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Development of bio-based nanoemulsions to improve physical and chemical stability of omega-3 fatty acids

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Título

Desenvolvimento de nanoemulsões baseadas em biopolímeros para melhorar a estabilidade física e química de ácidos gordos de ómega-3

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There's an old saying "That what doesn't kill you, makes you stronger" I don't believe that. I think the things that try to kill you make you angry and sad. Strength comes from the good things – your family, your friends, the satisfaction of hard work. Those are the things to old on when you're broken.

Jax Teller, Sons of Anarchy

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Abstract

Development of bio-based nanoemulsions to improve physical and chemical stability of omega-3 fatty acids

Nowadays there is a high interest by the food industry to develop nutritional food products. This concept promoted the development of bio-based structures to encapsulate bioactive compounds and thus enhance their physical and chemical stability from storage up until consumption. Omega-3 (ω-3) is known for its functional properties such as improving cardiovascular health, decrease inflammation, increase cognitive function. However, ω-3 fatty acids are highly susceptible to oxidation, have intense odour and low water solubility, which makes its direct application in foods extremely difficult. In order to reduce these problems, nanoencapsulation, through nanoemulsions can be used.

With this in mind, lactoferrin (Lf), a protein derived from milk with a wide range of reported biological activities (e.g. antioxidant, antimicrobial, cancer prevention) was used as natural emulsifier for the development of oil-in-water nanoemulsions for ω-3 encapsulation. The nanoemulsions were characterized and assessed by physical and chemical stability during storage. Nanoemulsions were also dried by freeze-drying and nanospray-drying and further characterized.

ω-3 nanoemulsions were successfully produced through high pressure homogenization, with results showing that the concentration of Lf influenced size and superficial charge of the nanoemulsions droplets obtained. Nanoemulsions revealed physical stability when stored at 4 °C for 69 days, while presenting instability at room temperature. The antioxidant capacity of the nanoemulsions did not show significative alterations over storage while a significative increase in oxidation was registered. Only the nanoemulsions dried by nanopray-drying presented defined structures. The rehydration of the powders resulted from freeze-drying was possible while powders obtained by nanospray-drying were not able to be rehydrated.

Overall the results suggest that ω-3 nanoemulsions with physical stability can be produced using Lf while chemical stability was not achieved. The Lf nanoemulsions can also be dried to obtain powders with defined submicron particles but its rehydration its limited. This work provides important information that can be useful for the design of nanoemulsions and dry capsules aiming the encapsulation of lipophilic compounds for pharmaceutical and food applications.

Keywords: ω-3; Lactoferrin; Nanoemulsion; Oxidation; Stability; Freeze-drying; Nanospray-drying.

Resumo

Desenvolvimento de nanoemulsões baseadas em biopolímeros para melhorar a estabilidade física e química de ácidos gordos de ómega-3

Hoje em dia existe um grande interesse da industria alimentar no desenvolvimento de produtos alimentares com elevado valor nutricional. Este conceito tem promovido o desenvolvimento de estruturas de origem natural para encapsular compostos bio-ativos, permitindo melhorar a sua estabilidade química e física, desde o armazenamento até ao seu consumo. O ómega-3 (ω-3) apresenta importantes propriedades funcionais tais como a melhoria de saúde cardiovascular, diminuição de inflamação, aumento de funções cognitivas. No entanto, é extremamente suscetível a oxidação, tem um odor intenso e uma baixa solubilidade em água, o que faz com que a sua aplicação direta em alimentos seja extremamente difícil. A nanoencapsulação deste composto surge como uma alternativa para solucionar tais problemas.

A Lactoferrina (Lf) uma proteína derivada do leite, com inúmeras propriedades biológicas (p.e. antioxidante, antimicrobiana, prevenção de cancro) foi usada como emulsionante natural na produção de nanoemulsões óleo-em-água para a encapsulação de ω-3, as quais foram posteriormente caracterizadas e avaliadas a nível da estabilidade física e química durante o armazenamento. Estas foram ainda submetidas a 2 diferentes processos de secagem (liofilização e *nanaopray-drying*).

Produziram-se com sucesso nanoemulsões através de homogeneização de alta pressão, tendo-se verificado que a concentração de Lf influencia o seu tamanho e carga superficial. As nanoemulsões, revelaram estabilidade física quando armazenadas (69 dias) a 4 °C, mas não quando armazenadas à temperatura ambiente. A capacidade antioxidante, quando armazenadas (4 °C durante 35 dias), não apresentou alterações significativas, enquanto que os valores de oxidação aumentaram. Apenas as nanoemulsões submetidas ao processo de *nanaopray-drying* apresentaram uma estrutura resultante bem definida, e apenas as nanoemulsões submetidas ao processo de liofilização foram possíveis de ser rehidratadas.

A presente tese permitiu verificar que nanoemulsões de ω-3, com estabilidade física, podem ser produzidas, usando Lf, no entanto não foi possível obter estabilidade química. A sua secagem permite a obtenção de estruturas definidas. Este trabalho permitiu obter informação relevante para o desenvolvimento de nanoemulsões que tenham como objetivo a encapsulação de compostos lipofílicos para aplicações alimentares e farmacêuticas.

Palavras-chave: ω-3; Lactoferrina; Nanoemulsão; Oxidação; Estabilidade; Liofilização; *Nanospray-drying*.

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List of Nomenclature

Abbreviations

ALA – α-linoleic acid

ATR-FTIR - Attenuated total reflection-Fourier transform infrared

DLS - Dynamic Light Scatterin

EE - Encapsulation efficiency

EFSA - European Food Safety Authority

ENMs - Engineered nanomaterials

EPA - Eicosapentaenoic acid

FA - Fatty acids

FAO - Food and Agriculture Organization

FDA - Food and Drug Administration

DHA - Docosahexaenoic acid

GRAS - Generally Recognized as Safe

HLB - Hydrophilic-lipophilic balance

IC_{50} - Inhibitory concentration of 50 %

IDF - International Dairy Federation

Lf - Lactoferrin

O/W - Oil-in-water

O/W/O - Oil-in-water-in-oil

PdI - Polydispersity Index

PUFAS - Polyunsaturated fatty acids

PV - Peroxide Value

ROS - Oxygen Reactive Species

SEM - Scanning Electron Microscopy

TEM - Transmission Electron Microscopy

T_{in} - Inlet Temperature

T_{out} - Oultlet Temperature

UV/VIS - Ultraviolet/Visible

WHO - Wold Health Organization

W/O - Water-in-oil

W/O/W - Water-in-oil-in-water

WPC - Whey protein concentrate

WPI - Whey protein isolate

List of Symbols

α - alpha

β - beta

ω - omega

ζ - zeta

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Part I – Introduction

Chapter 1. State of the art

1.1 ω-3: health benefits and associated problems

Fatty acids (FA) are organic compounds constituted by a carboxyl group and a hydrocarbonated chain which can be saturated (containing only simple bonds) or unsaturated (containing at least one double bond) (Rubio-Rodríguez et al. 2010). Long chain PUFAs are FA characterized for the presence of more than two double bounds, and include omega-3 (ω -3) PUFAs which have the first double bound in the third position counting from the "n or ω " methyl end of the hydrocarboneted chain (Scorletti & Byrne 2013). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are two of the most important ω -3 PUFAs (Figure 1), due to their long-term health benefits (e.g.improve cardiovascular health, decrease inflammation, increase the cognitive function and enable a better neurological and visual development). They are also positively associated with prevention and/or treatment of some diseases (e.g. diabetes, Alzheimer, allergies, Arthritis, Asthma and some types of cancer) (Arab-Tehrany et al. 2012; Falkeborg & Guo 2015; Lane et al. 2016; Sodeifian et al. 2017).

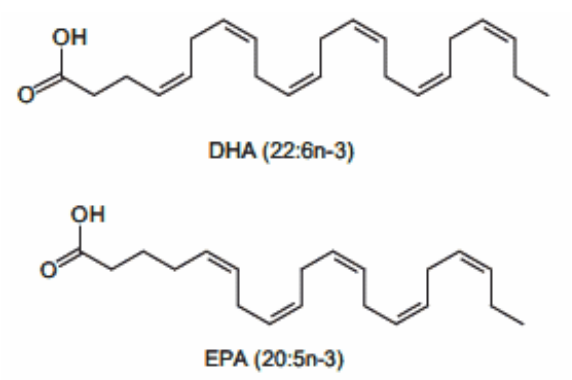

Figure 1. Chemical structure of the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) ω-3 PUFAs. In www.sigmaaldrich.com (BioFiles 2007).

EPA and DHA can be internally synthesized using essential α-linoleic acid (ALA) as a precursor, which can be present in diets from a diversity of plant sources (e.g. nuts, flax, chia seeds) while EPA and DHA presents in diet are mostly from marine sources, namely fish oils. However, despite the fact that ALA is the most abundant ω-3 present in the majority of diets, its internal conversion rate into EPA and DHA is very low, only 8 to 12% is converted to EPA, and even less is converted to DHA (approximately 1%) (Baker et al. 2016). Thus, to accomplish the health benefits described

above the both EPA and DHA require a proper and direct dietary intake, which as lead the Food and Agriculture Organization (FAO), the World Health Organization (WHO), the European Food Safety Authority (EFSA), among other scientific organizations and authoritative bodies to establish a dietary intake recommendation of EPA and DHA higher than 250 mg per day, and especially higher than 250 mg per day for persons with critical health conditions (e.g. cardiovascular diseases) (Dellarosa et al. 2015; Cook et al. 2016). Taking into consideration these dietary requests and the health benefits associated, food industries have increased their interest in enriching foods with ω-3 PUFAs, with an estimation that the world's demand for ω-3 ingredients to be worth 1595 million dollars and predicted to exceed the 4000 million dollars by 2018 (Eratte et al. 2015; Comunian & Favaro-Trindade 2016; Ghorbanzade et al. 2017).

Nowadays, the socioeconomic conditions as well the life styles developed over the last centuries have led the population of Western countries to acquire dietary habits that resulted in an unbalanced intake of polyunsaturated fatty acids (PUFAs). The increased intake of omega-6 (ω-6) and the reduced consumption of ω -3 caused not only an unbalance in the ratio between ω-6/ω-3 but it also implied a lack of ω -3 in daily diets (Dellarosa et al. 2015; Scoditti et al. 2014). Most of developed countries have an intake of PUFAs, with a 15/1 consumption ω-6/ω-3 ratio, when ideally, the ratio between ω-6/ω-3 should be, at a maximum, 2/1. Given that ω-6 and ω-3 compete for the same enzymes in their metabolic pathways, with an excess of ω-6 in diets its metabolization is favored, leading to the enhancement of its eicosanoids metabolic products (e.g. prostaglandins, thromboxanes and leukotrienes). These specific eicosanoids, resultant from ω-6 metabolozation, are biologically active in small amounts, but when present in high amounts they contribute to the development/aggravation of chronic conditions (Brenna et al. 2015; Marriott et al. 2014; Simopoulos 2016; Simopoulos 2008).

One of the major problems associated with the addition of ω-3 in food products is their high oxidation. The unsaturations of this kind of lipids are itself triggers for oxidation reactions, considering that the double bonds make the C-H bonds weaker making the removal of hydrogen easier (Kamal-Eldin & Min 2008). Such degradation process affects food's nutritional value, caloric content and loss of functionality, resulting in the production of toxic compounds (oxidation products), diminished shelf-life, and negative organoleptic properties associated to rancidity (production of off-flavors, unpleasant aroma, darkness of fats, lightening of pigments, loss of flavor,

among others), features that contribute to a decreased acceptability by consumers (Arab-Tehrany et al. 2012; Dellarosa et al. 2015; Comunian & Favaro-Trindade 2016).

1.1.1 The autoxidation pathway

Autoxidation, also known as peroxidation, is the spontaneous process of the reaction between atmospheric oxygen and lipids (Arab-Tehrany et al. 2012; Shahidi & Zhong 2010). Although this is the most common mechanism, other pathways for lipid oxidation can also be considered, namely (Shahidi & Zhong 2015):

- Photooxidation: when energy transfer occurs from an excited photosensitizer to lipids or oxygen molecules during the oxidation process;
- Thermal oxidation: when oxidation rates are enhanced by energy transfer in the heat form;
- Enzymatic oxidation: when enzymes such as lipoxygenases are catalyzing the oxidation reactions.

Lipid oxidation goes through a free radical chain mechanism that occurs in three different stages: initiation, propagation, and termination (Figure 2).

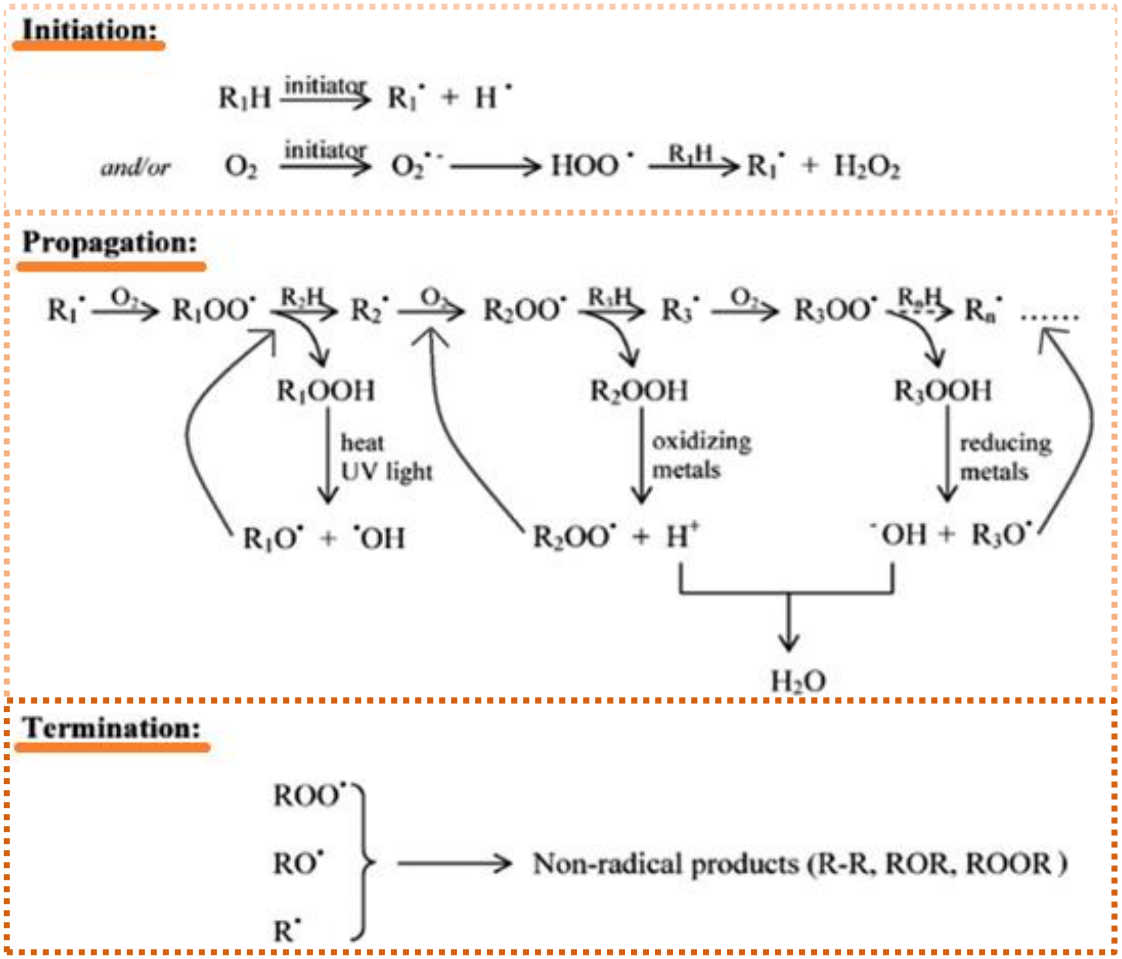

Figure 2. Schematic representation of the three oxidation phases: initiation, propagation and termination. Adapted from Okpala et al. 2016.

During the initiation stage, an activation energy is required to remove an electron from a lipid or an oxygen molecule in order to form a lipid alkyl radical. Alternatively, can occur a transformation of a triplet oxygen molecule in a singlet oxygen molecule (i.e. when an electron of the oxygen molecule inverts its spin) enabling its direct addition to a double bond in a lipid chain. In the propagation stage, the lipid alkyl radicals previously formed react with oxygen forming reactive peroxyl radicals, at a controlled diffusion rate. These peroxyl radicals remove hydrogen from adjacent lipid molecules leading to the formation of lipid hydroperoxides and the increase of the lipid alkyl radicals while maintaining the free radical chain (Okpala et al. 2016; Schaich et al. 2013).

More oxygen molecules are added to the new lipid alkyl radicals, promoting the formation of peroxyl radicals until no more hydrogens are available. In this phase, the reaction rate is maintained relatively low, however in the presence of specific conditions such as temperature, UV light or metals, occurs the degradation of hydroperoxides. During this reaction, different radicals are

formed such as alkoxy, peroxyl and hydroxyl radicals, enhancing the recycling rate of the chain reaction. Besides this, hydroxyl and alkoxyl are radicals that react faster than peroxyl radicals, increasing the oxidation rate (Arab-Tehrany et al. 2012; Okpala et al. 2016; Shahidi & Zhong 2015).

Finally, in the termination stage, the oxidation rate is reduced because the radical quenching processes starts to overlap the rate of new chain production. The hydroperoxides start to break down and reactions like radical recombination or alkoxyl radical scission take place, leading to the formation of non-radical products which can be volatile or non-volatile compounds. Ethers, aldehydes, ketones, alcohols, hydrocarbons, alkyl peroxides, organic acids and polymeric products are some of the secondary oxidation products that can be formed in this phase. However, it is necessary to keep in mind that this termination does not stop the overall oxidation (Kamal-Eldin & Min 2008; Halvorsen & Blomhoff 2011; Arab-Tehrany et al. 2012; Barriuso et al. 2013; Shahidi & Zhong 2015; Okpala et al. 2016).

1.1.2 Pro-oxidants versus antioxidants

In food matrices lipid oxidation can be initiated or accelerated by several factors, such as light and heat exposure, both of which are common steps in the processing of foods or in their storage, and that can contribute to accelerated lipid oxidation (Arab-Tehrany et al. 2012; Shahidi & Zhong 2015). Other compounds exist that contribute to this degradation process, such as pro-oxidants, which are chemicals that either induce oxidative stress (normally trough the formation of radicals) or inhibit antioxidants systems, and these can be enzymes (e.g. lipoxygenase), preformed radicals (e.g. oxygen reactive species (ROS)), heme proteins (e.g. myoglobin) or transition metals (e.g iron) (Shahidi & Zhong 2010; Arab-Tehrany et al. 2012; Schaich et al. 2013; Shahidi & Zhong 2015).

On the other hand, there are antioxidants with ability to delay, control or prevent the oxidative degradation process. These substances can be divided into two main groups: primary and secondary antioxidants. The primary antioxidants contribute to the stabilization of free radicals, either by hydrogen donation or free radical acceptation, while secondary antioxidants act as suppressors of the pro-oxidants (Shahidi & Zhong 2015). Antioxidants can be naturally occurring or synthetic produced, being tocopherols, polyphenols, anthocyanins, carotenoids, flavonoids some naturally occurring antioxidants, while ethylenediamine tetraacetic acid, butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tert-butylhydroquinone are some synthetic

antioxidants that have been previously used in food (Arab-Tehrany et al. 2012; Shahidi & Zhong 2015). The antioxidants can act through several mechanisms, such as scavenging of free radicals, chelation of metal ions, quenching of singlet oxygen species, quenching of secondary oxidation products, inhibition of pro-oxidative enzymes, among others. Their efficiency can be influenced by several factors such as their structural features and concentration, type oxidation of the substrate, physical state of the system, their location in the system, temperature and presence of pro-oxidants (Kristinová et al. 2009; Arab-Tehrany et al. 2012; Schaich et al. 2013; Falkeborg & Guo 2015; Shahidi & Zhong 2015).

1.2 Encapsulation: a protection and a delivery strategy

One of the strategies to reduce the lipid oxidation associated with ω-3 compound is encapsulation. Encapsulation is being used to improve the bioaccessibility of lipophilic compounds (i.e. improving their solubility in aqueous medium) and improve the stability of these compounds when exposed to different environmental conditions (e.g. light, temperature). (Comunian & Favaro-Trindade 2016; Komaiko et al. 2016; Alvarenga et al. 2017; Garti 2008; Pandit et al. 2016; Gomez-Estaca et al. 2012; Rodríguez et al. 2016). The encapsulation of lipophilic compounds, is defined as a process where droplets of lipid compound(s) are embedded or trapped in a coating that can consist in a homogeneous or heterogeneous matrix, forming capsules. The surrounding material forms a physical barrier that separates and protects the compounds from the environment, and, under certain conditions, enables the bioactive compound release (Pandit et al. 2016; Rodríguez et al. 2016; Dias et al. 2017; Encina et al. 2016).

1.2.1 Encapsulation structures

Over the years several systems have been developed for encapsulation of lipophilic compounds at both nano and micro scale (Table 1). Capsules with size between 1-1000 μm are considered micro capsules while capsules with size below 100 nm are considered nanocapsules, although these values generate some controversy with different authors pointing out different numbers for the same definitions of micro and nanocapsules (Bilia et al. 2014; Pereira et al. 2014; Rodríguez et al. 2016; Gupta et al. 2016; Pandit et al. 2016; Davidov-Pardo et al. 2015). Encapsulation structures can be differently sized (micro or nano sized) and can be polymer (e.g nanospheres, nanocapsules, microparticles) or lipid based (e.g. solid lipid nanoparticles, nanostructured lipid carriers, nanoemulsions, nanoliposomes, and liposomes and multi-layered emulsions at the micro

scale). There are also other ways to encapsulate lipophilic compounds which do not belong to either lipidic or polymeric systems which is the case of films, cyclodextrins inclusion complexes, co-crystallization (Figure 3) (Pereira et al. 2014; Pandit et al. 2016; de Souza Simões et al. 2017).

Table 1. Examples of structures for encapsulation already developed, and the materials applied.

STRUCTURE	ENCAPSULATYING AGENTE	ACTIVE COMPOUND	REFERENCE
Spray dried emulsions	Sodium caseinate Gum arabic	Fish oil	(Binsi et al. 2017)
Nanoemulsions	Tween-40 Sodium saseinate Soya lecithin	DHA algae oil	(Karthik & Anandharamakrishnan 2016)
Nano-liposomes	Lecithin	Fish oil	(Ghorbanzade et al. 2017)
Nanoemulsions	Sunflower phospholipids	Fish oil	(Komaiko et al. 2016)
Nanofibers	Whey protein isolate Fish protein hydrolysate Poly(vinyl) alchool	Fish oil	(García-moreno et al. 2016)
Solid lipid particles	Lecithin	Fish oil	(Salminen et al. 2017)
Emulsions	Whey protein isolate	Fish oil	(Horn et al. 2013)
Nanoemulsions	Marine lecithin	Fish oil	(Belhaj et al. 2010)
Nanoemulsions	Whey protein isolate	Fish oil	(Nejadmansouri et al. 2016)
Nanoemulsions	Mesquite gum	Fish oil	(García-márquez et al. 2017)
Nanoemulsions	Lecithin Quillaja saponin Tween-80 Sodium dodecyl sulfate	Fish oil ethyl esters	(Uluata et al. 2015)
Nanoemulsions	Soy lecithin Tween-40	ALA or DHA	(Lane et al. 2016)

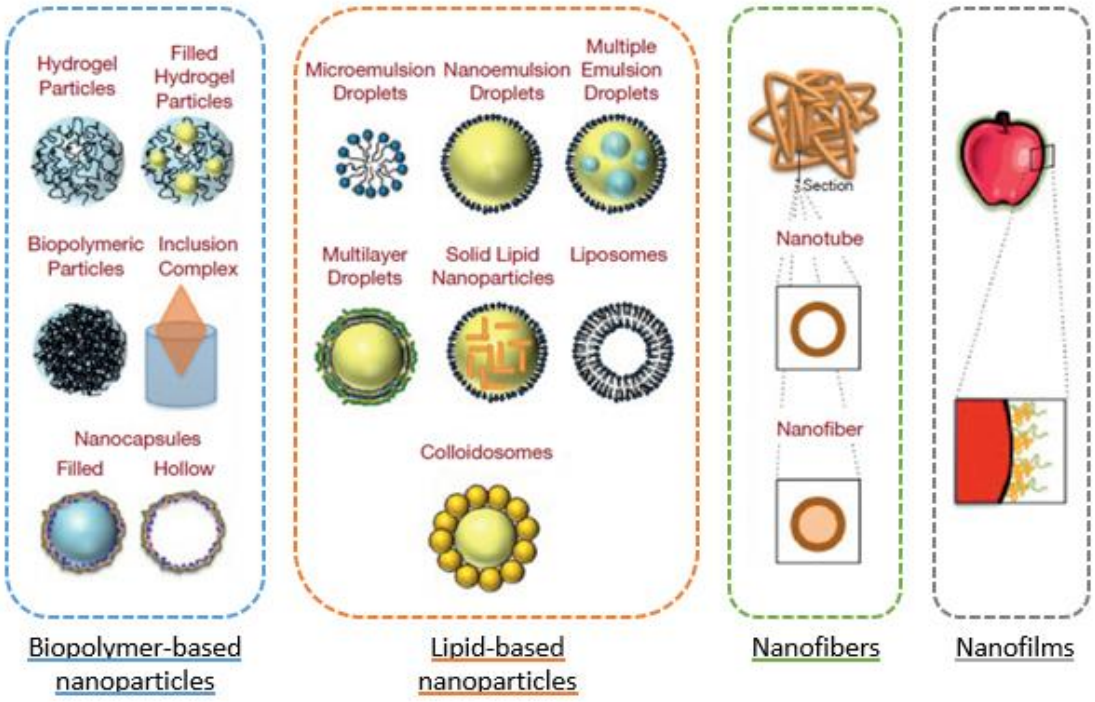

Figure 3. Representation of encapsulation structures that could be applied in food functionalization. Adapted from (Joye et al. 2016).

Nano systems production techniques are generally divided into two main categories (Figure 4), the "top-down" approaches which start with bigger structures that are reduced in size and modulated in form through the application of energy, and the "bottom-up" approaches that consist in techniques that create nanosystems through the self-assembly of smaller molecules like atoms or monomers, with these processes controlled through physical-chemical factors such as pH, temperature and molecule concentration (Chen et al. 2006; Ezhilarasi et al. 2013; Joye & McClements 2014; Paredes et al. 2016; Santiago & Castro 2016). Micro systems production techniques (such as microencapsulation) are not typically categorized, and the most used techniques include spray drying, spray chilling, fluidized bed drying, freeze drying, extrusion, coacervation, in situ polymerization, and several of these methods must be preceded by emulsification processes (Nedovic et al. 2011; C. J. Barrow et al. 2013; Dias et al. 2017).

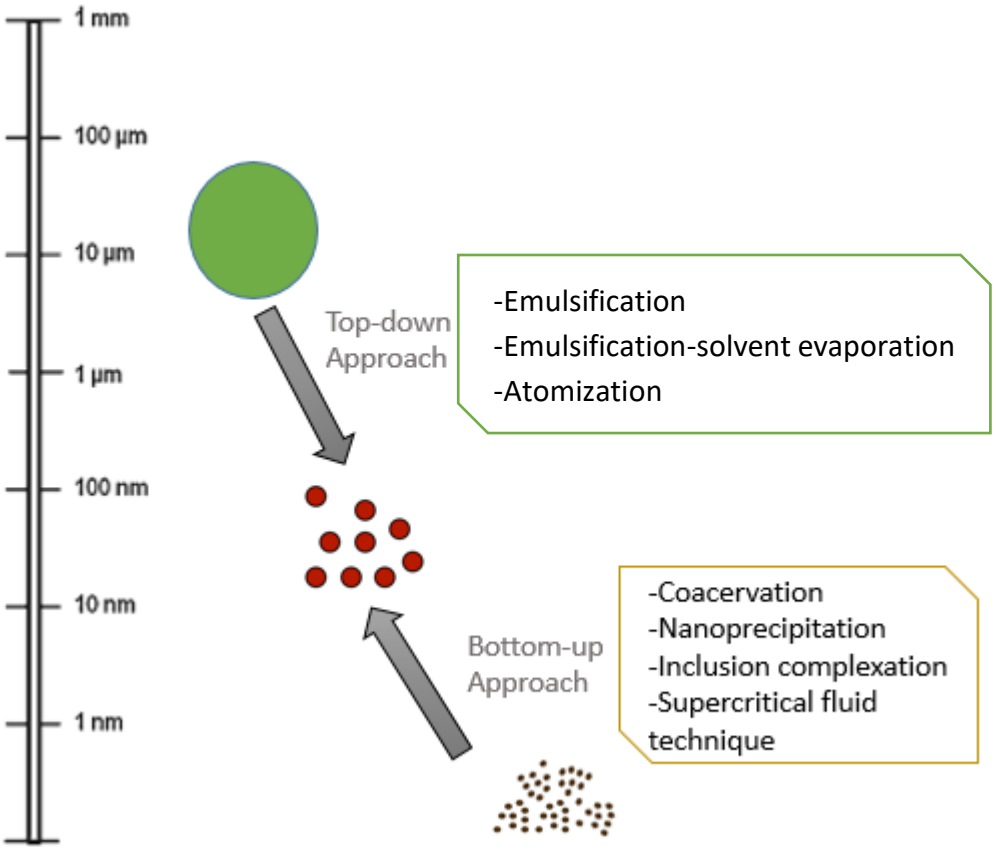

Figure 4. Schematic representation of "top-down" and "bottom-up" approach and the technics that could be used for development of nanostructures for encapsulation of active compounds. Adapted from Ezhilarasi et al. 2013.

1.3 Emulsification systems

Emulsions are heterogeneous systems composed by two or more immiscible liquids, usually water and oil, with one of the liquids dispersed in the other in the form of small droplets, the dispersed liquid composes the dispersed phase while the other the continuous phase of the system (Lam & Nickerson 2013; Abdolmaleki et al. 2016). Generally, emulsions can be divided in two types, oil-in-water (o/w) and water-in-oil (w/o), nevertheless there are also the multiple phase emulsions systems, which can be either oil-in-water-in-oil (o/w/o) (oil droplets entrapped in water droplets which in turn are dispersed in a continuous oil phase) or water-in-oil-in-water (w/o/w) (water droplets inside oil droplets that are dispersed in a continuous water phase). This classification is based on the extent of each phase (the larger one is typically the continuous phase) and on their spatial organization (McClements & Rao 2011; Barrow et al. 2013; Rodríguez et al. 2016). Nevertheless, there is also the Bancroft's rule which claims that the type of an emulsion is determined by the nature of the emulsifying agent used, with the continuous phase the one where

the emulsifying agent is more soluble. However, it has already been verified that the Bancroft rule is not always valid (Ruckenstein 1996; Besnard et al. 2014).

Food products like soup, mayonnaise, salad dressings, ice creams, beverages, milk, and butter are themselves or contain emulsions (Lam & Nickerson 2013; Serdaroğlu et al. 2015). However, emulsions typically have either a lack of thermodynamic or kinetic stability. Macroemulsions and nanoemulsions are thermodynamically unstable while microemulsions are thermodynamically stable but less kinetically stable than nanoemulsions (McClements 2004; Burguera & Burguera 2012; Gupta et al. 2016). To better realize the differences between emulsion systems it is useful to understand the concepts of thermodynamic and kinetic stability. Consider a system composed by two different energy states (one low energy and one high energy) that will be occupied by a large number of molecules, which have a tendency to occupy the state with lowest free energy. The distribution of the molecules between the two states at a thermodynamic equilibrium is given by Boltzmann distribution:

$$\frac{n_{\mathrm{low}}}{n_{high}} = exp\left(-\frac{(G_{low} - G_{high})}{kT}\right) \quad \textbf{Equation 1}$$

n_{low} and n_{high} are the number of molecules that occupy the low (G_{low}) and the high (G_{high}) energy state, respectively, k is the Boltzmann's constant (k = 1.38 x 10^{-23} J K^{-1}), and T is the absolute temperature. The higher the difference between the two free energy levels compared to the thermal energy of the system (given by kT), the higher the number of molecules in the state of lower energy will be. When a free energy barrier (given by $G_{low} - G_{high}$) between the two states exists the system may not be able to reach equilibrium in the moments following its formation, and thus is a thermodynamically unstable system. When this energy barrier is large enough to ensure that the thermodynamic instability of the system remains constant over time, the system can be considered kinetically stable (McClements 2004). In other words, the stability of an emulsion is its ability to remain unchanged over time. A thermodynamic stable system (e.g. microemulsion) is a system in equilibrium but very sensitive to physical and chemical changes (e.g. temperature) (Gupta et al. 2016; Abdolmaleki et al. 2016).

1.3.1 Emulsifiers and stabilizers

Emulsifiers are surface-active molecules which have both hydrophobic and hydrophilic components that adsorb to the interface between oil and water (McClements & Gumus 2016; Silva

et al. 2015). They facilitate the formation of emulsion droplets by lowering the tension between the two immiscible phases thus facilitating the droplet breakup, while also absorbing rapidly to the new droplets formed before they have the possibility to re-aggregate. They also play an important role stabilizing emulsions through the formation of an interfacial layer that generates strong repulsive forces (steric or electrostatic interactions) around emulsion droplets preventing them from aggregation over time. Without emulsifiers, an emulsion system would rapidly breakdown and return to the two oil and water immiscible phases (Msagati 2012; Silva et al. 2015; Bai et al. 2016; McClements & Gumus 2016; Chang & Julian 2016).

Some of the most common emulsifiers used in the food industry are: surfactants, phospholipids, proteins, and polysaccharides and these can be classified according to their electrical properties. Ionic surfactants, which are positively or negatively charged (e.g. sodium dodecyl sulfate, lauric arginate), non-ionic surfactants (e.g. tween, span) and zwitterionic (e.g. lecithin) (McClements & Rao 2011; Burguera & Burguera 2012). Emulsifiers can also be classified according to their lipophilic or hydrophilic character in a system, known as the hydrophilic-lipophilic balance (HLB). Nevertheless, the use of HLB in food industry is low due to the fact that it is only applicable for some emulsifiers (ie. It is not applicable for proteins, which are normally used as an emulsifier) (Barrow et al. 2013).

Emulsifiers also have an important role in the chemical stability of nanoemulsions. The interfacial film formed by emulsifiers can reduce the oxidation rate by affecting the localization and reactivity of pro-oxidant species presents in emulsion systems. Transition metals, for example, are pro-oxidant species usually present in aqueous phases, and by applying an emulsifier that provides positive charge to the emulsion droplets their positive superficial charge will in turn repeal the cationic metals from interacting with lipids susceptible to oxidation. (Uluata et al. 2015; Chityala et al. 2016).

Due to consumer concerns regarding the safety, toxicity and metabolic concerns associated with most of the widely used synthetic emulsifiers, the food industry now has a need for the use of "label friendly" ingredients, that in the case of emulsions consists in the use of natural emulsifying agents capable of forming and stabilizing nanoemulsions as or more effectively than traditional synthetic surfactants, which has led to an increase in interest in the use of polysaccharides and proteins as emulsifiers (Adjonu et al. 2014).

1.3.1.1 Proteins as emulsifiers

Proteins are biopolymers composed by a specific number and sequence of different amino acids (which are linked through peptide bonds and can be aromatic, aliphatic, positively or negatively charged or polar) which enables them to present different molecular and physicochemical properties and as so they can have very different functionalities, ranging from emulsification to gelation as well as foaming, thickening effects, and water binding ability. Some functionalities are intrinsic while others are due to the environmental factors to which the protein is subjected (e.g. pH, temperature and ionic strength) (Davidov-Pardo et al. 2015; de Souza Simões et al. 2017).

The emulsifying ability of proteins is particularly important for nanoemulsions production and stability, as proteins contain both polar and non-polar amino acids residues, and therefore have an amphiphilic character which enables them to adsorb to oil-water interfaces enabling the reduction of the superficial tension between the two phases (Ali et al. 2016; Lam & Nickerson 2013). During this process, proteins rearrange themselves (van der Waals forces, hydrophobic and electrostatic interactions are some of the forces that lead the conformational alterations), some unfolding occurs which leaves hydrophobic amino acids residues exposed and positioned to the oil phase while hydrophilic residues are positioned to the aqueous phase and the adsorbed proteins establish covalent and non-covalent interactions. At this point a strong visco-elastic layer at the interface of the emulsion droplets is formed, that offers them mechanical resistance against aggregation. These droplets can be positive or negatively charged, depending if the pH of the emulsion is above or below the isoelectric point of the used protein. The electrostatic repulsions between the droplets are higher when the interface is highly charged (Ali et al. 2016; Lam & Nickerson 2013).

The use of emulsifiers with antioxidant activity incorporated can be important to add chemical stability to nanoemulsions, and some proteins have antioxidant abilities (McClements & Gumus 2016). Beyond the emulsifying and stabilizing properties, food proteins also have an important nutritional value. They are materials General Recognized as Safe (GRAS) which is a requirement for applications in food industry, and are associated to high biocompatibility, being easy to use and have a low production cost. Once they suffer biodegradation from proteases the release of the encapsulated lipophilic compounds in the human gastrointestinal tract is also guaranteed. A diversity of proteins has already been applied (Figure 5) in the development of emulsified systems

with whey protein the most widely explored. However, its is necessary to be aware that some proteins have allergenicity (He et al. 2011; Adjonu et al. 2014; Raynes et al. 2014; Nejadmansouri et al. 2016).

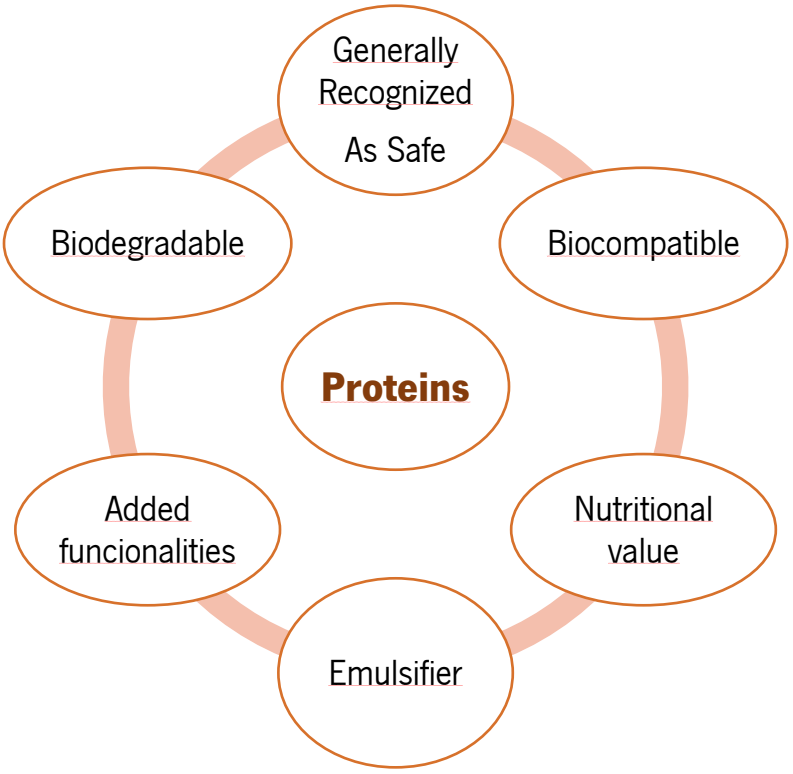

Figure 5. Functional and biological properties of proteins.

1.3.1.1.1 Milk proteins: the lactoferrin case

Milk contains a diversity of proteins with different properties and functionalities and has a high nutritional value due to the richness in essential amino acids which makes them particularly interesting for the development of bio-based products by the food industry. Among milk proteins, whey proteins are the most frequently used, including in its composition α-lactalbumin, β-lactoglobulin, bovine serum albumin, lactoferrins, and immunoglobulins (Abd El-Salam & El-Shibiny 2012; Ramos et al. 2015; Nejadmansouri et al. 2016).

Lactoferrin is a glycoprotein of the transferrin family with a molecular weight of 80 kDa and other than milk it can be found in other external mammalian secretions and it has the ability to bind two ferric ions in two structural lobes (Figure 6), having two different denaturation temperatures at 60 and 85 °C (Wei et al. 2001; Ye & Singh 2006). These proteins have an unusually high isoelectric point (pI) around 8.0-8.5 due to the high level of basic amino acids

presents in its sequence, meaning that in a vast range of pH values (above 8) it presents a positive charge which distinguishes it from most other milk proteins that have pI values between 4.5-5.5 and as such at neutral pH have a negative charge. Such characteristic is an advantage for its use in the stabilization of emulsion systems and in addition, lactoferrin also have very important biological functionalities (e.g. immuno-modulatory, anti-inflammatory, anticarcinogenic, antimicrobial, promotion of good gut flora, regulation of iron metabolism and antioxidant capacity, among other) (Wei et al. 2001; Ye & Singh 2006; Tokle & McClements 2011; Bourbon et al. 2016; Teo et al. 2016).

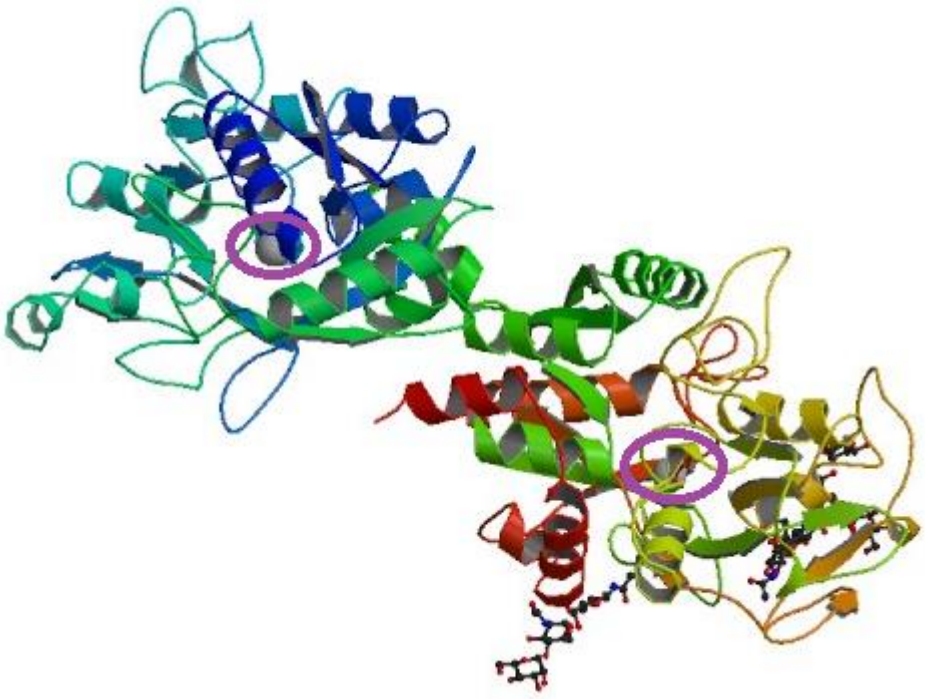

Figure 6. 3-D Structure of the bovine lactoferrin, with two ferric irons bind represented inside the purple circumference. Image obtained from PDB ID: 1BLF (Moore et al. 1997).

1.3.1.2 Other stabilizer agents

In order to facilitate the formation of nanoemulsions and enhance its stability, it is sometimes useful apply a combination of emulsifiers or an emulsifier and other stabilizers, such as cosurfactants, cosolvents, texture modifiers, weighting agents and ripening retarder agents. Cosurfactants are amphiphilic molecules with small polar head groups and so they are incapable of stabilizing an emulsion themselves although they possess surface activity. Cosolvents, are highly polar molecules and do not have surface activity but are able to change the surface activity of emulsifiers. Both cosurfactants and cosolvents are generally required for the formation of

nanoemulsions through low-energy methods of production (McClements 2004; Grumezescu & Oprea 2017).

The remaining stabilizers mentioned are normally used to enhance the stability of formed emulsions. The texture modifiers have the ability to retard droplets movements by thickening or gelling the aqueous phase and they can for example simply be used to alter the textural characteristics of a product providing, for example, creaminess. The weighting agents are added to oil droplets with the purpose of enhancing its density to match the aqueous phase density promoting stability against gravitational phase separation. Ripening agents are highly hydrophobic molecules that can be incorporated in oil droplets in order to prevent Ostwald ripening (McClements & Rao 2011).

1.3.2 Nanoemulsions advantages

Recently, nanoemulsions, especially o/w nanoemulsions, have taken an important role in the food industry mainly for the encapsulation of lipophilic bioactive compounds. (McClements et al. 2007; Serdaroğlu et al. 2015). Nanoemulsions are stable emulsion systems constituted by small droplets with a diameter below 200 nm, high surface-to-volume ratio, high bioavailability, high resistance to physical and chemical degradation over time (kinetically stable systems) (Figure 7). Such properties as well as a simple fabrication process differentiate these systems from other emulsions and makes them particularly suitable for food applications (Tadros et al. 2004; Silva et al. 2012; Walker 2015; Bai et al. 2016). O/w nanoemulsions are therefore particularly promising systems for encapsulation of ω-3 since they meet the required characteristic for application in food matrices such as enhanced water solubility, enhanced oxidative stability provided by an interfacial layer surrounding the oil droplets (and thus separating the lipophilic core from the pro-oxidative species highly present in aqueous phases), high bioavailability (efficient delivery systems that enable a rapid release of the lipophilic core and its rapid absorption)(Horn et al. 2013; Adjonu et al. 2014; Rodríguez et al. 2016).

Depending on the droplet sizes (below 100 nm) emulsions can have high optical clarity with conventional emulsions containing droplets with sizes close to the wavelength of visible light and so they scatter light while for nanoemulsions, particle sizes are significantly lower than the

wavelength of light that the light waves are poorly scattered which allows these systems to have a transparent or slightly turbid appearance (Mason et al. 2006; McClements & Rao 2011).

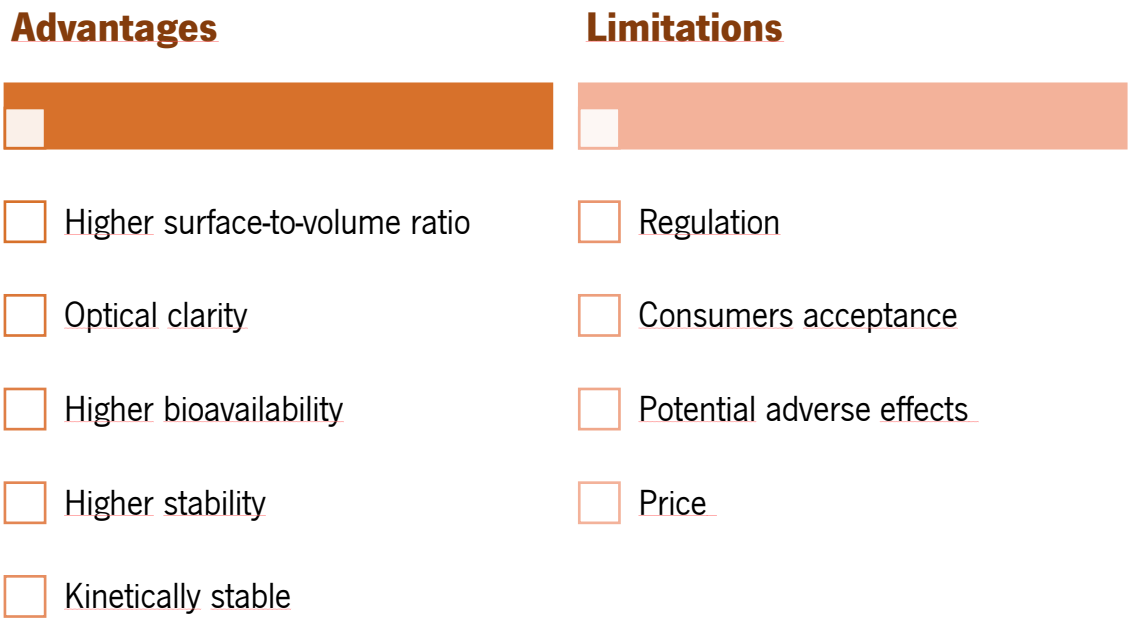

Figure 7. Advantages and limitation of nanoemulsions comparatively to conventional emulsions.

1.3.2.1 Nanoemulsions destabilization mechanisms

Nanoemulsions are, kinetically, highly stable systems, and thus thermodynamically unstable, and as such, they can breakdown even after a long-term stable period. The main mechanisms through which an emulsion can be disrupted are gravitational phase separation, droplet aggregation, and Ostwald ripening. Phase inversion of a nanoemulsion, consists in the inversion of the continuous and dispersed phases, can be considered a destabilization mechanism, although it is not usual and is also a process that can be intentionally used for nanoemulsions production (Heurtault et al. 2003; McClements & Rao 2011; Ali et al. 2016).

The dispersed and the continuous phase of an emulsion system do not have the same relative density and so a gravitational force acts on the emulsion droplets. When droplets have a lower density than the liquid surrounding they move upward and creaming is the gravitational mechanism that occurs (this tend to occur in o/w emulsion) (McClements & Rao 2011; Solans et al. 2005). On the other hand, when the droplets have a higher density they tend to move downwards and sedimentation is the separation mechanism (usually the case of w/o emulsions). However, the movement of the emulsion droplets can also be due to Brownian forces which are related with the

thermal energy of the system. Once nanoemulsions have reduced droplet sizes their movements are dominated by Brownian motion forces which enables the system to have enhanced stability against gravitational phase separation (Karthik et al. 2015; McClements & Rao 2011; Wooster et al. 2008; Solans et al. 2005).

Since emulsion droplets are in constant movement they collide several times and depending on the attractive and repulsive forces between them they can remain together or separate. The aggregation of emulsion droplets depends on the mechanisms responsible for the approximation, on the tendency of the thin layer separating the particles to rupture and on the existing hydrodynamic and colloidal interactions (van der Waals and hydrophobic interactions act as attractive forces while electrostatic and steric interactions act as repulsive forces) (McClements 2004; Karthik et al. 2015; Ali et al. 2016; Singh et al. 2017). From the collision between droplets, two outcomes are possible, flocculation or coalescence. In the first case, there is an association between two or more droplets but the individual integrity of each droplet is maintained and it normally occurs when the attractive interactions between the droplets overcome the long range repulsive interaction but not the short range repulsive interactions, this process can also be reversible, depending on the strength of the flocculation (David J. McClements 2004; Karthik et al. 2015; Ali et al. 2016; Singh et al. 2017). Coalescence, in turn, results from the junction of two or more droplets that merge forming a single larger droplet, and the rate of this destabilizing mechanism is closely related with the ability of the surfactant to surround the newly formed droplet and is influenced by several factors (e.g. salt concentration and pH). This process is irreversible and therefore accelerates phase separation (in the case of o/w emulsions lead to the formation of an oil layer on the top of the sample). Nanoemulsions tend to have better stability against these aggregation processes since the reduced size of the droplets is also associated with a decrease of attractive forces, with the steric repulsions less sensitive to the droplets size (McClements 2004; Karthik et al. 2015; Ali et al. 2016; Singh et al. 2017).

Ostwald ripening is a process that consist in the growth of the larger droplets in the emulsion due to the molecular diffusion of the dispersed phase from the smaller droplets through the continuous phase. In the case of o/w nanoemulsions, this mechanism is due to the water-solubility of an oil contained in a spherical droplet being higher in smaller droplets (Solans et al. 2005; Karthik et al. 2015; Singh et al. 2017). This effect is known as the Kevin effect and is caused by

the higher Laplace pressure associated with ultra-low size droplets. During the process, the increased number of oil molecules solubilized in the aqueous continuous phase surrounding the smaller droplets tends to move according to a concentration gradient around the larger droplets (where oil concentration is higher) leading to an increase of the mean size of the droplets over time, being this process considered the main destabilization mechanism occurring in nanoemulsions. The determining factor to its occurrence is the water solubility of the oil phase, with long-chain triglycerides (e.g. corn or fish oil) being the less water-soluble oils (Solans et al. 2005; Karthik et al. 2015; Singh et al. 2017).

The mechanisms described so far are related with changes of nanoemulsions physical properties, but these kinds of systems can also suffer chemical instability. When a nanoemulsion suffers chemical degradation, alterations occur in the type of molecules present mainly through oxidation or hydrolysis processes. The smaller the size of an emulsion droplets greater the surface area, which for the case of o/w nanoemulsions, can be a problem if the chemical degradation of oil occurs primarily at the interface of the droplets due to their reduced size which would contribute to the increase of the oxidation rate. Additionally, nanoemulsions that suffer chemical degradation by light are also more susceptible to destabilization, given that they can be easily penetrated by light. Thus, chemical degradation of an emulsion is majority related with the properties of the interfacial layer (McClements 2004; McClements & Rao 2011).

1.3.2.2 Nanoemulsions production methods

There is a broad range of approaches that can be used for nanoemulsions production, and these can be categorized as either high-energy or low-energy approaches (Gupta et al. 2016; McClements & Rao 2011; Silva et al. 2012). The high-energy approaches resort to mechanical forces generated by high-energy devices and such intense disruptive forces are capable of disrupting and intermingling the oil and aqueous phases into small oil droplets, thus forming nanoemulsions. On the other hand, the low-energy approaches make use of the intrinsic physicochemical properties of the materials (e.g. surfactants, oil phase) used in the system formulation. In these approaches, when specific changes on the solution composition or environmental properties take place, leading to the nanoemulsions formation (Tadros et al. 2004; McClements & Rao 2011; Silva et al. 2012; Gupta et al. 2016).

1.3.2.2.1 High-energy approaches

High-energy approaches are nowadays the most common techniques applied to prepare nanoemulsions in food industry. These methods are already well established for the production of conventional emulsions, are effectively at high production scales and can be applied using a diversity of starting materials (e.g. surfactants, phospholipids, proteins, polysaccharides, synthetic emulsifiers). In the high-energy approaches, there are three main categories according to the devices used in the process, which can be high-pressure homogenizers, high-speed devices and ultrasonic devices (McClements & Rao 2011; Yang et al. 2012; Komaiko et al. 2016).

High-pressure homogenizers are most commonly devices applied currently in food industry, using high pressures to force the mixture (oil phase, aqueous phase, and emulsifier) to pass through a valve which causes shear, impact and cavitation forces that lead to the deformation and disruption of droplets, increasing its surface area, and simultaneously leading to the adsorption of emulsifiers and thus to the stabilization of droplets. (Silva et al. 2012; Silva et al. 2015). High-pressure valve homogenizers make use of a pump that forces the feed emulsion through a chamber where in the end is a valve by which the feed is forced to pass, forcing the droplets to suffer disruptive forces that cause its breaking. Microfluidizers are devices similar to high-pressure valve homogenizers, with the difference between the two in the design of the channels through which the emulsion is forced to pass. The flow is divided through a channel into two streams, each being directed to a different finer channel, and at the end, the two streams are directed at one another in an interaction chamber (McClements & Rao 2011; Silva et al. 2012; Adjonu et al. 2014). These high-pressure homogenization techniques are more effective in the reduction of particles sizes when pre-existing coarse emulsions are used instead of a feed constituted by two separated liquids. The coarse emulsion can be efficiently formed by using a high-speed device (e.g. Ultra Turrax), which are not very efficient in the nanoemulsions formation by themselves given that the energy generated by them is mostly dissipated through heat form (Silva et al. 2012; Ali et al. 2016).

Ultrasonic homogenizers resort to generate the intense disruptive forces required for nanoemulsions formulation through high-intensity ultrasonic waves, forming micro-bubbles droplets by cavitation, which is the phenomena resulting from the motion induced in particles in the medium through a series of compressions and rarefactions caused by sound waves under fluctuating pressure, resulting in the collapse of the bubbles initially formed and causing intense

disruptive forces in the vicinity of the sonicator probe leading to nanoemulsions formation (McClements & Rao 2011; Abbas et al. 2013).

1.3.2.2.2 Low-energy approaches

In low-energy approaches, nanoemulsions formation takes place through phase transitions based on alterations of the physicochemical properties of the materials and can be categorized in two main groups, the spontaneous phenomena, which comprise processes such as spontaneous emulsification (or self-emulsification like is also known), solvent displacement, and the phase inversion techniques which comprise phase inversion temperature and phase inversion composition (Solans & Solé 2012; Adjonu et al. 2014). These approaches can be more effective in producing emulsions with reduced sizes than the previous high-energy approaches, but they are very dependent on the material properties, thus limiting the possibilities for systems composition, and often require the use of high amounts of surfactants. Nanoemulsions produced by these techniques also tends to suffer from long-term instability, and these factors limit its industrial application (McClements & Rao 2011; Yang et al. 2012).

Spontaneous emulsification is a technique that can be performed at a different range of temperatures and only requires stirring, making use of the chemical energy released due to a dilution process (McClements & Rao 2011; Silva et al. 2012). The phase to be dispersed (e.g. oil), as well as an emulsifier, are added to the continuous phase under constant stirring, and when the two phases are brought in contact the original location of some components shifts and leading to the diffusion of the miscible components with the continuous phase (e.g. water). During this process the oil-water interface increase, the interfacial turbulence is enhanced and the spontaneously formation of the emulsion droplet occurs (e.g. o/w) (Qadir et al. 2016; Solans & Solé 2012; Solans et al. 2016). The size of droplets is variable and dependent on the systems composition (e.g. ratio between oil and dispersed phase), environmental conditions (e.g. temperature) and mixing conditions (e.g. stirring speed). In the solvent displacement technique, a water miscible organic solvent (e.g. acetone, ethanol) is required in order to dissolve the oil phase followed by its addition to a water phase containing an emulsifier. The rapid diffusion of the organic phase leads to the formation of nanoemulsions, with a solvent removal step necessary at the end of the process (McClements & Rao 2011; Qadir et al. 2016; Silva et al. 2012; Solans & Solé 2012; Solans et al. 2016).

Phase inversion techniques consist in the manipulation of the temperature or the system composition and does not require the use of solvents, making use of the ability of some surfactants to change water and oil affinities due to temperature or composition change, provoking changes in the physicochemical properties of the surfactant causing its geometric alteration. These changes occur in the surfactant spontaneous curvature, and when curvature occurs from negative to positive the produced emulsions are o/w type, and if the alteration is from a positive to a negative curvature, w/o emulsions are formed. (McClements & Rao 2011; Silva et al. 2012; Solans & Solé 2012).

1.4 Drying techniques applied to nanoemulsions

In the food industry encapsulation techniques, such as emulsification are usually applied in combination with drying techniques, in order to ensure microbial stability and prevent chemical or biological degradation enabling the improvement of the suspensions stability as well as the extension of shelf-life while also enabling reduced storage and transport costs of the final product. There are several drying techniques that can be applied, being spray drying and freeze drying the most common ones (Davidov-Pardo et al. 2015; Ezhilarasi et al. 2013; Gharsallaoui et al. 2007; Sances et al. 2015; Zhao et al. 2015).

1.4.1 Spray dryer

The traditional spray dryer method consists of four crucial steps: (a) atomization of the liquid feed, (b) spray-air contact, (c) formation of dry particles by the drying of the spray and (d) separation and collection of the dry product (Lee et al. 2011). The liquid is sent to the drying chamber through an atomizer or nozzle that can be a rotary atomizer, a single-fluid high-pressure nozzle or a two-fluid nozzle, energy is applied and the atomization step takes place. The resultant spray, composed by fine droplets, enters in contact with the hot drying gas and solvent evaporation occurs, resulting in a solid and dry powder. A cyclone or a bag filter is then utilized to separate the powder from the drying gas, and small particles are accumulated in a glass collector (Lee et al. 2011; Jacobs 2014; Sosnik & Seremeta 2015).

Spray-drying is a well-established process largely used by pharmaceutical and food industries. Despite the high temperature required in this technique, it can be applied even in heat sensitive materials considering that the drying speed is extremely high and thus the heat exposure time is extremely low. Also, the temperature at the encapsulation surface is not as high as the gas flow

temperature due to the cooling effect of the evaporating solvent (Bürki et al. 2011; Li et al. 2010). This method is an attractive option for the production of nanoparticles being an easy, cheap and highly reproducible process that is easy to scale up at the industrial level. It also allows the adjustment of some capsules properties such as size and bulk density through the manipulation of process parameters (e.g. the feed solution viscosity) and spray dryer configuration (e.g. atomizer type, spray flow rates, feed rates, inlet temperature). Considering that spray drying is a technique that highly depends on the materials properties its parameter optimization is usually done as a trial and error approach. For this purpose, a laboratory scale system of spray dryer, is usually used to achieve the best spray drying conditions to a certain product before scaling up the drying process (Fathi et al. 2014; Lee et al. 2011; Paredes et al. 2016).

1.4.1.1 Nanospray dryer

Recently, a new generation of laboratory scale spray dryer system was developed to produce nanoparticles by spray dryer. This equipment developed by BÜCHI (Flawil, Switzerland), is the Nano Spray Dryer B-90. It is equipped with new technologies that enable the production of powders in the nano size range by using an improved atomization process composed by a vibrating mesh technology which consists in a stainless steel mesh with thin perforations (precise micron-sized holes of 4, 5.5 or 7 μm) coupled to a piezoelectric actuator. The ultrasonic frequency vibrations produced in the actuator enable the injection of the smaller droplets comparatively to conventional atomization processes, while the electrostatic particle collector system allows for the recovery of smaller particles contrary to conventional cyclone collectors where particles smaller than 2 μm are rarely recovered (Lee et al. 2011). The dried powder is directed to an electrostatic precipitator composed by a grounded start electrode and a cylindrical particle collecting electrode oppositely charged which creates an electrostatic field directing the powder particles deposition onto the inner wall of the cylindrical collector, where the nanocapsules are recovered with a rubber spatula. The device also uses a laminar drying gas flow which enables a mild, uniform and instant heating. This production technique has already been applied in the production of protein nanoparticles and the drying of bioactive compounds among other applications (Table 2). Furthermore, the Nano Spray Dryer B-90 was appointed as a one-step solution-based alternative with great yields (Bürki et al. 2011; Lee et al. 2011; Paredes et al. 2016; Sosnik & Seremeta 2015).

Table 2. Example of systems already developed using the nanospray dryer. Materials applied and the propose of nanospray drier application.

WALL MATERIALS	ENCAPSULATED COMPOUND	STRUCTURE DEVELOPED / PROPOSE	REFERENCE
Arabic gum Cashew nut gum Sodium alginate Sodium carboxymethyl cellulose Ammonium methacrylate copolymer (Eudragit)	Vitamina B12	Nanocapsules / production	(Oliveira et al. 2013)
Chitosan/pectin	Insulin	Nanocapsules / drying	(Al-Azi et al. 2014)
Sodium chloride	None	Nanoparticles / production	(Moncada et al. 2015)
Pectin/LDL	Curcumin	Nanogels / drying	(Zhou et al. 2016)
Silk	None	Nanoparticles / drying	(Kazemimostaghim et al. 2014)
Polyvinylpyrrolidone	Rifampicin	Nanocapsules / production	(Noraizaan & Wong 2017)
Magnesium sulfate	None	Nanoparticles / production	(Sarma et al. 2014)
Carboxymethyl cellulose	Soybean	Nanocapsules / production	(Gaudio et al. 2016)
Soy lecithin Sodium caseinate Pectin	None	Solid lipid nanoparticles / drying and coating	(Wang, Hu, Zhou, Xue, et al. 2016)
Lecithin Gum arabic	Eugenol	Nanoemulsions / drying	(Hu, Gerhard, et al. 2016)
Gallic acid-chitosan conjugate Gum arabic Polyethylene glycol	None	Nanoparticles / production	(Hu, Wang, et al. 2016)
Chitosan Tween 20	Curcumin	Nanocapsulesles / production	(Toole et al. 2012)
Sodium alginate Pectin	Gentamicine sulphate	Nanocapsules / production	(Cicco et al. 2014)
Chitosan	None	Nanoparticles / production	(Demir & Degim 2016)

Polycaprolactone Sodium deoxicholate	Dexamethasone	Nanocapsules / production	(Beber et al. 2014)
Beta-Galactosidase Trehalose	None	NAnoparticles / drying	(Bürki et al. 2011)

1.4.2 Freeze dryer

Freeze drying, also known as lyophilization is a process widely used to dehydrate diverse materials, especially heat-sensitive materials. This technique is applied at low temperatures and in the absence of air, which prevents the degradation of the active compounds encapsulated either by chemical or biological alterations, while also enabling the preservation of both the shape and primary structure of the drying products without significate volume losses. However, when compared to spray drying techniques this method has higher costs and longer production times and the resulting powders are usually more porous and therefore less stable, resulting in higher storage and transportation costs (Ratti 2001; David Julian McClements 2004; Anwar & Kunz 2011).

This method is based on the dehydration by sublimation of a frozen material and can be divided in three stages, freezing, primary drying, and secondary drying (Abdelwahed et al. 2006). Freezing as the first stage is the step of solidification of the liquid product, with the formation of, water crystals during the cooling of the liquid and increasing the concentration of the remaining liquid constituents. This concentration increase is accompanied by a viscosity enhancement which inhibits further crystallization. Next, in the primary drying, the ice sublimation takes place. At low pressures, a heat transfer to the frozen solution causes the ice sublimation. The water vapor passes through the dried portion of the material, reaches the surface and is transferred to the condenser where it condenses. The porous are formed at this stage due to the empty space left by the sublimated ice crystals in what is the longest step of the entire process. Finally, a second drying is required, as the product still contains 10-35 % of bound water, occurring through the desorption of the unfrozen water (Abdelwahed et al. 2006; Ezhilarasi et al. 2013; Morais et al. 2016).

1.5 Limitations of using nanotechnology on food industry

The term nanotechnology emerged in 1974 by the scientist Norio Taniguchi (Raynes et al. 2014). Nanotechnology is now the field of science responsible for the production and

characterization of structures, devices, and systems with shape and size controlled with one or more dimensions between 1 and 100 nanometers, although this terminology is not stringently enforced (Cerqueira et al. 2014; Raynes et al. 2014). At the nanometer scale materials acquire new proprieties and functions that can be explored allowing the production of food with improved quality for consumers and competitive advantages for enterprises. As so, nanotechnology offers many potential advances in food industry, such as improvement of food properties (e.g. bioavailability, color, texture, and flavor), protection and delivery mechanism (e.g. encapsulation) that allow the nutritional improvement of foods, extension of products shelf-life, and development of biosensors enabling the improvement of quality and safety of food products (Cerqueira et al. 2014; Chaudhry & Castle 2011; Contado 2015; Raynes et al. 2014).

Even with the great potential of nanotechnology, there are also safety issues and risks that can be associated with engineered nanomaterials (ENMs), these concerns are essentially related with the potential adverse effects that these materials with reduced size can cause in human health and environment. Scarce information is known about the side effects of ENMs but with the growing number of products containing ENMs the exposure of the general population and environment is increasing, as well as consumers concerns (Martirosyan & Schneider 2014). However, in the lack of knowledge has made difficult the establishment of guideline rules, leading to different countries with guidelines, that are not always in concordance between them. (Rossi et al. 2014). The EFSA establish that the analysis of parameters and approaches to assess the dangers of ENMs should be established in a case-by-case basis (EFSA Scientific Committee 2011). Every year EFSA publishes a report to improve and harmonize the guidance for ENMs containing the novel data acquired in the study of individual nanomaterials, the new methodologies developed for risk assessment, while the approaches or understandings still unreached are reviewed and actualized.

With growing demands on food properties and related benefits, agri-food industries are increasingly searching for new solutions namely in the nanotechnology field. Despite the lack of certainty regarding its regulations, it is expected that nanotechnology based products reach a market value of one trillion US dollars (), with food encapsulation estimated to reach a market value of 39 billion US dollars. Presently, there are already a lot of nanoemulsions for nutritional purposes available on the market, such as Syngenta, a company in Switzerland with the Primo Maxx nanoemulsions; Aquanova in Germany which has developed the NovaSol® Beverages; and

Improveat, a Portuguese company that has developed the BioNutriCoat a coating formulation with both nutritional and packaging functions where is possible the use of nanoemulsions (Cerqueira et al. 2017).

Chapter 2. Motivation and objectives

Nowadays people are increasingly concerned about how their health is affected, both on a daily basis and on the long term, with prevention efforts taking a key role. With these concerns in mind consumers demands beneficial food solutions that offer health advantages so, food industry searches for ways to enhance foods nutritional benefits while increasing its quality and organoleptic properties.

ω-3 fatty acids have a well-known association to a diverse range of long term health benefits, but are present in Western diets in extremely low amounts. These fatty acids are also very sensitive to oxidation and have an intense odour and low dispersibility in water, features that makes its addiction to food matrixes a challenge. Food industry has so a great interest in finding solutions that enable the application of ω-3 into foods without altering its organoleptic properties (e.g. appearance and odor), and encapsulation of ω-3 through the application of nanotechnology is a promise way to reduce or eliminate its limitations, allowing for its application in food for adequate levels of daily consumption.

With all of this on mind, the main objectives of this thesis are:

- Development of bio-based nanoemulsions using the milk protein Lf as emulsifier;
- Characterization of the nanoemulsions produced evaluating the effect of the Lf concentration applied on its properties;
- Evaluation of different storage conditions;
- Assessment of physical and chemical stability;
- Encapsulation efficiency of the nanoemulsions;
- Ability of nanoemulsions drying by different techniques (freeze-drying and nnanospray drying);
- Characterization of the obtained powders;
- Evaluation of the rehydration ability and characterization of the rehydrated solutions.

Part II – Experimental Work

Chapter 3. Materials and Methods

3.1 Materials

Lf purified powder was purchased from DMV International (USA), with a composition expressed as dry weight percentage of 96 % protein, 0.5 % ash, 3.5 % moisture and an iron content around 120 ppm. ω-3 (Biofil, Portugal) was purchased, with a reported composition expressed weight percentage of 35 % (EPA), 25 % (DHA) and 28 % Vitamin E (acting as natural antioxidant).

Hydrochloride acid 37 % (v/v), methanol and hydrogen peroxide 30 % (w/w) were obtained from CHEM-LAB (Belgium). 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), iron (II) sulfate 7-hydrate and ammonium thiocyanate were purchased from Sigma-Aldrich (UK, USA and Germany). Bariu chloride dihydrate and Isooctane were obtained from Merck (Germany). Isopropanol and chloroform were purchased from Fisher Chemical (UK). Phosphate buffer and iron (III) chloride 6-hydrate were obtained from Panreac (Spain). Sodium hydroxide was obtained from José Manuel Gomes dos Santos (Portugal).

3.2 Methods

3.2.1 Nanoemulsions production

The oil-in-water emulsions were developed according to Pinheiro et al. (2016) and Acevedo-Fani et al. (2017) with some adjustments. Briefly, nanoemulsions containing ω-3 were produced using Lf as emulsifier. For homogenization 5 % (w/w) of ω-3 oil with 95 % (w/w) aqueous emulsifier solution (Lf was used at concentrations 0.2, 0.6, 1, 2, 3, 4 and 5 % (w/w)) were used. A pre-mixing was applied during 2 min at 12000 rpm using a high-shear blender (Ultra-Turrax T25, Ika-Werke, Germany), which was followed by high-pressure homogenization (Nano DeBEE, BEE International, USA) performed at 20,000 Psi (137.9 MPa), during 5 cycles. A refrigeration system was used during the homogenization ensuring that the temperature was maintained below 60 °C in order to prevent Lf denaturation and oil oxidation. The resulting emulsions were then adjusted to pH 4 using 0.1 M hydrochloride acid and 0.1 M sodium hydroxide, and stored at 4 °C in the dark.

3.2.2 Nanoemulsions physical properties

3.2.2.1 Size and PdI

Size and polydispersity index (PdI) of the nanoemulsions were determined by dynamic light scattering (DLS) in a Zetasizer Nano ZS (Malvern Instruments Limited, UK) equipped with a He-Ne laser at a wavelength of 663 nm. All the measurements were performed at 25 °C using 12 mm square polystyrol/polystyrene cuvettes and a detection angle of 173° (Malvern 2013). Before the measurements the nanoemulsions were diluted at a ratio of 1:1000 using distilled water in order to avoid multiple scattering effects. The mean diameter of the droplets was calculated by the instrument through the measurement of the rate at which scattered light fluctuates using the Stokes-Einstein equation, which assumes the sphericity of the detected droplets (Malvern 2013). All the samples were performed in triplicate with three readings for each sample, and the results are expressed as the average ± standard deviation.

3.2.2.2 ζ- potential

The ζ-potential of the nanoemulsions were determined through dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Limited, UK) equipped with a He-Ne laser at a wavelength of 663 nm. All the measurements were performed at 25 °C using disposable capillary cells (DTS1070) and a detection angle of 17° (Malvern 2013). Before the measurements the nanoemulsions were diluted at a ratio of 1:1000 using distilled water. The ζ-potential is calculated through the measurement of the direction and velocity of the droplets movement in the applied electric field using the Smoluchowski model (Malvern 2013). All the samples were performed in triplicate with three readings for each sample, and the results are expressed as the average ± standard deviation.

3.2.2.3 Interfacial tension

The interfacial tension of the nanoemulsions solutions were measured by the Ring method represented in Figure 8 and described by Souza et al. (2017) using a KRÜSS K6 Tensiometer (KRÜSS GmbH, Hamburg, Germany) equipped with a 1.9 cm De Noüy platinum ring. All the

measurements were performed at room temperature. The results are expressed as the average ± standard deviation of five measurements.

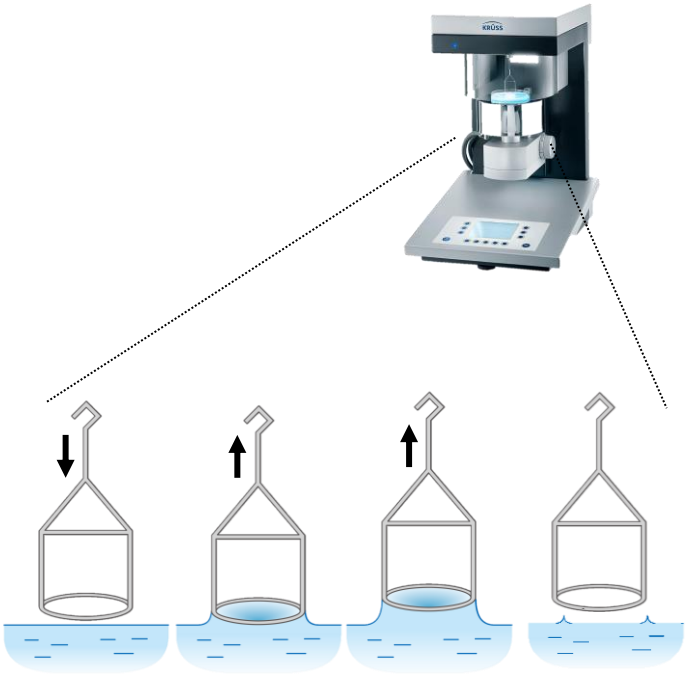

Figure 8. Representative scheme of the Ring method for interfacial tension measurements.

3.2.2.4 TEM

The nanoemulsions morphologic characterization was achieved by transmission electron microscopy (TEM). For the observation, 10 µL of nanoemulsions were deposited onto TEM grids (ultra-thin carbon film on Lacey carbon support film, 400 mesh, Copper, Ted Pella Inc., USA) and the liquid excess removed with a filter paper. Afterwards they were negatively stained with 1% (w/v) uranyl acetate for observation and samples were air-dried before analyses. The samples were observed using a JEM-2100 transmission (JEOL, Japan) electron microscope operating at a 200 kV accelerating voltage.

3.2.3 Nanoemulsions stability

Nanoemulsions were stored in the dark at two different temperatures: 4 °C and room temperature for 66 days and its size, PdI and ζ-potential were monitored through DLS as described above (sections 3.2.2.1 and 3.2.2.2). All the selected formulations were prepared freshly and in triplicate. Three readings were performed for each sample, and the results are expressed as the average ± standard deviation.

For chemical stability assessment, the selected nanoemulsions were freshly prepared in triplicate and stored in the dark at 4 °C during 35 and 36 days for DPPH• free radical scavenging activity and peroxide value determination, respectively.

The nanoemulsions ability to scavenge the free cationic radical 2, 2'-azino-bis 3-ethylbenzthiazoline-6- sulfonic acid (ABTS) was also investigated, however the application of this assay was not successful even after adjustments (data not shown).

3.2.3.1 Radical scavenging capacity

The DPPH• free radical scavenging assay was performed according to Ballesteros et al (2015) with some modifications. For each sample, a dilution series (six different concentrations) was prepared using methanol. The reaction was carried out in a 96-well microplate containing 25 µL of sample and 200 µL of 150 µM DPPH• solution (DPPH• was dissolved in 80% methanol to an absorbance value of 0.700 at 515 nm). The prepared microplate containing the reaction mixture was kept in agitation in the dark at room temperature during 30 min (Du et al. 2014; Okoh et al. 2014; Yang et al. 2009). The absorbance was then measured at 515 nm in an Elisa Biotech Synergy HT (Biotek, USA) microplate reader. A calibration curve was prepared with a standard solution of Trolox diluted in methanol (40, 80, 100, 300, 400 and 600 µM). DPPH• inhibition percentage data was plotted as a function of sample concentration to obtain IC_{50} values, which denote the concentration of sample required to scavenge 50 % of DPPH• radicals. The DPPH• inhibition percentage was calculated according to the follow equation:

$$Inhinition\ percentage = \frac{A_0-(A-A_b)}{A_0} \times 100 \quad \textbf{Equation 2}$$

Where A_0 is the absorbance at 515 nm of DPPH• without sample, A is the absorbance at 515 nm of samples and DPPH• and A_b is the absorbance at 515 nm of samples without DPPH•.

3.2.3.2 Peroxide values

The peroxide value of the nanoemulsions was determined by the International Dairy Federation (IDF) method using the procedures described by Shantan & Decker (1994) and Karthik et al (2016) in combination. The ω-3 was extracted from the nanoemulsions by adding 300 µL of these to 1.5 mL of isooctane/isopropanol (3:2 v/v) solution. This mixture was allowed to stand for 30 minutes after been vortexed 10 seconds, three times each sample. To determine the peroxide value a proportion of the clear upper solvent layer (30 to 100 µL, depending on the extent of

peroxidation) was collected and added to a solution of chloroform/methanol (7:3 v/v), making a total volume of 3 mL in a glass tube. After, 15 μL of ammonium thiocyanate were added to the mixture and vortexed by 5 seconds, followed by the addition of 15 μL of iron (II) solution and vortexed again for 5 seconds. The mixture obtained was then incubated at room temperature in a dimmed lit compartment for 5 minutes and the absorbance was measured at 500 nm by UV/VIS Spectrophotometer (Jasco V560, USA) using a quartz cuvette. A blank that contain all the reagents except the sample was also read and a calibration curve of Fe^{3+} (concentration *versus* absorbance) was performed. The peroxide value was calculated in milliequivalents (meq) of peroxide per kilogram of sample using the equation:

$$Peroxide\ value = \frac{(A_s - A_b) \times m}{55.84 \times m_0 \times 2} \quad \textbf{Equation 3}$$

where, A_s represents the absorbance of the sample, A_b the absorbance of the blank, m the slope of the calibration curve and m_0 is the mass in grams of the sample.

3.2.4 Encapsulation efficiency

The encapsulation efficiency (EE) of the nanoemulsions was determined by measuring the amount of ω-3 that was not encapsulated as previously reported (Azevedo et al. 2014). The non-encapsulated ω-3 was separated from the encapsulated through a membrane separation method using an Amicon® Ultra-0.5 centrifugal filter device (Amicon® Ultra - 0.5 mL filter with 10 kDa cut-of Millipore Corp., Ireland). Thus, 0.5 mL of nanoemulsions were applied in the Amicon® and centrifuged at 14 000 g for 15 min. the resulting filtrate contained the free ω-3 while the membrane retained a concentrate of the particles with ω-3 encapsulated. For the EE calculation, the free ω-3 in the filtrate was evaluated spectrophotometrically at 250 nm in an Elisa Biotech Synergy HT (Biotek, USA) microplate reader. The ω-3 was determined from a calibration curve consisting of different ω-3 concentrations prepared with heptane and also measured at 250 nm (García-Moreno et al. 2017). The calculation was performed according to the following equation:

$$EE\ \% = \frac{\omega 3_{total} - \omega 3_{free}}{\omega 3_{total}} \times 100 \quad \textbf{Equation 4}$$

Where, $\omega 3_{total}$ represents the total amount of ω-3 added to the system while $\omega 3_{free}$ represents the ω-3 free accounted in the filtrate.

3.2.5 Drying of nanoemulsions

3.2.5.1 Freeze-drying of nanoemulsions

Nanoemulsions freshly prepared were protected from light and were first frozen for 24 hr at -20 °C, and then frozen at -80 °C. Freeze drying was carried out in a pilot scale freeze dryer (CHRIST - Alpha 1-4 LD plus, Germany). The freeze-drying operation temperature was maintained at -40 °C for a drying period of 24 hr. The samples powders obtained were stored at room temperature in a desiccator until further use.

3.2.5.2 Nanospray-drying of nanoemulsions

The nanoemulsions freshly prepared were dried using a Nano Spray Dryer B-90 (BÜCHI Labortechnik AG, Flawil, Switzerland) schematically represented in Figure 9. The equipment has a piezoelectric vibrating membrane with thin perforations (spray mesh) in the spray head, which atomizes the feed. The feed was diluted 1:200 with distillated water in order to facilitate the spray drying process and also to prevent blockage of the recirculating system. During the process, the feed was kept at a constant magnetic stirring and protected from light. The liquid sample was fed to the spray head by a pump and the actuator functioning at around 60 kHz. The Inlet temperature (T_{in}) was kept at 80 °C, the spray relative rate at 100 % and the size of the spray cap utilized was 7.0 μm. Compressed air was used as the drying gas and its flow ranged between 100-120 L/min. The outlet temperature (T_{out}) varied between 40-46 °C and the pressure between 30-34 mbar. The dried particles were then accumulated by an electrostatic particle collector and later recovered with a particle scraper. The samples powders obtained were stored at room temperature in a desiccator until further use.

A different T_{in} were also tested (data not show), maintaining the remaining conditions unaltered. In order to promote the integrity of the protein, which is in the surface of the emulsion droplets and therefore more exposed to the temperatures of the drying chamber, only the particles obtained with a T_{in} 80 °C were utilized in further tests since this temperature is below the second melting temperature of lactoferrin (Wei et al. 2001; Ye & Singh 2006).

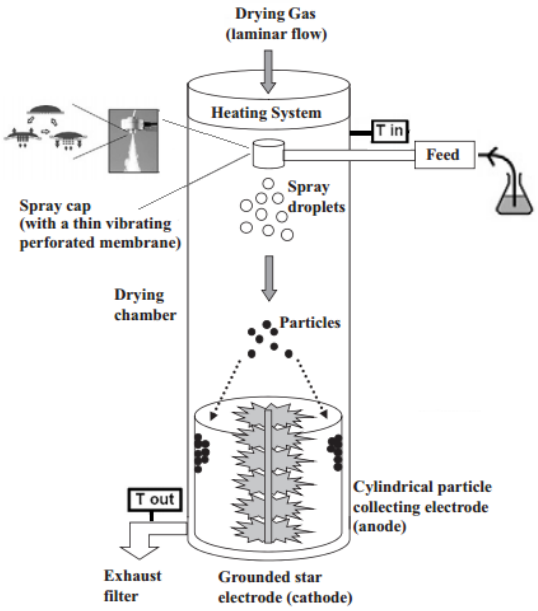

Figure 9. Schematic representation of the laboratoty-scale Nano Spray Dryer B-90. Adapted from Lee et al. (2011).

3.2.6 Rehydration of the dried nanoemulsions

The powders obtained by freeze-drying were rehydrated with distillated water and kept under magnetic stirring overnight. The rehydration solutions were then centrifuged at 1400 rpm for 15 minutes. The clear upper layer was recovered and used in further tests. The amount of water applied was considered to allow comparison of the results obtained for this rehydration solutions with the one obtained for the initial nanoemulsions, taking into account their composition.

The powders obtained by nanospray-drying were also rehydrated with distillated water and maintained under magnetic stirring overnight. It was verified by particles in suspension that the powders had not rehydrated and sonication (Sonicator 5510E-MT Bransonic, USA) was performed for 15 minutes in an attempt to facilitate rehydration, which had not occurred. An ultra-centrifugation was then performed (Micro-Ultracentrifuge MX120+ Thermo Fisher Scientific, Japan, at 26000 rpm for 30 minutes and the water solvent recovered and replaced by phosphate buffer 0.25 M pH 5.2. The new rehydration solutions were again sonicated for 15 minutes. Even though several particles remained in suspension, the solutions were kept and used in further tests.

The solutions obtained by the rehydration of the powders dried by the different drying techniques were analyzed by DLS to obtain information's about the size, PdI and superficial charge,

as described above. In situations where the need arose the solutions were filtrated using a 0.2 μm syringe filter (acetate cellulose membrane).

The rehydration solutions of the freeze-dried powders were also subjected to the radical scavenging capacity assay and peroxide value determination, both applied as described above. Given that the powders obtained by nanospray-drying were not properly rehydrated and were not dispersible in the reagents used in both assays, its proper application was not possible (data not shown).

The morphologic characterization of the solution resulting from the rehydration of the powders obtained by both freeze-drying and nanospray-drying was performed by TEM as described before (section 3.2.2.4).

3.2.7 Scanning electron microscopy (SEM)

The morphologic characterization of the dry particles obtained after booth freeze-drying and nanospray-drying were performed by Scanning electron microscopy (SEM) using a Quanta FEG 650 (FEI, USA). Dry samples were affixed on aluminum stubs covered by carbon ribbon, and then the samples were coated with gold and observed using an accelerating voltage of 5 kV under vacuum conditions.

3.2.8 Data analysis and statistical treatment

The data analyses were performed using Microsoft Windows Excel (Microsoft Office 365 ProPlus) and the free software ImageJ (version 1.50i). The statistical analyses were carried out using the software STATISTICA version 10.0 (StatSoft Inc. 2011). The statistical significance of differences among treatment was evaluated by a factorial ANOVA test followed by the Tukey HSD test with significance at $p<0.05$.

Chapter 4. Results and Discussion

The main objective of this work was the development of a biobased nanostructures with ability to protect ω-3 fatty acids from serious degradation issues, allowing its easier and safe application in foods. Lf nanoemulsions containing 5 % (w/w) fish oil were prepared and further characterized. The nanoemulsions formulations presenting better results were then evaluated in terms of stability during storage under different temperatures (4 °C and 25 °C). The emulsions with higher stability under storage were then evaluated in terms of encapsulation efficiency. Some of these nanoemulsions were also dried by two different processes obtaining particles at both nano and micro scale, which were morphologically characterized. The properties after a rehydration process of such particles were also assessed and compared to the ones of the starting nanoemulsions.

4.1 Nanoemulsions produced with different Lf concentration

Nanoemulsions with different Lf concentration were produced through a high-energy process (high pressure homogenization) and the differences in its physical properties were analyzed. The influence of Lf concentration in nanoemulsions was evaluated in terms of superficial charge (ζ-potential), size and polydispersity index (PdI). These parameters are extremely relevant concerning to the stability and homogeneity of the droplets of an emulsion (McClements & Rao 2011; Silva et al. 2012). Furthermore, the interfacial tension and the viscosity of the different formulations were accessed, once they can influence the nano-spray drying process (McClements & Rao 2011; Silva et al. 2012). The morphological properties of the different formulations were also analyzed by TEM.

4.1.1 Size and superficial charge properties

The use of different amounts of Lf in the emulsions production influenced significantly (p<0.05) the size and superficial charge of the emulsion droplets obtained (Figure 10). In general, high Lf concentrations lead to nanoemulsions with low size and higher superficial charge. All emulsions present sizes in the nano range (diameter lower than 200 nm) except the emulsions developed with 0.2 % (w/w) Lf which had a diameter of 223.2 ± 8.1 nm. The smallest size obtained was 145.8 ± 2.3 nm at a Lf concentration of 5 % (w/w). These results suggest that for lower Lf concentrations the surface of the droplets disrupted during the homogenization is not fully covered allowing their increase by process such as coalescence. With higher amounts of protein present in the aqueous phase the coating of the newly formed droplets in the homogenization process is

facilitated and quickly therefore preventing the growing of oil core (Acevedo-Fani et al. 2017). These results are in agreement with the obtained by Acevedo-Fani et al. (2017) which also used Lf as emulsifier in the formulation of the first layer of a multilayer emulsion system developed for the entrapment of resveratrol using a corn oil phase of 5 % (w/w). These authors observed that using higher protein concentrations a decrease in nanoemulsions size is promoted (Acevedo-Fani et al. 2017). On the other hand, the results presented by Ye and Singh (2006) only show a significant size reduction with the increase of Lf concentration until rich 1 % (w/w). For higher Lf concentrations the differences are not significant. This difference can be explained by the oil phase of the system that is 30 % (w/w) while in this work is 5 % (w/w) (Ye & Singh 2006).

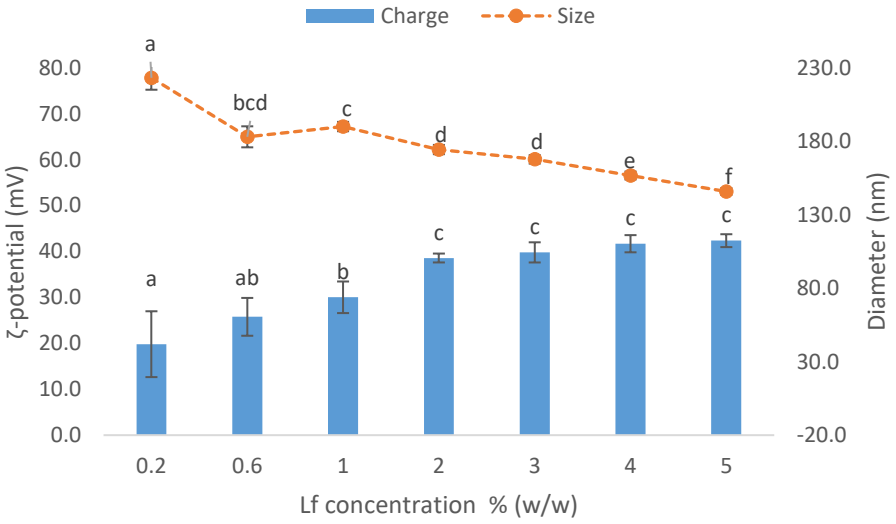

Figure 10. Effect of lactoferrin (Lf) concentration on size (line) and superficial charge (ζ-potential) (bars) of emulsions droplets. Each data point and each bar are the results of the measurements average and the error bars show the standard deviation; different letters represent statistically significant differences (p<0.05).

In all the formulations, the superficial charge of the emulsion droplets obtained is highly positive but the differences were not significant (p>0.05) above 2 % (w/w) Lf concentration. The positive electric charge of the obtained droplets confirms that Lf adsorbed to the oil/water interface, once it presents cationic charge at pH 4 (below its pI of 8.0-8.5). The emulsions with Lf concentration above 2 % (w/w) presented a ζ- potential above +30 mV, varying the values between 38.6 ± 1.0 mV (for Lf concentration 2 % (w/w)) and 42.4 ± 1.4 mV (for Lf concentration 5 % (w/w)), which provides strong repulsive forces between the droplets giving them stability against aggregation mechanisms (Silva et al. 2012). These results are in agreement with Acevedo-Fani et al. (2017) and Ye & Singh (2006) for the formulations with adjustment to pH 3. Ye and Singh (2006) also

analyzed formulations adjusted to pH 7.0 revealing a significant increase in the ζ- potential with increase of Lf concentration.

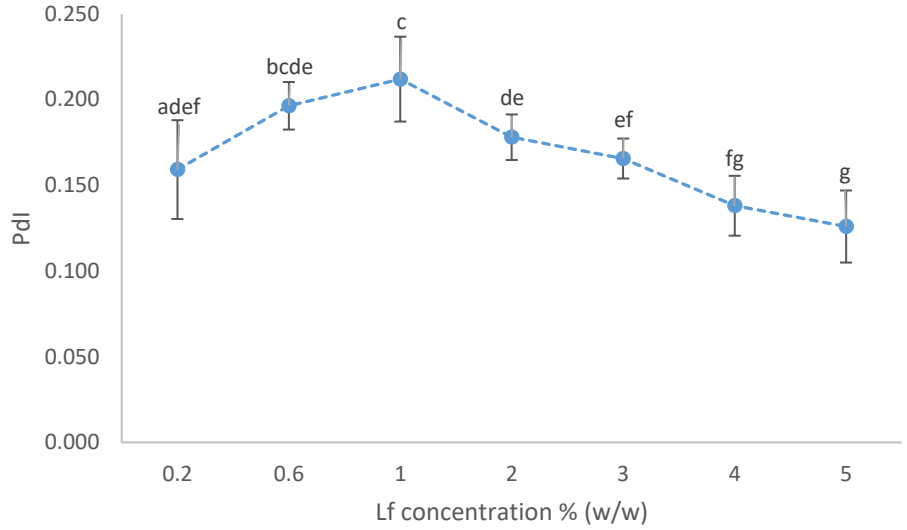

Figure 11. Effect of lactoferrin (Lf) concentration on polydispersity index (PdI) of emulsions droplets. Each data point is the result of the measurements average and the error bars show the standard deviation; different letters represent statistically significant differences (p<0.05).

Regarding PdI the influence of Lf concentration was not so evident (Figure 11). The PdI values vary between 0.212 ± 0.025 and 0.126 ± 0.021 for Lf concentrations of 1 % and 5 % (w/w), respectively, which indicates that in general the emulsions present good homogeneity. The formulations with Lf concentration higher than 1 % (w/w) showed a decrease in the PdI values associated to the increase of protein concentration, suggesting that, the differences between the droplets are lower. Nonetheless, all the size distributions obtained were generally monomodal, despite some formulations revealed a very slight intensity peak at the micrometric range (Figure A1).

4.1.2 Interfacial tension

The nanoemulsions formulations produced with less Lf concentration showed higher superficial tension (Table 3). Moreover, during size analyzes it was observed that lower Lf concentrations lead to the increase of nanoemulsions size. This also mean that the nanoemulsions production associated with great size droplets are associated to greater superficial tension Interestingly, these results are contrary to the ones obtained by Nejadmansouri et al. (2016), which study fish oil nanoemulsions stabilized by WPI and produced by ultrasounds. These authors concluded that the increase of WPI: Fish oil weight ratio (maintaining the oil phase fixed at 1 % (w/w)) was associated

with the decrease of size droplets and with the increase of superficial tension (between 39.75 ± 0.06 and 41.54 ± 0.30 mN/m) (Nejadmansouri et al. 2016). This could be explained by the fact that the proteins are not homogeneously incorporated on emulsion droplets surface, being this distribution influenced by the conformation/structure presented by each protein (Nejadmansouri et al. 2016). Furthermore, different environmental conditions and production methods can affect the surface hydrophobicity of the proteins which alter its affinity to the oil droplets surface. Therefore the different methods applied for the production of the nanoemulsions in both studies could be the reason to the differences noted in the interfacial tension obtained (Nejadmansouri et al. 2016).

Table 3. Influence of Lf concentration on the superficial tension of ω-3 nanoemulsions. Standard deviation (n = 5).

Lf concentration % (w/w)	Superficial Tension (mN/m)
0.2	47.8 ± 0.06
0.6	47.2 ± 0.03
1	46.0 ± 0.21
2	44.7 ± 0.23
3	45.1 ± 0.27
4	45.4 ± 0.29
5	45.8 ± 0.40

4.1.3 TEM

TEM images confirmed the development of organized structures in all produced formulations (Figure 12). The nanoemulsions with Lf concentrations between 0.2 and 1 % (w/w) showed structures without a well-defined shape, while nanoemulsions with the 0.2 and 0.6 % (w/w) Lf revealed a structural disorganization more noticeable. The remaining formulations (Figure 12 d, e, f and g) present the most well defined nanoemulsions droplets with spherical shapes surrounded by discrete protein interfaces. These results are similar to the ones obtained by other authors that also used Lf or milk proteins as emulsifiers for the production of nanoemulsions (Pinheiro et al. 2016; Acevedo-Fani et al. 2017; Hwang et al. 2017).

From TEM images it is notorious the aggregation process that occurs between some of the oil droplets which were probably caused by the processing that samples endure (dehydration of the nanoemulsions), in order to perform TEM analysis. Such processing factors can be the cause for the reduction in the droplets sizes verified (Table A1) relatively to the sizes obtained in DLS measurements (Figure 10) (Bhattacharjee 2016).

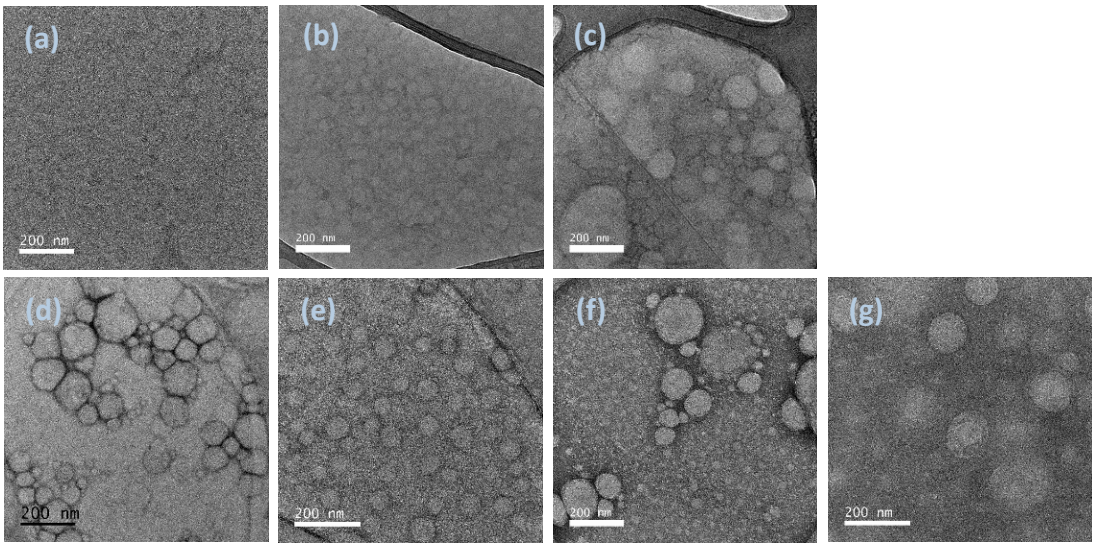

Figure 12. TEM images of nanoemulsions produced with (a) 0.2 %, (b) 0.6 %, (c), 1 %, (d) 2 %, (e) 3 %, (f) 4 % and (g) 5 % (w/w) Lf concentrations. All the images have a 200 nm scale.

4.2 Nanoemulsions stability under different storage conditions

Nanoemulsions with ζ- potential values higher than 30 mV, diameter smaller than 200 nm and spherical shapes were selected to access its physical and chemical stability under storage. For this propose the nanoemulsions produced with Lf concentrations of 2, 3, 4 and 5 % (w/w) were stored protected from light at 4 °C and at room temperature for 69 days and its size, PdI and ζ- potential were followed, in order to verify their physical stability.

The same nanoemulsions stored at 4 °C, were also followed regarding its antioxidant capacity (through DPPH assay) and primary oxidation products presence (through peroxide value determination), over 35 and 36 days, respectively. These tests are based on chemical reactions associated with prevention and measurement of oxidation first products, respectively, so they can be used to assess the existence of chemical alterations occurring in the nanoemulsions formulations during the storage.

4.2.1 Physical properties variations

The average droplet sizes and ζ- potential of all the nanoemulsions formulations stored at 4 °C are showed in Figure 13. The nanoemulsions produced with 2 % and 3 % (w/w) of Lf did not suffer statistically significant differences in its average droplet size during storage (p>0.05) (the values vary between 174.4 ± 3.15 and 196.1 ± 6.18 nm, and between 167.7 ± 2.89 and 186.8 ± 12.57 nm, for 2 % and 3 % (w/w) of Lf, respectively). Despite formulations with 4 % and 5 % (w/w) of Lf showed statistical significant differences in some of the days of evaluation, overall the average droplet size did not suffer great alterations (the values vary between 156.7 ± 2.70 and 179.50 ± 5.84 nm, and between 145.8 ± 2.34 and 168.1 ± 4.61 nm, for 4 % and 5 % (w/w) of Lf, respectively). These results suggest that the formulations when storage at 4 °C remain stable for at least 69 days.

The evaluation of ζ- potential values reveled that all the formulations suffered statistically significant modifications during the storage in terms of surface charge. The formulation with 2 % (w/w) of Lf was the one with more significant alterations, varying the ζ- potential value between 38.7 ± 1.99 and 26.0 ±0.51 mV, revealing a trend to a superficial charge reduction.

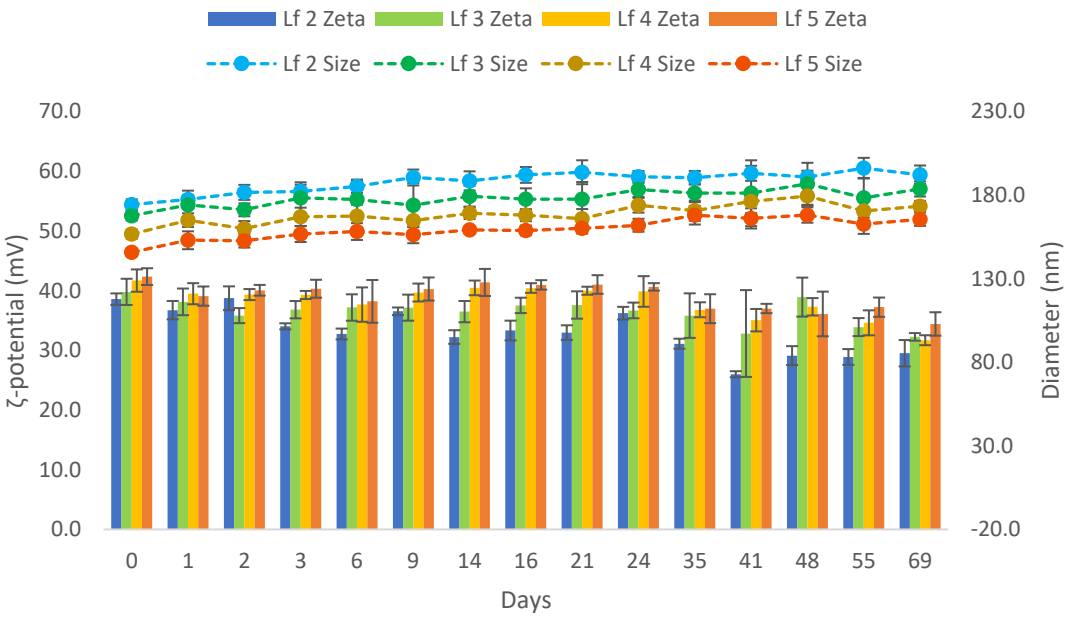

Figure 13. Size (lines) and superficial charge (ζ- potential) (bars) of nanoemulsions produced with lactoferrin (Lf) concentrations of 2, 3, 4 and 5 % (w/w) stored during 69 days at 4 °C. Each data point and each bar are the results of the measurements average and the error bars show the standard deviation.

Regarding PdI, none of the nanoemulsions showed statistically significant differences during the storage period (p>0.05), being the PdI values generally bellow 0.200 (Figure 14), indicating that the nanoemulsions solutions remain homogeneous.

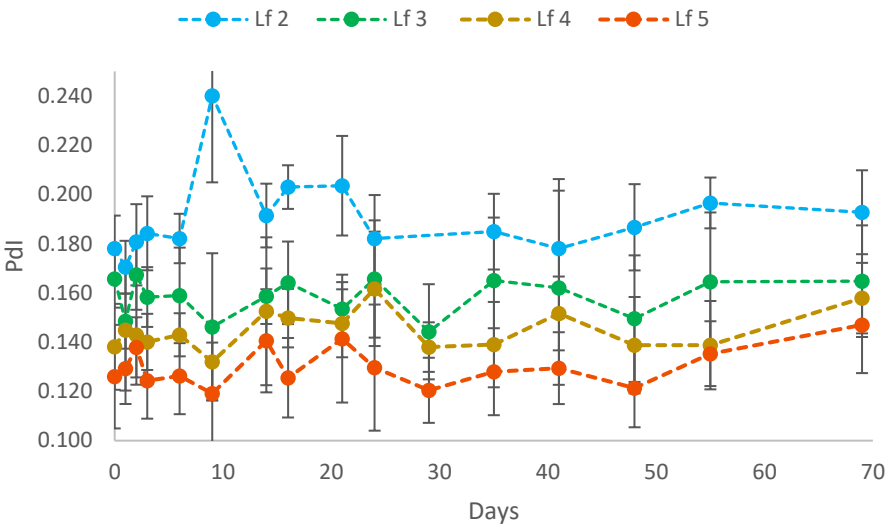

Figure 14. Polydispersity index (PdI) of ω-3 nanoemulsions produced with lactoferrin (Lf) concentrations of 2, 3, 4 and 5 % (w/w) stored during 69 days at 4 °C. Each data point is the result of the measurements average and the error bars show the standard deviation.

The average droplet sizes and ζ- potential values obtained for nanoemulsions storage at room temperature are depicted in Figure 15. The formulation produced with 2 % (w/w) Lf concentration showed an increase in the size droplets between the 16 and 24 days of storage (from 215.9 ± 15.33 nm to 670.7 ± 120.69 nm) followed by a reduction, near to the previous size, in day 35 (186.2 ± 3.65 nm). After 69 days of storage the formulation with 3 % (w/w) Lf concentration also showed a notorious increase in the droplets size (from 180.8 ± 11.17 nm to 642.4 ± 46.69 nm) (p<0.05). In general, the remaining formulations (4 and 5 % (w/w)) did not reveal statistically significant differences in the average droplets sizes over the storage period. The alteration in the mean droplets size of the formulation with 2 % (w/w) Lf concentration between days 16 and 24 could be associated to a reversible process known as flocculation in which occur an association between two or more droplets but the individual integrity of each droplet is maintained, and as so they can separate again (McClements & Rao 2011). In the case of the formulation with 3 % (w/w) Lf concentration the situation could be similar and nanoemulsions could be suffering a similarly process and decrease its size in a longer storage period or the alteration process could be the type of an irreversible one such as Oswald Ripening which is common between nanoemulsions formulations. Besides these, Chalothorn & Warisnoicharoen (2012) also reported an increase in the size of WPI ω-3 oil emulsions after 60 days of storage at room temperatures (Chalothorn & Warisnoicharoen 2012).

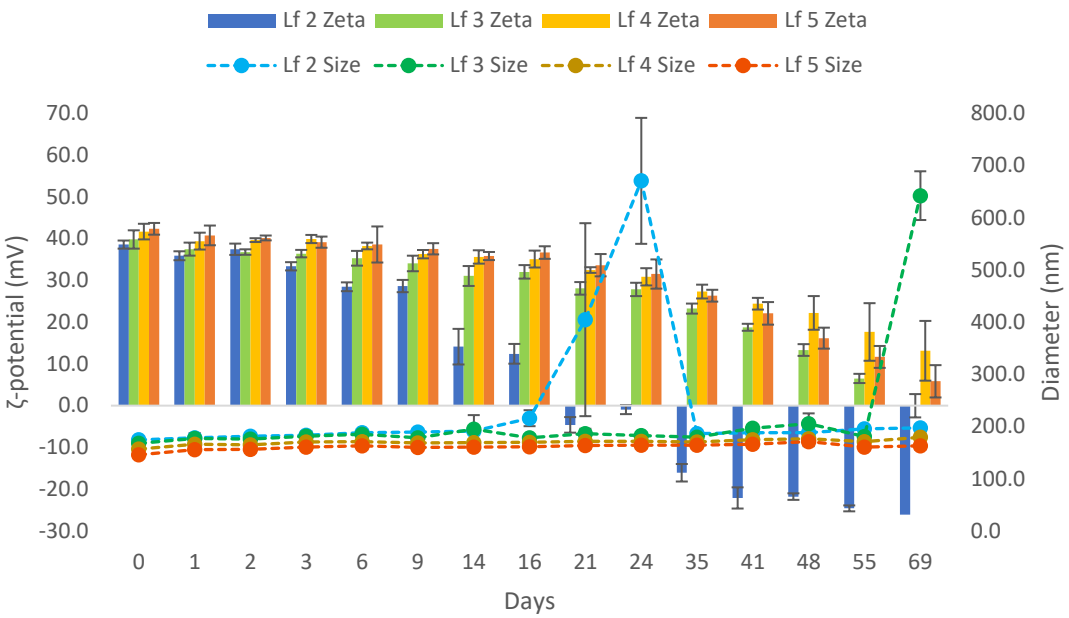

Figure 15. Size and superficial charge (ζ- potential of ω-3) nanoemulsions produced with lactoferrin (Lf) concentrations of 2, 3, 4 and 5 % (w/w) stored during 69 days at room temperature. Each data point and each bar are the results of the measurements average and the error bars show the standard deviation.

During storage, all the formulation suffered a reduction in ζ- potential values (Figure 15) (p <0.05). The obtained data suggest that exist a relation between the Lf concentration and the ζ- potential reduction. The formulation with 2 % (w/w) of Lf suffered the faster reduction in ζ- potential value (from 38.6 ± 0.97 mV to -26.1 ± 0.71 mV), followed by the formulation of 3 % (w/w) Lf concentration (from 39.8 ± 2.19 mV to 0.0 ± 2.79 mV). With regard to 4 % and 5 % (w/w) Lf concentrations, the reduction was smaller which allow the values to remain positive (from 41.7 ± 1.87 mV to 13.1 ± 7.16 mV and from 42.4 ± 1.40 mV to 5.8 ± 3.87 mV, respectively). Acevedo-Fani et al. (2017) also verified a decrease in ζ- potential of nanoemulsions produced with Lf for encapsulation of resveratrol and storage for 4 weeks at room temperature. The decrease in the superficial net charge of the nanoemulsions droplets it is thought to be related with conformational alterations caused by unfolding phenomena occurred at the o/w interface, which can lead to the formation of disulfide ligations between the proteins among others crosslink mechanisms (Acevedo-Fani et al. 2017). Another possible cause is the interaction between the proteins and reactive species such as transition metals or oxygen. Another study with Lf nanoemulsions stored at 55 °C also found a change in ζ- potential from positive to negative values (Zhao et al. 2015; Qiu et al. 2015).

Results also showed that during the storage, the formulations with 2 % and 3 % (w/w) Lf concentrations revealed a higher size of the droplets when ζ- potential values are close to zero. Such results support the theory of flocculation suggested previously, since it is a process decided by a balance between a series of attractive and repulsive forces acting between the droplets that in solution are constantly colliding. The net charge close to zero could have led to a period of aggregation followed by the separation when the net ζ- potential increase (McClements & Rao 2011). Nevertheless, it would be necessary to extend the time of stability evaluation of nanoemulsions produced with 3 % (w/w) Lf to verify if the grow on size droplets is also follow by a decrease such as the one verified in nanoemulsions produced with 2 % (w/w) of Lf. This would help to corroborate that the destabilizing process affecting nanoemulsions is indeed a reversible one, probably occurring through a flocculation mechanism.

With respect to the PdI values, the formulations with 2 % and 3 % (w/w) Lf concentrations presented statistically significant differences over all the storage period (p<0.05), while the formulation with 4 % (w/w) Lf concentration only showed significant differences from day 55 of storage and the formulation with 5 % (w/w) Lf concentration revealed differences from day 48 (p<0.05) (Figure 16).

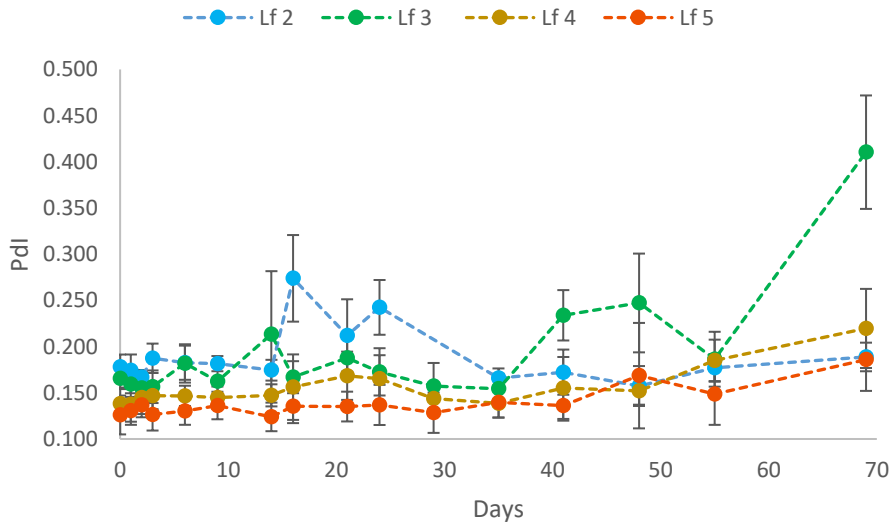

Figure 16. Polydispersity index (PdI) of ω-3 nanoemulsions produced with lactoferrin (Lf) concentrations of 2, 3, 4 and 5 % (w/w) stored during 69 days at room temperature. Each data point is the result of the measurements average and the error bars show the standard deviation.

Comparing the data obtained at both storage temperatures (4 °C and room temperature) the formulation with 2 % (w/w) Lf concentration only revealed statistically significant differences at days 21 and 24, which match the days of instability detected at room temperature storage, and the formulation with 3 % (w/w) Lf concentration almost only differ statistically at day 69, which also match the instability registered at room temperature. On the other hand, the formulations with 4 % and 5 % (w/w) Lf concentrations presented statistically significant differences (p<0.05) in several days during the storage period. These results indicate that the temperature of storage have an important impact on the stability of nanoemulsions, being that influence greater in the formulations with 2 % and 3 % (w/w) Lf concentrations for which clear instability processes are noticed at room temperature and not at 4 °C. These results are also supported by the ζ- potential results, since all the formulations suffered a reduction much more pronounced at room temperature storage, being the formulations with 2 % and 3 % (w/w) Lf concentrations the ones that showed the more accentuated reductions. In addition, the PdI values revealed that the nanoemulsions solutions maintain homogeneity in the stored at 4 °C which generally was not verified for the formulations keep at room temperature.

4.2.2 Chemical properties variations

The ω-3 fish oil used in the production of nanoemulsions is already marketed with a mixture of antioxidant (α-tocopherol). Its antioxidant capacity was evaluated by the DPPH test and an IC_{50} of 231.99 ± 6.16 mg of emulsion/ mL of solution was obtained. The IC_{50} obtained for the commercial antioxidant Trolox used as standard was 0.07 mg/mL. All the nanoemulsions at 4 °C were evaluated during a period of 35 days, revealing an IC_{50} bellow the IC_{50} of the free oil. These results indicate that a smaller amount of nanoemulsions is required for the scavenging of 50 % of the free radical DPPH comparatively to free oil, which mean that the nanoemulsions formulations have higher antioxidant capacity when compared to the free fish oil. These results are in agreement with the ones obtained by Lou et al. (2017), where they study the antioxidant capacity of essential oils obtained from *Citrus medica L. var. sarcodactylis*, in the free form and emulsified with Tween 80 by a spontaneous emulsification process. These authors observed that the emulsified oils have a greater scavenging ability of the free radical DPPH comparatively to free oils (Lou et al. 2017), and suggested that this fact could be related to the insolubility of the oil in aqueous systems.

During the storage, was not observed significant differences on antioxidant capacity of the nanoemulsions (Figure 17). The IC_{50} values obtained for different nanoemulsions ranged between 145.69 ± 3.62 and 221.81 ± 38.92 µl of emulsion/ ml of solution (p<0.05). Acevedo-Fani et al. (2017) also studied the antioxidant capacity of Lf primary nanoemulsions containing resveratrol for 4 weeks and showed that no significant change hapens in the antioxidant capacity of the nanoemulsions during storage. Besides, these authors also reported that some biopolymers with known antioxidant activity (e.g. Lf) may contribute to the overall radical activity of emulsions when present at the o/w interface (Acevedo-Fani et al. 2017; Tokle & McClements 2011).

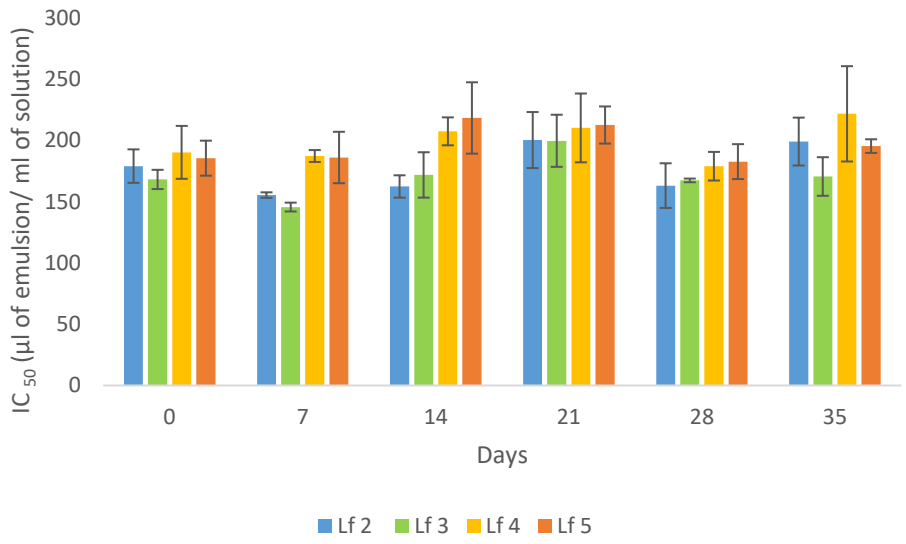

Figure 17. Antioxidant capacity of ω-3 nanoemulsions produced with lactoferrin (Lf) concentrations of 2, 3, 4 and 5 % (w/w) stored during 35 days at 4 °C. Data are expressed as the amount of nanoemulsions (µl of emulsion/ ml of solution) capable of scavenging 50% of the free DPPH radicals presents (IC_{50}). Each data bar is the result of the measurements average and the error bars show the standard deviation.

The oxidation state of the purchased fish oil was assessed immediately after air exposition and a peroxide value (PV) of 0.68 ± 0.03 meq/kg of oil was obtained. After 1 day of storage the peroxide value of the nanoemulsions was evaluated, being the obtained values (between 8.50 ± 0.20 and 14.48 ± 2.40 meq/kg of oil, Figure 18) higher (p<0.05) than the values of the free oil. The results showed that differences in the peroxide values obtained for the different nanoemulsions formulations are notorious after 15 days of storage (p<0.05) (Figure 18). From day 15 forward the formulations with 2 % and 3 % (w/w) Lf concentrations showed lower PV comparatively to formulations with 4 % and 5 % (w/w) (p<0.05) Lf concentrations. After 36 days of storage the formulation with low PV registered was the 2 % (w/w) Lf concentrations followed by the 3 % (w/w)

Lf concentrations (125.13 ± 6.20 and 136.21 ± 9.48 meq/kg of oil, respectively). These results suggest that may exist a relation between the increase on peroxide value and the application of higher amounts of Lf in the nanoemulsions formulations at long-term storage periods. Other studies with fish o/w nanoemulsions also reported an increase of peroxide values over the storage period (Walker et al. 2015; Karthik & Anandharamakrishnan 2016; Cheong et al. 2017). Furthermore, other authors had reported that the rate of the oxidation can be related with the size of the emulsions droplets, being the rate greater in the smaller ones (Haahr & Jacobsen 2008). However, the overall results presented by Walker et al. (2015) revealed that the size could interfere in the oxidation rate but is not a major factor, which can explain the alterations observed in our study between the different nanoemulsions after 15 days of storage, since the greater concentrations of Lf (4 % and 5 % (w/w)) are associated to smaller droplets sizes.

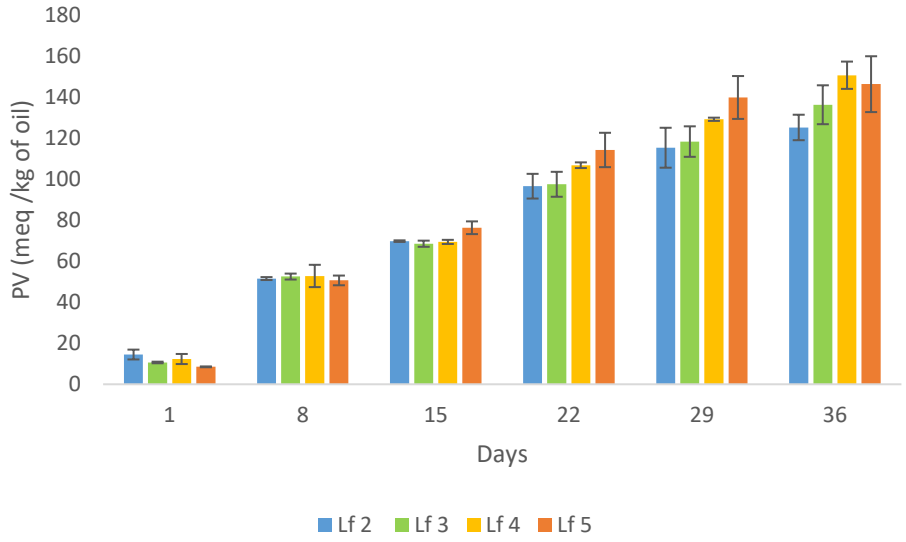

Figure 18. Peroxide value (PV) of ω-3 nanoemulsions produced with lactoferrin (Lf) concentrations of 2, 3, 4 and 5 % (w/w) stored during 35 days at 4 °C. Each data bar is the result of the measurements average and the error bars show the standard deviation.

The oxidation of PUFAs such as ω-3 could be caused by a high number of factors (e.g. light, heat, metals, oxygen). In our study, we verified that the nanoemulsions have the capacity of scavenging free radicals such as DPPH, but despite this it was also verified that the nanoemulsions produced were associated to high peroxide values which suffered an increase over storage (quality standard guideline of fish oil indicates that the PV should not overcome the 10 meq/kg oil) (Beasley & Temple 2015). Additionally, Lf is a protein known to be able of chelate transition metals (e. g. ferric ions) (Ye & Singh 2006) but has not been described as having the ability of inactive other

species capable of causing oxidation such as reactive oxygen species, which could be the cause of the high peroxides values registered for nanoemulsions, since it was not possible to isolate the oil from the air during the nanoemulsions production.

4.3 Encapsulation efficiency

The encapsulation efficiency (EE) of the nanoemulsions was higher than 99 % for all the tested formulations. Ghorbanzade et al. (2017) evaluated the encapsulation of fish oil in nano-liposomes composed by soy lecithin and observed an EE around 92 %. Sari et al (2015) also reported an EE of 90.56 % for nanoemulsions composed by whey protein concentrate with curcumin encapsulated. Studies using other for ω-3 encapsulation, such as nanofibers revealed smaller EE (68%) (García-Moreno et al. 2017).

4.4 Dehydration of nanoemulsions

The nanoemulsions used in the drying processes were selected according to their size, superficial charge and morphology. The nanoemulsions with smaller sizes, superficial tension above 30 mV and with more defined and delimited spherical individual shapes, were selected. In these case nanoemulsions produced with 2, 3 and 4 % (w/w) Lf.

The main objective of drying nanoemulsions was to produce powders which are more stable and easy to incorporate in some kinds of foods (e.g breakfast cereals). Two different drying techniques were evaluated: freeze-drying and nanospray-drying. While freeze-drying is a technique traditionally applied (e.g. fruits, egg yolk, coffe, proteins) (Ciurzyńska & Lenart 2011), nanospray-drying is an emerging technology developed to dry even susceptible active components and to allow the production of particles in nano-size range (Lee et al. 2011; Li et al. 2010).

4.4.1 Morphologic characterization of dried nanoemulsions

4.4.1.1 Nanoemulsions after freeze-drying

The nanoemulsions dried by freeze-drying originated amorphous structures (Figure 19). The structures obtained are similar to the ones obtained by other author in related studies (Abdelwahed et al. 2006; Hu, Gerhard, et al. 2016), Esquerdo et al. (2015) also dried ω-3 nanoemulsions but developed with chitosan, and obtained similar structures.

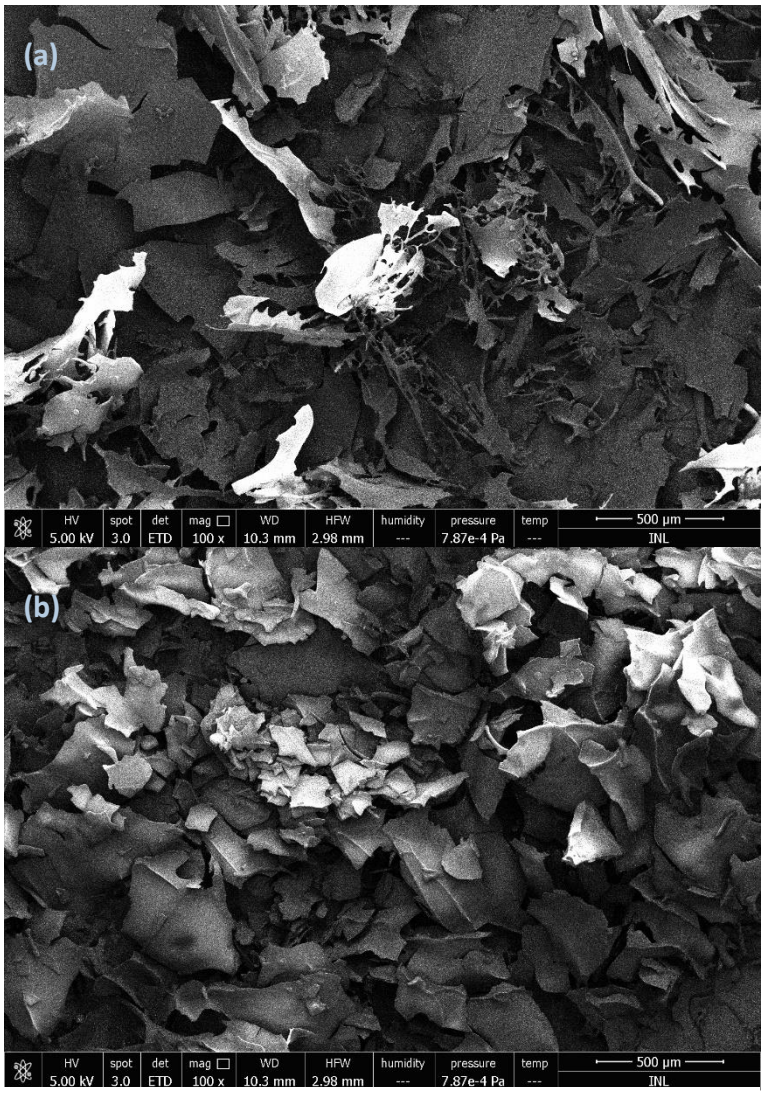

Figure 19. SEM images of ω-3 nanoemulsions formulated with 2 % (a) and 4 % (b) lactoferrin concentrations and dried by freeze-drying. All the images have a 500 µm scale.

4.4.1.2 Nanoemulsions after nanospray-drying

All the nanoemulsions submitted to nanospray-drying were successfully dried and originated smooth defined particles with submicron sizes generally below the 500 nm (Figure 20) which are sizes much smaller compared to the ones obtained by traditional spray drying techniques (10-25 µm) (Hu, Gerhard, et al. 2016). The powders derivatives of nanoemulsions produced with 2 and 3 % (w/w) Lf appear to have more defined spherical shape compared to 4 % (w/w) Lf formulation, among these the powder of the 3 % (w/w) Lf formulation has less agglomeration. This smooth surface verified is in agreement with the results obtained by other authors in the drying of

nanoemulsions or other particles by nanospray-drying (Hu, Gerhard, et al. 2016; Lee et al. 2011; Li et al. 2010; Pérez-masiá et al. 2015).

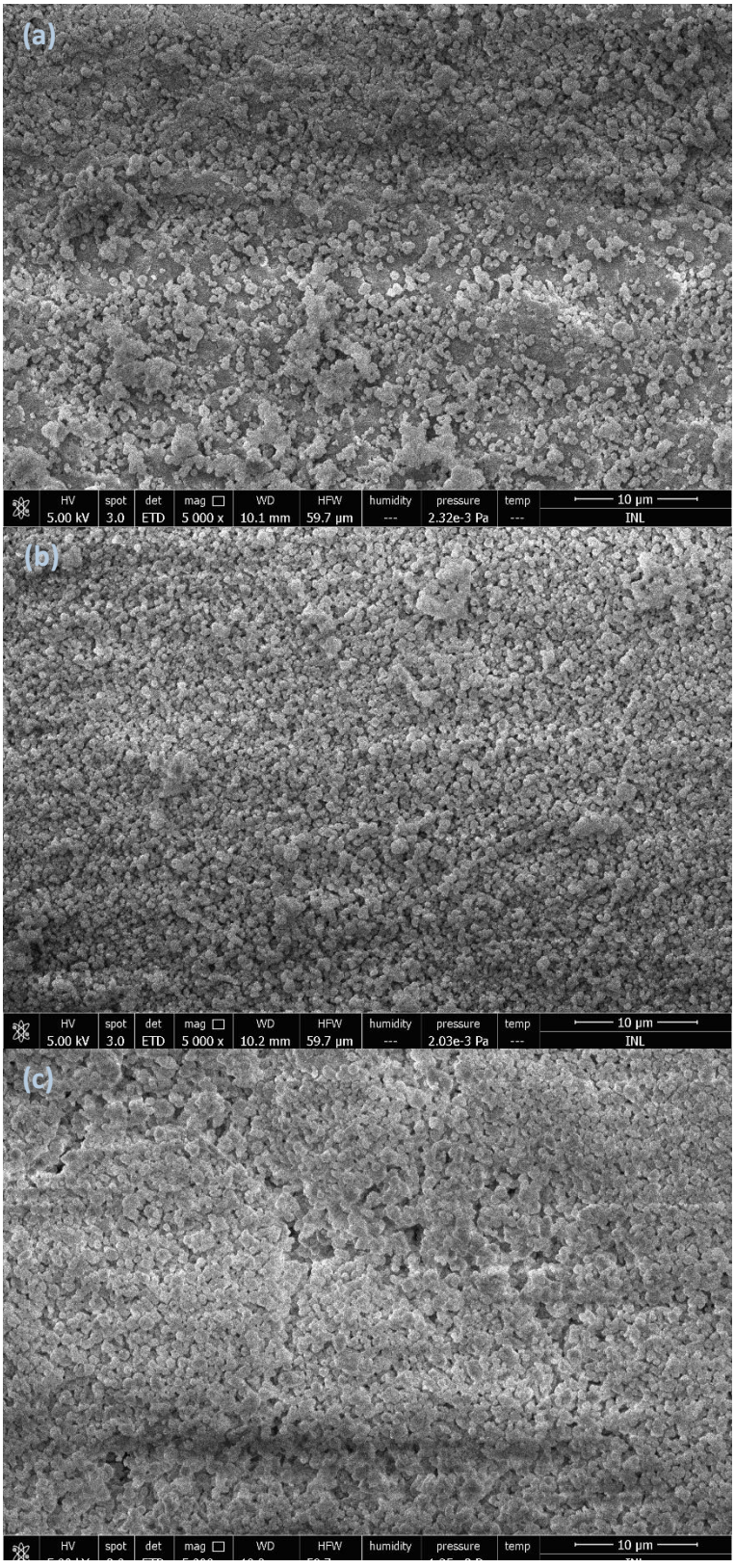

Figure 20. SEM images of ω-3 nanoemulsions formulated with 2 % (a), 3 % (b) and 4 % (c) lactoferrin concentrations and dried by nanospray-drying. All the images have a 10 µm scale.

4.4.2 Characterization of dried nanoemulsions after rehydration

The ω-3 nanoemulsions that were dried by the two different methodologies: freeze and nanospray drying (reported in the section 4.4.1.1 and 4.4.1.2, respectively) were posteriorly rehydrated in order to verify if there were changes in the properties of the rehydrated dry material comparatively to the initial nanoemulsions. The powders obtained were rehydrated and the physical properties of the solutions were evaluated (size, superficial charge and morphology). Chemical modifications were also analyzed through antioxidant capacity assay (DPPH assay) and the formation of primary oxidation products were evaluated by peroxide value assay. The results obtained were then compared to the ones obtained to the naonoemulsions formulation at the day 0 or 1 of storage.

4.4.2.1 Nanoemulsions dried by freeze-drying

The nanoemulsions dried by freeze drying were rehydrated and the obtained results took into account the amount of water used in the rehydration. The rehydration was performed in order to obtain nanoemulsions solutions with concentration similar to the initial conditions (5 % (w/w) oil phase and 95 % (w/w) aqueous phase, with the latter containing a determined amount of Lf, according to each formulation).

The DLS size measurements distributed by intensity percentage revealed the existence of more than one population in all the formulations analysed, especially in the formulation produced with 3 % (w/w) Lf (Figure 21). The correlograms of the results were assessed (Figure A2) and a defined exponential decay was observed, as expected, when the results obtained are properly (the program itself has a quality criteria tool that evaluate the quality of each measurement, and in the presented results the criteria indicated that the measurement have good quality) (Bhattacharjee 2016; Malvern n.d.). Therefore, the results were also expressed by number percentage distribution (Figure 21), what result from the conversion of the intensity measurements through the application of Mie theory. The results by number allow to correlate the contribution in terms of the actually number of spherical droplets present to each intensity peak detected (e.g. smaller intensity peak could be caused by an equal number of particles causing a high intensity peak, corresponding the latter to a population of bigger particles), concluding thus that the particles obtained after rehydration are in general smaller (around or below 100 nm) than the droplets of the starting nanoemulsions although the results obtained by intensity distribution (Table 4) reveal higher sizes. These higher

sizes can be releated to the presence of some aggregates that had not rehydrated properly causing a big scattering of light which is visible in the intensity distribution graphics through the presence of more than one peak, which are not observed in the graphics of number distribution. Such singular aggregates can promote differences between the two graphics and also justify the large PdI values obtained, all superior to 0.200, contrary to the starting nanoemulsions PdI values.

The superficial charge of the rehydrated solutions was also analysed with results closer to the values obtained for the starting nanoemulsions. The results of size distribution by number and the similar superficial charge suggest that the freeze-drying process allows the preservation of the physical properties of the nanoemulsions, with its shell structure remaining unaltered. Such results are in agreement with other previous studies which reported that also freeze-dried nanoemulsions produced with gum arabic and lecithin, having subsequently rehydrated them and analyse, concluding that the drying process did not change its shell structure (Abdelwahed et al. 2006; Hu, Gerhard, et al. 2016).

However, it should be noted that despite the relatively easy rehydration (visually checked) of the powders obtained by freeze-drying, a slight centrifugation was sufficient to visualize a considerable deposit of particles that had not undergone a properly rehydration (Figure A5), and as such were not used in the assays performed. Other authors such as Rampino et al (2013) who studied chitosan nanoparticles and Prestrelski et al (1993) who studied protein solutions freeze dried reported that the rehydration process was not possible (Prestrelski et al. 1993; Rampino et al. 2013a). Additionally, Prestrelski et al (1993) verified by infrared spectroscopy that the proteins analysed suffered irreversible conformational alterations. They also concluded that when the freeze-drying process of their samples is performed in the presence of stabilizers the resulting powder could be rehydrated. Additionally, they verified by infrared spectroscopy analysis that conformational alterations occurred in the proteins subjected to the drying process (Prestrelski et al. 1993).

When observed by TEM the rehydration solutions revealed the absence of defined structures and the existence of aggregates (Figure 22). Despite the results of DLS obtained and discussed above, it was verified by imaging that the structures of starting nanoemulsions were not achieved

after rehydration, being the DLS results probably caused by loose oil particles or protein agglomerates.

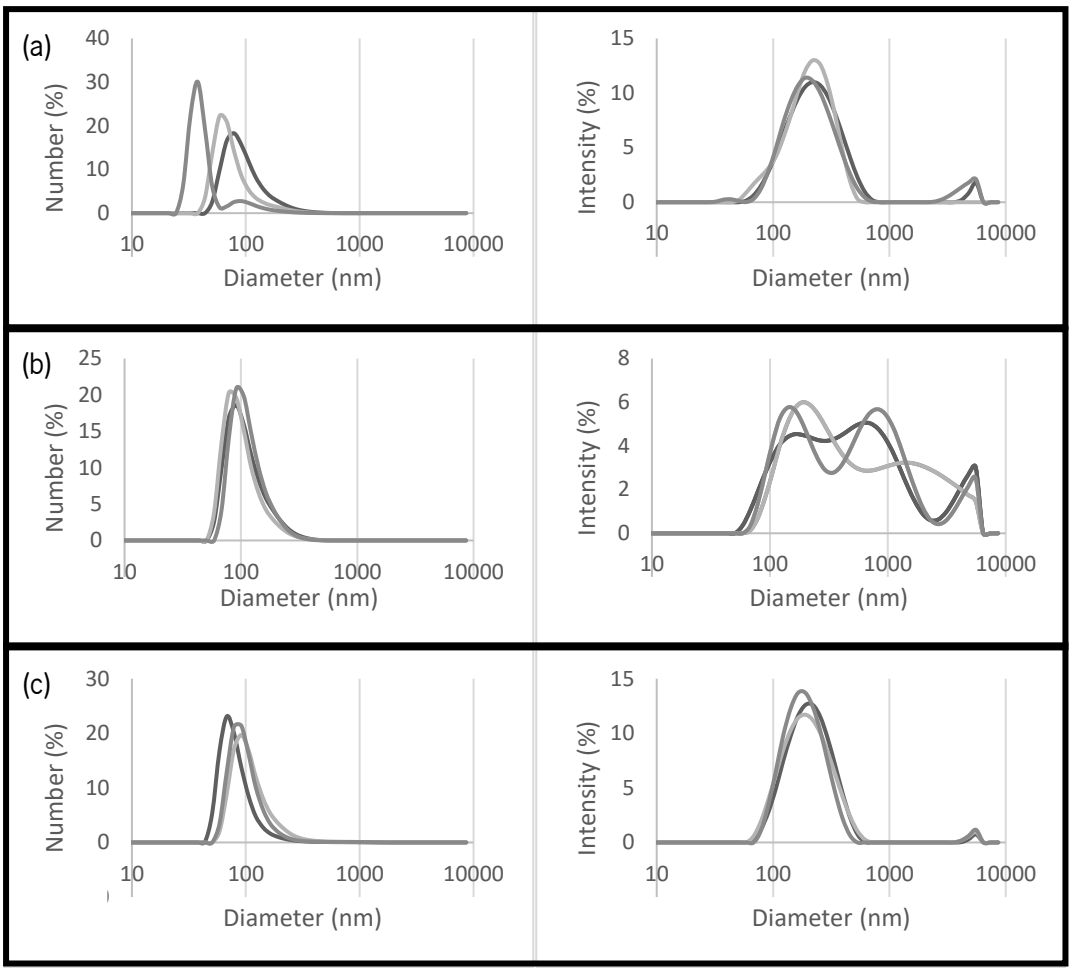

Figure 21. Size distribution of the ω-3nanoemulsions formulations with Lf concentrations of 2 (a), 3 (b) and 4 (c) % (w/w) dried by freeze-drying, after rehydration. The results were obtained by three DLS measurements of each sample and are expressed by both number and intensity percentages.

Table 4. Mean values of size, PdI and superficial charge (ζ-potential) obtained by three DLS measurements of each sample for ω-3nanoemulsions formulations with different Lf dried by freeze-drying, after rehydration.

Lf concentration % (w/w)	Size (nm)	PdI	Superficial charge (mV)
2	211.3	0.328	36.2
3	340.5	0.476	37.1
4	186.3	0.224	33.9

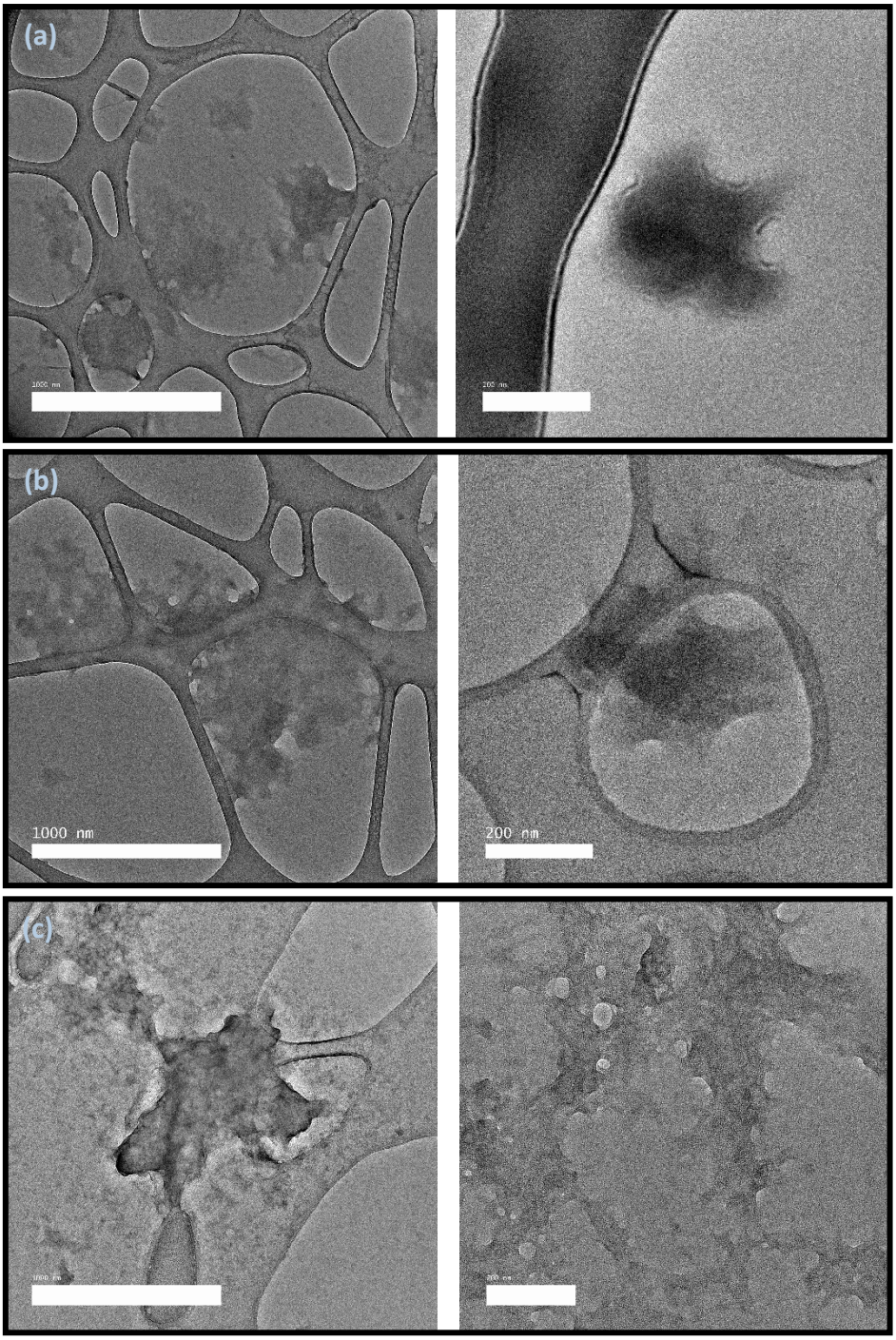

Figure 22. TEM images of the ω-3nanoemulsions formulations with Lf concentrations of 2 (a), 3 (b) and 4 (c) % (w/w) dried by freeze-drying, after rehydration. All the images of left have a 1000 nm scale while all the images on right have a 200 nm scale.

The antioxidant capacity assay of the freeze-died rehydrated powders revealed higher IC_{50} for the formulation produced with 2 % (w/w) Lf concentration, a similar value for the formulation produced with 3 % (w/w) Lf and a smaller IC_{50} for the formulation produced with 4 % (w/w) Lf concentration (Table 5). The higher IC_{50} indicates a loss of antioxidant capacity after the drying and

rehydration processes, with higher amount of rehydration solution to achieve 50% inhibition of the DPPH radicals applied in the assay comparatively to the starting nanoemulsion, however the opposite deduction is made in respect to the formulation developed with 4 % (w/w) Lf. In terms of the oxidation assessments, higher peroxide values were obtained for all the formulation comparatively to the freshly prepared starting nanoemulsions (Table 5). In their study, Prestrelski et al (1993) also verified the preservation of enzymatic functionality of the protein lactate dehydrogenase after a freeze-drying and rehydration and concluded that the activity was lost during the processing (Prestrelski et al. 1993).

The functional properties of proteins are highly dependent of the spatial conformation of molecule (folding/unfolding state) and their association states (the interactions stablished between neighboring proteins and between these and other molecules) which are very susceptible to the environmental conditions (e.g. water, other molecules, pH ionic strength) which in turn are highly influenced by physical and chemical processing (Dehnad et al. 2016). This suggest that the freeze-drying and rehydration processing applied in ω-3 nanoemulsions produced with different Lf interfered with the nanostructures allowing a limited recovering of the starting formulations, that is reflected for both re-hydration limitation and dissimilar chemical responses to the same assays.

Table 5. Chemical properties obtained for freeze-dried ω-3 nanoemulsions produced with different lactoferrin (Lf) concentrations, after rehydration. Antioxidant capacity is expressed as the amount of rehydrated emulsions (μl of emulsion/ ml of solution) capable of scavenging 50% of the free DPPH radicals presents (IC_{50}). The oxidation of rehydrated emulsions is evaluated by peroxide value determination. Each data is the result of three measurements average.

Lf concentration % (w/w)	IC_{50} (μl of emulsion/ ml of solution)	Peroxide Value (meq /kg of oil)
2	291.60	80.79
3	166.62	80.50
4	94.42	72.47

4.4.2.2 Nanoemulsions dried by nanospray-drying

The nanoemulsions dried by nanospray-drying were rehydrated without knowing the concentration since the powder obtained was not possible to weigh due to the fact that the amount of powder produced for each formulation did not reach the minimum weight detectable by the available scale. Initially, powders were placed in water with stirring for 24 hours, which did not

achieve rehydration, so the rehydration solutions were subject to an unsuccessful sonication. A final attempt of rehydration was made replacing the solvent water for phosphate buffer at pH 5.2 (a value below the pI value of Lf, which is of 8.0-8.5, to ensure that it did not precipitate). After a new cycle of stirring and sonication a proper rehydration of the powder continued to not occur, remaining present a visible suspension, containing some macroaggregates as well as some deposit of powder (Figure A6).

The solutions resulting from the rehydration with phosphate buffer were studied by DLS, with the results of the size distribution obtained by both number and intensity percentages present in Figure 23. The graphics obtained for size distribution by intensity reveal several peaks and the size distribution by number revealed some conclusions clearly showing a single peak (only one population of particles is detected) or at most two, which is the case for the formulation produced with 4 % (w/w) Lf concentration. Despite these results, the correlograms obtained (Figure A3) for these distributions do not have the exponential decay that allows proper DLS measurements. Additionally, the program itself indicate that quality criteria were not achieved in these measurements. In attempt to achieve somehow more illuminating results a filtration of the sample through a 0.2 μm syringe filter was performed and the size distributions measurement repeated. However, the correlograms obtained (Figure A4) still did not fit the expected profile in correct DLS measurements and the program continue to diagnose that the measurements did not achieve quality standards. With regards to the superficial charge the measurements were not possible to perform due to high conductivity of the samples (caused by the presence of salts from the buffer), which was higher than allowed for the use of the equipment.

The morphologic analyses of the rehydration solutions the results obtained by DLS, demonstrating that there was no rehydration of the powders obtained by nanospray dryer (Figure 24), maintaining the spherical structure of the solid particles. Some fragments of proteins were

probably dried separately during the drying process and originate the agglomerates observed in the right image of the Figure 24 (a).

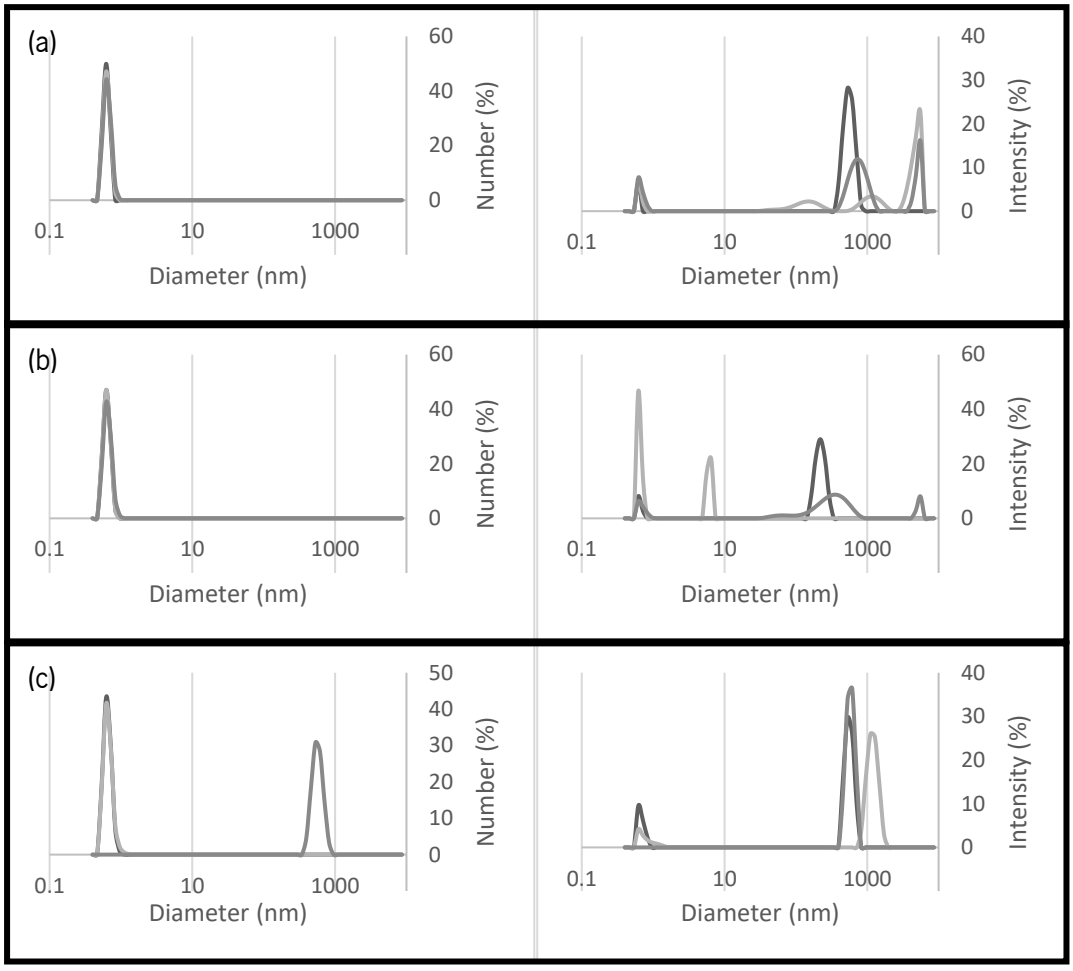

Figure 23. Size distribution of the ω-3nanoemulsions formulations with Lf concentrations of 2 (a), 3 (b) and 4 (c) % (w/w) dried nanospray-drying, after rehydration in phosphate buffer. The results were obtained by three DLS measurements of each sample and are expressed by both number and intensity percentages.

Table 6. Mean values of size and PdI obtained by three DLS measurements of each sample for ω-3 nanoemulsions formulations with different Lf dried by freeze-drying, after rehydration.

Lf concentration % (w/w)	Size (nm)	PdI	Superficial charge (mV)
2	1863.0	0.985	–
3	2622.8	0.844	–
4	3114.7	0.700	–

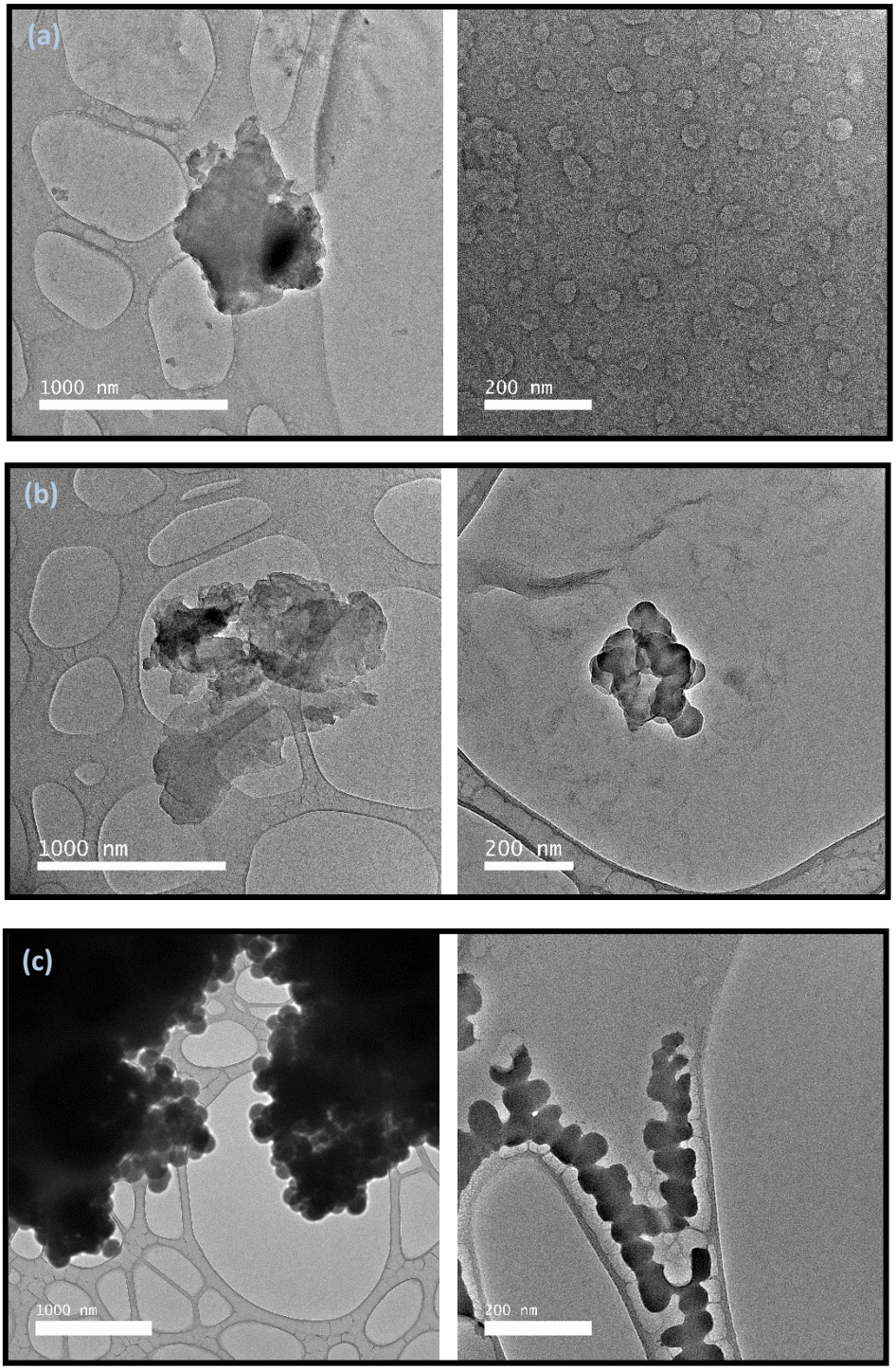

Figure 24. TEM images of the ω-3nanoemulsions formulations with Lf concentrations of 2 (a), 3 (b) and 4 (c) % (w/w) dried by nanospray-drying, after rehydration. All the images of left have a 1000 nm scale while all the images on right have a 200 nm scale.

Due to the impossibility of rehydration of the nanospray-drying powders, their chemical analyses in terms of antioxidant capacity and primary oxidation extension where not possible to perform.

Hu et al (2016) reported a successful rehydration process of nanoemulsions produced with gum arabic and lecithin, dried by nanospray dryer (Hu, Gerhard, et al. 2016; Hu, Wang, et al. 2016). Other work from the same authors showed that nanoparticles of gallic acid-chitosan conjugate and gum arabic dried by nanospray-drying were successfully rehydrated, while dried nanoparticles formulated as controls produced with only chitosan and gum Arabic, where not rehydratable. Rampino et al (2013) produced chitosan nanoparticles and added three types of cryoprotectants to the nanoparticles preparation before the nanosparay-drying process, and verified that the two formulations produced with trehalose and polyethylene glycol were re-dispersible while the formulation produced with mannitol were not properly re-dispersed leaving in suspension some macroaggregates. On the other hand, Wang et al (2016) successfully rehydrated solid lipid nanoparticles produced with sodium caseinate, glyceryl behenate, soya lecithin and coated with pectin and dried by nanospray-drying, needed a heating step at 80 °C for rehydration. It is clear the diversity behaviours according to the nanostructures and polymeric solutions dried by nanospray-drying, regarding the rehydration, showing that the formulations and materials used have an important role in the rehydration capacity.

As already mentioned the processing and environmental conditions surrounding a protein largely influence the proteins structures and consequently its features and properties (Dehnad et al. 2016). The spray dryer process itself has already been associated with diverse stress phenomena (e.g. stress adsorption, shear stress, thermal stress and dehydration stress) that could cause unfolding, aggregation or denaturation of proteins. The nanospray-drying in particular has been related to shear stresses such as shaking, pumping, noozle and atomization (Dehnad et al. 2016). In fact, Pérez-Masiá et al (2015) has already reported structural alterations in ATR-FTIR spectrum of whey protein concentrate caused by nanospray dryer processing, which can affect the molecular bonds in the WPC chains (Pérez-masiá et al. 2015). These suggest that such factors could be the ones influencing and causing the lake of rehydration ability verified in the powders obtained by nanospray-srying in the present study, which, in addiction, mat have led to the formation of an outer skin during the drying as has been reported by other authors that carried out studies of protein drying (Dehnad et al. 2016; Ghribi et al. 2015).

Chapter 5. Conclusions and Future Work

The main objective of this work was to develop stable bio-based nanoemulsions for encapsulation of ω-3, to be incorporated in food matrices. With this in mind, the nanoemulsions were characterized and its physical and chemical stability during storage was assed. The nanoemulsions systems were dried by two different techniques (freeze-drying and nanospray-drying) and the resulting powders characterized before and after a rehydration process.

Regarding ω-3 nanoemulsions the main results achieved were as follows:

- Nanoemulsions for ω-3 incorporation were successfully produced by high pressure homogenization using the milk protein Lf as emulsifier;
- The use of different concentrations of Lf in the nanoemulsions productions influenced the physical and morphological properties of the resulting nanoemulsions;
- The nanoemulsions stored at 4 °C remained physically stable during the 69 days of evaluation;
- The nanoemulsions stored at room temperature revealed instability in the 69 days period evaluated;
- The antioxidant activity of the nanoemulsions stored at 4 °C was not affected over the 35 days of assessment;
- The oxidation of the ω-3 increased significantly over the 36 days of storage at 4 °C;
- The ω-3 was encapsulated with EE higher than 99 %;
- Freeze-drying of ω-3 nanoemulsions originated amorphous powders;
- Nanospray-drying of ω-3 nanoemulsions resulted in powders constituted by defined spherical particles with submicron sizes;
- The powders resulting from freeze-drying were able to be rehydrated but the starting form of the nanoemulsions droplets was not recovered;
- The nanospary-drying powders were not able to be rehydrated.

Despite the achievement of the main objectives of this thesis, there is still some work to be done in an attempt to improve nanoemulsions and its functionalities. The addiction of another biopolymer, such as a polysaccharide, could contribute to the improvement of the chemical stability

by means of an improved interfacial layer, which could contribute to a reduction in the increase of oxidation over storage. Moreover, the addiction of a polysaccharide could also add benefits to the drying process, helping to protect the developed nanostructures during drying processes, as well as improve the rehydration processes, allowing for a better recovery of the initial structure of the developed nanostructures.

More knowledge of the nanoemulsions characterization in terms of the interactions between oil and Lf in the three distinct stages of the formation of nanoemulsions: before drying, after drying, and after rehydration would also be of interest. Information regarding the release profile of the ω-3 nanoemulsions and its bioaccessibility in human tract and properties such as its toxicity, is also of extreme importance.

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Appendixes

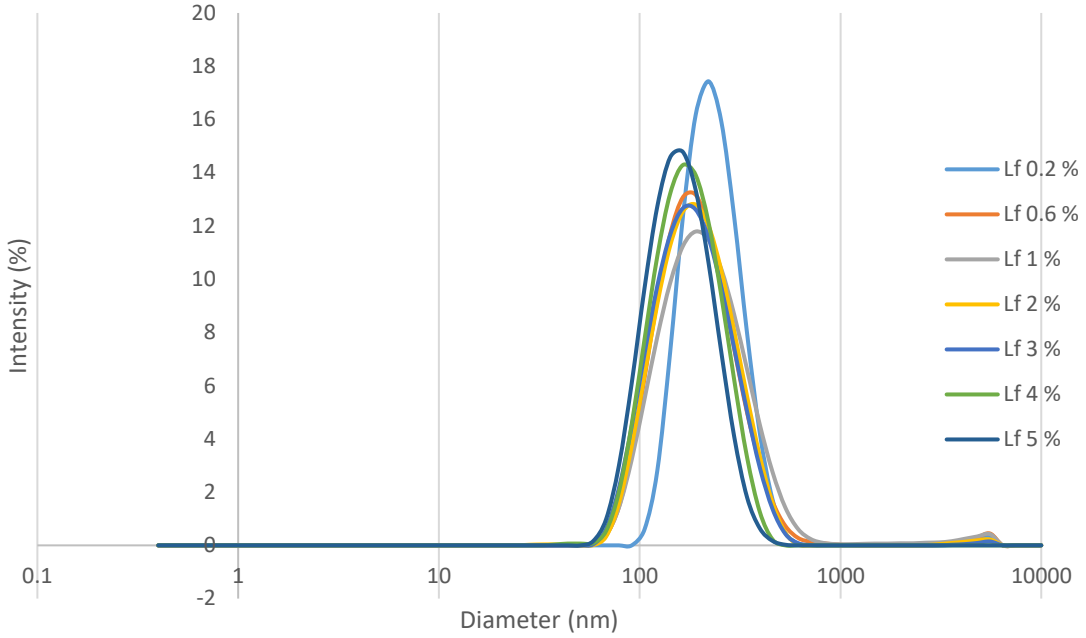

Figure A1. Size distribution of ω-3 nanoemulsions produced with different concentrations of lactoferrin (Lf) % (w/w).

Table A1. Size of the droplets of ω-3 nanoemulsions produced with different concentrations of lactoferrin (Lf) % (w/w). The values were obtained as the mean of the measurement of ten droplets in each TEM imagen obtained for the different nanoemulsions formulations (in the formulation of 5 % (w/w) Lf it was only possible to delimit 7 droplets). The size measurements were due using the free software *ImageJ*.

Lactoferrin concentration % (w/w)	0.2	0.6	1	2	3	4	5
Size (nm)	–	–	83.7	66.0	56.6	81.0	84.5

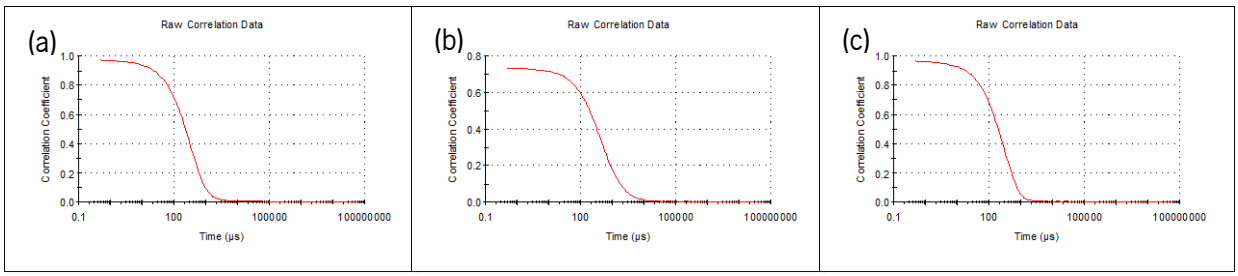

Figure A2. Correlograms of the size distributions obtained by DLS for ω-3nanoemulsions formulations with Lf concentrations of 2 (a), 3 (b) and 4 (c) % (w/w) dried by freeze-drying, after rehydration.

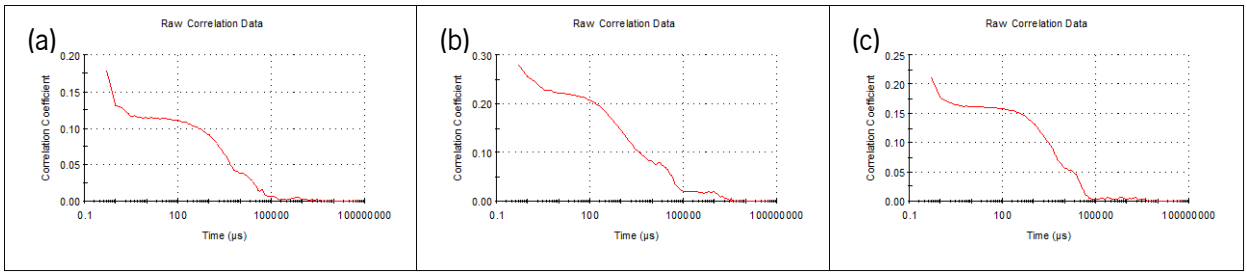

Figure A3. Correlograms of the size distributions obtained by DLS for ω-3nanoemulsions formulations with Lf concentrations of 2 (a), 3 (b) and 4 (c) % (w/w) dried by nanospray-drying, after rehydration.

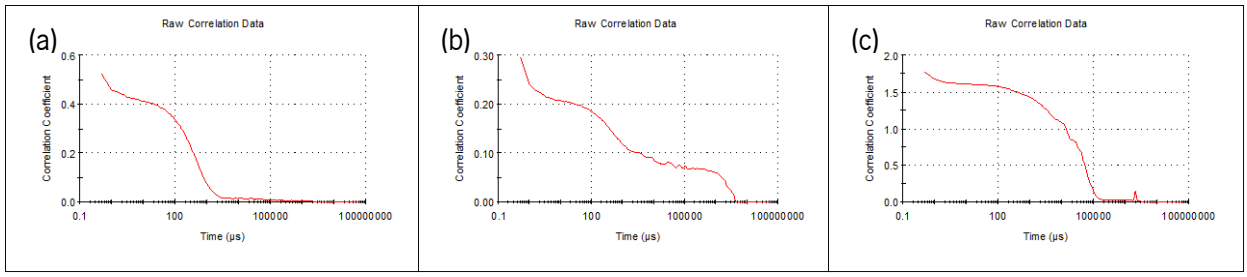

Figure A4. Correlograms of the size distributions obtained by DLS for ω-3nanoemulsions formulations with Lf concentrations of 2 (a), 3 (b) and 4 (c) % (w/w) dried by nanospray-drying, after rehydration and filtration through syringe filters.

Table A2. DLS results obtained for ω-3nanoemulsions formulations with different lactoferrin (Lf) dried by nanospray-drying, after rehydration and filtration through syringe filters.

Lf concentration % (w/w)	Size (nm)	PdI	Superficial charge (mV)
2	320.2	0.33	–
3	677.2	0.641	–
4	4422.9	0.862	–

Figure A5. Image of a freeze-dried sample after rehydration and centrifugation. A considerable deposit of particles that had not undergone a properly rehydration is showed.

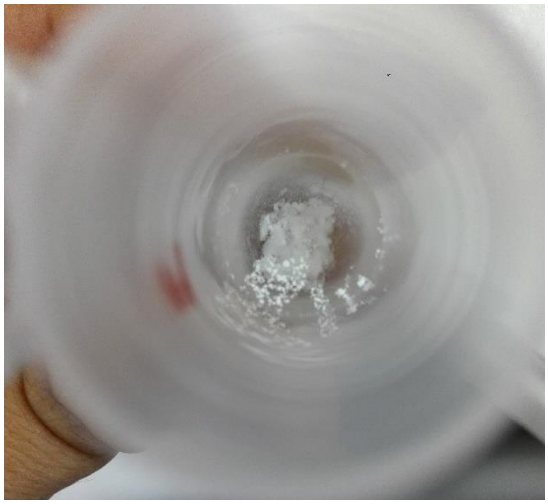

Figure A6. Image of a nanospray-dried sample after rehydration in phosphate buffer and submission to sonication. Is visible a remaining suspension containing some macroaggregates as well as some deposit of powder.