Title: Omega–3 encapsulation by PGSS-drying and conventional drying methods. Particle characterization and oxidative stability

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Abstract

Particles from Gas-Saturated Solutions (PGSS)-drying has been used as a green alternative to encapsulate omega-3 polyunsaturated fatty acids (n-3 PUFAs) at mild, non-oxidative conditions. PGSS-dried particles have been compared to those obtained by conventional drying methods such as spray-drying and freeze-drying, finding encapsulation efficiencies (EE) up to 98% and spherical morphology for PGSS- and spray-dried particles. Freeze-dried powders showed irregular morphology and EE from 95.8 to 98.6%, depending on the freezing method. Differential scanning calorimetry (DSC) analysis revealed glass-transition and melting peaks of OSA-starch and a cold-crystallization peak corresponding to the encapsulated n-3 PUFA concentrate. Compared to conventionally dried powders, PGSS-dried microparticles showed lower primary and secondary oxidation after 28 days of storage at 4 ºC. Ascorbic acid addition combined with the mild processing conditions of PGSS-drying yielded particles with a maximum peroxide value of 2.5 meq O₂/kg oil after 28 days of storage at 4 ºC.
1. Introduction

An adequate intake of omega–3 polyunsaturated fatty acids (n–3 PUFAs) is recommended in healthy diet guidelines due to their important benefits (Ruxton, Reed, Simpson, & Millington, 2004). Long-chain n–3 PUFAs, mainly eicosapentaenoic (EPA, 20:5 n–3) and docosahexaenoic (DHA, 22:6 n–3) acids are eicosanoid precursors, which are immunomodulatory molecules with a key role in the inflammatory response. EPA and DHA are claimed to contribute to the normal brain, eye and cardiovascular functions in adults and help in the normal development of the eyes, the brain and the nervous system in children (EFSA, 2010).

The perceived health benefits of these compounds have created a strong demand for EPA and DHA concentrates in the pharmaceutical and food industries. However, n–3 PUFAs are unstable and very prone to oxidation, easily generating lipid hydroperoxides and free radicals under oxidative conditions. These species negatively affect sensory properties, since they can decompose into low-molecular-weight volatile compounds that are perceived as rancid, and what is more, they present potentially cytotoxic, carcinogenic and mutagenic effects (Niki, 2009; Uluata, McClements, & Decker, 2015).

For these reasons, n–3 PUFA concentrates are often encapsulated in order to protect them from light and oxygen during shelf life; and natural antioxidants such as tocopherols, phospholipids, ascorbic acid, or their mixtures are usually added (Baik et al., 2004; Lölliger & Saucy, 1994).

Materials of different nature can be used as n–3 PUFA encapsulating agents: proteins such as whey protein isolate, sodium caseinate or gelatin, phospholipids such as lecithin, or polysaccharides such as gum Arabic, carboxymethyl cellulose, maltodextrin, chitosan, or modified starch are some examples of carrier materials for microencapsulation of oils rich in n–3 PUFAs (Encina, Vergara, Giménez, Oyarzún-
Among them, $n$-octenyl-succinic-anhydride modified starch (OSA-starch) has been chosen in this work because it presents good emulsifying properties and is suitable to encapsulate oils rich in $n$–3 PUFAs, as well as other bioactive compounds such as essential oils and hydrophobic compounds (Carneiro, Tonon, Grosso, & Hubinger, 2013; de Paz, Martín, Bartolomé, Largo, & Cocero, 2014; Drusch, Serfert, Scampicchio, Schmidt-Hansberg, & Schwarz, 2007; Jafari, Assadpoor, He, & Bhandari, 2008; Varona, Martin, & Cocero, 2011).

Different encapsulation techniques can be used to encapsulate $n$–3 PUFAs, such as emulsification, spray-drying, freeze-drying, coacervation, in situ polymerization, extrusion, or fluidized-bed coating (Bakry et al., 2016). Among these, the most widely used technique in the food and pharmaceutical industries is spray-drying, followed by freeze-drying. Freeze-drying is often applied to thermolabile and easily oxidizable compounds due to the protective low temperatures and vacuum conditions involved in the process. Its main drawback is the energy consumption, linked to the low temperature and high vacuum conditions as well as the long residence times required to completely dry the product, which in turn translate into high processing costs. On the contrary, spray-drying is a low-cost microencapsulation technology which operates in a relatively simple and continuous way, thus it is commonly used at industrial scale (Bakry et al., 2016).

Prior to the drying step, the non-soluble $n$–3 PUFAs need to be dispersed into the encapsulating agent solution, obtaining an oil-in-water (O/W) emulsion. Several methods can be used to prepare O/W emulsions, such as conventional emulsification (colloid milling, high speed blending and high-pressure homogenization), ultrasound (US) assisted emulsification, membrane emulsification, and micro-channel emulsification (Chatterjee & Judeh, 2015). Among them, US-assisted emulsification has
grown in importance among the pharmaceutical, cosmetic, and food industries, thanks to its versatility and the possibility of obtaining high quality food products with enhanced functional properties (Abbas, Hayat, Karangwa, Bashari, & Zhang, 2013). US-assisted emulsification can be applied to improve stability and bioavailability of the dispersed bioactive compounds and, in particular, it can be used to obtain O/W emulsions with nanometric droplet size and narrow size distribution. Typically, US-assisted emulsification consists on applying low-frequency sound waves of 20-100 kHz through a metallic sonotrode immersed in the liquid medium, in order to generate disruptive forces that break down the macroscopic phases to nanosize droplets. The nano-scale emulsions obtained present interesting functional properties and enhanced stability against oxidation (Abbas et al., 2013).

Supercritical fluids, and particularly supercritical carbon dioxide (SC-CO₂), are a convenient medium to produce particles loaded with bioactive compounds. Carbon dioxide is an inert, non-toxic solvent, and is completely released from the product as a gas once back to atmospheric conditions. Besides, the accessibility of the supercritical state of carbon dioxide (Tₐ = 31.1 °C; pₐ = 73.8 bar) and its advantageous physical properties (high density and diffusivity, and low viscosity) make SC-CO₂ the solvent of choice in many particle formation processes. (Türk, 2014). Among the several available techniques, the Particles from Gas Saturated Solutions (PGSS) process overcomes the problems of solubility limitations and high gas consumption of other particle formation methods using SC-CO₂ (Türk, 2014). This technique can be used for drying aqueous solutions, dispersions or, as in this work, O/W emulsions, in the so-called PGSS-drying process (Türk, 2014).

Basically, the PGSS-drying technique consists on mixing an aqueous solution with supercritical carbon dioxide upon saturation, and subsequently expanding the gas-
saturated solution down to atmospheric pressure through a nozzle. This technique can be used as an alternative to conventional spray-drying, achieving a more efficient atomization due to the sudden vaporization of the dissolved CO₂ and the expansion of gas bubbles in the solution during depressurization from supercritical to atmospheric conditions. Both effects improve the atomization of the sprayed solution forming small droplets, thus reducing the particle size of the dried powder and enhancing the drying process (Martín & Weidner, 2010; Weidner, 2009). Besides, and because of the intense and deep cooling caused by the Joule-Thomson effect, it is possible to dry the product at low temperature (40-80 °C) (de Paz, Martín, & Cocero, 2012; Weidner, 2009). The mild-temperature conditions, combined with the intrinsically inert atmosphere due to oxygen displacement, prevent, or at least delay, oxidative degradation of the encapsulated bioactive compounds (de Paz et al., 2012; Weidner, 2009). Operating conditions in the spray tower, particularly temperature and gas-to-product ratio (GPR), must be taken into account in order to operate above the dew line of the carbon dioxide–water system (Martín & Weidner, 2010), and ensure the complete drying of particles.

In this work, an n–3 PUFA enriched fish oil has been encapsulated by the alternative and green technology Particles from Gas Saturated Solutions (PGSS)-drying. The main hypothesis of the study is to explore whether or not the potential benefits of supercritical carbon dioxide technologies applied to particle formulation and encapsulation may affect particle properties and oxidative stability of heat-sensitive and easily oxidizable compounds such as n–3 PUFAs, compared to other conventional drying methods. This way, the PGSS-dried particles have been compared to those obtained by spray-drying and freeze-drying, which are commonly applied in the pharmaceutical, cosmetic, and food industries to dry aqueous solutions and dispersions. Characterization of the particles obtained by the different drying methods has been
performed in terms of particle morphology, residual humidity, and particle size
distribution of the reconstituted particles. Besides, encapsulation efficiency and
oxidative stability (primary and secondary oxidation) of the encapsulated n–3 PUFA
concentrate have been monitored over time in the particles formulated with each of the
drying methods. Additionally, an antioxidant (ascorbic acid) has been added to some of
the formulations as a strategy to potentially enhance the oxidative stability of the
encapsulated n–3 PUFA concentrate.

2. Materials and methods

2.1. Materials

n–3 PUFA concentrate from fish oil, Algatrium™ Plus, was kindly donated by Brudy
Technology S.L. (Spain). It has been stored at 4 °C in darkness and N₂ atmosphere. Hi-
Cap™ 100, an octenyl-succinic-anhydride modified starch (OSA-starch) derived from
waxy maize, was provided by Ingredion Inc. (Germany). Carbon dioxide (99.9%) was
provided by Air Liquide S.A. (Spain). Ascorbic acid (L(+)-Ascorbic acid, AA) was
purchased from Panreac AppliChem (Spain).

37% hydrochloric acid (HCl), diethyl ether, 1-butanol, 2-propanol, methanol, 2-
thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were provided by VWR
Chemicals (Germany). Hexane, absolute ethanol, Iron(II) sulphate heptahydrate, and
ammonium thiocyanate were purchased from Merck KGaA (Germany). 2,2,4-
trimethylpentane (isooctane) and barium chloride dihydrate were supplied by Macron
Fine Chemicals (France) and Panreac AppliChem (Spain), respectively. Cumene
hydroperoxide and 1,1,3,3-tetraethoxypropane (TEP) standards were purchased from
Sigma Aldrich (USA).
2.2. Characterization of the \(n\)-3 PUFA concentrate

Neutral lipid profile of the \(n\)-3 PUFA concentrate has been analyzed by normal-phase HPLC (NP-HPLC). Separation was carried out at room temperature in a Lichrospher Diol column (5 mm, 4 mm×250 mm) and detection was performed by evaporative light scattering (ELSD) (Agilent Technologies 1200 Series, USA) at 35 °C and 3.5 bar. Solvent gradient and calibration procedure have been reported elsewhere (Solaesa, Sanz, Falkeborg, Beltrán, & Guo, 2016).

Fatty acid profile of the \(n\)-3 PUFA concentrate has been determined according to the AOAC Official Method (AOAC International, 2012) in a Hewlett Packard gas chromatograph (6890N Network GC System) equipped with an auto-sampler (7683B series) and a flame ionization detector (FID). The separation was carried out in a fused silica capillary column (Omegawax-320, 30 m×0.32 mm i.d.) with helium (1.8 mL/min) as carrier gas. Injection and detection temperatures, as well as ramp conditions have been previously reported (Rebolleda, Rubio, Beltrán, Sanz, & González-San José, 2012). Most of the fatty acids were identified by comparison of their retention times with those of chromatographic standards (Sigma Aldrich). As indicated by the AOAC Official Method (AOAC International, 2012), an internal standard (methyl-tricosanoate, C23:0) was used for quantification purposes.

HPLC with diode array detection (HPLC-DAD) of the \(n\)-3 PUFA concentrate was carried out in order to detect tocopherol isomeric forms and other vitamin E analogs added to the \(n\)-3 PUFA concentrate, as their presence was reported by the provider. The analytical method is based on the IUPAC official method (Pocklington & Dieffenbacher, 1988) with slight modifications, as reported in Rebolleda et al., (2012).
Separation was performed in an ACE 5 silica 250 mm × 4.6 mm column with 1 mL/min of hexane:2-propanol (99:1) as the mobile phase. An isocratic gradient was used, and the total run time was 15 min. α-, β-, γ-, and δ-tocopherols were monitored at λ = 296 nm. For identification and quantification of each tocopherol isomer, a calibration curve with different amounts of the respective standard compound (Sigma Aldrich) was constructed.

2.3. Ultrasound-assisted emulsification

O/W emulsions were formulated in a weigh proportion of 70:24:6 (water:carrier:ω–3 PUFA concentrate), which in preliminary experiments was found to be the optimal in terms of obtaining the smallest droplet size. First, an aqueous solution of the encapsulating agent was prepared by dissolving 24.0 g of Hi-Cap™ 100 in 70.0 mL of distilled water. Subsequently, 6.0 g of ω–3 PUFA concentrate were added drop by drop to the carrier solution under continuous stirring. Then, the mixture was stirred for 5 minutes to obtain a pre-emulsion, which was subsequently processed in a 20 kHz 750 W ultrasonic liquid processor Vibra-Cell 75043 (Sonics & Materials Inc.) with a Ø13 mm titanium alloy sonotrode. Based on previous studies, amplitude was set at 100 % and sound waves were delivered in pulses (5 s On/5 s Off) in order to avoid excessive heating of the sample, for a total processing time of 180 s. O/W emulsions were produced in batches of 100 g.

2.4. PGSS-drying

O/W emulsions were processed using the PGSS-drying technique in order to remove water and obtain a solid powder with the encapsulated ω–3 PUFA concentrate loaded
into the OSA-starch microparticles. Fig. 1 presents the schematic flow diagram of the
PGSS-drying apparatus, in which CO$_2$ was fed by a membrane pump (LEWA) and
preheated using a silicone bath before injection into the static mixer, where it was mixed
with the O/W emulsion at the selected pressure and temperature. The CO$_2$ mass flow
rate was measured with a Coriolis flow meter (Danfoss) with an accuracy of ± 0.1 kg
CO$_2$/h. Temperature before and after the static mixer was measured by means of Pt100
thermoresistances (accuracy of ± 0.1 K), being the later under PID control. Pressure in
the CO$_2$ line and after the static mixer was measured with pressure transmitters (DESIN
Instruments) with an accuracy of ± 0.05 MPa. Bourdon manometers (Nuova Firma)
were installed to provide secondary lectures of the operating pressure.

The O/W emulsion was pumped into the static mixer by a GILSON 305 piston pump
(max. flow rate: 25 ± 0.1 mL/min). The gas-saturated emulsion was then expanded into
the spraying tower through a capillary nozzle with an internal diameter of 400 µm
(Spraying Systems Co., Ref.: PF1650-SS). The spraying tower was made of PVC and
heated by electrical resistances. Temperature in the spray tower was also measured with
a Pt100 probe and controlled using a PID. CO$_2$ was vented off the spraying tower and
passed through a water vapor condenser before final release. As security elements, a
rupture disk, check valves, and a relief valve were installed at different points in the
high-pressure circuit.

Typically, a PGSS-drying experiment began with the preheating of the system up to the
desired temperature in the static mixer, fixed at 110 °C, and in the spraying tower,
which was set at 55 °C. When temperature was achieved, CO$_2$ was pumped up to the
desired pressure, which was fixed at 10.0 MPa. Pressure in the static mixer and
temperatures in the static mixer and the spraying tower were selected based on previous
studies (Varona et al., 2011). Once temperature and pressure conditions were stable, the
emulsion pump was started at a flow rate such that the desired GPR, which was selected at 30 g/g, was obtained. After all the O/W emulsion was processed, CO2 was allowed to flow through the system at the same pressure and temperature conditions during 15 minutes in order to completely dry the particles. After that, the system was depressurized and particles were collected from the walls and bottom of the spraying tower and stored in darkness and refrigeration at 4 ºC for subsequent analyses.

2.5. Spray-drying

Spray-drying is a conventional, well-known drying technique which is widely used in the pharmaceutical, cosmetic and food industries; thus, it was chosen to compare the characteristics of the powder that may be obtained conventionally to those of the powder obtained by the alternative PGSS-drying process. The spray-drying process was carried out in a commercial Buchi B-290 mini Spray-dryer. The O/W emulsion, obtained as described in section 2.3, was fed into the spray-drying apparatus at an inlet temperature of 155 ºC, and %pump of 8 %, which was equivalent to a mass flow of emulsion of 3.0 g/min. Outlet temperature was 100 ºC. The emulsion was sprayed through a nozzle with 1.5 mm diameter and dried under a N2 flow of 360 L/h.

2.6. Freeze-drying

O/W emulsions obtained by the US-assisted method described in section 2.3 were submitted to two different freezing methods: (1) conventional at -20 ºC overnight, and (2) freezing with liquid nitrogen (-196 ºC). Samples were then equilibrated at -80 ºC for 2 h and submitted to freeze-drying in a Labconco Freeze Dry System at 1.5·10^{-4} mbar during 48 h. These two different freezing methods were chosen in order to evaluate the
effect of the freezing step, since the slower conventional freezing process is more likely
to form large crystals of water, which could adversely affect the emulsion stability and
structure, whereas the rapid freezing achieved with liquid nitrogen could better preserve
the physical structure of the emulsion.

2.7. Characterization of the O/W emulsion

2.7.1. Droplet size analysis of the O/W emulsions

The droplet size distribution of the O/W emulsions (original and reconstituted) was
measured by a Laser Diffraction (LD) equipment (Malvern Mastersizer 2000). A small
amount of sample was suspended in the suspension container filled with distilled water
under gentle agitation. In the case of the reconstituted O/W emulsions, the dried
powders were firstly dissolved in distilled water, maintaining the original ratio of

Droplet size measurements are reported as relative volume distribution and defined by
the mean diameter over volume (DeBroukere mean, D[4,3]) and the volume/surface
mean diameter (Sauter mean, D[3,2]), calculated as in Eqs. 1 and 2, respectively.

\[ D[4,3] = \frac{\sum D^4_{i,n}}{\sum D^3_{i,n}} \]  
\[ D[3,2] = \frac{\sum D^3_{i,n}}{\sum D^2_{i,n}} \]  

where \( D_i \) is the diameter of the \( i \)th particle.
The median particle size ($d_{0.5}$), defined as the maximum particle diameter below which 50% of the sample volume exists, is also reported. The span value, defined in Eq. 3, was also calculated.

$$\text{span} = \frac{d_{0.9} - d_{0.1}}{d_{0.5}}$$  \hspace{1cm} (3)

where $d_{x}$ is the maximum particle diameter below which $x\%$ of the sample volume exists. Span values near to 1 indicate a narrow particle size distribution (PSD).

2.7.2. **Emulsion stability**

Physical stability of the O/W emulsion was analyzed by static multiple scattering in a vertical scan analyzer Turbiscan Lab Expert (Formulaction Inc.) with ageing station AGS. By means of two optical sensors, the instrument measures the light transmitted through the emulsion (180° from the incident light, transmission, T) and the light backscattered by the emulsion droplets (45° from the incident light, backscattering, BS). The scanning process is made vertically along the glass cell from bottom to top, and the T/BS are each plotted as a function of the emulsion height in the glass cell. By monitoring the T/BS profiles at different time intervals, physical changes in the emulsion can be followed over time, which gives a detailed overview of dispersion stability or instability. In the current work, the stability of the original emulsion was monitored at 4 h intervals during 24 days. Emulsion samples were kept in the ageing station at a constant temperature of 25 °C. As variations in T profiles were lower than 2%, only BS profiles at different storage times were analyzed in this study.
2.7.3. Density of the O/W emulsions

Density of the O/W emulsions was measured in an Anton Paar DMA 5000 instrument at 25 ºC. Measurements were carried out in triplicate.

2.8. Characterization of the dried powders

2.8.1. Yield, moisture, encapsulation efficiency and bioactive loading

Yield of particles was calculated as the ratio between the mass of collected particles \( (m_{\text{collected \ particles}}) \) and the theoretical mass fed to the PGSS-drying, spray-drying, or freeze-drying apparatus \( m_{\text{initial \ feed}} \), expressed as weight percentage (Eq. 4).

\[
\text{Yield (\%)} = \left( \frac{m_{\text{collected \ particles}}}{m_{\text{initial \ feed}}} \right) \cdot 100
\] (4)

Moisture content of the dried particles was determined gravimetrically. Samples (ca. 0.5 g) of particles obtained by the different methods used in this work were weighed before and after drying in an oven at 120 ºC until constant weight.

Encapsulation efficiency (EE) was determined according to the method described by Wang et al. (Y. Wang, Liu, Dong, & Selomulya, 2016) with some modifications. For the non-encapsulated oil determination, samples (ca. 1.0 g) of particles obtained by the different methods used in this work were suspended with 25 mL of hexane in a Falcon centrifuge tube, which was vortexed for 15 s at room temperature and centrifuged at 3000 rpm during 20 min. Immediately afterwards, the supernatant was taken and filtered, and its oil content was measured spectrophotometrically at \( \lambda = 286 \) nm. The same procedure was repeated two additional times to extract the potentially remaining non-encapsulated oil. A calibration curve was previously constructed with known quantities of \( n-3 \) PUFA concentrate dissolved in hexane.
Total oil in the dried particles obtained by the different methods used in this work was
determined by acid digestion of approximately 1.0 g of powder with 37% HCl, and
subsequent extraction with diethyl ether and petroleum ether, following the AOAC
20 min, the solvent phase with the extracted oil was taken and transferred to tared
round-bottom flasks in order to evaporate the solvent under vacuum (Heidolph rotary
evaporator). Total oil in the samples was determined by mass difference of the initial
clean round-bottom flask and that containing the extracted oil residue. As a blank, the
same procedure was also followed with known quantities (ca. 1.0 g) of the carrier alone
(Hi-Cap™ 100). The fat traces found in the carrier were subtracted from the total oil
content of the powders.

Encapsulation efficiency (EE) was calculated from Eq. 5.

\[ \text{EE} \% = \frac{\text{TO} - \text{nEO}}{\text{TO}} \times 100 \]  (5)

where TO is the total oil content and nEO is the non-encapsulated oil.

The bioactive loading, which is also an important parameter of microencapsulated
bioactive compounds (Encina et al., 2016), has been also calculated. It can be referred
as to the total oil content (TO), expressed as mg oil/g sample.

2.8.2. Particle size analysis of the dried powders

Particle size analysis of the dried powders was carried out in a Malvern Mastersizer
2000 equipment, following the same procedure as in the original O/W emulsion (see
section 2.7.1), yet dispersing the particles in absolute ethanol to avoid dissolution of the
encapsulating agent.
2.8.3. Scanning electron microscopy (SEM)

Morphology of the dried particles was observed in a Scanning Electron Detector microscope JEOL JSM-6460LV with Energy Dispersive X-ray (JEOL Ltd. Japan) operating at 20 kV. Samples were gold-sputtered and observed with magnifications of 1500, 5000 and 10000x for PGSS- and spray-dried particles, and 50, 400 and 2000 or 3000x for the freeze-dried powders.

2.8.4. Differential scanning calorimetry (DSC)

A TA Instruments Q200 differential scanning calorimeter with refrigerated cooling system (RCS90) and nitrogen purge gas was used. Melting point and enthalpies of indium were used for temperature and heat capacity calibration. Samples (ca. 10 mg) were placed in TA Tzero 40-μL aluminum pans and closed with hermetic aluminum lids with a pinhole. An empty pan closed with pinholed lid was used as a reference. Starting temperature of the DSC analysis was set at 40 °C, and held for 30 min. Then, the system was cooled down to -80°C at 10°C·min⁻¹. After an isothermal period of 30 min, samples were heated from -80 °C to 350 °C at a constant heating rate of 10°C·min⁻¹. DSC thermograms were recorded and analyzed with the Advantage v. 5.5.20 software (TA Instruments).

2.9. Measurement of lipid oxidation

Oxidative status of the dried powders was determined in terms of primary oxidation (peroxide value, PV) and secondary oxidation (Thiobarbituric Acid Reactive Substances, TBARS). To observe the effect of each drying method, PV and TBARS
were determined in the \( n \)-3 PUFA concentrate, as well as in the O/W emulsions before drying.

For the dried powders, PV and TBARS were measured right after each drying method (PGSS-drying, spray-drying, and freeze-drying) and monitored over a 28-day storage period. Dried powders were placed in closed containers and stored at 4 °C in darkness. Samples were withdrawn at 7-day intervals and dissolved in distilled water to obtain reconstituted emulsions with the original water:carrier:\( n \)-3 PUFA concentrate proportion (70:24:6 wt.). PV and TBARS analyses were carried out as described below.

### 2.9.1. Peroxide Value

PV was measured spectrophotometrically with a Hitachi U-2000 apparatus and following the method described by Shanta et al. (Shantha & Decker, 1994) with slight modifications. In brief, 10-50 mg of oil or 0.025-1.0 mL of emulsion, depending on the expected PV, were taken in a centrifuge tube and mixed with 1.5 mL of isooctane:2-propanol (3:1 v/v). The tube was vortexed for 15 s and centrifuged at 5000 rpm during 10 min. Immediately afterwards, 0.2 mL of the supernatant were transferred to a new centrifuge tube and 2.8 mL of methanol:1-butanol (2:1 v/v) were added. After vortexing for 15 s, 15 \( \mu \)L of 3.94 M ammonium thiocyanate and 15 \( \mu \)L of a \( \text{Fe}^{2+} \) solution were added. The \( \text{Fe}^{2+} \) solution was obtained by mixing 0.132 M barium chloride in 0.4 M HCl and 0.144 M Iron(II) sulphate heptahydrate (1:1 v/v), centrifuging at 5000 rpm for 10 min, and taking the supernatant. Samples were vortexed again for 15 s and kept in darkness for 20 min. Blanks were prepared the same as above with 0.3 mL of distilled water instead of the oil or emulsion sample. Hydroperoxide concentration was determined spectrophotometrically at \( \lambda = 510 \) nm. A calibration curve was constructed.
using known concentrations of cumene hydroperoxide, ranging from 0.13 to 3.28 mM. Results were expressed in milliequivalents of oxygen per kg of \( n\text{-3} \) PUFA concentrate (meq O\(_2\)/kg oil).

2.9.2. **TBARS analysis**

TBARS present in the \( n\text{-3} \) PUFA concentrate were determined following the method described by Ke and Woyewoda (Ke & Woyewoda, 1979). Briefly, 10 mg of \( n\text{-3} \) PUFA concentrate were weighed in a screw-capped glass test tube. 5 mL of TBA work solution, which was prepared by mixing 0.04 M 2-thiobarbituric acid in glacial acetic acid, chloroform, and 0.3M sodium sulphite (12:8:1 v/v), were also added to the screw-capped glass test tube. The mixture was vortexed for 15 s and incubated in a water bath at 95 °C during 45 min. After cooling down the test tubes under running cold water, 2.5 mL of 0.28 M trichloroacetic acid were added to the samples, which were then mixed by inversion. Samples were then centrifuged at 2500 rpm for 10 min in order to separate the pink aqueous phase from the chloroform phase. Absorbance of the aqueous phase was measured at \( \lambda = 538 \) nm in a Hitachi U-2000 spectrophotometer. Blanks were prepared the same as above, yet without the oil, and subtracted from the absorbance measurement.

TBARS analysis of the original and reconstituted O/W emulsions was carried out following the method described by Mei *et al.* (Mei, McClements, Wu, & Decker, 1998) with slight modifications. Briefly, 0.025-1.0 mL of emulsion, depending on the expected oxidative status, were taken in screw-capped glass test tubes. Distilled water was used to complete to 1.0 mL if necessary. Subsequently, 2 mL of a TCA/TBA mixture – which was prepared by dissolving 7.5 g of TCA into 10 mL of 0.25M HCl,
adding this solution to 0.1875 g of TBA and completing to volume with 0.25 M HCl in a 50 mL volumetric flask – were added and the glass test tube was tightly closed, vortexed for 15 s and immersed in a water bath at 95 °C during 15 min. Then the vials were cooled down under running cold water and centrifuged at 5000 rpm during 10 min. Immediately afterwards, the supernatant was collected and its absorbance measured in a Hitachi U-2000 spectrophotometer at \( \lambda = 538 \) nm. Blank runs were also performed the same as above, but without adding the emulsion, and its absorbance subtracted from the measurements. TBARS concentration in the emulsion and the \( n-3 \) PUFA concentrate samples was determined using a TEP standard curve with concentrations ranging from 2.5 to 20 nM. Results were expressed in mg malondialdehyde equivalents (MW = 72.06 g/mol) per kg of \( n-3 \) PUFA concentrate (mg MDA/kg oil).

2.10. Statistical analysis

All results reported in this work represent the average of at least three independent measurements. Drying experiments performed in this work have been duplicated. Statistical analyses were performed using Statgraphics Centurion XVII software. Statistical significance was determined by analysis of variance (ANOVA) using the Fisher’s least significant difference test. Results were deemed as statistically significant when \( p < 0.05 \).
3. **Results and discussion**

### 3.1. Characterization of the $n$–3 PUFA concentrate

Results obtained in the characterization analysis are summarized in Table S-1 of the provided supplementary material. As it can be seen from Table S-1a, the fatty acid profile of the $n$–3 PUFA concentrate is constituted by more than 90 % $n$–3 PUFAs, being 73.49 % identified as DHA. Neutral lipid profile of the $n$–3 PUFA concentrate (Table S-1b) showed that more than the 75 % of the neutral lipids in the $n$–3 PUFA concentrate are in the form of triacylglycerides, with 21.6 % being in the form of fatty acid ethyl-esters. Traces of diacylglycerides and monoacylglycerides (1.2% and 0.7 %, respectively) were also found. The high content of triacylglycerides is an important feature of the $n$–3 PUFA concentrate, since these compounds are the natural form of food lipids and they may present better bioavailability and stability against oxidation (Rubio-Rodríguez et al., 2010). Tocopherol analysis by HPLC-DAD revealed a racemic mixture of tocopherol added as antioxidant (again, this is in consonance with consumer’s preference for natural sources). $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-tocopherol isomers were identified and quantified. Results are showed in Table S-1c.

### 3.2. Characterization of the O/W emulsion

#### 3.2.1. Droplet size of the O/W emulsions

Results from the analysis of droplet size distribution are reported in Table 1 for the original and reconstituted O/W emulsions. In general, similar values for D[4,3] and D[3,2] were found in all samples, with the exception of the conventionally freeze-dried powder that showed significantly higher values for both D[4,3] and D[3,2], which
means that the reconstituted emulsion from the conventionally freeze-dried powder presented larger mean diameters both in volumetric and surface basis, respectively.

Median droplet size by volume \(d_{0.5}\) of the emulsion is sub-micrometric, with \(d_{0.5} = 0.114\ \mu m\) and a \(D[4,3]\) and \(D[3,2]\) of 0.144 \(\mu m\) and 0.114 \(\mu m\), respectively. On the other hand, the drying methods proposed in this work significantly increased \(d_{0.5}\) after reconstitution, with the exception being the freeze-dried particles with liquid \(N_2\), in which no statistically significant differences were found with the original emulsion \((p < 0.05)\). Still, most droplet populations were around 0.130 \(\mu m\) for particles obtained by PGSS-drying, freeze-drying and spray-drying methods, which demonstrates that the proposed drying methods do not produce aggregation of oil droplets. The span values followed the same trend as \(d_{0.5}\), with original emulsion < freeze-drying (liq \(N_2\)) < spray-drying \(\approx\) freeze-drying (-20 \(^\circ\)C) < PGSS-drying. The higher span values in the reconstituted emulsions may be due to higher polydispersity.

3.2.2. Emulsion stability

Physical stability of the US-assisted O/W emulsion was analyzed by static multiple scattering. Changes in the backscattering profile (\(\Delta BS\)) of the O/W emulsion sample were recorded every 4 h during 24 days of storage at 25 \(^\circ\)C and plotted vs. time. Results are provided as supplementary material in Fig. S-1. As shown in this figure, \(\Delta BS\) in the top-section reached 5% increment on day 2 and started to decrease in the lower section (\(|\Delta BS| > 2\%) on day 5, indicating creaming destabilization due to phase separation and migration of the lighter oil droplets to the top zone. Moreover, a slight BS increase over time in the middle section of the glass cell can be seen (Fig. S-1), indicating emulsion droplet size slightly increased over the 24-day storage period.
3.2.3. Density of the O/W emulsions

Density measurements were carried out for the original emulsion as well as for the reconstituted dried powders. Results obtained are shown in Table 1.

No statistical difference ($p < 0.05$) was found between the densities of the original and reconstituted emulsions, being those the means of three independent measurements. Thus, the average value of 1.091281 g·cm$^{-3}$ was used for the volume-to-mass transformations necessary in the PV and TBARS calculations.

3.3. Characterization of the dried powders

3.3.1. Yield and bioactive loading

Calculated yield of particles and loading of fish oil concentrate of each of the proposed drying methods is showed in Table 1. The spray-drying method exhibits the lowest yield, which is because the particles deposited on the wall of the spraying tower were collected separately and finally not considered due to its low oxidative quality (results not shown). In the PGSS-drying method, some of the finer particles were blown away by the vented CO$_2$ and deposited in the condenser. This wet powder was not collected, slightly reducing the final yield. In the case of the freeze-dried particles, the observed yield is very close to unity. This trend was also observed by other authors (Lévai et al., 2017) and may be attributed to the one-pot processing and the preservation of the emulsion structure during freezing.

Regarding the bioactive loading, it is close to the maximum theoretical loading of 200 mg/g sample in all cases, and no statistical differences ($p < 0.05$) are observed no matter the drying method used to obtain the particles. Nevertheless, the spray-dried
particles present a slightly lower average value, which may be attributed to the higher moisture content that will be discussed next. On the other hand, the freeze-dried particles present the highest fish oil concentrate loading, which is possibly linked to the aforementioned preservation of the emulsion integrity.

3.3.2. Moisture content

The moisture content of the particles prepared with different drying methods is showed in Table 1. The spray-dried particles showed the highest residual humidity, whereas the PGSS-drying technique gave the lowest moisture value. Humidity values for the spray-dried particles found in this work are higher than those reported in the literature, which are usually around 1-3% (Carneiro et al., 2013; Hogan, McNamee, O’Riordan, & O’Sullivan, 2001). In the case of the freeze-dried particles, no significant difference in the final humidity was found ($p < 0.05$), no matter the freezing method used (conventional at -20°C or with liquid nitrogen).

3.3.3. Encapsulation efficiency

Encapsulation efficiency is one of the most important quality parameters in encapsulated fish oil and $n$–3 PUFA concentrates. The presence of free oil may adversely affect the physical properties of the final product, such as flowability and bulk density, and would also enhance lipid oxidation (Y. Wang et al., 2016). Table 1 shows the initial encapsulation efficiency of the drying methods proposed in this work.

In general, high initial encapsulation efficiencies, no matter the drying method used, were obtained. It can be noticed that the powder obtained by freeze-drying with conventional -20°C freezing presents a significantly lower ($p < 0.05$) initial
encapsulation efficiency, with EE = 95.8 ± 0.2 % (Table 1). As it has been previously mentioned, it is likely that partial destabilization of the emulsion and release of small amounts of n–3 PUFA concentrate may have happened, probably due to the mechanical and hygroscopic forces caused by the growing of large water crystals during the slow freezing process. By comparison, freeze-dried particles obtained with liquid nitrogen present the highest encapsulation efficiency with 98.6 ± 0.1 % (Table 1), which reflects that the emulsion casting is preserved with a rapid and deep freezing step. Similar results have been also obtained by Lévai et al. (Lévai et al., 2017) dealing with freeze-dried quercetin encapsulated in soybean lecithin. Still, more than 95 % of the total n–3 PUFA concentrate loading was encapsulated by conventional freeze-drying, and almost 98 % encapsulation efficiency was obtained by PGSS-drying (97.9 ± 0.3 %), which is similar to the EE value of the spray-dried microparticles (97.5 ± 0.1 %). Carneiro et al. (Carneiro et al., 2013) compared combinations of maltodextrin and Hi-Cap and other wall materials to encapsulate flaxseed oil by spray-drying, finding Hi-Cap as the best in terms of EE, with 95.7 %. Results obtained in this work are slightly higher in all cases except for conventionally freeze-dried particles, which may be attributed to the optimized US-assisted emulsification process.

Surface oil of the dried particles has been analyzed over time during 28 days of storage at 4ºC in darkness and ambient oxygen concentration to check if some of the n–3 PUFA concentrate could have been released. Results obtained are summarized in Fig. S-2 of the supplementary material. As Fig. S-2 shows, spray-dried particles released around 2% of the total encapsulated n–3 PUFA concentrate during the first 7 days and then the release continued at a lower rate, down to 94 % encapsulated oil after 28 days. In the case of the conventionally freeze-dried particles, a slight decrease in the encapsulated oil can be seen after the second week of storage; whereas for the PGSS- and freeze-
dried particles frozen with liquid N\textsubscript{2}, no significant changes in the encapsulation efficiency were noted during the first 21 days and only a slight decrease started to occur after the fourth week of storage.

**3.3.4. Particle Size Analysis**

The particle size distribution plot of PGSS-dried and spray-dried particles is provided in Fig. 2. Particle mean diameters ($d_{0.5}$) varied from 28.605 µm for PGSS-dried particles to 35.375 µm for the spray-dried particles. The span value of the PGSS-dried particles (1.663) was also lower than that of the spray-dried particles (6.082).

The microparticles produced by spray drying showed a bimodal distribution with a group of particles centered around 30 µm and a second population around 250 µm. This justifies the high span value and may be linked to particle swelling during drying as well as to agglomeration due to the higher moisture content. This agglomerated clusters are also visible in the SEM images showed in Fig. 3b and discussed in the next section.

On the other hand, the PGSS-dried particles show a monomodal particle size distribution with smaller mean diameter. As it has been reported in previous works (de Paz et al., 2012), the effective atomization caused by CO\textsubscript{2} vaporization may have led to the production of smaller and monodisperse particles.

**3.3.5. Particle morphology (SEM)**

Visual morphology of the dried powders can be observed in the SEM micrographs (Fig. 3). Both PGSS- and spray-dried particles present spherical morphology. For the PGSS-dried particles, small spheres with diameters ranging from 2 µm to 5 µm can be
observed together with some larger agglomerates around 10-20 µm diameter (Fig. 3a). Fractured particles are also seen in some micrographs, showing a porous internal structure in which the n–3 PUFA concentrate is probably encapsulated. As it has been also observed in the particle size analysis, spray-dried particles show more variance in size. A small population of microparticles around 2 µm was detected together with some specimens larger than 20 µm and particle clusters around 150 µm (Fig. 3b), which is also in accordance with the results obtained in the particle size analysis (section 3.3.4). This variety in size has been also reported in the literature (Carneiro et al., 2013), and seems to be a typical characteristic of particles produced by spray drying. Spray-dried particles also showed a rougher surface than PGSS-dried samples, with more imperfections or ‘teeth’. These surface depressions are associated to the collapse of the particle hollow core once the crust is formed during the initial stages of drying. Similar morphological characteristics have been also found in the literature, either with OSA-starch as encapsulating agent (Carneiro et al., 2013), or with other materials such as β-glucans (Salgado, Rodríguez-Rojo, Alves-Santos & Cocero, 2015).

In the case of the freeze-dried particles, larger and more irregular particles have been produced. Conventionally freeze-dried powder presents a flakey or scaly appearance, forming planar structures with some dimensions being larger than 100 µm (Fig. 3c). Some dents can be seen in the surface of several particles, probably corresponding to the voids left by water crystals after sublimation. In larger magnifications (3000x) a porous internal structure can be also appreciated, being the n–3 PUFA concentrate likely encapsulated inside these vesicles. In the case of the freeze-dried powder frozen with liquid N₂ (Fig. 3d), a powder finer than the conventionally frozen (Fig. 3c) has been obtained. Some particles show an alveolar structure, which may have been formed.
by liquid nitrogen boiling during freezing of the O/W emulsion. These alveolar holes present diameters around 5-7.5 µm.

3.3.6. Differential Scanning Calorimetry (DSC)

DSC runs of PGSS-dried particles, modified OSA-starch (Hi-Cap 100) used as a carrier, and n–3 PUFA concentrate revealed cold-crystallization, glass-transition (gelatinization) and melting peaks. The peak temperatures of these thermal events are summarized in Table 2. Endothermic peaks near 80 ºC were observed in the PGSS-dried and Hi-Cap 100 samples, which probably correspond to the glass transition (gelatinization) of OSA-starch. A second endothermic peak was found around 220 ºC in both PGSS-dried particles and Hi-Cap 100, which may be linked to the melting of OSA-starch. Similar glass-transition and melting temperatures have been reported in the literature for this polymer (Yu & Christie, 2001).

In the lower temperature range, an exothermic cold-crystallization peak was noticeable for the n–3 PUFA concentrate and for the PGSS-dried particles, which may correspond to some lipid compound of the n–3 PUFA concentrate transitioning from liquid to solid state. This assumption can be corroborated by the studies of Tolstorebrov et al. (Tolstorebrov, Eikevik, & Bantle, 2014), in which cold-crystallization peaks in the range -75 to -55 ºC have been reported for some olein-, linolenin-, and linolein-containing tryacylglycerides, which are minoritary constituents of the n–3 PUFA concentrate (Table S-1a). The slightly lower crystallization temperature observed in the PGSS-dried particles compared to the n–3 PUFA concentrate alone (Algatrium™ Plus) is probably linked to the particle shell offering heat transfer resistance to the encapsulated oil, and thus delaying the cold crystallization event.
3.4. Oxidative stability of the dried powders

Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) have been systematically determined in the PGSS-dried powders with and without ascorbic acid (AA) during 28 days of storage at 4 ºC and dark conditions. In order to determine the initial oxidative status, PV and TBARS were measured in the \( n-3 \) PUFA concentrate and in the original emulsion right after US-assisted emulsification. With the purpose of comparing the different drying methods used in this work, PV and TBARS of the spray-dried and the freeze-dried particles were measured after formulation of the powders (day 0) and after 28 days of storage under the same conditions as the PGSS-dried particles (4ºC, darkness). Results obtained are summarized in Fig. 4.

Fig. 4a shows that PV increases from 1.64 ± 0.05 meq O\(_2\)/kg oil in the \( n-3 \) PUFA concentrate up to 5.6 ± 0.3 meq O\(_2\)/kg oil during the US-assisted emulsification process, which slightly surpasses the maximum limit of 5 meq O\(_2\)/kg oil for fish oil concentrates intended for direct human consumption (Codex Alimentarius Comission, 2017). It is likely that the high energy input involved in the ultrasonication process promoted a temperature increase that may negatively affect the oxidative status of the \( n-3 \) PUFA concentrate (Abbas et al., 2013). As a strategy to prevent primary oxidation during US-assisted emulsification, 20 mM ascorbic acid (AA) was added to the emulsion formulation. AA concentration was selected based on Uluata et al. (Uluata et al., 2015) studies on lipid oxidation in O/W emulsions.

As it can be seen in Fig. 4a inset (O/W emulsion), the antioxidant successfully protected the \( n-3 \) PUFA concentrate and even reduced the PV of the emulsion down to 0.19 ± 0.03 meq O\(_2\)/kg oil. This behaviour has been also observed by Uluata et al. in O/W emulsions with AA (Uluata et al., 2015) and it is likely related to AA’s ability to
inactivate free radicals such as lipid hydroperoxides. Other mechanisms can be also involved in the observed antioxidant activity, since AA can act as an oxygen scavenger thanks to the enediol group in carbons 2 and 3 (Johnson, 1995; Liao & Seib, 1988), or even play a synergistic role by means of regenerating other antioxidants such as the tocopherol originally present in the n–3 PUFA concentrate (Reische, Lillard, & Eitenmiller, 2008). However, it is not easy to determine which of these pathways is taking place in any given food system (Uluata et al., 2015) and it is likely that all of them occur simultaneously.

If we focus on the PV results obtained after formulation of the dried particles (Fig. 4a day 0), it can be seen that PGSS-drying promoted a slight PV increase up to 5.9 ± 1.5 meq O_2/kg oil in the emulsion without AA, although this value is not significantly different (p < 0.05) from the PV of the original emulsion. Furthermore, AA addition had a significant (p < 0.05) effect on the PV of the PGSS-dried particles, since only a slight increase from 0.19 ± 0.03 to 0.5 ± 0.1 meq O_2/kg oil was observed in the PGSS-dried particles with antioxidant (Fig. 4a day 0). On the other hand, the spray-drying process yielded particles with much lower oxidative quality (PV = 28.0 ±1.6 meq O_2/kg oil). As some authors have pointed out for the spray-drying process (Drusch & Berg, 2008; H. Wang et al., 2011), it is likely that the rapid formation of the particle shell increased the resistance to evaporation of water trapped inside the particle core, promoting a rapid temperature increase in the particles and prolonging the n–3 PUFA exposure to high temperatures, thus promoting oxidation and increasing the PV after spray-drying formulation. The freeze-drying process with liquid nitrogen achieved good results, with PV = 4.6 ± 1.8 meq O_2/kg oil, which is not statistically different (p < 0.05) from that of the original emulsion (Fig. 4a day 0). This result can be related to the freeze-drying process being a degradation-free technology, since the samples are not submitted to
high processing temperatures and processed in absence of light and in an almost inert atmosphere due to vacuum conditions. Unexpectedly, the conventionally frozen emulsion did overcome oxidation despite the favourable processing conditions, showing a PV = 12.4 ± 1.5 meq O₂/kg oil (Fig. 4a day 0). This is likely due to oxygen contact during the conventional freezing step, in which the samples were held overnight at -20ºC under ambient oxygen concentration.

In view of the results (Fig. 4a day 0), we can infer that PGSS-drying is a suitable method to formulate dried particles loaded with n-3 PUFAs, more so if we combine the mild processing conditions with the addition of an antioxidant such as AA. As it has been previously stated, the short residence time of the O/W emulsion in the PGSS-drying system as well as the inert CO₂ atmosphere prevent the loaded bioactive compounds from degradation (Weidner, 2009) and as such, the n-3 PUFA concentrate can be successfully protected from oxidation.

Oxidative stability of the PGSS-dried particles was monitored during 28 days of storage in darkness at 4 ºC (Fig. 4a days 1-28). Results obtained showed a sustained increase of primary oxidation, reaching values of PV = 25.2 ± 0.7 meq O₂/kg oil after 28 days of storage (Fig. 4a). On the other hand, AA successfully protected the PGSS-dried particles from primary oxidation during storage, being the values found significantly lower ($p < 0.05$) than those of the PGSS-dried particles without antioxidant. The highest PV was found after 14 days of storage and was 2.5 ± 0.5 meq O₂/kg oil, still below the maximum allowable limit according to legislation, and remained with no significant changes ($p < 0.05$) during the rest of the 28-day storage period, reaching a final value of 2.2 ± 0.3 meq O₂/kg oil (Fig. 4a).
Comparing the primary oxidation of the particles obtained by the different drying methods after 28 days of storage, we can see the same trend as in the PV analysis after formulation, although PV increased in all samples (Fig. 4a days 1-28). Freeze-dried particles frozen with liquid nitrogen maintained a relatively low PV of 16.9 ± 0.8 meq O₂/kg oil, which is likely linked to the good encapsulation efficiency and the preservation of the physical structure of the emulsion thanks to the fast and deep-cooling effect of liquid nitrogen. The same was not true for the conventionally freeze-dried particles, with PV = 37.7 ± 3.7 meq O₂/kg oil after 28 days of storage. Spray-dried particles showed the highest PV with 66.0 ± 0.4 meq O₂/kg oil after 28 days of storage. The higher oxidation rates of these two samples (spray-drying and conventional freeze-drying) are probably due to the high starting PV (day 0) as well as their lower encapsulation efficiency, which implies more oil in the particle surface susceptible to oxidation. A similar encapsulation efficiency vs. oxidation rate inverse relationship has been observed by other authors (Yang & Ciftci, 2017). However, PGSS-dried and freeze-dried particles with liquid nitrogen exhibited high encapsulation efficiencies (up to 98%), and still encapsulated n–3 PUFA concentrate was not fully protected against primary oxidation (PV after 28 days = 25.2 ± 2.2 and 16.9 ± 0.8 meq O₂/kg oil, respectively). This trend can be explained by taking into account not only the oxidation of the oil present in the particle surface, but also oxygen diffusion through the encapsulating material. It must be also pointed out that the fish oil concentrate used in this work is extremely rich in n–3 PUFAs, which are highly prone to oxidation. This highly sensitive-to-oxidation fatty acid profile may also offer an explanation to the higher oxidation rates obtained in this work compared to other studies, even with no accelerated storage (Carneiro et al., 2013; Yang & Ciftci, 2017).
TBARS analysis results are summarized in Fig. 4b. Although there is no legal maximum limit for this parameter in food products, we can take the values of 10 µmol MDA equiv/kg fish and 1-2 µmol MDA equiv/g fat given in the FAO guidelines (Huss, 1995) as an orientative basis to evaluate rancidity of the n–3 PUFA concentrate (1 µmol MDA equiv/g fat corresponds to 72.06 mg MDA/kg oil). From Fig. 4b we can see that initial TBARS of the n–3 PUFA concentrate lay below this rancidity limit (TBARS = 41.1 ± 2.7 mg MDA/kg oil). US-assisted emulsification slightly increased the TBARS value up to 54.8 ± 0.6 mg MDA/kg oil in the formulation without AA, whereas the addition of AA yielded particles with TBARS = 42.8 ± 1 mg MDA/kg oil (Fig. 4b day 0). In view of the results, AA addition slowed down secondary oxidation during the ultrasonication step since no significant difference (p <0.05) between the AA-added emulsion and the n–3 PUFA concentrate was found (Fig. 3b inset).

Among the dried powders (Fig. 4b, day 0), spray-dried particles showed the highest secondary oxidative status with a TBARS value of 88.5 ± 6.0 mg MDA/kg oil, which is above the FAO rancidity limit (Huss, 1995). PGSS-drying process slightly increased TBARS up to 59.4 ± 4.4 mg MDA/kg oil, whereas the addition of AA did not make any statistically significant difference (p < 0.05). Both PGSS-drying with and without AA, and freeze-dried powder with liquid N₂ showed no statistically significant differences with the original emulsion, which gives an idea of the protective effect of these drying techniques against secondary oxidation. On the contrary, the conventionally frozen particles were not successfully protected, and TBARS increased up to 74.5 ± 3.5 mg MDA/kg oil after the conventional freeze-drying process.

Secondary oxidation products were also monitored in the PGSS-dried particles during the 28-day storage period. In Fig. 4b (days 1-28), we can see that TBARS in the PGSS-dried particles without AA did not significantly increase (p < 0.05) up to the second
week of storage, when TBARS value raised from $69.2 \pm 1.4$ up to $110.9 \pm 1.8$ mg MDA/kg oil, reaching a final value of $141.0 \pm 1.9$ mg MDA/kg oil after 28 days of storage. On the other hand, AA addition delayed secondary oxidation for the first 14 days of storage, obtaining significantly lower ($p < 0.05$) TBARS values than those of the control sample without antioxidant, yet increasing thereafter and even exceeding the control after 28 days of storage ($TBARS = 141.0 \pm 1.9$ mg MDA/kg oil). As previously mentioned, this behavior has been observed by other authors when studying the effect of ascorbic acid on lipid oxidation in O/W emulsions, especially in presence of transition metals such as iron and copper (Mei et al., 1998; Uluata et al., 2015). Uluata et al. (Uluata et al., 2015) provide an explanation related to the ability of AA to reduce metal ions, making them more reactive towards peroxides and hydroperoxides. According to this proposed mechanism, reduced metallic species would decompose peroxides and hydroperoxides into secondary oxidation products, increasing the observed TBARS and preventing the accumulation of primary oxidation intermediaries (Uluata et al., 2015). This behavior has been also observed in this work, although no metals were added to the O/W emulsion. However, and according to inductively coupled plasma mass spectrometry (ICP-MS) analysis (Table S-2), metal traces are present in the encapsulating material, enabling this hypothesis. Additionally, it has been found that spray-dried and conventionally freeze-dried particles underwent secondary oxidation during the 28-day storage period, with final TBARS values of $137.2 \pm 4.7$ mg MDA/kg oil and $166.6 \pm 0.3$ mg MDA/kg oil, respectively (Fig. 4b days 1-28). Again, this high secondary oxidation status might be linked to the poorer encapsulation efficiency of those methods. On the other hand, freeze-dried particles frozen with liquid N$_2$ showed good stability against secondary oxidation.
oxidation during storage, maintaining a TBARS value of 79.6 ± 2.4 during 28 days of storage at 4°C.

4. Conclusion

Particles from Gas-Saturated Solutions (PGSS)-drying has been used to encapsulate omega-3 polyunsaturated fatty acids (n-3 PUFAs) into octenyl-succinic-anhydride (OSA) starch, obtaining a solid powder with high bioactive load.

Similar encapsulation efficiencies (EE) and spherical morphologies have been obtained by PGSS and spray-drying.

Freeze-dried particles showed irregular morphology. Slow conventional freezing destabilizes the O/W emulsion and negatively affects EE. DSC analysis of the PGSS-dried particles successfully identified cold crystallization of the n-3 PUFA concentrate as well as gelatinization and melting peaks of OSA-starch.

PGSS-drying method offers low drying temperature and an intrinsically inert atmosphere, which avoid oxidative degradation of n-3 PUFAs during processing, as demonstrated by the oxidative stability analyses. Conventional freeze-drying method yielded particles with low oxidative stability, whereas freezing with liquid N₂ resulted in a powder with oxidative stability comparable to PGSS-dried particles. Combined with the addition of natural antioxidants such as ascorbic acid, the PGSS-drying technique rises as a suitable method to formulate n-3 PUFAs in solid form and protect them against oxidation during shelf life.
Acknowledgements

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Table 1. Summary of experimental results.

<table>
<thead>
<tr>
<th>Emulsion /drying method</th>
<th>Density (g·cm⁻³)</th>
<th>Yield (%)</th>
<th>EE (day 0) (%)</th>
<th>Bioactive loading (mg/g)</th>
<th>Moisture (%)</th>
<th>Droplet size analysis</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D[4,3]</td>
</tr>
<tr>
<td>Original</td>
<td>1.091281⁺</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.144ᵃ</td>
</tr>
<tr>
<td>PGSS-drying</td>
<td>61 ± 1</td>
<td>97.9ᵇ ± 0.3</td>
<td>191 ± 8</td>
<td>3.3 ± 0.3ᵃ</td>
<td>0.227ᵇ</td>
<td>0.116ᵃ</td>
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<tr>
<td>Spray-drying</td>
<td>30 ± 1</td>
<td>97.5ᵇ ± 0.1</td>
<td>187 ± 3</td>
<td>5.6 ± 0.2ᶜ</td>
<td>0.197ᵇ</td>
<td>0.112ᵃ</td>
</tr>
<tr>
<td>Freeze-drying (-20°C)</td>
<td>99 ± 1</td>
<td>95.8ᶜ ± 0.2</td>
<td>192 ± 2</td>
<td>4.66 ± 0.05ᵇ</td>
<td>0.567ᶜ</td>
<td>0.121ᵇ</td>
</tr>
<tr>
<td>Freeze-drying) (liq N₂)</td>
<td>99 ± 1</td>
<td>98.6ᵃ ± 0.1</td>
<td>192 ± 2</td>
<td>4.7 ± 0.1ᵇ</td>
<td>0.146ᵃ</td>
<td>0.107ᵃ</td>
</tr>
</tbody>
</table>

⁺ Standard uncertainty is $\sigma(\rho) = \pm 0.000002 \text{ g·cm}^{-3}$

ᵃᵇᶜᵈ Different upper-scripts in the same column denote statistically significant differences at $p < 0.05$
Table 2. Peak temperatures of the thermal events observed in the PGSS-dried powder loaded with \( n-3 \) PUFA concentrate (PGSS-drying), the carrier alone (Hi-Cap\(^{TM}\) 100), and the \( n-3 \) PUFA concentrate alone (Algatrium\(^{TM}\) Plus).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cold crystallization</th>
<th>Glass transition</th>
<th>Melting</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGSS-dried particles</td>
<td>-72.99</td>
<td>76.57</td>
<td>223.55</td>
</tr>
<tr>
<td>Hi-Cap(^{TM}) 100</td>
<td>n.d.</td>
<td>78.83</td>
<td>217.05</td>
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<tr>
<td>Algatrium(^{TM}) Plus</td>
<td>-71.42</td>
<td>n.d.</td>
<td>n.d.</td>
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</table>

n.d.: not detected
Figure 1. Schematic diagram of the PGSS-drying apparatus. VE-1: O/W emulsion vessel, VE-2: static mixer, VE-3: a) spraying tower, b) condenser, VE-4: CO₂ vessel, P-1: O/W emulsion pump, P-2: CO₂ pump, V-: process valve, E-: heat exchanger, R-: electrical resistance, PI: pressure indicator, TI(C): temperature indicator (and controller).
Figure 2. Particle size distribution plot of the particles obtained by PGSS-drying and by spray-drying.
Figure 3. SEM micrographs of the dried powders. a) powder obtained by PGSS-drying, b) powder obtained by spray-drying; from left to right, 1500, 5000 and 10000x magnifications. c) powder obtained by conventional freeze-drying (50, 400 and 3000x). d) powder obtained by freeze-drying with liquid N\textsubscript{2}; (50, 400 and 2000x).
Figure 4. a) Peroxide Value (PV) and b) Thiobarbituric acid reactive substances (TBARS) content of the powders obtained by the different drying methods right after drying (day 0) and during storage at 4°C in darkness and ambient oxygen conditions (days 1-28). Samples were reconstituted the day of analysis keeping the water:carrier:$\omega$–3 PUFA concentrate proportion the same as the original (70:24:6 wt.). Different letters denote statistically significant differences at $p < 0.05$. Insets: PV and TBARS of the $\omega$–3 PUFA concentrate (Algatrium™ Plus), and the original US-assisted O/W emulsions without and with ascorbic acid (AA).