Emulsion-based delivery systems as a strategy to improve functionality of β-carotene

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Emulsion-based delivery systems as a strategy to improve functionality of β-carotene

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"*Winning isn't about finishing in first place. Isn't about beating the others. Winning means surpassing yourself and turning your dreams into reality*".

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RESUM

Els compostos bioactius s'associen amb la promoció de la salut així com amb la prevenció de malalties, i és per això que estan despertant l'interès actual dels consumidors, les indústries alimentàries i els investigadors. No obstant això, la baixa solubilitat, estabilitat i biodisponibilitat d'alguns compostos bioactius de caràcter lipofílic, com el β-carotè, comprometen la seva incorporació en la majoria de productes alimentaris. Avenços recents en l'encapsulació i la protecció d'aquests compostos bioactius, han obert noves portes al desenvolupament d'aliments enriquits, amb especial atenció als sistemes d'alliberament de compostos bioactius basats en emulsions. Així mateix, la composició d'aquests sistemes determina clarament les seves propietats fisicoquímiques i la seva funcionalitat. Per tant, l'objectiu principal d'aquesta Tesi Doctoral es utilitzar diferents tipus d'estabilitzants per formular sistemes basats en emulsions i aconseguir un alliberament del β-carotè controlat i exitós.

Les nanoemulsions de β-carotè formulades amb diferents emulsionants (Tween 20, lecitina, caseïnat de sodi i palmitat de sacarosa) a diverses concentracions (2-8%) van ser digerides a través d'un tracte gastrointestinal *in vitro* i es van determinar les seves propietats fisicoquímiques, digestibilitat i finalment, la bioaccessibilitat del β-carotè. Les nanoemulsions estabilitzades amb lecitina al 8% van presentar l`alliberació d'àcids grassos lliures més elevada (100%) després de la digestió intestinal i, al mateix temps, la bioaccessibilitat de β-carotè més alta (23.5%). A més a més, es va avaluar la permeabilitat de les nanoemulsions de β-carotè a través de dos tipus de barrera intestinal (Caco-2 i Caco-2 / HT29-MTX). Les nanoemulsions estabilitzades amb lecitina van presentar una concentració de β-carotè en les cèl·lules Caco-2 significativament més elevada (2.28%) en comparació amb les que contenien caseïnat de sodi (1.72%). Per contra, la concentració de β-carotè va ser significativament menor en les cèl·lules Caco-2 / HT29-MTX (≥0.74%), sense diferències significatives entre els dos emulsionants.

A les nanoemulsions enriquides en β-carotè es van afegir diferents concentracions de fibra de mandarina (0-2%). Les nanoemulsions van presentar propietats fisicoquímiques similars al llarg de les diferents fases del tracte gastrointestinal *in vitro*, així com valors de digestibilitat molt semblants entre elles. Tot i això, es va observar un augment significatiu de la bioaccessibilitat de β-carotè en aquelles nanoemulsions que contenien fins a l'1% de fibra de mandarina.

L'emulsió terciària (lactoferrina / alginat / ε-poli-L-lisina) es va desenvolupar per encapsular el β-carotè i estudiar la seva eficàcia com a sistema d'alliberament. Es va avaluar la seva estabilitat física i contingut en β-carotè sota diverses situacions externes (canvis de temperatura, pH i força iònica), així com la seva funcionalitat. L'emulsió terciària va presentar un augment de mida de partícula després de ser sotmesa a temperatures extremes (≥70 ºC), condicions àcides (≤4) o amb l'addició de sal (≥0.1 M). A més, el contingut de β-carotè en les emulsions terciàries va disminuir només un 40% quan aquestes van ser sotmeses a temperatures inferiors a 70 ° C, en condicions àcides i per sota de 0.3 M de NaCl. Finalment, les emulsions terciàries van presentar,

no només una digestibilitat més elevada (83.59%), sinó que també una bioaccessibilitat de β-carotè més alta (70.10%) en comparació amb les emulsions primàries i secundàries.

Els resultats obtinguts en aquesta Tesi Doctoral proporcionen informació útil sobre com la composició dels sistemes basats en emulsions enriquits amb β-carotè pot influir en les seves propietats fisicoquímiques, en l'estabilitat al llarg del tracte intestinal i en les condicions externes, així com, en la digestibilitat i en l'alliberació de β-carotè. Per tant, aquests sistemes es poden considerar vehicles transportadors de compostos lipofílics i al mateix temps, representen un punt de partida per al disseny d'aliments funcionals amb beneficis per la salut.

RESUMEN

Los compuestos bioactivos son sustancias antioxidantes que aportan diversos beneficios a la salud, por lo que han despertado el interés de los consumidores, las industrias alimentarias y los investigadores. Sin embargo, la baja solubilidad, estabilidad y biodisponibilidad de algunos compuestos bioactivos lipofílicos, como el β-caroteno, comprometen su incorporación en los alimentos. Recientes avances en técnicas de encapsulación y protección de estos compuestos bioactivos han abierto nuevas posibilidades para el desarrollo de nuevos alimentos enriquecidos, con especial atención a los sistemas de liberación basados en emulsiones. Sin embargo, la composición de estos sistemas determina claramente sus propiedades fisicoquímicas y su funcionalidad. Así, el objetivo principal de esta Tesis Doctoral es utilizar diferentes tipos de estabilizantes para formular sistemas basados en emulsiones para una liberación efectiva del β-caroteno.

Las nanoemulsiones de β-caroteno formuladas con diferentes emulsionantes (Tween 20, lecitina, caseinato de sodio y palmitato de sacarosa) a diversas concentraciones (2-8%) se sometieron a una digestión *in vitro* y se determinaron sus propiedades fisicoquímicas, digestibilidad, así como la bioaccesibilidad del β-caroteno. Las nanoemulsiones estabilizadas con lecitina al 8% presentaron la mayor liberación de ácidos grasos libres (100%) después de la digestión y, a su vez, exhibieron la mayor bioaccesibilidad de β-caroteno (23.5%). Además, se evaluó la permeabilidad de las nanoemulsions de β-caroteno a través de dos modelos intestinales (Caco-2 y Caco-2 / HT29-MTX). Las nanoemulsiones estabilizadas con lecitina presentaron una concentración de β-caroteno en las células Caco-2 significativamente más elevada (2.28%) en comparación con las de caseinato de sodio (1.72%). Por el contrario, la concentración de β-caroteno fue significativamente menor en las células Caco-2 / HT29-MTX (≥0.74%), sin diferencias significativas entre ambos emulsionantes.

Por otro lado, se añadieron diferentes concentraciones de fibra de mandarina (0-2%) a las nanoemulsiones enriquecidas con β-caroteno. Las nanoemulsiones exhibieron propiedades fisicoquímicas similares a lo largo de las diferentes fases del tracto gastrointestinal *in vitro* y una digestibilidad parecidad entre las nanoemulsiones. Sin embargo, se observó un aumento significativo en la bioaccesibilidad de β-caroteno en aquellas nanoemulsiones que contenían hasta 1% de fibra de mandarina.

La emulsión terciaria (lactoferrina / alginato / ε-poli-L-lisina) se desarrolló para encapsular el β-caroteno y determinar su eficacia como sistema de liberación. Se evaluó su estabilidad física y contenido de β-caroteno bajo situaciones externas (cambios de temperatura, pH y fuerza iónica), así como su funcionalidad. La emulsión terciaria presentó un aumento de tamaño de partícula después de temperaturas extremas (≥70ºC), condiciones ácidas (≤4) o adición de sal (≥0.1M). Además, el contenido de β-caroteno en las emulsiones terciarias disminuyó solo un 40% cuando éstas se sometieron a temperaturas ≤70 °C, en condiciones ácidas y por debajo de NaCl 0.3 M. Finalmente, las emulsiones terciarias exhibieron una mayor digestibilidad

(83.59%) y bioaccesibilidad de β-caroteno (70.10%) en comparación con las emulsiones primarias y secundarias.

Los resultados obtenidos en esta Tesis Doctoral proporcionan información útil sobre cómo la composición de los sistemas enriquecidos con β-caroteno basados en emulsiones, pueden influir en sus propiedades fisicoquímicas, en su estabilidad en condiciones de estrés y tracto intestinal, así como la digestibilidad y liberación de β-caroteno. Por tanto, estos sistemas se consideran como vehículos transportadores de compuestos lipofílicos, lo que podría abrir nuevas perspectivas para diseñar alimentos funcionales con beneficios para la salud.

ABSTRACT

Bioactive compounds are of great interest for consumers, food industries and researchers due to their potential health benefits. However, the low aqueous solubility, stability and bioavailability of some lipophilic bioactive compounds, such as β-carotene, greatly compromise successful incorporation within foodstuffs. Recent advancements in encapsulation and protection of bioactive compounds has opened new possibilities for the development of enriched novel food products, with special attention to emulsion-based delivery systems. However, composition of these systems clearly determines their physicochemical properties and functionality. The main goal of this Doctoral Thesis was to use stabilisers of different nature to formulate emulsion-based systems for a successful β-carotene delivery.

β-carotene nanoemulsions formulated with different emulsifiers (Tween 20, lecithin, sodium caseinate and sucrose palmitate) at various concentrations (2-8%) were subjected to an *in vitro* gastrointestinal tract (GIT) and their stability, lipid digestibility as well as bioaccessibility of β-carotene were determined. Lecithin-stabilised nanoemulsions at 8% presented the highest release of free fatty acids (100%) after lipid digestion, and in turn, exhibited the highest β-carotene bioaccessibility (23.5%). Furthermore, the permeability across two intestinal barrier models (Caco-2 and Caco-2/HT29-MTX co-cultures) was evaluated. Nanoemulsions stabilised with lecithin had a β-carotene concentration in Caco-2 cells lysates significantly higher (2.28%) compared with those containing sodium caseinate (1.72%). On the contrary, the concentration of β-carotene was significantly lower in Caco-2/HT29-MTX co-cultures cell lysates (≥0.74%) compared to Caco-2 cells, with no significant differences between both emulsifiers.

Different mandarin fiber concentrations (0-2%) were added to β-carotene-enriched nanoemulsions with the goal of assist on their stabilisation but also to strength their nutritional properties. Nanoemulsions exhibited similar physicochemical properties along the different phases of the *in vitro* gastrointestinal tract (GIT) and lipid digestibility. However, a significant increase in β-carotene bioaccessibility was observed for those nanoemulsions containing until 1% of mandarin fiber.

Tertiary emulsion (lactoferrin/alginate/ε-poly-L-lysine) was developed as a β-carotene carrier. Its physical stability and β-carotene content under external stresses (temperature, pH and ionic strength changes), as well as functionality were evaluated. Tertiary emulsion presented a great particle size after extreme temperatures (≥70ºC), acidic conditions (≤4) or with salt addition (≥0.1M). In addition, the maximum β-carotene content in tertiary emulsions was obtained when emulsions were subjected at temperatures <70 °C, in acidic conditions and below 0.3 M NaCl. Finally, tertiary emulsions exhibited higher lipid digestibility (83.59%) and β-carotene bioaccessibility (70.10%) compared with primary and secondary emulsions.

Results obtained in the present Doctoral Thesis provide useful information about how composition of emulsion-based systems enriched with β-carotene might have an influence on

their physicochemical properties under *in vitro* GIT and stress conditions, as well as, lipid digestibility and β-carotene delivery. These systems can be considered as promising vehicles for lipophilic compounds, which might open new perspectives for designing functional food products with outstanding benefits for consumers.

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INTRODUCTION

LITERATURE REVIEW:
Nanostructured lipid-based delivery systems as a strategy to increase functionality of bioactive compounds

Ariadna Gasa Falcon, Isabel Odriozola Serrano, Gemma Oms Oliu, Olga Martín Belloso
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Abstract

Acquisition of a healthy lifestyle through diet has driven the food manufacturing industry to produce new food products with high nutritional quality. In this sense, consumption of bioactive compounds has been associated with a decreased risk of suffering chronic diseases. Nonetheless, due to their low solubility in aqueous matrices, high instability in food products during processing and preparation as well as poor bioavailability, the use of such compounds is sometimes limited. Recent advancements in encapsulation and protection of bioactive compounds has opened new possibilities for the development of novel food products. In this direction, the present review is attempting to describe encapsulation achievements, with special attention to nanostructured lipid-based delivery systems, i.e., nanoemulsions, multi-layer emulsions and liposomes. Functionality of bioactive compounds is directly associated with their bioavailability, which in turn is governed by several complex processes, including the passage through the gastrointestinal tract and transport to epithelial cells. Therefore, an overview of recent research on the properties of these nanostructured lipid-based delivery systems with a strong impact on the functionality of bioactive compounds will be also provided. Nanostructured lipid-based delivery systems might be used as a potential option to enhance the solubility, stability, absorption and, ultimately, functionality of bioactive compounds. Several studies have been performed in this line, modifying the composition of the nanostructures, such as the lipid-type or surfactants. Overall, influencing factors and strategies to improve the efficacy of encapsulated bioactive compounds within nanostructures have been successfully identified. This knowledge can be used to design effective targeted nanostructured lipid-based delivery systems for bioactive compounds. However, there is still a lack of information on food interactions, toxicity and long-term consumption of such nanostructures.

Keywords: nanoemulsions; multi-layer emulsions, liposomes; bioactive compounds; stability, lipid digestibility, bioaccessibility, absorption, functionality.

1. Introduction

The World Health Organization (WHO) reported that by 2020, chronic diseases will be responsible for nearly three-quarters of all deaths worldwide, including ischaemic heart disease, deaths due to stroke and diabetes. The fact that eating habits are relevant in either the appearance or the prevention of these chronic illnesses provides food for thought. People's awareness of nutrition habits is increasing and has derived into a growing demand for foodstuffs with specific functions for overall good health and well-being.

Not being synthesized by the human body, bioactive compounds need to be incorporated into our daily diet since they are involved in several biological processes, such as the regulation of gene expression in cell proliferation and apoptosis, as well as intermediation in hormone metabolism (Sashirekha, Mallikarjuna, & Rajaratham, 2015). In addition, they exhibit antioxidant and anti-inflammatory activities, as well as antibacterial properties, which have drawn the interest of the scientific community studying these compounds (Rodriguez-Casado, 2016).

In an attempt to satisfy the consumer's requests, the food industry is trying to use and incorporate these bioactive compounds within foodstuffs (Bonilla, Vargas, Oliveira, Aparecida-Makishi, & Amaral-Sobral, 2015). Nonetheless, they present several limitations, including low water solubility, stability and bioavailability, which can compromise successful incorporation and functionality. The common interest of both consumers and the food industry for healthier products has been translated into an in-depth search of tools in order to overcome these limitations (Asioli et al., 2017). Researchers have been exploring the possibilities for incorporating bioactive compounds into food and beverages as well as for strengthening their positive health benefits. In this sense, nanotechnology has been envisaged as a potential approach that offers several applications in the food field, such as animal feed, novel foods and food additives. Nanotechnology has been established as a key enabling technology (KET) by the European Commission because it facilitates forward-looking possibilities for the development of food innovative products (Peters et al., 2016). Specifically, nanostructured delivery systems consist of tiny droplets within the nanometric range, with the proven ability to carry and protect bioactive compounds. So far, different types of carrier systems have been developed, including nanoemulsions (Ozturk, Argin, Ozilgen, & McClements, 2015), double emulsions (Teixé-Roig, Oms-Oliu, Velderrain-Rodríguez. Odriozola-Serrano, & Martín-Belloso, 2018), multi-layer emulsions (Acevedo-Fani, Silva, Soliva-Fortuny, & Martín-Belloso, 2017), liposomes (Singh, Thompson, Liu, & Corredig, 2012) and solid lipid nanoparticles (Triplett, & Rathman, 2009), among others. These systems allow the solubilisation, encapsulation and protection of bioactive compounds, but also improve their stability under certain environments such as heat, extreme pH and gastrointestinal fluids (Oehlke et al., 2014). Thus, delivery of bioactive compounds through the gastrointestinal tract is guaranteed and, consequently, their bioavailability and functionality are enhanced. The outstanding properties concerning these systems facilitate the incorporation of bioactive compounds to foodstuffs, thereby conferring an extra nutritional value.

This review will be focused on discussing the use of nanostructured lipid-based delivery systems for enhancing the functionality of lipophilic bioactive compounds.

2. Lipophilic bioactive compounds

In the last decade, there has been a significant growing interest toward the development of nutraceuticals, which are foodstuffs that provide health and medical benefits (Daliu, Santini, & Novellino, 2018; Santini, & Novellino, 2017). Therefore, it is very common in the food and beverage industry to extract bioactive compounds molecules from its original sources and purify them for several applications, including dietary supplements or food fortification (Kumar, Yadav, Kumar, Vyas, & Dhaliwal, 2017). Bioactive compounds are non-essential for human life, but they have been categorised as disease-prevention ingredients due to their antioxidant, anti-tumour, anti-mutagenic, anti-proliferative and anti-inflammatory properties. Numerous studies have proven these health benefits both *in vitro* (tissue culture studies) (Clark, Lee, & Lee, 2015; Hadad, & Leyy, 2012) and *in vivo* (animal and human intervention trials) (Grainger et al., 2008; Horváth et al., 2015). These bioactive compounds are mainly found in fruits and vegetables, but also in legumes, nuts, herbs and spices, among others. According to some studies, the European population does not meet the daily recommended intake of fruits and vegetables, which has been set in 400 g/day (Yngve et al., 2005; Diethelm et al., 2012). In addition, special attention has been paid to lipophilic bioactive compounds (carotenoids, flavonoids and vitamins E, D and K), since their incorporation to aqueous-based food products is limited. Furthermore, these compounds are light, temperature and/or oxygen sensible, which can negatively affect their efficacy as health-related agents. Either manufacturing or storage of foodstuffs as well as the extreme conditions found through the digestive tract can lead to degradations of these lipophilic compounds (Xianquan, Shi, Kakuda, & Yueming, 2005). For instance, high temperature during food processing or acidic environments during the stomach phase can lead to the transformation of β-carotene into oxidation products, such as carotenoid carbocation, which is indicative of compound degradation (Boon, McClements, Weiss, & Decker, 2010). Besides that, digestive fluids are also aqueous, which can affect their transport throughout the gastrointestinal tract and the transfer to mixed micelles for further absorption. Thus, a combination of all these factors might imply the loss of lipophilic bioactive compounds and, consequently, compromise the intestine reach, absorption and postprandial delivery to target organs, meaning poor functionality. Nanostructured lipid-based delivery systems might help address these problems concerning bioactive compounds.

3. Nanostructured lipid-based delivery systems

Research studies focused on nanostructured lipid-based delivery systems as a strategy to protect lipophilic bioactive compounds have extensively increased. A brief description of the main nanostructured lipid-based delivery systems for encapsulating such compounds, including nanoemulsions, multi-layer emulsions and liposomes (Figure 1), is outlined below.

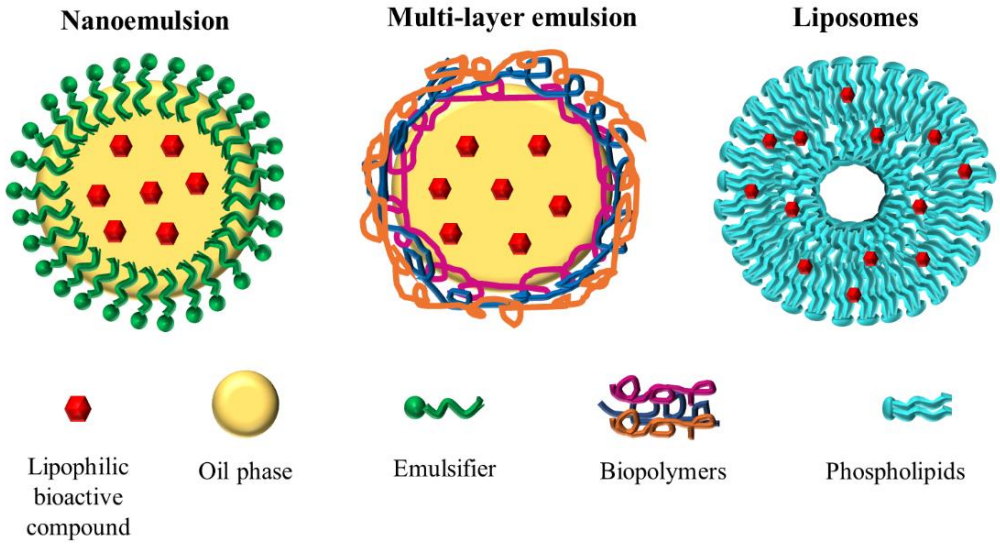

Figure 1. Nanostructured lipid-based delivery systems for encapsulating lipophilic bioactive compounds.

3.1. Nanoemulsions

Nanoemulsions are oil-in-water emulsions containing nanometric-size oil droplets ranging from 50 to 500 nm (Solans, Izquierdo, Nolla, Azemar, & Garcia-Celma, 2005). The main components for nanoemulsions formation are oil (lipid phase) and water (aqueous phase). On one hand, lipophilic bioactive compounds are solubilized within the oil before the formation of nanoemulsions. Different oils have been employed, including corn oil (Artiga-Artigas, Lanjari-Pérez, & Martín-Belloso, 2018), essential oils (Artiga-Artigas, Acevedo-Fani, & Martín-Belloso, 2018) and medium-chain triglycerides (MCT) (Miglyol® 810 or Miglyol®812) (Ozturk, Argin, Ozilgen, & McClements, 2015). On the other hand, there are different components that can be incorporated within the aqueous phase, such as emulsifiers (Tweens, phospholipids, proteins), texture modifiers (sodium alginate, pectin, carrageenan), preservatives and/or antioxidants (parabens, tocopherols), among others. Both the lipid and aqueous phases are immiscible, so they need to be combined in order to disperse the lipid phase (oil droplets) within the aqueous phase (water surrounding oil droplets). For this, the presence of emulsifiers is important since they are surface-active compounds that are adsorbed at the oil–water interface of the droplets, thereby reducing the interfacial tension between both phases and assisting in the stabilisation of the system (McClements, Bai, & Chung, 2017). Their ability to adsorb at the oil–water interface is also subjected to the molecular structure of each emulsifier (Silva, Cerqueira, & Vicente, 2015). Finally, homogenization of both phases with all the components is an important process in order to form nanoemulsions. For this process, the use of high-energy (high-pressure homogenization, sonication) or low-energy methods (self-emulsification, phase inversion) is required (Yang, Marshall-Breton, Leser, Sher, & McClements, 2012). High energy

methods are widely employed for producing these systems, which consist of applying mechanical forces (shear, disruptive) in order to create small oil droplets through their break down (Schultz, Wagner, Urban, & Ulrich, 2004). Alternatively, low-energy methods consist of spontaneous emulsification by using the internal chemical energy of the system (Marzuki, Wahab, & Hamid, 2019).

The final properties of nanoemulsions will be determined not only by the nature and characteristics of each component forming part of the system (Artiga-Artigas et al., 2018; Jo, Kwon, 2014; Komaiko, & McClements, 2015) but also by the fabrication employed for producing nanoemulsions (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2014).

3.2. Multi-layer emulsions

Multi-layer emulsions are oil-in-water emulsions with oil droplets covered by at least two layers of biopolymers that are electrostatically charged. Several stabilisers, including surfactants (lecithin, sodium dodecyl sulphate, Tween 20) and biopolymers (sodium alginate, pectin, chitosan, β-lactoglobulin) have been extensively used to produce multi-layer emulsions with different lipophilic bioactive compounds, such as lutein (Xu, Aihemaiti, Cao, Teng, & Li, 2016), resveratrol (Acevedo-Fani et al., 2017), curcumin (Silva et al., 2019) and β-carotene (Gasa-Falcon, Acevedo-Fani, Oms-Oliu, Odriozola-Serrano, & Martín-Belloso, 2019).

In general, a single-layer emulsion is firstly done, thereby homogenizing the lipid phase (oil containing lipophilic bioactive compounds) and the aqueous phase (charged surfactant or biopolymer). Then the layer-by-layer (LbL) method is used to produce several multilayers by placing biopolymers oppositely charged around the oil droplets.

It should be stated that fabrication of these emulsions is a delicate procedure as instability processes (e.g., bridging flocculation or depletion flocculation) occur when there is either an excess or lack of polyelectrolytes (Guzey & McClements, 2006). In this sense, there are different strategies that can be employed to produce stable multi-layer emulsions: (1) the saturation method, to empirically determine (ζ-potential and/or particle size) the concentration of the biopolymers needed to cover the oil droplets; (2) the centrifugation method, to remove the excess non-adsorbed biopolymers by centrifuging; or (3) the filtration method, whereby the excess of non-adsorbed biopolymers is removed by membrane filtration.

When a suitable concentration of each polyelectrolyte is employed to form multi-layer emulsions, there are two types of stabilisation mechanisms that contribute to the equilibrium of the whole system. On one hand, steric stabilisation occurs when oil droplets are coated with biopolymers that provide a layer around them, which is thick enough to overcome attractive forces between droplets. On the other hand, charges of the stabilisers covering the oil droplets contribute to the electrostatic stabilisation, which avoids that the oil droplets come close to each other (Berton-Carabin, Sagis, & Schroën, 2018). Apart from biopolymer concentration, the pH of the solution needs to be controlled during all stages of the preparation process, so that the biopolymers are charged enough to avoid destabilisation processes (Guzey & McClements, 2006).

In the literature, multi-layer emulsions with two (Silva et al., 2019), three (Acevedo-Fani et al., 2017) and up to four layers (Tokle, Mao, & McClements, 2013) of biopolymers have been produced. The properties of multi-layer emulsions strongly depend on the characteristics of the biopolymers used to assemble the different layers. For instance, Mun and co-authors reported that the particle size of a bi-layer emulsion (Tween 20-chitosan) was affected by the chitosan's degree of deacetylation (DDA), presenting lower particle sizes than those emulsions containing the chitosan with the lowest DDA (Mun, Decker, & McClements, 2006).

3.3. Liposomes

Liposomes are spherical structures consisting of polar lipids, which have hydrophilic and lipophilic groups within the same molecule (Singh, Thompson, Liu & Corredig, 2012). Hydrophobic tails of lipids interact with each other, thereby forming bilayer structures that have an aqueous interior and at the same time are surrounded in aqueous media. Therefore, hydrophilic and lipophilic compounds can be simultaneously encapsulated in the aqueous core and the phospholipid bilayer, respectively (Tsai & Rizvi, 2017).

According to the number of phospholipid bilayers contained within each structure and the size of the liposome, they are classified as small unilamellar vesicles (single layer and <0.1 μm), large unilamellar vesicles (single layer and 0.1–1 μm) and multilamellar liposomes (several layers and >1 μm) (Taylor, Davidson, Bruce, & Weiss, 2005). Different lipids have been employed for liposome formation, including phosphatidylcholine, sphingomyelin, phosphatidylserine, phosphatidylinositol and soybean lecithin. Methods to produce liposomes are based on the use of organic solvents, such as film hydration, ethanol injection and reverse-phase evaporation. However, liposomes prepared with these techniques normally present large particle sizes and a broad size distribution. The incorporation of an additional step (extrusion, sonication or microfluidization) might be used as an efficient strategy to produce liposomes with small particle size and with high encapsulation capacity (Dordevic et al., 2014). Solvent-free methods, such as pH-driven (Cheng et al., 2017) and heating-hydrated components from liposomes in the presence of glycerol (Mozafari, 2005), are alternatives that can be used for liposome formation and further food application. However, this type of nanostructures is difficult to scale up due to the several steps needed for the preparation. Besides, liposome fabrication techniques have a considerable influence on the encapsulation efficiency of bioactive compounds. Dehydration/rehydration techniques have shown high efficiency in incorporating lipophilic bioactive compounds, such as retinol (Mohammadi, Ghanbarzadeh, & Hamishehkar, 2014), while a significant decrease on encapsulation efficiency of resveratrol in liposomes from 97% to 44% was observed when they were prepared by the extrusion technique and sonication, respectively (Isailovic et al., 2013).

Recently, LbL techniques employed in emulsions have also been used for coating liposomes with different biopolymers, such as chitosan, pectin and carrageenan (Chun, Choi, Min, & Weiss, 2013). Another type of liposome is a niosome, which is formulated with non-ionic emulsifiers such as Span 40, Tween 20 or Tween 40. They are often chosen over liposomes because they are more stable and economic. However, there are only a few studies available on

the formulation of niosomes containing lipophilic bioactive compounds (Palozza, Muzzalupo, Trombino, Valdannini, & Picci, 2006).

4. Enhancing functionality of nanostructured lipid-based delivery systems

The successful delivery of encapsulated lipophilic bioactive compounds might be related to several factors, including the susceptibility of these nanostructures to undergo physical alteration when they are incorporated into foodstuffs or subjected to an *in vitro* gastrointestinal digestion. At the same time, effective nanostructures as delivery systems might be associated with their stability and digestibility, as well as bioaccessibility and absorption of health-related compounds. Thus, focusing research on studying all these processes is of great importance in order to cope with problems that might affect the overall functionality of nanostructures.

4.1. Enhancing stability of lipophilic bioactive compounds

Food processing and food matrix characteristics involve subjecting nanostructures to external environments that can negatively affect their integrity. To simulate these situations, nanostructures are normally exposed to different external or storing conditions (pH, ionic strength, light, temperature), followed by a monitorization of compound stability and/or physical stability of the system. Different studies about nanostructures under these conditions are described in Table 1.

On one hand, a high content of the lipophilic bioactive compound after exposure to external environments is important so as to maintain an overall functionality of the system. The principal advantage of nanostructured systems is their nanometric size, meaning that oil droplets have a large surface area. However, they are highly reactive with the surrounding media, and thus significant degradation of the encapsulated compound might occur. Teo and co-authors reported that the lutein degradation rate was strongly associated with the particle size of nanoemulsions, being higher as their particle size decreased from 147.3 nm to 68.8 nm (Teo, Lee, Goh, & Wolber, 2017). Stabilisers also play a fundamental role in the stability of the encapsulated compound because they are deposited at the interface, thereby covering the oil droplets that have the lipophilic compound solubilised. It was reported a faster β-carotene degradation in nanoemulsions stabilised with Tween 20 than those with β-lactoglobulin (Qian, Decker, Xiao, & McClements, 2012). Opposite results were observed by Young and co-authors, reporting a higher curcumin content in emulsions containing Tween 20 (>97.5%) compared to those containing whey protein (WPI) (<89.5%) (Young, Basiana, & Nitin, 2018). The electrical charge of oil droplets, which is mainly attributed to the stabilisers employed to formulate nanostructures, might have a direct impact on the stability of the compounds. Negatively charged oil droplets (anionic particles) are easily reactive to metal ions present in the aqueous phase, leading to degradation processes of the encapsulated compounds.

Another way to retard encapsulated lipophilic compound degradation is to strengthen the physical barrier by covering the oil droplets with several stabiliser layers (Xu et al., 2016). For

instance, it was observed that adding a layer of chitosan to lecithin-covered oil particles containing citral (multilayer emulsions) was effective to retain a twofold amount of citral compared to the lecithin emulsions (single layer emulsions) after four weeks of storage (25 °C) (Yang, Tian, Ho, & Huang, 2012). Accordingly, other authors found higher retention of β-carotene in multilayer emulsions (WPI-gum arabic) than in single layer emulsions (WPI) at all the storage temperatures tested (25 °C, 37 °C and 45 °C) during 70 days of storage (Lim, Griffin, & Roos, 2014). Similarly, applying a coating of pectin in the liposomes resulted in higher retention of resveratrol than in uncoated liposomes, avoiding oxidation and hydrolyzation of phospholipids processes and, therefore, leakage of encapsulated resveratrol (Shao, Wang, Niu, & Kang, 2018). Finally, the addition of water-soluble antioxidants, such as ascorbic acid, or oil-soluble antioxidants like coenzyme Q10, can delay the degradation of the encapsulated compounds in nanostructured systems by scavenging free radicals (Weigel, Wang, Niu, & Kang, 2018; Qian, Decker, Xiao, & McClements, 2012). Some of the stabilisers employed in nanostructured systems production, including proteins and some biopolymers, have antioxidant capacity. Accordingly, it was suggested that the antioxidant capacity of guar gum used to formulate β-carotene-enriched liposomes contributed to avoid β-carotene degradation, leading to values of retention ≥88% after 95 days under refrigeration conditions (Toniazzo, et al., 2014).

On the other hand, instability processes occurring in nanostructures can be associated with a breakdown of the structure, resulting in a no longer encapsulation of lipophilic compounds. Consequently, unprotected compounds would be more susceptible to undergoing degradation processes. Therefore, the physical stability of nanostructured systems needs to be evaluated under different situations in order to understand their behaviour, so as to design stable systems. The nanometric size of nanostructured systems is associated with a reduction in the gravity force and Brownian motion, as well as with the prevention of destabilisation processes (flocculation, coalescence, sedimentation, creaming). Lee and co-authors observed that emulsions with a low particle size (≈66 nm) had better stability under different pH conditions (3–8), ionic strength (0–500 mM), thermal treatment (30–90 °C) and freezing/thawing (−4 °C, 48 h followed by 25 °C, 6 h) (Lee, Choi, Li, Decker, & McClements, 2011). However, physical stability also lies on the composition of the nanostructures. Artiga-Artigas et al. (2018) observed that the physical stability of curcumin-enriched nanoemulsions stabilised with lecithin strongly depended on the concentration of the emulsifier used, being those nanoemulsions containing concentrations over 1% the most stable emulsion. In the case of sucrose palmitate emulsions, they presented destabilisation processes (clarification, sedimentation, flocculation and coalescence) just hours after preparation. Because of the numerous layers deposited around the droplets, multi-layer emulsions might improve physical stability over a wide range of conditions. The physical stability of emulsions subjected to ionic strength changes (0–500 mM NaCl) was improved when they were covered with two (sodium dodecyl sulphate-chitosan) and three layers (sodium dodecyl sulphate-chitosan-pectin membranes) (Aoki, Decker, & McClements, 2005). Another investigation concluded that the physical stability of multi-layer emulsions at different heat treatments highly depended on the biopolymer type employed for formulating these systems. Specifically, secondary emulsions containing lactoferrin-alginate

were prone to droplet aggregation at temperatures over 60 °C, while droplet size remained unchanged up to temperatures of 90 °C when they contained lactoferrin-pectin (high and low methoxyl). Authors attributed these differences to the branches that pectin has, which would have provided both steric and electrostatic stabilisation (Tokle, Lesmes, & McClements, 2010).

Table 1. Recent studies about stability of nanostructured lipid-based delivery systems under different stressing conditions

	Lipophilic compound	Materials	Preparation method	Stressing conditions	Main findings	References
Nanoemulsions	Curcumin	Lipid phase: corn oil Emulsifier: Tween 20, lecithin and sucrose monopalmitate	MF	Storage: 86 days at 25 ℃	Lecithin-stabilised nanoemulsions were the most stable, while the rest undergone destabilisation processes after 24h preparation.	(Artiga-Artigas et al., 2018)
	Ergocalciferol	Lipid phase: soybean oil Emulsifier: modified lecithin (ML), sodium caseinate (SC), decaglycerol monooleate (MO-7S)	HPH	pH conditions: 2-8 Ionic strength: 0-500 mM NaCl Thermal treatment: 80, 100 and 120 ℃ Dark storage 25 ℃ and 55 °C.	Physical stability depended on the emulsifier type. Stability of ergocalciferol in emulsion system decreased in order of ML>MO-7S≫SC during storage (55 ℃ for 30 days).	(Shu et al., 2016)
	Vitamin E	Lipid phase: SCT, MCT and LCT Emulsifier: Tween 80	LEM	Ionic strength: 0-500 mM NaCl pH: 2-8.5 Thermal treatment: 30-90 ℃ Storage: light/dark at 4, 25 and 40 ℃	Nanoemulsions were physically stable at high temperature (≈90 °C), high ionic strength (≈500 mM) and long-term storage (60 days) under light and dark conditions (4–40 °C)	(Hategekimana et al., 2015)

	β-carotene	Lipid phase: soybean oil Emulsifier: Ulva fasciata (UFP)	MF	Thermal treatment: 70, 80, 90 and 100 °C pH: 3-7 α-tocopherol: 0-500 mg/kg EDTA: 0-500µl/L	β-carotene was highly sensitive to acidic conditions and extreme temperatures. Addition of EDTA or α-tocopherol increased the stability of β-carotene	(Shao et al., 2017)
Multi-layer emulsions	Astaxanthin	Lipid phase: flaxseed oil Coatings composition: Q-naturale-pectin-chitosan	LbL	Thermal treatment: 20, 30, 50 and 80 °C Ionic strength: 50-1000mM NaCl pH: 2-8 Storage: quantification of astaxanthin for 15 days.	Multi-layer-coatings improved astaxanthin stability during storage, as well as physical stability at elevated ionic strengths and temperatures.	(Liu et al., 2019)
	β-carotene	Lipid phase: corn oil Coatings composition: lactoferrin-alginate-ε-poly-l-lysine	LbL	pH conditions: 2-11 Ionic strength: 0-500 mM NaCl Thermal treatment: 30-90 °C.	β-carotene content decreased only 40% when emulsions were subjected at temperatures ≤70 °C, in acidic conditions and below 0.3 M NaCl.	(Gasa-Falcon et al., 2019)

Liposomes	Resveratrol	Lipid phase: phospholipid, cholesterol Coatings: low and high methoxy pectin	FD	pH conditions: 3, 5 and 7.4 Ionic strength: 0-200mM NaCl Thermal treatment: 4 and 25 °C for 7 weeks	Low methoxy pectin improved physical stability of liposomes as well as resveratrol retention under different stress conditions.	(Shao et al., 2018)
	Curcumin	Lipid phase: phosphatidylcholine (98.1%) and lysophosphatidylcholine (0.7%) Modifier: Pluronic F127, F87 and P85	TFE + DHPM	pH conditions: 7.4, 8, 10 and 12 Thermal stability: 80 °C	Adding pluronics improved thermal and pH stability of liposomes.	(Li et al., 2019)

SCT: short-chain triglycerides; MCT: medium-chain triglycerides; LCT: long-chain triglycerides; EDTA: ethylenediaminetetraacetic acid; DTA, MF: microfluidizer; HPH: high pressure homogenizer; LEM: low-energy method (emulsion phase inversion: catastrophic phase inversion); LbL: layer-by-layer technique; FD: film dispersion method; TFE: thin film evaporation; DHPM: dynamic high pressure microfluidization.

4.2. Enhancing lipid digestibility and *in vitro* bioaccessibility of lipophilic bioactive compounds

Lipids present in nanostructured systems are important components because their hydrolysis will affect the release of encapsulated lipophilic compounds and, therefore, its bioaccessibility. Lipid digestion occurs mainly during the intestinal phase and consists of the hydrolysis of oil droplets, which produces free fatty acids, diacylglycerols and monoacylglycerols (Bauer, Jakob, & Mosenthin, 2005; Favé, Coste, & Armand, 2004). These products together with bile salts and other digestion components are responsible for producing mixed micelles, which incorporate and solubilise the released lipophilic bioactive compounds for further absorption (Maldonado-Valderrama, Wilde, Maclerzanka, & Mackie, 2011; Sarkar, Ye, & Singh, 2016). Thus, comprehension about bioaccessibility of lipophilic bioactive compounds requires knowledge about the lipid digestion of the nanostructured systems.

Different factors related to nanostructures might influence on lipid digestibility and bioaccessibility, including their physicochemical properties and composition (Table 2). As a general rule, emulsions with an initial small particle size present a higher lipid digestibility and bioaccessibility compared with emulsions with larger particle sizes (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013). However, Lee et al. 2011 observed that free fatty acids release was slightly higher in emulsions with a high particle size whereas no major differences in lipid digestibility between nanoemulsions (200 nm) and emulsions (10,000 nm) were found in another investigation (Ahmed, Li, McClements, & Xiao, 2012). These contradictory results might be related to the interfacial properties of the nanostructured systems, which in turn define their physicochemical properties. Furthermore, these properties might be associated with the susceptibility of nanostructures to undergo physical changes when they are subjected to an *in vitro* gastrointestinal tract (GIT). Specifically, nanostructured systems might be destabilised along the GIT because of the pH conditions during the different digestion stages, as well as the presence of salts and enzymes (Singh, Ye, & Horne, 2009). All these changes modify the initial properties of nanostructured systems and could probably affect lipid digestion (Golding & Wooster, 2010) and, therefore, the bioaccessibility of the encapsulated compounds. In a recent study, it was observed that nanoemulsions stabilised with different emulsifiers (Tween 20, lecithin, sodium caseinate and sucrose palmitate) and concentrations (2%, 4% and 8%) behaved differently during lipid digestion, but a similar final lipid digestibility was obtained regardless of the composition of the nanoemulsions. However, the bioaccessibility results were strongly influenced by the emulsifier nature rather than by the lipid digestibility results, obtaining the higher β-carotene bioaccessibility in lecithin-stabilised nanoemulsions (23.5%) (Gasa-Falcon, Odriozola-Serrano, Oms-Oliu, & Martín-Belloso, 2019). Similarly, it was reported that the highest vitamin E bioaccessibility was achieved when adding phospholipids to intestinal fluids (Yang, Decker, Xiao, & McClements, 2015). Mixed micelles are composed of bile salts, phospholipids from intestinal fluids as well as lipid digestion products from enzyme action during the intestinal phase. The fact that phospholipids were present, either in nanoemulsions

or in intestinal fluids, would have contributed to the formation of mixed micelles and therefore increase their solubilisation capacity.

Nanostructures formulated with protein-based emulsifiers tend to present values of lipid digestibility similar (Gasa-Falcon et al., 2019) or even higher (Mun, Decker, & McClements, 2007) compared to other emulsifiers, but poor bioaccessibility of the encapsulated compounds. Indeed, proteins are known to form complexes with some lipophilic compounds such as carotenoids, thereby promoting aggregation and precipitation of mixed micelles (Wackerbath, Stoll, Gebken, Pelters, & Bindrich, 2009). Poor digestibility has also been associated with the low stability of nanostructured systems along the GIT. If these systems became highly unstable and their particle size increase, the oil droplet surface exposed to enzymes would be lower, leading to low digestibility and bioaccessibility (Golding & Wooster, 2010; McClements, Decker, Park, & Weiss, 2008). In this sense, biopolymers, such as mandarin fibre, modified starch or corn fibre (texturizing/gelling agents), can be added to emulsions in order to increase the stability of these systems under digestive conditions and, therefore, increase both the lipid digestibility and bioaccessibility of the encapsulated compounds (Feng et al., 2017; Liang, Shoemaker, Yang, Zhong, & Huang, 2013; Gasa-Falcon, Odriozola-Serrano, Oms-Oliu, & Martín-Belloso, 2017). An increase in lipid digestibility in protein-stabilised nanoemulsions containing dietary fibre has also been observed, thereby preventing droplet aggregation during *in vitro* GIT digestion, maintaining the integrity of the system, and thus ensuring lipase could easily access the oil droplets (Qiu, Zhao, Decker, & McClements, 2015; Chang & McClements, 2016). On the contrary, it was observed that the initial rate of lipid digestion and the final amount of free fatty acids (%FFA) release was reduced when emulsions contained pectin (0.5%). This behaviour was explained because biopolymers can interact with calcium ions, reducing diffusion processes essential for complete lipid digestion. Alternatively, biopolymers might increase the thickness of the interfacial layer covering the oil droplets, thereby preventing their hydrolysation (Zhang, Zhang, Zhang, Decker, & McClements, 2015).

Biopolymers, including pectin, alginate and chitosan, can be used to assemble the different layers that cover the oil droplets of nanostructures (Scholten, Moschakis, & Biliaderis, 2014; Zeeb, Lopez-Pena, Weiss, & McClements, 2015; Li et al., 2010). However, there is controversy about whether biopolymer layers have a negative or positive effect on the bioaccessibility of the encapsulated compounds. Some authors have observed no significant correlation between the number of layers and lipid digestibility (Tokle et al., 2013; Huang, Wang, Li, Xia, & Xia, 2018). Silva and co-workers reported a similar total release in free fatty acids after *in vitro* dynamic digestion for curcumin-loaded nanoemulsions (WPI) (96.14%) and multilayer nanoemulsions (WPI–chitosan) (95.52%) (Silva et al., 2019). Nonetheless, a recent study reported that tertiary emulsions (lactoferrin/alginate/ε-poly-L-lysine) presented significantly higher β-carotene bioaccessibility (70.1%) compared to primary (30.2%) and secondary emulsions (35.26%) (Gasa-Falcon et al., 2019). On the contrary, other authors have suggested that the deposition of extra layers at the droplets interface might decrease free fatty acids release during lipid digestion (Li et al., 2010; Gudipati, Sandra, McClements, & Decker, 2010). Less free fatty acids were released in secondary emulsions (lecithin–chitosan) compared with

primary emulsions (chitosan) (Mun, Decker, Park, Weiss, & McClements, 2006). Accordingly, multi-layer emulsions formed with chitosan and soy protein isolate (SPI) presented a lower amount of free fatty acids (87.9%) than multi-layer emulsions prepared with alginate and SPI (104.1%) (Zhang et al., 2015). Therefore, using chitosan in nanostructured systems can negatively affect lipid digestion. Nonetheless, the deposition of a chitosan layer around liposomes (chitosomes) would have controlled the release of encapsulated carotenoids during GIT, favouring its bioaccessibility (Tan, Feng, Zhang, Xia, & Xia, 2016).

Other contributors or suppressor factors of lipid digestibility and bioaccessibility have been studied, including the chain length and degree of unsaturation of lipids used for nanostructure production. In general, no significant differences have been reported in the extent of lipid digestion for nanoemulsions containing long-chain triglycerides (LCT) or medium-chain triglycerides (MCT) (Qian et al., 2012; Salvia-Trujillo, Sun, Um, Park, & McClements, 2015). Conversely, a higher rate and extent of lipid digestion as well as bioaccessibility was reported for LCT–vitamin E emulsions in comparison with MCT–vitamin E emulsions (Yang & McClements, 2013). A recent study reported that vitamin D_3-loaded nanoemulsions containing monounsaturated fatty acids (corn oil) were digested quicker than those oils containing polyunsaturated fatty acids (flaxseed and fish oil), which was explained by the conformation that the polyunsaturated fatty acids can adopt, thereby restricting enzyme action. Moreover, vitamin D_3 bioaccessibility was higher in nanoemulsions formulated with corn oil (78%) than those formed with the rest of the oils (≈43%) (Schoener, Zhang, Lv, Weiss, & McClements, 2019). Similarly, liposomes from phospholipids derived from milk fat globule membrane (MFGM) showed a lower free fatty acids release compared to those derived from soybean, which was explained because of the higher amount of saturated fatty acids that MFGM contained (Liu, Ye, Liu, Liu, & Singh, 2012).

Finally, bioaccessibility of the encapsulated compounds can also be determined by the properties of the lipophilic compound. For instance, the intestinal release of different carotenoids loaded in liposomes changed depending on the type of carotenoid: lutein and β-carotene were released at a slower rate than lycopene and canthaxanthin (Tan et al., 2014). This study concluded that polar carotenoids with a perpendicular orientation within the phospholipid bilayer of liposomes (i.e., lutein and β-carotene) would have favoured the carotenoid transfer to mixed micelles for further absorption.

Table 2. Recent studies about *in vitro* lipid digestibility and bioaccessibility of encapsulated lipophilic compounds in nanostructured lipid-based delivery systems.

	Lipophilic compound	Materials	Gastrointestinal model	Main findings	References
Nanoemulsions	β-carotene	Lipid phase: corn oil Emulsifier: Tween 20	Static *in vitro* gastrointestinal tract (GIT)	Lipid digestibility and β-carotene bioaccessibility increased from 34% up to 59% with decreasing particle size of emulsions.	(Salvia-Trujillo et al., 2013)
	Curcumin	Lipid phase: corn oil Emulsifier: Tween 20, SDS or DTAB	Dynamic *in vitro* gastro-intestinal model (TIM)	Behaviour of nanoemulsions during *in vitro* digestion depended on the charge of the emulsifier	(Pinheiro et al., 2013)
	Vitamin D3	Lipid phase: MCT, corn oil, fish oil, mineral oil, or orange oil Emulsifier: Q-Naturale	Static *in vitro* gastrointestinal tract (GIT)	Long chain triglycerides (corn oil and fish oil) were most effective at increasing vitamin bioaccessibility (≈80%).	(Ozturk et al., 2015)
	DHA algae oil	Lipid phase: DHA algae oil Emulsifier: Tween 40, sodium caseinate, soya lecithin	Static *in vitro* gastrointestinal tract (GIT)	Encapsulated DHA in nanoemulsions showed higher FFA release (40-50%) compared to bulk DHA (≈20%).	(Karthik et al., 2016)

Multilayer emulsions	Fish oil	Lipid phase: fish oil Layers assembly: Citrem-chitosan-alginate	Dynamic *in vitro* gastro-intestinal model (TIM)	Lipid digestion rate was decreased with multilayer coating	(Gudipati et al., 2010)
	Carotenoids	Lipid phase: MCT oil Layers assembly: soy protein isolate-alginate-chitosan	Static *in vitro* gastrointestinal tract (GIT)	Alginate coating had no effect on lipid digestibility (≈100%) and bioaccessibility of carotenoids (≈11%).	(Zhang et al., 2015)
	Curcumin	Lipid phase: MCT oil Layers assembly: WPI-chitosan	Dynamic *in vitro* gastro-intestinal model (TIM)	The deposition of a chitosan layer did not affect lipid digestion (≈96%), but increased curcumin bioaccessibility (37.2%) compared to nanoemulsions (29.8%).	(Silva et al., 2019)
Liposomes	Curcumin	Lipid phase: cholesterol, phospholipid Modifiers: Pluronic F127, F87 and P85	Static *in vitro* gastrointestinal tract (GIT)	Curcumin loaded in Pluronics modified liposomes possessed increased bioaccessibility from 26.9% up to 43.3%.	(Li et al., 2018)

	Curcumin	Lipid phase: phosphatidylcholine and α-phosphatidic acid Coating composition: chitosan	Static *in vitro* gastrointestinal tract (GIT)	Uncoated and coated liposomes presented similar results in cucrumin bioaccessibility (≈70%).	(Cuomo et al., 2018)

Sodium dodecyl sulphate (SDS) ; dodecyltrimethylammonium bromide (DTAB); MCT: medium chain triglyceride oils; WPI: whey protein isole; DHA: docosahexaenoic acid

4.3. Enhancing absorption of lipophilic bioactive compounds in epithelial cells

There are several lines of evidence to conclude that lipid-based nanostructures might be potential delivery systems for bioactive compounds. However, the efficacy of these bioactive compounds is directly associated with their bioavailability, which is the fraction of the bioactive compound that reaches the systemic circulation. Bioavailability of compounds involves several steps, including their release from the matrix, solubilisation within mixed micelles, permeability, absorption and secretion to the blood system (Holst & Williamson, 2008). A critical step of absorption is permeability (or cellular uptake), which can be defined as the lipophilic bioactive compound amount that enters within epithelial cells for later passage to bloodstream and target organs.

Before carrying absorption assays and after digestion processes, it is important to determine the cytotoxicity of the nanostructured systems so as not to compromise the survival of cells during tissue culture studies. Sadhukha and co-workers investigated the cytotoxicity of the digested nanoemulsions, which were composed by pellets, oil droplets and an aqueous fraction, the latter one being the most important part of the digesta since it has solubilised the lipophilic compound (micellar fraction). Surprisingly, the aqueous fraction was the most toxic to cells, which was explained by the free fatty acids generated during *in vitro* GIT digestion (Sadhuka, Layek, & Prabha, 2018). Besides that, other hydrophilic components from nanostructured systems, such as emulsifiers, have also been reported to affect the viability of cells (Ujhelyi et al., 2012; Dimitrijevic, Shaw, & Florence, 2000). Both studies analysed the cytotoxicity of several non-ionic emulsifiers in Caco-2 cells using the diphenyltetrazolium bromide test (MTT) and lactate dehydrogenase test (LDH). Polyethene glycols (Tweens and Labrasol) were more toxic in a dependent manner than polyethene glycols, being those with the highest HLB (Tween 20 and 60) the most toxic.

Many lines of evidence have demonstrated that the use of differentiated monolayers (Caco-2 and Caco-2/HT29-MTX cocultures) with tight junctions best represent the morpho-functional features of the intestinal barrier to model the absorption of lipophilic bioactive compound in epithelial cells (Guri, Gülseren, & Corredig, 2013). This process consists of applying the digested sample at a suitable concentration in the apical compartment and incubate for at least 2 h. Finally, fractions from the apical (upper chamber), cell monolayer (in-between chamber) and basolateral compartment (lower chamber) are collected and analysed.

Numerous studies have investigated which properties of nanostructures might have a major influence on absorption of encapsulated compounds in tissue culture studies (Table 3). For instance, Andar et al. (2014) observed a higher cellular uptake in Caco-2 cells for liposomes with small particle sizes (between 40 and 73 nm) compared to larger sizes (between 97 and 277 nm). Similarly, nanoemulsions exhibited a higher cellular uptake of lutein as their particle size diminished from 147.3 to 68.8 nm (Teo et al., 2017). A great permeation through the cell monolayer as well as the large surface area would explain the high cell absorption in nanostructured delivery systems with low particle sizes.

Interfacial properties also have a strong influence on cellular uptake and absorption processes. In this sense, a recent study reported that the cellular uptake in Caco-2 cells of astaxanthin-loaded liposomes was improved when liposomes were formulated with 70% phosphatidylcholine (Sangsuriyawong, Limpawattana, Siriwan, & Klaypradit, 2018). On the other hand, another study described that cellular uptake of β-carotene in undifferentiated gastrointestinal epithelial cells (Caco-2 cells) depended on the emulsifier type added to the nanoemulsions rather than on the initial particle size of the nanoemulsions (Lu, Kelly, & Miao, 2017). Investigations about different emulsifiers and combinations in oil-in-water emulsions concluded that they had a strong influence on the cellular uptake of carotenoids by the HT-29 cells. In this sense, oil-in-water emulsions formulated with a combination of whey protein (Biozate) and lecithin or sucrose laurate (L-1695) enhanced more than threefold the cellular uptake of lutein and astaxanthin, respectively (Ribeiro et al., 2006; Frede et al., 2014). Since the interfacial properties of the nanostructured systems govern the cellular uptake, a positive charge of the multi-layer emulsions containing chitosan would have increased the uptake efficiency compared to those containing WPI, which had a negative charge (Silva et al., 2019). Hydrophilic polyelectrolytes, such as chitosan, can enhance the transport of these systems through Caco-2 cells via specific interaction between the nanostructured systems and the intestinal epithelium. Lipid composition is another parameter that might have a substantial influence on the permeability of lipophilic compounds. The impact of lipid phase-type (MCT, medium-chain triglycerides; and LCT, long-chain triglycerides) on bioavailability of vitamin E-encapsulated in quillaja saponin emulsions was investigated and it was concluded that LCT emulsions presented higher bioavailability (11.7%) than MCT emulsions (4.3%) (Yang, Xiao, & McClements, 2017). Opposite results were reported by Liu and co-authors, presenting a higher permeability coefficient of pterostilbene across the cell monolayer in those nanoemulsions containing MCT (8.21×10^{-6} cm s^{-1}) compared to those with LCT ($\approx 5.10 \times 10^{-6}$ cm s^{-1}) (Liu, Chen, Qin, Jiang, & Zhang, 2019)..

Co-culturing Caco-2 cells with HT-29MTX adds a further layer of mucus complexity to more closely resemble the *in vivo* environment (Arranz, Guri & Corredig, 2016), but reduces permeability rates, which may hamper compound detection. Accordingly, a lower permeability of β-carotene from liposomes was reported using an *in vitro*, 21 days differentiated co-culture model with Caco-2/HT29-MTX compared to 21 days differentiated Caco-2 cells (Li, Arranz, Guri, & Corredig, 2017). Interaction with the mucus produced by the Caco-2/HT29-MTX cell line reduces the permeability of mucoadhesive lipophilic molecules or large molecules due to steric blocking (Sigurdsson, Kirch, & Lehr, 2013). Detection of the target compound after absorption experiments by determining their bioactivity using other cells is an alternative that it has been used by Sabouri et al. (2018).

Table 3. Recent *in vitro* studies about different nanostructured lipid-based delivery systems encapsulating lipophilic compounds in epithelial cells

	Lipophilic compound	**Composition**	***In vitro* GIT digestion**	**Epithelial cells**	**Main findings**	**References**
Nanoemulsions	Conjugated linoleic acid (CLA)	Lipid phase: Soybean oil (14% v/v) Emulsifier: Soy protein isolate (4% v/v)	Yes	Differentiated Caco-2 cells	No significant differences on CLA bioavailability for all emulsion treatments.	(Fernandez-Avila & Trujillo, 2017)
	Curcumin	Lipid phase: soy oil (40% v/v) Emulsifier: Tween 20 (4%v/v) or Poloxamer 407 (4% v/v)	No	Differentiated Caco-2 cells	Curcumin uptake was significantly affected by the type of interface, being higher when emulsions were stabilised with Poloxamer 407.	(Gülseren, Guri, & Corredig, 2014)
	β-carotene	Lipid phase: sunflower oil (10% w/w) Emulsifier (1% w/w): whey protein isolate (WPI), sodium caseinate (SCN) and Tween 80 (TW80).	Yes	Undifferentiated Caco-2 cells	Sodium caseinate-stabilised emulsion showed the highest cellular uptake of β-carotene, followed by TW80- and WPI-stabilised emulsions.	(Lu et al., 2017)

	Vitamin D	Lipid phase: canola oil (0.5, 1, 2.5, 5% w/v) Emulsifier: pea protein (1, 5, 10% w/v)	No	Differentiated Caco-2 cells	Cellular uptake was higher for small sized nanoemulsions (233 nm) and protein-based-nanoemulsions.	(Waila & Chen, 2020)
Multilayer emulsions	Curcumin	Lipid phase: medium chain triglycerides (MCT) (10% w/w) Emulsifier: whey protein isolate (WPI) (1.5%w/w) Coating composition: chitosan	No	differentiated Caco-2 cells	Chitosan layer significantly increased the apparent permeability coefficient of curcumin through Caco-2 cells by 1.55-folds.	(Silva et al., 2019)
Liposomes	Epigallocatechin-3-gallate	Lipid phase: soybean oil (7%) Emulsifier: sodium caseinate (0.35%) Stabiliser: high metoxyl pectin (0, 0.45%)	No	Differentiated Caco-2 cells and coculture Caco-2/ HT29-MTX	Mucus layer was associated with a lower recovery of Epigallocatechin-3-gallate after uptake experiments	(Li et al., 2017)

Curcumin	Lipid phase: phospholipid, cholesterol Emulsifier: Tween 80	No	Undifferentiated Caco-2 cells	Curcumin loaded in nanoliposomes exhibited lower curcumin cellular uptake than free curcumin	(Chen et al., 2015)
Astaxanthin	Lipid phase: soybean with phosphatidylcholine (PC)	No	Differentiated Caco-2 cells	Cellular uptake of astaxanthin-loaded liposomes containing 70% PC was significantly higher than that of 23% PC-containing liposomes	(Sangsuriyawong et al., 2018)

5. Application of nanostructured lipid-based delivery systems

Functional foods are defined as food products that have been fortified or enriched with compounds that provide health benefits. Because of the promising properties that nanostructured systems offer, they can be applied in several foods either in a dry/solid or liquid form to produce functional foods.

Nanostructured systems can be transformed into a dehydrated or solid state by spray-drying or freeze-drying techniques. Both processes consist of drying the product so as to obtain a powder with the encapsulated and entrapped compound of interest. In spray drying, water is evaporated by using hot air, while in freeze drying samples are frozen and then removing the liquid by sublimation under a vacuum (Anwar & Kunz, 2011). One study obtained a lycopene powder through spray drying for further application within cakes. As a result, a pigmented cake was obtained, indicating that lycopene was released from the powder during cake preparation and coloured the dough homogenously (Rocha, Fávaro-Trindade, & Grosso, 2012). Other authors have found evidence on using freeze drying to encapsulate bioactive compounds for an efficient food functionality. In this sense, yogurt fortified with freeze-dried capsules containing carotenoids and polyphenols exhibited a high retention of both bioactive. Furthermore, good acceptability scores in terms of colour and appearance were obtained for fortified yogurts (Seregelj et al., 2019).

Alternatively, nanostructured systems can be incorporated into foodstuffs either as such (beverages) or as edible coatings (fruits and vegetables). On one hand, Marsanasco et al. (2011) incorporated both vitamin E- and vitamin C-enriched liposomes to an orange juice and observed that the antioxidant activity of both vitamins was protected before and after pasteurisation treatments. Furthermore, orange juice organoleptic properties were not altered, and its microbial stability was improved for over a month. On the other hand, edible coatings consist of a thin layer of nanostructured emulsion systems that are applied to foods by different techniques, such as spraying, dipping and electrospraying. This layer can act as a physical barrier and block oxygen, moisture and solute movement, without affecting the properties of the food (Hassan, Chatha, Hussain, Zia, & Akhtar, 2018). For instance, multi-layered emulsions have been used as edible coatings to provide a better quality and visual appearance to strawberries (Velickova, Winkelhausen, Kuzmanova, Alves, & Moldao-Martins, 2013). Nanoemulsions containing eugenol and Aloe vera were also applied to shrimps, resulting in a quality improvement of this food product (drip loss, colour changes, textural disintegration and deterioration was reduced) as well as in a retardation of oxidative reactions during seven days of storage (Sharifimehr, Soltanizadeh, & Hossein-Goli, 2019). In addition, nanostructured systems are a feasible approach to extend the shelf life of food as well as add extra nutritional value. The microbial stability of low-fat cheese was improved, and additional nutritional characteristics were provided to the food product by applying a nanoemulsion-based edible coating, carrying oregano essential oil and mandarin fiber (Artiga-Artigas et al., 2017).

6. Concluding remarks

Bioactive compounds are health-related compounds of interest not only because they are involved in biological processes but also for their specific functions for health and well-being. It is widely recognized that nanostructured lipid-based delivery systems are a feasible option to encapsulate and improve the functionality of such lipophilic bioactive compounds. At the same time, the successful delivery of bioactive compounds might be associated with the stability and digestibility of nanostructured systems, as well as bioaccessibility and absorption. Different characteristics from nanostructured lipid-based delivery systems can either strengthen or reduce their functionality, including interfacial properties of and/or carrier oil-type, among others.

However, most of the *in vitro* models used for studying delivery of encapsulated compounds within nanostructured systems do not include the large intestine step, where interaction with microbiota occurs. The need for accurate *in vitro* methods to study digestion and fermentation processes has become increasingly important given the recently recognised role of the large intestine in vital functions, nutrition and gut health. In addition, most of these nanostructures have only been developed at the scale laboratory level, which limits its extrapolation to *in vivo* studies. Further *in vivo* studies (animal tests) are needed not only to validate the results obtained from *in vitro* experiments but also to obtain information prior to embarking on intervention trials with human subjects.

Another latent concern is the lack of information about possible interactions between nanostructured lipid-based delivery systems and other food components, as well as their accumulation, toxicity and long-term consumption. Even though nanostructured delivery systems have been associated with the quality, safety and functionality enhancement of encapsulated bioactive compounds, there is a global concern regarding both the toxicological and regulation aspects when these systems are applied to foodstuffs and then consumed. Now that the food and beverage industry is continuously developing novel food products in which nanotechnology is involved, the implication of authorities is essential in order to establish a law when using nanotechnology along the food chain for future food applications.

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HYPOTHESIS AND OBJECTIVES

The hypothesis underlying the current research was that encapsulating bioactive compounds within emulsion-based delivery systems can protect and control the release of such compounds, and thus enhance their functionality. However, the nature of the stabilisers used to form these systems largely determines the physicochemical properties, stability and functionality of emulsion-based delivery systems.

Therefore, the main goal of this doctoral thesis was to study the impact of using different type of stabilisers on the formation, stability and functionality of these emulsion-based delivery systems considering physicochemical properties, lipid digestibility, bioaccessibility and delivery of β-carotene across the intestinal barrier. To achieve this main objective, the following specific objectives were proposed:

1. To evaluate the influence of the interfacial layer (emulsifier nature and concentration) of β-carotene-enriched nanoemulsions on their physicochemical properties and stability, as well as lipid digestibility and bioaccessibility.

2. To ascertain the impact of interface composition (emulsifier nature) on the delivery of β-carotene encapsulated within nanoemulsions to the intestinal barrier by coupling an *in vitro* gastrointestinal digestion with two different *in vitro* cell culture models (Caco-2 or co-culture of Caco-2/HT29-MTX).

3. To study the influence of mandarin fiber addition as a stabiliser on physicochemical properties of β-carotene nanoemulsions, especially those aspects concerning their gastrointestinal digestion, including stability, lipid digestibility and β-carotene bioaccessiblity.

4. To determine the feasibility of using tertiary emulsions as delivery systems of β-carotene in terms of physical stability and compound degradation under stress conditions (temperature, pH and ionic strength) as well as the lipid digestibility and β-carotene bioaccessibility after *in vitro* digestion.

MATERIALS AND METHODS

The common materials and equipment employed for experiments as well as general information about protocols are described in this section. Further details would be provided in the corresponding chapter in publications section.

1. Materials

This section lists materials, reagents or consumables used for the different experiments carried out during this doctoral thesis. Ingredients used in the preparation of each emulsion-based delivery system are included in Table 1. Table 2 describes chemicals employed during the simulation of the *in vitro* gastrointestinal tract. Finally, materials used to evaluate the permeability of emulsion-based delivery systems across intestinal barrier and their functionality are listed in Table 3. All other chemicals used will be specified in the corresponding chapter of each experiment.

Table 1. List of ingredients used for the preparation of emulsion-based delivery systems and their corresponding supplier.

Ingredients	**Supplier**
β-carotene	Sigma-Aldrich, Spain
Corn oil	Koipe Asua (local supermarket)
Tween 20	Sigma-Aldrich, Spain
Lecithin	Acros Organics (Geel, Belgium)
Sodium caseinate	Alfa Aesar (Karlsruhe, Germany)
Sucrose palmitate	Alfa Aesar (Karlsruhe, Germany).
Mandarin fiber	Indulleida S.A (Alguaire, Spain)
Lactoferrin	Friesland Campina DOMO (Wageningen, The Netherlands)
Alginate	IMCD España Especialidades Químicas, S.A. (Barcelona, Spain)
ε-poly-L-lysine	Handary S.A (Brussels, Belgium)

Table 2. Chemicals and product details used during the simulation of the *in vitro* gastrointestinal tract (GIT). All of them were sourced from Sigma-Aldrich unless specified.

Ingredients	**Product details**
Porcine pepsin	P7012
Bile extract porcine	B8631
Porcine pancreatin	P7545
Lipase from porcine pancreas	L3126
Pefabloc® SC (4-(2-Aminoetyl) benenesulfonyl fluoride	76307
$CaCl_2(H_2O)_2$	1023820
NaOH	106462
HCl	320331
KCl	104936
KH_2PO_4	15578634 (J.T. Baker)
$NaHCO_3$	106329
NaCl	106404
$MgCl_2(H_2O)_6$	105833
$(NH_4)_2CO_3$	379999

Table 3. Materials used to evaluate the permeability of emulsion-based delivery systems across the intestinal barrier and their functionality.

Materials	**Product details/Supplier**
Caco-2 cell line	European Collection of Cell Cultures (ECACC 86010202)
HT-29 cell lines	American Type Culture Collection (ATCCHTB-38)
Human monocyte THP-1	European Collection of Cell Cultures (ATCCTIB-202)
Dulbecco's modified Eagle's medium-high glucose (DMEM)	D5796 (Sigma-Aldrich, Ireland)
Fetal bovine serum (FBS)	F7524 (Sigma-Aldrich, Ireland)
Penicillin-Streptomycin	P4333 (Sigma-Aldrich, Ireland)
Trypsin-EDTA	T4049 (Sigma-Aldrich, Ireland)
Dulbecco's Phosphate Buffered Saline (PBS)	D8537 (Sigma-Aldrich, Ireland)
Hanks' Balanced Salt Solution	H8264 (Sigma-Aldrich, Ireland)
RPMI 1640 medium	R7388 (Sigma-Aldrich, Ireland)
12-O-tetradecanoyl phorbol-13-acetate (TPA)	P1585 (Sigma-Aldrich, Ireland)
Lipopolysaccharides (LPS)	L2630 (Sigma-Aldrich, Ireland)
Transwell® plates	Costar, Cambridge, MA
ELISA Kit	R&D Systems, Minneapolis, USA

2. Methods

2.1. Formation of emulsion-based delivery systems

Firstly, lipid phase and aqueous phase of each emulsion-based delivery system were prepared separately. On one hand, lipid phase was obtained by enriching corn oil with β-carotene at 0.5% (w/w). For this, both ingredients were subjected to sonication (ultrasound bath, JP Selecta, SA, Spain) for 1 min and then heating (≈45 ºC, 5 min) to ensure a complete dissolution. On the other hand, aqueous phase was prepared by dispersing in Milli-Q water the emulsifiers, texture modifiers or polyelectrolytes, depending on the experiment. Then, both phases were mixed by using a high-speed blender (Ultra-Turrax, Janke & Kunkel, Staufen, Germany) and a coarse emulsion was obtained. Finally, different processes were applied for obtaining either nanoemulsions or multi-layer emulsions. Composition of each emulsion-based delivery system is included in Table 4. In the case of nanoemulsions containing mandarin fiber, the aqueous phase was filtered in order to remove the fiber in excess.

Table 4. Composition of nanoemulsions and tertiary emulsions.

Lipophilic bioactive compound	Emulsion-based delivery system	Lipid phase	Aqueous phase
β-carotene *(0.5% w/w)*	Nanoemulsions	Corn oil *(4% w/w)*	*Emulsifiers (2-8 w/w):* Tween 20 Lecithin, Sodium caseinate Sucrose palmitate *Texture modifier (0.5-2% w/w)*: Mandarin fiber
	Tertiary (multi-layer) emulsions	Corn oil *(2.5% w/w)*	*Polyelectrolytes*: lactoferrin *(0.62% w/w)*, alginate *(0.25 % w/w)* and ε-poly-L-lysine *(0.18% w/w)*

2.1.1. Nanoemulsions

Coarse emulsions were treated with high pressure homogenizers (microfluidizers or high-pressure valve homogenizers) (Figure 1). The operating principle of both equipment is to pump

the liquid through a small and narrow gap, resulting in a breakdown of oil droplets and subsequently dispersion of them within the aqueous phase.

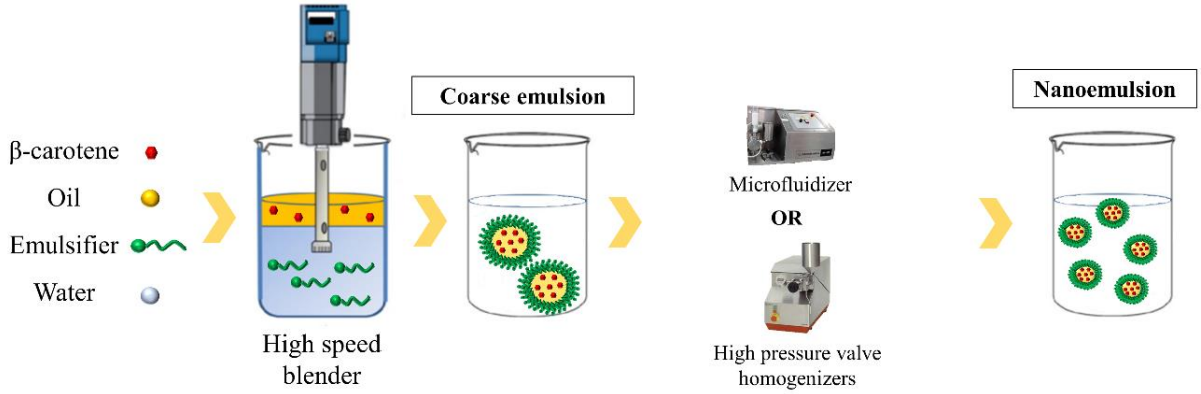

Figure 1. Schematic representation of coarse emulsions and nanoemulsions formation.

Specifically, microfluidization (M-110P, Microfluidics, Newton, MA, USA) consists on passing coarse emulsions through an interaction chamber, which has microchannels. As the pump forces the liquid through these channels at high velocity and high shear, impact forces are created for obtaining tiny oil droplets. This type of homogenizers can reach pressures of 2,068 bar (30,000 psi) (Schultz et al., 2004). In the case of high-pressure valve homogenizers (APV 1000, SPX Flow Technology, North Carolina, USA), the liquid enters the valve area at high pressure and low velocity. As the product enters the adjustable, close clearance area between the valve and seat, there is a rapid increase in velocity with a corresponding decrease in pressure. The intense energy release causes turbulence and localized pressure differences, which will tear apart the particles. High pressure valve homogenizers work at maximum pressure of 1,500 bars (21,750 psi) (Lee & Norton, 2013).

2.1.2. Tertiary (multi-layer) emulsions

Tertiary emulsions were developed by using the layer-by-layer technique (LbL), which consisted on the deposition of charged biopolymers (polyelectrolytes) around oil droplets, thereby forming layers (Bortnowska, 2015; Guzey & McClements, 2006). In the present doctoral thesis, a primary (lactoferrin), secondary (lactoferrin-alginate) and tertiary emulsion (lactoferrin-alginate-ε-poly-L-lysine) were obtained (Figure 2). It was necessary to optimize the polyelectrolyte concentration for each emulsion layer prior to perform the subsequent experiments. Firstly, primary emulsion was prepared by mixing the lipid phase with the aqueous phase containing different concentrations of lactoferrin. Then, this emulsion was passed through a microfluidizer to obtain the primary emulsion (lactoferrin). Finally, to prepare secondary and tertiary emulsions, different concentrations of alginate or ε-poly-L-lysine were

added at a fixed dripping rate and under continuous agitation to the primary or secondary emulsion, respectively. The pH of all polyelectrolyte solutions and emulsions was 5 since this is the pH at which polyelectrolytes are strongly oppositely charged and can form electrostatic complexes around oil droplets.

The most widely used method to fabricate multi-layer emulsions is by adding the exact amount of polyelectrolyte needed to saturate the oil droplets interface (Acevedo-Fani, Soliva-Fortuny & Martín-Belloso, 2017). In this experiment, finding the polyelectrolyte concentration where oil droplets are completely covered was done empirically. This process consisted on testing different concentrations of the corresponding polyelectrolyte and monitor the particle size and ζ-potential of emulsions until stabilisation. Further details are described in chapter IV.

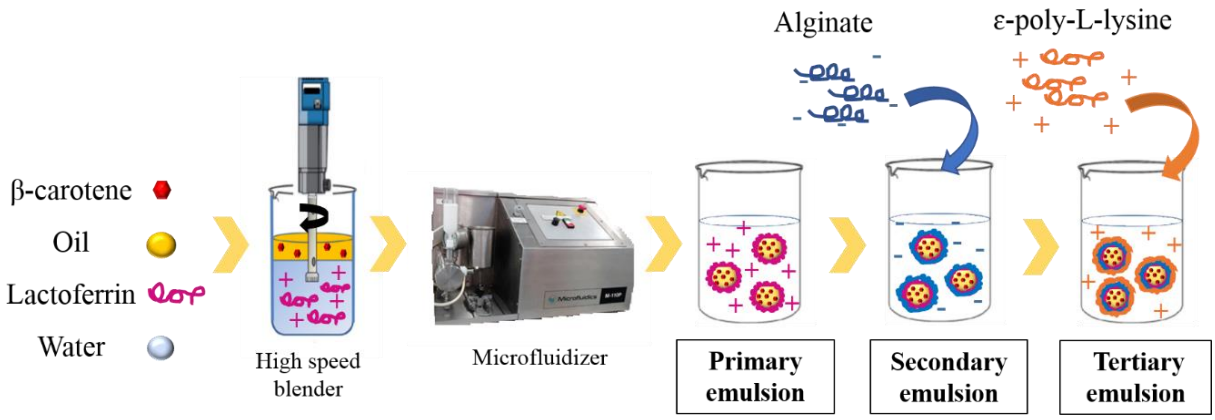

Figure 2. Schematic representation of primary, secondary and tertiary emulsions formation. (Adapted from Burgos-Díaz, Wandersleben, Marqués, & Rubilar, 2016)

2.2. Physicochemical characterization of emulsion-based delivery systems

In general, physicochemical properties of emulsion-based based delivery systems were determined just after preparation and along the different phases of the *in vitro* gastrointestinal tract. In the case of multi-layer emulsions, the most suitable polyelectrolyte concentration to create primary, secondary and tertiary emulsions was determined by measuring different parameters. For primary emulsions, protein surface load, particle size and ζ-potential were measured, whereas for secondary and tertiary emulsions, particle size and ζ-potential were determined. Furthermore, optical images were used to observe destabilisation processes during the interfacial layer's assembly. In addition, TEM images were used to provide information about emulsions' structure.

Finally, particle size and ζ-potential were also determined in tertiary emulsions exposed to different stressing conditions (temperature, pH and ionic strength changes). Temperatures ranged from 30 °C to 90 °C, emulsions were adjusted at different pH conditions from 2 to 11, and ionic strength was modified by adding different NaCl concentrations to tertiary emulsions

(0–0.5 M). In addition, the amount of β-carotene in tertiary emulsions when these were subjected at these stressing conditions was quantified as explained in section 2.2.6.

The parameters that were measured in each case will be detailed in the corresponding chapter in publications section.

2.2.1. Particle size

Particle size was measured using two different techniques depending on the range of droplet sizes and the experiment (McClements, 2005).

On one hand, particle size was measured using DLS technique with a Zetasizer (Malvern Instruments Ltd Worcestershire, UK), working at 633 nm at 25ºC and equipped with a backscatter detector (173º). Samples were previously diluted within the appropriate solution and their particle size was measured by their Brownian motion, which is based on the speed of these particles randomly moved in a liquid (Krieger, 1972). Results were reported as z-average size, which is an intensity-weighted mean diameter derived from the cumulants analysis and it is expressed in nanometres (nm).

On the other hand, a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK) was employed to determine particle size thereby using SLS technique. Samples were diluted in distillate water and stirred in the dispersion unit at 1700 rpm. As the sample passes through the measurement area, a light source illuminates the droplets. As a result, the detectors measure the intensity of the light scattered by these droplets. Results were reported as the surface area mean diameter ($d_{3,2}$) or as the volume mean diameter ($d_{4,3}$), both in micrometres (µm). In addition, a particle size distribution was obtained as well as expressed in terms of span, following equation (1):

$$Span = \frac{D_{(v,0.9)} - D_{(v,0.1)}}{D_{(v,0.5)}} \qquad (1)$$

where $D_{(v,0.9)}$, $D_{(v,0.5)}$, $D_{(v,0.1)}$ correspond to diameters at which 90%, 50% and 10% of the particles' volume are of a smaller size, respectively. Thus, lower span values are related to more monodisperse distributions.

2.2.2. ζ-potential

The ζ-potential was determined with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK). This equipment measures ζ-potential by combining two techniques: electrophoresis and laser doppler velocimetry. ζ-potential is a function of the surface charge of the particle, any adsorbed layer at the interface, and the nature and composition of the surrounding suspension medium (Hunter, 2001). Therefore, this type of analysis can be used to study the stability/instability processes occurring in colloidal

suspensions. Results were expressed in millivolts (mV) and values ±30 mV might indicate that suspension has a good physical stability as enough repulsive forces between particles are present (Mishra, Shaal, Müller, & Keck, 2009). It should be note that ζ-potential is not only affected by the properties of emulsion-based delivery systems, but also by the nature of the solution, including pH and ionic strength.

2.2.3. Microscope images

Images of samples were obtained by optical microscope or transmission electron microscopy (TEM) depending on the type of emulsion-based delivery system.

Optical images of nanoemulsions were obtained by using an optical microscope (Olympus BX41, Olympus America Inc., Melville, NY, USA) with a 100x objective lens. A drop of each sample was placed on a slide, covered using a cover slip and images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus). This technique provided valuable information about instability processes occurring in nanoemulsions, but specific information such as droplet size or shape could not be obtained.

Transmission electron microscopy (TEM) (Jeol JEM-1010, Tokyo, Japan) was used to observe the layer deposition in primary, secondary and tertiary emulsions. Samples were deposited onto a carbon-coated grid, and negatively stained with 2% (w/v) uranyl acetate for observation. TEM images provided more detailed information about microstructure of emulsions, by observing the interface thickness of the droplets, which in turn, might be related to layer deposition in multi-layer emulsions.

2.2.4. Viscosity

A SV-10 vibro-viscometer (A&D Company, Tokyo, Japan) vibrating at 30 Hz was used to measure the viscosity of 10 mL-aliquots of samples at room temperature (≈25ºC). Two thin sensor plates in a tuning fork arrangement are driven with electromagnetic force to vibrate at a frequency of 30 Hz within the liquid sample. Viscosity is then calculated based on the proportional relationship between the viscous resistance of the liquid sample and the amount of electric current required to drive and maintain the sensor plates at a constant vibration amplitude. Results were reported in m·Pas.

2.2.5. Physical stability

Physical stability of nanoemulsions was determined with an analytical centrifuge (LUMiSizer, L.U.M. GmbH, Berlin, Germany). Instrumental parameters used for physical stability analysis were: speed 2,186 rcf; time interval 20 seconds; exposure time 10,000 seconds; temperature 25 ºC. Results are analysed using the software package SEPView 6.0 (Lum GMBH) whichcalculates instability index. This index gives information about the overall demixing of

each sample (values near 0 indicates no or very slow demixing, while values near 1 represent completely or almost demixed samples).

2.2.6. Amount of β-carotene

Amount of β-carotene was determined using two different techniques: UV-vis and high-performance liquid chromatography (HPLC).

UV-vis is a fast, simple and inexpensive method to determine the concentration of a certain compound in solution. Extraction of β-carotene for quantifying such compound with an UV-vis was carried out following a method described previously (Salvia-Trujillo et al., 2013). Briefly, aliquots of 5 mL of sample were mixed with 5 mL of chloroform, vortexed (1800 rpm, 3 min) and centrifuged (1750 rpm, 10 min, 4ºC). The bottom layer containing the β-carotene was collected, while the top layer was again mixed with 5 mL of chloroform and the same procedure was repeated until all β-carotene was extracted. All the chloroform fractions collected were combined and analysed spectrophotometrically at 450 nm (Ultrospec 3000 pro GE Health Sciences, USA). Pure chloroform was used as a blank.

High-performance liquid chromatography (HPLC) technique was also used to quantify β-carotene (Yuan, Gao, Zhao, & Mao, 2008). Briefly, the sample (0.5 mL) was filtrated (0.45 µm) and mixed with ethanol (1mL) and hexane (1.5 mL). Then, it was shaken with a vortex and centrifuged (4000 rpm, 5 min, 5ºC) (Heraeus Megafuge 1.0, Massachusetts, USA). The upper fraction was collected and dried under N_2 for further quantification. Each sample extract was dissolved in 200 µL of the injection solvent ACN: MeOH 7:3 (v/v): acetone 6.7:3.3 (v/v) and filtered through Millex 0.2 µm nylon membrane syringe filters (Millipore, Bedford, MA). High-performance liquid chromatography system (Waters Xevo TQ-S, Milford, USA) equipped with a photodiode array detector (HPLC-PDA) at 450 nm was used for β-carotene quantification. Analysis was performed with a column ACQUITY UPLC® (C18 BEH 130 Å, 1.7 µm, 2.1 × 150 mm) (Waters) set at 30°C with a flow rate of 0.85 mL/min. Mobile phase consisted of solvent A: Acetonitrile (ACN): Methanol (MeOH) 7:3 (v/v) and solvent B: water 100%, and the flow was isocratic (100% ACN/MeOH 7/3). β-carotene was quantified by comparison with external standards.

2.3. *In vitro* simulated gastrointestinal tract (GIT) digestion

Emulsion-based delivery systems were subjected to a simulated *in vitro* static gastrointestinal tract (GIT) consisting of gastric and intestinal phases that mimics the adult human upper gut. The INFOGEST standardised method (Brodkorb et al., 2019; Minekus et al., 2014) was followed with minor modifications. The main characteristics of the simulated *in vitro* static gastrointestinal tract (GIT) will be outlined below and are summarised in Figure 3.

First, gastric phase consisted of mixing 5 mL of each emulsion with 2 mL of simulated gastric fluid (SGF) and pepsin (8,000 U/mL). Then, the mixture pH was adjusted to 3.0 using HCl (1

M) and incubated for 2 h at 37°C with continuous shaking in a rotator. After 2h, pepsin was inactivated by increasing pH to 6.5 using NaOH (1 *M*). For intestinal phase, 10 mL gastric chyme was mixed with 20 µl $CaCl_2$ (0.3 M), 4 ml bile, 2.5 mL pancreatin (based on trypsin activity) and bovine bile extract (630 g/mol). Based on Verkempinck et al. (2017), extra lipase (2.5 mL) was added in order to reach a final lipase activity of approx. 420 U/mL. Finally, the pH of the mixture was adjusted to 7.0, the volume brought up to 20 mL with milli-Q water, and the mixture was again incubated for 2 h at 37°C with continuous shaking. After this time, the digestion was stopped by adding protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride at a final concentration of 1 mM.

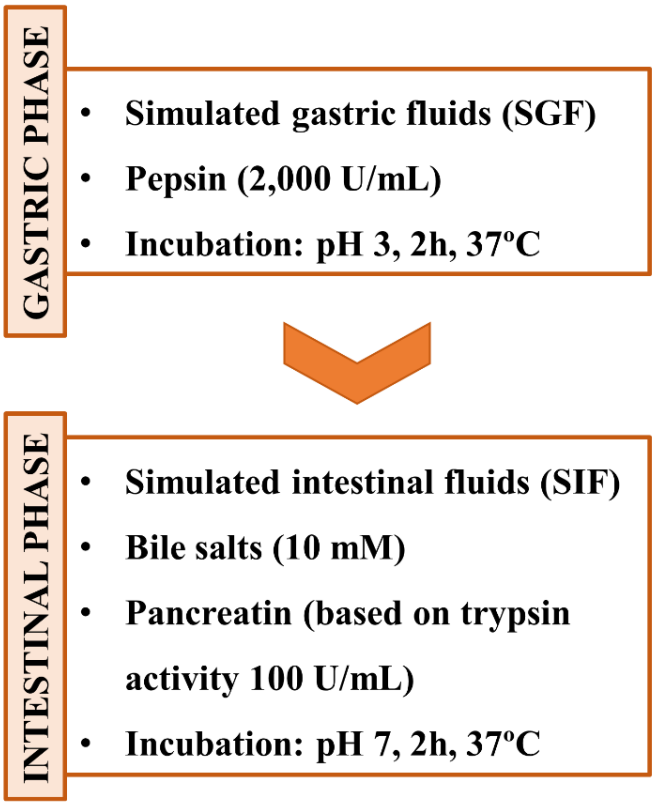

Figure 3. Flow diagram of conditions in each digestive phase during a simulated *in vitro* static gastrointestinal tract. (GIT) (Adapted from Brodkorb et al. 2019).

In some experiments of this doctoral thesis, a pH-stat (Metrohm, Riverview. FL, USA) was used to simulate intestinal conditions (Figure 4). This device is normally used to calculate the lipid digestibility of emulsion-based delivery systems (Li & McClements, 2010).

The release of free fatty acids (FFA) cause pH reduction in the samples and the constant titration with NaOH maintain the pH at 7 during 2h. The total volume of NaOH employed during intestinal digestion is used to calculate the lipid digestibility through total FFA release content, using equation (2).

$$FFA(\%)=\frac{V_{NaOH}\times C_{NaOH}\times M_{oil}}{2\times m_{oil}}\times 100 \qquad (2)$$

Where V_{NaOH} is NaOH volume (L) used during the digestion, C_{NaOH} is NaOH molarity (0.25 mol/L), M_{oil} is oil molecular weight (800 g/mol), m_{oil} is corn oil total weight present in the sample.

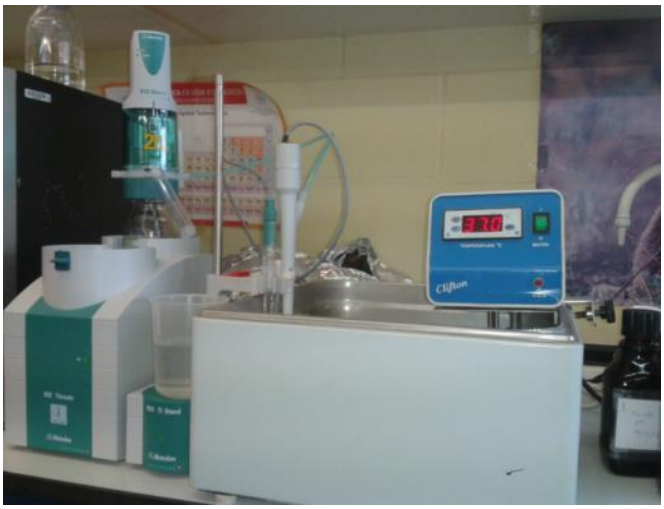

Figure 4. pH-stat Device. Metrohm USA, Riverview, FL.

2.3.1. Bioaccessibility of β-carotene

The bioaccessibility of β-carotene was determined after emulsion-based delivery systems were subjected to the simulated *in vitro* static GIT, using a previously described method (Qian et al., 2012). Digested samples were centrifuged (AVANTI J-25, Beckman Instruments Inc., Fullerton, CA, USA) at 4000 rpm for 40 min at 4ºC. The supernatant part of the centrifuged liquid was considered as the micellar fraction, in which the mixed micelles formed during the *in vitro* digestion contain the solubilised β-carotene. In some samples, a layer of oil could be observed on top of the liquid, which was dismissed since it was considered non-digested oil after *in vitro* digestion (Figure 5).

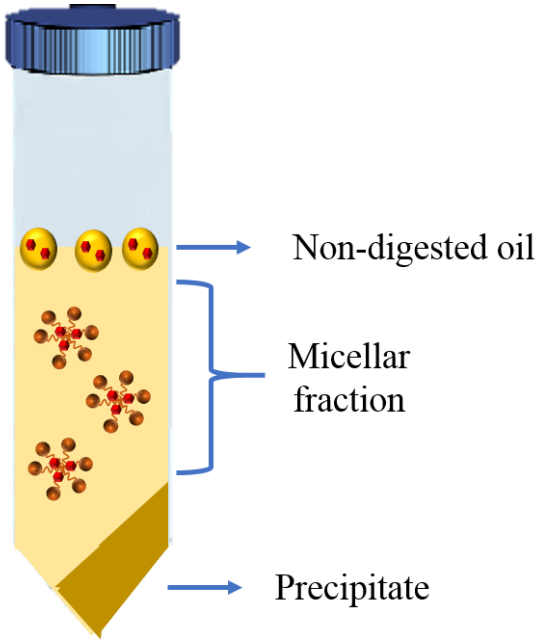

Figure 5. Fractions obtained after centrifuging digested samples.

Finally, amount of β-carotene was determined as described in section 2.2.6 and bioaccessibility was calculated using equation 3.

$$\text{Bioaccessibility}(\%) = \frac{C_{micelle}}{C_{initial}} \times 100 \qquad (3)$$

where $C_{micelle}$ is the carotenoid concentration of the micelle fraction and $C_{initial}$ the initial carotenoid concentration of emulsion.

2.4. *In vitro* cell-based assays

Three different cell lines were used to perform the different *in vitro* cell-based assays, which in turn, required different maintenance, including culture media and manipulation of such cells. Caco-2 and HT29-MTX cell lines were grown in 75 cm^2 tissue culture flasks in a humidified 37 °C incubator with a 5% CO_2 air atmosphere. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. At 80% confluency, cells were trypsinated with 0.25% trypsin/EDTA, diluted DMEM medium, and reseeded in flasks. Media was changed three times a week.

Human monocytes THP-1 were cultured in RPMI 1640 culture medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere.

2.4.1. Cytotoxicity assay

When performing *in vitro* assays using cell cultures, it is important to first determine the cytotoxocity level of the emulsion sample after the simulated *in vitro* GIT process, i.e. micellar fraction. Cytotoxicity is an essential factor to consider since micellar fraction contain several components that can damage the cells (Arranz et al., 2016). Cytotoxicity assay consists on applying different dilutions of the sample and then evaluate the cell viability, which is a good indicators of cell health (Bahadar, Maqbool, Niaz, & Abdollahi, 2016).
In the present doctoral thesis, Caco-2 cells were seeded at a density of 8×10^4 cells/well in 96-well plate. After 24h of incubation (37 °C in a 5% CO2 humidified atmosphere), cells were washed with PBS (100 µl). GID micellar fractions were filtrated (0.45 µm), diluted in complete DMEM (ranging from 1:2 to 1:35) and 80 µl were added to each well. Subsequently, 20 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent was added to each well and cells were incubated for 2 h (37 °C in a 5% CO2 humidified atmosphere). This method is based on the reduction of MTS tetrazolium compound by viable cells to generate a coloured formazan product. After 2h, the quantity of formazan produced was measured spectrophotometrically at 490 nm in a microplate reader (Synergy HT BioTek, Winooski, VT, USA). Results were expressed as the percentage of cellular viability relative to a control group (cells with DMEM medium) versus the micellar fraction concentration (%, v/v).

2.4.2. Permeability across intestinal barrier

Permeability across intestinal barrier of nanoemulsions was determined by using a 12-well Transwell® plates with inserts located in each well (0.4 µm pore size, inserts of 1.2 cm diameter, Costar, Cambridge, MA). Each insert has a microporous membrane, above which cells grow in order to produce the cell monolayer, which divides each well in two compartments: apical (upper compartment) and basolateral (lower compartment) (Figure 6).

In this doctoral thesis, two *in vitro* cell culture models were employed to perform permeability of β-carotene-enriched nanoemulsions. Caco-2 cells were seeded at a density of 6×10^4 cells per insert, while co-cultures of Caco-2 and HT29-MTX were grown separately and then seeded at a ratio of 75:25, to a final density 6×10^4 cells per insert. Caco-2 cells and Caco-2 cells co-culture with MTX cells at 29-41 and 53-67 passes, respectively were used.

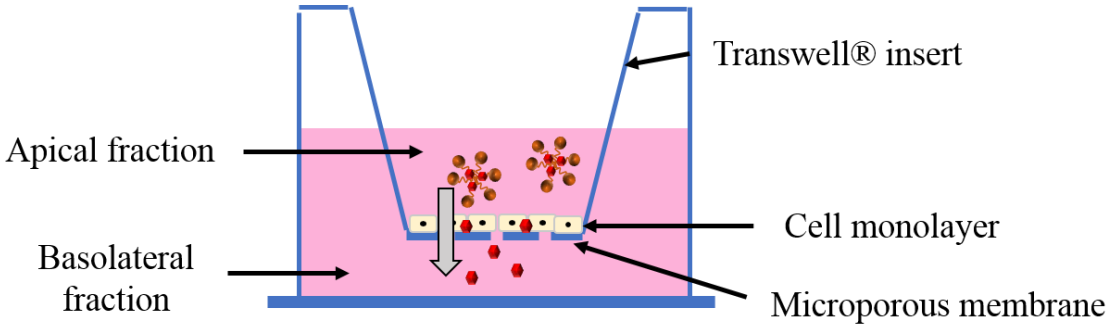

Figure 6. Schematic representation of the different parts of the inserts contained in Transwell® plates.

Culture media of each well was changed every two days for 21 days, until cells were fully differentiated and the monolayer was formed. For this, the integrity of the cell monolayer was quantitatively monitored by measuring the transepithelial electrical resistance (TEER) ($\Omega \cdot cm^2$) using a Millicell-ERS Voltohmmeter (Merck Millipore, Carrigtwohill, County Cork, Ireland (Figure 7).

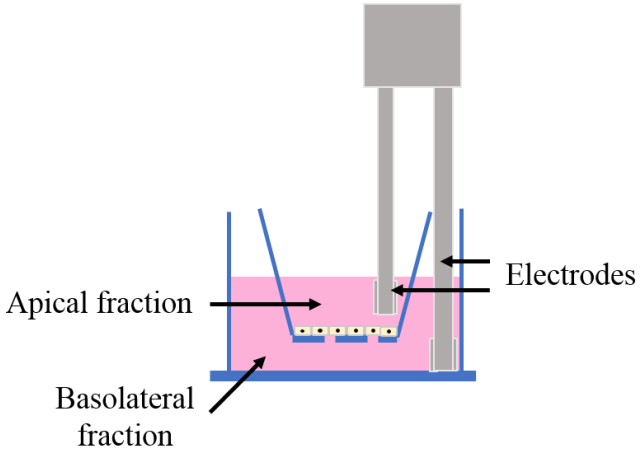

Figure 7. Schematic representation of Millicell-ERS Voltohmmeter for measuring the transepithelial electrical resistance (TEER).

On day 21, apical and basolateral compartments were washed three times with PBS, and micellar fractions as well as DMEM were added to apical and basolateral compartments, respectively. Cells were kept at 37ºC, 5% CO_2 during the following 2h, and the integrity of the monolayer was monitored by measuring TEER. After absorption experiment, apical and basolateral fractions as well as cell monolayer were then collected and stored at −80ºC for further analysis.

Finally, amount of β-carotene was determined as explained in section 2.2.6. Results of cellular uptake were calculated as in equation 4.

$$Cellular\ uptake\ (\%)\ \frac{C_{2h}}{C_{0h}}x100 \qquad (4)$$

Where C_{2h} is the concentration of β-carotene present in the cell monolayer after permeability experiment, and C_{0h} is the concentration of β-carotene present in the apical fraction at time 0h.

2.4.3. Anti-inflammatory activity of basolateral fraction

Co-culturing Caco-2 cells with HT-29MTX adds a further layer of mucus complexity to more closely resemble the *in vivo* environment (Arranz, Corredig, & Guri, 2016), but reduces permeability rates, which may hamper compound quantification. Detection of β-carotene after permeability experiment across the intestinal barrier by determining their bioactivity using other cells is an alternative that it can be used. Therefore, the anti-inflammatory activity of basolateral fractions was performed with macrophages, which were obtained from Human monocytes THP-1 differentiation.

Cells were added at a density of 5 x 10^5 cells/mL in 24 well plates. Differentiation to macrophages was induced by adding 1 µg/µL 12-O-tetradecanoyl phorbol-13-acetate (TPA) to cells followed by 48h incubation (37 °C in a 5% CO_2 humidified atmosphere). After differentiation to macrophages, cells were washed twice with PBS. LPS (0.05 µg/mL) and Caco-2 basolateral samples were added to each well. After 24h incubation (37 °C in a 5% CO_2 humidified atmosphere), the culture medium was collected and frozen at −80 °C for subsequent TNF-α and IL-1β quantification. Release of TNF-α and IL-1β was measured in the supernatants of THP-1 cells using ELISA kits (R&D Systems, Minneapolis, USA), according to manufacturer's instructions. Multiscanner autoreader (Synergy HT BioTek) was used to read the absorbance of the plates at 450 nm (Figure 8).

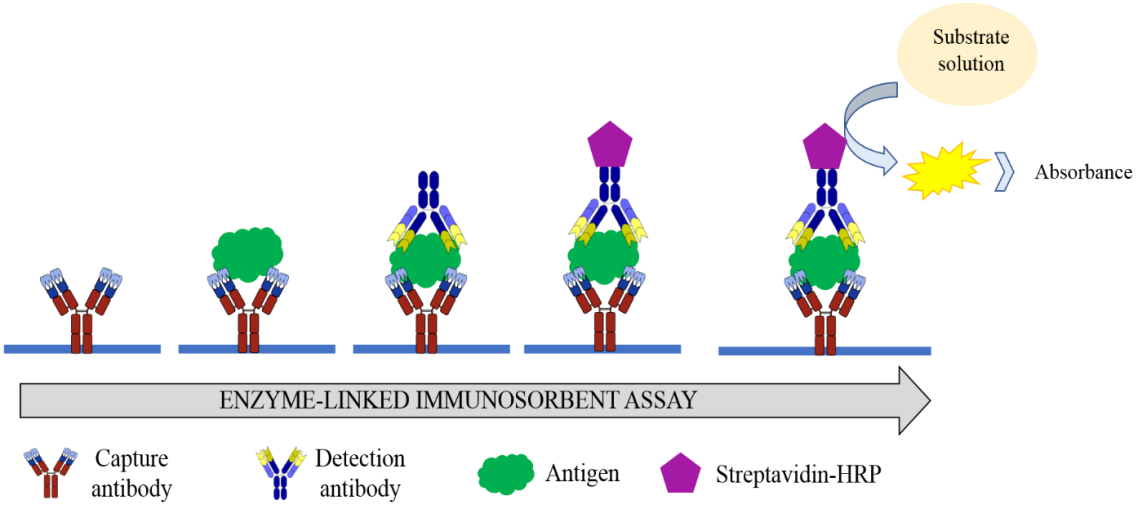

Figure. 8. Schematic representation of an enzyme-linked immunosorbent assay (ELISA).

2.5. Statistics

All experiments were carried out in duplicate and at least three replicate analyses were done for each parameter. The analysis of variance (ANOVA) was conducted using Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md, USA) to identify samples with significant differences ($p \leq 0.05$ was considered significant).

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PUBLICATIONS

CHAPTER I:
Impact of emulsifier nature and concentration on the stability of β-carotene-enriched nanoemulsions during *in vitro* digestion

Ariadna Gasa Falcon, Isabel Odriozola Serrano, Gemma Oms Oliu, Olga Martín Belloso.

Food & Function (2019); 10: 713-722

Abstract

The presence of emulsifiers facilitates the formation of nanoemulsions and assists in their stabilisation. Moreover, behaviour of nanoemulsions along the gastrointestinal tract primarily depends on their composition, affecting the bioaccessibility of the encapsulated compound. The goal of this work was to study how β-carotene-enriched nanoemulsions prepared with different emulsifiers (Tween 20, lecithin, sodium caseinate, sucrose palmitate) at various concentrations (2%–8%) would affect their stability (particle size and ζ-potential) during an *in vitro* gastrointestinal tract (GIT) digestion. In addition, the lipid digestibility and β-carotene's bioaccessibility of nanoemulsions was determined. Nanoemulsions stabilised with Tween 20, lecithin and sodium caseinate did not present any variation in particle size under stomach phase. After the intestinal GIT phase, all nanoemulsions experienced physical changes, *i.e* either increase or decrease in their particle size depending on the nature and concentration of the emulsifier used. The ζ-potential of all nanoemulsions was maintained negative throughout the GIT digestion; moreover, it was less negative after the stomach GIT phase (between −24.2 and −1.4 mV). Lecithin-stabilised nanoemulsions presented the highest number of free fatty acids when the emulsifier concentration increased from 2% to 8%. In this sense, nanoemulsions containing 8% of lecithin exhibited the highest β-carotene bioaccessibility (23.5%), suggesting that lecithin can enhance lipid digestion and bioaccessibility of β-carotene encapsulated within nanoemulsions. This study elucidates the importance of not only the emulsifier's nature but also the concentration used when designing nanoemulsions as delivery systems for lipophilic compounds.

Keywords: nanoemulsions, emulsifiers, β-carotene, *in vitro* digestion, bioaccessibility

1. Introduction

Fortifying foods and beverages with lipophilic compounds is a challenge that the food industry is facing in order to develop functional foods and satisfy consumers' demand for healthier products. Lipophilic compounds, such as carotenoids, have important biological functions in our body and provide multiple health benefits (Cooperstone & Schwartz, 2016). However, a majority of food matrices are primarily composed of water, which makes incorporation of these compounds difficult because of their low water solubility. In addition, external factors such as exposition to light and high temperatures can contribute to degradation of lipophilic compounds during food manufacturing and storage processes (Chen, Peng, & Chen, 1996; Shi, Maguer, Bryan, & Kakuda, 2003). Nanoemulsions can be used to encapsulate lipophilic compounds, which would not only make the incorporation of these compounds within aqueous environments possible but also prevent and/or delay their degradation and maintain their functionality and bioactivity. Adding emulsifiers to nanoemulsions helps in their formation and stabilisation as they are surface-active compounds that get adsorbed at the oil–water interface of the droplets. Based on the structure and properties of different emulsifiers, they act and deposit at the interface of the droplets differently, thereby defining the properties of nanoemulsions. Furthermore, susceptibility of nanoemulsions to undergo physical and chemical changes when they are subjected to an *in vitro* gastrointestinal tract (GIT) digestion is related to the properties of the emulsifier covering the droplets. During an *in vitro* GIT digestion, pH of the different digestion phases, digestive fluids and enzymes are added, all of which are responsible for constant changes suffered at the interface of the droplets. These changes can affect the physicochemical stability of nanoemulsions, promoting or preventing destabilisation processes such as flocculation or coalescence. In the past few years, certain studies have investigated the stability of nanoemulsions along the GIT and processes that occur within it (Hur, Decker, & McClements, 2009; Karthik & Anandharamakrishnan, 2016; Lu, Kelly, & Miao, 2017; Mun, Kim, Shin, & McClements, 2015; Ozturk, Argin, Ozilgen, & McClements, 2015; Qian & McClements, 2011). Moreover, few studies have focused their investigation on how the emulsifier concentration can affect the release of free fatty acids during the GIT intestinal phase (Joung et al., 2016; Li & McClements, 2011; Vinarov et al., 2012). To the best of our knowledge, only one study has determined the digestive stability of β-carotene-enriched nanoemulsions containing different concentrations of a particular emulsifier (L-α-phosphatidylcholine) (Verrijssen et al., 2015). Thus, conducting studies using emulsifiers with different structures (biosurfactants, phospholipids, biopolymers, and colloidal particles), properties (low and high mass), and from various sources (synthetic and natural) to understand the ways in which the composition of nanoemulsions influences the lipid digestion processes would provide knowledge for designing effective nanoemulsions as targeted delivery systems for lipophilic compounds.

Therefore, the goal of this study was to determine how different emulsifiers (Tween 20, lecithin, sodium caseinate, and sucrose palmitate) and concentrations (2%–8%) would impact the stability (particle size and ζ-potential) of nanoemulsions as they passed through a simulated *in*

vitro GIT. Moreover, the lipid's digestibility and bioaccessibility of β-carotene-enriched nanoemulsions was evaluated.

2. Materials and methods

2.1. Materials

Tween 20, pepsin from porcine gastric mucosa, pancreatin from porcine pancreatin, sodium phosphate monobasic and β-carotene were purchased from Sigma Aldrich. Lecithin and sucrose palmitate were obtained from Alfa Aesar. Sodium caseinate, magnesium chloride hexahydrate and potassium phosphate monobasic were purchased from Acros Organics. Hydrochloric acid (HCl) and sodium chloride (NaCl) were purchased from Poch S.A. Bile, sodium azide, calcium chloride dihydrate and chloroform were obtained from Fisher. Sodium hydroxide (0.25 M) and potassium chloride were purchased from Panreac. Corn oil (Koipe Asua) was purchased from a local market. Milli-Q water was used to prepare all nanoemulsions.

2.2. Methods

2.2.1. Preparation of nanoemulsions

Primary oil-in-water emulsions were prepared by mixing 4% (w/w) of the lipid phase (corn oil enriched with 0.5% of β-carotene) with 96% (w/w) of the aqueous phase containing the emulsifier (Tween 20, lecithin, sodium caseinate, and sucrose palmitate) at different concentrations (2%, 4% and 8% w/w). Both phases were mixed with an ultraturrax (Janke & Kunkel, Staufen, Germany) at 9500 rpm for 2 min. Once the primary oil-in-water emulsion was formed, it was passed 5 times through a microfluidizer (Microfluidics M-110P) equipped with a 75 μm ceramic interaction chamber (F20Y) at a pressure of 30,000 psi during the treatment.

Apparent viscosity of nanoemulsions was determined using a viscometer (SV-10, A&D Company, Tokyo, Japan) vibrating at 30 Hz (Table 1).

It should be noted that some nanoemulsions prepared in this study contain high amounts of emulsifier (8%), which could limit further commercial applications of these systems.

Table 1. Viscosity of nanoemulsions stabilised with different emulsifiers.

Emulsifier	Concentration (w/w)	Apparent viscosity (m·Pas)
Tween 20	2%	1.12 ± 0.01
	4%	1.31 ± 0.02
	8%	1.57 ± 0.02
Lecithin	2%	1.11 ± 0.01
	4%	1.38 ± 0.03
	8%	2.24 ± 0.02
Sodium caseinate	2%	1.73 ± 0.01
	4%	2.97 ± 0.05
	8%	7.53 ± 0.30
Sucrose palmitate	2%	1.71 ± 0.01
	4%	2.56 ± 0.02
	8%	7.86 ± 0.20

2.2.2. *In vitro* digestion

Nanoemulsions were subjected to a simulated *in vitro* gastrointestinal tract (GIT) digestion, which takes into account the stomach and the intestinal phase. This procedure was adapted from a standardised method (Minekus et al., 2014). For the stomach phase, each nanoemulsion was mixed with simulated gastric fluids (SGF) containing pepsin (2000 U mL^{-1} in the final mixture), $CaCl_2(H_2O)$ (0.3 M), Milli-Q water and HCl (1 M), and were incubated (Incubator OPAQ, OVAN, Barcelona, Spain) for 2 h at 37 °C with continuous agitation (100 rpm). The sample from the stomach phase was then placed in a water bath (37 °C) to simulate the intestinal phase using a pH-stat (Metrohm USA Inc., Riverview, FL, USA). Moreover, simulated intestinal fluids (SIF) (0.150 M NaCl and 0.01 M $CaCl_2$), bile extract (54 mg mL^{-1}) and pancreatin (75 mg mL^{-1}) were added to the sample. During the intestinal phase, conversion of triacylglycerols and diacylglycerols from the oil present in nanoemulsions into free fatty acids (FFA) takes place due to the lipase action. Release of these FFA was the cause of pH reduction in the samples, which was maintained at a pH of 7 by adding NaOH (0.25 M) during the 2 h.

The total volume of NaOH spent to maintain the pH at 7 during the intestinal phase was used to calculate the lipid digestibility of nanoemulsions, thereby obtaining the total FFA release using equation (1).

$$\text{FFA (\%)} = \frac{V_{NaOH} \times C_{NaOH} \times M_{oil}}{2 \times m_{oil}} \times 100 \qquad (1)$$

Where V_{NaOH} is NaOH volume (L), which is used to compensate the FFAs during the digestion; C_{NaOH} is NaOH molarity (0.25 mol L^{-1}); M_{oil} is corn oil molecular weight (800 g mol^{-1}); and m_{oil} is corn oil total weight present in the nanoemulsions (g).

In order to obtain accurate results, aqueous phases containing emulsifiers were subjected to the simulated *in vitro* GIT digestion. The percentage of FFA obtained for aqueous phases was took away from the FFA initially obtained of the corresponding nanoemulsion.

2.2.3. Determination of physicochemical properties

Physicochemical properties of nanoemulsions before and during the different phases of the simulated *in vitro* GIT digestion (stomach and intestine) were determined in terms of particle size and ζ-potential.

2.2.3.1. Particle size

Particle size was obtained using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK). Nanoemulsions were added in the form of drops to the dispersion unit, which contained distillate water. To disperse these droplets and deliver them to the optical unit, a constant stirring (1700 rpm) was applied to the liquid. As the sample passed through the measurement area, a light source illuminated the droplets. As a result, the detectors measured the intensity of the light scattered by these droplets. Results were reported as the surface area mean diameter (d_{32}) in micrometres (μm). The refractive index was fixed at 1.473 for corn oil (dispersed phase) and 1.333 for water (continuous phase).

2.2.3.2. Optical microscopy

Images of nanoemulsions were obtained using an optical microscope (Olympus BX41, Olympus America Inc., Melville, NY, USA) with a 100× objective lens. A drop of each sample was placed on a slide and covered using a cover slip. Finally, images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus).

2.2.3.3. ζ -potential

The ζ-potential was determined using a Zetasizer (Malvern Instruments Ltd Worcerstershire, UK). Previously to the measurement, nanoemulsions were diluted 1/10 using Milli-Q water, simulated gastric fluids or simulated intestinal fluids, depending on the digestion phase analysed. Moreover, the samples were equilibrated inside the equipment for 60 seconds.

2.2.4. Determination of β-carotene bioaccessibility

The fraction obtained after the intestinal phase was centrifuged (AVANTI J-25, Beckman Instruments Inc., Fullerton, CA, USA) at 4000 rpm for 40 minutes at 4 °C (Qian et al., 2012). The upper part of the centrifuged liquid was collected and considered as the micelle fraction, in which the mixed micelles formed during the *in vitro* digestion containing the solubilised β-carotene were present. In certain samples, a layer of oil could be observed on top of the liquid, which was dismissed because it was not digested during the *in vitro* digestion. The concentration of β-carotene in nanoemulsions and in the micelle fraction was determined using a previously reported method (Salvia-Trujillo et al., 2013). The absorbance was spectrophotometrically measured (CECIL CE 2021; Cecil Instruments Ltd, Cambridge, UK) at 450 nm using chloroform as a blank. Lastly, β-carotene's bioaccessibility was calculated using equation (2):

$$\text{Bioaccessibility}(\%) = \frac{C_{micelle}}{C_{initial}} \times 100 \qquad (2)$$

Where $C_{micelle}$ and $C_{initial}$ are β-carotene concentrations of the micelle fraction and the initial nanoemulsions, respectively.

2.2.5. Statistical analysis

The analysis of variance (ANOVA) was conducted using a Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md, USA). *In vitro* digestions were performed in duplicate for each of the nanoemulsions prepared. Particle size and ζ-potential were analysed in triplicate for each nanoemulsion and the data was reported as the mean ± standard deviation. The least significant difference (LSD) test was performed to determine significant differences ($p \leq 0.05$) among nanoemulsions, which contained different emulsifiers and concentrations at a 5% significance level.

3. Results and discussion

3.1. Stability of nanoemulsions during *in vitro* digestion

3.1.1. Particle size

Increasing the emulsifier concentration from 2% to 8% resulted in a significant reduction in particle size of the nanoemulsions stabilised using Tween 20 (from 0.35 to 0.30 μm), lecithin (from 0.36 to 0.25 μm) and sodium caseinate (from 0.62 to 0.47 μm) (Figures 1A, B and C). In general, evidence of tiny droplet formation when using these three emulsifiers could be observed using microscopic images. However, nanoemulsions stabilised at high concentrations of sodium caseinate showed certain droplet aggregation (Figure 2).

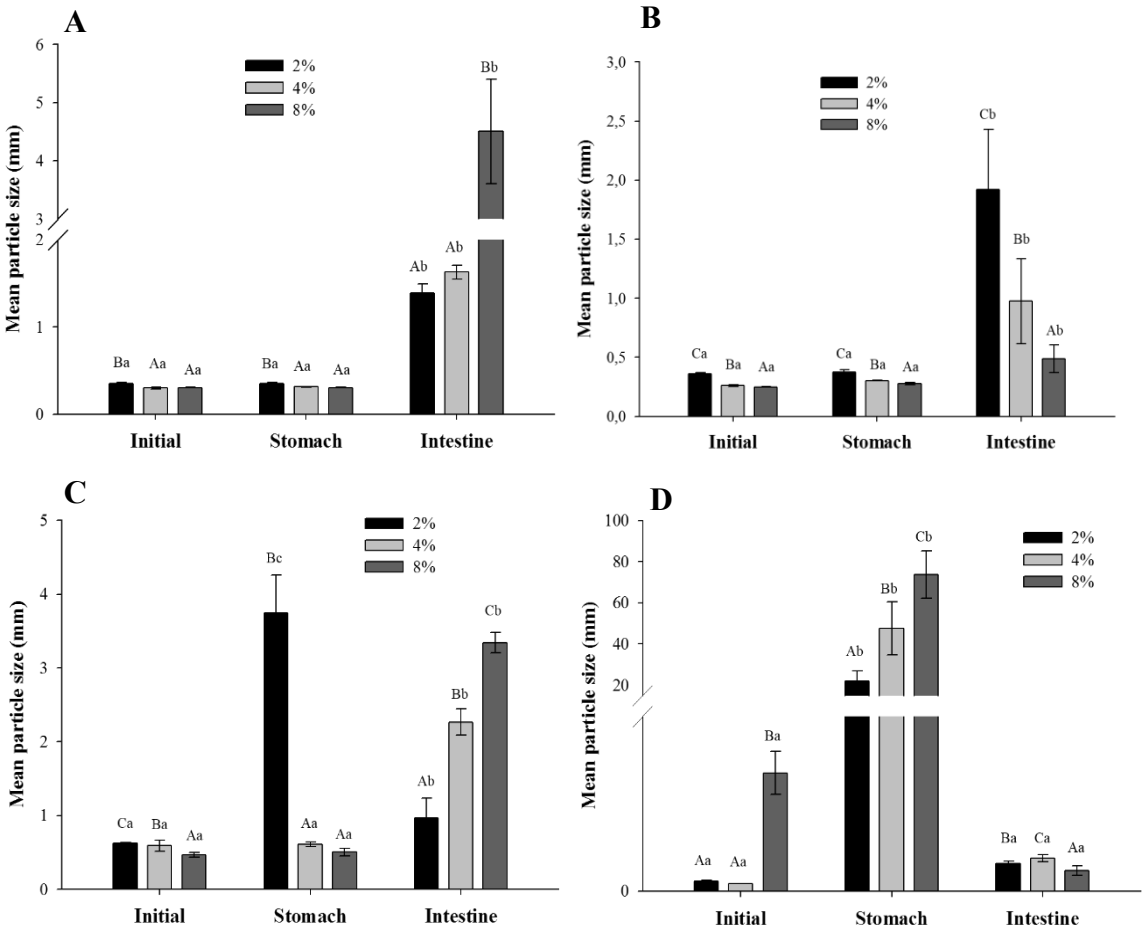

Figure 1. Particle size (μm) of β-carotene-enriched nanoemulsions stabilised with Tween 20 (A), Lecithin (B), Sodium Caseinate (C), or Sucrose palmitate (D) at different concentrations (2%, 4% and 8%) initially and during *in vitro* digestion phases (stomach, intestine). Different capital letters indicate significant differences ($p<0.05$) of nanoemulsions during the digestion phases, while different lowercase letters indicate significant differences ($p<0.05$) between nanoemulsions within the same digestion phase.

Thus, low mass emulsifiers, such as Tween 20 and lecithin, were more effective for producing small sizes than sodium caseinate, which is known to be a high mass emulsifier with a complex and large molecular structure. In addition, low mass emulsifiers have the ability to quickly adsorb at the droplets' surface during the formation of nanoemulsions, preventing the re-coalescence (McClements & Gumus, 2016).
On the other hand, particle size of sucrose palmitate-stabilised nanoemulsions remarkably increased from 0.29 to 4.73 μm when the emulsifier concentration increased from 2% to 8% (Figure 1D). Results showed that adding 4% sucrose palmitate to the nanoemulsions was enough to cover the entire surface of the formed droplets, assuming that a maximum number of tiny droplets were reached and that the particle size could not be reduced any further. Microscopic images confirmed that adding high concentrations (8%) could have led to non-adsorbed molecules in the media, generating attractive forces between droplets and resulting in droplet–droplet interactions (depletion flocculation) (Figure 2).

After the stomach GIT phase, the particle size of Tween 20- and lecithin-stabilised nanoemulsions remained unchanged irrespective of the emulsifier concentration (Figures 1A and 1B). Some non-ionic emulsifiers, such as Tween 20, were quite stable to droplet aggregation beyond low pH (Piorkowski & McClements, 2013). In particular, its high stability under acidic conditions is attributed to the polyoxyethylene head group, which produces steric repulsion between the droplets. Moreover, lecithin contains a mixture of phospholipids with phosphatidylcholine as its major component. Formation of lamellar structures at the oil–water interface, in which two layers of phosphatidylcholine were deposited (Pichot, Watson, & Norton, 2013; Van Nieuwenhuyzen & Szuhaj, 1998), might have provided stability to nanoemulsions against simulated gastric fluids (SGF) and to the drastic reduction of pH during the stomach phase. Nanoemulsions stabilised with 2% of sodium caseinate presented a sharp increase in particle size from 0.62 μm (undigested) to 3.74 μm (after stomach phase), while those stabilised with higher concentrations (4% and 8%) were more resistant under gastric conditions (Figure 1C). The emulsifying behaviour of sodium caseinate was associated with β-casein, which has 50 hydrophilic amino acid residues projected towards the aqueous phase as tails (external layer) and 159 hydrophobic residues attached to the droplet surface-forming trains (inner layer) (Dickinson, 1999). The layer thickness around the oil droplets is attributed to the high mass properties of protein and steric stabilisation of β-casein, which is primarily provided by the phosphoserine residues present in the protein. However, phosphoserine residues can suffer conformational changes when calcium ions are present, thereby resulting in a reduction of the layer thickness covering the droplets and stabilisation of nanoemulsions (Dickinson, Radford & Golding, 2003). In this study, the low stability of sodium caseinate-stabilised nanoemulsions at 2% under acidic conditions, suggested the formation of a thinner layer around droplets compared to nanoemulsions containing a higher amount of sodium caseinate. Addition of gastric fluids together with the presence of pepsin, made the nanoemulsion stabilised with 2% of sodium caseinate more prone to underwent flocculation processes as the hydrolysis of the sodium caseinate layer occurred. Microscopic images

confirmed that flocculated droplets were detected after the stomach phase (Fig. 3). On the contrary, particle size of sodium caseinate-stabilised nanoemulsions at 4% and 8% slightly changed after the stomach phase owing to the formation of a dense and thick layer around the droplets because of the β-casein deposition and the presence of phosphoserine residues. Sucrose palmitate-stabilised nanoemulsions presented a notable increase in the particle size after stomach GIT phase (Fig. 1D) and evidence of large particle aggregation could be observed in the microscopic images (Fig. 3). This enhancement might be related to the interaction between pepsin and sucrose esters and/or that sucrose head from sucrose palmitate could have changed its orientation at the interface of the droplets, resulting in flocculation and/or coalescence of particles (Verkempinck, Salvia-Trujillo, Moens, Charleer, Van-Loey, et al., 2018). In addition, Rao & McClements (2011) studied the stability of sucrose monopalmitate nanoemulsions at different pH and reported that when the pH was below the pKa of the carboxylic acid group from the palmitic acid (4.9), the acid lost its charge (Moran, Burczynski, Cheek, Bopp, Forker, 1987). In this situation, droplets were more prone to aggregate because attractive forces were more dominant than the repulsive ones.

After the GIT intestinal phase and regardless of the emulsifier concentration, nanoemulsions suffered a steep increase in their particle size, except for those stabilised with sucrose palmitate. In the latter case, flocculated droplets formed during the stomach phase because of the drastic change in pH could have been re-dispersed during the intestinal phase (Bellesi, Martinez, Pizones Ruiz-Henestrosa, & Pilosof, 2016). However, large values of particle size obtained for the rest of nanoemulsions could be attributed to various reasons. First, partial or total displacement of emulsifier molecules from the droplet surfaces when free fatty acids were produced, in addition to their hydrolysis by intestinal enzymes, would have resulted in a single emulsifier molecule being attached to the surface of more than one droplet, provoking aggregation phenomena (bridging flocculation) (Mun et al., 2007). Second, deposition of free fatty acids at the oil droplet interface and the presence of partially digested lipid droplets would have led to coalescence. Finally, all types of particles such as mixed micelles, vesicles and insoluble calcium complexes were formed during the lipid digestion (Zhang, Zhang, Zhang, Decker, & McClements, 2015), which would have contributed to the increase in the particle size.

Different particle size behaviour was observed for nanoemulsions stabilised with Tween 20, lecithin and sodium caseinate after the GIT intestinal phase. Tween 20- and sodium caseinate-stabilised nanoemulsions presented a larger particle size as the emulsifier concentration increased; it reached values of around 3.50 μm in all samples (Figures 1A and C). The high amount of emulsifier added initially would have formed complex aggregates and/or particles with other digestion components, further boosting the destabilisation processes during the intestinal phase. An opposite trend was observed for lecithin-stabilised nanoemulsions, decreasing the particle size to 0.48 μm with the addition of 8% lecithin (Figure 1B), which might be related to the hydrolysis of triglycerides from lipid droplets.

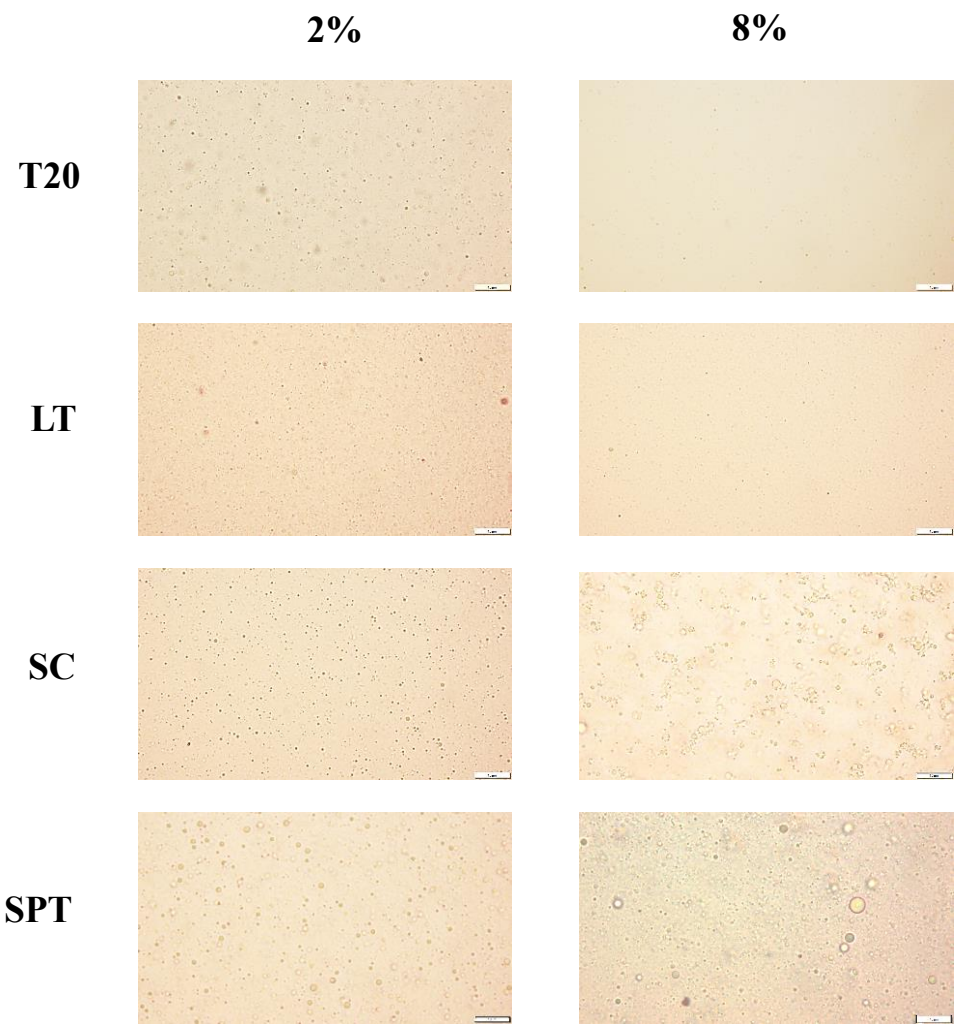

Figure 2. Images of β-carotene enriched-nanoemulsions stabilised with different emulsifiers (T20: Tween 20; LT: lecithin; SC: sodium caseinate; SPT: sucrose palmitate) at two concentrations (2% and 8%). Scales bar are 10µm long.

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Simultaneous and complex physicochemical processes occurring during lipid digestion, such as interactions and associations between all the particles present at that moment and the formation of new species (Ahmed et al., 2012), could have influenced the particle size of nanoemulsions. Therefore, microscopic images of nanoemulsions after intestinal phase showed the presence of two types of particles: some had an irregular shape and others were so small that they could barely be observed in the microscopic images (Figure 3). When analysing the particle size of nanoemulsions with a light scattering technique (such as Mastersizer) and microscopic images, complementary information was obtained. Using both approaches is of great importance when determining the particle size characteristics of nanoemulsions.

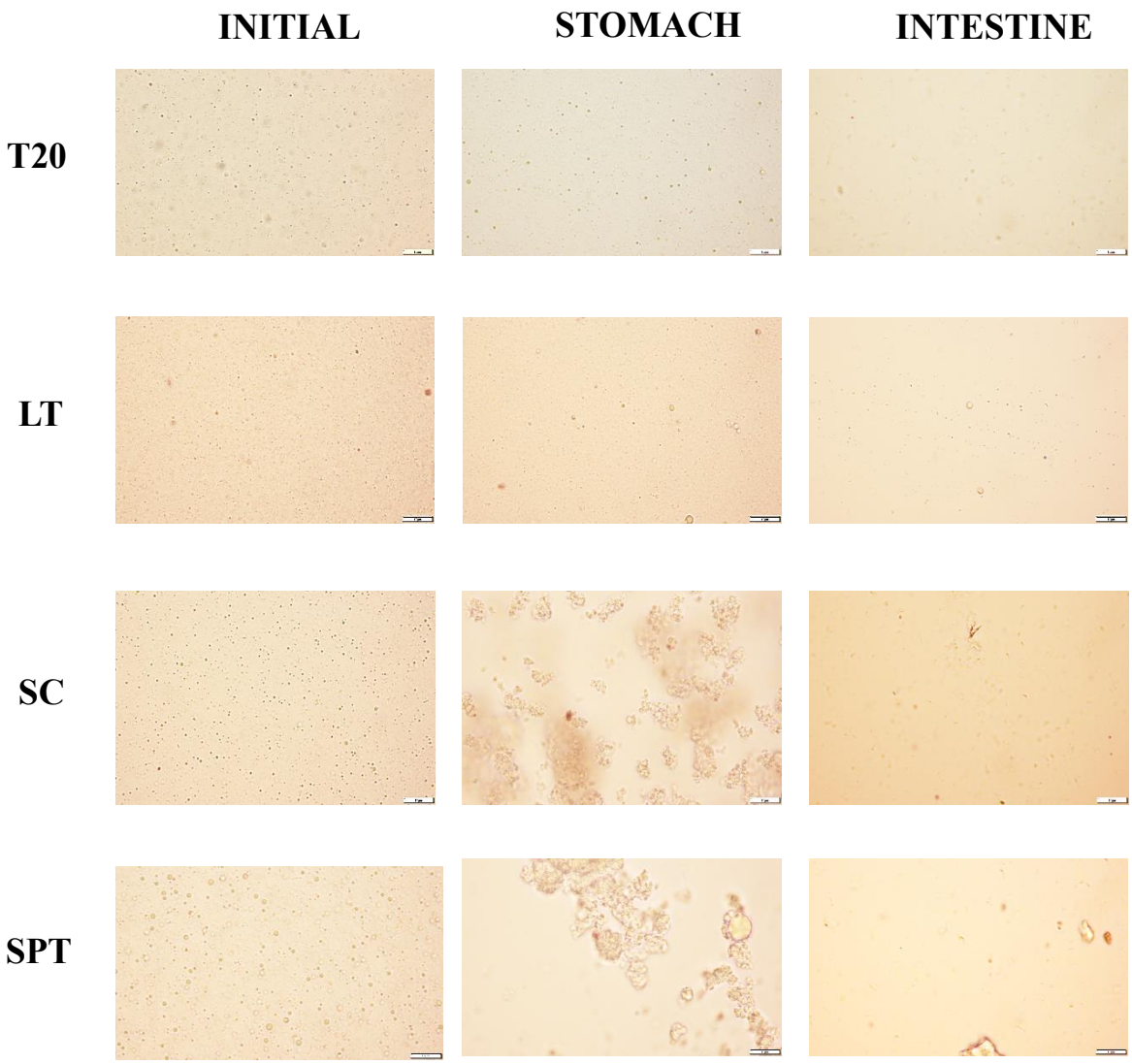

Figure 3. Representative microscope images of β-carotene-enriched nanoemulsions stabilised with different emulsifiers (T20: Tween 20; LT: lecithin; SC: sodium caseinate; SPT: sucrose palmitate) at 2% along the different phases (stomach and intestine) of the *in vitro* gastrointestinal tract (GIT). Scales bar are 10 μm long.

3.1.2. ζ -potential

All nanoemulsions presented negative values of ζ-potential irrespective of the emulsifier nature and concentration. The most negative values were exhibited by lecithin-stabilised nanoemulsions with values of around −80 mV. Phosphate groups from the different types of phospholipids present in the lecithin were the reason for the elevated negative charge (Artiga-Artigas et al., 2018). Although Tween 20 and sucrose palmitate are non-ionic emulsifiers, the ζ-potential of undigested nanoemulsions stabilised with both emulsifiers were negative, *i.e.*, around −24 mV and −49 mV, respectively, at all emulsifier concentrations (Figures 4A and D).

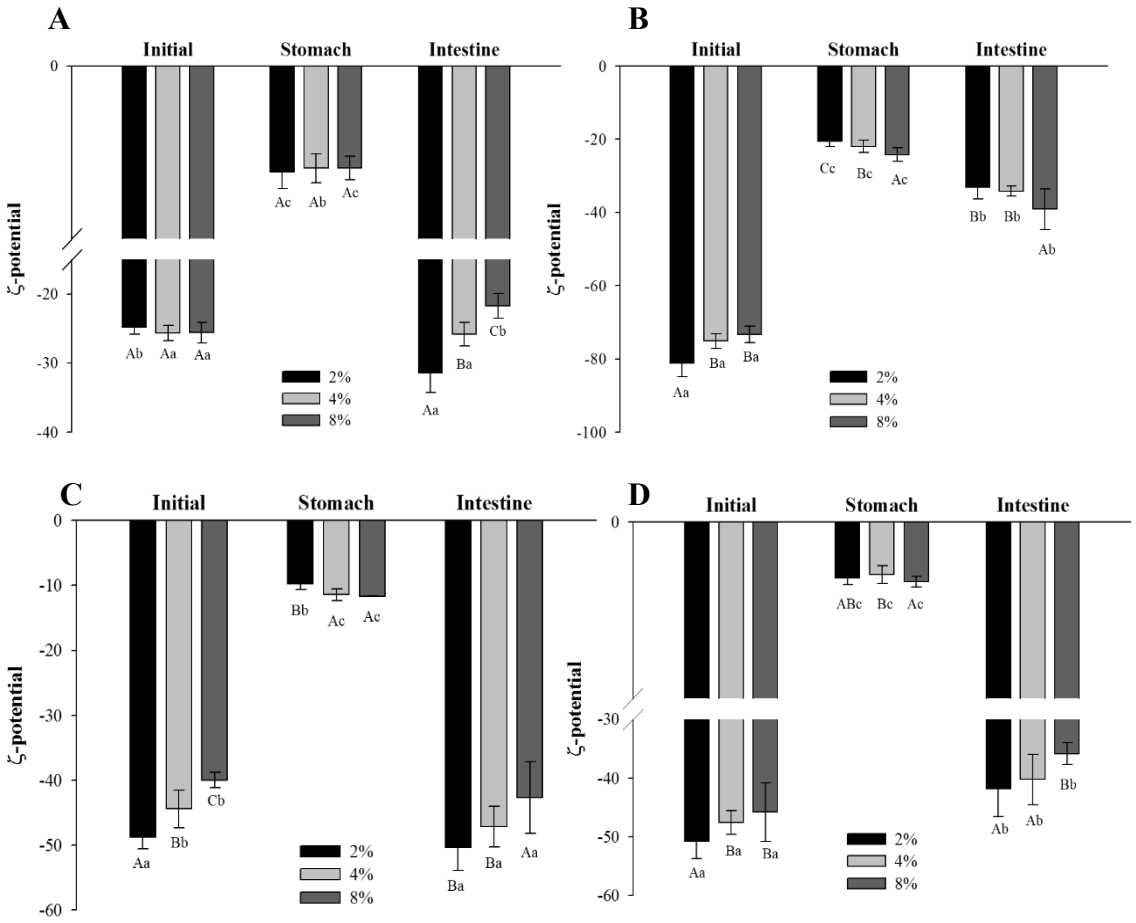

Figure 4. Changes in ζ-potential (mV) of β-carotene-enriched nanoemulsions stabilised with Tween 20 (A), Lecithin (B), Sodium Caseinate (C), or Sucrose palmitate (D) at different concentrations (2%, 4% and 8%) initially and during *in vitro* digestion phases (stomach, intestine). Different capital letters indicate significant differences ($p<0.05$) of nanoemulsions during the digestion phases, while different lowercase letters indicate significant differences ($p<0.05$) between nanoemulsions within the same digestion phase.

One argument to explain these results would be the adsorption of OH^- species from the aqueous phase to the interface of the oil droplets. Alternatively, cationic species of the oil (Yin, Chu, Kobayashi, & Nakajima, 2009), the presence of residual non-esterified fatty acids in the sucrose ester (Klang, Matsko, Raupach, El-Hagin, & Valenta, 2011) or impurities (palmitic acid) (Henry, Fryer, Frith, & Norton, 2009) in the case of sucrose palmitate could have been the reason for the negative charges. The ζ-potential values of sodium caseinate-stabilised nanoemulsions ranged between −40 and −48 mV, and they were slightly less negative as the emulsifier concentration increased. The addition of sodium caseinate to nanoemulsions might have decreased the pH of the system at around isoelectric point of caseinate (4.5). In this situation, there were a sufficient number of amino groups of caseinate positively charged, which would have increased the ζ-potential from −48 to −39 mV (Figure 4C).

Regardless of the emulsifier nature and concentration, the ζ-potential of all nanoemulsions became less negative after the stomach phase with values between −24.2 and −1.5 mV. The simulated gastric fluids used in this study contained free ions and the acidic pH during the stomach phase would have attenuated the charges of the nanoemulsion droplets (electrostatic screening effect) (Israelachvili, 2011). Interestingly, the negative ζ-potential (−9.70 mV) obtained for sodium caseinate-stabilised nanoemulsions at 2% confirmed that some of the sodium caseinate covering the droplets was partially displaced from the interface during the stomach GIT phase. However, nanoemulsions with higher concentrations of sodium caseinate presented values of ζ-potential that were slightly more negative (around −11.75 mV), suggesting that the interface of the droplets changed slightly. It should be noted that when the pH of the stomach phase (2.5) is below the isoelectric point of the proteins, a positive ζ-potential is expected. In this case, it was assumed that changes in the interface of the droplets, consisting of the displacement of sodium caseinate covering the droplets and the absorption of negatively charged particles, would have been the reason for the negative values. Furthermore, ζ-potential of all nanoemulsions became slightly negative after being subjected to intestinal conditions, reaching values similar to those of undigested nanoemulsions. The production of different particles in the process of lipid digestion (undigested lipid droplets, vesicles or micelles) and the presence of digestion components (bile, pancreatin, and calcium) (Wu, Yan, Chen, & He, 2017) could have had an impact on the ζ-potential of nanoemulsions after the intestinal phase (Figure 4). Simultaneous processes occurring during lipid digestion (enzyme hydrolysis, formation of new species, interactions between components, among others) resulted in a constant change in the interfacial properties of nanoemulsion droplets, affecting their electric charge. In addition, the neutral pH during the intestinal phase, similar to the undigested nanoemulsions, might be another reason why similar values of ζ-potential were obtained.

3.2. Oil digestibility

Depending on the emulsifier nature and concentration used to elaborate nanoemulsions, different free fatty acids profiles were observed. In general, there was a quick increase in the release of free fatty acids during the first 10–15 min of the intestinal phase for all

nanoemulsions. Then, there was a period of time where the free fatty acids release remained constant until the end of the intestinal phase (Figure 5).

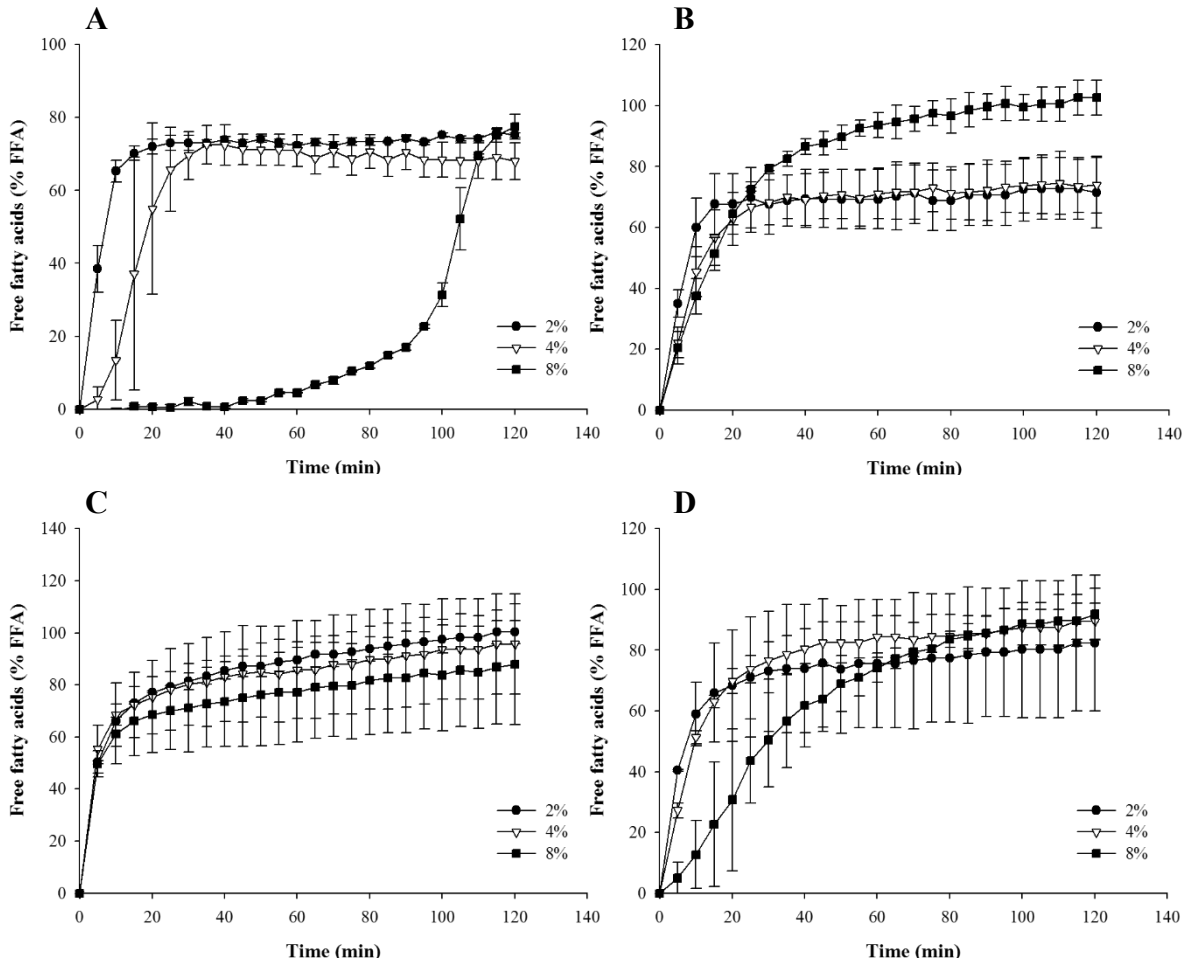

Figure 5. Influence of emulsifier nature (Tween 20 (A), Lecithin (B), Sodium Caseinate (C), Sucrose palmitate (D)) and concentration (2%, 4% and 8%) on the free fatty acids (FFA) release from β-carotene-enriched nanoemulsions during the intestinal phase.

Free fatty acids (FFA) are produced during the hydrolysis of the triacylglycerides from lipid droplets by the lipase present in the pancreatin. These FFA are considered to be surface-active components, which can be deposited and accumulated at the surface of the droplets as they are produced (Reis, Holmberg, Watzke, Leser, & Miller, 2009; Troncoso, Aguilera, & McClements, 2012). In this situation, new FFA were not generated because droplet interface was collapsed by the FFA that were already produced, inhibiting the deposition and action of the pancreatin at the interface of the droplets. As an exception, nanoemulsions stabilised with Tween 20 at concentrations ≥4% presented an interval period at the beginning of the intestinal phase (40–60 minutes) when no free fatty acids were produced (Figure 5A). Presumably, the interval period was the time required for the bile to be placed on the surface of the droplets and

displace the Tween 20. Furthermore, other studies have observed an interval period without the production of free fatty acids for nanoemulsions stabilised with lower concentrations of Tween 20 (0.6% and 1%) (Mun et al., 2007; Troncoso et al., 2012).

Despite observing different profiles of FFA during the lipid digestion among nanoemulsions and independently of the large values of particle size observed for certain nanoemulsions after stomach phase (section 3.1.1), no significant differences in the total amount of free fatty acids were observed at the end of the intestinal GIT phase. The particle size of nanoemulsions entering the intestinal phase was supposed to be an important factor for influencing the digestibility of nanoemulsions (Salvia-Trujillo et al., 2013). However, our results suggested that the large particle size of these nanoemulsions before entering the intestine was due to droplet flocculation, indicating that weak bonds linked the droplets together. As nanoemulsions were exposed to intestinal conditions, these bonds were broken down, resulting in the re-dispersion of droplets, which could have facilitated the digestion of lipids.
Interestingly, nanoemulsions stabilised with 8% of lecithin presented a higher lipid digestibility (100%) compared with those nanoemulsions stabilised with lower concentrations (around 73% of lipid digestibility). For this purpose, Yang, Decker, Xiao, & McClements, (2015) suggested that the addition of phospholipids to the simulated intestinal fluids (36 mg) increased the final extent of lipid digestion because phospholipids may facilitate the ability of lipase to interact with the emulsified triglycerides.

3.3. Bioaccessibility of β-carotene-enriched nanoemulsions

Based on the results obtained, both the emulsifier nature and their concentration in nanoemulsions had an impact on β-carotene's bioaccessibility (Figure 6). For Tween 20-stabilised nanoemulsions, β-carotene bioaccessibility was around 16% without significant differences regardless of the emulsifier concentration. On the contrary, for sodium caseinate-stabilised and sucrose palmitate-stabilised nanoemulsions, β-carotene's bioaccessibility significantly decreased when higher concentrations of emulsifier (8%) were added.
As the quantity of free fatty acids released during the lipid digestion was similar among the nanoemulsions prepared with these emulsifiers, different hypothesis were suggested to explain this tendency. First, not all the free fatty acids produced might have participated in the formation of mixed micelles because certain interactions with the digestion products may have occurred. Second, mixed micelles formed during digestion may not have incorporated and solubilised β-carotene, assuming that if β-carotene is not present within the mixed micelles, is not available to be absorbed. Third, interactions between the β-carotene itself and/or mixed micelles containing β-carotene with the emulsifier could have occurred. Indeed, proteins are known to form complexes with carotenoids *via* hydrophobic interactions (Wackerbarth, Stoll, Gebken, Pelters & Bindrich, 2009) and promote aggregation and precipitation of mixed micelles (Mun et al., 2015). Lecithin-stabilised nanoemulsions presented a greater β-carotene bioaccessibility as the emulsifier concentration increased, raising from 9.9% to 23.5%. These results agreed with the lipid digestibility results (section 3.2): the high number of free fatty acids produced for

nanoemulsions with 8% lecithin could lead to the formation of a large number of mixed micelles, enhancing both the lipid digestibility and the β-carotene bioaccessibility. Mixed micelles are composed of bile salts, phospholipids from the bile and pancreatic juices as well as lipid digestion products from the action of lipases such as free fatty acids (Porter, Trevaskis, & Charman, 2007; Harjinder Singh et al., 2009). Thus, lecithin, a nontoxic emulsifier generally recognized as safe (GRAS) that predominantly contains phospholipids, could have contributed to the formation of these particles and increase their solubilisation capacity (McClements & Decker, 2009; Yang et al., 2015).

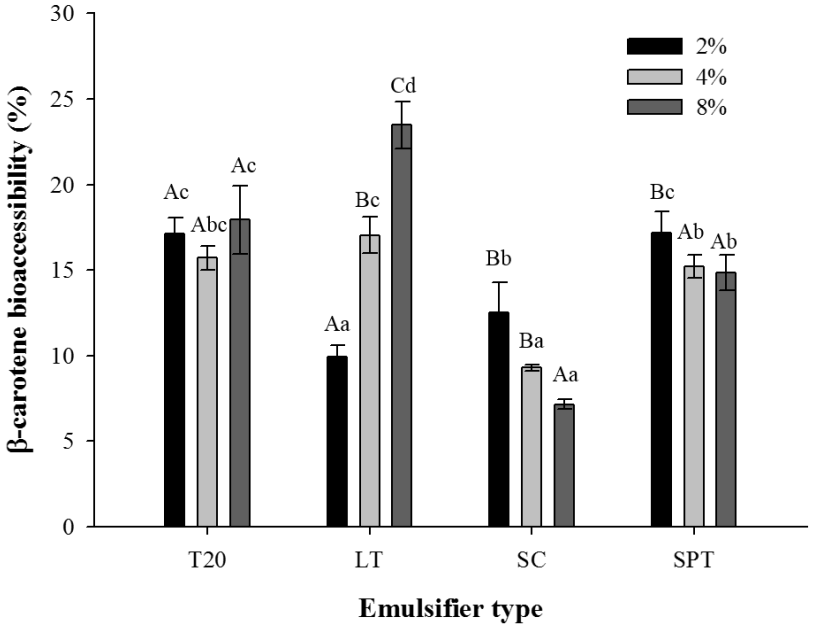

Figure 6. Influence of emulsifier nature (T20: Tween 20; LT: lecithin; SC: sodium caseinate; SPT: sucrose palmitate) and concentration (2%, 4% and 8%) on the bioaccessibility of β-carotene enriched nanoemulsions. Different capital letters indicate significant differences ($p<0.05$) of β-carotene bioaccessibilty taking into account the same emulsifier at different concentrations, while different lowercase letters indicate significant differences ($p<0.05$) between nanoemulsions containing different emulsifier type but at the same concentration.

4. Conclusions

Results obtained in this study indicated that β-carotene-enriched nanoemulsions presented different initial physicochemical properties and behaviours along the GIT owing to the emulsifier nature and concentration. However, nanoemulsions presented similar lipid digestibility results, with the exception to those elaborated with lecithin at 8%. In turn, these latter nanoemulsions showed the highest β-carotene bioaccessibility after being digested through an *in vitro* GIT. This study revealed that using lecithin could be a good option when

designing nanoemulsions as delivery systems of lipophilic compounds. Further investigation with *in vivo* studies (animal and human) are required to elucidate the importance of nanoemulsion composition-relationship with digestive processes and bioaccessibility of encapsulated compounds.

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CHAPTER II
Delivery of β-carotene to the *in vitro* intestinal barrier using nanoemulsions with lecithin or sodium caseinate as emulsifier

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Abstract

To increase the intestinal delivery of dietary β-carotene, there is a need to develop nanostructured food systems to encapsulate this fat soluble bioactive. The aim of this study was to evaluate the bioacessibility and bioavailability across the intestinal barrier of βcarotene-enriched nanoemulsions stabilised with two emulsifiers (lecithin or sodium caseinate) by coupling an *in vitro* gastrointestinal digestion with two *in vitro* cell culture models (Caco-2 or co-culture of Caco2/HT29-MTX). Nanoemulsions stabilised with lecithin had significantly higher β-carotene in the gastrointestinal digested micellar fraction, lower β-carotene in the Caco-2 (and Caco-2/HT29-MTX) apical compartment and significantly higher β-carotene in Caco-2 cellular content compared to β-carotene-enriched nanoemulsions stabilised with sodium caseinate. Finally, to assess anti-inflammatory activity of digested nanoemulsions, lipopolysaccharide stimulated macrophages were exposed to Caco- 2 basolateral samples with levels of TNF-α and IL-β, subsequently quantified. A TNF-α response from stimulated THP-1 macrophages was elicited by basolateral samples, regardless the emulsifier used to formulate nanoemulsions. This study demonstrated that β-carotene permeability is influenced by the food derived emulsifier used for stabilising nanoemulsions, indicating that composition may be a critical factor for β-carotene delivery

Keywords: β-carotene; nanoemulsions*; in vitro* digestion; intestinal barrier

1. Introduction

β-carotene is a vitamin A precursor with poor water-solubility (0.0006 g/L at 25ºC). Positive health benefits associated with β-carotene consumption include lower incidence of cancer, cardiovascular diseases and degenerative disorders (Goralcyk, 2009). These health attributes have been related to β-carotene's antioxidant and immunomodulatory bioactivities, proven both *in vitro* (Bai et al., 2005) and *in vivo* (Zhou et al., 2018).

Although the recommended dietary allowance (RDA) for β-carotene has not been set, the U.S. Food and Drug Administration and the European Food Safety Authority derive a RDA for vitamin A of 900-700 μg of retinol activity equivalents (RAE) daily and a population reference intake of 750-650 μg REA daily, respectively. In addition, β-carotene appears to be degraded by the acidic environment of the stomach (Boon, McClements, Weiss, & Decker, 2010) which undoubtedly reduces β-carotene concentration in the intestine.

As a solution, recent studies have investigated nanostructured delivery systems such as nanoemulsions to encapsulate and protect β-carotene after oral consumption and enhance its delivery to intestinal barrier (Chen, Li, Li, McClements, & Xiao, 2017; Gasa-Falcon et al., 2020; Yi, Zhong, Zhang, Yokoyama, & Zhao, 2015). β-carotene enriched nanoemulsions stabilised with pectin, lecithin, sodium caseinate, Tween 20 or sucrose palmitate, have been subjected to *in vitro* gastrointestinal digestion (GID) and the subsequent release of β-carotene has been determined (Gasa-Falcon et al., 2019; Salvia-Trujillo et al., 2013; Teixé-Roig et al., 2020). However, less information is currently available about the use of either proteins or phospholipids-based emulsifiers on β-carotene transit across the intestinal barrier and subsequent bioactivity from nanoemulsions post gastrointestinal digestion (GID)**.** For instance, Lu, Kelly, & Miao (2017) described that the permeability of β-carotene in undifferentiated naïve gastrointestinal epithelial cells is depended on the emulsifier type added to the nanoemulsions rather than initial particle size of the nanoemulsions.

To model absorption *in vitro*, the use of differentiated monolayers expressing tight junctions, best represent the morpho-functional features of the intestinal barrier (Guri, Gülseren, & Corredig, 2013). However, *in vitro* monolayers present their own challenges since digestive fluids and nanoemulsions post GID (micellar fractions) are cytotoxic at relatively low concentrations (Arranz, Corredig, & Guri, 2016). As a result, quantification of compounds on the basolateral side can be challenging.

Thus, the aim of this study was to evaluate the permeability of β-carotene-enriched nanoemulsions stabilised with two different emulsifiers across across 21 days differentiated Caco-2 and Caco-2/HT29-MTX co-cultures, post *in vitro* static GID. These 21 days old Caco-2/HT29-MTX co-cultures best represent the mature intestinal mucus barrier. Emulsifiers (lecithin and sodium caseinate) were selected based on their different properties (low (758 g/mol) and high (≈10-50 KDa) molecular weight) and sources (synthetic and natural) and previous physiochemical characterisation of nanoemulsions stabilised with these emulsifiers in our group (Gasa-Falcon et al., 2019). Furthermore, the subsequent basolateral anti-inflammatory activity was assessed by quantification of TNF-α and IL-β in lipopolysaccharide (LPS) stimulated macrophages (THP-1 cells).

2. Material and Methods

2.1. Materials

Corn oil (Mazola, ACH Food Companies Inc., Memphis, TN) was purchased from a local market. β-carotene (synthetic, ≥93% (UV), powder) was sourced from Sigma–Aldrich (Ireland). Lecithin was obtained from Alfa Aesar (Karlsruhe, Germany). Sodium caseinate (NaCas) (≥92% purity) was from Acros Organics (Geel, Belgium). The Caco-2 cell line was purchased from the European Collection of Cell Cultures (ECACC 86010202) and the human monocyte THP-1 (ATCCTIB-202) and the human colon adenocarcinoma HT-29 cell lines (ATCCHTB-38) were purchased from American Type Culture Collection. This latter cell line was differentiated to HT-29-MTX following the protocol described by Guri et al. (2013). Tissue culture plastics were sourced from Sarstedt Ltd. (Wexford, Ireland). CellTiter 96 AQueus One Solution reagent was purchased from Promega (MyBio, Kilkenny, Ireland). Milli-Q water was used to prepare all nanoemulsions. All other chemicals were sourced from Sigma–Aldrich (Ireland) unless specified otherwise.

2.2. Preparation of nanoemulsions

Primary emulsions were prepared by mixing 4% (w/w) of the lipid phase (corn oil enriched with 0.5% w/w of β-carotene) with 96% (w/w) of the aqueous phase containing the emulsifier (lecithin or NaCas) at 2% (w/w). Both phases were mixed with an Ultra-Turrax (IKA, Staufen, Germany) at 9500 rpm for 3 minutes. Then, primary emulsions were passed through an APV 1000 (SPX Flow Technology, Charlotte, NC, USA) at 500 bars for 3 cycles to obtain nanoemulsions.

2.3. Determination of nanoemulsions properties

Particle size of nanoemulsions was determined using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK). The results were reported as the surface mean diameter (d_{43} (μm)) and the width of the distribution. The refractive index of the corn oil and water employed to perform the analysis were 1.34 and 1.33, respectively.

The emulsions ζ-potential was determined using a Zetasizer NanoZS (Malvern Instruments Ltd, Worcerstershire, UK). Samples were previously diluted (1/100) and equilibrated prior to analysis.

Physical stability of β-carotene enriched nanoemulsions was determined with an analytical centrifuge LUMiSizer 6112 (L.U.M. GmbH, Berlin, Germany) that accelerates destabilisation of samples. Results were analysed using the software package SEPView 6.0 (L.U.M. GMBH) that records transmitted light across the sample length and calculates the instability index that 126 ranges from 0 to 1, with the greatest instability at 1. Instrumental parameters used for physical stability analysis were: speed 2,186 rcf; time interval 20 seconds; exposure time 10,000 seconds; temperature 25ºC.

2.4. *In vitro* static simulated digestion

Nanoemulsions were subjected to a simulated *in vitro* static GID (gastric and upper intestinal phases) that mimics the adult human upper gut. The INFOGEST standardised method (Brodkorb et al., 2019) was followed with minor modifications. Briefly, gastric phase consisted of 5 mL of nanoemulsion with simulated gastric fluid containing porcine pepsin (EC 3.4.23.1) (3925.3 U/mg); pH was adjusted to 3.0 using HCl (1 *M*) and volume to 10 mL (Milli-Q water). The mixture was incubated for 2 h at 37°C with continuous shaking in a rotator. After 2h, pH was increased to 6.5 using NaOH and 20 µL $CaCl_2$ (0.3 M), 4 ml bile (630 g/mol, EC232-369-0), and 2.5 mL pancreatin based on trypsin activity (8.13 U/mg; EC232.468.9) were added. Based on Verkempinck et al. (2017), extra lipase (pancreatin and lipase) was added to reach 420 U/mL. The pH of the mixture was adjusted to 7.0, the volume to 20 mL with milli-Q water, and the mixture was incubated for 2 h at 37°C. The digestion was then stopped by adding protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1 mM). To obtain the micellar fraction, the digested fractions were centrifuged (Heraeus Megafuge 1.0, Massachusetts, USA) at 4000 rpm for 40 minutes at 4 °C (Garrett, Failla, Sarama, &Craft, 1999). Samples were stored at −80°C for further experiments.

2.5. *In vitro* cell based assays

Caco-2 and HT29-MTX cell lines were grown in 75 cm^2 tissue culture flasks in a humidified 37 °C incubator with a 5% CO_2 air atmosphere. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. At 80% confluency, cells were trypsinated (0.25% trypsin/EDTA), diluted 1:6 in DMEM medium, and reseeded in flasks. Caco-2 and HT29-MTX cell lines in this study were used at passage number 29-41 and 53-67, respectively.

2.5.1. Cytotoxicity of micellar fractions

Caco-2 cells were seeded at a density of 8×10^4 cells/well in 96-well plate. After 24 h of incubation, cells were washed with PBS. GID micellar fractions were filtrated (0.45 µm), diluted in complete DMEM (between 2-16% v/v) and 80 µL were added to each well. Subsequently, 20 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent was added to each well and cells were incubated for 2 h. After 2 h, the quantity of formazan produced was measured spectrophotometrically at 490 nm in a microplate reader (Synergy HT BioTek, Winooski, VT, USA). Results were expressed as the percentage of cellular viability relative to a control group (cells with DMEM medium) versus the micellar fraction concentration (%, v/v). Cytotoxicity of pure β-carotene dissolved in DMSO was also evaluated (0.05-10 µg/mL).

2.5.2. Permeability across intestinal barriers

Caco-2 cells were seeded at a density of 6×10^4 cells per insert in 12-well Transwell® plates (0.4 μm pore size, 1.2 cm diameter, Costar, Cambridge, MA). In co-culture experiments, Caco-2 and HT29-MTX were grown separately and then seeded at a ratio of 75:25, to a final density 6×10^4 cells per insert.

Culture media of each plate was changed every two days for 21 days. The integrity of the cell monolayer was monitored by measuring the transepithelial electrical resistance (TEER) ($\Omega \cdot cm^2$) using a Millicell-ERS Voltohmmeter (Merck Millipore, Carrigtwohill, County Cork, Ireland). On day 21, apical and basolateral compartments were washed three times with PBS and 470.6 μL and 1500 μL DMEM were added to apical and basolateral compartments, respectively. Then, micellar fractions (29.4 μL) were added to apical compartment and incubated for 2 h. During this 2 h, the TEER value did not change significantly (data not shown). After permeability experiment, apical and basolateral samples were collected. Moreover, cell monolayer was washed three times with PBS, scraped and collected. Cells were centrifuged (Heraeus Megafuge 1.0) for 3 min at 215xg and the supernatant discarded. Cells were stored at -80 ºC for further analysis.

2.6. Determination of β-carotene

Extraction of β-carotene from samples (apical, cells and basolateral) was performed as described by Yuan, Gao, Zhao, & Mao (2008) with minor modifications. Briefly, the samples were filtrated (0.45 μm) and mixed with ethanol and hexane, followed by a centrifugation (4000 rpm, 5 min, 5ºC) (Heraeus Megafuge 1.0). The upper fraction was collected, dried under N_2 and stored at -80 ºC. Each sample extract was dissolved in 200 μL of the injection solvent acetonitrile (ACN): methanol (MeOH) 7:3 (v/v): acetone 6.7:3.3 (v/v) and filtered through 0.2 μm nylon filters (Millipore, Bedford, MA). High-performance liquid chromatography system (Waters Xevo TQ-S, Milford, USA) equipped with a photodiode array detector (HPLC-PDA) at 450 nm and a column ACQUITY UPLC® (C18 BEH 130 Å, 1.7 μm, 2.1 × 150 mm) (Waters) (30°C and flow rate 0.85 mL/min) were used. Mobile phase consisted of solvent A: ACN: MeOH 7:3 (v/v) and solvent B: water 100%, and the flow was isocratic (100% ACN/MeOH 7/3). β-carotene was quantified by comparison with external standards. Results were reported as ng/mL and cellular uptake as percentage of detected β-carotene in cells versus apical samples at time 0 h.

2.7. Anti-inflammatory activity of basolateral samples

Human monocytes THP-1 were cultured in RPMI 1640 culture medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Cells were added at a density of 5 x 105 cells/mL in 24 well plates. Differentiation to macrophages was induced by adding 1 μg/μL 12-O-tetradecanoyl phorbol-13-acetate (TPA) to cells followed by 48h incubation. After differentiation to macrophages,

cells were washed with PBS. LPS (0.05 µg/mL) and Caco-2 basolateral samples were added to each well. After 24h incubation, the culture medium was collected for subsequent TNF-α and IL-1β quantification.

Release of TNF-α and IL-1β was measured in the supernatants of THP-1 cells using ELISA kits (R&D Systems, Minneapolis, USA), according to manufacturer's instructions. Multiscanner autoreader (Synergy HT BioTek) was used to read the absorbance of the plates at 450 nm.

2.8. Statistical analysis

Experiments were performed in triplicate on at least two different days and data was expressed as the mean with standard deviation of three experiments. To determine the statistically significant differences between samples, one-way ANOVA followed by Bonferroni test ($p \leq 0.05$) was conducted with SigmaPlot 11.0.

3. Results and discussion

3.1. Physicochemical properties of nanoemulsions

Both nanoemulsions containing lecithin and NaCas had a monomodal particle size distribution (Fig. 1A) and exhibited particle sizes in the nanometer range (0.35 and 0.29 µm, respectively). Nanoemulsions had negative ζ-potential values, with lecithin displaying the highest negative value (Table 1), in line with our previous published results (Gasa-Falcon et al., 2019). NaCas-stabilised nanoemulsions exhibited the lowest end point instability index (0.603 ± 0.006) compared with lecithin-stabilised nanoemulsions (0.773 ± 0.001) (Fig. 1B).

Table 1. Particle size (µm) and ζ-potential (mV) of nanoemulsions stabilised with different emulsifiers (LE: lecithin; NaCas: sodium caseinate). Differences among nanoemulsions were compared using one-way ANOVA followed by Bonferroni test. Different letters indicate statistically significant differences within the parameter tested ($p<0.05$).

Emulsifier	Particle size (µm)	ζ-potential (mV)
LE	0.35 ± 0.001^{a}	-58.81 ± 2.56^{a}
NaCas	0.29 ± 0.001^{b}	-53.41 ± 1.83^{b}

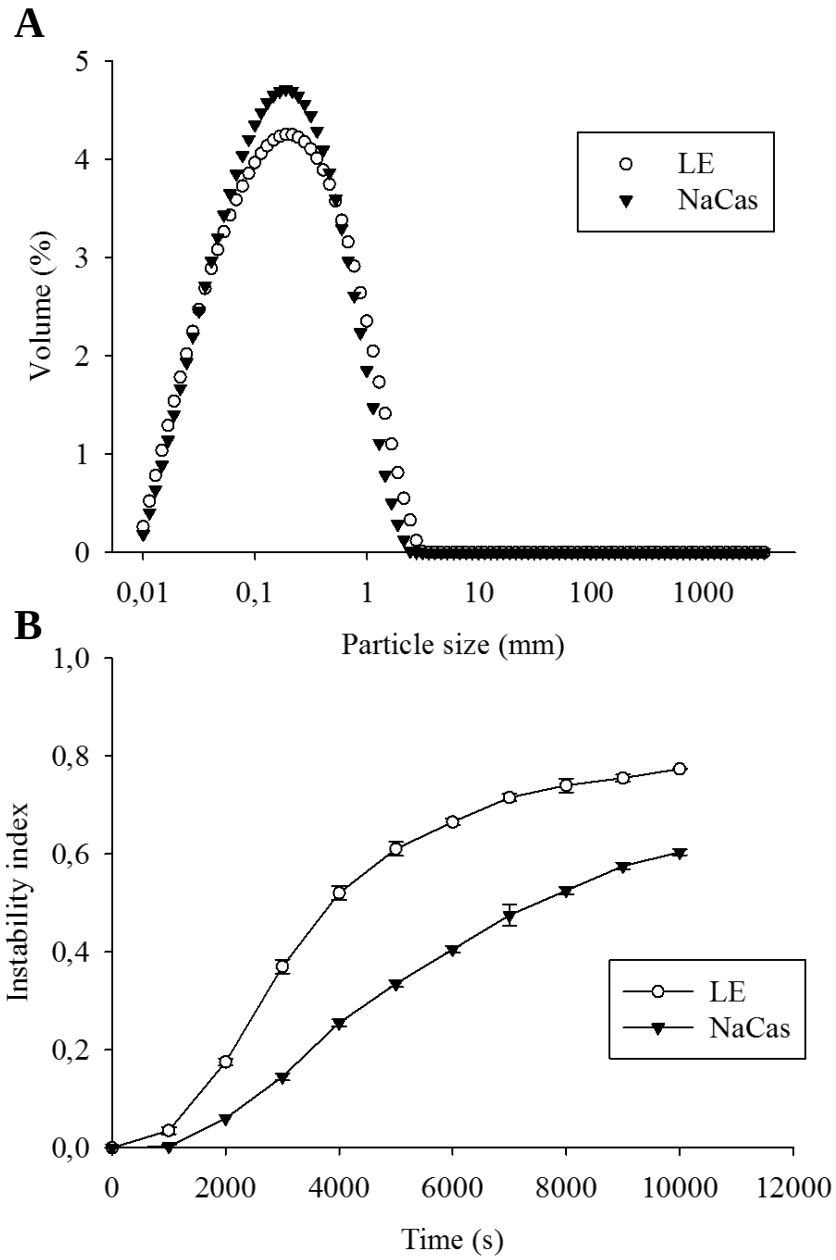

Figure 1. (A) Particle size distribution and (B) instability profile of β-carotene-enriched nanoemulsions (0.02% β-carotene w/w, 4% corn oil) stabilised with 2% of lecithin (LE) or sodium caseinate (NaCas).

Emulsifiers with a low molecular weight and/or with a high hydrophilic–lipophilic balance (HLB) are associated with a high efficiency at producing small particle sizes in oil-in-water emulsions (Jo & Kwon, 2014). Lecithin has an HLB value of 8 while NaCas has a value of 14. Thus, the intermediate-low HLB value of lecithin could explain why nanoemulsions stabilised with this emulsifier exhibited the highest particle size and instability index compared to nanoemulsions with NaCas (Iyer et al., 2015). In addition, the mass of NaCas (≈10-50 KDa)

(Ozturk & McClements, 2016), its gelation behaviour (Rodriguez-Patino & Pilosof, 2011) and the thick interfacial layer covering oil droplets (McClements et al., 1993) undoubtedly contributed to its nanoemulsion stability over the accelerated centrifugation process. The large negative ζ-potential values observed for lecithin and NaCas nanoemulsions (-58.81 mV and -53.41 mV, respectively) could be attributed to the phospholipid head groups from lecithin, and the fact that the nanoemulsion pH of ≈6.5 differs to the NaCas isoelectric point (pI=4.6) respectively (Chang & McClements, 2016). Interestingly, ζ-potential of lecithin stabilised β-carotene nanoemulsions became less negative after *in vitro* GID, while NaCas emulsions post GID have a stronger negative value (Gasa-Falcon et al., 2019). In that previous study, particle size after *in vitro* GID in lecithin nanoemulsions was higher compared to NaCas.

3.2. Cytotoxicity of micellar fractions

The maximum non-toxic concentration of micellar fractions was different depending on the emulsifier used (Fig. 2). Micellar fractions with lecithin showed no cell toxicity (>90% cell viability) when Caco-2 cells were exposed to concentrations below 6% (v/v) (Fig. 2A), while for those containing NaCas no cell toxicity was observed at concentrations under 10% (v/v) (Fig. 2B).

In agreement to our results, several studies have demonstrated that emulsifiers are cytotoxic in a concentration-dependent manner and that toxicity of nanoemulsions depends on the nature of emulsifier employed (Buyukozturk, Benneyan, & Carrier, 2010; Ujhelyi et al., 2012). Furthermore, Sadhukha, Layek & Prabha (2018) observed that the aqueous fraction of digested lipid-based delivery systems was responsible for cytotoxicity in MDCK kidney cells, reducing cell viability by 40%. It has been previously reported that monoglycerides induce dose-dependent apoptosis in mammalian cells (murine thymocytes), which consisted on a rapid reduction in mitochondrial transmembrane potential, production of reactive oxygen species, among other processes (Philippoussos, Arguin, Fortin, Steff, & Hugo, 2002). In our study, micellar factions of nanoemulsions were likely to contain lipid digestion products (i.e. free fatty acids and monoglycerides), and together with emulsifiers contributed to damage the cell integrity.

Similar cell viability results were obtained when control micellar fractions (without β-carotene) were tested in Caco-2 cells, but interestingly control micellar fractions derived from lecithin-stabilised nanoemulsions were 60% less toxic compared to micellar fractions with β-carotene. This suggests that β-carotene plays a role in cell cytotoxicity of Caco-2 monolayers. Indeed, Wooster et al. (2017) observed that the presence of β-carotene in LCT nanoemulsions (IC_{50}= 51 μg/mL) increased four times their toxicity in differentiated Caco-2 cells compared to empty nanoemulsions (IC_{50}= 257 μg/mL).

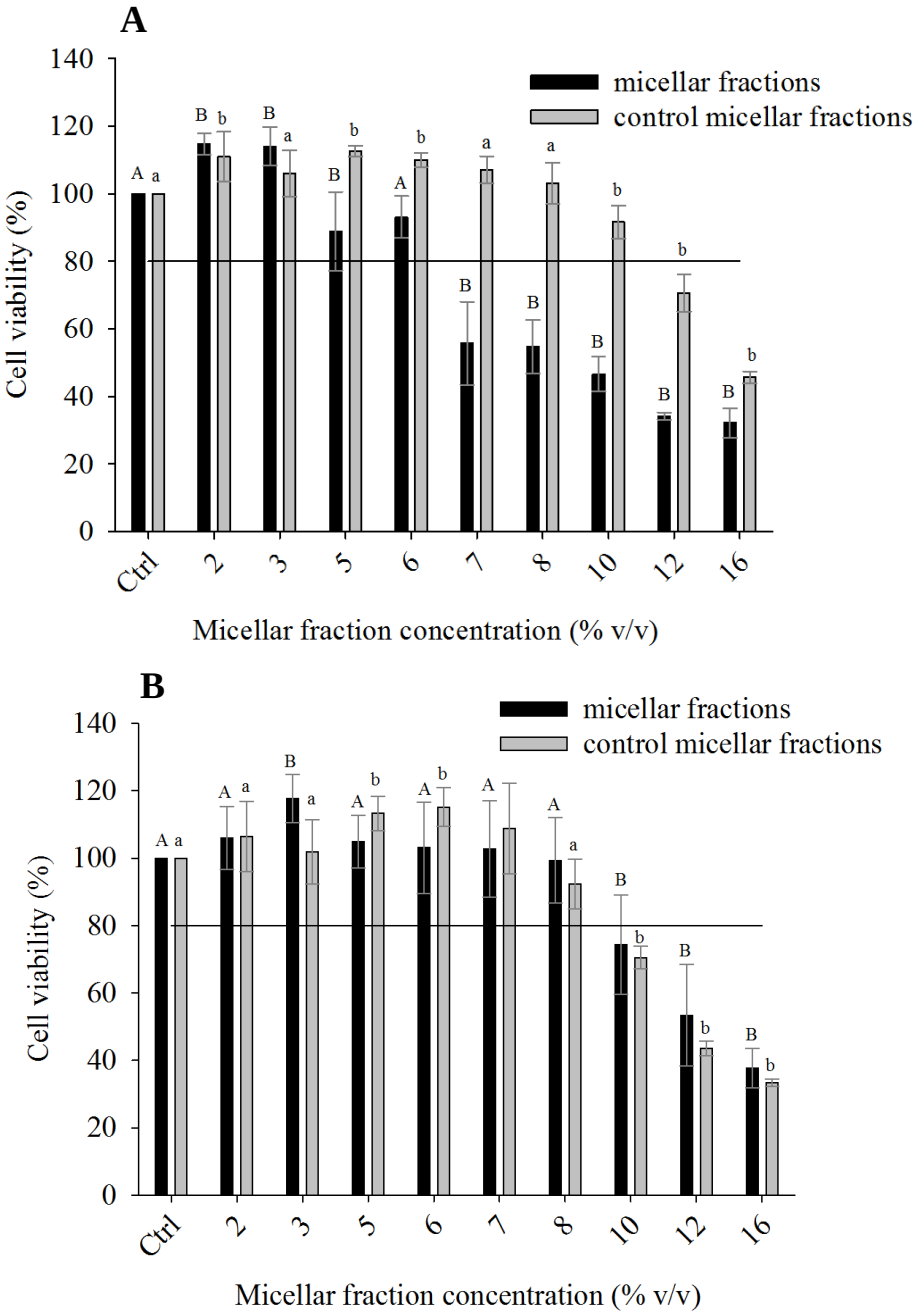

Figure 2. Cell viability (%) of Caco-2 cells after 2h with micellar fractions (with β-carotene) (% v/v) and control micellar fractions (without β-carotene) (% v/v) obtained after *in vitro* digestion of nanoemulsions stabilised with different emulsifiers. Micellar fractions containing lecithin (A) and NaCas (B) were diluted at different concentrations with complete DMEM. Control cells (Ctrl) were grown in media with no treatment (100% viability). Different uppercase and lowercase letters indicate significant differences to control cells for micellar fractions and control micellar fractions, respectively. Statistical analysis was performed using one-way ANOVA followed by Bonferroni test (*$p<0.05$). Percentage of cell viability above 80% was considered as non-cytotoxic.

In contrast, a preliminary study by our laboratory indicated that pure β-carotene present in nanoemulsions was not cytotoxic to undifferentiated Caco-2 cells (between 0.05 µg/mL and 10 µg/mL) (data not shown), suggesting that Caco-2 cell monolayers with tight junctions are more sensitive to GID β-carotene-enriched nanoemulsion than undifferentiated Caco-2 cells. Certainly, oxidation products of β-carotene could have been generated during *in vitro* GID, specifically due to the acidic pH of the gastric phase (Failla, Chitchumronchokchai, Ferruzzi, Goltz, & Campbell, 2014).
Oxidation of β-carotene can produce carotenoid aldehyde breakdown products, which have documented toxic effects on numerous cell lines (K562, RPE 28 SV4 and ARPE-19) at concentrations between 10–20 µM (Hurst, Saini, Jin, Awasthi, & Van Kuijk, 2005).
To investigate the bioavailability of β-carotene from nanoemulsions, permeability experiments were performed with lecithin and NaCas micellar fractions at a concentration of 6% (v/v).

3.3. Permeability of β-carotene nanoemulsions

After *in vitro* GID, β-carotene concentration present in the micellar fractions was significantly higher in lecithin nanoemulsions compared to those prepared with NaCas (Table 2). There was a significant reduction of β-carotene in apical compartment after 2 h incubation compared to time zero regardless of emulsifier used. Interestingly after 2 h incubation, the apical of NaCas-stabilised nanoemulsions had a significantly higher amount of β-carotene compared to lecithin-stabilised nanoemulsions. β-carotene content in cell lysates of Caco-2 cells was 3 times higher than in Caco-2/HT29-MTX co-cultures. In addition, the β-carotene concentration in Caco-2 cells lysates was significantly higher in lecithin-stabilised nanoemulsions (2.28%) compared with nanoemulsions containing NaCas (1.72%). Concentration of β-carotene was significantly lower in Caco-2/HT29-MTX co-cultures cell lysates (≥0.74%), with no significant differences between both emulsifiers.
The reason why NaCas nanoemulsions had less β-carotene in the micellar fraction, the apical sample at time zero and the cell lysate (Caco-2) may be explained by the fact that proteins such as NaCas, can interact hydrophobically with carotenoids and create complexes that act as a physical barrier for gastrointestinal digestive enzymes access (Wackerbarth, Stoll, Gebken, Pelters, & Bindrich, 2009). Hence, β-carotene may have remained entrapped within these complexes and not solubilised within mixed micelles, a process which is essential for permeability across the intestinal barrier (Baskaran, Sugawara, & Nagao, 2003). Moreover, Yang, Decker, Xiao, & McClements (2015) observed that the addition of 36 mg phospholipids (eg. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine) within the digestive fluids increased the degree of lipid digestion after *in vitro* GID of vitamin-E emulsions. This may explain why lecithin-nanoemulsions had higher concentrations of β-carotene within the micellar fraction. Another study observed that the maximum cellular uptake of carotenoids (β-carotene and lutein) in differentiated Caco-2 cells was obtained when micelles contained 50 µmol/L of lysophosphatidylcholine (phospholipid derived from phosphatidylcholine present in lecithin) (Sugawara et al., 2001).

In agreement with the present study, Li. Arranz, Guri & Corredig (2017) reported a lower permeability of β-carotene from liposomes using 21-day old Caco-2/HT29-MTX co-cultures compared to Caco-2 monolayers. Interaction with mucus produced by HT29-MTX cell line reduces permeability of mucoadhesive lipophilic molecules, such as β-carotene (Sigurdsson, Kirch, & Lehr, 2013). Co-culturing Caco-2 cells with HT-29MTX adds a further layer of mucus complexity to more closely resemble the *in vivo* environment (Arranz. Corredig & Guri, 2016), but reduces permeability rates which may hamper compound detection. Thus, in our study, the use of Caco-2/HT29-MTX cell line resulted in lower recovery of β-carotene in the cell lysates regardless of the emulsifier.

The failure to detect β-carotene in basolateral compartment underlined the limitations of the experiment due to upper concentration limits imposed by cytotoxicity data and inadequate sensitivity of detection instrumentation. Also, β-carotene may not have arrived at the basolateral within the 2 h incubation period.

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Table 2. β-carotene concentration (ng/mL) quantified by high-performance liquid chromatography (HPLC-PDA) in micellar fractions (after *in vitro* digestion), apical samples and basolateral samples from absorption experiments after 2h incubation with micellar fractions obtained from an *in vitro* digestion of nanoemulsions stabilised with lecithin (LE) and sodium caseinate (NaCas). ND = not detected, i.e., below detection limit of 10 ng/ml. Within a row, different lowercase letters indicate statistically significant differences ($p<0.05$) between emulsifiers. For an emulsifier, a statistical difference between apical t=0 and apical t=2h is denoted by *. Statistical analysis was performed using one-way ANOVA followed by Bonferroni test (*$p<0.05$).

	Caco-2 model (ng/mL)		**Caco-2/HT29-MTX model (ng/mL)**	
Emulsifier	**LE**	**NaCas**	**LE**	**NaCas**
Micellar fraction	3643 ± 665^{a}	32858 ± 70^{b}	37370 ± 521^{a}	32759 ± 102^{b}
Apical t=0h	$2171 \pm 39^{a*}$	$1930 \pm 4^{b*}$	$2198 \pm 40^{a*}$	$1927 \pm 6^{b*}$
Apical t=2h	251 ± 14^{a}	339 ± 65^{b}	263 ± 36^{a}	365 ± 44^{b}
Cells	49.6 ± 5.4^{a}	33.2 ± 8.4^{b}	15.1 ± 5.7^{c}	18.1 ± 11.2^{c}
Basolateral	ND	ND	ND	ND

3.4. Immune functionality of basolateral samples

Basolateral samples from permeability assays with β-carotene NaCas nanoemulsions and β-carotene lecithin nanoemulsions significantly increased TNF-α secretion (112% and 124% respectively, $p<0.05$) compared to basolateral sample control (positive control =2291.2±138.5 pg/mL of TNF-α) from LPS activated THP-1 macrophages (Fig. 3A). However, IL-1β levels were unchanged regardless of basolateral samples (4769.4±145.3 pg/mL) (Fig. 3B).

Previous studies have demonstrated that β-carotene reduces levels of TNF-α and IL-1β levels secreted from LPS-stimulated RAW264.7 cells (murine macrophage cell line) and from LPS-treated peritoneal macrophages (Li, Hong, & Zheng, 2019) as well as from serum of BALB/c mice, intraperitoneally injected with β-carotene (10 mg/kg) plus LPS (4 mg/kg) (Bai et al., 2005). This discrepancy with our results may be explained by the bypass of the gut and the use of different test material (β-carotene alone versus basolateral samples of Caco-2 monolayers treated with β-carotene-enriched nanoemulsions). Applying β-carotene directly to LPS stimulated THP-1 cells will dose dependently reduce secreted levels of TNF-α (data not shown). It is important to note that our results do not confirm the presence of β-carotene or metabolites in the basolateral compartment. It is possible that other GID components in the micellar fraction may be capable of modulating TNF-α, although previous studies have shown that lecithin, sodium caseinate or emulsions with different fatty acid composition do not up-regulate cytokine production (Mukhopadhya et al., 2014, Reimund et al., 2004, Treede et al., 2009). However, β-carotene can be metabolised to high molecular weight products (β-apo-8'-carotenal, β-apo-10'-carotenal, β-apo-12'-carotenal, β-apo-14'-carotenal, β-apo-15'-carotenal) and short-chain products (hcyclocitral, β-ionone, ionene, 5,6-epoxi-β-ionone, dihydroactinidiolide and 4-oxo-ionone) (Siems et al., 2005), which themselves may directly or indirectly act as pro-inflammatory agents (Yeh, Wang, Chen, & Wu, 2009).

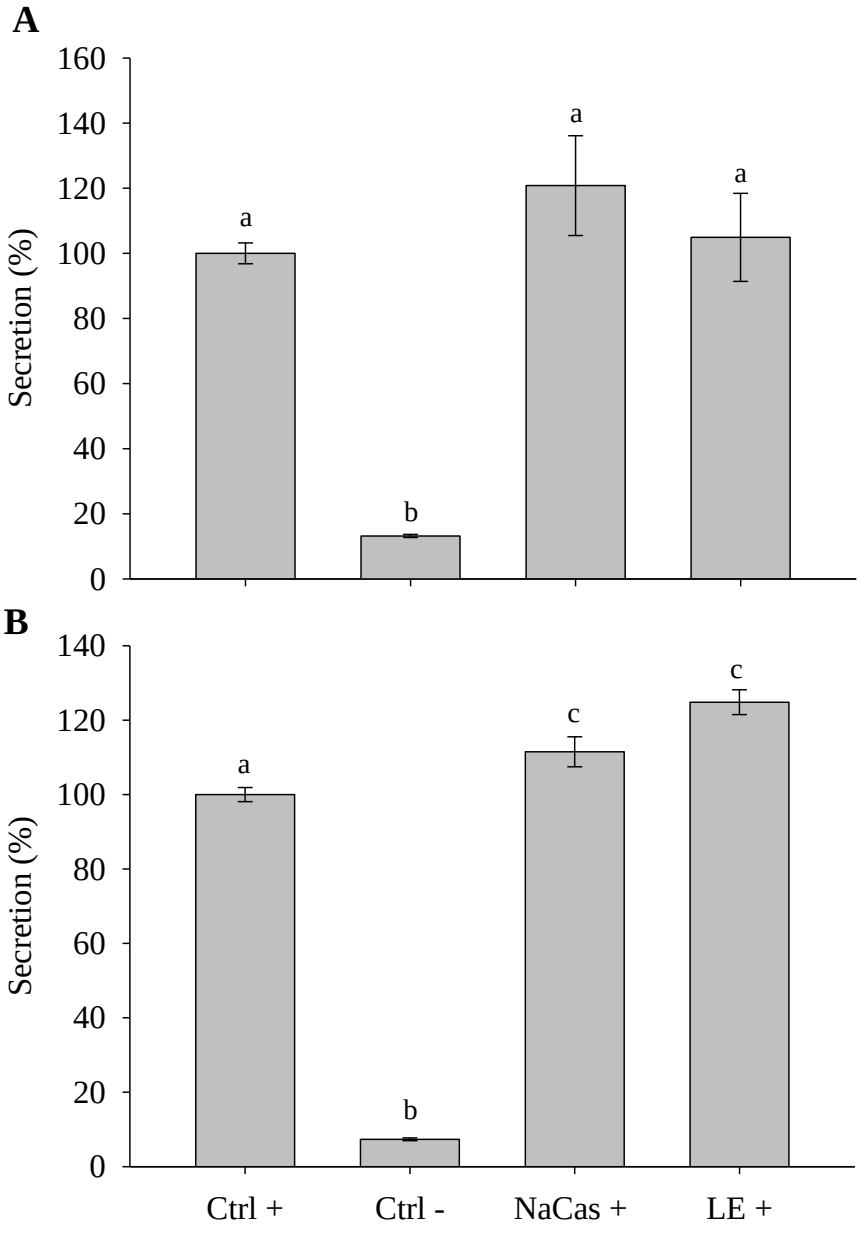

Figure 3. Effects of basolateral samples resulted from permeability experiments with β-carotene-enriched nanoemulsions emulsified with either 2% sodium caseinate (NaCas+) or lecithin (LE+) on the secretion (% ± SEM) of TNF-α (A) and IL-1β (B) in lipopolysaccharide (LPS)-simulated THP-1 cells. Positive controls (Ctrl +) were LPS-stimulated THP-1 cells and negative controls (Ctrl -) were non-stimulated THP-1 cells. Both controls were incubated with basolateral samples collected from control Caco-2 monolayers. Different letters indicate significant differences. Statistical analysis was performed using one-way ANOVA followed by Bonferroni test (*$p < 0.05$).

4. Conclusions

Bioaccessibility of GID β-carotene in a Caco-2 model was enhanced when β-carotene-enriched nanoemulsions were stabilised with lecithin compared to those stabilised with NaCas. Caco-2 basolateral samples from both nanoemulsions elicited a TNF-α response from stimulated THP-1 macrophages. This study elucidates the importance of nanoemulsion composition for *in vitro* cellular permeability assays and the hurdles faced by concentration limits. Nanostructured food systems using lecithin as emulsifier might be a potential tool to increase uptake of dietary β-carotene.

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CHAPTER III
Influence of mandarin fiber addition on physico-chemical properties of nanoemulsions containing β-carotene under simulated gastrointestinal digestion conditions

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Abstract

There is a lack of knowledge about how soluble fiber used as stabilizer may influence physicochemical properties of nanoemulsions during the lipid digestion process. In this study, different concentrations of mandarin fiber (0.5, 1.0, 1.5, 2.0 g/100 g) were added to nanoemulsions containing oil enriched with β-carotene (4 g/100 g emulsion) and Tween 20 (1.5 g/100 g emulsion). As nanoemulsions were subjected to the different phases of an *in vitro* simulated gastrointestinal tract (GIT), its particle size was gradually increased. Furthermore, the higher the mandarin fiber content in the nanoemulsion, the greater the particle size. Nanoemulsions containing concentrations of mandarin fiber over 1.5 g/100 g showed the lowest ζ-potential, meaning that the droplets may become unstable and were likely to aggregate. Besides, adding until 1 g of mandarin fiber/100 g was effective to enhance bioaccessibility of the β-carotene incorporated in nanoemulsions. The present study provides valuable information on the phenomenology of incorporating mandarin fiber within β-carotene-enriched nanoemulsions.

Keywords: mandarin fiber; nanoemulsion; β-carotene, *in vitro* digestion, bioaccessibility

1. Introduction

Carotenoids are natural lipophilic pigments found in many natural sources, like fruits and vegetables, with β-carotene being one of the most important for its pro-vitamin A activity (Ferreira & Rodriguez-Amaya, 2008; Yonekura & Nagao, 2007). Protecting cells from free radicals (Kiokias & Gordon, 2004; Maiani et al., 2009), and preventing several chronic diseases, such as cancer, heart disease and aging (Wang, Liu, Mei, Nakajima, & Yin, 2012), are some of the attributed features to β-carotene owing to its strong antioxidant activity. However, incorporating β-carotene in foods can be challenging due to its low water-solubility but also poor stability (Boon et al., 2010). An outstanding technology based on nanoemulsion delivery systems as a way to encapsulate and protect lipophilic compounds dispersed in aqueous media could be a possible method to design healthier foodstuffs. The small droplets of nanoemulsions present advantages such as physical stability, improvement of optical quality and increased bioaccessibility of lipophilic compounds (Nik, Langmaid, & Wright, 2012). Preventing nanoemulsions from structure breakdown can be possible by incorporating stabilizers (Odriozola-Serrano, Oms-Oliu, & Martín-Belloso, 2014). Dietary fibers (DF) are important components of the human diet that are being applied in emulsion-based foods thanks to their stabilizing, texturizing properties, as well as health-promoting ingredients (Dikeman & Fahey, 2006; Eastwood & Morris, 1992; Elleuch et al., 2011). On one hand, chronic illnesses like diabetes or obesity besides coronary heart and gastrointestinal diseases are linked to a low intake of DF (Anderson et al., 2009). On the other hand, its consumption in certain amounts may inhibit lipid absorption (Jenkins, Kendall, & Ransom, 1998; Lairon, 1996; Torcello-Gómez & Foster, 2016; Yokoyama et al., 2011), causing a decrease on the bioaccessibility of health-related compounds easily absorbed when lipids are present (Chawla & Patil, 2010). DF can be found not only in fruits and vegetables, but can also be obtained from fruit by-products, and mandarin fiber is especially rich in DF soluble fraction and thus, a feasible option to be used as a emulsion stabilizer. Hence, the purpose of this work was to analyze the effect of mandarin fiber on lipid digestibility of oil-in-water nanoemulsions containing β-carotene by subjecting them through an *in vitro* simulated GIT. Changes in the physicochemical properties of nanoemulsions during digestion were also characterized.

2. Materials and methods

2.1. Materials

β-carotene, Tween 20, mucin (from porcine stomach), pepsin (from porcine gastric mucosa), lipase (from porcine pancreas), bile extract (porcine) and all the solvents were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Corn oil was from a local supermarket. Mandarin fiber was kindly donated by Indulleida S.A. (Alguaire, Spain). Its proximate analysis was provided by the manufacturer and is presented in Table 1. Milli-Q water was used to prepare emulsions and reagents of the experiment.

Table 1. Proximate analysis of mandarin fiber.

Composition	**(g/kg)**
Soluble fiber (pectin)	289.10
Total carbohydrate	531.28
Protein	101.87
Total fat	9.70
Ashes	37.12

2.2. Methods

2.2.1. Nanoemulsions preparation

Firstly, β-carotene was dispersed in corn oil (0.5 g/100 g) by sonicating (1 min) and heating (<50 °C, 5 min) to obtain the lipid phase. Then, mandarin fiber at different concentrations (0.5, 1.0, 1.5 or 2.0 g/100 g) was solubilized in Milli-Q water using a homogenizer (Ultra-Turrax, Janke & Kunkel, Staufen, Germany) at 9500 rpm for 5 min, obtaining the aqueous phase. Once both phases were ready, a coarse emulsion was prepared by putting together 4.0 g lipid phase, 1.5 g Tween 20 and 94.5 g aqueous phase per 100 g of emulsion, and mixing with a homogenizer (9500 rpm, 2 min). Lastly, a microfluidizer (M-110P, Microfluidics, Newton, MA, USA), equipped with a 75 μm ceramic interaction chamber (F20Y) at an operational pressure of 30,000 psi, was used to form the nanoemulsions by passing the coarse emulsions for 5 cycles.

The main goal of the experiment was to study the mandarin fiber addition effect on the physicochemical properties along an *in vitro* GIT, as well as, lipid digestibility and β-carotene bioaccessibility. Finally, it should be noted that the specific effect of the microfluidization process was studied by preparing the coarse emulsion and nanoemulsion without mandarin fiber, so as to determine the influence of particle size on β-carotene bioaccessibility. The term "emulsions" has been used through the text to involve all nanoemulsions and coarse emulsions, irrespective of their composition.

2.2.2. *In vitro* digestion

An *in vitro* gastrointestinal tract (GIT) was used to mimic the digestion process, adapting the proceeding proposed by Salvia-Trujillo, Qian, Martín-Belloso, & McClements, (2013).

For mouth stage, 50 mL of simulated saliva fluid, which contained mucin (0.03 g/mL) and various salts (Sarkar, Goh, & Singh, 2009), was mixed with 50 mL of emulsion, adjusting its pH to 6.8. Finally, it was incubated in an orbital shaker for 10 min at 100 rpm and 37 °C. Following, the liquid proceeding from the mouth phase was mixed with gastric fluids (Sarkar, Goh, Singh, & Singh, 2009) at the same volume ratio to simulate the gastric stage. The pH of the mixture was adjusted to 2.5 and placed inside an orbital shaker at 37 °C for 2 h with a constant agitation (100 rpm). The gastric fluids were prepared by adding 2 g of NaCl, 7 mL of HCl and 0.0032 mg/mL of pepsin in 1 L of Milli-Q water. Finally, to simulate intestinal stage a pH-stat (Metrohm USA Inc., Riverview, FL, USA) was used (McClements & Li, 2010). The sample proceeding from the gastric phase was placed in a water bath at 37 °C. Solutions prepared using phosphate buffer (0.005 mol/L, pH 7) consisting of 9.3 mL of bile extract (46.87 mg/mL) and 1.0 mL of calcium chloride (110 mg/mL), were added to the sample followed by an adjustment of pH to 7.0. Afterwards, 2.5 mL of lipase (24 mg/mL) dissolved in phosphate buffer was also added. To compensate the free fatty acids (FFAs) that were released during the lipid digestion, the pH was constantly maintained at 7.0 by adding dropwise a NaOH solution. The volume of NaOH was recorded after 2 h, employed to calculate the lipid digestibility defined by the production of FFA (%) in the intestinal phase, using equation (1).

$$\text{FFA (\%)} = \frac{V_{NaOH} \times C_{NaOH} \times M_{oil}}{2 \times m_{oil}} \times 100 \qquad (1)$$

Where V_{NaOH} is NaOH volume (mL) used to compensate the FFAs during the digestion, C_{NaOH} is NaOH molarity (0.25 mol/L), M_{oil} is corn oil molecular weight (800 g/mol), m_{oil} is corn oil total weight present in the emulsions (g).

2.2.3. Physicochemical and rheological properties

The physicochemical properties of emulsions before and during the different stages of the *in vitro* GIT (mouth, stomach, intestine) were determined with respect to droplet size, electrical charge and viscosity.

To determine nanoemulsions droplet size, a Zetasizer NanoZS (Malvern Instruments Ltd, Worcerstershire, UK) was used, working at 633 nm and 25 °C, equipped with a backscatter detector. This device performs size measurements using a process called Dynamic Light Scattering (DLS), which measures Brownian motion and related it to the size of the particles. Prior to analysis, emulsions were diluted (1/10) in the appropriate solutions: saliva fluids for the mouth phase, gastric fluids for the stomach phase and buffer phosphate (pH 7) for the intestinal phase. Meanwhile, the droplet size of the coarse emulsion without mandarin fiber was determined using a Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK), reporting the particle size as the surface area mean diameter (d_{32}). The Mie theory is employed for calculate the size of emulsions, fixing a refractive index of the corn oil of 1.473 and 1.333 for the water.

The emulsions electrical charge (ζ-potential) was determined using a Zetasizer NanoZS (Malvern Instruments Ltd, Worcerstershire, UK). Emulsions were previously diluted (1/10) as in the droplet size tests.
The viscosity was studied by a SV-10 vibro-viscometer (A&D Company, Tokyo, Japan).

2.2.4. Carotenoid extraction and quantification

To extract and quantify the carotenoids present in the emulsions, the method described by Morales-De La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, (2011) was used with some changes. Fifteen grams of emulsions were put together with magnesium hydroxide carbonate, butylated hydroxytoluene (BHT) and ethanol-hexane solution (4:3 v/v) under N_2 atmosphere. After agitation, the liquid was filtered under vacuum, washed with ethanol-hexane solution (4:3 v/v) and filtered with solvents. Then, all filtrates were washed with 100 g/L sodium chloride solution and water. The organic part was evaporated in a rotary evaporator and the residue was put under N_2 atmosphere, adding diethyl ether (DE) and methanolic KOH (0.5 mol/L) with BHT (1 g/L). Then, DE was added again and the solution was evaporated until dryness. The residue was dissolved by adding DE, evaporated it under N_2 and stored at −18 °C until the chromatographic analysis. Before injecting into the HPLC, the carotenoid extract was reconstituted with 1 mL of methanol:*tert*-butyl methyl ether solution (70:30 v/v). β-carotene was identified using a reverse-phase C18 Spherisorb ODS2 (5 μm) stainless steel column (4.6 mm 250 mm), with the need of a gradient elution in order to separate β-carotene. The mobile phase consists of methanol/ammonium acetate 0.1 mol/L, milli-Q water, methyl *tert*-butyl ether and methanol. The flow rate was established at 1 mL/min during 60 min and the column was fixed at 30 °C. UV-vis spectral data and their retention times were used to determine the β-carotene present in the vials (Cortés, Esteve, Frígola, & Torregrosa, 2004; Mouly, Gaydou, & Corsetti, 1999) being quantified by comparing them with external β-carotene standards.

2.2.5. Bioaccessibility determination

A fraction resulting from the intestinal phase was centrifuged (AVANTI J-25, Beckman Instruments Inc., Fullerton, CA, USA) at 4000 rpm for 40 min at 4 °C (Qian et al., 2012). The upper part of the resulted liquid was collected and considered being the micelle fraction in which the β-carotene was present. In some samples, a layer of non-digested oil could be observed in the top, not considering it. Lastly, the β-carotene bioaccessibility was calculated using equation (2).

$$\text{Bioaccessibility}(\%) = \frac{C_{micelle}}{C_{initial}} \times 100 \qquad (2)$$

where $C_{micelle}$ is the carotenoid concentration of the micelle fraction and $C_{initial}$ the initial carotenoid concentration of emulsion.

The carotenoid concentration present in the micelle fraction was determined following the procedure explained above (2.2.4).

2.2.6. Statistical analysis

All tests were evaluated in duplicate while three replicate determinations were measured for each parameter. Interpretation of the variance (ANOVA) was examined using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md, USA).

3. Results and discussion

3.1. Particle size

In the absence of mandarin fiber, the particle size of the coarse emulsion was 19200.0 ± 1686.4nm, and it was efficiently lowered to 166.7 ± 8.1 nm, after microfluidization (Table 2, Figure 1). Microfluidization treatment consists of pressurising the emulsions by passing them through an interaction chamber which provokes impact forces and cavitation phenomena to reduce the emulsions droplet size (Maa & Hsu, 1999). As can be seen in Figure 1, the mean particle size of initial nanoemulsions when mandarin fiber was added did not appreciably change, but statistically significant differences existed when they contained more than 1.5 g of mandarin fiber/100 g of nanoemulsion compared to those with less mandarin fiber concentration. It is known that fiber is not attracted to the surfaces of non-ionic surfactants due to electrostatic and steric repulsion forces. Therefore, the increase of particle size in initial nanoemulsions containing high concentration of mandarin fiber can be attributed to the creation of an osmotic attraction between droplets (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, Narváez-Cuenca, & McClements, 2014) due to the exclusion of non-absorbed mandarin fiber from the droplets surface.

Table 2. Mean particle size of initial coarse emulsion during the different stages of the *in vitro* gastrointestinal tract (GIT).

Phase digestion	Mean particle size (nm)
Initial	19200.0 ± 1686.4a
Mouth	19850.0 ± 1456.7a
Stomach	22860.0 ± 7895.5b
Intestine	28400.0 ± 7185.5b

Different letters mean significant differences of particle size along digestion phases ($p < 0.05$).

During mouth and stomach phases, nanoemulsions droplets were quite resistant to coalescence and breakdown because they presented similar size to initial nanoemulsions (Figure 1). In the present work, nanoemulsions were stabilized using Tween 20, a non-ionic surfactant defined to

be highly surface-active because of the hydrophilic head group that form the molecule. Surface-active substances were present in the oral and gastric fluids, and so the surfactant incorporated seemed to rest at the droplets surface (Qian et al., 2012). After the intestinal phase, all the nanoemulsions increased of particle size up to 305.8 nm, indicating that flocculation or coalescence phenomena occurred. In agreement with our results, other authors concluded that nanoemulsions droplets were more susceptible to coalescence during the intestinal phase, the stage where the lipid digestion takes place (Salvia-Trujillo et al., 2013). Moreover, other surface-active substances such as phospholipids, bile salts and lipase could have displaced Tween 20 molecules initially present in the droplets surface, bringing out a reduce on droplet stability. However, nanoemulsions with 0.5 and 1.0 g of mandarin fiber/100 g presented lower mean particle size in the intestinal phase compared to those nanoemulsions with a higher concentration of fiber. High pectin concentration from the mandarin fiber may have formed hydrogel structures when calcium ions are present in the intestine.

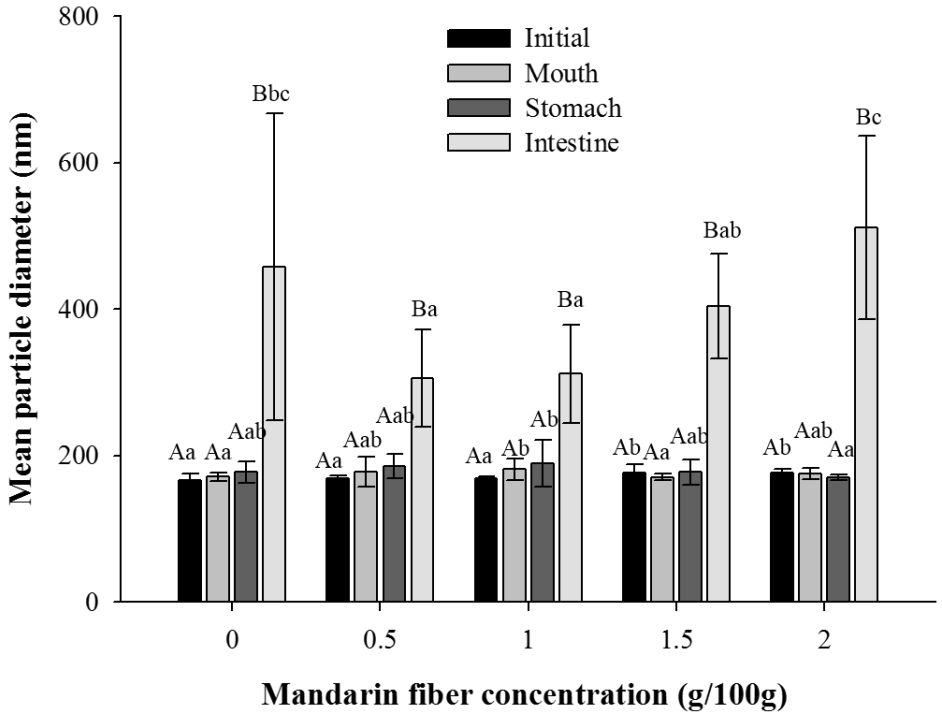

Figure 1. The impact of the *in vitro* digestion phases (initial, mouth, stomach, intestine) on the droplet size of nanoemulsions with β-carotene containing different concentrations of mandarin fiber (g/100 g). Equal capital letters indicate no significant differences ($p < 0.05$) of nanoemulsions droplet size during digestion phases. Equal lowercase letters indicate no significant differences ($p < 0.05$) of the droplet size among nanoemulsions within the same digestion phase. The values are the mean values of two experiments with standard deviation error bars.

The mean particle size of the coarse emulsion without mandarin fiber changed significantly after stomach and intestinal phases, reaching values of 22860 and 28400 nm, respectively (Table 2). It might be expected that lipid digestion reduces droplets size, but droplet aggregation can occur as a consequence of changes in interfacial characteristics (Ozturk, Argin, Ozilgen, & McClements, 2015). Indeed, this droplet aggregation could lead to an increase of particle size, explained by the formation of micelles, undigested lipids droplets, insoluble calcium soaps, among other particles (Yao, Xiao, & McClements, 2014; Zou et al., 2016).

3.2. Electrical charge

Emulsions had negative surface charges with values ranging between −16.8 and −25.8 mV (Figure 2). The surfactant utilized (Tween 20) would not be supposed to confer any charge to the droplets. However, some studies have concluded that non-ionic surfactants tended to have a considerable negative charge thereby the presence of free fatty acids not only from the oil but also from the surfactant, as well as the adsorption of hydroxyl ions (OH^-) present in the aqueous phase of emulsions (Chang & McClements, 2016; Mun, Decker, & McClements, 2005). The addition of high amounts of negatively charged mandarin fiber in the emulsions (≥1.5 g/100 g), results in a decrease in negative repulsive forces between oil droplets. Competitive adsorption of mandarin fiber molecules and other species (hydroxyl ions, anionic impurities or free fatty acids) combined with compounds present in the emulsions, could have induced a neutralization of the electrical charge values.

The electrical charge along the GIT was similar in all emulsions. Generally, the emulsions ζ-potential after the exposure to the mouth had similar values to the initial emulsions (Figure 2). Other authors have suggested that an appreciable increase of the negative charge could be caused by the absorption of mucin molecules negatively charged present in the saliva fluids to the droplet surfaces (Sarkar et al., 2009). The particles' electrical charges after the stomach phase were less negative (between −4.3 and −7.3 mV) than those obtained after mouth phase. The salts present in the water of emulsions when they are in the stomach phase are known to screen electrostatic interactions and consequently increase the ζ-potential (Mun et al., 2006). Meanwhile, the ζ-potential of all emulsions after the intestinal phase was significantly more negative, reaching electrical charges like those of the mouth and initial phases. This tendency could be attributed to the presence of surface-active anionic substances at droplets surfaces like bile salts and phospholipids from intestinal fluids or free fatty acids formed during lipid digestion (Pouton & Porter, 2008; Reis et al., 2009; Singh et al., 2009). In addition, nanoemulsions with ≥1 g of mandarin fiber/100 g exhibited values of ζ-potential less negative in gastric and intestinal phases than those nanoemulsions with fewer mandarin fiber concentration. These results suggest that mandarin fiber, which is rich in pectin, might associate with the oil droplets by forming electrostatic interactions under a gastric environment (Espinal-Ruiz et al., 2014), which can be due to the less negative charge of the droplets and mandarin fiber at pH 2.5.

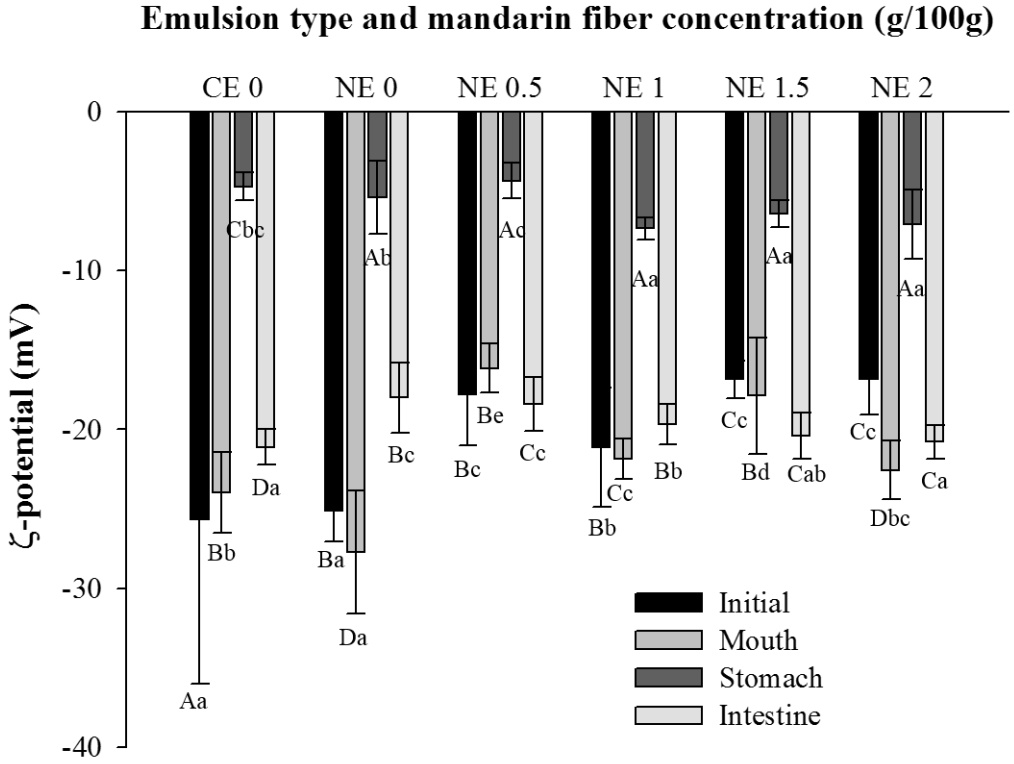

Figure 2. Influence of *in vitro* digestion phases (initial, mouth, stomach, intestine) on the ζ-potential of coarse emulsion without mandarin fiber (CE 0) and nanoemulsions (NE) containing different concentrations of mandarin fiber (g/100 g). Equal capital letters indicate no significant differences ($p < 0.05$) of the ζ-potential of an emulsion during digestion phases. Equal lowercase letters indicate no significant differences ($p < 0.05$) of the ζ-potential among emulsion types within the same digestion phase. The values are the mean values of two experiments with standard deviation error bars.

3.3. Viscosity

Regarding viscosity, no significant differences were noted between the initial coarse emulsion (1.04 mPa·s) and its nanoemulsion (1.08 mPa·s), both without mandarin fiber. Adding to nanoemulsions 1 and 2 g of mandarin fiber/100 g increased significantly its viscosity. The higher the concentration of mandarin fiber, the greater the viscosity of the emulsions was, reaching values of 9.11 mPa·s in nanoemulsions containing 2 g of mandarin fiber/100 g (Figure 3). Mandarin fiber is rich in soluble fibers and has been applied in food and beverage industry during many years as thickening and gelling agent, as well as colloidal stabilizer. Actually, this type of components have several unique properties that capacitate them to be used as a matrix for the entrapment and/or delivery of different substances (Sáenz, Estévez, & Sanhueza, 2007).

Emulsions presented similar viscosity throughout the GIT, regardless of the mandarin fiber concentration (Figure 3). Nanoemulsions with lower amount of 0.5 g of mandarin fiber/100 g and coarse emulsions without mandarin fiber presented the highest viscosity after the mouth phase because of the mucin addition. Otherwise, nanoemulsions containing over 0.5 g of mandarin fiber/100 g presented the lowest viscosity under simulated intestinal conditions. Generally, viscosity tended to decrease as nanoemulsions passed through the GIT, which can be due to the progressive dilution that occurs when in every phase of the GIT the relevant fluids are added.

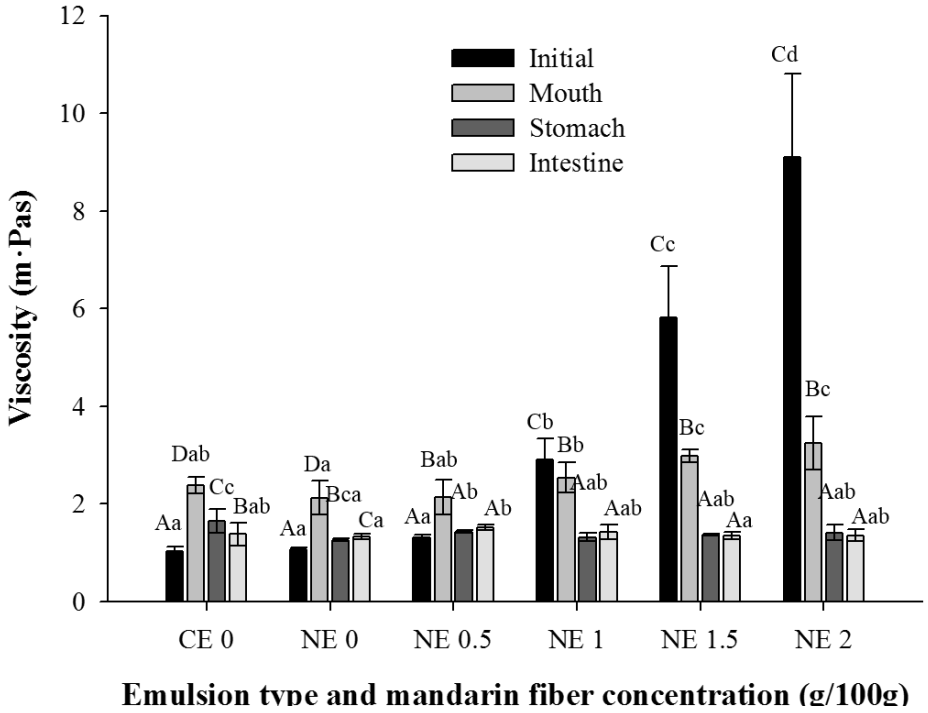

Figure 3. Influence of *in vitro* digestion phases (initial, mouth, stomach, intestine) of coarse emulsion without mandarin fiber (CE 0) and nanoemulsions (NE) containing different concentrations of mandarin fiber (g/100 g). Equal capital letters indicate no significant differences ($p < 0.05$) of the viscosity of an emulsion during digestion phases Equal lowercase letters indicate no significant differences ($p < 0.05$) of the viscosity among emulsion types within the same digestion phase. The values are the mean values of two experiments with standard deviation error bars.

3.4. Oil digestibility

In general, it was observed an initially rapid increase in the total free fatty acids (FFA) released from emulsions throughout the first minutes of intestinal phase digestion, followed by a

progressive increment until the end. Accordingly, other authors reported that the major amount of the corn oil in emulsions with pectin was digested within the first 10 min of intestinal phase (Zhang et al., 2015).

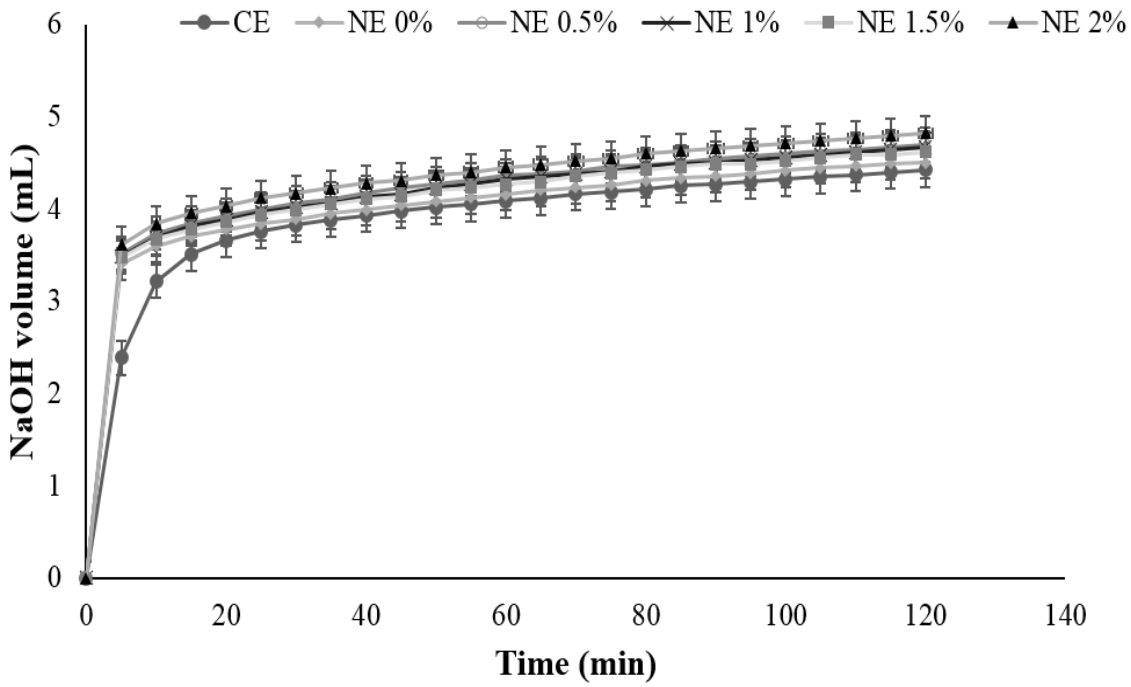

Figure 4. Impact of emulsion droplet size (coarse emulsion and nanoemulsion) and mandarin fiber concentration (g/100 g) on the volume (mL) of NaOH during the intestinal phase to maintain the pH to 7. Equal capital letters indicate no significant differences ($p < 0.05$) of NaOH volume used to maintain the pH 7 among emulsion types. (CE: coarse emulsion without mandarin fiber; NE 0: nanoemulsion without mandarin fiber; NE 0.5: nanoemulsion with 0.5 g mandarin fiber/100 g; NE 1: nanoemulsion with 1 g mandarin fiber/100 g; NE 1.5: nanoemulsion with 1.5 g mandarin fiber/100 g; NE 2 : nanoemulsion with 2 g mandarin fiber/100 g). Error bars are based on the standard deviation of two experiments

In Figure 4 it can be appreciated that the initial rate along the first 15 min of intestinal phase was higher in nanoemulsions than in coarse emulsions, irrespective of their composition. However, statistically significant differences only existed during the first 5 min of the process. Surface area of oil droplets exposed to lipase changed according to particle size of emulsions. Hence, nanoemulsions would favour the contact between the oil droplets and lipase, promoting the digestibility of the oil droplets. Nevertheless, the total number of FFAs released after 2 h of intestinal digestion was similar for coarse emulsion and nanoemulsions (74.7 and 74.6%, respectively), both without mandarin fiber.

Adding mandarin fiber to nanoemulsions produced a decrease of FFAs realised during the intestinal digestion, with values ranging from 59.6% to 62.2% (Figure 5). Espinal-Ruiz et al. (2014) reported a depletion in the rate and extent of lipid digestion when high concentrations of hydrocolloids such us pectin are present, resulting in a decrease in the total FFAs. In the present study, nanoemulsions were prepared with mandarin fiber, which contains soluble pectin, as it is shown in Table 1. The presence of pectin in nanoemulsions could possibly affect lipid digestion: (i) binding free calcium ions thereby reducing the amount available to remove long chain FFAs from droplet surfaces; (ii) forming a coating round the droplets, by which could inhibit the access of lipase to the lipid droplets; (iii) aqueous phase viscosity may increase in consequence of mixing and/or diffusion processes; (iv) influence the way of lipase to the oil droplets as a result of modified floc formation and structure (Zhang et al., 2015).

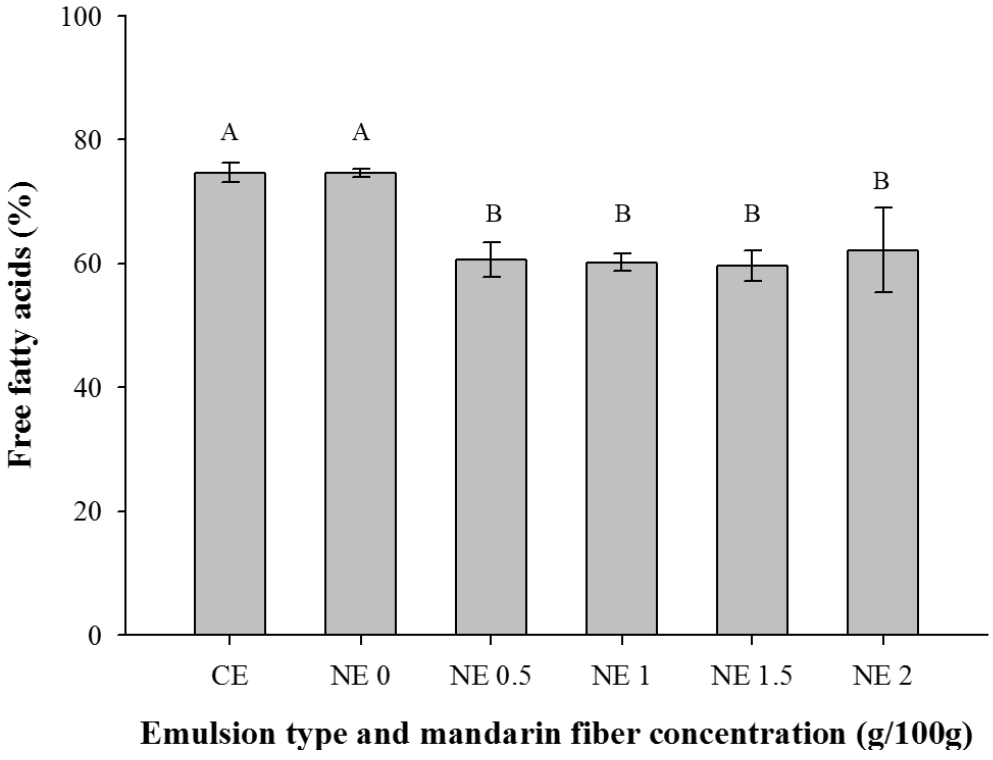

Figure 5. Total free fatty acids (FFA) produced in the intestinal phase of the coarse emulsion without mandarin fiber (CE 0) and nanoemulsions (NE) containing different concentrations of mandarin fiber (g/100 g). Equal capital letters indicate no significant differences ($p < 0.05$) of the oil digestibility among emulsion types. Error bars are based on the standard deviation of two experiments.

3.5. β-carotene bioaccessibility

The bioaccessibility of β-carotene was found to significantly increase when decreasing the droplet size of emulsions. In this sense, β-carotene bioaccessibility increased from 24% in coarse emulsion up to 33% in nanoemulsion, both without mandarin fiber (Figure 6). In the same way, Salvia-Trujillo et al. (2013) observed that the β-carotene bioaccessibility of coarse emulsion was lower than in nanoemulsions, presenting values of 34% and 59%, respectively. For the latter, spectrophotometer was used in the β-carotene quantification which could be the main cause of the different bioaccessibility results.

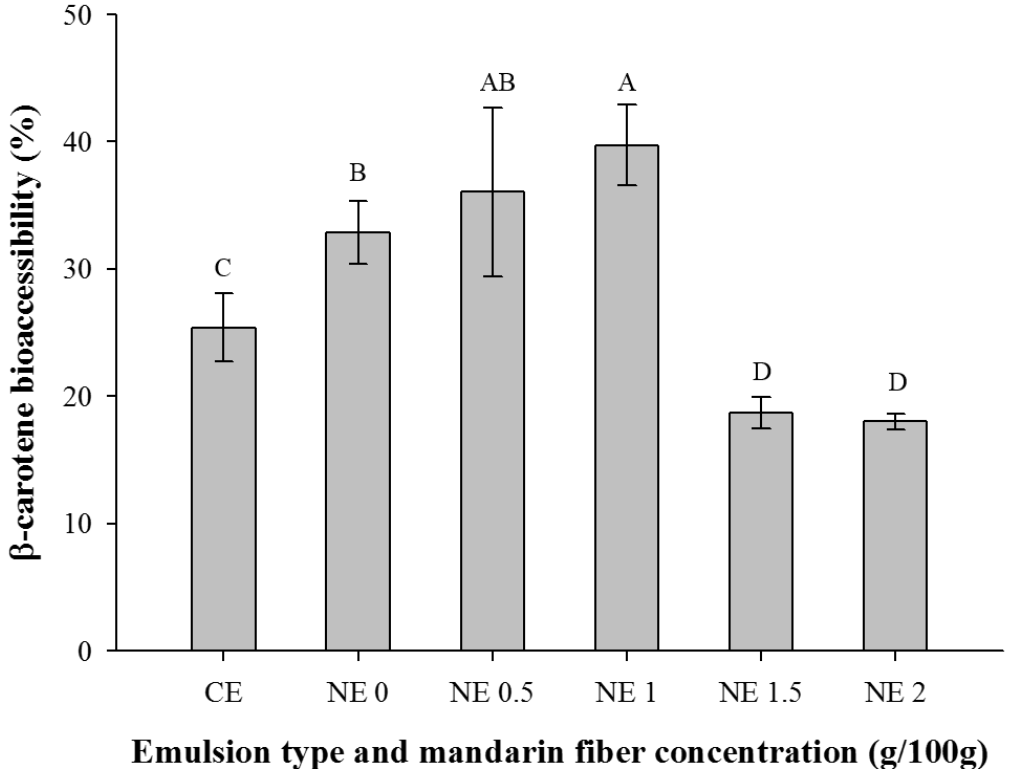

Figure 6. β-carotene bioaccessibility (%) of coarse emulsion without mandarin fiber (CE 0) and nanoemulsions (NE) containing different concentrations of mandarin fiber (g/100 g). Equal capital letters indicate no significant differences ($p < 0.05$) of β-carotene bioaccessibility between emulsion types. Error bars are based on the standard deviation of two experiments.

Particle size as well as digestibility would be supposed to have influence over bioavailability of encapsulated lipophilic nutraceuticals (McClements & Xiao, 2012). Besides, bioactive components present in coarse emulsions may be captured within the non-digested lipid fraction and consequently not be released. Furthermore, substances produced as a result of the digestion such as free fatty acids and monoacylglycerols, can form mixed micelles that can solubilize and

transport lipophilic components to the enterocytes where they are absorbed (Cho et al., 2014). In our study, coarse emulsions and nanoemulsions without mandarin fiber showed similar digestibility, thus the number of mixed micelles present to solubilize the β-carotene should be similar. The low β-carotene bioaccessibility of coarse emulsions could be associated to the changes produced in the particles structure after lipid digestion, changing their solubilizing capacity (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013). As can be seen in Figure 6, bioaccessibility of β-carotene tended to increase with the addition until 1 g of mandarin fiber/100 g nanoemulsion, but higher concentrations led to lower bioaccessibility levels, being reduced until 18.7% and 18.0% for nanoemulsions containing 1.5 and 2 g of mandarin fiber/100 g respectively, without statistical difference between them. These results can be explained by rheological changes in the nanoemulsions when adding high concentrations of mandarin fiber (≥1 g mandarin fiber/100 g nanoemulsion). Actually, the presence of a certain amount of fiber could affect the absorption of carotenoids (Riedl, Linseisen, Hoffmann, & Wolfram, 1999). In this way, dietary fiber is supposed to decrease bioaccessibility by (i) hiding the contact with micelles and small intestine, (ii) interacting with bile salts and lipase (iii) and slowing down the transport of digestive enzymes because of the viscosity increase (Verrijssen et al., 2014).

4. Conclusions

Particle size and ζ-potential of nanoemulsions during the GIT were similar irrespective of the mandarin fiber content. Conversely, viscosity of nanoemulsions appreciably increased when mandarin fiber was present at high concentrations. Based on the results observed, emulsion droplet initial size has an important role within lipid digestion. In fact, the initial digestion rate during the intestinal phase of nanoemulsions was faster than coarse emulsion. However, adding mandarin fiber to nanoemulsions produced a decrease of FFAs released. Finally, β-carotene bioaccessibility of emulsions increased appreciably as the initial droplet size decreased and until 1 g of mandarin fiber/100 g emulsion was added. The present work provides important phenomenological information of nanoemulsions containing mandarin fiber when they are subjected to different gastrointestinal phases using an *in vitro* method. However, more studies are required to define whether the presence of mandarin fiber in nanoemulsions could influence the β-carotene absorption, by using *in vivo* models.

5. Acknowledgements

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CHAPTER IV:
Development, physical stability and bioaccessibility of β-carotene-enriched tertiary emulsions

Ariadna Gasa-Falcon, Alejandra Acevedo-Fani, Isabel Odriozola-Serrano, Gemma Oms-Oliu, Olga Martín-Belloso.

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Abstract

The aim of this study was to develop a β-carotene-enriched tertiary emulsion (lactoferrin/alginate/ε-poly-L-lysine). Then, its physical stability as well as β-carotene content under external stresses (temperature, pH and ionic strength changes) was evaluated. Furthermore, lipid digestibility and β-carotene bioaccessibility of primary, secondary and tertiary emulsions were determined. Tertiary emulsion underwent a substantial particle size increase up to 13 μm after extreme temperatures, acidic conditions and with salt addition. However, small particle size (0.41 μm) and negative ζ-potential (−43.4 mV) was observed at basic pH. In addition, β-carotene content in tertiary emulsions decreased only 40% when emulsions were subjected at temperatures ≤70 °C, in acidic conditions and below 0.3 M NaCl. After in *vitro* digestion, tertiary emulsion presented higher lipid digestibility (83.59 ± 11.81%) and β-carotene bioaccessibility (70.10 ± 5.26%) compared with primary and secondary emulsions. This study provides knowledge to understand the behaviour of β-carotene-loaded tertiary emulsions under different conditions, valuable for further application in foodstuffs.

Keywords: tertiary emulsions; β-carotene; stability, external stresses, bioaccessibility

1. Introduction

Among carotenoids, β-carotene is one of the most extensively studied because of its health-related benefits, including anti-inflammatory (Li, Hong, & Zheng, 2019) and antioxidant activities (Zhou et al., 2018). Nonetheless, it has been reported that β-carotene is degraded by several factors including light, oxygen and heat, among others (Boon et al., 2010). Furthermore, β-carotene incorporation to aqueous-based foods is hindered because of its low-water solubility. Several techniques have been used to overcome the solubility issue, including lipid-based nanocarriers (nanoemulsions, nano-structured phospholipid carriers), nature-inspired nanocarriers (caseins, cyclodextrins), special equipment-based nanocarriers (electrospinning, electrospraying), biopolymer particles (single or complexed biopolymer nanoparticles) and miscellaneous nanocarriers (nanoparticles made from chemical polymers, nano-structured surfactants) (Assadpour & Jafari, 2018). While a professional equipment is needed to produce the majority of these nanocarriers, a common and more available equipment can be used for producing nanoemulsions. Indeed, nanoemulsions have been proposed as a promising solution although they are highly reactive to external stimulations because of their nanometric particle size. Alternatively, multi-layer emulsions are oil-in-water emulsions containing oil droplets coated by at least two layers of polyelectrolytes. Because of the numerous layers lying around the oil droplets, multi-layer emulsions present improved physical stability over a wide range of conditions, including extreme pH, high ionic strength and thermal processing (Guzey & McClements, 2006). At the same time, the thick layer covering the oil droplets might provide chemical stability to encapsulated lipophilic compounds (Xu et al., 2016), as well as decrease lipid oxidation rates (Katsuda, McClements, Miglioranza, & Decker, 2008).

The assembly of layers is carried out following the layer-by-layer technique (LbL), in which polyelectrolytes with positive or negative charge are alternately deposited around the oil droplets (Bortnowska, 2015). Multi-layer emulsions fabrication is a delicate procedure as instability processes (e.g. bridging flocculation or depletion) could occur when an excess or lack of polyelectrolyte molecules is present (Guzey & McClements, 2006).

Literature reports multi-layer emulsions prepared with oil concentrations ranging from 0.09 to 2% w/w (Acevedo-Fani et al., 2017; Tokle, Mao, & McClements, 2013). Indeed, oil concentration is one critical point when designing multi-layer emulsions, since each layer deposition involves the dilution of the sample. When these systems are envisaged for delivering lipophilic bioactive compounds, the low oil concentration will allow incorporating only small amounts of bioactive compound within oil droplets. From a technical point of view, working with low oil concentrations in multi-layer emulsions makes difficult to assess their potential bioaccessibility after simulated *in vitro* digestions. Besides that, there is a controversy over whether the number layers covering multi-layer emulsions have an impact on lipolysis during *in vitro* digestion, thereby decreasing (Silva et al., 2018) or increasing (Silva et al., 2019) bioaccessibility of lipophilic compounds.

In terms of applicability, multi-layer emulsions can be employed as functional ingredients to improve nutritional quality of foods and beverages. However, processing and manufacturing conditions, as well as food characteristics might affect multi-layer emulsions properties. Thus,

investigating how different external conditions affects physical stability of these emulsions would provide information about in which type of foodstuffs would be better to use multi-layer emulsions.

Previous studies have demonstrated that coated emulsions with at least two layers of polyelectrolytes are physically more stable under external conditions than uncoated emulsions (Zhang et al., 2015). Thus, the main purpose of this work was to develop β-carotene-enriched tertiary emulsions (lactoferrin-alginate-ε-poly-L-lysine) with a high oil concentration (2.5% w/w). Then, the effect of external stressing conditions (temperature, pH, and ionic strength changes) on the physical stability as well as on the β-carotene content of the tertiary emulsion was investigated. In addition, lipid digestibility and β-carotene bioaccessibility of the primary, secondary and tertiary emulsions after an *in vitro* digestion were evaluated.

2. Materials and methods

2.1. Materials

Lactoferrin was kindly donated by Friesland Campina DOMO (Wageningen, The Netherlands). Sodium alginate (MW≈26,000 g/mol, 60–65% Manuronic acid, 35–40% Guluronic acid) was provided by IMCD España Especialidades Químicas, S.A. (Barcelona, Spain). The ε-poly-L-lysine (Epolyly®, MW 30 kDa) was from Handary S.A (Brussels, Belgium). Corn oil (Koipe Asua) was purchased from a local market. Pepsin from porcine gastric (EC 3.4.23.1), pancreatin from porcine pancreatin (EC 232.468.9), sodium phosphate monobasic and β-carotene (synthetic, ≥93% (UV), powder) were from Sigma Aldrich. Magnesium chloride hexahydrate and potassium phosphate monobasic were from Acros Organics. Hydrochloric acid (HCl) and sodium chloride (NaCl) were from Poch S.A. Bile (EC 232.369.0), sodium azide, calcium chloride dehydrate were obtained from Fisher scientific. Sodium hydroxide (0.25 N) and potassium chloride were from Panreac. Milli-Q water was used to prepare all emulsions.

2.2. Methods

2.2.1. Preparation of emulsions

The polyelectrolytes combination for developing primary (lactoferrin), secondary (lactoferrin-alginate) and tertiary (lactoferrin-alginate- ε-poly-L-lysine) emulsions were selected based on a previous work carried out in our group (Acevedo-Fani et al., 2017). However, a suitable polyelectrolyte concentration for each interfacial layer deposition was optimized since the β-carotene emulsions contained a higher oil concentration, compared to our previous study.

First, the lipid phase containing 0.5% w/w β-carotene was prepared by mixing the bioactive compound with corn oil through sonicating (1 min) (Hielscher Ultrasound Technology, Teltow, Germany, UP-400S) and stirring (5 min, 45 ºC). To prepare the primary emulsions, the lipid phase (20% w/w) and the aqueous phase (80% w/w) with different lactoferrin concentrations (2.5–7.5% w/w), were mixed with an ultraturrax (Janke & Kunkel, Staufen, Germany) at

5000 rpm for 3 min. After that, this coarse emulsion was passed through a Microfluidizer (Microfluidics M−110P) at a pressure of 100 MPa for 5 cycles. The term primary emulsion was referred to the emulsion containing oil droplets coated by lactoferrin. The optimal formulation selected was used to formulate secondary emulsions.

To prepare secondary emulsions, 10 mL of primary emulsion were added to 10 mL of alginate solutions under continuous agitation (200 rpm) at a dripping rate of 10 mL/min. Afterwards, secondary emulsions were stirred (500 rpm) for 15 min in order to disrupt any flocs formed. The term secondary emulsions was referred to emulsions containing oil droplets coated with two layers (lactoferrin-alginate). The resulting secondary emulsion contained 0.25% (w/w) β-carotene, 10% (w/w) corn oil, 2.5% (w/w) lactoferrin and different concentrations of alginate (between 0.8% w/w and 1.2% w/w). The optimal formulation selected after analysing the data was used to formulate tertiary emulsions.

To prepare tertiary emulsions, 5 mL of secondary emulsion were added to 15 mL of ε-poly-L-lysine solutions under continuous stirring (200 rpm), at a dripping rate of 10 mL/min. Then, tertiary emulsions were stirred (500 rpm) for 15 min and then sonicated during 2 min (cycle 0.5, 40 Hz) (Hielscher Ultrasound Technology, Teltow, Germany, UP-400S). This helped to disrupt flocs formed during the process. The term tertiary emulsion was referred to emulsions containing oil droplets coated by three layers (lactoferrin-alginate-ε-poly-l-lysine). The resulting tertiary emulsion contained 0.0625% (w/w) β-carotene, 2.5% (w/w) corn oil, 0.62% (w/w) lactoferrin, 0.25% (w/w) alginate and different concentrations of ε-poly-l-lysine, ranging from 0 to 0.25% (w/w).

The pH of all polyelectrolyte solutions and emulsions was 5. This is the pH at which polyelectrolytes are strongly oppositely charged and can form electrostatic complexes around oil droplets.

2.2.2. Optimization of polyelectrolytes concentration in emulsions

The most suitable polyelectrolyte concentration to create primary, secondary and tertiary emulsions was determined by measuring different parameters. For primary emulsions, protein surface load, particle size and surface charge were measured, whereas for secondary and tertiary emulsions, particle size and surface charge were determined. Furthermore, optical images were used to observe destabilisation processes during the interfacial layer's assembly.

To have more information about optimized primary, secondary and tertiary emulsions, their encapsulation efficiency (Davidov-Pardo & McClements, 2015) as well as span index (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK) (Velderrain-Rodríguez, Acevedo-Fani, González-Aguilar, & Martín-Belloso, 2019) were determined. In addition, TEM images were used to provide information about emulsions' structure and morphology.

2.2.2.1. Protein surface load determination

Protein surface load was determined in primary emulsions using a method adapted from Ye (2010). This analysis consists on determining the minimum concentration of polyelectrolyte (in this case lactoferrin) needed to create a coating on the oil droplets surface of a primary emulsions. Briefly, primary emulsions were centrifuged for 30 min, at 1000 rpm (AVANTI J-25, Beckman Instruments Inc., Fullerton, CA, USA) and the aqueous phase (bottom layer of the centrifuge tube) was separated from the lipid phase (top layer). This procedure was repeated by re-dispersing the lipid phase in Milli-Q water and centrifuging again. This was done to ensure all lactoferrin of aqueous phase was extracted. The aqueous phase obtained after the first and second centrifugation were joined and passed through a 0.22 µm filter. The protein concentration was measured by the Bradford method (Thermo Scientific Pierce Coomassie Protein Assay Kit), using lactoferrin as standard (Bradford, 1976). Then, protein surface load in emulsions was calculated following equation (1) and (2).

$$C_{adsorbed} = C_{inital} - C_{aqueous\,phase} \qquad (1)$$

where $C_{adsorbed}$ is the lactoferrin concentration adsorbed at the oil droplets surface, $C_{initial}$ is the lactoferrin concentration added to the emulsions and $C_{aqueous\,phase}$ is the lactoferrin concentration quantified in the aqueous phase.

Therefore, protein surface load (ſ), which indicated lactoferrin mass adsorbed at the droplets interface, was calculated as follows:

$$\text{ſ} = \frac{d_{32} \times C_{adsorbed}}{6 \times \phi} \qquad (2)$$

where ϕ is the oil volume, ſ is the surface load of lactoferrin (kg/m^2), d_{32} is the oil droplet diameter (meters) and $C_{adsorbed}$ is the amount of lactoferrin adsorbed at the oil droplet surface per emulsions volume (kg/m^3).

2.2.2.2. Physicochemical determination

Particle size was determined using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK), reporting results as the surface are mean diameter, $D_{[3,2]}$ (µm). The refractive index of the corn oil and water were 1.47 and 1.33, respectively.

Oil droplets surface charge (ζ-potential) was determined using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcerstershire, UK). Samples were previously diluted 1/10 with Milli-Q water (pH ≈ 6.5). Temperature was set to 25 °C for all analyses.

For optical microscopy images of emulsions, a drop of freshly prepared emulsion was placed on a glass slide and a cover slip on top. Images were taken using an optical microscope BX41 (Olympus, Shinjuku, Japan), linked to a digital camera Olympus DP74 (Olympus, Shinjuku, Japan) at a magnification of 100×. For TEM images, samples were prepared by depositing each emulsion on a carbon-coated copper grid, and negatively stained with 2% (w/v) uranyl acetate

for observation. Samples were air-dried before observation with a transmission electron microscopy (TEM) (Jeol JEM-1010, Tokyo, Japan) working at 80 kV.

2.2.3. Tertiary emulsions stability to external stressing conditions

Tertiary emulsions were exposed to different external conditions such as temperature, pH and ionic strength changes. Temperatures ranged from 30 °C to 90 °C, emulsions were adjusted at different pH conditions from 2 to 11, and ionic strength was modified by adding different NaCl concentrations to emulsions (0–0.5 M).

Physical stability of tertiary emulsions under these conditions was assessed after 24 h exposure to these conditions as described in section 2.2.2.2. Amount of β-carotene in tertiary emulsions was quantified spectrophotometrically (Ultrospec 3000 pro GE Health Sciences, USA) (Salvia-Trujillo et al., 2013) as well as presence of creaming was visually checked after 24 h of treatment.

2.2.4. Emulsions functionality

2.2.4.1. Lipid digestibility

An *in vitro* static model of digestion was used to subject primary, secondary and tertiary emulsions throughout gastric and intestinal conditions (Minekus et al., 2014). For the stomach phase, each emulsion was mixed with simulated gastric fluids (SGF) containing pepsin (2000 U/mL in the final mixture), $CaCl_2$ (H_20) (0.3 M), Milli-Q water and HCl (1 M) and incubated (Incubator OPAQ, OVAN, Barcelona, Spain) during 2 h at 37 °C with continuous agitation (100 rpm). Afterwards, the sample from the stomach phase was placed in a water bath (37 °C) to simulate the intestinal digestion and monitor lipolysis using a pH-stat (Metrohm USA Inc., Riverview, FL, USA). Simulated intestinal fluids (SIF) (0.150 M NaCl and 0.01 M $CaCl_2$), bile extract (54 mg/mL) and pancreatin (75 mg/mL) were added to the sample. During intestinal digestion, lipase hydrolyses ester bonds from lipid droplets (triacylglycerols) into several lipid digestion products, including diacylglycerols (DAG), monoacylglycerds (MAG), free fatty acids (FFA) and glycerols. The release of these FFA cause pH reduction in the samples and the constant titration with 0.25 M NaOH maintain the pH at 7 during 2 h. The total volume of NaOH employed during intestinal digestion was used to calculate the lipid digestibility through total FFA release content, using equation 3.

$$\text{FFA (\%)} = \frac{V_{NaOH} \times C_{NaOH} \times M_{oil}}{2 \times m_{oil}} \times 100 \qquad (3)$$

where V_{NaOH} is NaOH volume (L) used to compensate the FFAs during the digestion, C_{NaOH} is NaOH molarity (0.25 mol/L), M_{oil} is corn oil molecular weight (800 g/mol), m_{oil} is corn oil total weight present in emulsions (g).

2.2.4.2. β-carotene bioaccessibility

Micellar fraction was obtained by centrifuging (AVANTI J-25, Beckman Instruments Inc., Fullerton, CA, USA) the liquid collected after the *in vitro* digestion at 4000 rpm for 40 min at 4 °C (Qian, Decker, Xiao, & McClements, 2012). The upper part of the centrifuged liquid was the micellar fraction, in which the mixed micelles formed during the *in vitro* digestion containing the solubilised β-carotene were suspended. In the centrifuged samples, a layer of oil could be observed on top of the liquid, which was dismissed since it was considered non-digested oil after *in vitro* digestion.

The concentration of β-carotene in undigested emulsions (initial) and micellar fraction was determined using a previously reported method (Salvia-Trujillo et al., 2013). Briefly, samples were mixed with chloroform, vortexed and centrifuged. This process was repeated until the upper part of the mixture became colourless. The bottom chloroform part containing the solubilised β-carotene was measured by UV–visible spectrophotometry (Ultrospec 3000 pro GE Health Sciences, USA) at 450 nm, using chloroform as a blank. Lastly, the β-carotene bioaccessibility was calculated using equation 4.

$$\text{Bioaccessibility}(\%) = \frac{C_{micelle}}{C_{initial}} \times 100 \qquad (4)$$

where $C_{micelle}$ and $C_{initial}$ are the β-carotene concentration of the micellar fraction and the undigested emulsion, respectively.

2.2.5. Statistical analysis

The analysis of variance (ANOVA) was conducted using Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md, USA) to identify samples with significant differences ($p \leq 0.05$ was considered significant). All parameters were performed in triplicate and data was reported as the mean with standard deviation.

3. Results and discussion

3.1. Primary emulsion formation: Effect of lactoferrin concentration

Primary emulsions presented particle sizes ($D_{3,2}$) below 0.41 μm and positive ζ-potential (between +51.9 mV and +55.3 mV) regardless the lactoferrin concentration. A significant reduction in particle size until 0.29 μm was observed when 5% (w/w) of lactoferrin was used to prepare the emulsion (Table 1). In fact, these results were in agreement with surface load results, in which a maximum value of 10.22 mg/m^2 was reached at a lactoferrin concentration of 5% (w/w), indicating that there were enough lactoferrin molecules covering the oil droplets of primary emulsion, thereby preventing droplet aggregation (Mao & McClements, 2011).

Table 1. Particle size ($D_{3,2}$(μm)), ζ-potential (mV) and surface load (mg/m^2) of primary emulsions containing different concentrations of lactoferrin (LF). Different letters mean significant differences ($p < 0.05$) between emulsions with different concentrations of lactoferrin.

LF (%)	$D_{3,2}$ (μm)	ζ-potential (mV)	Surface load (mg/m^2)
2.5	0.41 ± 0.02^{c}	52.85 ± 1.95^{a}	2.60 ± 0.46^{a}
4	0.39 ± 0.01^{bc}	52.13 ± 0.50^{a}	6.04 ± 0.04^{b}
4.5	0.39 ± 0.01^{bc}	53.15 ± 3.19^{a}	6.32 ± 0.01^{b}
5	0.29 ± 0.03^{a}	51.90 ± 2.08^{a}	10.22 ± 0.31^{cd}
6	0.37 ± 0.01^{b}	55.35 ± 1.15^{a}	9.91 ± 0.83^{c}
7.5	0.39 ± 0.02^{bc}	52.56 ± 5.75^{a}	10.94 ± 0.42^{d}

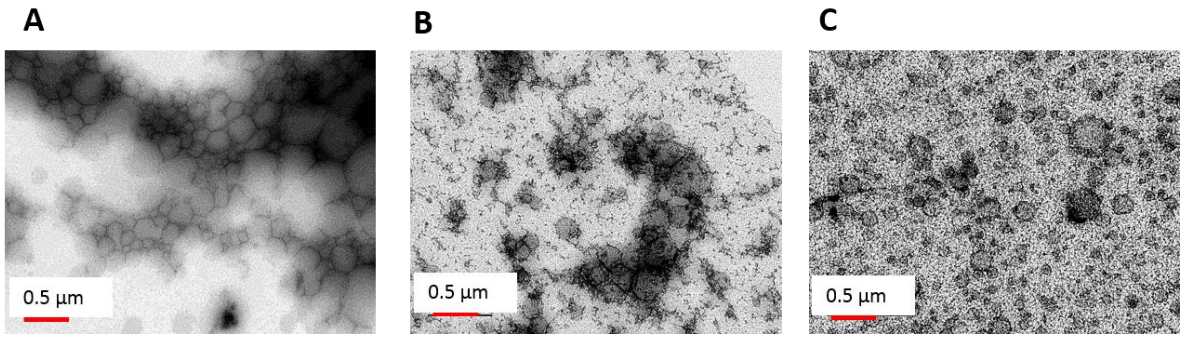

Figure 1. Images of primary (A), secondary (B) and tertiary emulsion (C) obtained by Transmission Electron Microscopy (TEM). The magnification was 40.000x.

In addition, TEM images confirmed the formation of primary emulsions, which showed a spherical shape and a fine interface, corresponding to lactoferrin coating (Figure 1A). The high molecular weight of lactoferrin (around 80 kDa) together with its distant isoelectric point (≈8) from pH of emulsions (≈5), would have stabilised oil droplets by a combination of both steric and electrostatic forces (Tokle & McClements, 2011). At higher lactoferrin concentrations (>5% w/w), emulsions underwent a significant increase of particle size, which may indicate that part of the extra lactoferrin added would have remained unabsorbed. This extra lactoferrin

prevailed in the aqueous phase producing attractive forces between droplets, which led to droplet-droplet interactions (Israelachvili, 2011).
Therefore, the primary emulsion containing 5% w/w of lactoferrin was selected as the optimal formulation to proceed with the formation of the secondary emulsion.

3.2. Secondary emulsion formation: Effect of alginate concentration

A significant increase in particle size of secondary emulsions from 0.29 μm to 0.68 μm was observed when alginate was added. The mininum particle size of secondary emulsions was observed at 1% (w/w) alginate (0.45 μm) and adding concentrations of alginate above 1% (w/w) resulted in a significant increase of the particle size (Figure 2A). Indeed, optical microscope images of secondary emulsions stabilised with low (0.8% w/w) and high (1.2% w/w) concentrations of alginate presented some droplet aggregation, while no destabilisation phenomena was observed in emulsions that contained alginate at 1% (w/w) (Figure 2B). On one hand, when the polyelectrolyte content in the emulsion was insufficient to cover the entire droplet surface, a single molecule of polyelectrolyte would have been adsorbed at the surface of different droplets simultaneously, linking them together and resulting in large particle size (bridging flocculation) (Guzey & McClements, 2006). On the other hand, depletion flocculation effect driven by an excess of polyelectrolyte would have been induced by the unadsorbed molecules surrounding the droplets, resulting in attractive forces and consequent droplet aggregation (Zeeb, Thongkaew, & Weiss, 2014).

Regarding the ζ-potential, adding alginate to primary emulsions resulted in a change of ζ-potential from positive (+42 mV) to negative values (−56 mV) (Figure 2A). As alginate and lactoferrin have opposite charges (anionic and cationic, respectively), alginate would have been adsorbed to lactoferrin-coated oil droplets surface by electrostatic interactions, leading to the formation of a second interfacial layer.
Based on these results, the optimal alginate concentration for formulating the secondary emulsion was 1% (w/w), as it presented the lowest values of particle size (0.45 μm) and a strong negative ζ-potential (−55.20 mV). According to TEM images, secondary emulsion also presented spherical shape and a slightly dark line around the droplets, which was the lactoferrin-alginate coating (Figure 1B).

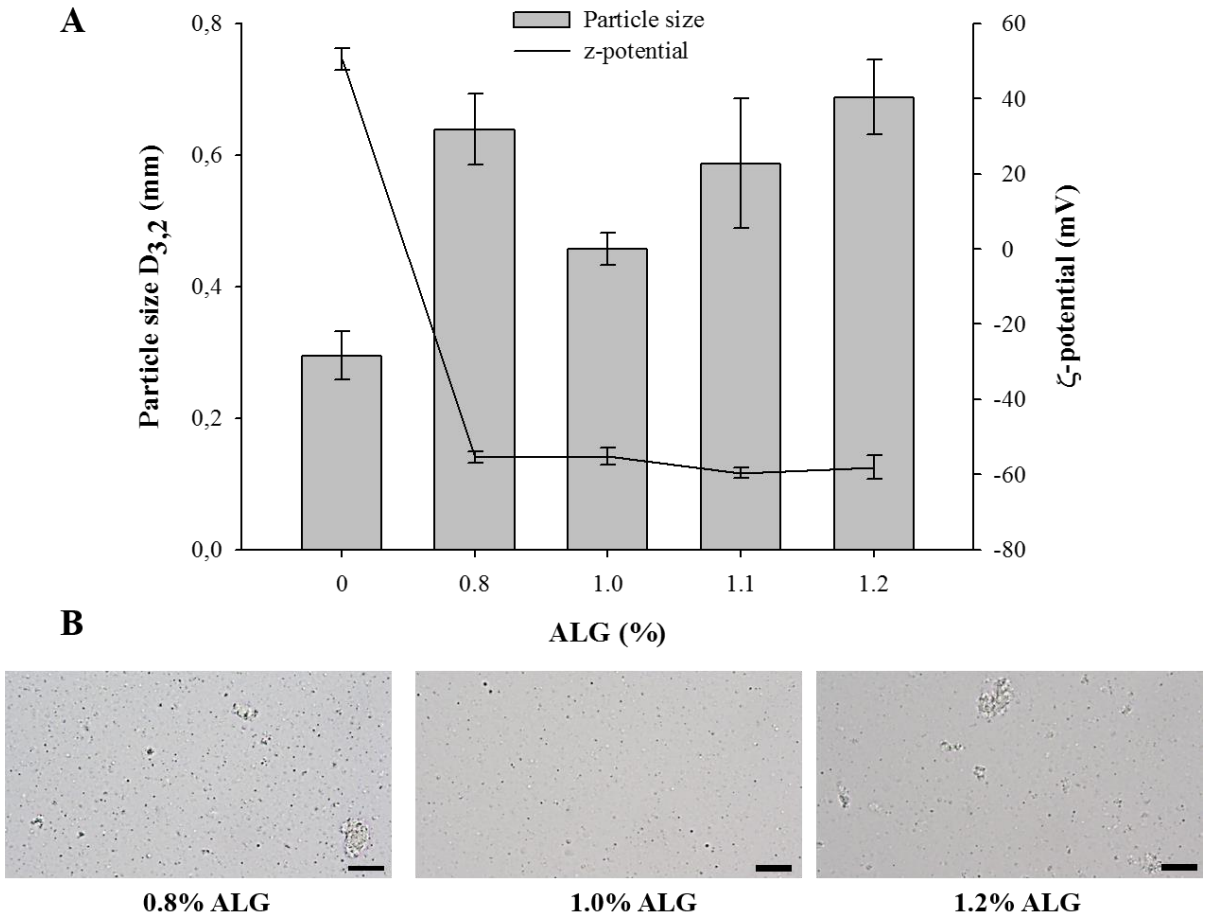

Figure 2. (A) Particle size ($D_{3,2}$(μm)) and ζ-potential (mV) of secondary emulsions containing different concentrations of alginate (ALG). (B) Optical microscopy images of secondary emulsions. Scale bars were 10 μm long.

3.3. Tertiary emulsion formation: Effect of ε-poly-L-lysine concentration

Tertiary emulsions with a low ε-poly-L-lysine concentration (0.12% and 0.15% w/w) presented particle sizes around 0.30 μm, whereas adding concentrations up to 0.17% w/w significantly increased the particle size of emulsions (≈1.85 μm). Furthermore, the initial ζ-potential of these secondary emulsions increased from −55 mV to −9.36 mV when tertiary emulsions contained up to 0.17% (w/w) of ε-poly-L-lysine (Figure 3A). These results indicated that there were not enough ε-poly-L-lysine molecules covering the lactoferrin/alginate-coated droplets and that alginate was the polyelectrolyte prevalent at the droplets interface. When tertiary emulsions contained concentrations of ε-poly-L-lysine between 0.18% (w/w) and 0.19% (w/w) a significant reduction in particle size was observed (0.59 μm and 0.58 μm, respectively).

Furthermore, these emulsions exhibited positive ζ-potential indicating that ε-poly-L-lysine had been deposited at the droplets surface. However, a steep increase in particle size until 14 μm and droplet aggregation (using optical microscope images) was detected at higher concentrations (0.20% and 0.25% w/w) (Figure 3A and 3B). High ε-poly-L-lysine concentrations in tertiary emulsion would have favoured depletion flocculation and thus, droplet aggregation (Benjamin, Silcock, Leus, & Everett, 2012).

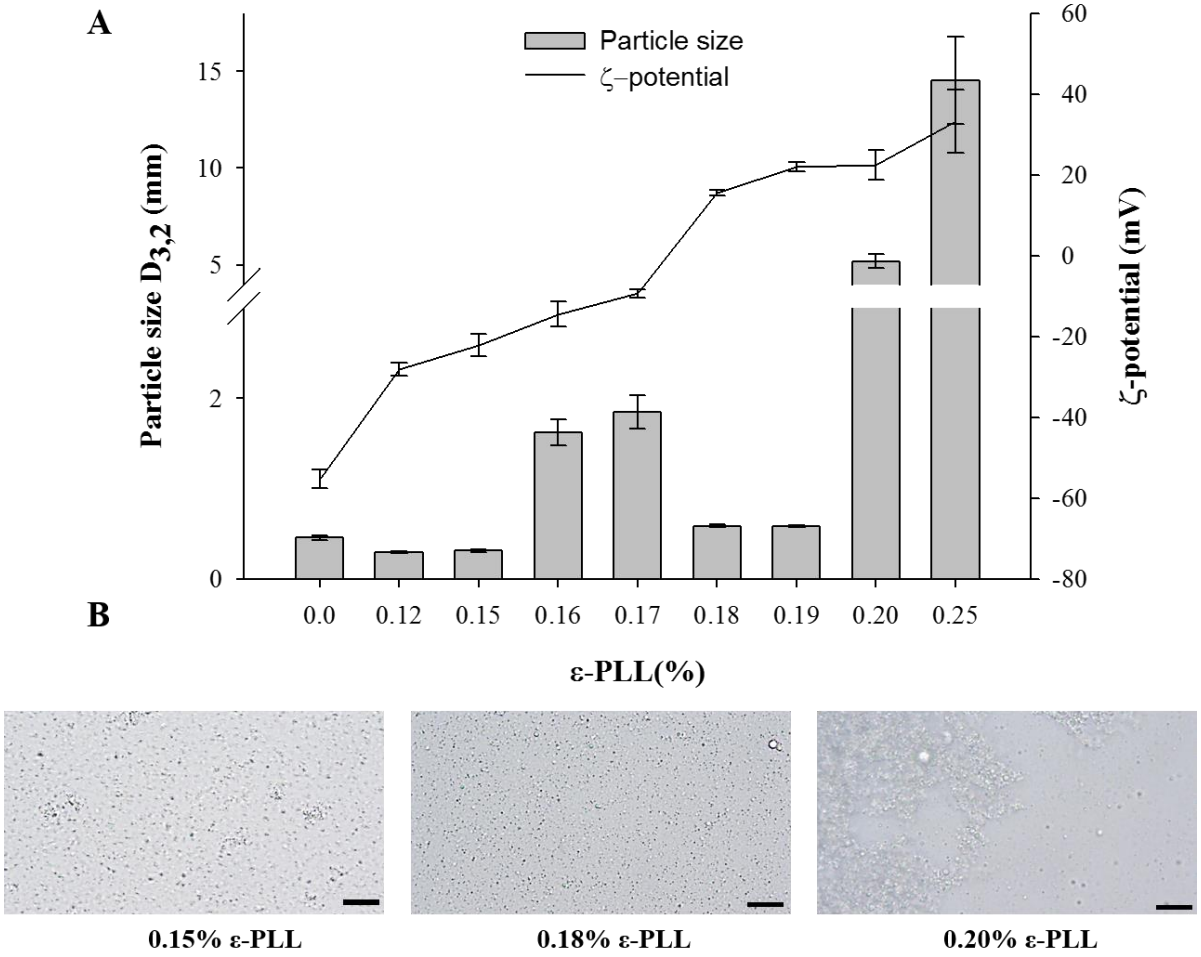

Figure 3. (A) Particle size ($D_{3,2}$(μm)) and ζ-potential (mV) of tertiary emulsions containing different concentrations of ε-poly-L-lysine (ε-PLL). (B) Optical microscopy images of tertiary emulsions. Scale bars were 10 μm long.

Taking into account all the results, two different concentrations of ε-poly-L-lysine (0.18% w/w and 0.19% w/w) could be selected as optimal concentration in the formation of the tertiary emulsion. However, tertiary emulsion containing 0.18 w/w of ε-poly-L-lysine presented high encapsulation efficiency and good span index indicating homogeneity (Table 2). TEM images confirmed the lactoferrin-alginate- ε-poly-L-lysine coating because of the intense black

coverage around the droplets (Figure 1C). Moreover, the lowest content of ε-poly-L-lysine in tertiary emulsions (0.18% w/w) could be related to economically and environmentally sustainable food systems. The following experiments of the study were carried out with the tertiary emulsion obtained after optimization process.

Table 2. Encapsulation efficiency of β-carotene and span index for primary, secondary and tertiary emulsions. Different letters mean significant differences (p<0.05) between emulsions with different interfacial layers.

Emulsions	β-carotene encapsulation efficiency (%)	Span index
Primary	82.52 ± 0.60^{a}	1.32 ± 0.05^{a}
Secondary	96.63 ± 1.50^{b}	1.42 ± 0.05^{b}
Tertiary	96.06 ± 0.35^{b}	2.19 ± 0.15^{c}

3.4. Tertiary emulsions stability to external stressing conditions

3.4.1. Temperature

Tertiary emulsions were stable at temperatures ≤ 60 °C since there were no changes in the particle size and ζ-potential as well as no evidence of droplet aggregation. On the other hand, tertiary emulsions presented a progressive particle size increase and some droplet aggregation at higher temperatures (70–90 °C) (Figure 4B). At the same time, ζ-potential of these tertiary emulsions significantly decreased from + 13 mV to + 3.45 mV (Figure 4A). Large particle sizes in emulsions heated above 60 °C could be related with a partial detachment of polyelectrolytes molecules from the droplets, thereby sharing one polyelectrolyte molecule with more than one droplet (bridging flocculation) (Guzey & McClements, 2006). Furthermore, proteins are heat sensitive molecules, while polysaccharides such as alginate tend to be temperature independent (Dickinson, 2003). Thermal denaturation of lactoferrin (between 70 °C and 85 °C) could have derived into protein unfolding, thus promoting hydrophobic attraction between droplets (Teo, Lee, & Goh, 2017). In addition, high temperatures could have changed ε-poly-L-lysine structure, particularly to β structures (Mirtič & Grdadolnik, 2013) characterised to have a compact structure and thus, less ε-poly-L-lysine amine groups charged (Lee, 2016).

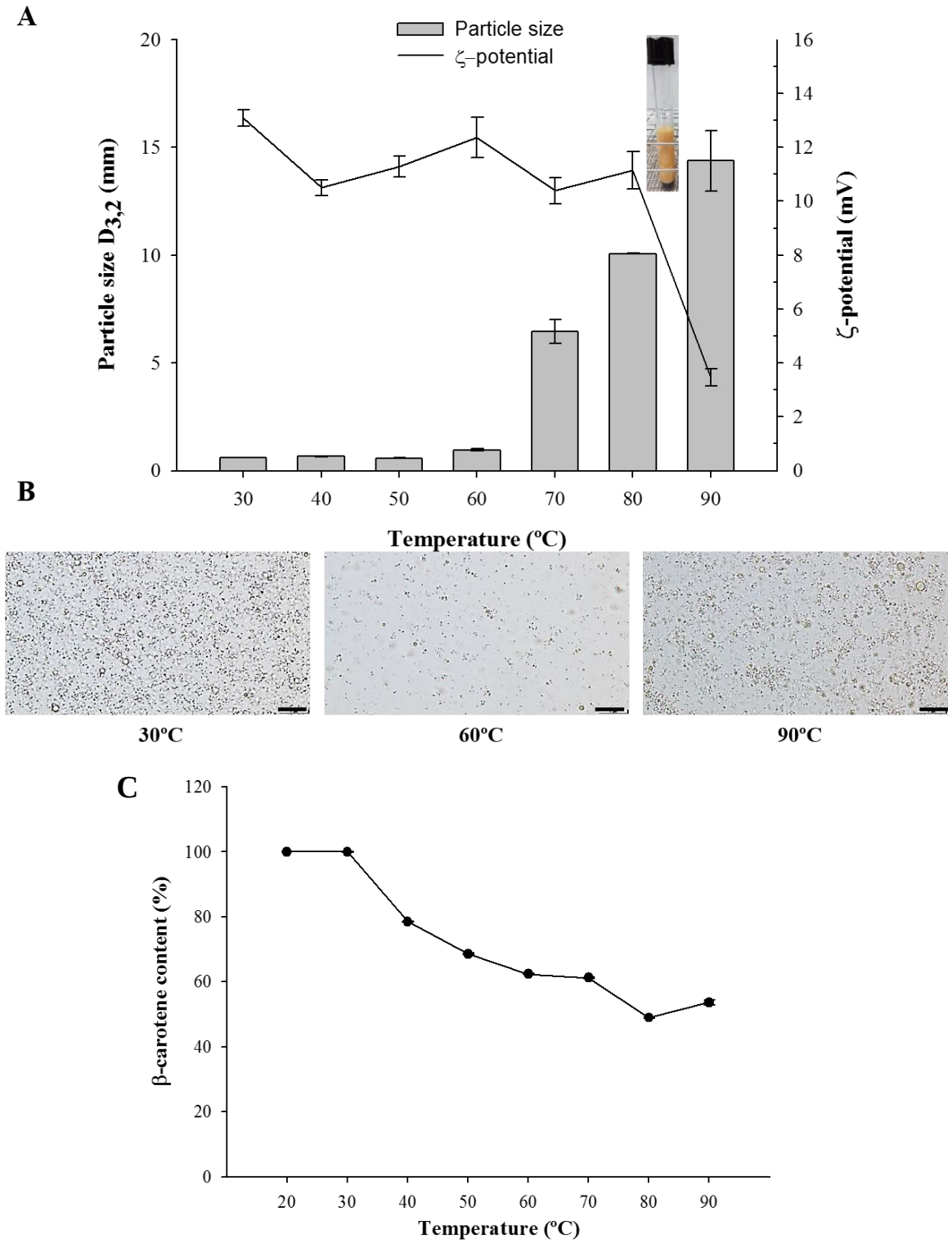

Figure 4. (A) Influence of different temperatures on particle size ($D_{3,2}$(μm)) and ζ-potential (mV) of tertiary emulsions. (Inset) Visual image of multi-layer emulsions after 24h treatment. (B) Optical microscopy images of tertiary-layer emulsions at different temperatures. Scale bars were 10 μm long. (C) Influence of different temperatures on β-carotene content (%) within tertiary emulsions.

These phenomena could explain the reduction of tertiary emulsion ζ-potential to nearly zero values, leading to poor electrostatic stabilisation and further droplet aggregation. However, no visual creaming in any of the tertiary emulsions was observed after 24 h treatment (Figure 4A). The creaming process not only depends on the particle size, but also on the density difference between the continuous and dispersed phase. When the density of the dispersed phase (oil) is lower than the density of the continuous phase (water), oil droplets move upwards thereby creating a phase separation. In this study, tertiary emulsion could have retarded the creaming process because the layers covering oil droplets would have reduced the density contrast between dispersed and continuous phases, decreasing the gravitational separation of both phases (McClements, 2005). Because of its unsaturated structure, β-carotene is susceptible to degradation under high temperatures. Accordingly, β-carotene was constantly degraded at temperatures above 40 °C (Figure 4C), although its content in tertiary emulsion remained always above 50%, irrespective of the temperature applied.

3.4.2. pH

The particle size of tertiary emulsions progressively increased from 0.57 μm to 11.91 μm as the pH of emulsions decreased from 5 to 2, while no significant changes in particle size were observed at higher pH values (from 6 to 11). However, none of the emulsions exhibited creaming after 24 h treatment. Regarding ζ-potential, tertiary emulsions presented a positive charge at pH below 5, being highly positive at pH 2 (+38 mV). On the other hand, when the pH of tertiary emulsions increased from 6 to 11, ζ-potential changed progressively from +15 mV to −43 mV. Specifically, emulsions presented ζ-potential close to 0 mV when the pH was between 7 and 8, and then it changed to negative when the pH of tertiary emulsions further increased (pH > 9) (Figure 5A).

Under acidic environments (pH between 2 and 5), the pH of emulsions was below the pK_a of ε-poly-L-lysine (≈9) and close to alginate pK_a (≈3.5) (Lee & Mooney, 2012). In this situation, electrostatic attraction between ε-poly-L-lysine (highly charged) and alginate (partially uncharged) would have been reduced, leading to a weak attachment between both components. These results suggested that the thickness of the interface was reduced, and ε-poly-L-lysine stabilisation via steric repulsion was not enough to overcome attractive forces. Indeed, droplet aggregation could be observed in optical microscope images when emulsions had acidic pH (Figure 5B).

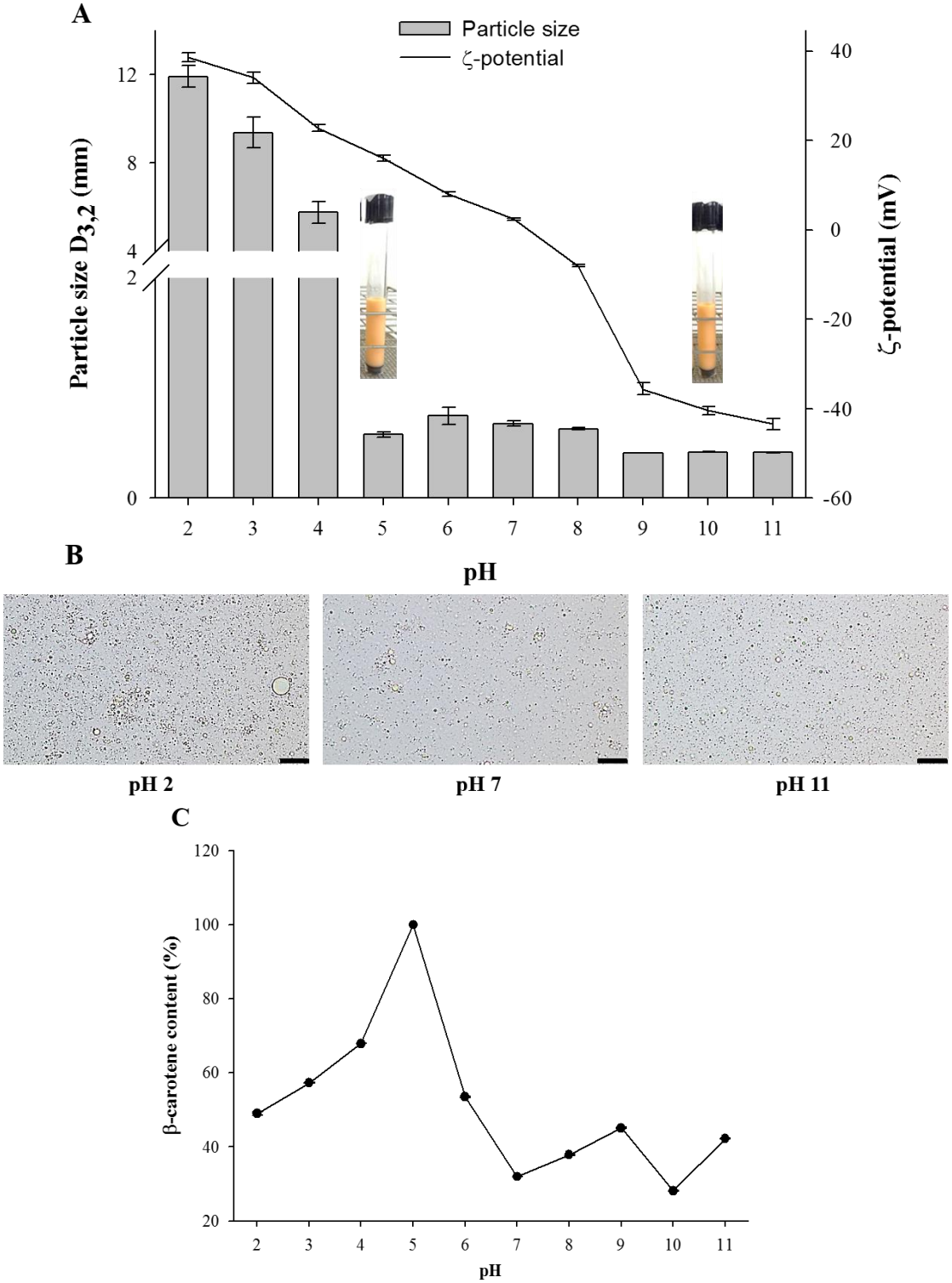

Figure 5. (A) Influence of pH conditions on particle size ($D_{3,2}$(μm)) and ζ-potential (mV) of tertiary emulsions. (Insets) Visual images of tertiary emulsions after 24h treatment. (B) Optical microscopy images of multi-layer emulsions at different pH. Scale bars were 10 μm long. (C) Influence of different pH conditions on β-carotene content (%) within tertiary emulsions.

When basic conditions were present (pH between 8 and 11), ε-poly-L-lysine lost its charge ($pK_a \approx 9$) and thus the electrostatic attraction with alginate might have been reduced, leading to a detachment of ε-poly-L-lysine from alginate-covered droplets. In this case, alginate was the polyelectrolyte that predominated at the interface of the droplets, contributing on the obtained negative ζ-potential. Either a particle size increase or droplet flocculation in tertiary emulsions were not observed in this situation (Figure 5B), which might be attributed to the thick and charged layer of alginate covering the droplets, providing both steric and electrostatic stabilisation to the system (McClements, 2004). Under neutral pH conditions (pH between 6 and 7), tertiary emulsions presented very low or almost no surface charge and no droplet aggregation. These results suggested that the stability of these tertiary emulsions was not determined by charge (electrostatic repulsion), but by the polyelectrolyte coating around the oil droplets (steric repulsion).

In general, β-carotene content was reduced when pH changed from the original pH employed to form the tertiary emulsion (≈5) to either acidic (49% of β-carotene) or basic conditions (28% of β-carotene) (Figure 5C). The interfacial layers of tertiary emulsion might act as a physical barrier to avoid the interaction between β-carotene and free radicals and/or oxygen, which would lead to compound degradation. Although tertiary emulsions were physical stable under basic environments, changes in the interfacial layer of the emulsion would have not been enough to prevent β-carotene degradation.

3.4.3. Ionic strength

With increasing NaCl concentration, particle size of tertiary emulsions was significantly incremented (≈7 μm), resulting in extensive droplet aggregation (Figure 6A, 6B). Furthermore, ζ-potential exhibited a progressive decrease from +10 mV to +4.5 mV when NaCl concentration increased from 0 to 0.5 M (Figure 6A). These results suggested that the presence of ions in the aqueous solution from NaCl (Cl^-) might have interacted with positively charged amino groups of ε-poly-L-lysine molecules, leading to either a partial desorption of ε-poly-L-lysine from the droplets surface and a charge reduction. Both situations might have derived to a poor electrostatic and steric stabilisation of the system, so attractive forces between droplets would have contributed on aggregation processes. The presence of a cream layer (top) and a serum layer (bottom) in tertiary emulsions with NaCl concentration over 0.3 M after 24 h treatment could be related to droplet aggregation as well as their low viscosity (data not shown). Subsequently, β-carotene content significantly decreased to values under 10% at NaCl concentrations over 0.3 M, which might be related to instability processes observed under these conditions (Figure 6C). Gravitational separation of emulsions can be reduced by increasing the viscosity of the continuous phase, reducing movement of oil droplets (Zinoviadou, Scholten, Moschakis, & Biliaderis, 2012).

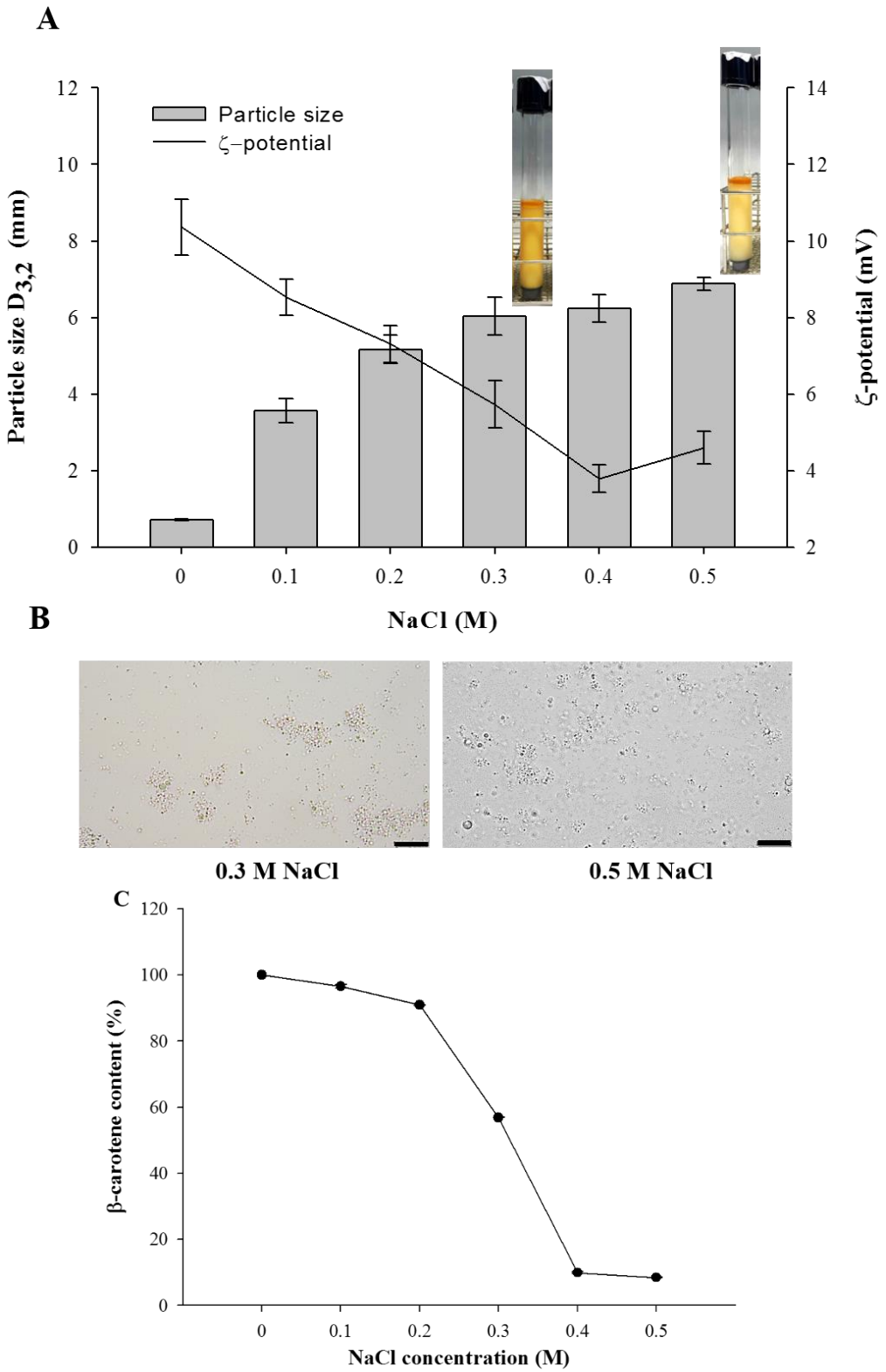

Figure 6. (A) Influence of salt addition (NaCl) on particle size ($D_{3,2}$(μm)) and ζ-potential (mV) of tertiary emulsions. (Insets) Visual images of tertiary emulsions after 24h treatment. (B) Optical microscopy images of tertiary-layer emulsions at different NaCl concentrations. Scale bars were 10 μm long. (C) Influence of salt addition (NaCl) on β-carotene content (%) within tertiary emulsions.

3.5. Lipid digestibility and β-carotene bioaccessibility

The final number of free fatty acids released was higher as the number of the layers in the emulsion increased: primary emulsion (34.61%), secondary emulsion (61.03%) and tertiary emulsion (83.79%) (Figure 7).

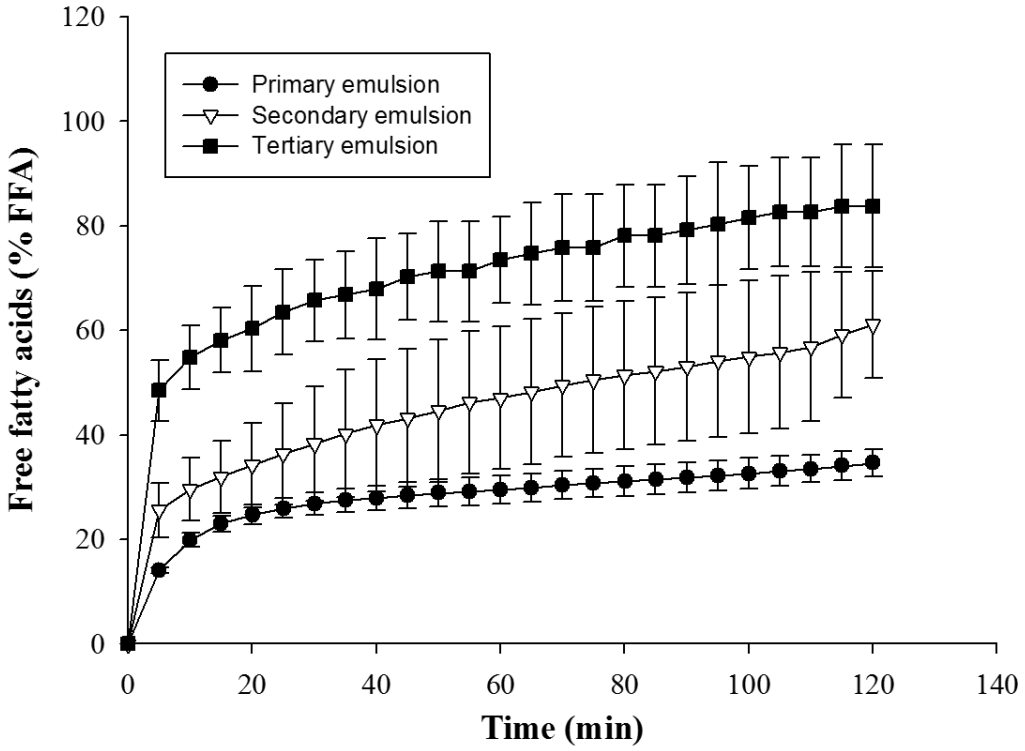

Figure 7. Free fatty acids release (%FFA) from primary, secondary and tertiary emulsions during intestinal phase

Although ε-poly-L-lysine might inhibit lipid digestion (Kido et al., 2003), the low amount of this polyelectrolyte present in tertiary emulsion (0.18% w/w) might not have been enough to affect the lipid digestion negatively. The acid pH conditions from the stomach phase (≈2.5) would have weakened the electrostatic attraction between ε-poly-L-lysine and alginate, third and second layer of the tertiary emulsion, respectively (section 3.4.2). In addition, bile salts in combination with chymotrypsin and trypsin, would have displaced lactoferrin from the droplets interface during intestinal phase (Pilosof, 2017) facilitating lipid digestion.

Alternatively, dilution of emulsions with simulated gastric fluids and the continuous agitation during this stage might have produced a redistribution of droplets and a reduction of droplet aggregation, contributing on physical stability of tertiary emulsion in stomach (Golding,

Wooster, Day, Xu, Lundin, Keogh, & Clifton, 2011). Furthermore, ε-poly-L-lysine is able to inhibit the activity of pepsin at low pH conditions (Lawton & Mekras, 1985). Consequently, it would be proposed that tertiary emulsions presented a high physical stability under stomach conditions, and therefore their particle size would have remained small when entering within the intestinal phase. When these emulsions reached the intestine (neutral pH), the different layers would have been completely detached from the surface droplets. Acevedo-Fani, Salvia-Trujillo, Soliva-Fortuny, & Martín-Belloso, (2015) reported a reduction of the surface ζ-potential of multilayer systems as the amount of adsorbed material in the layer diminishes, because there are less ionizable moieties that contribute on ζ-potential. Consequently, a less negative ζ-potential under neutral pH conditions (section 3.4.2) could be associated with a decrease in thickness of the layer covering oil droplets, suggesting desorption of the different layers. Combination of the small particle size of tertiary emulsion when reaching the small intestine, together with the layer's desorption from oil droplets, would have facilitated the lipase action and therefore, lipid digestion of tertiary emulsion.
Indeed, β-carotene bioaccessibility significantly increased as emulsions had more interfacial layers, rising from 30.24% and 35.26% (primary and secondary emulsions, respectively) to 70.1% for tertiary emulsions.

These results suggested that bioaccessibility might be associated with lipid digestibility, showing a higher β-carotene bioaccessibility as the free fatty acids release increased. The high β-carotene bioaccessibility observed for tertiary emulsions suggest that most of the β-carotene released from the lipid phase was solubilised within the mixed micelles formed during the intestinal digestion. Compared to other studies (Pinheiro, Coimbra, & Vicente, 2016; Silva et al., 2018; Tokle et al., 2013; Zhang et al., 2015), our bioaccessibility results were substantially greater. For instance, (Tokle et al., 2013) obtained a β-carotene bioaccessibility below 2% for a multi-layer emulsion containing a similar amount of oil (2%) but with different emulsifiers (lactoferrin/β-lactoglobulin/lactoferrin). Low bioaccessibility results were attributed to the interaction between lactoferrin and β-carotene, remaining β-carotene entrapped in the sediment phase and not being solubilised within mixed micelles. Furthermore, studies about nanoemulsions (single-layered emulsions) have reported bioaccessibility results between 1.5 and 35% (Feng et al., 2017; Gasa-Falcon et al., 2019), suggesting that higher number of layers covering droplets (multi-layered emulsions) would improve bioaccessibility of encapsulated compounds. In order to explain the high β-carotene bioaccessibility obtained in this study, a combination of different hypothesis was proposed. First, lipid digestibility as well as bioaccessibility might have been favoured because the presence of lipid content in tertiary emulsions was not in excess. Both lipase-to-lipid and bile-to-lipid ratio was high and thus, enough mixed micelles to incorporate the released β-carotene would have been generated. Second, antioxidant effects from lactoferrin (Huang, Satué-Gracia, Frankel, & German, 1999), alginate (Xue, Yu, Hirata, Terao, & Lin, 1998) and ε-poly-L-lysine (Scheffler, Wang, Huang, Gonzalez, & Yao, 2010) molecules would have protected β-carotene from degradation along the *in vitro* simulated gastrointestinal tract, but mostly from the acidic environment in stomach phase. Third, desorption of ε-poly-L-lysine, alginate and lactoferrin from droplets surface under

neutral conditions could have facilitated the access of lipase to the oil droplets. Bioaccessibility of lipophilic compounds such as β-carotene primarily depends on the amount of released compound from the lipid phase during digestion. Thus, the relatively high free fatty acids liberated (70%), might have indicated that lipids from tertiary emulsion were hydrolysed and that β-carotene was released from oil droplets.

4. Conclusions

A tertiary emulsion (lactoferrin/alginate/ ε-poly-L-lysine) containing a high oil concentration (2.5% w/w) was successfully developed. Selecting the optimal polyelectrolyte concentration of each layer was essential in order to avoid destabilization processes. Tertiary emulsions of this work would be most suitably used in neutral foods and beverages, in thermal processes below 60 °C as well as food products without salt due to its high physical stability. In addition, β-carotene content of around 30% was maintained under these conditions. Finally, although the numerous layers of polyelectrolyte covering the oil droplets, tertiary emulsions presented a greater lipid digestibility and β-carotene bioaccessibility compared to primary and secondary emulsions, suggesting that multilayered emulsions might be used as a new approach to enhance delivery of encapsulated lipophilic compounds. An in-depth study is needed in order to evaluate the application of this tertiary emulsion within food products.

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GENERAL DISCUSSION

Emulsion-based delivery systems

A number of positive health benefits have been associated with β-carotene consumption, including lower incidence of cancer, cardiovascular diseases and degenerative disorders (Goralczyk, 2009). However, its low solubility in aqueous-based matrices as well as its high susceptibility to be degraded at extreme conditions (high temperatures, pH changes and/or light exposure), complicates even further the obtention of lipophilic-enriched food products. Emulsion-based delivery systems might be used as a potential option to enhance the solubility, stability, absorption and, ultimately, functionality of bioactive lipophilic compounds, such as β-carotene (Fathi, Mozafari, & Mohebbi, 2012; Odriozola-Serrano, Oms-Oliu, & Martín-Belloso, 2014.). In this direction, the successful delivery of encapsulated lipophilic bioactive compounds might be related to the susceptibility of these systems to undergo physical alterations when they are subjected to external environments, such as gastrointestinal tract or stressing conditions. Instability processes can be associated with a breakdown of the emulsion structure, resulting in a no longer encapsulation of the bioactive compound.
An effective delivery of lipophilic bioactive compounds can be directly associated with stability and digestibility of emulsion systems, as well as bioaccessibility and absorption of such health-related compounds Therefore, these present doctoral thesis aims to provide novel insights in the formulation of emulsion-based systems for the delivery of β carotene so as to enhance its functionality.

1. Oil-in-water nanoemulsions

A wide range of water-soluble components such as stabilising agents, are often incorporated into emulsion-based systems for preventing their structure breakdown. Composition and characteristics of such stabilisers might have direct implications in physicochemical properties of nanoemulsions as well as in an effective delivery of encapsulated compounds (McClements, Bai, & Chung, 2017).

Therefore, the starting point of this doctoral thesis consisted of investigating how different stabilising agents might influence on digestion processes and bioaccessibility of β-carotene-enriched nanoemulsions.

1.1. Stabilising nanoemulsions with emulsifiers

Emulsifiers are one of the most important type of stabilisers used in any emulsion formulation because they have the ability to prevent the structure breakdown. Emulsifiers are typically amphiphilic molecules that have both hydrophilic and hydrophobic groups on the same molecule, such as small molecule surfactants, phospholipids, proteins, polysaccharides, and other surface-active polymers (McClements and Mahdi-Jafari, 2018). Most of the effective emulsifiers currently used are synthetic, although health-conscious consumers have driven to use natural alternatives. In addition, the nature of the emulsifier determines the ease of emulsion formation, and thus on final physicochemical properties as well as on functionality. Conducting

studies on nanoemulsions stabilised with emulsifiers having different structures and properties as well as from various sources, would provide knowledge for designing effective delivery systems for lipophilic bioactive compounds.

In chapter I, β-carotene enriched nanoemulsions were formulated with different emulsifiers and their stability along the *in vitro* gastrointestinal tract (GIT), lipid digestibility as well as bioaccessibility were determined. Furthermore, permeability of β-carotene-enriched nanoemulsions after *in vitro* GIT across two intestinal barrier models (Caco-2 or co-cultures of Caco-2/HT29-MTX) was evaluated (chapter II).

1.1.1. Effect of interfacial composition on physicochemical properties of nanoemulsions

Firstly, β-carotene-enriched nanoemulsions with different interfacial properties defined by emulsifier nature (Tween 20, lecithin, sodium caseinate, sucrose palmitate) and concentration (2, 4 and 8%), were characterized in terms of particle size and ζ-potential.

Increasing the emulsifier concentration from 2% to 8% resulted in a significant reduction in particle size of nanoemulsions stabilised with Tween 20 (from 0.35 to 0.30 μm), lecithin (from 0.36 to 0.25 μm) and sodium caseinate (from 0.62 to 0.47 μm). However, particle size in sucrose palmitate-stabilised nanoemulsions remarkably increased from 0.29 to 4.73 μm when the emulsifier concentration increased from 2% to 8%.

Low mass emulsifiers, such as Tween 20 (MW≈1228 g/mol), lecithin (MW≈758 g/mol) and sucrose palmitate (MW≈509 g/mol) were more effective in producing small particle sizes at low concentrations (2% w/w) than sodium caseinate, which is a high mass emulsifier (MW≈90000 g/mol) with a complex and large molecular structure (McClements & Gumus, 2016). Interfacial tension in nanoemulsions stabilised with Tween 20, lecithin and sodium caseinate might have been decreased with increasing concentrations of such emulsifiers, thus allowing a more efficient droplet break-up during emulsification (Pichot, Spyropoulos, & Norton, 2010). On the other hand, results showed that adding 4% sucrose palmitate to nanoemulsions was enough to cover the entire surface of the formed droplets, assuming that a maximum number of tiny droplets were reached, and that the particle size could not be reduced any further. Adding higher concentrations (8%) could have led to non-adsorbed molecules in the media, generating attractive forces between droplets and resulting in droplet–droplet interactions (depletion flocculation) (Israelachvili, 2011), as it could be observed in optic microscope images.

In terms of ζ-potential, all nanoemulsions presented negative values irrespective of the emulsifier nature and concentration. The most negative values were exhibited by lecithin-stabilised nanoemulsions with values of around −80 mV. Phosphate groups from the different types of phospholipids present in the lecithin were the reason for the elevated negative charge (Artiga-Artigas, Lanjari-Pérez, & Martín-Belloso, 2018). Although Tween 20 and sucrose palmitate are non-ionic emulsifiers, their ζ-potential was negative, *i.e.*, around −24 mV and −49 mV, respectively, and with any statistically significant differences between both emulsifiers. Adsorption of OH^- species from the aqueous phase to the interface of the oil droplets, cationic species of the oil (Yin, Chu, Kobayashi, & Nakajima, 2009), the presence of residual non-esterified fatty acids in the sucrose ester

(Klang, Matsko, Raupach, El-Hagin, & Valenta, 2011) or impurities (palmitic acid) (Henry, Fryer, Frith, & Norton, 2009), in the case of sucrose palmitate, could have contributed on the negative charges. The ζ-potential values of sodium caseinate-stabilised nanoemulsions ranged between −40 and −48 mV, and they were slightly less negative as the emulsifier concentration increased. The addition of sodium caseinate to nanoemulsions might have decreased the pH of the system at around isoelectric point of caseinate (≈4.5). In this situation, there were enough amino groups of caseinate positively charged, which would have increased the ζ-potential up to -40 mV.

1.1.2. Effect of interfacial composition on stability of nanoemulsions along *in vitro* GIT digestion

Nanoemulsions were subjected to an *in vitro* gastrointestinal tract (GIT) digestion consisting of gastric and intestinal phases in order to determine their physical stability by monitoring their particle size, aggregation state (optic microscope images) and ζ-potential.

After the stomach GIT phase, particle size of Tween 20- and lecithin-stabilised nanoemulsions remained unchanged with respect to those undigested, regardless the emulsifier concentration.

The polyoxyethylene head group of Tween 20 together with the fact of not having charged groups (Teo et al., 2016) would have contributed on nanoemulsions stability under gastric conditions. Similarly, the formation of lamellar structures at the oil-water interface in lecithin-stabilised nanoemulsions would have conferred stability to the system (Pichot, Watson, & Norton, 2013). Stability of sodium caseinate-stabilised nanoemulsions after gastric conditions was defined by the protein concentration, being less stable those containing 2% of sodium caseinate (3.7 μm) compared with nanoemulsions containing higher concentrations at 4 and 8% (about 0.5 μm). It was suggested that oil droplets were covered by a thinner layer of protein at low concentrations of sodium caseinate, which in turn, was more prone to aggregation processes (flocculation) due to gastric conditions (pH 1-3, ionic strength 150 mM) as well as the presence of pepsin (Golding, Wooster, Day, Xu, Lundin, Keogh, & Cliftonx, 2011). Sucrose palmitate-stabilised nanoemulsions presented a notable increase in the particle size and aggregation processes after stomach GIT phase at all emulsifier concentrations (between 21.9 μm and 73.6 μm). Pepsin might have interacted with sucrose molecules, resulting in a change of orientation of the emulsifier at the interface of the droplets, which drove into aggregation phenomena (Verkempinck et al., 2017). In addition, sucrose palmitate molecules might have lost their charge because the pH of stomach phase (2.5) was below the pK_a of the carboxylic acid group from the palmitic acid (4.9) (Moran, Burczynski, Cheek, Bopp, & Forker, 1987; Rao & McClements, 2011). Therefore, droplets were more prone to aggregate because attractive forces were more dominant than the those repulsive.

After the intestinal GIT phase, nanoemulsions suffered a steep increase in their particle size except for sucrose palmitate-stabilised nanoemulsions, regardless the emulsifier concentration. Flocculated droplets formed during stomach GIT phase from these latter nanoemulsions might have been re-dispersed under intestinal conditions due to drastic pH change (from pH 1-3 up to 7) (Bellesi, Martinez, Pizones Ruiz-Henestrosa, & Pilosof, 2016). Large particle size formed in most nanoemulsions could be attributed to various reasons. First, partial or total displacement of emulsifier molecules from the droplet surfaces during lipid digestion processes, would have resulted in a single

emulsifier molecule attached to the surface of more than one droplet, provoking aggregation phenomena (bridging flocculation) (Mun, Decker, & McClements, 2007). Second, deposition of free fatty acids at the oil droplet interface and the presence of partially digested lipid droplets would have led to coalescence. Finally, all types of particles such as mixed micelles, vesicles and insoluble calcium complexes formed during the lipid digestion (Zhang, Zhang, Decker, & McClements, 2015b), would have contributed to the increase in the particle size.

Regardless the emulsifier nature and concentration, the ζ-potential of all nanoemulsions became less negative after the stomach GIT phase with values ranging from −24.2 to −1.5 mV. Simulated gastric fluids used in this study containing free ions (Na^+, K^+ and Cl^-) and their acidic pH (1-3), would have attenuated the charges of the nanoemulsion droplets (electrostatic screening effect) (Israelachvili, 2011). It should be noted that when the pH of the stomach phase (2.5) is below the isoelectric point of the proteins (sodium caseinate ≈4.5), a positive ζ-potential is expected. Interestingly, sodium caseinate-stabilised nanoemulsions presented negative ζ-potential. It was assumed that changes in the interface of the droplets, consisting of the displacement of sodium caseinate covering the droplets and the absorption of negatively charged particles, would have been the reason for the negative values. Furthermore, ζ-potential of all nanoemulsions became slightly more negative after being subjected to intestinal conditions, reaching values similar to those of undigested nanoemulsions. The production of different particles in the process of lipid digestion (undigested lipid droplets, vesicles or micelles) and the presence of digestion components (bile, pancreatin, and calcium) (Wu, Yan, Chen, & He, 2017) could have had an impact on the ζ-potential of nanoemulsions after the intestinal GIT phase. Simultaneous processes occurring during lipid digestion (enzyme hydrolysis, formation of new species, interactions between components, among others) resulted in constant changes in the interface of nanoemulsion droplets, affecting their electric charge. In addition, their ζ-potential would be similar to those undigested nanoemulsions since pH in both situations was neutral.

1.1.3. Effect of interfacial composition on lipid digestibility

Lipid digestion consists of the hydrolysis of oil droplets, which produces free fatty acids (FFAs), diacylglycerols (DAGs) and monoacylglycerols (MAGs). Several factors have been reported to have a strong impact on lipid digestion behaviour of nanoemulsions, such as initial particle size (Salvia-Trujillo et al., 2013), carrier oil type (Salvia-Trujillo et al., 2013b) as well as oil droplets aggregation state along the gastrointestinal tract (McClements, 2018). However, in our study it was suggested that initial particle size of nanoemulsions or stability/instability processes occurring along the different phases of the GIT were not associated with lipid digestibility results. Alternatively, emulsifier covering the oil droplets can restrict the action of enzymes, and therefore, lipid digestion processes might be incomplete. When comparing the emulsifier concentration, different profiles of free fatty acids release were observed among nanoemulsions. On one hand, Tween 20- and sucrose palmitate stabilised nanoemulsions presented an initial slow release of FFAs as emulsifier concentration increased from 2 to 8%. Indeed, these nanoemulsions presented an interval period at the beginning of the intestinal phase (40-60 minutes) where no free fatty acids were produced. This period might have been the time required for the bile to be placed on the surface of oil droplets and displace Tween 20 and

sucrose palmitate. On the other hand, lecithin- and sodium caseinate-stabilised nanoemulsions exhibited similar FFAs profiles during lipid digestion, suggesting that emulsifier nature and concentration did not affect release of FFAs.

At the end of intestinal GIT phase, all nanoemulsions presented a final lipid digestibility above 70%. The large particle size of certain nanoemulsions entering the intestinal phase (*i.e* sucrose palmitate-stabilised nanoemulsions) was supposed to restrict lipid digestion of nanoemulsions, but our results suggested that this large particle size was due to droplet flocculation (weak bonds linked the droplets together). As nanoemulsions were exposed to intestinal conditions, these bonds were broken down, resulting in the re-dispersion of droplets, which could have facilitated the digestion of lipids. Finally, adding lecithin to nanoemulsions from 2% to 8% significantly increased the total free fatty acids release, from ≈73% up to 100%. Accordingly to another study, the presence of phospholipids in simulated intestinal fluids increased the final extent of lipid digestion (Yang, Decker, Xiao, & McClements, 2015). In our study, lecithin, that predominantly contains phospholipids, would have facilitate the ability of lipase to interact with the emulsified triglycerides.

1.1.4. Effect of interfacial composition on bioaccessibility of β-carotene

Lipid digestion products together with bile salts produce mixed micelles, which incorporate and solubilise the released lipophilic functional ingredient. All these events taking place during lipid digestion will directly influence on the release of encapsulated lipophilic bioactive compounds from nanoemulsions to became available for absorption (bioaccessiblity).

According to lipid digestibility results of this study, lecithin-stabilised nanoemulsions presented a higher β-carotene bioaccessibility as emulsifier concentration increased from 2% to 8%, reaching bioaccessibilities of ≈24%. Tween 20-stabilised emulsions exhibited similar β-carotene bioaccessibility regardless the emulsifier concentration (≈16%), while for nanoemulsions containing sodium caseinate and sucrose palmitate, β-carotene's bioaccessibility significantly decreased with increasing the emulsifier concentration.

In order to explain these results, different hypotheses were proposed. First, not all the free fatty acids produced might have participated in the formation of mixed micelles because certain interactions with the digestion products may have occurred. Second, β-carotene might have not been incorporated within mixed micelles, not being available for absorption. Finally, a high number of free fatty acids produced in lecithin-stabilised nanoemulsions could lead to the formation of a large number of mixed micelles, enhancing β-carotene bioaccessibility. Mixed micelles are composed of bile salts, phospholipids from the bile and pancreatic juices as well as lipid digestion products from the action of lipases such as free fatty acids. Thus, lecithin predominantly contains phospholipids, could have contributed to the formation of these particles and increase their solubilisation capacity.

It should be note that even though nanoemulsions containing sodium caseinate at 8% exhibited similar FFAs release as lecithin nanoemulsions at 8% w/w (≈100%), bioaccessibility results were different. Indeed, nanoemulsions containing sodium caseinate at 8% exhibited the lowest values of bioaccessiblity (≈7%). Interactions between the β-carotene itself and/or mixed micelles containing β-carotene with the emulsifier could have occurred. Indeed, proteins are known to form complexes with carotenoids via hydrophobic interactions (Wackerbarth, Stoll, Gebken, Pelters, & Bindrich,

2009) and promote aggregation and precipitation of mixed micelles. Therefore, it was suggested that bioaccessibility values were defined by both emulsifier nature and/or their concentration in nanoemulsions instead of amount of free fatty acids generated at the end of the intestinal phase.

1.1.5. Effect of interfacial composition on delivery of β-carotene to the intestinal barrier

The use of differentiated monolayers (Caco-2 or Caco-2/HT29-MTX cocultures) expressing tight junctions, best represent the morpho-functional features of the intestinal barrier to evaluate the delivery of lipophilic bioactive compounds (Guri, Gülseren, & Corredig, 2013). Previously, it is important to determine the cytotoxicity of digestied nanoemulsions so as not to compromise the survival of cells during tissue culture studies.
Therefore, digested nanoemulsions stabilised with different emulsifiers (Tween 20, lecithin, sodium caseinate, sucrose palmitate) were diluted at different concentrations (between 2% v/v and 50% v/v) with Dulbecco's Modified Eagle Medium (DME) and applied to undifferentiated colon epithelial cell line (Caco-2 cells) in order to establish the maximum non-toxic concentration of digested nanoemulsions. Results indicated that emulsifiers were cytotoxic in a concentration-dependent manner and that toxicity of nanoemulsions depends on the nature of emulsifier employed. On one hand, digested nanoemulsions containing Tween 20 or sucrose palmitate were highly cytotoxic to cells at relatively low concentrations (<3% v/v). On the other hand, digested nanoemulsions with lecithin and sodium caseinate showed no cell toxicity (>90% cell viability) when Caco-2 cells were exposed to concentrations below 6% (v/v) and 10% (v/v), respectively. Therefore, lecithin and sodium caseinate were selected at 2% (w/w) to perform permeability experiment since they were allowed to be used at higher concentrations, which would facilitate β-carotene detection in subsequent analyses.

β-carotene content was determined in apical and basolateral samples as well as in cell lysates after 2h incubation (37 °C in a 5% CO_2 humidified atmosphere) in order to study the permeability of β-carotene across the intestinal barrier.
β-carotene cellular uptake in Caco-2 cells lysates was significantly higher in lecithin-stabilised nanoemulsions (2.28%) compared with nanoemulsions containing sodium caseinate (1.72%).
Proteins such as sodium caseinate, can interact hydrophobically with carotenoids and create complexes that act as a physical barrier for gastrointestinal digestive enzymes access (Wackerbarth, Stoll, Gebken, Pelters, & Bindrich, 2009). Hence, β-carotene may have remained entrapped within these complexes and not solubilised within mixed micelles, a process which is essential for permeability across the intestinal barrier (Baskaran, Sugawara, & Nagao, 2003). Moreover, Yang, Decker, Xiao, & McClements (2015) observed that the addition of 36 mg phospholipids (eg. 1,2-Dioleoyl-sn-glycero-3-phosphocholine) within the digestive fluids increased the degree of lipid digestion after *in vitro* digestion of vitamin E emulsions. Another study observed that the maximum cellular uptake of carotenoids (β-carotene and lutein) in differentiated Caco-2 cells was obtained when micelles contained 50 μmol/L of

lysophosphatidylcholine (phospholipid derived from phosphatidylcholine present in lecithin) (Sugawara et al., 2001).
When the same experiment was carried out with Caco-2/HT29-MTX co-cultures, cellular uptake of β-carotene was significantly lower (≤0.74%), with no significant differences between both emulsifiers. Co-culturing Caco-2 cells with HT-29MTX adds a further layer of mucus complexity to more closely resemble the *in vivo* environment (Arranz, Corredig, & Guri, 2016), but reduces permeability rates which may hamper compound detection.

β-carotene in basolateral fractions was not detected, indicating that it may not have arrived at the basolateral within 2h incubation period. However, detection of the target compound in basolateral compartment after tissue culture experiments by determining their bioactivity using other cells is an alternative that can be used. Therefore, to indirectly ascertain the β-carotene presence after permeability study, THP-1 macrophages were treated for 24 h with basolateral fractions and secreted levels of the major cytokines (TNF-α and IL-1β) were subsequently quantified using an ELISA kit.
Basolateral samples increased TNF-α secretion, while IL-1β levels were unchanged. A priori, the TNF-α response may suggest that β-carotene was not present in the basolateral fractions from nanoemulsions. In constrast, previous studies have concluded that β-carotene reduces levels of TNF-α, both *in vitro* (Kim et al., 2013) and *in vivo* (Bai et al., 2005). In addition, applying pure β-carotene directly to LPS stimulated THP-1 cells dose dependently reduced secreted levels of TNF-α. This discrepancy may be explained by the bypass of the gut and the use of different test material (β-carotene alone versus basolateral fractions of Caco-2 monolayers treated with β-carotene-enriched nanoemulsions). Indeed, β-carotene can be metabolised to high molecular weight products (β-apo-8'-carotenal, β-apo-10'-carotenal, β-apo-12'-carotenal, β-apo-14'-carotenal, β-apo-15'-carotenal) and short-chain products (hcyclocitral, β-ionone, ionene, 5,6-epoxi-β-ionone, dihydroactinidiolide and 4-oxo-ionone) (Siems et al., 2005), which themselves may directly or indirectly act as pro-inflammatory agents (Yeh, Wang, Chen, & Wu, 2009). Nevertheless, other digestive components in the micellar fraction might be capable of modulating TNF-α, although previous studies have shown that lecithin, sodium caseinate or emulsions with different fatty acid composition do not up-regulate cytokine production (Mukhopadhya et al., 2014; Reimund et al., 2004; Treede et al., 2009).

1.2. Mandarin fiber as a stabiliser in nanoemulsions

Dietary fibers consists of non-starch polysaccharides and other components such as cellulose, inulin, lignin, pectins and oligosaccharides among others, with many beneficial effects when consumed. Indeed, prevention, reduction and treatment of chronic diseases (gastrointestinal disorders, cancer, cardiovascular disease), as well as promotion of physiological functions (lowering blood triglycerides and glucose control) are some of the important benefits of dietary fibers (Figuerola, Hurtado, Estévez, Chiffelle, & Asenjo, 2005). Processing of citrus fruits represents the obtention of by-products such as mandarin fiber, that can act as potential nutraceutical resources. Due to their easy availability, such by-products can offer a low-cost

nutritional source for the production of novel nutraceuticals. In addition to health benefits, dietary fibers presents functional properties, including water holding capacity, viscosity or gel formation, emulsion stabilisation as well as enhancer of shelf-life (Rafiq et al., 2018).

Therefore, dietary fibers are important components of the human diet that can be applied in emulsion-based foods thanks to their stabilizing, texturizing properties, as well as health-promoting benefits (Dikeman & Fahey, 2006; Eastwood & Morris, 1992; Elleuch et al., 2011). However, its consumption in certain amounts may inhibit lipid absorption, causing a decrease on the bioaccessibility of health-related compounds easily absorbed when lipids are present (Torcello-Gómez & Foster, 2016).

This study (chapter III) was carried out with a β-carotene-loaded nanoemulsion previously formulated within the research group (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013). With the goal of strengthen the nutritional properties of emulsion-based systems as well as assist on their stabilisation, different concentrations of mandarin fiber (0, 0.5, 1, 1.5 or 2%) were added to this β-carotene-loaded nanoemulsion. Then, the effect of mandarin fiber addition to nanoemulsions stability along the gastrointestinal tract (GIT), their lipid digestibility and β-carotene bioaccessibility were determined.

1.2.1. Effect of mandarin fiber addition on stability of nanoemulsions along an *in vitro* gastrointestinal (GIT) digestion

Although dietary fibers are thickening agents, they can also be partially adsorbed at the oil-water interface in certain conditions and modify interfacial properties of emulsions (Dickinson, 2009). Such adsorption was not expected since nanoemulsions of this study were formulated with a strong amphiphilic surfactant (Tween 20). Mandarin fiber contained high molecular weight components (mainly pectin), whereas Tween 20 is a low-molecular weight surfactant (MW≈1228 g/mol), which would have been rapidly absorbed to oil droplets interface during nanoemulsions formation (McClements & Gumus, 2016).

Adding mandarin fiber did not appreciably change the initial physicochemical properties of nanoemulsions, although a slight increase of particle size from ≈168 nm to ≈176 nm was observed when they contained mandarin fiber concentrations over 1%. Because of the thickening and gelling capacity of dietary fibers (Mesbahi, Jamalian, & Farahnaky, 2005), viscosity of nanoemulsions significantly raised from 1.08 m·Pas (nanoemulsions 0-0.5% mandarin fiber) up to values ≥3 m·Pas (nanoemulsions with mandarin fiber ≥1%). This viscosity enhancement might have lowered the efficiency of the high-pressure homogenizer device used to produce tiny oil droplets during nanoemulsions formation (Jafari, Assadpoor, He, & Bhandari, 2008; Qian & McClements, 2011). Alternatively, pectin molecules from mandarin fiber might have induced depletion flocculation and subsequent coalescence in emulsions, thus resulting in particle size increase (McClements, 2000; McClements, 2005; Neumann, Schmitt, & Iamazaki, 2003). Finally, pectin might have certain surface activity at oil-water interface since it contains functional units (acetyl and methyl groups). These groups have interfacial activity and facilitate adsorption of pectin chains at the interface of oil droplets.

Hence, pectin chains present in the aqueous phase can compete with Tween 20 molecules, and cause the displacement of these previously-adsorbed surface active species, resulting in less effectiveness of interfacial tension reduction (Alba & Kontogiorgos, 2017; Jafari, He, & Bhandari, 2007).

All nanoemulsions presented negative ζ-potential, which was attributed to Tween 20 employed for nanoemulsions formation. Some studies have concluded that non-ionic surfactants tended to have a considerable negative charge thereby the presence of free fatty acids not only from the oil, but also from the surfactant, as well as the adsorption of hydroxyl ions (OH-) present in the aqueous phase of nanoemulsions (Chang & McClements, 2016; Mun, Decker, & McClements, 2005).

Addition of negatively charged mandarin fiber molecules to nanoemulsions resulted in less negative ζ-potential values, changing from -25 mV to ≈-17 mV. Due to the electrostatic repulsion between the negatively oil droplets and anionic molecules of mandarin fiber, it was assumed that mandarin fiber remained unabsorbed in the aqueous phase of nanoemulsions. Indeed, the fact that ζ-potential was less negative as mandarin fiber was added to nanoemulsions, might indicate that mandarin fiber remained unabsorbed in the aqueous phase rather than in the interface of the oil droplets. The ζ-potential in such systems is determined by the Brownian motion of the droplets when voltage is applied, indicating that the higher the absolute value of ζ-potential, the higher is the electrophoretic mobility of droplets. Assuming that mandarin fiber was in the aqueous phase of nanoemulsions, oil droplets moved slower and therefore, the ζ-potential of nanoemulsions containing mandarin fiber was less negative. Alternatively, the electrophoretic mobility of droplets is directly influenced by particle size of oil droplets. As observed before, addition of mandarin fiber resulted in an increase of nanoemulsions particle size, resulting in a slower movement of the oil droplets and thus, to a less negative ζ-potential (Celus et al., 2018).

Behaviour of nanoemulsions during *in vitro* simulated gastrointestinal tract (GIT) digestion might be used as supportive information to understand later lipid digestion processes. It was hypothesised that interactions of nanoemulsions with digestive components might be different when mandarin fiber was present, thereby altering interfacial properties and aggregation state of nanoemulsion oil droplets. In this study, nanoemulsions with different concentrations of mandarin fiber behaved similar to that without mandarin fiber throughout the different phases of the GIT. In general, particle size remained unchanged during oral and gastric phase while a steep increase was observed after intestinal phase. The formation of a high density and self-organised Tween 20 monolayer around oil droplets (Shen, Guo, & Zhu, 2011), as well as the hydrophilic head group that forms the molecule, would have prevented instability processes in upper gut phases. After intestinal phase though, particle size of all nanoemulsions was increased regardless the mandarin fiber concentration. However, the extent of particle size growth was subjected to the amount of dietary fiber initially added. Indeed, particle size after intestinal phase was lower for nanoemulsions containing mandarin fiber concentrations below 1% (≈300 nm) compared to those with higher concentrations (≥1.5%) (≈500 nm). Complex structures and interactions might have been formed between all the components from the emulsion and digestive fluids, contributing to the large particle

sizes. Furthermore, high concentrations of mandarin fiber might have formed hydrogel structures in the presence of calcium ions within the small intestinal fluids (Chang & McClements, 2016).
Regarding the ζ-potential, all nanoemulsions presented similar values after oral phase (≈ -20 mV), while a decrease until values close to 0 mV was observed in gastric phase. Simulated gastric fluids have a low pH (≈2.5) and high ionic strength (150 mM) (Golding & Wooster, 2010), which may result in a reduction in droplet ζ-potential due to electrostatic screening effects (Israelachvili, 2011). This effect consists on the accumulation of counter-ions around the droplet surfaces which results in values of ζ-potential less negative. It should be noted that although droplets were not charged enough to prevent attractive forces, no evidence of droplet aggregation was observed after stomach GIT phase in these nanoemulsions. The continuous agitation during this stage might have produced a redistribution of flocculated droplets (reversible), reducing particle aggregation (Golding, Wooster, Day, Xu, Lundin, Keogh, & Clifton, 2011). Meanwhile, all nanoemulsions presented high negative charges after intestinal phase (between -18 mV and -21 mV). Surface-active species (bile salts, free fatty acids, enzymes) would have displaced Tween 20 from the droplets surface, contributing on constant changes at the interface. In addition, the presence of various types of anionic particles in the digested fractions, such as undigested lipid droplets, micelles, or vesicles might have also contributed on negative charges.

1.2.2. Effect of mandarin fiber addition on lipid digestibility and β-carotene bioaccessibility of nanoemulsions

In scientific literature, there are contradictory results whether presence of dietary fibers in emulsions might either slow down (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, Narváez-Cuenca, & McClements, 2014; Zhang, Zhang, Zhang, Decker, & McClements, 2015a) or enhance lipid digestion processes (Qiu, Zhao, Decker, & McClements, 2015). In our study, adding mandarin fiber to nanoemulsions decreased the final amount of free fatty acids (FFA) released, from 74.6% to ≈60%, without statistically significant differences among nanoemulsions with different mandarin fiber concentrations. Dietary fibers may alter lipid digestion in a number of ways: (i) binding free calcium ions thereby reducing the amount available to remove long chain FFAs from droplet surfaces: (ii) forming a protective coating around the droplets, thereby inhibiting access of lipase to the encapsulated lipids; (iii) increasing the viscosity of the aqueous phase, thereby reducing mixing and diffusion processes; (iv) altering floc formation and structure, thereby influencing the access of lipase to the lipid droplet surfaces (Espinal-Ruiz et al., 2014).
Lipid digestibility is closely associated to bioaccessiblity of lipophilic compounds since lipid digestion products (free fatty acids and monoacilglycerols) determine the total amount of lipophilic compound that can be incorporated within mixed micelles formed (Nik, Langmaid, & Wright, 2012). Based on lipid digestibility results, it could be expected a decrease in β-carotene bioaccessibility when adding mandarin fiber to nanoemulsions. Interestingly, bioaccessibility of β-carotene increased from 32.8% (nanoemulsion without mandarin fiber) up to ≈40% with the addition of low mandarin fiber concentrations (≤1%). On the contrary, higher concentrations of mandarin fiber led to low bioaccessibility levels, being 18.7% and 18.0% for nanoemulsions containing 1.5% and 2% of

mandarin fiber, respectively. These results suggest that when mandarin fiber was added to β-carotene-loaded nanoemulsions above a certain concentration, β-carotene bioaccessibility was reduced. Cervantes-Paz et al. (2016) observed that low pectin concentrations (0.14%) favoured carotenoid incorporation to mixed micelles, while pectin at higher concentrations (1%) significantly reduced carotenoid micellarization. Specifically, they concluded that low concentrations of pectin with low molecular weight and degree methyl-esterification reduced the negative effect of pectin on carotenoid micellarization. However, bile salts may bind to fiber molecules and become entrapped in a gel structure instead of participating in micelle formation, reduce the number of mixed micelles formed during lipid digestion and therefore, decrease bioaccessiblity of encapsulated compounds (Verrijssen, Verkempinck, Christiaens, Van Loey, & Hendrickx, 2015).

In general, it has been concluded that the role of dietary fiber in lipid digestion as well as bioaccessibility processes is unknown and it might be subjected to the properties of pectins, including length, embranchment, degree methyl-esterification, molecular weight, hydrophobicity, among others.

2. Tertiary (multi-layer) emulsions

Multi-layer emulsions are oil-in-water emulsions with oil droplets covered by at least two layers of biopolymers electrostatically charged (polyelectrolytes). Assembly of layers is based mostly on electrostatic interactions of polyelectrolytes, although other forces such as steric might also influence on stability of the system. Furthermore, oil concentration is one critical point when designing multi-layer emulsions, since each layer deposition involves the dilution of the sample. When these systems are envisaged for delivering lipophilic bioactive compounds, the low oil concentration will allow incorporating only small amounts of bioactive compound within oil droplets. From a technical point of view, working with low oil concentrations in multi-layer emulsions makes difficult to assess their potential bioaccessibility after simulated i*n vitro* digestion.

In this regard, chapter IV of the present doctoral thesis consisted on developing a β-carotene-enriched multi-layer emulsion with three interfacial layers containing high oil concentration (2.5% w/w). The polyelectrolytes combination for developing primary (lactoferrin), secondary (lactoferrin-alginate) and tertiary (lactoferrin-alginate-ε-poly-L-lysine) emulsions were selected based on a previous multi-layer emulsion formulated in our group consisting of: 0.0002% resveratrol, 0.09% corn oil, 0.03% lactoferrin, 0.02% alginate and 0.036% ε-poly-L-lysine (Acevedo-Fani, Silva, Soliva-Fortuny, Martín-Belloso, & Vicente, 2017). However, the suitable polyelectrolyte concentration for each interfacial layer deposition was optimized since the emulsions contained different oil concentration.

2.1. Effect of polyelectrolytes concentration on formation of tertiary emulsion systems

Multi-layer emulsions fabrication is a delicate procedure as instability processes (e.g. bridging flocculation or depletion flocculation) could occur when an excess or lack of polyelectrolyte molecules is present (Guzey & McClements, 2006). In this study, different concentrations of polyelectrolytes for a tertiary emulsion formation (lactoferrin-alginate-ε-poly-L-lysine) were tested and then, physicochemical properties (particle size and ζ-potential) as well as protein surface load were measured in order to choose the suitable concentration for avoiding instability processes and obtain a stable system.

2.1.1. Primary emulsion: effect of lactoferrin concentration

Protein surface load consisted on determining the minimum concentration of lactoferrin needed to create a coating on the oil droplets surface of a primary emulsion and therefore, avoid instability processes. Surface load of lactoferrin reached its maximum value (10.22 mg/m^2) when primary emulsions contained a lactoferrin concentration of 5% w/w, which in turn, presented the lowest particle size (0.29 μm). The high molecular weight of lactoferrin (around 80 kDa) together with the high positive ζ-potential (+51.9 mV), would have stabilised oil droplets by a combination of both steric and electrostatic forces (Tokle & McClements, 2011). Typically, protein concentration employed to stabilise emulsions ranges between 1 and 3% w/w. Using higher concentrations of protein might be related to the fact that the primary emulsions prepared in this work contained high amount of oil (20% w/w), which would require more amount of lactoferrin to cover the entire surface of droplets. In addition, not all the proteins have the same properties/structure, so their deposition at the interface of droplets might change. For instance, Lesmes, Sandra, Decker, & McClements, (2010) observed that more amount of lactoferrin was required to saturate the oil droplets compared with caseinate, due to the different affinity that both proteins have for oil droplets surfaces, which was associated to their structure (flexible vs globular) and molecular weight (low vs high).

2.1.2. Secondary emulsion: Effect of alginate concentration

The mininum particle size of secondary emulsions was observed at 1% w/w alginate (0.45 μm) and adding concentrations of alginate under or above 1% w/w resulted in a significant increase of the particle size (≈ 0.6 μm), which was confirmed with optical microscope images (droplet aggregation). On one hand, when the alginate content in the emulsion was insufficient to cover the entire droplet surface, a single molecule of alginate would have been adsorbed at the surface of different droplets simultaneously, linking them together and resulting in large particle size (bridging flocculation) (Guzey & McClements, 2006). On the other hand, depletion flocculation effect driven by an excess of alginate would have been induced by the unabsorbed molecules surrounding the droplets, resulting in attractive forces and consequent droplet aggregation (Zeeb, Thongkaew, & Weiss, 2014). Adding alginate to primary emulsions resulted in a change of ζ-potential from positive (+52 mV) to negative values (-56 mV). As alginate (anionic) and lactoferrin (cationic) have opposite

charges, electrostatic interactions between both polyelectrolytes would have resulted in the deposition of alginate at lactoferrin-coated oil droplets, forming secondary emulsions.

2.1.3. Tertiary emulsion: effect of ε-poly-L-lysine concentration

Tertiary emulsions presented a similar behaviour to secondary emulsions when ε-poly-L-lysine was added to them. On one hand, tertiary emulsions with a low ε-poly-L-lysine concentration (≤0.15% w/w) presented particle sizes around 0.30 μm, whereas adding concentrations up to 0.17% w/w significantly increased the particle size of emulsions (≈1.85 μm). Furthermore, ζ-potential of tertiary emulsions were less negative (−9.36 mV) when they contained up to 0.17% w/w of ε-poly-l-lysine. These results indicated that there were not enough ε-poly-l-lysine molecules covering the lactoferrin/alginate-coated droplets and that alginate was the polyelectrolyte prevalent at the droplets interface. On the other hand, when adding higher concentrations of ε-poly-l-lysine (≥0.18% w/w), a positive ζ-potential was achieved (above +15 mV), which revealed that cationic ε-poly-L-lysine molecules would have successfully deposited to lactoferrin/alginate-coated droplets. However, when tertiary emulsions contained concentrations of ε-poly-L-lysine over 0.19% w/w, a steep increase in particle size until 14 μm and droplet aggregation was detected (optical microscope images). High ε-poly-l-lysine concentrations in tertiary emulsion would have favoured depletion flocculation and thus, droplet aggregation (Benjamin, Silcock, Leus, & Everett, 2012). However, when tertiary emulsions contained concentrations of ε-poly-l-lysine between 0.18% w/w and 0.19% w/w, a low particle size was observed (0.59 μm and 0.58 μm, respectively). Furthermore, these emulsions exhibited high and positive ζ-potential indicating that ε-poly-l-lysine had been deposited at the droplets surface.

The tertiary emulsion chosen for the following experiments contained 0.0625% w/w β-carotene, 2.5% w/w corn oil, 0.62 % w/w lactoferrin, 0.25% w/w alginate and 0.18% w/w ε-poly-L-lysine.

2.2. Effect of external stressing conditions on stability of tertiary emulsions

In terms of applicability, multi-layer emulsions can be added to food and beverages so as to improve their nutritional quality. Processing and manufacturing conditions, as well as food characteristics might affect multi-layer emulsions properties. In this sense, it is important to understand stability/instability patterns of the emulsions to visualise their behaviour in further processing steps. Thus, investigating how different external conditions affect stability of these emulsions would provide information about in which type of foodstuffs would be better to use multi-layer emulsions. Hence, previously optimized tertiary emulsions containing β-carotene were subjected at different external stress conditions including heat treatment, acidic and basic environments as well as ionic strength changes.

Multi-layer emulsions stability is achieved by a combination of electrostatic and/or steric repulsion forces between droplets. These repulsion forces are provided by the polyelectrolytes forming the interfacial layer around the oil droplets, preventing to come closer to each other and therefore droplet-droplet interactions. However, polyelectrolytes are highly susceptible to

undergone structural and/or charge changes under different environments, thereby affecting to emulsion physical stability (Guzey & McClements, 2006). In this study, instability processes in tertiary emulsions were observed when they were subjected at high temperatures (≥70ºC), acidic conditions (pH≤5) or NaCl concentration over 0.1M. Destabilisation processes of tertiary emulsions were reflected by either a particle size increase, aggregation phenomena (optical microscope images) and ζ-potential changes.

Thermal denaturation of lactoferrin (between 70ºC and 85ºC) contained in tertiary emulsions could have derived into protein unfolding, thus promoting hydrophobic attraction between droplets (Teo, Lee, & Goh, 2017) and therefore, a particle size increase (14 μm). High temperatures (90ºC) might have changed ε-poly-L-lysine structure, particularly to β structures (Mirtič & Grdadolnik, 2013), characterised to have a compact structure and thus, less ε-poly-L-lysine amine groups charged providing electrostatic forces to droplets of tertiary emulsions (≈ +3 mV) (Lee, 2016).

The pH of all the solutions when developing multi-layer emulsions is carefully selected so as the polyelectrolytes are sufficiently charged to be adsorbed at the interface of the droplets and form the interfacial layers of multi-layer emulsions. Changes in pH might also be associated with instability processes since the degree of polyelectrolytes absorption at the droplets interface, their interfacial deposition and electrostatic and/or steric repulsion forces might be modified. This behaviour is explained because pKα of polyelectrolytes are extremely related to pH. In our study, under acidic environments the pH of emulsions (≤4) was below the pKα of ε-poly-L-lysine (≈9) and close to alginate pKα (≈3.5) (Lee & Mooney, 2012). In this situation, electrostatic attraction between ε-poly-L-lysine (highly charged) and alginate (partially uncharged) would have been reduced, leading to a weak attachment between both components. These results suggested that the thickness of the interface was reduced, and ε-poly-L-lysine stabilisation via steric repulsion was not enough to overcome attractive forces, leading to droplet aggregation (≈11μm). On the contrary, when basic conditions were present, ε-poly-L-lysine lost its charge (pKα ≈9) and thus the electrostatic attraction with alginate might have been reduced, leading to a detachment of ε-poly-L-lysine from alginate-covered droplets. In this case, alginate was the polyelectrolyte that predominated at the interface of the droplets, contributing on the obtained negative ζ-potential (-43 mV). Interestingly, no particle size increase was observed in this situation, which might be attributed to the thick and charged layer of alginate covering the droplets, providing both steric and electrostatic stabilisation to the system (McClements, 2004). Under neutral pH conditions, tertiary emulsions presented very low or almost no surface charge (≈ +2 mV) and no droplet aggregation (microscope images). In this case, stability of these tertiary emulsions was not determined by charge (electrostatic repulsion), but by the polyelectrolyte coating around the oil droplets (steric repulsion).

Finally, the characteristics of a polyelectrolyte may also be altered ionic strength changes in the aqueous solution of multi-layer emulsions. Increasing the ionic strength weakens the attractive interactions between polyelectrolyte molecules in different layers thereby causing an increase in interfacial porosity. In this study, the presence of ions in the aqueous solution from NaCl (Cl^-) might have interacted with positively charged amino groups of ε-poly-L-lysine molecules, leading to either a partial desorption of ε-poly-L-lysine from the droplets surface and a charge

reduction (+ 3.7 mV). Consequently, poor electrostatic and steric stabilisation of oil droplets in tertiary emulsions might have derived into attractive forces between droplets, contributing on particle size increase (≥ 3 μm).

The β-carotene content in processed foodstuffs is essential in nutritional and commercial terms for the industry since fruits and vegetables are the principal sources of β-carotene and vitamin A precursor. Therefore, it is important to understand the influence of external stresses on the stability of encapsulated β-carotene, as it is expected to maintain its functionality under a wide range of conditions, such as processing, storage, transport, and application of emulsion-based delivery systems. However, β-carotene is susceptible to degradation and oxidation due to its unsaturated structure (Pénicaud, Achir, Dhuique-Mayer, Dornier, Bohuon, 2010). Indeed, it has been reported that β-carotene contact with oxygen, light and high temperatures dramatically increases its degradation rates.

In the present study, β-carotene was constantly degraded at temperatures above 40 °C, reaching a maximum content of 50% when tertiary emulsions were subjected at extreme temperatures (90ºC). In addition, β-carotene content was reduced when pH changed from the original pH employed to form the tertiary emulsion (≈5) to either acidic (49% of β-carotene) or basic conditions (28% of β-carotene). Finally, β-carotene content significantly decreased to values under 10% at NaCl concentrations over 0.3 M, which might be related to instability processes observed under these conditions.

On one hand, the β-carotene degradation observed when tertiary emulsions were exposed under different stress condition can be explained by different reasons. In general, carotenoids may degrade through various mechanisms, such as oxidation and isomerization, depending on their composition and storage conditions (Boon et al., 2010). The different external stress conditions at which tertiary emulsions were subjected, might be related to structural changes of the polyelectrolytes adsorbed at oil droplets surfaces, thereby altering their ability to stabilise and protect the encapsulated β-carotene (Qian, Decker, Xiao, McClement, 2012; Davidov-Pardo, Gumus, McClements, 2016).

On the other hand, the interfacial layers of tertiary emulsion might act as a physical barrier to avoid the interaction between β-carotene and free radicals and/or oxygen, which would lead to compound degradation. In addition, the antioxidant properties from the polyelectrolyte layers forming tertiary emulsions (lactoferrin, alginate and ε-poly-l-lysine) (Huang, Satué-Gracia, Frankel, & German, 1999; Xue, Yu, Hirata, Terao, & Lin, 1998; Scheffler, Wang, Huang, Gonzalez, & Yao, 2010) would have avoid the total loss of β-carotene present in such emulsions.

2.3. Effect of interfacial layers on lipid digestibility and β-carotene bioaccessibility

There is a controversy over whether the number layers covering multi-layer emulsions have an impact on lipolysis during *in vitro* digestion, thereby decreasing (Silva et al., 2018) or increasing (Silva et al., 2019) bioaccessibility of lipophilic compounds.

In this study, the rate and extent of free fatty acids (FFA) released was higher as the number of the layers in the emulsion increased: primary emulsion (34.61%), secondary emulsion (61.03%) and tertiary emulsion (83.79%). Lipid digestion might have been influenced by the structure and composition of adsorbed polyelectrolytes of emulsions. Specifically, structural changes in oil droplets interface as well as physical stability of tertiary under the *in vitro* GIT digestion process might have contributed on facilitating lipid digestion of such emulsions. Although tertiary emulsions were covered by at least three polyelectrolyte layers, the acidic environment during the stomach phase emulsions (Golding, Wooster, Day, Xu, Lundin, Keogh, & Clifton, 2011), the neutral pH of intestine (Acevedo-Fani, Salvia-Trujillo, Soliva-Fortuny, & Martín-Belloso, 2015) as well as proteolytic enzimes (chymotrypsin and trypsin) (Pilosof, 2017), would have displaced the different layers covering the oil droplets, facilitating the action of lipases.

Accordingly, β-carotene bioaccessibility significantly increased as emulsions had more interfacial layers, rising from 30.24% and 35.26% (primary and secondary emulsions, respectively) to 70.1% for tertiary emulsions.

In order to explain the high β-carotene bioaccessibility obtained in this study, a combination of different hypothesis was proposed. First, a high lipid digestibility is positively correlated to great bioaccessibility results. Thus, the relatively high free fatty acids liberated during intestinal phase in tertiary emulsion (83.79%), might have indicated that lipids from this emulsion were hydrolysed and that β-carotene was released from oil droplets. Secondly, bioaccessibility might have been favoured because the presence of lipid content in tertiary emulsions was not in excess. The ratio of lipase-to lipid and bile-to-lipid decreases as the lipid content increases, which might negatively affect lipid digestion and therefore, β-carotene bioacessiblity (Li, Hu, & McClements, 2011). In this study, primary and secondary emulsions had a lower lipase-to-lipid and bile-to-lipid ratio compared to tertiary emulsions. Thus, high number of mixed micelles to incorporate the released β-carotene would have been generated during lipid digestion of tertiary emulsions. Third, antioxidant effects from lactoferrin (Huang, Satué-Gracia, Frankel, & German, 1999), alginate (Xue, Yu, Hirata, Terao, & Lin, 1998) and ε-poly-L-lysine (Scheffler, Wang, Huang, Gonzalez, & Yao, 2010) molecules would have protected β-carotene from degradation along the *in vitro* simulated gastrointestinal tract, but mostly from the acidic environment in stomach phase.

The high β-carotene bioaccessibility observed for tertiary emulsions suggest that most of the β-carotene released from the lipid phase was solubilised within the mixed micelles formed during the intestinal digestion. Compared to other studies (Pinheiro, Coimbra, & Vicente, 2016; Silva et al., 2018; Tokle, Mao, & McClements, 2013; C. Zhang et al., 2015), our bioaccessibility results were substantially greater. For instance, Tokle et al. (2013) obtained a β-carotene bioaccessibility below 2% for a multi-layer emulsion containing a similar amount of oil (2%) but with different emulsifiers (lactoferrin/β-lactoglobulin/lactoferrin). This low bioaccessibility results were attributed to the interaction between lactoferrin and β-carotene, remaining β-carotene entrapped in the sediment phase and not being solubilised within mixed micelles.

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CONCLUSIONS

Based on the results obtained in the present Doctoral Thesis and from their interpretation, it can be concluded that composition of emulsion-based delivery systems has a great impact on their physicochemical properties and stability along gastrointestinal tract. In addition, bioaccessibility of β-carotene encapsulated in both oil-in water-nanoemulsions and tertiary emulsions was directly affected by their interfacial composition.

Oil in water nanoemulsions: effect of interfacial composition

- All nanoemulsions presented particle sizes within the nanometric range as well as negative ζ-potential, irrespective of the emulsifier nature and concentration used to form nanoemulsions. When emulsifier concentration was increased from 2% to 8% (w/w), those nanoemulsions containing Tween 20, lecithin and sodium caseinate efficiently decreased their particle size.

- In general, Tween 20- and lecithin-stabilised nanoemulsions were physically stable under gastric conditions, while nanoemulsions containing sodium caseinate and sucrose palmitate presented a notable increase in particle size through stomach phase. After intestinal phase, all nanoemulsions suffered a steep increase in their particle size, except for sucrose palmitate-stabilised nanoemulsions. The ζ-potential of nanoemulsions along the gastrointestinal tract was similar, regardless the emulsifier nature and concentration.

- Different free fatty acids release profiles were observed during intestinal digestion, depending on the emulsifier nature and concentration used to form nanoemulsions. Tween 20- and sucrose palmitate-stabilised nanoemulsions presented an initial slow release of free fatty acids (FFAs) as emulsifier concentration increased from 2 to 8% w/w, while lecithin- and sodium caseinate-stabilised nanoemulsions exhibited similar FFAs profiles during lipid digestion. At the end of intestinal GIT phase, all nanoemulsions presented a lipid digestibility above 70%. Adding lecithin to nanoemulsions from 2% to 8% significantly increased the total free fatty acids release, from ≈73% up to 100%.

- Lecithin-stabilised nanoemulsions presented a higher β-carotene bioaccessibility as emulsifier concentration increased from 2% to 8% w/w, reaching bioaccessibilities of ≈24%. Therefore, bioaccessibility results were defined by emulsifier concentration in nanoemulsions instead of amount of free fatty acids generated at the end of the intestinal phase. This study revealed that using lecithin could be a good option when designing nanoemulsions as delivery systems of lipophilic compounds.

- Delivery of β-carotene to the intestinal barrier (Caco-2 cells) was significantly higher when nanoemulsions were stabilised with lecithin compared with sodium caseinate. When co-culture of Caco-2/HT29-MTX was employed as a cell culture model, a reduction in the cellular uptake was observed for both emulsifiers, with no significant differences between them.

Oil in water nanoemulsions: effect of mandarin fiber addition

- Mandarin fiber was successfully incorporated to β-carotene nanoemulsions stabilised with Tween 20. Addition of mandarin fiber to this system resulted in a slight increase of particle size as well as in less negative ζ-potential of nanoemulsions. Because of the thickening and gelling capacity of dietary fibers, viscosity of nanoemulsions significantly raised from 1.08 m·Pa up to values ≥3 m·Pas when mandarin fiber was added at concentrations over 1%.

- Particle size of nanoemulsions stabilised with mandarin fiber remained unchanged during oral and gastric phase while a steep increase was observed after intestinal phase. However, the extent of particle size growth was subjected to the amount of dietary fiber initially added. Regarding the ζ-potential, nanoemulsions presented similar values after oral phase, while a decrease until values close to 0 mV was observed in gastric phase. Finally, nanoemulsions presented highly negative charges after incubation in the small intestine phase.

- Adding mandarin fiber to nanoemulsions produced a decrease of free fatty acids (FFAs) release during the intestinal digestion from 74.6% to ≈60%. However, β-carotene bioaccessibility appreciably increased from 32.8% (nanoemulsion without mandarin fiber) up to ≈40% with the addition of low mandarin fiber concentrations (≤1%). On the contrary, higher concentrations of mandarin fiber (>1.5%) led to low bioaccessibility levels (≈18%).

Tertiary (multi-layer) emulsions: effect of interfacial layers

- A selection of the optimal polyelectrolyte concentration of each layer was essential in order to avoid destabilization processes. Stable tertiary emulsion (lactoferrin/alginate/ε-poly-L-lysine) was successfully developed containing; 0.0625% (w/w) β-carotene, 2.5% (w/w) corn oil, 0.62% (w/w) lactoferrin, 0.25% (w/w) alginate and 0.18 (w/w) ε-poly-L-lysine.

- Instability processes in tertiary emulsions were observed when they were subjected at high temperatures (≥70ºC), under acidic conditions (pH≤5) or at NaCl concentration over 0.1M. Therefore, tertiary emulsions of this work would be most suitably used in neutral foods and beverages, in thermal processes below 60 °C as well as food products without salt. However, content of β-carotene decreased around 80% when tertiary emulsions were subjected at different stress conditions.

- The rate and extent of free fatty acids (FFAs) released during lipid digestion was higher as the number of the layers in the emulsion increased (primary emulsion< secondary emulsion<tertiary emulsion). Although the numerous polyelectrolyte layers covering the oil droplets, tertiary emulsions presented a greater β-carotene bioaccessibility (≈70%) compared to primary and secondary emulsions (≈30%).

FUTURE RESEARCH

The results obtained in this Doctoral Thesis have shown that emulsion-based delivery systems, can be used as carriers of lipophilic bioactive compounds, such as β-carotene. Nevertheless, there is a lack of information about the application of these systems in the food and beverage industry. Therefore, further investigation for emulsion-based delivery systems are described below:

- Use more accurate *in vitro* methods, thereby including the large intestine step in order to study digestion and fermentation processes

- Validate the efficacy of nanoemulsions and multi-layer emulsions as delivery systems of lipophilic bioactive compounds using *in vivo* models.

- Incorporate emulsion-based delivery systems along different processes of the food supply chain, such as processing, storage and transport, as well as describe their interactions.

- Evaluate the shelf-life and functional properties of food products enriched with emulsion-based delivery systems.

- Evaluate consumers' acceptance of food products containing emulsion-based delivery systems.

- Assess the accumulation, toxicity and long-term consumption of food products containing delivery systems.