THESIS FOR THE DEGREE OF LICENTIATE OF ENGINEERING

Protein Yield and Protein Isolate Quality when applying pH-shift Processing on Cod, Haddock and Blue Whiting Fillets

Effects of replacing centrifugation with filtration

HELGI NOLSØE

Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
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Department of Chemical and Biological Engineering
Chalmers University of Technology
SE-412 96 Gothenburg
Sweden
Telephone +46 (0)31-772 10 00

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ABSTRACT

A more sustainable fishery and a better utilization of the harvested fish raw material are currently of great importance to prevent overexploitation of our marine resources. Production of protein isolates to use for example as surimi has for many years been an important way of better utilizing low-value fish species and fish rest raw materials. In the pH-shift protein isolation technique, fish muscle proteins are solubilized in water at strong acid or alkaline conditions, which allows for removal of non-soluble materials like bones, skin, cartilage and fat in a first separation step. The purified solubilized proteins are then precipitated at pH 5.5 and are recovered as a protein isolate in a second separation step. Traditionally, the two separation steps are carried out with centrifugation, a method that can be very costly in large scale. Also, several previous studies have reported on the formation of large sediments in the first centrifugation step containing not only non-soluble materials, but also nearly solubilized and solubilized proteins. These sediments are hard to recover, and reduces the final protein yields. The main aim of the present thesis was therefore to evaluate filtration as an alternative to centrifugation in the first and a second separation step of the acid and alkaline versions of the pH-shift process. The responses were total protein yield, basic composition of the protein isolates and quality of surimi gels produced from the isolates. The raw materials tested were fresh cod and haddock as well as fresh and frozen blue whiting.

Using filtration instead of centrifugation in the first separation step significantly increased the protein yields; for cod and haddock with 26% and for blue whiting with 38-60%. Changing separation method in the second step did not significantly influence the protein yield. The main compositional difference between isolates produced with filtration instead of centrifugation in the first separation step was a higher lipid content with the former, most likely as filtration separates according to particle size instead of density. Further, surimi gels derived from the filtration based process were in most cases weaker and less elastic than gels derived from the centrifugation based process. Slightly lower protein content in the surimi gels caused by a higher lipid content could be one reason. Another could be the slightly higher levels of very high molecular weight myofibrillar proteins like titin in isolates produced with centrifugation. For both isolates and surimi gels there was a tendency towards higher whiteness values when acid solubilization and centrifugation-based separation in the first step was used.

In conclusion, the filtration based pH-shift method is very promising alternative when protein yield is in focus. The impact of the higher lipid content and lower gelation capacity will be linked to the final use of the protein isolates. Many applications do not include a requirement for gelation.

Keywords: *Protein isolate, pH-shift processing, protein yield, cod, haddock, blue whiting, centrifugation, filtration, surimi*

ABBREVIATIONS

ATP	-Adenosine triphosphate
PCB	-Polychlorinated biphenyls
DTX	-Diarrheic shellfish toxins
FAO	-Food and Agriculture Organization of the United Nations
WHO	-World Health Organisation
UNU	-United Nations University
TBARS	-Thiobarbituric Acid Reactive Substances
STPP	-Sodiumtripolyphosphate
EDTA	-Ethylenediaminetetraacetic acid
NADH	-Nicotinamide adenine dinucleotide
BHA	-Butylated hydroxyanisole
SDS PAGE	-Sodium dodecyl sulfate polyacrylamide gel electrophoresis
KA	-Surimi grade
KB	-Surimi grade
A	-Surimi grade

LIST OF PUBLICATIONS

This thesis is based on the work in the following papers, of which Paper 3 is a review article:
Paper 1)
Nolsøe, H.; Imer, S.; Hultin, H. O.
Study of how phase separation by filtration instead of centrifugation affects protein yield and gel quality during an alkaline solubilisation process - different surimi-processing methods
International Journal of Food Science and Technology **2007**, 42, 139-147.

Paper 2)
Nolsøe, H.; Marmon, S.K; Undeland, I.
Application of Filtration to Recover Solubilized Proteins During pH Shift Processing of Blue Whiting (*Micromesistius poutassou*); Effects on Protein Yield and Qualities of protein isolates
The Open Food Science Journal **2011**, vol 5, 1-9.

Paper 3)
Nolsøe, H.; Undeland, I.
The Acid and Alkaline Solubilization Process for the Isolation of Muscle Proteins: State of the Art
Food Bioprocess Technology **2009**, 2, 1-27.

TABLE OF CONTENTS

1. INTRODUCTION 1
2. OBJECTIVES 3
3. BACKGROUND 4
3.1 Structure of fish muscle 4
3.2 Composition of fish muscle 6
3.2.1 Water 6
3.2.2 Protein 6
3.2.3 Lipids 6
3.2.4 Minor components 6
3.3 The pH-shift process 7
3.3.1 Basic principle 7
3.3.2 Changes in net charge and conformation during pH-shift processing 8
3.3.3 Potential to remove lipids 9
3.3.4 Process parameters influencing protein yield 10
3.3.5 Possibilities of substituting centrifugation with filtration to separate dissolved from undissolved matter 10
3.4 Important characteristics of the protein isolates 10
3.4.1 Lipid content 10
3.4.2 Moisture content 11
3.4.3 Polypeptide and amino acid composition 11
3.4.4. Gel-forming properties 12
3.4.5 Color 12
3.4.6. Microbial stability 13
3.4.7. Lipid oxidation 13
3.4.8 Frozen storage stability 15
4. STUDY DESIGN 17
4.1 Overview of measurements of moisture, protein, fat, color and gel quality 17
5. METHODOLOGICAL CONSIDERATIONS 19
5.1 Fish raw material 19
5.2 Basic procedure for performing the pH-shift process 19
5.3 Tests related to the substitution of the first centrifugation with filtration 19
5.4 Tests related to the substitution of the second centrifugation with filtration 20
5.5 Determination of protein yield 20
5.6 Qualitative analyses of the protein isolates 21
5.6.1 Determination of moisture content 21
5.6.2 Determination of lipid content 21
5.6.3 Surimi gel preparation and evaluation of gel strength 21
5.6.4 Polypeptide analysis by SDS polyacrylamide gel electrophoresis 21
5.6.5 Analysis of color of raw material, protein isolates and gels 22

5.7 Statistics 22
6. RESULTS & DISCUSSION 23
6.1 Effects of the substitution of the first centrifugation with filtration 23
6.1.1 Choice of mesh-sizes 23
6.1.2 Sizes of centrifugation sediment and filter cake during the first separation step of pH-shift processing 23
6.1.3 Protein yield 26
6.1.4 Character of the blue whiting protein isolates 26
6.1.4.1 Basic composition 26
6.1.4.2 Color of protein isolates 28
6.1.4.3 Surimi gel quality 29
6.1.4.4 Color of surimi gels from blue Whiting 30
6.1.4.5 Evaluation of polypeptide pattern by electrophoresis 32
6.2 Substituting the second centrifugation with filtration 35
7. CONCLUSIONS 38
8. FUTURE OUTLOOK 40
9. ACKNOWLEDGEMENTS 41
10. REFERENCES 42

APPENDIX 1. Examples of surimi specifications 47

1. INTRODUCTION

A more sustainable fishery is of great importance. Equally important, however, is the development of new processing methods that can help improve the utilization of the raw-materials. Every conscientious fish processer is trying to utilize the raw materials as well as possible. Although this is an ongoing process, it does not necessarily involve trying new methods.

A better protein yield from the processed fish is of major importance for the processer. One processing method that wastes much protein is traditional surimi processing by which minced fish is washed in several washes with water or a slightly alkaline solution, thereby removing water soluble proteins in the washing water. Protein yields reported from traditional surimi processing varies from about 38 % to 62 % (Paper 3). This means that as much as 38-62% of the muscle proteins in the fish raw materials are wasted.

Looking globally, about 2-3 million metric tonnes of fish are used for traditional surimi production annually [1]. This means that a substantial amount of fish proteins are wasted in surimi wash water. This is an irresponsible waste of raw-material, and is not acceptable in a world where increasing populations will require more proteins in the future.

Considering the various strategies developed to improve the protein yield during traditional surimi processing (e.g. precipitation of the proteins in the wash water), the so-called pH shift processing method appears to be a good alternative, as it is known to recover both sarcoplasmic ("water soluble") and myofibrillar ("salt soluble") proteins. In this process, fish mince is mixed with three to ten parts of water and homogenized. After the homogenization, the pH is adjusted to either a high or a low pH value, in the alkaline and the acidic processing method, respectively. At these extreme pH values most of the proteins dissolve. The dissolved proteins are then isolated from the un-dissolved material before they are precipitated and dewatered at the isoelectric point. The isolated proteins can be used for surimi or other food products.

The most common method to isolate dissolved proteins from non-dissolved material during pH-shift processing is centrifugation. One drawback with this technique, when used in the first separation step, is that the sediment formed sometimes becomes very large and also has a high moisture content [2]. Part of the sediment sometimes consists of a weak gel formed during centrifugation. Within this gel a large part of the solubilized proteins are trapped and thus removed with the sediment, resulting in a reduced protein yield. An alternative processing procedure could be replacement of centrifugation with filtration in the first separation step of the pH shift process (Paper 1 and Paper 2). The filter cake from such a process is expected to be much smaller in volume than the centrifugation sediment and thus would trap less protein.

This thesis study was designed to investigate protein yield as well as quality and composition of the resulting protein isolates when centrifugation vs. filtration was used in the first and second separation step of the pH-shift process. The research was carried out using fillets from cod (*Gadus morhua*) (Paper 1), haddock (*Melanogrammus aeglofinnus*) (Paper 1) and blue whiting (*Micromesistius potassou*) (Paper 2). The blue whiting was used both in a fresh and frozen state. The main reason for including blue whiting in this study was an interest articulated by the industry for a more remunerative exploitation of this particular species for human consumption because the fish has a good taste, has relatively white meat and is suitable for lucrative surimi production. All the parallel tests within each experiment were carried out using raw material from the

same batch of fish. This was done to minimize the influence e.g. from seasonal differences, including sexual maturation stage and feeding pattern. Within each batch, it is however still expected that there are fish-to-fish differences linked for example to age and sex. Biochemical differences that may occur as a result of the listed biological factors include e.g. different post mortem muscle pH´s and different post mortem activity of enzymes like ATPases, cathepsins B, D, H, L, and X, calpains, peptidases, collagenases, decarboxylases, trimethylaminoxid-oxidoreductases, trimethylaminoxid-demethylases, lipases, phospholipases, and different lipoxygenases [3]. It has previously been suspected that highly active acid proteases may be a reason behind unwanted proteolysis during the acid version of the pH-shift method [4;5].

2. OBJECTIVES

The main objectives of this thesis were:
to compare the protein yield obtained during pH-shift processing using:

- centrifugation vs. filtration in the first separation step
- centrifugation vs. filtration in the second separation step
- acid vs. alkaline protein solubilization
- fresh vs. frozen fish raw material

to compare basic composition (water, lipids, and total proteins), polypeptide pattern, color and gelation capacity of protein isolates produced with:

- centrifugation vs. filtration in the first separation step
- centrifugation vs. filtration in the second separation step
- acid vs. alkaline protein solubilization
- fresh vs. frozen fish raw material

3. BACKGROUND

3.1 Structure of fish muscle

The muscle tissue in fish is arranged in bundles of muscles, called myofibrils, on both sides of the fish from the backbone out to the skin and separated horizontally by a septum in an upper and a lower muscle bundle. The muscle cells are arranged longitudinally and are interrupted by a row of membranes (myocommata) composed of connective tissue separating the muscles in segments (myotom), as illustrated in the **Figures 1** and **2** [6;7].

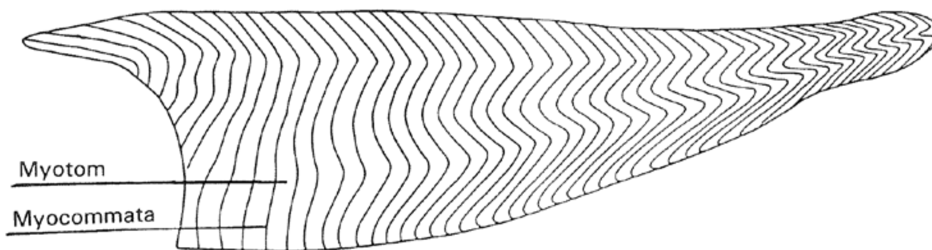


Figure 1. *Cod fillet (from Knorr, 1974 [7])*

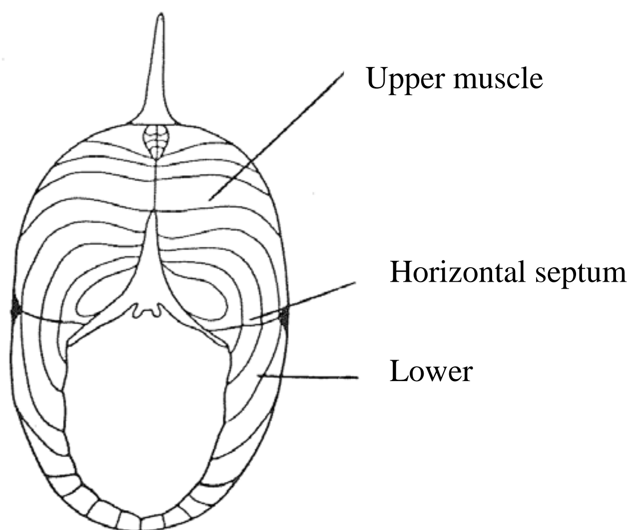
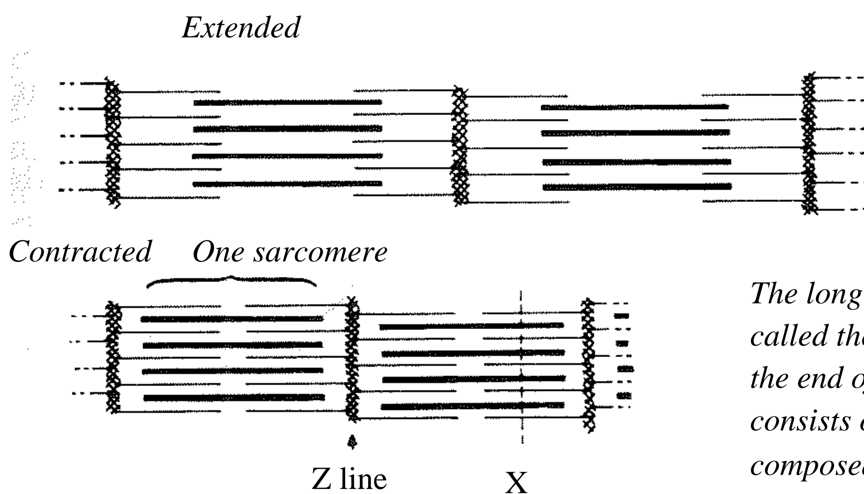


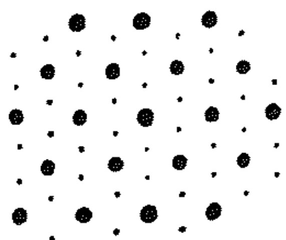
Figure 2. *Cross section of cod (from Knorr, 1974 [7])*

The myofibrils are arranged as shown in **Figure 3.** The thin filaments are made up of the protein actin and smaller amounts of tropomyosin and troponin. The thick filaments are made up of the protein myosin.

a) The arrangement of the thick and thin filaments

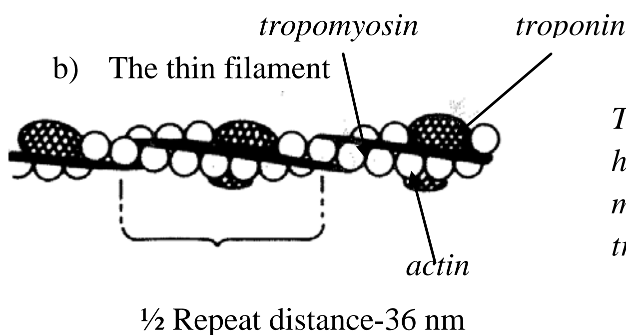


The longitudinal unit of the myofibril is called the sarcomere. The Z lines define the end of the sarcomeres, which consists of numerous short filaments, composed mostly of α-actinin that link neighboring sarcomeres.



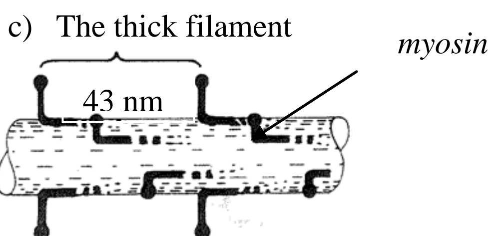
Cross-section at X showing hexagonal array of thin filaments around the thick filaments.

b) The thin filament



The double chain of actin molecules has two strands of tropomyosin molecules lying in the grooves and also troponin molecules at regular intervals.

c) The thick filament



Myosin heads protrude from the filaments in six directions and fit to interact with the actin molecules.

Figure 3. *The myofilaments of skeletal muscle (from T.P. Coultate [3], with modifications). In a) the thick and thin filaments are shown extended and contracted. In b) the double chain of actin moleculeswith with two strands of tropomyosin molecules and troponin is shown. In c) the thick filament with myosin heads protruding is shown.*

There are two kinds of fish muscle, light and dark muscle [8]. In white fish like cod, haddock and blue whiting, there is a layer of dark muscle just under the skin on both sides running beneath the lateral line.
Like mammals, the muscle tissue in fish is composed of striated muscles. The basic functional units in a muscle are myosin and actin molecules arranged in filaments or fibers. The myosin and actin molecules convert chemical energy to mechanical energy. The filaments are arranged in sarcomeres that form the myofibrils. The myofibrils are united in muscle fibers that are arranged in bundles that again are arranged in bigger muscle bundles comprising the muscles surrounded by a connective tissue membrane called sarcolemma.

The distribution of light and dark muscle varies from one species to another. Both light and dark muscles are striated muscles. The chemical composition of dark and light muscle is rather different [6]. The content of hemoglobin, glycogen and many vitamins is greater in dark muscle. What might be of importance in lean species, such as cod, haddock and blue whiting, is that the dark muscle contains lipids, thus increasing the potential for oxidation.

3.2 Composition of fish muscle

3.2.1 Water

The main component of fish muscle is water. The water content of fillets from lean fish like cod, haddock and blue whiting is about 80%. In fresh fish muscle, the water is bound to the proteins in the muscle structure and cannot be expelled easily even under high pressure. During chilled storage and especially after frozen storage, the proteins are less able to retain all the water.

3.2.2 Protein

The protein content in fish muscle is generally between 16 and 21%, but values from 6 to 28% are seen in the literature [6]. The proteins in fish muscle can be divided into three groups [9]. One group consist of the functional proteins actin, myosin, tropomyosin and actomyosin, which represent about 65% of the total proteins in fish muscle [6]. Another group is the cytoplasma proteins myogen, myoalbumin, globulin and enzymes, representing about 25-30% of the proteins in fish muscle [6]. A third group is the stroma protein (collagen), which is about 3% of the muscle protein in bony fish and 10% in Cartilaginous fish [6].

The proteins are composed of amino acids that are linked together in long molecules. There are about twenty amino acids, some of which are essential to the human diet. Fish proteins have a good combination of various amino acids, including the essential amino acids, and are well suited for human nutritional requirements.

3.2.3 Lipids

In white fish like cod, haddock and blue whiting, the lipid content of the muscle is always low, usually under 1% [8]. Seasonal variations in the fat content in lean fish like cod, haddock and blue whiting are mainly seen in the liver where most of the fat is stored.

3.2.4 Minor components

Fish muscle also contains small amounts of carbohydrates, minerals and vitamins and some extractable substances.

3.3 The pH-shift process

In 1999, Hultin and Kelleher patented the acid solubilization process as a way to improve the yield and stability of muscle protein isolates. A few years later, a similar process based on alkaline processing was patented [10]. The main advantages with the acid and alkaline solubilization processes over regular surimi production are that the muscle does not need to be mechanically removed from the bones or skin before processing. Crushed or minced material can be directly solubilized and all materials with a different density than the proteins, e.g., lipids, can be removed by gravity, e.g., through centrifugation. As a consequence, the risks of rancidity of the blood rich and pro-oxidative material are reduced. Another major advantage of the pH-shift method, compared to traditional surimi production, is the recovery of the sarcoplasmic proteins and the subsequent substantial increase in protein yield.

One other important reason for replacing traditional surimi processing with the pH-shift process is the reduction in organic material removed with the waste water and a reduction in the use of water [11]. Approximately 40-50 g/100 g of minced fish muscle are lost in the entire washing process in traditional surimi processing [12]. This has to be compared to what is lost in the supernatant after separation of precipitated proteins, which is about 3-5 g/100 g of minced muscle [5]. The surimi processing of white-fleshed fish requires large amounts of water. The average consumption of washing water in surimi processing is about 27 tonnes per tonne of surimi [11]. As a consequence, wastewater is a big problem for the surimi industry [11]. In sharp contrast, only 4 to 10 tonnes of water per tonne of isolate is used in the pH-shift process.

3.3.1 Basic principle

The main feature in the pH-shift process is the exploitation of pH-influence on the solubility of comminuted fish proteins in hydrated and homogenized material. **Figure 4** shows the process as it is usually performed in the laboratory [10].

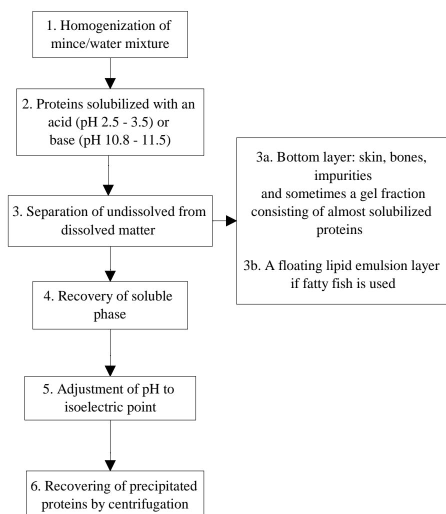


Figure 4. *The ph-shift process for protein isolation as performed in the laboratory. In step 1, minced muscle and water are homogenized. In step 2, the homogenate is solubilized by adding a base or an acid. In step 3, the homogenate is centrifuged and undissolved material (3a & 3b) is separated from the dissolved materials. In step 5, the pH of the dissolved material is adjusted to the isoelectric point. In step 6, the precipitated proteins are recovered by a second centrifugation.*

In the traditional pH shift processing, as described by Hultin and Kelleher [13] and shown in **Figure 4**, the dissolved proteins are separated from undissolved material by centrifugation in a batch type centrifuge. The homogenate is filled into centrifuge bottles and during the centrifugation the separation of materials with different density is accomplished by the application of strong centrifugal force.

3.3.2 Changes in net charge and conformation during pH-shift processing

Proteins are macromolecules that can change shape according to changes in the environment. At extreme acid or alkaline pH values, strong positive and negative charges, respectively, on the myofibrillar and cytoskeletal proteins drive them apart by repulsion. A possible shape is called a conformation. Changes in the environment can be changes in temperature, pH, ion concentration, voltage, pressure, etc. The conformation of a protein is also dependent on the type and sequence of the amino acids in the peptide chains that fold to minimize the free energy [14]. As proteins get a overall higher net charge, they gradually start electrostatic interactions with water [15]. By this-interaction, solubilization can be obtained (Paper 3).
An important feature of muscle proteins subjected to extreme pH values is that the proteins partly unfold (Paper 3). This leads to considerable changes in the conformation and structure of the proteins, and this again leads to different properties after refolding [16;17]. In the research conducted by Kristinsson et al. [16-18] conformational, structural and functional changes of hemoglobin and myosin were studied during exposure to low and high pH and after subsequent refolding at the isoelectric point. The pH-induced changes of Hb are determinant for lipid oxidation. Contrarily, the changes in the myosin molecules influence functionalities such as water holding capacity, gel forming ability and emulsification ability of protein isolates [18]. Kristinsson's results indicate that Hb was fully dissociated at low pH and that the heme group lost contact with the distal and proximal histidine even though it was not detached The globin partially unfolded into the molten globular state. The myosin was partially or fully dissociated at pH 2.5, but not at pH 11 [18]. The tertiary structure was lost at both these pH values, suggesting the "molten globular" configuration of the head [18]. After refolding, the pattern of the myosin rod, head and light chains was different from the native myosin. Some differences were also noted in the myosin head group following alkaline vs. acid processing. The refolded myosin was thermally more unstable, a phenomenon ascribed to the misfolded head [18]. Acid processing induced more misfolding of the head than alkaline processing. Myosin exposed to extreme acid and alkaline pH values therefore gelled at lower temperatures.

Mohan, et al. [19] got similar results in a study of the head group of myosin from mullet (*Mugil cephalus*), showing that the head group was more affected by acidification and alkalization than the rod part. When refolding, the tertiary structure of the acid treated myosin remained partially disrupted, a result that was not seen for the alkali-treated myosin. Kristinsson [18] also found higher solubility at 0-600 mM KCl of cod myofibrillar proteins that had been exposed to high and low pH, compared to native myofibrillar proteins. This was possibly because some structural proteins, like chaperon proteins normally keeping the myofibrillar structure collected, had been solubilized. Also, the emulsifying capacity of myofibrillar proteins subjected to extreme pH values was improved, and gelation was seen at lower temperatures than for native myofibrillar proteins Generally speaking, the functionality of the alkali-treated myosin and myofibrillar proteins was slightly more improved than the acid-treated myosin and myofibrillar proteins [18].

3.3.3 Potential to remove lipids

The opportunity for lipid reduction using the acid/alkaline solubilization process is implicit in whichever process is being performed wherein protein containing material is comminuted and mixed with water and homogenized. Water and lipids have different densities and will separate when subjected to centrifugation. In the classic pH-shift process, the first separation step is performed with high-speed centrifugation. In this separation, neutral lipids, like triglycerides, can float to the top, normally in the form of an emulsion with proteins, while the heavier membrane-bound phospholipids collect in the sediment. The amount of removable lipids in this step is influenced by the following factors: the lipid content of the starting material, the amount of water added, the viscosity of the pH-adjusted homogenate, and the centrifugation speed in the first centrifugation step (Paper 3). The co-sedimentation of membrane lipids with unsolubilized material is particularly influenced by centrifugation speed (Paper 3).

Based on the literature available on pH-shift processing, it is evident that this technique is more efficient in lipid reduction than traditional surimi production. Comparing acid and alkaline processing, three of four studies showed a higher lipid reduction for alkaline processing (Paper 3). In one study by Kristinsson et al. [20], lipid reduction using alkaline, acid and traditional surimi processing was compared. The highest lipid reduction was obtained by alkaline processing (88.6%), followed by acid processing (85.4%), and finally traditional surimi processing (58.3%). The authors also tested the lipid reduction when avoiding the first centrifugation step in production of alkaline and acid protein isolates. The lipid reductions were then 61.2% and 45.4%, respectively. Kristinsson and Liang [21] investigated lipid reduction in acid and alkaline processing of protein isolates and traditional surimi processing using Atlantic croaker fillets. The lipid reductions were 68.4%, 38.1% and 16.7%, respectively. The big differences between the traditional surimi processing and the acid and alkaline method was ascribed to the first centrifugation step [22]. Undeland et al. [23] investigated the lipid reduction during acid processing of herring mince with/without a 1:3 pre-wash, and with/without high-speed centrifugation in the first separation step. In the pre-wash, the lipid content was reduced by 39%. When unwashed and pre-washed minces were further processed with high-speed centrifugation, the total lipid reductions of the mince were 51% and 56%, respectively. Without the high speed centrifugation, the lipid content of the isolates became higher than the original mince.

The ability of the pH-shift processing method to remove membrane phospholipids is a quite unique property and has been the subject of a series of studies of the sedimentation behavior of membranes following acidification and alkalization [24;25]. The studies showed that treatment with $CaCl_2$, $MgCl_2$ and citric acid before the homogenization step could remove up to 90% of the membrane lipids at 4000 x *g* in acidified muscle homogenate samples. Citric acid might play a role as a binding agent to the residues of the basic amino acids of cytoskeletal proteins competing with the acidic phospholipids of membranes (Paper 3). Vareltzis and Undeland [26] compared lipid reduction following acid and alkaline processing of a mince from blue mussel that contained 13.5% lipids on a dry-weight basis, and a lipid to protein ratio of 0.3. They found that the acid-process gave isolates with lower lipid content than the alkaline-process, 11% vs. 18%, respectively on a dry weight basis. The lipid to protein ratio became 0.15 and 0.3, respectively. By adding 5 mM citric acid and 10 mM calcium chloride, the lipid reduction could be improved, so the lipid to protein ratio was reduced down to 3% vs. 5% for the acid and alkaline processes, respectively.

3.3.4 Process parameters influencing protein yield

One very important factor during the production of protein isolates is the protein yield. The protein yield in pH-shift processing is determined by the solubility of the proteins at the applied pH values for solubilization, the sizes of sediments or filter residues formed during the first centrifugation or filtration, respectively, and the amount of proteins staying in solution after separating out the precipitated proteins.

Based on a thorough investigation of the literature (Paper 3), it appears that the acid-process gives higher protein yields than the alkaline-process. A possible explanation for this is that the sarcoplasmic proteins are more difficult to precipitate and recover following alkaline solubilization, compared to acid solubilization [17;20;27].

One tested method to increase the protein yield in pH-shift processing of fish protein isolates is the use of flocculants prior to the second separation. Taskaya & Jaczynski [28], investigated different flocculants and their influence on the optical density of the process water removed after the second separation step. The test was done during alkaline-processing of rainbow trout by-products by the addition of flocculants to the protein solution, adjusted to pH 5.5. The results showed that the application of anionic flocculants with high molecular weight gave the fastest formation and separation of the protein flocks from the process water, indicating a very high separation of the precipitated fish muscle proteins. The tests also showed that the protein from double-frozen raw material took very little time to flocculate. It should be stressed that the flocculants used for these tests were not food grade. Further tests have been performed aimed at the determination of the safety of fish proteins recovered using flocculants [28].

3.3.5 Possibilities of substituting centrifugation with filtration to separate dissolved from undissolved matter

To avoid large protein losses as a result of centrifugation, filtration could be an interesting strategy to separate soluble proteins from un-solubilized materials. Many types of filters are available. Broadly classified, these are vacuum, pressure, pre-coat and depth filters, which again can be split into further sub-classifications [29]. However, a slurry like the pH-adjusted protein homogenate may be difficult to filter for a variety of different reasons. For example, some particles can be quite small and tend to block the filter apertures, or the chemistry of the particles can instigate gel formation, or the particles stick to the surface of the filter medium [29].For the studies presented here, standard sieves were selected for the test work. For future up-scaling of the process, automatic, time-saving cleaning equipment could be employed to clean the filter media.

3.4 Important characteristics of the protein isolates

3.4.1 Lipid content

Factors influencing the lipid content of the protein isolates are discussed above (see 3.3.3). The absolute lipid reduction may be of importance for the development of lipid oxidation in the protein isolates (Paper 3). However, as long as strong pro-oxidants, like Hb, are present, it seems likely that extremely low lipid levels have to be reached to avoid lipid oxidation. Richards and Hultin [30] found severe blood-mediated rancidity odor in washed cod mince with as low as 0.1% lipid content (wet weight ww). In a study comparing acid and alkali-processed protein isolates and conventionally processed surimi [20], the lipid reduction obtained in channel catfish muscle was 88.6%, 85.4% and 58.3% for alkaline/acid-processing of protein isolates and conventional surimi processing, respectively. In the same study, lipid reduction was also tested during the

processing of the protein isolates without the first centrifugation step. The obtained lipid reductions were 61.2% and 45.4% for the alkaline/acid-processes, respectively.

Lipid removal during pH-shift processing can indirectly be an important factor in the removal of lipid-soluble dioxins and toxins. Two recent studies [26;31] have shown that up to 80% of the original content of dioxins and dioxin-like PCBs, as well as DTX-1 toxin, could be removed from Baltic herring and mussels with pH-shift processing.

3.4.2 Moisture content

If the protein isolate is to be dried, the relative moisture content of the processed protein isolate is extremely important with regard to energy consumption during the drying process. However, it is also important in the production of surimi that the moisture content should remain around 75-76% after the addition of cryoprotectants [1].

3.4.3 Polypeptide and amino acid composition

The polypeptide composition of the protein isolate is important because it can affect the functional properties. The major myofibrillar protein in fish muscle is myosin. It is a 520 kDa hexamer consisting of two 220 kDa polypeptides called myosin heavy chains [32]. Each myosin heavy chain is attached non-covalently to two 18-25 kDa light chains [16]. The myosin heavy chain has a globular head section and a rod-like tail section [32]. Myosin heavy chains are of utmost importance because of their functional properties such as their water-holding capacity, gel forming ability and emulsifying properties [33]. From a human and animal health perspective, the biological value of a protein isolate is determined by the presence of all the nine, essential amino acids. Gehring et al. [32] investigated the amino acid composition in raw material and in protein isolates processed from fish by-products from carp using both acid and alkaline processing. The results showed that alkaline processing generally gave a higher amount of essential amino acids in the isolates than acid processing. This was probably because of less pH-induced proteolysis at alkaline pH [32]. Cleavage by hydrolysis may result in partially-hydrolyzed proteins and free amino acids, which typically are water soluble and consequently can be lost in the second separation step. Examples of hydrophilic amino acids that can be lost are lysine and threonine. These are easily lost following proteolysis [34]. Marmon & Undeland [4] investigated the amino acids in herring mince and in acid and alkaline protein isolates made there from. They found that the overall amino acid composition of the protein isolates from both acid and alkaline processing included essential amino acids well above FAO/WHO/UNU recommendations for adults [4;35].

Gehring et al [32] found that proteins derived from by-products of fish processing through solubilization and precipitation had higher biological value compared to a soy protein concentrate, and similar values to that of the milk protein casein. The concentration of lysine in a protein isolate is often considered a limiting factor [32]. The concentration of lysine in a protein isolate derived from carp by-products by solubilization/precipitation is at the same level as the lysine in a whole egg, and even higher in proteins derived from whole carp and whole krill [32].

3.4.4 Gel-forming properties

In the processing of protein isolate for surimi or similar products where quality of the gel is critical, the handling of the fish raw material (e.g. *post mortem* storage time and temperature) is of exceeding

importance. Fish proteins can denature at any temperature, but the denaturation rate is slowed at lower temperatures [36]. Rapid chilling of the catch is therefore of great importance when the fish are intended for products requiring a high gel quality. Comparing gels from proteins produced with different processing methods requires that the gels are comparable in terms of moisture content, salt content and pH. Gel quality can be determined by texturometry testing [1]. In Paper 3, 16 studies were reviewed regarding the results of gel quality. Gels from acid and alkaline pH-shift processing were compared in some of the studies. Comparing the gel quality of protein isolates processed from fillets of catfish, mackerel, mullet and croaker, Kristinsson and Demir [22] found that the alkaline process gave better gel quality than the acid process.
This was also the result in a study by Undeland et al. [5], comparing protein isolates from fresh herring, while the opposite result was seen with isolates from herring stored in ice for six days. It was hypothesized that there was a higher retention of more myosin heavy chains with the alkaline process, because a certain degree of hydrolysis occurred during acid processing [5]. When six-day-old herring was used, the gels were generally of inferior quality, a decomposition ascribed to denatured proteins initiated by lipid free-radicals [5]. The effects of cold storage and the freezing of ground meat from catfish muscle before the isolation of acid- and alkali-processed proteins has also been studied by Davenport et al. [37]. They found that alkaline processing gave stronger gels, and that gels from fresh raw materials gave higher gel strength, followed by cold stored, and then frozen gels. Park et al. [38] tested the gel quality of protein isolates from acid- vs. alkaline-processed protein isolates from horse mackerel, Japanese mackerel and redlip croaker. The results showed better gel quality from the alkaline processing in all cases. Kim et al. [39] got the same result comparing the gel quality of protein isolates from Pacific whiting. Identical results were seen with isolates from rockfish [39;40], Atlantic croaker [21;41], menhaden [42] and Pacific whiting [43].

In summary it is clear that both fish species and processing method have great influences on the gel strength. Comparing the gel strength of gels made from acid and alkali processed protein isolates, in most cases the gels from the alkali processed isolates had the highest gel strength (paper 3).

3.4.5 Color

Color is an important measure of the quality of protein isolates and the products made there from, especially in surimi production. The whiter the color, the higher the quality [1]. Generally, the market is most interested in protein isolates that are as white as possible [44]. The color parameters are therefore important considerations when comparing different processing methods for production of protein isolates and surimi. However, the end product to be produced from the isolate of course also influences the importance of the color. As for gelation capacity, the source and quality of the raw material are important determinants of color. Different fish species have different colors of their muscle meat. Moreover, the color can be affected by the amount of dark muscle, the presence of blood and pigments like melanin. Melanin can come from the skin, from the eyes and the dark membrane in the belly cave.

The color results found in different studies of acid- and alkali-processed protein isolates were compared in Paper 3. Protein isolates from fresh herring showed higher whiteness when the alkaline method was used compared to when the acid method was used [5],. Similar results were seen for protein isolate and gel from catfish muscle [22]. Croaker protein isolates on the other hand had higher whiteness after acid processing. However, the opposite was seen regarding whiteness of the gel [22]. For mullet, the whiteness was higher for alkali-processed protein isolate, while the whiteness for the gel was higher from acid-processed protein isolate [22]. For Spanish mackerel, alkali-processed isolate gave a higher whiteness both for protein isolate

and gel [22]. For rockfish, higher whiteness was seen for alkali-processed gels [40]. Also for channel catfish, protein isolate from alkaline processing gave a higher whiteness [20]. This was also the result for gel made from menhaden protein isolate [42]. For Atlantic croaker, a comparison of whiteness showed higher whiteness for acid-processed fresh protein isolate, while the gels showed higher whiteness from alkali-processed fresh protein isolate. For the frozen isolate, however, the alkali-processed isolate showed higher whiteness, while the frozen gel from acid-processed protein isolate showed a higher whiteness [21].

The above mentioned studies show that no generalization can be made about which process yields a higher whiteness; it has to be determined by testing each species.

3.4.6 Microbial stability

Microbial stability is of great importance during the processing and storage of food products. Kristinsson and Demir [45] compared microbial growth on catfish mince, acid- and alkali-processed protein isolates, as well as conventionally-processed surimi. There was a reduction in aerobic plate count directly after all three processes, but the largest reduction was seen for alkali-processed isolate followed by acid-processed isolate. The question, 'how does the extreme pH values in acid and alkaline processing affect the presence of microbes', has partially been answered in two recent studies by Landsowne et al. investigating the survival of *Listeria innocua* [46] and the survival of *Escherichia coli* [47] after solubilization and precipitation of fish protein. The essential objective of each of the studies was to determine if the microbes noted above survived the extreme pH levels to which they were subjected to in the acid and alkaline processes. In both investigations, the microbes were inoculated by mixing them into mince from fresh fillets of rainbow trout (*Onchorynchus mykiss*) for about two minutes before processing. The tested pH values were 2 and 3 for the acid process and 11.5 and 12.5 for the alkaline process. Precipitation was performed at pH 5.5. For the test with *Listeria innocua*, the greatest bacterial reduction in the protein fraction was seen when the pH was shifted to 2 and the least reduction was seen after solubilization at pH 11.5. Thus, the test showed that *Listeria innocua* is more resistant to alkaline conditions than to acid conditions. In the case of *Escherichia coli*, the opposite effect was observed with the greatest reduction occurring when the pH was shifted to 12.5. Future studies by the researchers will focus on organic acid for protein solubilization [46].

Thus, the above shows, that different effects are seen with different microbes for acid and alkaline processing with the biggest reduction for *Listeria innocua* at acid pH and the biggest reduction for *Escherichia coli* at alkaline pH .

3.4.7 Lipid oxidation

An important destructive factor in the processing of fish protein isolates is lipid oxidation. Minced fish products are especially exposed to oxidation because of the large surface area of the mince. Also, the presence of heme proteins, like hemoglobin and myoglobin, are believed to create a strong pro-oxidative environment in fish muscle foods [48].

A series of studies describing lipid oxidation during and after pH-shift processing were reviewed in Paper 3. From these studies, it is evident that oxidation in protein isolates can be delayed by the exogenous addition of antioxidants [49]. The acid process for protein isolation has been attributed a higher risk for lipid oxidation than the alkaline process. This is due to the fact that pro-oxidants, like the heme proteins, can be activated at low pH [18]. It has also been observed that the subjection of washed cod mince to low pH made

the muscle more prone to Hb-mediated oxidation than washed 'native' or non-pH-adjusted mince [18]. One study [50] showed that protein isolates from cod muscle processed using the acid process were significantly more susceptible to lipid oxidation than protein isolates derived with the alkaline process. Lipid oxidation was studied during the processing of acid and alkaline protein isolates, as well as during processing of conventional surimi from fillets of catfish, croaker, mullet and Spanish mackerel [22]. The results showed that the lipid oxidation in conventional surimi was at a lower level than for alkaline- and acid-processed isolates, except for one case of mackerel isolate previously stored for 6 days on ice. The acid-processed protein isolate was most vulnerable to oxidation, while the alkali-processed isolates were between the other two methods. Muscle from channel catfish was investigated for lipid oxidation by Kristinsson et al. [20]. The authors analyzed lipid oxidation products of ground muscle after conventional processing of surimi and after acid and alkaline processing of protein isolates with/without the first centrifugation step. The authors found that none of the processes gave significantly higher values of TBARS than the starting raw material. It should be noted, however, that alkaline processing without the first centrifugation step yielded an isolate with exceptionally low TBARS value.

Lipid oxidation in unprocessed ground muscle, conventional surimi and acid- and alkaline-processed protein isolates from Atlantic croaker was also studied by Kristinsson and Liang [21]. Samples were kept ice cooled for 14 days during which they were analyzed periodically for TBARS. The highest start value for TBARS was found for acid-processed protein isolate followed by ground muscle. The conventionally processed surimi sample had decreased in TBARS during processing, compared to the start raw material. The alkali-processed protein isolate and the conventionally processed surimi maintained a low TBARS value over the storage period studied, while the other samples, and especially the acid-processed protein isolate, had a substantial increase in TBARS. The study included lipid oxidation of cryoprotected protein isolate and gel directly after production and after 1 week of frozen storage. For this part of the study, the highest increase in TBARS was also found for acid-processed protein isolate followed by conventional surimi and alkali-processed protein isolate. The fastest increase in TBARS for the acid-processed protein isolates is most likely caused by the increased pro-oxidative potential of the heme proteins when subjected to low pH values [51].

Undeland et al. [23] studied the progression of lipid oxidation under the acid processing of herring fillet mince. The authors tested how changes in the process and the application of different antioxidants influenced lipid oxidation both during the processing itself and subsequent ice storage of protein isolates. The processing parameters tested were with/without a 1:3 pre-wash of the mince, exposure time to pH 2.7 (4, 30, and 75 min), with/without high speed centrifugation, and addition of antioxidants. The antioxidants applied were one reducing agent (0.2%, ww, erythorbate), two metal chelators (0.2% sodiumtripolyphosphate (STPP), or 0.044% ethylenediaminetetraacetic acid (EDTA)), and one protein concentrate that might act as a radical sink (4% milk proteins). The antioxidants (not the milk protein) were added in the pre-wash or the homogenization step. The milk protein was added to the final protein isolates. The results showed that without high speed centrifugation and without antioxidants extensive oxidation occurred under the process. When added in the pre-wash or homogenization step, erythorbate alone, or combined with STPP or EDTA reduced the lipid oxidation substantially, both under processing and subsequent ice storage. The best stabilities were obtained when fortifying both steps and with erythorbate in combination with EDTA.

Lipid oxidation during alkaline and acid processing of Spanish mackerel homogenates with added antioxidants was tested by Petty and Kristinsson [52]. The antioxidants tested were ascorbic acid and tocopherol, which were tested separately and combined at a 0.1% and 0.2% (ww basis) level in the homogenate. The homogenates were adjusted to high and low pH values. Analysis of primary and secondary oxidation products was performed after 0, 2, 4, 8 and 12 hours. Both antioxidants were more efficient in high concentrations and at low pH. Comparing acid and alkaline processing, there was no need to apply antioxidants during alkaline processing because no oxidation occurred in this case, even without the addition of antioxidants. In the acid process, however, oxidation developed rapidly without antioxidants.

Vareltzis and Hultin [50] studied inhibition of lipid oxidation during acid and alkaline isolation of cod muscle proteins. In their study, centrifugation of the solubilized homogenate was omitted. It was shown that both acid and alkaline processing enhanced the oxidative stability of protein isolates. The authors showed that acid-treated isolated cod membranes were resistant to Hb-mediated and/or NADH-dependent lipid oxidation, showing longer lag phase and lower oxidation rates under these conditions.

Vareltzis, et al. [48] evaluated how citric acid and calcium chloride influenced lipid oxidation in ground cod muscle. The results suggested that adding citric acid and calcium chloride to minced cod muscle could significantly improve the oxidative stability of protein isolates. It was suggested that citric acid and calcium chloride could behave like reducing agents, as well as cause higher in situ aggregation of membranes, when compared to muscle treated only with citric acid. Testing the oxidative stability of acid- and alkali-processed protein isolates from cod muscle with different antioxidants, Raghavan and Hultin [49] showed that the best results were obtained with propyl gallate followed by BHA and δ-tocopherol.

According to a more recent study, seaweed-derived antioxidants might very well be used to help inhibit oxidation in protein isolates [53]. Wang et al. investigated the potential of subfractions rich in oligomeric phlorotannins to be used as new natural antioxidants in fish and fish products, and found that these were able to suppress the onset of lipid oxidation. The oxidation set up was prepared according to Richards and Hultin [54] and according to Undeland, et al. [55].

In summation, the results indicate that lipid oxidation and lipid level for the different processes are not proportional. The better stability of conventional surimi, compared to the pH-shift processes, might be due to the lack of subjection to $pH < 7$ so that the Hb/Mb-activation is avoided. However, it is possible to avoid lipid oxidation for all processes by the addition of antioxidants, such as erythorbate, EDTA, citric acid and calcium chloride and others.

3.4.8 Frozen storage stability

Stability under frozen storage is a main advantage with cryoprotected surimi. Nevertheless, very little research regarding the frozen stability of cryoprotected, pH-shift produced protein isolate has been carried out. However, an important question is how the frozen storage stability of cryoprotected pH-shift process produced protein isolates compares to the stability of traditional surimi. Thawornchinsombut and Park [56] investigated the frozen storage stability of alkali-produced rockfish protein isolates at pH 5.5 and pH 7 under different storage conditions. In the test, cryoprotected and unprotected protein isolates stored for 4 weeks at -80 °C were subjected to three freeze and thaw cycles at -18 °C and 4 °C. Additionally, one cryoprotected sample at each pH level was stored at -80 °C for a period of four weeks. The texture properties of the gels

made from alkali-processed protein isolates showed that gels made from cryoprotected protein isolate and that were not exposed to freeze-thaw cycles had the best gel quality following frozen storage. These gels were followed by cryoprotected gels that were subjected to freeze-thaw cycles, and lastly unprotected gels.

The investigations showed the importance of using cryoprotectants for alkali-processed protein isolates and the storing of unprotected isolates at the higher pH level.

4. STUDY DESIGN

The study design used for this thesis is described in Figure 5. The general scope has been to evaluate how the protein yield and the properties of the protein isolates are affected by the separation techniques used in pH-shift processing In Paper 1, muscle from fresh cod and haddock was used in alkaline processing wherein filtration and centrifugation were tested in the first and second separation step. In that study, the different processes were evaluated based on loss of protein in the process water, protein yield, gel quality and analysis of the gels by peptide profile analysis using SDS-PAGE. In Paper 2, muscle from fresh and frozen blue whiting was used in acid and alkaline processing wherein filtration and centrifugation were tested in the first separation step. The composition and color of the protein isolates were evaluated by measuring the protein content, polypeptide pattern, moisture content, lipid content, L* values, a* values, b* values and whiteness. The same analyses were done on the raw material. Gel quality was determined by measuring the gel strength, deformation values and color.

4.1 Overview of measurements for moisture, protein, fat, color and gel quality

Figure 5 illustrates in which processing steps the samples for measurement of moisture content, polypeptide pattern, protein content, fat content, color and gel quality were taken.

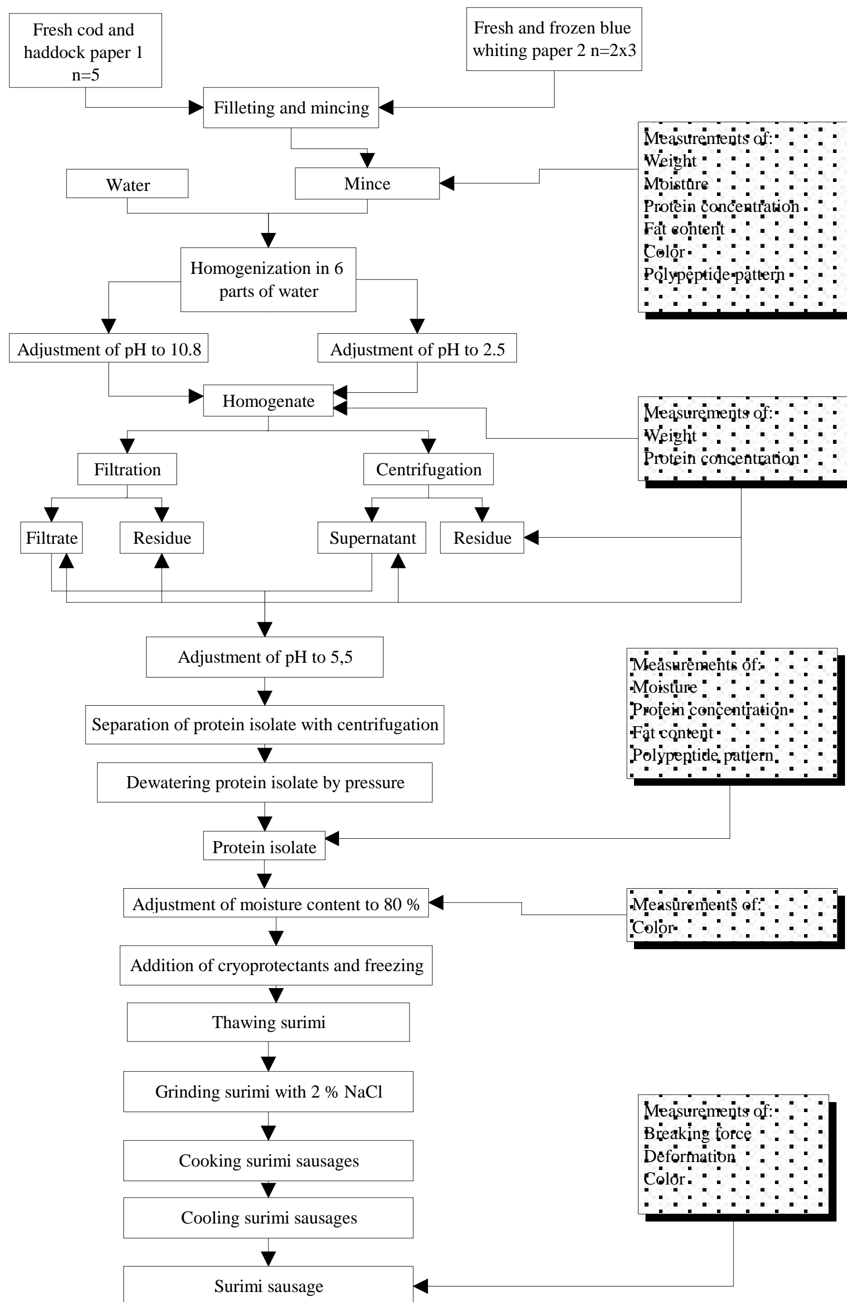


Figure 5. *Overview of the procedure and sampling in Paper 1 and 2. Fillets from fresh cod and haddock (Paper 1), as well as fresh and frozen blue whiting (Paper 2), were minced and homogenized in 6 parts de-mineralised water and pH adjusted. Cod and haddock were taken through alkaline processing only, while blue whiting was taken through both acid and alkaline processing. Weighing and analysis were made as indicated by the shadowed boxes. The precipitated proteins were generally separated from the supernatant with centrifugation, followed by pressure dewatering in a screw press. A few trials were however done also here to replace centrifugation with filtration (Paper 1, see* ***Figure 6****). The moisture content of the protein isolate was adjusted by addition of water, followed by addition of cryoprotectants and freezing. The frozen surimi was partially thawed before addition of 2% salt and grinding. After the grinding with salt, the surimi sausages were prepared and cooked. After cooking, the surimi sausages were cooled down and kept at room temperature for 18-24 hours.*

5. METHODOLOGICAL CONSIDERATIONS

5.1 Fish raw material

Fresh fillets of cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) were bought from John B. Wright Fish Company, Inc. in the summer and autumn of 2004 for the tests made in Gloucester, Massachusetts (Paper 1). Generally, the *postmortem* age of the fillets was between 12 and 36 hours upon arrival at the laboratory. The fillets were used either at once or after storage on ice for 1-2 days.

Fresh whole blue whiting (*Micromesistius poutassou*), caught in the period from January to May 2008 and 2009, were collected onboard trawlers immediately upon their arrival at port (Paper 2). The *postmortem* age of the raw material was from 12 to 48 hours. The fish length was generally from 23 to 40 cm. The fish were kept iced during the one hour transport to the laboratory. At the laboratory, the fish were manually beheaded and gutted. The fish for the frozen fish experiments were packed in plastic freezing bags in 2 kg portions and frozen at -80 °C and were kept stored for about 4 months at -80 °C. Before the pH-shift processing, the frozen fish were thawed out overnight in a refrigerator. Just before the processing of protein isolates, the fish were manually filleted, de-skinned and ground. A comparison of the nutritive value of the blue whiting caught in January-February and May, the results showed higher protein and lipid contents in the winter catch.

5.2 Basic procedure for performing the pH-shift process

The procedure utilized when performing the pH-shift process is shown in **Figure 5**. Ground muscle was first mixed with six parts of cold demineralised water and homogenized. The pH of the homogenate was adjusted, from generally between 6.5 and 7, to pH 2.5 and pH 10.8 for acidic and alkaline protein isolates, respectively. This was done by drop-wise addition of, respectively, 2 M HCL and 2 M NaOH. The homogenate was kept ice-cooled during the processing. The pH-adjusted homogenates were then split into two portions, one for centrifugation and one for filtration. The centrifugation was performed at 10 000 x *g* for 20 minutes in a cooling centrifuge. The temperature of the centrifuge was kept at 4 °C during the centrifugation. The filtration was done using a standard testing sieve with 1 mm openings.

The precipitation was performed by adjusting the pH of the supernatant and filtrate to pH 5.5 by drop-wise addition of 2 M HCl and 2 M NaOH to the alkaline and acid samples, respectively. After precipitation, the material was kept refrigerated until centrifugation at 10 000 x *g* for 20 min. After centrifugation the supernatant was poured off from the centrifuging bottles, and the protein isolate was collected in a container and kept in a refrigerator at 4 °C until all four batches were centrifuged. The moisture content of the protein isolate was reduced by wrapping it in a double layer of cheese cloth and subjecting it to pressure in a screw press. It should be noted that all materials were kept cool with ice during the pH-shift processing. Samples were weighed prior to each test step, and mass balance calculations at the relevant processing steps were carried out, as shown in **Figure 5**.

5.3 Tests related to the substitution of the first centrifugation with filtration

To avoid significant loss of proteins trapped in the sediment gel that is formed during the first centrifugation, filtration could be considered as a possible solution to separate solubilized proteins from unsolubilized proteins, bones, connective tissue and membranes. To test filtration as an alternative separation method, some initial tests were performed using test sieves with mesh sizes 2 mm, 1.5 mm, 1 mm, 0.8 mm, 0.5 mm

and 0.4 mm. These initial tests formed a basis for the investigations of the cod and haddock fillets, as well as the blue whiting fillets.

To compare the influence of centrifugation vs. filtration on the protein concentrations in the supernatant and the filtrate, parallel tests were performed using the same raw material for each test. Only alkaline, pH-shift processing was tested with cod and haddock, while both acid and alkaline pH-shift processing were tested with blue whiting. The yields for centrifugation and filtration were measured by taking out samples at different processing steps and calculating their protein concentrations and weight.

5.4 Tests related to the substitution of the second centrifugation with filtration

To compare how filtration vs. centrifugation in the second separation step influences the loss of proteins after the precipitation, a series of tests were performed using filters with different mesh sizes (20-73 µm) and centrifugations with different *g* values (2511, 4069, 10000 *g*) (Paper 1). **Figure 6** illustrates the experimental setup.

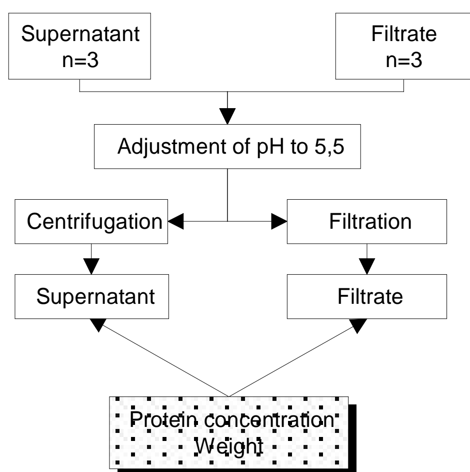


Figure 6. *An overview of the procedure and sampling in Paper 1. Supernatants and filtrates were subjected to pH adjustment from pH 10.8 to 5.5 and the proteins were recovered by centrifugation or filtration.. Centrifugations were performed at 2511, 4069 and 10000 g. The filtrations were performed with different mesh sizes from 20 to 73 µm.*

5.5 Determination of protein yield

The protein concentrations of the raw material, the homogenate, the filtrate, the supernatant, the residues left after filtration and the isolates were measured using the Biuret method [57]. The protein yield in the first separation step was determined as:

$$\text{Protein yield centrifugation} = \frac{\text{Protein concentration in supernatant x Weight of supernatant x 100 \%}}{\text{Protein concentration in homogenate x Weight of homogenate}}$$

$$\text{Protein yield filtration} = \frac{\text{Protein concentration in filtrate x Weight of filtrate x 100 \%}}{\text{Protein concentration in homogenate x Weight of homogenate}}$$

The total protein yield in the whole process was determined as:

Protein yield = $\frac{\text{Protein concentration of protein isolate x Weight of protein isolate x 100 \%}}{\text{Protein concentration of homogenate x Weight of homogenate}}$

5.6 Qualitative analyses of the protein isolates

5.6.1 Determination of moisture content

The moisture content of the raw materials and protein isolates was measured on ~3 g samples using a moisture balance.

5.6.2 Determination of lipid content

The lipid content of raw materials and protein isolates was determined by extraction with chloroform and methanol, according to Lee et al. [58]. The chloroform:methanol ratio was 1:2.

5.6.3 Surimi gel preparation and evaluation of gel strength

To compare the quality of protein isolates from different processing versions that had been processed in the same run (Paper 1 and 2), surimi was prepared from the different protein isolates. The moisture content of the dewatered protein isolate was adjusted to 80 % by the addition of water. The pH of the isolates was adjusted to pH 7 by adding 2 M NaOH drop-wise to the water used for adjusting the moisture content.

The following cryoprotectants – 4% sucrose, 4% sorbitol and 0.3% sodium tripolyphosphate –were added to the protein isolates before they were frozen at -80° C in surimi blocks.

When the gels were prepared, the frozen surimi blocks were partially thawed and cut into 20 mm pieces, mixed with 2 % NaCl (w/w), and ground in a mixer equipped with a rotating knife. The grinding was performed until the temperature of the material reached 7-8 °C. Then the material was stuffed into 27 mm plastic casings. The casings were cooked at 90 °C for 40 min. After the cooking the casings were immersed into cold running water and kept there for at least one hour. After that, the casings were stored at room temperature for 18-24 hours before they were tested.

The gel strength was measured by using a Rheo Tex model AP – 83 rheometer. Cylindrical specimens of surimi gel 30 mm thick cut from a plastic casing 27 mm in diameter were subjected to a pressure test to determine elasticity and penetration. This procedure for testing the gel properties is described in the Japanese grading standards (see Kim et al. [59]). A folding test, as described by Suzuki [60], supplemented the rheometer measurements.

5.6.4 Polypeptide analysis by SDS polyacrylamide gel electrophoresis

In Paper 1, an attempt was made to study the composition of polypeptides present in the loose gel layer formed in the first centrifugation. This was done by homogenizing 10 gm from the top portion of the sediment fraction in five volumes of distilled water for 20 sec. and centrifuging at 10 000 x *g* for 20 min. Following centrifugation, samples were removed from the supernatants and the sediments for polypeptide profile analysis. The samples were pre-diluted with distilled water to a protein concentration of approximately 2.5 mg/ml.

In Paper 2, samples were taken for polypeptide profile analysis from the raw material and from the four protein isolate versions. Samples were pre-diluted with 0.1 M NaOH until the protein concentration of the sample was approximately 2 mg/ml.

In both Paper 1 & 2, diluted samples were mixed with, respectively, ProteoGel^{TM} sample buffer and Laemmlli buffer prepared with 5% β-mercaptoethanol. Denaturation was performed by heating in boiling water for about 5 minutes. In both papers, protein profiles were analyzed using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). Pre-cast 4-20% gradient gels were used. The gels were run in vertical electrophoresis tools. After the runs, the gels were washed in distilled water, fixed and treated with staining reagent and de-staining. In Paper 1, the gel was captured with a digital camera, and in Paper 2 it was scanned into a GS-800 Calibrated Densitometer.

5.6.5 Analysis of color of raw material, protein isolates and gels

To analyze the colors (a*, b* & L*) of the raw material, the protein isolates and the gels, a Konica Minolta CR-300 Chroma Meter with DP-301 Data Processor was used. The colors of the raw material and the isolates were measured by filling plastic beakers with ground material and measuring at 10 different points on the surface. The moisture content of the isolates was adjusted to 80 % before color measurements. Color measurements of the surimi gels were performed when the gel strength measurements were performed by measuring the color of the surface of the 30-mm thick cylindrical specimens at 10 different points on the gel surface. The whiteness of the materials was calculated by the formula [61]:

$$W = 100-((100-L^*)^2 + a^{*2} + b^{*2})^{1/2}.$$

5.7 Statistics

In Paper 1, each process version was repeated five times with different batches of raw material. The number of replicate samples varied from 7-14. The tests for protein content in the supernatant and filtrate were conducted on three different batches of raw material. The number of replicate samples varied from 8 to 16 for one batch, and 12 for the other two batches. The statistics in Paper 1 were generated with SIGMASTAT 3.1. In Paper 2, each process version was repeated three times with both fresh and frozen raw materials. When color and gel strength were analyzed, 10 replicate samples from each test run were tested. For protein, lipid, polypeptide and moisture analyses, three replicate samples were tested. The statistical calculations were performed using PASW Statistics 18. All the data shown are mean values ± standard deviation. Significant differences between groups were determined by Tukey´s HSD multiple rank test. Differences were regarded as significant with p values < 0.05. A t-test was used to determine differences between the paired groups. The graphics were made with ABC Flow Charter and Microsoft Excel.

6. RESULTS AND DISCUSSION

The overall aim of these papers has been to investigate the possibilities for improvement in protein yield in pH-shift processing as described by Hultin et al. [10;13] by substituting both the first and second centrifugations by filtration. The main reasons for modifying the pH-shift process are: 1) to achieve a better utilization of the proteins trapped in the bottom sediment in the first centrifugation step [2], and 2) to develop a processing method that is more relevant for large-scale industrial production. The bottom sediment referred to is shown in **Figure 7** together with the supernatant and a vague floating layer. In the second paper, two additional courses of actions were studied in terms of their effect on protein yield and protein isolate quality. These were acid vs. alkaline solubilization and fresh vs. frozen raw material.

6.1 Effects of the substitution of the first centrifugation with filtration

6.1.1 Choice of mesh sizes

Based on comparisons of sieves with mesh sizes of 2 mm, 1.5 mm, 1 mm, 0.8 mm, 0.5 mm and 0.4 mm, a mesh size of 1 mm was found to be the most suitable dimension for filtrating homogenates from cod, haddock and blue whiting. The best suitability of 1 mm mesh size was because 2 mm and 1.5 mm mesh sizes had a tendency to let un-solubilized connective tissue pass through the sieve. This was not the case with 1 mm mesh size. Regarding the finer mesh sizes, these blocked up easily and would not let any material pass through the sieve. To avoid pressing connective tissue and un-solubilized material through the sieve, only gravity was utilized as the driving force in the creation of the filtrate. A simple nylon brush was used to keep the apertures open and free of blockage.

6.1.2 Sizes of centrifugation sediment and filter cake during the first separation step of pH-shift processing

Based on the 6 experiments with blue whiting, the weight of the sediments formed via centrifugation was typically from 18% to 40%, while the weight of the filter residue formed via filtration was 3 to 11% of the homogenate mass. The mean values of the protein concentrations (mg/ml) of the homogenates, supernatants, sediments, filtrates and filter residues are shown in **Table 1**. The protein concentrations of the supernatant and the filtrate after the first separation step were at the same level. Moreover, the protein concentrations of the sediment after the first centrifugation and of the residue after filtration were at the same level. Since there was considerably more centrifugation sediment than filtration residue, this illustrates that less solubilized proteins can be recovered with centrifugation.

Table 1*. Average protein concentration (mg/ml) ± standard deviation of homogenate, supernatant, filtrate, sediment and filter residue (n=12).*

Initial homogenate	Supernatant	Filtrate	Centrifugation sediment	Filter residue
23.5 ±4.3	22.9±4.5	22.7 ±2.7	28.6 ±11.2	28.1 ±7.2

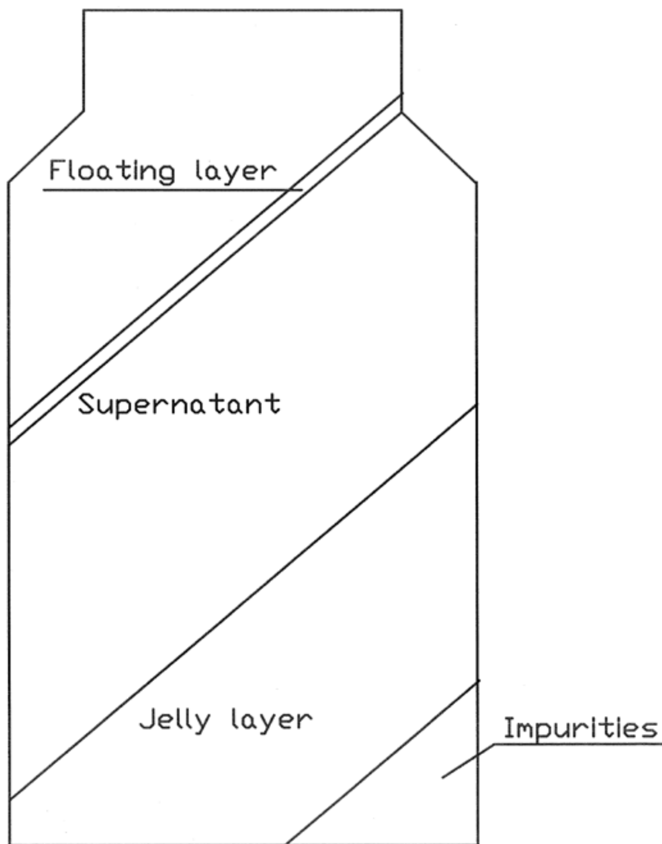


Figure 7. Illustration of the different fractions obtained during centrifugation of an acidified or alkalized homogenate. The lower sediment layer ("impurities") consists e.g. of membranes, connective tissue and skin and bone fragments. The upper part of the sediment ("Jelly layer") is largely comprised of solubilized or nearly solubilized swollen proteins. The middle layer ("supernatant") consists of solubilized proteins in water. On the surface there can be a floating lipid layer.

Figure 8 shows a mix of the unsolubilized sediment and the floating layer material collected after centrifugation by pouring the contents of the centrifuge bottle through double cheese cloth. The relatively big bottom sediment traps a lot of solubilized proteins in a weak gel formed by gravity force during the centrifugation. In **Figure 9** a sieve with filter residue from homogenized cod muscle is shown.

Figure 8 *Sediment and floating layer collected after centrifugation of homogenized cod muscle at pH 10.8. Centrifugation was performed at 10 000 x g for 20 min.*

Figure 9 *Filter residue collected after filtration of homogenized solubilized cod muscle at pH 10.8 through a standard test sieve with 1-mm mesh size.*

The reason for that the centrifugation sediments get larger than the filtration residues from the sieves is that in addition to containing connective tissue, skin and bone fragments, it contains a weak gel that is formed during the centrifugation of the homogenate. This weak gel traps a lot of the solubilized proteins, so they not can be utilized in the primary production. Undeland et al. [2] found that the formation of this week gel was closely linked to the consistency of the alkalized or acidified herring light muscle homogenate. The higher the consistency, the larger was the week gel layer. Consistency on the other hand was positively correlated e.g. to increased *post mortem* age and freezing of the herring. It was believed that these observations were

due to the fact that age/freezing caused a certain aggregation of protein monomers and aggregate which makes them larger.

6.1.3 Protein yield

From the tests performed using the alkaline process on cod and haddock (Paper 1), protein yields after the first separation step were significantly lower (p=0.008) with centrifugation than with filtration; 71 ±7% vs. 90 ±2%. As shown later under 6.2, the protein recovery during the second separation step varied from 94-97%. The total yield thereby became 65-68% and 84-87% when centrifugation and filtration respectively was used in the first separation step. These results were slightly higher than the total protein yields obtained for alkali processed fresh and frozen blue whiting (Paper 2); 51% and 81-83% using centrifugation and filtration, respectively (**Table 2**). It is thought that the main reason for this difference is the lower protein solubility of the blue whiting mince at pH 10.8 (about 70%) compared to the solubility for cod mince (about 93%). Another possible reason for the lower yield with blue whiting could be differences in the connective tissue content in the mince of different fish species [62;63].

For both fresh and frozen blue whiting, the difference in total protein yield between the centrifugation and the filtration process was significant (p=0.001). No significant difference in the total protein yield was measured between the use of alkaline vs. acid pH-shift processing.

Table 2 *Protein yields (mean ± SD) obtained with either centrifugation or filtration in the first separation step with alkaline or acid pH-shift processing of fresh and frozen blue whiting. "n" is the number of runs made with each process version. Results within the same column carrying different letters indicate a significant difference (p<0.05).*

Solubilization Method	Separation Method	Total protein yield %	
		Fresh blue whiting	Frozen blue whiting
Alkaline	Centrifugation, n=3	51.3±15.2^{a,b}	51.1±1.7^{a}
	Filtration, n=3	81.3±8.6^{b}	82.7±3.9^{b}
Acid	Centrifugation, n=3	47.2±9.7^{a}	58.7±12.6^{a}
	Filtration, n=3	73.4±6.2^{a,b}	81.2±0.9^{b}

Table 2 demonstrates a significant potential for a better utilization of the fish raw materials by replacing centrifugation with filtration during pH-shift processing. In paper 3, seven studies of traditional surimi processing were reviewed for total protein yields. An average value of 55.9% was found. Thus, the modified pH-shift process evaluated here gave much higher yields than traditional washing-based techniques for protein isolation.

6.1.4 Character of the blue whiting protein isolates

6.1.4.1 Basic composition

Table 3 shows the basic composition of the blue whiting raw material and the four different isolates produced. No significant differences in the final moisture content were seen between the different isolates, however, there was a tendency towards higher moisture content in the isolates made with acid processing. This might stem from a higher water-holding capacity of the acid-treated proteins, something which in turn can be linked to the specific unfolding-refolding pattern of the proteins when subjected to pH 2.5 vs. pH 10.8 before adjustment to pH 5.5. Also Undeland et al. [5] found a higher moisture content of acid-produced

protein isolates compared to alkali-produced ones when using herring light muscle; 90.1% and 87.6%, respectively.

Table 3. *Amounts of water, protein and lipids (mean ± SD) for blue whiting mince and protein isolates derived from either centrifugation or filtration in the first separation step with alkaline or acid pH-shift processing of fresh and frozen blue whiting. "n" is the number of runs of each process version with frozen and fresh raw material. Results within the same column carrying different letters indicate a significant difference (p<0.05)*

Samples (n=3)	Isolation Method	Separation Method	Moisture (%)		Protein (%) dw		Lipid (%) dw	
			Fresh fish	Frozen fish	Fresh fish	Frozen fish	Fresh fish	Frozen fish
Mince	-	-	79.4±0.1^{a}	78.6±2.2^{a}	86.3±1.0^{a}	81.4±8.8^{a}	4.7±0.7^{ab}	2.8±0.6^{a}
Protein isolate	Alkaline	Centrifugation	71.8±2.5^{a}	69.8±5.9^{b}	94.3±1.8^{a}	87.8±14.4^{a}	2.3±0.6^{c}	2.3±1.6^{a}
		Filtration	69.8±2.3^{a}	70.5±1.4^{b}	89.1±1.4^{a}	91.4±7.4^{a}	6.2±0.1^{bd}	5.3±2.0^{ab}
	Acid	Centrifugation	75.9±7.1^{a}	74.7±0.8^{ab}	92.7±1.6^{a}	93.7±1.7^{a}	3.6±1.0^{ac}	5.7±1.9^{ab}
		Filtration	77.6±2.1^{a}	73.2±1.1^{ab}	89.9±0.9^{a}	92.5±10.7^{a}	7.0±0.6^{d}	7.4±1.0^{b}

A trend towards protein enrichment could be seen following acid and alkaline processing of fresh and frozen blue whiting. However, no significant differences could be established between the raw material and the four isolates. These results are in disagreement e.g. with the results of Marmon & Undeland [31] and Vareltzis & Undeland [26], where the protein contents increase by 30.35% using whole gutted herring and mussel meat. A likely reason is the fact that the blue whiting fillets used here contained less non-protein compounds compared to the more complex gutted herring and mussels. Thus, the relative difference in protein content before and after processing becomes limited.

Comparison of the lipid content on a dry weight basis using fresh blue whiting showed that isolates made from the centrifugation process had lower lipid content than the starting mince, while isolates made via the filtration process had higher lipid levels than the starting mince. The latter is probably due to a greater removal of non-lipids than lipids in the filtration process, and is in agreement with the results of Undeland, et al. [23] from a study where acid processing of herring mince was tested without any centrifugation applied.

In general the results show that more lipids are removed during centrifugation than during filtration. As a result, the lipid levels in the isolates from the centrifugation process were in 3 out of 4 cases significantly lower than in isolates from the filtration process. It has been showed earlier [48] that membrane lipids can be trapped in the bottom sediment from centrifugation. However, since a small floating layer was also obtained after centrifugation, it is expected that some of the neutral lipids partitioned into this layer. Among reasons for the disability of the sieving process to remove lipids is the fact that in sieving, the separation is only based on size differences, while in centrifugation, one can take advantage of density differences between lipids, water and proteins.

Whether the higher lipid content in the isolates made with filtration versus centrifugation is an advantage or not depends on the intended use of the product, and the level of oxidation prevention applied (i.e. antioxidants). The likelihood is small that the differences in lipid levels between isolates made with

centrifugation vs. filtration (2.3% vs. 5.3% dw) will affect the development of rancidity. Undeland et al. [64] showed that hemoglobin (Hb)-mediated ‘painty’ odor and TBARS developed equally in washed cod mince fortified with either 0%, 7.5% or 15% non-stabilized fish oil.

6.1.4.2 Color of protein isolates

Color measurements of fresh and frozen blue whiting raw materials and isolates made thereof showed that all isolates had higher whiteness than the starting mince (**Table 4 and 5**). This was mainly shown by higher measured lightness (L*) and lower redness (a*) in the isolates. This could indicate that blood and melanin present in the mince was removed with the process water, and/or in the sediments/filter residues. The measurements also showed a tendency to higher levels of whiteness for acid-processed isolates, compared to alkali-processed isolates, especially when fresh blue whiting was used. This trend is in contrast to most previous studies on the pH-shift process (paper 3), and no clear explanation can be given. There was also a small tendency for lower whiteness-values in the isolates derived from filtration rather than centrifugation. As for the lipid contents, this could be explained by the different separation principles operating under centrifugation and filtration. Small pigment molecules (melanins, heme-proteins) would not be captured in the sieve unless they have co-precipitated with certain larger proteins/protein aggregates. In the centrifugation process on the other hand, they have the potential to be removed based on higher density than the soluble proteins.

The differences in b*-values (yellowness) between mince and isolates, as well as between the different isolates were in all cases non-significant for fresh blue whiting, and in nearly all cases also for frozen blue whiting. This is in line with the fact that there are no clear expectations of yellow pigments that could be removed during pH-shift processing of blue whiting. Among described groups of yellow pigments in fish muscle are “Schiff´s bases” formed from lipid oxidation products that react with free amino groups [65] and caroteinoids. None of these groups would be expected to play a major role here.

Table 4. *Measured lightness (L*), redness (a*), yellowness (b*) and whiteness (W) of blue whiting mince and protein derived from centrifugation and filtration in the first separation step with alkaline and or acid pH-shift processing of fresh blue whiting. “n” is the number of runs of each process version. The results within the same column carrying different letters indicate a significant difference (p<0.05).*

Samples (n=3)	Isolation Method	Separation Method	L*	a*	b*	W
Mince	-	-	45.0 ± 3.5^{a}	1.4 ± 1.1^{a}	6.7 ± 1.5^{a}	44.6 ± 3.7^{a}
Protein isolate	Alkaline	Centrifugation	56.4 ± 8.3^{ab}	0.1 ± 1.8^{a}	6.0 ± 2.3^{a}	55.9 ± 8.2^{ab}
		Filtration	53.4 ± 5.0^{ab}	-0.1 ± 0.4^{a}	6.1 ± 0.2^{a}	53.0 ± 4.9^{ab}
	Acid	Centrifugation	66.2 ± 2.2^{b}	-0.4 ± 0.7^{a}	6.7 ± 1.6^{a}	65.5 ± 2.1^{b}
		Filtration	62.6 ± 4.8^{b}	-0.7 ± 0.2^{a}	6.9 ± 1.0^{a}	62.0 ± 4.9^{b}

Table 5. *Lightness (L*), redness (a*), yellowness (b*) and whiteness (W) for blue whiting mince, as well as protein isolates derived from either centrifugation or filtration in the first separation step with alkaline or acid pH-shift processing of frozen blue whiting. "n" is the number of runs of each process version. Results within the same column carrying different letters indicate a significant difference (p<0.05).*

Samples (n=3)	Solubilization Method	Separation Method	L*	a*	b*	W
Mince	-	-	47.6±2.8[a]	2.0±0.4[a]	7.2±0.8[a]	47.0±2.8[a]
Protein isolate	Alkaline	Centrifugation	62.3±0.3[b]	1.9±0.1[a]	6.3±0.1[ab]	61.7±0.3[b]
		Filtration	59.6±0.8[b]	0.3±0.3[b]	5.7±0.4[abc]	59.2±0.8[b]
	Acid	Centrifugation	61.7±0.8[b]	-1.1±0.2[c]	4.4±0.8[c]	61.4±0.7[b]
		Filtration	60.7±1.5[b]	-0.7±0.2[c]	5.4±0.8[bc]	60.3±1.4[b]

In a comparison of a*-values between isolates made from fresh and frozen raw material, the alkali-processed isolates had slightly higher *a-values when frozen raw material was used than when fresh raw material was used. Frozen storage can cause aggregation of heme/heme-proteins with other muscle components making them more difficult to extract [65]. As an example it has been described how *postmortem* oxidation of heme-proteins can lead to liberation of the hydrophobic heme-group which then can partition into the lipid interior of cellular membranes [66].

6.1.4.3 Surimi gel quality

Values of breaking force and deformation of surimi gels produced from various protein isolates are shown in **Table 6**. It should be noted here that the data from cod- and haddock-based surimi gels are treated as one group. When the alkali-based filtration process was used, it was found that the breaking force and deformation values of this surimi gel group were, on average, 70% and 85%, respectively, of the breaking force and deformation values obtained with the alkali-based centrifugation process. Based on folding tests, surimi gels produced with both process versions exhibited double folding without any cracks of the folded specimens (Paper 1). For fresh blue whiting (Paper 2) the corresponding results for breaking force and deformation values were 67% and 89% for surimi gels made of alkali-processed isolate and 117% and 85% for surimi gels made of acid-processed isolate. For surimi gels made of frozen raw material, the corresponding values were 83% and 80% for alkali-produced isolates and 80% and 85% for acid-produced isolates.

Table 6. *Values of breaking force (g) and deformation (mm) of surimi gel produced of protein isolates from cod and haddock (Paper 1) and fresh and frozen blue whiting (Paper 2). The gels made from, respectively, fresh and frozen blue whiting contain 21.6% and 27.5% total dry matter why data on these two gel types are not directly comparable. Different letters in column indicate statistically significant differences (p=0.05).*

Separation Method	Solubili-zation Method	Fresh cod & haddock (n=5)		Fresh blue whiting (n=3)		Frozen blue whiting (n=3)	
		Breaking force (*g*)	Defor-mation (mm)	Breaking force (*g*)	Defor-mation (mm)	Breaking force (*g*)	Defor-mation (mm)
Centrifugation	Alkali	841 ±52 [a]	10.6 ±0.6	331±180[a]	10.7±0.8	622±264[a]	10.4±1.8
Filtration	Alkali	588 ±34 [b]	9.0±0.5	223±22[a]	9.5±1.4	515±104[a]	8.3±1.0
Centrifugation	Acid			149±63[a]	9.8±1.1	717±224[a]	10.8±0.8
Filtration	Acid			174±54[a]	8.3±0.9	573±29[a]	9.2±1.0

Combining the experimental gel results obtained from studying fresh and frozen blue whiting, three out of four cases showed lower breaking force and deformation values for surimi made from isolates produced by filtration than those produced by centrifugation. This is also consistent with the results for cod and haddock. A comparison of the breaking force and the deformation values of surimi from alkaline- vs. acid-processed protein isolates revealed no significant differences. Because of differences in dry matter content in the surimi gels made from fresh and frozen blue whiting, the absolute data for breaking force and the deformation values are not directly comparable.

Among reasons explaining the lower breaking force for surimi made from isolates produced by filtration compared to centrifugation can be a somewhat lower protein content. This is caused by the higher lipid content in the isolates produced by filtration (**Table 3**). When the surimi is made, the addition of cryoprotectants and water is based on the dry matter content of the protein isolates, which in turn is determined by the lipid and protein content. This implies that the final protein content of the surimi gels made from filtration produced isolates is lower than the protein content of the surimi gels made from centrifugation produced isolates. Another tentative reason for the lower breaking force of surimi gels obtained from the filtration process compared to the centrifugation process could be qualitative protein differences. This is further discussed under 6.1.4.5.

The importance of high gel strength is of course dependent on the intended use of the product. Investigations performed by Webb Foodlab [67] indicated that use of low gel strength surimi was as suitable as high gel strength surimi for most applications in processed meat products. Comparing the gel strength of the centrifugation and filtration processed frozen blue whiting gels with standard surimi specifications [1], the breaking force of the gels made with the centrifugation-based process are on the level of KA while the breaking force of the gels made with filtration are on the level of KB. The deformation value required for a KA-grade gel is 11.5 mm and for KB 10 mm. When comparing the blue whiting surimi gels with shore plant processed Pollock surimi, the blue whiting gels made with the centrifugation-based process are in the level from KA to A, while gels made with filtration are in the level from KB to KA. Different fish species have different values for gel strength and deformation, which can be seen in Appendix 1.

6.1.4.4 Color of surimi gels from blue whiting

When fresh blue whiting was used, all surimi gels obtained had higher whiteness values than the protein isolates (See **Table 7** and compare to **Table 4**). This was mainly seen as higher L*-values in the surimi gels. As for the crude protein isolates, the measurements also showed a tendency to higher whiteness for surimi gels from acid-processed isolates than for gels from alkali-processed isolates. Also, there was an indication that surimi produced from isolates made by filtration yielded lower whiteness values, than surimi gels produced from isolates made by centrifugation. Higher whiteness for heat treated gels has also been shown by Kristinsson et al. [21] for conventional surimi, and surimi made from acid and alkali processed isolates made from fillets of Atlantic croaker.

Table 7. *Lightness (L*), redness (a*), yellowness (b*) and whiteness (W) for blue whiting surimi gels derived from either centrifugation or filtration in the first separation step with alkaline or acid pH-shift processing of fresh blue whiting. "n" is the number of runs of each process version. Results within the same column carrying different letters indicate a significant difference (p<0.05)*

Samples (n=3)	Isolation Method	Separation Method	L*	a*	b*	W
Surimi gels*	Alkaline	Centrifugation	69.5±6.8^{a}	-3.5±0.1^{a}	1.5±0.7^{a}	69.2±6.7^{a}
		Filtration	69.6±1.3^{a}	-2.1±0.2^{b}	5.6±1.2^{b}	69.0±1.2^{a}
	Acid	Centrifugation	75.8±0.2^{a}	-3.2±0.7^{ab}	2.2±1.8^{ab}	75.4±0.2^{a}
		Filtration	72.5±2.2^{a}	-2.6±0.5^{ab}	4.3±1.9^{ab}	72.0±2.3^{a}

*surimi gels contained 21.6% total dry matter.

Table 8. *Lightness (L*), redness (a*), yellowness (b*) and whiteness (W) for surimi gels derived from isolates made either with centrifugation or filtration in the first separation step with alkaline or acid pH-shift processing of frozen blue whiting. "n" is the number of runs of each process version. Results within the same column carrying different letters indicate a significant difference (p<0.05)*

Samples (n=3)	Isolation Method	Separation Method	L*	a*	b*	W
Surimi gels*	Alkaline	Centrifugation	56.0±1.9^{a}	-3.0±0.9^{a}	0.4±1.8^{a}	55.9±1.9^{a}
		Filtration	60.3±0.9^{b}	-1.8±0.3^{b}	4.8±0.4^{bc}	59.9±0.9^{b}
	Acid	Centrifugation	61.0±1.2^{b}	-3.0±0.0^{a}	2.5±1.1^{ab}	60.8±1.1^{b}
		Filtration	61.3±1.4^{b}	-1.9±0.3^{b}	5.5±0.3^{c}	60.8±1.4^{b}

*Surimi gels contained 27.5 % total dry matter.

The whiteness of surimi gels produced from frozen raw material (**Table 8**) were in general at the same level as that of the corresponding isolates (**Table 5**). Further, significantly lower b*-values of surimi gels were found when centrifugation was used in the protein isolation processing of frozen raw material, as compared to when filtration was used. As stated earlier, this is probably related to the higher ability of centrifugation than filtration to remove small pigment molecules.

When comparing **Tables 7** and **8** statistically, the surimi gels from fresh blue whiting raw material showed significantly higher L*-values and whiteness values than surimi gels from frozen raw material. Denaturation of certain proteins like hemoglobin and myoglobin caused by the frozen storage can be one reason. Difficulties to extract heme with extended storage time were discussed under 6.1.4.2. Another possibility that could contribute to the b*-value is formation of aldehydes from lipid oxidation products [68]. Chaijan et al. found lower whiteness in natural actomyosin (NAM) extracted from frozen fillets than from fresh fillets of blue fish. NAM extracted from frozen fish showed a clear decrease in whiteness with myoglobin included [68].

Color is an important attribute of protein isolates and surimi. Generally, the higher the whiteness values, the higher the market interest and resultant value of the product. Based on the presented results, it can thus be regarded a small disadvantage of alkaline solubilization as well as filtration-based separation that these features created surimi with slightly lower whiteness. It is however clear from the literature that isolates/surimi from different fish species can respond very differently e.g. to solubilization method. In

paper 3, whiteness values resulting from acid and alkaline processing as well as from conventional surimi processing are compared for different fish species. In four out of 17 cases the highest whiteness values were obtained for acid processed protein isolates/gels. This was with croaker, Atlantic croaker [21;22] and mullet [22].

A method described in the literature for improving the color properties of protein isolates incorporates the addition of titanium dioxide. Titanium dioxide is commonly used as a whitening agent in surimi [69]. Taskayet al. [69] tested the application of titanium dioxide as a whitening agent for protein isolate produced from whole-gutted silver carp (*Hypophthalmichthys molitrix*) using the alkaline pH-shift process. The color parameters L*, a*, b* and the whiteness values were investigated by adding from 0.1 to 0.5 % titanium dioxide to cryoprotected protein isolate. By adding 0.2 % titanium dioxide, the L* value could be increased from 63.96 to 79.94 and the whiteness equaled that for Alaska Pollock surimi (about 76). These results show that titanium dioxide can be used as whitening agent.

6.1.4.5 Evaluation of polypeptide pattern by electrophoresis

The differences in gel strength between protein isolates made with centrifugation and filtration samples (Paper 1 and Paper 2) might be attributed to the soft gel sediment that is removed in centrifugation (**Figure 7**), but not in filtration. The gel fraction contained about 50% more protein than the supernatant. To determine if there were any specific proteins in the gel fraction that were not present in the supernatant fraction when using cod (Paper 1), a SDS-PAGE analysis of samples from different steps of the pH-shift process was conducted using 4-20% gradient gels. The results are shown in **Figure 10**.

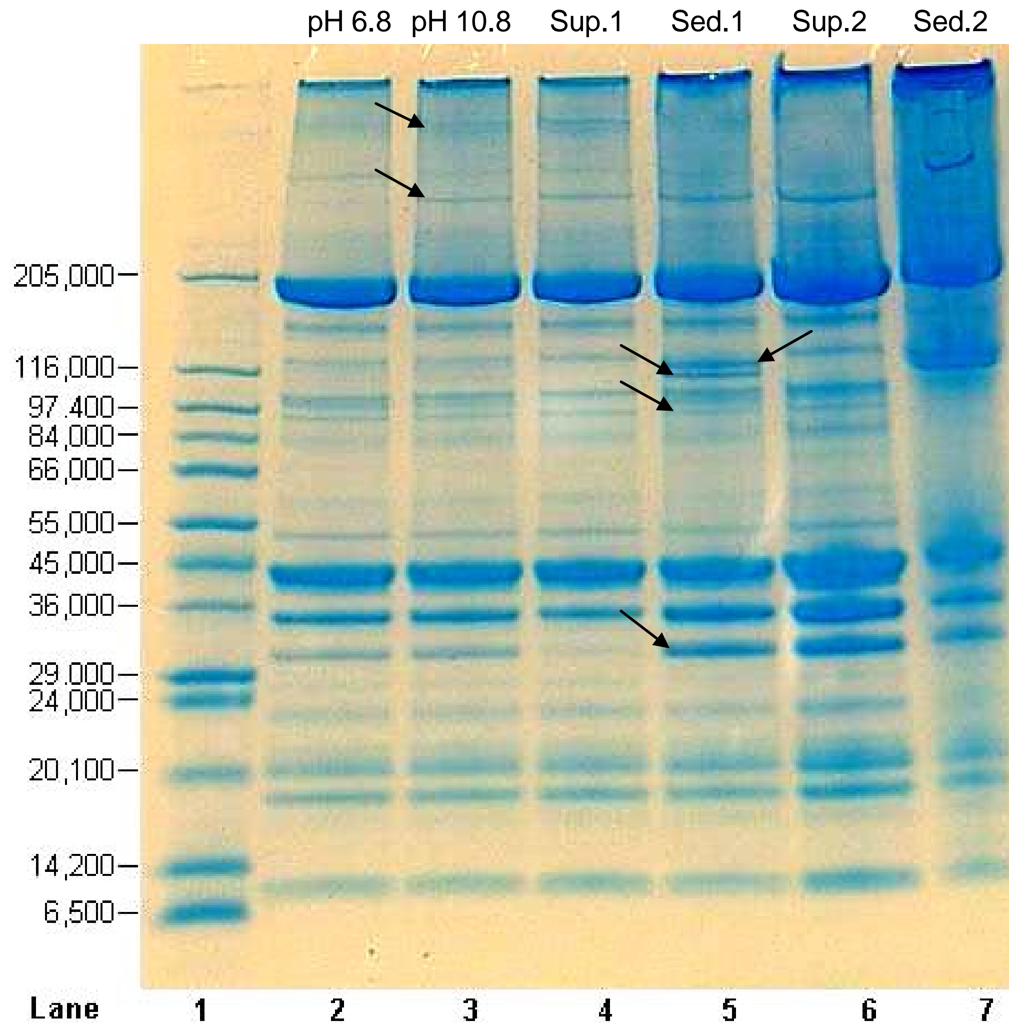


Figure 10 *Alkaline-treated cod muscle proteins analyzed by SDS-PAGE: Lane 1: molecular weight markers; Lane 2: homogenized, whole cod muscle (pH 6.8); Lane 3: homogenized, whole cod muscle at pH 10.8; Lane 4: supernatant from centrifugation of homogenized, whole cod muscle at pH 10.8; Lane 5: sediment from centrifugation of homogenized, whole cod muscle at pH 10.8; Lane 6: supernatant from centrifugation of the homogenized sediment (shown in Lane 5) in 5 parts distilled water; Lane 7: sediment from centrifugation of the homogenized sediment (shown in Lane 5) in 5 parts distilled water.*

Lane 2 is native cod muscle homogenized at pH 6.8, and Lane 3 is the homogenized cod muscle after adjustment to pH 10.8. Lane 4 and 5 are the supernatant and sediment gel fractions of the solubilized cod muscle protein after centrifugation at 10 000 g for 20 min. At pH 10.8 (**Figure 10**, Lane 3), titin-1 (also called α-connectin, ~2800 kDa), was partially broken down to a large sub-fragment, titin-2 (also called β-connectin, ~2100 kDa, upper arrow in lane 3) [70]. Both titin fractions were observed concentrated in the supernatant fraction of the 10.8 solution after centrifugation (**Fig. 10**, Lane 5). Moreover, a polypeptide was formed at pH 10.8, which was situated between nebulin (600-900 kDa) and the myosin heavy chain (220 kDa each chain) in the molecular mass (lower arrow in lane 3). This polypeptide did not appear to fractionate during centrifugation.

The polypeptide band patterns of Lane 4 and Lane 5 were similar in many respects, but there were four polypeptide bands that were substantially more concentrated in Lane 5 (the sediment) than in Lane 4 (the supernatant fraction). These polypeptides appeared at approximately 109 kDa, 116 kDa, 95 kDa, 31 kDa, and are pointed out by the arrows (**Figure 10**). The high concentration of these components suggests the possibility that they may be involved in fostering aggregation of the other proteins by some means, e.g. cross-linking. For example, the 96 kDa polypeptide may represent α-actinin and the 31 kDa polypeptide troponin T. These proteins are involved with binding proteins of the Z-disk and the thin filaments.

When the sediment formed by centrifugation of the protein solution at pH 10.8 was re-suspended in 5 parts water, homogenized and centrifuged once again under the same conditions, the polypeptide patterns of the supernatant (Lane 6) and second sediment (Lane 7) were obtained. The sediment obtained from the second centrifugation was relatively low in volume and had a great deal of smudging in the electrophoretogram. At this point, it was observed that a large part of the 31 kDa polypeptide and the 95 kDa polypeptide indicated by arrows in lane 5 were present in the supernatant fraction (Lane 6), while the polypeptides at 109 and 116 kDa remained in the sediment fraction (Lane 7). It would need to be confirmed in another trial if the removal of this polypeptide doublet improved the gel characteristics of the protein isolate prepared from the solubilised proteins and thus, could be a contributing factor to better gel characteristics of protein isolates prepared by centrifugation.

To determine if there were any qualitative protein differences in the blue whiting protein isolates made by centrifugation vs. filtration, and by the acid vs. alkaline process (Paper 2), a SDS-PAGE analysis of the isolates was made. The results are shown in **Figure 11**. The referenced differences are marked with arrows. The experimental results indicated a slight tendency towards more dense bands in the very high molecular weight region (> 2000 kDa) for alkali-derived isolates (Lanes 3-4), than for the acid-derived isolates (Lanes 5-6), especially with centrifugation as the separation method (Lane 3) (Paper 2). The acid-derived protein isolates also showed more dense bands at about 83 kDa and 152 kDa (Lane 5 and 6). There was also a band in all the isolates around 72 kDa, which was not found in the raw material (Lane 2). In the isolate derived by alkaline filtration processing (Lane 4), a band at 32 kDa, tentatively identified as tropomyosin α, was nearly absent. It is possible that the very high MW-proteins like titin, which connects the Z line to the M line in the sarcomere [71], plays a positive role for the gel characteristics. Westphalen et al. [72] suspected that proteins like actin, titin, tropomyosin, troponin and nebulin have a strong influence on the viscoelastic properties of meat gels by influencing the formation of the myosin gel matrix.

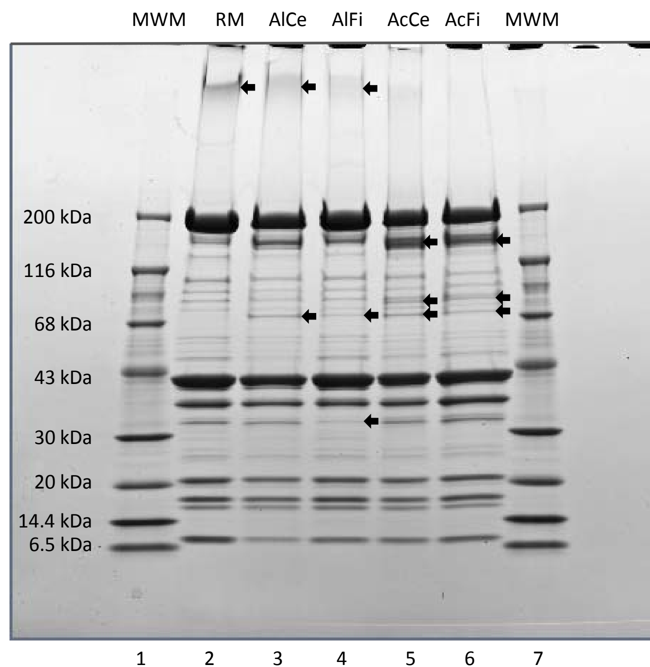


Figure 11. *SDS-PAGE (4-20% linear gradient) of blue whiting muscle protein isolates produced with different versions of the pH-shift method. Lane 1 (MWM): Molecular weight standards 6.5-200 kDa. Lane 2 (RM): Blue whiting mince. Lane 3 (AlCe): Alkaline-processed centrifuged protein isolate. Lane 4 (AlFi): Alkaline-processed filtrated protein isolate. Lane 5 (AcCe): Acid-processed centrifuged protein isolate. Lane 6 (AcFi): Acid-processed filtered protein isolate. Lane 7 (MWM): Molecular weight markers 6.5-200 kDa. 15 µg of protein was loaded into each well, except for the standards, where 10 µg was loaded. Polypeptides marked with an arrow are mentioned in the results and discussion.*

6.2 Substituting the second centrifugation with filtration

In the investigations on cod and haddock (Paper 1), a preliminary test was performed to determine how different separation methods affected the protein content in the soluble fraction obtained after precipitating out the proteins at pH 5.5. In these trials, a 1 mm test sieve was used to separate proteins soluble at 10.8 from the non-soluble matter. After precipitating out the proteins of the soluble fraction at pH 5.5, sieves having a mesh size of 420 µm and 106 µm, respectively were compared to centrifugation at 4 000 *g* for 20 min. The results showed that 15.8% (sieve 420 µm), 6.9% (sieve 106 µm) and 6.8% (centrifugation) of the protein content of the initial alkalized homogenate, respectively, were found in the precipitation supernatant. This was followed up by a series of experiments employing different filter sizes (30 µm, 40 µm and 51 µm) and different *g* values for centrifugation (2511 *g*, 4069 *g* and 10 000 *g* for 20 min) in the second separation step. These tests were performed with different batches of cod, and the first separation step was also in these trials was also performed with 1 mm test sieves. The results are shown in **Table 9**.

Table 9 *Protein concentrations in cod homogenate adjusted to pH 10.8, and in the soluble fraction collected with centrifugation or filtration after precipitating out the proteins at pH 5.5. The latter is also shown as percent of the protein concentration in the initial alkalized homogenate. "n" is the number of replica in each sample. In these trials, the first separation step was carried out with 1 mm test sieves.*

Separation method in step 2	Protein in pH 10.8 homogenate (mg/ml)	Protein in pH 5.5 supernatant or filtrate (mg/ml)	Protein in pH 5.5 supernatant or filtrate in %
Centrifugation 2511 x g (n=12)	26.2 ± 2.2	1.35 ± 0.05	4.4
Centrifugation 4069 x g (n=12)	24.3 ± 4.2	1.04 ± 0.28	3.7
Centrifugation 10 000 x g	26.3 ± 1.6 (n=8)	1.29 ± 0.16 (n=16)	4.6
Filtration 51 µm (n=36)	26.2 ± 2.2	1.2 ± 0.15	4.1
Filtration 40 µm (n=12)	24.3 ± 4.2	0.99 ± 0.24	3.5
Filtration 30 µm (n=12)	24.3 ± 4.2	1.02 ± 0.29	3.6

Generally, the results showed that the protein content of the soluble fraction obtained when centrifugation and filtration was applied in the second separation step was essentially equivalent. The results showed that the variation in the protein content of the initial homogenates (which was made from different batches of cod) was greater than the variation among the various treatments. These results show that the protein loss into the process water was at the same level when separating the precipitated proteins with filtration and centrifugation. In practice this means that the most economical solution would be selected. There is also a possibility to combine filtration and centrifugation to optimize the protein yield.

Three trials measuring the time for filtration of 500 ml alkali-processed homogenate through filters with different mesh sizes were performed to obtain an estimate of the time needed for filtration. The homogenate was produced from 1 part of cod muscle mixed with 6 parts of water that was homogenized and the pH was adjusted to 10.8. The pH adjusted homogenate was filtered through a 1 mm standard test sieve. The pH of the filtrate was adjusted to 5.5 for precipitation of the proteins. Filtration of the pH 5.5-adjusted filtrate was then carried out in a 120 mm diameter Buchner funnel subjected to a vacuum during filtration. In one case, the pH-adjusted filtrate was instead subjected to centrifugation at 10 000 *g* (20 min).

Table 10. *Mean filtration time with filters with mesh sizes 73, 59, 51, 40, 30 and 20µm compared to centrifugation at 10.000 g for 20 min. Protein concentration (µg/ml) and std in filtrate and supernatant. n= number of replica in each sample. The filtration was performed with a 120 mm Buchner funnel subjected to vacuum and protein concentration in filtrate.*

Process	Mean time (min) (n=6)	Protein concentration of filtrates or centrifugation (µg/ml) (n=6)
Filtration 73µm	37±9.8	53.3±1.3
Filtration 59µm	29±17.0	57.5±1.6
Filtration 51µm	12±3.5	56.5±2.3
Filtration 40µm	19±14.4	49.6±5.1
Filtration 30µm	26±13.5	54.8±1.2
Filtration 20µm	8±2.1	52.7±1.4
Centrifugation 10000 *g*	20	53.6±1.8

As can be seen from **Table 10**, different mesh sizes give different filtration times, but there is no direct relation beween the mesh size and the filtration time. The smallest mesh size (20µm) gave the fastest filtration time, but the second fastest filtration time was obtained with the 4th smallest mesh size (51µm). This indicates that certain mesh sizes are more easily obstructed. This phenomena has been studied previously and is described by Wakeman and Tarleton [29]. The following figure illustrates how different particle sizes can block the filters in different ways. Analysis of the involved particle sizes would give a better foundation to select the right filter.

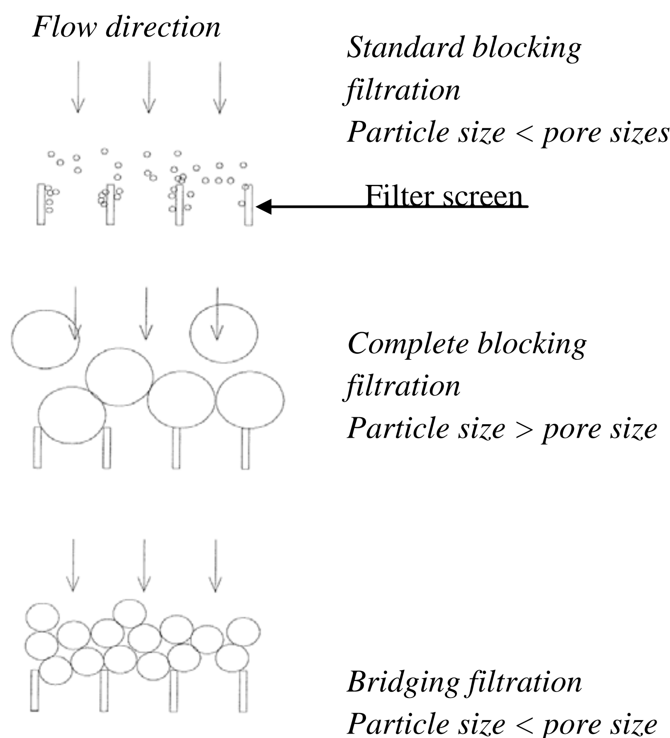


Figure 12. *Different mechanism of filtration. Filter blocking can occur in different ways dependent on the particle size and the pore size.With modifications from Wakeman and Tarleton [29].*

The moisture content after filtration was about 94% regardless of mesh size. This high moisture content would require an additional dewatering e.g. pressure dewatering. The protein concentration of the filtrates and supernatant show no systematic variation with filter sizes.

7. CONCLUSIONS

In the processing of protein isolate utilizing a modified pH-shift process, an improved protein yield can be achieved by substituting centrifugation with filtration in the first separation step.–The increase in protein yield was over 26% in tests conducted on cod and haddock using filtration rather than centrifugation (Paper 1). Experiments with blue whiting (Paper 2) resulted in 55 - 60% higher protein yields during alkaline processing of fresh and frozen fillets as well as during acid processing of fresh fillets. When acid processing was applied to frozen fillets the protein yield was about 38% higher. The higher yield is explained by the large protein containing sediments formed during centrifugation that are not used in the further steps of the pH-shift process.

Applying filtration instead of centrifugation in the second separation step did not result in any difference in the amount of protein lost in the processing waste water (Paper 1).

Comparing the influences of acid vs. alkaline processing as well as, the use of fresh vs. frozen blue whiting on the total protein yield revealed no significant differences (Paper 2).

There was no significant difference in moisture content among the four types of protein isolates obtained from the four different processing methods using fresh blue whiting. When using frozen raw material, however, the alkaline process yielded isolates with significantly lower moisture content (Paper 2).

The lipid content of the protein isolates processed with centrifugation was less than those processed with filtration. This is because centrifugation separates materials with different density while the filtration separates material with different particle size (Paper 2).

The color measurements of fresh blue whiting and isolates produced thereof indicated that all protein isolates had higher whiteness values than the starting blue whiting mince. The measurements also showed a tendency to higher whiteness values for acid-processed protein isolates, compared to alkali-processed isolates, and a lower whiteness value for filtration-processed isolates, compared to centrifugation-processed protein isolates (Paper 2).

All surimi gels obtained higher whiteness values than protein isolates when using fresh blue whiting. The measurements also showed a tendency toward higher whiteness for surimi gels made from acid processed isolates and lower whiteness values for surimi gels made from filtration-processed isolate.
When using frozen blue whiting raw material, the surimi gels processed were at the same whiteness levels, or, in two cases, at a lower level than the corresponding protein isolates. The surimi gels from fresh blue whiting showed higher whiteness values than surimi gels from frozen blue whiting.

Comparing the gel quality of surimi gels made of protein isolates processed with centrifugation and filtration show that gels made from centrifugation-produced isolates in most cases were stronger and more elastic than gels made from filtration-produced isolates. In Paper 1, this difference was significant while in Paper 2 it was not. Among tentative explanations for this finding could be the slightly lower protein content of surimi from filtration produced isolates caused by the higher lipid content of these isolates. When the surimi gels are made, the dry matter content of the isolates is used to determine the amounts of water and

cryoprotectants added to the surimi. Thus, with the same dry matter content in surimi derived both from the centrifugation and filtration processes, higher lipid content in the latter gives a lower protein content, which in turn reduces the gel strength. Another possibility is that small differences in the polypeptide pattern between these two types of isolates yields different gelation capacities. Using blue whiting (Paper 2) SDS PAGE-analyses revealed that slightly more dense bands at the very high MW-region (>>2000 kDa) were present in centrifugation derived isolates, especially during alkaline processing. It has been described that high MW-proteins like titin may affect visco-elastic properties of meat gels positively.

8. FUTURE OUTLOOK

This thesis provides insight into the opportunities for improving the protein yield in the processing of protein isolates for surimi production by applying a modified pH-shift process.

Interesting future studies on gel quality improvement could investigate optimal mesh size of filter screens, pressure techniques, and rheology enhancing additives, such as transglutaminase and carrageenan. The results of these investigations might well lead to revised processing methods in future surimi production.

The opportunities surrounding the use of frozen raw material in surimi production should be investigated more thoroughly. Moreover, applied research and development on advanced techniques to improve color should be conducted.

Another important aspect of the pH-shift method is the reduced discharge of organic material in the processing waste water. The possibility of running wastewater through a bio-filter in a pH-shift surimi process in a land-based pH-shift surimi plant should be investigated, beginning with a small-scale pilot test. In the long-term, this could result in cleaner surimi production, where recycling of the process water becomes routine and an industry best practice.

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Apendix 1. Examples of surimi specifications

(Source: Pascal Guenneugues, Oregon State University Surimi School) from Vidal-Girad and Chateau [1]

POLLOCK SURIMI

FACTORY TRAWLER

GRADE	Moisture	GEL STRENGTH		WHITENESS		Impurities
		GS	Depth	L*	b*	/40g
SA	75%	>1000	>1.40	>75	<4	<10
FA	75%	>900	>1.35	>75	<4	<14
AA	75%	>900	>1.30	>75	<4	<23
A	75%	>750	>1.25	>74	<4	<28
KA	75%	>600	>1.1	>72	<6	<35
KB	75%	>500	>1.0	>70	<10	<40
RA	75%	>400	>1.0	>70	<10	<60

SHORE PLANT

GRADE	Moisture	GEL STRENGTH		WHITENESS		Impurities
		GS	Depth	L*	b*	/40g
SA	75%	>1000	>1.30	>75	<4	<7
FA	75%	>900	>1.20	>75	<4	<14
AA	75%	>750	>1.15	>74	<4	<14
A	75%	>600	>1.05	>74	<4	<14
KA	75%	>500	>1.00	>72	<6	<23
KB	75%	>400	>0.9	>70	<8	<30
RA	75%	>200	>0.8	>70	<10	<40

PACIFIC WHITING

FACTORY TRAWLER

GRADE	Moisture	GEL STRENGTH		WHITENESS		Impurities
		GS	Depth	L*	b*	/40g
SA	75%	>1000	>1.25	>75	<4	<10
FA	75%	>900	>1.15	>75	<4	<14
AA	75%	>800	>1.10	>75	<4	<23
A	75%	>700	>1.05	>74	<4	<28
KA	75%	>500	>1.00	>72	<6	<35
KB	75%	>400	>0.95	>70	<10	<40
RA	75%	>300	>0.90	>70	<10	<60

SHORE PLANT

GRADE	Moisture	GEL STRENGTH		WHITENESS		Impurities
		GS	Depth	L*	b*	/40g
FA	75%	>900	>1.20	>75	<4	<14
AA	75%	>700	>1.10	>75	<4	<14
A	75%	>600	>1.05	>74	<4	<14
KA	75%	>400	>1.00	>74	<6	<23
KB	75%	>300	>0.90	>70	<8	<30
RA	75%	>200	>0.80	>70	<10	<40

NORTHERN BLUE WHITING

FACTORY TRAWLER

GRADE	Moisture	GEL STRENGTH		WHITENESS		Impurities
		GS	Depth	L*	b*	/40g
SA	75%	>1200	>1.50	>75	<5	<10
FA	75%	>1000	>1.40	>75	<5	<15
A	75%	>900	>1.30	>74	<6	<30
KA	75%	>600	>1.15	>72	<8	<40
KB	75%	>400	>1.00	>70	<10	<60

ITOYORI

THAILAND

GRADE	Moisture	GEL STRENGTH		WHITENESS		Impurities
		GS	Depth	L*	b*	/40g
AA	75%	>600	>0.95	>76	<10	<40
A	76%	>500	>0.90	>76	<12	<40
KA	76%	>300	>0.85	>76	<12	<40

INDIA

GRADE	Moisture	GEL STRENGTH		WHITENESS		Impurities
		GS	Depth	L*	b*	/40g
SSA	76%	>1000	>1.30	>78	<6	<40
SA	76%	>900	>1.20	>76	<6	<40
FA	76%	>750	>1.15	>75	<6	<40
AA	76%	>600	>1.10	>75	<8	<40
A	76%	>400	>1.05	>75	<8	<40
KA	77%	>300	>1.00	>74	<10	<60

JACK MACKEREL

GRADE	Moisture	GEL STRENGTH		WHITENESS		Impurities
		GS	Depth	L*	b*	/40g
FA	75%	>600	>1.00	>72	<10	<15
A	75%	>400	>0.80	>70	<10	<20
KA	75%	>300	>0.70	>70	<12	<40
B	76%	>200	>0.60	>68	<14	<60