# Use of olive and sunflower protein hydrolysates for the physical and oxidative stabilization of fish oil-in-water emulsions

Running title: Upgrading of oilseed by-products as emulsifiers in fish oil-in-water emulsions

Ospina-Quiroga, J. Lizeth, Coronas-Lozano, Cristina, García-Moreno, Pedro J., Guadix, Emilia M., Almécija-Rodríguez, M. Carmen, Pérez-Gálvez, Raúl[1*]

Department Of Chemical Engineering, University of Granada, Spain.

## ABSTRACT

**BACKGROUND**: Olive and sunflower seeds are by-products generated in large amounts by the plant oil industry. The technological and biological properties of plant-based substrates, especially protein hydrolysates, have increased their use as functional ingredients for food matrices. This paper evaluates the physical and oxidative stabilities of 50 $g \cdot kg^{-1}$ fish oil-in-water emulsions where protein hydrolysates from olive and sunflower seeds were incorporated at 20 g protein$\cdot kg^{-1}$ as natural emulsifiers. Our goal was to investigate the effect of protein source (i.e. olive and sunflower seeds), enzyme (i.e. subtilisin and trypsin), and degree of hydrolysis (5%, 8% and 11%) on the ability of the hydrolysate to stabilize the emulsion and retard lipid oxidation over a 7-day storage period.

**RESULTS:** The plant protein hydrolysates displayed different emulsifying and antioxidant capacities when incorporated into the fish oil-in-water emulsions. The hydrolysates with DH 5%, especially those from sunflower seed meal, provided higher physical stability, regardless of the enzymatic treatment. For instance, the average D[3,2] values for the emulsions containing sunflower subtilisin hydrolysates at DH 5% only slightly increased from 1.21 ± 0.02 μm (day 0) to 2.01 ± 0.04 μm (day 7). Moreover, the emulsions stabilized with sunflower or olive seed hydrolysates at DH 5% were stable against lipid oxidation throughout the storage experiment, with no significant variation in the oxidation indices between days 0 and 4.

**CONCLUSIONS**: These results support the use of sunflower seed hydrolysates at DH 5% as natural emulsifiers for fish oil-in-water emulsions, providing both physical and chemical stability against lipid oxidation.

**Keywords**: Sunflower seeds, olive seeds, protein hydrolysates, fish oil-in-water emulsions, physical stability, oxidative stability

[1*]Corresponding author: rperezga@ugr.es. Department of Chemical Engineering. University of Granada, 18071 Granada, Spain. Tel: +34 958243360

# 1. INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are fatty acids (FA) containing two or more double bonds in the carbon chain. Several studies report the beneficial health effects associated with the dietary intake of PUFAs, especially long chain (LC) ω-3 PUFAs like eicosapentaenoic acid (EPA; 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3), among others [1–3]. The regular consumption of these FA has been linked to lower the risk of cancer[1], cardiovascular disease[4], type 2 diabetes (by increasing insulin sensitivity)[5], as well as mental disorders such as Alzheimer's, dementia [6], or depression[7], among others. Although LC PUFAs can be obtained metabolically from the precursor α-linolenic acid (ALA; 18:3 ω-3) their conversion rate is less than 4 %[3], being necessary to incorporate ω-3 PUFAs in the diet[8]. The major dietary sources for EPA and DHA are fatty fish and seafood[9], whose intake is usually deficient in Western diets[10]. As a reference, an intake of nearly 0.3-0.45 g of EPA and DHA per day (around two servings of fatty fish per week) is recommended[3]. On this basis, functional foods enriched with LC ω-3 PUFAs have been gaining attention as an effective option to increase the amount of ω-3 PUFAs in the diet. Nonetheless, incorporating fish lipids as ingredients into dietary supplements, pharmaceuticals or functional foods is challenging[11] due to their low water solubility and physical and chemical instability during processing and storage [12]. In this regard, fish oil has a high trend to oxidize owing to the multiple double bonds in ω-3 PUFAs, and their oxidation products could lead to undesirable rancid off-flavors and even be harmful after frequent intake[13]. To overcome this, some successful techniques have been developed to encapsulate fish oils into colloidal systems like oil-in-water (O/W) emulsions, providing better dispersibility, stability, and bioavailability[14].

Simple emulsion systems generally are formed by high shear mixing of oil and water, adding synthetic surfactants or animal protein-derived compounds as emulsifiers[11]. Emulsions are thermodynamically unstable systems where oily and aqueous phases tend to separate, being necessary to incorporate an emulsifier/surfactant to provide kinetic stability. These compounds with amphiphilic properties are capable to adsorb at the oil-water interface, reducing interfacial tension and providing physical stability to the emulsion by steric hindrance or electrostatic repulsions [12]. In addition, is common to oxidatively stabilize emulsions by adding synthetic antioxidant compounds able to retard different lipid oxidation mechanisms. However, despite their high availability and low production costs, the consumption of synthetic antioxidants has been related to adverse side effects (e.g., skin allergies, gastrointestinal disorders, or even cancer risk associated with long-term intake)[10,14]. This drawback, and the clean-label trend in the food industry, has increased the interest in searching novel emulsifiers and antioxidants of natural origin[15] like peptides with bioactivity and technofunctional characteristics. The last exerts multiple advantages such as biodegradability, sustainable production, and suitable performance in extreme media conditions of pH, temperature, and salinity[16]. Moreover, the properties of the interface are crucial, since this environment is the frontier where transition metals and lipid hydroperoxides interact, forming free radicals that initialize oxidative reactions[17]. Therefore, the use of amphiphilic peptides, which are located at the interface and

also exhibit antioxidant properties, is an effective approach to control lipid oxidation in emulsions, reducing the number of ingredients used[12].

Peptides with emulsifying and antioxidant properties traditionally are obtained through chemical hydrolysis of food-source proteins[18] where strong acids or bases are employed to cleave peptide bonds at high temperatures and prolonged reaction times. However, is not a preferred method since has several limitations like salt and toxic compounds generation, loss of nutritional value due to amino acid degradation, and poor selectivity and control of the reactions. In contrast, enzymatic hydrolysis is a well-known technique employed to modify and enhance the limited technological properties of native plant proteins under mild conditions with high specificity and no residual organic solvents and toxic chemicals present in the final product[19–21]. Enzymatic proteolysis generates short fragments of proteins with enhanced solubility, which might also exhibit superior amphiphilicity and antioxidant properties[22,23]. For example, Chang Liu et al.[24] investigated the effects of enzymatic hydrolysis (DHs from 0 to 15%) of fava bean protein by Alcalase on the physical and oxidative stability of 5% rapeseed oil-in-water emulsions. They concluded that limited hydrolysis by Alcalase (DH <10%) led to an improvement in the physical and oxidative stability of emulsions and recommended avoiding extended hydrolysis as it might negatively impact the emulsifying activity of the hydrolysates.

Different high-protein sources have been explored and employed to obtain peptides with bioactive and techno-functional characteristics, with those from animals as the most source studied (e.g., meat, milk, egg, fish, etc.)[25 26]. In contrast, protein-peptides from vegetable sources (e.g., cereals, fruits, oilseeds, legumes, etc.) have gained attention due to their sustainabilityas well as an increased interest to shift animal sources towards vegetarian/vegan diets[25].

Likewise, the current concern about the negative environmental impact of processes has led to a growing interest in a circular economy model where wastes could reincorporate into the process or be the star point to a new one as raw materials. Considering this, agro-industrial wastes and by-products, like those from oilseed processing, are a protein-rich source that also has been considered as an alternative to obtaining bioactive peptides[26]. For instance, the vegetable oil industry generates cakes and/or meals as by-products, once the extraction of oil from oleaginous seeds is carried out, with an important presence of protein, mineral, and special constituents that can be valorized as a source of several industrial important compounds[27]. In the case of olive oil, almost 75% of the global annual production comes from European Union countries near the Mediterranean Sea, bringing with it environmental pollution by olive mill wastes as they contain high amounts of organic materials and complex substances hardly biodegradable. Nevertheless, these wastes have been considered an economic resource to obtain valuable products like antioxidants, enzymes, and biogas fuel. The cake obtained after pressing, de-oiled, and drying has approximately 130 $g \cdot kg^{-1}$ of lipids, 160 $g \cdot kg^{-1}$ of proteins, 20 $g \cdot kg^{-1}$ of sugars, and 15 $g \cdot kg^{-1}$ of tannins[28]. As with the olive oil industry, the production of sunflower oil generates different by-products such as sunflower defatted meal, whose protein content can reach 660 $g \cdot kg^{-1}$ [29]. In the same way that oilseeds production is constantly increasing to meet human needs (food, feed, and biodiesel) so do their by-products, estimating a yearly outcome

of around 351 million tons worldwide. Thus, there is an increasing interest in their protein valorization via enzymatic hydrolysis to produce peptides with bioactive and techno-functional characteristics[30–33]. To our knowledge, only a few works[32,34,35] have investigated the techno-functional and antioxidant *in vitro* properties of olive or sunflower seed hydrolysates, although both substrates are generated extensively by the plant oil industry and could be upgraded as a source of natural additives for cosmetic, pharmacological, and food preparations.

Therefore, this work aimed to investigate the potential of protein hydrolysates from olive and sunflower seed meals to physiochemically stabilize fish oil-in-water emulsions. To this end, the meals were hydrolyzed at three different degrees of hydrolysis (DH 5%, 8%, and 11%) by employing subtilisin or trypsin as single enzymes. These hydrolysates were evaluated as antioxidant emulsifiers in fish oil-in-water emulsions, which were studied for their physical and oxidative stabilities over a storage period of seven days at 25 °C. Hence, this work investigates the influence of the enzymatic process (e.g. DH and type of enzyme) on the emulsifying and antioxidant properties of olive and sunflower protein hydrolysates when applied to a food model system such as fish oil-in-water emulsions.

# 2. MATERIALS AND METHODS

## 2.1. Materials

All chemicals were of analytical grade and purchased from Sigma Aldrich (Merk, New York, NJ, USA). Olive (*Olea europaea*) and Sunflower (*Helianthus annuus*) seed meals, were purchased from Q'omer (Valencia, Spain) and Bernabé Campal (Salamanca, Spain), respectively. Previous studies have tested the antioxidant properties of protein hydrolysates from both plant substrates when incorporated as ingredients (20 $g \cdot kg^{-1}$) in 5% fish oil-in-water emulsions.[36]Plant meals were analyzed for their protein content, presenting average values of 209 $g \cdot kg^{-1}$ and 246 $g \cdot kg^{-1}$, respectively. As for their lipid content, it ranged between 80 – 140 $g \cdot kg^{-1}$ for the olive meal and less than 40 $g \cdot kg^{-1}$ for the sunflower meal. The enzymes Alcalase 2.4 L (subtilisin EC 3.4.21.62) and PTN 6.0S (trypsin 3.4.21.4), purchased from were Novozymes (Bagsvaerd, Denmark), were employed to obtain protein hydrolysates from both plant substrates as described in section 2.3. The fish oil-in-water emulsions were prepared with refined fish oil (Omega Oil 1812 TG Gold) acquired from BASF Personal Care and Nutrition GmbH (Illertissen, Germany).

## 2.2. Characterization of the plant meals and hydrolysates

The protein content of the plant meals and the protein hydrolysates was estimated through elemental analysis. To this end, the samples were subjected to complete combustion and a thermal conductivity detector identified the electrical signal of combustion products ($CO_2$, $H_2O$, $N_2$, and $SO_2$) which is proportional to each elemental concentration (C, H, N, and S). A Flash 2000 CHNS/O elemental analyzer (Thermo Scientific, Waltham, MA, USA) was employed assuming a nitrogen-to-protein content factor of 5.3[37].

## 2.3. Enzymatic hydrolysis

Protein hydrolysates at degree of hydrolysis (DH) 20% were obtained from the plant meal substrates, following the protocol described by Ospina-Quiroga [36]. To this end, a solution containing 25 $g \cdot kg^{-1}$ of protein was prepared by mixing the different plant meals with distilled water at 50ºC. The solution was then transferred to a jacketed glass reactor of 1 L capacity. The pH was set to 8 and maintained constant throughout the reaction by a pH-stat titrine (718 STAT Titrino, Metrohm, Switzerland) employing 0.5 M sodium hydroxide as the titration agent. Subtilisin or trypsin was employed singly as the catalyst, with an enzyme-to-substrate ratio of 10 $g \cdot kg^{-1}$. The hydrolysis was allowed until a certain DH (5, 8, and 11%). The DH was estimated as a function of the amount of NaOH consumed during the enzymatic hydrolysis to maintain pH constant[14], according to the pH-Stat method[38]. Then, the reaction was stopped by heating at 90 °C for 10 min, as described in previous literature. Finally, the inactivated mixture was vacuum-filtered through 8 mm cellulose paper and then freeze-dried in a LyoMicron lyophilizer (Coolvacuum Technologies, Barcelone, Spain). These powdered hydrolysates were employed as emulsifiers to prepare the emulsions.

## 2.4. Plant meal solubility and protein recovery

The solids retained after vacuum filtration of the plant meal hydrolysates were dried at 105 °C for 2 h, defining the percentage solubilization as described in a previous work[36]:

$$Solubilization\ (\%) = [1-(m_R / m_0)] \times 100$$

where $m_R$ (g) represents the mass of dried solids retained on the filter paper, and $m_0$ (g) is the mass of plant meal dissolved in distilled water at the start of the reaction. The protein solubilization of the meal after hydrolysis was estimated by the protein recovery index, following the equation:

$$Protein\ recovery\ (\%) = (m_H y_H)/ (m_0 y_0) \times 100$$

where m (g) and *y* represent mass and protein content (g of protein · g of dried $sample^{-1}$) of the plant protein hydrolysate ($m_H$, $y_H$) and plant meal ($m_0$, $y_0$), respectively.

## 2.5. Size exclusion chromatography (SEC) of the hydrolysates

Powdered plant protein hydrolysates were dissolved in distilled water at a concentration of 10 $mg \cdot mL^{-1}$. 500 µL of each solution was injected into a Superdex Peptide 10/300 GL column (GE HealthCare, Uppsala, Sweden) for elution with distilled water as the mobile phase at 0.5 $mL \cdot min^{-1}$. The absorbance of the eluted sample was measured at 280 nm. The molecular weight distribution of PPHs was related to a calibration curve prepared with L-Tyrosine (217.7 Da), Vitamin $B_{12}$ (1355.4 Da), Aprotinin (6512 Da), Cytochrome C (12384 Da), and Ribonuclease A (13700 Da) as standards, as described in previous literature[36,39,40]. For a specific range of molecular weight, the content of peptides was expressed as the percentage area under the curve.

## 2.6. Preparation of the emulsions

Twelve fish oil-in-water emulsions were prepared containing 50 $g \cdot kg^{-1}$ of fish oil and stabilized with 20 $g \cdot kg^{-1}$ of protein from the plant protein hydrolysates, following the recipe employed in our previous works[36,41–43]. The emulsions were coded as EO-XY (olive seed meal hydrolysate) and ESF-XY (sunflower seed meal hydrolysate). The X letter is related to enzyme treatment (S: subtilisin, T: trypsin), and Y to the final degree of hydrolysis reached (5, 8, or 11%). As an example, the emulsions stabilized with sunflower seed hydrolysates produced with subtilisin were coded as ESF-S5, ESF-S8, and ESF-S11. Firstly, the aqueous phase containing the hydrolysate was brought to pH 8.0 and left stirring overnight at room temperature to allow solubilization and rehydration of the protein. Pre-emulsions were prepared by dispersing the fish oil in the aqueous phase employing an Ultra Turrax mixer (IKA Werke GmbH & Co., Staufen, Germany) at 2000×g for 2 min. Then, homogenization was conducted in a high-pressure homogenizer (Panda Plus 2000, GEA Niro Soavi., Lübeck, Germany) at 450/75 bar, running 3 passes. To accelerate lipid oxidation 100 µM of ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was added to the emulsions. Sodium azide 0.5 $g \cdot kg^{-1}$ was also added to the emulsions to avoid microbial growth. The emulsions were stored in amber glass jars at 25 °C in the dark and samples were taken on days 0, 2, 4, and 7 to evaluate their physical and oxidative stabilities.

## 2.7. Zeta potential

The zeta potential (ζ), which is an index to evaluate the net repulsion between oil droplets within the emulsion, was measured on day 1 after preparation, following the protocol described in a previous work[36]. To this end, the emulsions were diluted 500-fold in distilled water and pH was adjusted to pH 8.0. Measurements of zeta potential (mV) were conducted at room temperature on a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) in a range between −100 and 50 mV, and employing a DTS-1060C cell. Measurements were carried out in triplicate.

## 2.8. Droplet size distribution

The droplets constituting the disperse phase present a size distribution which can be characterized by means of the average statistical diameters. In our case, the *Sauter* diameter (D[3,2]), based on the surface/volume of oil droplets, was chosen as reference. The size distribution of emulsion droplets was obtained by laser diffraction, employing a Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK), following the same procedure described in our previous work[36]. The samples were dispersed in recirculating distilled water at 3000 rpm until reaching an obscuration in the range of 12–15%. The Refractive index was 1.481 for the dispersed phase (fish oil), and 1.330 for the dispersant (distilled water). Measurements were carried out in triplicate.

## 2.9. Turbiscan® stability

The physical stability of the emulsions was additionally evaluated by multiple light scattering in an optical analyzer Turbiscan™ LAB (Formulaction, Toulouse, France), following the procedure described by Wang et al.[44].To this end, 25 mL of each emulsion was kept in a glass cell to perform measurements. The analysis was executed considering the change of backscattering (ΔBS) between the initial and final day of the study.

## 2.10. Oxidative stability of the emulsions

PV is one of the most common chemical methods to test the oxidative deterioration of oils which reflects the content of primary oxidation productos (e.g. hydroperoxides). Normally, it is combined with another method of monitoring secondary oxidation products in order to produce a fuller picture of the oxidation progress. In that sense, the measurement of *p*-anisidine index is a reasonable way to estimate secondary oxidation products. This reagent reacts with unsaturated aldehydes and the formed products (2-alkenals) can be detected at 350 nm. Both PV and AV measures are commonly used together for describing the total extent of oxidation by the Totox value, which is the sum of AV and twice the PV. Totox is an empirical parameter that connects two parameters with different units[45]. Based on the results for physical stability, lipid oxidation was measured in selected emulsions over the storage time. For that, the formation of primary and secondary oxidation products was evaluated by determining peroxide (PV)[46] and *p*-anisidine (AV)[47] values, as previously described[14]. Briefly, a mixture of hexane and 2-propanol was employed to extract the oil fraction from the emulsions. For the peroxide index, oil extracted (~ 20 mg) was mixed with 2-propanol, ammonium thiocyanate, and iron II dissolutions. Then, samples were vortexed and incubated for 5 min at 25 °C to measure absorbance at 485 nm in a Genesys™ 30 visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The results are presented in milliequivalents of peroxide per kilogram of oil. For p-anisidine value, oil extracted (~150 mg) was mixed with hexane, vortexed, and separated into two equal volumes. The first volume was mixed with anisidine dissolution in acetic acid, while the second volume was employed as the control. All of them were covered and left in the dark for 10 min before measuring absorbance at 350 nm. The results are expressed as a 100-fold increase in absorbance of a test solution that was reacted with anisidine at certain conditions stipulated by the ISO method. Four measurements for both peroxide and *p*-anisidine were carried out.

## 2.11. Statistical Analysis

Statistical analysis was carried out on Statgraphics Centurion XVI ™. To study the effects of storage time, DH, and enzyme on the physical and oxidative stability of the emulsions, one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were conducted. Data are presented as the mean ± of the standard deviation (SD) with a 95% confidence level.

# 3. RESULTS AND DISCUSSION

## 3.1. Plant meal solubility and protein recovery

Cakes and meals by-products from oilseed processing are a valuable source of proteins (~150-320 $g \cdot kg^{-1}$)[48,49] which could be valorized for food and pharmaceutical applications[31]. The weight protein content of the hydrolysates (Table 1) was determined on the powder samples after vacuum filtration and freeze-drying. Moreover, as solubility is one of the most relevant properties of proteins due to their largest impact on overall usefulness in food systems [50], this table also presents the solubilization percentage of plant meals and protein attained after the enzymatic treatment. We concluded that the enzymatic treatments assayed were effective to solubilize the plant meals (269-546 $g \cdot kg^{-1}$), as well as to release peptides from the vegetal matrix, despite the low DH values reached. Moreover, meal solubilization and protein recovery improved with increasing DH, attaining a maximum at DH 11% for both enzymes. The latter was expected, since poor functional properties of seed proteins (e.g. solubility, foaming, emulsifying properties) have been reported to improve through limited enzymatic hydrolysis (e.g., DH between 1 – 10 %)[51]. In the reaction, the ionizable groups encrypted in the native protein are liberated, promoting protein-water over protein-protein interactions, and thus enhancing their solubility. Nevertheless, it is not the DH by itself the main responsible for the functional properties of resulting peptides. In this sense, more focus should be placed on the structural properties of the peptides formed (e.g., molecular mass, hydrophobicity, and amount of charged groups)[50].

**Table 1**. Protein content of the hydrolysates, meal solubilization, and protein recovery after the enzymatic treatments. Samples are coded as O and SF, respectively, followed by enzymatic treatment (S: subtilisin, T: trypsin) and DH (5, 8, or 11%). Different letters (a,b,c) indicate significant differences ($p < 0.05$) among DH for the same enzyme and substrate. (+) indicates differences ($p < 0.05$) between enzymes, at the same DH and substrate. (*) indicates significant differences ($p < 0.05$) between substrates at the same DH and enzyme.

| Sample | Protein Content (%) | Meal solubility (%) | Protein recovery (%) |
|---|---|---|---|
| OS5 | $16.2 \pm 3.9^{a}$ | $38.7 \pm 5.3^{a}$ | $32.2 \pm 9.0^{a}$ |
| OS8 | $26.6 \pm 1.2^{b}$ | $44.8 \pm 3.0^{a}$ | $60.0 \pm 4.8^{b}$ |
| OS11 | $23.4 \pm 1.0^{ab}$ | $51.5 \pm 2.6^{a}$ | $65.3 \pm 4.3^{b}$ |
| OT5 | $21.9 \pm 1.5^{a}$ | $37.3 \pm 4.6^{a}$ | $48.7 \pm 6.8^{a}$ |
| OT8 | $24.2 \pm 1.1^{a}$ | $39.4 \pm 2.2^{a}$ | $52.2 \pm 3.8^{a}$ |
| OT11 | $24.1 \pm 1.2^{a}$ | $54.6 \pm 2.7^{b}$ | $65.8 \pm 4.6^{b}$ |
| SFS5 | $35.3 \pm 1.4^{a,*}$ | $26.9 \pm 3.6^{a}$ | $31.5 \pm 4.4^{a}$ |
| SFS8 | $40.8 \pm 2.8^{a,*}$ | $32.4 \pm 1.1^{a}$ | $50.7 \pm 3.9^{ab}$ |
| SFS11 | $43.2 \pm 2.9^{a,*}$ | $34.9 \pm 3.9^{a,*}$ | $63.5 \pm 8.3^{b}$ |
| SFT5 | $36.6 \pm 2.9^{a,*}$ | $27.5 \pm 3.8^{a}$ | $39.4 \pm 6.3^{a}$ |
| SFT8 | $44.0 \pm 2.2^{a,*}$ | $30.1 \pm 2.7^{ab}$ | $39.4 \pm 4.1^{a}$ |
| SFT11 | $45.4 \pm 2.4^{a,*}$ | $44.2 \pm 5.1^{b}$ | $59.1 \pm 7.5^{b}$ |

Regarding protein content, the sunflower meal was richer in protein, yielding hydrolysates with contents from 353 $g \cdot kg^{-1}$ (SF-S5) to 454 $g \cdot kg^{-1}$ (SF-T11). Similar protein content is observed in the work of Karefyllakis et al[52] with native mixtures derived from sunflower seeds. In contrast, olive meal hydrolysates presented on average a lower protein content varying from 162 $g \cdot kg^{-1}$ (O-S5) to 266 $g \cdot kg^{-1}$ (O-S8), which is like the olive protein content present in the stones (up to 22%)[53]. Regardless of the enzymatic treatment, olive seed presented higher values of meal solubilization and protein recovery, with OT-11 reaching the maximum values of 658 $g \cdot kg^{-1}$ and 546 $g \cdot kg^{-1}$, respectively. Similar results were observed by Qin et al[51] who reported 27% solubility for defatted sunflower seed meal. The authors improved this value by 22% after a combined treatment of resin adsorption and enzymatic hydrolysis employing alcalase as catalyst.

The limited solubility of these plant meals could be related to their content in globulins, which are the major storage proteins in oilseeds[54], as well as other compounds such as fibers[55,56]. Moreover, defatted oilseed by-products may present poor technological properties due to the binding of proteins to phenolic compounds or their denaturation after high oil extraction temperatures[29,57]. To overcome this issue, enzymatic hydrolysis has been extensively employed to improve both the solubility and emulsifying capacity of native proteins[50,54,57,58]. In this work, two serin endopeptidases, subtilisin and trypsin, were selected as proteases for the hydrolysis of the oilseed meals. Both enzymes have been reported to improve emulsifying properties of plant-based proteins[59]. Subtilisin is a broad-spectrum endoprotease of bacterial origin that cleaves preferably peptide bonds involving aromatic and methionine amino acids while trypsin is extracted from animals and cleaves only when the carbonyl group is followed to positively charged amino acids arginine and/or lysine. Both enzymes release peptides with hydrophobic or polar residues which exhibit surface active properties[38,60–62]. When it comes to comparing the enzyme treatment effect on meal solubility and protein recovery at the same DH, no significant differences were found for both olive and sunflower hydrolysates. Protein hydrolysates can display different structures and functionalities depending on the enzyme employed. Nevertheless, the substrate should also be considered as functionality will depend on the distribution over the peptide chain of amino acids with hydrophobic side chains as well as their positive or negative charges. Besides, protein solubility is also affected by other properties like pH and ionic strength[50].

## 3.2. Molecular weight distribution of the hydrolysates

The molecular weight distribution of the olive and sunflower hydrolysates (Figure 1) comprises four peptide fractions, namely A (> 10 kDa), B(5-10 kDa), C(1-5 kDa) and D(<1 kDa) The fractions A (~64% area under the curve) and D (~20%) were the most abundant for olive seed hydrolysates. According to the statistical analysis, neither enzymatic treatment nor DH had a significant effect on the molecular weight distribution of the olive seed hydrolysates, except for the hydrolysate OS5 whose content in peptides below 1 kDa was the highest in the experimental series. In contrast, the fraction of medium-sized peptides C (1-5 kDa) was the most abundant (~50%) in the sunflower hydrolysates, irrespective of the enzymatic treatment and the DH,

while fractions A and D were minoritary. The significant differences between the molecular weight profiles may suggest that the protein substrates were degradated by proteases following different mechanisms. The MW distribution of olive hydrolysates, where large peptide species (> 10 kDa) and short-chain peptides/free amino acids (< 1 kDa) were predominant, is compatible with the zipper mechanism of proteolysis, where the polypeptide chain is unfolded to a large extent under experimental conditions. This structure is highly accessible to enzyme attack, releasing large peptides which are quickly hydrolyzed into oligopeptides and free aminoacids. On the contrary, the high content of intermediate peptides in sunflower hydrolysates is compatible with a one-by-one mechanism, where the protein presents a globular structure which limits the expore of the target peptide bonds to endoproteases[63,64].

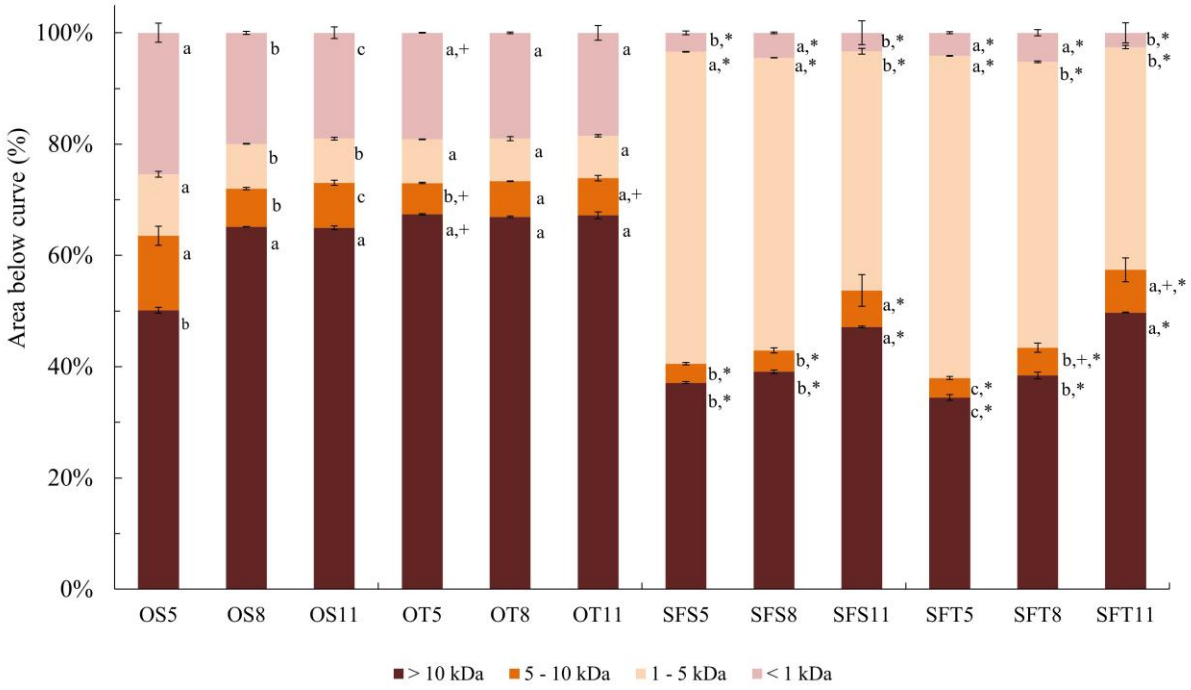


**Figure 1**. Molecular weight distribution (percentage area under the curve) of the olive and sunflower seed hydrolysates. The hydrolysates are coded as O or SF, respectively, followed by the enzymatic treatment (S: subtilisin, T: trypsin) and the DH reached (5, 8, or 11%). Different letters (a,b,c) indicate significant differences ($p < 0.05$) among DH for the same enzyme and substrate. (+) indicates differences ($p < 0.05$) between enzymes, at the same DH and substrate. (*) indicates significant differences ($p < 0.05$) between substrates at the same DH and enzyme.

The extent of the proteolysis has a direct impact on the MW distribution of the hydrolysates, and therefore their expected functional properties, due to three phenomena: average reduction in the peptide size, increase of the amount of ionizable groups and higher exposure of hydrophobic groups[65]. According to Figure 1, the content of large peptides (> 10 kDa) increased with DH for both substrates, except for tryptic olive hydrolysates where no significant differences were observed. This unexpected trend conforms with previous authors who have reported no changes in the molecular weight distribution or even an increase of high molecular weight fractions as DH increases[15,66]. This was attributed to the formation of soluble aggregates

between peptides and other non-protein compounds by hydrogen bonds, as reported for other plant substrates such as chickpea hydrolyzed with subtilisin[67] in the range of DH (4-14%).

The potential emulsifying properties of hydrolysates are dependent on several properties of the peptides adsorbed in the interface, such as secondary structure, amino acid composition or chain length . To this regard, short peptides and free amino acids are commonly reported to exhibit poor emulsifying properties due to their low concentration of hydrophobic sites, which reduces its interfacial adsorption. On the contrary, medium-sized and large peptides are able to unfold at the interface, exposing their hydrophobic domains to the lipophilic phase[68,69]. In this regard, Yang et al[70] investigated the incorporation of different peptide fractions (<5 kDa, 5-10 kDa, and >10 kDa) from rice glutelin tryptic hydrolysates on the stability of 100 $g \cdot kg^{-1}$ oil-in-water emulsions at pH 7.0. The best surface hydrophobicity and emulsion stability were achieved for large peptides (>10 kDa), followed by intermediary peptides (5 – 10 kDa), while short peptides displayed poor interfacial properties. Considering this, the abundance of large and medium-sized peptides in the olive and sunflower hydrolysates supports their use as emulsifiers.

## 3.3. Physical stability of the emulsions

### 3.3.1. Zeta potential

Zeta potential is the main criterion to evaluate the repulsion forces between the oil droplets dispersed in the emulsion. Its magnitude is a direct indicator allowing to predict emulsion destabilization by flocculation and coalescence phenomena. The zeta potential in emulsions stabiliced with protein emulsifiers is largely influenced by pH, as well as concentration, net charge and interfacial properties of the peptides adsorbed[71]. In our case, the differences among zeta potential values are mostly attributable to the protein source, since all the emulsions were stabilized at pH 8.

Figure 2 shows the zeta potential of the emulsions stabilized by olive and sunflower seed hydrolysates. Olive and sunflower seed hydrolysates All the emulsions displayed negative net charges as the carboxyl ($–COO^–$) groups present in the peptides are de-protonated at high pH values above the isoelectric point[15]. Both sunflower and oilseed hydrolysates at DH 5% presented an average isoelectric point around pH 2 (results not published), carrying negative charge under experimental conditions (pH 8). The ζ-potential of the emulsion will be affected by the net charge of the peptides, as well as their ability to adsorb onto the oil-water interface. The net charge of protein emulsifiers prevents oil droplet coalescence by electrostatic repulsion. In this sense, absolute ζ values higher than 30 mV are commonly required for providing physical stability to emulsions[70,72]. In general, emulsions prepared with olive seed hydrolysates showed zeta potential values ranging from -39.1 ± 0.8 to -56.6 ± 1.4 mV while those prepared with sunflower hydrolysates exhibited less negative values, ranging from -34.2 ±1.0 to -37.4 ± 0.9 mV. Nevertheless, all emulsions displayed absolute ζ values higher than 30 mV, which may suggest sufficient electrostatic repulsions to assure the physical stability of emulsions.

Regarding the emulsions with olive seed hydrolysates, the absolute ζ values increased significantly with DH. The enzymatic treatment with trypsin led to absolute ζ values significantly higher than those observed for the subtilisin treatment (DH 8 and 11%). On the contrary, no significant differences were found for the emulsions prepared with sunflower seed hydrolysates, independent of both the DH and enzyme treatment, except for the hydrolysate at DH 11%.

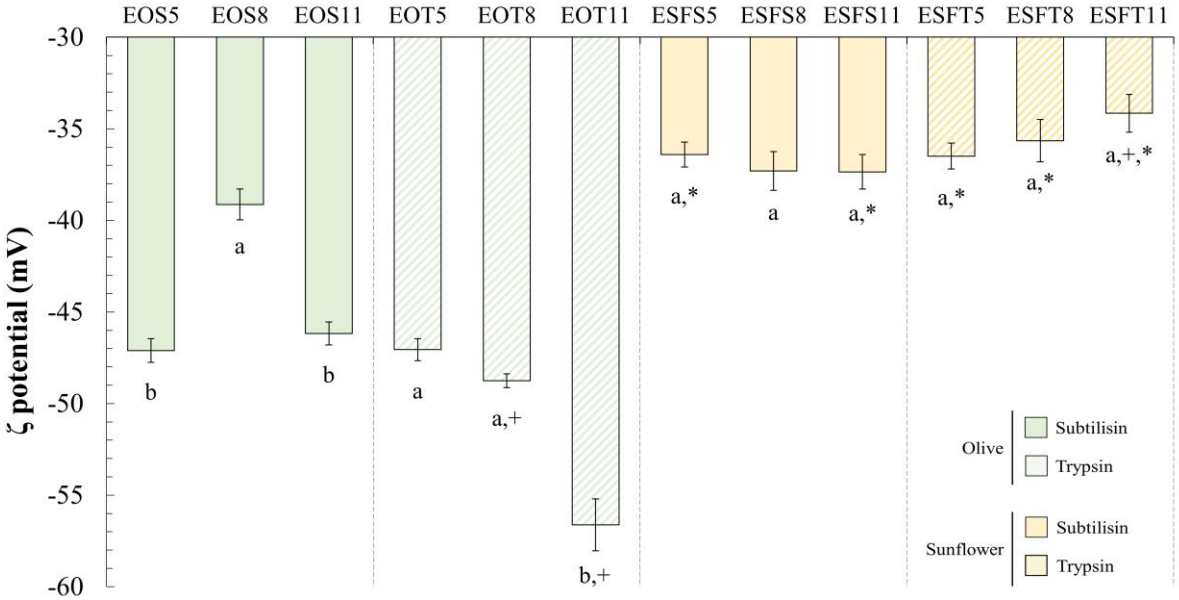


**Figure 2**. Surface net charge (on day 1 at pH 8.0) for the 5% fish O/W emulsions stabilized with 2% of olive and sunflower seed hydrolysates. Samples are coded as EO or ESF, respectively, followed by the enzymatic treatment (S: subtilisin, T: trypsin) and DH (5, 8, or 11%). Different superscript letters (a,b,c) indicate significant differences ($p < 0.05$) among DH for the same enzyme and substrate. (+) indicates differences ($p < 0.05$) between enzymes, at the same DH and substrate. (*) indicates significant differences ($p < 0.05$) between substrates at the same DH and enzyme.

The average zeta potential values were significantly lower for sunflower seed hydrolysates, for all the DHs and enzymatic treatments assayed. Similar values of zeta potential (-36 to -50 mV) were reported by Tamm et al[73] in 100 $g \cdot kg^{-1}$ rapeseed oil-water emulsions stabilized with 20 $g \cdot kg^{-1}$ of pea protein hydrolysates at DH 1% - 6% and pH 8.0. The differences observed between the emulsions stabilized with olive and sunflower could be related to the differences in net charge and pI of the peptides present in the hydrolysates, as well as the type and the local concentration of peptides/proteins adsorbed at the interface[74].

Karefyllakis et al[52] studied the emulsifying potency of different protein fractions (i.e. cold-press sunflower cake, pure protein, fibre-based) from sunflower seeds. The storage triacylglycerols present in sunflower and other oleaginous plants are normally surrounded by phospholipids and proteins, mainly oleosins, building intracellular organelles known as oil bodies. The ζ potential of the fractions was measured through the pH interval from 3 to 10, reporting for all the samples

a plateau (- 20 mV) at pH 7, similar to the average ζ values found in this work for the sunflower emulsions. The sunflower samples presented a typical zero charge point at pH 4.0-4.5, which was explained by the negatively charged domains of oleosins present at the oil body membranes, as well as their interaction with storage proteins. It was found that increasing contents of fibre in the plant mixture shifted the zero charge point towards lower pH, even below pH 3 for ratios fibre:protein higher than 4. An increasing content of fibres conferred larger negative charge to the sunflower protein mixtures, suggesting electrostatic protein-polysaccharide complexation. This interaction has been also described for proteins from soy or sugar beet .[75,76]

In our case, the more negative ζ potentials observed for the emulsions stabilized with olive seed protein hydrolysates could be attributed to their significantly higher content in dietary fibre (530-610 $g \cdot kg^{-1}$) compared to that of sunflower (~ 230 $g \cdot kg^{-1}$), according to the data provided by the supplier.

### 3.3.2. Turbiscan scattering and oil droplet size distribution

The physical stability of the emulsions during the storage time was initially monitored through the Turbiscan change of backscattering (ΔBS) as a function of the cell height, for the emulsions stabilized with olive (Figure 1S) and sunflower seed hydrolysates (Figure 2S). For example, the emulsions prepared with olive hydrolysates at DH 5% (i.e. EOS5 and EOT5 in Figure 1S) displayed an increasing negative ΔBS at the bottom of the cell as the days elapsed, which indicates that emulsions in that zone become less concentrated in oil droplets. On the contrary, ΔBS at the top of the cell increased, suggesting a major concentration of oil droplets at the top. On this basis, creaming could be the principal phenomenon destabilizing those emulsions. However, in the middle of the cell, ΔBS signal changes through time, which suggests that not only oil droplet migration but also size variations lead to physical destabilization. Overall, it was observed that the emulsions prepared with subtilisin and trypsin sunflower hydrolysates at the DH 5% (i.e. ESFS5 and ESFT5 in Figure 2S) displayed the best stability, compared to their counterpart emulsions with olive seed hydrolysates. Irrespective of the plant substrate, the backscattering profiles showed a marked trend toward destabilization at increasing DH.

This is in line with the tendency observed in the oil-droplet size distribution of the emulsions at day 0 and end of the storage period (day 7), reported as D[3,2] mean diameters (Figure 3). Olive protein hydrolysates produced with subtilisin led to emulsions with initial D[3,2] ranging from 1.4 ± 0.02 μm to 9.4 ± 0.67 μm, observing significant differences in D[3,2] values at day 0 among DH. These initial values increased significantly throughout the storage period, attaining a maximum of 12.3 ± 0.16 μm for EOS11. The emulsions stabilized with olive trypsin-hydrolysates presented a significant increase in the D[3,2] during the storage period, attaining final mean diameters at day 7 in the interval from 5.7 ± 0.03 μm to 18.0 ± 6.54 μm. Overall, the enzymatic treatment led to no significant differences among olive-hydrolysate emulsions at the same DH. Regarding the emulsions stabilized with sunflower subtilisin-hydrolysates, D[3,2] values at day 0 ranged from 1.2 ± 0.02 μm to 12.6 ± 0.61 μm, where D[3,2] for emulsion

ESFS11 was significantly higher compared to those stabilized with DH5% and DH8% hydrolysates.

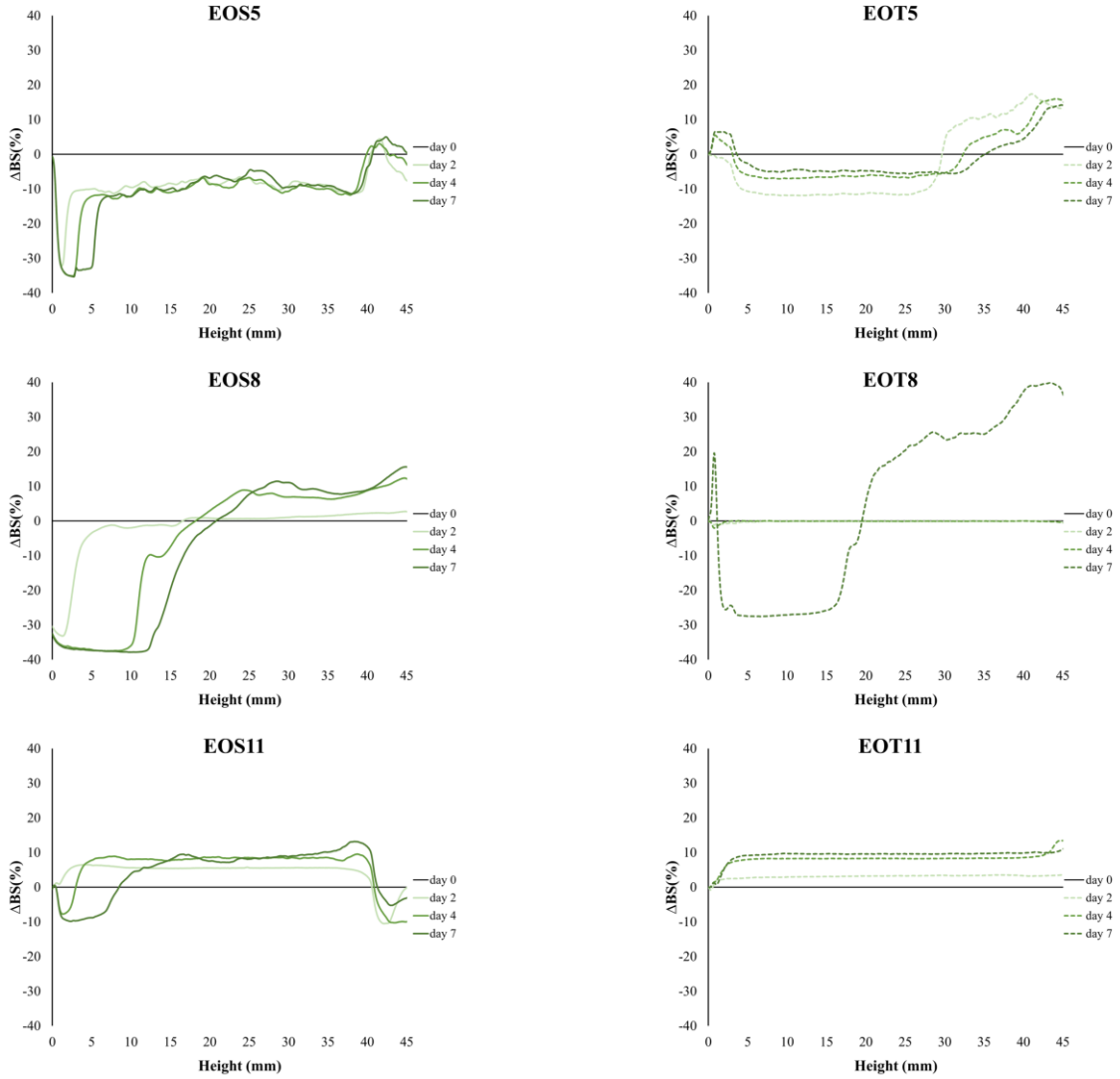


**Figure 1S**. Turbiscan change of backscattering (ΔBS) for the fish O/W emulsions stabilized with olive seed hydrolysates, measured at day 0, day 2, day 4 and day 7. Samples are coded as EO followed by the enzymatic treatment (S: subtilisin, T: trypsin) and DH (5, 8, or 11%).

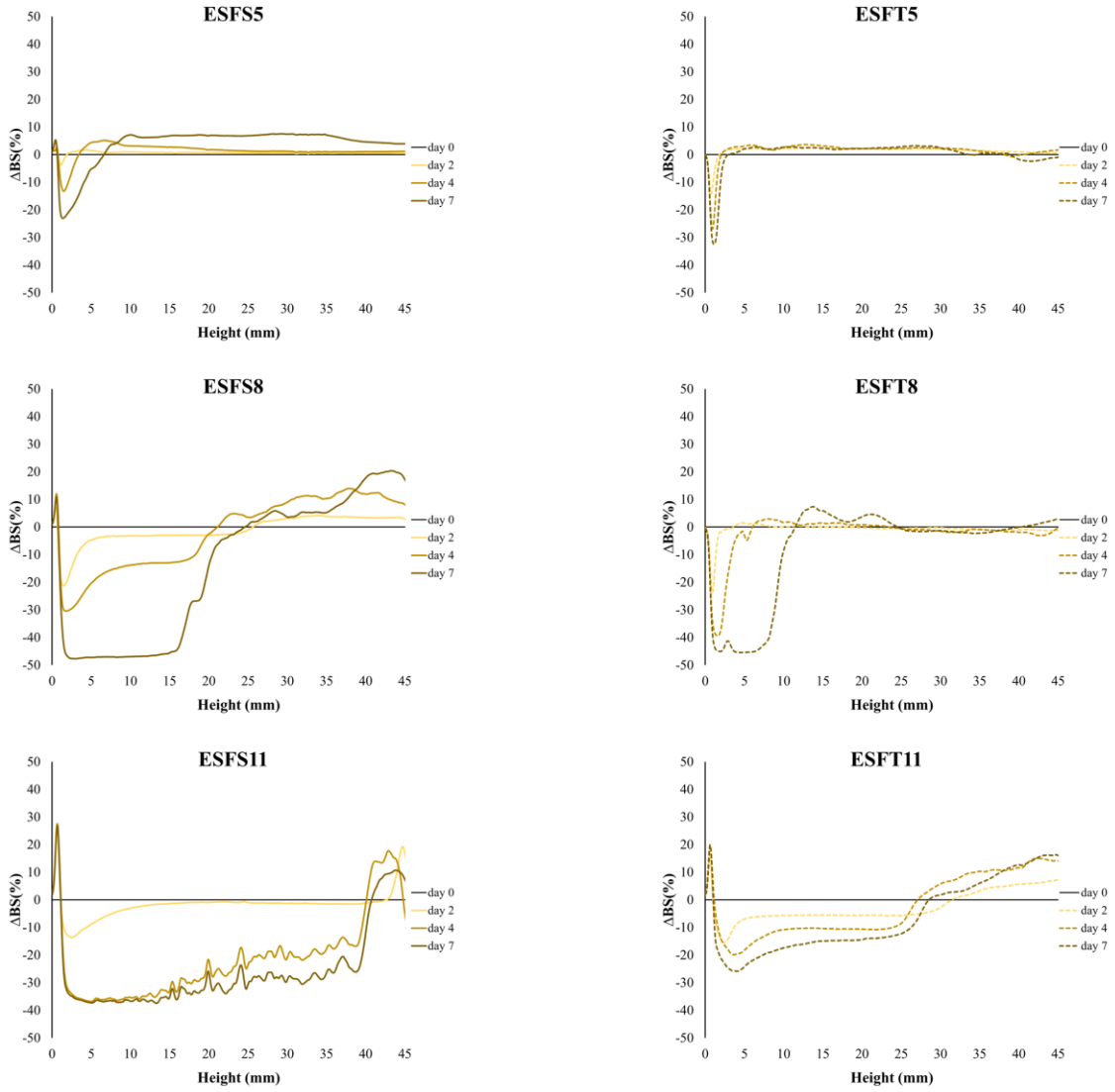


**Figure 2S**. Turbiscan change of backscattering (ΔBS) for the fish O/W emulsions stabilized with sunflower seed hydrolysates, measured at day 0, day 2, day 4 and day 7. Samples are coded as ESF followed by the enzymatic treatment (S: subtilisin, T: trypsin) and DH (5, 8, or 11%).

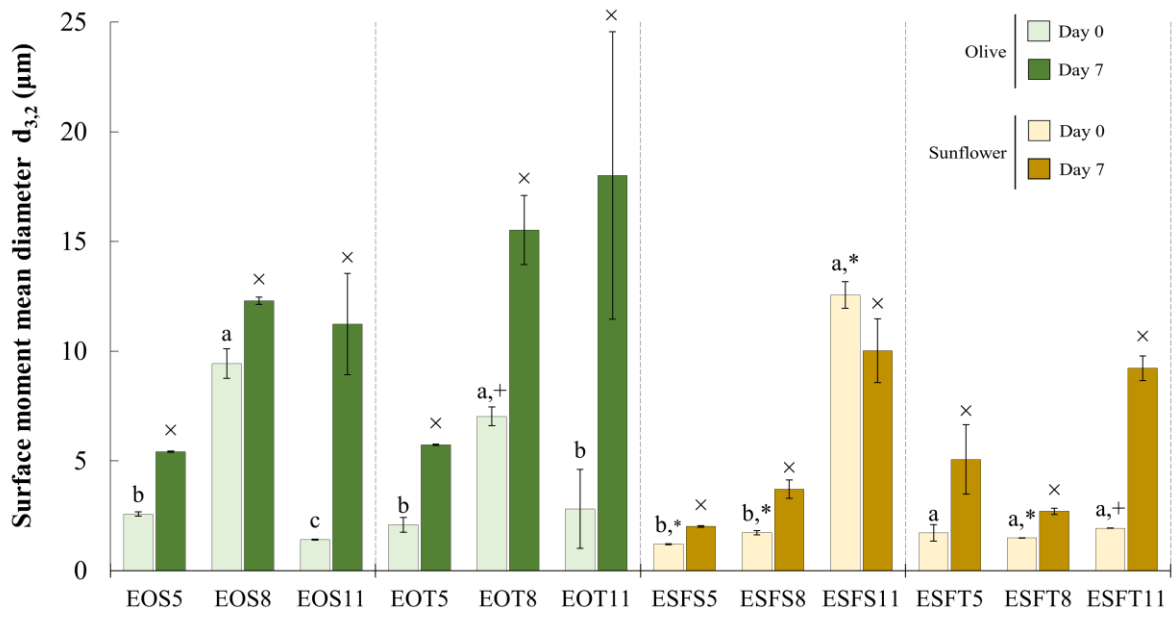


**Figure 3.** Droplet size surface moment mean diameter D[3,2] for the 5% fish O/W emulsions stabilized with 2% protein of olive and sunflower seed hydrolysates. Samples are coded as EO and ESF, respectively, followed by the enzymatic treatment (S: subtilisin, T: trypsin) and DH (5, 8, or 11%). Different superscript letters (a,b,c) indicate significant differences ($p < 0.05$) among DH for the same enzyme and substrate. (+) indicates differences ($p < 0.05$) between enzymes, at the same DH and substrate. (*) indicates significant differences ($p < 0.05$) between substrates at the same DH and enzyme. (×) indicates significant differences ($p < 0.05$) between day 0 and day 7.

Regarding the D[3,2] at the end of the storage study, an increase in droplet size between days 0 and 7 was observed for all the emulsions, except for ESFS11. This increase in droplet size was significantly lower for the emulsions stabilized with sunflower hydrolysates, irrespective of DH and enzymatic treatment. For instance, the emulsions stabilized with sunflower-trypsin hydrolysates presented an average D[3,2] value of 1.7 ± 0.10 μm at day 0, which increased signicantly over the storage period up to 2.7 ± 0.15 μm (ESFT8) and 9.2 ± 0.56 μm (ESFT11).

Taken altogether, we concluded that among variables studied in this work (e.g., substrate, enzyme treatment, and DH) the main factor affecting the physical stability of emulsions was the source of protein, with sunflower protein hydrolysates leading to more physically stable emulsions (i.e. lower droplet size) when compared to olive protein hydrolysates. Physical stability of emulsions could be related to several factors such as the molecular weight distribution of hydrolysates or the adsorption rates of the amphiphilic peptides towards the interface, among others.

As shown in Figure 1, sunflower hydrolysates presented a MW distribution where the fraction between 1-5 kDa was the most abundant, followed by large peptides above 10 kDa, while short-chain peptides below 1 kDa were minority. Emulsifying activity is related to amphiphilic peptides with sufficient length (e.g., above 15 amino acid residues, depending on the secondary

structure β-strand or α-helix, respectively) and with significant hydrophobic patches that could unfold at the oil/water interface[77,78]. Thus, our results indicate that the fraction [1-5 kDa], which was the most abundant in the sunflower hydrolysates, was rich in amphiphilic peptides which conferred better emulsifying properties. Interestingly, emulsions stabilized with olive or sunflower protein hydrolysates with DH 5%, presented lower D[3,2] values when compared to DH 8 and 11% (except for ESFT5), which is in agreement with previous studies reported a superior emulsifying activity when limited hydrolysis (i.e. larger average peptide chain length) is carried out[8,79]. For instance, Chang Liu et al[24] investigated the effect of fava bean protein hydrolysates produced with subtilisin at different DH to stabilize rapeseed oil-in-water emulsions at pH 8.0. They reported that emulsions stabilized with low DH hydrolysates (< 4%) presented lower D[3,2] during the 7 days of storage, in contrast with those emulsions containing hydrolysates with higher DH (9 and 15%).

The formation and stabilization of interfacial protein layers is also related to the concentration of amphiphilic peptides and their rate of adsorption. Although the formation of stable interfacial layer requires a sufficient concentration of protein in the solution, droplet size is mostly affected by the rate at wich amphiphilic peptides adsorb onto the interface. To this regard, peptides presenting high adsorption rates (e.g. due to higher exposure of hydrophobic sites) are able to cover oil-water interfaces rapidly, avoiding droplet coalescence at early stages. This results in the formation of emulsions with smaller droplet size [80].

This fact could explain why the emulsions stabilized with sunflower hydrolysates presented lower droplet size compared to those containing olive hydrolysates. Although the latter exhibited more negative ζ-potential values, implying stronger repulsive forces between oil droplets, their emulsions presented larger droplet size, which could be related to the slow adsorption of the olive proteins onto the interface. This has been attributed to the steric hindrance caused by fibre or protein-fibre interactions. To this regard, Karefyllakis et al[52] suggested that the formation of protein-fibre complexes may reduce their mobility, delaying their diffusion towards the interface. This results in larger droplet size (e.g. 1-10 μm).

## 3.4. Oxidative stability of selected emulsions

Based on the previous results, the emulsions employing olive and sunflower hydrolysates at DH 5% (i.e. ESFS5, ESFT5, EOS5 and EOT5), which showed physical stability throughout the storage period, were evaluated for their ability to redard lipid oxidation. Such reactions generally occur faster in emulsion systems than in bulk oil, due to the exposure to air and generation of free radicals during emulsion processing (i.e. sonication, homogenization), as well as the creation of a large interfacial area where pro-oxidant species make contact with the lipid phase [81].

The Figure 4a shows the peroxide value of the emulsions during storage. No significant differences were observed in the initial peroxide value (PV at day 0) of the four emulsions evaluated in this study, presenting an average value of 6.6 ± 0.6 meq $O_2 \cdot kg^{-1}$ of oil. This initial value is indicative of the level of oxidation reached during emulsion production, and has been

related to the incorporation of oxygen during homogenization as well as pre-oxidation of the lipids naturally occurring in the plant meals [23].

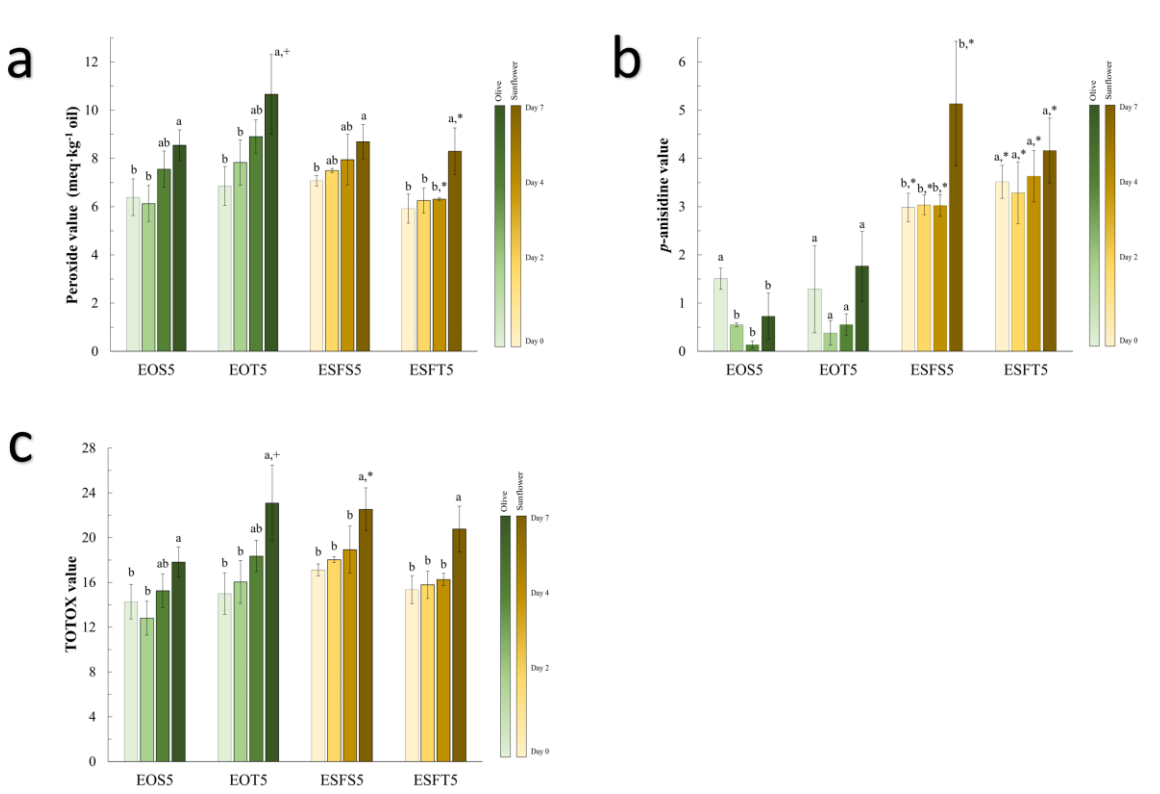


**Figure 4.** Peroxide index (a) p-anisidine index (b) and Totox value (c) for the 5% fish O/W emulsions stabilized with 2% protein of olive and sunflower seed hydrolysates at DH 5%. Samples are coded as EO or ESF, respectively, followed by the enzymatic treatment (S: subtilisin, T: trypsin). Different superscript letters (a,b,c) indicate significant differences ($p < 0.05$) among day of storage for the same substrate and enzyme. (+) indicates differences ($p < 0.05$) between enzymes, at the same substrate and storage time. (*) indicates significant differences ($p < 0.05$) between substrates at the same enzyme and storage time.

PV value increased during the storage period for all the emulsions, observing significant differences at the end of the experiment. Overall, protein source and enzymatic treatment led to no significant differences between the samples, except for the emulsions employing trypsin hydrolysates (ESFT5 and EOT5), where it was observed that the emulsion stabilized with sunflower hydrolysates presented significantly lower values of PV at day 7. These emulsions were less oxidized than those reported in a previous work[14], were sunflower and olive seed hydrolysates (DH 20% employing a mixture 1:1 of subtilisin and trypsin) were incorporated as protein antioxidants (2 g protein·kg$^{-1}$ emulsion) in fish oil emulsions stabilized with Tween20™. In our current study, both hydrolysates were added as emulsifiers at concentrations 10 times higher, which could explain the higher oxidative stability. Similarly, rapeseed hydrolysates were incorporated at 200 μM as antioxidants in rapeseed oil emulsions stabilized with Tween20™ and incubated at 40ºC for 27 days, reporting average PV values around 30 meq $O_2$·kg$^{-1}$ of oil at day 6 of storage, significantly higher than our results[82].

These findings could be explained by the higher average droplet size (D[3,2]) observed in this work. This implies lower specific interfacial area and thus less exposition to pro-oxidant species (e.g. metal ions, free radicals). Furthermore, peptides solubilized in the aqueous phase or adsorbed onto the oil/water interface could retard lipid oxidation by two main mechanisms: (i) chelating metal ions, which act as catalyzers of lipid oxidation reactions, (ii) scavenging free radicals present in the aqueous phase or in the proximity of the interfacial layer[36,81,83]. To this regard, previous works have reported the *in vitro* radical scavenging and metal chelating activities of olive and sunflower meal hydrolysates due to their content in hydrophobic (Val, Leu, Ile), aromatic (Tr, Tyr) or sulfur-containing amino acids. [36,84]

*P*-anisidine index evaluates the decomposition of hydroperoxides (primary oxidation products) into secondary oxidation products which are relatively more stable, like aldehydes. In this work, emulsions stabilized with olive protein hydrolysates exhibited significantly lower values (on average 0.85) when compared to emulsions stabilized with sunflower protein hydrolysates (on average 3.6) (Fig. 4b). Olive emulsions were able to retard significantly hydroperoxide degradation, which could be related to (i) their larger average droplet size compared to their sunflower counterparts, which implies lower specific surface area and thus lower exposition to pro-oxidant species (ii) their lower ability to adsorb at the interface. The latter fact implies that olive peptides will be mostly solubilized in the aqueous phase, where they could chelate metal ions, which catalyze the degradation of hydroxyperoxides. Metal chelation has been related to the presence of amino acid residues with carboxyl groups at the side chain such as Glu, Asp or His[69,85].

The PV and AV results can be related through the total oxidation value (TOTOX), which indicates the overall oxidation state of the samples by the equation TOTOX=2PV+AV[86]. Although there are no oxidation limits established by governments for PUFAs products, some entities like the Global Organization for EPA & DHA Omega-3s (GOED), which represents the worldwide industry of PUFAs products, have suggested a maximum TOTOX level for raw or limited processed oils of 26 meq $O_2 \cdot kg^{-1}$ of oil, based principally on palatability. TOTOX values above this are considered highly oxidized oils which significantly have reduced the PUFAs concentration and therefore their efficacy[13,87,88]. The TOTOX value for the olive and sunflower hydrolysates emulsions were similar, remaining stable up to the 4$^{th}$ day of storage (12.8 ± 1.5) and undergoing a significant increase at day 7 (23.1 ± 3.38) (Figure 4c). It should be noted that although emulsion ESFS5 presented a slightly but significantly higher TOTOX at day 7 when compared to EOS5 (Fig. 4c), the superior physical stability of the sunflower-stabilized emulsion supports its utilization as emulsifier with antioxidant properties for fish oil-in-water emulsions.

## 4. CONCLUSIONS

Protein hydrolysates from olive and sunflower seed meals, obtained enzymatically with either subtilisin or trypsin at DH 5% were able to stabilize fish oil/water emulsions. Sunflower peptides showed higher adsorption to the oil/water interface, yielding stable emulsions with

lower droplet size (e.g. 2.01 μm after 7 days of storage for ESFS5). This fact was attributed to their high content (50.2 %) of medium-sized peptides in the range [1-5 kDa], which are reported to exhibit good amphiphilic properties, providing physical stability in emulsion systems.

Both sunflower and olive protein hydrolysates at DH5% provided oxidative stability to the emulsions over the storage period, observing peroxide and *p*-anisidine values inferior to those reported for other protein emulsifiers. In the emulsions stabilized with olive protein hydrolysates, the generation of secondary oxidation products was significantly inferior, which was attributed to their larger droplet size and presence of metal chelating peptides in the aqueous phase. Nevertheless, according to the TOTOX value, which accounts for primary and secondary oxidation products, no significant differences were observed between sunflower and olive seed hydrolysates. Overall, all the emulsions were stable up to 4$^{th}$ day of storage (TOTOX value 12.8 ± 1.5), observing a significant increase at day 7 (23.1 ± 3.38).

Despite the better *p*-anisidine values observed for the emulsions stabilized with olive protein hydrolysates, it should be noted that the use of sunflower seed hydrolysates at DH 5% presented similar TOTOX value and provided significantly higher physical stability. This supports its utilization as emulsifier with antioxidant properties for fish oil-in-water emulsions

## 5. ACKNOWLEDGEMENTS

This research was funded by the project PY20_00021 from the Regional Ministry of Economic Transformation, Industry, Knowledge, and Universities of Andalusia (Spain). J.L. Ospina. acknowledges the grant from the Colombian Ministry of Science, Technology and Innovation for funding her doctoral studies. The authors are very grateful to Novozymes™ for kindly providing the proteases used in this work.

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