Extraction of Functionally Active Collagen from Fish By-Products.

A thesis submitted for the award of the degree of Master of Biotechnology at Flinders University of South Australia

By

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Declaration:

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

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Abbreviation:

°C	Celsius
ADGs	Australian Dietary Guidelines
Ala	Alanine
Arg	Arginine
ASC	Acid soluble collagen
Asn	Asparagine
Asp	Aspartic acid
BSE	Bovine spongiform encephalopathy
$Ca(OH)_2$	Calcium hydroxide
CCD	Central composite design
Cr	Chromium
Cys	Cysteine
DSC	Differential scanning calorimetry
e.g.	Example
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
Eq.	Equation
FACIT	Fibril-associated collagens with interrupted triple helices
FAO	Food and agriculture organization
SIDS	Several small island developing states
FMD	Foot mouth disease
FRG	Federal Republic of Germany
FTIR	Fourier-transform infrared spectroscopy

g	Force of gravity, RCF
G	Gram
Gln	Glutamine
Glp	Pyroglutamatic
Glu	Glutamic acid
Gly	Glycine
h	Hours
HCl	Hydrochloric acid
His	Histidine
Hyp	Hydroxyproline
Ile	Isoleucine
kDa	Kilodalton
kg	Kilogram
kHz	Kilohertz
Leu	Leucine
Lys	Lysine
M	Molar
Met	Methionine
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PAGE	Polyacrylamide gel electrophoresis
pH	Potential of hydrogen
Phe	Phenylalanine
PPTT	Polyproline II type
Pro	Proline

PSC	Pepsin soluble collagen
RSM	Response surface methodology
SDGs	Sustainable development goals
SDS	Sodium dodecyl sulphate
Ser	Serine
Std.	Standard
t	Tonnes
T_d	Denaturation temperature, °C
Thr	Threonine
Tris-base	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl) amino methane hydrochloride
Trp	Tryptophan
TSE	Transmissible spongiform encephalopathy
Tyr	Tyrosine
USD	United states dollars
Val	Valine
Vit	Vitamin

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ABSTRACT

The skin of salmon (*Salmo salar*), a waste product of the filleting process line, could serve as a good source of aquatic collagen. Until to date, however, the standard extraction and purification of collagen is time-consuming, delivering relatively low yields. Ultrasound can improve the extraction efficiency of many materials. To find a green and advanced method for collagen extraction, this study investigated the suitability of ultrasound for the extraction of acid-soluble collagen from salmon skin and compared yields and purities with the conventional extraction method. Salmon skin was pre-treated for both methods with 0.5 M acetic acid. The collagen subunits extracted by these processes were then analysed by sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) to determine the peptide chains of α1, α2 and β. For extraction of acid-soluble collagen using ultrasound, the following parameters settings were investigated to optimise performance: amplitude (80 and 100%), sample:solvent ratio (1:10-1:30), concentration of acetic acid (0.5 and 1 M) and extraction time (30-120 min). The collagen yield for the conventional method was 34.5%, while ultrasound-assisted extraction increased yields to 46%. SDS-PAGE analysis confirmed that ultrasound-assisted processing did not change the main component of the collagens, α1, α2 and β chains. A soluble collagen assay confirmed that collagen was extracted. The techno economic analysis showed that ultrasound extraction of collagen from salmon skin returned a three-times higher net income ($1,382 million per year) compared to the conventional method ($499,000 per year) at a sales price of 510 USD/kg but was not economically feasible regardless of extraction method used at sales prices of 219 USD per kg or less. Since ultrasound-intensified extraction of collagen has advantages over the conventional method due to reduced extraction time and improved yield, losses generated at a sales price of 219 USD per kg were three-times lower than for the conventional method. Given the large capital investment required to build the plant, it would

take nine years to break even based on the most profitable scenario. It is therefore concluded that the adoption of ultrasound-assisted production of collagen from salmon skin is economically feasible and preferable over the conventional extraction method, if a processing facility that could be retro-fitted with the required equipment, already existed close to the filleting industry. If the processing plant has to be built completely anew, the waiting period for returns on investment would dissuade investment.

CHAPTER 1

1. INTRODUCTION

1.1. Background

Collagen is the most remarkable and abundant protein found in the human body. It accounts for approximately 30-35% of total body protein, with 70-80% being a skin component (Li et al., 2020). As the use and applications of collagens are widespread and growing on a commercial scale, research and development focuses on alternative collagen sources and greener processing strategies. Due to its safety, bioavailability, bioactivity and biocompatibility, collagen is extensively used in the pharmaceutical, cosmetic, and food and beverage industries. Important properties of collagen are: water absorption and holding capacity, low viscosity, moisturising effects, emulsification and gel strength, foam formation, stabilisation, adhesion and cohesion (Petcharat et al., 2020, Zou et al., 2017). There are two major sources for collagen production: farmed mammals and fish, but mammalian collagen is the primary source for industrial production. Although characteristics of mammalian- and fish-derived collagen are slightly different from each other, mammalian-derived collagen carries the risk to transmit various diseases like bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot mouth disease (FMD) (Jongjareonrak et al., 2005), leading to queries with regards to safety in human applications. Religious believes also limits the use of mammalian collagen, e.g., bovine-derived collagen cannot be used by Hindus and Sikhs, while porcine-derived collagen cannot be consumed by Muslims and Jews. Collagen derived from fish is less cross-linked, improving solubility (Nagai et al., 2000) which helps preserve the macromolecule structure during the extraction process (Fernandes et al., 2008). Due to limitations and biosafety concerns, finding an alternative source to mammalian collagen had become a necessity (Ri et al., 2007). The collagen extraction

method currently used in the industrial sector is lengthy and inefficient. Thus, this study aimed to explore ultrasound-intensified processing for the production of collagen from an underutilised marine by-product (salmon skin) to reduce processing time and solvent-requirements.

1.2. Problem statement

Fish waste is an abundant source of collagen (Mahboob, 2015). Globally, each year about 20 million tonnes, i.e. about 25% of fish waste is generated (Kim et al., 2006). Besides being an underutilised resource, the waste causes environmental concerns, as it is discarded in landfills. Thus, turning this waste into a valuable product can be economical and environmentally friendly (Rustad, 2003). As collagen is of commercial interest, research has focussed on optimising its extraction. Despite, industrial extraction and purification is still a time-consuming procedure. Therefore, this research focussed on optimising ultrasound-intensified extraction to reduce extraction time and make the process more economical and environmentally friendly by saving on solvent use.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Collagen

Collagen is the most commonly found protein in the human body, accounting for about 30-35% of total body protein, with skin containing up to 70-80% of the total collagen (Li et al., 2020). As an insoluble fibrous glycosylated structural protein (Goldberga et al., 2018, Shoulders et al., 2009, Tang et al., 2020), it is additionally a component of tendons, cartilage, bones, teeth and other connective body tissues (Deshmukh et al., 2016) (Fig. 2.1). The extracellular matrix (ECM) contains collagen as a significant component (Zou et al., 2017). The location of collagen in the body defines its biological function (Schmidt et al., 2016). Collagen's basic function is to protect and support organs (Chiquet et al., 2014), being responsible for body tissue strength by forming a network in the cellular structures. The size of human collagen can range from 662 to 3152 amino acids (Ganceviciene et al., 2012).

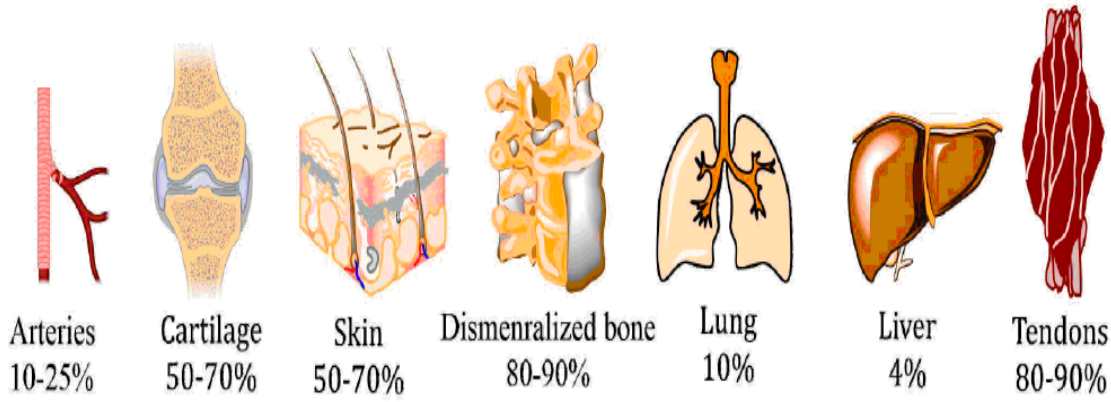

Figure 2.1. Collagen distribution and the amount present in different parts of the body (Jafari et al., 2020).

2.1.1. The collagen family

The collagen family is functionally classified by its complex and diverse structure, location, splice variants and non-helical domains. With the help of various receptor families, collagens can connect with other cells to control proliferation, migration, and differentiation. There are 27 types of collagen with different molecular and structural properties (Ali et al., 2018), which can be categorised as: fibril forming, basement membrane, microfibrillar, anchoring, fibril-associated collagen with an interrupted triple helix (FACIT), transmembrane, and multiplexins (Table 2.1). Of the different types of collagen, the one used commercially is type I collagen (Jiang et al., 2016).

Table 2.1. Classification of collagen types (Gelse et al., 2003).

Family	Type
Fibril-forming	I
	II
	III
	V
	XI
Basement membrane	IV
Microfibrillar	VI
Anchoring	VII
FACIT	IX
	XII
	XIV
	XIX
	XX
	XXI
Transmembrane	XIII
	XVII
Multiplexins	XV
	XVI
	XVIII

Fibril-forming collagens are the most abundant types, representing about 90% of the total collagen. Types I and type V collagen fibrils help with bone structure and types II and type XI collagens form a fibrillary network of the articular ligament. The tensile strength and stability of fibrillar collagen dictates stability of tissues (Gelse et al., 2003). Type IV collagens with a more adaptable triple helix form a confined network in the basement membrane. The microfibrils in collagen type VI are cross-linked by disulphide bonds (Gelse et al., 2003, Von der mark et al., 1984). Fibril-associated collagen with interrupted triple helices (FACIT) like types IX, type XII, and type XIV connect multiple collagen fibrils and probably contribute to determine the diameter of collagen fibrils. Types VIII and type X collagens form hexagonal networks (Gordon et al., 2010). Type XIII and type XVII are transmembrane collagens, while type XV, type XVI and type XVIII are the multiplexins (Avila Rodríguez et al., 2018).

2.1.2. Collagen structure

Structurally, collagen is composed of three polypeptides chains that are glycosylated on lysine and hydroxyl-lysine residue (Tang et al., 2020). The functional significance of this glycosylation is unknown (Jürgensen et al., 2011). Collagens are either homotrimers composed of three identical polypeptide α chains arranged in a right-handed triple helix or heterotrimers, consisting of two or more non-identical polypeptide chains. The most commonly found collagen types I, IV, V, VI, IX and X are heterotrimers, while the remaining are homotrimers (Fallas et al., 2009). Collagens are also structurally classified as three polypeptide strands, type polyproline II (PPTT), left-handed helical, arranged parallel to each other, forming a right-handed helical conformation coil (Shoulders et al., 2009). Two α1 chain and one α2 chain polypeptide, arranges themselves in the middle axis such that the glycine is orientated to the middle of the axis. Other amino acid side chains arrange around the glycine. Each chain

consists of a repeated motif of Gly-X-Y where Gly, X and Y are glycine, proline, and hydroxyproline, respectively (Patino et al., 2002) (Fig. 2.2).

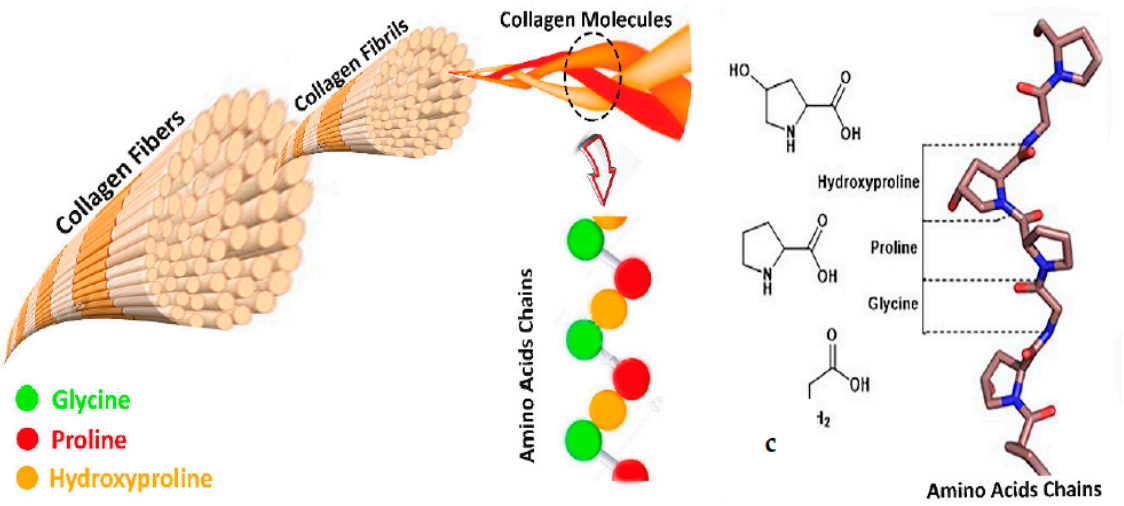

Figure 2.2. Generalised molecular representation of collagen fibres, collagen fibrils and amino acids chains with amino acid residues: hydroxyproline (Hyp), Glycine (Gly), and proline (Jafari et al., 2020).

Some proline and lysine residues are modified by post-translational enzymatic hydroxylation, depending on the form of collagen. 4-hydroxyproline is essential for improving the stability of intramolecular hydrogen bonds and contributes to the triple-helical conformation. Between different types of collagen, the length of the triple helical component varies significantly. In fibril-forming collagens (I, II, III), except for collagen types XXIV and XXVII, the $(Gly\text{-}X\text{-}Y)_n$ repeats itself in the triple helical structure and the types are found in the matrix. Fibril forming collagen is nearly 1,000 amino acids long with a perfect structure of Gly-X-Y. In non-fibrillar collagens, the Gly-X-Y motif has at least one interruption of the motif repeat (Gordon et al., 2010).

The amino end in fibrillar collagen is the N-peptide or N-propeptide. It usually consists of at least one small triple-helical domain, called the minor helix. After the major triple helix is made, the amino and carboxyl terminals are processed, the processed molecules are adjusted in an alignment of a quarter stagger in the developing fibril. Type V and XI collagens nucleate fibrils of types I and type II collagens, respectively (Atiakshin et al., 2020, Kadler et al., 2008). The N-peptides of collagen type V and type XI are retained after being processed, which help to manage fibril diameter. For a normal tissue to function properly, the regulation of its fibril diameter is important. Collagen type XXIV and type XXVII in the fibrillar collagen are uncommon as they consist of major but shorter triple-helical structure and have at least one or two interruptions compared to the other fibrillar collagen members (Bella et al., 2017, Gordon et al., 2010).

2.1.3. Collagen sources

Collagen can be extracted from - bovine, human, rodents, porcine, avian, or marine origins and can also be produced from recombinants (Fig 2.3). Slaughtered animal by-products, like skin, cartilage, bones and tendons, are rich in collagen, the reason why collagen is commercially produced from bovine and porcine (Schmidt et al., 2016). The first kind of raw material used in the early 1930s was porcine skin, and, up to date, porcine is considered a primary source for the large-scale production of collagen (Gómez-Guillén et al., 2002).

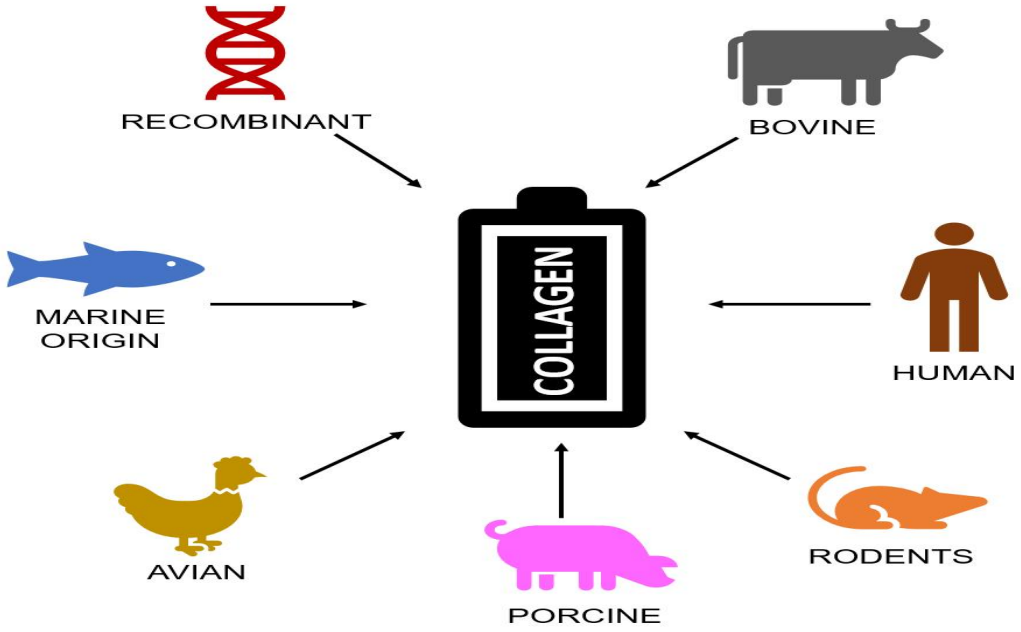

Figure 2.3. Different sources of collagen (Davison-Kotler et al., 2019).

Despite these economic advantages, extracting collagen from animal sources has certain drawbacks. These include the possibility of transferring zoonotic illnesses, like transmissible spongiform encephalopathy (TSE) foot and mouth disease (FMD) and bovine spongiform encephalopathy (BSE). Also, avian flu transmission limits collagen production from poultry source (Raman et al., 2018a, Huang et al., 2016). In addition, strict opinions of the use of cow products, has raised negativity and worries among purchasers who buy and consume mammalian collagen. To find alternative sources for mammalian collagen, plenty of research has been carried out, and fish-based collagen has the most comparable characteristics to mammalian collagen. Consequently, fish collagen produced from fish waste could be an economically viable alternative to mammalian collagen (Chinh et al., 2019, Nagai et al., 2001).

Aquatic collagen has been shown to possess certain advantages over animal-based collagen (Yamamoto et al., 2014). Aquatic collagen source are marine sponges, jellyfish, squid and fish. Skin, bones, blades, heads and scales, the under-used part of the fish filleting industry (Fig.2.4),

constituting about 75% of the total fish weight, which should offer stable and cheaper supplies of collagen resources (Silva et al., 2014).

Figure 2.4. Fish processing waste; fish frame, scales and skin (Jafari et al., 2020)

Aquatic collagen is derived from both invertebrates and vertebrates; they are readily bioavailable and obtainable, have a small particle size, high absorption rate and lower molecular weight compared to mammalian collagen (Fig 2.5). Fish collagen is recommended as an ingredient in for production of food, makeup, biomedical and drug applications because of the outlined characteristics. To check the safety of aquatic collagen, Liang et al. (2012) carried out a clinical trial to measure potential chronic adverse effects and tolerability and found no significant adverse effects, suggesting that aquatic collagen is a safe, functional ingredient. In addition, the FDA has granted aquatic collagen GRAS status (generally recognised as safe) (Raman et al., 2018b).

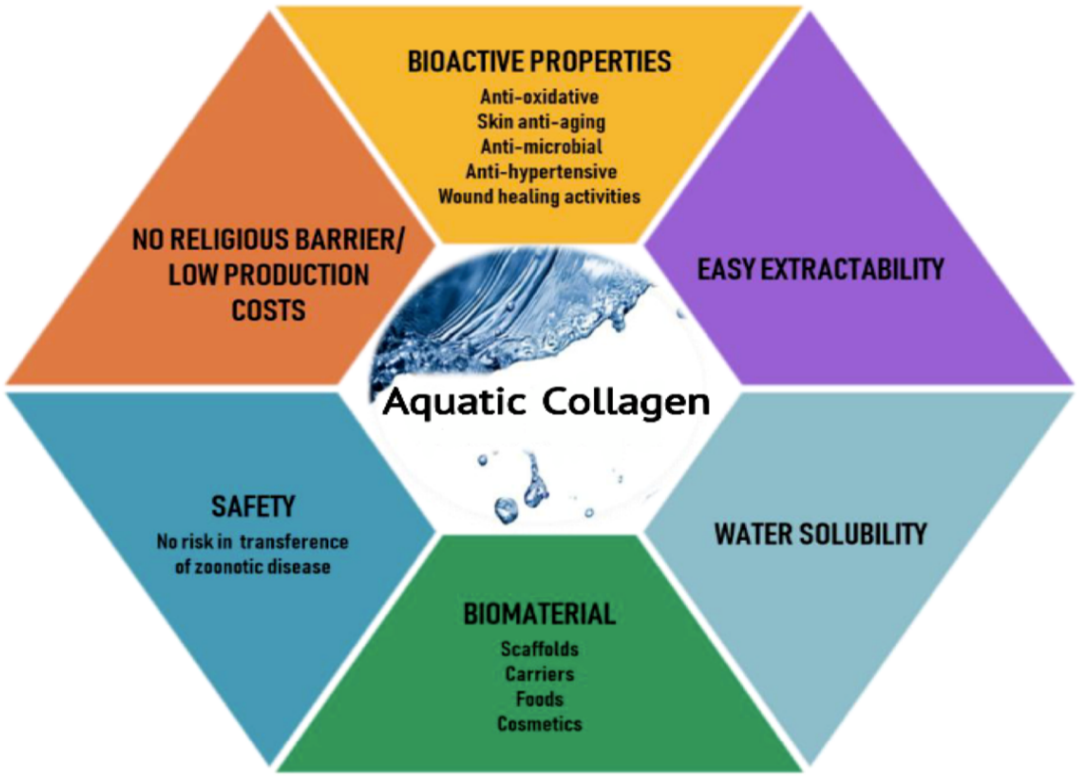

Figure 2.5. Benefits of aquatic collagen over terrestrial animal collagen (Lim et al., 2019).

As fish is considered an alternative source for collagen production, many fish species have been investigated to determine their potential. All species have different denaturation temperature (T_d), making optimisation of extraction conditions for improving yields difficult. Red Tilapia and Seabream (Ikoma et al., 2003), Sheepshead and Black Drum (Ogawa et al., 2004), Grass Carp and Deep-Sea Redfish (Wang et al., 2008a) were broadly studied for extraction of collagen from their scales. Fish fins obtained from fish processing units producing canned fish were proposed as an economical and superior source, but yields are low (Aewsiri et al., 2008). Research on collagen production from fish offal taken from marinated or salted herring or cold-smoked salmon found that smoking improved denaturation temperatures of collagen in comparison to other fish-derived sources of collagen (Gómez-Guillén et al., 2011). In countries like China, Thailand and the southern United States, alligator bones are produced as waste in large amounts and are used to produce collagen. Collagens from alligator bones are

type I collagens with near identical characteristics to collagen from exotic fish species like the Black Drum and Sheepshead (Gómez-Guillén et al., 2011, Wood et al., 2008). The Giant Red Sea cucumber was also studied as a potential source of collagen and was discovered to be of type I where it can be used for drug applications, but had low amino acid when compared to other cold water fishes (Liu et al., 2010). Poultry is also used to produce type I and type III collagen (Cliche et al., 2003).

2.2. Fish as a waste source

More than 50% of waste is generated by processing of fish processing with regards total production (Mo et al., 2018). Some portion of this waste is used as a raw material for protein extraction from fish, but most of this waste remains completely unutilised, with the vast majority being disposed in landfills, raising concerns for the environment and the stability of the planet's climate. Numerous applications have been identified for fish processing waste, such as animal feed, enzyme isolation, biodiesel/biogas production, dietic items like chitosan, Cr immobilisation, extraction of pigments, makeup (collagen), soil manure and as a moisture controller in food products (hydrolysates) (Arvanitoyannis et al., 2008). The yield of collagen extracted from fish waste can be more than half in dry mass, although the number of fish studied for this purpose are fewer than the number of fish species utilised in the food industry. Ergo, such waste material can be used as an eco-friendly alternative and an inexpensive collagen source (Arnesen et al., 2007).

Globally, fish production reached 171 million tonnes in 2016, with 47 % contributed by aquaculture, and 53 % utilised in the food industry (FAO, 2020b) (Table 2.2 and Fig. 2.6). In 2016 fisheries and aquaculture were worth USD 362 billion, of which USD 232 billion was

from the aquaculture sector. Between 1961 and 2016, the average yearly expansion in worldwide food fish consumption was 3.2% increasing more than that of population growth (1.6%) (Fig. 2.7). Fish consumption increased from 9.0 kg to 20.2 kg per capita which is nearly about 1.5% per year. In 2015, 17% of total animal protein consumed was fish protein (FAO, 2020b).

Table 2.2. Production and utilisation of fish (million tonnes) of world fisheries and aquaculture

Category	2011	2012	2013	2014	2015	2016
Production						
Capture						
Inland	10.7	11.2	11.2	11.3	11.4	11.6
Marine	81.5	78.4	79.4	79.9	81.2	79.3
Total Capture	92.2	89.5	90.6	91.2	92.7	90.9
Aquaculture						
Inland	38.6	42.0	44.8	46.9	48.6	51.4
Marine	23.2	24.4	25.4	26.8	27.5	28.7
Total Capture	61.8	66.4	70.2	73.7	76.1	80.0
Total world fisheries and aquaculture	154.0	156.0	160.7	164.9	168.7	170.9
Utilisation						
Human consumption	130.0	136.4	140.1	144.8	148.4	151.2
Non-food uses	24.0	19.6	20.6	20.0	20.3	19.7
Population (*billions*)c	7.0	7.1	7.2	7.3	7.3	7.4
Per capita apparent consumption (kg)	18.5	19.2	19.5	19.9	20.2	20.3

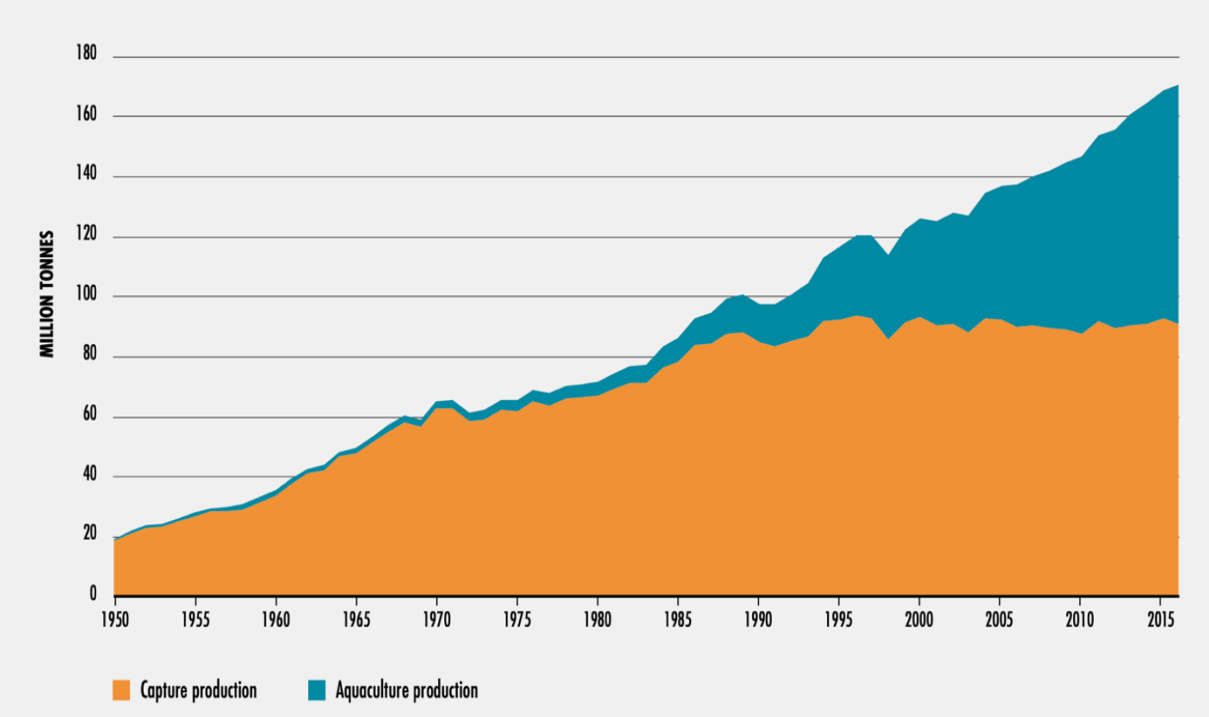

Figure 2.6. Global capture and aquaculture production (FAO, 2020b).

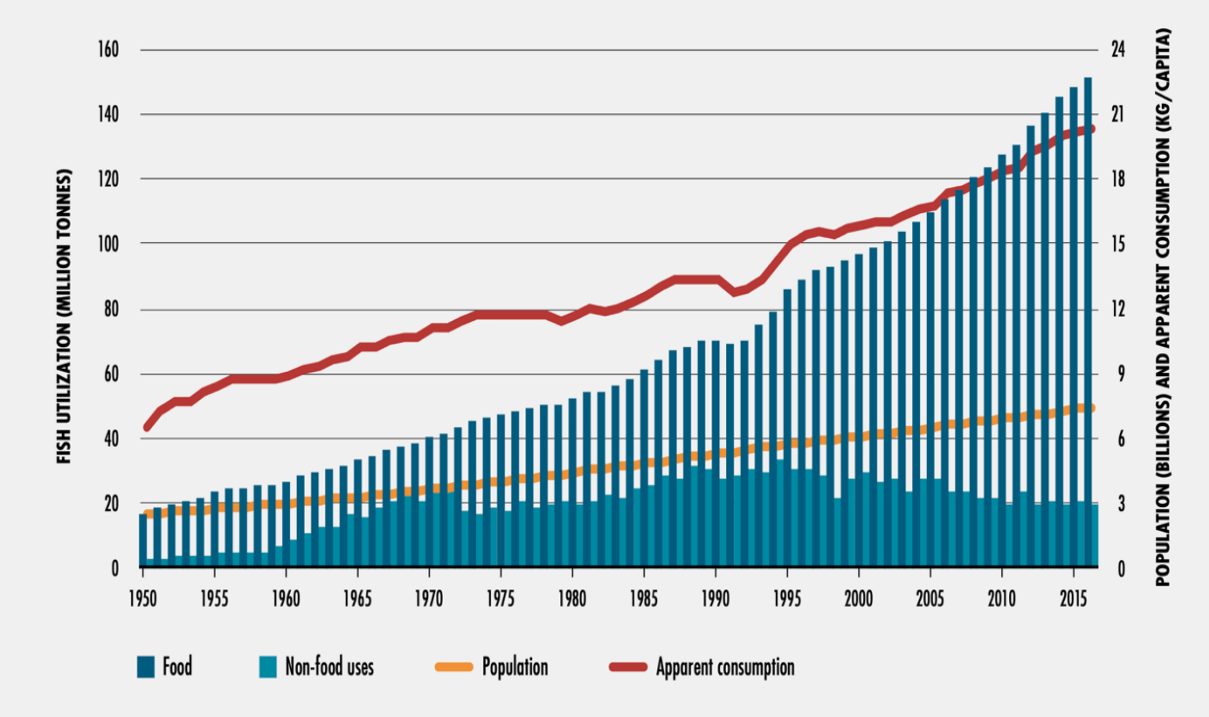

Figure 2.7. Global fish supply and utilization (FAO, 2020b).

Fish contributes to food production of 3.2 billion people, making up to 20 % of the animal protein per capita. While fish is a less consumed food product worldwide, in developing countries, there is a higher intake of fish. Several small islands developing states (SIDS), mostly in Oceania, have a per capita consumption of 50 kg, but consumption can be as little as 2 kg in Central Asia and landlocked countries per year. The Australian Dietary Guidelines (ADGs) recommend a weekly intake of fish of 140-280 g, to benefit health. Incorporation of fish in our diets helps to reduce the risk of cardiovascular disease, macular degeneration, dementia and stroke. Fish food consumption is also influenced by fish species and processing (e.g. filleted and or marinated, smoked etc.). The per-capita fish consumption has increased to 45% between 1995 and 2011/12, based on the number of fish consumed, bought and percent of fish shoppers of the total population (Fig 2.8).

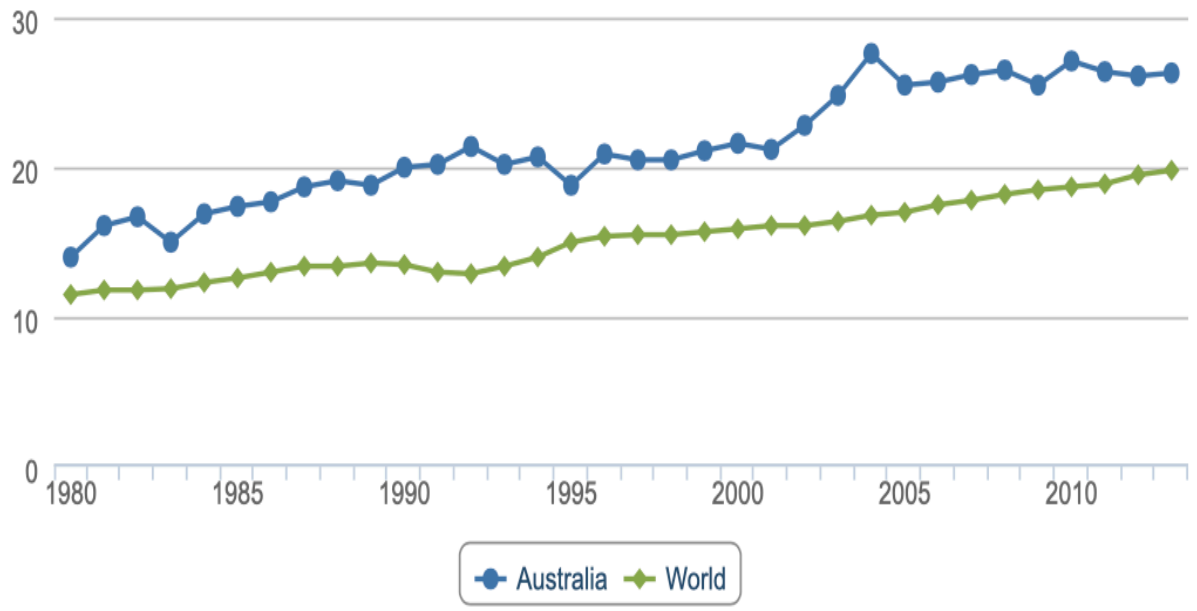

Figure 2.8. Per capita supply of fish and fishery products (kg) in Australia (FAO, 2015).

In Australia, the production of captured and aquacultured fishes increased to 184,445 t and 96,798 t from 121,691 t and 8,994 t (1989-2018) (Fig 2.9 and Fig 2.10). The most significant

rise has been in canned and otherwise processed fish, by 11% and 89%, respectively with buyers of canned fish and processed fish increased by 77% and 100%, respectively, relative to the total population. Globally, ecological sustainability of fish production businesses is controlled by imposing laws and guidelines, provided by national and international committees, including Regional Fishery Management Authorities and National Aquaculture Council, and guidelines such as the Code of Conduct for Responsible Fisheries. For most wild-catch fisheries, 'sustainability' is officially surveyed through government-led stock evaluations, which are included in the half-yearly State of World Fisheries and Aquaculture reports. Minimising food loss and waste in the fishing industries and the steps taken in order to improve sustainability is the objective of the Sustainable Development Goals (SDGs) (target 12.3). The Australian Government has focused on this objective through the National Food Waste Strategy implemented in 2017. Under this procedure, the National Food Waste Baseline Assessment report assessed that in 2016/17, Australia created 7.3 million tonnes (t) of food waste; 31% in the first stage of production, 25% in the manufacturing area and 31% at the household level. The evaluation did not estimate the seafood waste for the first stage of production; however, 50,080 t of fish waste is to be estimated in the manufacturing stage, and 11,400 t in the wholesaling stage (FAO, 2015). With the increase in the population, the demand for seafood consumption is increasing. Australia has observed a nearly 50-60% increase in the total per capita seafood consumption since 1995 (Bogard et al., 2019), but concomitant waste production has exceptional ecological problems, including land debasement, ozone-depleting, substance discharges, water use and biodiversity misfortune (Bogard et al., 2019). The Australian fishing industries' create 20,000 t of waste per year (Howieson et al., 2017) The amount of waste produced can fluctuate, depending on the type of fish; however, it typically ranges between 40-60% of the total fish production. To combat ecological issues generated by

fish waste, this study focussed on developing a green and economical pathway for extraction of collagen from the skin of salmon (Muralidharan et al., 2013)

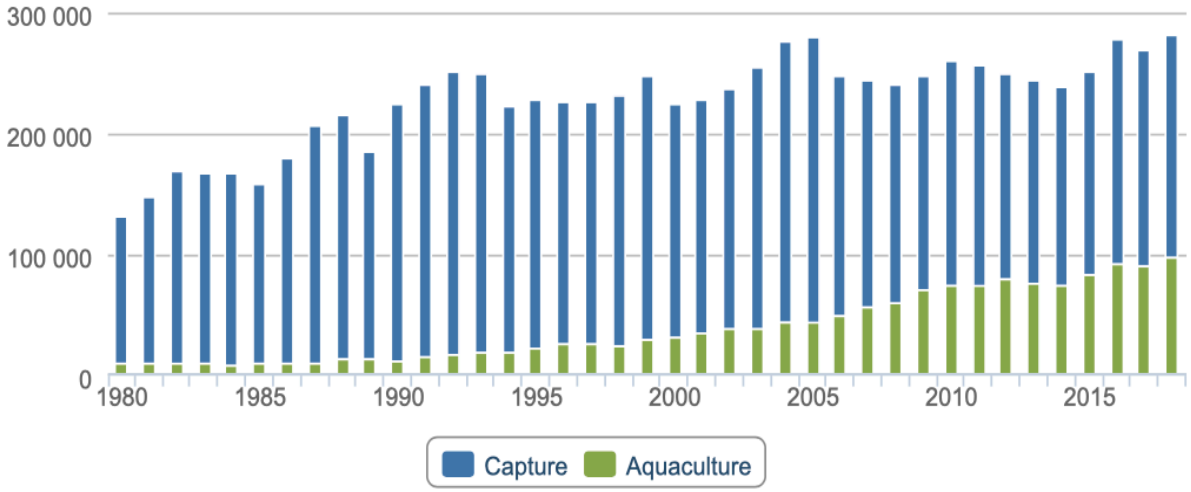

Figure 2.9. Total production of captured and aquaculture fishes in Australia in tonnes (FAO, 2015).

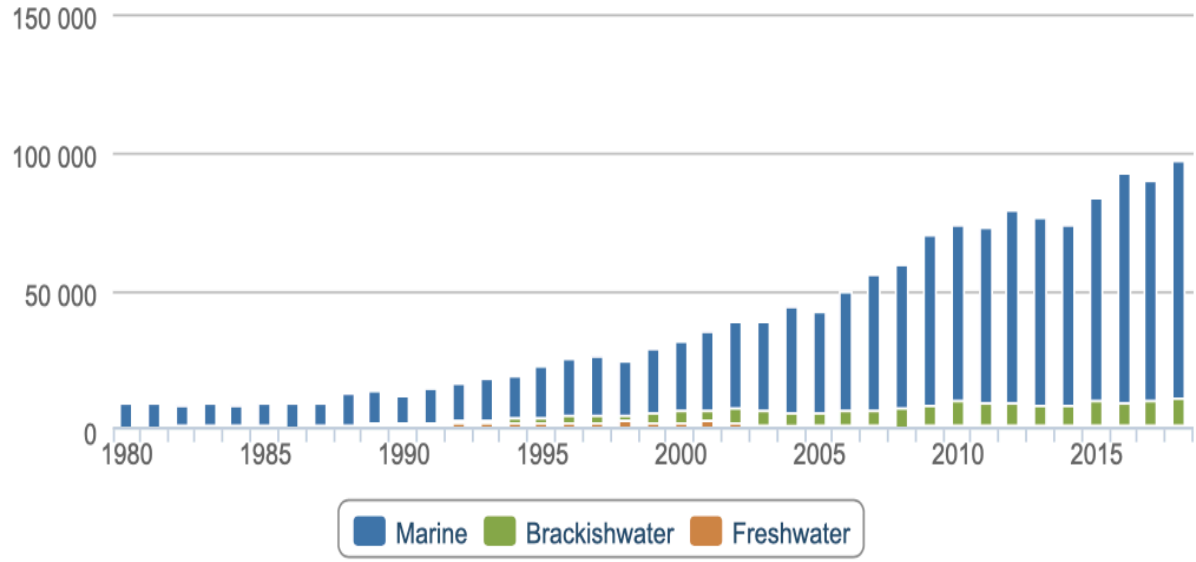

Figure 2.10. Total production of wild fishery in Australia in tonnes (FAO, 2015).

2.3. Salmon production, skin rate and main production state

Salmon is the most common species consumed and production is experiencing fast growth (Kobayashi et al., 2015). The north-western countries like Belgium, Denmark, the Federal Republic of Germany (FRG), the Netherlands, Norway, Sweden, Switzerland, Asa-Pacific, and the United Kingdom are the biggest producers and the highest consumer of salmon (FAO, 2020a). Salmon aquaculture is one of the most prominently growing industries, accounting for 70% (2.5 million metric tons) of the total market (WWF, 2020). Global salmonid production (salmon, trout and smelt) was 4.5 million tonnes in 2017, with 3.5 million tonnes contributed from the aquaculture (Mobsby et al., 2020). Norway and Chile are the world two largest producers of salmonids, which contributed 37 and 22%, respectively to the global supply. In contrast, the aquaculture salmonid production of Australia accounts for around 2% of the global production (60,000 tonnes) with a value of $862 million in 2019–2020, expected to reach 71,061 tonnes by 2023–2024 (Mobsby et al., 2019, Mobsby et al., 2020). Although salmon is typically sold gutted or whole, a significant quantity of salmon is processed to supply fillet products to the market. The rate of salmon by-products generated from a typical automated filleting line for salmon with an average body weight of 5-6 kg is 37-41%; of which 9-15%, 10-12% and 1-2% are salmon frame, head and trimmings (Howieson et al., 2017, Liaset et al., 2003). About 3.5% of the weight of salmon is skin which is usually removed as by-product during processing (Stevens et al., 2018).

2.4. Physio-chemical properties of fish collagen

The physio-chemical properties of collagen are different for collagen of different sources. Cod, Atlantic salmon and Alaska pollock are cold-water species that were studied on a physio-

chemical and functional basis. Cold-water species have essentially lower denaturation temperatures (4-17 °C) in contrast to exotic species (18-29 °C) like Nile perch, red tilapia, channel catfish, yellowfin fish, skate or grass carp (Gómez-Guillén et al., 2011).

2.4.1. Thermal stability

The thermal stability of fish collagen is lower than that of mammalian collagen, which reduces application scope. Thus, to be an alternative to mammalian collagen, the thermal stability of fish collagen should be equivalent—the thermal stability of collagen is positively correlated by hydroxyproline and proline contents, due to higher cross-linking density. Collagens from freshwater fishes have higher thermal stability (Pati et al., 2010).

The thermal denaturation temperature of collagens from different sources is positively correlated with content of the amino acids proline and hydroxyproline. The triple helical structure of a collagen is stabilised by intra and intermolecular cross-linking. The T_d of pig- and calf-derived skin collagen is 37 and 40.8 °C, respectively, but cold-water fish collagens have a low T_d since their hydroxyproline, and proline contents are lower.

2.4.2. Amino acid composition

The amino acid composition of fish and mammalian collagen are marginally different (Alves et al., 2017). Hydroxyproline is only present in collagen and strongly influences the molecule's stability, 13% of collagen total weight is hydroxyproline. Specifically, the ratios of hydroxyproline to proline varies with fish species. Around 34% of glycine is typically present in fish skin-, scale-, bone- and calf skin collagen. According to the study carried by Duan et al.

(2009) and Tylingo et al. (2016), the proline and hydroxyproline ratio in collagen derived from salmon skin was 121/1000 and 146/1000. The higher the hydroxyproline content, the higher the denaturation temperature, increasing the thermal stability as hydrogen bonding between polypeptide chains is increased. In salmon skin, the most abundant amino acids are proline, hydroxyproline, glycine, and alanine (Moreno et al., 2012, Wu et al., 2011).

Table 2.3. Amino acid composition of collagen extracted from salmon (Alves et al., 2017).

Amino Acid	**Salmon Collagen (moL%)**
Asp	47
Thr	16
Ser	56
Glu	71
Gly	365
Ala	121
Cys	3
Val	18
Met	28
Ile	6
Leu	24
N leu	27
Tyr	1
Phe	16
Lys	12
His	9
OH Lys	25
Arg	36
HPro	48
Pro	73
Total	1000

2.5. Collagen and its applications

Collagen loss in the body begins at the age of 18-29, and around 1% is lost every year at an age of 40 and over. Collagen synthesis can decrease by 75% at the age of 80 (Ganceviciene et al., 2012). Other factors such as free radicals, deficiency of nutrition, smoking and alcoholism also contribute to the reduction of collagen synthesis in the body (Schagen et al., 2012). The role of collagen in the body is vital because it facilitates organ development; healing of wounds and tissues; repair of the cornea, gums, and scalp. Collagen elastic fibres and hyaluronic acid are the skin's main structural components, the largest organ in the human body. The skin protects the body from external injury, maintains the temperature and performs other body functions (León-López et al., 2019). Aging is a normal process, involving anatomical, structural and functional degradation of the skin. The development of lines and wrinkles are minimised by collagen and elastin fibres (León-López et al., 2019). In the cosmetic industry, skin ageing regulation is a problem, but hydrolysed collagen has proven to be an alternative way to slow down the effects of ageing. Hydrolysed collagen from milkfish scales demonstrated excellent capacity for water preservation, moisture absorption and retention, and anti-skin ageing and anti-melanogenic capabilities, demonstrating a potential active ingredient in skincare products (Chen et al., 2018). In the dermis, hydrolysed collagen works in two distinct forms; free amino acids provide the building blocks for producing fibres of collagen and elastin in the first action. Collagen oligopeptides serve as ligands in the second action, binding to receptors on the membrane of fibroblasts and stimulating the development of new collagen, elastin and hyaluronic acid (Alves et al., 2017).

Beauty and appearance are an essential aspect of society. Healthy and young skin has become a standard for defining beauty. Age-induced degradation of collagen manifests itself in the loss

of elasticity, dullness, wrinkles, etc. (Ganceviciene et al., 2012). Type I collagen helps in the regeneration and replacement of dead and old cells, hence it is the most prominent type of collagen used in all cosmetics products (Purohit et al., 2016). Loss of type I skin collagen leads to skin lightening, aging, skin thinning etc. (Quan et al., 2015). This results in increased research and development to find alternatives and solutions for improving skin texture and integrity to maintain young and healthy skin.

2.5.1. Oral collagen supplementation

Oral collagen supplementation has become popular in recent years as it has been advertised as an anti-aging product to customers, since it enters the deeper layers of the skin and enhances skin physiology and appearance, increasing hydration, elasticity, firmness, reduction of wrinkles and rejuvenation of the skin (Alves et al., 2017). In vivo studies in women aged 40 to 60 years with oral hydrolysed collagen supplementation over 12 weeks showed a substantial improvement in hydration, wrinkling, and skin elasticity (Kim et al., 2018). In women between 35 and 65 years of age, hydrolysed collagen as an oral nutrient supplement has been shown to increase dermal thickness, skin firmness, and elasticity after treatment of three months (Addor et al., 2018). Another 90-day study of 120 subjects consuming daily oral supplementation of hydrolysed collagen -containing liquid nutraceuticals resulted in enhanced skin texture and elasticity, also a protective effect was observed on the joints (Czajka et al., 2018). After 28 days of oral supplementation, a study of 60 healthy female subjects aged between 40 and 50 years found that this works on skin elasticity and has a more pronounced effect on dermal echogenicity, reducing skin pores, enhancing hydration, texture, elasticity and skin firmness (Maia Campos et al., 2019). An oral supplement made of hydrolysed collagen and a combination of fruit extract, vitamins A, C, E and zinc and biotin ELASTEN®, a drinking

ampoule product was administered for 12 weeks to 36 healthy women aged 35 years or older, resulting in higher hydration, elasticity, smoothness and density of their skin (Bolke et al., 2019). Thus, oral collagen supplementation shows various benefits in the aspects of health and beauty (León-López et al., 2019).

2.5.2. Food industry

Hydrolysed collagen has antioxidant and antimicrobial activity, so it can also be used as a functional ingredient in food supplements (Gómez-Guillén et al., 2011). Collagen hydrolysates can bind calcium ions, enhancing their bioavailability, so hydrolysed collagen can be used to manage mineral deficiencies in functional food ingredients (Guo et al., 2015). Hydrolysed collagen functions as an anticoagulant because it helps mitigate the harm caused by low temperatures in cells and tissues, so it may be useful in foods that need cold or freezer storage (Wang et al., 2015). Hydrolysed collagen has been used to prepare various items such as meat products, drinks, soups, and others. It helps to improve and sustain their physical, chemical, and sensory properties. Hydrolysed collagen has been used to substitute 50% of pork fat in processed foods, such as sausages, because it has higher water holding capacity, better consistency after cooking, and improved texture, such as hardness and chewiness (Sousa et al., 2017). When buffalo patties with and without hydrolysed fish collagen were compared, patties with hydrolysed collagen showed a higher protein content, lower fat content and improved texture (Ismail-Fitry et al., 2018). Hydrolysed collagen from fish can be added to drinks such as orange juice (2.5%) and has been shown to boost the food's nutritional and functional properties with higher protein content, bioavailability, low viscosity and high-water solubility (Bilek et al., 2015). A fermented milk drink produced using hydrolysed collagen added ricotta

cheese whey as a functional ingredient showed low syneresis and sedimentation, with strong physical-chemical and microbiological properties. Hydrolysed collagen, açaí pulp, and cheese milk have demonstrated greater sensory acceptability, positively impacting viscosity after 28 days of storage (León-López et al., 2019). Application of collagen improves the quality of the food prepared.

2.5.3. Biomaterial

Collagen provides good biocompatibility and biodegradability, so it is safe and effective as a biomaterial and has been used in tissue engineering and clinical applications (Ramshaw, 2016). Hydrolysed collagen has a key benefit compared to native collagen, with higher solubility; besides, hydrolysed collagen extraction is easy and does not require a multi-step extraction process and because of the peptides' low molecular weight, hydrolysed collagen does not form scaffolds by itself, but it can be combined with other biopolymers, such as cellulose and chitosan (Ramadass et al., 2014). Films prepared with a cellulose-hydrolysed collagen blend showed good clarity, good absorption of ultraviolet radiation, and outstanding cell adhesion and proliferation support. High biocompatibility dictates that, in the biomaterial field, the films will have promising applications (Pei et al., 2013). Collagen-hydrolysed collagen films produced from leather waste were very transparent and had excellent UV light barrier properties, and studies such as Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) showed absolute miscibility between both polymers (Ocak, 2018). Due to the low molecular weight of hydrolysed collagen, the production of a hydrolysed collagen biomaterial may be useful for treating bone and joint disorders. By encouraging collagen production, it is more bioavailable and causes greater osteointegration. Chitosan sponges are alternate biomaterials containing hydrolysed collagen. The sol-gel transfer

technique demonstrates porous morphology, enhanced biostability, adequate capacity for water absorption, excellent biocompatibility and antimicrobial activity (Ficai et al., 2013, Ramadass et al., 2014). Hydrolysed collagen has been used to deliver drugs such as insulin and methylene blue in hydrogel applications, demonstrating lower water absorbency. At pH 2, the hydrogel delivery was quicker. It is useful for oral drug applications for drugs prone to degradation in the acid and proteolytic condition of gastric fluids (León-López et al., 2019, Noppakundilograt et al., 2018).

2.6. Current industrial extraction process of collagen

Collagen can be produced by chemical and enzymatic hydrolysis. Chemical hydrolysis is more widely used in industry, but when products with high nutritional value and enhanced functionality are required, biological processes that use the addition of enzymes are more promising. Besides, enzymatic processes produce less waste and can decrease processing time, but they are costly. To extract collagen, numerous covalent intra- and intermolecular cross-links makes the process very complicated (See et al., 2015). Pre-treatment is carried out using an acid or alkaline procedure, which varies according to the raw materials' origin before collagen can be extracted. In order to achieve higher yields, pre-treatment aims to remove all non-collagenous materials. Collagen solubility in neutral saline solutions, acidic solutions with or without enzymes are the most widely used extraction methods (Gómez-Guillén et al., 2011, Schmidt et al., 2016).

2.6.1. Pre-treatment

Collagen dissolves very slowly, even in boiling water (Zubal et al., 2018). In order to break cross-links in collagen fibres or between collagen fibres and other filamentous proteins before extraction, mild chemical treatment is required, using diluted acids and bases (Schmidt et al., 2016). For more sensitive raw material with less interconnected collagen fibres, such as pig and fish skins, the acidic process which uses NaOH and $Ca(OH)_2$ in varying concentrations like (0.01, 0.1, 0.2, and 0.5 mol/L) is more suitable (Zhou et al., 2005). The alkaline and acidic method consists of treating the raw material for a few hours to a few days with a simple solution, usually sodium hydroxide (0.1 M NaOH). This approach is used for thicker materials, such as bovine, to penetrate more aggressively. Calcium hydroxide ($Ca(OH)_2$) are also used for pre-treatment, but NaOH is ideal for pre-treating skin because it induces substantial swelling by increasing the transfer rate of mass in the tissue matrix that promotes collagen extraction (Schmidt et al., 2016).

The effect of alkaline pre-treatment on the extraction of acid soluble collagen (ASC) from grass carp skin was evaluated in a report (Liu et al., 2015). NaOH concentrations of 0.05 to 0.1 M effectively eliminated non-collagenous proteins without ASC loss and structural alteration at 4, 10, 15 and 20 °C. However, 0.2 and 0.5 M NaOH caused considerable ASC losses, at 15 and 20 °C and 0.5 M NaOH resulted in the structural modification of the collagen. Besides, to produce products with various properties, the use of acids and bases, enzymes or chemicals may also be used to cleave cross-linking bonds (Schmidt et al., 2016).

2.6.2. Chemical hydrolysis

Neutral saline solutions, such as sodium chloride (NaCl), Tris-HCl (Tris (hydroxymethyl) aminomethane hydrochloride), phosphates or citrates, are used to remove collagen that is soluble in salt. Given that most collagen molecules are cross-linked, the use of these procedures is limited.

Organic acids such as acetic acid, citric acid and lactic acid, and inorganic acids such as hydrochloric acid can be used. Organic acids are, however, more potent than inorganic acids. Organic acids are capable of solubilising non-cross-linked collagen and also of breaking some of the inter-strand collagen cross-links, resulting in higher collagen solubility during the phase of extraction. Therefore, to extract collagen, acidic solutions, especially acetic acid, are widely used (Liu et al., 2015).

The pre-treated material is added to the acid solution, usually 0.5 M acetic acid, to extract acid-soluble collagen and maintained for 24 to 72 h under continuous stirring at 4°C, depending on the raw material (Nagai et al., 2015).

During the extraction stage, filtering is carried out to separate the liquid and solid phase, collagen is present in the liquid phase. The liquid phase is typically subjected to precipitation with NaCl, to obtain collagen precipitate. The precipitate is then sedimented by centrifugation and then dissolved in a minimum volume of 0.5 M acetic acid and then dialysed in distilled water overnight (Schmidt et al., 2016).

In a study by de Moraes et al. (2013), the maximum concentrations of soluble proteins obtained from treatments was achieved at 80°C and a pH below the isoelectric point, s. Extreme pH conditions (3 and 10) or high temperatures (60 and 80 °C) fully denatured the collagen. Collagen with reduced molar mass was produced from extractions using an acidic pH and elevated temperature. In particular, the hydrolysates formed firmer gels when obtained with acidic extractions. Except for the hydrolysates obtained at high pH (7 and 10) and above the denaturation temperature (80 °C), the water retention ability of the gels was around 100% (Schmidt et al., 2016).

(Wang et al., 2008b) optimised the conditions for the extraction of acid-soluble collagen from grass carp (*Ctenopharyngodon idella*) skin, investigating the effects of acetic acid concentrations (0.3, 0.5 and 0.8 M), temperature (10, 20 and 30 °C) and extraction time (12, 24 and 36 h). The three tested variables demonstrated a significant impact on collagen extraction yields and a positive correlation between extraction time and collagen yields was found. Increased concentrations of acetic acid up to xxxx and temperature from yyy to zzz increased yields to a 18.6% which then declined to 12.8%. The optimum conditions for achieving the highest yields of acid-soluble collagen derived from grass carp skin were an acetic acid concentration of 0.54 M for 32.1 h at a temperature of 24.7°C (Sinthusamran et al., 2013). Similarly, high acid-soluble collagen yields from pre-treated skin and swimming bladder of barramundi (*Lates calcarifer*) were obtained with an extraction time of 48 h at 4°C with 0.5 M acetic acid. Acid-soluble collagen yields from the swim bladder were almost two-fold higher (28.5%) compared to yields from skin (15.8%) (Sinthusamran et al., 2013). In both cases, certain variations in the primary structure of collagen type I was recognised.

In general, by regulating process variables such as concentration of solvents, pH, temperature, and process time, chemical hydrolysis processes pursue optimum conditions for obtaining greatest functional yields of acid-soluble type I collagen.

2.6.3. Ultrasound-assisted extraction

Ultrasound is commonly used in wet processing, to increase homogenisation through mixing, dispersion and extraction of various components from different bioresources to enhance the transfer of mass. Ultrasound is a system that utilises the energy produced by sound waves at a higher frequency than the hearing ability of human beings (higher than 16 kHz). In liquid systems, ultrasound generated high- and low-pressure waves result in the quick generation and breakdown of cavitation bubbles. The collapse of cavitation bubbles results in a rise of temperature and pressure, in the cavitation region, which creates turbulence and shearing (Ali et al., 2018, Jiang et al., 2016).

The extraction of acid-soluble collagen from the skin of Japanese sea bass (*Lateolabrax japonicus*) showed increased yields and decreased extraction time after ultrasonic treatment at 20 kHz in 0.5 M acetic acid (Kim et al., 2012) and the α1, α2 and β chains of the extracted collagen were not altered. Ultrasound-intensified collagen extraction generally provided higher yields than the traditional extraction method with 0.5 M acetic acid and lowered required acid concentration to 0.01 M) (Kim et al., 2013). Moreover, increasing treatment time and ultrasound amplitude achieved significantly higher yields of collagen extracted from the skin of Japanese sea bass (*Lateolabrax japonicus*) (Kim et al., 2012). Studies of the impact of ultrasound on the function of enzymes, however, are limited. Li et al. (2009) and Yu et al.

(2014) indicated that ultrasound treatment can change the activity of the enzymes papain and pepsin, primarily due to changes in their secondary and tertiary structures; Papain activity was inhibited, while pepsin activity was activated. Ultrasound treatments for long periods can result in elevated temperatures, shear strength, and high pressures due to cavitation within the medium, severing hydrogen bonds and van der Waals forces in the polypeptide chains, leading to protein/enzyme denaturation (Schmidt et al., 2016).

2.7. Optimisation

Optimisation studies are carried out to study effects of and optimise different processing variables. Therefore, an effective experimental design is needed for complex processes where several factors may show significant interaction, influencing outcomes. The efficiency of extracting collagen from fish waste is affected by many process variables, such as acid concentration, extraction time, temperature, pH, sample to solvent ratio. Thus, an effective experimental design is needed to examine the interactive effects of multiple variables (Wang et al., 2009). Response surface methodology (RSM), Box-Behnken designs and central composite designs (CCD) are mainly used to approximate a second-order polynomial regression to a response variable.

2.8. Factors affecting collagen extraction

2.8.1. Temperature

Collagen is a protein that is considered thermo-unstable, as it quickly denatures at room temperature (Bozec et al., 2011). Temperature sensitivity is type- and species-dependent, i.e. affected by the varying content of amino acids (particularly proline and hydroxyproline) (Sotelo et al., 2016). Compared to those with higher hydroxyproline levels, fish species with lower hydroxyproline levels have lower denaturation temperatures, because hydroxyproline is capable of producing hydrogen bonds within the collagen molecule, creating a more stable molecular structure (Coppola et al., 2020). Denaturation temperatures of collagen from cold-water fish such as cod, ayu and chum salmon have relatively low denaturation temperatures, 15°C, 29.7°C and 19.4°C respectively (Nagai et al., 2000). Thus, to preserve the native structure of collagen, regulation of the extraction temperature is essential. Collagen may gel at higher temperatures increasing extracted collagen yields. Appropriately high temperatures, however, decrease collagen solubility by inducing conformational changes. Therefore, for grass carp, temperatures exceeding 30°C dramatically reduced collagen yields (Wang et al., 2008a). The optimal collagen extraction temperature for grass carp is 24.7°C and the denaturing temperature is 28.4°C. It is apparent that optimal extraction temperatures depend on the species-specific collagen denaturing temperature and need to be determined experimentally.

2.8.2. Acetic acid concentration

Solubilising collagen in acid is one of the important parameters for collagen extraction, as it increases collagen solubility by assisting entry of water into the collagen fibres, causing electrostatic swelling (Kiew et al., 2013). The existence of intermolecular cross-links in collagen molecules is demonstrated by the partial solubility of fish skins, which is affected by acid concentration. The first step of collagen solubilisation is hydration of the fibrous collagen through interaction with acids. As the acid concentration increases, the protein's structure and its electrostatic interactions change, since pH influences the protein charge density of the protein (Skierka et al., 2007, Wang et al., 2008a).

Different concentrations of acetic acid, ranging from 0.1 M to 0.9 M, were used to remove collagen from the skin of *Hybrid clarias*. Yields increased to a maximum of 26.7% when acetic acid concentrations were raised from 0.1 M to 0.7 M (Kiew et al., 2013). However, acetic acid concentrations at 0.9 M reduced the yield to 20.4 %. For collagen extraction from grass carp, the optimal acetic acid concentration was 0.54 M in a study carried by Wang et al. (2008a). An increase in acetic acid concentration to 0.9 M triggered a decrease in the hydration of collagen, due to repulsion of acetic acid interaction with positively charged amine groups of collagen. In addition, collagen fibres shrink at pH values below 2.0, making protein hydration unachievable (Wang et al., 2008a).

2.8.3. Extraction time

A study on the isolation of collagen from the Baltic cod backbone (*Gadus morhua*), showed that collagen solubility increased by 5 to 10% by extending the extraction time from 48 to 72

h (Żelechowska et al., 2010) the . In contrast, only 1.9% of collagen was dissolved in repeated 24 h extractions with 0.5 M acetic acid from the skin and bone of bigeye snapper (*Priacanthus tayenus*) (Kittiphattanabawon et al., 2005). The optimum extraction time for collagen from grass carp was 32.1 h (Wang et al., 2008b). In contrast 0.5 M acetic acid did not completely solubilise collagen even for treatments of 3 days from Japanese sea bass skin collagen, chub mackerel and bullhead shark. Adding 2 days to the extraction time improved yields to 50%. Yields further improved further when extracted in 0.5 M acetic acid for 72 h twice, followed by another 48 h to decalcify the bones of Japanese sea bass (*Lateolabrax japonicas*) and Yellow seabream (*Dentex tumifrons*) (Nagai et al., 2000). For the Japanese sea bass caudal fin, 0.5 M EDTA was used for decalcification and relatively high collagen yield of 36.4% was obtained (Nagai et al., 2000). Mass transfer rates of analytes is an important factor affecting the efficacy of extraction, which is typically dependent on diffusion and hence influenced by extraction time. Therefore it extraction time affects the yield of collagen (Wang et al., 2008a).

CHAPTER 3

3. METHODOLOGY

3.1. Materials

3.1.1. Chemicals, reagents, and buffers

Acetic acid (CH_3COOH) (Sigma-Aldrich Pty Ltd, New South Wales, Australia)

Acrylamide (Bio-Rad, California, United States)

Bis-acrylamide (Bio-Rad, California, United States)

Broad range protein standard ((Bio-Rad, California, United States)

BSA standard (Sigma-Aldrich Pty Ltd, New South Wales, Australia)

Butanol ($C_4H_{10}O$) (Sigma-Aldrich Pty Ltd, New South Wales, Australia)

Chloroform ($CHCl_3$) (Sigma-Aldrich Pty Ltd, New South Wales, Australia)

Collagen Standard (MET-5016, Soluble collagen assay, Cell Biolabs, Inc., San Diego, United States)

Distilled Water (MilliQ water, Millipore Academic MilliQ water, Millipore)

Extraction Solution (MET-5016, Soluble collagen assay, Cell Biolabs, Inc., San Diego, United States)

Glycerol (Sigma-Aldrich Pty Ltd, New South Wales, Australia)

Mercaptoethanol (βME) (Bio-Rad, California, United States)

Methanol (CH_3OH) (Sigma-Aldrich Pty Ltd, New South Wales, Australia)

Phosphate Buffered Saline (PBS) (MET-5016, Soluble collagen assay, Cell Biolabs, Inc., San Diego, United States)

Sirius Red Reagent (MET-5016, Soluble collagen assay, Cell Biolabs, Inc., San Diego, United States)

Sodium Chloride (NaCl) (Sigma-Aldrich Pty Ltd, New South Wales, Australia)

Sodium Hydroxide (NaOH) (Sigma-Aldrich Pty Ltd, New South Wales, Australia)

Tris-base (Tris (hydroxymethyl) aminomethane) (Sigma-Aldrich Pty Ltd, New South Wales, Australia)

Tris-HCl (Tris (hydroxymethyl) aminomethane hydrochloride) (Sigma-Aldrich Pty Ltd, New South Wales, Australia)

3.1.2. Instruments used

15, 50 mL Microcentrifuge tubes (Corning®, New South Wales, Australia)

96 well microtiter plate (Corning® Costar®, round bottom plate, New South Wales, Australia)

Centrifuge (Eppendorf, Centrifuge 5810 R, New South Wales, Australia)

Centrifuge (J2-21, Beckman Coulter, Indianapolis, United States)

Electronic balance (SB12001, Mettler Toledo, Victoria, Australia)

Freeze-drier (VirTis Benchtop K, BTEKEL, Quantum Scientific, New York, United States)

Incubator (Ratek Instruments PTY LTD, Victoria, Australia)

Laminar flow (Clyde- Apac air filteration, New South Wales, Australia)

Magnetic stirrer (IKA® RCT, Selangor, Malaysia)

Micropipettes with disposable tips (Axygen, Corning Life Science, Wujiang, China)

Omega plate reader (Ω) (FLUOstar Omega, BMG Labtech, Victoria, Australia)

Orbital platform shaker (Innova 2300, New South Wales, Australia)

Sonicator (VCX-750, SONICS Vibra cell™, Connecticut, United States)

Vacuum rotary evaporator (Ratek, Adelab Scientific, Victoria, Australia)

Vertical electrophoresis system (Bio-Rad, California, United States)

Vortex Mixer (Ratek, Adelab Scientific, Victoria, Australia)

3.1.3. Raw materials

Salmon skin was collected from the Flinders Medical Centre, Flinders University. The fish skin was cut into 1cm x 1 cm pieces with a sharp butcher knife. The skin was washed with tap water and frozen until use. The fish skin was thawed thoroughly at room temperature before extraction.

3.2. Methods

3.2.1. Preparation of collagen

Collagen from salmon skin was prepared using the conventional (control) and the ultrasound assisted extraction method. The conventional method followed the protocol from (Kittiphattanabawon et al., 2005). All preparations and experiments outlined below were carried out in triplicates at 4°C or lower, unless stated otherwise, and the means ± standard deviation (SD) is reported in the result section.

3.2.2. Analysis for composition of fish skin

Proximate analyses of fish skin have been carried out using methods identified by the AOAC (1990). Crude protein of the fish skin was determined by the Kjeldahl method for total nitrogen estimation AOAC (1990), crude fat determination followed Folch et al. (1957) described in AOAC (1990), moisture content was analysed freeze drying to constant weight. Collagen

concentration was determined using a soluble collagen assay kit (Cell Biolabs) following the manufacturer's protocol.

3.2.3. Pre-treatment of the fish skin

3.2.3.1. Removal of non-collagenous protein

Fish skin pieces were extracted with 0.1 N NaOH at a sample to solvent ratio of 1:10 (w/v). The mixture was stirred (IKA® RCT) at the speed of 200 g for 6 h, and the solution was changed every 2 h. The extracted skin was washed with water until the pH was neutral. The solution was kept aside to determine extracted total protein and lipid.

3.2.3.2. Removal of fats

Fats were extracted from the deproteinized skin with 10 % butyl alcohol at a sample to solvent ratio of 1:10 (w/v). The mixture was stirred (IKA® RCT) at a speed of 200 rpm for 18 h, and the solution was changed every 6 h. The defatted skin was washed to remove any solvent residue. The solution was kept aside to determine extracted total protein and lipid.

3.2.4. Extraction of acid soluble collagen

3.2.4.1. Conventional method

The pre-treated salmon skin was extracted with 0.5 M acetic acid at a sample to solvent ratio of 1:30 (w/v), using a magnetic stirrer (IKA® RCT) at 480 g for 24 h. The solution was changed

every 2 h. The mixture was filtered using a two layered cheese cloth. Residues were re-extracted with 0.5 M acetic acid at the same sample:solvent ratio and identical extraction and filtration conditions. Filtrates were combined and stored in a glass bottle. The filtrate was precipitated with 2.6 M NaCl in presence of 0.1 M Tris(hydroxymethyl)aminomethane (Tris-base) at pH 7.0 - 7.5. The precipitate was collected and centrifuged (J2-21, Beckman Coulter) at 10,000 g for 2 h. The pellet was dissolved in 0.5 M acetic acid and dialysed against distilled water (MilliQ water, Millipore Academic MilliQ water, Millipore) for 24 h. The dialyzed pellet was freeze dried.

The collagen extract was stored at 4°C. Analytical grade chemical reagents were used for the procedures.

3.2.4.2. Ultrasonic method

The pre-treated skin was extracted with 0.5 M acetic acid. The skin/solvent mixtures were subjected to the ultrasonication waves with various condition as mentioned in Table 3.1 Extraction temperature was maintained at 4°C using an ice bath. The mixture was filtered through a double layered cheese cloth. The mixture was precipitated as described in **3.2.4.1** and centrifuged at 2,767 g (Eppendorf, Centrifuge 5810 R) for 30 min. Pellets were dissolved in 0.5 M acetic acid and dialyzed against distilled water (MilliQ water, Millipore Academic MilliQ water, Millipore) overnight. The dialyzed pellets were freeze dried (VirTis Benchtop K, BTEKEL, Quantum Scientific).

The freeze-dried collagen was stored at 4°C. Analytical grade chemical reagents were used throughout the process.

Table 3.1. Ultrasound assisted parameters setting for Collagen extraction from deproteinated and de-fatted salmon skin.

Amplitude	80 %, 100 %
Pulse on/off (s)	5/10
Temperature (°C)	4
Concentration of acetic acid (M)	0.5, 1
Extraction time (min)	30, 45, 60, 120
Sample:solvent ratio	1:10, 1:15, 1:20, 1:30

3.1.4. Estimation of extracted collagen yield

Collagen yield was calculated by determining the dry weight of the extracted collagen divided by the dry weight of the de-proteinated and de-fatted salmon skin. Collagen yield is expressed in % (Eq. 1).

$$\text{Collagen \%} = \frac{\text{Dry weight of freeze dried collagen}}{\text{Dry weight of salmon skin}} \times 100 \qquad \text{Eq.1}$$

3.1.5. Collagen determination

3.1.5.1 Spectrophotometric collagen quantification

Collagen was quantified colourimetrically using a collagen assay kit (Cell Biolabs, San Diego, California, USA). Collagen standards and unknown samples were added to 96 well plates and dried at 37°C overnight. Once dried, the wells were washed with distilled water (MilliQ water,

Millipore Academic MilliQ water, Millipore) four times and tapped dry on a paper towel. Sirius Red Reagent was added to stain the triple helical structure [Gly-x-y] of the collagen and incubated for 60 min on an orbital shaker (Innova 2300, Platform shaker). Wells were washed four times with 5% acetic acid and then tap-dried on a paper towel. Then extraction solution was added to each well containing standards and sample and incubated on an orbital shaker (Innova 2300, Platform shaker) for 30 min. Eluted samples and standards were transferred into a new plate and measured using a spectrophotometer (Omega Plate reader) (Ω) FLUOstar Omega, BMG Labtech) at 595 nm. Collagen concentrations of samples were determined using the linear regression equation derived from a collagen standard curve.

3.1.5.2 EZQ protein quantification for collagen:

EZQ protein assay kit helps to determine the concentration of protein in the solution. This assay kit is based on the Fluorescence as there is no interference of detergents, reducing agents, urea, and tracking dyes for quantification of the protein. This assay kit is mostly used to determine the protein before the polyacrylamide gel electrophoresis. Proteins are spotted on the assay paper provided with the kit and stained with the EZQ protein quantification reagent. Standard curve is used to estimate the protein concentration, effective within the range of 0.02–5 mg/mL, or 0.02–5 μg per spot. A 2.0 mg/mL stock solution of ovalbumin and collagen was prepared.

3.1.6. Simulation of collagen extraction for conventional and ultrasound-intensified processing

Up-scaling production of functional and nutraceutical collagen from Australian salmon skins were carried out using the SuperPro Designer software version 10.0 developed by Intelligen

Inc. (Petrides et al., 2002). The software built-in tools and updateable databank were used to draw process diagrams and estimate total capital investment, total production costs, and profitability of the simulated processes. It is assumed that industrial-scale units perform as well as laboratory-scale units, and both the yield and extraction time of the large-scale processes are the same as the laboratory-scale ones when keeping the processing parameters constant. Since ultrasound is still an emerging technology, not all updated data and equipment units for this technology are included in the software databank and built-in tools. Therefore, the ultrasonic extraction was simulated based on the conventional process with some reasonable suggestions from Keil et al. (1999) and Vinatoru (2001) that the ultrasonic extractors at industrial scales were produced by bonding the ultrasonic transducers to the external walls of the tanks. Purchasing cost of the ultrasonic extractor used in this study was estimated by adding the ultrasonic transducer cost of 28,200 USD to the conventional extractor (Vieira et al., 2013). Diagram processes of these conventional and ultrasonic extractions are shown in Figs 3.1 & 3.2. Additionally, full capacity of the industrial processing plant was assumed at 7,920 h per year as the default time, allowing for the calculation of number batches per year for the simulated process based on recipe batch time and cycle time.

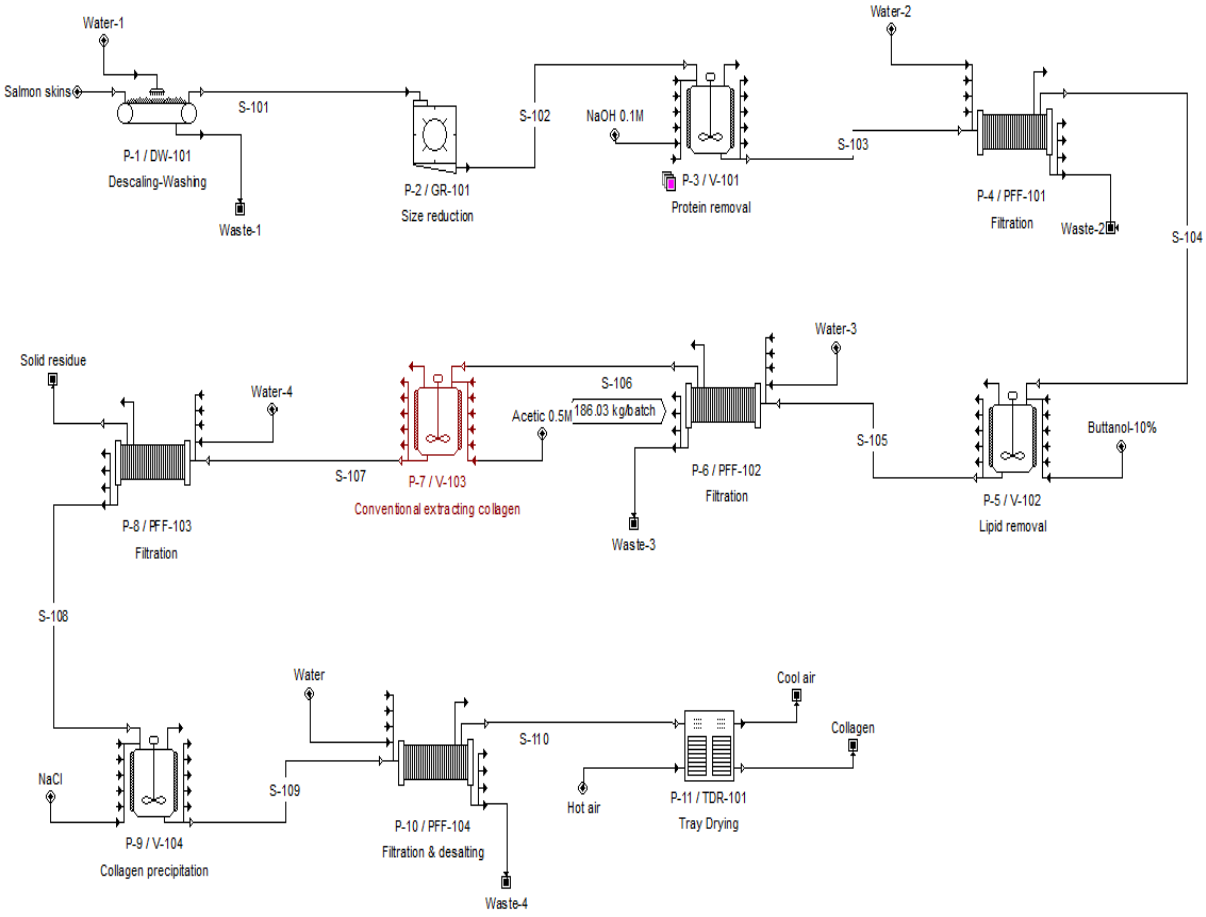
Water-1
Salmon skins
S-101
P-1 / DW-101
Descaling-Washing
Waste-1
S-102
P-2 / GR-101
Size reduction
NaOH 0.1M
P-3 / V-101
Protein removal
S-103
Water-2
P-4 / PFF-101
Filtration
Waste-2
S-104
Solid residue
Water-4
P-8 / PFF-103
Filtration
S-107
P-7 / V-103
Conventional extracting collagen
Acetic 0.5M
S-106
186.03 kg/batch
Water-3
P-6 / PFF-102
Filtration
Waste-3
S-105
P-5 / V-102
Lipid removal
Buttanol-10%
S-108
NaCl
P-9 / V-104
Collagen precipitation
S-109
Water
P-10 / PFF-104
Filtration & desalting
Waste-4
S-110
Hot air
Cool air
P-11 / TDR-101
Tray Drying
Collagen

Figure 3.1 Simulation of conventional extraction of collagen from salmon skins

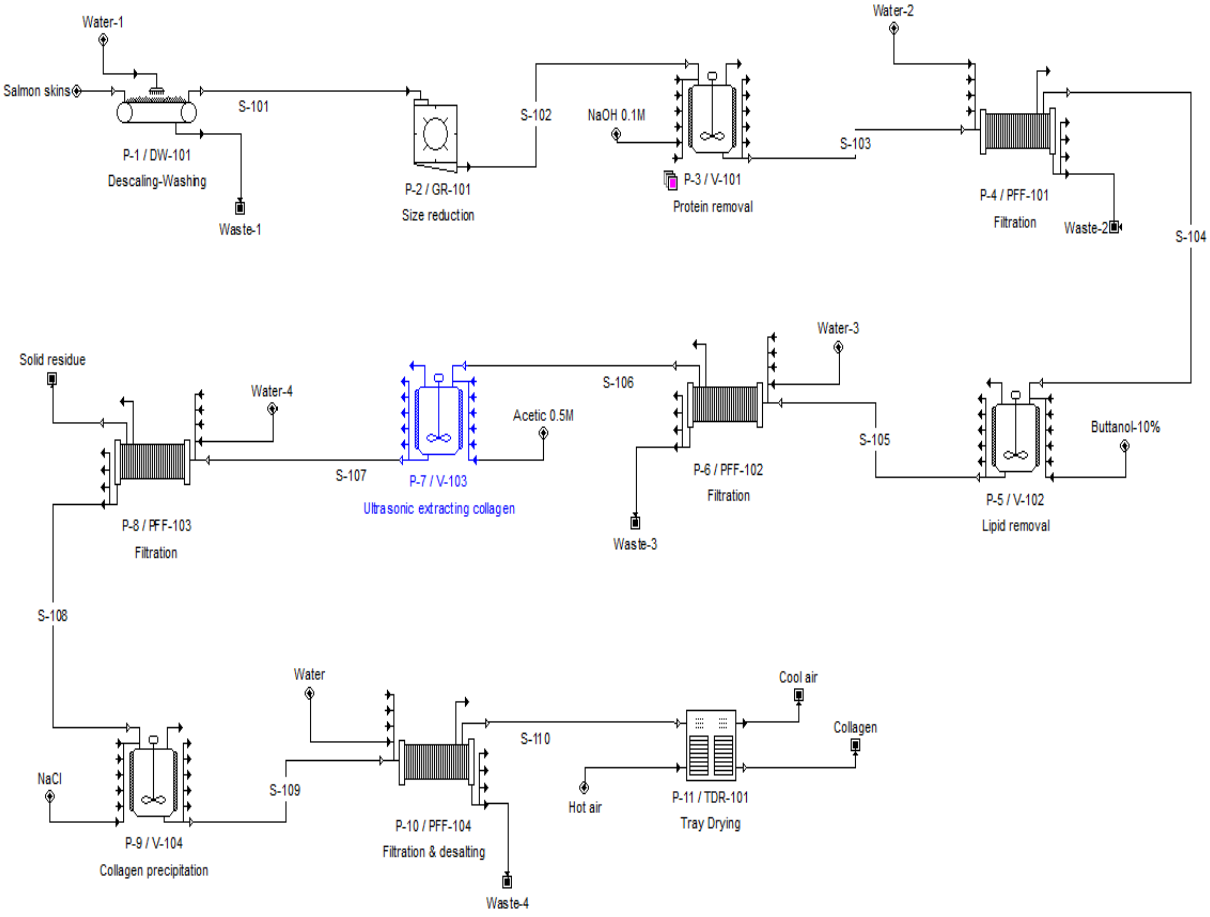

Figure 3.2 Simulation of ultrasonic extraction of collagen from salmon skins

3.1.7. Analysis of collagen by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

Protein patterns of collagen samples were analysed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5 % stacking gel and 7.5 % resolving gel following the method defined by Laemmli (1970). Samples of collagen were dissolved in 0.1 M acetic acid. Five times-concentrated sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol) and 10% (v/v) mercaptoethanol (βME) were combined

with the solubilized collagen samples, at a sample to buffer ratio of 1:4 (v/v). The samples were incubated at 95°C in a heating block. Twenty µl of sample was transferred to each well of the gel. Electrophoresis was carried out at a constant voltage of 300 V. Gels were stained with 15 % (v/v) methanol and 5 % (v/v) acetic acid with 0.05 % (w/v) Coomassie blue R-250. To estimate the molecular weights of proteins, a high-molecular-weight marker kit was used (66.4 - 212 kDa).

3.1.8. Statistical Analysis

Extractions were carried out in triplicate. The mean values with standard deviations (SD) were reported. Data were assessed in R software (The R Core Team, Auckland, New Zealand) for normal distribution using the Shapiro-Wilks test (Appendix C) and homogeneity of variances were determined using the Welch Two Sample t-test for two variances of different amplitude, F-test to compare two variances of different concentration, and the Bartlett test for comparing multiple variances (Appendix D). The Welch Two t-test was used to compare two means and the Kruskal-Wallis analysis to compare multiple means (Appendix E). Drivers of significance were determined via HSD Tukeys post hoc tests (Appendix F) in R software (The R Core Team, Auckland, New Zealand).

CHAPTER 4

4. RESULTS

4.1 Biochemical composition of salmon skin and isolation of collagen

The salmon skin had a high moisture content (80 %), reasonably good protein (13 %) and lipid contents (5.7 %) and a very low ash content (0.86 %), as shown in Table 4.1. Collagen content of the skin was 4 %, while the isolated collagen yield from the skin was 3% (Table 4.2)

Pre-treatment of salmon skin was carried out for all treatments to reduce the presence of non-collagenous proteins and lipids.

Table 4.1 Biochemical composition of salmon skin.

Skin	Salmon
Moisture content (%)	80.066 ± 0.21
Ash weight (%)	0.86 ± 0.023
Lipid content (%)	5.7 ± 0.34
Protein content (%)	12.76 ±0.26

All values are mean ± standard deviation of triplicate analysis.

Table 4.2 Analysis for the collagen content in salmon skin determined spectrophotometrically using a collagen kit:

Fish	Salmon
Collagen content present in the skin	3.47 ± 0.19
Collagen content isolated from the skin	2.49 ± 0.04

All values are mean ± standard deviation of triplicate analysis.

4.2 Collagen yields

4.2.1. Conventional method

The collagen extracted using the conventional method currently used in industries yields 0.697 ± 0.103 g and a percentage yield of 34.5%.at a sample:solvent ratio of 1:30, a concentration of acetic acid 0.5 M and an extraction time of 48 h

4.2.2. Ultrasonic Method

Collagen was extracted using 0.5 M acetic acid unless stated otherwise. Highest collagen yields were obtained at an extraction time of 30 min (Table 4.3). Yields gradually decreased from 46% to 38% when extraction times were increased up to 120 min. In comparison to the conventional method, the maximal collagen was 11% higher (Fig. 4.1)

Table 4.3 Ultrasound assisted collagen extraction with different duration of extraction.

Time [min]	Sample to Solvent Ratio	Dry weight ± SD [g]	% yield ± SD
30	1:30	0.92 ± 0.009	46.0 ± 0.009
45	1:30	0.913 ± 0.009	45.7 ± 0.009
60	1:30	0.843 ± 0.008	42.2 ± 0.008
120	1:30	0.761 ± 0.007	38.0 ± 0.007

All values are mean ± standard deviation of triplicate analysis.

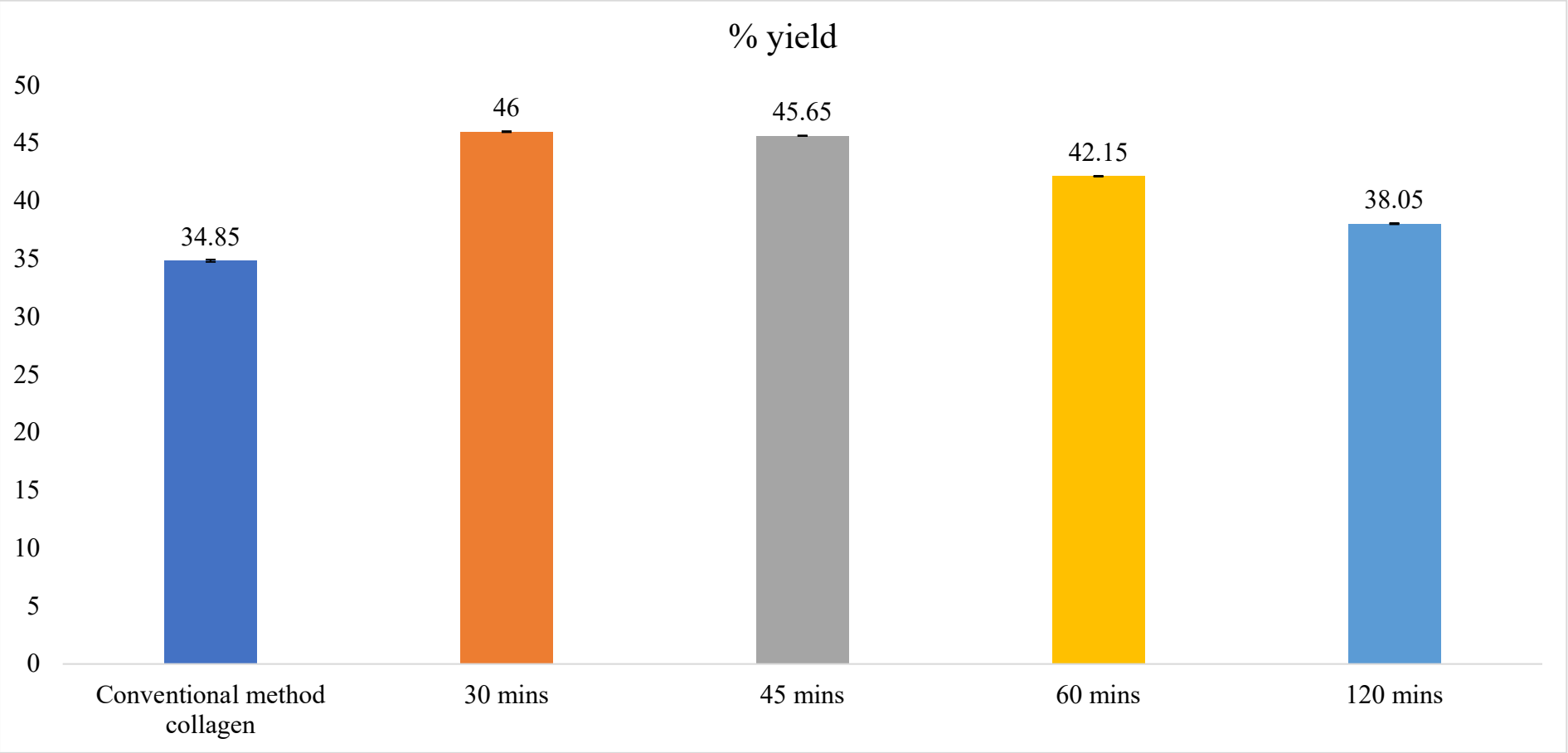

Figure 4.1 Comparison of yields (%) of extracted collagen between conventional and ultrasound-assisted methods and the effect of extraction time on ultrasound-intensified extraction yields.

4.3 Factors affecting extraction and purity of collagen

Increased extraction times of the ultrasound treatment gradually decreased collagen yield (%) from 46 to 38% and purity (%) from 16 to 0.7% (Fig. 4.2A). The sample:solvent ratio had no large effect on % collagen yield with a maximal yield of 55% achieved at a ratio of 1:20, while % purity was strongly affected, with highest purity of 93% obtained at 1:15 and lowest purity of 16% obtained at 1:30 (Fig. 4.2B). The use of 1 M concentration of acetic acid decreased % collagen yield from 55 to 43% and % purity from 72 to 20% (Fig. 4.2C). Ultrasound amplitude had no large observable effect on % yield which ranged from 46 to 43%, but % purity strongly declined with increase in amplitude from 16 to 9%, respectively (Fig. 4.2D).

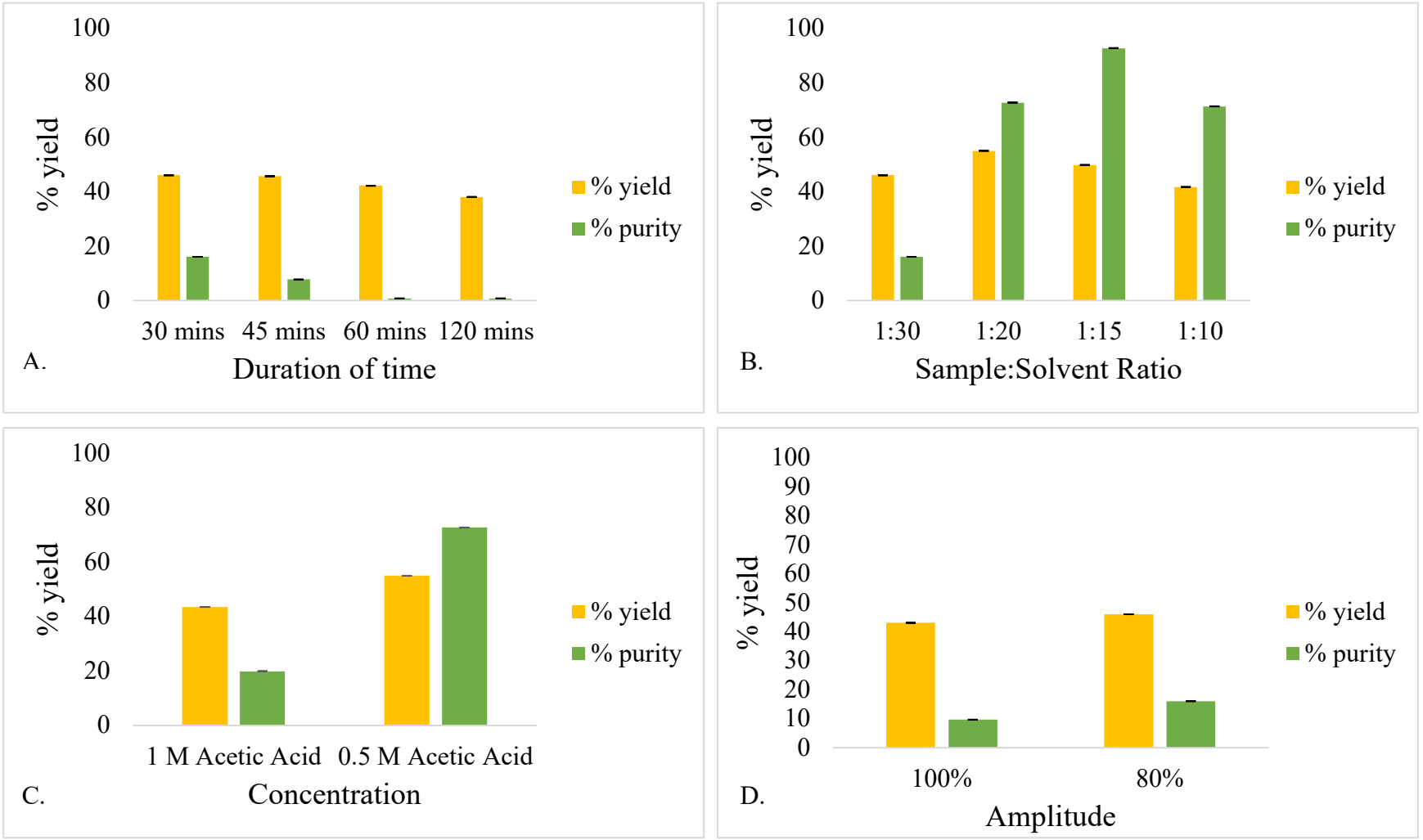

Figure 4.2 Effect of extraction time (A), sample to solvent ratio (B), concentration of acetic acid (C) and ultrasound amplitude (D) on yield and purity of ultrasound-extracted collagen

4.4 SDS-PAGE

SDS-PAGE was used to determine the protein profile of the extracted collagen. Commercially available Type I calf collagen was used as a control. Based on protein molecular weight markers (first lane of the gel), the molecular weights of the collagen chains was calculated. The SDS-PAGE pattern showed three bands, two bands for the α chains and one band for the β chain (Fig. 4.3). The molecular weight for type I collagen from calf skin used as control for the α1 and α2 chains was 129 kDa and 109 kDa, respectively, and 237 kDa for the β chain (Fig. 4.3 I. A) From the SDS PAGE it was confirmed that the collagen extracted from the above-mentioned processes are type I collagen (Nagai et al., 2001, Skierka et al., 2007). Fig 4.2 I. B shows the peptide pattern of collagen extracted with the conventional method with the molecular weight of bands of α1, α2 and β chain being 145, 112 and 215 kDa. Molecular weights of collagen extracted using ultrasound assisted extraction at different extraction times (30-, 45-, 60-, and 120-min) (Fig. 4.3 I. B-E) were between 180-220 kDa for α1, 97-105 kDa for α2, and 150-220 kDa for the β chain. However, the key components of collagen, more precisely the α1, α2 and β chains, were not altered by ultrasound extraction in the extraction duration of 30, 60 and 120 min but at 45 min only one chain with molecular weight of 235 kDa was extracted as shown in Fig 4.3 I. D. Collagen extracted with the ultrasound-assisted method at amplitudes of 100 and 80% (Fig. 4.3 I. G and H) showed molecular weights of the α1 at 95 and 125 kDa, for the α2 at 85 and 105 kDa and for the β chain 165 and 245 kDa. Molecular weights for salmon skin collagen extracted at sample:solvent ratios of 1:30, 1:20, 1:15, 1:10 (Fig 4.2 II A to D) showed a range of 120-110 kDa for the α1 chain, , 97-105 kDa for the α2 chain, and 190-220 kDa for the β chain .Similarly, the molecular weights of collagen extracted

at acetic acid concentration of 1 and 0.5 M (Fig. 4.2 II E and F) were 116-110 kDa for the α1 chain, 97-115 kDa for the α2 chain, and 200-235 kDa for the β chain.

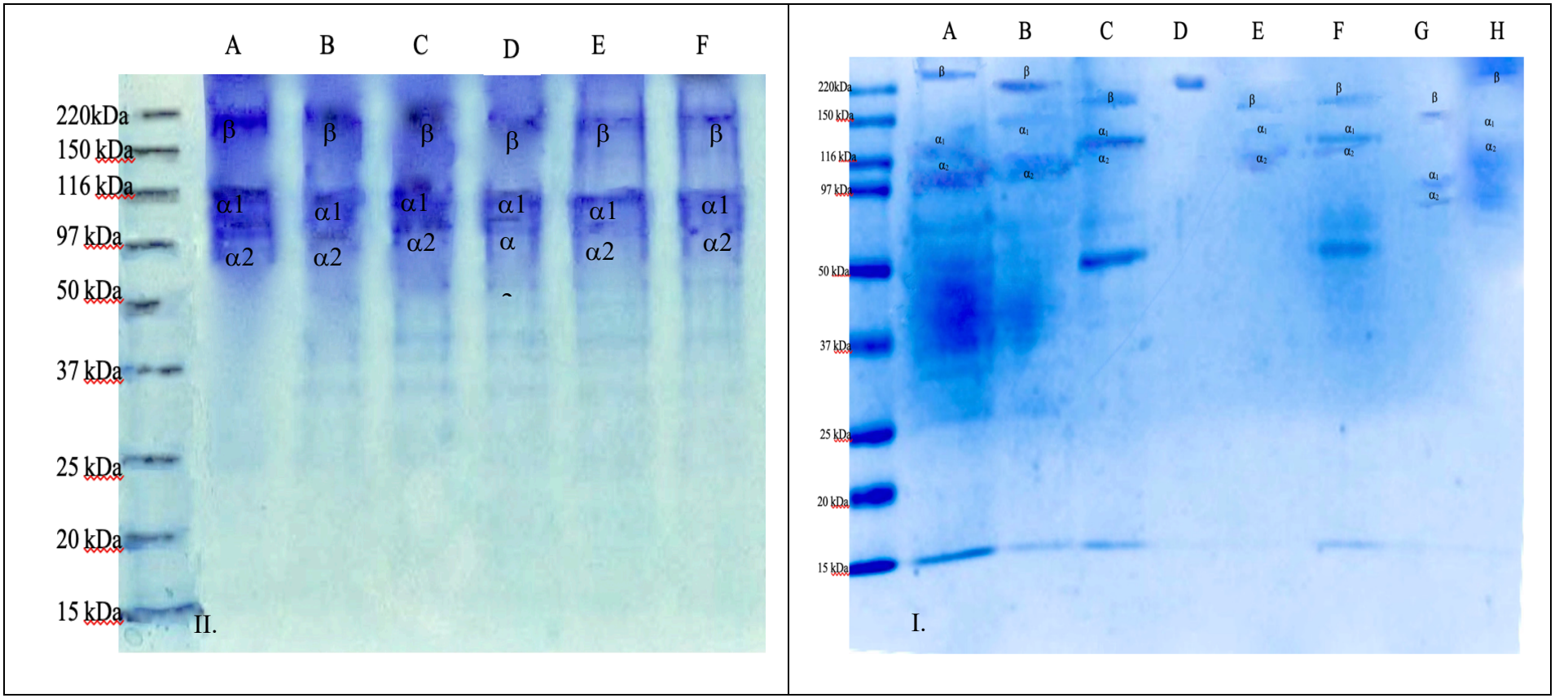

Figure 4.3 SDS PAGE protein patterns of extracted collagen. **I)** standard mammalian (calf) collagen. A) Convention method extraction B) ultrasound-assisted extraction with 30 min extraction time C) ultrasound-assisted extraction with 45 min extraction time D) ultrasound-assisted extraction with 60 min extraction time E) ultrasound-assisted extraction with 120 min extraction time F) ultrasound-assisted extraction with 100% amplitude G) ultrasound-assisted extraction with 80% amplitude H). **II)** ultrasound-assisted extraction of 1:30 sample:solvent ratio A) ultrasound-assisted extraction of 1:20 sample:solvent ratio B) ultrasound-assisted extraction of 1:15 sample:solvent ratio C) ultrasound-assisted extraction of 1:10 sample:solvent ratio D) ultrasound-assisted extraction using 1 M acetic acid E) ultrasound-assisted extraction using 0.5 M acetic acid F)

4.5 EZQ protein quantification for collagen:

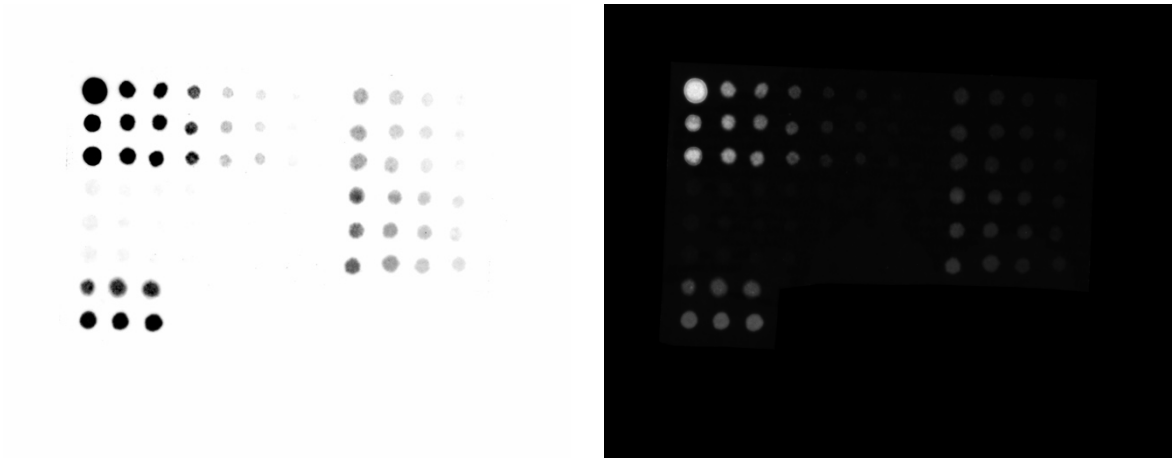

Figure 4.4 SYPRO RUBY image of the EZQ protein quantification of collagen.

When scanned under UV- Lights the collagen did not bind with the staining dye and did not form any stain, thus did not work for the collagen quantification of the collagen.

4.6 Statistical Analysis:

The Kruskal-Wallis test determined that there was a significant effect of extraction time on collagen yield ($p < 0.001$). A Tukey's post hoc test determined that significant differences in yields were driven by extraction times of 30 min being significantly lower than at 60 ($p = 0.017$) and 120 min ($p = 0.002$) and 45 min being different to 120 min ($p = 0.007$) (Appendix F).

Although there were trends in the data for solvent:sample ratio, acetic acid concentration, and amplitude, these were not statistically significant ($p > 0.05$, Appendix C, D, E, F).

5. TECHNO ECONOMIC FEASIBILITY

5.1 Economic evaluation

The economic feasibility of the developed processes was analysed to determine profitability and financial attractiveness aside from providing the necessary support for implementation at industrial scale. Evaluating both the capital and operational costs in correlation to the respective assemblance and operation of the processing plant is the focus of the analysis (Rostagno et al., 2013). For uptake of processing strategies by industry, it is paramount that the economic evaluation demonstrates profitability (Van Dael et al., 2015).

5.1.1 Capital investment

5.1.1.1 Fixed capital estimation

For sustainable development and significantly lower operational costs, the industrial processing plant would be assumingly constructed at a strategic site in Tasmania where 99% of Australia salmons are annually produced (Mobsby et al., 2019). As such, the direct fixed capital cost (DFC, the fixed assets of an investment including plant and equipment) was calculated for the plan constructed in Tasmania. The DFC includes total plant direct cost (TPDC, cost components directly related to an investment); total plant indirect cost (TPIC, cost elements indirectly related to an investment such engineering and construction); and contractor's fee & contingency (CFC, referring to miscellaneous costs). All the capital investments related to each section of a process were calculated as shown in Table 5.1 using a

factor-based method which was set up in SuperPro Designer as default. Default values that were assigned to these factors are reasonable for most cases (Intelligen, 1991).

Table 5.1 Direct fixed capital estimation computed by SuperPro Designer as the default setting

DFC = TPDC + TPIC + CFC			
TPDC	=	(1) + (2) + (3) + (4) + (5) + (6) + (7) + (8) + (9)	
	(1)	Equipment purchase cost (PC)	LPC + UPC
	(2)	Installation	ILE + IUE
	(3)	Process piping	0.35*PC
	(4)	Instrumentation	0.4*PC
	(5)	Insulation	0.03*PC
	(6)	Electrical	0.10*PC
	(7)	Buildings	0.45*PC
	(8)	Yard improvement	0.15*PC
	(9)	Auxiliary facilities	0.4*PC
TPIC	=	(10) + (11)	
	(10)	Engineering	0.25*TPDC
	(11)	Construction	0.35*TPDC
CFC	=	(12) + (13)	
	(12)	Contractor's fee	0.05*(TPDC+ TPIC)
	(13)	Contingency	0.1*(TPDC+ TPIC)

The two components of equipment purchase costs (PC) are listed as equipment purchase cost (LPC) and unlisted equipment purchase cost (UPC), UPC = 0.25*LPC. The PCs of most units used in the simulated process were estimated based on available data in the built-in databank of SuperPro. Estimation was also applied for some equipment units where published data were not available due to the novelty of ultrasonic extractors in industrial-scale processing. In such case, information provided by industrial suppliers and suggestions from related studies were employed to calculate the PCs of the desired units according to their capacity and scale. By default, installation costs are composed of installation of listed equipment (Bilek et al.) and installation of unlisted equipment (IUE) in which the IUE = 0.5*ILE. The estimated DFCs of different simulated processes are shown on Table 5.2. The ultrasound process requires a larger DFC than the conventional process due to the cost of the ultrasonic transducers.

Table 5.2 Direct fixed capital (DFC) of the conventional and ultrasonic processes for large-scale production of collagen from Australian salmon skins

Processes	**DFC distribution**			**DFC (USD)**
	TPDC (USD)	**TPIC (USD)**	**CFC (USD)**	
Conventional	7,226,000	4,336,000	1,734,000	**13,296,000**
Ultrasound	7,096,000	4,258,000	1,703,000	**13,057,000**

5.1.1.2 Total capital investment

The total amount of money needed to supply the necessary plant, manufacturing facility, and working capital for operation is defined as total capital investment (TCI). The TCI for an industrial production plant was described by Turton et al. (2008) including DFC, working capital, and start-up cost as illustrated in Table 5.3. Total capital investment estimations charged to the two simulated processes were obtained using the default setting of SuperPro, which were automatically adjusted according to the year of commencement (2020) with a 30-month construction period and a 4-month start-up period for a 15-year project lifetime.

Table 5.3 Total capital investment (TCI) for the conventional and ultrasonic productions of collagen in a large scale from Australian salmon skins

Processes	**Capital investment distribution**			**TCI (USD)**
	DFC (USD)	**Working capital (USD)**	**Start-up cost (USD)**	
Conventional	13,296,000	29,000	665,000	**13,990,000**
Ultrasound	13,057,000	23,000	653,000	**13,733,000**

5.1.1.3 Operating cost estimation of conventional & ultrasonic processes (databank & collected data)

The facility-dependent costs such as equipment maintenance, depreciation, insurance, property taxes, and possible other overhead costs, which depend on the facility set-up, were estimated using the software default settings. Utility costs were set at the current price (11/2020) applied

for the intended location in Tasmania with AU$0.266 per kWh for electricity (0.194 USD/kWh) (Gudova, 2020) and AUS$1.1 per kilolitre for water (0.803 USD/m^3) (Tastewater, 2019). The rate for basic labourers of AU$24.85 per hour (18.14 USD/h) for the Tasmania market was used while the rate of AU$38 per hour (27.74 USD/h) was set for the specific types of operators, the associated supervision, supplies, administration, and overhead costs. These labour rates were obtained from the website www.payscale.com accessed on 23 November 2020. The default value of 15% was also included in the labour costs for covering quality assurance and checking. Due to the fact that Australian seafood processing by-products are currently discarded with a disposal cost of AU$150 per ton (He et al., 2016), each ton of salmon skin utilised for collagen production is assumed to obtain a financial support of AU$150 for collecting, storing, and transporting skins from salmon processing facilities to the collagen production plant. Therefore, no material cost for utilisation of salmon skins in collagen production is assumed in this scenario. Costs for other materials used in the simulated processes were estimated according to the quotes provided by commercial suppliers as described in Table 5.4. Since acetic acid 0.5M, NaOH 0.1 M, butanol 10%, and NaCl 2.6 M were assumed to be diluted from the concentrated solutions or pellets carried out on site, their costs were estimated according to the sum of the mixed components. The currency rate used for conversion is 1AUD = 0.73 USD obtained from the website https://www.westpac.com.au/personal-banking/services/currency-converter accessed date 23 November 2020.

Table 5.4 The buying price of materials used for cost estimation and the suggested selling price of collagen for (Appendix G-L)

Materials – Buying price

Materials		Unit	Price (USD)	Suppliers	References
Feeding materials	Sodium hydroxide	MT	73		(Millford, 2019)
	Butanol	MT	525		(Alibaba, 2020)
	Acetic acid	MT	625		(Tarun, 2016)
	Sodium chloride	MT	50		(Skylark, 2020)
Consumable materials	Filtration fabric	m^2			
	Dialysis membrane	m^2			

Marine collagen – Selling price

Collagen	Suggested price	Reference products	Price (USD)	Product unit & suppliers	References
Product 1	US$510 per kg	Bioglan Marine Collagen Powder	US$20.4 (A$27.95)	40g Jar, Chemist warehouse	(Chemist, 2019)
Product 2	US$219 per kg	Advanced Marine Collagen	US$65.7 (A$89.95)	300g bottle, GelPro	(Gelpro, 2019)
Product 3	US$196 per kg	Marine Collagen	US$55.4 (A$75.95)	280g pack, Nutraviva	(Nutraviva, 2019)
Product 4	US$109 per kg	Pure Marine Collagen Powder	US$108.8 (A$149)	1kg pack, CollagenX	(CollagenX, 2019)

Waste disposal costs were neglected in these simulated processes since the alkaline effluent water generated from the protein removal step could be neutralised by the acidic effluent derived from the collagen extraction step. Wastewater generated from other outlets were

neither hazardous, nor expensively treated. In addition, the removal of unwanted parts and biological residues generated from these processes was assumed to be without charge due to the fact that these can be further utilised in commercial production of aquafeeds, biofertilizer, compost or soil conditioners. Labour charges and labour not directly associated with production were estimated by the simulator. Other company costs such as packaging, marketing, and research and development were not included in these calculations. The actual annual operating costs calculated for these two processes are illustrated on Table 5.5.

Table 5.5 Annual operating costs (AOC) of the large-scale production of collagen from Australian salmon skin in the conventional and ultrasonic processes

Processes	AOC distribution			AOC (USD)
	Raw materials (USD)	Labour depend (USD)	Facility depend & utilities (USD)	
Conventional	61,000	243,000	2,516,000	2, 820,000
Ultrasound	54,000	190,000	2,470,000	2,715,000

5.1.1.4 Revenues and profitability analysis

The profitability of collagen extraction using the conventional and ultrasound-assisted method was compared using different sales price for collagen as shown in the table 5.6. At a sales price at 510 USD per kg collagen, the profitability of ultrasound-assisted extracted collagen was three-times higher than for collagen obtained with the conventional method, therefore being economically feasible (Appendix K and L). At lower sales price of 219 and 109 USD per kg collagen, net profits were, however, negative indicating losses, therefore these methods were not economically feasible at these sales prices (Appendix G-J).

Table 5.6 Profitability of different simulated processes for production of collagen from Australian salmon skins with different selling price (Appendix G-L)

Collagen selling price (USD/kg)	Processes	Revenues (USD/Year)	AOC (USD/Year)	Gross profit* (USD/Year)	Net profit** (USD/Year)
510	Conventional	2,056,000	2, 820,000	-764,000	499,000
	Ultrasound	2,950,000	2,715,000	235,000	1,382,000
219	Conventional	883,000	2, 820,000	-1,937,000	-675,000
	Ultrasound	1,267,000	2,715,000	-1,448,000	-208,000
109	Conventional	439,000	2, 820,000	-2,381,000	-1,118,000
	Ultrasound	631,000	2,715,000	-2,086,000	-844,000

*Gross profit = Revenues - AOC
**Net profit = Gross profit – Taxes (40 %) + Depreciation (10 %)

5.1.1.5 Economic feasibility of industrial production of collagen

Different economic indicators, like net predicted value (NPV), return on investment (ROI %) and payback time in years, are used to determine economic viability of a project. At a sales price of 510 USD per kg of collagen, the return on investment for the ultrasound-assisted extraction of collagen was ten years while for the conventional method, it was at least 28 years. Lower sales price of 219 USD or lower had, as expected, a negative impact on return of investment (Table 5.7).

Table 5.7 Economic indicators of different simulated processes for production of collagen from Australian salmon skins applied for different selling price (Appendix G-L)

Collagen selling price (USD/kg)	Processes	NPV (USD)	GM (%)	ROI (%)	Payback time (Years)
510	Conventional	-9,997,000	-37.19	3.56	28.06
	Ultrasound	-3,744,000	7.99	10.06	9.94
219	Conventional	-18,057,000	-219.98	-4.82	NA
	Ultrasound	-14,472,000	-114.27	-1.51	NA
109	Conventional	-21,198,000	-541.89	-9.99	NA
	Ultrasound	-18,980,000	-330.51	-6.14	NA

6. DISCUSSION

6.1 Effect of extraction method on collagen yield and purity

Compared to the conventional method (Control) which achieved collagen yield of 35% when 0.5 M acetic acid was used for a duration of 48 h, the ultrasound-assisted method achieved 49.8% using the sample:solvent ratio of 1:15, at amplitude 80% and with the duration time of 30 min. This was the optimal condition found for the highest % yield and %. The yield observed were higher than results obtained by Alves et al. (2017) and Zhang et al. (2009) which was 20% and 17%, respectively, using the conventional method for extracting collagen from salmon skin, but comparable to results obtained by Kołodziejska et al. (1999) and Nagai et al. (1999) with yields of 53 and 46.4% extracted from the skins of squid and edible jellyfish, respectively. This suggests that sonification aids the hydration of collagen fibrils, thereby promoting acid hydrolysis (Kim et al., 2012). For the extraction of collagen from salmon skin, ultrasound-assisted extraction was more successful than conditions used in current industrial collagen extraction processes, which was likely due to cavitation-induced loosening of the collagen fibres in the pre-treated skin (Zou et al., 2017). As shown in the Fig. 4.1 the extraction yield increased by 11% at an extraction time of 30 min, which is much shorter than conventional extraction methods which take more than 24 h ((Nagai et al., 2010). Nonetheless, a study carried out by Kim et al. (2012) used an extraction time of 24 h despite using ultrasound, which is way in excess of the optimal conditions determined in this research (Fig. 4.2). The molecular weights of type I calf collagen shows the effect of extraction parameter settings on the peptide pattern of the extracted collagen similar to Zhang et al. (2009) and Li et al. (2009).

The molecular weight of the collagen helps to identify the purity of the collagen, but the presence of lower molecular weight bands can suggest that the collagen has been hydrolysed, while the presence of α chains and β chain at the correct molecular weights is indicative of collagen purity. Most extractions showed similar molecular weights to the study by Khong et al. (2018). β-chains had molecular weights of around 200 kDa, and α1 and α2-chains were in the vicinity of 115–180 kDa. Ali et al. (2018) showed that β-chains had molecular weights of ≥ 210 kDa and α1 and α2 were ∼117 and ∼108 kDa, respectively. For an extraction time of 45 min only one chain with molecular weight of 235 kDa was extracted (Fig 4.3 I. D), which may suggest that collagen could have disintegrated during the extraction or that insufficient sample was loaded to visualise the α lower concentration -bands. Similarly, 100% amplitude (Fig 4.3 I G.) resulted in bandsof lower molecular weights compared to controls, which could indicate some strand breakages induced by high ultrasonic power (Ali et al., 2018).

6.2 Factors affecting ultrasound-assisted collagen extraction

6.2.1 Effect of extraction time

Extractions using the conventional method showed yields increased with increasing extraction times. Żelechowska et al. (2010) showed that the yield increased up to 32 h but decreased after that due to slow degradation caused by acetic acid. The extraction of acid-soluble collagen using the ultrasonic-assisted method from the skin of clown featherback (*Chitala ornata*) in a study by Petcharat et al. (2020) showed an increased yield of 57.35% for ultrasonic treatment at 20 kHz frequency in 0.5 M acetic acid for 30 min and at 80% amplitude with. The % yield of collagen extracted is lower than yields reported by Petcharat et al. (2020). In contrast to the

conventional method, increased extraction time decreased collagen yields in ultrasound-assisted extractions, which may be due to effects of cavitation, higher pressure and temperature in localised areas of the treated sample leading to collagen degradation (Ali et al., 2018, Nagai et al., 2000). Under the optimal conditions also with respect preventing collagen degeneration, ultrasound-assisted extraction is 96 times faster than achievable with the conventional method. From the SDS-PAGE, we can say the bands formed by the collagen extracted with 60 min and 120 min are lower than the band formed at 30 mins. This could be due breakage of structure with long exposure of the ultrasound treatment (Kim et al., 2012).

6.2.2 Effect of sample:solvent ratio

In Arumugam et al. (2018), extraction of collagen from fish skin was performed at different solvent:solid ratios (8–16 ml/g). A maximum collagen yield of 18.358 mg/g of fish skin was obtained at a solvent/solid ratio of 10 ml/g of fish skin, while higher solvent:solid ratios decreased yields. In contrast, a study carried out by Yu et al. (2018) showed that increasing the sample:solvent ratio significantly increased collagen yields. In the conducted study presented here, no significant increase in collagen yield was observed, rather yields decreased slightly at solvent:sample ratios from 1:30 and higher (Fig 4.2). An increase in the solvent:sample ratio assists in cleaving of the collagen, which should result in increased yields (Yu et al., 2018). As shown in Fig 4.3, the molecular weight of the β chain (200-210 kDa), the α1 chain (110-120 kDa), and the α2 (100 kDa) were those reported, but the peptide pattern does suggest some degradation with reducing the ratio.

6.2.3 Effect of Amplitude

Ali et al. (2018) and Kim et al. (2012) carried out a study to determine the effect of amplitude in the collagen extraction process. They concluded that increasing the amplitude up to 80% increased the yield, as it enhances the solubility and the swelling of the raw material which cleaves the cross linkage of the collagen with minimal damage (Khong et al., 2018). Similarly, in the presented study increasing the amplitude to 100% decreased collagen yields to 43.05%, but yields were higher at an amplitude setting of 80% (46%) than reported by Ali et al. (2018) and Kim et al. (2012). It can therefore be concluded that excessive power damages the cross linkage in the collagen reducing the extraction yield. This conclusion is supported by results obtained by SDS PAGE (Fig. 4.3 I. G) as the bands for the β, α1, and α2 chains have a lower molecular weight than reported and shown for intact chains of type I calf collagen.

6.2.4 Effect of Acetic Acid Concentration

Most of the extraction of acid-soluble collagen used a concentration of 0.5 M, as higher concentrations would cause the peptides to degrade, thereby reducing the final product's yield and purity. Using a range of concentrations between 0.2 and 1 M, the effect of acetic acid concentration on the collagen yield of sole fish skin was studied by Arumugam et al. (2018), while all other experimental variables were kept constant. The yield of collagen increased to 16 mg of collagen/g of fish skin at an acetic acid concentration of 0.6 M. Due to the degradation effect caused by excess acid, the collagen yield decreased to 12.5 mg of collagen/g of fish skin for the concentration above 0.6 M. Similarly, in Kiew et al. (2013) investigated the effect of acetic acid concentration (0.1-0.9 M) on collagen yield, which increased up to 0.7 M and then declined at higher concentrations. This is in line with results obtained here, as collagen yield

decreased to 43.55% from 55% at a concentration of 1 M acetic acid. Despite the impact of acetic acid concentration on yield, the molecular weight of the polypeptide chains was not significantly affected (Fig 4.3), with increase in the acid concentration of acetic acid, the structure of collagen degrades but as the pH was not affected it might protect the molecular structure of collagen, thus forming peptide bonds with no change in the molecular weight for SDS-PAGE analysis (Skierka et al., 2007, Wang et al., 2008b).

6.3 Techno-economic feasibility

The direct fixed capital of the conventional and ultrasound-assisted method was somewhat similar with 13,296,000 and 13,057,000 USD with total investment of 13,990,000 and 13,733,000 USD, respectively. The annual operating cost was 2,820,000 and 2,715,000 USD, respectively. Thus, the analysis showed that when collagen was sold at 510 USD per kg the net profit of ultrasound-assisted was three-times higher compared to the conventional method, with the return of investment of 10% in 10 years while the conventional method had just 3.5% return of investment but take too long with 28 years. This is explained by the much shorter processing time and, to a lesser extent improved yields, for ultrasound-assisted extraction of collagen from salmon skin.

When collagen is sold at 219 USD per kg or lower prices, the business would incur losses instead of profits, with no return on investment.

Thus the ultrasound-assisted method was not economically feasible at lower prices, but higher prices or decreasing the total capital investment might help to increase the gross profit with more return of investment in less time.

7. CONCLUSION

As shown by the techno-economic analysis, the ultrasound-assisted collagen extraction method from salmon skin gave higher yields and comparable purity, but time saved on extraction led to a significantly better period for return on investment compared to the conventional extraction method. In addition, the developed process reduced the amount of acetic acid used for collagen extraction. Comparison of the extracted collagen with purchased calf collagen type I showed that the purity of collagen using ultrasound was comparable with purities achieved with the conventional method for most parameter settings. Consequently, this ultrasonic procedure may be an environmentally sustainable and efficient form for industrial-scale collagen extraction. Since the NPVs of the developed processes have negative values at sales prices of 219 USD per kg and lower, the process is not economically feasible at low market prices, but having a semi-equipped suitable facility close by would change this outcome. Furthermore, economic feasibility could be improved by further simplifying the process, and full utilisation of current other protein and lipid waste streams for the production of co-products in a biorefinery approach to increase revenues.

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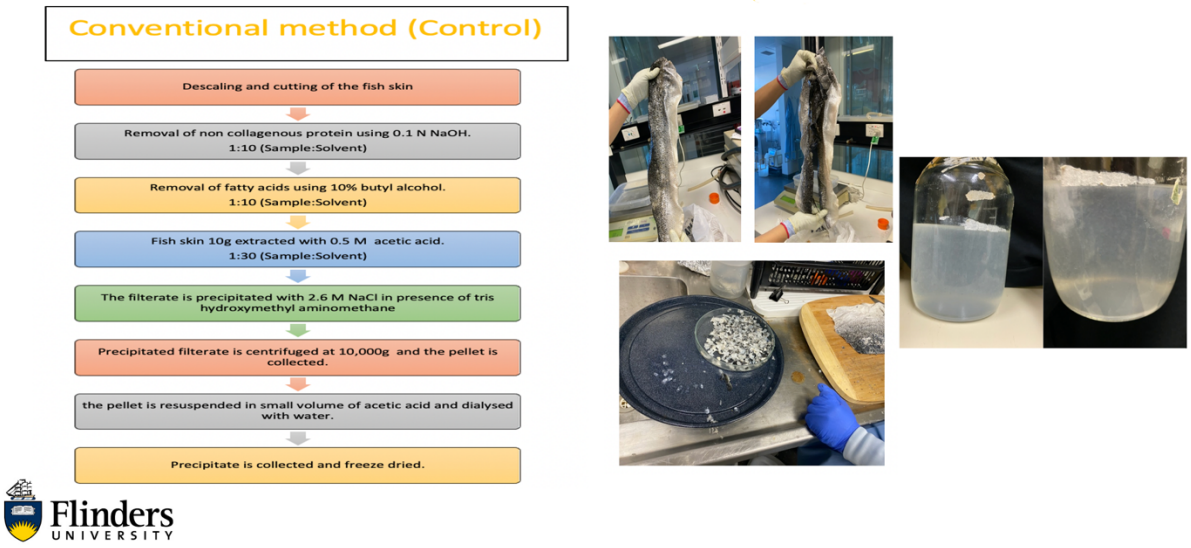
9. APPENDICES

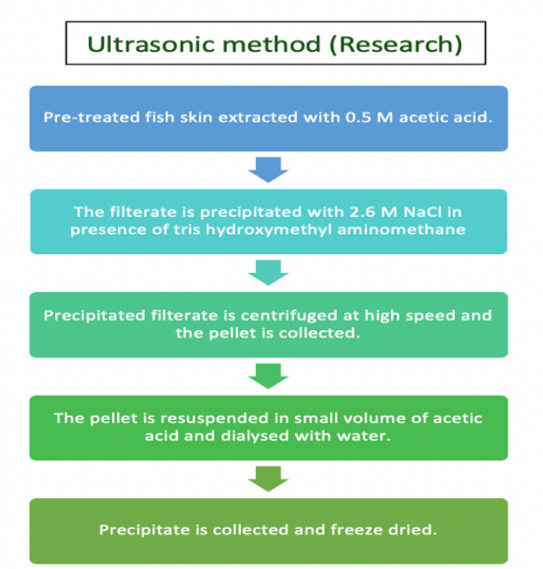
Appendix A

Flow chart for the extraction of conventional method for collagen extraction used in the industrial sector.



Appendix B

Flow chart for the extraction of ultrasound-assisted method for collagen extraction.



https://www.sonics.com/liquid-processing/products/vibra-cell-processors/vcx-500-vcx-750/

Flinders UNIVERSITY

Appendix C

1. Test for normal distribution

1.1 Test for normal distribution of yield with different amplitudes

```
> yield_a = c(43.05, 43.55, 46, 46.4)
> amplitude = c(100, 100, 80, 80)
> data_a = data.frame(amplitude, yield_a)
> shapiro.test(data_a$yield_a)
```

Shapiro-Wilk normality test

data: data_a$yield_a

W = 0.85239, p-value = 0.234 ==>Normal distribution

1.2 Test for normal distribution of yield with different concentrations

```
> yield_c = c(43.55, 44.5, 55, 49.35)
> concentration = c(1, 1, 0.5, 0.5)
> data_c = data.frame(concentration, yield_c)
> shapiro.test(data_c$yield_c)
```

Shapiro-Wilk normality test

data: data_c$yield_c

W = 0.90869, p-value = 0.4755 ==> Normal distribution

1.3 Test for normal distribution of yield with different ratios

```
> yield_r = c(47.5, 46, 45, 55, 49.75, 49.8, 43, 41.7)
> ratio = c(30, 30, 20, 20, 15, 15, 10, 10)
> data_r = data.frame(ratio, yield_r)
> shapiro.test(data_r$yield_r)

        Shapiro-Wilk normality test

data:  data_r$yield_r
W = 0.96242, p-value = 0.8328
```

==> Normal distribution

1.4 Test for normal distribution of yield with different extraction time

```
> yield_t = c(46, 47.5, 45.65, 44, 42.15, 41.5, 38.05, 39)
> time = c(30, 30, 45, 45, 60, 60, 120, 120)
> data_t = data.frame(time, yield_t)
> shapiro.test(data_t$yield_t)

        Shapiro-Wilk normality test

data:  data_t$yield_t
W = 0.95106, p-value = 0.7219
```

==> Normal distribution

Appendix D

1. **Homogeneity of variance**

1.1 Comparing two variances (two groups)

a. Extraction with 2 different applitudes

var.test(yield_a ~ amplitude, data = data_a)

Welch Two Sample t-test

data: yield_a by amplitude

F = 0.64, num df = 1, denom df = 1, p-value = 0.8591 ==>No significant difference between the two variances

alternative hypothesis: true ratio of variances is not equal to 1

95 percent confidence interval:

9.87976e-04 4.14585e+02

sample estimates:

ratio of variances

0.64

b. Extraction with 2 different concentrations

var.test(yield_c ~ concentration, data = data_c)

F test to compare two variances

data: yield_c by concentration

F = 35.371, num df = 1, denom df = 1, p-value = 0.2121 ==>No significant difference between the two variances

alternative hypothesis: true ratio of variances is not equal to 1

95 percent confidence interval:

 5.460295e-02 2.291307e+04

sample estimates:

ratio of variances

        35.37119

1.2 Comparing multiple variances (more than two groups)

a. Extraction with different ratios

bartlett.test(yield_r ~ ratio, data = data_r)

        Bartlett test of homogeneity of variances

data: yield_r by ratio

Bartlett's K-squared = 9.2335, df = 3, p-value = 0.02634 ==>Significant difference among the variances

b. Extraction with different extraction time

bartlett.test(yield_t ~ time, data = data_t)

        Bartlett test of homogeneity of variances

data: yield_t by time

Bartlett's K-squared = 0.68266, df = 3, p-value = 0.8773 ==>No significant difference among the variances

Appendix E

1. **Analysis of variance**

1.1 Comparison of two means (two groups)

a. Extraction with 2 different applitudes

t.test(yield_a ~ amplitude, data = data_a)

Welch Two Sample t-test

data: yield_a by amplitude

t = 9.0581, df = 1.9081, p-value = 0.01385 ==>Significant difference

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

1.456874 4.343126

sample estimates:

mean in group 80 mean in group 100

46.2 43.3

b. Extraction with 2 different concentrations

t.test(yield_c ~ concentration, data = data_c)

Welch Two Sample t-test

data: yield_c by concentration

t = 2.845, df = 1.0565, p-value = 0.2043 ==>No significant difference

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-23.95651 40.25651

sample estimates:

mean in group 0.5 mean in group 1

52.175 44.025

1.2 Comparison of multiple means (more than two groups)

a. Extraction with different ratios

Kruskal-Wallis rank sum test

data: yield_r by ratio

Kruskal-Wallis chi-squared = 4.6667, df = 3, p-value = 0.1979 ==>No significant difference

b. Extraction with different extraction time

> av_t = aov(yield_t ~ time, data = data_t)

> summary(av_t)

Df Sum Sq Mean Sq F value Pr(>F)

time 1 71.34 71.34 45.53 0.000517 *** ==>Significant difference

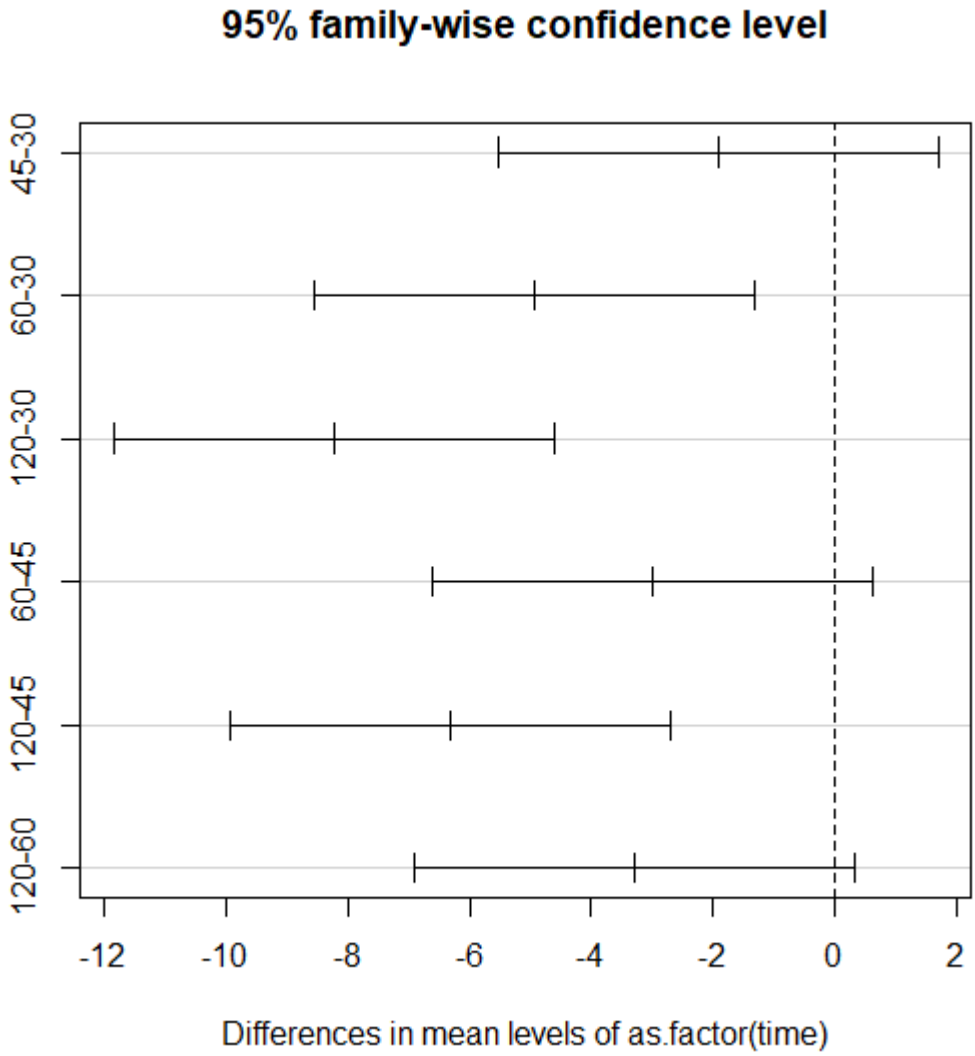
Residuals 6 9.40 1.57

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix F

1. **Posthoc analysis**

```
> av_t = aov(yield_t ~ as.factor(time), data = data_t)

> TukeyHSD(av_t)

  Tukey multiple comparisons of means

    95% family-wise confidence level


Fit: aov(formula = yield_t ~ as.factor(time), data = data_t)


$`as.factor(time)`

         diff        lwr        upr     p adj

45-30  -1.925  -5.536809  1.6868086 0.2732808

60-30  -4.925  -8.536809 -1.3131914 0.0175033  ==>Significant difference

120-30 -8.225 -11.836809 -4.6131914 0.0026246 ==>Significant difference

60-45  -3.000  -6.611809  0.6118086 0.0885645

120-45 -6.300  -9.911809 -2.6881914 0.0071684 ==>Significant difference

120-60 -3.300  -6.911809  0.3118086 0.0664432
```


95% family-wise confidence level
45-30
60-30
120-30
60-45
120-45
120-60
-12
-10
-8
-6
-4
-2
0
2
Differences in mean levels of as.factor(time)

Appendix G

Economic Evaluation Report

for Convention_1470kg_109USD

February 1, 2021

1. EXECUTIVE SUMMARY (2020 prices)

Total Capital Investment	13,990,000 $
Capital Investment Charged to This Project	13,990,000 $
Operating Cost	2,820,000 $/yr
Revenues	439,000 $/yr
Batch Size	18.58 kg MP
Cost Basis Annual Rate	4,031 kg MP/yr
Unit Production Cost	699.66 $/kg MP
Net Unit Production Cost	699.66 $/kg MP
Unit Production Revenue	109.00 $/kg MP
Gross Margin	- 541.89 %
Return On Investment	- 7.99 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	- 21,198,000 $

MP = Total Flow of Stream 'Collagen'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2020 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	82,000	82,000
		Rated Throughput = 720.30 kg/h		
2 / 0 / 0	V-101	Blending Tank	279,000	558,000
		Vessel Volume = 8819.23 L		
1 / 0 / 0	PFF-101	Plate & Frame Filter	140,000	140,000
		Filter Area = 38.76 m2		
1 / 0 / 0	V-102	Blending Tank	240,000	240,000
		Vessel Volume = 3014.45 L		
1 / 0 / 0	PFF-102	Plate & Frame Filter	31,000	31,000
		Filter Area = 6.26 m2		
1 / 0 / 0	V-103	Blending Tank	267,000	267,000
		Vessel Volume = 6439.28 L		
1 / 0 / 0	PFF-103	Plate & Frame Filter	57,000	57,000
		Filter Area = 13.97 m2		
1 / 0 / 0	V-104	Blending Tank	269,000	269,000
		Vessel Volume = 6876.45 L		
1 / 0 / 0	PFF-104	Plate & Frame Filter	62,000	62,000
		Filter Area = 15.41 m2		
1 / 0 / 0	TDR-101	Tray Dryer	70,000	70,000
		Tray Area = 1.20 m2		
		Unlisted Equipment		444,000
			TOTAL	2,220,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2020 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	2,220,000
2. Installation	833,000
3. Process Piping	777,000
4. Instrumentation	888,000
5. Insulation	67,000
6. Electrical	222,000
7. Buildings	999,000
8. Yard Improvement	333,000
9. Auxiliary Facilities	888,000
TPDC	7,226,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	1,807,000
11. Construction	2,529,000
TPIC	4,336,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	11,562,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	578,000
13. Contingency	1,156,000
CFC = 12+13	1,734,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	13,296,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	41.72	5,816	242,652	100.00
TOTAL		5,816	242,652	100.00

5. MATERIALS COST - PROCESS SUMMARY

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Air	0.000	13,250	kg	0	0.00
Butanol 10%	0.053	518,786	kg	27,236	44.67
CH3COOH (0.5 M)	0.019	1,212,071	kg	22,726	37.27
Fish skins	0.000	318,990	kg	0	0.00
NaOH 0.1M	0.000	3,119,505	kg	892	1.46
Sodium Chloride	50.000	183	MT	9,168	15.04
Water	0.803	1,184	m3(STP)	951	1.56
TOTAL				60,974	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

6. VARIOUS CONSUMABLES COST (2020 prices) - PROCESS SUMMARY

THE CONSUMABLES COST IS ZERO.

7. WASTE TREATMENT/DISPOSAL COST (2020 prices) - PROCESS SUMMARY

THE TOTAL WASTE TREATMENT/DISPOSAL COST IS ZERO.

8. UTILITIES COST (2020 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.100	46,697	kW-h	4,670	38.11
Steam	12.000	2	MT	27	0.22
Glycol	0.350	21,592	MT	7,557	61.67
TOTAL				12,254	100.00

9. ANNUAL OPERATING COST (2020 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	61,000	2.16
Labor-Dependent	243,000	8.60
Facility-Dependent	2,504,000	88.80
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	12,000	0.43
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	2,820,000	100.00

10. PROFITABILITY ANALYSIS (2020 prices)

A.	Direct Fixed Capital	13,296,000 $
B.	Working Capital	29,000 $
C.	Startup Cost	665,000 $
D.	Up-Front R&D	0 $
E.	Up-Front Royalties	0 $
F.	Total Investment (A+B+C+D+E)	13,990,000 $
G.	Investment Charged to This Project	13,990,000 $
H.	**Revenue/Savings Rates**	
	Collagen (Main Revenue)	4,031 kg /yr
I.	**Revenue/Savings Price**	
	Collagen (Main Revenue)	109.00 $/kg
J.	**Revenues/Savings**	
	Collagen (Main Revenue)	439,382 $/yr
1	Total Revenues	439,382 $/yr
2	Total Savings	0 $/yr
K.	**Annual Operating Cost (AOC)**	
1	Actual AOC	2,820,000 $/yr
2	Net AOC (K1-J2)	2,820,000 $/yr
L.	**Unit Production Cost /Revenue**	
	Unit Production Cost	699.66 $/kg MP
	Net Unit Production Cost	699.66 $/kg MP
	Unit Production Revenue	109.00 $/kg MP
M.	Gross Profit (J-K)	- 2,381,000 $/yr
N.	Taxes (40%)	0 $/yr
O.	Net Profit (M-N + Depreciation)	- 1,118,000 $/yr
	Gross Margin	- 541.89 %
	Return On Investment	- 7.99 %
	Payback Time	N/A

MP = Total Flow of Stream 'Collagen'

Appendix H

Economic Evaluation Report

for Ultrasound_1470kg_109USD

February 1, 2021

1. EXECUTIVE SUMMARY (2020 prices)

Total Capital Investment	13,733,000 $
Capital Investment Charged to This Project	13,733,000 $
Operating Cost	2,715,000 $/yr
Revenues	631,000 $/yr
Batch Size	26.54 kg MP
Cost Basis Annual Rate	5,785 kg MP/yr
Unit Production Cost	469.25 $/kg MP
Net Unit Production Cost	469.25 $/kg MP
Unit Production Revenue	109.00 $/kg MP
Gross Margin	- 330.51 %
Return On Investment	- 6.14 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	- 18,980,000 $

MP = Total Flow of Stream 'Collagen'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2020 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	82,000	82,000
		Rated Throughput = 720.30 kg/h		
2 / 0 / 0	V-101	Blending Tank	279,000	558,000
		Vessel Volume = 8819.23 L		
1 / 0 / 0	PFF-101	Plate & Frame Filter	140,000	140,000
		Filter Area = 38.76 m2		
1 / 0 / 0	V-102	Blending Tank	240,000	240,000
		Vessel Volume = 3014.45 L		
1 / 0 / 0	PFF-102	Plate & Frame Filter	31,000	31,000
		Filter Area = 6.26 m2		
1 / 0 / 0	V-103	Blending Tank	281,000	281,000
		Vessel Volume = 4362.37 L		
1 / 0 / 0	PFF-103	Plate & Frame Filter	41,000	41,000
		Filter Area = 9.36 m2		
1 / 0 / 0	V-104	Blending Tank	256,000	256,000
		Vessel Volume = 4816.01 L		
1 / 0 / 0	PFF-104	Plate & Frame Filter	46,000	46,000
		Filter Area = 10.75 m2		
1 / 0 / 0	TDR-101	Tray Dryer	70,000	70,000
		Tray Area = 1.70 m2		
		Unlisted Equipment		436,000
			TOTAL	2,182,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2020 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	2,182,000
2. Installation	813,000
3. Process Piping	764,000
4. Instrumentation	873,000
5. Insulation	65,000
6. Electrical	218,000
7. Buildings	982,000
8. Yard Improvement	327,000
9. Auxiliary Facilities	873,000
TPDC	7,096,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	1,774,000
11. Construction	2,484,000
TPIC	4,258,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	11,353,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	568,000
13. Contingency	1,135,000
CFC = 12+13	1,703,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	13,057,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	41.72	4,560	190,265	100.00
TOTAL		4,560	190,265	100.00

5. MATERIALS COST - PROCESS SUMMARY

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Air	0.000	18,451	kg	0	0.00
Butanol 10%	0.053	521,177	kg	27,362	51.00
CH3COOH (0.5 M)	0.019	811,771	kg	15,221	28.37
Fish skins	0.000	320,460	kg	0	0.00
NaOH 0.1M	0.000	3,133,880	kg	896	1.67
Sodium Chloride	50.000	184	MT	9,210	17.17
Water	0.803	1,198	m3(STP)	962	1.79
TOTAL				53,651	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

6. VARIOUS CONSUMABLES COST (2020 prices) - PROCESS SUMMARY

THE CONSUMABLES COST IS ZERO.

7. WASTE TREATMENT/DISPOSAL COST (2020 prices) - PROCESS SUMMARY

THE TOTAL WASTE TREATMENT/DISPOSAL COST IS ZERO.

8. UTILITIES COST (2020 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.100	43,205	kW-h	4,320	38.04
Steam	12.000	3	MT	38	0.34
Glycol	0.350	19,999	MT	7,000	61.63
TOTAL				11,358	100.00

9. ANNUAL OPERATING COST (2020 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	54,000	1.98
Labor-Dependent	190,000	7.01
Facility-Dependent	2,459,000	90.60
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	11,000	0.42
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	2,715,000	100.00

10. PROFITABILITY ANALYSIS (2020 prices)

A.	Direct Fixed Capital	13,057,000 $
B.	Working Capital	23,000 $
C.	Startup Cost	653,000 $
D.	Up-Front R&D	0 $
E.	Up-Front Royalties	0 $
F.	Total Investment (A+B+C+D+E)	13,733,000 $
G.	Investment Charged to This Project	13,733,000 $
H.	**Revenue/Savings Rates**	
	Collagen (Main Revenue)	5,785 kg /yr
I.	**Revenue/Savings Price**	
	Collagen (Main Revenue)	109.00 $/kg
J.	**Revenues/Savings**	
	Collagen (Main Revenue)	630,581 $/yr
1	Total Revenues	630,581 $/yr
2	Total Savings	0 $/yr
K.	**Annual Operating Cost (AOC)**	
1	Actual AOC	2,715,000 $/yr
2	Net AOC (K1-J2)	2,715,000 $/yr
L.	**Unit Production Cost /Revenue**	
	Unit Production Cost	469.25 $/kg MP
	Net Unit Production Cost	469.25 $/kg MP
	Unit Production Revenue	109.00 $/kg MP
M.	Gross Profit (J-K)	- 2,085,000 $/yr
N.	Taxes (40%)	0 $/yr
O.	Net Profit (M-N + Depreciation)	- 844,000 $/yr
	Gross Margin	- 330.51 %
	Return On Investment	- 6.14 %
	Payback Time	N/A

MP = Total Flow of Stream 'Collagen'

Appendix I

Economic Evaluation Report

for Convention_1470kg_219USD

February 1, 2021

1. EXECUTIVE SUMMARY (2020 prices)

Total Capital Investment	13,990,000 $
Capital Investment Charged to This Project	13,990,000 $
Operating Cost	2,820,000 $/yr
Revenues	883,000 $/yr
Batch Size	18.58 kg MP
Cost Basis Annual Rate	4,031 kg MP/yr
Unit Production Cost	699.66 $/kg MP
Net Unit Production Cost	699.66 $/kg MP
Unit Production Revenue	219.00 $/kg MP
Gross Margin	- 219.48 %
Return On Investment	- 4.82 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	- 18,057,000 $

MP = Total Flow of Stream 'Collagen'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2020 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder Rated Throughput = 720.30 kg/h	82,000	82,000
2 / 0 / 0	V-101	Blending Tank Vessel Volume = 8819.23 L	279,000	558,000
1 / 0 / 0	PFF-101	Plate & Frame Filter Filter Area = 38.76 m2	140,000	140,000
1 / 0 / 0	V-102	Blending Tank Vessel Volume = 3014.45 L	240,000	240,000
1 / 0 / 0	PFF-102	Plate & Frame Filter Filter Area = 6.26 m2	31,000	31,000
1 / 0 / 0	V-103	Blending Tank Vessel Volume = 6439.28 L	267,000	267,000
1 / 0 / 0	PFF-103	Plate & Frame Filter Filter Area = 13.97 m2	57,000	57,000
1 / 0 / 0	V-104	Blending Tank Vessel Volume = 6876.45 L	269,000	269,000
1 / 0 / 0	PFF-104	Plate & Frame Filter Filter Area = 15.41 m2	62,000	62,000
1 / 0 / 0	TDR-101	Tray Dryer Tray Area = 1.20 m2	70,000	70,000
		Unlisted Equipment		444,000
			TOTAL	2,220,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2020 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	2,220,000
2. Installation	833,000
3. Process Piping	777,000
4. Instrumentation	888,000
5. Insulation	67,000
6. Electrical	222,000
7. Buildings	999,000
8. Yard Improvement	333,000
9. Auxiliary Facilities	888,000
TPDC	7,226,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	1,807,000
11. Construction	2,529,000
TPIC	4,336,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	11,562,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	578,000
13. Contingency	1,156,000
CFC = 12+13	1,734,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	13,296,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	41.72	5,816	242,652	100.00
TOTAL		5,816	242,652	100.00

5. MATERIALS COST - PROCESS SUMMARY

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Air	0.000	13,250	kg	0	0.00
Butanol 10%	0.053	518,786	kg	27,236	44.67
CH3COOH (0.5 M)	0.019	1,212,071	kg	22,726	37.27
Fish skins	0.000	318,990	kg	0	0.00
NaOH 0.1M	0.000	3,119,505	kg	892	1.46
Sodium Chloride	50.000	183	MT	9,168	15.04
Water	0.803	1,184	m3(STP)	951	1.56
TOTAL				60,974	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

6. VARIOUS CONSUMABLES COST (2020 prices) - PROCESS SUMMARY

THE CONSUMABLES COST IS ZERO.

7. WASTE TREATMENT/DISPOSAL COST (2020 prices) - PROCESS SUMMARY

THE TOTAL WASTE TREATMENT/DISPOSAL COST IS ZERO.

8. UTILITIES COST (2020 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.100	46,697	kW-h	4,670	38.11
Steam	12.000	2	MT	27	0.22
Glycol	0.350	21,592	MT	7,557	61.67
TOTAL				12,254	100.00

9. ANNUAL OPERATING COST (2020 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	61,000	2.16
Labor-Dependent	243,000	8.60
Facility-Dependent	2,504,000	88.80
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	12,000	0.43
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	2,820,000	100.00

10. PROFITABILITY ANALYSIS (2020 prices)

A.	Direct Fixed Capital	13,296,000 $
B.	Working Capital	29,000 $
C.	Startup Cost	665,000 $
D.	Up-Front R&D	0 $
E.	Up-Front Royalties	0 $
F.	Total Investment (A+B+C+D+E)	13,990,000 $
G.	Investment Charged to This Project	13,990,000 $
H.	**Revenue/Savings Rates**	
	Collagen (Main Revenue)	4,031 kg /yr
I.	**Revenue/Savings Price**	
	Collagen (Main Revenue)	219.00 $/kg
J.	**Revenues/Savings**	
	Collagen (Main Revenue)	882,795 $/yr
1	Total Revenues	882,795 $/yr
2	Total Savings	0 $/yr
K.	**Annual Operating Cost (AOC)**	
1	Actual AOC	2,820,000 $/yr
2	Net AOC (K1-J2)	2,820,000 $/yr
L.	**Unit Production Cost /Revenue**	
	Unit Production Cost	699.66 $/kg MP
	Net Unit Production Cost	699.66 $/kg MP
	Unit Production Revenue	219.00 $/kg MP
M.	Gross Profit (J-K)	- 1,938,000 $/yr
N.	Taxes (40%)	0 $/yr
O.	Net Profit (M-N + Depreciation)	- 675,000 $/yr
	Gross Margin	- 219.48 %
	Return On Investment	- 4.82 %
	Payback Time	N/A

MP = Total Flow of Stream 'Collagen'

Appendix J

Economic Evaluation Report

for Ultrasound_1470kg_219USD

February 1, 2021

1. EXECUTIVE SUMMARY (2020 prices)

Total Capital Investment	13,733,000 $
Capital Investment Charged to This Project	13,733,000 $
Operating Cost	2,715,000 $/yr
Revenues	1,267,000 $/yr
Batch Size	26.54 kg MP
Cost Basis Annual Rate	5,785 kg MP/yr
Unit Production Cost	469.25 $/kg MP
Net Unit Production Cost	469.25 $/kg MP
Unit Production Revenue	219.00 $/kg MP
Gross Margin	- 114.27 %
Return On Investment	- 1.51 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	- 14,472,000 $

MP = Total Flow of Stream 'Collagen'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2020 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	82,000	82,000
		Rated Throughput = 720.30 kg/h		
2 / 0 / 0	V-101	Blending Tank	279,000	558,000
		Vessel Volume = 8819.23 L		
1 / 0 / 0	PFF-101	Plate & Frame Filter	140,000	140,000
		Filter Area = 38.76 m2		
1 / 0 / 0	V-102	Blending Tank	240,000	240,000
		Vessel Volume = 3014.45 L		
1 / 0 / 0	PFF-102	Plate & Frame Filter	31,000	31,000
		Filter Area = 6.26 m2		
1 / 0 / 0	V-103	Blending Tank	281,000	281,000
		Vessel Volume = 4362.37 L		
1 / 0 / 0	PFF-103	Plate & Frame Filter	41,000	41,000
		Filter Area = 9.36 m2		
1 / 0 / 0	V-104	Blending Tank	256,000	256,000
		Vessel Volume = 4816.01 L		
1 / 0 / 0	PFF-104	Plate & Frame Filter	46,000	46,000
		Filter Area = 10.75 m2		
1 / 0 / 0	TDR-101	Tray Dryer	70,000	70,000
		Tray Area = 1.70 m2		
		Unlisted Equipment		436,000
			TOTAL	2,182,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2020 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	2,182,000
2. Installation	813,000
3. Process Piping	764,000
4. Instrumentation	873,000
5. Insulation	65,000
6. Electrical	218,000
7. Buildings	982,000
8. Yard Improvement	327,000
9. Auxiliary Facilities	873,000
TPDC	7,096,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	1,774,000
11. Construction	2,484,000
TPIC	4,258,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	11,353,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	568,000
13. Contingency	1,135,000
CFC = 12+13	1,703,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	13,057,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	41.72	4,560	190,265	100.00
TOTAL		4,560	190,265	100.00

5. MATERIALS COST - PROCESS SUMMARY

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Air	0.000	18,451	kg	0	0.00
Butanol 10%	0.053	521,177	kg	27,362	51.00
CH3COOH (0.5 M)	0.019	811,771	kg	15,221	28.37
Fish skins	0.000	320,460	kg	0	0.00
NaOH 0.1M	0.000	3,133,880	kg	896	1.67
Sodium Chloride	50.000	184	MT	9,210	17.17
Water	0.803	1,198	m3(STP)	962	1.79
TOTAL				53,651	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

6. VARIOUS CONSUMABLES COST (2020 prices) - PROCESS SUMMARY

THE CONSUMABLES COST IS ZERO.

7. WASTE TREATMENT/DISPOSAL COST (2020 prices) - PROCESS SUMMARY

THE TOTAL WASTE TREATMENT/DISPOSAL COST IS ZERO.

8. UTILITIES COST (2020 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.100	43,205	kW-h	4,320	38.04
Steam	12.000	3	MT	38	0.34
Glycol	0.350	19,999	MT	7,000	61.63
TOTAL				11,358	100.00

9. ANNUAL OPERATING COST (2020 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	54,000	1.98
Labor-Dependent	190,000	7.01
Facility-Dependent	2,459,000	90.60
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	11,000	0.42
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	2,715,000	100.00

10. PROFITABILITY ANALYSIS (2020 prices)

A.	Direct Fixed Capital	13,057,000 $
B.	Working Capital	23,000 $
C.	Startup Cost	653,000 $
D.	Up-Front R&D	0 $
E.	Up-Front Royalties	0 $
F.	Total Investment (A+B+C+D+E)	13,733,000 $
G.	Investment Charged to This Project	13,733,000 $
H.	**Revenue/Savings Rates**	
	Collagen (Main Revenue)	5,785 kg /yr
I.	**Revenue/Savings Price**	
	Collagen (Main Revenue)	219.00 $/kg
J.	**Revenues/Savings**	
	Collagen (Main Revenue)	1,266,947 $/yr
1	Total Revenues	1,266,947 $/yr
2	Total Savings	0 $/yr
K.	**Annual Operating Cost (AOC)**	
1	Actual AOC	2,715,000 $/yr
2	Net AOC (K1-J2)	2,715,000 $/yr
L.	**Unit Production Cost /Revenue**	
	Unit Production Cost	469.25 $/kg MP
	Net Unit Production Cost	469.25 $/kg MP
	Unit Production Revenue	219.00 $/kg MP
M.	Gross Profit (J-K)	- 1,448,000 $/yr
N.	Taxes (40%)	0 $/yr
O.	Net Profit (M-N + Depreciation)	- 208,000 $/yr
	Gross Margin	- 114.27 %
	Return On Investment	- 1.51 %
	Payback Time	N/A

MP = Total Flow of Stream 'Collagen'

Appendix K

Economic Evaluation Report

for Convention_1470kg_510USD

February 1, 2021

1. EXECUTIVE SUMMARY (2020 prices)

Total Capital Investment	13,990,000 $
Capital Investment Charged to This Project	13,990,000 $
Operating Cost	2,820,000 $/yr
Revenues	2,056,000 $/yr
Batch Size	18.58 kg MP
Cost Basis Annual Rate	4,031 kg MP/yr
Unit Production Cost	699.66 $/kg MP
Net Unit Production Cost	699.66 $/kg MP
Unit Production Revenue	510.00 $/kg MP
Gross Margin	- 37.19 %
Return On Investment	3.56 %
Payback Time	28.06 years
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	- 9,997,000 $

MP = Total Flow of Stream 'Collagen'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2020 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	82,000	82,000
		Rated Throughput = 720.30 kg/h		
2 / 0 / 0	V-101	Blending Tank	279,000	558,000
		Vessel Volume = 8819.23 L		
1 / 0 / 0	PFF-101	Plate & Frame Filter	140,000	140,000
		Filter Area = 38.76 m2		
1 / 0 / 0	V-102	Blending Tank	240,000	240,000
		Vessel Volume = 3014.45 L		
1 / 0 / 0	PFF-102	Plate & Frame Filter	31,000	31,000
		Filter Area = 6.26 m2		
1 / 0 / 0	V-103	Blending Tank	267,000	267,000
		Vessel Volume = 6439.28 L		
1 / 0 / 0	PFF-103	Plate & Frame Filter	57,000	57,000
		Filter Area = 13.97 m2		
1 / 0 / 0	V-104	Blending Tank	269,000	269,000
		Vessel Volume = 6876.45 L		
1 / 0 / 0	PFF-104	Plate & Frame Filter	62,000	62,000
		Filter Area = 15.41 m2		
1 / 0 / 0	TDR-101	Tray Dryer	70,000	70,000
		Tray Area = 1.20 m2		
		Unlisted Equipment		444,000
			TOTAL	2,220,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2020 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	2,220,000
2. Installation	833,000
3. Process Piping	777,000
4. Instrumentation	888,000
5. Insulation	67,000
6. Electrical	222,000
7. Buildings	999,000
8. Yard Improvement	333,000
9. Auxiliary Facilities	888,000
TPDC	7,226,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	1,807,000
11. Construction	2,529,000
TPIC	4,336,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	11,562,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	578,000
13. Contingency	1,156,000
CFC = 12+13	1,734,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	13,296,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	41.72	5,816	242,652	100.00
TOTAL		5,816	242,652	100.00

5. MATERIALS COST - PROCESS SUMMARY

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Air	0.000	13,250	kg	0	0.00
Butanol 10%	0.053	518,786	kg	27,236	44.67
CH3COOH (0.5 M)	0.019	1,212,071	kg	22,726	37.27
Fish skins	0.000	318,990	kg	0	0.00
NaOH 0.1M	0.000	3,119,505	kg	892	1.46
Sodium Chloride	50.000	183	MT	9,168	15.04
Water	0.803	1,184	m3(STP)	951	1.56
TOTAL				60,974	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

6. VARIOUS CONSUMABLES COST (2020 prices) - PROCESS SUMMARY

THE CONSUMABLES COST IS ZERO.

7. WASTE TREATMENT/DISPOSAL COST (2020 prices) - PROCESS SUMMARY

THE TOTAL WASTE TREATMENT/DISPOSAL COST IS ZERO.

8. UTILITIES COST (2020 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.100	46,697	kW-h	4,670	38.11
Steam	12.000	2	MT	27	0.22
Glycol	0.350	21,592	MT	7,557	61.67
TOTAL				12,254	100.00

9. ANNUAL OPERATING COST (2020 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	61,000	2.16
Labor-Dependent	243,000	8.60
Facility-Dependent	2,504,000	88.80
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	12,000	0.43
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	2,820,000	100.00

10. PROFITABILITY ANALYSIS (2020 prices)

A.	Direct Fixed Capital	13,296,000 $
B.	Working Capital	29,000 $
C.	Startup Cost	665,000 $
D.	Up-Front R&D	0 $
E.	Up-Front Royalties	0 $
F.	Total Investment (A+B+C+D+E)	13,990,000 $
G.	Investment Charged to This Project	13,990,000 $
H.	**Revenue/Savings Rates**	
	Collagen (Main Revenue)	4,031 kg /yr
I.	**Revenue/Savings Price**	
	Collagen (Main Revenue)	510.00 $/kg
J.	**Revenues/Savings**	
	Collagen (Main Revenue)	2,055,823 $/yr
1	Total Revenues	2,055,823 $/yr
2	Total Savings	0 $/yr
K.	**Annual Operating Cost (AOC)**	
1	Actual AOC	2,820,000 $/yr
2	Net AOC (K1-J2)	2,820,000 $/yr
L.	**Unit Production Cost /Revenue**	
	Unit Production Cost	699.66 $/kg MP
	Net Unit Production Cost	699.66 $/kg MP
	Unit Production Revenue	510.00 $/kg MP
M.	Gross Profit (J-K)	- 765,000 $/yr
N.	Taxes (40%)	0 $/yr
O.	Net Profit (M-N + Depreciation)	499,000 $/yr
	Gross Margin	- 37.19 %
	Return On Investment	3.56 %
	Payback Time	28.06 years

MP = Total Flow of Stream 'Collagen'

Appendix L

Economic Evaluation Report

for Ultrasound_1470kg_510USD

February 1, 2021

1. EXECUTIVE SUMMARY (2020 prices)

Total Capital Investment	13,733,000 $
Capital Investment Charged to This Project	13,733,000 $
Operating Cost	2,715,000 $/yr
Revenues	2,950,000 $/yr
Batch Size	26.54 kg MP
Cost Basis Annual Rate	5,785 kg MP/yr
Unit Production Cost	469.25 $/kg MP
Net Unit Production Cost	469.25 $/kg MP
Unit Production Revenue	510.00 $/kg MP
Gross Margin	7.99 %
Return On Investment	10.06 %
Payback Time	9.94 years
IRR (After Taxes)	1.80 %
NPV (at 7.0% Interest)	- 3,744,000 $

MP = Total Flow of Stream 'Collagen'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2020 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder Rated Throughput = 720.30 kg/h	82,000	82,000
2 / 0 / 0	V-101	Blending Tank Vessel Volume = 8819.23 L	279,000	558,000
1 / 0 / 0	PFF-101	Plate & Frame Filter Filter Area = 38.76 m2	140,000	140,000
1 / 0 / 0	V-102	Blending Tank Vessel Volume = 3014.45 L	240,000	240,000
1 / 0 / 0	PFF-102	Plate & Frame Filter Filter Area = 6.26 m2	31,000	31,000
1 / 0 / 0	V-103	Blending Tank Vessel Volume = 4362.37 L	281,000	281,000
1 / 0 / 0	PFF-103	Plate & Frame Filter Filter Area = 9.36 m2	41,000	41,000
1 / 0 / 0	V-104	Blending Tank Vessel Volume = 4816.01 L	256,000	256,000
1 / 0 / 0	PFF-104	Plate & Frame Filter Filter Area = 10.75 m2	46,000	46,000
1 / 0 / 0	TDR-101	Tray Dryer Tray Area = 1.70 m2	70,000	70,000
		Unlisted Equipment		436,000
			TOTAL	2,182,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2020 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	2,182,000
2. Installation	813,000
3. Process Piping	764,000
4. Instrumentation	873,000
5. Insulation	65,000
6. Electrical	218,000
7. Buildings	982,000
8. Yard Improvement	327,000
9. Auxiliary Facilities	873,000
TPDC	7,096,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	1,774,000
11. Construction	2,484,000
TPIC	4,258,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	11,353,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	568,000
13. Contingency	1,135,000
CFC = 12+13	1,703,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	13,057,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	41.72	4,560	190,265	100.00
TOTAL		4,560	190,265	100.00

5. MATERIALS COST - PROCESS SUMMARY

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Air	0.000	18,451	kg	0	0.00
Butanol 10%	0.053	521,177	kg	27,362	51.00
CH3COOH (0.5 M)	0.019	811,771	kg	15,221	28.37
Fish skins	0.000	320,460	kg	0	0.00
NaOH 0.1M	0.000	3,133,880	kg	896	1.67
Sodium Chloride	50.000	184	MT	9,210	17.17
Water	0.803	1,198	m3(STP)	962	1.79
TOTAL				53,651	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

6. VARIOUS CONSUMABLES COST (2020 prices) - PROCESS SUMMARY

THE CONSUMABLES COST IS ZERO.

7. WASTE TREATMENT/DISPOSAL COST (2020 prices) - PROCESS SUMMARY

THE TOTAL WASTE TREATMENT/DISPOSAL COST IS ZERO.

8. UTILITIES COST (2020 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.100	43,205	kW-h	4,320	38.04
Steam	12.000	3	MT	38	0.34
Glycol	0.350	19,999	MT	7,000	61.63
TOTAL				11,358	100.00

9. ANNUAL OPERATING COST (2020 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	54,000	1.98
Labor-Dependent	190,000	7.01
Facility-Dependent	2,459,000	90.60
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	11,000	0.42
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	2,715,000	100.00

10. PROFITABILITY ANALYSIS (2020 prices)

A.	Direct Fixed Capital	13,057,000 $
B.	Working Capital	23,000 $
C.	Startup Cost	653,000 $
D.	Up-Front R&D	0 $
E.	Up-Front Royalties	0 $
F.	Total Investment (A+B+C+D+E)	13,733,000 $
G.	Investment Charged to This Project	13,733,000 $
H.	**Revenue/Savings Rates**	
	Collagen (Main Revenue)	5,785 kg /yr
I.	**Revenue/Savings Price**	
	Collagen (Main Revenue)	510.00 $/kg
J.	**Revenues/Savings**	
	Collagen (Main Revenue)	2,950,425 $/yr
1	Total Revenues	2,950,425 $/yr
2	Total Savings	0 $/yr
K.	**Annual Operating Cost (AOC)**	
1	Actual AOC	2,715,000 $/yr
2	Net AOC (K1-J2)	2,715,000 $/yr
L.	**Unit Production Cost /Revenue**	
	Unit Production Cost	469.25 $/kg MP
	Net Unit Production Cost	469.25 $/kg MP
	Unit Production Revenue	510.00 $/kg MP
M.	Gross Profit (J-K)	236,000 $/yr
N.	Taxes (40%)	94,000 $/yr
O.	Net Profit (M-N + Depreciation)	1,382,000 $/yr
	Gross Margin	7.99 %
	Return On Investment	10.06 %
	Payback Time	9.94 years

MP = Total Flow of Stream 'Collagen'

10. Addendum:

Vortex Fluidic Device (VFD)-assisted extraction is based on the concept of dynamic thin film rotating tube processor (RTP). VFD in the past has contributed to the field of green chemistry, to improve and develop the process of transesterification (Britton et al., 2014). VFD uses a high-speed rotation, creating high shear stress which assists cell rupture, but efficacy depends on the tilt angle of the 20 mm external diameter rotating tube (θ). A VFD (Fig. 1) with a RTP of 6 cm diameter and tube length of 30 cm reduced the relative amount of solvent required and capital outlay, as well as offering control of the residence time of the liquid (Sitepu et al., 2018). The VFD operates under turbulent flow conditions. The VFD can be used in continuous flow as well as in confined mode (Britton et al., 2014). In confined mode, the VFD can operate with finite amount of liquid which is spun at high speed (1,000 – 3,500 rpm). For continuous flow operation, jet feeds are attached which deliver the liquid to the base of the tube under the same rotation conditions. With rapid rotation of the liquid in the tube, it forms a dynamic thin film of a thickness of *ca.* 200 μm. The thickness of the film formed depends on the tilt angle (θ), rotational speed, and flow rates or volume of the liquid when used in confined mode, where the Stewartson/Ekman layer arises from the liquid being driven up the rotating tube with gravity forcing the liquid back (Luo et al., 2016). The continuous flow mode of operation of the VFD imparts additional shear relative to the confined mode ($\theta > 0°$) which arises from the viscous drag as the liquid whirls along the tube prior to exiting at the top. Overall, the VFD is a versatile microfluidic platform which imparts controlled mechanical energy into the dynamic thin film (Luo et al., 2016). This controlled and energetically favourable processing platform has been applied to a number of biological and chemical processes such as refolding of proteins, synthesis of graphene from graphite, coating a magnetic responsive polymer or graphene onto

microalgae cells, synthesis of silica xerogel, microencapsulation of bacteria cells in graphene oxide, slicing of carbon nanotubes, controlling organic reactions, enhancing enzymatic reactions and more (Sitepu et al., 2018).

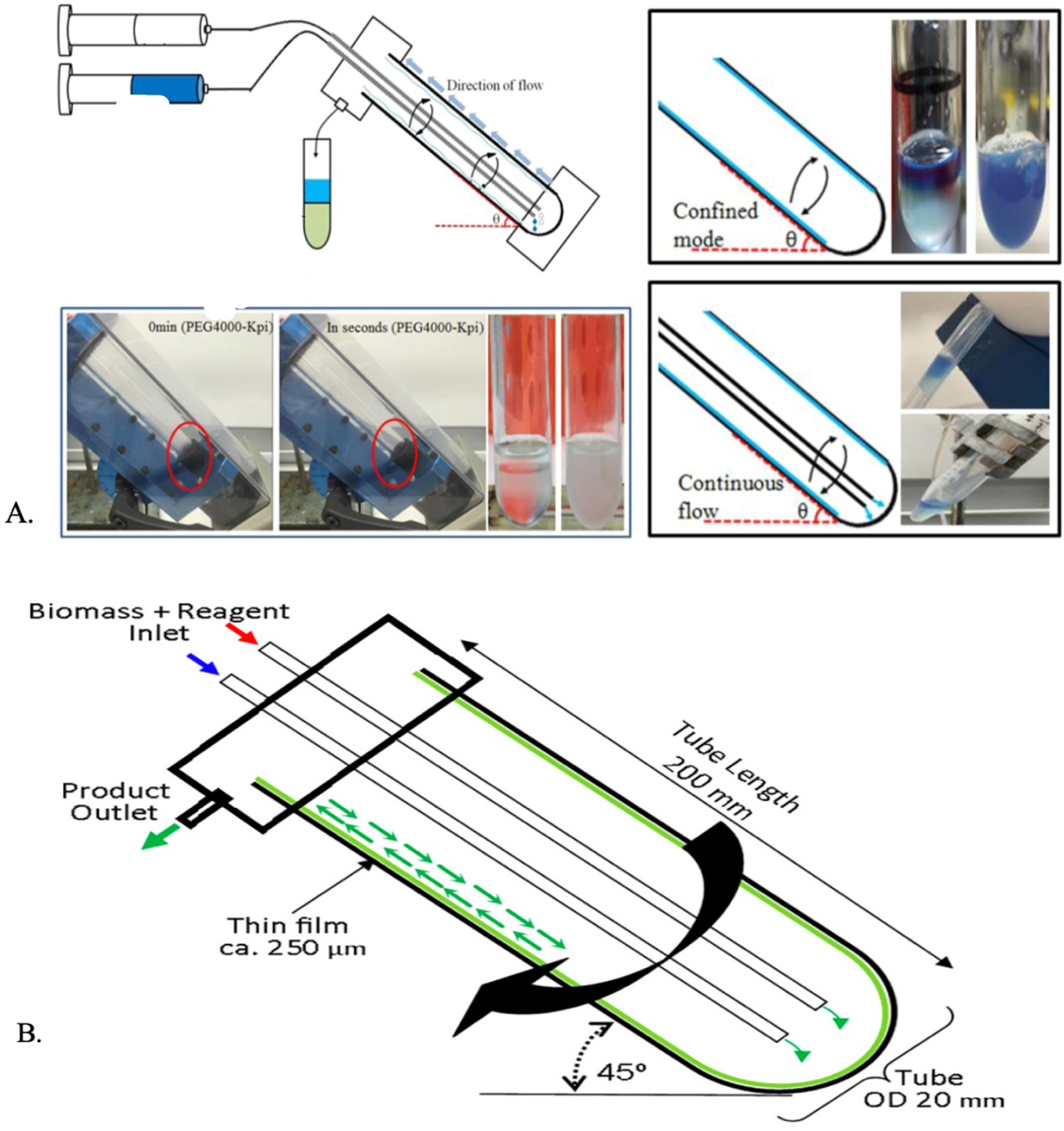


Fig 1. Operation and working mechanism of the Vortex Fluidic Device (VFD) (Luo et al., 2016, Sitepu et al., 2018).

Thus, **aim** of this study was to test the suitability of the VFD as a green and novel process to extract collagen from Salmon skin based on the hypothesis that collagen produced by this process would have a high yield and interesting functionalities for cosmetical applications.

Methodology:

The Salmon skin was pre-treated as detailed in chapter 3 (3.2.3.).

Extraction of collagen was carried out using optimized parameters derived from Sitepu et al. (2018). The pre-treated skin was extracted with 0.5 M acetic acid. 150 mg of the dry/wet biomass were extracted with 1,200, 1,000, 750 and 550 µL of 0.5 M acetic acid at sample to solvent ratios of 1:3.7, 1:5. 1:6.7 and 1:8 (w/v) in the VFD tube (20 mm borosilicate tube) at rotational speeds of 2,000, 4,000, 6,000, and 8,000 rpm for 15, 30, 45, 60, and 75 min. The mixture was filtered through a double layered cheese cloth. The mixture was precipitated as described in **3.2.4.1** and centrifuged at 2,767 g (Eppendorf, Centrifuge 5810 R) for 30 min. Pellets were dissolved in 0.5 M acetic acid and dialyzed against distilled water (MilliQ water, Millipore Academic MilliQ water, Millipore) overnight. The dialyzed pellets were freeze dried (VirTis Benchtop K, BTEKEL, Quantum Scientific).

Results:

The process did not work successfully as the skin of the fish swelled and firmly adhered to the bottom the borosilicate tube, only the acetic acid solvent formed a thin film but failed to extract the collagen from biomass, for both dry and wet biomass.

Future research directions exploring alternative methods for VFD operation:

To overcome the stickiness problem of Salmon skin, the borosilicate tube could be coated with Sigmacote. Sigmacote is a solution of a chlorinated organopolysiloxane in heptane that readily forms a covalent, microscopically thin film on glass. The film repels water, retards the clotting of blood or plasma, and prevents surface adsorption of many basic proteins. So the skin may not stick to the bottom of the tube (Sigma-Aldrich, 2016).