THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Cross-Processing Fish Co-Products with Plant Food Side Streams or Seaweeds Using the pH-Shift Method

- a new sustainable route to functional food protein ingredients stable towards lipid oxidation

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Abstract

The seafood value chain is highly inefficient as 50-60% of the fish weight end up as co-products in the filleting operation. Despite their abundance in high-quality proteins, fish co-products mainly go to low value products such as fodder. The pH-shift process, *i.e.*, acid/alkaline solubilization followed by isoelectric precipitation, is an opportunity to instead recover these proteins in a food grade manner while maintaining their functionality. A challenge when subjecting hemoglobin-rich fish raw materials to pH-shift processing is however oxidation of polyunsaturated fatty acids (PUFAs).

This thesis investigated, for the first time, cross-processing of fish co-products with antioxidant-containing support materials ("helpers") to protect the fish protein isolates from lipid oxidation in a clean label and sustainable manner. The helpers, including locally sourced plant food side streams (press cakes from lingonberry (LPC) and apple, barley spent grain, oat fiber residues), shrimp shells, and seaweeds, were also expected to introduce new characteristics to the protein isolates.

All helpers, except shrimp shells, reduced lipid oxidation in herring/salmon co-products when added at 30% (dw/dw) at start of the pH-shift process. LPC was the most effective, and even at 2.5% addition it prevented volatile aldehyde formation during production of herring protein isolates while at 10% addition, the isolates were also stable towards oxidation for $\geq$8 days on ice. When the 10% LPC instead was added during protein precipitation, the oxidation lag phase was extended to 21 days. The oxidative stability of protein isolates correlated with their total phenolic content, and the very high antioxidant ability of LPC's was mainly attributed to anthocyanins, *e.g.*, ideain and procyanidin A1.

LPC also improved the water solubility, emulsifying activity, and gel-forming capabilities of herring protein isolates, expanding their potential applications in food products. The water solubility and emulsifying activity were also boosted by adding shrimp shells and *Ulva*, while the gel-forming ability was also enhanced by apple press cake. LPC-derived anthocyanins resulted in red isolates under acidic conditions and dark-colored isolates under neutral/alkaline conditions. *Ulva* resulted in green isolates due to the presence of chlorophyll. The color of protein isolates was also affected by oxidation of fish-derived pigments like Hb and astaxanthin. The addition of helpers also influenced the composition of protein isolates. LPC added at the start of the process reduced lipid content, while shrimp shells and LPC added during precipitation increased it. Seaweeds raised ash content by introducing minerals.

Additionally, the organic acids of LPC saved the use of HCl in acid-aided protein solubilization and in isoelectric precipitation of alkali-solubilized proteins. During the latter, adding 30% LPC decreased HCl usage by as much as 61%. Opposite, alkaline protein solubilization in presence of LPC required more NaOH than the control, but this issue was naturally less pronounced at low LPC additions. Another challenge of introducing helpers was that they reduced total protein yield in the pH-shift process. This was however successfully mitigated by optimizing solubilization/precipitation pH, increasing water addition, and employing more powerful high shear homogenization and ultrasound techniques.

In summary, this thesis introduced a completely new concept of cross-processing fish co-products with antioxidant-containing food materials, significantly reducing lipid oxidation and enhancing protein isolate techno-functionalities. Herring co-products paired with 10% LPC was particularly promising. Beyond its technical advantages, cross-processing can add economic value to side streams of both fish and other food industries, while stimulating circularity and industrial symbiosis. Altogether, these features reduce food chain losses and promote a more sustainable food system.

Keywords: fish protein, by-products, pomaces, press cakes, fruit, berry, valorization, lipid oxidation, natural antioxidants, protein gels, protein techno-functionality.

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LIST OF PUBLICATIONS

This dissertation is based on the work described in the following papers:

Paper I Abdollahi, M., Olofsson, E., **Zhang, J.**, Alminger, M., and Undeland, I. (2020). Minimizing lipid oxidation during pH-shift processing of fish co-products by cross-processing with lingonberry press cake, shrimp shells or brown seaweed. *Food Chemistry, 327*: 127078.

Paper II **Zhang, J.**, Ström, A., Bordes, R., Alminger, M., Undeland, I., and Abdollahi, M. (2023). Radial discharge high shear homogenization and ultrasound assisted cross-processing of herring co-products for maximum protein yield and functionality. *Food Chemistry, 400*: 133986.

Paper III **Zhang, J.**, Alminger, M., Abdollahi, M., and Undeland, I. (2022). Cross-processing herring and salmon co-products with agricultural and marine side-streams or seaweeds produces protein isolates more stable towards lipid oxidation. *Food Chemistry, 382*: 132314.

Paper IV **Zhang, J.**, Hong, B., Abdollahi, M., Wu, H., and Undeland, I. Towards circular food production: Investigating the effects of lingonberry press cake on oxidative stability and yield in herring co-product protein isolation. *Submitted*.

Paper V **Zhang, J.**, Abdollahi, M., Ström, A., and Undeland, I. (2023). Lingonberry (*Vaccinium vitis-idaea*) press-cake as a new processing aid during isolation of protein from herring (*Clupea harengus*) co-products. *Food Chemistry: X, 17*: 100592.

OTHER RELEVANT PUBLICATIONS

In addition to the appended papers, I have authored or co-authored the following published papers not included in this dissertation:

Paper 1 Coelho, C. R. V., Peters, G., **Zhang, J.**, Abdollahi, M., and Undeland, I. (2023). Fish beyond fillets: Life cycle assessment of cross-processing herring and lingonberry co-products into a food product. *Resources, Conservation and Recycling, 188*: 106703.

Paper 2 Coelho, C. R. V., Peters, G., **Zhang, J.**, Hong, B., Abdollahi, M., and Undeland, I. (2022). A comparative life cycle assessment of cross-processing herring side streams with fruit pomace or seaweed into a stable food protein ingredient. *Future Foods, 6*: 100194.

CONTRIBUTION REPORT

Paper I Jingnan Zhang (JNZ), as a co-author, participated partially in the experimental work, and contributed to data analysis, result interpretation, and manuscript writing.

Paper II JNZ, as the first author, was involved in the study design, executed the experimental work, collected and analyzed the data, interpreted the results, and drafted and revised the manuscript.

Paper III JNZ, as the first author, was involved in the study design, executed the experimental work, collected and analyzed the data, interpreted the results, and drafted and revised the manuscript.

Paper IV JNZ, as the first author, made partial contributions to the experimental work and supervised a master's student who carried out the remaining experiments. JNZ conducted data analysis, interpreted the results, and drafted and revised the manuscript.

Paper V JNZ, as the first author, was involved in the study design, executed the experimental work, collected and analyzed the data, interpreted the results, and drafted and revised the manuscript.

ABBREVIATIONS

ANS	1-anilino-8-naphthalene sulfonate
DNPH	2,4-dinitrophenylhydrazine
HHE	4-hydroxy-(E)-2-hexenal
HNE	4-hydroxy-(E)-2-nonenal
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
ANOVA	Analysis of variance
APC	Apple press cake
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAR/PDMS	Carboxen/polydimethylsiloxane
DHA	Docosahexaenoic acid
dw	Dry weight
EPA	Eicosapentaenoic acid
EAI	Emulsifying activity index
ESI	Emulsion stability index
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and agriculture organization
GAE	Gallic acid equivalents
GC-MS	Gas chromatography-mass spectrometry
Hb	Hemoglobin
HS-SPME	Headspace solid-phase microextraction
HPLC-UV/VIS	High-performance liquid chromatography-ultraviolet/visible
HSMH	High shear mechanical homogenization
CIE	International commission on illumination
pI	Isoelectric point
LPC	Lingonberry press cake
LOX	Lipoxygenase
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LC PUFAs	Long chain n-3 polyunsaturated fatty acids
LMW	Low molecular weight
MDA	Malondialdehyde
Mb	Myoglobin
MHC	Myosin heavy chain

PV	Peroxide value
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
STPP	Sodium tripolyphosphate
SPME	Solid phase microextraction
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TPC	Total phenolic content
TCA	Trichloroacetic acid
WHC	Water holding capacity
ww	Wet weight

CONTENTS

1

Introduction

The global demand for high-quality protein sources is constantly increasing due to the rapid growth of the human population and rising awareness of the importance of healthy and sustainable food choices [1]. Fish from sustainable wild stocks or aquaculture provides valuable sources of protein, and also offer long chain n-3 polyunsaturated fatty acids (LC n-3 PUFAs) and important micronutrients [2], thereby possessing considerable potential to meet the growing protein demand [1]. However, the seafood industry primarily focuses on fish fillets, consequently generating significant amounts of highly nutritious co-products such as heads, viscera, and frames [3]. While some of these co-products are used for the production of low- or medium-value products such as animal feed, aquafeed, and pet food, others are discarded [4].

A promising approach to valorize fish co-products is the extraction of functional proteins via the pH-shift method, alternatively referred to as pH-driven solubilization and isoelectric precipitation [5].

This method is based on the high solubility of muscle proteins at extreme acid or alkaline pH levels, and conversely, their low solubility at the isoelectric point [6, 7]. The process thus allows for the selective separation of solubilized proteins from non-soluble fractions such as bones, connective tissue and lipids present in fish co-products[5, 8].

pH-shift processing of fish has been extensively researched over the years in terms of its ability to up-concentrate proteins with preserved or improved amino acid composition, as well as retained protein functionality and digestibility [5, 9]. However, a primary concern is the high susceptibility of fish lipids to heme-mediated oxidation, which can lead to rancidity, off-flavors, and reduced nutritional value [8]. Moreover, oxidation can affect the color and techno-functional properties of extracted proteins, including solubility, emulsification, and gelation [5, 9]; all crucial for their applicability in various food products. A handful of previous studies have tackled oxidation during pH-shift processing using erythorbate, sodium tripolyphosphate (STPP), ethylenediaminetetraacetic acid (EDTA) [10], and polyphosphate [11]. Promising results have been revealed both during the process itself [10] and in subsequent ice/frozen storage of isolates [10, 11]. However, the use of these compounds does not align with current consumer wishes for clean label, *i.e.*, no or few food additives.

To address the challenge of pH-shift process-induced lipid oxidation in a completely new and clean label manner, this thesis investigates, for the first time, the hypothesis that cross-processing of fish co-products with various non-fish support raw materials ("helpers") rich in natural antioxidants could protect the fish protein isolates against lipid oxidation while also bringing in other values. Considering the production volumes in Sweden as well as the levels of phenolic compounds, carotenoids, and other molecules with antioxidative properties, berry and fruit pomaces (*i.e.*, juice press cakes), barley spent grain, oat fiber residues, shellfish shells as well as seaweeds were considered promising helper candidates at the start of this project. These support materials were expected to also impart new nutrient characteristics and techno-functionality to the protein isolates, paving the way for value-added applications. Additionally, it was hypothesized that *e.g.*, phenolic compounds and organic acids of the helpers could also benefit protein precipitation. On a societal level, the cross-processing of *e.g.*, plant and fish co-products was expected to stimulate industrial symbiosis and circular economy. Also, by developing new efficient and sustainable food processing strategies, this thesis may contribute knowledge to enhance the value of currently underutilized marine and agricultural resources and promote food security in the face of increasing global demands.

2

Objectives and hypotheses

The goal of this PhD thesis was to investigate, for the first time, the potential of adding natural antioxidant-containing support materials ('helpers') during pH-shift processing of fish co-products to limit lipid oxidation and introduce new characteristics to the resulting protein isolates in a sustainable and clean-label manner.

Based on their local production volumes in Sweden as well as their antioxidant potentials, selected helpers were lingonberry press cake (LPC), apple press cake (APC), barley spent grain, oat fiber residues, shrimp shells, brown seaweed (*Saccharina latissima*), and green seaweed (*Ulva fenestrata*).

The specific objectives of the thesis were:

- **To investigate if adding helpers during the pH-shift process could:**
    - **Mitigate lipid oxidation during the processing *per se* and subsequent ice storage of protein isolates:** The hypothesis was that *e.g.*, polyphenols and carotenoids extracted from the helpers with water/acid/base and high shear mechanical homogenization can limit fish lipid oxidation.
    - **Influence protein yields:** The hypothesis was that helper-derived phenolics can cause the precipitation of fish proteins, thereby hampering protein solubilization if added at the start of the process but facilitating protein precipitation if added during the precipitation step.
    - **Change consumption of acid and base solutions:** The hypothesis was that helpers rich in organic acids, *e.g.*, LPC can change the amount of acid/base solution required in the process depending on the step at which they are added.
    - **Influence color of protein isolates:** The hypothesis was that helper-derived pigments could directly change the color of isolates and indirectly protect fish-derived pigments from oxidation.
    - **Alter composition of protein isolates:** The hypothesis was that helper-derived compounds *e.g.*, phenolic compounds, polysaccharides, salts and acids could en-

hance the lipid removal capacity of pH-shift process, while also potentially diluting the protein content of isolates.

- **Influence techno-functional properties of protein isolates:** The hypothesis was that helpers can modify the dynamics of protein unfolding/refolding, and introduce *e.g.*, phenolic compounds, polysaccharides, and phospholipids, which can interact with proteins and/or influence protein-protein plus protein-water interactions.

- **To identify the most promising combinations of fish co-products and helpers.** All factors influenced by the helpers were synthesized in order to achieve minimal lipid oxidation and acid/base solution consumption as well as maximal protein yields, protein up-concentration and protein isolate techno-functionalities. Regarding color, no specific direction was predetermined, although lightness is typically preferred in the seafood industry.

3

Background

3.1 Fish consumption growth: confronting supply limitations and sustainability

Over the past few decades, there has been a significant increase in global fish consumption, driven primarily by population growth, rising incomes, and urbanization [1]. Fish and seafood are important dietary components due to their high nutritional value, providing LC n-3 PUFAs, high-quality proteins, vitamins, and minerals crucial for human health [2]. Overall, increased awareness of the health benefits associated with the consumption of fish and seafood has further contributed to the rising demand [1]. According to the Food and Agriculture Organization (FAO), the average global per capita fish consumption has increased from 9.0 kg in 1961 to 20.5 kg in 2018, with projections indicating a continuous upward trend in the coming years [1].

Despite the growing demand for fish-based seafood, the global fish supply has been facing increasing constraints. Wild fisheries have stagnated at around 90 million tonnes for many years (see Figure 3.1), and further attempts to increase it could lead to the collapse of fish stocks. FAO reported that 35.4% of the assessed fish stocks were overfished in 2019, while only 64.6% were within biologically sustainable levels [1]. Although aquaculture has emerged as a viable alternative to meet the rising demand, it also faces challenges such as disease outbreaks and resource competition for feed ingredients [1]. Thus, the limited availability of fish supply has become a pressing concern in the context of global food security and nutrition [1].

Despite an increasing demand for fish and seafood, a considerable portion of the global fish catch is still diverted to the production of fishmeal and fish oil, which are utilized as essential ingredients in aquaculture, livestock, and pet food industries [12]. In 2018, approximately twenty million metric tons of captured fish (around 27% of the total catch) were directly used for the production of fishmeal and fish oil [1]. On top of this comes all the filleting rest raw materials emerging in the processing of the fish dedicated to food. In Sweden, as much as 85% of the landed fish is converted to feed; when counting both so-called “fodder fish” and the rest raw materials emerging from seafood processing [13]. This practice exacerbates the pressure on wild fish stocks and reduces the direct availability of fish for human consumption [13].

Moreover, the fact that fishmeal and fish oil production is mainly built upon the use of small pelagic fish species that are critical to marine ecosystems and serve as a primary food source for numerous marine predators is problematic from an ecological perspective [13]. Consequently, the large-scale use of captured fish for non-food purposes has implications for both food security and the sustainability of marine ecosystems [1, 13].

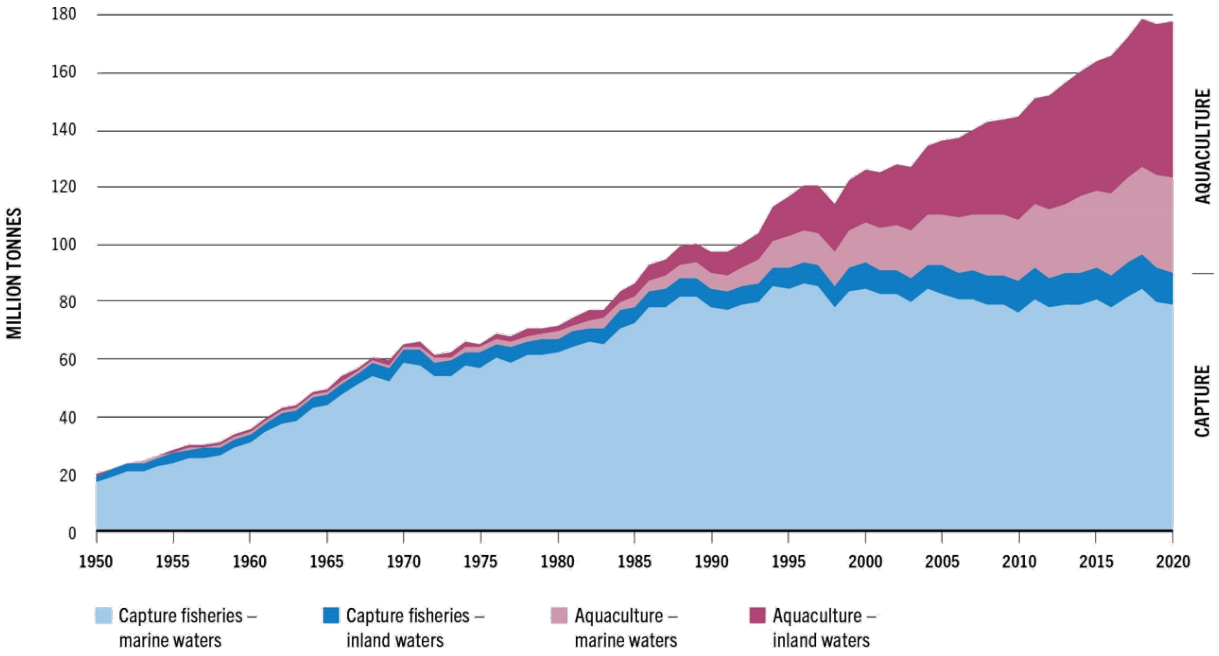

Figure 3.1: Annual production of fisheries and aquaculture from the years 1950 to 2020 [1].

3.2 Catch, harvest and early handling of wild-caught and farmed fish

The processes involved in early handling of wild caught fish *vs.* harvested aquacultured fish differ significantly, with implications for product quality, safety, and sustainability. Wild-caught fish targeted for human consumption are harvested from many different habitats, including oceans, seas, and inland water bodies. Various fishing techniques, such as trawling, purse seining, longlining, and gillnetting, are hereby employed [14]. Proper handling and storage practices, including rapid chilling and icing, are crucial during the early stages of capture to ensure product quality and safety [15]. However, the efficiency of these practices may be influenced by factors such as fishing duration, weather conditions, and vessel facilities [15] Additionally, bycatch and discarding of non-target species are common issues in wild capture fisheries, raising concerns about environmental sustainability and resource waste [4]. Fish produced through aquaculture is a more controlled process, not least in terms of feed. The quality thereby becomes more even than for wild caught fish. The harvesting of farmed fish typically involves techniques such as seine netting, dip netting, or fish pumps, followed by immediate stunning and bleeding to ensure product quality and minimize stress [16]. For both wild caught and aquacultured fish, proper handling during transportation and storage is essential, as any temperature fluctuations

or physical damage will affect the quality and shelf life of the final product [15, 16].

3.3 Fish filleting co-products

Filleting has become an increasingly used process step in the preparation of fish for human consumption, yielding desirable clean fish meat portions while generating a significant amount of co-products [4]. These co-products can make up 30-85% (w/w) of the whole fish [5], the exact amount depending on species and filleting technique, which include heads, viscera, skins, and bones (Figure 3.2) [5]. Although traditionally considered waste, there is now a growing recognition of the potential value of these co-products in diverse applications such as fishmeal, fish oil, animal feed, pet food, functional compounds such as bioactive peptides, chitin and chitosan, and biodegradable materials production [17]. This is because they are rich sources of high-quality proteins, LC n-3 PUFA, enzymes, vitamins and minerals [17, 18]. However, a significant portion of the co-products emerging globally remains underutilized for food production [1]. One reason for this is the high sensitivity of fish co-products towards lipid oxidation, stemming from the abundance of blood-derived and other pro-oxidants, lipids, and hydrolytic enzymes [19]. Often, all the different co-product cuts are mixed in one container, enhancing this problem further. Thus, in order to successfully apply valorization technologies to co-products like meat/bone separation, enzymatic hydrolysis and the pH-shift process, such processes much be combined with active cooling, pre-sorting technologies, and tailored antioxidant strategies [15, 16].

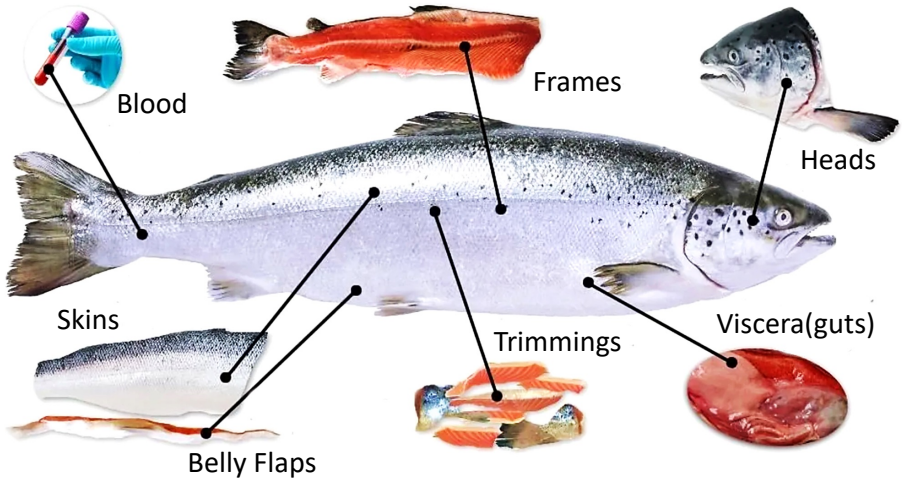

Figure 3.2: Different parts of salmon co-products [20].

Annually, Swedish seafood processing industries produce around twenty-five thousand metric tons of co-products [13] which primarily derive from herring (*Clupea harengus*) and salmon (*Salmo salar*) [13].

Herring is a significant commercial fish species with substantial global catches, particularly from the Atlantic Ocean and the Baltic Sea [1]. Herring ranks among the top species in terms

of global fish catch, with a live weight of 1.82 million tons in 2018 [1]. Herring co-products can make up to approximately 60% of the fish [19].

Salmon is a fatty fish primarily produced through aquaculture operations [1]. It was one of the major species produced in world aquaculture in 2018, accounting for 2.44 million tons [1]. When consumed as food, typically only the fillets, which make up around 55% of the total fish, are used, resulting in 45% of salmon co-products [20]. There is a growing emphasis on hand-scraping of mince from salmon backbones and on extracting value-added compounds for various applications.

3.4 Post-mortem changes in fish and fish co-products

Post-mortem changes in fish and fish co-products significantly affect their quality, nutritional value, and shelf life [21]. Understanding and controlling these changes is crucial for optimizing the utilization of fish and their co-products, ensuring food safety, and minimizing waste.

Autolytic changes refer to the biochemical processes initiated by the fish's endogenous enzymes after death [21]. These enzymes, such as proteases, lipases, and glycolytic enzymes, can degrade proteins, lipids, and carbohydrates, respectively, leading to the deterioration of texture, flavor, and nutritional value [21]. In fish co-products, autolytic changes can also affect the extraction and functionality of proteins, peptides, and lipids [22]. To minimize autolytic changes, rapid cooling and proper storage conditions are essential [21, 22]. Moreover, the application of enzyme inhibitors or the inactivation of endogenous enzymes through heat or other processing methods can help control autolytic degradation in fish and fish co-products [17, 21, 22].

Oxidative changes, particularly lipid oxidation, are significant concerns for fish and fish co-products, as they contain high levels of PUFA that are susceptible to oxidation combined with powerful pro-oxidants [2, 19]. Lipid oxidation can lead to off-flavors, changes in texture and color, as well as nutrient loss, all of which negatively affect the quality, shelf life, and nutritional value of fish and fish products [23, 24]. In fish co-products, oxidation can also impair the stability and functionality of extracted compounds, such as oils, proteins, and bioactive peptides [22]. To control oxidative changes, fish and fish co-products should be handled, processed, and stored under conditions that minimize exposure to oxygen, pro-oxidants (*e.g.*, via bleeding), light, and high temperature [21]. Additionally, the use of antioxidants, either natural or synthetic, can be employed to protect raw materials from oxidative deterioration [19, 25].

Microbial spoilage is a primary cause of quality deterioration in fish and fish co-products, as various bacteria, yeasts, and molds can grow and produce metabolites that adversely affect the sensory properties and safety of the products [21]. The limited *postmortem* drop in pH of fish/shellfish muscle compared to *e.g.*, beef or poultry muscle make seafood extra vulnerable to microbial spoilage [21]. Other important factors are temperature, especially for cold water species, water activity, and initial microbial load on the fish surface (*i.e.*, skin) and in the co-products [21]. Effective strategies to minimize microbial spoilage include strict hygiene practices during catch, harvest, and processing, rapid chilling, and the use of preservation methods, such as modified atmosphere packaging, vacuum packaging, or the application of antimicrobial agents and natural preservatives [21].

3.5 FISH MUSCLE PROTEINS

Proteins are the primary constituents of fish muscle, contributing to its nutritional value, texture, and functional properties such as water holding [26]. Muscle proteins can be categorized into three main groups: myofibrillar proteins, stromal proteins, and sarcoplasmic proteins [26]. Each group has distinct characteristics and roles within the fish muscle, and their interactions and properties influence the quality and processing attributes of fish products.

Myofibrillar proteins, also known as contractile proteins, are the primary structural components in fish muscle fibers, accounting for approximately 50-60% of total muscle proteins [26]. The most abundant among these proteins are myosin and actin, which are responsible for muscle contraction [26, 27]. The solubility of myofibrillar proteins is affected by factors such as pH, salt and temperature [26, 28]. The myofibrillar proteins gradually dissolve below and above the isoelectric point (pI) at pH 5.5 [29]; at pH 3 and pH 11, $> 90\%$ can be soluble (see Section 5.1). In salt solutions, myosin and actin can spontaneously bind, forming a complex called "actomyosin" [28]. The thermal stability of actomyosin depends on pH and ionic strength, with proteins from warm-water species being more thermally stable than their cold-water counterparts [28]. Additional myofibrillar proteins, like tropomyosin, troponin, and titin, have regulatory roles in muscle contraction and relaxation [27, 28]. During fish processing, myofibrillar proteins play a critical role in developing desirable textural properties, water-holding capacity, and gelation characteristics [26, 28]. Moreover, they contribute to the formation of protein networks that affect the structure and stability of various processed fish products, such as surimi-based products, fish patties, and sausages [26, 28].

Stromal proteins, commonly referred to as connective tissue proteins, account for 10-15% of the total muscle proteins in fish [26]. These proteins form the extracellular matrix, which provides structural support and integrity to muscle fibers [26]. The primary stromal proteins include collagen, elastin, and reticulin, all of which are water-insoluble, regardless of the solution's pH, temperature, or ionic strength [28]. Collagen, the most abundant stromal protein, is predominantly found in the form of type I and type III collagen, contributing to the muscle's tensile strength and elasticity [26]. Texture and tenderness of muscle are highly influenced by the properties of stromal proteins, particularly collagen [26, 28]. The soft texture of fish muscle compared to *e.g.*, red meat is the result of very low levels of collagen [26, 28]. During thermal processing, collagen denatures and turns into gelatin through a process known as gelatinization, which affects the WHC, juiciness, and mouthfeel of muscle [26, 28]. On the other hand, elastin remains unaffected by heat [26]. When subjected to dry heat such as during roasting and grilling, stromal proteins can become hard and chewy, impacting the texture of fish-based food products[28].

Sarcoplasmic proteins, which account for approximately 25-30% of total fish muscle proteins, are water-soluble and play crucial roles in various metabolic processes [26]. These proteins include enzymes such as glycolytic enzymes, proteases, and lipases, as well as myoglobin (Mb) and regulatory proteins like calmodulin and calcium-binding proteins [26]. Due to their water solubility, sarcoplasmic proteins can be easily isolated through simple pressing or extraction with a low ionic strength salt solution [26, 28]. In food applications, the water solubility of sarcoplasmic proteins is minimally affected by ionic strength [26]. Sarcoplasmic proteins also contribute to the color, flavor, and oxidative stability of fish muscle [26]. Mb, for example, to-

gether with Hb of the blood, plays a significant role in determining fish muscle color [26]. Both of these proteins are especially enriched in the dark muscle [26, 28]. Furthermore, sarcoplasmic enzymes such as proteases and lipases can influence post-mortem proteolysis and lipolysis as well as spoilage in fish products [26].

3.6 Fish lipids and lipid oxidation

3.6.1 Fish lipids

Fish lipids contribute to the nutrition, taste, and quality of fish and products thereof [30]. Their levels vary greatly and can be influenced by various factors such as fish species, maturity, catch season, and feeds [31]. There are two main types of fish lipids: neutral lipids and polar lipids [32]. Neutral lipids consist primarily of triglycerides and are primarily used for energy storage, while polar lipids, like phospholipids, make up cell membranes [32]. Triglyceride levels differ largely among fish species and while lean fish muscle mainly has membrane lipids, fatty fish contain substantial levels of triglycerides [33, 34]. Lipid distribution also varies across muscle types, with most fish having higher lipid concentrations in their dark muscle compared to their white muscle since the former depends on oxidative metabolism [35]. Gadoid fish like cod and haddock store most neutral lipids in their liver [35, 36], while fatty fish such as herring and salmon have neutral lipids as droplets both inside and outside their muscle cells [35].

Fish lipids remain liquid at low temperatures due to the presence of PUFAs [37]. Up to 40% of fish lipids can be LC PUFAs, predominantly from the n-3 family [37]. This makes fish an excellent source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have been recognized for their preventive effects *e.g.*, towards inflammation [37]. Some fish lipids, like those of herring, also contain significant amounts of monounsaturated fatty acids like gondoic acid and cetoleic acid [3]. These have been ascribed effects on reducing the risk of metabolic syndrome and cardiovascular disease [38].

3.6.2 Lipid oxidation

Fish and products thereof are prone to lipid oxidation due to their high levels of PUFAs and the presence of pro-oxidants *e.g.*, Hb [23, 19]. Small pelagic fish such as herring are more prone to lipid oxidation compared to white fish due to their higher levels of heme-proteins, partly related to the larger ratio of dark muscle [39, 40].

Free radical-mediated initiation (pathway A in Figure 3.3) involves the formation of a highly reactive and short-lived fatty acid radical (L•) when a hydrogen atom is abstracted from an unsaturated fatty acid by a free radical [41, 42]. This hydrogen abstraction takes place at the methylene groups at the carbon atom between the double bonds, leading to a conjugated double bond in the fatty acid [41, 43]. Blood enzymes such as xanthine oxidase, superoxide dismutase (SOD), and peroxidases can facilitate radical-driven lipid oxidation by generating reactive oxygen species (ROS) [44]; particularly the highly reactive hydroxyl radical, •OH. Lipid oxidation can also be initiated by the enzyme lipoxygenase (LOX) (pathway B), which is present in animal tissues such as fish [25, 42]. LOX catalyzes the incorporation of oxygen into unsaturated fatty

acids (LH) [23] (Figure 3.3). LOX activity is more pronounced in the gills and under the skin of many species and is therefore concentrated in certain co-product fractions as the head [45]. LOX contributes to lipid oxidation mainly in non-frozen fish [46], and its activity is reported to be inhibited above 40°C [47]. Photo-oxidation (Pathway C) is driven by the generation of highly reactive, short-lived singlet oxygen molecules ($^1O_2$) (Figure 3.3). These molecules are formed when normal ground state oxygen molecules ($^3O_2$) absorb energy from excited photosensitizers such as heme, chlorophyll, and riboflavin (photo-oxidation type II) [48, 49]. Unsaturated fatty acids can directly react with singlet oxygen through a cyclo-addition mechanism, which is distinct from the non-reactive ground state oxygen that is restricted by the rule of spin conservation [48, 49]. In type I photooxidation, the excited photosensitizer produces ROS such as superoxide anion ($O_2^{\bullet -}$) and the protonated superoxide radical ($^{\bullet}OOH$), which then initiate lipid oxidation [49].

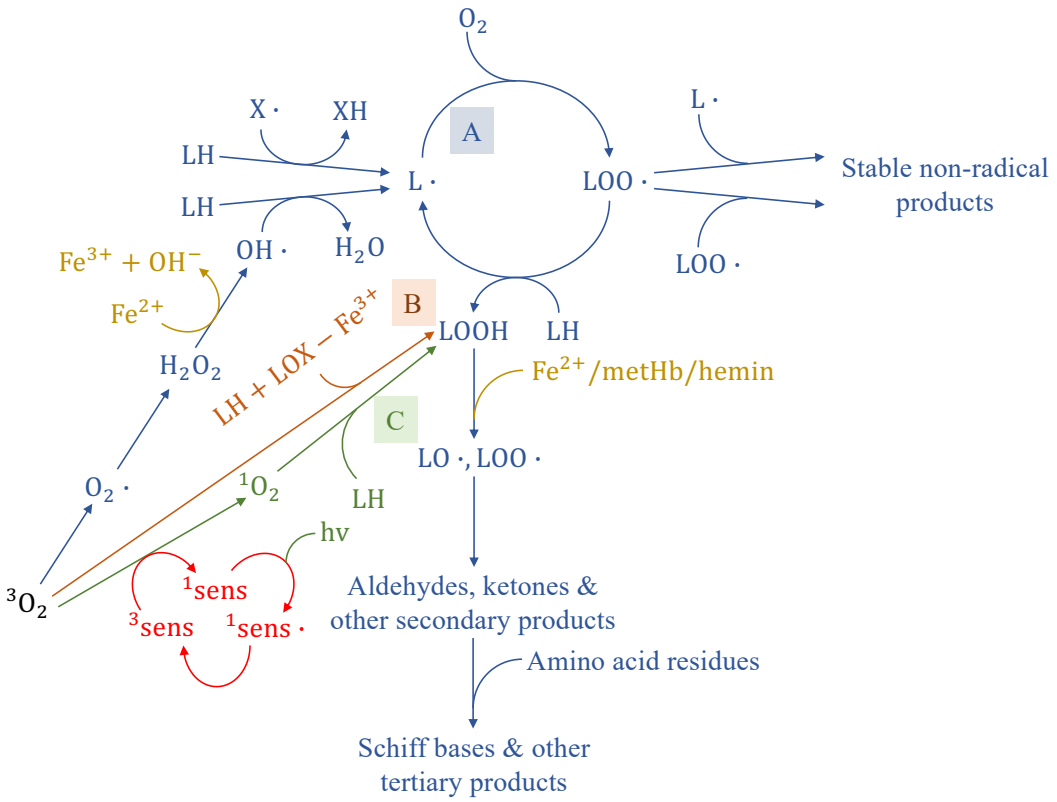

Figure 3.3: The lipid oxidation reaction including (A) radical driven initiation, (B) LOX catalyzed lipid oxidation, and (C) photooxidation. Figure adopted from Ghirmai [50].

The propagation stage of lipid oxidation is marked by a series of continuous reactions that lead to the formation of primary oxidation products [41, 42]. In this stage, L• reacts with oxygen, generating lipid peroxyl radicals (LOO•) [41] (Figure 3.3). These peroxyl radicals can readily abstract hydrogens from other fatty acids, producing LOOH and a new L• [23, 41]. This ongoing chain reaction is crucial to the lipid oxidation process, with the rate-limiting step being the formation of LOO• radicals [41]. As the propagation step unfolds and the subsequent

breakdown of hydroperoxides takes place, there is a notable increase in the number of radicals within the lipid phase [42]. This rise in radicals leads to an elevated rate of reactions with oxygen, further driving and intensifying the lipid oxidation process [41, 42]. Lipid alkoxyl radicals (LO•) are formed in the subsequent cleavage of hydroperoxides by *e.g.*, trace elements or heme, supplying the oxidation with further radicals, and resulting in the formation of more or less volatile aldehydes (*e.g.*, hexanal, octanal, malondialdehyde (MDA), 4-hydroxy-(E)-2-hexenal (HHE), and 4-hydroxy-(E)-2-nonenal (HNE)), (see Figure 3.4) ketones, and alcohols, which lead to the unpleasant odor and taste of oxidized lipids [41].

The termination stage of lipid oxidation involves the reaction between LOO• and L• or other LOO• in the presence of oxygen (Figure 3.3). As these radicals interact, they form stable non-radical products, effectively ending the lipid oxidation process [41, 42]. In oxygen-limited conditions, the termination stage can also lead to the formation of fatty acid dimers. These dimers are created when two L• react with each other, resulting in non-radical products that contribute to stabilizing the lipid oxidation reaction [41, 42]. This termination step plays a crucial role in halting the propagation of lipid oxidation and preventing further breakdown of lipids [24].

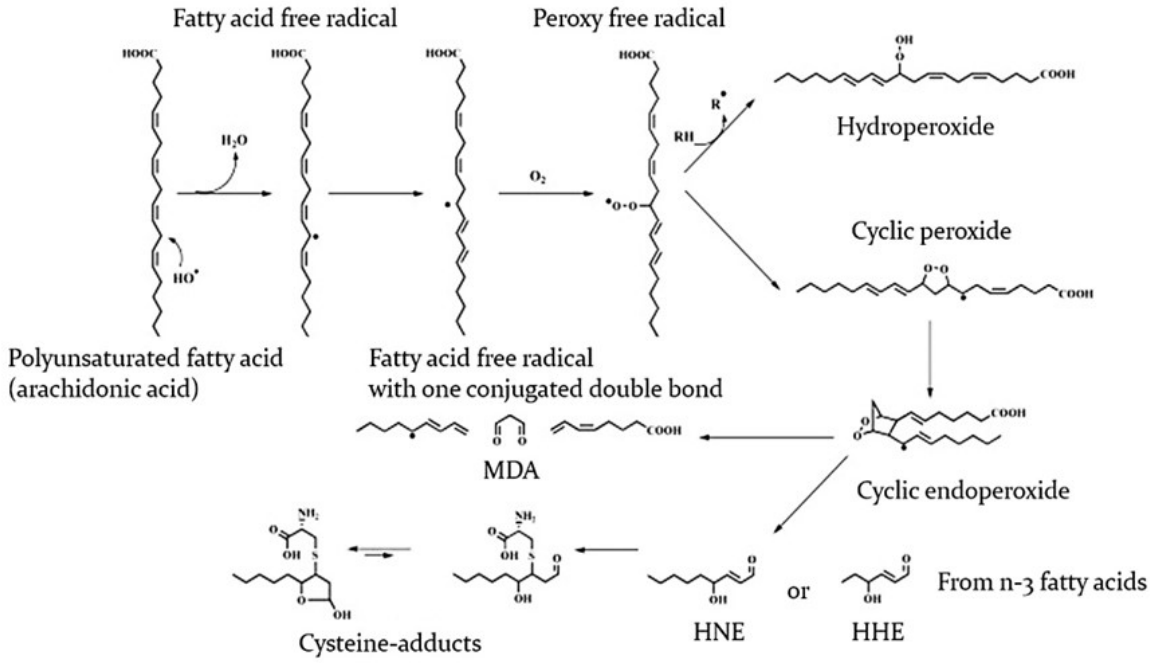

Figure 3.4: Proposed reaction mechanism for MDA, HHE, and HNE formation from C20:4 n-6. Figure adopted from Tullberg (2016) [51]; originally from Bocci, et al. (2021) [52].

3.7 Approaches to recover proteins from fish co-products

Several approaches can be employed to recover proteins from fish co-products, such as mechanical meat bone separation, enzymatic hydrolysis and pH-shift technology.

3.7.1 Mechanical meat bone separation

Mechanical meat bone separation is a conventional technique that automates the removal of skin, scales, and bones from fish meat, usually by applying pressure to squeeze the meat through holes in a cylinder [22]. This process effectively extracts protein-rich fish mince from cuts that have a high muscle-to-bone ratio, *e.g.*, herring backbones [3]. However, it's less efficient on cuts with a high proportion of non-muscle tissues like heads and intestines [3]. One notable drawback of this method is the potential inclusion of small bone fragments, scales, and other undesired components in the resulting mince, compromising its quality [22]. Additionally, the high external surface area of mechanically separated fish mince makes it less stable, with greater exposure to oxygen and resultant oxidation [28]. Therefore, mechanically separated fish mince is usually frozen immediately after processing [22].

3.7.2 Enzymatic hydrolysis

Enzymatic hydrolysis uses proteolytic enzymes to break down fish proteins into smaller peptides and free amino acids. This method is reported to efficiently extract proteins from fish co-products while simultaneously producing bioactive peptides with potential health benefits [53]. However, the resulting hydrolysates lack other functional properties such as the proteins' ability to form a gel [28]. Additionally, the resulting hydrolysates can have a bitter taste [54], which may restrict their use in certain food applications.

3.7.3 pH-shift technology

3.7.3.1 Introduction to pH-shift technology

The pH-shift technology, also known as solubilization and isoelectric precipitation, is an efficient method for isolating muscle proteins from terrestrial and marine animals[55]. This technique was developed based on differences in muscle protein solubility as a function of pH [56]. These muscle proteins are highly soluble in water at low pH (pH $\leq$ 3) and high pH (pH $\geq$ 10.5) while being insoluble at the pI [8] forms the fundament of the process. The entire process is conducted at low temperatures, preferably below 4°C, allowing the recovered proteins to retain several techno-functional properties, such as the ability to form a gel, foam, and emulsion [57].

An outline of the pH-shift process as applied to fish co-products is shown in Figure 3.5. The theoretical foundation of pH-driven protein solubilization involves the formation of net positive or negative charges on proteins at low and high pH, respectively [58] (Figure 3.6). This process creates strong electrostatic repulsive forces between protein molecules, which increases the hydrodynamic volume due to swelling and expansion, and promotes protein-water interactions, enhancing solubilization [5]. It has been clearly described earlier how the solubility of myofibrillar proteins depends both on their electrostatic and hydrophobic interactions within a protein solution [59]. Following the protein solubilization step, centrifugation is employed to separate insoluble fractions such as skin, bones, cartilage, and impurities from soluble myofibrillar, cytoskeletal, and sarcoplasmic proteins [56]. Then, the soluble proteins are precipitated by adjusting the pH of the solution to the protein's pI, which is typically close to pH 5.5 for fish

muscle proteins [8]. At the pI, protein-water interactions weaken, causing reduced solubility and WHC, promoting hydrophobic protein-protein interactions through simultaneous protein molecule refolding, leading to protein precipitation [58, 60].

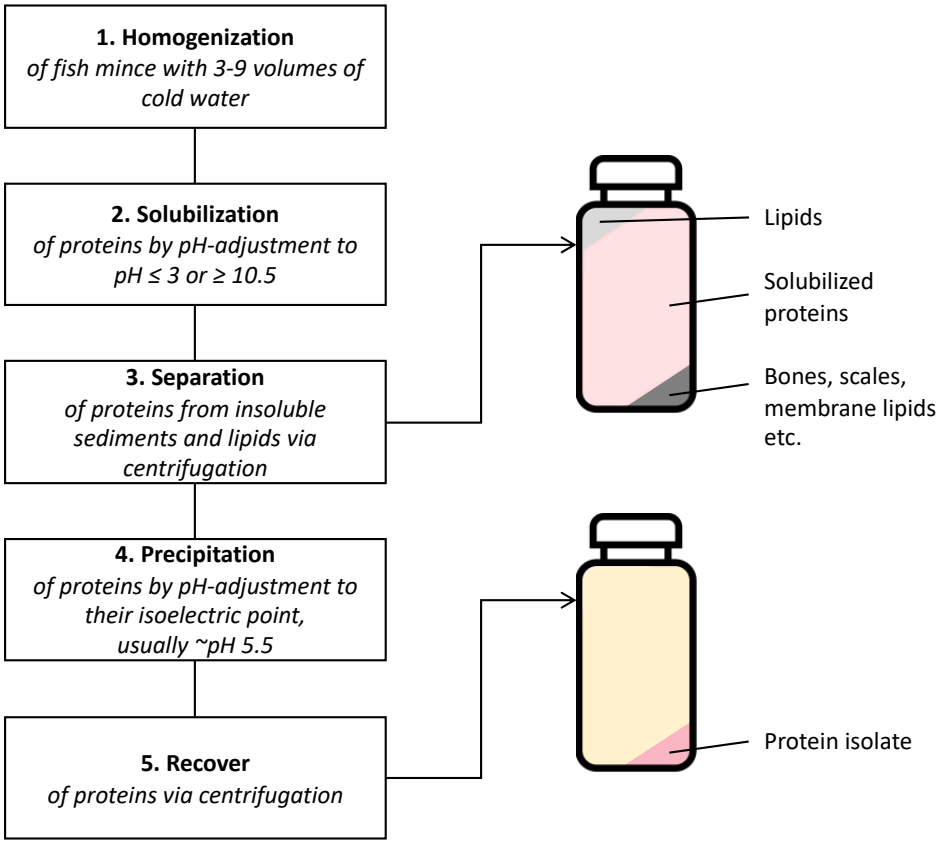

Figure 3.5: Protein recovery from *e.g.*, fish co-products by applying the pH-shift method.

3.7.3.2 Advantages of pH-shift technology over mechanic and enzymatic techniques

Compared to mechanical meat bone separation and enzymatic hydrolysis, the pH-shift technology offers several advantages. First, it can be applied to more complex materials compared to mechanical separation, the latter which requires pure muscle on bone structures to yield acceptable minces, leaving out *e.g.*, heads and guts [8]. Second, it ensures better protein purity, as it effectively separates proteins from other co-product components, such as lipids, bones, and scales, resulting in a purer protein isolate with fewer impurities [9, 61]. Third, due to the unfolding-refolding process, the recovered proteins generally exhibit improved functionality, including superior solubility, emulsifying, and gelation capacities compared to proteins obtained through mechanical separation or enzymatic hydrolysis [62, 63]. Compared to the latter, indeed also the absence of proteolysis is an important fundament behind retained gelation capacity of proteins isolated with pH-shift processing [62, 63].

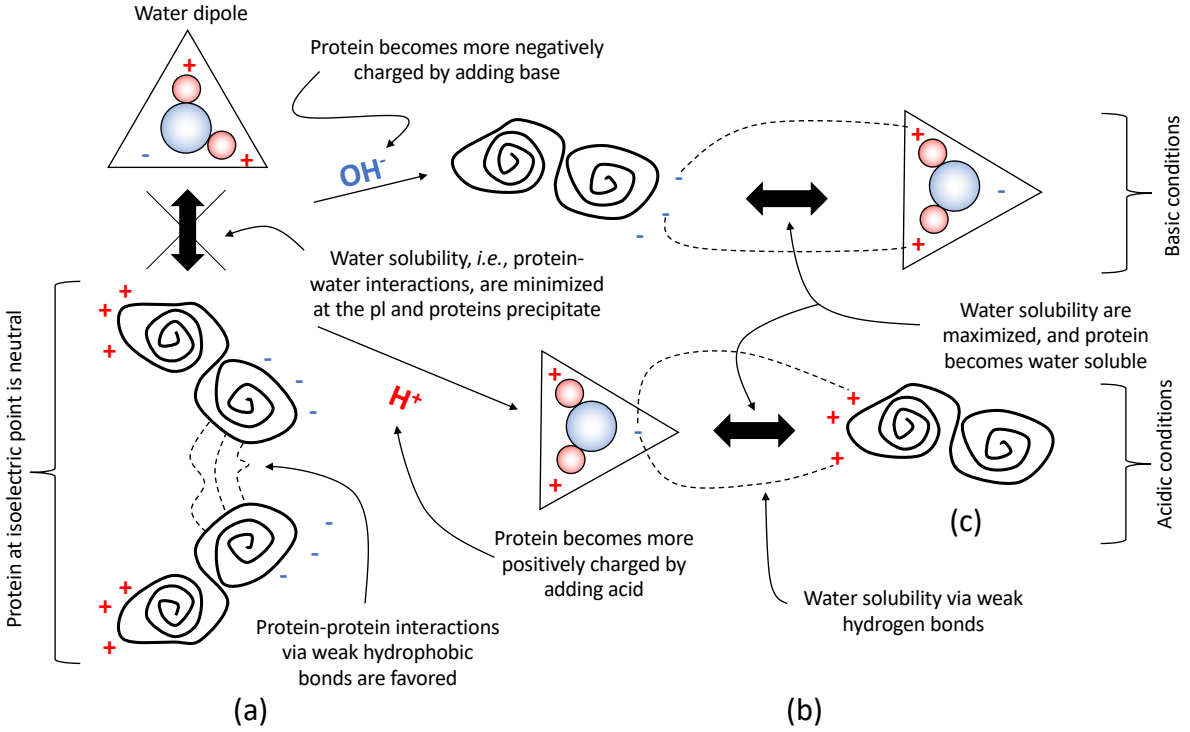

Figure 3.6: Changes in protein charges during the pH-shift process [58].

3.7.3.3 Key parameters during pH-shift processing

Protein solubility and yield. The pH-dependent protein solubility in water is a crucial parameter during pH-shift processing as it contributes to the protein yield of the first process step. It is influenced by the hydrophobic or polar nature of the amino acids, the pI-pH relationship, and the structural status or denaturation of the proteins [64]. However, of great importance are also the sizes of the first sediment and floating layers, as they will trap solubilized proteins, preventing them from being transferred to the second step where solubilized proteins are precipitated [65]. The total protein yield is calculated by determining the amount of proteins that are precipitated in the second sediment of the process and relating it to the initial total amount of protein in the raw material [56]. The total yield of protein reported in the literature for the pH-shift process ranges from 26% to 91% [5], and several factors are reported to influence it. As an example, to minimize the amounts of floating fat layer and sediment during the first centrifugation, it has been found essential to lower the viscosity of the acidified/alkalized homogenates [65]. With high viscosity at the point of centrifugation, a larger floating lipid layer forms, as well as an extra "jelly layer" on top of the regular sediment, both suppressing the recovery of solubilized fish muscle proteins [56]. The increased electrostatic repulsion causes the protein aggregates to swell, which significantly increases their effective volume and viscosity. In most cases, these aggregates collapse when the protein-protein repulsion is strong enough, reducing the homogenate viscosity at the point of centrifugation. However, *e.g.*, with freeze-thawed fish, crosslinks can be present which prevents the final collapse of the swollen network, thereby

maintaining high viscosity [8]. Also, other processing conditions such as salt can affect the exposure and charge of amino acid side chains, and thereby homogenate viscosity [5]. Apart from reducing the size of the first sediment, a lower viscosity reduces the resistance for neutral lipids to rise to the surface during centrifugation which in turn helps oil separation [56] At the same time, the higher the lipid content in the homogenate, the higher emulsification and the larger the floating fat layer, thus reducing the protein yield [55].

Acid and base consumption. This parameter directly impacts the cost of the process, as well as its environmental footprint. The consumption of acids and bases during pH-shift processing can be influenced by several factors, including the protein source, *i.e.*, the amino acid profile, the initial pH of the medium, and the overall buffering capacity of the system affected *e.g.*, by bones [5, 8].

Lipid removal efficiency. A reduction in lipid content of over 80% has been reported with pH-shift processing [8]. During this process, the pH-induced protein solubilization leads to the separation of storage and membrane lipids from the proteins, the former floating on top as triglycerides and the latter precipitating as phospholipids, allowing removal during the first centrifugation step [66]. However, the relative lipid reduction depends on the starting lipid content of the raw material, the fish species used, the version of the pH-shift process employed (acid or alkaline) and the exact process settings [5]. For example, increasing the g-force during the first centrifugation step to $\geq 10,000$ g has been found to reduce the lipid content of the protein isolate, and is also required if aiming at sedimentation of some of the phospholipids [67].

Lipid oxidation. Lipid oxidation is another critical parameter to monitor during the pH-shift processing of fish co-products, especially when the starting material is rich in heme-proteins [68]. Although the pH-shift process has been reported to reduce levels of both lipids and active pro-oxidants from the protein isolates to a great extent, low levels of residual lipids are still enough to serve as substrates for oxidation [8]. This results in considerable losses in the quality and storage stability of the produced protein isolate [8].

3.8 Approaches to limit lipid oxidation in fish and fish co-products

Several approaches can be used to limit lipid oxidation in fish and fish co-products. For example, rapid chilling after catch or harvest, maintaining low storage temperatures, minimizing blood contamination and minimizing light and oxygen exposure are important measures [15, 21]. For the latter, *e.g.*, modified atmosphere packaging and vacuum packaging are commonly used [69, 70].

Also, the use of antioxidants is an important strategy to mitigate lipid oxidation in fish and fish co-products during processing and storage [10, 11, 71]. Antioxidants can limit lipid oxidation through *e.g.*, scavenging of free radicals, decomposition of peroxides, reducing local oxygen concentrations, or neutralizing oxidation initiators like Hb, Mb, LOX, or low molecular weight (LMW) metals [72]. Antioxidants can neutralize radicals and peroxides through *e.g.*, hydrogen atom transfer, proton-coupled electron transfer, sequential proton loss electron transfer, single electron transfer followed by proton transfer, radical adduct formation, or sequential proton loss hydrogen atom transfer [73]. Several antioxidants have been reported to be effective

during pH-shift processing of fish and fish co-products. Examples include erythorbate, ascorbic acid, STPP, EDTA [10], polyphosphate [11], α-tocopherol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate [71]. When it comes to direct stabilization of fish and fish co-products towards lipid oxidation, *i.e.*, without pH-shift processing, promising results have been seen when using polyphenol-rich extracts from various herbs and spices (*e.g.*, oregano, thyme, star anise [74], rosemary, basil [75] and turmeric [76]), fruits (*e.g.*, apple [77], hawthorn berries [78], sea buckthorn berry [79], and grape [80, 81]) and seaweed (*e.g.*, *Palmaria palmata* and *Fucus vesiculosus* [82]). Notably, the methods of adding such extracts play a fundamental role for the achieved activity, as recently reviewed by Wu et al. (2022) [25]. Direct mixing into minces, as well as glazing, dipping, soaking, spraying, coating and injection into fillets are together active antioxidant-containing packaging all routes which have been described [25]. In some studies, used antioxidants have also been encapsulated to be better protected against degradation [25].

3.9 Cross-processing: a new approach to produce stable protein isolates from fish co-products

Currently, the most common way of using natural antioxidants from plants is to extract them through various techniques, mechanical procedures, solvent extraction, ultrasound-assisted extraction, accelerated solvent extraction, microwave-assisted extraction, molecular distillation, and supercritical fluid extraction [83, 84]. The extracts are then added to fish and fish co-products as described above (Section 3.8) in order to limit lipid oxidation. However, the high costs of producing pure antioxidant extracts present significant barriers to their large-scale implementation and integration into the food industry [25]. This is especially true for low value bulk materials such as fish co-products. Additionally, adding these extracts into fish minces or protein isolates requires extensive and intensive mixing to achieve homogeneous distribution, especially when added in small quantities. However, prolonged mixing duration could affect the physical and chemical properties of the ground mince, potentially promoting oxidation [25]. Also, significant levels of oxidation may then develop already *before* their addition, *i.e.*, during protein isolation processes [10]. If circumventing this by adding extracts at the start of for example pH-shift processing, large additions must be made to reach a certain concentration in the fish-in-water homogenate, something which raises costs.

In response to some of these challenges, studies have been conducted to add whole antioxidant-containing materials into fish mince. For example, Damerau et al. (2020) [85] demonstrated that adding press cakes from Finnish berries to herring mince resulted in similar or greater antioxidant activity compared to traditional antioxidants *e.g.*, α-tocopherol and ascorbic acid over a 10-month frozen storage period. However, the lack of an extraction process may limit the antioxidants´ contact with neutral lipids and cellular membranes [85].

When this thesis project commenced, there were no published studies that addressed the co-extraction of antioxidants from non-fish materials along with the extraction of fish proteins. To accomplish this, we here for the first time explored pH-shift-based cross-processing of fish co-products with antioxidant-containing support materials ('helpers') coming from plants, shellfish or seaweeds. This new and clean-label approach was expected to facilitate the strategic

placement of polyphenols or other antioxidants within the fish matrix, precisely where needed, thereby mitigating lipid oxidation. It is known from before that the interphases of cellular membranes and oil droplets towards the aqueous phase constitute particularly sensitive oxidation substrates in fish, while the protein Hb is a key pro-oxidant [56].

The pH-shift process starts with high shear mechanical homogenization, during which antioxidants can be extracted into either fish lipids or water. During the protein solubilization step, given that helper and fish tissues are thoroughly homogenized, lipophilic, acid-, or alkali-soluble antioxidants from helpers, can thus partition more easily into oil droplets or interact with proteins/phospholipids via non-covalent (hydrophobic, ionic, and hydrogen bonding) or covalent bonds [86] to form protein-polyphenol conjugates. The formation of covalent conjugates is often favored in food applications due to their strong and stable interactions [25]. Under alkaline conditions, polyphenols can then oxidize into semiquinone radicals, which rearrange into quinones [87]. These radicals then react with protein side chain residues such as methionine, lysine, tryptophan, and cysteine, forming covalent crosslinks (C–N or C–S) that enhance antioxidant activity [88]. Additionally, cross-processing is also expected to contribute to an efficient distribution of antioxidants into the aqueous phase of the resultant protein isolates, which usually have a moisture content of 80-90% (w/w) [5], thereby further enhancing the antioxidant potential of the final product.

From a Swedish food industry perspective, productions of lingonberry juice, apple juice, beer, and oat-based products (*e.g.*, Oatly®) offer large volumes of interesting plant-derived side streams as potential helpers in the form of press cakes, barley spent grain, and oat fiber residues. These raw materials are all abundant in phenolic compounds such as anthocyanins, anthocyanidins, flavonols, tannins, and phenolic acids [77, 89, 90, 91] as well as in terpenoids such as carotenes and xanthophylls [92]. In the marine sector, shrimp shells present a viable option for cross-processing due to their high astaxanthin content [93], which exhibits potent antioxidative properties [93]. Furthermore, the brown seaweed *Saccharina latissima*, commonly known as sugar kelp, is rich *e.g.*, in phlorotannins and fucoxanthin [94] that have demonstrated effectiveness in mitigating lipid oxidation in various foods [94], including fish mince [94, 95]. Additionally, *Ulva fenestrata*, a green seaweed species cultivated along the Swedish West coast, contains terpenoids, chlorophyll, and sulfated polysaccharides, all with antioxidant potential [96, 97], further expanding the range of potential helpers.

It is important to stress that the helpers were hypothesized to not only contribute with natural antioxidants, but also to provide additional components, such as pigments, polysaccharides, organic acids, vitamins and minerals, which impart novel characteristics, including color, texture, nutrient composition, sensorial attributes, and techno-functionalities, to the cross-processed protein isolates. Some compounds, *e.g.*, phenolic compounds and organic acids, are also hypothesized to help the protein precipitation step by contributing to precipitation *per se*, and by lowering pH, respectively. In addition, by combining rest materials streams, available raw material volumes would increase at individual food processors, motivating investments in process equipment.

4

Methodological approaches

4.1 Study design

This PhD thesis is comprised of six studies. The study design is outlined in Figure 4.1.

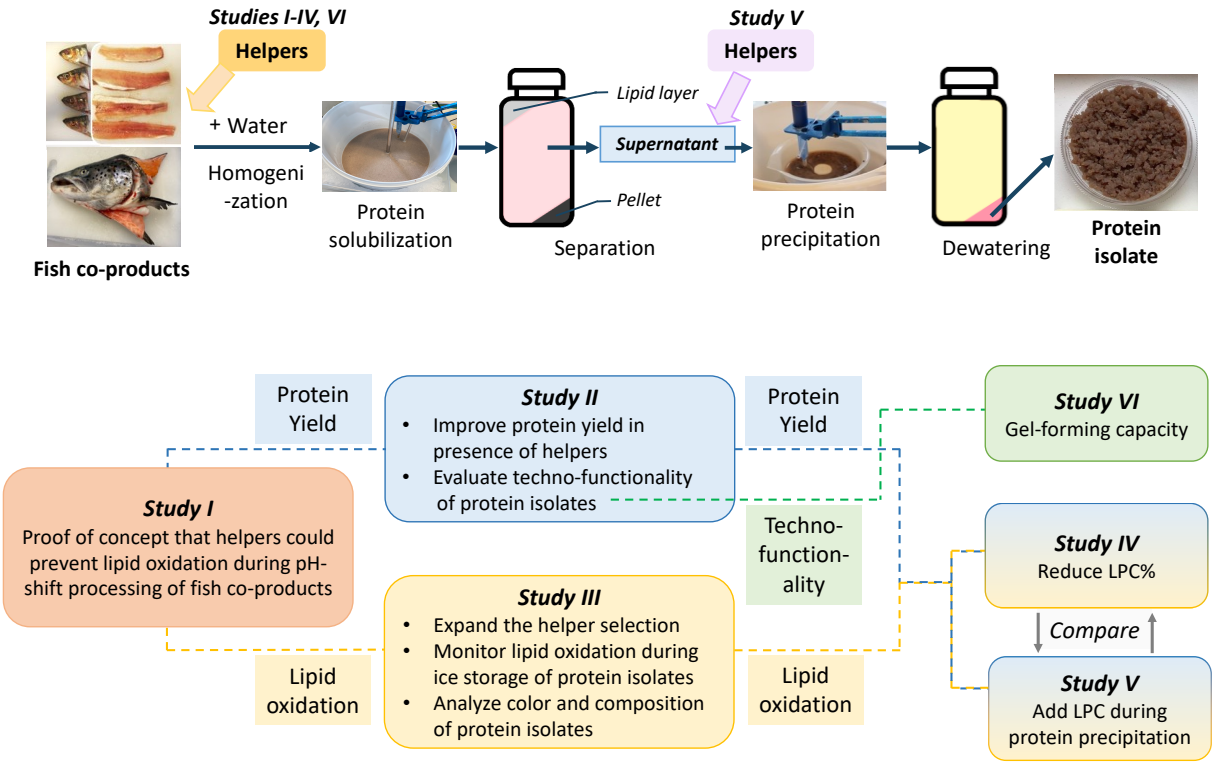

Figure 4.1: Overview of the study design.

Study I was a proof-of-concept study that cross-processed herring and salmon co-products with helpers including LPC, shrimp shells, and Saccharina to prevent oxidation of fish lipids.

The study measured lipid oxidation, protein solubility, protein yield, and consumption of acid and base solutions during the pH-shift process, and characterized the resulting protein isolates in terms of composition and appearance.

Study II addressed the issue of reduced total protein yield during processing due to the addition of helpers by testing four strategies, including optimizing solubilization and precipitation pH's, increasing the water-to-raw material ratio, and replacing single-stage toothed with radial discharge high shear mechanical homogenization (HSMH) without and with ultrasonication. Beyond the effects of these strategies on protein solubility and yield, their effects on lipid oxidation during processing, as well as the crude composition, color, and protein structural and functional properties of the protein isolates were also assessed.

Study III expanded the selection of helpers to also include APC, barley spent grain and oat fiber residues, and further investigated the ability of cross-processing to limit lipid oxidation during the storage of protein isolates on ice. The effects of various types of helpers on the composition and coloration of cross-processed protein isolates and their color changes during ice storage were also investigated.

In ***Study IV***, the inclusion ratio of LPC, which was found to be the most effective helper in ***Studies I-III***, was reduced from 30% (dw/dw) due to the decreased protein yield and the dark color of the protein isolates. The impact of this LPC% reduction on protein solubility and yield, acid and base solution consumption during processing, as well as the lipid oxidative stability, appearance, and composition of the resulting protein isolates were studied.

In ***Study V***, an alternative way of combining herring co-products and LPC during the pH-shift process was examined. Instead of being added at the start, LPC was added to the alkali-solubilized herring co-product proteins during their precipitation. Lipid oxidation products, acid/base consumption, color and protein solubility and yield were measured and compared with the results of ***Study IV***.

Finally, motivated by the improved gel-forming capacity of the cross-processed protein isolates in ***Study II***, ***Study VI*** was conducted to evaluate the seven helpers for their ability to enhance the gel strength and WHC after cross-processing with herring co-products. LPC, APC, and *Saccharina* were selected based on their potential to enhance gel-forming capacity and limit lipid oxidation (***Study III***) for a further investigation where their additional ratio was reduced to 10% (dw/dw).

4.2 Raw materials

4.2.1 Fish filleting co-products

This thesis comprized co-products from two species of fish: herring and salmon. They were selected due to their high importance for both the Scandinavian and global seafood sectors [1]. The herring co-products were provided by *Sweden Pelagic AB* (Ellös, Sweden), while the salmon co-products were provided by *Fisk Idag AB* (Gothenburg, Sweden).

In ***Study I***, heads and backbones from herring and salmon were used. ***Study III*** also used herring heads and backbones, but focused only on salmon heads since there are already established procedures to manually scrape off residual muscle from the salmon backbones within

the fish industry. ***Studies II, IV-VI*** were solely focused on herring heads and backbones, as the beneficial role of helpers was most pronounced for this raw material in the earlier studies.

In all studies, fresh fish heads and backbones were packed separately and directly transported from Ellös or Gothenburg fish harbor on ice to Chalmers University of Technology. They were then minced with a meat grinder to create homogenous raw materials for the different sub-studies, and were stored at -80°C until use. The fish co-products were analyzed for their moisture, protein, lipid, ash (see Table 4.1) and Hb content (see Section 5.4).

Table 4.1: Proximate composition of fish co-products and helpers. Protein, lipid, and ash data are given as g/100 g on the dry weight with data showing mean values ± standard deviation (n≥3). Except for the herring co-products, all other raw materials have the same batch used throughout the thesis. The data for herring co-products in the table is based on the batch from March 2020.

Source	**Moisture**	**Protein**	**Lipid**	**Ash**
Herring heads and backbones	71.0 ± 0.2	48.7 ± 1.2	32.7 ± 1.5	14.5 ± 0.2
Salmon heads and backbones	61.3 ± 0.5	31.1 ± 0.2	56.2 ± 0.5	11.6 ± 0.3
Salmon heads	62.5 ± 0.2	34.3 ± 0.7	56.4 ± 3.0	9.4 ± 0.7
Lingonberry press cake	75.6 ± 0.1	5.6 ± 0.1	14.4 ± 2.1	2.0 ± 0.2
Apple press cake	80.6 ± 0.2	2.3 ± 0.0	4.2 ± 0.6	2.7 ± 0.5
Barley spent grain	70.6 ± 0.6	14.8 ± 0.3	5.7 ± 0.2	3.6 ± 0.0
Oat fiber residues	62.6 ± 0.5	36.1 ± 0.4	10.7 ± 0.5	5.8 ± 0.0
Shrimp shells	90.3 ± 0.4	39.8 ± 0.1	7.7 ± 0.5	48.1 ± 0.8
Saccharina	82.1 ± 0.1	11.8 ± 0.1	3.2 ± 0.1	23.8 ± 0.1
Ulva	81.9 ± 0.5	15.4 ± 0.1	7.0 ± 0.1	28.3 ± 0.1

4.2.2 Helper raw materials

The thesis utilized seven different types of helpers, which were classified into three groups: plant food side streams, marine side streams, and seaweeds (Figure 4.2). These helpers were selected based on their antioxidant potential, availability, and abundance in Sweden, as discussed in the Section 3.9 of the thesis. The helpers were characterized in terms of their moisture, protein, lipid, ash content (see Table 4.1), total phenolic content (TPC) (see Section 5.4), and their effects on changing the pH of the fish co-product homogenates (see 5.1). The seaweeds were also analyzed for their salt contents, while LPC was analyzed for its anthocyanin profiles (see 5.3).

Both laboratory-produced and industrial press cakes of lingonberry (*Vaccinium vitis-idaea*)

Figure 4.2: Helpers involved in this thesis [98, 99].

were used in this thesis. The laboratory-produced LPC was prepared using frozen lingonberries purchased from a local food store. The frozen lingonberries were defrosted, mixed using a hand blender and centrifuged (5,000×g, 10 min, 10 °C) to obtain pellets. Laboratory-produced LPC was used in ***Study I*** and ***V***. Motivated by the promising outcomes of ***Study I***, LPC generated during industrial lingonberry juice production at *Grangärde AB* in Sweden was used in ***Studies II-VI***. Unlike laboratory-produced LPC, which was produced solely from the actual lingonberries and therefore consisted mainly of peels, leftover flesh, and seeds, industrial LPC also consisted of leaves (9%, wet weight (ww)), stems, and leftover flesh.

The apple (*Malus domestica*) press cake, including peels, leftover flesh as well as core with seeds and stems, was obtained from *Kiviks Musteri AB* (Kivik, Sweden) in October 2019. Barley spent grain, including pale malt with small percentages of caramel malts, flaked oats, and rice hulls, was provided by *Beerbliotek* (Majorna, Sweden) in September 2019. Oat fiber residues were provided by *Oatly® AB* (Lund, Sweden) in June 2018.

Shrimp shells, including cephalothorax, legs, peels, and tails were provided by *Räkor och Laxgrossisten AB* (Gothenburg, Sweden) in March 2018.

Saccharina and *Ulva* were cultivated at the Sven Lovén Centre for Marine Infrastructure (Tjärnö, Sweden) and were harvested in June 2018 and November 2019, respectively. The fresh-harvested seaweeds were transported on ice to Chalmers University of Technology within one day. Prior to their use the seaweed was ground with a meat grinder.

Upon arrival in Chalmers, all helper materials were stored at -80°C until use. Prior to their inclusion during pH-shift processing of fish co-products, certain helpers underwent pretreatment. The industrial LPC, Saccharina, *Ulva*, and shrimp shells were ground to smaller pieces using the same method as the fish co-products to facilitate homogenization. All helpers were stored at -80°C until they were needed. The ground seaweeds were osmo-shocked by soaking them in ice-cold distilled water for 15 minutes before being used as helpers. The soaked seaweeds, along with the water used for soaking, were added together.

4.3 Cross-processing of fish co-products and helpers by the pH-shift method

The steps of the cross-process as run with herring co-products are illustrated in Figure 4.1. In all studies, the amount of helpers added was calculated based on the dry weight of the fish co-products. ***Studies I & III*** investigated both the alkaline and acid versions of the pH-shift process, while ***Studies II, IV-VI*** focused only on the alkaline version due to its more favorable results. In all studies except for ***Study V***, the helpers were added to the minced fish co-products at the beginning of the process. In ***Study V***, LPC was added to the alkali-solubilized herring proteins during the precipitation step.

In ***Study I***, the protein solubilization pH was 11.5 and 2.5 for the alkaline and acid versions, respectively, while the precipitation pH was 5.5. The amount of water added at the process start was calculated to achieve the same dry weight to moisture ratio in control samples (*i.e.*, fish co-product samples without helpers) as in samples with helpers added. The raw materials were homogenized with water using a single-stage toothed high shear homogenizer (T18 digital Ultra-Turrax, IKA, Staufen, Germany) at 10,000 rpm for 60 s.

In ***Study II***, several modifications to the process were tested to compensate for the reduction in total protein yield induced by helpers in ***Study I***. The optimal solubilization pH for the alkaline pH-shift process version was found to be pH 12 for all combinations of raw materials while the optimal precipitation pH was helper-dependent; pH 5 for plant helpers and shrimp shells, but pH 4.5 for seaweeds. Further, the amount of water was increased to six times the combined wet weight of the fish and helper raw materials. Also, the single-stage toothed high shear homogenizer was replaced by a radial discharge high shear homogenizer (L5M-A, Silverson, Chesham, UK). These modifications were then applied in ***Studies III, IV, VI*** when cross-processing was conducted.

4.4 Protein solubilities and yields during processing

Protein solubility and protein yield were determined based on the protein content of homogenates and supernatants obtained during pH-shift processing. The protein concentration was determined by a modified version of the Lowry method as described in Section 4.6.

4.5 Ice storage of protein isolates

Ice storage trials (Figure 4.3) were conducted in ***Studies III-V*** with the cross-processed protein isolates to further investigate the antioxidant potential of the helpers. As both moisture content and pH are known to affect lipid oxidation [100, 101], these parameters were adjusted in the freshly made isolates to approximately 80% and pH 7, respectively. To prevent microbial growth, streptomycin was added to the protein isolates [102]. The protein isolates were then stored at the bottom of 250 mL Erlenmeyer flasks as a thin layer, approximately 5-6 mm, to allow oxygen penetration. The flasks were tightly sealed, wrapped in aluminum film to avoid exposure to light, and placed on ice in insulated cooler boxes located in a 4°C cold room. At each storage time point, about 0.7 g of sample was taken from the thin layer, wrapped in aluminum foil, and stored at -80°C until the analyses of lipid oxidation. Additionally, a sensory screening of the odor of the samples was performed daily using a scale from 0 to 100 [103]. Sampling was stopped when the rancid odor was detected for two consecutive days or, for very stable samples, when microbial growth was sensorially detected.

Figure 4.3: Ice storage trials of cross-processed fish protein isolates.

4.6 ANALYSES OF SOLUBILIZED PROTEINS

The Lowry method quantifies solubilized protein within a 5–100 μg range [104, 105]. It calculates protein concentration via the reaction of peptide nitrogen with Copper ions under alkaline conditions and subsequent reduction of Folinciocalteay phosphomolybdic phosphotungstic acid to Heteropolymolybdenum blue via copper-catalyzed oxidation of aromatic acids [105]. This blue color is detectable between 650 nm and 750 nm [105]. In this thesis, the heteropolymolybdenum blue's concentration was measured at 660 nm [104]. The Lowry method outperforms the bicinchoninic acid assay or Bradford assays by being nearly 100 times more sensitive in determining protein concentration [105].

4.7 ANALYSES OF LIPID OXIDATION

4.7.1 Chemical measurements

Several tests are available to study lipid oxidation, but no single test can detect all the products that are formed in the different reaction steps; *i.e.*, primary, secondary and tertiary products. In this thesis, four analytical methods targeting different lipid oxidation products were employed, in order to provide a more comprehensive assessment of the lipid oxidation kinetics in the samples studied and to circumvent analytical problems deriving from highly pigmented molecules of the helpers.

4.7.1.1 Analysis of peroxide value (PV)

In ***Study I***, the ferric thiocyanate method was utilized to assess PV, an early-stage lipid oxidation indicator [106]. This simple, quick method determines lipid hydroperoxides through colorimetric measurement in the chloroform phase of lipid extraction, transforming ferrous to ferric iron ions oxidized by hydroperoxides [106]. The ferric thiocyanate method, requiring a smaller sample ($\sim$0.1 g lipid), outperforms the standard iodometric method (requiring $\sim$5 g lipids for PV below 10 meq O_2/kg and $\sim$1 g for higher PVs) [107]. While PV can accurately represent lipid oxidation levels for lowly oxidized samples (PV$<$50 meq O_2/kg) under mild conditions, high levels of certain metals may result in an underestimated PV due to peroxide decomposition [107, 108]. For a more accurate assessment, secondary oxidation product measures, such as chain-shortened aldehydes, are often coupled with PV [109]. However, PV proved unsuitable for cross-processed protein isolates in ***Study I*** due to red pigment interference, leading to PV overestimation [110]. Also, high trace element content, particularly iron, in some samples, such as seaweeds, resulted in elevated background absorbance in the ferric thiocyanate method, leading to PV not being measured in subsequent studies [110].

4.7.1.2 Analysis of Thiobarbituric acid reactive substances (TBARS)

The TBARS assay is a widely used method for analyzing lipid oxidation-derived carbonyls in fish tissue due to its simplicity and relatively high sensitivity [111]. The assay involves extraction with TCA (trichloroacetic acid) [112] or with chloroform/methanol [87] followed by

reaction between carbonylic groups and TBA yielding a pink complex which absorbs light at 532 nm [113]. Quantification is normally done based on a standard curve from MDA, derived from TEP [114] and thus results are expressed as MDA equivalents. However, in ***Study I***, the TBARS assay was found unsuitable for analyzing lipid oxidation in protein isolate samples produced by cross-processing due to the presence of red pigments brought in by the helpers, such as anthocyanins from LPC [115], which can interfere with the characteristic pink color. Furthermore, the TBARS assay is not specific for lipid-oxidation derived carbonyls such as MDA but can also react with non-lipid oxidation compounds such as sugar degradation products and browning reaction products, leading to overestimation [116]. Due to these drawbacks and limitations, the TBARS assay was not used in later studies.

4.7.1.3 Analysis of MDA, HHE and HNE

In ***Studies I-III***, extraction of the samples with 0.25 M HCl followed by carbonyl derivatization and Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was employed to quantify selected aldehydes in the cross-processed fish protein isolates. The aldehydes targeted here were MDA, HHE and HNE [51]. The method was developed in-house to our LC-MS instrument (SCIEX QTRAP® 6500+) based on Pilz's study [117] and involved the reaction of carbonyl compounds with 2,4-dinitrophenylhydrazine (DNPH) to form the corresponding 2,4-DNPhydrazones [118]. In this reaction, the amine attacks the carbonyl carbon, forming a neutral tetrahedral intermediate called carbinolamine, which is in equilibrium with two protonated forms. The oxygen-protonated intermediate can form a protonated hydrazone that loses a proton to yield the hydrazone by eliminating H_2O. The formation of hydrazone is reversible, and the hydrazone derivatives can be hydrolyzed back to the carbonyl compound and DNPH in an acidic environment [118]. The DNPH derivatization method followed by LC-MS/MS analysis had several advantages, including the ability to selectively analyze several specific aldehydes simultaneously in a complex mixture at room temperature and with short running times (7 min).

4.7.1.4 Analysis of volatile aldehydes

In ***Studies IV & V***, lipid oxidation-derived volatiles were analyzed using headspace solid-phase microextraction (HS-SPME) paired with gas chromatography-mass spectrometry (GC-MS). A fiber coated with a specific adsorbent material was used to extract the volatiles (Figure 4.4). Although some compounds might over-saturate the SPME fiber [119], correct matching of the fiber coating and targeted volatiles minimized this [120]. A 75 μm carboxen/polydimethylsiloxane (CAR/PDMS)-coated SPME fiber (Supelco, Bellefonte, PA, USA) was chosen for extraction, based on the lipid oxidation derived volatile aldehydes' properties. Extraction involved stirring the sample at 60°C for 20 minutes and a 5-minute equilibration. Desorption into the GC inlet for analysis was at 300°C for 10 minutes. Separation was conducted with a fused silica ZB-1701 capillary column (30 m×0.32 mm, 1 μm) (Phenomenex, Torrance, CA, USA). The scan mode of the MS captured all analytes within 10-250 amu mass range to select lipid oxidation markers. This mode was used due to the unexpected nature of volatile compounds formed during oxidation of PUFAs-rich samples during processing or preservation [121, 122]. The most abundant volatiles were explored, and those confirmed to be lipid oxida-

tion products based on existing literature were chosen as markers. The lipid oxidation markers included hexanal, (E)-2-hexenal, heptanal, octanal, and 2,4-heptadienal, all commonly found in oxidized fish and other samples rich in n-3 or n-6 PUFAs [123, 124]. An internal standard (3-methyl-3-buten-1-ol) was used to mitigate potential volatile loss and matrix effects. This HS-SPME-GC-MS method correlated more closely with the development of rancid odor than the LC-MS/MS method.

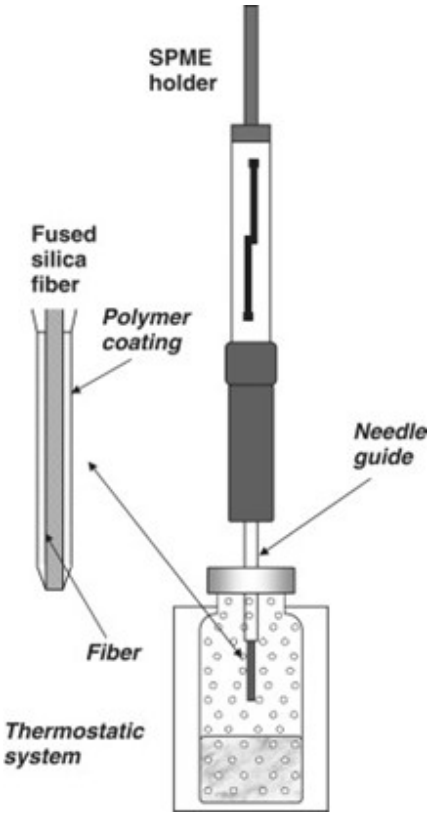

Figure 4.4: SPME for volatile compounds collection [125].

4.7.2 Sensory analysis of rancid odor

Rancid odors are produced as a result of lipid oxidation and can be easily detected by the human senses. Therefore, they are often used as an indicator of lipid oxidation in food [107]. In ***Studies III-V***, where ice storage trials were conducted, the rancid odor of each sample-containing Erlenmeyer-flask was daily assessed by using a rating scale ranging from 0 to 100 [103]. The results primarily guided the timing of sampling for chemical analyses, and were included in the supplementary materials of publications.

4.7.3 Color changes of protein isolates

The surface color of protein isolates stored on ice was monitored daily using a Minolta colorimeter (CR-400, Konica Minolta Sensing, Japan) from the bottom side of the Erlenmeyer

flasks. When storage was not employed, protein isolates were filled in a small petri dish (5-6 mm) for color measurement.

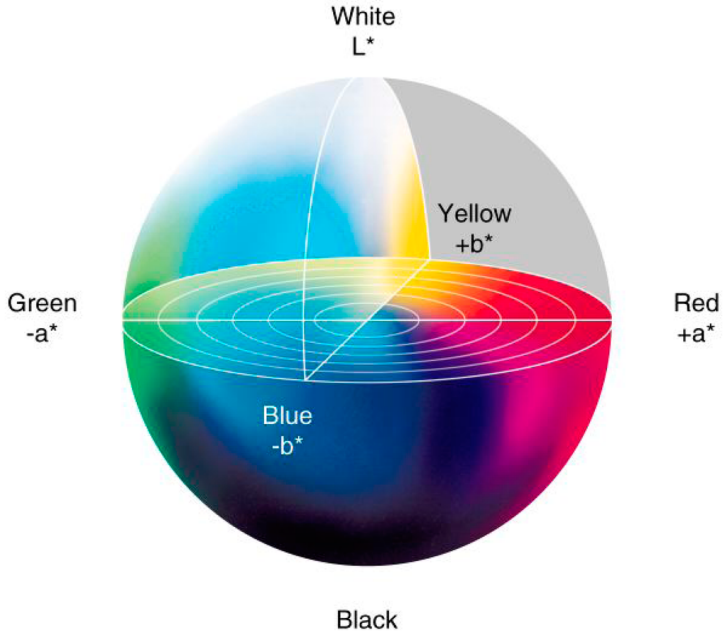

Figure 4.5: CIELAB 1976 color space [126].

Color was measured in the $L^*a^*b^*$ (CIELAB) color space, established by the international commission on illumination (CIE) in 1976 (Figure 4.5) [127]. In this model, L^* indicates lightness, a^* represents the position between red (positive values) and green (negative values), and b^* depicts the position between yellow (positive values) and blue (negative values) [127]. This model approximates human vision and covers the entire range of human photopic vision, outperforming the sRGB or CMYK models [127]. The color difference between the protein isolates was calculated using the CIEDE2000 formula, which employs L^*, a^*, and b^* values for improved industrial color difference computation compared to its forerunners, the CIELAB ΔE^*_{ab} and the CIE94 [128].

4.8 Proximate composition analyses

Moisture and ash content in various samples were determined using gravimetric methods [129]. A moisture balance (HA 300, Precisa) was used for rapid measurements, while prolonged analyses required overnight drying at 105°C [129]. No significant discrepancy was noted between these two methods. Ash content was measured by heating samples at 550°C for six hours [129].

The crude protein content was measured using a modified Dumas method [130], a procedure favored for its ease, automation, safety, and speed compared to the traditional Kjeldahl method [131], but requiring correction for non-protein nitrogen and protein type variation [131, 132]. A LECO TruMac-N nitrogen analyzer (St. Joseph, MI, USA) was utilized, and calibrated using EDTA, which contains $9.56 \pm 0.07\%$ of nitrogen. Nitrogen-to-protein conversion factors varied across samples: 5.58 for fish co-products and protein isolates, 5.45 for barley spent grain,

5.34 for oat fiber residues, 5.4 for LPC and APC [132], 4.88 for shrimp shells [133] and 5 for seaweeds [134].

The crude lipid content was determined using chloroform and methanol extraction [135]. The solvent ratios were altered based on the anticipated lipid content [135]. High-lipid samples, such as salmon and herring co-products, used a 2:1 chloroform:methanol ratio, while a 1:1 ratio was used for the protein isolates. Helpers, such as oat fiber residues and LPC, were also extracted using a chloroform:methanol ratio of 1:1. For APC, barley spent grain, shrimp shells, and seaweeds, which had an even lower expected lipid content, a chloroform:methanol ratio of 1:2 was used. The crude lipid content was determined by evaporating a known quantity of the chloroform phase under nitrogen gas.

4.9 Total Hb content of fish co-products

The Hornsey acid hematin method was used to estimate the Hb content of fish co-products based on analysis of total haem [136, 137, 138]. Haem was extracted with an acid acetone solution, which reduces the haem to hematin. The resulting hematin was then dissolved in an alkaline solution, and its absorbance is measured using a spectrophotometer. Quantification was made using a standard curve of bovine hemoglobin and results were expressed as μmol Hb/kg, dw, dry weight (dw).

4.10 TPC

To measure the TPC, the Folin-Ciocalteu colorimetric method was utilized [139], which involves the reduction of the Folin-Ciocalteu reagent by phenolic compounds, resulting in a blue color that can be measured using spectrophotometry [131, 139]. The TPC results were reported as gallic acid equivalents (GAE) per gram of the sample.

4.11 Anthocyanin profile analysis

The anthocyanins in LPC were analyzed using high-performance liquid chromatography-ultraviolet/visible (HPLC-UV/VIS) detection, which is widely used for separating and quantifying anthocyanins in plant extracts [140]. The sample was extracted using acidified methanol, followed by sonication, centrifugation, and storage at -20°C until the HPLC-UV/VIS analysis [141, 142].

4.12 Active and total sulfhydryl groups measurement

Ellman's method was used to determine the concentration of both active and total sulfhydryl groups in protein isolates pre-dissolved in a tris-glycine buffer containing EDTA and urea [143]. This method involves reacting the sulfhydryl groups with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), resulting in the production of a yellow-colored product that can be measured spectrophotometrically at 412 nm. The tris buffer maintained a constant pH, which was crucial for

the reaction of DTNB with sulfhydryl groups, and glycine was added to break disulfide bonds. EDTA bound to metal ions to prevent interference with the assay, and urea was added to denature the protein and expose the sulfhydryl groups, making them more accessible to the DTNB reagent [143]. For measuring total sulfhydryl groups, 2-mercaptoethanol was used as a reducing agent to break disulfide bonds, and TCA was added to remove interfering substances and concentrate the protein sample [143].

4.13 SURFACE HYDROPHOBICITY ANALYSIS

Kato's method [144] was used to measure the surface hydrophobicity (H0) of proteins in the isolates. This method is based on the binding of the hydrophobic dye 1-anilino-8-naphthalene sulfonate (ANS) to exposed hydrophobic patches on the protein surface [144]. A higher H0 value indicates greater hydrophobicity on the protein surface [144]. Kato's method is highly sensitive and can detect changes in protein surface hydrophobicity caused by various factors such as temperature, pH, ionic strength, and ligand binding [145].

4.14 POLYPEPTIDE PROFILES

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), a widely used method to separate polypeptides with molecular masses between 5 and 250 kDa [146, 147], utilizes SDS and polyacrylamide to enable the separation of polypeptides based on their molecular weight. SDS masks the proteins' intrinsic charge, rendering other charges insignificant. Under the influence of an electric field, polypeptides migrate at speeds determined by their mass [146, 147]. In ***Study II***, SDS-PAGE was performed using precast mini linear gels 4–20% (Bio-Rad, USA) to examine the polypeptide patterns of fish co-products, helpers and cross-processed proteins. Myosin heavy chain (MHC) and actin were identified based on their well-known molecular weight and high abundance in muscle ($\sim$200 and $\sim$42 kDa, respectively) [9], while all other proteins were tentatively identified based on size and relative abundance or discussed as unknown and referred to by their molecular weight.

4.15 WATER SOLUBILITY OF PROTEIN ISOLATES

The protein isolates were dissolved in distilled water to determine their water solubility in an intuitive and simple way. After sufficient stirring, the solutions were subjected to centrifugation. The resulting supernatant was collected and analyzed for protein content using the Lowry method where after it was related to the initial protein content of the used sample to calculate solubility (%).

4.16 EMULSIFICATION PROPERTIES OF PROTEIN ISOLATES

Emulsifying activity index (EAI) and emulsion stability index (ESI) of protein isolates were analyzed by creating an oil-in-water emulsion system [148]. The protein isolates were dispersed

in distilled water, and sunflower oil was added at a ratio of 1:3 (oil to water), and the mixture was then homogenized using single-stage toothed homogenizer. EAI was determined by measuring the absorbance at 500 nm after diluting the emulsions with 0.1% SDS. ESI was evaluated by observing the stability of the emulsions for 10 minutes and then measuring the absorbance at 500 nm. The EAI and ESI values were calculated based on the absorbance measurements obtained [148].

4.17 Heat-induced gelation of protein isolates

4.17.1 Gel preparation

Thermal processing is a widely employed method for producing gels from protein isolates obtained through pH-shift processing [62]. In ***Study II & VI***, a protocol mainly based on the study by Abdollahi et al. (2020) [149] was utilized to prepare gels, which involved the addition of 2% salt and adjustment of the isolates to 80% moisture and pH 7. Chopping of the protein paste was done using an electric mini food chopper (B0BBYY98PX, Budstfee, Shenzhen, China). The cooking loss during the production of the gels was recorded in ***Study VI***, which refers to the weight loss that occurs due to the release of moisture, lipids, and other compounds [92].

4.17.2 Gel quality analyses

To assess the quality of the gels, gel strength and WHC tests were monitored as they reflect the overall sensory and functional properties of the gels [92]. To measure gel strength, the puncture test was performed using a texture analyzer with a 5-mm spherical probe, while measuring the probe's penetration force and distance. To measure WHC, the gel was weighed before and after centrifugation (3000 ×g, 10 min, 20°C), and the difference was used to calculate its WHC.

4.18 Statistical analysis

The thesis reports data as mean $\pm$ standard deviation, and uses one-way analysis of variance (ANOVA) to determine significant differences between sample groups. Post-hoc comparisons were conducted using Duncan's multiple range test [150], a commonly used multiple comparison procedure that compares sets of means using the studentized range statistic q_r [150, 151, 152]. In this thesis, the statistical analysis was performed using SPSS Statistics software (version 27; IBM, New York, NY, USA) and p-values less than 0.05 were considered significant.

5

Results and discussions

5.1 PROTEIN SOLUBILITY AND YIELD IN PRESENCE OF HELPERS

Total protein yield is a crucial parameter in the pH-shift method. The total protein yield is determined by the protein solubilization yield and protein precipitation yield. To achieve a high protein solubilization yield, it is preferable to have high protein solubility during the solubilization step. Conversely, during the protein precipitation step, low protein solubility is preferred to ensure successful protein precipitation.

The addition of seven types of helpers at the start of the pH-shift process, including LPC, APC, barley spent grain, oat fiber residues, shrimp shells, *Saccharina*, and *Ulva* at an inclusion ratio of 30% (dw/dw), reduced protein solubilization and total protein yield. This result was found regardless of the type of helper, fish co-product (herring or salmon), or version of the pH-shift process version (acid or alkaline) (***Studies I & III***). A main explanation was the precipitation of the fish proteins by phenolic compounds and/or polysaccharides in the plant-/seaweed helpers [153, 154]. A similar effect may have been achieved by calcium carbonate from the shrimp shells [155].

The impact of adding 30% helpers was further investigated by monitoring the protein solubility and solubilization yield of herring co-product proteins over a broad pH-range in absence and presence of the helpers. Typical U-shaped curves were observed for protein solubility and yield in all samples, but with slightly different characteristics (Figure 5.1). The herring co-products alone exhibited the lowest protein solubility at pH 5.5 and the highest values at extreme acidic and alkaline conditions, consistent with previous findings [156, 157]. The addition of helpers in most cases significantly reduced the protein solubility and solubilization yield, regardless of the type of helper or the pH level (acidic or alkaline). Shrimp shells and seaweeds most drastically decreased protein solubility and yield under acidic conditions. Several reasons could explain the effect from seaweed, such as the protein precipitating role of seaweed phlorotannins [92], salt pushing the pH downwards [92] and the competition for water between seaweed-derived polysaccharides and herring proteins, given the high water holding capacity of the former at low pH. Regarding shrimp shells, the strong buffering capacity of cal-

cium carbonate of the shells was a likely reason. Therefore, it was concluded that both acid- and alkali-aided protein solubilization were suitable when cross-processing herring co-products with plant helpers, but only alkali-aided solubilization was compatible with shrimp shells or seaweeds.

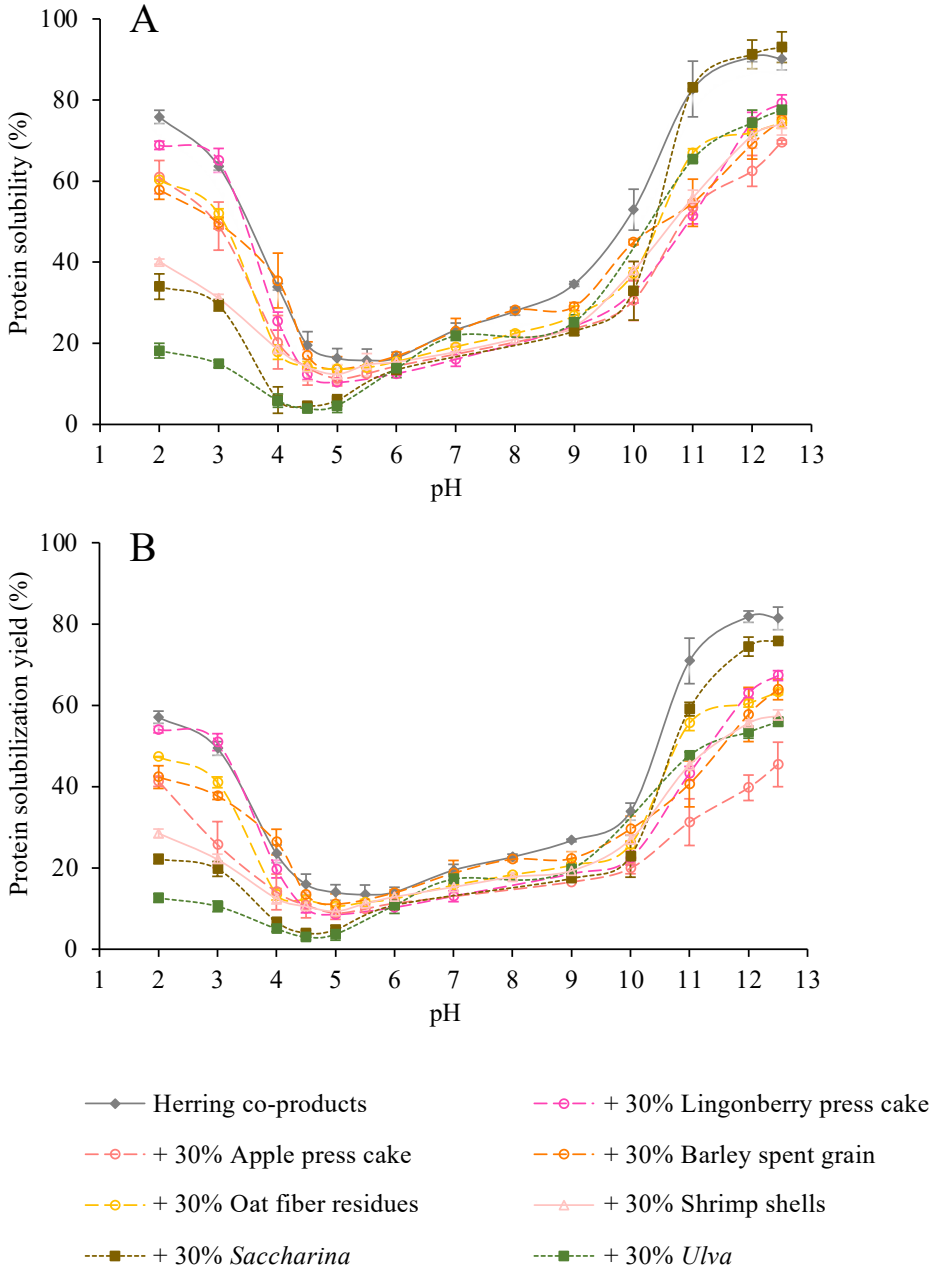

Figure 5.1: Protein solubility (A) and solubilization yield (B) as a function of pH for herring co-products alone and its combinations with plant helpers, shrimp shells and seaweeds.

5.2 STRATEGIES TO IMPROVE PROTEIN SOLUBILITY AND YIELD

5.2.1 Optimization of solubilization and precipitation pHs

The pH values used for cross-processing in the presence of helpers were optimized based on the results presented in Figure 5.1 (***Study II*** and unpublished results). To enhance protein solubilization in the alkaline process version, the solubilization pH was increased from 11.5 to pH 12 for all combinations of helpers and fish co-products. Regarding pH to precipitate alkali-solubilized proteins, it was decreased from 5.5 to 5 when using plant helpers and shrimp shells, while for seaweeds, it was lowered to pH 4.5. For the acid process version, the solubilization and precipitation pH values were set at 2.5 and 5.5, respectively, for all helpers.

5.2.2 Increasing water to raw material ratio

Deeper investigation of modes to improve protein solubility and yield was done for three selected helpers: LPC, shrimp shells and *Ulva*, representing each of the three helper groups, *i.e.*, plant food side streams, marine side streams and seaweeds. Going in depth with multiple helpers was seen as crucial for a comprehensive understanding of their impact on yield improvement.

Increasing the ratio between water and biomass improved the protein solubilization and total protein yield when herring co-products were combined with all three types of helpers (***Study II***). This was likely due to water's ability to swell the protein structure which thereby expose amino acid side chains, promoting dissolution [92]. Additionally, the increased water amount may have facilitated the formation of hydrogen bonds between water molecules and polar groups of the proteins, further enhancing solubilization [92]. Based on the results, an increased water addition was used in ***Studies III-IV***.

5.2.3 Replacing mode of homogenization; from single-stage toothed to radial discharge HSMH

Changing the homogenization mode at the start of the pH-shift process significantly improved protein solubilization, protein precipitation, and total protein yields in the presence of shrimp shells or *Ulva*, reaching levels equivalent to those obtained with herring alone (***Study III***). These improvements can be attributed to the finer dispersion of raw materials generated by radial discharge HSMH, which resulted in reduced particle size and thus, increased surface area, that facilitated protein extraction [92]. Moreover, the improved protein solubilization yield in the combination of herring co-products with *Ulva* could be due to a promoted extraction also of seaweed proteins by radial discharge HSMH, as reflected by the SDS-PAGE patterns. The promising results of radial discharge HSMH with shrimp shells and *Ulva* motivated its use in ***Studies III, IV & VI***.

5.2.4 Use of ultrasonication

Ultrasonication significantly enhanced the protein solubilization yield and total protein yield when cross-processing herring co-products with LPC or *Ulva* (***Study II***). This could be attributed to less polysaccharide aggregation of less protein-polysaccharide precipitates, as evidenced by the largely reduced viscosity of homogenates. As stated earlier, polysaccharides can co-precipitate proteins that then end up in the sediment after the first centrifugation and subsequently are removed [154]. The positive effect of ultrasonication on cross-processing with *Ulva* was not caused by enhanced protein solubility, but by an increased recovery of supernatant after the first centrifugation; from 81.7 $/pm$ 2.4 to 87.6 $/pm$ 0.4 ml per 100 ml of homogenate, reflecting reduced swelling of sediments. In addition, the reduction in seaweed particle size can facilitate the extraction of seaweed proteins [92]. In contrast, ultrasonication reduced the solubilization yield and total protein yield when shrimp shells were used as helpers. This was possibly due to the promoted release of calcium carbonate and other shrimp shell constituents as chitin that induced protein precipitation during the protein solubilization step [155].

5.2.5 Reducing the amount of helper used

For LPC, which proved to be the most powerful antioxidant (see Section 5.4.3), further optimizations of the addition ratio were also done to evaluate its effect on protein solubilization and protein yield. Decreasing the LPC addition ratios from the initially used 30% down to 2.5% mitigated the reduction in herring protein solubilization without affecting protein precipitation. Altogether, this increases the total protein yield (***Study IV***). Compared to 30% LPC addition, 20% and 10% LPC addition increased the total protein yield by five and thirteen percent units, respectively. LPC addition at 5% and 2.5% showed no significant effect on protein solubility and protein yields, compared to the control sample without LPC (***Study IV***).

5.2.6 Changing process step of adding helpers

Shifting the addition point of the most antioxidative helper (LPC) from the beginning of the alkali-aided pH-shift processing of herring co-products to the protein precipitation step avoided the LPC-induced reduction in protein solubilization (***Studies I-III***) and showed no negative effects on the protein precipitation (***Study V***), therefore significantly improved the total protein yield (***Study V***).

5.3 ACID AND BASE CONSUMPTION DURING PROCESSING

The high consumption of acid and base solutions has been reported as a drawback of the pH-shift method [5, 8]. To address this issue, the effects of adding helpers on the consumption of 2 M HCl (acid) and 2 M NaOH (alkali) during the acid and alkaline process versions were examined. This was based on the fact that some of the helpers contain *e.g.*, organic acids or calcium carbonate.

Table 5.1: Initial homogenate pH with different combinations of raw materials. Data show mean values ± standard deviation (n≥3).

Type of fish co-products	Type of helper	Helper addition ratio (%, dw/dw)	Initial pH of homogenate	
Herring heads and backbones	/	/	6.7 ± 0.0^{d}	
	Lingonberry press cake	30	5.2 ± 0.1^{i}	▼
		20	5.7 ± 0.1^{h}	▼
		10	6.2 ± 0.0^{f}	▼
		5	6.5 ± 0.0^{e}	▼
		2.5	6.7 ± 0.0^{d}	
	Apple press cake	30	6.0 ± 0.0^{g}	▼
	Barley spent grain	30	6.8 ± 0.1^{d}	
	Oat fiber residues	30	6.7 ± 0.1^{d}	
	Shrimp shells	30	8.2 ± 0.0^{b}	▲
	Saccharina	30	6.7 ± 0.1^{d}	
	Ulva	30	6.5 ± 0.2^{de}	
Salmon heads and backbones	/	/	6.2 ± 0.1^{f}	
	Lingonberry press cake	30	4.8 ± 0.1^{j}	▼
	Shrimp shells	30	8.5 ± 0.1^{a}	▲
	Saccharina	30	7.1 ± 0.1^{c}	
Salmon heads	/	/	6.8 ± 0.1^{d}	
	Lingonberry press cake	30	5.0 ± 0.2^{ij}	▼

Data within the same column carrying a different superscript letter are significantly different ($p < 0.05$). ▲ and ▼ indicate a significant increase and decrease in the homogenate pH, respectively, compared to the fish co-products alone.

Results revealed that helpers having a low endogenous pH, such as LPC (pH=3), significantly affected required acid and base volumes since it brought in *e.g.*, benzoic acid, citric acid, malic acid [158, 159, 160], cinnamic acid [160], tartaric acid, fumaric acid and shikimic acid [159] to the system. Thus, when added to fish co-products at 30%, LPC significantly decreases the pH of the initial homogenate (Table 5.1), benefiting the acid version of pH-shift processing by saving 27% and 29% 2 M HCl for herring and salmon heads and backbones, respectively (***Study I*** and unpublished results). However, for the alkaline process version, the decreased

initial pH increased the consumption of 2 M NaOH in the alkaline process version. Adding 30% LPC to herring co-products doubled the usage of 2 M NaOH in the protein solubilization step (***Studies I, IV*** and unpublished results). To mitigate this drawback, two strategies were employed: i) reducing the LPC addition ratio (***Study IV***), and ii) adding LPC during the precipitation step rather than at the start, without and with increasing the precipitation pH from 5.5 to 6.5 (***Study V***). By lowering the LPC addition from 30% to 2.5%, the consumption of 2 M NaOH in alkaline pH-shift processing was reduced by 40% (***Study IV***). Adding LPC during the precipitation step had dual advantages in that it not only circumvented the increased 2M NaOH usage during protein solubilization, but also reduced the 2M HCl consumption in the precipitation *per se* as it lowered the pH of the recovered alkaline supernatant. At 30% LPC addition, the requirement for 2M HCl was reduced by 61% (***Study V***). In addition, precipitating the proteins at pH 6.5 rather than 5.5 further decreased the consumption of 2 M HCl by up to 54% at 30% LPC addition (***Study V***).

Contrary to when having LPC as helper, adding 30% shrimp shells to fish co-products increased the pH of the homogenate (Table 5.1), contributing to a higher HCl consumption for the acid-aided processing of herring and salmon co-products; by 55% and 57%, respectively (***Study I***). The increased acid requirement was also attributed to the presence of calcium carbonate in shrimp shells being a strong buffering agent, *i.e.*, reacting with HCl.

5.4 Role of helpers for lipid oxidation during pH-shift processing and ice storage of protein isolates

5.4.1 Lipid oxidation during pH-shift processing without helpers

Significant increases in the monitored lipid oxidation markers; PV as well as individual aldehydes including MDA, HHE, HNE, hexanal, (E)-2-hexenal, heptanal, octanal, and 2,4-heptadienal, were observed in freshly prepared herring protein isolates compared to in the crude herring co-product raw materials (***Studies I-V***). A similar increase was observed when pH-shift processing salmon heads (***Study II***), but not with a mixture of salmon heads and backbones (only MDA measured) (***Study I***). This difference could be due to the lower Hb content in the mixture of salmon heads and backbones (84.99 $\pm$ 1.01 μmol/kg, dw) compared to salmon heads alone (144.35 $\pm$ 1.77 μmol/kg, dw) since gills serve as a significant blood source. Hb is a strong pro-oxidant which according to several mechanisms, such as the decomposition of lipid hydroperoxides into free radicals, can stimulate lipid oxidation [161, 162, 163]. Herring co-products were generally found to be more susceptible to lipid oxidation during pH-shift processing than salmon co-products, whether using salmon heads alone or a mixture of salmon heads and backbones (***Studies I & III***). Likely reasons were the higher Hb content in herring heads and backbones (270.61 $\pm$ 0.87 μmol/kg dw) than in the salmon raw materials, as well as the presence of astaxanthin in salmon muscle, which is a strong antioxidant [164].

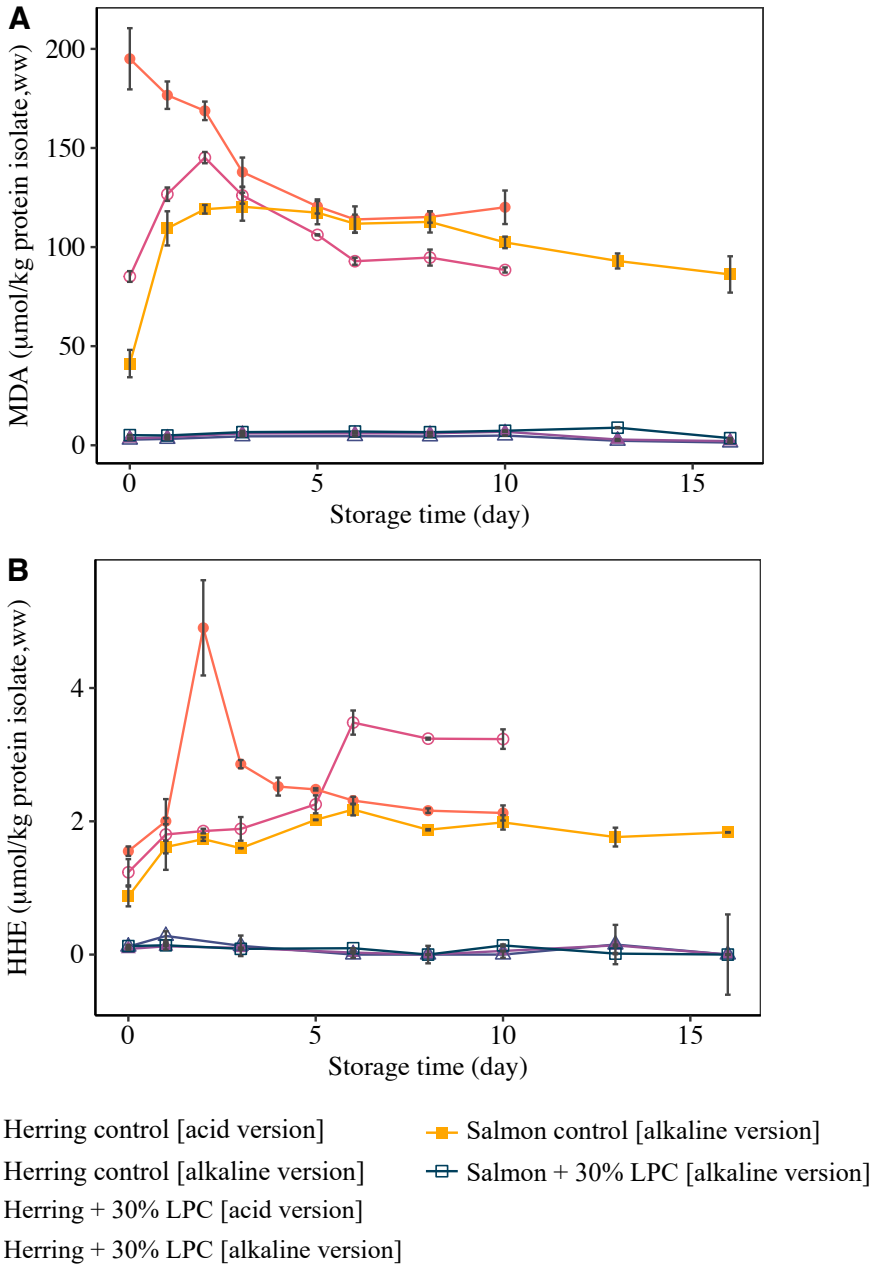

Figure 5.2: Malondialdehyde (MDA -A) and 4-hydroxy-(E)-2-hexenal (HHE -B) measured in protein isolates derived from herring and salmon co-products. These isolates were produced through both alkaline and acid versions of the pH-shift process, with or without the addition of lingonberry press cake (LPC). The storage conditions were under extended duration on ice.

Lipid oxidation observed during the pH-shift processing of herring and salmon co-products can be attributed to several factors. First, the addition of water to fish co-products at the beginning of the process dilutes the endogenous aqueous antioxidants *e.g.*, ascorbic acid and uric acid that are proven to limit lipid oxidation [39, 103]. Second, high-speed homogenization dis-

rupts the organized fish muscle microstructure and removes connective tissues (endomysium), leading to increased exposure of membrane phospholipids to pro-oxidants like Hb [165]. Third, along with membrane removal, α-tocopherol is partially removed during the first centrifugation step [19]. Lastly, and perhaps most significantly, the low pH (5.5) applied during protein precipitation can induce Hb-deoxygenation and metHb-formation, promoting the pro-oxidative activity of Hb through heme group exposure and hemin loss [162, 163]. Sarcoplasmic proteins like Hb have been reported to co-precipitate with myofibrils and/or membranes at pH 5.5, resulting in close proximity between Hb/hemin and phospholipids [166].

During ice storage of herring protein isolates made without helpers, the elevated MDA initially decreased followed by stabilization from Day 5 (***Study III***). The HHE content on the other hand peaked on Day 2, followed by a decline (***Study III***). In salmon isolates, MDA and HHE levels increased during storage, with peaks reached on Day 2 and Day 6, respectively (***Study III***). Differently from MDA and HHE, the elevated levels of volatile aldehydes that were detected in freshly made herring co-products isolates continuously increased for another 3 days when sampling stopped due to a strong rancid odor (***Studies IV & V***).

5.4.2 Role of pH-shift process versions for lipid oxidation

Contrary to previous findings [167], both ***Studies I*** and ***III*** reported higher MDA levels in alkali-produced herring protein isolates compared to in isolates from the acid process version. This discrepancy may arise from the complex interplay of two opposing factors that influence lipid oxidation during pH-shift processing. Firstly, acidic conditions increase heme-protein pro-oxidative activity due to autoxidation, heme-loss, and hydroperoxide breakdown, thereby accelerating oxidation [164, 168]. However, lipid oxidation may be slowed down at extremely acidic pHs ($\leq$3.5) as a result of membrane aggregation [169], despite the activation of Hb as a pro-oxidant at slightly acidic pHs (5.7-7). The net effect of these opposing factors depends on variables such as initial raw material quality, heme-protein levels, and holding times at various pHs. Further to this, MDA is reported to be more reactive below its pKa-value of pH 4.46 where it exists in equilibrium between the protonated enol-aldehyde form and the dialdehyde form [170]. Thus it, may be formed but not detected in the free form at low pH due to binding *e.g.*, to proteins.

5.4.3 Role of the type of helper

LPC was the most effective in limiting lipid oxidation during both the production and ice storage of fish protein isolates (***Studies I-III***). The addition of 2.5-30% LPC to herring/salmon co-products at the beginning of processing completely inhibited the formation of MDA and HHE as well as volatile aldehydes during the isolate production, regardless of whether acid or alkaline versions of pH-shift processing were used (***Studies I-IV***). Moreover, at a 30% LPC addition, the formation of MDA, HHE and volatile aldehyde markers was entirely inhibited in cross-processed herring protein isolates during 16 days of ice storage (***Studies II & IV***).

Table 5.2: Ability of different helpers to preventing lipid oxidation, taking the alkaline version of the pH-shift processing of herring co-products as an example. MDA= malondialdehyde, HHE= 4-hydroxy-(E)-2-hexenal.

Helper	**Helper addition ratio (%, dw/dw)**	**Lipid oxidation marker**	**Ability to reduce the max. level of the marker**	**Extension of lag phase in achieving the max. level *vs.* control (day)**
Lingonberry press cake	30	MDA	Yes	>16
	30	HHE	Yes	>16
Apple press cake	30	MDA	Yes	3
	30	HHE	Yes	1
Saccharina	30	MDA	No	2
	30	HHE	Yes	1
Ulva	30	MDA	No	2
	30	HHE	Yes	2
Oat fiber residues	30	MDA	No	3
	30	HHE	No	5
Barley spent grain	30	MDA	No	1
	30	HHE	No	4
Shrimp shells	30	MDA	No	0
	30	HHE	No	0

The outstanding antioxidant capacity of LPC can be attributed to its high concentration of aqueous antioxidants, *e.g.*, proanthocyanidins, flavonols, anthocyanins and benzoic acid [90, 159, 171, 172, 173]. When using the alkaline process version (***Studies I-IV***), alkali-soluble molecules that remain soluble at approximately pH 5 or that co-precipitate with the protein isolate are expected to be key antioxidants. The TPC of the LPC used in this thesis was 3.1 $\pm$ 0.0 g gallic acid equivalents (GAE)/100g, dw. To further study the potential of LPC phenolic compounds in limiting Hb-mediated lipid oxidation, LPC was pH-shift processed alone, and the resulting fractions (pellet 1 and 2, supernatant 1 and 2) were added to Hb-fortified washed cod mince, which was then stored on ice to monitor lipid oxidation (unpublished results). The most active fraction (Supernatant 2) was separated based on polarity and subjected to anthocyanin analysis, identifying cyanidin-3-galatoside ("ideain") and procyanidin A1 as the most potent antioxidants in LPC (unpublished results). Anthocyanin profiling of crude LPC used in this thesis also showed that ideain was the most abundant molecule, followed by cyanidin-3-rutinoside ("keracyanin"), delphinidin-3-rutinoside ("tulipanin"), and malvidin-3,5-diglucoside ("malvin"). All of these anthocyanins have previously shown promising antioxidant capacity in various *in vitro* assays, including DPPH, FRAP, and ABTS [172, 173, 174]. An outstanding an-

tioxidant capacity of lingonberries has also been reported in other studies. For example, *in vitro* assays revealed that ethanol-water (60:40, v/v) extracts of lingonberries effectively scavenged DPPH radicals, while ethyl acetate extracts exhibited strong cupric-reducing antioxidant capacity (CUPRAC) [171]. Moreover, lingonberry leaves and stems have been proposed as good sources of natural antioxidants due to their high antioxidant capacity, as measured by the DPPH assay [90]. Additionally, when 3% (w/w) dried industrial LPC was added directly to minced de-skinned herring fillets, it significantly decreased the formation of volatile aldehydes during a 10-month frozen storage period [85]; the antioxidant ability was ascribed to proanthocyanidins and flavan-3-ols [85, 175].

Table 5.3: Anthocyanin profile of lingonberry press cake. The results are presented in μmol/g dry weight, dw (mean values $\pm$ standard deviation (n=2)).

Anthocyanin compound	Common name	Concentration (µmol/g, dw)
Cyanidin-3-galatoside	Ideain	671.67 ± 56.57
Cyanidin-3-rutinoside	Keracyanin	79.91 ± 8.53
Delphinidin-3-rutinoside	Tulipanin	44.26 ± 2.58
Malvidin-3,5-diglucoside	Malvin	39.92 ± 2.33
Delphinidin-3-galactoside		3.24 ± 0.71
Pelargonidin-3-rutinoside		2.77 ± 0.61
Delphinidin 3-glucoside	Myrtillin	0.54 ± 1.47
Cyanidin		n.d.
Cyanidin-3,5-diglucoside	Cyanin	n.d.
Cyanidin-3-glucoside	Kuromanin	n.d.
Cyanidin-3-arabinose		n.d.
Delphinidin		n.d.
Malvidin		n.d.
Malvidin-3-galactoside		n.d.
Malvidin-3- glucoside	Oenin	n.d.
Pelargonidin		n.d.
Pelargonidin-3-glucoside	Callistephin	n.d.
Peonidin		n.d.
Peonidin-3-arabinoside		n.d.
Peonidin-3-galactoside		n.d.

n.d. = Not-detected.

Beyond LPC, the most effective helpers in reducing MDA levels when added at 30% level

were: APC > barley spent grain > *Saccharina* > oat fiber residues = *Ulva* (***Study III***). A similar ranking was observed regarding HHE-prevention (***Study III***). In presence of all these helpers, apart from oat fiber residues, aldehyde levels initially increased and then declined. APC and *Ulva* however significantly reduced the maximum level of MDA formed, while the other helpers delayed the formation of MDA. The MDA level peaked on Day 2 with barley spent grain, on Day 3 with *Saccharina*, and on Day 4 with oat fiber residues. The kinetics of HHE contents were generally similar but with a few differences. First, the maximum HHE level was significantly reduced when adding APC and seaweeds, while adding barley spent grain and oat fiber residues, the HHE peak was delayed to Day 5 (***Study III***).

The considerable antioxidant potential of APC during pH-shift processing can be explained by its rich content of phenolic compounds, such as tannins, phlorizin, chlorogenic acid, epicatechin, and quercetin glycosides [77]. Also, earlier researchers have documented antioxidant effects of apples and apple side streams in fish. Bitalebi et al. (2019) effectively incorporated apple peel water extracts made with ultrasound into minced rainbow trout, which delayed lipid oxidation (PV and TBARS values), protein oxidation (protein carbonyls), and total sulfhydryl group losses during 96 hours of cold storage [176]. Sun et al. (2017) discovered that adding an ethanol extracted from young apples using microporous resins successfully slowed down lipid oxidation in grass carp surimi over seven days of refrigerated storage, as evidenced by decreases in PV and TBARS values. Chlorogenic acid was identified as the main preservative component in the apple extract [77].

Possibly the antioxidant potential of APC, barley spent grain and oat fiber residues was limited by their lower TPC and the inefficient extraction of their antioxidants *e.g.*, phenolic compounds, by water under the pH-cycle used in the pH-shift process. Hereby, antioxidants would be lost into the pellet from the first centrifugation. There was a positive correlation between the lipid oxidative stability of cross-processed protein isolates and their TPC. The protein isolate produced with LPC had a notably higher TPC (828.0 $\pm$ 16.1 mg GAE/100 g dw) in comparison to the protein isolate produced with APC; 338.8 $\pm$ 18.9 mg GAE/100 g dw. Isolates with barley and oat co-products only had 252.6 $\pm$ 14.0 and 263.5 $\pm$ 18.9 mg GAE/100 g dw, respectively (***Study II***). Chemical and physical pretreatments could likely be applied to these helpers to achieve higher antioxidant capacity. Methods such as enzymatic hydrolysis [91], maceration, ultrasound, and microwave treatments [89] have been utilized for extracting aqueous phenolic compounds from barley and oat side-streams. Additionally, different ratios of polar to non-polar antioxidants (phenolic and non-phenolic) in the various plant helpers likely impacted their antioxidant activity, given that the pH-shift system is primarily aqueous, aside from the presence of fish lipids, which can also function as extractants. It is also possible that the specific antioxidants found in apple, oat, and barley by-products were less effective against the predominantly Hb-mediated oxidation that occurs during pH-shift processing of fish co-products [166]. As described above, earlier in vitro studies of antioxidants in these raw materials have not tested their specificity towards Hb, but rather towards low molecular weight (LMW) Fe, Cu and synthetic radicals.

Saccharina is recognized for containing considerable amounts of polyphenols, such as phlorotannins, and the carotenoid fucoxanthin [94]. Brown seaweed phlorotannins have been shown to scavenge free radicals, peroxyl and nitric radicals, as well as to chelate ferrous ions *in vitro* [94]. Regarding *Ulva*, sulfated polysaccharides have earlier been reported to have strong

DPPH·, ABTS·+, hydroxyl, and superoxide radical scavenging capabilities, along with ferrous ion chelating ability *in vitro* [96, 97]. However, the issue of inefficient antioxidant extraction may apply also to the tested seaweeds. Seaweed cell walls and cuticles are chemically and structurally more complex and heterogeneous than those of land-based plants, due in part to the presence of polysaccharides like alginates and carrageenan [94]. Additionally, the high concentration of salts and minerals in seaweeds may act as pro-oxidants within the fish/water/alkali system [94], counteracting the potential antioxidant capacity of seaweeds. Unpublished results from our group (Harrysson et al., unpublished) revealed that the addition of press juice from *Saccharina* to minced trout muscle resulted in a net pro-oxidative effect on TBARS/PV-formation during ice storage.

Shrimp shells added at 30% did not show significant potential in limiting lipid oxidation during the production or storage of protein isolates, regardless of the type of fish co-products (herring or salmon) or the pH-shift process version (acid or alkaline). Instead, MDA levels in alkali-produced herring plus shrimp peel protein isolates appeared to peak during the actual processing step and then continuously decreased during ice storage (***Studies I-III***). Similar lipid oxidation kinetics have earlier been observed following acid pH-shift processing of herring fillets [10], and during ice storage of *e.g.*, minced herring co-products [138] and Hb-fortified washed cod mince [56, 177]. A likely reason is the high reactivity of many carbonyls with proteins and phospholipids, forming Schiff bases[178]. The lack of antioxidant capacity from cross-processing with shrimp peels could be linked to inadequate extraction of the lipophilic and/or protein bound [93] astaxanthin into the protein isolate. It could even be seen how some astaxanthin appeared in the floating emulsion layer and first sediment from the first centrifugation, which were subsequently discarded. In an earlier study, astaxanthin was extracted from shrimp peels at a yield of 50.6 ± 0.30% [93] using pH-shift processing and the recovered protein isolates exhibited *in vitro* DPPH and ABTS radical scavenging properties [93].

5.4.4 Role of LPC to fish ratio

LPC, recognized as the most effective helper in limiting lipid oxidation, was studied using different addition ratios, ranging from 2.5% to 30%. Adding 2.5% LPC was sufficient to prevent the formation of volatile aldehydes, including hexanal, (E)-2-hexenal, heptanal, octanal, and 2,4-heptadienal, during alkali-aided pH-shift processing of herring by-products (***Study IV***). Throughout ice storage, there was then a clear correlation between the hexanal development lag phase and the LPC addition. The lag phase was 4 days with 2.5% LPC, 5 days with 5% LPC, 9 days with 10% LPC, and 12 days with 20% LPC. The 30% LPC addition prevented the formation of hexanal throughout the entire 16 days of ice storage. Similar patterns were observed for other volatile aldehyde markers (***Study IV***). These results were strongly associated with TPC of the protein isolates (***Study IV***).

5.4.5 Role of helper addition point during the process

Adding 10% and 30% LPC during the precipitation step instead of at the beginning of the alkaline pH-shift process significantly enhanced the stability of herring co-product protein isolates towards lipid oxidation. In fact, the monitored volatile aldehyde markers were not detected

throughout the entire 21 days of ice storage, whereas when added at the beginning, 10% and 30% LPC only prevented volatile aldehyde formation for 8 and 15 days on ice, respectively (***Studies V-VI***). These results were clearly associated with the TPC of the protein isolates. Protein isolates produced with 10% or 30% ILP added during the precipitation step exhibited TPC levels of 1.2 $\pm$ 0.1 and 2.4 $\pm$ 0.0 g GAE/100g, dw, respectively which was notably higher than those produced with LPC added from the start (0.43 $\pm$ 0.01 g GAE/100g, dw and 0.87 $\pm$ 0.02 GAE/100g, dw, respectively) (***Studies V-VI***). Adding LPC at the beginning of the process only transfers alkali-soluble antioxidants, such as ideain and procyanidin A1, to the isolate. In contrast, adding LPC during the precipitation step retains most of the LPC-derived antioxidants, including those bound to peels and seeds, in the protein isolates.

5.5 Composition of protein isolates

5.5.1 Crude protein content

The pH-shift process has been demonstrated to effectively remove collagenous materials, such as bone, skin, and connective tissues, as well as lipids from fish co-products [5, 167, 64, 179]. Aligned with this, the protein content measured in protein isolates was higher than in their corresponding raw materials (***Studies I-V***). The fish co-product type, *i.e.*, salmon or herring, and the pH-shift process version, *i.e.*, acid or alkaline, however played significant roles in determining the exact protein content of protein isolates (***Studies I & III***). For herring co-products, the alkaline version up-concentrated the protein more compared to the acid version (***Studies I & III***), while for salmon, the difference between the two process versions was less pronounced (***Studies I & III***). This pattern persisted even when helpers were added (***Studies I & III***).

The addition of different helpers at the start of the pH-shift process did not affect the up-concentration of proteins in a systematic manner (***Studies I-IV***). For example, protein content was increased with the addition of 30% LPC in alkali-produced salmon isolates and acid-produced herring isolates (***Studies I & III***) but in alkali-produced salmon protein isolates made with 30% *Saccharina* and shrimp shells (***Study I***), or alkali-produced herring protein isolates made with 30% LPC, APC, *Saccharina*, and *Ulva*, protein content was decreased (***Studies I-III***). This reduction was possibly due to partitioning of *e.g.*, carbohydrates and phenolics in the precipitated protein isolate (***Studies I-III***).

The addition of LPC at varying levels (***Study IV***) and at different stages of the process (***Study V***) also influenced the protein content of the alkali-produced herring protein isolates. This was due to proteins being diluted to different degrees by *e.g.*, carbohydrates and phenolics of the LPC (***Studies IV-V***). When reducing the addition of LPC at the start of the pH-shift process from 30% to 2.5%, the protein content of the isolate increased from 70.2 $\pm$ 0.8 to 81.5 $\pm$ 3.6 g/100, dw (***Study IV***). When 2.5-30% LPC was added during the precipitation step, the protein content decreased from 82% to 63% with increasing LPC addition (***Study V***).

The protein content was also impacted by the processing settings used. For example, replacing the single-stage toothed by the radial discharge HSMH increased the protein content in the final protein isolate made with 30% *Ulva*. This could be explained *e.g.*, by more effective removal of bones and lipids from the fish co-products (***Study II***). Ultrasonication, on the other

hand, decreased the protein content of isolates produced with 30% LPC (***Study II***). This was attributed to its ability to co-extract non-protein compounds such as carbohydrates from the LPC, diluting the protein of the final isolates.

5.5.2 Crude lipid content

The lipid content in protein isolates was generally lower than in their corresponding raw materials (***Studies I-V***), which was consistent with previous studies reporting an effective lipid-removal ability of the pH-shift method [5, 167, 64, 179]. Both the type of fish co-product (salmon or herring) and the pH-shift variant (acidic or alkaline) however had considerable influence on the precise lipid content of protein isolates (***Studies I & III***). Salmon co-products contained higher lipid content than herring co-products, resulting in higher lipid content in salmon-derived protein isolates (***Studies I & III***). Additionally, the acid version of the process produced isolates with a higher lipid content compared to the alkaline version (***Studies I & III***), which aligns with previous findings [180].

The addition of different helpers also had varying effects on the lipid content. Combining 30% LPC, *Saccharina* and *Ulva* with herring or salmon co-products during the alkaline pH-shift process version showed a high potential for lipid removal, possibly due to an increase in polarity of the aqueous phase from polyphenols and salts [92] (***Studies I-III***). Possibly LPC also stimulated lipid emulsification and thereby larger removal to the lipid layer [66, 92, 181]. When the addition of LPC was gradually reduced from 30% to 2.5%, the lipid content of the protein isolates increased, confirming that adding LPC improved the lipid removal capacity of the process (***Study IV***). In contrast, the addition of 30% shrimp shells increased lipid content, potentially due to the visually fragile and unstable floating lipid layer formed after the first centrifugation (***Studies I-III***).

Changing the addition of LPC from the process start to the precipitation step increased the lipid content of isolates; more so when more LPC was added (***Studies IV-V***). This was attributed to the LPC itself, which consists of 14% lipid (dw basis), remaining in the final protein isolates. (***Study V***). Lipids are mainly found in the seeds, wax layers and left-over flesh of the LPC [182].

The lipid content was also influenced by the principle of HSMH applied to the raw materials. In particular, radial discharge HSMH resulted in a notable reduction in lipid content compared to single-stage toothed HSMH, especially when *Ulva* was added (***Study II***). This reduction was attributed to the improved solubilization and emulsion capacity of proteins, which subsequently enhanced the size of a stable floating lipid layer which can be easily removed [5, 66]. However, when radial discharge HSMH was combined with ultrasonication, the floating lipid layers were visually weak and protein isolates exhibited a higher lipid content for all raw materials (***Study II***). This observation might stem from the greater effectiveness of ultrasonication in dispersing the material and promoting higher curvature of lipid droplets [183, 184].

5.5.3 Ash content

The ash content of fish protein isolates was lower compared to their corresponding raw materials (***Studies I-V***). This finding aligns with previous studies reporting that the pH-shift process

effectively removes mineral-dense components such as bones and shells from fish co-products [5, 167, 64, 179].

Protein isolates from the acid-aided pH-shift process exhibited higher ash content than those from the alkaline version (***Studies I & III***). This observation was in line with previous studies [185] and was likely due to the higher volume of acid and base consumed during acid processing creating salt and also extracting more minerals from the bones into the final protein isolates. Moreover, herring-derived protein isolates had lower ash content than salmon-derived ones, even though the crude herring co-products contained more ash (***Studies I & III***). Thus, the pH-shift process was more effective for herring than salmon co-products.

When seaweeds were added as helpers, the ash content of herring and salmon protein isolates increased (***Studies I-III***) due to the naturally high ash content in seaweeds. In line with this, changing from single-stage toothed to radial discharge HSMH led to a further increase in the ash content of protein isolates, which was amplified even more by ultrasonication (***Study II***).

5.6 Color of protein isolates

5.6.1 Color of fresh protein isolates

The color of fish protein isolates was influenced by the pH-shift process version, the type of fish co-products and the type of helpers.

The acid-produced isolates were visually lighter, reflected by higher L^*-values, than their alkali-produced counterparts (***Studies I & III***). This observation was consistent with the findings of Yongsawatdigul and Park (2004) [186] and can be attributed to either a more effective removal of hemeproteins during alkaline pH-shift processing [166] or higher moisture content in the acid-produced isolates (***Study I***).

Salmon-derived isolates exhibited higher L^*-, a^*-, and b^*-values than those from herring (***Studies I & III***), primarily due to the presence of astaxanthin in the residual muscle of salmon co-products along with less heme-proteins; the latter easily changing to the brown met-form and deceasing lightness of herring-derived isolates [100, 187].

The addition of helpers impacted the color of protein isolates primarily due to the co-extraction of pigments (Figure 5.3). For example, the addition of *Ulva* resulted in a green color, *i.e.*, reduced a^*-value, attributable to the presence of chlorophyll [188]. A parallel increase in b^*-value could be explained by the presence of xanthophyll [188] (***Study III***). *Saccharina* has fucoxanthin as its major pigment [94], thus rendering a more yellow color to the isolates, reflected by a higher b^*-value (***Studies I & III***). Shrimp shells contributed with pinkish-red color, likely from astaxanthin [189, 190] (***Studies I & III***). LPC imparted a purple shade (***Studies I, III-V***) due to anthocyanins [191], which change color depending on the surrounding pH [192] and shift from bright reddish at low pH to darker purple $\geq$ pH 7 because of the reversible chalcone-flavanone conversion [193]. An additional effect from the antioxidant-containing helpers like LPC was that they could prevent the formation of brown metHb or metMb during the protein precipitation step at pH 5 [19].

Figure 5.3: Color of protein isolates produced from herring co-products in the absence and presence of helpers by the alkaline version of pH-shift process. The protein isolates have a pH of 7 and a moisture content of 80%.

5.6.2 Color changes during ice storage

During ice storage, salmon-derived alkali-produced protein isolates experienced an increase in L^*-values and a decrease in a^*-values after seven days of storage (***Study III***). This result aligned with previous studies [39, 194] and was mainly attributed to astaxanthin bleaching. However, the loss of oxy-Hb in favor of the brownish-grey met-Hb and/or possible heme-ring destruction could also decrease a^*-values, both of which can occur alongside lipid oxidation [92, 100].

For the protein isolates produced from herring co-products alone, the alkali-produced isolates decreased in a^*-values and increased in b^*-values after ten days of storage (***Study III***). The latter was likely due to the formation of tertiary lipid oxidation products, which can turn into yellow pigments [23, 41, 44]. On the other hand, the acid-produced isolates displayed continuous increases in L^*- and b^*-values throughout the ice storage, and decreases in a^*-values from Day 2. The reduction in a^*-values was correlated with the onset of MDA/HHE-formation and is likely a result of Hb-oxidation [100] (***Study III***).

With the addition of 30% LPC, the herring protein isolates showed an increase in b^*-values during storage, regardless of the pH-shift version used. This change appeared non-connected to lipid oxidation (***Study III***). Protein isolates produced with barley spent grain and oat fiber residues remarkably lost a^*-values during ice storage from Day 10 along with increased L^*-values; the former also showed increased b^*-values (***Study III***). The decreased a^*-values were also found in the protein isolates produced with *Saccharina* and shrimp shells, from Day 8 and Day 10, respectively (***Study III***). The a^*-value losses in alkali-produced herring isolates did however not follow lipid oxidation development, and appeared linked to other phenomena, *e.g.*, co-oxidation/bleaching of helper-derived pigments (*e.g.*, carotenoids) (***Study III***).

5.7 Active and total sulfhydryl groups

Adding 30% LPC, shrimp shells, and *Ulva* at the start of the alkali-aided pH-shift processing of herring co-products generally resulted in a higher content of active and total sulfhydryl groups in the final protein isolates (***Study II***). In the presence of shrimp shells and *Ulva*, substituting single-stage toothed with radial discharge HSMH led to higher contents of active and total sulfhydryl groups and a lower ratio of active to total sulfhydryl groups (***Study II***). This could be due to protein unfolding, exposing internal sulfhydryl groups [195]. In contrast, when LPC was added, the use of radial discharge-HSMH raised the ratio of active to total sulfhydryl groups from 63% to 76% (***Study II***). This suggested better exposure of sulfhydryl groups to the solvent, indicating more extensive protein conformational changes during the pH-shift-based cross-processing [196]. Moreover, ultrasonication generally increased the content of active and total sulfhydryl groups of protein isolates recovered from herring co-products without and with LPC addition (***Study II***). A likely explanation was the breakage of intermolecular disulfide bonds and exposure of buried sulfhydryl groups caused by cavitation [197]. In contrast, for protein isolates produced with shrimp shells and *Ulva*, ultrasonication decreased the content of active and total sulfhydryl groups (***Study II***). In addition, partial aggregation of proteins caused by the high ultrasound power could lead to re-encapsulation of some sulfhydryl groups [198].

5.8 Surface hydrophobicity

The addition of 30% LPC, shrimp shells and *Ulva* to herring co-products at the beginning of the alkaline pH-shift process significantly enhanced the surface hydrophobicity of produced protein isolates (***Study II***). This finding suggested that helpers either stimulate molecular unfolding, revealing previously concealed hydrophobic groups [197], or facilitate the co-extraction of proteins or peptides with high surface hydrophobicity from the helpers.

Replacing the single-stage toothed by radial discharge HSMH led to a considerable decrease in the surface hydrophobicity of proteins obtained with LPC and *Ulva* (***Study II***). On the other hand, ultrasonication was found to enhance the surface hydrophobicity of protein isolates produced with LPC, from 68.1 to 82.1 (***Study II***). This enhancement indicated that cavitation and shear stress generated during the ultrasound treatment induce protein conformational changes. Similarly, Sun et al. (2014) [199] discovered that long and intense ultrasound treatment decreased protein surface hydrophobicity of milk proteins, which was attributed to the aggregation and re-polymerization [199].

5.9 Polypeptide profile

The role of helpers for the electrophoretic patterns of protein isolates under reducing conditions is presented in Figure 5.4. The shown protein isolates were produced from herring co-products through alkali-aided pH-shift processing, without and with 30% LPC, shrimp shells, and *Ulva* added at the start. Just as the non-processed herring co-products, all protein isolates were abundant in MHC (∼205 kDa), myosin light chain (∼25 kDa), and actin (∼42 kDa) (***Study II***),

Table 5.4: Sulfhydryl groups (SH) and surface hydrophobicity of protein isolates produced from herring co-products without and with different helpers. Data are given as mean values ± standard deviation (n=3). SST= single-stage toothed, RD= radial discharge, HSMH= high shear mechanical homogenization, US= Ultrasonication.

Source	Treatment	Active SH (µmol/g)		Total SH (µmol/g)		Ratio of active to total SH (%)	Surface hydrophobicity	
Herring co-products	SST-HSMH	11.5 ± 0.1^{h}		15.9 ± 0.2^{i}		72.2	46.1 ± 4.2^{h}	
	RD-HSMH	13.6 ± 0.1^{g}		22.5 ± 0.4^{h}		60.4	42.9 ± 5.0^{h}	
	RD-HSMH +US	15.0 ± 0.1^{f}		23.7 ± 0.3^{gh}		63.3	46.6 ± 2.7^{h}	
+ 30% Lingonberry press cake	SST-HSMH	16.6 ± 0.3^{e}	▲	26.2 ± 0.0^{fg}	▲	63.4	106.1 ± 5.3^{a}	▲
	RD-HSMH	13.9 ± 0.3^{g}		18.3 ± 0.4^{i}	▼	76.1	68.1 ± 6.3^{g}	▲
	RD-HSMH +US	17.0 ± 0.4^{e}	▲	25.9 ± 0.8^{fg}		65.7	82.1 ± 2.8^{de}	▲
+30% Shrimp shells	SST-HSMH	21.5 ± 0.0^{b}	▲	31.2 ± 1.7^{cd}	▲	68.9	98.4 ± 1.0^{ab}	▲
	RD-HSMH	21.0 ± 0.3^{bc}	▲	36.1 ± 0.7^{b}	▲	58.1	97.0 ± 1.8^{abc}	▲
	RD-HSMH +US	13.4 ± 0.0^{g}	▼	28.1 ± 0.2^{ef}	▲	47.8	86.2 ± 3.1^{cde}	▲
+ 30% *Ulva*	SST-HSMH	20.2 ± 0.1^{c}	▲	29.0 ± 0.2^{de}	▲	69.7	91.7 ± 1.2^{bcd}	▲
	RD-HSMH	27.8 ± 0.4^{a}	▲	42.1 ± 1.2^{a}	▲	66.1	79.7 ± 3.3^{ef}	▲
	RD-HSMH +US	18.4 ± 0.3^{d}	▲	32.6 ± 1.7^{c}	▲	56.6	68.9 ± 0.6^{fg}	▲

Different small letters in each column show a significant difference ($p<0.05$).

▲ and ▼ indicate a significant increase and decrease, respectively, compared to the fish co-products when using the same treatments.

demonstrating a polypeptide pattern similar to that previously reported for gutted herring proteins [180]. This finding revealed that cross-processing with helpers did not significantly affect the overall polypeptide composition of herring proteins. The exception was LPC which possessed a proteolysis-inhibiting and cross-linking effect. Protein isolates derived through classic pH-shift processing of herring co-products +/- *Ulva* displayed a few bands below MHC (***Study II***), indicating minor proteolysis. This phenomenon was counteracted by LPC, which on the other hand darkened the area above MHC, signifying potential protein crosslinking (***Study II***). The latter was most likely due to polyphenols.

The transition to radial discharge HSMH resulted in a more intense MHC band, which was subsequently diminished by ultrasonication (***Study II***) as shown by minor MHC degradation on the herring controls (***Study II***). This was likely attributable to acoustic cavitation and streaming, leading to violent shear force between solvent and protein molecules [183]. Another explanation could be the activation of endogenous protease(s) [200]. Nonetheless, this effect was not seen when applying ultrasound in the presence of helpers (***Study II***).

5.10 Water solubility of protein isolates

The water solubility of proteins is a critical characteristic that influences various protein functionalities, such as emulsifying, foaming, and gelation properties [92]. The water solubility of herring protein isolates, produced through alkali-aided pH-shift processing in presence or absence of 30% LPC, shrimp shells, or *Ulva*, was investigated at pH 7 and pH 11 (***Study II***). The former pH represents physiological and early post mortem conditions of many biological systems and is significant to understand for industrial applications of food proteins in which these interact with each other and with other molecules [92]. On the other hand, alkaline conditions induce changes in protein conformation, charge distribution, and molecular interactions, which can enhance the water solubility of protein isolates compared to neutral conditions [92].

A significantly lower water solubility was observed for all protein isolates at pH 7 compared to pH 11. However, absolute numbers were lower, which was in line with the findings of Marmon et al. (2012) [165]. Herring protein isolates generated without helpers exhibited 6-8% solubility at pH 7 and 36-46% at pH 11 (***Study II***). The lower water solubility at pH 11 for the isolate compared to native muscle could be due to the formation of hydrogen bonds, hydrophobic interactions, and S−S bridges [165]. Neither changing the homogenizer nor employing ultrasonication impacted the water solubility of protein isolates at pH 7 (***Study II***). However, at pH 11, protein solubility increased considerably when substituting single-stage toothed by radial discharge HSMH and utilizing ultrasonication (***Study II***). This phenomenon could be attributed to the cavitation effect of ultrasonication, which disrupts Van der Waals forces, hydrogen bonding, and dipole attractions among molecules [197].

The addition of 30% LPC, shrimp shells and *Ulva* in general increased the water solubility of protein isolates up to 2-3-fold at both pH 7 and pH 11 compared to herring isolate controls made without helpers (***Study III***). It is likely that the helpers affected protein unfolding and refolding patterns during the pH-shift process, resulting in greater exposure of polar groups [197]. Change to radial discharge HSMH significantly reduced the water solubility of isolates made with LPC at both pH levels (***Study III***). For combinations with shrimp shells or *Ulva*, radial discharge

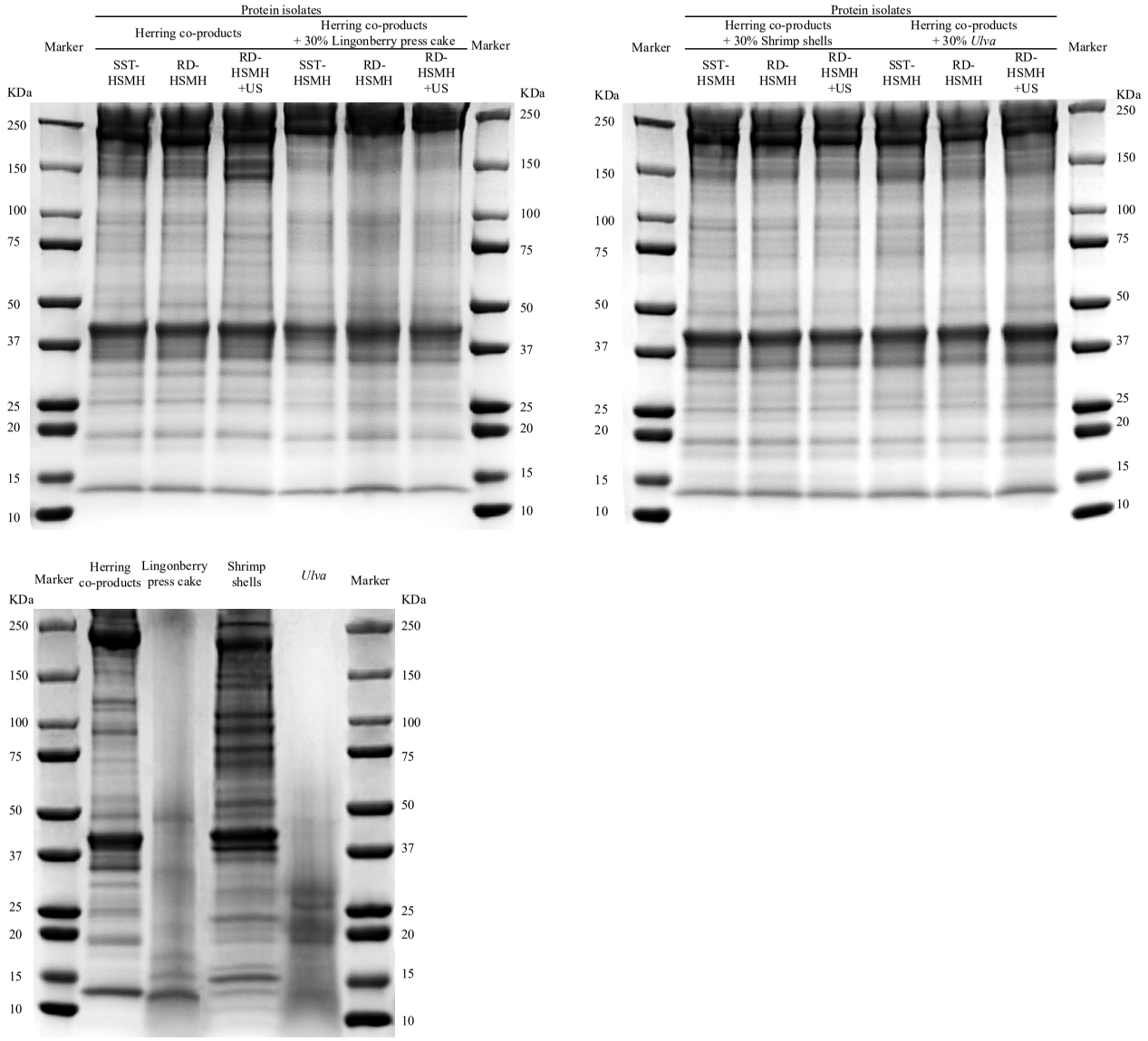

Figure 5.4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) patterns of herring co-products, lingonberry press cake, shrimp shells and *Ulva*, and the protein isolates produced from herring co-products without and with different helpers. SST= single-stage toothed, RD= radial discharge, HSMH= high shear mechanical homogenization, US= Ultrasonication.

HSMH significantly lowered water solubility only at pH 11, which was further diminished by ultrasonication (***Study III***). Thus, there were interaction effects between homogenizer principle and helpers.

5.11 Emulsifying properties of protein isolates

The herring protein isolates, produced using the alkaline version of pH-shift processing with and without the addition of 30% LPC, shrimp shells, or *Ulva*, were examined for their EAI and ESI at pH 3, 5, 7, 9, and 11 (***Study II***). The EAI values, which indicate the proteins' ability to cover the oil-water interface [92], were lower for all isolates at pH 7 compared to pH 11 (***Study II***). The results are related to the water solubility and suggest that deviating from the pI (approximately pH 5), which increases in the net charge of protein molecules, weakens protein-protein interactions and consequently enhances their emulsifying properties [201]. The addition of helpers, regardless of the helper type, generally increased the EAI of protein isolates at pH 7 and 11 (***Study II***). This observation aligns with the capacity of helpers to enhance both water solubility and surface hydrophobicity, the latter of which reduces the energy barrier at the oil-water interface and thus augments the adsorption kinetics [92]. Other components extracted from the helpers during processing, *e.g.*, phospholipids, may also contribute to the increased EAI [92].

The use of ultrasonication generally decreased ESI (***Study II***), which could be attributed to the generation of LMW polypeptides or peptides [92]. Previous research indicates that high molecular weight (HMW) peptides stabilize emulsions [202], whereas LMW peptides and amino acids are less effective in reducing interfacial tension due to a lack of unfolding and reorientation at the interface [203].

5.12 Heat-induced gelation of protein isolates

5.12.1 Gel strength

The addition of helpers at the start of the process, in most cases, impacted both the breaking force and distance of the 5-mm spherical probe into the gels (*i.e.*, elasticity)(Figure 5.5). The effect varied depending on the type of helper used and their additional ratio (***Studies II & VI***). LPC showed the most promising results in improving the breaking force of the gels, followed by APC. This effect was more pronounced at a 10% addition compared to 30%, most likely due to the increase in protein content at 10% (***Study VI***). In contrast, the breaking force decreased with the addition of *Ulva* and oat fiber residues (***Studies II & VI***). This decrease could be attributed to the significantly reduced protein content of *Ulva*-produced isolates and the significantly increased lipid content in oat fiber residues (***Studies II & VI***). The positive effects of LPC and APC were likely related to the presence of pectin, which was supported by the results showing improved gel strength with the addition of pectin (unpublished results). Regarding elasticity, the addition of all tested helpers had a decreasing effect (***Studies II & VI***).

Additionally, the impacts of the mode of HSMH and the use of ultrasound during isolate production were investigated in ***Study II***. Radial discharge HSMH generally resulted in the highest

Table 5.5: Water solubility, emulsifying activity index (EAI) and emulsion stability index (ESI) of protein isolates produced from herring co-products without and with different helpers. Data are given as mean values ± standard deviation (n=3). SST= single-stage toothed, RD= radial discharge, HSMH= high shear mechanical homogenization, US= Ultrasonication.

Source	Treatment	Water solubility at pH 7 (%)	Water solubility at pH 11 (%)	EAI at pH 7 (m^2/mg)	EAI at pH 11 (m^2/mg)	ESI at pH 7 (min)	ESI at pH 11 (min)
Herring co-products	SST-HSMH	7.7 ± 0.4^{c}	36.9 ± 1.3^{g}	22.1 ± 0.2^{e}	53.2 ± 1.9^{f}	4.6 ± 0.0^{cd}	101.4 ± 8.1^{fg}
	RD-HSMH	7.4 ± 0.8^{c}	41.5 ± 4.6^{fg}	20.8 ± 1.6^{f}	53.5 ± 1.3^{f}	8.1 ± 1.0^{b}	211.4 ± 0.5^{ef}
	RD-HSMH +US	6.8 ± 0.2^{c}	45.8 ± 2.1^{f}	13.1 ± 0.3^{h}	66.4 ± 2.1^{e}	5.8 ± 0.2^{c}	64.8 ± 0.4^{fg}
+ 30% Lingberry press cake	SST-HSMH	12.6 ± 0.3^{a} ▲	88.6 ± 0.8^{b} ▲	32.6 ± 0.8^{a} ▲	87.8 ± 5.5^{d} ▲	9.8 ± 0.4^{b} ▲	18.0 ± 2.2^{g}
	RD-HSMH	9.7 ± 0.1^{b} ▲	79.6 ± 0.5^{c} ▲	28.3 ± 0.9^{bc} ▲	93.5 ± 2.4^{c} ▲	19.5 ± 1.3^{a} ▲	1012.1 ± 13.0^{c} ▲
	RD-HSMH +US	9.3 ± 0.8^{b} ▲	70.9 ± 1.0^{d} ▲	22.7 ± 0.9^{e} ▲	68.8 ± 0.4^{e}	9.0 ± 1.4^{b} ▲	10.8 ± 1.3^{g}
+ 30% Shrimp shells	SST-HSMH	10.4 ± 0.4^{b} ▲	94.7 ± 1.7^{a} ▲	19.1 ± 0.1^{g} ▲	87.1 ± 0.9^{d} ▲	3.1 ± 0.0^{d}	497.3 ± 3.3^{d} ▲
	RD-HSMH	10.4 ± 0.2^{b} ▲	74.7 ± 0.9^{cd} ▲	21.9 ± 0.3^{e} ▲	34.2 ± 0.2^{g} ▼	9.4 ± 1.1^{b}	120.5 ± 7.1^{fg}
	RD-HSMH +US	9.1 ± 0.3^{b} ▲	58.6 ± 2.0^{e} ▲	20.7 ± 0.2^{f} ▲	99.0 ± 0.4^{ab} ▲	5.5 ± 0.0^{c}	1201.7 ± 26.4^{b} ▲
+ 30% *Ulva*	SST-HSMH	12.2 ± 0.0^{a} ▲	80.5 ± 1.4^{c} ▲	27.5 ± 0.6^{cd} ▲	97.3 ± 1.8^{bc} ▲	2.5 ± 0.3^{d}	74.5 ± 19.3^{fg}
	RD-HSMH	13.0 ± 0.5^{a} ▲	64.0 ± 1.8^{e} ▲	29.0 ± 0.2^{b} ▲	102.2 ± 0.6^{a} ▲	3.5 ± 0.1^{cd} ▼	3944.0 ± 154.6^{a} ▲
	RD-HSMH +US	10.0 ± 0.1^{b} ▲	36.6 ± 0.1^{g} ▼	27.3 ± 0.1^{d} ▲	57.2 ± 6.3^{f} ▼	3.0 ± 0.0^{d} ▼	326.7 ± 16.2^{e} ▲

Different small letters in each column show a significant difference ($p<0.05$).
▲ and ▼ indicate a significant increase and decrease, respectively, compared to the fish co-products when using the same treatments.

breaking force compared to the use of single-stage toothed HSMH. This difference could be attributed to the lower lipid content of protein isolates obtained from the radial discharge treatment [204]. Interestingly, the application of radial discharge HSMH could largely compensate for the negative effects of cross-processing with shrimp shells and *Ulva* on the breaking force. However, the use of ultrasound decreased the breaking force, most likely due to the induction of protein aggregations, myosin degradation, and increased lipid content (***Study II***).

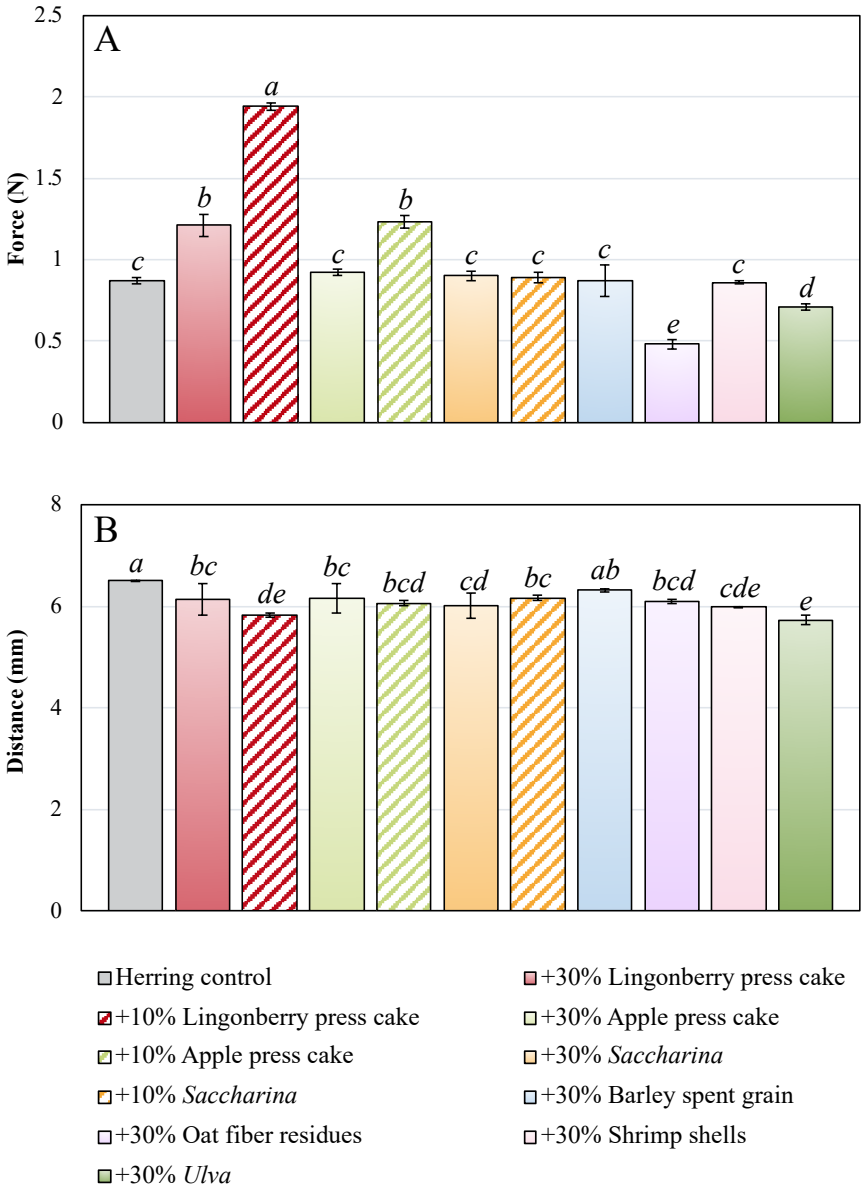

Figure 5.5: Breaking force of the produced gels (A), and the distance that the 5-mm spherical probe entered the gel (B) measured by puncture test. Different small letters in each column show a significant difference ($p<0.05$).

5.12.2 WHC and cooking loss

The WHC of protein gels (Figure 5.6) increased with the addition of 30% LPC and APC, which was consistent with the breaking force results. However, when the concentration of LPC and APC was reduced to 10%, a significant decrease in WHC was observed, despite the fact that the 10% LPC or APC gels exhibited higher breaking force results. The ability of LPC and APC to increase WHC could be linked *e.g.* to pectin or other polysaccharides of these helpers. The decrease in WHC was likely related to the cooking loss (Figure 5.4), which is defined as the quantity of moisture and soluble components released from a protein gel during the cooking process [92]. When the LPC and APC addition was reduced from 30% to 10%, the cooking loss dramatically increased, resulting in protein gels with lower moisture content and a higher concentration of proteins in the final gel. The concentrated proteins formed a more condensed network [92], which is expected to increase WHC.

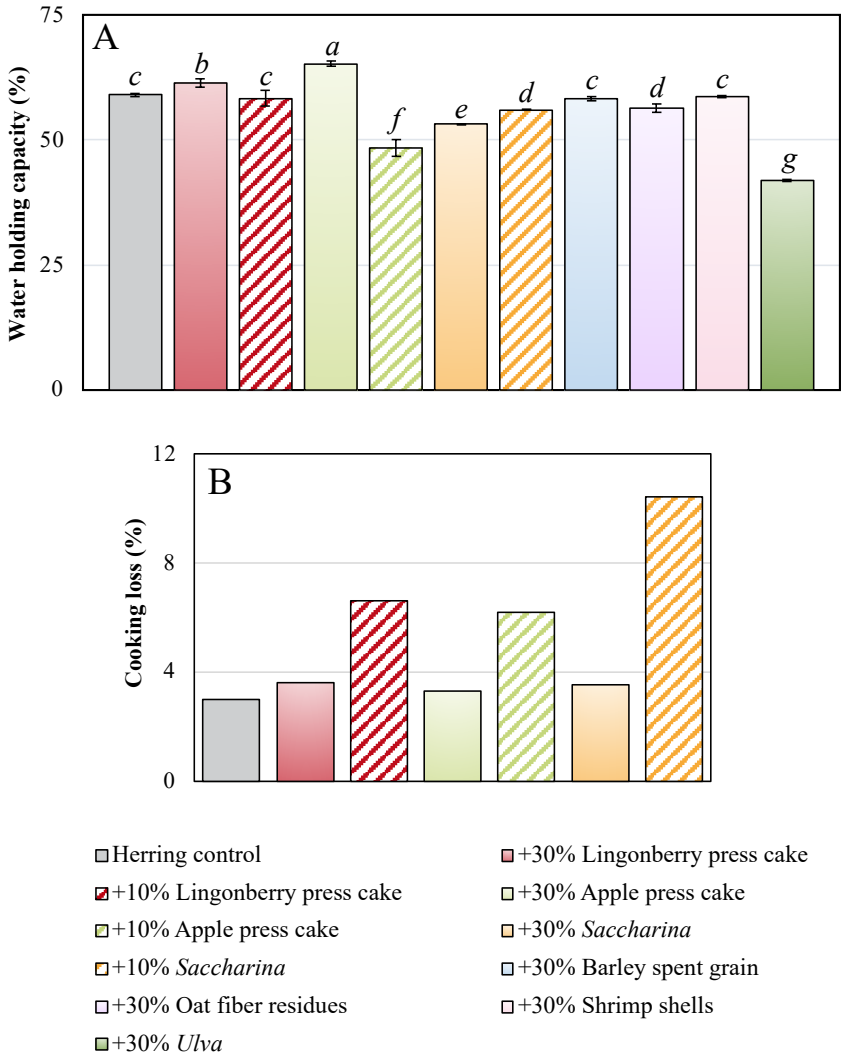

Figure 5.6: Water holding capacity (A) and cooking loss (B) of protein gels. Different small letters in each column show a significant difference ($p<0.05$).

In contrast, addition of 30% oat fiber residues, *Saccharina* and *Ulva* reduced the WHC; especially *Ulva*. These results agreed with the results of the gel strength tests where the high salt content of the seaweed was one of the explanations. This was further supported by the fact that reducing the addition ratio of *Saccharina* from 30% to 10% increased the WHC. However, this done-response effect could also be a result of lower levels of polysaccharides disturbing protein interactions and thereby protein network formation.

5.13 POTENTIALS AND LIMITATIONS OF THE CROSS-PROCESSING CONCEPT

Overall, the completely new cross-processing concept, *i.e.*, pH-shift processing of fish co-products with the addition of antioxidant-containing helpers, particularly LPC, presents several advantages. Primarily, the cross-processing enhances the lipid oxidative stability, water solubility and emulsifying activity of the fish protein isolates in accordance with a clean label principle. The oxidation stabilization is ascribed both to the introduction of antioxidants *per se*, but also to an enhanced contact between lipids and antioxidants during protein solubilization/-precipitation. Introducing various helpers also enables the development of protein isolates with diverse colors and textures, fostering product innovation and differentiation. Although not analyzed in depth in this thesis, a broader palette of nutrients; *e.g.*, vitamins, minerals and dietary fibers, is also expected from cross-processing compared to processing of the fish raw material alone. Based on its abundance in organic acids and polyphenols, LPC further aids the protein precipitation step, limiting the need for acid solutions, *i.e.*, HCl, during alkaline pH-shift processing. By using side streams of both seafood and plant-based foods, cross-processing indeed also encourages a more efficient use of currently underutilized food resources thereby supporting circular economy. Regarding seaweed, which is a highly sustainable food raw material on the rise in Europe, cross-processing shows a new application area. As public environmental awareness grows, the demand for foods with low environmental impact is rising, making the cross-processing concept appealing to both consumers and manufacturers. Industrial symbiosis is also stimulated as side streams of one industry can become raw material for another; preferably within a limited geographic area to minimize transport. Such symbiosis can also raise raw material volumes for small companies which otherwise would not manage large investments in process equipment. Additionally, the implementation of cross-processing can contribute to the ongoing dietary protein shift and to food security by providing alternative food protein ingredients with a high sustainability profile, helping to alleviate pressure on overexploited fisheries and farmland, as well as fostering responsible food production practices [155, 156].

However, there are also some drawbacks discovered, such as reduced protein yield (all helpers), as well as trade-offs in protein content (LPC, APC, seaweeds) and techno-functionalities of protein isolates (*Ulva* and oat fiber residues). Although the variety in colors is mentioned above as potentially advantageous, it can also be a disadvantage since many consumers have white or salmon-colored pink as standards in their fish eating. The dark color produced with LPC for example may limit possibilities for traditional products; the same with the green color from *Ulva*. Further to this, it could be expected that the introduction of *e.g.*, fibers, phytic acid and polyphenols into the cross-processed isolate could hamper protein digestibility and mineral accessibility. The former was subject to preliminary *in vitro* studies

using protein isolates made with herring co-products and 5-30% (dw/dw) LPC. Results reveled no negative effects on protein degree of hydrolysis (DH%), but results need to be confirmed in a more thorough study together with mineral accessibility studies (see Chapter 7).

Thus, to fully release the potential of cross-processed protein isolates, communicating their environmental benefits to consumers [155, 156], and addressing the mentioned trade-offs beyond what was done in this thesis are therefore expected to be crucial.

6

Conclusions

The established goal of this PhD thesis was successfully achieved by exploring, for the first time, whether locally sourced helpers containing antioxidants could minimize lipid oxidation and improve isolate characteristics during the pH-shift processing of fish co-products. The conclusions drawn from the thesis are summarized as follows:

- **Lipid oxidation mitigation:**
    - Except for shrimp shells, all helpers limited lipid oxidation during pH-shift processing and ice storage of protein isolates. Lingonberry press cake (LPC) was by far the most potent, followed by apple press cake (APC) and *Saccharina*.
    - The oxidative stability of protein isolates in most cases correlated to their total phenolic contents with the strong antioxidant ability of LPC being attributed to specific anthocyanins such as ideain and procyanidin A1.
    - Adding LPC during the protein precipitation step, rather than at the process start, was more effective in limiting lipid oxidation.
- **Protein yields:** Adding 30% (dw/dw) helpers at the start of the pH-shift process reduced total protein yields. However, several strategies were effective in mitigating this reduction:
    - **Optimizing protein solubilization/protein precipitation pH:** For alkaline cross-processing with plant food side streams or shrimp shells: pH 12/pH 5 but with seaweeds pH 12/4.5. For the acid process: pH 2.5/5.5.
    - **Increasing the water-to-raw-material ratio to 6:1 (w/w).**
    - **Changing from single-stage toothed to the more powerful radial discharge HSMH.**
    - **Use of ultrasound.**

  - **Changing helper addition ratio and point:** Reducing the LPC addition ratio from 30% to 2.5% or adding LPC during the precipitation step rather than at the start.

- **Acid/base solution consumption:** LPC lowered HCl requirements in acid-aided protein solubilization but increased NaOH demand in alkali-aided solubilization. The latter was mitigated by reducing the LPC addition ratio or changing the addition point; *e.g.*, adding 2.5% instead of 30% LPC decreased NaOH usage by 40% and adding 30% LPC during the protein precipitation step rather than at the start reduced acid consumption by up to 61%. Shrimp shells and seaweed increased HCl usage when added at the start of acid-aided pH-shift processing.

- **Color of protein isolates:** LPC-derived anthocyanins rendered protein isolates red under acidic conditions and dark under neutral/alkaline conditions whereas Ulva-derived chlorophyll resulted in green isolates. Storage-induced oxidation of Hb and astaxanthin of the fish co-products *e.g.*, reduced redness and increased lightness.

- **Composition of protein isolates:** When added at the start, LPC reduced the lipid content of isolates, while shrimp shells increased it. Increased lipid levels were also seen when adding LPC during the precipitation step. Seaweeds brought in minerals to the system and thereby increased the ash content. Process modifications, *i.e.*, the use of radial discharge HSMH and ultrasonication, decreasing and increasing lipid levels, respectively.

- **Techno-functional properties of protein isolates:**

  - The addition of 30% LPC, shrimp shells and *Ulva* at the beginning of alkali-aided processing increased water solubility and emulsification activity of protein isolates.
  - The addition of 30% LPC and APC at the beginning of alkali-aided processing improved the strength and WHC of the fish protein gels.
  - Changing from single-stage toothed to radial discharge HSMH in most cases improved emulsifying properties but decreased the water solubility of the protein isolates. Additionally, the strength of fish protein gels was enhanced.

- **Optimal combination of fish co-products and helpers:** Herring co-products with 10% LPC provided an ideal balance across all considered parameters; including lipid oxidation limitation and acceptable protein yields, acid/base solution consumptions as well as color, protein-content and techno-functionalities of the protein isolates. This combination shows great promise for further development.

Overall, this thesis has generated completely new understanding of the advantages gained when cross-processing fish co-products with locally sourced antioxidant-containing food raw materials that are either underutilized (side streams) or emerging (seaweed). The addition of helpers, particularly LPC, can together with the pH-shift process, clearly add value to fish co-products in a clean label manner and thereby pave the way for innovative, sustainable, and high-value food protein ingredients while contributing to a more sustainable and circular bioeconomy. This thesis has also elucidated the pivotal role of process parameters, laying a solid foundation for future optimization. Ultimately, this thesis advances the prospects of achieving food sustainability and security in the future.

7

Future perspectives

Several potential directions for future research and development can be identified, including:

- Deeper evaluation of the nutritional value of protein isolates as a function of the new cross-processing concept; this would for example involve analyzing amino acid composition, mineral content, vitamin content and the presence of bioactive peptides. The latter could be derived from the autolysis mediated by endogenous proteases [205]. As stated initially, the helpers brought numerous non-fish nutrients to the system, but it was not fully clarified how much of these partitioned into the protein isolates.

- Allergenic potential of protein isolates; this would involve evaluating changes in allergenic proteins' structure and immunoreactivity due to the addition of helpers. Examples of known fish allergens are parvalbumin, collagen, gelatin, β-enolase, aldolase and tropomyosin [206], which could be altered quantitatively/qualitatively due to presence of helper-derived molecules.

- Shelf-life evaluation; this will involve monitoring of the helpers´ impact on quality changes beyond lipid oxidation, *e.g.*, physicochemical, microbiological, and sensory properties of the protein isolates during storage under various conditions. It is for example known that polyphenols also can be antimicrobial [207] in addition to strong antioxidant effects.

- Development of novel applications for the cross-process-derived fish protein isolates; this involves investigating their potential use in various food applications, such as meat alternatives, protein supplements, and functional foods, assessing their compatibility with different food matrices and processing conditions to develop innovative products that meet consumer demands.

- Deeper investigation of the underlying mechanisms behind the ability of the most efficient helpers, *i.e.*, LPC, AP, and SL, to improve lipid oxidative stability; this involves exploring specific interactions between molecules of the helpers and fish proteins. Examples could

be to examine the antioxidant properties of helper fractions or individual molecules in a fish model system with known pro-oxidants, such as the Hb-fortified washed cod mince (WCM) model.

- Deeper investigation of the underlying mechanisms behind improved protein techno-functionalities (solubility, gelation, emulsification) induced *e.g.*, by LPC and AP; this could involve investigating the structural changes in proteins using *e.g.*, nuclear magnetic resonance (NMR) spectroscopy, fourier transform infrared (FTIR) spectroscopy, dynamic light scattering (DLS) and scanning electron microscopy (SEM), their interactions with helper-derived molecules, as well as the role of specific processing parameters in modulating these functionalities.

- Investigations of the role of helpers for other protein techno-functionalities, *e.g.*, foaming capacity and film-forming ability; this will provide a broader understanding of the potential applications of the cross-protein isolates in various food products and their compatibility with different food matrices.

- Investigating the digestibility of proteins in the cross-processed protein isolates. It has earlier been found that pH-shift processing *per se* does not affect herring protein digestibility *in vitro* [208]. Preliminary trials have also indicated that the same applies when cross-processing with LPC. However, this is an important point to address further given the known ability of polyphenols to react with proteins and induce cross-linking [209].

- Deeper sensorial investigation of cross-processed protein isolates. Preliminary trials within this thesis revealed certain bitterness in *sous vide* cooked isolates made with LPC and seaweeds. It is crucial to investigate this further in more realistic product prototypes where isolates may be mixed with mince, surimi or other ingredients.

- Scalability and economic feasibility of cross-processing; this will involve analyzing factors such as requirements for new logistics (*e.g.*, stabilization of the helpers), equipment requirements, and yield, as well as identifying potential bottlenecks and opportunities for optimization.

- Expanding the range of fish co-products and helpers; this could lead to the discovery of new combinations that further enhance protein functionality and reduce lipid oxidation of isolates while at the same time omitting problems with *e.g.*, dark color and bitterness.

By effectively addressing these challenges, the innovative processing approach evaluated in this thesis has significant potential to contribute to the development of sustainable, clean-label and high-quality protein ingredients for a variety of food products. Altogether, this will pave the way for a larger contribution of marine proteins to the ongoing protein shift and also to global food security.

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