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Gelatine and Oil Extraction from Atlantic Salmon (*Salmo salar*) Viscera

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I. Preface

This Master's thesis was written in the Department of Biotechnology at the Norwegian University of Science and Technology (NTNU), in collaboration with SINTEF Fisheries and Aquaculture AS.

First of all, I would like to thank my supervisors in this project, Prof. Turid Rustad and Dr. Rasa Šližytė. They have guided me throughout the planning and completion of this project, answering all my questions and I would not be here without their support and knowledge.

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I must say thank you to my parents for always being there and providing me with the opportunities that led me to this point in life. I will always be thankful to them. Finally I would like to thank my better half, Angel, for his support and encouragement during these two years.

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Rebeca Garcia Alvarez

II. Summary

The production of marine rest raw materials in Norway amounted to 909 742 tons in 2016. Around half of these materials are used for silage production, and only 10% was used for human consumption. Viscera represent the largest fraction of rest raw materials obtained in the farming of Atlantic Salmon (*Salmo salar*) and contains a high content of lipids and proteins that can be used in the production of value-added products.

The objective of this study was to evaluate the potential of salmon viscera as a raw material for the extraction of high quality oils and gelatine. Heat treatment at different temperatures was used to separate the oil, and two gelatine extraction methods (salt pre-treatment and acid pre-treatment) were used to extract the gelatine.

The oil yield after heat treatment was high (≥84.4%) and did not seem to be affected by the different temperatures used in the extraction of oil. The quality of the oils extracted was low (high peroxide values and free fatty acid content) but within the normal range for crude oils. Oil was extracted from salmon viscera with and without roe and milt, and the content of lipids was found to be higher in the viscera without roe and milt. In addition, for extraction temperatures below 48.5 °C, the viscera with roe and milt had higher peroxide values.

The gelatine extracts obtained were analysed to determine the effect of the pre-treatments in the yield and purity of the gelatine extracted from viscera without roe and milt. The viscera were divided into two parts, one was subjected to separation of oil prior to gelatine extraction and the other part was used directly (with oil) for the gelatine extraction. In regard to hydroxyproline yield, the salt pre-treatment was more effective in the extraction of viscera with oil, and the acid pre-treatment was more effective in the extraction of viscera without oil. The SDS-PAGE analysis of the freeze dried gelatine extracts showed that the salt pre-treatment has more degraded protein components than the acid pre-treatment.

The results of this study suggest that in regard to yield, salmon viscera are a viable source of lipids and gelatine, but since this raw material is prone to deterioration, measures should be taken to inhibit endogenous enzymatic activity and lipid oxidation.

III. List of abbreviations

AFDGE	Acid pre-treated freeze dried gelatine extract
APT	Acid pre-treatment
ddH_2O	Doubly distilled water
dH_2O	Distilled water
FDGE	Freeze dried gelatine extract
FFA	Free Fatty Acid
FPH	Fish protein hydrolysate
HPLC	High-performance liquid chromatography
PV	Peroxide Value
RRM	Rest Raw Materials
SFDGE	Salt pre-treated freeze dried gelatine extract
SPT	Salt pre-treatment
TLP	Total Lipid Content

Contents

1. Introduction

Rest raw materials (RRM) are the remaining raw materials after the production of the main product (e.g. Fillets in fish) (Rustad, Storrø, & Slizyte, 2011), these include the heads, skins, backbones, trimmings and viscera. These RRM can be used in the production of animal feed, biogas, natural pigments, ingredients for the food industry, nutraceuticals, etc. (Arvanitoyannis & Kassaveti, 2008).

In 2016, 909 742 tons of marine RRM were produced in Norway, 46% of these materials were used for silage and only 10% was used for human consumption. In the same year, the RRM obtained from the farming of salmon and trout represented a 31.9% of the total harvest, with viscera representing the biggest fraction (Richardsen, Nystøyl, Strandheim, & Marthinussen, 2017).

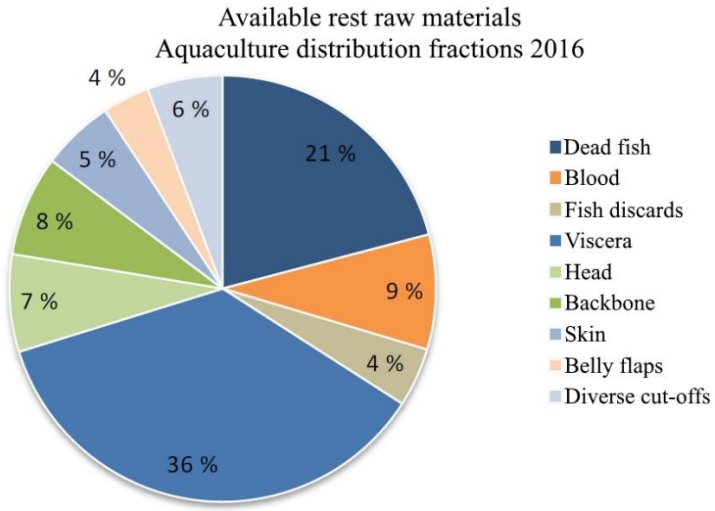

Figure 1. Distribution of rest raw materials obtained from the farming of Salmon and Trout in 2016. Figure modified from Richardsen et al., 2017.

The main focus of this work of investigation has been the optimization of processing parameters for the extraction of oil and gelatine from rest raw materials (specifically viscera) obtained during the processing of Atlantic salmon (*Salmo salar*). The objectives were both to improve the yield and purity of the gelatine, and also to extract the oil fraction without reducing the yield and properties of the gelatine.

Thermal extraction was used to separate the oil in the viscera and two extraction techniques, with different the pre-treatments and extraction conditions were compared to determine their efficacy regarding gelatine yield and quality.

The optimization of the extraction method for fish gelatine aims to produce a gel with better properties, thereby increasing the value and expanding the uses of these materials, e.g. for human consumption.

1.1 Fish viscera

Viscera contain the intestines, stomach, liver, kidneys gall bladder, roe and milt of the fish (Šližytė, Opheim, Storrø, & Sterten, 2017). Fish viscera is of interest to the industry due to its high content of lipids and proteins, which due to their properties have the potential to be used as raw material in the production of value added products (Villamil, Váquiro, & Solanilla, 2017).

Fish oils are a good source of polyunsaturated fatty acids (PUFA), e.g. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Hamilton et al., 2005). The consumption of these ω-3 fatty acids has been shown to reduce cardiovascular diseases (Kris-Etherton, Harris, & Appel, 2002). The oil from salmon viscera contains a higher content of PUFAs than the fillet, which indicates that the viscera is a better candidate for oil extraction than muscle tissues (Sun, Pigott, & Herwig, 2002). Fish oils also contain phospholipids, squalene, and cholesterols (Rustad et al., 2011).

The protein fraction of fish by-products can also be used in the production of fish protein hydrolysates (FPH), which are the peptides of varying sizes obtained after hydrolysis (chemical or enzymatic) of proteins (Rustad, 2007; Vidanarachchi et al., 2014).

FPHs can be obtained through acid, alkali and enzymatic hydrolysis of material rich in fish protein (Villamil et al., 2017). Enzymatic hydrolysis is the preferred method since the FPHs obtained through this technique are of high quality and maintain their functionality (Vidanarachchi et al., 2014). FPHs have been shown to have bioactive and nutritional properties, and can be further processed into higher value products (Aspmo, Horn, & Eijsink, 2005; Rustad et al., 2011). FPHs from viscera have the potential to be used as emulsifiers, antioxidants, ACE inhibitors (for their antihypertensive properties), and peptone production (Villamil et al., 2017).

When using viscera as a source for FPHs, the lipid fraction should be extracted before hydrolysis, since the presence of lipids during hydrolysis leads to FPHs with low quality (unpleasant smell and taste, and possible form of toxic compounds) (Šližytė et al., 2017; Villamil et al., 2017).

Fish rest raw materials are also a source of useful enzymes, such as proteases, lipases, transglutaminases, and others. The enzymes from fish that inhabit cold environments have adapted to function optimally at low temperatures, and therefore are of interest for processes where low temperature is crucial (Venugopal, 2016).

Fish viscera contain high amounts of digestive enzymes, and proteolytic enzymes tend to have a high commercial value. In particular, pepsin from fish viscera has been said to be a competitive alternative to the commercial pepsin extracted from hogs (Kim & Dewapriya, 2014).

Although viscera have potential to be raw material of several products with added value, there is also a disadvantage when it comes to stability. The high content of lipids (especially PUFAs) and endogenous enzymes means that fish by-products containing viscera are prone to lipid oxidation and protein degradation. Therefore, the correct handling and preservation of these by-products is important if these are to be used for further processing (e.g. extraction of FPHs and fish oil) (Rustad et al., 2011).

1.1.1 Collagen and gelatine from fish viscera

Gelatine, a product derived from the hydrolysis of collagen, is an important ingredient in the food industry due to its ability to form thermoreversible gels and improve textural properties of food. Gelatine can be used as a film-forming, emulsifying, foaming or gelling agent. It is also used to increase the protein content in food. Outside of the food industry, gelatine is used in the field of medicine and pharmaceutical production (as a stabilizer for medical compounds and in microencapsulation processes), and in photography (as a binding, emulsifying agent, that can act as a matrix for the chemical reactions occurring in the development of photographic content) (Boran & Regenstein, 2010; Gómez-Guillén et al., 2009; Haug & Draget, 2009; Karim & Bhat, 2009; Schrieber & Gareis, 2007).

The extraction of collagen and gelatine from marine sources has been of interest in the food industry as new challenges have arisen with the use of more commercially available mammalian alternatives. Collagen and gelatine are usually extracted from the skin and bones of pigs and cattle. The consumption of products derived from these sources is not allowed for some religious groups (Islam, Judaism and Hinduism), but marine sources are. The increase in the number of mad cow disease cases (bovine spongiform encephalopathy) in the 1990s resulted in an increase of consumer concern for products obtained from animals that could

potentially be contaminated with these pathogens (Karim & Bhat, 2009; Wang et al., 2014). The use of marine by-products as a source of collagen and gelatine is advantageous, not only from an environmental perspective but also because they have a high protein content (especially in skin and bones) which is preferred for the extraction of these products (Gómez-Guillén et al., 2002).

Bones, skins and fins have been studied previously as sources for extraction of collagen and gelatine due to the high collagen content in these tissues in comparison to other by-products (Boran & Regenstein, 2010; Gómez-Guillén et al., 2002). A previous report by Sandnes, Pedersen, and Hagen (2003), evaluated the chemical composition of salmon by-products. In relation to the whole fish, the highest by-product component is the viscera. The viscera (minced) contains less amount of proteins and ash than the other by-products (heads, backbones, skins, and belly flaps). In this report it was also observed that the hydrolysates obtained from heads and backbones contained the highest amounts of hydroxyproline/proline compared to viscera (which had about half the amount).

Salmon viscera is an interesting candidate in the production of value added products, such as oils, gelatine and FPHs. To understand its potential in comparison to other rest raw materials, it is necessary to study the efficacy of extraction methods and quality of the products obtained using this by-product.

1.2 Collagen

Collagens are a very diverse family of proteins, the basic structure is comprised of three polypeptide chains, which form a triple helix (Brinckmann, 2005). This insoluble protein is present in the connective tissue of animals and is extracted primarily from collagen-rich tissues such as skin, fins, scales and bones. In these tissues, collagen forms fibrils that give stability to the structure, and have other functions such as organization and filtration (Kadler, Baldock, Bella, & Boot-Handford, 2007). The hierarchical structure of a tendon can be seen in Figure 2.

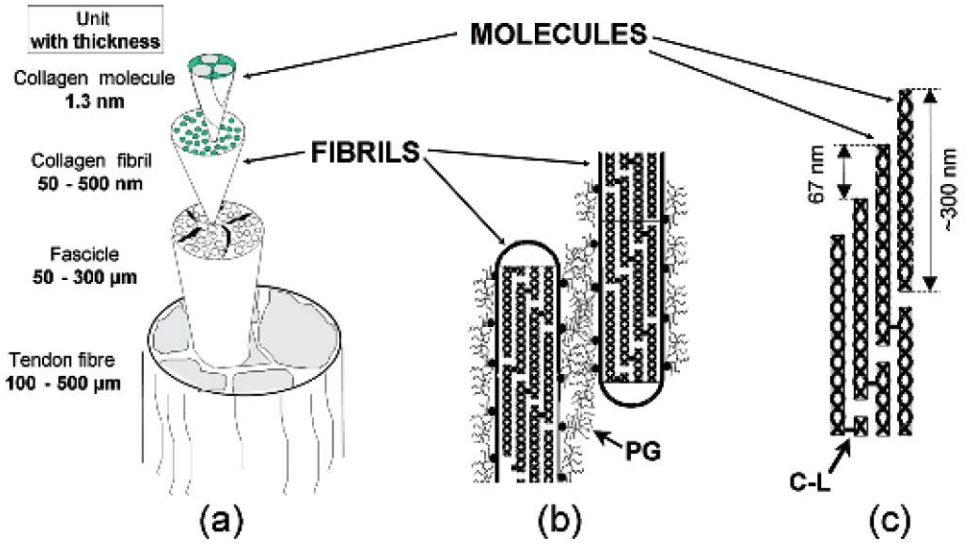

Figure 2. Tendon composition showing the a) fibre, fascicle, fibril and collagen molecule with their respective sizes; b) interaction between fibrils (PG =proteoglycan matrix); c) interaction between collagen molecules (C-L = crosslinks between collagen molecules). (Figure obtained from Fratzl and Weinkamer 2007 with modifications by Fratzl, 2008)

The structure of type I collagen (archetypal collagen) is a right-handed triple helix, where each individual α-chain is a left-handed helix with three amino acids per turn. These individual chains are mainly composed of a repeating sequence of Gly-X-Y (X=mostly proline, Y=mostly hydroxyproline), with the glycine residues facing the inside of the superhelix (Engel & Bächinger, 2005).

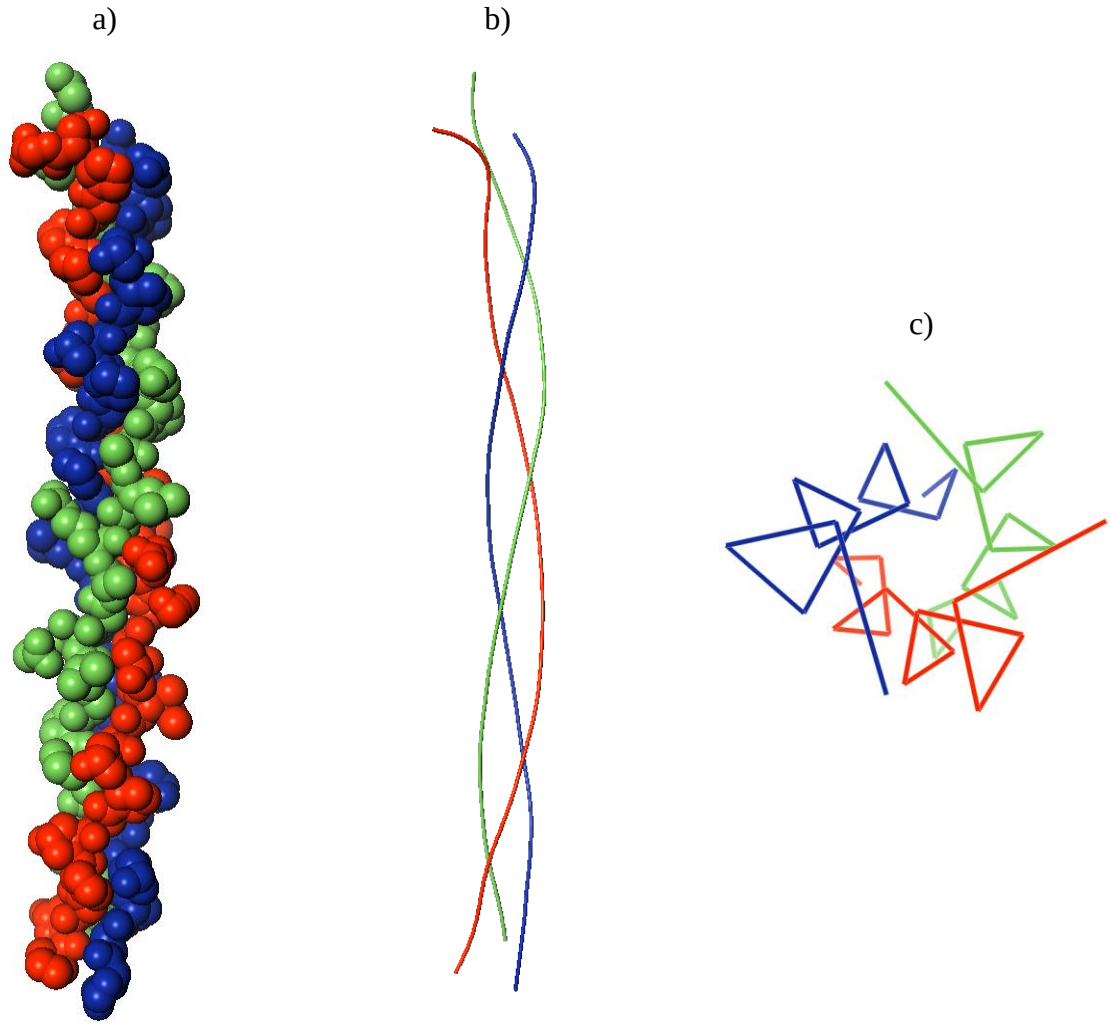

Figure 3. Triple-helical structure of collagen where each α chain is represented by a different colour. a) Right handed triple helix collagen molecule (space-filling representation). b) Right handed triple helix (cartoon representation). c) Triple helix seen from molecular axis, left-handed α-chains can be observed. (Figures obtained using PyMOL Molecular Graphics System, PDB ID: 1BKV, Kramer, Bella, Mayville, Brodsky, and Berman (1999))

Inter- and intra-chain hydrogen bonds give stability to the collagen structure. The presence of hydroxyproline is key for the stability due to its hydroxyl group. The hydroxyl group in hydroxyproline is able to bind to water molecules and allows for the creation of the ordered water shell around the triple-helix, providing structural stability. (Brodsky & Ramshaw, 1997)

The definition of what a "collagen" is can vary. For a molecule to be classified as a "collagen", it must have a triple-helical motif, however, not all molecules containing this motif are considered collagens (Hulmes, 2008). Different variations of collagen types exist and are classified depending on structure and function. To this date, 28 types of collagens have been discovered, and they are categorised into fibrillar and non-fibrillar collagens (seen in Table 1).

Table 1. Collagen types, classification and function in connective tissues (Kadler et al., 2007; O'Reilly et al.; Wang et al., 2014)

Category	Subcategory	Collagen types	Function
Fibrillar	Fibril-forming collagens	I, II, III, V, XI, XXIV, XXVII	Tensile strength
Non-fibrillar	FACIT (Fibril-associated collagens with interrupted triple helices)	IX, XII, XIV, XVI, XIX, XX, XXI, XXII	Attachment to fibrils
	Network-forming collagens	IV, VIII, X	Filtration through mesh structure in basal membrane
	Transmembrane collagens	XIII, XVII, XXIII, XXV, Ectodysplasin A, Gliomedin	Cell surface receptors
	Endostatin-producing collagens	XV, XVIII	Release endostatin (fragment of collagen XVIII) which inhibits tumor growth
	Anchoring fibrils	VII	Integrity of basement membrane (dermoepidermal junction)
	Baded-filament-forming collagen	VI, XXVI, XXVIII	Formation of links between cells

1.3 Gelatine

Gelatine is a product derived from the processing of collagen extracted from animal tissues. Hydrolysis of collagen results in the breaking of the hydrogen bonds of the secondary structure of the protein. The released chains (α-,β-,or γ-chains, each with varying molecular weight), then rearrange into coils, which are soluble in water (Haug & Draget, 2009).

1.3.1 Structure and gelling properties

The gelation process occurs when the temperature of the gelatine solution is below the critical helix-to-coil point. Below this temperature (and at the correct concentration), the random coil structures (α-chains) will rearrange into triple helical structures, the cross-linkages between these structures will result in the formation of a gel network (Haug & Draget, 2009), as can be seen in Figure 4.

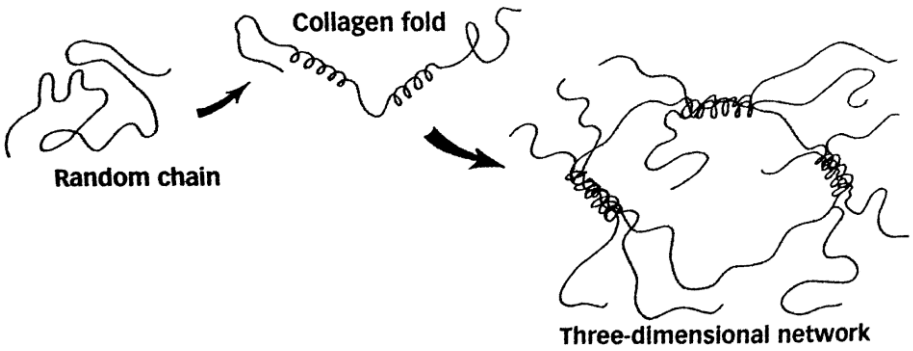

Figure 4. Gel formation upon cooling Figure obtained from Schrieber and Gareis (2007).

The mechanism of gelation can be seen in the figure below (Figure 5). This rearranging process will depend on the concentration of the α-chains in solution and the cooling rate. The formation of intrachain and interchain linkages are affected by these conditions. Lower concentration of α-chains will lead to more intrachain interaction, and higher concentrations allow for the different triple helix structures to form the networks that will contribute to the gelation process. Lower cooling rates lead to the formation of a molecule resembling the collagen structure, whereas a high cooling rate will lead to a higher presence of random coils (Belitz, Grosch, & Schieberle, 2009).

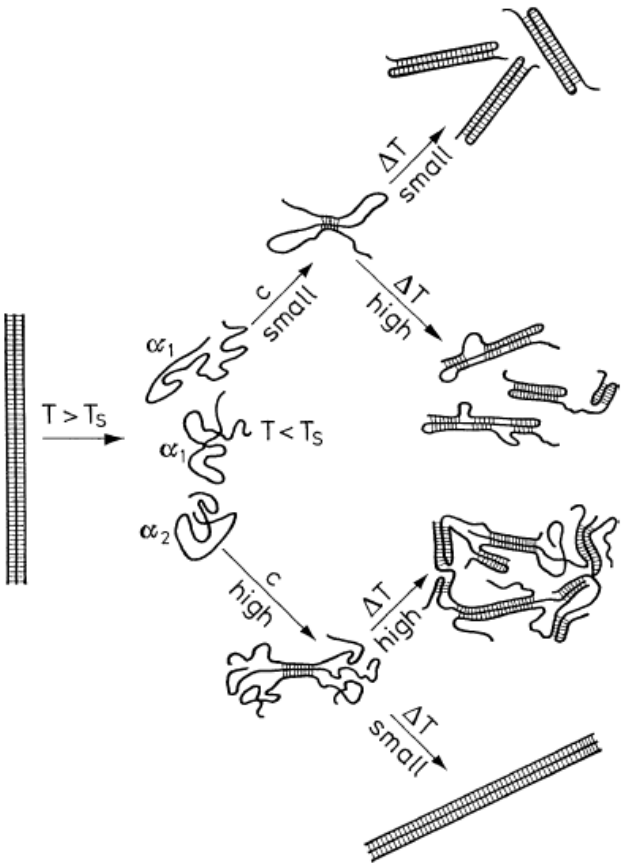

Figure 5. Collagen conversion into gelatine. Ts: shrinkage temperature, T: temperature, c: concentration. Figure obtained from Belitz et al., 2009.

The main factors that determine the gel strength of a gelatine are its structure and hydroxyproline content which vary between the animal species from which the collagen is extracted, and extraction conditions (alkali/acid concentration, time and temperature). Extreme extraction conditions tend to degrade the collagen further, producing weaker gels. (Gómez-Guillén et al., 2002; Haug & Draget, 2009) .

The content of proline and hydroxyproline is a determining factor for the gelling properties and thermal stability of gelatine due to its role as a structural amino acid. (Haug & Draget, 2009). As with collagen, the hydroxyproline content contributes with the increase in strength of gelatine due to its -OH residues, these interact with water molecules allowing for the creation of hydrogen bonds. The pyrrolidine ring present in proline and hydroxyproline give rigidity to the triple helix structure, which contributes to the thermal stability of the gelatine (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011; Haug & Draget, 2009; Wasswa, Tang, & Gu, 2007).

1.3.2 Gelatine extraction

According to Haug and Draget (2009), the extraction process is divided in several steps: first the collagen-rich raw material is selected and washed to remove impurities. After washing, a pre-treatment is used in the raw material, this is usually an acid or alkaline pre-treatment. Depending on the pre-treatment used, different gelatines are obtained. An acid pre-treatment will give rise to a type A gelatine, whereas an alkaline will give rise to a type B gelatine.

1) Pre-treatments

a. Acid pre-treatment (Type A gelatines)

The material is immersed in acid (pH 1.5-3.0) for 8-30 hours, then it is washed with water until neutralised and reaching the desired pH level. Some examples of used acids are: acetic acid, hydrochloric acid, citric acid and sulphuric acid. (Zhou & Regenstein, 2005). The acid pre-treatment is milder than the alkaline pre-treatment. It is used in tissues where the crosslinks of collagen molecules is not as extensive, such as fish skins (Karim & Bhat, 2009).

b. Alkaline pre-treatment (Type B gelatines)

The material is immersed in an alkaline substance (usually NaOH or $Ca(OH)_2$) for a period of 20 days – 6 months, with agitation. It is then washed with water until neutralised, and finally treated with dilute acid to reach the desired pH level.

Unlike the acid pre-treatment (which does not affect the amino acid profile of collagen), the alkali pre-treatment used to obtain type B gelatine causes deamidation of glutamine and asparagine residues (Haug & Draget, 2009). This is why type A gelatines are positively charged and have an isoelectric point between pH 6.0 – 8.0, whereas type B gelatines are negatively charged (glutamate and aspartate present) and their isoelectric point lies between pH 4.7 – 5.3 (Wang et al., 2014).

After the pre-treatment process, the gelatine extraction process begins, followed by concentration of the material. The pre-treated material is subjected to a series of extractions with hot water. The temperature of the water is usually 40 - 80°C for fish, and 55 - 100°C for mammalian gelatine, although it has been observed that water at room temperature can be used in the gelatine extraction from for cold water fish species (Eysturskarð, Haug, Elharfaoui, Djabourov, & Draget, 2009). These extractions will produce the fragments mentioned previously (α-, β-, γ-chains). The concentration of the solution is done by evaporation, and the

resulting product will be very viscous. Following the concentration process, the extract is filtered, sterilised, and dried in a sterile, de-humidified environment. Once dried, it is milled into particles reaching up to 10 nm.

1.3.3 Gelatine quality

Gelatine is used in a wide range of different applications, even within the same industry. Each of these applications requires gelatines with specific properties. The different aspects used to determine the quality of gelatine and some of the methods used for this purpose are listed below.

1.3.3.1 Gel strength

The gel strength of gelatines is measured by the bloom test. According to the standardised method (Gelatine Manufacturers of Europe, 2017), the gel strength (or Bloom) is determined by preparing a 6.67% (w/v) gelatine solution, which is then matured at 10 °C for 17 hours. A 4 mm plunger is then depressed into the gel, and the mass used is measured. The plunger, gelometer, and bloom jar used are standardised, as well as the dilution process of the gelatine solution. Arnesen and Gildberg (2007) studied the gel strength of gelatine extracted from Atlantic salmon skin. The authors pointed out that the bloom test does not accurately represent the potential gel strength of fish gelatines, which show a higher strengthening rate during storage compared to porcine gelatine. Moreover, the maturation time and temperature of this method is not compatible with some gelatines from cold water fish, which do not gel at these conditions (Haug & Draget, 2009).

1.3.3.2 Viscosity

The viscosity of gelatine depends on its molecular weight distribution (high fractions lead to higher viscosities), however, this does not affect the gel strength (Boran & Regenstein, 2010). Different products will require different viscosities, low viscosity gelatines are preferred in moulded desserts, while a gelatine with high viscosity is preferred as a stabilising agent. The viscosity of a gelatine solution can be measured (as a function of time and temperature) to determine the thermal stability of the gelatine, this is done using rheometers/viscometers (Schrieber & Gareis, 2007). The Gelatine Manufacturers of Europe (2017) describe the standard determination of viscosity as the measurement of flow time of a 6.67% gelatine solution (100 ml at 60°C) through a standard bloom pipette.

1.3.3.3 Melting and gelling points

Gelatine is very useful in the food industry because it melts easily in the mouth, improving the texture and flavour releasing properties of foodstuff (Wang et al., 2014). In addition to the gelling temperature, the gelling time is important in industries where the gel must set rapidly (e.g. photography) (Schrieber & Gareis, 2007). Cold water fish species tend to yield gelatines with lower gelling and melting temperatures than warm water fish species (Gómez-Guillén et al., 2011). The melting point and gelling point of gelatine can be determined using rheological methods such as temperature sweep tests (Boran & Regenstein, 2010; Gudmundsson, 2002).

1.3.3.4 Molecular weight distribution

Because gelatine is obtained from the hydrolysis of collagen into different sized fractions, gelatine solutions are considered polydisperse. Milder conditions during extraction lead to a higher content of high molecular weight fractions (Gómez-Guillén et al., 2011). Eysturskarð, Draget, Ulset, and Haug (2009) suggested that gelatines with a high content of high molecular weight molecules, and low polydispersity index have better gelling properties. This is due to the low molecular weight fractions counteracting the network-forming process. The molecular weight distribution of gelatines is determined by chromatographic methods (GPC, SEC-MALLS), although the SDS-PAGE technique is also used (not quantitatively) (Boran & Regenstein, 2010; Haug & Draget, 2009).

1.3.3.5 Amino acid composition

Since hydroxyproline is found in collagenous tissues, the determination of this imino acid is used as a quantitative and qualitative analysis of gelatine products. The acid hydrolysed sample is analysed in a photometric assay where the hydroxyproline molecules produce a red pigment after a series of reactions (Leach, 1960; Schrieber & Gareis, 2007).

Although hydroxyproline is the main indicator of quality in gelatines, other amino acids are also of interest due to their involvement in electrostatic interactions in the gelling process. Glutamate, aspartate, lysine, hydroxylysine, arginine and histidine are examples of these amino acids (Boran & Regenstein, 2010). The presence of these charged amino acids allows for gelatine to be dissolved in water (Haug & Draget, 2009). The properties of these amino acids (hydrophobicity and structure) also affect

other aspects of gelatines, such as film formation, water vapor permeability and surface properties (Gómez-Guillén et al., 2011). The amino acid composition is usually obtained using high-performance liquid chromatography (HPLC) analysis.

The pH, water content, colour/lightness (measured with a spectrophotometer), ash content, yield, and texture (Texture Profile Analysis with a texturometer) are other characteristics of gelatine used to determine quality.

1.3.4 Functional properties of gelatine

Gelatine has different functional properties that are of interest depending on the application. Apart from its gelling property, the most important functions of gelatine rely on its surface, film-forming and microencapsulating properties.

1.3.4.1 Surface properties

The hydrophobic and hydrophilic amino acids present in gelatine allow it to act as a stabilizer, reducing surface tensions. In the case of foaming and emulsifying, hydrophobicity is one of the most important properties since it allows for the proteins to interact in these hydrophobic areas and form a film between the air/oil and aqueous phase (Belitz et al., 2009). Gelatine is used as a foaming and emulsifier agent because it contains the necessary hydrophobic and hydrophilic amino acids. In addition, gelatine also contributes with increasing the viscosity of the solutions, which improve its foaming properties (Schrieber & Gareis, 2007).

1.3.4.2 Film-forming properties

Biodegradable films for the packaging of foods are being evaluated as an alternative to plastic based films. These films protect the food from external agents that could increase deterioration. Fish gelatine films have been studied for this purpose due to their desirable qualities (transparency, water solubility and extensibility) (Wang et al., 2014). Since fish gelatine contains lower amounts of imino acids, it is more hydrophobic than mammalian or warm water fish gelatine. This property makes fish gelatines films have a lower water vapor permeability than other gelatines, but also increases their deformability. Other biopolymers, cross-linkers, antioxidants or antimicrobial materials can be added to gelatine films to improve their performance for the use of packaging of foodstuffs (Gómez-Guillén et al., 2009).

1.3.4.3 Microencapsulation

Microencapsulation is the process where a material (core) is encapsulated in a layer of another material (shell), as means to protect it or control its release (Madene, Jacquot, Scher, & Desobry, 2006). One way to create this shell material is by using the principle of coacervation, where a mixture of polyelectrolytes with opposite charges neutralize each other and separate from the liquid phase, surrounding the core material (Benita, 2005). Gelatine can be used in this technique, usually along gum arabic, because below pH 5 gelatine will have a positive charge while gum arabic is negatively charged (Schrieber & Gareis, 2007). Microencapsulation has many uses, among these: flavours and volatile products can be encapsulated for flavour release, oily substances can be protected from oxidation, and drugs and probiotic bacteria can be protected from the conditions in the stomach or during manufacturing (Gómez-Guillén et al., 2011). Fish gelatine is useful in microencapsulation processes because it allows for a reduction in time and temperature during these reactions, which is also an advantage when temperature sensitive molecules are involved (Piacentini, Giorno, Dragosavac, Vladisavljević, & Holdich, 2013).

It is important to remember that these functional properties depend on the amino acid composition and molecular weight distribution of the gelatines. The amino acid composition will vary between species, and it is affected by the season and feeding patterns of the animals used as raw materials. Also, the extraction treatments affect the molecular weight distribution of the gelatines.

1.3.5 Fish gelatine

Collagen from cold water fish has a lower content of hydroxyproline than warm water fish and mammalian gelatine. Due to this, there are less intra- and interchain hydrogen bonds in the collagen coming from these species, and these molecules are more susceptible to degradation during extraction treatments (Gómez-Guillén et al., 2002). The content of hydroxyproline in connective tissues varies depending on the temperature of the species' habitat, colder temperatures lead to lower amount of hydroxyproline (Arnesen & Gildberg, 2002).

The gel strength of a gelatine is greatly influenced by the amount of hydroxyproline in the collagen, this is why cold water fish yields gelatines with lower gel strength than warm water fish or mammals (Haug, Draget, & Smidsrød, 2004).

Gelatine films obtained from fish show a greater elasticity and lower water vapor permeability, although less strength than films from mammalian gelatine. The lower water vapor permeability is expected due to the higher hydrophobicity in gelatines from cold water fish (Gómez-Estaca, Montero, Fernández-Martín, & Gómez-Guillén, 2009).

Cold water fish collagen has also shown a lower melting temperature than mammalian collagen, due to this, the temperature needed to extract gelatine from cold water fish is much lower than for mammalian gelatine (Eysturskarð, Haug, Elharfaoui, et al., 2009; Joly-Duhamel, Hellio, & Djabourov, 2002). Gudmundsson (2002) suggested that cod gelatine can be used as a thickening agent, because of its high viscosity.

1.1 Aim of the project

The aim of this project was to evaluate heat treatment as a method for oil extraction and compare two gelatine extraction methods using salmon viscera as a raw material, with a focus on yield and quality.

2. Materials and methods

2.1 Oil extraction

Thermal treatment of the raw material (salmon viscera with and without roe and milt) was carried out to evaluate the effect of temperature in the separation of the lipids from the raw material.

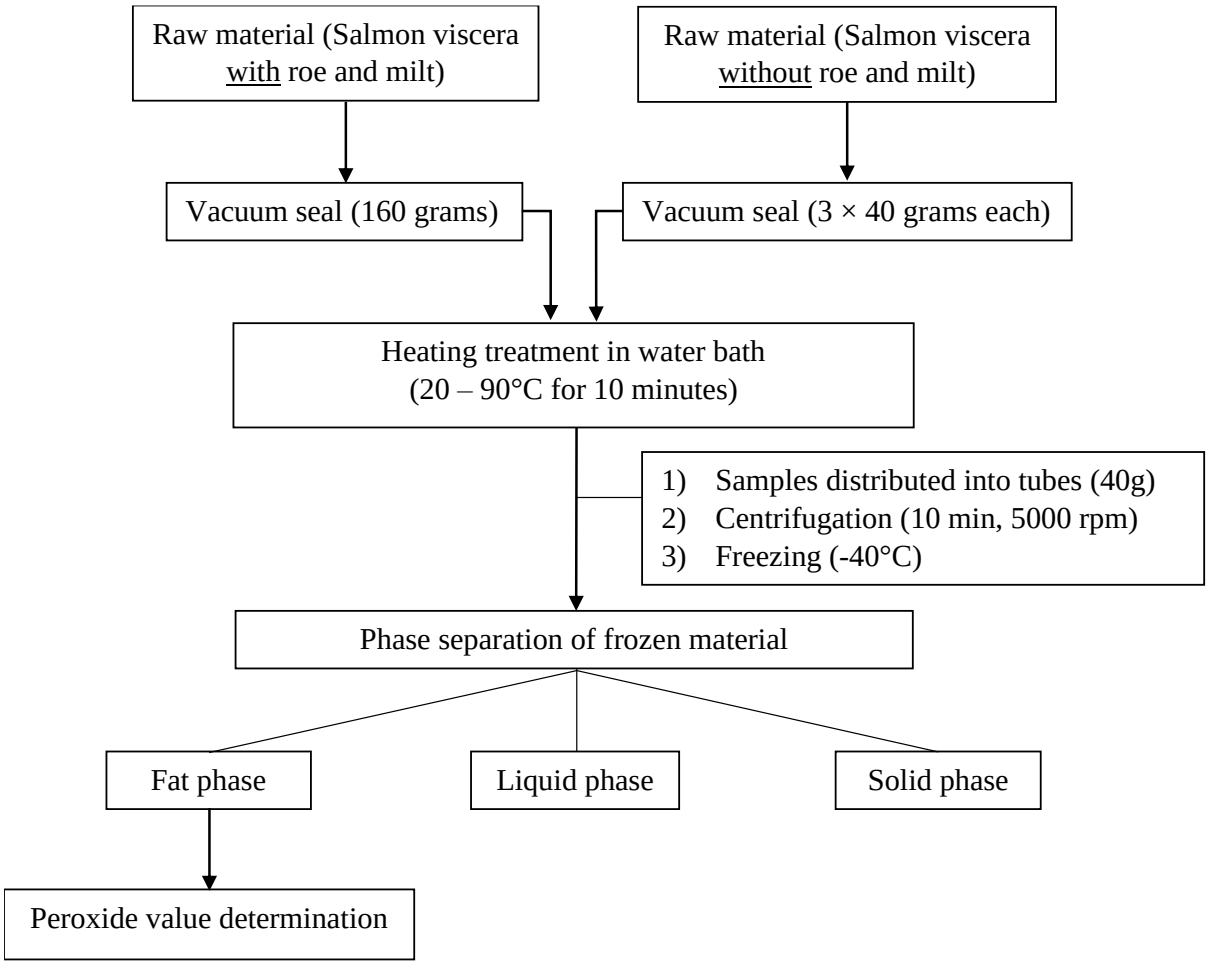

Figure 6. Flowchart of the heat pre-treatment procedure for the salmon viscera (with/without roe and milt) prior to the extraction and analysis of oils.

For the heating treatment of the viscera without roe and milt, the raw material (160 g) was packed in a vacuum bag before heating. After heating, the treated material was distributed into three centrifuge tubes (40 g each). In the heating treatment of the viscera with roe and milt, the raw material was distributed in vacuum bags before heating (40 g in each bag). Once packed, the bag with the sample was placed in a water bath for 10 minutes. The temperatures tested were: 20, 30, 40, 50, 60, 70, 80, and 90 °C. The samples heated to 90°C were heated using a

microwave instead of a water bath due to problems obtaining a sufficiently high temperature using the water bath.

After 10 minutes of heating, the bag was opened and the temperature of the sample (in the bag) was measured and registered. The sample was transferred to a centrifuge tube and centrifuged (10 minutes, 2300×g) using the corresponding temperature (up to 40 °C). The centrifuge used had an upper limit in temperature at 40°C, therefore the samples heated at 40 °C and above were centrifuged at 40°C. The tubes with the centrifuged sample were frozen (-40°C) overnight and the phases (fat, liquid and solid) were manually separated from the frozen sample. The amount of each phase obtained was registered, and the percentage of dry matter was determined for all liquid and solid phases.

The oil phase obtained for each temperature was collected, nitrogen gas (N_2) was added to the tubes and the samples were stored at -80°C until further analysis.

2.1.1.1 Proximate composition and mass balance

The percentage dry matter for each phase obtained during the heat treatment of the salmon viscera (both with and without roe and milt), and the chemical composition of the raw materials were calculated. The dry matter content was determined gravimetrically by drying the samples at 105°C overnight.

The mass balance was calculated for both types of raw material and for each temperature used in the heat treatment. The dry matter and amounts obtained of each phase were used to obtain the mass balance. The following formula was used to calculate the mass balance:

Equation 1. Mass balance calculation

$$\begin{aligned} &\%DM\ in\ raw\ material\ \times wet\ raw\ material\ (g) \\ &\quad = Fat\ phase(g) \times \%DM\ in\ fat\ phase \\ &\quad + Emulsion\ phase\ (g) \times \%DM\ in\ emulsion\ phase \\ &\quad + Liquid\ phase\ (g) \times \%DM\ in\ liquid\ phase \\ &\quad + Solid\ phase\ (g) \times \%DM\ in\ solid\ phase \end{aligned}$$

Where:

%DM = percent dry matter

Proximate composition of raw material

The dry matter of both types of raw material was obtained gravimetrically, the viscera sample (with oil) was dried overnight at 105°C (4 parallels were used). In addition, the viscera were heat treated to separate the solid, liquid and oil phases and calculate the dry matter for each phase. The heating of viscera without roe and milt was done at 25°C, and dry matter for each phase was determined gravimetrically. In the case of the viscera with roe and milt, the heat treatment was done at 20°C and 30°C. The dry matter values for each phase (obtained at 20°C and 30 °C) were determined gravimetrically, and the values were averaged for each phase.

The protein content (%) in the raw material was assumed to amount to the dry matter content left after separation of the oil. The dry matter of the solid and liquid phases was used as an estimation of the protein content in the raw material. The protein content and the total lipid content (obtained using the Bligh & Dyer method, 2.1.2) were summed to obtain the "calculated" mass balance. This value was compared to the dry matter of the raw material previously obtained gravimetrically (drying the viscera with oil in the sample).

2.1.2 Total lipid content

The total lipid content (TLP) of the sample was determined to calculate the yield of lipids extracted with the thermal treatment of the raw material at various temperatures. The method used for the determination of TLP in the raw material is a modified version of the Bligh and Dyer (1959) method.

The lipid was extracted from the salmon viscera (with and without roe and milt) and the samples were analysed in duplicate. For each sample, 10 g were weighed out in centrifugation bottles and these bottles were placed on ice thereby decreasing evaporation. Distilled water (16 ml), methanol (40 ml), and chloroform (20 ml) were added to the bottles and homogenised with an ULTRA-TURRAX for two minutes. Chloroform (20 ml) was then added and the mixture was homogenised for 40 seconds. Finally, distilled water (20 ml) was added and the mixture was homogenised for 40 seconds. The centrifugation bottles were then balanced using distilled water and centrifuged at 2300×g for 15 minutes.

To determine the percentage of total lipids, two parallels (2 ml) of the chloroform phase were pipetted into pre-weighed tubes. These tubes were placed in a heat block at 60°C and the chloroform was evaporated using nitrogen gas (N_2). After vaporization, the samples are cooled

down in a desiccator overnight, and weighed. To calculate the percentage of total lipids, the following equation was used:

Equation 2. Percentage of total lipids calculation in sample of salmon viscera

$$\%\, Total\ lipids = \frac{oil\ after\ evaporation\ (g) \times 40\ ml\ \times 100}{2\ ml\ \times sample\ used\ for\ extraction\ (g)}$$

2.1.3 Peroxide value determination

The peroxide value (PV) of the oils extracted from thermal treatment of viscera was determined. This value was used to observe the effect of the extraction temperature on the oxidation and therefore the quality of the oils.

The PV was determined by iodometric titration, which corresponds to the AOCS Official Method Cd 8b-90(1). The instrument used in this method is a SI Analytics titrator (TL 6000/7000) with a platinum electrode (Pt 62/61).

2.1.3.1 Sample preparation for PV determination

The oils extracted from thermal treatment of the viscera were used for PV determination. Each analysis was done in triplicate. Oil samples (5 g) were placed in centrifuge tubes and double distilled water (0.15 g) was added and mixed. The samples were centrifuged (15 minutes, 7500 ×g) and the upper layer was filtered (Whatman 0.2µm).

2.1.3.2 PV determination

Each oil sample (2 g), was dissolved in the solvent (30 ml, acetic acid/chloroform 3/2 mixture). Next, a freshly prepared saturated solution of potassium iodine (0.5 ml, 1 g KI in 1.3 g ddH_2O) was added and the mixture mixed for 60 seconds. Doubly distilled water (30 ml) was then added, the electrode and the titration tip immediately placed in the sample solution and the titration was performed. The titration agent was a freshly prepared 0.01 M sodium thiosulphate ($Na_2S_2O_3$) solution.

The peroxide value (meqO_2/kg oil) was calculated using the following equation:

Equation 3. Peroxide value calculation from oil sample

$$PV = \frac{(V - B) \times 0.01M \times 1000}{oil\ sample\ weight\ (g)}$$

Where:

V = volume of titrant consumed during iodometric titration of the sample (ml)

B = volume of titrant consumed during titration of blank (ml)

2.1.4 Free fatty acid content

The free fatty acid (FFA) content of the oil extracted from the heat treatment of viscera with roe and milt was determined using the method by Bernárdez, Pastoriza, Sampedro, Herrera, and Cabo (2005). The samples were analysed in triplicate.

The oil sample (around 0.1 g) was weighed out into a Kimax tube, 5 ml of isooctane was added and the mixture was vortexed. To the tubes, 0.5 ml of the aqueous reagent (5% cupric acetate-pyridine) was added and the tubes were closed, the mixture was vortexed (30 seconds) and centrifuged (5 minutes, 2000 ×g). The absorbance of the upper layer was then measured at 715 nm against isooctane. The oleic standards were prepared (from 0 to 20 µmol oleic acid) and analysed using the same steps described previously.

The FFA content (% FFA as oleic acid) was obtained using the following equation:

Equation 4. Calculation of Free fatty acid (FFA) content

$$\%\ FFA = \frac{(Abs - B) \times 282.46}{A \times m\ \times 10000}$$

Where:

Abs = absorbance of the sample

B = y-intercept of the linear regression (Absorbance of blank)

282.46 = molar weight of oleic acid (g/mol)

A = slope of the linear regression (1/µmol)

m = mass of the sample (g)

10000 = conversion units to obtain %

2.2 Gelatine extraction

The following gelatine extractions were performed on the salmon viscera without roe and milt. Two methods of gelatine extractions were used: gelatine extraction using salt pre-treatment (method by Kołodziejska et al. (2008) with modifications) and gelatine extraction using acid pre-treatment (method by Arnesen and Gildberg (2007) with modifications). In previous investigations of extraction from salmon skins, these methods have shown a higher gelatine yield compared to others (Unpublished data).

To evaluate the effect of the presence of oil on the efficiency of the gelatine extraction, the gelatine was extracted from viscera with the fat phase present and from viscera where the fat phase had been separated through thermal treatment

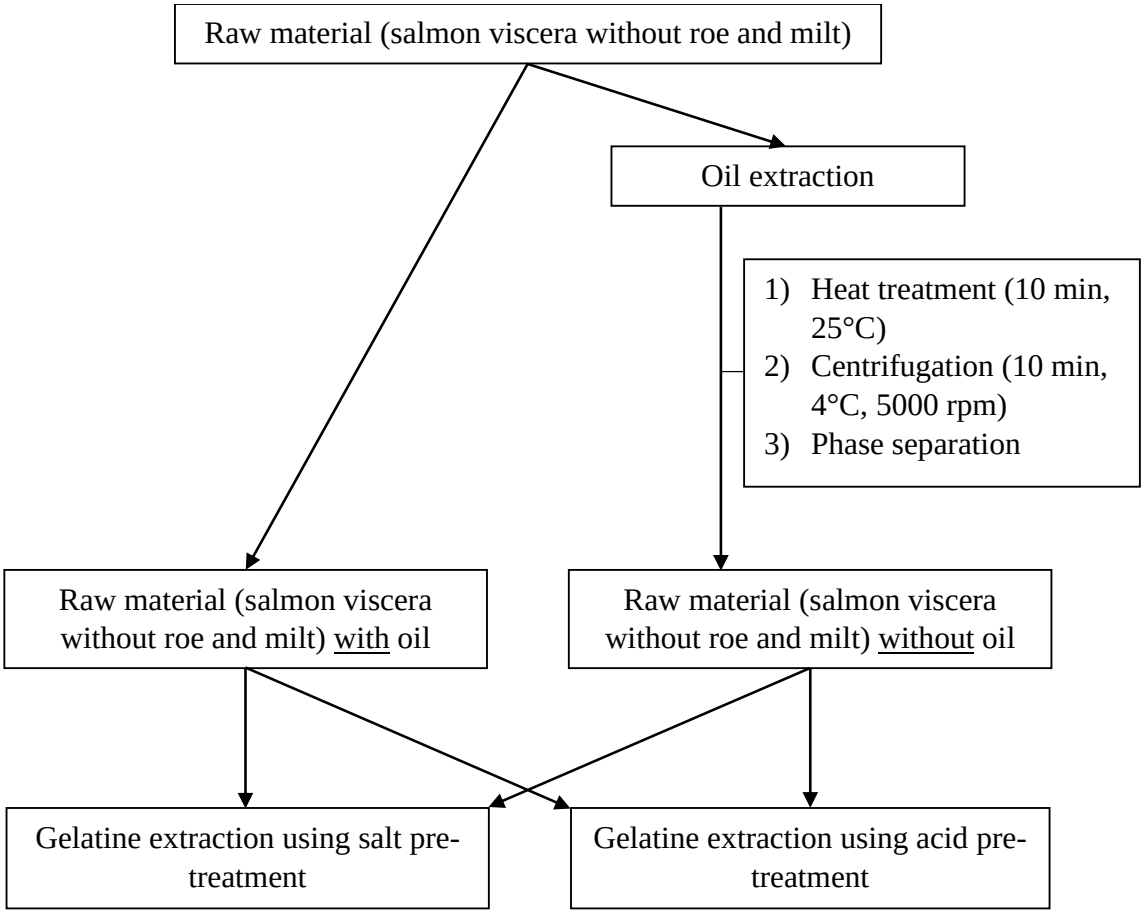

Figure 7. Flowchart of the preparation of the raw material prior to gelatine extraction. A portion of the salmon viscera (without roe and milt) was heat-treated to extract the fat phase. Both viscera (oil and no oil) were used in gelatine extractions with different pre-treatments.

The viscera without roe and milt was divided into two parts. On one part, the extraction process was done directly on the raw material. On the other part, thermal treatment was used to separate the fat phase before extraction. The viscera were heated (25 °C for 10 minutes) and centrifuged (10 minutes, at 4°C and 2300×g), the fat phase was separated and stored (-40°C) and the liquid and solid phases were gathered for gelatine extraction.

The following procedures (2.2.1 and 2.2.2) were conducted in duplicate, the pre-treatment was conducted at 4°C in a cold room and all solutions used were cooled overnight at 4°C. These solutions were added to a 6:1 v/w ratio in the salt pre-treatment and 3:1 v/w ratio in the acid pre-treatment. During the filtration steps, the discarded washes (solutions of NaCl, NaOH,

ethanol etc.) were weighed, and a portion was used to determine the percentage dry matter in the discarded wash.

2.2.1 Gelatine extraction with salt pre-treatment

The first pre-treatment method for gelatine extraction used was salt washing of the raw material. The purpose of the pre-treatment process is to remove non-collagenous proteins and to facilitate the extraction of the collagen molecule (Boran & Regenstein, 2010). The hydration shell of the collagen molecule is destabilised by the ions in the salt, allowing for the structure to be available during extraction (Giménez, Gómez-Guillén, & Montero, 2005).

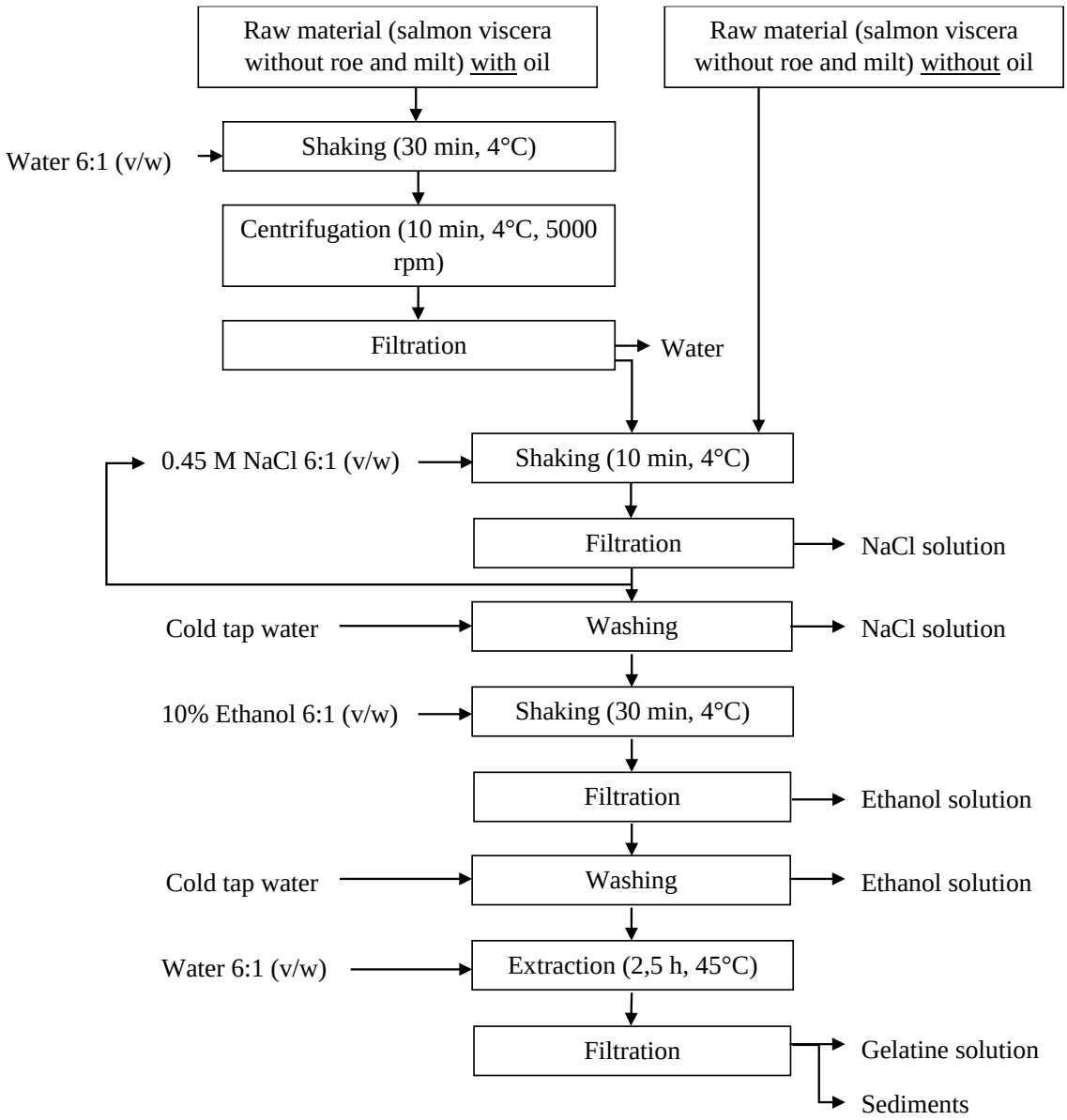

Figure 8. Flowchart of the procedure used to extract gelatine from the salmon viscera. The viscera (oil and no oil) was pre-treated with a salt solution and ethanol prior to the extraction of gelatine.

The raw material containing the oil phase was treated with water before the salt pre-treatment. Cold tap water was added to the raw material and the mixture was shaken (30 min). The mixture was centrifuged (10 minutes, at 2300×g) before filtration, after which the wash water solution was weighed and discarded. The remaining sediments were used in the following procedures.

A salt solution (0.45 M NaCl) was added to the viscera and the mixture was shaken for 10 minutes, then filtered and the salt wash was weighed and discarded. This step was repeated two more times. The viscera were then washed with cold tap water (for two minutes) to remove the excess salt solution. Following this, an ethanol solution (10%) was added to the viscera and the mixture was shaken for 30 minutes, then filtered and the ethanol wash was weighed and discarded. The viscera were then washed with cold tap water (for two minutes) to remove the excess ethanol solution.

Following this pre-treatment, the gelatine in the viscera was extracted by the addition of water and heating the solution in a water bath (at 45°C, for 2.5 hours) with shaking every 30 minutes. After the extraction step was completed, the mixture was filtered and the gelatine solution obtained was weighed and kept. A portion of the gelatine solution, and the remaining sediments was used to calculate percentage dry matter.

The gelatine solution was filtered through glass wool, frozen overnight and lyophilised to obtain the freeze-dried gelatine extract (SFDGE) used in further analyses.

2.2.2 Gelatine extraction with acid pre-treatment

The second pre-treatment method used was acid pre-treatment, in which an acid solution was used to disrupt the crosslinks in the collagen molecule and make it available for extraction (Schrieber & Gareis, 2007).

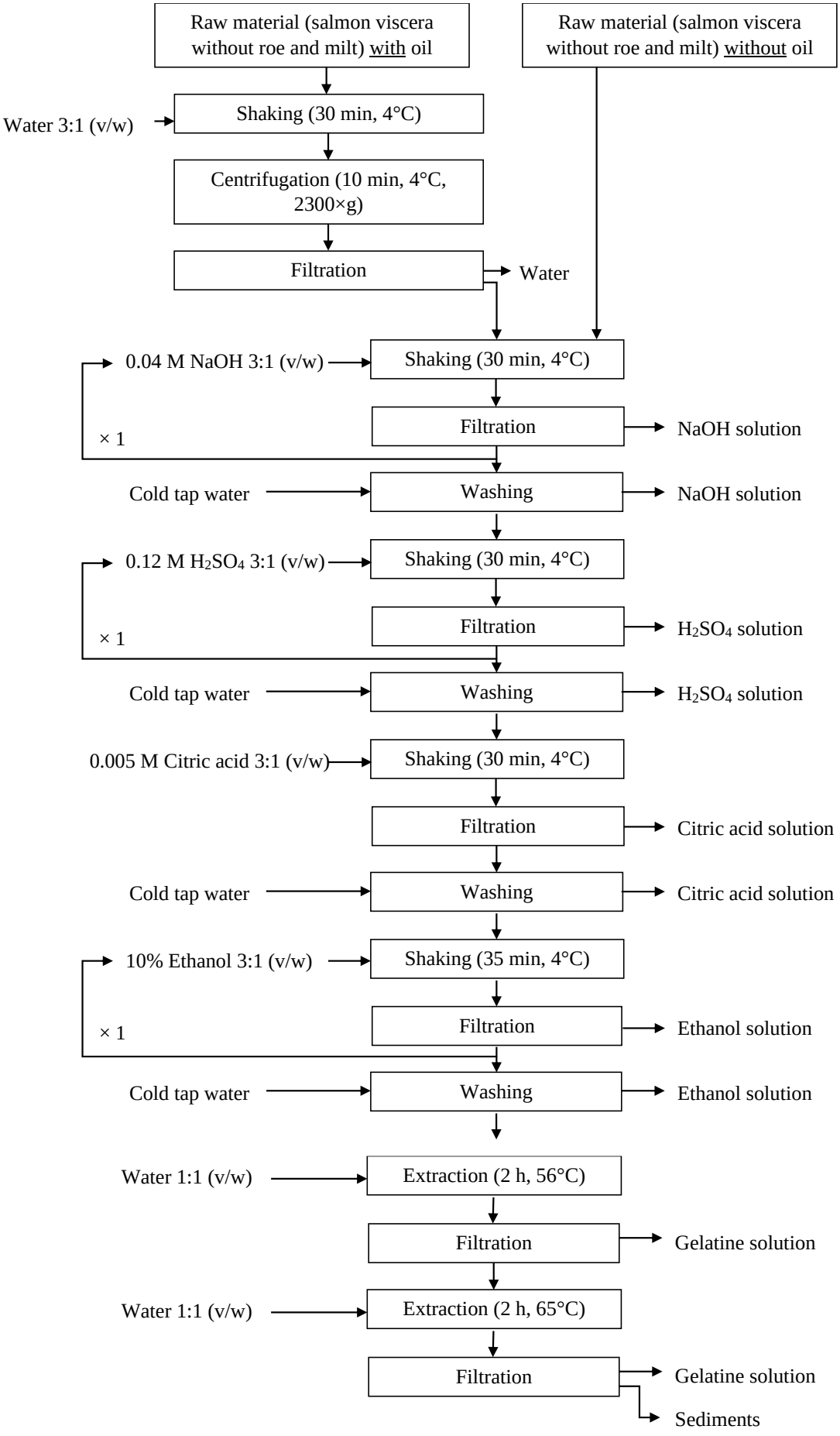
Raw material (salmon viscera without roe and milt) with oil
Raw material (salmon viscera without roe and milt) without oil
Water 3:1 (v/w)
Shaking (30 min, 4°C)
Centrifugation (10 min, 4°C, 2300×g)
Filtration
Water
0.04 M NaOH 3:1 (v/w)
Shaking (30 min, 4°C)
Filtration
NaOH solution
× 1
Cold tap water
Washing
NaOH solution
0.12 M H2SO4 3:1 (v/w)
Shaking (30 min, 4°C)
Filtration
H2SO4 solution
× 1
Cold tap water
Washing
H2SO4 solution
0.005 M Citric acid 3:1 (v/w)
Shaking (30 min, 4°C)
Filtration
Citric acid solution
Cold tap water
Washing
Citric acid solution
10% Ethanol 3:1 (v/w)
Shaking (35 min, 4°C)
Filtration
Ethanol solution
× 1
Cold tap water
Washing
Ethanol solution
Water 1:1 (v/w)
Extraction (2 h, 56°C)
Filtration
Gelatine solution
Water 1:1 (v/w)
Extraction (2 h, 65°C)
Filtration
Gelatine solution
Sediments

Figure 9. Flowchart of the procedure used to extract gelatine from the salmon viscera. The viscera (oil and no oil) was pre-treated with base, acids and ethanol prior to the extraction of gelatine.

Similarly to method 2.2.1, the raw material containing the oil phase was treated with water before pre-treatment. In this case, cold tap water was added to the raw material in a 3:1 (v/w) ratio and the mixture was shaken for 30 minutes before centrifugation (10 minutes at 4°C) and filtration.

In the acid pre-treatment, a 0.04 M NaOH solution was added to the raw material, the mixture was shaken for 30 minutes, and filtered. This process was repeated one more time. Cold tap water was then used to wash the excess NaOH from the raw material (for two minutes). A solution of 0.12 M H_2SO_4 was added to the raw material, the mixture was shaken for 30 minutes and filtered. This process was repeated one more time. Cold tap water was used to wash the excess H_2SO_4 from the raw material (for two minutes). A 0.005 M citric acid solution was added to the raw material, the mixture was shaken for 30 minutes and filtered, and cold tap water was used to wash the excess citric acid (for two minutes). The final steps of the pre-treatment were two ethanol washes. A 10% ethanol solution was added to the raw material, the mixture was shaken for 35 minutes and filtered. Before the gelatine extraction, the excess ethanol in the raw material was washed with cold tap water (for two minutes).

In this method, two successive gelatine extractions were performed. In the first extraction, water was added to the pre-treated material (1:1 v/w) and the mixture was heated to 56°C for 2 hours (shaken every 30 minutes). The first gelatine solution was then obtained by filtering the mixture. The remaining material was used for the second extraction, in which water was added (1:1 v/w) and the mixture was heated to 65°C for 2 hours. The mixture was then filtered to obtain the second gelatine solution and remaining sediments. Both gelatine solutions were weighed and a portion taken to analyse the percentage of dry matter.

The gelatine solutions were filtered through glass wool, frozen overnight and lyophilised to obtain the freeze-dried gelatine extract (AFDGE) used in further analyses.

2.2.3 Gelatine analysis

The FDGE were hydrolysed and the hydroxyproline contents of the hydrolysed raw material and gelatine extracts obtained after extraction were determined using the method Neuman and Logan (1950) with modifications by Leach (1960).

2.2.3.1 Hydrolysis

The FDGE samples were hydrolysed by weighing 50 mg and adding 1 ml HCl (6 M) to the sample in a flat-bottomed tube. The tube was kept closed at 105°C for 22 hours. After this, the contents were transferred to a 10 ml beaker and the pH neutralised (pH = 7.0). The solution was filtered through a Whatman glass microfibre filter (GF/F) and the volume of each sample was increased to 10 ml with distilled water.

The raw material was pre-treated prior to hydrolysis to separate the oil and moisture. The raw material was heated to 25 °C for 10 minutes in a water bath and then centrifuged (10 minutes, 4°C, 2300×g). After the oil separation, the raw material was freeze dried and blended to obtain a homogeneous mix of sediments. This was done to get more representative results after hydrolysis. The hydrolysis process of the raw material was the same as the hydrolysis of the FDGEs, but 100 mg of raw material and 2 ml HCl (6M) were used in the first step.

2.2.3.2 Hydroxyproline content

A stock solution of L-hydroxyproline was prepared (100 µg/ml) and diluted to 3, 5, 10 and 15 µg/ml to be used as standards. Triplicates of the samples (hydrolysed FDGEs and raw material) and the standards (0.5 ml) were placed in separate test tubes. To these the following was added: 0.05 M $CuSO_4$ (0.5 ml) and 2.5 M NaOH (0.5ml), the mixture was shaken. The tubes were covered and placed in a water bath (40°C) for 5 minutes. Then, a freshly prepared solution of 6% H_2O_2 (0.5 ml) was added to the tubes and shaken immediately. The tubes were placed in a water bath (40°C) for 10 minutes. After cooling to room temperature, solutions of 1.5 M H_2SO_4 (2 ml) and 5% *p*-dimethylaminobenzaldehyde in 1-propanol (1 ml) were added to each sample/standard and the tubes shaken immediately. The tubes were placed in a water bath (70°C) for 16 minutes, then cooled to room temperature and mixed, and left for two minutes at room temperature before measuring the OD at 555 nm.

2.2.3.3 SDS-PAGE

The freeze dried gelatine extracts (FDGE) were analysed using gel electrophoresis to observe the molecular weight distribution obtained in the samples. The SDS-PAGE system used was the Dual Cool electrophoresis system DCX-700. The gels used were ClearPAGE precast gels (4-20%). The running buffer used was the ClearPAGE SDS Standard TEO-Tricine Running Buffer. The gels were stained using InstantBlue Protein Stain. The protein molecular weight standards used were the Amersham Low Molecular Weight Calibration Kit and Amersham

High Molecular Weight Calibration Kit, both by GE Healthcare. The component of the standards can be seen in the Tables 11 and 12 in the appendix.

To prepare the samples, distilled water was used to dissolve and dilute the FDGEs to a concentration of 16 mg/ml. The amount of sample obtained from the second extraction using viscera with oil and the acid pre-treatment (AwO2) was too low to use in the gel. The sample buffer (10mM Tris/HCl, 1 mM EDTA, 5% SDS, 2% DTT) was used to dilute the samples further (1:2, 0.5 ml sample buffer and 0.5 ml sample). The mixture was placed in a water bath (70 °C, 10 minutes). After heating, glycerol (0.1 ml) was added to each sample and mixed.

The running buffer was prepared (800 ml) according to the directions from the provider (1:20 dilution of running buffer). The inner chamber of the unit was filled with 200 ml, and the rest was added to the outer chamber. The samples were loaded into the wells (10 µl) and run at 175 V for 45 minutes. After the samples reached the bottom of the gel, the gel cassettes were removed from the unit and rinsed, the gel was removed from the cassette and stained overnight.

3. Results

3.1 Proximate composition and mass balance

The proximate composition of the raw material used in this study was determined. The calculated mass balance (i.e. the sum of the protein content and the TLC, refer to 2.1.1.1) was compared to the dry matter content obtained gravimetrically. The raw material lipids (%) in Table 2 were determined by the Bligh and Dyer (1959) method.

Table 2. Proximate composition and mass balance for salmon viscera (with/without roe and milt)

Raw material	Dry matter (%)	Lipids (%)	Protein (%)	Mass balance[1]
Viscera with roe and milt	53±11	31±1	15±4	46 (53)
Viscera without roe and milt	63±1	46±2	19±1	65 (63)

[1]units: % calculated (% dry matter obtained gravimetrically)

In Table 2 it is seen that both type of viscera (with and without roe and milt) show a higher content of lipids than protein (approximately twice the amount). The viscera with roe and milt has a lower percentage of dry matter and lipids, and a higher percentage of proteins compared to the viscera without roe and milt. The ash content in the raw material is assumed to be less than 1% (Šližytė et al., 2017).

3.2 Oil extraction

3.2.1 Total lipid content

After the heat treatment of the salmon viscera at different temperatures, the amount of oil obtained was compared to the total amount of lipids present in the untreated viscera, which was determined using the Bligh and Dyer (1959) method.

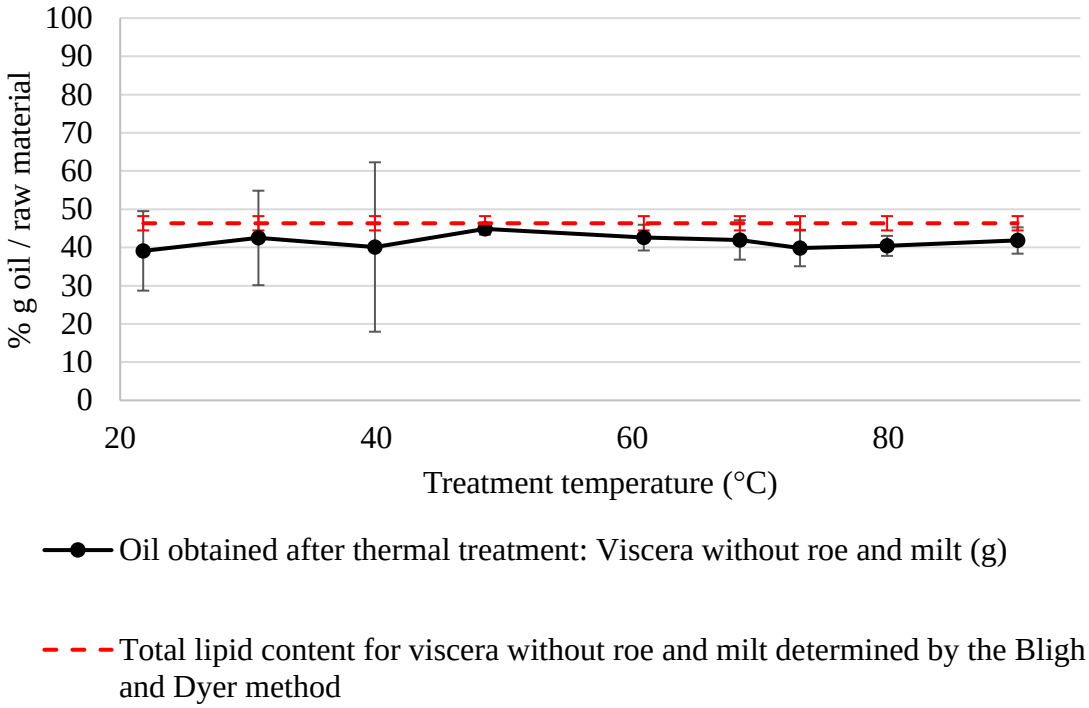

Figure 10. Oil extraction in salmon viscera without roe and milt. The total lipid content (%) of the viscera (determined by the Bligh and Dyer method) is shown with a red dashed line. The percentage of oil obtained by heating at various temperatures is shown with a black line.

In Figure 10, the percentage of oil obtained from the heat-treatment of the viscera without roe and milt is seen along with the total amount of lipids present in the sample. The highest amount of oil was obtained at 48.5°C (44.8%). The TLP in the salmon viscera without roe and milt was found to be 46.3% using the Bligh and Dyer (1959) method.

The standard deviation seen at 39.9°C is larger than the rest due to the non-homogeneous nature of the samples. At this temperature, the heat-treated viscera were divided into three different tubes and an uneven distribution of fat and sediments was obtained in each tube. With the increase of the pre-treatment temperature, the sample homogeneity increased, which is why the standard deviation is lower at temperatures of 48.5 °C and above.

Table 3. Oil yield after thermal pre-treatment of viscera without roe and milt

Pre-treatment temperature (°C)	**Oil yield (%)**
21.8	84.4±22.5
30.8	91.7±26.7
39.9	86.6±47.8
48.5	96.8±3.3

60.9	91.9±7.3
68.4	90.6±11.1
73.1	86.0±10.3
79.9	87.2±5.7
90.1	90.3±7.4

The yields from the extraction of oil from viscera without roe and milt were high. All values obtained were above 84% of TLP. The highest standard deviations were found in the lowest temperatures (21.8 – 39.9 °C), this is in relation to the previously mentioned variation between parallels.

In the heat pre-treatment of the viscera with roe and milt, the raw material was divided into three parallels before heating. This was done to obtain more representative parallels and avoid the uneven division of fat and sediments after heating.

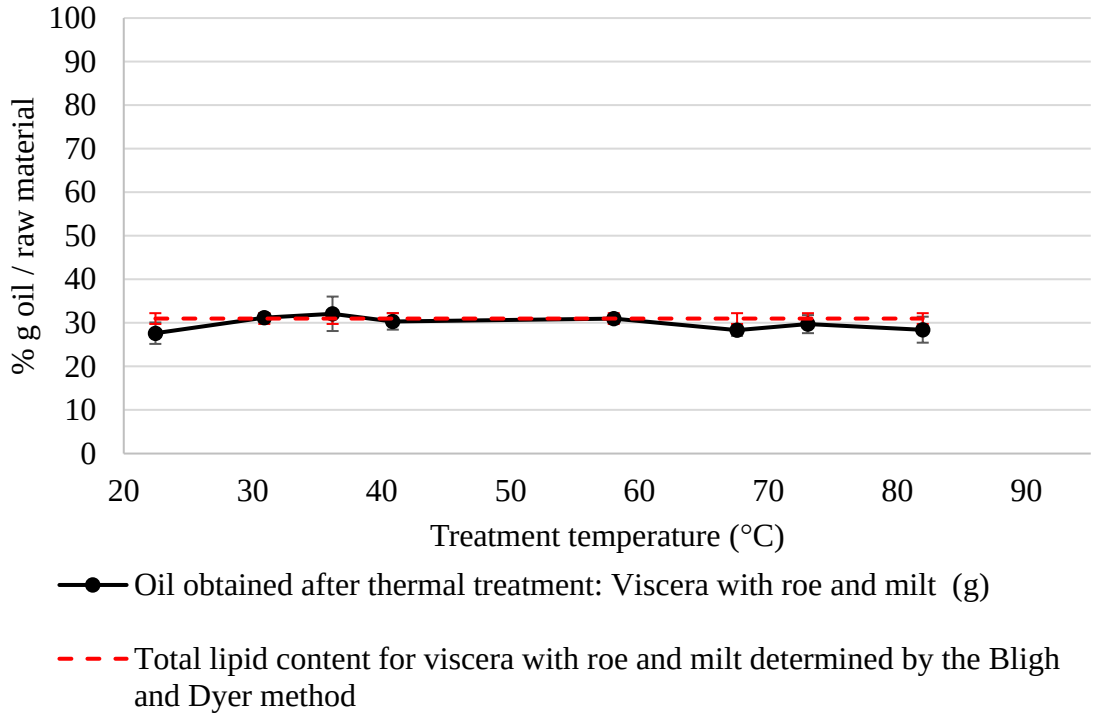

Figure 11. Oil extraction in salmon viscera with roe and milt. The total lipid content (%) of the viscera (determined by the Bligh and Dyer method) is shown with a red dashed line. The percentage of oil obtained by heating at various temperatures is shown with a black line.

In Figure 11, the highest percentage of oil was obtained at 36.2 °C (32.1%). The TLP of the sample was determined to be 31% g oil in the salmon viscera with roe and milt using the Bligh and Dyer (1959) method.

Table 4. Oil yield after thermal pre-treatment of viscera with roe and milt.

Pre-treatment temperature (°C)	Oil yield (%)
22.5	89.2±7.9
30.9	100.7±3.4
36.2	103.5±12.8
40.9	97.9±6.1
58.0	99.9±2.1
67.6	91.3±4.1
73.1	95.9±6.7
82.0	91.8±9.6

The yields from the extraction of oil from viscera with roe and milt were higher than the viscera without roe and milt. All yield values obtained were above 89% of TLP.

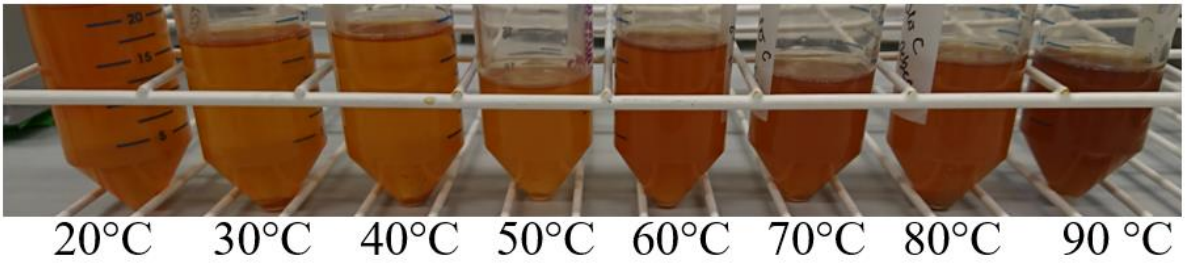

Figure 12. Coloration of oils extracted from viscera (with roe and milt) ranging from 20 °C to 90°C

For both types of viscera (with and without roe and milt), the increase of the extraction temperature caused the oil samples to become darker and darker, this can be seen in Figure 12.

3.2.2 Peroxide value determination

Following the heat pre-treatment of the raw material, the peroxide value of the extracted oil was determined to evaluate the effect of the treatment in the quality of the oil.

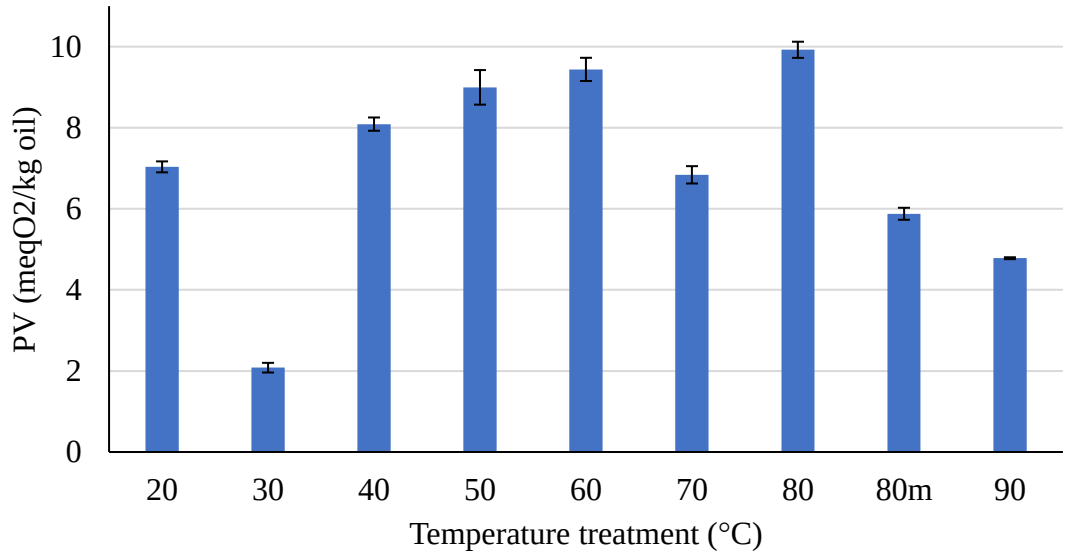

Figure 13. Peroxide value (meqO2/kg) oil of oil samples obtained after heat-treatment of the salmon viscera (without roe and milt) as a function of temperature. The sample named 80m was heated using a microwave instead of the water bath.

The peroxide value (PV) of the oils extracted from the viscera without roe and milt are seen in Figure 13, the highest PV was obtained (9.92 meqO2/kg) when the oil was extracted at 80 °C using the water bath, the lowest PV was seen in the oil extracted at 30°C (2.08 meqO2/kg).

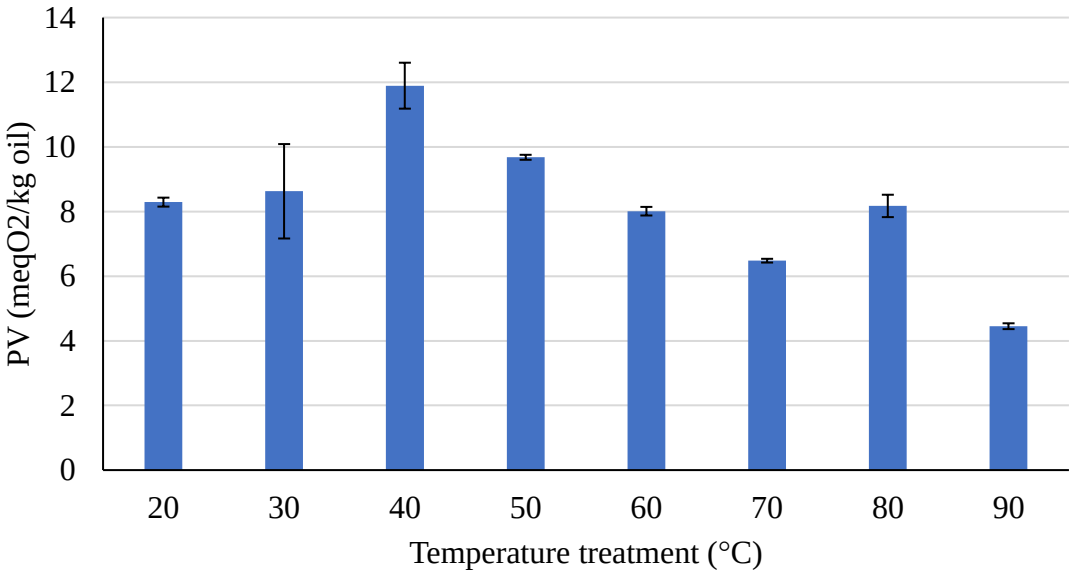

Figure 14. Peroxide value (meqO2/kg) of oil samples obtained after heat-treatment of the salmon viscera (with roe and milt) as a function of temperature.

The PV of oils extracted from salmon viscera with roe and milt can be seen in Figure 14. The oil with the highest PV was obtained at 40 °C (11.89 meqO2/kg), and the lowest was obtained at 90°C (4.45 meqO2/kg).

3.2.3 Free fatty acid content

The free fatty acid (FFA) content is an indication quality in oils. The FFA content of the oil extracted from the heat treatment of viscera with roe and milt was determined.

Table 5. FFA content (%FFA) in oil extracted from the heat treatment of viscera with roe and milt from 22.5 °C – 40.9 °C

Pre-treatment temperature (°C)	FFA (%)
22.5	4.34±0.04
30.9	4.10±0.00
36.2	4.03±0.02
40.9	4.12±0.02

The values of the oils extracted at 60 °C and above had high absorbance levels (outside of standard range). The amount of oil used in this assay was the minimum recommended in the protocol (0.1 g). Therefore, the oil samples contained concentrations of FFA equivalent to more than 20 µmol oleic acid.

3.3 Gelatine extraction

To compare the different pre-treatments and extraction methods used, the freeze-dried gelatine extracts (FDGE) were analysed with regards to yield, purity and molecular weight distribution.

3.3.1 Yield

To evaluate the efficacy of both extraction methods, the yields of freeze dried gelatine obtained from each technique were compared in Figure 15.

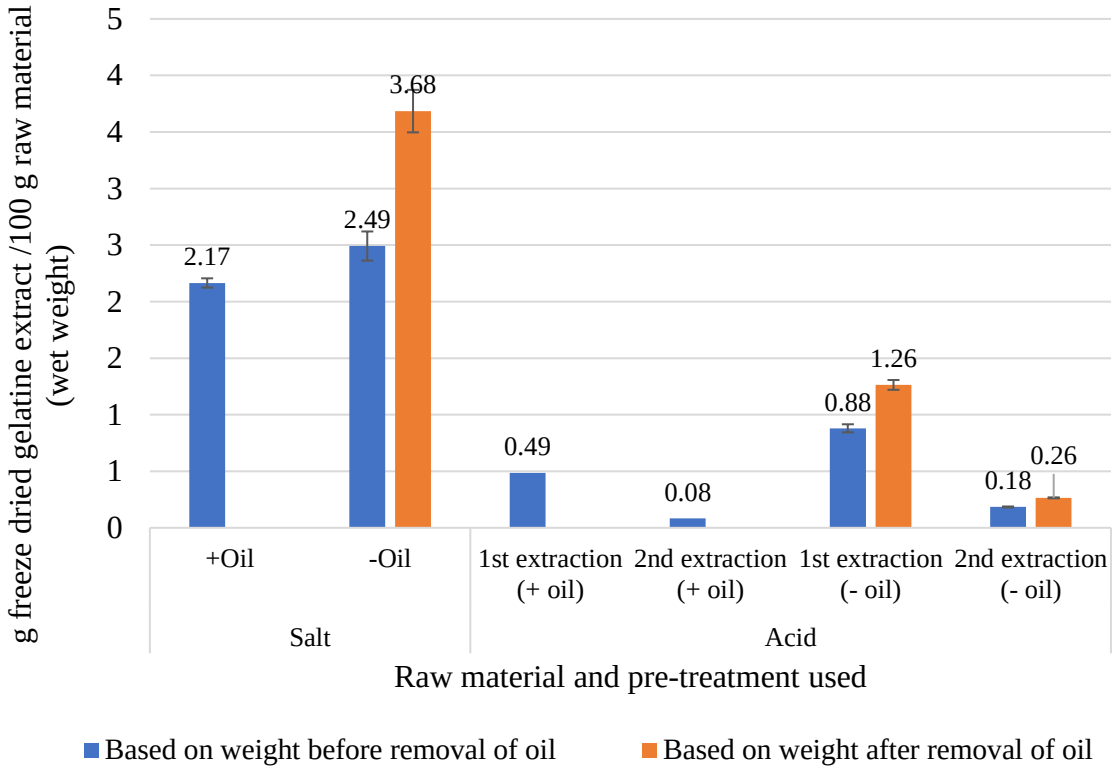

Figure 15. Freeze-dried gelatine extract (g) obtained from (100 g) salmon viscera (with and without oil) using different pre-treatments prior to extraction. The values were calculated based on the wet weight of the sample before the removal of oil (blue), and on the weight of the sample after the removal of oil (orange).

As seen in Figure 15, the salt pre-treatment (SPT) method yielded a higher amount of freeze-dried gelatine extract (FDGE) than the acid pre-treatment (APT) method. In all cases, the viscera containing oil yielded less amount of FDGEs in comparison to their respective viscera where the oil was extracted. The values are calculated based on wet weight of the raw material used in the extractions, both before and after oil separation. As expected, the second extraction in the APT method yields a lower amount of FDGE than the first extraction.

3.3.2 Hydroxyproline content

The hydroxyproline content of gelatine is an indication of its gelling strength and therefore quality. The hydroxyproline content of the gelatine extracts obtained in this study was determined to compare the purity and yield of the gelatine obtained from each pre-treatment and extraction method.

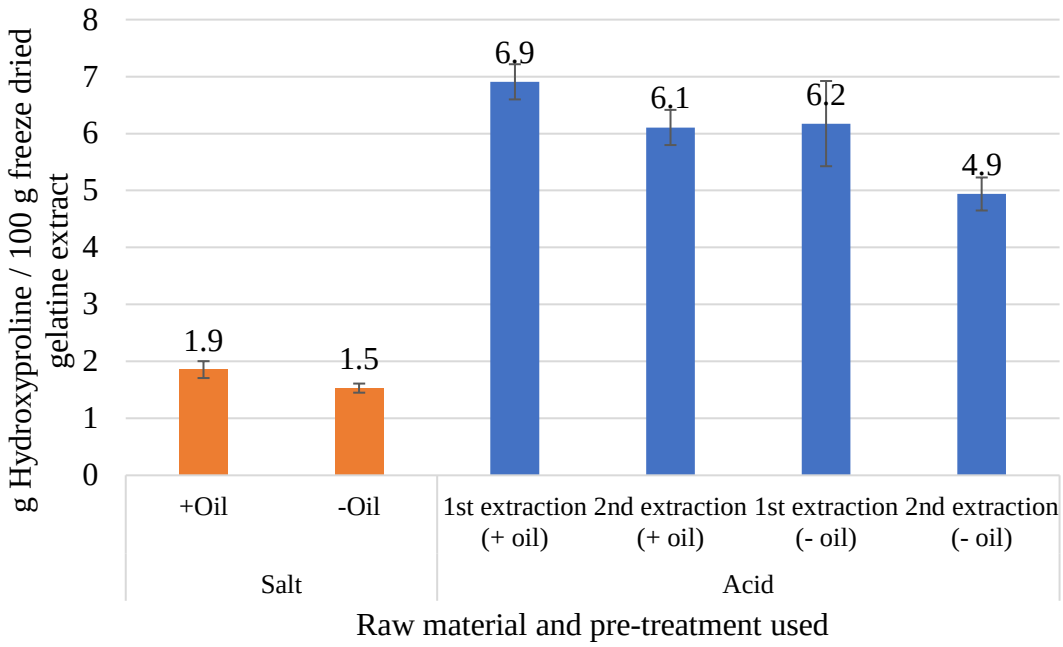

Figure 16. Content of hydroxyproline (g) in the freeze-dried gelatine extract obtained from salmon viscera (with and without oil) using different pre-treatments prior to extraction.

In Figure 16, the content of hydroxyproline in the freeze-dried gelatine extract (FDGE) is shown. The acid pre-treatment extraction method yielded the FDGE with a higher percentage of hydroxyproline than the FDGE obtained with the salt pre-treatment. In both methods, the viscera containing oil yielded FDGE with a higher percentage of hydroxyproline than the viscera from which the oil had been separated. The FDGE sample containing the highest percentage of hydroxyproline was obtained from the first extraction in the acid pre-treatment method, using viscera containing oil.

3.3.2.1 Hydroxyproline yield

The hydroxyproline content in the FDGE was compared to the total amount of hydroxyproline in the raw material.

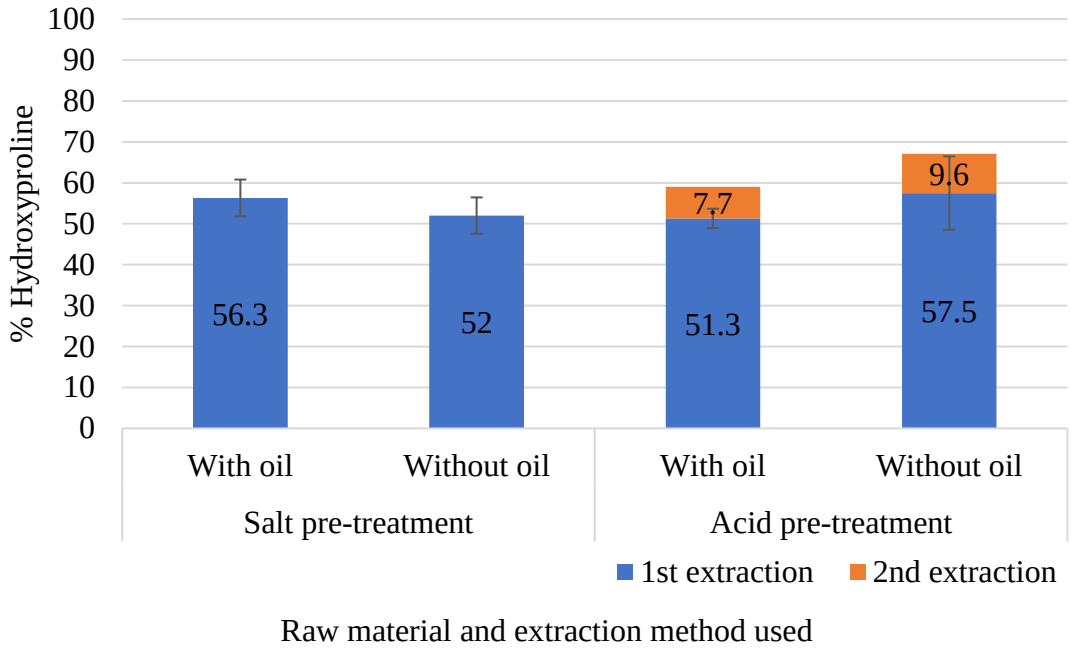

Figure 17. Yield of hydroxyproline from salmon viscera (with and without oil) using different pre-treatments prior to gelatine extraction. The yield is given as % hydroxyproline (hydroxyproline in FDGE compared to hydroxyproline in raw material, based on dry weight). In the samples pre-treated with acid, the blue bars represent the first extraction, the orange represent the second extraction.

Figure 17 shows the yield of hydroxyproline obtained after the pre-treatment and extraction procedures of salmon viscera. In the samples obtained using the APT method, the viscera with oil yielded 59% (of the total hydroxyproline), whereas the viscera without oil yielded 67.1%. The salt pre-treatment was more effective in the viscera with oil, and the opposite was the case for the acid pre-treatment. Only considering the first extraction, the yields are similar between pre-treatments. As expected, the second extraction yield was much lower than the first extraction.

3.3.3 SDS-PAGE analysis

Gel electrophoresis was used to analyse the freeze dried gelatine extracts (FDGE), using a low molecular weight (LMW) and a high molecular weight (HMW) standards.

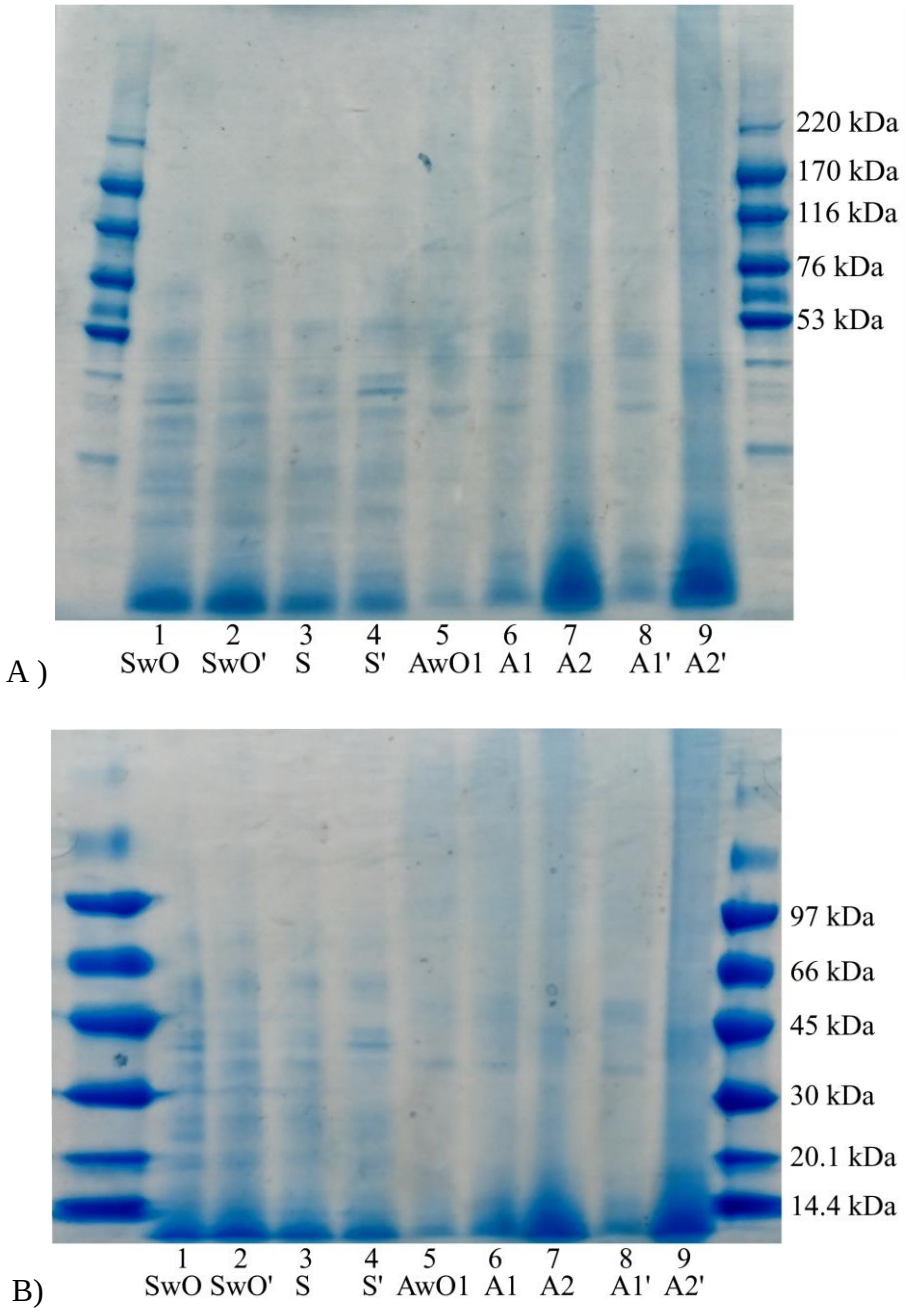

Figure 18. SDS-PAGE patterns of gelatine extracts from salmon viscera obtained with the salt and acid pre-treatments. A) with a HMW standard and B) with a LMW standard. Lanes 1 – 4: salt pre-treatment (S). Lanes 5 – 9: acid pre-treatment (A). Lanes 1,2,5: viscera with oil (wO). Lanes 3,4, 6 – 9: viscera without oil. Parallel samples are marked with an apostrophe (ʻ). Refer to Table 6 for a summary of the contents in the gel.

Table 6. SDS PAGE analysis of FDGEs – Well content and abbreviation

Lane	Pre-treatment	Sample	Abbreviation
1	Salt	+ oil I	SwO
2		+ oil II	SwO’
3		- oil I	S
4		- oil II	S’
5	Acid	+ oil 1st extraction	AwO1
6		- oil I 1st extraction	A1
7		- oil I 2nd extraction	A2
8		- oil II 1st extraction	A1’
9		- oil II 2nd extraction	A2’

The protein pattern is similar for the FDGE samples where the salt pre-treatment was used (lanes 1 – 4). The samples pre-treated with salt (S) have a larger number of bands than the acid pre-treated samples (A). This is expected due to the method used, which solubilizes non-collagenous proteins as well (Regenstein & Zhou, 2007). The samples with oil (lanes 1 and 2) show more bands and more degradation products than the samples from which the oil was separated prior to extraction (lanes 3 and 4).

The samples where acid pre-treatment was used (lanes 5 – 9) show a higher presence of high molecular weight components than the salt pre-treated samples (lanes 1 – 4), especially the second extractions (lanes 7 and 9).

Faint bands can be seen over the 76 kDa mark (Figure 18A), these could represent the non-degraded α-chains. There are no clear high molecular bands seen in the gels, which indicates a high degree of collagen degradation.

4. Discussion

In recent years, the has been an increasing interest in the improvement of methods for the better use of rest raw material from salmon processing (Šližytė et al., 2017). The extraction of oils and of gelatine from salmon viscera has been studied in this investigation.

4.1 Chemical composition and mass balance

The raw material analysed in this study contained a higher content of lipids than previously found. The lipid content in viscera from farmed salmon has been found to range from 22.1 % to 44 % (Aursand, Bleivik, Rainuzzo, Leif, & Mohr, 1994; Deepika et al., 2014; Šližytė et al.,

2017; Sun et al., 2002). The lipid content in fish tissues has been known to vary depending on temperature, diet and season (Halver, 1980). Changes in the cultivating conditions of fish used in these studied may be the reason for this variation. The fat content in farmed fish has also increased during the years (Turid Rustad, personal communication, May 11, 2018), which could be another reason why the content found in this study was higher compared to previous studies.

The moisture content in the gonads of Atlantic salmon is over 80% (Aursand et al., 1994), this could be the reason why by removing the roe and milt from the raw material, the percentage of dry matter increases to 63%.

4.2 Oil

All the oil extractions from viscera without roe and milt were >84.4% and from viscera with roe and milt were >89.2% of the total lipid content in the raw material. The temperature used in the extractions does not appear to greatly influence the yield in this temperature range (20 – 90°C). When thawed, oil in the raw material began to separate (before the heat treatment). Because of this, the handling of the raw material led to some oil loss between transfers, and the determination of TLP in the raw material may have resulted in lower values than in the intact material. In addition, high levels of uncertainty due to the non-homogenous nature of the raw material contribute to obtaining yields based on the TLP higher than 100% (Table 4).

The peroxide value in the extracted oil samples vary between the types of viscera (with and without roe and milt) when heat treated with temperatures below 50 °C, but become more similar once the temperature is increased above 50 °C as can be seen in Figure 19.

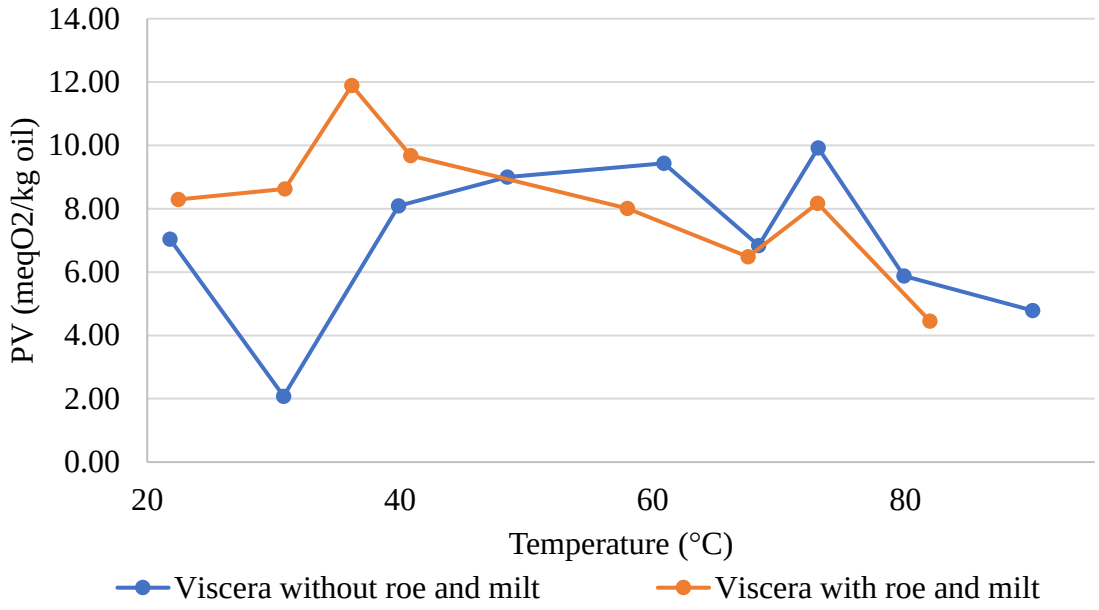

Figure 19. Peroxide values of oil obtained from viscera (with and without roe and milt)

The PVs of the oil separated at 50°C and above were found to be higher in viscera without roe and milt than the viscera with roe and milt. The heating of oils during extraction promotes oxidation of lipids with high degrees of unsaturation (Haq, Ahmed, Cho, & Chun, 2017) which could the reason why the PV increase with extraction temperature in the case of the viscera without roe and milt. The PV was expected to increase with the increasing temperature, since the heating of the tissue will lead to the denaturation of proteins and the release of prooxidants such as iron (Chantachum, Benjakul, & Sriwirat, 2000).

The PVs of the oil obtained from the viscera with roe and milt were higher in the oils separated at lower temperatures (30-40°C). Roe and milt have a high moisture content (>80%) (Aursand et al., 1994), the increase in moisture may contribute to the oxidation process by acting as a solvent for prooxidants (Ladikos & Lougovois, 1990). The viscera with roe and milt has a higher moisture content than the viscera without roe and milt, this increase in moisture could be the reason why oxidation is higher at the lower temperatures. In a recent study, Šližytė et al. (2017) determined the PV in oil extracted from salmon viscera using a thermal pre-treatment (heating at 40 °C for 5 minutes) to be 6.8±1.4 meq/kg. This study used longer heating times (10 minutes), this could have been the reason why the PVs in this study were higher.

Fish oil contains high amounts of polyunsaturated fatty acids which are prone to oxidation. The phospholipids in the extracted oil can increase oxidation of oils by acting as emulsifiers, allowing the interaction of prooxidants (iron and lipid hydroperoxides) with any available

substrate present (Cui & Decker, 2016). It is important to note that the behaviour of phospholipids as antioxidants or prooxidants depends on the system.

The lower PV seen at higher temperatures could also be caused by the production of melanoidins through the Maillard reaction, which have previously shown antioxidative activity (Yilmaz & Toledo, 2005). The darkened colour of the oils as the heat temperature increases (Figure 12) is likely caused by these molecules. Fish tissues contain other endogenous antioxidants that could prevent oxidation such as tocopherol (vitamin E). Tocopherols act as antioxidants by scavenging peroxy radicals, slowing down the rate of lipid oxidation (Belitz et al., 2009).

Farmed Atlantic salmon is usually fed a diet containing α-tocopherol as an antioxidant supplement and also to reduce post-mortem lipid oxidation. In a study by Parazo, Lall, Castell, and Ackman (1998), it was found that α-tocopherol is mainly stored in the liver, testes, and serum of parr. Naturally the tocopherol molecule is found in the cell membrane, which is rich in phospholipids (Gutiérrez et al., 2003). The lipids in roe and milt in other fish species (cod and other gadiform species) has been shown to consist of more than 60% phospholipids (Falch, Rustad, & Aursand, 2006).

The presence of these antioxidative components in the viscera may affect the oxidation rate. The lipid composition of milt and roe from farmed Atlantic salmon has not been studied in detail, therefore it is difficult in this case to draw conclusions based on the possible effect of phospholipids and α-tocopherol on lipid oxidation. Previous studies done in herring oil point out the inefficiency of α-tocopherol at temperatures above 50°C, because this condition favours hydroperoxide decomposition over the consumption of the antioxidant (Aidos, Lourenco, Padt, Luten, & Boom, 2002).

The decrease in PV is most likely caused by the decomposition of hydroperoxides, which is promoted by an increase in temperature and the presence of metal ions. At this point, the degradation of hydroperoxides is faster than the formation. An increase in temperature above 50°C changes the mechanism and the products of lipid decomposition (Kamal-Eldin, 2003), and even when denatured, enzymes containing metal groups can continue catalysing the oxidation reactions (Sikorski & Kolakowska, 2010). In this case, the higher temperatures (≥50°C) and the presence of metal ions could have been the contributing factors for the degradation of lipids.

The FFA analysis showed high values of %FFA for oils extracted from the viscera containing roe and milt. All the oil samples from the viscera with roe and milt contained FFA values above 4%. Šližytė et al. (2017) obtained a much lower %FFA in the heat treated viscera (0.29±0.06). Although high, the values found in this study are considered to be within the acceptable range for crude oils (EFSA Panel on Biological Hazards, 2010). Aidos et al. (2002) found a similar trend in the quality of herring oils at 50°C, the PV had decreased while the secondary oxidation products increased. Fish viscera has a high lipase activity, this enzyme catalyses the hydrolysis of lipids even at low temperatures, contributing to the production of FFAs (Sovik & Rustad, 2005). The lipases found in salmon viscera were deactivated after heating at 70°C for 5 minutes (Šližytė et al., 2017). In this study, the activity of this enzyme is very likely to contribute to the increase in %FFA in the salmon viscera.

The storage of the frozen oil samples is an important factor when preserving the quality of oils, and the samples in this study went through several freezing/thawing cycles during the analysis process. This occurred due to troubleshooting during PV determination to obtain lower deviation values between parallels. The change of solvents (from isooctanol to chloroform), the sample preparation process and adjusting the amount of oil used were the steps necessary to decrease the deviation. These freeze/thawing cycles could have promoted the autooxidation process and cause higher PVs than in an otherwise freshly extracted sample.

The change in the method used to extract the oil (i.e. heating the three parallels separately for the viscera with roe and milt) decreased the variation between the parallels. In future projects this should be taken in consideration.

Since the heating in this method is done using a water bath, the control of the sample temperature (in the bag) is not completely accurate. The highest temperatures (80 and 90°C) were difficult to reach using the water bath, and a microwave had to be used to increase the temperature. The use of the microwave did not affect the yield of the oil but a difference between the PV in the oil samples obtained from heating the raw material in the water bath versus the microwave could be observed, the oil obtained at 80°C using the microwave had a lower PV. It is possible that by heating the samples using the microwave, the time necessary to reach the intended temperature was lowered, and thus the endogenous enzymes could have been deactivated earlier in comparison to the samples in the water bath. These variations in extraction methods need to be considered when evaluating the PV. The non-homogenous

nature of the raw material also affects the analyses done in this study since a representative amount is difficult to obtain.

4.3 Gelatine

In regard to yield of the freeze dried gelatine extract (FDGE), the salt pre-treatment (SPT) extraction produced a higher amount than the acid pre-treatment (APT) extraction, but the hydroxyproline content obtained was lower. The higher amount of FDGE obtained was expected since the method used solubilizes other salt-soluble proteins other than collagen, and the volume of extraction solution allows for a higher concentration of proteins to be solubilized.

This is also the reason why the concentration of hydroxyproline in the FDGE obtained by salt pre-treatment (SFDGE) is lower than what was obtained by acid pre-treatment (AFDGE). The APT removes non-collagenous proteins, and the resulting extract is therefore richer in hydroxyproline. The higher hydroxyproline content indicates that the extracts are of higher purity, however, the acid pre-treatment method yields less amount of extract.

As expected, the AFDGE obtained with a subsequent extraction was much lower in amount in both types of viscera (with and without oil) than the first extraction. Even with a lower yield of the freeze dried extract, the hydroxyproline content obtained in the second extraction was similar to those in the first extraction.

The addition of a second extraction step caused the hydroxyproline yield to increase from 51.3% in samples with oil and 57.5% in samples without oil to 59% and 67.1% respectively. The use of several consecutive extractions steps has previously been used by Sadowska, Kołodziejska, and Niecikowska (2003) to successfully extract 89% of total collagen from cod skin. The degradation of proteins needs to be considered when evaluating the use of several extraction steps, with each extraction the gelatine obtained is more degraded.

The presence of oil in the raw material had different effects on the yield of FDGE and hydroxyproline content. The yield of FDGE is higher when the oil is removed prior to extraction, but the content of hydroxyproline is lower in these samples. The purity of the samples is lowered when oil is present, it is possible that by extracting samples from raw material with oil, the gelatine extracts obtained are contaminated with other molecules (increasing the weight and yield of extracts but lowering purity).

When considering the hydroxyproline yield extracted from the viscera, oil caused the opposite effect for the SPT (higher yield) compared to the APT (lower yields). Important interactions

between lipids and proteins occur due to electrostatic forces through their charged groups, hydrogen bonding and hydrophobic interactions (Shenouda & Pigott, 1975). The interactions between the lipid and proteins may affect the availability and stability of the collagen during pre-treatment.

The presence of lipids during the pre-treatment could have led to the formation of protein-lipid interactions, inhibiting the solubilization of proteins. This would then make the extraction of the FDGE less effective. The hydroxyproline content in the FDGEs indicate that the oil presence had a positive effect, this could be due to the interaction between collagen and the lipids. Collagen has been shown to interact with phospholipids and increase the stabilisation of liposomes (Ghannam, Mady, & Khalil, 1999). This stabilising interaction may lead to the protection of the collagen protein preventing the collagen to be washed out during pre-treatments.

Previously, SPT and APT have used to extract gelatine from the skin of Atlantic salmon obtaining total hydroxyproline yields (%) of 52.9±2.3 and 77.4±0.7 respectively (unpublished data). The results from this study indicate that the SPT is as effective for the viscera (52%) as it is on the skin. The APT seems to be the most efficient method in both cases.

The protein patterns obtained using SDS PAGE analysis showed that the SFDGEs have a more varied protein composition than the AFDGEs. Both SFDGEs (with and without oil) have similar protein patterns, but the samples with oil show a higher amount of degraded proteins. The most likely reason for the presence of more protein fractions in the SFDGEs compared to the AFDGEs is the solubilisation of non-collagenous proteins.

The protein patterns of the AFDGEs are similar in the first extraction of both raw materials (with and without oil). Contrary to the case of the SFDGEs, the AFDGE containing oil shows less degradation of products than the oil containing viscera. This could be due to the presence lipid-collagen interaction preventing the degradation of the collagen molecule. As expected, the highest amounts of degradation products were seen on the AFDGEs obtained in the second extraction. The proteins are more degraded with each extraction of the raw material.

The expected molecular weight of α-chains for collagen is between 80 – 125 kDa (Boran & Regenstein, 2010). The protein patterns show a faint band above the 76 kDa mark that could represent α-chains. While studying gelatine from fish skin, Zhou, Mulvaney, and Regenstein (2006) obtained faint bands at the 80 kDa mark and attributed them to degradation products after intrachain breakage. There are no distinct bands in the high molecular weight regions that

could represent β- or γ- chains. This is expected to be due to the low level of inter- and intrachain interactions between chains in collagens from cold water species, this causes the collagen to be more easily degraded.

The LMW components (<α-chains) in these FDGEs are an indication of low quality gelatine, since these components have been shown to decrease gel strength, and to low viscosity and gelling/setting time (Eysturskarð, Haug, Ulset, Joensen, & Draget, 2009; Muyonga, Cole, & Duodu, 2004). These LMW components are also observed in higher amounts in gelatines extracted from frozen skins than gelatine from fresh skins (Gudipati & Kannuchamy, 2014)

The degree of degradation in these samples may be due to the presence of proteases in the viscera that are able to cleave the collagen molecules. The native collagen protein can be cleaved by specific collagenases (Schrieber & Gareis, 2007). Sovik and Rustad (2006) showed that the collagenases obtained from of cod viscera have a maximum activity at 50°C, although they will drastically lose activity if kept for 10 minutes at this temperature. These samples give an indication the deactivation of cold-adapted collagenases, although the study was done in extracts which may increase the stability of the enzyme in comparison to the intact viscera. The authors suggested the use of viscera for protein hydrolysate production due to the high proteolytic activity of the enzymes present in this by-product. It is very likely that the content of proteases in the viscera contributed to the LMW components seen in the SDS PAGE analysis. Šližytė et al. (2017) evaluated the use of the protein fraction in viscera as a source for fish protein hydrolysates (FPH) and by separating the oil prior to enzymatic hydrolysis, obtained a high-quality oil fraction.

The reduction of processing times and reduced use of chemicals is of interest if these methods are to be adopted industrially. Only taking into account the first extractions of the APT, the hydroxyproline yield is similar between the SPT and APT methods. Although the second extraction in this method yields a similar amount of hydroxyproline content in the extract, the SDS PAGE shows a high content of degraded components. These results indicate that in practice, the use of a second extraction is not the best use of resources, since the extract amount of FDGE obtained will be very low and of low quality.

Viscera has a high content of enzymes that promote degradation of proteins and lipids. This represents a problem for the use of viscera as a raw material. High temperatures and pH changes can be used to inactivate the endogenous enzymes (Hayes & McKeon, 2014) prior to extraction, but these treatments may negatively affect the quality of the oil and proteins.

The quality of the oil and gelatine extracts obtained could be improved by using fresh/intact viscera and by pre-treatment it (separating the oil fraction and inactivation of collagenases) prior to gelatine extraction. Lower temperatures during extractions than those used in this study (<45°C) may yield gelatines with less degradation. Eysturskarð, Haug, Elharfaoui, et al. (2009) suggested the use of room temperature during extraction of gelatine from cold water species.

Protease inhibitors (EDTA disodium salt, phenylmethanesulfonyl fluoride and pepstatin) have also previously been used to successfully prevent degradation of gelatine extracted from fish skin (Zhou & Regenstein, 2005). However, the use of these inhibitors may not be economically favourable on an industrial scale. The study of collagenase activity from salmon viscera would provide information on the conditions necessary for the inactivation of the enzyme in this species. Sovik and Rustad (2006) found slight differences in the activity of collagenase depending on season and fishing ground of cod. The activity of collagenase from salmon viscera could be studied based on season/fishing ground to evaluate if these factors can dictate which technologies will be best suited for the utilisation of viscera as a raw material.

Viscera is a delicate by-product that is prone to decomposition. The separation of lipid and inactivation of proteases may lead to better results on the quality of the products (less oxidation of oils, no degradation of proteins). Factors such as light, oxygen and temperature must be taken in consideration during the processing of this raw material, as this may be critical for the quality of the potential products.

5. Conclusion

The use of viscera as a raw material for the extraction of lipids and gelatine was evaluated, and the results suggest that in regard to yield, it is a viable source of lipids and gelatine, but since this raw material is prone to deterioration, measures should be taken to inhibit endogenous enzymatic activity and lipid oxidation.

The separation of oil prior to extraction was done at various temperatures and the extraction temperature did not seem to influence the yields of oils, which were very high (≥84.4%) for all extractions. The quality of the oils was suboptimal, as PV and FFA content was high but still within the range of accepted values for crude oils. The lower quality of the oil was attributed to the presence of endogenous enzymes and prooxidants in the raw material.

The two gelatine extraction methods used produced good yields of hydroxyproline (lowest value was 51.3%). The SDS PAGE analysis of the FDGEs show faint bands only in the lower molecular weight range (≤80 kDa) which indicated that the gelatines obtained had a high level of degradation. The presence of oil in the raw material seems to have a positive effect on the hydroxyproline yield for the SPT extraction and a negative effect for the APT extraction. According to these results, the best method for the oil and gelatine extractions (based on hydroxyproline yield and purity of the sample) using salmon viscera, was a combination of oil separation prior to the acid pre-treatment and extraction of gelatine. The freeze dried gelatine extract obtained using this treatment combination produced samples with lower levels of low molecular weight components than the other combinations, with the exception of the APT sample without prior oil separation.

6. Suggestion for further work

The use of salmon viscera as a source of gelatine has not been extensively researched. The characterization of the rheological properties of gelatine would allow for a more informed evaluation of the use of viscera as a gelatine source or if this by-product should be utilised in the production of other useful products such as FPHs or for the extraction of digestive enzymes.

There are also many studies focusing on improving the functional characteristics of fish gelatines, for example the use of ultraviolet irradiation (Bhat & Karim, 2009), or coenhancers for improving gel quality (Koli, Basu, Nayak, Kannuchamy, & Gudipati, 2011). These methods could be explored with a focus on gelatine from fish viscera.

7. References

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Appendix

I. Mass balance heat-treated samples

Table 7. Mass balance data from heat-treated samples of viscera with roe and milt

Treatment temperature (°C)	Dry matter (%)			Amount obtained (g)				Dry Matter (Sum) (%)	Dry Matter (Raw Material) (%)	Deviation (%)
	Liquid	Solids	Emulsion	Oil	Emulsion	Liquid	Solids			
20	20.2	26.3	-	10.4	0.0	0.4	3.6	55.3	52.7	-4.8
30	21.5	27.3	30.1	12.6	3.5	0.8	3.1	51.1	52.7	3.1
40	17.7	26.0	29.1	12.8	3.1	0.8	2.8	50.4	52.7	4.4
50	20.3	28.6	29.0	12.0	2.4	1.5	2.9	49.8	52.7	5.5
60	17.5	28.7	35.0	12.4	1.7	1.8	3.1	49.7	52.7	5.8
70	15.0	31.3	35.6	11.2	1.6	1.4	4.0	48.0	52.7	8.9
80	14.5	30.3	33.5	11.8	2.4	1.0	3.8	49.4	52.7	6.3
90	13.9	33.5	36.9	11.2	1.9	1.3	4.1	48.8	52.7	7.4

Table 8. Mass balance data from heat-treated samples of viscera without roe and milt

Treatment temperature (°C)	Dry matter (%)			Amount obtained (g)				Dry Matter (Sum) (%)	Dry Matter (Raw Material) (%)	Deviation (%)
	Liquid	Solids	Emulsion	Oil	Emulsion	Liquid	Solids			
20	26.6	26.5	-	15.3	-	2.7	3.6	55.2	54.1	-2.0
30	29.6	26.5	-	16.5	-	3.5	2.8	58.6	54.1	-8.3
40	23.7	26.8	-	15.3	-	2.9	2.9	54.9	54.1	-1.5
50	23.9	28.6	-	17.9	-	3.6	1.4	60.5	54.1	-11.7
60	23.1	27.9	79.7	16.5	1.0	2.8	2.5	58.7	54.1	-8.4
70	20.4	27.4	57.4	16.2	1.0	1.7	3.4	57.6	54.1	-6.4
80	24.4	27.8	-	15.9	-	2.4	3.9	55.7	54.1	-2.9

80m	21.2	26.3	47.8	16.0	1.2	1.8	3.3	56.3	54.1	-4.0
90	18.3	31.7	27.0	16.2	1.6	1.1	3.5	57.5	54.1	-6.3

II. PV determination

Table 9. PV of oil samples extracted from viscera without roe and milt after heat treatment for 10 minutes.

Theoretical temperature (°C)	Measured temperature (°C)	Average PV (meqO2/kg oil)
20	21.8	7.03±0.14
30	30.8	2.08±0.12
40	39.9	8.09±0.16
50	48.5	9.00±0.43
60	60.9	9.44±0.29
70	68.4	6.84±0.21
80	73.1	9.92±0.20
80m	79.9	5.88±0.15
90	90.1	4.78±0.02

Table 10. PV of oil samples extracted from viscera with roe and milt after heat treatment for 10 minutes.

Theoretical temperature (°C)	Measured temperature (°C)	Average PV (meqO2/kg oil)
20	22.5	8.29±0.14
30	30.9	8.63±1.46
40	36.2	11.89±0.71
50	40.9	9.68±0.08
60	58.0	8.01±0.13
70	67.6	6.48±0.06
80	73.1	8.17±0.35
90	82.0	4.45±0.09

III. Protein standard components

Table 11. Components of the LMW standard used in the SDS PAGE analysis

Protein	Molecular weight (kDa)
Phosphorylase b	97
Albumin	66
Ovalbumin	45
Carbonic anhydrase	30
Trypsin inhibitor	20.1
α-Lactalbumin	14.4

Table 12. Components of the HMW standard used in the SDS PAGE analysis

Protein	Molecular weight (kDa)
Thyroglobulin	669
Ferritin	440
Catalase	232
Lactate dehydrogenase	140
Albumin	66