Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Evelyn H. Fossen

Chemical and structural composition of gelatine extracted from defatted Atlantic salmon *(Salmo salar)* skin

Master's thesis in Biotechnology
Supervisor: Turid Rustad, IBT
August 2020

NTNU
Norwegian University of
Science and Technology

Evelyn H. Fossen

Chemical and structural composition of gelatine extracted from defatted Atlantic salmon *(Salmo salar)* skin

Master's thesis in Biotechnology
Supervisor: Turid Rustad, IBT
August 2020

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

NTNU
Norwegian University of
Science and Technology

PREFACE

This master's thesis is the final work of a two-year biotechnology program (MSBIOTECH) in the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology (NTNU).

First and foremost, I would like to thank my main supervisor, Prof. Turid Rustad, for exceptional guidance throughout the planning and completion of this thesis. Secondly, I would like to give thanks to Siri Stavrum whose positive demeanour and willingness to help made every day at the lab a pure joy, despite all the frustrating complications that arose. I would also like to thank my co-supervisor, Kurt Ingar Draget, for good inputs in the planning stage and excellent taste in t-shirts, as well as for connecting me with Olav Andreas Aarstad - who deserves his own thanks for performing the SEC-MALLS analysis. Additionally, I would like to thank Trond Viggo Pettersen for aiding me in the water activity analysis on one of his last days working at NTNU - may he find peace and prosperity at E.C. Dahls Brewery. I would also like to thank all the other master's students at the lab for lending an ear or hand whenever needed. And lastly, an honourable mention to my significant other who have endured throughout this somewhat bumpy ride (..because science!)

ABSTRACT

Atlantic salmon (*Salmo salar*) is one of the main fish species harvested from aquaculture. During processing, a large amount of rest raw materials (RRMs) containing valuable components are disposed of or used for low value products. Many of these RRMs have the potential to be used for gelatine production, which could be a way to valorise the RRMs, especially in terms of RRM fractions that contain considerable amounts of collagen, such as skin and bones.

The first objectives of this thesis were to perform a simple screening experiment and use the obtained data to develop a gelatine extraction procedure that would be appropriate for extraction of gelatine from Atlantic salmon skins. The emphasis was on finding the most optimal method for defatting the salmon skins, as it is known that high lipid contents can be detrimental to the properties of the gelatine. In short, the developed gelatine extraction procedure was as follows; pre-treatment for 24 hours at 4 °C using 0.1 M NaOH, defatting for 3×2 hours at 4 °C using 10 % (v/v) 1-butanol, and extraction at 22 °C using 0.1 M acetic acid as the extraction medium. The objective of the main experiment was to investigate how different extraction times (6, 12 and 18 hours) affected the chemical and structural composition of the gelatines.

All three extraction times yielded approximately 20 g freeze-dried extract/100 g raw material (wet weight). However, the 12-hour extractions (99.7 ± 0.6 % collagen) resulted in extracts that had a significantly higher ($p \leq 0.006$) purity, compared to the 6-hour (94.6 ± 3.4 % collagen) and 18-hour extractions (82.3 ± 1.4 % collagen). The extraction efficiency, calculated as the percent ratio of gelatine yield compared to the amount of dry matter in the raw material, was higher than the theoretical maximum of 39 % for all three extraction times. The proximate composition of the freeze-dried gelatines varied and had seemingly no relationship with the extraction times. All the freeze-dried gelatine extracts had low residual moisture contents (0.7 ± 0.1 – 2.2 ± 0.5 %), and low water activity ($a_w <$ 0.5), suggesting high stability and good shelf-life.

The molecular weights of the salmon skin gelatines all showed similar, albeit narrow distributions. However, the weight average molecular weight (M_w) was significantly lower ($p \leq 0.001$) for the 18-hour extractions (M_w 152.0 ± 0.9 kg/mol) than for the 6- and 12-hour extractions (M_w 161.2 ± 2.8 kg/mol and M_w 159.4 ± 1.3 kg/mol, respectively). Seen in conjunction with the low purity in the 18-hour extractions, the lower M_w was suggested to be due to differential extraction of non-collagenous proteins with low molecular weights. Alternatively, it could be a result of increased hydrolysis of the gelatine chains during longer extractions. The M_w was found to be lower than in gelatine extracted from saithe skin under similar conditions, which suggest that the collagen in salmon skin is less thermostable than collagen in saithe skin. If true, that also indicates that gelatine could be

extracted from salmon skins at temperatures lower than 22 °C, which might lead to gelatine with higher M_w and better functional properties.

Overall, this work demonstrated that gelatines with high purity can be extracted from salmon skin, and with adequate yield compared to skins from other fish species. However, further investigations are needed to determine the functional properties of the salmon skin gelatines.

SAMMENDRAG

Atlanterhavslaks (*Salmo salar*) er en av de viktigste fiskeartene i havbruk. Store mengder restråstoff oppstår under prosessering, og disse kan inneholde verdifulle komponenter som enten forblir uutnyttet eller blir utnyttet i produkter av lav verdi. Mye av dette restråstoffet har potensiale til å bli benyttet til gelatinproduksjon, dette gjelder spesielt restråstoff-fraksjoner som inneholder betydelige mengder kollagen, som skinn og bein. Å utnytte disse fraksjonene til gelatinproduksjon kan være en god løsning for å gi dette restråstoffet økt verdi.

De første målene i denne masteroppgaven var å gjennomføre et enkelt screening-eksperiment, og benytte data fra denne til å utarbeide en prosedyre for ekstraksjon av gelatin fra skinn av atlanterhavslaks. Hovedfokuset med dette var å finne best mulige metode for å avfette lakseskinnet, da det er kjent at høyt fettinnhold kan være ugunstig for egenskapene til gelatinet. Følgende metode ble utarbeidet: forbehandling med 0,1 M NaOH i 24 timer ved 4 °C, avfetting med 10 % (v/v) 1-butanol i 3 × 2 timer ved 4 °C og ekstraksjon ved 22 °C med 0,1 M eddiksyre som ekstraksjonsmedium. Målet med hoved-eksperimentet var å undersøke hvordan ulike ekstraksjonstider (6, 12 og 18 timer) påvirket den kjemiske og strukturelle sammensetningen i gelatinet.

Gelatinekstraksjon fra avfettede lakseskinn med metoden utviklet basert på screening-eksperimentet viste lovende resultater med hensyn til gelatinutbytte og ekstraksjonseffektivitet. Alle ekstraksjonstider ga et omtrentlig utbytte tilsvarende 20 gram frysetørket gelatin per 100 gram lakseskinn (våtvekt). 12-timersekstraksjon (99,7 ± 0,6 % kollagen) resulterte dog i ekstrakter som hadde signifikant høyere ($p \leq 0.006$) renhet, sammenlignet med 6-timersekstraksjon (94,6 ± 3,4 % kollagen) og 18-timersekstraksjon (82,3 ± 1,4 % kollagen). Ekstraksjonseffektiviteten, beregnet som forholdet (i %) mellom gelatinutbytte og tilgjengelig tørrstoff i råmaterialet, var høyere enn det teoretiske maksimum på 39 % for alle tre ekstraksjonstidene. Gelatinprøvene hadde variert sammensetning og viste ingen tegn til å være korrelert med ekstraksjonstiden. Alle de frysetørkede gelatinekstraktene hadde lavt fuktighetsinnhold (0,7 ± 0,1 – 2,2 ± 0,5 %), og lav vannaktivitet ($a_w < 0,5$), noe som tilsier at de er stabile og bør ha god holdbarhet.

Molekylærvektene i lakseskinngelatin viste lignende, om enn smale distribusjoner. Imidlertid førte 18-timersekstraksjon (M_w 152,0 ± 0,9 kg/mol) til en signifikant ($p \leq 0.001$) lavere vektgjennomsnittlig molekylærvekt (M_w) sammenlignet med 6- og 12-timersekstraksjon (henholdsvis M_w 161,2 ± 2,8 kg/mol og M_w 159,4 ± 1,3 kg/mol). Sett i sammenheng med den lave renheten i 18-timersekstraktene, kunne dette tyde på at lavere M_w skyldtes differensiell ekstraksjon av ikke-kollagenøse proteiner med lavere molekylærvekt. Alternativt, kan det ha blitt forårsaket av økt hydrolyse av gelatinkjedene ved lengre ekstraksjonstid. M_w i lakseskinngelatin var lavere enn for gelatin ekstrahert fra skinn av sei (*Pollachius virens*) under lignende forhold, noe som kan indikere at kollagenet

i lakseskinn er mindre varmestabilt enn kollagenet i skinn fra sei. Dette kan igjen tyde på at gelatin kan ekstraheres fra lakseskinn ved lavere temperaturer enn 22 °C, noe som kan resultere i gelatin med høyere M_w og bedre funksjonelle egenskaper.

Dette arbeidet viste at gelatin med høy renhet kan bli ekstrahert fra lakseskinn, og med adekvat utbytte sammenlignet med skinn fra andre fiskearter. Dog kreves videre undersøkelser for å bestemme de faktiske funksjonelle egenskapene til gelatinet.

TABLE OF CONTENTS

APPENDIX

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1. HISTORICAL USES OF GELATINE

Gelatine has been used by humans for thousands of years. There is evidence of its use as a biological adhesive all the way back to when humans still lived in caves. The scholar Hildegard von Bingen (1098-1179) recommended in her catalogue of scientific and medicinal properties of plants and animals that "frequent and adequate" portions of broth made from calves' hooves could ease joint pains, a recommendation that has been proven true by modern science. Henry VIII frequently had pickled dishes with "glittering calves"-foot jelly on the menu of his banquets, and during the British blockade of French ports during the Napoleonic era, French scientists and politicians were forced to find alternative sources of proteins for the population due to a shortage of meat proteins, which, in turn, resulted in the industrial production of gelatine. Around the same time, the French pharmacist Mothes was granted a patent for the manufacture of gelatine capsules, which enabled medicines to be dosed more easily and be better protected against the environment. It also helped with sealing in the bitter taste of the medicine, thus making it easier to ingest. Gelatine also had a big part in making photography available to the masses. George Eastman introduced in 1888 his easy-to-use Kodak number 1 camera, which utilised films instead of plates. The films consisted of photographic paper coated in high quality gelatine and this made it possible for amateur photographers to produce photos at a reasonable cost. Gelatine became ubiquitous in the mid-to-late 19th century when granulated gelatine became readily available, and furthermore when the fruit-flavoured gelatine dessert, JELL-O, was introduced to the market. Today, gelatine is an essential element of our daily lives, with a wide variety of applications ranging from normal culinary uses to cosmetics, and ballistic gelatine. (Schrieber & Gareis, 2007, pp. 1–11)

1.2. SOURCES OF GELATINE

Normally, gelatine is manufactured from by-products of the meat and leather industries, with bovine and porcine sources being the preferred raw materials. This is due to a number of factors, such as tradition, a steady supply of raw materials and well-established quality control. (Hattrem & Draget, 2014, p. 19) With gelatine having such a wide range of useful applications, the global demand for gelatine has been increasing over the years, giving rise to a search for alternative sources other than bovine and porcine. This demand for gelatine derived from alternative sources is compounded by the concerns from religious groups and people with alternative dietary lifestyles. Both Jews and Muslims are forbidden to consume porcine-related products, and Hindus do not consume bovine-related products. In addition, there has been a general concern for the overall safety of bovine-derived gelatine due to bovine spongiform encephalitis (BSE, also referred to as mad cow disease). However, the

gelatine production process has been shown to be an effective barrier against possible BSE prions and the risk associated with bovine bone gelatine is nearly non-existent. (Benjakul, Kittiphattanabawon, & Regenstein, 2012, p. 388; Karim & Bhat, 2009) Still, only a small amount (2-3 % of the global annual production) of gelatine is produced from fish and poultry sources, partly due to a lack of stability in the supply of good quality raw material and, more importantly, due to the sub-optimal properties of gelatine derived from some of these sources. Gelatine from warm-water fish species is almost interchangeable with gelatine derived from mammalian sources, but gelatine from cold-water fish species show significant differences in both gelling and melting temperatures, as well as resulting in gels with poorer mechanical properties. (Hattrem & Draget, 2014, p. 19)

2. COLLAGEN AND GELATINE

2.1. COLLAGEN COMPOSITION AND STRUCTURE

Considering that all gelatine is derived from collagen, it is pertinent to describe collagen before discussing gelatine. Collagen is a term given to a family of distinct proteins that are the major constituents of all animal connective tissue, including skin, bone, tendon, and cartilage. Approximately 10 % of mammalian muscle protein consist of collagen, but the collagen fraction in fish is generally much lower. (Foegeding, Lanier, & Hultin, 1996, p. 902) Each protein in the collagen family has specific structures, functions and tissue distribution in the extracellular matrix. (Ramshaw, Peng, Glattauer, & Werkmeister, 2009) To date, some 27 different types of collagen have been identified (Schrieber & Gareis, 2007, p. 45). Table 1 shows a simple classification of the different collagen types. Collagen can further be divided into two classes; fibrillar collagens and non-fibrillar collagens. Type 1 collagen is by far the most common, being the principal component of the skin and bones, and, together with type II and III collagen, it makes up most of the fibrillar collagens. The non-fibrillar collagens (shown as "other" in Table 1) are present in only small amounts and therefore has minimal impact on this paper. (Hulmes, 2008, pp. 16–19)

Table 1: Classification of collagen types and their distribution (Table modified from Benjakul, Nalinanon, & Shahidi, 2012, p. 366; Karim & Bhat, 2009, p. 564; Schrieber & Gareis, 2007, p. 45).

Collagen type	Peptide chains	Description
I	α1, α2	Occurs widely, primarily in connective tissue, such as skin, bone, tendons, and muscle (epimysium).
II	α1	Occurs almost exclusively in cartilage tissue.
III	α1	Occurs in foetal skin, cardiovascular vessels, uterus, inner organs, and muscle (perimysium). Strongly dependent on age; very young skin can contain up to 50 % but is reduced to 5-10 % with age.
Other		Occurs in placental membranes, lens capsules, cardiovascular system, and muscle (endomysium). These other types of collagen are present in very low amounts and are mostly organ specific.

What all types of collagen have in common, is a triple-helical motif as part of its tertiary structure (Ramshaw et al., 2009). A single collagen molecule (tropocollagen) is composed of three α-chains wound counter-clockwise around each other, resulting in a rope-like structure as shown in Figure 1-C. The α-chains may form homo- or heterotrimers, resulting in different types of collagen. (Benjakul, Nalinanon, et al., 2012, p. 366; Eysturskarð, 2010; Hulmes, 2008, p. 15; Ramshaw et al., 2009) The tropocollagen molecule is mainly held together by multiple intermolecular hydrogen bonds between neighbouring aldehyde (-CO) and amino (-NH) groups (Benjakul, Nalinanon, et al., 2012, p. 366; Eysturskarð, 2010; Karim & Bhat, 2009). Tropocollagen subunits of the fibril-forming collagens can further assemble into fibre bundle networks that are stabilised by specific covalent cross-links, mainly between lysine and hydroxylysine residues found on the non-helical tails (telopeptides) of neighbouring tropocollagen molecules, as shown in Figure 1-D (Gómez-Guillén et al., 2002; Ramshaw et al., 2009). The degree of crosslinking is highly variable, depending both on collagen type, tissue type, species, age etc. (Gómez-Guillén et al., 2002). The non-fibril forming collagens, on the other hand, often form different structures, such as networks (Ramshaw et al., 2009).

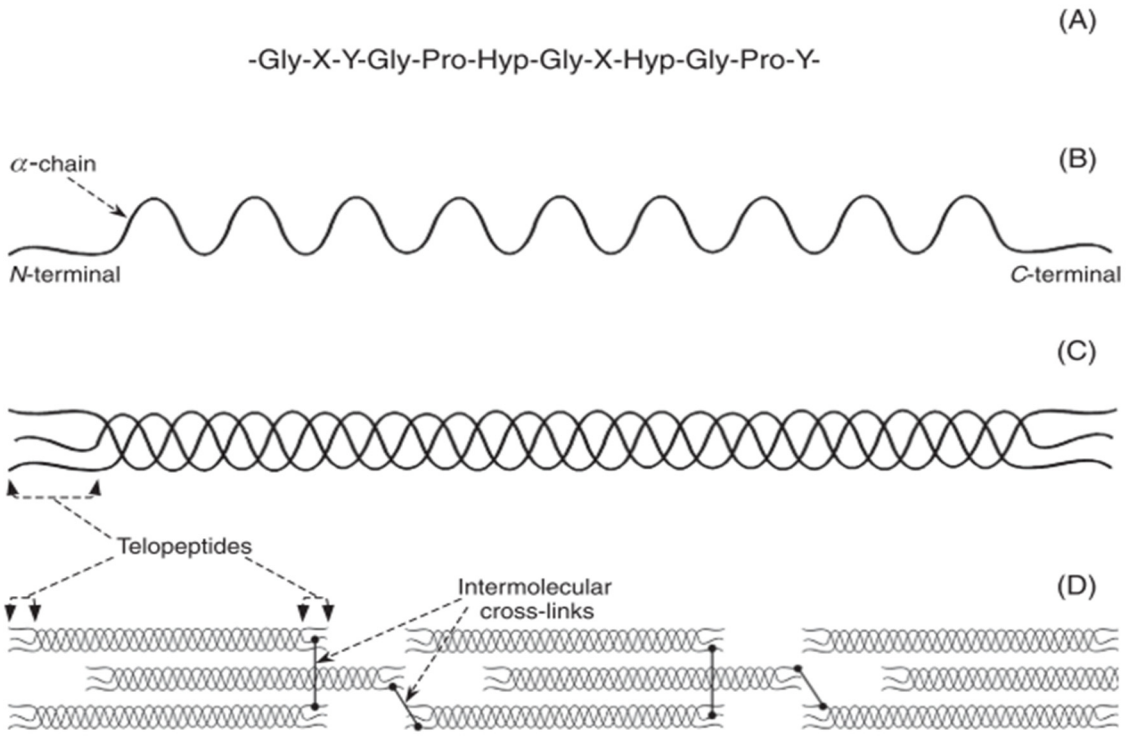


Figure 1: The arrangement of collagen fibril in collagen fibers. (A) shows the amino acid composition (primary structure) of a collagen polypeptide, (B) shows an α-chain (secondary structure) collagen polypeptide. (C) shows a tropocollagen molecule (tertiary structure). (D) shows a collagen fibril (quaternary structure). Figure taken from Benjakul, Nalinanon, et al., 2012, p. 366.

Each α-chain contains around 1050 amino acids with 337-343 regions (depending on collagen type) characterised by the repeating amino acid motif Gly-X-Y, and it is this motif that allows the α-chains to form a triple helix with three residues per turn (Eysturskarð, 2010; Hulmes, 2008, p. 17; Ramshaw et al., 2009). Glycine is found in every third position as it is the only amino acid small enough to pack into the centre of the structure. The X- and Y-positions are exposed on the surface of the triple helix and can be filled by any amino acid, but is frequently occupied by the imino acid[1] proline, which, in the Y-position, is usually altered by post-translational modifications to 4-hydroxyproline. (Hulmes, 2008, p. 24; Ramshaw et al., 2009) The presence of proline and hydroxyproline stabilises the helical structure by steric restrictions, because the imino rings in proline and hydroxyproline impose rigid constraints on rotational movement about the N-C_α bond in the collagen backbone (Akita et al., 2020; Foegeding et al., 1996, p. 903). The post-translational modification of proline results in an enhanced stability of the triple-helix, most likely due to the two following effects: (i) increased hydrogen-bonding and (ii) the electron-withdrawing effect of the hydroxyl group, thus, enabling mammalian collagens to be stable at physiological temperatures. (Hulmes, 2008, p. 24; Ramshaw et al., 2009) If hydroxylation of proline is prevented in some way, the denaturation (or helix-to-coil transition) temperature of collagen can drop with approximately 30 °C, which shows that hydroxyproline is an essential element in the thermostability of the molecule (Hulmes, 2008, p. 24), and since the amino acid composition of gelatine naturally is very close to that of its parent collagen, the amount of hydroxyproline will also affect the structural properties of gelatine (Karim & Bhat, 2009).

[1] Imino acid is an obsolete term, according to IUPAC. However, it is still widely used and so will be used in this thesis for both convenience and eligibility

*Table 2: Approximate amino acid composition of collagen and gelatine from various fish and mammalian sources. Values are listed as residues/1000 amino acids; * indicates values that are converted from mole percent. Sources: A* - Arnesen & Gildberg (2007) p. 56; B - Duan, Zhang, Du, Yao, & Konno (2009) p. 704*; C* - Jongjareonrak et al., (2010) p. 164; *D* - Giraud-Guille, Besseau, Chopin, Durand, & Herbage (2000) p. 901*; E* - Ikoma, Kobayashi, Tanaka, Walsh, & Mann (2003) p. 201

Amino acid	**Salmon skin [A]**	**Cod skin [A]**	**Cod skin [B]**	**Giant catfish [C]**	**Calf skin [D]**	**Porcine skin [E]**
	Gelatine*	**Gelatine***	**Collagen**	**Gelatine**	**Collagen**	**Collagen**
Ala	104	103	107	106	119	115
Arg	53	53	54	63	50	48
Asx	54	52	53	15	45	44
Cys	-	-	-	-	-	-
Glx	74	71	80	62	72	72
Gly	366	358	342	359	341	341
His	13	12	8	4	7	7
Hyl	-	-	7	5	7	7
Hyp	60	56	51	87	94	97
Ile	9	11	12	13	11	10
Leu	19	20	22	23	23	22
Lys	24	27	29	32	26	27
Met	18	17	15	10	06	06
Phe	13	12	12	13	03	12
Pro	106	98	103	124	121	123
Ser	46	63	59	36	33	33
Thr	23	23	23	24	18	16
Trp	-	-	-	-	-	-
Tyr	3	5	4	3	3	1
Val	15	17	19	22	21	22

Table 2 shows how the amount of the imino acids proline and hydroxyproline differs between cold-water fish (salmon and cod) versus mammals and warm-water fish (giant catfish). The varying amount of imino acids in collagen from various species is connected to the temperature of the animal's habitat; mammals and warm-water fish need more hydroxyproline to maintain stability in their collagen helices, while cold-water fish do not. This difference results in a significantly lower melting and gelling temperature of gelatine derived from cold-water species as well as a lower denaturation temperature of its parent collagen, when compared to those derived from warm-water species and mammals. (Gilsenan & Ross-Murphy, 2000; Gómez-Guillén et al., 2002; Kołodziejska, Kaczorowski, Piotrowska, & Sadowska, 2004) Table 3 gives a summary of the findings from a recent

study by Akita et al. (2020). In this study, they found that habitat temperature, collagen denaturation temperature and imino acid content were positively correlated with the triple-helix content and, consequently, the thermostability of the collagens. Conversely, they found that serine content was negatively correlated with both habitat temperature, collagen denaturation temperature and triple-helix content.

*Table 3: Summary of habitat temperature (Tp) of select warm- and cold-water fishes, and shrinkage temperature (Ts), denaturation temperature (Td), focused amino acid content and Pro + Hyp content of acid-soluble skin collagen. Warm-water fish are indicated by pink cell shading, cold-water fish are indicated by blue cell shading. The amino acid content is given as residues per 1000 amino acid residues. *indicate a significant difference ($p < 0.05$) between warm- and cold-water fish. ND = not determined. Table taken from* Akita et al. (2020).

Common name	T_p (°C)	Pro (res/1000)	Hyp (res/1000)	Ser (res/1000)	Pro+Hyp (res/1000)	T_s (°C)	T_d (°C)
Double-lined fusilier	28.0	103	76	33	179	56.2	34.9
Common dolphinfish	27.4	110	69	39	179	ND	29.5
Fivespot flounder	26.0	99	72	46	171	ND	26.8
Ridged-eye flounder	20.7	101	65	58	166	49.0	23.5
Blue mackerel	18.7	105	71	44	176	53.2	27.5
Roughear scad	16.6	112	69	41	181	56.2	30.4
Average	**22.9 ± 4.9***	**105 ± 5***	**70 ± 4***	**43 ± 8***	**175 ± 6***	**53.6 ± 3.4***	**28.8 ± 3.8***
Okhotsk atka mackerel	12.8	93	58	71	151	41.3	18.1
Deepwater arrowtooth eel	3.4	93	47	61	140	41.7	19.9
Giant grenadier	2.6	88	49	73	137	34.5	19.2
Pacific grenadier	2.4	84	49	73	133	42.2	18.0
Scaly wolf eelpout	0.4	87	44	77	131	43.4	20.2
Average	**4.3 ± 4.9***	**89 ± 4***	**49 ± 5***	**71 ± 6***	**138 ± 8***	**40.6 ± 3.5***	**19.1 ± 1.0***

To explain these correlations, Akita et al. (2020) suggest that serine residues might substitute for hydroxyproline residues in cold water fish collagen because serine provides a greater degree of freedom in the C_α-N and C_α-C bonds of the peptide backbone than the

cyclic hydroxyproline residues. Further, they theorise that this substitution might be an evolutionary strategy to adapt to the cold-water habitat as serine might provide greater flexibility in the collagen triple helix, while maintaining some degree of stability with hydrogen bonds driven by the hydroxyl group of serine.

2.2. GELATINE

Gelatine is a versatile biopolymer obtained from partial denaturation of collagen by acid treatment, alkali treatment, high-temperature treatment, and/or enzymatic hydrolysis (Schrieber & Gareis, 2007, p. 45). A description of the different methods for gelatine extraction can be found in Chapter 3.

Due to its unique functional and technological properties, gelatine has a wide range of applications in the food-, pharmaceutical-, cosmetic-, and photographic industries (Karim & Bhat, 2009). The global demand for gelatine has been increasing over the years, and recent reports indicate that the annual global production of gelatine will reach an estimated 516.8 thousand metric tons in 2020 and 696.1 thousand metric tons by 2027 (NASDAQ OMX's News Release Distribution Channel, 2020). Gelatine derived from fish accounted for less than 1.5 % of the annual global production in 2007[2], while the most abundant raw materials utilised for gelatine production were pig skin (46 %), bovine hide (29.4 %), and pork and cattle bones (23.1 %). It should be noted that the percentage for fish gelatine in 2007 was double that of the gelatine market data in 2002, which is a good indication that gelatine production from alternative, non-mammalian sources had gained some attention. (Gómez-Guillén et al., 2009) But due to the lack of easily accessible and more recent data it is difficult to tell if this trend has continued.

2.2.1. CHEMICAL AND STRUCTURAL PROPERTIES

As previously mentioned, the amino acid composition of gelatine is relatively similar to that of its parent collagen, which again is dependent on the habitat temperature of the organism it is obtained from (Akita et al., 2020). Additionally, the main factors that determine the thermostability of the parent collagen are also applicable to the structural properties of gelatine. The small differences in amino acid composition between collagen and gelatine are a result of the employed pre-treatment and extraction procedures (Johnston-Banks, 1990, p. 238). To avoid repetition, the reader will be referred to Chapter 2.1 for the general amino acid composition of gelatine and its effect on the structural

[2] More recent data could not be accessed

properties, and this chapter will focus mainly on the changes that occur during the conversion of collagen into gelatine.

Pre-treatment methods using either acid or alkali will result in gelatine with slightly different amino acid compositions and, thus, different properties. Treating the collagen with alkali nearly completely converts the amino acids asparagine and glutamine into aspartic and glutamic acid, respectively. This increases the number of carboxyl groups and consequently lowers the isoelectric point (pI) of the gelatine molecule, resulting in the so-called gelatine type B. Acidic pre-treatments, on the other hand, preserves most of the amidated forms of asparagine and glutamine, resulting in gelatine type A which will have an amino acid composition that is virtually identical to that of its parent collagen. (Haug & Draget, 2011, p. 101; Johnston-Banks, 1990, pp. 238–249; Schrieber & Gareis, 2007, p. 48) Because of the limited chemical alterations of side-groups during processing, the isoelectric point of type A gelatines is close to that of the parent collagen (Johnston-Banks, 1990, pp. 247–248). The difference in chemical alterations means that type A gelatines consist of cations with a broad isoelectric point in the range 6-9, while type B gelatines consist of anions with a more narrow isoelectric point in the range 4.7-5.3 (Haug & Draget, 2011, p. 101; Karim & Bhat, 2009; Wang et al., 2014, p. 217). The isoelectric point influences the applications of gelatine as many physical properties displays either a minimum or a maximum at the isoelectric point. For example, using a type B gelatine (pI 4.7-5.3) in aspic (pH 5-6) may result in a gel that has a lower degree of clarity due to the proximity of the pI to the pH in the aspic, compared to type A gelatines (pI 6-9). (Johnston-Banks, 1990, p. 248) Additionally, type B gelatines tend to have a higher hydroxyproline content and lower tyrosine content, compared to type A gelatines (Eastoe & Leach, 1977).

With an exception for tryptophan and cysteine, all amino acids commonly found in proteins are present in gelatine (Eastoe & Leach, 1977). Although cysteine residues are present in the telopeptide regions of collagen, especially in type III collagens where a higher cysteine content is thought to compensate for a lower hydroxylysine content, these telopeptides and the accompanying cysteine residues are mostly removed during pre-treatment due to cross-link cleavage (Johnston-Banks, 1990, pp. 237–238). The presence of cysteine residues in gelatines can also be a result of the co-extraction of keratin and other non-collagenous proteins (Eastoe & Leach, 1977). Cysteine content can therefore be used as an additional determinant of the purity of the gelatine, which is of outmost importance in the production of photographic gelatine where the presence of cysteine can have a detrimental effect on the quality of the end product (Lestra, 1985).

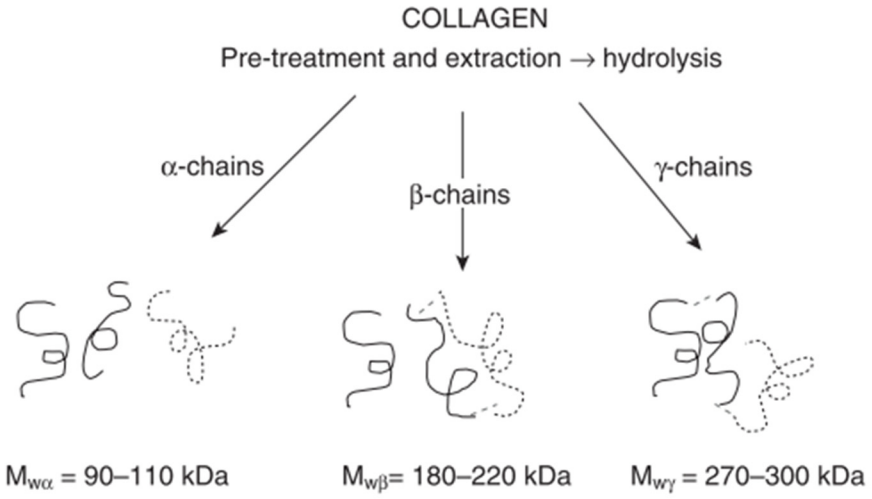


Figure 2: Various polypeptide chains produced during the conversion of collagen to gelatine. Figure taken from Haug & Draget, 2011, p. 95.

The structural properties of gelatine are not only influenced by its amino acid composition but also the distribution of molecular weights, structure, and compositions of its subunits. During the conversion of collagen to gelatine, the effects of acid and/or alkali as well as thermal energy produce various polypeptide fragments with different compositions and molecular weights, as shown in Figure 2. The dominating fragments found in gelatine are: free α-chains, β-chains (two α-chains that are covalently linked) and γ-chains (three α-chains that are covalently linked). Free α-chains may also be depolymerized into sub-α-chains with lower molecular weights than the intact α-chain. (Haug & Draget, 2011, p. 95; Karim & Bhat, 2009; Papon, Leblond, & Meijer, 2006, p. 201) Consequently, gelatine displays a pronounced polydisperse character and the polydispersity index (PDI) is usually over 2 (Schrieber & Gareis, 2007, p. 50). The PDI is calculated as the ratio of the weighted average molecular weight (M_w) to the number average molecular weight (M_n) (Karim & Bhat, 2009; Schrieber & Gareis, 2007, p. 50).

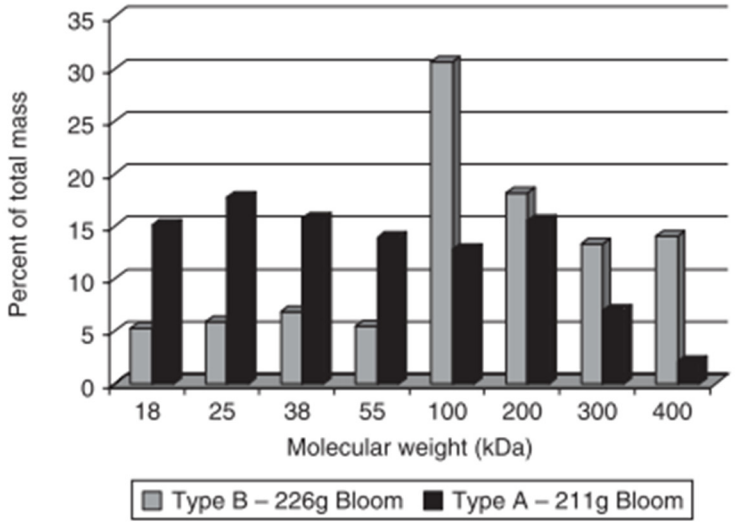


Figure 3: The molecular weight distribution of two gelatines with similar Bloom values. The average M_w for the type B gelatine is listed as 171 kg/mol, and the average M_w for the type A gelatine is listed as 94 kg/mol. Figure taken from Haug & Draget (2011).

The type and intensity of the gelatine extraction process determines in large part the molecular weight distribution of the gelatine. For the alkaline-treated type B gelatine, a major part of the molecular weight fractions is normally in the region of 100 kg/mol (Figure 3), which corresponds to the α-chain. The molecular weights of the acid treated type A gelatine displays a much wider distribution, which is a result of the hydrolysis of specific acid-labile peptide bonds within the helical part of the collagen molecule. (Schrieber & Gareis, 2007, pp. 49–51) During the extraction step, the pH of the extraction medium, the temperature(s) used, and the duration of the extraction will exert an influence on the molecular weight distribution as well. More severe treatments will generally lead to a higher degree of hydrolysis and, thus, a lower average molecular weight. (Eysturskarð, 2010; Johnston-Banks, 1990, pp. 243–245)

2.2.2. Functional properties

Gelation, viscosity and texture are closely related properties determined mainly by the structure, molecular size and temperature of the system (Gomez-Guillen, Gimenez, Lopez-Caballero, & Montero, 2011). When gelatine is dissolved in water or other appropriate solvents and heated above the melting temperature, the intra- and intermolecular hydrogen bonds stabilising the triple helical structures are broken, the gelatine chains are converted to the coil conformation, and a colloidal solution (or sols) is formed. When cooled to below the gelling temperature, the gelatine chains change conformation, forming helices again and the sols convert to gels, and vice versa (Figure 4). (Haug & Draget, 2011, pp. 104–105; Papon et al., 2006, pp. 189–201; Schrieber & Gareis, 2007, pp. 52–54) The thermo-

reversibility of gelatine gels is a function of the nature of the bonds between the polymer chains. In thermo-reversible gels, such as gelatine, the interactions between the polymer chains are weak (van der Waals interactions and hydrogen bonds), and breaking and reforming these interactions do not degrade the polymers as they do in the case of gels that are not thermo-reversible. (Alberto & Gabriela, 2012, p. 86; Papon et al., 2006, p. 191) It should be noted that the denaturation temperature of the parent collagen is not identical to the gelling and melting temperature of the derived gelatine, as both of these will in actuality lie below the denaturation temperature because only a certain fraction of the helices are needed to form a gel network (Haug & Draget, 2011, pp. 205–206). Also, the gelling and melting temperatures of gelatine gels exhibits hysteresis, i.e. when gelatine is in the gel state and is re-heated, it does not revert to the sol state at the exact same temperature where gelation occurred (Papon et al., 2006, p. 192).

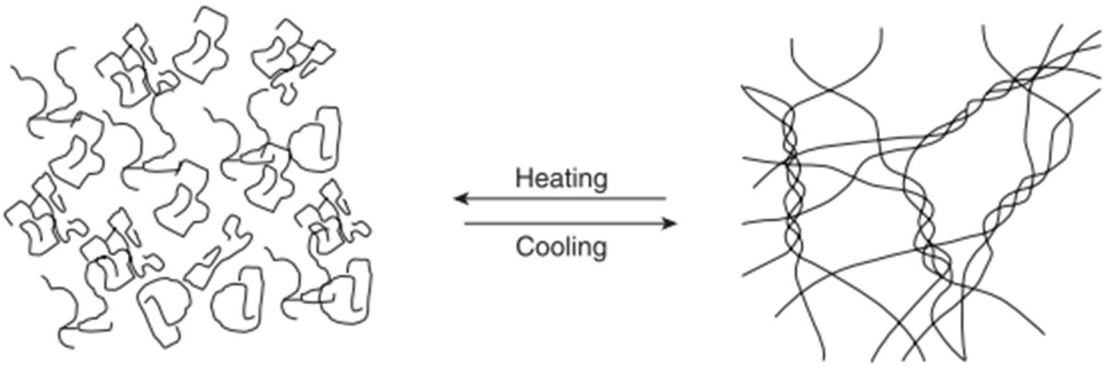


Figure 4: The gelation mechanism of gelatine. Left: gelatine in random coil conformation (sol state). Right: gelatine in helical conformation (gel state). Figure taken from Haug & Draget (2011).

As already mentioned, the content and distribution of proline and hydroxyproline are major factors determining the thermostability of collagen and, consequently, the physical properties of gelatine. The gelatine chains are made up of interspersed polar and non-polar regions, making it an amphipathic molecule. The non-polar regions mainly consist of the repeated amino acid sequence Gly-Pro-Y, where Y is a non-polar amino acid, predominantly hydroxyproline. (Haug & Draget, 2011, p. 101) These non-polar regions are the sites most likely to participate in the helical regions (junction zones) of the gelatine network due to their ability to form extensive hydrogen bonds (Johnston-Banks, 1990, pp. 258–259). Just as hydroxyproline is essential in the thermostability of collagen, the hydroxyl group of hydroxyproline is equally important in gel formation due to its ability to form interchain hydrogen bonds via a bridging water molecule as well as direct hydrogen bonds with a carbonyl group (Wong, 1989). It stands to reason that gelatine with a low content of imino acids will form fewer and/or less stable junction zones and, as a consequence, form weaker gels with lower melting points, compared to gelatines with higher content of imino acids. In addition to hydrogen bonded junction zones, hydrophobic

and ionic interactions are also involved in the gelation mechanism (Figure 5) (Benjakul, Kittiphattanabawon, et al., 2012, p. 392).

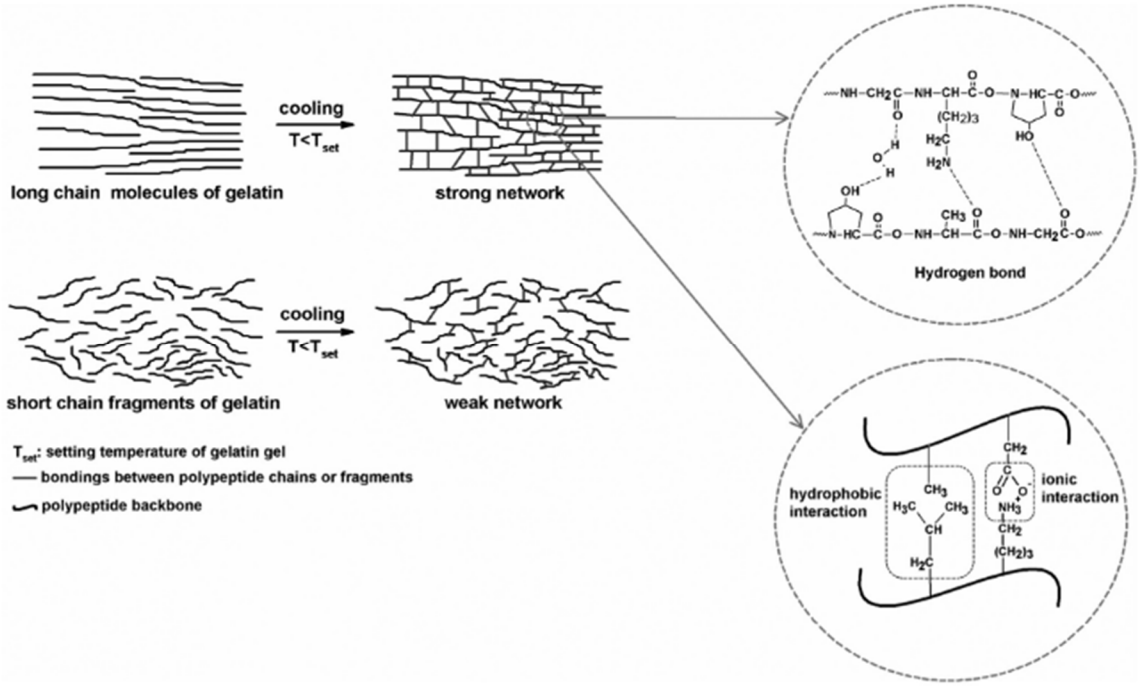


Figure 5: Gelatine network associated with hydrogen bonds, hydrophobic interactions, and ionic interactions. Figure taken from Benjakul, Kittiphattanabawon, et al. (2012).

Figure 5 also illustrates the effect that molecular weight has on the gel properties of gelatine. According to Schrieber & Gareis (2007), the gel strength is mainly dependent on the proportion of fractions having a molecular weight of around 100 kg/mol, while viscosity primarily is a function of the proportion of fractions within the molecular weight range of 200 to over 400 kg/mol. Studies have shown that gelatine fragments with low molecular weight (LMW) are negatively correlated with gel strength (Bloom value), while α-chains, β-chains and high molecular weight (HMW) molecules are positively correlated with gel strength. This might be due to LMW fragments ability to enter into junction zones without adding functionality to the gel network since they are too short to connect to other junction zones. (Eysturskard, Haug, Ulset, & Draget, 2009; Eysturskard, Haug, Ulset, Joensen, & Draget, 2010; Haug & Draget, 2011, p. 103)

The inherent properties of gelatine and other proteins make them excellent starting materials for films and coatings. The interactive forces created by the distribution of charged, polar and non-polar amino acids along the gelatine chain results in cohesive gelatine films, stabilised through electrostatic interactions, hydrogen bonds and van der Waals interactions. Due to the various amino acid functional groups, gelatine have multiple

sites for chemical interactions which can allow for modifications to further improve the functional properties of the films and coatings. The film properties are affected by the intrinsic properties of the components used and the extrinsic processing factors. The gelling properties of gelatine combined with its foaming properties translates to it being a good edible film former. (Dangaran, Tomasula, & Qi, 2009, pp. 26–33)

The surface properties of gelatine are based on the presence of charged groups in the protein side chains, and the presence of hydrophilic and hydrophobic regions in the molecule. The hydrophobic regions tend to migrate towards the oil/water- or liquid/air interface, thus reducing the surface tension of aqueous systems. Gelatine can also form identically charged films around the components of the dispersed phase, preventing them from coalescing. This property can be further strengthened by gel formation. (Gomez-Guillen et al., 2011; Schrieber & Gareis, 2007, p. 59)

Emulsions are colloidal systems of two immiscible liquids, where one phase is dispersed or suspended in another phase (the continuous phase) (Coultate, 2009, p. 141), while foams are colloidal systems containing air bubbles dispersed in an aqueous continuous phase (Damodaran, 2005). Without stabilising agents, emulsions and foams are unstable and will eventually return to their initial two-phase states. But they can be stabilised by addition of surface-active agents (surfactants) that contain hydrophobic and hydrophilic regions which can adsorb at colloidal interfaces. Surfactants adsorbing at the colloidal interface strengthens the mechanical stability of the dispersed phase and creates barriers that hinders the dispersed phase from coalescing. (Papon et al., 2006, p. 325) Gelatine can act as a surfactant due to the interspersed hydrophobic regions on the peptide chains but is generally a weaker emulsifier than other macromolecular surfactants, like gum Arabic. On its own, gelatine produces relatively large droplets during homogenisation and should therefore either be modified by attachment of non-polar side groups or used in conjunction with anionic surfactants to improve its emulsifying properties. (Karim & Bhat, 2009) The emulsifying properties of gelatine depends on concentration and molecular weight. LMW gelatine emulsions have been shown to result in larger droplets and exhibit more destabilisation than HMW gelatine emulsions. (Gomez-Guillen et al., 2011) Gelatine exhibits suitable foaming properties, even without gelling, because it is able to reduce the surface tension at the liquid/air interface in addition to increasing the viscosity of the continuous phase, thus, inhibiting the coalescence of the dispersed phase (Schrieber & Gareis, 2007, p. 59). Foaming properties depend on the characteristics of the raw materials. Adsorption at the oil/water interface is correlated with the surface hydrophobicity of the molecules, while adsorption at the liquid/air interface is correlated with the total hydrophobicity of the molecule because the proteins unfold more extensively at the latter interface (Townsend & Nakai, 1983).

The ionisable groups give gelatine a net charge after being dissolved in water. This property gives gelatine an affinity for electrostatically charged molecules which can potentially

cause the formation of coacervates. This property is exploited in clarification and taste improvement of alcoholic beverages, where gelatine is added to form coacervate with tannins and other bitter compounds. The formed precipitates sediment to the bottom of the container and can easily be removed. This process can also be used in the microencapsulation of both solids and liquids. (Hattrem & Draget, 2014, p. 23)

3. GELATINE EXTRACTION METHODS

Gelatine manufacturing procedures generally consists of several processes; cleaning of raw material, pre-treatment, extraction of gelatine, filtration, concentration/evaporation, sterilisation and drying (Eysturskarð, 2010; Johnston-Banks, 1990, pp. 242–245). Cleaning and pre-treatments are performed to increase the purity of the extracted gelatine, as well as to break intra- and intermolecular bonds within and between the helices in collagen (Hattrem & Draget, 2014, p. 20; Karim & Bhat, 2009). Alkaline and/or acidic pre-treatments are employed to enhance the gelatine extraction efficiency, and the choice of pre-treatment will determine if the resulting gelatine is type A or type B, as described in Chapter 2.2.1 (Benjakul, Kittiphattanabawon, et al., 2012, p. 390; Haug & Draget, 2011, p. 101; Johnston-Banks, 1990, pp. 238–249; Schrieber & Gareis, 2007, p. 48).

3.1. PRE-TREATMENTS

The raw materials used in gelatine manufacture contain a variety of substances that are classified as impurities, many of which have an adverse effect on the properties of the resulting gelatine. These include non-collagenous proteins, lipids and other cell components, as well as inorganic impurities in the form of intrinsic minerals, such as calcium, sodium, potassium and iron. (Eastoe & Leach, 1977) Some of these impurities are removed when cleaning the raw materials with water, while others require chemical treatments (Haug & Draget, 2011, p. 93).

In principle, gelatine can be extracted by long-term heating in water, but because of the cross-linked nature of collagen, it dissolves very slowly even when heated and subjecting the raw material to long periods of higher temperatures has a negative influence on all the parameters that affect the quality of the gelatine (Schrieber & Gareis, 2007, p. 72). Employing pre-treatments before extraction enhances the cleavage of the cross-links, thus affecting the swelling and solubilisation of the collagen (Benjakul, Sae-leaw, & Simpson, 2020, pp. 188–189).

Swelling of collagen in acid and alkaline solutions is mainly governed by cohesion of the protein, and the osmotic pressure differences arising between the protein phase and the external solution. High cohesiveness in collagen, i.e. high degree of intra- and

intermolecular bonds and cross-linking, opposes swelling. (Bowes & Kenten, 1950) pH and ionic strength are important factors for the swelling of collagen fibres. Kaye & Lloyd (1924) reported that the swelling maxima appeared at pH = 2.2 and pH = 11.8. Ranganayaki, Asghar, & Henrickson (1982) found that swelling was minimal in the pH range from 6.0-9.0 but that addition of 6 % NaCl increased the swelling in this pH range, while decreasing the swelling at pH levels above and below. The high collagen swelling below pH 6.0 and above pH 9.0 is a result of the protein unfolding due to disruption of non-covalent bonds by the increased H^+ and OH^- concentrations, respectively. This disruption leads to the development of a Donnan membrane potential inside the collagen fibres, causing water to flow into the fibres and, thus, making them swell. (Ranganayaki et al., 1982) The swelling of the raw material is important because it lowers the denaturation temperature of the collagen and permits the gelatine to be extracted at milder conditions (Stainsby, 1987).

Although use of pre-treatments generally enhances the subsequent gelatine extraction, it may also lead to lower yields if not conducted under appropriate conditions. Excessive swelling and solubilisation during pre-treatment promote leaching and results in the loss of collagen in the pre-treatment solution. (Benjakul et al., 2020, pp. 188–189) Thus, a gentle chemical pre-treatment is necessary to break down the cross-links while, at the same time, reducing loss of quality in the final gelatine.

3.1.1. Alkaline pre-treatment for type B gelatine

Alkaline pre-treatments are usually employed for collagen tissues that are highly cross-linked, like cattle hides and ossein prepared from cattle bones. The raw materials are treated with a 1 % sodium hydroxide solution at 20 °C for a few days, or with supersaturated milk of lime for up to four months. The quality of the final gelatine can be a result of the relationship between sodium hydroxide concentration, temperature and duration of the pre-treatment. Stronger pre-treatments with alkali normally results in a higher viscosity but the yield will be lower due to the collagen becoming soluble in cold water, resulting in loss of collagen when the raw material is washed in between treatment steps. (Schrieber & Gareis, 2007, pp. 73–74) Alkaline treatments can also result in random hydrolysis of peptide bonds and degradation of some amino acids, which leads to a product of variable quality with a broad molecular weight distribution (Slade & Levine, 1987).

According to Zhou & Regenstein (2005), alkaline pre-treatments can remove considerable amounts of non-collagenous proteins with minimal loss of collagen. The type of alkali does not have a significant impact on the quality of the gelatine, but the concentration of alkali is critical (Zhou & Regenstein, 2005). Sato et al. (1987) found that NaOH concentrations between 0.01-0.5 N resulted in little to no loss of collagen in the alkali-soluble fraction,

and that a concentration of 0.1 N NaOH is sufficient to inactivate endogenous proteases that may induce enzymatic degradation of intrachain peptide bonds.

3.1.2. Acid pre-treatment for type A gelatine

Acidic pre-treatments are generally milder than the alkaline pre-treatments, and is normally used for less cross-linked collagen tissues, like skin from young pigs. The acid pre-treatment process offers a significant advantage over alkaline pre-treatments as it requires a much shorter treatment period. The raw materials are treated with 2-5 % inorganic or organic acid for 10-48 h at 15-20 °C. After the acid treatment, the pH is raised to about 2-4 by adding alkali and the formed salts and excess acid are washed out. (Eysturskarð, 2010; Schrieber & Gareis, 2007, pp. 74–75)

Acid pre-treatments can also inactivate or remove endogenous proteases and lower the enzymatic degradation of intrachain peptide bonds during extraction. But Zhou & Regenstein (2005) found that using 0.05 M acetic acid resulted in a less efficient removal of non-collagenous proteins and a much higher loss of collagen compared to various alkaline pre-treatments. Mild acid pre-treatments disrupt acid-labile cross-links with minimal peptide bond hydrolysis and amino acid degradation, although it may lead to lower yields, especially in collagen that is highly cross-linked (Slade & Levine, 1987). However, using acid treatments in conjunction with pepsin or other appropriate proteases can increase the extraction yield. Nalinanon, Benjakul, Visessanguan, & Kishimura (2008) studied the effects that bigeye snapper pepsin (BSP) in combination with protease inhibitors had on the extraction efficiency and characteristics of gelatine from bigeye snapper skin. The endogenous enzymes of the bigeye snapper skin were inactivated using either heat treatments or specific protease inhibitors. They found that the yield increased with increasing concentrations of BSP, from 22.2 ± 0.35 % without the aid of BSP to 40.3 ± 0.44 % with 15 units BSP/g treated skin. Also, they found that using soybean trypsin inhibitor (0.1 μM) during the gelatine extraction markedly reduced the degradation of the α-chains, thereby resulting in gelatine with higher bloom strength.

3.1.3. Defatting

Raw materials with high lipid contents are associated with a negative effect on the properties of the extracted gelatine. Fish skin contains lipids and fatty acids with high degrees of unsaturation that are vulnerable to oxidation both during storage and during gelatine extraction, especially at higher temperatures. Lipid oxidation causes the development of undesirable odours and flavours in the resulting gelatine, thereby limiting its applications. (Sae-leaw & Benjakul, 2015) Several studies have been performed to assess the effect that defatting has on the properties of gelatine extracted from fish skin.

Sae-leaw, Benjakul, & O'Brien (2016b, 2016a) found that the gel strength was lower in gelatine extracted from defatted seabass skins than gelatine extracted from non-defatted seabass skins, while the fishy odour and abundance of volatile compounds associated with lipid oxidation was lower in defatted skins. Sae-leaw, Benjakul, O'Brien, & Kishimura (2016) found that gelatine from defatted seabass skins had higher foam expansion and stability than those extracted from non-defatted skins, additionally, emulsions containing gelatine from defatted skins had smaller droplet size and displayed higher stability during storage than those from non-defatted skins. Sae-leaw, Benjakul, & O'Brien (2016a) found that the efficacy of phospholipid removal from seabass skin decreased as the concentrations of ethanol or isopropanol increased, and the highest reduction of phospholipids was observed in skin pre-treated with citric acid, followed by defatting using 30 % isopropanol. The skins pre-treated with citric acid and defatted with 30 % isopropanol also showed lower amounts of volatile compounds associated with fishy odour, compared to the other gelatines in the study (Sae-leaw, Benjakul, & O'Brien, 2016a).

Defatting has been performed in different ways using a wide range of solvents: Muyonga et al. (2004) performed a simple defatting in bone of Nile perch by tumbling in warm water (35 °C), Eysturskard et al. (2009) removed lipids from the skins of haddock, cod and saithe using 10 % butyl alcohol, and Sae-leaw & Benjakul (2018) used lipase extract from liver of seabass to remove fat which resulted in a higher efficacy compared to defatting using 30 % isopropanol.

3.1.4. DEMINERALISATION

Demineralisation is a treatment step employed for certain types of raw material, such as bones or scales. The aim of this step is to remove calcium and other inorganic substances to facilitate the extraction of gelatine and increase the purity. Treatment with dilute hydrochloric acid solutions dissolves the calcium phosphates present in bones and scales, thus removing these impurities from the raw material. (Waldner, 1977) According to Jones (1977) the maximum accepted ash content of gelatine intended for food applications is 2.6 %, and a lower ash content contributes to a higher quality gelatine. However, Schrieber & Gareis (2007) states that most pharmacopeia and food regulations specify less than 2 % ash, and that many applications in the pharmaceutical industries require less than 1 %.

3.2. EXTRACTION

When subjected to heating, collagen fibres shrink to about one quarter of their original length at a critical temperature known as the shrinkage temperature (T_s) (Foegeding et al., 1996, p. 905; Miles & Bailey, 1999). During the shrinking, the fibres are disassembled and the triple-helical arrangement of the polypeptide subunits of the collagen collapses. These

changes also occur when collagen is heated in solution, but at a lower temperature, known as the denaturation temperature (T_d)[3]. During the conversion of collagen to gelatine, many non-covalent bonds are broken along with some covalent inter- and intramolecular bonds and a few peptide bonds. This results in a structural rearrangement, from helical collagen to amorphous gelatine. (Foegeding et al., 1996, p. 905)

The extraction process should be optimised to obtain maximum yield in combination with the desirable physical properties. The pH of extraction can be selected either for maximum extraction rate (low pH) or for the maximum in physical properties (neutral pH), or a compromise between the two. The pre-treatments will influence the extraction conditions; longer pre-treatments can facilitate extraction from highly cross-linked collagen at neutral pH and lower temperatures, while extraction conditions must be more acidic and performed at higher temperatures to achieve acceptable extraction rates when shorter pre-treatments are employed. Basically, more efficient pre-treatments conditions allow for milder extraction conditions, and this will, in turn, result in gelatines with better properties. (Johnston-Banks, 1990, pp. 243–244)

In typical industrial practice, gelatine is dissolved out from the raw material in several stages, each at successively higher temperatures (50 to 100 °C). Each stage takes from four to seven hours, and the successive extractions will yield gelatines with decreasing quality in terms of bloom strength and colour due to increased hydrolysis and Maillard reactions respectively. (Johnston-Banks, 1990, pp. 243–244; Schrieber & Gareis, 2007, pp. 75–78)

3.3. PURIFICATION AND DRYING

After extraction, the gelatines are filtered and deionised to remove suspended or insoluble matter and inorganic salts left from the pre-treatment. Diatomaceous earth or activated carbon can be used to clarify the gelatine solutions. Finally, the gelatine solution is subjected to evaporation, sterilisation and drying. (Johnston-Banks, 1990, p. 245)

[3] To make it entirely clear: Shrinkage temperature (native collagen) > denaturation temperature (collagen in solution)

4. REST RAW MATERIALS FROM THE FISH INDUSTRY AS A SOURCE OF GELATINE

Rest raw materials (RRMs) can be defined as every part of the animal that traditionally is not viewed as the primary product during the utilisation of raw materials. This includes both edible and inedible materials, such as by-catch, guts, heads, roe, blood, trimmings, skin and scales. (Bekkevold & Olafsen, 2007, p. 21; Richardsen & Nystøyl, 2017, pp. 3–14) RRMs can arise at any point in the capture fisheries and aquaculture value chain. A substantial amount (5-15 % depending on region – see Figure 6) is lost already in the fisheries, mainly due to the discarding of unwanted fish (Gustavsson, Cederberg, Sonesson, van Otterdijk, & Meybeck, 2011, pp. 8–9). FAO estimated that annual discards in the global marine capture fisheries was around 9.1 million tons in the years 2010-2014 (Pérez Roda et al., 2019, p. 10).

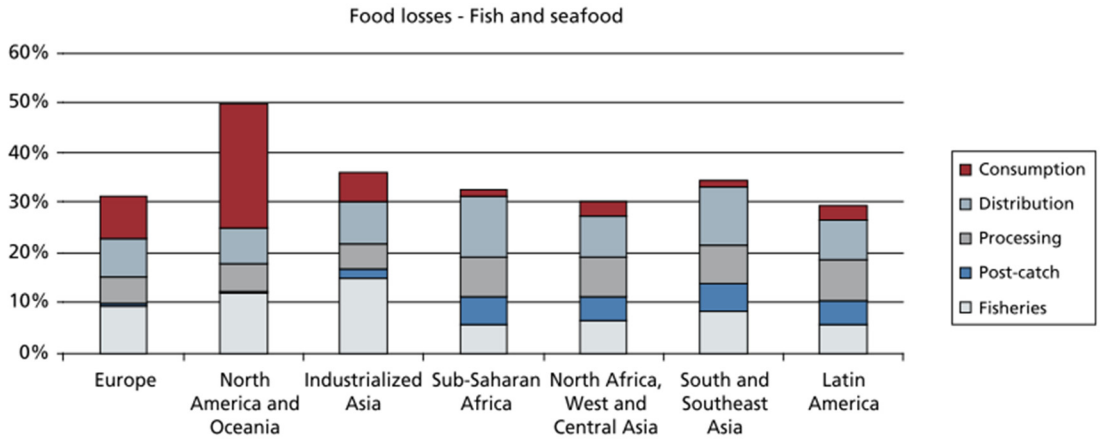


Figure 6: Food losses from fish and seafood in 2007, divided by region and points in the value chain (Gustavsson et al., 2011, p. 8).

A significant amount of RRMs arises during processing and transportation of fish and fish products, where some parts are unavoidable (e.g. cuttings and trimmings from filleting) and others stem from sub-optimal handling and storage (FAO, n.d.). Either way, all RRMs derived from the fish industry contain valuable components that can be utilized, be it directly or indirectly. As an example; it is estimated that 10-20 % of the total proteins in fish can be found in the RRM fraction (Harnedy & Fitzgerald, 2013, p. 7).

Over the years, the fraction of the world's total fish production utilised for direct human consumption has increased significantly, from 67 % in the 1960s to 88 % in 2016, as illustrated in Figure 7. The remaining 12 % (corresponding to about 20 million tons) was mainly utilised in the production of fishmeal and fish oil, with the rest largely being reduced to feed used in aquaculture and the raising of livestock and fur animals, as well as materials for pharmaceutical uses and ornamental purposes. (FAO, 2018, p. 47)

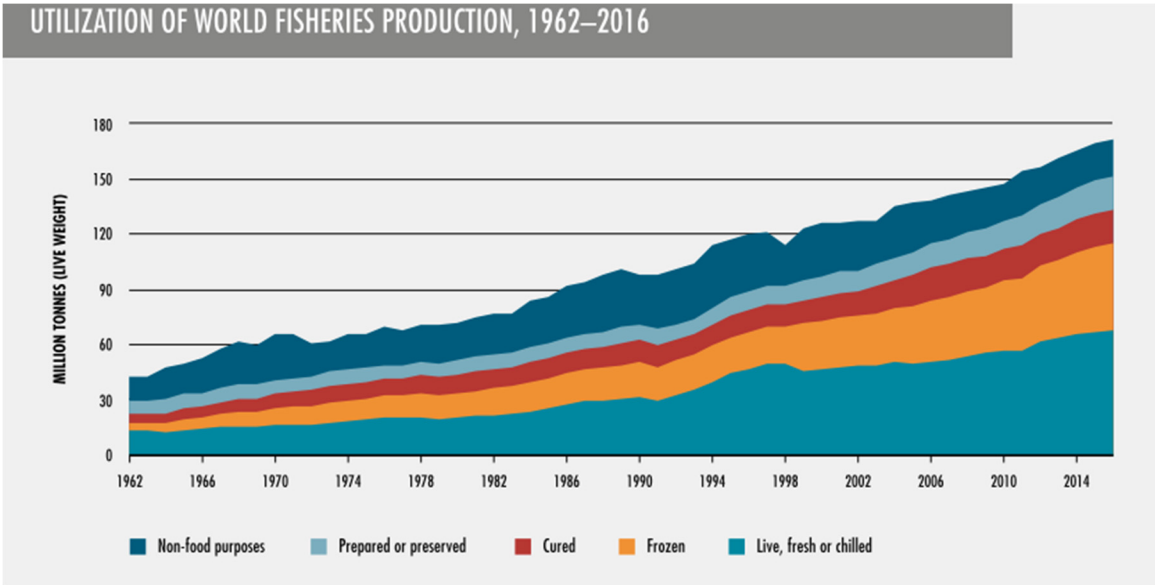


Figure 7: The utilization of the world's fisheries production, 1962-2016. The top blue area represents the amount of the world's total fish production that is utilized for non-food purposes, the remaining areas represent the amount that is in some way utilized for food purposes. (FAO, 2018, p. 48).

About 30 % of RRMs from fish processing consists of skin and bones with high collagen content (Gómez-Guillén et al., 2002). As an example, Dave et al. (2019) reported that RRMs (skin, fins, scales and frames) from Atlantic salmon farming in Newfoundland contained between 2.31 % (frames) and 51.11 % (scales) collagen (as percent of total dry matter). Collagenous by-products from the fishing industry could therefore prove to be a valuable source for gelatine and the answer to the overall increasing global demand for gelatine, as well as being a safe alternative for consumers with dietary restrictions or concerns for the safety of mammalian gelatine (Benjakul, Kittiphattanabawon, et al., 2012, p. 365; Karim & Bhat, 2009).

4.1. COLD-WATER FISH GELATINE: PROPERTIES AND CHALLENGES

Gel strength, viscosity, and thermal stability (gelling and melting temperatures) are the three most commercially important properties for gelatine, which are governed by molecular weight, amino acid composition (especially proline and hydroxyproline) and ratio of α/β-chains. This basically means that the properties of gelatine are influenced by two main factors: the characteristics of the native collagen (which is species-specific) and the gelatine extraction process. (Alfaro, Balbinot, Weber, Tonial, & Machado-Lunkes, 2014; Gomez-Guillen et al., 2011; Haug, Draget, & Smidsrød, 2004; Karim & Bhat, 2009) In gelatine derived from mammalian sources, the imino acids, hydroxyproline and proline, make up around 30 % of the total amino acids. The imino acid content of gelatines extracted from warm-water fish (Nile perch and tilapia) is slightly lower compared to mammalian

gelatine and amount to 22-25 % of the total amino acids. The lowest content of imino acids can be found in gelatines extracted from cold-water fish (cod), where it makes up around 17 % of the total amino acids. (Muyonga et al., 2004) Avena-Bustillos et al. (2006) found that gelatines from cold-water fish have significantly less hydroxyproline, proline, valine, and leucine than gelatine from mammalian sources, and significantly more glycine, serine, threonine, aspartic acid, methionine, and histidine than mammalian gelatines. The remaining amino acids was found in to be in the same proportions as for mammalian gelatines. These differences in amino acid composition is likely, as mentioned, mainly a result of evolutionary adaptations to different habitat temperatures (Akita et al., 2020). Imino acid content is positively correlated with gel strength and thermal stability, with higher contents resulting in gels that are stronger and has higher gelling temperatures due to the formation of more stable network structures, and vice versa (as described in Chapter 2). (Alfaro et al., 2014; Gomez-Guillen et al., 2011; Haug et al., 2004; Karim & Bhat, 2009) The comparatively low content of imino acids means that gelatine from cold-water fish does not gel at room temperature, like mammalian gelatine does, and instead behaves like a viscous liquid. The gelling point of cold-water fish gelatine is below 8-10 °C, which limits its use in the food industry. (Karim & Bhat, 2009; Milovanovic & Hayes, 2018) However, the inferior properties associated with a low content of imino acids can in part be remedied by enzymatic or chemical cross-linking that increases the stability of the gel network (Gomez-Guillen et al., 2011; Huang et al., 2019; Karim & Bhat, 2009). Huang et al. (2019) have published a comprehensive review of fish gelatine modifications. In certain food systems, the suboptimal properties of cold-water fish gelatines may be desired, as the relatively low gel strengths and melting temperatures can lead to better sensory characteristics due to faster and stronger flavour release, compared to mammalian gelatine (Boran & Regenstein, 2010, pp. 127–139; Choi & Regenstein, 2000).

Due to the limitations posed by its suboptimal physical and chemical properties, gelatine from cold-water fish cannot replace mammalian gelatine in every application. But the higher amount of hydrophobic amino acid found in cold-water fish gelatine, compared to mammalian gelatine, could in certain cases offer an advantage (Milovanovic & Hayes, 2018). In previous studies by Avena-Bustillos et al. (2011, 2006) comparing the properties of gelatine films from various sources, they found that gelatine films from mammalian sources and warm-water fish contained helical structures and had higher tensile strength, percent elongation and puncture deformation compared to gelatine films from cold-water fish. Gelatine films from cold-water fish were amorphous due to the films being dried at a temperature well above their helix-to-coil transition temperature. But although the mechanical properties were poorer for cold-water fish gelatine films, the oxygen and water vapour permeability of the films were significantly lower than those for mammalian gelatine films. The difference in water vapour and oxygen permeability was explained based on the comparatively higher amount of hydrophobic amino acids and the lower amount of hydroxyproline in cold-water fish gelatine. It is likely that the helical structures of gelatine provides an orientation of hydrophilic groups that are more favourable to water

binding than amorphous gelatine chains (Chiou et al., 2009; Tanioka, Tazawa, Miyasaka, & Ishikawa, 1974), and that higher moisture contents results in increased mobility of gelatine chains, which leads to greater oxygen diffusivity and higher permeability (Avena-Bustillos et al., 2011).

Another thing to consider is that collagenous material from fish skins are highly susceptible to degradation due to the lower content of intra- and intermolecular cross-links, compared to the more stable mammalian collagen. The freshness and quality of the raw material is one of the key factors determining the properties of fish gelatine, thus, making proper handling and preservation of RRMs intended for gelatine production essential. (Alfaro et al., 2014) Because of the suboptimal properties associated with the characteristics of the native collagen in cold-water fish, care must be taken to avoid lowering the properties further. Processing conditions for gelatine extraction from cold-water fish should be optimised specifically to avoid further reductions in properties.

4.2. SCOPE OF THE THESIS

The first objective of the thesis was to investigate the effect of various pre-treatments and defatting on the lipid, dry matter, and ash content of skin of Atlantic salmon (*Salmo salar*) using a screening experiment. High lipid content is known to have a negative effect on both the physio-chemical and sensory properties of gelatine (Sae-leaw, Benjakul, & O'Brien, 2016a, 2016b; Sae-leaw, Benjakul, O'Brien, et al., 2016), and since Atlantic salmon is a fatty fish it is necessary to reduce the amount of lipids in the raw material before extraction to yield gelatine with better properties. Food and other industrial regulations demand that gelatine contain no more than 2.6 % ash (Jones, 1977), so this must factor into the extraction process to avoid limiting the gelatine's applications. The second objective was to process the data from the screening experiment and implement the obtained results in the main experiment. The objective of the main experiment was to determine the effect of different extraction times on the yield, purity and molecular weight distribution of gelatine extracted from defatted Atlantic salmon skin.

5. MATERIALS AND METHODS

5.1. PREPARATION OF RAW MATERIAL

Fresh salmon skins were obtained from Nutrimar AS (Frøya, Norway). The skins were placed on ice and cooling elements in a Styrofoam container and sent with Kystbudet (Hitra, Norway) on the morning 19th of November 2019 and was received at the Department of Biotechnology and Food Science (NTNU, Trondheim, Norway) around noon the same day. Due to conflicts with a lecture, the container had to sit in a cold room (4 °C) until the afternoon. Then, one third of the skins were cleaned by manually removing scales and remaining flesh and rinsed in tap water. The clean skins were cut into approximately 2 cm^2 squares with a box cutter and portioned out in zip-lock bags before being stored in a freezer at -20 °C. The remaining uncleaned skins were frozen at -80 °C in case of need in further experiments.

5.2. SCREENING

An experimental design with five factors was developed to investigate how different combinations of pre-treatments and defatting affected the composition of the raw material. The five factors (or independent variables) were: OH concentration, pre-treatment time, pre-treatment temperature, butanol concentration and defatting time. Each of these factors were tested at two levels (see Table 4). The dependent variables were: dry matter, ash and lipid content. Total number of runs were 32 with no replicates. The full factorial screening design along with observed data can be found in Appendix A. Untreated salmon skins were analysed in the same manner as the treated skins to be used as a comparison in evaluating the efficacy of the treatments. An additional aim of the screening experiment was to determine what effect OH concentration, pre-treatment time and temperature had on the swelling of the skins.

*Table 4: Independent variables and their levels in the 5-factor, 2-level factorial (2^5) screening experiment. *Pre-treatment and defatting solutions were changed every hour.*

Factor	Level	
	-	+
OH concentration (mol/L)	0.1	0.2
Pre-treatment time (h)	3 × 1 *	24
Pre-treatment temperature (°C)	4	Room temp.
1-Butanol concentration (%)	10	20
Defatting time (h)	3 × 1 *	24

Both pre-treatment and defatting were performed under gentle shaking on an orbital shaker (Orbital Shaker, PSU-10i, Grant Bio, 130 rpm) to ensure that the raw material was treated evenly. The w/v-ratio was kept constant at 1:10. Since defatting with 1-butanol had to be performed in a fume hood for safety reasons, the temperature variable for this step was neglected as the agitation of the solution was mistakenly considered to be more important than the temperature. In retrospect, the optimal solution would have been to use a cold-water bath with an integrated orbital shaker. Quantification of dry matter, ash and lipid content in the samples were performed using methods described in 5.4.2 and 5.4.3.

5.2.1. ADDITIONAL SCREENING FOR DEFATTING

Considering the low thermal stability of collagen from cold-water fish, all pre-treatments should optimally be performed at a temperature that is close to the habitat temperature of the animal to reduce thermal denaturation and premature solubilisation of the collagen. In the first screening procedure the emphasis was placed on the agitation of the defatting solution at the expense of temperature, since the defatting had to be performed in a fume hood. It was rationalised that since the rather non-polar butanol were to be diluted with polar water, agitation would prevent complete separation of the liquids and that this would increase the defatting efficiency due to better contact between the raw material and the solvent. It was later decided to do a new experimental setup to test the efficacy of defatting in a cold-water bath (4 °C) without agitation.

Clean, untreated skins were defatted using 1-butanol (VWR International LTD, Oslo, Norway) at concentrations of 10 and 20 % (v/v) using a solid/solvent ratio of 1:10 at 4 °C using a water bath (Comfort Heto Chill Master, model CB-8-30E). The skins were defatted for 2, 2×2, 3×2 (solutions changed every two hours) and 24 hours before being removed from the solvent and washed with 10 volumes of tap water. Solution samples were collected after each round of defatting. The samples were analysed in duplicates for lipid content using the modified Bligh & Dyer method described in 5.4.3.

5.3. GELATINE EXTRACTION PROCEDURE

All processes were carried out at 4 °C, either in cold room or in cold water baths. All solutions and distilled water for washing were stored at 4 °C. Samples of all solutions were collected and frozen for determination of protein content, dry matter etc. pH was measured before, during and after each treatment step. A flowchart of the developed gelatine extraction process for salmon skins can be seen in Figure 8.

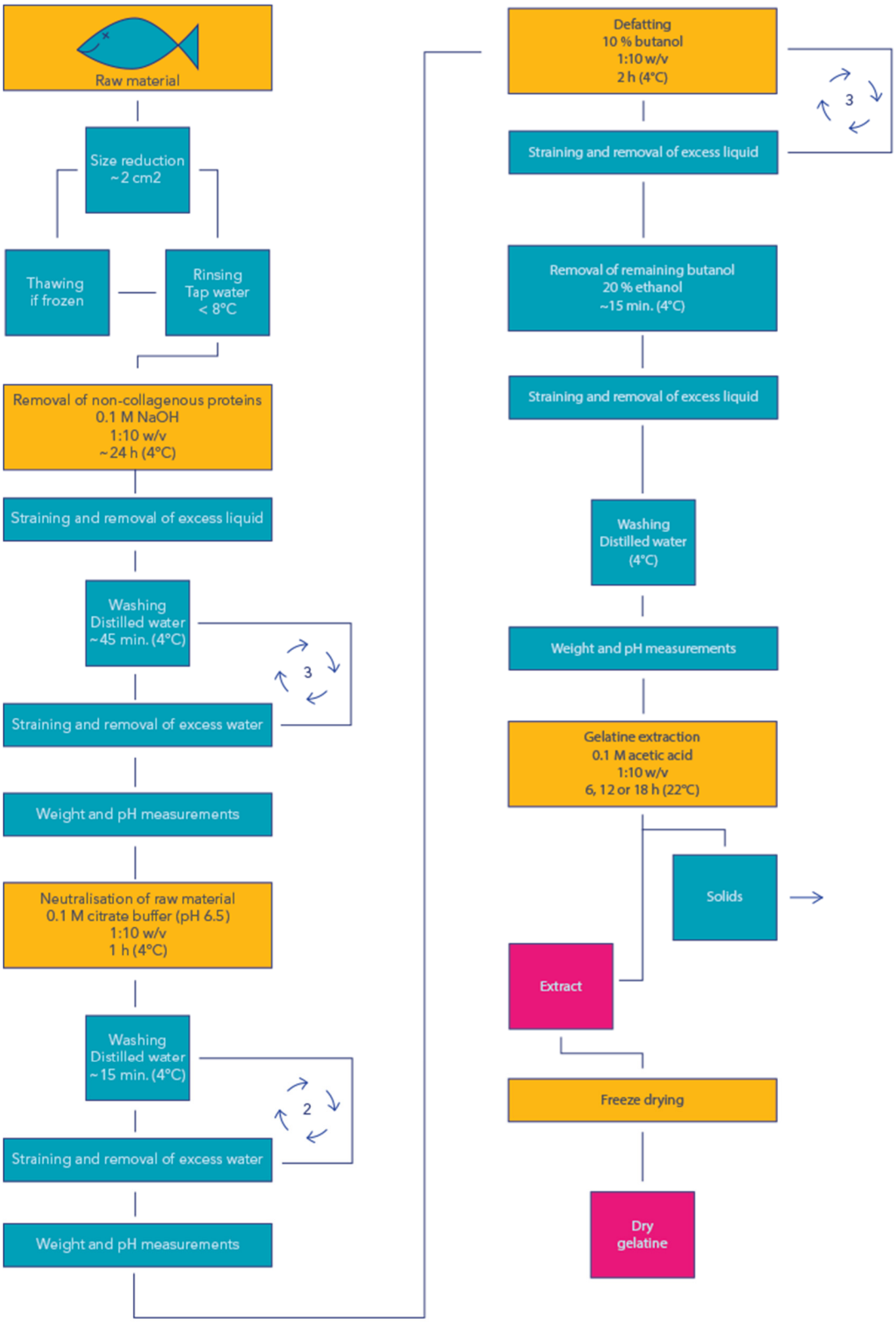


Figure 8: Flowchart of the developed gelatine extraction process. Yellow boxes indicate the main steps, while the blue boxes indicate intermediary steps (like washing etc.)

5.3.1. PRE-TREATMENT

Rinsed salmon skins were soaked in 0.1 M NaOH (VWR International LTD, Oslo, Norway) with a solid/solvent ratio of 1:10 (w/v) for 24 hours with gentle shaking (Orbital Shaker, PSU-10i, Grant Bio, 130 rpm). After the alkali pre-treatment was finished, the skins were separated from the NaOH solution using a fine mesh sieve and excess NaOH was squeezed out using a cheese cloth. To obtain a neutral pH in the alkali-treated skins, the raw material was soaked in distilled water for around 45 minutes with gentle shaking. The raw material was then separated from the wash water using a fine mesh sieve and excess water was squeezed out using a cheese cloth. This step was repeated three times. Then, the skins were treated with 0.1 M citrate buffer (pH 6.5, 1:10 w/v) for one hour, before being separated from the buffer solution with a fine mesh sieve. Finally, the skins were soaked in distilled water twice for 15 minutes, before excess water was squeezed out using a cheese cloth. The raw materials were then weighed and transferred to zip-lock bags and stored at -20 °C.

5.3.2. DEFATTING

Pre-treated salmon skins were defatted for 3×2 hours using 10 % 1-butanol with a ratio of 1:10 (w/v) at 4 °C using a cold-water bath without agitation. The butanol solution was exchanged every two hours. After the defatting procedure, the raw material was separated from the butanol solution using a fine mesh sieve and excess water was squeezed out using a cheese cloth. The raw material was subsequently soaked in 20 % ethanol for 15 minutes to remove remaining butanol and, thus, reduce the butanol odour. Then, the raw material was separated from the ethanol solution and washed with cold distilled water until the wash water was neutral in pH. Excess water was squeezed out using a cheese cloth, the raw material was weighed and transferred to zip-lock bags before being stored at -20 °C.

5.3.3. EXTRACTION

Gelatine was extracted from pre-treated and defatted salmon skins with 0.1 M acetic acid (VWR International Ltd, Oslo, Norway) with a ratio of 1:10 (w/v) for 6, 12 or 18 hours at 22 °C using a water bath. After extraction, the solids were separated from the extract using a fine mesh sieve and excess liquid was gently squeezed out using a cheese cloth. 80 mL of the extraction liquid was frozen at -20 °C for use in analysis of dry matter content etc. The remaining extraction liquid was transferred to freeze-drying vessels. The solids were weighed and frozen at -20 °C. A summary of the actual gelatine extraction conditions is given in Chapter 6.2 (Table 9).

5.4. ANALYSES

All chemicals used were of analytical grade.

5.4.1. YIELD, EXTRACTION EFFICIENCY AND MASS BALANCE

The yield was calculated as total extraction-, protein- and gelatine yield as a function of extraction time. Total extraction yield was determined gravimetrically, protein content was determined by the Lowry method (see Chapter 5.4.4.), and gelatine yield was calculated based on total extraction yield and the gelatine sample purity obtained from hydroxyproline determinations (see Chapter 5.4.6).

The extraction efficiency was calculated as the amount of dry gelatine compared to the amount of dry matter in the raw material, in accordance with Arnesen & Gildberg (2007), using Equation 1:

$$Extraction\ efficiency\ = \frac{Weight\ of\ freeze{-}dried\ gelatine\ (g)}{Weight\ of\ dry\ matter\ in\ raw\ material\ (g)} \times 100 \qquad \textit{Equation 1}$$

The mass balance was determined by measuring the amount of protein, gelatine and dry matter removed from the salmon skins during the extraction process, including remaining dry matter in left-over solids after extraction. The sum of this was then compared to the dry matter content of untreated salmon skins. For the determination of dry matter removed during the pre-treatments, the estimated dry matter contribution of the NaOH was subtracted from the total. During the gelatine extraction process, the weight of the raw material fluctuated due to absorption/repulsion of water and chemicals, therefore the determination of mass balance was based on the weight of the matter since basing it on percentages of wet weight would be highly uncertain.

5.4.2. DRY MATTER AND ASH

The dry matter and ash content were determined gravimetrically by accurately weighing out 0.5-4 grams or 5.0 mL (depending on sample type) of sample in dried and pre-weighed crucibles. The crucibles were then placed in a drying cabinet holding 105 °C for approximately 24 hours. Dried samples were placed in a desiccator until they reached room temperature before being weighed again. Percent dry matter was then calculated according to Equation 2:

$$Dry\ matter\ or\ ash\ (\%)\ = \frac{(b-D)}{s} \times 100 \qquad \textit{Equation 2}$$

Where b = weight of crucible and dry sample or ash (g); D = crucible (g); s = sample (g)

The crucibles were then transferred to a muffle furnace and left at 550 °C overnight, before being cooled to room temperature in a desiccator and weighed. Percent ash was calculated in the same manner as for dry matter.

5.4.3. LIPID CONTENT

The lipid content of the pre-treated and defatted skin samples from the screening procedure were determined according to a modified version of the lipid extraction method described by Bligh & Dyer (1959).

For each sample, 5-10 g were weighed out in centrifugation bottles and placed on ice to decrease evaporation. 16 mL distilled water, 40 mL methanol and 20 mL chloroform were added to the centrifugation bottles. The next step deviates from the original modified version which calls for homogenisation with an ULTRA-TURRAX for two minutes. Due to complications arising from the toughness of the skins during the homogenisation steps, it was decided that homogenisation had to be replaced by manual stirring to avoid significant loss of sample. Since most of the lipids are deposited on the inner surface of the skins, it was assumed that stirring would be adequate to dissolve most of these lipids and would in turn give less uncertainty in the results than the alternative.

After stirring for two minutes, 20 mL chloroform was added, and the mixture was stirred for 40 seconds. Then, 20 mL distilled water was added, and the mixture was stirred for another 40 seconds. The sample bottles were then centrifuged at 5000 G for 15 minutes at 0-10 °C.

To determine the percentage of total lipids, three parallels á 2 mL of the chloroform phase were transferred to pre-weighed tubes. The tubes were placed in a heat block at 60 °C and flushed with nitrogen gas for evaporation of the chloroform. After evaporation, the tubes were capped and placed in a desiccator overnight for cooling, before being weighed anew. The percentage of total lipids in the samples were calculated using equation 3.

$$\%\ total\ lipids\ = \frac{a \times b \times 100}{c \times v} \qquad \textit{Equation 3}$$

Where: a = oil after evaporation (g); b = added chloroform (mL); c = evaporated chloroform phase (mL); v = sample used for extraction (g)

The lipid content of the freeze-dried gelatine was to be determined using a micro version of the modified Bligh & Dyer procedure:

Approximately 30 mg of sample was weighed out in small centrifugation bottles and placed on ice. 0.8 mL distilled water, 2 mL methanol and 1.0 mL chloroform were added to the centrifugation bottles and the sample was homogenised using an ULTRA-TURRAX for one minute. Then, 1.0 mL chloroform was added, and the solution was homogenised for

20 seconds. Lastly, 1 mL distilled water was added, and the solution was homogenised for another 20 seconds. The tubes were then centrifuged at 5000 G for 10 minutes at 0-10 °C. After the centrifugation step, the chloroform phase is transferred to a new tube held on ice, before transferring exactly 0.5 or 1.0 mL of the chloroform phase to a pre-weighed tube. The last few steps follow the normal procedure described above, and the lipid content is calculated using the same equation.

5.4.4. PROTEIN DETERMINATIONS

The protein concentration in the pre-treatment solutions and freeze-dried gelatine was determined by the method of Lowry, Rosebrough, Farr, & Randall (1951). Bovine serum albumin (BSA; Sigma Aldrich, Oslo, Norway) was used as a standard with a measuring range of 12.5-300 µg/mL, and samples were diluted to fall within this range. Three replicates were made for each sample.

To tubes containing either 0.5 mL sample, distilled water (blank) or standard, 2.5 mL alkaline copper reagent was added, followed by immediate mixing and incubation at room temperature for 10 minutes. Then, 0.25 mL Folin-Ciocalteu reagent was added and mixed in, before the samples were incubated at room temperature for 30 minutes. The sample absorbance was determined at 750 nm using a spectrophotometer (Ultrospec 2000 UV/Visible spectrophotometer, Pharmacia Biotech, Uppsala, Sweden) using the blank sample as a reference.

A standard curve was plotted based on the absorbance of BSA at the given concentrations, and the determined slope and intercept was used to calculate the protein concentration of the samples.

5.4.5. AMINO ACID COMPOSITION

The amino acid composition of the freeze-dried gelatines was analysed using reversed-phase Ultra High-Performance Liquid Chromatography (RP-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), and pre-column derivatization using the o-phtalaldehyde (OPA) method.

Samples from each batch of gelatine were hydrolysed in triplicates in accordance with the method described by Blackburn (1968). Approximately 50 mg of freeze-dried gelatine was completely hydrolysed with 6 M HCl for 22 hours at 105 °C, pH adjusted to around 7.0, and filtered using suction (Whatman glass microfibre filter GF/C, 1.2 µm). Filtrates were transferred to 10 mL volumetric flasks and the volume was filled up with doubly distilled water. Samples intended for HPLC analysis was further diluted to 1:500 using doubly

distilled water and filtered through a syringe filter (0.2 µm, Whatman, F30/0.2 CA-S) before being transferred to HPLC sample glasses.

The RP-HPLC procedure was performed by Siri Stavrum at the Department of Biotechnology and Food Science (NTNU). It should be noted that this method cannot detect proline, hydroxyproline, hydroxylysine and cysteine, nor can it distinguish between glycine and arginine. Methods for determination of hydroxyproline and cysteine content are described in Chapter 5.4.6 and 5.4.7, respectively.

During the acid hydrolysis process, acid-labile amino acids such as serine and threonine tend to be destroyed before measurement can be made (Blackburn, 1968, p. 11; Darragh, Garrick, Moughan, & Hendriks, 1996). Buňka, Kříž, Veličková, Buňková, & Kráčmar, (2009) studied the effect of acid hydrolysis time on amino acid determination in casein and stated that approximately 14 and 3 % of serine and threonine, respectively, was destroyed after 24 hours hydrolysis. Hence, the loss rate for these two amino acids was corrected for by using correction factors of 1.14 (serine) and 1.03 (threonine).

5.4.6. HYDROXYPROLINE CONTENT

The imino acid hydroxyproline is almost exclusively found in collagen and can therefore be used to determine the amount of gelatine (or collagen) in a sample using a species-specific conversion factor (Boran & Regenstein, 2010, p. 123). The amount of hydroxyproline in a sample is used to estimate its purity, i.e. how much of the sample is actually gelatine.

Hydroxyproline content in hydrolysed gelatine samples was determined according to the method described by Leach (1960), which is a modification of a method by Neuman & Logan (1950). L-hydroxyproline (Sigma Aldrich, Oslo, Norway) was used as a standard with a measuring range of 5-15 µg/mL, and samples were diluted to fall within this range. Three replicates were made for each hydrolysed sample. The method was performed using specific conditions for fish samples.

To test tubes containing either 0.5 mL distilled water (blank), standard or sample, 0.5 mL 0.05 M $CuSO_4$ and 0.5 mL 2.5 M NaOH was added and mixed immediately. Test tubes were covered using marbles and placed in a water bath at 50 °C for 10 minutes. Then, 0.5 mL 6 % H_2O_2 was added and mixed in, before the tubes were covered again and placed in a water bath at 50 °C for another 10 minutes with occasional shaking. The samples were then cooled to around room temperature and placed in a fume hood. There, 2 mL 1.5 M H_2SO_4 and 1 mL 5 % p-dimethylaminobenzaldehyde in 1-propanol were added to the test tubes and mixed in immediately. Then, the test tubes were covered again and placed in a water bath at 70 °C for 16 minutes. After this, the samples were cooled to room temperature, shaken, and left at room temperature for two minutes. The optical density

(OD) were determined at 555 nm with a spectrophotometer using the blank sample as a reference.

A standard curve was plotted, and the determined slope and intercept was used to calculate the concentration of hydroxyproline. To convert the amount of hydroxyproline into collagen/gelatine content, a factor of 11.42 was used (Sato, Ohashi, Ohtsuki, & Kawabata, 1991).

5.4.7. THIOL CONTENT

Quantification of thiol content in the hydrolysed gelatine samples was performed to further assess the purity of the gelatine. Three replicates were made for each sample.

800 µL 8 M urea (pH = 7.4) and 100 µL Ellman's reagent (5,5'dithiobis(2-nitrobenzoate); DTNB; pH = 7.4) was added to Eppendorf tubes containing either 100 µL sample or distilled water (blank), before being mixed thoroughly. The tubes were then incubated at room temperature for 30 minutes and then centrifuged (Centrifuge 5415 R, Eppendorf AG, Hamburg, Germany) at room temperature for 3 minutes at 13 200 G. The sample absorbance was determined at 412 nm with a spectrophotometer using the blank as a reference.

The concentration of thiols in the samples were calculated using Equation 4.

$$C = \frac{A \times total\ volume\ in\ cuvette\ (mL)}{b \times E \times sample\ volume\ in\ cuvette\ (mL)} \qquad \textit{Equation 4}$$

Where: C = thiol concentration (M); A = absorbance at 412 nm; b = 1 (cuvette width or length of light path); E = extinction coefficient for TNB: 14 290 ($M^{-1}cm^{-1}$)

It was assumed that all thiols found in the samples were cysteine residues, and the molar concentration of thiols was converted to mg cysteine/g sample (dry weight).

5.4.8. PH AND WATER ACTIVITY

pH measurements were performed with a Mettler Toledo MP220 pH meter (Mettler Toledo instruments Co., Zurich, Switzerland). The pH was measured at each step throughout the whole production process, as well as in the final gelatine samples. The pH of the salmon skin gelatines was measured using 1 % solutions cooled to 45 °C (Johnston-Banks, 1990, p. 251) to get an indication of which type (A or B) of gelatine is obtained from the extraction process.

Water activity in the freeze-dried salmon skin gelatines were measured at 25 °C with Novasina LabMaster-aw (Novasina AG, Zurich, Switzerland). All six samples were measured twice.

5.4.9. SEC-MALLS

The weight average molecular weight (M_w), number average molecular weight (M_n), polydispersity index (PDI), and fractions of α- and β/γ-chains of the freeze-dried gelatine samples were determined using size-exclusion chromatography with multi-angle static laser light scattering (SEC-MALLS). The principle of size-exclusion chromatography is that when suitable samples are injected into the size-exclusion column, the porous packing material in the column will hinder the elution of the molecules based on their hydrodynamic volumes. The smaller molecules are slowed because they can penetrate the porous packing material in the column to a larger extent than larger molecules, making the larger molecules elute prior to the smaller ones. Multi-angle static laser light scattering measures the intensity of the light scattered by molecules and is directly proportional to their molar mass × concentration. (Eysturskarð, 2010)

The M_w gives the average molecular mass of the individual peptide fragments in the polymer sample (i.e. the weight fraction), while M_n gives the mole fraction of molecules in the polymer sample (i.e. the total molecular weight of the sample divided by the number of molecules in the sample) (Eysturskarð, 2010). M_w and M_n can be calculated using Equation 5 and 6, respectively.

$$M_w = \frac{\sum_i c_i M_i}{\sum_i c_i} = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} \qquad \textit{Equation 5}$$

$$M_n = \frac{\sum_i n_i M_i}{\sum_i n_i} = \frac{\sum_i c_i}{\sum_i c_i / M_i} \qquad \textit{Equation 6}$$

Where n_i is the number and c_i is the concentration of molecules (g/L) of molecules with the molecular weight M_i (g/mol)

The PDI is calculated as the ratio of the weight average molecular weight and the number average molecular weight (M_w/M_n). It is a measure of the polydispersity of the polymer mixture, which gives an indication of how widely the range of molecular weights are distributed. (Eysturskarð, 2010; Karim & Bhat, 2009; Schrieber & Gareis, 2007, p. 50)

Samples for SEC-MALLS analysis were prepared by dissolving freeze-dried gelatine in MILLI-Q water to 4 mg/mL and diluting them 1:1 (v/v) using eluting buffer (0.1 M Na_2SO_4, 0.02 M Na_2EDTA, pH 9). Two replicates were made for each gelatine, and the individual replicates were analysed twice.

Size-exclusion chromatography (SEC) with online multi-angle static laser light scattering (MALLS) were performed at 40 °C on an HPLC system consisting of a solvent reservoir, on-line degasser, column oven from Waters and Agilent 1260 infinity ii pump and automatic sample injector. The samples were separated on serially connected TSk gel pre-column and Pwxl 6000+ 5000 main columns from Tosoh bioscience. The column outlet was connected to a Dawn HELEOS-II multi-angle laser light scattering photometer (Wyatt, U.S.A.) ($\lambda 0$=663.8 nm) followed by Shodex RI-501 refractive index detector. The eluent was 0.1 M Na_2SO_4 with 0.02 M EDTA (pH=9.0) and the flow rate was 0.5 mL/min. Samples (2mg/mL) were filtered (pore size 0.45 µm) before injection and each sample were analysed twice with injection volume 50 µL. Pullulan (137 kDa) and BSA were included as standards. Dextran (11.2 kDa) was used for normalisation. Data were collected and processed (with dn/dc=0.190 mL/g for gelatine) using the Astra (v. 7.3.0) software (Wyatt, U.S.A.).

The SEC-MALLS procedure and initial data processing was performed by Olav Andreas Aarstad at the Department of Biotechnology and Food Science (NTNU).

5.4.10. STATISTICAL ANALYSIS

Data from the screening experiments and the main experiment were statistically analysed using IBM SPSS Statistics 25. Assumption of normality and equal variance was tested before analysis. Data that fit these assumptions were analysed using the appropriate analysis of variance procedures (ANOVA/MANOVA) to determine whether there was a significant difference between group means. If *p-values* < 0.05, a pairwise comparison (Tukey HSD post hoc test) was used to determine the differences between the means of each pair of treatments. Data that resulted in significant interaction terms were further analysed using simple main effects tests. Data that violated the assumption of normality and equal variance was analysed using the more robust Welch's ANOVA, with Dunnett's T3 post hoc test for pairwise comparisons. For comparisons between two groups, the independent samples t-test procedure was used.

6. RESULTS AND DISCUSSION

6.1. SCREENING

6.1.1. PROXIMATE COMPOSITION AND SWELLING

The average responses from the 2^5-factorial screening experiment is shown in Table 5. Five independent variables were investigated in an attempt to identify their effect on the content of dry matter, ash and lipids in the raw materials. The full factorial design along with the observed data can be found in Appendix A.

It should be kept in mind that the calculated average values from the screening experiment has quite high standard deviations. Most of these deviations are normal and expected, considering that the averages are calculated from data obtained from 16 runs with only one common variable and treatment level. E.g. data from all 16 treatment combinations using 0.1 M NaOH were used to calculate the average dry matter, ash, and lipid content for that level of OH concentration, and so on. Although it is likely that a large proportion of the deviations are a result of the nature of the screening experiment, some of it could also stem from inconsistencies during the removal of excess water after the treatments and the fact that the raw materials are not uniform.

Table 5: Proximate composition of salmon skins subjected to different treatment combinations. Values are listed as percent of wet tissue (mean ± SD, n=16). For dry matter, the % reduction is also included (indicated by ↓). The values were calculated as the average of all runs with one common variable. [a] denotes significant differences ($p < 0.05$) between treatment levels.

Independent variable	Level	Dry matter (% ± SD) (↓% reduction)	Ash (% ± SD)	Lipid content (% ± SD)
	Control	39.4	2.7	7.3 ± 0.1
OH concentration	0.1 M NaOH	24.10 ± 3.1 (↓38.8)	2.03 ± 0.8[a]	1.4 ± 0.7
	0.2 M NaOH	21.68 ± 4.7 (↓45.0)	1.34 ± 0.9[a]	1.2 ± 0.7
Pre-treatment duration	3×1 h	24.07 ± 2.8 (↓38.9)	1.98 ± 0.8	1.4 ± 0.8
	24 h	21.71 ± 4.9 (↓44.9)	1.39 ± 0.9	1.2 ± 0.7
Pre-treatment temperature	4 °C	25.11 ± 2.3 (↓36.3)[a]	1.91 ± 0.8	1.5 ± 0.7[a]
	22 °C	20.67 ± 4.4 (↓47.5)[a]	1.46 ± 0.9	1.0 ± 0.7[a]
Butanol concentration	10%	22.33 ± 4.8 (↓43.3)	1.62 ± 0.9	1.4 ± 0.7
	20%	23.45 ± 3.4 (↓40.5)	1.75 ± 0.9	1.2 ± 0.7
Defatting duration	3×1 h	23.03 ± 3.1 (↓41.5)	1.73 ± 0.9	1.6 ± 0.6[a]
	24 h	22.75 ± 5.0 (↓42.3)	1.64 ± 1.0	1.0 ± 0.7[a]

For dry matter, only the pre-treatment temperature variable resulted in a significant difference *($p = 0.002$)* between the two levels, with an average value of 25.11 ± 2.3 % dry matter in the samples treated at 4 °C and 20.67 ± 4.4 % in the samples treated at room temperature (22 °C). This difference might be attributed to the relatively high swelling exhibited when pre-treated at room temperature (see Table 6), i.e. higher moisture content,

or it could be due to a more efficient removal of non-collagenous proteins and other impurities at higher temperatures. It could also indicate a loss of collagenous proteins during pre-treatment due to disruption of the cross-links in the collagen fibrils. Zhang & Regenstein (2017) found that higher pre-treatment temperatures and higher H^+ concentrations may destroy the crosslinks between the collagen molecules, resulting in a more destabilised fibril structure that is more easily dissolved in the pre-treatment solution. Zhou & Regenstein (2004) reported that pre-treating Alaska pollock skins at room temperature led to a lower extraction yield due to a high loss of gelatine during the pre-treatment.

As reported by Dave et al. (2019), collagen amounts to 27.45 ± 1.38 % of the dry matter in Atlantic salmon skin but they have used a different factor (conversion factor = 8) when converting the hydroxyproline content to collagen content. Recalculating their results using the factor 11.42 given by Sato et al. (1991) gives an estimated collagen content of around 39 % of the total dry matter. Which means that in an optimal setting where everything except the collagen is removed during the pre-treatment, the maximum reduction in dry matter would be no more than around 60 %. The highest reduction (47.5 %) was seen in the samples pre-treated at 22 °C but based on the previously mentioned studies it is likely that a good portion of this reduction is a result of loss of collagen (Zhang & Regenstein, 2017; Zhou & Regenstein, 2004). Considering that the gelatine ended up being extracted at 22 °C with good efficiency gives substantial credence to this assumption.

Table 6: Swelling of skins subjected to various combinations of pre-treatments. [a] denotes significant differences ($p < .05$) between treatment levels. Swelling was calculated as % increase on a wet weight basis.

Independent variable	Level	Swelling (% mean ± SD wet weight)
OH concentration	0.1 M NaOH	50 ± 10
	0.2 M NaOH	51 ± 23
Treatment duration	3×1 h	56 ± 23
	24 h	45 ± 5
Temperature	4 °C	39 ± 8[a]
	22 °C	62 ± 16[a]

The OH concentration had a significant effect ($p = 0.026$) on the removal of inorganic substances, with 2.0 ± 0.8 % ash remaining in the samples treated with 0.1 M NaOH and 1.3 ± 0.9 % ash remaining in the samples treated with 0.2 M NaOH. On the other hand, both averages are lower than the recommended 2.6 % (Jones, 1977). However, if the standard deviation is included, the average for 0.1 M NaOH (2.0 ± 0.8 %) exceeds it somewhat. When seen in conjunction with the OH concentrations effect on the dry matter, the lower ash content of samples treated with 0.2 M NaOH could be explained by increased solubilisation and subsequent loss of collagen. Although OH concentration had no significant effect ($p = 0.098$) on the dry matter content at the chosen significance level ($\alpha = 0.05$), 0.2 M NaOH still yielded one of the lowest averages with 21.7 ± 4.7 % dry matter remaining in the samples. This corresponds to a 45 % reduction in dry matter, which is comparable to the reduction seen when pre-treated at 22 °C. As mentioned, Zhang &

Regenstein (2017) reported that increased pre-treatment temperatures and H^+ concentrations lead to a higher likelihood of collagen loss during the pre-treatment. The OH^- concentration has a similar effect on the swelling and solubilisation of collagen as H^+ concentration (just on the opposite sides of the pH spectra) (Ranganayaki et al., 1982). This makes it likely that the decrease in dry matter for 0.2 M NaOH is also a result of collagen loss during the pre-treatment. Therefore, to be on the safe side with regards to yield, 0.1 M NaOH was chosen for the pre-treatment in the main experiment.

Both pre-treatment temperature and defatting duration had significant effects (with $p = 0.045$ and $p = 0.008$, respectively) on the lipid removal efficiency. Samples pre-treated at 4 °C had an average of 1.5 ± 0.7 % lipids remaining after treatment, while samples pre-treated at 22 °C had an average of 1.0 ± 0.7 % lipids. As the defatting was performed at room temperature in all runs, the difference in lipid content of the samples pre-treated at 4 and 22 °C could be a result of an increased saponification effect by the NaOH at higher temperatures. Samples defatted for 3 × 1 hours had an average of 1.6 ± 0.6 % lipids remaining after treatment, while samples defatted for 24 hours had an average of 1.0 ± 0.7 %. Even though both treatments were performed at 22 °C, the making of and exchange of solutions every hour during the shorter treatment resulted in a lower average temperature of the system. For the short treatment, the solution temperature started at around 10 °C (tap water temperature) but due to the short treatment intervals, the solution never reached room temperature. The solution temperature for the long treatment also started at around 10 °C but reached room temperature before the end of the treatment. Thus, the difference between the defatting duration levels might be explained more by the temperature differences than by the differences in treatment durations.

6.1.2. ADDITIONAL SCREENING FOR DEFATTING

The lipid content of samples treated at 4 °C with different concentrations of butanol for various amounts of time is shown in Table 7. The lipid content of samples treated with 10 % butanol were significantly lower ($p < 0.05$) than the samples treated with 20 % butanol at every duration, except in the 2-hour treatment. For 10 % butanol, the defatting efficiency was significantly better ($p < 0.05$) when treated for 3×2 hours than both the 2- and 24-hour treatments. And although it was not significantly different from the 2×2-hour treatment it still resulted in the overall lowest lipid content (1.9 ± 0 %), which corresponds to a 74 % reduction in lipids.

Table 7: Lipid content of skins treated with different concentrations of butanol (10 and 20 %) for various durations. Values are listed as average lipid content ± SD (%) on a wet weight basis, values in brackets give the % reduction in lipids compared to non-defatted skins. Values in the same column that share letters are significantly different ($p < 0.05$) from each other, while values in the same row that share numbers are significantly different ($p < 0.05$) from each other.

	Lipid content (% mean ± SD wet weight)	
Defatting duration (h)	**10 % butanol**	**20 % butanol**
2	3.8 ± 0.28^{a} (↓48 %)	4.4 ± 0.24^{ab} (↓40 %)
2×2	2.6 ± 0.55^{b1} (↓64 %)	5.0 ± 0.16^{cd1} (↓32 %)
3×2	1.9 ± 0^{ac2} (↓74 %)	6.6 ± 0.47^{ac2} (↓10 %)
24	4.5 ± 0.28^{bc3} (↓38 %)	7.3 ± 0.49^{bd3} (↓0 %)

For samples treated with 20 % butanol, the lipid content increased with increasing treatment time, from 4.4 ± 0.24 % after 2 hours treatment to 7.3 ± 0.49 % after 24 hours. The samples treated with 20 % butanol for 24 hours thus had a lipid content comparable with non-defatted skins (Table 5). No reasonable explanation for the negative correlation between solvent concentration and lipid removal efficiency could be found. However, Saeleaw, Benjakul, & O'Brien (2016a) reported similar results when investigating the effect of solvent type (ethanol and isopropanol) and concentration (30, 50, and 70 %) on the removal of phospholipids from seabass skin. They found that the efficacy of both ethanol and isopropanol decreased as the concentrations increased but gave no theories about as to why. Extended defatting (24 hours) using 10 % butanol also resulted in lower efficiency, although not as drastic as for 20 % butanol, with a final lipid content of 4.5 ± 0.28 %. It is uncertain why this is the case, but it might be due to the development of a solubility equilibrium after a certain amount of time. Or, since there was no constant agitation involved in the defatting, the better part of the butanol rapidly separated from the water phase and rose to the top of the solution. The raw material, on the other hand, remained in the lower half of the water phase. So, it could be explained by the fact that the raw material only was in direct contact with the butanol phase for a miniscule amount of time. Further investigations using a water bath with an integrated orbital shaker could aid in the understanding of this phenomenon.

6.1.3. SCREENING SUMMARY

Based on literature and data obtained from the screening experiments, it was decided to use the following conditions for the pre-treatment and defatting steps in the main experiment (Table 8):

Table 8: Determined pre-treatment conditions based on both the results from the screening experiments and relevant literature.

OH concentration	0.1 M
Pre-treatment duration	24 h
Pre-treatment temperature	4 °C
Butanol concentration	10 %
Defatting duration	3 × 2 h
Defatting temperature	4 °C

Although pre-treatments using 0.2 M NaOH resulted in significantly lower contents of inorganic substances, 0.1 M NaOH was deemed to be the safer choice of pre-treatment to avoid harsher conditions that may have a negative effect on both the extraction yield and the properties of the gelatine. Since the pre-treatment duration had no significant effects on any of the dependent variables, it was decided to follow the procedure of Eysturskarð et al (2009) and pre-treat the raw material for 24 hours. The significant reduction in dry matter when performing pre-treatments at room temperature was likely a result of loss of collagen in the pre-treatment solution, and it was decided to perform both pre-treatment and defatting at 4 °C to avoid the potential thermal denaturation and subsequent loss of collagen in the pre-treatment and defatting solutions. The results clearly showed that the lower concentration (10 %) of butanol was more efficient in removing lipids from the raw material, and that treatment durations longer than 3 × 2 hours had a negative effect on the lipid removal efficiency. Thus, the defatting during the main experiment was to be performed using 10 % butanol for 3 × 2 hours.

6.2. GELATINE

Some of the parameters in the gelatine extraction procedure deviate from the procedure described in Chapter 5.3. These deviations are indicated in the summary of the gelatine extraction process (pink cells in Table 9). Originally, all batches should have been pre-treated for 24 hours but due to restrictions on laboratory use during the Covid-19 pandemic, the pre-treatment durations of the individual batches are not identical. The extraction time for G6 also got slightly prolonged due to force majeure.

Table 9: Summary of the gelatine extraction process. The solid/solvent ratio was 1:10 for all processes. The pink cells indicate parameters that deviated from the procedure described in Chapter 5.3.

Batch	Pre-treatment			Defatting			Extraction		
	[NaOH]	Time (h)	Temp.	[Butanol]	Time (h)	Temp.	[Acetic acid]	Time (h)	Temp.
G1	0.1 M	21	4 °C	10 %	3×2	4 °C	0.1 M	6	22 °C
G2	0.1 M	17	4 °C	10 %	3×2	4 °C	0.1 M	6	22 °C
G3	0.1 M	19	4 °C	10 %	3×2	4 °C	0.1 M	12	22 °C
G4	0.1 M	19	4 °C	10 %	3×2	4 °C	0.1 M	12	22 °C
G5	0.1 M	24	4 °C	10 %	3×2	4 °C	0.1 M	18	22 °C
G6	0.1 M	21	4 °C	10 %	3×2	4 °C	0.1 M	18.5	22 °C

6.2.1. PROXIMATE COMPOSITION OF FREEZE-DRIED GELATINE

The proximate compositions of the salmon skin gelatines are given in Table 10. All six gelatine batches displayed very low moisture contents (0.7–2.2 %). According to the Gelatin Manufacturers Institute of America (GMIA, 2019), the moisture content of typical commercial gelatines is 10.5 ± 1.5 %, which means that the freeze-drying procedure used in this project has been more than adequate.

Table 10: Proximate composition of freeze-dried gelatine. Values are listed as percent ± SD (wet weight). The protein content in the gelatines was determined using the Lowry assay. Lipid content could not be determined. For dry matter and ash: n = 2, for protein: n = 3.

Extraction time	Batch	Moisture (mean ± SD %)	Ash (mean ± SD % ww)	Protein (mean ± SD % ww)	Lipid
6 hours	G1	2.2 ± 0.5	1.9 ± 0.2	97.1 ± 3.3	ND
	G2	1.2 ± 0.1	0.4 ± 0.3	98.4 ± 5.2	ND
12 hours	G3	0.7 ± 0.8	1.0 ± 0.2	101.2 ± 9.9	ND
	G4	0.7 ± 0.1	0.8 ± 0.2	98.5 ± 7.7	ND
18 hours	G5	0.8 ± 0.3	2.1 ± 0.4	102.0 ± 4.0	ND
	G6	1.4 ± 0.2	0.7 ± 0.3	101.7 ± 1.1	ND

The ash contents in the salmon skin gelatines were highly variable, ranging from 0.4 ± 0.3 to 2.1 ± 0.4 % on a wet weight basis and was seemingly independent on extraction time. Since all batches were pre-treated using the same conditions, the difference in ash contents is likely a result of initial differences in the raw materials used for the extractions. Although the ash contents were variable, all values were lower than the recommended maximum of 2.6 %, as stated by Jones (1977). However, the ash content in some of the gelatines are tangent to (G1) or exceed (G5) the recommendation of less than 2 % ash, as stated by Schrieber & Gareis (2007). This means that their applications would be limited, especially in the pharmaceutical industries where they typically require less than 1 % (Schrieber & Gareis, 2007, p. 83). However, neither the raw material nor the gelatine was subjected to demineralisation in this study. The addition of a demineralisation step either before or after extraction might be favourable even when extracting gelatine from raw materials with a relatively low initial ash content.

The protein content of the salmon skin gelatines were determined using the method of Lowry et al. (1951) with BSA as the reference protein, as described in Chapter 5.4.4. It must be mentioned that there are some limitations to using this method for actual quantification of protein content in gelatines. In a study by Zhou & Regenstein (2006), they found that the colour response of gelatine tends to differ from the colour response of BSA at the same concentrations. They also found that the colour responses of gelatines from different sources varied significantly, and the difference in hydroxyproline content was thought to be the main reason for this variation. These findings mean that the protein concentration of the salmon skin gelatines cannot be quantified with certainty using the Lowry assay with BSA as the reference protein. However, the hydroxyproline contents of the salmon skin gelatines in this study should be similar enough to give comparable responses.

The protein content in the salmon skin gelatines ranged from 97.1 ± 3.3 to 102.0 ± 4.0 % on a wet weight basis, as determined by the Lowry assay. The samples for the assay was in this case prepared by diluting 1 % (10 mg/mL) aqueous gelatine solutions to 250 µg gelatine (wet weight) per mL. The average colour response of the gelatine samples corresponded to 242 - 255 µg protein per mL sample, which gives an indication that the difference in the colour responses of these gelatines, compared to BSA, might not be as significant as in the study by Zhou & Regenstein (2006). In some samples, the protein content was found to be above 100 %, which is likely due to errors during the sample preparations or the abovementioned uncertainties in the assay itself. Nonetheless, for the remaining discussions it will be presumed that the protein determinations are relatively correct and that the gelatines consist of nearly pure protein.

A problem arose when attempting to determine the lipid content in the gelatines, as the gelatine samples gelled during the centrifugation step of the Bligh & Dyer procedure (see Chapter 5.4.3 for method description). When inspecting the tubes after the centrifugation was complete, it seemed that around half of the fluids had been trapped in an opaque gel network. The remaining liquid phases was transferred to new tubes for visual inspection, but no separate phases could be discerned by eye. In one tube, the gel looked less opaque and when perturbed the gel ruptured causing fluids to leak out. This fluid was transferred to the tube containing the previously removed liquid phase and immediately sank to the bottom, indicating that the fluid trapped in the gel network was the chloroform phase. When perturbing the more opaque gels in the remaining tubes, nothing happened, so it seemed that the gelatine in one tube had only encapsulated the chloroform phase, while the others had formed more uniform gels that could retain the fluids. Considering that it is the chloroform phase that is used to determine the lipid content, the method proved to be unfeasible in this situation and needs further modifications to be applicable for gelatine. Since cold-water fish gelatines gel around 8-10 °C (Karim & Bhat, 2009; Milovanovic & Hayes, 2018), it might be enough to elevate the temperature slightly during the centrifugation step to prevent gelling, although this may lead to increased chloroform

evaporation. Alternatively, the lipid content can be determined using a different method, like the Soxhlet method. Due to time restrictions, alternative methods for lipid determination was not tested and the lipid content in the salmon skin gelatines was not determined.

6.2.2. WATER ACTIVITY OF FREEZE-DRIED GELATINE

Water activity (a_w) is a measure of the effective concentration of water in a food material that can actually participate as a chemical agent in various biological and chemical processes, hence it can be used to predict the microbial and chemical stability of the material. In the a_w range of 0.2-0.5, the enzymatic activity and rate of chemical reactions (lipid oxidation, etc.) of food materials are generally low, and at $a_w < 0.6$ there is no microbial proliferation. (Damodaran, 2017, pp. 48–62)

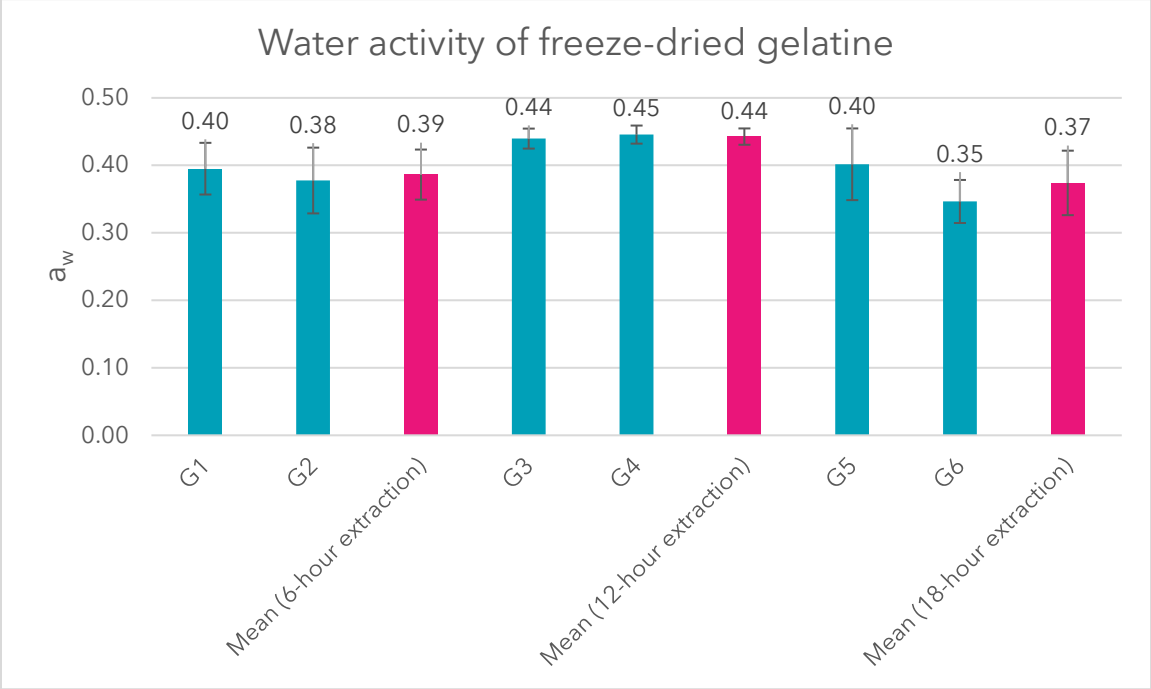


Figure 9: Water activity (a_w) of freeze-dried gelatines extracted for 6, 12 or 18 hours. Values are listed as means per batch (G1-G6, n = 2) and means as a function of extraction time (6-18 hours, n = 4).

In general, the water activity in the freeze-dried gelatine samples was low ($a_w < 0.5$), which gives a good indication that the gelatines are relatively stable and should have a good shelf-life. Figure 9 shows the average a_w-values of the individual gelatine batches (blue bars) and the average a_w-values as a function of extraction time (pink bars). It should be noted that the water activity was measured on an unusually hot and humid day, which is why there is much spread in the measurements. For G1-G2 and G5-G6 the a_w increased with 13-21 % from the first to the second measurement, most likely due to absorption of

moisture from the environment between the readings. G3 and G4 displayed more stability and only had an increase in a_w of around 5 % between the readings, which is probably because these samples had a higher a_w initially.

6.2.3. pH

The pH of a 1 % gelatine solution can give an indication of whether the obtained gelatine is type A or type B. According to GMIA (2019), type A gelatines (acid method) typically have a pH in the range of 3.8-5.5, while type B gelatines (alkali method) lie in the pH range of 5.0–7.5. The pH changes in the salmon skins during the gelatine extraction process and the pH of the liquid extract and in the 1 % gelatine solutions is shown in Table 11.

Table 11: Summary of pH changes in the raw material during the gelatine extraction process, in addition to the pH of the liquid extract and of the 1 % gelatine solutions. pH was only measured once per treatment step.

Batch	pH					
	Salmon skins				Gelatine	
	Untreated salmon skins	After pre-treatment	After washing and neutralisation	After defatting	Liquid extract	Gelatine solution (1 % w/v)
G1	7.1	13.2	7.2	7.1	4.0	5.0
G2	7.1	13.1	7.1	7.1	4.0	4.7
G3	7.1	13.1	7.5	7.4	3.9	4.7
G4	7.1	13.0	7.3	7.3	4.0	4.7
G5	7.1	12.8	7.4	7.3	4.0	5.1
G6	7.1	13.0	7.3	7.2	3.9	4.8

The pH determinations in the 1 % gelatine solutions did not give a good indication of whether the obtained gelatines were type A or type B. All the gelatines either lie in or slightly below the pH range where type A and type B overlap (pH 5.0-5.5). As the isoelectric points of the gelatines were not determined, nothing conclusive can be said about the type.

6.2.4. Purity (collagen content)

The purity of the gelatine extracts was calculated using data from the hydroxyproline determinations (see Chapter 5.4.6.). The results are presented as the percent average collagen content of the freeze-dried extracts as a function of extraction time (Table 12). The lowest purity was found in the samples extracted for eighteen hours, with 82.3 ± 1.4 % collagen. The samples extracted for six hours had a significantly higher ($p = 0.006$) collagen content (94.6 ± 3.4 %) than the 18-hour extraction samples but displayed more variance. The samples extracted for twelve hours showed not only more consistency in collagen content, but also yielded nearly pure collagen (99.7 ± 0.6 %).

*Table 12: Mean purity (collagen content) as a function of extraction time. The values are listed as mean collagen content ± SD (%) of dry gelatine extract. The collagen content was determined from hydrolysates of each extraction batch, with three replicates per hydrolysate, altogether 18 per extraction time. [a] denotes significant difference ($p < 0.05$) between the sample groups. *one hydrolysate lost during filtration.*

Extraction time	n	Mean collagen content ± SD (% dw)
6 hours	18	94.6 ± 3.4[a]
12 hours	15*	99.7 ± 0.6[a]
18 hours	18	82.3 ± 1.4[a]

The protein content of the freeze-dried extracts was determined using the Lowry assay described in Chapter 5.4.4, and was presumed[4] to be nearly 100 % (g protein/g dry sample) for all three extraction times (Table 13). For some samples, the protein content was found to be above 100 %, which is likely due to uncertainties in the sample preparation and/or the Lowry assay itself. Statistical analyses showed that there were no significant differences between the three groups in terms of protein content.

Table 13: Mean protein content as a function of extraction time. The values are listed as mean protein content ± SD (%) on a dry weight basis. The protein content was determined from three parallels of each extraction batch, altogether six per extraction time.

Extraction time	n	Mean protein content ± SD (% dry weight)
6 hours	6	99.4 ± 3.4
12 hours	6	100.5 ± 8.1
18 hours	6	102.9 ± 2.6

Considering that all three extraction times resulted in extracts that presumably consist of nearly pure protein, the significant difference in purity between the three groups must be a result of differential extraction of non-collagenous protein. Disregarding the lower purity of the 6-hour extraction samples (which display a much higher variance than the other two and is therefore less certain), there seems to be a cut-off point during the extraction process where the extraction medium stops taking up collagen and instead takes up other proteins. This might mean that all the available collagen in the raw material is extracted during the first twelve hours without saturating the extraction medium, so that the extraction of other acid-soluble proteins start to take place at some point after that. Or it might indicate that the non-collagenous proteins in the salmon skin have a higher thermal stability than the

[4] See Chapter 6.2.1. for discussions regarding the uncertainties associated with using BSA as a reference for protein determinations in gelatine.

collagen and, thus, require a more prolonged exposure to heat before they become soluble in the acidic extraction medium.

6.2.5. YIELD AND EXTRACTION EFFICIENCY

The average total extraction-, protein-, and gelatine yield as a function of extraction time is presented in Figure 10. In terms of total extraction yield, the 18-hour extractions produced a slightly higher amount (20.9 ± 2.0 g/100 g skin wet weight) than both the 6-hour extractions (19.9 ± 0.5 g/100 g skin wet weight) and the 12-hour extractions (20.5 ± 0.1 g/100 g skin wet weight). But the gelatine yield, on the other hand, was lower for the 18-hour extractions (17.2 ± 1.7 g) compared to the 6- and 12-hour extractions that yielded 18.8 ± 0.7 g and 20.4 ± 0.1 g per 100 g skin (wet weight), respectively. Compared to gelatine extraction from mammals, the yield is lower when extracted from fish skins, with yields between 6 and 19 g dry gelatine/g skin on a wet weight basis (Karim & Bhat, 2009). All three extraction times in this work resulted in gelatine yields that are either near the top of or above the range reported by Karim & Bhat (2009). This suggests that the extraction method used in this work is more than adequate in terms of gelatine yield. The low yield of fish gelatine compared to mammalian gelatine is thought to be due to the incomplete hydrolysis of collagen or loss of collagen during pre-treatment or washing (Alfaro et al., 2014; Karim & Bhat, 2009). All three extraction times resulted in extracts that presumably consisted of nearly 100 % protein (Chapter 6.2.4). Statistical analyses revealed no significant differences in any yields between the three groups, probably because of the high variance in the 18-hour extraction group. Further investigations with higher sample numbers is needed to determine if the seemingly lower gelatine yield (g) for the 18-hour extractions represents an actual effect.

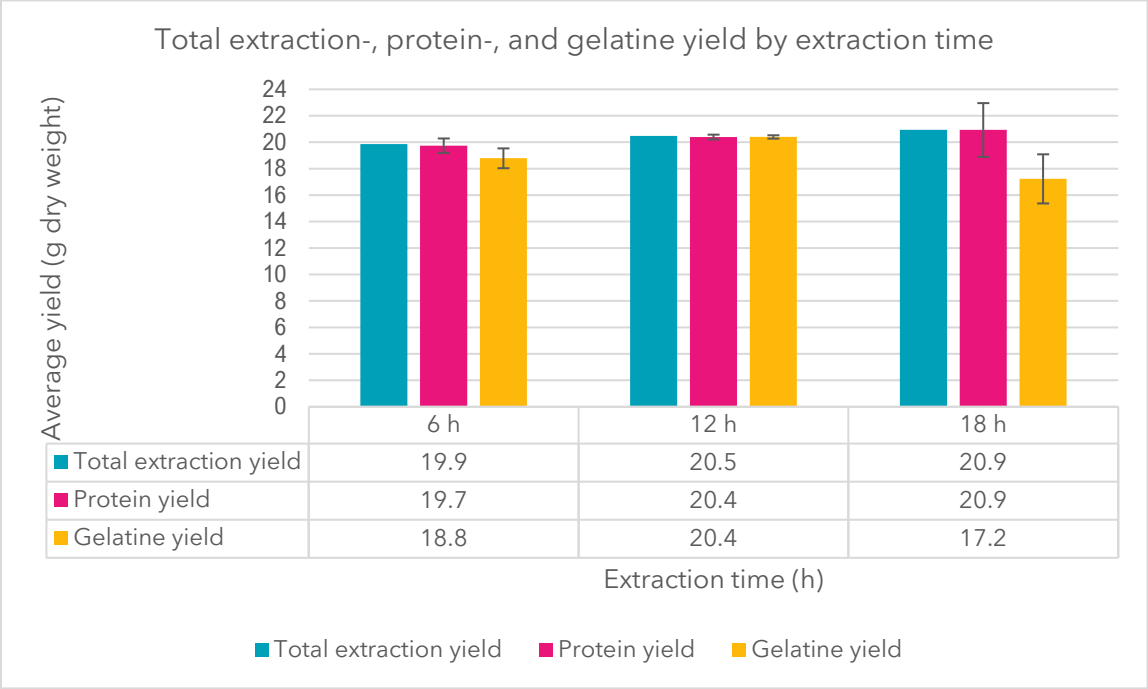
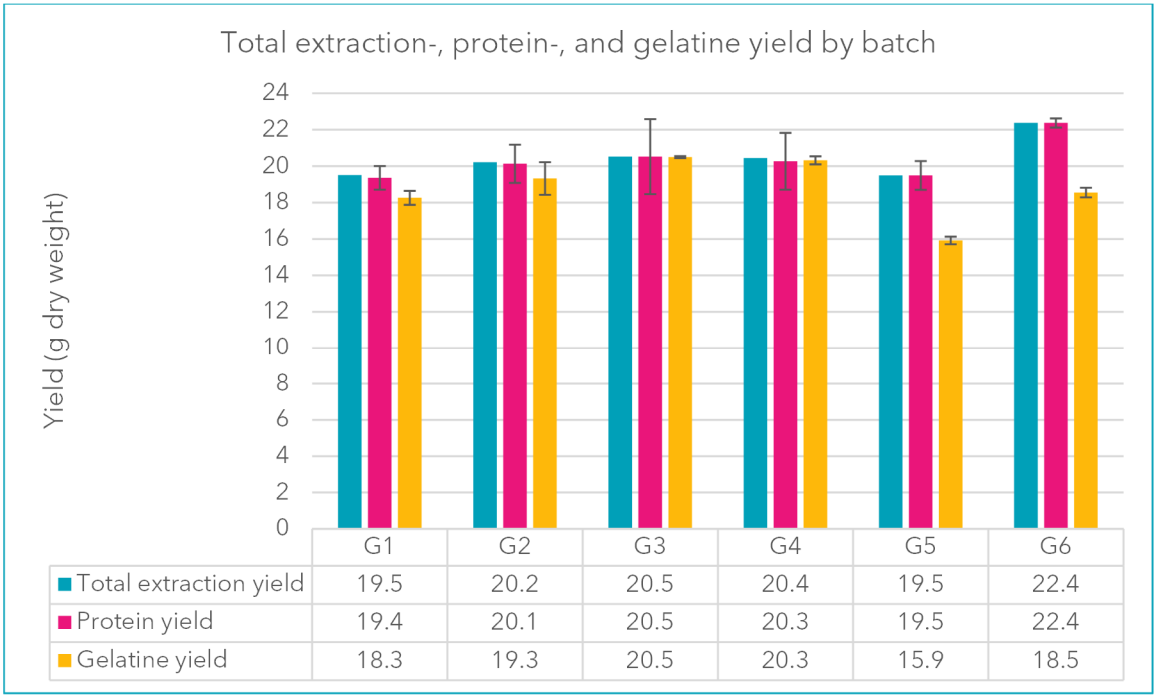


Figure 10: Total extraction-, protein-, and gelatine yield as a function of extraction time (n = 2). Yields are listed in g dry sample/100 g raw material (wet weight).

Although no statistically significant difference between the different extraction times was found, a clear trend can be seen when inspecting the same data for the individual gelatine batches (see Figure 11). The two batches extracted for six hours (G1 and G2) differ slightly in terms of overall yields but the proportions of protein and gelatine are nearly equal. G5 and G6 (18-hour extractions) follow the same trend, although G5 had a much lower overall yield than G6. G3 and G4 (12-hour extractions) are virtually identical, both in terms of overall yield and the proportions. The difference in yield for the two 18-hour extractions (G5 and G6) is a bit of a conundrum and cannot reasonably be explained by the 30 minute difference in extraction time alone (see Chapter 6.2). But it might be explained by a difference in moisture content of the raw material used for the extraction, i.e. that although 100 g raw material were used for all extractions the actual amount of dry matter could have been significantly different. According to Boran & Regenstein (2010), longer extraction times should result in higher extraction- and protein yields at the expense of purity, compared to shorter extraction times, so it is not unreasonable to assume that G6 is more representative for the 18-hour extractions than G5 is.



	G1	G2	G3	G4	G5	G6
Total extraction yield	19.5	20.2	20.5	20.4	19.5	22.4
Protein yield	19.4	20.1	20.5	20.3	19.5	22.4
Gelatine yield	18.3	19.3	20.5	20.3	15.9	18.5

Figure 11: Total extraction-, protein-. and gelatine yield by batch. Extraction times: G1 and G2 = 6 hours; G3 and G4 = 12 hours; G5 and G6 = 18 hours. Yields are listed in g (dry weight) per 100 g raw material (wet weight).

The extraction efficiency was calculated as gelatine yield compared to the amount of dry matter in the raw material, and is shown in Table 14. The dry matter of the untreated salmon skins (39.4 %) was determined only once in the beginning of the project and is therefore subject to some uncertainty. According to Dave et al. (2019), the dry matter of skin from Atlantic salmon (farmed in Newfoundland) is 43.53 ± 1.38 %. The value used for calculations of extraction efficiency is within three standard deviations from the value reported by Dave et al. (2019). Arnesen & Gildberg (2007), on the other hand, stated that Atlantic salmon skin contained 36 % dry matter. The value used for the calculation of extraction efficiency is thus approximately equal to the average of the values reported by Arnesen & Gildberg (2007) and Dave et al. (2019), and should be a reasonably realistic value to base the calculations on.

Table 14: Extraction efficiency by extraction time. Extraction efficiency was calculated as the percent gelatine yield compared to the amount of dry matter in the raw material. The last column lists the average extraction efficiency (% ± SD) as a function of extraction time, n = 2.

Extraction time	Batch	Extraction efficiency (gelatine yield as % of total dry matter in raw material)	Average extraction efficiency
6 hours	G1	46.3 %	47.7 ± 1.9 %
	G2	49.1 %	
12 hours	G3	52.0 %	51.8 ± 0.3 %
	G4	51.6 %	
18 hours	G5	40.4 %	43.7 ± 4.7 %
	G6	47.1 %	

Dave et al. (2019) also reported that around 39 %[5] of the dry matter in the skin of Atlantic salmon consisted of collagen, which in theory means that the maximum gelatine extraction efficiency should be no higher than around 39 %. In this project, the lowest extraction efficiency was 43.7 ± 4.7 % (18-hour extractions), while the highest extraction efficiency was 51.8 ± 0.3 % (12-hour extractions). The 6-hour extractions resulted in an extraction efficiency of 47.7 ± 1.9 %. No statistically significant difference between the extraction times was found, probably due to the low sample number (n = 2) and the high variance in some of the groups. But considering that all extraction times resulted in an extraction efficiency that exceeded the theoretical maximum of 39 %, it can at least be concluded that the used extraction method is satisfactorily efficient.

6.2.6. MASS BALANCE

The mass balances in Table 15 was determined by summing all matter removed during the whole extraction process (pink cells), adding the dry matter in the solids remaining after extraction (blue cells), and comparing the total to the predetermined dry matter in the initial raw material (grey cells). The mass balances are given as percent of the total available dry matter in the salmon skins. The cells with yellow shading show the total matter removed assuming that the reduction in lipids during the defatting in the main experiment was equal to the 74 % reduction determined in the screening experiment (Chapter 6.1.2.). The actual amount of lipids removed during defatting could not be determined (see explanation below). The last column shows the theoretical mass balance, including the presumed 74 % reduction in lipids.

The total dry matter removed during pre-treatments was calculated based on a single determination of dry matter in samples of the pre-treatment solutions and scaled up to

[5] Recalculated because the authors had used a lower conversion factor than the one used in this project – see Chapter 6.1.1.

represent the total volume. The 0.1 M NaOH's contribution to the dry matter was corrected for by subtracting 4 mg/mL from the total determined dry matter. The dry matter in the solids remaining after extraction were also scaled to represent the total, based on a single determination. Considering that there is no way of being certain that the samples used for these determinations are representative, the presented mass balance should be considered as an estimation with high uncertainty.

Table 15: Mass balance of all six salmon skin gelatine extractions. The grey cells show the predetermined available dry matter in the raw materials, the pink cells show the estimated amount of dry matter removed either during the pre-treatments or in the extraction step, and the yellow cells show the theoretical amount of dry matter removed presuming that 5.4 g lipids were removed during the defatting step. The second-to-last column shows the mass balance as the percent difference between the initial dry matter in the raw material and the estimated matter removed during the gelatine extraction process, including the dry matter in the solids remaining after extraction. The last column shows the theoretical mass balance presuming that 5.4 g lipids were removed during defatting.

Batch	Raw material	Pre-treatment	Extraction	Total		Remaining solids	Mass balance	
	Available dry matter (g/100 g)	Dry matter removed (g)	Total extraction yield (g dry weight)	Removed matter (g)	Theoretical removed matter (g)	Dry matter in remaining solids (g)	Difference (% of initial dry matter in raw material)	Theoretical difference (% of initial dry matter)
G1	39.4	2.7	19.5	22.2	27.6	9.1	-20.5	-6.8
G2	39.4	2.9	20.2	23.1	28.5	7.1	-23.4	-9.6
G3	39.4	3.0	20.5	23.5	28.9	7.5	-21.3	-7.5
G4	39.4	2.0	20.4	22.4	27.9	5.3	-29.7	-16.0
G5	39.4	3.9	19.5	23.4	28.8	9.0	-17.7	-4.0
G6	39.4	3.8	22.4	26.2	31.6	9.4	-9.7	+3.9

During the whole gelatine extraction procedure, the majority (56-66 %) of the available dry matter was removed from the raw materials, either during the pre-treatments or in the extraction step. The solids remaining after extraction made up 13-24 % of the available dry matter, and the remaining 10-30 % dry matter is unaccounted for (Table 15).

A simple determination of lipid content in the defatting solutions was attempted using the dry matter method (Chapter 5.4.2) but proved to be unfeasible as the presence of 1-butanol meant that it had to be done in a heating block placed in a fume hood instead of a heating cabinet. The heating block was not able to evaporate the water/butanol solution even after six hours of constant heating, so the tubes were left for passive evaporation instead. After all the fluids had evaporated, the sample tubes were weighed to determine the amount of dry matter, but the tubes weighed the same as when empty. This indicates that the samples taken during the defatting procedure consisted of mostly water. Since the lipid content of the salmon skin gelatines could not be determined either (see Chapter 6.2.1), the lipid contribution to the mass balance is unaccounted for.

A theoretical mass balance including the lipid contribution is shown in Table 15 nonetheless. In this scenario it is assumed that the lipid removal efficiency during the main experiments equalled the lipid removal efficiency determined in the screening experiment (Chapter 6.1.2). This means that 5.4 g (or 74 %) of the lipids in the raw material is assumed removed during the defatting. Adding this value to the total removed matter (see yellow cells) reveals that 69-80 % of the available dry matter in the raw material was removed either during pre-treatments or in the extraction step. The theoretical mass balance then accounts for 84-104 % of the available dry matter.

6.2.7. AMINO ACID COMPOSITION

The amino acid composition of the salmon skin gelatines was determined using the RP-HPLC procedure described in Chapter 5.4.5, and the average composition as a function of extraction time is shown in Figure 12. It should be noted that the measurements of glycine/arginine and glutamic acid had particularly high variability. This variability was not only between the two gelatines that shared extraction time, but also between the individual replicates. For glutamic acid, the first replicate of every gelatine consistently showed a concentration that was nearly a tenfold of the other two replicates. However, the areas in the HPLC chromatogram were similar, indicating that something probably had gone awry at some point during the data processing. To alleviate some of the variability, the most deviant replicate for each gelatine was swapped with data from a previous HPLC analysis done on the same gelatines. Still, there is much variability in the measurements of certain amino acids (as indicated by the error bars in Figure 12), which makes the results highly uncertain. Therefore, the discussion regarding the amino acid compositions of the gelatines will be limited to brief comparisons between the groups. As a reminder, the

content of hydroxyproline and cysteine was determined using different methods (Chapter 5.4.6 and 5.4.7, respectively) and did not show the same spread as for the amino acids measured by the HPLC. The raw data for the individual gelatines is given in Appendix B, including the deviant replicates.

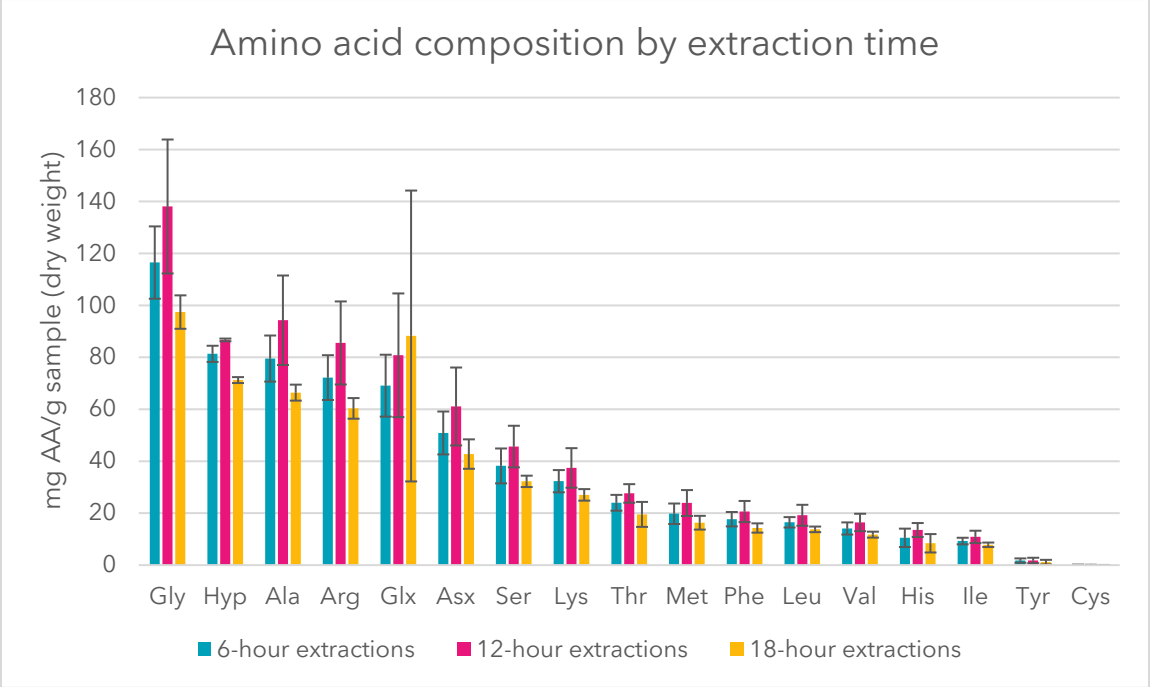


Figure 12: Amino acid composition as a function of extraction time. Values are listed as mean ± SD mg AA/g sample on a dry weight basis. n = 6 for 6- and 18-hour extractions, n = 5 for 12-hour extractions.

Proline is one of the major constituents in gelatine and amounts to 10-12 % of the total amino acid residues of gelatine, depending on source (Arnesen & Gildberg, 2007; Duan et al., 2009; Giraud-Guille et al., 2000; Ikoma et al., 2003; Jongjareonrak et al., 2010). Proline could not be measured in this study; hence the amino acid composition of the salmon skin gelatines is reported as mg amino acid per g sample (dry weight) instead of mole percent or amino acid residues per 1000 residues. Converting the measured molar concentrations to mole percent or residues per 1000 residues would give a skewed picture, considering the abovementioned lack of proline measurements. And, as previously mentioned, the HPLC procedure used in this study cannot separate between glycine and arginine. Considering that glycine normally represents over 30 % of the total number of amino acid residues in gelatine, while arginine only represents around 5 % (Karim & Bhat, 2009), it was assumed that glycine would represent roughly 80 % of the combined molar concentration determined by the HPLC. Hence, the molar mass quantified by the HPLC was split 80:20 (glycine:arginine) and their individual concentrations (in mg AA/g dry sample) was calculated based on this ratio.

Figure 12 show that most prevalent amino acid in all the salmon skin gelatines, was glycine (see also Table 16). The estimated average glycine content based on extraction time seems to follow the same trend as for hydroxyproline content, which lends some credence to the measurement. The gelatine samples that have higher purities, would naturally have a higher glycine content too, considering nearly every third amino acid residue in gelatine is glycine, as mentioned above (Karim & Bhat, 2009). The least prevalent amino acid was cysteine, which had a maximum concentration of 0.1 ± 0.1 mg/g sample on a dry weight basis. This is in agreement with the literature that states that the cysteine residues found in the telopeptide regions of collagen are mostly removed during pre-treatment due to cross-link cleavage (Johnston-Banks, 1990, pp. 237–238). Despite the uncertainties in the measurements, the relationship between the three extraction groups is relatively constant and in line with the previous results. The 12-hour extractions consistently show the highest amount of amino acids, and the 18-hour extractions consistently show the lowest amount. With a notable exception for glutamic acid/glutamine (Glx), but this deviation from the trend is likely due to the uncertainties mentioned in the first paragraph in this chapter.

Table 16 shows the focused amino acid content (serine, glycine and hydroxyproline) along with the total amount of amino acids based on extraction times. The total amount of amino acids, as determined by RP-HPLC and hydroxyproline determinations, was surprisingly low with concentrations ranging between 579.1 ± 46 mg AA/g sample (dry weight) for the 18-hour extractions, and 764.4 ± 130.8 mg AA/g sample (dry weight) for the 12-hour extractions. Some of this can be explained by proline not being measured but there are still some discrepancies which cast some doubts about the actual protein content in the salmon skin gelatines. However, some inferences can be made in conjunction with the protein content as measured by the Lowry assay (Chapter 6.2.4). It is likely that the actual protein content of the gelatines lies somewhere between the concentrations measured by the two methods. I.e. the protein content of the gelatines extracted for six hours is between 65–99 % (dry weight), while the 12-hour extractions have a protein content between 76–100 % (dry weight) and the 18-hour extractions have protein contents between 58–100 % (dry weight).

*Table 16: Total amount of amino acids detected by HPLC analysis, and focused amino acid content (serine, glycine/arginine and hydroxyproline). Listed as the average ± SD (mg AA/ g sample on a dry weight basis) per extraction time (6-, 12- and 18 hours). *Serine was corrected for loss during hydrolysis using correction factor 1.14* (Buňka et al., 2009).

	Serine*	**Glycine**	**Hydroxyproline**	**Total AA**
	mg AA/g sample (dry weight)	mg AA/g sample (dry weight)	mg AA/g sample (dry weight)	mg AA/g sample (dry weight)
6-hour extraction	38.2 ± 6.7	116.5 ± 13.9	81.4 ± 3.1	654.1 ± 77.8
12-hour extraction	45.7 ± 8.0	138.1 ± 25.8	86.7 ± 0.6	764.0 ± 130.8
18-hour extraction	32.3 ± 2.2	97.4 ± 6.4	71.3 ± 1.1	579.1 ± 46.0

As mentioned in Chapter 2.1, Akita et al. (2020) found that serine content was negatively correlated with the triple-helix content in collagen, which again is directly related to the physical properties of gelatine. The serine content in the salmon skin gelatines is consistently lower than the hydroxyproline for all three extraction groups, which could indicate that the extracted gelatines may have decent structural properties. However, comparisons with the literature is made difficult by the fact that the amino acid composition is presented as ratios (i.e. amino acid residues per 1000 residues), which is not directly comparable with the measurement unit used in this work. However, compared to cod skin gelatine from a study by Gudmundsson & Hafsteinsson (1997) which yielded an average serine content of 64.3 ± 1.9 mg/g protein, the content of the salmon skin gelatines from this study is low (32.3 ± 2.2 – 45.7 ± 8.0 mg serine/g dry gelatine). Arnesen & Gildberg (2007) found that the physical properties of salmon skin gelatine were like those of cod skin gelatine extracted under the same conditions, except that the salmon skin gelatine had slightly higher gel strength and melting points. The authors suggested that the different gel strengths and melting points was due to differences in hydroxyproline and serine content. Compared to the serine content in tilapia gelatine (31.2 mg/g protein) as reported by Grossman & Bergman (1992), the salmon skin gelatine in this study has a slightly higher serine content. But, considering that tilapia is a warm-water fish, the slight difference is remarkable. This might be an indication that the physical properties of the salmon skin gelatines may be more like those of tilapia gelatine than those of cod gelatine. However, the difference in hydroxyproline between tilapia and salmon gelatine speaks to the contrary. According to Grossman & Bergman (1992), gelatine from tilapia contains 103.3 mg hydroxyproline/g protein, which is significantly more than the salmon skin gelatines in this study which contained between 71.3 ± 1.1 (18-hour extractions) and 86.7 ± 0.6 (12-hour extractions) mg hydroxyproline/g sample. Conversely, the hydroxyproline content in cod skin gelatine is slightly lower, compared to the salmon skin gelatines in this study, with 67.2 ± 2.1 mg hydroxyproline/g protein (Gudmundsson & Hafsteinsson, 1997). From this it can be assumed that the physical properties of the gelatines from this study likely lies somewhere in between those of cod and tilapia.

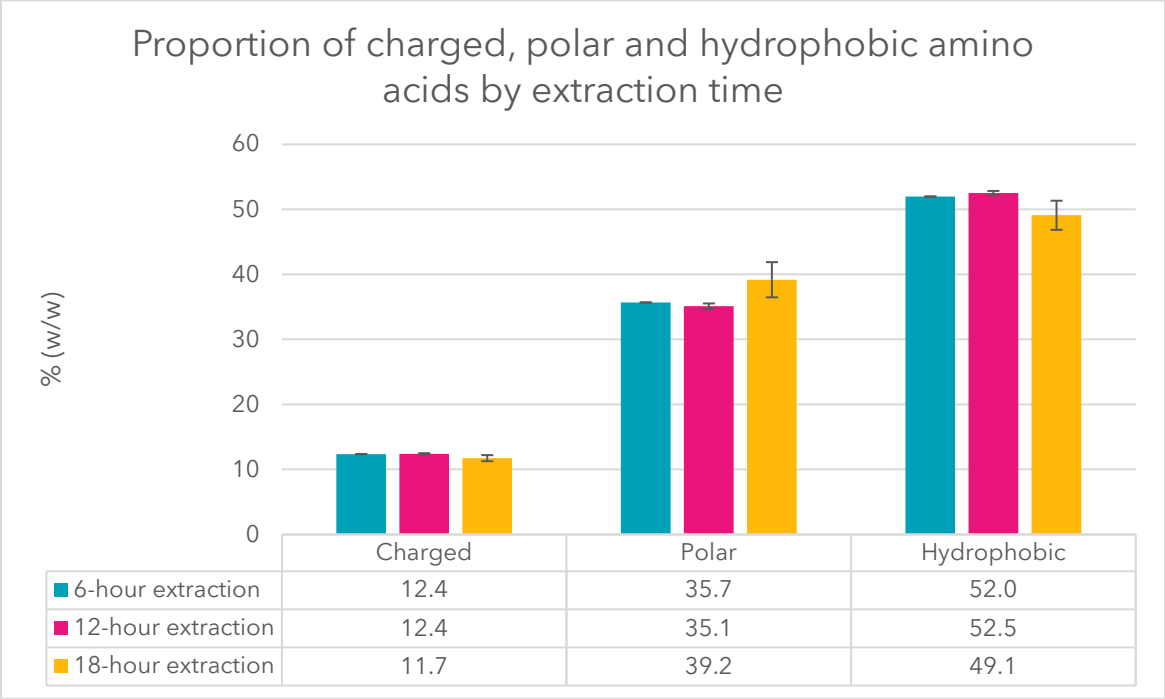


Figure 13: Proportion of charged, polar and hydrophobic amino acids by extraction time. Results are listed as the percent average ± SD, n = 6 for 6- and 18-hour extractions, n = 5 for 12-hour extractions.

The estimated proportion (% w/w) of charged, polar and hydrophobic amino acids is shown in Figure 13. Hydrophobic amino acids make up the majority of the amino acids for all gelatines, regardless of extraction time. This indicates that the salmon skin gelatines might be good stabilising agents for foams and emulsions, as these properties are associated with the total- and surface hydrophobicity of the molecule, respectively (Townsend & Nakai, 1983, pp. 589–590). It also indicates that the gelatines could result in films with lower water vapour permeability, compared to films made of mammalian or warm-water fish gelatine (Avena-Bustillos et al., 2006). However, further investigations are needed to determine their actual physical properties.

6.2.8. MOLECULAR WEIGHT DISTRIBUTION

All the salmon skin gelatine samples were analysed twice during the SEC-MALLS procedure (Chapter 5.4.9), with approximately 15 hours difference. The samples were kept at around 40 °C between the injections, and the difference in the elution profiles between the first (pink line) and second (blue line) injection showed evidence of hydrolysis in between measurements (Figure 14). Further data processing revealed a systematic decrease in M_w of around 10 kg/mol between the first and second injection. Because of this, the data for the second injections was not included in the results given below.

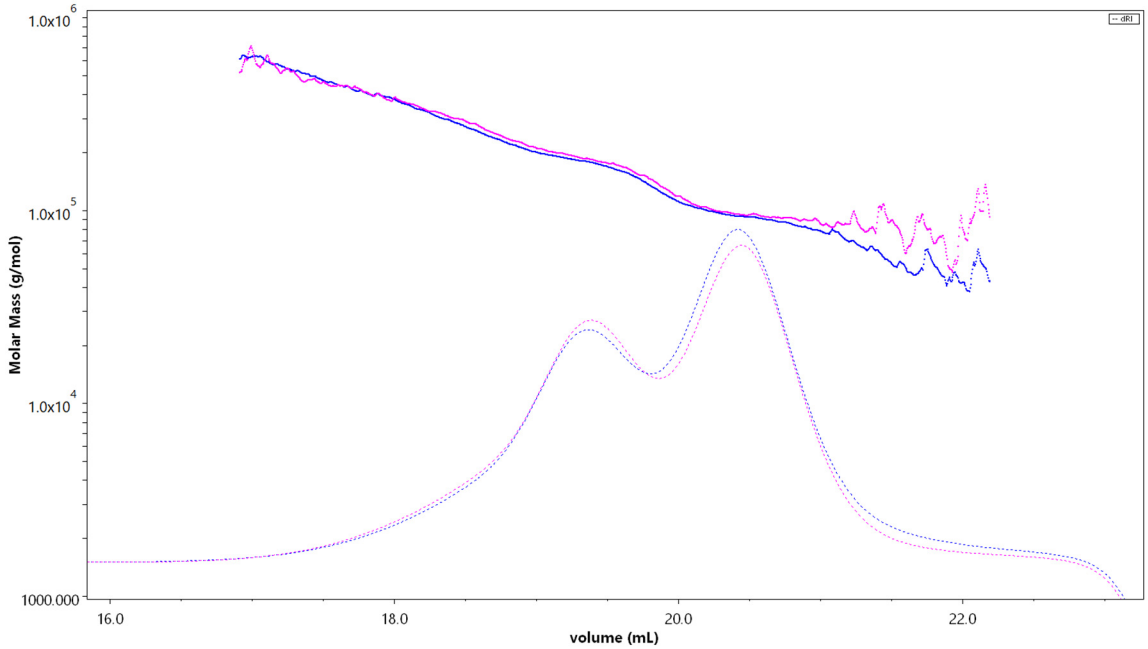


Figure 14: Hydrolysis of gelatine samples during SEC-MALLS. The figure shows the elution profiles of G1 injected in the column with around 15 hours' time difference. The pink line shows the first injection, the blue line shows the second.

As shown by the SEC-MALLS elution profiles in Figure 15, all the extracted salmon skin gelatines show comparable molecular weight distributions (MWDs). This indicates that six-hour extractions at 22 °C using 0.1 M acetic acid as the extraction medium is sufficient to disassemble the triple-helical arrangement of the salmon skin collagen and solubilise it in the form of gelatine. The MWD of all the gelatines exhibit two distinct peaks, where the rightmost peak can be assigned to the α-chains (M_w ~100 kg/mol) and the leftmost peak to the β-chains (M_w ~ 200 kg/mol). The β-peak has a shoulder to the left, which indicates the presence of molecules with higher molecular weights, such as γ-chains (M_w ~ 280 kg/mol).

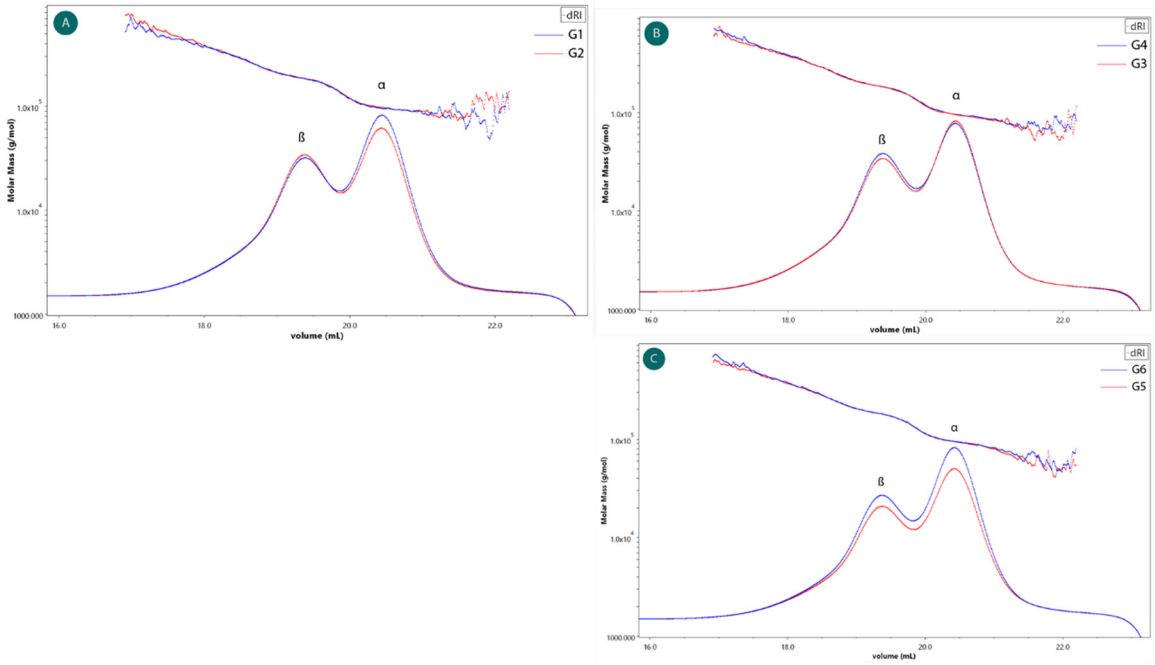


Figure 15: SEC-MALLS elution profiles of salmon skin gelatines grouped by extraction time. A: 6-hour extractions (G1 = blue line; G2 = red line); B: 12-hour extractions (G4 = blue line; G3 = red line); C: 18-hour extractions (G6 = blue line; G5 = red line). The left peak indicates β-chains (M_w ~200 kg/mol), the right peak indicates α-chains (M_w ~100 kg/mol).

The PDI indicates that the gelatines have relatively narrow MWDs, with PDIs in the range of 1.2 – 1.3 (Table 17). As mentioned in Chapter 2.1, the PDI of a monodisperse polymer is 1, while the PDI of gelatine is usually over 2 (Schrieber & Gareis, 2007, p. 50). According to Eysturskarð et al. (2009), the relatively low PDI may indicate some degree of selective hydrolysis of peptide bonds, which is consistent with previously published data showing that certain amino acids, such as glycine and serine, are more frequently found on the terminal ends of degraded gelatine chains (Courts, 1954). Although the MWD of the salmon skin gelatines is more narrow than usual for gelatine, it still gives an indication that the extracted gelatines are of type B, as type A gelatines normally display a much wider MWD (Schrieber & Gareis, 2007, pp. 48–50). The fraction of α-chains was found to be around 51-53.5 % (w/w) for all gelatines.

Table 17: Polydispersity index (PDI) and fraction of α- and β+γ-chains for salmon skin gelatines extracted for 6, 12, or 18 hours (n = 4).

Extraction time	PDI ± SD (M_w/M_n)	α-chains ± SD (% w/w)	β+γ-chains ± SD (% w/w)
6 hours	1.2 ± 0.00	51.0 ± 1.2	49.0 ± 1.2
12 hours	1.2 ± 0.01	51.5 ± 0.6	48.5 ± 0.6
18 hours	1.3 ± 0.01	53.5 ± 0.6	46.5 ± 0.6

The M_w and M_n of the salmon skin gelatines, as determined by SEC-MALLS (see Chapter 5.4.9.), is shown in Figure 16. The 6-hour extractions resulted in an average M_w of 161.2 ± 2.8 kg/mol, which was not significantly different ($p = 0.391$) from the average M_w for the 12-hour extractions (159.4 ± 1.3 kg/mol). However, the average M_w for the 18-hour extractions (152.0 ± 0.9 kg/mol) was significantly different ($p \leq 0.001$) from both the 6- and the 12-hour extractions. This could indicate that the 18-hour extractions resulted in increased hydrolysis of the gelatine chains, a theory which is somewhat supported by the minor decrease in M_w between the 6- and the 12-hour extractions as well. Or, if seen in conjunction with the lower purity of the 18-hour gelatines (Chapter 6.2.4.), the difference in M_w could be due to differential extraction of non-collagenous proteins with lower molecular weights. However, further investigations are needed to see if this decrease in M_w with increasing extraction times is an actual trend or just random.

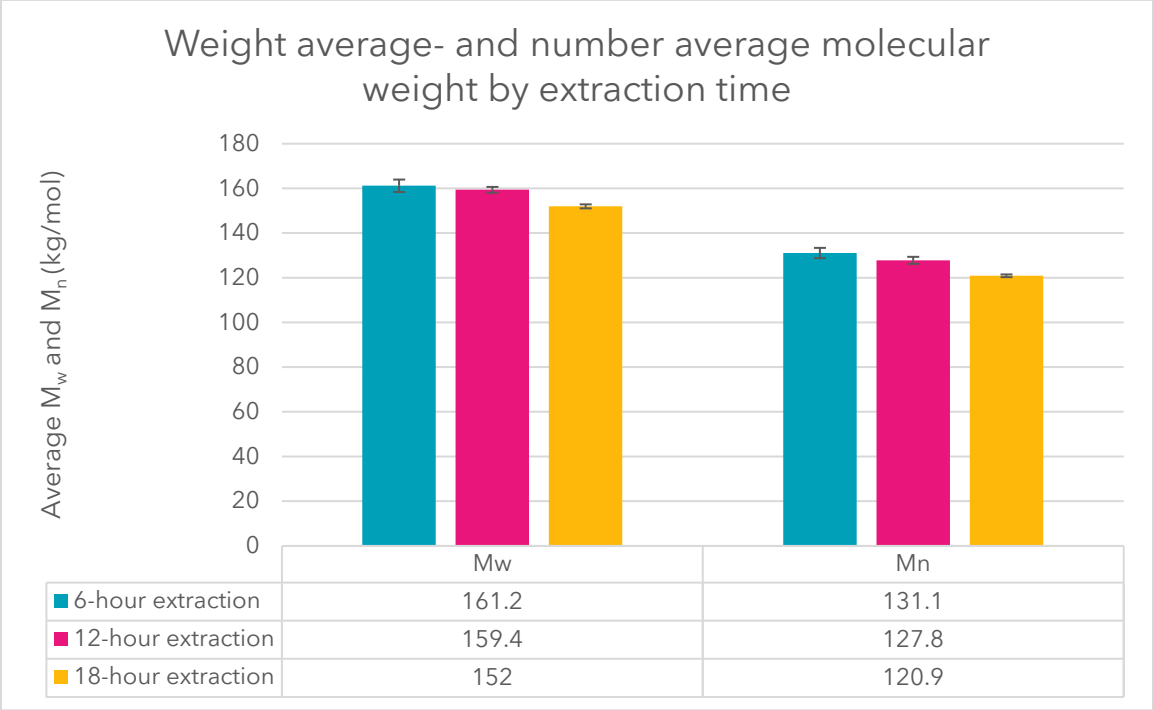


Figure 16: Weight average molecular weight (M_w) and number average molecular weight (M_n) measured by SEC-MALLS for salmon skin gelatine extracted for 6, 12, or 18 hours. The results are given as average M_w and M_n ± SD (kg/mol), n = 4.

Eysturskarð et al. (2009) conducted a study on the structural and mechanical properties of saithe skin gelatines as a function of extraction conditions. In that study, gelatine was prepared in a similar way as in this work, except for a longer defatting duration and an added bleaching step. The M_w of the saithe gelatines extracted at 22 °C was between 220 – 270 kg/mol, which is considerably higher than the M_w of 150-160 kg/mol for the gelatines extracted in this study. This suggests that the inter- and intramolecular crosslinks in salmon collagen might be less thermostable than in collagen from saithe. If this is a valid

suggestion, it might indicate that gelatine might be extracted from salmon skins at lower temperatures, which could lead to gelatine with higher M_w and better functional properties.

Arnesen & Gildberg (2007) characterised gelatines extracted from salmon skins at 56 °C and 65 °C. It should be noted that they used an acid extraction process given by Gudmundsson & Hafsteinsson (1997), which is different from the method used in this work. Although Arnesen & Gildberg (2007) did not report M_w, the results they gave might be used to infer the properties of the salmon skin gelatines extracted in this study. As evidenced by the increased hydrolysis at 40 °C during the SEC-MALLS analysis in this study, it is likely that the M_w of the salmon gelatines extracted at 56 and 65 °C by Arnesen & Gildberg (2007) was lower than the M_w of the gelatines in this present work. They found that the Bloom strength of salmon gelatines extracted at 56 °C (Bloom value ~ 110 g) was higher than the ones extracted at 65 °C (Bloom value ~ 75 g), which suggest that salmon gelatines extracted at 22 °C may result in even higher Bloom values. They also reported that the viscosity of salmon gelatines extracted at the lower temperature (56 °C) was higher than the ones extracted at 65 °C, which indicate that salmon gelatines extracted at 22 °C might display even higher viscosity. However, further investigations are needed to test the validity of these suggestions.

7. CONCLUSION

Extraction of gelatine from defatted skin of Atlantic salmon (*Salmo salar*) using the developed extraction procedure gave promising results in terms of yield and extraction efficiency, regardless of extraction time. The extraction efficiency, calculated as the percent ratio of gelatine yield compared to the amount of dry matter in the raw material, was higher than the theoretical maximum of 39 % for all three extraction times. However, the purity (collagen content) of the extracts indicated a relationship with extraction time, where extraction times longer than 12 hours resulted in extracts with lower purity. The proximate composition of the freeze-dried gelatines varied and had seemingly no correlation with the length of the extractions. The ash contents were near or close to the recommended maximum of 2 %, which showed that the addition of a demineralisation step either before or after extraction would be favourable to avoid further limitations in its applications. All the freeze-dried gelatine extracts displayed low residual moisture contents and the water activity was low enough to prevent microbial proliferation, which suggested that the gelatines were relatively stable and would have good shelf-lives.

The molecular weights of the salmon skin gelatines all showed similar, albeit narrow, distributions. However, the weight average molecular weight (M_w) of the gelatines indicated a negative correlation with extraction time. When seen in conjunction with the purity of the extracts, the lower M_w was suggested to be caused by differential extraction of non-collagenous proteins with low molecular weights.

Overall, this work demonstrated that gelatines with high purity can be extracted from salmon skin, and with adequate yield compared to skins from other fish species. However, further investigations are needed to determine the functional properties of the salmon skin gelatines.

8. SUGGESTIONS FOR FURTHER WORK

The screening experiment did not include extraction and characterisation of gelatine from the salmon skins subjected to different pre-treatment and defatting combinations, as it would have required more time and resources than what was realistic for this study. Conclusions were drawn based solely on the remaining dry matter, ash and lipid content in the samples, and it would be interesting to see if the theories proposed in this study were realistic. Future screening experiments should also seek to investigate the molecular weights of the proteins removed during the pre-treatment step to determine if it mainly removes non-collagenous proteins. Hydroxyproline determinations in proteins from the pre-treatment solutions could also be a possibility in this regard. Furthermore, although 1-butanol gave good results in terms of lipid reduction, it proved to be very difficult to remove the butanol odour from the raw materials after defatting. This makes the raw materials more complicated to handle considering that butanol vapours can cause irritation of the airways, among other things. Studies have also shown that defatting might be more efficient if mixed/binary solvents are used (Kudre & Benjakul, 2013; Sae-leaw & Benjakul, 2018). Therefore, defatting using membrane lipid removal ($CaCl_2$) in conjunction with milder and less hazardous solvents should be investigated.

Future work should also include rheological tests on salmon skin gelatine to determine its functional properties, especially in terms of the commercially important properties such as gel strength and viscosity. Additionally, the isoelectric points of the gelatines should be established, and the total- and surface hydrophobicity should be determined along with investigations into the film-forming, emulsifying and foaming properties of the gelatines. Additionally, future work should investigate the possibility of extracting gelatine from salmon skins at temperatures lower than 22 °C and see if that results in gelatine with higher M_w.

CITATIONS

Akita, M., Nishikawa, Y., Shigenobu, Y., Ambe, D., Morita, T., Morioka, K., & Adachi, K. (2020). Correlation of proline, hydroxyproline and serine content, denaturation temperature and circular dichroism analysis of type I collagen with the physiological temperature of marine teleosts. *Food Chemistry*, *329*(March), 126775. https://doi.org/10.1016/j.foodchem.2020.126775

Alberto, M., & Gabriela, M. (2012). Hydrodynamic Properties of Gelatin - Studies from Intrinsic Viscosity Measurements. In C. J. R. Verbeek (Ed.), *Products and Applications of Biopolymers*. Rijeka, Croatia: InTech. ISBN: 978-953-51-0226-7. https://doi.org/10.5772/34401

Alfaro, A. da T., Balbinot, E., Weber, C. I., Tonial, I. B., & Machado-Lunkes, A. (2014). Fish Gelatin: Characteristics, Functional Properties, Applications and Future Potentials. *Food Engineering Reviews*, *7*(1), 33–44. https://doi.org/10.1007/s12393-014-9096-5

Arnesen, J. A., & Gildberg, A. (2007). Extraction and characterisation of gelatine from Atlantic salmon (Salma salar) skin. *Bioresource Technology*, *98*(1), 53–57. https://doi.org/10.1016/j.biortech.2005.11.021

Avena-Bustillos, R. J., Chiou, B., Olsen, C. W., Bechtel, P. J., Olson, D. A., & Mchugh, T. H. (2011). Gelation, Oxygen Permeability, and Mechanical Properties of Mammalian and Fish Gelatin Films. *Journal of Food Science*, *76*(7), 519–524. https://doi.org/10.1111/j.1750-3841.2011.02312.x

Avena-Bustillos, R. J., Olsen, C. W., Olson, D. A., Chiou, B., Yee, E., Bechtel, P. J., & McHugh, T. H. (2006). Water vapor permeability of mammalian and fish gelatin films. *Journal of Food Science*, *71*(4), 202–207. https://doi.org/10.1111/j.1750-3841.2006.00016.x

Bekkevold, S., & Olafsen, T. (2007). *Råvarer med muligheter*. (S. L. Olsen, Ed.) (1st ed.). Trondheim: RUBIN. Retrieved from http://www.rubin.no/files/documents/rubin_lavoppl._m.omslag.pdf

Benjakul, S., Kittiphattanabawon, P., & Regenstein, J. M. (2012). Fish Gelatin. In B. K. Simpson (Ed.), *Food Biochemistry and Food Processing* (2nd ed., pp. 388–405). Oxford, UK: Wiley-Blackwell. ISBN: 9781118308035. https://doi.org/10.1002/9781118308035

Benjakul, S., Nalinanon, S., & Shahidi, F. (2012). Fish Collagen. In B. K. Simpson (Ed.), *Food Biochemistry and Food Processing* (2nd ed., pp. 365–387). Oxford, UK: Wiley-Blackwell. ISBN: 9781118308035. https://doi.org/10.1002/9781118308035

Benjakul, S., Sae-leaw, T., & Simpson, B. K. (2020). Byproducts from Fish Harvesting and Processing. In B. K. Simpson, A. N. A. Aryee, & F. Toldrá (Eds.), *Byproducts from Agriculture and Fisheries: Adding Value for Foos, Feed, Pharma, and Fuels*

(1st ed., pp. 179–217). John Wiley & Sons Ltd. ISBN: 9781119383956. https://doi.org/10.1002/9781119383956.ch9

Blackburn, S. (1968). *Amino acid determination: methods and techniques*. New York, U.S.A.: Dekker.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917.

Boran, G., & Regenstein, J. M. (2010). Fish gelatin. In *Advances in Food and Nutrition Research* (1st ed., pp. 119–143). Elsevier Inc. https://doi.org/10.1016/S1043-4526(10)60005-8

Bowes, J. H., & Kenten, R. H. (1950). The swelling of collagen in alkaline solutions. 1. Swelling in solutions of sodium hydroxide. *Biochemical Journal*, *46*(1), 1–8. https://doi.org/10.1042/bj0460001

Buňka, F., Kříž, O., Veličková, A., Buňková, L., & Kráčmar, S. (2009). Effect of acid hydrolysis time on amino acid determination in casein and processed cheeses with different fat content. *Journal of Food Composition and Analysis*, *22*(3), 224–232. https://doi.org/10.1016/j.jfca.2008.10.023

Chiou, B. Sen, Avena-Bustillos, R. J., Bechtel, P. J., Imam, S. H., Glenn, G. M., & Orts, W. J. (2009). Effects of drying temperature on barrier and mechanical properties of cold-water fish gelatin films. *Journal of Food Engineering*, *95*(2), 327–331. https://doi.org/10.1016/j.jfoodeng.2009.05.011

Choi, S. S., & Regenstein, J. M. (2000). Physicochemical and sensory characteristics of fish gelatin. *Journal of Food Science*, *65*(2), 194–199. https://doi.org/10.1111/j.1365-2621.2000.tb15978.x

Coultate, T. (2009). *FOOD: The Chemistry of its Components* (5th ed.). Cambridge: The Royal Society of Chemistry. ISBN: 978-0-85404-111-4.

Courts, A. (1954). The N-Terminal Amino Acid Residues of Gelatin. *Biochem. J.*, *58*(1), 70–74.

Damodaran, S. (2005). R : Concise Reviews / Hypotheses in Food Science: Protein Stabilization of Emulsions and Foams. *Journal of Food Science*, *70*(3), 54–66.

Damodaran, S. (2017). Water and Ice Relations in Foods. In S. Damodaran & K. L. Parkin (Eds.), *Fennema's Food Chemistry* (5th ed., pp. 19–90). Boca Raton, U.S.A.: CRC Press. ISBN: 9781482208122.

Dangaran, K., Tomasula, P. M., & Qi, P. (2009). Structure and function of protein-based edible films and coatings. In M. E. Embuscado & K. C. Huber (Eds.), *Edible films and coatings for food applications* (1st ed., pp. 25–56). New York, U.S.A.

Darragh, A. J., Garrick, D. J., Moughan, P. J., & Hendriks, W. H. (1996). Correction for

amino acid loss during acid hydrolysis of a purified protein. *Analytical Biochemistry*, *236*(2), 199–207. https://doi.org/10.1006/abio.1996.0157

Dave, D., Liu, Y., Clark, L., Dave, N., Trenholm, S., & Westcott, J. (2019). Availability of marine collagen from Newfoundland fisheries and aquaculture waste resources. *Bioresource Technology Reports*, *7*(June), 100271. https://doi.org/10.1016/j.biteb.2019.100271

Duan, R., Zhang, J., Du, X., Yao, X., & Konno, K. (2009). Properties of collagen from skin, scale and bone of carp (Cyprinus carpio). *Food Chemistry*, *112*(3), 702–706. https://doi.org/10.1016/j.foodchem.2008.06.020

Eastoe, J. E., & Leach, A. A. (1977). Chemical constitution of gelatin. In A. Courts & A. G. Ward (Eds.), *The science and technology of gelatin* (pp. 73–107). London, UK: Academic Press. ***As cited in**:* Avena-Bustillos, R. J., Olsen, C. W., Olson, D. A., Chiou, B., Yee, E., Bechtel, P. J., & McHugh, T. H. (2006). Water vapor permeability of mammalian and fish gelatin films. *Journal of Food Science*, *71*(4), 202–207. https://doi.org/10.1111/j.1750-3841.2006.00016.x

Eysturskarð, J. (2010). *Mechanical properties of gelatin gels: Effect of molecular weight and molecular weight distribution*. Norwegian University of Science and Technology (NTNU).

Eysturskarð, J., Haug, I. J., Elharfaoui, N., Djabourov, M., & Draget, K. I. (2009). Structural and mechanical properties of fish gelatin as a function of extraction conditions. *Food Hydrocolloids*, *23*, 1702–1711. https://doi.org/10.1016/j.foodhyd.2009.01.008

Eysturskard, J., Haug, I. J., Ulset, A. S., & Draget, K. I. (2009). Mechanical properties of mammalian and fish gelatins based on their weight average molecular weight and molecular weight distribution. *Food Hydrocolloids*, *23*(8), 2315–2321. https://doi.org/10.1016/j.foodhyd.2009.06.007

Eysturskard, J., Haug, I. J., Ulset, A. S., Joensen, H., & Draget, K. I. (2010). Mechanical properties of mammalian and fish gelatins as a function of the contents of α-chain, β-chain, and low and high molecular weight fractions. *Food Biophysics*, *5*(1), 9–16. https://doi.org/10.1007/s11483-009-9138-3

FAO. (n.d.). Food Loss and Waste in Fish Value Chains. Retrieved November 3, 2019, from http://www.fao.org/flw-in-fish-value-chains/en/

FAO. (2018). *The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals*. Rome: Food and Agriculture Organization of the United Nations. ISBN: 9789251305621. Retrieved from http://www.fao.org/state-of-fisheries-aquaculture

Foegeding, E. A., Lanier, T. C., & Hultin, H. O. (1996). Characteristics of edible muscle tissues. In O. R. Fennema (Ed.), *Food Chemistry* (3rd ed., pp. 879–942). New York,

U.S.A.: Marcel Dekker. https://doi.org/10.5005/jp/books/18033_5

Gilsenan, P. M., & Ross-Murphy, S. B. (2000). Rheological characterisation of gelatins from mammalian and marine sources. *Food Hydrocolloids*, *14*(3), 191–195. https://doi.org/10.1016/S0268-005X(99)00050-8

Giraud-Guille, M. M., Besseau, L., Chopin, C., Durand, P., & Herbage, D. (2000). Structural aspects of fish skin collagen which forms ordered arrays via liquid crystalline states. *Biomaterials*, *21*(9), 899–906. https://doi.org/10.1016/S0142-9612(99)00244-6

GMIA. (2019). *Gelatin Handbook*. *Gelatin Manufacturers Institute Of America*. New York, U.S.A. Retrieved from http://www.gelita.comhttp//www.nitta-gelatin.comhttp://www.pbleiner.comhttp://www.rousselot.comhttp://www.weishardt.com

Gómez-Guillén, M. ., Turnay, J., Fernández-Díaz, M. ., Ulmo, N., Lizarbe, M. ., & Montero, P. (2002). Structural and physical properties of gelatin extracted from different marine species: a comparative study. *Food Hydrocolloids*, *16*(1), 25–34. https://doi.org/10.1016/S0268-005X(01)00035-2

Gomez-Guillen, M. C., Gimenez, B., Lopez-Caballero, M. E., & Montero, M. P. (2011). Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids*, *25*(8), 1813–1827. https://doi.org/10.1016/j.foodhyd.2011.02.007

Gómez-Guillén, M. C., Pérez-Mateos, M., Gómez-Estaca, J., López-Caballero, E., Giménez, B., & Montero, P. (2009). Fish gelatin: a renewable material for developing active biodegradable films. *Trends in Food Science and Technology*, *20*(1), 3–16. https://doi.org/10.1016/j.tifs.2008.10.002

Grossman, S., & Bergman, M. (1992). Process for the production of gelatin from fish skins. *U.S. Patent 5,093,474*. ***As cited in:*** Gudmundsson, M., & Hafsteinsson, H. (1997). Gelatin from cod skins as affected by chemical treatments. *Journal of Food Science*, *62*(1), 37–40. https://doi.org/10.1111/j.1365-2621.1997.tb04363.x

Gudmundsson, M., & Hafsteinsson, H. (1997). Gelatin from cod skins as affected by chemical treatments. *Journal of Food Science*, *62*(1), 37–40. https://doi.org/10.1111/j.1365-2621.1997.tb04363.x

Gustavsson, J., Cederberg, C., Sonesson, U., van Otterdijk, R., & Meybeck, A. (2011). *Global Food Losses and Food Waste: Extent, Causes and Prevention*. Rome, Italy.

Harnedy, P. A., & Fitzgerald, R. J. (2013). Bioactive Proteins and Peptides from Macroalgae, Fish, Shellfish and Marine Processing Waste. In S.-K. Kim (Ed.), *Marine Proteins and Peptides: Biological Activities and Applications* (pp. 5–39). Chichester, England: John Wiley & Sons Inc. https://doi.org/10.1002/9781118375082.ch2

Hattrem, M. N., & Draget, K. I. (2014). Gelatins - Physiochemical Properties, Source Dependence and Applications. In P. Williams & G. Phillips (Eds.), *Gums and Stabilisers for the Food Industry 17: The Changing Face of Food Manufacture: The Role of Hydrocolloids* (pp. 19–27). Royal Society of Chemistry. ISBN: 9781782621300. https://doi.org/10.1039/9781782621300-00019

Haug, I. J., & Draget, K. I. (2011). Gelatin. In P. A. Williams & G. O. Phillips (Eds.), *Handbook of Food Proteins* (pp. 92–115). Elsevier Science. ISBN: 9781845697587.

Haug, I. J., Draget, K. I., & Smidsrød, O. (2004). Physical and rheological properties of fish gelatin compared to mammalian gelatin. *Food Hydrocolloids*, *18*(2), 203–213. https://doi.org/10.1016/S0268-005X(03)00065-1

Huang, T., Tu, Z. cai, Shangguan, X., Sha, X., Wang, H., Zhang, L., & Bansal, N. (2019). Fish gelatin modifications: A comprehensive review. *Trends in Food Science and Technology*, *86*(November 2017), 260–269. https://doi.org/10.1016/j.tifs.2019.02.048

Hulmes, D. J. S. (2008). Collagen Diversity, Synthesis and Assembly. In P. Fratzl (Ed.), *Collagen: Structure and Mechanics* (pp. 15–47). Boston, MA: Springer US. ISBN: 9786611512996.

Ikoma, T., Kobayashi, H., Tanaka, J., Walsh, D., & Mann, S. (2003). Physical properties of type I collagen extracted from fish scales of Pagrus major and Oreochromis niloticas. *International Journal of Biological Macromolecules*, *32*(3–5), 199–204. https://doi.org/10.1016/S0141-8130(03)00054-0

Johnston-Banks, F. A. (1990). Gelatine. In P. Harris (Ed.), *Food Gels* (pp. 233–290). Springer Netherlands. ISBN: 9789400907553. https://doi.org/10.1007/978-94-009-0755-3

Jones, N. R. (1977). Uses of gelatine in edible products. In A. G. Ward & A. Courts (Eds.), *The science and technology of gelatin* (pp. 366–394). London, UK: Academic Press. ISBN: 0127350500. ***As cited in:*** Mahmoodani, F., Ardekani, V. S., See, S. F., Yusop, S. M., & Babji, A. S. (2014). Optimization and physical properties of gelatin extracted from pangasius catfish (Pangasius sutchi) bone. *Journal of Food Science and Technology*, *51*(11), 3104–3113. https://doi.org/10.1007/s13197-012-0816-7

Jongjareonrak, A., Rawdkuen, S., Chaijan, M., Benjakul, S., Osako, K., & Tanaka, M. (2010). Chemical compositions and characterisation of skin gelatin from farmed giant catfish (Pangasianodon gigas). *LWT - Food Science and Technology*, *43*(1), 161–165. https://doi.org/10.1016/j.lwt.2009.06.012

Karim, A. A., & Bhat, R. (2009). Fish gelatin : properties , challenges , and prospects as an alternative to mammalian gelatins. *Food Hydrocolloids*, *23*(3), 563–576. https://doi.org/10.1016/j.foodhyd.2008.07.002

Kaye, M., & Lloyd, D. J. (1924). A Histological and Physico-Chemical Investigation of

the Swelling of a Fibrous Tissue. *Proceeding of the Royal Society of London. Series B, Containing Papers of a Biological Character*, *96*(677), 293–316.

Kołodziejska, I., Kaczorowski, K., Piotrowska, B., & Sadowska, M. (2004). Modification of the properties of gelatin from skins of Baltic cod (Gadus morhua) with transglutaminase. *Food Chemistry*, *86*(2), 203–209. https://doi.org/10.1016/j.foodchem.2003.08.036

Kudre, T. G., & Benjakul, S. (2013). Effects of binary organic solvents and heating on lipid removal and the reduction of beany odour in Bambara groundnut (Vigna subterranean) flour. *Food Chemistry*, *141*(2), 1390–1397. https://doi.org/10.1016/j.foodchem.2013.04.047

Leach, A. A. (1960). Notes on a modification of the Neuman and Logan method for the determination of the Hydroxyproline. *The Biochemical Journal*, *74*(01), 70–71.

Lestra, Y. (1985). The importance of photographic gelatin. *Nature*, *313*(6000), 333–333. https://doi.org/10.1038/313333a0

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurment with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265–275. Retrieved from https://www.jbc.org/content/193/1/265

Miles, C. A., & Bailey, A. J. (1999). Thermal Denaturation of Collagen Revisited. *Proceedings of the Indian Academy of Sciences - Chemical Sciences*, *111*(1), 71–80. https://doi.org/10.1007/BF02869897

Milovanovic, I., & Hayes, M. (2018). Marine gelatine from rest raw materials. *Applied Sciences (Switzerland)*, *8*(12), 1–20. https://doi.org/10.3390/app8122407

Muyonga, J. H., Cole, C. G. B., & Duodu, K. G. (2004). Extraction and physico-chemical characterisation of Nile perch (Lates niloticus) skin and bone gelatin. *Food Hydrocolloids*, *18*(4), 581–592. https://doi.org/10.1016/j.foodhyd.2003.08.009

Nalinanon, S., Benjakul, S., Visessanguan, W., & Kishimura, H. (2008). Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor. *Food Hydrocolloids*, *22*(4), 615–622. https://doi.org/10.1016/j.foodhyd.2007.01.012

NASDAQ OMX's News Release Distribution Channel. (2020, July 10). Global Gelatin Industry. Retrieved July 6, 2020, from https://www.reportbuyer.com/product/5478473/global-gelatin-industry.html?utm_source=PRN

Neuman, R. E., & Logan, M. A. (1950). The determination of hydroxyproline. *The Journal of Biological Chemistry*, *184*(1), 299–306. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1204050/pdf/biochemj01017-0078.pdf

Papon, P., Leblond, J., & Meijer, P. H. E. (2006). *The physics of phase transitions: Concepts and applications* (2nd ed.). Berlin, Heidelberg: Springer Berlin Heidelberg. ISBN: 3-540-33390-8. https://doi.org/10.1007/3-540-33390-8

Pérez Roda, M. A., Gilman, E., Huntington, T., Kennelly, S. J., Suuronen, P., Chaloupka, M., & Medley, P. (2019). *A third assessment of global marine fisheries discards. FAO Fisheries and Aquaculture Technical Paper No. 633.* Rome, Italy. Retrieved from www.fao.org/

Ramshaw, J. A. M., Peng, Y. Y., Glattauer, V., & Werkmeister, J. A. (2009). Collagens as biomaterials. *Journal of Materials Science: Materials in Medicine*, *20*(SUPPL. 1). https://doi.org/10.1007/s10856-008-3415-4

Ranganayaki, M. D., Asghar, A., & Henrickson, R. L. (1982). Influence of Anion and Cation on the Water-Holding Capacity of Bovine Hide Collagen at Different pH Values. Effect of Sodium Chloride and Polyphosphates on Hydration. *Journal of Food Science*, *47*(3), 705–710. https://doi.org/10.1111/j.1365-2621.1982.tb12695.x

Richardsen, R., & Nystøyl, R. (2017). *Analyse marint restråstoff, 2016.* Tromsø, Norway.

Sae-leaw, T., & Benjakul, S. (2015). Physico-chemical properties and fishy odour of gelatin from seabass (Lates calcarifer) skin stored in ice. *Food Bioscience*, *10*, 59–68. https://doi.org/10.1016/j.fbio.2015.02.002

Sae-leaw, T., & Benjakul, S. (2018). Lipase from liver of seabass (Lates calcarifer): Characteristics and the use for defatting of fish skin. *Food Chemistry*, *240*(February 2017), 9–15. https://doi.org/10.1016/j.foodchem.2017.07.089

Sae-leaw, T., Benjakul, S., & O'Brien, N. M. (2016a). Effect of Pretreatments and Defatting of Seabass Skins on Properties and Fishy Odor of Gelatin. *Journal of Food Biochemistry*, *40*(6), 741–753. https://doi.org/10.1111/jfbc.12267

Sae-leaw, T., Benjakul, S., & O'Brien, N. M. (2016b). Effects of defatting and tannic acid incorporation during extraction on properties and fi shy odour of gelatin from seabass skin. *LWT - Food Science and Technology*, *65*, 661–667. https://doi.org/10.1016/j.lwt.2015.08.060

Sae-leaw, T., Benjakul, S., O'Brien, N. M., & Kishimura, H. (2016). Characteristics and functional properties of gelatin from seabass skin as influenced by defatting. *International Journal of Food Science and Technology*, *51*, 1204–1211. https://doi.org/10.1111/ijfs.13085

Sato, K., Ohashi, C., Ohtsuki, K., & Kawabata, M. (1991). Type V Collagen in Trout (Salmo Gairdneri) Muscle and Its Solubility Change During Chilled Storage of Muscle. *Journal of Agricultural and Food Chemistry*, *39*(7), 1222–1225. https://doi.org/10.1021/jf00007a005

Sato, K., Yoshinaka, R., Sato, M., & Shimizu, Y. (1987). Isolation of Native Acid-Soluble

Collagen from Fish Muscle. *Nippon Suisan Gakkaishi*. https://doi.org/10.2331/suisan.53.1431

Schrieber, R., & Gareis, H. (2007). *Gelatine Handbook: Theory and Industrial Practice*. Wiley-VCH. ISBN: 9783527315482.

Slade, L., & Levine, H. (1987). Polymer-Chemical Properties of Gelatin in Foods. In A. M. Pearson, T. R. Dutson, & A. J. Bailey (Eds.), *Advances in Meat Research: Volume 4: Collagen as a food* (pp. 251–266). Westport: AVI publ. ***As cited in:*** Galea, C. A., Dalrymple, B. P., Kuypers, R., & Blakeley, R. (2000). Modification of the substrate specificity of porcine pepsin for the enzymatic production of bovine hide gelatin. *Protein Science*, *9*(10), 1947–1959. https://doi.org/10.1110/ps.9.10.1947

Stainsby, G. (1987). Gelatin gels. In A. M. Pearson, T. R. Dutson, & A. J. Bailey (Eds.), *Advances in Meat Research. Vol. 4. Collagen as a food.* (pp. 209–222). New York, U.S.A.: Van Nostrand Reinhold Co. Inc. ***As cited in:*** See, S. F., Ghassem, M., Mamot, S., & Babji, A. S. (2013). Effect of different pretreatments on functional properties of African catfish (Clarias gariepinus) skin gelatin. *Journal of Food Science and Technology*, *52*(2), 753–762. https://doi.org/10.1007/s13197-013-1043-6

Tanioka, A., Tazawa, T., Miyasaka, K., & Ishikawa, K. (1974). Effects of water on the mechanical properties of gelatin films. *Biopolymers*, *13*(4), 753–764. https://doi.org/10.1002/bip.1974.360130410

Townsend, A.-A., & Nakai, S. (1983). Relationships Between Hydrophobicity and Foaming Characteristics of Food Proteins. *Journal of Food Science*, *48*(2), 588–594. https://doi.org/10.1111/j.1365-2621.1983.tb10796.x

Waldner, R. H. (1977). Raw material. In A. G. Ward & A. Courts (Eds.), *The science and technology of gelatin* (pp. 295–314). London, UK: Academic Press. ISBN: 0127350500. ***As cited in:*** Benjakul, S., Kittiphattanabawon, P., & Regenstein, J. M. (2012). Fish Gelatin. In B. K. Simpson (Ed.), *Food Biochemistry and Food Processing* (2nd ed., pp. 388–405). Oxford, UK: Wiley-Blackwell. ISBN: 9781118308035. https://doi.org/10.1002/9781118308035

Wang, C., Chang, T., Shi, L., Yang, H., Cui, M., & Tambalu, L. (2014). Seafood Processing By-products. In S.-K. Kim (Ed.), *Seafood Processing By-products: Trends and Applications* (1st ed., pp. 207–242). New York, NY: Springer New York. 9781461495895.

Wong, D. W. S. (1989). *Mechanism and theory in food chemistry*. New York, U.S.A.: Van Nostrand Reinhold. ISBN: 0442207530. ***As cited in:*** Benjakul, S., Kittiphattanabawon, P., & Regenstein, J. M. (2012). Fish Gelatin. In B. K. Simpson (Ed.), *Food Biochemistry and Food Processing* (2nd ed., pp. 388–405). Oxford, UK: Wiley-Blackwell. ISBN: 9781118308035. https://doi.org/10.1002/9781118308035

Zhang, Y., & Regenstein, J. M. (2017). Yield and Gel Strength of Gelatin Extracted from Smoked Salmon (Salmo salar) Skins. *Journal of Aquatic Food Product Technology*,

26(5), 553–565. https://doi.org/10.1080/10498850.2016.1236868

Zhou, P., & Regenstein, J. M. (2006). Determination of total protein content in gelatin solutions with the Lowry or Biuret assay. *Journal of Food Science*, *71*(8). https://doi.org/10.1111/j.1750-3841.2006.00151.x

Zhou, P., & Regenstein, J. M. (2004). Optimization of Extraction Conditions for Pollock Skin Gelatin. *Journal of Food Science*, *69*(5), C393–C398. https://doi.org/10.1111/j.1365-2621.2004.tb10704.x

Zhou, P., & Regenstein, J. M. (2005). Effects of Alkaline and Acid Pretreatments on Alaska Pollock Skin Gelatin Extraction. *Journal of Food Science*, *70*(6), c392–c396. https://doi.org/10.1111/j.1365-2621.2005.tb11435.x

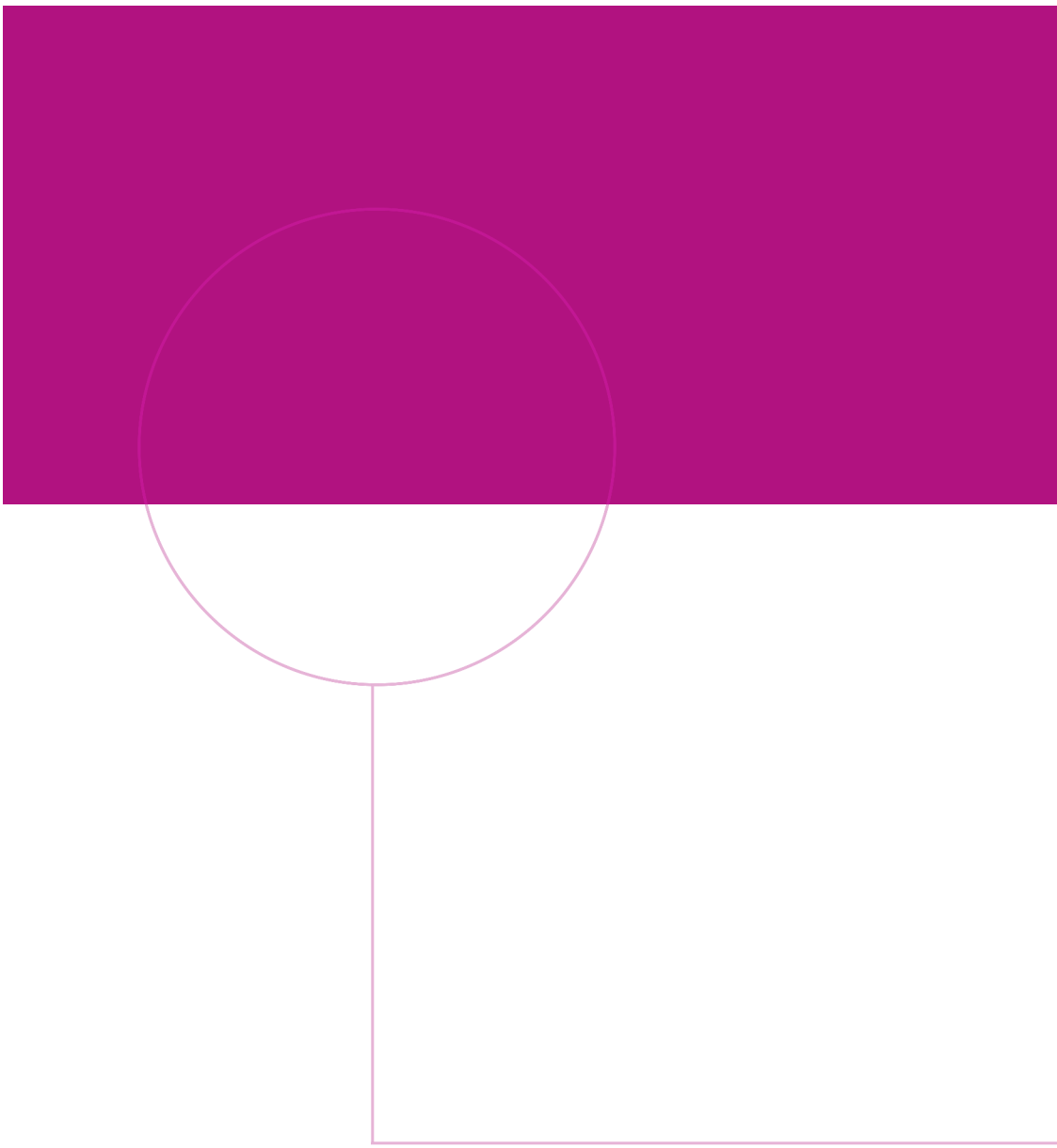
A. Full factorial screening design together with observed raw data

	Independent variables					Observed data		
Run	OH conc.	Pre-treatment time	Pre-treatment temp.	Butanol conc.	Defat time	Dry matter (%)	Ash (%)	Lipid (%)
1	+	+	+	+	+	15.1	0.20	1.02
2	-	+	+	+	+	25.9	0.52	0.44
3	+	-	+	+	+	23.9	1.32	0.24
4	-	-	+	+	+	22.2	2.67	0.12
5	+	+	+	-	+	12.8	0.02	0.10
6	-	+	+	-	+	15.9	1.62	1.01
7	+	-	+	-	+	17.0	1.51	0.50
8	-	-	+	-	+	22.1	2.32	1.32
9	+	+	+	+	-	17.8	0.73	0.73
10	-	+	+	+	-	25.9	1.62	2.27
11	+	-	+	+	-	22.4	2.76	1.11
12	-	-	+	+	-	26.4	3.09	2.40
13	+	+	+	-	-	15.0	0.55	0.84
14	-	+	+	-	-	22.4	1.68	1.21
15	+	-	+	-	-	22.7	1.11	1.93
16	-	-	+	-	-	23.2	1.64	1.11
17	+	+	-	+	+	26.2	1.99	1.43
18	-	+	-	+	+	24.1	2.26	0.71
19	+	-	-	+	+	24.5	2.19	0.51
20	-	-	-	+	+	29.3	0.23	1.63
21	+	+	-	-	+	22.7	1.60	0.99
22	-	+	-	-	+	28.4	2.77	0.61
23	+	-	-	-	+	27.4	2.07	2.80
24	-	-	-	-	+	26.5	2.96	1.76
25	+	+	-	+	-	21.5	2.22	1.93
26	-	+	-	+	-	22.5	1.71	1.92
27	+	-	-	+	-	24.3	2.15	0.65
28	-	-	-	+	-	23.2	2.33	1.49
29	+	+	-	-	-	27.3	0.10	1.68
30	-	+	-	-	-	23.9	2.63	2.26
31	+	-	-	-	-	26.3	0.91	1.99
32	-	-	-	-	-	23.7	2.47	2.15

B. Raw data for the amino acid content (in mg AA/g sample dry weight) as determined by RP-HPLC, including deviant measurements

	G1 replicates				G2 replicates				G3 replicates			
Amino acid (mg/g sample dry weight)	**1**	**2**	**3**	**Deviant**	**1**	**2**	**3**	**Deviant**	**1**	**2**	**3**	**Deviant**
Asp	55.5	38.5	52.2	53.2	62.0	45.0	51.9	49.9	89.1	52.9	52.8	74.8
Glu	73.0	47.3	70.7	398.2	80.9	66.6	61.1	644.7	115.8	94.2	60.1	962.5
Asn	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0
His	9.7	3.9	13.5	10.5	12.8	10.6	12.6	9.8	18.7	12.7	12.7	14.9
Ser	28.7	26.0	40.9	33.6	33.0	33.0	39.7	31.7	48.2	39.7	39.6	47.8
Gln	2.3	2.6	2.5	3.7	1.9	2.9	2.8	2.7	3.0	2.2	5.0	5.6
Gly/Arg	208.2	169.8	231.9	197.6	234.1	200.5	232.7	183.5	341.3	239.7	236.2	281.9
Thr	23.9	18.8	26.8	23.5	26.3	22.5	25.9	20.2	32.7	25.5	28.3	31.3
Ala	76.6	65.2	87.6	75.0	85.8	75.1	86.8	70.4	125.5	90.4	88.3	107.7
Tyr	1.6	0.2	2.1	2.1	2.4	2.4	1.7	0.3	1.0	2.3	2.5	0.0
Aba	0.3	1.0	1.0	0.9	0.3	0.1	0.6	0.6	0.4	1.2	0.7	0.8
Met	19.5	12.5	21.8	17.3	23.5	19.0	22.4	18.5	33.7	21.2	22.5	27.3
Val	14.4	10.0	15.9	13.2	15.8	13.0	15.7	12.3	22.8	14.2	15.4	18.5
Phe	17.6	13.2	20.0	15.6	19.7	15.8	20.0	15.1	28.5	18.7	19.0	23.6
Ile	9.7	7.1	10.0	8.5	10.6	8.6	9.8	7.7	15.6	10.2	9.5	11.9
Leu	17.0	13.2	17.5	14.1	18.7	15.3	17.5	14.1	27.0	18.0	17.5	20.9
Lys	32.8	25.1	35.1	27.8	36.5	29.5	35.0	26.1	52.5	35.1	34.2	38.6
Total	590.9	454.4	649.5	895.1	664.3	559.9	636.3	1107.8	956.2	678.2	644.3	1668.1

	G4 replicates				G5 replicates				G6 replicates			
Amino acid (mg/g sample dry weight)	**Lost sample**	**2**	**3**	**Deviant**	**1**	**2**	**3**	**Deviant**	**1**	**2**	**3**	**Deviant**
Asp	-	51.0	53.3	67.2	48.3	38.8	40.0	42.7	51.3	37.9	40.1	48.7
Glu	-	61.7	63.6	873.9	58.3	193.2	55.4	473.2	63.0	99.3	48.3	709.1
Asn	-	0.1	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.1	0.0
His	-	10.9	13.0	13.2	10.1	9.8	9.5	8.2	10.7	9.2	1.3	11.5
Ser	-	27.4	41.0	44.4	26.1	31.4	29.8	25.8	27.2	27.6	28.0	29.8
Gln	-	2.1	4.0	4.2	1.2	1.5	2.2	2.2	1.7	2.9	2.4	2.9
Gly/Arg	-	200.8	242.5	253.1	185.9	162.3	177.2	157.1	195.7	169.8	177.0	181.0
Thr	-	22.0	28.6	28.6	22.6	10.4	20.9	17.3	23.9	20.0	19.5	21.4
Ala	-	73.4	91.2	97.0	66.5	64.4	66.0	59.8	72.2	63.4	66.1	69.0
Tyr	-	0.0	2.7	2.2	0.1	2.0	1.2	0.0	2.1	1.6	0.0	1.9
Aba	-	0.3	1.0	0.6	0.3	1.2	0.6	0.8	0.3	1.2	0.5	0.9
Met	-	19.9	22.7	23.8	19.0	13.9	16.2	13.3	20.0	15.4	13.5	17.5
Val	-	13.4	16.1	16.7	12.7	10.4	11.8	9.5	13.4	11.3	10.9	12.1
Phe	-	16.8	20.6	20.3	15.8	13.1	14.4	12.2	16.9	13.8	11.9	14.5
Ile	-	9.2	10.2	10.9	8.6	7.4	7.5	6.7	9.2	6.9	7.6	7.8
Leu	-	15.5	18.1	19.2	14.6	13.0	13.3	12.2	15.7	12.9	13.4	13.8
Lys	-	31.0	36.0	35.9	29.0	25.3	26.1	22.0	30.7	25.2	26.2	25.3
Total	-	555.3	664.5	1511.2	519.0	598.2	491.9	863.0	553.9	518.5	466.6	1167.1



NTNU

Norwegian University of
Science and Technology