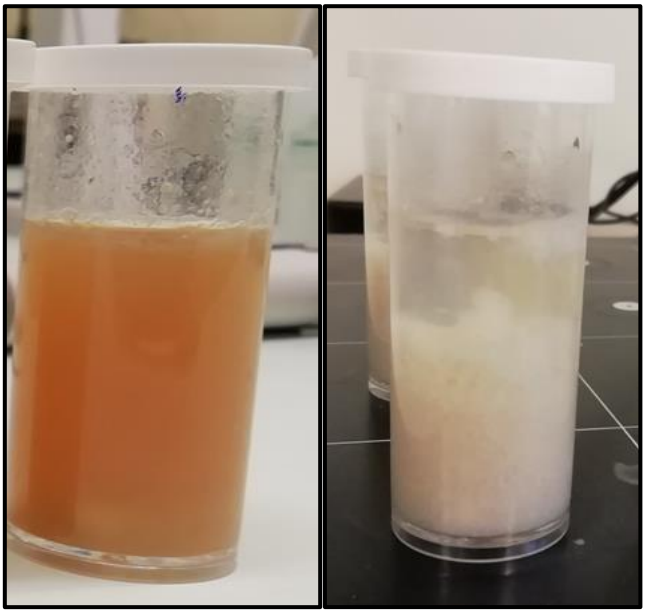
CHALMERS
UNIVERSITY OF TECHNOLOGY



Flocculation of protein in seafood process water

Evaluation of flocculants in the presence of high salt content

Master thesis in Food Sciences

MARTIN HAGSTRÖM

Department of Food and Nutrition Sciences

Institution of Biotechnology and Biology

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2022

www.chalmers.se

MASTER'S THESIS, 2022.

Flocculation of protein in seafood process water

Evaluation of flocculants in the presence of high salt content

MARTIN HAGSTRÖM

CHALMERS
UNIVERSITY OF TECHNOLOGY

Department of Food and Nutrition Sciences

Food Science

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2022

Flocculation of protein in seafood process water

Supervisor: Romain Bordes, Chalmers University of Technology
Supervisor: Anna Ström, Chalmers University of Technology
Supervisor: Bita Forghani Targhi, Chalmers University of Technology
Examiner: Ingrid Undeland, Chalmers University of Technology

Master's Thesis 2022
Department of Food and Nutrition Sciences
Food Science
Chalmers University of Technology
SE-412 96 Gothenburg
Telephone +46 31 772 1000

Cover: Illustrative representation of flocculant performance in herring process waters, left raw sample and right treated sample.

Photograph, Martin Hagström.

Gothenburg, Sweden 2022

Acknowledgements

Firstly, I would like to thank family and friends that have supported me, assisted me, and most importantly motivated me throughout the whole education at Chalmers. Thank you!

Furthermore, I would want to seize the opportunity to thank everyone, from head of unit to fellow degree-workers at the Department of Food and Nutrition Sciences at Chalmers University of Technology. Thank you for your warm and respectful demeanors, making me feel included and a part of a bigger team. Thank you for all interesting discussions and helpful tips shared in the common breakrooms. In particular I would like to express my appreciation to Mehdi Abdollahi, although you were not my supervisor, for support and always going out of your way to help with any issues encountered in the laboratory rooms.

Thank you to Romain Bordes and Anna Ström, two of my supervisors, for all the valuable information and tips regarding the chemistry surrounding this work.

Finally, I would like to address a special thank you to Bita Forghani Targhi, my main supervisor, and Ingrid Undeland, my examiner, for all the support throughout my thesis work and for the freedom I was given to discuss and try out ideas.

Martin Hagström, Gothenburg 2022.

Abstract

Water is a necessity to produce marinated herring. One application of water is in the various brines used for pre-salting, marinating and maturing the herring to achieve the sought-after quality. During these processes high quality nutrients from herring inevitably leach out into the water and will subsequently be lost. Recovery of these nutrients, via food-grade flocculation, would ameliorate sustainability whilst simultaneously decrease effluent taxation cost.

Flocculation was performed on two pre-salting brines (5% and 13%) from the herring industry using chitosan, alginate, carrageenan, silica, and crystal nanocellulose (CNC) as flocculants. Responses measured were relative sediment height (RSH) and protein sedimentability (%) after one hour sedimentation at room temperature. First the influence of pH on flocculation with the above-mentioned flocculants was analyzed. Flocculation with 0.45 g/L carrageenan and 0.5 g/L alginate performed the best at pH 4 in the 5% brine, while chitosan performed the best at pH 4.5. Flocculation with CNC at 0.5 g/L showed effect on the 5% brine, but no optimal pH could be determined, whilst flocculation with silica at 0.5 g/L showed no effect. None of the flocculants displayed any flocculation in the 13% brine. Secondly, the influence of the concentration of carrageenan and alginate on flocculation was investigated. Flocculation with carrageenan (0.09-0.9 g/L) at pH 4 reduced RSH the most, indicating efficient sedimentation of flocs. Optimal carrageenan concentrations were 0.675 g/L and 0.9 g/L, which yielded the greatest protein sedimentability, ~99%. Flocculation, at pH 4, with alginate (0.1-1.0 g/L) gave the second highest reduction of RSH whilst protein sedimentability reached around 98%. No specific concentration proved optimal, and rather alginate was effective over the whole concentration range.

With the intention of creating two model systems for optimizing flocculation of herring brines, protein extractions of herring mince at low (0.05 M) and high (0.7M) ionic strength were done. The optimal extraction-liquid to mince ratio was 10:1 with respect protein recovery (%) and ease of use. SDS-PAGE showed enrichment of sarcoplasmic proteins in low salt extracts and of myofibrillar proteins in high salt extracts. It was concluded that optimization of flocculation would be done on the brines themselves and that artificially built model systems would be more suitable for evaluating flocculation mechanisms.

Keywords; Flocculation, Protein recovery, Food grade flocculant, Chitosan, Alginate, Carrageenan, Silica, Crystal nano cellulose, Seafood process water.

Table of contents

1. Background

During handling and processing of herring, water is a necessity for processing and delivering a high-quality end-product. Water is used for storage of whole herring, for washing of fillets and for pre-salting and marinating to develop desired taste and texture, the later use of water is typically as brines. During processing and marinating, inorganic and organic matter is transferred from the herring into the water. Thus, a loss in raw material occurs and, accordingly, an increased need for effluent treatment before disposal. Today effluent disposal differs worldwide due to different regulations. It is not uncommon that process waters are discharged into the surrounding nature, either in any body of water or landfills. In Sweden, however, regulations require treatment of the waters, which is commonly done on-site at the factory, where the water is pre-cleaned, and at a water treatment plant for final treatment of the pre-cleaned water.

1.1 Flocculation

Flocculation, as a mean to treat wastewaters/effluents, has been applied since the early Egyptian and Roman civilizations. However, by then little was known about mechanisms and about the chemistry involved. (1) The basic principle of flocculation is via addition of a flocculant to destabilize the colloidal system within the wastewater, which enables particle agglomeration into larger complexes, so called flocs, initially micro flocs. These micro flocs can further aggregate creating larger flocs which are generally denser than the surrounding water and these flocs are allowed to sediment (flocs can also be lighter and, thus, float to the surface). (2,3) As a result, two phases will be created; an upper phase (supernatant) where the treated, preferably, clear liquid purified from suspended particles and a so-called sludge, a mass of sedimented particles with high moisture content. These two phases can subsequently be separated. The sedimentation is governed by gravitational forces, where Stoke's law can be utilized to understand the rate of sedimentation (4), but it can be aided by for example magnetism, provided that the flocculant has magnetic properties. Although the whole picture of how flocculation works is not fully understood to this date, especially on more complex systems, the major principles have been theorized. The two major mechanisms regulating flocculation will be described in separate sections, and factors affecting flocculation as one section. The mechanisms may work independently or at the same time. (5)

1.1.1 Charge neutralization

One of the main mechanisms exploited in a flocculation system is the principle of charge neutralization. This principle is based on the knowledge that suspended particles in any given wastewater system are generally negatively charged, thus, a flocculant with opposite charge would, with advantage, be used. Thereby, attractive forces will prevail leading to agglomeration of particles. (6) If on the other hand to much flocculant is added to the system a switch in particle charge may be induced (from negative to positive) and thus causing re-stabilization of the system preventing aggregation. (7) One may also adjust the pH, past (either by reduction or increase of pH) the isoelectric point of the particles and/or the flocculant in the solution, this will cause a charge inversion of the particles and/or flocculant. A too large pH adjustment may cause the particles to have the same charge as the flocculant, preventing aggregation. By reducing the pH below the isoelectric point of the proteins in the solution (making them positively charged) enables the use of negatively charged flocculants, facilitating the use of food grade flocculants (many of which are negatively charged). To further understand the interactions between the charges the DVLO-theory (Figure 1) is a helpful tool in understanding the net forces involved. The theory describes the relation between repulsive and attractive forces of colloidal particles as a sum between, mainly, the electrostatic repulsion and Van Der Waals forces, and accounts for their interparticle distance dependency.

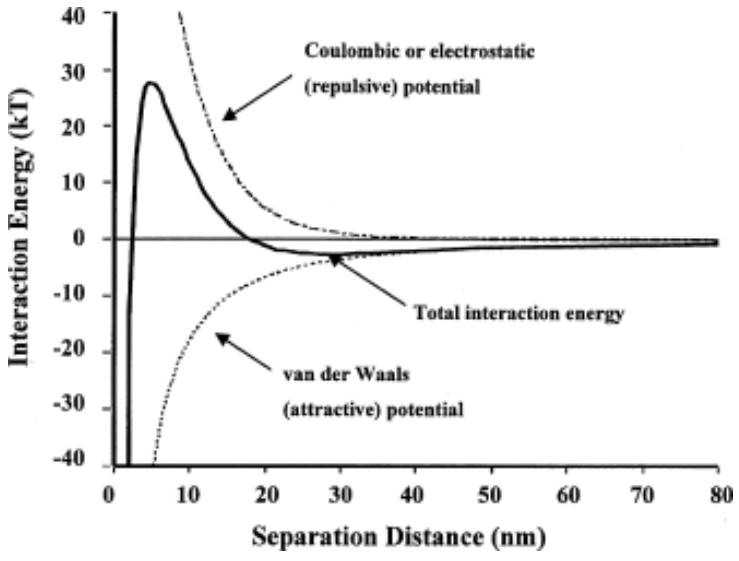


Figure 1. Visual representation of the two main forces and the subsequent interaction which the DLVO aims to describe. Source; https://www.sciencedirect.com/topics/pharmacology-toxicology-and-pharmaceutical-science/dlvo-theory

Even though the DLVO-theory predicts suspended particle aggregation at higher salt concentration, in some high ionic strength (ionic strength above critical coagulation

concentration) systems this prediction is not accurate. Instead, some systems containing higher salt than the critical coagulation concentration might become stable where the DLVO- theory does not predict stability and particle aggregation is hindered. (8)

1.1.2 Bridging

Another property of the flocculant, other than the charge, is the size/length of the flocculant. Swenson et al. (1998) indicates that a higher molecular weight and longer molecule can flocculate efficiently without the need for charge neutralization, labeling it as steric stabilization. The authors propose that the larger size of the flocculant means that it can interact with itself and bridge flocculants together. (9) The network of polymers is based on the few sites on the particles at which the polymer might bind to. Thus, a high molecular weight polymer has long molecule structures that are non-bound and these structures are referred to as tails and loops. These structures tend to reach out into the liquid surrounding the polymer, which allows the flocculant to interact with more particles and at a greater distance. Similarly to charge neutralization, bridging is also distance dependent, a close enough distance is required for the particles to interact with the loops and tails and thus creating a bridge between them.(10) Fellows and Doherty (2006) describes another structural property of the polymer that they claim is essential for efficient bridging; the dynamics of chain relaxation. For polymers of high molecular weight and with long chains, the chains must be long enough to extend well past the thickness of the double layer (Figure 2).

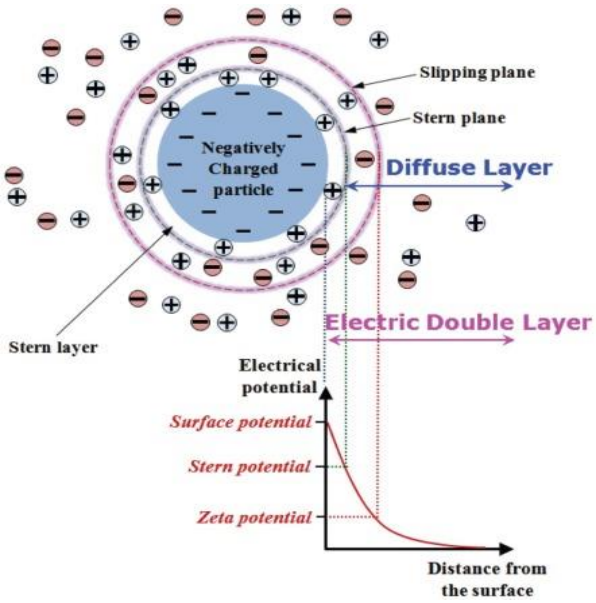


Figure 2. Overview of the different layers of the double layer, including Stern layer and diffuse double layer. Source;https://ars.els-cdn.com/content/image/1-s2.0-B9780123750495000013-gr17.jpg

Furthermore, the chains must stay rigid, and for a long enough time, to surpass the average interparticle collision time. (11) As a result, particles will get entrapped, even at lower concentrations of polymer, thus, leading to an increased rate of sedimentation. (9, 10, 11)

1.1.3 Factors affecting flocculation

There are several factors affecting the efficiency of flocculation, one of them is temperature. Fitzpatrick et al. (2004) turns to viscosity as an underlying factor of how quick sedimentation occurs; at lower temperatures the viscosity increases (Figure 3) and thus decreases the speed of sedimentation. Furthermore, a lower temperature will affect the rate of aggregation itself. (12)

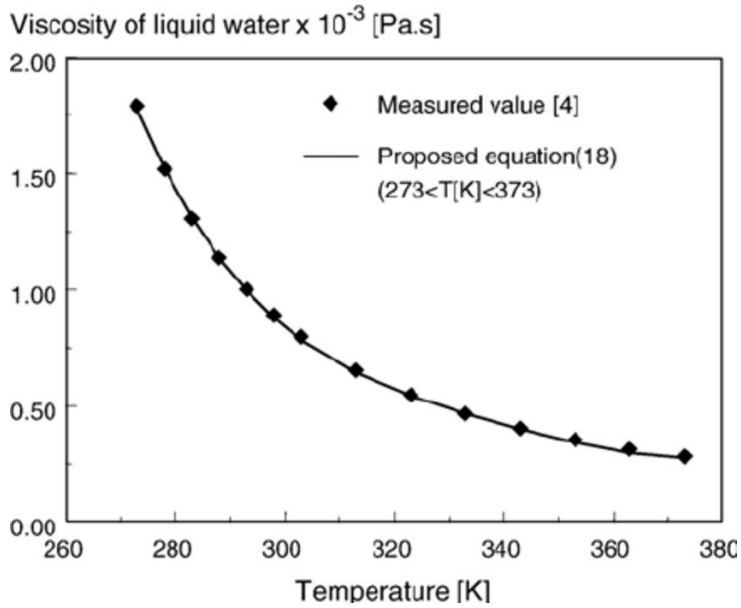


Figure 3. Graphical description of viscosity of water as a function of temperature Source;https://www.researchgate.net/publication/222573141_Enhanced_modeling_of_moisture_equilibrium_and_transport_in_cementitious_materials_under_arbitrary_temperature_and_relative_humidity_history/download

This was further studied by Xiao et al. (2009) where alum was used as a flocculant. The rate of aggregation was found to be higher at 22 °C than at 2°C. They found a decrease of aggregation ratio of 1-8% per °C in the interval between 2-22 °C. The authors stated that the reduced rate of perikinetic collisions was the reason for these findings. (13)

Another important parameter is pH, which can affect the properties of the particles to be flocculated, especially proteins. In a study, by Kristinsson and Hultin (2003), the effect of pH treatment on cod muscle proteins was investigated. Their results indicate that low and high pH increased the solubility of the protein (regardless of the ionic strength) (Figure 4). Low and high

pH conditions cause incomplete folding of the proteins, this will change the properties of the proteins enabling higher surface area to interact with surrounding liquid/particles, unfolding also causes increased surface hydrophobicity which promote interaction with other particles. (14)

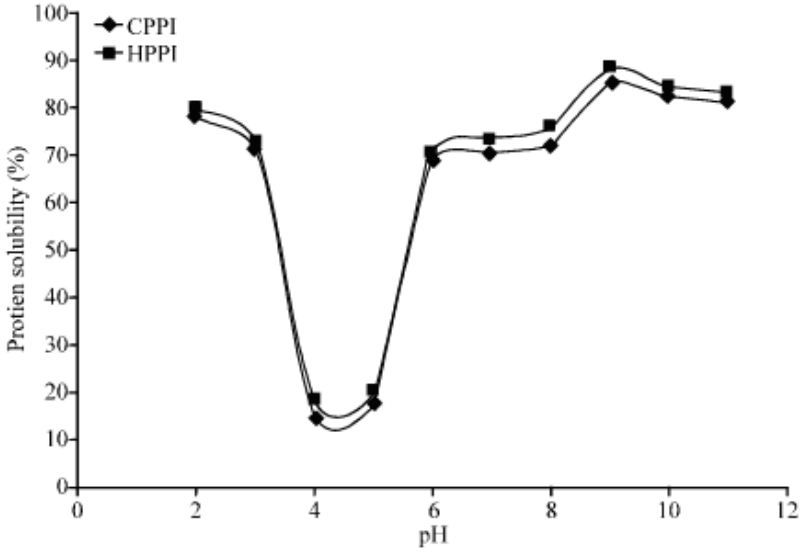


Figure 4. Graphical depiction of changes in protein solubility as a function of pH. CPPI represents cold pressed peanut protein isolate and HPPI denotes heat pressed peanut protein isolate. Source; https://scialert.net/fulltext/?doi=ajft.2009.47.55

The change in solubility is due to that the pH affects the charge distribution of the proteins rendering the proteins more positively/negatively charged which promotes interaction with water molecules. Furthermore, Shi et al. (2005), found the pH to be responsible for changes in many of the chemical properties of proteins, such as the net charge and the characteristics of the charge as well as charged groups, all of which determines interactions with other molecules. (15)

The final parameter to be unveiled is the effect of salt on flocculation, through an understanding of how salt affects colloidal systems. For flocculation to occur, according to Fleer and Lyklema (1974), a specific amount of salt is needed. The salt is needed to affect the Stern layer, which denotes the highest potential in the double layer and is located at the surface of the particle, and to decrease the size of the diffuse double layer. For bridging this needs to happen so that the diffuse double layer does not inhibit the loops of the polymers to bind together and create 'bridges' to entrap particles. (16, 17) However, Trulsson et al. (2006) describes a system where there is an excess of salt. At those conditions the salt concentration indirectly causes molecule charge inversion. What the authors described was that at a certain salt concentration, labelled as an excess salt concentration, the interaction of two oppositely charged particles can shift

from attractive to repulsive. (18) The potential reason for the shift to repulsion is described by Sjöström and Åkesson (1996), who states that the salt concentration in the double layer is larger than the salt concentration in the surrounding liquid. This affects the entropy of the system. (19)

Thus, these three parameters, pH, temperature and salt, are important for the flocculation efficiency, by either affecting solubility of proteins, flocculation mechanisms, the rate of sedimentation and the entropy of the system, all of which will affect how or if inter-particle interactions happen.

1.2 Natural Flocculants

Recently, the search for natural (organic) flocculants, e.g. chitosan, alginate, carrageenan, as a safer, more economical (organic flocculants are generally more expensive than inorganic flocculants, e.g. chitosan, derived from shrimp, cost almost 2000 SEK/100 g versus ferric chloride priced at around 10 SEK/kg) and a more sustainable alternative to synthetic (inorganic) flocculants, e.g. alum, ferric chloride, Magnafloc, has increased. There are some significant advantages of natural flocculants compared to previously used flocculants. These advantages include safety (non-hazardous), biodegradability, availability and cost effectiveness. (20) Furthermore, natural flocculants will create lower sludge volumes, much accredited to the lesser need of flocculant dosage. Additionally, natural flocculants will also alter the ionic load in the treated water to a lesser extent than conventional/synthetic flocculants. (21) Regarding the safety of the natural flocculants, the addition of the flocculant should not alter the safety to consume the product, thus it should be of food grade quality, nor should impose health issues either for humans or other life. To state one conventional flocculant that has been linked to health concerns: alum. This flocculant has been associated to development of Alzheimer's disease. (22) The aspect of availability is not only limited to market availability but also regional or industrial availability. Chitosan, derived from chitin, is an example of industrial availability where shellfish industries can produce chitin, and subsequently chitosan, along with their conventional production, thus, adding value to their production (23). Other aspects of availability may include, but not limited to, bacterial protein/polymer production or plant-based co-product utilization such as, for example, starch.

Previously, extensive research has been performed on flocculation of dairy, dye and sewage effluents with synthetic/conventional flocculants. Forghani et. al. (2019) analyzed the flocculation capacities of natural flocculants on shrimp process waters. They studied alginate, carrageenan and carboxymethyl cellulose in shrimp boiling water after a reduction of pH to

around 5. All three flocculants achieved good protein removal from shrimp boiling water, where carrageenan was the best performing flocculant which resulted in up to 86% protein removal from the shrimp boiling water. (24) There have been many studies performed on flocculation with chitosan (highly interesting since chitosan is positively charged and thus not requiring any pH reduction of the process water), one of these studies were performed by Divakaran et. al. (2001) where chitosan's flocculating capacities were evaluated in kaolinite suspensions. The results indicated that chitosan, at pH 7.5, was able to remove turbidity by 90% and indicated a quick sedimentation, a near complete sedimentation within minutes. (25) Another study, by Ahmad et. al. (2005) compared chitosan's flocculation capacity against activated carbon and bentonite in palm oil mill wastewater. The authors discovered that chitosan was able to reduce the amount of oil by 99%, higher reduction than either bentonite or activated carbon. For reduction of suspended solids chitosan also found to outperform the other two, reducing the initial load (4000mg/L) to 25 mg/L, compared to 35 mg/ml and 70 mg/ml for activated carbon and bentonite, respectively. Furthermore, this higher reduction was done using a chitosan dosage of 0.5 g/L, whereas the dosages for activated carbon and bentonite where several fold higher, 8 and 10 g/L, respectively. (26) Morantes et al. (2019) studied the flocculation capacities of cationic cellulose nanocrystals (CNC) in a model system, based on a silica suspension to simulate process waters. Their findings indicated that cationic CNC's had promising flocculation capacities compared to conventional/synthetic flocculants. At a mere concentration of 2 ppm, the cationic CNC achieved a turbidity reduction of the silica suspension of 99.7%. (27) In a study performed by Demchenko et al. (2008) polyacrylamide (PAA) grafted silica's flocculation performance in kaolin suspensions was evaluated. The authors compared PAA-grafted silica with shorter grafts, PAA-grafted silicas with longer grafts, PAA alone and Magnafloc. The PAA-grafted silicas with longer grafts were concluded to be the most efficient flocculant in their study. This flocculant showed the most promising efficiencies regarding, amongst other parameters, quicker sedimentation rate and the reduced optical density of the supernatant compared to the other evaluated flocculants. (28)

1.3 Herring constituents and herring process water

The fish used for this thesis is herring (*Clupea harengus*), a small pelagic fish, which is rich in dark muscle. The macro nutrient composition, especially the fat content, is subject to large variations, both seasonally and geographically. The fat content can vary from 2% up to more than 20 %, the protein content is generally between 16-21 %. (29) The myofibrillar proteins

constitute about 66-77% of the total muscle proteins. To this group of muscle proteins myosin, actin, regulatory proteins (e.g. tropomyosin) are counted. These proteins are considered relatively insoluble at physiological conditions (0.05M), but are more soluble, and therefore more easily extracted, at higher ionic strengths (0.6 M and upwards). (30) The other major group of muscle proteins is the sarcoplasmic fraction, which makes up to 35% of the total protein in fish. The main groups are globular (e.g. myoglobin) and rod-shaped proteins and they are recognized as water soluble. (31) Thus, in this work extraction of myofibrillar proteins will be at an ionic strength above 0.6M (0.7M) and extraction of sarcoplasmic proteins at physiological condition (0.05M). During processing of herring, the ionic strength of the brine, the duration of marinating and the cut of herring will affect what nutrients (proteins and fats mainly) and the amount of the nutrients that will leach out from the herring into the brine. This was analyzed in a study performed by Osman et.al. (2015), which indicated that the herring cut into smaller pieces released more protein into the brine compared to herring which had not been cut as small, at the same ionic strength. Generally, the leaching of proteins was found to increase with increasing ionic strength and marinating time. However, the authors indicated that in some cases cut pieces of herring could leach out more proteins compared to un-cut herring, even when the brine had a lower ionic strength and a shorter marinating time. This was probably due to higher surface area of the herring and therefore higher exposure to the surrounding liquid. Additionally, for the effect of ionic strength the authors found that the higher salt content brines (5% and 8% brines) leached out relatively more of the myofibrillar proteins than lower salt content brine (3%). However, the authors clarified that the salt content, duration of marinating and cut size all affected each other on the total amount of protein that could be leached out. (32)

1.4 Knowledge gap

To the best of my knowledge there is a limited number of scientific reports regarding the use of natural/food grade flocculants to recover a nutrient-rich biomass from herring marinating process waters. Osman et.al. (2015) (32) performed electro-flocculation and ultrafiltration of herring process waters to attempt to recover the nutrients that are leach out into the brine. Another study, with some similarities to this work, was performed by Forghani et.al.(2020) (24) in which similar flocculants were evaluated in shrimp boiling water. With the use of conventional flocculants (in-organic), for example Alum, to treat these waters, the extracted biomass will not be classified as food grade. Thus, there is a need to evaluate organic, food grade, flocculants in search of finding one food grade flocculant with efficient enough

flocculation efficacy, whilst retaining food grade quality to add value to the overall process. As a result, the herring industry will be able to achieve a higher amount of end-product versus influx amount of raw material, thus making the process more sustainable, at least from a raw material conversion perspective. In addition, the raw material use could become more ethical (more high-quality products will be extracted and by so reducing unnecessary waste). Furthermore, retaining food grade quality of the process water might facilitate or allow for further on-site treatment, such as removal of salt, other metals, and fats, to be able to reduce the freshwater need. This would allow the herring marinating industry to be more sustainable regarding their water usage, since freshwater is a decreasingly available natural resource. If one or both of the following aspects, food-grade flocculation and on-site water recycling, would prove to be technically and economically feasible it could provide the industry the means to become circular. This project for the first time addressed food-grade flocculants as a method to recover a nutrient-rich biomass from herring brines. Although not initially food grade, silica was also addressed since this molecule can be modified into food-grade.

1.5 Aims

- To design two herring process water model systems, one enriched in sarcoplasmic proteins and one enriched in myofibrillar proteins originating from herring fillets to better understand the flocculation effect in relation to type of muscle protein and salt concentration.
- To evaluate the flocculation capacities of a selection of flocculants, mainly food grade, in herring pre-salting brines under different pH-conditions and flocculant concentrations.

1.6 Materials

1.6.1 Herring for extraction and model system

Fresh herring fillets were provided by Scandic Pelagic Ellös AB, and the fillets were minced and packed in plastic bags of 300-400 g which were stored at -80 °C until use.

1.6.2 Process waters

Two waters from a primary producer, (Scandic Pelagic Ellös AB) were tested, these were a 5% and a 13% salt brine. The 5% salt brine was collected 13th of September 2018 and the herring had been marinating for 14 hours as whole fillets. The 13% salt brine was collected 13th of

September 2018, where the herring had been cut into pieces and marinated for 20 hours. The brines were stored at -80°C until use.

1.6.3 Flocculants

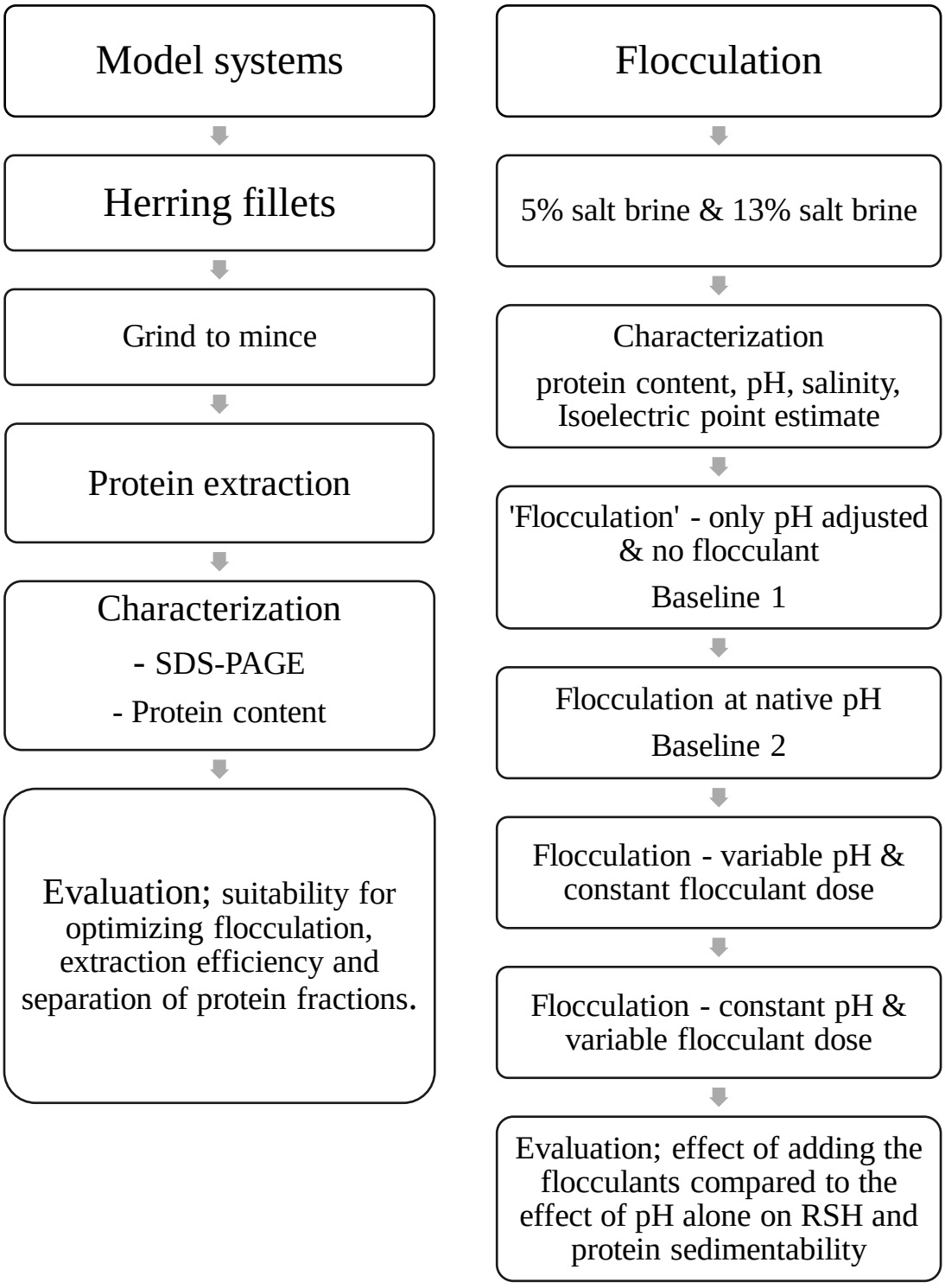
- Chitosan (China).
- Alginate, Manucol® DM, and λ Carrageenan ((Viscarine® GP-109NF) were provided by FMC Food and Nutrition (PA, USA).
- Silica (Sweden).
- Crystal nanocellulose was provided from Celluforce (Canada).

1.6.4 Equipment

- pH-meter; PHM210 (STANDARD pH METER), Meterlab, Radiometer analytical (France). Equipped with a HAMILTON Double Pore (Switzerland) probe.
- Spectrophotometer; Cary60 BIO UV–vis spectrophotometer (Varian Australia Pty Ltd., Victoria, Australia)
- Centrifuge; Sorvall Lynx 6000 centrifuge, Thermo Scientific (USA)
- Homogenizer; Silverson, L5M (USA)
- Polytron; IKA T18 digital Ultra Turrax. (Germany)
- Conductivity meter; CDM210 (Conductivity meter), MeterLab, Radiometer analytical. (France).
- SDS-PAGE set-up; a plastic container fitted with a slot where-in a cassette can be inserted. This cassette, with two electrodes, is where the gel is placed. The container has a lid which will connect the electrodes on the cassette to a power source, BIO-RAD POWER PAC 300 (Sweden), where voltage and current can be adjusted. For the staining a plastic tray and a tabletop pivot, Stuart Scientific PLATFORM STR8 coupled to a Stuart scientific DRIVE UNIT STR8 (UK), was used.

2. Experimental design

Below follows a brief overview of the main steps in the experimental design by which this work has been performed.



3. Methods

3.1 Fat content analysis of process waters

Fat content of process water samples was analyzed through a chloroform:methanol extraction, see Appendix A. Two ml of process water sample was mixed with 20 ml ice cold chloroform:methanol solution (one part chloroform and two parts methanol) and vortexed. Thereafter 8 ml ice cold 1M NaCl solution was added to the sample and then vortexed for 1 minute again and mixture was then centrifuged at 2000 × g for 6 min at 4°C. 3 ml of the chloroform phase, i.e. the lower phase, were transferred into pre-weighed glass tubes and left to dry under nitrogen gas until completely dry. The dried glass tubes were then weighed, and the fat content was calculated as follows;

For fat in grams in the 3ml sample;

$$Weight\ of\ tube_{dried} - weight\ of\ empty\ tube \quad (1.A)$$

For fat in percent;

$$\frac{\frac{Fat}{3/6.67}}{g_{sample}} * 100 \quad (1.B)$$

*6.67 is a proportion factor, the extraction was performed at 1:2 ratio ($CHCl_3$:MeOH) and from the total volume (20 ml) added to the extraction, the fraction of $CHCl_3$ is 6.67 ml. g_{sample} is the weight of the process water sample used for the extraction (approximately 2 g).

3.2 Protein measurement – the modified Lowry method

For protein content determination the modified Lowry method according to Markwell (1978) was applied (see Appendix B). (33) 0.33 ml of sample (process water and supernatants from flocculation, protein solubility and protein extraction) was mixed with 1 ml of reagent C, followed by a 30 min incubation. Thereafter 0.1 ml of 1:1 Folin-Ciocalteu' phenol reagent: milliQ water was added, vortexed and incubated for 45 min. Thereafter the absorbance was measured at 660nm. Bovine serum albumin standard curves were created in the range of 10-100µg/ml (Figure 5) and protein concentrations were calculated from these standard curves. The samples were in several cases diluted to fit within the range of the standard curve absorbance of 0.1-0.45.

Before protein content determination a solubilization step was applied to the untreated process water samples as these contained aggregated and insolubilized proteins; where 1 part of sample was mixed with 1 part of 1N NaOH. The mixture was vortexed for 30 seconds and incubated for 15 min and then vortexed again for 30 sec.

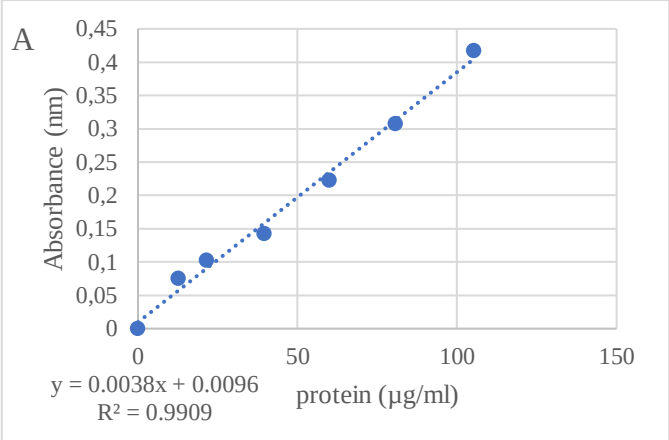


Figure 5. BSA standard curve was utilized for the process water characterization part. In appendix B the STD curve for protein content determination in the supernatants collected from flocculation tests is visualized.

Corresponding calculation, equation 2, for protein content from the standard curve:

$$\frac{Abs-0.0096}{0.0038} * tentative\ dilution \qquad (2)$$

*Tentative dilution denotes the dilution that was used during Lowry sample preparation.

3.3 Protein solubility – p*I* estimate

The protein solubility of the 5% and 13% salt brines as a function of pH was analyzed between pH 3 and pH 11, with a step size of one unit of pH. Under magnetic stirring pH was adjusted, and at each new pH the solution was left to mix and re-adjust to the new pH for three minutes. Thereafter samples were collected and centrifuged at 10,000 × g for 15 min at 4 °C. From the resulting two phases the supernatant was collected, and protein content was determined through the modified Lowry method. Solubility, in percentage, was then calculated according to equation 3. From the solubility curves, a rough estimate of both brine's p*I* could be made.

$$\frac{Protein\ concentration_{supernatant}}{Protein\ concentration_{\ brine}} * 100 \quad (3)$$

3.4 Ionic strength determination

For ionic strength determination of the process waters the conductivity was measured. A dilution series of different molar of NaCl, from 0 M to 2.5 M, was used as a standard curve, which are presented in Figure 6.

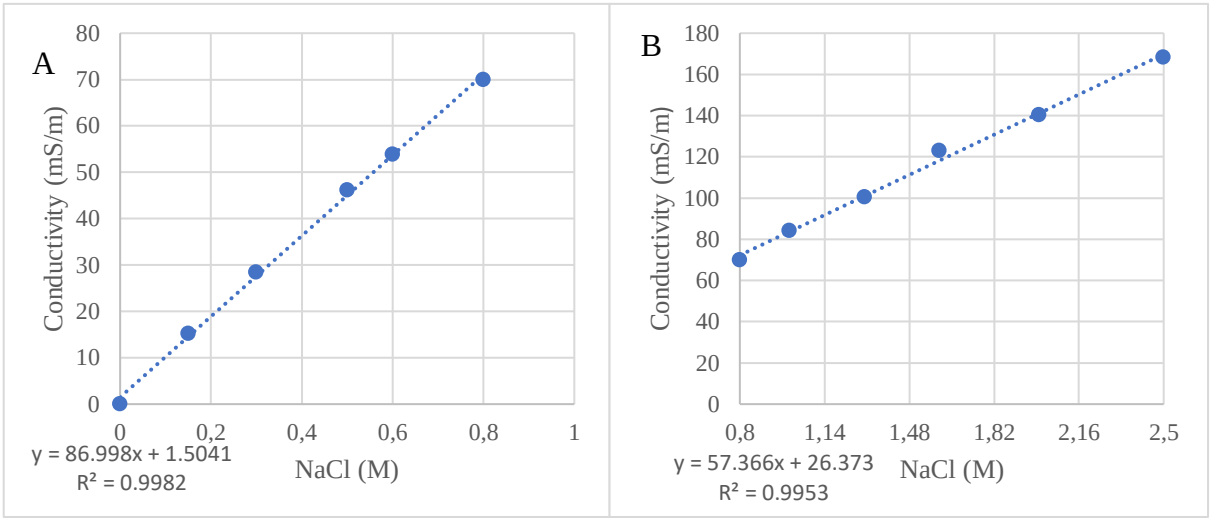


Figure 6. Ionic strength standard curves from 0-0.8 M NaCl, graph 6.A, and from 0.8M to 2.5M NaCl, graph 6B. Two curves were created due to the linearity changes with increased molarity.

The subsequent calculations for salinity, expressed as NaCl-equivalents, were done;

For the lower range (0-0.8 M)

$$\frac{conductivity - 1.5041}{86.998} * M_{NaCl} \qquad (4.A)$$

For the higher range (0.8-2.5 M)

$$\frac{conductivity - 26.373}{57.366} * M_{NaCl} \qquad (4.B)$$

3.5 Flocculation in process waters

Flocculation tests were carried out in 30 ml test tubes, where approximately 20 ml of process waters were mixed with a flocculant solution. Immediately after addition of flocculant the mixture was stirred; at 200 rpm for 2 min and then 100 rpm for 1 min. Thereafter the samples were left to sediment for 1 hour in room temperature. Each flocculant-sample combination was carried out in duplicates. For each test the relative sediment height (RSH) was measured. The RSH was calculated by dividing the height of the sediment by the initial height of the sample in the tube.

$$RSH = H_{sediment}/H_{liquid\ prior\ to\ sedimentation} \quad (5.A)$$

Where a RSH close to 1 represent no sedimentation (no phase separation between supernatant and sediment) and a RSH close to zero represent a high level of sedimentation (high phase separation with a large supernatant and small and densely packed sediment). A sample from the supernatant, after flocculation and sedimentation, was collected for protein content determination to examine how much protein was left in the supernatant and had not been sedimented, referred as residual protein concentration. Finally, the sedimentability was calculated as follows:

$$Sedimentability\ (\%) = (1 - \frac{Protein\ concentration_{supernatant}}{Protein\ concentration_{brine}}) * 100 \quad (5.B)$$

For the flocculation experiments the following flocculant solutions were used; 1% chitosan solution was made by suspending chitosan powder in 70°C in 1% acetic acid. Alginate and carrageenan solutions were prepared the same way but without acetic acid. CNC was provided in 1% solution and Silica in 30% solution (diluted 30 times to achieve 1% solution). Firstly, controls in the pH range 4-6.5 were made without the addition of flocculants to investigate the effect of pH *per se* on sedimentation and protein content in supernatant. Secondly, experiments were designed to evaluate the effect of the flocculants in the brines, where an interval of pH-values was used to which a fixed amount of flocculant solution was added. The added flocculant concentrations, based on literature: Forghani et. al. (2019) (24), and their pH ranges (based on flocculant charge) are described in Table 1.

Table 1. Flocculant dosage and pH range applied for each flocculant in both pre-salting brines.

Flocculant	**Concentration (g/L)**	**pH-range**
Chitosan	***0.5***	***4.5 -6***
Alginate	***0.5***	***4-5.5***
Carrageenan	***0.45***	***4-5.5***
CNC	***0.5***	***4-5***
Silica	***0.5***	***4-6***

For alginate and carrageenan additional experiments were designed to evaluate the effect of flocculant concentration at their respective optimum pH. The concentration ranges were created

from the fixed concentrations used when pH was variable (0.5 g/L for alginate and 0.45 g/L for carrageenan). The following concentration set points for alginate were 0.1, 0.25, 0.5, 0.75 and 1.0 g/L at pH 4. For carrageenan the concentration set points were 0.09, 0.225, 0.45, 0.675 and 0.9 g/L at pH 4. Each individual flocculation test was performed in duplicates.

3.6 Extraction of sarcoplasmic and myofibrillar proteins from herring mince

Sarcoplasmic and myofibrillar protein extraction was performed using minced herring fillets (see Appendix C). Twenty grams of herring mince was weighed out for the protein extraction. Extraction was performed with four different extraction liquid: herring mince ratios, 2:1, 6:1, 10:1 and 20:1. For the sarcoplasmic protein extraction the ionic strength was adjusted to 0.05M by addition of 1M NaCl in MilliQ according to each liquid:mince ratio. The mixture of liquid and mince was then homogenized and left to incubate for 30 min on ice, followed by centrifugation at 10,000 × g for 15 min at 4 °C. The supernatant was collected and stored in -80°C. For extraction of myofibrillar protein the pellet from the sarcoplasmic protein extraction was re-suspended in 1M NaCl and milliQ water. The same liquid: mince ratios (based on initial weight of herring sample) were done but had the ionic strength adjusted to 0.7M. The pellet and liquid mixture was homogenized, left to incubate on ice for 30 min and then centrifuged at the same conditions as the centrifugation step for sarcoplasmic protein extraction. Finally, the supernatant was collected and stored in -80°C. The collected supernatant samples from both protein extractions were used for determination of protein content, total protein recovery, and polypeptide profiling using SDS-PAGE. Protein recovery was calculated as follows;

$$Protein\ recovery\ (\%) = \frac{(protein_{content\ of\ supernatant} * volume\ of\ supernatant)(g)}{g_{sample} * 0.18} * 100 \quad (6)$$

*g_{sample} is the weight of the sample (approximately 20 g) and 0.18 is conversion factor (18 % estimate of protein in raw herring).

3.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

For polypeptide profiling of the process water model system samples SDS-PAGE was applied. The samples were diluted to have a protein concentration of 1.33 mg/ml, to normalize protein amount across all samples. Samples for loading the wells in the gel were prepared by mixing 25 µl normalized samples with 25 µl of dye solution. Thereafter, the mixture was subject to a boiling step at 95 °C for 5 min. Gels were prepared, by loading 15 µl of the mixed solution in each well, then the gels were put into the electrophoresis vessel and a voltage of 100 V was

applied. The gels were left under the applied voltage until the protein bands had traversed to the bottom of the gel. Gels were then carefully removed from their plastic covers and transferred to small plastic boxes placed on a pivoting table. Gels were stained according to the Coomassie blue staining procedure. The staining solution was added and put on a pivoting table for 30 min. Followed by a de-staining step with the de-staining solution, this was repeated, 20 min for each time, until the liquid around the gel was un-colored. Pictures were finally taken of the gel. (See Appendix D)

3.8 Statistics

To determine if any of the treatments with flocculants had any significant effect on flocculation in the process waters, ANOVA was used. The tested parameter was the resulting RSH of each treatment. Each flocculant treatment RSH was tested against pH control RSH. T-test assuming unequal variances was used, with null hypothesis; hypothesized mean difference equal to zero, to identify possible significant treatment levels (e.g., a certain concentration or pH). The significance level was set to $\alpha = 0.05$ for both types of tests.

4. Results and Discussion

4.1 Characteristics of herring pre-salting brines

The characteristics of the 5% pre-salting brine and 13% pre-salting brine at the point of collection are presented in Table 2.

Table 2. Characteristics of the process waters, 5% salt brine and 13% salt brines. The herring fillets had been cut into pieces for the 13% brine but left as whole fillets for the 5% brine. The incubation time for the two brines were 14h and 20h, respectively.

Process Waters	pH	Protein (mg/ml)	Fat content (%)	Salinity (M)
13% salt brine	6.4	12.97 ± 0.3	0.36	0.65 ± 0.07 (3.77%)
5% salt brine	6.3	7.07 ± 0.6	0.35	0.83 ± 0.2 (4.76%)

The fat content of both process waters is fairly low. No technique to concentrate the fat, such as flotation, has been applied, the waters are therefore in their native state. However, seasonal variations as well as the duration of which the herring has incubated in the brine might change the amount of fat. What stands out in both brines is the salinity of the waters, comparing the initial salinity of the brine with the salinity of the water when it was collected. The 5% brine had, as its name implies, an initial salinity of 5% and the collected water of ~4.87%. The 13% brine had an initial salinity of 13% and a final salinity of ~3.81%. Osman et al. (2015) describes the effect salt has on the herring flesh. When the salinity of the marinating water is higher than the physiological salt level in the fish (>0.9 %) an osmotic pressure gradient is present. The gradient cause migration of salt into the flesh and a counteracting flux of water out of the flesh. Another factor that may contribute to the difference in salt may be the size and cuts of the herring, pieces or fillets. If the fillets are cut into pieces the surface area of each individual piece would be increased compared to a whole fillet. The higher surface area may contribute to a higher rate of absorption of salt into the flesh which contributes to a higher protein leakage, especially myofibrillar proteins. Furthermore, cutting the fillets into piece will also disrupt the structure of the proteins in the flesh further boosting leakage of proteins. (32) Even though the fillets in the 5% brine had been cured for six more hours than the 13 % brine, the combination of higher osmotic pressure and the lower total area of herring fillets (and subsequent higher structural integrity) the herring fillets had not absorbed nearly as much salt. Which probably explains the lower amount of protein leakage, which is represented by the lower protein concentration, 7 mg/ml in the 5% brine versus almost 13 mg/ml in the 13% brine.

4.1.1 Protein solubility in the pre-salting brines as a function of pH

As can be seen in Figure 7. the solubility of the proteins in both brines was low in the lower pH range, with a subsequent minimum for both brines at around pH 4. When increasing pH from pH 4 the protein solubility increased and at pH 6-7 that increase stagnated, as well as when dropping pH closer to pH 3 solubility increased, which was as expected (shown in Figure 4.).

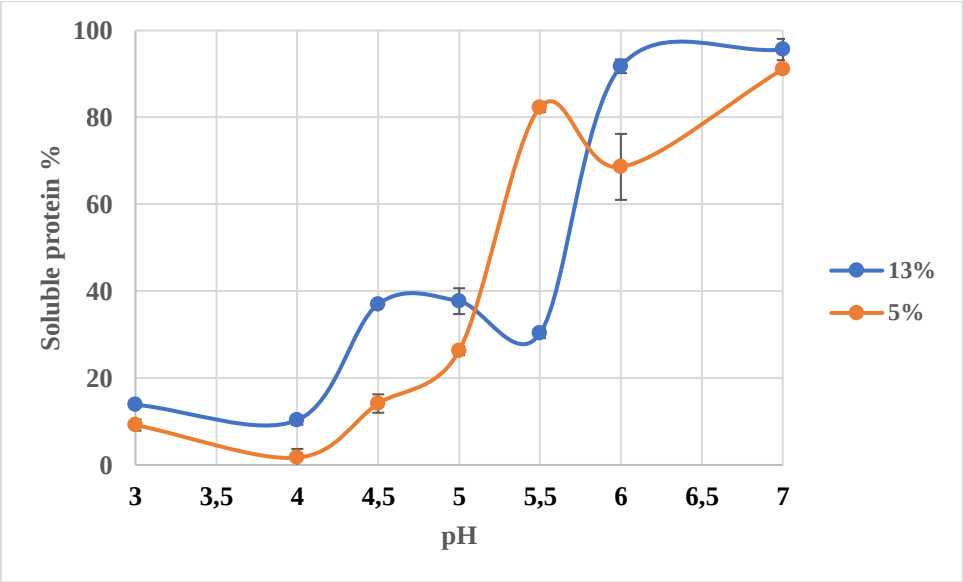


Figure 7. A solubility curve 5% brine and 13% brine to approximate the isoelectric point of the two brines, protein measurements were made in duplicates.

The p*I* estimate for both brines by this method, which is done by analyzing the effect that pH has on the solubility of the proteins in the given solution, is, however, a rough estimation. When attempting to estimate the p*I* of any protein and salt rich liquid solution by this method there are some factors to be aware of that affects the solubility of the proteins. The purity of the proteins in the solution, the amount of proteins and the salt content influences the p*I* of the solution. Where more charged amino acids and higher concentration of ions (higher salt content) affects when the point of neutral charge, and thus solubility, is achieved. (34) In this case, for the 5% and 13% brine, the higher salt concentration in the 5% brine (4.8% versus 3.8%) and the relative amount of acidic amino acids might balance each other so that the p*I* estimations for both brines are close to each other at around pH 4.

4.2 Flocculation treatments of herring pre-salting brine

4.2.1 Effect of pH on sediment height and protein flocculation

For both brines and for each pH tested, a pH control was made; this control recorded the three flocculation makers by the effect of pH alone, this is presented in Table 3. The sediment, in the cases where separation had occurred, was loosely packed and since the supernatant was small the risk of unintentionally collecting particles during pipetting, this caused the analysis of residual protein in the supernatant to vary (not represented in the table, but observational remarks) in several runs. The highest protein sedimentability was recorded at pH 4 reaching 91.5%. As expected, the changes in pH seems to only affect the solubility of the proteins (as previously described in section 4.1) which subsequently causes the observed minor phase separation. The pH had a slight, but significant, effect on the RSH where a lowered pH resulted in a lower RSH in both brines (p-values<0.05 for both brines). The only flocculation efficiency marker from the pH controls which was determined to be reliably used for comparing flocculant treatments versus the effect of pH was the RSH. The RSH from the pH controls is henceforth labelled 'RSH-pH control' in upcoming graphs.

Table 3. Evaluation of the effect of pH alone on the two pre-salting brines. Denotes the residual protein concentration in the supernatant after one hour sedimentation.*

pH control 5% pre-salting brine				
pH	RSH	Res. protein Sup.(mg/ml)*	Volume supernatant (ml)	Protein sedimentability (%)
4	0.90±0.0	0.60±0.00	1.96±0.0	91.5±0.01
4.5	0.95±0.0	5.83±0.22	0.98±0.0	17.6±3.07
5	0.96±0.0	5.06±0.27	0.74±0.0	28.4±3.89
5.5	1±0.0	7.07±0.0	0±0.0	0±0.0
6	1±0.0	7.07±0.0	0±0.0	0±0.0
pH control 13% pre-salting brine				
pH	RSH	Res. protein Sup.(mg/ml)*	Volume supernatant (ml)	Protein sedimentability (%)
4	0.90±0.0	2.36±0.08	1.96±0.0	81.8±0.62
4.5	0.90±0.0	1.80±0.01	1.84±0.0	86.1±0.10
5	0.90±0.0	3.38±0.03	1.35±0.0	73.9±0.23
5.5	0.95±0.0	5.18±0.02	1.47±0.0	60.1±0.13
6	1.00±0.0	12.97±0.0	0±0.0	0±0.0

Figure 8 shows one of the pH control runs. Unfortunately, the lighting at the time of photography made the supernatants appear more turbid than they were. However, it is still possible to observe the supernatant, in the three leftmost tubes, which were the three most acidic ones (pH 4, pH 4.5 and pH 5). Additionally, apart from a small phase separation, a whitening colour effect can be observed, the lower the pH the whiter the appearance of the brine becomes. In these controls no instantaneous formations of flocs were observed.

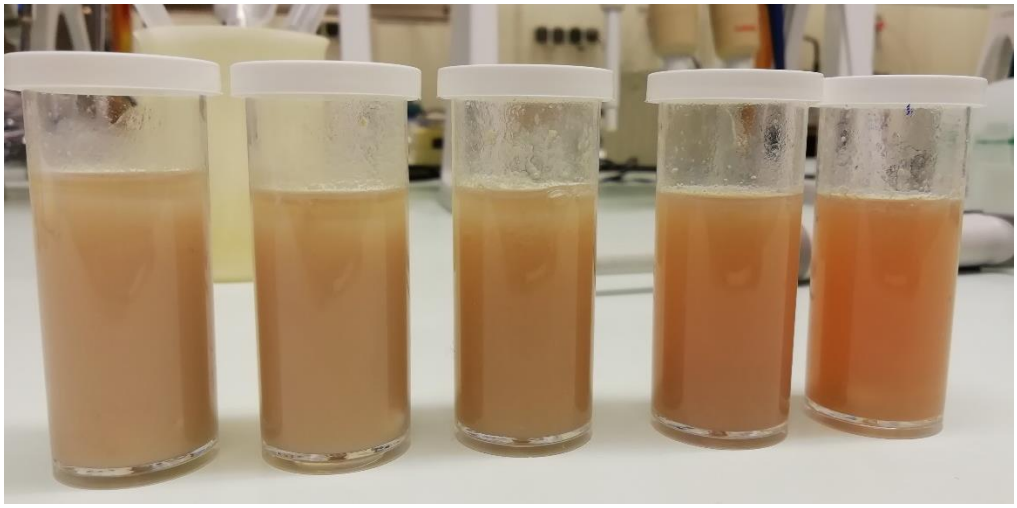


Figure 8. Effect of pH alone on flocculation in the 5% pre-salting brine.

4.2.2 Effect of flocculants on sediment height and protein flocculation at varied pH

The results from the flocculation experiments are presented and discussed in the terms of two of the flocculation performance markers, RSH and protein sedimentability (%). Each flocculant is presented individually for how they affected the performance markers. In Appendices F, G and H the complete datasets are presented.

4.2.2.1 Effect of chitosan addition

The effect of flocculation with 0.5 g/L chitosan at varied pH in both pre-salting brines are presented in Figure 9. For all pH points the addition of chitosan reduces the RSH compared to the pH control in the 5% pre-salting brine, whereas in the 13% pre-salting brine the addition of chitosan does not affect RSH to the same extent. ANOVA analysis shows $p<0.05$ for the effect of chitosan on RSH in the 5% and $p>0.05$ for the effect on RSH in the 13% brine. No significant difference was found between addition of chitosan at pH 4.5 and pH 5 on the effect on RSH, but a significant effect of both pH points with chitosan compared to the effect of their respective

pH on its own was found. Furthermore, in the 5% brine the protein sedimentability seemed to follow a linear dependency, with decreased protein sedimentability the higher the pH. However, the same observations could not be seen when 0.5 g/L of chitosan was added to the 13% brine. Nor could any visual observations of creation of flocs be seen in the 13% brine, whereas it was observed at pH 4.5 and 5 in the 5% brine.

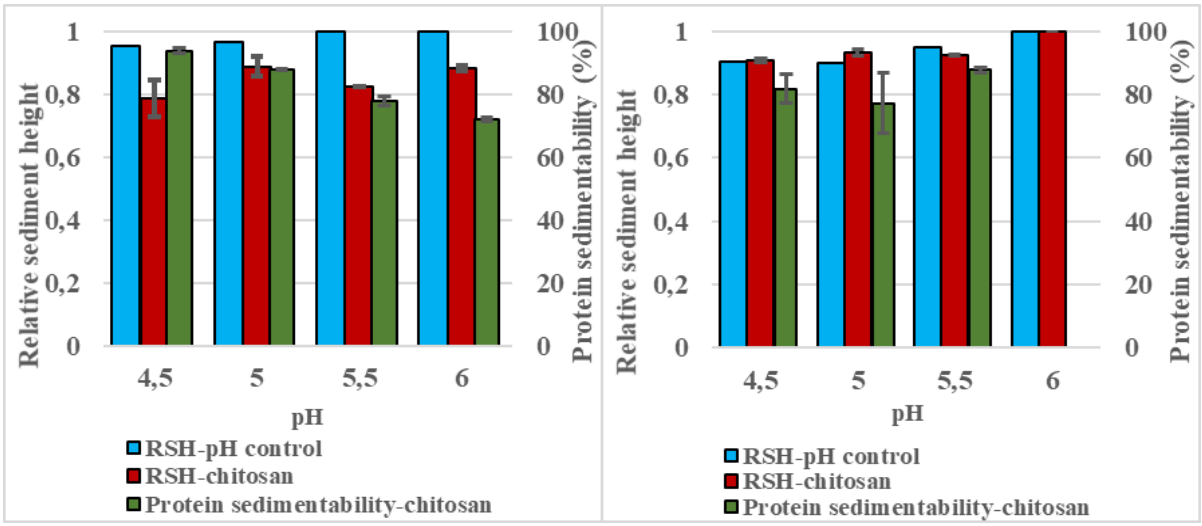


Figure 9. Flocculation in both pre-salting brines with 0.5 g/L of added chitosan. pH control contains no chitosan. Left panel: 5% brine and right panel: 13% brine.

In Figure 10 one of the flocculation runs with chitosan is visualised. It represents the experiments with chitosan carried out at a fixed concentration, 0.5 g/L, and at pH 4.5, 5 and 5.5. A noteworthy difference from the photo of the effect of pH is in the leftmost tube, where chitosan addition at pH 4.5 caused an greater phase separation and an observable creation of flocs (small in size, thus, not clearly visible in the photo), indicating that flocculation occured.

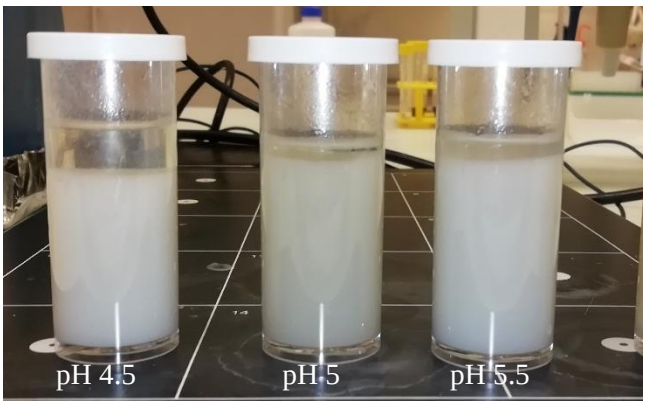


Figure 10. Effect of the addition of chitosan at 0.5 g/L at different pHs on flocculation in the 5% pre-salting brine.

4.2.2.2 Effect of alginate addition

Displayed in Figure 11 is the effect of adding 0.5 g/L alginate to both brines. The effect on RSH in the 5% brine was non-significant, p-value>0.05, whereas addition of alginate was found to have a significant effect on the 13% brine, p-value<0.05. However, the directional effect on RSH seemed to be opposite desirable effect (an increased RSH). Similarly, to the results found for addition of chitosan the protein sedimentability followed the same tendencies with the addition of alginate in the 5% brine. Regarding pH 4 in the 5% brine a visual formation of flocs could be seen, with a reducing effect on RSH, which was significant compared to pH alone, and higher protein sedimentability compared to pH control at pH 4.

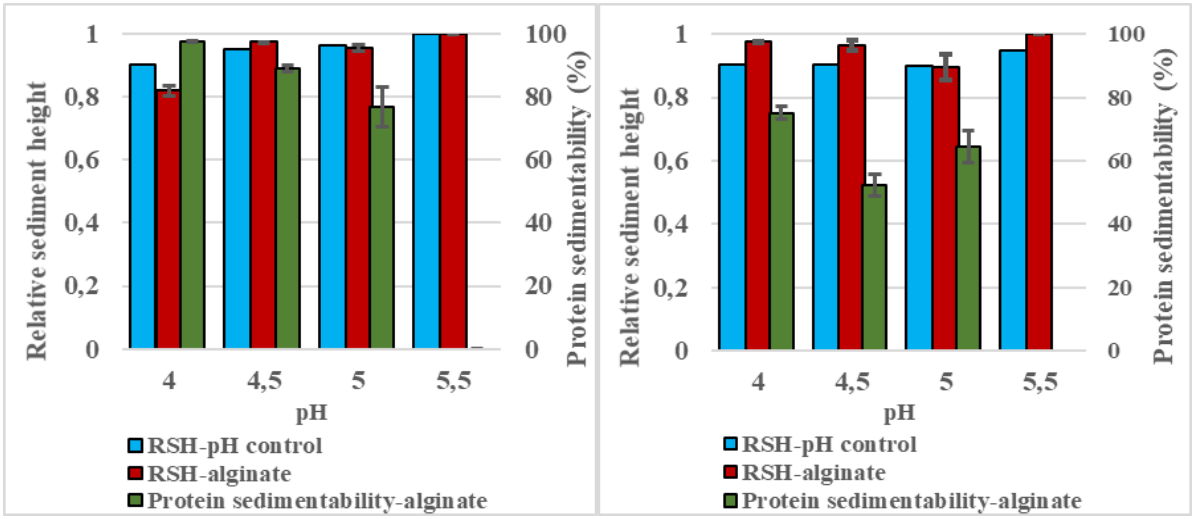


Figure 11. Flocculation in both pre-salting brines with 0.5 g/L of added alginate. pH control contains no alginate. Left panel: 5% brine and right panel: 13% brine.

4.2.2.3 Effect of carrageenan addition

Presented in Figure 12 are the results from adding carrageenan (0.45 g/L) to both brines. As can be observed addition of carrageenan slightly reduces RSH in the 5% brine, but not enough to be significant (p-value>0.05). For carrageenan addition into the 13% a significant increase of RSH can be observed at all pH points. Only pH 4 in the 5% brine showed promising results; reducing effect on RSH and simultaneously recording the greatest protein sedimentability (~98 %). The pH 4 was also visually observed to have formation of flocs showing to be significant compared to RSH-pH control.

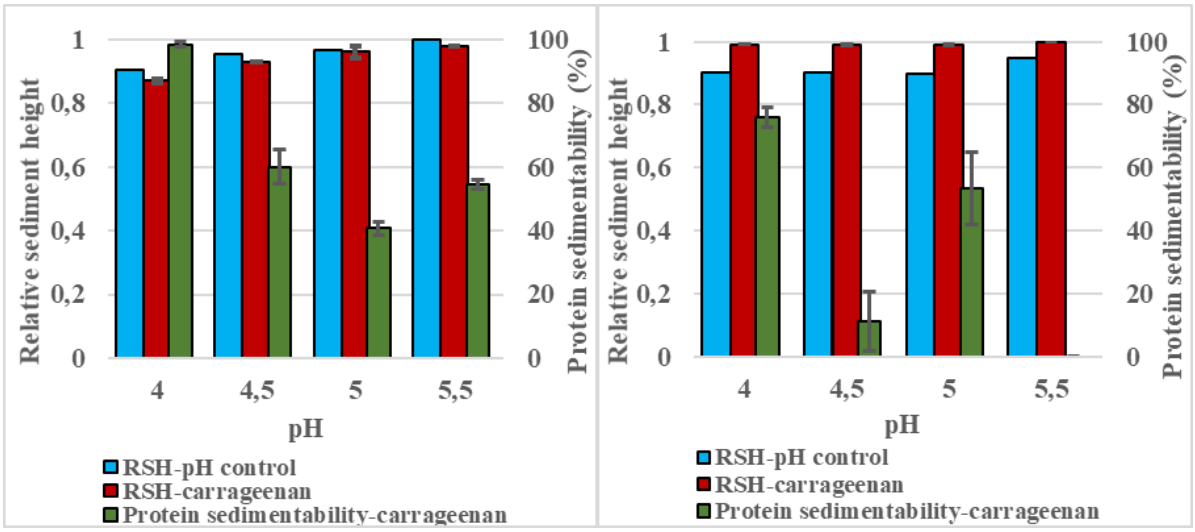


Figure 12. Flocculation in both pre-salting brines with 0.45 g/L of added carrageenan. pH control contains no carrageenan. Left panel: 5% brine and right panel: 13% brine.

4.2.2.4 Effect of CNC addition

In Figure 13 the results from flocculation runs with 0.5 g/L of CNC added, at varied pH, are presented. In the 5% brine addition of CNC was determined to have a significant influence on the RSH, p-value<0.05, reducing the RSH. pH 5, which had the most affected RSH the most was checked for significance, revealing that addition was non-significant (p>0.05) as compared to RSH-pH control. In the 13% brine addition of CNC had a significant increasing effect on RSH. The obtained protein sedimentability did not indicate that any flocculation had occurred compared to pH control, and no visual observations were documented.

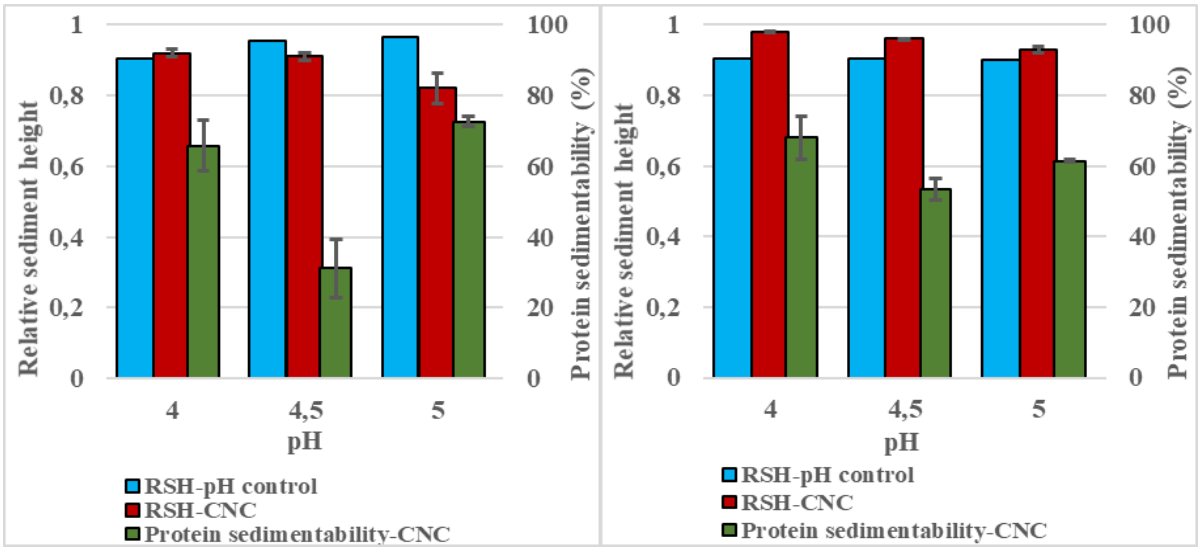


Figure 13. Flocculation in both pre-salting brines with 0.5 g/L of added CNC. pH control contains no CNC. Left panel: 5% brine and right panel: 13% brine.

4.2.2.5 Effect of silica addition

In Figure 14 the data-points from flocculation with 0.5 g/L of silica, at varied pH, are visualized. In the 5% brine addition with silica had a non-significant influence on the RSH, p-value>0.05, compared to the RSH-pH control and the addition of silica in the 13 % brine had a significant increasing effect on RSH p-value<0.05. Furthermore, the protein sedimentability nor any visual observations pointed towards that flocculation had occurred compared to pH control.

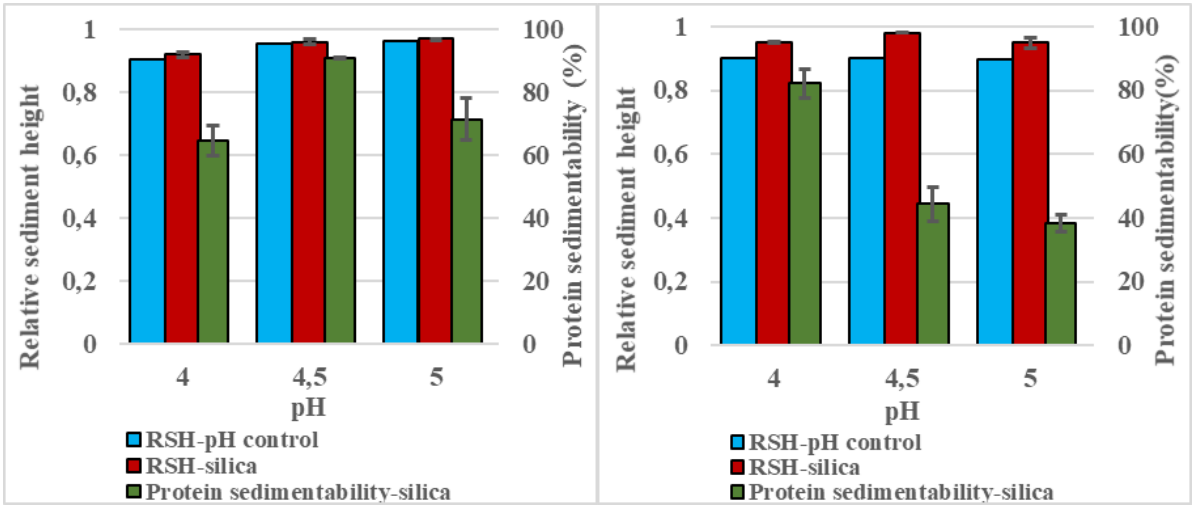


Figure 14. Flocculation in both pre-salting brines with 0.5 g/L of added silica. pH control contains no silica. Left panel: 5% brine and right panel: 13% brine.

4.2.2.6 Discussion on flocculation at a fixed concentration

The results from flocculation with chitosan, alginate and carrageenan indicate that the p*I* estimate for both brines was slightly inaccurate, as expected. The p*I* estimate, for both brines, was placed at pH 4, which would not be accurate based on the notion of charge, thus, alginate nor carrageenan would function at pH 4. Thus, the real p*I* would be slightly higher than pH 4 but lower than pH 4.5. Regarding the flocculation, addition of 0.5 g/L or 0.45 g/L (for carrageenan) of every flocculant in the 13% brine seemed to increase the RSH, i.e., worsening flocculation performance. A potential reason for this occurrence could be that flocculant dosage was not normalized to the initial protein concentration in the brine. 0.45/0.5 g/L of added flocculant worked fine for 7.07 mg/ml protein concentration but was not enough for 12.97 mg/ml. The dosage of added flocculant should have been 0.825 g/L for carrageenan and 0.92 g/L for the remaining four flocculant (based on gram flocculant per gram protein in the

solution). Flocculation depends on de-stabilization of the system for suspended particles to aggregate. Thus, the addition of a too low amount of flocculant might not have been enough to cause enough of a de-stabilization to allow for flocculation. (7) The probability of salt affecting this observed performance drop of flocculation in the 13% brine versus the 5% is low. Trulsson et al. (2006) indicated that higher salt concentrations could cause a charge inversion, and that the higher valency of the ions provokes charge inversion at lower concentrations, of the suspended particles and thus, prevent flocculation of said particles. (18) To relate their findings to the results in this study, even though the sodium and chloride ions are monovalent, the salinity of the 13 % brine is roughly one percentage unit lower than the salinity of the 5% brine (Table. 2). However, the flocculation efficiency is lower in the 13% brine which has the lowest salt content, which seems contradictory to findings reported by Trulsson et al. (2006). Potentially indicating that charge inversion, caused by excess salt, might not be as prevalent when analysing systems with monovalent ions. The 'critical' concentration might be higher than salt concentration in for example the analysed pre-salting brines in this report. In a study, Gamage et.al (2007), three types of chitosan were produced through deacetylated chitin and evaluated for the flocculation capacity on Bovine serum albumin. Their created chitosan recorded almost 100% protein recovery at low protein concentrations (2mg – 4 g/mL) but performance dropped by 35 units of percent when protein concentration was increased to 6-8 mg/mL. (35) Even though chitosan was evaluated at lower pH in this study (pH 4.5/5 versus pH 7 in Gamage et.al.'s study (2007) a similar trend can be seen, that when the protein concentration in the process water increases chitosan's' performance drops off. Silica can be modified, as exemplified by Demchenko et. al (2010) (26), where modifications could include changing the functional groups to facilitate interaction between flocculant and protein. CNC, which had a significant effect on RSH but no statistically optimal pH, can also be modified. In a study by Blockx et.al. (2020) CNC was modified with functional groups, pyridinium and methylimidazolium and at different degrees of substitution, to harvest microalgae. The authors tested an unmodified CNC as a control in their flocculation runs, and flocculation with the unmodified CNC was found to not induce flocculation. Whereas the two groups of modified CNCs were able to reach 95% flocculation efficiency (optical density measurements) in the dosage range from 10 to 50 mg/L. (36) This study does not relate to the findings or procedure in this report, but it does, however, point towards CNC being a highly modifiable flocculant. With the knowledge that CNC can be modified and the significant reduction of RSH in this report CNC does show potential, although not the most promising. Carrageenan and alginate

were found to have significant effects, so they underwent concentration dependent flocculation, thus, they are discussed in the following section.

4.2.3 Effect of flocculant concentration on sediment height and protein flocculation

From the optimum pH setting that the flocculants performed the most efficient the concentration dependency was investigated. This has been done for both carrageenan and alginate in the 5% pre-salting brine presented in the sections below. The optimum pH for both was deemed to be at pH 4, where both the protein sedimentability was the greatest and the reduction of RSH was the most efficient. Each flocculant is presented individually for how they affected the protein sedimentability and the RSH levels.

4.2.3.1 Effect of added carrageenan concentration at fixed pH

In Figure 15 the data from varied added concentration of carrageenan in the 5% brine is presented. The general trend regarding the RSH is that with higher added concentration of carrageenan the more reduced the RSH became. These changes were significant p-value<0.05, indicating that flocculation with carrageenan is concentration dependent. Furthermore, all concentrations were found to be significant on reducing the RSH compared to the respective RSH-pH control. The difference between the low concentrations and high concentrations had a significant difference suggesting that higher concentrations resulted in more reduced RSH. Thus, a t-test was performed between concentrations 0.675 and 0.9 g/L to identify the potentially optimal concentration. However, the difference between the two treatments was non-significant, rendering it impossible to distinguish between the two treatment levels. Both treatment levels were also similar with respect to protein sedimentability (~98.7% at 0.675 g/L versus ~99.1 % at 0.9 g/L) further proving the difficulty of separating both treatment levels.

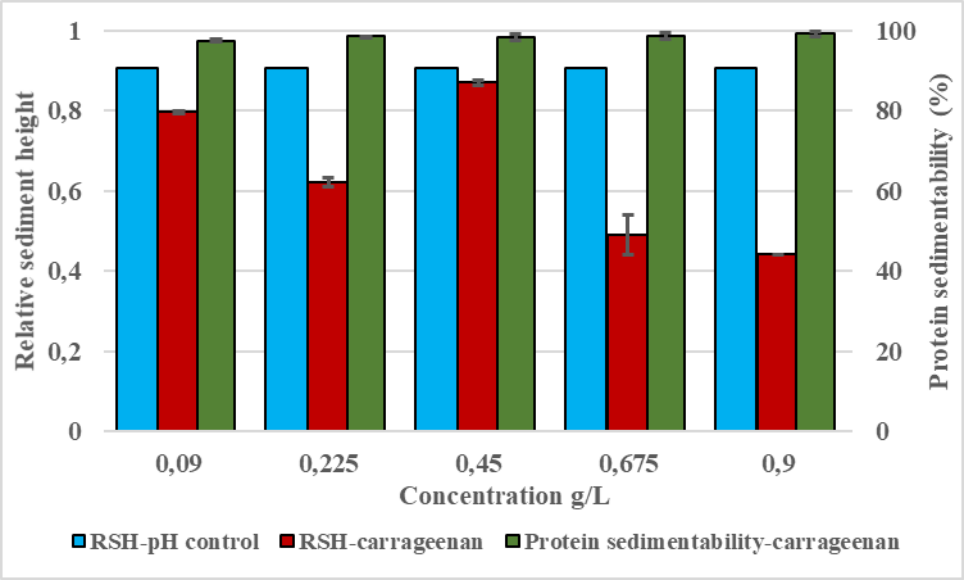


Figure 15. Flocculation in 5% pre-salting brine at pH 4 with varied added concentration of carrageenan. pH control contains no carrageenan.

The photograph, Figure 16, is taken during the one-hour sedimentation phase, as can be observed separation had already occurred. Furthermore, the sediment has a granular resembling appearance, these are the flocs. At the end of the sedimentation time the flocs that are suspended in the liquid had settled and the liquids' appearance was clear. The concentration 0.09 g/L is not included in the picture, it also had an observable creation of flocs but affected the phase separation to a lesser extent.

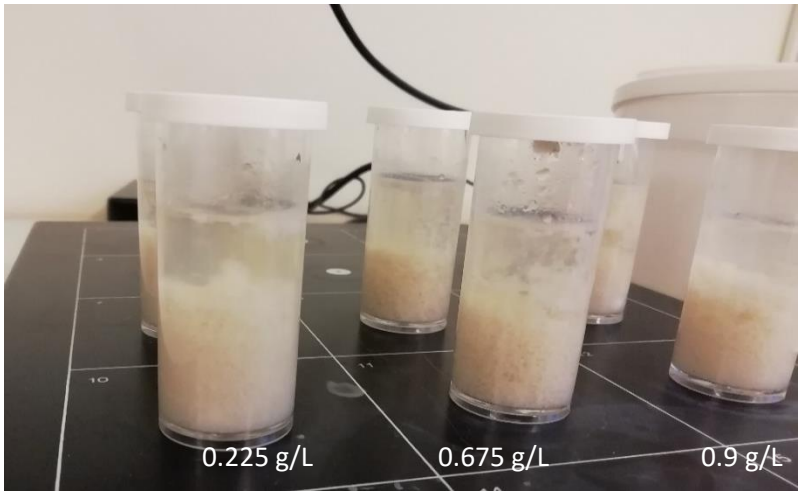


Figure 16. Effect of the different carrageenan concentrations at pH 4 on flocculation in the 5% pre-salting brine.

4.2.3.2 Effect of added alginate concentration at fixed pH

In Figure 17 the results from varied added concentration of alginate in the 5% brine is presented. The effect of added alginate at different concentrations on RSH was found to be statistically significant with p-value<0.05 compared to the pH control for pH 4. However, a change in concentration did not seem to have any effect on the RSH. A t-test determined that the changes in concentration were non-significant. Additionally, the different concentrations, lower versus higher, tended to yield similar protein sedimentability (~97.5 - 98 %).

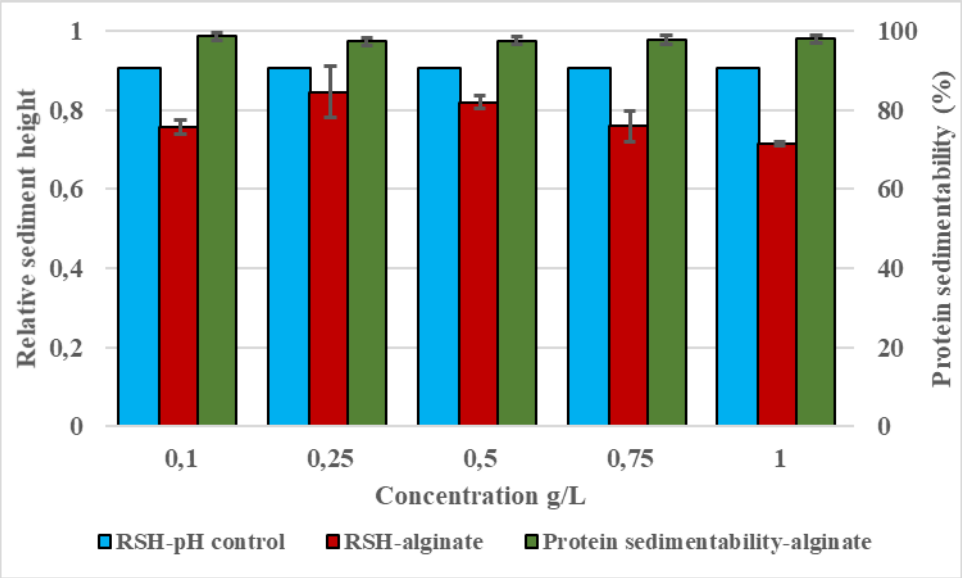


Figure 17. Flocculation in 5% pre-salting brine at pH 4 with varied added concentration of alginate. pH control contains no alginate.

Figure 18 shows the effect of adding different concentrations of alginate at pH 4 in the 5% brine after one hour of sedimentation. From the left to right are the incremental concentrations: 0.1, 0.25, 0.75 and 1.0 g/L. From the photograph, Figure 18, it can be observed that phase separation was distinct, that the supernatant was transparent and a creation of flocs, although the flocs were visually smaller in size than the flocs created upon carrageenan addition at pH 4.

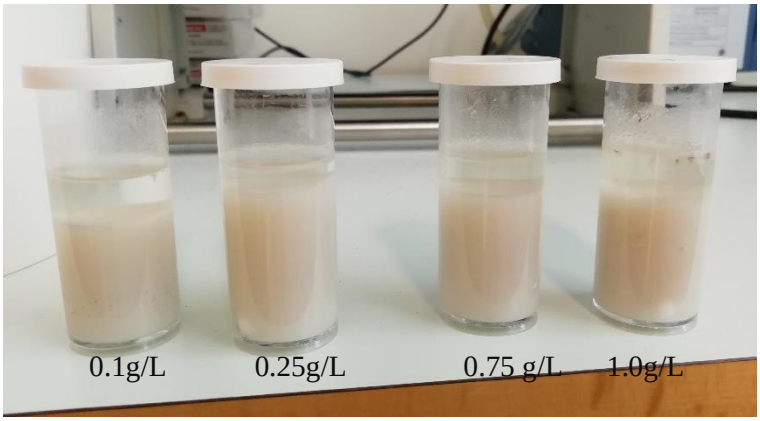


Figure 18. Effect of the different alginate concentrations at pH 4 on flocculation in the 5% pre-salting brine.

4.2.3.3. Discussion on flocculation at a fixed pH with variable flocculant concentration

Flocculation is concentration dependent up to a concentration at which the treatment re-stabilizes and the process of particles aggregating ceases. (7) When reviewing alginate and carrageenan for their respective concentration dependent effect on flocculation it can be seen in the case of alginate that reduction of the RSH was not significantly affected by different concentrations. Furthermore, residual protein concentration in the supernatant (appendix H) and the protein sedimentability (%) were similar across all concentrations. Thus, it cannot be concluded that flocculation with alginate at pH 4 is concentration dependent or concentration independent, raising the possibility that pH is the governing factor. Regarding the concentration dependency of flocculation at pH 4 with carrageenan, a statistically significant reduction of RSH and lowered residual protein concentration (appendix H) with higher added concentration of carrageenan, it seems to be concentration dependent. At least between lower (0.09 and 0.225 g/L) concentrations and higher (0.675 and 0.9 g/L) concentrations. The statistically non-significant t-test comparing 0.675 and 0.9 g/L of added carrageenan could potentially indicate that the concentrations are close to maximum performance. Thus, there is a likelihood that higher than 0.9 g/L concentrations will not improve flocculation but instead cause an involuntary re-stabilization of the system in the 5% brine.

Forghani et.al. (2019) investigated the flocculation efficiency, defined as protein recovery, residual protein reduction and RSH, of alginate and carrageenan in shrimp boiling water, which had a protein content (10.9 mg/mL) during flocculation experiment which is close to the 13% brine analysed in this report (12.97), and at similar added concentrations and pH ranges. Their findings indicated that carrageenan flocculation capacity was optimal at pH 4, and that it was more effective than alginate, recovering 86% of protein. (24) This supports the findings done

in this work where carrageenan was more efficient than alginate, although the best performance was recorded in the 5 % brine which had a lower (7.07 mg/mL) protein content. The lower protein content in the 5% could be one of several reasons why a higher protein sedimentability (~99%) was recorded in this study. In a study performed by Hao et.al. (2016) the optimal pH for flocculation and protein recoveries of chitosan, guar, alginate, and carrageenan was evaluated in fishmeal wastewater. Similar to the results found in this report and those reported by Forghani et.al. (2019), Hao et.al. (2016) also concluded that carrageenan performed better than both chitosan and alginate, with optimum pH at pH 4 and that it recorded the highest protein recovery (70%). Furthermore, the authors reported that electrostatic interactions had a significant effect on flocculation performance and thereby raising the possibility that alginate would not achieve the same flocculation as carrageenan. Carrageenan's higher performance was reasoned to be a result of stronger functional group whereas alginate had weaker carboxyl groups. Thus, alginate attracted proteins to a lesser extent than what carrageenan could. (37)

4.3 Herring process water model system

4.3.1 Extraction of sarcoplasmic and myofibrillar proteins to create a model system

From the data displayed in Table 4, it could be speculated that freezing the mince prior to extraction improves the extractability (due to increased solubility of the proteins). This could account for the higher protein concentration for the sarcoplasmic fraction for extraction on frozen mince versus fresh mince. However, this cannot conclusively be stated for the 20:1 mince-liquid ratio nor could it be stated for the myofibrillar fraction. Marino et al. (2009) explored the difference in extraction between denaturing, D, (containing urea, thiourea, DTT, CHAPS, NP-40, glycerol and tris-HCL) and non-denaturing, ND, solutions (KCL phosphate buffer). Their findings suggested that the efficiency of myofibrillar extraction between ND and D solutions might be depending on the species. They stated that the two methods did not differ for hake or sea bass, but that the D-method was more efficient in sole. (38) Parallels are hard to draw since the results in this report are based on herring, but using a denaturation extraction solution, similar to Marinos et.al. (2009), in combination with freezing, might improve the extractability of the myofibrillar protein fraction. Furthermore, the most advantageous liquid to mince extraction solution, when considering ease of use and results, would be the 10:1 extraction solution. The more concentrated extraction solutions' 2:1 and 6:1 yielded a higher protein concentration, as expected, but not necessarily higher protein recovery (%) as a result in the frozen mince. Furthermore, the more diluted solutions (10:1 and 20:1) increased the ease of use due to comparatively larger supernatant and more distinct phase separation between supernatant and pellet, with the 10:1 solution yielding a higher protein recovery than the 20:1 solution. Noteworthy are two pair-wise unexpected results; both on the fresh mince extraction, where a more diluted extraction gave a higher protein content. This discrepancy is hard to explain, possibly experimental error, but the mince would be used for every extraction and, thus, had to be frozen and the same discrepancy was not observed on the frozen mince extractions.

Table 4. Sarcoplasmic and myofibrillar protein extraction from herring fillet. Fresh mince extraction was performed once (presented values are averages ± SD (E=1 and n=3)) and frozen mince extraction was performed three times (values are averages ± SD (E=3 and n=3)).

Extraction solution	Mince:liquid ratio	Protein content (mg/ml)	Protein recovery (%)
Fresh mince			
Sarcoplasmic (0.05M NaCl*)	2:1	7.97 ± 0.05	13.3 ± 0.08
	6:1	1.83 ± 0.21	7.10 ± 0.81
	10:1	2.25 ± 0.02	13.75 ± 0.12
	20:1	1.1 ± 0.04	12.8 ± 0.47
Myofibrillar (0.7M NaCl**)	2:1	14.8 ± 3.5	24.6 ± 5.83
	6:1	1.32 ± 0.11	5.15 ± 0.43
	10:1	0.1 ± 0.04	0.54 ± 0.25
	20:1	1.1 ± 0.004	12.3 ± 0.05
Frozen mince			
Sarcoplasmic (0.05M NaCl*)	2:1	8.93 ± 4.9	14.9 ± 8.31
	6:1	3.33 ± 1.6	12.9 ± 6.04
	10:1	2.6 ± 0.33	15.6 ± 2.01
	20:1	1.1 ± 0.48	12.7 ± 5.66
Myofibrillar (0.7M NaCl**)	2:1	7.2 ± 0.44	12.0 ± 0.73
	6:1	2.45 ± 1.4	9.51 ± 5.6
	10:1	1.72 ± 0.52	10.5 ± 3.17
	20:1	0.63 ± 0.26	7.38 ± 3.09

** Extraction solution was directly used for the herring mince.*

*** Extraction solution was used for re-suspending the pellet from first extraction.*

4.3.2 SDS-PAGE of sarcoplasmic and myofibrillar fractions

In Appendix E the complete photos of the gels from the SDS-PAGE on herring mince protein extractions can be viewed. As can be seen from Figure 19. the separation of myofibrillar and sarcoplasmic protein groups were distinct. Furthermore, the SDS-PAGE verifies that the extractions, in section 4.3.1, worked as intended. One can observe for columns 3 and 4 (myofibrillar fractions) a clear band at around 220 kDa this identifies as heavy chain myosin. Additionally, one can observe, in columns 3 and 4, several bands between 30-45 kDa and one distinct band at 17kDa. The bands at 45-17 kDa are presumably actin (45 kDa), tropomyosin (~38), troponins (~35-20 kDa) and myosin light chains components (~17 kDa). (39) In columns 1 and 2 (sarcoplasmic fractions) the first clear band, at ~70 kDa is observed this band could tentatively represent albumin (69 kDa) (40). A much larger occurrence of protein bands, compared to the myofibrillar fraction, was seen in the kDa range of 20-55 kDa. The bands, based on earlier studies of chicken proteins, could tentatively be identified as: 55 kDa phosphoglucose isomerase, 47 kDa enolase, 43 kDa creatine kinase, 39 kDa aldolase, 36 kDa glyceraldehyde phosphate dehydrogenase, 34 kDa lactate dehydrogenase, 25kDa phosphoglycerate mutase and 23 kDa triosephosphate isomerase.(41) Furthermore, the SDS-PAGE verifies that the extractions, in section 4.3.1, worked as intended, indicating very little contamination (little or no presence of sarcoplasmic proteins in the myofibrillar fraction and vice versa).

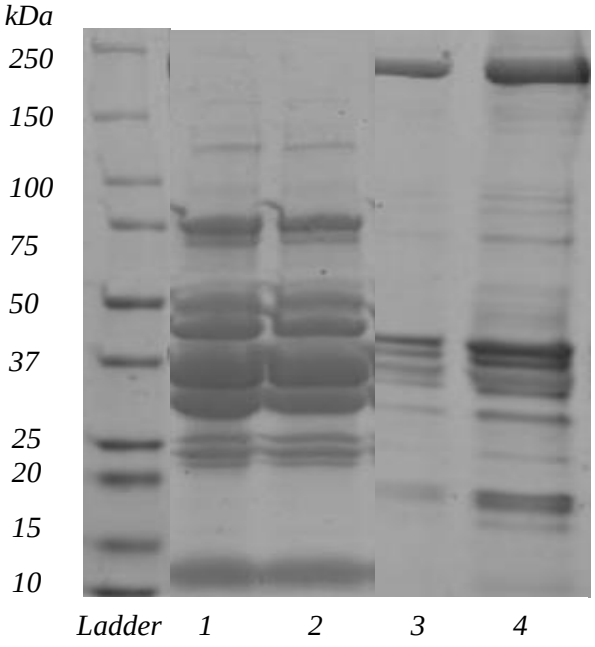


Figure 19. SDS-PAGE results. The ladder used as reference on the very most left, column 1&2 are from sarcoplasmic protein extractions (extraction liquid:mince ratios 2:1 & 6:1). Column 3&4 are myofibrillar extractions (extraction liquid:mince ratios 2:1 & 6:1).

However, despite the successful separation and profiling of the proteins in both extractions the model system was not deemed to be used for optimizing flocculation in the pre-salting brines. One reason was that trying to optimize flocculation performance in the model system, with lower complexity than the pre-salting brines, might not be directly carried over to the brines. Furthermore, the high complexity of the brines is down to several factors, such as, osmotic pressure, structural integrity of the herring flesh, proteolytic enzyme activity and marinating time, which contributes to the final protein/peptide content in the brine and can be hard to model for. This led to the decision that flocculation optimization would be done on the brines themselves. Whereas, creating artificial, pure, model systems the complexity could easily be increased and decreased. The model systems could instead be used for analyzing flocculant-protein interactions and determining the extent of the effect pH might have on flocculation. Additionally, an artificially made model systems would facilitate the possibility of adjusting the salinity of the solution, to create a simplistic model of the effect of salt on flocculation mechanisms.

5. Conclusions

The extraction of the sarcoplasmic and myofibrillar fractions were, although yielding relatively low protein recovery, successful corroborated by the polypeptide profiling through SDS-PAGE. However, given the complexity of the brines and that the polypeptide profile in the brines is dependent on cut, salinity, endogenous enzyme activity and length of marinating it would be beneficial to work with pure, artificially built, model systems only to determine the flocculation mechanisms of the flocculants. Whereas real herring brines would preferably be used to evaluate the quantitative performance of the different flocculants.

Based on the studies of real herring brines, alginate and carrageenan showed the most promising flocculation results, however, flocculation efficacy was only found in the 5% brine. None of the flocculants worked in the 13% brine:

- For carrageenan, the best added concentrations were: 0.675 and 0.9g/L at pH 4, where both concentrations recorded a protein sedimentability around 99%. The higher concentrations yielded a more powerful reduction of RSH than the lower concentrations, but all concentrations performed better than the respective pH control. However, since the protein sedimentability for the higher concentrations was only ~1-1.5 percent units higher than that recorded for 0.09 g/L and 0.225 g/L, the two latter would be the most financially sound choices.

- Regarding alginate, it was only effective at pH 4, independent of concentration added to the 5% brine. All tested concentrations recorded a more efficient RSH reduction and a higher protein sedimentability at ~98%, compared to the corresponding pH controls. The concentration independency could, however, indicate that sedimentation was mainly governed by pH rather than alginate.

- For chitosan, treatment with 0.5 g/L at pH 4.5 gave an increased protein sedimentability (93.8%) and also recording a more efficient reduction of RSH than the respective pH control. Trials evaluating the effect of higher concentrations of chitosan is, however, advised.

6. Future prospects

There are other food grade flocculants available on the market that should be evaluated on similar waters to examine if these are more competitive, both on performance and economics, versus conventional flocculants. As an example, proteins from *Moringa peregrina* and *Moringa olifeira* seeds have been shown to have promising flocculation capacity on proteins in water treatment. (42)

The experiments in this thesis have only been done in laboratory scale and the next experimental step is upscaling. Upscaling, in its itself, imposes several challenges both rheological and dynamic issues. Can one ensure uniform distribution of proteins and flocculants when stirring, to avoid areas within the process water where the flocculant-to-protein ratio is larger or smaller than desired? Another equally important factor, especially for industries, is the cost of the process. Can already existing infrastructure or technology be applied or is there a need for rebuilt infrastructure or novel technologies? The most desirable solution would be that a flocculation system could be integrated into the current infrastructure of the factory.

Furthermore, will the dosage of added flocculant follow a linear dependency in relation to the total volume of the liquid and not only by increasing amount of protein, would there be a change in rheological characteristics between different sizes of volumes that could influence the flocculant-to-protein ratio required? Could a combination of flocculants, as previously described for grafted silica, improve the flocculation capacity, by for example giving rise to a cocktail-effect. The idea of a flocculant combination system could be even more desirable if different flocculants target different proteins (as previously described); e.g., flocculant A is most effective on sarcoplasmic proteins and flocculant B on myofibrillar proteins. Then the flocculants A and B might achieve better results when combined than both individually. Thus, potentially reducing the cost of the process water treatment.

Additionally, the flocculation mechanism (bridging, charge neutralization etc.) which is the major driving mechanism of flocculation in high salt process waters should be determined to further understand the complexity of flocculation as a treatment technique. Furthermore, flocculation governed by isoelectric precipitation and gravity alone, could be aided by e.g., centrifugation. Aiding sedimentation would ultimately decide how much and how easily proteins can be extracted. By aiding sedimentation, the proteins, and other nutrients, that have interacted with the flocculant, the so-called sludge, will be more compressed and, thus, more

easily separated from the water phase. Furthermore, the more compact the sludge is the less water is in it, making the proteins higher in concentration and reducing the need to de-water the sludge as to extract the sought-after nutrients.

Finally, to be able to address the issue of water usage one needs to characterize the treated process water and identify the remaining contaminants, i.e. minerals, non-recovered protein and lipids. Since the treated waters will be of food grade quality it would be beneficial to evaluate the possibility to incorporate, on-site, a final treatment step to bring the process water back to such a quality that it can be re-used in new brines/marinades. Furthermore, an economical assessment, in the long-term, must be performed to determine if this would be cost-efficient for the industry, i.e., that the decreased cost for freshwater and reduced cost in form of water treatment plant taxation would out-weigh the long-term cost of infrastructure and treatment processes.

7. References

1. *Bratby, J. Coagulation and Flocculation in Water and Wastewater Treatment. Third edition. p. 1. IWA Publishing. 2016.*

2. *Renualt, F. Sancey, B. Badot,P.M, Crini, G. Chitosan for coagulation/flocculation processes – An eco-friendly approach. European Polymer Journal. Vol. 45. p. 1337-1348. 2009.*

3. *Hogg, R. Flocculation and dewatering. International Journal of Mineral Processing. Vol. 58. p. 223-236. 2000.*

4. *Taskaya, L. Jaczynski, J. Flocculation-enhanced protein recovery from fish processing by-products by isoelectric solubilization/precipitation. LWT Food Science and Technology. Vol. 42. p. 570-575. 2008.*

5. *Runkana, V. Somasundaran, P. Kapur, P.C. Mathematical modeling of polymer-induced flocculation by charge neutralization. Journal of Colloid and Interface Science. Vol. 270. p. 347-358. 2004.*

6. *Chong,M.F. Direct Flocculation Process for Wastewater Treatment. Advanced in Water Treatment and Pollution Prevention. 2012.*

7. *Roselet, F. Vandamme, D. Roselet, M. Muylaert, K. Abreu, P.C. Effects of pH, Salinity, Biomass Concentration, and Algal Organic Matter on Flocculant Efficiency of Synthetic Versus Natural Polymers for Harvesting Microalgae Biomass. Bioenergy Research. Vol. 10. p. 427-437. 2017.*

8. *Molina-Bolivar, J.A. Galisteo-Gonzalez, F. Hidalgo-Alvarez, R. Colloidal stability of protein-polymer systems: A possible explanation by hydration forces. The American Physical Society. Vol. 55. p. 4522-4529 1997.*

9. *Swenson, J. Smalley, M.V. Hatharasinghe, H.L.M. Mechanism and Strength of Polymer Bridging flocculation. Physical Review Letters. Lett. 81. 1998*

10. *Ebeling, M.J, Rishel, L.K, Sibrell, L.P. Screening and evaluation of polymers as flocculation aids for the treatment of aquacultural effluents. Aquacultural Engineering. Vol. 33. p. 235-249. 2005.*

11. *Fellows, M. C. Doherty, O.S. W. Insights into Bridging Flocculation. Macromolecular Symposia. Vol. 231. p. 1-10. 2006.*

12. *Fitzpatrick, C.S.B, Fradin, E. Gregory, J. Temperature effects on flocculation, using different coagulants. Water Science and Technology. Vol. 50. p. 171-175. 2004.*

13. *Xiao, F. Huang, J-C. H. Zhang, B-j. Cui, C-w. Effects of low temperature on coagulation kinetics and floc surface morphology using alum. Desalination, Vol. 237. p. 201-213. 2009.*

14. *Kristinsson, H.G. Hultin, H.O. Effect of Low and High pH Treatment on the Functional Properties of Cod Muscle Proteins. Journal of Agricultural and Food Chemistry. Vol. 51. p. 5103-5110. 2003.*

15. *Shi, Q. Zhou, Y. Sun, Y. Influence of pH and Ionic Strength on the Steric Mass-action Model Parameters around the Isoelectric Point of Protein. Biotechnology Progress. Vol. 21. p.516-523. 2005.*

16. *Fleer, G.J. Lyklema, J. Polymer adsorption and its effect on the stability of hydrophobic colloids. II. The flocculation process as studied with the silver iodide-polyvinyl alcohol system. Journal of Colloid and Interface Science. Vol. 46. p. 1-12. 1974.*

17. *Park, S-J. Seo, M-K. Interface Science and Technology. Vol 18. p. 1-57. Chapter 1 – Intermolecular Force. 2011.*

18. *Trulsson, M. Jönsson, B.G. Åkesson, T. Forsman, J. Labbez, C. Repulsion between Oppositely Charged Surfaces in Multivalent Electrolytes. Physical Review Letters. Lett. 97. 2006.*

19. *Sjöström, L. Åkesson, T. the Stability of Charged Colloids: Attractive Double layer Forces Due to Asymmetric Charge Distribution. Journal of Colloid and Interface Science. Vol. 181. p. 645-653. 1996.*

20. *Mishra, A. Bajpai, M. Flocculation behavior of model textile wastewater treated with a food grade polysaccharide. Journal of Hazardous Materials. Vol. 118. p. 213-217. 2005.*

21. *Prado, H.J. Matulewicz, M.C. Bonelli, P.R. Cukierman, A.L. Potential use of novel modified seaweed polysaccharide as flocculating agent. Desalination. Vol. 281. p. 100-104. 2011.*

22. *Iqbal, A. Hussain, G. Haydar, S. Zahara, N. Use of new local plant-based coagulants for turbid water treatment. International Journal of Environmental Science and Technology. Vol. 16. 2018.*

23. *No, H.K. Meyers, S.P. Crawfish Chitosan as a Coagulant in Recovery of Organic Compounds from Seafood Processing Streams. Journal of Agricultural and Food Chemistry. Vol. 37. p. 580-583. 1989.*

24. *Forghani, B. Borges, R. Ström, A. Undeland, I. Recovery of a protein-rich biomass from shrimp (Pandalus borealis) boiling water: A colloidal study. Food Chemistry. Vol. 302. 2019.*

25. *Divakaran, R. Sivasankara Pillai, V.N. Flocculation of kaolinite suspensions in water by chitosan. Water Research. Vol. 35. p. 3904-3908. 2001.*

26. *Ahmad, A.L, Sumathi, B.H, Hameed, B.H. Residual oil and suspended solid removal using natural adsorbents chitosan, bentonite and activated carbon: A comparative study. Chemical Engineering Journal. Vol 108. p. 179-185. 2005*

27. *Morantes, D. Muños, E. Kam, D. Shoseyov, O. Highly Charged Cellulose Nanocrystals Applied as A Water Treatment Flocculant. Nanomaterials. Vol. 9. 2019.*

28. *Demchenko, O. Novyk, A. Zheltonozhskaya, T. Syromyatnikov, V. Polyacrylamide-Grafted Silica for Environmental Protection. Molecular Crystals and Liquid Crystals. Vol. 486. p. 306-315. 2008.*

29. *Marmon, S. Protein isolation from herring (Clupea harengus) using the pH-shift process. pHd Chalmers University of Technology. 2012.*

30. *Tahergorabi, S.V. Hosseini, J. Jaczynski, J. Seafood proteins. Handbook of Food Proteins. Edition 1. 2011.*

31. *Jafarpour, A. Gorczyca, E.M. Contribution of Sarcoplasmic Proteins to Myofibrillar Proteins Gelation. Journal of Food Science. Vol. 77. p. R73-R81. 2012.*

32. *Osman, A. Gringer, N. Svendsen, T. Yuan, L. Hosseini, S.V. Baron, C.P. Undeland, I. Quatification of biomolecules in herring (Clupea harengus) industry processing waters and their recovery using electroflocculation and ultrafiltration. Food and Bioproducts Processing. Vol. 96. p. 198-210. 2015*

33. *Markwell, M.A.K. Suzanne, M.H. Bieber, L.L. Tolbert, N.E. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Analytical Biochemistry. Volume 87. p.206-210.1978*

34. *Przylecki, S.J. Effect of addition of salts on the isoelectric point of proteins. I. Biochemical Journal. Vol. 25. p. 713-727. 1931.*

35. *Gamage,A. Shahidi, F. Use of chitosan for the removal of metal ion contaminants and proteins from water. Food Chemistry. Vol. 104. p. 989-996. 2007.*

36. *Blockx, J. Verfaillie, A. Eyley, S. Deschaume, O. Bartic, C. Muylaert, K. Thielemans, W. Cationic Cellulose Nanocrystals for Flocculation of Microalgea: Effect of Degree of Substitution and Crystallinity. Applied Nano Materials. Vol. 2. p. 3394-3403. 2019*

37. *Hao, Y. Liu, L. Feng, G. Jin, Q. Zou, X. Xie, D. Wang, X. Polysaccharides as Coagulants for the Recovery of Protein in Fishmeal Wastewater. Journal of Aquatic Food Product Technology. Vol. 25.2016.*

38. *Marino, R. della Malva, A. Albenzio, M. Santillo, A. Russo, D. Figliola, L. Caropresse, M. Methods for Extraction of Muscle Proteins from Meat and Fish Using Denaturing and Nondenaturing Solutions. Journal of Food Quality. Vol. 2018. 2018.*

39. *Saleem, R. Hasnain, A. Ahmad, R. Solubilisation of muscle proteins from chicken breast muscle by ultrasonic radiation in physiological ionic medium. Cogent Food & Agriculture. Vol.1. 2015.*

40. *Vann, G.V. et.al. Skeletal Muscle Protein Composition Adaptations to 10 Weeks of High-Load Resistance Training in Previously-Trained Males. Frontiers in Physiology. Vol 11. 2020.*

41. *Brian, B. Hong, Z. Relationship between muscle exudate protein composition and broiler breast meat quality. Poultry science. Vol. 92.p. 1385-92. 2013.*

42. *Nouhi, S. Kwaambwa, H.M. Gutfreund, P. Rennie, A.R. Comparative study of flocculation and adsorption behaviour of water treatment proteins from Moringa peregrina and Moringa oleifera seeds. Scientific Reports (9). 2019.*

8. Appendix

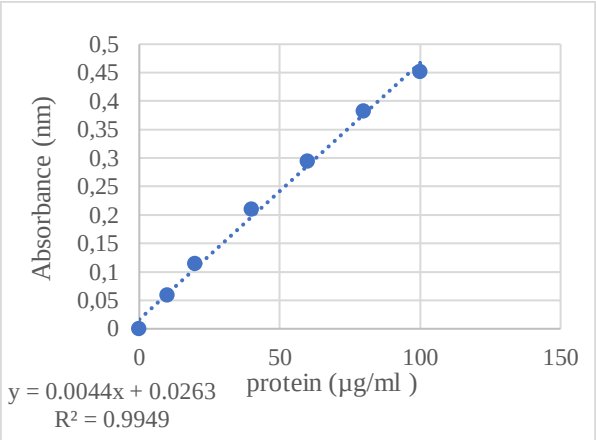
A. Fat content analysis of process waters

- Liquid extraction by chloroform and methanol; at a ratio of 1:2, chloroform:methanol.
- Weigh approximately 2 g of sample in a 50 ml tube.
- Add 20 ml of $CHCl_3$:MeOH (ratio 1:2).
- Vortex
- Add 8 ml 0.5% NaCl solution
- Vortex
- Centrifuge at 2000 × g for 6 min at 4 °C.
- Remove 3ml of the chloroform part (bottom phase after centrifugation) to a pre-weighed glass tube.
- Do triplicates for each sample.
- Weigh a cap and use it for all glass tubes. Cap is used to close the tube if one needs to transport the tube outside fume hood.
- Weigh tube + $CHCl_3$+ cap.
- Dry under nitrogen.
- Weigh the tube once completely dried.
- If stored in fridge over the night, dry again under nitrogen to remove any moisture.

B. The Lowry method modified by Markwell (33)

- Preparation of Reagent A; 2% Na2CO3, 0,4% NaOH, 0.16% P-Na-tartarate and 1 % SDS
- Preparation of reagent B; 4 % CuSO4 in H2O (distilled)
- Preparation of reagent C; mix 1 part reag. B into 100 parts reag. A.
- Mix 0.33 ml sample with 1 ml reagent C in a 1.5 ml Eppendorf tube
- Incubate for 30 mins at room temperature
- Prepare phenol reagent; 1 part distilled water and 1 part Folin-Ciocalteu phenol reagent.
- After incubation add 0.1 ml of phenol reagent to sample, vortex immediately and thoroughly.
- Incubate for 45 mins at room temperature. Keep in dark.
- Blanks were created the same way but with 0.33 ml 0.1M NaOH instead of sample
- Set-up the spectrophotometer to 660 nm and read the samples.
- A bovine serum albumin standard curve in the range 0-100 µg/ml was created to be able to determine protein concentration of samples.
-

BSA STD-curve



$$\frac{Abs - 0.0096}{0.0038} * tentative\ dilution$$

*Tentative dilution denotes the dilution that was used during Lowry sample preparation.

C. Extraction of sarcoplasmic and myofibrillar protein from herring mince

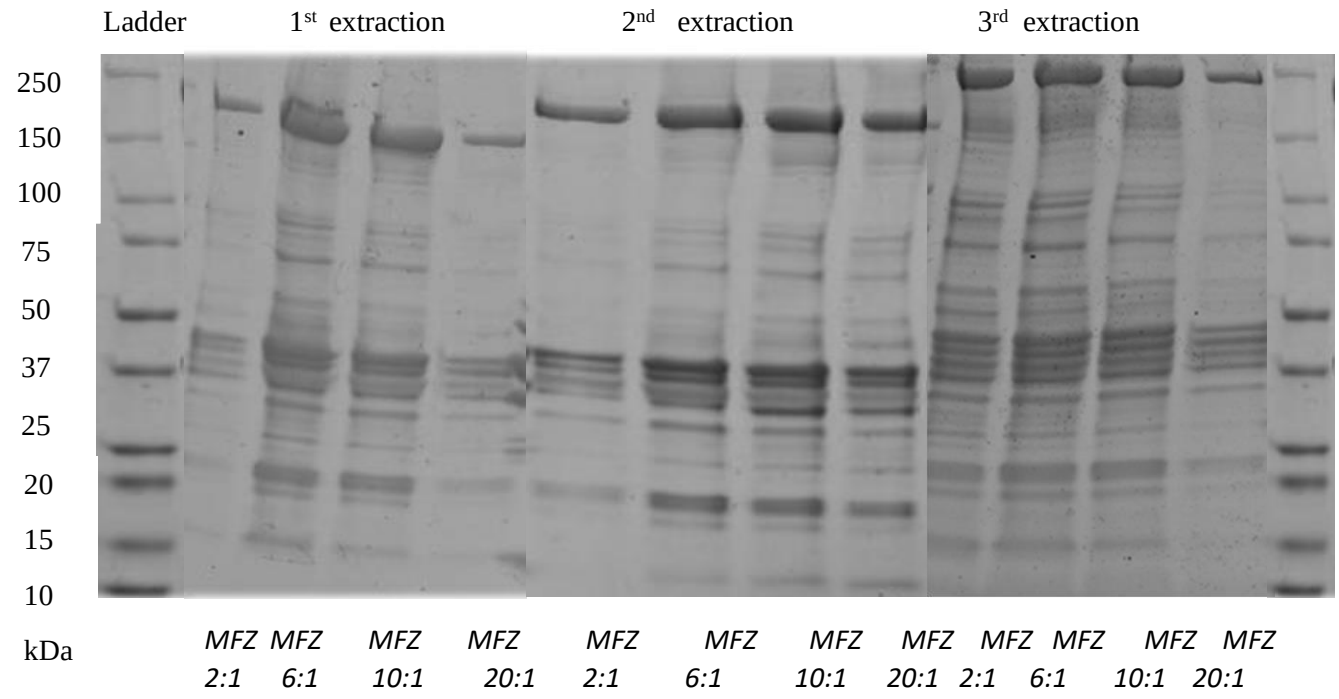
- Weigh approx. 20 g of sample (herring mince)
- For sarcoplasmic protein extraction; 4 sample to liquid ratio; 1:2, 1:6, 1:10 and 1:20 using MilliQ water and 1M NaCl and simultaneously adjusting the ionic strength to 0.05M. Used: C1*V1=C2*V2, and assuming C1 to be the physiological ionic strength of herring.
- Measure pH, control that pH was not changed between samples.
- Homogenize (on ice) 10000 rpm, 1 min using a Polytron.
- Incubate for 30 min on ice
- Centrifuge at 4°C, 10000xg, 15 min.
- Supernatant – store and discard overflow. Store samples in -80 C freezer, for later protein content determination and SDS-PAGE.
- Weigh the pellet
- Re-suspend the pellet with 1M NaCl solution and MilliQ water to reach an ionic strength of 0.7M and at the same mince:liquid ratios as for the sarcoplasmic extraction.
- Homogenize, as previously described.
- Incubate 30 min on ice
- Centrifuge, as previously described.
- Store supernatant in -80 °C and discard the pellet. Used later for protein determination and SDS-PAGE.

D. SDS - PAGE

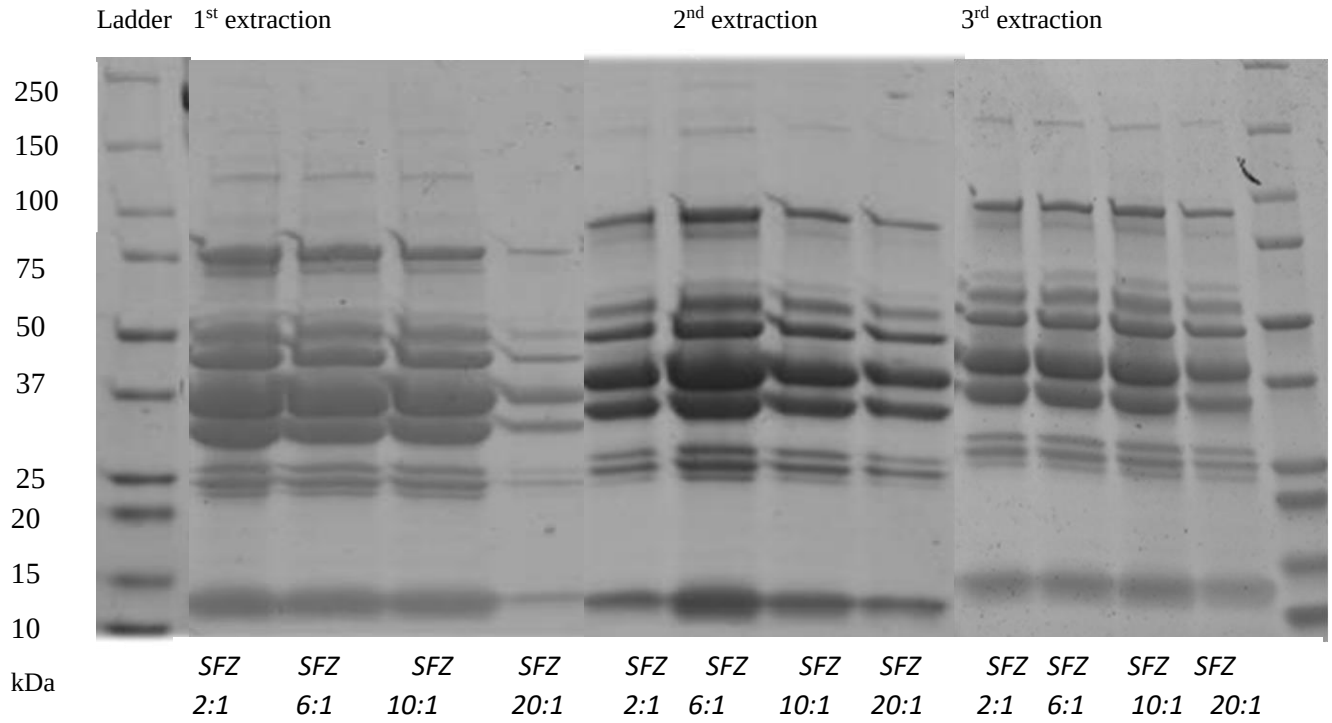
- Dilute samples to 1.33µg/ml using MilliQ water to a total of 25 µl.
- Prepare the dye solution; take one part Beta-mercaptomethanol into 19 parts of dye.
- Mix 25 µl of the diluted samples with 25 µl of dye solution
- Boil at 95 C for 3-5 min, in a water bath.
- Let samples cool down
- Prepare the gel, by removing it from its package and rinsing it in water and then fitting the gel into the slot in the SDS-PAGE set-up. My recommendation is to mark on a corner of the gel with a small cut, used for reference when documenting.
- Fill up the containers with working buffer. Check so that they are not leaking. Completely cover the gel. Be careful not to break the wire at the bottom of the gel-rack.
- Check if the machine is working. Plug in the electrodes (Black to black and red to red) and turn on the machine (press button with running person). If bubbles arise from the bottom it is working
- Turn off and unplug.
- Load the wells, starting from the left, with 15 microliters of your sample. Put the reference sample (ladder) in the outer wells (3 microliters is enough).
- Plug in the electrodes and adjust voltage to 100V (400 mA) and turn on the machine.
- Let the sample traverse downwards to the bottom of the gel, approx. 30-60 min. Then turn off the machine. NOTE; if the samples are not moving downwards the electrodes are plugged in in the wrong order.
- Prepare staining solution proportions; mix 0.025% Coomassie blue (powder), 45% methanol, 10 % acetic acid and 45% milliQ water. Put it on a magnetic stirrer to make sure the Coomassie is evenly distributed and dissolved. (Be careful with the powder, it has very strong color). Color should be almost black.
- Prepare staining solution proportions; 40% methanol, 10% acetic acid and 50% milliQ water.
- Transfer the gels to plastic boxes and place them on the ‘moving machine’ in microlab.
- Soak the gels completely with the staining solution. Turn on the machine. Run for 30 min.
- Repeat the process with destaining solution at least 3 times. Put tissue paper in the corners to soak up more excessive dye.
- Discard staining and destaining solutions in acidic waste.
- Discard working buffer in alkaline waste.
- Rinse the containers with water. (be careful with the wire)
- Rinse the gels with milliQ water once the background is clear and color is washed away.
- Take picture or store cold in zip bags for later documentation (remove air bubbles and droplets).

E. SDS-PAGE results on extracts enriched in sarcoplasmic and myofibrillar proteins

Myofibrillar protein extractions on frozen mince.



Sarcoplasmic protein extractions from frozen herring mince.



2:1, 6:1, 10:1 and 20:1 represent the different liquid (water/salt solution) to solid (herring mince) ratio.

F. Flocculation with fixed concentration 5% pre-salting brine

Table F. Data of all flocculation markers for each flocculant in the 5% pre-salting brine.

Chitosan 0.5g/L 5% pre-salting brine				
pH	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	***Protein sedimentability (%)***
4.5	***0.79±0.06***	***0.44±0.05***	***4.91±1.39***	***93.8±0.69***
5	***0.89±0.03***	***0.85±0.01***	***2.45±0.69***	***88.0±0.08***
5.5	***0.82±0.0***	***1.57±0.09***	***3.33±0.0***	***77.9±1.31***
6	***0.88±0.01***	***1.99±0.04***	***2.58±0.17***	***71.9±0.62***
Alginate 0.5g/L 5% pre-salting brine				
pH	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	***Protein sedimentability (%)***
4	***0.82±0.02***	***0.18±0.01***	***3.44±0.35***	***97.5±0.19***
4.5	***0.97±0.00***	***0.78±0.07***	***0.49±0.0***	***89.0±0.95***
5	***0.96±0.01***	***1.63±0.44***	***0.86±0.17***	***77.0±6.32***
5.5	***1.00±0.0***	***7.07±0.0***	***0±0.0***	***0±0.0***
Carrageenan 0.45g/L 5% pre-salting brine				
pH	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	***Protein sedimentability (%)***
4	***0.87±0.01***	***0.12±0.05***	***2.58±0.17***	***98.4±0.74***
4.5	***0.93±0.0***	***2.83±0.38***	***1.47±0.0***	***60.0±0.39***
5	***0.96±0.02***	***4.19±0.15***	***0.74±0.35***	***40.7±2.16***
5.5	***0.98±0.0***	***3.22±0.11***	***0.49±0.0***	***54.4±1.50***
Silica 0.5g/L 5% pre-salting brine				
pH	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	***Protein sedimentability (%)***
4	***0.92±0.01***	***2.49±0.35***	***1.35±0.17***	***64.8±4.89***

4.5	***0.96±0.01***	***0.63±0.02***	***0.86±0.17***	***91.0±0.27***
5	***0.97±0.00***	***2.01±0.48***	***0.49±0.0***	***71.5±2.10***
CNC 0.5g/L 5% pre-salting brine				
pH	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	***Protein sedimentability (%)***
4	***0.92±0.01***	***2.42±0.51***	***1.35±0.17***	***65.8±7.25***
4.5	***0.91±0.01***	***4.87±0.58***	***1.60±0.17***	***31.1±8.23***
5	***0.82±0.04***	***1.94±0.10***	***3.19±0.69***	***72.6±1.40***

G. Flocculation with fixed concentration in 13% pre-salting brine

Table G. Data of all flocculation markers for each flocculant in the 13% pre-salting brine.

Chitosan 0.5g/L 13% pre-salting brine				
pH	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	***Protein sedimentability (%)***
4.5	***0.91±0.01***	***2.35±0.59***	***1.84±0.17***	***81.9±4.55***
5	***0.93±0.01***	***2.95±1.23***	***1.35±0.17***	***77.2±9.52***
5.5	***0.93±0.00***	***1.59±0.10***	***1.47±0.0***	***87.8±0.74***
6	***1.00±0.0***	***12.97±0.0***	***0±0.0***	***0±0.0***
Alginate 0.5g/L 13% pre-salting brine				
pH	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	***Protein sedimentability (%)***
4	***0.98±0.00***	***3.22±0.26***	***0.49±0.00***	***75.1±1.99***
4.5	***0.96±0.02***	***6.18±0.46***	***0.74±0.35***	***52.4±3.53***
5	***0.90±0.04***	***4.61±0.66***	***2.09±0.87***	***64.5±5.11***
5.5	***1.00±0.0***	***12.97±0.0***	***0±0.0***	***0±0.0***
Carrageenan 0.45g/L 13% pre-salting brine				
pH	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	***Protein sedimentability (%)***
4	***0.99±0.0***	***3.12±0.38***	***0.25±0.0***	***76±2.96***
4.5	***0.99±0.00***	***11.5±1.23***	***0.25±0.0***	***11.1±9.49***
5	***0.99±0.00***	***6.05±1.50***	***0.25±0.0***	***53.4±11.54***
5.5	***1.00±0.0***	***12.97±0.0***	***0±0.0***	***0±0.0***
Silica 0.5g/L 13% pre-salting brine				
pH	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	***Protein sedimentability (%)***
4	***0.95±0.00***	***2.31±0.58***	***0.98±0.0***	***82.2±4.44***

4.5	***0.98±0.0***	***7.22±0.70***	***0.49±0.0***	***44.3±5.39***
5	***0.95±0.02***	***8.01±0.33***	***0.98±0.35***	***38.3±2.54***
CNC 0.5g/L 13% pre-salting brine				
pH	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	***Protein sedimentability (%)***
4	***0.98±0.00***	***4.15±0.79***	***0.49±0.00***	***68.0±6.08***
4.5	***0.96±0.00***	***6.05±0.40***	***0.74±0.00***	***53.4±3.08***
5	***0.93±0.01***	***5.01±0.05***	***1.35±0.17***	***61.4±0.40***

H. Flocculation at fixed pH in 5% pre-salting brine

Table H. Data of all flocculation markers for alginate and carrageenan from concentration dependent flocculation in the 5% pre-salting brine.

Carrageenan at pH 4				
Concentration g/L	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	**Protein sedimentability (%)**
0.09	***0.80±0.00***	***0.18±0.03***	***3.93±0.00***	***97.5±0.39***
0.225	***0.62±0.01***	***0.10±0.00***	***7.36±0.35***	***98.6±0.03***
0.45	***0.87±0.01***	***0.12±0.05***	***2.58±0.17***	***98.4±0.74***
0.675	***0.49±0.05***	***0.09±0.06***	***9.57±1.04***	***98.7±0.80***
0.9	***0.44±0.0***	***0.06±0.05***	***10.6±0.0***	***99.1±0.76***
Alginate at pH 4				
Concentration g/L	***RSH***	***Res. protein Sup.(mg/ml)****	***Volume supernatant (ml)***	**Protein sedimentability (%)**
0.1	***0.76±0.06***	***0.1±0.02***	***4.79±1.21***	***98.6±0.24***
0.25	***0.85±0.01***	***0.19±0.07***	***3.07±0.17***	***97.3±0.93***
0.5	***0.82±0.02***	***0.18±0.01***	***3.44±0.35***	***97.5±0.19***
0.75	***0.76±0.00***	***0.16±0.04***	***4.79±0.17***	***97.7±0.55***
1.0	***0.71±0.02***	***0.15±0.00***	***5.65±0.35***	***97.8±0.05***

*

DEPARTMENT OF FOOD AND NUTRITION SCIENCE
INSTITUTION OF BIOTECHNOLOGY AND BIOLOGY
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden
www.chalmers.se