Food Chemistry 194 (2016) 1208–1216

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Functional and antioxidant properties of hydrolysates of sardine (*S. pilchardus*) and horse mackerel (*T. mediterraneus*) for the microencapsulation of fish oil by spray-drying

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ARTICLE INFO

Article history:
Received 11 March 2015
Received in revised form 14 August 2015
Accepted 28 August 2015
Available online 29 August 2015

Keywords:
Fish hydrolysates
Fish oil
Microencapsulation
Spray-drying
Antioxidant properties

ABSTRACT

The functionality of fish protein hydrolysates (FPH) for the microencapsulation of fish oil was investigated. Muscle protein from sardine (*Sardina pilchardus*) and horse mackerel (*Trachurus mediterraneus*) was hydrolysed using Alcalase or trypsin. Physically stable emulsions suitable for spray-drying were obtained when using FPH with a degree of hydrolysis of 5%. Microencapsulation efficiency amounted to 98 ± 0.1% and oxidative stability of the encapsulated oil over a period of twelve weeks was in a similar range as it is reported for other matrix systems. Therefore, the suitability of FPH for use in spray-dried emulsions has been shown for the first time. Since no clear correlation between the antioxidative activity of the FPH and the course of lipid oxidation could be established future research is required to more specifically characterise the molecular structure of the peptides and its impact on protein alteration and role in lipid oxidation.

1. Introduction

Fish industry produces a large amount of by-products among whom discards are considered the most significant waste of fish resources (Davies, Cripps, Nickson, & Porter, 2009). The term discard refers to "the portion of the total organic material of animal origin in the catch, which is thrown away, or dumped at sea for whatever reason" and in 2004 the average amount of discards was estimated around 7.3 million of tonnes/year (Kelleher, 2005). Discards do not only have a great ecological impact due to the alteration of trophic channels but also considerably affect the economic viability of the fishing sector. Due to this critical situation, the European Commission is undertaking an extensive reform in the common fisheries policy, adopting a set of measures towards the complete elimination of discards. However, the adaptation to the new policy requires technical solutions allowing the use of discards as raw materials for the production of added-value compounds.

In this context, functional and bioactive properties (antihypertensive, antioxidant or antimicrobial) of fish protein hydrolysates gain importance. Non-hydrolysed fish protein does not possess these properties because bioactive and functional peptide sequences are poorly accessible (Kim & Wijesekara, 2010). Another functional ingredient for promoting health is fish oil with its unique composition, namely its high content of polyunsaturated fatty acids (PUFA) of the omega-3 family. More specifically, eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA) have been described as substances with antithrombotic, anti-arrhythmic and anti-inflammatory effects (Ruxton, 2011). However, due to the high level of unsaturation of omega-3 PUFA they get easily oxidised resulting in the loss of their good organoleptic and nutritional properties. Hence, the stabilization of the oil against autoxidation is essential for its industrial use.

The addition of a combination of antioxidants with different mode of action to bulk fish oils (Drusch, Groß, & Schwarz, 2008) or emulsions (Serfert, Drusch, & Schwarz, 2009) achieved efficient stabilization. In addition, microencapsulation of fish oil by spray-drying in presence of antioxidants can further extend the shelf life of fish oil (Serfert et al., 2009). The enhancement of the stability is due to the amorphous structure of the carbohydrate-based glassy-state matrix of the microcapsules reducing the molecular mobility of oxygen and thus slowing down the rate of lipid oxidation. Regarding the use of antioxidants, it has been shown that antioxidants efficient in stabilizing liquid systems do not necessarily increase the stability of an oil encapsulated by spray-drying (Serfert et al., 2009). Furthermore, the location of the antioxidants in the disperse system has impact on its antioxidant activity,

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http://dx.doi.org/10.1016/j.foodchem.2015.08.122
0308-8146/

ideally being positioned at the oil/water-interface with its specific microenvironment where autoxidation begins (Oehlke, Heins, Stöckmann, Sönnichsen, & Schwarz, 2011). Hence, there is increasing interest in the utilisation of emulsifying compounds with antioxidant properties like proteins or protein hydrolysates. Recently it was shown that whey protein hydrolysates can increase the stability of microencapsulated fish oil compared to microcapsules stabilized with non-hydrolysed whey protein (Tamm et al., in press).

The antioxidant properties of FPH have been studied for a wide range of species and enzymes (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; García-Moreno et al., 2014; Klompong, Benjakul, Kantachote, & Shahidi, 2007; Wu, Chen, & Shiau, 2003). The antioxidant activity of the hydrolysates is affected by the protein source and the conditions of hydrolysis (i.e., degree of hydrolysis; enzyme, pH, substrate to enzyme ratio, etc.), since these factors determine the peptide profile being essentially responsible for the antioxidant and functional activities (Chalamaiah et al., 2012). Some of the discard species of Alboran Sea, such as sardine (*Sardina pilchardus*) or horse mackerel (*Trachurus mediterraneus*) have been described as an adequate substrate for the production of hydrolysates with antioxidative properties when treated with Alcalase, trypsin or their mixture even when achieving a relative high DH (>14%). These species exhibited DPPH radical scavenging activity, reducing power and iron chelating activity, however a high DH may involve too small peptides with no suitable functional properties for the stabilisation of emulsions (García-Moreno et al., 2014). The antioxidant and functional properties of FPH have been studied for hydrolysates of yellow stripe trevally (*Selaroides leptolepis*) at different degree of hydrolysis (DH) and employing different enzymes (Klompong et al., 2007). However, the functional properties were described in terms of emulsifying or foaming activity, but did not include experiments on the stability of FPH emulsions or the behaviour during spray-drying. With respect to the use of FPH for the physical and oxidative stabilisation of emulsions, different molecular weight fractions of cod hydrolysates have been employed; nonetheless, the physical stabilisation of emulsions was conducted by adding emulsifiers such as Citrem instead of taking advantage of the interfacial properties of the FPH (Farvin et al., 2014). Physical stability of emulsions prepared using cod hydrolysates has been described, but no research on the oxidative stability of the emulsified oil was conducted (Petursson, Decker, & McClements, 2004). To the best of the authors' knowledge, there are no previous studies related to the microencapsulation of fish oil stabilised with FPH.

In this work, two discarded species of the Alboran Sea, namely sardine (*S. pilchardus*) and horse mackerel (*T. mediterraneus*) were hydrolysed to a degree of hydrolysis (DH) 5% and 10% (FPH(5) and FPH(10)) with two different enzymes: Alcalase and trypsin. FPH were used as emulsifiers and antioxidants in the production of emulsions (oil load 5%) and subsequent spray-drying to yield microcapsules with an oil load of 14.33%. Finally the course of lipid oxidation in these microcapsules (stored at 20 °C and 33% relative humidity) was monitored for a period of 12 weeks. The aim of this work was to study both functional and antioxidative properties of FPH to evaluate the potential of the use of FPH for the microencapsulation of sensitive lipophilic substances.

2. Materials and methods

Raw sardines (*S. pilchardus*) and horse mackerels (*T. mediterraneus*) were purchased from the fishing harbour of Motril (Spain) in March 2014. They were kept in ice during the transportation and storage. Muscle meat was manually prepared by retiring bones, skin and viscera, grinded in a cutter SK-3 (Sammic, Guipúzcoa, Spain) and kept at −80 °C until use. Refined fish oil (Omevital 18/12 TG Gold; 21% of EPA and 14% of DHA) was acquired from BASF Personal Care and Nutrition GmbH (Illertissen, Germany). For the enzymatic hydrolysis, two serine endoproteases were employed; subtilisin EC 3.4.21.62 of bacterial origin, and pancreatic trypsin, EC 3.4.21.4 from animal sources. Both enzymes were provided by Novozymes (Bagsvaerd, Denmark) namely Alcalase 2.4 L and PTN 6.0 S, respectively. Glucose syrup (DE38, C*Dry 1934) and MCT oil (CremerCOOR MCT 60/40) were purchased from Cargill Germany GmbH (Krefeld, Germany) and Cremer Oleo GmbH & Co. KG (Hamburg, Germany), respectively. All other chemicals and solvents used were of analytical grade.

2.1. Characterisation of the composition of the fish muscle meat and the fish protein hydrolysate

The proximate composition of the muscle meat and lyophilized hydrolysates was determined following the official methods of the AOAC (2006). Briefly, ash and moisture content were gravimetrically measured by heating the samples until constant weight at 550 °C and 103 °C, respectively. Protein content was analysed following the Kjeldahl method and employing a nitrogen-to-protein conversion factor of 6.25. Total lipid fraction was evaluated according to the Soxhlet extraction method.

Amino acid composition of the protein hydrolysates was determined by complete acid digestion of the samples coupled with separation by reversed phase chromatography (Liu, Chang, Yan, Yu, & Liu, 1995). To this end, 100 mg of powdered sample were dissolved in 5 mL of 6 M HCl for 22 h at 112 °C. This reaction was conducted under nitrogen atmosphere to avoid amino acid oxidation. After cooling down, the hydrolysate was diluted 50-fold with MilliQ water and filtered through a 0.45 μm syringe filter (Cronun 25 mm PES FFPS2545, EMD Millipore, Billerica, MA, USA) to remove suspended particles. An aliquot of 10 μL of this filtrate were mixed with 70 μL of borate buffer and 20 μL of the derivatisation reagent (AccQ·Fluor Reagent WAT052880, Waters Corporation), consisting of a solution of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in acetonitrile. The reaction took place at 55 °C for 10 min, forming asymmetric urea derivatives which present high fluorescence emission.

The derivatives were subsequently separated by reversed-phase chromatography, employing a column of 2.1 mm (inner diametre) × 100 mm (length) packed with silica-based particles of 1.7 μm (AccQ·Tag Ultra RP Column 186003837, Waters Corporation, Milford, Massachusetts, USA).

The sample was eluted in a mobile phase composed of AccQ·Tag Eluent A, acetonitrile and water at 37 °C and the derivatives were detected after separation by UV detection at 248 nm.

To analyse the molecular weight distribution of the peptides produced, a tris-tricine SDS–PAGE under reducing conditions was conducted as described elsewhere (Tamm et al., in press). Samples were diluted with tris-tricine buffer (Bio-Rad Laboratories GmbH, München, Germany). In order to detect the presence of disulphide bonds, these analyses were conducted in the presence and absence of 1 M DL-dithiothreitol (DTT) solution (Sigma Aldrich, Taufkierchen Germany) as reducing agent.

Samples were then heated to 90 °C for 5 min and loaded on a 16.5% tris-tricine gel (Bio-Rad cat# 345-0065). The separation was conducted employing tris-tricine running buffer (Bio-Rad, cat#161-0744). The gels fixation was conducted using an aqueous methanol/acetic acid (40/10%) solution with 0.025% Coomassie brilliant blue G250 (Serva Electrophoresis GmbH, Heidelberg, Germany) were used to fix gels. Finally, as marker a commercial mixture of seven peptides, which covered the range between 2.5 and 17 kDa (cat# MWSDS17S, Sigma Aldrich) was employed.

2.2. Enzymatic hydrolysis of fish muscle meat

An aliquot of muscle meat, containing 50 g of protein, was homogenised with demineralised water until reaching a final volume of 1 L. For both degrees of hydrolysis (DH 5 and DH 10) the enzyme-substrate ratio was set to 0.5 for Alcalase and 0.125 for trypsin, whereas the temperature was fixed to 50 °C and the pH to 8. Hydrolysis was conducted in an automatic titrator 718 Stat Titrino (Metrohm AG, Herisau, Switzerland) and the DH was estimated with the pH-stat-method, as a function of the base consumption (Eq. (1)):

$$\mathrm{DH} = \frac{B \cdot N_b}{\alpha \cdot m_p \cdot h_{TOT}} \cdot 100 \quad (1)$$

In the Eq. (1), B refers to the amount of base consumed, N_b to the normality of the base, α to the average degree of dissociation of the α-NH_2 amino groups released during the hydrolysis, m_p to the mass of protein in the substrate and h_{TOT} to the number of equivalents of peptide bonds per gram of protein. At pH 8 and temperature of 50 °C, the 88.5% of the amino groups are dissociated, while h_{TOT} was assumed to be 8.6 meq/g of protein, as reported in literature (Nissen, 1986). A solution of 1 M sodium hydroxide was employed to automatically adjust the pH. Samples were heated to 100 °C for 15 min to deactivate the enzyme and then centrifuged (20,000g, 15 min) to extract manually the residual oil and remove the remaining solids. Finally the sample was filtered under vacuum (pore size: 10 μm). The purification step was carried out twice and samples were stored at −20 °C until they were lyophilized in a Labconco freeze drying system (Kansas City, MO, USA).

In the text, sardine and horse mackerel protein hydrolysates produced with Alcalase are referred as SAH and HAH respectively; whereas in the case of trypsin they are denoted as STH and HTH. When necessary the DH is indicated in brackets, e.g., SAH(5) or SAH(10) for sardine hydrolysates produced with Alcalase at DH 5 and 10 respectively.

2.3. Determination of the antioxidant activity and the interfacial tension of the fish protein hydrolysates

Antioxidant activity was measured employing three methods: DPPH scavenging activity, Fe^{3+} reducing power and iron (Fe^{2+}) chelating activity.

In order to evaluate the antioxidant activity, the evolution of the DPPH scavenging activity of the hydrolysates the method described by Picot et al. (2010) was used. Briefly, 1 mL of each protein hydrolysates solutions (ranging from 1 to 30 mg/mL) was mixed with 1 mL of 0.1 mM DPPH in methanol. The mixture was stirred and incubated for 30 min in the dark. Finally, the absorbance was measured at 517 nm. Furthermore, A blank was run in the same way by using distilled water instead of sample, and a sample control, using methanol instead of DPPH solution, was made for each sample. All measurements were done in duplicate. DPPH scavenging activity was calculated by Eq. (2):

$$\text{DPPH scavenging activity},\% = \left(1 - \frac{A_{sample} - A_{control}}{A_{blank}}\right) \quad (2)$$

The reducing power was determined according to the method of Oyaizu (1986). An aliquot of 2 mL of each hydrolysate (1–30 mg/mL) were mixed with 2 mL of 0.2 mM phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide. Then, samples were incubated (50 °C, 20 min). After that, 2 mL of 10% TCA was added and they were centrifuged at 1,500×g for 10 min. Finally, 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride were added to 2 mL of supernatant. After 10 min, the absorbance was measured at 700 nm. As a control, instead of sample distilled water was used. Analyses were carried out in triplicate.

The iron chelating activity of the fish protein hydrolysates was estimated by the method described by Decker and Welch (1990). In short, 1 mL of hydrolysate solution (0.2–1.2 mg/mL) was mixed with 3.7 mL of distilled water and 0.1 mL of ferrous chloride 2 mM. After 3 min, by adding 0.2 mL of ferrozine 5 mM the reaction was inhibited. Then, sample were stirred and incubated at room temperature during 10 min. Finally absorbance was measured at 562 nm. As blank water was used instead of sample and as for the sample control ferrozine was not added. Duplicate measurements were done. The chelating activity was calculated with Eq. (3):

$$\text{Metal chelating activity},\% = \left(1 - \frac{A_{sample} - A_{control}}{A_{blank}}\right) \quad (3)$$

The interfacial tension at the oil/water-interface was measured using a drop tensiometre OCA20 (Dataphysics GmbH, Filderstadt, Germany). The oil phase employed was MCT oil treated with magnesium silicate (Florisil® 60–100 mesh) to remove surface-active compounds. A drop of the dissolved protein hydrolysate (0.01–2% by weight (wt%); pH: 2, 3 and 8 adjusted with a solution of 0.1 M HCl) was created at the tip of a needle using a computer-controlled dosing unit. The interfacial tension was automatically calculated from the drop shape and was monitored for 30 min. All experiments were conducted at 20 °C in duplicate.

2.4. Preparation of FPH-stabilised emulsions and evaluation of the stability of the liquid emulsions

For optimising the composition of emulsions with fish protein hydrolysates (FPH) at DH 5 and DH 10 the influence of the protein content (0.5; 1 and 2 wt%) and pH (2, 3 and 8) was varied, whilst the load of MCT oil was fixed at 5 wt%. In all cases the pH was adjusted by addition of 0.5 M HCl and the solution was stirred over night to allow complete rehydration of the protein. Then, a pre-emulsion was prepared by shear homogenisation (22,000 rpm, 90 s, Ystral GmbH, Ballrechten-Dottingen, Germany). Finally, the emulsion was homogenised in a high-pressure homogeniser (Panda 2K; Niro Soavi Deutschland, Lübeck, Germany) at a pressure range of 300/50 bar and applying 2 passes.

The oil droplet size distribution (ODSD) was determined by laser diffraction using a LA-950 (Horiba Jobin Yvon GmbH, Unterhaching, Germany). Results of the volume distribution are presented as the 10th, 50th and 90th percentiles of the oil droplets. The zeta potential was determined employing electrophoretic light scattering (Zetasizer Nano-ZS, Malvern Instruments GmbH, Herrenberg, Germany) once emulsions were diluted 20-folds in water. Both measurements were conducted in triplicate.

Stability against creaming was evaluated as described by Pettersson et al. (2004). Emulsions were store in glass tubes at room temperature for 7 days and the creaming index was calculated as the percentage of phase separation. This parametre is used as an indirect measurement of the droplet aggregation. Emulsions were qualitatively characterised using light microscopy with 40× increment using a Motic B3 series microscope (Motic Deutschland GmbH, Wetzlar, Germany) to visually check whether there was coalescence or aggregation in the emulsions. Finally, conductivity of emulsions was measured with a WTW conductivity-metre LF96 (WTW GmbH, Weilheim, Germany).

2.5. Emulsion preparation and spray-drying of emulsions for microencapsulation of fish oil

Emulsions for subsequent spray-drying were prepared with 2 wt% of FPH(5), 5% of fish oil and 28% of glucose syrup as

described in Section 2.4, glucose syrup was added prior to emulsification. The pH was adjusted to 2.0 by adding 0.5 N HCl. Spray-drying was conducted on a Mobile Minor (Niro A/S, Copenhagen, Denmark) at 180/70 °C inlet/outlet temperature and 4 bar with rotary atomization resulting in 22,000 rpm.

ODSD of fresh and reconstituted emulsions; and zeta-potential (ζ-potential) and conductivity of reconstituted emulsions were measured as explained in Section 2.4. The efficiency of the encapsulation can be measured by extracting the non-encapsulated oil, which is mainly located at the particle surface or in interstitial voids located close to the surface (Drusch & Berg, 2008). The extractable oil content was determined gravimetrically after extraction of the fat with petrol ether (Westergaard, 2004). Results are expressed in wt% of the extracted mass against the total fat content of the powder.

2.6. Storage and determination of the hydroperoxide content of microencapsulated fish oil over time

To examine the stability of the encapsulated oil the microcapsules were stored in the dark in desiccators over a saturated solution of magnesium chloride (resulting in 33% of relative humidity in the headspace) at 20 °C. The hydroperoxide content was analysed once per week during 12 weeks. Extraction of the oil was conducted by blending the rediluted powder with a mixture of 2-propanol/isooctane (1:1, v/v). The hydroperoxide content was measured using the thiocyanate assay as described by Drusch et al. (2012). Briefly, a mixture of the extracted oil was diluted with 2-propanol and incubated at 60 °C for 30 min after the addition of iron-II-chloride and ammonium thiocyanate solution. After cooling down the sample to room temperature the absorbance was measured at 485 nm (Novaspec II Spektralphotometer, Uppsala, Sweden). Extractions were done in duplicate and hydroperoxide determination of each extract at least with two replicates.

2.7. Statistical analysis

The Statgraphics software (version 5.1) was used to conduct a one way analysis of variance (ANOVA) on the data. Mean values were compared by employing Tukey's test. Differences between means were considered significant at $p \leqslant 0.05$. Furthermore, Grubb's test was employed for identifying outlier values of the ODSD.

3. Results and discussion

3.1. Proximate composition and amino acid composition of fresh muscle meat and lyophilized FPH

The fresh muscle meat of both species showed similar proximate composition with low percentages of oil and ash (Table 1). These data slightly differ from those of the whole fish (including bones, skin and viscera) published by García Moreno, Pérez-Gálvez, Morales-Medina, Guadix, and Guadix (2013). The content of ash found in the literature was higher, which is due to the presence of bones and skin in the whole fish sample but not in purified muscle meat. With respect to the FPH, all samples exhibited a similar content of protein, whilst the amount of oil is almost double in horse mackerel samples. The ash content was, in all cases, higher compared to the fresh muscle, this may be due to the increase of salts in the media caused by the addition of 1 M sodium hydroxide solution during hydrolysis.

The amino acid composition of the raw protein and hydrolysates is shown in Table 1. All samples contained aspartic and glutamic acids as major amino acids, representing almost 45 wt% of the total amino acid content. Lysine, arginine and leucine were also present in relatively high amounts. These data are in accordance with those reviewed by (Chalamaiah et al., 2012) where species like capelin (*Mallotus villosus*), pacific whiting (*Merluccius productus*) or red salmon (*Oncorhynchus nerka*) also contained aspartic and glutamic acid in a high proportion. Nevertheless, in the case of herring (*Clupea harengus*) the content of lysine is higher than glutamic acid. On the contrary, it is also common in fish protein and FPH to show low levels of cysteine and tryptophan (Chalamaiah et al., 2012). There was a considerable difference between the average recovered mass of hydrolysates with DH 5 (22.6 ± 1.5 g) and with DH 10 (31.9 ± 4.1 g). This variation may be related to the increase of the solubility of hydrolysates with the DH (Klompong et al., 2007). However, the amino acid composition remained almost constant for the non hydrolysed protein and hydrolysates with both DHs (data not shown).

Table 1
Proximate composition and amino acid composition of muscle of horse mackerel and sardine as well as lyophilized hydrolysates thereof hydrolysed with Alcalase or trypsin.

	Sardine muscle	H. mackerel muscle	SAH(5)	STH(5)	HAH(5)	HTH(5)
Proximate composition, wt%						
Protein	18.8	21.4	85.8	86.6	86.3	87.6
Oil	1.2	1.0	0.3	0.5	0.8	0.8
Water	78.1	77.5	3.0	5.3	4.2	6.7
Ashes	1.5	1.5	12.7	10.9	12.4	11.2
Amino acids composition, wt%						
Asp	27.6	29.4	24.0	30.0	28.6	28.8
Ser	2.9	2.5	3.8	2.5	2.7	2.6
Glu	15.0	15.7	16.4	17.3	16.4	16.3
Gly	5.2	3.5	4.4	3.6	3.8	3.7
His	1.9	2.3	3.8	2.7	3.3	2.8
Arg	6.1	5.5	5.6	5.7	5.9	6.2
Thr	3.4	3.3	3.4	2.9	3.3	3.3
Ala	4.4	4.4	5.0	4.6	4.4	4.4
Pro	2.9	2.3	2.5	2.2	2.4	2.4
Cys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tyr	3.9	2.8	2.4	2.0	2.1	2.3
Val	4.0	4.3	4.3	3.7	3.9	4.0
Met	2.1	2.2	1.9	1.7	1.8	1.8
Lys	7.7	8.8	9.6	9.7	9.7	9.4
Ile	3.6	3.9	3.5	3.2	3.2	3.4
Leu	6.0	6.2	6.5	5.8	6.0	6.0
Phe	3.2	3.1	2.9	2.4	2.5	2.6

3.2. Molecular weight distribution of muscle and FPH by SDS–PAGE

As depicted in Fig. 1 hydrolysis led to significant changes in the molecular weight distribution of both proteins for all FPH. Non-hydrolysed sardine and horse mackerel muscle contained a high amount of peptides larger than 17 kDa with a slight band at around 10.6 kDa. FPH(5) showed a continuous band from 2.5 to 10.6 kDa. Among FPH(10) exclusively SAH(10) presented a slight band between 3.5 and 8.2 kDa, in the other samples the absence of band may indicate that all peptides were smaller than 2.5 kDa and diffused through the gel. No differences between patterns prepared in the presence and absence of a reducing agent (DL-dithiothreitol, DTT) were found (data not shown). These findings are supported by the results of the amino acid composition (Table 1), as no cysteine was detected in any of the samples. Accordingly no disulphide bonds can be formed in the muscle protein and in the FPH.

The molecular weight distribution of the hydrolysates depends not only on the conditions of hydrolysis but also on the intensity of the purification step. For instance, in the case of the hydrolysis

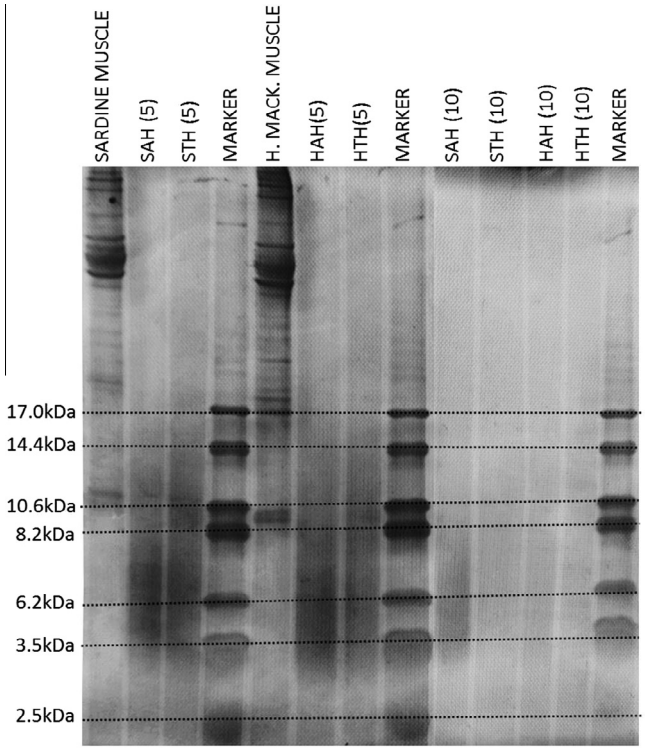

Fig. 1. SDS–PAGE of sardine and horse mackerel raw protein and hydrolysates produced with Alcalase and trypsin (DH 5 and DH 10).

of yellow stripe trevally using Alcalase (50 °C, pH 8.5) with subsequent purification at 2,000*g*, the molecular weight profile contained a high proportion of large peptides compared to the hydrolysates of the present study at DH 5, which were purified at 20,000*g* (Klompong, Benjakul, Kantachote, Hayes, & Shahidi, 2008).

3.3. In vitro antioxidant properties of FPH

Since antioxidants can act by several mechanisms, three different in vitro assays were conducted: DPPH radical scavenging activity, Fe^{2+} chelating activity and Fe^{3+} reducing power (Fig. 2). DPPH determines the ability of a substrate to transfer electrons or hydrogen atoms which can react with free radicals to form more stable compounds. The chelation of metal ions may be an effective tool to decrease the amount of metals available in the media and to avoid their contact to reactive oxygen species. It is well known that, metals catalyse lipid oxidation due to their ability to produce alkyl radicals and reactive oxygen species. Furthermore, they decompose hydroperoxides by accelerating the autoxidation process (Shahidi & Zhong, 2010). Reducing power is related to the ability of compound to donate an electron to free radicals converting them into more stable compounds. By this electron donation, an oxidised antioxidant molecule can be regenerated (Shahidi & Zhong, 2010). However, they could also act as pro-oxidants by reducing metals present in the medium. Ferrous iron, considered the most powerful pro-oxidant, is much stronger (100-fold) than ferric iron due to its higher solubility and reactivity (Shahidi & Zhong, 2010). Additionally, by means of the Fenton reaction, reduced metal ions (mainly cuprous and ferrous) can produce hydroxyl radicals, which are considered the most harmful reactive oxygen species with respect to lipid oxidation (Shahidi & Zhong, 2010).

Among the range of concentrations studied, all antioxidant activities showed a dose-dependent behaviour. In the case of DPPH, the activity increased linearly ($r^2 > 0.99$) and with a sharp slope in case the concentrations ranged between 1 and 10 mg/mL, whereas for higher concentrations the slope was much lower. On the contrary, for reducting and chelating capacities, the slope remained constant ($r^2 > 0.95$). As a general trend, DPPH activity and reducing power decreased significantly ($p < 0.05$) with the increase of DH (data not shown) whereas the opposite behaviour was found for the chelating activity. The effect of the enzyme and the fish species on the antioxidant properties were exclusively significant at DH 5.

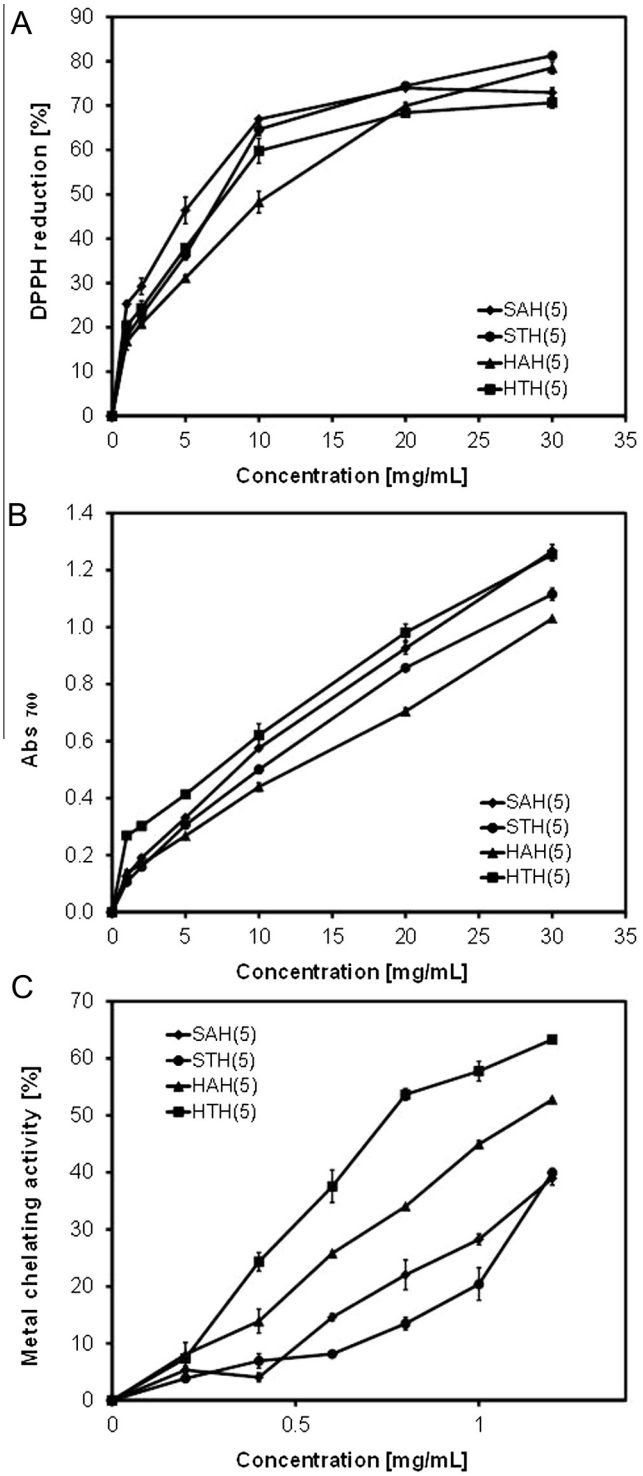

Fig. 2. In vitro antioxidant activity of FPH(5) produced with Alcalase or trypsin (A) DPPH radical scavenging activity, (B) chelating activity and (C) reducing power.

These current results are in line with those reported for horse mackerel (*T. mediterraneus*) and sardine (*S. pilchardus*) treated with mixtures of Alcalase and trypsin (García-Moreno et al., 2014). On the contrary, yellow stripe trevally hydrolysed with Alcalase or Flavourzyme (Klompong et al., 2007) exhibited lower activities.

Since all FPH tested showed scavenging and reducing properties, they may contain peptides capable of transferring electrons or hydrogen atoms and may be able to react with free radicals to form more stable compounds. The difference of the activities that samples presented may be related to the amino acid composition and the peptides size. High DPPH radical scavenging ability is associated to the presence of hydrophobic amino acids (Suetsuna, Ukeda, & Ochi, 2000). Peptides containing amino acid residues as Val, Leu, Ile, Ala, Phe, Cys or Lys at the N-terminal position have been reported to act as good scavengers (Suetsuna et al., 2000). These amino acids are present in a similar proportion (Table 1) being their average content 29.8 ± 0.6 wt%. Moreover, Tyr residues, especially when located at the C-terminal, present strong scavenging and histidine residues have also shown antioxidant properties which may be related to their indolic and imidazole groups (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). As it is shown in Table 1, the average percentage of Tyr and His is 5.1 ± 0.4 wt%. On the other hand, some carboxyl (Glu, Asp) and amino groups (Lys, Arg) at side chains can also act as chelators (Q. Liu, Kong, Xiong, & Xia, 2010). Glutamic and aspartamic acids are the predominant amino acids, their average sum being 45.6 ± 1.5 wt%, increasing up to 53.8 ± 1.0 wt% when taking into account lysine and arginine (Table 1). This percentage is considerably higher than that of possible scavenger amino acids and may explain the increased chelating activity compared to the radical scavenging activity. Additionally, the position of the amino acids plays an important role in the chelating activity. His residues, whose imidazole ring may coordinate with iron, showed higher affinity when located at the N-terminus rather than at C-terminus (Chen et al., 1998). Hence, the differences observed in the chelating activity of the FPH may be due to the size and position of amino acids.

3.4. Interfacial activity of FPH

The impact of protein content and pH on the interfacial tension of SAH(5) solutions is illustrated in Fig. 3A. At acidic pH (2, 3) and for concentrations above 0.1 wt% the interfacial tension values were much lower than at pH 8. These variations may be mainly related to the influence which pH exerts on solubility: in the case of sardine non-hydrolysed protein at pH 8 only 20% is soluble whereas at pH 2 this value increases to 80% (Batista, Pires, & Nelhas, 2007). Despite the increased solubility by intensifying the enzymatic treatment, non-hydrolysed and hydrolysed proteins follow the same trend with pH, as indicated by the isoelectric pH remaining constant (Klompong et al., 2007). Moreover, these differences could be related to the solvent composition (i.e., pH and ionic strength), which influences the ability of the protein to adsorb to interfaces.

Among all FPH at pH 2, SAH(5) with a concentration of 2 wt% exhibited the lowest interfacial tension (5.3 ± 0.3 mN/m) followed by HTH(5) (6.6 ± 0.1 mN/m) and STH(5) (6.8 ± 0.1 mN/m) (Fig. 3B). The protease employed in the enzymatic treatment had a significant effect on the interfacial tension in the case of sardine but not for horse mackerel. On the other hand, comparing data with a concentration of 0.1 wt%, the STH(5) (10.8 ± 0.1 mN/m) followed by SAH(5) (11.5 ± 0.5 mN/m) showed the lowest interfacial activity. The influence of the enzyme may be explained by the selectivity of the proteases employed during hydrolysis: trypsin cleaves at a few specific sites of the amino acid chain while Alcalase cleaves less specific. A more detailed description of the specific cleaves sites of each enzyme has been summarised by Tamm et al. (in press).

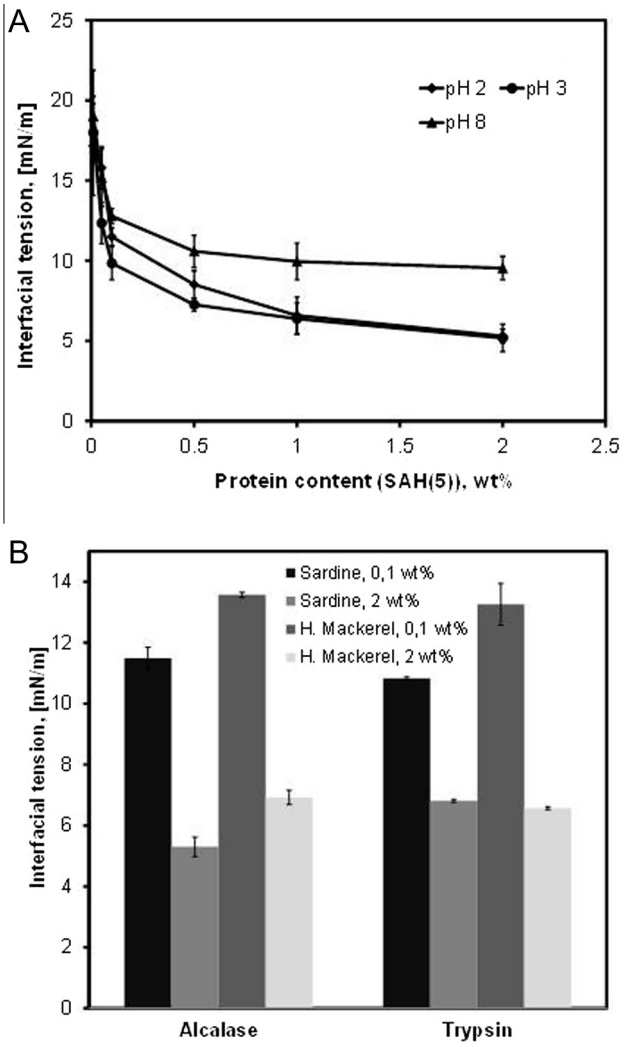

Fig. 3. Interfacial tension of fish protein hydrolysates with a degree of hydrolysis of 5% at the oil/water-interface. (A) Impact of protein (SAH(5)) content and pH. (B) Impact of enzyme type at pH 2 at different concentrations.

3.5. Selection of pH and composition of the emulsion for microencapsulation by spray-drying

Among all samples of protein, only when using FPH(5) stable emulsions for spray-drying could be produced. The solubility of the lyophilised unmodified substrate was found poor and the peptides in FPH(10), most of them less than 2.5 kDa as shown in Fig. 1, are too small for adapting themselves to the interface with good emulsifying properties. Apparently, a relatively high amount of peptides larger than 2 kDa is required for avoiding emulsion instability, e.g., hydrolysates possessing only peptides smaller than 500 Da did not give stable emulsions (Caessens, Daamen, Gruppen, Visser, & Voragen, 1999).

Different emulsion formulations were tested in the present study with respect to the physical stability, varying the pH (2, 3 and 8) and the protein content of FPH(5) (0.5, 1 and 2 wt%). FPH (5) stabilized emulsions at pH 2 and 3 but not at 8 (data not shown). The stability of the emulsions increased as the percentage of protein was increased until a maximum of 2 wt%. At acidic pH, the emulsions with the lower percentage of protein (0.5%) presented creaming (6.2%) at the first day, which was mainly caused by coalescence and minor aggregation. The ODSD of fresh emulsions showed a median of 0.60 ± 0.10 μm and 0.48 ± 0.07 μm at

pH 2 and pH 3, respectively. As a consequence of coalescence and aggregation, the median increased to 0.94 ± 0.01 μm and 2.00 ± 0.09 μm after one day of storage. In the case of emulsions with 1 wt% of protein less creaming (3%) and coalescence occurred. The ODSD increased from 0.58 ± 0.08 and 0.40 ± 0.02 μm to 1.22 ± 0.06 and 1.50 ± 0.2 μm at pH 2 and pH 3, respectively. In the higher protein concentration tested (2 wt%), emulsions at pH 2 were the most stable with no creaming during 7 days. Furthermore, the median increased from 0.36 ± 0.00 to 0.44 ± 0.05 μm during storage.

At acidic pH, the ζ-potential of all emulsions ranged from 40 and 55 mV. These results are to some extent higher than those obtained with cod protein hydrolysate at pH 2 (30 mV) (Petursson et al., 2004). High absolute values of the ζ-potential are important for stabilisation against aggregation by means of electrostatic repulsion. Independent from the protein content, the ζ-potential slightly increased with pH, e.g., emulsions with 1 wt% of FPH(5) at pH 3 presented: 53.0 ± 0.9 mV and at pH 2: 45.0 ± 0.1 mV. In addition, all emulsions produced at pH 2 showed higher conductivity and hence, higher ionic strength. Although, it has been described that an increase in the ionic strength may result in electrostatic screening effects with a consequent reduction in the ζ-potential, in the present study the opposite was observed. This can be explained when taking into account that the ζ-potential increases with the concentration of protein as well as the ionic strength of media (due to the addition of 0.5 N HCl). It seems that the ζ-potential of the emulsions in the present study is more affected by the increase of the protein content rather than electrostatic screening effects. For emulsions stabilised with FPH, pH plays an important role on their stability because it may govern not only solubility but interfacial properties and, consequently, the steric conformation at the interface. In summary, emulsions stabilized with 2% of hydrolysates at pH 2 were the most stable, they were selected for the process of microencapsulation.

3.6. Characterisation of fresh emulsions, microcapsules and reconstituted emulsions after spray-drying

Focusing on ODSD of emulsions before spray-drying (Table 2), SAH(5) presented the smallest droplets in all the percentiles. With regard to sardine hydrolysates, the effect of enzyme was only significant in the 90th percentile, whereas for horse mackerel it was significant in all percentiles. Since all samples had the same protein:oil ratio, the ODSD is mainly affected by the functional properties of the peptides, i.e., by their molecular weight distribution and by their amino acids composition. As all emulsions presented median values smaller than 1.5 μm, they were stable enough for spray-drying purposes (Drusch, Serfert, Scampicchio, Schmidt-Hansberg, & Schwarz, 2007).

When comparing the ODSD of fresh and reconstituted spray-dried emulsions, no significant changes were observed for hydrolysates treated with Alcalase, whereas a significant increase was found for samples hydrolysed using trypsin. The extractable oil content for all microcapsules was 2.0 ± 0.1% resulting in a high microencapsulation efficiency of 98.0 ± 0.1% (data not shown); hence despite the changes of ODSD in samples treated with trypsin the integrity of the interface was maintained during spray-drying and all hydrolysates effectively stabilised the oil droplets during atomisation and drying.

3.7. Lipid oxidation of fish oil in spray-dried emulsions stabilised by FPH

The degree of oxidation of the fresh oil employed for the production of emulsions and microcapsules was below the detection limit, whereas fresh microcapsules contained up to 5.5 ± 0.9 mmol/kg oil in the case of STH(5). This increment is in accordance with previous works describing how lipid oxidation can take place during the microencapsulation process due to homogenisation and drying (Serfert et al., 2009). The homogenisation of the emulsion involves intense mechanical stress and turbulences, which can lead to oxygen inclusion and to a better distribution of pro-oxidant species. Additionally, as previously stated, the presence of shear forces during atomisation (Vega & Roos, 2006) as well as the heat of the drying process can to some extent oxidise the lipids (Serfert et al., 2009). The course of the lipid oxidation in the microcapsules stabilised with FPH(5) is illustrated in Fig. 4. After 12 weeks of storage, the hydroperoxide content ranged from 114 ± 18 to 136 ± 17 mmol/kg oil for HAH(5) and HTH(5) respectively. The slope of the oxidation curves provide a rough value of the oxidation rate, SAH(5) with 1.42 mmol/kg oil per day being the fewer value followed by HAH(5) with 1.48 mmol/kg oil per day. Microcapsules stabilised with FPH produced with trypsin, regardless the substrate, showed similar slope values: ~1.61 mmol/kg oil per day. All curves presented a highly linear behaviour ($r^2 > 0.97$) for the entire time of study, with no lag or exponential phase.

Since no stable emulsions could be prepared using the non-hydrolysed protein, a direct evaluation of the release of antioxidative peptides through hydrolysis is not possible. Concerning the general protective effect of the microencapsulation, a comparison with the available literature shows that the lipid oxidation is a similar magnitude as it occurs in other systems. E.g., a similar behaviour was found in the case of microcapsules stabilised with nOSA starch, in which the oxidation presented a linear drift

Table 2
Zeta potential, conductivity and oil droplet size distribution of oil in water emulsions (fresh and reconstituted after spray-drying) stabilised with hydrolysates with a degree of hydrolysis of 5% of sardine and horse mackerel produced with Alcalase or trypsin.

Sample	Percentile of oil droplet size (μm)			Span	Conductivity (mS/cm)	Zeta potential (mV)
	10th	50th	90th			
	Liquid emulsions before spray-drying					
SAH(5)	0.18 ± 0.04^{a}	0.35 ± 0.06^{a}	1.13 ± 0.15^{a}	2.43	n.d.	n.d.
STH(5)	0.19 ± 0.01^{ab}	0.44 ± 0.01^{bc}	1.54 ± 0.10^{bc}	3.10	n.d.	n.d.
HAH(5)	0.21 ± 0.01^{bc}	0.48 ± 0.04^{c}	1.45 ± 0.01^{b}	2.60	n.d.	n.d.
HTH(5)	0.26 ± 0.01^{d}	0.73 ± 0.03^{d}	1.58 ± 0.07^{bc}	1.82	n.d.	n.d.
	Reconstituted emulsion after spray-drying					
SAH(5)	0.17 ± 0.03^{a}	0.37 ± 0.08^{ab}	1.12 ± 0.12^{a}	2.57	1.8	46.9 ± 0.9^{a}
STH(5)	0.24 ± 0.01^{cd}	0.67 ± 0.04^{d}	1.76 ± 0.03^{c}	2.29	1.5	45.1 ± 0.3^{b}
HAH(5)	0.21 ± 0.05^{ab}	0.45 ± 0.08^{bc}	1.44 ± 0.03^{b}	2.71	1.7	48.9 ± 0.4^{c}
HTH(5)	0.27 ± 0.01^{d}	0.82 ± 0.03^{e}	1.69 ± 0.13^{c}	1.72	1.5	44.1 ± 0.2^{d}

Values within a column with different superscript letters indicate significant differences ($p < 0.05$).

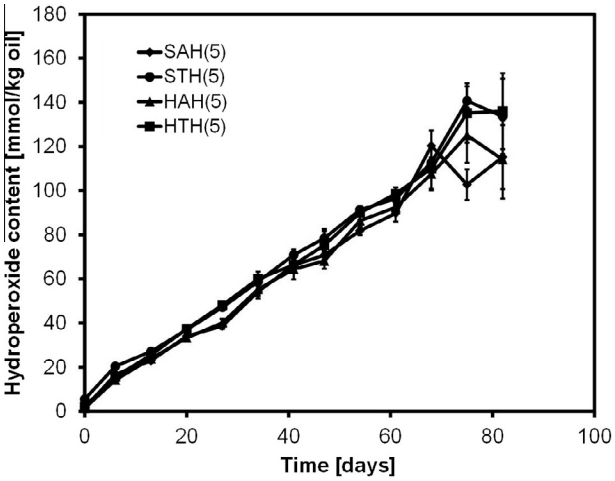

Fig. 4. Development of hydroperoxide content in microcapsules stabilised with FPH produced with Alcalase or trypsin in a degree of hydrolysis of 5%.

(r^2 = 0.96) with a similar slope as FPH(5) produced with Alcalase (1.4 mmol/kg oil per day) (Drusch et al., 2009). Additionally, fish oil has been microencapsulated by using milk proteins (sodium caseinate and hydrolysed casein, DH 7.6) at different protein concentrations (0.25–5 wt%) with glucose syrup and 18 wt% (wet basis) of oil (Drusch et al., 2012). The encapsulation efficiency for microcapsules produced with sodium caseinate was similar to those obtained in this work, whereas casein hydrolysates presented lower values.

More specifically, the current results can be compared to those obtained for the microencapsulation by spray-drying of emulsions stabilised using unhydrolysed and hydrolysed whey proteins (Tamm et al., in press). Microcapsules in the cited study were produced under the same conditions using the same source of fish oil. In the case of non-hydrolysed whey proteins the hydroperoxide content after 11 weeks amounted to 113 mmol/kg oil showing a lag phase of 20 days and was in a similar range like the data for the spray-dried FPH-stabilised emulsions in the present study. Emulsions prepared with whey protein hydrolysates produced with trypsin showed much lower values of 55 and 40 mmol/kg for DH 3 and 6, respectively and thus more effectively protected against oxidation than those stabilised with FPH(5). In general this difference might be related to several factors: the ODSD of parent emulsions, the encapsulation efficiency, antioxidative properties of the hydrolysates, degradation of microcapsules or to physical protection of oil due to the protecting barrier formed around the oil (Elias, Kellerby, & Decker, 2008). The molecular weight profile of the hydrolysates, the amino acids composition and their distribution within the backbone are the parametres which govern the aforementioned properties. Although differences in the molecular weight profile occur, the ODSD and the encapsulation efficiency are similar in both studies. Hence, the differences between oxidation rates might be related to the antioxidative properties of the hydrolysates, a consumption of radicals through radical attack of proteins or to the efficiency of the physical barrier at the interface as discussed in the following section.

Apart from the positive overall performance of the FPH(5)-stabilised emulsions, no correlation between rate of oxidation and antioxidative properties of the FPH was found: SAH(5) and HAH(5) presented similar values of scavenging activity whereas SAH(5) presented better reducing capacity, however the degree of oxidation of both microcapsule was similar. The absence of a clear correlation might be related to several factors, as for instance the narrow range of variation of the antioxidant properties. Furthermore, antioxidant activity in food systems depends on several factors as physical location of the antioxidant, environmental conditions (e.g., pH), or interactions with other food components. However in vitro test often correlate to a specific antioxidant activity against a unique oxidant agent, hence a considerable simplification of the real system is conducted (Decker, Warner, Richards, & Shahidi, 2005). In this study, the emulsions were produced at pH 2 whereas the antioxidant tests were evaluated around neutral pH (as specified in the analysis protocol), hence the structure of the protein can vary and consequently the antioxidant activity. Chelating activity tested in vitro has been found to decrease at acidic pH whereas DPPH radical scavenging activity of ferric reducing power was reported to remain constant (Klompong et al., 2008). In addition, in vitro tests are usually performed in the absence of lipids; the conformation of the protein may vary when located at the interface (Decker et al., 2005) and, hence, the antioxidant properties. So as to determine whether the in vitro antioxidant tests can be a useful tool for the prediction of oxidation of microencapsulated oil, further comparisons between in vivo and in vitro test must be done. Finally apart from an antioxidative effect, consumption of radicals through reaction with proteins may retard lipid oxidation. Oxidation pathways of proteins have been reviewed in the literature, i.e., electrophilic radicals can produce the cleavage of the polypeptide backbone as well as aldehydes can react with histidine, lysine or cysteine by Michael addition (Elias et al., 2008). By means of the hydrolysates oxidation, which can happen if available amino acids are more oxidatively labile than the unsaturated fatty acids, the oil is protected. Further characterisation of hydrolysates should be conducted so as to determinate the degree of degradation of the proteins, i.e., analysing the protein thiol and protein carbonyl groups as indicators of protein oxidation (Eymard, Baron, & Jacobsen, 2009).

4. Conclusions

Hydrolysis of fish protein is a possibility to improve its functional properties. However, type of enzyme and degree of hydrolysis heavily affect the performance of the FPH. FPHs with a degree of hydrolysis of 5% were capable to physically stabilise emulsions, in contrast to the non-hydrolysed protein and FPH(10). Emulsions stabilised with FPH(5) were also physically stable after the homogenisation and subsequent microencapsulation by spray-drying. During storage the encapsulated oil exhibited a similar course of hydroperoxide content as it was reported for microencapsulated fish oil prepared from other matrix systems showing that FPH may serve as an alternative matrix constituent. No differences among substrates (species of fish) and type of enzyme were found. Future research should focus on a more specific characterisation of hydrolysates with a DH lower than 5% and possibilities to produce and purify hydrolysates with a well-defined, tailor-made peptide profile to define a compromise between solubility, emulsifying activity and functionality in spray-dried systems. Hydrolysates may provide additional stability through their localisation at the interface and their antioxidative activity. More basic research on the complex interplay between protein, its alteration and lipid oxidation is required to fully understand the chemical background and its impact on functionality of proteins in these amorphous systems.

Acknowledgements

R. Morales-Medina acknowledges a FPI grant from the Spanish Ministry of Science and Innovation (code EEBB-I-14-08499) and an international exchange grant for young researchers from the DAAD (German Academic Exchange Service; code

91525301-50015537). The present work was funded by the Spanish National Plan I+D+i (CTQ 2011-23009).

The authors gratefully acknowledge the skillful help of Jörg Knipp.

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