁻³ NaOH, 0–48 h at 4 °C)	Acid-enzyme hybrid (0.5 kmol m ⁻³ acetic acid with 0.1% pepsin, 24–72h at 38 °C); precipitation (2.6 kmol m ⁻³ NaCl); centrifugation (7000 × g, 30 min at 4 °C); redissolution and dialysis (acetic acid)	[81]
No pretreatment detailed	Enzyme hydrolysis (1 g pepsin in 100 mL buffer (pH 2.0) at 37 °C for 15 min); second enzyme addition (0.1 U pepsin, 1–120 min); neutralization (1 kmol m ⁻³ NaOH); centrifugation (1000 × g, 15 min)	[100]
Pig		
Degrease in ultrasonic bath (75% sodium dodecylbenzene (SDBS)), skin to SDBS 1:2.5 volume ratio at 25 °C, 120 W); non-collagen removal (1% NaCl for 6 h)	Conducted at 4 °C. Acid-enzyme hybrid (2000 U g ⁻¹ pepsin in 0.5 kmol m ⁻³ acetic acid, 18 h); precipitation (NaCl, 8–12 h); centrifugation; redissolution and dialysis (both acetic acid, and water for second dialysis)	[56]

Table 3. Cont.

Pretreatment	Extraction and Isolation	Reference
No pretreatment detailed	Alkaline treatment (3–7% NaOH in 6% NaCl solution, 24 h); neutral salt wash (NaCl); neutralization (7% acetic acid); vacuum freeze dry (75–90% moisture removal)	[94]
Fat removal (1:3 <i>w/v</i> petroleum ether at 30 °C, 1 h)	Enzyme hydrolysis (microfluidizer) (2400 U g ^{−1} pepsin (pH 7, 50 °C) and 3000 U g ^{−1} Alcalase (pH 8.5, 60 °C)); centrifugation (10,000 × <i>g</i> , 4 °C, 20 min); dialysis and freeze dry	[65]
Fat removal (hexane, 60 g skin per 400 mL); drying (24 h at 60 °C, −76 mm Hg)	Hydrolysis with rotary evaporator (125 g L ^{−1} of acid with pH 3 or alkali with pH 12 at 60 °C for 1 h at rotary speed scale 6); centrifuge (8000 × <i>g</i> , 10 min); freeze dry	[66]
Fat removal (petroleum ether); non-collagen removal (1% <i>w/v</i> NaCl, 6 h); alkali pretreatment (2% g g ^{−1} NaOH with 2.5 g skin mL ^{−1} solution ratio, 1 h); ultrasound (in alkali solution, at 25 kHz at 290 W, 40 min)	Maintain basic pH (0.1 kmol m ^{−3} phosphate buffer with pH 8.0); enzyme hydrolysis (Alcalase 1:100 <i>w/v</i> at 55 °C, varying hydrolysis times); centrifugation	[64]
Fat removal (10% Na ₂ CO ₃ ; hot water bath 45 °C)	pH adjustment (pH 8); enzyme hydrolysis (2 h at 40 °C, enzyme not mentioned); filtration and centrifugation; freeze dry (supernatant)	[20]
In a rotating drum: Wash (0.3% peregal at 30 °C for 3 h); fat removal (manual defleshing; 300% float with 2.5% Na ₂ CO ₃ and 0.5% peregal at 30 °C for 3 h); dehair (2.5% trypsin (250 µg mg ^{−1}) coated on flesh side at 25 °C overnight)	Alkali-enzyme hydrolysis (not detailed); freeze dry (−5 °C for 5 h) (stirred at 4 °C); acid hydrolysis (2 kmol m ^{−3} acetic acid with 1:50 <i>w/v</i> , 6 h) centrifugation (20,000 × <i>g</i> , 30 min); neutralization (NaOH addition until pH 7.5); precipitation (1.5 kmol m ^{−3} NaCl, refrigerated desiccator for 12 h); centrifugation; dialysis (water until neutral pH, 30 kDa molecular weight cutoff membrane; freeze dry; acid-enzyme hybrid (0.5 kmol m ^{−3} acetic acid solution containing 1.5% pepsin with 1:20 <i>w/v</i> ratio for 3 days); centrifugation and precipitation; dialysis (2×); freeze dry	[82] Based on [101] and patent CN1569260
Fat and flesh removal (mechanical removal); wash (phosphate-buffered saline)	Decellularized collagen: Supercritical CO ₂ vessel system (75% ethanol, 30–50 °C and 200–350 bar for 40 min); neutralization (0.1–1 kmol m ^{−3} NaOH); drying and sterilization (γ-irradiation 25 kGy). Atelocollagen: Milling (freeze milled with liquid nitrogen, 50–200 µm); acid and enzyme digestion (0.01 kmol m ^{−3} HCl containing 1 g L ^{−1} pepsin, stirred for 16–18 h at 25 °C); filtration (1) 0.1–0.4 µm, (2) >150 kDa, (3) 0.2 µm; fibrillogenesis (3 mg mL ^{−1} acidic atelocollagen solution with 0.2 kmol m ^{−3} phosphate buffer at a ratio of 9:1 <i>v/v</i>); centrifugation (7000 × <i>g</i> , 30 min at 4 °C); freeze dry (precipitate)	[102]
Hot water bath (1:9 <i>w/v</i> at 60 °C, 30 min)	Acid/alkali-enzyme hydrolysis ((1) 762 U g ^{−1} pepsin, (2) trypsin and (3) Alcalase with 1 kmol m ^{−3} HCl or NaOH at their respective optimal temperature and pH, 4 h); inactivate enzyme, neutralization, filtration, dialysis and freeze dry	[103]
Alkali treatment (0.1 kmol m ^{−3} NaOH at 1:5 volume ratio, 3 days)	Acid hydrolysis and filtration (0.5 kmol m ^{−3} acetic acid for 3 days, 2×. Filtrate was collected separately); freeze and precipitated (frozen 24 h; added in 0.9 kmol m ^{−3} NaCl for 12 h). Centrifugation; dissolution and dialysis (acetic acid; dialysis at 1:10 for 2 days); freeze dry	[58]
Hot water bath (90 °C, 1 min)	Control mixture (5:100 volume ratio skin to solution (water)); enzyme hydrolysis (1) Alcalase, (2) Flavorzyme, (3) Neutrase, (4) bromeline, (5) Protamex, (6) papain-at 1:100 enzyme substrate ratio with hydrolysis times 1–24 h at optimal temperature; inactivation and cooling; centrifugation (4000 × <i>g</i> , 15 min); filtration (3 kDa, 60 psi nitrogen gas at 20 °C); freeze dry	[67]
Cattle		
No pretreatment detailed	Alkali hydrolysis (3–5% NaOH, stirred at 60 °C); filtration (filtrate collected); neutralization (acetic acid); dialysis	[78]

Table 3. Cont.

Pretreatment	Extraction and Isolation	Reference
Neutral salt wash (10 volumes (<i>v/w</i>) 0.5 kmol m ^{−3} NaCl for 2 h); lime treatment (dehair) (40 g L ^{−1} lime suspension at 1:10 <i>w/v</i> for 4 days); non-collagen removal (2% NaOH at 1:10 <i>w/v</i> for 5 days)	Conducted at 4 °C, stirred. Acid-enzyme hybrid (0.5 kmol m ^{−3} acetic acid at 1:100 <i>w/v</i> with pepsin addition at 1:20 <i>w/v</i> , 2 days); centrifugation (20,000 × <i>g</i> , 20 min), precipitation (2 kmol m ^{−3} NaCl, 6 h); centrifugation (15 min); dialysis (14 kDa MW, deionized water for 4 days); freeze dry	[83]
Hair removal (1) sulfide solution [104]; (2) oxidative process [105]; relime [104]; acid pretreatment (0.5% acetic acid first at pH 7.1 for 3 h and then at pH 5.4 for 4 h). Neutral salt wash (400% water containing 4.35% NaCl for 2 h, twice); lime treatment (400% water containing 0.3% lime for 2 h, thrice); acetone treatment (400% acetone, 12 h repeated 5–7 times); air dry and frozen; (pulverized)	Conducted at 4 °C. Acid hydrolysis (200–250 mg hide powder in 100 mL 0.5 kmol m ^{−3} acetic acid, 3 days); centrifugation and freeze dry	[106]
No pretreatment detailed	Skin-buffer mixture (100 g L ^{−1} skin to solution, 0.1 kmol m ^{−3} phosphate buffer and 0.15 kmol m ^{−3} NaCl with pH 7.5); alkali addition (0.001 kmol m ^{−3} NaOH and 3.5g NaBH ₄ , incubated for 24 h at 25 °C); acid addition (glacial acetic acid, adjusting pH to 3); centrifugation (23,000 × <i>g</i> for 30 min, pellet collected); freeze dry	[95]
Hair removal (0.5 kmol m ^{−3} NaOH, 24 h); non-collagen removal (3 different samples prepared: (1) 1:10 <i>w/v</i> 0.1 kmol m ^{−3} NaOH, 6 h; (2) 20 vol 0.1 kmol m ^{−3} NaOH and 0.1 kmol m ^{−3} NaCl, 24 h; (3) 1:20 <i>w/v</i> 0.1 kmol m ^{−3} NaOH and 0.1 kmol m ^{−3} NaCl, 6h); fat removal/demineralization ((1) 1:10 <i>w/v</i> 10% butyl alcohol, 10 h; (2) 0.1 kmol m ^{−3} HCl and 0.1 kmol m ^{−3} NaCl 1:20 <i>w/v</i> , 24 h; (MAES) 0.1 kmol m ^{−3} HCl and 0.1 kmol m ^{−3} NaCl 1:20 <i>w/v</i> , 24 h)	Acid/acid-enzyme hydrolysis: (1) 30 vol. 0.5 kmol m ^{−3} acetic acid, 24 h, (2) 20 vol 0.5 kmol m ^{−3} acetic acid with 1% <i>w/w</i> pepsin, 24 h, (3) 20 vol 0.7 M acetic acid with 1% <i>w/w</i> pepsin, 48 h; filtration (4 mm and 250 µm filter); precipitation: (1) 2.5 kmol m ^{−3} NaCl, (2) 2.5 kmol m ^{−3} NaCl, (3) 2.5 kmol m ^{−3} NaCl; centrifugation (various times); dialysis (14 kDa MW, acetic acid and water); freeze dry (−52 °C, 48–72 h)	[59]
Lime treatment (100 g hide in 1.5 g Na ₂ S and 5 g CaO solution, soaked for 2 days); de-lime treatment (2 g NH ₄ Cl and 4 mL concentrated HCl); wash (rinsed with water until neutral pH); fat removal (10 volumes 10% butanol for 24 h)	Acid hydrolysis (30 volumes 0.5 kmol m ^{−3} acetic acid solution containing 1% pepsin, 2 days stirred periodically); filtration and centrifugation (2-layer cheesecloth; 10,000 <i>g</i> for 20 min). Precipitation (3 kmol m ^{−3} NaCl); centrifugation and dissolution (acetic acid); dialysis (tris buffer)	[60]
Hot-water bath (70 °C for 15 min, dehair)	Solutions (500 g hide each sample, calcium hydroxide and acetic acid solutions were added to each beaker). Samples and concentrations (1) control, not pretreated; (2) 5% <i>w/v</i> Ca(OH) ₂ ; (3) 15% <i>w/v</i> Ca(OH) ₂ ; (4) 5% <i>w/v</i> Ca(OH) ₂ and 5% <i>v/v</i> CH ₃ COOH; (5) 15% <i>w/v</i> Ca(OH) ₂ and 5% <i>v/v</i> CH ₃ COOH. Hydrolyzed for 4 days; hot water bath (60–70 °C for 24 h); filtration and dehydration (filtrate collected, 60 °C oven for 24 h)	[107]
De-lime treatment (2% NH ₄ Cl and 0.5% HCl, 60 min); neutralization (0.5% HCl until pH 6–7; rinsed with distilled water)	Acid-enzyme hybrid (30 volumes 0.5 kmol m ^{−3} acetic acid containing 1% pepsin, 48 h, 4 °C); centrifugation (10,000 × <i>g</i> at 4 °C, 15 min); precipitation (3 kmol m ^{−3} NaCl); centrifugation; dissolution and precipitation (0.5 kmol m ^{−3} acetic acid and 0.7 kmol m ^{−3} NaCl); dissolution and dialysis (0.5 kmol m ^{−3} and 0.1 kmol m ^{−3} acetic acid, respectively)	[84]

The pretreatments listed in Table 3 usually have the purpose of removing some of the non-collagenous material but also serve the important function of “opening up” the skin to enable easier penetration of the reagents that are used to solubilize collagen. In some

cases, the pretreatment might include similar chemistry to the subsequent treatment, but generally with lower concentrations of reagents. The main options described in Table 3 may be summarized pictorially, as in Figure 4.

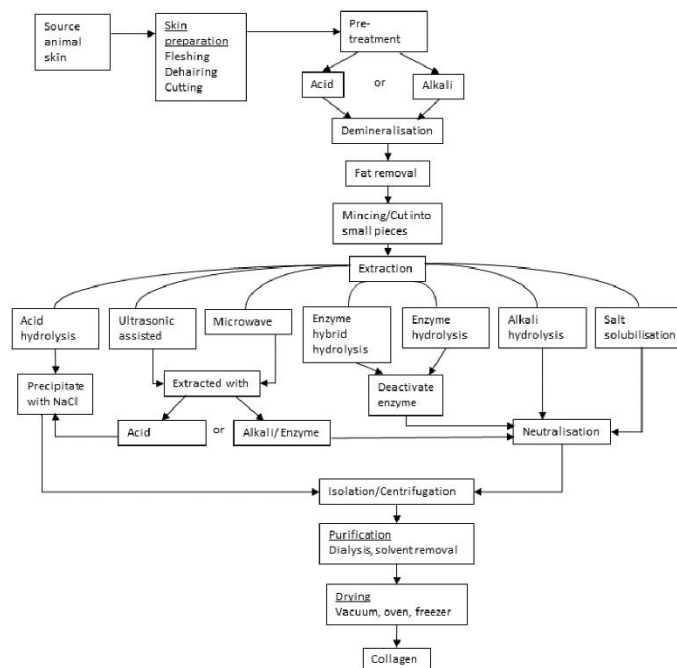


Figure 4. Overview of processing options for extraction of collagen from skin (based on Table 3).

Future Considerations

While a range of methods for extracting collagen from animal skins has been described, these do not cover the skin from all commercially reared meat animals. Skin from some animal species have not been investigated in detail, notably sheep and goats, which represents an opportunity for further study and exploitation. The nature of the collagen resulting from the extraction processes has often not been well characterised. For example, do the collagen fibrils remain intact, or do they reform spontaneously, or are they degraded partially? Also what is the impact of these factors on the mechanical properties of materials manufactured using the extracted collagen? This is an area where further knowledge would be useful.

5. Conclusions

A wide variety of collagen extraction and separation methods have been described for animal skins from a number of meat-producing species. These have been categorized and summarized here showing the options available to produce a collagen extract. Multiple choices are clearly available for the commercial production of collagen from various animal skins.

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D2.Publication 2 in Polymers: Gelatin and Collagen from Sheepskins



Article

Gelatin and Collagen from Sheepskin

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Simple Summary: Simple Summary: With the low demand for leather from sheepskin, New Zealand slaughterhouses dispose of most byproduct sheepskin from meat production as solid waste. This study aimed to develop a use for these sheepskins as a source of gelatin or collagen. Three extraction methods were evaluated. The extracted material was characterized for gelatin and collagen composition and structure. Sheepskin was found to be a useful alternative source of gelatin–collagen material.

Abstract: Abattoirs dispose of sheepskins as solid waste due to low price and poor demand for sheepskin leather. In principle, as an alternative to being disposed of in landfill, sheepskins can serve as a source of the protein collagen or the hydrolysis product, gelatin. In this research, sheepskins collected from abattoirs were used as a source of collagen. Three extraction methods were compared: acid extraction, acid with enzymes, and alkali extraction. The extracted material was characterized using scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR), small angle X-ray scattering (SAXS), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The collagen and gelatin extraction yield ranged from 3.1% to 4.8% with the product purity determined by hydroxyproline, ranging from 7.8% for the alkali process to 59% and 68% for the acid and acid-enzyme processes. SDS PAGE showed that the acid process produced fragments with molecular weights in the range 100 to >250 kDa, while acid-enzyme resulted in smaller fragments, below 30 kDa. The FTIR region of the amide I band at 1800–1550 cm^{−1}, which was used as an indicator of the collagen and gelatin content, showed that the gelatin dominated in the acid extracts, and the alkaline extract contained a large portion of keratin. SAXS was found to be a sensitive method for showing the presence of intact collagen fibrils in materials from all of the extraction methods, albeit at low concentrations. Herein, sheepskin is shown to be a useful source for collagen–gelatin material of varying molecular weights.

Keywords: gelatin; collagen; extraction; sheepskin



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1. Introduction

Collagen is the most abundant structural protein in animals [1]. Collagen can be extracted from animal tissue such as skins from animals slaughtered for meat and further processed for diverse applications. Gelatin is a hydrolysis product of collagen, and maybe readily obtained from skins, sometimes in combination with collagen, wherein the two may grade into each other with a range of molecular weights.

Collagen and gelatin are used in cosmetics [2], medical and pharmaceutical products [3], and food formulation and packaging [4]. Methods used to extract collagen from tissue [5] include hydrolysis with acids [6] and alkalis [7], solubilization with salts [8], and enzymatic hydrolysis [9]. Often, these chemical treatments are assisted with ultrasound [10] and microwaves [11].

This work compares gelatin and collagen extraction from sheepskins using acid hydrolysis, acid-enzyme hydrolysis, and alkali hydrolysis. The aim was to identify a suitable process for collagen recovery from sheepskins, in the form of gelatin or collagen, for possible commercial systems.

2. Materials and Methods

Whole sheepskins from unknown sheep breeds were collected from a local abattoir (ANZCO Foods, Bulls, New Zealand). The sheepskins were stored at $-20\text{ }^{\circ}\text{C}$ until they were processed for extraction. Other materials and chemicals used were acetic acid (99.7%, Thermo Fischer Scientific, Waltham, MA, USA), sodium chloride (99%, Dominion Salt, Lake Grassmere, New Zealand), EDTA-4Na (Hamilton Chemicals, Hamilton, New Zealand), butanol (99.5%, RP Normapur, Merck, Darmstadt, Germany), sodium hydroxide (Scharlau, Sentmenat, Spain), sodium acetate (Scharlau), pepsin (Merck), dialysis bags (8–14 kDa molecular weight cutoff), molecular weight markers (Bio Rad Precision Plus Protein Kaleidoscope Prestained Protein Standards and Dual Xtra Standards), Protein Gel Criterion Tris-HCl Precast Gels (Bio Rad, Hercules, CA, USA), Laemmli sample buffer (Bio Rad), tris/glycine/SDS buffer (Bio Rad), 2-mercaptoethanol (Bio Rad), sodium acetate trihydrate (Merck), citric acid (Merck), chloramine-T (Sigma Aldrich), n-propanol (Unilab, Mandaluyong, Philippines), p-dimethylaminobenzaldehyde (Merck), 70% perchloric acid (AnalaR), and L-hydroxyproline (Merck).

2.1. Pretreatment

The sheepskins were thawed overnight in a fridge ($4\text{ }^{\circ}\text{C}$). The skins were checked for adhering flesh, fat, and wool and, if present, these were trimmed. The skins were washed and cut into $120 \pm 5\text{ g}$ (fresh weight) samples. The pretreatment was conducted at $4\text{ }^{\circ}\text{C}$ with continuous stirring. The various pretreatment steps are shown in Figure 1. All skin samples underwent the same pretreatment with a skin to solution ratio of 1.2:10 kg skin:L solution.

Non-collagenous protein was removed by soaking the skins in 0.1 M NaOH for 6 h; the NaOH solution was changed every 3 h. The skins were then rinsed until the pH of the wash was neutral. The swollen skins were then manually dehaired with tweezers and washed with water. The washed skin was cut into 1 cm^2 pieces and demineralized by soaking in EDTA-4Na solution (pH 7.4) for 48 h, changing the solution every 16 h. The demineralized skins were washed and defatted by soaking in 10% *v/v* butanol for 16 h. The pretreated skin pieces were processed using one of the three extraction methods identified below.

2.2. Acid Extraction

The pretreated skin samples underwent acid hydrolysis to extract the acid-soluble collagen. The skins were immersed in 0.5 M acetic acid (a skin-to-acid ratio of 1.2:10 *w/v*) at $30\text{ }^{\circ}\text{C}$ for 4–7 days in sealed Erlenmeyer flasks held on a shaker platform (150 rpm agitation speed).

2.3. Acid-Enzyme Extraction

For the acid-enzyme extraction, the skin pieces (Section 2.1) were suspended in 0.5 M acetic acid and 1 g L^{-1} pepsin, with a skin-to-solution ratio of 1.2:10 *w/v*. This suspension was agitated at 150 rpm at $30\text{ }^{\circ}\text{C}$ for 4–7 days until the skins dissolved then heated at $90\text{ }^{\circ}\text{C}$ for 10 min to deactivate the enzyme.

2.4. Alkali Extraction

Alkali soluble gelatin and collagen was extracted by suspending the skins in 0.5 M NaOH with a skin-to-alkali ratio of 1.2:10 *w/v*. The suspension was maintained at $60\text{ }^{\circ}\text{C}$ (hotplate), with continuous stirring (150 rpm) for 10–12 h until the skins dissolved.

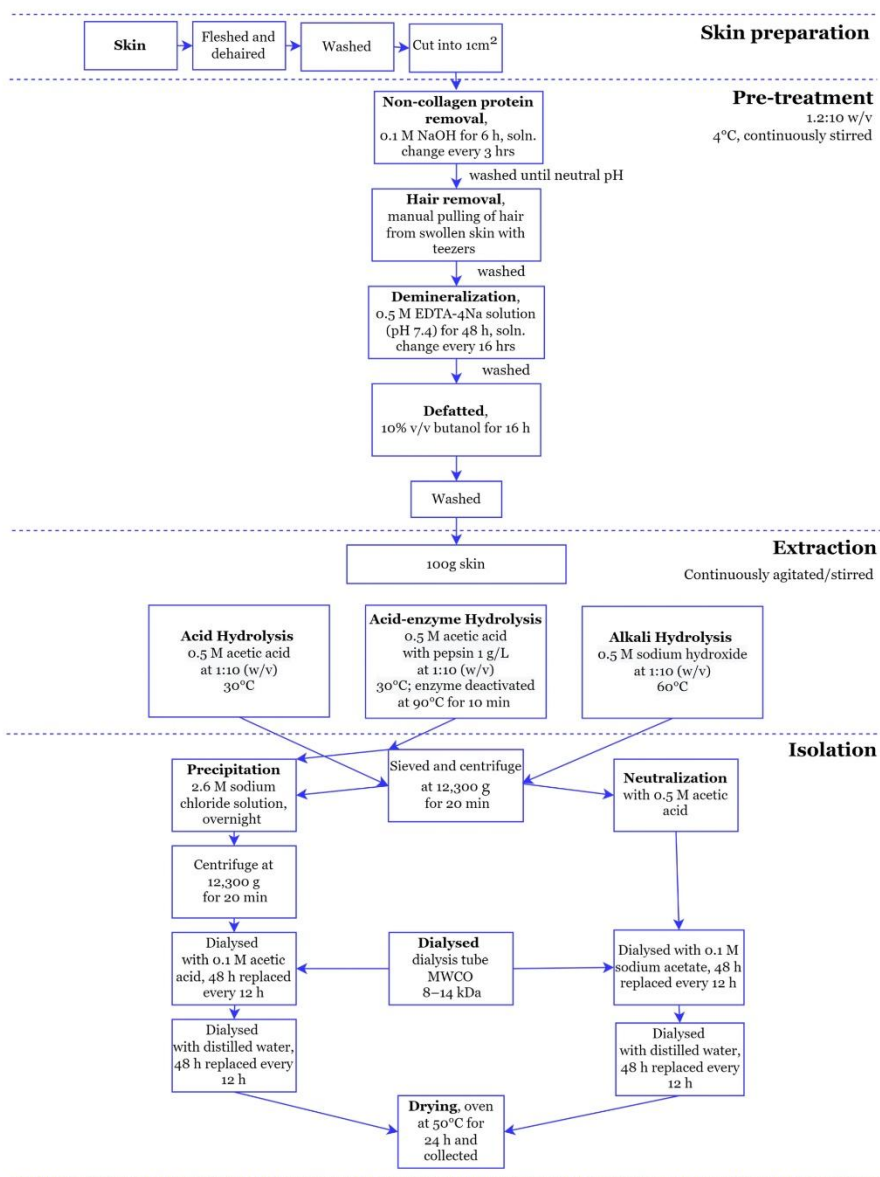


Figure 1. Flowchart of the three extraction methods.

2.5. Isolation of Collagen and Gelatin

The collagen and gelatin were isolated from the extracts of the acid (Figure 1) first by centrifugation ($12,300 \times g$, 20 min), with the supernatant subjected to salt precipitation by adding sodium chloride to attain a final concentration of 2.6 M, and allowed to stand overnight at room temperature ($\sim 23^\circ\text{C}$). The precipitate formed was recovered by centrifugation ($12,300 \times g$, 20 min). The recovered precipitate was dissolved in 0.5 M acetic acid at a ratio of 1:9 v/v , transferred to a dialysis tube, and dialyzed against 0.1 M acetic acid for 48 h at room temperature. The 0.1 M acetic acid dialysate was replaced with distilled water and dialyzed for 48 h. Both dialysate solutions were replaced every 12 h.

For the alkali extraction, the sample was sieved and centrifuged ($12,300 \times g$, 20 min) (Figure 1). The supernatant was collected and neutralized using 0.5 M acetic acid. The neutralized sample is dialyzed with 0.1 M sodium acetate (48 h), and distilled water (48 h), with the dialysate solution replaced every 12 h. Sodium acetate rather than acetic acid was used in order to have a similar pH on either side of the dialysis membrane.

The concentrated solution from inside the dialysis bags was placed in trays and dried at 50°C for 24 h.

2.6. Extractable Yield

The extractable yield was calculated as the mass of the dry solids ($M_{\text{recovered solids}}$) recovered from the extracts divided by the mass of the dry skin (M_{skin}) used in extraction [12], with weighing taking place immediately after drying.

2.7. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of the dried and ground collagen samples were recorded (Thermo Scientific Nicolet iS5 with iD7 ATR spectrometer; Thermo Fisher Scientific, Waltham, MA, USA) over the wavenumber range of 400 to 4000 cm^{-1} with a resolution of 2 cm^{-1} and data spacing of 0.241 cm^{-1} .

2.8. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE followed Laemmli's methodology [13] to determine the molecular weights of the collagen and gelatin extracted by the different methods. The collagen or gelatin was dissolved in the buffer to obtain a concentration of 1 mg mL^{-1} . Afterwards, $10\text{ }\mu\text{L}$ of the denatured sample and $5\text{ }\mu\text{L}$ of a protein standard (molecular weight markers) were loaded into wells at the top of a precast gel (4–20% Criterion Tris-HCl Protein Gel, 18 well, $30\text{ }\mu\text{L}$). Coomassie Blue R250 stain was used.

2.9. Hydroxyproline Quantification

Due to the consistent hydroxyproline–collagen ratios in sheepskin, hydroxyproline quantification can be used to provide an assessment of the total collagen and gelatin content [14]. The hydroxyproline in collagen samples was quantified using an adaptation of a published method [15]. Briefly, 0.1 g of a sample (either the extracted collagen or raw skin) in a 50 mL capped vial was digested with 10 mL of 6 M HCl for 24 h in an oven at 110°C . After hydrolysis, the samples were cooled to room temperature, and 6 M NaOH was added until the pH was 6.0. The resulting solution was filtered, and the volume was adjusted to 50 mL with deionized water. A $50\text{ }\mu\text{L}$ portion of this solution was mixed with $450\text{ }\mu\text{L}$ of chloramine-T (0.056 M chloramine-T in acetate-citrate buffer, pH 6.5) and allowed to stand for 25 min at room temperature in a capped vial. A $500\text{ }\mu\text{L}$ portion of Ehrlich's reagent was added to the vial and it was held in a 65°C water bath for 20 min to allow the color to develop. The absorbance value of the sample was measured against a blank (distilled water) in a UV spectrophotometer at 550 nm . The readings were converted to concentration of hydroxyproline using a calibration plot that had been prepared using standard solutions of hydroxyproline.

2.10. Small-Angle X-ray Scattering (SAXS)

SAXS diffraction patterns were recorded on the Australian Synchrotron SAXS/WAXS beamline using a high intensity undulator source. The energy resolution was 10^{-4} using a cryo-cooled Si(111) double crystal monochromator. The beam size (FWHM focused on the sample) was $250 \times 80 \mu\text{m}$ with a total flux of approximately 2×10^{12} photons s^{-1} . A Pilatus 1 M detector with an active area of $170 \times 170 \text{ mm}$, a sample detector distance of 3371 mm, and an X-ray of 8 or 11 keV, was used to record all the diffraction patterns. The exposure time for diffraction patterns was in the range of 1–5 s. Data were processed using Scatterbrain 2.82 software [16].

3. Results and Discussion

3.1. Extracted Collagen

The samples extracted using the different methods differed in appearance and texture (Figure 2). The acid-extracted samples had a lighter off-white appearance (Figure 2a,b) compared to the alkali-extracted sample (Figure 2c). The acid processed samples comprised larger aggregates with smoother surfaces and sharp edges, typical of gelatin (Figure 2, SEM images), whereas the alkali processed sample had solid particles with rougher surfaces.

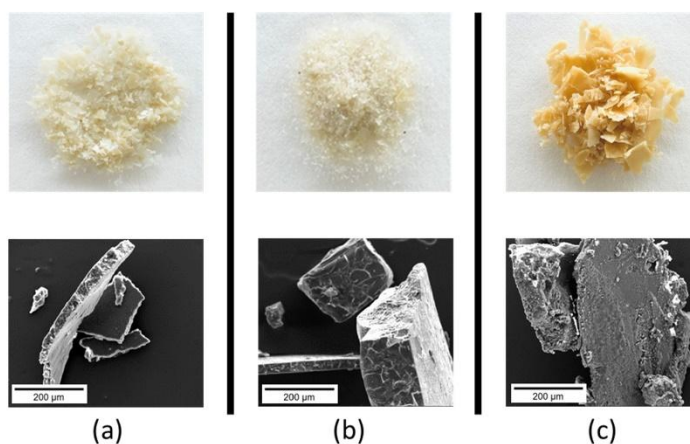


Figure 2. Dried gelatin–collagen (top, optical images; bottom, SEM images) from the different extraction methods: (a) acid hydrolysis, (b) acid-enzyme, (c) alkali hydrolysis.

3.2. Extractable Yield

The extraction yield of gelatin–collagen using the different methods are as follows (average values $\pm$ standard deviation (% w/w); three replicates): 3.1 ± 1.6 for acid extraction; 4.8 ± 1.9 for acid-enzyme extraction; and 4.1 ± 7.7 for alkali extraction (Table 1). Based on the Student's *t*-test, the yields from the acid and acid-enzyme extractions were not significantly different ($p > 0.05$). Including enzymes had no positive benefit on the extraction yield, which is in contrast to other studies that have shown improved extraction of collagen from cattle skin and fish skin in the presence of enzymes [12,17].

Table 1. Hydroxyproline and gelatin–collagen content of the material recovered using the different extraction methods (acid, acid-enzyme, alkali) and in raw sheepskin.

Sample	Hydroxyproline Content (mg g ^{−1} Dry Product)	Gelatin–Collagen in Dry Product (%)	Gelatin–Collagen Extracted (mg g ^{−1} Dry Raw Skin)	Extracted Gelatin–Collagen Relative to the Total Amount of Collagen in the Raw Skin (%)
Acid	74.2 ± 8.1	59.3 ± 6.5	10.7	3.1
Acid-enzyme	85.1 ± 25.3	68.1 ± 20.2	16.8	4.8
Alkali	9.7 ± 2.1	7.8 ± 1.7	14.3	4.1
Raw sheepskin	19.4 ± 7.3	15.5 ± 5.8	-	-

3.3. Hydroxyproline Quantification

Collagen molecules have a specific characteristic motif of Gly-X-Y (Gly = glycine, X = proline, Y = 4-hydroxyproline) that stabilizes their triple-helix structure. The hydroxyproline component in the motif is also uniquely abundant in collagen compared to other proteins. The abundance of hydroxyproline in an extract is used when estimating its collagen content. The measured data (Section 2.10) were used in the following equation to calculate the hydroxyproline content:

$$\text{Hydroxyproline } (\mu\text{g g}^{-1} \text{ sample}) = \frac{\text{Hydroxyproline } (\mu\text{g mL}^{-1}) \times \text{Dilution volume (mL)}}{\text{Sample weight (g)}} \quad (1)$$

The collagen content was then estimated using the following equation:

$$\text{Collagen content } (\mu\text{g g}^{-1} \text{ sample}) = \frac{\text{Hydroxyproline } (\mu\text{g g}^{-1} \text{ sample}) \times \text{Conversion factor}}{1000} \quad (2)$$

For sheepskin collagen extracts and the raw sheepskin samples, it is known that ca. 12.5% of the collagen mass is hydroxyproline [18]. This is slightly less than in the hides of other animals such as cattle, deer, and goat. Using the percentage of hydroxyproline in sheepskin, the collagen content of the samples was calculated using a conversion factor of 8 (100/12.5) in Equation (2). The hydroxyproline and collagen content of the samples are shown in Table 1. The material extracted by acid extracted material contained the most gelatin–collagen. The acid extract had 7.6-fold more gelatin–collagen than the gelatin–collagen content of the alkali-processed sample (Table 1). Although the alkaline solution had a high extraction of material, it is also known that the alkali treatment will readily hydrolyze collagen into smaller peptides [19]. The relatively high extraction temperature (60 °C) for the alkali method may also have been a factor in hydrolyzing the collagen into small molecular weight components. These small molecular weight components would have been mostly removed during dialysis using the membrane with a molecular weight cutoff of 8–14 kDa; and therefore, are not included in the apparent yield. Nevertheless, such small molecular weight components are not useful for most gelatin applications; and therefore, it is useful not to include this component in the apparent yield calculation.

The hydroxyproline contents in the materials produced by acid and acid-enzyme extraction here are similar to or greater than those which have been reported for cattle skin [12,20] and fish skin [21] subjected to similar treatments.

The extraction yield from sheepskin is marginally lower than that obtained from cattle hide [12]. Notably, sheepskin has a higher fat content (30–50% in the dermis alone) compared to bovine hide (2–3% fat) and goatskin (3–10% fat) [22]. The extra fat, which was not effectively removed in the defatting stage, may have contributed to the lower extraction yield of collagen by providing the collagen with some protection (by hydrophobicity) from the digestion chemicals.

The extraction yield of gelatin–collagen from skin could perhaps be improved by modifying the pretreatment conditions to better “open up” the skin matrix, enabling better penetration of the hydrolysis chemicals. In the leather industry, an initial stage of liming the

skins and hides is performed to remove the hair, solubilize the fat, and swell the skin, which opens up the fibers for better chemical penetration at subsequent processing stages [23].

3.4. FTIR

The FTIR spectra of the material extracted using the different extraction protocols had some major differences (Figure 3). A summary of the positions of the main absorption bands and their assignment is provided in Table 2. Absorption bands that can be assigned to collagen or gelatin and bands due to keratin are observed in all samples, but the proportions of each differs between the samples. The material from the alkali extraction contained more intense peaks, which is due to the keratin.

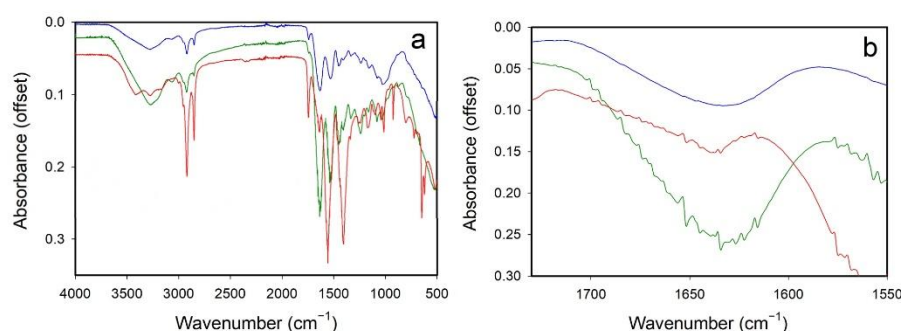


Figure 3. FTIR spectra of collagen recovered by acid (blue line), acid-enzyme (green line), and alkali (red line) extraction: (a) full spectrum; (b) expanded region that assists in distinguishing between gelatin and collagen [24]. See Table 2 for details of peak assignments.

Table 2. FTIR absorption bands of amide vibration modes from the literature [25] and measured in this work, and the keratin bands identified in these samples which were assigned using the literature [26].

Band	Literature Band (Wavenumber cm^{-1})	Absorption Band Positions from This Work for Different Extraction Methods (Wavenumber, cm^{-1})			Functional Group and Vibration Modes
		Acid	Acid Enzyme	Alkali	
Collagen and gelatin					
Amide A	3300–3500	–	–	–	NH stretching
Amide B	~3100	3065	3065	3163	NH stretching
Amide I	1600–1800	1630	1635	1638	C=O stretching
Amide II	1470–1570	1530	1528		CN stretching
Amide III	1250–1350	1334	1334	1339	CN stretching, NH bending
Keratin specific (keratin also displays amide bands above)					
	2931	2921	2923	2919	(CH ₃) symmetric stretch
	2875	2851	2852	2850	(CH ₂) symmetric stretch
		1743	1743	1743	unassigned
	1551			1559 (overlaps with collagen band)	(NH) bend; (CN) stretch
	1420	1409	1407	1408	(CH ₃) deformation
Water		3275	3275	3275	OH stretching (water)

Whether the material contains collagen or gelatin can be observed from the amide I bands, which are from C=O stretching in hydrogen-bonded carbonyls of proteins. These bands are sensitive to changes in the secondary structure of proteins. Amide II bands from C–N stretching are, to a lesser extent, also affected by changes in the secondary

structure of proteins [27]. Amide III bands result from C–N and N–H planar deformation vibrations and other effects associated with carbon-nitrogen stretching and the bending of the nitrogen-hydrogen. These bands are sensitive to changes in the triple helix structure and the polarity of the protein.

To distinguish between collagen and the hydrolysis product gelatin it has been shown elsewhere that the amide I absorption bands between 1700 and 1600 cm^{-1} can be used as described in the literature [24]. Collagen has been shown to have a peak at 1659–1651 cm^{-1} , whereas in gelatin the peak is at 1643–1633 cm^{-1} , depending on the temperature during the measurement (lower wavenumber at higher temperature). This region of the FTIR recorded here is displayed in Figure 3b. In these samples there is just one broad peak centered at 1630 cm^{-1} for the acid and acid-enzyme extracted material and a peak at 1638 cm^{-1} for the alkali extracted. These may indicate that gelatin dominates over collagen in all of these extracted materials and, due of the shift in band position, could suggest that the alkali extracted material is hydrolyzed further than the acid extracted material, although the poor resolution of this region limits the conclusions that can be drawn.

The FTIR makes it clear that these materials are not pure collagen or pure hydrolysis products of collagen. There are intense peaks associated with keratin, and these are more prominent in the alkali extracted material. The low hydroxyproline content of the alkali material showing that it is less than 8% gelatin and the intense FTIR peaks for keratin show that the alkaline extracted material is likely to be largely keratin.

3.5. SDS PAGE

The developed SDS PAGE gel shows the molecular weights of the extracted proteins (Figure 4). The globular protein molecular weight standards are known to be unfavorable molecular weight standards for the fibrous collagen components, possible due to different SDS binding, so are presented only as an approximate range standard. Collagen extraction by acid hydrolysis resulted in products with molecular weights in the range of 100 to greater than 250 kDa. When enzyme was used in conjunction with the acid hydrolysis, much smaller molecular weight components resulted, mostly 13–30 kDa. For the alkali extraction process indistinct bands were observed in the SDS PAGE.

The simple acid hydrolysis therefore results in material that contains more of the longer collagen chains and less material that has been broken into smaller fragments of gelatin. The α 1-chains and α 2-chains, specific to collagen-1, produce bands at around 116 kDa [11], as observed here. Furthermore, the higher molecular weight bands observed here are the result of either two crosslinked α -chains (β -band), or three crosslinked α -chains (γ -band).

The acid hydrolysis with enzymes results in the collagen being hydrolyzed into small fragments, in other words, gelatin. The dialysis membrane used for separation after extraction had a cutoff of 8–15 kDa so the range observed here in the SDS PAGE of 13–30 kDa is limited by this separation and it is probable that smaller polypeptide fragments were also produced by the acid-enzyme hydrolysis. Although pepsin is considered to digest only the non-helical parts of the procollagen [28], acid hydrolysis (as used here) is nonspecific.

The samples extracted with alkali, did not have distinct bands in the SDS PAGE. However, from the hydroxyproline analysis and the FTIR, it was clear that this material was largely keratin and contained only a small proportion (7.8%) of collagen or collagen hydrolysis products. In other studies, alkali-based treatments have been found effective for extracting collagen under milder conditions than in the present work [29].

Current market conditions suggest that “collagen peptides” or “collagen hydrolysate” may represent an increasing market sector wherein the smaller components have distinct value. If these components could be efficiently recovered by methods such as those studied here, high-quality peptides may be produced in greater quantities and higher value than low bloom strength gelatin.

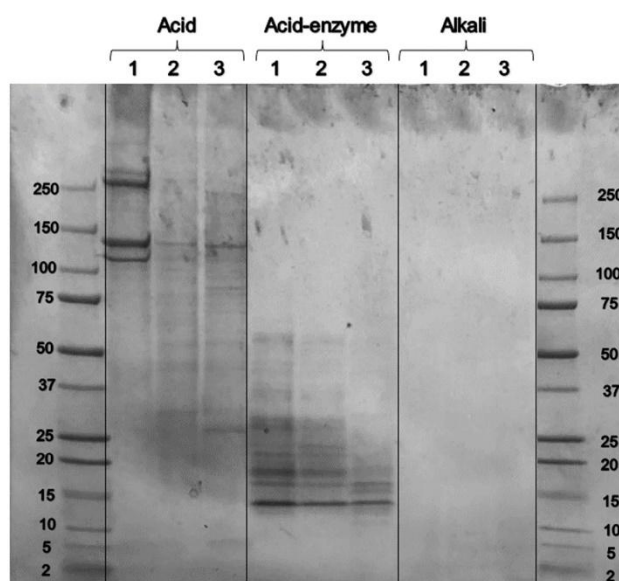


Figure 4. SDS PAGE of collagen from acid, acid-enzyme, and the alkali extraction processes. The lanes on extreme left and right show the globular protein molecular weight markers (2–250 kDa). There are three replicate lanes for each treatment. The figure has been modified to remove some lanes on the right-hand side not discussed in this work (full original SDS PAGE available).

3.6. SAXS

The SAXS diffraction patterns of the gelatin–collagen material recovered by the three extraction methods are shown in Figure 5. The diffraction peaks (Bragg peaks) are due to banding in the fibrillar structure of collagen [30]. The presence of this diffraction feature is definitive evidence for the presence of collagen fibrils in the samples. The distance between the bands (i.e., the *D*-spacing) in the collagen molecules is calculated from the diffraction data. Diffraction also occurs from the triple-helix, at around 0.287 nm [31]. Triple-helix structures exist in gelatin or collagen-like peptide fragments [32]. We did not measure diffraction at this scale, only the diffraction angle at which we can see fibrillar banding at around 63 nm.

The presence of these *D*-spacing diffraction peaks in the acid-enzyme and the alkaline samples is a little surprising considering the SDS PAGE results showing little material of higher molecular weight in these samples (although there is high molecular weight material in the acid extracted material). This perhaps is an indication of the sensitivity and specificity of SAXS for detecting fibrillar collagen.

The *D*-spacing is barely affected by the method of collagen extraction (Table 3) and is similar to that present in native tissues of various types, for example [33].

Table 3. The *D*-spacing of collagen extracted by acid or alkali.

Extraction Method	<i>D</i> -Spacing (nm)
Acid	63.0
Acid-enzyme	63.0
Alkali	62.9

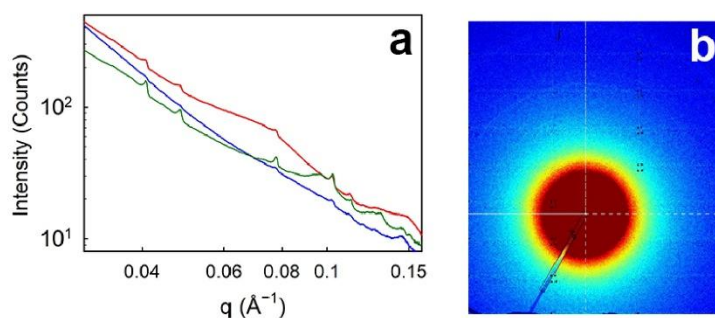


Figure 5. (a) SAXS integrated scattering intensity for the extract obtained by acid (blue line), acid-enzyme (green line), and alkali (red line) extraction, showing sharp peaks for diffraction from the collagen fibril D-banding; (b) an example of the SAXS pattern (acid extracted material) with the faint rings visible due to the collagen diffraction (the color scale represents diffraction intensity).

3.7. Commercialization

The adaptation of this process for commercialization would require some changes to what is described here in this laboratory study. For example, demineralization with EDTA is not required and the initial processing of the skins could be a conventional beamhouse operation in which the skins are dehaired and defatted to produce pickled pelts.

4. Conclusions

This study trialed three different methods for extracting collagen from sheepskin. The key difference with sheepskin from other skins is the high fat content that must be removed at the start of the process. A gelatin–collagen mix was successfully extracted using both an acid process and an acid-enzyme process. All three approaches give distinct and different products with the potential to adapt and give three different commercially relevant materials. Longer chain polypeptides were obtained from the acid process, while the acid-enzyme process broke the collagen into small fragments considered to be gelatin. An amount of fibrillar collagen was observed in all extracts. The alkaline extraction was dominated by keratin which may find markets in haircare. Small angle X-ray scattering, was shown to be sensitive in detecting small quantities of intact fibrillar collagen in the extracts. The range of collagen derived molecules with varied molecular weights in these extracts, and the ability to select this range, informs of the uses of these materials in varied applications.

Author Contributions: Conceptualization, R.G.H., K.L.P., M.R.W., Y.C., A.M.E.M.; methodology, A.M.E.M.; formal analysis, A.M.E.M.; investigation, A.M.E.M.; writing—original draft preparation, A.M.E.M.; writing—review and editing, Y.C., K.L.P., M.R.W., R.G.H.; visualization, A.M.E.M.; supervision, R.G.H., Y.C., M.R.W., K.L.P. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article.

Acknowledgments: Part of this research was undertaken on the SAXS/WAXS beamline at the Australian Synchrotron, part of ANSTO.

Conflicts of Interest: The authors declare no conflicts of interest.

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Gelatin and Collagen from Sheepskin

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Introduction

Collagen is the most abundant structural protein in animals [1]. Collagen can be extracted from animal tissue such as skins from animals slaughtered from meat and further processed for diverse applications. Gelatin is a hydrolysis product of collagen and can be classified according to molecular weights. Both are used in cosmetics [2], medical and pharmaceutical products [3], and food formulation and packaging [4]. Methods used to extract collagen from tissue include hydrolysis with acids and alkalis, solubilization with salts, and hydrolysis with enzymes [6]. This research compares gelatin and collagen extraction from sheepskins using acid hydrolysis, acid with enzyme, and alkali hydrolysis. The aim was to identify a suitable process for collagen recovery from sheepskin in the form of gelatin or collagen for possible commercial systems.



Figure 1. Structural difference between collagen and gelatin (reproduced with permission from Kito Gelatin Inc.) [5].

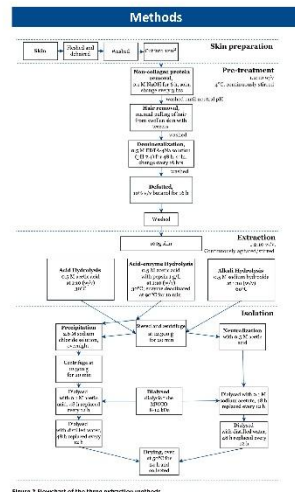


Figure 2. Flowchart of the three extraction methods.

1. Appearance and surface topography

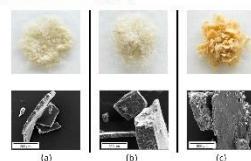


Figure 3. Dried gelatin-collagen (top, optical images; bottom, SEM images) from the different extraction methods (a) acid hydrolysis, (b) acid-enzyme, (c) alkali hydrolysis.

- Difference in color and surface texture were observed from the oven-dried gelatin-collagen extracts from the different extraction methods.

2. Extracted yield and collagen content

Sample	Yield (mg/g dry product)	GC to dry product (%)	GC extracted (mg/g dry product)	GC relative to the total amount of collagen in the raw skin (%)
Acid	71.7 ± 5.1	50.5 ± 6.5	36.1	5.7 ± 1.5
Acid-enzyme	85.1 ± 25.3	60.1 ± 30.2	50.8	4.8 ± 1.3
Alkali	37.4 ± 2.4	7.8 ± 2.7	2.9	1.4 ± 0.7
Raw sheepskin	19.1 ± 8.3	19.5 ± 8.3		

- Based on the Student's *t*-test done on extracted GC material relative to skin weight, the yields from the acid and acid-enzyme extractions were not significantly different ($p > 0.05$).
- Abundance of hydroxyproline in the collagen helix was utilized to estimate the collagen in the samples. The conversion factor was used is 8 from the 12.5% v/v content of sheepskin from literature [7].
- Acid extract had 7.6-fold more GC than alkali-processed sample.
- High extraction temperatures in the alkali extraction have partially degraded the GC material.
- Hydroxyproline content in acid and acid-enzyme extraction similar to cattle and fish skin treatments. Extraction yield, however, is lower due to the higher fat content of sheepskin compared to cattle and fish skins – contains 30-40% fat on-dry-mass alone.

3. Fourier Transform Infrared Spectroscopy

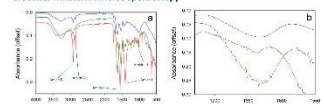


Figure 4. FTIR spectra of collagen extracted by acid (blue line), acid-enzyme (green line) and alkali (red line) extraction. A: full spectrum; B: expanded region that assists in distinguishing between gelatin and collagen [24]. See Table 2 for details of peak assignments.

- Collagen/gelatin and keratin absorption bands observed in all samples, proportions vary.
- Shifts in the Amide I peak shown in Figure 4b, particularly for the alkali extract indicates the presence of gelatin in the sample. Intense keratin peaks were also observed, which are more prominent in alkali-extracted material.
- Low hydroxyproline content in alkali material (< 8%) and rates less than 8% gelatin, intense FTIR peaks for keratin suggest alkali extracted material is largely keratin.

Results and Discussions

(FTIR continued)

Table 2. Amide vibration modes for collagen [8]. Keratin bands identified in these samples FTIR absorption bands assigned in brackets [9] and absorption bands observed in this work.

Band	Band wavenumber (cm ⁻¹)	Acid	Acid-enzyme	Alkali	Functional group and vibration modes
Amide A	3300-3500 (2931)	- (2921)	- (2923)	- (2919)	N-H stretching (CH ₂ symmetric stretch)
Amide B	3100 (2875)	3065 (2851)	3065 (2852)	3103 (2851)	N-H stretching (CH ₂ symmetric stretch)
Amide I	1600-1800	1592 (1743)	1633 (1743)	1638 (1743)	C=O stretching (C=O stretching)
Amide II	1470-1570 (1551)	1530 (14)	1528 (14)	One-up at 1558	CH ₂ stretch (C-H stretch)
Amide III	1260-1360 (1340)	1294 (1305)	1294 (1305)	1392 (1308)	C-N stretching (C-N stretching)

4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

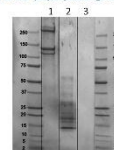


Figure 5. SDS-PAGE of collagen from (1) acid, (2) acid-enzyme and (3) alkali extraction processes. The lanes on the left and right show the gelatin protein molecular weight markers (2-200 kDa). Results were not shown (30-40 kDa) due to analysis tubes used (8-14 kDa).

- Alkali treatment hydrolyzes collagen into smaller peptides due to high extraction temperature effectively contributed to hydrolyzing collagen.
- Acid hydrolysis produces more longer collagen chains, less smaller gelatin fragments.
- Acid hydrolysis yields products with molecular weights ranging from 100 to 250 kDa but also smaller molecular weight components.
- Alkali extraction results in indistinct bands in SDS-PAGE. It is largely keratin with small proportion (7.8%) collagen or hydrolysis products.
- Acid-enzyme hydrolysis results in collagen hydrolyzed into small gelatin fragments.

5. Small-angle X-ray diffraction (SAXS)

Table 3. The D-spacing of collagen extracted by acid or alkali.

Extraction method	D-spacing (nm)
Acid	63.0
Acid-enzyme	63.0
Alkali	62.9

- Diffraction peaks (Bragg peaks) and corresponding rings (Figure 6) reappeared, banding in fibrillar structure of collagen and confirms the presence of collagen fibrils.
- Collagen fibrillar banding observed at around 63 nm. The D-spacing values observed for the different extraction methods are presented in Table 3.
- Presence of D-spacing diffraction peaks in acid-enzyme and alkali samples surprising given SDS-PAGE results showing little material of higher molecular weight.
- SAXS results indicates sensitivity and specificity for detecting fibrillar collagen.

(SAXS continued)

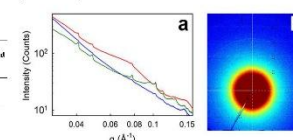


Figure 6. SAXS integrated scattering intensity for extract obtained by acid (blue line), acid-enzyme (green line), and alkali (red line) extraction showing sharp peaks for fibrillar collagen (Bragg banding). A: example of the SAXS pattern (and extracted material) with the last ring visible due to the collagen extraction.

Conclusions and Future Work

- Three methods were tested for extracting collagen from sheepskin.
- Sheepskin has a high fat content that needs to be removed initially.
- GC mix was successfully extracted using acid and acid-enzyme processes.
- Each approach yielded distinct products with potential commercial applications.
- Longer chain polypeptides were obtained from the acid process.
- Acid-enzyme process resulted in collagen broken into small gelatin fragments.
- All extracts contained fibrillar collagen.
- Small angle X-ray scattering detected small quantities of intact fibrillar collagen.
- The collagen extracts had a range of molecules with varied molecular weights, offering versatility for different applications.
- Modify pre-treatment conditions could improve collagen extraction yield, possibly using liming stage in leather industry to open up skin matrix for better chemical penetration for extraction.

References

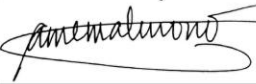
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Appendix E: Statement of Contribution Forms

E1. Publication 1 in Biology: Collagen Extraction from Animal Skin

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We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.			
Student name:	Andrea Marie Evangelista Matinong		
Name and title of main supervisor:	Prof. Richard Haverkamp		
In which chapter is the manuscript/published work?	Chapter 2 Literature review 1: Collagen		
Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work: ¹ The conceptualization of the paper was agreed upon by me (the student) and my supervisors. I wrote the first full draft independently, following the guidance of my supervisors, and co-edited subsequent versions of the article with my supervisory team.			
Please select one of the following three options:			
☒	The manuscript/published work is published or in press Please provide the full reference of the research output: Matinong, A. M. E., Chisti, Y., Pickering, K. L., & Haverkamp, R. G. (2022). Collagen Extraction from Animal Skin. <i>Biology</i> , 11(6), 905. DOI: https://doi.org/10.3390/biology11060905		
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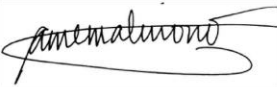
E2. Publication 2 in Polymers: Gelatin and Collagen from Sheepskin



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STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Andrea Marie Evangelista Matinong		
Name and title of main supervisor:	Prof. Richard Haverkamp		
In which chapter is the manuscript/published work?	Chapter 4 Gelatin and collagen from sheepskin		
Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:¹ The conceptualization of the paper was agreed upon by me (the student) and my supervisors. I prepared all the samples, performed all the experiments, processed and analyzed all the data and wrote the first full draft independently, following the guidance of my supervisors. I also co-edited subsequent versions of the article with my supervisory team.			
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