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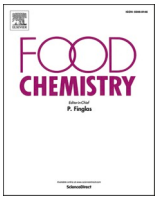

Enzyme-assisted aqueous extraction of fish oil from Baltic herring (*Clupea harengus membras*) with special reference to emulsion-formation, extraction efficiency, and composition of crude oil

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ABSTRACT

Enzyme-assisted aqueous extraction (EAAE) is a green, and scalable method to produce oil and protein hydrolysates from fish. This study investigated the role of different parameters on emulsion formation, oil recovery, and the composition of crude oil during EAAE of Baltic herring (*Clupea harengus membras*). Fatty acid compositions, lipid classes, tocopherols, and oxidation status of the EAAE crude oils were studied. Compared to solvent-extracted oil, EAAE resulted in a lower content of phospholipids accompanied by a 57% decrease in docosahexaenoic acid. Changing fish to water ratio from 1:1 to 2:1 (w/w) with ethanol addition led to the greatest reduction (72%) of emulsion, which resulted in an increase in oil recovery by 11%. The addition of ethanol alone, or reduction of enzyme concentration from 0.4% to 0.1% also reduced emulsion-formation significantly. Overall, emulsion reduction resulted in higher content of triacylglycerols and $n-3$ polyunsaturated fatty acids in the crude oil extracted.

1. Introduction

Baltic herring (*Clupea harengus membras*) is one of the most important fish catch of the Baltic Sea region. In Finland alone, 74 000 tons was caught in 2021 (Natural Resources Institute Finland, 2022). Despite the large availability and healthiness of Baltic herring, its domestic consumption is only 3 000–4 000 tons, while most of the catch is used in feed production, and a small portion is exported (Natural Resources Institute Finland, 2020). Food utilisation of especially the smallest fish categories and use of filleting co-products should be advanced. It is therefore of great interest to develop methods to extract different food-grade fractions such as Baltic herring oil, which can be used in foods and as food supplements. Enzyme-assisted aqueous extraction (EAAE) as a green extraction method to produce oil has been studied for many fish species (Aitta et al., 2021; Araujo et al., 2021; Carvajal et al., 2015; Vázquez et al., 2020; Wang et al., 2019). In addition to whole fish, it can also be applied to fish co-products, such as heads, fins, skin and viscera (Aitta et al., 2021; Głowacz-Różyńska et al., 2016; Šližyte et al., 2018). Extraction without hydrolysis would require the use of heat and/or solvents as used in conventional production of fish oil, which also leads to an exposure of PUFAs to light, heat, and other pro-oxidants. EAAE provides a green alternative to the conventional methods avoiding solvents harmful to the environment and health. Further, the use of heat and/or solvents causes denaturation of proteins and unfavourable changes in protein quality.

Baltic herring is a fatty fish, consisting of 4–10% of fat depending on the season (Aitta et al., 2021; Aro et al., 2000; Rajasilta et al., 2022). The oil consists mainly of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Omega-3 fatty acids ($n-3$ FAs) form up to 26–28% of the total FAs, the major ones being docosahexaenoic acid (DHA) (10.5–11.5%) and eicosapentaenoic acid (EPA) (6.5–7.2%) (Aitta et al., 2021; Kakko et al., 2022). EPA and DHA have shown to improve neuronal, retinal, brain, and cardiovascular health, as well as immune function (Ghasemi Fard et al., 2019). Although the health benefits of $n-3$ PUFAs are well known, their intake in the diet is low, especially from the Western diets (Molendi-Coste et al., 2011). Baltic herring would be

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an excellent raw material for oil production due to its high EPA and DHA contents, especially as is estimated that the increase in water temperatures due to climate change could lead to a 10 to 58% loss of globally available DHA by the year 2100 (Colombo et al., 2020). The fat content of Baltic herring has decreased from an average of 7–8% to 2–3% during the last few decades but on the other hand, the proportion of $n-3$ PUFAs of the total FAs have steadily increased (Rajasilta et al., 2022).

In fish oil, FAs are found as free fatty acids (FFA) or most commonly in different lipid classes, such as monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs), and phospholipids (PLs). TAGs are found in the adipose tissue of the fish whereas PLs occur mostly in cell membrane structures and cell signalling molecules. The distribution of FAs in different lipid molecules in fish tissue varies depending on the species. For example, EPA + DHA are bound to PLs and TAGs in a ratio of 40:60, respectively, in Atlantic salmon muscle (Polvi & Ackman, 1992). The PL content in Baltic herring have shown a wide range of 7.6–41.3% of the total lipids, and it is dependent on differences in feeding and spawning seasons. In their study, Linko et al. (1985) measured the lowest PL contents in the autumn and highest in the summer. Recently, Kakko et al. (2022) reported a PL content of 18.8% of the total lipids from Baltic herring caught in autumn. Lipid classes in Baltic herring have been studied using a semi-quantitative method, showing effect of EAAE on lipid class composition of Baltic herring oil (Kakko et al., 2022).

PLs are often removed from crude oil by a degumming process 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, fish PLs are rich in $n-3$ PUFAs, and some studies suggest that EPA and DHA may have a higher bioavailability when they are present in PLs instead of TAGs or ethyl esters (Rossmeisl et al., 2012; Wijendran et al., 2002). Therefore, the removal of PLs will result in a loss of $n-3$ PUFAs and may compromise both the nutritional value of the oil and the bioavailability of the important $n-3$ PUFAs. PLs in Baltic herring could emulsify with peptides formed during EAAE, trapping a proportion of the oil within the emulsion, and thus, significantly decreasing oil recovery during the extraction. In our previous study (Aitta et al., 2021), we showed that EAAE with various commercial enzymes are promising green technology for oil extraction from Baltic herring. In the current study, we investigated the yield and lipid composition of crude oil from several batches of Baltic herring caught from different seasons with varying oil content. Our special focus was placed on various parameters and measures for reducing emulsion-formation and increasing oil recovery as well as their impact on the composition of crude oil. Especially, lipid class composition of EAAE crude oil from Baltic herring as impact of different extraction parameters was studied for the first time. As the fat content decreases, the fish contains less TAGs as the main component of storage fat, which leads to an increase in the ratio of PLs to TAGs. The research hypothesis was that phospholipids emulsify with peptides during the EAAE, thus decreasing oil recovery, and that the effect could be decreased by optimising the process and by studying different emulsion-breaking methods. Our findings suggested that seasonal variation and batch to batch difference in the composition of fish raw material significantly influence the oil extraction efficiency during EAAE. In lean fish, high phospholipid/triacylglycerol ratio leads to a formation of emulsion and low extraction yield, which can be mitigated by careful optimisation of the process parameters of EAAE.

2. Materials and methods

2.1. Materials, chemicals and reference compounds

Baltic herring for the optimisation of the EAAE was purchased in January 2021 and a second batch for the emulsion-breaking trials was purchased in May 2022 from Martin Kala Oy (Turku, Finland). The batch of fish was kept frozen at − 80 °C before extractions. Alcalase® (from *Bacillus licheniformis*), Neutrase® (from *Bacillus amyloliquefaciens*) and Protamex® (from *Bacillus* sp.*)* were purchased from Novozymes (Bagsvaerd, Denmark). LC-MS quality isopropanol, water, methanol, *n*-hexane, and heptane were purchased from Honeywell Riedel-de-Haën AG (Seelze, Germany). HPLC-grade chloroform was purchased from Fisher Chemical (Fisher Scientific, Loughborough, UK). Ammonium formate, ammonium thiocyanate, iron dichloride, iron trichloride, and 1,4-dioxane were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and formic acid from VWR Chemicals (Radnor, PA, USA). 1-Decanol was purchased from Merck KGaA (Darmstadt, Germany), and diethyl ether from Acros Organics (Geel, Belgium). Sodium chloride and citric acid were from Alfa Aesar GmbH & Co (Karlsruhe, Germany). Ethanol (ETAX A, 96.5%) was purchased from Anora Industrial Oyj (Rajamäki, Finland).

Lipid standards monopalmitin (MAG 1 × 16:0), monostearin (MAG 1 × 18:0), monolinolein (MAG 1 × 18:2), dipalmitin (DAG 2 × 16:0), distearin (DAG 2 × 18:0), dilinolein (DAG 2 × 18:2), hexadecanoic acid (FFA 16:0), octadecanoic acid (FFA 18:0), 9(*Z*)-12(*Z*)-octadecadienoic acid (FFA 18:2), trimyristin (TAG 3 × 14:0), tripentadecanoin (TAG 3 × 15:0), tripalmitin (TAG 3 × 16:0), tripalmitolein (TAG 3 × 16:1), tristearin (TAG 3 × 18:0), triolein (TAG 3 × 18:1), trilinolein (TAG 3 × 18:2), triheneicosanoin (TAG 3 × 21:0), cholesteryl stearate, cholesteryl palmitate, and cholesteryl linoleate were purchased from Larodan AB (Solna, Sweden). Polar lipids 1,2-dioctadecanoyl-*sn-glycero*-3-phosphate, PA (18:0/18:0); 1-palmitoyl-2-oleolyl-*glycero*-3-phosphocholine, PC (16:0/18:1$n-9$); 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphoethanolamine, PE (16:0/18:1$n-9$); 1,2-dioctadecanoyl-*sn-glycero*-3-phospho-(1′racglycerol), PG (18:0/18:0); and 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phospho-L-serine, PS (16:0/18:1$n-9$); phosphatidylinositol (PI) and phosphatidylserine (PS) from soybean and phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and sphingomyelin (SM) from chicken egg yolk were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Lipid standards Supelco 37 Component FAME Mix was purchased from Supelco Inc. (Bellafonte, PA, USA), and 11 A, 68 D, and GLC-490 from Nu-Chek Prep (Elysian, MN, USA). α-, β-, γ- and δ-tocopherol standards were purchased from Sigma–Aldrich (Buchs, Switzerland) while α-, β-, γ- and δ-tocotrienol standards were purchased from Supelco Inc.

2.2. Solvent extraction

Modified Bligh & Dyer extraction was used as a reference method for the oil extraction (Aitta et al., 2021). 10 g of homogenised fish was mixed with 8 mL of MQ-water, 20 mL of chloroform, and 40 mL of methanol. The mixture was homogenised for 1 min while being held on ice. Next, 20 mL chloroform was added and then the mixture was homogenised for 30 s. Lastly, 20 mL of MQ-water was added and the mixture was homogenised for 30 s. The homogenate was centrifuged for 10 min at 4075 *g* (Eppendorf 5810 R, Eppendorf AG, Hamburg, Germany), the aqueous layer was removed, and the organic (bottom) phase was collected and evaporated with a rotary evaporator. The extraction was conducted in triplicate.

2.3. Enzyme-assisted aqueous extraction

The extraction protocol was modified from Aitta et al. (2021) to optimise the extraction in terms of oxidation and oil recovery. Three *endo*-proteases Alcalase®, Neutrase® and Protamex® were used. The optimisation parameters included homogenisation size (mincing vs chopping with a knife) magnetic stirrer agitation speed (100, 190 and 280 rpm), centrifugation temperature (chilled on ice for 1 h or after inactivation at 60–65 °C), extraction time (35, 70 or 105 min), and enzyme concentration (0.4, 1 and 2% of the fish weight, Neutrase and Protamex only). Fish was defrosted at 4 °C overnight (16 ± 1 h). The fish was chopped or minced (Kenwood Titanium XL Chef, Kenwood Limited, Havant, UK) and mixed with water (1:1 w/w). The pH of Alcalase-

treated samples was adjusted to 9.0 (±0.2) with 1 M NaOH whereas it was not adjusted for Neutrase and Protamex. The starting pH of the fish homogenate was close to neutral pH. For Neutrase and Protamex no pH adjustment was carried out. Samples were heated to 50 °C (Neutrase) or 55 °C (Alcalase and Protamex) in a water bath, and the enzyme was added. After the hydrolysis, enzymes were inactivated at 90 °C for 15 min. The hydrolysates were centrifuged at 4075*g* for 20 min (Eppendorf 5810 R, Eppendorf AG). Oil and emulsion layers were collected by pipetting, centrifuged again at 1810*g* for 5 min to remove water and impurities, collected in vials, flushed with nitrogen and stored at − 80 °C. Each treatment was conducted in triplicate.

In a second trial, different methods were used to reduce or break emulsion to increase oil recovery rate. In these trials, Protamex was used with a hydrolysis time of 105 min (time selected based on trial one). The control treatment had enzyme concentration of 0.4% and fish to water ratio of 1:1 (w/w): 50 g of fish and 50 mL of water. 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 overnight at − 20 °C then thawing at RT for 1.5–2 h before centrifugation (modified from Nilsuwan et al., 2022), addition of 10 mL of ethanol (20% of the raw material weight) after inactivation, addition of 2 mL of 0.9% NaCl solution before centrifugation (modified from Iberahim et al., 2020), or addition of 0.1% (of the raw material weight) of 100% citric acid before the extraction (modification of the method for thermal treatment from Carvajal et al., 2014). All tests were conducted in triplicate.

Optimised parameters and best emulsion-breaking method were used with all three enzymes, later marked as “final samples”. The conditions were: 0.4% enzyme, 105 min extraction time, fish:water ratio 2:1 (w/w) and addition of 10 mL of ethanol after the inactivation step. Three replicates were prepared with each enzyme.

2.4. Oil recovery and emulsion layer

Oil recoveries were measured after the extractions by collecting the oil by pipetting. The oil was transferred into an empty vial, which was weighed empty and together with the oil. Emulsion layers of the emulsion test samples and final samples were determined by measuring the emulsion layer after the second centrifugation from 15 mL centrifugation tubes.

2.5. Peroxide value

Peroxide values (PV) of enzymatically extracted oils were analysed with ferric thiocyanate method from Lehtonen et al. (2011). Each biological replicate (n = 3) from the optimisation set was measured once and the final samples were measured in duplicate.

2.6. Volatile secondary oxidation products

Volatile secondary oxidation products (VSOPs) of the enzymatically extracted oils from the optimisation tests were analysed with head-space solid-phase micro extraction combined with gas chromatography and mass spectrometry (HS-SPME-GC–MS). For the analysis, 20 mg of oil diluted in hexane was pipetted into a vial, and evaporated to dryness under nitrogen gas flow. VSOPs were collected with a 1 cm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm, Supelco Inc.) fibre at 45 °C for 30 min under agitation using a TriPlus RSH multipurpose autosampler (Thermo Scientific™, Waltham, MA, USA). The GC–MS analyses were conducted using a Trace 1310 Gas Chromatograph (Thermo Scientific™) with an SPB®-624 Fused Silica Capillary Column (60 m × 0.25 mm × 1.4 μm, Merck KGaA). The GC was coupled to an ISQ 7000 Single Quadrupole Mass Spectrometer (Thermo Scientific™). Incubation and extraction times of the samples were 20 min and 30 min, respectively. Samples were agitated at 40 °C. The fibre was cleaned at 250 °C for 2 min prior to extraction and 5 min after extraction. The VSOPs were desorbed from the GC injector port for 5 min at 240 °C using splitless mode. Helium was used as a carrier gas in the gas chromatography at a flow rate of 1.4 mL/min. The oven temperature was programmed to hold at 40 °C for 6 min, after which it was increased to 200 °C at a rate of 5 °C/min and held at 200 °C for 10 min. Mass spectra were recorded in electron-impact (EI) mode at 70 eV within the mass range *m/z* 40–300. Chromeleon 7.2.10 (Thermo Scientific™) was used to operate the system. Each biological replicate (n = 3) of the optimisation samples was measured once.

The final samples were analysed in duplicate 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 min. 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 min. Helium flow rate was 1.6 mL/min. Other parameters were as described in the previous chapter. Compounds were identified based on our previous study (Aitta et al., 2021), 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 (version 2.3, National Institute of Standards and Technology, Gaithersburg, MD, USA).

2.7. Fatty acids

Fatty acids were analysed with gas chromatography coupled with flame ionization detection (GC-FID). Fatty acid methyl esters were prepared by an acid-catalysed method (Christie, 2003). 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 K_2CO_3 was added, followed by 1 mL of *n*-hexane. The samples were agitated at centrifuged at 1000*g* for 3 min, and the top layer was collected dissolved in *n*-hexane. The fatty acid methyl esters were analysed with a gas chromatograph (Shimadzu GC-2010 equipped with AOC-20i auto injector, flame ionization detector, Shimadzu Corporation, Kyoto, Japan). 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 inlet temperature was 270 °C and detector temperature 280 °C. The oven temperature was kept at 130 °C for 1 min, followed by an increase of 6.5 °C/min to 170 °C, then by 2.75 °C/min to 205 °C where it was held for 18 min, when it was increased by 30 °C/min to 230 °C and held for 2 min). FAs were identified using external standards, and the quantification of the FAs was conducted based on the internal standard area, concentration, and correction factors. Tripentadecanoin (TAG 3 × 15:0) was used as an internal standard (Larodan AB). Each biological replicate (n = 3) from the optimisation set was measured once and the final samples were measured in duplicate.

2.8. Lipid classes

The oil samples were fractioned into polar and neutral fractions, and Sep-Pak Silica 6 cc Vac Cartridges (500 mg, 55–105 μm, Waters co.) were used in the fractionation. The cartridge was 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 0.30 mg of triheneicosanoin (TAG 3 × 21:0) and 0.12 mg of PG 18:0/18:0, evaporated, dissolved in 1 mL of hexane:diethyl ether and applied into the column. The sample was applied to the column, and the sample tube was washed with 2 mL of hexane:diethyl ether, applied to the column. Neutral lipids were eluted with 9 mL of the hexane:diethyl ether. This neutral lipid fraction (NF) contained mainly triacylglycerols. The second fraction containing MAGs, DAGs, FFAs and PLs was eluted with 8 mL of chloroform:methanol:water (5:3:2 v/v). The second fraction is later referred to as polar fraction (PF), although it also contained

MAGs and DAGs. 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 in NF and PF were separated and detected with Shimadzu 8045 ultra-high performance lipid chromatography coupled with triple quadrupole mass spectrometer (UHPLC-MS) equipped with an ESI source, LC-40D XS pumps, SIL-40C XS autosampler and CTO-40C column oven (Shimadzu Corporation, Kyoto, Japan). The column for lipid class quantification was CORTECS UPLC C18, 1.6 μm, 2.1 × 150 mm (Waters Co., Milford, MA, USA). The injection volumes were 0.5 and 0.3 μL for the NF and PF fractions, 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 binary gradient was adopted from Knittelfelder et al. (2014). The starting condition was 55% of A and 45% of B. In the first 30 min, the concentration of B was increased to 90%, and in the next two minutes increased further to 100%, where it was held for 10 min. Within the next minute, the concentration of B was decreased from 100% to 45% and kept there for 7 min, the total sample run time being 50 min. 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 N_2, 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, MAG 1 × 18:2, 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). 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 Fig. 1). FFAs and lysophospholipids (LPLs) were semi-quantified by using peak areas and internal standard concentration.

Identification of PLs was aided by using a Cortecs UPLC HILIC, 1.6 μm, 2.1 × 150 mm column (Waters Co.) which separated different PL classes. The sample mixture used for initial method screening consisted of natural extracts of PA (egg), PC (egg), PG (egg), PE (egg), PI (soybean), PS (soybean) and SM (egg), 20 μg/mL each (Supplementary Fig. 2). 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 min, the concentration of A was increased to 32%, and in the next 0.5 min to 80% where it was kept for 9.5 min. Within the next 1 min, the concentration of A was dropped to 0%, where it was kept for 6 min. Total analysis 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.

2.9. Tocopherols and tocotrienols

Tocopherol and tocotrienol contents of the final samples were analysed with normal phase (NP) high performance liquid chromatography (HPLC) with fluorescence detector (FLD). The method was adapted and optimised from the method of Schwartz et al. (2008) to be an UHPLC method to save solvent and reduce analysis time. The final 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, and 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). The column temperature was 30 °C and tray cooler temperature 4 °C. The sample injection volume was 10 μL and the compound were separated isocratically within 8 min 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 corresponding tocotrienols) were quantified using calibration curves constructed by analysis of external standards (α-, β-, γ- and δ-tocopherols) at concentrations of 1–50 μg/mL. Tocotrienols were identified with α-, β-, γ- and δ-tocotrienol standards. The final samples and solvent-extracted oil (n = 3) were measured in duplicate.

2.10. Statistical analysis

Significant differences in oil recoveries, PV, FA, VSOPs, lipid classes, and tocopherol and tocotrienol contents 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 are reported for $p < 0.05$.

3. Results and discussion

3.1. The composition of oil extracted from Baltic herring

The two fish batches caught in January 2021 and May 2022 used in

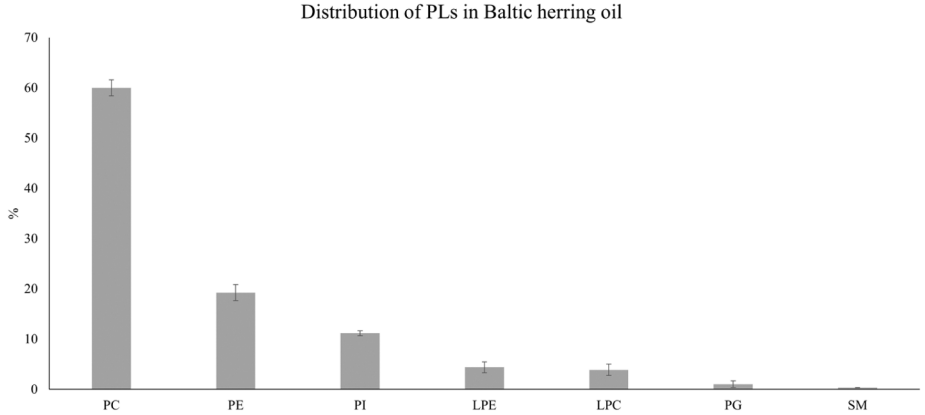

Fig. 1. The relative abundance of different groups of phospholipids (PLs) in Baltic herring oil extracted with solvent-extraction method and quantified with UHPLC-MS. PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, LPE = lysophosphatidylethanolamine, LPC = lysophosphatidylcholine, PG = phosphatidylglycerol, and SM = sphingomyelin.

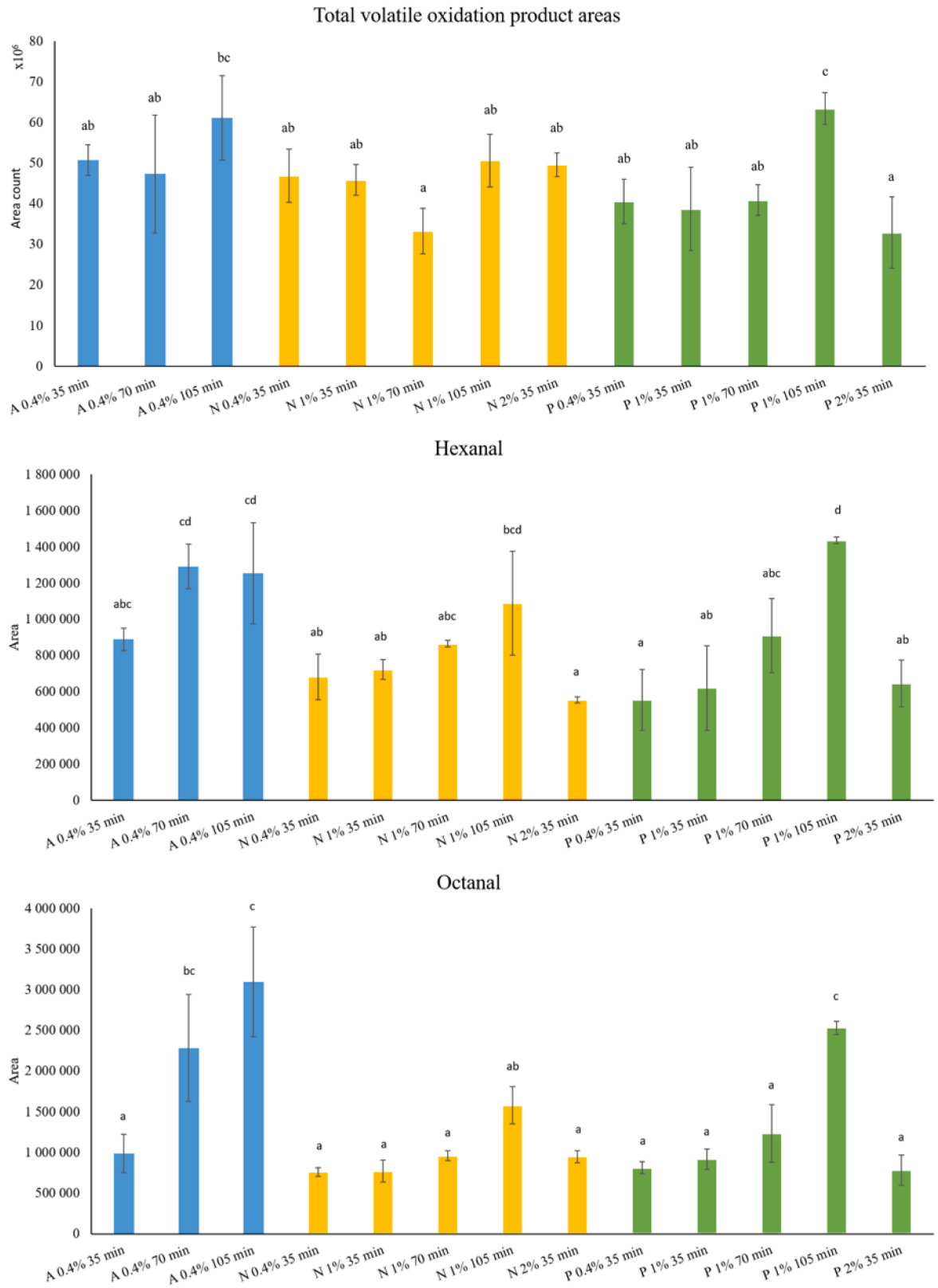

Fig. 2. Total volatile secondary oxidation products, hexanal, and octanal areas in the oils extracted from Baltic herring with enzyme-assisted aqueous extraction. The results are presented as mean value ± standard deviation (n = 3). Significant differences between the samples are indicated with different letters a–d (one way ANOVA with Tukey's post hoc test, $p < 0.05$). Samples: A = Alcalase (blue), N = Neutrase (yellow), and P = Protamex (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this study contained 3.8% and 2.8% of lipids, respectively (Table 1). The fat content was significantly less than that in our previous study, where the lipid content of the whole fish was as much as 9.0% (Aitta et al., 2021). Lower lipid content of Baltic herring has been reported before, likely due to a decrease in the Baltic Sea salinity and increase sea temperatures during winter, which affect the nutrition of Baltic herring (Rajasilta et al., 2022). However, the oil was rich in the MUFAs and PUFAs comprising 75% of all FAs, being especially rich in $n-3$ PUFAs, which add up to 27% of the total FAs (detailed results on Supplementary Table 1, summary of important FAs on Table 2). The EPA and DHA contents were 6.2% and 11.5%, respectively, which was similar to our previous study. In comparison, sprat and herring have been reported EPA values of 6% and 10%, respectively, and DHA values of 4% and 7%, respectively (Keinänen et al., 2017). Salmon is known for its high fat content and EPA and DHA contents. Percentage values of 21.0%, 24.1% and 24.6% of EPA + DHA of the total fatty acids have been reported for fish oil extracted from the head, skin and backbone, respectively, of wild salmon, while the corresponding values for farmed salmon were 18.9%, 16.9% and 16.4%, respectively (Głowacz-Różyńska et al. 2016).

The lipid classes were analysed with a novel lipid class method developed for UHPLC-MS (Supplementary Fig. 1). 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 ionization of PLs by TAGs. The solvent-extracted fish oil contained 9.5% of PLs, of which the identified phospholipids consisted of lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), phosphatidyl inositol (PI), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and sphingomyelin (SM). The most abundant PL 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% (Fig. 1). Phosphatidylserines (PS) or phosphatidic acids (PA) were not identified in the oil. According to the review article by Lu et al. (2011), the most abundant PL group in marine sources is PC, followed by either PE or LPC depending on the species. For example, in rainbow trout PC comprises 53.6%, PE 22.9%, PI 8.3%, cardiolipin 6.2%, SM 4.9%, and PS 4.1% of the PLs. On the contrary, krill, which is known for its high PL content, has 86.0% of its PLs in the PC form, whereas the other groups contain 1–6% each. The results shown here are in line with the literature presented above, and it is the first time a more specific information about Baltic herring phospholipids is presented. This indicates that the PL composition of Baltic herring is similar to that reported for other fish species. In addition, the consistent findings with the previous report on PL in other fish species serve as an indicator that the method optimised was suitable for quantifying PLs.

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 2). The results are presented as percentage of the lipid sample applied for fractioning, and therefore some part may be lost during the process. 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 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).

3.2. The effect of homogenisation size, stirring and centrifugation temperature on oil recovery

Previously, we reported enzymatic extraction of oil from Baltic herring with a high fat content (9% as determined by solvent extraction). The oil recovery was up to 68 and 69%, respectively, of the total oil in the raw material with Protamex and Neutrase using a 70 min extraction time (Aitta et al., 2021). However, with leaner fish batches with the fat content under 4% fresh weight, the recovery was lower, and a higher amount of emulsion was formed (pre-tests, results not shown here). The emulsion-formation occurred during centrifugation, although lipid-protein complexes were likely forming already during the hydrolysis as no freely floating lipid droplets were visible. After centrifugation, the fish hydrolysate consisted of a thin layer of oil, loose emulsion layer, supernatant, and sludge.

To begin the optimisation of EAAE for whole Baltic herring with a low fat content of 3.8%, the effect of stirring speed, centrifugation temperature and homogenisation size were studied. Decreasing stirring speed from 280 rpm down to 100 rpm increased 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 & Goosen, 2023). However, the reaction kinetics were not measured in this study, so the exact mechanism remains still 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 & Maneeintr, 2018).

3.3. The effect of enzymes, enzyme concentrations and extraction times on oil recovery

The impact of different enzymes, enzyme concentrations and extraction times on oil recovery was studied using Alcalase, Neutrase and Protamex. Oil recovery rates were statistically influenced neither by the enzyme concentration nor by the extraction time (Table 1). The oil recoveries were 37.0–58.1% of the total oil amount extractable by the solvent-extraction method, or 1.4–2.2 g/100 g of the raw material weight. The longest extraction time of 105 min increased recoveries by 0.6–0.8 g/100 g compared to 35 and 70 min extractions with all enzymes. The different dosages of the enzymes of either 0.4, 1 or 2% of enzyme did not affect recoveries significantly, which supports the use of the lowest dosage to minimise the cost of the process. Some studies have used a combination of different enzymes, such as Papain and Bromelain, or Flavourzyme in combination with Neutrase or Alcalase. However, the combination of Papain and Bromelain did not show improvements in oil recovery (Šližyte et al., 2016) but the combination of Flavourzyme with different enzymes affected the flavour of protein hydrolysates (Muzaifa et al., 2012).

3.4. The effect of enzymes, enzyme concentrations and extraction times on fatty acid and lipid class compositions

There were no significant differences in EPA contents between the different enzymatic extractions or compared to the solvent-extracted oil. The enzymatically extracted samples contained between 4.8 and 6.6% EPA (Table 2). However, there was a significant drop in the DHA content in the enzymatically extracted oils (5.0–6.0%) as compared to the solvent-extracted oil (11.5%). The change was also visible in the total PUFA and $n-3$ contents, which were significantly lower in most of the enzymatically extracted oils compared to the content in the control oil. EPA and DHA are found in TAGs and PLs 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 an earlier study using cod, hydrolysed proteins formed protein–lipid complexes, especially with phospholipids (Šližyte et al., 2004).

Nearly all PLs were lost during the enzymatic extractions (Table 2).

Table 1
Oil recoveries, emulsion layer thicknesses, peroxide values and sum of tocopherols and tocotrienols of enzymatically extracted Baltic herring oils and solvent-extracted oil.

1. Optimisation tests	**Solvent extraction**	**Alcalase**	**Alcalase**	**Alcalase**	**Neutrase**	**Neutrase**	**Neutrase**	**Neutrase**	**Neutrase**	**Protamex**	**Protamex**	**Protamex**	**Protamex**	**Protamex**
Enzyme (%)	–	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
Extration time (min)	–	35	70	105	35	35	70	105	35	35	35	70	105	35
Extracted oil content (g/100 g)	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	40 ± 10	37 ± 10	58 ± 11	44 ± 20	42 ± 12	42 ± 14	58 ± 18	47 ± 22	39 ± 3	39 ± 11	37 ± 9	55 ± 11	38 ± 6
Peroxide value (meq/kg)	N/A	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
2. Emulsion tests	**Protamex Control**[1]													
Samples	**1**	**0.1% enzyme 2**	**Fish:water (2:1) 3**	**+NaCl 5**	**+Ethanol 7**	**+Citric acid 6**	**Freezing 4**	**Fish:water (2:1) + ethanol 8**						
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 ± 4.6d	5.3 ± 2.5abcd	9.5 ± 4.1 cd	7.7 ± 4.0bcd	5.0 ± 3.4abc	6.0 ± 1.7abcd	2.0 ± 1.0a	3.0 ± 0.0ab						
3. Final samples	**Solvent extraction**	**Alcalase**	**Neutrase**	**Protamex**										
		0.4% enzyme, 105 min, fish:water (2:1) + ethanol												
Extracted oil content (g/100 g)	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										
Peroxide value value (meq/kg)	–	5.20 ± 0.65	8.29 ± 3.87	8.20 ± 3.15										
Tocopherols & tocotrienols (μg/g of oil)	1578 ± 64	1539 ± 20	1531 ± 43	1567 ± 49										

The results are presented as mean value ± standard deviation (n = 3). The data set consists of optimisation samples with different enzymes, enzyme concentrations and extraction times (1.), emulsion tests with different methods to reduce or break emulsion (2.), and final samples (3.). Peroxide values of each biological replicate (n = 3) was measured once for the optimisation test samples, and for the final samples in duplicate. Tocopherols and tocotrienols of the final samples (n = 3) and solvent-extracted oil were measured in duplicate. Significant differences between the samples are indicated with different lower case letters a–f (one way ANOVA with Tukey's post hoc test or Independent-Samples Kruskal-Wallis Test, $p < 0.05$).

[1] Control sample parameters: enzyme concentration 0.4%, extraction time 105 min, and fish:water ratio 1:1 (w/w).

Table 2
Fatty acids and lipid classes of oils extracted with enzyme-assisted aqueous extraction, and solvent-extracted reference oil from Baltic herring.

	Solvent extraction	Alcalase	Alcalase	Alcalase	Neutrase	Neutrase	Neutrase	Neutrase	Neutrase	Protamex	Protamex	Protamex	Protamex	Protamex
Enzyme (%)		**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**
Fatty acid analysis														
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
ΣSFAs	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
ΣMUFAs	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
ΣPUFAs	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
$n-3/n-3$	2.89	2.09	2.31	2.51	2.23	2.29	2.08	2.55	2.24	2.63	2.66	2.10	2.55	2.61
Lipid class analysis														
FFAs	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
MAGs	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
DAGs	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
TAGs	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
PLs	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). Lipid class data is presented as percentage of the oil sample (weight %) ± standard deviation (n = 3). Significant differences between the samples are indicated with different lower case letters a–b (one way ANOVA with Tukey's post hoc test, $p < 0.05$ for the fatty acid results, FFAs, MAGs, DAGs and TAGs, Dunnett's C for the PLs). 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, FFAs = free fatty acids, MAGs = monoacylglycerols, DAGs = diacylglycerols, TAGs = triacylglycerols, and PLs = phospholipids.

The solvent-extracted sample contained 9.5% PLs whereas the enzymatically extracted oils contained only 0.01–0.02% (Supplementary Fig. 3). On the contrary, the relative abundance of TAGs was higher in the samples compared to the solvent-extracted oil, the difference being significant in the 35 min extraction of Alcalase (0.4% enzyme), and Neutrase and Protamex (2% enzyme). Without optimising the hydrolysis for leaner fish, a large part of important $n-3$ FAs were lost, even though the removal of PLs is desirable in oil production as they easily hydrolyse to FFAs. The amounts of identified MAGs, DAGs and FFAs were low in all of the samples and there were no significant differences between the treatments.

3.5. The effect of enzymes, enzyme concentrations and extraction times on oxidation

Oxidation of the oils was measured with PV, and VSOPs using HS-SPME-GC–MS. The PV values of the optimisation samples ranged between 3.8 and 11.8 meq/kg oil (Table 1), which were significantly smaller values than those in our previous study determined using a titration-based method (Aitta et al., 2021). Higher fat content and storage conditions (–20 °C instead of –80 °C) likely explain the higher oxidative status of the oils reported in the previous study. The Codex standard for refined fish oil is PV ≤ 5 meq/kg (Joint FAO/WHO Codex Alimentarius Commission, 2017). The PV of oils obtained using 0.4% Alcalase with 35 min extraction time, or Neutrase 0.4 and 1% with 35 min were under the Codex standard value (3.8, 4.2, and 4.5, respectively) whereas the highest PV values (8.0–11.9 meq/kg) were observed in the oils from 70 and 105 min extractions (Joint FAO/WHO Codex Alimentarius Commission, 2017). Crude oils undergo refining steps, such as degumming, deacidification and deodorisation which lower the degree of oxidation and extend the storage period of the oil. Based on the results shown here, oils extracted from Baltic herring with EAAE should be refined to increase the oxidative stability, especially after longer extraction times. 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 2 h (Głowacz-Różyńska et al., 2016), whereas in another study using mixed fish co-products, the hydrolysis resulted in a 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 oil extracted from the raw material (Hathwar et al., 2011). EAAE can therefore result in very different levels of oxidation depending on raw material, extraction time and in some cases the enzyme.

The VSOPs of the oils extracted with different conditions were analysed with an SPB-624 column (Supplementary Figure 4). A total of 21 compounds were identified from the samples (Supplementary Table 2); the most abundant compounds were 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 with 105 min extraction times had significantly larger total VSOP contents compared to all other samples (Fig. 2). Different enzymes can lead to different volatile compositions as they produce proteins and peptides that can have anti- 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 industry 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-

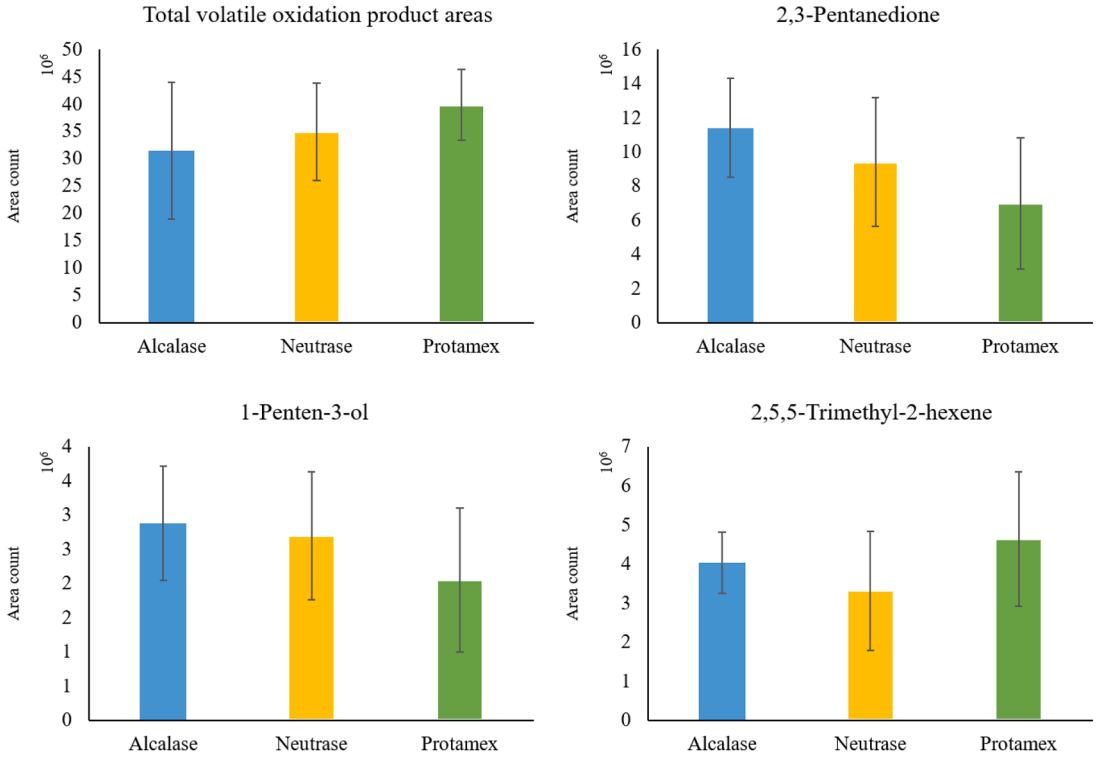

Fig. 3. Total volatile secondary oxidation products, 2,3-pentanedione, 1-penten-3-ol and 2,5,5-trimethyl-2-hexene areas in the enzymatically extracted oils from Baltic herring where emulsion-formation was reduced. The results are presented as mean value ± standard deviation (n = 3).

heptenal, 2-methylbutanal and hexanal. Increasing the extraction time correlated with an increasing level of oxidation as was also shown in our previous study (Aitta et al., 2021), but the different enzyme concentrations did not contribute to differences in oxidation.

3.6. Emulsion reduction methods

Emulsion reduction tests were done using Protamex with an enzyme concentration of 0.4% (except one test using only 0.1%) and 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 1). 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 effect of ethanol on emulsion systems can vary depending on protein size and molecular weight of the surfactant. In some cases, ethanol has been 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 density, which affect 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). 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, which were most likely caused by the small sample sizes.

3.7. The composition of oils with reduced emulsion

Finally, all three enzymes 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. In the emulsion reduction tests, using 0.1% of enzyme reduced the amount of emulsion, however, it did not improve the oil recovery. Therefore, the concentration of 0.4% was used for the final sample set, the same as used in our previous study (Aitta et al., 2021). Oil recoveries of the samples were 0.7–0.8 g/100 g fish while the fat content of the raw material was 2.8 g/100 g extractable by the solvent-extraction (Table 1). Therefore, the recovery percentages were 26–28%, lower than those from the previous samples. Throughout the study, oil recoveries were lower than in our previous study (Aitta et al., 2021), 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, 2.8% in those used for the emulsion tests and the final samples while the raw material in our previous study contained 9.0% of fat. EAAE is widely used for the production of protein hydrolysates, which have also shown bioactive functionalities (Mäkinen et al., 2022). With lean fish material, the focus of the process could be the production of peptides, while in fattier raw material the method could be optimised for the production of oil rich in PUFAs. In both cases, the removal of lipids from the protein hydrolysate is desirable in order to minimise oxidation, and both fractions can be used as added-value products. In the current study, our aim for performing the solvent extraction using the method of Bligh & Dyer was to determine the content and composition of lipids in the fish raw material, in order to measure the oil recovery and extraction efficiency by EAAE with different enzymes and parameters. Since chloroform and methanol were used, chloroform being especially toxic for humans and harmful to environment, such extraction process is not in use for industrial oil extraction for food use. Therefore, our aim was not to compare the quality e.g. oxidation status of the solvent-extracted oil with the quality of the oils extracted with EAAE.

The fatty acid analysis showed no significant differences in the FA compositions between the three enzymes (Supplementary Table 3). The most abundant fatty acid was oleic acid (18:1$n-9$c) accounting for 25.1–25.5% of the total fatty acids, 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 fatty acids) 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 those in the oil samples enzymatically extracted before using the optimisation of the method (Table 2), but the level of DHA was lower than in the solvent-extracted oil. The results show that the emulsion reduction improved the DHA content of the oil. In our previous study, the EPA contents in enzymatically extracted oils from Baltic herring were 6.1–7.3%, while the DHA contents were 9.4–10.8% (Aitta et al., 2021), showing some differences compared to the results obtained with the optimised method in the current study. However, there were major differences in the lipid contents between the batches used in the studies, which likely partly explains the varying levels of DHA in the oils.

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 PL contents were only 0.02–0.03% (Supplementary Table 3). The amount of TAGs was remarkably larger than in the previous set of samples but the amounts of PLs remained as low (Table 2). The results indicate that the emulsion reduction led to an increased TAG content in the oil, whereas PLs were still trapped in the remaining emulsion or other phases. The increased TAG content was likely related to the increased EPA and DHA contents in the oil as well, as these FAs were also present in TAGs. The amounts of FFAs, MAGs and DAGs remained low in the oils: 0.01–0.04% indicating a low level of lipid hydrolysis during extraction.

3.8. The oxidation status of oils with reduced emulsion

Peroxide values of the final samples with reduced emulsion were 5.2–8.3 meq/kg (Table 1). Alcalase resulted in the lowest PV but the difference was not statistically significant compared to Neutrase and Protamex. In the volatile analysis, 17 VSOPs were identified from the final samples using the DB-WAX column (Supplementary Figure 4). Protamex resulted in the highest value of total VSOP areas but there were no statistically significant differences between the samples prepared with enzymes (Fig. 3). The most abundant VSOP compounds

Table 3
Tocopherols and tocotrienols (μg/g of oil) in in the oils extracted from Baltic herring with enzyme-assisted aqueous extraction, using a method to reduce emulsion. Solvent-extracted oil was used as a reference.

	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

The results are presented as μg/g of oil ± standard deviation (n = 3). Significant differences between the samples are indicated with different lower case letters a–b (one way ANOVA with Tukey's post hoc test, $p < 0.05$).

identified in the samples 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., 2020; Lee et al., 2003).

The total contents of tocopherols and tocotrienols in the final samples varied between 1530 and 1570 μg/g of oil while the solvent-extracted oil contained 1580 μg/g (Table I). 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. 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. (2022) 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 sample 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 the enzymes or between the enzymatically and solvent-extracted oil were not statistically significant. The results of all quantified tocopherols and tocotrienols are shown in Table 3.

4. Conclusions

Overall, there is a decreasing trend in oil content in Baltic herring likely due to the climate change. In addition, Baltic herring fish caught from different seasons and different batches within the seasons vary greatly in oil content, which poses challenges to oil extraction using enzyme-assisted aqueous extraction (EAAE) as a green oil extraction technology. In the present study, various extraction parameters were studied for the first time to reduce the emulsion formation and oil recovery of EAAE from Baltic herring. This is the first study showing the impact of different parameters of EAAE on lipid class composition and content of $n-3$ FAs of crude oil. EAAE alone is not an efficient method to extract oil from Baltic herring fish with low oil content due to the high emulsion formation. Baltic herring used in this study contained less oil than the typical lipid content reported in this species. The oil recoveries from EAAE using Alcalase, Neutrase and Protamex were low (37–58%). Low oil recoveries were accompanied with up to 57% loss of DHA. The oxidative stabilities of the oils were under 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, especially aldehydes hexanal and propanal. Combining water reduction with the addition of ethanol was most efficient in both reducing the emulsion and increasing oil recovery. The optimised method increased the content of TAGs, EPA, and DHA in the extracted crude oils, but the PL content remained low in all crude oil samples extracted with EAAE. In future studies, the partition of lipids in different fractions should be studied to better understand which compounds are responsible for the formation of emulsions. It would also be important to find ways to collect and further break down the emulsion to recover the phospholipids and important $n-3$ FAs. Peptide hydrolysate is another important fraction produced in the process, and it should also be collected and utilised due to the potential bioactivities of Baltic herring peptides. Furthermore, our results also indicated that different strategies should be adopted for value-addition of fish materials of different composition and quality.

Like all other research, this study has strengths and weaknesses. To avoid complication caused by lipid oxidation in raw materials during long-term frozen storage of fish, we used fresh fish catch of each season as raw materials for this study. Due to the variation in oil content among different fish batches, it was difficult to compare the oil recoveries obtained with different batches of fish raw materials. On the other hand, involving different fish batches caught within the season and catches from different seasons revealed the variation in oil content and the impact of such variation on oil extraction process, such as formation of emulsion, recovery of lipids, and fatty acid composition of the extracted lipids, which should be considered as a strength of this study providing novel findings. Furthermore, to the best of our knowledge, this was the first study, where different methods were studied to reduce the formation of emulsions, and where the profiles of different lipid classes were analysed as a result of different enzymes and extraction parameters used for fish oil extraction using EAAE.

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CRediT authorship contribution statement

Ella Aitta: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Annelie Damerau:** Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing. **Alexis Marsol-Vall:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Mikael Fabritius:** Methodology. **Lumi Pajunen:** Methodology, Formal analysis. **Maaria Kortesniemi:** Methodology, Supervision, Writing – review & editing. **Baoru Yang:** Conceptualization, Project administration, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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