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Optimization of ultra-sonicated homogenization conditions of fish oil emulsions to improve stability, efficiency and bioaccessibility of ω -3 microcapsules

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ABSTRACT

This study aimed to improve the quality characteristics of fish oil microcapsules from monolayer and multilayer emulsions by optimizing the ultra-sonicated homogenization conditions to obtain a stable source of omega-3 fatty acids to add in food matrix. For that, a response surface methodology trial was firstly conducted to get optimum ultra-sonicated conditions that increase stability of emulsions and efficiency of microcapsules. Thus, optimum combinations of power of (141.01 and 141.21 kg m^2/s^3), time (15 and 5 min), pulse (80%) and emulsion volume (100 and 200 mL) were obtained for monolayered and multilayered emulsions, respectively. In comparison to homogenization by high-pressure, the use of ultrasounds improved most quality characteristics of emulsions (stability and drop size) and microcapsules (microencapsulation yield, total oil, microencapsulation efficiency, quantities and bioaccessibility of EPA and DHA, lipid oxidation, size, and distribution), especially in monolayered ones. It worth noting the increase in microencapsulation efficiency in multilayered and the improvement in emulsion stability and bioaccessibility of EPA and DHA in both types of microcapsules.

1. Introduction

A diet rich in omega-3 polyunsaturated fatty acids (PUFA ω-3), principally eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been shown to have a wide range of beneficial health effects, including proper neurological development, especially in early developmental stages (Czyż et al., 2016), regulation of blood glucose levels by incorporating into cell membrane phospholipids, stimulating insulin receptors or reduction of risk factors for the development of various inflammatory and cardiovascular diseases by regulating triglyceride concentrations and increasing cholesterol transport to the liver by high-density lipoproteins (HDL) (Lavie et al., 2009).

The beneficial effects of PUFA ω-3 together with the insufficient intake of these have led to a growing interest in the development of dietary supplements and foods enriched in PUFA ω-3 in recent years, which has led a European regulation with nutritional claims related to these fatty acids, such as "source of ω-3 fatty acids" (0.3 g of ALA or 40 mg of the sum of EPA + DHA per 100 g and per 100 Kcal) (EU. Commision Regulation, 2010).

Different methods have been investigated to microencapsulate PUFA ω-3, being spray drying the most used in the food industry as it is a flexible, economical process, easy to scale up and giving rise to a powder of good quality. This process involves the preparation of a simple oil-in-water (O/W) or double oil-in-water-in-oil (O/W/O) emulsion, having the O/W emulsions a single layer of emulsifier around each drop of oil. From here, techniques based on the superimposition of wall materials have been developed to increase the protection of ω-3 PUFAs, giving rise to multilayer emulsions (Jiménez-Martín et al., 2015, 2016), where the oil droplets are surrounded by multiple layers of wall materials, made up of a combination of an ionic emulsifier (i.e., lecithin) to stabilize the oil particles in the aqueous phase and one or more polyelectrolytes of opposite charges (i.e., chitosan and maltodextrin), joined by the electrostatic deposition technique "layer by layer". The choice of one or the other wall materials is influenced by the final application, depending on the food matrix likely to be enriched, the desired moment of release of the bioactive compound and the environmental conditions at which the

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Table 1
Quality characteristics of fish oil monolayered emulsions and their corresponding microcapsules as affected by ultrasonic homogenization conditions.

		Emulsion		Microcapsules		
		CI (%)	**↑ Tª (°C)**	**MY (%)**	**MEE (%)**	**TBARs (mg MDA/kg sample)**
Amplitude (%)	**60**	2.22 ± 0.69	15.70 ± 0.40	53.81 ± 5.56	75.01[a] ± 1.46	16.74 ± 5.73
	75	2.00 ± 0.78	14.73 ± 5.52	58.11 ± 8.51	62.96[ab] ± 13.41	12.89 ± 2.87
	90	1.50 ± 0.30	19.05 ± 0.35	65.50 ± 1.02	85.83[a] ± 2.56	11.70 ± 0.43
	p	0.736	0.800	0.199	0.014	0.120
Power (kg m^2/s^3)	**140**	2.23 ± 0.68	15.70 ± 5.90	53.81 ± 8.80	75.01[a] ± 13.03	16.74 ± 3.08
	200	2.00 ± 1.00	13.75 ± 0.70	59.70 ± 2.46	67.93[b] ± 4.30	14.09 ± 4.44
	260	2.00 ± 0.00	14.92 ± 0.23	57.80 ± 2.66	61.97[b] ± 16.25	12.65 ± 1.62
	p	0.897	0.926	0.348	0.030	0.199
Time (minute)	**10**	1.62[b] ± 0.68	18.29[a] ± 6.90	57.00 ± 8.80	70.66[a] ± 13.03	13.27 ± 3.08
	5	3.00[a] ± 1.00	4.4[b] ± 0.70	61.92 ± 2.46	65.2[a] ± 4.30	13.66 ± 5.44
	15	3.00[a] ± 0.00	8.97[ab] ± 0.23	64.08 ± 2.66	49.45[ab] ± 16.25	12.52 ± 1.62
	p	0.031	0.033	0.289	0.046	0.916
Emulsion Volume (ml)	**100**	1.15 ± 0.31	16.30 ± 6.21	57.71 ± 8.34	69.88[a] ± 12.24	13.32 ± 3.50
	200	0.00 ± 0.00	8.97 ± 0.23	64.08 ± 2.66	49.45[b] ± 16.25	12.52 ± 1.62
	p	0.096	0.243	0.210	0.016	0.703
Pulse (%)	**50**	2.12[b] ± 0.94	14.08[b] ± 6.35	59.21 ± 8.89	67.36[b] ± 13.33	13.70 ± 3.65
	20	3.00[b] ± 0.00	3.56[c] ± 0.15	53.27 ± 3.39	53.57[b] ± 11.07	10.69 ± 0.37
	80	0.00[a] ± 0.00	35.10[a] ± 0.30	59.47 ± 5.06	80.85[a] ± 10.90	12.87 ± 0.73
	p	<0.001	<0.001	0.509	0.055	0.355

* Creaming index (CI); increase of temperature after the homogenization process (↑ Tª); microencapsulation yield (MY); microencapsulation efficiency (MEE); lipid oxidation (TBARs). Values are expressed as mean ± standard deviation. Bars with different letters (a, b, c) within the same parameter studied show significant differences ($p < 0.05$) due homogenization condition applied.

microcapsules will be subjected.

Fish oil emulsions are generally made by rotor–stator systems that allow the adsorption of emulsifiers to the oil particles and the reduction of interfacial tension, avoiding the aggregation of particles, however, the emulsions obtained have size ranges of extremely variable particles (80 nm to 100 μm), being necessary to prolong the stability of these emulsions through a mechanical reduction of the size of the fat globules. In this context, the use of high pressure has been reported in previous studies as a promising strategy to improve the quality characteristics of emulsions and microcapsules (McClements, 2004; Pérez-Palacios et al., 2019), requiring the optimization of different homogenization parameters (Solomando et al., 2019). Moreover, among the scientific literature the use of ultrasound has also been proposed as a potential tool to encapsulate bioactive compounds, based on the ability of this technology to reduce the size of the emulsion droplets, however, the information available on the use of this technique to encapsulate bioactive compounds is limited (Mura et al., 2015) and most of the researches has focused on the optimization of the emulsion preparation, there being a great variability in the applied conditions (amplitude, power, time, pulse and emulsion volume). Apart from the influence on the emulsion stability, the homogenization of the emulsions is also a crucial effect on the quality characteristics of the microcapsules, especially the microencapsulation efficiency (Pérez-Palacios et al., 2019) thus, being reasonable the optimization of the homogenization procedure. However, no studies about the effect of the homogenization by ultrasonicated of fish oil emulsions on the bioaccessibility of microcapsules (McClements, 2004) have been found among the scientific literature. To optimize the quality characteristics of fish oil microcapsules univariate methodologies are not always satisfactory, because they do not consider the interactions between the different conditions applied, making it necessary to implement response surface methodologies as a viable alternative to resolve these limitations. These procedures allow exploring the relationship between several variables and one or more responses through a mathematical model capable of predicting the values of the responses and selecting the optimal conditions.

Therefore, the main objective of the present study was to improve the quality characteristics of fish oil microcapsules from monolayer and multilayer emulsions by optimizing the ultra-sonicated homogenization conditions, mainly focusing on stability, efficiency and bioaccessibility of EPA and DHA.

2. Material and methods

2.1. Experimental-statistical design

A preliminary assay was conducted to evaluate the influence of ultrasonicated homogenization parameters on the quality characteristics of the monolayered fish oil emulsions and their corresponding microcapsules, which were obtained by spray drying, as detailed below. In this assay, the ultra-sonicated homogenization parameters applied (amplitude (60, 75 and 90%), power (140, 200 and 260 kg m^2/s^3), time (5, 10 and 15 min), pulse (20, 50 and 80 %), and volume of homogenized emulsion (100 and 200 mL)) were set accordingly previous researcher (Soares et al., 2019). Table 1 shows the results of the creaming index and temperature changes were measured in the emulsions, and the microcapsules were evaluated by means of microencapsulation yield, microencapsulation efficiency and lipid oxidation. Triplicate experimental samples (n = 3) of emulsions and microcapsules batches were produced and analyzed by duplicate. The effect of each ultrasonic homogenization parameter was analyzed by one-way ANOVA using IBM SPSS Statistics v.27. This assay allowed the selection of most influencing parameters to further optimize ultrasonic homogenization conditions.

Following, a response surface methodology trial conducted to optimize the ultrasonic homogenization parameters for two types of emulsions (monolayer and multilayer) to achieve the best quality characteristics of emulsions and their corresponding microcapsules. For that, it was applied a full factorial central design, consisting of a complete 2^4 factorial design with five center points and one axial point on the axis of each design variable at $\alpha = 1$ from the design center. Therefore, the entire design had 30 combinations, including 5 replicates of the center point. Experimental parameters were power (varying from 140 to 260 kg m^2/s^3), time (varying from 5 to 15 min), pulse (varying from 20 to 80 %), and volume of homogenized emulsion (varying from 100 to 200 mL) and two responses were evaluated: creaming index and microencapsulation efficiency. Table 2 shows the complete experimental design, which was performed by Design Expert Trial-Version 7 (Stat-Ease Inc., Minneapolis, MN).

Finally, emulsions obtained under optimized ultra-sonicated conditions and their corresponding microcapsules were characterized by means of (a_w, moisture content, bulk and tapped density, Carr's index, Hausner ratio, dissolution rate, microencapsulation yield, microencapsulation efficiency, lipid oxidation, instrumental color and EPA and DHA quantity), comparing the results with those obtained in monolayer and multilayer emulsions and microcapsules produced under optimized conditions of high pressure homogenization using one-way analysis ANOVA using IBM SPSS Statistics v.27.

2.2. Materials and reagents

Fish oil from cod liver was kindly provided by Biomega Nutrition

Table 2
Coded and uncoded values of the independent variables and responses obtained of the central composite design for optimization of the ultrasonic homogenization conditions of monolayered and multilayered fish oil emulsions.

	Independent variables								Responses			
	Coded				Uncoded				Monolayered		Multilayered	
Run	Power	Time	Pulse	Volume	Power	Time	Pulse	Volume	CI (%)	MEE (%)	CI (%)	MEE (%)
1	0	0	0	0	200	10	50	150	0.33 ± 0.25	89.30 ± 1.48	13.50 ± 0.55	66.94 ± 0.84
2	−1	−1	+1	+1	140	5	80	200	1.33 ± 0.15	78.23 ± 0.67	7.00 ± 1.10	78.29 ± 1.68
3	+1	−1	+1	+1	260	5	80	200	1.67 ± 0.55	70.48 ± 5.03	13.50 ± 1.50	76.90 ± 4.86
4	0	0	0	0	200	10	50	150	1.00 ± 0.00	85.27 ± 1.51	9.50 ± 0.85	72.63 ± 0.18
5	0	0	0	0	200	10	50	150	1.67 ± 0.45	81.82 ± 1.80	7.00 ± 1.45	68.22 ± 6.50
6	−1	+1	+1	−1	140	15	80	100	0.33 ± 0.25	82.70 ± 7.95	10.00 ± 2.10	68.52 ± 5.47
7	−1	+1	+1	+1	140	15	80	200	0.33 ± 0.15	85.69 ± 3.36	6.50 ± 1.05	66.41 ± 0.26
8	0	0	0	−1	200	10	50	100	1.33 ± 0.45	79.13 ± 2.36	10.50 ± 1.55	58.23 ± 0.75
9	1	−1	−1	−1	260	5	20	100	6.33 ± 0.80	70.54 ± 1.29	7.00 ± 0.90	66.64 ± 2.00
10	+1	−1	−1	+1	260	5	20	200	6.33 ± 0.55	61.09 ± 1.08	5.50 ± 1.00	56.41 ± 7.91
11	0	0	0	+1	200	10	50	200	3.67 ± 0.25	69.58 ± 3.04	8.50 ± 1.65	58.12 ± 6.18
12	−1	−1	+1	−1	140	5	80	100	1.33 ± 0.15	73.47 ± 1.51	6.50 ± 1.45	68.92 ± 0.84
13	+1	+1	+1	−1	260	15	80	100	0.00 ± 0.00	65.27 ± 6.18	9.50 ± 1.00	75.51 ± 5.70
14	0	0	0	0	200	10	50	150	2.67 ± 0.25	70.90 ± 0.92	5.00 ± 0.40	73.13 ± 2.27
15	0	0	−1	0	200	10	20	150	6.00 ± 1.35	74.63 ± 5.97	10.50 ± 1.70	63.27 ± 1.58
16	−1	−1	+1	+1	140	5	20	200	5.00 ± 0.90	67.81 ± 3.29	9.00 ± 1.90	60.14 ± 4.21
17	0	+1	0	0	200	15	50	150	1.33 ± 0.10	78.16 ± 0.53	16.50 ± 1.65	65.39 ± 1.34
18	0	0	0	0	200	10	50	150	3.00 ± 0.35	75.41 ± 1.73	6.00 ± 0.25	77.40 ± 0.52
19	+1	+1	+1	+1	260	15	80	200	0.00 ± 0.00	75.30 ± 0.86	5.50 ± 1.00	80.88 ± 0.78
20	+1	+1	−1	−1	260	15	20	100	0.67 ± 0.10	86.21 ± 0.59	11.50 ± 0.55	73.91 ± 1.42
21	−1	+1	−1	+1	140	15	20	200	6.00 ± 0.60	76.03 ± 1.41	11.00 ± 1.10	70.56 ± 0.03
22	−1	−1	−1	−1	140	5	20	100	5.67 ± 0.75	77.29 ± 2.20	10.50 ± 1.25	75.73 ± 1.96
23	0	0	+1	0	200	10	80	150	0.00 ± 0.00	90.87 ± 4.81	10.00 ± 1.55	75.84 ± 1.84
24	+1	+1	−1	+1	260	15	20	200	5.33 ± 0.40	69.39 ± 2.13	11.50 ± 1.60	62.87 ± 3.05
25	0	−1	0	0	200	5	50	150	4.33 ± 0.35	71.85 ± 6.57	8.50 ± 1.05	71.75 ± 5.70
26	+1	−1	+1	−1	260	5	80	100	0.00 ± 0.00	90.42 ± 0.94	3.50 ± 0.60	58.33 ± 1.05
27	−1	0	0	0	140	10	50	150	3.33 ± 0.15	75.43 ± 0.37	5.50 ± 1.05	66.99 ± 1.45
28	−1	+1	−1	−1	140	15	20	100	4.33 ± 0.80	79.22 ± 3.09	11.00 ± 1.10	55.53 ± 0.30
29	0	0	0	0	200	10	50	150	3.00 ± 0.70	92.30 ± 2.35	5.50 ± 0.75	76.14 ± 1.59
30	+1	0	0	0	260	10	50	150	4.67 ± 1.10	81.68 ± 1.45	9.50 ± 0.60	70.02 ± 1.07

CI, creaming index; MEE, microencapsulation efficiency. Values are expressed as mean ± standard deviation.

(Galicia, Spain). Soybean lecithin (Across Organics, Madrid, Spain), chitosan with 95 % of deacetylation (Chitoclear FG 95, kindly provided by Trades, Murcia, Spain), maltodextrin with a 12 % dextrose equivalent (Glucidex 12, kindly provided Roquette, Lestrem, France), and food-grade glacial acetic acid (Scharlau, Barcelona, Spain) were used for the preparation of the emulsions. Commercial buffer solutions (Crison, Barcelona, Spain) at pH 4.0 and 7.0 were used to calibrate the pH meter. Hydrochloric acid and petroleum ether (Scharlau) were used for the oil extraction of the microcapsules. 1-butanol and isopropanol (Scharlau) were used as solvents and 2-thiobarbituric acid (TBA, Serva, Heidelberg, Germany), trichloroacetic acid (Scharlau), and 2,6-di-*tert*-butyl-4-methylphenol 99% (BHT, Across Organics, Madrid, Spain) as reagents for the oxidative stability. Sulphuric acid, methanol, and hexane (Scharlau) were used for the transesterification of fatty acids. α-amylase from human saliva type IX-A, 1000–3000 U mg^{-1} protein (EC 3.2.1.1), pepsin from porcine gastric mucosa 3200–4500 U mg^{-1} protein (EC 3.4.23.1), porcine pancreatic lipase 350 U mg^{-1} protein (EC 3.1.1.3) and porcine bile extract (EC. 232–369-0) (Sigma-Aldrich, Madrid, Spain) were used for the simulated digestion and sodium chloride, potassium chloride, monopotassium phosphate, sodium bicarbonate, magnesium chloride hexahydrate, ammonium carbonate, calcium chloride tetrahydrate (Sigma-Aldrich) were used as electrolytes in simulated salivary, gastric, and intestinal fluids; sodium hydroxide and hydrochloric acid (Scharlau) were used to adjust the pH.

2.3. Elaboration of fish oil emulsions and microcapsules

Two different types of fish oil emulsions, monolayered (Mo) and multilayered (Mu), and their corresponded microcapsules were prepared following the methodology described by Jiménez-Martín et al., (2014) with slight modifications. Fish oil (20 g) and lecithin (6 g) were mixed with a magnetic stirrer overnight under controlled environment conditions of temperature and relative humidity (18 °C and 40%, respectively). Then, water was added until a total weight of 200 g and homogenized (20000 rpm, 10 min) using an Ultraturrax T-18 basic (IKA, Germany). In this way, the primary emulsion was obtained and then homogenized by ultrasounds with a 24 KHz probe, model S24d (Hielscher UP400St, Germany) under the different conditions tested in this study. For the emulsions homogenized by high pressures, an SPX homogenizer, model APV-200a(Silkeborg, Denmark), was used under previously optimized conditions in a previous work (Solomando et al., 2019). The homogenized primary emulsion was blended with 200 g of water, in the case of Mo, and with 200 g of 1 % of chitosan (w/w) in acetic acid 1 %, in the case of Mu, by slowly agitation with a magnetic stirrer for 15 min. In both types of emulsions, the final step consists of adding 400 g of maltodextrin solution (120 g maltodextrin + 280 g water), to obtain the feed emulsion (800 g), which was dried in a laboratory-scale spray drier equipped with a 0.5-mm nozzle atomizer (Mini spray-dryer B-290, Buchi, Switzerland). The emulsions, maintained at room temperature, were constantly and gently agitated in a magnetic stirrer during the spray drying process. The aspirator rate was adjusted at 80 %, feed rate was 1 L/h, inlet temperature was 180 °C, and outlet temperature ranged 85–90 °C. The collected dried powders were stored in containers at −80 °C until being analyzed.

2.4. Analysis of emulsion characteristics

2.4.1. Microscopic observations

Samples of the feed emulsions were observed at the microscope to provide assessment of their respective microstructures. Immediately after the production and before spray drying process, emulsions were kept under gently agitation on a magnetic stirrer to maintain

homogeneity, and a drop of sample was then taken with a Pasteur pipette, placed on a microscope slide, and covered by a cover slip. An optical microscope Axiovert 25 CFL (Prolabo, France) was used to examine the microstructure of the feed emulsions through a × 100 oil immersion objective. Digital image files were acquired with a Nikon F90X camera connected to a digital image processing system (AxioVision Release 4.8, Zeiss, Germany).

2.4.2. pH

The pH of the feed emulsions was determined with a glass electrode pH meter model CyberScan pH 510 (Eutech Instruments, Illkirch, France), testing a 10 mL volume taken immediately after the production. The pH meter was calibrated with commercial buffer solutions (Crison, Barcelona, Spain) at pH 4.0 and 7.0 prior to the samples.

2.4.3. Creaming index (CI)

Emulsions were transferred into a tube sealed with a plastic cap and stored for 1–7 days at room temperature. The cream layer (HC) and the total height (HE) of the emulsions were measured. Creaming index was calculated as CI = 100 * (HE − HC/HE).

2.4.4. Density and viscosity

Density (ρ) was determined using a 25 mL Gay-Lussac pycnometer, and viscosity (η) was measured using a Selecta STS-2011 viscometer (J. P. SELECTA, Spain). A spindle TL1 with APM adaptor was used at 50 rpm for viscosity determination in 100 mL of sample. Both analyses were performed in triplicate at 25 °C, using an incubator FTC E90 (VELP Scientifica Srl, Italy).

2.5. Analysis of microcapsules characteristics

2.5.1. Moisture content and water activity (a_w)

Moisture was analyzed by following AOAC (2000) reference method 935.29 and water activity was analyzed using a Novasina aw-Center meter (Novasina AG, Lanchen, Switzerland).

2.5.2. Instrumental color

Instrumental color was measured with a transparent film on a cross section of the sample, determining luminosity (L*), redness (a^*) and yellowness (b^*). It was used a Minolta CR-300 colorimeter (Minolta Camera Corp., Meter Division, Ramsey, NJ) with illuminant D65, a 0° standard observer and one port / display area of 2.5 cm. The colorimeter was calibrated before use with a white tile having the following values: L* = 93.5, a^* = 1.0 and b^* = 0.8.

2.5.3. Bulk (ρ_B) and tapped (ρ_T) density

For bulk density (ρ_B), each powder sample (2 g) was filled in a 25 mL measuring glass cylinder (diameter 2.5 cm) and the cylinder was slightly tapped to remove the powder sticking to the walls. The volume (Vo) was read directly from the cylinder and bulk density was calculated by using following formula: $\rho_B = m/Vo$. For tapped density (ρ_T), the cylinder was tapped manually approximately 50 times on marble solid surface from a height of 10 cm to measure final volume (Vn) and it was calculated by using following formula: $\rho_T = m/Vn$.

2.5.4. Carŕs index and Hausner ratio

The flow characteristics of the microencapsulated powders were evaluated using the Carŕs index and Hausner ratio that were determined according to the procedure described by Turchiuli et al., (2005). Carŕs index indicates the compressibility and Hausner ratio the cohesion of the powder and were calculated using the following equations: Carŕs index $= [(\rho_T - \rho_B)/\rho_T] \times 100$ and Hausner ratio $= \rho_T/\rho_B$.

2.5.5. Dissolution rate

The dissolution rate was determined according to the procedure described by Millqvist-Fureby et al., (2001). The absorbance at 620 nm was measured in a spectrophotometer Jenway 7305 (Roissy, France) using glass cuvettes with a light path length of 1 cm, following the increase in optical density at 620 nm (OD_{620}) with time until being constant.

2.5.6. Microencapsulation efficiency

Microencapsulation efficiency (MEE) was determined as a function of the encapsulated oil (within the core of the microcapsules) related to the total oil content of the microcapsules (no encapsulated oil plus oil within the core of the microcapsules). For that, the external (no encapsulated oil) and total oil of the microcapsules were quantified, and the MEE was calculated with the following equation, according with (Jiménez-Martín et al., 2015).

For the quantification of the external fish oil, 2 g of the powders were placed into an Erlenmeyer flask with 25 mL of petroleum ether, and softly stirred. The flask was sealed using a cap with an air condenser and introduced in a bath at 65 °C during 20 min. After that, filtration was carried out using Whatman no 3 filter. Finally, solvent was evaporated, and external oil content was determined gravimetrically and expressed as g of external oil/100 g of microcapsules.

For the extraction of total fish oil, 5 g of powders were placed into a sealed flask with 50 g of hydrochloric acid 1 M and, after digestion during 20 min at 96 °C, filtration was carried out using Whatman no 1 filter. Finally, solvent was evaporated, and total oil content was determined gravimetrically and expressed as g of total oil/100 g of microcapsules.

$$Microencapsulation\ \ efficiency\ \ (\%) = \frac{total\ \ oil - external\ \ oil}{total\ \ fish\ \ oil} x100$$

2.5.7. Lipid oxidation

The lipid oxidation of the microcapsules was determined by the thiobarbituric acid reactive substances method, as described by Hu and Zhong (2010). First, a TBA stock solution was obtained by mixing 0.8 g of TBA dissolved in 100 mL of a ternary solvent mixture (1-butanol/isopropanol/HCl 0.5 mol/L (2:2:1, v/v/v)). Samples (10 mg) were placed into centrifuge tubes and mixed with 3.16 mL of ternary solvent and 6.8 mL of TBA stock solution using a vortex. Then, tubes were placed in a heater at 20 °C for 24 h and in darkness. At the same time, a standard curve was prepared with solutions of TMP in the ternary solvent in a range between 0.2 and 10 μM, which were processed as the samples. Finally, absorbance at 532 nm was measured in a Jenway 7305 spectrophotometer (Jenway, Roissy, France) using ternary solvent mixture as a blank and glass cuvettes with a light path length of 1 cm. Malondialdehyde (MDA) equivalence was calculated from the calibration curve, and TBARS level was expressed as mg MDA kg^{-1} of oil. Absorbance data were the averages of at least triplicate determinations.

2.5.8. Particle size

Mean diameter of microcapsules was measured using a laser light diffraction instrument, Mastersizer 3000 (Malvern Instruments, Malvern, UK) in dry dispersion. Results were given in percentage volume, surface area mean diameter (D [3;2]), and percentile 10 and 90% (Dv 10 and 90, respectively).

2.5.9. Quantification of fatty acids

Fatty acid methyl esters (FAME) were obtained by acidic transesterification following the method described by Sandler and Karo (1992), using 100 mg of microcapsules. FAME were analyzed by gas chromatography, using an Agilent 6890 N gas chromatograph, equipped with a flame ionization detector (FID). Separation was carried out on a cyanopropyl column (ZEBRON ZB-FAME, Phenomenex, California, USA) (20 m × 0.18 mm i.d. × 0.15 μm film thickness) with split injection (100:1). Oven temperature programming started at 150 °C. Immediately, it was raised 10 °C min^{-1} to 180 °C. This was held for 1 min and increased again at 7 °C min^{-1} to 205 °C which is maintained for 2 min.

Injector and detector temperatures were 250 °C. The carrier gas was helium at a flow rate of 2.7 mL min^{-1}. Individual FAME peaks were identified by comparison of their retention times with those of standards (Supelco 37 component FAME mix, Merck) and quantified using tridecanoic acid (C13:0) as internal standard. Results were expressed as mg FAME/g microcapsule.

2.6. Simulated in vitro digestion

The release of fatty acids from the microcapsules was evaluated in simulated digestion conditions, including three sequential steps: oral, gastric, and intestinal. Stock solutions, simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared following the methodology of Minekus et al., (2014). All solutions were prepared daily and pre-warmed at 37 °C before use. Additionally, salivary α-amylase (oral phase) was prepared in SSF to a final concentration of 75 U/mL, pepsin (gastric phase) in SGF to a final concentration of 2000 U/mL, as well as pancreatin and bile salts (intestine phase) in SIF to a final concentration of 100 U/mL and 10 mM, respectively. Fish oil microcapsules (2 g) were weighed in a falcon tube and mixed thoroughly with 1.4 mL SSF solution, 0.2 mL of salivary α-amylase solution, 10 μL of 0.3 mol/L $CaCl_2$ and 300 μL of distilled water. The mixture was homogenized by vortex during 1 min and stirred at 300 rpm during 2 min at 37 °C. The supernatant was separated from the residue by extraction with hexane (5 mL) and centrifugation (4000 rpm, 20 min). Gastric digestion was continued by immediate addition of 3 mL of SGF solution, 0.64 mL of pepsin, 2 μL of 0.3 mol/L $CaCl_2$, and 0.278 mL of distilled water to the oral bolus, and pH was adjusted to 3.0 with 80 μL volume of 6 M HCl. The mixture was incubated at 37 °C with shaking at 300 rpm during 2 h. Again, the supernatant was extracted with 10 mL of hexane. Then, intestinal digestion was followed by the addition to the gastric extract of 4.4 mL of SIF, 2 mL of pancreatin, 0.12 mL of bile salts, 0.12 mL of pancreatic lipase, 6.4 μL of 0.3 M $CaCl_2$ and 0.564 mL distilled water; pH was adjusted to 7 with 60 μL of 1 M NaOH and kept under agitation (120 rpm) at 37 °C for 2 h. The supernatant was separated from the residue by extraction with hexane (5 mL) and centrifugation (4000 rpm, 20 min). All supernatants were extracted in weighted glass tubes and the FAMEs of the three steps were obtained by applying acidic *trans*-esterification and analyzed by gas chromatography, as previously described.

2.7. Scan electron microscopy

The morphology of the microcapsules was explored by using a scanning electron microscope (FEI Quanta 3D FEG; FEI Company, Hillsboro, OR USA) in high-vacuum conditions mode using an Everhart–Thornley detector to explore the morphology of the microcapsules. Powder samples were added on stubs, fixed with a conductive double-sided adhesive carbon sheet, and then subjected to metallization (sputtering) with a thin layer of a conductive gold coating for 8 s to amplify the secondary electron signal. After metallization, the samples were imaged operating at 3 kV with a focused electron beam of Ga^{+} (current of $< 6 \times 10^{-4}$ Pa) and observed with magnifications of between 2500× and 20000×.

3. Results and discussion

3.1. Prediction adequacy of models

Table 1 shows results on quality characteristics of monolayered fish oil emulsions (CI and ↑ T^a after homogenization process) and their corresponding microcapsules (MY, MEE and TBARs) as affected by ultrasonic homogenization conditions (amplitude, power, time, pulse, and emulsion volume). MEE was significantly influenced by most studied variables (amplitude, power, time, and emulsion volume), while CI and ↑ T^a were only affected by time and pulse and MY and TBARs values did not changes with the studied variables. Considering these findings and that amplitude and power are related parameters, next step was trying to optimize homogenization variables (power, time, pulse, and volume) to maximize CI in monolayered and multilayered fish oil emulsions, and MEE in microcapsules by response surface methodology. Table 2 exposes the four full-factorial central composite design, specifying coded and uncoded values of the studied variables and the obtained responses for each one of the 30 combinations. Table 3 shows results of the variance analysis by means of Fisher's *F* test for CI and MEE. In the case of monolayered emulsions, the model F values were 6.39 and 1.41 for CI and MEE, respectively. This indicates the significance of the model for CI, with a 0.03% chance, that a so large model F value could occur due to noise. Lower values than 0.05 were found for B (time), C (pulse) and D (volume) in CI, and for C in MEE which indicates that they are significant terms. The lack of fit F values of 1.38 and 1.02, respectively for CI and MEE, are good and show that the model fits. There is a 38.33% and 53.67% chance, respectively, for CI and MEE, that a so large lack of fit F value could take place due to noise. Thus, the response surface quadratic models for CI and MEE of monolayered fish oil microcapsules are adequate and significant.

Table 3
Analysis of variance for response surface model for the creaming index (CI) and microencapsulation efficiency (MEE) of ultrasound homogenized monolayered and multilayered fish oil emulsions and microcapsules.

		Monolayered		Multilayered	
Dependent variable	Source	F value	Prob. > F	F value	Prob. > F
CI	Model	6.39	0.0003	0.80	0.6334
	A	0.032	0.8605	0.00	1
	B	4.57	0.0458	2.84	0.1082
	C	48.26	<0.0001	1.41	0,2496
	D	4.57	0.0458	0.023	0,8798
	AB	1.75	0.2017	0.059	0.8100
	AC	0.036	0.8522	0.42	0.5233
	AD	1.75	0.2017	0.54	0.4734
	BC	0.32	0.5775	0.95	0.3417
	BD	0.89	0.3567	1.49	0.2377
	CD	1.75	0.2017	0.24	0.6314
	Residual				
	Lack of fit	1.38	0.3833	0.87	0.620
MEE	Model	1.41	0.0247	1.43	0.2401
	A	1.01	0.3280	0.13	0.7177
	B	0.73	0.4037	0.053	0.8211
	C	3.50	0.0469	5.21	0.0331
	D	1.95	0.1789	0.11	0.7470
	AB	2.42	0.1363	4.57	0.0458
	AC	0.084	0.7752	0.19	0.6684
	AD	0.022	0.8834	0.023	0.8817
	BC	2.70	0.1169	0.034	0.8552
	BD	0.23	0.6362	0.037	0.8495
	CD	1.50	0.2358	3.96	0.0613
	Residual				
	Lack of fit	1.02	0.5367	3.11	0.1081

A = Power; B = Time; C = Pulse; D = Volume.

Regarding multilayered emulsions, the model F value of 0.80 and 1.43 for CI and MEE, respectively, implied the insignificance of models for these parameters. This lack of fit indicates the no existence of relationship between the parameters of power, time, pulse and volume of ultra-sonicated homogenization and the response variables, which could be related to the multilayer wall (lecithin-maltodextrin and chitosan) of this type of microcapsules, giving rise to a much more compact structure and less susceptible to changes than the lecithin-maltodextrin monolayer wall that should be more susceptible to changes due to variations in the ultra-sonicated homogenization parameters. In fact, this finding is in accordance with a previous study that showed a major influence of the homogenization by high pressures on monolayered than in multilayered microcapsules (Solomando, Antequera, Ruiz-Carrascal, et al., 2020).

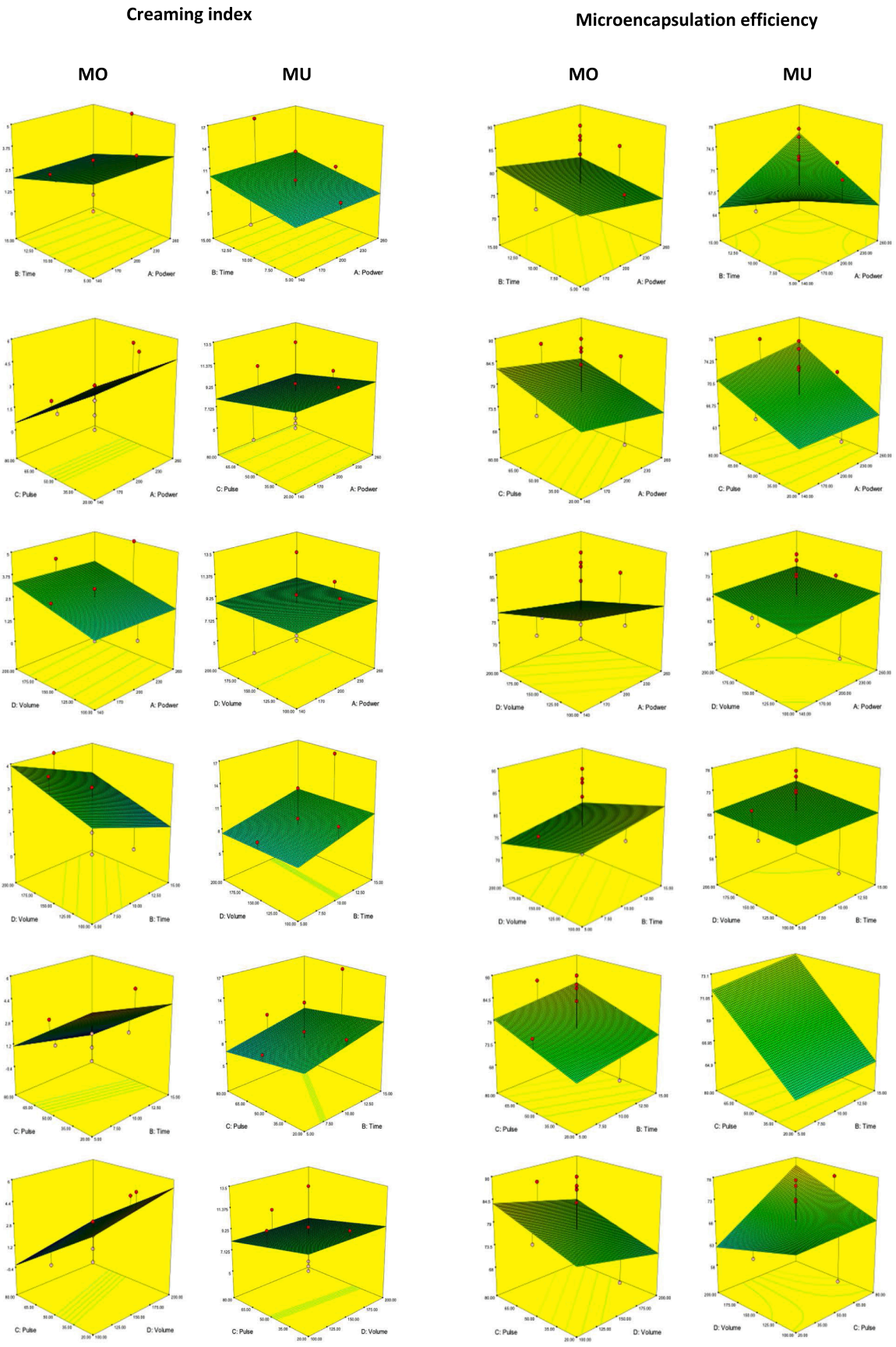

Fig. 1. Response surfase plots on the creaming index and microencapsulation efficiency of monolayered (Mo) and multilayered (Mu) fish oil emulsions and their corresponding microcapsules as affected by ultra-sonicated homogenization conditions (power, time, pulse and volume).

Table 4
Values of experimental and predicted responses in monolayered and multilayered fish oil emulsions homogenized by ultrasounds under optimized conditions.

			Monolayered	Multilayered
Optimum homogenization conditions		Power (kg m^2/s^3)	141.01	141.21
		Time (minute)	15	5
		Pulse (%)	80	80
		Emulsion volume (ml)	100	200
Responses	Creaming index (%)	Experimental	0.2 ± 0.08	6.00 ± 1.08
		Predicted	0	6.63
	Microencapsulation efficiency (%)	Experimental	82.70 ± 7.94	76.90 ± 4.86
		Predicted	87.15	77.50

Table 5
Quality characteristics of monolayered and multilayered emulsions and their corresponding microcapsules obtained by spray drying as affected by ultrasonic (US) and high-pressure (HP) homogenization.

	US			HP		$p_{US\ Vs\ HP}$	
	Mo	**Mu**	**p**	**Mo**	**Mu**	**Mo**	**Mu**
Emulsions							
pH	6.27 ± 0.01	3.72 ± 0.04	***	6.54 ± 0.00	4.08 ± 0.01	**	**
Creaming index (%)	0.2 ± 0.08	6.00 ± 1.08	***	2.00 ± 0.00	9.30 ± 0.58	***	***
Density (g/cm^3)	1.05 ± 0.00	1.05 ± 0.00	n.s.	1.04 ± 0.00	1.05 ± 0.00	n.s.	n.s.
Viscosity (Pa·s)	0.04 ± 0.00	0.06 ± 0.00	*	0.04 ± 0.00	0.06 ± 0.00	n.s.	n.s.
Microcapsules							
Water activity (aw)	0.16 ± 0.00	0.19 ± 0.00	*	0.11 ± 0.00	0,12 ± 0.00	**	***
Moisture content (%)	1.22 ± 0.06	1.75 ± 0.61	n.s.	0.60 ± 0.09	1.09 ± 0.23	**	n.s.
Bulk density (g/cm^3)	0.31 ± 0.00	0.29 ± 0.02	n.s.	0.30 ± 0.01	0.30 ± 0.01	n.s.	n.s.
Tapped density (g/cm^3)	0.58 ± 0.01	0.50 ± 0.01	*	0.56 ± 0.02	0.54 ± 0.03	n.s.	n.s.
Carŕs index (% compressibility)	45.94 ± 0.68	42.53 ± 0.37	**	46.42 ± 0.31	44.44 ± 0,24	n.s.	**
Hausner ratio (HR)	1.85 ± 0.02	1.74 ± 0.01	*	1.86 ± 0,01	1.80 ± 0.02	n.s.	*
Dissolution rate (% of OD_{max}/min/mg)	0.81 ± 0.02	0.41 ± 0.08	***	0.85 ± 0.05	0.34 ± 0.12	n.s.	n.s.
Microencapsulation yield (%)	58.41 ± 2.14	41.36 ± 1.79	***	51.54 ± 2.89	39.47 ± 3,61	**	n.s.
Total oil (%)	11.37 ± 0.61	11.42 ± 0.16	n.s.	9.18 ± 0.51	7.44 ± 0.23	**	***
External oil (%)	1.99 ± 0.44	4.55 ± 0.28	***	1.37 ± 0.26	3.65 ± 0.56	n.s.	n.s.
Microencapsulation efficiency (%)	82.70 ± 7.94	76.90 ± 4.86	*	85.06 ± 2.94	50.94 ± 9.09	n.s.	**
Lipid oxidation (mg MDA/kg sample)	13.22 ± 1.94	11.47 ± 1.43	n.s.	32.91 ± 3.94	30.41 ± 5.87	***	***
mg EPA + DHA/g microcapsule	15.28 ± 1.56	15.3 ± 1.67	n.s.	12.3 ± 0.37	10.04 ± 0.51	**	***
Instrumental color							
L*	96.30 ± 0.62	97.84 ± 0.49	n.s.	99.72 ± 0.42	99.38 ± 0.56	**	*
*a**	−0.12 ± 0.04	−0.44 ± 0.06	**	0.29 ± 0.09	0.53 ± 0.10	**	***
*b**	4.54 ± 0.67	4.49 ± 0.83	n.s.	5.58 ± 0.51	6.74 ± 0.61	n.s.	n.s.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. non-significant.

3.2. Selection of the optimum conditions

The surface and contour plots on each response function (CI and MEE) as affected by four studied variables (power, time, pulse, and volume of ultrasound homogenization) for monolayered and multilayered fish oil microcapsules are shown in Fig. 1. In the case of monolayer microcapsules, CI decreased with longer times (Fig. 1 a1, a4, a5), higher pulses (Fig. 1a2, a5, a6) and lower volumes (Fig. 1 a3, a4, a6). The MEE increased with lower powers (Fig. 1c1, c2, c3) and volumes (Fig. 1c3, c4, c6), longer times (Fig. 1 c1, c4, c5) and higher pulses (Fig. 1c2, c5, c6). In the case of multilayer microcapsules, CI decreased with shorter times (Fig. 1b1, b4, b5) and higher pulses (Fig. 1b2, b5, b6), and MEE increased with lower powers (Fig. 1d1, d2, d3), shorter times (Fig. 1d1, d4, d5) and higher pulses (Fig. 1d2, d5, d6). Next, optimization of these variables was developed to achieve the lowest values for CI and the highest percentage for MEE (Table 4), resulting on power of 141.01 kg m^2s^{-3}, time of 15 min, pulse of 80 % and 100 mL of emulsion volume in the case of monolayer emulsions and 141.21 kg m^2s^{-3}, time of 5 min, pulse of 80 % and 200 mL of emulsion volume in the case of multilayer emulsions.

3.3. Validation of model prediction

To confirm the validity of the experimental models, the emulsion stability and microencapsulation efficiency were analyzed in homogenized monolayer and multilayer emulsions elaborated under the selected optimal conditions and in their respective microcapsules, respectively, with a triplicate set. The experimental values obtained were compared with those predicted values (Table 4), finding a high consistency (Table 4) for CI and MEE in both, monolayer (0.2 *vs* 0, and 82.70 *vs* 87.15) and multilayer samples (6 *vs* 6.63, and 76.90 *vs* 77.50, respectively).

3.4. Characterization of optimum ultra-sonicated emulsions and microcapsules

After optimization, ultra-sonicated homogenized emulsions and microcapsules were characterized and compared with results from high-pressure homogenized emulsions and microcapsules that were prepared under conditions set in previous studies (Table 5) (Solomando, Antequera, Ruiz-Carrascal, et al., 2020).

The results of the quality characteristics of the ultra-sonicated emulsions indicated that the type of wall materials influences three of the four characteristics studied, showing monolayer fish oil emulsions higher pH values and lower CI and viscosity values than Mu ones, which agree with previous studies (Solomando et al., 2019), while no differences were found in the density of the emulsions. The additional layer of chitosan in Mu explains its lower pH, due to it is dissolved in acetic acid 1%, and higher viscosity, while the higher CI in Mu, the stability was lower in this type of emulsions. Since there is a positive correlation between CI and particle size (Klinkesorn et al., 2005), this result can be explained by the lower influence of the ultra-sonicated homogenization

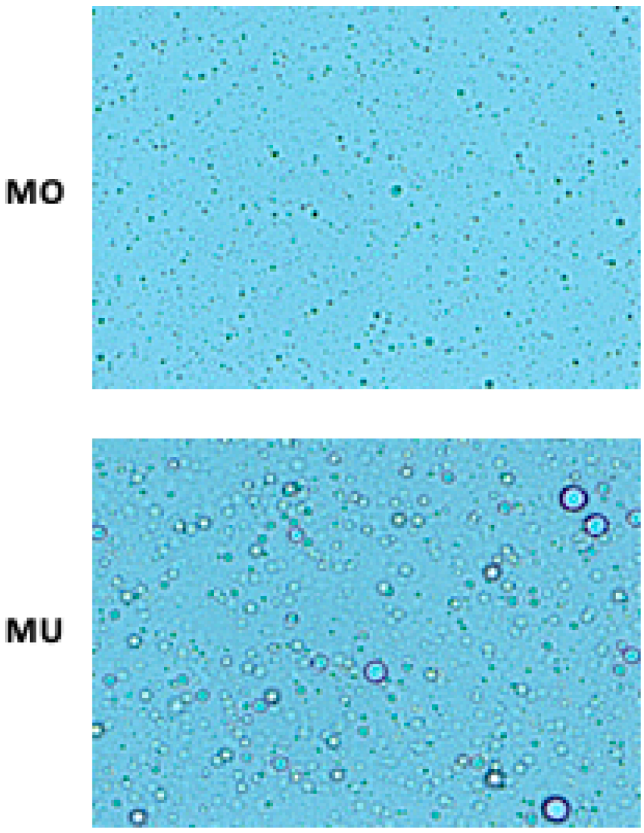

Fig. 2. Optical microscopic images of monolayered (Mo) and multilayered (Mu) fish oil primary emulsions homogenized by ultra-sonicated and observed at 100x magnifications.

in Mu than in Mo, as previously indicated, not achieving Mu emulsion the same size reduction in the oil drops as Mo. In fact, optical microscope images of ultra-sonicated emulsion (Fig. 2) reflected this fact, observing smaller oil drops in Mo in comparison to Mu emulsions. The type of homogenization method applied to Mo and Mu fish oil emulsions did not significantly influence density and viscosity, which was expected because these parameters are specially related to the wall materials, lower pH and CI values were found in Mo and Mu emulsions homogenized by ultrasound (0.2% and 6%, respectively) compared to those emulsions homogenized by high-pressure (2% and 9.30%, respectively), having more stable emulsions when homogenizing by ultrasounds.

This positive influence of ultra-sonicated as a homogenization method can be attributed to the cavitation effect that is transmitted through waves that compress and expand the molecular structure of the medium (emulsion) through which it passes. In addition, as the bubbles in the cavitation grow and quickly collapse, cause high shear forces that implode at a high speed and produce droplets of the immiscible liquid phase to break, giving rise to an emulsion composed of small droplets. The lower pH in ultra-sonicated emulsions may be also related its significant lower CI and support the lower size of oil droplets, having in these emulsions a high superficial area that requires more maltodextrine (in Mo) and maltodextrine-quitosan (in Mu), which are positively charged, to surround the oil droplets, leading to less positive charge in the emulsion.

The results on quality characteristics of fish oil microcapsules (Table 5) showed that the type of wall materials and the homogenization method influenced in nine of the sixteen characteristics studied, showing Mo fish oil microcapsules lower values of a_w and percentage of external oil (not encapsulated), and higher tapped density, Carŕs index, Hausner ratio, dissolution rate, MY, MEE and instrumental color coordinate a^*, compared to Mu, while no differences were found in moisture content, bulk density, total oil, lipid oxidation, quantity of EPA plus DHA and L and b^* instrumental color coordinates.

Regarding the homogenization method, it significantly influenced in eight and nine of the sixteen quality characteristics studied in Mo and Mu, respectively, finding that both types of microcapsules homogenized by ultrasound had higher values of water activity (a_W), moisture, MY (in Mo), total oil, MEE (in Mu) and quantity of EPA plus DHA, and lower values of Carŕs index and Hausner ratio (in Mu), lipid oxidation and instrumental color coordinates (L and a*) compared to those homogenized by high-pressure.

In relation to water activity and moisture content, despite the significant differences, the values for these parameters are within a narrow range (0.11–0.16 and 0.60–1.75 %, respectively), lower than those required in microcapsules to guarantee physico-chemical, microbiological, and oxidative stability (0.4 and 5%, respectively) (Klinkesorn et al., 2005). This may indicate that the use ultrasounds gives rise to appropriate emulsions to be corrected desiccated during the spray-drying process, and allows, consequently, obtaining stable microcapsules (McClements et al., 2007) which is reflected in the lipid oxidation values obtained, being the ultra-sonicated homogenization method the most favorable for both types of microcapsules and the measurement of reactive substances to thiobarbituric acid an excellent indicator to see the stability and useful life of the encapsulated oil (Jafari et al., 2008).

Similarly, despite some significant differences between microcapsules batches in tapped density, Carŕs index, Hausner ratio, and L* and a* instrumental color coordinates, it can also be observed a narrow range of values for these parameters (0.50–0.58 g cm^{-3}, 42.53–46.42, 1.74–1.86, 96.30–99.72 and −0.44–0.53). Bulk and tapped densities are related to particle size and geometry and influence on the stability of the microcapsules during package, transport, storage, and marketing. Thus, spherical and low size microcapsules may have high density values (>1 g cm^{-3}), which supposes lower air incorporation during microcapsule production and therefore greater stability against oxidation as well as the storage of larger quantities in smaller containers (Turchiuli et al., 2005). Densities values of microcapsules of the present studies are lower than those desired, however, it seems not have a negative impact on oxidation stability because low TBARs values have been found in the analyzed samples. Regarding the flowing properties, Carr's index evaluates the flow properties of powders, being the limit between free flow (granular) and non-free flow (powder) around 20 to 25% compressibility, and the Hausner ratio indicates the cohesiveness and ability to flow freely, with Hausner ratio higher than 1.34 related to poor flow characteristics of powders (Turchiuli et al., 2005). Therefore, all batches of microcapsules of this study presented poor fluidity. Other authors (Turchiuli et al., 2005) who also obtained not so appropriate flowing properties have related this aspect with high oil content, which could also apply to the finding in the work. No marked differences were also found for the instrumental color coordinates, with values near to 100, in the case of L*, and to 0 for a* and b*, which may indicate high luminosity and white color in the analyzed batches of microcapsules.

Higher dissolution rate was obtained in Mo than in Mu, which is explained by the different wall materials between these two types of microcapsules (maltodextrine and maltodextrine-chitosan). This finding deserves special attention when microcapsules are used for food enrichment since the quantity of water in the food may have effect on the microcapsule stability.

In ultra-sonicated microcapsules, Mo obtained significant higher microencapsulation yield than Mu. Besides, the effect of the homogenization technique was also significant in Mo, finding higher percentages with ultrasounds in comparison to high-pressure. Thus, the highest MY was found in microcapsules from Mo emulsions homogenized by ultrasounds, being noted an important increase in this parameter from microcapsules of previous studies (50.71% as the most) (Solomando et al., 2019). In fact, the influence of the emulsion preparation on microencapsulation yields has been previously reported (Gallardo et al., 2013). In the present work, the homogenization by ultra-sonicated may

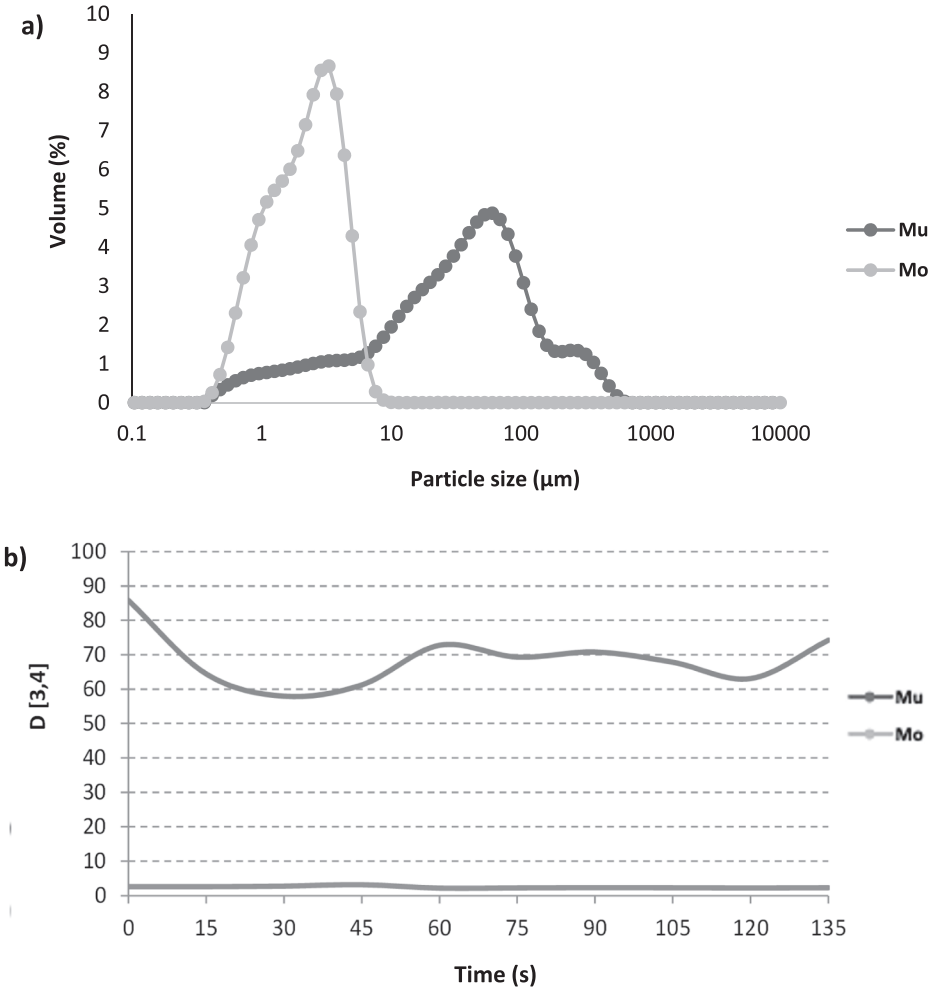

Fig. 3. Particle size (a) mean distribution (b) of monolayered (Mo) and multilayred (Mu) fish oil microcapsules obtained from fish oil emulsions homogenized by ultra-sonicated optimized conditions.

achieve an appropriate reduction and homogenization of the oil drop size, improving the emulsion atomization and, consequently, the microencapsulation yield, which is a notable parameter from an economic point of view (Gallardo et al., 2013).

The homogenization technique also influenced significantly in the percentage of total oil and the quantity of EPA and DHA, with ultra-sonicated homogenization increasing the percentage of total oil as well as the quantity of EPA plus DHA in Mo and Mu (9.18 and 7.44 %; 15.28 and 12.3 mg EPA + DHA/g microcapsule; respectively) in comparison to high-pressure (11.37 and 11.42 %; 15.3 10.04 mg EPA + DHA/g; respectively). In addition, in the case of Mu, the homogenization technique significantly influences on the MEE, with higher percentages when applying ultra-sonicated in comparison to high-pressure (76.90 *vs*. 50.72 %), while no differences were found in Mo between ultra-sonicated and high-pressure. This finding is quite remarkable because, in a previous study (Solomando et al., 2019), the authors of this work achieved to improve the MEE of Mo microcapsules by high-pressure while no improvements were obtained in Mu, however, the use of ultra-sonicated have allowed it. Despite this, higher MEE has been found in Mo than in Mu, in agreement with previous studies (Jiménez-Martín et al., 2015; Solomando et al., 2019). Although MEE is a parameter with a high variability (Klinkesorn et al., 2006), it can be influenced by the wall materials, being favored when the covering is formed at lower times and the diffusion of the encapsulated oil is avoided. Longer times for the formation the crust during spray drying may favor the release of some oil to the surface of the formed microcapsules. Thus, according to these facts and results of the present study, the homogenization by ultra-sonicated of monolayered emulsions with maltodextrine as wall material may achieve oil drops of so small size that allow a rapid crust formation with poor diffusion of the oil to the surface of the microcapsules. In fact, as previously indicated, optical microscope images of ultra-sonicated emulsion showed smaller oil drops in Mo in comparison to Mu emulsions (Fig. 2).

Regarding the lipid oxidation values, although all batches of microcapsules have low TBARs values, they were significantly influenced by the homogenization method, being lower when using ultra-sonicated than high-pressure. This can be related to the temperature of the emulsions, while the homogenization with ultra-sonicated is performed a refrigeration (with ice), in high pressure homogenization it is not possible, reaching the emulsions a temperature close to 50 °C.

Particle size curves and mean diameter of Mo and Mu microcapsules optimized by ultra-sonicated are shown in Fig. 3. Mo is shown as monomodal, with a peak that represents the predominant size, while Mu shows a bimodal distribution (Fig. 3a). Furthermore, a smaller and more homogeneous particle size is observed in Mo (D [3;4] = 2.31 µm, Dv (10) = 0.78 µm and Dv (90) = 4.16 µm) compared to Mu (D [3;4] = 68 µm, Dv (10) = 2.97 µm and Dv (90) = 166 µm) (Fig. 3b). These findings can be explained by the additional layer of chitosan in Mu, are in concordance with that observed in Mo and Mu homogenized by high pressure (Solomando et al., 2019) and, as previously pointed out, may indicate the higher influence of ultra-sonicated in Mo in comparison to Mu.

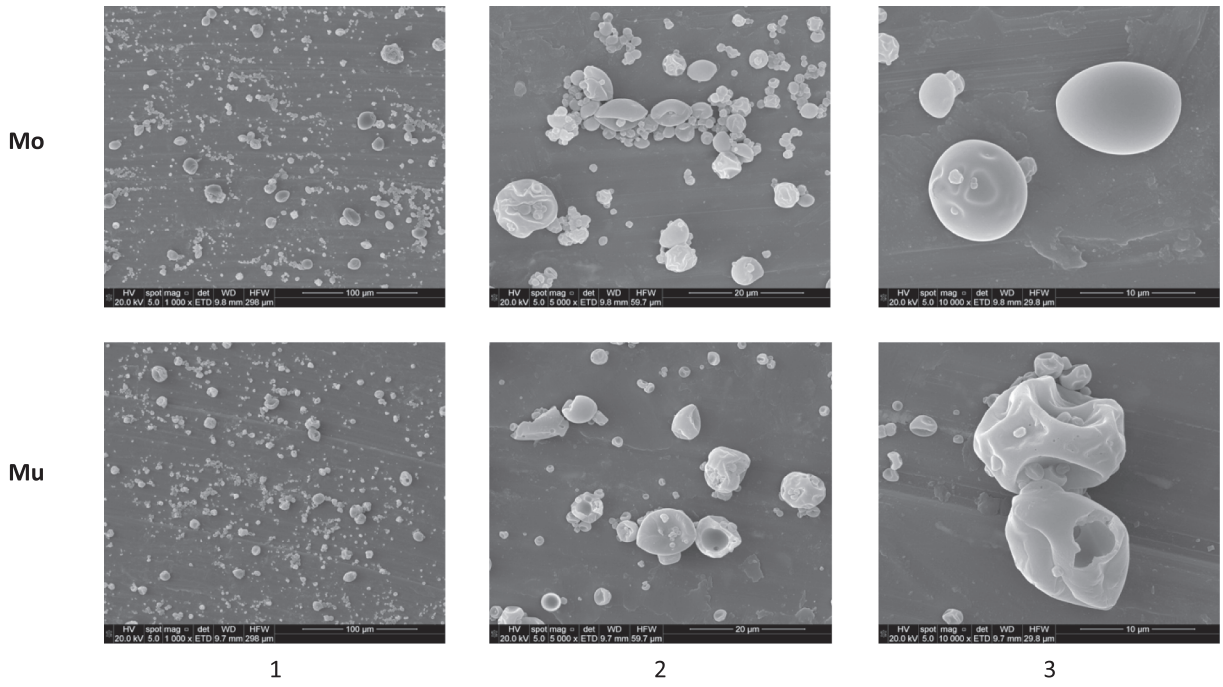

Fig. 4. Scanning electron microscopy images of monolayered (Mo) and multilayered (Mu) fish oil microcapsules from fish oil emulsions homogenized by ultra-sonicated and observed at 1000× (1), 10,000× (2) and 20,000× (3) magnifications.

Table 6
Release of fatty acids composition(mg FAMEs/g microcapsule) through in vitro digestion of monolayered (Mo) and multilayered (Mu) fish oil microcapsules.

Fatty acids	Oral digestion phase			Gastric digestion phase			Intestinal digestion phase		
	Mo	Mu	*p*	Mo	Mu	*p*	Mo	Mu	*p*
C14:0	0.89 ± 0.29	0.48 ± 0.04	*	1.09 ± 0.04	0.53 ± 0.05	***	1.30 ± 0.41	1.21 ± 0.08	n.s.
C14:1	0.05 ± 0.02	0.03 ± 0.00	n.s.	0.05 ± 0.02	0.02 ± 0.00	n.s.	0.07 ± 0.03	0.00 ± 0.00	*
C15:0	0.16 ± 0.05	0.08 ± 0.00	n.s.	0.19 ± 0.00	0.09 ± 0.01	***	0.23 ± 0.06	0.21 ± 0.01	n.s.
C15:1	0.06 ± 0.02	0.03 ± 0.00	n.s.	0.07 ± 0.00	0.03 ± 0.00	**	0.07 ± 0.03	0.09 ± 0.01	n.s.
C16:0		2.51 ± 0.20	**	6.10 ± 0.19	3.11 ± 0.28	***	6.32 ± 1.01	7.34 ± 0.39	n.s.
C16:1	0.93 ± 0.34	0.78 ± 0.04	n.s.	1.81 ± 0.06	0.83 ± 0.01	***	1.96 ± 0.72	2.28 ± 0.13	n.s.
C17:0	0.20 ± 0.05	0.11 ± 0.03	n.s.	0.29 ± 0.00	0.14 ± 0.01	**	0.30 ± 0.11	0.30 ± 0.02	n.s.
C17:1	0.08 ± 0.03	0.04 ± 0.02	n.s.	0.10 ± 0.00	0.04 ± 0.01	**	0.09 ± 0.03	0.10 ± 0.01	n.s.
C18:0	1.34 ± 0.54	0.68 ± 0.16	n.s.	1.59 ± 0.04	0.82 ± 0.06	***	1.68 ± 0.52	2.25 ± 0.08	n.s.
C18:1n-9	5.31 ± 1.74	2.72 ± 0.23	**	6.35 ± 0.24	3.15 ± 0.32	***	6.68 ± 2.24	8.16 ± 0.41	n.s.
C18:2n-6	2.21 ± 0.89	0.68 ± 0.14	**	2.66 ± 0.14	1.62 ± 0.16	***	2.32 ± 0.52	4.84 ± 0.37	**
C18:3n-6	0.06 ± 0.02	0.03 ± 0.01	n.s.	0.06 ± 0.00	0.02 ± 0.00	**	0.05 ± 0.02	0.03 ± 0.00	n.s.
C18:3n-3	0.33 ± 0.12	0.11 ± 0.02	n.s.	0.40 ± 0.02	0.23 ± 0.02	**	0.34 ± 0.09	0.66 ± 0.05	*
C20:0	0.08 ± 0.03	0.04 ± 0.00	n.s.	0.10 ± 0.00	0.04 ± 0.00	**	0.11 ± 0.04	0.05 ± 0.02	n.s.
C20:1	2.23 ± 0.73	1.17 ± 0.09	*	2.64 ± 0.09	1.26 ± 0.14	***	2.62 ± 0.48	3.19 ± 0.21	n.s.
C20:2	0.16 ± 0.06	0.08 ± 0.00	n.s.	0.20 ± 0.00	0.08 ± 0.01	***	0.21 ± 0.07	0.22 ± 0.01	n.s.
C20:4n6	0.68 ± 0.21	0.37 ± 0.03	*	0.85 ± 0.03	0.40 ± 0.04	***	1.02 ± 0.39	1.06 ± 0.07	n.s.
C22:0	0.15 ± 0.04	0.08 ± 0.00	n.s.	0.16 ± 0.00	0.07 ± 0.01	***	0.22 ± 0.12	0.19 ± 0.01	n.s.
C22:1	0.39 ± 0.13	0.20 ± 0.01	*	0.47 ± 0.01	0.22 ± 0.03	***	0.51 ± 0.17	0.57 ± 0.04	n.s.
C20:5n-3	1.52 ± 0.47	0.79 ± 0.06	*	1.84 ± 0.06	0.88 ± 0.06	***	2.22 ± 0.89	2.16 ± 0.14	n.s.
C24:0	0.31 ± 0.09	0.18 ± 0.04	n.s.	0.39 ± 0.02	0.20 ± 0.02	**	0.45 ± 0.09	0.56 ± 0.05	n.s.
C24:1	0.37 ± 0.14	0.17 ± 0.01	n.s.	0.41 ± 0.01	0.18 ± 0.02	***	0.46 ± 0.14	0.45 ± 0.03	n.s.
C22:6n-3	5.90 ± 1.15	3.13 ± 0.16	**	7.20 ± 0.23	3.39 ± 0.37	***	10.40 ± 1.39	10.55 ± 0.67	n.s.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. non-significant.

Fig. 4 shows SEM images of ultra-sonicated Mo and Mu fish oil microcapsules. In general, no evidence of agglomeration was observed in any of the two types of microcapsules studied. Mo shows some wrinkles, but without pores, which could indicate that the ultra-sonicated homogenization of the emulsion gives rise to flexible microcapsules, favoring the protection of the core material. Instead, Mu showed breaks in the wall that could be explained by the inclusion of acetic acid and chitosan, which lowers the pH value and strengthens the coating layer, giving rise to less flexible drops that are susceptible to fragmentation. A similar morphology was observed in a previous study with microcapsules from high-pressure homogenized emulsions but noting some signs of agglomeration in high-pressure Mu microcapsules (Jiménez-Martín et al., 2015; Solomando et al., 2019) that were not observed in this work.

3.5. Fatty acids released through in vitro digestion of ultra-sonicated fish oil microcapsules

Table 6 exposes the quantities of fatty acids release from ultra-sonicated Mo and Mu fish oil microcapsules at the end of oral, gastric, and intestinal phases of the in vitro digestion assay. In general, palmitic (C16:0) and oleic acids (C18:1n-9) were the major, followed by stearic acid (C18:0), gadoleic acid (C20;1), EPA and DHA, with the rest being

minor ones. This profile is quite in agreement with the fatty acid composition of the fish oil used in this work (Jiménez-Martín et al., 2016). As expected, the highest quantities of fatty acids released were found in the intestinal phase in both types of microcapsules, followed in decreasing order by gastric and oral phases. These results agree with previous studies of fat digestion that have shown higher percentages of hydrolysis in the intestinal phase compared to the oral and gastric phase (Friedman & Nylund, 1980; Solomando, Antequera, & Perez-Palacios, 2020), which is consistent with the secretion of lipolytic enzymes such as pancreatic lipase, phospholipase, and sterol esterase, at the intestinal phase, hydrolyzing most lipid compounds. In general, the main fatty acids releases were docosahexaenoic acid (C22:6n-3, DHA), following in decreasing order by oleic (C18:1n-9c), palmitic (C16:0), linoleic (C18:2n-6) and gadoleic acid (C20:1), which according to the fatty acid profile of the fish oil used in this work and described in previous studies (Jiménez-Martín et al., 2016). On the other hand, the type of fish oil microcapsules significantly influences the amounts of fatty acids released. In the oral phase, higher release was observed in 10 of the 24 fatty acids identified in Mo compared to Mu; in the gastric phase all fatty acids except for *trans*-tetradecenoic (C14:1) showed higher release in Mo compared to Mu in the intestinal phase differences were only observed in 3 of the 24 fatty acids identified, being the release of linoleic (C18:2n-6) and α-linolenic acid (C18:3n-3) higher in Mu than in Mo and of C14:1 higher in Mo than in Mu. These differences could be explained by the extra coating of chitosan in Mu, providing a major protection of the encapsulated fish oil, specially from the acidic environment of the stomach, at pH between 1 and 3, chitosan tends to be positively charged, having a high electrostatic attraction with anionic molecules such as maltodextrin. However, chitosan loses its positive charge at pH values above 6.5, not presenting electrostatic interaction (McClements & Li, 2010). Thus, during acid digestion in the stomach, a high electrostatic interaction can take place between chitosan and lipid droplets, decreasing the amount of lipid surface exposed to enzymatic activity, but in the phase of intestinal digestion (pH between 6 and 7,5), the electrostatic interaction decreases, which would favor the release of lipid molecules, remaining at this time available to be digested by the enzyme pancreatic lipase. Focusing on the fatty acids of interest in the present study, EPA and DHA, despite the significant differences at oral and gastric, the release of fatty acids was quite similar in Mo and Mu at the intestinal phase, being the bioaccessibility of EPA + DHA (expressed as the percentage of fatty acids at the intestinal phase in relation to initial quantities) 82.6% and 83.07% for Mo and Mu, respectively, This may point out that the fatty acid released at oral and gastric phases should not be hydrolyzed, reaching the intestinal phase, in comparison to fish oil microcapsules from emulsion homogenized at high pressure that showed higher bioaccessibility of EPA and DHA in Mu (around 50%) than in Mo (around 40%) (Solomando, Antequera, & Pérez-Palacios, 2020). The ultra-sonicated homogenization supposes a notable improvement.

In the case of Mo, the single wall of maltodextrin may prevent the rapid absorption of water of oral fluids, providing partial protection against oxidizing agents in the early stages of the gastrointestinal tract. However, at the gastric level, the decrease in pH catalyzes the hydrolysis of the glycosidic bonds that join the monosaccharide molecules, which may induce the degradation of the microcapsule wall. However, the fatty acids of Mo may not be completely digested until the intestinal phase by the action of the enzyme pancreatic lipase (Damodaran et al., 2008).

4. Conclusions

This work demonstrates the importance of optimizing the ultrasonic homogenization conditions of monolayer and multilayer fish oil emulsions to improve the quality characteristics of the microcapsules.

Optimum combination of power (141.01 and 141.21 kg m^2/s^3), time (15 and 5 min), pulse (80%) and emulsion volume (100 and 200 mL) has been achieved for monolayer and multilayer fish oil emulsions, respectively, giving rise to microcapsules with a higher content of encapsulated oil.

The homogenization method influences the quality characteristics of emulsions (creaming index) and microcapsules (lipid oxidation, microencapsulation yield and microencapsulation efficiency), showing the ultra-sonicated homogenization of monolayer and multilayer fish oil emulsions better quality characteristics to high-pressure homogenization.

The simulated digestion of both types of microcapsules released the highest quantities of fatty acids in the intestinal phase, however, the combination of maltodextrin-chitosan in multilayer microcapsules provides higher resistance in the first phases of digestion compared to simple maltodextrin coating on monolayer microcapsules.

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CRediT authorship contribution statement

Juan Carlos Solomando: Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Teresa Antequera:** Methodology, Resources, Writing – review & editing. **Jorge Ruiz:** Project administration, Supervision, Writing – review & editing. **Francisco De La Haba:** Formal analysis, Investigation. **Trinidad Perez-Palacios:** Conceptualization, Funding acquisition, Project administration, Software, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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