DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

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To know what you know and what you do not know, that is true knowledge.

Confucius

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ABSTRACT

Baltic herring (*Clupea harengus membras*) is a species of herring (*Clupea harengus*) found exclusively in the Baltic Sea. In Finland, it is the most valuable commercially caught species in both volume and value; however, it is highly underutilised as food. Of the approximately 100 million kilograms of Baltic herring caught annually from the Finnish Sea areas, only 3–4 million kilograms are used as food, while most of the catch is processed for feed. The small size of the fish makes conventional processing more challenging, which is why new technologies for the utilisation of the important proteins and omega-3 fatty acids are essential to create added value from the fish resources.

In the first part of the thesis research (publication **I**), pH-shift method was used to extract proteins from Baltic herring using alkaline processing method (solubilisation pH 11.2 and precipitation pH 5.4). The protein isolate obtained from the pH-shift process was incorporated into two food models: surimi-type gel and fish balls. The functionality of the protein isolate in these models was studied based on composition, colour, texture through texture profile analysis, and volatile compounds with HS-SPME-GC-MS. Furthermore, two different pH-shift methods (changed solubilisation (pH 11.5) and precipitation (pH 6.5) pH values, respectively) were applied to study their effects on the colour of the protein isolate. The results showed that the surimi-type gel had a hardness value comparable to commercial surimi, while the fish balls were significantly softer than commercial products. However, the colour of the surimi was significantly darker than the commercial surimi typically made of white-muscle fish species. The changed solubilisation or precipitation modifications did not improve the whiteness of the gels. According to the volatile analysis, increasing solubilisation pH increased the total content of volatile compounds, as well as the amounts of many important secondary oxidation compounds, which indicates increased oxidation compared to the method using a lower solubilisation pH. Conversely, increasing the precipitation pH decreased the total volatile content, indicating a decreased level of oxidation but it lead to a significantly softer surimi-type gel.

In the second part of the research (publication **II**), enzyme-assisted aqueous extraction was used to extract oil from whole Baltic herring and filleting co-products. Different commercial proteases (Alcalase, Neutrase & Protamex) were used with two different extraction times (35 and 70 min), and the effect on oil stability and quality were studied by determining peroxide value, *para*-anisidine value, volatile compounds, fatty acid composition, and oil recovery. Overall, longer extraction times led to better extracted recoveries of oil, but also increased oxidation. The highest oil yields from whole fish were from the 70-minute extractions with Neutrase and Protamex. The extraction for 35 minutes using

Protamex resulted in the highest contents of eicosapentaenoic acid (EPA; 20:5*n*–3) and docosahexaenoic acid (DHA; 22:6*n*–3), but it also increased oxidation compared to the extractions with other enzymes. The highest extracted oil content from the co-products was obtained from the 70-minute extraction with Protamex. However, there were no significant differences in EPA and DHA contents among the oils extracted with different enzymes. Oxidation was the lowest in the oil produced with 35-minute treatment using Neutrase and Protamex. This study showed the potential of using proteolytic enzymes in the extraction of crude oil from Baltic herring and its co-products.

The third part of the research (publication **III**) focused on the optimisation of enzyme-assisted aqueous extraction from lean Baltic herring. Pre-trials showed that Baltic herring with low lipid content (<4%) led to significant emulsion formation with low oil recovery. Lowering stirring speed during extraction, centrifuging while the hydrolysate was still hot (compared to cooling down), and cutting the fish raw materials into smaller sizes before extraction were all effective methods to improve recovery. However, different enzymes, treatment times, or enzyme concentrations did not have significant effects on oil stability, recovery, or composition in this study. Furthermore, lipid classes were analysed using a novel UHPLC-MS-based method. The study revealed that nearly all phospholipids were lost during the enzyme-assisted aqueous extraction, together with a partial loss of DHA, likely due to the emulsion formation. Different methods were applied to reduce emulsion: changing the fish-to-water ratio from 1:1 to 2:1 (w/w) together with an addition of ethanol was the best method to both reduce emulsion and improve oil recovery. As a result of the emulsion reduction, more DHA was recovered, and the oil contained more triacylglycerols while the phospholipid content remained low.

SUOMENKIELINEN ABSTRAKTI

Silakka on Itämeressä esiintyvä sillin alalaji, ja se on kalastusmäärältään ja markkina-arvoltaan Suomen tärkein kaupallisesti kalastettu kalalaji. Vaikka Suomessa pyydystetään vuosittain lähes 100 miljoonaa kiloa silakkaa, vain 3–4 miljoonaa kiloa päätyy suomalaisten lautasille, kun taas suurin osa saaliista käytetään eläinten rehuksi. Suurimmat syyt silakan käytön vähenemiselle on sen nopeasti heikkenevä laatu, sesongittainen saatavuus ja kuluttajien muuttuneet käyttötottumukset. Silakka on kuitenkin terveellinen kala: se sisältää runsaasti terveydelle hyödyllisiä omega-3-rasvahappoja ja proteiinia sisältäen kaikki ihmiselle välttämättömät aminohapot. Lisäksi sen kalastus on tärkeää Suomen kalataloudelle ja poistaa samalla Itämerestä ravinteita. Näiden syiden takia silakkaa täytyisi hyödyntää nykyistä paremmin ja kehittää uusia teknologioita mahdollistamaan sen laajemman hyödyntämisen ihmisten ravinnoksi sekä lisäarvon luomisen alihyödynnetylle kalalajille.

Tässä tutkimuksessa tutkittiin kahta erilaista ympäristöystävällistä teknologiaa silakan hyödyntämiseksi. Väitöskirjan ensimmäisessä osatutkimuksessa (**I**) peratun silakan proteiinit eristettiin pH:n vaihteluun perustuvalla tekniikalla, ja tuotettua proteiini-isolaattia käytettiin kahdessa eri mallielintarvikkeessa: geeliytetyssä suriminkaltaisessa tuotteessa sekä kalapullissa. Lisäksi tutkittiin kahta eri variaatiota pH-muunnosmenetelmästä: korkeampaa proteiinien liuotus- tai saostus-pH-arvoa, joilla ajateltiin olevan vaikutusta proteiini-isolaatin väriin. Tutkimuksessa selvitettiin proteiini-isolaatin vaikutus tuotteiden rakenteisiin, väriin sekä haihtuviin yhdisteisiin, mitkä kertovat sekä tuotteiden aromeista että härskiintymisestä. Tulosten mukaan silakasta uutettu proteiini-isolaatti toimi hyvin geeliytettynä, sillä surimi oli rakenteeltaan kaupallisten surimien kaltaista. Kalapullat olivat puolestaan pehmeämpiä kaupalliseen kalapullaan verrattuna, mutta niiden vedensidontakyky oli hyvä, ja pullat pitivät muotonsa kypsennyksen aikana. Proteiini-isolaatista tehty suriminkaltainen geeli oli kuitenkin väriltään huomattavasti tummempaa kuin kaupalliset surimit, mikä johtuu silakan lihaskudoksen sisältämistä pigmenteistä. pH-muunnosmenetelmän variaatiot eivät parantaneet proteiini-isolaatin väriä. Haihtuvien yhdisteiden analyysin mukaan korkeamman liuotus-pH:n käyttö lisäsi kuitenkin haihtuvien yhdisteiden kokonaismäärää sekä useiden sekundääristen hapettumistuotteiden määriä verrattuna verrokkimenetelmään. Alhaisemman saostamis-pH-arvon käyttö puolestaan alensi haihtuvien yhdisteiden määriä, mikä kertoo alhaisemmasta hapettumisesta prosessin aikana.

Toisessa osatutkimuksessa (**II**) perehdyttiin öljyn uuttamiseen sekä kokonaisesta silakasta että fileoinnin sivuvirtatuotteista. Öljy uutettiin kolmea eri kaupallista proteaasientsyymiä hyödyntäen (Alcalase, Neutrase ja Protamex) ja

öljyn laatua tutkittiin hapettumisen ja rasvahappokoostumuksen kannalta. Lisäksi tutkimuksessa selvitettiin entsyymien ja kahden eri käsittelyajan (35 ja 70 min) aiheuttamia eroavaisuuksia öljysaantoihin ja öljyn koostumukseen. Tutkimuksessa havaittiin eroja sekä entsyymien, käsittelyaikojen että raaka-aineiden välillä. Kokonaisen kalan käsittelyssä Neutrase-entsyymillä saavutettiin paras öljysaanto mutta Alcalase-entsyymillä saavutettiin alhaisin hapettumisaste. Sivuvirtatuotteiden kohdalla Protamex-entsyymi 70 minuutin käsittelyajalla oli paras saannon ja hapettumisen kannalta, kun taas Alcalase lisäsi hapettumista. Kaikissa öljyissä oli korkea monityydyttymättömien rasvahappojen pitoisuus ja parhaimmillaan öljyn saanto ylsi 70 %:in.

Kolmannessa osatutkimuksessa (**III**) tutkittiin vähärasvaisen kokonaisen silakan öljyjen uuttoa. Öljyn saanto oli uudella raaka-aine-erällä erittäin alhaista ja sen sijaan prosessin aikana muodostui huomattava määrä emulsiota. Saannon parantamiseksi uutto-olosuhteita optimointitiin raaka-aineen pilkkomiskoon, sentrifugointilämpötilan ja sekoitusnopeuden optimoinnilla, jonka jälkeen tarkasteltiin entsyymipitoisuuksien ja eri uuttoaikojen vaikutusta öljyn saantoon ja koostumukseen. Tutkimusta varten kehitettiin lipidiluokka-analyysimenetelmä, jolla selvisi, että lähes kaikki fosfolipidit katosivat entsymaattisessa uutossa ja öljyn omega-3-rasvahappopitoisuus oli myös alhaisempi kuin liuottimilla uutetussa kontrolliöljyssä. Tutkimuksessa selvitettiin, miten eri keinot vähentävät prosessissa muodostuvaa emulsiota. Lisätyn veden määrän vähentäminen ja etanolin lisääminen uuton aikana vähensivät emulsiokerrosta huomattavasti, mikä johti myös korkeampiin triasyyliglyserolien ja omega-3-rasvahappojen määriin öljyssä. Fosfolipidien määrä jäi kuitenkin edelleen alhaiseksi, mutta tutkimuksessa ei selvitettty, jäävätkö fosfolipidit emulsiokerrokseen vai muihin uuton faaseihin. Tutkimuksen aihe oli tärkeä, sillä silakan rasvapitoisuus vaihtelee huomattavasti vuodenaikojen ja vuosien välillä, mikä täytyy ottaa huomioon sen prosessoinnissa.

LIST OF ABBREVIATIONS

AV	*para*-Anisidine value
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
EAAE	Enzyme-assisted aqueous extraction
EPA	Eicosapentaenoic acid
FA	Fatty acid
FFA	Free fatty acid
FPI	Fish protein isolate
GC-FID	Gas chromatography-flame ionization detector
DW	Dry weight
HS-SPME-GC-MS	Headspace-solid phase microextraction-gas chromatography-mass spectrometry
MAG	Monoacylglycerol
MUFA	Monounsaturated fatty acid
NL	Neutral lipid
n–3	Omega-3 fatty acid
n–6	Omega-6 fatty acid
PCA	Principal component analysis
PA	Phosphatidic acid
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
PV	Peroxide value
SFA	Saturated fatty acid
SM	Sphingomyelin
SPI	Surimi from protein isolate
TAG	Triacylglycerol
TBARS	2-Thiobarbituric acid reactive substances
TOTOX	Total oxidation products
TPA	Texture profile analysis
UHPLC-MS	Ultra high precision liquid chromatography-mass spectrometry
VSOP	Volatile secondary oxidation product

LIST OF ORIGINAL PUBLICATIONS

I. Kakko, T.; Aitta, E.; Laaksonen, O.; Tolvanen, P.; Jokela, L.; Salmi, T.; Damerau, A.; Yang, B. Baltic herring (*Clupea harengus membras*) protein isolate produced using the pH-shift process and its application in food models. *Food Res. Int.,* **2022**, 158, 111578.

II. Aitta, E.; Marsol-Vall, A.; Damerau, A.; Yang, B. Enzyme-assisted extraction of fish oil from whole fish and by-products of Baltic herring (*Clupea harengus membras*). *Foods*, **2021**, 10(8), 1811.

III. Aitta, E.; Damerau, A.; Marsol-Vall, A.; Fabritius, M.; Pajunen, L.; Kortesniemi, M.; Yang, B. Enzyme-assisted aqueous extraction of fish oil from Baltic herring (*Clupea harengus membras*) with special reference to emulsion-formation, extraction efficiency, and composition of crude oil. *Food Chem.,* **2023**, 424, 136381.

1 INTRODUCTION

Baltic herring (*Clupea harengus membras*) is a pelagic fish found in the Baltic Sea. It is the most commercially caught fish species in Finland, however, it is widely underutilised as food, and mostly used in feed production. Baltic herring is known for its high fat content: 4–10% depending on the season, and it is a good source of protein, vitamins and minerals. New technologies need to be developed to utilise the valuable oils and proteins from the fish, as it is currently highly under-utilised as food. It is important to utilise the fish as a whole, including its processing co-products in regards to the circular economy approach, and as the growing global population will require more protein and omega-3 fatty acid sources. Baltic Sea also suffers from eutrophication, so the removal of Baltic herring simultaneously removes nutrients, such as phosphorus and nitrogen, from the sea. This thesis research was conducted as a part of a national program to develop methods for creating added value for under-utilised fish species, including Baltic herring. Therefore, the research is significant for the domestic fishing sector.

Green extraction techniques avoid the use of high temperatures and toxic solvents, which may destroy heat-sensitive nutritional components or leave harmful residues in the products. These techniques include, for instance, fermentation, enzymatical hydrolysis (also known as enzyme-assisted aqueous extraction, EAAE), pH-shift process, supercritical fluid extraction using carbon dioxide, and subcritical fluid extraction using water or ethanol. The methods can be optimised for the production or extraction of specific components, such as proteins, peptides or lipids. pH-Shift process can be used to extract proteins from fish without compromising their functional properties, such as gelling and water-holding capacities. Therefore, the produced protein isolate can be incorporated into different food matrices, for example, fish balls or surimi. EAAE is another green technique, which is often used to produce protein hydrolysates, but it can also be used in the extraction of oil as the enzymes break down the fish raw material and thus, enable the release of lipids. These two methods were chosen for this thesis research due to their suitability for industrial up-scaling, and previous scientific knowledge about the processes.

In the first part of the thesis research (Study **I)**, pH-shift was used to extract soluble proteins from gutted and beheaded Baltic herring, and the produced fish protein isolate (FPI) was then used in two different food models. The feasibility of FPI was investigated in fish balls and surimi in terms of texture, colour and volatile compounds, which can be used to analyse the volatile aroma and oxidation. As a second method, EAAE was used in the extraction of oil from whole Baltic herring and its filleting co-products. This consisted of two parts: in Study **II**, the suitability of the EAAE on whole fish and filleting co-products was

studied in terms of oxidative stability, crude oil yield, and oil composition. In Study **III**, the method was further optimised for lean fish, as the newer fish batches resulted in emulsion formation and significant decrease in oil recovery. The declining fat content of Baltic herring has been reported, and evidently the changing raw material composition also compromises the efficiency of the EAAE. Different techniques were used to first optimise the process in terms of crude oil yield and composition, and secondly, to decrease the amount of emulsion formed during the process. A novel method was developed to study the lipid classes of the extracted oils and to understand which lipid classes play a role in the extraction efficiency. The literature review part introduces Baltic herring as a food ingredient, focusing especially on the lipids and proteins. Also, different green processing techniques are discussed, with special attention to the pH-shift process and EAAE.

2 REVIEW OF THE LITERATURE

2.1 Baltic herring

Baltic herring (*Clupea harengus membras*) is a pelagic fish found exclusively from the Baltic Sea. It is a subspecies of the Atlantic herring (*Clupea harengus*). The typical size of Baltic herring is 14–18 cm but it can grow up to 35 cm long, and its weight varies between 30–100 g. Baltic herring and herring are so similar that they are generally classified based on their fat content: Baltic herring contains less than 10% of fat, while herring contains more than 10%. Baltic herring is one of the most important fish catches in the Baltic Sea region, and historically, it has been an important source of protein for the population living in the coastal areas of Finland and the Baltic. In Finland alone, 74 million kilograms of Baltic herring were caught in 2021 (Natural Resources Institute Finland, 2022) while the fishing quota was set at 103 million kilograms for 2022 (Ministry of Agriculture and Forestry of Finland, 2021). Despite the large availability and healthiness of Baltic herring, its domestic consumption is only 3–4 million kilograms, while most of the catch is used in feed production, and a small portion is exported (Natural Resources Institute Finland, 2020).

2.2 Lipids in Baltic herring

2.2.1 Lipid content

The lipid content of Baltic herring is 4–10% depending on the season (Aro et al., 2000; Rajasilta et al., 2022, 2019). The lipid content is highest during winter and lowest during the spawning season at spring. According to the recent study from Rajasilta et al. (2022), the average lipid content in Baltic herring caught in winter has decreased from the average of 7–8% to 2–3%, while the average of spawning season fish has decreased from 3–4% to 1–2% during the last 20–30 years. The lipids in the oil consist of mainly monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Omega-3 fatty acids (*n*−3 FAs) form up to 26–28% of the total fatty acids, the major ones being docosahexaenoic acid (DHA) (10.5–11.5%) and eicosapentaenoic acid (EPA) (6.5–7.2%) (Kakko et al., 2022). EPA and DHA (Figure 1) have shown to improve neuronal, retinal, brain, and cardiovascular health, as well as immune function (Dyall and Michael-Titus, 2008; Ghasemi Fard et al., 2019; Russo, 2009; Zhang et al., 2019). The lipid content of Baltic herring has decreased drastically during the past 20–30 years, but interestingly, the *n*−3 FA content has steadily increased (Rajasilta et al., 2022). The declining salinity, and the effect of global warming on increasing winter temperatures of the Baltic Sea have been associated with these changes,

as they reduce diatom biomass and change phytoplankton fatty acid profile, which affect the food chain (Lind et al., 2018).

The total amount of *n*−3 FAs in Baltic herring muscle is approx. 1 g/100 g fw, of which 0.3 g/100 g is EPA and 0.4 g/100 g DHA (Finnish Food Safety Authority, 2014). The Finnish National Nutrition Council recommends that at least 1% of the total energy consumption for adults and over 2-year-old children should come from *n*−3 FAs, which equals to approx. 2 grams in a diet consisting of 2000 calories. In Finland, the recommended daily consumption for DHA is 200 mg, whereas EPA does not have a specific recommendation (Valtion ravitsemusneuvottelukunta, 2014). Therefore, Baltic herring is an excellent source of *n*−3 FAs, and the recommended daily amount of DHA can be fulfilled with a 50-gram portion of fresh fish.

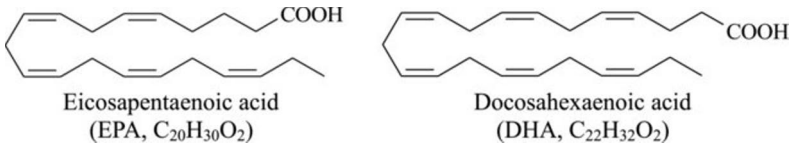

Figure 1. Chemical structures of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Li et al., 2014).

Fatty acids can be attached to many different lipid structures, such as monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs), and phospholipids. TAGs are found in the adipose tissue of the fish whereas phospholipids occur mostly in cell membrane structures and cell signalling molecules. The distribution of fatty acids and different lipid molecules in fish tissue vary depending on the fish species. For example, EPA and DHA are bound to phospholipids and TAGs in a ratio of 40:60 in Atlantic salmon muscle (Polvi and Ackman, 1992). Figure 2 displays example structures of TAGs and a phospholipids.

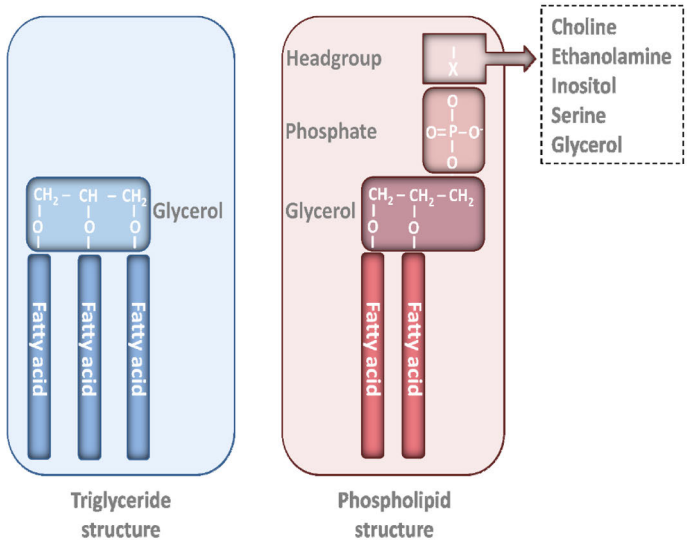

Figure 2. Schematic structures of a triacylglycerol (triglyceride) and a phospholipid (Burri et al., 2012).

2.2.2 Acylglycerols

Acylglycerols consist of a glycerol backbone, where either one (MAG), two (DAG) or three (TAG) fatty acids are attached with an ester bond. MAGs and DAGs mainly act as cell signalling molecules, while TAGs are mainly functioning as storage lipids (Mérida et al., 2008). TAGs are the main lipid class in medium-fat and fatty fish species. The proportion of TAGs out of all lipids in freshwater salmonids, for example, is 43.4–89.7% (Sushchik et al., 2020), whereas marine species arrow-tooth flounder, golden pompano and freshwater whitefish have respective values of 80%, 90% and 51.5% (He et al., 2019; Oliveira and Bechtel, 2006; Suomela et al., 2016). The proportion of TAGs in Baltic herring is prone to fluctuations depending on the season, and have been reported values of 54–91% of total lipids (Linko et al., 1985). In a more recent study, 78% of the Baltic herring lipids were TAGs, while the amount of MAGs and DAGs were 0.2% and 2.1%, respectively (Kakko et al., 2022). The amounts of MAGs and DAGs in different mesopelagic fish species is 0.1–0.4%, and 0.3–0.4% of the fish muscle dry weight (dw), respectively, whereas TAGs have concentrations of 0.9–10.6% dw. (Voronin et al., 2022)

Fatty acids are attached to the glycerol backbone of a TAG in *sn*-1, *sn*-2 and *sn*-3 positions (Figure 3). In nature, different fatty acids are more prone to attach either the *sn*-1 and *sn*-3 (outer), or the *sn*-2 position (middle), a phenomenon known as regiospecificity, which is caused by the position and/or fatty acid specificity of the enzymes involved in the biosynthesis of TAGs. In salmon, 50% of EPA and 80% of DHA is located to the *sn*-2 position. (Ruiz-Lopez et al., 2015).

In another study, EPA was mainly distributed in the *sn*-2 position in anchovy, tuna and salmon oils, whereas DHA was evenly distributed within the different positions (Zhang et al., 2018).

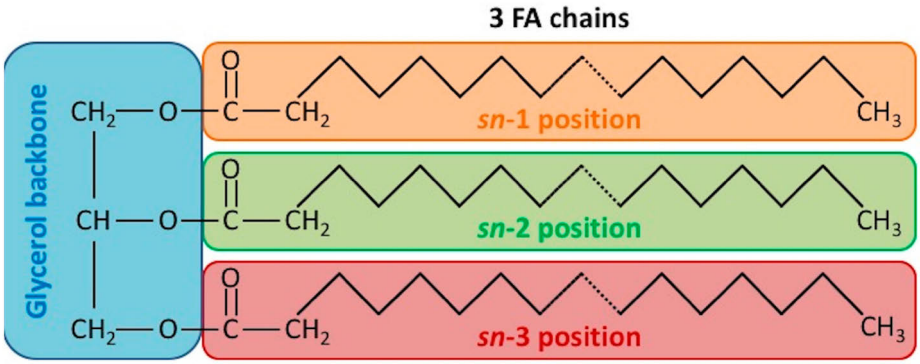

Figure 3. Schematic structure of a triacylglycerol. Fatty acids are attached to the glycerol backbone in *sn*-1, *sn*-2, and *sn*-3 positions. (Alfieri et al., 2018)

2.2.3 Cholesterol

Cholesterol can be found in fish tissue, but in a significantly smaller concentrations than in meat from land animals. The roles of cholesterol in fish are: as a part of membrane structures, hormone synthesis, insulation, and vitamin D synthesis (Arts and Kohler, 2009). Baltic herring has been reported to contain 0.6–1.9% of cholesterol of the total lipids (Linko et al., 1985), although another study reported only a negligible amount (Aro et al., 2000).

2.2.4 Ethyl esters

The total content of *n*−3 PUFAs in refined fish oil TAGs is typically around 20–30%, and the contents are dependent on the fish species and parts, extraction method and refining method. It is desirable to increase the levels of *n*−3 PUFAs while reducing SFAs and MUFAs, as this ensures the health benefits associated with EPA and DHA, particularly. Some clinical studies reviewed by Watanabe and Tatsuno (2020) are using dosages of as much as 4 g/d of EPA, DHA or a combination of both. Therefore, enrichment of *n*−3 PUFAs, especially EPA and DHA, have been an active field of research in order to concentrate these valuable lipids (Marsol-Vall et al., 2021). Ethyl esters are synthetic forms of lipids consisting of fatty acids esterified with ethyl groups, which are created by a reaction between a free fatty acid (FFA) and ethanol. Many omega-3 supplements on the market are in the ethyl ester form, however, their

bioavailability has shown to be lower than the natural TAG form (Dyerberg et al., 2010).

Most commonly used industrial methods for enriching PUFAs in the forms of natural TAGs are enzymatic purification, urea complexation, and low temperature crystallisation. Other techniques for concentrating *n*−3 PUFAs are liquid and supercritical fluid chromatography, and supercritical fluid extraction, which are described more in detail in the review by Bonilla-Mendez and Hoyos-Concha (2018). In addition, studies on *n*−3 PUFA enrichment using pressurised liquids have also emerged (Castejón and Señoráns, 2019).

2.2.5 Free fatty acids

FFAs are formed when the ester bonds in lipids are hydrolysed by heating or enzymatic action in the presence of water. FFAs are often formed in animal tissue after death due to enzymatic reactions, and during processing, especially at high temperatures. (Bernárdez et al., 2005). In one study, the FFA content of Baltic herring fillets straight after catching was 2.6% (% of oleic acid) and the value increased to 3.6% with time increasing after catching. The FFA content of gutted Baltic herring, on the other hand, was 2.9% straight after catching, and 3.8% once the fish had been transported to the factory (Aro et al., 2000).

FFAs are prone to oxidation, which can cause oils to become rancid rapidly. Therefore, deacidification is an important step in oil refining to remove FFAs. The conventional deacidification process involves adding sodium hydroxide to neutralise the FFAs, followed by precipitating them as soap. The soaps are then removed through centrifugation or washing. However, deacidification can result in a significant loss of oil due to the alkali hydrolysis of TAGs. Non-traditional neutralising agents, such as sodium carbonate and sodium bicarbonate, have also been studied for their removal of FFAs in vegetable oils, although sodium hydroxide was shown to be the most efficient method (De and Patel, 2010). Alternative approaches to the conventional deacidification method for fish oil processing include enzymatic esterification with ethanol or glycerol using a commercial lipase, as well as membrane-assisted solvent extraction as reviewed by Marsol-Vall et al. (2021).

2.2.6 Phospholipids

Phospholipids are a major lipid class in fish. Their structure consists of a hydrophobic tail made up of fatty acids, and a hydrophilic head made up of a phosphate group (Figure 1). The structure allows the formation of a bilayer, and the integration of the molecule in the cell membrane. Phospholipids are important for maintaining the structural integrity and fluidity of cell membranes

– being especially important in cold-adapted fish species. The membrane fluidity can be altered by changing the fatty acid composition of the phospholipids, for example, adding long-chain fatty acids with many double bonds leads to a less packed (more fluid) membrane structure (Farkas et al., 2001).

The phospholipid content has shown a wide range of 7.6–41.3% of the total lipids in Baltic herring (Linko et al., 1985). The amount of phospholipids is dependent on differences in the feeding and spawning periods of the fish within seasons: the lowest phospholipid contents have been measured in the autumn and highest in the summer. Recently, a phospholipid content of 18.8% was reported in Baltic herring caught in autumn (Kakko et al., 2022). The major phospholipid class in marine sources is phosphatidylcholine (PC), followed by phosphatidylethanolamine (PE), then phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), and lysophosphatidylcholine (LPC). In salmon, PS and SM are more abundant than PI and LPC, whereas frigate and yellowfin muscles show the opposite division between these classes. The specific phospholipid division has not been reported for Baltic herring.

Phospholipids are often removed from the crude oil by degumming to create a more stable oil, since they are easily hydrolysed to FFAs and other compounds that may compromise the stability of the oil (Marsol-Vall et al., 2021). However, some studies suggest that EPA and DHA may have a higher bioavailability when they are linked to phospholipids instead of TAGs or ethyl esters (Rossmeisl et al., 2012; Wijendran et al., 2002). Therefore, the removal of phospholipids during oil extraction and processing may compromise both the nutritional value of the oil by decreasing the bioavailability of the important fatty acids.

2.2.7 Wax esters

Wax esters are fatty acids esterified with alcohols. They are usually relatively low in fish, however, there are some marine species, such as *Calanus finmarchicus*, a type of copepod, where they can be found. In *C. finmarchius*, more than 85% of the lipids are wax esters, and the most abundant fatty acids found in the wax esters are long-chain fatty acids cetoleic acid (22:1*n*–11) and gondoic acid (20:1*n*–9) (Schots et al., 2020). According to Linko et al. (1985), there are no significant amounts of wax esters in Baltic herring.

2.3 Proteins in Baltic herring

2.3.1 Protein content

Fish is a good source of protein due to its balanced amino acid content. Baltic herring contains 14–15% of protein and it contains all essential amino acids

(Kakko et al., 2022; Nisov et al., 2020). In herring, the most predominant essential amino acids are lysine, threonine, valine and leucine while the most predominant non-essential amino acids are arginine, glycine, alanine, glutamic acid and aspartic acid (Wu et al., 2022a).

2.3.2 Types of proteins

Fish proteins are classified to myofibrillar, stroma and sarcoplasmic proteins. The myofibrillar proteins, mainly myosin and actin, are the building blocks of the muscle fibre. About two thirds of the proteins in fish meat consist of myofibrillar proteins: carp 70–72%, and cod up to 76%. Sarcoplasmic proteins include heme proteins, such as myoglobin and haemoglobin, globulin, parvalbumin, and different enzymes, and they make up to 20–30% of the proteins in fish. Stroma proteins consist of primarily collagen and other connective tissue proteins and they constitute approx. 3% of the fish proteins. (Love, 1970).

2.3.3 Light and dark muscle tissue

Fish muscle tissue can be divided into light and dark muscle, which have different roles in fish physiology. Light muscle tissue is responsible for fast movements and it uses glycogen as its main energy source, while dark muscle tissue is responsible for slower movements. Baltic herring, as other herrings and small pelagic fish, can be called dark muscle fish due to high proportion of dark muscle tissue. In herring, the fillets consist of around 20% of dark muscle tissue (Undeland et al., 1998). The darker muscle tissue contains higher concentrations of pigments, fat and vitamins, which are beneficial for health in terms of human consumption, but the higher contents of fat, myoglobin and haemoglobin can also lead to increased lipid oxidation, causing rancid flavours and a quickly decreasing quality (Park, 2013; Sajib et al., 2021; Undeland et al., 1998; Wu et al., 2022b).

2.3.4 The role of proteins in gelation

Gelation allows the conversion of a fluid to a solid. It is an important phenomenon in many food products, such as surimi, tofu, seitan, and other products relying on gluten, as well as yogurts and gelatine-based foods. In fish, the main protein responsible for gelation is myosin. The tail portions of myosin form hydrophobic interactions in the early stages of gelation, which is later accompanied by disulphide linkages between the head parts. Other proteins may be involved, but the exact mechanisms are also species-dependent. Myofibrillar proteins are largely insoluble in water, but the addition of salt enhances solubility,

and therefore induces gel formation. Stroma proteins, on the other hand, are almost fully insoluble in water or saline, while the sarcoplasmic proteins are soluble in pure water at low ionic strengths. The effect of sarcoplasmic proteins on gelling properties is controversial as both adverse and favourable effects on gelling ability have been reported. Sarcoplasmic proteins are washed away during a traditional surimi processing, which leads to a loss of proteins but improves the gel structure especially during storage. Further, the washing step removes heme proteins that can cause unpleasant colours to the gel when denatured. (Marmon, 2012; Park, 2013).

It is essential to use fresh fish in the processing, since endogenous proteolytic activity after death reduces the extractable actomyosin levels (Stone and Stanley, 1992). On the other hand, freezing may also interfere with the gelation due to protein denaturation. Therefore, additives, such as sucrose, sorbitol, and phosphates are often used as cryoprotectants if the raw material is frozen before processing. Herrings are abundant in heme pigments which belong to sarcoplasmic proteins that lack gel-forming capacity (Abdollahi and Undeland, 2019). Protein gels made of herring co-products have shown worse water-holding capacity and breaking force compared to gels made of salmon or cod.

2.4 Challenges in the utilisation of Baltic herring as food

Baltic herring is the most abundant fish species in the Baltic Sea, but widely underutilised for human consumption. The biggest reasons amongst consumers for not eating Baltic herring are: “bad taste”, “not used to consume or prepare it”, and “poor availability” (Pihlajamäki et al., 2019). As Baltic herring is prone to oxidation, the chain from catching to consumers’ plates should be quick to ensure good quality. As Baltic herring is a small fish, its use in automated filleting processes can be challenging. Further, nowadays consumers also tend to favour fish products that are easy to use, which is why it is important to study alternative methods for utilising the important oils and proteins from small fishes, as well as the processing co-products. Baltic herring is typically processed into fillets or fillets preserved in acid-based marinade, but fish fingers and fish balls have also been developed for the Finnish market. Alternative method for the utilisation of Baltic herring proteins could be extracting them, and use in surimi-type gels or fish balls, while the oil rich is *n*–3 FAs could be used as supplements, or as spray-dried products, which have a wide range of product applications.

Lipid oxidation is the main cause of quality deterioration in muscle foods, such as fish. Haemoglobin, lipoxygenases and tocopherol content have shown strong correlations to lipid oxidation in herring (Wu et al., 2022b). The head parts are especially prone to oxidation because they have the highest haemoglobin content, and lipoxygenase activity, while the viscera and belly flaps are more

protected by higher α-tocopherol contents. The utilisation of fish co-products and by-catch is needed to achieve sustainability and circular economy, however, sorting of the different parts is needed because different parts oxidise easier, and may be better suited to different technological applications. For example, herring frames resulted in the highest protein yield and best gel-forming capacity using the pH-shift method to extract proteins compared to other fractions, while the head fraction resulted in the lowest protein yield and gel-forming capacity, as well as the darkest colour (van Berlo et al., 2023). The differences in gel-forming capacity were most likely due to the proteolytic activity in the head fraction, and the darker colour resulted from the higher concentrations of heme and melanin.

The addition of natural antioxidants has attracted a lot of interest in the research field to improve the stability of aquatic products. Incubating herring co-products in 0.5% rosemary extract before ensilaging inhibited the rise in peroxide value (PV) and 2-thiobarbituric acid reactive substances (TBARS) (Sajib et al., 2021). Further, lingonberry press-cake, apple pomace and seaweed *Ulva fenestrate* decreased lipid oxidation during pH-shift processing and ice storage of herring and salmon co-products, although the lingonberry press cake also changed the colour of the protein isolate significantly (Zhang et al., 2022). Natural additives, such as CO_2-extracted plant extracts and berry press cakes from juice processing can also be used in fish mince to reduce lipid oxidation, and they may function better than conventionally used synthetic antioxidants (Damerau et al., 2020b). However, their concentration in the fish minces need to be optimised to ensure consumer acceptance.

Environmental toxins, such as dioxins and polychlorinated biphenyl compounds, have been a big concern over the use of Baltic herring. The levels of these toxins have decreased during the 21st century to a level that does not pose a threat to the general population any longer (Airaksinen et al., 2014). The levels of polychlorinated dioxins and furans, and polychlorinated biphenyl compounds, have decreased by 80%, from approx. 20 to 5 pg/g fresh weight (fw) (expressed as toxic equivalence quantity) during 1978–2009. Dioxin values of 0.5 to 4.8 pg/g were recently reported in Baltic herring fillets, where the study raw material consisted of a pool of 10–20 fish caught from different locations in the Baltic Sea, and the mean length of the caught fish was approx. 15–16 cm (Rajasilta et al., 2022). The maximum level for these contaminants in fishery products is 6.5 pg/g fw set by the Commission Regulation (Commission Regulation 2006/1881/EC, 2006).

2.5 Green processing technologies

Green techniques have emerged in order to reduce solvents, time, and energy consumption in the extraction of natural compounds (Picot-Allain et al., 2021).

The development of environmentally friendly extraction and processing methods is crucial for enhancing processing efficiency, improving the quality of food products, and minimizing the environmental impact of the processes (Chemat et al., 2012). Marsol-Vall et al. (2021) conducted a review of different green technologies for producing oils rich in *n*−3 PUFAs intended for human consumption. Several green methods, such as supercritical fluid and pressurized liquid extraction, EAAE, and fermentation, have shown to enhance oil quality and stability by preserving natural antioxidants and reducing oxidation when utilising marine sources, such as fish and algae (Gallego et al., 2018; Ozogul et al., 2018; Yang et al., 2011). Fish oil is most commonly produced by wet pressing, which involves heating to coagulate the proteins, allowing the liberation of oil and water, and separation of the oil containing liquid and solid material by pressing. The oil is removed from the sludge by centrifugation, and fish meal is achieved as another product in the process. Conventional methods of producing fish oil can lead to the degradation of labile PUFAs as a result of the application of harsh conditions, such as high temperatures (Fournier et al., 2006). In some processes, solvents, such as hexane, acetone, ethanol or isopropanol are used, which can leave harmful residues in the final product and cause environmental pollutions.

In fish oil extraction, supercritical fluid extraction requires high initial investment and maintenance costs due to the specialised equipment. However, the processing may be profitable for producing high-quality omega-3 fish oil, as reviewed by Jamalluddin et al. (2022). Pressurised liquid extraction, also known as accelerated solvent extraction, or pressurised fluid extraction, can be used to extract lipids using water or ethanol. Accelerated solvent extraction has shown to improve the antioxidant capacity of rainbow trout and sole skin and head extracts, when used as a pre-treatment (Wang et al., 2021). However, most of the studies on pressurised liquid extraction in the production of omega-3 oils focus on microalgae, instead of fish (Gallego et al., 2018; Pieber et al., 2012; Señoráns et al., 2020). Fermentation, on the other hand, is commonly used method to treat fish and fish co-products, especially in the production of many traditional foods, such as the Swedish surströmming, or fish sauce and other products in Asia. The challenges with fermentation are mostly related to contaminations from pathogenic micro-organisms, but also related to the current trends in foods: consumers today prefer mild taste and low salt products, whereas traditional fermentation techniques produce foods with strong flavours (Martí-Quijal et al., 2020a; Rajauria et al., 2016). One of the most studied techniques to extract oils, and to produce protein hydrolysates is EAAE, which is described in detail in chapter 2.5.2.

In addition to oil, green extraction techniques, such as enzymatic protein hydrolysis, fermentation, and pH-shift process can be used to produce a range of

protein products with different functionalities. Enzymatic hydrolysis, ensilaging, and fermentation result in hydrolysed proteins and peptides that can have bioactive functions (Mäkinen et al., 2022; Martí-Quijal et al., 2020b; Sajib et al., 2020). The pH-shift process produces proteins in their native folding, which can have good gelling and water-holding capacities (Abdollahi and Undeland, 2019; Gehring et al., 2011). Different techniques result in products with different functionalities, for example, surimi, protein concentrate, isolate or hydrolysate, and collagen. Traditional surimi production consumes a lot of water and usually results in a loss of myofibrillar proteins due to the repeated washing of the fish mince. Protein concentrates and hydrolysates can be produced from the fish meal after wet pressing, but they can also be produced by silaging, fermentation, acid or alkaline hydrolysis or EAAE. The pH-shift process is described in detail in chapter 2.5.1, whereas applications of different protein fractions are described in chapter 2.6.

2.5.1 pH-shift

pH-shift process, which is also called isoelectric precipitation, is a method used for extracting proteins. The method is based on changes in protein interactions at different pH levels (Figure 4). In alkaline conditions, proteins become negatively charged because the side chains of tyrosyl, tryptophanyl, cysteinyl, lysyl, argininyl or histidinyl deprotonate (Gehring et al., 2011). On the other hand, in the acidic process, aspartyl and glutamyl undergo protonation and thus, the proteins become positively charged. The charged proteins form weak hydrogen bonds with water, becoming soluble. At the isoelectric point of proteins (pI), the protein has zero net electrostatic charge and it favours protein-protein interactions that are formed by weak hydrophobic bonds, causing proteins to aggregate. The pI of fish proteins is close to 5.5 but it can vary slightly depending on the exact protein composition. The lowest solubility of herring has studied to be 5.4 (Marmon, 2012).

Production of protein isolates using the pH-shift method has been suggested as a potential way to provide added value for under-utilised fish species, such as small pelagic fishes. The process can be applied for both whole fish and co-products (Abdollahi and Undeland, 2019; Chomnawang and Yongsawatdigul, 2013; Marmon and Undeland, 2010; Surasani, 2018). In the pH-shift process, fish proteins are collected by first solubilising the proteins at high > 10.8 (alkaline process) or low < 3.0 (acidic process) pH, and then precipitating at the isoelectric point of the proteins. The alkaline process has been shown to be more favourable in terms of yield (Abdollahi and Undeland, 2019), lipid removal (Zhong et al., 2016), and gel forming ability (Phetsang et al., 2021). In addition, most studies have reported the alkaline process to result in protein isolates with

a lower degree of oxidation (Kristinsson and Hultin, 2004; Zhong et al., 2016), although contradictory findings have also been reported (Abdollahi et al., 2020).

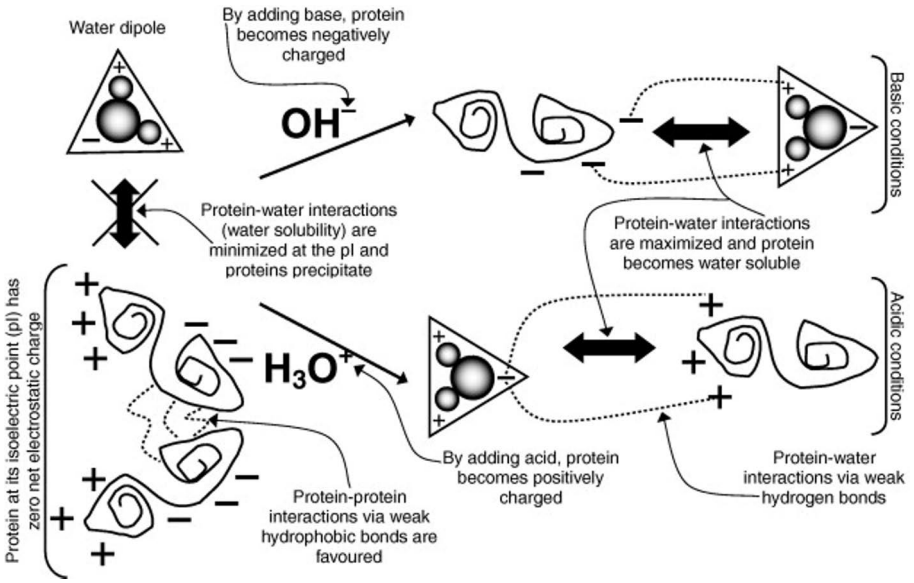

Figure 4. Protein interactions during the alkaline and acidic pH-shift process (Gehring et al., 2011).

Majority of the raw material lipids are removed during the pH-shift extraction of fish proteins. High removal of the lipids is desired as lipids are sensitive to oxidation, compromising stability and sensory properties. There is a significant correlation between the total lipid hydroperoxide content and rancid off-odour in the dark muscle of fish, and the extent of lipid removal is likely to improve the odour and flavour by removing fishy odours (Sohn et al., 2005). The pH-shift process was shown to remove 69% of lipids from herring light muscle and the removal efficiency was affected by holding time on ice before centrifugation — longer holding time resulted in better lipid removal efficiency (Undeland et al., 2002).

The benefit of pH-shift process is that proteins retain their functional properties, such as gelling and water-holding capacity, as the process does not involve heating. The pH-shift process is seen as an alternative for the conventional production of surimi, in which the fish muscle is first mechanically separated and then washed, leading to a partial loss of soluble proteins (Nolsøe and Undeland, 2009). The pH-shift has also applied to recover the proteins lost in the surimi washing, resulting in a precipitation of 63% of the proteins (Iwashita et al., 2016). Chang et al. (2017) compared surimi washing and both acidic and alkaline pH-shift processes in the recovery of bighead carp proteins. The alkaline process led to a higher breaking force than the conventional surimi

washing process, but the deformation value was lower. It was noticed that especially myosin exhibited conformational changes, which affected the rheological properties of the gels extracted by the pH-shift. Further, the surimi had higher whiteness value than the gels produced by the pH-shift due to the loss of some pigment proteins.

Protein recovery from the pH-shift process is largely dependent on the fish species and fish parts (Table 1). The lowest recovery using acidic processing has been reported for tilapia frames (16.8%) (Chomnawang and Yongsawatdigul, 2013), while the best recovery has been achieved with Atlantic croaker (78.7%) (Kristinsson and Liang, 2006). In alkaline processing, on the other hand, the lowest reported recovery has been 19.2% with tilapia frames (Chomnawang and Yongsawatdigul, 2013), while the best recovery has been 74% with herring frames (van Berlo et al., 2023). In studies comparing acidic and alkaline processing using the same raw material, acidic processing seems to result in higher protein yields with Atlantic croaker (Kristinsson and Liang, 2006) and gutted herring (Marmon and Undeland, 2010), but opposite results were observed with cod, salmon, and herring (Abdollahi and Undeland, 2019), as well as silver carp co-products (Zhong et al., 2016).

The high content of dark muscle in Baltic herring is challenging for the colour and texture of potential applications. Firstly, dark muscle tissue contains more sarcoplasmic proteins and lipids, which may interfere with gelation properties (Abdollahi and Undeland, 2019). The high heme pigment content can also cause a darker colour in the extracted protein isolate. Alkaline processing seems to also produce darker FPIs than the acidic processing (Chen and Jaczynski, 2007; Kristinsson and Liang, 2006; Marmon and Undeland, 2010; Panpipat and Chaijan, 2017; Yongsawatdigul and Park, 2004), although opposite results have also been reported (Zhong et al., 2016). Both processes remove pigments and lead to increased whiteness compared to the raw material, but the acidic process might be better at removing or destructing pigments, such as melanin, myoglobin and haemoglobin. It has been suggested that an addition of sodium chloride could increase the whiteness of FPIs due to the light scattering of water entrapped in the gel matrix. However in their study, Panpipat and Chaijan (2017) saw no effect on colour with or without added sodium chloride. Van Berlo et al. (2023) used different fractions of herring to see their effect on gelation and colour: gutted herring led to a clearly lighter coloured FPI (57.4) than other parts, especially head (37.5), which is rich in heme. The finding highlights the importance of selecting suitable raw materials to the process.

Table 1. pH-shift process (isoelectric precipitation) in the production of fish protein isolate.

Fish	*Fish part*	*Fish: water*	*pH (solubilisation)*	*pH (precipitation)*	*Centrifugation force (g)*	*Composition/ Protein recovery*	*Whiteness*	*Other effects*	*Reference*
Acidic pH-shift									
Atlantic croaker (*Micropogonias undulates*)	Fillet	1:9	2.5	5.5	10 000	78.7% protein recovery	75.2[a]	38.1% lipid reduction	Kristinsson and Liang, 2006
Bigeye snapper (*Priacanthus tayenus*)	Head	1:9	2.5	5.5	8 000		34.1	Gel-forming ability ↓ compared to alkaline process	Panpipat and Chaijan, 2017
Bighead carp (*Aristichthys nobilis*)	Gutted	1:6	3.0	5.5	10 000		68.7	Breaking force, deformation ↓ compared to alkaline process	Chang et al., 2017
Cod (*Gadus morhua*)	Head, tail & backbone	1:6	2.0	5.5	8 500	~20% protein recovery (estimated from graph)			Abdollahi and Undeland, 2019
	Head, tail & backbone	1:6	2.5	5.5	8 500	~25% protein recovery (estimated from graph)			Abdollahi and Undeland, 2019
	Head, tail & backbone	1:6	3.0	5.5	8 500	~15% protein recovery (estimated from graph)			Abdollahi and Undeland, 2019
Herring (*Clupea harengus*)	Light muscle	1:9	2.7	5.5	18 000	92% solubilisation of which 96% were recovered	63.0	69% lipid removal	Undeland et al., 2002
	Gutted	1:9	2.7	5.4	8 000	59.3% protein recovery	61.0		Marmon and Undeland, 2010
	Filleting co-products	1:6	2.0	5.5	8 500	~35% protein recovery (estimated from graph)			Abdollahi and Undeland, 2019
	Filleting co-products	1:6	2.5	5.5	8 500	~40% protein recovery (estimated from graph)			Abdollahi and Undeland, 2019
	Filleting co-products	1:6	3.0	5.5	8 500	~45% protein recovery (estimated from graph)			Abdollahi and Undeland, 2019
Rainbow trout (*Oncorhynchus mykiss*)	Filleting co-products	1:6	2.5	5.5	20 000		93.3		Chen and Jaczynski, 2007
Rockfish (*Sebastes flavidus*)	Muscle	1:9	2.5		10 000/6 000		64.8	Breaking force and deformation (mm) ↑ compared to alkaline process	Yongsawatdigul and Park, 2004
Salmon (*Salmo salar*)	Head, tail, backbone	1:6	2.0	5.5	8 500	~40% protein recovery (estimated from graph)			Abdollahi and Undeland, 2019
	Head, tail, backbone	1:6	2.5	5.5	8 500	~43% protein recovery (estimated from graph)			Abdollahi and Undeland, 2019

Fish	*Fish part*	*Fish: water*	*pH (solubilisation)*	*pH (precipitation)*	*Centrifugation force (g)*	*Composition/ Protein recovery*	*Whiteness*	*Other effects*	*Reference*
	Head, tail, backbone	1:6	3.0	5.5	8 500	~37% protein recovery (estimated from graph)			Abdollahi and Undeland, 2019
Silver carp (*Hypophthalmichthys molitrix*)	Processing co-products	1:9	2.0	5.5	10 000	78.8% protein, 8.3% fat, 4.6% ash (dry basis); 46.6% protein recovery	61.0	Protein recovery, lipid removal ↓ compared to alkaline process	Zhong et al., 2016
Silver carp (*Hypophthalmichthys molitrix*) + kilka (*Cluponella cultiventris*)	Mince from gutted fish	1:6	2.5	5.5	8 500			Haemoglobin removal ↓ compared to acidic process	Abdollahi et al., 2017
Tilapia	Frames	1:9	2.0	5.5	10 000	87.7% protein, 1.9% fat (dry basis); 16.8% protein recovery	14.7	Breaking force and deformation ↓ compared to alkaline process	Chomnawang and Yongsawatdigul, 2013
Alkaline pH-shift									
Atlantic croaker (*Micropogonias undulates*)	Fillet	1:9	11.0	5.5	10 000	65.0% protein recovery	72.6*	68.4% lipid reduction	Kristinsson and Liang, 2006
Bigeye snapper (*Priacanthus tayenus*)	Head	1:9	2.5	5.5	8 000		27.8	Gel-forming ability ↑ compared to acidic process	Panpipat and Chaijan, 2017
Bighead carp (*Aristichthys nobilis*)	Gutted	1:6	3.0	5.5	10 000		68.6	Breaking force, deformation ↑ compared to alkaline process	Chang et al., 2017
Cape hake (*Merluccius capensis*)	Portioning cutoffs					90% protein, 0.5% fat, 1.4% ash (dry basis)			Pires et al., 2011
Cod (*Gadus morhua*)	Head, tail & backbone	1:6	11.5	5.5	8 500		71.8	Glycine, proline ↓ compared to raw material, odour intensity ↓ compared to salmon and herring	Abdollahi and Undeland, 2018
Cod (*Gadus morhua*)	Head, tail & backbone	1:6	11.5	5.5	8 500	~55% protein recovery (estimated from graph)	N/A		Abdollahi and Undeland, 2019
Cod (*Gadus morhua*)	Head, tail & backbone	1:6	12.0	5.5	8 500	~62% protein recovery (estimated from graph)	57.0		Abdollahi and Undeland, 2019
Cod (*Gadus morhua*)	Head, tail & backbone	1:6	12.5	5.5	8 500	~45% protein recovery (estimated from graph)	55.7		Abdollahi and Undeland, 2019
Herring (*Clupea harengus*)	Light muscle	1:9	10.8	5.5	18 000	89% solubilisation of which 94% were recovered	65.5	69% lipid removal	Undeland et al., 2002
Herring (*Clupea harengus*)	Gutted	1:9	11.2	5.4	8 000	57.3% protein recovery	57.4		Marmon and Undeland, 2010

Fish	*Fish part*	*Fish: water*	*pH (solubilisation)*	*pH (precipitation)*	*Centrifugation force (g)*	*Composition/ Protein recovery*	*Whiteness*	*Other effects*	*Reference*
Herring (*Clupea harengus*)	Head, tail & backbone	1:6	12.0	5.5	8 500		54.8	Glycine, proline ↓ compared to raw material	Abdollahi and Undeland, 2018
Herring (*Clupea harengus*)	Filleting co-products	1:6	11.5	5.5	8 500	~45% protein recovery (estimated from graph)	N/A		Abdollahi and Undeland, 2019
Herring (*Clupea harengus*)	Filleting co-products	1:6	12.0	5.5	8 500	~55% protein recovery (estimated from graph)	49.8		Abdollahi and Undeland, 2019
Herring (*Clupea harengus*)	Filleting co-products	1:6	12.5	5.5	8 500	~65% protein recovery (estimated from graph)	51.9	Protein yield, heme removal, breaking force ↑ compared to solubilisation at 11.5	Abdollahi and Undeland, 2019
Herring (*Clupea harengus*)	Frames	1:6	11.5	5.5	8 000	74% protein recovery	49.8–50	Oxidation ↓, gel-forming capacity ↑ compared to other raw materials	van Berlo et al., 2023
Herring (*Clupea harengus*)	Head	1:6	11.5	5.5	8 000	39% protein recovery	37.5	Oxidation ↑ compared to other raw materials	van Berlo et al., 2023
Herring (*Clupea harengus*)	Head & frame	1:6	11.5	5.5	8 000	61% protein recovery	43.2		van Berlo et al., 2023
Herring (*Clupea harengus*)	Head, frame & tail	1:6	11.5	5.5	8 000	50% protein recovery	46.7		van Berlo et al., 2023
Herring (*Clupea harengus*)	Co-products	1:6	11.5	5.5	8 000	52% protein recovery	46.1–50.5		van Berlo et al., 2023
Mackerel (*Rastrelliger kanagurta*)	Mince from fillet	1:9	10.8	5.5			63.6	Surimi prepared with conventional washing process showed better gelling properties	Chaijan et al., 2006
Rainbow trout (*Oncorhynchus mykiss*)	Processing co-products	1:6	13.0	5.5	20 000		90.7		Chen and Jaczynski, 2007
Rockfish (*Sebastes flavidus*)	Muscle	1:9	2.5	11.0	10 000/6 000		59.2	Breaking force and deformation (mm) ↓ compared to alkaline process	Yongsawatdigul and Park, 2004
Salmon (*Salmo salar*)	Head, tail, backbone	1:6	12.0	5.5	8 500		66.1	Glycine, proline ↓ compared to raw material	Abdollahi and Undeland, 2018
Salmon (*Salmo salar*)	Head, tail, backbone	1:6	11.5	5.5	8 500	~65% protein recovery (estimated from graph)	N/A		Abdollahi and Undeland, 2019
Salmon (*Salmo salar*)	Head, tail, backbone	1:6	12.0	5.5	8 500	~70% protein recovery (estimated from graph)	67.2		Abdollahi and Undeland, 2019
Salmon (*Salmo salar*)	Head, tail, backbone	1:6	12.5	5.5	8 500	~75% protein recovery (estimated from graph)	64.3	Protein yield ↑, breaking force ↓, colour ↓ compared to solubilisation at 11.5	Abdollahi and Undeland, 2019

Fish	*Fish part*	*Fish: water*	*pH (solubilisation)*	*pH (precipitation)*	*Centrifugation force (g)*	*Composition/ Protein recovery*	*Whiteness*	*Other effects*	*Reference*
Sardine (*Sardinella gibbosa*)	Mince from fillet	1:9	10.8	5.5			65.7	Prewashing + alkaline processing removed myoglobin effectively Surimi prepared with conventional washing process showed better gelling properties	Chaijan et al., 2006
Silver carp (*Hypophthalmichthys molitrix*)	Processing co-products	1:9	12.0	5.5	10 000	82.2% protein, 6.2% fat, 4.3% ash (dry basis); 70.9% protein recovery	68.5	Protein recovery, lipid removal ↑ compared to alkali-made isolate	Zhong et al., 2016
Silver carp (*Hypophthalmichthys molitrix*) + kilka (*Cluponella cultiventric*)	Mince from gutted fish	1:6	11.5	5.5	8 500			Hb removal ↑ compared to acidic process	Abdollahi et al., 2017
Tilapia	Frames	1:9	12.0	5.5	10 000	90.0% protein, 1.3% fat (dry basis); 19.2% protein recovery	9.5	Breaking force and deformation ↑ compared to alkali-made gel	Chomnawang and Yongsawatdigul, 2013

* Expressed as L* value, not whiteness.

2.5.2 Enzyme-assisted aqueous extraction

EAAE is typically used to produce hydrolysed proteins, but it has also been studied in the extraction of oil (Table 2) (Araujo et al., 2021; Bruno et al., 2019; Carvajal et al., 2014b; de Oliveira et al., 2017; Gbogouri et al., 2006; Głowacz-Różyńska et al., 2016; Hathwar et al., 2011; Mbatia et al., 2010a; Šližyte et al., 2018, 2005). In addition to whole fish, it can also be applied to fish processing co-products, such as heads, fins, skin, liver, and viscera (Głowacz-Różyńska et al., 2016; Šližyte et al., 2018, 2005; Wang et al., 2020). The utilisation of fish processing co-products (also called side-streams or by-products) is important for the circular economy approach.

In the production of fish oil, EAAE utilises protease enzymes to break down the raw material with mild temperature conditions and an appropriate fish/water ratio to maximise the extraction efficiency. Some of the most commonly studied commercial proteases are Alcalase, Bromelain, Corolase, Flavourzyme, Neutrase, Papain and Protamex. Proteolytic enzymes hydrolyse proteins, therefore causing a breakdown of tissue, and consequent release of cellular contents, including lipids, into the matrix. Different phases, such as lipids, soluble peptides, and insoluble material can then be separated by centrifuging. Industrial processing may not be as efficient in terms of oil recovery and quality compared to laboratory scale processes. A study by Vázquez et al. (2020) determined that the oil recovery was considerably lower in an industrial pilot plant compared to laboratory scale experiments, due to the formation of emulsion between the liberated oils and hydrolysed proteins. Additionally, the utilisation of a tricanter instead of a centrifuge resulted in inadequate separation of the oil phase, likely due to its lower speed.

Optimisation of the hydrolysis process is needed for specific types of raw materials to improve the recovery and quality of the *n*−3 PUFA rich oils. The hydrolysis process can be significantly influenced by various factors related to the composition and quality of the raw materials. These include, for example, protein and lipid contents, as well as the levels of pro-oxidants and anti-oxidants. In some studies, EAAE resulted in an increased lipid oxidation compared to thermal treatment (Carvajal et al., 2014a). To mitigate oxidation levels, the incorporation of antioxidants before and during the processing (Carvajal et al., 2014a) or conducting the process in oxygen-free conditions in combination with antioxidants has been found to be effective (Sarteshnizi et al., 2019).

Water-addition is an important parameter of EAAE. According to Šližyte et al. (2018), extraction without added water resulted in the lowest oil recovery but the least amount of emulsion, while Y. Liu et al. (2021) observed the opposite effect with salmon co-products. In their study, extraction without water addition led to the best recovery and highest quality of oil. Emulsion formation is undesirable in the oil extraction as it is formed by lipids, mainly TAGs, and peptides, therefore trapping

some of the oil which may lead to a loss of important *n*−3 FAs (Šližyte et al., 2004). While TAGs are found from the emulsion phase, part of the phospholipids end up in the protein-rich phases, such as sludge. In some studies, almost all phospholipids had disappeared from the oils produced by EAAE. In a study using herring, the raw material contained 11.2–11.4% of phospholipids while the oils contained only 0.1–0.5% (Carvajal et al., 2014b). On the other hand, the sediment phase from the hydrolysis contained 30.1–40.3% of phospholipids out of all lipids. The used enzyme affected the results as well: the sediments produced by hydrolysis with Papain and Bromelain contained more PC (81.0%) compared to Alcalase (69.6%). Phospholipids are considered to be excellent emulsifiers, and therefore it is interesting, that some studies have rather identified TAGs as the main lipids in emulsion-phases. However, there are no conclusive studies identifying how much of each lipid class is found in each phase after EAAE, and why some fish species and raw-materials lead to more emulsion formation than others.

According to de Oliveira et al. (2017), Alcalase has demonstrated better oil recovery rates in extracting oil from tuna co-products compared to solvent and wet reduction methods. The crude oil obtained through Alcalase contained a higher proportion of PUFAs, and lower oxidation levels when compared to conventional methods. Additionally, Gbogouri et al. (2006) found that Alcalase outperformed Neutrase and Protamex in extracting oil from salmon heads, while Y. Liu et al. (2021) reported better performance of Alcalase compared to Flavourzyme and SEBPro in combined extraction from salmon heads and bones. Moreover, Wang et al. (2020) observed that Alcalase was more effective than pepsin and trypsin (though not as effective as papain) in extracting oil from Cobia liver. Each protease enzyme has specificity for peptide bonds adjacent to certain amino acid residues, which results in different kinds of peptides. This specificity can then affect the efficiency of the release of cellular contents, such as lipids.

The study by Bruno et al. (2019) investigated the pre-treatment of rohu (*Labeo rohita*) heads using microwave and ultrasound techniques. The results showed that both microwave and ultrasound pre-treatments improved oil recovery rates. The control sample without pre-treatment extracted 55.9% of the lipids, while the microwave and ultrasound pre-treatments achieved recovery rates of 69.8% and 68.1%, respectively. The ultrasound pre-treatment also enhanced the PUFA content. However, microwave pre-treatment negatively affected the oxidative stability of the oil, increasing PV and AV significantly. Scanning electron microscopy analysis revealed that the pre-treated samples had more disrupted cells compared to the control sample, leading to an increased matrix porosity and facilitating protein hydrolysis and subsequent release of intracellular lipids. Ultrasound was found to be more efficient than microwave, as microwave-induced heat caused protein unfolding and aggregation, interfering with the protein hydrolysis.

Despite the potential increase in costs, enzymatic treatments are being more commonly employed in industrial scale to separate oils and proteins. However, EAAE changes the protein structure and can potentially lead to oxidation and/or emulsion formation, which is why innovative solutions are needed to address these challenges and to obtain value-added products from the oil and protein fractions.

Table 2. Enzyme-assisted aqueous extraction in the extraction of oil from different fish species.

Fish	*Fish part*	*Pre-treatment/ additions*	*Enzyme*	*Enzyme concentration (%)*	*Extraction time (h)*	*Extraction temperature (°C)*	*Oil recovery (%)*	*Other effects*	*Reference*
Catla (*Catla catla*) + rohu (*Labeo rohita*)	General visceral co-products		Protease-P-Amano6	0.5	2	40	74.9		Hathwar et al., 2011
Catla (*Catla catla*) + rohu (*Labeo rohita*)	General visceral co-products		Alcalase®	0.5	2	40	61.7		Hathwar et al., 2011
Catla (*Catla catla*) + rohu (*Labeo rohita*)	General visceral co-products		Protex 7L®	0.5	2	40	~70 (estimated from graph)		Hathwar et al., 2011
Catla (*Catla catla*) + rohu (*Labeo rohita*)	General visceral co-products		Neutrase®	0.5	2	40	~64 (estimated from graph)	Highest acid value compared to other enzymes	Hathwar et al., 2011
Cobia (*Rachycentron canadum*)	Liver		Alcalase®	0.5	2	50	32.7		Wang et al., 2020
Cobia (*Rachycentron canadum*)	Liver		Papain	0.5	2	30	37.0		Wang et al., 2020
Cobia (*Rachycentron canadum*)	Liver		Pepsin	0.5	2	37	16.0		Wang et al., 2020
Cobia (*Rachycentron canadum*)	Liver		Trypsin	0.5	2	37	8.4		Wang et al., 2020
Herring (Clupea harengus)	Heads, tails, belly flaps, backbones, and viscera		Alcalase®2.4 L FG	0.1	1	55	N/A	FFA content 0.4%	Carvajal et al., 2014b
Herring (*Clupea harengus*)	Heads, tails, belly flaps, backbones, and viscera		Bromelain + Papain	0.05 + 0.05	1	55	N/A	FFA content 0.2%	Carvajal et al., 2014b
Mixed fish material	Medium-sized fish heads (e.g., salmon), skins, viscera, mangled muscles of fish, small fishes, molluscs, such as squid and mussels		Alcalase®2.4 L	0.94–4.68 AU/1	3	50	75% with the highest enzyme concentration		Araujo et al., 2021

Fish	*Fish part*	*Pre-treatment/ additions*	*Enzyme*	*Enzyme concentration (%)*	*Extraction time (h)*	*Extraction temperature (°C)*	*Oil recovery (%)*	*Other effects*	*Reference*
Nile perch (*Lates niloticus*)	Head		Protex 30L	0.05	1	55	81.0		Mbatia et al., 2010b
Rainbow trout (*Oncorhynchus mykiss*)	Head		Bromelain + Papain	0.05	1	50	89.6		Kvangarsnes et al., 2021
Rainbow trout (*Oncorhynchus mykiss*)	Head		Bromelain + Papain + BHT (antioxidant)	0.05	1	50	92.0		Kvangarsnes et al., 2021
Rainbow trout (*Oncorhynchus mykiss*)	Head	Addition of H_2O_2 and Fe2+, stored for 1 week at 4 °C	Bromelain + Papain	0.05 + 0.05	1	50	93.7	Oxidation ↑ compared to non-oxidised samples	Kvangarsnes et al., 2021
Rainbow trout (*Oncorhynchus mykiss*)	Head	Addition of H_2O_2, Fe2+ and BHT, stored for 1 week at 4 °C	Bromelain + Papain	0.05 + 0.05	1	50	93.2		Kvangarsnes et al., 2021
Rohu (*Labeo rohita*)	Head		Protamex®	1.0	2	55	55.8		Bruno et al., 2019
Rohu (*Labeo rohita*)	Head	Thermal treatment	Protamex®	1.0	2	55	32.0–39.0	PV and AV ↓	Bruno et al., 2019
Rohu (*Labeo rohita*)	Head	Microwave	Protamex®	1.0	2	55	60.5–69.8	PUFA, PV, TBARS, AV and FFAs ↑	Bruno et al., 2019
Rohu (*Labeo rohita*)	Head	Ultrasound	Protamex®	1.0	2	55	58.7–67.5	MUFA, PUFA and PLs ↑	Bruno et al., 2019
Salmon (*Salmo salar*)	Head		Alcalase®2.4L	5.0	2	55	91.2		Gbogouri et al., 2006
Salmon (*Salmo salar*)	Head		Neutrase®0.8L	5.0	2	50	67.0		Gbogouri et al., 2006
Salmon (*Salmo salar*)	Head		Protamex 90 AU/kg		2	50	67.9		Gbogouri et al., 2006
Salmon (*Salmo salar*)	Head		Bromelain	0.5	1	55	65.0		Mbatia et al., 2010b

Fish	*Fish part*	*Pre-treatment/ additions*	*Enzyme*	*Enzyme concentration (%)*	*Extraction time (h)*	*Extraction temperature (°C)*	*Oil recovery (%)*	*Other effects*	*Reference*
Salmon (*Salmo salar*)	Head		Protex 30L	0.05	1	55	88.0		Mbatia et al., 2010b
Salmon (*Salmo salar*)	Head		Alcalase®2.4L	5.0	2	55	72.1	PLs ↑ compared to heat extraction	Głowacz-Różyńska et al., 2016
Salmon (*Salmo salar*)	Backbone	Thermal treatment	Corolase® PP	0.1	0–2	50	67 + 13.1–31.4*		Šližyte et al., 2018
Salmon (*Salmo salar*)	Backbone	Thermal treatment	Corolase® 7089	0.1	0–2	50	67 + 13.7–22.9*		Šližyte et al., 2018
Salmon (*Salmo salar*)	Backbone	Thermal treatment	Protamex®	0.1	0–2	50	67 + 13.7–21.1*	PV ↑ compared to other enzymes	Šližyte et al., 2018
Salmon (*Salmo salar*)	Backbone	Thermal treatment	Papain FG + Bromelain	0.05 + 0.05	0–2	50	67 + 13.7–26.3*		Šližyte et al., 2018
Salmon (*Salmo salar*)	Backbone	Thermal treatment	Protex 6L	0.1	0–2	50	67 + 15.4–25.1*		Šližyte et al., 2018
Salmon (*Salmo salar*)	Backbone	Thermal treatment	Sea-B-Zyme L 200	0.1	0–2	50	67 + 11.4–25.7*		Šližyte et al., 2018
Salmon (*Salmo salar*)	Backbone	Thermal treatment	Trypsin	0.1	0–2	50	67 + 11.4–28.0*		Šližyte et al., 2018
Salmon (*Salmo salar*)	Head, frames, viscera		Alcalase 2.4 L	0.1/1.0	2/4	55		Water addition, 1.0% enzyme, 4h extraction time → PUFA ↓, PLs ↑, *n*−3 FAs ↑	Y. Liu et al., 2020
Salmon (*Salmo salar*)	Head and frames or viscera		Alcalase 2.4 L	0.1/1.0	2/4	55	Head and frames: 96.3% (1% enzyme, 4h extraction)	Oxidation ↓ compared to Flavourzyme and SEBPro. Water addition led to ↓ oil yield and quality	Y. Liu et al., 2021
Salmon (*Salmo salar*)	Heads and frames or viscera		Flavourzyme	0.1/1.0	2/4	50	Viscera: 93.6% (1% enzyme, 2h extraction, no water addition)	Water addition led to ↓ oil yield and quality	Y. Liu et al., 2021

Fish	*Fish part*	*Pre-treatment/ additions*	*Enzyme*	*Enzyme concentration (%)*	*Extraction time (h)*	*Extraction temperature (°C)*	*Oil recovery (%)*	*Other effects*	*Reference*
Salmon (*Salmo salar*)	Heads and frames or viscera		SEBPro	0.1/1.0	2/4	50		Water addition led to ↓ oil yield and quality	Y. Liu et al., 2021
Salmon (*Salmo salar*)	Heads and frames		Immobilised Alcalase	44.4 U/mL	1	65	88.3	TOTOX 5.7; immobilised enzyme can be reused up to 3 times	Y. Liu and Dave, 2022
Salmon (*Salmo salar*)	Heads and frames		Alcalase 2.4 L	0.1	1	65		TOTOX 8.9	Y. Liu and Dave, 2022
Yellowfin tuna (*Thunnus albacares*)	Head		Alcalase	0.05	2	60		EPA and DHA ↑ compared to solvent-extraction and wet pressing	de Oliveira et al., 2017

* 67% of the total oil was separated after thermal pre-treatment by centrifugation. The percentages after that value indicate how much of the remaining fat was able to be recovered during 0–2 h of the hydrolysis.

The abbreviation used in the table are: AV = *para*-anisidine value EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, FFA = free fatty acid, n−3 FA = omega-3 fatty acid, MUFA = monounsaturated fatty acid, PL = phospholipid, PUFA = polyunsaturated fatty acid, PV = peroxide value, and TOTOX = total oxidation value.

2.6 Different fractions in food

Different green extraction techniques result in fractions with different functionalities, as well as nutritional and sensory properties. Protein fractions from enzymatic hydrolysis, ensilaging and fermentation can be processed into wet or dried hydrolysates, concentrates, collagen, and gelatine, while the pH-shift results in protein isolates with good gel-forming capacities. Potential applications of protein isolate produced by the pH-shift include: the use as an emulsifier (Petursson et al., 2004), as a functional food ingredient to increase the nutritional value of ice cream (Shaviklo et al., 2011), or as the main ingredient in products such as fish balls or fish burgers (Shaviklo et al., 2016, 2010), and sausages (Moosavi-Nasab et al., 2018; Pires et al., 2009) to replace surimi or minced fish (Table 3). Some factors limiting the use of FPI, and related products, are challenges in industrial up-scaling, production costs, choosing the right solubilisation and precipitation pH values, lipid and protein oxidation during processing, choosing the right amount in food products to minimize or completely avoid negative effect on odour and texture, storage stability and sensory quality (Shaviklo and Etemadian, 2019). Fish protein isolate is especially sensitive to oxidation and protein degradation during frozen storage, therefore, the addition of natural antioxidants and cryoprotectants should be considered.

Dried protein concentrates and hydrolysates may be used as nutraceutical ingredients, as many of them have shown biological activities or emulsifying, stabilizing, foaming or gelling properties, which have a vast range of applications in the food industry (review by Gao et al., 2021). Gelatine and collagen are other products, which can be extracted from fish skin, scales and bones. A market for different dietary supplementations with fish collagen already exists, but other applications, such as wound healing membranes, which have shown positive effects in the healing process in *in vivo* studies, have also attracted interest (de Souza et al., 2022; Subhan et al., 2021). Fish gelatine, which is derived from collagen, can be used in baking and other food applications instead of the typical gelatine produced from mammals. Also, gelatine can be used to make edible films that could be utilised in food packaging, as well as clarification of juices, encapsulation, and production of sweets (Alfaro et al., 2014).

Table 3. Examples of the addition of fish protein isolate in different food models.

Fish ingredient	***Food model***	***Effect***	***Ref.***
Cape hake by-products, protein isolate from alkaline pH-shift	Frankfurter-type sausage	Sausages with 20% of protein, 5% pork fat, and 27.5% water; and with 20% of protein, 17.5% pork fat, and 20% were best in terms of colour, texture and sensory evaluation. These sausages had lighter colour and softer texture than pork sausages. Addition of 3.5% cod liver oil to the first formulation increased EPA and DHA but did not affect flavour or overall acceptability.	Pires et al., 2009
Cod protein	Oil-in-water emulsion (5% wt corn oil)	0.2% wt of protein produced at pH 3.0 produced an emulsion that was stable to droplet flocculation and creaming at pH≤4 and NaCl concentrations ≤150 mM when stored at room temperature.	Petursson et al., 2004
Haddock cut-offs, fish protein isolate	Fried fish balls	Replacing 50% of fish ball mass with fish protein isolate caused better forming ability but grainy texture and more frozen storage flavour. Fish ball with only 25% of protein isolate was more similar to the control fish ball.	Shaviklo et al., 2010
Lantern fish, protein isolate from alkaline pH-shift	Sausage	Protein isolate was added at 2% and 4% concentrations. Sausage with 2% isolate showed food quality and sensory acceptance, whereas sausage with 4% isolate resulted in decreased acceptability and worse scores in texture, colour, flavour and odour. Sausage with 4% isolate showed higher scores in stiffness and adherence.	Moosavi-Nasab et al., 2018

Fish ingredient	*Food model*	*Effect*	*Ref.*
Saithe, freeze-dried protein from surimi	Ice cream	Freeze-dried protein powder was added at 0, 30 and 50 g/kg; ice creams with fortification had higher protein and solid-non-fat contents. After production, sensory quality, except colour, was similar to control. After two-month storage, sandiness increased, sweetness decreased and fish flavour and odour increased.	Shaviklo et al., 2011
Trout, gutted, protein isolate from pH-shift	Heat-set gel	Good texture and gelation properties.	Tahergorabi et al., 2012
Tuna, co-products, protein isolate	Fish burger	Burger with 20% tuna protein isolate with carp mince had similar sensory qualities after two-month storage compared to control burger with only carp mince. After four months, the burger with isolate showed degradation of lipids and proteins which increased rancid and frozen storage odours and flavours.	Shaviklo et al., 2016

Oil fractions, on the other hand, can be further processed to produce high-quality fish oils that can be processed into fish oil supplements. Fish oils can also be spray-dried and microencapsulated to be used in food applications. After refining, fish oil can be concentrated to have higher EPA and DHA contents, and the fatty acids are often transesterified from TAGs into ethyl esters, which are used for producing food supplements, mainly in the form of capsules (Yi et al., 2023). The global omega-3 market has been valued at 2.43 billion USD in 2022 (Grand View Research, Inc., 2023), but at the same time, it has been estimated that the global fish sources are currently not adequate to meet the demand for EPA and DHA (Salem and Eggersdorfer, 2015).

Besides capsules, fish oil can be incorporated into foods to increase the omega-3 content (Table 4). In one study, fish oil was coated with either pea, soy or sunflower proteins in combination with whey protein or maltodextrin in spray-drying processes and used in chocolate and cookies (Damerau et al., 2022a). The whey-protein formulations were best during storage in terms of sensory properties and oxidative stability. In another study, micro-encapsulated fish oil together with garlic essential oil was incorporated into chicken nuggets, in which it inhibited lipid oxidation and microbial growth, most likely due to the effects of the garlic essential oil (Raeisi et al., 2021). Addition of 4% (w/w) of fish oil + garlic essential oil was better accepted in sensory study compared to adding 8%. Microencapsulated salmon oil was also added to strawberry-flavoured yogurt, however, sensory evaluation was not conducted (Estrada et al., 2011). The coating material and processing parameters used in microencapsulation affect oil droplet sizes, stability and volatile compounds (Damerau et al., 2022b). For example, rice protein has shown to be a good matrix for microencapsulation of Baltic herring oil. At pH 3, best emulsion efficiency was achieved, but it also led to the lowest oxidative stability. The best oxidative stability was achieved when emulsions were prepared at pH 6.5 with either of the coating materials used in the study (rice protein concentrate and whey protein concentrate). In addition to encapsulation, fish oil has been added to food products, such as sausages, as an emulsion to increase the $n-3$ PUFA content, and improve the $n-6/n-3$ ratio (Cáceres et al., 2008; Valencia et al., 2008).

Table 4. Examples of fish oil incorporated into food models.

Fish species/oil source	*Pre-treatment*	*Food model*	*Effect*	*Ref.*
Cod liver oil	Encapsulation by spray-drying with pea, soy or sunflower protein in combination with whey protein or maltodextrin	Chocolate and cookies	Whey protein improved oxidative stability compared to maltodextrin, but whey caused the number of cracks in outer shell of the particles. In both food models, pea protein resembled the reference product best, but fortification caused fishy flavour and odour, and affected volatile profiles with all formulations.	Damerau et al., 2022a
Fish oil (Sigma-Aldrich) + garlic essential oil	Encapsulation with chitosan and Tween 80	Chicken nuggets	Addition of encapsulated fish oil + garlic essential oil at 4 and 8% w/w delayed lipid oxidation and microbial spoilage. 8% sample gave the best antioxidative and antimicrobial properties but 4% was better accepted by sensory study.	Raeisi et al., 2021
Fish oil (omega-3 18/12 from Lysi, Iceland)	Emulsification with soya protein	Pork sausage	Fish oil increased EPA and DHA significantly but affected flavour and acceptability. Addition of green tea catechins as an antioxidant improved the sensory properties	Valencia et al., 2008

Fish species/oil source	*Pre-treatment*	*Food model*	*Effect*	*Ref.*
Fish oil	Emulsification with caseinates and water	Mortadella (Spanish sausage)	Fish oil addition (1−6%) lead to a better *n*−6/*n*−3 ratio. High levels of fish oil caused a more compact structure analysed with scanning electron microscopy, and a slight increase in hardness and shear force. There were no significant changes in TBARs. All sausages were well accepted by sensory evaluation.	Cáceres et al., 2008
Salmon oil	Microencapsulated with Arabic gum, maltodextrin and water	Strawberry-flavoured yogurt	Emulsification and spray-drying increased oxidation of the oil. The addition of microencapsulated oil did not alter pH, colour or water-holding capacity of the yogurt. The *n*−3 fatty acid content decreased significantly during 4-week storage.	Estrada et al., 2011

2.7 Concluding remarks

Baltic herring is a good raw ingredient for different extraction applications due to its healthiness, being especially rich in *n*−3 PUFAs, and availability in the Baltic Sea. However, challenges regarding its high amount of dark muscle tissue, relatively small size, fluctuating lipid content and challenges with oxidation should be considered, when the extraction techniques and food applications are developed.

The pH-shift process is suitable for the extraction of proteins, which still retain their functional properties, such as gelling and water-holding capacity. The FPIs produced by the pH shift can be used in many types of food, for instance surimi-type gelled products and fish balls. Different fish species or fish parts have a big impact on what kind of FPI is produced in terms of colour, oxidation and gelling capability. Further, extraction parameters, such as precipitation and solubilisation pH-values, the addition of additives or antioxidants, as well as centrifugation speed affect the final product. Although some research has been conducted for the use of the pH-shift on protein extraction from herring, a close relative to Baltic herring, the resulting protein isolates have not been studied in food applications.

EAAE can be used to produce protein hydrolysates but also to extract oils. EAAE has shown high extraction efficiencies with many fish species and different fish parts, but the efficiency is dependent on the type of enzyme, fish to water ratio, pre-treatment, extraction time, and separation speed. Some studies have also shown that EAAE can result in high amounts of emulsion, which decreases the oil recovery rate. There is a lack of scientific literature on the efficiency of EAAE in the extraction of oil from Baltic herring, especially related to different extraction parameters. It is especially important to understand how seasonal changes affect the raw material and its behaviour during the extraction process.

3 AIMS OF THE STUDY

The aim of this thesis was to study two different environmentally friendly techniques to extract oils and proteins from Baltic herring. pH-Shift method was used to produce fish protein isolate, which was further applied to two different food models: surimi and fish balls. The suitability of the protein isolate was studied in terms of texture, colour and volatile compounds. EAAE was used to extract oils from whole Baltic herring and its filleting co-products. The effect of different enzymes, treatment times and other optimisation parameters on oil recovery, oxidation, and composition were assessed. Further, different techniques were studied to reduce emulsion formation during the hydrolysis.

The aims of the separate studies were:

I. To study the feasibility of pH-shift method in the production of fish protein isolate from gutted Baltic herring and its applications in food models.

II. To study the suitability of EAAE in the production of fish oil from Baltic herring and its filleting co-products, and the effect of different enzymes and treatment times in the composition and oxidative status of the oil.

III. To optimise the parameters of EAAE to improve oil recovery and quality of crude oil using whole Baltic herring, focusing especially on emulsion formation, oxidation and lipid composition including fatty acids and lipid classes.

4 MATERIALS AND METHODS

4.1 Raw materials

The raw materials used in the study were gutted and beheaded Baltic herring from Martin Kala Oy (Turku, Finland) caught in March 2019 (Study **I**), whole Baltic herring and filleting co-products caught in November 2019 (Study **II**), and whole Baltic herring caught in January 2021 and May 2022 (Study **III**).

4.2 pH-shift method for the production of fish protein isolate (Study I)

The preparation of Baltic herring protein isolate (FPI) using the pH-shift, and its use in food models is shown in Figure 5. The fish was stored at −80 °C and thawed at 4 °C overnight before the protein extraction. Defrosted fish were rinsed and homogenised with water (100 g fish + 800 g water) using a professional mixer (Raw Mix 2, Rawmix Oy, Helsinki, Finland). The pH-shift process was performed in a double-jacketed glass reactor (1 L, 100 mm diameter, Lenz Laborglas GmbH & Co. KG, Wertheim, Germany) cooled to 4 °C. Stirring (800 rpm) was achieved with a teflon plade impeller, and acid/base addition was conducted using a precision pump (Alaris Asena GH MK III, Becton, Dickinson and Company, Franklin Lakes, USA) at a constant rate of 30–40 mL/h. To solubilise the proteins, 10–15 mL of 1 M NaOH was added for approx. 15–25 minutes until the target pH 11.2 (± 0.05), chosen based on literature (Marmon & Undeland, 2010) and a pre-test on yield (data not shown), was reached. The homogenate was then centrifuged (Cellsep 6/720R, MSE, Nuaill´e, France) at 4000*g* for 20 minutes at 5 °C. The supernatant containing the solubilised muscle proteins was separated from the sediment and lipid emulsion layer by decanting and filtering through a 4-fold cotton cheesecloth (Decola, Jyvaskylä, Finland) and a metal sieve. Proteins were precipitated by adding 12–17 mL of 1 M HCl within approx. 20–30 min, until pH 5.4 (± 0.05) was reached, after which the mixing was continued for further 5–10 min. The mixture was centrifuged again (4000*g*, 20 min, 5 °C), and the precipitate was collected by removing the solid lipid emulsion layer and the supernatant by decanting. The sediment containing the precipitated proteins was collected as FPI, and cryoprotectants were added to the FPI according to intended use. For surimi-type gels, a total amount of 8% (w/w) of cryoprotectants (4% fructose and 4% sorbitol) was used, whereas for fish balls, a lower amount of 4% (w/w) (2% fructose and 2% sorbitol) was used. The pH of the isolate was adjusted to 7.0 using 4 M NaOH while mixing manually, after which it was frozen at −80 °C.

In addition, modifications to the process were studied to increase the whiteness value of the FPI. The first modification included precipitation of the proteins at pH 6.5, instead of 5.4, and the second modification was solubilisation at pH 11.5, instead of 11.2. Other stages of these processes were conducted as described above.

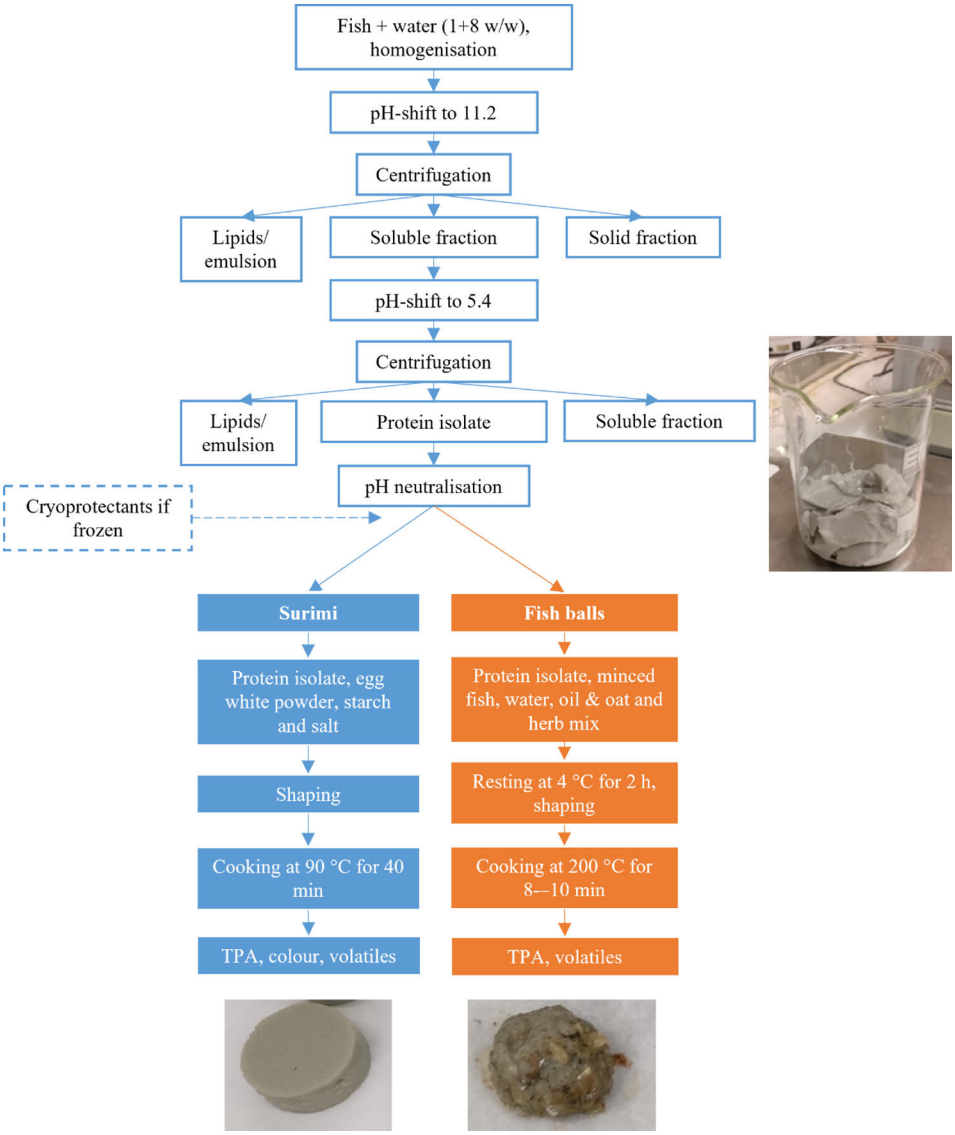

Figure 5. A flowchart of the production of fish protein isolate using pH-shift method, and its use in food models. TPA = texture profile analysis.

4.3 Food models using the protein isolate (Study I)

The FPI with a total content of 8% cryoprotectants (4% fructose and 4% sorbitol) was used to prepare the surimi-type gels, whereas the FPI with 4% of cryoprotectants (2% fructose and 2% sorbitol) was used as an ingredient in fish balls. Different amounts were used according to the typical dosage of cryoprotectants in surimi, but the amount was decreased for the fish ball application to reduce sweetness. For the surimi-type gels, the FPI (89.5% of total mixture) was mixed with 6.0% of potato starch, 3.0% of egg white powder (Munax Oy, Laitila, Finland), and 1.5% of table salt. Ingredients of the surimi-type gels were mixed for 3 × 10 seconds at full speed with Bamix® Mono (ESGE Ltd, Mettlen, Switzerland) at room temperature. The mass was cooked in 50 mL centrifuge tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) immersed in a water bath at 90 °C for 40 min. The surimi-type gels are later referred to as SPI (surimi-type gel from FPI), SPI6.5 (surimi-type gel from FPI produced by precipitation at pH 6.5 instead of 5.4), and SPI11.5 (surimi-type gel from FPI produced by solubilisation at pH 11.5).

To prepare the fish balls, different amounts of FPI (Table 5) were combined with a flavour and oat mixture (Kalaneuvos Oy, Sastamala, Finland), canola oil (Bunge Finland Oy, Raisio, Finland) and minced Baltic herring fillets (Martin Kala Oy, Turku, Finland). Freshly prepared fillets were brought to the laboratory on ice and minced using a food processor with a meat grinder attachment (Kenwood Limited, Havant, UK). Mixing of ingredients was conducted manually. The amount of minced fish, oil and dry ingredients were kept at constant weight for samples including 0–15% FPI, but their amounts were reduced in the sample containing 28 and 50% of the FPI. Compared to FB0, the amount of added water was reduced in all the fish ball mass samples with added FPI. The fish ball mixture was kept at 4 °C for 2 h prior to cooking. Fish balls (diameter approximately 3 cm, weight 15 g) were cooked in a 200 °C oven for 8–10 min, until the inside temperature reached 76 °C (± 2 °C). The cooked fish balls and surimi-type gels used for texture profile analysis (TPA) and volatile analysis were stored at 4 °C overnight after cooking, and their temperature was allowed to reach room temperature before TPA measurement. For other analyses the samples were stored at −80 °C.

Table 5. Compositions of fish ball mixtures.

Sample	*Protein isolate (w-%)*	*Baltic herring mince (w-%)*	*Canola oil (w-%)*	*Dry ingredients (w-%)*	*Water (w-%)*
FB0	0	55	10	7	28
FB5	5	55	10	7	23
FB15	15	55	10	7	13
FB28	28	52	9	7	4
FB50	50	38	7	5	0

4.3.1 Composition

The protein contents of the raw material and the FPI were analysed using the Kjeldahl method by the AOAC International (Latimer, 2016). The protein contents were determined using the nitrogen conversion factor of 6.25. The lipid content of the raw material and FPI was analysed by using a modified Folch extraction method (Folch et al., 1957). In the first phase separation, chloroform, methanol and 0.88% potassium chloride were added in a ratio of 32:16:15 (v/v/v). The lower phase containing lipids was further washed with KCl:MeOH (1:1, v/v) and evaporated in a rotary evaporator. The moisture contents of homogenised Baltic herring, FPI (2% sorbitol and 2% fructose) and fish balls with 0, 15 and 50% FPI added were determined by drying the samples in an oven at 105 °C to a constant weight (Memmert Loading Model 100–800, Germany).

4.3.2 Texture profile analysis

Texture profile analysis (TPA) was conducted using a QTS25 texture analyser (CNS Farnell Company, Borehamwood, UK), and measurements were carried out at room temperature. The samples were cut to 1.5 × 1.5 × 1.5 cm pieces and measured in triplicate. A cylindrical probe with a 3.8 cm diameter was used to penetrate the sample at the rate of 60 mm/min to a total deformation of 50% of the sample length. The compression cycle was repeated twice for each sample. The measured attributes were hardness, cohesiveness, springiness and chewiness. The prepared surimi-type gels and fish balls were compared to commercial reference samples (CS1, CS2, and CFB).

4.3.3 Colour

The whiteness of commercial surimi and the surimi-type gels prepared with the FPI were determined by an Eoptis CLM-194 colorimeter (EOPTIS SRL, Trento, Italy). The L*, a*, and b*, representing the black to white, red to green and yellow to blue axes, respectively, were used to determine whiteness (W) values by using the following formula:

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

4.3.4 Volatile compounds

Volatile compounds in fish balls and surimi-type gels were analysed with head space solid-phase microextraction combined with gas chromatograph and mass spectrometer (HS-SPME-GC–MS) using TRACE 1310 GC coupled with a ISQ7000 single quadrupole MS and TriPlus RSH autosampler (Thermo Fisher Scientific Waltham, MA, USA). The surimi-type gels and fish balls were cut to 1 cm^3 cubes. Incubation and extraction times of the samples were 20 and 30 minutes, respectively, while the samples were agitated at 40 °C. The fibre was cleaned at 250 °C for 2 minutes prior the extraction and 5 minutes after the extraction. The fibre (1 cm DVB/CAR/PDMS, Supelco Inc., St. Louis, MO, USA) was desorbed for 5 minutes at 240 °C in the injection port of the GC, which was operated in a splitless mode. Helium was used as a carrier gas in the gas chromatography at a flow rate of 1.4 mL/min. SPB-624 (60 m × 0.25 mm i.d., 1.4 µm) column was used for the separation of the compounds (Supelco Inc.). Oven temperature was held at 40 °C for 6 min, followed by an increase of 5 °C /min to 200 °C, and held there for 10 min. The MS was operated in electron ionisation mode. Transfer line and ion source temperatures were 220 °C and 250 °C, respectively, and the spectra were collected in a mass range of 40–300 amu. The data was processed using Chromeleon 7.0 (Thermo Scientific™), and the NIST database (https://webbook.nist.gov/chemistry/).

4.4 Enzyme-assisted aqueous extraction in the extraction of oil (Studies II & III)

Endo-proteases Alcalase®, Neutrase® and Protamex® (mixture of Alcalase and Neutrase) (Novozymes, Bagsvaerd, Denmark) were used in Studies **II** & **III**. The chosen enzymes are commercially available and they resulted in the highest oil recoveries in pre-testing. In Study **II**, two different extraction times (35 and 70 minutes) were used. The fish was chopped to 2 cm pieces, mixed with water and 0.4% of enzyme and heated to 55 °C, while agitation was set to 280 rpm. After the extraction, enzymes were inactivated at 90 °C for 15 minutes, after which the hydrolysates were cooled down on ice for 1 h. The samples were centrifuged at 4500 rpm for 20 minutes with cooling set to 4 °C (Avanti jxn-26, Beckman Coulter, Brea, CA, USA). The oil layers from each triplicate were pooled together to limit the number of samples for analyses. The combined oil sample was centrifuged again at 3000 rpm for 5 minutes (Eppendorf Centrifuge 5804, Eppendorf AG, Hamburg, Germany). The sample tubes were flushed with nitrogen gas and stored at −80 °C.

To improve the oil extraction yield, the optimisation parameters in the Study **III** included chopping size (mincing vs chopping with a knife) agitation speed during hydrolysis (100, 190 and 280 rpm), centrifugation temperature (chilled on ice for 1h, or soon after inactivation at 60–65 °C), extraction time (35, 70 or 105 minutes), and enzyme concentration (0.4, 1 and 2% of the fish weight, Neutrase and Protamex only). The fish raw material was defrosted at 4 °C overnight (16 ± 1 h). Then it was chopped or minced (Kenwood Titanium XL Chef, Kenwood Limited, Havant, UK) and mixed with water (1:1 w/w). The pH of the Alcalase-treated samples was adjusted from 7.0 to 9.0 (± 0.2) with 1 M sodium hydroxide. The samples were heated to 50 °C (Neutrase) or 55 °C (Alcalase and Protamex) in a water bath, and the enzyme was added once the hydrolysis temperature was reached. After the hydrolysis, the enzymes were inactivated at 90 °C for 15 min. The hydrolysates were centrifuged at 4500 rpm (Eppendorf 5810 R, Eppendorf AG) for 20 minutes after they had cooled down to approx. 60–65 °C. Oil and emulsion layers were collected by pipetting, centrifuged again at 3000 rpm for 5 minutes to remove water and impurities, the oil was collected in vials, flushed with nitrogen gas and stored at −80 °C (Study **III**).

Different methods were used to reduce or break emulsion to increase oil recovery rate in Study **III**. Protamex was chosen for the tests as it is a mixture of Alcalase and Neutrase, and would therefore be well suited to find the optimal process that works with all three enzyme products. The control treatment had an enzyme concentration of 0.4% and fish to water ratio of 1:1 (w/w). The methods for reducing or breaking emulsion were: reducing enzyme to 0.1% w/w, changing fish:water ratio to 2:1 (w/w), freezing the hydrolysate for overnight at −20 °C then thawing at RT for 1.5–2 h before centrifugation, adding 10 mL of ethanol (20% of the raw material weight) after inactivation, adding 2 mL of 0.9% sodium chloride solution before centrifugation, or adding 0.1% of citric acid (w/w) before the extraction.

Optimised parameters and best emulsion-breaking method were used with all three enzymes, later referred to as “samples with reduced emulsion” (Study **III**). The conditions were: 50 g of minced fish, 0.4% of enzyme, extraction time of 105 min, fish:water ratio 2:1 (w/w), and addition of 10 mL of ethanol (20% of the raw material weight) after the inactivation step.

4.5 Oxidation (Studies II & III)

4.5.1 Peroxide value (Studies II & III)

In Study **II**, PVs of the oil samples were determined with a titration-based method (AOCS official method Cd 8−53). For each analysis, 2.0 g of oil was

weighed and dissolved in 10 mL of chloroform in which 15 mL of glacial acetic acid and 1 mL of potassium iodide were added, and mixed for 1 minute. The flask was kept at room temperature protected from light for exactly 5 minutes, after which 75 mL of MQ-water was added. The mixture was titrated with 0.01 N sodium thiosulphate until colourless using starch solution (10 g/L) as indicator. The sodium thiosulphate was checked with freshly prepared starch solution before analysis: 10 mL of potassium iodate was placed in an Erlenmeyer bottle. 1.0 g of solid potassium iodide was added, followed by 5 mL of 2 N sodium chloride. The mixture was titrated with 0.01 N sodium thiosulphate until light yellow. 1 mL of starch (10 g/L) was added and the mixture was titrated until colourless.

In Study **III**, the PVs were determined by a spectrophotometric method (Lehtonen et al., 2011). Oil dilution in hexane containing 15 mg of the fish oil was pipetted into a glass tube, filled to 0.2 mL with hexane, then 9.8 mL of solvent mixture (methanol:1-decanol:hexane, 3:2:1) was added. After this, 50 µL of ammonium thiocyanate was added followed by a brief vortexing, then 50 µL of iron(II) chloride followed by vortexing for 5 seconds. After exactly 5 minutes of the iron (II) chloride addition, absorbance was measured at 500 nm. The PVs were quantified by using a standard line containing 0−40 µg of iron(III) chloride.

4.5.2 *p*-Anisidine value (Study II)

Para-anisidine values (AVs) in Study **II** were determined spectrophotometrically (AOCS official method Cd 18−90). For the analysis, 0.5 g of the oil sample was diluted in 25 mL of iso-octane, and 5 mL of the dilution was pipetted into a test tube followed by 1 mL of *p*-anisidine reagent and shaking. After exactly 10 minutes, the absorbance of the sample (A_s) was measured at 350 nm using iso-octane as a blank (A_b). The AV was calculated by using the following formula:

$$AV = \frac{25\ x\ (1.2A_s - A_b)}{m}$$

4.5.3 Volatile secondary oxidation products (Study II & III)

Volatile secondary oxidation products (VSOPs) of the extracted crude oils were analysed with head-space solid-phase micro extraction combined with gas chromatography and mass spectrometry (HS-HPME-GC-MS) based on the method from Damerau et al. (2020a). For the analysis, 50 mg (**II**) or 20 mg (**III**) of the oil sample diluted in *n*-hexane was pipetted in 10 mL SPME vial and evaporated under nitrogen gas. The VSOPs of the oils extracted in Study **II** were analysed as described in section 4.3.4. In Study **III**, the samples were analysed

with a DB-WAX column (60 m, 0.25 mm, 0.25 µm; Agilent Technologies, Santa Clara, CA, USA). VSOPs were collected with a 2 cm DVB/CAR/PDMS fibre (50/30 µm, Supelco Inc.) at 40 °C for 30 minutes. The oven temperature was programmed to hold at 40 °C for 3 min, after which it was increased to 220 °C at a rate of 8 °C/min and held at 220 °C for 10 minutes. Helium flow rate was 1.6 mL/min. The compounds were identified based on literature, retention indices (RI) calculated using a homologous series of short (C5–C12) and long (C7–C30) *n*-alkane standards purchased from Supelco Inc., and NIST MS Search library.

4.5.4 Tocopherols and tocotrienols (Study III)

Tocopherols and tocotrienols were analysed with a UHPLC coupled with a fluorescence detector (FLD). Oil samples were diluted in heptane at concentration of 20 µg/mL, and filtered with a 0.2 µm PTFE filter. The equipment was Shimadzu Nexera XR LC-30 with LC-20AD XR pump, SIL-20AC autosampler, RF-20A prominence fluorescence detector, CTO-20AC prominence column oven (Shimadzu Corporation, Kyoto, Japan), using Restek Pinnacle DB Silica UHPLC column (100 × 2.1 mm, 1.9 µm, Bellefonte, PA, USA). Column temperature was 30 °C and tray cooler temperature 4 °C. Sample injection volume was 10 µL and the compounds were separated isocratically within 8 minutes using a mobile phase containing 2% 1,4-dioxane and 98% heptane with a flow rate of 0.4 mL/min. Chromatograms were recorded at 280 and 360 nm. Different compounds (α-, β-, γ- and δ-tocopherols and -tocotrienols) were quantified using calibration curves constructed by running external tocopherol standards (Sigma-Aldrich, Buchs, Switzerland) using concentrations of 1−50 µg/mL. Tocotrienols were identified with α-, β-, γ- and δ-tocotrienol standards but quantified with the tocopherol standards, because the concentrations of the tocotrienol standards were unknown (Supelco Inc.).

4.6 Fatty acid analysis (Studies II & III)

Fatty acids were analysed from the oil samples as fatty acid methyl esters (FAMEs) prepared using a sodium methoxide catalysed method (Study **II**) (Christie, 1982) or an acid catalysed method using acetyl chloride (Study **III**) (Christie, 2003). In the first method, the oil sample (0.5 mg) and internal standard TAG 3×15:0 (5% of the lipid sample) from Larodan AB (Solna, Sweden) were dissolved in 1 mL of sodium-dried diethyl ether. Then, 25 µL of methyl acetate and 25 µL of 1 M sodium methoxide were added and the sample was agitated. After 5 minutes, 6 µL of glacial acetic acid was added with a brief agitation, and the sample was centrifuged at 3100*g* for 5 minutes (Eppendorf 5810 R, Eppendorf AG). The supernatant was collected, evaporated and dissolved in 1

mL of *n*-hexane (Christie, 1982). In the acid catalysed method, the sample and internal standard were mixed with 2 mL of acetyl chloride:methanol (1:10), shaken, sealed and placed in an oven at 50 °C overnight. Next day, the samples were cooled down and 2 mL of potassium carbonate was added, followed by 1 mL of *n*-hexane. The sample was agitated at centrifuged at 1000*g* for 3 min, and the top layer was collected (Christie, 2003).

FAMEs were analysed with a gas chromatograph (Shimadzu GC-2010 equipped with AOC-20i auto injector, and flame ionization detector, Shimadzu Corporation). The injection (0.5 µL) was operated in a splitless mode with a sampling time of 1 min. Helium was used as the carrier gas. The column was a DB-23 (60 m × 0.25 mm i.d., liquid film 0.25 µm, Agilent Technologies). The following temperatures were used: inlet temperature 270 °C; detector temperature 280 °C; oven temperature 130 °C for 1 min, followed by increases of 6.5 °C/min to 170 °C, 2.75 °C/min to 205 °C (held 18 min), and 30 °C/min to 230 °C (held 2 min). The enzymatically extracted samples included triheptadecanoin (TAG 3×15:0) and the solvent extracted samples included triheneicosanoin (TAG 3×21:0) as internal standards. Fatty acids were identified using external standards Supelco 37 Component FAME mix (Supelco Inc.), and 68D and GLC-490 (Nu-Check-Prep, Elysian, MN, USA). Correction factors were calculated using the external standards. The quantification of the fatty acids was conducted based on the internal standard area, concentration and correction factors.

4.7 Lipid class analysis (Study III)

Oil samples were fractioned into polar and neutral fractions by using Sep-Pak Silica 6 cc Vac Cartridges (500 mg, 55−105 µm, Waters Co., Milford, MA, USA). The cartridges were first conditioned with 5 mL of hexane:diethyl ether (1:1 v/v) (waste fraction). 6 mg of fish oil dissolved in hexane was pipetted into a single use test tube together with 0.3 mg of triheneicosanoin (TAG 3×21:0, Larodan AB) and 0.12 mg of phosphatidylglycerol (PG 18:0/18:0, Avanti Polar Lipids Inc., Alabaster, AL, USA), evaporated, dissolved in 1 mL of hexane:diethyl ether and applied into the column. The sample was let to elute into the column, and the sample tube was washed with 2 mL of hexane:diethyl ether, applied to the column and finally, neutral lipids were eluted with 9 mL of the hexane:diethyl ether. This fraction contained mainly triacylglycerols (neutral fraction, NF). A new test tube was applied, the sample tube was washed with 2 mL of chloroform:methanol:water (5:3:2, v/v), applied into the column, and the second fraction containing MAGs, DAGs, FFAs and phospholipids was eluted with 8 mL of chloroform:methanol:water. The second fraction is later referred to as polar fraction (PF), although it also contains other than polar lipids. Both

fractions were evaporated in a heating block at 50 °C under nitrogen gas flow. The NF was dissolved in 1 mL of isopropanol:hexane (1:1, v/v) and diluted 1:200, and the PF was dissolved in 1 mL of chloroform:methanol (2:1, v/v).

Lipid classes were separated and detected with Shimadzu 8045 ultra-high performance lipid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-MS) equipped with an electrospray ionisation (ESI) source. The column was CORTECS UPLC C18 (1.6 µm, 2.1 × 150 mm, Waters Co.). The injection volumes were 0.5 and 0.3 µL for the NF and PF samples, respectively. The mobile phases were A: water:methanol (1:1, v/v) with 0.1% of formic acid and 10 mM ammonium formate, and B: isopropanol:water (95:5, v/v) with 0.1% of formic acid and 10 mM ammonium formate. The flow rate was set to 0.2 mL/min and the column oven temperature was 50 °C. The starting condition was 55% of A and 45% of B. In the first 30 minutes, the concentration of B was increased to 90%, and in the next two minutes increased further to 100%, where it was held for 10 minutes. Within the next minute, the concentration of B was decreased from 100% to 45% and kept there for 7 minutes, the total sample run time being 50 minutes. The mass ranges were 340–1100 *m/z* on the positive mode and 150–1100 *m/z* on the negative mode; scan time 0.2 sec. Nebulizing, heating and drying gas flows were 2, 15 and 5 L/min, respectively, using nitrogen gas, while the interface and desolvation temperatures were set to 300 and 526 °C, respectively. For the quantification of different lipid classes, calibration curves were prepared by analysing different concentrations of TAG 3×18:2, DAG 2×18:2, and MAG 1×18:2 (Larodan AB), as well as PC 16:0-18:1*n*−9, PS 16:0-18:1*n*−9, PE 16:0-18:1*n*−9, PI (soybean), and SM (egg) (Avanti Polar Lipids Inc.). For PI and SM, the intensities of the most abundant ion was used for constructing the calibration curves. The analysed concentrations were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10 and 20 µg/mL with an injection volume of 1 µL (Supplementary Figure 1). FFAs and lysophospholipids (LPLs) were semi-quantified by using peak areas and internal standard concentration.

Identification of phospholipids was aided by using a Cortecs UPLC HILIC, 1.6 µm, 2.1 × 150 mm column (Waters Co.) which separated different phospholipid classes. The sample was a mixture of PA (egg), PC (egg), PG (egg), PE (egg), PI (soybean), PS (soybean) and SM (egg), 20 µg/mL each. The mobile phases were A: methanol:water (80:20, v/v) with 0.2% of formic acid and 10 mM of ammonium formate, and B: acetonitrile:methanol:water (90:7:3, v/v) with 0.2% of formic acid and 10 mM of ammonium formate. The flow rate was 0.15 mL/min and the starting conditions were 0% of A and 100% of B, which were kept for one minute. During the next 24 minutes, the concentration of A was increased to 32%, and in the next 0.5 minutes to 80% where it was kept for 9.5 minutes. Within the next 1 min, the concentration of A was dropped to 0%, where it was kept for 6 min. Total running time was 44 min. The mass range in

the negative mode was 150–1500 *m/z*, and the event time was 0.2 s. Gas flows, and interface and desolvation temperatures were as above.

4.8 Statistical analysis

Significant differences in volatile compounds of surimi-type gels and fish balls (**I**), colour of the surimi-type gels (**I**), oil recoveries (**II** & **III**), PV (**II**), FA (**II** & **III**), VSOPs (**II** & **III**), lipid classes (**III**) and tocopherol and tocotrienol contents (**III**) between different enzymatically extracted oils were analysed with one-way analysis of variance (ANOVA) and Tukey's test (IBM SPSS Statistics, version 28.0.0.0, IBM, New York, USA). Dunnett's C test was used when the data had unequal variances, and independent samples' Kruskal-Wallis test was performed for data that was not normally distributed. Significant differences were reported for $p < 0.05$. The fatty acid data of Study **II** was also analysed with two-way ANOVA to study the effects of hydrolysis times and different enzymes.

5 RESULTS AND DISCUSSION

5.1 pH-shift method for the production of fish protein isolate (Study I)

5.1.1 Composition

According to the results of Study **I**, gutted and beheaded Baltic herring contained 14.7% of protein, 6.1% of lipids, and 80.2% of moisture (Table 6). Nisov et al. (2021) reported similar protein contents of 14.2 and 14.4%, lipid contents of 2.2 and 2.3%, and moisture contents of 80.3 and 81.8% for whole and gutted Baltic herring, respectively. The fish protein isolate (FPI) from Baltic herring including 4% of cryoprotectants contained 9.2% of protein, 2.7% of lipids, and 85.3% of water. On dry matter basis, the protein contents were 74% and 85% for the raw material and FPI (cryoprotectants excluded), respectively. In a previous study on pH-shift processing of herring, the protein content of an isolate produced with alkaline pH-shift method was 17.8% fw, while the moisture content was 78% and the lipid content was 3.9% (Marmon and Undeland, 2010). Nisov et al. (2021) reported a protein content of 78% (dry matter basis) for Baltic herring protein isolate produced using the alkaline pH shift..

Table 6. Protein, lipid and moisture compositions of the raw material (gutted Baltic herring) and fish protein isolate.

	Protein (%)	***Lipids (%)***	***Moisture (%)***
Gutted & beheaded Baltic herring	14.7 ± 0.8	6.1 ± 0.1	80.2 ± 0.0
Protein isolate (incl. 4% of cryoprotectants)	9.2 ± 0.3	3.7 ± 0.0	85.3 ± 0.0

During processing, 37% of the lipids present in the raw material were removed. Alkaline pH shift has been reported to have better lipid removal efficiency compared to the acid-aided process. Marmon and Undeland (2010) achieved approx. 50% lipid removal from herring using alkaline pH shift, whereas the acid-aided process removed 38%. Higher efficiencies of lipid removal have been reported by Kristinsson et al. (2006), who observed reductions of 85 and 89% by acid and alkali aided processes, respectively, on Atlantic croaker, and by Taskaya et al. (2009), who reported lipid removals of 88–89% and 94–97% from silver carp using the acid and alkali aided processes, respectively. Some of the variation

in lipid removal efficiencies can likely be explained by different centrifugation speeds (8000–10000*g* used by Kristinsson et al. (2006), Taskaya et al. (2009), and Marmon and Undeland (2010) compared to 4000*g* in this study) and different composition between fish species, especially related to the proportions of unsaturated fatty acids and phospholipids (Kakko et al., 2022; Marmon and Undeland, 2010).

5.1.2 Volatile compounds

In the case of the surimi-type gels, the total peak area of all identified volatile compounds was significantly ($p < 0.05$) higher in SPI11.5 compared to the SPI and SPI6.5 gels, the latter having the lowest total peak area ($p < 0.05$) (Figure 6). Propanal, 1-penten-3-ol, 2,3-pentanedione, and hexanal were among the most prominent volatile compounds in all samples. Most of these compounds are secondary lipid oxidation products derived from *n*−3 FAs (Gómez-Cortés et al., 2015), while hexanal can be produced also from *n*–6 FAs (Medina et al., 1999). 1-Penten-3-ol, 2,3-pentanedione, and hexanal followed the same trend with total areas, SPI11.5 having the largest peaks ($p < 0.05$), followed by SPI, and SPI6.5. Some compounds, such as (*E,Z*)-2,4-heptadienal and 3,5-octadien-2-one, did not differ significantly between SPI, and SPI6.5, but their peak areas were significantly higher in SPI11.5 ($p < 0.05$). The slight increase in solubilisation pH from 11.2 (SPI) to 11.5 (SPI11.5) caused a significant increase in the total peak area of identified volatile compounds as well as the abundance of individual volatile products of lipid oxidation. Increasing pH has been shown to increase lipid oxidation in water–oil systems (Kim et al., 2016), which might explain the differences in the oxidation-related volatile contents. On the other hand, SPI6.5 had statistically significantly lower areas of hexanal, 2,3-pentanedione, and, 1-penten-3-ol compared to the other two surimi-type gels, in which proteins were precipitated at pH 5.4. This indicates precipitation proteins at higher pH induces less lipid oxidation. Marmon et al. (2012) also showed that using herring as a raw material, precipitation at pH 6.5 led to less oxidation compared to precipitation at pH 5.5.

In fish balls, the most influential volatile compounds were related to the flavour mixture added to the fish ball mass, and many of the relevant odour-contributing compounds of the FPI were not detected. The compounds originating from the flavour mixture included terpenes, such as β-pinene, α-phellandrene, β-phellandrane, 3-carene, D-limonene, and o-cymene, which are common in herbs and spices (Kruma et al., 2011; Sonmezdag et al., 2015). The most important compounds related to lipid oxidation or contributing to FPI odour are presented in Figure 7. FB0 had significantly lower ($p < 0.05$) peak areas of

2-ethylfuran, pentanal, heptanal and hexanal compared to FB50. On the contrary, the area of 3-methylbutanal was significantly higher — the compound is associated to baked, fresh Baltic herring (Aro et al., 2003). FB0 contained a higher proportion of minced Baltic herring compared to FB50 (Table 3). These results indicate that the addition of FPI led to an increased amount of compounds related to oxidation, but sensory evaluation should be conducted to study whether these changes are detected by consumers.

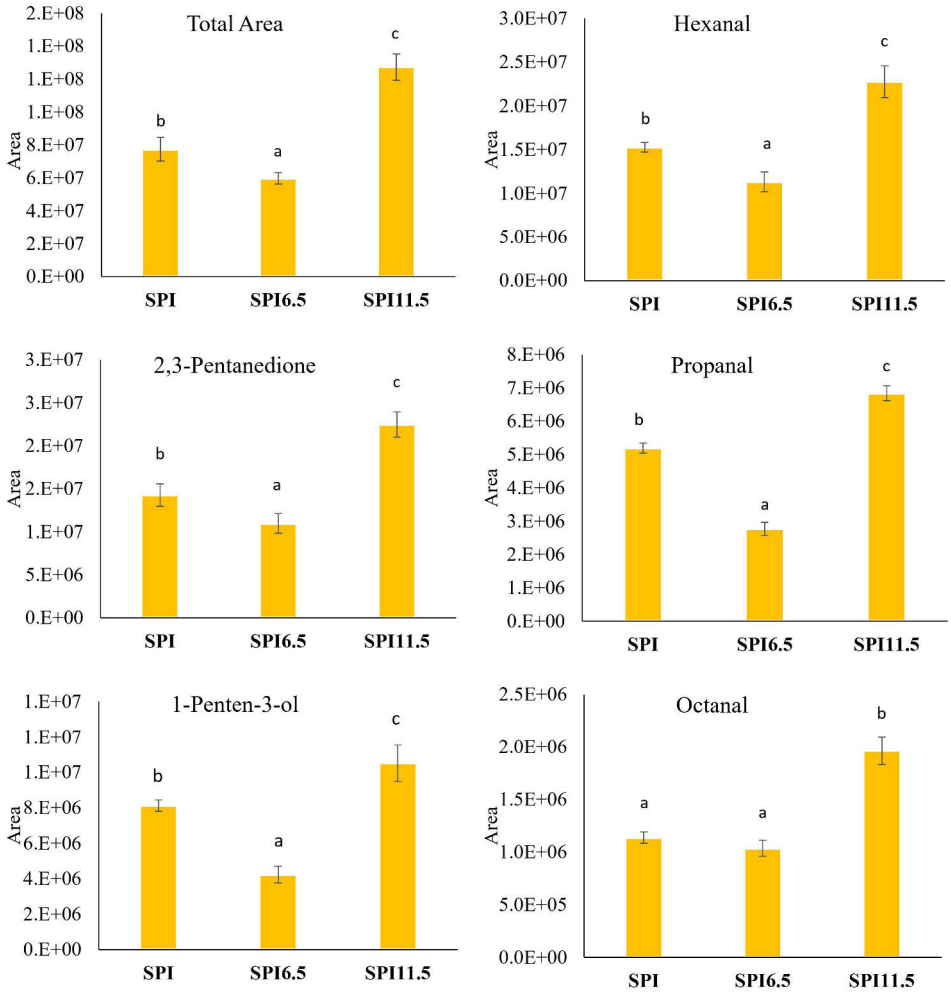

Figure 6. Total peak area, and areas of important volatile compounds hexanal, 2,3-pentanedione, propanal, 1-penten-3-ol, and octanal of surimi-type gels made of Baltic herring protein isolate. SPI was prepared from protein concentrate isolate with solubilisation pH 11.2 and precipitation pH 5.4, in SPI6.5 the protein concentrate isolate was produced by precipitation at pH 6.5 (solubilisation pH 11.2), and in SPI11.5 the protein concentrate isolate was produced by solubilisation at pH 11.5 (precipitation pH 5.4). Different letters indicate a statistically significant ($p < 0.05$) difference between the samples (n=3).

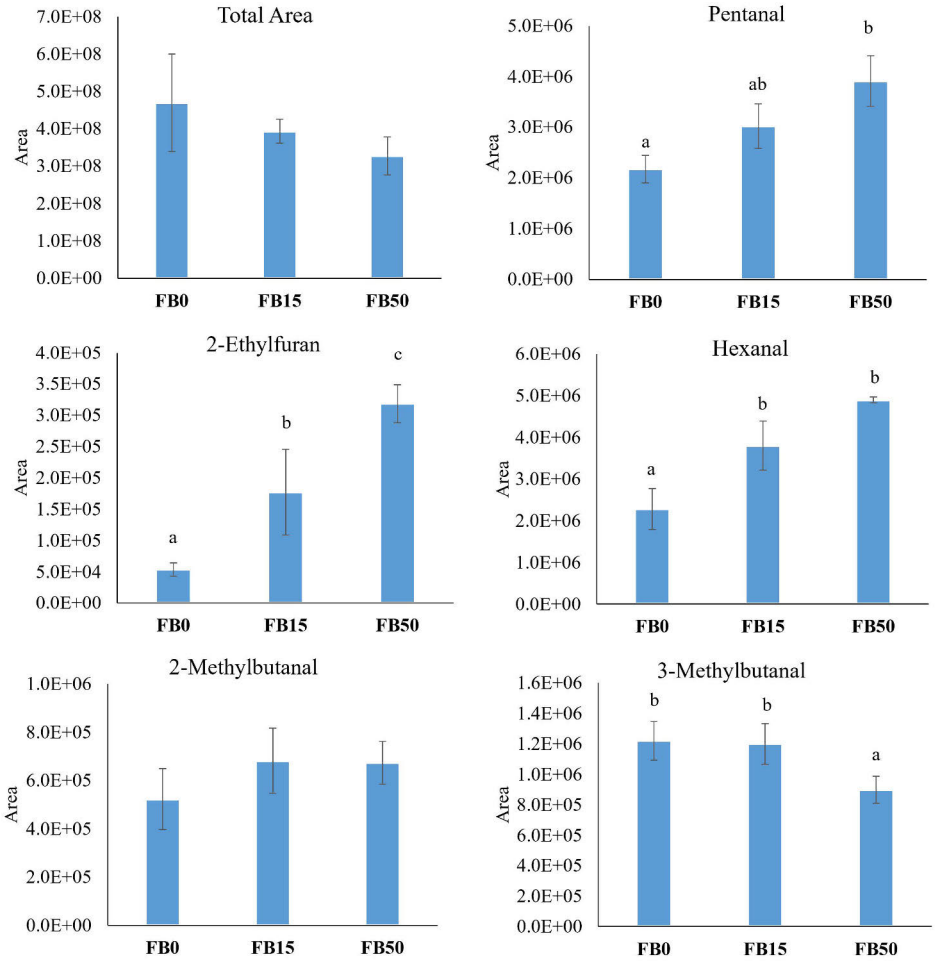

Figure 7. Total peak area, and areas of important volatile compounds hexanal, pentanal, 2-ethylfuran, 2-methylbutanal and 3-methylbutanal of fish balls with different amounts of protein isolate from Baltic herring; control fish ball without protein isolate (FB0), fish ball with 15% protein isolate (FB15), and fish ball with 50% protein isolate (FB50). Different letters indicate a statistically significant ($p < 0.05$) difference between the samples (n=3).

5.1.3 Texture profile analysis

According to the TPA results, SPI was within the range of the commercial surimi in terms of hardness (Figure 8). However, changing the precipitation pH to 6.5 or solubilisation pH to 11.5 significantly softened the gel. All SPIs had higher springiness values than the two commercial surimi. Springiness describes the rate at which a deformed sample returns to its original shape, thus, the SPIs were significantly more elastic in texture compared to the commercial surimi. Cohesiveness evaluates the strength of the internal bonds in the sample. Based on the results, SPIs with modified pH values were significantly less cohesive compared to the other samples. The softening of the SPI11.5 may have resulted from an increased degree of protein denaturation, and as a result decreased renaturation after precipitation and neutralisation (Abdollahi and Undeland, 2019). In addition, as the isoelectric point of the muscle proteins was not reached, the proteins in SPI6.5 were likely more charged, holding more water leading to an increased water content. However, the water contents of these isolates were not determined, so the exact reason is not clear. As with hardness and cohesiveness, SPI6.5 and SPI11.5 had significantly lower chewiness values than SPI, while the SPI was significantly higher in chewiness than the commercial surimi samples. Based on the results, Baltic herring FPI resulted in somewhat comparable gel with the commercial products in terms of texture properties, but the modifications to the precipitation and solubilisation pH values caused softening.

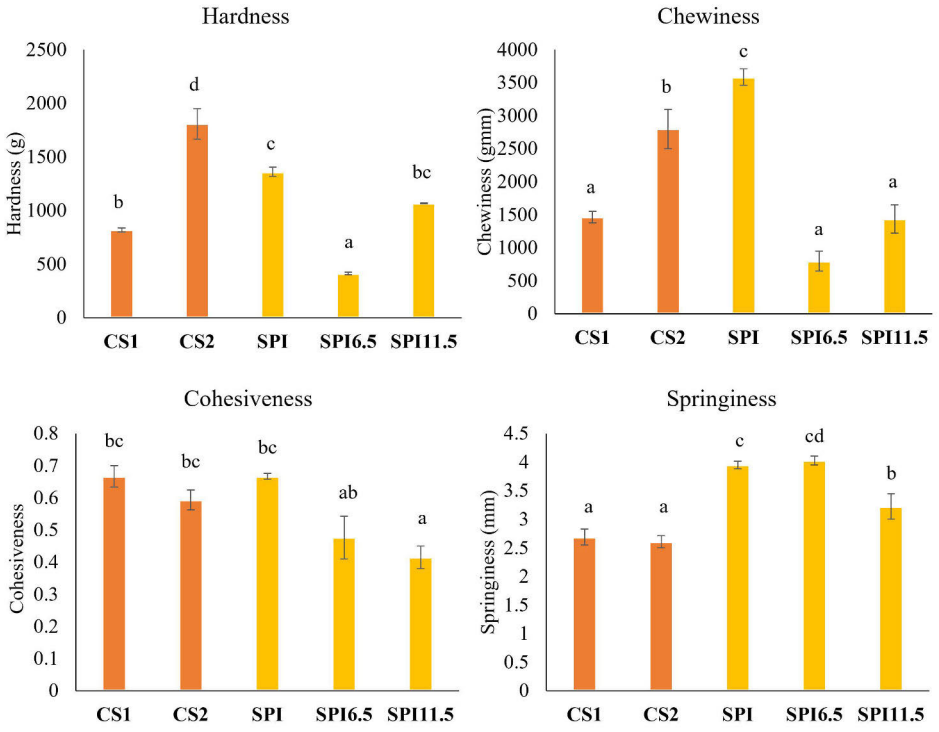

Figure 8. Texture profile analysis attributes hardness, chewiness, cohesiveness and springiness of surimi-type gels made of Baltic herring protein isolate, and two commercial surimi (CS1 & CS2). SPI was prepared from protein concentrate isolate with solubilisation pH 11.2 and precipitation pH 5.4, in SPI6.5 the protein concentrate isolate was produced by precipitation at pH 6.5 (solubilisation pH 11.2), and in SPI11.5 the protein concentrate isolate was produced by solubilisation at pH 11.5 (precipitation pH 5.4). Different letters indicate a statistically significant ($p < 0.05$) difference between samples (n=3).

The aim of the FPI addition was to evaluate whether it can replace some of the minced fish and improve the textural quality of the fish balls. The textural properties of Baltic herring fish balls were further compared to a commercial fish ball (CFB). The addition of 5–50% of Baltic herring FPI affected hardness, springiness and chewiness of the fish ball samples analysed by the TPA (Figure 9). The addition of 50% of FPI resulted in a harder fish ball, but the difference to other Baltic herring fish balls (FB0, FB5, FB15, and FB28) was not statistically significant when the commercial fish ball (CFB) was included in the comparison. The lowest springiness was observed in FB5, while the highest chewiness was observed in FB28. Based on moisture contents, the addition of 50% of FPI increased the water content from 60% to 64% compared to the FB0. The increased moisture content, however, did not cause leaking or softer texture. Based on hardness, water leaking during cooking (visual observation), and the

roundness of the fish balls (visual observation), the addition of FPI improved the texture of the fish balls. It can also be considered a potential food model in which proteins isolated from Baltic herring can be easily utilised.

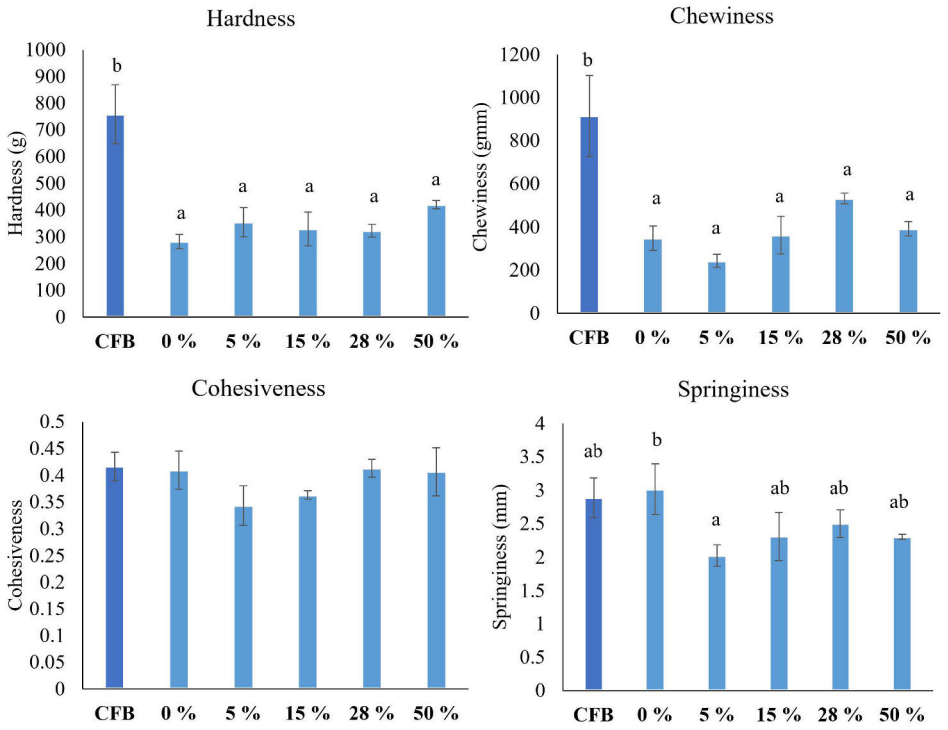

Figure 9. Texture profile analysis attributes hardness, cohesiveness, chewiness and springiness of fish balls containing Baltic herring and protein isolate; control fish ball with no protein isolate, fish ball with 5% of mince replaced with the protein isolate, fish ball with 15% of mince replaced, fish ball with 28% of mince replaced, and fish ball with 50% of mince replaced (n=3). A commercial fish ball (CFB) was included as a reference sample. Different letters indicate a statistically significant ($p < 0.05$) difference between the samples.

5.1.4 Colour

Pelagic fish, including Baltic herring, are abundant in dark muscle tissue, which is rich in lipids and pigments, such as haemoglobin and myoglobin. The presence of haemoglobin can diminish the whiteness of proteins and result in a yellow or brown coloration. Darker tissue leads to darker protein mass, whereas the fish species conventionally used in surimi production, such as Alaska pollock (*Theragra chalcogramma*), have white flesh (Park, 2013). The whiteness values of SPIs were significantly lower than those of commercial surimi (Figure 10), due to the use of fish rich in dark muscle tissue. Abdollahi and Undeland (2019)

achieved a slightly lighter herring gel by increasing the solubilisation pH from 11.5 to 12.5, due to more effective removal of heme pigments. However, in Study **I**, altering the solubilisation pH from 11.2 to 11.5 or the precipitation pH from 5.4 to 6.5 did not enhance the whiteness of the FPI. In one study, an acidic pH shift resulted in increased whiteness compared to alkaline processing of herring (57 vs. 61) (Marmon and Undeland, 2010). Conversely, another study found that alkaline processing, particularly with precipitation at pH 6.5, lead to a more efficient removal of haemoglobin (Abdollahi et al., 2016). Novel approaches have been explored to improve the whiteness of surimi, such as treatment with ozone water, which improved the whiteness of grass carp surimi (C. Liu et al., 2021).

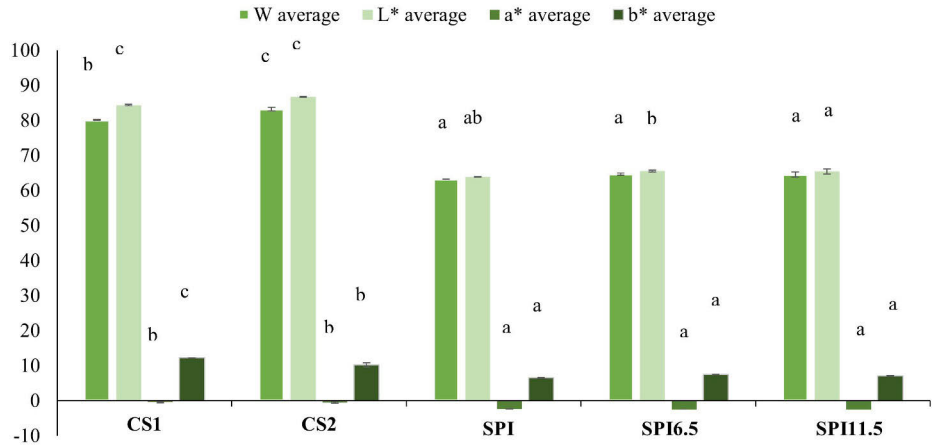

Figure 10. Colour of commercial surimi (CS1 and CS2) and surimi-type gels prepared from Baltic herring protein isolate. SPI was prepared from protein isolate with solubilisation pH 11.2 and precipitation pH 5.4. In SPI6.5, the protein isolate was produced by precipitation at pH 6.5 (solubilisation pH 11.2), and in SPI11.5, the protein isolate was produced by solubilisation at pH 11.5 (precipitation pH 5.4). W = whiteness, L* = lightness, a* = red−green axis, and b* = yellow−blue axis. Different letters above the bars (within the same colour parameter) indicate a statistically significant ($p < 0.05$) difference between the samples (n=3).

5.2 Enzyme-assisted aqueous extraction in the production of fish oil (Studies II & III)

5.2.1 The effect of raw material on oil recovery (Studies II & III)

The lipid contents of whole Baltic herring and its filleting co-products used in Study **II** were 9.0% and 9.5%, respectively, extracted by a solvent extraction method (Table 7). The lipid content of the fish caught in autumn 2019 was higher than those reported in most previous studies, especially considering the findings of Rajasilta et al. (2019), who reported a decrease in the lipid content of Baltic herring muscle from 5–6% to 1.5% during 1987–2014. However, the contents were comparable to Baltic herring fillets from fish caught in autumn (10 %) (Aro et al., 2000). Baltic herring has higher fat contents in autumn compared to the spring season due to their spawning season being in spring and early summer (Keinänen et al., 2017). The lipid content of the fish used in Study **II** was also significantly higher than 4.5 g/100 g fw extracted from a catch from autumn of 2020, which was analysed by the authors (data not published). There seems to be a high variation in fat content of Baltic herring not only between seasons, but also between years. Further, the samples may be caught from different areas which might also cause batch-to-batch variation. The two fish batches caught in January 2021 and May 2022 used in Study **III** contained 3.8% and 2.8% of lipids, respectively (Table 7), which was significantly less than that in Study **II**. Lower lipid content of Baltic herring has been reported before, likely due to the decrease in the salinity of the Baltic Sea, and increased sea temperatures during winter, which affect the nutrition of Baltic herring (Rajasilta et al., 2022).

Table 7. Oil recoveries of enzymatically extracted Baltic herring oils from whole fish (Studies **II** & **III**) and filleting co-products (Study **II**), and solvent-extracted reference oil. The abbreviations used in the table are: SE = solvent extraction, A= Alcalase, N = Neutrase and P = Protamex. The results are presented as mean value ± standard deviation (n=3).

	SE	***A***	***A***	***A***	***N***	***N***	***N***	***N***	***N***	***P***	***P***	***P***	***P***	***P***
Enzyme (%)	***-***	***0.4***	***0.4***	***0.4***	***0.4***	***1.0***	***0.4 (II) / 1.0 (III)[1]***	***1.0***	***2.0***	***0.4***	***1.0***	***0.4 (II) / 1.0 (III)[1]***	***1.0***	***2.0***
Extration time (min)	***-***	***35***	***70***	***105***	***35***	***35***	***70***	***105***	***35***	***35***	***35***	***70***	***105***	***35***
Whole fish (II)														
Extracted oil content (g/100g)	9.00 ± 0.46	4.09	3.85	-	5.39	-	6.25	-	-	4.52	-	6.08	-	-
Oil recovery (%)	100.0	45.4	42.8	-	59.9	-	69.4	-	-	50.2	-	67.6	-	-
Co-products (II)														
Extracted oil content (g/100g)	9.46 ± 0.23	4.57	5.13	-	5.17	-	5.44	-	-	4.57	-	6.14	-	-
Oil recovery (%)	100.0	48.3	54.2	-	54.7	-	57.5	-	-	48.3	-	64.9	-	-
Whole fish, Optimisation tests (III)														
Extracted oil content (g/100g)	3.84 ± 0.26	1.52 ± 0.40	1.42 ± 0.37	2.23 ± 0.41	1.70 ± 0.78	1.60 ± 0.51	1.60 ± 0.52	2.22 ± 0.69	1.81 ± 0.83	1.49 ± 0.11	1.50 ± 0.32	1.52 ± 0.34	2.13 ± 0.41	1.47 ± 0.24
Oil recovery (%)	100.0	39.5 ± 10.4	37.0 ± 9.6	58.1 ± 10.6	44.3 ± 20.2	41.7 ± 12.0	41.7 ± 13.6	57.8 ± 18.1	47.3 ± 21.7	38.8 ± 3.00	39.1 ± 11.3	37.1 ± 8.82	55.4 ± 10.7	38.2 ± 6.3

[1]The enzyme concentration used in Study II with the 70-minute extraction was 0.4%, whereas in Study III, a concentration of 1.0% was used.

5.2.2 The effect of homogenisation size, stirring and centrifugation temperature on oil recovery (Study III)

In Study **II**, the highest oil recoveries were 68% and 69% of the total lipids in the raw material, achieved using Protamex and Neutrase, respectively, with a 70-minute extraction time (Table 7). However, for leaner fish batches with a lipid content below 4% fw used in Study **III**, the recovery was lower, and a higher amount of emulsion was formed (pre-tests, results not shown here). In Study **II**, the fish raw material was cut into 2 cm pieces, stirred at 280 rpm, and cooled down after hydrolysis. The samples were then centrifuged with cooling set to 4 °C. To improve the lipid recovery for the whole Baltic herring with a low lipid content of 3.8%, the effect of stirring speed, centrifugation temperature and homogenisation size were studied. The emulsion formation occurred during centrifugation, although lipid–protein complexes were likely forming already during the hydrolysis as no freely flouting lipid droplets were visible. After centrifugation, the fish hydrolysate consisted of a thin layer of oil, loose emulsion layer, supernatant, and sludge. Decreasing stirring speed from 280 rpm down to 100 rpm increased the oil recovery by 50%. Higher stirring speed may induce the interactions between peptides and oil, and improve the degree of protein hydrolysis, all of which can lead to larger amount of emulsion (Chiodza and Goosen, 2023). However, the reaction kinetics were not measured in this study, so the exact mechanism remains to be studied. Secondly, mincing, instead of chopping to 2 cm pieces, improved the recovery by 21% which is most likely due to the increased surface area, which leads to a faster hydrolysis of proteins (Jayasinghe et al., 2013). Thirdly, centrifugation soon after the inactivation step while the hydrolysate was approx. 60 °C compared to cooling down on ice improved the recovery by 26%. At higher temperature, oil emulsions are less stable due to decreased viscosity which results in the increased oil recovery (Juntarasakul and Maneeintr, 2018).

5.2.3 The effects of raw materials, enzyme concentrations, and extraction times on oxidation (Studies II & III)

The AVs, PVs and total oxidation products (TOTOX = 2 × PV + AV) of enzymatically extracted oils are presented in Table 8. There were differences in AVs and PVs between the treatments of the samples in Study **II**: a longer extraction time resulted in a higher PV when using Alcalase or Protamex. In addition to enzymes and extraction times, there was evidently an influence by the raw materials on the PVs of the crude oils. The difference can be explained by interference of endogenous enzymes in the proteolytic process or differences in the haemoglobin contents (Sajib et al., 2021). Further, the fish raw material

was stored at –20 °C in Study **II**, while it was kept at –80 °C in Study **III**. In the extractions from whole fish, the lowest PV was in the oil from 35-minute extraction with Alcalase, whereas the highest value was from the 70-minute extraction with Protamex. When using the co-products, the lowest PV was obtained from the 35-minute extraction with Neutrase, which also had the lowest AV. The highest PV, on the other hand, was in the 70-minute Alcalase-extracted oil, which also had the highest AV. According to the TOTOX values, the 35-minute extraction with Alcalase was the best for whole fish in terms of oxidative stability, while the 35-minute extraction with Neutrase was the best for the co-products. The Codex standard for refined fish oil states that the acceptable limits are PV $\leq$ 5 meq/kg, AV $\leq$ 20 and TOTOX $\leq$ 26 (Joint FAO/WHO Codex Alimentarius Commission, 2017). In relation to these limits, all oil samples had a compromised oxidative status. However, the Codex standard applies for refined oils, whereas the oil studied here is crude oil which can have higher PV values. In the industry, oils are typically refined with activated carbon filtration or molecular distillation, which are methods to remove undesired compounds from the crude oil.

The PV values from the optimisation part of Study **III** ranged between 3.8–11.8 meq/kg oil (Table 8), which were significantly smaller values than those in Study **II** determined using a titration-based method. Higher lipid content, endogenous enzyme activity, and storage conditions likely explain the higher oxidative status of the oils reported in Study **II**. The PVs of oils obtained using 0.4% Alcalase with 35 minute extraction time, or Neutrase 0.4% and 1% with 35 minute extraction time were under the CODEX standard of 5 meq/kg for refined oils (3.8, 4.2, and 4.5, respectively). The highest PV values (8.0–11.9 meq/kg) were observed from the oils from 70 and 105 minute extractions. In a study using salmon co-products, the PV value of the oil extracted with Alcalase was 1.6 meq/kg using an enzyme concentration of 5% and extraction time of two hours (Głowacz-Różyńska et al., 2016), whereas in another study using mixed fish co-products, the hydrolysis resulted in PV values of 26.9, 28.9, 37.6 and 39.4 meq/kg using Neutrase, Alcalase, F protease and Protex 7L, respectively, compared to 8.7 meq/kg in the oil extracted from the raw material (Hathwar et al., 2011). Therefore, EAAE can result in very different levels of oxidation depending on raw material, extraction time, and in some cases, the enzyme.

Table 8. *p*-Anisidine values (AV), peroxide values (PV, meq/kg) and total oxidation product (TOTOX) values of enzymatically extracted oils from whole Baltic herring (Studies **II** & **III**) and filleting co-products (Study **II**).

	Alcalase 35 min	***Alcalase 70 min***	***Neutrase 35 min***	***Neutrase 70 min***	***Protamex 35 min***	***Protamex 70 min***
Whole fish (II)						
AV	11.10 ± 0.61a	16.87 ± 2.77bc	16.91 ± 0.78bc	15.25 ± 0.03ab	21.08 ± 1.31cd	25.84 ± 0.54d
PV[1]	22.95 ± 1.92a	53.66 ± 6.90b	54.44 ± 7.27b	43.65 ± 2.89b	57.58 ± 6.50b	75.65 ± 7.40c
TOTOX	54.88	124.19	125.80	102.56	136.23	177.14
Co-products (II)						
AV	16.34 ± 0.37bc	25.88 ± 5.80c	5.60 ± 1.14a	13.35 ± 0.29ab	12.05 ± 0.30ab	9.94 ± 0.03ab
PV[1]	49.67 ± 1.53c	68.43 ± 1.01d	23.56 ± 0.19a	52.05 ± 2.00c	35.21 ± 0.68b	36.77 ± 0.64b
TOTOX	115.69	162.74	52.72	117.44	82.46	83.48

Whole fish (III), optimisation	***Alcalase 0.4% 35 min***	***Alcalase 0.4% 70 min***	***Alcalase 0.4% 105 min***	***Neutrase 0.4% 35 min***	***Neutrase 1.0% 35 min***	***Neutrase 1.0% 70 min***	***Neutrase 1.0% 105 min***	***Neutrase 2.0% 35 min***	***Protamex 0.4% 35 min***	***Protamex 1.0% 35 min***	***Protamex 1.0% 70 min***	***Protamex 1.0% 105 min***	***Protamex 2.0% 35 min***
PV[2]	3.81 ± 1.39a	8.90 ± 0.09cdef	9.36 ± 1.18def	4.19 ± 0.49a	4.47 ± 0.91ab	9.66 ± 2.99def	11.76 ± 1.08f	5.27 ± 1.47abc	5.91 ± 1.04abcd	7.21 ± 0.14abcde	10.95 ± 1.49ef	7.95 ± 1.93bcde	8.13 ± 0.97bcdef

Whole fish (III), Final samples[3]	**Alcalase 0.4% 105 min**	**Alcalase 0.4% 105 min**	**Alcalase 0.4% 105 min**
PV[2]	5.20 ± 0.65	8.29 ± 3.87	8.20 ± 3.15

The results for AV and PV are shown as mean value ± standard deviation (n=3). Significant differences ($p < 0.05$) were determined by one-way ANOVA and Tukey HSD. Different letters indicate significant differences ($p < 0.05$).

[1] PVs were determined with a titration-based method.

[2] PVs were determined with a spectrophotometric method.

[3] Samples with reduced emulsion.

A total of 41 volatile compounds were tentatively identified from the oil samples of Study **II** based on the database NIST MS and comparison to external standards (Table 9). Retention indices (RIs) of the compounds with retention times of 12.93 minutes or higher were calculated based on the retention times and *n*-alkane standards. Most of the identified compounds were secondary oxidation products formed from lipid oxidation, such as propanal, butanal, 1-penten-3-ol, hexanal, heptanal and nonanal. A larger volatile content indicates a higher degree of lipid oxidation in fish oil, because the majority of the volatile compounds were oxidation products. The oils extracted from whole Baltic herring had a larger total content of volatile compounds compared to those from the co-products, indicating a higher degree of oxidation, which was also seen in the PV, AV and TOTOX values when using Neutrase and Protamex (Figure 11).

Based on the total ion chromatogram of the HS-SPME-GC-MS analysis, the most abundant volatiles in the oils extracted from whole fish were 2-propenal, 2-ethylfuran, 1-penten-3-ol, hexanal and two unidentified compounds (unknown 1 and 2). In the oils extracted from co-products, the most abundant volatile compounds were propanal, 2,3-butanedione, 2-ethylfuran, 1-penten-3-ol, 2,3-pentanedione, unknown 2, and (*E,E*)-2,4-heptadienal. Hexanal and 1-penten-3-ol are typical oxidation products from *n*–3 PUFA degradation and they are both related to unpleasant odours and flavours (Fu et al., 2009; Rahmani-Manglano et al., 2020). Both of these compounds have shown increases in Baltic herring during storage, indicating oxidation of long-chain unsaturated fatty acids (Aro et al., 2003). (*E,E*)-2,4-Heptadienal is another oxidation compound related to rancid and fishy odours. It has also been detected in oxidised herring oil and could be a good indicator for the oxidative status of fish oil (Aidos et al., 2002). Furthermore, 2-ethylfuran has been detected in fish, for instance, triploid rainbow trout, and it is also derived from the oxidation of *n*–3 PUFAs. The odour of the compound is described as “rubber”, “pungent” and “green bean” (Ma et al., 2020; Medina et al., 1999). Out of all the identified volatile compounds, 17 were aldehydes including acetaldehyde, 2-propenal, propanal, butanal, 2-methylbutanal, 3-methylbutanal, (*Z*)-2-butenal, (*E*)-2-pentenal, hexanal, 2-methyl-4-pentenal, (*E*)-2-hexenal, heptanal, (*E,E*)-2,4-hexadienal, (*Z*)-2-heptenal, octanal, (*E,Z*)-2,4-heptadienal and nonanal. Aldehydes are secondary oxidation products, which can also be measured with AV.

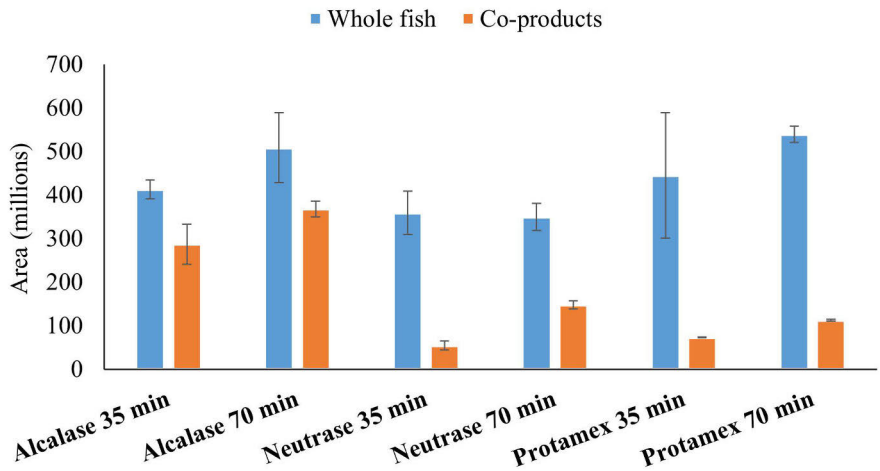

Figure 11. Total peak areas of identified volatile compounds (n=41) in different enzymatically extracted oils from whole fish (blue) and co-products (orange) of Baltic herring analysed with HS-SPME-GC-MS in Study **II**. The results are shown as mean value ± standard deviation (n=3).

Table 9. Volatile compounds in enzymatically extracted oils from Baltic herring analysed by HS-SPME-GC-MS (Study **II**). The compounds were identified based on the literature, NIST library and external standards (STD). Retention indices are calculated by using a series of *n*-alkanes. The column was SPB®-624 Fused Silica Capillary Column 60 m × 0.25 mm × 1.4 µm.

Compound number	***Compound***	***Identification method***	***RT (min)***	***RI***
1	Acetaldehyde	STD	6.51	
2	2-Propenal	MS	9.79	
3	Propanal	STD	9.97	
4	Butanal	STD	14.55	638
5	2,3-Butanedione	MS	14.64	640
6	Unknown 1	-	14.84	644
7	Formic acid	MS	15.96	667
8	1,3-Butanediol	MS	14.64	676
9	Acetic acid	STD	17.44	696
10	3-Methylbutanal	MS	17.67	700
11	Heptane	STD	17.84	704
12	2-Methylbutanal	MS	18.05	709
13	(*Z*)-2-Butenal	MS	18.13	711
14	2-Ethylfuran	STD	18.85	727
15	1-Penten-3-one	MS	19.31	738
16	1-Penten-3-ol	STD	19.44	741
17	2,3-Pentanedione	MS	19.68	746
18	Cyclopentanol	MS	19.73	747
19	Propanoic acid	MS	21.67	787
20	(*E*)-2-Pentenal	STD	22.41	801
21	Hexanal	STD	24.44	851
22	2-Methyl-4-pentenal	MS	24.48	852
23	3-Hexanol	MS	25.71	880
24	2,4-Hexadien-1-ol	MS	26.84	905
25	(*E*)-2-Hexenal	STD	27.37	920
26	Heptanal	STD	28.69	954
27	(*E,E*)-2,4-Hexadienal	STD	30.05	988
28	Unknown 2	-	30.58	1002
29	2-Pentylfuran	STD	31.23	1020
30	(*Z*)-2-Heptenal	MS	31.48	1027
31	(*E*)-2-(2-Pentenyl)furan	MS	31.72	1034
32	Octanal	STD	32.55	1058
33	Hexanoic acid	MS	32.62	1060
34	(*E,Z*)-2,4-Heptadienal	MS	33.22	1076
35	(*E,E*)-2,4-Heptadienal	MS	33.81	1091
36	(*E,E*)-3,5-Octadien-2-one	MS	35.57	1146
37	2-Nonanone	MS	35.73	1151
38	Nonanal	STD	36.08	1161
39	5-Ethyl-2(5H)-furanone	MS	36.45	1173
40	(*E,E*)-3,5-Octadien-2-one	MS	36.52	1175
41	Nonanoic acid	MS	41.94	1334

The VSOPs of the oils extracted with different optimisation parameters in Study **III** were analysed with an SPB-624 column. A total of 21 compounds were identified from the samples (Table 10), the most abundant compounds being 3-methylpentane, 2,4-dimethylhexane, and 1-octen-3-ol. 3-Methylpentane is likely an impurity coming from *n*-hexane, which was used as a solvent to prepare the sample dilutions and evaporated before analysis. 1-Octen-3-ol, on the other hand, is produced by the degradation of linoleic acid (Kunyaboon et al., 2021). Aldehydes, such as hexanal, heptanal and octanal were also present in the oils and their increasing concentrations correlated with increasing extraction times. Protamex 1% and Alcalase 0.4% with 105 minute extraction times had significantly larger total VSOP contents compared to the other samples. Different enzymes can lead to different volatile compositions as they produce proteins and peptides that can have anti-oxidative or pro-oxidative effects during EAAE. In their study, Wang et al. (2020) found differences in the DPPH radical scavenging properties and ferrous-ion chelating activities of cobia liver hydrolysates between Alcalase, Papain, Pepsin and Trypsin. In another study using EAAE to extract oils from fish co-products, Protex 7L and F protease resulted in higher PVs compared to Neutrase and Alcalase, highlighting that different enzymes can lead to differences in oxidation (Hathwar et al., 2011). Peinado et al. (2016) studied the effect of different enzymes on aroma formation in fish hydrolysates with and without added fish oil. The results indicated that the different enzymes led to distinctive differences in volatile compositions. For example, Flavopro 750 was distinguished from Flavopro Umami 852, the most important differences being in the concentrations of 1-octen-3-ol, 4-heptenal, 2-methylbutanal, and hexanal. In Study **III**, there were no significant differences between Neutrase and Protamex samples when comparing the different enzyme concentrations. Therefore, increasing the extraction time correlated with an increasing level of oxidation as was also shown in Study **II**, but the different enzyme concentrations did not contribute to differences in oxidation.

Table 10. Identified volatile secondary oxidation products of enzymatically extracted Baltic herring oil using HS-SPME-GC-MS (Study **III**). The compounds were identified based on the literature, Study **II**, NIST library (MS), and retention indices. Retention indices (RI) were calculated in regard to a series of *n*-alkanes. The columns were SPB®-624 Fused Silica Capillary Column (60 m × 0.25 mm × 1.4 µm) used for the optimisation tests, and DB-WAX (60 m, 0.25 mm, 0.25 µm) used in the samples with reduced emulsion.

SPB-624				***DB-WAX***			
Nbr.	***Compound***	***Identification***	***RI***	***Nbr.***	***Compound***	***Identification***	***RI***
1	Acetaldehyde	MS, Ahonen et al. 2022, Study II		1	2-Methyl-3-pentanone	MS	1055
2	2-Propenal	MS, Ahonen et al. 2022, Study II		2	2,3-Pentanedione	MS	1059
3	Propanal	MS, Ahonen et al. 2022, Study II		3	2-Hexanone	MS	1083
4	3-Methylpentane	MS		4	1-Penten-3-ol	MS	1164
5	2-Ethylfuran	MS, Ahonen et al. 2022, Study II	722	5	Heptanal	MS	1192
6	Methylcyclohexane	MS	737	6	3-Hexanol	MS	1203
7	2,3-Pentanedione	MS, Study II	743	7	2-Hexanol	MS	1228
8	(*E*)-2-Methyl-2-butenal	MS	796	8	2,3-Octanedione	MS	1329
9	2,4-Dimethylhexane	RI, Damerau et al., 2022a	825	9	1-Hexanol	MS	1362
10	3-Hexanone	MS	831	10	1-Octen-3-ol	MS	1457
11	2-Hexanone	MS	839	11	Acetic acid	MS	1465
12	3-Hexanol	MS	844	12	(*E,Z*)-2,4-Heptadienal	MS	1481
13	Hexanal	MS, Study II	847	13	3,5,5-Trimethyl-2-hexene	MS	1494
14	2-Hexanol	MS	849	14	(*E,E*)-2,4-Heptadienal	MS	1506
15	2,4-Dimethyl-1-heptene	MS	851	15	(*E,E*)/(*E,Z*)-3,5-Octadien-2-one	MS	1533
16	2,3-Dimethylheptane	MS	863	16	(*E,E*)/(*E,Z*)-3,5-Octadien-2-one	MS	1591
17	4-Methyloctane	MS	867	17	(*E,Z*)-2,6-Nonadienal	MS	1607
18	(*E*)-2-Hexenal	MS, Ahonen et al. 2022	915				
19	Heptanal	MS, Study II	950				
20	1-Octen-3-ol	MS	1027				
21	Octanal	MS, Study II	1053				

5.2.4 The effects of raw material, enzyme concentrations and extraction times on lipid composition (Studies II & III)

The fatty acid compositions of different enzymatically extracted oils and solvent-extracted oil from whole Baltic herring and its co-products are presented in Table 11 (Study **II**). The amount of SFAs, MUFAs, and PUFAs of the identified fatty acids were 26%, 38%, and 36%, respectively. Further, the *n*−3 and *n*−6 PUFA contents were 28% and 8%, respectively. The lipid composition of whole Baltic herring was similar to shown in literature, where the content of *n*−3 PUFAs have ranged from 25 to 30%, and *n*–6 PUFAs from 8 to 9% (Aro et al., 2000; Damerau et al., 2022b). The 35-minute extraction with Alcalase and the 70-minute extraction with Neutrase resulted in statistically lower contents of PUFAs compared to other treatments. Together with the 70-minute extraction with Protamex, these treatments led to lower *n*−3 FA contents, whereas the other enzymatic treatments resulted in a PUFA content similar to or better than the levels obtained with the solvent extraction. The *n*–6 FAs were found in significantly higher concentrations in the enzymatically extracted oils than in the solvent-extracted oil. The most dominant SFA was palmitic acid, which had a concentration varied of 17.6% to 18.4% depending on the extraction. Further, the most dominant MUFA was oleic acid (C18:1*n*–9c), which had a concentration of 23.3% to 24.8%. The fatty acid results presented here are in line with previous studies on Baltic herring oil composition (Damerau et al., 2022b; Kakko et al., 2022; Szlinder-Richert et al., 2010).

The most interesting findings in the fatty acid data concern the differences in PUFAs, especially in EPA and DHA contents. The EPA content in the oil from whole fish was 6.1–7.3%, the lowest values being from 70-minute extractions with Neutrase and Protamex, and the highest value from the 35-minute extraction with Protamex. The two-way ANOVA also shows that both the enzymes and hydrolysis times as well as their interaction (enzyme*time) had a significant impact on the *n*−3 PUFA content. The DHA content was significantly higher in the solvent-extracted oil, reaching 11.5%, whereas in the enzymatically extracted oils, it ranged from 9.4% to 10.8%. The lowest DHA values were obtained from the 35-minute extractions with Neutrase and Alcalase, as well as the 70-minute extraction with Protamex. The highest values were achieved with the 70-minute extraction with Alcalase, and the 35-minute extraction with Protamex. Again, the two-way ANOVA showed significant differences between the enzymes and hydrolysis times as well as the impact of their interaction. In another study, DHA was the most abundant PUFA in Baltic herring oil with a content of 20.2 ± 4.9% (Szlinder-Richert et al., 2010), which is significantly more than in Study **II**. However, The DHA contents presented here were comparable to Baltic herring fillets (7.8%) and gutted Baltic herring (9.4%) reported by Damerau et al.

(2020b). The differences in these compositions may be partly from oxidation processes during the extraction because oxidation causes degradation of long-chain PUFAs. Further, differences in the composition of lipid classes could cause differences in fatty acid profiles, as different lipid molecules have varying contents of different fatty acids (Linko et al., 1985). The solvent-extraction used as a reference method in the study extracts all lipids whereas the emulsion-formation during EAAE may result in differences in lipid class, and thus, FA composition of the crude oils.

In contrast to whole fish, the EPA and DHA contents in the oils extracted from the co-products did not significantly differ between the samples (Table 11). Further, there were no significant differences in the compositions of SFAs, MUFAs, PUFAs or *n*−3 FAs between the treatment groups. The most abundant fatty acids from SFAs, MUFAs, and PUFAs were palmitic acid (17.8–19.4% of total FAs), oleic acid (24.7–25.5%) and DHA (8.3–9.3%), respectively. Statistical differences ($p < 0.05$) were seen in the *n*–6 PUFAs according to one-way ANOVA (all samples) but the two-way ANOVA (only enzymatically extracted samples) did not show significant differences between the enzymes or extraction times. The FAs contributing to the biggest differences between the different enzymatic extractions were myristoleic acid (14:1*n*–5) and palmitoleic acid (16:1*n*–6), which were significantly affected by the different enzymes and hydrolysis times.

The enzymatically extracted samples in Study **III** using whole Baltic herring contained 4.8–6.6% of EPA (Table 12), however, there were no significant differences in the contents between the different enzymatic extractions or compared to the solvent-extracted oil. Remarkably, there was a significant drop in the DHA content in the enzymatically extracted oils (5.0–6.0%) compared to the solvent-extracted oil (11.5%). The change was also visible in the total PUFA and *n*−3 FA contents, which were significantly decreased in most of the enzymatically extracted oils compared to the content of the solvent-extracted oil. EPA and DHA are found in TAGs and phospholipids in different ratios depending on the fish species but their distribution in Baltic herring lipids have not been studied. We hypothesised that the DHA is lost with phospholipids that emulsify during the hydrolysis. In a study using cod, hydrolysed proteins formed protein–lipid complexes, especially with phospholipids (Šližyte et al., 2004).

Table 11. Fatty acid compositions of enzymatically extracted oils using whole fish and co-products of Baltic herring, and solvent-extracted oil (reference) (Study **II**).

	2-way ANOVA			*Solvent*	*Alcalase*		*Neutrase*		*Protamex*	
Fatty acid	***Enzyme***	***Time***	***E×T***	***extraction***	***35 min***	***70 min***	***35 min***	***70 min***	***35 min***	***70 min***
Whole fish										
EPA	*	**	**	6.46 ± 0.08b	6.11 ± 0.06a	6.93 ± 0.03c	6.74 ± 0.03bc	6.06 ± 0.09a	7.33 ± 0.20d	6.06 ± 0.16a
DHA	*	*	**	11.50 ± 0.11d	9.68 ± 0.10a	10.73 ± 0.05c	10.23 ± 0.04b	9.45 ± 0.15a	10.76 ± 0.29c	9.43 ± 0.27a
∑SFA	*	n	n	25.81 ± 0.26	25.72 ± 0.24	25.56 ± 0.17	26.02 ± 0.07	25.93 ± 0.32	25.84 ± 0.39	26.23 ± 0.44
∑MUFA	n	*	**	8.14 ± 0.28b	38.06 ± 0.18b	35.92 ± 0.10a	35.86 ± 0.03a	37.25 ± 0.29b	35.83 ± 0.53a	37.56 ± 0.44b
∑PUFA	n	n	**	36.04 ± 0.29a	36.2 ± 0.40a	38.52 ± 0.27b	38.13 ± 0.07b	36.83 ± 0.56a	38.33 ± 0.91b	36.21 ± 0.89b
∑*n*–3	n	*	**	28.25 ± 0.25bc	27.21 ± 0.30ab	29.31 ± 0.17c	28.97 ± 0.07c	27.23 ± 0.38a	29.27 ± 0.71c	27.09 ± 0.69ab
∑*n*–6	*	*	n	7.79 ± 0.05a	9.01 ± 0.10b	9.21 ± 0.10b	9.16 ± 0.12b	9.60 ± 0.21c	9.06 ± 0.22b	9.12 ± 0.20b
Co-products										
EPA	n	n	n	5.62 ± 0.18	5.83 ± 0.01	5.36 ± 0.76	5.53 ± 0.06	5.35 ± 0.10	5.87 ± 0.01	5.88 ± 0.01
DHA	n	n	n	9.26 ± 0.34	9.18 ± 0.05	8.27 ± 1.39	8.76 ± 0.09	8.48 ± 0.13	9.28 ± 0.03	9.03 ± 0.03
∑SFA	n	n	n	26.48 ± 0.17	26.06 ± 0.06	27.81 ± 3.41	26.05 ± 0.26	27.34 ± 0.67	25.84 ± 0.08	25.77 ± 0.04
∑MUFA	*	n	n	38.00 ± 0.66	38.35 ± 0.04	39.15 ± 1.29	37.79 ± 0.10	38.26 ± 0.22	37.79 ± 0.06	37.72 ± 0.10
∑PUFA	n	n	n	35.52 ± 0.75	35.59 ± 0.02	33.04 ± 4.69	36.16 ± 0.24	34.40 ± 0.72	36.37 ± 0.05	36.51 ± 0.11
∑*n*–3	n	n	n	26.45 ± 0.74	26.43 ± 0.04	24.25 ± 3.56	26.41 ± 0.30	25.20 ± 0.48	26.90 ± 0.06	26.89 ± 0.11
∑*n*–6	n	n	n	9.07 ± 0.05ab	9.16 ± 0.03ab	8.78 ± 1.14a	9.75 ± 0.05b	9.21 ± 0.25ab	9.47 ± 0.01ab	9.62 ± 0.03ab

The values are presented as mass percentages of the identified fatty acids ± standard deviation (n=3). Different letters indicate significant differences ($p < 0.05$) detected by one-way ANOVA and Tukey HSD. * = $p < 0.05$, ** = $p < 0.001$, and n = not significant as determined by statistical comparisons with two-way ANOVA.

Table 12. Fatty acid and lipid class composition of enzymatically extracted oils (optimisation test) and solvent-extracted oil (reference) from whole Baltic herring (Study **III)**.

	Solvent extraction	***Alcalase***	***Alcalase***	***Alcalase***	***Neutrase***	***Neutrase***	***Neutrase***	***Neutrase***	***Neutrase***	***Protamex***	***Protamex***	***Protamex***	***Protamex***	***Protamex***
Enzyme dose (%)		***0.4***	***0.4***	***0.4***	***0.4***	***1.0***	***1.0***	***1.0***	***2.0***	***0.4***	***1.0***	***1.0***	***1.0***	***2.0***
Time (min)		***35***	***70***	***105***	***35***	***35***	***70***	***105***	***35***	***35***	***35***	***70***	***105***	***35***
EPA	6.20±0.23	4.80±1.23	5.59±0.93	5.64±0.80	5.58±0.64	5.63±0.68	5.26±0.99	5.83±0.76	5.62±0.68	6.29±1.87	6.61±2.11	5.53±1.06	5.85±0.74	6.26±1.74
DHA	11.47±0.25b	4.96±0.47a	5.68±0.27a	5.71±0.95a	5.54±0.60a	5.59±0.59a	5.83±0.24a	6.04±0.88a	5.57±0.45a	5.74±1.15a	5.81±1.06a	5.74±0.45a	5.87±0.90a	5.76±1.04a
ΣSFA	25.00±0.23	24.63±0.05	25.10±0.36	24.69±0.31	24.99±0.20	24.84±0.26	23.78±0.50	23.53±0.23	24.88±0.48	23.49±1.89	24.24±0.93	25.01±1.55	23.74±0.32	23.72±1.59
ΣMUFA	38.64±0.05	45.30±1.60	44.44±2.66	46.50±2.18	44.46±2.85	44.23±2.52	42.90±3.31	46.80±2.89	44.59±2.50	47.24±4.34	46.79±2.89	42.21±4.36	47.10±2.66	47.56±4.20
ΣPUFA	36.36± 0.19b	30.07± 1.58ab	30.46± 2.57ab	28.81± 1.98a	30.55± 2.71ab	30.93± 2.34ab	33.32± 2.83ab	29.67± 2.98ab	30.53± 2.16ab	29.28± 2.56ab	28.98± 2.02a	32.78± 3.08ab	29.16± 2.66ab	28.72± 2.61a
Σ*n*−3	26.96± 0.26b	20.32± 0.24a	21.26± 0.07a	20.61± 1.77a	21.09± 1.30a	21.54± 0.78a	22.50± 0.58ab	21.32± 2.75a	21.11± 0.64a	21.22± 2.80a	21.06± 2.16a	22.22± 1.32ab	20.95± 2.46a	20.77± 2.39a
Σ*n*−6	9.40±0.31	9.74±1.77	9.21±2.51	8.20±0.25	9.46±1.49	9.39±1.56	10.82±2.60	8.36±0.24	9.41±1.59	8.06±1.38	7.92±1.70	10.56±2.34	8.21±0.20	7.95±1.45
FFA	0.09 ± 0.02	0.05 ± 0.00	0.05 ± 0.00	0.06 ± 0.01	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.00	0.06 ± 0.01	0.06 ± 0.01
MAG	0.06 ± 0.01	0.09 ± 0.03	0.11 ± 0.01	0.10 ± 0.03	0.07 ± 0.00	0.08 ± 0.02	0.12 ± 0.01	0.12 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.11 ± 0.01
DAG	0.02 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
TAG	43.71 ± 4.38a	58.85 ± 7.22bc	53.48 ± 3.03abc	56.85 ± 6.03abc	57.90 ± 4.41bc	57.68 ± 1.40abc	49.39 ± 4.55ab	56.57 ± 2.11abc	65.03 ± 0.46c	62.78 ± 3.83bc	58.78 ± 8.33bc	59.27 ± 3.75bc	57.60 ± 4.92abc	61.33 ± 5.07bc
PL	9.46 ± 0.95b	0.02 ± 0.00a	0.02 ± 0.01a	0.02 ± 0.00a	0.02 ± 0.00a	0.01 ± 0.00a	0.02 ± 0.01a	0.01 ± 0.00a	0.02 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.01a	0.01 ± 0.00a	0.01 ± 0.01a
PL/ TAG	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

The results are presented as mass percentages of the identified compounds (m-%) ± standard deviation (n=3). Significant differences ($p < 0.05$) are marked with different letters. Abbreviations: SFAs = saturated fatty acids, MUFAs = monounsaturated fatty acids, PUFAs = polyunsaturated fatty acids, *n*–3 = omega-3 fatty acids, *n*–6 = omega-6 fatty acids, FFA = free fatty acids, MAG = monoacylglycerols, DAG = diacylglycerols, TAG = triacylglycerols, and PL = phospholipids.

Lipid classes were analysed with a novel lipid class method developed for UHPLC-MS. The method was developed to quantify lipid classes, and the same analysis method was used for both neutral and polar lipid fractions. Prior fractionation of the samples was necessary to avoid suppression of ionisation of phospholipids by TAGs (Araujo et al., 2016). The method separated compounds based on their fatty acid chain length and it enabled precise quantification, which was determined by creating standard curves with different lipid compounds. The solvent-extracted fish oil contained 9.5% of phospholipids, of which the identified phospholipids consisted of lysophosphatidylethanolamine (LPE), LPC, PI, PC, PG, PE, and SM. The most abundant phospholipid group in the solvent-extracted oil was PC comprising 60.0% of the identified compounds, followed by PE with 19.2%, PI with 11.2%, LPE with 4.4%, LPC with 2.9%, PG with 1%, and SM with 0.3% (Figure 12). PS or phosphatidic acid were not identified from the oils. According to the review by Lu et al. (2011), the most abundant phospholipid group in marine sources is PC, followed by either PE or LPC, depending on the species. For example, PC comprises 53.6%, PE 22.9%, PI 8.3%, cardiolipin 6.2%, SM 4.9%, and PS 4.1% of the phospholipids in rainbow trout. On the contrary, krill, which is known for its high phospholipid content, has 86.0% of its phospholipids in the PC form, whereas the other groups contain 1–6% each. The results shown here are in accordance to the literature on phospholipid class division in fish, but it is the first time a more specific information about Baltic herring phospholipids is presented.

Nearly all phospholipids were lost during the enzymatic extractions (Table 12), as the enzymatically extracted oils contained only 0.01–0.02% of phospholipids. On the contrary, the relative abundance of TAGs was higher in the enzymatically extracted samples compared to the solvent-extracted oil. Even though the removal of phospholipids is desirable in oil production as they easily hydrolyse to FFAs, it simultaneously removed important fatty acids attached to them, such as DHA and EPA (Marsol-Vall et al., 2021). The amounts of identified MAGs, DAGs and FFAs were low in all of the samples and there were no significant differences between the enzymatic treatments.

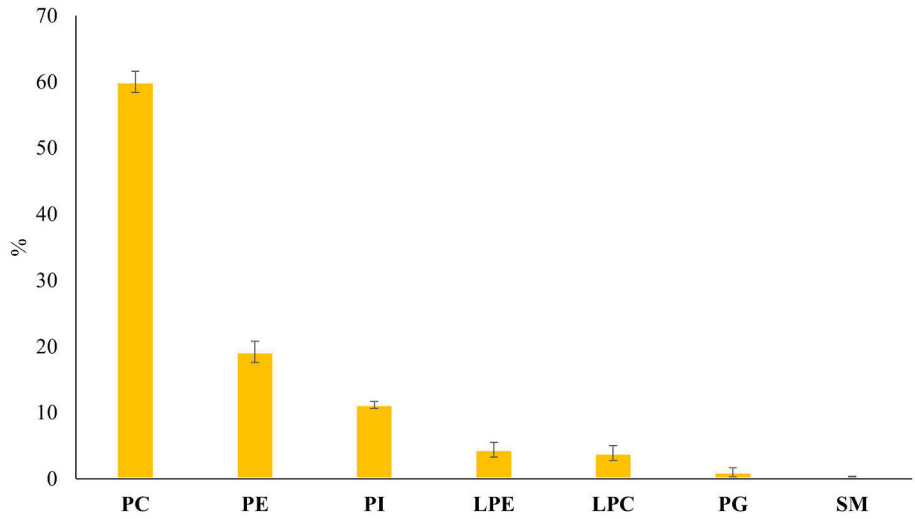

Figure 12. The percentage of different groups of phospholipids out of all identified phospholipids in Baltic herring oil extracted with a solvent extraction method and quantified with UHPLC-MS. PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, LPE = lysophosphatidylethanolamine, LPC = lysophosphatidylcholine, PG = phosphatidylglycerol, and SM = sphingomyelin.

TAGs comprised 42.8% of the lipids in the oil sample while MAGs, DAGs, and FFAs represented 0.06%, 0.02% and 0.09%, respectively (Table 12). The results are presented as percentage of the lipid sample applied for fractioning, and some lipids may have lost during the fractioning. TAGs are the main lipid class in medium-fat and fatty fish species. In freshwater salmonids, for example, the proportion of TAGs is 43.4–89.7% of total lipids (Sushchik et al., 2020), whereas in marine species golden pompano and freshwater whitefish the values are 90% and 51.5%, respectively (He et al., 2019; Suomela et al., 2016). The proportion of TAGs in Baltic herring is prone to fluctuations depending on the season, and has been reported to be 54–91% of the total lipids (Linko et al., 1985). In a more recent study, 78% of the Baltic herring lipids were TAGs, while the amount of MAGs and DAGs were 0.2% and 2.1%, respectively (Kakko et al., 2022).

5.2.5 Emulsion reduction methods (Study III)

Different methods were studied to reduce or break emulsion during EAAE to improve oil recovery. All the tests were done using Protamex with an enzyme concentration of 0.4% (except one test using only 0.1%), and an extraction time of 105 min. Protamex was chosen for the tests as it is a mixture of Alcalase and Neutrase, and would therefore be well suited to find the optimal process that works with all three enzyme products. Freezing the hydrolysate before centrifugation was the best method, reducing emulsion by 86%. However, almost all of the oil was trapped either in the hydrolysate or sediment, therefore only a small amount was collected despite the reduction of emulsion formation using the optimised method (Table 13). In their study, Nilsuwan et al. (2022) were able to increase oil recovery with repeated freeze-thawing cycles.

Other methods were also effective in reducing the emulsion layer: addition of salt reduced it by 45%, citric acid by 57%, reducing enzyme concentration from 0.4% to 0.1% by 62%, addition of ethanol by 64%, and the combination of water reduction and ethanol addition reduced as much as 72% of the emulsion compared to the control sample. The best method both to improve oil recovery and to reduce emulsion was the combination of water reduction and addition of ethanol. The highest recovery rates were achieved with the addition of ethanol, or combining fish:water ratio change from 1:1 to 2:1 (w/w) to the addition of ethanol. However, there were no statistically significant differences ($p < 0.05$) in the oil recoveries of the emulsion test samples due to high standard deviations. The effect of ethanol on emulsion systems can vary depending on protein size and molecular weight of the surfactant. In some cases, ethanol has shown to stabilise emulsions but it can also cause protein to aggregate and therefore lose stability in emulsion systems. Further, ethanol can cause the emulsion droplet size to decrease and lower its density, which affects the emulsion stability (Ferreira et al., 2020). The reduction or elimination of water has shown to reduce emulsion and to be a key element in increasing the separation of oil in EAAE (Šližyte et al., 2005).

Table 13. Oil recovery (% of the control sample) and emulsion layer (mm) of enzymatically extracted oils (Study **III**). The control sample parameters were: enzyme concentration 0.4%, extraction time 105 minutes, and fish:water ratio 1:1 (w/w).

	Control	***0.1% enzyme***	***Fish:water (2:1)***	***+NaCl***	***+Ethanol***	***+Citric acid***	***Freezing***	***Fish:water (2:1) + ethanol***
Oil recovery (% compared to control)	100	80 ± 14	90 ± 52	51 ± 33	102 ± 60	53 ± 3	15 ± 26	111 ± 56
Emulsion layer (mm)	14.3 ± 4.6b	5.3 ± 2.5a	9.5 ± 4.1ab	7.7 ± 4.0ab	5.0 ± 3.4a	6.0 ± 1.7ab	2.0 ± 1.0a	3.0 ± 0.0a

The results are presented as mean value ± standard deviation (n=3). Significant differences between the samples are indicated with different letters (one way ANOVA with Tukey's post hoc test, $p < 0.05$).

5.2.6 The effect of emulsion reduction on the quality of oil (Study III)

In Study **III**, Alcalase, Neutrase and Protamex were used with the concentration of 0.4% and extraction time of 105 min, while the fish to water ratio was 2:1 (w/w) and ethanol was added after the inactivation step to study the effect of emulsion reduction on the quality of the crude oils. The oil recoveries of the samples were 0.7–0.8 g/100g fish, while the lipid content of the raw material was 2.8 g/100g extractable by the solvent-extraction (Table 14). Therefore, the recovery percentages were 26–28%, lower than those from the previous samples (optimisation part). Throughout Study **III**, the oil recoveries were lower than in Study **II**, and even the optimisation of the parameters and reducing emulsion did not improve them. The two different batches of fish used in this study had low lipid contents: 3.8% in the batch used for the optimisation tests, and 2.8% used for the emulsion tests and the samples with reduced emulsion, while the whole Baltic herring used in Study **II** contained 9.0%.

Table 14. Extracted oil contents (g/100g of fish) and emulsion layers (mm) of enzymatically extracted oils with reduced emulsion (Study **III**). The extraction conditions for enzymatic extractions were: 0.4% enzyme concentration, 105 min, fish:water (2:1) + ethanol.

	Solvent extraction	***Alcalase***	***Neutrase***	***Protamex***
Extracted oil content (g/100g)	2.84 ± 0.08b	0.74 ± 0.17a	0.81 ± 0.17a	0.77 ± 0.19a
Emulsion layer (mm)	-	5.3 ± 1.2	3.7 ± 0.6	3.3 ± 0.6

The results are presented as mean value ± standard deviation (n=3). Significant differences between the samples are indicated with different letters (one way ANOVA with Tukey's post hoc test, $p < 0.05$).

The fatty acid analysis showed no significant differences in FA compositions between the three enzymes (Table 15). The most abundant FA was oleic acid (18:1*n*–9c) accounting for 25.1–25.5% of the total FAs, followed by palmitic acid (16:0) (17.5–17.7%), and palmitoleic acid (16:1*n*–7) (11.3–11.6%). The extracted oils were rich in unsaturated fatty acids (71.4% of all FAs) including *n*–3 FAs (24.1–24.4%) and *n*–6 FAs (6.9–7.8%). The proportions of EPA and DHA were 8.2% and 8.8–8.9%, respectively. The percentages of EPA and DHA were both higher than in the "optimisation" samples of Study **III** (Table 12), but the levels of DHA were lower than in the solvent-extracted oil. In Study **II**, the EPA contents in enzymatically extracted oils from whole Baltic herring were

6.1–7.3%, while the DHA contents were 9.4–10.8% (Study **II**) showing some differences compared to the samples with reduced emulsion in Study **III.** However, there were major differences in the lipid contents between the batches used in the studies, which likely explains the varying levels of DHA.

The lipid class results of the enzymatically extracted oils with reduced emulsion show that 87.8–92.2% of the oil consisted of TAGs which is a desired oil fraction, whereas the phospholipid contents were only 0.02–0.03% (Table 15). The amount of TAGs is remarkably larger than in the optimisation samples but the amounts of phospholipids remained low (Table 12). The results indicate that the emulsion reduction led to an increased TAG content in the oil, whereas phospholipids were still trapped in the remaining emulsion or other phases. The increased TAG content is likely related to the increased EPA and DHA contents in the oil as well, as these fatty acids are abundant in TAGs. The amounts of FFAs, MAGs and DAGs remained low in the oils: 0.01–0.04%.

The PV of the samples with reduced emulsion were 5.2–8.3 meq/kg (Table 8). Alcalase resulted in the lowest PV but the difference was not statistically significant compared to Neutrase and Protamex. In the VSOP analysis, 17 compounds were identified from the samples using the DB-WAX column (Table 10). Protamex resulted in the highest total area of VSOP, but there were no statistically significant differences between the enzymes (Figure 13). The most abundant VSOP identified from the oils were 2,3-pentanedione, 3,5,5-trimethyl-2-hexene, and 1-octen-3-ol, which are known compounds in fish related to oxidation and spoilage (Duflos et al., 2010). Alcalase treatment led to the highest areas of 2,3-pentanedione and 1-penten-3-ol, whereas Protamex had highest area of 2,5,5-trimethyl-2-hexene, however, the differences were not significant between the enzymes. 2,4-Heptadienal and 1-penten-3-ol, both found from the samples, are produced by the degradation of DHA and EPA, and they have shown potential as indicator compounds for lipid oxidation of oils rich in EPA and DHA (Damerau et al., 2020b; Lee et al., 2003).

Table 15. Fatty acid compositions and lipid classes of oil samples extracted enzymatically from Baltic herring, where emulsion formation was reduced (Study **III**). The fatty acid results are presented as mass percentages of the identified compounds (m-%) ± standard deviation (n=3) whereas the lipid class results are presented as percentage of the lipid sample (%) ± standard deviation (n=3). The extraction conditions were: enzyme concentration of 0.4% (w/w), extraction time of 105 minutes, fish:water ratio of 2:1 (w/w) and additional ethanol added after inactivating enzymes.

	Alcalase	***Neutrase***	***Protamex***
Fatty acids			
EPA	8.22 ± 0.25	8.20 ± 0.26	8.22 ± 0.16
DHA	8.85 ± 0.94	8.88 ± 1.19	8.81 ± 0.84
ΣSFA	22.68 ± 0.86	22.69 ± 0.80	22.79 ± 0.98
ΣMUFA	40.09 ± 4.17	40.26 ± 4.74	40.57 ± 3.41
ΣPUFA	31.32 ± 3.36	31.21 ± 3.90	30.81 ± 2.52
Σ*n*−3	24.40 ± 1.84	24.16 ± 2.26	24.06 ± 1.29
Σ*n*−6	6.93 ± 1.57	7.04 ± 1.77	6.75 ± 1.39
n−3/ *n*−6	3.52	3.45	3.56
Lipid classes			
FFA	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
MAG	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
DAG	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
TAG	92.27 ± 5.46	94.68 ± 5.15	87.80 ± 5.32
PL	0.03 ± 0.02	0.02 ± 0.00	0.03 ± 0.01
PL/TAG	0.00	0.00	0.00

Abbreviations: SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid, *n*−3 = omega-3 fatty acid, *n*−6 = omega-6 fatty acid, FFA = free fatty acid, MAG = monoacylglycerol, DAG = diacylglycerol, TAG = triacylglycerol, and PL = phospholipid.

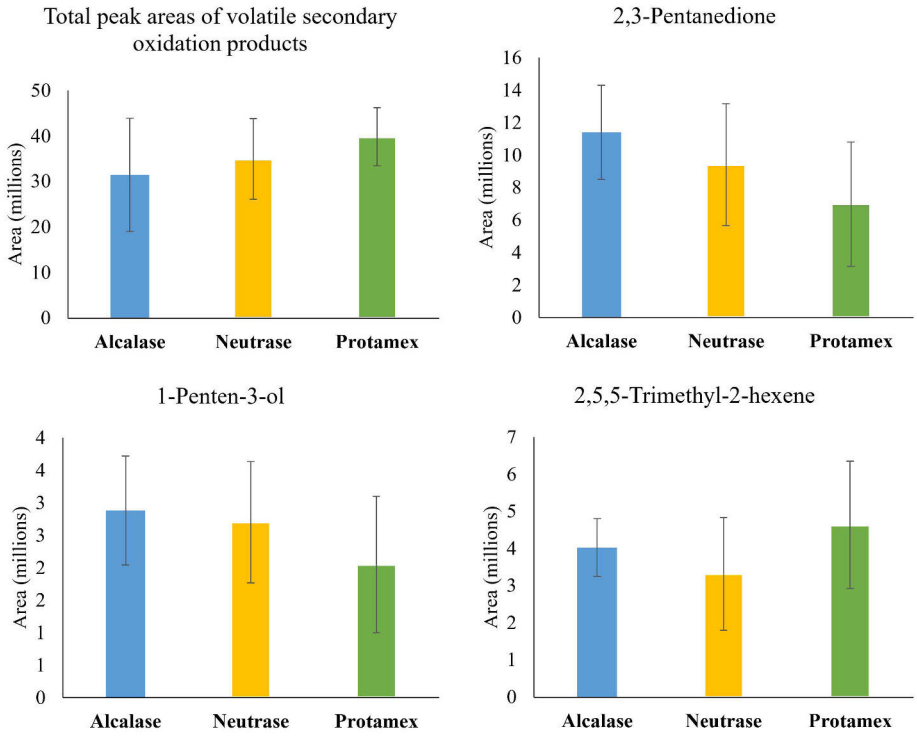

Figure 13. Total peak area of volatile secondary oxidation products, and 2,3-pentanedione, 1-penten-3-ol and 2,5,5-trimethyl-2-hexene areas in the enzymatically extracted oils from Baltic herring, where emulsion formation was reduced (Study **III**). The results are presented as mean value ± standard deviation (n=3).

The total contents of tocopherols and tocotrienols in the samples with reduced emulsion varied between 1530–1570 µg/g of oil, while the solvent-extracted oil contained 1580 µg/g (Table 16). The identified compounds were α-tocopherol, γ-tocopherol, δ-tocopherol, α-tocotrienol, and γ-tocotrienol. The largest quantified peak (815–1018 µg/g) was an unidentified compound assumed to be a mixture of oxidation products of α-tocopherol as they eluted before the α-tocopherol standard. Possible compounds could be α-tocopheryl quinone and 5-formyl-γ-tocopherol, that have previously been identified as α-tocopherol oxidation products (Tang et al., 2020). The amount of α-tocopherols, however, was only 116–130 µg/g. Wu et al. (2022b) identified α-tocopherol from different parts of herring but other tocopherols were not shown in the analysis. Based on the results shown here, the α-tocopherol was likely consumed during the extractions, or even before the processing (after catching, before the fish was frozen), because the solvent-extracted oil contained only a small amount, although significantly more than Alcalase- and Neutrase-extracted oils. The

samples contained γ-tocopherols and γ-tocotrienols in concentrations of 589–598 µg/g and 640–659 µg/g, respectively, but the differences between samples were not significant.

Table 16. Tocopherols and tocotrienols (µg/g of oil) in enzymatically extracted Baltic herring oil with reduced emulsion, and solvent-extracted oil used as a reference. The results are presented as µg/g of oil ± standard deviation (n=3). Significant differences between the samples are indicated with different letters (one way ANOVA with Tukey's post hoc test, $p < 0.05$). The sum of tocopherol and tocotrienols does not contain the unknown compound.

	Solvent extraction	***Alcalase***	***Neutrase***	***Protamex***
Unknown	1018 ± 258	814 ± 95	926 ± 114	970 ± 199
α-Tocopherol	130 ± 9b	116 ± 3a	115 ± 4a	121 ± 9b
α-Tocotrienol	141 ± 15	130 ± 6	133 ± 8	137 ± 13
γ-Tocopherol	593 ± 15	596 ± 3	589 ± 13	597 ± 7
γ-Tocotrienol	658 ± 25	643 ± 8	640 ± 19	658 ± 19
δ-Tocopherol	54 ± 3	51 ± 1	52 ± 2	52 ± 4
SUM	1578 ± 64	1539 ± 20	1531 ± 43	1567 ± 49

5.3 General discussion

5.3.1 Limitations and strengths of the study

Study **I** was the first study about the utilisation of Baltic herring protein isolate in food models familiar to consumers. The study revealed important insights into the texture and volatile compounds, essential for determining the viability of the pH-shift process for protein extraction from Baltic herring. Additionally, it demonstrated that modifying the solubilization and precipitation pH values did not impact the colour of the protein isolates, which remains an unresolved issue. However, Study **I** had certain limitations, most importantly the lack of sensory evaluation to analyse consumers' acceptance to the addition of the protein isolate in food models in terms of texture, aroma and flavour, and whether the grey colour of the protein isolate is perceived as a negative attribute.

Study **II** uncovered variations between different commercial enzymes and treatment times in the extraction of oil from whole Baltic herring and its filleting co-products using EAAE. Both the choice of raw material and different enzymes resulted in differences in oxidative stabilities, likely influenced by varying levels of oxidative compounds, such as heme protein, and the impact of enzymes on

the generation of pro-oxidants and antioxidants during hydrolysis. The strengths of the studies were the use of three different methods for assessing the oxidative stability as it was the first time EAAE was studied in the extraction of oil from Baltic herring. Further, the study concluded that filleting co-products, which are usually regarded as waste, can be used to produce oils rich in *n*−3 FAs.

Study **III** was not originally planned as a part of this doctoral thesis, but the topic seemed highly important when problems occurred in the extraction using leaner raw material. The strength of the study was the development of a new method (UHPLC-MS) to study the lipid classes, and therefore, a deeper comprehension of oil composition alterations resulting from various extraction parameters utilized in EAAE. However, different phases resulting from EAAE, such as the sediment and supernatant should have been analysed to determine the distribution of different lipids in each phase. Also, a combined method to produce both oil rich in *n*−3 FAs, and a functional protein hydrolysate should have been assessed.

5.3.2 Relevance and significance of the work

Baltic herring is the most important fish catch in Finland both in terms of volume and value. Unfortunately, a major portion of this catch is used as feed, and its consumption within Finland has also decreased dramatically in the last decades. Being a healthy fish rich in protein and PUFAs, it is important to develop new techniques to utilise it for human consumption. It is generally accepted, that the optimal human diet should have an *n*−6/*n*−3 ratio of approximately 1. However, the Western diet has changed drastically over the last 100 years, and nowadays people are getting more *n*−6 FAs from their diets, typically from vegetable oils. As a result, the *n*−6/*n*−3 ratio in the Western diet is approx. 15/1–16.7/1. Such elevated ratios of *n*−6/*n*−3 FAs have been associated with various health-related issues, such as cardiovascular disease, cancer and inflammatory and autoimmune diseases. Individuals with diets rich in fish ,or supplementation with DHA and EPA, have been associated with reductions in cardiovascular diseases and related mortality compared to those who do not consume fish (Russo, 2009; Simopoulos, 2002). According to the fatty acid compositions presented in this research, the *n*−6/*n*−3 ratio in Baltic herring oil was 3.6/1 in the whole fish and 2.9/1 in the co-products, highlighting that Baltic herring is a good source of *n*−3 FAs.

5.3.3 Future prospects

The future of Baltic herring and its utilisation in food applications depends on one main factor: the healthiness of the fish stock. The Baltic Sea has a unique ecosystem due to its low salinity, but it is also very vulnerable to changes, global

warming being one of its biggest threats. Baltic herring is a healthy fish but due to changes in its fat composition and size, its industrial processing can be difficult and fluctuating. The key research topics on Baltic herring are: how to develop transportation and processing methods to preserve its quality better, and how to modify technological applications to treat both high-fat and low-fat fish. Further, the grey colour of Baltic herring, and technologies to mask it in food applications, should be further studied together with consumer and sensory tests. EAAE is widely used for the production of protein hydrolysates, which have also shown bioactive functionalities. With lean fish material, the focus of the process could be the production of peptides, or the use in protein extraction with pH-shift or other methods, while the fish with higher lipid content would be used for the production of PUFA-rich oils. In both scenarios, removing lipids from the protein fraction is essential to minimise oxidation, resulting in two valuable fractions that can be used as added-value products.

6 SUMMARY AND CONCLUSION

This thesis showed that proteins and lipids can be extracted from Baltic herring using the pH-shift method and enzyme-assisted extraction, respectively. Further, it showed that both whole fish and filleting co-products are good raw materials to produce oils rich in *n*–3 FAs. The study also showed the significance of the fluctuating oil content of Baltic herring to the efficiency of the EAAE, and highlighted the importance of optimisation of the process parameters depending on the raw material composition. In terms of protein isolation, Baltic herring produced protein isolate with good functional properties, but its grey colour might pose challenges in product development. In conclusion, the thesis is an important part of the national project "Blue Products" in developing methods to use underutilised fish species.

In Study **I**, the pH shift was shown to have potential as a protein extraction method for Baltic herring. The produced FPI was successfully used as an ingredient in two food models: surimi-type gels and fish balls. The surimi-type gels prepared from the protein isolate had similar texture properties to commercial surimi-based products, whereas the addition of FPI improved the shape and water holding ability of fish balls, although resulting in a softer product compared to a commercial reference. Yet, the colour of the SPI was significantly darker than in the commercial products due to the abundancy of dark muscle tissue in Baltic herring. The reduced fishy odour seen for the FPI compared to the raw material could have a positive effect on sensory perception of the final products. However, a consumer acceptability study of the developed food models is needed to confirm this.

In Study **II**, the best conditions for the enzymatic treatment were dependent on both the enzyme and the raw material. For whole Baltic herring, Neutrase resulted in the best oil recovery, and the 70-minute extraction time was better than 35 minutes. Neutrase also led to a smaller total volatile content than the other two enzymes; however, treatment with Alcalase gave the smallest TOTOX values. In contrast, extraction with Protamex resulted in the most oxidised oils. As for the co-products, Protamex with a 70-minute extraction time led to the highest oil recovery. Both hydrolysis times with Protamex resulted in oils with lower total volatile contents compared to treatment with Alcalase, and the longer extraction time with Neutrase.

In Study **III**, lean fish raw material caused increased amount of emulsion formation and simultaneous reduction in the oil recovery. The Baltic herring used in the first part of the study contained only 3.8% of lipids which is a lower than the typical lipid content reported in this species, and much lower than the 9.0% used in Study **II**. The oil recoveries using Alcalase, Neutrase and Protamex were

significantly lower (37–58%) than in Study **II**. The recoveries increased with increasing extraction time (35 to 105 min) but were not affected by the type or concentration of the enzymes. Low oil recoveries were accompanied with high (up to 57%) loss of DHA and almost a total loss of phospholipids. The oxidative stabilities of the oils were within the Codex standard (PV < 5) when using the shortest extraction time (35 min) with Alcalase and Neutrase, whereas the longest extraction times significantly increased the PV and VSOPs. Combining water reduction with an addition of ethanol was the most efficient method to reduce emulsion formation. The oil recoveries from the samples with reduced emulsion were low (26–28%) even with optimised extraction method, partly due to the even lower fat content of the raw material used in the second part of the study (2.8%). However, the optimised method increased the content of TAGs, EPA, and DHA in the extracted oils, but the phospholipid content remained low. Study **III** showed that the optimisation of the oil extraction using enzymes is crucial with different fish raw materials to improve the recovery rate and the quality of the oil.

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