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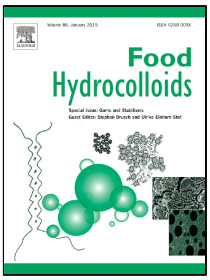

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1 **Encapsulation of fish oil in soybean protein particles by emulsification and spray drying**

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Abstract

This manuscript reports a systematic study of encapsulation of fish oil into soybean microparticles by emulsification and subsequent spray drying in order to protect it from lipid autoxidation and/or facilitate its handling for incorporation into healthy food products. In particular, the effect of the formulation and the emulsification technique on the physicochemical properties of the resulting emulsions and encapsulates was evaluated. Two emulsifying processes and four protein:oil ratios were analyzed. Soy proteins managed to encapsulate the fish oil, masking its characteristic odor and its oily texture into matrix type microcapsules, as was observed by confocal microscopy. The emulsification process was determinant in the efficiency of drying and encapsulation as well as in the protection exerted on the oil oxidative stability. Although both,emulsifying and drying processes caused certain initial oil oxidation (verified by peroxide and TBA indexes), some of the studied systems showed a good perspective of oxidative stability over time(studied by accelerated rancimat test).

Keywords: microencapsulation, fish oil, soybean protein, O/W emulsion, spray drying, **lipid oxidation**.

1. Introduction

It is well established that omega-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), provide important health benefits (Arab-Tehrany et al., 2012; Valenzuela, Tapia, González, & Valenzuela, 2011). Numerous clinical studies and *in vitro* and *in vivo* experiments have confirmedtheir beneficial effect against cancer (De Deckere, 1999; Hull, 2011), cardiovascular and inflammatory diseases (Bays, 2008; Wang et al., 2006; Yokoyama et al., 2007; Goldberg & Katz, 2007; Kim, 1996; Wall, Ross, Fitzgerald, & Stanton, 2010), and immune and neurological disorders (Damsgaard et al., 2007; Dyall & Michael-Titus, 2008; Amminger et al., 2010).

Endogenous synthesis of EPA and DHA from α-linolenic acid (ALA) occurs at a very slow rate (Kralovec, Zhang, Zhang, & Barrow, 2012), therefore their incorporation through diet is recommended (Lands, 2005; Valenzuela et al., 2011) and healthy foods supplemented with these acids are gaining importance in the food market.Marine oils contain large amounts of omega-3 polyunsaturated fatty acids (Bakry et al., 2016). However, due to their low solubility in aqueous solvents, undesirable aroma and high susceptibility to oxidative deterioration, their incorporation into food is a significant challenge (Aghbashlo, Mobli, Madadlou, & Rafiee, 2012, 2013; Aghbashlo, Mobli, Rafiee, & Madadlou, 2012). Microencapsulation of fish oil deals to protect unsaturated fatty acids against oxidation and other unwanted reactions caused by environmental conditions (light, temperature, oxygen, humidity), masking of the unpleasant fish oil flavours and improving the stabilization and delivery of omega-3 fatty acids in foods (Ixtaina, Julio, Wagner, Nolasco, & Tomás, 2015; Encina, Vergara, Giménez, Oyarzún-Ampuero, & Robert, 2016, Bakry et al., 2016).

Various processes such as spray drying, coacervation and extrusion (Drusch & Berg, 2008; Jiménez-Martín, Gharsallaoui, Pérez-Palacios, Carrascal, & Rojas, 2015), and shell materials such as chitosan, maltodextrin, sugars, starch, milk, alginates, plant gums,whey proteins and gelatin have been used to encapsulate fish oil (Garrett, 1965; McClements, 2012).

Spray-drying technique is extensive used in food industry as it is simple, low-cost, reproducible and easy to scale up. Moreover, it is useful for encapsulating heat-sensitive materials such as fish oil because of its short drying times (5-

30 s) (Encina et al., 2016) but some active compounds remain exposed on the microparticle surface (de Vos, Faas, Spasojevic, & Sikkema, 2010). Lipophilic molecules of fish oil requires the previous formation of oil-in-water emulsions, whose stability during spray-drying process plays an important role in the retention and stability of fish oil (Drusch, Serfert, Van Den Heuvel, & Schwarz, 2006; Drusch, 2007; Keogh et al., 2001). Proteins have good emulsifying and film-forming properties (Encina et al., 2016) and consequently they are potential encapsulating agents. During emulsion formation, protein molecules are rapidly adsorbed at the newly formed oil-water interface, and the resulting steric-stabilizing layer prevents oil droplet coalescence and provides physical stability to the emulsion (Vega & Ross, 2006). Protein nature determines the strength and flexibility of the layer. Milk proteins (whey and caseins) (Vega, Dalgleish, & Goff, 2005) and gelatin (Bruschi, Cardoso, Lucchesi, & Gremião, 2003) have been the most used in encapsulation but those of plant origin such as soybean, wheat, sunflower begun to be more and more frequently used (Nesterenko, Alric, Silvestre, & Durrie, 2013). Soybean proteins appear to be suitable for the encapsulation of fish oil since they have acceptable water solubility, good **adsorption** at the water/oil interfaceandadvantageous gelling and film forming properties (Gu, Campbell, & Euston, 2009). Soybean protein concentrates and isolates have been used as encapsulating material of orange oil, palm oil, paprika oleoresin and others (Kim & Morr, 1996; Rusli, Sanguansri, & Augustin, 2006 and Rascón, Beristain, García, & Salgado, 2011) alone or in blends with other biopolymers such as maltodextrin, gelatin, pectin, and others (Yu, Wang, Yao, & Liu, 2007; Favaro-Trindade, Santana, Monterrey-Quintero, Trindade, & Netto, 2010; Mendanha et. al., 2009). As far as we know, **only Nasrin & Anal (2015) encapsulated fish oil in culled banana resistant starch-soy protein isolate based microcapsulesdried by lyophilization.**

The aim of this work was to encapsulate fish oil with soybean protein isolate by emulsification and subsequent spray drying in order to protect it from lipid **oxidation** and/or facilitate its handling for incorporation into healthy food products. In particular, the effect of the formulation and the emulsification technique on the physicochemical properties of the resulting emulsions and encapsulates was evaluated.

2. Materials and Methods

2.1. Materials

A commercial soybean protein isolate (**SPI**) SUPRO 500E, kindly supplied by DuPont N & H (Brazil), was used as encapsulating material. The protein content of SPI, as measured by the Kjeldahl method, was 85 ± 2% (w/w on dry basis; N x 5.71). Fish oil (C22:6 docosahexaenoic acid, DHA, >22%, C20:5eicosapentaenoic acid, EPA, >7% with vitamin E, rosemary extract and BHA as antioxidant)was kindly supplied by OmegaSur (Argentina). All the other reagents used in this study were of analytical grade.

2.2. Emulsions preparation

Oil in water emulsions with different protein:oilmass ratio: 1:1, 2:1, 3:1 and 4:1 were prepared from an aqueous dispersion of SPI (5% w/v) and fish oil addition. They were prepared by two different process: i) using only the high-shear probe mixer Ultra-Turrax T-25 (IKA®, Werke GmbH & Co. KG, Germany) with a dispersion tool S25N-18G (rotor diameter **13.4**mm) operated at 13,500 rpm for 90 s; **and ii) with a second additional step performed with an ultrasonic homogenizer (SONICS Vibra Cell VCX 750, Vibra Cell Sonics Materials Inc., USA) with a standard tip (13 mm diameter) immersed 2/3 in a beaker of 70 mm diameter (250 ml volume) in agitation with a magnetic stirrer and in an ice bath. Each formula was sonicated at an output intensity of 50% (375 W) with 30-second on and 30-second off pulse for 5 min.**

Two individually prepared replicates were at least assayed for each condition to confirm the repeatability of the process.

Sodium azide (0.1%, w/v) was added to the emulsions to prevent microbial growth.

2.3. Emulsion characterization

2.3.1.Morphology of the droplets

Morphology of the droplets was observed by opticalmicroscopy. Each emulsion (20 µL) was placed on a slidewith a cover and immediately examined with an optical microscope equipped with a Leica DC100 digital camera (Bensheim, Germany) using a 100X magnification.

2.3.2. Particle size distribution and mean diameters

Particle size distribution, and De Broucker ($d_{4,3}$) and Sauter ($d_{3,2}$) (Jafari, He, & Bhandari, 2007b) mean diameters of particles of the emulsions were determined by **static light scattering (SLS)** with a particle size analyzer Malvern Mastersizer 2000E (Malvern Instruments, Worcestershire, UK) at room temperature (20°C). About 1 mL of sample was diluted to 600 mL of water and the pump rate in dispersion unit was set at 2000 rpm (Hydro 2000MU, Malvern Instruments, UK). The refractive indexes of the dispersed and continuous phases were 1.47 and 1.33 respectively.

Also, specific surface area was calculated according Equation 1:

$$SSA = \frac{6\,\Phi}{d_{32}} \qquad \text{(Equation 1)}$$

Where: SSA is the specific surface area, ϕ is the phases ratio, $d_{3,2}$ is the Sauter mean diameter(µm).

Measurements were carried out in sixfold.

2.3.3. Rheological behaviour

Rheological measurements were carried out with a Haake RS600 controlled stress oscillatory rheometer (Haake, Germany) using a plate–plate sensor system **with 1.0** mm gap between plates. Measurements were performed in triplicate at constant temperature (25 ± 0.5°C). The samples were subjected to a logarithmic increasing shear rate with a continuous ramp from 1 to 500 s^{-1} in 2 min, followed by a steady shear at 500 s^{-1} for 1 min, and finally a decreasing shear rate from 500 to 1 s^{-1} in 2 min.

Flow behavior of emulsions was described by fitting the experimentally measured data **to the** Newton model:

$$\tau = \eta D \qquad \text{(Equation 2)}$$

Where: τ is the shear stress (Pa), $D$is the shear rate (s^{-1}), and η is the viscosity (Pa.s).

124 ***2.3.4. Emulsion stability***

125 Stability of emulsions was determined by measurements of dispersed light with a Vertical Scan Analyzer (Quick Scan)
126 (Coulter Corp., Miami, FL, USA) according to Cabezas, Madoery, Diehl & Tomás (2012). The emulsions were
127 transferred to cylindrical glass measurement cells immediately after the emulsification. The backscattering of
128 monochromatic light (λ = 850 nm) from the emulsions was determined **every** 1 min for 2 h as a function of the height
129 of the sample tube (*ca.* 65mm), at room temperature (20°C).
130 Determinations were conducted at least in duplicate.
131

132 ***2.4. Preparation of microcapsules by spray-drying***

133 The spray-drying process was performed in a laboratory-scale Mini Spray Dryer Büchi B-290 (BüchiLabortechnik AG,
134 Switzerland) equipped with an atomizer nozzle of **700 μm** diameter. The emulsions were fed into the main chamber
135 (70 cm diameter) through a peristaltic pump and the feed flow rate was controlled by the pump rotation speed (3.1
136 mL/min). Drying air flow rate was 357 L/h and compressor air pressure was 6-8 bars. Inlet and outlet air temperature
137 were 180±2°C and 96±8°C, respectively.
138 The spray-dried microcapsules were collected and stored in caramel colored glass containers and wrapped with
139 aluminum foil in the dark at room temperature (20°C) for further analysis.
140 Two individually prepared replicates were assayed for each condition.
141

142 ***2.5. Characterization of microcapsules***

143 ***2.5.1. Moisture content and recovered solid yield***

144 Moisture content (MC) of microcapsules was determined gravimetrically by drying in a vacuum oven (Arcano,
145 Argentina) at 70°C and 20 mmHg until constant weight (for 48 h). The sample moisture content analysis was carried
146 out immediately after the drying process.

The recovered solid yield (SY) was calculated as the ratio of the powder weight collected after each drying experiment (W_f, in dry basis) and the initial amount of components in the prepared emulsions (except water) (W_i, in dry basis), as indicated in Equation 3:

$$SY\ (\%) = \frac{W_f}{W_i} 100\% \qquad \text{(Equation 3)}$$

Where: SY is the recovery solid yield (%), W_i is the initial weight of emulsion (except water) (g, in dry basis), and W_f is the powder weight collected after each drying experiment (g, in dry basis).

Determinations were carried out in duplicate.

2.5.2. Protein content

The percentage of protein content of microcapsules was determined according to the Kjeldahl method (f = 5.7)(AOAC 920.53). Determinations were carried out in duplicate.

2.5.3. *Total oil content, free oil content and encapsulation efficiency*

Total oil (TO) content of microcapsules was based on the Soxhlet method (AOAC Method 920.39) using ethyl ether as extraction solvent. The TO extracted was weighed and expressed as a percentage of oil with respect to the weight (dry basis) of the microcapsules.

The free oil (FO) content (non-encapsulated oil fraction) of microcapsules was determined according to Bae & Lee (2008) with some modifications. Briefly, ethyl ether (7.5 mL) was added to 0.5 g powder and the mixture stirred in a glass jar with a lid for 2 min at room temperature (20°C). The mixture was filtered through a Whatman filter paper nº1 and the powder collected on the filter was rinsed three times with 10 mL of ethyl ether. Then, the solvent was left to evaporate at room temperature until constant weight. The FO extracted was weighed and expressed as a percentage of oil with respect to the weight (dry basis) of the microcapsules.

Encapsulation efficiency (EE) was determined by calculating the ratio of the total oil contained in the microcapsules (TO) and the free oil (FO) located on its surface, according to Equation 4:

$$EE\ (\%) = \frac{(TO - FO)}{TO} 100\% \qquad \text{(Equation 4)}$$

Where: EE is the encapsulation efficiency (%), TO is the total oil content of microcapsules (%) and FO is the free oil content of microcapsules (%), determined as previously described.

All determinations were performed at least in duplicate.

2.5.4.Morphology

The spray-dried microcapsules obtained were photographed with a digital camera (Kodak M853, USA)in order to reveal their macroscopic aspects.

Particle size and morphology were evaluated by scanning electronmicroscopy (SEM). Powders were mounted on aluminum stubs using a double-sided tape and were coated with a thin gold layer using a cool sputter system (SCD 005, BAL–TEC, Switzerland). SEM images were acquired with a scanning electron microscope (SEM 505, Philips, Netherlands) under high vacuum with a 20 kV acceleration voltage. Samples were observed with magnifications of 500x and 1000x. ImageJ (ImageJ, National Institutes of Health, USA) was used to determine microcapsules size.

The structure and morphology of the fish oil powders were observed using a confocal scanning laser microscope (CSLM) (LEICA TCS SP5, Mannheim, Germany). Fluorescein and nile red were used as fluorescent probes to stain protein and oil respectively. For labeling, a 5% w/v SPI solution was prepared and fluorescein, FITC, (10 mg/ml) solution was slowly added into the protein solution while gently stirring. Staining of the oil was done by dissolving Nile red in the oil at 0.2 mg/ml. Then, they were mixed to form the emulsion and obtain the micro-particles for spray drying. The system was equipped with an Argon laser. Samples containing FITC and Nile red were excited at 488 nm and detected sequentially at 500-550 (FITC green channel), to detect the protein phase, and 600-700 nm (Nile red channel) to detect the oil phase. The powders were placed in a cover slip and observed with a 63x oil immersion objective.

2.5.5. Oxidative stability of fish oil

Lipid oxidation was evaluated by determination of the peroxide value and Thiobarbituric acid assay (TBA) and by analysis of the oxidative stability under accelerated conditions through a RANCIMAT test.

Peroxide value. The oil was extracted according to the method described by Partanen, Hakala, Sjövall, Kallio & Forssell (2005). A sample of each powder (0.5 g) wasweighed into a test tube and suspended in 5mL of distilledwater.The tubewas shaken until complete powder dissolution. A 300 µL portion was taken and vortexed 3 times for 10 s with 1.5 mL of an isooctane/isopropanol (3:1 v:v) mixture. The phases were separated andthe upper phase was taken for analysis. Fish oil (300 µL) was used as control. Peroxide value was determined spectrophotometrically, according to Mancuso, McClements & Decker (1999) method. A portion of the extraction medium (10–200 µL) was added to 2.8 mL of a methanol/butanol (2:1 v:v), followed by 15 µL of thiocynate solution (3.94 mol/L) and 15 µL ferrous iron (0.072 mol/L acidic solution). The sample was briefly vortexed, reacted in the dark for 20 min, and the absorbance was measured at 510 nm with a Beckman DU650 spectrophotometer (Beckman, Germany). Lipid hydroperoxide concentrations were determined using cumene hydroperoxide (Sigma-Aldrich Inc., St. Louis, USA) standard curve. Measurements were done in duplicate.

Thiobarbituric acid assay (TBA). The number of 2-thiobarbituric acid (TBA) was determined according to the method proposed by Tironi, Tomás, & Añón (2007) with some modifications. A sample of powder (0.22 g) was homogenized with 1.78 mL of 5% w/v trichloroacetic acid (TCA, Cicarelli, Argentina), and after 30 min it was centrifuged at 10000 xg, 10 min, 20°C (HERMLE Z 326 K, Germany). Supernatant (0.5 mL) was mixed with 0.5 mL of 0.5% w/v TBA (Sigma-Aldrich, USA) aqueous solution in closed tubes. The mixture was incubated 30 min at 70°C with gently agitation, and then absorbance at 532 nm was determined using Beckman DU650 spectrophotometer (Fullerton, CA, USA).TBA values were expressed as mg of malondialdehyde/kg of oil according to Equation 5:

$$TBA = \frac{AbsMVsVe\ 1000}{\varepsilon lm} \qquad \text{(Equation 5)}$$

Where: TBA is the TBA number (mg of malondialdehyde/kg of oil); Abs is the absorbance at 532 nm; M is the malondialdehyde molar mass (72 g/mol); Vs is the sample volume (0.5 mL); Ve is the extract volume (1.78 mL); **ε is** the molar extinction coefficient of the colored complex (**1.56** x10^5 M^{-1}); l is the optical path (1 cm); and m is the sample mass (g).

Measurements were done in duplicate.

Oxidative stability analysis under accelerated conditions (RANCIMAT test). An accelerated oxidation test of the bulk fish oil and the microcapsules was performed in a Rancimat (Metrohm743, Switzerland) apparat **using** 1 g of fish oil or microcapsules at 90°C with continuous bubbling of an air stream at 20 L/h. Stability was expressed as induction time (IT). This assay was performed in duplicate for each sample.

2.6 Statistical analysis

Results were expressed as mean ± standard deviation and were analyzed by analysis of variance (ANOVA). Means were tested with the Tukey's HSD (Honestly Significant Difference) test for paired comparison, with a significance level α=0.05, using the Statgraphics Plus version 5.1 software (Statgraphics, USA).

3. Results and Discussion

3.1. Effect of protein:oil ratio and emulsifying process on emulsion characteristics

The protein:**oil mass** ratio and the emulsification techniques determined the initial characteristics and the stability of the corresponding O/W emulsions. Figure 1 shows optical microscope imagesof the studied emulsions. It is worth noting that, as in all cases a SPI dispersion 5% w/v was used as aqueous phase, the emulsions with a higher protein:oil mass ratio have a lower amount of oil.Those processed only by Ultra-Turrax (UT) showed **a lower number of droplets but of higher size** than those prepared by Ultra-Turrax and Ultrasound (UT+US), and seemed to have different size distributions. This was confirmed by **SLS** analysis, shown in Figure 2 and Table 1. The emulsions prepared by UT presented different behaviors according to their protein:oil mass ratio. Emulsions with lower protein proportions (1:1 and 2:1) showed bimodal size distributions with two defined populations, a predominant one with dp ≈ 20 µm and another minority with dp ≈ 0.2 µm; while those with higher protein proportions (3:1 and 4:1) presented monomodal size distributions with dp ≈ 28 µm. Likewise, the 1:1 and 2:1 emulsions showed a greater difference between De Brouckere ($d_{4,3}$) and Sauter ($d_{3,2}$) mean diameters (Table 1), evidencingthat they were

247 composed bymore heterogeneous droplets in size than the 3:1 and 4:1 emulsions. The specific surface areas(SSA) in
248 these emulsions were minimal (Table 1). **In these cases, the amount of protein seems to be enough to cover the**
249 **new generated interface. This not occurred for lower protein:oil mass ratio (1:1 and 2:1 emulsions), in which the**
250 **generated interfacial area increased about twenty times.**
251 On the other hand, all emulsions prepared by UT+US presented bimodal particle size distributions with two defined
252 populations with dp ≈ 0.25 µm and 2.5 µm respectively (Figure 2), regardless of the emulsions formulation. These
253 particle size distributions were considerably narrower than those of the emulsions prepared only by UT as had the
254 lowest difference between $d_{4,3}$ and $d_{3,2}$, indicating greater uniformity in the droplet sizes. More energy (as
255 mechanical work) was applied to the system when adding the US emulsification step, and a greater SSA than that
256 obtained for the corresponding emulsions prepared by UT was generated (Table 1). These results could be explained
257 taking into account differences between both emulsification mechanisms. Drops are formed by shear stress in
258 laminar flow and/or shear and inertial stress in turbulent flow when emulsifying by UT. **Meanwhile acoustic**
259 **cavitation and power dissipation can break droplets during ultrasound emulsification and yield emulsions with**
260 **particles in the sub-micron range (Gavahian, Chen, Khaneghah, Barba, & Yang, 2018).** In this sense, Jafari, He, &
261 Bhandari (2007a) also described that fish oil emulsions using mixtures of maltodextrin and whey proteins as
262 surfactant (4:1 wall/core ratio) obtained by UT+US showed lower particle **diameters** than those prepared only by UT.
263 The emulsions rheological behavior conditions their stability and further processing. Table 1 also summarizes the
264 rheological characteristics for fish oil-in-water emulsions prepared with different SPI protein:oil mass ratios
265 processed by UT and UT+US. The experimental values for the shear stress as a function of strain rate gradient were
266 satisfactorily adjusted to the Newton model ($R>0.97$). The emulsions prepared by UT almost doubled the viscosities
267 of those prepared by UT+US, regardless to the emulsion formulations. Tatar, Sumnu, & Sahin (2017) describe the
268 effect of several factors, including volume fraction, droplet size, and colloidal interactions on emulsions rheology. It
269 seems that emulsions containing smaller droplets (UT+US) have the ability to pack more efficiently than those with
270 larger droplets (UT), decreasing their viscosity. But no correlation between emulsion viscosity and volume fractions

were found in this work, possibly due to that the studied volume fraction correspond with dilute emulsions ($0.01<\Phi<0.05$).

The emulsions stability, shown in Figure 3, was determined by comparing the percentages of initial backscattered light (immediately after the emulsion was formed) and after two hours in order to ensure the necessary time to process them by spray. The emulsions prepared by UT showed ≈50-55 % of BSo regardless of the formulation; while in those processed by UT+US, the % BSo decreased from 70 to 50% when increasing protein:oil mass ratio. Thus those emulsions with highest oil proportions (1:1 and 2:1) and the smallest oil droplets size (prepared by UT+US) showed the highest % BSo (> 60%), according to the dependency of BS with the oil droplet size and their concentration. On the other hand, in the emulsions with lower oil concentration (3:1 and 4:1), the effect of droplet size due to different processes was not observed in the % BSo.

After two hours, all the emulsions prepared by UT diminished their BS ≈10-20%, suggesting the occurrence of creaming-flocculation mechanisms although no evidence of phase separation was observed. The migration of oil droplets from the bottom to the top of the emulsion sample could lead to a progressive reduction of oil droplet concentration at the bottom of the emulsion sample, and a concurrent decrease in the intensity of backscattered light. But no evidence of phase separation was observed even on the top of the tube. On the other hand, the emulsions prepared by UT+US remained stable after 2 h without significantly modifying their % BSo value. Taking into account Stokes' law, differences in the emulsion stability could be attributed mainly to droplets sizes that in this work would be determined by the emulsification processes (Wang, Liu, Chen, & Selomulya, 2016; Wang, Liu, Chen, & Selomulya, 2016; Day, Xu, Hoobin, Burgar, & Augustin, 2007).

3.2. Characterization of fish oil-soybean protein microencapsulates

The emulsion characteristic and the encapsulating agent affect the properties of fish oil microparticles, such as the encapsulation efficiency, peroxide value, particle size, morphology and moisture content (Encina et al., 2016).

3.2.1. Chemical composition and encapsulation efficiency

All the studied emulsions could be spray dried. The drying conditions were set in preliminary tests, using an inlet air temperature of 180°C (Jafari, Assadpoor, Bhandari, & He, 2008) and outlet temperatures of 96±5°C. In all cases, light yellowish powders were obtained, which did not present an oily appearance at touch nor the characteristic odor of the fish oil. Table 2 shows their color parameters. Emulsifying process seemed not to influence the powder coloration as the formulation did. When increasing the protein proportion, encapsulates clarity increased (greater L*), and their yellowish coloration (lower b*) and total coloration change (ΔE*) decreased.

Table 3 shows the chemical composition of microencapsulates, recovered solid yield (SY) and encapsulation efficiency (EE) of each process. Moisture content also seemed to be dependent only by formulation as, for both emulsifying process, encapsulates prepared with 3:1 protein:oil mass ratio showed the highest MC value. The protein:oil mass ratio resulting after drying remains similar to that of the initial formulation only for samples emulsified by both UT+US. Those processed only by UT, showed higher protein:oil ratios (1.8:1; 2.8:1; 4:1 and 5.6:1) than their corresponding emulsions (1:1; 2:1; 3:1 and 4:1 respectively), implying that during drying lipids were lost to a greater extent than proteins. Probably due to the lower stability of emulsions formed by higher oil droplets. These results were reflected in SY values. Emulsions prepared by UT+US presented the highest SY (>46%), regardless of their protein:oil mass ratio while those processed only by UT showed a lower SY that increased with the protein:oil mass ratio from ≈ 23to≈43% (being this last value statistically similar to those treated by UT+US). In all cases mass losses occurred by the product adhesion to the drying chamber walls and later to the cyclone`s, according to Sosnik & Seremeta (2015) who reported typical SY for the spray drying process from 20% to70% depending on both process conditions and sample characteristics.

On the other hand, the encapsulation efficiency for all the studied formulations was maintained between 57% and 69% except for the 4:1 emulsion prepared by UT+US, which presented the highest encapsulation efficiency (EE≈ 90%). It is evident that the protein:oil mass ratio and the emulsifying process played a fundamental role, as the same formulations treated only by UT or lower protein:oil mass ratios processed by UT+US showed significantly lower EE. Indirectly, droplets size, emulsion viscosities and oil and protein concentration are responsible of these results. Thus, increasing protein concentration, lower free oil was present in the products. Wang et al. (2016), also observed

higher EE when increasing whey proteins concentration that attributed to the increased oil retention principally by reducing the time to forma semi-permeable crust at the droplet-air interface during spray drying, making it difficult for the oil to diffuse to the particle surface during drying. Jafari et al. (2008) also observed that increasing whey protein isolate content led to the increase of the emulsion viscosity, thus reducing the circulation movement inside the droplets during drying and resulting in higher oil retention. Although in this work the viscosity increase was not observed, the samples with highest protein concentrations emulsified by UT+US showed the highest encapsulation efficiency.

3.2.2. Morphology of the fish oil-soybean protein microencapsulates

Figure 4 shows SEM images of the spray-dried fish oil-soybean protein microcapsules. Their mean apparent diameters -estimated from the images- **were** 15-20 μm for all samples. However, wide size distributions were observed (from 5 to 40 μm). These size dispersions are typically found in spray dried particles (Carneiro, Tonon, Grosso, & Hubinger, 2013). Protein:oil mass ratio and emulsions characteristics such as particle size distribution and stability, seemed not to influence the microcapsules size, which was mainly determined by the spray-dryer nozzle diameter used. Differences in emulsions viscosities observed in this work seemed not to affect particle size either, even though it has been reported the increase in particle size due to the formation of larger droplets during atomization when working with emulsions of higher viscosity (Drusch, Serfert, Scampicchio, Schmidt-Hansberg, & Schwarz, 2007).

Particles showed a smooth spherical shape characterized by the presence of surface dents (indentations) (Figure 4). According to **I Ré (1998)**, such dents/indentations are formed with slow processes of film formation during drying of the atomized droplets, being these surface depressions the consequence of droplets collapse during the early stages of drying process. In our study, the formation of these dents on the outer surfaces seemed to increase with protein:oil mass ratio, according to Fäldt & Bergenståhl (1996), who reported that this morphology is typical of oil encapsulates with high protein concentrations.

No evidence of cracks, fissures or holes was found by SEM observations (Figure 4), which is an advantage and suggests that soybean proteins were efficient to encapsulate the fish oil. However, it was possible to find a few broken microcapsules, as that prepared from 3:1 UT+US emulsion shown in Figure 4, that allowed to observethe typical inner topography of the studied particles. Inside, the microcapsules were hollow and the fish oil droplets were adhered to the surface or embedded in the wall materials matrix. The formation of central voids in spray-dried particles was attributed to the microcapsule expansion (or "ballooning") which is caused by fast fixing of the particle structure in the early stage of drying with subsequent steam formation in the interior of the particle and inflation of the particle (Rosenberg, Talmon & Kopelman, 1988; Nijdam & Langrish, 2006; Jafari et al., 2008). Wang et al. (2016) also observed hollow particles with irregular dents appearing on the surfaces when encapsulating DHA-containing fish oil with whey protein isolate, and related the mechanisms for the formation of the central hollow structure to the expansion of the particles during the later periods of the drying process, and the dents to shrinkage at the early stages of the drying process (Botrel, de Barros Fernandes, Borges, & Yoshida, 2014).

Fish oil was dispersed within the wall of the microcapsule in the form of small droplets with, similar size (≈1.4 μm) **than** those dispersed in the parent-emulsion as observed in Table 1 and Figure 2. This may be attributed to both, the fast crust formation (or solidification) that prevents the coalescence of the dispersed droplets and to the good emulsion stability during atomization and subsequent drying (I Re, 1998). Jafari et al. (2008) reported that a fast crust formation could be associated with low levels of surface oil content as it was difficult for the fish oil droplets to come onto the surface of particles.

The location of each component in the microcapsules was tried to be elucidatedby CLSM using specific fluorescent probes that differentially stained proteins and lipids (Figure 5). Soybean proteins were co-localized with the fish oil drops, suggesting that the resulting microcapsules should be matrix-type where the oil and the wall material were integrated (Raybaudi-Massilia & Mosqueda-Melgar, 2012; Bakry et al., 2016). Central voids into the particles also could be observed. These results agree with SEM observations and those reported by Wang et al. (2016) who described similar matrix-type microcapsules composed of fish oil and whey proteins.

370 *3.2.3. Oxidative stability of fish oil-soybean protein microencapsulates*

371 The oxidation state of both, the original fish oil and the resulting powder encapsulates was analyzed. Lipid
372 autoxidation is a chemical alteration reaction that occurs by free radicals through chain reactions. Peroxides are the
373 primary products produced at the beginning of this alteration that then are degraded into other products, such as
374 alcohols, ketones, aldehydes and volatile compounds, responsible for undesirable odors in food (Barrow, Wang,
375 Adhikari, & Liu, 2013). These secondary oxidation compounds can be measured by thiobarbituric acid reactive
376 compounds (Jiménez-Martín et al., 2015; Shaw, McClements, & Decker, 2007) among other methods. Figure 6 shows
377 the peroxide index and, thiobarbituric acid value for the studied samples. Fish oil had a low peroxide content (PI = 1
378 meq /kg) and absence of self-oxidation byproducts, indicating that lipid autoxidation had not begun or was in its
379 early stages. **This could probably be attributed to the presence ofantioxidants in the commercial fish oil**
380 **sample.** Emulsifying and spray drying processes as well as the formulation used for encapsulation affected the
381 oxidative stability of fish oil. Unexpectedly all encapsulating systems showed a higher peroxide index and TBA values
382 than those corresponding to free fish oil. Encina et al. (2016) mentioned that although manystudies have reported an
383 improvement in fish oil stability when it is microencapsulated,there are some works where a lower fish oil stability
384 from microparticles has been described when compared to bulk oil. Thus, Kolanowski & Laufenberg 2006 found that
385 the encapsulation of fish oil with methylcellulose did not significantly improve its oxidative stability with respect to
386 bulk fish oil, as evaluated by peroxide value measurements, despite high encapsulation efficiency (over 80%), and
387 attributed itto the higher surface area of the microparticles than bulk fish oil. Serfert, Drusch, & Schwarz, (2009)
388 reported that fish oil encapsulated with modified starch and glucose syrup added with different antioxidants showed
389 higher peroxide indexes thanthose of bulk oil.
390 Wang et al. (2016) also observed higher peroxide values of whey encapsulates than the oil blank in the initial storage
391 stage that attributedto the oxidation of the particle surface/near surface oil duringpreparation when it was exposed
392 to air; and mentioned previous researchesthat suggested that the auto-oxidation of encapsulated and non-
393 encapsulatedcore oil likely occurred during the drying process ifa wall material with strong film forming properties
394 was used,which could catalyze further oxidation during storage (Drusch & Berg, 2008; Wang, Tian, & Chen, 2011).

And they explained that quick formed shell possibly increased the resistance of evaporation, resulting in the rapid increaseof particle temperature during drying. Thus, the period of particle free oil exposure in the high temperature was prolonged; resulting in peroxides increased, above than the unprocessed crude fish oil at the early stage of storage. Furthermore, other authors observed that particle ballooning during drying leads to air inclusion in the particle and oxidation reactions (Drusch et al., 2006; Keogh et al., 2001).

Encapsulates emulsified by UT presented a significantly higher peroxide index and TBA values than those processed by UT+US, with the exception of those with a protein:oil mass ratio of 4:1 processed only by UT that showed a similar PI than those processed by UT+US. But as this sample showed the highest TBA value, these PI value would correspond to an advanced period of oxidation reaction, when unstable peroxides oxidized themselves to form other compounds, such as aldehydes and ketones (Drusch et al., 2007). During UT emulsification a certain amount of air is incorporated into the dispersion, and together with light it would facilitate the beginning of the oxidation reaction. Then the US stage could remove part of the incorporated oxygen, delaying the autoxidation. Oxidation could also be accelerated with the increase oftemperature during spray drying. But although the drying T was set at 180°C, the effective temperature of the drops was lower (≅ 60°C) and the residence time in the equipment was minimal (≅ 1-2 s).

Furthermore all the formulations prepared by UT + US showed PI values lower than 10 meq / Kg oil that is the maximum PI allowed by the Argentine Food Code (CAA) for the commercialization of fish oil (www.anmat.gov.ar).

Also unexpectively, for both processing technologies PI and TBA indexes increased with the protein:oil mass ratio. When reducing the proportion of fish oil in the encapsulates, TBARS values increased markedly (≈200% for UT vs. ≈300% for UT+US).It should be expected that the 4:1 sample processed by UT+US that showed significantly higher encapsulation efficiency would show an improvement in the oxidative stability of fish oil. In this sense, Drusch & Schwarz (2006) reported that theamount of surface oil, which probably represented a high proportionof the non-encapsulated oil was less significant on oil oxidation and shelf-life stability than the particle structure and shell properties. Wang et al. (2016) observed that encapsulates with higher protein concentration showed a lower oxidation stability before 20 days, indespite of higher encapsulation efficiency, and attributed it to the faster shell

formation process ofthe high protein content emulsions, that possibly increased the resistance of evaporation, resulting in the increase of particle temperatureduring drying and to the fact that the lower protein contentin the high oil samples somehowavoided the free oil being exposedin high temperature for such long period; producing higher initial oxidation rates as the protein:oil mass ratio increased.

Furthermore soy protein isolates contains low amounts of divalent cations, such as copper and iron (http://www.guia-nutricion.com), which could catalyze the autoxidation of unsaturated fatty acids in fish oil. Working with other biopolymers, other authors tried to improve the stability of the encapsulated oil by incorporation of chelating agents like citric acid (Drusch & Berg, 2008).

In order to study the oxidative stability of encapsulates during storage,the Induction Time (IT) obtained from Rancimat accelerated oxidation test was determined and showed in Figure 7.The accelerated Rancimat test has gained acceptance as an indirect measure of oil stability, as the oily sample is treated under controlled conditions in which the lipoperoxidative process reaches its final steps and lipids are oxidized to short-chain volatile acids which are collected in distilled water increasing its conductivity. **Finally, the sample turns out to be more stable as the time required to increase the conductivity increases.**The induction time (IT) for control fish oil was 3.9 h. All encapsulated samples processed by UT were more susceptible to oxidation than those processed by UT+US, since they had the shortest induction times. For both emulsification techniques, formulations 1: 1 and 4:1 presented the longest induction times; being only the one prepared by UT+US similar to that of non-encapsulated fish oil. It seemed that protein:oil mass ratio 1:1 samples prepared by UT+US manage to protect the fish oil during its microencapsulation (by the initial values of peroxides and TBA) and also maintain its oxidative stability; while the 4:1 samples prepared by UT+US, which showed the highest EE, also maintain their oxidative stability, despite presenting the highest values of PI and TBARS for the samples processed by UT+US.

4. Conclusions

Soy proteins managed to encapsulate the fish oil, masking its characteristic odor and its oily texture. The emulsification process was determinant in the efficiency of encapsulation as well as in the protection exerted on the

oil oxidative stability. Although emulsifying and drying processes caused certain initial oil oxidation (verified by peroxide and TBA indexes), some of the studied systems showed a good perspective of oxidative stability over time. Microparticles prepared with a protein: oil ratio of 4: 1, emulsified by UT + US to oildrop sizes smaller than 2.5 µm and good stability showed encapsulation efficiencies of 88% and managed to maintain the oil oxidative stability, as presented similar induction times (measured by rancimat test) than free oil. This microparticles should be use to vehiculate healthy fish oil, rich in omega 3 fatty acids, into functional foods.

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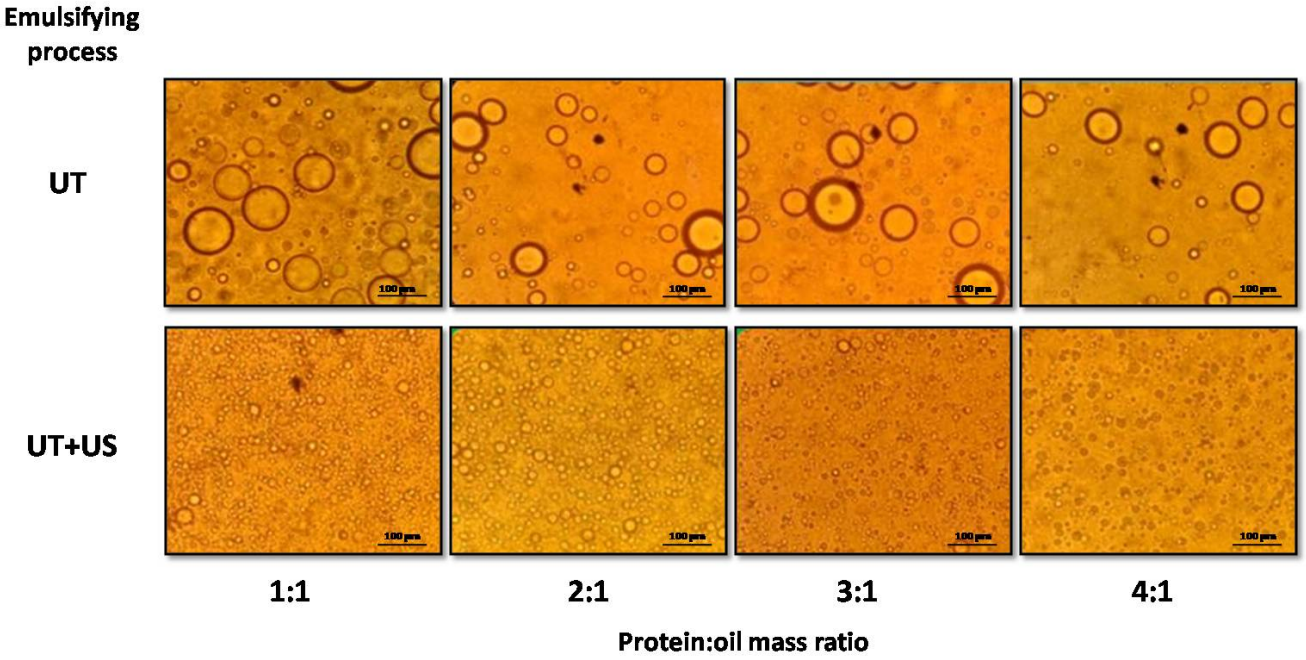


Figure 1. Optical microscope images (100X) of the fish oil-in-water emulsions with different protein:oil mass ratios (1:1, 2:1, 3:1, and 4:1 w/w) and processed by Ultraturrax (UT) and Ultraturrax+Ultrasound (UT+US).

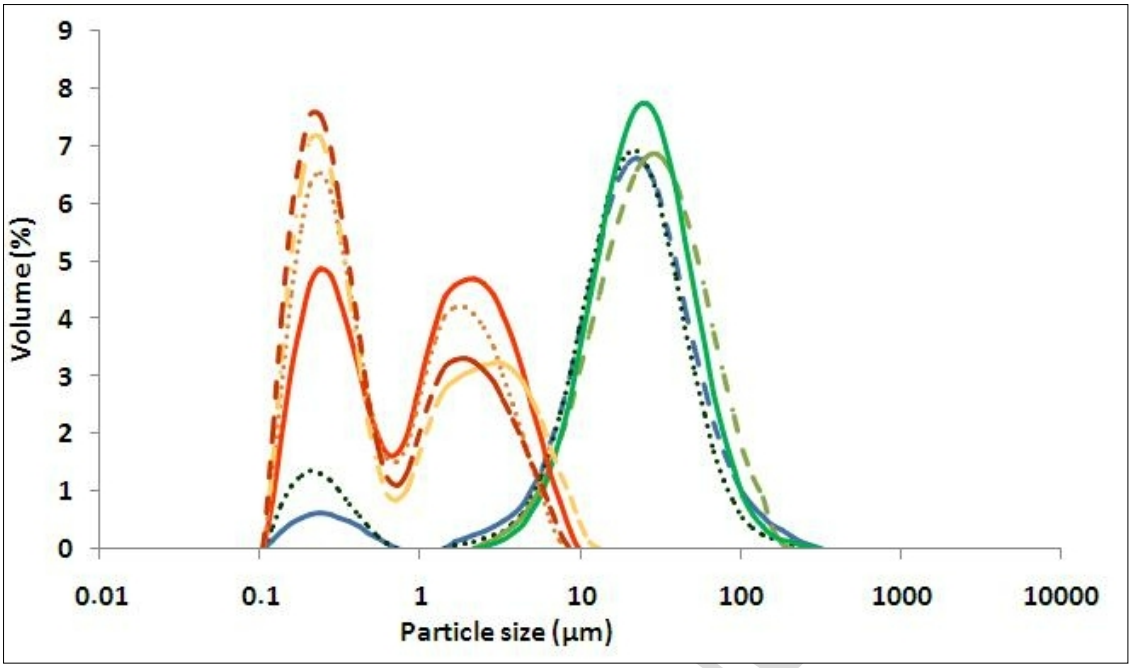


Figure 2. Profiles of particle size distributions of the fish oil-in-water emulsions prepared with different protein:oil mass ratios: 1:1 (----), 2:1 (…), 3:1 (-.-.-), and 4:1 (___) w/w) and processed by Ultraturrax (UT) ( ) and Ultraturrax+Ultrasound (UT+US) ( ). Distributions were expressed in percentage of oil volume.

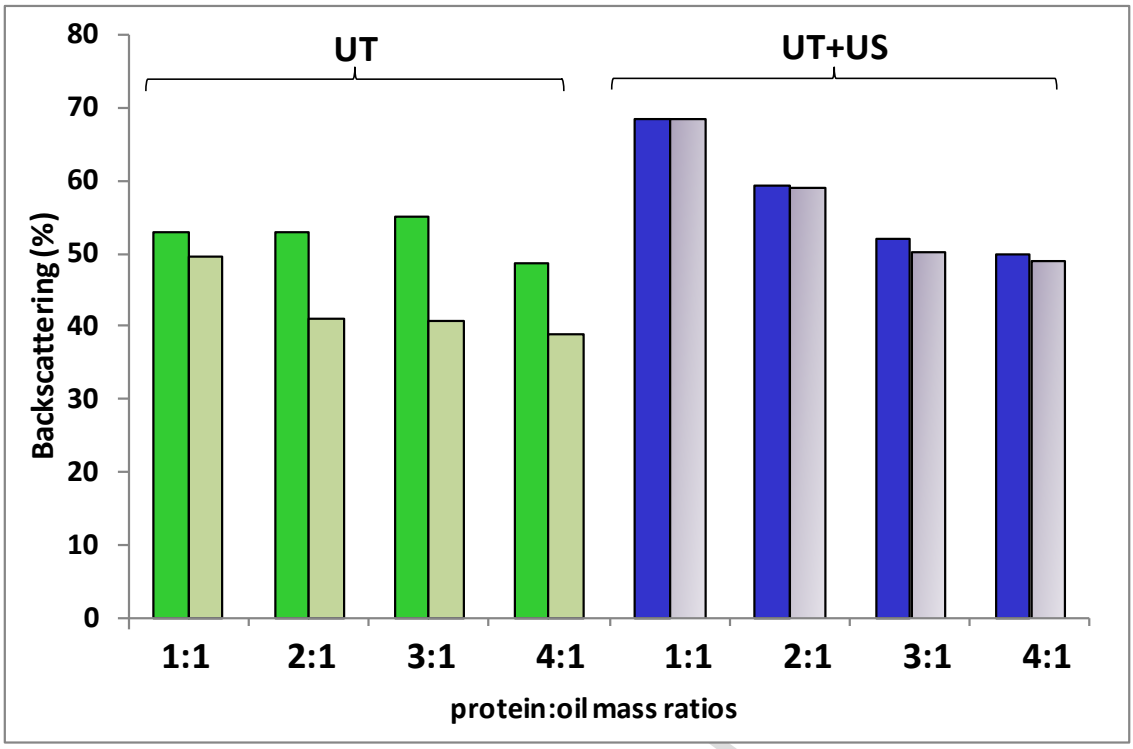


Figure 3. Destabilization kinetics (clarification, zone 10–15 mm of test tube), corresponding to fish oil-in-water emulsions prepared with different protein:oil mass ratios (1:1, 2:1, 3:1, and 4:1 w/w) prepared by Ultraturrax (UT) and Ultraturrax+Ultrasound (UT+US). Figure shows the initial Backscattering (■ and ■) and its value after 2 h of storage at room temperature (■ and ■), corresponding to the maximum time for spray drying of emulsions.

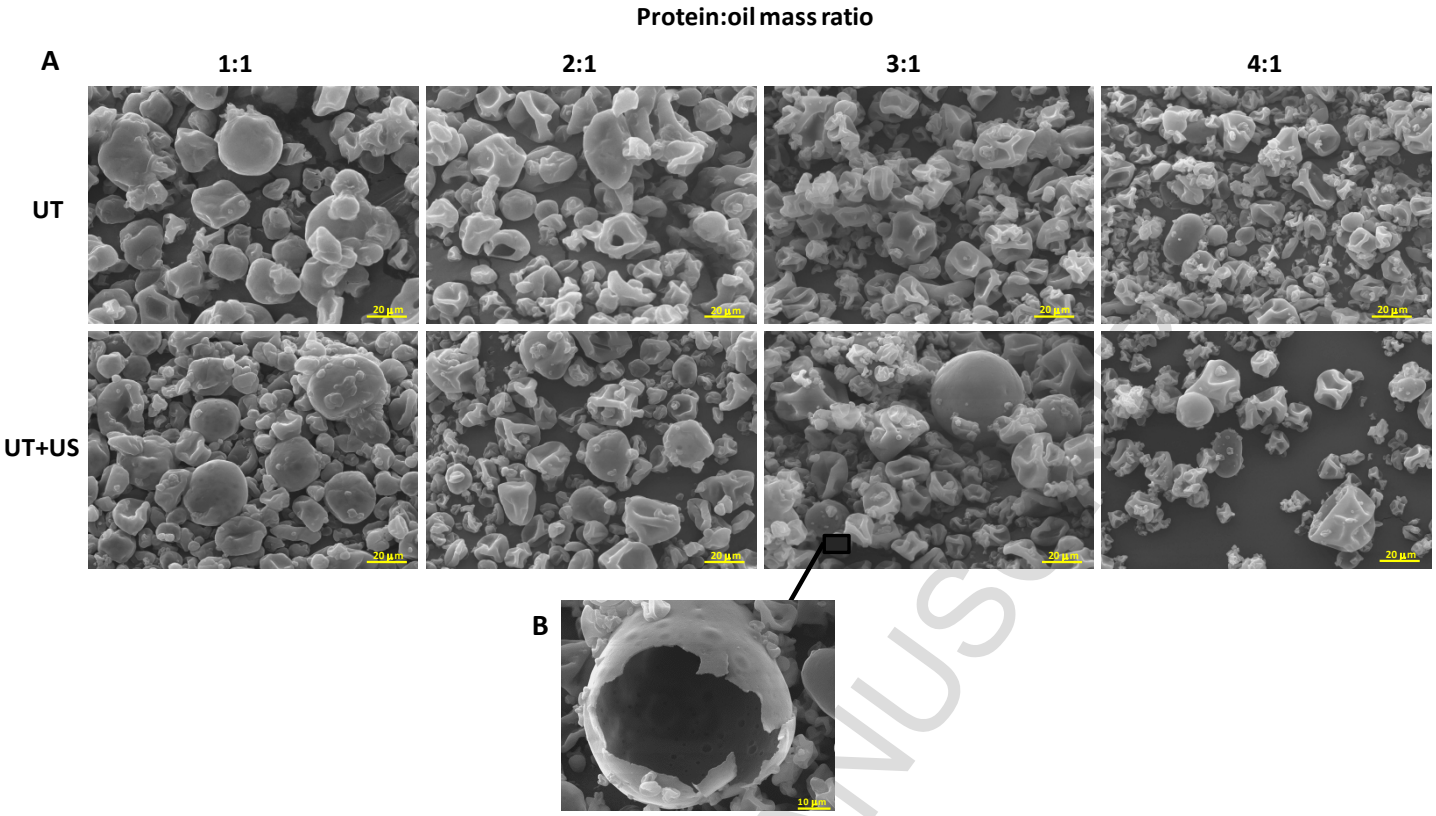


Figure 4. A) Scanning electron micrographs (SEM) of the outer morphology (topography) of the fish oil microcapsules obtained after spray-drying of fish oil in water emulsions prepared with different protein:oil mass ratios (1:1, 2:1, 3:1, and 4:1 w/w) and processed by Ultraturrax (UT) and Ultraturrax+Ultrasound (UT+US). SEM at 500x magnification. **B)** Typical inner morphology (topography) of the fish oil microcapsules obtained after spray-drying of fish oil in water emulsions prepared with protein:oil mass ratios of 3:1 w/w and processed by Ultraturrax+Ultrasound (UT+US). SEM at 1000x magnification.

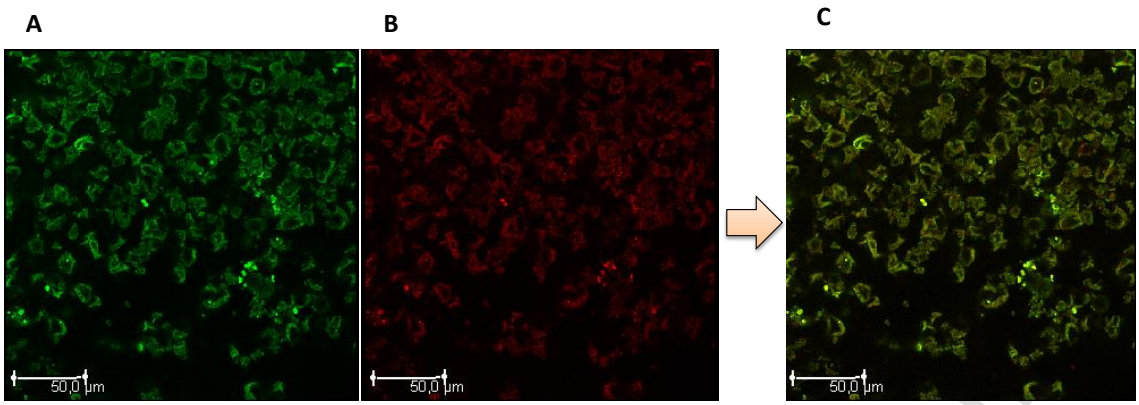


Figure 5. Confocal laser scanning micrograph (CLSM) of fish oil microcapsules obtained after spray-drying of fish oil in water emulsions prepared with protein:oil mass ratio of 4:1 w/w and processed by Ultraturrax+Ultrasound. CLSM at 63x magnification.

Double staining of O/W emulsion with: (A) Green colour represents the soybean protein stained by Fluorescein. (B) Red colour represents the fish oil stained by Red Nile. (C) Represent the colocalization of both colorants.

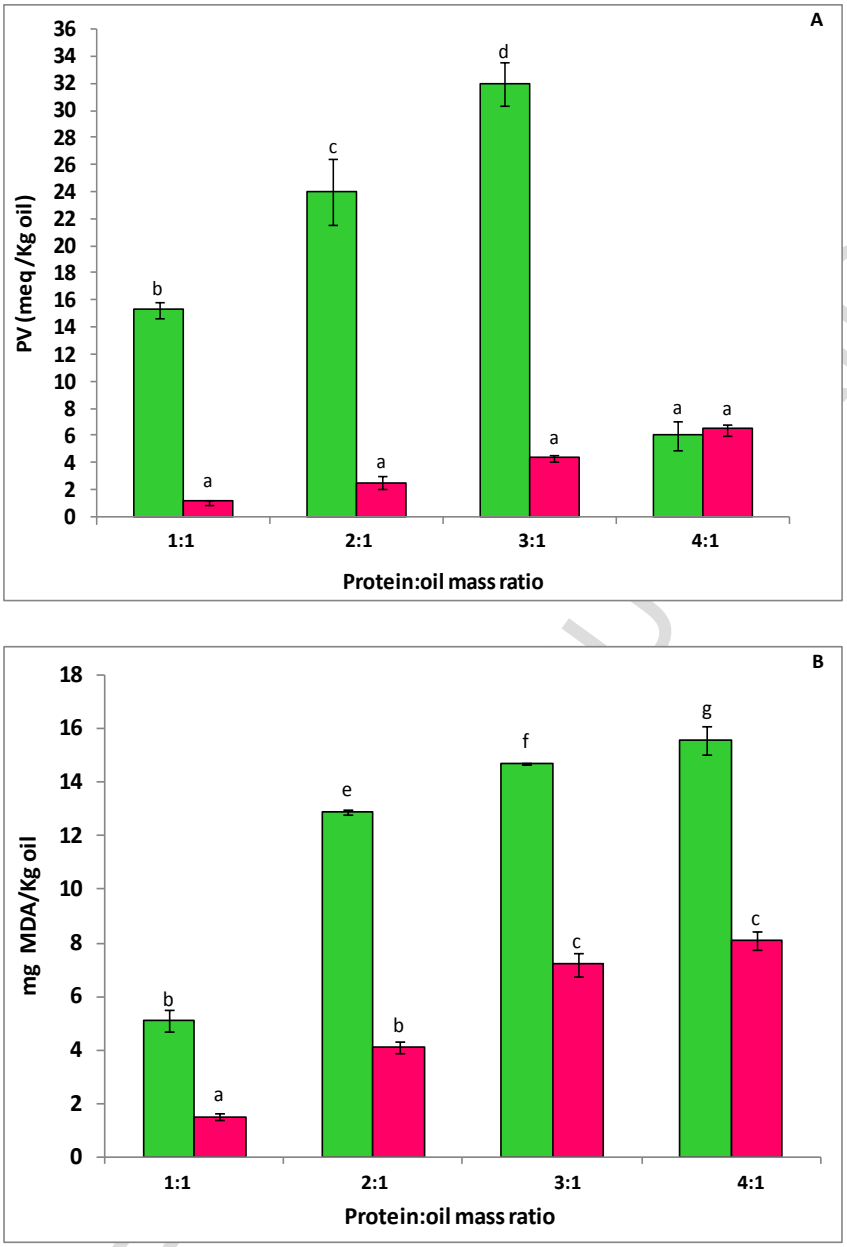


Figure 6. Peroxide value (A) and TBA number (B) of the fish oil-soybean proteins encapsulates prepared from spray dried O/W emulsions with different protein:oil mass ratios (1:1, 2:1, 3:1, and 4:1 w/w) and processed by Ultraturrax (UT) (■) and Ultraturrax+Ultrasound (UT+US) (■). Peroxide value of the fish oil: 1,067±0,009 PV/Kg oil.

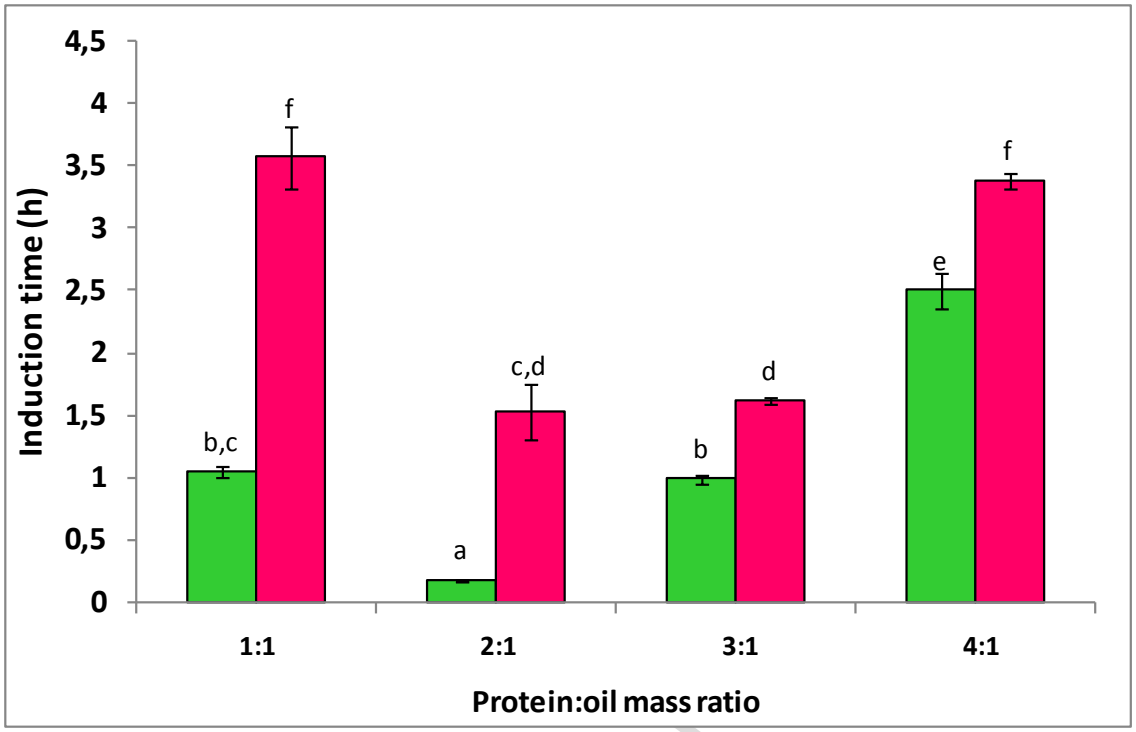


Figure 7. Induction time of the fish oil encapsulates in soybean protein matrices prepared from spray dried O/W emulsions with different oil:protein mass ratios (1:1, 2:1, 3:1, and 4:1 p/p) and processed by Ultraturrax (UT) (■) and Ultraturrax+Ultrasound (UT+US) (■). Induction time of the fish oil: 3,91 ± 0,25 h.

Highlights

- Soy proteins managed to encapsulate fish oil masking its characteristic odor and oily texture.

Or: Soy proteins managed to encapsulate fish oil.

- These microparticles would facilitate fish oil incorporation into healthy food products.
- **The effect of emulsifying process and protein : oil ratio on capsules properties and oil stability were studied or Two emulsification procedures and several protein-to-oil ratios were analyzed.**
- Some systems showed a good perspective of oil oxidative stability over time.

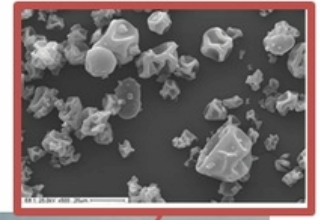
ENCAPSULATION OF FISH OIL IN SOYBEAN PROTEIN PARTICLES

Fish oil

Soy protein dispersion

Emulsification

Spray drying



Soy protein particles containing fish oil to carry healthy fish oil, rich in omega 3 fatty acids, into functional foods.

Table 1. Initial parameters of the fish oil-in-water emulsions prepared with different protein:oil mass ratios (1:1, 2:1, 3:1, and 4:1 w/w) and processed by Ultraturrax (UT) and Ultraturrax+Ultrasound (UT+US).

Emulsifying process	*Protein:oil mass ratio*	$d_{4,3}$ *(μm)*	$d_{3,2}$ *(μm)*	*Difference* ($d_{4,3}$ - $d_{3,2}$)	*Specific surface area* (μm^{-1})	*η (mPa.s)*
UT	1:1	26.44 ± 0.86^{c}	3.21 ± 0.21^{c}	23.23	0.093 ± 0.006^{b}	7.20 ± 0.40^{b}
	2:1	22.54 ± 0.51^{b}	1.62 ± 0.13^{b}	20.92	0.093 ± 0.008^{b}	5.80 ± 1.50^{b}
	3:1	32.45 ± 0.85^{e}	18.22 ± 0.27^{e}	14.23	0.005 ± 0.001^{a}	6.00 ± 0.50^{b}
	4:1	28.64 ± 1.09^{d}	17.04 ± 0.58^{d}	11.60	0.004 ± 0.001^{a}	7.60 ± 0.60^{b}
UT+US	1:1	1.56 ± 0.01^{a}	0.47 ± 0.01^{a}	1.09	0.633 ± 0.001^{f}	3.60 ± 0.50^{a}
	2:1	1.14 ± 0.01^{a}	0.38 ± 0.01^{a}	0.76	0.399 ± 0.001^{e}	2.60 ± 0.30^{a}
	3:1	1.42 ± 0.07^{a}	0.35 ± 0.01^{a}	1.07	0.282 ± 0.005^{d}	2.80 ± 0.10^{a}
	4:1	1.03 ± 0.02^{a}	0.32 ± 0.01^{a}	0.71	0.232 ± 0.002^{c}	3.40 ± 0.20^{a}

Reported values for each emulsion are means ± standard deviation (n=4). Different letters in the same column indicate significant differences between samples ($p<0.05$), according to Tukey´s test.

Table 2. CIE-Lab color parameters (*L**, *a** and *b**), total color difference (*ΔE**) of the fish oil microcapsules obtained after spray-drying of fish oil in water emulsions prepared with different protein:oil mass ratios (1:1, 2:1, 3:1, and 4:1 w/w) and processed by Ultraturrax (UT) and Ultraturrax+Ultrasound (UT+US).

Emulsification technique	*Protein:oil ratio*	*ΔE**	*L**	*a**	*b**
UT	1:1	10.46 ± 1.21^{c}	92.81 ± 1.22^{a}	-0.46 ± 0.13^{c}	$11.10 \pm 0.86^{c,d,e}$
	2:1	$9.94 \pm 1.81^{b,c}$	92.90 ± 1.72^{a}	$-0.58 \pm 0.12^{b,c}$	$10.54 \pm 1.17^{b,c,d}$
	3:1	$9.49 \pm 1.63^{b,c}$	$93.35 \pm 1.71^{a,b}$	-0.43 ± 0.18^{c}	$10.25 \pm 1.04^{b,c}$
	4:1	7.38 ± 0.56^{a}	$94.95 \pm 0.62^{b,c}$	$-0.69 \pm 0.03^{a,b}$	8.64 ± 0.48^{a}
UT+US	1:1	10.78 ± 0.65^{c}	92.77 ± 1.06^{a}	$-0.69 \pm 0.08^{a,b}$	$11.39 \pm 0.80^{d,e}$
	2:1	$8.80 \pm 0.56^{a,b}$	$93.71 \pm 0.49^{a,b,c}$	-0.83 ± 0.06^{a}	$9.67 \pm 0.62^{a,b}$
	3:1	10.79 ± 0.51^{c}	$94.22 \pm 0.57^{a,b,c}$	-0.63 ± 0.08^{b}	12.01 ± 0.43^{e}
	4:1	$8.50 \pm 0.33^{a,b}$	95.31 ± 0.38^{c}	-0.82 ± 0.05^{a}	9.91 ± 0.34^{b}

Reported values for each encapsulate are means ± standard deviation (n=9). Different letters in the same column indicate significant differences between samples ($p<0.05$). according to Tukey´s test.

Table 3. Moisture (MC), protein (PC), total oil (TO) and free oil (FO) contents, recovered solid yield (SY) and encapsulation efficiency (EE) of the fish oil microcapsules obtained after spray-drying of fish oil in water emulsions prepared with different protein:oil mass ratios (1:1, 2:1, 3:1, and 4:1 w/w) and processed by Ultraturrax (UT) and Ultraturrax+Ultrasound (UT+US).

Emulsifying process	*Protein:oil mass ratio*	*MC (%)*	*Protein (%)*	*Total Oil (%)*	*Free Oil (%)*	*SY(%)*	*EE(%)*
UT	1:1	$5.10 \pm 1.21^{b,c}$	$52.36 \pm 1.12^{b,c}$	27.82 ± 2.43^{c}	11.57 ± 1.79^{c}	23.24 ± 3.65^{a}	57.82 ± 6.27^{a}
	2:1	$2.32 \pm 0.01^{a,b}$	62.95 ± 0.09^{d}	$21.84 \pm 2.39^{b,c}$	6.65 ± 0.95^{c}	$31.04 \pm 3.57^{a,b}$	$69.20 \pm 5.50^{a,b}$
	3:1	8.29 ± 1.21^{d}	$64.27 \pm 0.94^{d,e}$	15.69 ± 0.67^{a}	4.86 ± 0.80^{b}	$29.15 \pm 5.31^{a,b}$	$69.10 \pm 5.56^{a,b}$
	4:1	$3.88 \pm 0.55^{a,b}$	68.24 ± 2.35^{e}	12.14 ± 1.94^{a}	4.35 ± 1.82^{b}	$42.60 \pm 4.22^{b,c}$	65.19 ± 11.04^{a}
UT+US	1:1	$3.92 \pm 0.08^{a,b}$	38.78 ± 0.39^{a}	48.77 ± 1.20^{d}	16.06 ± 2.46^{c}	50.22 ± 4.90^{c}	$66.93 \pm 4.55^{a,b}$
	2:1	1.62 ± 0.49^{a}	49.63 ± 1.34^{b}	28.94 ± 1.49^{c}	12.23 ± 1.48^{c}	50.26 ± 2.22^{c}	57.73 ± 1.83^{a}
	3:1	9.23 ± 0.25^{d}	56.10 ± 0.39^{c}	$20.16 \pm 0.85^{a,b}$	6.06 ± 0.71^{b}	$47.21 \pm 0.78^{b,c}$	$69.82 \pm 4.89^{a,b}$
	4:1	$6.80 \pm 0.79^{c,d}$	60.64 ± 0.23^{d}	15.21 ± 1.49^{a}	1.26 ± 0.42^{a}	$46.04 \pm 0.38^{b,c}$	88.74 ± 3.15^{b}

Reported values for each encapsulate are means ± standard deviation (n=2). Different letters in the same column indicate significant differences between samples ($p<0.05$), according to Tukey´s test.