**Protein-based emulsion electrosprayed micro- and submicroparticles
for the encapsulation and stabilization of thermosensitive hydrophobic
bioactives**

Laura G. Gómez-Mascaraque, Amparo López-Rubio*

Food Quality and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7,
46980 Paterna, Valencia, Spain

*Corresponding author: Tel.: +34 963900022; fax: +34 963636301

E-mail address: amparo.lopez@iata.csic.es (A. López-Rubio)

ABSTRACT

This work shows the potential of emulsion electrospraying of proteins using food-grade emulsions for the microencapsulation and enhanced protection of a model thermosensitive hydrophobic bioactive. Specifically, gelatin, a whey protein concentrate (WPC) and a soy protein isolate (SPI) were compared as emulsion stabilizers and wall matrices for encapsulation of α -linolenic acid. In a preliminary stage, soy bean oil was used as the hydrophobic component for the implementation of the emulsion electrospraying process, investigating the effect of protein type and emulsion protocol used (i.e. with or without ultrasound treatment) on colloidal stability. This oil was then substituted by the ω -3 fatty acid and the emulsions were processed by electrospraying and spray-drying, comparing both techniques. While the latter resulted in massive bioactive degradation, electrospraying proved to be a suitable alternative, achieving microencapsulation efficiencies (MEE) of up to ~70%. Although gelatin yielded low MEEs due to the need of employing acetic acid for its processing by electrospraying, SPI and WPC achieved MEEs over 60% for the non-sonicated emulsions. Moreover, the degradation of α -linolenic acid at 80°C was significantly delayed when encapsulated within both matrices. Whilst less than an 8% of its alkene groups were detected after 27 hours of thermal treatment for free α -linolenic acid, up to 43% and 67% still remained intact within the electrosprayed SPI and WPC capsules, respectively.

39 KEYWORDS

40 Emulsion electrospraying; spray-drying; emulsion; encapsulation; omega-3; fatty acid;
41 linolenic acid; functional food

¹ ABBREVIATIONS:

WPC: Whey protein concentrate
SPI: Soy protein isolate
MEE: Microencapsulation efficiency
ALA: α -linolenic acid
O/W: Oil in water
GRAS: Generally Recognized as Safe
Gel: Gelatin
SBO: Soy bean oil
DLS: Dynamic light scattering
CI: Creaming index
SEM: Scanning electron microscopy
FT-IR: Fourier transform infrared
TGA: Thermogravimetric analysis
DTG: Derivative thermogravimetric curves
HSH: High-speed homogenization
US: Ultrasound

1. Introduction

One of the most promising approaches to preserve hydrophobic bioactive ingredients in food systems is their nano- or microencapsulation within protective matrices (Dube, Ng, Nicolazzo, & Larson, 2010), as they act as barriers, thus limiting direct contact of the bioactives with the detrimental agents of the environment (Ye, Cui, Taneja, Zhu, & Singh, 2009). Moreover, microencapsulation can also help overcoming the incompatibility between the hydrophobic compounds and the aqueous matrix of many food products, potentially increasing their bioavailability (Braithwaite et al., 2014). However, it also represents an additional challenge, given that the use of aqueous media for the dissolution or suspension of the polymers to be used as encapsulating matrices is almost imperative for the production of edible products, in order to avoid toxicity issues (López-Rubio & Lagaron, 2012). A plausible strategy to disperse the lipophilic bioactive into the aqueous polymer solution is to prepare oil-in-water (O/W) emulsions prior to microencapsulation. Although O/W emulsions are, in general, thermodynamically unstable (McClements, 2012) there are several strategies which can be used to increase their stability and the subsequent encapsulation efficiency (Bock, Dargaville, & Woodruff, 2012), such as reducing the size of the oil droplets or addition of tensioactive compounds (Malaki Nik, Wright, & Corredig, 2010). Spray-drying is the most commonly used technology in the food industry to obtain dry encapsulation structures from emulsions (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). However, spray-drying involves the use of a hot gas stream to rapidly dry the fine droplets produced in its initial atomization step, which results detrimental for hydrophobic thermosensitive bioactives such as ω -3 fatty acids. In contrast, electrospraying (i.e. a technique based on the electrohydrodynamic processing of polymer melts, solutions or dispersions) can be performed under mild conditions

(López-Rubio & Lagaron, 2012), so it has recently been proposed as an alternative for the microencapsulation of labile bioactive agents (Bock et al., 2012) with promising applications in the field of functional foods (Gómez-Mascaraque, Lagarón, & López-Rubio, 2015; Pérez-Masiá, Lagaron, & Lopez-Rubio, 2015; Pérez-Masiá et al., 2015). This technology allows the production of nano- and microencapsulation structures by subjecting the polymeric fluid, which is pumped through a conductive capillary, to a high voltage electric field. As a result, a charged polymer jet is ejected towards the opposite electrode, which is broken down into fine droplets during the flight, generating dry polymeric particles upon solvent evaporation before being deposited on the collector (Bhardwaj & Kundu, 2010; Bhushani & Anandharamakrishnan, 2014; Chakraborty, Liao, Adler, & Leong, 2009). Emulsion electrospraying has been recently proposed for drug encapsulation (Wang, Zhang, Shao, & Wang, 2013) and for the development of cytocompatible microcapsules (Song, Chan, Ma, Liu, & Shum, 2015) using carbohydrate matrices, but to the best of our knowledge only the electrospraying of whey protein concentrate (WPC)-stabilized emulsions has been reported for the microencapsulation and protection of bioactive compounds of interest in functional foods (Pérez-Masiá et al., 2015) to date.

Proteins are particularly interesting molecules for emulsion electrospraying, as their amphiphilic structures allow their use as effective emulsifiers (McClements, 2004) in addition to their primary function as wall materials. Indeed, proteins are often used as ingredients in food emulsions, providing both electrostatic and steric stabilization, in addition to their own nutritional properties (Malaki Nik et al., 2010).

In this work, three different protein types, specifically gelatin, a whey protein concentrate (WPC) and a soy protein isolate (SPI) were used as encapsulation matrices of α -linolenic acid (ALA) as a model hydrophobic bioactive by the emulsion

electrospraying technique, with the aim of comparing their protection ability against oxidation. ALA was chosen for this purpose as, apart from being one of the most relevant ω -3 fatty acids playing an important role in the regulation of cellular functionality (Crawford et al., 2000) and the preservation of the cardiovascular, neurovascular and mental health (Nguemeni, Gouix, Bourourou, Heurteaux, & Blondeau, 2013), it is highly susceptible to oxidative degradation when exposed to oxygen, light and/or heat (Umesha, Monahar, & Naidu, 2013). In fact, ALA is considered to be the most important precursor of flavor reversion (i.e. development of off-flavors) (Frankel, 1980) and, thus, its high instability can compromise not only the nutritional value of ALA-enriched food products but also their sensorial properties, reducing their shelf-life (Habib, Amr, & Hamadneh, 2012). Furthermore, the well-established spray-drying technique was used to dry the emulsions for comparison purposes. Two emulsification protocols were carried out prior to microencapsulation using both techniques, and the influence of emulsion properties, drying technique and type of protein on the microencapsulation efficiency and on the stabilization of ALA against degradation at high temperatures were studied.

2. Materials and Methods

2.1. Materials

Whey protein concentrate (WPC), under the commercial name of Lacprodan[®] DI-8090 and with a w/w composition of ~80% protein, ~9% lactose and ~8% lipids, was kindly donated by ARLA (ARLA Food Ingredients, Viby, Denmark). Soy protein isolate (SPI) was kindly donated by The Solae Company (Switzerland). Type A gelatin from porcine skin (Gel), with reported gel strength of 175 g Bloom, soy bean oil (SBO), α -linolenic

acid ($\geq 99\%$) (ALA), Tween[®] 20 and potassium bromide FTIR grade (KBr) were obtained from Sigma-Aldrich. 96% (v/v) acetic acid (Scharlab) was used as received.

2.2. Preparation of oil in water (O/W) emulsions

The aqueous phase of each emulsion consisted of a protein solution/dispersion. Three different proteins were used to prepare the O/W emulsions: gelatin (Gel), soy protein isolate (SPI) and whey protein concentrate (WPC). In a preliminary stage, soy bean oil (SBO) was used as the oily phase in order to optimize the production of the encapsulating structures containing lipophilic compounds, using an inexpensive oil. Afterwards, SBO was substituted by a ω -3 fatty acid, linolenic acid (ALA), as a model functional oil. In all cases, the oil was added in a proportion of 10% (w/w) with respect to the total mass of non-volatile compounds in the capsules. The use of a surfactant, Tween20[®], was also considered for the stabilization of some of the emulsions, as described below. The preparation of the O/W emulsions using each of the three different proteins was slightly different, as illustrated in Figure 1.

The emulsification step itself was conducted using two different procedures. The first one consisted of a one-step high-speed homogenization process conducted using an IKA T-25 Digital ULTRA-TURRAX[®] equipped with a S 25N - 25F dispersing element whose stator diameter was 25 mm (Germany) at 6000 rpm during 2 min. The other approach included a second step consisting of an ultrasonication treatment, which was aimed at reducing the drop size of the oil phase. For this purpose, an ultrasonic probe (Bandelin electronic, Germany) was used at an amplitude of 10% and a frequency of 20 kHz for 2 min, in intervals of 30 s to avoid excessive heating. An ice bath was also used to prevent overheating of the samples.

Figure 1. Scheme of the preparation of O/W emulsions

2.2.1. Preparation of O/W emulsions using gelatin

Gelatin aqueous solutions (8% w/v) were prepared as described in (Gómez-Mascaraque, Lagarón, & López-Rubio, 2015) and cooled down to room temperature before preparation of the emulsions. The use of a surfactant for the formation of stable emulsions was not necessary in this case. In fact, preliminary optimization tests showed that the addition of either Tween20® or soy lecithin as surfactants resulted in the coalescence of the oil droplets, due to the aggregation of the protein and the surfactant molecules at acidic pH. Conversely, the emulsions were stable for weeks when gelatin was used alone, both as emulsifier and as wall matrix for the capsules. Consequently, the oil phase was directly incorporated to the premix and the emulsions were prepared following both approaches described in Figure 1.

2.2.2. Preparation of O/W emulsions using SPI

SPI (10% w/v) was dissolved in distilled water and denaturation of the protein was carried out to improve its electrosprayability (Pérez-Masiá, Lagaron, & López-Rubio, 2014), by heating the solution to 90°C for 30 min. Then, the solution was cooled down to room temperature in an ice bath before preparation of the emulsions. The addition of the surfactant Tween20® (5% w/v) was necessary to obtain stable emulsions in this case. Lastly, the oil phase was added to the premix and the emulsions were prepared following both approaches described in Figure 1.

2.2.3. Preparation of O/W emulsions using WPC

The preparation of SBO/WPC emulsions has already been reported for the encapsulation of lipophilic bioactive ingredients (Pérez-Masiá, Lagaron, & Lopez-Rubio, 2015). Based on this work, an aqueous surfactant solution was first prepared by dissolving 5 % (w/v) of Tween20® in distilled water. Afterwards, the oil phase was added, and pre-emulsions were prepared following both approaches described in Figure 1. Lastly, the required mass of WPC to achieve a protein concentration of 20% (w/v) in the aqueous phase was added to the preformed emulsions and magnetically stirred until a homogeneous emulsion was obtained.

2.3. Characterization of the emulsions

The rheological behaviour of the emulsions at 20°C ± 0.1°C was studied using a rheometer model AR-G2 (TA Instruments, USA) with a parallel plate geometry, using the methodology described in Gómez-Mascaraque et al. (2015) after equilibrating the samples for 2 min. All measurements were made at least in triplicate.

In addition, optical microscopy images were taken using a digital microscopy system (Nikon Eclipse 90i) fitted with a 12 V, 100 W halogen lamp and equipped with a digital camera head (Nikon DS-5Mc). Nis Elements software was used for image capturing.

2.4. Stability of the emulsions

The stability of the emulsions was assessed following the creaming index method proposed in (Surh, Decker, & McClements, 2006). Briefly, each emulsion was transferred into a sealed tube and stored for 5, 24 and 48 h at room temperature. When the emulsions separated into two different phases, the height of the top opaque ('cream') layer was measured (H_c), and the creaming index (CI) was calculated following Eq. (1):

$$CI = 100 (H_c/H_E) \quad \text{Eq. (1)}$$

where H_E is the total height of each emulsion in the tube.

2.5. Production of microencapsulation matrices by spray-drying

The emulsions were diluted 20-fold in distilled water prior to their processing by spray-drying, to avoid too high viscosities which would block the spraying head. The emulsions were subsequently fed to a Nano Spray Dryer B-90 apparatus (Büchi, Switzerland) equipped with a 7.0 μm pore diameter cap. The inlet air temperature was set at 90°C, as it proved to be enough to achieve complete drying of the particles at an inlet air flow of 146 ± 4 L/min and a reduced pressure of 50 ± 3 mbar. Under these conditions, the outlet air temperature varied between 50 and 65°C. The spray-dried

powders were deposited on the collector electrode by means of an applied voltage of 15 kV.

2.6. Production of microencapsulation matrices by emulsion electrospraying

The emulsions were processed without further dilution using a homemade electrospraying/electrospraying apparatus, equipped with a variable high-voltage 0-30 kV power supply. The emulsions were introduced in a 5 mL plastic syringe and were pumped at a flow-rate of 0.15 mL/h through a stainless-steel needle (0.9 mm of inner diameter). The needle was connected through a PTFE wire to the syringe, which was placed on a digitally controlled syringe pump. Processed samples were collected on a stainless-steel plate connected to the cathode of the power supply and placed facing the syringe in a horizontal configuration, at a distance of 10 cm. The applied voltage was 15 kV for the gelatin emulsions and 17 kV for SPI and WPC emulsions. The above processing parameters were selected from preliminary tests in order to attain stable electrospraying, avoiding dripping of the solution.

2.7. Morphological characterization of the particles

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 8-9 mm. Samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination. Particle diameters were measured from the SEM micrographs in their original magnification using the ImageJ software. Size distributions were obtained from a minimum of 200 measurements.

2.8. Fourier transform infrared (FT-IR) analysis of the samples

FT-IR spectra were collected in transmission mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment following the methodology described in Gómez-Mascaraque et al. (2015).

2.9. Microencapsulation efficiency

The microencapsulation efficiency (MEE) of the ALA-loaded capsules was determined based on FT-IR absorbance measurements. A calibration curve was obtained for each encapsulation matrix ($R_{Gel}^2 = 0.999$, $R_{SPI}^2 = 0.993$, $R_{WPC}^2 = 0.986$) from the spectra of protein/ALA mixtures of known relative concentrations (0, 5, 10 and 15 % w/w of ALA). The relative absorbance intensities of the peaks at 3012-3013 cm^{-1} (attributed to ALA) and at 1541-1543 cm^{-1} (corresponding to the Amide II band of the proteins) were plotted against the ALA concentration in the mixtures. The intact ALA content in the capsules was interpolated from the obtained linear calibration equations. The MEE of the ALA-loaded particles was then calculated using Eq. (2):

$$MEE (\%) = \frac{\text{Content of ALA in the capsules}}{\text{Content of ALA initially added to the emulsions}} \times 100 \quad \text{Eq. (2)}$$

2.10. Thermal Properties of the materials

Thermogravimetric analysis (TGA) was performed with a TA Instruments model Q500 TGA. The samples (ca. 8 mg) were heated from 25°C to 600°C with a heating rate of

10°C/min under dynamic air atmosphere. Derivative TG curves (DTG) express the weight loss rate as a function of temperature.

2.11. Accelerated oxidation assays for free and microencapsulated ALA

Non-encapsulated and microencapsulated ALA was subjected to thermal treatment at 80°C in order to evaluate the protective effect of each wall material. After selected time intervals, FT-IR spectra were recorded for each sample, and the absorbance intensity of the band at 3012-3013 cm⁻¹, corresponding to ALA, was measured. The decrease in the relative intensity of the aforementioned band was related to the extent of degradation of ALA within the capsules or in its native form, as previously reported (Torres-Giner, Martinez-Abad, Ocio, & Lagaron, 2010).

2.12. Statistical analysis

A statistical analysis of experimental data was performed using IBM SPSS Statistics software (v.23) (IBM Corp., USA). Significant differences between homogeneous sample groups were obtained through two-sided t-tests (means test of equality) at the 95% significance level ($p < 0.05$). For multiple comparisons, the p-values were adjusted using the Bonferroni correction.

3. Results and discussion

3.1. Characterization of O/W emulsions

Two different procedures were used for the preparation of the emulsions, as illustrated in Figure 1. The first approach consisted of a simple high-speed homogenization treatment, while the second one included a second ultrasonication step aimed at reducing the droplet size of the emulsions (Leong, Wooster, Kentish, & Ashokkumar, 2009). In general, smaller droplets lead to increased stability of emulsions and improved bioavailability of the active ingredients (McClements, 2011, 2012), also facilitating their inclusion and dispersion within the fine microencapsulation structures to be produced. However, ultrasonic treatments may heat the emulsions, potentially leading to partial degradation of thermosensitive bioactives. Therefore, both approaches were used for the preparation of the emulsions and the impact of the ultrasonication treatment was studied. Figure 2 shows the images obtained by optical microscopy for the different emulsions produced using SBO as a model oily phase.

Figure 2. Optical micrographs for the different emulsions produced using SBO. Scale bars represent 20 μm

The appearance of the emulsions produced using the first approach (single high-speed homogenization step) was dramatically different for each protein. Gel and WPC led to well dispersed droplets, which were significantly smaller for Gel, while flocculated droplets were observed for SPI emulsions. These differences were also manifested in their rheological behaviour, i.e. while the emulsions prepared using Gel and WPC exhibited quite a Newtonian behaviour in the range of study, the emulsion prepared in

the single high-speed homogenization step using SPI showed a manifest shear thinning behaviour (cf. Figure S1 of the Supplementary Material), usually associated to a high degree of droplet flocculation (McClements, 2007; Surh et al., 2006). This strong shear thinning behaviour, together with the high viscosity of the emulsion suggested that the mechanism of flocs formation was by bridging flocculation, which occurs when the protein chains or aggregates are shared between two droplets (Malaki Nik, Wright, & Corredig, 2010). A plausible explanation for the occurrence of bridging flocculation when using SPI involves the previous denaturation step which is carried out for this protein as a requirement for the subsequent electrospraying process (Pérez-Masiá et al., 2014). Denaturation leads to protein unfolding and thus to increased exposure of its non-polar amino acids. This may promote droplet flocculation in oil-in-water emulsions through increased hydrophobic attraction between protein chains adsorbed onto different droplets (McClements, 2004).

When the second emulsification approach was applied (i.e. including an ultrasonication treatment) the droplet size was indeed greatly reduced for the three protein formulations tested. Moreover, the SPI-stabilized emulsion turned Newtonian, with a substantial decrease in its viscosity, suggesting that the flocs were disrupted. Previous studies had shown a decrease in the extent of droplet aggregation and apparent viscosity upon ultrasound treatments, in addition to a reduction of the mean particle size (Surh et al., 2006).

3.2. Creaming stability of the emulsions

The creaming index of an emulsion after a particular time lapse is an indicative of its stability to gravitational separation. As the density of the oil droplets in an O/W emulsion is lower than that of its aqueous environment, they tend to move upwards unless efficiently stabilized (McClements, 2007). The creaming index (CI) of the emulsions prepared in this work after 5, 24 and 48 h are summarized in Table 1, and the appearance of the emulsions with or without a cream layer is shown in Figure S2 of the Supplementary Material.

Table 1. Creaming index (CI) of the emulsions, calculated according to Eq. 1

Emulsion		5 h	24 h	48 h
Protein	Emulsion procedure			
Gel	HSH	0%	0%	1%
Gel	HSH+US	0%	0%	0%
SPI	HSH	4%	4%	4%
SPI	HSH+US	0%	0%	0%
WPC	HSH	0%	24%	15%
WPC	HSH+US	0%	13%	9%

Five hours after preparation the SPI emulsion prepared through high-speed homogenization (without ultrasound treatment) already experienced creaming, which was not surprising taking into account the presence of big flocs in this sample (cf. Figure 2). The rest of the emulsions did not show signs of gravitational separation during these first 5 hours, meaning that they were stable to creaming during at least the time required to electrospray them. Gelatin was the most efficient protein system evaluated for the stabilization against gravitational separation of the emulsions, only experiencing subtle creaming after 48 h when no ultrasound treatment was applied, due to the bigger droplet sizes. On the other hand, sonication had a positive effect on the stability of SPI-based emulsions, as the disruption of the flocs together with the droplet

size reduction avoided the creaming phenomenon during at least 48 h. Regarding WPC, a thick cream layer appeared after 24 h, which further compacted during the following day reaching smaller CIs at 48 h. This cream layer was obviously thicker for the non-sonicated emulsion, due to its considerably bigger droplet size. Thick cream layers are usually caused by bridging flocculation, as strong attractive forces yield less packed flocs (McClements, 2007). However, unlike the SPI emulsion produced through procedure 1, WPC emulsions did not show signs of flocculation during the first hours after preparation. The reason for this late flocculation might be related to the conformational changes that globular proteins may suffer resulting from adsorption to an interface (McClements, 2004), consequently exposing their non-polar and cysteine residues to the aqueous phase. This phenomenon has been described for whey proteins, where disulphide cross-linking can occur at the interface (Malaki Nik et al., 2010). In SPI, as denaturation was forced through the previous thermal treatment, a much earlier flocculation was observed until the emulsion was ultrasonicated. Conversely, the ultrasound treatment in the WPC-stabilized emulsion was applied before the addition of the protein, so it could not affect its reassembling.

3.3. Morphology of electrosprayed capsules from O/W emulsions

The production of microcapsules from protein-stabilized emulsions by spray-drying has been studied in a number of works. However, the emulsion electrospraying approach has only recently been proposed for the microencapsulation of functional ingredients (Pérez-Masiá et al., 2015). Thus, in order to ascertain the feasibility of producing electrosprayed microencapsulation structures from the prepared emulsions (i.e. using

SBO as a model oil), these were subjected to hydrodynamic processing (cf. Section 2.6. for process parameters) and the obtained structures are shown in Figure 3.

Figure 3. SEM images of electrosprayed structures obtained from the protein-stabilized SBO/W emulsions prepared using Procedure 1 (left) and Procedure 2 (right). Scale bars correspond to 10 µm.

From the micrographs it was concluded that gelatin was the only protein which yielded proper microparticulate structures when the first emulsification procedure was used. The structure obtained using SPI showed signs of dripping and wetted particles while WPC yielded a mixed structure exhibiting a continuous polymeric surface below some

spherical microcapsules. This can be explained in the light of the properties and droplet size distribution of each emulsion. While gelatin exhibited the smallest droplet sizes using Procedure 1 (cf. Figure 2), SPI emulsion formed large floccules and the size the majority of the droplets in the WPC emulsion was too big to be encapsulated within the generated smaller microcapsules. Although the effective volume of the droplet is significantly increased by the absorbed proteins on their surface (Howe & Pitt, 2008; Malaki Nik et al., 2010), which means that the actual oil volume is smaller than the apparent droplet size, some of the droplets in WPC and the floccules in SPI were still too big for microcapsule formation.

Upon ultrasound treatment, the droplet size was dramatically reduced for the three protein systems, so that all of them could yield neat microcapsules when electrosprayed, even though the particle size also decreased (except for WPC). The size and morphology of these particles varied from one protein to another, and this can be attributed not only to the characteristics of the emulsion droplets but also to the properties of the proteins themselves. WPC dispersions in the absence of added oils or bioactive compounds usually give rise to bigger particles than gelatin or SPI (Gómez-Mascaraque et al., 2015; Pérez-Masiá et al., 2014) for similar concentrations and electrospraying conditions as the ones used in this work.

3.4. Morphology of ALA-loaded electrosprayed and spray-dried capsules

Once the feasibility of the emulsion electrospraying technique had been confirmed for the three proteins, the model SBO was substituted by the bioactive ω -3 fatty acid, ALA. The emulsions were prepared using the second procedure, including the ultrasound treatment, as it proved to be more adequate for the encapsulation of oil droplets in the

previous section, and they were processed both by electrospraying and spray-drying. Emulsions prepared using Procedure 1 were also electrosprayed for comparison purposes. Figure 4 shows the micrographs of the obtained structures and Figure S3 of the Supplementary Material summarizes the particle size distribution for each sample.

Figure 4. SEM images of ALA-loaded electrosprayed and spray-dried structures obtained from the protein-stabilized emulsions. Scale bars correspond to 10 μ m.

Surprisingly, both the emulsions prepared through Procedures 1 and 2 allowed the production of micro- or submicroparticles through electrospraying. These results suggest smaller droplet sizes of the emulsions prepared with ALA in comparison with the ones prepared using the model oil, SBO. In fact, although ALA is water insoluble, it has a polar head in its structure which provides enhanced compatibility through reorganization of the lipid molecules to expose their carboxyl groups to the water interface, fact which can contribute to increased stability of the emulsions and decreased

droplet size. Also, the extent of protein unfolding at the interface is usually larger for more non-polar oils (McClements, 2004), so the flocculation of the emulsions prepared with the globular proteins might have been prevented. As a result, the six emulsions yielded ALA-loaded microencapsulation structures through electrospraying.

In general, the ultrasonicated emulsions led to smaller particle size distributions, with the exception of WPC, where little differences in the particle diameters were observed. This can be attributed to the increase in surface tension and electrical conductivity (Jaworek & Sobczyk, 2008) found after the ultrasound treatment (cf. Section 2.1.3.), and also to their expected smaller droplet sizes. The structures obtained through spray-drying showed bigger mean diameters than the ones obtained through electrospraying, as observed in previous works (Pérez-Masiá et al., 2015).

3.5. Molecular organization

FTIR spectroscopy was used to characterize the molecular organization of the microencapsulation structures. For this purpose, the spectra of emulsion electrosprayed and spray-dried ALA-loaded particles were compared to those of the raw proteins and the free fatty acid. The spectra of the unloaded particles were also obtained.

The IR spectrum of commercial ALA showed its most representative bands centered at 3013 cm^{-1} (stretching of *cis*-alkene groups -HC=CH- in PUFAs) and 1711 cm^{-1} (C=O stretching in fatty acids) (Moomand & Lim, 2014). Other relevant bands of the bioactive compound were found at 2965, 2932 and 2856 cm^{-1} due to the methyl asymmetrical stretching, the methylene asymmetrical stretching and the methylene symmetrical stretching vibrations, respectively (Guillen & Cabo, 1997). On the other hand, the spectra of the three as-received proteins showed the characteristic bands

ascribed to the vibration of the bonds in their amide groups, referred as the Amide A (N-H stretching), Amide B (asymmetric stretching vibration of $=C-H$ and $-NH_3^+$), Amide I (C=O stretching), Amide II (N-H bending and stretching) and Amide III (C-N stretching) bands (Aewsiri, Benjakul, Visessanguan, Wierenga, & Gruppen, 2010; Nagarajan, Benjakul, Prodpran, Songtipya, & Nuthong, 2013). Also noticeable are the bands observed around 2960 cm^{-1} and 2930 cm^{-1} , corresponding to the $-CH_2$ asymmetric and symmetric stretching vibrations, respectively (Nagiah, Madhavi, Anitha, Srinivasan, & Sivagnanam, 2013).

Regarding the microencapsulation structures, differences in their characteristic bands were observed in comparison with the as-received proteins. Table 2 shows a summary of the wavenumbers at which each of these characteristic bands were found, and the complete spectra are provided as supplementary data (cf. Figure S4). Interestingly, ALA-loaded particles produced using different emulsification protocols (i.e. with or without the ultrasonication step) yielded similar infrared spectra, only differing in the intensity of the peak at 3013 cm^{-1} , due to the presence of ALA. Thus, for simplification purposes only the results for the materials obtained following the first emulsification approach are displayed. The spectral data of unloaded electrosprayed particles are also provided.

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Table 2. Characteristic FTIR absorption bands (wavelengths in cm⁻¹) of as-received proteins and microencapsulation structures thereof

	Gel (raw)	Gel ES (unloaded)	ALA- loaded Gel ES	ALA- loaded Gel SD	SPI (raw)	SPI ES (unloaded)	ALA- loaded SPI ES	ALA- loaded SPI SD	WPC (raw)	WPC ES (unloaded)	ALA- loaded WPC ES	ALA- loaded WPC SD
Amide A	3430	3312	3313	3313	3294	3289	3289	3289	3297	3293	3293	3293
Amide B	3085	3080	3080	3080	3075	3075	3075	3075	3078	3078	3077	3079
Amide I	1642	1653	1653	1653	1653	1653	1653	1655	1651	1654	1654	1654
Amide II	1543	1541	1541	1541	1534	1542	1543	1543	1540	1540	1543	1543
Amide III	1244	1244	1244	1244	1240	1251	1250	1250	1262	1249	1248	1248
-CH ₂	2960, 2928	2961, 2939	2961, 2931	2961, 2938	2961, 2928	2956, 2925	2957, 2927	2957, 2927	2963, 2924	2959, 2927	2959, 2927	2958, 2926
ALA	-	-	3013	-	-	-	3012	-	-	-	3013	-

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ES = Electrosprayed; SD = Spray-dried

444 After processing the proteins, both by emulsion electrospraying and spray-drying, a
445 general narrowing of the peaks was observed, which has already been described in
446 previous works (Gómez-Mascaraque et al., 2015; López-Rubio & Lagaron, 2012).
447 Moreover, the Amide A band shifted to significantly lower wavenumbers for the three
448 proteins, indicating changes in the hydrogen bonding structure of the proteins (Doyle,
449 Bendit, & Blout, 1975). These changes have been previously attributed to the removal
450 of the structural water during the rapid drying process in the formation of electrosprayed
451 gelatin particles (Gómez-Mascaraque et al., 2015), and this hypothesis could be
452 extended to the other proteins as inferred from the TGA analysis (see below). The
453 Amide B band only experienced a displacement towards lower wavenumbers for
454 gelatin, not being significantly affected upon processing of SPI or WPC. This is
455 attributed to the processing conditions of gelatin in diluted acetic acid and subsequent
456 protonation of its amino groups, being this band partially due to the vibration of $-\text{NH}_3^+$
457 groups. Gelatin also showed the most significant changes in the displacement of the
458 Amide I band upon processing, which is attributed to changes in the secondary structure
459 of proteins (Ebrahimgol, Tavanai, Alihosseini, & Khayamian, 2014). Although the
460 denaturation of SPI prior to processing did not lead to displacements in the Amide I
461 band, which only narrowed, it did have an impact on the Amide II band, which is also
462 conformationally sensitive (Aceituno-Medina, Mendoza, Lagaron, & López-Rubio,
463 2013; Long et al., 2015)), producing a considerable shift towards higher wavenumbers,
464 as previously observed (Pérez-Masiá et al., 2014). The Amide III band also experienced
465 changes in its shape for both WPC and SPI, but not for gelatin, although the complex
466 mixture of globular proteins present in these samples limits the interpretation of these
467 results. Finally, the relative intensities of the bands corresponding to the asymmetric
468 and symmetric stretching vibrations of methylene groups and their peak maxima

changed upon processing of the proteins, especially for SPI and WPC (which contained the surfactant). It is worth noting that these general comments are valid for both electrosprayed and spray-dried capsules, which exhibited little differences between them in terms of the bands ascribed to the proteins.

The presence of the ω -3 fatty acid in the electrosprayed encapsulation microstructures was evidenced by the existence of its characteristic absorption band at 3013 cm^{-1} in these samples. However, this peak was not detected for any of the spray-dried materials, suggesting that the bioactive compound was completely degraded during processing through the latter technique, due to the high temperatures required for the production of the encapsulation structures in this case. As the aforementioned band is related to the presence of *cis*-alkene groups in the samples, its disappearance implies that these double bonds were no longer present in the samples, i.e. they had been oxidized. Therefore, regardless of the protein used as wall material, electrospraying proved to be an effective technique for the microencapsulation of the thermosensitive bioactive while spray-drying resulted in complete ALA degradation. Other works had reported the successful encapsulation of ALA-rich oils, such as linseed oil (Gallardo et al., 2013) or chia oil (Rodea-González et al., 2012), within proteins and polyssacharides through spray-drying. However, pure ALA (>99%) showed extreme sensitivity to heat and thus it could not be detected in any of the spray-dried samples. Therefore, only the electrosprayed capsules will be considered in the following sections.

3.6. Microencapsulation efficiency

The band at 3013 cm^{-1} was also used to estimate the microencapsulation efficiency (MEE) for the electrosprayed materials, as it did not overlap with any of the bands of

the proteins and it was a good indicator for the integrity of the bioactive compound. Hence, the MEE estimated in this work is based on the presence of intact double bonds in the bioactive fatty acid rather than the mere content of oil (oxidized or not) which is measured in other works by gravimetric techniques (Jiménez-Martín, Gharsallaoui, Pérez-Palacios, Carrascal, & Rojas, 2014; Rodea-González et al., 2012; Wang, Adhikari, & Barrow, 2014). Calibration curves were constructed for each ALA-protein system ($R^2_{\text{Gel}} = 0.999$, $R^2_{\text{SPI}} = 0.993$ and $R^2_{\text{WPC}} = 0.986$) using physical mixtures of the unloaded electrosprayed proteins with known relative concentrations of the fatty acid, and using the Amide II band of each protein as a reference. The MEE of the ALA-loaded particles was then calculated using Eq. (2) and the results are summarized in Table 3.

Table 3. Microencapsulation efficiencies for the electrosprayed materials. Different letters (a-e) within the same column indicate significant differences at $p < 0.05$ among the samples

Electrosprayed sample		
Protein	Emulsion procedure	MEE (%)
Gel	HSH	$40 \pm 4^{a,c}$
Gel	HSH+US	23 ± 12^a
SPI	HSH	61 ± 4^b
SPI	HSH+US	30 ± 3^a
WPC	HSH	67 ± 5^b
WPC	HSH+US	$50 \pm 3^{b,c}$

HSH = High-speed homogeneization; US = Ultrasonication

In general, the estimated MEE was considerably higher when no ultrasound was used for emulsion preparation. This was directly related to the heating of the emulsions during the ultrasonication treatment, which must have partially degraded the thermosensitive fatty acid. In fact, despite the short duration (i.e. 30 s) of the treatment intervals and the use of an ice bath to cool down the samples, the temperature of the

emulsions after de ultrasonication treatment raised up to 45 °C. Hence, although the second emulsification approach proved to be useful to decrease de droplet size of the emulsions and increase their stability, it caused partial oxidation of the bioactive oil before encapsulation.

Concerning the MEE of the different proteins, gelatin yielded the lowest values regardless of the emulsification protocol employed. This was attributed to the low pH of the gelatin solution prepared in diluted acetic acid, which might have contributed to the greater degradation of the fatty acid before and during processing. Recent studies also found greater extents of lipid oxidation in ALA-containing SBO at acidic pH than at neutral pH (Kapchie, Yao, Hauck, Wang, & Murphy, 2013), supporting this hypothesis.

The best results were achieved when either SPI or WPC were used as wall matrices and the first and simplest emulsification approach was employed, both samples belonging to the same statistical group (cf. Table 3). Remarkably, those two emulsions were the most unstable when SBO was used as a model oil, even leading to bridging flocculation. However, the differences in the model oil and the bioactive oil structures might have led to improved stability of the latter, as commented above.

3.7. Thermogravimetric analysis of the materials

Thermogravimetric analysis of the raw proteins and the free ALA, as well as the unloaded and ALA-loaded electrosprayed particles, was conducted in oxidative conditions at 10°C/min in order to assess potential thermostability changes of the bioactive ingredient upon microencapsulation. A physical mixture of the unloaded electrosprayed materials and ALA (10% w/w) was also analysed. Figure 5 shows the obtained DTG curves.

Figure 5. DTG curves of raw ALA, as-received proteins, e-sprayed particles and their mixtures for gelatin (a), SPI (b) and WPC (c).

The main degradation stage (major weight loss) for free ALA had its temperature of maximum degradation rate ($T_{\max}$) at 228 °C. In contrast, the main degradation stage for the three proteins took place in the range of 250-400°C for all the materials. Thus, the degradation of both components could be distinguished in the physical mixtures. For instance, a small peak at $T_{\max}$ =219 °C was observed for SPI and a small shoulder at $T_{\max}$ =215 °C for WPC, which were both attributed to the presence of ALA, as they were not present in the unloaded electrosprayed proteins alone. The decrease in the thermal stability of ALA in these mixtures could be attributed to its increased exposed surface when physically absorbed on the proteins. For gelatin, its physical mixture with the fatty acid exhibited a small weight loss well below the $T_{\max}$ of the free bioactive, which was absent in the neat electrosprayed gelatin, and hence was similarly attributed to the degradation of free ALA absorbed on the gelatin surface. The exceptional decrease in stability in this case could be motivated by the presence of residual acetic acid in the capsules.

The DTG curves of the ALA-loaded encapsulation structures showed similar degradation profiles to those of the unloaded particles. Both exhibited slight changes in the degradation profile of the main stage when compared to the as-received proteins. As previously reported for gelatin (Gómez-Mascaraque et al., 2015), the $T_{\max}$ of the main degradation stage of the three protein systems increased upon electrospraying, although the onset temperature decreased (i.e. the degradation started at lower temperatures). These changes have been attributed to the structural changes caused by the electrospraying process and the reduction of the particle size, which results in an increase of the specific area and, consequently, of their susceptibility to thermal degradation (Gómez-Mascaraque et al., 2015). Regardless, the weight losses attributed to ALA in the mixtures were not found in the ALA-containing encapsulation structures,

suggesting an increase in the thermal stability of the bioactive fatty acid upon encapsulation, as its degradation was delayed until its protective matrices themselves degraded.

Another interesting feature, also observed in previous works dealing with electrospraying of proteins (Gómez-Mascaraque et al., 2015), is that their fast drying triggered the removal of their structural water, so that only losses of absorbed water were afterwards detected in the processed materials (up to 100°C), while the as-received proteins prolonged the weight loss attributed to solvent (water) evaporation up to around 125°C in SPI and WPC and even close to 200°C for gelatin. This would have an impact on the structural changes detected by infrared spectroscopy.

3.8. Accelerated oxidation/degradation assays

The protective effect of the different emulsion electrosprayed encapsulation structures on the oxidative stability of ALA was assessed through an accelerated degradation assay at 80°C. For this purpose, the relative intensity of the infrared band attributed to the presence of alkene groups (3012-3013 cm^{-1}) was measured after different time periods at this temperature. The decrease in the relative absorbance of this band was related to the extent of ALA degradation (Torres-Giner et al., 2010). Results were normalized to the initial ALA content in the capsules for a better comparison of the different matrices, and they are shown in Figure 6.

Figure 6. Degradation profiles at 80°C for free and encapsulated ALA. Different letters (a-c) within the same time period indicate different statistical groups with significant differences among them at $p < 0.05$

The peak of interest was not detected in any of the gelatin samples after only 3 hours of thermal treatment, emphasizing that not only this matrix did not protect ALA from thermal oxidation, but it also accelerated its degradation, most probably due to the presence of residual acetic acid in the capsules.

Regarding the globular proteins, the degradation of ALA within the WPC matrices did not show significant differences with that of free ALA during the first 3 h, although the stability of the encapsulated fatty acid was significantly improved during the following days. This fast, initial degradation could be attributed to the fraction of the bioactive allocated on the surface or very close to the surface of the particles.

A similar degradation profile was found for ALA-loaded SPI capsules. However, in this case during the first hours of high temperature exposure, a greater extent of degradation was observed in comparison with that of free ALA. This might be attributed to the increased specific area of the ALA domains located on the surface of the particles. Compared to free ALA in bulk, whose specific area exposed to air was low, the

encapsulated fatty acid was fragmented into very small domains (droplets) by emulsification prior to electrospraying, greatly increasing its specific area. Thus, if a fraction of this oil remained on the surface of the particles, it would be more exposed to the environment. This statement would also be applicable to WPC, meaning that the fraction of oil on the surface of the protein particles would be lower for the WPC than for SPI. This difference could be attributed, among other factors, to the bigger particle sizes of the WPC capsules, which thus had smaller specific surface area. After the first three hours, the degradation of encapsulated ALA, both within SPI and WPC particles, was significantly delayed with respect to free ALA, highlighting the effective protection of these electrosprayed matrices against oxidation at high temperatures.

While the protection exerted by the WPC capsules was enhanced compared to the SPI particles during the first hours of treatment, no significant differences were observed among the samples after 2 days. Furthermore, the procedure used for the preparation of the emulsions had little effect on the degradation profiles, despite its impact on the encapsulation efficiency. Only WPC showed a significant difference between both methodologies after the first 27 hours of treatment, most probably due to the bigger droplet sizes of the non-ultrasonicated emulsion, which led to a bigger fraction of non-encapsulated or superficial oil, taking into account that the particle size of the capsules was very similar for both approaches.

4. Conclusions

A novel emulsion electrospraying technique has been used to develop protein-based microencapsulation structures for the protection of ALA (used as a model thermosensitive hydrophobic bioactive compound) and compared with a well-

established technology used in the food industry such as spray-drying. Being ALA a thermosensitive compound, spray-drying was inappropriate for this purpose, completely degrading the ω -3 fatty acid. As hypothesised, the electrospraying technique proved to be a satisfactory alternative, achieving microencapsulation efficiencies of up to 67% $\pm$ 5%. It was also found that the low pH required for processing gelatin through electrospraying resulted in quick degradation of the encapsulated bioactive, while the ultrasound treatment for emulsion preparation also decreased the MEE due to heating. Thus, the best results were achieved using the globular proteins (WPC and SPI) and the simple homogenization procedure for the preparation of the emulsions, significantly delaying ALA oxidation during accelerated degradation assays at 80°C. The overall results of the present work demonstrate the potential of electrospraying of protein-stabilized emulsions for the microencapsulation and enhanced protection of thermosensitive and hydrophobic bioactive ingredients, specifically ω -3 fatty acids, offering an improved alternative to traditional technologies used in the food industry such as spray-drying, which gives rise to oxidative degradation and does not significantly protect ω -3 fatty acids (Kolanowski, Ziolkowski, Weißbrodt, Kunz, & Laufenberg, 2006). Further research will be needed to extend the applicability of these results to a wider range of hydrophobic bioactive ingredients and wall materials.

Acknowledgements

Laura G. Gómez-Mascaraque is recipient of a predoctoral contract from the Spanish Ministry of Economy and Competitiveness (MINECO), Call 2013. The authors would like to thank the Spanish MINECO project AGL2012-30647 for financial support. Sara Díaz Cuesta is also acknowledged for experimental support. Authors would also like to

thank the Central Support Service for Experimental Research (SCSIE) of the University of Valencia for the electronic microscopy service.

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GRAPHICAL ABSTRACT