THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Protein isolation from herring (*Clupea harengus*) using the pH-shift process

Protein yield, protein isolate quality and removal of food contaminants

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Cover: TEM micrographs of minced herring fillet (pH 6.5) and protein isolate (pH 5.5),
by Annika Krona

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ABSTRACT

Herring (*Clupea harengus*) contain valuable proteins but is difficult to process into high-quality foods due to its small size and high content of bones, heme-proteins and lipids. Herring is among the most abundant fish species in the world, but is currently utilized largely for fish meal and oil production.

The work presented in this thesis has aimed at evaluating pH-shift processing as a method to isolate proteins from herring and thereby increase its potential as a food raw material. The pH-shift process solubilizes muscle proteins at low or high pH (pH ≤3 or ≥10.8) whereafter impurities can be removed and the solubilized purified proteins are precipitated near the isoelectric point (~pH 5.5). The focus has been the yield and quality of the proteins. Specific aims have been to investigate: i) possible differences between the acid and alkaline version of the pH-shift process, ii) the possibility to remove dioxins and PCBs, and iii) the effect of alkaline pH-shift processing on the microstructure, salt solubility and *in vitro* digestibility of the proteins.

The acid and alkaline versions of the pH-shift process performed similarly when applied to gutted herring, with protein yields of 57-59%. The protein isolates had significantly higher protein concentration and less ash and lipids than the gutted herring, and also a significantly improved color and a well-balanced amino acid profile. The two process versions isolated proteins with similar ability to form a gel, but the acid process version induced proteolysis. Furthermore, the pH-shift process was highly efficient at removing dioxins and PCBs from contaminated Baltic herring, which was correlated to the removal of lipids. The microstructure analyses of the alkali-processed herring proteins revealed a loose protein network, with no remaining myofibrillar structure. The salt solubility of the proteins was significantly decreased after processing, and this was mainly due to exposure to low pH (5.5) during precipitation of the proteins. Precipitation at pH 6.5 was therefore evaluated and resulted in higher protein salt solubility, less lipid oxidation and higher gelation ability of the proteins compared to precipitation at pH 5.5. Despite the changes in protein salt solubility and microstructure, the *in vitro* digestibility of the alkali-processed proteins precipitated at pH 5.5 remained the same as that of the herring raw material.

To conclude, pH-shift processing is a promising tool to isolate proteins from herring and other small pelagic fish species, resulting in high protein yield and an isolate with good gelation capacity, nutritional characteristics, and low content of lipophilic contaminants. Protein isolation using the pH-shift process therefore has the potential to enhance the value of small pelagic fish species and increase their use for human consumption.

Keywords: pH, acid, alkaline, solubilization, precipitation, herring, protein, dioxins, lipids, salt solubility, gelation, transmission electron microscopy, *in vitro* digestion

LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers:

I.   **Sofia Marmon** and Ingrid Undeland. (2010) Protein isolation from gutted herring (*Clupea harengus*) using pH-shift processes. *Journal of Agricultural and Food Chemistry*, 58 (19), 10480-10486.

II.   **Sofia Marmon**, Per Liljelind and Ingrid Undeland. (2009) Removal of lipids, dioxins, and polychlorinated biphenyls during production of protein isolates from Baltic herring (*Clupea harengus*) using pH-shift processes. *Journal of Agricultural and Food Chemistry*, 57 (17), 7819-7825.

III.   **Sofia Marmon,** Annika Krona, Maud Langton and Ingrid Undeland. Changes in salt solubility and microstructure of proteins from herring (*Clupea harengus*) after pH-shift processing. *Submitted manuscript.*

IV.   **Sofia Marmon** and Ingrid Undeland. Effect of alkaline pH-shift processing on *in vitro* gastrointestinal digestion of herring (*Clupea harengus*) fillets. *Submitted manuscript.*

Related publications not included in the thesis:

Helgi Nolsøe, **Sofia Marmon** and Ingrid Undeland. (2011) 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*, 5, p. 1-9.

Britt G Gabrielsson, Johannes Wikström, Robert Jakubowicz, **Sofia Marmon**, Nils-Gunnar Carlsson, Nina Jansson, Li-Ming Gan, Ingrid Undeland, Malin Lönn, Agneta Holmäng, Ann-Sofie Sandberg. (2012) Dietary herring improves plasma lipid profiles and reduces atherosclerosis in obese *low-density lipoprotein receptor*-deficient mice. *International Journal of Molecular Medicine*, 29 (3) p. 331-37.

CONTRIBUTION REPORT

Paper I: The author, Sofia Marmon (SM), participated in the study design, performed the experimental work, participated in evaluation of the results, and was responsible for writing the manuscript.

Paper II: SM participated in the study design, performed most of the experimental work, participated in evaluation of the results, and was responsible for writing the manuscript.

Paper III: SM was responsible for the study design, performed most of the experimental work, participated in evaluation of the results, and was responsible for writing the manuscript.

Paper IV: SM was responsible for the study design, performed the experimental work, participated in evaluation of the results, and was responsible for writing the manuscript.

ABBREVIATIONS

ACE	angiotensine I-converting enzyme
CLSM	confocal laser scanning microscopy
DH	degree of hydrolysis
DHA	docosahexaenoic acid
DNPH	2,4-dinitrophenylhydrazine
dl-PCB	dioxin like PCB
dw	dry-weight
EPA	eicosapentaenoic acid
GI	gastrointestinal
Hb	hemoglobin
LC	long chain
Mb	myoglobin
MDA	malondialdehyde
MHC	myosin heavy chain
PCB	polychlorinated biphenyls
PCDD	polychlorinated dibenzodioxins
PCDF	polychlorinated dibenzofurans
PDCAAS	protein digestibility corrected amino acid score
PUFA	polyunsaturated fatty acids
PV	peroxide value
TBARS	thiobarbituric acid reactive substances
TCDD	2,3,7,8-tetraklordibenzo-p-dioxin
TEM	transmission electron microscopy
TEQ	TCDD equivalents

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INTRODUCTION

The world fish stocks are exploited to a level where no increase in the catch can be expected in any near future. At the same time, a growing population experience food shortages and there is thus a need for high quality protein products. Many of the most abundant fish species of the world are small and rich in dark muscle, bones and fat. Due to these characteristics they are largely used for production of fish meal and oil, and not as much for direct human consumption. Examples are herring (*Clupea harengus*), anchoveta (*Engraulis ringens*) and sprat (*Sprattus sprattus*) [1].

The processing of small pelagic fish is associated with difficulties. The size makes total removal of bones difficult. The low post mortem pH and the high content of heme-proteins and lipids makes these fish especially susceptible to oxidation, which deteriorates the quality of the fish [2, 3]. An additional problem with these fish is the high pigmentation that lowers their market value. This is caused by the abundance of myoglobin, hemoglobin and melanin [3]. Further, a more geographically isolated problem is that herring and sprat from the Baltic Sea are restricted in their use due to contamination with dioxins and PCBs [4, 5]. Methods for processing small pelagic fish that counteract these difficulties and facilitate utilization of their valuable proteins have been missing.

A promising method for protein isolation from small pelagic fish is the pH-shift process, which was developed in the late 1990s. The process uses high or low pH to solubilize muscle proteins and separate them from bones, scales, skin and a large part of the lipids by centrifugation. The solubilized isolated proteins are then precipitated by adjustment to a pH close to the isoelectric point (pI) of the proteins [6, 7]. Early studies of the process showed that protein yields from fish fillets were high. In addition, the isolated proteins had retained, and in some cases even had improved, functional properties after being subjected to the pH-shift process [6, 7]. The isolated proteins have also been reported to have lower lipid content compared to the starting material [6-8]. On the other hand, variations in the yield and quality of the proteins have been reported to depend on whether the acid or the alkaline version of the pH-shift process has been used. The process version that performs best seems to differ among raw materials [6].

The functional properties of proteins are a major interest as they affect the usability of the proteins in different food applications. Early studies on isolated cod (*Gadus morhua*) myosin showed the tertiary structure to be modified after pH-shift processing, and differently so after the acid and alkaline version of the process [9]. Structural changes, including increased hydrophobicity [10, 11], a decreased amount of sulhydryl groups [10, 12] and lowered salt solubility [13] have also been shown. The latter has often been used as an index of protein functionality, *i.e.* a low protein salt solubility has been related to inferior protein functionality [14, 15]. However, pH-shift produced protein isolates have

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shown a high gelation capacity in spite of a low protein salt solubility, and the reasons for this discrepancy have not been fully understood.

It is also of great importance for proteins to have good nutritional characteristics, *i.e.* having both a high content of essential amino acids, and a high digestibility [16]. An extraction of proteins such as in pH-shift processing discriminates between different proteins, and thus the amino acid composition of the protein isolate may differ from that of the raw material. Furthermore, structural changes in the proteins can result in an altered digestibility [17]. To elucidate possible differences in protein nutritional quality induced by pH-shift processing is thus highly relevant.

With this background, the work presented in this thesis has evaluated the possibility to apply the pH-shift process on gutted whole herring as a way to increase its direct use for human consumption. When the work was initiated, the pH-shift process had been applied only on fish fillets including herring light muscle [7, 18] and herring fillets [19], but no studies had been published on any more complex raw materials.

OBJECTIVES

The overall aim of the work presented in this thesis was to investigate the possibilities to use pH-shift processing to isolate functional protein from herring as a means to increase the usage of herring for food. Protein yield, qualitative aspects of the protein isolates and removal of lipophilic contaminants were focus areas.

Specific aims were:

- To evaluate the possibility to use such a complex material as whole gutted herring for pH-shift protein isolation (**Study I**)

- To elucidate differences in protein yield and quality between the acid and alkaline version of the pH-shift process when applied on whole gutted herring (**Study I**).

- To investigate the potential for the pH-shift protein isolation process to separate dioxins, PCBs and lipids from herring proteins (**Study II**).

- To study the effect of the alkaline version of the pH-shift process on protein microstructure and salt solubility, in relation to protein gelation characteristics (**Study III**).

- To establish whether a higher precipitation pH could be used to minimize the decrease in salt solubility observed after alkaline pH-shift processing, and furthermore investigate the effect of this higher pH on protein yield and protein isolate characteristics (**Study III).**

- To investigate whether pH-shift protein isolation affects the degradation of herring proteins during a static *in vitro* gastrointestinal (GI) digestion (**Study IV**).

BACKGROUND

Fish resources

Fish is man's single most important source of high-quality protein [20]. In addition to proteins, fish contain important fatty acids, vitamins and minerals [21]. The fishing industry increased until the 1970s and has since leveled off. In recent years, the annual world catch of wild fish and invertebrates has stabilized around 90 million tons. This amount cannot be foreseen to increase as only 15% of the world's monitored fish stocks are considered utilized such that the catches may increase from current levels. Over 30% of the world fish stocks are considered overexploited [1]. The production of fish from aquaculture has on the other hand followed an increasing trend, and was in 2009 estimated at 55 million tons.

Of the world's total fish production, about 80% is directed towards food while the rest is used mainly for fish meal and oil production, as well as for pharmaceutical use, bait, ensilage, animal feed *etc.* [1]. During automated filleting about 30-40% of the fish is turned into fillets while the rest is processing discards or by-products [22]. There is also a problem with unreported by-catch and discards, which is estimated at an additional 7 million tons per year [1]. The discards are generally hauled back into the ocean, dumped inland or burned [23]. Considering the large amount of fish being used for purposes other than human consumption, the discarded fish and the amount of fish by-products, there is great room for improvement in the usage of fish proteins.

Small pelagic fish rich in dark muscle

Some fish species are underutilized in the sense that they could be further used for human consumption. Such fish is often from the group of small pelagic fish that live in schools and are present in large abundance. Examples are anchoveta (*Engraulis ringens*), herring (*Clupea harengus*), blue whiting (*Micromesistius poutassou*) and chub mackerel (*Scomber japonicus*), all on the top ten list of most fished species [1]. However, due to their size they are hard to fillet, and many small bones are included in the fillet. Many, but not all of these fish (*e.g.* anchoveta, herring and chub mackerel but not blue whiting), are also rich in dark muscle. Although a simplification, in this thesis, the expression "small pelagic fish" will refer to the group of small pelagic fish that are rich in dark muscle. Due to above mentioned process difficulties and to consumer attitudes, these fish species are to a large degree used for fish-meal and oil production [1]. In addition to their small size and many bones, these fish have a high lipid content (often with large seasonal variations) and a high heme protein content, often leading to problems of both colour and rancidity of the final products. Small pelagic fish also often reach a low post mortem pH [24], leading to increased lipid oxidation [25], lowered water holding capacity [26], and thus to poor quality. Additionally, belly bursting sometimes occurs in some of these fish, which negatively affects the quality [27].
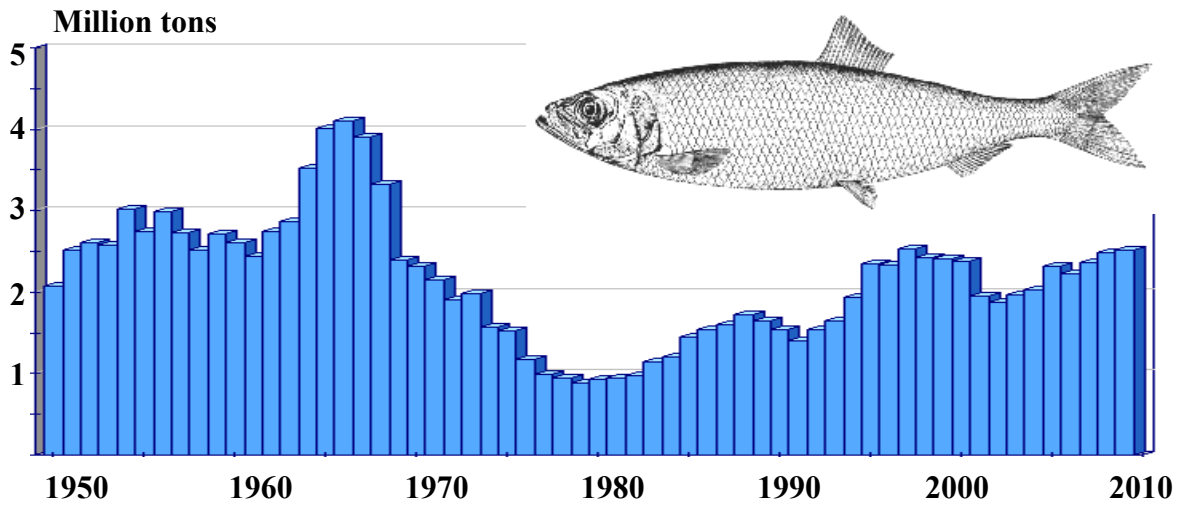
Herring

Figure 1. Herring world catches 1950-2009 [28] and Atlantic herring, drawing by H.L. Todd. [29]. Both reproduced with permission by the Food and Agriculture Organization of the United Nations.

Herring (**Figure 1**) is a small pelagic fish rich in dark muscle. Its lipid content shows large seasonal and geographical variations, and ranges from 2% to over 20% [30-32]. Herring lives in large schools in the northern part of the Atlantic Ocean, as well as in the Baltic Sea and the Barents Sea [29]. It can be up to 45 cm long (usually 20-25 cm), weigh 700 g and live for over 20 years. It feeds mainly on plankton such as copepods and is in turn preyed upon by many kinds of larger fish, such as cod (*Gadus morhua*), salmon (*Salmo salar*) and mackerel (*Scomber scombrus*) [29, 33]. Herring is one of the most abundant fish species in the world. In 2008, herring was the third most caught fish, with a catch of 2.5 million tons [1]. The amount of herring landed in Sweden 2011 was ~60,000 tons, of which approximately 50% were directed toward human consumption [34]. The proportion of the herring catch used for human consumption varies considerably between fisheries and countries, and *e.g.* where herring is caught as part of the roe industry the discards of males and spent females can be high. Several of the herring stocks are fully exploited, many are well managed, but some stocks are depleted [1].

Fish muscle

Based on weight, about 50-65% of the fish are muscle [35]. The main constituents in fish muscle are proteins (16-21%), lipids (0.2-25%), ash (1.2-1.5%) and water (58-82%). The amount of carbohydrates is low, normally below 0.5% [36]. The combined content of lipid and water is generally constant, and when the lipid content is increased, the water content is lowered and *vice versa* [37]. Depending on *e.g.* fish species and stress level of the fish [26], the post mortem pH of fish muscle varies from 6.8-7 (*e.g.* cod and flounder) and 6-6.2 (*e.g.* mackerel and bluefish) [24].

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Fish muscle consists of dark (slow) and light (fast) muscle which, in contrast to in mammals, are well separated. The light muscle constitutes up to 99% of the fish muscle depending on species [38]. It is mainly responsible for rapid movements as those used to attack a prey or flee a predator. The main energy source for the light muscle is glycogen, which is metabolized anaerobically [36]. The dark muscle cells are narrower than the light muscle cells. They follow the lining of the fish, are responsible for slow constant movement such as cruising, and are abundant in schooling fish that move large distances. The proportion of dark to light muscle increases with the fish swimming activity [36]. For example, herring fillets contain around 20% dark muscle [39]. Dark muscle uses aerobic
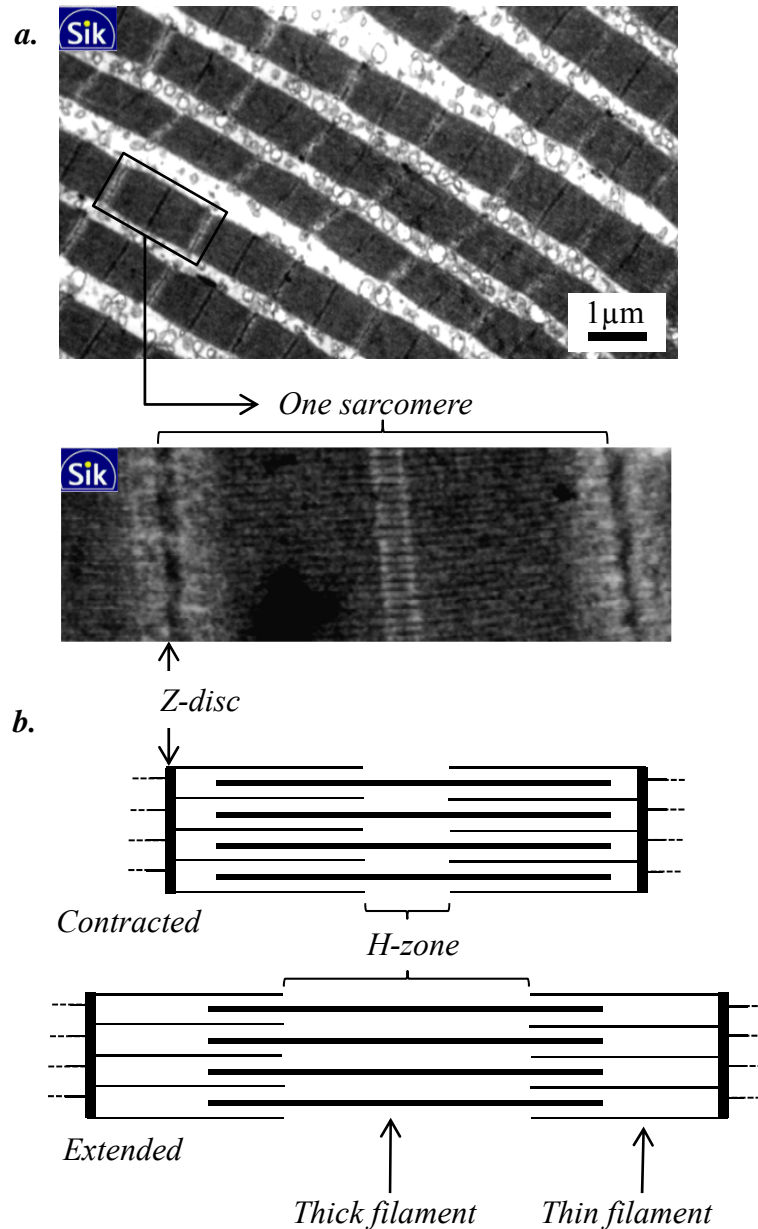

Figure 2. The fish muscle sarcomere (longitudinal view)
a. TEM micrographs of herring muscle.
b. Representation of contracted and extended sarcomere.

respiration, with lipids as the energy supply. The dark muscle is therefore rich in lipids, lipases, blood capillaries and myoglobin (Mb). Light muscle on the other hand contains very little Mb even in dark-fleshed species such as mackerel [40]. Further, herring has different isoforms of myosin in its light and dark muscle (about 200 and 210 kDa, respectively) [41].

There are three main groups of proteins in fish, first, the *myofibrillar proteins*. They are the structural proteins that build up the contractile unit (sarcomere) of the myofibril (the muscle fiber). They are the most abundant proteins, making up between 70 and 80% of the total proteins. The second protein group is the *sarcoplasmic proteins*, such as myoalbumin, globulin, heme proteins, parvalbumin and enzymes. This protein fraction is about 20-30% of the total proteins. Third is the *stroma proteins*, mostly collagen and other connective tissue proteins, that only constitute about 3% of the muscle proteins in fish [36, 42]. These three protein groups can be separated by their different solubility properties in salt solution. At a neutral pH, sarcoplasmic proteins are soluble in pure water and at low ionic strengths, while myofibrillar proteins are soluble in pure water and at higher salt concentrations (0.5-1 M) [14]. The stroma proteins are insoluble regardless of ionic strength [43].

Myofibrillar proteins

The myofibrils are built up of many different proteins, with myosin and actin being the two major ones [44]. Myosin is the most abundant of the structural proteins and is the most important protein in gel formation and water holding [43]. It consists of two large subunits (myosin heavy chains, MHC, around 200kDa) and four smaller (light chains, about 17-20 kDa). The MHC has one globular "head" and one fibrous "rod" each, and the rods are bound together in a coiled coil structure [45]. The heads contain binding sites for actin and active sites for ATPase [46]. In the sarcomere (**Figure 2**), myosin molecules are bound together forming the thick filaments. Actin monomers are globular (G-actin), and the monomers can build up filaments (F-actin), as in the thin filaments of the sarcomere [47]. *In vivo*, the myosin head binds the actin filaments and uses ATP to bend its head and contract the sarcomere [47]. *Post mortem*, actin and myosin bind tightly to each other and form actomyosin [43]. Tropomyosin together with troponin are important proteins for the regulation of sarcomere contraction and relaxation [43]. Other important structural proteins in muscle are: titin, a very large protein that is important for the structural arrangement of thin and thick filaments; nebulin, which is found in association with the thin filaments; α-actinin, which is part of the Z-disc and acts as an actin-binding protein; desmin, which is part of the Z-disc; and myomesin which acts as a scaffolding protein for actin and myosin [48].

Sarcoplasmic proteins

The sarcoplasmic proteins consist mostly of enzymes involved in different parts of the cell metabolism. The enzymes include proteinases and peptidases, such as the cathepsins,

which are important during processing of the fish since they can cause protein degradation and *e.g.* softening of the fish tissue [49].

Two sarcoplasmic proteins worth mentioning further due to their involvement in fish quality and relevance for human health is Mb (myoglobin) and parvalbumin. Mb is an iron-containing protein that binds oxygen. It functions in the muscle by receiving oxygen from the blood and storing it for use in aerobic respiration [50]. Parvalbumin is a small (~12 kDa) heat-stable protein involved in calcium-signaling. This is the proteins that most of the persons allergic to fish are sensitive to [51].

Sarcoplasmic proteins have long been thought to act adversely on protein gel formation [52, 53]. However, the field is now divided since several researchers have shown sarcoplasmic proteins to instead increase gel strength [54, 55], and the topic has recently been reviewed without solving the question [53].

Lipids

Fish contains two types of lipids: neutral lipids and polar lipids. The neutral lipids, mostly triglycerides, are generally found as depot fat while the polar lipids, mostly phospholipids, are the main constituents of the cell membranes [56, 57]. The amount of triglycerides can differ greatly between fish types. Lean fish such as cod contains mostly membrane fat, about 0.5-1% (w/w), which thus consists mainly of phospholipids. Fatty fish, like herring, has roughly the same amount of membrane fat, but in addition a large amount of neutral fat [56, 58]. Not only the amount, but also *where* the depot fat is situated differs between fish species. All fish have more lipids in their dark muscle than in their white muscle [59]. Gadoid fish species, *e.g.* cod and haddock, have most of their neutral storage lipids in the liver [56]. Fatty fish such as herring and salmon have neutral lipids as droplets in the muscle, especially in the dark muscle close to the mitochondria, and in the connective tissue close to the muscle cells and in the belly flaps [42].

The fish lipids differ from mammalian, avian and plant lipids in being richer in long-chain poly-unsaturated fatty acids (LC-PUFA), especially from the n-3 (omega-3) family. The two most common n-3 PUFA in fish are EPA (20:5 n-3) and DHA (22:6 n-3,) [60]. These have gained a great deal of attention due to their positive effect on human health [21, 61, 62]. The PUFA are however easily oxidized due to their high content of double bonds [60, 63]. Fish also has a high abundance of mono-unsaturated fatty acids [59]. Herring specifically contains significant amounts of gondoic acid (20:1, n-9) and cetoleic acid (22:1 n-11) due to their consumption of copepods [64].

Low molecular weight substances

Low molecular weight substances of fish muscle include for example important anti-oxidative compounds such as ascorbic acid [65], glutathione and uric acid. The low molecular weight pool of the muscle also contains pro-oxidants such as iron and copper ions and free heme [66].

Fish muscle also contains a significant amount of non-protein nitrogen compounds, such as free amino acids, nucleotides, peptides and urea. These compounds are important since they contribute both to sensory properties and muscle deterioration [52]. Fish is noteworthily high in the free sulfonic acid taurin [52], thought to have a positive effect on human health [67]. Fish rich in dark muscle, especially fish of the *Scombridae* family such as tuna and mackerel, can also have a high content of the free-amino acid histidine, Histidine can be decarboxylated to histamine, which can trigger allergy-like symptoms if consumed in high amounts [52, 68].

Blood and bones

Blood and bones are not part of the muscle cell but are integral parts of fish fillets from small pelagic fish. The blood contains white and red blood cells, the latter of which has a high concentration of the protein hemoglobin (Hb) used to transport oxygen [69]. Hb is a tetrameric protein in which each subunit holds a heme group containing iron. Hb is of great relevance for the quality of fish products, since it both promotes oxidation and affects the color of the product [40]. Bones are rich in minerals such as calcium.

Factors affecting utilization of small pelagic fish

Pigmentation

The color of a fish product is often of great importance for the consumer and affects the price. White fish achieves the highest prices on the world market together with some colored fish species such as tuna and salmon [70]. Consumers often consider brownish and grey colors to be unappealing. This kind of pigmentation of fish is therefore considered a problem. By-products and whole fish contain more pigments than fillets. The reason for this is that viscera and backbone *etc.* contain considerable amounts of blood, and thereby Hb, as well as melanin [23]. The color of both Hb and Mb depends on the chemical state of their heme-iron (*i.e.* ferric or ferrous), which also affects their role as pro-oxidants. The type of ligands bound to the heme and the state of the globin protein also influence the color [71].

Melanin is a dark brown to black pigment found in eyes, skin and peritoneal lining of some fish species (*e.g.* herring). It sometimes forms non-harmful, but unappealing, black spots on crustacean species [72]. This pigment might also negatively affect the color of fish-products. Melanin is the same pigment as the one responsible for brown skin color in humans. It is produced by a multi-step reaction from tyrosine via enzymatic oxidation and polymerization to produce the polymer of melanin [72].

A third type of pigmentation in muscle products can come from non-enzymatic browning. This is a phenomenon partially caused by Maillard reactions [73]. In fish, a similar reaction can occur when lipid oxidation products interact with free amino groups of proteins or phospholipids [74, 75].

To remove different unwanted pigments, physical methods such as washing can be used [76]. Both Mb and Hb can be partly removed from fish-mince with that method. The Hb content can also be reduced from large fish by bleeding [40, 77]. Processes to bleach the fish with hydroperoxide also exist [78] as well as coloring of products with *e.g.* titanium dioxide [79].

Lipid oxidation

Rancidity is one of the main reasons why fish and other seafood can become unacceptable to the consumer [80]. Rancidity is the result of lipid oxidation and is sensed primarily as a bad smell. The odor is often described as "painty", "fishy" or "metallic", but texture and color changes can also be a result of lipid oxidation [23]. Fish is especially sensitive to rancidity since, compared to other food, it contains high amounts of n-3 LC-PUFA with up to six double bonds [80]. The latter makes PUFA more susceptible to oxidation than more saturated lipids.

Oxidation of lipids is a complex process, leading to a vast variety of oxidation products as well as lower levels of n-3 PUFA and anti-oxidants. Details on the lipid oxidation process are beyond the scope of this thesis. In short, the process follows a free radical mechanism and therefore, once started, propagates and catalyzes further oxidation of lipids and proteins, including Hb. The reaction can be divided into three main steps. *Initiation* – where radicals are formed, often by interaction of lipids with different active oxygen forms or with transition metals. *Propagation* – where the lipid free radical reacts with oxygen to form a peroxy radical which then reacts with an intact fatty acid, producing new lipid radicals and lipid hydroperoxides. After this step, chain breaking can happen, where lipid hydroperoxides are cleaved to volatiles and new radicals by low molecular weight (LMW) metals or heme. *Termination* – this is the step where two radicals are combined into a new stable compound [81, 82]. The lipid oxidation reaction is thus catalyzed by many different compounds including heme components, LMW trace elements and enzymes such as lipoxygenases. The most important metal ions involved in lipid oxidation are Cu^+, Fe^{2+} and Fe^{3+} [75, 83, 84]. Lipid oxidation is also affected by temperature, light, water activity, pH and chemical environment, such as the level of oxygen present [57, 81].

Lipid oxidation is generally a greater problem in small pelagic fish than in white fish such as cod and Alaska pollock (*Theragra chalcogramma*). The concentration of lipids and the pro-oxidants Hb and Mb is, as earlier mentioned, much higher in the muscle of pelagic fish, especially the dark muscle. The lower post mortem pH found in small pelagic fish also promotes lipid oxidation [24]. At a lower pH, Hb de-oxygenation (the Bohr effect) and Hb autoxidation that leads to the formation of metHb (contains Fe^{3+} instead of Fe^{2+}) are increased [25]. Deoxygenized Hb and MetHb are more prone to induce oxidation than reduced oxy-Hb [85]. MetHb also more easily loses its heme-groups which are hydrophobic and easily enter into the lipid-membranes where they can catalyze the oxidation [86]. Mb is thought to act in a similar fashion as Hb [50].

Protein oxidation

Oxidative processes may alter the structure of proteins, and protein and lipid oxidation often occurs simultaneously [87, 88]. Protein oxidation is thought to follow a free radical mechanism similar to that of lipid oxidation, and also produces a large variety of end products. Protein oxidation can be induced and catalyzed by *e.g.* heme-compounds and iron [88]. The most common results of protein oxidation are protein cross-linking, amino-acid side chain modifications and protein fragmentation [88]. Formation of carbonyl compounds is a common amino acid side chain modification that is often studied and used as a measure of protein oxidation. Loss of sulfhydryl groups and subsequent formation of S-S crosslinks within or between proteins can result in changed functional properties such as lowered water holding capacity of the proteins [89, 90]. Oxidation may also lead to covalent bonds between proteins and lipids, altering their functionality [91].

Lipophilic toxins

FAO/WHO has recently stated that in most cases the benefits of fish consumption is greater than the risks from contaminants present in the fish [21]. However, there are cases where the risks may not be negligible, and these include consumption of fish with a high content of dioxins, especially during pregnancy [21].

Among the most well studied lipophilic toxins are the dioxins, *i.e.* polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), and PCBs (polychlorinated biphenyls). They are persistant organic pollutants. Human exposure to dioxins and PCBs is over 90% through food, with fish as one of the main food classes [92].

Dioxins and a subgroup of the PCBs called dioxin-like (dl-PCBs) bind to the aryl hydrocarbon receptor, which then mediates an array of responses resulting in negative effects on the central nervous system, hormone levels, immune defense and reproduction rate [93]. High levels are also thought to cause cancer [94]. In humans, the developing fetus is likely to be at the greatest risk following exposure [21, 92].

The TEQ concept

There are 75 congeners with different chlorination patterns for PCDD and 135 for PCDF. Further, there are 209 congeners of PCB, of which some are considered dl-PCBs. The differences between the congeners give them different toxicity. To handle these different toxicities during risk evaluations *etc.*, a concept called TCDD (2,3,7,8-tetraklordibenzo-*p*-dioxin) equivalents has been developed. TCDD is the most toxic of the congeners and the toxicity of the other congeners is expressed as fractions of that. The total toxicity of the different congeners is added together to get a TCDD-equivalent (TEQ) [93]. This concept is based on the assumption that all dioxins act through the same mechanism in an additive manner [93]. The toxicity of dl-PCBs is expressed in the same, or a separate, TEQ value. The general structures of PCDD, PCDF and PCB are shown in **Figure 3**.

TEQ limits within EU

According to the recently updated EU commission directive [5], fish shall not contain more than 6.5 ng TEQ dioxins and dl-PCBs per kg fresh weight, and not more than 3.5 ng TEQ per kg of dioxins alone. The Baltic Sea is a problematic area with high contamination levels, and fatty fish from this region, such as herring and salmon, sometimes exceeds these values. Generally, Baltic herring smaller than 17 cm have TEQ values below EU limits

Figure 3 . General structure of polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs). Numbers, except (5), indicate different possible chlorination sites.

[95, 96]. Even though releases of dioxins into nature have decreased, the levels in herring have not been seen to be significantly lowered compared to in the 1980s, while the PCB levels are slowly decreasing [96]. Fish exceeding the EU values are not allowed to be sold inside EU but Sweden and Finland have an exception from the EU directives [5]. Fish with high TEQ values are however only allowed to be sold on the domestic market or to non EU-member states. The exception is allowed since Sweden and Finland have detailed recommendations on how often to eat contaminated fish [97]. The dioxins in Baltic fish are continuously measured by different national programs and independent scientist groups.

In addition to direct human exposure to dioxins, high levels of dioxins in animal feed (*e.g.* as fish meal and oil) can theoretically be a problem to human health. Since these contaminants accumulate in fatty tissue, feed-derived dioxins and can result in high levels of dioxins in the muscle or milk of the farmed animal, which then may pose a risk for humans. As an example of this, concentrations of dioxins and dl-PCBs have in some cases been higher in farmed than wild fish due to contaminated feed [98, 99].

Dioxin removal

Different approaches to remove dioxins from fish and fish products have been evaluated. Since dioxins and PCBs are very lipophilic, methods that remove fat generally lower the TEQ-value. Since the fat of the fish in unevenly distributed, removal of fatty parts can be effective. For example removal of skin reduces the weight of herring fillets by 10-12% and the fat content of about 23% [100]. During cooking of the fish, the dioxins may be further removed together with fat that is released from the fish. The reduction observed when cooking fish has been proportional to the loss of lipid [101].

To minimize the negative impact of fish meal and oil on human health, methods have been developed to remove dioxins and PCBs from these products. For fish meal, methods that minimize lipid content by solvent extraction are generally used although degradation
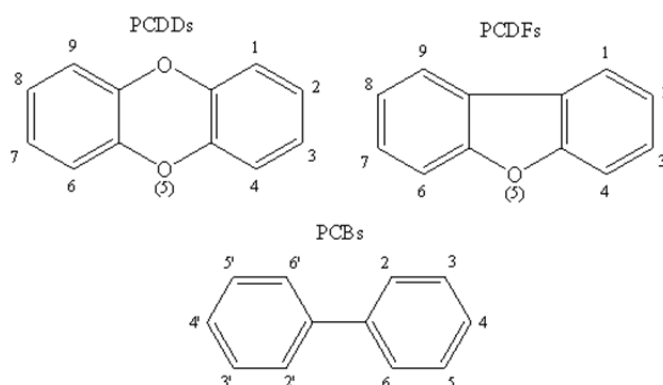
methods using UV-light [102] and enzymes (oxidoreductase) [103] have also been tested. Removal of dioxins from fish oil has been done using short path distillation [104] or solid phase extraction using active carbon, the latter either alone [105, 106], or combined with supercritical CO_2 extraction [99].

Current uses of small pelagic fish for human consumption

Small pelagic fish are to a certain degree used directly for human consumption; fresh, frozen or marinated. Marinated herring products are particularly popular in the Scandinavian countries. Some small pelagic fish are also used for roe production. To set the pH-shift protein isolation method into perspective, two other processing methods that can be used to upgrade small pelagic fish into foods or food ingredients, as compared to fish meal, are shortly summarized below: production of surimi and protein hydrolysates. It should though be stressed that it is only the second of these that can be applied directly onto whole fish or by-products. Surimi production requires a pre-filleting step or other mechanical separation where the meat is separated from skin and bones. The latter can be done with deboning equipment such as where the raw material is pressed through perforations of a rotating drum. However, small bones, cartilage and skin may not be fully removed [6].

Surimi

Surimi is an intermediate frozen product that is further used for processing into gelled products. The gel is formed by the myofibrillar proteins after heating. Surimi is used in traditional Japanese "kamaboko" [107] and the market for surimi products is huge, especially in Asia. The most common form of surimi product in Sweden is "crab stick". The cold temperature during surimi processing together with addition of cryoprotectants before freezing enables the proteins to avoid denaturation and thus to retain their functionality. However, as mentioned above, surimi processing is only suitable for bone-free minces such as from fish fillets. [107, 108].

In conventional surimi production, the fish is headed, gutted and deboned after which the muscle is minced. The fish mince is then washed with water or a slightly alkaline solution to get rid of sarcoplasmic proteins and other water soluble compounds. Traditionally three washes with a mince:water ratio of 1:5 to 1:10 have been used [109]. However, research has been done to minimize the waste water, and two to three washings with a lower water ratio, typically 1:3, are now common [108]. The washed fish mince is dewatered and mixed with cryoprotectants, most commonly sucrose and sorbitol. The surimi is then frozen in blocks and ready for further processing [109].

Fish used for surimi-production is traditionally cold living white fish species such as Alaska pollock and Pacific whiting (*Merluccius productus*). Some tropical species such as threadfin bream (*Nemipterus virgatus*) and pelagic species such as jack mackerel (*Trachurus murphi*) are also used [110]. However, the surimi quality is generally lower when including species rich in dark muscle, whereas specific removal of the dark muscle

increases the quality [2, 111]. Several reasons for this have been discussed, including dark muscle having a higher content of lipids and sarcoplasmic proteins [2]. Surimi from mackerel light muscle has been shown to have a similar quality as surimi from Alaska pollock [3].

Fish protein hydrolysates

Protein hydrolyates can, as mentioned above, be produced from complex raw materials such as whole fish. In a hydrolysate the proteins are enzymatically or chemically hydrolyzed to smaller peptides (*i.e.* peptide bonds are cleaved). Hydrolysates have a high solubility in water, typically 90-100%, but have lost other functional properties such as the proteins' ability to form a gel. The good solubility makes hydrolysates interesting in certain food applications. One problem however is the bitter taste commonly found for protein hydrolysates [112]. The bitterness is coupled to the overall hydrophobicity of the peptide, *i.e.* the amino acid composition of the peptides. The size of the peptides also affects bitterness and only peptides of a size below 6 kDa can be sensed as bitter [113]. Limited and extensive hydrolysis typically results in less bitterness than intermediate hydrolysis [114].

Furthermore, protein hydrolysates are of interest since bioactive functions have been found for a large amount of peptides. Most research on the bioactivity of peptides has been performed using milk protein hydrolysates. Studies on fish protein hydrolysates have shown that fish derived peptides can have important features such as ACE-(Angiotensine I-converting Enzyme) inhibitory and antioxidative effects [115, 116]. Protein hydrolysates can be made chemically, with acid or alkaline hydrolysis, or enzymatically with either endogenous or added enzymes [114]. Fish protein hydrolysates made using endogenous enzymes (*i.e.* autolysis) are an old product and have been made for thousands of years (*e.g.* fish sauce).

The pH-shift method for protein isolation

The pH-shift protein isolation method, also called the acid or alkaline solubilization and precipitation method, or isoelectric solubilization/precipitation method, was invented and patented in the late 1990s/early 2000s [6, 7, 117, 118]. The method is based on the differences in solubility that muscle proteins in water exhibit at different pH values. It has the advantage over surimi-production that it can be applied to complex starting materials such as whole fish or fish by-products [8]. Compared with fish meal or hydrolysate production it has the advantage that the end product contains whole, functional proteins that may be used for food applications. The method, as generally performed in the laboratory scale, is stepwise summarized in **Figure 4.** In the pH-shift method, the muscle source is first mixed with water to obtain a medium in which to solubilize the proteins

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Figure 4. *Overview of the pH shift process as it is generally performed in the laboratory.*

(step 1, homogenization). The proteins are homogenized to increase the interaction surface between the proteins and the water. To avoid heat-induced denaturation and enzymatic degradation of proteins, the whole pH-shift process is performed at cold temperatures, with a maximum temperature of 10°C [117]. At physiological pH and ionic strength, the insoluble myofibrillar proteins are held together in aggregates. Both sarcoplasmic and myofibril proteins also interact with the water through polar amino acids. At physiological pH, most muscle proteins are negatively charged. By reducing the pH the proteins first become neutral and then positively charged by hydrogenation of *e.g.* glutamate and aspartate (**Table 2**). In a similar way, the proteins become more negatively charged by addition of base and subsequent dehydrogenation of *e.g.* cysteine, tyrosine and lysine (**Table 2**). The overall increased protein charge results in an increase of protein-water interactions. Similarly charged amino acids on the proteins also repel each other, causing the individual proteins to separate from each other. In this way they attract more water, and partly unfold, becoming more soluble (Step 2, solubilization) [9]. On the way towards solubilization, the protein aggregates swell by the water attraction, and the viscosity of the solution is drastically increased. Attracting enough water, most of the

Table 1. *Some definitions of high relevance for the pH-shift process.*

Concept	Definition
pH	The pH is a measure of the concentration of hydrogen ions, H^+, and is defined as $pH = -log[H^+]$
pI	The isoelectric point. The pH at which a molecule contains no net charge, *i.e.* the number of positive charges equals the negative
pK_a	The inversed logarithm of the dissociation constant, K_a, The pH at which *e.g.* an acid, base or functional group is half dissociated, and the different forms exist in equal amounts

muscle proteins become fully soluble and the viscosity is again lowered [18]. Under these conditions, the proteins can be separated from insoluble matter such as bones, scales, some insoluble proteins and neutral lipids (depot fat) using *e.g.* centrifugation [117, 118]. Including a high centrifugation force step at this point has been shown to also remove parts of the cellular membrane phospholipids [119]. After centrifugation, a three phase system is observed (Step 3, separation): a sediment in the bottom containing insolubles *e.g.* bones, scales and parts of the phospholipids; the major phase of solubilized proteins; and, depending on the fat content of the muscle source, a floating fat (emulsion) layer. Sometimes also a fourth gelatinous layer is formed on top of the bottom sediment which is thought to be a mix of solubilized and non-soubilized proteins [2]. The purified proteins that remain in solution are then adjusted to a pH close to the pI of the muscle proteins, generally between pH 5 and 6 (step 4, precipitation) [14, 121]. During this pH adjustment, unfolded proteins start to refold, although not necessarily to their native conformation. At the pI, there is a minimum of interaction between water and proteins. Salt bridges are also thought to form between amino acids of opposite charge, and a vast majority of the muscle proteins precipitate at this pH. The precipitated proteins, called protein isolate, are generally collected from the solution via a second centrifugation (Step 5, isolation).

The field of pH-shift protein isolation has during the time of this work changed from the first published studies on fish fillets and purified proteins to be used on complex raw materials that are much more demanding. Currently, in addition the whole gutted herring studied in this thesis, complex aquatic raw materials that have been used for pH-shift protein isolation include whole Antarctic krill (*Euphausia superba*) [122], rainbow trout (*Oncorhynchus mykiss*) by-products [123], whole gutted silver carp (*Hypophthalmichthys molitrix*) [124], shrimp processing discards [125] and surimi wash water [126].

Table 2. *pKₐ values of acid and basic amino acids (free form) and protein terminal groups [120]. The absolute pKₐ value of an amino acid side chain is dependent on the protein.*

Amino acid	pK_a
Aspartic acid	3.65
Terminal COOH	3.75
Glutamic acid	4.25
Histidine	6.00
Terminal NH_2	7.8
Cysteine	8.18
Tyrosine	10.07
Lysine	10.53
Arginine	12.48

Protein yield

One of the most important factors for evaluating the pH-shift process is the protein yield. Generally, pH-shift processing gives a high protein yield compared to conventional surimi processing [6] which is linked to the retention of sarcoplasmic proteins and the fact that all parts of the fish muscle can be utilized. According to a recent review [6], protein yields of 55-80% are common, generally with the acid version of the pH-shift process giving somewhat higher yield than the alkaline version. Many factors affect the exact protein yield obtained during the pH-shift process with muscle source (*e.g.* species, freshness and the use of fillets vs. whole fish), water addition, separation technique(s) and pH values used for solubilization and precipitation being important ones. These factors can affect the protein solubilization (high solubility desired), the size of sediment and floating layer following the first separation step (low size desired) or the protein precipitation (low protein solubility desired).

Solubilization and precipitation pH

The solubilization pH is of high importance for the protein yield. Generally a more extreme pH value leads to higher protein solubility. However, at very high and low pH-values this increase in protein solubility then comes at a cost of higher use of acid and base, and also possibly an increase in structural changes of the proteins. It is also of importance to reach a pH value were the viscosity of the protein solution is low, as high viscosity impairs the separation during centrifugation. Typically the viscosity increases at the pH-ranges where there are the greatest changes in protein solubility. Once beyond these pH values, the viscosity is generally lowered [18].

While the solubilization pH is chosen as a balance between protein solubility and other factors, the precipitation pH has in contrast been chosen solely based on where the solubility is lowest, which is close to the pI of most muscle proteins. This pH is not optimized in many studies, and pH 5.5 is then used.

The structural changes induced during subjection to acid or alkaline pH may modify the solubility of the proteins. After alkaline or acid solubilization, sarcoplasmic proteins obtained lower solubility at both pH 5.5 and 7 compared to native sarcoplasmic proteins. This increases the protein yield during pH-shift processing. The acid version of the pH-shift process further reduced the solubility at pH 5.5 and 7 compared with the alkaline process version [127]. Sarcoplasmic proteins have also been found to co-precipitate together with myofibrillar proteins at pH 5.5, leading to a higher retention of sarcoplasmic proteins in the protein isolate [128].

Water

The amount of water added in the pH-shift process is important for the protein yield. The protein concentration affects the viscosity during solubilization and thereby the effectiveness of the first separation [2]. After the first centrifugation, the sediment and the floating layer contain considerable amounts of water. This trapped water contains

solubilized proteins, presumably at an equal concentration as in the supernatant. These trapped proteins are thus lost during removal of the sediment and floating layer [7, 18]. An increased water to muscle ratio leads to lower protein concentration, and thus a lower protein concentration in the water trapped in the sediment and floating layer as well. Thus higher water content leads to higher protein yield after the first separation in the pH-shift process. However, water is a limited resource that comes at a cost, and high water content also leads to more waste water at the end of the process. The amount of water used in the process is therefore a very important practical consideration. Reuse of the water has been suggested as a mean to minimize water usage [2].

Separation technique

Batchwise centrifugation is most commonly used in laboratory scale pH-shift protein isolation. A continuous mode has been proposed [22], but the effect on yield has not been evaluated. To avoid loss of proteins into sediments formed during the first centrifugation, trials have been done to switch the centrifugation step to a filtration step [129, 130]. Filtration as a separation technique during the first separation has led to increased protein yield [129, 130] but has also resulted in lower functionality [129] and less white color [130] of the retrieved proteins. Filtration in the second step of separation did not lead to any change in protein yield as compared to centrifugation [129]. It can be noted that these tests have been performed on fillets of white-fleshed fish. Filtration may thus be less useful for small dark-fleshed fish species or by-products due to the risk of lowered whiteness.

Effect of the pH-shift process on protein structure

A thorough study on purified cod myosin has been performed to investigate how the pH-shift method affect protein structure [9]. On the basis of the results, the authors proposed that the myosin head went in to a so called 'molten globular' state at alkaline pH, accompanied with dissociation of the light chains, and that the refolded head had a more loose structure than the native structure. A similar restructuring of the head occurred at acid pH, but the refolded structure was less loose than after the alkaline process. The coiled coil of the rods of the two myosin heavy chains also dissociated during acid pH, but that structure reformed during neutralization [9]. The anion used for acidification had no effect on myosin secondary structure but did have an effect on myosin gel rheology, indicating increased myosin head deformation. PO_4^{3-} gave the lowest deformation, SO_4^{2-} intermediate and Cl^- the highest deformation, which is in agreement with their different positions in the Hofmeister series. Also, lower solubilization pH increased myosin head deformation [131].

Several studies on whole muscle have shown that proteins subjected to pH-shift processing to have an increased surface hydrophobicity [10, 11], reduced ATPase activity [12], increased amount of reactive sulfhydryl groups [11, 132] and a reduced content of total sulfhydryl groups [10, 12, 133, 134]. Taken together, this shows that pH-shift

processing induces di-sulfur bridges and changes the conformation of proteins to reveal amino acids that are natively hidden in the interior of the proteins.

Conformational studies have also been made with sarcoplasmic proteins from striped catfish (*Pangasius hypophthalmus*) [127] and Hb [135, 136] following subjection to pH-shift processing. Sarcoplasmic proteins unfolded, revealing hydrophobic residues during alkaline solubilization, while, during acid solubilization, the surface hydrophobicity was thought to induce clustering of the proteins. The sarcoplasmic proteins also had a decrease in sulfhydryl groups, indicating formation of di-sulfur bridges during both acid and alkaline pH-shift processing [127]. Hb unfolded at acidic pH (pH 1.5-3.5), and more rapidly so at pH 1.5. This unfolding was not observed at pH 10 or 11, but to some degree at pH 12. These conformational changes were reflected in the ability of Hb to reform during pH-adjustment to 5.5 (*i.e.* larger changes during solubilization resulted in less ability to reform during neutralization). The acid process resulted in a lowered Hb solubility at pH 5.5, which was not seen after alkaline solubilization at pH 10 or 11 [135, 136].

Protein functionality

The ability of proteins to induce favorable characteristics to a food product other than nutritional values is called protein functionality. Protein functionality includes *e.g.* solubility in salt solution or water, emulsification, foaming, gelation and sensory properties. For protein isolates made with the pH-shift process the main functional property evaluated is gelation. The color of the protein isolates may also be regarded as a functional property and has been well studied.

During studies of gelation properties, the acid and alkaline versions of the pH-shift process have often been compared to each other and/or to conventional surimi production. Several different parameters of the gels produced have been compared including gel strength, deformability, elasticity, water holding capacity and color. In general the alkaline pH-shift process has produced stronger and more deformable gels than the acid pH-shift process [6]. Comparisons of the alkaline pH-shift process with conventional surimi production has yielded more varying results [6]. In the cases where protein isolates from the alkaline pH-shift process version has performed better than conventional surimi, this has been ascribed to the partial denaturation with increased surface hydrophobicity or S-S bridges, resulting in more protein-protein interactions and stronger gels [133, 137, 138]. The lower strength of gels made with the acid pH-shift version has been suggested to be due to proteolysis [12]. In relation to gelation, another effect of the pH-shift process is that the resulting proteins in some cases produced improved gels when no salt was added [139]. Salt addition is required to induce gelation of conventionally made surimi products. This feature thus gives the possibility for low-salt surimi-products.

The whiteness of gels made from protein isolates has generally been higher after the alkaline pH-shift version than the acid pH-shift version. However, conventional surimi production have often given even better results [6]. pH-shift processing of small pelagic

fish, as well as by-products, also led to improvement of color compared to the starting material. However, due to high pigment and blood content, the color of gels made from protein isolates from these sources has been regarded as poor with low whiteness values [8].

In addition to having effects on gel formation and color, pH-shift processing has been shown to increase the emulsifying properties of cod myofibrillar proteins [137] and threadfin bream sarcoplasmic proteins [140], as well as the foaming properties of chicken dark meat [132].

Lipid removal

One major advantage of the pH-shift protein isolation process compared to conventional surimi production is an increased removal of lipids. Lipids are removed with both the floating layer and the sediment during the first centrifugation. Reductions in lipid content of more than 80% have been reported following pH-shift processing [6, 134]. However, the exact reduction depends on the material's starting lipid content, fish species and the version of the pH-shift process used. Including a high *g*-force during the first centrifugation step ($\geq 10,000g$) have been shown to reduce the lipid content and also remove parts of the phospholipids, which are then found in the sediment [119].

Addition of $CaCl_2$ and citric acid during solubilization has been shown to reduce the phospholipid content in the protein isolate, since the membranes to a larger extent end up in the sediment after the first separation. The effect was presumably achieved by rupture of linkages between membranes and cytoskeletal proteins. [141, 142]. Addition of chitosan has also been shown to reduce the membrane lipid content [143]. However, both $CaCl_2$+citric acid and chitosan caused a lower protein yield [141-143].

Oxidation induced during pH-shift processing

Although lipids are removed during pH-shift processing, there are some lipids left in the protein isolate. Lipid oxidation products can develop in fish at a lipid content as low as 0.1% (wet weight) when blood-derived Hb is present [144]. Hb is partly dissolved into the supernatant of the second centrifugation during pH-shift processing, with more Hb remaining soluble after the alkaline version of the pH-shift process than after the acid version [145]. More Mb has also been shown to be removed during the pH-shift process than during conventional surimi production [76, 146]. Since the removal of neither lipids nor pro-oxidants is complete, there are still problems with lipid oxidation during the pH-shift process, particularly during the acid version of the process [19, 138]. The low pH promotes lipid oxidation through changes in the Hb molecule that then becomes more pro-oxidative [135, 136]. In addition, a pH of 5.5, normally used for precipitation of the proteins in both the acid and alkaline version of the pH-shift process, is acidic enough to make Hb more pro-oxidative [25]. Indeed, alkaline pH-shift processing of chicken dark muscle has been shown to increase the production of secondary oxidation products [147]. However, studies of white fish muscle have shown lower or equal amounts of secondary

lipid oxidation products in isolated proteins made with the alkaline pH-shift process compared to the fish muscle [138, 145]. In one of these studies, no oxidation was either induced during the acid pH-shift process [145]. Addition of anti-oxidants during production [19] and storage [148] of protein isolates has been found to be effective in inhibiting lipid-oxidation.

Uses of pH-shift produced protein isolates

The amino acid composition of fish suggests that protein isolates thereof can be used to fortify the nutritional value of foods that naturally have lower protein quality, such as cereal-based foods. It can also be used as a base for protein powders. Due to the good gelling ability of proteins isolated with the pH-shift method, especially the alkaline process version, most research has been done on using the proteins for surimi-like products. The good gelling abilities also suggest that pH-shift produced protein isolates can be used as a functional ingredient in other products. It has also been suggested that the proteins can be used for injection marinades, *i.e.* a solution that is injected into a fillet to increase the water holding capacity and thus the juiciness. A study comparing marinades with proteins solubilized at pH 2.5 or phosphates however showed phosphates to be superior to the proteins [149]. Some different companies produce protein isolates using the pH-shift method. One of them is ProteusIndustries that have a product called NutriLean®. They use the acid process to produce protein isolates as coatings for fried products. On their homepage they state that this product minimizes water loss during frying, thereby giving a fried product with lower fat content (25-75%) and better sensory properties than with normal coating [150]. There are also some other companies in North America and Iceland: MPF, MPF Iceland and Iceprotein, that are using the pH-shift protein isolation process, presumably working mainly with the alkaline version of the process.

Since protein hydrolysates produced from complex raw materials often suffer *e.g.* from high pigmentation and from being susceptible to rancidity, attempts have also been made to use pH-shift produced protein isolates to make hydrolysates [151-154]. These studies have shown that hydrolysates from pH-shift isolated proteins can have antioxidative [151, 152] and ACE-inhibiting [153, 154] properties. The advantage of starting the hydrolysis with a pure and uniform protein source such as a pH-shift made protein isolate in contrast to *e.g.* by-products is emphasized in the studies.

Furthermore, two different animal studies have been performed on pH-shift produced protein isolates. These studies investigated the effect of sardine proteins on rats with metabolic syndrome [155] and the effect of krill proteins on renal injury [156]. In which the consumption of protein isolates were compared to that of casein, and the results indicated positive effects on exchanging casein to sardine or krill protein isolates. Positive results on health are likely to increase the value of pH-shift made protein isolates and thereby their applicability.

Protein nutritional value

The focus in most studies of pH-shift protein isolation has been on protein yield, purity and functionality. However, the nutritional quality of the proteins is also of great importance but has so far been less studied.

Protein is an essential part of the human diet needed for growth and maintenance of body functions. Proteins act *e.g.* as enzymes, hormones and antibodies and give structure to the cells but can also provide energy. The protein intake for adults that is regarded as safe is 0.83 g protein per kg bodyweight (*i.e.* this protein content is expected to meet the requirement of the vast majority of healthy adults) whereas children have higher recommendation in order to maintain growth [16]. The individual need however varies *e.g.* with age, size and physical exercise [157, 158]. The body does not store protein, and during excess consumption protein is used as energy, and can also be converted to fat and stored in the adipose tissue of the body [157].

Not all proteins are alike considering nutritional quality. Proteins that are both readily digestible and have a high enough content of essential amino acids are considered to be of high quality [16, 157]. Proteins of animal source including fish are typically high quality proteins [159].

Proteins consist of 21 amino acids and modifications of these [120]. For humans, nine amino acids are considered essential, since the human body cannot synthesize them but needs to gain access to them through the diet [158]. Comparing the amino acid profile of a protein source against the amino acid requirements can show whether the protein source sufficiently covers human nutritional needs or, if limiting, which amino acid is the single most limiting. Lysine is often a limiting amino acid in plant foods [17]. The amino acid composition of pH-shift made protein isolates from trout by-products [123], gutted silver carp [160] and whole krill [122] have been analyzed and fulfills human adult requirements.

Digestibility

In addition to the amino acid composition, the protein digestibility and the bioavailability of amino acids affect the nutritional quality of proteins. Today, the best recognized method to evaluate protein quality is the protein digestibility corrected amino acid score (PDCAAS) [161]. A single study has shown krill proteins isolated with the pH-shift method to have a PDCAAS of 1.0, the same as for casein [162]. This method combines the information on limiting amino acid with true digestibility. The true digestibility is determined by how well the protein can maintain growth in a rat model compared to a reference protein. Fish protein has a true digestibility of 94 ± 3%, compared to the reference protein casein which has a true digestibility of 95 ± 3% [159].

To briefly summarize, human digestion of proteins starts in the mouth. The chewing results in mechanical decomposition of the protein-containing food and in mixing with saliva. In the stomach the proteins are further mixed with the gastric juice that contains

HCl and has a very low pH (1-3.5). The low pH may denature protein, and it further helps to activate the enzyme pepsin (secreted in the inactive form pepsinogen). Pepsin is an endo-peptidase with a broad specificity. After release of the partly digested proteins from the stomach, the digestion continues in the small intestine. Here the pH is neutralized by secretion of pancreatin and bile, and the pepsin from the stomach is inactivated. Several other peptidases, including trypsin and chemotrypsin, continue the protein digestion. At the brush border additional peptidases are available, breaking down remaining larger peptides into amino acids and di- or tri-peptides [163]. The proteins are mainly absorbed into the body as tri- and di-peptides, and secondly as free amino acids, but larger peptides may also be absorbed [164]. The digestibility of proteins is affected by anti-nutritional factors present in the food and also the tertiary and secondary protein structure [17]. The actual uptake is also affected by *e.g.* health and nutritional status of the individual [158].

Process-induced changes affecting the nutritional quality of protein

Processing can lead to chemical modifications of the proteins resulting in altered nutritional quality [17]. Reduced nutritional quality can be a result of changes in the amino acid content, following *e.g.* oxidative modifications or racemization. Racemization from the L to D form of amino acids (different chirality of the molecules; only the L form is synthesized in nature) can both render the protein resistant to enzymatic cleavage, and also make the amino acids non-metabolizable and unable to participate in *in vivo* protein synthesis [17, 120]. Protein oxidation may modify amino acid side chains, which can have a negative effect on the nutritional value due to a loss of essential amino acids as lysine, histidine, arginine and threonine [88]. Further, changes in secondary or tertiary protein structure by *e.g.* crosslinking can render the protein less accessible to the digestive enzymes and result in reduced digestibility [90, 120, 165]. Thus processing effects on protein conformation and oxidative status are of great importance for the nutritional quality.

STUDY DESIGN AND METHODOLOGICAL CONSIDERATIONS

Study overview

The thesis is based on four separate studies that investigate the effect of pH-shift protein isolation from herring. An overview of the studies is given in **Figure 5.**
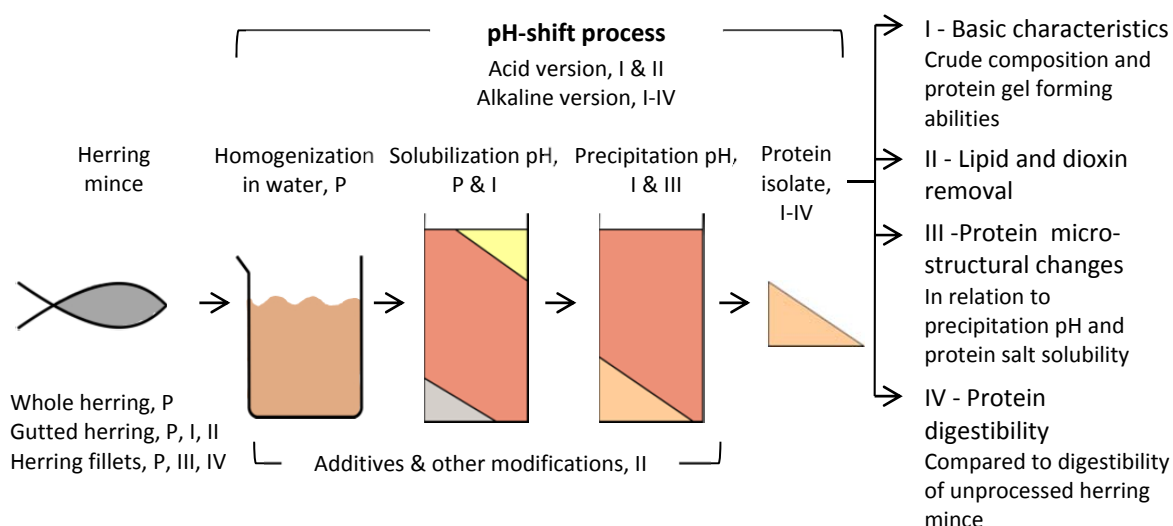

Figure 5. Overview of the study design. Modifications and evaluations of the different process steps as well as parameters studied for the protein isolate are indicated. P denotes pre-study and I-IV denote studies I-IV.

Pre-study

The aim of the pre-study was to gather basic information that would facilitate the choice of processing parameters for the following studies. The use of the mince to water ratio (1:6 or 1:9) was evaluated. Further, a comparison was made between herring fillets, gutted herring and whole herring and the solubility of proteins from whole and gutted herring at various pH values was investigated.

Study I

The main aim of study I was to evaluate the possibilities of using gutted herring as a raw material in pH-shift processing and to distinguish between possible differences between the acid and alkaline version of the process. When the study was initiated, no previous study using a more complex material than fish fillets had been reported. The choice of pH for the solubilization and precipitation steps of the process was made based on protein solubility over a range of different pH values, with the aim to optimize the protein yield. The crude composition of the protein isolate was determined. In addition, content of selected minerals, amino acid content, protein gel characteristics and protein salt solubility were established.

Study II

The aim of study II was to investigate the possibility of using the pH-shift protein isolation as a method to remove dioxins. Different ways of modifying the process (**Figure 6**) and the impact on removal of lipids, dioxins and PCBs were investigated. The most promising modification was then further studied.

The relation between lipids, dioxins and PCBs during removal was investigated as well as congener profiles and in which sub-fractions the lipids and the contaminants were removed.

Study III

The aim of study III was to further understand the reasons for the

Figure 6. Overview of the pH-shift process and process modifications used during study II.

drastically lowered protein salt solubility of protein isolates, and investigate whether this lowering was correlated to microstructural changes. It was also of interest to see how large a part of the lowered salt solubility was due to the alkaline solubilization step, and how much was due to the decrease in pH from 6.5 to 5.5 during the last part of the precipitation. Furthermore, the effect of precipitating the proteins at pH 6.5 instead of pH 5.5 on yield and some protein qualitative aspects was investigated.

Study IV

The aim of study IV was to investigate whether the pH-shift induced microstructural changes of alkali-solubilized proteins also affected the proteins' accessibility to digestive enzymes in a static *in vitro* gastrointestinal (GI) model. Heat treated and raw mince from herring fillets and alkali-made protein isolate were compared. Factors studied to explain possible differences were protein and lipid oxidation as well as protein salt solubility. The *in vitro* digestibility was determined as the amount of peptide bonds broken (%DH) and as changes in the polypeptide profile using gel electrophoresis. Further, the amount of free amino acids and amount of peptides below 3 kDa formed during digestion of the raw herring mince and protein isolate were compared.
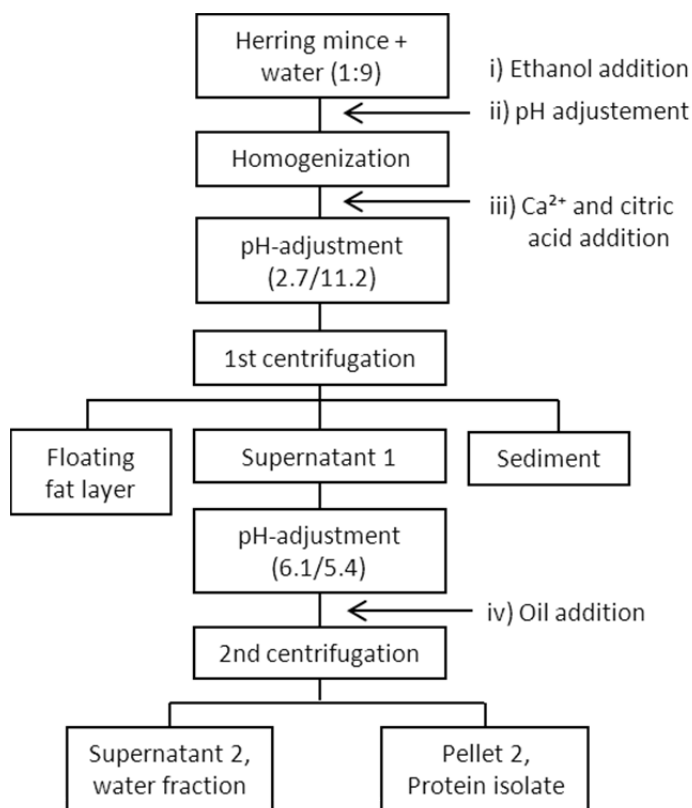
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Methodological considerations

This section describes methodological principles, limitations and the rationale for selecting certain methods. The analytical methods as such are reported in each paper and are thus not described in detail here.

Raw material and pH-shift process version

During the pre-study whole herring, gutted herring and herring fillets were compared. For studies I and II, whole Baltic herring was obtained from Gävlefisk, since the dioxin content is known to be elevated in the region of the Baltic Sea where this company operates. The herring was manually gutted in the lab to minimize the microbial load and presence of enzymes. Further, consumer acceptance was thought to be higher if the starting material was without intestines. For studies III and IV skinned herring fillets were used. For the investigation of protein microstructure and the digestibility of the proteins, it was considered important that the results were due to changes in the proteins and not as much to the removal of *e.g.* skin and bones. Further, fillets are generally regarded as the edible portion of herring.

In all studies a larger batch of the raw material was minced and frozen at -80°C to ensure accessibility to a homogenous starting material with *e.g.* equal lipid content. Storage at -80°C have previously been shown to effectively inhibit lipid and protein oxidation in rainbow trout fillets for as long period as 13 months [166]. In the current studies, no difference was observed in any parameter with extended storage time at -80°C. Furthermore, one experiment was typically performed on the herring mince before freezing, to allow for comparison with the results obtained from the frozen raw material.

Studies I and II examined both the acid and the alkaline versions of the pH-shift process, as differences between the process versions were among the end-points of these studies. Several modifications of the process were tested in study II, and these modifications were all performed on the alkaline process version since alkali-made protein isolates seemed to have lower lipid content. For studies III and IV, only the alkaline version of the process was chosen, since lipid oxidation has been shown to be a problem after acid pH-shift processing of herring [19].

As described in the results section (page 35) pH 11.2 was used for solubilization and pH 5.4 for precipitation of the proteins for the alkaline version of the pH-shift process, while the corresponding pH values for the acid version of the process were pH 2.7 and 6.1. These pH values were used in studies I, II and IV, while alkaline solubilization at pH 11.2 followed by precipitation at either pH 6.5 or 5.5 was used in study III. pH 6.5 was chosen to correspond to the pH of the raw material and pH 5.5 was chosen as this is the most commonly used precipitation pH. The force used during centrifugation was not evaluated, and 8,000*g* was used throughout the studies.

Analysis of proteins and amino acids (I-IV)

For the protein analysis, the Folin phenol reaction as described by Lowry [167] and modified by Markwell [168] was used. The method was chosen since it is rapid and simple, and with high sensitivity compared to *e.g.* the Biuret method [167]. Further, the Markwell modification allows for improved determination of membrane proteins [168]. The method is based on a two-step reaction in which proteins are first solubilized in alkali and SDS and allowed to react with copper. Secondly the copper-protein complex is allowed to react with Folin-Ciocalteu phenol reagent, forming a blue color proportional to the amount of peptide bonds of the sample, which is then measured using a spectrophotometer [168]. Since peptide bonds are broken during digestion, no Markwell protein measurement was routinely made after the *in vitro* GI digestion but the protein content rather calculated based on the dilution of the original sample. Furthermore β-mercaptoethanol (as well as some other molecules) interferes with the Markwell protein analysis and, for the extended solubility test in study III, samples containing β-mercaptoethanol were dialyzed before analysis. The protein concentration is calculated on the basis of a standard curve of a protein of known concentration. In specific cases, protein content after digestion and subsequent ultrafiltration was analyzed by an accredited company using the Kjeldahl method [169]. This is a standard method that measures total nitrogen, which is then converted to protein using a conversion factor of 6.25 (for fish). This method is therefore insensitive to the presence or absence of peptide bonds. Similarly, analyses of amino acid composition (study I) and free amino acids (study III) were performed by an accredited company. These analyses of amino acids were done on single samples, with a 10% standard variation reported. Thus only differences >20% are discussed.

Lipid content (I-IV)

To determine total lipid content, a chloroform methanol extraction followed by gravimetrical determination according to the method of Lee *et al.* [170] as modified by Undeland *et al.* [171] was used. Due to the presumed high lipid content of the herring mince in studies I, II and IV, a chloroform:methanol ratio of 2:1 was used, while for the leaner herring in study III the ratio used was 1:1. Lee *et al.* recommend a ratio of 1:2 for lipid contents below 2%, but the ratio 1:1 was used for the protein isolates as well to achieve large enough chloroform samples to also allow for analysis of lipid oxidation products.

Ash and water content (I-IV)

Water and ash content was measured gravimetrically. Two different methods were used to analyze the water content, depending on how quickly the results were needed. A moisture balance was used for fast results during *e.g.* surimi production and preparation for *in vitro* digestion. For all other analyses, weights were noted manually before and after heating at 105°C overnight. After heating, the samples were allowed to cool in a

desiccator to avoid samples attracting moisture before measuring their weight. No differences between the methods were noted.

Dried samples were used in certain cases for further analysis of ash (study I), which was also measured gravimetrically following heating at 400°C. This temperature is thought to combust carbon, but to avoid gasification of inorganic compounds.

PCBs and dioxins (II)

PCBs and dioxins were analyzed in collaboration with the Department of Chemistry at Umeå University, using a version of the standard method SS-EN 1948-3. The process uses a stepwise extraction in which first lipids and the lipid-soluble contaminants are isolated, followed by lipid removal and separation of dioxins and PCBs into two entities depending on their coplanar conformation using active carbon. The extracted and isolated contaminants are then identified and quantified using GC-MS, and compared to internal standards.

Metals (I)

Ash samples were further analyzed for metal ions using ion chromatography as described by Fredrikson *et al.* [172]. The method was chosen as it is sensitive (down to 5 ppb), easy and reasonably rapid. It is also cheaper than the more sensitive inductively coupled plasma optical emission spectrometry and inductively coupled plasma mass spectrometry.

Color (I, III and IV)

The color of proteins is an important consumer aspect and an indicator of the chemical state of Mb and Hb. Color was measured using a colorimeter according to the CIE L*a*b* scale, in which the L* value describes the black to white axis, the a* value describes the red to green axis and the b* value correspond to the yellow-blue axis (**Figure 7**). This color space is designed to evaluate colors

*Figure 7. The CIE L*a*b* color space.*

in a similar fashion as in human vision. However, as the color is a point in a three-dimensional space, comparisons are rendered difficult. The L*, a* and b* values are therefore often converted to a whiteness (W) value when evaluating the quality of *e.g.* surimi. Two different formulas are commonly used for this conversion [107];

$$W = L^* - 3b^* \quad \text{or} \quad W = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

As both the a* value and the b* value were thought to be important contributors to the color of the studied samples, the second formula was chosen.

The color obtained during measuring is affected by the structure of the sample as well as its thickness, water content and lipid content. Therefore, samples should ideally be compared at the same water content.

During these studies (I, III and IV) herring mince and protein isolates were filled in a small plastic cylinder onto which the colorimeter probe was pressed. This procedure was developed to ascertain a constant sample thickness and distance to sample. The color was mainly measured in samples with their original water content, but in some cases protein isolates in which the water content had been adjusted were used in order to facilitate comparisons.

Protein gelation (I and III)

One of the most important functional properties of muscle proteins is their ability to form a gel. This is also the most frequently studied functional property for pH-shift produced protein isolates. There are however differences between studies in the exact gel preparation procedure. When comparing eight published studies [7, 124, 129, 138, 139, 173, 174], as well as two protocols given by personal communication, many variations on the same theme were found. Factors that differed were *e.g.* moisture content (adjusted to 78-83% before or after addition of cryoprotectants), type of cryoprotectants (sucrose and/or sorbitol to a total of 8-10%, sodium tripolyphosphate 0-0.4%, and sometimes more additives), pH (pH 6.9-7.4, including how and when it was adjusted), salt content (0-3%), chopping conditions, and heating procedure used to induce the gel (20-60 min at 70-90°C). Evaluating different gel processing details was beyond the scope of this work, and thus a protocol based mainly on the study by Undeland *et al.* [7] was used, with chopping temperatures as described in the code of practice section of the book Surimi and surimi seafood [107].

One source of variation worth further mention is the addition of salt. It has been noted that, in contrast to conventional surimi, pH-shift produced protein isolates have not needed salt to form a gel [139]. However, different studies have given inconclusive results in this matter [173, 175] and it was therefore decided that 2% salt would be used during the studies in this thesis.

Several tests can be applied to evaluate gel quality. A torsion test or a punch test is typically performed to evaluate gel strength and deformability, alone or in combination with other tests [176]. In studies I and III, the most common test, the punch test (**Figure 8**), was chosen, in which a spherical metal probe is pushed into a sample, measuring the depth it penetrates into the sample and the force it needs until rupture of the gel. The probe in these studies was 6.35 mm (¼ inch), which resulted in a somewhat higher gel-strength and deformation compared to the more commonly used 5-mm probe. [176] The punch test was

Figure 8*. Measurement of gel strength and deformation.*

combined with other commonly used methods for evaluating gel quality, the folding test, and determination of expressible moisture [107, 176]. In the folding test, a 3-mm slice of the gel is folded and receives a score from 1 to 5, where 5 is the highest score and corresponds to a gel that can be folded twice without cracking [107].

Protein salt solubility (I, III and IV)

Protein solubility in a salt solution is often used as a measure of protein quality, and loss in solubility has been linked to lower functional properties of the proteins [14, 15]. Many studies use 0.6 M NaCl, typically dissolved in a buffer of ~7 pH, to evaluate protein salt solubility. A reference for the method is seldom included. To optimize the salt solubility test for fish muscle, Kelleher and Hultin [177] evaluated different salts (NaCl, KCl and LiCl), ion strengths and homogenization times. According to their study, 1M LiCl at pH 7.2 and intermediate mixing gave the highest solubility. Further, LiCl gave more stable results over a range of different pH values, compared to the other salts [177]. A second evaluation [178] of the same ions for salt solubility determination indicated that 0.8 NaCl resulted in higher salt solubility and better resolution of the solubilized proteins when analyzed with gel electrophoresis. However, also in this study, 1M LiCl gave the most stable results over a range of pH values [178]. In studies I, III and IV, a version of the LiCl method was used. To be able to analyze the total protein content and the salt soluble protein content of the same sample, the protein was first mixed in water, and subsamples were then mixed with either LiCl to a final concentration of 1.0 M or with NaOH to a final concentration of 1M. The NaOH sample was used for analysis of total protein

An extended solubility test was performed in study III to further try to distinguish between the bonds formed between proteins that inhibit the salt solubility. This test was done according to the method suggested by Matsumoto [179] as described by Perez-Mateos *et al.* [180].

Polypeptide pattern (I, III and IV)

Due to the very large variation in size (5-200kDa) between the proteins present in fish muscle, a polyacrylamide gradient gel was used to facilitate good separation and visualization. Gradient gels of 4-16% or 4-20% were judged to give the best separation. As there were problems in fully destaining gels in study I, different gel systems were evaluated. Page® Gold from Lonza with coomassie R-250 and the BioRad protocol for staining/destaining usually used for peptides were finally applied. Study III used mini-protean TGX-gels from BioRad, which behaved in a similar way as the Page® Gold-gels during destaining but has a much longer shelf life.

Myosin heavy chain (MHC) and actin were identified based on their well-known molecular weight and high abundance in muscle (~200 and ~42 kDa, respectively) [48]. All other proteins were tentatively identified based on size and relative abundance or discussed as unknown and referred to by their molecular weight.

Protein carbonyls (IV)

In this work, protein oxidation was measured as protein carbonyls. Formation of carbonyls is considered a hallmark of protein oxidation in general and metal catalyzed protein oxidation in particular [181]. Protein carbonyls form during oxidation of lysine, arginine, and proline residues, as well as during some protein cleavage reactions [182]. The analytical method of choice for this work was the 2,4-dinitrophenylhydrazine (DNPH) method. This is currently the most common way to determine protein carbonyls, in which DNPH reacts with the carbonyls and covalently binds the proteins [88, 181]. The bound DNPH can then be quantified spectrophotometrically as it absorbs UV light at 370 nm.

Lipid oxidation products (III and IV)

During the very complex reaction of lipid oxidation, lipid hydroperoxides are formed which are referred to as *primary* oxidation products. These are decomposed to produce an array of *secondary* products, including aldehydes, alkanes, alkenes and alcohols [82]. Several tests exist to study lipid oxidation, but none covers all the products formed. To avoid the limitation that the use of a single method contains, a combination of two different methods investigating primary and secondary lipid oxidation products respectively, was used. For primary oxidation products, the ferrithiocyanate method, giving a so called peroxide value (PV), was used as described by Undeland *et al.* [171]. The method can be applied directly to the chloroform fraction from the lipid extraction previously described. Secondary products were measured as thiobarbituric acid reactive substances (TBARS) using malondialdehyde (MDA) as standard. The method used was that of Schmedes and Hølmer [183]. This version of the TBARS method was chosen since it can be conveniently applied to the water-methanol phase resulting from the lipid extraction.

Microscopy studies (III)

Confocal Laser Scanning Microscopy (CLSM) analysis (unpublished)

CLSM with double protein and lipid staining was used to investigate the microstructure of proteins and the presence of lipids in the protein isolates and herring mince. The CLSM results are not included in any of the manuscripts and the method is therefore described in more detail. The minced herring fillet and protein isolates were from study III.

The microstructure of the herring mince and the protein isolates were examined using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany). Alexa 488 and Nile Red were used as fluorescent dyes binding to protein and lipid, respectively. The dyes were mixed and allowed to dry on a cover glass that was later placed on top of the sample. Samples were kept cold (4°C) during microscopic examination. The light sources were Ar laser using λex = 488 nm (Alexa 488) and HeNe laser using λex = 594 nm (Nile Red). Signals from the samples were captured at

wavelengths of 496-540 and 605-675 nm for Alexa 488 and Nile Red, respectively. Pictures were taken at magnifications of 63x, zoom 2, format 1024x1024.

Nile red is an uncharged hydrophobic dye mainly used to stain lipids, but it also stains hydrophobic protein surfaces. It is photo stable and insensitive to pH in the range of 4.5-8.5 [184]. Alexa is a commonly used protein dye. It was chosen as a photostable and highly fluorescent dye, which is also insensitive to pH in the range of 4-10. It is hydrophilic and negatively charged and may in some cases bind unspecifically to positively charged structures [185].

Transmission electron microscopy (TEM) (III)

TEM uses electrons instead of light to produce an image, which improves the resolution due to the much smaller wavelength of the electrons. This makes TEM a high resolution (nm) method compared to CLSM (μm). TEM uses very thin sections of sample through which the electrons are sent (therefore the word transmission). Due to this, TEM can give more information on the structure and network formation of the proteins than CLSM. The information given by TEM differs from scanning electron microscopy (SEM) in that SEM detects reflected signals and gives an image of the surface of the sample. TEM is therefore more suitable for studying protein networks.

Evaluation of protein digestibility (IV)

An investigation was made of whether the changes in microstructure and salt solubility shown to be induced by pH-shift processing influenced the possibility for the proteins to be broken down by gastrointestinal enzymes. For this purpose, a static *in vitro* GI digestion method was used. The GI digestion set-up was a version of that described by Svelander *et al.* [186]. It was however modified by addition of a homogenizing step and exclusion of antioxidants. Homogenization was included to mimic chewing. Further, for simplicity, the lipase was added during the homogenization.

A combination of methods was chosen to evaluate protein digestibility: gel electrophoresis to evaluate if there were detectable differences in the non-digested peptide fraction and degree of hydrolysis (%DH) to evaluate how large a part of the protein peptide bonds had been broken. The theoretic maximum DH value of 100% corresponds to the state when all peptide bonds have been broken and the sample consists of free amino acids. To determine the DH, a spectrophotometric method using trinitro-benzene-sulfonic acid (TNBS) was chosen [187]. This method is commonly used and safer than the more accurate o-phthaldialdehyde method [188] which includes high concentrations of teratogenic sodium borate.

Since small peptides escaped the SDS-PAGE gel, another approach was used to detect whether the mince and protein isolate gave rise to different amounts of small peptides during digestion. An easily adoptable procedure for ultrafiltration at 3kDa with spin centrifugation was chosen. It should be noted that, although a specific molecular weight is stated, the cutoff of a filter is normally a range also affected by other physicochemical

characteristics than molecular weight. Free amino acids were analyzed in the digests of the two samples as an additional measure of potential digestibility differences.

Statistical analysis (I-IV)

Univariate analyses were performed with Excel (study I), PASW Statistics 18.0 (study II) and SPSS 19.0 (studies III and IV). For the data in the thesis, the un-paired t-test was used for comparison of two samples, while one-way ANOVA followed by Tukey's test was used in comparing three or more samples using SPSS 19.0. A level of $p<0.05$ was considered significant. Data are reported as mean ± standard deviation. Tukey's test is a commonly used *post hoc* ANOVA, and simultaneously compares pairwise differences between the means of all included samples. It can be used in groups of unequal size, as it assumes homogeneity of variance within different samples.

RESULTS AND DISCUSSION

Protein yield

The first publications on pH-shift protein isolation [7, 12] and most studies reported since then have used a water-to-raw material ratio of 9:1. Since it would be positive to minimize water usage, some studies of this process have been made with a ratio 6:1 or lower, instead [11, 129, 189, 190]. A comparison in this thesis (P) showed that the yield of protein from herring fillets was 46-55% using 6 volumes of water, and 61-65% using 9 volumes of water (unpublished results). There were only small differences in the solubility of the proteins and the main difference in yield was connected to the fact that more proteins were lost in the sediment and floating layer of the first centrifugation when the lower water content was used. All further experiments were therefore made with the larger amount of water.

The pre-study also showed that a higher protein yield was obtained from whole herring than from gutted herring using both the acid and alkaline process versions (**Figure 9)**. After solubilization and centrifugation at pH 11-12, gutted herring yielded a large floating fat layer and a smaller sediment. The opposite was seen after solubilization at acidic pH. This difference might be of importance if a separation technique other than centrifugation is to be used. Whole herring on the other hand formed a small floating fat layer and a small sediment at both acidic and alkaline pH. The smaller sediment and floating layers resulted in higher protein yield, probably due to a lower emulsification capacity of the proteins. Despite the increased protein yield with whole herring, gutted herring was chosen as the raw material for studies I and II to minimize the microbial load and the content of enzymes from the digestive tract. Throughout study P and I, relatively large differences in protein yield were obtained between different batches of gutted herring, which was likely due to different qualities of the raw material. Previous studies have demonstrated that reduced freshness of the fish as well as freezing greatly reduces the protein yield [18, 154].

To determine what pH to use for protein solubilization and precipitation for Baltic herring in studies I and II, solubilization tests were done at several pH values. Based on these tests pH 2.7 and 11.2 were chosen for solubilization in the acid and alkaline process versions, respectively. pH 2.7 was a peak value in protein solubility, while pH 11.2 was a compromise between a use of excess base and solubility. The pH after alkaline solubilization that gave the lowest protein solubility was pH 5.4, which is in agreement with most other studies. The pH at which the protein solubility was lowest after the acid solubilization (pH 6.1) was higher than used in other studies. In a previous study testing precipitation at pH 4.8-5.9, herring light muscle proteins had its lowest solubility at pH 5.9 after acid solubilization [7]. Myosin has its pI at about pH 5.5 at physiological ionic strength [191]. However, at very low salt concentrations, myosin and actomyosin gain a higher pI [191]. During the current studies (I-IV), 9 volumes of distilled water were added
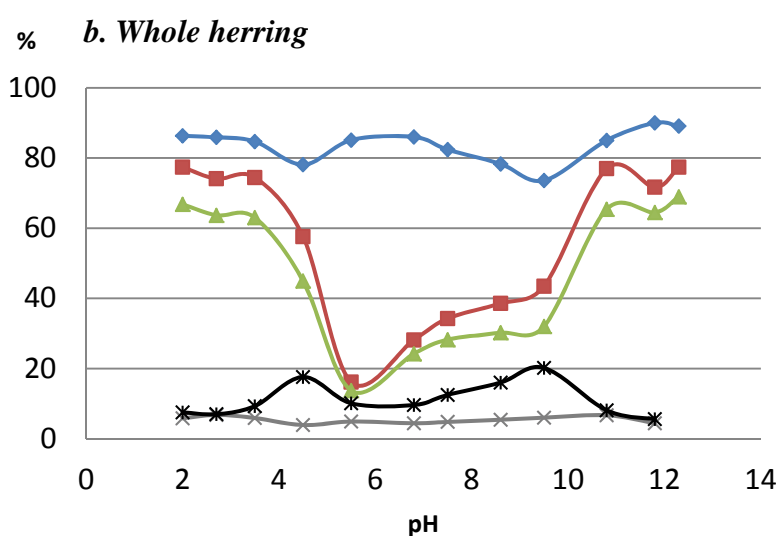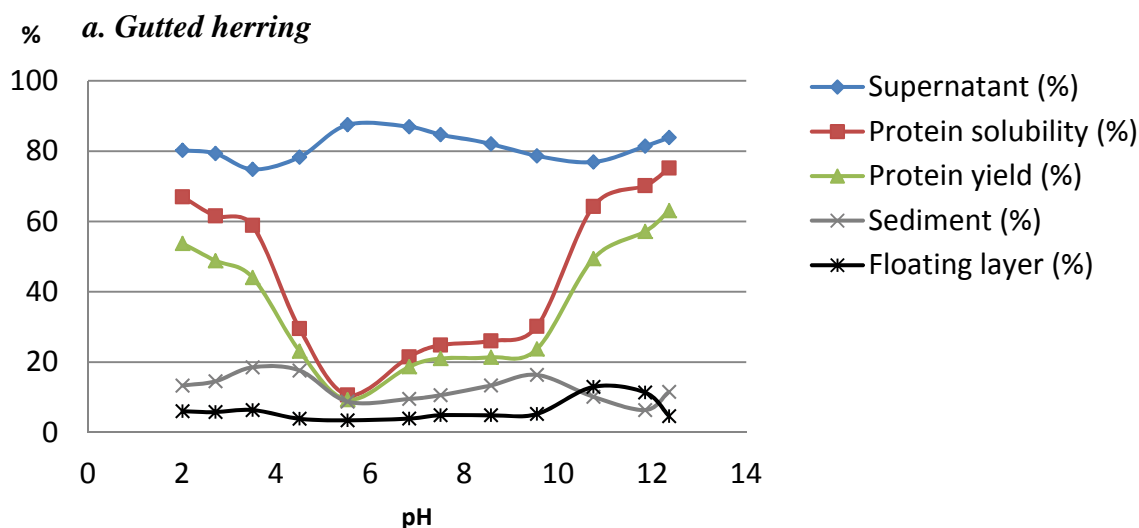
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a. Gutted herring

b. Whole herring

Figure 9. *Protein solubility of* ***a****. gutted herring and* ***b****. whole herring, depending on pH. Protein solubility and protein yield after centrifugation is in % of total protein, Also the relative distribution (%) of supernatant, sediment and floating layer at each pH is presented.*

in the initial phase of the pH-shift process, and the physiological salt concentration (~150 mM) [191] was thus tenfold diluted. The addition of HCl and NaOH then resulted in formation of NaCl (0.017 ± 0.002 M and 0.028 ± 0.002M for the alkaline and acid pH-shift process, respectively, when applied to gutted herring), giving a theoretical final salt content of about 0.03-0.05M in the protein isolates. This low salt content might thus affect the pI [191]. However, it is not clear why an increased pI was seen after acidic and not alkaline solubilization. Removal or breakdown of proteins with low pI could theoretically have resulted in a higher total pI for the protein mixture. Bound ions such as calcium could also be removed to different extents, possibly affecting the pI. Both gutted herring and herring light muscle contain bones that may release calcium ions into solution. Indeed the amount of calcium and magnesium differed significantly between the protein isolates, with a higher concentration in the acid-made protein isolates.

The protein yields obtained using the final processing parameters are presented in **Table 3**. There was no significant difference in protein yield between the acid and the alkaline pH-shift process versions, both gave total protein yields of 57-59%. This protein yield was lower than what has been previously reported for herring fillets and herring

light muscle [7, 19] but was similar to what has been obtained for gutted silver carp [192]. The lower yield compared to previous herring studies might be due to more non-muscle proteins being present in a more complex raw material like whole gutted fish, and these proteins may be removed during the process.

The solubility of both gutted herring proteins and herring fillet proteins at the applied precipitation pH (**Table 3**) was generally higher than for proteins from herring light muscle [7]. Possibly the dark muscle of herring contains a higher content of proteins not being precipitated at pH 5.4-6.5. When comparing the solubility during the precipitation step in study III at pH 5.5 and 6.5 (*i.e.* not including results from study I) it was shown that at the higher precipitation pH a larger amount of proteins remained soluble ($p<0.05$, t-test). However this increased precipitation pH did not affect the protein yield, which has not been previously shown. That the protein yield was unaffected by the higher precipitation pH was a result of a higher water content of the resulting protein isolate (**Table 4**), which thus retained a larger portion of soluble proteins.

Many studies have used pH 5.5 for the precipitation of proteins without reporting any optimization of this part of the process. The current results show that several process parameters may affect what is the optimal precipitation pH. It is therefore suggested to choose the precipitation pH carefully in order to optimize protein yield in the pH-shift process.

Table 3. *Protein yield and solubility (%).*

Study	Process version, starting material and precipitation pH	Centrifugation 1		Centrifugation 2		Total Yield (%)
		Solubility (%)	Yield (%)	Solubility (%)	Yield (%)	
I	Alkaline process, gutted herring, pH 5.4	79 ± 5.5^a	65 ± 2.3^a	11 ± 2.8^{ab}	90 ± 2.6^a	57 ± 2.5^a
	Acid process, gutted herring, pH 6.1	77 ± 2.8^a	65 ± 2.8^a	9 ± 1.8^a	92 ± 1.7^a	59 ± 3.0^a
III	Alkaline process, herring fillet, pH 5.5	-	-	11 ± 1.3^{ab}	90 ± 1.1^a	-
	Alkaline process, herring fillet, pH 6.5	-	-	14 ± 1.3^b	89 ± 1.0^a	-

Different letters within a column indicate significant differences ($p<0.05$),

Protein purity and safety

Protein, water, ash and lipid content

The protein content of the protein isolates was 17.8% or higher when expressed based on 78% water content (*i.e.* the water content of the gutted herring) as shown in **Table 4**. This corresponds to a protein content of 80-95% on a dry-weight basis (dw), which could be compared to the gutted herring, which contained ~56% protein (dw) and the herring fillets from study III that contained about 50% protein (dw). Interestingly, the protein content was dependent on the raw material, and the use of herring fillets compared to gutted herring led to protein isolates with fewer impurities.

The water content of the protein isolates was significantly higher than that of the herring raw materials (**Table 4**). The water content of the herring raw materials differed a great deal depending on lipid and ash content. However, when the protein isolates were precipitated at their pI the water content was about 89% regardless of starting material or process version, which is similar to what previous studies report [7, 160]. The alkali-made protein isolate precipitated at pH 6.5 had significantly higher water content (93%), as the higher pH resulted in more negatively charged proteins.

The ash content (**Table 4**) was investigated in study I and was significantly lower in the protein isolates than in the gutted herring. When compared on a dry-weight basis the alkaline pH-shift processing reduced the ash content from 8.8 ± 1.0 of the gutted herring to 1.1 ± 0.3 in the protein isolate, *i.e.* a reduction of over 85%. The ash was presumably removed mainly with the sediment formed after the first centrifugation, as has been shown for gutted silver carp

Table 4. Major constituents of herring samples and protein isolates.

Study and sample	Moisture (%)	Protein[1] (%)	Lipids[1] (%)	Ash[1] (%)
I, Gutted herring	78.0 ± 0.4^b	12.4 ± 0.7^{ab}	7.9 ± 0.9^c	2.0 ± 0.3^b
I, Alkali-made isolate, pH 5.4	89.3 ± 0.9^c	17.8 ± 1.4^c	3.9 ± 0.6^b	0.2 ± 0.1^a
I, Acid-made isolate, pH 6.1	89.3 ± 0.9^c	17.9 ± 1.4^c	5.0 ± 1.2^b	0.3 ± 0.1^a
II, Alkali-made isolate with 5% ethanol, pH 5.4	89.8 ± 0.2^c	12.0 ± 1.0^a	1.2 ± 0.1^a	-
III, Herring fillet	71.4 ± 0.4^a	14.7 ± 0.3^b	14.4 ± 1.1^d	-
III, Alkali-made isolate, pH 5.5	89.5 ± 0.9^c	21.2 ± 0.9^{de}	1.5 ± 0.5^a	-
III, Alkali-made isolate, pH 6.5	93.3 ± 1.0^d	22.0 ± 0.7^e	1.1 ± 0.3^a	-
IV, Herring fillet	77.8 ± 0.5^b	17.5 ± 0.8^c	4.9 ± 0.6^b	-
IV, Alkali-made isolate, pH 5.4	88.3 ± 0.9^c	18.9 ± 1.1^{cd}	1.6 ± 0.2^a	-

[1]Data for protein isolates are expressed on 78% water content, i.e. the same as the gutted herring. Data for herring raw materials are expressed on their original water content.
Different letters within a column indicate significant differences ($p<0.05$)

[160]. In that study the alkaline pH-shift process gave a protein isolate with significantly lower ash content than the acid process. In the present study, the alkali-made isolate had the lowest ash content, but it was not significantly different from the acid-made protein isolate.

The lipid contents of the protein isolates were significantly lower than in the starting materials (**Table 4**). Further, lower lipid contents were obtained in the protein isolates made from herring fillets than in those made from gutted herring. That the herring fillets in studies III and IV had very different lipid contents did not influence the final lipid content in the alkali made protein isolates, indicating that the additional amount of lipids in the fattier herring used in study III was easily removed. To visualize remaining lipids CLSM was used for the first time on pH-shift-made protein isolates. The CLSM micrographs of minced herring fillet and protein isolate (**Figure 13**, page 44) showed lipid droplets remaining in the isolates, and these were considerably smaller than the ones

present in the herring mince. These droplets were likely remaining neutral lipids, as the membrane bilayers are of too small a magnitude to be visualized with CLSM. The small lipid droplets in the protein isolates were always observed in close proximity to the proteins, and typically had a size of 1-3 μm. Areas with many droplets and without droplets were observed, with the micrograph in **Figure 13** having an intermediate number of droplets.

The reasons behind the lower lipid removal from gutted herring compared to herring fillets have not been identified, but it could be speculated that the high amount of other components from the bones and also skin leads to more lipid-protein interactions, and possibly the formation of an emulsion that lowered the possibility for a successful lipid-protein separation during the first centrifugation of the pH-shift process. However, other authors using complex raw materials like gutted silver carp [160] have reported lower lipid contents in their protein isolates than those reported here. The study of gutted silver carp included a 10 min incubation before the two centrifugations, which has been shown to lower the viscosity of the solution and allow better separation [18]. It is possible that inclusion of such a step would have been beneficial for the removal of lipids from gutted herring. Higher force (10,000g) was also used during centrifugation in the study mentioned. Still, the process differences discussed above do not fully explain the difference in lipid removal between the gutted herring and herring fillets as starting material. Since herring is the only species that has been used for pH-shift processing both as whole gutted fish and as fillet it is difficult to draw conclusions on what differences are caused by different complexities of the raw material and what differences are due to variations between species.

Polypeptide profile

The polypeptide profile of different steps in the acid and alkaline version of the pH-shift process shows that most of the muscle proteins were retained throughout the process (**Figure 10**). It is apparent that the acid version of the pH-shift process induced proteolysis of certain proteins. Loss of a very large molecular weight protein (possibly titin or nebulin) is observed as is partial breakdown of MHC (~190 kDa). The degradation was accompanied by the development of new polypeptides of lower size, and several bands are seen between 68 and 180 kDa. It was previously shown that several cathepsines (proteases) were active at and after acid pH-shift processing of Pacific whiting [12]. If such acid proteases are active also during acid pH-shift processing of herring proteins, they could be responsible for the proteolytic degradation of the acid produced herring protein isolate. There was no apparent proteolysis during the alkaline pH-shift process, and the relative amount of MHC was slightly increased in the protein isolate compared to the herring mince. Another difference between the alkaline and the acid versions of the pH-shift process was that there were two proteins (51 and 9 kDa) that remained soluble to a larger extent after the alkaline version (lane 10, **Figure 10**). Thus these proteins were largely removed together with the supernatant after the second centrifugation. The molecular weights indicate that the proteins presumably could be desmin and
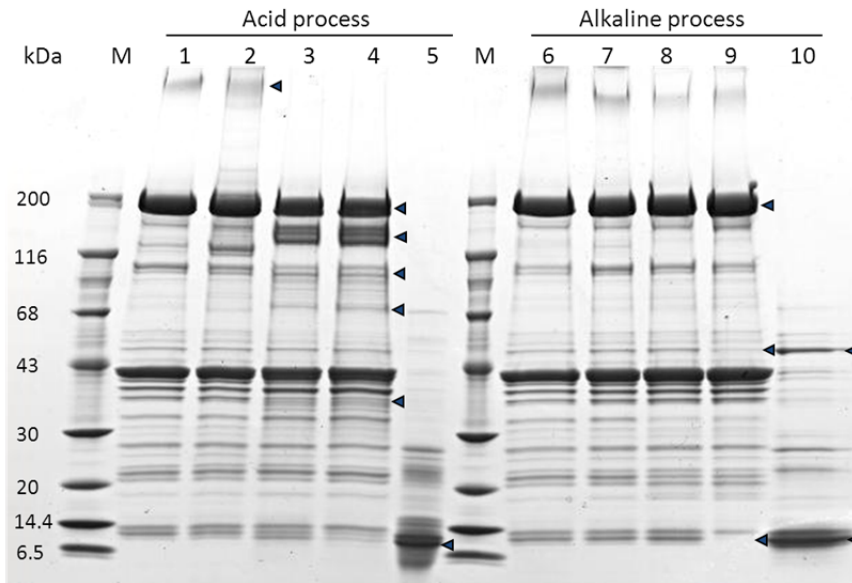
Figure 10. Polypeptide profile of gutted herring at selected stages of acid and alkaline pH-shift processing. Lanes: M, molecular weight marker; 1, herring mince homogenate; 2, homogenate after adjustment to pH 2.7; 3, supernatant 1 of the acid process; 4, acid-made protein isolate; 5, supernatant 2 of the acid process; 6, herring mince homogenate; 7, homogenate after adjustment to pH 11.2; 9, supernatant 1 of the alkaline process; 9, alkali-made protein isolate; 10, supernatant 2 of the alkaline process. Arrowheads indicate proteins that are discussed.

parvalbumin, respectively. Desmin has been proposed as a solubility-inhibiting muscle protein [193]. Regarding parvalbumin, a previous study identified a small protein (~6kDa) remaining soluble after alkaline pH-shift processing as this protein [194]. Parvalbumin is the protein to which most persons allergic to fish react [51], and removal could reduce the allergenicity of the proteins. However, since some of the soluble proteins remain in the water fraction of the protein isolates, the removal is not complete.

Oxidation products

Lipid oxidation has been described as a problem, especially after the acid pH-shift process version (see *Oxidation induced during pH-shift processing*, page 21). The current results (**Table 5**) show that lipid oxidation was also initiated during the alkaline pH-shift process (studies III and IV). This has also been shown for chicken dark muscle [147], which like herring is rich in Hb, Mb and lipids. Both Hb and Mb become more pro-oxidative at pH values below neutrality [25, 50], which is also the case in the last part of the precipitation step of the alkaline pH-shift process. This can be one of the reasons for the development of lipid oxidation products during the alkaline process as well. This is further supported by the fact that precipitation of the proteins at pH 6.5 instead of 5.5 led to less lipid oxidation. General features that could boost oxidation during the pH-shift process could be dilution of endogenous anti-oxidants [65] and the fine homogenization, allowing oxygen increased access to the lipids [40]. There are presumably differences in the susceptibility of different raw materials to oxidation during the pH-shift process as neither acid nor alkaline pH-shift processing induced lipid oxidation in channel catfish

(*Ictalurus punctatus*) [145]. The amount of lipid oxidation products formed after alkaline pH-shift processing in this thesis is comparable to the amount of lipid oxidation products previously measured for acid-made pH-shift isolates produced with inclusion of anti-oxidants [19], although the starting TBARS value of the herring mince was higher (9.3) in the study mentioned. Further, PV and TBARS values determined in studies III and IV were low compared to what has been measured after long-term frozen storage of herring [195].

Table 5. *Lipid and protein oxidation products before and after alkaline pH-shift processing.*

	Study III			Study IV	
	Herring fillet, minced	Alkali-made isolate, pH 5.5	Alkali-made isolate, pH 6.5	Herring fillet, minced	Alkali-made isolate, pH 5.4
TBA (μmol/kg wet weight)[1]	3.0 ± 0.5^a	16 ± 1.1^c	8.5 ± 3.4^b	4.1 ± 0.6^{ab}	16 ± 2.5^c
PV (μmol/kg wet weight)[1]	30 ± 13^a	190 ± 20^{cd}	130 ± 36^{bc}	80 ± 30^{ab}	270 ± 44^d
Protein carbonyls (nmol/mg protein)	-	-	-	2.4 ± 0.5^a	2.5 ± 0.1^a

Different letters within a row indicate significant differences ($p<0.05$) [1]Values are expressed at 78% water content of the protein isolates.

Although lipid oxidation has been considered a concern, protein oxidation products have not been studied before. However, despite the induction of lipid oxidation, there were no formation of protein carbonyls during alkaline pH-shift processing (**Table 5**). Thus, under the conditions of this process, it seems that lipid and protein oxidation did not occur in parallel, which on the other hand has been recorded in several other systems, as recently reviewed [88].

Dioxin removal

Table 6. *Toxicity (TEQ) before and after pH-shift processing compared to EU limits.*

Sample	dioxin-TEQ (pg/g)	dl-PCB-TEQ (pg/g)	∑ TEQ (pg/g)
Gutted herring	6.3 ± 0.3^c	3.7 ± 0.1^c	10.0 ± 0.5^c
Alkali-made isolate, pH 5.4[1]	2.2 ± 0.3^b	0.9 ± 0.3^{ab}	3.1 ± 0.5^b
Acid-made isolate, pH 6.1[1]	2.4 ± 0.1^b	1.2 ± 0.2^b	3.6 ± 0.3^b
Alkali-made isolate with ethanol, pH 5.4[1]	1.3 ± 0.1^a	0.6 ± 0.2^a	1.9 ± 0.3^a
EU maximum limit [5]	3.5	-	6.5

[1]Data for protein isolates are expressed on 78% water content, the original water content of the gutted herring mince. Different letters within a column indicate significant differences ($p<0.05$).

Since dioxins and PCBs are generally removed from fish if the lipid content is reduced [100, 101] the pH-shift process was for the first time investigated as a means to reduce the load of these contaminants (study II). The acid and the alkaline versions of the pH-process were compared in Baltic gutted herring. Modifications of the alkaline process

were tested to see whether a further decrease in dioxin and PCB content could be obtained. An overview of the modifications initially tested is shown in **Figure 6** (page 26). The most promising modification was an addition of 5% ethanol which was then further tested and is reported together with the original acid and alkaline process results. All versions of the pH-shift process significantly reduced the contaminant load compared to the starting material (**Table 6**), and the levels in the final isolates were well below the newly updated EU maximum limits [5].

The acid and the alkaline versions were equally successful in the removal of lipids, dioxins and PCBs, while the removal seemed further improved by addition of 5% ethanol. Ethanol has previously been used to extract lipids and dioxins from fish meal [103]. However, based on toxicity per gram of proteins, there was no significant difference between the protein isolates made with the acid process, the alkali process and alkali process with 5% ethanol. The reduction in toxicity per unit of protein was 70-80% based on the contaminant load of the starting material.

The pH shift process resulted in three different fractions after the first centrifugation: the floating fat layer, the sediment and the soluble protein fraction. After precipitation and the second centrifugation, the latter fraction was converted into two, the protein isolate and a water fraction (**Figure 4**, page 16), thus forming a total of four fractions after the full pH-shift process. The relative distribution of dioxins and dl-PCBs (total TEQ) between these fractions during alkaline pH-shift processing is shown in **Figure 11**. A majority of the toxic compounds were found in the floating fat layer and the sediment after the first centrifugation, while very little of the contaminants was found in the water fraction from
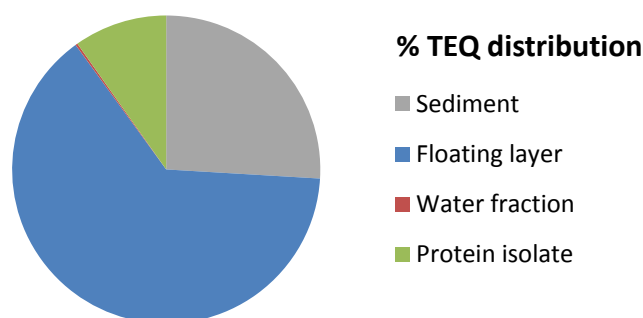

% TEQ distribution
- Sediment
- Floating layer
- Water fraction
- Protein isolate

Figure 11. The relative distribution of the sum of PCB TEQ and dioxin TEQ between the fractions formed during the alkaline pH-shift process.

the second centrifugation. Similar results were obtained for all the different dioxin and PCB congeners investigated, and no difference in congener profiles was found between the samples. A larger sediment and smaller floating layer formed after the acid version of the pH-shift process, which suggest that the distribution of lipids and dioxins between these fractions differed. The dioxins, dl-PCBs and lipid contents in herring mince and the different sub-fractions produced during pH-shift processing were highly correlated ($p<0.01$), with a Pearson correlation of $R^2=0.996$ between lipids and dl-PCBs, and $R^2=0.952$ between lipids and dioxins.

These results show that the pH-shift method can be used as an effective means to reduce the load of dioxins and PCBs from fatty fish. This is of great importance in order to use fish with elevated contaminant levels. It is also likely that the results of this study can be generalized to other lipophilic contaminants and that the removal of lipophilic contaminants can be further optimized. Since the removal of lipids from herring fillets was higher than from gutted herring throughout this thesis (**Table 4**, page 38), it is possible that a pre-filleting step could further increase the removal of contaminants from the proteins. The herring lipid content varies largely over seasons. However, when the Baltic herring has it highest lipid content, the contaminants are somewhat diluted and their concentration per g lipid is lower [196]. Since a similar lipid content was found in the protein isolates regardless of the lipid content of the herring fillet (**Table 4**), it is suggested that the lowest concentration of PCB and dioxins would be found in protein isolates made from herring fillets of high lipid content.
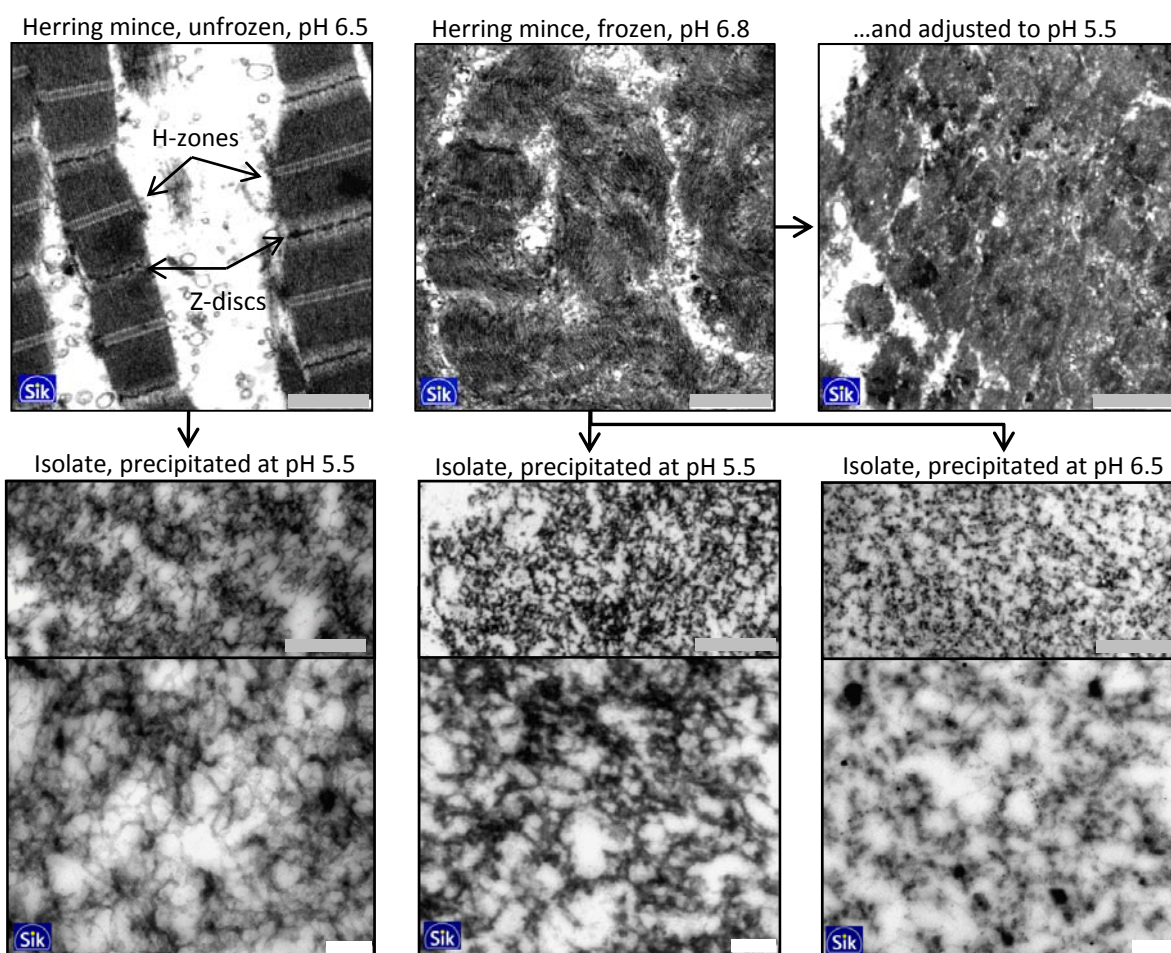
Protein microstructure

Figure 12. TEM micrographs of herring mince and protein isolates thereof. White scalebar corresponds to 200 nm and grey scalebar to 1000 nm. Isolate denotes protein isolate made by the alkaline pH-shift method.

The microstructure of protein isolates and herring mince as visualized by TEM (**Figure 12**) indicates that large differences were induced by alkaline pH-shift processing (study III). Unfrozen herring mince had clearly visible myofibrillar structures with evident light H-zones with semi-gray M-lines, and darker Z-discs [48]. The freezing however ruptured the myofibrils. The striated structure remained, but only vague remnants of M-lines and z-bands were observed. Adjustment of the herring mince to pH 5.5 delocalized the proteins even more, and very little structure remained. Further restructuring was induced by the alkaline pH-shift method. No structure that resembles the myofibril was observed in the protein isolates. Instead there was a loose, homogenous network consisting of both thin and denser strands, as well as protein aggregates. The protein network of isolates made from unfrozen herring included more fine strands. The small strands presumably consisted of myosin and/or actin as these proteins are able to form filaments. Further, the protein isolates precipitated at pH 5.5 had a coarser structure with more large pores than the protein isolate precipitated at pH 6.5, which was more homogenous.
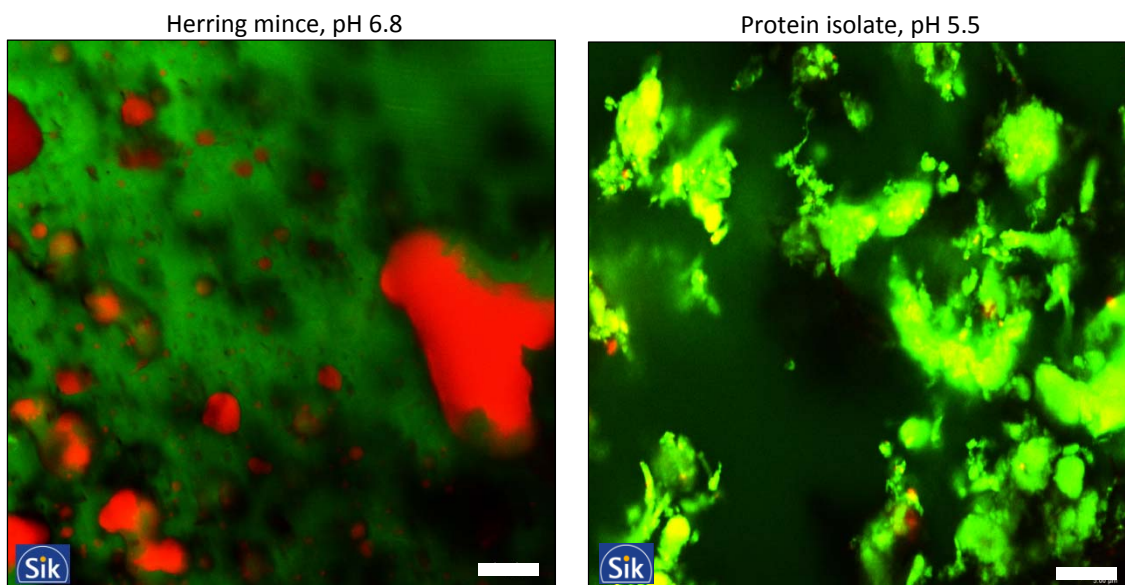

Figure 13. Confocal microscopy picture of herring mince (pH 6.8) and alkali-made protein isolate (pH 5.5). Samples are double stained with the protein stain Alexa (green) and the stain Nile red (red) that binds to hydrophobic areas. The combination of both stains is seen as yellow. White scalebar corresponds to 10 μm. The herring mince has been frozen, and later thawed, while the protein isolate was made from unfrozen herring.

The protein structure of isolates and herring mince was also analyzed with confocal microscopy. Double staining to visualize both proteins and lipids was used. The picture (**Figure 13**) of herring mince at original pH shows a smooth, homogenous protein mixture (green) with large and abundant lipid droplets (red). The protein isolate shows more structuring and aggregation of the proteins. Furthermore, the hydrophobic dye also bound to protein regions of the protein isolates, indicating an increased protein surface hydrophobicity. Confocal microscopy is not a standard test to determine protein hydrophobicity, but Nile red has previously been shown to interact with both lipids and

hydrophobic patches of protein [184]. It has also been used to measure protein aggregation [197]. Previous studies of pH-shift produced protein isolates have shown alkaline solubilization to increase the surface hydrophobicity of proteins soluble in salt solution [11, 13, 198]. However, the samples used for CLMS contained both salt soluble and salt insoluble proteins. CLSM might therefore be an alternative way to study protein hydrophobicity in relation to loss of protein solubility.

Both the TEM and CLSM micrographs showed that the protein structure was drastically altered after alkaline pH-shift protein isolation and indicated that some protein aggregation occurred between the proteins at pH 5.5.
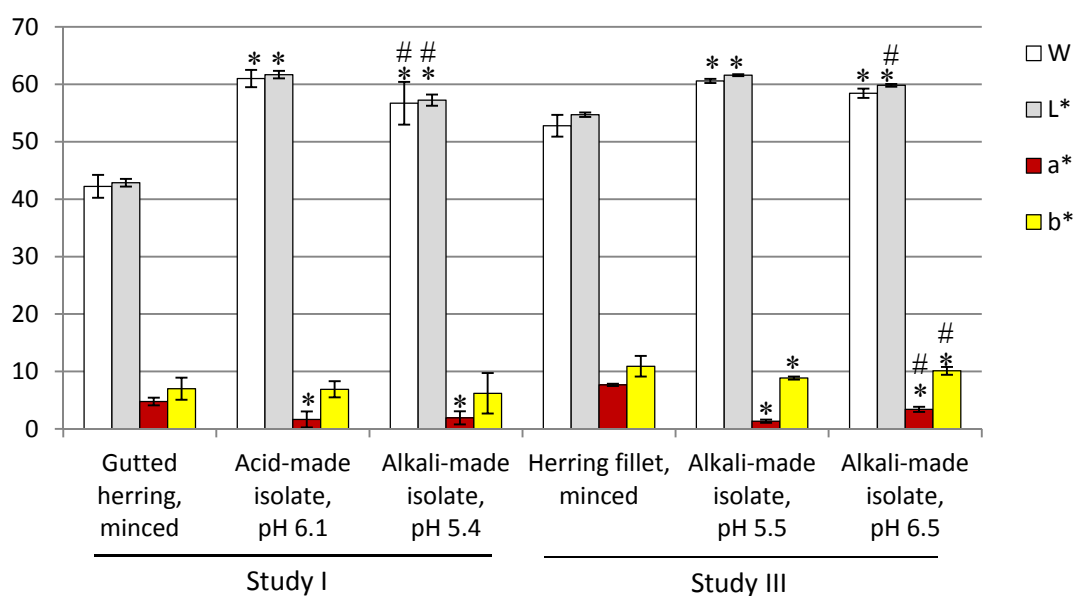
Protein functionality

Color

*Figure 14. Color of raw material and protein isolates from studies I and III. * indicates a significant color difference (p<0.05) from the respective raw materials, and # indicates a significant difference (p<0.05) from protein isolate within the same study. Protein isolates of study III were adjusted to 80% water content, while the other samples were analyzed at their original water content (Table 4). W- whiteness, L*-lightness, a*- redness, b*- yellowness.*

Protein isolates from both gutted herring (study I) and herring fillets (study III) were significantly whiter than the color of the respective raw materials (**Figure 14**), indicating removal of pigments during the pH-shift processing. Further, the pre-study (P) showed that protein isolates from gutted herring had better color compared to the isolates from whole herring. In this study (P) the acid process version gave protein isolates with a whiteness of 64, 59 and 54 from fillets, gutted herring and whole herring, respectively. The alkaline process version gave whiteness values of 66, 58 and 55, respectively, for the

same starting materials (un-published results). It should be pointed out that the protein isolates presented in **Figure 14** are not directly comparable, due to their different water contents. If equal water content had been used in the two studies, protein isolates from herring fillets would be expected to have higher whiteness and lightness values then protein isolates from gutted herring, as was demonstrated in the pre-study. The color is presumably improved during pH-shift processing due to removal of pigments such as melanin present in the peritoneal lining of the gutted herring. The sediment after the first centrifugation was greyish, and it contained a large amount of dark pigments especially during processing of gutted herring. Further the pH-shift process has previously been shown to remove Hb [145] and Mb [76, 146]. Moreover, the pH shifts may affect their chemical state with low pH presumably inducing formation of metHb and metMb. Although the redness did not differ between the acid and alkali-made protein isolates in study I, the color of the second supernatant was, when visually inspected, markedly different depending on whether acid or alkaline solubilization had been applied. The color was reddish after alkaline processing and yellowish after acid pH-shift processing. In addition to the removal of pigments, changes in the structure of the proteins may affect the color, as aggregates become more opaque and are perceived as whiter. Differences in lipid content can also affect the color, as a lipid emulsion may scatter light and increase the lightness [199]. Although the color of the herring proteins was significantly improved by subjection to pH-shift processing, the whiteness of the isolates is still low compared to surimi standards and protein from whitefish. The color of gels made from proteins isolated from whole fish and fish by-products have also been judged as poor in work reported by other authors [8].

Gelation

The ability of fish proteins to form a heat-induced gel is considered very important. It has therefore been the most well studied functional property in pH-shift produced protein isolates. Work presented in this thesis (studies I and II, **Table 7**) shows that proteins isolated from both gutted herring and herring fillets can form gels of moderate to high strength and with high elasticity, as shown by the top score after the folding test. There was no significant difference between the alkali-made and acid-made protein isolates from gutted herring.

Table 7. Protein gel characteristics.

	Study I		Study III	
	Alkali-made protein isolate	Acid-made protein isolate	Alkali-made protein isolate	Alkali-made protein isolate
Precipitation pH	5.4	6.1	6.5	5.5
Gel strength (g)	810 ± 30^a	830 ± 50^a	1390 ± 40^c	1170 ± 110^b
Deformation (mm)	9.8 ± 0.7^a	10.7 ± 0.7^{ab}	13.6 ± 0.9^c	11.5 ± 0.9^b
Folding (scoring)	5 ± 0^a	5 ± 0^a	5 ± 0^a	5 ± 0^a
Water content (%)	70.8 ± 0.2^a	71.3 ± 0.3^a	71.2 ± 0.4^a	70.7 ± 0.3^a
Expressible moisture (%)	3.0 ± 0.1^a	3.6 ± 0.3^a	2.8 ± 0.3^a	3.1 ± 0.2^a

Different letters within a row indicate significant differences ($p<0.05$)

Both the gels made from herring fillet proteins had higher gel strength than the gels made from gutted herring proteins. This may be because the proteins isolated from gutted herring were less pure, with a higher lipid content (**Table 4**, page 38). Further, the gel made from herring fillet proteins precipitated at pH 6.5 was significantly stronger than the other gels that were made from proteins precipitation at the pI. According to the TEM-micrographs of the protein isolates before gel production (**Figure 11**, page 42), the protein isolate precipitated at pH 6.5 had a more homogenous protein network with smaller pores and thinner protein strands in the network than the proteins precipitated at pH 5.5. This improved network was thought to be the reason for the increased gel strength. As earlier reviewed [6, 8], previous studies have most often shown increased gel strength after alkaline pH-shift processing compared to acid pH-shift processing. However no such difference was seen in study I, which was the only part of this thesis where gels from alkali-made and acid-made protein isolates were compared.

Protein salt solubility

The salt solubility of proteins isolated by pH-shift processing has been shown in this thesis (studies I, III and IV, **Figure 15**) and several other studies to be drastically lowered [13, 134, 200]. The results of study III showed that the pH to which the proteins have been subjected (the "pH history") highly influenced the salt solubility. Solubilization of the proteins at pH 11.2 followed by precipitation at pH 6.5, *i.e.* a pH close to that of the un-processed herring mince, resulted in a drop in salt solubility of about 20 percentage
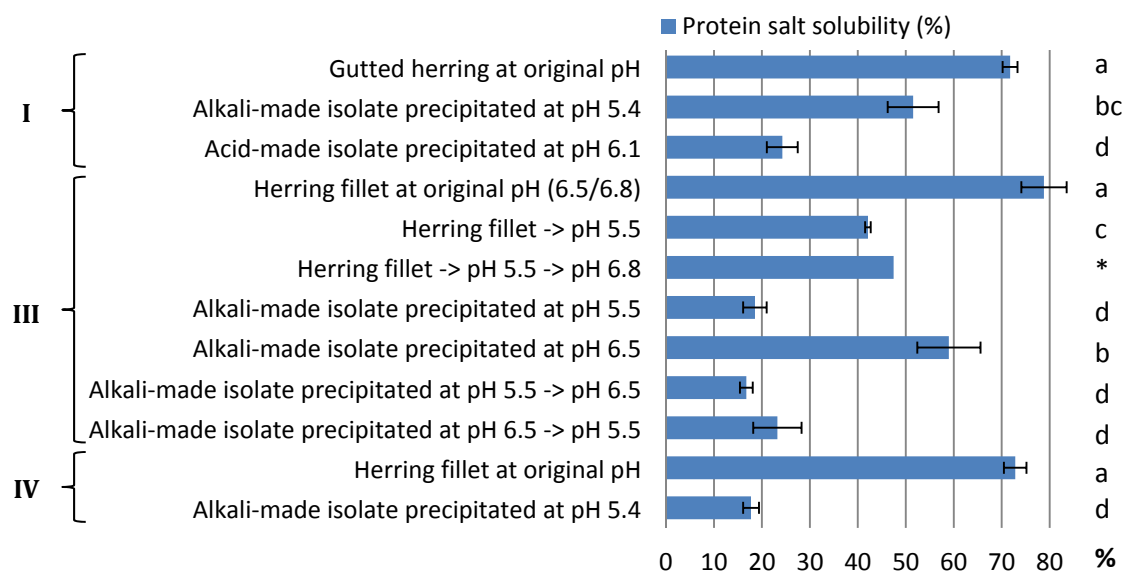

*Figure 15. Proteins' salt solubility (i.e. solubility in a 1.0 M LiCl solution after homogenization and centrifugation) in % of total protein concentration. I, III and IV denotes the study. Different letters indicate significant differences (p<0.05), * indicates a single sample that was excluded from statistical analysis. -> indicates a pH adjustment.*

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points. A subsequent lowering of the pH to precipitate proteins at pH 5.5 (*i.e.* according to the "classic" pH-shift method) further lowered the salt solubility by approximately 40 percentage points. A similar reduction in salt solubility was seen when the herring mince was adjusted from original pH to pH 5.5. Further, adjusting the proteins precipitated at pH 6.5 down to pH 5.5 led to a similar decrease in salt solubility. This last test ruled out the possibility that precipitation of different proteins at pH 5.5 and 6.5 causes the lowering in salt solubility. The lowering to pH 5.5 *per se* also seems to be a more important factor for the altered salt solubility than the alkaline solubilization process. Other authors have discussed the changed protein structure induced during the solubilization to be the reason for the low salt solubility [13, 134, 200] which based on these results seems only partly valid. It was also noted that readjustment of a protein isolate precipitated at pH 5.5 or herring mince subjected to pH 5.5 to a higher pH did not recover the salt solubility.

As part of study III, the lowered salt solubility of the protein isolates was further investigated using an extended solubility test (**Figure 16**). The lower salt solubility seen in protein isolates precipitated at pH 6.5 compared to the herring mince was a result of an increase in hydrophobic interactions ($p<0.05$). This was in line with the CLSM results (**Figure 13**) as well as other studies, showing that both the alkaline and acid solubilization caused proteins to increase their surface hydrophobicity [10, 11, 127]. Alkaline solubilization followed by precipitation of proteins at pH 5.5 further induced a significant ($p<0.05$) increase in hydrogen bonds, hydrophobic interactions and S-S bridges as well as a decrease in electrostatic bonds, see **Figure 16**.
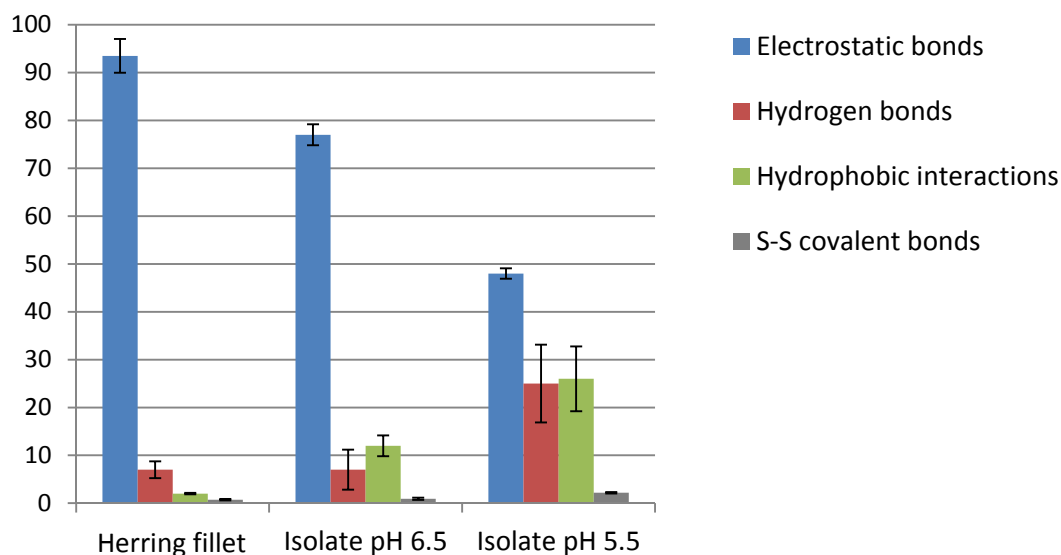

Figure 16. *Distribution of bonds between proteins in herring fillet (pH 6.8) and alkali-made protein isolates precipitated at pH 6.5 and 5.5. The bonds were identified by sequential solubilization of proteins in 1. 0.6M NaCl, 2. 0.6 M NaCl and 1.5M urea, 3. 0.6M NaCl and 8M urea, and 4. 0.6M NaCl, 8M urea and 0.5 M β-mercaptoethanol.*

Considering that the "pH history" of proteins seems to be a very strong determinant for protein salt solubility, it is surprising that the alkali-made protein isolate from study I had a salt solubility that was only about 20 percentage points lower than the gutted herring mince used in the process (**Figure 15**). It is possible that the lipids and components in the ash (**Table 4**, page 38) present in the gutted herring protein isolate have interfered with the interactions between proteins, and thereby have hindered the formation of bonds that reduce the salt solubility. The acid-made protein isolate of study I, however had a lower salt solubility. It is therefore possible that somewhat different mechanisms operate in the acid and alkaline pH-shift process versions. It has been suggested that sarcoplasmic proteins aggregate during acid solubilization, but not during alkaline solubilization [127]. The protein interactions resulting after acid and alkaline solubilization may therefore differ. The sarcoplasmic proteins could possibly act as a link between proteins and facilitate the protein-protein interactions after acid solubilization. More research would be needed to determine this.

The salt solubility of muscle proteins has been considered an index of their functionality, the latter being related to protein structure and denaturation [14, 15]. However, despite a very low salt solubility, the proteins isolated with the pH-shift process still had good gelling properties (see *protein functionality*, page 20). Data in this thesis suggest that the reduced salt solubility of proteins isolated with the pH-shift process was related to the formation of bonds stronger than electrostatic, but only to a certain degree to protein microstructure (**Figure 12**), gel forming ability (**Table 7**), and protein digestibility in an *in vitro* model (later discussed). Although previous studies of *e.g.* frozen storage of fish muscle have linked protein salt solubility to protein functionality, the link appears to be lower for heavily restructured proteins such as the protein isolates. The use of salt solubility as a crude measure of protein functionality and quality is thus questioned and should be further studied.

Further, the protein functionality aspect investigated during the course of this thesis has been protein gel formation, in which the proteins are heated. Heating could break many of the non-covalent bonds formed between proteins at pH 5.5, masking potential differences that would possibly show up in other functionality tests. It has been suggested that the right balance between salt solubility, hydrophobicity and sulfhydryl content of a protein mixture is more important for its functional properties than high values of each of these individual parameters [201]. Different combinations of freezing, pH adjustment and heating of the salt soluble protein fraction isolated from rockfish (*Sebastes*) led to differences in functional properties. Higher pH (pH 6.5) lead to improved emulsifying properties, while lower pH (pH 5.5) led to increased fat binding properties [201]. As the precipitation pH largely affected the salt solubility of the protein isolate, precipitation at different pH values during pH-shift processing may be a way to optimize a specific functional property.

Precipitation of proteins at pH 6.5 instead of 5.5 also appear promising, as it did not significantly alter the protein yield (**Table 3**), and resulted in a more homogenous

microstructure of the proteins (**Figure 12**), less lipid oxidation (**Table 5**) and improved gelation properties (**Table 7**). Further, precipitation at pH 6.5 after alkaline solubilization leads to less use of acid, and a lower content of base is needed during gel production to achieve a neutral pH.

Nutritional aspects of pH-shift protein isolation

Amino acid composition

The gutted herring and the protein isolates thereof had a high content of all essential amino acids (**Table 8**), well above amino acid requirements for adults [16]. This has also been shown for protein isolates from rainbow trout by-products [123], gutted silver carp [160] and whole krill [122]. It can be noted that the amount of all essential amino acids was slightly increased in both the acid-made and alkali-made protein isolates compared to

Table 8. Amino acid composition of gutted herring and protein isolates compared to human (adult) amino acid requirements.

	Gutted herring (mg/g protein)	Alkali-made isolate (mg/g protein)	Acid-made isolate (mg/g protein)	Recommendation for adults (mg/g protein) [16]
essential amino acids				
Histidine	25	26	27	15
Isoleuine	43	51	50	30
Leucine	70	82	81	59
Lysine	83	86	95	51
Methionine and cysteine[1]	41 (31+10)	47 (36+11)	44 (34+10)	22 (16+6)
Phenylalanine and tyrosine[1]	71	73	79	30
Threonine	40	46	44	23
Tryptophan	11	14	12	6
Valine	51	56	56	39
non-essential amino acids				
Alanine	61	53	58	
Arginine	59	64	60	
Aspargine and aspartic acid[2]	89	102	98	
Glutamine and glutamic acid[2]	130	128	143	
Glycine	65	35	38	
Proline	43	33	32	
Hydroxyproline[3]	11	<1	<1	
Serine	41	42	39	
∑ essential amino acids	436	481	488	
∑ non-essential amino acids	500	457	469	
∑ amino acids	935	938	957	

[1]Cysteine and tyrosine are conditionally essential; they can be synthesized in the body with methionine or serine and phenylalanine, respectively, as precursors [158]. [2]These amino acids are not separated in the analysis. [3]A post-translational modified proline.

the gutted herring. There were larger differences between the herring mince and the protein isolates in terms of the non-essential amino acids than the essential amino acids. Most notably, glycine and hydroxyproline were both reduced in the protein isolates (>30% change). The proline content was also reduced in both protein isolates while the tryptophan content increased in the alkali-made protein isolate compared to the gutted herring (>20% change). Hydroxyproline is a post-translational modified proline that is almost only present in collagen. Collagen is also rich in un-modified proline and glycine [202, 203]. The decrease in hydroxyproline specifically but also in glycine and proline are therefore strong indicators that collagen, that is present in large amounts in the skin, has been effectively removed during the pH-shift process. This is of high relevance when using whole fish and by-products with skin and bones. It has previously only been presumed that the skin is removed during the pH-shift process, but in this thesis removal of specific skin components was actually demonstrated. The removal of collagen has led to an increased concentration of muscle proteins, which is likely the cause for the small increase in essential amino acids. For rainbow trout and gutted silver carp, the alkaline process version resulted in isolation of a significantly higher proportion of all essential amino acids than the acid version [123, 160]. Further, solubilization at pH 13 resulted in a higher concentration of several essential amino acid than solubilization at pH 12 [123]. Thus a higher solubilization pH than pH 11.2 might have been needed to elucidate any difference between the acid and alkaline process versions for gutted herring.

In addition to the amino acids bound in proteins, herring contains a certain amount of free amino acids. Study IV revealed 13 free amino acids of ≥0.05g/kg in herring fillet, with the highest content found for taurine (more correctly a sulfonic acid), histidine and alanine (1.4, 1.1 and 0.4g/kg, respectively). However, the alkali-made protein isolate from this herring contained only about 10% of the histidine and taurine, and no other free amino acids were above the detection limit. It is expected that the free amino acids were diluted during the pH-shift process, as they are likely to be diluted in the added water and then to a large extent removed with the second supernatant. However, as the water contents of the protein isolates were high, a small fraction of the free amino acids remained. As taurine is ascribed positive effects against cardiovascular disease [67], its removal constitutes an unavoidable drawback of the pH-shift process. Removal of free histidine on the other hand could be valuable due to its involvement in histamine poisoning [68], especially if processing fish of the *Scromboid* family.

Digestion

To ascertain wheter the documented changes induced during the alkaline pH-shift process resulted in any effect on the digestibility of the proteins, minced herring fillets and alkali-made protein isolate were subjected to an *in vitro* GI digestion (study IV). There was no difference however in the DH (degree of hydrolysis) between the digests from herring mince and alkali-made protein isolate (**Figure 17**). Further there was no significant difference in DH between the mildly heat treated and raw samples after digestion. In

addition, a control experiment revealed that the proteolysis of the herring-derived samples was similar to that of casein (DH 14.7%) using the same *in vitro* digestion method.
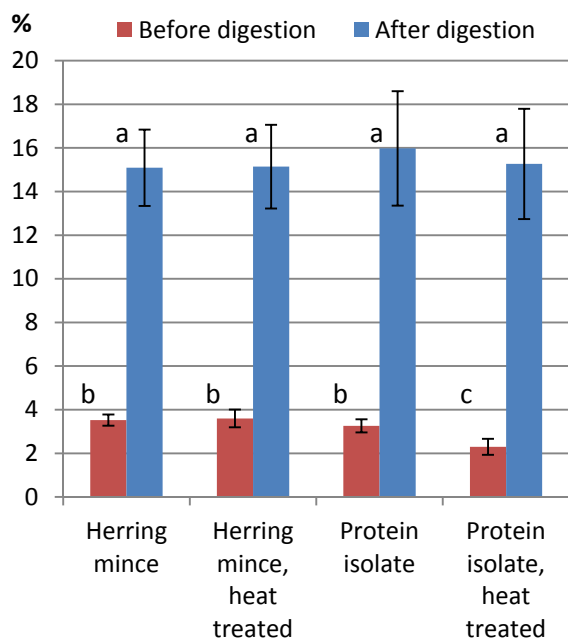

Figure 17. The DH (%) of proteins before and after in vitro gastrointestinal digestion. Different letters indicate significant differences (p<0.05).

The polypeptide profile of herring mince and alkali-made protein isolate before digestion, after the stimulated stomach step and after full digestion is shown in **Figure 18**. These results indicate that some peptides were broken down to different extents during digestion of unprocessed herring mince and alkali-made protein isolates. Some differences were observed already after the stomach step, and after the full digestion two peptides of 33 and 36 kDa remained to a larger extent in the digested protein isolate. These peptides might have undergone some modification during the alkaline pH-shift process that rendered them less accessible to the digestive enzymes.

SDS-PAGE at higher protein concentrations indicate that the 33 and 36 kDa peptides were also present in the herring mince digests but at lower concentration. It is therefore likely that the modification did not totally inhibit the proteolytic degradation. As S-S bonds were shown to lower the salt solubility in protein isolates subjected to pH 5.5 (**Figure 16**, page 48), these bonds are possibly the cause for the lower digestibility of certain peptides after alkaline pH-shift processing.

The SDS-PAGE analysis also indicated a lower content of very small peptides in the digest of the herring mince than in the protein isolate digest, possibly a result of smaller peptides in the former passing through the gel. To investigate this, the content of peptides <3kDa and free amino acids was determined in the digests of raw herring mince and protein isolate. The results showed that the release of small peptides and free amino acids (corrected for the amount of free amino acids in the starting material) during digestion was similar and thus did not indicate any differences in digestibility between the herring mince and protein isolate.
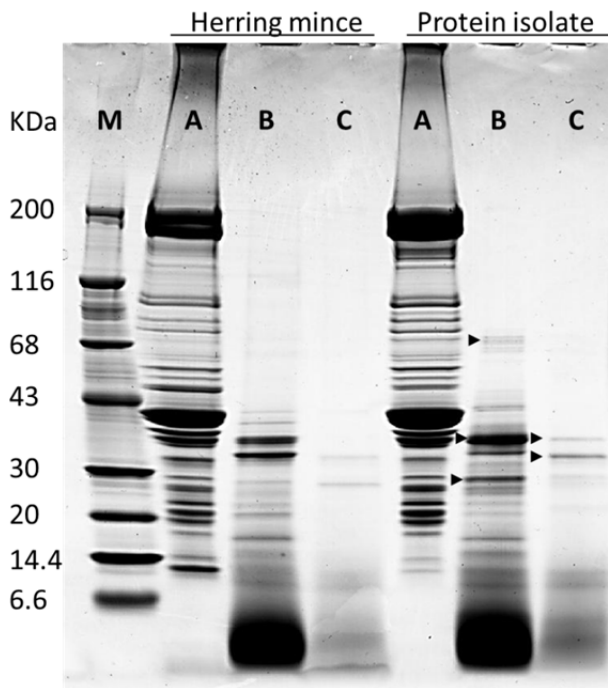
Figure 18. *Polypeptide profile of herring mince and protein isolate from different steps of in vitro GI digestion.*
M –Molecular weight marker,
A – sample before digestion, B – sample after simulated stomach step, i.e. digestion with pepsin, C – sample after full in vitro gastrointestinal digestion, i.e. stepwise digestion with pepsin and pancreatin. Arrowheads indicate bands mentioned in the discussion

The present results were in accordance with a previous animal study on alkali-made protein isolate from krill, where the PDCAAS was the same as that of the reference protein casein [162]. The results of this thesis generally showed that there were no differences between herring mince and alkali-made protein isolate in DH, release of free amino acids and peptides <3kDa, indicating that fish proteins retain good digestibility after alkaline pH shift processing. This in combination with the high content of essential amino acids suggests that the pH-shift process is very promising as a method to isolate proteins of high nutritional quality.

CONCLUSIONS

Protein isolation with the pH-shift process is a promising strategy to increase utilization of small pelagic fish for human consumption. Protein isolates should generally be of high protein content, free from environmental contaminants, and have good functional and nutritional properties. The results presented in this thesis suggest that a pH-shift produced herring protein isolate can meet all these demands. Specifically:

o Protein isolation from whole gutted herring by pH-shift processing gave high protein yields (57-59%), and a significant reduction of lipid and ash content. No significant differences were observed between the acid and the alkaline versions of the pH-shift process regarding protein yield and major constituents of the proteins isolates. Furthermore, the protein isolates had similar gel-forming abilities and the color of the pH-shift-produced proteins had an increased lightness and whiteness compared with the raw material. This effect was more pronounced in acid-made isolates.

o The pH-shift process was an efficient way to remove dioxins and PCBs from gutted herring proteins, and the removal was highly correlated to the removal of lipids. Furthermore, all dioxin and PCB congeners studied were similarly removed during the pH-shift process. Addition of 5% ethanol led to a lower lipid and contaminant content in the protein isolate.

o The microstructure of herring proteins was greatly changed by the alkaline pH-shift process and a loosely structured protein network with no remaining myofibrillar structure was formed. The microstructure of the herring mince was also largely affected by freezing, which ruptured the myofibril structure.

o The protein salt solubility was drastically lowered by the pH-shift process, which was primarily a result of subjecting the proteins to a pH close to their pI (pH 5.5) and only secondly to the alkaline solubilization process. The alkaline solubilization process increased the hydrophobic interactions between the proteins, while precipitation at pH 5.5 led to the formation of hydrogen bonds, hydrophobic interactions and S-S bridges.

o The alkaline pH-shift process with precipitation at pH 6.5 produced a protein isolate superior to that produced at pH 5.5 with a higher protein salt solubility. It also improved gelation capacity and gave less lipid oxidation.

o The pH shift process had little effect on the essential amino acid composition of the herring proteins. Furthermore the alkaline pH-shift process did not induce protein oxidation and had a minor effect on the protein digestibility. These results indicate that the high nutritional quality of fish proteins is retained after alkaline pH-shift processing.

FUTURE OUTLOOK

The pH-shift process is a promising method for isolating proteins from small pelagic fish and by-products and likely has a future in increasing the usability of such complex materials. During the years of the work on this thesis, the amount of research on the pH-shift process has greatly increased and also changed, from focusing on isolated myosin and fillets to complex materials. There are many aspects of the process in which continued research is suggested. It is also time for the pH-shift protein research to be further adopted by the industry and pH-shift-made protein isolates to be included in their product development. Furthermore, consumer acceptance should be considered, which might call for research in another discipline than food science.

Research on the pH-shift process has mainly focused on the protein fraction of the fish. However, there are also by-products of this process. The ultimate goal must be to use everything, and especially not to waste the lipids. Hopefully in the future the sediment and floating fat layer will be further processed into edible oil and a mineral-rich fraction for *e.g.* animal feed. The proteins in the waste water should also be isolated and the water reused.

There are certain factors that need to be improved to further increase the use of complex materials in the pH-shift process, among which color is one of the most important. It would be very valuable to increase the lightness and whiteness of the isolated proteins without impairing other parameters. Taste and odor are other sensory attributes of pH-shift-produced protein isolates where very little research has been done, and where it is needed.

Methods to further reduce the lipid content would be of interest. Addition of ethanol was proven promising, but needs further evaluation. Lower lipid contents are needed than the ones obtained in these studies to perform animal or human studies on possible health effects of herring protein isolates without the confounding factor of n-3 PUFA.

The microstructure of the protein isolates indicated that there was a difference between isolates made from fresh and frozen raw material. It would be of interest to see the effect of raw material treatment on the functionality and stability of the proteins. Pre-freezing has been shown to impair the yield in pH-shift processing, but the effect on the functionality of the isolated proteins has not been studied.

Further investigating whether salt solubility of proteins isolated using the pH-shift process is related to other functional properties such as foaming and emulsification is another area of interest. Studies in this area should start with optimization of the salt solubility method for such restructured materials as the protein isolates.

The digestibility results showed at least two peptides that were less degraded after pH-shift protein isolation than in the herring mince. It would be valuable to identify these peptides and how they are modified.

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