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Characterization of Cod (*Gadus morhua*) frame composition and its valorization by enzymatic hydrolysis

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Abstract

This study aimed at evaluating proximate composition, phosphorous content, amino acid (AA) and fatty acid (FA) profiles of cod (*Gadus morhua*) frame at five different sampling times (March 2017 to March 2018). Furthermore, the valorization possibility of cod frame by application of enzymatic hydrolysis was investigated using the samples from September 2017. In terms of protein content, this sample showed a significantly ($P<0.05$) higher level (16.5%) compared to the other samples, whereas lipid and phosphorous contents varied in a narrow range of 0.9-1.1% and 2.9-4.4%, respectively, ($P<0.05$). Furthermore, the total amino acids (AAs) content varied from about 98 to 155 mg/g in minced cod frame. Enzymatic hydrolysis of minced cod frame (MCF) and heated cod meat (HCM) was carried out by application of Alcalase and Neutrase, either individually or sequentially to obtain fish protein hydrolysate (FPH) and bone powder rich in phosphorus and calcium. The protein content of FPH varied from 76% to 84% and soluble-nitrogen in

trichloroacetic acid (SN-TCA) index varied from 30.6-40.3%, resulting in similar trends for yield and nitrogen recovery. Considerable amounts of phosphorus and calcium (330 and 583 g/kg, respectively) were recovered from the cod frame bones after enzymatic hydrolysis. This study showed that it is possible to produce bone powder rich in phosphorous and calcium as well as peptides from the cod frame. Thus, the cod frame side-stream can be converted from its current use as mink feed ingredient into higher value products for human consumption, without generating new waste products.

Keywords**:** cod frame composition, amino acid profile, free fatty acid, fish protein hydrolysate, minerals

Introduction

Globally, the direct share of human consumption and non-food products (mainly fish meal and fish oil) from marine catches and aquaculture productions is reported to be 151 and 20 million tonnes, respectively, in 2016 (FAO, 2018). More than 60% of the aquatic biomass end up as by-products, including the head, skin, trimmings, fins, frames, viscera and roe from seafood processing factories (Chalamaiah et al., 2012). Hence, there is a great potential for taking advantage of these apparent waste or low value materials, by turning them into actual authentic value added components such as lipids, chitin and chitosan, calcium, nucleic acids, pigments, and biologically active peptides. In cod fillet production, as much as 60% of the whole fish is by-products, the backbone yielding about 15% of the fish weight (Gildberg, 1993). Fish backbone is rich in minerals such as calcium and phosphorous (Toppe et al., 2007) and contains residual fish meat that was not removed during the filleting process.

Despite general awareness of health benefits of elements such as calcium and phosphorus, most societies still suffer from deficiencies in their diets (Soetan et al., 2010). Calcium deficiency is associated with osteoporosis, which affects 26 million lives annually (Melton, 1995). Dairy products are considered the major source of calcium. However, due to some consumption inhibitory factors like lactose intolerance, saturated fat and cholesterol alternative sources of calcium such as marine by-products have attracted serious attention. Recently, apart from protein isolation and peptide recovery, the utilization of organic components or minerals from the fish bone have been reported (Kim and Jung, 2006; Nemati et al., 2017; Terzioğlu et al., 2018). The most commonly reported recovery method of calcium and phosphorus from the fish backbone is boiling of fish frame with subsequent grinding to obtain a bone powder (Toppe et al., 2007). However, in other studies, alkaline solubilization has been combined with boiling as an efficient approach for fish bone powder recovery (Nemati et al., 2017).

In terms of protein/peptide recovery, application of enzymatic hydrolysis is considered as an environmental friendly treatment to produce a large and diversified range of products from fish side-streams compared to less efficient mechanical and chemical methods. Enzymes are highly versatile biocatalysts that have evolved to function under optimized conditions for the host organism. Consequently, different enzymes have different activity profiles spanning broad activity ranges, highly specific ranges, and even extreme ranges in terms of e.g. temperature and pH (Robinson, 2015). Therefore, application of enzymatic hydrolysis in the food industry is increasing, as it is possible to find enzymes with high proteolytic activity under the applied processing conditions (Oliveira et al., 2015). Protein rich fish by-products have been subject to different proteases for digestion of intact proteins and liberating potential bioactive peptides in the form of fish protein hydrolysate (FPH) (Guérard and Shahidi, 2007). While some proteases show

unselective specificity towards cleaving of peptide bond in a variety of substrates, others show high specificity for particular sequences, which influence the functional properties of generated peptides in different food systems. However, protease specificity is one side of the story; another side is the variation in substrate characteristics and its quality due to different variables such as species, sex, seasonal variation and catchment region. Apparently, physiochemical properties of fish muscle can be influenced by seasonal variation, which can subsequently affect its processing and functional properties (Ingolfsdottir et al., 1998). However, information on influence of different catchment periods on nutritional quality parameters such as amino acids, fatty acids profile and mineral content of cod frame is not available. Such information will give a better knowledge of the potential of using cod frame as an underutilized side-stream for various food applications. Furthermore, heat treatment to facilitate the separation of the meat remaining on the backbone could be an obstacle in recovery of functional proteins, due to protein denaturation. Therefore, enzymatic hydrolysis could be a practical approach for recovery of functional FPH after application of heat treatment on fish backbone. Thus, it would be necessary to evaluate the impact of heating of fish backbone on efficiency of enzymatic recovery and functionality of obtained FPH, as it has not been studied to any detail, previously.

The current study was performed to increase the knowledge of possible variation in nutritional composition of cod (*Gadus morhua*) frame from different catchment periods. The second aim was to examine a practical short-term hydrolysis process on cod frame using two commercial proteases, Alcalase (Alc) and Neutrase (Neut), either individually or sequentially, and to evaluate their efficiency in producing FPH powder and bone powder, rich in phosphor and calcium. Moreover, since the rigid structure of the cod frame was a major obstacle in the grinding process, this study also aimed at investigating whether heating of fish frames, for facilitating the meat separation

process, could affect the extraction efficiency and functionality of recovered FPH powder and the resulting calcium and phosphorus content in the bone powder.

Materials and Methods

Cod frames were received from Espersen A/S, (Klaipeda city-Lithuania) in batches of 7 kg at five different sampling times (March 2017, June 2017, September 2017, December 2017 and March 2018). The captured cods were processed (filleted) on-site and the frames (side-stream) were packed, transported to the National Food Institute (Kgs. Lyngby, Denmark) in frozen condition, and stored at -40°C upon arrival. Alcalase® 2.4 L FG (Alc; declared activity of 2.4 AU/g) and Neutrase® 0.8 L (Neut; declared activity 0.8 AU/g), both as endopeptidase with broad specificity and high activity in range of pH and temperature, were provided by Novozymes (Bagsværd, Denmark). All chemical reagents used for experiments were of analytical grade.

Preparation of cod frames for characterization

For each sampling point, the 7 kg block was thawed overnight in the fridge prior to the analysis. The frames were separated and one frame was selected and cut into smaller pieces. Three samples were made out of one frame. The small pieces were grinded into a homogenous mass using liquid nitrogen. The samples were stored at -40 °C until further analysis

Dry matter

Homogenized thawed cod frame sample (approx. 2 g) were dried for 20-24 h in an oven at 102-105°C. All analyses were carried out in triplicate samples (n=3). The dry matter [%] was calculated based on weight of wet and dry sample.

115

116 *Lipid extraction and determination of oil content*

117 The lipid content of cod frames was measured gravimetrically according to Bligh and Dyer method
118 with reduced amount of solvent (Bligh and Dyer, 1959; Iverson et al., 2001). For the extraction,
119 chloroform:methanol (1:1, v/v) was used by fixing the centrifuge tube containing 30 mL methanol
120 to its holder on the Ultra Turrax. Then 15 ml chloroform were added and mixed for 30 seconds
121 with a speed of 15000 rpm. Subsequently, another 15 ml chloroform were added and the solution
122 was mixed again for 30 seconds, followed by centrifugation at 1665 ×g and 18 °C for 10 minutes.
123 After centrifugation, the methanol:water phase (upper phase) was removed and extract
124 (chloroform phase) was filtered down into a 50 ml Pyrex bottle. Extracts (10 g) were left overnight
125 at room temperature, dried (oven 1-2 h, 105 °C) and weighed. All analyses were carried out in
126 triplicate samples (with two analytical replicates; n=3×2).

127

128 *Fatty acid composition*

129 The lipid extract was used to determine the fatty acid composition. Lipid extract was evaporated
130 to dryness under nitrogen and re-dissolved in internal standard (100 µL, C23:0 in heptane), 200
131 µL heptane with BHT and 100 µL toluene. Boron trifluoride reagent (20%) was added for lipid
132 transesterification in a one-step procedure using a microwave (Multiwave 3000 SOLV, Rotor:
133 64MG5, Anton Paar, Graz, Austria). Samples were heated for 5 min at 500 W and cooled down
134 for 10 min. Then samples were mixed with 1 mL saturated NaCl solution and 0.7 mL heptane. The
135 top layer was used for fatty acid composition analysis by gas chromatography with flame
136 ionization detection (GC-FID; Column: DB-wax column (10 m×ID 0.1 mm × 0.1 µm film
137 thickness, J&W Scientific, Folsom, CA)) according to AOCS official Method Ce 1b-89. Results

were reported as percentages of total fatty acids. All analyses were carried out in triplicate samples (with two analytical replicate; n=3×2).

Protein content

The protein content was measured based on total nitrogen content analyzed by Dumas (Rapid MAX N exceed cube N/protein analyzer, Elementar Analysensysteme GmbH, Germany). Depending on the sample type, 250 mg-500 mg of sample was used for the determination. Crude protein was estimated in all samples by multiplying the total nitrogen content (%) by a factor of 6.25. All analyses were carried out in triplicate samples (with two analytical replicates; n=3×2).

Amino acid composition

The amino acid composition was determined by HPLC-MS, following hydrolysis and derivatization using EZ:faast amino acid kit (Phenomenex, Torrance, CA, USA) (Ghelichi et al., 2017). The acid hydrolysis was applied in order to release the amino acid using 6 M HCl at 110 °C for 18 h in oven. The subsequent neutralized samples were purified by a solid-phase extraction sorbent tip and derivatization was performed following the injection of sample aliquots into an Agilent HPLC 1100 instrument (Santa Clara, CA, USA) coupled to an Agilent ion trap mass spectrometer. The amino acids were identified by comparing retention time and mass spectra of an external standard mixture. Calibration curves were prepared and analyzed by HPLC-MS for quantification. All analyses were carried out in triplicate samples (with two analytical replicate; n=3×2).

Phosphorous content measured by spectrophotometry

161 Before measuring the phosphorous content in the cod frames, the samples were burnt in an oven
162 (30 min at 200 °C, 30 min at 300 °C, 1h at 400 °C and 12h at 600 °C) and the ashes were used for
163 determination of phosphorous. Ash samples were weighed and sulfuric acid (5.6 M) and
164 ammonium molybdate (2.5%) were added for reaction. Under acidic conditions, phosphate is
165 converted to phosphomolybdic acid. Under reduction (stannous chloride, 2.5%) a blue colored
166 complex was formed. The intensity of the complex was measured spectrophotometrically at 690
167 nm after 15-25 min and quantified using a standard curve prepared from a phosphate standard
168 solution (Sodium dihydrogen phosphate dodecahydrate, 1.05 mM containing 0.1 mg PO_4^{3-}/mL or
169 0.033 mg P/mL). All analyses were carried out in triplicate samples (with two analytical replicates;
170 n=3×2).
171
172 **Preparation of cod frame hydrolysates**
173 Following Liaset et al. (2000), proteolysis variables such as temperature, pH, E/S ratio and time
174 related to application of Neut and Alc enzymes were chosen. Cod frames (Sep. 2017) were cut into
175 smaller parts and divided in two batches; minced cod frame (MCF) which was chopped in a blender
176 (Waring blender, model 32BL80, USA) by aid of liquid nitrogen, and heated cod meat (HCM).
177 The HCM fraction was obtained by boiling of cod frame at 95°C in a water bath for 20 min with
178 subsequent separation of heated meat from the bones fraction. The samples were mixed with 0.1
179 M sodium phosphate buffer at pH 7.4 in ratio of 1:1 (w:v) and homogenized for 3 minutes. In
180 individual process, enzymes were added into the sample solution at E/S ratio of 1.5% (based on
181 sample protein content as determined by Dumas) with subsequent shaking (80 rpm) in water bath
182 at 50°C for 3h. In the sequential process, sample solution was subjected to Neut for 3 h and then
183 Alc was added and incubated for the next 3h. The hydrolysis was terminated by heating the

solution at 95°C for 20 min (Guerard et al., 2002). The hydrolysates were then centrifuged at 6700 ×g for 20 min (Merck, Beta 1-8, Martin Christ® GmbH, Germany). The supernatant was collected and freeze-dried and grinded into a fine powder, manually, and stored at 4°C in dark bottles for further experiments. The process is illustrated in Fig. 1.

SN-TCA index (%)

The soluble nitrogen content as an indicator of hydrolysis efficiency was calculated by application of trichloroacetic acid (TCA) method as described by Hoyle & Merritt (1994). Accordingly, 20% TCA (w/v) solution was prepared and 20 mL of collected supernatant was added to 20 mL of TCA (10% final concentration). The mixture was centrifuged at 7800 ×g for 15 min. The supernatant (soluble protein) was then analyzed for nitrogen content using Dumas instrument (n = 2).

$$SN - TCA\ index = \frac{(\%)\ SN\ in\ 10\%\ TCA\ supernatant}{(\%)\ TN\ in\ raw\ material} \times 100$$

Where *SN* is soluble nitrogen, *TN* is total nitrogen in cod frame meat

Nitrogen recovery

Nitrogen recovery (NR) in the soluble fraction was calculated using the following formula:

$$NR\ (\%) = \frac{N\ (\%) in\ FDHP \times its\ weight\ (g)}{TN\ (\%) in\ raw\ material\ \times its\ weight\ (g)} \times 100$$

Where *N* is nitrogen percentage, *FDHP* is freeze-dried hydrolysate powder and *TN* is total nitrogen in cod backbone meat. Nitrogen was determined by the Dumas method as described above.

Yield

The yield was calculated as percentage ratio of weight of the initial substrate (cod frame) in gram to the weight of the hydrolysates obtained in gram.

$$Yield\ (\%) = \frac{FDHP\ weight\ \ (g)}{initial\ weight\ of\ raw\ material\ (g)} \times 100$$

Where *FDHP* is freeze-dried hydrolysate powder.

FPH Solubility

In order to measure the FPH powder relative solubility, 200 mg of powder were dispersed in 20 mL of 0.1 M sodium phosphate buffer (pH 7.4) and mixed thoroughly (stirring for 10 sec.), then mixtures were left at room temperature while shaking (80 rpm) for 30 minutes and finally centrifuged at 7500 ×g for 15 minutes. The protein/peptide content of each supernatant was determined using the Dumas instrument (Brinton et al., 2005). Protein/peptide solubility was calculated as;

$$Solubility\ (\%) = \frac{Protein/peptide\ content\ in\ supernatant}{Total\ protein/peptide\ content\ in\ FPH} \times 100$$

Water holding capacity (WHC)

Following the method of Diniz and Martin (1997) with some modification, the WHC was determined by adding 100 mg FPH powder in 1000 µl of distilled water followed by mixing with a magnet stirrer. The protein suspension was then centrifuged at 1800 ×g for 20 min at 22 °C. The supernatant was decanted, and the tube drained at 45° angle for 10 min. WHC, as mL of water absorbed per g of FPH, was calculated based on the difference between initial volumes of distilled water added to the protein sample and the volume retrieved.

227

228 **Oil absorption capacity (OAC)**

229 For measuring OAC parameter, an emulsion was prepared by dispersing 100 mg of FPH sample
230 in 1000 µl of rapeseed oil for 30 sec following Foh et al., (2010) with slight modification by
231 increasing the protein concentration to 10% instead of 5%. The resulting emulsion was incubated
232 at room temperature for 30 minutes, and then centrifuged at 13600 ×g for 10 minutes at 25 °C,
233 while Foh et al., (2010) used 2800 ×g for 25 min without mentioning the temperature. The
234 supernatant decanted and drained at 45° angle for 15 minutes to determine the volume of absorbed
235 oil. OAC was calculated in the same way as WHC.

236

237 **Bulk Density**

238 Bulk density of freeze-dried cod frame FPH was measured following Foh et al. (2010) with slight
239 modification. Approximately 5 g of each sample were packed into 50 mL graduated cylinders by
240 gently tapping on the lab bench 10 times. The volume was recorded and bulk density was reported
241 as g/mL of the sample.

242

243 **Color parameters**

244 The color of the FPH powders was evaluated using the Hunter Lab Miniscan XE colorimeter
245 (Reston, Virginia, USA). The CIE $L^*a^*b^*$ color parameters were used: L^*; indicating lightness
246 from black (0) to white (100); a^*; indicating redness from green (- 120/ negative values) to red
247 (+120 / positive values); and b^*; indicating yellowness going from blue (-120 / negative values)
248 to yellow (+120 / positive values) (Hashemi and Jafarpour, 2016). Whiteness was calculated as:

249 $$Whiteness = 100 - \sqrt[2]{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

Phosphorus and calcium content of peptides and bone fractions

Analysis of calcium and phosphorus in the bone powder was done using ICP-MS (iCAPq, Thermo-Fischer, Germany) in KED mode (helium as cell gas) following digestion of the samples with concentrated nitric acid (SPS Science, France) in a microwave oven (Multiwave 3000). Quantification was done using external calibration with standards made from certified stock solutions of calcium and phosphorus (SPS Science, France) and using rhodium as internal standard (SPS Science, France). A certified reference material DORM-4 (NRCC, Canada) was analyzed together with the samples and the obtained values were in good agreement with the reference values.

Amino acid profile of protein hydrolysates by middle-down proteomics

Freeze-dried protein hydrolysates were prepared using a sodium deoxycholate (SDC) in-solution digestions protocol according to Zhou et al. (2015), without applying the tryptic digestion step. Alkylated peptides were purified using C-18 StageTips (Rappsilber et al., 2007; Yanbao Yu et al., 2014), dried down, and re-suspended in 0.1% (v/v) trifluoroacetic acid, 2% acetonitrile (v/v) for analysis. Peptide analysis was performed using an automated LC–ESI–MS/MS, consisting of an EASY-nLC system (Thermo Scientific) on-line coupled to a Q Exactive HF mass spectrometer (Thermo Scientific,) equipped with a Nanospray Flex ion source (Thermo Scientific). Peptides were loaded onto a reverse phase (RP) Acclaim Pepmap Nanotrap column (C18, 100 Å, 100 μm. × 2 cm, nanoViper fittings (Thermo Scientific)) followed by separation on a RP Acclaim Pepmap RSLC analytical column (C18, 100 Å, 75 μm. × 50 cm, nanoViper fittings (Thermo Scientific) as described in (García-Moreno et al., 2020).

Proteomics analysis was performed using MaxQuant 1.6.0.16 (Cox and Mann, 2008; Tyanova et al., 2016) using a reference proteome constructed by combining the two available GenBank (Benson et al., 2017; Sayers et al., 2019) assemblies (as of December 10th, 2018) for *Gadus morhua* (GCA_000231765.1 and GCA_900302565.1) (Star et al., 2013) and removing redundant entries by applying a 90% identity cutoff. The analysis was performed as unspecific digestion with peptide length from 3 to 65 AAs and applying a false discovery rate of 1% on both peptide and protein level.

The sample-level molar amino acid frequency f_{AA}^{sample} for each AA was approximated using the peptide-level data output using an MS1 intensity-weighted normalization of the peptide level for each amino acid and summed up over all identified peptides after filtration of reverse hits and potential contaminant peptides according to:

$$f_{AA}^{sample} = \sum_{pep=1}^{n} f_{AA}^{p} * I_{rel}^{p}$$

Where f_{AA}^{p} is the integral frequency of a given AA in peptide *p* and I_{rel}^{p} is the MS1 intensity of peptide *p* divided by the sum of intensities for all *n* peptides. To approximate the amount of each AA per 100g of samples (i.e. soluble peptides), the sample-level molar AA frequency was multiplied with the MW of the AA (subtracted for water cleaved during peptide bond formation), divided by the weighted average AA MW (110 g/mol) (Kim et al., 2018) and multiplied by 100.

Statistical analysis

293 The current experiment performed in a completely randomized design test and obtained data were
294 analyzed by one-way ANOVA in SPSS (v. 16.0). Multiple comparison among means was
295 calculated in Tukey as a *post hoc* test, while setting the confidence level at 95%.
296
297 **Results and Discussion**
298 ***Characterization of cod frames at different sampling months***
299 Table 1 shows proximate composition of cod frame along with its phosphorus contents as a main
300 mineral component from Mar. 2017 to Mar. 2018 with 3 months sampling intervals. Accordingly,
301 the protein content of cod frame varied from 13% to 17%. No significant difference ($P>0.05$) was
302 observed with the exception of the sample from Sep. 2017 ($P<0.05$), which had the highest protein
303 content. On average, these values are in line with study conducted by Gildberg et al. (2002), who
304 reported the protein content of cod frame as 16%. Generally, it is expected that a higher protein
305 content will be obtained for fish caught during the summer because the higher temperature will
306 cause blooming in late summer of free-floating algae, specifically cyanobacteria (Groetsch et al.,
307 2016). Hence, more food sources are available for nourishing the fish during the summer season.
308 However, in the current study, higher protein content in sample Sep. 2017 might be related to
309 inefficiency of fillet trimming machine, which could result in a higher proportion of meat
310 remaining on the bone in the batch received in this sampling month. The support for this claim is
311 that the ash and phosphorous content do not correlate with the protein content in sample Sep. 2017;
312 otherwise, these values should be higher than those recorded for sample Sep. 2017. The low protein
313 content of cod frame from Mar. 2017 to Jun. 2017 is in line with the study reported by Ingolfsdottir
314 et al. (1998), who attributed the lowest protein content in North Atlantic cod muscle to the period
315 of spawning of cod during March to May.

316 In terms of phosphorus (P) content, the sample from Sep. 2017 contained the lowest amount of
317 2.2%, while the highest amount was determined in Mar. 2018 followed by Dec. 2017 (4.4% and
318 3.7%, respectively) ($P<0.05$). This trend coincides well with the lower content of other fractions
319 such as protein and lipid. This can result in higher percentage of P in calculated proximate
320 composition of cod frame. However, the P content of cod frame in our study is not in agreement
321 with those reported by Toppe et al. (2007) and Malde et al. (2010) as 113 g/kg and 180 g/kg,
322 respectively. The difference in reported P values is mainly due to the reason that in the current
323 study, the P content (Table 1) was analyzed on the ash fraction and calculated based on the whole
324 cod frame. In contrast, by measuring the P content based on the dried bone powder (Table 8) its
325 value was 170 g/kg, which is in line with that reported by Malde et al. (2010). Cod is classified as
326 a lean fish (with lipid content of lower that 2%) (Zeng et al., 2010), and the lipid content of samples
327 in our study recorded a low value of around 1% during different sampling months. However, the
328 slight changes in lipid content of cod frame in our study showed no correlation with the typical
329 lipid content of fish muscle during spawning and feeding seasons. The lipid content of the cod
330 frame (0.98 % to 1.13 %) was higher than those obtained by Gildberg et al. (2002) and Zeng et al.
331 (2010), who reported the lipid content of cod backbone and cod flesh as 0.3% and 0.8%,
332 respectively. Furthermore, Ingolfsdottir et al. (1998) reported that the values for the fat content of
333 North Atlantic cod muscle varied from less than 0.15% in the autumn to higher than 0.35% in the
334 late spring. On the other hand, according to Toppe et al. (2007), the lipid content of cod backbone
335 was reported as 2.3%, which is significantly higher than those recorded in our study and the study
336 conducted by Ingolfsdottir et al. (1998). Obviously, the lipid content of cod frame in our study is
337 surprisingly high as it stems from residual cod meat on the frame. Most of the frame must be bone,
338 so if the lipid content in the frame is higher than what was reported by others in pure cod muscle,

339 then it means that the meat left on the cod bone investigated in our study contains more lipid than
340 the cod filet itself.
341 In case of dry matter, the highest content was determined in the samples from Dec. 2017 and Mar.
342 2018 (about 22%) and the lowest amount in the sample of Jun. 2017 (19.4%, $P<0.05$). As
343 aforementioned, the different content of dry matters may be related to the exact ratio between meat
344 and bone and not necessarily be directly related to seasonal variation. Therefore, the fluctuations
345 in proximate composition of cod frame in our study cannot be attributed to the feasting (March-
346 June) and fasting (September-December) seasons. In other words, the highest protein and lipid
347 content and lowest dry matter and phosphorus content were recorded for fish caught in Sep. 2017
348 (fasting season), whereas, protein and lipid content showed a declining trend during feasting
349 season. However, for proper interpretation of seasonal variation in proximate composition of cod
350 frame, it would be necessary to consider real seasonality versus differences in filleting efficiency,
351 which may in fact be influenced by seasonal changes in cod meat texture and functional properties
352 (Malcolm Love, 1979). However, to unveil the true influence of seasonality on the proximate
353 composition, the amino acids and fatty acid profile of samples during different seasons should be
354 analyzed, which will be discussed in following sections. Consequently, based on the type of
355 application, i.e. targeting mainly protein or phosphorus, cod frame can be selected from different
356 seasons as a valuable source for valorization process which creates new high value ingredients
357 preferably without generating new wastes.
358
359 ***Amino acid profile of cod frame in different seasons***
360 Amino acids are considered as precursors for synthesis of a broad range of biologically important
361 substances including nucleotides, peptide hormones, and neurotransmitters. Total amino acid

362 content of cod frame is presented in Table 2, and is divided into two sections: essential amino acids
363 (EAA) and non-essential amino acids (NEAA). A significant seasonal variation is observed among
364 the AAs with different patterns. For instance, there is a sinusoidal pattern in the content of Thr,
365 Val, Ser, Gly, Ala, Asp and Glu with an increase in the first half of year (Mar. to Sep. 2017)
366 followed by a decline up to the end of year (Dec. 2017) and again a rise in Mar. 2018. Whereas,
367 for Met, Phe, His, Tyr and C-C a slight fluctuation was recorded during different sampling months.
368 On the other hand, Ile and Arg presented a sharp increase during spring (Mar.-Jun. 2017) followed
369 by a sharp decline during the rest of the sampling months and nearly the same pattern was observed
370 for Leu. The highest content of total AAs (155.83 mg/g) was found in the sample from Sep. 2017,
371 while the lowest content of 97.93 mg/g was recorded for sample Mar. 2017. This corresponds well
372 with the protein content of cod frame determined by proximate composition analysis. With the
373 exception of the cod frame sample from Mar. 2017, which showed nearly equal amount of EAAs
374 and NEAAs, the content of EAAs was remarkably lower than the NEAA content. The EAA
375 occurring in the highest amount was Val with its highest values in samples Jun. and Sep. 2017
376 (15.02 and 15.42 mg/g, respectively) while the EAA which occurred in lowest amounts (2.16-
377 3.32 mg/g) was His. In case of NEAAs, Glu had the highest abundance (14-22.12 mg/g), while the
378 C-C content was lower than 1.0 mg/g in all samples.
379 Toppe et al. (2007) reported that AAs profile of cod bone is rich in Gly>Glu>Arg>Pro>Asn, and
380 poor in Trp<His<Tyr<Ile<Phe, which was relatively in agreement with our results. Thus, cod
381 frame in our study contained large amounts of Glu>Gly>Val followed by nearly same amounts of
382 Ala, Asp, Pro, Leu and Lys, with their peak values in sample Sep. 2017. The possible explanation
383 for the difference in the content of some AAs in these two studies might be due to the nature of
384 analyzed sample. Toppe et al. (2007) used a farmed codfish, while in our study the frame of wild

385 codfish was examined. Furthermore, it is noteworthy that regarding the Glu and Asp content in
386 our study, they are determined as sum components of Glu+Gln and Asp+Asn due to conversion
387 during hydrolysis prior to analysis. Higher amounts of Gly, Ala and Pro compared to other NEAA
388 content of cod frame, especially in sample Sep. 2017, could indicate high amounts of collagenous
389 peptides in the raw material (de Paz-Lugo et al., 2018).
390
391 **Fatty acid (FA) profile**
392 It is generally accepted that highly unsaturated n-3 fatty acids (n-3 HUFA) or long-chain n-3
393 polyunsaturated fatty acids (LC n-3 PUFA), particularly 20:5 n-3 (eicosapentaenoic acid [EPA])
394 and 22:6 n-3 (docosahexaenoic acid [DHA]) positively affect human health such as early
395 development, and the prevention of some diseases (Vonder Haar et al., 2016). According to the
396 FA profile of cod frame (Table 3), the most abundant FAs was determined as DHA (23.0-24.9%),
397 followed by palmitic acid (C16:0, 13.5–17.5%), EPA (11.5–15.6%) and oleic acid (C18:1n9, 11.1–
398 11.8%). FAs with lower frequency included eicosenoic acid, stearic acid isomer of oleic acid,
399 lignoceric, palmitoleic acid, dihomo-gamma-linolenic acid and cetoleic acid, while the rest were
400 ≤1.0% at all sampling points.
401 These results do not coincide with Toppe et al. (2007), where the most abundant FAs of farmed
402 cod backbone was reported to be oleic acid followed by palmitic acid, DHA and EPA. In another
403 study conducted on farmed Atlantic cod fillet by Zeng et al. (2010), the authors reported DHA as
404 the most abundant FA (31.0%), and a total of 48,8% of n-3 PUFAs in the total lipid of cod muscle.
405 The same trend but lower content was observed in our study, most possibly due to the differences
406 in the raw material used, i.e, cod muscle in Zeng et al. (2010) study, versus cod frame with
407 remaining meat in our study. Furthermore, all analyzed samples contained higher amounts of n-3

PUFAs (ranging from 37.66 to 40.64%) than n-6 PUFAs (ranging from 2.5 to 2.8%) (Table 3). Toppe et al. (2007) reported these values as 12.6% and 1.9% for n-3 PUFAs and n-6 PUFAs, respectively. In our study, apart from a slight increase in n-3 PUFAs, starting from Mar. 2017 and reaching to its highest value in Dec. 2017, no profound seasonal variation was observed on PUFAs profile.

Referring to the proximate composition of cod frame with emphasis on protein and phosphorus content and also with respect to high levels of EAAs and NEAAs in different months, cod frame could be considered as a valuable source for further valorization experiments in order to recover bioactive compounds such as peptides, calcium and phosphorous without producing more waste. In accordance with the aforementioned statement, this study evaluated the release of potentially bioactive peptides by enzymatic hydrolysis and recovery of calcium and phosphorous from the remaining bones as added-value components.

Valorization of cod frames

SN-TCA index

For a given substrate and enzyme at a given pH, the comparison of different hydrolysates is based on the degree of hydrolysis (DH) as it was measured in terms of SN-TCA in our study. DH depends on the experimental variables such as the type of enzyme, substrate and applied conditions (e.g. time, temperature, pH, E/S ratio,) (Williams, 2004). As shown in Table 4, application of Alc and Neut, either separately or sequentially on two types of fish substrate, resulted in 30-40% SN-TCA index. Alc was more efficient compared to Neut on both MCF or HCM, whereas, Neut and Alc did not show any substrate preference, as the resulting SN-TCA index was not significantly

different between these two substrates ($p>0.05$). However, sequential application of Neut&Alc to the MCF caused significantly higher SN-TCA index compared to HCM ($p<0.05$).

In our study, the main experimental variables were the enzyme and substrate types, which affected the dependent variables differently. In addition, applied enzymes were calculated based on the same concentration (1.5% w/w). Thus, they cannot be directly compared as Alc has a specified activity three times higher than Neut (2.4 AU/g vs. 0.8 AU/g), i.e, to make this comparison, three times more Neut should have been added. Therefore, we cannot claim that application of Alc on the cod backbone meat was more efficient compared to Neut treatment. Nevertheless, the higher efficiency of Alc compared to Neut was reported by Gildberg et al. (2002). These authors stipulated that the lower efficiency of Neut could be related to higher susceptibility to the inhibitory activity of protease inhibitors found in cod meat. Therefore, it is expected that application of preheating treatment on the substrate before enzymatic hydrolysis, could enhance the efficiency of Neu treatment. However, this is not in agreement with results of our study as Neut still showed low efficiency in case of HCM (including pretreatment at 95°C for 20 min) (further investigation needed). On the other hand, Liaset et al. (2000) reported higher DH in case of Neut (23%) compared to pepsin (15%) and Alc (20%) on salmon and cod frame, but these authors applied Neut enzyme with E/S ratio of 30 AU/kg protein versus 3.6 AU/kg protein in our study.

When running the enzymatic hydrolysis process, it is also important to figure out the amount of recovered protein and nitrogen after enzymatic hydrolysis. It is noticeable that despite the higher yield obtained with the MCF treatment (Table 4), its protein content was significantly lower than HCM ($p<0.05$) most probably due to the difference in substrate composition as MCF contains fish bones while HCM is only comprised of fish meat. However, in terms of nitrogen recovery percentage (NR%), individual application of Alc and Neut resulted in significantly higher NR%

453 in MCF compared to HCM (P<0.05), whereas subjecting MCF and HCM to sequential application
454 of Netu&Alc caused no significant difference in NR% (P>0.05). In terms of yield percentage the
455 same trend as for NR% was observed, i.e highest yield was obtained by application of Alc either
456 individually or along with Neut on both MCF and HCM substrate (P<0.05), mainly due to its
457 higher applied AU/g compared to Neut in our study.
458
459 *Color parameters of FPH powder*
460 Color parameters of recovered hydrolysate powder from cod frame is presented in Table 5. In
461 terms of substrate type, peptide powder from enzymatic hydrolysis of HCM resulted in higher *L**
462 value, but lower *a** and *b** values compared to MCF substrate, which resulted in whiter recovered
463 FPH powder from HCM (P<0.05). Among all examined treatments, Alc showed highest ability to
464 result in lighter and consequently whiter powder from HCM substrate. By comparing the MCF
465 data, even though, the Neut treatments caused higher *L** values, the whiteness of the obtained
466 powder was significantly lower compared to the rest of treatments (P<0.05). This is mainly due to
467 its higher redness and yellowness values, which are negatively correlated to the whiteness index.
468 In a study conducted by Šližyte et al. (2009) on cod backbone, color parameters of recovered FPH
469 powder were recorded as *L**= 87.8, *a**= -0.7, and *b**=17.5. In comparison, the trends in color
470 values in our study is generally in line with values reported by Šližytė et al. (2009) as nearly the
471 same yellowness, higher lightness and lower redness was recorded for recovered FPH from cod
472 frame. This will positively affect its customer popularity and market acceptability. Apart from the
473 broad specificity of Alc and Neut, the differences in color values can be attributed to the difference
474 in cleavage site of enzymes in peptide chain, which could contribute differently to the color of the
475 obtained peptide powder. Furthermore, apart from the enzyme type, several other parameters such

476 as differences in color measuring instruments, E/S ratio and types, enzyme concentration etc.,
477 could influence the color of obtained FPH powder.
478
479 *FPH Solubility and water holding capacity (WHC)*
480 The solubility of the FPH powders at a certain concentration (1% w/v) are shown in Table 6, and
481 ranged from 93% to 100% which is in agreement with other studies (Klompong, Benjakul,
482 Kantachote, & Shahidi, 2007; Nalinanon, Benjakul, Kishimura, & Shahidi, 2011; Pacheco-Aguilar
483 et al., 2008; Taheri et al., 2013). In comparison, administration of Alc on both MCF and HCM and
484 also Neut&Alc treatment on HCM resulted in significantly higher solubility ($P<0.05$) than the
485 other treatments, while lowest solubility was determined for the hydrolysate from Neut treatment
486 on MCF ($P<0.05$), followed by Neut&Alc on MCF and Neut on HCM.
487 Protein solubility to a large extent depends on pH values, showing its lowest solubility close to the
488 isoelectric point (pI) (Chobert et al., 1988; Linder et al., 1996). The pH influences the charge on
489 the weakly acidic and basic side-chain groups with subsequent effects on the protein solubility
490 (Gbogouri et al., 2004) which is proportional to the square of the net charge on the protein (Shaw
491 et al., 2001). Improved solubility of the hydrolyzed protein compared to its original form can be
492 attributed to the degradation of the proteins leading to increased repulsive interactions between
493 peptides and a subsequent increase in hydrogen bonding with water molecules (Souissi et al.,
494 2007). The hydrolysis degrades proteins to peptides, which are generally more soluble. However,
495 the increased solubility of the peptides is largely caused by the fact that peptides hydrophobic
496 domains is lower compared to the intact protein molecule.
497 Due to the high solubility of the hydrolysates, the WHC of obtained FPH powders was close to
498 zero. In contrast to our results, Taheri et al. (2012) reported high WHC (5.1 mL/g) for fish

hydrolysate powder recovered from rainbow trout viscera using Alc, which possibly is related to the higher DH in their study compared to ours. According to Kristinsson and Rasco (2000), the increased concentration of polar groups such as COOH and NH_2 that is caused by extensive enzymatic hydrolysis has a substantial positive effect on the amount of adsorbed water. Apart from different substrate types, the differences in reported studies can possibly be explained by the number and ratio of polar and nonpolar groups (hydrophilic and hydrophobic amino acids), and the amino acid composition of the FPH recovered with each enzyme. According to Trevino et al. (2007), Asp, Glu, and Ser contribute more favorably to protein solubility than the other hydrophilic amino acids especially at high net charge. In our study, the inability of obtained FPH to imbibe water molecules can be possibly attributed to the dominant portion of released hydrophilic amino acid residues with low net charge, which results in weak hydrogen bonds that could not retain it against the gravitational force in the protein matrix. In addition, the common practice in protease hydrolysis in aqueous solution is to collect the supernatant after enzymatic process by centrifugation, which cause a major loss of hydrophobic amino acids that goes to the precipitated sediment. Consequently, peptides are more prone to hydration compared to undenatured protein. However, depending on the type of FPH application, both solubility and WHC are considered as important FPH functionality parameters as these can influence other functionalities such as emulsification and foaming properties.

Oil absorption capacity (OAC) and bulk density (BD)

The highest OAC was obtained for hydrolysates produced by Alc treatment of both MCF and HCM substrates ($P<0.05$), while the lowest values were related to the individual application of Neut and sequential application of Neut&Alc on either MCF or HCM ($P>0.05$) (Table 6). It is

522 believed that OAC is mainly dependent on physical entrapment of oil, which is directly related to
523 the hydrophobic surface of hydrolyzed proteins (Kristinson & Roscoe, 2000), protein mass density,
524 and their amino acid composition.
525 BD represents the physical property of a product, which influence the packaging requirements of
526 a product. Therefore, to obtain higher BD, higher degree of hydrolysis is required, which in turn
527 lower the molecular size of resulting peptides (may also drastically change the biological, physical
528 and chemical properties of the hydrolysate) and consequently a finer powder with less porosity
529 would be obtained.
530 In terms of BD values, there was a significant difference ($P<0.05$) among the various samples
531 studied (Table 6). HCM hydrolyzed sequentially with Neut&Alc showed significantly higher BD
532 of 0.40 g/mL followed by MCF sample (0.35 g/mL) ($P<0.05$). However, HCM hydrolyzed with
533 Neut and Alc separately, showed the lowest values of 0.19 g/mL and 0.25 g/mL, compared to MCF
534 samples with BD values of 0.31 and 0.28 g/mL, respectively ($P<0.05$). Considering the fact that
535 MCF samples contained bone, this can likely explain the difference as these treatments may get
536 different collagen derived peptides along with a likely higher content of minerals, which contribute
537 to a higher density of the sample as a whole. Foh et al. (2010) studied the functionality of FPH
538 from hydrolyzed Tilapia (*Oreochromis niloticus*) and reported that BD of resulting peptide powder
539 from Neut heat treated fish mince was significantly lower compared to Alc treatment which is in
540 accordance with the current study.
541
542 *Amino acid profile of FPH by middle down proteomics*
543 The AA profile of cod frame protein hydrolysates (g/100 g of soluble peptide) is presented in Table
544 7. It should be noted that the AA profile is an approximation by means of peptide-level MS1

intensity weighing, and that peptide MS1 intensities are not in all cases proportional to peptide abundance due to variability in peptide physiochemical properties. Furthermore, the AA profile presented here only takes into account AAs found in (potentially bioactive) peptides (3-65 AAs) released by enzymatic hydrolysis and does not take into account free AAs nor larger protein fragments. Consequently, it is challenging to compare directly to other studies, where it has been common to analyze either free AA or total AA profiles, where both free AAs and total AAs from non-hydrolyzed proteins are included. Although analysis of total AA content provides valuable insight into e.g. nutritional value of a substrate, our approach provides a novel and alternative way of characterizing protein hydrolysates in terms of peptide AA composition.

As seen in Table 7, apart from the sequential application of Neut&Alc on HCM, all other treatments resulted in release of peptides with higher content of EAAs from both MCF and HCM substrates compared to the original Sep. 2017 sample. The most abundant AAs in the identified peptides were Glu, Leu, Asp, Pro and Phe, in all treatments. The highest content of the EAAs were Leu, Phe and Val and was found in peptides released from the cod frame by the sequential application of Neut&Alc on both MCF and HCM, while application of Neut was responsible for significantly higher content of Thr, Lys and Gln. In case of NEAAs, the highest content of Gly and Pro was found in peptides released by Alc, while hydrolysis by Neut resulted in release of peptides richest in Ala and Glu for both MCF and HCM. Interestingly, the content of Lys, Gln, Glu, and Cys was over two-fold higher in hydrolysates only treated with Neut compared to other treatments. This trend was also seen to a lower extent for Thr and Asn. This could indicate that treatment with Alc may be responsible for release of these AAs in free form to a much higher extent or those peptides rich in these AAs are to a larger extent hydrophobic and hence not found in the FPH. Furthermore, the peptides obtained using sequential hydrolysis with Neut&Alc had a

568 significantly lower content of His and Arg, indicating that this treatment may result in increased
569 release of free positively charged AAs. The increased release of charged AAs in free form, could
570 contribute to an increased antioxidant activity of the peptides obtained with sequential Neut&Alc
571 treatment, as these AAs are reported to be positive contributors to peptide antioxidant activity
572 compared to other AAs (Udenigwe and Aluko, 2011). The lower content of Cys in the sequential
573 hydrolysate peptides may impair antioxidant activity; however, as free Cys is known to have
574 significant antioxidant activity by itself due to the high redox potential of the thiol group, the total
575 antioxidant potential of the sequential hydrolysate may be significant. This aspect is currently
576 under investigation.
577 As the obtained hydrolysate powder in the current study was prepared from the Sep. 2017 sample,
578 it is reasonable to compare the AAs profile of these two samples (Table 7). Accordingly, the most
579 abundant EAAs in the intact proteins from the cod frame were Val, Lys, Leu and Ile, whereas, the
580 most abundant AA in released peptides by different enzymatic treatments were Leu, Phe and Val.
581 The relatively low content of His in the hydrolysates corresponded well with the analysis of the
582 cod frame from Sep. 2017 sample. Nevertheless, the hydrolysate peptides had, in general, a higher
583 content of EAAs compared to the total AA analysis of Sep. 2017 sample (Table 7), indicating that
584 the majority of EAAs are found in peptides and not free AA form. In terms of NEAAs, total AA
585 analysis revealed, by far, the highest abundance of Glu and Gly (14.2 and 11.2 g/100g). The high
586 content of Glu was also seen in the hydrolysate peptides (11.9 g/100g soluble peptide on average),
587 while the Gly content in the peptides was somewhat more moderate (5.31 g/100g soluble peptide
588 on average), indicating that Gly is released to a high extent as free AA in the hydrolysates or found
589 in insoluble peptides. By direct comparison, total AA analysis of Sep. 2017 sample appear to have
590 a general enrichment of Gly and Ala while the peptides from hydrolysis are slightly enriched in

591 Pro, Asp, and Tyr. Moreover, Cys (in the disulfide form (C-C)) was not detected in the cod frame
592 total AA analysis and only found in very low amount in the hydrolysate (0.11 g/100g soluble
593 peptide on average). Hyp was not included in the proteomics analysis of hydrolysates, while Asn
594 was not included in the AA analysis of the cod frame.
595 In this study, the most abundant EAA in hydrolysate peptides was Leu (11.7 g/100g soluble peptide
596 on average), while the EAA with lowest abundance was His (1.14 g/100g soluble peptides on
597 average). This corresponds well with the findings of Tan et al. (2018) who studied the nutritional
598 properties of enzymatic hydrolysates of cod bone proteins. According to these authors, the most
599 abundant AAs were as Glu, Gly, Lys, Arg, Ala, Thr and Leu, which is largely in agreement with
600 the cod frame AA profile. In our study, in case of NEAAs, highest abundance was observed for
601 Glu with 11.9 g/100 g, followed by Asp and Pro. According to Cao et al. (2008), Lys and Arg are
602 connected with the healthy function of FPH and in our study, these AAs were found in highest
603 abundances (4.27 to 7.85 g/100g soluble peptide) in Neut-driven FPH, which is good indication
604 of health benefits of prepared FPH from cod frame using Neut hydrolysis.
605 Sabeena Farvin et al. (2016) stated that the predominant amino acids in industrially prepared cod
606 hydrolysate were Glu (14.5%), Gly (13.4%), Lys (8.4%) and Ala (7.7%) of the total amino acids
607 content. However, as it was mentioned earlier, the AA profile in our study is not directly
608 comparable with others as we here only determine the AA composition in FPH peptides.
609
610 *Calcium and phosphorus content*
611 Calcium and phosphorus contents in either bone powders from MCF and HCM treatments or its
612 residual in FPH powder are presented in Table 8. On top of that, the amount of protein content in
613 dried bone powder of cod frame was measured to be in the range of 30-43% (data not shown). In

614 case of phosphorus and calcium content, bone-Neut&Alc sample on MCF resulted in significantly
615 higher concentrations (330 and 583 g/kg of phosphorus and calcium, respectively), compared to
616 those from the other treatments ($p<0.05$). Statistically, no significant difference was observed
617 between control sample (bone-boiling) with those obtained by individual application of Neut and
618 Alc on MCF ($P>0.05$). This result is an indication of the efficiency of sequential application of
619 Neut&Alc on recovery of minerals from leftover bone fractions after enzymatic hydrolysis, while
620 containing substantial amount of protein too. Interestingly, there was no significant difference in
621 phosphorus content of FPH powders ($P>0.05$). On the contrary, the calcium content of FPH
622 powder from HCM was significantly higher than that from MCF treatments, indicating loss of a
623 minor part of calcium (around 0.1%) in obtained bone powder after boiling of fish frame compared
624 with bone powder obtained after enzymatic treatment. Thus, application of enzymatic hydrolysis
625 on cod frame before separating meat and bone fraction can results in significantly higher value of
626 calcium and phosphorus in obtained bone powder ($P<0.05$), while its protein content was lower
627 compared to dried bone powder obtained by drying at 50 °C from the frame without enzymatic
628 hydrolysis. However, further experiments are needed to clarify the effect of type of treatments on
629 the bioavailability of obtained minerals and protein.
630 Fish bone consists of both organic and inorganic (mineral) parts. Kim and Jung (2006) reported
631 the inorganic mineral portion as 69.5% on dry basis, which was mainly composed of 59.7% of
632 calcium (Ca) and 35.8% of phosphorus (P) with the Ca/P mole ratio of 1.67. In the current study,
633 the Ca/P mole ratio was between 1.6 to 1.7, which is close to the desired ratio for human bones,
634 and in agreement with the results reported by other researchers (Kim et al., 2018; Logesh et al.,
635 2012; Nemati et al., 2017). According to Toppe et al. (2007), P and Ca content of cod bone are
636 113 and 190 g/kg of lipid free dry matter, respectively. In another study, conducted by Malde et

637 al. (2010), these minerals were reported as 180 and 248 g/kg of boiled cod bone, which is in nearly
638 comparable with P and Ca values recorded in our study as 170 and 297 g/kg of boiled cod bone,
639 respectively. However, the mineral content of bone-Neut&Alc sample was as twice and triple as
640 those reported by Malde et al. (2010) and Toppe et al. (2007), respectively. The findings of the
641 present study indicated that cod frame is rich in calcium, phosphorus and protein, which can be
642 considered as a potential source in fortification of food products for human consumption.
643
644 **Conclusions**
645 In this study, the proximate composition, phosphorous content, amino acid and fatty acid profiles
646 of cod frame from five different catchment periods were evaluated. Apart from some amino acids,
647 no profound variation was observed in proximate composition of cod frame among different
648 catchment periods. Therefore, it can be considered as a steady and valuable side-stream source
649 based on its high content of calcium, phosphorus, protein, and low fat content (dominantlyn-3 fatty
650 acids, namely, EPA and DHA), as well as potentially bioactive peptides by enzymatic hydrolysis.
651 Further studies are needed to evaluate the functional properties of the obtained peptides and the
652 bioavailability of calcium and phosphorous of the resultant bone powder.
653
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659

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