THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Valorization of herring filleting co-products to silage

Control of protein hydrolysis and lipid oxidation during ensilaging and possibilities for separating herring silage into multiple products

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"Sometimes things turn out differently from what you expect"

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Abstract

Industrial processing of herring (*Clupea harengus*) into convenience products such as fillets generates around 60% co-products being rich in both protein and n-3 polyunsaturated fatty acids (PUFAs). A promising cost-efficient strategy to valorize these raw materials into food and/or feed ingredients would be to apply ensilaging, i.e., proteolysis mediated by endogenous proteases under acidic conditions. Although an ancient technique, very little is still known about factors affecting the protein degree of hydrolysis (DH) and lipid oxidation during ensilaging. In this work, the effect of e.g. temperature and time on DH and lipid oxidation during ensilaging of herring co-products was investigated with the aim to maximize the DH while keeping the formation of unwanted free amino acids (FAA) and lipid oxidation to a minimum. Also, the role of hemoglobin (Hb) in ensilaging-induced lipid oxidation was studied, along with different ways of introducing antioxidants to the silage. Finally, possibilities to separate pilot scale-produced herring silage into oil and hydrolysates were investigated.

Ensilaging for 1-7 days between 7-47°C revealed that the highest DH was noticed at 32°C, but the DH increased over time at all studied temperatures, which also applied to FAA. At ambient temperature (i.e. 22°C), being the main focus in this thesis, DH and FAA reached 60% and 14%, respectively, at day 7. (**Paper I**) Heat-treating the silage for 30 min at 85°C prior to storage (0-6 months; 4°C or 22°C) stopped hydrolysis, and is thus a route to minimize FAA formation. (**Paper IV**) Lipid oxidation proceeded during ensilaging at all temperatures, however, ≥ 22°C, the secondary oxidation product marker malondialdehyde (MDA) underwent hydrolytic cleavage or interacted with proteins/peptides/amino acids, preventing its accumulation. Instead, non-enzymatic browning reaction products e.g. 2-ethylfuran and 2-pentylfuran developed, along with saturated aldehydes as pentanal and hexanal. (**Paper II**) Upon adjusting the pH from physiological to 3.5 (i.e. ensilaging pH), it was documented that trout oxyHb changed to metHb, facilitating heme group release, suggesting heme-mediated peroxide cleavage was a dominating mechanism behind the ensilaging-induced lipid oxidation. (**Paper III**) To minimize lipid oxidation, two different strategies were evaluated; (i) pre-incubating the co-products in antioxidant solutions, or (ii), direct addition of 0.25-1.25% antioxidants at the start of ensilaging (**Paper IV**). Both strategies were effective, and among all the antioxidants studied, the commercial rosemary extract-based antioxidant Duralox MANC-213 provided best protection against lipid oxidation during ensilaging, heat-treatment and storage of silage. The total volatile basic nitrogen (TVB-N) level in silages remained below the acceptable limit of 30 mg TVB-N/100 g fish for human consumption, suggesting that silage can be used in both food and feed. (**Paper III**, **IV**) Scaling up the ensilaging to a 1500-liter batch size, ~38% DH was recorded after 2 days at ~22°C, which was similar to the DH found in lab-scale (40% after 2 days; 22°C), suggesting lab-scale data well simulated what happens in pilot scale. Subjecting the silage to centrifugation (3000-8500 x g; 2-20 min) showed that it can be successfully separated into fish oil and protein hydrolysates. (**Paper V**)

To summarize, these studies provide valuable information on optimal process parameters to use in on site valorization of herring co-products into a high-quality peptide-rich silage, and/or fish oil and hydrolysates, paving the way for silage based-biorefining of herring side streams into multiple products.

Keywords: Herring (*Clupea harengus*), by-products, valorization, ensilaging, silage, lipid oxidation, antioxidants, separation, fish oil, protein hydrolysis, pilot-scale

List of papers

This thesis is based on the work presented in the following papers.

I. **Sajib, M.**, Albers, E., Langeland, M. and Undeland, I., 2020. Understanding the effect of temperature and time on protein degree of hydrolysis and lipid oxidation during ensilaging of herring (*Clupea harengus*) filleting co-products. *Scientific reports*, 10(1), pp.1-13.

II. **Sajib, M.** and Undeland, I., 2020. Towards valorization of herring filleting by-products to silage 2.0: Effect of temperature and time on lipid oxidation and non-enzymatic browning reactions. *LWT*, 127, p.109441.

III. **Sajib, M.**, Wu, H., Fristedt, R. and Undeland, I. Hemoglobin-mediated lipid oxidation of herring filleting co-products during ensilaging and its inhibition by pre-incubation in antioxidant solutions. *In review*.

IV. **Sajib, M.**, Langeland, M. and Undeland, I. Effect of antioxidants on lipid oxidation in herring (*Clupea harengus*) co-product silage during its production, heat-treatment and storage. *In review*.

V. **Sajib, M.**, Trigo, J.P., Abdollahi, M. and Undeland, I. Pilot-scale ensilaging of herring filleting co-products and subsequent separation of fish oil and protein hydrolysates. *Draft manuscript*.

Other papers, not included in this thesis:

VI. Wu, H., **Sajib, M.** and Undeland, I., 2021. Controlling hemoglobin-mediated lipid oxidation in herring (*Clupea harengus*) co-products via incubation or dipping in a recyclable antioxidant solution. *Food Control*, 125, p.107963.

VII. Wu, H., Forghani, B., **Sajib, M.** and Undeland, I. A recyclable dipping strategy to stabilize herring (*Clupea harengus*) co-products during ice storage. *In review*.

The author's contributions to the papers

I. The author, Mursalin Sajib (MS), planned the work together with coauthors, carried out the experimental work, analyzed results, wrote the first draft of the manuscript, and revised the manuscript together with coauthors.

II. MS planned the work together with Ingrid Undeland (IU), carried out the experimental work, analyzed results, wrote the first draft of the manuscript, and revised the manuscript together with IU.

III. MS planned the work together with coauthors and carried out the experimental work. MS analyzed results, wrote the first draft of the manuscript, and revised the manuscript together with coauthors.

IV. MS planned the work together with IU, carried out the experimental work, analyzed results, wrote the first draft of the manuscript, and revised the manuscript together with coauthors.

V. MS planned the work together with IU, coordinated the experiments, and carried out major part of the experimental work. MS analyzed results together with coauthors, wrote the first draft of the manuscript, and revised the manuscript together with coauthors.

List of abbreviations

AOCS	The American Oil Chemists' Society
APCI	Atmospheric pressure chemical ionization
p-AV	p-Anisidine value
BHT	Butylated hydroxytoluene
CAGR	Compound annual growth rate
CAR/PDMS	Carboxen/polydimethylsiloxane
DH	Degree of hydrolysis
DHA	Docosahexaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FFA	Free fatty acid
FAA	Free amino acid
GC-MS	Gas chromatography-mass spectrometry
Hb	Hemoglobin
oxyHb	Oxyhemoglobin – the oxygenated state of Hb
deoxyHb	Deoxyhemoglobin – the deoxygenated state of Hb
metHb	Methemoglobin – the Fe^{3+} (ferric) state of Hb
HHE	4-Hydroxy-2-hexenal
HNE	4-Hydroxy-2-nonenal
HPLC	High-performance liquid chromatography
HS-SPME	Headspace solid-phase microextraction
LC-MS	Liquid chromatography-mass spectrometry
LC n-3 PUFA	Long-chain n-3 polyunsaturated fatty acid
LC n-6 PUFA	Long-chain n-6 polyunsaturated fatty acid
LOX	Lipoxygenase
MDA	Malondialdehyde
MUFA	Monounsaturated fatty acid
OPA	o-Phthaldialdehyde
PC	Phosphatitylcholine
PCA	Principal component analysis
PLS	Partial least squares regression
PV	Peroxide value
PUFA	Polyunsaturated fatty acid
SEC	Size exclusion chromatography
SEM	Standard error of the mean
SFA	Saturated fatty acid
TBARS	2-Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TMA	Trimethylamine
TOTOX	Total oxidation value
TVB-N	Total volatile basic nitrogen

Table of Contents

1 Introduction

Industrial processing of herring (*Clupea harengus*) into convenience products e.g. fillets generates around 60% co-products, accounting for around 12,000 tons annually in Sweden. On a global basis, exact data on herring co-product generation is missing, and it is also hard to find exact data on total global amounts of fish co-products. However, postharvest losses was estimated to be around 25% of the catch according FAO [1], which would mean around 45 million tonnes fish co-products based on the latest FAO-data on fish/shellfish production (i.e. 179 million tonnes in 2018 [2]). But, also higher numbers have been reported [3]. Such co-products have traditionally been used for fish meal/fish oil production or as feed for fur animals like mink. In case of Sweden, herring co-products are primarily exported to Denmark for fish meal/oil production which makes the region lose the opportunity to sustainably utilize the whole herring catches and generate revenue linked to value-adding. Also, utilization of these co-products locally could offer the possibilities of new entrepreneurships, job creation and competence build-up. Today, however, the Swedish herring industry does not have proper strategies to minimize lipid oxidation, value adding processes and logistics for taking care of these co-products in place.

This thesis addresses acid-induced ensilaging of herring co-products as a route to create local value-adding chains. Ensilaging carried out with acid is a traditional "green" method used for preventing bacterial growth and for producing so called silage. Based on its simplicity and low energy demand it can be implemented locally on site where the fish co-products are generated. During ensilaging, fish proteins are hydrolyzed into its smaller units e.g. peptides and free amino acids forming a peptide-rich product [4]. The positive outcomes of short-chain, potentially bioactive, peptides in food applications are well-known; e.g. peptides containing 2–20 amino acids [5] and molecular weight below 6 kDa [6] may possess e.g. anti-oxidative, anti-inflammatory, and antihypertensive activities, among others [7-9]. In case of feed applications, even though detailed studies are lacking, it is generally accepted that short-chain peptides are more bioavailable to e.g. fish than larger ones [10-12]. Generation of free amino acids is however not preferred neither in food nor feed applications since this can induce chemical reactions as the Maillard reaction, reduce the bioactive properties of the silage and also, e.g. in feed applications, hamper bioavailability as free amino acids are less absorbed than oligopeptides and intact proteins [10]. Therefore, it is important to tune the ensilaging process in a way that will produce a silage rich in short-chain peptides and low in free amino acids. In depth understanding of how to achieve such tuning is however lacking today, and is therefore one of the challenges addressed in this thesis.

Herring co-products, being rich in both long-chain polyunsaturated fatty acids (LC PUFAs) and blood-derived hemoglobin (Hb) [13], are highly susceptible to lipid oxidation (i.e. rancidity) [13], which limits its high-value applications. The negative consequences of lipid oxidation are well-known both in food and feed applications; e.g., in case of food applications, reduced nutritional value [14], sensorial quality [15], increased risk of tumor and atherosclerosis development [16], and in case of feed applications, a lower feed conversion ratio and reduced carcass quality [17] have been reported. Due to the high pro-oxidative activity of Hb under reduced pH settings [18], the acidic

pH used in ensilaging (i.e. pH 3.5) makes the production of high-quality herring co-product silage challenging **(Paper II)**. To solve this, a deepened understanding about the nature of lipid oxidation products formed during ensilaging under varying process parameters is needed. Studies in model systems have shown that the most used secondary lipid oxidation product marker malondialdehyde (MDA) – often measured in the 2-thiobarbituric acid reactive substances (TBARS) test – undergoes hydrolytic cleavage and/or react further with proteins/peptides/amino acids forming cross-links and various potential (toxic) adducts at elevated temperature (e.g. 40°C) [19-22]. Such reactions can thus be expected if ensilaging is done above ambient temperature. Since the quality of silage largely depends on the quality of the starting raw material **(Paper III-IV)**; it is imperative to apply antioxidative strategies as early as possible, preferably right after generation of the co-products. Altogether, techniques to minimize lipid oxidation are thus needed before, during and after ensilaging. This thesis will address two different antioxidative strategies; namely pre-incubation of the co-products in antioxidant-containing solutions and direct antioxidant addition at the start of ensilaging.

Herring co-product silage can be a good source of both LC n-3 PUFA-rich fish oil and a protein hydrolysates rich in short-chain peptides. The global market value for n-3 PUFAs was USD 2.49 billion in 2019, and is expected to grow at a compound annual growth rate (CAGR) of 7% over the period 2020–2027 [23]. Corresponding values for fish protein hydrolysates was USD 407 million in 2019, which is expected to grow at a CAGR of 4.7% over the period 2019–2027 [24]. To date, however, detailed understanding on simultaneous recovery of fish oil and protein hydrolysates from herring co-product silage as a function of process parameters is lacking, and is therefore addressed in this thesis.

While very little is known about degree of hydrolysis (DH) and lipid oxidation development during ensilaging of herring co-products on lab scale [25-28], even less is documented during pilot scale ensilaging. To allow industrial implementation of lab scale research results, the ability to extrapolate these to larger scale is essential. Therefore, DH and lipid oxidation data are in this thesis retrieved both during lab- and pilot-scale ensilaging.

To summarize, by creating in depth understanding of biochemical reactions such as protein hydrolysis and lipid oxidation during ensilaging, and by developing strategies to prevent the latter, the results presented in this thesis are expected to pave the way for successful biorefining of herring filleting co-products into multiple high-value products.

2 Knowledge gaps

The overall knowledge gap addressed in this thesis was the lack of published results on biochemical reactions taking place during herring co-product ensilaging, measures to improve herring silage quality and methods for its subsequent separation into oil and hydrolysates.

On a more detailed level, knowledge gaps encountered were the scarcity of information on:

- the link between on the one hand different ensilaging process parameters and on the other DH and silage quality (e.g. lipid oxidation and total volatile basic nitrogen (TVB-N) formation).
- the (exact) nature of lipid oxidation products formed during herring co-product ensilaging under varying process settings.
- the role of Hb in lipid oxidation development during herring co-product ensilaging process settings (i.e. pH ~ 3.50).
- antioxidative strategies suitable for herring ensilaging conditions.
- separation settings to use for optimal yield and quality of oil and hydrolysates from herring co-product silage.

Knowledge gaps outside the scope of the thesis, which rendered the exact tuning of the ensilaging process difficult were e.g.:

- the desired protein DH to strive for during ensilaging, and the impact of free amino acids on different health end points when used as feed or food.
- the type, amount, activity and, seasonal variation of endogenous proteases found in herring co-products.
- the simultaneous cascade-like reactions – where multiple enzymes work simultaneously to varying extent – (taking place) during ensilaging.
- the impact of seasonal variability in herring co-product composition e.g. lipid content, on the extent of e.g. lipid oxidation, something which would allow adjustments e.g. of antioxidant concentrations.

3 Aim of this thesis

The overall aim of this thesis was to deepen the understanding of (i) biochemical reactions, mainly DH and lipid oxidation during production of herring co-product silage, (ii) stabilization strategies to minimize lipid oxidation during the silage production and (iii) process settings to use for recovery of fish oil and protein hydrolysates from the silage, allowing build-up of a silage-based biorefinery.

The specific aims were to:

- better understand how herring co-product ensilaging process parameters e.g. temperature and time affects the DH and free amino acid formation.
- explore how above-mentioned process parameters affect the nature of lipid oxidation products formed during herring co-product ensilaging in absence of antioxidants.
- investigate the role of Hb for lipid oxidation development during herring co-product ensilaging.
- unravel the effectiveness of pre-incubating the herring co-products in antioxidant solutions vs adding antioxidants at the start of ensilaging on lipid oxidation development during herring silage production.
- investigate the effect of silage storage temperature and time on TVB-N formation.
- elucidate how well lab-scale ensilaging of herring co-products can predict DH and lipid oxidation achieved during pilot scale ensilaging.
- investigate the possibility of recovering fish oil and protein hydrolysates from herring co-product silage produced in pilot scale.

4 Background

4.1 Increased global demand for animal-derived protein ingredients

Fish is an important source of animal-derived protein [29]. Together with shellfish it contributes to around 6% of the global protein supply [30]. It is projected that the demand for animal-derived protein will double by 2050 [29], which is mainly due to the estimated population growth to 9.5 billion by 2050 and partly due to factors like urbanization, changes in consumption patterns and increased awareness about the role of protein in a healthy diet, e.g. its importance for a healthy aging. [31-33].

4.2 Global fish supply and contribution of wild catch and aquaculture fisheries

The global fish supply increased with an average growth rate of 3.2% during the last 50 years, which is higher than world's population growth rate (1.7%), resulting in an increase in the per capita fish supply by 25.6% over a 14-year period from 2000 to 2014 [34,35]. In 2018, the total global fish production was 178.5 million tons, of which 96.4 million tons (54%) came from wild catches and 82.1 million tons (46%) from aquaculture [2]. The world's wild-catch fish resources are however limited, and according to a survey [36], 90.1% of the global fish stocks were fully or over-exploited in 2011, meaning that the wild-catch fish has already reached its limits and cannot be expanded more. It is expected that aquaculture will make up the gap between demand and supply, and aquacultured fish and shellfish is therefore expected to represent roughly 2/3 of the global supply by 2030 [37].

4.3 Processing of seafood co-products

To meet the growing demand for fish and especially fish-based convenience products, around 70% of all caught/harvested fish is processed to e.g. headed/gutted fish, fillets, mince or surimi before sale [34]. The amounts of co-products generated in such processes vary from 20-80% (w/w) depending on various factors e.g. type of fish, season of catch, size of fish and level of processing employed [34,38,39]. In this thesis, co-products refer to the remaining parts of fish after filleting which consists of head, backbone, tail, skin, belly flap and intestinal organs (**Figure 1**).

Figure 1. Herring filleting co-product parts

4.4 Herring filleting co-products generated in Sweden and its current use

In Sweden, the amount of co-products generated by seafood processing industries was between 30,000-60,000 tons in the year 2014, depending on method of estimation (e.g. interviews with companies or official statistics on catches and uses). These co-products are handled as low-value raw materials and are used as feed for minks or exported to Denmark for fish meal and oil production for subsequent animal feed production [40]. In case of herring (*Clupea harengus*), around 20,000 tons are processed into e.g. fillets every year in Sweden. This processing generates around 60% co-products, accounting for around 12,000 tons annually. To date, there is no plant in Sweden to valorize these co-products, meaning no value chains are created.

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

Post-mortem changes in fish can generally be divided into four categories: rigor mortis, autolytic changes, bacteriological changes, as well as lipid oxidation and lipid hydrolysis [41]. Among these, the most intense is the onset of "rigor mortis", which is characterized by a succession of muscle stiffness. After death of fish, the oxygen supply ceases, and the glycogen reserve present in the muscle before death is converted through enzymatic activity into lactic acid. The latter, in addition to ATP breakdown, contributes to a reduction in the pH. These events also lead to an energy depletion preventing the thick and thin filaments of the sarcomere to detach. Thus, while the muscles are totally relaxed and elastic in the pre-rigor phase, they begin to stiffen after some hours, and continues until reaching to its maximum, which is known as rigor mortis. The stiffness lasts for hours or days depending on several factors e.g., glycogen reserves, temperature, stunning and killing method used etc. [41]

Autolytic changes, i.e. endogenous proteolysis, contribute in varying degrees to the overall post-mortem changes in fish [42]. In herring, proteolytic breakdown of muscle by endogenous proteases leaking out e.g., during belly-bursting can lead to autolytic proteolysis. This phenomenon is most predominant in the summer months when pelagic fish are feeding heavily [41]; however, physical handling practices have also been reported to cause belly-bursting [43]. Also, the pyloric caeca has been identified as in important organ enriched in proteolytic enzymes, being of importance e.g. during herring ripening [44]. Several proteolytic enzymes are present in the fish, but pepsins and cathepsins have been studied the most. The cathepsins are usually found in lysozomes, while pepsins are mainly found in the stomach. Cathepsins D and L are responsible for autolytic degradation of fish tissue because of their wide pH activity range. Cathepsins L has been reported to contribute more to autolysis of fish muscle than cathepsins D because of its activity at neutral pH. "Calpains" or "calcium activated factor (CAF)" is another group of intracellular proteases responsible for post-mortem autolysis of fish muscle. [41]

In live and newly caught fish, most microorganisms are present on the skin, gills and in the intestines. In newly caught fish, the immune system prevents the bacteria from growing on the flesh. However, once the fish dies, the immune system is not functioning anymore, which allows the bacteria to multiply easily. The bacteria on the skin surface usually colonize in the scale pockets, which later attack the flesh by moving between the muscle fibers. The surface

properties of fish are important factors affecting the rate of spoilage. Fish e.g. cod (*Gadus morhua*) spoil rapidly compared to flatfish species e.g. plaice as they have a very fragile integument. [41] Flat fish, in addition, has a thick slime layer which contains antibacterial components e.g. antibodies and bacteriolytic enzymes [45,46].

Changes in the lipid fraction during storage could occur either because of chemical action (e.g., lipid oxidation) or due to enzymes as lipases or lipoxygenases (LOX). Triglycerides (TG) and phospholipids present in the depot fat and membranes, respectively, may be hydrolyzed by triglyceride lipase or phospholipases yielding an increase in the amount of free fatty acids (FFA) over storage time. This phenomenon is more pronounced in ungutted fish compared to gutted fish as most lipases are present in the gut. [41]

Based on the high ratio of enzymes to muscle in fish co-products; autolysis of the protein and lipid fraction is expected to occur fast when the co-product parts are mixed, such as under normal conditions in the factory. The time elapsing between filleting and subsequent ensilaging will therefore impact both the DH and lipid quality at start of the ensilaging, affecting e.g. the specific peptide pattern and amount of FAA of the final silage.

4.6 Fish muscle proteins, proteases, and hydrolysis of proteins

4.6.1 Fish muscle proteins

Fish muscle consists of three principal tissues to varying degrees: white muscle, dark muscle, and connective tissue. The majority of a fish fillet consists of white muscle, which is dependent on anaerobic metabolism, and contains myofibrillar protein molecules e.g. myosin and actin. The dark muscle, which is used for long distance swimming, is oxygen dependent and is enriched in the heme pigments e.g. myoglobin, and hemoglobin. The connective tissue mainly consists of collagen, and is both distributed throughout the musculature and holds it together; the latter in the form of the skin. [47]

The proteins in the fish muscle tissue can roughly be divided into three main groups; myofibrillar proteins, sarcoplasmic proteins and stroma proteins [48]. Myofibrillar proteins constitute around 65-80% of the total muscle protein. Myofibrillar proteins give the muscle its fiber-like structure and are responsible for the muscle contraction and relaxation. The major molecules are myosin, actin, tropomyosin and troponin. [49] Myofibrillar proteins are soluble in salt solutions >0.3-1M or at very low ionic strength (<0.3 mM) [50] as well as at pH-values <3.5 and >10.0 [50-52]. The isoelectric point (pI) of myofibrillar proteins is around pH 4.5-5.5. [52]

Sarcoplasmic proteins are usually found in the cell plasma where they act as enzymes and oxygen carriers, and constitutes around 25-30% of total fish muscle protein [49,53]. The amount of sarcoplasmic protein is usually higher in pelagic fish, e.g. herring, sardine and mackerel, and lower in demersal fish, e.g. plaice and snapper [53,54]. These proteins are soluble in water [53].

Stroma proteins are those making up the connective tissues, surrounding the muscle fibers and the skin; examples of such are collagen, elastin and connectin. These proteins contribute to

around 3-5% of total protein. Stroma proteins are resistant to solubilization except in strong salt solutions. [49,53]

4.6.2 Proteases and hydrolysis of proteins

Proteases belong to the class hydrolases – enzymes which split chemical bonds using water. Based on the bonds they are hydrolyzing, the class hydrolases is sub-classified into those cleaving ester bonds, glycosyl bonds, ether bonds, peptide bonds, and C-N bonds beyond those in the peptide bond. Proteases belong to the subclass peptide hydrolases because they hydrolyze peptide bonds. Further sub-classing of this enzyme category is based on the specificity for the site of the peptide bond cleaved. Exoproteases (also known as peptidases or exopeptidases) are those enzymes that cleave peptide bonds from the terminal end, either from the amino terminal or carboxyl terminal, of the protein; and endoproteases (also known as proteinases or endopeptidases), cleave peptide bonds at the "center" of the polypeptide chain. There is also an unknown subsubclass where the specificity of enzymes is still unknown. [55]

The group exoproteases is classified based on e.g. the site of cleaving, the terminal end of the performed protein hydrolysis (e.g. amino or carboxyl terminal), the size of peptide released (e.g. dipeptide, tripeptide etc.), and the site restrictions on the length of susceptible peptide. The group endoproteases is considered as the major group of industrially used proteases, and is classified, based on the chemical nature of the amino acid residues responsible for the catalytic activity of the enzyme, into serine-proteinases, cysteine-proteinases (or thiol-proteinases), aspartic-proteases (or carboxyl- or acidic-proteinases), and metalloproteinases. [55] **Table 1** shows a few examples of endoproteases, amino acids involved in the active site of the enzyme, and their optimal pH range.

Table 1. Examples of endoproteases, their active site and optimal pH.

Type	Example	Active site	Optimal pH*	Reference
Serine	Trypsin, chymotrypsin, subtilisin	Serine	7-11	Garcia-Carreon and Del Toro [55,56]
Cysteine	Papain, ficin, calpain, cathepsin B H L	Cysteine	5-7.5	
Aspartic	Penicillopepsin, pepsin, chymosin, cathepsin D	Aspartic acid	2-4	
Metallo	Collagenase, Carboxypeptidase	Zn^{2+}	6-8	

*Might vary depending on the source of enzyme

Figure 2 shows the hydrolysis mechanisms of proteases. A protease can perform hydrolysis either by a one-step (e.g. aspartyl and metalloproteases) or two-step mechanism (e.g. serine and cysteine proteases). In case of a one-step hydrolysis, the enzymes activate a water molecule to serve as the nucleophile, which then hydrolyses the protein. In case of a two-step hydrolysis, the active sites usually pair up with a proton-withdrawing group to promote nucleophilic attack on the peptide bond. This results in an intermediate where the enzyme attacks a peptide bond in the chain. In the next step, a water molecule is activated to hydrolyze this intermediate and

complete the catalysis. Other enzyme residues donate and accept hydrogen atoms and electrostatically stabilize the charge build-up along the reaction mechanism. Nevertheless, the overall process of peptide bond hydrolysis is principally the same for all protease classes. [57,58]

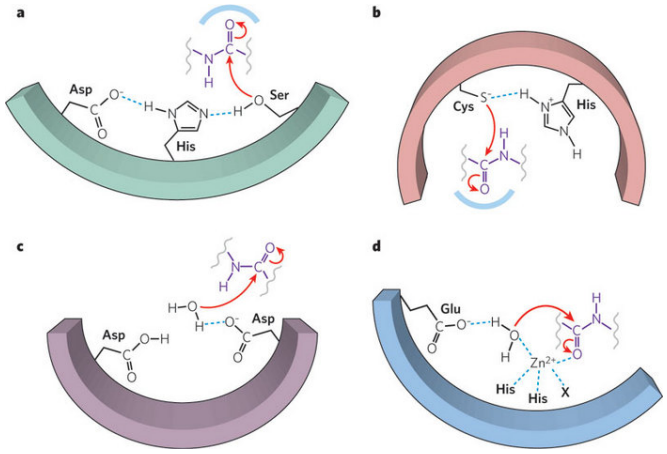

Figure 2. Hydrolysis mechanisms of proteases; (a) serine, (b) cysteine, (c) aspartyl, (d) metallo-proteases. Adopted from Neitzel [57].

4.7 Fish lipids, lipid oxidation and the role of hemoglobin (Hb) in lipid oxidation

4.7.1 Fish lipids

The lipid content of fish is highly variable, and is influenced by e.g. type of fish, maturity, catching season, food availability and feeding habit. Depending on the species, fat deposition occurs either in the muscle (e.g. herring, carp), in the liver (e.g. cod, saithe, haddock) or in the intestines (e.g. perch, pike, blue pike). [59] Lean species use their liver as the energy depots and thus have low fat content in the muscle, whereas the fatty species store lipids in fat cells throughout the body. [41]

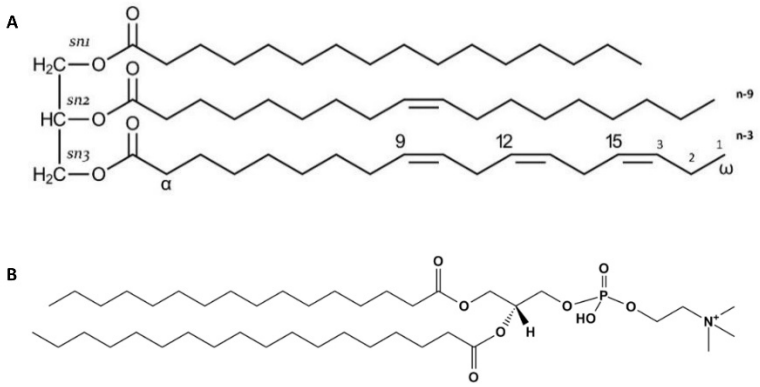

Figure 3. Chemical structure of a triglyceride (A) and phospholipids (B). The triglyceride contains a saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated n-3 fatty acid (n-3 PUFA) in the sn-1, -2, and -3 position, respectively. The phospholipid contains a choline and phosphate group attached to the glycerol backbone and is referred to as phosphatitylcholine (PC). Figure adopted from Tullberg [60].

Lipids present in fish may be divided into two major groups: triglycerides (triacylglycerols; **Figure 3A**) and phospholipids (**Figure 3B**). Triglycerides are lipids used for storage of energy in fat depots and are often termed as depot fat. They are present within special fat cells surrounded by a phospholipid membrane and a weak collagen network. [41] This type of lipids are often observed as oil globules either in the flesh, liver or in the intestine [49]. The second type of lipids, phospholipids are often called as structural lipids as they make up the integral structure of the unit membranes in the cells. They are present in the outer cell membrane, the endoplasmic reticulum and other intracellular tubule systems, and in the membranes of mitochondria. In addition to phospholipids, the membrane also contain cholesterol, which contributes to the membrane rigidity. [41]

Most fish lipids are usually liquid at low temperature, but it may vary depending on the amount of PUFAs. To keep the membranes fluid, fish of cold-water origin contains more PUFAs than e.g. fish of tropical origin. Fish lipids may contain up to 40% of long-chain (LC)-PUFAs (14-22 carbon atoms), a large part being of the n-3 family. Thus, fish is known as an important source of LC n-3 PUFAs, especially eicosapentaenoic acid (EPA; 20:5; **Figure 4A**) and docosahexaenoic acid (DHA; 22:6; **Figure 4B**) [41,59].

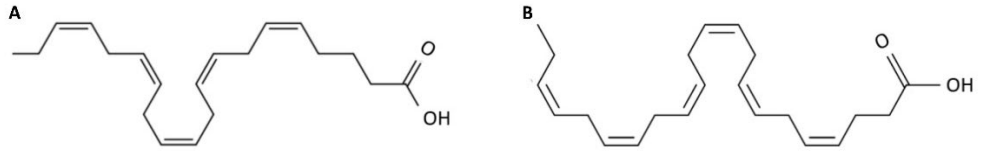

Figure 4. Chemical structure of EPA (A) and DHA (B). Adopted from Tullberg [60].

4.7.2 Lipid oxidation

Lipid oxidation is a free radical induced oxidative deterioration of PUFAs. Fish of marine origin is highly susceptible to lipid oxidation because of its high content of PUFA and its highly pro-oxidative heme pigments e.g. hemoglobin (Hb) [61,62]. The autocatalytic mechanism of lipid oxidation is shown in **Figure 5** below.

The process starts by abstraction of a hydrogen atom from a fatty acid acyl chain containing at least one double bond. The produced lipid radical (L●) reacts very quickly with atmospheric oxygen and produce a peroxy-radical (LOO●). This peroxy-radical (LOO●) later abstracts a hydrogen from another acyl chain and forms lipid hydroperoxides (LOOH) and a new radical (L●). This stage is known as the propagation stage and it continues until one of the radicals is removed either by reaction with another radical or with an antioxidant.

Fatty acid hydroperoxides may also be formed via enzymatic catalysis by LOX. A relatively high activity of this enzyme has been noticed in the gills and under the skin of many species, and possibly it may be enriched in a co-product fraction. Reported optimal LOX pH´s vary depending on species/organ, and examples reported are pH 7.5 [63] and 9-10 [64]. This enzyme has been found to contribute to lipid oxidation mainly in fresh fish [41], but is reported to be inhibited above 40°C [63].

The subsequent hydroperoxide cleavage is catalyzed by trace metal ions, heme-bound or free, resulting in the formation of secondary oxidation products of shorter carbon chain-length. These secondary reaction products are mostly aldehydes e.g. MDA, 4-hydroxy-2-hexenal (HHE), 4-hydroxy-2-nonenal (HNE) (see **Figure 6**), and among other ketones, alcohols, small carboxylic acids, and alkanes. The hydroperoxide cleavage also results in free radicals, which can re-initiate lipid oxidation reactions. [41,65,66]

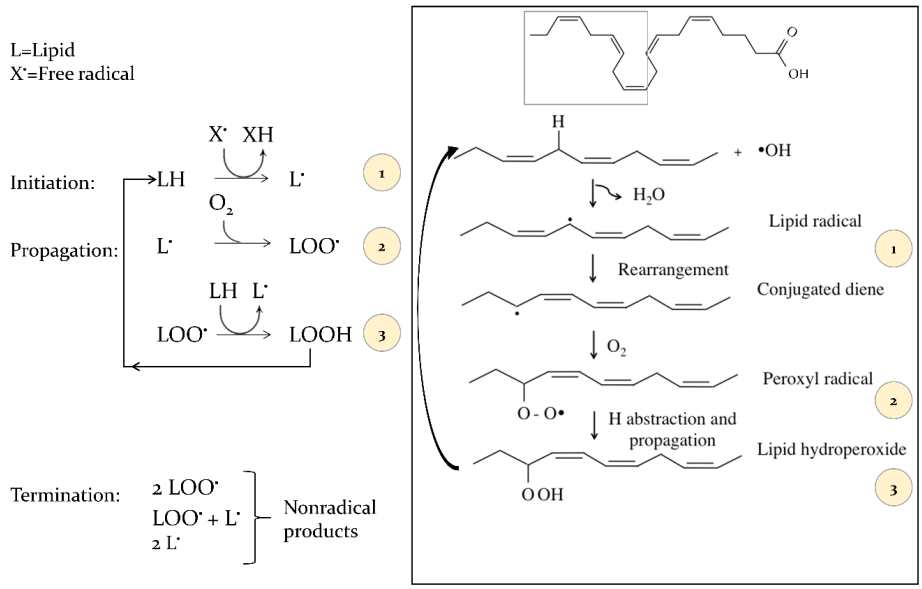

Figure 5. The lipid oxidation reaction mechanism. On the left - the three major steps of lipid oxidation reaction; on the right - an example of oxidation of a free fatty acid up to the stage of hydroperoxide formation. Figure adopted from Tullberg [60]; originally from Kostner and Frank [67].

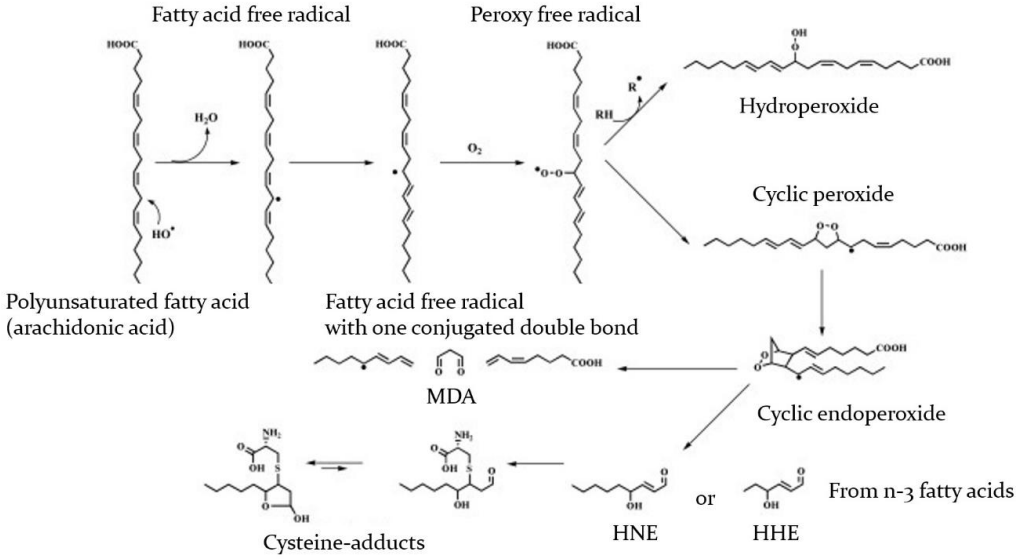

Figure 6. Proposed reaction mechanism for MDA, HHE, and HNE formation from C20:4 n-6. Figure adopted from Tullberg [60]; originally from Bocci, et al. [68].

Volatile compounds can form as a result of lipid hydroperoxide cleavage and/or non-enzymatic browning interactions, hence, the use of the terms 'lipid oxidation-derived volatile compounds' and 'non-enzymatic browning reactions-derived volatile compounds', respectively, can be used to classify the origin of the volatile compounds. Lipid oxidation-derived volatile compounds are thus secondary lipid oxidation products, whereas non-enzymatic browning reactions-derived volatile compounds are usually considered as tertiary lipid oxidation products. In protein containing matrices, the occurrence of both lipid oxidation and non-enzymatic browning reactions (e.g. the Maillard reaction) has been suggested to be interrelated [19,69]; meaning that the extent of one can influence the other and vice versa. For example, the secondary lipid oxidation product (E,E)-2,4-heptadienal might degrade further to give rise to the tertiary lipid oxidation product 4,5-(E)epoxy-2-(E)-heptenal with two oxygenated functional groups, which are reactive towards primary amine groups [70-74].

Oxidation of LC n-3 PUFAs, e.g. EPA (20:5n-3), and DHA (22:6n-3), gives rise to e.g. propanal, 1-penten-3-ol, 1-penten-3-one, (E)-2-pentenal, (E)-2-hexenal, (Z)-4-heptenal, 1-octen-3-one, 1,5-octadien-3-one, (E,E)-2,4-heptadienal, (E, Z)-2,6-nonadienal, and 2-ethylfuran [71,75,76]. 1-Penten-3-ol can form as a result of 15-lipoxygenase activity on EPA [76]. Further, autooxidation of EPA has been proposed to result in the formation of 2,4-heptadienal [76], which can be involved in the formation of tertiary lipid oxidation products under appropriate conditions [72].

Oxidation of n-6 PUFAs, e.g. LA (18:2n-6), AA (20:4n6) etc., gives rise to the formation of volatile compounds e.g. pentanal, hexanal, and 1-octen-3-ol etc. [76-78]. Autooxidation of LA can lead to the formation of pentanal and hexanal [79,80]. Hexanal can also be formed as a result of the degradation of other preformed volatiles e.g. 2-octenal and 2,4-decadienal [76]. Hexanal gives a grass-like odor, and has been reported to be the dominant aldehyde produced during lipid oxidation in muscle foods [80,81].

The highly reactive secondary lipid oxidation products, in particular 4-hydroxy-2-alkenals e.g. HHE and HNE, are probable precursors of 2-alkylfurans in foods; thus, 2-alkylfurans such as 2-ethylfuran and 2-pentylfuran can be formed from their corresponding 2-hexenal and 2-nonenal, respectively [19]. **Figure 7** shows a hypothesized reaction mechanism of 2-alkylfuran formation from (E)-2-alkenal. Briefly, the hydroxylation of a α, β-unsaturated aldehyde is followed by trans- to cis-isomerization of the double bond, which then cyclize into 4-hydroxy-2-alkenals in the presence of amino acids resulting in the formation of 2-alkylfurans [19]. Here, it is worth mentioning that some volatile compounds e.g. 2-ethylfuran, can form both via oxidative degradation of PUFAs e.g. α-linolenic acid (ALA, 18:3n-3), EPA, and DHA [76], and via a non-enzymatic pathway [19].

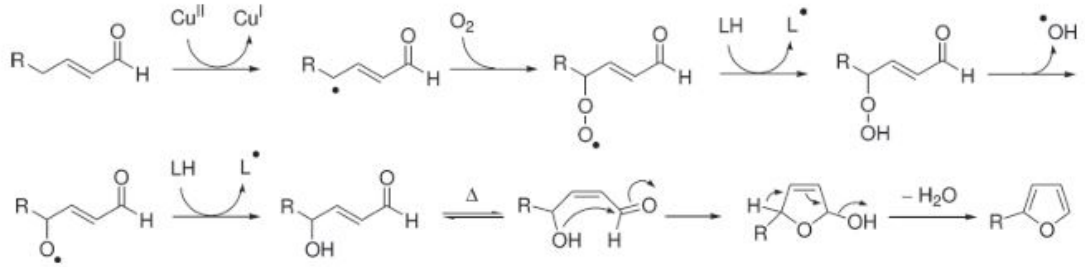

Figure 7. Proposed reaction mechanism of 2-alkylfuran formation from 2-alkenal. Adopted from Adams, et al. [19].

4.7.3 The role of Hb in lipid oxidation

The main pigment in red blood cells – hemoglobin (Hb) – contributes to lipid oxidation in post-mortem fish, according to several mechanisms, resulting in rancidity. Hb is a tetramer consisting of two α chains and two β chains (**Figure 8**). Each chain of Hb contains one porphyrin (heme) moiety. During Hb auto-oxidation, the reduced iron atom (Fe^{2+}) of each heme moiety is oxidized to the met state (Fe^{3+}) yielding metHb. The porphyrin-globin linkage in metHb is weakened, resulting in faster hemin loss than from oxyHb [82-85]. Hemin efficiently decomposes preformed lipid hydroperoxides into volatiles and free radicals and thereby promote lipid oxidation [82,83,86] . Other routes by which Hb can catalyze oxidation goes e.g. via the reaction of metHb with hydrogen peroxide (H_2O_2) or preformed lipid hydroperoxide (LOOH) to generate ferrylHb or ferrylHb radicals [87]. These can initiate oxidation reactions by abstracting H+ from a fatty acid or react with preformed lipid hydroperoxides and propagate lipid oxidation [88-91]. Several papers, e.g. Aranda IV, et al. [92], Undeland, et al. [18], and, Richards, et al. [93], have illustrated that there are large species differences when it comes to the pro-oxidative capacity of Hb. Fish Hbs are more pro-oxidative than avian and bovine Hbs, ascribed to the Root effect – i.e. decreased oxygen affinity at reduced pH – and differences in five specific amino acid sites [92]. Within the group of fish, there are also large differences, and e.g. Undeland, et al. [18] ranked four different species of fish Hb as follows; pollock > mackerel > menhaden > flounder.

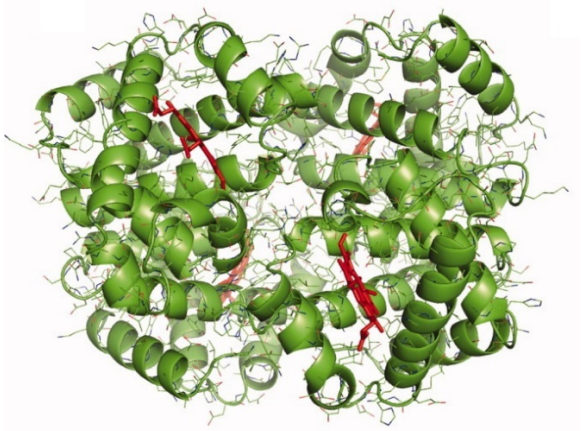

Figure 8. Tetrameric structure of bovine Hb at pH 5.7. Heme groups are shown in red. Adopted from Aranda IV, et al. [92].

Despite several publications showing that metHb-formation and heme-loss are accelerated at low pH [18,94]; no published studies have addressed Hb-mediated lipid oxidation during fish ensilaging.

4.8 Ensilaging, its advantages, and challenges

Ensilaging of fish processing co-products provides a viable option to preserve the co-products from bacterial growth and at the same time produce a product rich in short-chain peptides. The final product is a protein hydrolysates, traditionally known as "silage" and/or "fish silage". [4,26,95]

The ensilaging process can easily be done by adding acid to the ground raw materials (i.e. acid-induced ensilaging). The pH of the mix is kept below pH 4.0 to prevent microbial growth [28,96]. Endogenous enzymes present in the fish (mostly in the gut and partly in the muscle) will then work to liquefy the raw material in a temperature-dependent autolytic process [4,17,95].

There are however other ensilaging methods existing, e.g. bacterial fermentation (fermented silage) and ensilaging by adding base (i.e. alkali) e.g. ammonia etc. [17]. In case of fermented silage, the fermentation is initiated either by adding a starter culture to the raw material (i.e. fish co-products) or by adding a fermentable sugar to the raw material [97,98]. Fish silage can also be performed by adding alkali; however, this may result in degradation of lysine and cysteine, and may thus form lysinoalanine, which is considered as a carcinogenic substance which can cause poisoning in animals [17] . Further, free amino acids may become racemized from L- to D-forms under alkaline conditions. [17] In this thesis work, the acid-induced ensilaging method has been studied, and the terms ensilaging and/or silage will solely refer to the acid-induced ensilaging method.

In case of which acid to use, the choice varies between organic acid and inorganic acids (i.e. mineral acid) or a mixture of both. When using only inorganic acids, the pH of the silage has to be around 2.0 to prevent microbial growth [4]. The pH of such silage later has to be increased or neutralized before being used in e.g. feed [4]. This neutralization results in higher concentration of salts in the silage, which is nutritionally undesirable, and limits the use of inorganic acids. Organic acids are however quite useful at stabilizing higher pH values (e.g. around 4.0), and does not require pH neutralization prior to animal feeding. [99]

Organic acids, especially weak organic acids have a relatively low ionization at low pH. This allows weak organic acids to easily pass through cell membranes. Inside the cell, for example, where the pH is neutral, the acid will ionize resulting in a lower pH and accumulation of anions. These contribute to the anti-microbial effects of weak organic acids. The anti-microbial activity of an organic acid increases when the pH of the silage falls below the pKa-value of that acid. For example, the pKa-value of formic acid is 3.75; so, the silage pH should be maintained at a value lower than 3.75 to have more than 50% of its concentration in the dissociated anti-microbial form. [4,17,99-103]

The most commonly used organic acid is formic acid, and its use has been recommended by e.g. EFSA [104], NOFIMA [105,106] and Norwegian Scientific Committee for Food Safety [107]. Further, the use of formic acid in silage production, if no other additives of non-organic origin are used, allows to label the final product (i.e. silage) as an "organic" product [108].

When it comes to acid to fish co-product ratio, it mainly depends on the amount of acid required to bring the silage pH below a desired pH-value (i.e. pH 3.75). The initial pH of the fish raw material used, its bone (i.e. ash) content, the desired final pH of silage, and the concentration of acid used etc. are some of the factors that will dictate the amount of required acid. Immediately after acid addition, the pH-value tends to be lowered artificially, which is because the acid enters the cell structure within a short period. However, after some time, the pH-value tends to increase a bit for a short period of time due to the neutralization of bones and the formation of $CaCO_3$. [99,100] Thus, a higher bone content in the silage will lead to a higher increase in pH-value. Once all the bones are neutralized, the pH-value tend to remain more or less the same throughout the entire ensilaging period.

Fish silage can be made from any types of fish or fish co-products. Usually, co-products or by-catch of low commercial value are used for ensilaging. Fish silage can also be prepared by mixing co-products or by-catches from different species together. Here, it is important to note that, in case of feed applications, silage made of one species or mix of species should not be used to feed their respective species to avoid cannibalism.

Fish silage production has several advantages over fishmeal production. For example, fish co-products or by-catch which are not suitable for fishmeal production, due to e.g. shortage in quantity to make a viable fishmeal plant, can be used for ensilaging [17]. Ensilaging plants can also be placed anywhere where installation of a fishmeal plant is practically impossible. Ensilaging can be performed at any scale without affecting the economy of the process significantly [17]; this means that the batch volume can be adjusted anytime based on the co-products available. Acid preserved silage does not putrefy; a properly stored acid silage will give a fresh acidic smell even after several weeks of storage at tropical temperatures. The silage is considered as almost sterile; presence of pathogens like Salmonella are very rare in acid silage. [4,17,38,109] Further, fish processing co-products can often be classified as category 3 co-products, which are fit for human consumption [38]; thus, proving a controlled process, high-quality silage produced from such raw material can be used not only for feed, but also for food applications.

Although a series of papers have been published on lab-scale ensilaging of co-products from e.g. sprats, mackerel, sand-eels, herring, herring offal, white fish offal [28], plaice, sole, flounder, and whiting [109,110], reporting for example proximate composition, DH, and changes in amino acids, soluble nitrogen and oil, very little is still known about how wanted and unwanted biochemical reactions taking place during ensilaging are linked to specific process parameters; for example the actual proteolysis reaction as well as the formation of lipid oxidation products and total volatile basic nitrogen (TVB-N).

To further raise the value of fish silage, it can be separated into e.g. an oil, something which is reported for silage from klunzinger's ponyfish, gibel carp, sea bass, and nile tilapia [111-113]. The most common separation process applied is centrifugation, but temperatures, time, and g-forces have varied [111-113]. The retrieved oils have been characterized in terms of e.g. recovery yield, PV, p-AV, TOTOX, and TBARS with promising results emerging. So far, nothing is however published about recovery of fish oil and hydrolysates from herring co-product silage, and also systematic studies of yield as a function of optimal separation parameters are missing.

5 Methodological approaches

5.1 Study design

This thesis is based on five studies (i.e. papers I-V), and an overview of different studies can be found in **Figure 9.** In studies I-IV, lab-scale ensilaging was used to investigate different study variables and their effects on analyzed responses. In study V, pilot-scale ensilaging was performed, followed by lab-scale separation of silages into multiple products, and both silage and products derived thereof after separation were analyzed.

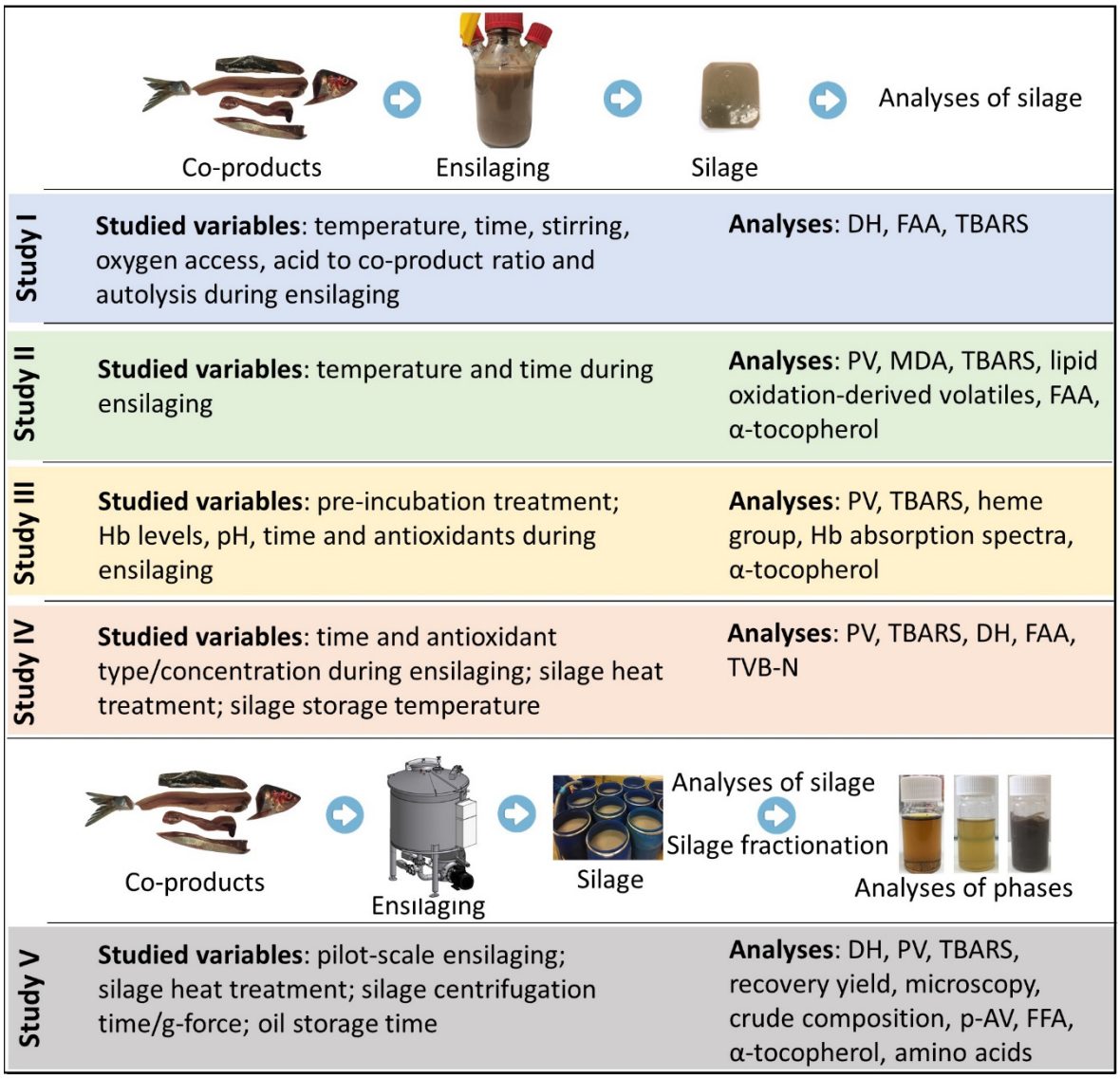

Figure 9. Schematic overview of studies I-V

In study I, effects of ensilaging process parameters e.g. temperature and time on DH, FAA and lipid oxidation were investigated.

In study II, effects of ensilaging temperature and time on the nature of lipid oxidation products formed were investigated.

In study III, the role of Hb for lipid oxidation development during ensilaging was investigated. Also, the effect of pre-incubating the co-products in different antioxidant solutions on lipid oxidation inhibition was studied.

In study IV, effects of different antioxidants and antioxidant concentrations on lipid oxidation inhibition during ensilaging, heat treatment and subsequent storage of silage were investigated. Also, the effects of heat treatment and storage time and temperature on DH, FAA and TVB-N were studied.

In study V, pilot-scale ensilaging was performed at conditions found optimum based on the results from studies I-IV. Thereafter, the possibilities of separating silage into multiple products were investigated in lab-scale.

5.2 Raw materials (Paper I-V)

Herring filleting co-product raw materials were kindly provided by Sweden Pelagic Ellös AB (Ellös, Sweden). The co-products used in the different studies presented in this thesis were from herring filleted on the 21st of August 2017 (batch 1), 27th of March 2018 (batch 2), 4th of September 2018 (batch 3), 21st of October 2019 (batch 4), 29th of October 2019 (batch 5), and 30th of September 2020 (batch 6); and are hereafter referred to as their batch number. The herring obtained was a mix of spring- and autumn spawners. The co-products contained a mixture of heads, frames, tails, skins, guts, belly flaps and other intestinal organs (see **Figure 1**). The co-products were collected immediately after filleting, transported to the lab covered by ice under cold conditions (5°C) within the same day, minced using a meat grinder (la Minerva, Italy) with either a 2.0 or 4.5 mm hole plate, and stored in aliquots of 500 g at -80°C until being used in ensilaging.

Formic acid (85%) was purchased from Fisher Scientific (Göteborg, Sweden). Antioxidants used were Duralox MANC-213 (Kalsec, Kalamazoo, Mich., UK), rosemary extract (Senyuan Bencao Natural Products Co., Ltd., Yuzhou, Henan, China), BORDANTIX LIQUID W/S and BORDANTIX LIQUID O/S (EVESA, Cádiz, Spain) as well as isoascorbic acid (Sigma-Aldrich, USA), butylated hydroxytoluene (BHT; Sigma-Aldrich, USA), propyl gallate (Sigma-Aldrich, USA) and ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, USA).

5.3 Ensilaging of herring filleting co-products

5.3.1 Lab-scale ensilaging (Paper I-IV)

The frozen co-products were first thawed in plastic bags under running cold water and then ensilaged by adding 2.5% v/w formic acid (85% purity) in 500-mL glass reactors equipped with overhead stirrer (see **Figure 10**). The co-product and acid mixture was then stirred at 10 rpm and ensilaged at ambient temperature (~22°C), unless otherwise specified, during the studied ensilaging period. This process setup is hereafter referred as the basic ensilaging protocol. In case of ensilaging at above or below ambient temperature, a water bath was used to adjust the temperature. Silage samples were collected at specified time intervals and stored in 5-mL Eppendorf tubes at -80°C until analysis.

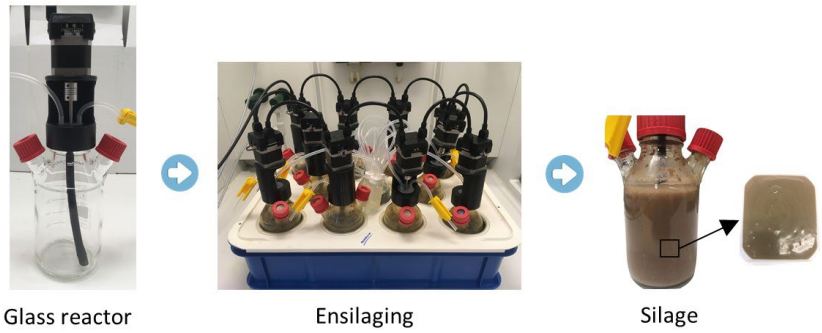

Figure 10. Lab-scale ensilaging setup

5.3.2 Pilot-scale ensilaging (Paper V)

Pilot-scale ensilaging was performed in a 2.0 m^3-capacity semi-automated BioChop ensilaging tank (**Figure 11**; Landia A/S, Lem, Denmark). The tank was equipped with a BioChop pump at the bottom of the tank, as well as a heating jacket for temperature control via hot/cold water circulation. Around 1420 kg herring filleting co-products were used for pilot-scale ensilaging at ambient temperature (i.e. 21-22°C). The co-products were first chopped at 1000 rpm for 15 min, followed by 8.0 kg antioxidant addition (0.5% w/w ratio; 1:1 ratio of BORDANTIX W/S and BORDANTIX O/S), mixed at 1000 rpm for 15 min, where after 35.5 liters formic acid (2.5 % v/w ratio) was added and mixed at 1000 rpm for 1 h. Thereafter, the silage was mixed every third hour at 200 rpm for 30 min. Samples were collected at specific time points, and put immediately into a freezer at -20°C. After 2 days of ensilaging, the silage was transferred to 100-liter plastic barrels and stored at 4°C. A portion of this silage was transported to the lab within the same day and stored at 4°C until further use for lab-scale separation (described later).

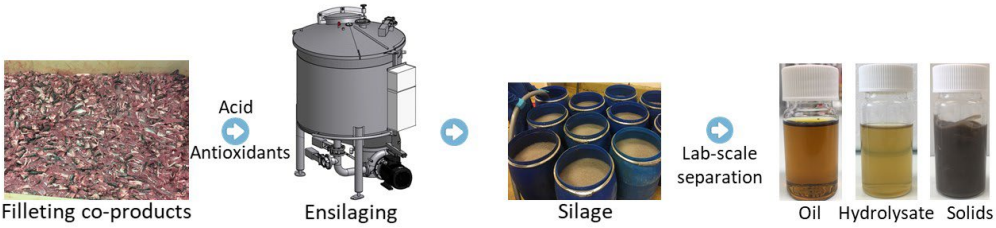

Figure 11. Pilot-scale ensilaging setup, followed by lab-scale separation of silage

5.4 Experimental setup for studies of DH and lipid oxidation

5.4.1 Effect of ensilaging process parameters on DH and lipid oxidation (Paper I-II)

The basic ensilaging protocol, as described earlier and in **Figure 10**, was used to investigate the effect of temperature (0-47°C), time (0-21 days), stirring (0-20 rpm), acid to co-product ratio (2.5 or 4.0% v/w), headspace oxygen removal and use of fresh vs frozen co-products on DH. To also study the contribution of autolysis to the DH, the control silage was heated at 95°C for 30 min in a water bath to inactivate the endogenous enzymes. Headspace oxygen was removed by flashing the headspace with N_2 for 30 sec. Samples collected at different time points were stored in 5-mL Eppendorf tubes at -80°C until analysis.

5.4.2 The role of Hb for lipid oxidation (Paper III)

The role of Hb for lipid oxidation development was studied by adding trout (*Oncorhynchus mykiss)* hemolysate to minced co-products at two different levels – i.e. double and triple the amount of endogenous Hb present in the respective silage controls. The controls were prepared by adding the same volume of tris buffer (0.1 M, pH 8.0) as the added volumes of hemolysate. The final Hb concentrations in the Hb-fortified silages were 114.4 and 147.1 µmole Hb/kg silage, respectively, and their respective controls contained 57.2 and 49.0 µmole Hb/kg silage, respectively.

To further understand the effect of ensilaging pH (i.e. pH 3.50) on Hb changes, a simple tris buffer model system (pH 3.5) fortified with trout Hb was used, and shifts in the Hb spectra were studied together with heme group release and Hb precipitation. To simulate the Hb concentration found in the herring co-products, trout hemolysate was added to the tris buffer (0.1 M, pH 8.0) at 68.6 µmole/L. The pH was then adjusted to 3.5 using formic acid (85% purity), and the system was stirred continuously at ambient temperature (i.e. 22°C). Samples were collected at specific time points and stored at -80°C until analysis.

5.4.3 Strategies to minimize lipid oxidation

5.4.3.1 Incubation treatment (Paper III)

One kilo of the freshly collected co-products were pre-incubated for either 30 sec, 20 min or 2 h in different treatments solutions listed in **Table 2** together with the ratio used. The treatment solutions were prepared in tap water and stored at 4°C until being used. After the incubations, the solution was drained off using a stainless-steel fine strainer. The co-products were then minced as described earlier, and a portion of the mince was collected and stored immediately at -80°C (referred to as "incubation" samples). The minced co-products were then ensilaged at ambient temperature (i.e. 22°C) for 7 days as described earlier, and silage samples were collected and stored at -80°C until further analysis (referred to as "silage" samples). For comparative purposes, a control sample without any incubation was also included in each trial.

Table 2. Incubation treatment solutions, ratio and time used in the different incubation trials. Herring co-products from batch 2, 3, 4 and 5 were used in trials 1, 2, 3, and 4, respectively. Treatment solutions were prepared in tap water.

Trials	Treatment solutions	Treatment details
1	Water, 0.9% NaCl, and 3% NaCl	Incubated for 30 sec or 2 h in treatment solutions (5:1 solution to co-products ratio)
2	Water, 0.9% NaCl, 5% Duralox MANC-213 in water, 0.2% isoascorbic acid with 0.04% EDTA in 0.9% NaCl, and 0.2% isoascorbic acid with 0.04% EDTA in water	Incubated for 20 min in treatment solutions (5:1 solution to co-products ratio)
3	Water, 2% Duralox MANC-213 in water, 2% isoascorbic acid in water	Incubated for 20 mins in treatment solutions (5:1 solution to co-products ratio)
4	0.5% rosemary extract in 0.9% NaCl, 2% isoascorbic acid in 0.9% NaCl	Incubated for 30 sec in treatment solutions (2:1 and 3:1 solution to co-products ratio)

5.4.3.2 Direct antioxidant addition at start of ensilaging (Paper IV)

To study the effect of antioxidant type and concentration on lipid oxidation inhibition during ensilaging, selected antioxidants were added to minced co-products at 0.25, 0.75, and 1.25% w/w concentrations, mixed for 10 min at 10 rpm, followed by addition of formic acid (2.5% v/w), and the mix was then ensilaged at ambient temperature (i.e. ~22°C) with continuous stirring (10 rpm) for up to 7 days as described earlier. Antioxidants used were Duralox MANC-213 (a mixture of rosemary extract, α-tocopherol,citric acid and ascorbic acid), BORDANTIX LIQUID W/S, BORDANTIX LIQUID O/S, isoascorbic acid, BHT and propyl gallate. A control without any antioxidant addition was also ensilaged. Samples were collected at specific time points and stored immediately at -80°C until further analysis. A portion of the day 7-silage was used for heat treatment and subsequent storage experiments as described later.

5.4.4 Heat treatment and storage trial (Paper IV)

To simulate the heat treatment which is often done industrially to stop autolysis and secure absence of pathogens, around 10 mL of the day 7-silage samples were subjected to heat treatment at 85°C for 30 min in a water bath. After the heat treatment, the silage samples were let to cool down to room temperature, and aliquots of samples were stored in -80°C until analysis.

To simulate a prolonged storage of silage, also likely to occur under industrial process parameters, either heat-treated or non-heat-treated silages containing 0, 0.25, 0.75, and 1.25% w/w Duralox MANC-213 were stored in 15-mL tubes at both 4°C and 22°C to simulate cold and ambient storage, respectively. Silages were vortexed for 30 sec before sampling at regular time points, and samples were stored in -80°C until analysis.

5.4.5 Silage separation study (Paper V)

Around 10 mL silage samples, either heat-treated or non-heat-treated, were transferred to 15-mL tubes and subjected to centrifugation at either 3000, 4500, or 8500 x g for 2, 10, or 20 min as specified in **Table 3**. Thereafter, the phases were collected, weighed, and stored in -80°C until further analysis. Recovery yields of different phases were calculated using the equation below.

$$\text{Yield} = (\text{weight of collected phase}/\text{weight of silage before centrifugation}) * 100$$

Table 3. Experimental plan for lab-scale separation of silage at 20°C. Herring co-products from batch 6 were used here.

g-force	Heat-treatment (85°C; 30 min)	Centrifugation time (min)		
3000 x g	Non-heat-treated	2	10	20
	Heat-treated			
4500 x g	Non-heat-treated	2	10	20
	Heat-treated			
8500 x g	Non-heat-treated	2	10	20
	Heat-treated			

5.5 Analysis of raw material and silages

5.5.1 Proximate composition (Paper I, V)

The NREL protocol (NREL/TP-510-42621) was followed to analyze the moisture content by drying the samples at 105°C for 24 h [114]. Crude protein content was calculated by converting the total nitrogen content, analyzed by a nitrogen analyzer (LECO), to protein content using a conversion factor of 5.58 [115]. Crude lipid content was determined gravimetrically using the chloroform phase from a chloroform:methanol (2:1) extraction according to Lee, et al. [116]. Ash content was determined by ashing the samples at 575°C for 4 h according to NREL protocol (NREL/ TP-510-42622) [117].

5.5.2 Protein degree of hydrolysis (DH) (Paper I, III-V)

DH was determined using the o-phthaldialdehyde (OPA)-reagent according to Nielsen, et al. [118] with slight modifications as described in **Paper I**. Briefly, 0.5 mL diluted sample was mixed with 3.75 mL OPA reagent, vortexed for 5 sec, incubated for 2 min at room temperature, and the absorbance was read at 340 nm using a spectrophotometer (Cary 60 UV–vis, Agilent technologies, 117 USA).

5.5.3 Free and total amino acids (Paper I-II, IV-V)

Samples for free amino acid analysis were prepared by centrifuging 0.9 g silage at 12,000 × g for 10 min (4 °C), followed by a 1:1 addition of 7.5% trichloroacetic acid (TCA) solution to the supernatant. Thereafter, the sample was held on ice for 15 min, centrifuged as described earlier, and the supernatant was then diluted with 0.2 M acetic acid and transferred to vials for LC/APCI-MS analysis as described in **paper II**. Samples for total amino acid analysis were prepared by adding 4 mL of 6 N HCl to 0.5 g silage in glass tubes, followed by headspace oxygen removal by flushing with N_2 for 30 sec, vortexing, and then hydrolysis at 110 °C for 24 h. Thereafter, the solution was quantitatively transferred to a 15-mL tube using 6 mL milli-Q water, followed by further dilution with 0.2 M acetic acid, and then transferred to vials for LC/APCI-MS analysis. Both free and total amino acids were then analyzed by LC/APCI-MS according to Özcan and Şenyuva [119] with slight modifications as described by Harrysson, et al. [120].

5.5.4 Molecular weight distribution of proteins and peptides (Paper I)

Molecular weight distribution of soluble proteins and peptides was analyzed according to Abdollahi, et al. [121] with slight modifications for sample preparation. Briefly, samples were diluted with the mobile phase to an equal protein concentration (e.g. 25 mg/mL), centrifuged at 10,000 x g for 10 min, and then the supernatant was used for HP-SEC analysis after filtration (0.45 µm, Fisher brand).

5.5.5 Peroxide value (PV) and TBARS (Paper I-V)

Around 2 g silage sample was first mixed with 20 mL ice-cold chloroform:methanol (2:1) containing 0.05% w/v BHT, vortexed for 60 sec, followed by addition of 8 mL ice-cold 0.5% NaCl, vortexed for 30 sec, and then centrifuged at 3000 x g for 6 min (4°C) [116]. The resulting lower and upper phases were then used for spectrophotometric (Cary 60 UV–vis, Agilent

technologies, USA) analysis of PV and TBARS, respectively, according to Undeland, et al. [122] (PV) and Schmedes and Hølmer [123] (TBARS).

5.5.6 p-anisidine value (p-AV) and TOTOX value (Paper V)

p-AV was analyzed according to AOCS's official method as described by Semb [124] with slight modifications for sample preparation. Briefly, 0.3 g fish oil was dissolved in 60 mL isooctane, vortexed to dissolve completely, followed by measurement of the first absorbance at 350 nm (Cary 60 UV–vis, Agilent technologies, 117 USA) against isooctane as the blank. Then, 0.5 mL p-anisidine reagent (0.25% w/v p-anisidine in glacial acetic acid) was added to 2.5 mL sample/blank, vortexed for 30 sec, incubated in dark for 10 min, followed by measurement of the second absorbance, and then p-AV was calculated as described by Semb [124]. TOTOX values were calculated according to the equation below:

$$\text{TOTOX} = 2\ \text{PV} + \text{p-AV}$$

5.5.7 Volatile compounds by HS-SPME-GC-MS (Paper I-II)

Volatile compounds derived from lipid oxidation and non-enzymatic browning reactions were determined by headspace solid-phase microextraction (HS-SPME) coupled with GC-MS according to a method by Iglesias and Medina [76] with slight modifications as described in **paper II**. Briefly, around 2 g silage samples were diluted using 8 mL milli-Q water in 20-mL SPME vials, followed by addition of 200 µl internal standard (3-methyl-3-buten-1-ol), and, vortexed for 30 sec. Volatile compounds were then equilibrated at 60°C for 5 mins with stirring at 500 rpm, followed by extraction using 75 µm Carboxen/polydimethylsiloxane (CAR/PDMS) coated SPME fiber (Supelco, USA) at 60°C for 20 mins with stirring, and then the fiber was injected in the GC injection port. Volatile compounds were separated on a fused silica ZB-1701 capillary column (Phenomenex, 30 m x 0.32 mm, 1 µm), and MS data acquisition was performed in scan mode in the mass range of 10-250 amu using a single quadrupole MS (ISQ, Thermo Scientific) operated in the electron impact mode. Volatile compounds were identified and quantified using external standards and in some cases by matching the mass spectra with MS-libraries.

5.5.8 Total Hb content in herring co-products (Paper III)

Total Hb content in the co-products was measured according to Hornsey [125] with slight modifications as described by Harrysson, et al. [126]. Briefly, 4 g minced co-products were mixed with 18 mL freshly made acidic acetone solution to a final concentration of 80% acetone, 2% HCl, and 18% water, incubated at 8°C for 60 min in the dark, centrifuged at 5000 x g for 10 min, and then the absorbance of the supernatant was read at 640 nm (Cary 60 UV–vis, Agilent technologies, USA). Hb content was then calculated using bovine Hb (Sigma-Aldrich, USA) as the standard.

5.5.9 Shift in Hb spectra under acid conditions (Paper III)

The shift in Hb spectra upon adjusting the pH of the trout hemolysate model system from 8.0 to 3.50 was analyzed by first centrifuging the samples at 16 000 x g for 5 min (4°C), and then scanning the samples in the range of 300-700 nm (Cary 60 UV–vis, Agilent technologies, USA).

5.5.10 Heme group release from Hb (Paper III)

Heme group release from Hb was measured according to Maestre, et al. [127] using ISOLUTE® C18 100 mg/mL cartridges (Biotage Sweden AB, Sweden). Briefly, 1 mL sample was added to preconditioned (using 2 mL of 1:1 methanol:water) cartridges, followed by elution of Hb using 4 mL milli-Q water, and then free heme was eluted using 1 mL methanol. The absorbance of this methanol portion was scanned in the wavelength range of 350-450 nm (Cary 60 UV–vis, Agilent technologies, USA), and the heme group concentration was calculated using a hemin standard (Sigma-Aldrich, Netherlands).

5.5.11 Hb precipitation (Paper III)

The resulting supernatant after centrifugation of the Hb-fortified buffer model system at 16 000 x g for 5 min (4°C) (see section 5.5.9) was used for protein content determination according to Lowry, et al. [128] to estimate Hb precipitation. In this study, the Bio-Rad DC protein assay kit was used together with Bovine Serum Albumin (Sigma) as the standard.

5.5.12 Total volatile basic nitrogen (TVB-N) (Paper I, IV)

TVB-N was analyzed using Conway diffusion cells according to Rawdkuen, et al. [129] with slight modifications as described in **paper I**. Briefly, around 2 g silage sample was mixed with 8 mL of 4% TCA solution, vortexed for 2 min, centrifuged at 3,000 × g for 15 min, and then 2 mL supernatant was used for the analysis. The Conway cells, containing 2 mL supernatant and 2 mL saturated potassium carbonate in the outer ring and 2 mL of 1% w/v boric acid containing 0.165% v/v methyl red and 0.0825% v/v bromocresol green in the inner ring, were incubated at 37°C for 60 min, and then the inner ring solution was titrated using 0.02 N HCl.

5.5.13 Microscopic analysis of emulsion formed during silage separation (Paper V)

Microscopic analysis of the emulsion, formed when centrifuging the silage, was performed according to Abdollahi, et al. [121] using a microscopy (Axiostar Plus, Carl Zeiss Microscopy, LLC, USA) with 40x magnification (A-Plan 40x/0.65 Ph2, Carl Zeiss Microscopy, LLC, USA). Emulsion images were taken with a camera (Canon PowerShot G9, 12.1 Megapixels) mounted on the top of the microscope with 6x optical zoom lens.

5.5.14 Color measurement (Paper II, V)

The color of oil samples was measured using a colorimeter (CR-400, Konica Minolta Sensing, Japan) in the CIE L∗a∗b∗ color space as described in **paper II**. Briefly, oil samples were placed on flat polystyrene plates, and the color readings were taken by placing the colorimeter probe against the bottom of the plates.

5.5.15 Fatty acid composition (Paper II)

To analyze fatty acid composition of raw material and silage oils, lipids were first extracted according to Lee, et al. [116], then methylated according to Cavonius, et al. [130], where after the fatty acid methyl esters (FAMEs) were analyzed using a GC-MS (7890A GC System couped with 5975 C triple-axis mass spectrometric detector; Agilent Technologies) according to a method described by Cavonius, et al. [130]. Heptadecanoic acid (C17:0) was used as an internal

standard, and FAMEs were identified and quantified using external fatty acid standard mixture GLC-463 (Nu-Chek Prep, Inc., USA).

5.5.16 Free fatty acid (FFA) (Paper V)

Samples for FFA analysis were prepared according to Abdollahi and Undeland [131] with slight modifications and then analyzed according to Lowry and Tinsley [132]. Briefly, 200 µl diluted oil sample was mixed with 1.8 mL isooctane [133], vortexed for 10 sec, and then 400 µl cupric acetate-pyridine reagent (5% w/w cupric acetate in milli-Q; pH adjusted to 6.0 using pyridine) was added, vortexed for 90 sec, centrifuged at 2000 x g for 10 min, and then the absorbance of the resulting upper phase was read at 710 nm (Cary 60 UV–vis, Agilent technologies, 117 USA).

5.5.17 α-Tocopherol (Paper II-III, V)

α-Tocopherol content in co-products and silages was analyzed by evaporating 8 mL chloroform extract from the chloroform:methanol extraction described earlier (see section 5.4.5) under N_2, followed by dilution in 1 mL methanol, centrifugation at 2000 x g for 3 min, and then the supernatant was used for analysis. Fish oil samples were simply dissolved in chloroform:methanol (2:1). α-Tocopherol content was analyzed using HPLC with fluorescence detection according to Larsson and Undeland [134], and quantification was performed using α-tocopherol standard (Sigma-Aldrich, USA).

5.5.18 Determination of total phenolics (Paper III)

Total phenolics content (TPC) of Duralox MANC-213 and the used rosemary extract was analyzed according to the Folin–Ciocalteu method [135] with slight modifications as described by Trigo, et al. [136]. Briefly, 0.1 g sample was mixed with 10 ml extraction solvent (methanol:water + trifluoroacetic acid; 70%:30% v/v + 1% v/v), vortexed for 30 sec, centrifuged at 5000 x g for 5 min. Thereafter, 50 µl supernatant was mixed with an equal volume of Folin–Ciocalteu reagent, followed by addition of 1 ml Na_2CO_3 (75 g/L) and 1.4 ml MilliQ-water. The mixture was then vortexed for 10 sec, incubated for 1 h in darkness at ambient temperature (i.e. 22°C), and then the absorbance was read at 750 nm. Gallic acid (Janssen Chimica, Belgium) was used as the standard, and TPC was expressed as mg gallic acid equivalent/g sample.

5.5.19 Determination of carnosic acid (Paper III)

Carnosic acid content of the same samples was analyzed by HPLC according to the method described by Zhang, et al. [137] with slight modifications. Briefly, 0.1 g sample was dissolved in 10 ml methanol, vortexed for 30 sec, centrifuged at 2000 x g for 3 min, and the resulting supernatant was used for HPLC analysis. Chromatographic separation of carnosic acid was performed on a HPLC column (Inertsil® ODS-3, 3 µm, 150 x 3 mm, GL Sciences Inc., Japan) at 40°C column temperature with 0.5 ml/min flow rate for 14 min run time with 10 µl sample injection volume. Eluents used were; (A) 1% acetic acid in Milli-Q water, and (B) 1% acetic acid in methanol. Eluent gradient conditions used were; 10% A and 90% B at 0 min, followed by gradual increase of A to 64% over 10 min, and then gradual decrease to 10% from 12 min and this ratio was maintained until the end of the run. Detection was performed using a UV

detector at 280 nm wavelength, and carnosic acid standard (Sigma Aldrich 91209, Supelco, analytical standard) was used for peak detection and quantification.

5.5.20 Statistical and multivariate analysis (Paper I-V)

Results of the different sub-studies I-V were expressed as mean values (n = 2, or, 3, or, 4) ± standard error of the mean (SEM). ANOVA analysis with Tukey's Honest Significant Differences (HSD) test was performed on RStudio software (https://www.rstudio.com/). Significant differences were accepted at $p < 0.05$. Selected data sets were also subjected to multivariate analysis using e.g. SIMCA software (version 15, Umetrics, Sweden) and MODDE Pro software (version 12.1, Sartorius Stedim Data Analytics AB, Sweden). For principal component analysis (PCA), data set was unit variance (UV)-scaled, and then PCA analysis was performed using SIMCA software. For the analysis of main and interaction effect of studied factors on the measured responses, selected data sets were analyzed using MODDE Pro software. The data set was subjected to full factorial (mixed) design and fitted with partial least squares regression (PLS). The responses were scaled and centered, and coefficient plots were presented. The size of coefficients – i.e. half of the effect – denotes a change in their respective responses upon changing a factor from its medium to high level, while keeping other factors at their average level.

6 Results and discussion

6.1 Proximate composition of the used herring filleting co-products (Paper I, V)

The composition of used herring co-products from two different catching seasons is given in **Table 4**. The main difference was in the moisture and lipid content, which is inversely related to each other [138,139]. The higher lipid content in autumn than spring is in line with earlier reports, possibly due to availability of more food in the autumn and use of fat reserves during the spawning season in the spring [139]. Although a slight, but significant ($p < 0.05$), difference in protein content between the two seasons was noticed; the recorded protein content of herring co-products was within the range of 11.0-16.9% as earlier reported by Aidos, et al. [139]. The ash content remained constant irrespective of the catching season.

Table 4. Proximate composition of herring co-products from batch 1 (autumn 2017) and 2 (spring 2018). Results are expressed as mean ± SEM (n=3).

Sampling	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)	Others (%)
Autumn 2017	66.70 ± 0.1	12.29 ± 0.1	17.85 ± 0.1	2.81 ± 0.1	0.3
Spring 2018	77.03 ± 0.2	14.95 ± 0.1	4.55 ± 0.2	2.69 ± 0.1	0.7

6.2 Protein hydrolysis during ensilaging of herring co-products (Paper I, III-V)

The extent of protein degree of hydrolysis (i.e. DH) during lab-scale ensilaging of herring co-products at 7-47°C for 1-7 days is shown in **Figure 12A**. Overall, the DH increased with ensilaging time, and, the highest DH was noticed at 32°C. It is apparent that free amino acids were also formed during ensilaging, and around 14% of the total amino acids were in free form after 7 days ensilaging at 22°C (**Figure 12B**). Thus, a strong correlation between DH and free amino acid formation was noticed (**Figure 12C**), which has also been reported by other authors [140].

That the highest DH was found at 32°C suggests that the used herring co-products' endogenous proteases were most active at this temperature. The maximum endogenous proteolytic activity of different tissue fractions from herring, e.g. muscle, intestine, and head, skin and bones, has been reported to be around 45-50°C at pH 3.2-3.8 using Hb as the substrate [141]. The same authors also reported lower proteolytic activity towards herring myofibrillar protein than herring Hb. In our study, a mix of herring filleting co-products was used, comprising both myofibrillar and sarcoplasmic proteins, which probably explains the observed difference in optimum activity. Stoknes, et al. [141] reported the highest protease activity in the intestine fraction of cod (*Gadus morhua*) and herring (*Clupea harengus*) at acidic pH, which probably suggests enrichment of aspartic proteases; this was also in agreement with other reports [28,142].

Apart from the specific fraction of co-products used as substrates for the proteolysis, the rate of DH might vary depending on e.g. the physiological condition of the fish at the time it was caught/ensilaged, the amount, type and activity of endogenous protease present in the mixed co-products etc. [17,99,143,144]. The optimum pH range for digestive proteases from e.g. cod viscera, assayed using Hb as the substrate, has been reported to be around pH 2.0-4.0 [145]; thus, it is likely that a variety of proteases are activated at the ensilaging pH ~ 3.5, supporting the formation of

both short-chain peptides and free amino acids during ensilaging (**Figure 12** and **Figure 13**).

As shown in **Figure 13**, proteins in the range of 17-366 kDa were hydrolyzed into short-chain peptides of 1.9-4.7 kDa already after 3 days of ensilaging, at a time point at which the level of free amino acids had reached only 8%. This type of information offers the flexibility to stop the hydrolysis when the desired DH and/or amounts of short-chain peptides has been achieved, while levels free amino acids are kept to a minimum.

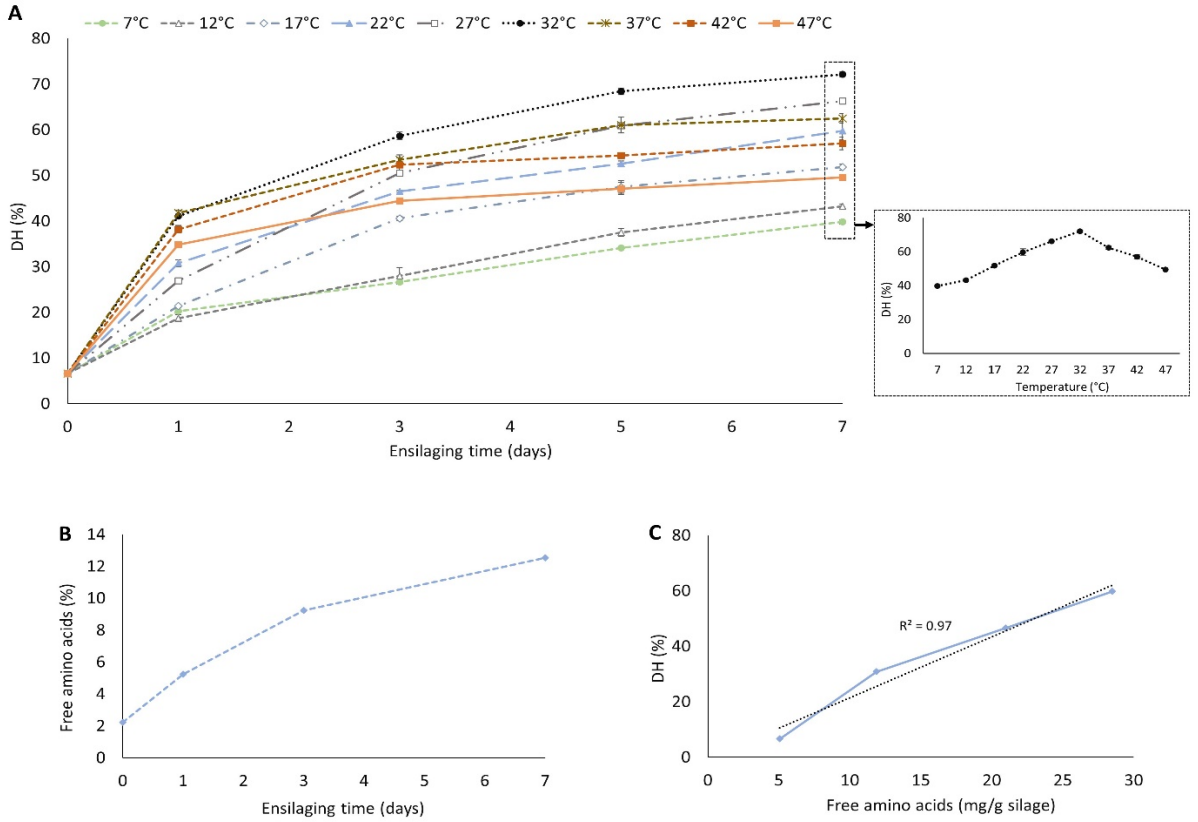

Figure 12. Effect of ensilaging temperature and time on DH (A), percentage free amino acid formation of total amino acids (B) during ensilaging at 22°C, and, correlation between DH and free amino acids (C). Herring co-products from batch 1 were used here. Results are expressed as mean ± SEM (n = 3).

Traditionally, ensilaging has primarily been used as a preservation technique to protect the co-products against microbial spoilage [17,109], and, thus, little attention has been paid on the formation of bioactive short-chain peptides and unwanted free amino acids. In this thesis, an attempt to upgrade the traditional ensilaging technique was done, to convert it more to a processing technique for simultaneous preservation and production of short-chain peptides. The exact time point to stop the autolysis to have an optimized combination of peptides and free amino acids, however, requires further investigations in humans and animals for food and feed applications, respectively. After stopping the autolysis by a heat treatment step, the silage remains stable without excessive formation of free amino acids (**Figure 14B**) for an extended

period of time, as shown in this thesis for up to 6 months. This gives a long window of time during which further processing and/or direct use in feed or food can be applied.

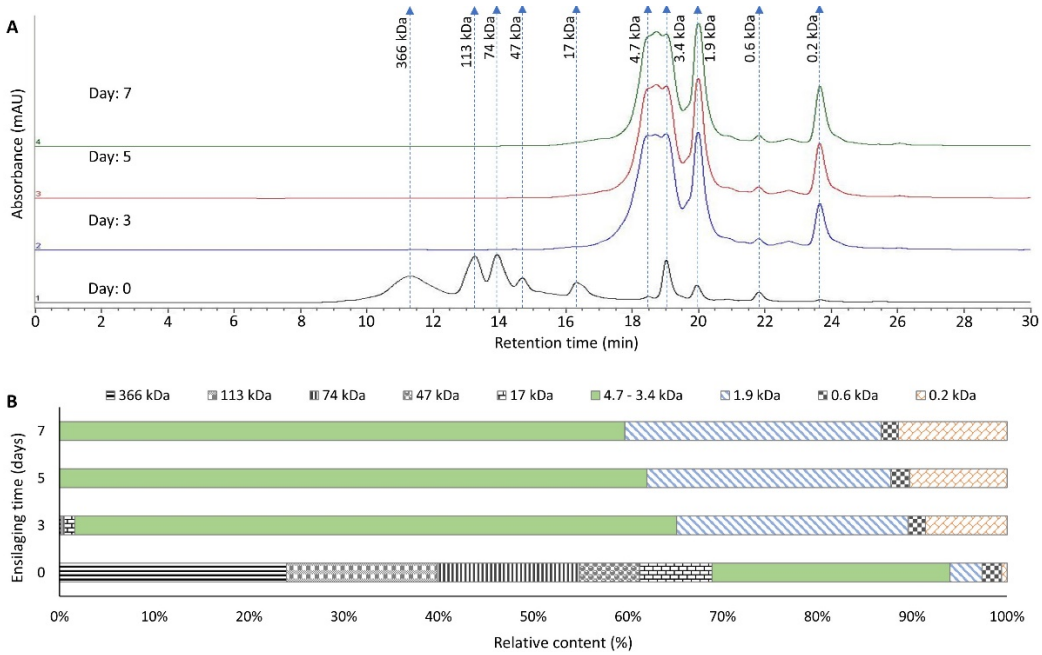

Figure 13. Changes in molecular weight distribution of proteins and peptides during 7 days ensilaging at 27°C; (A) SEC chromatogram, (B) relative content of proteins and peptides. Herring co-products from batch 2 were used here. The 0.2 kDa peak refers to peptides containing e.g. 2 amino acids, and peaks around 0.6-4.7 kDa refer to peptides containing e.g. 3-20 amino acids.

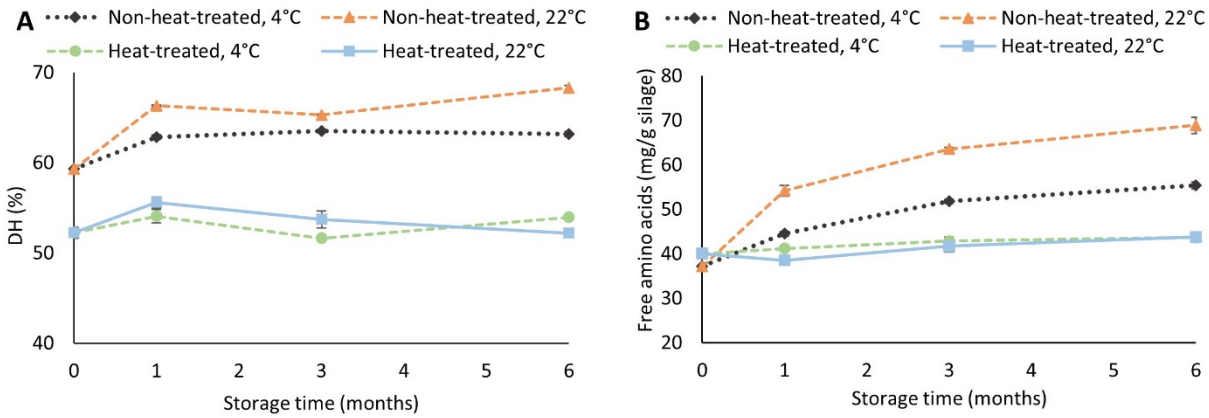

Figure 14. Effect of heat-treatment, storage temperature and time on DH (A) and free amino acids (B). Silage samples contain 0.75% w/w Duralox MANC-213. Herring co-products from batch 4 were used here. Results are expressed as mean ± SEM (n = 2).

6.3 Lipid oxidation during ensilaging of herring co-products (Paper I-V)

6.3.1 Lipid oxidation during ensilaging in absence of antioxidants (Paper II)

As described earlier, the herring co-products had the highest DH at 32°C; thus, to speed up the peptide formation, one might consider using this temperature for ensilaging. However, herring co-products also contain PUFAs, which are highly sensitive to lipid oxidation. **Figure 15** shows the formaiton of primary and secondary lipid oxidation products, i.e. PV and TBARS, respectively, during 1-7 days of ensilaging at 7-47°C. Overall, both PV and TBARS increased over time at all studied temperatures. Temperature caused an increased PV between 7 and 22°C, which then was followed by a decreasing trend between 22 and 47°C. Lipid hydroperoxides are known to be temperature sensitive [146] and might break down in presence of heme-proteins [147], explaining the decrease at elevated temperatures (i.e. > 22°C). That a decrasing trend was found also for TBARS at temperatures > 22°C required further investigations of the main carbonyl compound responding in the TBARS test, i.e. MDA. LC-MS analyses revealed that MDA was broken down > 22°C and gave rise to its hydrolyzed product acetaldehyde (**Figure 16A-B**). While the highly reactive MDA did not accumulate at 47°C, it gradually increased over time at 7°C, and at 22°C, it increased up to 3 days and then followed a decreasing trend. Similar trends were also noticed for another highly reactive aldehyde, HHE (**Figure 16C**).

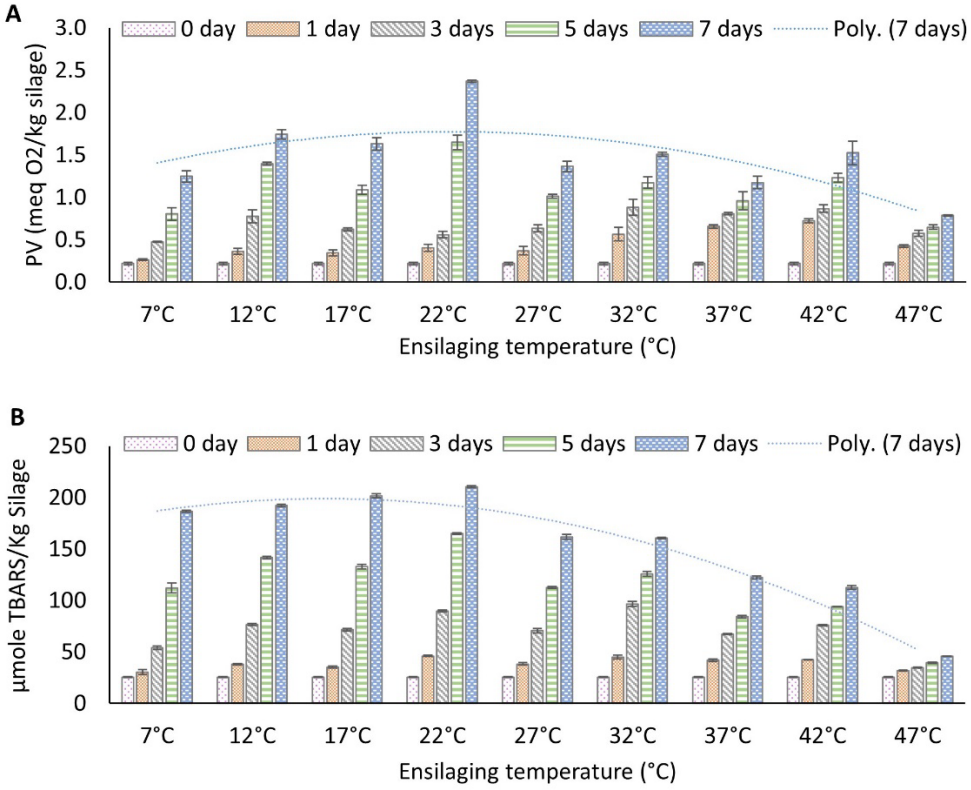

Figure 15. Effect of temperature and time on PV (A) and TBARS (B). Herring co-products from batch 1 were used here. Fig. A-B: the second order polynomial trendline provides a general visualization of the changes in values after 7 days of ensilaging at different temperatures.

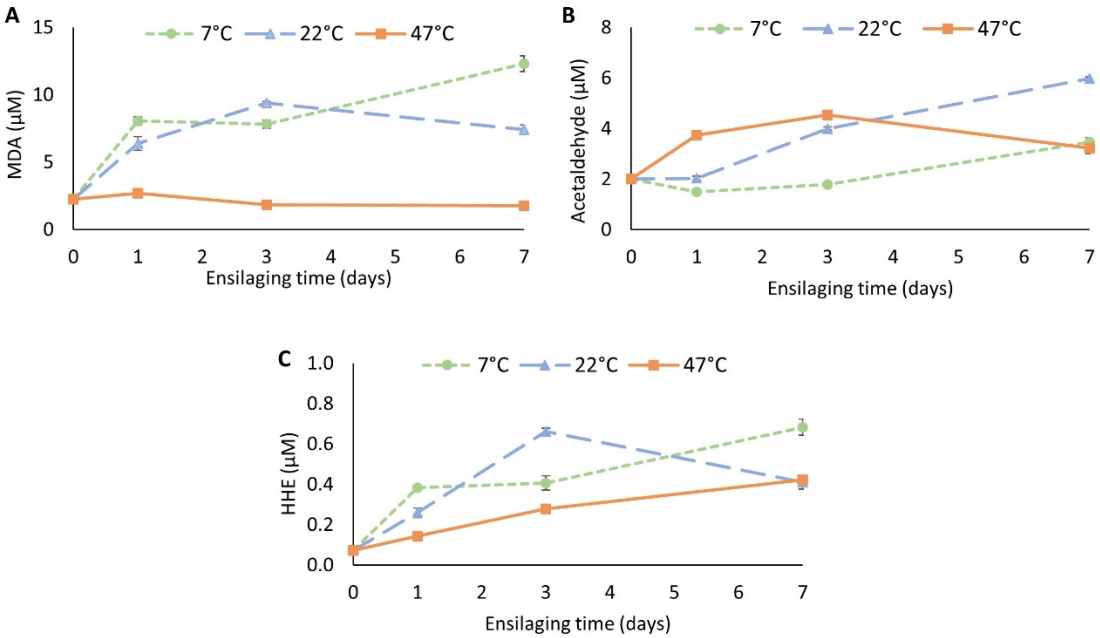

Figure 16. Effect of temperature and time on MDA (A), acetaldehyde (B), and HHE (C). Herring co-products from batch 1 were used here.

Thus, although the TBARS measurement is one of the most commonly used methods for secondary lipid oxidation product analysis, it does not represent the true extent of lipid oxidation in silage > 22°C, as the MDA undergoes hydrolytic cleavage. Although this has not earlier been reported in silage settings, the finding was in agreement with previously reported work using buffer- and emulsion-model systems, where the authors noticed a temperature-dependent dynamic equilibrium between MDA and its degradation products [22]. As both MDA and its hydrolysis product acetaldehyde have been ascribed highly toxic, carcinogenic, and mutagenic properties [148,149], linked to their high reactivity towards e.g. proteins/peptides/amino acids, their intake levels should indeed be kept to a minimum. In a protein-rich matrix like silage, the high reactivity of MDA and acetaldehyde was expected to form various adducts [19,150], which called for additional analyses to be revealed.

To further understand the nature of the oxidation products formed during ensilaging at 7, 22, and 47°C, lipid oxidation- and non-enzymatic browning-derived volatile compounds were analyzed using HS-SPME-GC-MS. In general, formation of a variety of volatile compounds was noticed at 22 and 47°C which increased with time as can be seen in **Figure 17**. Formation of saturated aldehydes e.g. pentanal and hexanal and unsaturated aldehydes e.g. (*E*)-2-hexenal and (*E,Z*)-2,6-nonadienal increased steadily over time at both 7 and 22°C, however the rate was higher at 22°C (**Figure 18A-D**). At 47°C, both saturated and unsaturated aldehydes increased up to 3 days, whereafter they followed a decreasing trend (**Figure 18A-D**). Contrary, non-enzymatic browning reactions-derived volatiles e.g. 2-ethylfuran and 2-pentylfuran, developed by higher rates in the order of 7 < 22 < 47°C, and the formation increased significantly ($p < 0.05$) with time (**Figure 18E-F**).

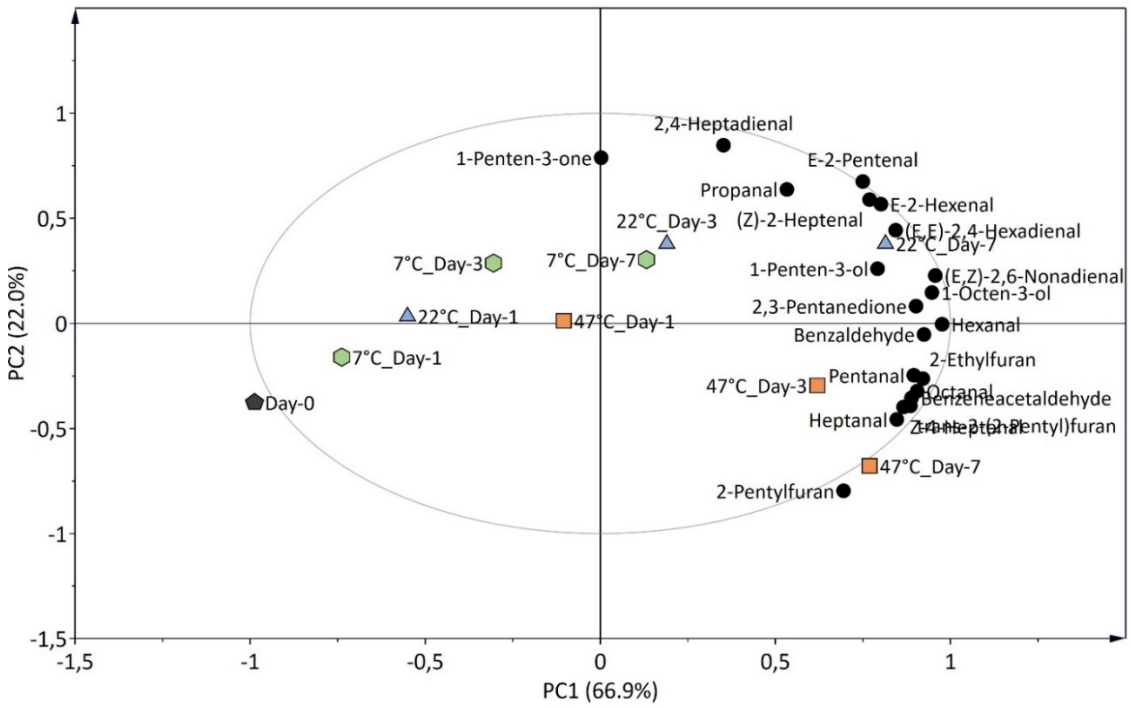

Figure 17. PCA bi-plot showing an overview of the volatile compounds formed during ensilaging. Colored symbols represent treatments (i.e. different temperatures with different time points), and black circles represent volatile compounds. Treatments located close to volatile compounds are high in these volatile compounds and are low in volatile compounds located far away. Herring co-products from batch 1 were used here.

Pentanal and hexanal can form from oxidation of n-6 PUFAs, e.g. LA (18:2n-6) and AA (20:4n6) [76,78]. Hexanal can also be formed from breakdown of other preformed volatiles e.g. 2-octenal and 2,4-decadienal [76]. Similar to this study, the formation of pentanal and hexanal upon storage of Baltic herring has been reported by Aro, et al. [151]. (*E*)-2-hexenal and (*E,Z*)-2,6-nonadienal can form from oxidation of n-3 LC PUFAs e.g. ALA (18:3n-3), EPA (20:5n-3), and DHA (22:6n-3) [76,152]. It has been reported that LOX action on EPA can produce 2,6-nonadienal [153] which can then be degraded into e.g. (*Z*)-4-heptenal [154], something which probably explains its decreasing trend after 3 days of ensilaging at 47°C. Further, autooxidation of EPA can produce 2,4-heptadienal [76], which then can be involved in the generation of tertiary lipid oxidation products [78].

The non-enzymatic browning reaction-derived volatile compounds e.g. 2-ethylfuran and 2-pentylfuran can be formed from e.g. 2-hexenal and 2-nonenal, respectively [19], and these compounds were also noticed in silages. The formation of precursors, i.e. 4-hydroxy-2-alkenals such as HHE and HNE is required to catalyze the formation of such 2-alkylfurans [19,74]. Thus, the observed relatively low amount of HHE (**Figure 16C**) and the absence of HNE at 47°C, as well as decreasing trend of (*E*)-2-hexenal (**Figure 18C**) and the absence of 2-nonenal at 47°C, was in line with the pronounced formation of 2-ethylfuran and 2-pentylfuran 47°C. Altogether, this confirmed increased interactions between secondary lipid oxidation products and proteins/peptides/amino acids present in silage at elevated temperatures (> 22°C). These

observations were also in agreement with earlier reports [19,72]. Here it is important to note that, besides the non-enzymatic browning reaction pathway, 2-ethylfuran and 2-pentylfuran can also be formed from oxidation of e.g. ALA, EPA, and DHA [76,152]. However, given the decline in precursors, and the enrichment of proteinaceous material in silage, makes the pronounced formation of these two alkylfurans at 47°C more likely to be the result of non-enzymatic browning reactions and/or a combination of both reaction pathways.

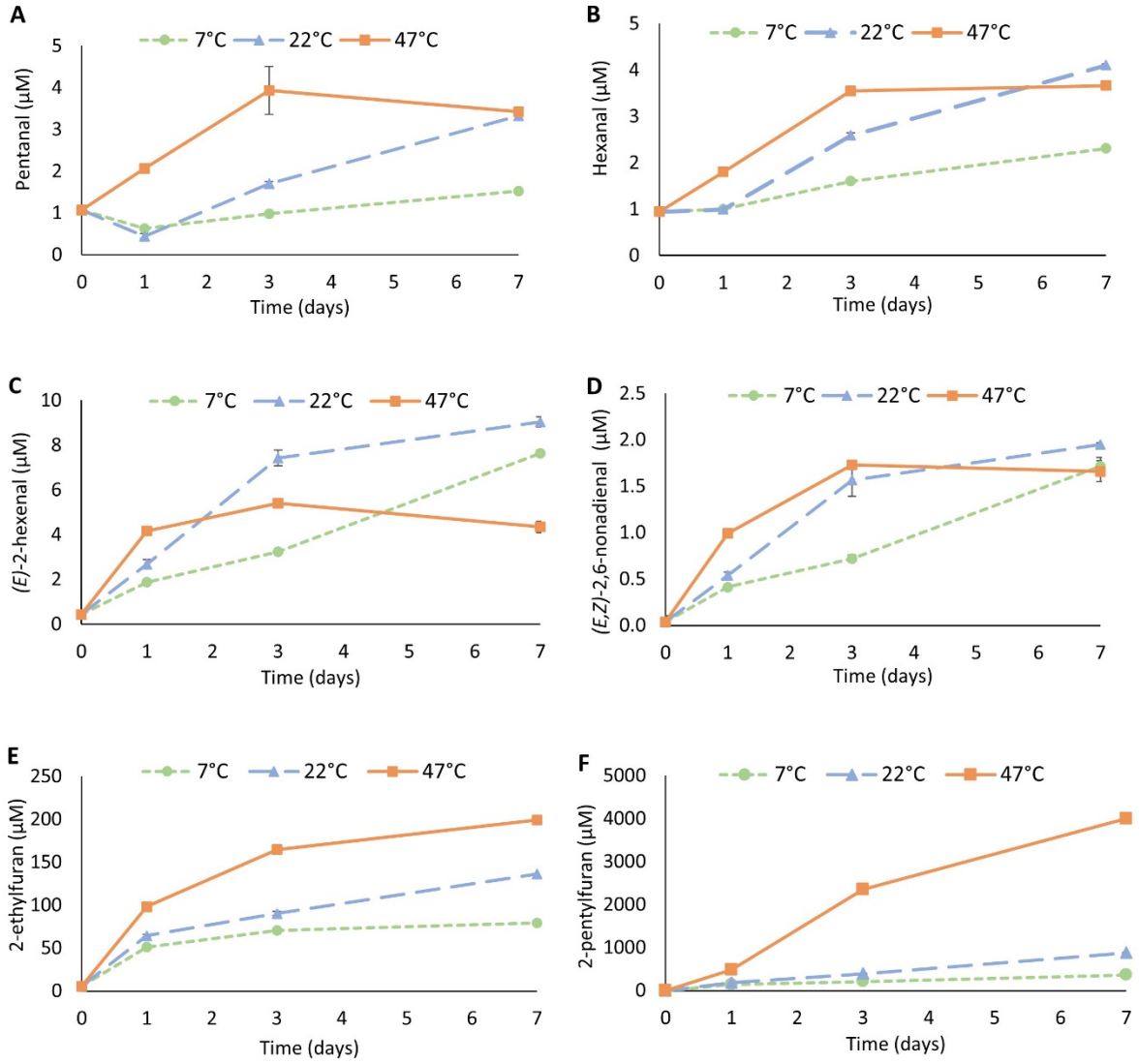

Figure 18. Effect of temperature and time on the saturated aldehydes pentanal (A), hexanal (B); the unsaturated aldehydes (E)-2-hexenal (C), (E,Z)-2,6-nonadienal (D), and the non-enzymatic browning reactions-derived volatiles 2-ethylfuran (E), 2-pentylfuran (F). Herring co-products from batch 1 were used here.

The more pronounced formation of products from lipid oxidation and non-enzymatic browning at 47°C than at 22°C and 7°C was also supported by a rapid consumption of the endogenous antioxidant α-tocopherol at 47°C (**Figure 19**). This suggests that other endogenous antioxidants present in herring co-products, and potentially antioxidative short-chain peptides [155] produced

during ensilaging were not sufficient to provide protection against lipid oxidation, calling for addition of exogenous antioxidants. Further, to limit lipid oxidation/browning, and also to minimize energy usage, it was decided to use ambient temperature for further ensilaging trials.

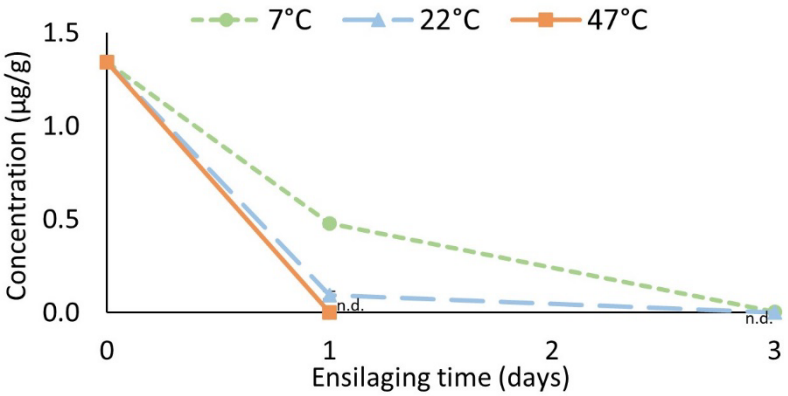

Figure 19. Effect of temperature and time on α-tocopherol consumption. n.d.: α-tocopherol was not detected after 1 day of ensilaging at 47°C and after 3 days of ensilaging at 22°C. Herring co-products from batch 1 were used here.

6.3.2 Role of Hb in lipid oxidation development during ensilaging (Paper III)

The role of Hb in lipid oxidation development during ensilaging was studied by adding Hb to herring co-products at the beginning of ensilaging. Significantly ($p < 0.05$) higher levels of PV and TBARS were noticed in Hb-fortified silages than control silages (**Figure 20**). At the end of 7 days ensilaging, the relative differences in PV´s between silages fortified with 2- and 3-fold higher levels of Hb and their respective controls were 15.9% and 29.8%, respectively. Further, a higher absolute level of PV was noticed in the silage fortified with a 2-fold higher Hb-level than that fortified with 3-fold higher Hb, i.e. 15.6 vs 11.7 meq O_2/kg silage, respectively. Contrary, a higher TBARS level was recorded in 3-fold Hb-fortified silages than the 2-fold Hb-fortified silage after 7 days of ensilaging, i.e. 1954.9 vs. 1777.0 µmole TBARS/kg silage, respectively. Also, the relative difference in TBARS values at the end of ensilaging, compared to the control, was higher for the former silage, i.e. 137.6% vs. 66.5% for the 3-fold vs. 2-fold Hb-fortified silage, respectively.

It was evident from this study that Hb fortification primarily resulted in an increased TBARS, with a smaller effect on PV, confirming the described ability of Hb and heme to react with lipid hydroperoxides generating ferryl heme protein radicals, lipid radicals and hydroxyl radicals [156], which altogether prevented a pronounced buildup of peroxides. The ratio between the maximum TBARS values reached during the 7 days ensilaging and the Hb levels added was 16.0 ± 2.2 µmole TBARS/µmole Hb tetramer (range 13.3 – 18.6, *n*=8), which was similar to previously reported studies in fish muscle, i.e. 13.6 ± 4.8 µmole TBARS/µmole Hb tetramer (range 6.4 – 24, *n* =15) [126,157]. This shows that the Hb/heme level present in the system strongly influences oxidation development, and thus can be a basis for predicting the degree of oxidation in both fish muscle and silage derived thereof.

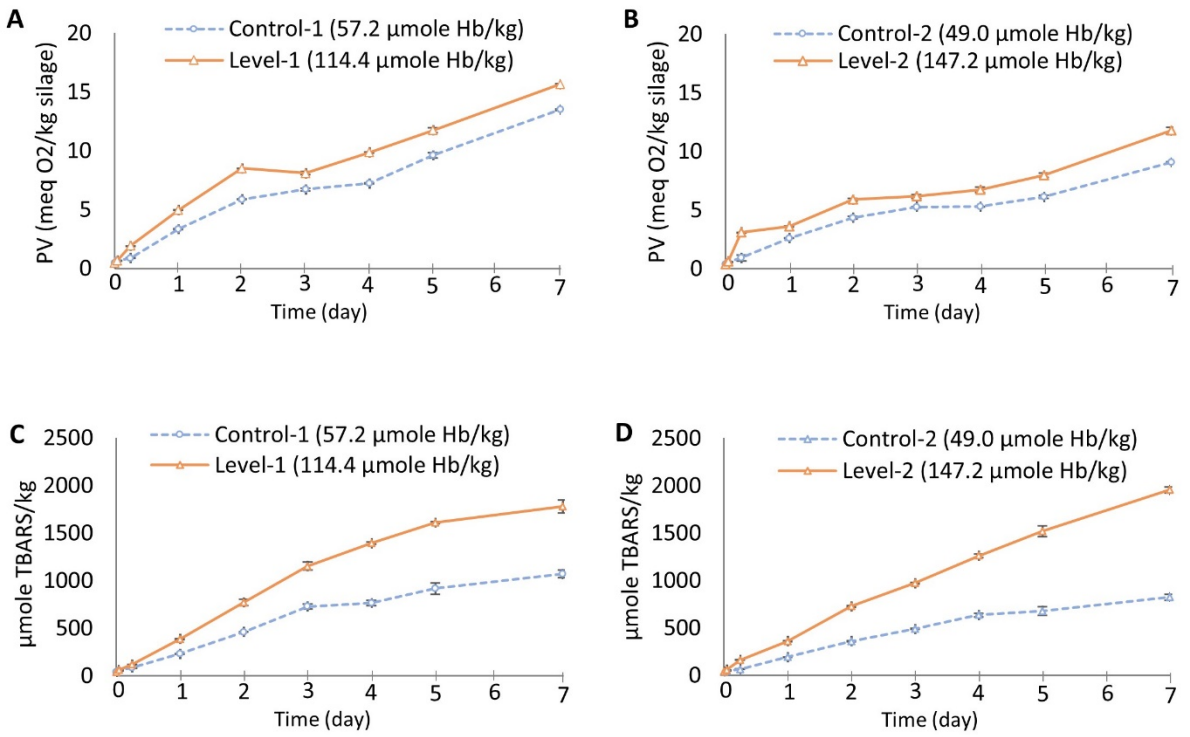

Figure 20. Effect of Hb fortification on PV (A-B) and TBARS (C-D) development during ensilaging of herring filleting co-products at 22°C. Controls refer to samples without Hb fortification, but with an equal amount of buffer added to compensate for the dilution caused by hemolysate. Time point zero (0) refers to the sample before ensilaging. Herring co-products from batch 3 were used here, and their endogenous Hb-level was 68.6 µmole Hb/kg. Control-1 and 2 contained 57.2 and 49.0 µmole Hb/kg silage, respectively; and, level-1 and 2 contained 114.4 and 147.2 µmole Hb/kg silage, respectively. Results are expressed as mean ± SEM (n = 2).

It was evident from this study that Hb fortification resulted in an increased TBARS; the effect on PV was however much smaller, suggesting the possibility of Hb and heme to react with lipid hydroperoxides generating ferryl heme protein radicals, lipid radicals and hydroxyl radicals [156], which altogether prevented a pronounced buildup of peroxides. The ratio between the maximum TBARS values reached and the Hb levels added was 16.0 ± 2.2 µmole TBARS/µmole Hb tetramer (range 13.3 – 18.6, *n*=8), which was similar to previously reported work, i.e. 13.6 ± 4.8 µmole TBARS/µmole Hb tetramer (range 6.4 – 24, *n* =15) [126,157]. This strongly suggests that Hb/heme level present in the system controls oxidation development, and thus can be a basis for predicting the degree of oxidation in both fish muscle and silage derived thereof.

The role of Hb in lipid oxidation development was further examined using a simple trout hemolysate model system simulating ensilaging conditions by adjusting the pH to 3.5. The bright red color of trout hemolysate changed into brown immediately after acidification, suggesting a change of the native oxygenated state of Hb (i.e. oxyHb) first to deoxyHb (Root effect) and then to metHb, something which was also confirmed by spectral changes (**Figure**

21A). The appearance of a peak around 375 nm further suggests release of the heme group (**Figure 21B**), and the subsequent gradual decrease of this peak over time suggests degradation of the heme group yielding release of free iron. It has been reported that heme release from Hb is around 60 times faster from metHb form than oxyHb and deoxyHb [84], which thus supports the significant ($p < 0.05$) increase in heme group release from the Hb immediately after appearance of the acid-induced shift in Hb spectra from oxyHb to metHb. The rapid increase in PV, followed by increased TBARS, in Hb-fortified silages is thus most likely linked to sequential acid-induced Hb-changes going via deoxygenation, heme-group exposure, autoxidation and subsequent heme-loss [158]. The protein content in the hemolysate model system remained constant throughout the studied period (data not shown), suggesting that Hb did not precipitate during ensilaging. Therefore, it is suggested that the likely mechanism by which Hb promoted lipid oxidation during ensilaging was via heme-mediated peroxide cleavage.

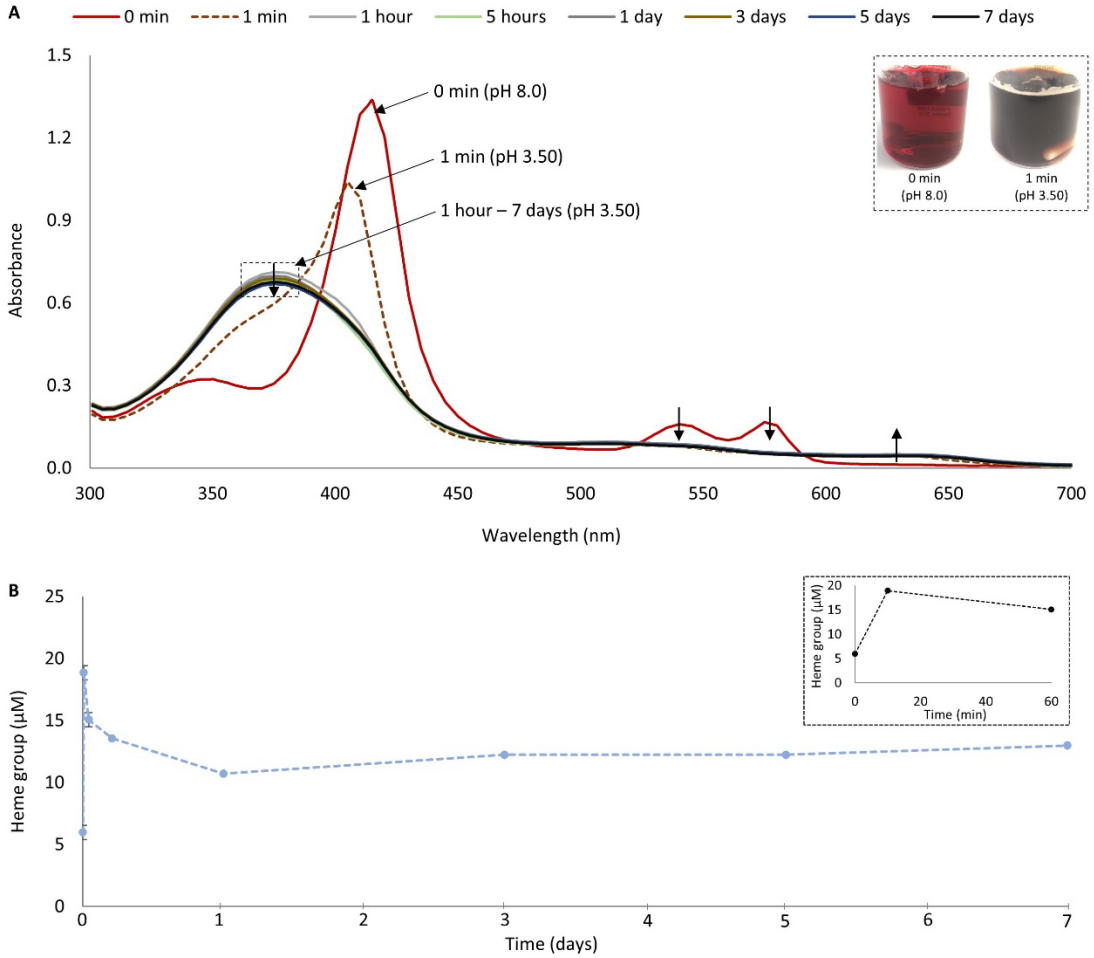

Figure 21. Effect of adjusting a solution of trout Hb (68.6 µmole/kg) in 0.1 M Tris buffer from pH 8.0 to pH 3.5 on shift in Hb absorption spectra (A) and heme group release (B) over time at 22°C. The insets in Fig. A and Fig. B show the changes in Hb color from red to dark brown and heme group release from the Hb, respectively, upon adjusting pH from 8.0 to 3.5. Time point zero (0) refers to a sample before adjusting pH to 3.5 (i.e. pH 8.0). Results are expressed as mean ± SEM (n = 2).

6.3.3 Use of antioxidants to minimize lipid oxidation before and during ensilaging (Paper III-IV)

6.3.3.1 Pre-incubating the co-products in antioxidant solutions to stabilize them against lipid oxidation (Paper III)

To simulate conditions where herring co-products need to be transported to another plant for ensilaging, or where they need a temporary holding time, the possibility of pre-incubating the co-products in different treatment solutions to limit oxidation was investigated. Incubating herring co-products for 20 min in physiological salt solution (i.e. 0.9% NaCl), to prevent the lysis of erythrocytes [159], has previously been shown to remove 6.6 – 18.0% Hb. The exact amount removed varied with the specific co-product part, i.e. 6.6, 10.3, 17.9, and 18.0% were removed from fin, head, backbone, and residuals, respectively [13]. Therefore, we hypothesized that removing Hb from the outer layer of the co-products, with or without covering this layer by antioxidants, could minimize lipid oxidation both before and during ensilaging. Incubation in 3.0% NaCl, simulating seawater, was also investigated since this is commonly used during industrial pre-storage of herring. Results suggest that the slight removal of pro-oxidative Hb by pre-incubation in 0.9% NaCl was counteracted by a simultaneous loss of endogenous α-tocopherol during the incubations (see **paper III**), thus, oxidation was not efficiently prevented. Using 3% NaCl, even a pro-oxidative effect was seen.

Therefore, it was decided to add antioxidants to the treatment solutions, in which the co-products were then incubated for 30 s or 20 min. The commercial rosemary extract-based antioxidant mixture Duralox MANC-213, also containing citric acid, ascorbic acid and α-tocopherol, as well as rosemary extract provided the best TBARS inhibition both pre-ensilaging and after 7 days of ensilaging when added at 5 and 2%, respectively (**Table 5**). Considering the need to label less ingredients, a short pre-incubation (30 sec) in 0.5% rosemary extract was proposed as the most promising strategy if the co-products cannot be ensilaged immediately. The carnosic acid – one of the most active antioxidative compounds of rosemary extract – level in the rosemary extract was much higher than in Duralox MANC-213 (57.84 ± 1.54 vs. 1.55 ± 0.06 mg/g), explaining its high activity even in the absence of other antioxidants such as those present in the Duralox MANC-213 mixture (i.e. ascorbic acid, citric acid and tocopherol). Also, its TPC was higher, but to a much smaller extent (102.53 ± 0.04 vs 69.63 ± 2.29 mg gallic acid eq./g), revealing that other phenolics than carnosic acid played a dominant role in Duralox MANC-213. Overall, the fact that the same incubation solution could be reused for up to 10 times without losing its activity, paves the way for a scalable and easily applicable technology to stabilize the herring filleting co-products against lipid oxidation *per se*, but also to produce a value-added product from them via ensilaging.

Table 5. Effect of pre-incubating the co-products in different treatment solutions on TBARS during the actual incubation and during subsequent ensilaging for 7 days. MANC: Duralox MANC-213. The ratios 5:1, 2:1 and 3:1 refer to the solution to co-product ratios used in different treatments. Herring co-products from batch 2, 3, 4 and 5 were used in trials 1, 2, 3, and 4, respectively.

Trial	Treatments	µmole TBARS/kg	
		After incubation	In final silage
1	**Control (i.e. no incubation)**	**6.3**	**114.6**
	0% NaCl (5:1 ratio); 30 sec	16.7	92.7
	0.9% NaCl (5:1 ratio); 30 sec	21.2	107.9
	3.0 % NaCl (5:1 ratio); 30 sec	10.7	115.6
	0% NaCl (5:1 ratio); 2 h	20.2	107.7
	0.9% NaCl (5:1 ratio); 2 h	19.3	104.5
	3.0 % NaCl (5:1 ratio); 2 h	124.0	97.5
2	**Control (i.e. no incubation)**	**62.2**	**423.8**
	Tap water (5:1 ratio); 20 min	80.3	380.3
	0.9% NaCl in Tap water (5:1 ratio); 20 min	56.4	360.9
	5% MANC in Tap water (5:1 ratio); 20 min	12.8	123.1
	0.2% isoascorbic acid with 0.04% EDTA in 0.9% NaCl (5:1 ratio); 20 min	19.1	269.5
	0.2% isoascorbic acid with 0.04% EDTA in tap water (5:1 ratio); 20 min	19.8	278.8
3	**Control (i.e. no incubation)**	**27.2**	**287.0**
	Tap water (5:1 ratio); 20 min	23.1	216.1
	2% MANC in tap water (5:1 ratio); 20 min	2.9	4.2
	2% Isoascorbic acid in tap water (5:1 ratio); 20 min	7.0	83.9
	2% MANC in tap water (5:1 ratio); 20 min; prolonged storage at 4°C for 24 h	5.4	8.0
4	**Control (i.e. no incubation)**	**13.0**	**147.5**
	0.5% Rosemary extract in 0.9% NaCl; 1st incubation (2:1 ratio); 30 sec	9.0	10.2
	0.5% Rosemary extract in 0.9% NaCl; 4th incubation (2:1 ratio); 30 sec	11.1	12.3
	0.5% Rosemary extract in 0.9% NaCl; 10th incubation (2:1 ratio); 30 sec	11.6	17.3
	0.5% Rosemary extract in 0.9% NaCl; 10th incubation (3:1 ratio); 30 sec	3.3	16.2
	2% Isoascorbic acid in 0.9% NaCl; 1st incubation (2:1 ratio); 30 sec	2.53	46.4
	2% Isoascorbic acid in 0.9% NaCl; 4th incubation (2:1 ratio); 30 sec	4.2	96.3

6.3.3.2 Direct antioxidant addition at the start of ensilaging (Paper IV)

To simulate the case where ensilaging can be performed on site at filleting plant, antioxidants were directly added to the co-products at the beginning of ensilaging. In a comparison among isoascorbic acid and several rosemary extract-based antioxidants, we noticed that Duralox MANC-213 provided the best protection against both PV and TBARS development during 0-7 days ensilaging at 22°C (**Figure 22** and **Figure 23**). Increasing the Duralox-MANC concentration from 0.25% to 0.75% and 1.25% lowered PV values from 0.4 to 0.2 and 0.06 meq O_2/kg silage, respectively, and TBARS values from 43.5 to 25.1 and 18.0 μmole TBARS/kg silage, respectively, after 7 days ensilaging (**Figure 22**).

The rosemary extract component of Duralox MANC-213 contains several phenolic compounds e.g. rosmarinic acid, carnosic acid, and carnosol; the latter two which have been reported to contribute to more than 90% of antioxidative properties of such extracts [160]. These two components are efficient radical scavenging o-diphenols [59,161]. Further, Frankel, et al. [162] reported that carnosic acid and carnosol provide better antioxidative protection at pH 4.0 than pH 7.0, as examined in a corn oil-in-water emulsion oxidized at 60°C for 4 days. Carnosol can also work as a LOX inhibitor [163]. The citric acid of Duralox MANC-213 provides protection by chelating metal ions [59] and the ascorbic acid by scavenging free radicals. The latter also provide excellent synergism with e.g. citric acid, tocopherol, and metal chelators [65]. Further, we (Wu, et al. [164]; not included in thesis) reported that Duralox MANC-213 specifically prevented Hb autoxidation and heme-loss, which according to above is an important antioxidative mechanism in the herring silage. The multiple mechanisms by which Duralox MANC-213 can inhibit oxidation in silage are further enhanced by the fact that it contains both hydrophilic and lipophilic antioxidants, which aids its partitioning both into the oil phase and to the oil-water interface under acidic ensilaging conditions [162].

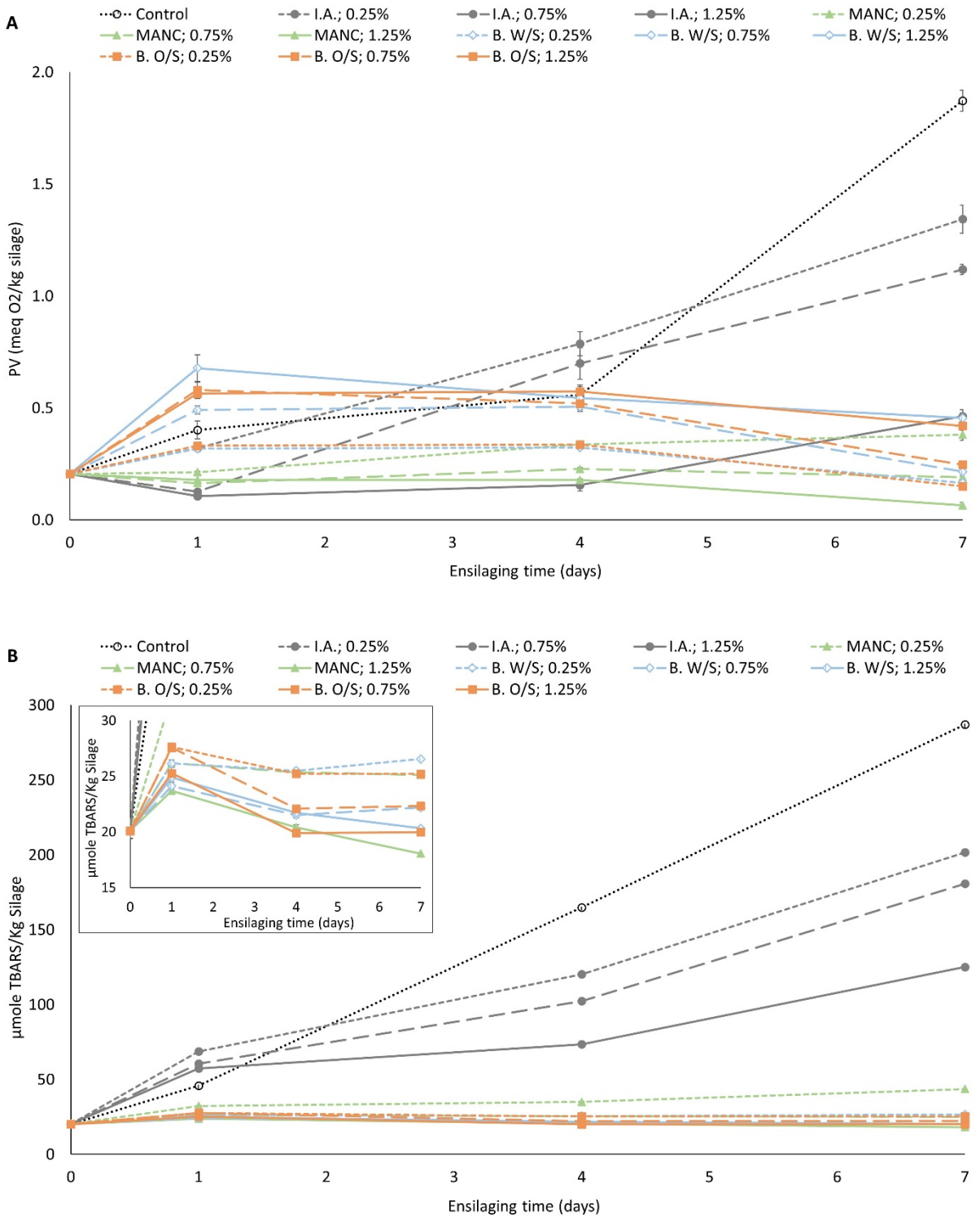

Figure 22. Effect of antioxidants in different concentrations on PV (A) and TBARS (B) during ensilaging of herring filleting co-products at 22°C. Control refers to silage without any antioxidant addition; and, time point zero (i.e. day 0) refers to minced herring co-products before ensilaging. Herring co-products from batch 4 were used here. The inset in Fig. B shows the differences in TBARS values in the range 15-30 µmole TBARS/kg silage. Results are expressed as mean ± SEM (n = 3). I.A.: isoascorbic acid; MANC: Duralox MANC-213; B. W/S: BORDANTIX W/S; B. O/S: BORDANTIX O/S.

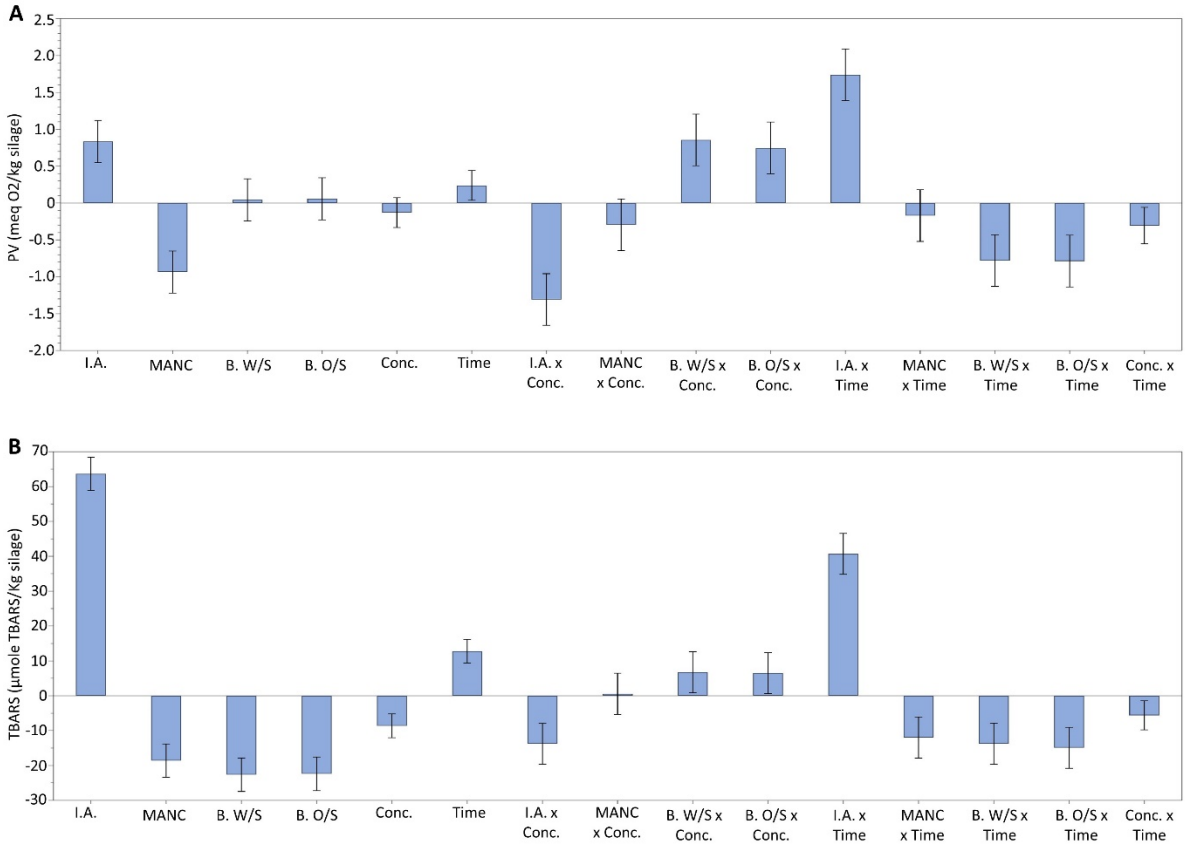

Figure 23. Coefficient plots showing responses from antioxidants in different concentrations on PV (A) and TBARS (B) during 0-7 days of ensilaging of herring filleting co-products at 22°C. Herring co-products from batch 4 were used here. Responses were scaled and centered; and, the size of coefficients represents the change in their respective responses when a factor varies from medium to high level, while keeping other factors at their average values. I.A.: isoascorbic acid; MANC: Duralox MANC-213; B. W/S: BORDANTIX W/S; B. O/S: BORDANTIX O/S; Conc.: concentration.

6.3.3.3 Effect of antioxidants on lipid oxidation inhibition during heat treatment of silages (Paper IV)

Silage needs to be heat treated to stop hydrolysis, inactivate thiaminases, and mitigate pathogen risks. However, it is well-known that heat-treatment can affect the status and activity of many pro-oxidants; for example, heme or low molecular weight transition metal ions (iron) might be released from proteins during heat-treatment and catalyze lipid oxidation [165]. The effect of heat-treating the silage at 85°C for 30 min on PV and TBARS development is shown in **Figure 24**. Among the three rosemary extract-based antioxidant studied, Duralox MANC-213 provided the best protection against both PV and TBARS development. There were no significant ($p > 0.05$) changes in PV after heat-treatment of silages containing 0.75% and 1.25% Duralox MANC-213. Also, no significant ($p > 0.05$) change in TBARS was noticed in silage containing 1.25% Duralox-MANC; however, significantly ($p < 0.05$) lower TBARS was noticed after heat-treatment of the silage containing 0.75% MANC.

The lower PV′s of silages after heat-treatment could be due the fact that peroxides are temperature sensitive and break down in presence of released heme/hemin **(Paper II)**. The increasing trend for PV after heat treatment can be explained by the fact that some peroxides are also formed during heat-treatment and/or that peroxide breaking species, such as heme, were stabilized by phenolic compounds [166,167]. The lower TBARS values after heat-treatment could be due to heat-induced breakdown of MDA and/or reaction between MDA and amino acids/peptides/ proteins forming non-enzymatic browning products **(Paper II)**, the latter which also can provide antioxidative protection [75]. Overall, silages with lower PV and TBARS before heat-treatment, which translated into silages containing higher antioxidant concentrations, had relatively lower PV and TBARS after heat-treatment, suggesting that adequate antioxidant amounts should be added at the start of ensilaging to provide better protection in later process steps.

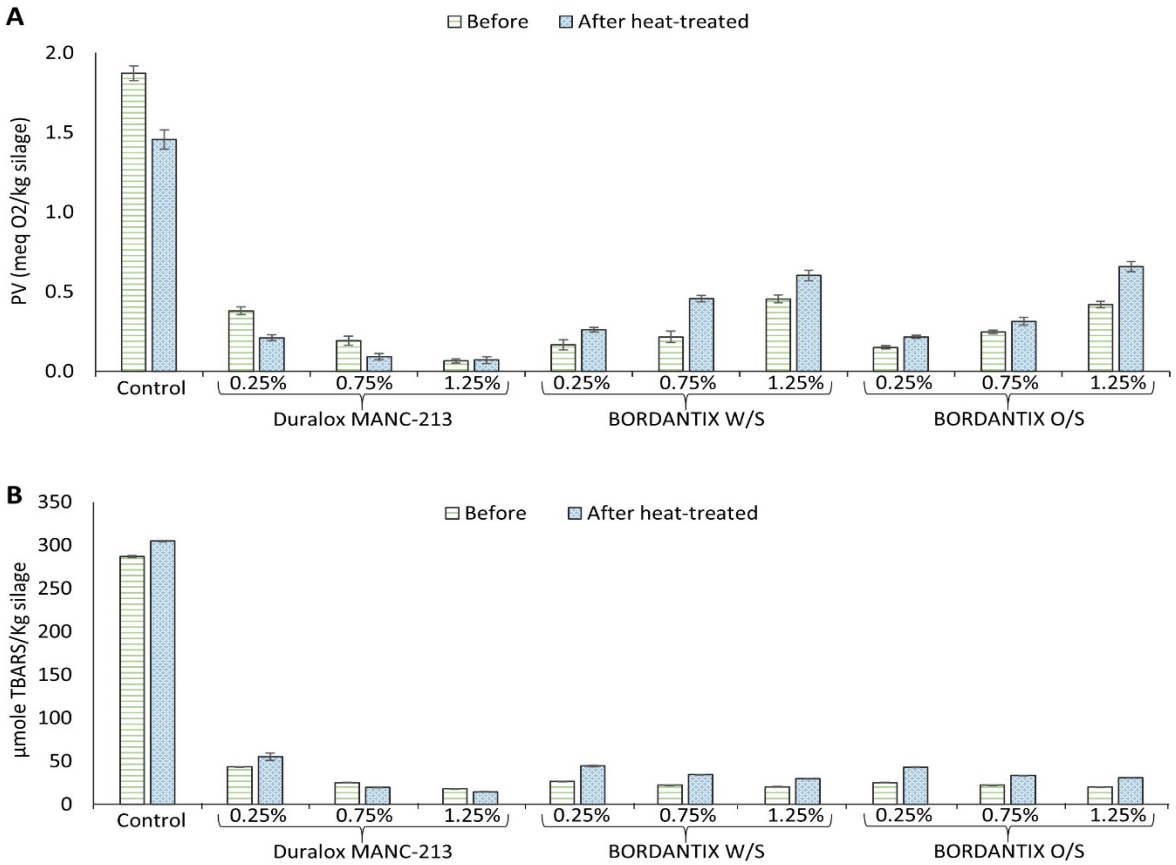

Figure 24. Effect of antioxidants in different concentrations on PV (A) and TBARS (B) before and after heat-treating the silage at 85°C for 30 min. Control refers to silage without any antioxidant addition. Herring co-products from batch 4 were used here. Results are expressed as mean ± SEM (n = 3).

6.3.3.4 Effect of antioxidant concentrations on lipid oxidation inhibition during storage of silages (Paper IV)

Silage might need to be stored for some time until further processing, which might affect its oxidative quality. Therefore, silages with Duralox MANC-213 concentrations at 0.25-1.25% levels were taken further to a 6-month storage trial at 4°C and 22°C (**Figure 25**). TBARS values increased significantly ($p < 0.05$) with storage time, but significantly ($p < 0.05$) lower TBARS values were noticed in silages containing higher Duralox MANC-213 concentrations. Heat-treatment at 80°C for 5 min has been reported to completely inactivate LOX in lake herring (*Coregonus artedi*) [168], and as mentioned above, carnosol of rosemary extracts can work as LOX inhibitor [163]; therefore, lower TBARS values were expected in Duralox MANC-213-containing heat-treated silages, compared to non-heat-treated ones. However, there was no difference in TBARS between heat-treated and non-heat-treated silage, suggesting that LOX most likely was not a major prooxidant in herring silages, which in further reinforces by the different pH-optima reported for fish LOX (pH 7.5-10) [63,64]. Based on the PLS analysis, no significant ($p > 0.05$) effects of heat-treatment and storage temperature on TBARS were noticed; rather, the storage time *per se* played the most important role in TBARS development. Further, higher Duralox MANC-213 concentrations provided better oxidative stability over storage, suggesting that antioxidants are consumed over time **(Paper II)**.

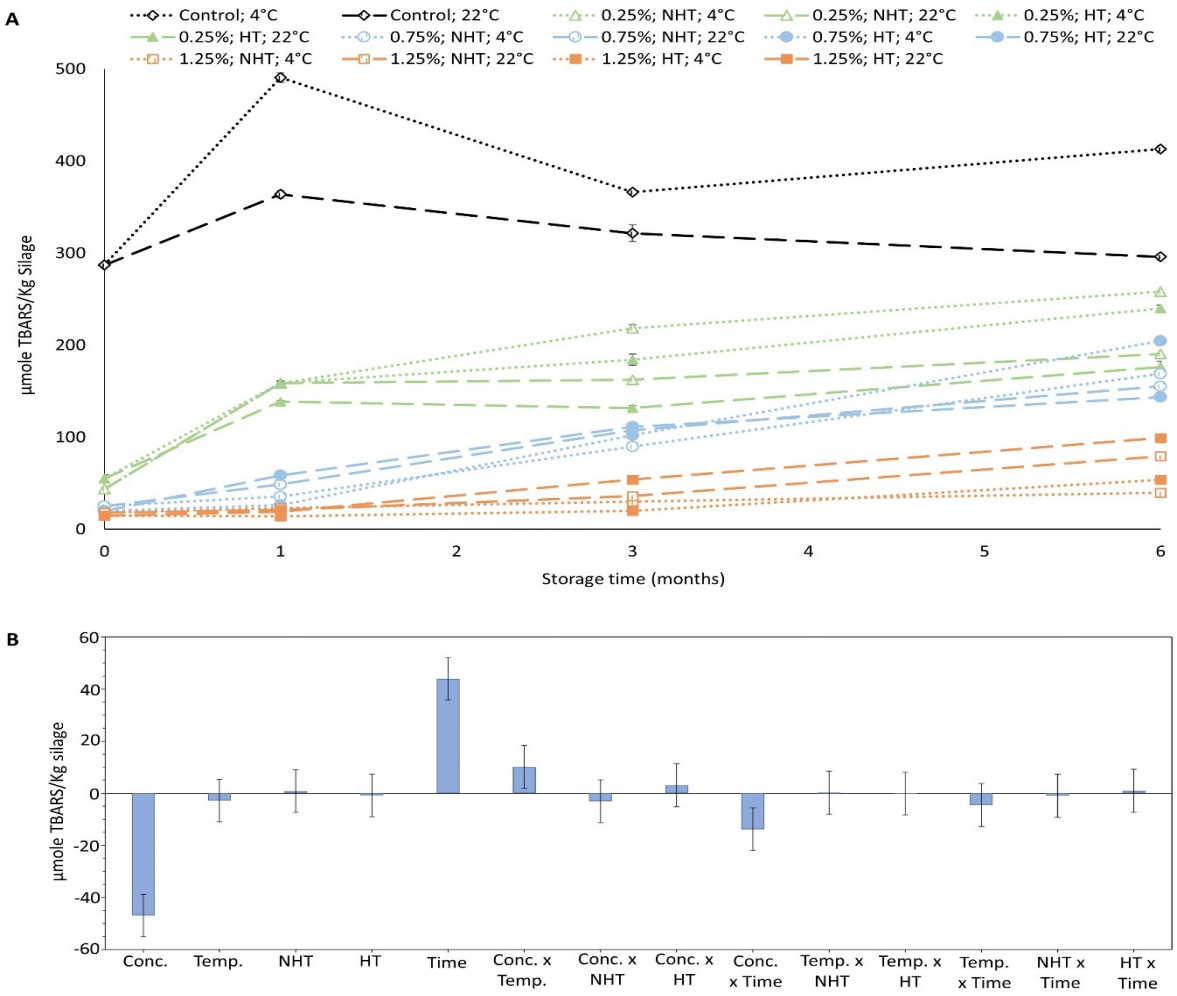

Figure 25. Effect of storage temperature, time, heat-treatment, and Duralox MANC-213 concentrations on TBARS (A-B). Control refers to silage without any antioxidant addition. Herring co-products from batch 4 were used here. Results are expressed as mean ± SEM (n = 3). Coefficient plot (B) refers to data presented in panel A, and shows responses of Duralox MANC-213 concentrations, heat-treatment, storage temperature and time on TBARS. Responses were scaled and centered; and, the size of coefficients represents the change in their respective responses when a factor varies from medium to high level, while keeping other factors at their average values. NHT: non-heat-treated; HT: heat-treated; Conc.: concentration; Temp.: temperature.

6.4 Development of total volatile basic nitrogen (TVB-N) in silages (Paper IV)

TVB-N is widely used as a quality indicator of fish and fish products. It measures degradation of proteins and non-protein nitrogenous compounds into volatile amines such as trimethylamine (TMA) and NH_3 [169]. In general, TVB-N values increased significantly ($p < 0.05$) both with time and a higher storage temperature (**Figure 26**), which agreed with earlier studies [109,169]. Higher TVB-N values were noticed in non-heat-treated silage than heat-treated ones stored at 22°C. This was possibly due to continued autolysis leading to the formation of NH_3 by deamination of amide-N groups containing amino acids such as asparagine and glutamine into NH_3 [109]. The recorded TVB-N values in this study were lower than previously reported TVB-N values in silages prepared from different combinations of plaice (*Pleuronectes platessa*), sole (*Solea solea*), flounder (*Platichthys flesus*), and whiting (*Merlangius merlangus*) [109]. Overall, TVB-N values of silages from all treatments were well below the acceptable limit of 30 mg TVB-N per 100 g fish for human consumption [169], even after 6 months storage at both 4°C and 22°C. This provides high flexibility regarding transportation of silage from an ensilaging plant to plants for further value-adding processing.

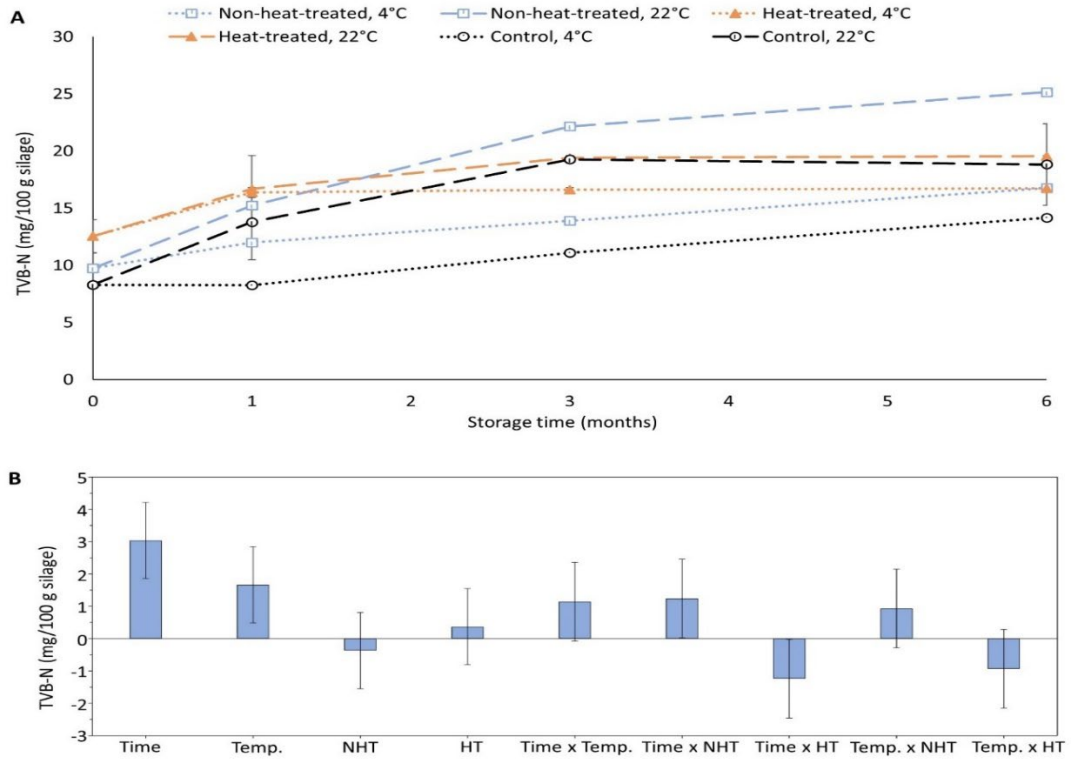

Figure 26. Effect of heat-treatment, storage temperature and time on TVB-N. Silage samples contain 0.75% w/w Duralox MANC-213 (A-B). Control refers to silage without any antioxidant addition. Herring co-products from batch 4 were used here. Results are expressed as mean ± SEM (n = 2). Coefficient plot (B), referring to data presented in panel A, shows TVB-N responses to heat-treatment, storage temperature and time. Responses were scaled and centered; and, the size of coefficients represents the change in their respective responses when a factor varies from medium to high level, while keeping other factors at their average values. NHT: non-heat-treated; HT: heat-treated; Temp.: temperature.

6.5 Separation of silage into multiple products - a biorefinery approach (Paper V)

The effect of heat-treating the silage at 85°C for 30 min, centrifugation g-force, and time on recovery yields of oil, hydrolysates, and solids is shown in **Table 6**. Heat-treating the silage resulted in a significantly ($p<0.05$) higher oil and hydrolysates recovery at all g-forces, compared to the non-heat-treated silage. Increasing the g-force had the same effect while increasing the centrifugation time only increased the recovery of hydrolysates phase. At 3000, 4500 and 8500 x g, the oil recovery was 6.5, 8.3 and 9.7% (w/w), respectively and increasing centrifugation time from 2 to 10 and 20 min increased oil recovery from 4.9 to 6.6 and 8.3% (w/w), respectively. The recovered oil yield was similar, or in some cases higher, than the oil recovery reported from alkaline pH-shift processing or conventional heat-based separation of herring co-products; 0.5 and 6% (w/w), respectively [131]. The interaction effects of the studied factors on yields were very small compared to the main effects, i.e., g-force, centrifugation time and heat treatment (**Figure 27**). Overall, the gain in oil recovery with increasing g-force from 4500 x g to 8500 x g was very small, i.e. 8.3% vs 9.7%; thus, considering industrial applicability, it was decided to continue further with 4500 x g for 20 min.

Table 6. Recovery yield of phases after centrifugation of non-heated/heated silage at different times and g-forces. Herring co-products from batch 6 were used here.

			Yield (%; w/w)			
g-force	**Time**	**Treatment**	**Oil (%)**	**Emulsion (%)**	**Hydrolysates (%)**	**Solids (%)**
3000	2	Non-heat	1.3 ± 0.0	12.4 ± 1.6	1.7 ± 0.2	84.6 ± 1.7
		Heat	5.7 ± 0.7	19.9 ± 1.4	9.4 ± 0.4	64.9 ± 1.6
	10	Non-heat	0.4 ± 0.0	14.9 ± 0.8	27.2 ± 0.4	57.5 ± 0.9
		Heat	5.5 ± 0.2	15.7 ± 0.8	38.2 ± 0.9	40.5 ± 1.1
	20	Non-heat	0.6 ± 0.0	14.45 ± 0.6	34.6 ± 0.3	50.3 ± 0.7
		Heat	6.5 ± 0.2	14.7 ± 0.7	40.5 ± 0.5	38.2 ± 0.6
4500	2	Non-heat	0.1 ± 0.0	33.1 ± 1.3	9.4 ± 0.3	57.4 ± 1.5
		Heat	4.9 ± 1.0	25.1 ± 1.4	29.4 ± 0.4	40.6 ± 1.5
	10	Non-heat	0.4 ± 0.0	23.2 ± 0.5	33.0 ± 0.2	43.3 ± 0.4
		Heat	6.6 ± 1.0	12.1 ± 0.6	45.4 ± 1.0	35.9 ± 0.8
	20	Non-heat	2.1 ± 0.1	16.0 ± 1.9	40.2 ± 0.3	41.7 ± 1.9
		Heat	8.3 ± 0.4	10.4 ± 1.0	47.1 ± 0.5	34.2 ± 0.7
8500	2	Non-heat	5.9 ± 0.8	9.6 ± 1.3	17.9 ± 0.3	66.6 ± 0.8
		Heat	9.0 ± 0.1	9.5 ± 0.3	42.1 ± 0.6	39.4 ± 0.4
	10	Non-heat	3.7 ± 0.1	11.7 ± 1.5	42.1 ± 0.9	42.4 ± 0.6
		Heat	9.5 ± 0.2	5.9 ± 0.7	51.7 ± 0.5	32.8 ± 1.0
	20	Non-heat	4.1 ± 0.0	9.2 ± 1.1	48.1 ± 0.1	38.6 ± 1.1
		Heat	9.7 ± 0.0	5.9 ± 0.6	53.1 ± 0.2	31.3 ± 0.3

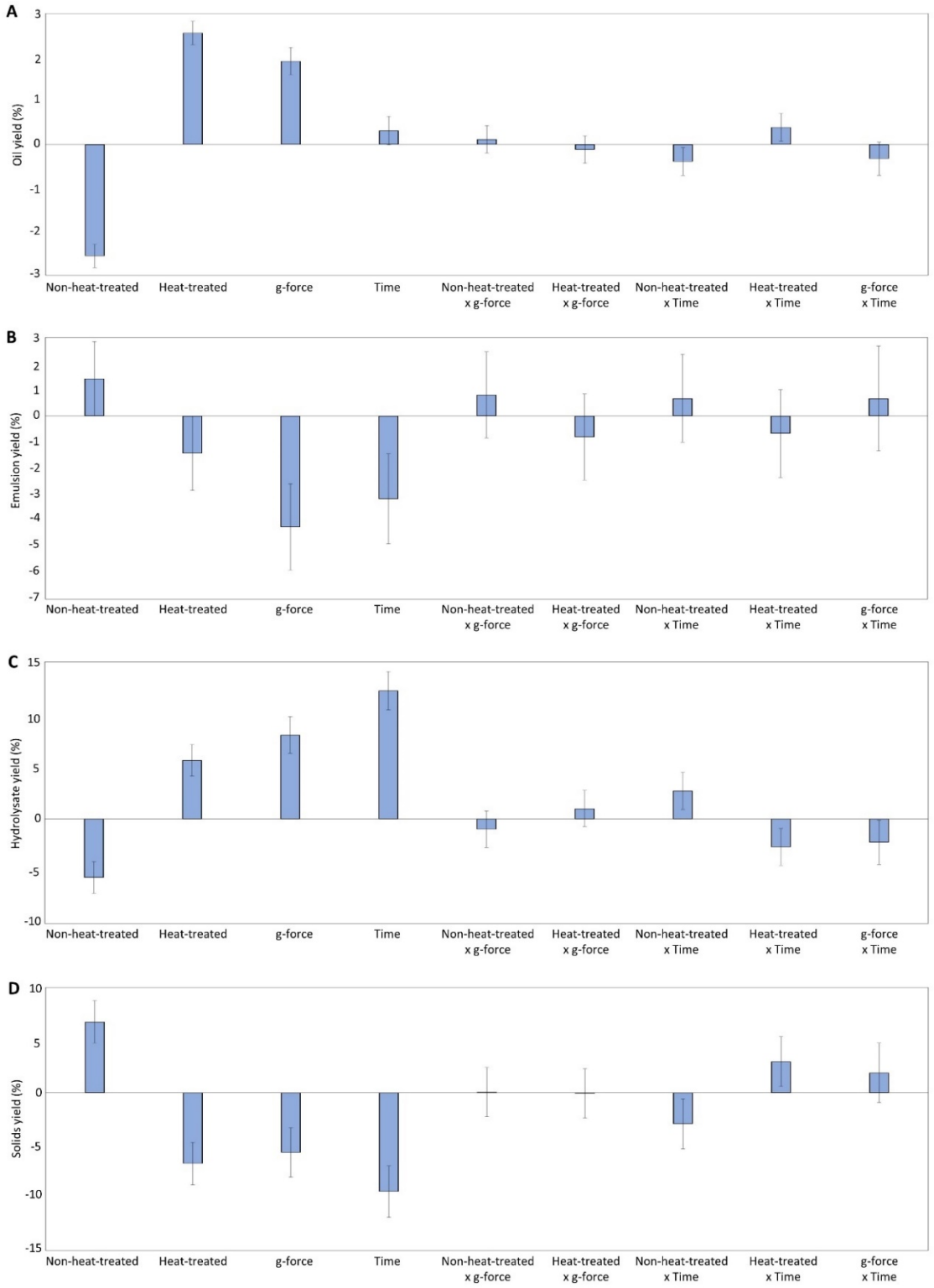

Figure 27. Coefficient plots showing responses of studied factors (i.e. heat-treatment, g-force, and centrifugation time) on yield of oil (A), emulsion (B), hydrolysates (C), and solids (D). Responses were scaled and centered; and, the size of coefficients represents the change in their respective responses when a factor varies from medium to high level, while keeping other factors at their average values. Herring co-products from batch 6 were used here.

The microscopic images of the emulsion phase formed between oil and hydrolysates phases after centrifugation at 4500 x g for 20 min is shown in **Figure 28**. The observed fewer oil droplets in the emulsion phase from the heat-treated silage could be due to destabilization of the emulsion by e.g. protein aggregation during heat treatment, which resulted in fewer oil droplets in the emulsion phase and thus facilitated a higher oil recovery after centrifugation. The observed larger oil droplets in the emulsion phase are possibly separated oil redispersed in the emulsion and/or droplets formed by coalescence. The latter is a phenomenon continuously happening in all emulsions at different rates destabilizing emulsions and causing phase separation [170].

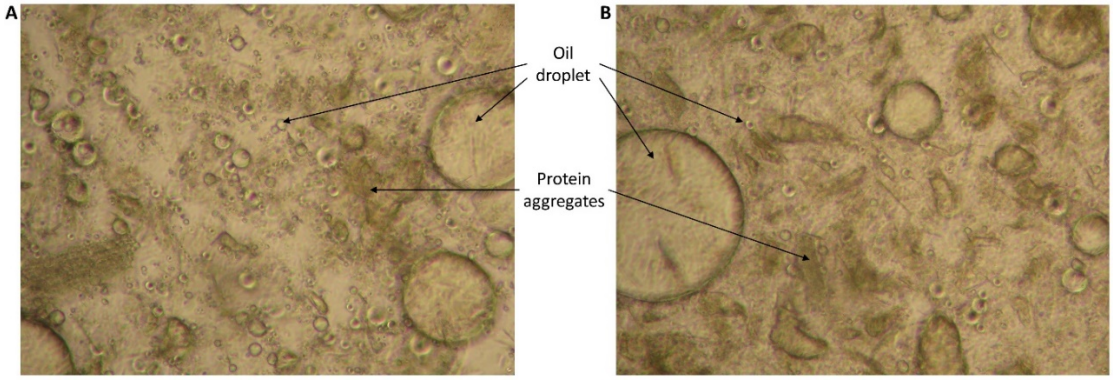

Figure 28. Light microscopy images of emulsion formed at the oil-hydrolysates interface after centrifugation. Centrifugation was performed at 4500 x g for 20 min. A: Non-heat-treated silage; B: Heat-treated silage. Herring co-products from batch 6 were used here.

The proximate composition of herring co-product, silage, and phases derived thereof after centrifugation at 4500 x g for 20 min is presented in **Table 7**. The small dilution of the co-product by acid and antioxidants at start of the ensilaging did not create a significantly ($p > 0.05$) different composition of the silage. Significantly ($p < 0.05$) higher protein contents, were noticed in the solids compared to the silage and hydrolysates. The hydrolysates contained similar ash contents to that of the co-products, silage and solids, suggesting that a portion of the ash remains soluble in the hydrolysates phase. There was no significant ($p > 0.05$) difference between oils from heat-treated and non-heat-treated silage. The emulsion layer, however, was largely affected by heat, with a clear reduction in amount.

Table 7. Proximate composition of co-products, silage, and phases derived of silages after centrifugation. Heat treatment was done at 85°C for 30 min. Centrifugation was performed at 4500 x g for 20 min. Results are expressed as mean ± SEM (n = 3). n.a.: not applicable.

Sample type	Treatment	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
Co-products	n.a.	73.3 ± 0.3	11.1 ± 0.4	11.3 ± 1.5	2.4 ± 0.1
Silage; day: 2	n.a.	72.2 ± 0.0	11.4 ± 0.0	13.4 ± 0.3	2.3 ± 0.0
Oil	Non-heat	0.0	0.3 ± 0.0	90.3 ± 0.2	0.0
	Heat	0.2 ± 0.3	0.2 ± 0.0	92.1 ± 1.4	0.0
Emulsion	Non-heat	32.0 ± 0.0	8.4 ± 0.0	60.7 ± 0.6	1.1 ± 0.0
	Heat	62.5 ± 0.1	13.9 ± 0.0	24.2 ± 0.2	1.9 ± 0.1
Hydrolysates	Non-heat	85.0 ± 0.0	10.9 ± 0.2	0.1 ± 0.1	2.4 ± 0.2
	Heat	84.4 ± 0.0	11.0 ± 0.1	0.6 ± 0.1	2.5 ± 0.1
Solids	Non-heat	75.6 ± 0.3	15.2 ± 0.0	6.6 ± 0.0	2.6 ± 0.1
	Heat	75.5 ± 0.0	16.7 ± 0.0	4.3 ± 0.2	2.6 ± 0.0

The color of food oils is often considered as an important quality indicator [131]. Oils from both heat-treated and non-heat-treated silage had similar lightness (L* = 46-50), redness (a* = 0.3-2.6) and yellowness (b* = 30-34) values. However, the oil from heat-treated silage had slightly, but, significantly ($p > 0.05$), higher yellowness value, compared to that from non-heat-treated silage, i.e. b* values were 34.3 vs. 30.1, respectively. This could possibly be due to formation of non-enzymatic browning reaction products [146] e.g. pyrroles [74], which requires further investigation.

To investigate the oxidative stability of recovered oils, these were stored in darkness at 4°C for 0-4 months, and changes in PV, TBARS, p-AV, TOTOX, FFA, and α-tocopherol were monitored (**Figure 29**). At day 0, the recovered oils had PV, p-AV and TOTOX values in the range of 3.6-3.7 meq/kg oil, 2.5-4.0, and 9.9-11.1, respectively, which were within acceptable limits of 5 meq/kg (PV), 20 (p-AV), and 26 (TOTOX), respectively, for human consumption specified by the GOED Voluntary Monograph [171]. These values were also in agreement with oil recovered from garfish, golden mullet, and shad using solvent extraction [172]. However, all measured parameters increased significantly ($p < 0.05$) during storage at 4°C. Similar observations have also been reported by Boran, et al. [172] during storage of oil extracted with hexane from horse mackerel, shad, garfish and golden mullet at 4°C. In our study, significantly ($p < 0.05$) lower PV was recorded in oil from heat-treated silage, compared to the non-heat-treated one at the end of the 4 months storage. In case of TBARS and p-AV, however, there were no such differences. The lower PV could be due to different oxidation kinetics caused e.g. by the lower FFA and tocopherol content in this oil at start of the storage. It was also seen that the endogenous α-tocopherol was significantly ($p < 0.05$) consumed during storage, supporting the enhanced oxidation. The observed significantly ($p < 0.05$) lower FFA values in the oil from heat treated silage (4-5%), compared to the non-heat-treated one (6-7%), could be due to reduction in lipase activities [168,173,174]. Although FFA values of oils from the heated silage were within the quality guidelines of 2-5% for crude fish oil followed by industries [175], further refining of this oil might be needed before being used in food applications to also remove e.g. off-flavor compounds, pigments and oxidation products [175].

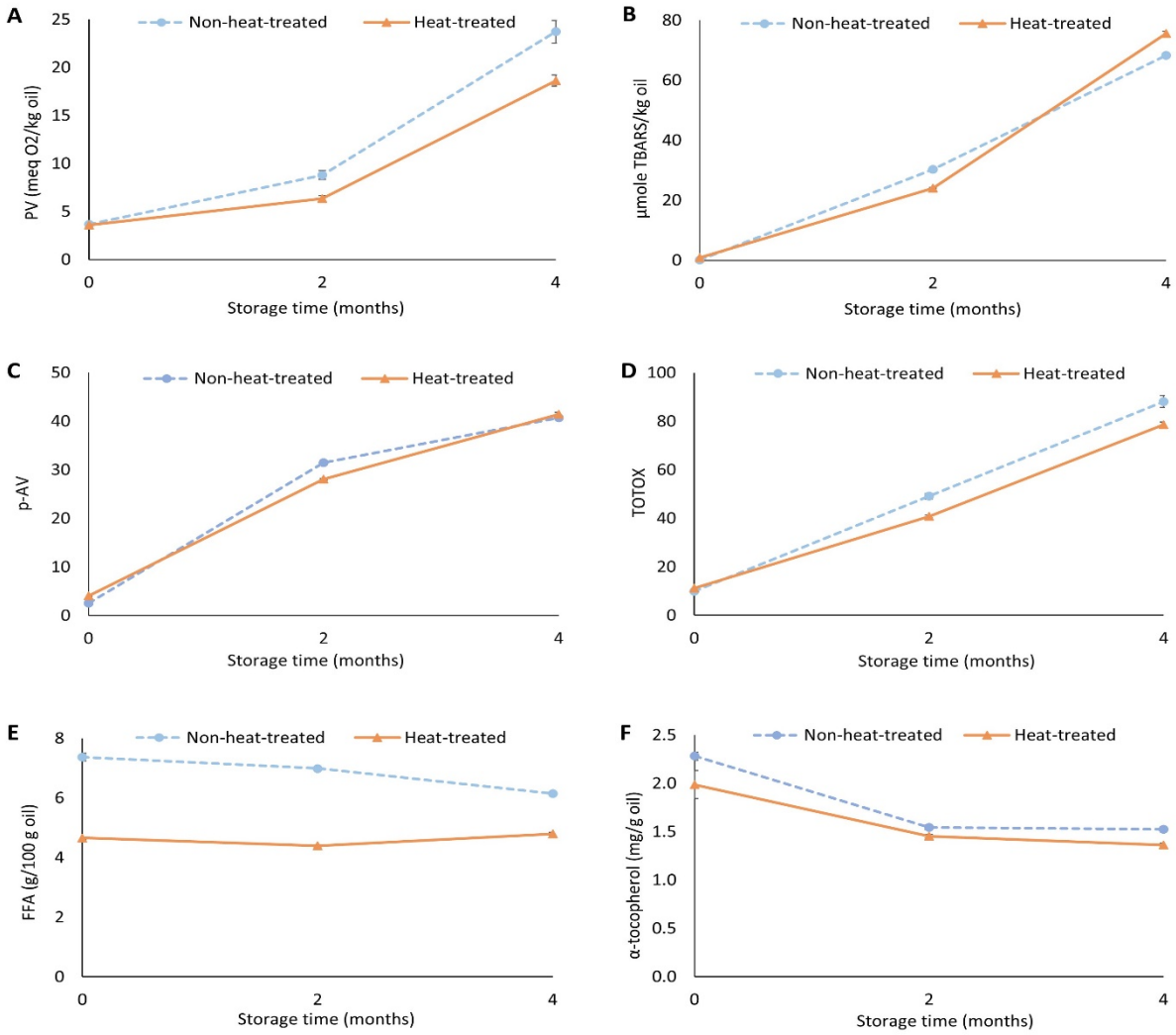

Figure 29. Changes in PV (A), TBARS (B), p-AV (C), TOTOX (D), FFA (E), and α-tocopherol (F) values during storage of oils from non-heated and heated silages at 4°C for 0-4 months. Results are expressed as mean ± SEM (n = 3). Herring co-products from batch 6 were used here.

The amino acid composition of the co-product raw material and silage, as well as hydrolysates and solids phases derived after centrifugation of non-heated/heated silage at 4500 x g for 20 min is presented in **Table 8**. No significant ($p > 0.05$) difference in total amino acid contents between co-products and silage was noticed, suggesting that co-product nutrients were well-preserved during ensilaging and that the small dilution by acid and antioxidant did not affect the nutrient density. The silage and products derived thereof contained all essential amino acids, except tryptophan which was not analyzed in the method used. Among the essential amino acids, lysine was the most abundant one in all samples, followed by leucine, valine, threonine, isoleucine, phenylalanine, methionine, and histidine. The fact that the separated solids contained significantly ($p < 0.05$) higher levels of total amino acids, compared to the hydrolysates, agrees with the crude protein data and suggests that these solids can also be used as a separate product after recovery of oil and hydrolysates.

Table 8. Amino acid composition of herring co-products, silage, and phases derived thereof after centrifugation of non-heated/heated silage at 4500 x g for 20 min. Herring co-products from batch 6 were used here. Results are expressed as mg amino acid/g of co-products, silage, hydrolysates, or solids (mean ± SEM; n = 3). TAA: total amino acids; TEAA: total essential amino acids.

Amino acid	**Co-products**	**Silage; Day-2**	**Hydrolysates**		**Solids**	
			Non-heat	**Heat**	**Non-heat**	**Heat**
Glycine	15.9 ± 1.0	18.4 ± 0.2	18.9 ± 0.7	21.0 ± 0.4	26.3 ± 1.0	25.6 ± 0.2
Alanine	18.2 ± 0.3	18.8 ± 0.1	18.9 ± 0.34	19.5 ± 0.2	24.9 ± 0.0	26.7 ± 0.6
Serine	12.6 ± 0.1	12.7 ± 0.2	12.5 ± 0.3	12.5 ± 0.3	16.7 ± 0.1	17.7 ± 0.5
Proline	11.6 ± 0.4	12.9 ± 0.1	14.1 ± 0.2	14.2 ± 0.1	17.5 ± 0.2	17.6 ± 0.3
Valine	15.8 ± 0.3	15.5 ± 0.2	15.7 ± 0.2	15.6 ± 0.1	21.5 ± 0.2	24.5 ± 0.5
Threonine	13.6 ± 0.1	13.2 ± 0.3	12.2 ± 0.3	12.2 ± 0.1	17.0 ± 0.1	18.6 ± 0.3
Isoleucine	11.8 ± 0.4	11.6 ± 0.1	10.7 ± 0.2	10.6 ± 0.1	16.5 ± 0.5	19.1 ± 0.5
Leucine	22.4 ± 0.5	21.4 ± 0.3	21.9 ± 0.4	21.9 ± 0.1	30.9 ± 0.3	35.4 ± 0.5
Aspartic acid	28.2 ± 0.5	28.1 ± 0.2	23.9 ± 0.4	23.7 ± 0.2	34.2 ± 0.3	37.2 ± 0.2
Lysine	26.5 ± 0.4	25.0 ± 0.1	26.1 ± 0.2	25.3 ± 0.4	32.9 ± 0.5	36.3 ± 0.5
Glutamic acid	39.9 ± 0.7	39.1 ± 0.4	35.5 ± 0.5	34.1 ± 0.2	49.6 ± 0.6	55.4 ± 1.2
Methionine	8.3 ± 0.1	7.8 ± 0.0	8.1 ± 0.1	8.3 ± 0.1	12.0 ± 0.0	13.6 ± 0.3
Histidine	7.4 ± 0.1	7.3 ± 0.1	6.9 ± 0.1	6.7 ± 0.0	8.5 ± 0.1	9.2 ± 0.3
Phenylalanine	11.5 ± 0.2	11.1 ± 0.0	10.5 ± 0.2	10.5 ± 0.0	17.1 ± 0.2	19.56 ± 0.3
Arginine	15.4 ± 0.3	16.4 ± 0.8	14.8 ± 0.2	14.8 ± 0.1	19.1 ± 0.5	19.9 ± 0.5
Tyrosine	8.9 ± 0.2	8.7 ± 0.2	6.6 ± 0.2	6.6 ± 0.2	10.7 ± 0.2	12.2 ± 0.5
TAA	**268.0** ± 1.67	**267.9** ± 1.5	**257.4** ± 3.8	**257.8** ± 1.2	**355.7** ± 1.0	**388.7** ± 6.5
TEAA	**117.4** ± 1.8	**112.9** ± 0.4	**112.0** ± 1.4	**111.1** ± 0.9	**156.6** ± 1.2	**176.3** ± 2.9
TEAA/TAA	**0.44**	**0.42**	**0.44**	**0.43**	**0.44**	**0.45**

6.6 Upscaling of ensilaging from lab- to pilot-scale (Paper V)

Changes in DH, PV, and TBARS during pilot-scale ensilaging of herring co-products are shown in **Figure 30**. The DH increased significantly ($p < 0.05$) over time and around 38% DH was recorded after 2 days ensilaging at ambient temperature (i.e. 21-22°C), which was similar to our lab-scale DH value of 40% (2 days; 22°C) **(Paper I)**. This suggests that our lab-scale DH values well simulated what happens in pilot-scale ensilaging. The observed unexpected increase in PV after 1 h ensilaging could be due to inefficient mixing of antioxidants and co-products in the beginning, or to sudden inhibition of their breakdown to secondary oxidation products. The latter corresponds to the slight, but significant ($p < 0.05$), decrease in TBARS value after 1 h of ensilaging. Indeed, this TBARS decrease could also be due to breakdown of MDA and/or reaction between MDA and protein/peptides/amino acids, as discussed earlier **(Paper II)**. Overall, the TBARS values were lower after 1-2 days pilot-scale ensilaging at 21-22°C with 0.5% BORDANTIX W/S and BORDANTIX O/S in a 1:1 ratio than during lab-scale ensilaging of herring co-products with 0.25-1.25% BORDANTIX W/S or BORDANTIX O/S antioxidant; i.e. 8-10 µmole TBARS/kg silage (pilot-scale; **Figure 30**) vs 20-28 µmole TBARS/kg silage (lab-scale; see **Figure 22**) **(Paper IV)**. This could be due to higher quality starting raw material used in the pilot-scale ensilaging, stressing the importance of ensuring a fresh starting raw material to produce a silage with low oxidation levels for further value-added processing.

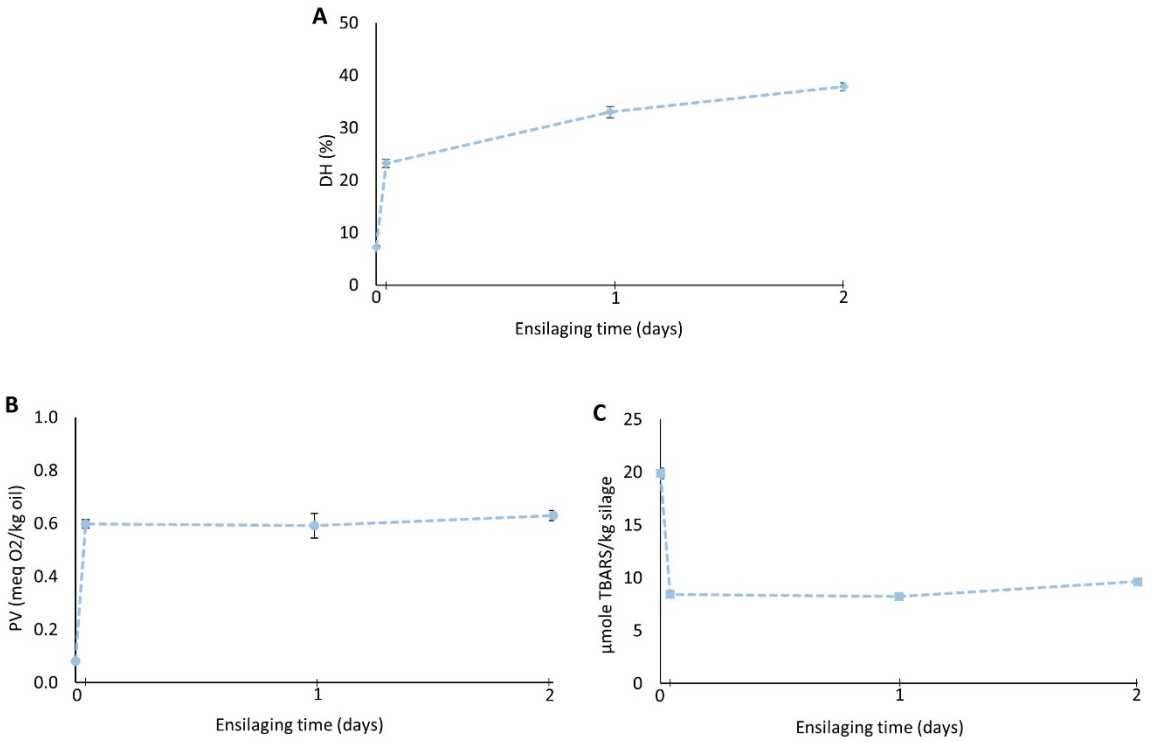

Figure 30. Changes in DH (A), PV (B), and TBARS (C) during pilot-scale ensilaging of herring co-products. Time point zero (i.e. day 0) refers to herring co-products before ensilaging. Herring co-products from batch 6 were used here. Results are expressed as mean ± SEM (n = 3).

6.7 Possible application areas of silage and products derived thereof

Considering that TBARS values in pilot-scale produced silage were well below the reported TBARS values of e.g. Atlantic mackerel (*Scomber scombrus*) fillets [176] and fish sauce prepared from Atlantic horse mackerel (*Trachurus trachurus*) [177], and that TVB-N-values of lab-scale produced silage were below the acceptable limit of 30 mg TVB-N per 100 g fish for human consumption [169], herring silage can be promising for food applications, in addition to applications as feed. The only change required for such applications would be replacement of formic acid by e.g. acetic acid or lactic acid. It is foreseen that silages could then be added to food products in a similar way as fish protein hydrolysates today are used, e.g. in a dried, semi-dried or wet state as fortification of drinks, soups and sauces [10], or into dietary supplements or sports nutrition products to provide them with potential added values. There are already several fish hydrolysates- and peptides-containing products available in the market claiming e.g. antioxidative, anti-stress, antihypertensive, glycemic index lowering and weight management properties [178]. Further, numerous *in vitro* studies are also available reporting on specific positive outcomes of short-chain peptides, e.g. anti-oxidative, antihypertensive, and anti-inflammatory activities [7-9].

The recovered fish oil from herring co-product silage had PV, p-AV and TOTOX values within acceptable limits for human consumption specified by the GOED Voluntary Monograph [171], suggests that also the oil part can be considered for food application. Currently, there is a massive market demand for EPA/DHA-rich oil driven by e.g. its negative association with cardiovascular disease (CVD) and CVD risk factors such as elevated plasma lipids and high blood pressure [179-181], as well as potential adjuvant therapeutic strategies for Covid-19 patients [182-184]. Thus, herring co-product silage or its oil could provide a cheaper alternative to traditionally produced fish oil, considering the fact that the ensilaging process is less energy-demanding compared to the heat-driven fish oil/meal production process.

In case of feed applications, fish silage and silage hydrolysates, can be used both as a feed nutrients and bioactive feed additives [4,185]. Several studies on e.g. Atlantic salmon, Atlantic cod, rainbow trout, shrimp, abalone, red seabream, orange-spotted grouper, Japanese sea bass, and Japanese flounder have reported positive outcomes such as improved growth and feed intake by replacing e.g. 10-25% fish meal with silage, and examples of such can be found elsewhere [4,110,186-198]. It is believed that the short-chain peptides (2-6 amino acids) of silage contributes to these positive outcomes. Also, short-chain peptides are available for protein synthesis for longer periods of time and reduce fluctuations in plasma amino acid levels [195,199-203]. In addition, both short-chain peptides and free amino acids of silage have been reported to improve health and welfare of Atlantic salmon (*Salmo salar*) by reducing the activation of caspase-3 in liver cells subjected to oxidative stress induced by H_2O_2 [4,204]. Further, the formic acid used in silage can work as growth promoter [4,205-208].

Just as in food applications, the recovered fish oil can also be used as a substituent of commercial fish oil in the formulation of fish feed. Fish oil has been reported to improve cellular immune function in e.g. South African abalone and Mozambique tilapia [209,210].

Finally, the solids from silage-separation can be used as a good source of essential amino acids providing an alternative to e.g. plant-based ingredient like soybean meal [4,211]. Altogether, the ensilaging process can thus be used as a basis for a green herring co-product biorefinery since it facilitates for the separation of different sub-fractions without the use of solvents.

7 Concluding remarks

The scope of this thesis was to deepen the understanding of biochemical reactions; proteolysis, lipid oxidation and TVB-N development, during herring co-product silage production, as well as to develop strategies to minimize lipid oxidation during this process. In addition, the possibility of separating silage into multiple products was investigated. Results showed that DH reached up to 72% after 7 days; the specific level however being controlled by e.g. temperature and time. Also, in absence of antioxidants, a broad range of lipid oxidation products were formed during ensilaging of the herring co-products; with Hb playing a major role. The oxidation could however be controlled by use of rosemary-based antioxidants at adequate dosages, yielding a silage and/or products derived thereof with comparable oxidative levels to that of fish-based food products. This opens up possibilities for use of silages in both food and feed applications. More precisely, the results can be summarized as:

- Temperature and time were the factors most strongly controlling the proteolysis during ensilaging. The highest DH rate was recorded at 32°C, and both DH and formation of free amino acids increased with ensilaging time. Heat treating the silage at 85°C for 30 min prevented an excess formation of free amino acids during up to 6 months storage at 4°C or 22°C, and thus, can be a route to produce a silage rich in short-chain peptides rather than free amino acids.

- Lipid oxidation developed both during ensilaging and subsequent storage. In absence of antioxidants, tertiary lipid oxidation products dominated at temperatures >22°C, which were not possible to measure using the classic PV and TBARS tests. For example, MDA, the main carbonyl compound responding in the TBARS test, broke down, yielding e.g. acetaldehyde. Also, saturated and unsaturated aldehydes followed a decreasing trend after 3 days ensilaging >22°C, resulting in the formation of non-enzymatic browning-derived products, suggesting interactions between lipid oxidation-derived α, β-unsaturated aldehydes. At lower temperatures (7 and 22°C), the formation of saturated and unsaturated aldehydes increased steadily during the whole 7-day period, without pronounced formation of browning products.

- The extent of lipid oxidation during ensilaging was related to the Hb/heme levels present in the system, suggesting they control oxidation development, possibly via heme-mediated lipid hydroperoxide cleavage. Pre-incubating the co-products in an antioxidant-containing solution could minimize Hb-mediated lipid oxidation both in the co-products and in silage.

- The commercial rosemary extract-based antioxidant mixture Duralox MANC-213 provided the best protection against both PV and TBARS development during ensilaging (0-7 days; 22°C), and during subsequent heat-treatment (30 min; 85°C), with the two highest concentrations (0.75 and 1.25%) reducing lipid oxidation the most. During prolonged storage of heat-treated and non-heat-treated silage at 4°C, 1.25% Duralox MANC-213 could almost completely prevent TBARS development during 6 months, while a level >1.25% seemed necessary to prevent TBARS formation at 22°C.

- TVB-N development during ensilaging reached up to 25 mg TVB-N/100 g silage, which was however lower than previously reported TVB-N values of silage prepared from e.g. plaice, sole, flounder, and whiting. The levels reported in this thesis work were also below the acceptable limit of 30 mg TVB-N per 100 g fish for human consumption, even after 6 months storage at 4 and 22°C, suggesting that silage can be used for food applications.

- The DH and lipid oxidation levels found in herring silage produced in 1500-liter scale agreed well with 0.5-liter lab-scale levels (e.g. 38% DH vs 40% DH after 2 days ensilaging at 21-22°C). This showed that data from lab-scale ensilaging can be extrapolated to larger scale.

- Herring silage was successfully separated into fish oil, hydrolysates, and solids. Heat-treating the silage prior to centrifugation and applying a higher g-force gave higher oil and hydrolysates recovery yields. The highest yields (w/w) were 10% and 53%, respectively.

8 Future outlook

The results of this thesis opened several new tracks which could be the subject for future studies. Examples of these are to investigate:

- the formation of short-chain peptides during the ensilaging process, i.e., their exact sizes and quantities, and how to tune the process to get a silage rich in short-chain peptides but with a minimum amount of free amino acids.

- the consumer acceptance of fish silage as food ingredients by e.g. applying sensory testing with a focus on bitterness, if any, from both bitter peptides and rancidity.

- the formation of bitter peptides during the ensilaging process, and how to minimize these with/without using exogenous protease during and post ensilaging. Examples of the latter are e.g. hydrolysis of bitter peptides into amino acids by proteases, protease-catalyzed condensation of peptides (i.e. reversed proteolysis) or the plastein reaction.

- how to shorten the ensilaging time to less than a day with maintained DH, to allow day-in-day-out ensilaging of co-products, which will facilitate resource use such as plant space.

- analysis of the polyphenol composition of aqueous and lipophilic rosemary extracts, which will help us better understand the antioxidative mechanism provided by their different compounds.

- the use of other natural, efficient and cost-effective antioxidant compounds to minimize lipid oxidation during pre-ensilaging, ensilaging and post-ensilaging storage while at the same time avoid the flavor of e.g. rosemary.

- the effect of seasonal variability in e.g. lipid content of the co-products on silage lipid oxidation, and how this might affect the required antioxidant dosage to provide maximum protection against lipid oxidation while minimizing the use of antioxidants.

- how to facilitate fast antioxidant distribution during large-scale ensilaging of herring co-products to provide better protection against lipid oxidation.

- cross linking of protein and formation of non-enzymatic browning reaction products, occurring when e.g. elevated temperatures are used in the ensilaging process and heat-treatment used at the end of the ensilaging process, and their effects on e.g. protein/peptide bioavailability and digestibility.

- the possibility of using other separation techniques to recover fish oil and protein hydrolysates from the silage.

- the economic feasibility of building a herring co-product silage-based biorefinery for production of multiple products; i.e., how does it compare to current state of the art where co-products are exported to Denmark for fish meal and fish oil production.

- High-throughput biomining for bioactive peptides in silage, for potential use in the pharma sector.

9 Acknowledgements

Maybe I should have written this part a bit earlier and not in the middle of the night the day before it goes for printing! Anyway, many people to thank! I have met many wonderful people during my PhD journey. So, if you are here reading this page, regardless I mention here or not, the latter is very likely considering the limitation of human brain (read my brain) to recall all those faces I met during my nearly 4.5 years PhD journey at this very last hour, thanks for being a part of this wonderful journey!

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