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# 1 **Characterization of Cod (*Gadus morhua*) frame composition and its** 2 **valorization by enzymatic hydrolysis**

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11

## 12 **Abstract**

13 This study aimed at evaluating proximate composition, phosphorous content, amino acid (AA) and  
14 fatty acid (FA) profiles of cod (*Gadus morhua*) frame at five different sampling times (March 2017  
15 to March 2018). Furthermore, the valorization possibility of cod frame by application of enzymatic  
16 hydrolysis was investigated using the samples from September 2017. In terms of protein content,  
17 this sample showed a significantly ( $P<0.05$ ) higher level (16.5%) compared to the other samples,  
18 whereas lipid and phosphorous contents varied in a narrow range of 0.9-1.1% and 2.9-4.4%,  
19 respectively, ( $P<0.05$ ). Furthermore, the total amino acids (AAs) content varied from about 98 to  
20 155 mg/g in minced cod frame. Enzymatic hydrolysis of minced cod frame (MCF) and heated cod  
21 meat (HCM) was carried out by application of Alcalase and Neutrase, either individually or  
22 sequentially to obtain fish protein hydrolysate (FPH) and bone powder rich in phosphorus and  
23 calcium. The protein content of FPH varied from 76% to 84% and soluble-nitrogen in

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

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

33

#### 34 **Introduction**

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

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

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

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

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

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

94

## 95 **Materials and Methods**

96 Cod frames were received from Espersen A/S, (Klaipeda city-Lithuania) in batches of 7 kg at five  
97 different sampling times (March 2017, June 2017, September 2017, December 2017 and March  
98 2018). The captured cods were processed (filleted) on-site and the frames (side-stream) were  
99 packed, transported to the National Food Institute (Kgs. Lyngby, Denmark) in frozen condition,  
100 and stored at -40°C upon arrival. Alcalase® 2.4 L FG (Alc; declared activity of 2.4 AU/g) and  
101 Neutrase® 0.8 L (Neut; declared activity 0.8 AU/g), both as endopeptidase with broad specificity  
102 and high activity in range of pH and temperature, were provided by Novozymes (Bagsværd,  
103 Denmark). All chemical reagents used for experiments were of analytical grade.

104

### 105 *Preparation of cod frames for characterization*

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

110

### 111 *Dry matter*

112 Homogenized thawed cod frame sample (approx. 2 g) were dried for 20-24 h in an oven at 102-  
113 105°C. All analyses were carried out in triplicate samples (n=3). The dry matter [%] was calculated  
114 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  $1665 \times g$  and  $18 \text{ }^\circ\text{C}$  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,  $105 \text{ }^\circ\text{C}$ ) and weighed. All analyses were carried out in  
126 triplicate samples (with two analytical replicates;  $n=3 \times 2$ ).

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  $\mu\text{L}$ , C23:0 in heptane), 200  
131  $\mu\text{L}$  heptane with BHT and 100  $\mu\text{L}$  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 $\times$ ID 0.1 mm  $\times$  0.1  $\mu\text{m}$  film  
137 thickness, J&W Scientific, Folsom, CA)) according to AOCS official Method Ce 1b-89. Results

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

140

#### 141 *Protein content*

142 The protein content was measured based on total nitrogen content analyzed by Dumas (Rapid  
143 MAX N exceed cube N/protein analyzer, Elementar Analysensysteme GmbH, Germany).  
144 Depending on the sample type, 250 mg-500 mg of sample was used for the determination. Crude  
145 protein was estimated in all samples by multiplying the total nitrogen content (%) by a factor of  
146 6.25. All analyses were carried out in triplicate samples (with two analytical replicates; n=3×2).

147

#### 148 *Amino acid composition*

149 The amino acid composition was determined by HPLC-MS, following hydrolysis and  
150 derivatization using EZ:faast amino acid kit (Phenomenex, Torrance, CA, USA) (Ghelichi et al.,  
151 2017). The acid hydrolysis was applied in order to release the amino acid using 6 M HCl at 110  
152 °C for 18 h in oven. The subsequent neutralized samples were purified by a solid-phase extraction  
153 sorbent tip and derivatization was performed following the injection of sample aliquots into an  
154 Agilent HPLC 1100 instrument (Santa Clara, CA, USA) coupled to an Agilent ion trap mass  
155 spectrometer. The amino acids were identified by comparing retention time and mass spectra of  
156 an external standard mixture. Calibration curves were prepared and analyzed by HPLC-MS for  
157 quantification. All analyses were carried out in triplicate samples (with two analytical replicate;  
158 n=3×2).

159

#### 160 *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<sub>4</sub><sup>3-</sup>/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

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

188

### 189 **SN-TCA index (%)**

190 The soluble nitrogen content as an indicator of hydrolysis efficiency was calculated by application  
191 of trichloroacetic acid (TCA) method as described by Hoyle & Merritt (1994). Accordingly, 20%  
192 TCA (w/v) solution was prepared and 20 mL of collected supernatant was added to 20 mL of TCA  
193 (10% final concentration). The mixture was centrifuged at 7800 ×g for 15 min. The supernatant  
194 (soluble protein) was then analyzed for nitrogen content using Dumas instrument (n = 2).

$$195 \quad SN - TCA \text{ index} = \frac{(\%) SN \text{ in } 10\% TCA \text{ supernatant}}{(\%) TN \text{ in raw material}} \times 100$$

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

197

### 198 *Nitrogen recovery*

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

$$200 \quad NR (\%) = \frac{N (\%) \text{ in } FDHP \times \text{its weight (g)}}{TN (\%) \text{ in raw material} \times \text{its weight (g)}} \times 100$$

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

203

### 204 *Yield*

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

$$207 \quad \text{Yield (\%)} = \frac{\text{FDHP weight (g)}}{\text{initial weight of raw material (g)}} \times 100$$

208 Where *FDHP* is freeze-dried hydrolysate powder.

209

### 210 **FPH Solubility**

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

217

$$218 \quad \text{Solubility (\%)} = \frac{\text{Protein/peptide content in supernatant}}{\text{Total protein/peptide content in FPH}} \times 100$$

219

### 220 **Water holding capacity (WHC)**

221 Following the method of Diniz and Martin (1997) with some modification, the WHC was  
222 determined by adding 100 mg FPH powder in 1000 µl of distilled water followed by mixing with  
223 a magnet stirrer. The protein suspension was then centrifuged at 1800 ×g for 20 min at 22 °C. The  
224 supernatant was decanted, and the tube drained at 45° angle for 10 min. WHC, as mL of water  
225 absorbed per g of FPH, was calculated based on the difference between initial volumes of distilled  
226 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 
$$Whiteness = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

250

251 **Phosphorus and calcium content of peptides and bone fractions**

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

260

261 **Amino acid profile of protein hydrolysates by middle-down proteomics**

262 Freeze-dried protein hydrolysates were prepared using a sodium deoxycholate (SDC) in-solution  
263 digestions protocol according to Zhou et al. (2015), without applying the tryptic digestion step.  
264 Alkylated peptides were purified using C-18 StageTips (Rappsilber et al., 2007; Yanbao Yu et al.,  
265 2014), dried down, and re-suspended in 0.1% (v/v) trifluoroacetic acid, 2% acetonitrile (v/v) for  
266 analysis. Peptide analysis was performed using an automated LC-ESI-MS/MS, consisting of an  
267 EASY-nLC system (Thermo Scientific) on-line coupled to a Q Exactive HF mass spectrometer  
268 (Thermo Scientific,) equipped with a Nanospray Flex ion source (Thermo Scientific). Peptides  
269 were loaded onto a reverse phase (RP) Acclaim Pepmap Nanotrap column (C18, 100 Å, 100 µm.  
270 × 2 cm, nanoViper fittings (Thermo Scientific)) followed by separation on a RP Acclaim Pepmap  
271 RSLC analytical column (C18, 100 Å, 75 µm. × 50 cm, nanoViper fittings (Thermo Scientific) as  
272 described in (García-Moreno et al., 2020).

273 Proteomics analysis was performed using MaxQuant 1.6.0.16 (Cox and Mann, 2008; Tyanova et  
274 al., 2016) using a reference proteome constructed by combining the two available GenBank  
275 (Benson et al., 2017; Sayers et al., 2019) assemblies (as of December 10<sup>th</sup>, 2018) for *Gadus morhua*  
276 (GCA\_000231765.1 and GCA\_900302565.1) (Star et al., 2013) and removing redundant entries  
277 by applying a 90% identity cutoff. The analysis was performed as unspecific digestion with peptide  
278 length from 3 to 65 AAs and applying a false discovery rate of 1% on both peptide and protein  
279 level.

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

284

$$285 \quad f_{AA}^{sample} = \sum_{pep=1}^n f_{AA}^p * I_{rel}^p$$

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

291

292 **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 Ingolfssdottir  
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 than 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, Ingolfssdottir 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 Ingolfssdottir 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  $\leq 1.0\%$  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

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

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

420

## 421 **Valorization of cod frames**

### 422 *SN-TCA index*

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

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

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

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

499 hydrolysate powder recovered from rainbow trout viscera using Alc, which possibly is related to  
500 the higher DH in their study compared to ours. According to Kristinsson and Rasco (2000), the  
501 increased concentration of polar groups such as COOH and NH<sub>2</sub> that is caused by extensive  
502 enzymatic hydrolysis has a substantial positive effect on the amount of adsorbed water. Apart from  
503 different substrate types, the differences in reported studies can possibly be explained by the  
504 number and ratio of polar and nonpolar groups (hydrophilic and hydrophobic amino acids), and  
505 the amino acid composition of the FPH recovered with each enzyme. According to Trevino et al.  
506 (2007), Asp, Glu, and Ser contribute more favorably to protein solubility than the other hydrophilic  
507 amino acids especially at high net charge. In our study, the inability of obtained FPH to imbibe  
508 water molecules can be possibly attributed to the dominant portion of released hydrophilic amino  
509 acid residues with low net charge, which results in weak hydrogen bonds that could not retain it  
510 against the gravitational force in the protein matrix. In addition, the common practice in protease  
511 hydrolysis in aqueous solution is to collect the supernatant after enzymatic process by  
512 centrifugation, which cause a major loss of hydrophobic amino acids that goes to the precipitated  
513 sediment. Consequently, peptides are more prone to hydration compared to undenatured protein.  
514 However, depending on the type of FPH application, both solubility and WHC are considered as  
515 important FPH functionality parameters as these can influence other functionalities such as  
516 emulsification and foaming properties.

517

#### 518 *Oil absorption capacity (OAC) and bulk density (BD)*

519 The highest OAC was obtained for hydrolysates produced by Alc treatment of both MCF and  
520 HCM substrates ( $P < 0.05$ ), while the lowest values were related to the individual application of  
521 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

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

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