Encapsulation of EPA and DHA concentrate from *Kilka* fish oil by milk proteins and evaluation of its oxidative stability

Hamed Hosseini¹ · Mohammad Ghorbani¹ · Seid Mahdi Jafari¹ · Alireza Sadeghi Mahoonak¹

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Abstract Glyceride product from lipase-catalyzed hydrolysis of fish oil (large-scale) is a rich source of n-3 PUFA (49.62%); but it is prone to oxidation. Our aim was to encapsulate this product by a mixture of whey powder and sodium caseinate (4:1) as a new wall material. The emulsification was done using ultrasonication in different powers (180–380 W) and times (1–3 min) and, then, the emulsions were freeze-dried to obtain the powders. Based on encapsulation efficiency, sonication (88–94%) could inhibit the presence of oil at the surface of powder particles in comparison with the samples prepared without sonication (control, 68%). The highest oxidation rate and the lowest L-value were found for the unencapsulated glyceride product stored in air atmosphere followed by the control powder, the powders from sonication treatment and the unencapsulated glyceride product under N₂, respectively. In the case of oxidative stability of the samples, the sonication time was more significant than sonication power. According to our results, a sonication treatment of 380 W for 3 min was recommended to prepare parent emulsions during fish oil encapsulation.

Keywords Fish oil encapsulation · Ultrasound-assisted emulsification · Oxidative stability · Scanning electron microscopy · Image processing

Introduction

The n-3 polyunsaturated fatty acids (PUFA), particularly eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) fatty acids, with some benefits for the human health have been widely studied (Kaur et al. 2014). Therefore, their consumption is beneficial in the human diet; however, they are very susceptible to oxidative deterioration resulting in an unpleasant odor (Jeyakumari et al. 2016); this situation may be worse when the oil is concentrated in n-3 fatty acids (FA) to provide the daily requirements of n-3 PUFA through lower overall fat intake (Xia et al. 2017). The oxidative stability of fish oils can be minimized or controlled using different strategies, including addition of antioxidants, modified atmosphere packaging, and encapsulation (Arab-Tehrany et al. 2012; Rathod and Kairam 2018; Huang et al. 2014).

In general, encapsulation of edible oils includes a primary emulsion preparation which is followed by a drying step (Yazicioglu et al. 2015). The encapsulation efficiency (EE) is affected by various factors including emulsion droplet size distribution (Jafari et al. 2008a), oil-to-wall material ratio (Chen et al. 2013), type of encapsulating agent (Jafari et al. 2008b), and drying technique (Karthik and Anandharamakrishnan 2013).

In recent years, milk proteins such as skimmed milk powder (Arab-Tehrany et al. 2012), whey protein isolate (Ramakrishnan et al. 2013; Chen et al. 2013), whey protein hydrolysate (Ramakrishnan et al. 2013), and sodium caseinate (NaCA) (Chen et al. 2013) alone or in combination with other biopolymers have been successfully used as encapsulating agents. Among dairy proteins, NaCA is a more efficient encapsulating agent than whey proteins and skimmed milk powder due to its higher emulsifying properties and a high heat resistance (Vega and Roos 2006).

✉ Hamed Hosseini
hosseini_hamed63@yahoo.com

¹ Faculty of Food Science and Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

Low-molecular-weight carbohydrates, like maltodextrin, and hydrocolloids used as common wall materials lack emulsifying properties; therefore, they need to be associated with an emulsifier over the emulsification process, while simple whey powder (WP) contains both emulsifiers (protein) and filler (mainly lactose) (Keogh et al. 2001). To the best of our knowledge, application of WP for encapsulation of fish oil has not been reported in the literature. In addition, WP is part of food formulations, such as biscuits and chocolate where they are usually considered to be enriched with n-3 FAs (Takeungwongtrakul and Benjakul 2017). Therefore, it can be beneficial to assess the applicability of WP for encapsulation of fish oils.

The droplets size of dispersed oil in the parent emulsion is another key parameter to be controlled, as well as type of wall material. Relevant studies indicate that a higher EE and oxidative stability can be achieved by decreasing the droplets size of emulsions (Turchiuli et al. 2014). Jafari et al. (2008a) used two high energy emulsifying techniques, including microfluidization (at 60 MPa) and ultrasonication (24 kHz for 100 s) to reduce the droplets size of emulsions containing fish oil (as core material), and modified starch, whey protein concentrate and maltodextrin (as wall materials). Their results showed a significant increase in the EE using these techniques as compared with rotor-stator emulsification method. They confirmed the positive role of microfluidization and ultrasonication techniques on the EE, as an important measure for extending the shelf-life of encapsulated oil products, through minimizing the oil droplet size in the parent emulsion.

Therefore, the aims of the present study were to evaluate WP in combination with NaCA as wall materials to encapsulate the glyceride product from large-scale hydrolysis of *Kilka* fish oil, followed by application of ultrasound energy with different powers and times in the emulsification process during encapsulation process. Finally, the oxidative stability of encapsulated glyceride product was compared with unencapsulated glyceride product stored under N₂ and air atmospheres.

Materials and methods

Materials

The *Kilka* (*Clupeonella delicatula*) fish was purchased from a fishing port in Babolsar (Iran) in winter and was shipped to the laboratory in ice within 3 h. Microbial lipase (*Candida rugosa* lipase,¹ CRL, type VII, ≥ 700 unit/mg solid) was purchased from Sigma–Aldrich Co. (Tokyo, Japan). Boron Trifluoride (BF₃), n-heptane and NaCA

(protein content 89%) were obtained from Sigma–Aldrich Co. (USA). Nitrogen gas (N₂, approximately 100% in purity) was purchased from Arham gas (Iran). Fresh whey powder (protein content 6%, lactose 70%, fat 1%) was provided by Golestan Pegah CO. (Iran). The rest of the chemicals were from Merck (Germany).

Extraction and purification of the fish oil

The crude oil was extracted from *Kilka* fish according to the method of Bispo et al. (2014) and, then, was purified as described by Sathivel and Prinyawiwatkul (2004) with some changes, including higher chitosan level (degree of deacetylation 83.3%, 7% of the oil weight) and operation temperature (28 °C). Given that the oil used in this study was extracted from fresh *Kilka* fish, no significant different ($p > 0.05$) was observed between quality indices (peroxide value, acidity and conjugated diene value) measured for crude oil (1.83 mEqO₂/kg, 0.46% and 6.73, respectively) and purified oil (1.79 mEqO₂/kg, 0.46% and 6.65, respectively), while their moisture content showed a significant ($p < 0.05$) difference.

Hydrolysis reaction procedure

The hydrolysis reaction procedure and conditions (water-to-oil ratio, the amount of enzyme and reaction time) used in the present study were based on our previous work performed on a laboratory-scale with 2 g of fish oil (Hosseini et al. 2018). *Kilka* oil (180 g) and 0.1 N phosphate buffer (pH = 7.20) with a 1:1 ratio were transferred into a 1 L round-bottomed flask and warmed to 37 °C under constant stirring (300 rpm) and, then, the CRL (2%, w/w, oil basis) was added to the reaction mixture. At the end of the reaction (1 h), enzyme activity was inhibited by adding 500 mL of 96% ethanol given that this lipase does not tolerate ethanolic conditions (Mbatia et al. 2011) and, then, the predetermined amount of NaOH was added to the flask. Next, 1 L hexane to solve mono-, di- and tri-acylglycerols, 2 L distilled water to remove FFA from the glyceride fraction, and 100 g anhydrous sodium sulphate to break the emulsion were added to the reaction mixture. The hexane layer was filtered through a bed of anhydrous sodium sulphate for water separation. The solvent was removed in a rotary evaporator (IKA RV05, Germany) and the glyceride product was centrifuged (10,000 g for 10 min at 10 °C), and then, stored under N₂ at – 20 °C until further analysis. AOCS Official Method was followed to determine acidity (Ca 5a-40/93) of the reaction mixture. The product was gravimetrically (Sartorius, 4 digits, Germany) measured and percentage yield calculated, based on the initial oil weight (Gamez-Meza et al. 2003; Bispo et al. 2014). The percentage of recovery for the fatty acids in the

¹ Lot: BCBNC....V, PCODE: 101555899.

glyceride product was calculated, based on Eq. (1) as follows Bispo et al. (2014):

$$\text{Recovery (\%)} = \%FFA_f \times \%W / \%FFA_i \quad (1)$$

where FFA_f is the FA percentage in the glyceride product, W is the weight percentage of the glyceride product, and FFA_i is the FA content (%) in the original oil.

Fatty acid profile

Fatty acid methyl esters (FAME) were prepared as described by AOCS Official Method Ch 1-91 (1998). FAME analysis was performed by gas chromatography (TGF 2552, Iran) equipped with flame ionization detector and capillary column (CP-Sil 88, 100 m length, 0.25 mm i.d., film thickness 0.25 μ m). The column temperature was set at 80 °C for 4 min and ramped at 3 °C/min to 240 °C. The injection and detector temperatures were maintained at 225 and 250 °C respectively. Hydrogen with a flow rate of 1.20 mL/min was used as carrier gas. The FAs were identified by comparing the relative retention times of the sample peaks with Supelco 37 component FAME mix² (Bellefonte, PA, USA).

Production of the encapsulated oil and encapsulation efficiency

The primary emulsions (oil-in-water type) containing enzymatically enriched glyceride product (7 g), as dispersed phase, and a mixture of WP and NaCA (4:1, w/w), as matrix, in a wall-to-oil ratio of 4:1 was prepared as described by Chen et al. (2013) with a modification, by means of Heidolph homogenizer (D91126, 130 W, Germany) at 13,000 rpm for 3 min. These ratios were chosen based on the primary experiments (data not shown) and the literature (Chen et al. 2013). The pre-emulsions with 30% solids content (WP and NaCA) were then further sonicated using a 20 kHz ultrasonic homogenizer (Topsonics, UP400-A, Iran) at powers of 180 and 380 W for 1 and 3 min. During the emulsification process, temperature of emulsions was maintained at 26 °C. Finally, the emulsions were dried using a freeze drier (Operon-FDB5503, South Korea) at − 42 °C and 80 mTorr. It is recommended that freeze-drying method as a low-temperature process is a suitable method for microencapsulation of fatty acids susceptible to high temperature, used in spray-drying technique, like DHA (Karthik and Anandharamakrishnan 2013). The control sample was obtained from ultrasound-unassisted emulsification. Since the freeze-dried samples had uneven forms with different contact surfaces, the dried samples were carefully and manually crushed and screened (mesh size 2) to obtain

particles with a uniform size so that the comparison between means could be carried out in same conditions (Heinzelmanna et al. 2000). Encapsulation efficiency of the powders was measured according to method of Goula and Adamopoulos (2012) and was calculated as follows:

$$\begin{aligned} \text{Encapsulation efficiency} \\ = [(Total \text{ extractable oil from powders} - \text{Extractable surface oil}) / Total \text{ extractable oil from powders}] \times 100 \end{aligned} \quad (2)$$

Oxidative stability

In order to evaluate the oxidative stability, the encapsulated samples (25 g for each type), as well as the unencapsulated glyceride product under N₂ or air (7 g for each), were stored at 40 °C and $a_w = 0.43$ (adjusted by saturated potassium carbonate solution) for 12 days. The glyceride products were extracted from the encapsulated samples as described by Karaca et al. (2013), then, they were stored at − 20 °C until further analysis. UV specific absorbances ($E_{1\text{ cm}}^{1\%}$) of the oils at 233 and 268 nm as a measure of conjugated dienoic (CDs) and trienoic acids (CTs) formation, respectively, were determined by reading in a T80 UV/VIS spectrophotometer (PG Instruments, UK) based on the method of Pegg (2005).

Colour analysis

Evaluation of colour parameters (R, G, B) for the powders stored in $a_w = 0.43$ at 40 °C for 12 days was carried out using image processing of sample images taken by a scanner (Hewlett Packard, Scanjet G3110, USA) as described by Ghorbani and Hosseini (2017). The scanner resolution was set at 1240 × 1240 pixels (1200 dpi). The colour coordinates ranging from $L = 0$ (black) to $L = 100$ (white), $-a$ (greenness) to $+a$ (redness), and $-b$ (blueness) to $+b$ (yellowness), were determined from scanner colour data by ImageJ 1.45 s software (National Institute of Health, USA). The overall colour difference (ΔE) was calculated as follows (Guine and Barroca 2012):

$$\Delta E = [(L \text{ standard} - L)^2 + (a \text{ standard} - a)^2 + (b \text{ standard} - b)^2]^{1/2} \quad (3)$$

Morphology

A scanning electron microscope (SU3500, Hitachi Co. Ltd., Tokyo, Japan) was used to investigate the morphology of the encapsulated powders. The particles were placed on the cylindrical stubs with a tape. The specimens were

² Lot: XA16568V. CRM47885.

subsequently coated with gold using a desk magnetron sputter coater (DSR1, Iran). Digital images ($\times 150$ and $\times 500$) were captured at an accelerating voltage of 10 kV.

Statistical analysis

All experiments were carried out in duplicate. Full factorial design was used to analyze the experimental data, using JMP 10 software (SAS Institute Inc., USA, 2012). Differences between the least square means were compared with Tukey test and reported to be significant when $p < 0.05$. Moreover, factorial design optimization was applied to determine the optimal condition (power and time) for ultrasound process.

Results and discussion

Large-scale production of the glyceride product

The fatty acid composition of purified *Kilka* fish oil and the glyceride product before and after CRL-catalyzed hydrolysis reaction (pH = 7.20, 37 °C), is presented in Table 1. Mild selective hydrolysis of ester bonds in tri-acylglycerols (TAGs) of the oil was successfully led to provide a glyceride product with high n-3 PUFA contents through the release of non n-3 PUFA from glycerol backbone of TAG in *Kilka* fish oil. The higher recovery levels of fatty acids in the glyceride product were obtained for the fatty acids with aliphatic chains longer than C₁₈. Generally, the CRL showed more preference for hydrolyzing either saturated or monounsaturated fatty acids (SFA or MUFA) than PUFA. Therefore, overall level of EPA + DHA in the original oil was significantly increased ($p < 0.05$) by nearly twofold during 1 h hydrolysis reaction as recovery levels of EPA and DHA in the glyceride product were $42.23 \pm 0.54\%$ and $74.60 \pm 0.80\%$, respectively. CRL-catalyzed hydrolysis has already been used successfully by other researchers, as they confirmed the capability of CRL to provide the products concentrated in n-3 PUFA (Hosseini et al. 2018). Among the non n-3 PUFAs, two major fatty acids in the original oil, C18:1 ($34.51 \pm 0.07\%$) and C16:0 ($16.56 \pm 0.47\%$), were respectively decreased 1.70-fold and 1.60-fold as shown in Table 1. A less steric hindrance for SFAs and MUFAs linked to glycerol backbone, when a TAG molecule is close to the active site of the enzyme, was proposed as a possible reason to explain the preference of CRL to SFA + MUFA in comparison with EPA + DHA (Gamez-Meza et al. 2003). Finally, the glyceride product was richer in n-3 fatty acids, though it was more susceptible to oxidative deterioration as compared with the initial oil or vegetable oils. Thus, it was required to encapsulate the final product in order to increase its oxidative stability by providing a barrier against oxygen and light.

Morphology

SEM images of the samples are presented in Fig. 1. A structure with highly porous surfaces (honeycomb-like structure) was observed for the freeze-dried samples (Fig. 1) similar to those described in another study (Karthik and Anandharamakrishnan 2013). In freeze drying process, sublimation of ice crystals formed in freezing step could create pores within the microcapsules (Anandharamakrishnan et al. 2010) which improves the rehydration properties of microcapsules, while oxygen will diffuse faster through the pores. Therefore, it is necessary to use a technique in order to enhance the oil shrouding in freeze-dried encapsulated oil. According to Fig. 1b, sonication leads to higher and finer pores in the fresh microcapsules than control (Fig. 1a) which were subsequently combined or destroyed during accelerated storage (Fig. 1d). This may partially be attributed to oxidation that occurred over 12 days storage period. Because, the products of oxidation reactions occurred during storage such as peroxides and free radicals could affect the flavor and texture (such as staling and toughening) of the powders by reaction with vitamins, amino acids and proteins (Senter et al. 1984). After microencapsulation of DHA by spray-freeze-drying, spray-drying and freeze-drying methods, Karthik and Anandharamakrishnan (2013) stated that the structure loss and reduction in pore size of solid matrix of the encapsulated oil was a result of changing the matrix from glassy state to the rubbery state due to changes in moisture content or increase in temperature. The control powder similarly exhibited a structure with pores (Fig. 1a), but with uneven and larger pores than ultrasound treatment either at beginning or end of the storage period. It is believed that the pores created in freeze-dried powders leads to an increase in contact surface of oil with air which it is associated with increase in oxidation reaction progress during storage (Zuidam and Shimoni 2009). Nevertheless, we think that in this study, sonication treatment could possibly inhibit the presence of oil at the surface of particles as a result of better distribution and retention of oil droplets within the matrix compared with the control. To confirm this claim, further analysis was carried out as follows.

Oxidative stability

The fishy odor was not detected for freshly produced encapsulated powders with sonication or without it. However, this odor was developed throughout the storage period, in particular for the control sample. By the encapsulation process, part of the core material that is not surrounded by the wall may strongly affect the acceptability of microcapsules containing core materials rich in PUFA (Drusch and Berg 2008) such as capsules produced in the current research. Thus, EE was determined as a very important factor for evaluating the shelf life of the powders.

Table 1 Fatty acid composition in *Kilka* oil and in concentrated glyceride product

Common name	Abbreviated sign	Amount (% w/w) ^a	
		Fresh oil	Product
Myristate	C14:0	1.36 ± 0.39 ^a	0.96 ± 0.07 ^a
Pentadecanoate	C15:0	0.32 ± 0.05 ^a	0.23 ± 0.02 ^a
Palmitate	C16:0	16.56 ± 0.47 ^a	9.14 ± 0.04 ^b
Heptadecanoate	C17:0	1.12 ± 0.02 ^a	1.04 ± 0.08 ^a
Stearate	C18:0	4.41 ± 0.64 ^a	3.10 ± 0.32 ^a
Arachidate	C20:0	0.26 ± 0.07 ^a	0.19 ± 0.01 ^a
Heneicosanoate	C21:0	2.53 ± 0.66 ^a	3.59 ± 0.28 ^a
Behenate	C22:0	0.60 ± 0.08 ^a	0.7 ± 0.05 ^a
Tricosanoate	C23:0	0.12 ± 0.01 ^b	0.44 ± 0.03 ^a
Palmitoleate (<i>cis</i> -9)	C16:1 n-7	4.17 ± 0.34 ^a	0.88 ± 0.01 ^b
Heptadecenoate (<i>cis</i> -10)	C17:1	0.54 ± 0.03 ^a	0.26 ^b
Oleate (<i>cis</i> -9)	C18:1	34.51 ± 0.07 ^a	18.87 ± 0.15 ^b
Eicosenoate (<i>cis</i> -11)	C20:1 n-9	1.62 ± 0.02 ^a	1.11 ± 0.01 ^b
Erucate (<i>cis</i> -13)	C22:1 n-9	0.77 ± 0.16 ^a	0.89 ± 0.01 ^a
Nervonate (<i>cis</i> -15)	C24:1 n-9	0.56 ± 0.04 ^b	2.71 ± 0.02 ^a
Linoleaidate (<i>trans</i> -9,12)	C18:2 n-6	0.37 ± 0.01 ^a	0.31 ± 0.01 ^b
Linoleate (<i>cis</i> -9,12)	C18:2 n-6	1.84 ± 0.03 ^a	0.45 ± 0.01 ^b
γ-linolenate (<i>cis</i> -6,9,12)	C18:3 n-6	0.26 ± 0.01 ^b	0.32 ± 0.01 ^a
Linolenate (<i>cis</i> -9,12,15)	C18:3 n-3	0.26 ± 0.02 ^b	0.53 ± 0.01 ^a
Eicosadienoate (<i>cis</i> -11,14)	C20:2 n-6	0.25 ± 0.01 ^b	0.41 ± 0.01 ^a
Eicosatrienoate (<i>cis</i> -8,11,14)	C20:3 n-6	0.18 ± 0.02	ND
Eicosatrienoate (<i>cis</i> -11,14,17)	C20:3 n-3	0.48 ± 0.02 ^b	0.81 ± 0.02 ^a
Arachidonate (<i>cis</i> -5,8,11,14)	C20:4 n-6	0.33 ± 0.06 ^b	0.54 ± 0.03 ^a
Docosadienoate (<i>cis</i> -13,16)	C22:2 n-6	0.23 ± 0.09	ND
Eicosapentaenoate (<i>cis</i> -5,8,11,14,17)	C20:5 n-3 (EPA)	6.79 ± 0.22 ^b	8.96 ± 0.22 ^a
Docosahexaenoate (<i>cis</i> -4,7,10,13,16,19)	C22:6 n-3 (DHA)	16.86 ± 0.13 ^b	39.33 ± 1.01 ^a
Σud (Unidentified fatty acids)		2.72 ± 0.85 ^a	4.24 ± 0.65 ^a
ΣSFA (Saturated fatty acid)		27.28 ± 0.90 ^a	19.38 ± 0.82 ^b
ΣMUFA (Monounsaturated fatty acid)		42.16 ± 0.47 ^a	24.72 ± 0.20 ^b
ΣPUFA (Polyunsaturated fatty acid)		27.84 ± 0.52 ^b	51.66 ± 1.28 ^a
Σ n-3 PUFA		24.38 ± 0.35 ^b	49.62 ± 1.27 ^a
EPA + DHA		23.64 ± 0.35 ^b	48.29 ± 1.23 ^a
n-3/n-6		7.05 ± 0.25 ^b	24.38 ± 0.49 ^a

^aValues are expressed as duplicate mean ± standard deviation. Means with the same letters within the same rows are not significantly different ($p < 0.05$)

Encapsulation efficiency of the glyceride product was significantly ($p < 0.05$) affected by the sonication power and time as shown in Table 2. The lowest EE ($68.24 \pm 1.75\%$) was obtained for the control. In addition, we didn't observe a significant difference ($p > 0.05$) among the EE of samples treated by sonication except for the one treated in 180 W for 1 min as seen in Table 2. The positive role of sonication treatment in our results can be justified by the literature, where the relevant investigations have shown that increase in the EE of fish oil was frequently affected by combination of two factors including the initial droplet size of emulsions and the wall-to-oil ratio, so that a decrease in droplet size distributions by

various techniques such as premix membrane emulsification (Ramakrishnan et al. 2013) and ultrasonic energy (Jafari et al. 2008a) would promote the EE through reductions in unencapsulated surface oil which consequently, will improve the oxidative stability of microcapsules over storage (Ramakrishnan et al. 2013).

Monitoring the formation of CDs and CTs which is directly related to the production of hydroperoxides in the lipids, in line or in place of PV can serve as a relative measurement of oxidation (Shahidi 2005). The daily variations in CD and CT values during storage period of glyceride product at different oxygen availability levels are presented in Fig. 2. According to the statistical analysis, formation of dienoic and trienoic

Fig. 1 Scanning electron micrographs ($\times 150$ and $\times 500$) for the freeze-dried encapsulated glyceride product from parent emulsions produced by homogenizer (13,000 rpm for 3 min) as control or

produced by both homogenizer and ultrasound (380 W—3 min) in fresh form (control: **a**, treatment: **b**) and at the end of the accelerated storage period (control: **c**, treatment: **d**)

acids was significantly ($p < 0.05$) affected by storage time, treatment and their interactions. The accelerated storage testing is a conditional method for assessment of the oil oxidative stability which indicates the induction period (IP) of an oil as

the time needed to reach a significant increase in the suitable parameter (Lopez-Duarte and Vidal-Quintanar 2009) like UV absorbance that was selected to determine IP in the current research. Based on the results of IP (Table 2), the oil stored

Table 2 The formation rate of oxidation products (k , 1/day) and induction period (IP, day) for the samples stored at 40 °C, as well as encapsulation efficiency (%) of the powders

Sample ^a		k_{CD} (1/day)	k_{CT} (1/day)	R^2	IP (day)	Encapsulation efficiency (%)
Oil ^b	Air	5.829 ± 0.002^A	2.483 ± 0.145^A	0.92	1	–
	N ₂	0.255 ± 0.001^G	0.069 ± 0.008^D	0.99	≥ 12	–
Sonication ^c	n.u. ^d	1.781 ± 0.024^B	1.239 ± 0.077^B	0.80	3	68.24 ± 1.75^C
	180 W—1 min	1.619 ± 0.034^C	0.520 ± 0.029^C	0.91	5	88 ± 0.37^B
	180 W—3 min	0.792 ± 0.079^E	0.407 ± 0.015^C	0.98	9	90.95 ± 0.60^{AB}
	380 W—1 min	1.357 ± 0.001^D	0.538 ± 0.006^C	0.96	5	92.79 ± 0.74^A
	380 W—3 min	0.499 ± 0.015^F	0.301 ± 0.003^{CD}	0.95	9	94.11 ± 0.07^A
Prob > F		< 0.0001	< 0.0001			< 0.0001

^aValues are expressed as duplicate mean $\pm$ standard deviation. Means with the same capital letters within the same columns are not significantly different ($p < 0.05$)

^bConcentrated glyceride product

^cSonication treatment conditions used to produce the encapsulated glyceride product

^dn.u. note used

Fig. 2 Comparing the changes in CD (a) and CT (b) values ($E_{1\text{ cm}}^{1\%}$) of the samples, including the glyceride product under N₂ (filled square) or air (filled diamond), and the powders from parent emulsions untreated (Δ) or treated by ultrasound energy, including 180 W—1 min ($\times$), 180 W—3 min ($-$), 380 W—1 min (filled circle) and 380 W—3 min ($+$) during storage at 40 °C and a_w value of 0.43

Fig. 3 Scanner images of different samples, including wall materials (WP and NaCA), and the powders from parent emulsions untreated (control) or treated by ultrasound energy stored at a_w value of 0.43 at

40 °C in fresh form (**a**) and at the end of the accelerated storage period (**b**, 12 days), with dimensions of 2200 × 3000 pixels (400 dpi)

under N_2 showed the highest oxidative stability as there were no significant ($p > 0.05$) changes in its CD and CT values during storage, while the oxidative indices for 380 W—3 min, 380 W—1 min, 180 W—3 min, 180 W—1 min, the control and air atmosphere were significantly ($p < 0.05$) increased after 9, 5, 9, 5, 3 and 1 days storage, respectively (Table 2).

Chen et al. (2013) used milk proteins, including whey protein isolate (protein content 92%) and NaCA (protein content 92.70%) in a ratio of 4:1 to produce microcapsules

containing fish oil. They prepared the emulsions by a homogenizer at 13,500 rpm for 1 min that followed by high pressure homogenization at 80 MPa to produce fine emulsions. In agreement with our results, they observed a significant increase in oxidation indices (peroxide and anisidine values) of the samples during storage period (7 days) at 45 °C and 30% relative humidity.

The slopes from plotting CD and CT values vs. storage time which show the constant rate of the formation of

Table 3 Colour features of the powders produced under different emulsification conditions

Color property ^a	Time (day)	WP + NaCA ^b	Control ^c	Sonication treatment (power–time)			
				180 W—1 min	380 W—1 min	180 W—3 min	380 W—3 min
L*	0	99.68 ± 0.01 ^{A,a}	98.63 ± 0.07 ^{A,c}	99.18 ± 0.03 ^{A,b}	99.21 ± 0.08 ^{A,b}	99.31 ± 0.04 ^{A,b}	99.23 ± 0.00 ^{A,b}
	12	99.14 ± 0.12 ^{B,a}	80.06 ± 0.04 ^{B,f}	94.38 ± 0.04 ^{B,e}	95.86 ± 0.02 ^{B,c}	95.38 ± 0.00 ^{B,d}	98.45 ± 0.06 ^{B,b}
a*	0	− 1.68 ± 0.04 ^{A,a}	− 5.63 ± 0.05 ^{B,c}	− 3.82 ± 0.11 ^{A,b}	− 3.85 ± 0.15 ^{A,b}	− 3.65 ± 0.14 ^{A,b}	− 3.77 ± 0.01 ^{A,b}
	12	− 4.22 ± 0.10 ^{B,b}	7.20 ± 0.12 ^{A,a}	− 6.23 ± 0.11 ^{B,c}	− 6.59 ± 0.03 ^{B,cd}	− 6.82 ± 0.09 ^{B,d}	− 7.41 ± 0.13 ^{B,e}
b*	0	4.47 ± 0.11 ^{B,c}	17.27 ± 0.07 ^{B,a}	10.97 ± 0.40 ^{B,b}	10.96 ± 0.51 ^{B,b}	10.31 ± 0.44 ^{B,b}	10.84 ± 0.03 ^{B,b}
	12	12.41 ± 0.04 ^{A,f}	51.82 ± 0.07 ^{A,a}	38.33 ± 0.15 ^{A,b}	32.18 ± 0.06 ^{A,d}	35.26 ± 0.31 ^{A,c}	23.87 ± 0.14 ^{A,e}
ΔE	0	3.17 ± 0.09 ^{B,c}	15.96 ± 0.09 ^{B,a}	9.49 ± 0.41 ^{B,b}	9.50 ± 0.52 ^{B,b}	8.85 ± 0.45 ^{B,b}	9.36 ± 0.03 ^{B,b}
	12	10.96 ± 0.01 ^{A,f}	53.32 ± 0.04 ^{A,a}	36.78 ± 0.16 ^{A,b}	30.69 ± 0.05 ^{A,d}	33.78 ± 0.33 ^{A,c}	22.78 ± 0.17 ^{A,e}
Source		Prob > F					
		L*	a*	b*	ΔE		
Treatment		< 0.0001	< 0.0001	< 0.0001	< 0.0001		
Time		< 0.0001	< 0.0001	< 0.0001	< 0.0001		
Treatment × time		< 0.0001	< 0.0001	< 0.0001	< 0.0001		

^aValues are expressed as duplicate mean ± standard deviation. Means with the same capital (in each colour parameter row) and small letters within the same columns and rows, respectively, are not significantly different ($p < 0.05$)

^bWall materials (whey powder + NaCA)

^cControl: the encapsulated glyceride product produced without sonication treatment

oxidation products (k_{CD} and k_{CT}) are shown in Table 2. The slopes included the points before reaching the propagation phase as stated by Lopez-Duarte and Vidal-Quintanar (2009). The indices were increased during storage period in a linear behavior with high R^2 values (Table 2). Power and time of sonication were significantly ($p < 0.05$) influenced k_{CD} and k_{CT} . Interaction of sonication power and time had no significant effect ($p > 0.05$) on k_{CD} , indicating that power and time independently affected k_{CD} (see “Appendix”). Based on k_{CD} and IP results (Table 2), the highest oxidation rate was observed for the glyceride product kept in contact with air followed by the control powder, 180 W—1 min, 380 W—1 min, 180 W—3 min, 380 W—3 min and the glyceride product packaged under N_2 atmosphere, respectively. During progression of oxidation in the lipids, CD moieties are formed in the structure of FAs with two double bonds that showed a maximum absorption at 233 nm, while in fatty acids containing three or more double bonds, CD moieties can be extended to include a more double bond resulting in formation of a conjugated triene with strong absorption at 268 nm. Therefore, CD moieties are formed at the beginning of the reaction followed by CT moiety formation (Pegg 2005). Since the analysis were performed only based on the induction phase, when considerable CT moieties were not formed yet, it could be explained why significant letters presented for k_{CD} were not perfectly compatible with those determined for k_{CT} (Table 2). In addition, this is why CT

values of the lipids are usually lower than their CD values (Pegg 2005).

Application of ultrasound energy could significantly ($p < 0.05$) affect the oxidative stability of powders containing the glyceride product rich in n-3 PUFA. Based on our results, it was shown that the sonication time was more important in comparison with the sonication power (see “Appendix”). Also it was found that contact surface of glyceride product with oxygen was a very effective factor on its shelf-life, as the highest oxidative stability was obtained by replacing the air with N_2 , while the encapsulation process could only limit the contact surface of glyceride product with oxygen, instead of strong inhibition of oxygen as seen for N_2 atmosphere.

Colour parameters

Colour parameters of R, G, B obtained from the prepared images with dimensions of 1242×1242 pixels and with resolution of 1200 dpi (Fig. 3; 2200×3000 pixels, 400 dpi) were respectively converted to L, a and b indices by ImageJ software in which the parameters were used to evaluate colour changes of the samples stored at 40 °C and $a_w = 0.43$ for 12 days (Table 3). The effect of storage period, treatment and their interactions on colour parameters of the samples including L, a, b and ΔE were significant ($p < 0.05$), so that during 12 days storage period, lightness (L-value) was significantly ($p < 0.05$) decreased

for all samples, while their yellowness (b-value) and ΔE parameter were showed a significant increase ($p < 0.05$) due to development of the deteriorative reactions. Significant increase ($p < 0.05$) in the redness (a-value) of the stored samples, as a measure of oxidation development, was only recorded for control powder (Table 3).

At the end of storage period, the highest lightness and lowest yellowness or ΔE corresponding to the higher oxidative stability were recorded for the wall material, as expected due to its very low lipid content (1%), followed by the samples prepared using ultrasound energy, including 380 W—3 min, 180 W—3 min, 380 W—1 min, 180 W—1 min, and the control, respectively (Table 3). Interestingly, the similar order was observed by monitoring oxidative indices (CD value and IP) of the samples (Table 2).

Christopoulos and Tsantili (2011) also reported a decrease in lightness and hue in walnut kernels stored at 1 and 20 °C under air atmosphere. It is believed that different factors including oxidation reaction, loss or reduction in antioxidant compounds (Guine et al. 2015) and Millard reaction (Christopoulos and Tsantili 2011) are predominant factors for higher redness (a-value) and lower lightness (L-value) in low water containing foods during storage. Qualitative development of red-golden colour for peanuts (Ghorbani and Hosseini 2017) and corn flour (Lopez-Duarte and Vidal-Quintanar 2009) kept in temperatures higher than 40 °C was previously reported as confirmed by the present work. In line with the results of chemical tests (Table 2), less colour changes were observed for the samples containing glyceride product when a prolonged ultrasound time (3 min) was associated with the different powers (180 or 380 W) as compared to those treated by a short time (1 min).

According to the above mentioned results, a factorial optimization was carried out to determine the sonication time (min) and power (W) required to minimum levels of oxidative indices (k_{CD} and k_{CT}) and ΔE value, as well as maximum levels of L-value and EE. The prediction profiler, indicating the optimum conditions, as well as related desirability values are shown in “Appendix”. It can be seen that the sonication treatment with a power of 380 W for 3 min was optimum condition for preparing the parent emulsions during fish oil encapsulation process (“Appendix”).

Conclusion

CRL-catalyzed hydrolysis reaction (pH = 7.20, 37 °C) was successfully led to provide a high n-3 PUFA glyceride product (49.62%) on a large scale. The results from UV absorbance, SEM and image processing revealed that the mixture of whey powder and NaCA can be appropriate to improve the oxidative stability of the glyceride product; however, there was need to apply ultrasound energy during

production of the encapsulated oil in order to enhance the oxidative stability. Based on the results, a practical procedure, including (A) concentration of fish oil by CRL-catalyzed hydrolysis, and (B) encapsulation of the product by whey powder and NaCA mixture, is proposed to be used on an industrial scale (specially, fishmeal plants). Moreover, the sonication treatment with a power of 380 W for 3 min was suggested to prepare parent emulsions during fish oil encapsulation process.

Appendix

Prediction profiler presenting the optimum values of the independent variables required to achieve maximum levels for both L-value and EE, as well as minimum levels for both oxidative indices (k_{CD} and k_{CT}) and ΔE .

Source	<i>p</i> Value/F-value				
	<i>k</i> _{CD}	<i>k</i> _{CT}	EE	ΔE	L*
Analysis of variance (ANOVA) data					
Power	0.0009/80.49	0.0193/14.33	0.0004/120	< 0.0001/1452.9	< 0.0001/4702.33
Time	< 0.0001/741.27	0.0001/226.35	0.0042/34.53	< 0.0001/3569.19	< 0.0001/7604.22
Power × time	0.6371/0.26	0.0058/28.74	0.0877/5.06	< 0.0001/294.95	< 0.0001/925.09

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