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Comparison of enzymatic and pH shift methods to extract protein from whole Baltic herring (*Clupea harengus membras*) and roach (*Rutilus rutilus*)

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ABSTRACT

This study aimed to establish the differences between enzymatically extracted hydrolysates and pH shifted protein isolates from whole Baltic herring and roach in terms of polypeptide patterns, functionality, sensory properties, microbial quality, yield, and composition. Alkaline extraction resulted in the highest yields, whereas the hydrolysates showed the highest protein contents. The hydrolysates showed higher protein solubility (86.0–88.5%) than the protein isolates (5.1–14.5%) as well as the higher foam capacity for Baltic herring. However, for roach, alkaline extracted protein isolates exhibited the highest foam capacity. All hydrolysates showed poor foam stability (0–13%) while the protein isolates showed notably higher stability (30–55%). The hydrolysates showed relatively low bitterness, whereas alkaline extracted roach proteins were perceived as bitter. This study demonstrated that it was possible to produce protein isolates and hydrolysates from whole fish with good microbial quality. However, both processes need to be optimised according to the food application and fish species.

1. Introduction

There is a growing global need to increase the proportion of fish in the diets to replace meat, but it must be done by avoiding overfishing. To manage the balance of sustainable fishery and the growing need for food fish, the catch should be used more completely than is currently done. Approximately 12 % of the global fish catch is destined for non-food uses, such as fish meal and oil (FAO, 2020) for feed. For instance, in Finland, majority of the food fish is imported while domestic wild fish are being used for non-food purposes. A market niche exists for novel food products and ingredients processed from Baltic herring (*Clupea harengus membras*) and roach (*Rutilus rutilus*), among others. Thus, solutions are needed to valorise these two fish species into high-value products for human consumption. Enzymatic hydrolysis and pH-shifting are well-known methods for valorising undervalued fish for food or other value-added use (Abdollahi et al., 2018; Egerton et al., 2018). These methods enable extraction of nutritionally valuable components (protein, polypeptides and oil) without the need for mechanical filleting step that produces substantial amounts of by-products.

Enzymatic hydrolysis allows the conversion of fish mass into value-added polypeptides with good functional properties, such as emulsification and foaming (Chalamaiah et al., 2010; Liu et al., 2014). These properties would enable the use of fish hydrolysates as emulsion or foam stabilisers in food products (Gao et al., 2021). Other relevant applications of fish hydrolysates in food are related to their bioactivities, which would enable their use as preservatives due to the anti-oxidation properties (Egerton et al., 2018; Klompong et al., 2007). However, a bitter taste as well as fishy odour or flavour may develop during the hydrolysis process, which may limit the use of hydrolysates as food ingredients. Selection of enzymes and optimising their activity during the processing are focal for proper sensory quality (Yarnpakdee et al., 2012) and functionality of the hydrolysates.

Extraction of proteins by pH shift process includes acidic or alkaline solubilisation and is followed by isoelectric precipitation of the proteins (Abdollahi & Undeland, 2018). The method is based on increased protein solubility induced by changing the pH, which allows the separation of protein from lipids and insoluble material, such as scales and bones. The resulted protein isolates differ from the hydrolysates, especially regarding the molecular weight of the polypeptides, and thus, their functionalities. Several studies have been conducted using gutted fish or

Abbreviations: DH, degree of hydrolysis; OPA, *ortho*-phthalaldehyde; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; ANS, 1-anilino-8-naphthalene-sulphonate.

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by-products of different fish species, such as Atlantic croaker (Kristinsson & Liang, 2006), herring, cod, and salmon (Abdollahi & Undeland, 2018; Marmon & Undeland, 2010). The process has not however been published for Baltic herring or roach, and the authors are not aware of studies where whole (ungutted) fish was used as a raw material.

Publications on microbial quality of protein fractions extracted by enzymatic treatment and pH-shift process are scarce. Studies by Lansdowne et al. (2009) reported the reduction of inoculated *Listeria innocua* and *Escherichia coli* during acidic (pH 2.0 and 3.0) and alkaline (pH 11.5 and 12.5) pH shift, but little data is available concerning other pathogens. Rodrigues Freitas et al. (2016) also observed that pH 2 and pH 12 in the pH-shift process inactivated microbes originating from the raw material.

This study aimed to develop a food-grade valorisation method for whole Baltic herring and roach. It focused on comparing the characteristics of protein hydrolysates and isolates produced by the two well-established protein extraction methods; enzymatic hydrolysis and pH shifting. To assess the chemical properties of the obtained hydrolysates and protein isolates, the protein solubility, polypeptide pattern, degree of hydrolysis (DH), surface hydrophobicity and zeta potential were analysed. In addition, foaming, gelation, colour and sensory properties were studied. It was hypothesised that limited enzymatic hydrolysis would result in high solubility, which is usually a prerequisite for such functional properties as foaming and gelation. Regarding pH shifting, it was assumed that the extracted proteins would result in low solubility and limited functionality, as the protein was recovered after isoelectric precipitation. Few studies (Abdollahi & Undeland, 2018; Aspevik et al., 2016) have reported the full sensorial descriptive profile of the protein isolates, which is why it was investigated in the current study. Furthermore, the aim was to use whole fish as a raw material to minimize by-product production and maximise the cost-effectiveness of the process. Therefore, also the microbiological quality of the protein fractions was analysed.

2. Materials and methods

2.1. Raw materials

Fresh Baltic herring (caught from the Archipelago Sea in September 2018) and descaled roach (caught from the Bothnian Sea in June 2018) were both provided by Arvo Kokkonen Oy (Finland). The ungutted Baltic herring and descaled and ungutted roach were ground by a kitchen mixer (CombiMax 600, Braun, Germany) with a maximum speed for 60 s. The fish were kept on ice during the grinding procedure and the ground fish mass was frozen (−22 °C) after grinding. The fish was ground and frozen within 24 h from catching for further processing by enzymatic hydrolysis or pH-shift method. The processing was conducted 3 and 1 months after freezing the roach and Baltic herring mass, respectively.

2.2. Enzymes and chemicals

Food grade proteolytic enzymes Protamex® and Neutrase® (Novozymes) and Corolase 7089® (AB Enzymes) were used for protein hydrolysis. The protease activity of the enzymes was determined at pH 7 using 1.2% casein as a substrate at 50 °C and dosed as nanokatals (nkat). All used reagents were at least analytical grade. For the determination of lipid content, potassium chloride (Merck, Darmstadt, Germany), chloroform (VWR Fontenay Sous Bois, France), and methanol (Sigma-Aldrich, Steinheim, Germany) were used.

2.3. Protein recovery and yield

2.3.1. Enzymatic hydrolysis

For enzyme-aided protein hydrolysis (Fig. S1), the frozen fish mass was thawed at 12 °C and mixed with fresh tap water (6 °C) (750 g fish and 750 g tap water). The mixture temperature was kept at 4 °C unless stated otherwise. A control sample for analysing the DH was collected at this point. The mixture was heated to 50 °C under constant stirring. After reaching the target temperature, the enzyme was dosed as 11.4 nkat/g of ground fish. The enzyme treatment time was selected as 30 min based on pre-trials with varying durations of the hydrolysis (data not shown). The hydrolysis was stopped by inactivating the enzyme at 75 °C for 15 min. A sample for analysing the DH was collected at this point. The mixture was cooled down to 4 °C and centrifuged at 4000*g* for 15 min (Sorvall lynx 4000, Thermo Scientific, USA). Due to a minute lipid layer, the supernatant of the roach samples was collected by pouring without any lipid phase separation. Baltic herring supernatant, oil phase and solid lipid-containing emulsion layer were separated from the solids by pouring. Then the centrifugation step was repeated for the supernatant, and it was collected with a laboratory-scale pump to separate it from the oil phase and solid lipid-containing emulsion layer. All supernatants were frozen immediately with a blast freezer, freeze-dried and stored at −22 °C. Three parallel treatments were conducted.

2.3.2. pH shift and subsequent isoelectric precipitation

For pH-shift process (Fig. S1), the frozen fish mass was thawed at 12 °C and mixed with fresh tap water (6 °C) (375 g fish and 1125 g water). The mixture temperature was kept at 4 °C. The pH of the mixture was adjusted to 2.5 ± 0.1 (acid solubilisation) or 11.5 ± 0.1 (alkaline solubilisation) for 20 min with 6 M HCl or NaOH, respectively, under constant stirring for an additional 15 min. After solubilisation, the mixture was centrifuged at 4000*g* for 15 min (Sorvall lynx 4000, Thermo Scientific, USA). Due to a minuscule lipid layer, the supernatant of the roach samples was collected by pouring without any lipid phase separation. Baltic herring supernatant, oil phase and solid lipid-containing emulsion layer were separated from the solids by pouring. Then the centrifugation step was repeated for the supernatant, and it was collected with a laboratory-scale pump to separate it from the oil phase and solid lipid-containing emulsion layer. The pH of the supernatant was adjusted to 5.2 with 6 and 1 M NaOH (for acidic supernatant) or 6 and 1 M HCl (for alkaline supernatant) to precipitate the proteins. The precipitated suspension was heated to 75 °C and held there for 1 min to hinder the possible microbial growth and cooled down to 4 °C immediately. The suspension was centrifuged at 4000*g* for 15 min. The precipitated proteins were collected, frozen immediately in a blast freezer, freeze-dried and stored at −22 °C. Three parallel treatments were conducted.

2.3.3. Extraction yields

Mass and protein yields of the different treatments were calculated according to the following equations:

$$Mass\ yield(wet\ basis) = \frac{Dried\ product(g)}{Ground\ fish(g)} 100\% \quad (1)$$

$$Protein\ yield(dm\ basis) = \frac{Protein\ in\ dried\ product(g)}{Protein\ in\ ground\ fish(g)} 100\% \quad (2)$$

2.4. Analyses

2.4.1. Protein content

Protein content was determined by a Kjeldahl autoanalyser (Foss Tecator Ab, Höganäs, Sweden) according to the AOAC method 2001.11. The nitrogen conversion factor of 6.25 for calculating the protein content was chosen according to the EU council directive on nutrition labelling for foodstuff (90/496/EEC). One measurement for each of three parallel treatments was conducted.

2.4.2. Lipid content

Lipid content was measured gravimetrically after modified Folch extraction (Folch et al., 1957). Lipids were extracted with chloroform:

methanol (2:1 v/v) and 8.8 % potassium chloride solution was used for phase separation. Lipid content was analysed in triplicate (once from each parallel treatment).

2.4.3. Moisture and ash

The moisture content was determined by drying the samples at 105 °C for 24 h. The ash content was quantified gravimetrically after combustion at 550 °C for 24 h in a muffle furnace (model N11, Nabertherm GmbH, Lilienthal/Bremen, Germany).

2.4.4. Degree of hydrolysis

The DH was defined as the percentage of cleaved peptide bonds broken (h) in relation to the theoretical total number of peptide bonds present in the protein substrate (h_{tot}) similarly to the study of (Xu et al., 2016):

$$DH = \frac{h}{h_{tot}} \cdot 100\% \quad (3)$$

The h and h_{tot} values were calculated as nitrogen concentrations determined by the Kjeldahl method using the equations according to the study of Nisov, Ercili-Cura, et al. (2020) to obtain the DH values. The amount of cleaved peptide bonds was determined by OPA (*ortho*-phthalaldehyde) method according to the study of Spellman et al. (2003) with slight modifications. The modifications were described in the study of Nisov, Ercili-Cura et al. (2020). Four parallels were measured from three parallel treatments.

2.4.5. Protein and polypeptide profile

The protein profiles were determined in reducing conditions by sodium dodecyl sulphide polyacrylamide gel electrophoresis (SDS-PAGE). A commercial Criterion TGX (Tris-glycine extended) stain-free precast gel (4–20%, 30 μl 18-well, Bio-Rad, Hercules, CA, USA) was used for the analysis. First, the extracted and freeze-dried protein powders were suspended in MQ water as 1.0 mg/mL protein content (w/w). Then the suspensions were treated according to the protocol described in the study of Nisov, Ercili-Cura, et al. (2020).

2.4.6. Solubility

The water-solubility of the proteins was determined according to the Bio-Rad DC protein assay kit (Bio-Rad, Richmond, CA, USA). Protein-water suspensions with 0.1% or 1% (w/v) concentrations were prepared and the pH was adjusted to 7. The suspensions were centrifuged at 4000×*g* for 10 min and the absorbance values of supernatants were analysed at 750 nm (UV-1800, UV–VIS Spectrophotometer, Shimadzu, Japan) according to the Bio-Rad instructions. The protein content was obtained by quantifying against a bovine serum albumin (BSA) standard curve. Triplicate absorbance values were measured from three parallel treatments.

2.4.7. Zeta potential

The surface charge of protein particles was measured as zeta potential using a Zetasizer Nano ZS equipment (Malvern Instruments Ltd., UK). Protein-water suspensions with 0.1% (w/w) concentrations were prepared and adjusted to pH 7. The suspensions were centrifuged at 4000×*g* for 10 min and the zeta potential was analysed from the supernatant. At least three parallels were measured from three parallel treatments.

2.4.8. Surface hydrophobicity

The surface hydrophobicity of the protein was determined by fluorescence spectroscopy using 1-anilino-8-naphthalene-sulphonate (ANS) as a probe. A 0.1% (w/v) protein-water suspension was prepared and adjusted to pH 7. The suspension was centrifuged at 4000×*g* for 10 min. The supernatant was collected and diluted to 50 mM phosphate buffer (pH 7.0) to obtain solutions with protein concentrations ranging from 0.02 to 0.5 mg/ml. The diluted samples were mixed with an ANS reagent and measured in a black polystyrene 96-well microplate as described by Nisov, Ercili-Cura, et al. (2020). In brief, the fluorescence intensity was measured with the excitation and emission wavelengths of 390 and 479 nm, respectively. Surface hydrophobicity was reported as the slope of the curve of the relative fluorescence intensity as a function of protein concentration.

2.5. Determination of functional properties

2.5.1. Foam formation

Foaming properties were analysed according to Nisov, Ercili-Cura, et al. (2020) with slight modifications. A 5% (v/w) protein-water suspension was prepared and adjusted to pH 7. Foam formation was assessed by whipping 10 mL of the suspension with a battery-operated whisk (AeroLatte AL-V1-SS Chef Kitchen Whisk, United Kingdom) in a 100 mL measuring cylinder. The volume of the foam and the drained liquid was analysed 0 and 20 min after whipping using the scale on the cylinder. Foam capacity (FC, %), drainage (DR, %) and foam stability (FS, %) were calculated according to the following equations:

$$FC = \frac{V_{tot} - V_0}{V_0} \cdot 100\% \quad (4)$$

$$DR = \frac{V_{DR}}{V_0} \cdot 100\% \quad (5)$$

$$FS = \frac{V_{F,somin}}{V_{tot,omin}} \cdot 100\% \quad (6)$$

$$V_{F,30min} = V_{tot,30min} - V_{DR,30min} \quad (7)$$

where V_{tot} is the total volume of a resulted foam and possible drainage (ml), V_0 is the aliquot sample volume before whipping (ml) and V_{DR} is the volume of the drained liquid (mL). One analysis was conducted for three parallel treatments.

2.5.2. Heat-induced gelation

Heat-induced gelation was analysed by the inverting tube method (Agboola et al., 2005). Protein-water suspensions with 5% (w/v) and 15 % (w/v) concentrations were prepared and the pH was adjusted to 7. The 1 mL sample was heated in an Eppendorf tube for 20 min at 98 °C. Samples were placed at 4 °C for overnight storage. After 24 h, the gelation ability was analysed by observing if the suspension was self-supporting i.e. gelled or leaking when the tube was inverted upside down. One observation from each of three parallel treatments was conducted.

2.5.3. Colour

The colour of the freeze-dried protein powders (3 g / petri dish with 50 mm diameter) was determined by a colourimeter (Minolta Chroma meter, CR-200 Handheld, Osaka, Japan). L* (lightness), a* (green–red) and b* (blue-yellow) values were recorded according to the CIELAB colour space system. The colourimeter was calibrated with a white plate provided by the manufacturer. Whiteness value was calculated according to the following equation (Pérez et al., 2016):

$$WI = \left((100 - L)^2 + a^2 + b^2\right)^{\frac{1}{2}}$$

Five replicate measurements from each of three parallel treatments were conducted.

2.5.4. Microbiology

The microbiological quality of the samples was analysed as described in Nisov, Aisala, et al. (2020). Briefly, 1 g of sample was homogenised using 9 mL of peptone saline and the numbers of lactic acid bacteria, aerobic heterotrophic bacteria, psychrotrophic bacteria, sulphite reducing bacteria, enterobacteria, coliforms, and sulphite reducing

Table 1
Chemical composition of the Baltic herring and roach raw materials (as is, %) and derived hydrolysates and protein isolates (dry matter basis, %) produced by enzymatic or pH shift methods, respectively.

	Baltic herring composition (%)			Roach composition (%)		
Sample	Protein	Lipids	Ash	Protein	Lipids	Ash
Raw material	63.1 ± 0.0	29.9 ± 0.4	8.9 ± 0.5	64.8 ± 0.3	15.9 ± 1.9	16.7 ± 3.3
Neutrase	87.9 ± 0.0	3.3 ± 0.2	11.1 ± 0.1	85.1 ± 1.5	6.0 ± 0.2	6.4 ± 0.1
Protamex	91.1 ± 1.2	3.2 ± 0.1	11.5 ± 0.1	84.0 ± 1.7	5.8 ± 0.1	6.8 ± 0.5
Corolase	88.7 ± 1.2	3.5 ± 0.2	10.8 ± 0.2	83.6 ± 1.5	6.3 ± 0.1	7.2 ± 0.1
Enz control*	81.3 ± 0.7	n.a.	18.5 ± 0.7	82.4 ± 1.7	n.a.	7.4 ± 0.2
Acid	80.4 ± 0.8	16.7 ± 1.0	4.2 ± 0.1	83.0 ± 1.6	11.1 ± 0.5	5.2 ± 0.3
Alkaline	77.9 ± 0.7	16.8 ± 1.8	3.8 ± 0.7	73.0 ± 2.0	19.4 ± 0.9	4.0 ± 0.5
pH control*	68.3 ± 1.0	n.a.	2.7 ± 0.1	72.9 ± 0.3	n.a.	3.6 ± 0.2

* Control sample was prepared similarly to the enzymatic and pH-shifted samples, but without added enzymes or pH adjustment n.a. = not analysed.

microbes and yeast and moulds determined.

2.5.5. Sensory properties

A generic descriptive sensory analysis was performed for Baltic herring samples by 11 trained assessors and for roach samples by 12 trained assessors. The sensory profile of the freeze-dried powders was analysed from aqueous solutions (1.2%, w/v). The samples were served at room temperature in plastic dishes as 20 mL portions covered with a plastic lid. Water and cucumber slices were offered between sample evaluations. The samples were coded with random 3-digit codes and presented in randomised order. The preliminary list of sensory attributes was formulated by four sensory experts, and the attribute list was finalised by the whole panel in a training session before the actual evaluations. Eight chosen attributes were fishy odour, fresh odour, cloudiness, fish flavour, saltiness, bitterness, rancidity and coarseness. The intensity of the attribute values was rated on a scale from 0 to 10 where 0 = attribute not detected and 10 = attribute very clear. The evaluation scores were collected by a Compusense Five data system, Version 5.4 (Compusense, Guelph, Canada). Two parallel evaluation sessions were conducted using a sample mixture from three parallel treatments.

2.6. Statistical analysis

The differences between the means in compositional, functionality and descriptive sensory data were subjected to one-way analysis of variance followed by Tukey's test ($p < 0.05$) using IBM SPSS Statistics Version 26 (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Chemical composition, mass yield and protein yield of different protein fractions

The chemical compositions (dry matter basis) of the ground ungutted Baltic herring and roach raw materials are presented in Table 1. As was expected, the ground Baltic herring showed higher lipid content than roach; however, the protein contents were similar.

The yields of protein and oil fractions separated from fish raw materials are the key factors when developing successful technological processes for industry. Fig. 1A and B show the mass (Equation (1)) and protein yields (Equation (2)) of Baltic herring and roach protein powders obtained by enzymatic (hydrolysates) and pH shift methods (protein isolates). Alkaline treatment showed the highest mass yields for both Baltic herring (9.1%) and roach (9.6%), respectively. Enzymatic and acidic treatments had insignificant differences in mass yields regardless of the fish species showing mass yields of 5.7–6.1% and 7.5–8.2% for Baltic herring and roach, respectively. Otherwise all the control treatments (without enzyme and pH shifting) with both fish species showed notably lower mass yields (3.3–3.4%), except for the roach control for enzymatic treatment that showed a mass yield of 6.2%. The yield was close to what was detected for the Corolase treated roach (7.5%), which indicated that endogenous enzymes were strongly active in roach samples and contributed to the protein extraction. This would partly explain the higher mass yields for roach samples regardless of the applied extraction method (Fig. 1). In addition, higher lipid content in Baltic herring raw material could explain the lower yields, as lipids can interfere with the protein extraction process by forming complexes with

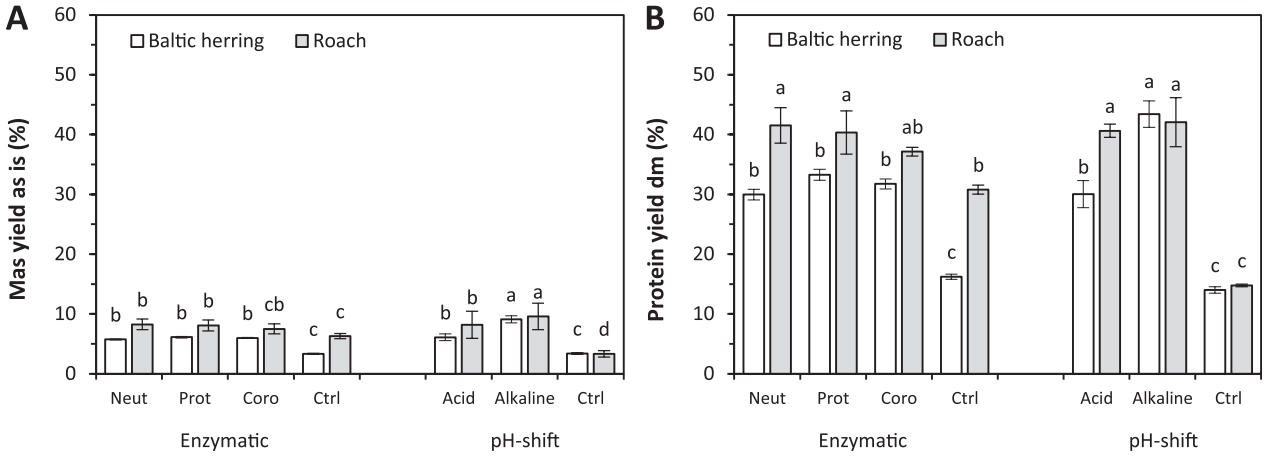

Fig. 1. Mass yield as wet weight (as is, %) (A), and protein yield as dry matter basis (dm, %) (B) of Baltic herring and roach proteins extracted by enzymatic and pH-shift methods. Different letters (a, b, c, d) indicate the significant differences ($p < 0.5$) within sample groups of Baltic herring and roach separately. Neut denotes for Neutrase, Prot for Protamex, Coro for Corolase and Ctrl for control sample (without pH shifting or added enzyme).

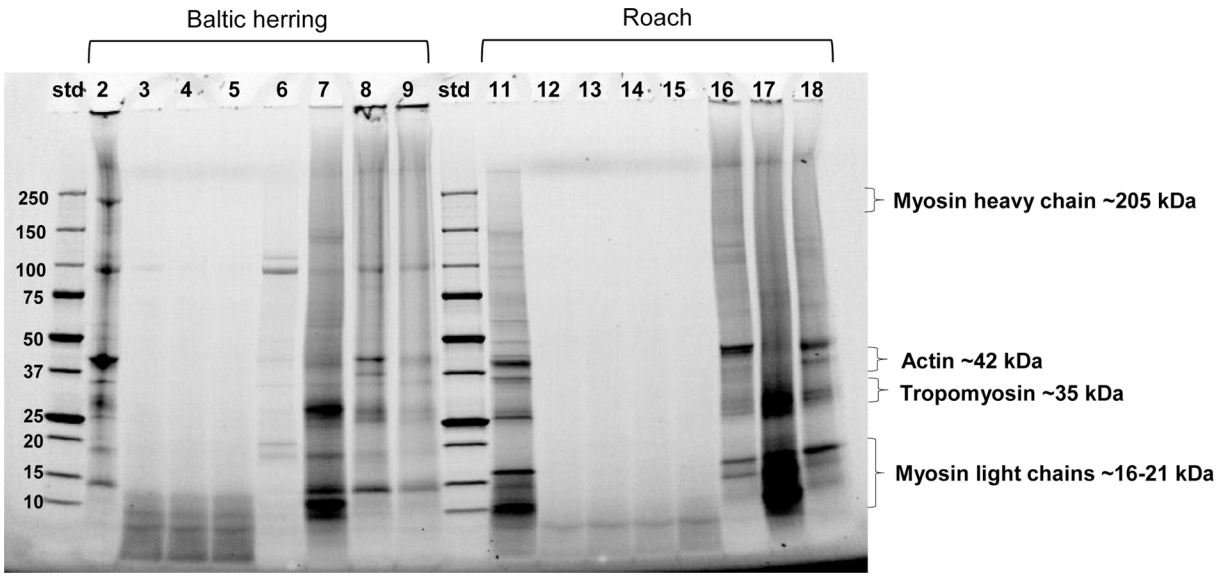

Fig. 2. Reducing SDS-PAGE image for Baltic herring and roach raw materials and protein fractions derived from them by enzymatic extraction and pH shifting. Lanes 2–9 denote for Baltic herring samples: 2) Ground Baltic herring mass 3) Neutrase, 4) Protamex, 5) Corolase, 6) enzyme control, 7) acid pH-shift, 8) alkaline pH-shift, 9) pH shift control. Lanes 11–18 denote for roach samples: 11) ground roach raw material, 12) Neutrase, 13) Protamex, 14) Corolase, 15) enzyme control, 16) acid pH shift, 17) alkaline pH shift 18) pH shift control. Standard is abbreviated as std.

proteins (Batista et al., 2010; Slizyte et al., 2005). Similar mass yields to the enzymatic process have been previously reported for the hydrolysed Atlantic cod backbone (4.6–6.3%) and carp meat (5.4–10.4%) with varying DH values of 21–24% and 6.7–13%, respectively, depending on the used enzyme (Elavarasan et al., 2014; Slizyte et al., 2016).

Similarly to the mass yield results, alkaline treatment resulted in the highest protein yields for both Baltic herring (43.4%) and roach (42.1%). However, the difference to other treatments was significant only for the Baltic herring samples showing notably lower protein yields of 30.0% (acidic) and 30.0–33.3% (enzymatic). A similar trend regarding the acid and alkaline treatment with lower protein yield for acidic treatment was observed by Abdollahi and Undeland (2019) when they studied salmon, cod and herring by-products during the pH-shift process. For herring by-products, they reported similar protein yields (45%) to our study at the same solubilisation pH of 11.5 whereas for salmon and cod by-products, they reported notably higher protein yields (50–60%). They reported protein yield values slightly under 40% for salmon and herring after acidic solubilisation (pH 2.5); however, for cod, they reported <30% protein yield. Clearly, the protein yield varies depending on the raw material and solubilisation pH; however, in the current study, the added heating step for pH treatment may have decreased the protein yield as well.

Although the highest mass and protein yields were obtained for protein isolates, the protein contents of the hydrolysates were generally higher and varied between 84.1–87.2% for Baltic herring (wet basis) and 79.3–81.1% for roach (wet basis) (Table 1, dm basis). The resulted protein contents in this study were considerably higher than those reported by Egerton et al. (2018) for whole blue whiting hydrolysates with values (wet basis) of 76.8% (Protamex) and 40.7% (Neutrase). The treatment conditions in the study of Egerton et al. (2018) were similar to the present study, and they reported similar lipid and ash contents. Thus, the explanation behind the differences in the protein contents remains unclear, however, one reason could be the different substrate specificities of the enzymes towards different fish species. In the current study, the protein isolates showed protein contents of 80.4% (acid) and 77.9% (alkaline) for Baltic herring and 83.0% (acid) and 73.0% (alkaline) for roach. The values for Baltic herring were slightly higher than those reported by (Marmon et al., 2009) for herring (*Clupea harengus*) protein isolates produced by acid (72%) and alkaline (64%) extractions, respectively.

The protein hydrolysates showed notably lower lipid contents (3.2–6.3%) than the protein isolates (11.1–19.4%) for both Baltic herring and roach. Previously, Liceaga-Gesualdo & Li-Chan (1999) have reported even lower lipid content of 0.77% for a freeze-dried herring protein hydrolysate (Alcalase 2.4 L). The lipid contents of the protein isolates in the current study were partly comparable to lipid contents reported by Marmon & Undeland (2010) for herring protein isolates produced by the acid (22.2%, dm) and alkaline (17.7%, dm) pH-shifting. Changes in temperature or pH during protein extraction may induce changes in pro-oxidants, such as haemoproteins (e.g. haemoglobin and myoglobin) present in fish, and increase the susceptibility of lipids to oxidation (Halldorsdottir et al., 2013). On the other hand, the products of lipid oxidation can promote protein oxidation, which induces protein unfolding and aggregation. This may influence physical and chemical properties of proteins, such as solubility, hydrophobicity, water-holding capacity, and gelation (Zhang et al., 2013).

3.2. Polypeptide profiles

Fig. 2 shows polypeptide profiles of roach and Baltic herring raw materials and the derived protein hydrolysates and isolates after enzymatic and pH-shift treatments. Regarding the SDS-PAGE results, there was a difference between the sample preparation of the ground fish raw materials and freeze-dried protein powders. While the polypeptide patterns of the fish raw materials include all possible SDS-soluble proteins, the hydrolysates and their control powder include only the supernatants after the separation process. Similarly, the protein isolates and their control powder include only the precipitated supernatant after the pH solubilisation. In other words, none of the analysed protein powders included the insoluble sediment. For example, only the water-soluble polypeptides were present in Baltic herring control hydrolysate whereas myosin heavy chain, actin and tropomyosin were either absent or very faint. As expected, the protein profile of the Baltic herring raw material was similar to what was detected for herring (*Clupea harengus*) filleting by-products by Abdollahi and Undeland (2019). Based on the polypeptide profiles reported by Abdollahi and Undeland (2019), the Baltic herring raw material in the current study most probably included myosin heavy chain band around 205 kDa, actin around 42 kDa, tropomyosin around 35 kDa and myosin light chains around 16–21 kDa. Additionally, two intense bands were detected at around 100 kDa, which have been previously identified as vitellin-like

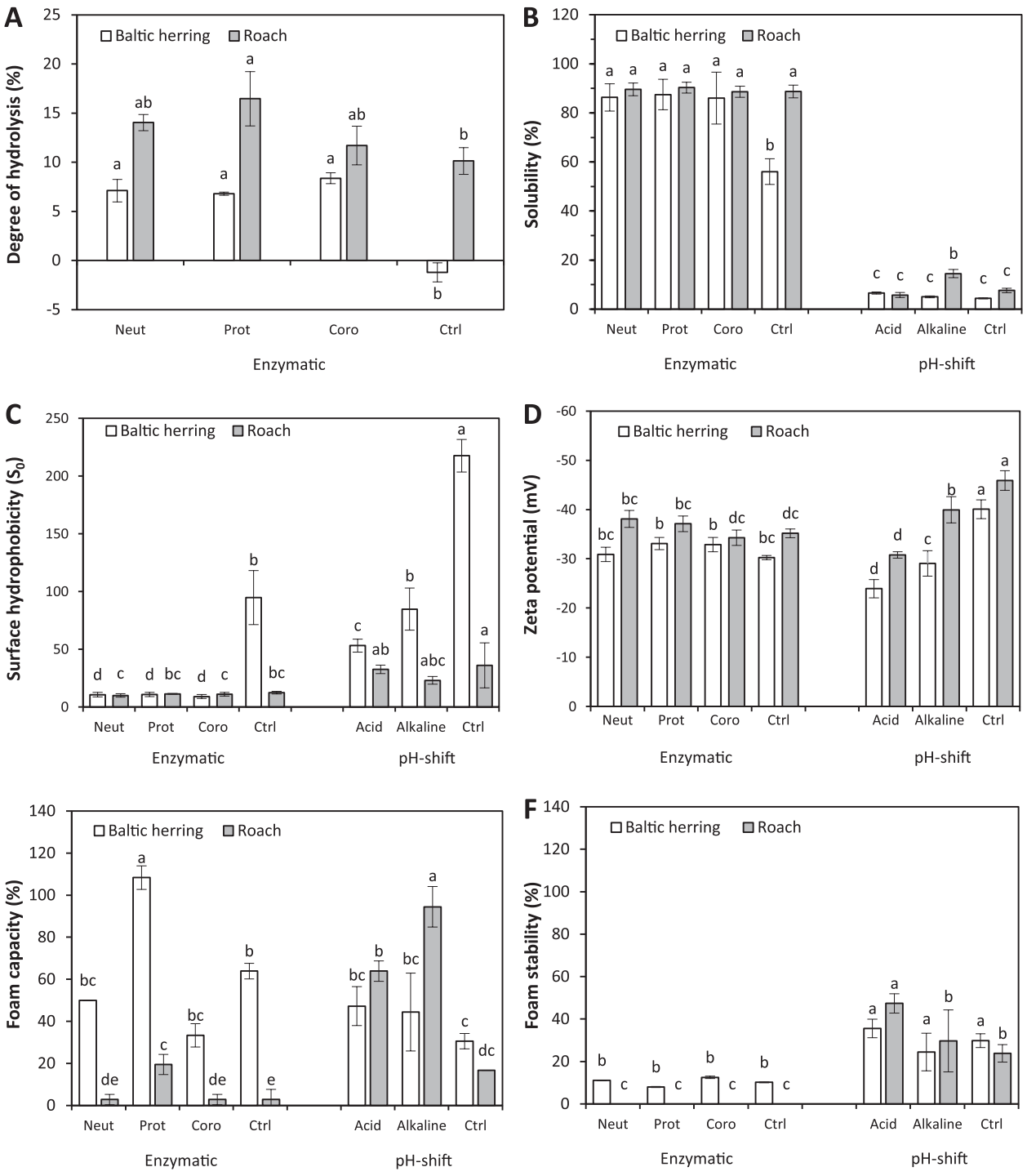

Fig. 3. Physicochemical and functional properties of Baltic herring and roach derived protein fractions produced by enzymatic and pH shift methods. A) Degree of hydrolysis of enzymatically extracted protein fractions. B) solubility, C) surface hydrophobicity, D) zeta potential, E) foam capacity and F) foam stability of protein fractions produced by pH shift and enzymatic extraction where Neut denoted for Neutrase, Prot for Protamex, Coro for Corolase and Ctrl for the control treatment (without pH shifting or added enzyme). Different letters (a, b, c, d, e) indicate the significant differences ($p < 0.5$) within sample groups of Baltic herring and roach separately.

protein (97 kDa) for salmon and sturgeon caviar (Al-holy & Rasco, 2006) as well as for yellowfin tuna roes (Lee et al., 2016). The protein profile of roach raw material differed from that of Baltic herring. The myosin heavy chain band was not detectable and the most intense bands could be detected at a low molecular weight range of 10 to 16 kDa. However, similarly to that found in Baltic herring, actin (42 kDa) and tropomyosin (35 kDa) bands could be detected also in the roach raw material. In addition, roach raw material showed an intense band around 26 kDa.

All hydrolysates (excluding the controls) for both raw materials showed similar polypeptide profiles with most intensive bands under 15 kDa indicating that the proteins were successfully hydrolysed to shorter peptides. The Baltic herring hydrolysates showed more intense bands with higher molecular weight than roach hydrolysates, and even one faint band could be detected at around 100 kDa. The polypeptide pattern of the Baltic herring control hydrolysate showed that it was not hydrolysed, since it lacked the short peptides under the size of 15 kDa. This

indicated that the Baltic herring sample lacked endogenous enzyme activity. On the contrary, the polypeptide pattern of the roach control hydrolysate was the same as the ones treated with enzymes. This again was a clear indication that the roach raw material had endogenous protease activity.

The size range of polypeptide bands in the protein isolates was clearly broader than in the hydrolysates. Alkaline extracted polypeptide pattern of Baltic herring sample resembled its control sample while the roach control resembled the peptide pattern of the acid extracted protein isolate. The acid extracted Baltic herring sample and alkaline extracted roach sample showed intense bands between 20 and 37 kDa as well as under 15 kDa, the latter ones indicating that hydrolysis had occurred. Usually, the endogenous enzyme activity in fish is optimum at slightly alkaline pH values, which would explain the peptide pattern of roach samples. It is also possible that the added heating step may have influenced the polypeptides so that non-enzymatic hydrolysis has occurred. This could explain the 15 kDa bands in Baltic herring samples, as there was no evidence of endogenous enzyme activity. The polypeptide patterns of the hydrolysates should be further studied on SDS-PAGE that resolves peptide bands under 10 kDa to gain more thorough understanding on the hydrolysates.

3.3. Degree of hydrolysis

The DH was measured only for the hydrolysates (Fig. 3A). In previous studies, a wide range of DH values (5–62%) have been reported for protein hydrolysates produced from different types of fish raw materials, including surimi processing by-products, minced roe and minced fish meat (Chalamaiah et al., 2010; Klompong et al., 2007; Liu et al., 2014). Previously, a trend with an increasing mass yield has been observed as a function of increasing DH when samples with varying DH values have been hydrolysed with the same enzyme (Chalamaiah et al., 2010; Elavarasan et al., 2014). However, if the application target for the protein hydrolysate is for human consumption, it is not desirable to produce hydrolysates with high DH values, since it is well known that also the bitterness increases with an increasing DH (Aspevik et al., 2016). Aspevik et al. (2016) reported that a bitter taste was perceived for short peptides with molecular weights between 0.5 and 2 kDa extracted from Atlantic salmon. In the current study, the target was to produce protein hydrolysates with limited DH, with the aim not only to minimise the bitterness, but also to produce more functional peptides (Klompong et al., 2007; Nisov, Ercili-Cura, et al., 2020). Reducing the molecular size of a protein makes it more flexible, and thus, more functional; however, if the protein hydrolysis exceeds a certain level, the resulting hydrolysate may lose the ability to form a stable foam or emulsion (Aspevik et al., 2016; Nisov, Ercili-Cura, et al., 2020). A wide range of DH values (5–62%) has been reported for protein hydrolysates produced from different types of fish raw materials, including surimi processing by-products, minced roe and minced fish meat (Chalamaiah et al., 2010; Klompong et al., 2007; Liu et al., 2014). The DH values in the present study varied between 7.1 and 14%, which was clearly at the lower range of what has been reported previously, however, the functional properties and bitterness will determine if the limited hydrolysis was successful or not.

The enzymatic treatments of roach material resulted in DH values of 14.0, 16.5 and 11.7% with Neutrase, Protamex and Corolase samples, respectively. Enzymatic treatments of Baltic herring resulted in considerably lower DH values of 7.1, 6.8 and 8.4% with Neutrase, Protamex and Corolase, respectively. All the DH results were in line with the SDS-PAGE patterns of the hydrolysates, as smaller peptides were detected for roach than for Baltic herring. Roach control resulted in a relatively high DH value of 10.1 %, which was higher than any values detected for Baltic herring, and almost as high as the DH values detected after the Corolase treatment. Again, this was a clear indication of an endogenous enzyme activity in roach raw material as was mentioned already regarding the yield and SDS-PAGE results. Polypeptides obtained in the control treatment (without added enzyme) of Baltic herring showed a negative DH value of −1.2%. Despite several attempts to replicate the DH analysis for the Baltic herring control, the results remained as negative values. The negative DH value can be explained by the inactivation step that was conducted at the end of the extraction process. The hypothesis is that when the proteins aggregated during the heating step in an aqueous environment, the OPA reagent (used in DH analysis) was unable to reach all free amino ends due to a more compact protein structure caused by the aggregation. Similar findings were reported by Slizyte et al. (2014) where DH values of herring hydrolysates decreased after an enzyme inactivation step. However, they hypothesized that the DH was reduced due to the Maillard reaction.

3.4. Protein solubility, surface hydrophobicity, zeta potential, foaming and gelation properties

Protein solubility in water at pH 7 was measured, as it is well known that solubility is a prerequisite for several functional properties (Wouters et al., 2016). Baltic herring and roach hydrolysates showed notably higher protein solubility values (86.0–90.3%) than the pH shifted protein isolates (5.1–14.5%) (Fig. 3B). This was expected as the pH shifted proteins were precipitated and dried at their isoelectric point (pH 5.5.) where most of the protein material was insoluble. Moreover, a heating step was included in this study following the pH-shift process to inhibit microbial growth, which in turn may have decreased the solubility due to protein denaturation and subsequent aggregation. On the other hand, in enzymatic extraction, the hydrolysates were collected as the soluble fractions (supernatants) in the process; thus it is logical for them to have high solubility. Also previous studies have reported that after shifting the pH to 7 after the isoelectric precipitation would not result in as high protein solubility as the enzymatic processes. For example, Rodrigues Freitas et al. (2016) studied the solubility of Argentine anchovy and whitemouth croacker protein isolates produced by acid and alkaline extraction whereas Abdollahi and Undeland (2018) studied the solubility of protein isolates produced by alkaline extraction from cod, herring, and salmon by-products. Depending on the fish species of these studies, acid and alkaline extracted proteins showed solubility values of 21–25% and 9–50% at pH 7, respectively (Abdollahi & Undeland, 2018; Rodrigues Freitas et al., 2016). Similar to the present study, solubility values of over 80% have been previously reported by Egerton et al. (2018) for hydrolysates from whole blue whiting produced using several different commercial enzymes (Alcalase, Protamex, Flavourzyme, Savinase), by Liu et al. (2014) for hydrolysates from silver carp by-products (flesh on bones, head, skin and viscera) using Alcalase and by Klompong et al. (2007) for hydrolysates of yellow stripe trevally minced meat using Alcalase. In the present study, a Baltic herring control for enzymatic process resulted in 56% solubility, which was significantly lower compared to the actual hydrolysates, whereas roach control resulted in 88.8% solubility with an insignificant difference to the actual treatments. Again, this indicated that the roach raw material exhibited endogenous enzyme activity leading to a hydrolysis of roach proteins, which improved the solubility without any added enzyme. No significant differences were detected within the solubility values of the pH shifted Baltic herring protein isolates. On the other hand, alkaline extracted roach protein isolate resulted in a significantly higher solubility value (14.5%) than the acid extracted proteins (5.7%) or the control proteins (7.6%). A similar observation of higher solubility values with alkaline extraction was reported by Rodrigues Freitas et al. (2016) for Argentine anchovy at pH 9 and for whitemouth croaker residues at pH 7 and 9.

All roach and Baltic herring hydrolysates as well as roach control showed low surface hydrophobicity values (9.0–12.3) regardless of the studied enzyme (Fig. 3C). Exceptionally, the Baltic herring control showed considerably higher surface hydrophobicity value of 94.7. A negative correlation between DH values and surface hydrophobicity could be detected. When the DH value increased, the surface

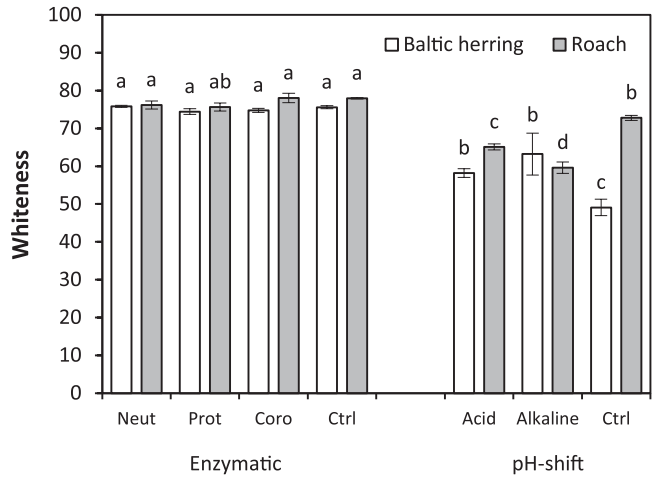

Fig. 4. The whiteness of Baltic herring and roach protein powders extracted by enzymatic and pH-shift methods. Neut denotes for Neutrase, Prot for Protamex, Coro for Corolase and Ctrl for control sample i.e. sample prepared similarly to the enzymatic and pH-shift method, but without any pH adjustment or added enzymes. Different letters (a, b, c, d) indicate the significant differences ($p < 0.5$) within sample groups of Baltic herring and roach separately.

hydrophobicity was low and vice versa. This could mean that the proteins in this study were hydrolysed into a molecular size that was too small for the ANS probe to be able to properly attach to the peptide and form the needed reaction for detection of hydrophobic groups as was discussed in the study of Nisov, Ercili-Cura, et al. (2020) for rice protein hydrolysates. Also, the pH shifted Baltic herring protein isolates showed substantially higher surface hydrophobicity values with an increasing trend from the acid extracted sample (53.2) to alkaline (84.7) and control (217.5). On the other hand, all pH shifted roach protein isolates showed notably lower surface hydrophobicity values of 32.5, 22.9 and 35.9 for acid, alkaline and control proteins, respectively. The surface hydrophobicity values measured in this study seemed low compared to what has been previously reported for fish protein hydrolysates (225–565) by Liu et al. (2014) and for alkaline extracted fish proteins (600–1200) reported by Kobayashi and Park (2017). Moreover, Kim et al. (2003) reported a surface hydrophobicity value of nearly 2000 for acid extracted Pacific whiting proteins. Liu et al. (2014) reported that a change in surface hydrophobicity during enzymatic hydrolysis depends on the used enzymes, as the values decreased with increasing DH when Protamex was used, but on the contrary, it increased when Alcalase was used. The values detected for the fish protein hydrolysates in this study were closer to those reported by Nisov, Ercili-Cura, et al. (2020) for rice protein hydrolysates (5–40) and those reported by Wu et al. (1998) for soy protein hydrolysates (1–30) with varying sizes. Nisov, Ercili-Cura, et al. (2020) reported a similar trend to this study with decreasing surface hydrophobicity when compared to the control samples, whereas, Wu et al. (1998) reported an increasing trend when compared to the control. The reason behind this trend remains unknown and needs further investigation.

Regardless of the fish species, the zeta potential (Fig. 3D) was lowest for the acid extracted samples followed by alkaline and control samples, respectively. Little variation was detected regarding the enzymatically treated samples for which the absolute zeta potential values varied from 30.2 to 33.1 mV for Baltic herring and from 34.3 to 38.1 mV for roach samples. Slightly lower absolute zeta potential values were reported by Liu et al. (2014) for protein hydrolysates derived from surimi processing products by Alcalase (24.0–29.2 mV) and Protamex (22.5–29.8 mV). The pH shifting showed an increasing trend in absolute zeta potential values from acid (23.9, 30.8) to alkaline (29.1, 39.9) and control (40.1, 45.9) treatment with both fish species.

Foegeding et al. (2006) reviewed that the surface hydrophobicity correlates with improved foaming properties. However, no clear correlation between surface hydrophobicity values and foaming properties was detected in the current study. All produced foams (Fig. 3E and F) were unstable, showed a high drainage, and collapsed almost immediately after whipping. Enzymatically extracted protein hydrolysates resulted in considerably lower foam stability than the pH-shifted proteins. This can be partly explained by the low molecular size of the hydrolysate peptides, however, it does not explain why also Baltic herring control showed low foam stability as it had molecular size ranging between 20 and 100 kDa. Enzymatically extracted roach hydrolysates showed clearly a lower foaming capacity than the Baltic herring hydrolysates or pH shifted protein isolates. This can be explained by the higher DH value being the result of endogenous enzyme activity in roach raw material. Moreover, none of the produced hydrolysates nor protein isolates were able to form rigid gel networks, which also suggests that the enzymatically extracted samples exhibited too high DH values and pH extracted proteins possessed too low solubility values. Thus, it can be concluded that the targeted limited enzymatic hydrolysis was not successful in terms of optimising functional properties, especially regarding the roach samples.

3.5. Colour

In general, all hydrolysates showed significantly higher whiteness values (74.4–78.8) than the pH shifted protein isolates (49.1–72.8) (Fig. 4). The difference in whiteness values of the hydrolysates was insignificant regardless of the applied method or raw material. Similar values were reported by Slizyte et al. (2009) for different types of fish protein hydrolysates derived from backbones of Atlantic cod with lightness values varying from 85.3 to 91.5 depending on the hydrolysis time and the freshness of the raw materials. They reported that the most significant effect on obtaining higher lightness values was using fresh fish instead of frozen fish. Regarding the protein isolates and their controls, significant differences in colour values were found depending on the extraction conditions as well as the used fish species. For example, the roach control produced using alkaline extraction resulted in the highest whiteness value of 72.8, which was close to the whiteness values of the hydrolysates whereas both acid and alkaline extracted roach protein isolates resulted in significantly lower whiteness values of 65.1 and 59.8, respectively. On the contrary, the Baltic herring hydrolysate control resulted in the lowest whiteness value of 49.1, whereas the acid and alkaline extracted proteins showed higher whiteness values of 58.2 and 63.2, respectively. Similar values to the alkaline and acid extracted Baltic herring proteins obtained in the present study were reported by Abdollahi and Undeland (2018) using alkaline (pH 12) extraction for herring, which resulted in a whiteness value of 54.8. In the same study, they reported whiteness values of 71.8 and 66.1 for alkaline extracted cod and salmon proteins, respectively. This demonstrated that the whiteness value is dependent on the fish species used, as was also noticed in our study. Rodrigues Freitas et al. (2016) reported whiteness values of 55.6 and 49.0 for acid and alkaline extracted proteins from Argentine anchovy residue and 56.6 and 47.4 for acid and alkaline extracted proteins from whitemouth croaker residue, respectively.

3.6. Microbiology

The lowest level of microbes was observed in the alkaline extracted protein isolate in which the level of psychrotrophic and heterotrophic microbes was <10 colony forming units (CFU)/g. In other samples, low levels of psychrotrophic and heterotrophic microbes were observed, 10–40 CFU/g. Levels of other microbial groups were below 100 CFU/g. Freitas et al. (2016) reported that the alkaline and acidic treatments, investigated in their study, were sufficient to inactivate microbes originating from raw materials. Microbes are known to adapt and produce e.g. acid-tolerant mutants. Therefore, Lansdowne et al. (2009) examined the efficacy of acid (pH 2.0 and 3.0) and alkaline (pH 11.5 and 12.5) pH

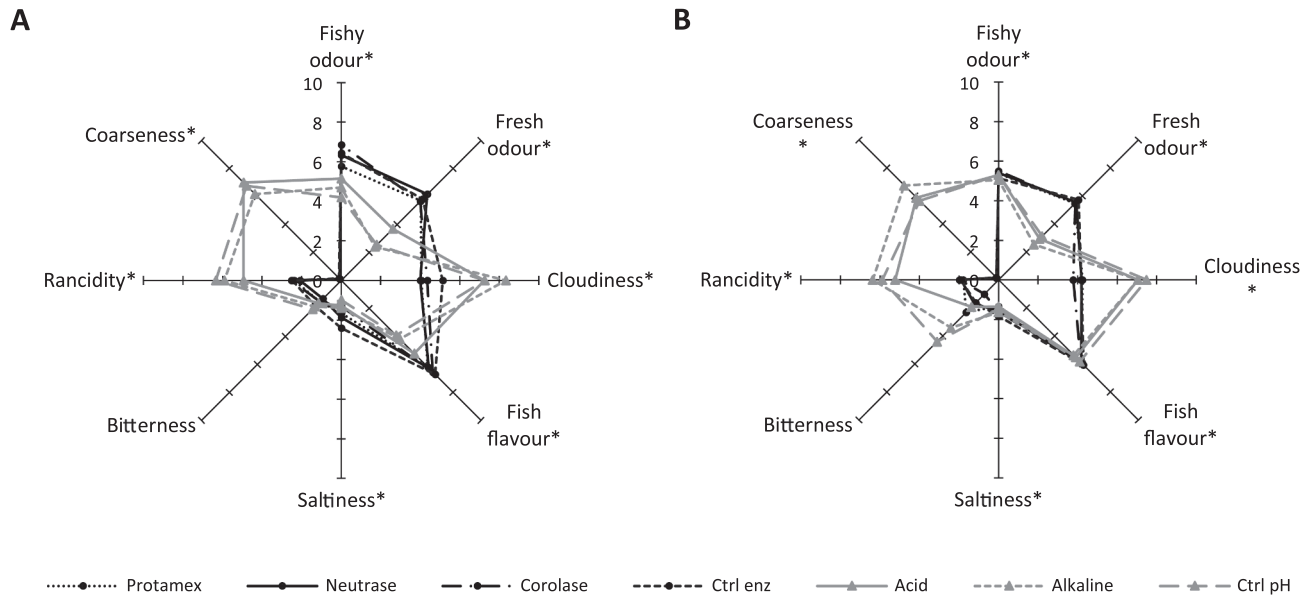

Fig. 5. Descriptive sensory profiles of A) Baltic herring and B) roach protein powders extracted by enzymatic and pH shift methods. The attributes marked with an asterisk (*) have statistically significant differences between samples ($p < 0.05$).

shift on the reduction of inoculated *Listeria innocua* and *Escherichia coli* during processing. They observed a reduction of the viability of the inoculated bacteria, but little data are available concerning other pathogens. In industrial-scale process, the food producers need to examine their products according to Regulation (EC) No 2073/2005 food safety criteria and examine also hygienic quality including *Salmonella*, *Listeria* and sulphite reducing clostridia. Raw material quality and process hygiene influence the microbiological quality of the final products. Therefore, the results of the current study indicated that the microbial quality of the produced hydrolysates and protein isolates was good.

3.7. Sensory profile

Both roach protein products produced by enzymatic and pH-shift methods resulted in moderately fishy odour, whereas, the odour of Baltic herring hydrolysates were perceived fishier when produced by the enzymatic method, and with some variation with different enzymes (Fig. 5). However, the freshness of the odour was perceived significantly higher for both fish species when the protein was extracted enzymatically and not by the pH-shift method. The cloudiness of all samples was perceived significantly higher in samples produced by pH shifting including the control. A similar moderate fish flavour was perceived in all roach samples, whereas Baltic herring hydrolysates were perceived to have more fishy flavour than the pH shifted counterparts. Rancidity, fishy flavour and fishy odour are common issues in fish proteins produced by the pH-shift method and by enzymatic extraction (Abdollahi & Undeland, 2018; Shaviklo et al., 2011; Yarnpakdee et al., 2012). Yarnpakdee et al. (2012) reported a decreased fishy odour and taste when the amount of pro-oxidants and lipids were reduced. None of the protein products was perceived as salty regardless of the used fish species or method. Surprisingly, none of the Baltic herring samples, roach hydrolysates or acid extracted roach samples were perceived bitter, whereas significantly higher bitterness was perceived for roach samples produced by alkaline extraction and by control pH shifting. This supported the findings regarding the endogenous enzyme activity in roach samples, especially in alkaline and control extracted samples. All pH shifted samples were perceived clearly more rancid than samples produced by enzymatic extraction. None of the hydrolysates was perceived coarse, whereas all pH shifted samples showed quite high coarseness values. The reason for this could be that the heating step in the pH-shift method denatures the proteins and subsequently forms aggregates that lead to coarse mouthfeel especially at pH 5.5 where the proteins are almost insoluble.

4. Conclusions

This study showed that it is possible to produce microbiologically good quality food-grade hydrolysates and protein isolates from whole Baltic herring and roach. However, both studied processes need optimisation regarding functional properties as the produced polypeptides and proteins showed poor foaming properties, and no gelation ability. The highest mass yield was obtained for alkaline extracted protein isolates; however, the highest protein content was detected for the hydrolysate produced using Protamex. Surprisingly, all enzymatically extracted hydrolysates exhibited low bitterness values while alkaline extracted proteins showed high bitterness values for roach. On the other hand, although the protein isolates resulted in low solubility, the foam capacity was comparable to the hydrolysates and showed even higher foam stability. Thus, it can be concluded that the selection between the two processes was not straightforward and requires preliminary investigations for each fish species depending on what properties are desired. Further studies are needed for improving the sensory quality and functional properties of the protein isolates and hydrolysates.

CRediT authorship contribution statement

Anni Nisov: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization. **Tanja Kakko:** Methodology, Validation, Investigation, Writing – review & editing. **Hanna-Leena Alakomi:** Methodology, Validation, Investigation, Writing – review & editing. **Raija Lantto:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Kaisu Honkapää:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131524.

References

Abdollahi, M., Rezaei, M., Jafarpour, A., & Undeland, I. (2018). Sequential extraction of gel-forming proteins, collagen and collagen hydrolysate from gutted silver carp (*Hypophthalmichthys molitrix*), a biorefinery approach. *Food Chemistry, 242*, 568–578. https://doi.org/10.1016/j.foodchem.2017.09.045

Abdollahi, M., & Undeland, I. (2018). Structural, functional, and sensorial properties of protein isolate produced from salmon, cod, and herring by-products. *Food and Bioprocess Technology, 11*, 1733–1749. https://doi.org/10.1007/s11947-018-2138-x

Abdollahi, M., & Undeland, I. (2019). Physicochemical and gel-forming properties of protein isolated from salmon, cod and herring by-products using the pH-shift method. *Lwt - Food Science and Technology, 101*, 678–684. https://doi.org/10.1016/j.lwt.2018.11.087

Agboola, S., Ng, D., & Mills, D. (2005). Characterisation and functional properties of Australian rice protein isolates. *Journal of Cereal Science, 41*, 283–290. https://doi.org/10.1016/j.jcs.2004.10.007

Al-holy, M. A., & Rasco, B. A. (2006). Characterization of salmon (*Oncorhynchus keta*) and sturgeon (*Acipenser transmontanus*) caviar proteins. *Journal of Food Biochemistry, 30*, 422–428.

Aspevik, T., Totland, C., Lea, P., & Oterhals, Å. (2016). Sensory and surface-active properties of protein hydrolysates based on Atlantic salmon (*Salmo salar*) by-products. *Process Biochemistry, 51*, 1006–1014. https://doi.org/10.1016/j.procbio.2016.04.015

Batista, I., Ramos, C., Coutinho, J., Bandarra, N. M., & Nunes, M. L. (2010). Characterization of protein hydrolysates and lipids obtained from black scabbardfish (*Aphanopus carbo*) by-products and antioxidative activity of the hydrolysates produced. *Process Biochemistry, 45*, 18–24. https://doi.org/10.1016/j.procbio.2009.07.019

Chalamaiah, M., Rao, G. N., Rao, D. G., & Jyothirmayi, T. (2010). Protein hydrolysates from meriga (*Cirrhinus mrigala*) egg and evaluation of their functional properties. *Food Chemistry, 120*, 652–657. https://doi.org/10.1016/j.foodchem.2009.10.057

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). Characterization of protein hydrolysates from blue whiting (*Micromesistius poutassou*) and their application in beverage fortification. *Food Chemistry, 245*, 698–706. https://doi.org/10.1016/j.foodchem.2017.10.107

Elavarasan, K., Naveen Kumar, V., & Shamasundar, B. A. (2014). Antioxidant and functional properties of fish protein hydrolysates from fresh water carp (*Catla catla*) as influenced by the Nature of Enzyme. *Journal of Food Processing and Preservation, 38*, 1207–1214. https://doi.org/10.1111/jfpp.12081

FAO. (2020). *The state of world fisheries and aquaculture 2020*. 10.4060/ca9229en.

Foegeding, E. A., Luck, P. J., & Davis, J. P. (2006). Factors determining the physical properties of protein foams. *Food Hydrocolloids, 20*, 284–292. https://doi.org/10.1016/j.foodhyd.2005.03.014

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry, 226*, 497–509. https://doi.org/10.1016/s0021-9258(18)64849-5

Gao, R., Yu, Q., Shen, Y., Chu, Q., Chen, G., Fen, S., … Sun, Q. (2021). Production, bioactive properties, and potential applications of fish protein hydrolysates: Developments and challenges. *Trends in Food Science and Technology, 110*, 687–699. https://doi.org/10.1016/j.tifs.2021.02.031

Halldorsdottir, S. M., Kristinsson, H. G., Sveinsdottir, H., Thorkelsson, G., & Hamaguchi, P. Y. (2013). The effect of natural antioxidants on haemoglobin-mediated lipid oxidation during enzymatic hydrolysis of cod protein. *Food Chemistry, 141*, 914–919. https://doi.org/10.1016/j.foodchem.2013.03.101

Kim, Y. S., Park, J. W., & Choi, Y. J. (2003). New approaches for the effective recovery of fish proteins and their physicochemical characteristics. *Fisheries Science, 69*, 1231–1239. https://doi.org/10.1111/j.0919-9268.2003.00750.x

Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry, 102*, 1317–1327. https://doi.org/10.1016/j.foodchem.2006.07.016

Kobayashi, Y., & Park, J. W. (2017). Biochemical and physical characterizations of fish protein isolate and surimi prepared from fresh and frozen whole fish. *LWT - Food Science and Technology, 77*, 200–207. https://doi.org/10.1016/j.lwt.2016.11.027

Kristinsson, H. G., & Liang, Y. (2006). Effect of pH-shift processing and surimi processing on Atlantic croaker (*Micropogonias undulates*) muscle proteins. *Food Chemistry and Toxicology, 71*, C304–C312. https://doi.org/10.1111/j.1750-3841.2006.00046.x

Lansdowne, L. R., Beamer, S., Jaczynski, J., & Matak, K. E. (2009). Survival of *Escherichia coli* after isoelectric solubilization and precipitation of fish protein. *Journal of Food Protection, 72*, 1398–1403. https://doi.org/10.4315/0362-028X-72.7.1398

Lee, H. J., Lee, G. W., Yoon, I. S., Park, S. H., Park, S. Y., Kim, J. S., & Heu, M. S. (2016). Preparation and characterization of protein isolate from Yellowfin tuna Thunnus albacares roe by isoelectric solubilization/precipitation process. *Fisheries and Aquatic Sciences, 19*, 1–10. https://doi.org/10.1186/S41240-016-0014-Z

Liceaga-Gesualdo, A. M., & Li-Chan, E. C. Y. (1999). Functional properties of fish protein hydrolysate from herring (*Clupea harengus*). *Journal of Food Science, 64*, 1000–1004. https://doi.org/10.1111/j.1365-2621.1999.tb12268.x

Liu, Y., Li, X., Chen, Z., Yu, J., Wang, F., & Wang, J. (2014). Characterization of structural and functional properties of fish protein hydrolysates from surimi processing by-products. *Food Chemistry, 151*, 459–465. https://doi.org/10.1016/j.foodchem.2013.11.089

Marmon, S. K., Liljelind, P., & Undeland, I. (2009). Removal of lipids, dioxins, and polychlorinated biphenyls during production of protein isolates from baltic herring (*Clupea harengus*) Using pH-shift processes. *Journal of Agricultural and Food Chemistry, 57*, 7819–7825. https://doi.org/10.1021/jf901266v

Marmon, S. K., & Undeland, I. (2010). Protein isolation from gutted herring (*Clupea harengus*) using pH-shift processes. *Journal of Agricultural and Food Chemistry, 58*, 10480–10486. https://doi.org/10.1021/jf101057q

Nisov, A., Aisala, H., Holopainen-Mantila, U., Alakomi, H.-L., Nordlund, E., & Honkapää, K. (2020). Comparison of whole and gutted Baltic herring as a raw material for restructured fish product produced by high-moisture extrusion cooking. *Foods, 9*, 1541. https://doi.org/10.3390/foods9111541

Nisov, A., Ercili-Cura, D., & Nordlund, E. (2020). Limited hydrolysis of rice endosperm protein for improved techno-functional properties. *Food Chemistry, 302*, 1–9. https://doi.org/10.1016/j.foodchem.2019.125274

Pérez, M. D. M., Ghinea, R., Rivas, M. J., Yebra, A., Ionescu, A. M., Paravina, R. D., & Herrera, L. J. (2016). Development of a customized whiteness index for dentistry based on CIELAB color space. *Dental Materials, 32*, 461–467. https://doi.org/10.1016/j.dental.2015.12.008

Rodrigues Freitas, I., Cortez-Vega, W. R., & Prentice, C. (2016). Physicochemical and functional properties of protein recovered from fish waste. *Journal of Aquatic Food Product Technology, 25*, 1034–1044. https://doi.org/10.1080/10498850.2015.1008714

Shaviklo, G. R., Thorkelsson, G., Arason, S., & Sveinsdottir, K. (2011). Characteristics of freeze-dried fish protein isolated from saithe (Pollachius virens). *Journal of Food Science Technology, 49*, 309–318. https://doi.org/10.1007/s13197-011-0285-4

Slizyte, R., Carvajal, A. K., Mozuraityte, R., Aursand, M., & Storro, I. (2014). Nutritionally rich marine proteins from fresh herring by-products for human consumption. *Process Biochemistry, 49*, 1205–1215. https://doi.org/10.1016/j.procbio.2014.03.012

Šližyte, R., Daukšas, E., Falch, E., Storrø, I., & Rustad, T. (2005). Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (*Gadus morhua*) by-products. *Process Biochemistry, 40*, 1415–1424. https://doi.org/10.1016/j.procbio.2004.06.033

Šližytė, R., Mozuraitytė, R., Martínez-Alvarez, O., Falch, E., Fouchereau-Peron, M., & Rustad, T. (2009). Functional, bioactive and antioxidative properties of hydrolysates obtained from cod (*Gadus morhua*) backbones. *Process Biochemistry, 44*, 668–677. https://doi.org/10.1016/j.procbio.2009.02.010

Slizyte, R., Rommi, K., Mozuraityte, R., Eck, P., Five, K., & Rustad, T. (2016). Bioactivities of fish protein hydrolysates from defatted salmon backbones. *Biotechnology Reports, 11*, 99–109. https://doi.org/10.1016/j.btre.2016.08.003

Spellman, D., McEvoy, E., O'Cuinn, G., & FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal, 13*, 447–453.

Wouters, A. G. B., Rombouts, I., Fierens, E., Brijs, K., & Delcour, J. A. (2016). Relevance of the functional properties of enzymatic plant protein hydrolysates in food systems. *Comprehensive Reviews in Food Science and Food Safety, 15*, 786–800.

Wu, W. U., Hettiarachchy, N. S., & Qi, M. (1998). Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modification and ultrafiltration. *Journal of the American Oil Chemists' Society, 75*, 845–850.

Xu, X., Liu, W., Liu, C., Luo, L., Chen, J., Luo, S., … Wu, L. (2016). Effect of limited enzymatic hydrolysis on structure and emulsifying properties of rice glutelin. *Food Hydrocolloids, 61*, 251–260.

Yarnpakdee, S., Benjakul, S., Kristinsson, H. G., & Maqsood, S. (2012). Effect of pretreatment on lipid oxidation and fishy odour development in protein hydrolysates from the muscle of Indian mackerel. *Food Chemistry, 135*, 2474–2482. https://doi.org/10.1016/j.foodchem.2012.07.037

Zhang, W., Xiao, S., & Ahn, D. U. (2013). Protein oxidation: Basic principles and implications for meat quality. *Critical Reviews in Food Science and Nutrition, 53*, 1191–1201. https://doi.org/10.1080/10408398.2011.577540