Two stage processing of salmon backbones to obtain high-quality oil and proteins

Running title: Two stages processing of salmon backbones

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Abstract

Traditional processing technologies for fish by-products containing significant amounts of oils usually either give high amounts of oil or maximised solubilisation of proteins. Due to lower yields and insufficient quality, the proteins or the oil are considered as secondary products. The proposed concept combines a gentle thermal separation of oil followed by enzymatic hydrolysis of the remaining protein rich fraction. The first stage, thermal treatment (40°C) of fresh salmon backbones, separated up to 85% of the oil from the raw material and gave high-quality oil (PV=0.2±0.0 meq/kg, 0.16±0.05% free fatty acids). Separation of a significant part of the oil gave reduced mass flow into the enzymatic stage, which then requires less enzymes and reduced energy consumption. Among the tested enzymes: Trypsin, Corolase PP and Papain and Bromelain mixture gave the highest yield of FPH, while use of Protamex and Corolase PP resulted in FPH with the best sensory properties leading to the lowest bitterness.

Key words: salmon backbones, quality, oil, hydrolysate, two stage processing

Introduction

Better utilisation and processing of by-products are important factors for sustainable and economically viable development of the seafood industry (Aspevik *et al.,* 2017). The fish processing industry produces several by-products, which are currently mainly used as low-value products or wasted without any attempt at recovery (Rustad *et al.,* 2011). Fish by-products provide a good source of nutrients such as high-quality marine oils and proteins, and can be used for many purposes, including production of novel and value-added products for feed, food, nutraceuticals, pharmaceuticals, and fine chemicals. The quantities of these by-products can be significant. In 2015, almost 900 000 tons of marine by-products were generated in Norway and more than 45 % of the amount came from aquaculture (Richardsen *et al*., 2016). With an estimated protein content of 15% and a lipid content of 20%, this valuable biomass contains more than 61 000 tons of proteins and about 81 000 tons of marine lipids. The main part (90%) of aquaculture by-products are utilised, but most of the biomass goes for production of silage and fish meals and only a small portion is processed for into products for human consumption. Traditional silage production is a simple process (Vieira *et al*., 2015) that is difficult to control and lasts over several days or weeks (Gildberg, 1993). During prolonged processing lipid oxidation occurs, leading to undesirable changes in flavor, color, and loss of nutritional value (Gildberg, 1993; Vieira *et al*., 2015) including loss of some amino acids like cysteine, tryptophan, methionine, histidine and proline (De Arruda *et al*., 2007; Ramasubburayan *et al*., 2013). Both thermal processing and controlled enzymatic hydrolysis with added commercial enzymes can be applied to recover both valuable fish oil and proteins from by-products under mild conditions (Aspevik *et al.,* 2017). However, traditional processing technologies usually focus on the yield and/or quality on the main product, sacrificing the yield and quality of the co-products. Fish meal and fish oil are commonly produced by the wet rendering method (Xu *et al.*, 2007), where fish are minced and heated at 90-95 ^{o}C to coagulate protein and liberate water and oil. High temperature and long heating time lead to reduction in fish oil quality (Xu

et al., 2007, Carvajal *et al*., 2015) and denaturation of proteins. Controlled enzymatic hydrolysis is a technological solution to recover both the valuable fish oil and the proteins from by-products in a mild and reproducible way. Enzymatically hydrolyzed proteins often have better bioactive properties compared to silage proteins and can be used in different diet formulations with several health beneficial properties (Ngo *et al*., 2012). One of the challenges when producing fish protein hydrolysates (FPH) is the bitter taste, which can be influenced by the composition of starting material, choice of enzymes or the hydrolysis process itself (Dauksas *et al.*, 2004; Slizyte *et al*., 2014).

Farmed salmon are filleted on land under good sanitary conditions resulting in very fresh and human food grade quality by-products, which can be used for production of valuable marine oil and proteins (Opheim *et al.,* 2015). To obtain more sustainable utilisation of these by-products, a techno-economically feasible process leading to high-quality products is needed. Thermally separated oil from fish by-products is more stable and has better oxidation status compared to oil obtained after enzymatic hydrolysis (Carvajal *et al*., 2015; Slizyte *et al*., 2017). In addition to higher quality oil, separation of oil before hydrolysis will reduce the amounts of raw material directed for hydrolysis. Combination of two technological solutions might give both economical and quality benefits. The aim of this work was to evaluate a two stage processing concept where oil from salmon backbones is thermally separated before the hydrolysis step leading to maximised yield and quality of both oils and proteins. The first part of the work was directed to the thermal treatment of the raw material to find the optimal temperature leading to maximal yield and quality of separated oil and low degree of protein denaturation. The second part shows the results of the combined technologies: thermal separation of oil using optimal temperature followed by enzymatic hydrolysis of protein rich fractions, remaining after oil separation.

Materials and methods

Raw material, enzymes and chemicals

Fish backbones from farmed Atlantic salmon (*Salmo salar*) of the same freshness (the same batch) and similar size were used for the experiments. The weight of the backbones varied from 600 to 700 g. The fish was farmed and harvested in central Norway by Lerøy and eviscerated and filleted by the specialized fish shop "Ravnkloa Fisk og Skalldyr" (Trondheim) 1 day after slaughter. Whole salmon backbones were minced in a HOBART mincer (model AE 200) with holes of 10mm. The minced raw material was distributed in plastic bags (600 g each) and spread out evenly in order to have a thin layer of minced material (thickness of about 1 cm) to ensure maximum heat transfer and uniform temperature throughout the raw material. The plastic bags were then sealed and vacuum packed. The plastic bags were kept on ice for about 30 minutes before the experiments.

Eight different proteolytic enzymes were used in the experiment. Corolase® PP and Corolase® 7089 (activity optimum at 45^{o}C and 55^{o}C respectively, both from AB Enzymes GmbH), Protamex® (optimal working conditions at 35-60^{o}C, Novozymes A/S), Papain FG and Bromelain 400 GDU/g (temperature optimum 25-70^{o}C and 50-60^{o}C respectively, both from Enzybel), Trypsin (Sigma Aldrich), Protex 6L (effective temperature range 25-70^{o}C, Genecor) and Sea-B-Zyme L 200 (optimal temperature range 60-70^{o}C, Specialty Enzymes & Biotechnologies) were received from the producers. Methanol, chloroform, formaldehyde hexane, diethyleter, and formic acid (Merck, Darmstad, Germany) were used for the chemical analysis. All chemicals used were of reagent grade.

Experimental set-up

In order to prove the benefits of the two stage processing concept, a randomized design was used in the tests. The experiments were performed as two screening experiments. The first was done to study the effect of temperature on the separation of oil (Initial thermal oil separation), temperature was used as input variable and yield and quality of oil was used as response variables. In the second experiment the effect of initial oil separation, use of different enzymes and time of hydrolysis on yield and quality of hydrolysates (Hydrolysis) was studied with different enzymes and time as input variables.

Initial thermal oil separation

To determine the effect of temperature on the yield and quality of oil, initial thermal separation of oil was tested at eight different temperatures ranging from 20-95°C. The water bath was heated to the defined temperatures between 20-95°C (23°C, 30°C, 39°C, 46°C, 58°C, 67°C, 77°C and 95°C) and the plastic bag with minced material (defined above) was placed in the water bath until the raw material reached the desired temperature and was kept at this temperature for 5 minutes. After the thermal treatment, the content of each plastic bag was placed into centrifugation tubes, centrifuged for 10 minutes at $2300 \times g$ and immediately frozen at (-80°C). Oil, water and sediments fractions were separated while frozen. The mass of separated oil was measured to determine the yield of separated oil.

Hydrolysis

Based on the yield and quality of separated oil from the thermal separation experiments, 40^{o}C was chosen for thermal separation of oil. Minced and vacuum-packed salmon backbones

prepared for hydrolysis tests were placed into water bath (42^{o}C) to warm up the raw material to approx. 40^{o}C, kept at this temperature for 5 min and centrifuged at *2300×g* for 15 min to separate the oil fraction. The mixture of stick water and sediments were used as a raw material for hydrolysis. The hydrolysis was performed as described by Slizyte *et al.*, (2016). Based on earlier experiments (Slizyte et al., 2016), the following experimental set-up was chosen. Six different commercially available enzymes (given below) and one mixture of two enzymes (Papain + Bromelain) was used. The raw material was mixed with warm (approx. 50°C) distilled water (1:1 of raw material mass). The enzymatic hydrolysis was started when the temperature of the mixture had reached 50 °C by adding enzyme dosed at 0.1% (w/w of raw material mixture). In the case of combination of enzymes, each enzyme was added at 0.05% + 0.05% (w/w) of raw material mixture. Two control hydrolyses were performed using Protamex: Control 1 (C1): Hydrolysis with raw material without initial separation of oil; Control 2 (C2): Hydrolysis of raw material after initial separation of oil and without addition of water. Representative samples of hydrolysate from each hydrolysis were taken at 0 (stick water, taken before addition of commercial enzymes), 20, 40, 60 and 120 min of hydrolysis.

Oil quality

Peroxide value (PV) in the oil samples was determined by the iodometric titration method described in a titration application issued by Radiometer Analytical SAS (TTIP02-01AFD/2002-06A, 2002). The application is based on to the AOCS official method (AOCS, 2003b; a) for PV determination in edible oils. The titration end point was assessed potentiometrically, using TitraLab980 automatic titrator (Radiometer Analytical SAS, Copenhagen, Denmark), coupled with a single platinum electrode (M21Pt, Radiometer Analytical ASA, Copenhagen, Denmark) and a reference electrode (REF 921, Radiometer

Analytical ASA, Copenhagen, Denmark). The analysis was performed with 4 – 6 parallels, and the results are expressed in meq/kg oil as a mean value with standard deviation of the mean.

Free fatty acid (FFA) content in oil samples was analysed according to a procedure proposed by Bernardez *et al*. (2005). Isooctane was used as a solvent for lipids, instead of cyclohexane. Oils samples (0.1-0.2g) were mixed on a vortex with 5 mL isooctane and 1 mL of 5% cupric acetate-puridine aqueous reagent was added. The mixture was vortexed for 30s and centrifuged at *2000×g* for 5 min. The upper layer was collected and absorbance measured at 715nm. Each sample was measured in four parallels. A standard curve prepared with oleic acid standard (0 – 20 µmol) was used for FFA content calculation. The analysis was performed with 4 – 6 parallels and the results are expressed as % of oleic acid ± standard deviation.

Sensory analysis

Ranking test was used for evaluation of bitter taste in freeze dried FPH obtained by different enzymes and for the study of process kinetics. The participants (16 persons) for the sensory panel were selected among the SINTEF Ocean (Norway) employees on the basis of their threshold level for bitter taste after training using caffeine solutions (0%, 0.006%, 0.014% and 0.027% in water). Six different samples with a concentration of 0.5% of freeze dried fish protein hydrolysate (FPH) and stick water samples dissolved in water was presented for panellist evaluation. The bitterness was evaluated in the range from 1 (the least bitter sample) up to 6 (the most bitter sample). Orders of serving were completely randomized. Bitterness of 0.027 caffeine solution was set to have 5 at our bitterness scale). Bitterness results are presented in Box plot indicating the median of the measurements, first and third quartiles as well as total distribution of the measurements.

Statistical analysis

Microsoft Office Excel 2010 were used for statistical analysis and data processing. Student T-test was used to test for significant differences between treatments. The means were accepted as significantly different at 95% confidence level ($p < 0.05$).

Results and discussion

Thermal separation of oil

Salmon backbones are by-products rich in oil (17.5±1.6% ww). The temperature used for the initial thermal separation of oil influences the yield and the quality (Table 1) of the separated oil. A significant increase ($p < 0.05$) in the amount of oil separated was observed from 20 to 40°C. More than 85% of the total oil content from the raw material was extracted at 40°C compared to approximately 55% at 20°C. However, no significant difference in the amount of oil separated by thermal separation was observed at temperatures above 40°C.

The quality of the thermally extracted oil was evaluated by amount of free fatty acids (FFA), which is the results of hydrolysis of lipids and primary oxidation products measured by peroxide value (PV). Low *PV values* indicate low oxidation of the oil and confirm high-quality of thermally separated oil (Table 1). However, temperature increase from 20^{o}C to 40^{o}C and from 70^{o}C till 95^{o}C led to significant increase ($p < 0.05$) in PV. This indicates that temperature is an important factor for oxidative quality of fish oil, even the oil as exposed to elevated temperatures only for a few minutes. The same tendency was observed with separation of oil from fresh herring by-products at different temperatures (Carvajal *et al.*, 2015) indicating that even 10^{o}C increase in oil separation temperature can lead to significant increase in oxidative status of the oil.

The amount of FFA is an indication of the activity of endogenous lipases, which hydrolyses the lipids and leads to FFA formation. The recommended limit for high-quality crude oil is 2-5 % of FFA (Bimbo, 2007). All salmon oils extracted from fresh backbones at different temperatures contained very low amount of FFA: 0.002- 0.107% (Table 1) indicating very high-quality oil. Lipolytic enzymes in fish are not stable at elevated temperatures and it could be expected that temperature above 60°C will lead to full inactivation of lipolytic enzymes (Sovik & Rustad, 2005). However, our results show that the amount of FFA increased with increasing separation temperatures (from 20 to 77°C) with the highest amount measured at around 77°C (Table 1). The same development pattern was observed in the crude oil extraction from fresh herring by-products where the highest amount of FFA was measured in the oil extracted at 80ºC (Carvajal *et al.*, 2015). The lipolysis could be caused by an activity of different lipolytic enzymes acting during the increase of temperature in the materials. To reach the inactivation temperature, the material goes through a temperature range where lipolytic activity is high leading to formation of FFA up to 80ºC.

The first visual changes in raw material were observed at 40°C. The raw material became less homogenous, more rigid, and started to flake. This effect was increasingly prominent as the temperature increased. In addition, the color of the raw material changed from bright red at temperatures up to 40°C to an increasingly grey and colorless appearance at higher temperatures. These changes were probably due to denaturation and aggregation of proteins, as proteins in raw material can denature and precipitate at elevated temperatures. Ofstad *et al*. (1993) found that salmon protein started to lose water at around 35°C and the maximum water loss was reached at a temperature between 45-50°C. This was ascribed to denaturation of myosin and shrinkage of collagen. Thermal denaturation and aggregation of actomyosin from Threadfin bream starts at around 35°C (Yongsawatdigul & Park, 2003). Denatured proteins were observed to be more resistant to enzymatic breakdown reducing the amount of proteins

that can be solubilized during hydrolysis and thereby the FPH yield (Slizyte *et al*., 2005b). Due to this, extensive denaturation of proteins is not desired in raw material designated for hydrolysis. The color of thermally treated stick water, obtained after centrifugation of heated raw material, also indicated that the temperature range around 50°C is critical for protein changes. The stick water is bright red up to 50°C and transparent above, probably due to denaturation of proteins like hemoglobin and myoglobin, and precipitation of these in the sediment fraction.

Determination and control of processing parameters like temperature and time of exposure during initial thermal separation of oil is critical when the raw material is intended for hydrolysis in the next processing step. Therefore, the temperature of 40°C for thermal separation of oil was chosen since it was the lowest temperature giving a high yield and quality of oil, at the same time showing minimum visual changes in the raw material indicating protein denaturation.

Hydrolysis

Mild initial thermal isolation of oil, tested in the first part of the work was combined with the enzyme screening test during the second part of the work. Thermal separation at 40°C temperature led to separation of 67±6% of the oil from the starting raw material. This is significantly lower compared to the amount of oil which was separated during the first part of the work by using the same separation temperature (85% of oil was then separated). The slight difference between experiments could be due to uneven distribution of the salmon mass in the plastic bags, also to slight variations in the experimental temperatures.

Hydrolysis with Trypsin, Sea-B-Zyme and a mixture of Papain and Bromelain resulted in a separation of 58, 54 and 54 % respectively of the remaining oil from the defatted starting

material after 120 min hydrolysis (Table 2). Hydrolysis without addition of water gave the lowest yield of separated oil probably due to the high viscosity of the hydrolysis mixture which restricts hydrolysis and where oil is entrapped in the viscous mixture hindering separation of oil by centrifugation. However, hydrolysis without addition of water gave the lowest amount of emulsion which is not a desirable product after hydrolysis: 0.6 g dry material/100 g start material (no water) compared to 1.4 g dry material/100 g start material (addition of water) and 1.9 g dry material/100 g of control hydrolysis without initial separation of oil before hydrolysis. Higher amount of oil in the raw material leads to the formation of undesirable emulsion and this tendency was also indicated during the hydrolysis of cod by-products (Slizyte *et al.*, 2005a).

In a one stage hydrolysis process (without initial separation of oil) the oil in the raw material is kept at elevated temperature and usually with access to oxygen for several hours. The oil makes up a significant amount of inert material during hydrolysis, increase process volume, requires energy for heating of the hydrolysis mixture as well as a higher capacity for transportation and separation units. From an economical point of view oil should be separated in order to increase profitability of the process. During hydrolysis of defatted raw material 15-30% of oil in raw materials was separated during hydrolysis, which is significantly less compared to the amount of oil separated from control hydrolysis: Pr-T (without defatting) (Table 2). In average all hydrolysis yielded 4.5±0.6 g/100 g raw material of oil after 120 min hydrolysis where Corolase PP and Trypsin were the most effective enzymes for oil separation. However, hydrolysis without addition of water gave the lowest yield of separated oil indicating that addition of water is not only important for optimal protein solubilisation process, but also for maximisation of oil separation after hydrolysis.

In addition to economic advantages of initial separation of oil before hydrolysis, the quality differences between "first stage" and "second stage" oil play an important role for process profitability. Oil obtained by thermal separation before hydrolysis (first stage) had

several times lower PV values indicating the significance of the oil separation prior to hydrolysis (Table 3). These results were in accordance with previous studies indicating that thermally separated oil from is more stable and has better oxidation status compared to oil obtained after enzymatic hydrolysis (Carvajal et al., 2015; Slizyte et al., 2017). Even oil obtained at a hydrolysis time of 0 (when the material is mixed with warm water and warmed up to the hydrolysis temperature) showed the first signs of oxidation, which increases with hydrolysis time leading to elevated PV value in the oil obtained after hydrolysis. This confirms that to obtain high-quality oil and at the same time reduce the amount of raw material for hydrolysis, the oil should be separated by gentle heating and centrifugation before the hydrolysis. Other antioxidative technological features like use of anoxic atmosphere or addition of antioxidants during hydrolysis may also be considered.

Amount of FFA was also significantly higher in the oil separated after enzymatic hydrolysis compared to thermally extracted oils (Table 3), but they are still below the limit for recommended PV and FFA values for crude oil (Bimbo, 2007). Amount of FFA further increases with the time of hydrolysis. In the case of hydrolysis with Protamex, crude oil extracted thermally showed higher FFA values compared to other thermally extracted oil. This could be due to different quality of raw material (backbones). Prolonged storage even at low temperatures could lead to increased amount of FFA due to the activity of endogenous lipolytic enzymes (Sovik & Rustad, 2005). These enzymes continue to release FFA during the following hydrolysis with the added commercial enzymes confirming the theory that oil loses quality during hydrolysis. Elevated temperatures, mixing, which lead to incorporation of additional oxygen into the system, are the main reasons leading to reduction of the quality during hydrolysis.

The hydrolytic efficiency of the enzymes was evaluated by the amount of dry material ending up in the hydrolysate fraction: the amount of dried fish protein hydrolysate (FPH). Table

2 shows that Trypsin, Corolase PP and mixture of Papain and Bromelain were the most efficient enzymes during the whole hydrolysis period. The other four studied enzymes (Protamex, Sea-B-Zyme, Protex 6L and Corolase 7089) gave similar, but lower yield of dried FPH after 120 min hydrolysis. The three enzymes: Protamex, Protex 6L and Corolase 7089 originate from Bacillus species, while the enzymes giving the best results were obtained from animal digestion tract or fruits. Control hydrolysis with Protamex indicated the necessity of addition of water before hydrolysis. Hydrolysis with Protamex without addition of water before hydrolysis gave less than half the amount of FPH compared to the corresponding hydrolysis with added water. The same tendency was reported for the hydrolysis with cod by-products (Slizyte *et al.*, 2005b) and can be related to the high viscosity of hydrolysates which hinders hydrolysis (Aspevik *et al.*, 2017) and restricts separation of the different fractions after hydrolysis. The most intensive hydrolysis was observed during the first 20-30 min of the hydrolysis (Table 2). The following hydrolysis led to a slower increase in FPH yield. The actual yield of FPH based on defatted raw material would be higher. A yield of 8-11% g of dried FPH was obtained from approx. 90 g raw material as 67±6% (approx. 10g) of oil was separated before hydrolysis. This would give about 10% increase in FPH yield.

Sensory analysis

Both process conditions and choice of enzyme may influence development of bitterness in FPH (Slizyte *et al*., 2014). All used commercial enzymes gave variable bitterness level of FPH after 60 min of hydrolysis (Figure 1). Protamex and Corolase PP gave the lowest and Protex 6L gave the highest median value. Hydrolysis with mixture of Papain and Bromelain increased the bitterness during the first minutes of hydrolysis (Figure 1), but the bitterness was then reduced slightly compared to samples obtained before hydrolysis (0 and 60/120 samples). Previous studies with herring by-products indicated a similar tendency: bitterness of FPH after 60 min

hydrolysis was reduced compared to time 0 (Slizyte *et al*., 2014). However, different raw materials can contain compounds, which can influence the bitterness in the produced hydrolysates (Dauksas *et al*., 2004), therefore development and kinetics of bitterness changes should be checked when new raw materials are introduced and new enzymes are applied.

Two stage thermo-enzymatic process

Techno-economical evaluation of the technological concept shows that the two stage thermo-enzymatic process is profitable and this concept shows advantage with value added, more expensive products compared to the fish meal concept (Nappa *et al*., 2013). Based on techno-economic evaluation and technological evaluation of the process, the two stage thermo-enzymatic process for processing the salmon by-products to intermediate products was proposed (Figure 2). It is known that bones and sediments fraction after hydrolysis are rich in marine phospholipids (Šližytė *et al.,* 2004; Dauksas *et al*., 2005; Carvajal *et al*., 2015) and therefore a third stage of the optimal processing of salmon by-products can be suggested.

Conclusions

A new approach for traditional hydrolysis of fish by-products containing significant amounts of oils is presented. The basis of two stage processing is the mild thermal separation of oil prior to hydrolysis of the de-fatted backbones. The oil obtained by thermal separation shows significantly higher quality compared to oil separated during hydrolysis with addition of commercial enzymes. The protein changes during the thermal separation step does not affect the hydrolysis of the proteins by the added proteases. Therefore, the two stage thermo - enzymatic processing where oil is separated using mild heating during the first step look very promising both with regard to economical (up till 85% of oil from raw material is separated

before hydrolysis) and quality aspects (oil contained low amount of free fatty acids and were not oxidised). The test of different proteases in the second stage of enzymatic hydrolysis shows that among the tested enzymes Trypsin, Corolase PP and mixture of Papain and Bromelain gave the highest yield of FPH after 40 min of hydrolysis, while Protamex and Corolase PP gave FPH with the lowest bitterness after 60 min hydrolysis.

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References

AOCS. (2003a). Official Methods and Recommended Practices of the American Oil Chemists' Society. Method Cd 8b-90.

AOCS. (2003b). Official Methods and Recommended Practices of the American Oil Chemists' Society Method Cd 8b-90.

Aspevik, T., Oterhals, A., Ronning, S.B., Altintzoglou, T., Wubshet, S.G., Gildberg, A., Afseth, N.K., Whitaker, R.D., Lindberg, D. (2017). Valorization of Proteins from Co- and By-Products from the Fish and Meat Industry. *Topics in Current Chemistry*, **375 (3:53)**, 1-28.

Bernardez, M., Pastoriza, L., Sampedro, G., Herrera, J.J.R. & Cabo, M.L. (2005). Modified Method for the Analysis of Free Fatty Acids in Fish. *Journal of Agricultural and Food Chemistry,* **53**, 1903-1906.

Bimbo, A.B. (2007). *Processing of marine oils*. In Breivik, H. (ed) Long-chain omega-3 specialty oils The Oily Press, Bridgwater, England. Pp. 80-84.

Carvajal, A.K., Šližytė, R., Storrø, I. & Aursand, M. (2015). Production of high quality fish oil by thermal treatment and enzymatic hydrolysis from fresh Norwegian spring spawning herring by-products. *Journal of Aquatic Food Product Technology*, **8**, 1-17.

Dauksas, E., Falch, E., Slizyte, R. & Rustad, T. (2005) Composition of fatty acids and lipid classes in bulk products generated during enzymic hydrolysis of cod (*Gadus morhua*) by-products. *Process Biochemistry*, **40**, 2659-2670.

Dauksas, E., Slizyte, R., Rustad, T. & Storro, I. (2004). Bitterness in fish protein hydrolysates and methods for removal. *Journal of Aquatic Food Product Technology*, **13**, 101-114.

De Arruda, L.F., Borghesi, R. & Oetterer, M. (2007). Use of fish waste as silage - A review. *Brazilian Archives of Biology and Technology*, **50**, 879-886.

Gildberg, A. (1993). Enzymatic Processing of Marine Raw-Materials. *Process Biochemistry*, **28**, 1-15.

Nappa, M., Kangas, P., Šližytė, R., Navickas, K., Venslauskas, K., Nekrošius, A. & Remman, T. (2013). Concepts and Profitability Forutilisation of Fish-Industry Side-Stream Rural development 2013: the sixth international scientific conference. Akademija, Aleksandras Stulginskis University. Pp. 95-101.

Ngo, D.H., Vo, T.S., Ngo, D.N., Wijesekara, I., and Kim, S.K. (2012). Biological activities and potential health benefits of bioactive peptides derived from marine organisms. *International Journal of Biological Macromolecules*. **51(4),** 378-383.

Ofstad, R., Kidman, S., Myklebust, R. & Hermansson, A.M. (1993). Liquid Holding Capacity and Structural-Changes during Heating of Fish Muscle - Cod (Gadus-Morhua L) and Salmon (Salmo-Salar). *Food Structure*, **12**, 163-174.

Opheim, M., Slizyte, R., Sterten, H., Provan, F., Larssen, E. & Kjos, N.P. (2015). Hydrolysis of Atlantic salmon (Salmo salar) rest raw materials-Effect of raw material and processing on composition, nutritional value, and potential bioactive peptides in the hydrolysates. *Process Biochemistry*, **50**, 1247-1257.

Ramasubburayan, R., Iyapparaj, P., Subhashini, K.J., Chandran, M.N., Palavesam, A. & Immanuel, G. (2013). Characterization and Nutritional Quality of Formic Acid Silage Developed from Marine Fishery Waste and their Potential Utilization as Feed Stuff for Common Carp Cyprinus carpio Fingerlings. *Turkish Journal of Fisheries and Aquatic Sciences*, **13**, 281-289.

Richardsen, R., Nystøyl, R., Strandheim, G. & Marthinussen, A. (2016). Analyse marint restråstoff, 2015. Analyse av tilgang og anvendelse for marint restråstoff i Norge. SINTEF Fisheries and Aquaculture, Trondheim.

Rustad, T., Storro, I. & Slizyte, R. (2011). Possibilities for the utilisation of marine by-products. *International Journal of Food Science & Technology*, **46**, 2001-2014.

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.

Slizyte, R., Dauksas, E., Falch, E., Storro, I. & Rustad, T. (2005a). Characteristics of protein fractions generated from hydrolysed cod (Gadus morhua) by-products. *Process Biochemistry*, **40**, 2021-2033.

Slizyte, R., Opheim, M., Storrø, I. & Sterten, H. (2017). Simple technologies for converting rest raw materials of Atlantic salmon (Salmo salar) into high quality, valuable and tasty feed ingredients. *Journal of Aquatic Food Product Technology*, in press.

Slizyte, R., Rustad, T. & Storro, I. (2005b). Enzymatic hydrolysis of cod (Gadus morhua) by-products - Optimization of yield and properties of lipid and protein fractions. *Process Biochemistry*, **40**, 3680-3692.

Šližytė, R., Van Nguyen, J., Rustad, T. & Storrø, I. (2004). Hydrolysis of cod (Gadus morhua) by-products: influence of initial heat inactivaton, concentration and separation conditions. *Journal of Aquatic Food Product Technology*, **13,** 31-48.

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.

Sovik, S.L. & Rustad, T. (2005). Effect of season and fishing ground on the activity of lipases in byproducts from cod (Gadus morhua). *LWT- Food Science and Technology*, **38**, 867-876.

TTIP02-01AFD/2002-06A (2002). Peroxide Number of Edible Oils (ISO 3960/2001) Titration applications - Redox titrations. Radiometer Analytical SAS, Villeurbanne Cedex, France.

Vieira, E.O., Venturoso, O.J., Reinicke, F., da Silva, C.C., Porto, M.O., Cavali, J., Vieira, N.T. & Ferreira, E. (2015). Production, Conservation and Health Assessment of Acid

Silage Vicera of Freshwater Fish as a Component of Animal Feed. *International Journal of Agriculture and Forestry*, **5**, 177-181

Yongsawatdigul, J. & Park, J.W. (2003). Thermal denaturation and aggregation of threadfin bream actomyosin. *Food Chemistry*, **83**, 409-416.

Xu, X., H-Kittikun, A., and Zhang, H. (2007). Enzymatic processing of omega-3 speciality oils. In: *Long-chain omega-3 speciality oils* (edited by H. Breivik). Pp. 141-164. The Oily Press, Bridgwater, England.

Legends to Figures

Figure 1. Relative bitterness of the hydrolysates (0.5% solution in water). Hydrolysates obtained after 0 (0) and 60 min of hydrolysis. Papain+Bromelain hydrolysis obtained after 0, 20, 60 and 120 min hydrolysis. Scale of relative bitterness: 1 (the least bitter sample) up to 6 (the most bitter sample). Box plot indicates the median of the measurements, first and third quartiles as well as total distribution of the measurements.

Figure 2. Two stage thermo-enzymatic processes for utilising salmon co-streams followed by possible extraction of phospholipids (third stage) from bones/sediments.

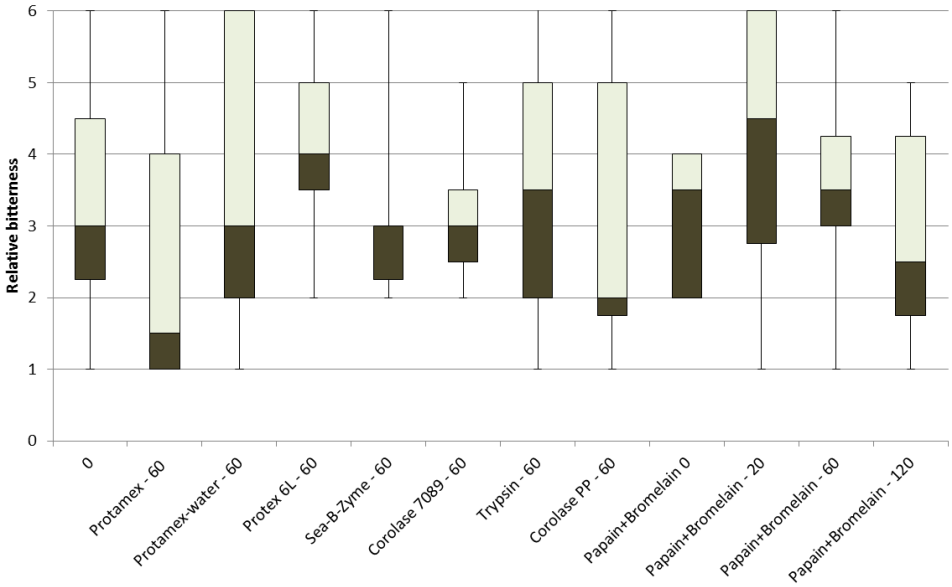
Relative bitterness
6
5
4
3
2
1
0
0
Protamex - 60
Protamex-water - 60
Protex 6L - 60
Sea-B-Zyme - 60
Corolase 7089 - 60
Trypsin - 60
Corolase PP - 60
Papain+Bromelain 0
Papain+Bromelain - 20
Papain+Bromelain - 60
Papain+Bromelain - 120

Figure 1.

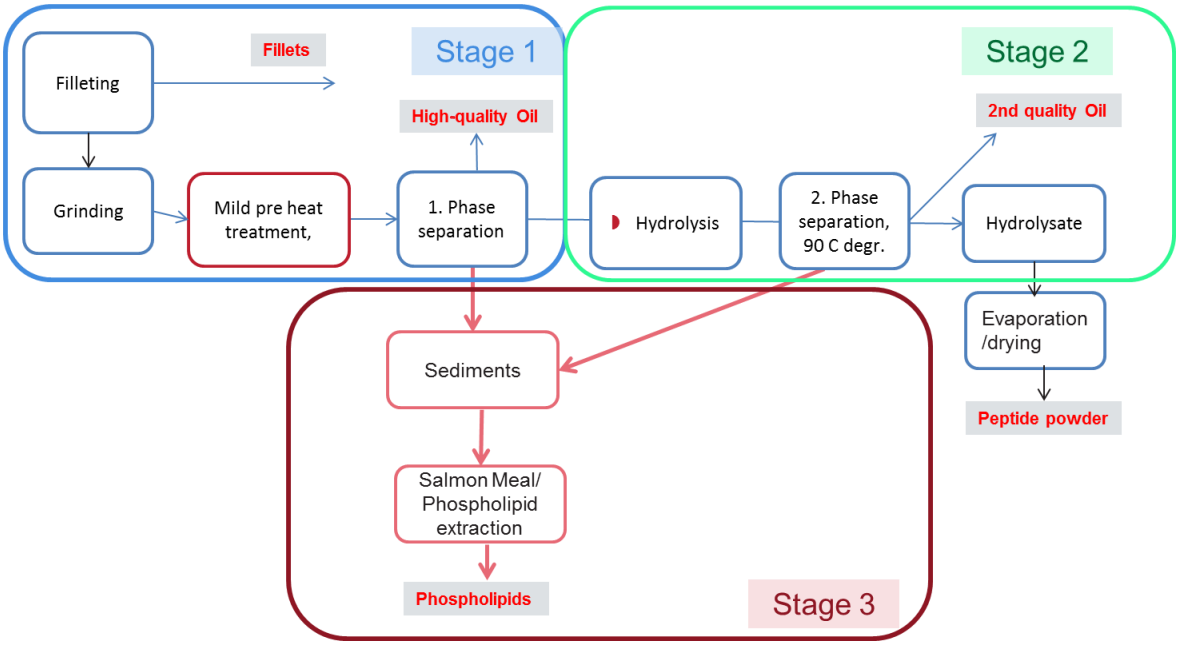
Filleting
Fillets
Stage 1
High-quality Oil
Grinding
Mild pre heat treatment,
1. Phase separation
Hydrolysis
2. Phase separation, 90 C degr.
Stage 2
2nd quality Oil
Hydrolysate
Evaporation /drying
Peptide powder
Sediments
Salmon Meal/ Phospholipid extraction
Phospholipids
Stage 3

Figure 2.

Tables

Table 1. The amount of oil (g/100g raw material) and amount of free fatty (FFA), peroxide value (PV) in oil separated from fresh raw material by thermal separation at different temperatures.

Temperature (°C)	Oil yield (g/100g raw material)	FFA (%)	PV (meq/kg oil)
23	9.6±0.5[a]	0.00±0.00[a]	0.12±0.01[a]
30	11.4±0.2[b]	0.00±0.00[a]	0.22±0.03[b]
39	14.4±0.8[c]	0.01±0.00[b]	0.48±0.04[c]
46	14.8±0.9[c]	0.02±0.01[b]	0.52±0.15[c]
58	15.4±0.5[c]	0.01±0.01[b]	0.59±0.13[c]
67	15.1±0.5[c]	0.03±0.01[c]	0.55±0.14[c]
77	14.8±1.4[c]	0.11±0.02[d]	1.07±0.22[d]
95	15.6±1.9[c]	0.04±0.03[c]	0.92±0.07[d]

Results are presented as mean ±SD; % of oleic acid ± SD and meq/kg oil as a mean value ± SD.
Means in columns are accepted as significant different at 95% level ($p<0.05$), values marked with same letters are not significantly different.

Table 2. Yield of oil and dried FPH as a function of hydrolysis time for the studied enzymes and control hydrolysis.

Hydrolysis time (min)	0		20		40		120	
Fraction	Oil	FPH	Oil	FPH	Oil	FPH	Oil	FPH
Corolase-PP	2.3	3.6	2.9	8.3	3.4	8.4	5.5	10.1
Trypsin	2.0	3.5	3.2	7.2	4.1	8.7	4.9	10.5
Protex 6L	2.7	3.5	3.4	6.2	3.8	6.6	4.4	8.1
Corolase 7089	2.4	3.6	3.2	5.9	3.1	6.9	4.0	8.9
Papain+Bromelain	2.4	3.4	3.3	6.3	3.0	8.0	4.6	10.3
Sea-B-Zyme	2.7	3.9	3.2	5.8	3.6	6.7	4.5	8.7
Protamex	2.4	4.0	2.8	6.4	2.9	7.4	3.7	8.4
Protamex-Thermal (C1)	5.4	5.5					17.7	11.5
Protamex-water (C2)	2.0	2.0	2.1	3.4	2.7	3.8	2.8	3.1

Values are presented as g oil/dried FPH from 100 g start raw material.

Table 3. Peroxide values (PV) and amount of free fatty acids (FFA) in the oil obtained by thermal separation and after enzymatic hydrolysis using different enzymes.

	Hydrolysis time (min)	PV (meq/kg)	FFA (%)
Thermal separation	-	0.2±0.0^{a}	0.06±0.05^{a}
Corolase PP	0	2.2±0.0^{b}	0.25±0.02^{b}
	40	2.8±0.2^{b}	0.76±0.03^{c}
	120	6.3±0.3^{c}	1.11±0.01^{d}
Corolase 7089	0	2.8±0.2^{b}	0.23±0.01^{b}
	40	5.8±0.2^{c}	0.30±0.01^{c}
	120	7.6±0.2^{d}	0.47±0.01^{d}
Sea-B-Zyme	0	2.2±0.4^{b}	0.29±0.01^{b}
	40	2.4±0.1^{bc}	0.46±0.03^{c}
	120	2.7±0.1^{c}	0.65±0.00^{d}
Protex 6L	0	2.0±0.2^{b}	0.35±0.01^{b}
	40	2.7±0.2^{b}	0.42±0.02^{c}
	120	2.0±0.2^{b}	0.62±0.01^{d}
Protamex - water	0	5.8±0.0^{b}	1.17±0.01^{b}
	40	1.2±0.1^{c}	1.29±0.01^{c}
	120	1.0±0.1^{c}	1.48±0.02^{d}
Protamex	0	3.4±0.1^{b}	0.93±0.07^{b}
	40	12.7±0.3^{c}	1.01±0.02^{c}
	120	16.7±0.2^{d}	1.21±0.02^{d}
Trypsin	0	3.6±0.5^{b}	0.20±0.01^{b}
	40	9.6±0.3^{c}	0.32±0.01^{c}
	120	10.5±0.3^{d}	0.47±0.01^{d}
Papain+Bromelain	0	3.8±0.4^{b}	0.32±0.01^{b}
	40	8.0±0.3^{c}	0.39±0.01^{c}
	120	8.2±0.1^{c}	0.59±0.02^{d}

Results are expressed in meq/kg oil as a mean value ± SDOM for PV and % of oleic acid ± SDOM for FFA. Means in columns for each treatments in intervals from 0 till 120 min are accepted as significant different at 95% level ($p<0.05$), values marked with same letters are not significantly different.