Masteroppgave

NTNU
Norges teknisk-naturvitenskapelige universitet
Fakultet for naturvitenskap
Institutt for bioteknologi og matvitenskap

Carl Henrik Grefstad

Extraction and Characterization of Collagen From Heads of Cod (*Gadus morhua*) and Pollock (*Pollachius virens*)

Masteroppgave i Industriell kjemi og bioteknologi
Veileder: Turid Rustad
Medveileder: Rasa Slizyte
Mai 2022

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Kunnskap for en bedre verden

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Preface

This master's thesis is for the course TBT4900 - Biotechnology, Master's Thesis. The thesis builds upon a project report written for the course TBT4500 - Biotechnology, Specialization Project, and as such, parts of the introduction material will be covering the same topics. The thesis was done in cooperation with SINTEF Ocean AS, as a part of the SUPREME project. The practical laboratory work was conducted at the food chemistry laboratory at NTNU during the fall of 2021.

I want to thank my supervisor, Professor Turid Rustad at NTNU, and my co-supervisor, Dr. Rasa Slizyte at SINTEF Ocean, for their remarkable guidance, support and counselling during this master's thesis. Their patience, suggestions and help have been very significant, and motivated me throughout the work. I would also like to thank Siri Stavrum at the food chemistry laboratory at NTNU for the advice and help she gave me with the practical tasks. I am grateful to all the other students working in the laboratory for making the days there delightful and contributing to a fun working environment. I also want to thank SINTEF Ocean for allowing me the opportunity to participate in this project. Lastly, I am very grateful to all my friends and family for their support and good wishes.

Abstract

A large amount of rest raw materials arise from capture fisheries and aquaculture globally each year. In 2019 a total amount of 964 000 metric tonnes of rest raw material was produced from white fish, pelagic fish, aquaculture and shellfish in Norway[1]. It was estimated that 811 000 tonnes were utilized in other food products, ensilage and bioenergy. However, 153 000 metric tonnes of rest raw material was not utilized at all, mainly arising in the white fish sector (78%), which includes Atlantic cod (*Gadus morhua*) and Atlantic Pollock (*Pollachius virens*). Due to a lack of processing solutions on board of sea-going vessels or economic incentives rest raw materials like heads, intestines and liver are discarded at sea and not brought back to shore.

The goal of this project was to study the possibility of extracting collagen from heads of Atlantic cod and pollock, to increase the utilization of these rest raw material and generate a high-value product that could contribute to meeting global demands. The investigated extraction method was a modified version of the one proposed by Nagai and Suzuki (2000)[2]. The method is fairly simple and should have the potential to be performed fishing vessels which is the place where the rest raw material is discarded. This project was carried out in cooperation with SINTEF Ocean As under the SUPREME project.

The raw material was prepared beforehand by SINTEF Ocean with a grinder, following this the minced heads were washed with cold water to remove blood and other water soluble impurities. Following this, a three step pretreatment was performed consisting of treating the raw material with NaOH, EDTA and ethanol to remove non-collagen proteins, ash and fats. The fish heads were then subjected to collagen extraction with acetic acid. The extractions were done in one and two steps, by storing and changing the extraction liquid halfway through the extraction time. Following extraction, the collagen was salted out with NaCl, redissolved in acetic acid and dialyzed against dilute acetic acid and pure water. Finally, the solvent was removed by freeze-drying to obtain isolated collagen. Following the extraction amino acid composition, collagen purity, molecular weight distribution and protein content were analysed by HPLC, determination of hydroxyproline content, SDS-polyacrylamide gel electrophoresis and C/N/S elemental analysis.

Extraction 1 showed the overall highest yield and purity, with 24 hours extraction time with milder pretreatment conditions, that is with higher

raw material to solvent ratio and skipping the ethanol washing step. The highest observed yield was 2.3%, using pollock head as raw material with one extraction step, while the highest collagen purity was 38.5% with cod head as raw material in two extraction steps. Both extraction yield and purity were lowered with longer extraction time, lower raw material to solvent ratio in the pretreatment and inclusion of the fat removal with ethanol. No collagen was extracted in extraction 4, with 72 hours extraction time and long pretreatment, as the product did not contain hydroxyproline. Protein bands of around 200 and 100 kDa were observed on the polyacrylamide gels with collagen product from extractions 1, 2 and 3, confirming that type 1 collagen was extracted.

Sammendrag

En stor mengde restråstoff oppstår fra fiskeri og akvakultur globalt hvert år. I 2019 ble det produsert en mengde på totalt 964 000 tonn restråstoff fra hvitfisk, pelagisk fisk, akvakultur og skalldyr i Norge[1]. Det ble anslått at 811 000 tonn ble brukt i andre matprodukter, ensilasje og bioenergi. Imidlertid ble 153 000 tonn restråstoff ikke utnyttet i det hele tatt, hovedsakelig fra hvitfisksektoren (78%), som inkluderer atlantisk torsk (*Gadus morhua*) og atlantisk sei (*Pollachius virens*). Grunnet mangel på prosesseringsløsninger om bord på sjøgående fartøyer eller økonomisk insentiv, blir råmaterialer som hoder, tarm og lever kastet på sjøen og ikke brakt tilbake til land.

Målet med dette prosjektet var å studere muligheten for å utvinne kollagen fra hoder fra atlantisk torsk og sei, for å øke utnyttelsesgraden av disse restråstoffene og å generere et høyverdiprodukt som kan bidra til å møte globale krav. Ekstraksjonsmetoden som ble undersøkt var en modifisert versjon av den som ble foreslått av Nagai og Suzuki (2000)[2]. Metoden er ganske enkel og har potensial til å bli utført på fiskefartøy som er stedet hvor restråstoffet kastes. Dette prosjektet ble gjennomført i samarbeid med SINTEF Ocean As under SUPREME-prosjektet.

Råstoffet ble forbredt av SINTEF Ocean med en kvern, deretter ble de hakkede hodene vasket med kaldt vann for å fjerne blod og andre vannløselige urenheter. Etter dette ble det utført forbehandling i tre trinn bestående av å behandle hodene med NaOH, EDTA og etanol for å fjerne ikke-kollagene proteiner, aske og fett. Fiskehodene ble deretter utsatt for kollagenekstraksjon med eddiksyre. Ekstraksjonene ble gjort i ett og to trinn, ved å lagre og bytte ekstraksjonsvæsken halvveis ute i ekstraksjonstiden. Etter ekstraksjon ble kollagenet saltet ut med NaCl, oppløst på nytt i eddiksyre og dialysert mot fortynnet eddiksyre og rent vann. Til slutt ble løsemiddelet fjernet ved frysetørking for å sitte igjen mend isolert kollagen. Etter ekstraksjonen ble aminosyresammensetning, kollagenrenhet, molekylvektfordeling og proteininnhold analysert ved HPLC, bestemmelse av hydroksyprolininnhold, SDS-polyakrylamid gelelektroforese og C/N/S elementæranalyse.

Ekstraksjon 1 hadde i alt høyest utbytte og renheten, med 24 timers ekstraksjonstid med mildere forbehandling, det vil si med høyere forhold mellom råmateriale og løsemiddel og uten etanolbehandling. Det høyeste observert utbytte var 2,3% ved bruk av seihode som råstoff med ett ekstraksjonstrinn, mens høyeste kollagenrenhet var 38,5% med torskehode som råmateriale i to

ekstraksjonstrinn. Både ekstraksjonsutbytte og renhet var lavere med lengre ekstraksjonstid, lavere forhold mellom råstoff og løsemiddel i forbehandlingen og inkludering av fettfjerning med etanol. Det ble ikke ekstrahert kollagen i ekstraksjon 4, med 72 timers ekstraksjonstid og lang forbehandling, da produktet ikke inneholdt hydroksyprolin. Proteinbånd på rundt 200 og 100 kDa ble observert på polyakrylamidgelene med kollagenprodukt fra ekstraksjoner 1, 2 og 3, som bekrefter at type 1 kollagen ble ekstrahert.

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1 Introduction

This master's thesis is a continuation of an earlier project completed at NTNU. As such, this introduction is based on and expands the introduction of this project[3].

1.1 Fish Rest Raw Material as a Collagen Source

Rest raw material (RRM), or by-products, can be defined as every part of the fish that is not viewed as the main product when utilizing the raw material. In the fish industry, the main product is the fillet, leaving both edible and non-edible RRMs like skin, bone, scale, heads, trimmings and viscera. It is estimated that in 2019 approximately 153 000 metric tons of RRMs from the seafood industry, including white fish, pelagic fish, aquaculture and shellfish, were not utilized in Norway. Even if these RRMs contain nutritional and valuable compounds, only up to 13% of the RRMs are used to make products for human consumption, the majority being processed into low-value products like fish meal, fertilizer or silage. About 76% of the unutilized RRM came from the white fish sector, totaling 117 000 metric tons of unused material. This is largely heads and intestines, and it arises during filleting and processing on seagoing vessels[1]. In an assessment from 2019, FAO estimated that between 2010-2014 the annual discard from global marine capture fisheries was 9.1 million metric tons[4]. The RRMs that are not utilized are discarded at landfills or in the oceans, and thus have a hazardous effect on the local environment.

RRMs can have a large content of protein, and it is estimated that between 10-20% of the total protein content in fish raw material is discarded. As shown in Table (1.1), the biggest potential for increasing the utilization of heads from white fish like cod and pollock is the proteins, including collagen. In addition to heads, fish parts like skin, scale and frames could become a potential substitute or excellent supplement to the traditional collagen sources of bovine or porcine skin and bone[5]. Furthermore, collagen and gelatin extracted from fish sources do not challenge the religious practices of Muslims and Jews for whom pigs are not considered kosher or halal, Hindu people who believe cows are sacred and should not be eaten nor the ethical choices of pescatarians who don't eat meat[6]. Jews, Muslims and Hindus make up a total of 38% of the global population[7]. In addition, there is a concern for bovine-derived products due to the risk of being vehicles for diseases like bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD)[8].

Table 1.1: Chemical composition (wt.%) of the heads of cod, pollock and haddock[9]

Species	Water (%)	Protein (%)	Lipid (%)	Ash (%)
Cod	79.3	14.2	4.1	6.4
Pollock	77.9	16.6	<1	5.3
Haddock	78.9	13.1	<1	7.0

1.2 Collagen Structure and Composition

Collagen is the main structural protein in the extracellular matrix of connective tissue (i.e., bone, skin, cartilage, scale and tendon) in the body. As such, it is the most abundant protein in animals and fish and can make up about a quarter of the total protein content in many animals[10]. To date, a total of 28 different types of collagen have been identified, with varying structures, functions and tissue distributions. Types of collagen are classified as either fibrillar or non-fibrillar collagen, depending on the quaternary protein structure[11]. Type I collagen has a fibrillar structure, and is the most common type being the main protein component in bone, skin, organs and tendon. Together with the fibrillar collagen types II and III, these three types are by far the most common, while other types are found in smaller quantities[12]. Descriptions of the five most common types of collagen are presented in Table (1.2). Extraction of collagen from animal and fish parts such as skin and bone mainly yields type I collagen[13].

The defining feature of all collagen types is that the tertiary structure contains a right-handed tripe helix motif[11]. The collagen monomer, tropocollagen, is a rod-shaped protein consisting of three polypeptide units (called α-chains) intertwined to form a triple helical structure. The α-chain is made up of around 1050 amino acids, and coils in a left-handed helix with three amino acids per turn. The three α-chains within a tropocollagen molecule are designated $\alpha 1$, $\alpha 2$ and $\alpha 3$ and may differ in their amino acid composition giving rise to different types of collagen, both heteromers and homomers. Typically the α-chains and entire triple helix have a molecular masses of about 100 kDa and 300 kDa respectively. Type I collagen consists of two $\alpha 1$ chains and one $\alpha 2$ chain with a different composition[14]. Every third amino acid on the α-chains is glycine (Gly), giving rise to a repeated amino acid sequence of Gly-X-Y. The X and Y position can be filled by any amino acid, however most common are the imino acids proline (Pro) and hydroxyproline (Hyp) respectively[15]. Gly is required in every third position because its

Table 1.2: Structures and descriptions of the most common collagen types. Table taken from (Benjakul, Soottawat and Nalinanon, 2012, p. 366)[14]

Collagen Type	Structure	Description
I	Fibrillar	Most common type of collagen, found in bone, skin, organs, tendons and muscles.
II	Fibrillar	Found almost exclusively in cartilage.
III	Fibrillar	Found in fetal skin, lung and blood vessel.
IV	Non-Fibrillar	Found in the basement membrane which are specialized structures at tissue boundaries.
V	Fibrillar	Found in association with type I collagen, with particulary high amounts in the cornea.
Other types		Include both fibrillar and non-fibrillar types. These collagens are present in low amounts and are mostly organ-specific.

small size allows it to fit in the center of the tight three-stranded helix, as it does not have a side chain. If Gly residues are replaced by other amino acids it interrupts the triple helix motif and results in rigid kinks or flexible hinges[11]. The polypeptide chains are primarily held together by hydrogen bonds between adjacent -CO and -NH_2 side groups[16]. The hydroxyl groups of the imino acids Pro and Hyp interact with the pyrrolidine ring. Furthermore, the side groups of Pro and Hyp are very rigid, which also provides stability to the tropocollagen by limiting rotation of the α-chains[10]. As such, the imino acids contribute to making the triple helix particularly stable, and increase the denaturation temperature and denaturation enthalpy of collagen.

Tropocollagen is the main subunit of collagen tissue and, it has a length and diameter of 300 nm and 1.5 nm respectively[17]. The collagen is flanked by short extrahelical telopeptides that lack the Gly-X-Y sequence and helical confrontation. To form collagen fibers tropocollagen molecules associate in adjacent rows, displaced by about one-fourth of its 300 nm length (a "quarter staggered" array). The protein structures of collagen, from its amino acid sequence to quarter staggered collagen fibers, are shown in Figure (1.1). During maturation, collagen fibers are strengthened and stabilized, primarily by covalent cross-linkages. It has been proposed that cross-link formation involves enzymatic oxidation of lysine and hydroxylysine, conversion of these

aldehydes to aldols and aldimines followed by stabilization of these products by further reduction or oxidation reactions[18]. The stereochemistry of collagen fibers derives from specific reactions between peptidyl aldehydes on the short extrahelical telopeptides in one tropocollagen molecule with side groups of lysine and hydroxylysine in the triple helical region of another molecule[19]. Furthermore, a multitude of different non-specific and spontaneous reactions with glucose and its oxidation products leads to advanced glycation end products[15]. These collagen fibers make up the structure in connective tissue, and the degree of cross-linkages varies greatly between species, age and types of tissue. Typically fish collagen has a lower degree of cross-linkages than mammalian collagen such as bovine and porcine. Collagen from young animals has a lower degree of stable cross-linkage in the fibril network and can be soluble in warm water, however from sources of higher age the amount of cross-linkage increases, thus making the collagen insoluble in water.[20].

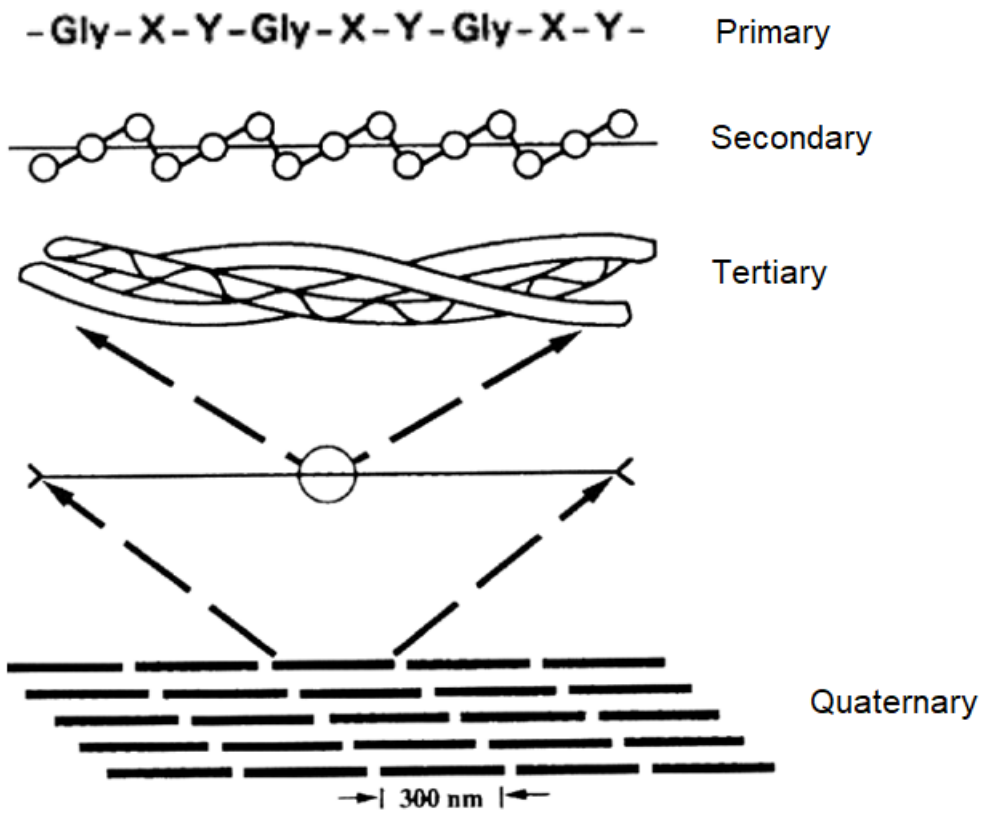

Figure 1.1: The primary protein structure of collagen is the amino acid sequence, secondary structure is a helical α-chain, tertiary structure is a tropocollagen molecule and the quaternary structure is collagen network. Figure taken from (D.R. Eyre, 1980, p. 1317) [21].

The repeated Gly-X-Y sequence in the triple helical domain of collagen dictates that about a third of the amino acid composition is made up of glycine.

In fact, it is only the 14 first amino acids from the N-terminus and first 10 from the C-terminus that do not follow the repetitive sequence[22]. Two amino acids which are almost exclusively found in collagen, hydroxyproline and hydroxylysine, can be located on the Y position. Meanwhile, their unhydroxylated counterparts, proline and lysine can be found in both the X and Y positions. The ordered association of tropocollagen molecules is attributed to the distribution of amino acids with polar and non-polar side groups in the X position. Regions of the α-chains where the imino acids are found on the X and Y position are non-polar, while segments containing other amino acids are mostly polar[14]. Apart from glycine, proline and alanine make up a large part of the amino acid composition of collagen. Meanwhile, the contents of tyrosine, histidine, cysteine and tryptophan are low, with the two latter amino acids being completely deficient in some collagen types. Since hydroxyproline is only present in collagen in significant amounts, it is used to estimate the collagen content in food or collagen purity[22]. In Table (1.3) the amino acid compositions of mammal, cold-water fish and warm-water fish collagen are presented, and as can be seen, it can vary greatly between species.

As stated, the imino acids contribute to increasing the stability and strength of the triple helical collagen molecule. Their sterical side groups restrict the conformation of the polypeptide chain, and the hydroxyl group of Hyp plays a major role in intramolecular hydrogen bonds[23]. The thermal stability of collagen also greatly depends on the content of these amino acids. The denaturation temperature or melting temperature of collagen, which can be measured by circular dichroism during heating or differential scanning calorimetry, is the temperature at which a sharp transition can be observed. The melting temperature is commonly a few degrees above the body temperature of whichever species the collagen originates from. If hydroxylation of proline is blocked, it results in a subsequent drop in the collagen denaturation temperature by approximately 30°C[12].

As can be seen in Table (1.3), mammalian collagen contains larger quantities of imino acids when compared with fish collagen. Furthermore, the amount of these amino acids are generally lower in cold-water fish than in warm-water fish. This can be attributed to the ambient temperature of the environment of cold-water fish. The low temperatures make the collagen stiffer and thus a larger content of imino acids would make it too rigid. In addition, water also provides structural support to the body of the fish, so its collagen does not require as much strength as that of land mammals[24]. The diverse amino

acid compositions of collagen give rise to the diverse characteristics as well. For example, the melting temperature of fish collagen range from 5°C to 30°C, where collagen from warm-water fish is in the upper range of that interval, and from mammalian sources, the melting temperature can be as high as 40°C[25].

Table 1.3: Amino acid compositions (in %) of collagen extracted from cold-water fish(cod), warm-water fish (big eye snapper and grass carp) and mammals. Imino acids include Pro and Hyp. Table taken from (Benjakul, et al., 2012, p.368)[14].

Amino Acid	Cod skin	Big eye snapper skin	Big eye snapper bone	Grass carp skin	Porcine dermis	Calf skin
Alanine	10.7	13.6	12.9	13.5	11.5	11.9
Arginine	5.4	6.0	4.6	5.7	4.8	5.0
Asparagine	5.3	5.1	4.7	4.2	4.4	4.5
Cysteine	0	0	0	0.4	0	0
Glutamine	8.0	7.8	7.4	6.1	7.2	7.5
Glycine	34.2	28.6	36.1	33.4	34.1	33.0
Histidine	0.8	1.0	0.6	0.5	0.7	0.5
Isoleucine	1.2	0.5	0.5	1.0	1.0	1.1
Leucine	2.2	2.4	2.5	2.2	2.2	2.3
Lysine	2.9	3.1	2.5	2.3	2.7	2.6
Hydroxylysine	0.7	1.0	2.0	0.8	0.7	0.7
Methionine	1.5	1.2	0.8	1.0	0.6	0.6
Phenylalanine	1.2	1.5	1.2	1.7	1.2	0.4
Hydroxyproline	5.1	7.7	6.8	6.5	9.7	9.4
Proline	10.3	11.6	9.5	12.1	12.3	12.1
Serine	5.9	3.6	3.4	3.9	3.3	3.3
Threonine	2.3	2.9	2.5	2.4	1.6	1.8
Tyrosine	0.4	0.4	0.2	0.2	0.1	0.3
Valine	1.9	2.2	1.7	3.1	2.2	2.1
Total imino acids	15.4	19.3	16.3	18.6	22.0	21.5

1.3 Collagen Production

Isolation of collagen is a process that consists of three stages, pretreatment of the raw material, extraction and purification. Raw material preparation includes size reduction, removing impurities and could also include breakdown of some intermolecular cross-linkage prior to extraction. Collagen is most commonly extracted as gelatin, which is partially hydrolysed collagen with a different peptide composition. Extraction is performed batchwise under constant stirring with various extraction liquid and proposed methods. These include salting out, alkali, acid and enzymatic methods, and combinations of these, like acid-enzymatic extraction[26]. During extraction, the intermolecular bonds between triple helical collagen molecules are hydrolysed. Thus, free tropocollagen trimers, dimers and monomers are released from the matrix of the raw material into the solvent, as free γ-, β- and α-chains respectively, see Figure (1.2). In gelatin extraction the intermolecular bonds between tropocollagen molecules in γ and β chains and bonds within free α-chains are cleaved. This results in peptide composition consisting of mainly α-chains and hydrolysed tropocollagen peptides called sub α-chains. Purification of collagen consists of separating the collagen from the extraction liquid and produceing a clean product.

The molar mass distribution, composition and structure of collagen depend on the processing conditions and the raw materials from which it is derived. For example, as shown in Table (1.3), the amino acid composition of a cold-water fish like cod differs from that of a warm-water fish like big eye snapper. Thus, collagen derived from these two fishes would have differences in their amino acid composition, importantly in their imino acid content which provides stability to the collagen. As such, it is likely that the less stable collagen from the cod would be hydrolysed to a higher degree than the collagen from big eye snapper, resulting in different mass distribution as well. Even within the same fish, there are differences in collagen found in the bone, skin and cartilage, when it comes to structure and chemical composition. There are differences in the matrices and impurities in skin and bone, and therefore they would require different pretreatments and processing conditions to reach the highest yield. Even when using the exact same type of raw material the process conditions would influence the final collagen product. Raising the concentration of the acid used as extraction liquid or lowering the ratio between raw material and extraction liquid would lead to a greater rate of hydrolysis, and thus change the mass distribution of the final product. As such, it is necessary to determine the optimal extraction process for each raw material in order to obtain collagen with the desired

functional characteristics and properties[27]. To optimize the process, parameters like extraction time, temperature, chemical concentration and solvent to material ratio can be tuned in order to maximize yield while avoiding excessive hydrolysis of the collagen. When working with cold-water fish as raw material, all steps of the process are carried out at a temperature of 4°C to prevent denaturation and contaminations[28].

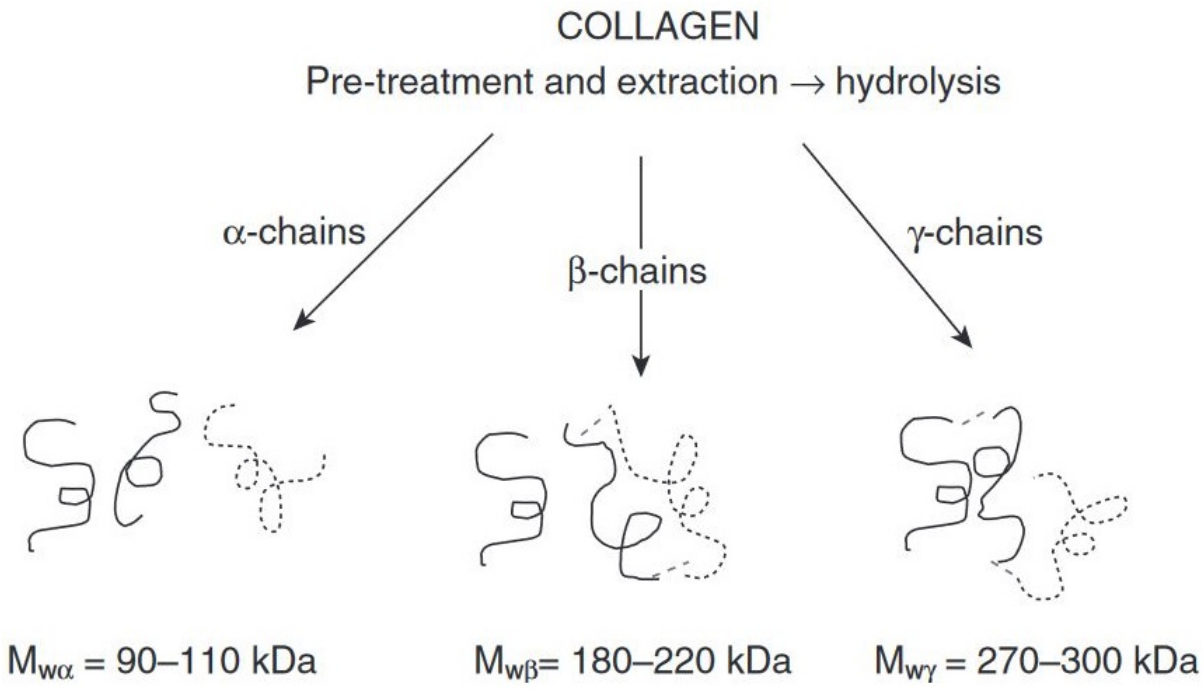

Figure 1.2: The types of peptide chains in extracted type 1 collagen, including ranges for their respective molecular weights. These include γ-, β- and α-chains which are monomers, dimers and trimers of tropocollagen molecules respectively. The red dotted lines are meant to illustrate intramolecular bonds between two tropocollagen peptides in β-chains and three tropocollagen peptides in γ-chains. Figure taken from (Haug and Draget, 2011, p.95) [29].

1.3.1 Raw Material Pretreatment

Collagen-rich raw material contains a number of different impurities that would result in a loss of quality in the collagen product if not removed prior to extraction. These impurities include ash (ie. inorganic minerals like calcium, phosphorous, zinc and iron), non-collagenous proteins, pigments and lipids[27]. One goal of the pretreatment is to remove these impurities to get higher purity in the end product. The impurities are present in larger or smaller quantities depending on the type of raw material used; bone contains a larger amount of ash than, for example skin. Furthermore, pretreatment can also facilitate better extraction conditions by breaking down some of the cross-linkages in the collagen fibrils with alkali, acids or enzymes. This increases the solubility of collagen in the extraction liquid and thus increases the overall yield. Collagen dissolves slowly even in boiling water due to the

cross-linkages, so in order to extract collagen at 4°C mild chemical breakdown of these is necessary[13].

Mechanical removal of residual tissue like muscle or fat is commonly done before any chemical treatments. The inclusion of this type of tissue in the collagen extraction process would introduce larger amounts of impurities, thus lowering the overall yield of the process and reducing the quality of the final product. This is followed by size reduction of the raw material to increase the surface area to volume ratio. Size reduction facilitates further chemical cleaning and later extraction by increasing the mass transfer area[28].

Removal of impurities from the raw material is done under mild conditions to avoid excessive breakdown of the collagen, and washing is done with water or dilute solutions of alcohol, alkali and acids. Non-collagenous proteins and pigments is commonly removed by washing the raw material with alkali or salt solutions. Collagen is more resistant to dissolution than other proteins and, as such, it is not washed away in this pretreatment step. Minor hydrolysis of the collagen cross-links occurs in alkali solutions, however, the product withstands excessive hydrolysis and resulting loss with the use of dilute alkali solutions. When working with fish raw material, NaOH with a concentration of 0.1 M is most often used[2][30]. Defatting is employed with raw materials such as skin tissue, and the most commonly used solutions are acetone and 10% or 15% butanol. Other alcohols and nonionic detergents can also be used to remove lipids[27][31][32]. Material such as bones and scales contain a large amount of ash, as such, demineralizing is used when working with these types of raw materials. Acids like EDTA and HCl dissolve calcium phosphates and other inorganic material, and removes these impurities. For fish raw material, EDTA at a concentration of 0.5 M is preferred, however, it has been shown that HCl is able to remove almost all minerals from cod bones at concentrations of 1 M and 0.5 M with a small loss of collagen[33]. Decalcification has the added benefit of making the raw material more porous, and thus increasing the surface area to volume ratio even further before extraction.

When the raw material is treated with dilute acid or alkali solutions, the collagen is also subject to partial hydrolysis, which maintains the collagen chains intact but the cross-links are cleaved[27]. During acidic pretreatment, the raw material is immersed in an acidic solution, which penetrates the

raw materials and causes it to swell. The acid solution also causes some of the non-covalent inter- and intra-molecular bonds to be cleaved. Pretreatment with acid is appropriate for raw material such as fish with less mature collagen fibers, that is, with a lower degree of covalent cross-links[34]. As mentioned above, pretreatment with acid also has the added effect of demineralizing. Alkaline pretreatment typically includes subjecting the raw material to NaOH or sometimes $CaOH_2$ solutions. This is more suitable for collagen with a high degree of cross-linkages such as bovine ossein or shavings, and it is more aggressive towards the collagen fibers. NaOH is normally preferred because it significantly contributes to swelling, thus increasing the mass transfer rate of collagen during extraction[34]. It has been reported that NaOH in concentrations between 0.05 to 0.1 M effectively removed non-collagenous proteins from grass carp skin at temperatures up to 20°C without a significant change in the structure or loss of collagen. However, a 0.2 M concentration resulted in a significant loss of collagen, and at 0.5 M, the structure of collagen was modified[35]. Leaching may occur during acid and alkali pretreatment, resulting in a loss of product and a lowered overall yield[36].

Pretreatments are preformed in batches with varying number of steps, treatment time, chemical concentration and solvent to material ratios. When using fish raw material two to three steps are common for each treatment, with duration between 2-48 hours[2][31][37][38].

1.3.2 Extraction

Collagen is extracted from the raw material after this has been pretreated. The solubility of collagen in cold water is low due to the strong intramolecular interactions in the triple helix of tropocollagen, and the cross-linkages present in collagen fibers. There are several possible extraction methods that can be applied to extract collagen. To date, the most successful methods when it comes to fish collagen is acid extraction and enzyme aided acid extraction, both of which require long processing time[28][39]. High acidity and temperatures can induce a higher degree of collagen chain degradation, especially when the raw material is subjected to these conditions over an extended period of time. As such, new technologies are investigated for collagen extraction with the goal of better preservation of the peptide chains and shortening the processing time. Some newly proposed approaches include deep eutectic solvent extraction, supercritical fluid extraction and extrusion combined with ultrasound-assisted extraction[10]. Apart from these proposed extraction processes, collagen could also be extracted using salt solutions like

NaCl, Tris-HCl, phosphate or citrate or alkali solutions, however, it has been reported that these are seen as less favorable[26].

Acid extraction has been widely used in the production of collagen, especially when working with fish. The extracted product is referred to as acid soluble collagen or ACS. Various acids can be used in the extraction process, including both organic (acetic, citric and lactic acid) and inorganic (HCl). Acids hydrolyse the cross-links between tropocollagen molecules in the triple helix, resulting in depolymerization into single α-chains and shorter peptides which are soluble[36]. Due to the acidic conditions, the positive charge of collagen becomes dominant, resulting in greater repulsion between collagen molecules and an increased solubility[14]. In a study by Skierka and Sadowska, the influence of different acids on extraction of collagen from cod skin was investigated. The best yields were obtained with 0.5 M acetic and lactic acids resulting in dissolution of 90% of the available collagen, while only 18% was dissolved using 0.15 M HCl[40]. Acetic acid is also used to extract collagen from animal sources with concentrations in the range of 0.5 to 1.0 M, which allow the cleavage of intra- and inter-molecular cross-links without affecting the structure of the collagen chains significantly. Fourier transform infrared (FTIR) spectra of collagen extracted with 0.5 M acetic acid from the skin of cold water fish arabesque greenling and warm water fish brownbanded bamboo shark showed that the triple helical structure of the tropocollagen molecules was conserved[41][42].

Acetic acid with a concentration of 0.5 M is generally used for collagen extraction, as increasing the concentration can lead to a loss in yield and purity due to degradation of the peptides[43]. The diffusion of collagen into the extraction liquid is a time-dependent process and increased extraction times lead to increased protein recovery. Normally the extraction time is kept between 24-48 hours. Further time increases either do not lead to significant increases in the yield or may even lead to excessive hydrolysis of the collagen, thus lowering the extraction yield[43][44]. The solvent to material ratio also has a positive correlation with collagen yield. As this ratio increases, the raw material is exposed to larger quantities of fresh extraction solvent, thus enhancing interactions between the acid and collagen. There is a wide range of solvent to material ratios that have been investigated from 10 to 60 ml/g[10]. As with extraction time, it has been reported that increasing this parameter may lead to insignificant increases in collagen yield or even result in excessive hydrolysis and lowered yield[43][44]. Anyhow, large solvent to material ratios does result in products richer in lower molecular weight peptide chains, thus changing the functionality of the extracted collagen[36].

The enzyme aided acid extraction is the second main method for collagen extraction, where peptide cleaving enzymes (pepsin) are added to the extraction acid. The extracted product is referred to as pepsin soluble collagen or PCS. The telopeptide of tropocollagen molecules can cause antigenicity, which limits the possible applications of the collagen in food and pharmaceutical industries[45]. Pepsin is able to cleave the telopeptide region of collagen, thus solving the challenge of antigenicity. Simultaneously the cross-link site on the telopeptides of individual tropocollagen molecules are removed, resulting in increased extraction efficiency[46]. Furthermore, the enzyme is able to hydrolyse potentially still present non-collagenous proteins, making them easy to remove by salt precipitation and dialysis in later purification steps, thus increasing the purity of the product. Pepsin is not able to cleave the helical part of the collagen molecule, however, cleavage of the telopeptide region likely results in conversion of intramolecular hydrogen bonds. This will result in lower amounts of γ and β chains, trimers and dimers, and a larger amount of α chains when comparing PCS to ACS[46]. Figure (1.3) show pepsin hydrolysis of non-collagenous proteins and cleavage of collagen telopeptides. The batch time of extraction can also be significantly reduced with the aid of enzymes. In a study by Skierka and Sadowsa, it was reported that pepsin was able to reduce the extraction time of collagen by 24 hours with an increase in collagen solubility from 55% to 95%[40].

Typically acetic acid with a concentration of 0.5 M is used in enzyme aided acid extraction, and it plays a similar role as in normal acid extraction. The two methods can be used in series where the first step is acid extraction, then the raw material residue is subjected to enzyme aided extraction in a second step[41][38]. In the first extraction, collagen with a lower degree of cross-links which is more soluble is obtained. Furthermore, because the raw material swells, its structure becomes looser and more porous, caused by positive charge repulsion. This results in an increase in pepsin penetration into the raw material matrix and enhances the enzyme's hydrolytic activity.[46]. Generally, pepsin isolated from porcine stomach is used in enzyme aided extraction, however, this has some limitations mainly based on religious constraints. As such, pepsin isolated from the stomach of fishes like big eye snapper and tuna have been investigated as potential substitutes to porcine pepsin for fish collagen extraction with promising results[14]. In a study by Nalinanon et al. collagen extracted with 24 hours of preswelling followed by 48 hours of enzyme aided extraction, the yield was higher when using big eye snapper pepsin (19.79%) than porcine pepsin (13.03%)[46]. One possible explanation is that collagen from various sources cleave collagen at

distinct sites resulting in different degrees of hydrolysis of the telopeptide[47].

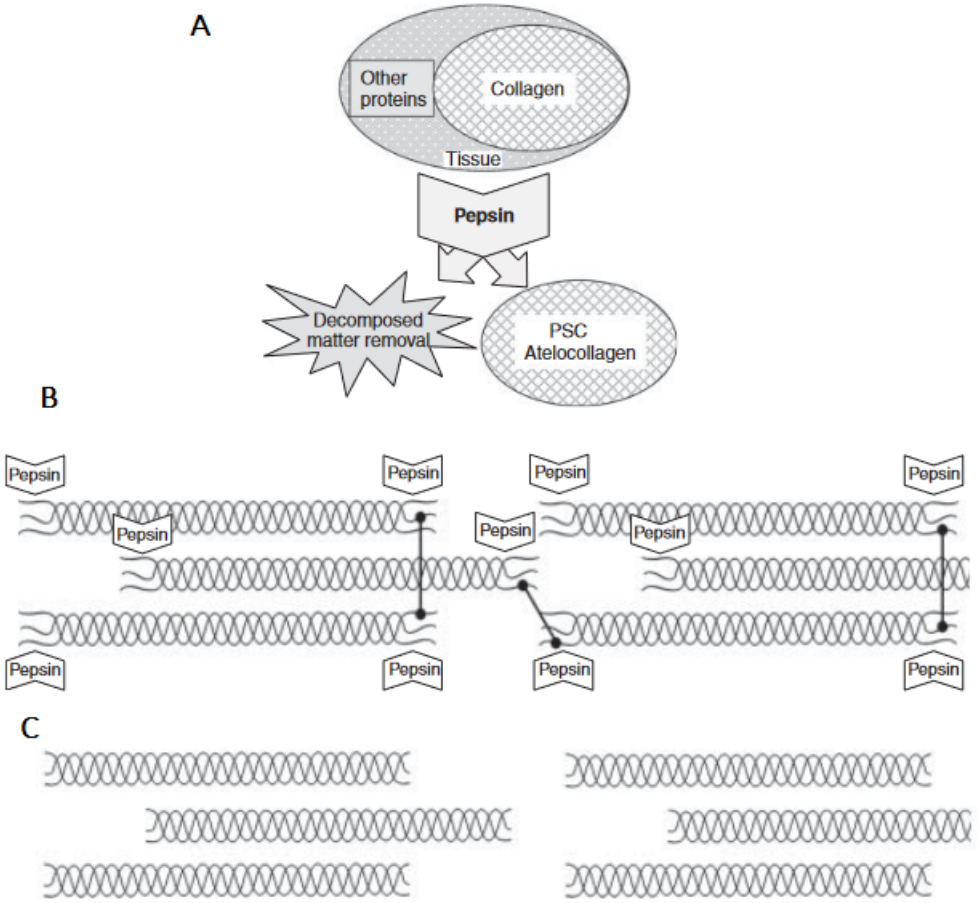

Figure 1.3: Pepsin aided acid extraction of collagen. A) Pepsin hydrolysis of collagen and non-collagenus proteins. B) Pepsin cleaves teleopeptide regions in collagen C) Pepsin soluble tropocollagen molecules. Figure taken from (S. Benjakul, 2012, p. 371)[14].

Pepsin extraction requires low temperatures, generally between 4 to 10°C, because the enzyme can selfdigest and lose its activity at higher temperatures. As it is common to pair enzyme aided extraction with acid the same time is normally spent on each step of the extraction with similar solvent to material ratios, between 24-48 hours and between 10 to 60 ml/g respectively. Pepsin concentration in the extraction liquid varies, however a concentration between 1 to 1.5% is common[31][48][49]. A higher concentration of enzymes leads to higher digestion rate, resulting in increased extraction efficiency and potentially lowering the extraction time. which would make the process more suitable for industrial scale-up. However, when the pepsin concentration is too high and all telopeptides have been cleaved, the enzymes would start degrading the solubilized collagen peptides[10].

1.3.3 Purification

Following extraction, collagen is separated from the extraction liquid using salt precipitation and purified with dialysis and lyophilization. The batch material is then centrifuged and the raw material precipitate is discarded, and the supernatant is kept. When extractions are performed in series the batch material is centrifuged between each step. The raw material is carried over to the next extraction batch, and each individual supernatant are kept and combined. Generally, the supernatant is then neutralized to around pH 7, and Tris-HCl 0.05 M is added, and the collagen is salted out with NaCl to a final concentration of 2.6 M[2][31][44]. It is also possible to add NaCl directly to the supernatant until the collagen precipitates, which occurs in the range of 0.7 to 2.6 M as final concentration[32][50]. The precipitated collagen is recovered by centrifugation, and the supernatant is discarded. The collagen is dissolved in 0.5 M acetic acid, using the minimum required volume, followed by two steps of dialysis first against 0.1 M acetic acid and then distilled water[42][47]. The dialysate is subsequently freeze-dried and the obtained collagen powder is collected and stored.

1.4 Functional Properties and Applications of Collagen

Collagen typically has different functional properties like thermal stability, molecular weight distribution and amino acid composition, which depends on its source and extraction process. As stated earlier, fish collagen typically has lower amounts of imino acids compared with mammalian collagen, and as such, its stability is lower which influences its possible applications. However, due to its high water solubility, easy extractability, biocompatibility , safety and low immunogenicity there are a wide range of possible uses for fish collagen[51].

Collagen has an important role in the formation of tissue and organs and in various expressions of cells. The main property of collagen that is exploited in biomedical applications is that it can form fibers with high strength and stability through self-aggregation and cross-linking[52]. Physical cross-linking techniques like irradiation and dehydrothermal treatment are preferred, as opposed to chemical techniques which result in potential cytotoxicity and poor biocompatibility[53]. There are several possible uses of collagen in the biomedical field such as wound healing, cell development, formation of new blood vessels and activation of platelets in the blood, as a drug delivery system, sponges for burns, mini pellets and tablets for protein delivery, as a

gel in sustained drug delivery, nanoparticles for gene delivery and as a basic matrix for cell cultures[53]. Furthermore, collagen also has applications in skin replacement, bone substitution and in hydrolysed form, can help with weight management[54].

The extracellular matrix (ECM) organizes cells, directs cell-specific regulation and provides environmental signals. Collagen is a very promising natural biopolymer as scaffold or carrier in tissue engineering, as it is abundant, biocompatible, biodegradable, resembles the components present in ECM and supports the connective tissues including skin, tendon, bone, cartilage, blood vessels, and ligaments[55]. The pore size, pore number, surface area and pore wall morphology of collagen make it a desirable biopolymer as a scaffold for cell seeding, migration, growth and new tissue formation in tissue engineering[56].

Collagen is used as a component in drug delivery systems, generally in the form of membranes. Important properties of collagen when used in drug delivery systems include in vivo instability, bioavailability, solubility and body tissue absorption with target-specific delivery and tonic effectiveness[8]. These systems diffuse the drug by exploiting collagen resorption, which is absorption into the circulatory system of cells or tissue. When the collagen is absorbed the drug is released from its matrix in a controlled manner. The most commonly used membrane is made up of collagen with chlorhexidine as an additional component. Tetracycline fibers impregnated in collagen fibers is another example of collagen in a membrane delivery system[57].

In dentistry, marine collagen has been used for a wide range of applications, mainly as membrane, bone graft material and drug delivery vehicle. As plugs, collagen can be used to control bleeding, dress oral wounds, function as extraction site closure and promote healing. Furthermore, collagen membranes are used in periodontal and implant therapy to prevent epithelial migration and support regeneration and repopulation of cells in the defective area[53]. Fish collagen has been added to dental gels used for lowering the healing time of tissue in the mouth due to its active peptides. The gel also creates a protective membrane that provide protection of damaged tissue, promotes regeneration, relieves pain and soothes irritation without causing unnecessary pressure to the soft tissue[58].

As a food additive or packing, collagen can also be used in the food industry.

Collagen is used as a nutritional additive to foods, as its production in the body is lowered with an unhealthy diet and older age[59]. Furthermore, it is used as additives to improve rheological properties and reduce fat consumption in sausages and frankfurters[60]. Collagen-based edible films and coatings are used to increase the shelf life of food products. The film protects the food product from migration of oxygen and moisture while providing structural integrity and vapor permeability to the food product. Moreover, it prevents fat oxidation, discoloration, microbial growth and preserves the sensory qualities[61].

1.5 Gelatin

Gelatin is a multifunctional biopolymer that is derived from type I collagen. It is a hydrolysate of collagen manufactured by cleavage of specific bonds to make collagen soluble. The main difference between collagen and gelatin is the molecular weight distribution. In extracted collagen, the main product is a mixture of peptides consisting of γ-, β- and α-chains. In comparison, these peptide chains are present in lower quantities in gelatin, which has a molecular weight distribution with a broader range. This is due to the presence of large amounts of lower molecular weight protein peptides in gelatin, or sub α-chains (lower MW than 100 kDa), which are caused by more excessive hydrolysis of the native collagen[62]. Gelatin is traditionally extracted from collagenous raw material by hydrolyzing it with acid or alkali, enzymes or high temperatures. There are two main types of gelatin, type A and type B, which are produced with acidic and alkali pretreatment respectively. The amino acid composition of type A gelatin is very similar to that of its native collagen, however, the alkali treatment used to produce type B gelatin transforms asparagine and glutamine into aspartic acid and glumatic acid respectively[29]. As with collagen, the properties of gelatin depend in large part on its amino acid composition and molecular weight distribution. The most important functional properties of gelatin are associated with its gelling and surface-active properties[63].

Gelling properties include gel strength, texture, viscosity, setting- and melting temperatures, while the surface active-properties are adhesion, wetting, film formation and abilities for foaming and emulsifying[13]. Gelatin dissolves in warm water, whereas the hydrogen bonds between monomers break and the peptides assume random coil formation, this is known as a colloidal solution or sol. When a sol is cooled down below its setting temperature, it leads to conformational changes for the monomers, and they assume helical for-

mations. Thus helical interactions can happen between monomers resulting in formation of hydrogen bonds and growing polymer clusters which grow into gel networks[64]. The sol to gel transformation is thermoreversible, thus subsequent heating to the melting temperature will dissolve the gel back to a sol. Figure (1.4) show the mechanism behind the thermoreversible gelling of gelatin.

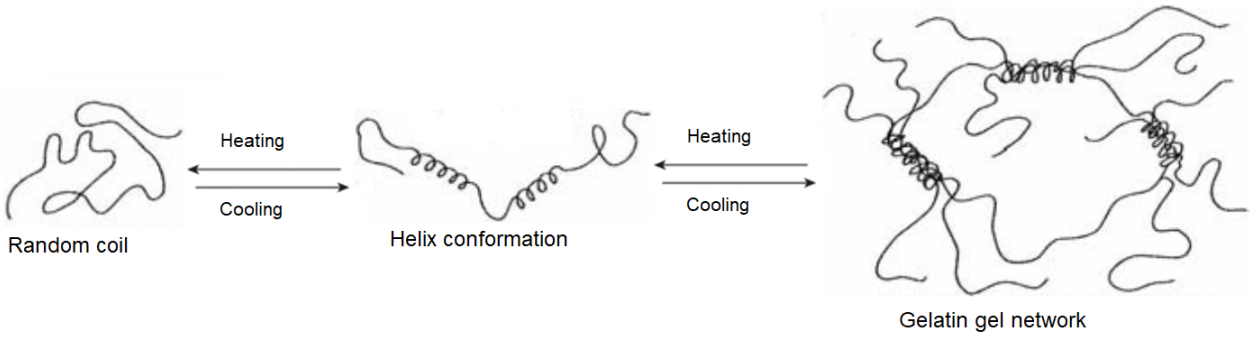

Figure 1.4: Gelatins gelling mechanism from monomer with random coil conformation to the left, to monomer with helical conformation in the middle to entire gel network to the right. Figure taken from (Schrieber and Gareis, 2007, p.54) [13]

Conformational changes and formation of hydrogen bonds are more likely to take place in regions with a large amounts of imino acids, which become important junctions in the gel network. As such, gelatin with low content of these amino acids forms weaker and less stable gels. The gel strength is an important property of commercial gelatin, beside the amino acid composition, it is also influenced by the molecular weight distribution. High gel strength is positively correlated with the amount of higher MW peptides, and as such it is beneficiary with high content of α-chains and especially β- and γ-chains[63]. The viscosity of gelatin is measured above gelling temperature, and it decreases with increasing temperatures. It depends on the concentration of gelatin, pH, molecular weight and size of the peptides, gelatin sols exhibit both Newtonian and non-Newtonian behaviour[29].

The surface-active properties of gelatin is a result of hydrophilic and hydrophobic amino acid side groups along its peptide chains. It can interact with a wide array of other compounds and as such, can be used as starting material for films and coating as well as agents for wetting, foaming, emulsifying and adhesion. Due to its non-toxicity and biodegradability it can be used in the pharmaceutical, cosmetics and food industries[64]. The hydrophilic and hydrophobic side groups on the gelatin chains tension at interfaces and as such, can produce and stabilize water/oil and liquid/air interfaces in emulsions and foams respectively[13]. Higher MW gelatin is

preferred for emulsions as they increase the viscosity of the solution. In turn, higher viscosity increases its stability and forms more stable membranes around droplets. For foams on the other hand, shorter MW gelatin is preferred as smaller peptides migrate faster to the liquid/air interface and as such, equilibrium is reached in shorter time which increases the stability of the foam[63].

1.6 Goals for the Project

SINTEF Ocean AS is a partner in the SUPREME project, which aims to increase the degree of utilization and value creation from white fish rest raw material by developing new solutions for onboard handling, logistics and processing of rest raw materials from the ocean-going fleet[65]. One important aspect of developing new processing solutions in the industry is to achieve a financial surplus, that is for the earnings to outweigh the costs of production. The main objective of this master's thesis was to investigate simple processing methods for collagen extraction using cod and pollock heads as raw material. The method chosen in this project was chosen due to the simplicity of the processing. The largest loss of lost raw material from cod and pollock fishing takes place during filleting and processing onboard of sea-going vessels. The simplicity of the chosen method could make it possible to perform on board fishing vessels, where space, storage and energy are large restrictions. The method has been carried out on fish raw materials in the past with promising results in regards to dissolving the available collagen[66][67][37].

Properties like extraction yield, product purity, amino acid composition and molecular weight distribution were considered when assessing the potential of the extraction method with cod and pollock heads as raw material. The raw material was prepared and provided by SINTEF Ocean. The amino acid composition of the collagen was determined using HPLC. Furthermore, the protein content was estimated using C/N/S elemental analysis and from the total amino acid content from HPLC. By determining the hydroxyproline content of the product its purity was estimated using a hydroxyproline conversion factor. Extracted collagen was run on SDS-page gels using electrophoresis to investigate the molecular weight distribution in the product. Pretreatment conditions and extraction times were varied to investigate optimization of the extraction process.

2 Materials and Methods

All experiments and analytical procedures were carried out with reagents of analytical grade.

2.1 Raw Material Preparation

The raw material used in this experiment were heads from the fish species Atlantic cod (*Gadus morhua*) and Atlantic pollock (*Pollachius virens*). The heads were ground using a grinder to increase the surface area. The minced fish heads were then washed with cold water to remove water soluble impurities like blood and proteins, to reduce the need for later washing steps. The wash water was kept to investigate its potential use in the production of a protein rich fraction. Afterwards, the ground fish heads were frozen and stored at -20°C until processing. The frozen fish heads were provided and prepared by SINTEF Ocean.

2.2 Collagen Extraction From Cod and Pollock Heads

The collagen extraction method that were used in this experiment was a modified version of the one proposed by Nagai and Suzuki (2000) for extraction of collagen from bones of several fish like sea bass, horse mackrel and sea beam[2]. The three main parts of the process are pre-treatment of raw material, collagen extraction and product purification. Processing parameters were changed in the pre-treatment and collagen extraction in order to investigate their effects on product yield and purity. All the solutions used for washing and extraction were prepared in advance and stored at 4°C. The experiment was carried out in a cold room with its temperature kept at 4°C.

2.2.1 Pretreatment

The homogenized fish head was weighed and placed in a beaker with 0.1M NaOH solution for 12 hours with a solid to solvent ratio of 1:6 (w/v) with slight stirring to remove non-collagenous proteins. The beakers were stirred slightly by using a stirring table. When renewing the solution and between sequential steps, the raw material was filtered using a fine mesh sieve and two layers of cheesecloth and washed with distilled water until neutral pH. The fish heads were then decalcified in a beaker with 0.5M EDTA (Disodium ethylenediaminetetraacetate dihydrate) for another 12 hours with a solid to solvent ratio of 1:6 (w/v) under stirring. Afterwards, fat was removed by washing the raw material with 10% ethanol for 12 hours with a solid to

solvent ratio of 1:6 (w/v) with stirring. A flowchart of the pretreatment process is shown below in Figure (2.1).

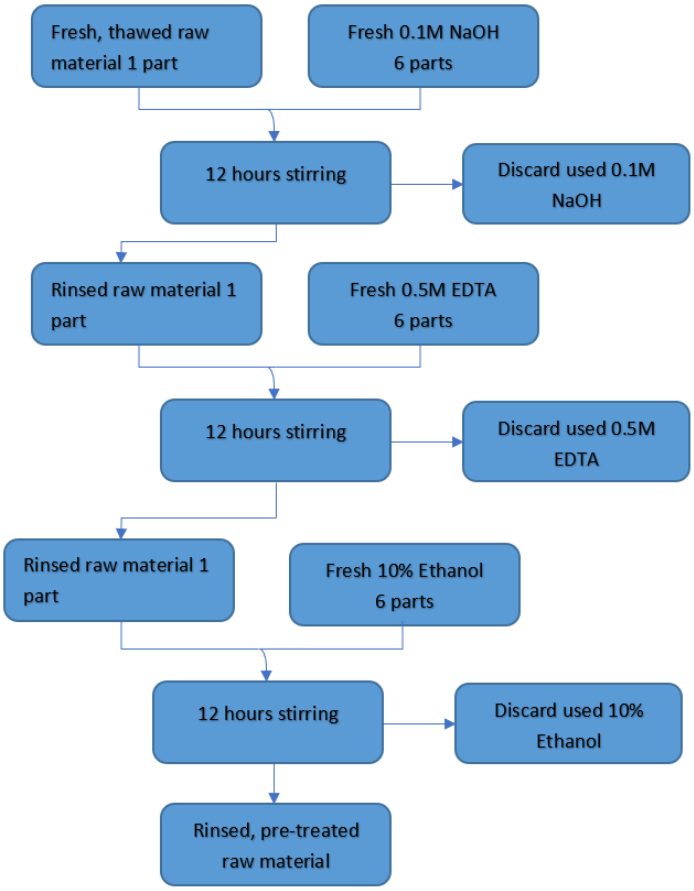

Figure 2.1: Flowchart of how the raw material is pretreated, showing the steps of protein removal with NaOH, ash removal with EDTA and fat removal with ethanol. The rinsed, pre-treated raw material is then used in the extraction step of the process.

2.2.2 Extraction

After pretreatment the raw material was placed in a beaker with 0.5M acetic acid with a solid to solvent ratio of 1:6 (w/v). Half the raw material was extracted for 24 hours with stirring, after which the extraction solution was filtered with a fine mesh sieve and a double layer of cheesecloth, and then stored. The other half was extracted for 12 hours, after which the extraction solution was filtered and stored, while the residual raw material was placed in fresh 0.5M acetic acid with a solid to solvent ratio of 1:6 (w/v) for another 12 hours. The two filtrates from the 12 hours extraction in series were combined. A small part of the used raw material was frozen and stored for analyzing its hydroxyproline content, while the rest was discarded. A flowchart of the extraction process is shown below in Figure (2.2).

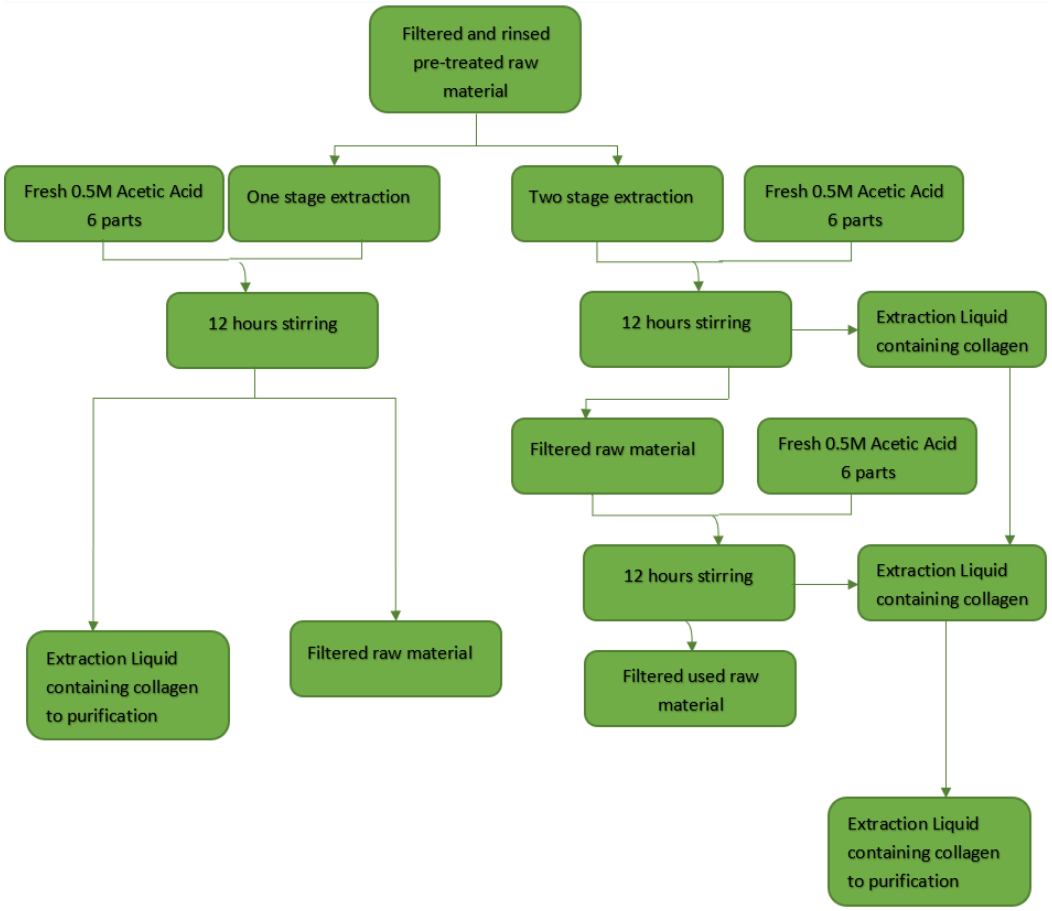

Figure 2.2: Flowchart of how the collagen is extracted, including both one step and two step extraction with acetic acid. For the two step extraction the two extraction liquids are combined.

2.2.3 Purification

After extraction, the total volume of the extraction solutions was measured. In order to achieve weight balance during centrifuging, 100 ml of extraction solution was measured out. Two separate 100 ml volumes were taken from the combined filtrate of the 12 hours in series extractions. Collagen was salted out of the filtrates by adding NaCl to a final concentration of 2.6M, at which point the collagen precipitated. To collect the precipitate centrifuging was used at 27 000 g for 20 minutes, after decantation the supernatant was discarded. The precipitate was dissolved in 0.5M acetic acid and dialyzed with 10 volumes for 24 hours against 0.1M acetic acid and distilled water, with a change of solution halfway. Afterwards, to remove the water, it was freeze-dried and the product collagen collected, weighed and then stored at -20°C. A flowchart of the extraction process is shown below in Figure (2.3).

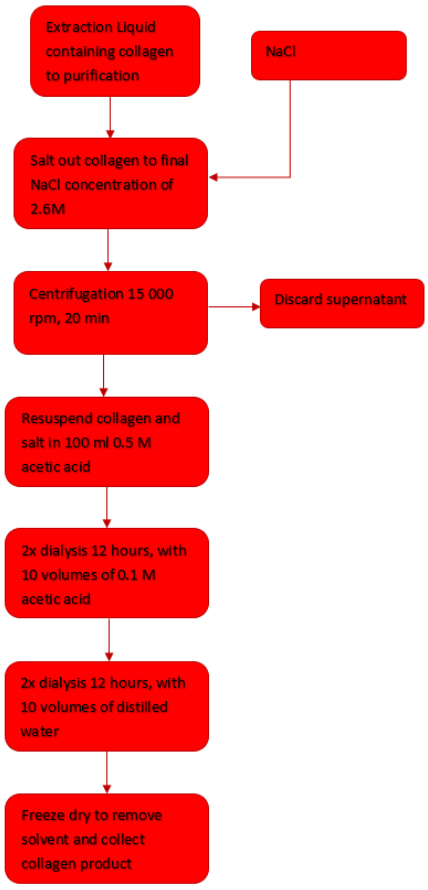

Figure 2.3: A flowchart of how the collagen is purified, showing salting out the collagen, centrifugation and decantation, resuspension and dialysis and freeze-drying to obtain the final collagen product.

2.3 Extraction Parallels

A total of four different extractions were carried out, using both cod- and pollock-heads as raw material in one- and two-stage extractions. The extractions have varying extraction time, and pretreatment conditions, with two parallels for each variation. An overview of all the extraction variations is presented in Table (2.1) below.

Table 2.1: An overview of the 4 different extractions that was performed in this experiment, each with 4 variations. For each variation 2 parallels was performed, thus a total of 32 extractions were carried out.

Parallel name	RRM	NaOH	EDTA	Ethanol	Extraction
1C 24h	Cod	1:4 12 hours	1:4 12 hours	-	24 hours
1P 24h	Pollock	1:4 12 hours	1:4 12 hours	-	24 hours
1C 2*12h	Cod	1:4 12 hours	1:4 12 hours	-	2*12 hours
1P 2*12h	Pollock	1:4 12 hours	1:4 12 hours	-	2*12 hours
2C 24h	Cod	1:4 12 hours	1:6 12 hours	1:6 12 hours	24 hours
2P 24h	Pollock	1:4 12 hours	1:6 12 hours	1:6 12 hours	24 hours
2C 2*12h	Cod	1:4 12 hours	1:6 12 hours	1:6 12 hours	2*12 hours
2P 2*12h	Pollock	1:4 12 hours	1:6 12 hours	1:6 12 hours	2*12 hours
C 48h	Cod	1:4 12 hours	1:6 12 hours	1:6 12 hours	48 hours
P 48h	Pollock	1:4 12 hours	1:6 12 hours	1:6 12 hours	48 hours
C 2*24h	Cod	1:4 12 hours	1:6 12 hours	1:6 12 hours	2* 24 hours
P 2*24h	Pollock	1:4 12 hours	1:6 12 hours	1:6 12 hours	2* 24 hours
C 72h	Cod	1:4 12 hours	1:6 12 hours	1:6 12 hours	72 72 hours
P 72h	Pollock	1:4 12 hours	1:6 12 hours	1:6 12 hours	72 72 hours
C 2*36h	Cod	1:4 12 hours	1:6 12 hours	1:6 12 hours	2* 36 hours
P 2*36h	Pollock	1:4 12 hours	1:6 12 hours	1:6 12 hours	2* 36 hours

2.4 Amino Acid Composition

The method for determining the amino acid composition of the collagen product was the same as described in Grefstad (2021)[3], the precursor project for this thesis. The amino acid composition of the collagen was analysed by high performance liquid chromatography (HPLC)(Dionex UltiMate® 3000 UHPLC+ focused, Dionex UltiMate® 3000 Autosampler, Dionex RF Fluorescence Detector, Thermo Scientific, USA) and Nova-Pak column (Nova-Pak C18 4 μm, 3.9·150 mm). HPLC is a method that utilizes the differences in interaction between molecules in a sample and a chromatographic packing material in a column. The sample mixture is passed through a column at high pressure, and as the sample passes through the column, it interacts with the packing material at different rates. Depending on the amount of interaction, molecules will exit the column at different times. At the end of the column there is a detector that determines the identity and concentration of exiting compounds[3].

The collagen samples were prepared using the method described by S. Blackburn (1968)[68]. One sample of 20 mg was weighed out from each batch of extracted collagen. The samples were dissolved in 0.5 mL of 6 M HCl in sample vials and placed in a heating cabinet at 105°C for 22 hours. After 30 minutes, the lids of the vials were tightened, and the samples were placed back into the heating cabinet. After 22 hours the pH of the samples was adjusted with NaOH and HCl to approximately neutral, pH values in the range of 6.5-7.5 were accepted. The samples were filtered with suction (Whatman glass microfiber filter GF/C, 1.2 μm), filtrates were transferred to 10 mL test tubes and doubly distilled water was added to a total of 10 mL[3].

For HPLC samples from the 10 mL test tubes were diluted to 1:500 using distilled water, and filtered through a syringe filter (0.2 μm, Whatman, F30/0.2 CA-S). Afterwards, 205 μL was transferred to a HPLC sample vial. The 10 mL test tubes with hydrolysate were stored in a freezer at -20°C to be used for determining hydroxyproline content[3].

The HPLC procedure was performed by Siri Stavrum at the Department of Biotechnology and Food Science at NTNU.

2.5 Hydroxyproline Content

The method for determining the hydroxyproline content of the collagen product, raw material and raw material residue was the same as described in Grefstad (2021), the precursor project for this thesis[3], and is described below. Hydroxyproline is an amino acid that characteristically is found almost exclusively in collagen. Thus, the amount of hydroxyproline in a collagen or gelatin sample can be utilized to determine its purity. The hydroxyproline content was determined according to the method described by Leach (1960), a modification of a method by Neuman and Logan (1950)[69][70]. In the method, an oxidation reaction with hydroxyproline takes place in a solution, resulting in a final oxidation product that has a pink colour. This allows the optical density of the solution containing the oxidation product to be measured to determine the original hydroxyproline content of the sample[3].

A stock solution of 100 μg/mL hydroxyproline was prepared by dissolving L-hydroxyproline in distilled water and HCl. From the stock solution samples were taken out and diluted to 5, 10 and 15 μg/mL to make a standard curve. Specific conditions for fish samples were used. Hydrolysed collagen

samples were made earlier for HPLC and stored in freezer until use. The hydroxyproline content of unused raw material and raw material residue taken after the extraction process was also determined. Frozen samples of used and unused fish heads were freeze-dried and crushed to a homogeneous powder, before it was hydrolysed with 6M HCl in the manner described in Section (2.2). Three parallels were made for each gelatin sample and standard[3].

Firstly, 0.5 mL of standard, sample and water (blank) were transferred to separate test tubes. Afterwards, 0.5 mL 0.05 M $CuSO_4$ and 0.5 mL 2.5 M NaOH was added to each tube and mixed by shaking. The tubes were covered with marbles and placed in a water bath at 50°C for 10 minutes. The marbles were removed and 0.5 mL of 6% H_2O_2 was added to each tube and immediately shaken before the tubes were placed back in the water bath at 50°C for another 10 minutes covered with marbles. The tubes were then cooled with running water to room temperature and, in a fume cupboard, 2 mL of 1.5 M H_2SO_4 and 1 mL of 5 % p-dimethylaminobenzaldehyde in 1-propanol was added to each tube before shaking them. The tubes were then covered with a marble and placed in a water bath at 70°C for 16 minutes. Then, the test tubes were cooled to room temperature, shaken and left to rest 2 minutes. The optical density was measured at 555 nm with a spectrofotometer[3].

The OD of the standard samples was used to make a standard curve. The function ofthe curve was determined and used to calculate the concentration and amount of hydroxyproline in the gelatin hydrolysate sample. The amount of hydroxyproline in the sample was multiplied with a factor of 14.7 to get the collagen content. This factor was used because collagen from cod skin has been determined to contain 14.7% hydroxyproline[71].

2.6 SDS-PAGE

The method for determining the molecular weight distribution of the collagen product was a modified version of the one described in Grefstad (2021), the precursor project for this thesis[3]. SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) was used to determine the molecular weight distribution of the extracted collagen. It is an electrophoresis system that separates proteins based on size. SDS is a negatively charged molecule that non covalently binds to proteins, denaturing them in the process. When associated with a protein the negative SDS molecule gives the protein negative charge with a fixed charge to mass ratio. In a gel exposed

to an electrical field the negatively charged polypeptide chains will migrate towards the anode. The polypeptide chains are retained in the gel depending on the molecular weight of the chain, thus separating them according to size[3].

Collagen samples of 25 mg were weighed out and dissolved in 20 mL of distilled water and left to dissolve overnight at room temperature. Afterwards, 0.5 mL of dissolved collagen was transferred to an eppendorf tube and mixed with 0.5 mL diluted buffer (Pierce™ LDS Sample Buffer, Non-Reducing (4X)), 2% DDT was added to the buffer right before use. The eppendorf tubes were placed in a water bath at 70°C for 10 minutes, and 0.1 mL of 10% glycerol was added to each tube and mixed. A high molecular weight standard (Amersham™, HMW Calibration kit for SDS Electrophoresis) was used. An 4-20% acrylamide gel (ClearPAGE™ SDS Gel, 4-20%, 12-well) was rinsed and placed into the apparatus. The running buffer (ClearPage™, SDS Run Buffer, 20x 500 ml, C.B.S Scientific) was mixed with distilled water in a 1:20 (w/v) ratio, and added to the inner and outer running chambers. Then, 10 μL of standard was added to the two outer wells on the gel and 10 μL of samples were added to each of the inner wells. The apparatus was connected to a PS300B 300 Volt Power Supply (AA Hoefer). The power supply was turned on with a constant voltage of 180 V for 45 minutes[3].

After the electrophoresis the gel was removed from the cassette and briefly rinsed with distilled water. Protein stain (InstantBlue Protein Stain™, Expedon) was added to stain the gels using a protein Staining System (eStain® L1 Protein Staining System, GenScript). Afterwards, it was rinsed in water and photographed.

2.7 C/N/S Elemental Analysis

CHNS elemental analysis is a method that is able to quickly determine carbon, hydrogen, nitrogen and sulfur in solid, liquid, volatile and viscous organic samples. The CHNS elemental analyser is a flexible instrument that can be configured to analyse various combinations of elements, such as CHNS, CNS and CHN.

The samples are weighed in tin containers which are loaded onto an auto sampler and then dropped into a combustion chamber. The analysis requires high temperatures (ranging from 1000-1800°C) and high levels of oxygen in

the combustion chamber. Combustion can be done with static conditions by introducing a set amount of oxygen to the chamber prior to combustion or under continuous conditions by introducing a continuous flow of oxygen into the chamber during combustion. The gasses N_2, NO_x, H_2O, SO_2, O_2 and CO_2 are produced from combusting the sample. Afterwards, the exhaust gasses are swept out of the combustion chamber by an inert carrier gas to a Cu column, which reduces nitrogen oxides to nitrogen gas, and removes any excess oxygen not consumed in the combustion. The gaseous mixture is then passed through an absorbent trap to remove all other gasses except N_2, H_2O, SO_2 and CO_2. Detection of the final gaseous mix is done by separation or partial separation by gas chromatography followed by thermal conductivity detection[72].

In this experiment, C/N/S elemental analysis was used to determine the amount of protein in the final collagen product from the extractions. Collagen samples of about 1 mg were weighed out and placed in tin containers. The C/N/S analysis was carried out by Gerd Inger Sætrom at the Department of Biotechnology and Food Science at NTNU using an Elementar vario El cube instrument.

3 Results

3.1 Collagen Extraction Yield

The dry collagen yield and hydroxyproline yields were calculated for the extractions. The dry yield is based on the weight of the extracted collagen and the dry weight of the raw material used, while the hydroxyproline yield is based on the amount of hydroxyproline in the raw material and the extracted collagen. Dry weight yield is determined by dividing the mass of the collagen product by the weight of the raw material used in the extraction. Hydroxyproline yield is determined by dividing the amount of hydroxyproline in the collagen product by the amount of hydroxyproline in the raw material. The yields from all extractions are presented in Table (3.1) below. The yields varied between each extraction method, and they were generally all quite low. The highest dry weight yield was achieved in extraction 1P 24h, using pollock as raw material, with mild pretreatment and a 24 hour extraction time, while the lowest were observed for C (cod) 72h and P (pollock) 72h, with long pretreatment and a 72 hour extraction time. The highest hydroxyproline yield was also observed for extraction 1P 24h. Meanwhile, the lowest was observed for all the extractions with a 72 hour extraction time, as the hydroxyproline content of the collagen product from this extraction were all observed to be zero.

Table 3.1: Extraction yields for all extractions given in a dry weight basis and theoretical hydroxyproline basis. C and P refer to the raw material used, cod head and pollock head respectively, in the extraction parallel. Dry weight yield is determined by dividing the mass of the collagen product by the weight of the raw material used in the extraction. Hydroxyproline yield is determined by dividing the amount of hydroxyproline in the collagen product by the amount of hydroxyproline in the raw material. The names of the parallels are explained in Table (2.1) in section 2.3.

Parallel name	Dry weight yield [%]	Hydroxyproline yield [%]
1C 24h	1.9	3.0
1P 24h	2.3	3.4
1C 2*12h	1.3	1.5
1P 2*12h	1.6	2.4
2C 24h	0.4	0.6
2P 24h	0.6	1.9
2C 2*12h	0.4	1.6
2P 2*12h	0.5	2.2
C 48h	0.2	0.1
P 48h	0.6	0.3
C 2*24h	0.2	0.4
P 2*24h	0.4	0.7
C 72h	0.1	-
P 72h	0.1	-
C 2*36h	1.3	0
P 2*36h	1.8	0

3.2 Hydroxyproline and Collagen Purity

The concentration of hydroxyproline showed a large variation over a wide range between each extraction. From extraction 4 with 72 hours extraction time there was no hydroxyproline detected for any of the eight samples. The highest amount of hydroxyproline was observed in the product from 1C 2*12h, at 26.2 μg/g sample, which thus was estimated to have 38.5 % collagen. Apart from extraction 4, the lowest amount of hydroxyproline and subsequently estimated collagen % was observed for extraction C 48h. To calculate the collagen % in the product, a hydroxyproline conversion factor of 14.7 was used[71]. The hydroxyproline amount and collagen purity in each extraction product is presented in Table (3.2) below.

Table 3.2: Amount of hydroxyproline and weight% of collagen in hydrolysed product samples. The names of the parallels are explained in Table (2.1) in section 2.3.

Parallel name	hydroxyproline [μg/g sample]	Collagen in product [%]
1C 24h	25.5	37.5
1P 24h	23.3	34.2
1C 2*12h	26.2	38.5
1P 2*12h	22.2	32.6
2C 24h	11.3	16.7
2P 24h	22.2	32.6
2C 2*12h	24.0	35.3
2P 2*12h	21.1	31.0
C 48h	2.6	3.9
P 48h	4.4	6.5
C 2*24h	10.3	15.2
P 2*24h	10.7	15.8
C 72h	-	-
P 72h	-	-
C 2*36h	0.0	0.0
P 2*36h	0.0	0.0

Hydroxyproline was also measured in the used raw material and unused raw material in order to compare them. For unused cod and pollock heads, the hydroxyproline levels were found to be 16.7 and 15.7 μg/g sample respectively on a dry basis. The largest change in hydroxyproline levels was observed in the used raw material from extraction P 2*36h, with a change of 7.9 μg/g sample, as such it also had the lowest measured hydroxyproline amount. The used raw material with the smallest change in its hydroxyproline content was C 2*36 in which the change was only 0.7 μg/g sample. The hydroxyproline content and change in hydroxyproline content for all extractions are presented in Table (3.3) below.

Table 3.3: Amount of hydroxyproline the raw material left after extraction and the difference in hydroxyproline level between the used raw material and unused raw material. For the unused raw material 16.7 and 15.7 μg/g sample was observed for cod and pollock respectively. The names of the parallels are explained in Table (2.1) in section 2.3.

Parallel name	hydroxyproline [μg/g sample]	Change hydroxyproline [μg/g sample]
1C 24h	11.7	5.0
1P 24h	11.5	4.2
1C 2*12h	13.0	3.7
1P 2*12h	11.3	4.3
2C 24h	9.4	7.3
2P 24h	11.6	4.0
2C 2*12h	11.7	5.0
2P 2*12h	13.5	2.2
C 48h	11.6	5.1
P 48h	12.1	3.6
C 2*24h	11.0	5.7
P 2*24h	14.2	1.4
C 72h	14.8	1.9
P 72h	8.9	6.8
C 2*36h	15.9	0.7
P 2*36h	7.7	7.9

3.3 Amino Acid Composition

The analysis of amino acid compositions for extraction methods 1 and 2 show similar distributions, as can be seen in Figures (3.1) and Figure (3.2) below however, there are some variations depending on the raw material used and number of extraction steps. For these two methods, glycine/arginine was the most abundant amino acid while histidine and tyrosine were the least. However, the compositions of collagen extracted with methods 3 and 4 are quite varying as can be seen in Figures (3.3) and Figure (3.4) below. Glutamic acid, aspartic acid, glycine/arginine and lysine are the most abundant amino acids depending on the method and raw material used.

For collagen extracted from pollock heads in one stage with 48 hours extraction time (P 48h) there are several amino acids which were not detected. The imino acids hydroxyproline and proline are not possible to quantify with the detection method used in this experiment. For the 72 hours extraction in one stage, there was not enough product to analyse with HPLC.

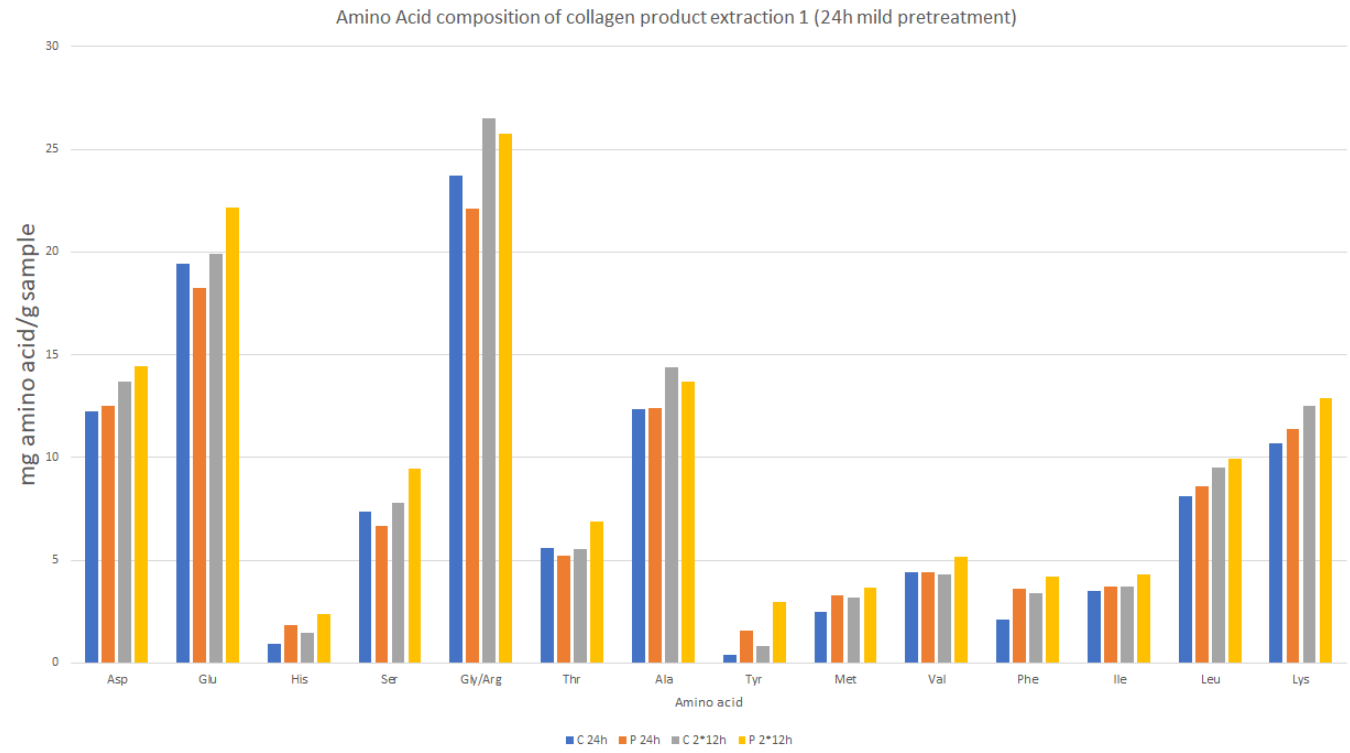

Figure 3.1: Amino acid composition of the collagen product from extraction 1, with mild pretreatment and 24 hours extraction time. Collagen from Cod heads (C) and Pollock heads (P) with one step extraction (24h) and two step extraction (2*12h) are included.

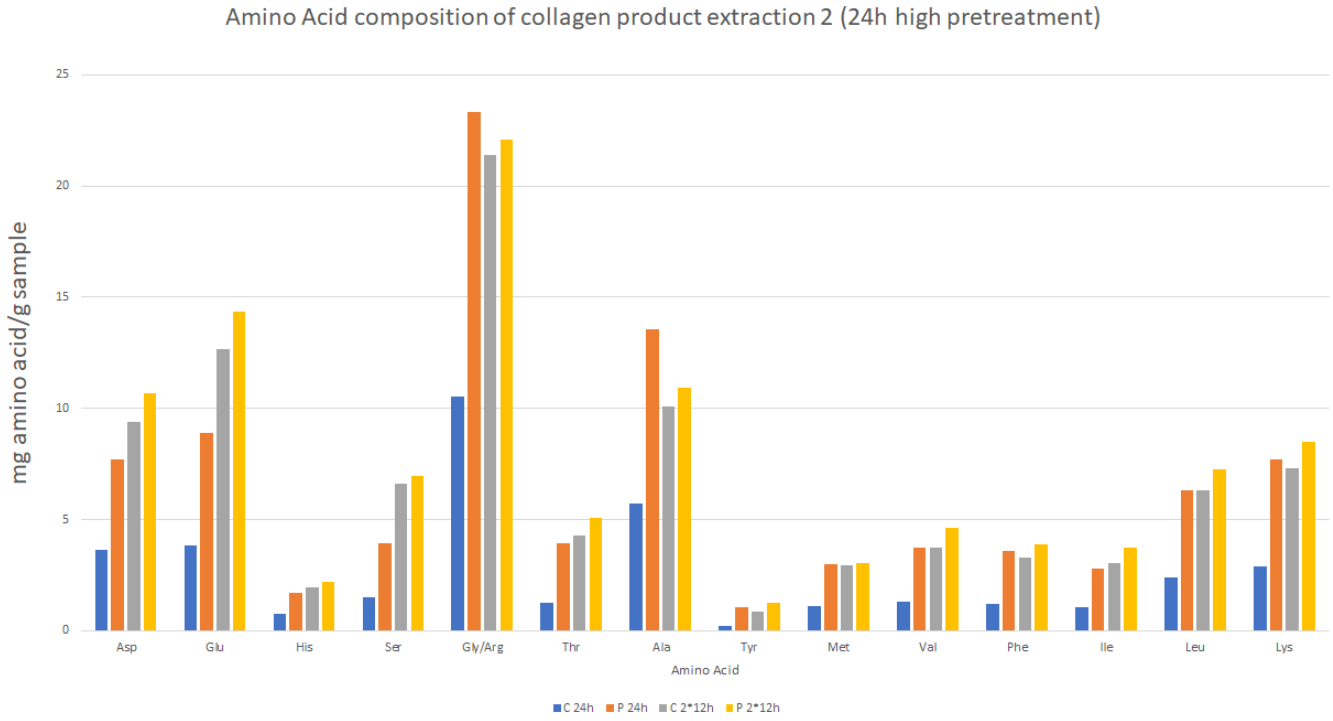

Figure 3.2: Amino acid composition of the collagen product from extraction 2, using 24 hours extraction time. Collagen from Cod heads (C) and Pollock heads (P) with one step extraction (24h) and two step extraction (2*12h) are included.

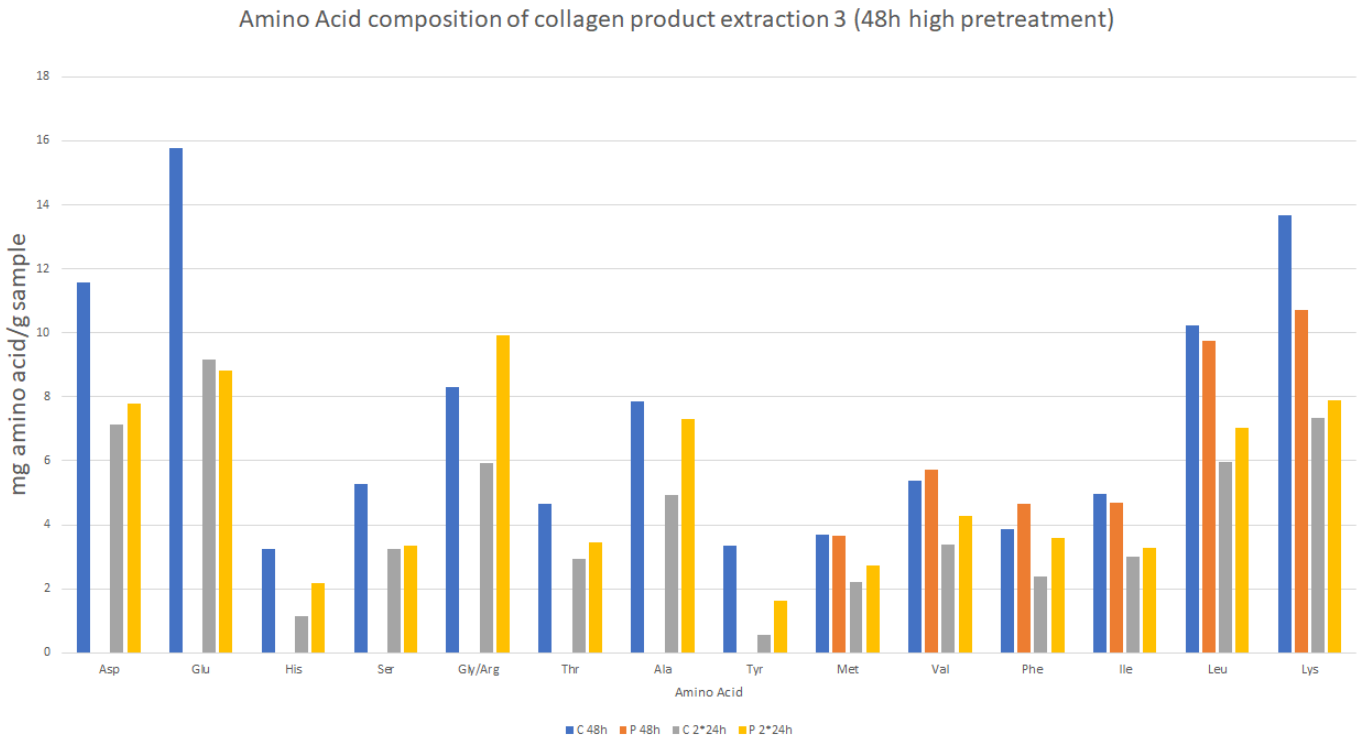

Figure 3.3: Amino acid composition of the collagen product from extraction 3, using 48 hours extraction time. Collagen from Cod heads (C) and Pollock heads (P) with one step extraction (48h) and two step extraction (2*24h) are included.

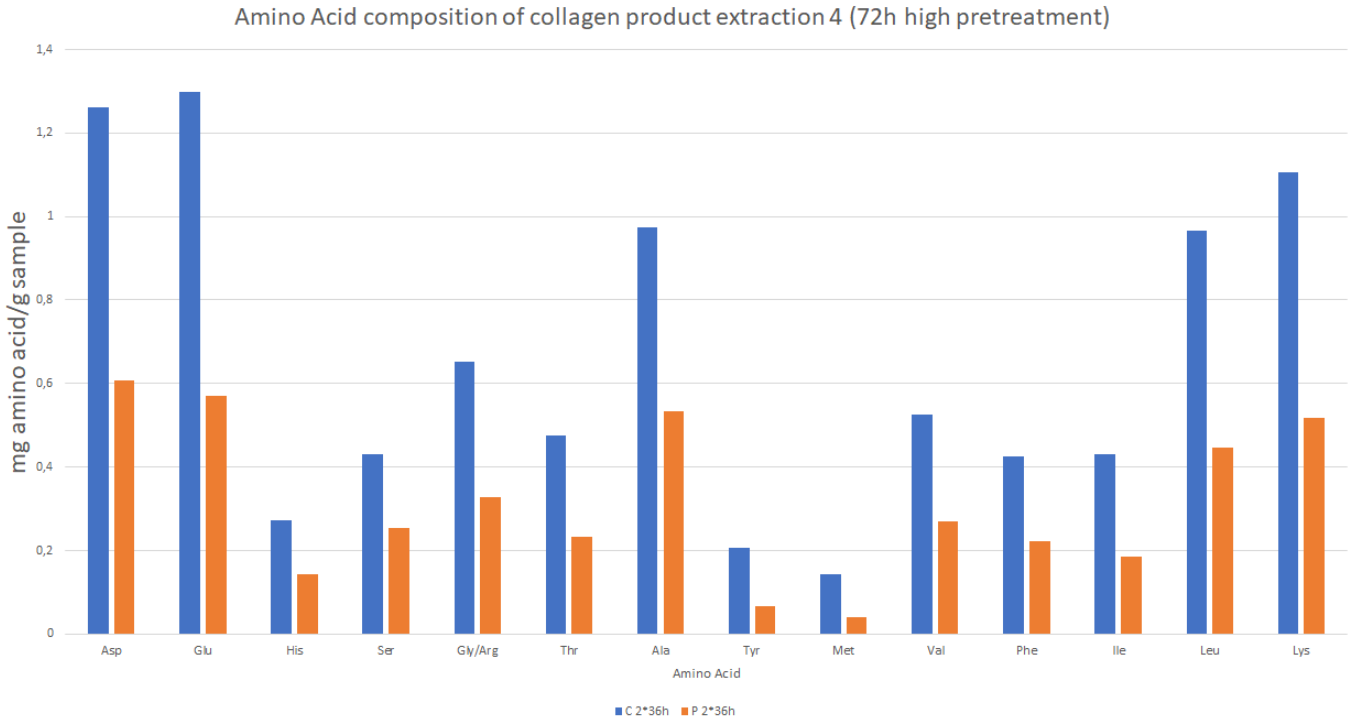

Figure 3.4: Amino acid composition of the collagen product from extraction 4, using 72 hours extraction time. Collagen from Cod heads (C) and Pollock heads (P) with one step extraction (72h) and two step extraction (2*36h) are included.

The mole fraction of glycine in each collagen sample is presented in Table (3.4) below. For collagen it is characteristic that 33% of the amino acids are glycine, as it makes up every third amino acid residue. The largest molar fraction of glycine observed was for parallel 2C 24h, with cod as raw material, 24 extraction time and harsher pretreatment conditions. The lowest glycine mole fraction was observed for P 48h, using pollock as raw material, 48 hours extraction time and harsh pretreatment condition which was determined to be 0%.

Table 3.4: The mole fraction of glycine in the extracted collagen

Extraction	mole fraction glycine [%]
1C 24h	22,2
1P 24h	20,6
1C 2*12h	22,3
1P 2*12h	20,2
2C 24h	29,1
2P 24h	26,6
2C 2*12h	24,2
2P 2*12h	22,6
C 48h	9,2
P 48h	0,0
C 2*24h	11,1
P 2*24h	14,8
C 72h	0,0
P 72h	0,0
C 2*36h	7,9
P 2*36h	8,0

The total amino acid content of each HPLC sample is presented in Table (3.5) below. This is a measure of the protein purity of the sample. A pure protein sample would have an expected amino acid content of 1000 mg amino acid/g sample. The highest total amino acid content was observed for the collagen extracted with pollock heads as raw material, with mild pretreatment, 24 hours extraction time in two stages, with a total of 138,1 mg aa/g sample. Thus, the overall amino acid content was low for all HPLC samples. The lowest detected amino acid content was observed with pollock as raw material with 72 hours extraction time in two stages, with a total of 4,4 mg aa/g sample.

Table 3.5: Total amount of amino acid detected from HPLC analysis of all the extracted collagen. The names of the parallels are explained in Table (2.1) in section 2

Extraction	Total mg aa/g sample
1C 24h	113.7
1P 24h	115.8
1C 2*12h	127.0
1P 2*12h	138.1
2C 24h	37.5
2P 24h	91.4
2C 2*12h	94.0
2P 2*12h	104.6
C 48h	101.9
P 48h	39.2
C 2*24h	59.4
P 2*24h	73.4
C 72h	-
P 72h	-
C 2*36h	9.2
P 2*36h	4.4

The amino acid composition of unused raw material is presented in Figures (3.5-3.9) below. The most abundant amino acid for all HPLC samples was glycine/arginine, while histidine was the least common amino acid. The distribution of amino acids is similar for the used raw material samples that were analysed using HPLC.

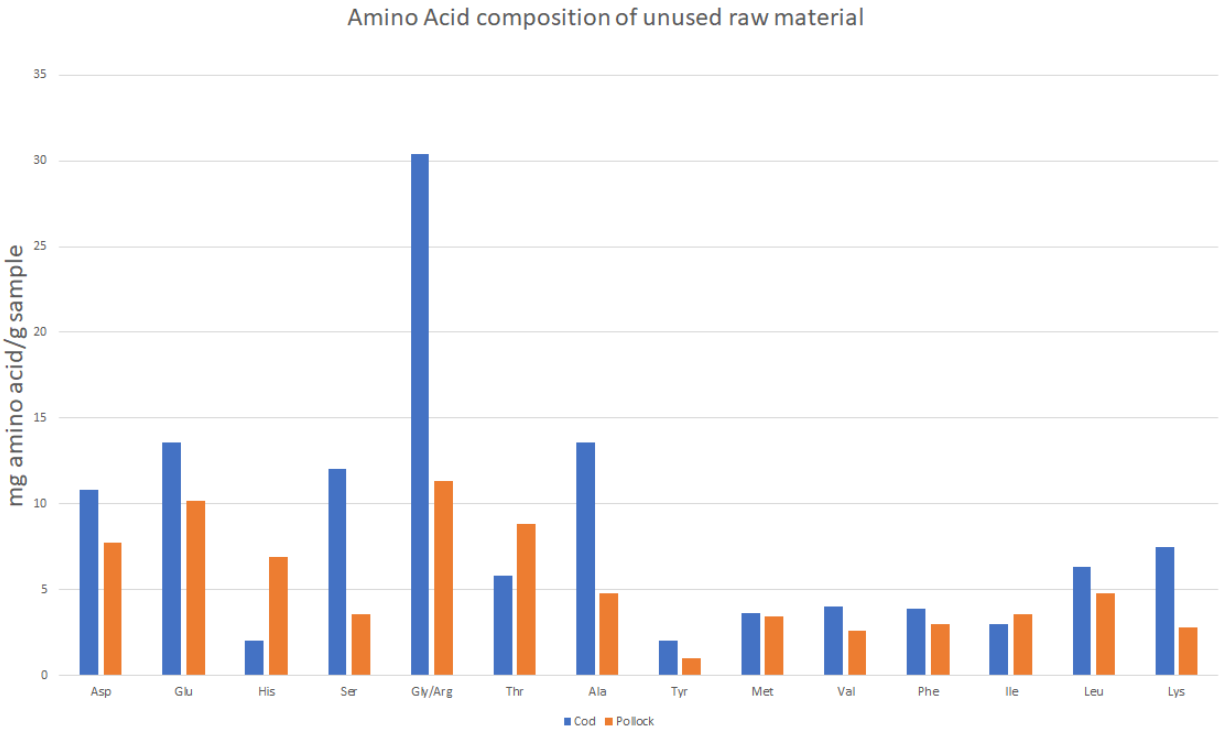

Figure 3.5: Amino acid composition of unused raw material. The C and P refer to the raw material used, cod and pollock heads respectively.

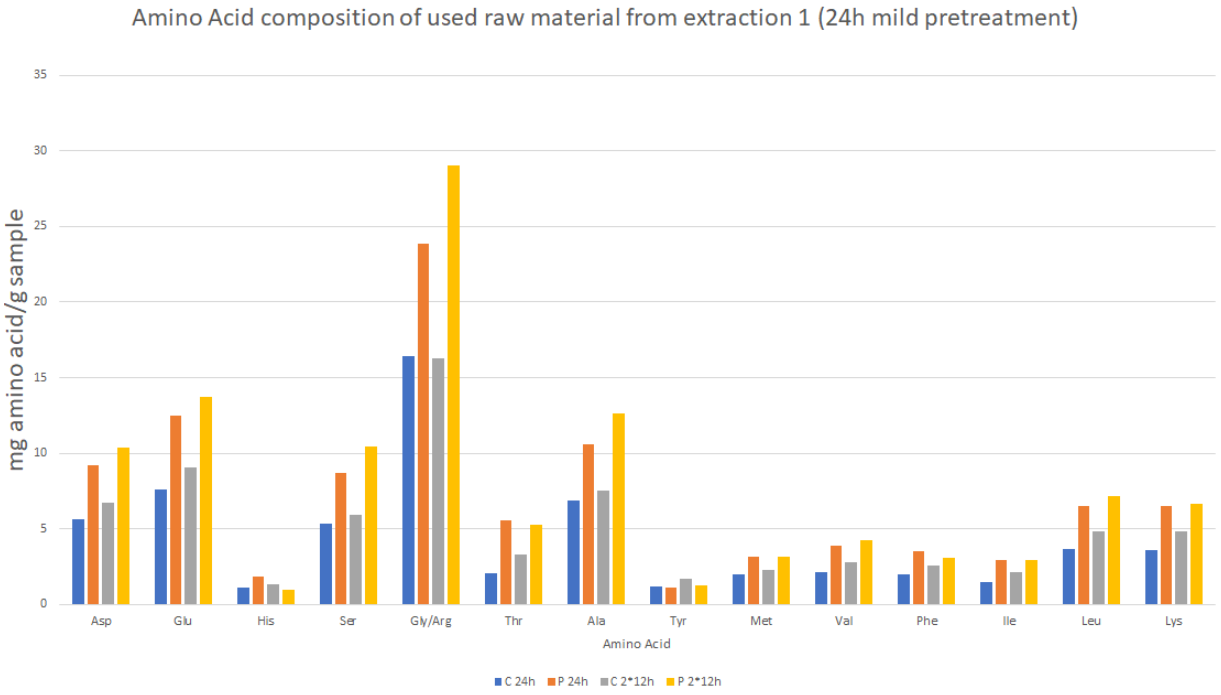

Figure 3.6: Amino acid composition of the used raw material from extraction 1, with mild pretreatment and 24 hours extraction time. Cod heads (C) and Pollock heads (P) with one step extraction (24h) and two step extraction (2*12h) are included.

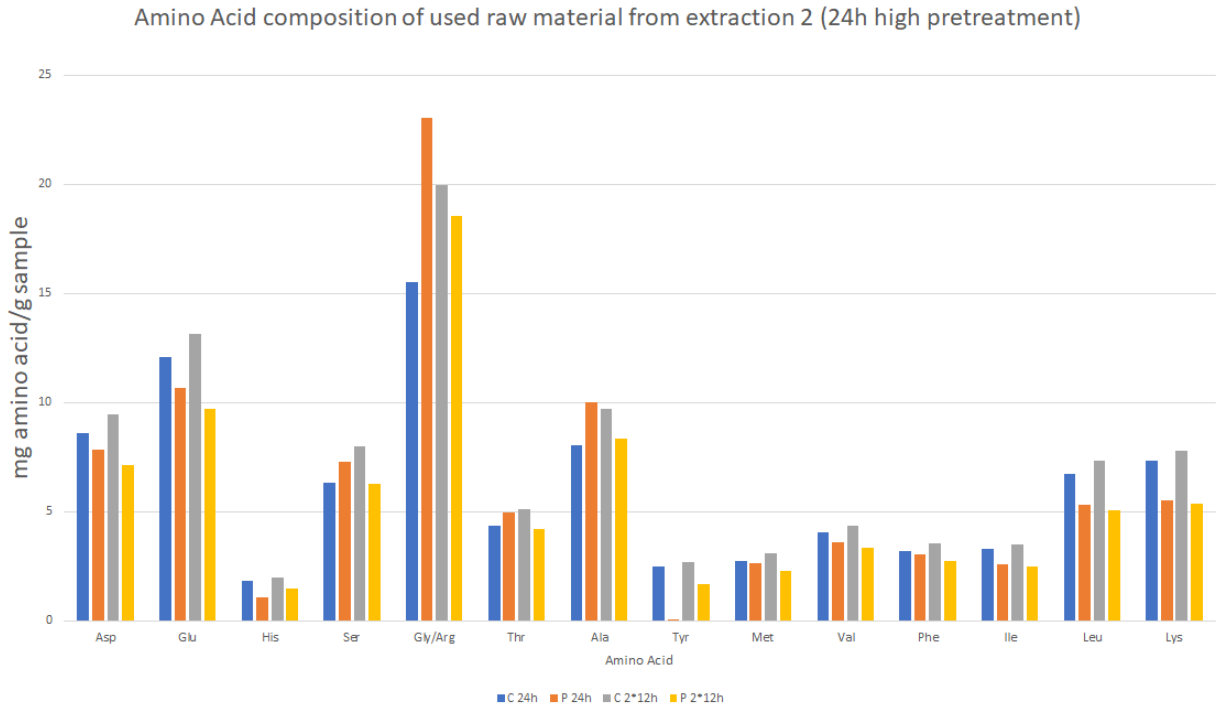

Figure 3.7: Amino acid composition of the used raw material from extraction 2, using 24 hours extraction time. Cod heads (C) and Pollock heads (P) with one step extraction (24h) and two step extraction (2*12h) are included.

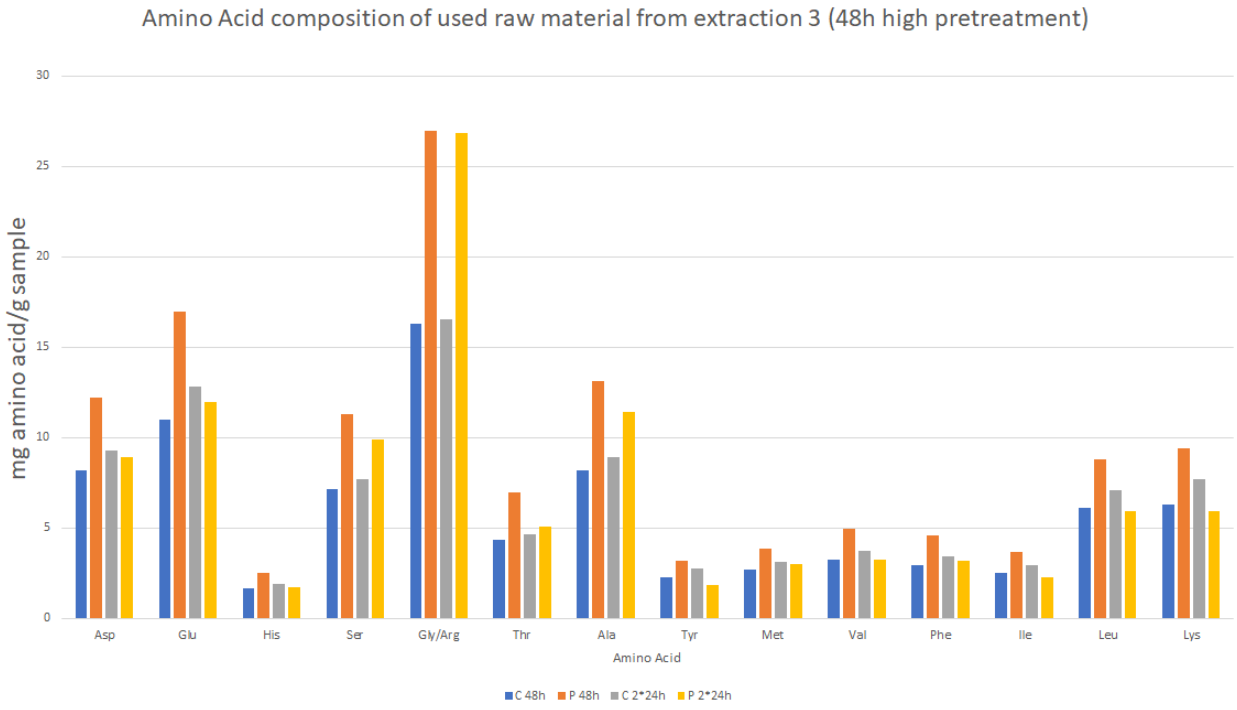

Figure 3.8: Amino acid composition of the used raw material from extraction 3, using 48 hours extraction time. Cod heads (C) and Pollock heads (P) with one step extraction (24h) and two step extraction (2*12h) are included.

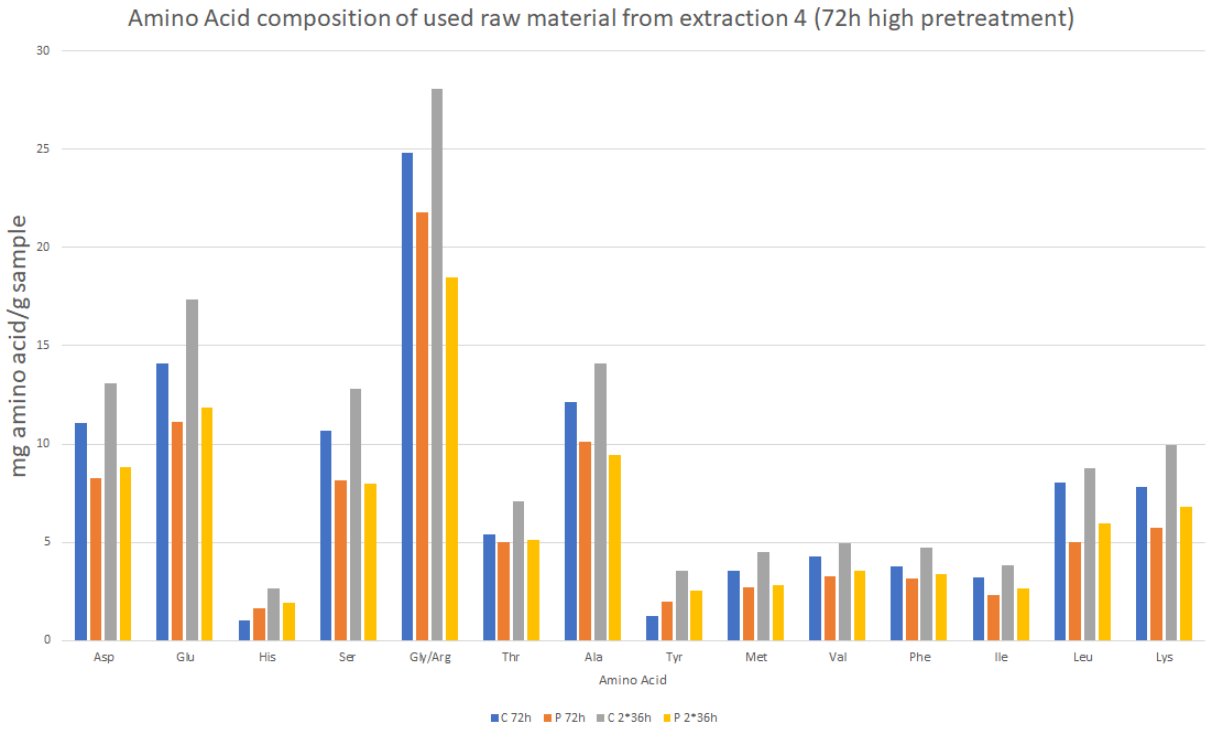

Figure 3.9: Amino acid composition of the used raw material from extraction 4, using 72 hours extraction time. Cod heads (C) and Pollock heads (P) with one step extraction (24h) and two step extraction (2*12h) are included.

3.4 Molecular Weight Distribution

All collagen product samples from the three first extractions were run on three separate polyacrylamide gels. The gels were run with a HMW protein standard in the two outer wells (Amersham™, HMW Caribration kit for SDS Electrophoresis), with typical bands appearing at 220, 170, 116, 76 and 53 kDa. On each separate gel, collagen samples from the same extraction were run. Collagen samples from all eight parallels of extraction 1 (mild pretreatment and 24 hours extraction time) were run on one gel shown in Figure (3.10) below. There is some noise between 170 kDa and 76 kDa on the gel. However, there are clear bands at about 200 kDa, as well as some bands above 220 kDa and around 53 kDa.

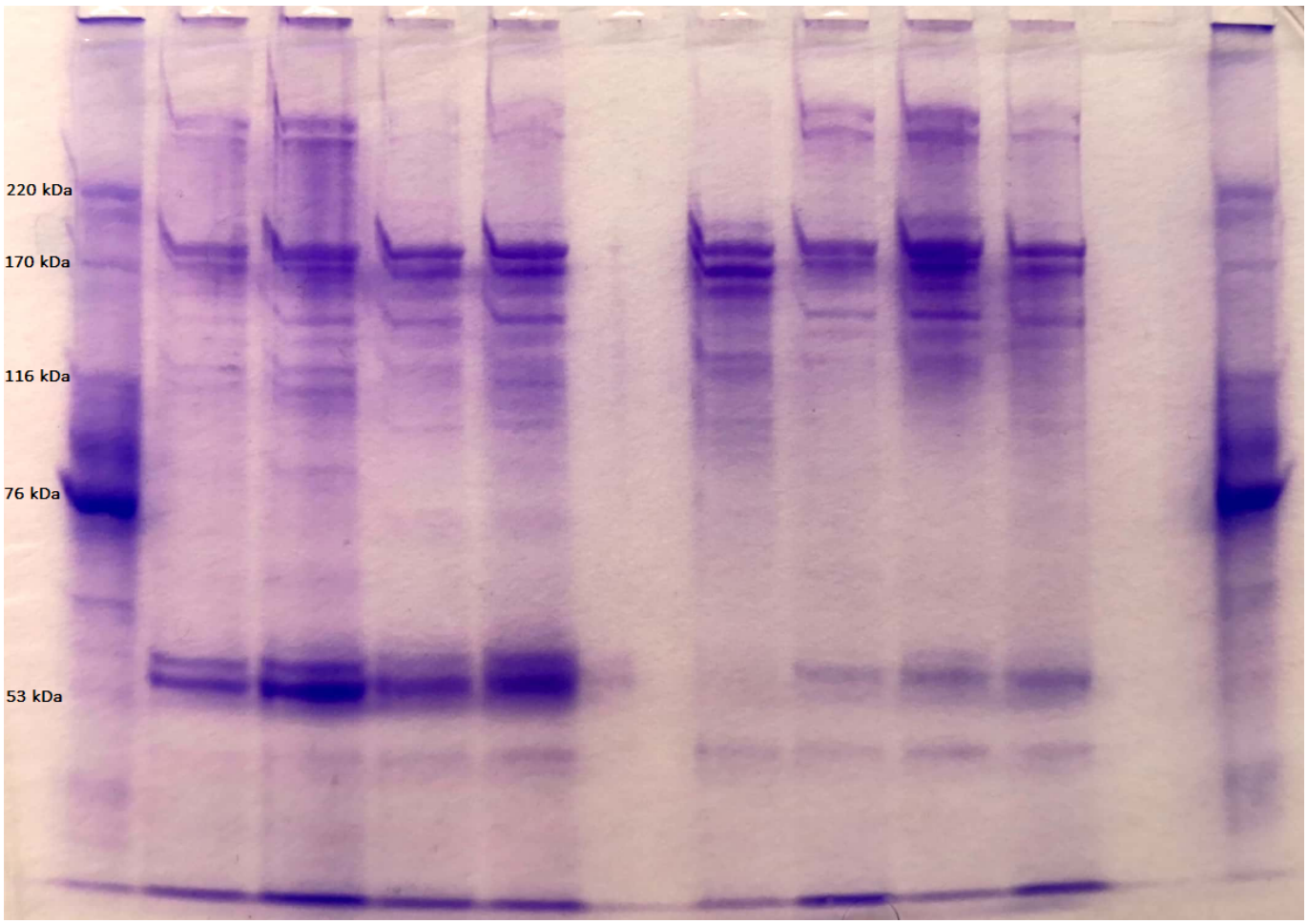

Figure 3.10: Polyacrylamide gels with HMW protein standard were deposited on the outer wells with bands between 220 and 53 kDa. The collagen samples are from extraction 1, with mild pretreatment and a total extraction time of 24 hours. From left to right the collagen samples deposited on the inner wells are 1P2 2*12h, 1P1 2*12h, 1C2 2*12h, 1C1 2*12h, 1P2 24h, 1P1 24h, 1C2 24h and 1C1 24h. C and P refer to the raw material used, pollock heads and cod heads respectively, while 2*12h and 24h refer to two-stage extraction with 12 hour steps and one-stage extraction with one 24 hour step.

Collagen samples from all eight parallels of extraction 2 (long pretreatment and 24 hours extraction time) were run on the same gel, which is shown in Figure (3.11) below. There are clear bands for all collagen samples at around slightly above 220 kDa, ca 220 kDa and below 170 kDa. Furthermore, there are several bands of lower MW, from about 60 kDa and lower.

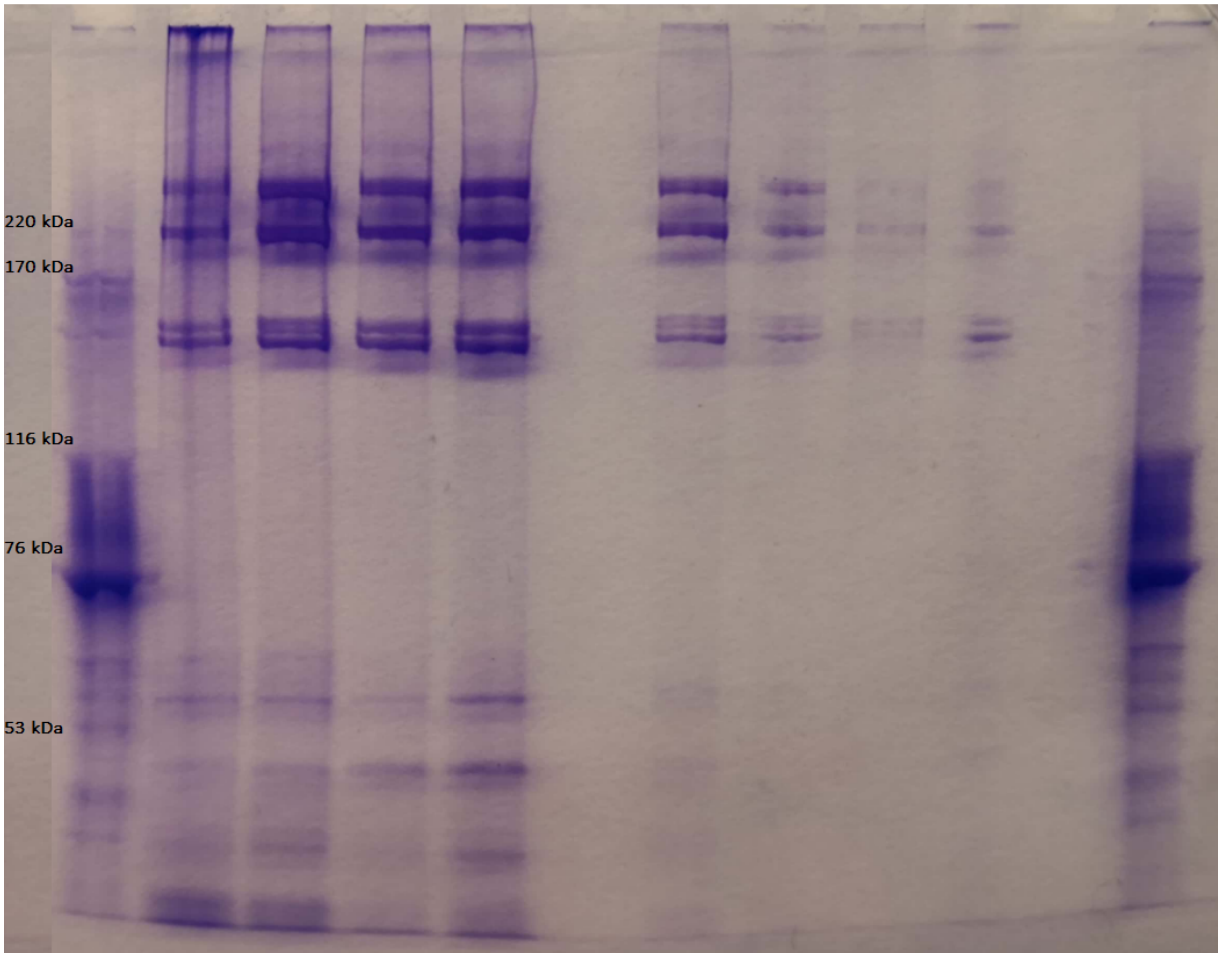

Figure 3.11: Polyacrylamide gels with HMW protein standard were deposited on the outer wells with bands between 220 and 53 kDa. The collagen samples are from extraction 2, with long pretreatment and a total extraction time of 24 hours. From left to right the collagen samples deposited on the inner wells are 2P2 2*12h, 2P1 2*12h, 2C2 2*12h, 2C1 2*12h, 2P2 24h, 2P1 24h, 2C2 24h and 2C1 24h. C and P refer to the raw material used, pollock heads and cod heads respectively, while 2*12h and 24h refer to two-stage extraction with 12 hour steps and one-stage extraction with one 24 hour step.

Collagen samples from six of eight parallels of extraction 3 (long pretreatment and 48 hours extraction time) were deposited together on the gel shown in Figure (3.12). The bands are quite faint, however, bands at about 200 kDa and between 170-116 kDa were visible for all collagen samples. There were also some bands visible at lower mw, from about 60 Kda and lower. There was not enough collagen from extraction C1 48h and P1 48h to do gel electrophoresis with.

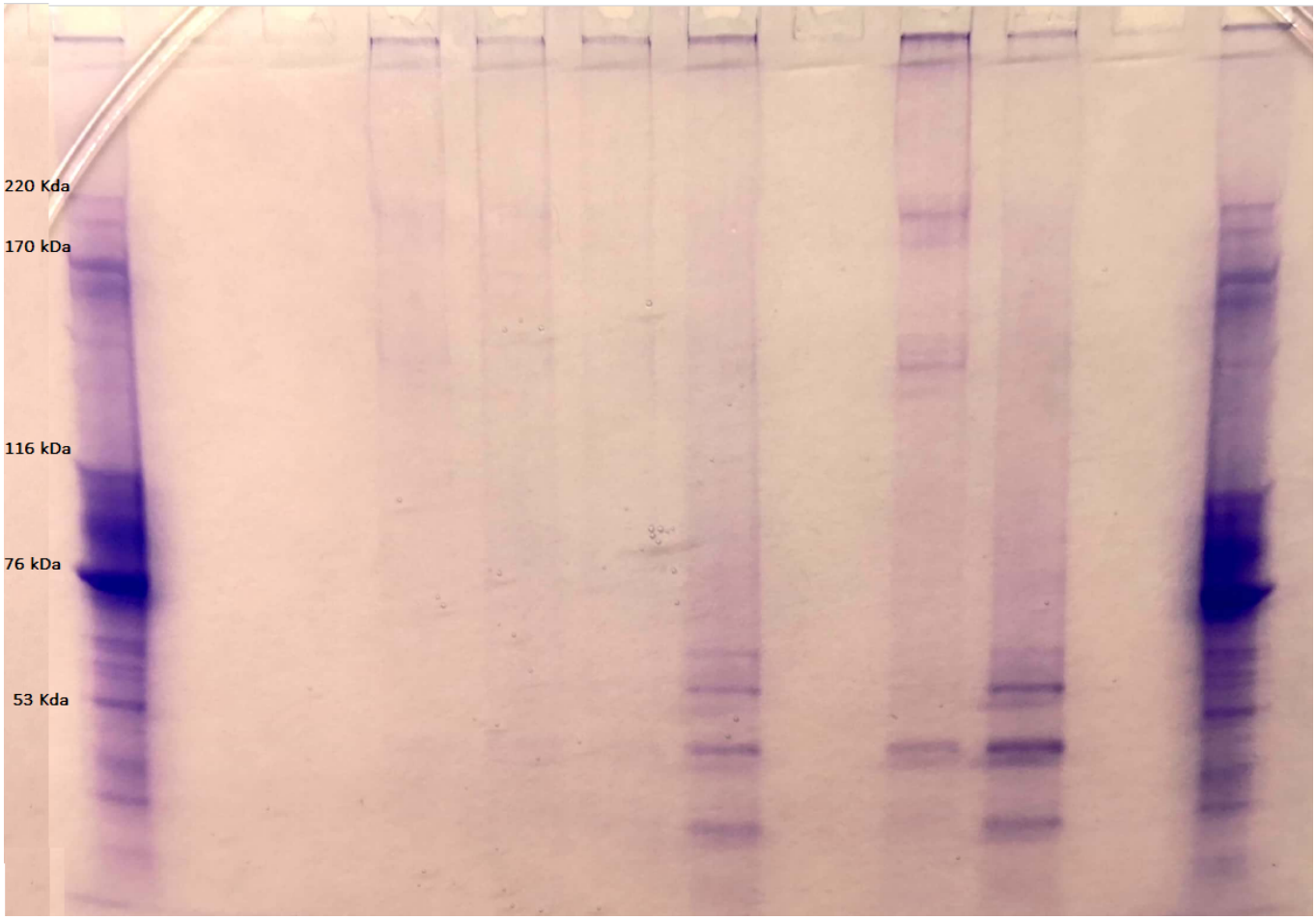

Figure 3.12: Polyacrylamide gels with HMW protein standard were deposited on the outer wells with bands between 220 and 53 kDa. The collagen samples are from extraction 3, with long pretreatment and a total extraction time of 48 hours. From left to right the collagen samples deposited on the inner wells are P2 2*24h, P1 2*24h, C2 2*24h, C1 2*24h, P2 48h and C2 48h. C and P refer to the raw material used, pollock heads and cod heads respectively, while 2*24h and 48h refer to two-stage extraction with 24 hour steps and one-stage extraction with one 48 hour step.

From extraction 4 (long pretreatment and 72 hours extraction) there was not enough collagen product to perform gel electrophoresis to determine the molecular weight distribution.

3.5 C/N/S elemental analysis

The results from the C/N/S elemental analysis are presented in Table (3.6). The highest amount of protein was observed in the collagen product from extraction 1C 24h, the extraction using cod as raw material, with mild pretreatment and 24 hours extraction time. The overall protein content is higher for the two extractions with 24 hours extraction time, than for the extractions with 48 and 72 hours extraction time. There is also some variation when using different raw materials and extractions steps within the same total extraction time. This can be clearly seen with the extractions that

Table 3.6: Results from the C/N/S elemental analysis, showing the content of carbon, nitrogen and sulphur in each sample, the ratio between carbon and nitrogen as well amount of protein present in the collagen products. The names of the parallels are explained in Table (2.1) in Section 2.3.

Parallel name	N [%]	C [%]	S [%]	C/N ratio	Prot. [%]
1C 24h	15.2	42.6	0.8	2.8	57.9
1P 24h	14.5	41.5	0.8	2.9	55.1
1C 2*12h	14.0	39.0	0.7	2.8	53.1
1P 2*12h	13.0	36.4	0.7	2.8	49.2
2C 24h	8.1	22.9	0.4	2.8	30.6
2P 24h	14.5	41.1	0.6	2.8	55.0
2C 2*12h	15.0	43.3	0.6	2.9	57.0
2P 2*12h	14.0	41.9	0.6	3.0	53.1
C 48h	12.7	41.0	0.8	3.2	48.2
P 48h	5.4	17.6	0.6	3.2	20.6
C 2*24h	4.8	15.8	0.4	3.3	18.3
P 2*24h	9.6	31.3	0.7	3.3	36.3
C 72h	11.5	37.2	0.8	3.2	43.8
P 72h	8.8	30.3	0.7	3.5	33.3
C 2*36h	0.7	7.1	0.3	12.8	2.8
P 2*36h	0.7	2.7	0.2	3.9	2.7

had a total extraction time of 48 hours, where C 48h is significantly higher than P 48h, C 2*24h and P 2*24h. The lowest observed protein content was observed in the product from extraction P 2*36h, where pollock was used as raw material, with long pretreatment and two stage extraction with total extraction time of 72 hours.

4 Discussion

4.1 Collagen Extraction Yield

The extraction yield was calculated using a dry basis and theoretical hydroxyproline yield. The dry basis uses the dry weight of the raw material, while the theoretical hydroxyproline yield is calculated using the amount of hydroxyproline available in the raw material. The yields are presented in Table (3.1) in section 3.1. Overall, both yields were quite low for all extraction parallels. The highest dry yield and hydroxyproline yield were observed for parallel 1P 24h, with 24 hours extraction time and mild pretreatment conditions which were determined to be 2.3 and 3.4% respectively. Extraction 1 had overall the highest yields for both dry weight and hydroxyproline. Extraction 4 had very low yields for the one step extractions, while the two stage extractions had relatively high dry weight yield with 1.3 and 1.8 %with cod and pollock as raw materials respectively, however the hydroxyproline yields were determined to be 0 for both. As such, it is likely that the product did not contain any collagen. Apart from these two parallels, the dry yield appears to decrease with longer extraction time and harsher pretreatment conditions. This could imply that longer extraction time and harsher pretreatment conditions leads to higher loss of product during the extraction, which could be explained by excessive hydrolyzation of the collagen. The yields were slightly higher when using pollock as raw material compared to the ones were cod was used. There is no observable difference between one and two step extractions in terms of the yields. For extraction 1 the one step extraction had slightly higher yields, while for extraction 2 and 3 the hydroxyproline yields were slightly higher for the two step extractions. These differences might be explained by the presence of impurities in the final collagen product.

The yields obtained in this experiment are a lot lower than the yields reported by Nagai and Suzuki (2000), which were in the range of 40.7 to 53.6% with various fish bones as raw material[66]. Furthermore, Ali et al. (2018) reported an extraction yield of 81.5% with golden carp skin using 0.5M acetic acid to extract collagen[73]. Fish heads have lower collagen content than fish skin and bones, it does not explain why the yields obtained in this project were so much lower than the reported yields. The collagen in the cold-water fish used in this experiment is less stable than the collagen of warm-water fish. As such, it is possible that the processing conditions used for warm-water fish collagen extraction is not suitable when using cold-water fish due to excessive hydrolysis.

4.2 Hydroxyproline and Collagen Purity

Hydroxyproline content was determined colorimetrically on hydrolysed collagen, except parallels C 72h and P 72h, since there was not enough product from these extractions to hydrolyse. Since hydroxyproline is almost exclusively found in collagen, it can be used to estimate the percentage of collagen in a sample. To convert hydroxyproline content to collagen purity a conversion factor of 14.7 was used for fish collagen[71]. Furthermore, hydroxyproline was also analysed in the unused raw material and the used raw material in order to compare the content of this amino acid before and after extraction. The amount of hydroxyproline and collagen purity of the extracted products are presented in Table (3.2), while the hydroxyproline content of the used raw material is presented in Table (3.3) in Section 3.2.

The collagen product with the highest estimated purity was from extraction 1, parallel 1C 2*12h which had 38.5% collagen. Overall the product from extraction 1, with 24 hours extraction time and mild pretreatment, had the highest purity, followed by extraction 2, with the same amount of extraction time and harsher pretreatment conditions. Apart from parallel 2C 24h, the collagen purity in the product from these two extractions was all above 30%. For extraction 3, 48 hours extraction time and harsh pretreatment conditions, the collagen that was extracted in two steps have a significantly higher purity than the ones extracted in one stage. This tendency was not observed for extraction 1, however, there is also a large difference in product purity between 2C 24h and 2C 2*12h. Furthermore, the products from extraction 3 have significantly lower purity than the products from extraction 1 and 2. As stated above, only collagen from the two stage parallels of extraction 4, 72 hours extraction time and harsh pretreatment conditions, had sufficient amount of product to perform hydrolysis and subsequently analyse with HPLC and determine hydroxyproline content. The hydroxyproline content was determined to be zero in the product from extraction 4, and thus there it probably does not contain collagen. As such, it appears that a longer extraction time have a negative effect on the product purity. Furthermore, extraction 3 and the cod head parallels of extraction 2 suggest that two step extraction is preferred in terms of product purity compared to one step extraction.

Hydroxyproline content in unused raw material was determined to be 16.7 and 15.7 μg/g sample for cod heads and pollock heads respectively. All the used raw material contained less hydroxyproline than the unused raw mate-

rial, which is to be suspected since collagen is extracted during the process. For extraction 1, there is not a lot of variation in the change of hydroxyproline content in the raw material during the process. However, the changes are overall not very large, the highest one being 1C 24h with a change of 5 μg/g sample. This could indicate that there is still a significant amount of collagen left in the raw material after it is used for extraction. Parallel 2C 24h had a large change in hydroxyproline, 7.3 μg/g sample, apart from that parallel the changes are similar between extraction 1 and 2. This indicates that a larger amount of collagen was extracted, or removed, from the raw material in the 2C 24h parallel than in the other parallels of extraction 1 and 2. This is curious given that the hydroxyproline yield for this parallel was the lowest out of all parallels in extraction 1 and 2, and it had the lowest product purity as well. This indicates that most of the collagen which was extracted from the raw material in 2C 24h was lost, either during extraction due to collagen degradation or was lost during the purification steps of the process. The raw material used in extraction 3 shows similarities to the ones used in extraction 1 and 2, although extraction 3 has significantly lower yields and product purity in comparison. This would also indicate that similar amounts of collagen was extracted from the raw material, but is lost during the processing. Since extraction 3 have 24 hours longer extraction time than extractions 1 and 2 this loss of product is probably caused by excessive acid hydrolysation of collagen. The hydroxyproline content in the raw material used in extraction 4 differs quite a lot from the other extractions. The change of this amino acid is very low in the cod head parallels, only 1.9 and 0.7 μg/g sample, while it is very large for the pollock head parallels 6.8 and 7.9 μg/g sample. Given that this is the extraction with the longest extraction time it was not suspected to see the least change in hydroxyproline, in other words, the least amount of collagen extraction, for any of its parallels. The hydroxyproline should probably have been determined a second time for this raw material, to confirm if this was indeed the case, especially since the change in hydroxyproline for the pollock head parallels were among the highest observed. The hydroxyproline content in the raw material from P 72h and P 2*36h, indicates that a large portion of the collagen was extracted, however the theoretical hydroxyproline yield for these parallels were 0, meaning the product did not contain collagen. This further indicates that the longer extraction time could lead to loss of product.

4.3 Amino Acid Composition

HPLC analysis was run on hydrolysed collagen samples from all extractions, except C 72h and P 72h, as there was not enough product to perform these

tests. The glycine mole fractions of each collagen product is presented in Table (3.4). The highest glycine mole fraction was observed for parallel 2C 24h, which was 29.1%. This is quite close to the 33% one would expect to see, and indicates a higher collagen purity than was determined by the hydroxyproline content. However, for other products from extraction 1 and 2 the mole fraction of glycine is about 20%. The collagen from extraction 3 and 4 had a significantly lower mole fraction of glycine, which indicates lower collagen purity. The amino acid composition of the extracted collagen are shown in Figure (3.1), Figure (3.2), Figure (3.3) and Figure (3.4) in section 3.3. The distributions of amino acids in the extracted collagen are quite similar for extraction 1 and 2, however there are significant differences in the composition of collagen from extraction 3 and 4. Given the fact that every third amino acid residue in collagen is glycine it is expected to make up 33% of the total amino acid composition. As observed in the collagen from extraction 1, glycine is the most abundant amino acid for each parallel. However, it is closely followed by glutamic acid and is not making up the expected 33% of the composition. Glutamic acid is the most abundant amino acid in the collagen from extraction 3, except for the collagen from P 48h, which appears to be missing several amino acids. This is also the case for the product from the C 2*36h and P 2*36h extractions. The fact that the expected amount of glycine was not observed for any collagen product suggests that there are non-collagen protein impurities in all the collagen extracted in this experiment.

The total amino acid in the hydrolysed collagen samples are shown in Table (3.5) in section 3.3. Overall, the amino acid content in all the samples was quite low, with the highest observed value being 138,1 mg amino acid/ g sample. For pure protein samples it is expected to get close to 1000 mg amino acid/g sample. As such, the low amount of amino acid is an indication of low collagen purity in the product. The amino acid content in the product from extraction 1 and 2 are quite high, apart from 2C 24h which was only 37,5 mg amino acid/g sample. Extraction 1, with 24 hours extraction time and mild pretreatment, has higher amino acid content than extraction 2 which has the same extraction time but with harsher pretreatment of the raw material. For extraction 3 the amino acid content is generally lower, with C 48h having the highest observed amount among the 48 hours extraction time collagen. The hydrolysed product from extraction 4, 72 hours extraction time, is very low and indicates that there is very little protein in the extraction product. Generally, it appears that the amino acid amount decreases with a longer extraction time. If this is the case, it indicates that

the longer extraction time resulted in a loss of product. This could be due to the acetic acid cleaving the protein chains in the collagen into small protein fragments which are lost during purification. The low amount of amino acids in the product from extraction 4 indicates that the product is mostly impurities, possibly NaCl which was not removed during dialysis.

The amino acid composition of unused and used raw material was also analysed and are shown in Figure (3.5), Figure (3.6), Figure (3.7), Figure (3.8) and Figure (3.9) in section 3.3. The most common amino acid for the unused and used raw material was observed to be glycine for all samples. This might indicate that there is still an abundance of collagen left in the used raw material that was not extracted. Glycine did not make up 33% of the amino acids in any of the used raw material samples, indicating the presence of other non-collagen protein impurities after pretreatment and extraction.

4.4 Molecular Weight Distribution

Polyacrylamide gel electrophoresis was run on samples from all the collagen products from extraction 1 and 2. Six collagen samples from extraction 3 were run, those being C2 48h, P2 48h, C1 2*24h, C2 2*24h, P1 2*24h and P2 2*24h, due to lack of product from the two excluded extractions. From the 72 hours extractions there was not sufficient amount of collagen product from any parallels to run gel electrophoresis with. The results from the electrophoresis are shown in Figure (3.10), Figure (3.11) and Figure (3.12) in section 3.4. The protein bands formed by the product sample from extraction 3 were quite faint. This could be due to issues regarding the staining of the protein or a result of lower collagen purity in the product from this extraction (see Table (3.2) and Table (3.6) thus the samples contained less protein compared to the product samples from extraction 1 and 2.

All samples showed protein bands at around 200 kDa, although they were quite faint for the collagen from extraction 3. These bands strongly indicate the presence of β-chains which typically have a molecular weight ranging from 200-250 kDa[74]. Furthermore, there are also visible bands for all the collagen samples between the standard bands of 116 and 170 kDa. These bands indicate that there are also α-chains present in the collagen product, whose molecular weight commonly ranges between 120-150 kDa[74]. In Figure (3.10) and Figure (3.11) several distinct protein bands are visible at around 140-150 kDa. These bands strongly suggest that it is type 1 collagen which has been extracted, since its tropocollagen molecule consists of

two α_1-chains and one α_2-chain with different molecular weight[10]. These two figures also show some protein bands of quite high molecular weight, far above the 220 kDa standard band. These bands could potentially be from γ-chains, that is collagen trimers. The protein bands observed in this experiment are similar to what has previously been reported for fish collagen.[75][32]

On all the gels that were run in this experiment, there are numerous protein bands of low molecular weight, below the 76 kDa standard band. This indicates that α-chains are cleaved during the collagen extraction and thus degraded into sub α-chains. If it is the case that α-chains are degraded during the extraction, this would probably lead to some loss of product, as the collagen is cleaved into small protein fragments. As such, the results from SDS-page gel electrophoresis might also suggest that the extraction method is too harsh when using cod and pollock heads as raw material.

4.5 C/N/S elemental analysis

Only a small amount of sample material is necessary to do C/N/S elemental analysis, and as such it was possible to do with the product from every extraction parallel. The results from the analysis is shown in Table (3.6) in section 3.5 and show the amount of nitrogen, carbon and sulphur in each sample, as well as the protein content. The products from extraction 1 and 2 have a significantly higher protein content than the products from extraction 3 and 4. Parallel 2C 24h is indicated to contain a lot less protein than the other products from extraction 2. Other than that, there is not a lot of variation in the products protein content between parallels in extraction 1 and 2. The collagen products from extraction 3 all contain less than 50% protein, with the highest one, C 48h, determined to have 48.2% protein which is significantly higher than the products from the other 48 hours extraction time parallels. The products with the lowest amount of protein are C 2*36h and P 2*36h which were 2.8 and 2.7% respectively. This indicates that the low level of collagen purity that was determined by hydroxyproline content is indeed correct, and that there is next to no collagen in the product. In comparison to these two, the product from the other 72 hours extraction time parallels, C 72h and P 72h, had much higher protein content. However, the yield was also very low for these two extractions.

The C/N/S elemental analysis indicates that a longer extraction time had a negative effect on the protein content in the product. The two extractions with 24 hours extraction time had a higher protein content than the ones

extracted for 48 and 72 hours, and the lowest were observed for the two step 72 hours extractions. The protein content determined by this method was overall higher than the collagen content determined by hydroxyproline content for all samples. This could indicate that there are a lot of non-collagen proteins in the final product, which means that the NaOH pretreatment to remove these impurities was not very effective. Another explanation for this observation is that a different conversion factor should be used for every type of raw material, since the amount of hydroxyproline in the collagen is different throughout the body. The conversion factor used in this experiment of 14.7 was determined for skin of Baltic cod[71]. As such, it is can only be used to give a rough estimate of the actual collagen content, when the collagen was extracted from heads of Atlantic cod and Atlantic pollock.

The protein content determined by C/N/S has a similar distribution as the total amino acid content for the extraction variations. Overall, extraction 1 had the highest protein and amino acid content, closely followed by extraction 2. There was a decrease in both protein and amino acid content when the extraction time was doubled from 24 to 48 hours. Amino acid content was not determined for the one-stage 72 hours extractions, so no comparison can be made. However, the two stage 72 hours extractions were the lowest in terms of bot protein and amino acid content. Although the distributions are similar, there is a discrepancy between the protein content found by C/N/S and the amino acid content determined by HPLC. According to the amino acid content the protein purity in the product from extraction 1 ranges from 11.4% to 13.4%. This is a lot lower than both the collagen purity determined by hydroxyproline content, which ranges from 32.6 to 38.5% and the protein purity determined by C/N/S which ranges from 49.2 to 57.9%. The same trend was observed for extraction 2 and 3 as well. It is not certain why the differences are so large between the two estimates for protein content. However, the protein content determined by C/N/S is compatible with the collagen purity estimated by the hydroxyproline content, which is not the case for the total amino acid estimates.

5 Conclusion

All collagen extractions that were performed in this experiment showed quite poor results in regards to low yields (0.1-2.3%) and product purity (0.0-38.5%), from Table (3.1) and Table (3.2). The results indicate that the methods used in this experiment would not be suitable in a commercial context, since the low yield makes economic gains infeasible and the purity of the product does not meet market standards.

The low yields achieved were probably caused by too harsh processing of the raw material, since the highest yield was observed for extraction 1, with the lowest extraction time and mildest pretreatment conditions. Since collagen is hydrolyzed in acid and alkali it is likely that the pretreatments with NaOH and EDTA, as well as the extraction with acetic acid, caused excessive degradation of the collagen which led to a loss of product. This can be seen especially in extraction 4, with long pretreatment and a 72 hours extraction time. No hydroxyproline was detected in any product from this extraction, and as such there was probably no collagen in the product. It is thus likely that the collagen extracted to the acetic acid in this parallel was overexposed to the acid, and was hydrolysed into too small protein fragments.

The product purity achieved in this experiment was not particularly high. From the hydroxyproline estimate of collagen purity the highest observed was 38.5%. The total amino acid content from HPLC and the C/N/S elemental analysis also give indications of product purity. It would be expected that a pure collagen sample would have 1000 mg amino acid/g sample, however the highest amino acid content observed was only 138.1 mg amino acid/g sample. The largest protein content observed in the C/N/S analysis was 57.9%. There is some difference between the collagen and protein contents that were observed, which would indicate the presence of protein impurities or shortages in the method used to determine collagen purity.

The results indicate that it is favorable with lower extraction time and milder pretreatment in terms of product yield and purity. The highest product purity and yields were observed in extraction 1, with mild pretreatment conditions and 24 hours extraction time. It was possible to extract collagen from the raw material using the method, which was confirmed by the gel electrophoresis and hydroxyproline content of the product.

6 Future work

Although the extraction method investigated in this experiment was able to extract collagen from heads of cod and pollock, with the exception of the 72 hours extraction time parallels, the obtained yields and product purity were quite low. The results suggest that extraction with milder processing conditions are favorable. As such, to further improve the method adjustments like lowering the concentration of solutions used for pretreatment and extraction, lowering the raw material to solution ratios, lowering processing time and outright removing steps of the process, as was done for extraction 1 which achieved the highest product purity even if the fat removal step of the pretreatment was skipped, could lead to higher yields and product purity. It appeared that there was a loss of product in the extractions with a longer processing time. By calculating the mass balance of collagen for the extraction, one could gain a better understanding of this.

Furthermore, there are other extraction methods which could be tested using cod and pollock heads as raw material with the potential for better results than the one used in this project. As mentioned in the introduction, enzyme aided acid extraction has been tested with fish raw material before, and generally results in higher solubility of collagen[40]. As such, adding pepsin to the extraction liquid could result in higher collagen yields when extracting collagen from cod and pollock heads. Addition of enzymes to the extraction liquid is a very simple modification of the process, and would not require significant additional resources in terms of storage, space and energy. Other methods that have been tested on marine raw material include supercritical fluid extraction, deep eutectic solvent extraction and ultrasound assisted extraction of collagen[10].

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A Equations

A.1 Hydroxyproline

To calculate the hydroxyproline content in hydrolysed collagen and raw material samples standard, a standard curve was prepared the same day, and an expression for the curve was found using regression. The standard curve was made with hydroxyproline solutions with concentrations of 5, 10 and 15 μg/ml, by thinning out a 100 μg/ml stock solution. The OD of the standard solution was measured at wavelength of 555 nm, and hydrolysed samples with an OD outside the interval of the standard curve were diluted and measured again. The mass of collagen in the product, M_{hyp}, was calculated using the measured OD_{555}, total sample volume (which was 10 ml), V and the expression for the standard curve which was linear on the form $ax + b$.

$$M_{hyp} = \frac{V(OD_{555} - b)}{a} \tag{A.1}$$

With the mass of hydroxyproline in the collagen product, the weight percentage of hydroxyproline in the product, $\mathrm{HYP}_{\%}$, could be calculated using the total mass of the product, M_{tot}.

$$HYP_{\%} = \frac{M_{hyp}}{M_{tot}} \tag{A.2}$$

By multiplying the mass and weight percentage of hydroxyproline in the product with the hydroxyproline conversion factor of 14.7 the mass of collagen and weight percentage of collagen in the product, $\mathrm{COL}_{\%}$ and M_{col}, could be estimated.

$$COL_{\%} = HYP_{\%} \cdot 14.7 \tag{A.3}$$

$$M_{col} = M_{hyp} \cdot 14.7 \tag{A.4}$$

A.2 Yields

To calculate the extraction yield on a dry basis it was necessary to determine the water content of the raw material. This was done by weighing the raw material before and after its water was removed by freeze-drying. The water content of the raw material was thus determined to be 72.9% and 72.5% for

cod and pollock respectively. As such, the dry weight of the raw material, M_{raw}, could be estimated and used to calculate the dry basis yield for the extraction.

$$\text{Dry Yield} = \frac{M_{col}}{M_{raw}} \cdot 100\% \tag{A.5}$$

The hydroxyproline yield is how much of the hydroxyproline, ie. collagen, present in the raw material was extracted during the process. To calculate this yield, the hydroxyoroline weight percentage of unused raw material and collagen product, $HYP_{raw\%}$ and $HYP_{col\%}$ and the masses of the raw material and product, M_{raw} and M_{col} were used.

$$\text{Hydroxyproline Yield} = \frac{M_{col} \cdot HYP_{col\%}}{M_{raw} \cdot HYP_{raw\%}} \tag{A.6}$$

A.3 Collagen mass balance

As suggested, it is possible to make a simple collagen mass balance for the system. To do so, one can imagine a system with collagen in the fresh raw material as its one feed. There would be three outlets consisting of collagen in the product, collagen still left in the used raw material and collagen lost due to bleeding. A simple schematic of the system and its flows is presented in Figure (A.1). It is thus possible to calculate the loss of product by calculating $M4_{collagen}$ which could give more insight into whether there was excessive hydrolysis of collagen or not. One can also calculate $M3_{collagen}$, the mass of collagen still left in the raw material after extraction to get an idea of the extraction efficiency. No collagen is generated during the process, and one can assume that collagen that is hydrolyzed into small protein fractions are found in stream 4, the bleed. As such, the mass of collagen into the system in Flow 1 should be equal to the mass going out of the system in Flow 2, Flow 3 and Flow 4. To calculate the loss of collagen, one would have to measure M1, M2 and M3, the weight of fresh raw material, the product and used raw material respectively. Furthermore, by analysing the hydroxyproline content of the fresh raw material, product and used raw material one can calculate $w1_{collagen}$, $w2_{collagen}$ and $w3_{collagen}$, the mass percentage of collagen in these flows. The mass of collagen in flow i, $Mi_{collagen}$, can then be calculated by the following equation

$$Mi_{collagen} = Mi \cdot wi_{collagen} \tag{A.7}$$

where Mi is the mass of flow i and $wi_{collagen}$ is the collagen mass percentage of flow. The loss of collagen in the bleed, $M4_{collagen}$, can then be calculated by the equation below.

$$M4_{collagen} = M1 \cdot w1_{collagen} - M2 \cdot w2_{collagen} - M3 \cdot w3_{collagen} \tag{A.8}$$

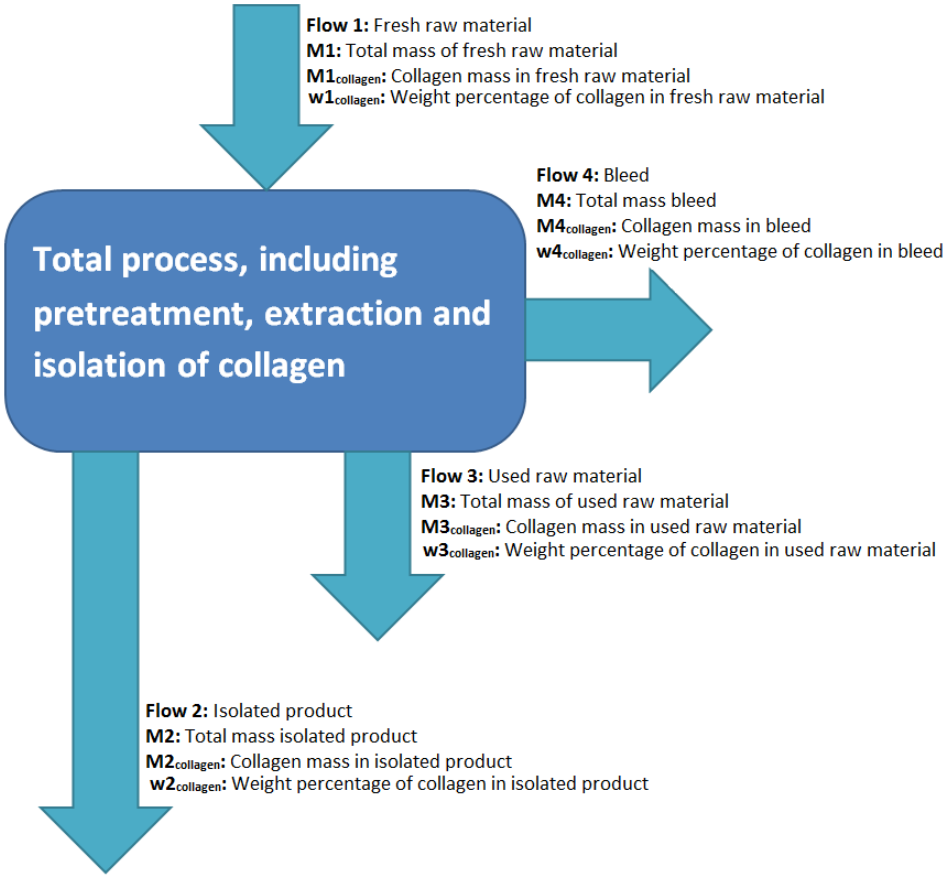
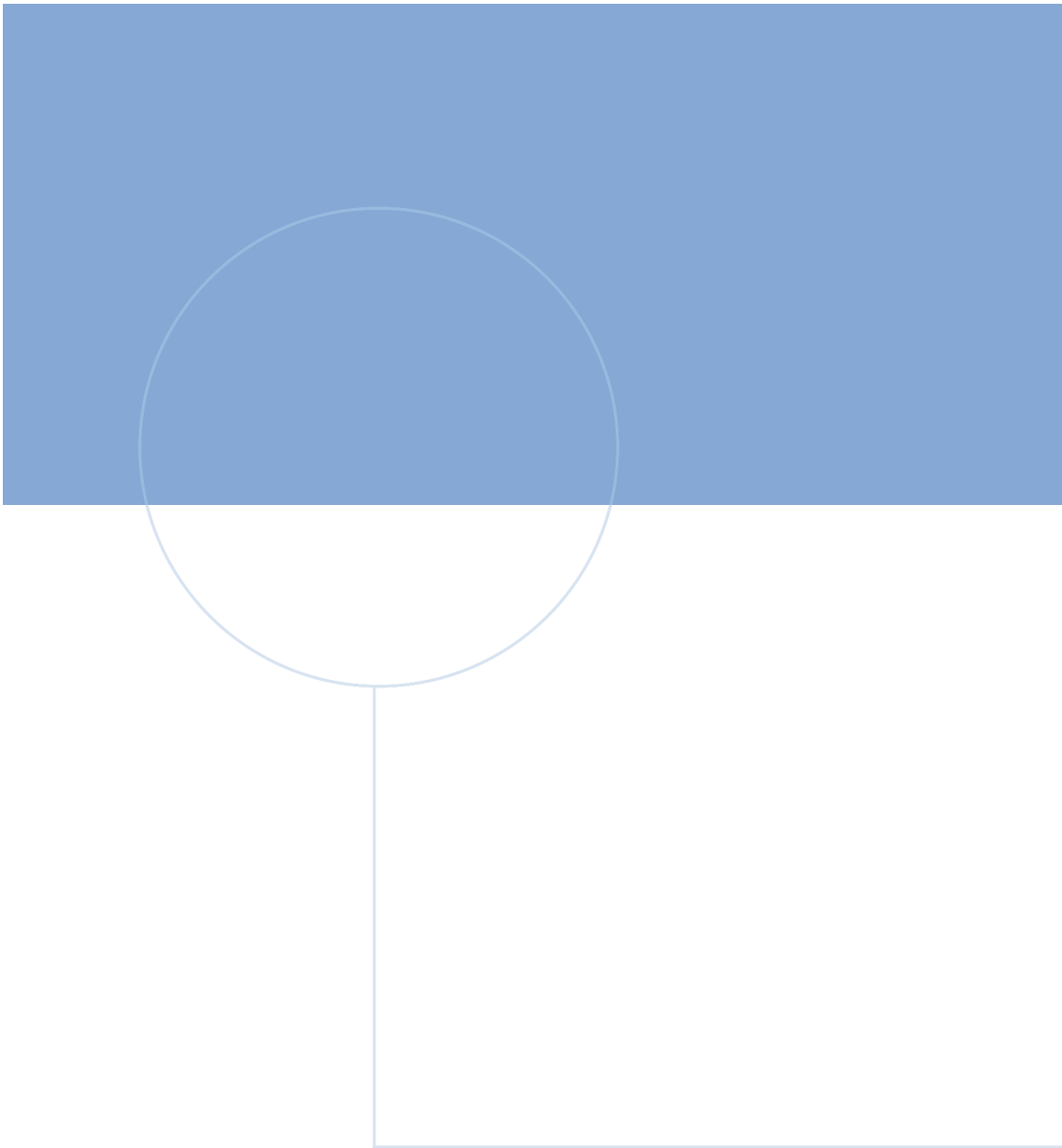


Figure A.1: A simple schematic of the system with its four flows. The feed flow is the fresh raw material, while the three outlets are the collagen product, used raw material after extraction and the bleed.



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