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Oxidative stability of pullulan electrospun fibers containing fish oil: effect of oil content and natural antioxidants addition

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Running title: Oxidative stability of fish oil-loaded pullulan fibers

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Abstract

The effect of oil content and addition of natural antioxidants on the morphology and oxidative stability of pullulan ultra-thin fibers loaded with fish oil and obtained by electrospinning was investigated. Pullulan sub-micron fibers containing 10 and 30 wt.% fish oil were prepared and both presented beads where the oil accumulated. The number of beads was significantly higher in 30 wt.% oil-loaded fibers. Moreover, fibers containing 30 wt.% fish oil had a higher oxidative stability when compared to 10 wt.% oil-loaded fibers, despite its lower encapsulation efficiency (EE) value (67.1±3.1 %). The oxidative stability of fibers loaded with 10 wt.% fish oil (EE=88.5±0.7 %) was significantly improved when adding δ-tocopherol (500 ppm) and rosemary extract (500 ppm) as antioxidants. However, higher concentration of antioxidants (2000 ppm δ-tocopherol and 1000 ppm rosemary extract) did not further improve the oxidative stability of 10 wt.% oil-loaded fibers, but had a pro-oxidant effect. Finally, the production of pullulan fibers containing 10 wt.% fish oil from formic acid solutions increased the oxidative stability of the fibers when compared to the same type of fibers obtained from water solutions. The latter was observed for fibers without and with antioxidants (500 ppm of δ-tocopherol and 500 ppm of rosemary extract).

Practical applications

Encapsulation of omega-3 polyunsaturated fatty acids and addition of antioxidants are the most efficient strategies to protect these lipids against oxidation when incorporating them into food matrices. These results show the feasibility to encapsulate fish oil in pullulan ultra-thin fibers and to improve their oxidative stability by adding natural antioxidants such as δ-tocopherol and rosemary extract. Therefore, this study might open up new opportunities for further technological development in the production of omega-3 nanodelivery systems, which have potential applications in different types of fortified foods.

1. Introduction

Long chain omega-3 polyunsaturated fatty acids (PUFA), particularly eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids, present numerous benefits on human health such as prevention of cardiovascular disease and development of neural and visual functions in infant [1]. As a result, the demand for omega-3 fortified products (e.g. infant formula) continues to increase in Europe, North America and Asia [2]. Nevertheless, omega-3 PUFA are highly prone to oxidation, which negatively affects the quality of the final product (e.g. nutritive value, odor, and flavor) [3]. In this context, it has been demonstrated that a successful approach for incorporation of omega-3 PUFA into food is the use of encapsulated lipids [4]. This allows the protection of the oil against prooxidants (e.g. oxygen, free radicals and metal ions) by creating a biopolymer barrier between the oil and the rest of the components in the food matrix. Moreover, encapsulation enables the masking of unpleasant odors and flavors of omega-3 PUFA (e.g. when added in the form of fish oil) [5].

Electrospinning is a straightforward and versatile technique for the encapsulation of bioactive ingredients in ultra-thin fibers, as reported for gallic acid [6], curcumin [7] and ferulic acid [8]. In the electrospinning process, a high-voltage electro-static field is used to charge the surface of a polymer solution droplet formed at the end of a capillary tube. When the electrical forces overcome the surface tension, an electrically charged jet of polymer solution is ejected from the tip of the formed Taylor cone to a grounded collector. On the way to the collector, the jet is stretched out due to bending instabilities, which favors the evaporation of the solvent resulting in dried fibers [9]. Encapsulates produced by electrospinning are gaining increasing attention since the process is carried out at room temperature, which reduces denaturation of the bioactives. Moreover, due to their small diameters, electrospun fibers present a high specific surface area, which favors the release of the active compound [10]. On the other hand, the incorporation of electrospun fibers into food systems has some challenges due to their morphological nature (e.g. continuous in length). In this regard, multi-layered food systems (e.g. granola bars) might be potential products for successful enrichment with sub-micron fibers, but this requires further evaluation.

Food-grade biopolymers need to be used as shell material for further incorporation of encapsulates into food matrices. Materials displaying appropriate viscoelastic properties, electric conductivity and surface tension are required for the production of fibers by electrospinning [11]. Recently, Mendes et al. [12] reviewed the key parameters affecting the electrospinning of food biopolymers including proteins and polysaccharides. In contrast to most proteins, which require the use of a carrier polymer in order to allow fibers formation (except for zein and gelatin), polysaccharides (e.g. pullulan, dextran and starch) are normally more easily electrospun when dissolved in food-grade solvents (e.g. water, formic or acetic acids).

Particularly interesting is the employment of pullulan, a linear glucan consisting of maltotriose units connected by α-1,6 glycosidic bonds, for the encapsulation of oxygen-sensitive compounds such as omega-3 PUFA. This is due to the excellent spinnability of pullulan in water solutions [13] and to the low oxygen-permeability of this biopolymer, although the latter is negatively affected at relative humidity conditions [14]. To the best of the authors' knowledge, ultra-thin fibers loaded with omega-3 PUFA have only been obtained by using zein [15] or a bio-compatible polymer such as poly-vinyl alcohol (PVA) [16]. Our group has recently reported the development of fish oil-loaded pullulan sub-micron fibers with a high oxidative stability after production [17]. Nevertheless, the ability of these fibers to protect fish oil against oxidation during storage remains to be investigated.

The high surface-to-volume ratio of ultra-thin fibers implies a high exposed surface area of the encapsulated oil to oxygen, which is detrimental in terms of oxidative stability. Thus, addition of antioxidants to omega-3 loaded sub-micron fibers, which has not yet been studied, seems appropriate as a parallel strategy to encapsulation in order to enhance the oxidative stability of the fibers. Serfert et al. [18] indicated that a combination of lipophilic and hydrophilic antioxidants with different partitioning is required for an efficient stabilization of fish oil in environments with multiple phases like emulsions (e.g. oil dispersed in the aqueous phase) and microencapsulates obtained by spray-drying (e.g. oil dispersed in the biopolymer matrix). Tocopherols are strong radical scavengers preventing lipid oxidation; particularly, the employment of δ-tocopherol has led to efficient

stabilization of bulk [19] and encapsulated fish oil [18]. Carnosic acid and carnosol (an oxidation product of carnosic acid), both present in rosemary extract, are also effective peroxyl radical scavengers, which prevent lipid oxidation [20]. Additionally, a synergistic effect has been reported when combining δ-tocopherol and carnosic acid, which reduces tocopheroxy radicals recycling tocopherol in the system [18,21].

Thus, this work aimed to study the oxidative stability during storage of fish oil-loaded pullulan fibers obtained by electrospinning. First, the influence of fish oil content (10-30 wt.%) on the properties of the electrospinning solutions (e.g. viscosity and droplet size) as well as on the morphology and oxidative stability of the fibers during storage was assayed. Secondly, the effect on the oxidative stability of the fibers of incorporating a hydrophilic antioxidant (e.g. water dispersible rosemary extract) to the pullulan solution and a lipophilic antioxidant (e.g. δ-tocopherol) to fish oil was evaluated. Finally, the influence of using formic acid as solvent instead of water on fibers morphology and oxidative stability was assayed.

2. Materials and Methods

2.1 Materials

Pullulan (molecular weight = 200,000 Da) was kindly donated by Hayashibara Co., Ltd. (Okayama, Japan). Commercial cod liver oil was kindly provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway) and stored at -40 ºC until use. The fatty acid composition (major fatty acids only) of the fish oil used was C16:0, 9.5%; C16:1, 8.7%; C18:1, 16.3%; C20:1, 12.6%; C20:5, 9.2% and C22:6, 11.4%. The tocopherol content of the fish oil was: α-tocopherol, 200±3 μg/g oil; β-tocopherol, 5±1 μg/g oil; γ-tocopherol, 96±3 μg/g oil and δ-tocopherol, 47±1 μg/g oil. The peroxide value (PV) of the fish oil used was 0.38±0.04 meq/kg oil. Tween-20, δ-tocopherol and formic acid of 96% purity were purchased from Sigma Aldrich (Brøndby, Denmark). Water dispersible rosemary extract with a content of 1.5% carnosic acid and 0.4% carnosol was kindly provided by Kalsec® (Denver City, Texas, United States). All other chemicals and solvents used were of analytical grade.

2.2 Preparation of pullulan solutions containing fish oil

Pullulan (17 wt.%) was dissolved in distilled water under constant stirring at room temperature. Tween-20 (20 wt.% with respect to fish oil) was added to the biopolymer solution and mixed using mechanical stirring for 10 min under nitrogen atmosphere. Subsequently, neat fish oil, accounting for 10 or 30 wt.% of the biopolymer, was dispersed in the previous solution using mechanical stirring for 20 min under nitrogen atmosphere. Two antioxidants; δ-tocopherol (lipid soluble) and rosemary extract (water dispersible) were added to pullulan solutions containing 10 wt.% fish oil in two different concentrations: i) 500 ppm of δ-tocopherol and 500 ppm of rosemary extract (medium concentration), and ii) 2000 ppm of δ-tocopherol and 1000 ppm of rosemary extract (high concentration). Antioxidant concentrations are given in mg/kg oil. Both antioxidants were dissolved in ethanol and δ-tocopherol was added to the oil, whereas rosemary extract was added to the pullulan solution containing Tween-20. Additional solutions containing 10 wt.% oil with and without antioxidants (medium concentration) were produced as commented above but using formic acid instead of distilled water as solvent. Samples were used immediately after production for electrospinning processing and subsequently stored (no more than three days) at 5 °C in the dark for further electrospinning processing and analysis of solution properties.

2.3 Characterization of electrospinning solutions

2.3.1 Viscosity

The viscosity of electrospinning solutions was measured using a stress controlled rheometer Stresstech (Reologica Instruments AB, Lund, Sweden) equipped with a CC25 standard bob cup system in a temperature vessel. Measurements were done at 20 ºC over a shear stress range from 0.1 to 100 Pa. Viscosity was measured in triplicate on each solution at day 1 after production and it was expressed in Pa·s.

2.3.2 Droplet size

Droplet sizes were measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). Solutions were diluted in recirculating water (3000 rpm), until it reached an

obscuration of 12%. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. Results are given in surface ($D_{3,2}$) and volume ($D_{4,3}$) mean diameters. Measurements were made in triplicate after production.

2.4 Electrospinning process

The pullulan solutions containing fish oil were placed in two syringes in a NE-4000 double syringe pump (NewEra Pump Systems, Inc., USA). Each syringe had a 16G needle (Proto Advantage, Canada) attached. The syringe pump delivered solutions with a flow rate of 0.015 ml/min. Using a high voltage power supply (Gamma High Voltage Research, USA), an electric field of 20 kV was applied between the spinneret of the syringe and a 15 × 15 cm collector plate made of stainless steel with alumina foil wrapped around it. The distance between the syringe tip and the collector plate was 15 cm. The electrospinning process was conducted at room temperature. Fibers were produced in batches during 30 min under nitrogen atmosphere.

2.5 Characterization of fibers

2.5.1 Morphology

The morphology of the fibers was investigated using scanning electron microscopy (SEM) (FEI Inspect, Hillsboro, OR, USA). Approximately 0.5×0.5 cm of the fiber sheet was placed on carbon tape and sputter coated with gold, 8 s, 40 mA utilizing a Cressington 208HR Sputter Coater (Cressington Scientific Instruments, Watford, England). The mean fiber or bead diameters were determined from the SEM images by image analysis (ImageJ, National Institutes of Health). For this purpose, the diameter of 100 randomly selected fibers and all the beads (if any) presented in the micrograph were measured using the ImageJ software (National Institutes of Health).

2.5.2 Lipid distribution and encapsulation

The droplet size distribution of the fish oil-loaded electrospun fibers after re-dispersion in distilled water was measured as previously described in Section 2.3.2. For that purpose, 200 mg of fibers were dissolved in 10 mL of distilled water at room temperature under magnetic stirring for 30 min. The resulting dispersion was filtered (pore size: 150 μm) in order to remove possible remains of fibers.

Encapsulation efficiency (EE) was determined by measuring the non-entrapped fish oil according to Moomand and Lim [15] with some modifications. NMS (50 mg) were submerged in heptane (10 mL) and gently shaken (100 rpm) for 15 min. The mixture was filtered and the absorbance of the liquid was measured at 250 nm (UV-1800, Shimadzu, Japan). The amount of oil present in the liquid was determined from a calibration curve (R^2=0.99), prepared by dissolving various quantities of fish oil in heptane. The EE was calculated as:

$$EE\ (\%) = \frac{A-B}{A} * 100 \qquad [1]$$

where A is the total theoretical amount of fish oil and B is the free amount of fish oil in the collection solution. Measurements were carried out in triplicate.

2.5.3 Oxidative stability

For lipid oxidation measurements, immediately after production, fibers were stored for 20 days in the dark in desiccators over a saturated solution of magnesium chloride (resulting in 33% of relative humidity in the headspace) at 20 ºC. Samples were taken at day 0, 5, 10, 15 and 20 for analysis.

2.5.3.1 Determination of PV

Lipids were extracted from 200 mg of fibers according to the Bligh and Dyer method using a reduced amount of the chloroform/methanol (1:1, w/w) solvent [22]. Three extractions were made from each sample. PV was determined on lipid extracts using the colorimetric ferric-thiocyanate method at 500 nm as described by Shantha and Decker [23].

2.5.3.2 Secondary oxidation products - dynamic headspace GC-MS

Approximately 100 mg of fibers and 30 mg internal standard (4-methyl-1-pentanol, 30 μg/g water) were weighted out in a 100 mL purge bottle, to which 5 mL of distilled water and 1 mL antifoam (Synperonic 800 μL/L water) were added. The bottle was heated to 45°C in a water bath while purging with nitrogen (flow 240 mL/min, 30 min). Volatile secondary oxidation products were trapped on Tenax GR tubes. The volatiles were desorbed again by heating (200°C) in an Automatic Thermal Desorber (ATD-400, Perkin Elmer, Norwalk, CN), cryofocused on a cold trap (-30°C), released again (220°C), and led to a gas chromatograph (HP 5890IIA, Hewlett Packard, Palo Alto, CA, USA;

Column: DB-1701, 30 m x 0.25 mm x 1.0 μm; J&W Scientific, CA, USA). The oven program had an initial temperature of 45°C for 5 min, increasing with 1.5°C/min until 55°C, with 2.5°C/min until 90°C, and with 12.0°C/min until 220°C, where the temperature was kept for 4 min. The individual compounds were analyzed by mass-spectrometry (HP 5972 mass-selective detector, Agilent Technologies, USA; electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250). The individual compounds were identified by both MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard) and by authentic external standard and quantified through calibration curves. The external standards employed were 2-ethylfuran, 1-penten-3-one, 1-penten-3-ol, hexanal, heptanal, (*E*,*E*)-2,4-heptadienal, octanal and nonanal (Sigma-Aldrich, Brøndby, Denmark).

2.6 Statistical analysis

Statgraphics Centurion XV (Statistical Graphics Corp., Rockville, MD, USA) was used for data analysis. Data were expressed as mean ± standard deviation. Firstly, multiple sample comparison analysis was performed to identify significant differences between samples. Secondly, mean values were compared by using the Fisher's least significant difference (LSD) test. Differences between means were considered significant at $p < 0.05$.

3. Results and discussion

3.1 Influence of oil content

First, the influence of fish oil content (10 or 30 wt.%) on solution properties as well as on fibers morphology and oxidative stability was evaluated. It is noteworthy that a high content of fish oil in the final encapsulate (e.g. pullulan sub-micron fibers) is desired since it would allow the enrichment with omega-3 of food products by incorporating less amounts of other unnecessary ingredients (e.g. biopolymers used as wall material in encapsulates). Nevertheless, increasing oil load may negatively affect the morphology and oxidative stability of encapsulates, which could have a detrimental effect on the quality of the final fortified product (e.g. texture and shelf-life).

3.1.1 Solutions properties: viscosity and droplet size

The properties of the electrospinning solutions (e.g. viscosity, conductivity and surface tension) are mainly determined by the type of biopolymer (e.g. molecular weight and concentration) and solvent employed as well as the oil load used [24]. Biopolymer solutions must have the sufficient polymer chain entanglements that prevent jet breakup because of electrostatic repulsions. On the other hand, solution viscosity should not be so high so it allows biopolymer motion induced by the electric field [12]. All pullulan solutions produced in this study presented Newtonian behavior with viscosity values varying from 1.6 to 2.9 Pa·s (Table 1). These values were higher than those previously reported by García-Moreno et al. [17] for 15% pullulan solutions containing 10 wt.% fish oil (0.9 Pa·s). This indicates that even a slight increase in pullulan content led to a considerable increase in solution viscosity; from 0.9 to 1.9 Pa·s for 15 and 17.5 wt.% pullulan solutions containing 10 wt.% fish oil, respectively. Moomand and Lim [25] also reported lower viscosity values (<0.03 Pa·s) for 20 wt.% zein alcohol-aqueous solutions containing up to 30 wt.% fish oil. This phenomenon can be explained by the fact that pullulan yields high viscosity solutions even at relatively low concentrations [26]. Table 1 also shows that the viscosity of the solution increased significantly ($p<0.05$) when increasing fish oil load from 0 to 10 and 30 wt.%. These results are in agreement with the rise in viscosity reported for zein solutions when increasing oil load from 0 to 17 and 30 wt.% [25]. This is due to the fact that fish oil has an inherent higher viscosity when compared to water or alcohol-water mixtures.

Lipid distribution in solutions, which is dependent on the approach used to incorporate the oil, will affect the physical stability of the solutions as well as the morphology of the encapsulates and their efficiency to entrap the oil. Results indicated that fish oil loading had a significant influence on the droplet size distribution in solutions (Table 2). Although no significant differences were observed in the surface mean diameter of solutions containing 10 and 30 wt.% oil (wt. % with respect to pullulan), the volume mean diameter of 10 wt.% fish oil-loaded solution was significantly higher (2.602±0.042 μm) than the one measured for the solution containing 30 wt.% oil (1.566±0.106 μm) (Table 2). Likewise, Hadnedev et al. [27] reported an increase in specific surface area when rising the oil load of

oil-in-water emulsions stabilized with 20 wt.% triethanolamine oleate (with respect to oil) using Ultra-Turrax.

3.1.2 Fibers characterization

3.1.2.1 Morphology, lipid distribution and encapsulation

Solution properties together with the selected processing conditions (e.g. applied potential, flow rate and spinning distance) are the variables affecting: i) the stability of the jet so it allows the formation of continuous fibers; and ii) the morphology of the fibers (e.g. diameter and appearance or not of beads) [28]. Differences in morphology were observed for pullulan fibers loaded with fish oil and control fibers (with no added oil). Control fibers were void of beads (Fig. 1a) whereas fibers loaded with 10 and 30 wt.% fish oil presented beads interspersed along the fibers (Fig. 1b,c). Control fibers had a completely different fiber diameter distribution and a significant lower average diameter when compared to both types of fish-oil loaded fibers (Fig. 1d and Table 2). Previous studies have also reported an increase in fiber diameter when incorporating fish oil, although fibers with smaller average diameter were obtained; 163±45 nm for 10.5 wt.% PVA fibers loaded with 10 wt.% fish oil [16] and <600 nm for 20 wt.% zein fibers loaded with up to 30 wt.% oil [25]. The appearance of beads in pullulan fibers loaded with fish oil is related to the partial incorporation of the oil along the fibers as well as to its accumulation within the bead-structures [17]. A similar effect was observed when increasing the load of emulsified fish oil or emulsified hexadecane in PVA fibers [16,29]. Despite the differences in viscosity and oil distribution of the solutions, fibers loaded with 10 and 30 wt.% did not present significant differences in their average fiber and bead diameters (Table 2). However, fibers loaded with 30wt.% had a significantly higher number of beads when compared to 10 wt.% oil-loaded fibers, which correlated well with the higher amount of oil added (Table 2).

Lipid distribution and encapsulation within the fibers are of great importance since they determine the amount of surface oil (e.g. unprotected oil) which is highly prone to oxidation. Droplet size distributions of re-dissolved oil-loaded pullulan fibers were significantly different (Table 2). Although the oil droplets might coalesce during re-dispersion of the fibers, no or very little coalescence is

expected to occur due to the stabilization of oil droplets by using Tween-20 and the short time required for the analysis. This is confirmed by $D_{4,3}$ values in Table 2. In fibers containing 10 wt.% oil, the oil was dispersed in the biopolymer matrix as smaller droplets when compared to 30 wt.% oil-loaded fibers, as shown by the surface and volume mean diameters (Table 2). This indicates a reduction in the size of large droplets present in 10 wt.% solution during the electrospinning processing, as $D_{4,3}$ was reduced from 2.602±0.042 to 1.144±0.026 μm (Table 1 and 2). This phenomenon needs further investigation since it was not observed when comparing the droplet size distribution of 30 wt.% oil-loaded re-dissolved fibers and its parent solution. The latter results were in line with previous studies on the incorporation of emulsified hydrophobic compounds in ultra-thin fibers [16,29]. Fish oil load also affected the oil entrapment in both types of fibers. Fibers containing 10 wt.% oil had a high EE value (88.5±0.7 %), which is within the range of previous results on fish oil encapsulation in PVA fibers (92.4±2.3 %) [16] and ferulic acid encapsulation in amaranth protein-pullulan fibers (83.7±0.1 %) [30]. On the other hand, 30 wt.% oil-loaded fibers had a significantly lower EE value (67.1±3.1 %) (Table 2). Moomand and Lim [15] reported EE values up to 95.88± 0.23 % for zein fibers containing 30 wt.% fish oil. This difference might be due to a higher ability of zein to interact with oil than pullulan because of zein's hydrophobic character. Nevertheless, caution should be taken when comparing our EE results with those reported by Moomand and Lim [15] since we immersed the fibers in an organic solvent (e.g. heptane) while shaking for 15 min, whereas in the study by Moomand and Lim [15] the fibers were immersed only for 30 s in hexane.

3.1.2.2 Oxidative stability

The oxidative stability of the fibers was determined by measuring their content of both primary (e.g. hydroperoxides) and secondary volatile oxidation products (e.g. alcohols, ketones and aldehydes). It was observed that independently of the oil content, fish oil-loaded pullulan fibers were only oxidized to a low degree after production, as shown by their low PV (<15 meq O_2/kg oil) and low content of volatiles such as 1-penten-3-ol and (*E,E*)-2,4-hepatidenal (Fig. 2). These results are in agreement with our previous work on pullulan fibers containing 10 wt.% of neat fish oil [17]. Nevertheless, an

increase in PV of fish oil-loaded pullulan fibers was observed during storage, in contrast to what was observed for pullulan fibers containing only Tween-20 (Fig. 2a). Moreover, significant differences were found between both types of oil-loaded fibers, with 10 wt.% oil fibers resulting in significantly higher PV than the PV of 30 wt.% oil-loaded fibers during storage (Fig. 2a). Contrary to fibers containing 30 wt.% oil, which increased their PV linearly (R^2=0.994) during storage, 10 wt.% fish oil-loaded fibers had a sharp increase in PV from day 0 to day 5 (Fig. 2a). Nonetheless, it should be mentioned that a possible overestimation of PV for 10 wt.% oil-loaded fibers could have occurred as a consequence of the lower oil content of these fibers when compared to 30 wt.% oil-loaded fibers combined with the low amount of fibers used for this analysis (e.g. 200 mg). Thus, further research on the minimization of the amount of sample for PV analysis is required in order to obtain a linear relation between the amount of oil analyzed and the absorbance measured [31]. In regard to secondary oxidation products, both types of fibers increased their content of 1-penten-3-ol and (*E*,*E*)-2,4-heptadienal, which are volatiles derived from the oxidation of omega-3 fatty acids, after 5 days of storage (Fig. 2b,c). This increase was not observed for pullulan fibers containing only Tween-20. However, contradictory results for 10 and 30 wt.% oil-loaded fibers were obtained when looking at different volatiles. Fibers containing 10 wt.% oil presented a significantly higher content of 1-penten-3-ol at days 15 and 20 when compared to 30 wt.% oil-loaded fibers (Fig. 2b). The same trend was observed for 1-penten-3-one, another volatile derived from omega-3 fatty acids, and for other volatiles obtained from the oxidation of omega-6 (e.g. hexanal) and omega-9 (e.g. octanal) fatty acids (data not shown). On the other hand, fibers loaded with 30 wt.% fish oil had a significantly higher content at day 20 of storage of other volatiles produced from the oxidation of omega-3 fatty acids such as (*E*,*E*)-2,4-heptadienal (Fig. 2c) and 2-ethylfuran (data not shown), but they were present in lower concentration than 1-penten-3-ol. In any case, the content of primary and secondary oxidation products of both types of fish oil-loaded pullulan fibers are considerably lower than those reported for PVA fibers containing emulsified fish oil [16].

Taken together, fibers loaded with 10 wt.% fish oil were more oxidized than fibers containing 30 wt.% oil. Nevertheless, the significantly lower PV and 1-penten-3-ol content of 30 wt.% oil-loaded fibers during storage when compared to fibers containing 10 w.t% oil did not correlate well with the EE values obtained for these fibers. As suggested by the significantly higher EE value for 10 wt.% oil loaded-fibers (Table 2), less surface oil was present in these fibers when compared to fibers containing 30 wt.% oil. This should have led to a better protection against oxidation of the oil encapsulated in 10 wt.% oil-loaded fibers, which was not observed. Hence, the results indicated that in pullulan fibers containing fish oil a faster oxidation of encapsulated oil occurred when compared to free surface oil (which was more abundant in 30 wt.% oil-loaded fibers). Likewise, Velasco et al. [32,33] reported a faster oxidation of fish or sunflower oils encapsulated in a sodium caseinate and $_D$-lactose matrix when compared to free oil present in the surface of the microcapsules. The authors attributed this phenomenon to the presence of encapsulated oil droplets very susceptible to lipid oxidation (e.g. if oxygen diffusion was not a limiting factor). Moreover, these authors pointed out that the ratio of surface area to volume of the free oil might be smaller than that of encapsulated oil in the dried particles.

3.2 Effect of antioxidants addition on oxidative stability of the fibers

In an attempt to further improve the oxidative stability of encapsulated fish oil in pullulan electrospun fibers, the addition of natural antioxidants (e.g. δ-tocopherol and rosemary extract) was evaluated. Pullulan fibers containing 10 wt.% fish oil were selected due to their significantly higher EE value when compared to 30 wt.% oil-loaded fibers (Table 2), which implied less free surface oil. As expected, the addition of antioxidants modified neither the morphology nor the EE values of 10 wt.% oil-loaded pullulan fibers (data not shown).

Fig. 3 shows the oxidative stability of pullulan fibers containing 10 wt.% oil without antioxidants or with medium (500 ppm δ-tocopherol plus 500 ppm rosemary extract) or high (2000 ppm δ-tocopherol plus 1000 ppm rosemary extract) concentration of antioxidants. It was observed that the addition of antioxidants did not reduce the increase in PV of the fibers from day 0 to day 5; but fibers containing

medium concentration of antioxidants had significantly lower PV than fibers without antioxidants at days 15 and 20 (Fig. 3a). Further, fibers containing medium concentration of antioxidants showed a significantly lower content of 1-penten-3-ol and (*E,E*)-2,4-heptadienal when compared to fibers without antioxidants at days 15 and 20 (Fig. 3a,b). These results are in agreement with those indicating an increase in the oxidative stability of fish oil microencapsulates obtained by spray drying when adding low-medium concentration of tocopherols (≤500 ppm) combined with: i) metal chelators such as EDTA [34], or ii) antioxidants showing a synergistic effect with tocopherols (e.g. ascorbic acid) together with metal chelators (e.g. lecithin) [35]. In the same line, Serfert et al. [18] reported that the addition of rosemary extract to ternary blends of tocopherols, ascorbyl palmitate and lecithin or Citrem significantly retarded autoxidation of spray-dried microcapsules containing fish oil. Conversely, fibers containing high concentration of antioxidants had significantly higher values of PV at day 5 (Fig. 3a), 1-penten-3-ol at days 10 and 15 (Fig. 3b) and (*E,E*)-2,4-heptadienal at day 15 (Fig. 3c) when compared to fibers without antioxidants or with medium concentration of antioxidants. This denoted a pro-oxidant effect of 2 wt.% δ-tocopherol when combined with 1000 ppm of rosemary extract. A similar effect was described in bulk fish oil when adding 100 ppm of ascorbyl palmitate to oil containing 2 wt.% δ-tocopherol; which might be due to the promotion of hydroperoxide scission by ascorbyl radicals [36].

It should be noted that a decrease was observed in the volatiles content of fibers with antioxidants after 10-15 days of storage (Fig. 3b,c). This can be explained by: a) a slower formation rate of volatiles in these samples while having a similar evaporation rate than the rest of the samples (e.g. in case that volatiles evaporated from the samples during storage), or b) to a further degradation of those secondary oxidation products to other compounds. For instance, the ozonolysis of 1-penten-3-ol has been described to produce lower molecular weight compounds such as formaldehyde, 2-hydroxybutanal and propanal [37]. Similarly, Damerau et al. [38] explained a low concentration of hexanal in microencapsulated sunflower oil because a possible decomposition of hexanal to hexanoic acid.

3.3 Fibers produced using formic acid as solvent

The influence of using formic acid as solvent on the morphology and oxidative stability of 10 wt.% fish oil-loaded fibers was assayed. Formic acid is approved by the FAO/WHO expert committee on food additives as a flavouring and preservative agent, with an acceptable daily intake of ≤ 3 mg/kg of body weight [39]. Due to its capacity to dissolve proteins with high hydrophobicity, formic acid has been employed for the production of zein or amaranth protein-pullulan fibers by electrospinning [40,41]. It is worth noting that the encapsulation of food bioactives such as ferulic acid, quercetin and curcumin has also been carried out in amaranth protein-pullulan sub-micron fibers using formic acid as solvent [30,42].

Fig. 4 shows the morphology and the diameter distribution of pullulan electrospun fibers loaded with 10 wt.% fish oil and produced using formic acid as solvent. Contrary to 10 wt.% fish oil-loaded pullulan fibers obtained from water solutions, fibers produced from a formic acid solution did not present beads where fish oil could accumulate (Fig. 4a). This denoted that the fish oil was distributed along the fibers, which might be a consequence of a better dispersion of fish oil in formic acid than in water due to the difference in polarity of the solvents. In addition, most of the fish oil was encapsulated within the pullulan matrix since these fibers presented a high EE value (90.9±2.0 %). In regard to average fiber diameter, no significant differences were found between these fibers and 10 wt.% fish oil-loaded pullulan fibers obtained from a water solution (Fig. 4b).

Interestingly, 10 wt. % fish oil-loaded fibers produced from formic acid solutions had a higher oxidative stability when compared to those fibers with the same amount of oil obtained from water solutions (Fig. 5). Fig. 5a shows that fibers from formic acid presented a significantly lower PV than fibers from water during storage. Moreover, the content of 1-penten-3-ol of fibers from water was significantly higher when compared to fibers from formic acid after 15 days of storage (Fig. 5b). Further, the addition of antioxidants (in medium concentration) to fibers from formic acid significantly reduced the PV after 10 days of storage (Fig. 4a). Although both types of 10 wt.% oil-loaded fibers (obtained from water or formic acid solutions) presented no significant differences in EE, the higher

protection of the oil in fibers from formic acid suggested that pullulan and fish oil interacted differently when dissolved in water or formic acid. This interaction could also explain the higher water resistance of the fibers from formic acid when compared to fibers obtained from water solutions. The latter fibers were completely dissolved in water, whereas fibers from formic acid formed agglomerates which did not dissolve when immersed in water at 45 ºC during 30 min while purging with N_2 (240 mL/min). The different solubility of both types of fibers open up the range of food matrices where these encapsulates can be incorporated. Nevertheless, further research is needed in order to confirm the water resistance of the fibers and to identify the phenomena involved.

4. Conclusions

Morphology and oxidative stability of pullulan electrospun fibers loaded with fish oil were affected by oil content and antioxidant addition. Pullulan fibers containing 30 wt.% fish oil presented similar fibers and beads diameters, but significantly higher number of beads than 10 wt.% fish oil-loaded fibers. The oxidative stability of 30 wt.% fish oil-loaded-fibers was higher when compared to fibers containing 10 wt.% oil, although a considerably lower EE value was found for fibers loaded with 30 wt.% oil. This can only be explained by a slower oxidation of free surface oil due to the presence of encapsulated oil droplets very susceptible to lipid oxidation. Nevertheless, an increase in the oxidative stability of fibers containing 10 wt.% fish oil was obtained when adding 500 ppm of δ-tocopherol and 500 ppm of rosemary extract. On the contrary, a pro-oxidant effect of antioxidants on the oxidative stability of 10 wt.% fish oil-loaded fibers was observed when adding them in higher concentrations (2000 ppm δ-tocopherol and 1000 ppm rosemary extract). Pullulan fibers containing 10 wt.% fish oil and produced using formic acid as solvent presented a significant higher oxidative stability than 10 wt.% oil-loaded fibers obtained from water solutions. The addition of antioxidants (500 ppm of δ-tocopherol and 500 ppm of rosemary extract) also increased the oxidative stability of these fibers. Moreover, an improved water resistance of fibers obtained from formic acid solutions when compared to those produced from water solutions was observed.

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Conflict of Interest

The authors have declared no conflict of interest.

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Tables

Table 1. Viscosity and droplet size distribution of electrospinning solutions.

Sample	**Viscosity** (Pa·s)	**Droplet size – $D_{3,2}$** (μm)	**Droplet size – $D_{4,3}$** (μm)
Pullulan	1.6±0.1[a]	-	-
Pullulan+10% oil	1.9±0.1[b]	0.508±0.054[a]	2.602±0.042[a]
Pullulan+30% oil	2.9±0.1[c]	0.442±0.056[a]	1.566±0.106[b]

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

Table 2. Results of fibers characterization.

Sample	Fiber diameter (μm)	Bead diameter (μm)	Number of beads*	Droplet size – $D_{3,2}$ (μm)	Droplet size – $D_{4,3}$ (μm)	Encapsulation efficiency (%)
Pullulan	0.509±0.075[a]	-	-	-	-	-
Pullulan+10% oil	0.786±0.094[b]	1.227±0.152[a]	34±7[a]	0.499±0.048[a]	1.144±0.026[a]	88.5±0.7[a]
Pullulan+30% oil	0.773±0.140[b]	1.225±0.222[a]	71±3[b]	0.693±0.030[b]	1.485±0.013[b]	67.1±3.1[b]

Values within a column with different superscript letters indicate significant differences ($p<0.05$).
*Beads were counted in ×5000 magnified SEM images for each type of fibers. Results are the average of triplicate determinations ± standard deviation.

Figures

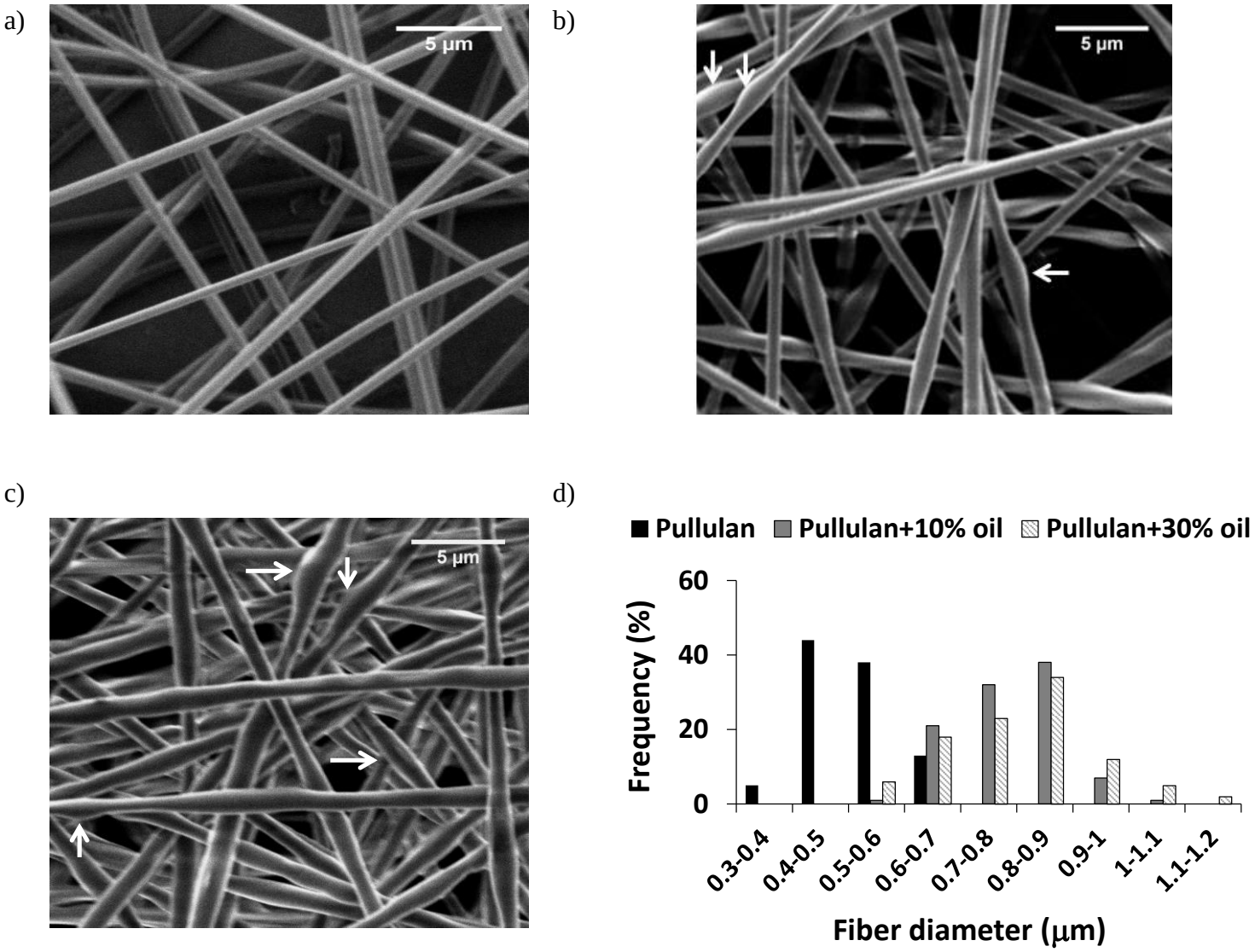
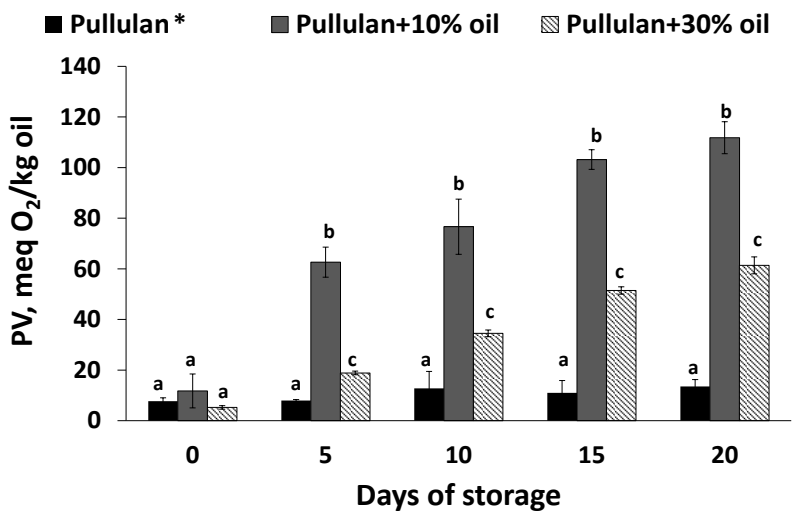
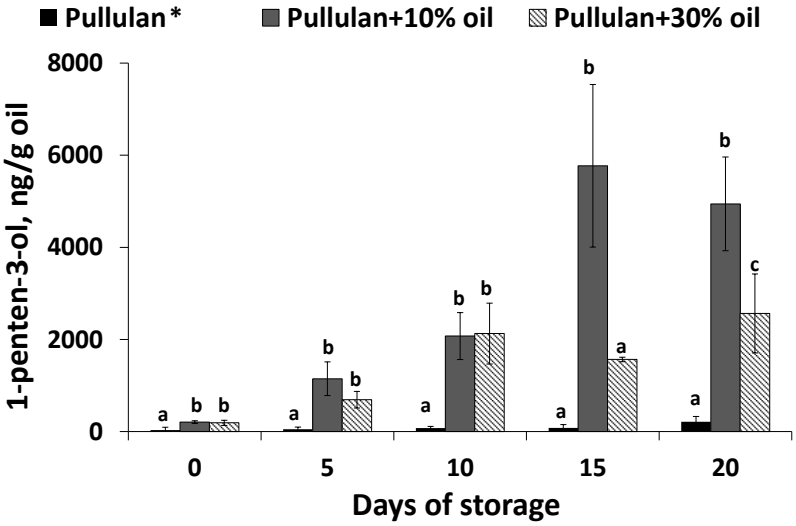


Figure 1. SEM micrographs of electrospun fibers: a) pullulan, b) pullulan+10% oil, c) pullulan+30% oil; and d) fiber diameter distribution.

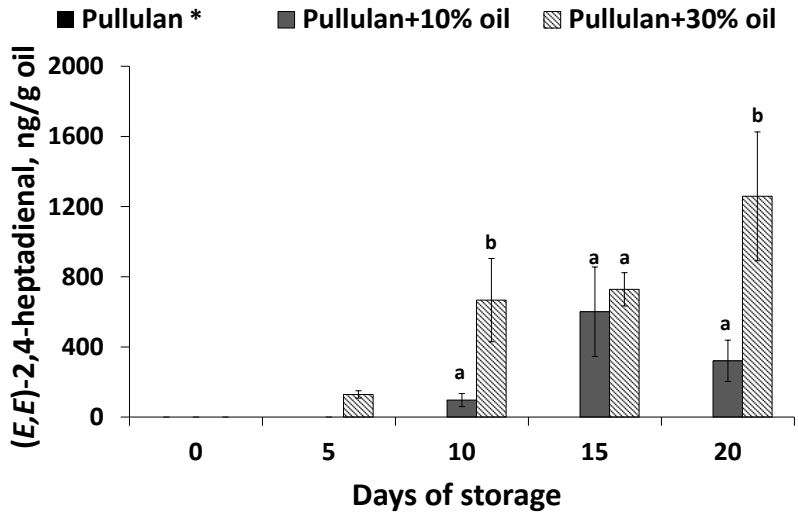
a)



b)



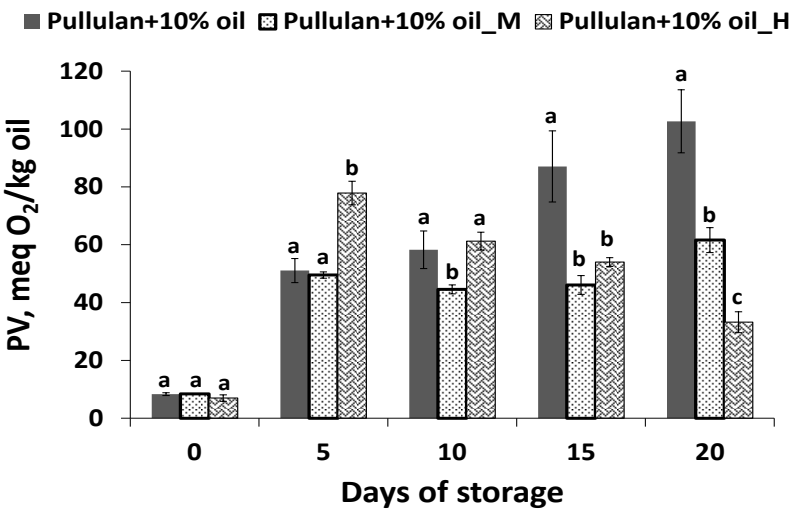
c)



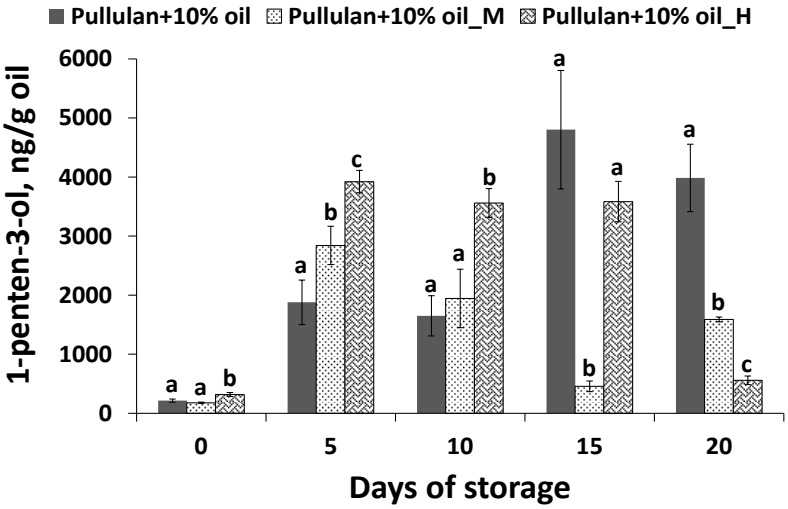
*Pullulan fibers containing the same amount of Tween-20 than 30 wt.% fish oil-loaded pullulan fibers.
Results are the average of triplicate determinations ± standard deviation. For each sampling point, values with different superscript letters indicate significant differences ($p<0.05$).

Figure 2. Oxidative stability of electrospun fibers with different oil content during storage at 20 ºC and 33% RH: a) peroxide value, b) 1-penten-3-ol, and c) (*E*,*E*)-2,4-heptadienal.

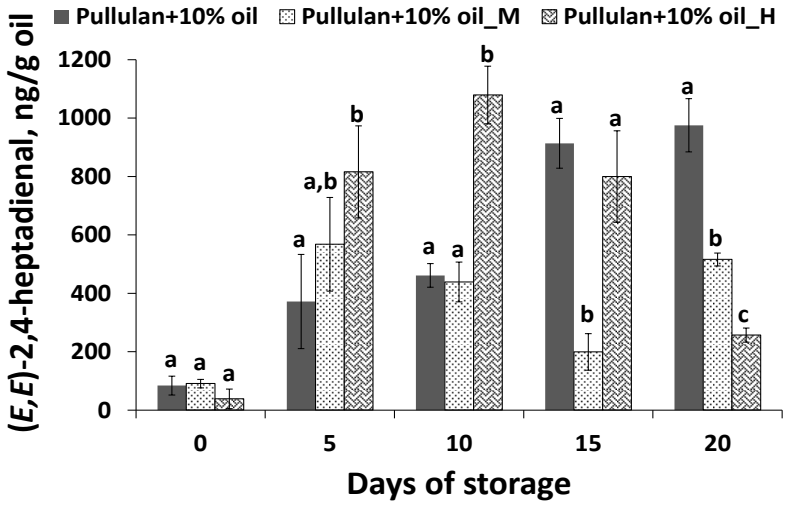
a)



b)



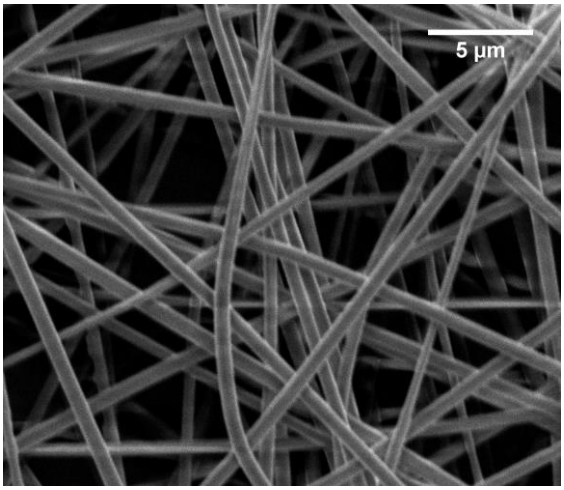
c)



10% oil_M: pullulan fibers loaded with 10% fish oil, containing 500 ppm of δ-tocopherol and 500 ppm of rosemary extract; *10% oil_H:* pullulan fibers loaded with 10% fish oil, containing 2000 ppm of δ-tocopherol and 1000 ppm of rosemary extract. Results are the average of triplicate determinations ± standard deviation. For each sampling point, values with different superscript letters indicate significant differences ($p<0.05$).

Figure 3. Oxidative stability of 10% fish oil loaded-electrospun fibers with antioxidants during storage at 20 ºC and 33% RH: a) peroxide value, b) 1-penten-3-ol, and c) (*E,E*)-2,4-heptadienal.

a)



b)

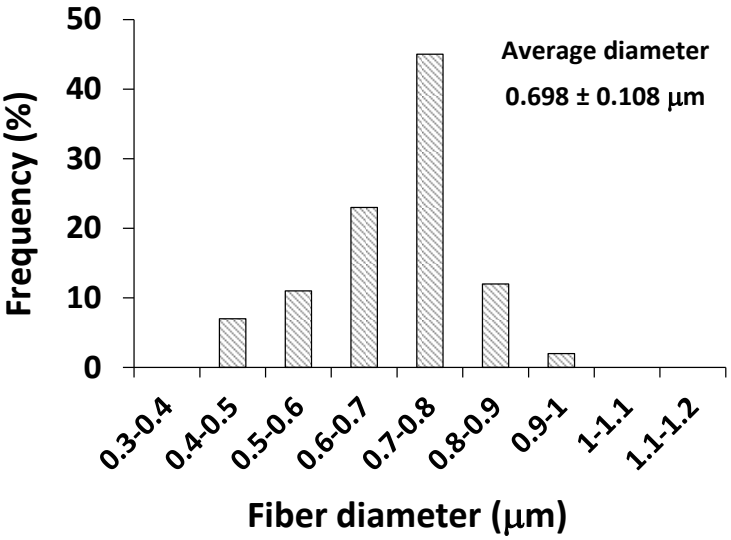
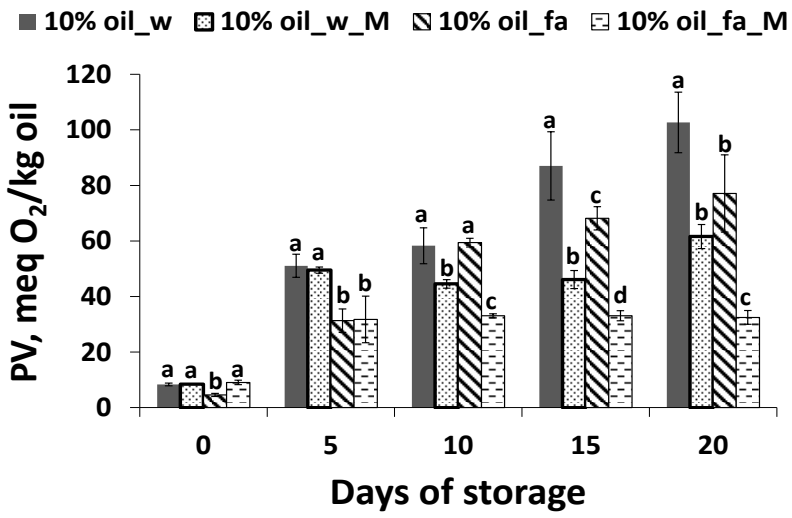
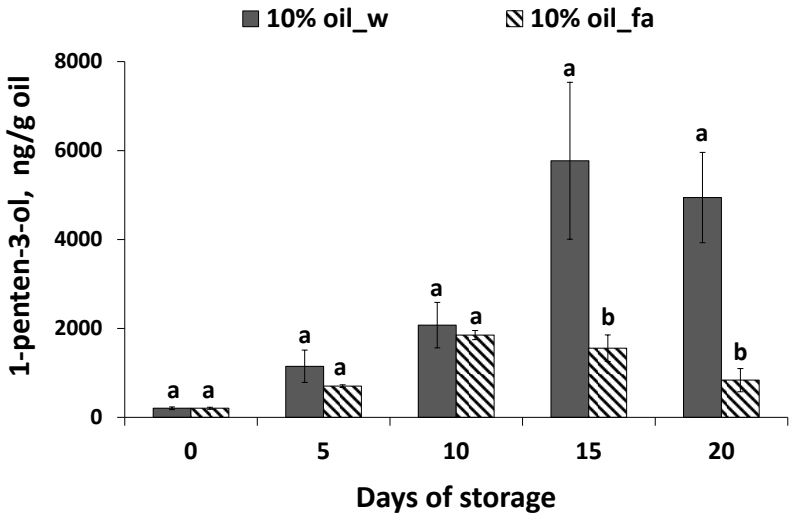


Figure 4. Morphology of 10% fish oil-loaded pullulan fibers produced using formic acid as solvent: a) SEM micrograph, and b) fiber diameter distribution

a)



b)



10% oil_w: pullulan fibers loaded with 10% fish oil and produced using water as solvent; *10% oil_w_M:* pullulan fibers loaded with 10% fish oil, containing 500 ppm of δ-tocopherol plus 500 ppm of rosemary extract, and produced using water as solvent; *10% oil_fa:* pullulan fibers loaded with 10% fish oil and produced using formic acid as solvent; *10% oil_fa_M:* pullulan fibers loaded with 10% fish oil, containing 500 ppm of δ-tocopherol plus 500 ppm of rosemary extract, and produced using formic acid as solvent. Fibers were stored at 20 ºC and 33% RH. Results are the average of triplicate determinations ± standard deviation. For each sampling point, values with different superscript letters indicate significant differences ($p<0.05$).

Figure 5. Oxidative stability of 10% fish oil loaded-electrospun fibers produced by using water or formic acid as solvent: a) peroxide value, and b) 1-penten-3-ol.