PROTEIN DERIVED EMULSIFIERS WITH ANTIOXIDANT ACTIVITY FOR STABILIZATION OF OMEGA-3 EMULSIONS

Marta Padial-Domínguez[a], F. Javier Espejo-Carpio[a]*, Pedro J. García-Moreno[a], Charlotte Jacobsen[b], Emilia M. Guadix[a]

[a] Department of Chemical Engineering, University of Granada, Granada, Spain

[b] National Food Institute, Technical University of Denmark, DK-2800 Lyngby, Denmark

*Corresponding author. Tel: +34958241329, E-mail: fjespejo@ugr.es

ABSTRACT

The performance of a whey protein hydrolysate (WPH) was compared to hydrolysates obtained from other sustainable protein sources such as soy (SPH) and blue whiting (BPH). The oxidative stability of hydrolysate-stabilized emulsions was greatly influenced by their physical stability. Emulsion stabilized with BPH suffered a constant increase in droplet size and BPH was not able to prevent omega-3 oxidation, showing high concentration of volatiles. The peroxide value of SPH emulsion increased after the first day of storage, but it had a lower concentration of volatiles. In contrast, WPH-stabilized emulsion, which did not had any change in droplet size during storage, showed the highest oxidative stability. Therefore, our results confirmed that WPH is an interesting option for physical and oxidative stabilization of omega-3 emulsions, while SPH could be used in emulsions with shorter storage time such as pre-emulsions for microencapsulation of omega-3 oils.

Keywords: Omega-3, protein hydrolysates, oil-in-water emulsion, physical stability, oxidative stability

1. INTRODUCTION

Omega-3 (ω-3) polyunsaturated fatty acids (PUFA´s) have been consistently reported to exhibit beneficial effects in human health (Shahidi & Ambigaipalan, 2018). Numerous studies confirmed the potential role of ω-3 PUFA, especially eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3), in inhibiting major chronic diseases, including cardiovascular disease, diabetes, cancer or Alzheimer's disease. The main ω-3 PUFAs are alpha-linolenic acid (ALA, 18:3 n-3), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3). Although alpha-linolenic acid (ALA, 18:3 n-3) serves as precursor to the synthesis of EPA and DHA) in the body, this conversion is limited to rates lower than 4% in men and 9% in women (Burdge & Wootton, 2002). Thus, EPA and DHA should be incorporated in the daily diet. However, there is increasing evidence that certain population groups may not be consuming enough long-chain ω-3 PUFAs (Eilander, Harika, & Zock, 2015). Dietary intake recommendations vary considerably between organizations and also depend on factors such as age or gender. As a reference, the Food and Agricultural Organization the United Nations recommends 0.25-2 g/day of EPA and DHA for adults. Additionally, an excessive consumption of LA decreases the conversion of ALA to EPA and DHA, which would have a negative health effect (Calder, 2005). According to the International Society for the Study of Fatty Acids and Lipids, a ratio of LA/ALA of 4:1 would be desirable; however, ratios around 11 are found in occidental countries. Therefore, food supplementation with oils with high content of EPA and DHA is required.

From a technological point of view, fortifying foods with oils containing high proportion of EPA and DHA is challenging, mainly due to lipid oxidation issues. Generally, the oils should be incorporated to aqueous-based foods as oil-in-water emulsion. Emulsions are often more prone to oxidation than bulk oils because of the high surface/volume ratio that facilitates interactions between the oil and water-soluble prooxidants. Oxidation of ω-3 PUFAs reduces significantly their biological potential and generates off-flavors and toxic reaction products (Berton-Carabin, Ropers, & Genot, 2014). Therefore, the stabilization of emulsions containing ω-3 PUFAs against lipid oxidation by using antioxidants is crucial for its industrial use. Antioxidants can delay, control, or prevent oxidative processes through different mechanism such as scavenging free radicals, quenching singlet oxygen, inactivating peroxides and chelating metal ions among others (Shahidi & Zhong, 2007). Apart from their intrinsic activity, the location of the antioxidant compounds in the emulsion system has an important effect on antioxidant efficiency (Shahidi & Zhong, 2011). In general, food components (e.g. trace metal ions) that promote oxidation are located in the aqueous phase. Thus, oxidation reactions are initiated and propagated at the oil/water interface (Berton-Carabin et al., 2014). Hence, the use of antioxidants that locate at the interface is an effective way of controlling lipid oxidation (McClements & Decker, 2018). One of the approach to achieve that is to use emulsifiers exhibiting antioxidant activity, which assures the location of the antioxidants at the interface. This will increase their antioxidant effect in emulsions systems. This also reduces the complexity of the system, since the use of one single ingredient (i.e. having emulsifying and antioxidant activities) allows both the physical and chemical stabilization of

69 emulsions. On the contrary, when using combinations of emulsifiers and antioxidants,
70 interactions between emulsifiers and antioxidants will determine the antioxidant
71 activity, which may even lead to an unexpected pro-oxidation effect (Oehlke, Heins,
72 Stöckmann, Sönnichsen, & Schwarz, 2011).
73 Recently, the chemical modification (e.g. conjugation with caffeic acid) of commonly
74 used emulsifiers (i.e. diacetyl tartaric acid esters of mono- and diglycerides (DATEM)
75 and phosphatidylcholine has been carried out to enhance their antioxidant activity
76 (Yesiltas et al., 2018). Moreover, these studies reported the positive effect of the
77 modified emulsifiers on the stabilization of omega-3 emulsions. However, consumers'
78 preference for less synthetic ingredients requires the development of natural
79 alternatives. In this regard, milk proteins having both emulsifying and antioxidants
80 properties have been traditionally used (Hu, McClements, & Decker, 2003). On the
81 other hand, for sustainability reasons, the use of alternative protein sources (e.g. plant
82 proteins or by-products rich in proteins) is desired. Nevertheless, non-diary proteins
83 have normally reduced emulsifying and antioxidant properties in their native form
84 when compared to whey protein or caseinates. Alternatively, protein hydrolysates
85 obtained by enzymatic hydrolysis present peptides with enhanced emulsifying (García-
86 Moreno, Guadix, Guadix, & Jacobsen, 2016; Gbogouri, Linder, Fanni, & Parmentier,
87 2004; Rahali, Chobert, Haertlé, & Guéguen, 2000) and antioxidant activities (i.e. radical
88 scavenging or metal chelating) (García-Moreno et al., 2014; Peng, Xiong, & Kong,
89 2009).
90 Although protein hydrolysates have previously been employed as antioxidants in oil-in-
91 water emulsions, they have been tested together with other compounds (e.g.

92 Tween20, citrem) used as emulsifiers (Farvin et al., 2014; Ghelichi, Sørensen, García-
93 Moreno, Hajfathalian, & Jacobsen, 2017). However, only few studies employed protein
94 hydrolysates exhibiting both emulsifying and antioxidant properties for the physical
95 and chemical stabilization of oil-in-water emulsions (García-Moreno et al., 2016; C. Liu,
96 Bhattarai, Mikkonen, & Heinonen, 2019).
97 The objective of this work was to investigate the physical and oxidative stabilities of
98 omega-3 oil-in-water emulsions stabilized with protein hydrolysates obtained from
99 non-dairy protein sources. Particularly, we compared the stability of omega-3
100 emulsions stabilized either with a fish protein or soy protein hydrolysates. Whey
101 protein hydrolysate was used as control due to their confirmed emulsifying and
102 antioxidant effects (Tamm et al., 2015). The fish protein hydrolysate was obtained
103 from by-products from the fishing industry. In this case, we used as raw material blue-
104 whiting (*Micromesistius poutassou)* discards, which are produced in the West-
105 Mediterranean sea. Finally, soy was selected as plant-protein source due to its low
106 environmental impact compared to animal proteins.

107 # 2. MATERIALS AND METHODS

108 ## *2.1. Materials*

109 Raw blue whiting (*Micromesistius poutassou*) was purchased from the fishing harbor of
110 Motril. Dewatered and defatted substrate was obtained after pressing the whole fish
111 according to the process described elsewhere (García-Moreno et al., 2014). The
112 protein cake was grinded in a Sammic cutter SK-3 (Guipúzcua, Spain) and the mix was

kept at -80°C until use. Whey protein concentrate (Wheyco GmbH, Hamburg, Germany) and soy protein isolate (Solae LLC, MO,USA) were also used as substrates. Subtilisin (EC 3.4.21.62) and trypsin (EC 3.4.21.4), both provided by Novozymes (Bagsvaerd, Denmark) were employed for enzymatic hydrolysis. Refined fish oil, Omevital 18/12 TG Gold was purchased from BASF Personal Care and Nutrition GmbH (Illertissen, Germany) with a minimum content of omega-3 fatty acids of 35% (18% of EPA and 12% of DHA).

2.2. Enzymatic hydrolysis

Preliminary studies were carried out for evaluating the effect of different enzymes treatments and degree of hydrolysis on the antioxidant and emulsifying activity of hydrolysates. According to those previous studies (unpublished data), in order to maximize both emulsifying and antioxidant activity, blue whiting was hydrolyzed by trypsin until final degree of hydrolysis (DH) of 4%, while soy protein and whey protein were hydrolyzed using subtilisin to final DH of 2 % and 10 %, respectively. In each case, a protein solution of 40 g protein/L was hydrolyzed in a stirred tank reactor at 50°C and pH 8, using an enzyme substrate ratio of 0.1%. Hydrolysis was monitored by an automatic titrator 718 Stat Titrino (Metrohm, Herisau, Switzerland), which maintained pH constant by adding NaOH (1M). DH was determined according to the pH-stat method (Adler-Nissen, 1986). When the desired DH was obtained, the reaction was immediately stopped by thermal deactivation of enzyme (90 °C, 5 min). Hydrolysates were freeze dried and stored at 4ºC until further use.

2.3. Characterization of hydrolysates

Amino acid profile was determined according to Liu et al. (1995), using a Waters Alliance 2695 system mounted with AccQTag column (Waters Corporation, Milford, MA, USA).

The molecular size distribution of the hydrolysates was determined by a fast protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) mounted with Superdex Peptide 10/300 GL column (GE Healthcare, Uppsala, Sweden). An aliquot of 500 μL was injected and eluted at 0.5 mL/min using 0.5 mL/min of water as mobile phase. The absorbance was recorded at 280 nm. Glycine (75 Da), alanine (89 Da), Phe-Gly-Gly (279 Da), $(Gly)_6$ (360 Da), vitamin B12 (1355 Da), insulin (5733 Da), aprotinin (6511 Da) and ribonuclease (13,700 Da) were used as standards. Each sample was run in triplicate.

The radical scavenging activity of hydrolysates was determined using the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the method described by Picot et al. (2010). The scavenging capacity was expressed as EC_{50} value, which is the concentration of hydrolysate that scavenges the 50% of the radical. The reducing power of hydrolysates was determined by the method described by Oyaizu (1988). The reducing power was expressed as EC_{50} value, which is the concentration of hydrolysate that gives an absorbance of 0.5. Finally, the Fe2+ chelating capacity of hydrolysates was determined according to the method of by Decker and Welch (1990). The EC_{50} value was estimated as the concentration of hydrolysate that was able to chelate the half of the iron ions in the assay.

2.4. Emulsion preparation and sampling

Hydrolysates were dissolved in distilled water and the pH was adjusted to the appropriate value which allows to maximize emulsifying properties. Blue whiting hydrolysate (BPH) was adjusted to pH 2 while soy and whey protein hydrolysates (SPH and WPH) were set at pH 8. Emulsions containing 2% (w/w) of hydrolyzed protein and 5% (w/w) of fish oil were produced similarly to García-Moreno et al. (2016). Firstly, the mixture was pre-emulsified by stirring in Ultra Turrax (IKA Werke GmbH &.Co., Staufen, Germany) at 16.000 rpm during 2 minutes and the fish oil was added during the first minute. After, the homogenization was done in a high pressure homogenizer (Panda Plus 2000, GEA Niro Soavi, Lübeck, Germany) at 450/75 bar, running 3 passes. To accelerate lipid oxidation, a solution of 100 μM $FeSO_4$ was added to the emulsions (4 μl per 1 g of emulsion). Emulsions were stored in the dark at 20°C for 10 days in 30 mL glass bottles. Each bottle contained approximately 15 mL of emulsion. Samples were taken at days 0, 1, 3, 6 and 10 for droplet size, creaming and oxidative stability measurements, while Zeta potential was determin

2.5. Physical stability of emulsion

The zeta potential was determined in a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Emulsions were diluted 1:20 in distilled water adjusting the pH to the original emulsion. Emulsions were placed in graduated tubes and the creaming index was calculated during storage as the percentage of phase separation (Petursson, Decker, & McClements, 2004). Droplet size distribution was determined by laser diffraction in a Malvern Mastersizer 2000 (Malvern Instruments Ldt., Wortcestershire,

178 UK). The emulsions samples were diluted in recirculating water (3000 rpm) until it
179 reached an obscuration of 12–15%. The refractive indexes of sunflower oil (1.469) and
180 water (1.330) were used for particle and dispersant, respectively. Results are given in
181 volume mean diameter (D4,3), Sauter mean diameter (D3,2) and percentile 90.

182 *2.6. Oxidative stability of emulsions*

183 2.6.1. Determination of hydroperoxide content.

184 Firstly, the oil was extracted by mixing in vortex for 5 min a mixture of 0.5 g of
185 emulsion and 7 mL of 2-propanol/hexane (1:1, v/v), the mixture was centrifuged 670 g
186 for 2 min. Afterwards, around 10 mg of extracted oil was diluted with 2-propanol and
187 mixing with iron-II-chloride and ammonium thiocyanate according to the method
188 described by Drusch et al. (2012). Samples were incubated for 30 min at 60 °C and
189 absorbance was measured at 485 nm. Each sample was extracted in duplicate and
190 each extracted oil was measured in quadruplicate.

191 2.6.2. P-anisidine value (AV).

192 The AV method is based on the reaction of p-anisidine diluted in acetic acid with the α
193 and β unsaturated aldehydes present in the extracted oil. Results were expressed as
194 100 times the increment of absorbance, measured at a wavelength of 350 nm in a 10
195 mm cell, of a test solution when reacted with p-anisidine under the test conditions
196 specified in the International Standard (ISO, 2006).

2.6.3. Secondary oxidation products – dynamic headspace GC–MS.

Approximately 4 g of emulsion and 30 mg internal standard (4-methyl-1-pentanol, 30 µg/g water) were weighed in a 100 mL purge bottle. To it, 5 mL of distilled water and 1 mL antifoam (Synperonic 800 µL/L water) were added. The bottle was heated in a water bath at 45°C while purging with nitrogen (flow 150 mL/min, 30 min). Volatile secondary oxidation products were trapped on Tenax GR tubes. The volatiles were desorbed again by heat (200°C) in an Automatic Thermal Desorber (ATD-400, Perkin Elmer, Norwalk, CN), cryofocused on a cold trap (-30°C), released again (220°C), and led to a gas chromatograph (HP 5890IIA, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m x 0.25 mm x 1.0 µm; J&W Scientific, CA, USA). The oven program had an initial temperature of 45°C for 5 min, increasing with 1.5°C/min until 55°C, with 2.5°C/min until 90°C, and with 12.0°C/min until 220°C, where the temperature was kept for 4 min. The individual compounds were analyzed by mass-spectrometry (HP 5972 mass-selective detector, Agilent Technologies, USA; electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250). The individual compounds were identified by both MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard) and quantified through calibration curves. The external standards employed were 3-methyl-butanal, 3-methyl-butanol, 1-penten-3-one, pentanal, 1-penten-3-ol, (*E*)-2-pentenal, 1-pentanol, hexanal, (*E*)-2-hexenal, heptanal, (*Z*)-4-heptenal, octanal, benzaldehyde, (E,*E*)-2,4-heptadienal, (*E,Z*)-2,6-nonadienal, (*E,E*)-2,4-octadiene, 2-ethylfuran and 2-pentylfuran. Measurements were made in triplicate in each sample.

2.7. Statistical analysis.

The analysis of variance (ANOVA) was carried out using Statgraphics (version 5.1.). Mean values were compared using the Tukey´s multiple range test. Differences between means were considered significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Characterization of hydrolysates

The protein content of the hydrolysates (Table 1) was similar to that of the original substrates employed. WPH presented significant lower protein concentration because of the high lactose content of the whey protein concentrate employed as substrate in hydrolysis. Similarly, lipid content of BPH was higher than the SPH and WPH. Although blue whiting usually has a relatively low proportion of lipids, it is usually higher than the fat in soy and whey protein concentrate.

All hydrolysates presented high concentrations of aspartic acid, glutamic acid, alanine, lysine and leucine (Table 1). Essential amino acids in BPH and WPH represented around 45% of the total while SPH had a slightly inferior value (41%). Lower values were obtained for other fish protein and plant protein hydrolysates with emulsifying properties (García-Moreno et al., 2016). The proportion of hydrophobic amino acids was 5% higher in WPH, these amino acids would have a positive influence in emulsifying activity of hydrolysates (Rahali et al., 2000).

Overall, the three hydrolysates studied presented high proportions of low molecular peptides (Fig. 1). Particularly, BPH had more than 60 % of the peptides between 0.5-3 kDa. Similar proportions of low molecular weight peptides was observed in fish

240 hydrolysates at low degree of hydrolysis (García-Moreno et al., 2016). This fraction
241 would be an important factor affecting the radical scavenging and metal chelating
242 properties of hydrolysates (Sila & Bougatef, 2016). SPH and WPH were obtained by
243 subtilisin hydrolysis at 4 and 10% of degree of hydrolysis, respectively. The lower
244 degree of hydrolysis reached for SPH is responsible for the higher proportion of high
245 molecular weight fraction obtained. This is important since the presence of large
246 peptides (above 2 kDa) usually would enhance the emulsifying properties of the
247 hydrolysate (Schröder, Berton-Carabin, Venema, & Cornacchia, 2017) because of their
248 capacity to adsorb and unfold at the oil/water interface. However, the emulsifying
249 properties will also depend on the composition and sequence of the amino acids.
250 The antioxidant capacity differs considerably between hydrolysates studied (Table 2).
251 In general, antioxidant activity of hydrolysates depends on their molecular size as well
252 as on their amino acids composition and sequence (Nwachukwu & Aluko, 2019). As
253 expected, the antioxidant activity increased when increasing the concentration of
254 hydrolysate (data not shown). BPH presented remarkable global antioxidant capacity,
255 having significantly higher radical scavenging activity than WPH and significantly higher
256 metal chelating capacity than SPH (Table 2). The high concentration of low molecular
257 weight peptides and the high proportion of amino acid with antioxidant potential, such
258 as alanine, tyrosine, histidine or methionine (Aluko, 2015), contribute to the good
259 antioxidant characteristic of BPH. Similar EC50 values were obtained for carp protein
260 hydrolysate (Ghelichi et al., 2017). However, lower values of radical scavenging activity
261 was detected in sardine hydrolysate used for omega-3 emulsification (García-Moreno
262 et al., 2016). WPH showed no significant differences in chelating capacity when

compared to BPH, but the radical scavenging activity of WPH was significantly lower when compared to the one obtained for BPH. This could be explained because of the low concentration of His in the WPH. Histidine has been reported to have excellent scavenging and chelating antioxidant properties (Aluko, 2015). Similar scavenging activity has reported in literature for whey protein hydrolysates even at much higher degree of hydrolysis (Peng et al., 2009). Finally, although SPH presented similar radical scavenging as BPH ($p>0.05$), it showed significantly lower reducing power and metal chelating activity when compared to BPH and WPH. This can be attributed to the low DH reached in SPH, which only permitted the exposure of a limited number of electron-donating amino acid side chain groups (Guan, Diao, Jiang, Han, & Kong, 2018). Moreover, SPH presented the highest proportion of peptides above 3 kDa, which has been related to lower reducing capacity (Kim, Liceaga, & Yoon, 2019)

3.2. Characterization and physical stability of emulsions

High absolute values of the ζ-potential (>30 mV) are desirable since electrostatic repulsion prevents aggregation and make emulsions more stable. The ζ-potential values obtained in the emulsions stabilized by hydrolysates were 36.3±0.06, -46.8±0.72 and -44.0±0.60 mV for BPH, SPH and WPH, respectively. It is worth noting that positive ζ-potential was obtained for the emulsions stabilized with BPH, since this emulsion was produced at pH 2 which is below the pI of the blue-whiting proteins. On the contrary, negative ζ-potential values were found for emulsions stabilized with SPH and WPH, which were produced at pH 8 (e.g. above the pI of these proteins). Similar values were obtained in cod (Petursson et al., 2004) and whey protein (Schröder et al., 2017)

285 hydrolysates-stabilized emulsions when compared to BPH and WPH-stabilized
286 emulsions. The lower absolute ζ-potential obtained for BPH would imply reduced
287 repulsions between oil droplets compared with oil droplets in emulsions stabilized
288 with SPH and WPH. Nevertheless, negative ζ-potential values may favor lipid oxidation
289 reactions, since the negative surface charges obtained in emulsions stabilized with SPH
290 and WPH attract metal ions, which catalyze lipid oxidation (Mei, McClements, &
291 Decker, 1999). However, as for other emulsifiers such as citrem, the iron chelating
292 capacity of the hydrolysates (both when present at the interface and in the aqueous
293 phase) also play a role in in preventing the oxidation catalyzed by metal ions (Sørensen
294 et al., 2008).
295 It is remarkable that the emulsions stabilized with hydrolysates did not present
296 creaming after 10 days of storage (data not shown). However, significant differences in
297 droplet size distribution were observed for the different hydrolysate-stabilized
298 emulsions (Fig. 2). Initially all emulsions presented monomodal distribution with no
299 significant differences in $D_{4,3}$ values (Fig. 2). After the first day of storage, BPH showed
300 a bimodal distribution with a second peak centered around 11 μm (data not shown).
301 The proportion of this second peak increased during storage, which explains the
302 increasing values of $D_{4,3}$ (Fig. 2) until reaching a value of 1.09 ± 0.13 μm after 10 days
303 of storage. This behavior could be explained because of the low proportion of high
304 molecular weight peptides, which would limit the emulsifying potential of BPH. Larger
305 size peptides are more likely to be amphiphilic enhancing their adsorption and
306 unfolding at the oil/water interface (Gbogouri et al., 2004). Similar initial $D_{4,3}$ was
307 obtained in emulsion (5% oil) stabilized by sardine hydrolysate of DH 3%, but in the

physical stability of this emulsion was higher, reaching a final $D_{4,3}$ of 0.349 ± 0.002 μm (Garcia, López-Hernandez, & Hill Jr., 2011).

Emulsion stabilized with SPH also showed an initial increase in $D_{4,3}$ after the first day of storage (Fig. 2). However, in this case, the size distribution remained stable from day 1 until the end of the storage time. Differently, the $D_{3,2}$ (see Fig S1 in Supplementary Material), which is not so affected by the presence of large droplets, only varied slightly during storage (average $D_{3,2}$ = 0.155 ± 0.003 μm). Smaller droplet size ($D_{3,2}$ = 0.052± 0.001 μm) were obtained for a similar emulsion (1% w/v hydrolysate and 5% w/v oil) stabilized by fava protein hydrolysate at DH4% (C. Liu et al., 2019). However, the physical stability of the emulsion was lower and after seven days of storage the droplets had two fold the initial size.

The emulsion prepared with WPH presented an excellent physical stability, the initial monomodal distribution ($D_{4,3}$ = 0.263 ± 0.008 μm and $D_{3,2}$ = 0.144 ± 0.002 μm) remained unaltered until the end of the storage period. Slightly larger droplet sizes were obtained for emulsions (10%wt oil) stabilized by a similar whey protein hydrolysates at the same protein concentration (Drapala, Mulvihill, & O'Mahony, 2018; Schröder et al., 2017), nevertheless these emulsion presented excellent stability.

3.3. Oxidative stability of emulsions

3.3.1. Peroxide Value (PV)

Initial PV of SPH and WPH emulsions were 2.3 and 3.3 mmol O_2/kg oil, respectively. These values were faintly higher than the values obtained for fresh oil (< 1 mmol O_2/kg oil). The high shear stress and the incorporation of oxygen produced during

330 emulsification would be responsible for the slight lipid oxidation observed (Horn,
331 Nielsen, Jensen, Horsewell, & Jacobsen, 2012). Particularly, BPH emulsion presented a
332 significant higher initial PV (11.44 ± 0.39 mmol O_2/kg oil). In this case, apart from the
333 lipid oxidation produced during emulsification, the oxidation of lipids present in the
334 hydrolysate would influence the PV values obtained. This is in agreement with previous
335 results reported for fish oil-in-water emulsions stabilized with fish protein
336 hydrolysates (García-Moreno et al., 2016).
337 The evolution of hydroperoxides content in emulsions during storage differed
338 significantly depending on the hydrolysate employed. BPH emulsion suffered an
339 intense oxidation during the first three days of storage. The oxidation process was
340 initialized in the hydrolysate lipids during emulsifying process. The later would confirm
341 the importance of having a fat free hydrolysate for producing oxidatively stable
342 emulsions. The acid pH of BPH emulsion could also contribute to increase the oxidation
343 due to the higher solubility of iron at low pH (Berton-Carabin et al., 2014). García-
344 Moreno et al. (2016) observed a sharp increase in PV for fish oil-in-water emulsions
345 stabilized with fish protein hydrolysates at pH 2. Differences in PV values between our
346 work and this study are due to the incubation process carried out in our analyses to
347 determine PV. Ghelichi et al. (2017) stabilized fish oil emulsions using citrem as
348 emulsifier and carp protein hydrolysate as antioxidant. The fat content of the carp
349 hydrolysate was high (~11%), however, because of the low concentration of
350 hydrolysate employed in the emulsions (2 mg per mL of aqueous phase) the PV of
351 these emulsions was maintained in relatively low values during storage.

352 For the emulsions stabilized with SPH, a lag-phase of 1 day was observed (Fig. 3a).
353 After 1 day of storage, the PV constantly increased until reaching a value of 31.46
354 mmol/kg oil at day 10. A similar trend in the evolution of the concentration of primary
355 oxidation compounds was observed in rapeseed oil emulsion stabilized with fava bean
356 hydrolysate (C. Liu et al., 2019). Fig. 3a shows that the lowest PV during storage was
357 obtained for the emulsion stabilized with WPH. For this emulsion, PV (3.49±0.76
358 mmol/kg oil) remained constant during 10 days storage. Other authors (Drapala et al.,
359 2018) found a significant increase in hydroperoxides after seven days for infant
360 formula emulsions stabilized with a commercial whey protein hydrolysate of 10.7 %
361 degree of hydrolysis. The higher PV values observed could be due to the high
362 concentration of iron salts used in the formulation of these emulsions (8 μg/mL)
363 compared to the 0.06 μg/mL employed in our study.

364 **3.3.2. Secondary oxidation products**

365 The anisidine index measures the secondary oxidation compounds, primarily 2-alkenals
366 and 2,4-alkadienals and it is more sensitive to unsaturated aldehydes. Similarly to PV,
367 BPH presented an initial anisidine index significantly higher than SPH and WPH, it
368 might be attributed to the previous oxidation of the lipids present in this hydrolysate
369 and to the lower pH in this emulsion. Moreover, the emulsion stabilized with BPH
370 showed a constant increase in the anisidine value during the storage (Fig. 3b). This
371 trend is similar to the increase observed in PV for this emulsion. In contrast, the
372 emulsions stabilized with SPH and WPH did not show a significant increase in anisidine
373 index during 10-day storage.

374 Likewise, results on secondary volatile oxidation products indicated that the emulsion
375 stabilized with BPH was the most oxidized during storage. BPH-stabilized emulsion
376 presented the highest initial content as well as the higher increase during storage for
377 the following volatiles 2-ethylfuran (Fig. 4a), 1-penten-3-ol (Fig. 4b), pentanal (Fig. 4c),
378 hexanal (Fig. 4d), (t,*t*)-2,4-heptadienal (Fig. 4e), 1-penten-3-one, (t)-2-pentenal,
379 heptanal, (*c*)-4-heptenal, benzaldehyde, octanal, nonanal, (t,*t*)-2,4-octadiene, (t,*c*)-2,6-
380 nonadienal (see Fig. S2 in Supplementary Material). Interestingly, the volatiles 2-
381 ethylfuran, 1-penten-3-one, 1-penten-3-ol, (t)-2-pentenal, (t)-2-hexenal, (t,*t*)-2,4-
382 heptadienal and (t,*c*)-2,6-nonadienal derives from the oxidation of omega-3 fatty acids,
383 which clearly suggests the higher oxidation of EPA and DHA in the BPH stabilized-
384 stabilized emulsion. The rest of the mentioned volatiles derived from the oxidation of
385 omega-6 (e.g. hexanal) and omega-9 fatty acids.
386 The emulsion stabilized with SPH was the second most oxidized, as observed for
387 pentanal (Fig. 4c) and hexanal (Fig. 4d). In addition, this emulsion, when compared to
388 the emulsion stabilized with BPH and WPH, presented higher content of the following
389 volatiles: 3-methyl-butanal (Fig. 4f), 3-methyl-1-butanol (Fig. 4g), 1-pentanol (Fig. 4h),
390 2-pentylfuran (Fig. 4i). It should be noted that 3-methyl-butanal and 3-methyl-1-
391 butanol are tertiary lipid oxidation products formed by the reaction of lipid-derived
392 reactive carbonyls (e.g. aldehydes) and amino groups of peptides. Indeed, the increase
393 in the concentration of these volatiles from day 6 of storage (Fig. 4f,g) fits with the
394 decrease observed in pentanal and hexanal after 6 days (Fig. 4c,d). These findings are
395 in agreement with previous studies reporting the presence of 3-methyl-butanal (Farvin
396 et al., 2014) and 3-methyl-1-butanol (Ghelichi et al., 2017) in fish oil-in-water

emulsions containing fish protein hydrolysates. It is also worth mentioning that 2-pentylfuran is present in higher concentration in the SPH-stabilized emulsion compared to WPH and BPH-stabilized emulsions (Fig. 4i). However, although 2-pentylfuran is derived from the oxidation of omega-3 fatty acids, its concentration in the SPH-stabilized emulsion is considerably lower when compared to the concentration of other volatiles derived from omega-3 fatty acids (e.g. 1-penten-3-ol) in the BPH-stabilized emulsion.

Taken altogether, the emulsion stabilized with WPH was the most oxidative stable. This cannot be attributed to a superior antioxidant activity for this hydrolysate (Table 2). Furthermore, the WPH-stabilized emulsion had a negative zeta potential, which may favor attraction of metal ions to the interface where they can interact with hydroperoxides and catalyze lipid oxidation. Moreover, WPH-stabilized emulsion presented the lowest droplet size (Fig. 2), which implies the highest specific surface area where oxidation can be initiated. Thus, the enhanced oxidative stability of the WPH-stabilized emulsion, when compared to BPH and SPH emulsions, may be explained by its superior physical stability (Fig. 2). Similarly, García-Moreno et al. (2016) attributed the superior oxidative stability of fish oil-in-water emulsions stabilized with sardine protein hydrolysates to a physical barrier effect of an enhanced interfacial protein layer. This correlates well with the higher content of larger peptides of WPH (Fig. 1), which can better adsorb and unfold at the interface providing both physical and oxidative stabilities (e.g. by reducing the diffusion of prooxidants) to the emulsion.

4. CONCLUSIONS

Food protein hydrolysates obtained from sustainable sources are an interesting alternative of stabilizing omega-3 fatty acids for its incorporation into food matrices. Hydrolysates of blue whiting (BPH), soy (SPH) and whey (WPH) protein exhibited high in vitro antioxidant activity (e.g. radical scavenging, metal chelating and reducing power). However, physical stability plays a crucial role in oxidative stability. The BPH emulsion increased its droplet size during storage, suffering a significant lipid oxidation during storage time (e.g. 1-penten-3-ol = 142 ± 11 ng/g after ten days storage). Differently, emulsion stabilized with SPH was physically stable and presented higher oxidative stability, at least during the first days of storage (e.g. 1-penten-3-ol = 11 ± 2 ng/g after ten days storage). Finally, WPH showed superior capacity for physical and oxidative stabilization of omega-3 emulsions. Although WPH is confirmed as the best option for stabilizing omega-3 emulsions, SPH have also the potential of stabilizing omega-3 emulsions. Especially interesting would be the use of SPH for stabilizing pre-emulsions used in microencapsulation of omega-3, where storage time is limited. Further research is needed for enhancing the physical and oxidative stabilities of omega-3 emulsions stabilized with BPH.

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Table 1. Proximal and amino acid composition of hydrolysates.

	BPH	**SPH**	**WPH**
Moisture, wt%	3.35 ± 0.06	2.75 ± 0.13	5.32 ± 0.17
Protein, wt%	76.76 ± 0.43	83.90 ± 0.91	32.65 ± 0.78
Lipid, wt%	9.35 ± 0.15	4.79 ± 0.06	2.29 ± 0.01
Ash, wt%	7.31 ± 0.08	5.75 ± 0.18	8.08 ± 0.11
Amino acid composition, molar%			
ASP	9.51 ± 0.23	11.35 ± 0.74	10.23 ± 0.72
SER	5.48 ± 0.13	6.44 ± 0.06	5.72 ± 0.52
GLU	12.29 ± 0.29	16.54 ± 1.09	14.19 ± 0.63
GLY	6.26 ± 0.33	4.48 ± 0.88	7.19 ± 1.42
HIS	12.57 ± 0.05	10.24 ± 0.36	3.23 ± 0.09
ARG	5.21 ± 0.05	5.37 ± 0.16	2.16 ± 0.25
THR	4.56 ± 0.06	4.03 ± 0.20	7.28 ± 0.33
ALA	8.31 ± 0.46	5.99 ± 0.53	6.99 ± 0.34
PRO	4.37 ± 0.05	5.82 ± 0.28	6.91 ± 0.10
TYR	2.61 ± 0.09	2.85 ± 0.20	2.12 ± 0.37
VAL	4.86 ± 0.00	4.60 ± 0.04	5.97 ± 0.19
MET	2.61 ± 0.01	1.01 ± 0.08	1.56 ± 0.06
LYS	7.63 ± 0.00	5.78 ± 0.13	8.17 ± 0.37
ILE	3.81 ± 0.02	4.37 ± 0.05	5.57 ± 0.12
LEU	7.09 ± 0.23	7.42 ± 0.09	10.14 ± 0.32
PHE	2.84 ± 0.09	3.72 ± 0.26	2.57 ± 0.14

Table 2. Antioxidant activity (EC_{50} values) of blue whiting hydrolysate (BPH), soy hydrolysate (SPH) and whey protein hydrolysate (WPH).

	DPPH Radical scavenging (mg/mL)	**Reducing Power (mg/mL)**	**Chelating Power (mg/mL)**
BPH	1.46 ± 0.20^{a}	11.00 ± 0.34^{a}	0.95 ± 0.01^{a}
SPH	1.26 ± 0.08^{a}	23.11 ± 5.90^{b}	1.11 ± 0.02^{b}
WPH	4.45 ± 0.00^{b}	7.95 ± 2.15^{a}	0.95 ± 0.01^{a}

Means with different superscripts in the same column are significantly different ($p < 0.05$)

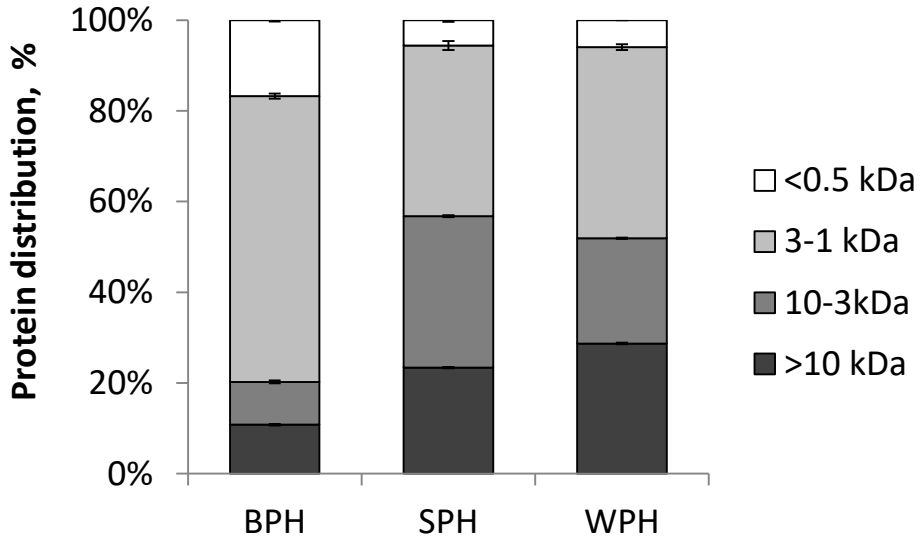

Figure 1. Molecular weight distribution of blue witting hydrolysate (BPH), soy hydrolysate (SPH) and whey protein hydrolysate (WPH).

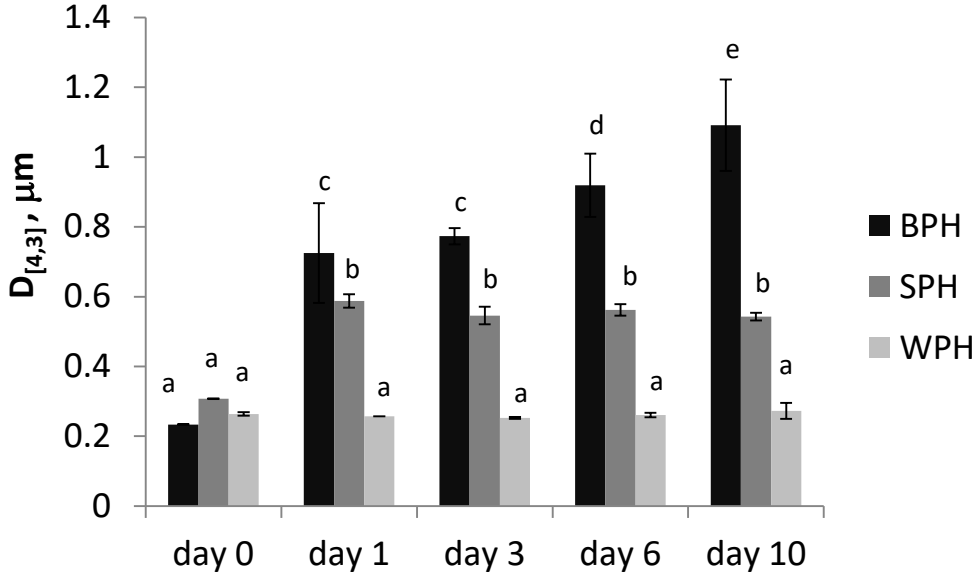

Figure 2. Droplet size of emulsions stabilized with blue whiting hydrolysate (BPH), soy protein hydrolysate (SPH) and whey protein hydrolysate (WPH) during storage at 20ºC.

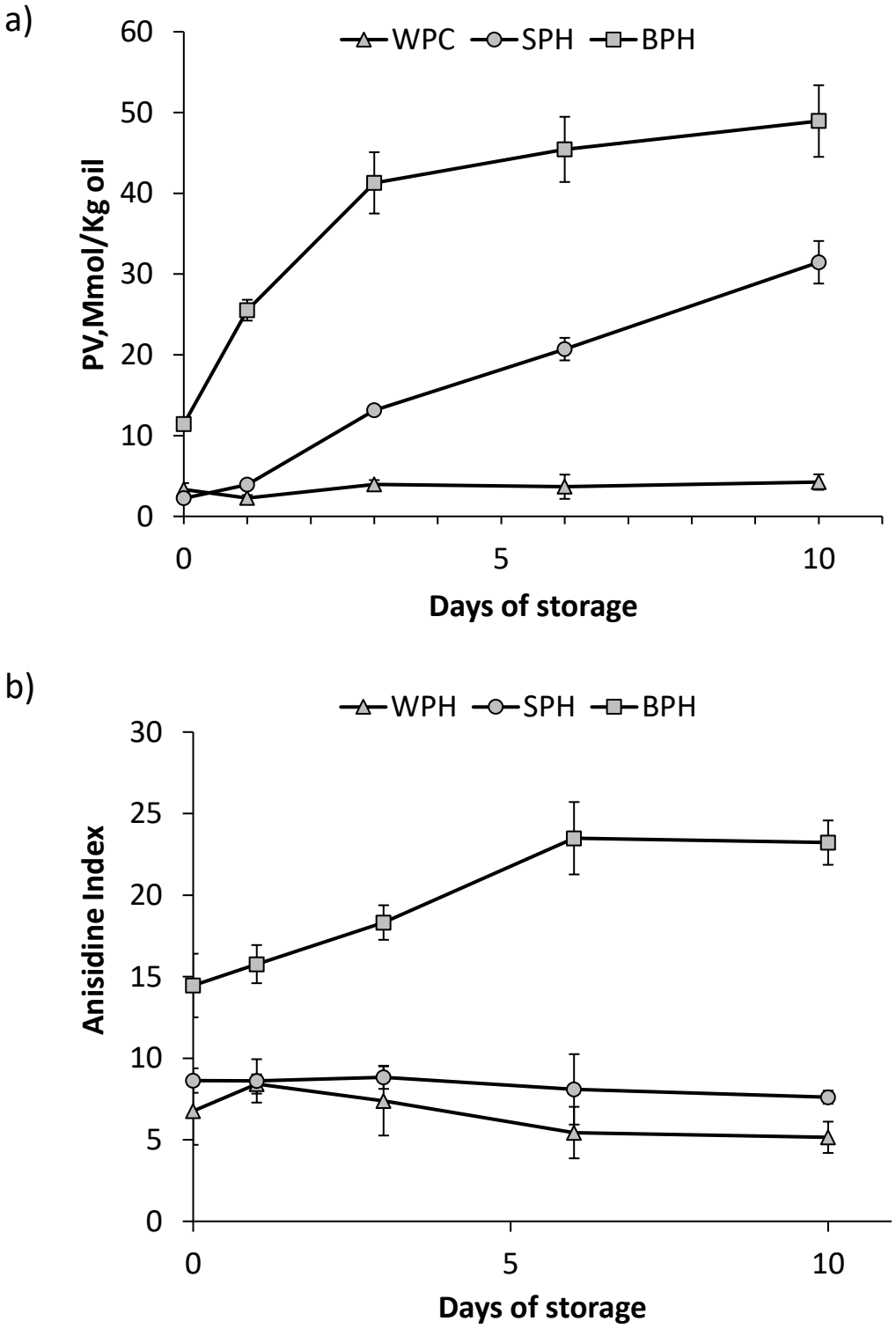

Figure 3. Peroxide value (PV) and anisidine index of emulsions stabilized with blue whiting (BPH), soy (SPH) and whey protein (WPH) hydrolysates in a ten-day storage period at 20 °C in the dark.

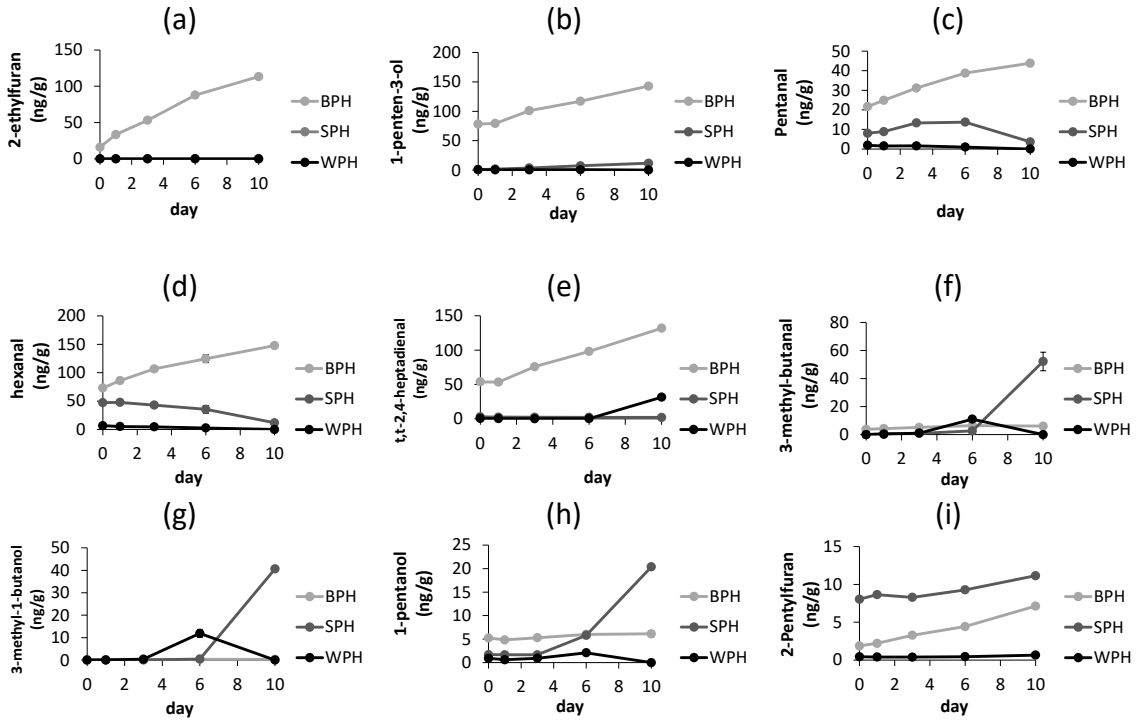

Fig. 4. Concentration of (a) 2-ethylfuran, (b) 1-penten-3-ol, (c) pentanal, (d) hexanal, (e) (*E,E*)-2,4-heptadienal, (f) 3-methyl-butanal, (g) 3-methyl-butanol, (h) 1-pentanol, and (i),2-pentylfuran (ng/g emulsion) in 5% fish oil-in-water emulsions stabilized with blue-whiting (BPH), soy (SPH) or whey protein (WPH) hydrolysates. 10-day storage at 20 °C in the dark. Results are the mean values of triplicate measurements ± standard deviations.

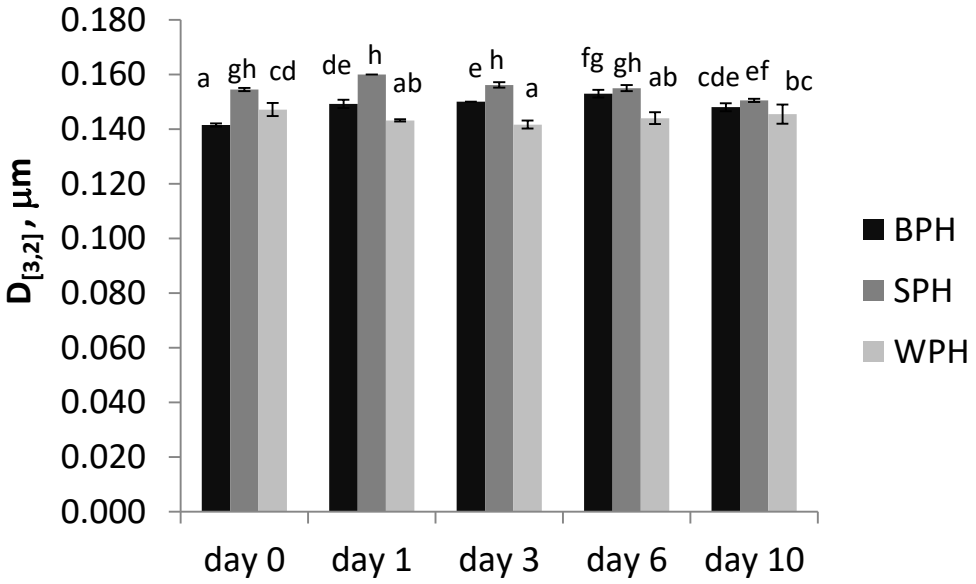

Figure S1. Droplet size of emulsions stabilized with blue whiting hydrolysate (BPH), soya protein hydrolysate (SPH) and whey protein hydrolysate (WPH) during storage at 20ºC.

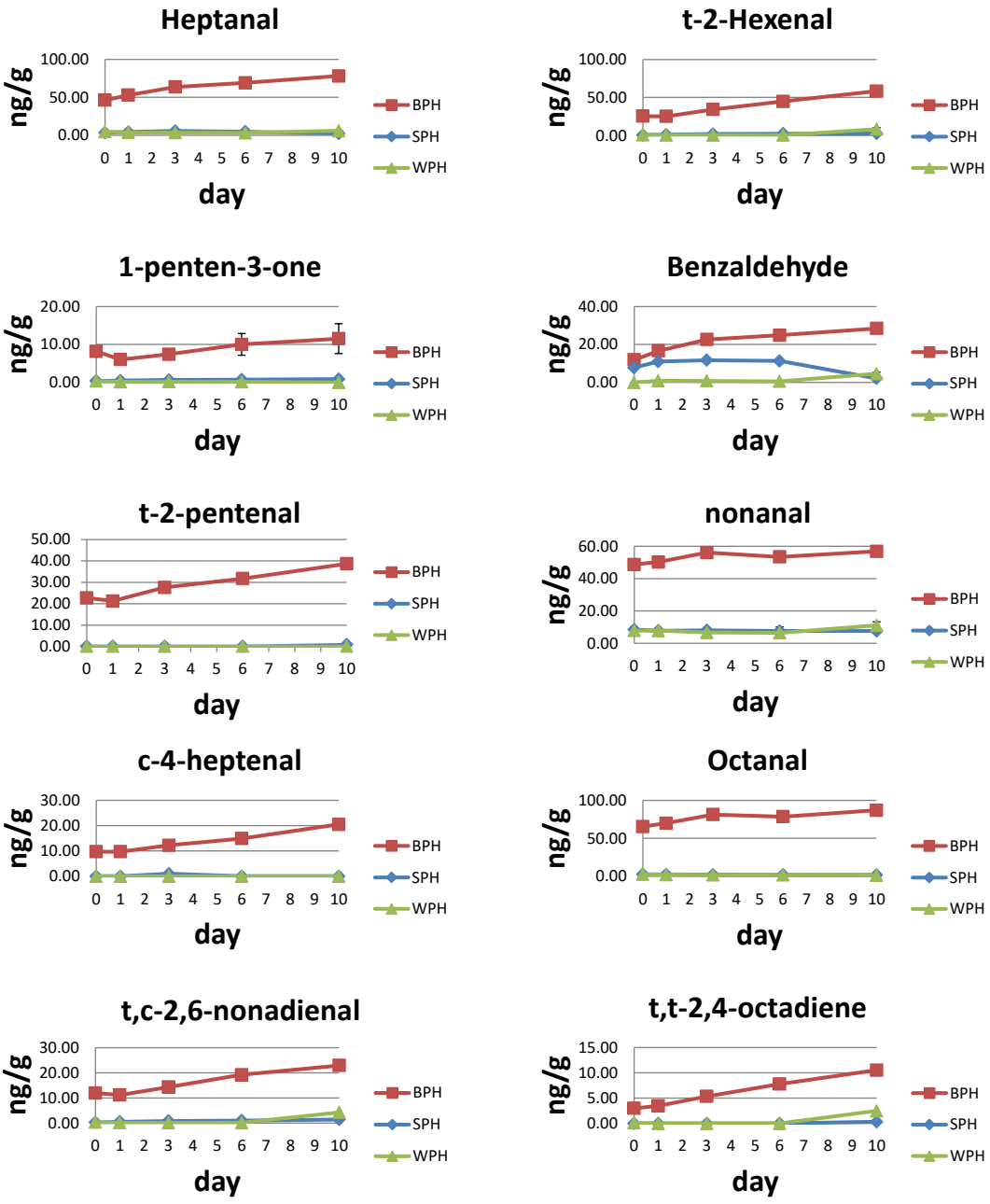

Figure S2. Concentration of heptanal, t-2-Hexenal, 1-penten-3-one, Benzaldehyde, t-e-pentenal, nonanal, c-4-heptenal, octanal, t,c-2,6-nonadienal and t,t-2,4-octadiene (ng/g emulsion) in 5% fish oil-in-water emulsions stabilized with blue-whiting (BPH), soy (SPH) or whey protein (WPH) hydrolysates. 10-day storage at 20 °C in the dark. Results are the mean values of triplicate measurements ± standard deviations.