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Development of emulsion-based delivery systems to improve the functionality of bioactive compounds *in vitro* and *in vivo*

Júlia Teixé Roig

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Universitat de Lleida

TESI DOCTORAL

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Development of emulsion-based delivery systems to improve the functionality of bioactive compounds *in vitro* and *in vivo*

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RESUM

Els compostos bioactius han mostrat tenir propietats biològiques diverses que aporten beneficis per a la salut humana. Desafortunadament, aquests compostos són degradats fàcilment i presenten una baixa absorció i biodisponibilitat. La investigació duta a terme en els últims anys ha revelat que els sistemes basats en emulsions (nanoemulsions, emulsions múltiples, emulsions altament concentrades, nanopartícules, etc.) són útils per a protegir aquests compostos, i augmentar-ne l'absorció. Tanmateix, sembla que la composició d'aquests sistemes pot tenir un gran impacte en les seves propietats i la seva funcionalitat. Per tant, l'objectiu d'aquesta tesis doctoral va ser estudiar com la composició (tipus d'emulsionant, concentració d'oli, presència de polisacàrids, tipus i estat físic de l'oli) afecta les propietats i estabilitat de diferents tipus d'emulsions, així com en la bioaccessibilitat i biodisponibilitat dels compostos bioactius encapsulats.

En general, es va observar que utilitzant emulsionants proteics (proteïna del sèrum i caseïnat sòdic) es van obtenir nanoemulsions amb una mida de partícula menor i una càrrega elèctrica menys negativa que utilitzant emulsionants lipídics (lecitina de soja), tot i que l'efecte va dependre d'altres paràmetres com la concentració d'oli. A més, les nanoemulsions que contenien emulsionants proteics van presentar una major digestibilitat lipídica, degut a les gotes de mida reduïda que presentaven a l'intestí, i una major bioaccessibilitat del compost *in vitro* (≈ 64% en el cas del β-carotè i ≈ 67% en el cas de la curcumina). Com a conseqüència, després de la seva administració oral en rates, les nanoemulsions proteiques van augmentar l'absorció del β-carotè i la biodistribució de la seva forma activa (retinol) en diferents òrgans, especialment al fetge (≈ 3250 ng/g). A més, es va observar que l'augment de la concentració d'oli en les nanoemulsions disminuïa la digestibilitat del sistema, i en conseqüència, la bioaccessibilitat del compost encapsulat. L'efecte de l'addició de polisacàrids va variar en funció del tipus utilitzat. L'addició de pectina va disminuir l'estabilitat de les nanoemulsions i va augmentar la bioaccessibilitat del β-carotè (en un 12%). En canvi, l'addició d'alginat va incrementar notablement l'estabilitat del sistema i el compost durant la digestió, però va reduir la digestibilitat lipídica i la bioaccessibilitat de la curcumina (en un 40%).

En emulsions dobles, l'efecte del tipus d'emulsionant també va ser notable. Es va

observar que les emulsions que contenien l'emulsionant Tween 20 presentaven una mida de partícula menor (4.4 μm) que aquelles que contenien lecitina de soja (5.7 μm). A més, la digestibilitat de les emulsions va ser més alta utilitzant Tween 20 (42%) que lecitina de soja (23%), però la bioaccessibilitat de les antocianines encapsulades (23%) no es va veure afectada pel tipus d'emulsionant. L'addició de polisacàrids en la fase aquosa externa de les emulsions dobles va reduir la mida de partícula i va incrementar la eficiència d'encapsulació i l'estabilitat dels sistemes al llarg del temps. A més, va millorar l'estabilitat dels sistemes durant la digestió gastrointestinal, evitant la degradació dels compostos encapsulats i incrementant-ne la bioaccessibilitat.

La concentració d'emulsionant (lecitina de soja) va ser determinant per aconseguir emulsions altament concentrades estables contenint curcuminoids, essent 0.1 el rati emulsionant-oli òptim. Les emulsions altament concentrades van incrementar la biodisponibilitat oral dels curcuminoids en rates 10.6 vegades, en comparació amb la suspensió control. La curcumina es va detectar en forma glucuronidada en plasma, observant-se les concentracions màximes (≈ 217-260 ppm) entre les 2 h i les 4 h post-administració. D'altra banda, la curcumina es va trobar majoritàriament en forma lliure (la que presenta major activitat biològica) en els òrgans, especialment al teixit adipós bru (193 ng/g).

En general, aquelles nanoemulsions contenint nanopartícules líquides a temperatura ambient (oli de blat de moro o miglyol) van presentar una mida de partícula menor que aquelles que contenien nanopartícules sòlides (oli de palma o oli de coco). A més, aquelles formulades amb olis de cadena mitja (miglyol o oli de coco) van presentar mides menors que aquelles contenint olis de cadena llarga (oli de blat de moro o oli de palma). L'estudi *in vivo* va revelar que, després de la administració oral de les nanoemulsions, aquelles formulades amb oli de blat de moro van ser les més efectives per incrementar l'absorció de curcumina en l'intestí de les rates així com per augmentar la distribució del compost en diferents òrgans. La curcumina es va trobar principalment en forma glucuronidada al plasma, essent 1612 ng/mL la màxima concentració observada. No obstant, en els diferents òrgans, la curcumina es va detectar majoritàriament en forma lliure, essent el teixit adipós bru l'òrgan amb major

acumulació (1400 ng/g).

Els resultats obtinguts en aquesta tesis doctoral proporcionen nou coneixement sobre el disseny de sistemes basats en emulsions per incrementar la biodisponibilitat de compostos bioactius amb alt valor biològic. Aquesta informació és de gran rellevància per seguir investigant en l'obtenció de sistemes que permetin protegir aquests compostos, incrementar-ne l'absorció i la biodistribució, especialment en òrgans on poden tenir un impacte positiu en la prevenció o tractament de malalties.

RESUMEN

Los compuestos bioactivos han mostrado poseer propiedades biológicas diversas que aportan beneficios para la salud humana. Desafortunadamente, estos compuestos son degradados fácilmente y presentan una absorción y biodisponibilidad bajas. La investigación realizada en los últimos años ha revelado que los sistemas basados en emulsiones (nanoemulsiones, emulsiones múltiples, emulsiones altamente concentradas, nanopartículas, etc.) son útiles para proteger estos compuestos y aumentar su absorción. Sin embargo, la composición de estos sistemas parece tener un gran impacto en sus propiedades y su funcionalidad. Por lo tanto, el objetivo de esta tesis doctoral fue estudiar cómo la composición (tipo de emulsionante, concentración de aceite, presencia de polisacáridos, tipo y estado físico del aceite) afecta a las propiedades y la estabilidad de distintos tipos de emulsiones, así como a la bioaccesibilidad y biodisponibilidad de los compuestos bioactivos encapsulados.

Por lo general, se observó que utilizando emulsionantes proteicos (proteína del suero y caseinato sódico) se obtuvieron nanoemulsiones con tamaño de partícula menor y carga eléctrica menos negativa que utilizando emulsionantes lipídicos (lecitina de soja), aunque el efecto dependió de otros parámetros como la concentración de aceite. Además, las nanoemulsiones que contenían emulsionantes proteicos presentaron una mayor digestibilidad lipídica, debido a las gotas de tamaño reducido que presentaban en el intestino, y una mayor bioaccesibilidad del compuesto *in vitro* (≈ 64% en el caso del β-caroteno y ≈ 67% en el caso de la curcumina). Como consecuencia, después de su administración oral en ratas, las nanoemulsiones proteicas aumentaron la absorción del β-caroteno y la biodistribución de su forma activa (retinol) en diferentes órganos, especialmente en el hígado (≈ 3250 ng/g). Además, se observó que incrementando la concentración de aceite en las nanoemulsiones disminuía la digestibilidad del sistema y, en consecuencia, la bioaccesibilidad del compuesto encapsulado. El efecto de la adición de polisacáridos varió en función del tipo utilizado. La adición de pectina disminuyó la estabilidad de las nanoemulsiones y aumentó la bioaccesibilidad del β-caroteno (en un 12%). Por el contrario, la adición de alginato incrementó notablemente la estabilidad del sistema y el compuesto encapsulado durante la digestión, pero disminuyó la

digestibilidad lipídica y la bioaccesibilidad de la curcumina (en un 40%).

En emulsiones dobles, el efecto del tipo de emulsionante también fue notable. Se observó que las emulsiones que contenían Tween 20 como emulsionante presentaban un tamaño de partícula menor (4.4 μm) que aquellas que contenían lecitina de soja (5.7 μm). Además, la digestibilidad de las emulsiones fue mayor utilizando Tween 20 (42%) que lecitina de soja (23%), pero la bioaccesibilidad de las antocianinas encapsuladas (23%) no se vio afectada por el tipo de emulsionante utilizado. La adición de polisacáridos en la fase acuosa externa de las emulsiones dobles disminuyó el tamaño de partícula e incrementó la eficiencia de encapsulación y la estabilidad de los sistemas a lo largo del tiempo. Además, mejoró la estabilidad de los sistemas durante la digestión gastrointestinal, evitando la degradación de los compuestos encapsulados e incrementando su bioaccesibilidad.

La concentración de emulsionante (lecitina de soja) fue determinante para conseguir emulsiones altamente concentradas estables conteniendo curcuminoides, siendo 0.1 el ratio emulsionante-aceite óptimo. Las emulsiones altamente concentradas incrementaron la biodisponibilidad oral de los curcuminoides en ratas 10.6 veces, en comparación con la suspensión control. La curcumina se detectó en forma glucuronidada en plasma, presentando los valores máximos (≈217-260 ppm) entre las 2 h y las 4 h post-administración. Por otro lado, en los órganos, la curcumina se observó mayoritariamente en forma libre (la que presenta mayor actividad biológica), especialmente en el tejido adiposo pardo (193 ng/g).

Por lo general, aquellas nanoemulsiones conteniendo nanopartículas líquidas a temperatura ambiente (aceite de maíz o miglyol) presentaron un tamaño de partícula menor que aquellas que contenían nanopartículas sólidas (aceite de palma o aceite de coco). Además, aquellas conteniendo aceites de cadena media (miglyol o aceite de coco) presentaron tamaños menores que aquellas formuladas con aceites de cadena larga (aceite de maíz o aceite de palma). El estudio *in vivo* reveló que, tras la administración oral de las nanoemulsiones, aquellas formuladas con aceite de maíz fueron las más efectivas para incrementar la absorción de curcumina en el intestino de las ratas y aumentar la distribución del compuesto en los órganos. La curcumina se halló

principalmente en forma glucuronidada en el plasma, siendo 1612 ng/mL la máxima concentración observada. Sin embargo, en los diferentes órganos, la curcumina se detectó mayoritariamente en forma libre, siendo el tejido adiposo pardo el órgano con mayor acumulación (1400 ng/g).

Los resultados obtenidos en esta tesis doctoral proporcionan conocimiento nuevo sobre el diseño de sistemas basados en emulsiones para incrementar la biodisponibilidad de compuestos bioactivos con alto valor biológico. Esta información es de gran relevancia para seguir investigando en la obtención de sistemas que permitan proteger estos compuestos, e incrementar su absorción y biodistribución, especialmente en órganos donde pueden tener un impacto positivo en la prevención o tratamiento de enfermedades.

ABSTRACT

Bioactive compounds have been shown to present multiple biological properties beneficial for human health. Unfortunately, these compounds are easily degraded and show low absorption and bioavailability. Research performed in recent years has shown that different emulsion-based systems (nanoemulsions, multiple emulsions, highly concentrated emulsions, nanoparticles, etc.) are useful to protect these compounds and increase their absorption. However, the composition of these systems seems to have a major impact on their properties and functionality. Therefore, this doctoral thesis aimed to study how the composition (emulsifier type, oil concentration, presence of polysaccharides, type and physical state of the oil) affects the properties and stability of different types of emulsions, as well as the bioaccessibility and bioavailability of the encapsulated bioactive compounds.

In general, by using protein emulsifiers (whey protein and sodium caseinate) nanoemulsions with smaller particle size and lower electrical charge were obtained rather than using phosphorlipid emulsifiers (soybean lecithin), although the effect depended on other parameters such as oil concentration. Moreover, nanoemulsions containing protein emulsifiers showed a higher lipid digestibility due to smaller droplet size in the gut, and a higher bioaccessibility of the encapsulated compound *in vitro* (≈ 64% for β-carotene and ≈ 67% for curcumin). As a result, after the oral administration in rats, protein nanoemulsions better increased the β-carotene absorption and the biodistribution of its active form (retinol) in different organs, especially in the liver (≈ 3250 ng/g). Furthermore, it was observed that by increasing the oil concentration in nanoemulsions the digestibility of the system was reduced and, consequently, the bioaccessibility of the encapsulated compound was lower. The effect of the polysaccharide was different depending on the type used. The addition of pectin decreased the stability of nanoemulsions and increased the bioaccessibility of β-carotene (by 12%). In contrast, the addition of alginate significantly increased the stability of the system and the compound during digestion but decreased lipid digestibility and curcumin bioaccessibility (by 40%).

In double emulsions, the effect of the emulsifier type was also noticeable. Double

emulsions containing Tween 20 as emulsifier presented smaller particle sizes (4.4 μm) than those containing soybean lecithin (5.7 μm). In addition, the digestibility of emulsions was higher using Tween 20 (42%) than lecithin (23%), but the bioaccessibility of the encapsulated anthocyanins (23%) was not affected by the type of emulsifier used. The addition of polysaccharides in the external aqueous phase of double emulsions reduced the particle size and increased the encapsulation efficiency and the physical stability of the systems over time. In addition, it improved the stability of the systems during gastrointestinal digestion, preventing the degradation of the encapsulated compounds and increasing their bioaccessibility.

The concentration of the emulsifier (soybean lecithin) was decisive to achieve stable highly concentrated emulsions containing curcuminoids, with 0.1 being the optimal surfactant-oil ratio. The highly concentrated emulsions increased the oral bioavailability of curcuminoids in rats by 10.6-fold compared to the control suspension. Curcumin was detected in the glucuronidated form in plasma, presenting the maximum concentration values (≈ 217-260 ppm) between 2 h and 4 h post-administration. Nevertheless, curcumin was mostly found in its free form (the form with the highest biological activity) in the organs, especially in brown adipose tissue (193 ng/g).

In general, nanoemulsions containing liquid lipid nanoparticles at room temperature (corn oil or miglyol) had a smaller particle size than those containing solid lipid nanoparticles (palm oil or coconut oil). In addition, those containing medium-chain oils (miglyol or coconut oil) presented smaller a smaller particle size than those containing long-chain oils (corn oil or palm oil). The *in vivo* study revealed that, after oral administration of nanoemulsions, those formulated with corn oil were the most effective in increasing the absorption of curcumin in the intestine of the rats and the biodistribution of the compound in the organs. Curcumin was mainly found in the glucuronidated form in plasma, with 1612 ng/mL being the highest concentration observed. However, in the different organs, curcumin was mostly detected in the free form, with brown adipose tissue being the organ with the highest accumulation (1400 ng/g).

The results obtained in this doctoral thesis provide novel insights into the design of

emulsion-based systems to increase the bioavailability of bioactive compounds with high biological value. This information is of great relevance to continue with the research on the development of systems to protect these compounds and increase their absorption and biodistribution, especially in organs where they can play a relevant role in the prevention or treatment of diseases.

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INTRODUCTION

Literature review

Emulsion-based delivery systems to enhance the functionality of bioactive compounds: towards the use of ingredients from natural, sustainable sources.

Júlia Teixé-Roig, Gemma Oms-Oliu, Isabel Odriozola-Serrano, Olga Martín-Belloso*

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Abstract

In recent years, the trend in the population towards consuming more natural and sustainable foods has increased significantly. This claim has led to the search for new sources of bioactive compounds and extraction methods that have less impact on the environment. Moreover, the formulation of systems to protect these compounds is also focusing on the use of ingredients of natural origin. This article reviews novel, natural alternative sources of bioactive compounds with a positive impact on sustainability. In addition, it also contains information on the most recent studies based on the use of natural (especially from plants) emulsifiers in the design of emulsion-based delivery systems to protect bioactive compounds. The properties of these natural-based emulsion-delivery systems, as well as their functionality, including *in vitro* and *in vivo* studies, are also discussed. This review provides relevant information on the latest advances in the development of emulsion delivery systems based on ingredients from sustainable natural sources.

Keywords: emulsions, delivery-systems, bioactive compounds, sustainability, plant-based

1. Introduction

Bioactive compounds such as carotenoids, polyphenols, or polyunsaturated fatty acids (PUFAs) have been found to reduce the risk of cardiovascular diseases, cancer, and other diseases (Bazzano et al., 2003; Potter, 2005). This positive effect has been correlated to the different biological activities of these compounds, which are present in plant-based foods such as fruits, vegetables, tea, and wine, among others (Eggersdorfer & Wyss, 2018; Liu, 2013). Moreover, promising novel sustainable sources of these compounds have emerged in recent years, with microalgae and agrifood residues being sources of high interest. However, most bioactive compounds are prone to degradation and present low bioavailability (Hu et al., 2018; Shishir et al., 2018). To overcome these problems, encapsulation techniques can be very useful, as they increase the stability and functionality of these valuable compounds (Baysal et al., 2022; Vieira et al., 2020). Among them, emulsion-based delivery systems have been widely used, with some examples being nanoemulsions (Fan et al., 2017; Yao et al., 2021; Zheng et al., 2019), highly-concentrated emulsions (Artiga-Artigas et al., 2019), or double emulsions (Aditya et al., 2015; Artiga-Artigas et al., 2019; Giroux et al., 2016). These encapsulation systems have been shown to provide high stability to encapsulated compounds, as well as to increase their bioaccessibility and bioavailability.

Initially, emulsion-delivery systems were obtained mostly by using synthetic ingredients, leading to high stability of the resultant systems. However, in recent years, consumers have become aware of the impact of synthetic ingredients on health and on the environment, increasing the demand for food products containing ingredients from natural sources. For that reason, the design of emulsion-based delivery systems must go forward with the use of components of natural origin, with emulsifiers being included in the main focus. Indeed, most synthetic emulsifiers used in the formulation of these systems have been proven to be associated with health problems and toxic symptoms with a long administration. It has been observed that these emulsifiers could bind to proteins, enzymes, and phospholipid membranes in the human body, producing alterations such as enzyme dysfunction or protein structure modification and phospholipids in the membrane cell (Dammak et al., 2020; Gao et al., 2016). In addition, the use of natural ingredients, which are obtained from plants and algae, can help to take

an important step towards more sustainable and clean-label products. While synthetic ingredients are associated with environmental issues due to their low biodegradability (Garcia et al., 2016), natural ingredients such as proteins, polysaccharides, phospholipids, or saponins that are obtained from renewable sources can be a feasible alternative (McClements et al., 2017).

Therefore, the aim of this article is, first, to provide a general overview of the most innovative sources from which to obtain bioactive compounds in a more sustainable way. Second, this paper also reviews recent advances in the use of natural emulsifiers to obtain stable emulsion-based delivery systems with optimal functionality.

2. Bioactive compounds

In recent years, purified extracts obtained from plants, fruits, and vegetables have been used as bioactive compounds, both lipophilic and hydrophilic. However, in recent years, the search for more sustainable foods has focused the attention on the use of alternative sources to obtain bioactive compounds. Among these natural alternatives, the use of microalgae seems to be a suitable source from which to obtain these bioactive compounds due to the fast growth, high yield, and short cultivation time of microalgae (Wang et al., 2022) (Figure 1). In addition, residues from the agrifood industry are promising sources of these compounds with biological activity. This form of residue valorisation would represent a new conceptualisation of sustainability in the food chain, moving from a linear to a circular economy and allowing the possibility of advancing the use of co-products to generate new and safe value-added products (Cádiz-Gurrea et al., 2020).

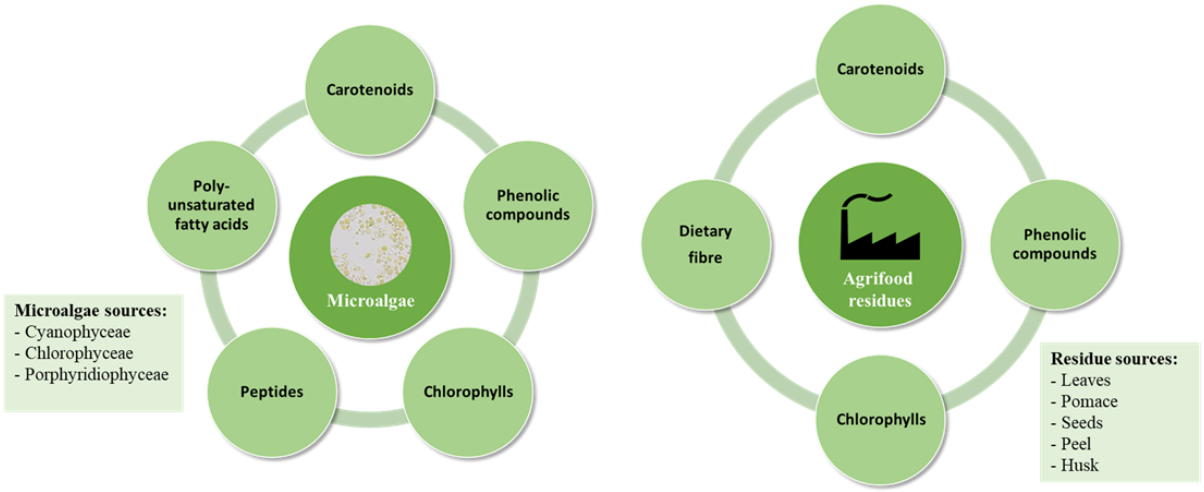

Figure 1. Bioactive compounds from microalgae and agrifood residues.

2.1. Novel Alternative Sources of Bioactive Compounds

2.1.1. Microalgae

These organisms produce metabolites which have been associated with relevant health benefits (Ampofo & Abbey, 2022). In general, these metabolites are produced as a response to environmental stress and include carotenoids, polyunsaturated fatty acids, phenolics, chlorophylls, and peptides, among others.

- Carotenoids: It has been found that microalgae can produce and accumulate carotenoids, with *Chlorophyceae* being the dominant carotenoid-producing group of xanthophylls and carotenes (Sui et al., 2021). Specifically, *Dunaliella salina* and *Haematococcus pluvialis* are commonly used for high-value carotenoid production due to their high content of carotenoids such as β-carotene and astaxanthin, which can represent up to 14% of the microalgae dry biomass (Tamaki et al., 2021). Other compounds such as lutein have been identified in *Muriellopsis sp*., although the concentrations were lower (0.4% to 0.6% per dry biomass) (D'Alessandro & Antoniosi Filho, 2016).

- Polyunsaturated fatty acids: In microalgae, fatty acids cover the largest percentage of

total lipids, with polyunsaturated fatty acids (PUFAs) representing 20-60% of the total lipids (Liang et al., 2019). *Spirulina* and *Chlorella* are valuable sources of PUFAs such as docosahexaenoic acid (DHA), arachidonic acid (ARA), alpha-lipoic acid (ALA), and eicosapentaenoic acid (EPA) (Zhou et al., 2022). Microalgae can represent an interesting vegan source of fatty acids that, up to date, have been obtained mostly from animal sources such as fish oil.

- Phenolic compounds: Although high concentrations have been observed in macroalgae, microalgae such as *Chlorella* or *Arthrospira* have been found to contain appreciable levels of phenolic compounds. However, according to the literature, the concentration in microalgae present significant variations due to species type, cultivation conditions, and techniques used for extraction, identification, and quantification (Ampofo & Abbey, 2022).

- Chlorophylls: These natural green pigments are crucial in photosynthetic organisms for harvesting energy from sunlight and can be classified as a, b, or c (Ampofo & Abbey, 2022). However, chlorophyll c is present only in brown algae and not in green algae. Among microalgae species, *Chlorella* is the main producer of chlorophyll, with other species such as *Spirulina* and *Arthrospira* producing limited concentrations (Khanra et al., 2018).

- Peptides: Microalgal proteins have been demonstrated to be a source of bioactive peptides after enzymatic hydrolysis. Due to their differentiated sequential, structural, and compositional properties, microalgae peptides exert a list of positive health effects such as antioxidant, antihypertensive, antitumor, and immunomodulatory effects (Hamidi et al., 2019; Zhou et al., 2022).

In summary, microalgae are a promising source of multiple bioactive compounds that can be isolated via various extraction methods. Moreover, they can grow in non-potable water and agriculturally non-productive land, in addition to presenting greater surface productivity and photosynthetic efficiency compared to terrestrial crops (Chisti, 2007; Larkum, 2010).

2.1.2. Co-products from agrifood industry

During the various stages of food production, the agrifood industry generates large amounts of residues which contain bioactive compounds that present beneficial biological activities for human health (Chaouch & Benvenuti, 2020; Chauhan et al., 2021; Lemes et al., 2022). The main bioactive compounds that can be obtained as agrifood co-products can be classified into carotenoids, phenolic compounds, chlorophylls, and dietary fibre.

- Carotenoids: Various agrifood residues such as tomato peel (Silva et al., 2019), guarana peel (Pinho et al., 2021) or peel, and the pulp of citrus fruits (Agócs et al., 2007) have been found to contain carotenoids such as lycopene, β-carotene, or lutein. According to these authors, these agrifood residues can contain variable concentrations that can be up to 60% carotenoids per unit of dry weight.

- Phenolic compounds: A variety of phenolic compounds such as flavonoids, phenolic acids, and lignans has been found in different agrifood residues from fruit and vegetables. The waste parts in which they have been identified include pomace (Castellanos-Gallo et al., 2022), leaves (Baccouri et al., 2023), seeds (da Costa et al., 2020), peel, or husk (Nazeam et al., 2020).

- Chlorophylls: These green pigment compounds, which can be classified as chlorophyll a, b, or c, have been found in residues from different vegetables, especially in the leaves. As an example, chlorophyll a and b, in concentrations ranging from 1132.33 to 1795.93 ppm, were detected as co-products from olive leaves (Bahloul et al., 2014; Flamminii et al., 2019). However, higher concentrations have been detected in the leaf residues from broccoli (Liu et al., 2018) (up to 4477.9 µg/g dry weight) or asparagus (Chitrakar et al., 2020) (up to 5096 µg/g dry weight).

- Dietary fibre: Both soluble and insoluble fibre have been found in residues from vegetables such as artichoke, carrot, or pepper (Vaz et al., 2022), as well as fruits such as guava or passion fruit (Casarotti et al., 2018). However, most of these residues contained higher amounts of the insoluble fraction rather than the soluble one. In addition, diverse cereal residues have been found to contain soluble (beta-glucans) and

insoluble dietary fibres (such as cellulose or lignin) in variable concentrations (Fărcaș et al., 2022).

Therefore, several bioactive compounds can be obtained as co-products of agrifood residues of diverse origins such as vegetables, fruits, or cereals. The use of these residues as co-products implies an improvement in the production process, focusing it on a circular economy of high value for more sustainable production.

2.2. Extraction methods of bioactive compounds

Many extraction methods have been developed to isolate bioactive compounds from various matrices. Among them, there are some called "conventional extraction methods", which have been widely used to obtain these valuable compounds. Some of them are Soxhlet, maceration, hydrodistillation, infusion, or decoction. However, these methods present some limitations such as the thermal destruction of compounds, the use of high amounts of solvent, long extraction times, and a negative impact on the environment. To overcome these challenges, novel methods that are also called "green extraction techniques" have been developed. These methods provide better environmental, health, and safety properties since they use lower temperatures during extraction, shorter times, higher extraction yield, and better extraction efficiency (Banožić et al., 2020). Some of the recent technologies that have shown less solvent and energy usage are ultrasound-assisted extraction, hydrodynamic cavitation-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, liquid biphasic flotation extraction, cloud point extraction, pulsed electric field, high voltage electrical discharge, and instant controlled pressure drops (More et al., 2022). Moreover, it has been found that the hybridisation of extraction by combining two or more green extraction technologies provides excellent separation of bioactive compounds.

It is well known that the efficiency of the extraction depends on several factors, including extraction technique, plant component matrix properties, extraction solvent, temperature, pressure, and time, among others (Drosou et al., 2015). Therefore, the extraction methods differ greatly depending on the matrix where the compounds have to be isolated. In general, conventional methods are the most widely used, although in recent years, the use of green technologies such as ultrasound-assisted extraction (Fan

et al., 2022), microwave extraction (Low et al., 2020), or supercritical CO_2 extraction has increased substantially. By using these techniques, the extraction of bioactive compounds from microalgae and agrifood residues has been shown to be highly efficient, and the extraction times have been reduced. Moreover, there are other technologies such as liquid biphasic flotation, pulsed electric fields, and high-voltage electrical discharge that are being investigated and have shown promising results, although some of them are still under lab-scale research (More et al., 2022).

3. Emulsion-based delivery systems to carry bioactive compounds

As mentioned, bioactive compounds are easily degraded by multiple factors, so various emulsion-based delivery systems have been investigated to protect them. One of the most relevant properties of these systems is that they need to present high biocompatibility. This means that they cannot be toxic, but they should also fulfil a planned role in the biological environment (Kubiak, 2022). These systems can be classified depending on their structure into different groups (Table 1 and Figure 2), with some of the most used systems to encapsulate bioactives as follows:

- Simple emulsions and nanoemulsions. Emulsions consist of two immiscible liquids, with one of the liquids dispersed as small spherical droplets in the other, and they have been used to encapsulate different bioactive compounds such as vitamin E (Wang et al., 2023) or curcumin (Zhang et al., 2023), among others. Nanoemulsions, which are emulsions containing nanometric-size oil droplets ranging from 50 to 500 nm, have been shown to present higher stability over time and higher digestibility than conventional emulsions due to the greater surface area exposed to intestinal enzymes (Mason et al., 2006; Salvia-Trujillo et al., 2013a). These systems have been widely studied to increase the bioavailability of poorly soluble drugs such as carotenoids (Luo et al., 2022; Teixé-Roig et al., 2023) or vitamin D (Kadappan et al., 2018) because authors have found that after reducing the particle size of fat globules, the drug solubility and absorption of the encapsulated compounds was greater (Harwansh et al., 2019). Moreover, nanoemulsions have been shown to have increased encapsulation stability during storage compared to conventional emulsions due to their more stable structure (Teixé-Roig et al., 2020). Simple emulsions can be formed by using low-energy or high-energy methods, with the latter being the most used to produce emulsion-based delivery systems. Low-energy

methods (phase inversion temperature method, phase inversion composition method, etc.) are based on the spontaneous emulsion formation under specific system compositions or environmental conditions. In contrast, high-energy methods are based on the use of intense mechanical forces to break up droplets into smaller droplets and are performed by using homogenisers such as high-shear mixers, high-pressure homogenisers, colloid mills, ultrasonic homogenisers, and membrane homogenisers. Nanoemulsions have been used to encapsulate bioactive compounds with low bioavailability to be administered via different routes such as parenteral, oral, nasal, or topical routes. Moreover, they have also been used as edible coatings and to encapsulate flavouring agents or preservatives in food products and have also been applied in the cosmetic and pharmaceutical fields (Ozogul et al., 2022).

- Double emulsions. These emulsions belong to the group of multiple emulsions since they contain an emulsion structure with coexisting water-in-oil (W/O) and oil-in-water (O/W) morphologies. Double emulsions can be prepared via a one-step or two-step emulsification procedure, with the latter being the most used. In the one-step emulsification method, strong mechanical agitation is required to induce phase inversion. In contrast, in the two-step emulsification method, the first step consists of the formation of a simple emulsion by using high-shear devices including ultrasonicators, high-pressure homogenisers or microfluidizers, among others. Afterwards, the third phase is added, and by using the same devices as in the first step, the double emulsion is formed (Muschiolik & Dickinson, 2017). Due to their structure, these systems allow the encapsulation of a lipophilic compound and a hydrophilic compound within the same emulsion, which can be an interesting strategy to encapsulate compounds with synergic activity (Aditya et al., 2015). These systems have been applied to encapsulate labile and/or bioactive compounds with low bioavailability (Han et al., 2022; Huang et al., 2022). Moreover, using double emulsions has been found to be an interesting strategy to produce fat-reduced products. However, these systems are sensitive and unstable, which can decrease the encapsulation efficiency. The main reasons for their instability are the free energy at the droplet level, osmotic pressure, and Laplace pressure (Heidari et al., 2022). To improve the stability of these systems, numerous strategies have been developed, such as adjusting the internal and external osmotic pressure by adding sugar or salts or increasing the viscosity (Eisinaite et al., 2018).

- Multi-layer emulsions. These emulsions present a simple emulsion structure (O/W or W/O) surrounded by multiple layers of biopolymers. For their preparation, the primary emulsion can be obtained by using the same methods that are used in simple emulsions. Afterwards, biopolymer layers of opposite charges, which act as a stabiliser, are normally constituted by the layer-by-layer electrostatic deposition technique (Kartal et al., 2017). These systems, which have been reported to contain two, three, or up to four layers of oppositely charged biopolymers, are prone to instability phenomena, with the solution pH being an important factor to prevent them during and after the formation of the interface (Gasa-Falcon et al., 2020; Guzey & McClements, 2006). To prevent instability processes such as bridging flocculation or depletion flocculation, which are observed when there is either an excess or lack of polyelectrolytes in the solution, different strategies can be employed: (1) the saturation method, to empirically determine the biopolymer concentration required to cover the oil droplets; (2) the centrifugation method, to remove the excess of non-adsorbed biopolymers by centrifuging; or (3) the filtration method, whereby the excess of non-adsorbed biopolymers is removed via membrane filtration (Gasa-Falcon et al., 2020). The application of multilayer emulsions has been limited compared with other emulsion-based systems. Few studies have investigated its potential to increase the bioavailability of bioactive compounds or control their release. However, due to their structure, they could be very useful in achieving concrete functional performances, offering a high level of control in triggering the release of the encapsulated compounds.

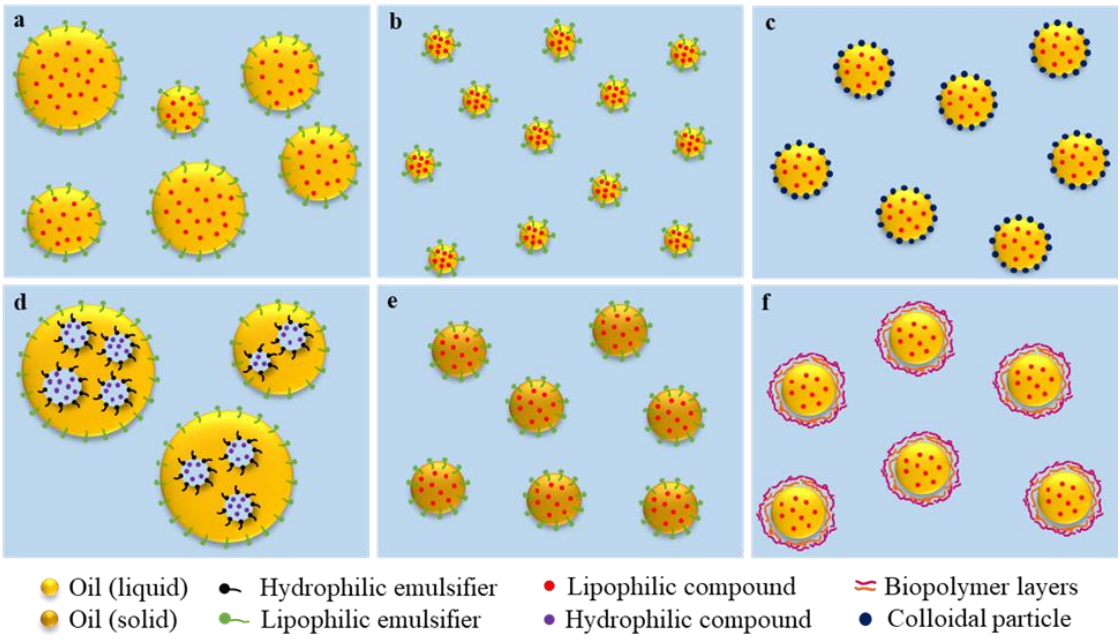

Figure 2. Different emulsion-based delivery systems to enclose bioactive compounds. Oil-in-water emulsions (a), oil-in-water nanoemulsions (b), Pickering emulsions (c), water-in-oil-in water double emulsions (d), solid-lipid nanoparticles (e), and multi-layer emulsions (f).

- Pickering emulsions. These encapsulation systems can present a simple or multiple emulsion structure and are not stabilised by surfactant molecules but by solid colloidal particles, either organic or inorganic, that should be partly wetted by oil and by water (Berton-Carabin & Schroën, 2015). As an example, they can be stabilised by using turmeric granules (Kubiak et al., 2021) or protein-based particles (Wang et al., 2022). To prepare Pickering emulsions, all emulsification processes that are used to prepare emulsions stabilised by surfactants can be applied. However, the most commonly used are rotor-stator homogenization, high-pressure homogenization, and sonication. Moreover, other techniques such as membrane emulsification and microfluidic emulsification have been recently applied. These emulsion-based systems present high stability and high biocompatibility without the addition of surfactants. Even under high stress, the shells covering the emulsion droplets have been found to remain in the systems (Khobaib et al., 2021; Mikkelsen & Rozynek, 2019; Wu & Ma, 2016). Moreover, they present an adjustable permeability, meaning that the release of the encapsulated bioactive compounds can be controlled under the action of external factors such as ultrasonic waves (Kubiak et al., 2020). Among the applications of Pickering emulsions, the encapsulation and protective storage of sensitive or low-bioavailable compounds

have been extensively investigated. Other applications include the construction of porous materials, three-dimensional (3D) printing, and their use as a substitute for partially hydrogenated oils (Wang et al., 2022).

- Solid-lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). SLN are simple emulsions that contain a solid lipid core. These systems are usually formed by using high-energy methods including the hot homogenization technique or cold homogenization technique. For the hot homogenization technique, the lipid is mixed with a hot aqueous solution by using a high-shear mixing device and treated with a high-pressure homogeniser above the melting point of the lipid to obtain nanoparticles. For the cold homogenization technique, the lipid is cooled and crushed into lipid microparticles that are dispersed in a cold aqueous solution. The mixture is then treated with a high-pressure homogeniser at room temperature or below to obtain nanoparticles. SLN have been shown to present some advantages over systems that contain a liquid oil phase, as SLN provide higher chemical stability of the encapsulated compound and are more stable against lipid coalescence. Moreover, these systems may allow a controlled release of compounds since the drug mobility in a solid lipid should be considerably lower compared with a liquid oil (Mehnert & Mäder, 2012). Indeed, the application of these systems as carriers of labile and low-bioavailable compounds has been tested *in vitro* and *in vivo* (Baek & Cho, 2017; Harde et al., 2011). However, SLN are prone to aggregation and present a highly ordered structure of the crystals within that can promote the expulsion of the encapsulated bioactives, making them more prone to degradation (Das et al., 2012). To overcome these problems, NLC containing liquid lipid mixtures rather than pure solid lipid have been developed. In these systems, the mixture of lipids generates a less ordered crystal structure that allows accommodating more lipophilic bioactive compounds, increasing the encapsulation efficiency and reducing the adverse excretion caused by the polymeric transition (Shu et al., 2022).

Table 1. Preparation techniques, advantages, and limitations of different emulsion-based delivery systems.

System Type	Preparation Techniques	Particle Size	Advantages	Limitations
Conventional emulsions	- High-energy (high-pressure homogenization, sonication, microfluidization). - Low-energy methods (self-emulsification, phase inversion, membrane emulsification).	500 nm–100 µm	- Ease of preparation. - Low cost. - These emulsions can be used in their wet state or be dried to form powders, facilitating their transport and utilisation in some applications.	- Prone to physical instability when exposed to environmental stresses such as heating, freezing, drying, pH extremes, etc. - Limited control over the ability to protect and control the release of the encapsulated compounds.
Nanoemulsions	- High-energy (high-pressure homogenization, sonication, microfluidization). - Low-energy methods (self-emulsification, phase inversion, membrane emulsification).	<500 nm	- They can be easily incorporated into food systems since they are usually optically translucent. - Higher physical stability than conventional emulsions to gravitational separation and aggregation. - Increased surface area compared with conventional emulsions that enhances the digestibility, compound bioaccessibility, and bioavailability. - Higher encapsulation efficiency and compound stability during storage time.	- Some of the synthetic emulsifiers used to obtain small droplet sizes may be associated with health issues. - These systems usually provide less chemical stability than structures with a solid lipid or multilayer emulsions. - Limited control over the release of encapsulated compounds compared with more complex structures.
Multiple emulsions	- 2-step procedure: emulsification of primary emulsion + addition of 3rd phase via homogenization (high-shear mixers, microfluidization, ultrasounds). - Low-energy methods (phase inversion).	Micrometric	- Higher ability to protect and control the release of the encapsulated compounds. - Possibility to reduce the overall fat content. - Possibility to encapsulate lipophilic and hydrophilic within the same system, especially interesting for compounds with synergic effect.	- Highly susceptible to breakdown during storage or when exposed to environmental stresses such as mechanical forces, thermal processing, etc. - More difficult and expensive to prepare than conventional emulsions. - The osmotic balance among the internal and external phases needs to be accurately controlled to avoid compound transfer.
Multilayer emulsions	- Obtention of the primary emulsion by homogenization + biopolymer deposition by using layer-by-layer approach.	Nanometric to micrometric	- Improved physical stability to environmental stresses via control of the composition and properties of the interfacial layer. - High chemical stability of encapsulated components. - Greater control over the release rate of encapsulated compounds due to the ability to manipulate the thickness and properties of the interfacial layer. - Ability to trigger release of functional agents in response to specific changes in environmental conditions.	- The formation of stable multilayer emulsions requires careful control over the system composition and preparation procedures in order to avoid droplet aggregation. - More ingredients and processing steps are required compared to conventional emulsion formation. - These systems are usually more diluted than other systems because of the tendency towards flocculation.
Pickering emulsions	- High-shear homogenization. - High-pressure homogenization. - Ultrasounds.	Nanometric to micrometric	- Droplets are stabilised by solid particles, avoiding the use of surfactants.	- Modification performed on the solid particle stabiliser to adjust particle wettability can be expensive, time-consuming, and partially effective.

	- Microfluidization. - Membrane emulsification.		- High control over the release of encapsulated compound, their contents can be controlled under the action of external factors such as ultrasonic waves. - Shells covering the emulsion droplets in these systems have been found to be even when they undergo high stress	- Some particle stabilisers need to be pre-treated via complex or toxic surface modification to be able to function as hydrophobic colloidal stabiliser.
Solid-lipid nanoparticles (SLN)	- High-energy (high-pressure homogenization, sonication, microfluidization). - Low-energy methods (self-emulsification, phase inversion, membrane emulsification).	50 nm–1 µm	- Improved chemical stability of labile compounds by trapping them within a structured solid matrix. - High ability to control the delivery of lipophilic functional components. - Possibility to obtain stable emulsion-based systems containing crystalline lipophilic components. - These promote oral bioavailability of the entrapped compounds through selective lymphatic uptake.	- SLN must be prepared at elevated temperatures to avoid crystallisation of the lipid phase during the homogenization process, which can promote the degradation of heat-sensitive lipophilic components. - The highly ordered crystalline structure can cause compound expulsion. - The lipid used should present a high degree of saturation which can have implications to human health.
Nanostructured lipid carriers (NLC)	- High-energy (high-pressure homogenization, sonication, microfluidization). - Low-energy methods (self-emulsification, phase inversion, membrane emulsification).	10 nm–0.5 µm	- The partial crystal structure facilitates more space to accommodate bioactive ingredients, leading to less expulsion and higher loading capacity and controlled release of encapsulated carotenoid compounds. - Higher encapsulation efficiency and high stability. - High chemical stability of the encapsulated compounds.	- Most formulations include synthetic lipids or surfactants which can be associated with health issues. - There is a lack of a proper method and scaleup techniques.

Adapted from (Albert et al., 2019; McClements et al., 2007; Muschiolik & Dickinson, 2017)

4. Natural-based stabilisers for emulsion-based delivery systems

Emulsifiers play two key roles in the obtention of emulsion-based systems: emulsion formation and emulsion stabilisation. For emulsion formation, they need to be rapidly adsorbed, decrease the interfacial tensions, and facilitate droplet breakup. Moreover, emulsion stabilisers have to generate strong repulsive forces and provide a resistant interfacial layer, preventing droplet aggregation (McClements & Gumus, 2016). A hydrophilic-lipophilic balance (HLB) value is assigned to each emulsifier, which indicates its solubility in oil and/or water phases. Usually, a surfactant with a low HLB value (3-6) is predominantly hydrophobic and is more likely to stabilise W/O emulsions. In contrast, a surfactant with a high HLB number (10-18) is predominantly hydrophilic and better stabilises O/W emulsions. Finally, surfactants with an intermediate HLB number (7-9) have no particular preference for either oil or water (McClements, 2005).

In recent years, synthetic emulsifiers have been the most used to produce emulsion-based delivery systems for bioactive compounds, as these molecules can be rapidly adsorbed at the interface, efficiently reduce the interfacial tension, and provide systems with high stability (Salvia-Trujillo et al., 2013b; Shakeel et al., 2008; Yuan et al., 2008). Nevertheless, the consumption of these synthetic emulsifiers might induce health problems and may cause toxic symptoms after long administration periods (Grumezescu, 2017). For that reason, researchers focus on emulsion stabiliser ingredients from natural sources, which can be classified depending on their chemical structure in proteins, phospholipids, polysaccharides, or saponins.

4.1. Proteins

Most proteins from natural sources present an amphiphilic structure since they contain a mixture of polar and non-polar amino acids, which means that can be adsorbed into oil-water interfaces stabilising lipid droplets in emulsions. These emulsifiers tend to be bulkier and diffuse slower to the interface than small molecule emulsifiers, and higher concentrations are needed rather than with smaller molecular weight. However, once at the interface, they provide a strong viscoelastic film that resists mechanical stresses and provides electrostatic and steric stabilisation (Lam & Nickerson, 2013). Nevertheless, these natural emulsifiers have been found to be highly affected by pH changes and high

ionic strength, which can cause bridging flocculation of droplets (Delahaije et al., 2013; Ozturk et al., 2014). Regarding natural proteins, whey proteins and caseins from bovine milk have been widely used as emulsifiers, as they are effective for the stabilisation of emulsion-based systems (Flores-Andrade et al., 2021; Qi et al., 2022; Yerramilli & Ghosh, 2017). Recently, some researchers have focused on the use of plant-based proteins such as those from peas, lentils, or rice, to stabilise emulsion-based delivery systems since they are better for human health, the environment, and animal welfare (Tan & McClements, 2021). As an example, some authors have reported that despite being a poorly soluble protein, pea protein can be used to stabilise vitamin-D-loaded nanoemulsions after a pH-shifting and sonication treatment (Jiang et al., 2019). In this work, the authors reported small particle sizes < 150 nm and high UV radiation stability of vitamin D3. This highlights that the functionality of these molecules as emulsifiers can be improved by treating them before incorporating them into the delivery systems. Alternatively, rice bran protein was used as an emulsifier of quercetin-loaded nanoemulsions achieving reduced particle sizes (200 nm) and showing relatively high stability (Chen et al., 2020). In addition, recent studies have investigated the emulsifying capacity of proteins from algae such as *Nannochloropsis gaditana*, *Tetraselmis impellucida*, and *Arthrospira platensis* (Böcker et al., 2021; Silva et al., 2022; Teuling et al., 2019). In these works, proteins extracted from algae were shown to form stable emulsions at similar concentrations to proteins from other sources such as dairy or legumes. Indeed, the minimum particle size that was achieved was observed to be similar when comparing algae proteins to those from milk (Teuling et al., 2019). Moreover, emulsions containing a protein-rich extract from *Arthrospira platensis* as an emulsifier were shown to present a good emulsifying capacity and provided emulsions with physical stability for up to 30 days. Thus, the use of protein-rich algae extracts as emulsifiers presents an added value since the proteins that they contain can act as emulsion stabilisers, but they also contain great amounts of bioactive compounds.

4.2. Phospholipids

Phospholipids have non-polar and polar regions within the same molecule, so they are amphiphilic molecules that can adsorb to oil-water interfaces and stabilise lipid droplets. Phospholipid-based emulsifiers used in the food industry are usually called lecithins.

This emulsifier type, which is a major component of cell membranes, can be obtained from both vegetal and animal sources. However, most of the research focused on emulsion-based delivery systems has been performed by using lecithins from vegetal sources, mainly soybean, sunflower, and cottonseed. The HLB of lecithins can be different depending on the phospholipid composition, but the values are usually approximately 8. This means that these emulsifiers can stabilise both O/W and W/O interfaces. Moreover, lecithins stabilise emulsion-based systems via electrostatic repulsion, so when they are adsorbed at the interface, they provide highly negative charges. As an example, Gao et al. (2020) observed extremely negative ζ-potential (-70 mV) and particle sizes < 250 nm when soy lecithin was used at concentrations higher than 2% in nanoemulsions that were based on fractionated coconut oil. Moreover, this emulsifier type has been found to be highly effective in reducing the interfacial tension. Indeed, soy lecithin has been found to be more effective than whey protein or gum Arabic in reducing the interfacial tension, showing the lowest particle size when preparing oil-in-water nanoemulsions that encapsulate paprika oleoresin (< 140 nm) (Flores-Andrade et al., 2021). Moreover, these authors reported that lecithin nanoemulsions were highly stable when exposed to temperatures (40-80 ºC) but were affected by the ionic strength, showing an increase in the particle size and loss of negative electrical charge. Lecithin nanoemulsions have been shown to be stable at a wide range of pH values, presenting no instability phenomena for 7 days at various studied pH values (Mantovani et al., 2013). Indeed, some authors have reported that lecithin emulsions presented a low particle size (< 200 nm) at a pH range of 3-8 and a negative ζ-potential, especially at a pH > 4, which was about -60 mV (Ozturk et al., 2014). Moreover, by using this emulsifier over 1% w/w, long-term stable nanoemulsions (up to 86 days) were obtained, which were able to efficiently entrap curcumin within, preventing its autoxidation and, hence, maintaining the antioxidant capacity of the bioactive compound (Artiga-Artigas et al., 2018). Soybean lecithin has been found to be also effective in stabilising the oil-water interface of double emulsions. Indeed, by using this emulsifier, emulsions with a particle size of about 4 μm and a phycocyanin encapsulation efficiency of 82% were achieved (Teixé-Roig et al., 2022). Therefore, lecithins seem to be a highly valuable emulsifier since they are highly efficient in reducing interfacial tension and providing systems with high stability over time. Moreover, emulsion-based delivery systems containing these emulsifiers seem to be

more stable to external factors such as pH or temperature compared to others such as proteins.

4.3. Polysaccharides

Some polysaccharides from natural sources can also be useful as emulsifiers since they present an amphiphilic structure that can adsorb at the water-in-oil interface and help to stabilise the system (Shao et al., 2020). Moreover, most of them are of vegetal origin, so they can be used in plant-based products. This type of emulsifiers generally present good pH, salt concentration, and temperature stability, but they need to be used in higher amounts to stabilise emulsion-based systems and produce small particles due to their large molecular weight and dimensions (McClements, 2005). When polysaccharides are adsorbed at the interface, they form relatively thick layers that provide steric repulsion, so they are less affected by changes in pH and ionic strength than proteins (McClements & Gumus, 2016). Among them, Arabic gum has been widely used and has been shown to reduce interfacial tension, providing emulsions with particle sizes < 1 μm. However, this polysaccharide seems to be less effective in reducing the particle size and preventing the degradation of the encapsulated carotenoids than others such as whey protein or lecithin (Flores-Andrade et al., 2021). Nevertheless, it provides emulsions with better flocculation stability at different pH values, high ionic strength, and high temperatures than those containing whey protein as an emulsifier due to their steric stabilising mechanism (Ozturk et al., 2015). Therefore, it seems that polysaccharides such as Arabic gum can be potential emulsifiers to obtain stable systems against external factors but present some disadvantages, such as the low stability of the encapsulated compound and higher particle sizes when compared with proteins or phospholipids. Moreover, a natural hydrocolloid exudated by the bark of *Cercidium praecox* tree (Brea gum) has been found to produce emulsions with even more stability than Arabic gum at the same concentration, which was attributed mainly to its higher viscosity (Castel et al., 2017). Another polysaccharide that is widely used in the food industry is pectin, which has been reported to present emulsifying properties, although the particle sizes that were achieved were not in the range of nanoemulsion (Verkempinck et al., 2018). However, a recent work has reported that extracts from avocado residues (from peel and seeds) that are rich in phenolic compounds presented a higher interfacial activity than that of low-methoxyl

pectin (Velderrain-Rodríguez et al., 2021). Thus, this work demonstrated the advantages of agrifood residues as a source of polysaccharides with emulsifying properties but with added value due to the high content of bioactive compounds that reduced lipid oxidation. In the same way, polysaccharides isolated from seaweed have also been tested as emulsifiers that are rich in bioactive compounds. As an example, polysaccharides from alga *Ulva fasciata* have been tested as emulsifiers in β-carotene-loaded emulsions, showing particle sizes of about 0.8 μm and < 10% of encapsulated compound degradation for 4 days at 4 ºC (Shao et al., 2017). Other algae polysaccharides such as fucoidan have been found to have a good emulsifying capacity, especially when isolated by using microwaves, presenting also antioxidant activity (Alboofetileh et al., 2022; Saravana et al., 2016). This polysaccharide has shown to form emulsions with higher stability and fucoxanthin encapsulation efficiency than Arabic gum (Oliyaei et al., 2022). Moreover, it has been used in combination with other biopolymer, forming complexes. As an example, Jamshidi et al. (2018) used whey protein-inulin-fucoidan complexes to stabilise double emulsions and concluded that the presence of fucoidan had a significant influence on the nutritional quality and oxidative stability.

4.4. Saponins

Saponins are relatively small amphiphilic molecules that are mostly obtained from plants and that consist of a hydrophobic aglycone and a hydrophilic sugar moiety (Augustin et al., 2011). These plant-based emulsifiers appear to be highly effective at forming small droplets that are stable over a wide range of conditions (pH, ionic strength, and temperature) (Ozturk et al., 2014). These emulsifiers, which have been shown to provide steric and electrostatic stabilisation, can form interfacial layers with a high dilatational elasticity, inhibiting droplet deformation and coalescence. Among them, saponins obtained from the bark of the *Quillaja saponaria* tree have been shown to reduce the interfacial tension in the oil-water interface faster and to a higher extent than other emulsifiers such as lecithin, whey protein, or Arabic gum, rendering to emulsions with a smaller particle size (Bai et al., 2016). The use of this emulsifier has been compared with saponins extracted from other plants: *Tribulus terrestris*, *Trigonella foenum-graecum*, and *Ruscus aculeatus* (Schreiner et al., 2021). These authors reported the best results by using the *Tribulus terrestris* extract and highlighted the use of saponin-rich

extracts as potential emulsifiers due to their similar or even additional functional properties than saponin pure forms, avoiding complex extraction and purification treatments. In another work, by using tea saponin extract from *Camellia lutchuensis* (51.8 wt% saponin content) stable emulsions were obtained in a pH range of 3-9 and thermal processing from 30 ºC to 90 ºC (Zhu et al., 2019).

5. Functionality of emulsion-based delivery systems containing natural emulsifiers

5.1. *In vitro* lipid digestibility and bioactive compound bioaccessibility

The lipid digestibility of emulsion-based delivery systems has a strong impact on the release and bioaccessibility of the encapsulated compounds. In that sense, it has been found that the emulsifier type can modulate the digestibility of emulsions. Depending on the emulsifier used, emulsions may present different initial properties (particle size, ζ-potential, viscosity, etc.), and their stability may vary during their pass through the gastrointestinal tract, affecting lipid digestibility (McClements & Li, 2010).

In recent years, the study of lipid digestibility and compound bioaccessibility in emulsions formulated by using natural emulsifiers has increased. Some authors have compared the use of natural emulsifiers with that of synthetics in the stability of emulsions and their gastrointestinal behaviour (Table 2). As an example, Lamothe et al. (2020) compared the digestibility of emulsions containing synthetic emulsifiers (cetyltrimethylammonium bromide (CTAB), Citrem) with that of natural emulsifiers (sodium caseinate, fish gelatin, Arabic gum, modified starch). These authors concluded that, by using some natural emulsifiers such as Arabic gum or sodium caseinate, the digestibility of emulsions was higher than by using synthetic emulsifiers such as CTAB or Citrem. In another work, soybean lecithin was shown to be more effective than synthetic emulsifiers such as Tween 20 or sucrose palmitate in increasing β-carotene bioaccessibility due to its contribution to the formation of mixed micelles and its solubilisation capacity (Gasa-Falcon et al., 2019). In contrast, Tan et al. (2020) observed that lipid digestibility was higher when synthetic Tween 20 or quillaja saponin were used, compared to the other studied natural emulsifiers (lysolecithin, caseinate, or Arabic gum). However, these authors reported that emulsions containing caseinate presented

the same β-carotene bioaccessibility as those with Tween 20 (about 60%), which was attributed to the ability of that protein to inhibit oxidation. These results highlight that bioaccessibility depends not only on lipid digestibility but also on other aspects such as the protection of the compound against degradation or its incorporation into the mixed micelles. The use of gypenosides as a natural emulsifier has been compared to that of Tween 20 in emulsions encapsulating astaxanthin. In that work, gypenoside-emulsion presented lower digestibility and astaxanthin bioaccessibility than emulsion with Tween 20, although the stability of the compound was the same for both emulsions (Chen et al., 2018). The authors attributed the lower bioaccessibility when using the natural emulsifier to the lower digestibility and the possible inhibition of micelle formation due to the presence of gypenoside molecules. In double emulsions, novel natural emulsifiers have also been tested as a substitute for those synthetics. As an example, black bean protein (BBP) has been proposed as an effective emulsifier to increase the bioaccessibility of insulin and quercetin by 2.6- and 4.56-fold, respectively, compared to unencapsulated compounds (Han et al., 2022). Moreover, these authors found that BBP-emulsion showed the same lipid digestibility as the emulsion formulated with synthetic Tween 80.

Many authors have based their research on the use of natural emulsifiers, especially on those of plant origin (Table 2). For instance, the use of plant-based emulsifiers such as Arabic gum and quillaja saponin has been compared with that of one animal-based emulsifier (whey protein isolate) in emulsions enclosing vitamin E (Lv et al., 2019). The results showed that lipid digestion was equal for emulsions containing whey protein or Arabic gum but lower for those with saponins. The authors correlated the results to the high surface activity of saponins, which may have inhibited their removal by bile acids and lipase. Moreover, the highest Vitamin E bioaccessibility was achieved by using the animal emulsifier (85%), followed by plant-based emulsifiers (65%). In another work, curcumin-loaded nanoemulsions containing soybean lecithin or whey protein as emulsifiers presented the same lipid digestibility. However, the bioaccessibility of the encapsulated compound was higher when whey protein was used than when soybean lecithin was used, which was attributed to the capacity of the proteins to better prevent the degradation of curcumin during digestion (Teixé-Roig et al., 2022). Comparison among different plant-based emulsifiers has also been performed by Yan et al. (2022),

who studied the digestibility of emulsions formulated with soybean lecithin (SBL) or hydrolysed rice glutelin (HRG) as emulsifiers. These authors reported that SBL-emulsion was more stable against flocculation under gastric conditions and presented higher digestibility than HRG-emulsion under intestinal conditions. In the same way, other authors have studied the lipid digestion of emulsions containing Arabic gum, ghatti gum, or sugar beet pectin as emulsifiers (Yao et al., 2016). In this work, most Arabic gum-stabilised droplets were digested, but the extensive flocculation and coalescence of undigested droplets were observed in emulsions stabilised with the two other studied emulsifiers. Moreover, the authors attributed the differences in digestion rate among emulsions to their stability in the stimulated intestinal juice and the resistance of the interfacial layer against displacement by bile salts. In addition, emulsions that contained polysaccharides from alga *Ulva fasciata* presented higher digestibility and β-carotene bioaccessibility than emulsions that were formulated by using other polysaccharides such as Arabic gum or beet pectin, which was attributed to the small particle sizes of the former in the intestinal environment (Shao et al., 2017).

Thus, these studies reveal that the use of emulsifiers of natural origin allows obtaining emulsion-based delivery systems with a functionality comparable to that of systems formulated with synthetic emulsifiers. In addition, differences are observed among the natural emulsifiers studied, as they present diverse properties depending on their structure and origin. Nevertheless, it seems that those of plant origin are not as effective as those from milk in increasing the bioaccessibility of encapsulated compounds, although minimal research has been performed with plant-derived emulsifiers.

Table 2. Recent studies on the lipid digestibility and compound bioaccessibility of emulsion-based delivery systems formulated with natural emulsifiers.

System Type	Emulsifiers Used	Encapsulated Compound	Main Findings	Reference
O/W emulsion	Soybean lecithin (SBL), hydrolysed rice glutelin (HRG)	None	SBL-emulsion was more stable against flocculation under gastric conditions and presented higher digestibility than HRG-emulsion.	(Yan et al., 2022)
O/W emulsion	Lysolecithin (LL), Arabic gum (AG), caseinate (SC), quillaja saponin (QS), Tween 20 (T20)	β-carotene	Digestibility was lower for the emulsions stabilised by LL or SC, than those stabilised by AG, QS, or T20. β-carotene bioaccessibility increased in the following order: LL < AG < SC < QS < T20.	(Tan et al., 2020)
O/W emulsion	Quillaja saponin (QS), Arabic gum (AG), whey protein isolate (WPI)	Vitamin E	Lipid digestion was slower in QS-emulsions, presumably because the high surface activity of saponins inhibited their removal by bile acids and lipase. Vitamin E bioaccessibility was higher in WPI- than in QS- or AG-emulsions.	(Lv et al., 2019)
O/W emulsion	Cetyltrimethylammonium bromide (CTAB), Citrem, sodium caseinate (SC), fish gelatin (FG), Arabic gum (AG), or modified starch (MS)	Linseed oil (rich in omega-3 PUFA)	Emulsions prepared with CTAB and GA were the most stable under gastric conditions, while those stabilised by proteins (SC or FG) and MS showed aggregation with partial coalescence in the gastric phase. AG-emulsion showed the highest FFA extent, followed by CTAB- and SC- emulsions.	(Lamothe et al., 2020)
O/W emulsion	Arabic gum (AG), ghatti gum (GG), or sugar beet pectin (SBP)	None	The digestion rate decreased in the following order: AG > SBP > GG. Differences were attributed to the stability of the emulsified lipid droplets in the stimulated intestinal juice and the resistance of interfacial layer against displacement by bile salts.	(Yao et al., 2016)
O/W emulsion	*Ulva fasciata* polysaccharide (UFP), Arabic gum (AG), or beet pectin (BP)	β-carotene	UFP-stabilised emulsion showed higher release extent of free fatty acids and bioaccessibility of carotenoids compared to BP and AG-stabilised emulsions.	(Shao et al., 2017)
O/W emulsion	Tween 80 (TW), phosphatidylcholine (PC), or citrus pectin (CP)	β-carotene	T80-emulsion presented a higher β-carotene bioaccessibility than those with PC or CP, and it was associated with the higher concentration of incorporated MAG and FFA into the micellar fraction by using T80-emulsion.	(Verkempinck et al., 2018)
W/O/W double emulsion	Lecithin (L), pectin (P), black bean protein (BBP), or Tween 80 (T80)	Insulin and quercetin	The BBP-stabilised double emulsion presented the lowest particle size during the GIT digestion. Moreover, it yielded a 2.60- and 4.56-fold increase in the bioaccessibility of insulin and quercetin, respectively, by increasing their chemical stability and solubility under simulated gastrointestinal conditions.	(Han et al., 2022)
W/O/W double emulsion	gelatin-epigallocatechin gallate (EGCG)-high methoxyl pectin ternary complex	Vitamin C	Gelatin-EGCG-high methoxyl pectin ternary complex had a better protective effect on vitamin C in the internal aqueous phase during in vitro simulated digestion. Compared with the W_1/O primary emulsion, the double emulsion effectively improved the bioavailability of vitamin C.	(Huang et al., 2022)

Multilayer emulsion	Quillaja saponin (QS), chitosan (CS), pectin (P)	Astaxanthin	Coating layers of CS and P improved the lipid stability during gastrointestinal digestion and reduced the release of free fatty acids (by nearly 20%). Meanwhile, the release of Astaxanthin was prolonged in the small intestine, and its final bioaccessibility was improved by the coating layers.	(Sun et al., 2023)
Multilayer emulsion	Sodium caseinate (SC), sulphated fucan (SF), ι-carrageenan (ICA), κ-carrageenan (KCA), or alginate (ALG)	None	All studied multilayer emulsions presented an increased digestibility compared to the primary emulsion. Moreover, the digestion rate and degree of multilayer emulsions decreased in the order of KCA > ALG ≈ ICA > SF.	(Shi et al., 2023)
Pickering emulsion	Nanochitin (NCh)	Vitamin D_3	NCh–Pickering emulsions presented lower digestibility and vitamin bioaccessibility than T80-emulsions as a consequence of flocculation, hindered access for lipase to reach lipid, and precipitation of mixed micelles.	(Zhou et al., 2020)
Pickering emulsion	Chitosan (CS)	Roasted coffee oil	CS nanoparticles were shown to be able to adsorb onto oil droplet surfaces, providing efficiency in encapsulating and protecting bioactive compounds during lipid digestion and increasing the bioaccessibility of phenolic compounds.	(Ribeiro et al., 2020)
Pickering emulsion	Nanofibrillated cellulose (NFC) or whey protein isolate (WPI)	Astaxanthin	Pickering emulsions containing 0.7% NFC presented higher compound stability during digestion than emulsions with 0.7% WPI. However, they presented the same astaxanthin bioaccessibility due to the reduced digestibility of NFC emulsions.	(Saechio et al., 2023)

5.2. *In vivo* bioavailability

In vitro studies are very helpful for obtaining information about the expected gastrointestinal stability and digestibility of emulsion-based delivery systems, as well as the bioaccessibility of the encapsulated compound. However, *in vivo* studies are essential to obtain stronger conclusions and information about the absorption and metabolism of the encapsulated compound. For all that, some authors have investigated the oral bioavailability of different compounds enclosed in emulsion-based delivery systems. To date, most research that has been conducted to increase the bioavailability of bioactive compounds such as curcumin (Nazari-Vanani et al., 2017), β-carotene (Meng et al., 2019), or resveratrol (Pandita et al., 2014) by using different emulsion-based delivery systems has been performed by using synthetic emulsifiers. However, in the last years, *in vivo* research has been focused on emulsions formulated by using emulsifiers of natural origin (Table 3).

Among the emulsifiers of natural origin, those from milk have been widely used to study the *in vivo* bioavailability of bioactive compounds enclosed in different systems (Table 3). As an example, the use of different β-carotene-loaded emulsion delivery systems containing whey protein as an emulsifier has been investigated (Chen et al., 2021). The authors found that the bioavailability of β-carotene was higher when using nanoemulsions than when using macroemulsions or non-encapsulated β-carotene due to their greater capacity to promote the transportation and absorption of the compound in the digestive tract. In the same way, the carotenoid bioavailability was also increased by using excipient emulsions containing sodium caseinate as an emulsifier (Yao et al., 2021). Moreover, this study revealed that by increasing the oil content from 0.2 to 1 g, the carotenoid concentration in the plasma of rats increased from ≈ 40 ng/mL to ≈ 110 ng/mL. Therefore, it indicates that emulsifiers from milk are effective in increasing the bioavailability of lipophilic compounds such as carotenoids and that the oil content and the particle size have an effect on their bioavailability. Moreover, curcumin oral bioavailability was increased by 5-fold compared to the suspension, when adding chitosan and carboxymethyl konjac glucomannan to a whey-protein-stabilised emulsion to form a multilayer emulsion. In contrast, when the primary emulsion was used, curcumin oral bioavailability was only 1.95-fold greater than when the suspension was

used (Wang et al., 2021). However, interest in plant-derived emulsifiers has increased in response to demands from consumers with milk protein allergies or intolerances or those with a vegan diet.

Plant-based emulsifiers such as soybean lecithin have been incorporated into nanoemulsions designed to increase the bioavailability of carotenoids from microalga *Dunaliella Salina* (Table 3) (Teixé-Roig et al., 2023). In this study, nanoemulsions containing soybean lecithin increased the bioavailability of carotenoids compared to the control suspension, but better results were observed when whey proteins (by 2.8-fold) rather than soybean lecithin (by 2.15-fold) were used. Other vegetal emulsifiers such as pea protein have been used in combination with chitosan in the formulation of EPA-loaded Pickering emulsions, and these vegetal emulsifiers were shown to be more effective in increasing the bioavailability of the enclosed compound than an emulsion containing Tween 80 (Ji et al., 2022). The use of saponins has been investigated in emulsions and nanoemulsions enclosing cholecalciferol (vitamin D3) or α-tocopherol (Vitamin E) that were orally administered to rodents. Parthasarathi et al. (2016) found that, by reducing the particle size of emulsions, the bioavailability of α-tocopherol was enhanced, being 3-fold higher in rats fed with the saponin-nanoemulsion rather than in those fed with the conventional saponin-emulsion. Moreover, saponin emulsions and nanoemulsions have been shown to increase the bioavailability of vitamin D3 by 36% and 73%, respectively (Kadappan et al., 2018), highlighting the relevance of emulsion particle size in increasing the bioavailability of closed compounds.

Table 3. Recent studies on the *in vivo* bioavailability of bioactive compounds enclosed in different emulsion-based delivery systems containing natural emulsifiers.

Bioactive Compound	Dose	System Type	Animal Model	Ingredients	Outcomes	Reference
β-carotene	1 mg/kg BW	O/W nanoemulsion	mice	10% corn oil; 2% whey protein isolate	Nanoemulsions increased transportation and absorption of β-carotene in the digestive tract compared to macroemulsions.	(Chen et al., 2021)
	60 mg/kg BW	O/W nanoemulsion	rat	30% corn oil; 12% whey protein isolate; or soybean lecithin	Nanoemulsions containing protein-based emulsifiers better increased the bioavailability of β-carotene than those containing soybean lecithin.	(Teixé-Roig et al., 2023)
Carotenoids (from fresh spinach puree)	1.0, 0.6, 0.2, and 0 g/kg BW	O/W nanoemulsion (excipient)	rat	10% oil (medium-chain triglyceride and long-chain triglyceride 1:1); 1% sodium caseinate	Carotenoid bioavailability was enhanced by increasing the lipid content due to the higher transfer efficiency of the carotenoids from spinach to fat droplets and mixed micelles.	(Yao et al., 2021)
Cholecalciferol (VD_3)	4000 IU kg^{-1} supplementation	O/W emulsion or nanoemulsion	mice	10% corn oil; 2% quillaja saponin	Nano-based delivery system improved the bioavailability and homogeneity of VD absorption.	(Kadappan et al., 2018)
Coenzyme Q10	30 mg/kg BW	O/W nanoemulsion	rat	10% soybean oil; 1–10% lecithin	Incorporation of Coenzyme Q10 to nanoemulsions increased the bioavailability of the bioactive compound by 1.8-fold.	(Niu et al., 2020)
Tangeretin	100 mg/kg BW	O/W emulsion	rat	20% medium-chain triglyceride oil, whey protein concentrate, and gum Arabic, or cinnamaldehyde, or hydroxypropyl methylcellulose	Tangeretin bioavailability increased from 4- to 20-fold after encapsulation, especially in the presence of hydroxypropyl methylcellulose.	(Hu et al., 2021)
α-tocopherol	100 mg/kg BW	O/W emulsion, submicron emulsion and nanoemulsion	rat	10% sunflower oil; 0.1% saponins	By reducing the particle size of emulsions, the bioavailability of α-tocopherol was enhanced, which was 3 times higher when the nanoemulsion was used than when the emulsion was used.	(Parthasarathi et al., 2016)
EPA	60 mg/kg BW	Pickering emulsion	mice	60% oil; 4% pea protein–chitosan nanoparticles	EPA-loaded Pickering emulsions containing pea protein — chitosan nanoparticles were shown to be more effective in increasing EPA bioavailability than an emulsion containing Tween 80.	(Ji et al., 2022)
Curcumin	12 mg/kg BW	Multilayer emulsion	mice	10% medium chain oil and 90% WPI aqueous solution (1%), 0.2% chitosan (CS), and 0.1 carboxymethyl konjac glucomannan (CKG)	Emulsions coater with CKG or CS + CKG conferred a higher C_{max} value and improved the bioavailability of curcumin by up to 5-fold compared with free curcumin.	(Wang et al., 2021)

6. Concluding remarks and future perspectives

In recent years, much research has been performed to obtain bioactive compounds from alternative sources that contribute to food sustainability. Among them, microalgae and agrifood residues appear to be promising sustainable sources of pure or rich extracts to be used as ingredients to design novel functional foods. Moreover, novel green extraction methods are being developed to obtain these valuable compounds in a more safe and environmental-friendly way. Various encapsulation systems are being designed to protect these valuable compounds, with emulsion-based delivery systems being effective systems to increase their stability, bioaccessibility, and oral bioavailability. In general, the selection of the emulsion-based delivery system depends on the properties of the compound and the required functionality. Among the existing methods, novel systems such as Pickering emulsions or NLC have been found to provide higher chemical stability and higher control over the release of the encapsulated compounds compared to other systems such as conventional emulsions.

Some of the most recent *in vitro* and *in vivo* studies have yielded results that reveal that emulsifiers from natural sources can be used as substitutes for those synthetics, improving the bioaccessibility and oral bioavailability of encapsulated compounds. In addition, although vegetal emulsifiers seem to provide emulsions with characteristics comparable to those of milk proteins, *in vitro* studies reveal that milk proteins seem to be more effective at increasing the bioaccessibility and bioavailability of the encapsulated bioactive compounds than those of vegetable origin. Nevertheless, most *in vivo* studies have been performed by using emulsions or nanoemulsions. Therefore, more research is required to evaluate the functionality of those more novel and complex systems containing ingredients from natural sources. Furthermore, in recent years, promising emulsifier molecules of plant and algae origin have been identified but have not yet been studied *in vivo*. Therefore, more studies are needed to determine the potential of these plant-derived molecules to increase the oral bioavailability of compounds with relevant biological properties.

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HYPOTHESIS AND OBJECTIVES

The hypothesis of this doctoral thesis was that emulsion-based delivery systems can be useful systems for increasing the stability and bioavailability of bioactive compounds, with the composition of these systems being a factor that could influence their properties and functionality.

Therefore, the main objective of this doctoral thesis was to study how the composition affects the properties, stability and functionality of different emulsion-based delivery systems enclosing bioactive compounds. To achieve this goal, the following specific objectives were proposed:

1. Prepare and optimize the different emulsion-based systems incorporating bioactive compounds.
2. Evaluate the physicochemical characteristics and the stability over time of the developed emulsion-based systems.
3. Assess the *in vitro* and *in vivo* stability of the emulsion-based systems along the gastrointestinal tract.
4. Evaluate the digestibility of the system lipid fraction and determine the *in vitro* bioaccessibility of the encapsulated compounds.
5. Determine the *in vivo* bioavailability and metabolism of the encapsulated compounds.

MATERIALS AND METHODS

1. Materials

This section includes materials or reagents used in the experiments carried out during this doctoral thesis. Table 1 lists the ingredients used in the formulation of the different emulsion-based delivery systems. All the reagents used for the *in vitro* simulated gastrointestinal digestion are included in Table 2.

Table 1. Physicochemical characteristics of ingredients used for the preparation of the emulsion-based delivery systems and their supplier. (nda: no data available).

Ingredient	Purity (%)	Molecular weight (g/mol)	Melting point (ºC)	Density (g/cm^3 25ºC)	Form	Supplier
Anthocyanins Medox ®	38%	270-330	nda	nda	Powder	Biolink Group (AS, Sandnes, Norway)
Phycocyanin	nda	nda	nda	nda	Powder	GNT (Derby, United Kingdom)
Curcumin	> 98	368.37	183	nda	Powder	Sigma-Aldrich (St. Louis, MO)/Acros organics
Alga *Dunaliella Salina*	Raw algae	nda	nda	nda	Powder	Monzón Biotech (Huesca, Spain)
β-carotene	≥ 95	536.87	174	1	Powder	Sigma-Aldrich (St. Louis, MO)
Corn oil	nda	≈ 536.9	-10	0.9	Liquid	Koipe Asua (Spain)
Miglyol 812 N ®	99.9	505-520	-5 to 6	0.93-0.96	Liquid	IOI Oleochemical GmbH (Hamburg, Germany)
Palm oil	100	695	35	0.9-0.93	Solid	Mystic Moments (Hants, UK)
Coconut oil	100	nda	25	nda	Solid	HMF (GmbH & Co. KG, Germany)
Tween 20	> 99	1127.72	nda	1.11	Viscous liquid	Sigma-Aldrich (St. Louis, MO)
Tween 80	> 99	1310	nda	1.07	Viscous liquid	Lab Scharlab (Sentmenat, Spain)
Soybean lecithin	≥ 95	758.08	nda	nda	Powder	Alfa Aesar
Glycerol	≥ 99.5	92.09	20	1.25	Viscous liquid	Danisco (DuPont, EEUU)
Polyglycerol polyricinoleate	nda	nda	nda	nda	Viscous liquid	Danisco (DuPont, EEUU)
Sodium caseinate	> 98	nda	nda	nda	Powder	Alfa Aesar (Karlsruhe, Germany)
Whey protein isolate	≈ 65	nda	nda	nda	Powder	Pastoret (Lleida, Spain)
Sodium carboxymethylcellulose	nda	nda	>300	1.59	Powder	Sigma-Aldrich (St. Louis, MO)
Arabic gum	> 95	nda	nda	1.35	Powder	Acros organics (Geel, Belgium)
Sodium alginate	nda	nda	>300	nda	Powder	FMC Biopolymer Ltd (Scotland, U.K.)
Pectin	100	nda	nda	nda	Powder	Acros organics (Geel, Belgium)

Table 2. List of ingredients used for the simulation of the *in vitro* gastrointestinal digestion and their supplier.

Ingredients	**Supplier**
Porcine pepsin	Sigma-Aldrich (St. Louis, MO)
Bovine bile	Sigma-Aldrich (St. Louis, MO)
Porcine pancreatin	Sigma-Aldrich (St. Louis, MO)
Lipase from porcine pancreas	Sigma-Aldrich (St. Louis, MO)
$CaCl_2(H_2O)_2$	Sigma-Aldrich (St. Louis, MO)
NaOH	Sigma-Aldrich (St. Louis, MO)
HCl	Sigma-Aldrich (St. Louis, MO)
KCl	Sigma-Aldrich (St. Louis, MO)
KH_2PO_4	J.T. Baker (USA)
$NaHCO_3$	Sigma-Aldrich (St. Louis, MO)
NaCl	Sigma-Aldrich (St. Louis, MO)
$MgCl_2(H_2O)_6$	Sigma-Aldrich (St. Louis, MO)
$(NH_4)_2CO_3$	Sigma-Aldrich (St. Louis, MO)

2. Methods

2.1. Formulation of emulsion-based delivery systems

2.1.1. Emulsions

To formulate the different oil-in-water emulsions, the lipid and aqueous phases were prepared separately. The lipid phase was obtained by adding the lipophilic compounds (β-carotene or curcumin) to the oil by stirring and sonicating. The temperature of the oil, stirring rate and sonication time were different depending on the lipophilic compound incorporated. To obtain the aqueous phase, polysaccharides and emulsifiers were added in milli-Q water and stirred until dissolution was complete. Afterwards, the lipid phase was mixed with the aqueous phase and homogenized using a high-speed blender (Ultra-Turrax, IKA, Staufen, Germany).

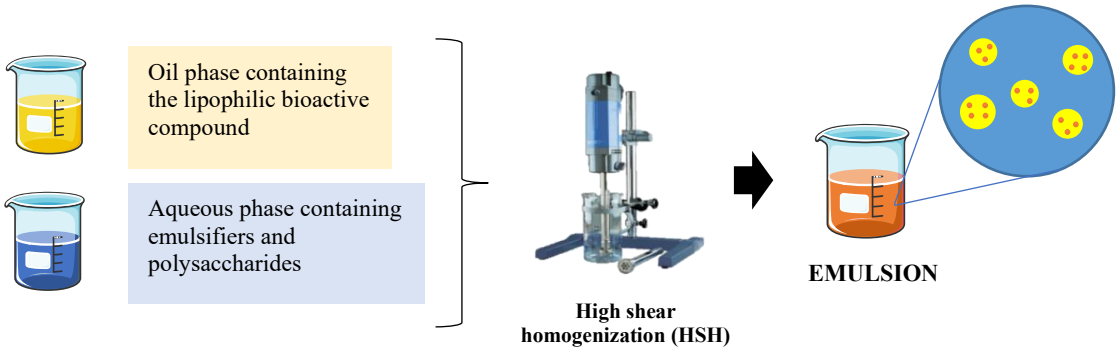


Figure 1. Methodology used for the formulation of emulsions.

2.1.2. Nanoemulsions

The obtained emulsions were subjected to a microfluidization or sonication process to obtain nanoemulsions. For the microfluidization process, emulsions were treated with a microfluidizer (LM10, Microfluidics, USA). For the sonication process, emulsions were treated with a P400S Hielscher sonicer (Hielscher Ultrasound Technology, Teltow, Germany), of 400 W nominal power and a frequency of 24 kHz equipped with a 22 mm sonotrode for 3 min at 100% of amplitude. Table 3 summarizes the different nanoemulsions formulated during this doctoral thesis, their ingredients and the treatment conditions used.

Table 3. Composition and treatment conditions for the different nanoemulsions formulated in this doctoral thesis.

Lipophilic bioactive compound	Lipid phase	Aqueous phase	Treatment conditions
β-carotene (commercial)	4% w/w corn oil (5 mg β-carotene/g oil)	4% w/w Tween 20 + 0, 1 or 2% w/w citrus pectin	HSH 9500 rpm for 2 min + microfluidization 100 mPa for 5 cycles
Curcumin	5% w/w corn oil (1 mg curcumin/g oil)	5% w/w soybean lecithin or whey protein + 0, 0.5, 1 or 1.5% w/w sodium alginate	HSH 11000 rpm for 2 min + sonication for 3 min at 100% amplitude
β-carotene (from alga Dunaliella Salina)	10 or 30% w/w corn oil (20 mg β-carotene/g oil	4 or 12 % w/w (SOR 0.4) soybean lecithin or whey protein isolate	HSH 1100 rpm for 2 min + microfluidization 130 mPa for 5 cycles
β-carotene (from alga Dunaliella Salina)	20% w/w corn oil (20 mg β-carotene/g oil)	8 % w/w soybean lecithin, whey protein isolate or sodium caseinate	HSH 1100 rpm for 2 min + microfluidization 130 mPa for 5 cycles

HSH: high shear homogenization

2.1.3. Double emulsions

For the formulation of the double emulsions, a two-step emulsification method described by Aditya et al. (2015) with some modifications was used. To obtain the primary W_1/O emulsion, the two phases were prepared separately. The W_1 phase, comprising the hydrophilic extract solution and glycerol, and the oil phase, consisting of corn oil and polyglycerol polyricinoleate (PGPR) or a mixture of PGPR and sodium caseinate as emulsifiers. Subsequently, the two phases were mixed and homogenized using a high-speed homogenizer (Ultra-Turrax, Janke & Kundel, Staufen, Germany), and a sonication process was performed on the primary W_1/O emulsion using a P400S Hielscher sonicer (Hielscher Ultrasound Technology, Teltow, Germany) at a frequency of 24 kHz. The external aqueous phase (W_2) was prepared adding the emulsifier (lecithin or Tween 20)

on the NaCl 0.1 M solution. In the polysaccharide-added emulsions, the studied polysaccharide (carboxymethyl cellulose, Arabic gum, pectin or alginate) was added and fully dissolved into the W_2 phase before adding the emulsifier. Finally, W_1/O and W_2 were mixed, homogenized using the high-speed homogenizer and sonicated at a frequency of 24 kHz.

2.1.4. Solid lipid nanoparticles

To obtain solid lipid nanoparticles, the lipid phase and the aqueous phase were prepared separately. The lipid phase was prepared by solubilizing the lipophilic compound in the lipid by magnetically stirring at 450 rpm for 30 min at 60 °C and sonicating for 15 min to ensure complete dissolution. To prepare the aqueous phase, 2.5% w/w of Tween 80 in milli-Q water were stirred at 60 °C. Afterwards, both the lipid phase (21% w/w) and aqueous phase (79% w/w) were mixed and homogenized using an Ultra-Turrax (IKA, Staufen, Germany) at 7600 rpm for 3 min. Then, nanoparticles were obtained by passing the coarse emulsions through a microfluidizer (LM10, Microfluidics, USA) at 100 MPa for 3 cycles. Finally, the temperature of nanoparticles was reduced to 4 °C for 2 h in order to allow fat recrystallization.

2.2. Physicochemical characterization of emulsion-based delivery systems

2.2.1. Particle size

The particle size of nanoemulsions was measured using a Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). Samples were diluted in ultrapure water and stirred in the dispersion unit with a constant speed of 1800 rpm. The mean particle size was expressed as surface area mean diameter (d_{32}) in micrometres (μm), fixing a refractive index of 1.333 for water.

2.2.2. ζ-potential

The ζ-potential was measured by phase-analysis light scattering (PALS) using Zetasizer laser diffractometer (NanoZS Malvern Instruments Ltd Worcestershire, UK). Prior to the analysis, nanoemulsions were diluted in ultrapure water. Gastric and intestinal samples were diluted maintaining its pH at 3 and 7, respectively. Then, the diluted samples were

placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles. The results were reported in millivolts (mV).

2.2.3. Viscosity

Apparent viscosity of nanoemulsions was determined using a SV-10 vibro-viscometer (A&D Company, Tokyo, Japan), which produces a vibration of 30 Hz and a constant amplitude of 0.4 mm at controlled room temperature. The results were expressed in mPa·s.

2.2.4. Microscope images

Optical microscope

Images of emulsions-based systems were obtained using an optical microscope (Olympus BX41, Olympus America Inc., Melville, NY, USA) with a 100x objective lens. The images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus).

Optical microscope using fluorescence

Emulsion-based systems were stained with Nile Red, previously dissolved at 0.1% (w/v) in ethanol. Then, micrographs were obtained using fluorescence with an optical microscope (Olympus BX41, Olympus, Göttingen, Germany) with a 100x objective lens. The images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus Göttingen, Germany).

Confocal microscope

Confocal microscope images were obtained using a confocal scanning laser microscopy (CSLM) (Olympus FV1000 Spectral Confocal Microscope Olympus, Melville, NY). Emulsions-based systems were stained with Nile Red dissolved at 0.1% (w/v) and examined with a 100x magnification lens and a laser with an excitation line of 559 nm.

2.2.5. Physical stability

The stability of nanoemulsions over time was assessed by the multiple light scattering technique using an optical scan analyzer Turbiscan MA 2000 (Formulaction, Toulouse, France), which can measure the static stability of samples without destruction and detect the cause of instability (flocculation, coalescence, sedimentation or creaming). After their preparation, 7 mL aliquots of each nanoemulsion were introduced into glass cylindrical cells and analyzed by a light beam emitted in near infrared wavelength, which scanned vertically from the bottom to the top of the sample cell. Two synchronous optical sensors receive light backscattered by the sample (45° from the incident radiation).

2.2.6. Encapsulation efficiency

Nanoemulsions

Encapsulation efficiency (EE) of oil-in-water nanoemulsions was measured using a previously reported method (Artiga-Artigas et al., 2018). Initially, 10 mL of nanoemulsion was placed inside a dialysis tubing cellulose membrane of 43 mm x 27 mm (Sigma-Aldrich, Darmstadt, Germany). Then, the membrane was inserted into a centrifuge tube containing 20 mL of ethanol and centrifuged at 2000 rpm for 10 min. Finally, the non-encapsulated lipophilic compound content was quantified using a UV-visible spectrophotometer (CECIL CE 2021; Cecil Instruments Ltd, Cambridge, UK). The encapsulation efficiency (%) was calculated using Eq. (1) (Surassmo et al., 2010):

$$\text{EE (\%)} = \frac{\text{Total amount of bioactive compound - Free bioactive compound}}{\text{Total amount of bioactive compound}} \times 100 \qquad (1)$$

where the total amount of bioactive compound is the initial concentration added in emulsions and the free bioactive compound is the concentration of the compound that was not loaded in emulsions.

Double emulsions

To determine the encapsulation efficiency of hydrophilic bioactive compounds in double emulsions, firstly, aliquots of 10 mL of emulsions were centrifuged at 4500 rpm for 10

min at 4 ºC. Then, the W_2 phase containing the non-encapsulated hydrophilic bioactive compound was collected and quantified. Finally, using the hydrophilic bioactive compound content in W_2, and the content in the total emulsion (previously quantified), the encapsulation efficiency was calculated using Eq. (1).

2.3. *In vitro* studies

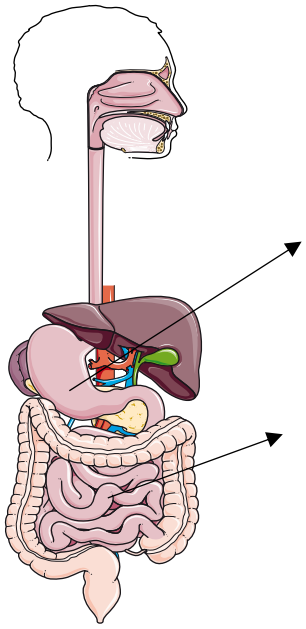
2.3.1. Gastrointestinal digestion and digestibility

Emulsion-based delivery systems were subjected to an *in vitro* static gastrointestinal tract digestion based on an international consensus method (Brodkorb et al., 2019; Minekus et al., 2014). The protocol included both gastric and small intestinal phases. The mouth phase was not performed since nanoemulsions were liquid. Figure 2 summarizes the main characteristics of the *in vitro* digestion method used.

To perform the gastric phase, 20 mL of nanoemulsion was mixed 1:1 with simulated gastric fluids (SGF) containing pepsin (2000 U/mL) and 10 μL of a $CaCl_2$ solution (0.3 M). Then, the pH was adjusted to 3 using HCl (1M) and the mixture was placed into an incubator at 37 °C for 2 h while shaking at 100 rpm. To simulate the intestinal phase, a pH-stat device was used. Once the gastric phase was completed, an aliquot of 20 mL of gastric sample was placed in a 37 °C water bath and mixed 1:1 with simulated intestinal fluids (SIF) containing 10 mM bile solution. Afterwards, the pH was adjusted to 7 with NaOH (1 M) and pancreatin and lipase enzymes were added to the final mixture to achieve 100 U/mL of trypsin and 2000 U/mL pancreatic lipase. The pH of the sample was maintained to 7 by adding NaOH (0.25 M) constantly for 2 h. The final volume of NaOH (0.25 M) was recorded and used to calculate the amount of free fatty acids (FFA) released during intestinal digestion. The FFA (%) was determined according to Eq. (2):

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

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



Gastric phase

- Simulated gastric fluids, porcine pepsin enzyme (2000 U/mL)
- Incubation 2 h at 37 ºC 100 rpm pH 3

Intestinal phase

- Simulated intestinal fluids, porcine pancreatin and lipase enzymes (100 U/mL of trypsin and 2000 U/mL pancreatic lipase), bovine bile enzyme (10 mM)
- Incubation 2 h at 37 ºC pH 7

Figure 2. Conditions in each digestive phase during the *in vitro* static gastrointestinal digestion.

2.3.2. In vitro bioaccessibility

The bioaccessibility of the encapsulated bioactive compounds was determined after emulsion-based delivery systems were subjected to the simulated *in vitro* static gastrointestinal digestion, using a previously described method (Qian et al., 2012). After the *in vitro* digestion process, digested emulsion-based delivery systems were centrifuged to obtain the micellar fraction. Then, an extraction and quantification of the bioactive compound content was performed in both the initial emulsion and micellar fraction. Finally, bioactive compound bioaccessibility was calculated according to Eq. (3):

$$\text{Bioaccessibility (\%)} = \frac{C_{micelle}}{C_{emulsion}} \quad (3)$$

where $C_{micelle}$ is the bioactive compound concentration ($mg \cdot mL^{-1}$) in the micellar fraction and $C_{emulsion}$ is the initial bioactive compound concentration ($mg \cdot mL^{-1}$) in the initial emulsion.

2.3.3. Compound degradation

The capacity of emulsion-based delivery systems to prevent the degradation of the encapsulated compound along the simulated gastrointestinal tract was investigated. For

that, 1 ml of digesta was collected at specific digestion times (0, 30, 60, 90, 120, 150, 180, 210 and 240 min). Then, the bioactive compound was extracted and quantified to obtain the bioactive compound content. Finally, the curcumin content in the aliquots collected at different digestion times was determined according to the Eq. (4):

$$\text{Bioactive compound content (\%)} = \frac{C_{digesta}}{C_{initial}} \times 100 \quad (4)$$

where $C_{digesta}$ is the compound concentration ($mg \cdot mL^{-1}$) in the digesta and $C_{initial}$ is the initial compound concentration ($mg \cdot mL^{-1}$) in the nanoemulsion.

2.4. *In vivo* studies

2.4.1. Pharmacokinetic studies

All the animal procedures were conducted in accordance with EU Directive 2010/63/EU guidelines for animal experiments and approved by the Animal Ethics Committee of Universitat de Lleida. Rats were housed at constant temperature (20 ± 0.5 °C) and humidity (50 ± 10%) and a 12:12 h day/night cycle. Animals were fasted for 12 h before the experiment with free access to water. The animals were fed with a single dose of one of the different nanoemulsions containing the bioactive compound or a suspension of the bioactive compound using a feeding needle. Blood samples were taken at 0, 30, 60, 120, 240, and 480 min after administration from the tail vein. Plasma was immediately separated by centrifuging the blood samples at 4000 rpm for 10 min at 4 ºC and stored at -80 ºC. Then, bioactive compounds and their metabolites were extracted and quantified in plasma samples.

The pharmacokinetic analysis was performed using a non-compartmental design. The area under the drug concentration versus time curve from 0 to 8 hours ($AUC_{0-8\ h}$) was calculated using the trapezoidal rule. The maximum plasma concentration of curcumin (C_{max}) and the time to reach maximum plasma concentration (T_{max}) were directly obtained from plasma analyses.

2.4.2. Gastrointestinal stability and biodistribution

All the animal procedures were conducted in accordance with EU Directive 2010/63/EU guidelines for animal experiments and approved by the Animal Ethics Committee of

Universitat de Lleida. Rats were fasted for 12 h before the experiment with free access to water. Animals were orally administered at a volume of 10 mL/kg with a feeding needle for the single dose experiments and 20 mL/kg for the multiple dose experiments. In the case of multiple dose experiments, sample administration was conducted in five times with 30 min intervals, and rats were sacrificed 30 min after the last administration. For the single dose experiments, rats were sacrificed at different times (2 and 4 h after administration).

In all cases, rats were anesthetised with isoflurane and blood samples were taken by cardiac puncture. Plasma was immediately separated by centrifuging the blood samples at 4000 rpm for 10 min at 4 ºC and stored at -80 ºC. Then, the rats were sacrificed by exsanguination and the stomach, duodenum, jejunum, ileum and colon were removed. The content of the gastrointestinal organs was collected and immediately characterized by microscopy as described in section 2.4.4. Afterwards, the mentioned tissues and, liver, kidneys, white adipose tissue and brown adipose tissue were collected, rinsed with phosphate buffered saline (PBS), weighted and stored at -80 ºC until analyses. Bioactive compounds and their metabolites were extracted and quantified in plasma and tissue samples as it follows.

2.4.3. Extraction and quantification of different bioactive compounds from plasma and tissues of rats

2.4.3.1. Carotenoids

Retinol and β-carotene extraction in plasma and tissue samples were performed according to a previously reported method with some modifications (Fan et al., 2017). Extractions were performed in a dark room to avoid compound degradation. For plasma samples, aliquots of 150 µL were mixed with the internal standard trans-β-apo-8'-carotenal and 600 µL of ethanol/hexane (1:2 v/v) containing 0.1% BHT. The mixture was vortexed at 1000 rpm for 1 min, centrifuged at 9000 rpm for 5 min at 4 ^{O}C, and the organic fraction was collected, evaporated under N_2 and stored at -80 ºC. For the tissue samples, 250 mg of tissue was mixed with 600 µL of milli-Q water and homogenized using an Ultra-Turrax at 9000 rpm for 1 min. After that, the tissue homogenates were mixed with the internal standard trans-β-apo-8'-carotenal and 1000 µL of

ethanol/hexane (1:2 v/v) containing 0.1% BHT. Then, the mixture was vortexed at 1000 rpm for 1 min, centrifuged at 9000 rpm for 5 min at 4 ^{O}C, and the organic fraction was collected. Afterwards, upper organic layers were evaporated under N_2 and stored at -80 ºC.

The quantification of β-carotene and retinol from plasma and tissue samples was performed using a Acquity UPLC with a photodiode array detector from Waters (Milford, MA, USA) equipped with a binary solvent delivery system. The analysis was performed using a reverse-phase C18 column (ACQUITY UPLC® BEH 1.7µm 150 x 2.1 mm) kept at constant temperature (32 ºC). The volume injected was 7.5 µL and the mobile phase consisted of a gradient of (A) acetonitrile: methanol (70:30 v/v) and (B) water: acetonitrile (95:5 v/v). The flow rate was 1 mL/min. The gradient profile of the mobile phase was set at 90% A and increased linearly to 100% A over 5.5 min with a 6.5-min hold, after which the mobile phase was changed back to 90% A over 2 min and the held for 2 min. β-carotene was detected at 450 nm and retinol at 325 nm.

2.4.3.2. Curcuminoids

Curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin glucuronide extraction in plasma samples was performed according to a previously reported method with some modifications (Li et al., 2011). Aliquots of 150 µL of plasma were mixed with the internal standard (Honokiol) and 600 mL of acetonitrile and vortexed at 1800 rpm for 1 min. Then, the mixture was submerged into an ultrasonic bath for 30 s, centrifuged at 9000 rpm for 10 min at 4 ºC, and the organic fraction was collected. Afterwards, upper organic layers were evaporated under N_2 and stored at -80 ºC until quantification. For tissues, a previously described method with some modifications was used (Chirio et al., 2019). Initially, samples were mixed with milli-Q water and homogenized with an Ultra-Turrax at 9000 rpm for 1 min to obtain the tissue homogenates. Then internal standard (Honokiol) and 1 mL of acetonitrile were added to tissue homogenates and the mixture was vortexed at 2000 rpm for 1 min. Then, the mixture was centrifuged at 9000 rpm for 5 min at 4 ºC, and the organic fraction was collected. Afterwards, upper organic layers evaporated under N_2 and stored at -80 ºC until quantification.

The quantification of curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin glucuronide was performed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI–MS/MS) using the method described by Liu et al. (2018), with some modifications. To perform the analyses an ACQUITY UPLC binary (Waters, Milford, MA, USA) coupled to a Xevo TQS (triple quadrupole) (Waters, Milford, MA, USA) was used. Chromatographic separation was performed on a 150 mm × 2.1 mm i.d., 1.6 µm CORTECS Phenyl Column (Waters, Milford, MA, USA) with mobile phase A [acetonitrile containing 0.1% (v/v) formic acid] and mobile phase B [98% water and 2% acetonitrile containing 0.1% (v/v) formic acid]. The injection volume was 2.5 µL and the flow rate was 0.4 mL/min. Separation was carried out in 5.5 min under the following conditions: 0 min, 60% B; 4 min, 25% B; 4.1 min, 60% B; 5.5 min, 60% B. The column was equilibrated for 10 min prior to each analysis. The column temperature was maintained at 40 ºC. The MS operated in electrospray ionization (ESI) in negative mode and nitrogen was used as the source gas in all cases.

2.4.4. Microstructure of nanoemulsions

Digesta obtained from the different part of the gastrointestinal tract of rats (stomach, duodenum, jejunum, ileum, and colon) were collected and stained with Nile Red, previously dissolved at 0.1% (w/v) in ethanol. Then, micrographs were obtained using fluorescence with an optical microscope (Olympus BX41, Olympus, Göttingen, Germany) with a 100x objective lens. The images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus Göttingen, Germany).

2.5. Statistical analysis

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 ($p < 0.05$ was considered significant).

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PUBLICATIONS

Chapter I

Improving the *in vitro* bioaccessibility of β-carotene using pectin added nanoemulsions

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Abstract

The intestinal absorption of lipophilic compounds such as β-carotene has been reported to increase when they are incorporated in emulsion-based delivery systems. Moreover, the reduction of emulsions particle size and the addition of biopolymers in the systems seems to play an important role in the emulsion properties but also in their behavior under gastrointestinal conditions and the absorption of the encapsulated compound in the intestine. Hence, the present study aimed to evaluate the effect of pectin addition (0%, 1%, and 2%) on the physicochemical stability of oil-in-water nanoemulsions containing β-carotene during 35 days at 4 ºC, the oil digestibility and the compound bioaccessibility. The results showed that nanoemulsions presented greater stability and lower β-carotene degradation over time in comparison with coarse emulsion, which was further reduced with the addition of pectin. Moreover, nanoemulsions presented a faster digestibility irrespective of the pectin concentration used and a higher β-carotene bioaccessibility as the pectin concentration increased, being the maximum of ≈ 36% in nanoemulsion with 2% of pectin. These results highlight the potential of adding pectin to β-carotene nanoemulsions to enhance their functionality by efficiently preventing the compound degradation and increasing the *in vitro* bioaccessibility.

Keywords: β-carotene, nanoemulsions, pectin, stability, bioaccessibility

1. Introduction

Carotenoids are important compounds that act as natural pigments and have been related to several potential health benefits as the prevention of some cancers, cardiovascular diseases, macular degeneration, or cataracts (Handelman, 2001; Krinsky, 1993; Von Lintig, 2010). β-carotene is among the main carotenoids present in the human diet (Johnson, 2002) and it provides the highest provitamin A activity (Wang et al., 2012). Nevertheless, the biological activity of β-carotene is highly dependent on its intestinal absorption, which is often inefficient as a consequence of entrapment in food matrices, and low stability under gastrointestinal conditions, among others (Boon et al., 2009; Ornelas-Paz et al., 2008; Yuan et al., 2008). Besides, the application of β-carotene in many food matrices is limited because the compound is poorly dispersed in water and its chemical stability is low, being especially sensitive to heat, oxidation, and light due their unsaturated chemical structures (Liu et al., 2016; Ribeiro et al., 2010).

Emulsion-based delivery systems can be produced using various emulsification processes and they have been widely used to protect bioactive compounds such as carotenoids from degradation, ameliorate its dispersion in an aqueous media, and increase its bioaccessibility (Hou et al., 2014; Liu et al., 2012; Mao & Miao, 2015; McClements & Li, 2010; Ribeiro et al., 2010; Verrijssen et al., 2015; Zhang et al., 2016). However, in the last years, oil droplet reduction in the range of 20–500 nm (Usón et al., 2004) showed several advantages over conventional emulsions. Nanoemulsion-based delivery systems have been reported to be more resistant to gravitational separation and aggregation than conventional emulsions (Mason et al., 2006; Wooster et al., 2008). Moreover, their high surface area seems to facilitate the digestive enzyme activity (McClements, 2018; Salvia-Trujillo et al., 2019) and increase the compound bioaccessibility (Acosta, 2009; Gasa-Falcon et al., 2019; Ribeiro et al., 2010; Salvia-Trujillo et al., 2013; Troncoso et al., 2012). Pectin is a naturally-sourced biopolymer composed of a group of complex polysaccharides rich in galacturonic acid units linked by α (1–4) bonds located in the cell wall of plants. This molecule has an amphiphilic character that helps to reduce the interfacial tension between oil and water phases, providing good emulsification properties (Dickinson, 2018; Guerra-Rosas et al., 2016), and also increases the viscosity of the aqueous phase (Zhang et al., 2015). Actually,

consumers are interested in more natural products, so the use of natural ingredients in the formulation of delivery systems is an actual area of interest. In that sense, pectin has been used as an emulsifying and stabilizing agent in food emulsions, but although the use of this biopolymer together with other emulsifiers and their possible interactions has been studied in emulsions, there is a lack of knowledge on its effect on nanoemulsions and, specifically on its behavior under gastrointestinal conditions. Moreover, the addition of biopolymers such as pectin in emulsion-based delivery systems not only can modify the initial emulsion characteristics, but also the behavior of the systems under gastrointestinal conditions and the bioaccessibility of the encapsulated compound (Beysseriat et al., 2006; Espinal-Ruiz et al., 2014; Feng et al., 2017; Gasa-Falcon et al., 2017; Klinkesorn & McClements, 2009; Li et al., 2010; Schmidt et al., 2015). Thus, pectin can have an impact on some health important factors as the satiety, the glycaemia control, and the prevention of some gastrointestinal diseases (Anderson et al., 2009). Hence, the present study aimed to evaluate the effect of pectin addition (0%, 1%, and 2%) on the physicochemical stability of oil-in-water nanoemulsions containing β-carotene during 35 days at 4 ºC, the oil digestibility and the compound bioaccessibility.

2. Materials and methods

2.1. Materials

β-carotene, Tween 20, pepsin (from porcine gastric mucosa), pancreatin (from porcine pancreas), bile extract (bovine), and all the solvents were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Corn oil was purchased from a local supermarket. Food-grade high methoxyl pectin from citrus peel with a degree of methylesterification from 67% to 71% was obtained from Acros Organics (Morris Plains, NJ, USA). Ultrapure water, obtained from Millipore milli-Q filtration system water was used to prepare emulsions and reagents of the experiment.

2.2. Methods

2.2.1. Coarse emulsion and nanoemulsion preparation

To obtain the lipid phase, β-carotene was dissolved in corn oil (5 $mg \cdot g^{-1}$) by sonicating (1 min) and stirring (45 ºC, 5 min). To formulate the aqueous phase, pectin (0%, 1%,

and 2%) was added into ultrapure water, previously heated at 70 ºC, and dispersed using an homogenizer (Ultra-Turrax, Janke & Kunkel, Staufen, Germany) at 9500 rpm for 5 min. The aqueous phase (containing water and pectin) was left for 1 h until it was at room temperature. Then, the lipid phase (4% w/w), Tween 20 (4% w/w) and the aqueous phase were homogenized at 9500 rpm for 2 min to obtain the coarse emulsion. Finally, a microfluidizer (M-110P, Microfluidics, Newton, MA, USA), equipped with a 75 μm ceramic interaction chamber (F20Y) at an operational pressure of 100 mPa, was used to form nanoemulsions by passing the coarse emulsion for 5 cycles.

2.2.2. Particle size

Particle size of emulsions was measured using a Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). Samples were diluted in ultrapure water and stirred in the dispersion unit at a constant speed of 1800 rpm. The particle size was expressed as surface area mean diameter (d_{32}) in nanometers (nm), fixing a refractive index of the corn oil of 1.473 and 1.333 for water. Moreover, due to the presence of large particles in some samples, d_{90} was also reported.

2.2.3. Electrical charge

The electrical charge (ζ-potential) was measured by phase-analysis light scattering (PALS) using a Zetasizer NanoZS (Malvern Instruments Ltd. Worcestershire, UK) to determine the surface charge at the interface of the droplets. Emulsions were diluted (1:10) in ultrapure water and placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles. The results were reported in millivolts (mV).

2.2.4. Stability

The stability of emulsions was studied using an optical scan analyzer Turbiscan MA 2000 (Formulaction, Toulouse, France) which is a non-destructive method that can measure the static stability of samples and detect the cause of instability (flocculation, coalescence, sedimentation, or creaming) by the multiple light scattering technique. A sample of 7 mL was introduced into a glass cylindrical cell and analyzed by a light beam emitted in near infrared wavelength, which scanned vertically from the bottom to the top

of the sample cell. Two synchronous optical sensors received light backscattered by the sample (45º from the incident radiation). In this study, the backscattering was measured during 35 days at 4 ºC to assess the stability of emulsions over time. The backscattering was analyzed at three different zones of the test tube (top, middle, and bottom) in order to study different instability phenomena throughout the tube such as creaming at the top, flocculation, or coalescence in the middle and sedimentation at the bottom.

2.2.5. β-carotene extraction and quantification

β-carotene determination was carried out according to a previously reported method with some modifications (Salvia Trujillo et al., 2013a). To extract the β-carotene, 5 mL of sample was mixed with 5 mL of chloroform, vortexed and centrifuged at 1750 rpm for 20 min at 4 ºC. After centrifugation, the bottom chloroform phase (orange colored) was collected, while the top layer was vortexed with another 5 mL of chloroform and centrifuged at 1750 rpm at 4 ºC during 20 min. The bottom layer, which contained β-carotene, was collected, while the top layer was mixed with an additional 5 mL of chloroform and the same procedure was repeated. Then, the absorbance of the collected bottom layers was measured at 450 nm using a UV-visible spectrometer (CECIL CE 2021; Cecil Instruments Ltd., Cambridge, UK), using chloroform as a blank. The β-carotene content from a sample was measured from a previously prepared calibration curve of absorbance versus β-carotene concentration in chloroform.

2.2.6 In vitro digestion

To simulate the human digestion process, an *in vitro* gastrointestinal tract (GIT) digestion based on an international consensus method (Minekus et al., 2014) with some modifications was used. The coarse emulsion and nanoemulsions were digested immediately after their preparation. The protocol included both gastric and small intestinal phases. Briefly, 20 mL of the sample was mixed with 18.2 mL of simulated gastric fluid (SGF) containing pepsin (2000 $U \cdot mL^{-1}$), 0.4 mL HCl solution (1 M), and 10 µL of a $CaCl_2$ solution (0.3 M). Finally, 1.39 mL of ultrapure water was added to reach a final volume of 40 mL. The mixture was placed into an incubator at 37 ºC for 2 h while shaking at 100 rpm. To simulate the intestinal phase, a pH-stat device was used. Once the gastric phase was completed, an aliquot of 30 mL of gastric sample was placed

in a 37 ºC water bath. Then, 3.5 mL of bile solution (54 $mg \cdot mL^{-1}$) and 1.5 mL of salt solution (NaCl 0.150 mM and $CaCl_2$ 0.01 mM) were added and the pH was adjusted to 7 with NaOH (1 M). Finally, 2.5 mL of pancreatin solution (75 $mg \cdot mL^{-1}$) was incorporated into the mixture. The pH of the sample was maintained to 7 by adding NaOH (0.25 M) constantly for 2 h. The final volume of NaOH (0.25 M) was recorded and used to calculate the amount of free fatty acids (FFAs) released during the intestinal phase. The FFA (%) was determined according to Equation (1):

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

where V_{NaOH} is NaOH volume (L) used during the intestinal digestion, C_{NaOH} is NaOH molarity (0.25 $mol \cdot L^{-1}$), M_{oil} is corn oil molecular weight (800 $g \cdot mol^{-1}$), and m_{oil} is corn oil total weight present in the emulsions (g).

2.2.7. Bioaccessibility determination

Aliquots of digested emulsions were centrifuged at 4000 rpm during 40 min at 4 ºC (Qian e al., 2012a) to obtain the micellar fraction. The concentration of β-carotene in the micellar fraction was determined following the method described in Section 2.2.5. Lastly, β-carotene bioaccessibility was calculated according to Equation (2):

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

where $C_{micelle}$ is the β-carotene concentration ($mg \cdot ml^{-1}$) in the micellar fraction and $C_{initial}$ the initial β-carotene concentration ($mg \cdot ml^{-1}$) in the emulsion.

2.2.8. Optical microscopy

Images of coarse emulsion and nanoemulsions were obtained using an optical microscope (Olympus BX41, Olympus America Inc., Melville, NY, USA) with a 100× objective lens. The images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus).

2.2.9. Statistical analysis

All experiments were assayed in duplicate and three repetitions of each analysis were carried out on each parameter in order to obtain mean values. Analysis of the variance (ANOVA) was performed to compare treatments. Least significant difference (LSD) test was employed to determine differences between means. The confidence interval was set at 0.95 and all results were analyzed using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, MD, USA).

3. Results and discussion

3.1. Physicochemical characterization

3.1.1. Particle size

Initially, the coarse emulsion presented a higher mean particle size *d*32 (7300 ± 307 nm) than nanoemulsion without pectin (313.4 ± 20.2 nm) (Table 1). Moreover, d_{90} value, which indicates the maximum particle diameter of 90% of the sample, showed that the largest droplets in each sample have a size of 25464 ± 1401 nm in the coarse emulsion and 540.7 ± 59.7 nm in the nanoemulsion without pectin. The particle size distribution showed that the microfluidization process not only reduced the mean particle size, but also makes the nanoemulsion less polydisperse compared to the coarse emulsion (Figure 1). A high pressure is applied during the microfluidization process to guide the flow stream through microchannels to the interaction chamber, where cavitation, along with shear and impact, produce a reduction of the emulsion particle size (Maa & Hsu, 1999). The addition of pectin significantly ($p < 0.05$) decreased the particle size of nanoemulsions, irrespective of the concentration used. Our results are in accordance with those reported by Verkempinck et al. (2018a), who also observed a particle size reduction in emulsions that contained a small molecule surfactant (Tween 80) and pectin as stabilizers. On the one hand, the observed decrease in the particle size when pectin was added in the nanoemulsions is suggested to be a consequence of a competitive effect between the polymer and the small molecule surfactant for the interface. Tween 20, which has a lower molecular weight than pectin, may move slightly faster to the interface, leading to small particle sizes (Verkempinck et al., 2018a). In that sense, we

hypothesize that Tween 20 has been efficiently adsorbed in the interface, but also part of the pectin added was present. On the other hand, the viscosity of the nanoemulsions increased from 1.23 ± 0.05 mPa·s in the nanoemulsion without pectin to 6.51 ± 0.10 mPa·s and 19.77 ± 0.26 mPa·s in nanoemulsions with 1% and 2% of pectin, respectively (Table 1). The increase in the viscosity of the continuous phase could enhance the reduction of the particle size by increasing the disruptive shear stresses (Qian & McClements, 2011).

3.1.2. Electrical charge

As it can be observed in Table 1, the electrical charge of the initial coarse emulsion was −32.1 ± 1.9 mV and became less negative after the microfluidization process (−20.9 ± 2.1 mV), as previously reported by other authors (Salvia-Trujillo et al., 2019). Although the systems are expected to have no negative charge because they are formulated using a non-ionic surfactant (Tween 20), the obtained negative charges can be a consequence of a preferential absorption of OH^- species from water to the oil-water interface (McClements, 2005; Mun et al., 2007). The ζ-potential became less negative when pectin was added, irrespective of the concentration used (Table 1). This anionic biopolymer has been reported to show negative charges when it is added in emulsions (Celus et al., 2018; Verkempinck et al., 2018b) due to the carboxyl groups present on its molecule (Burapapadh et al., 2010), but this fact is influenced by the pH. In our study, nanoemulsion without pectin showed a pH about 6.5, but pectin added nanoemulsions presented a low pH (≈ 3). At this pH, the pectin molecules lose most of the negative charges because the pK_a value of their anionic carboxylic groups is around 3.5, so the majority of carboxyl groups are protonated (-COOH) (Zhang et al., 2015). Moreover, no variations were observed between nanoemulsions with 1% and 2% of pectin. In that sense, due to the competence for the interface between Tween 20 and pectin, it seems that in both nanoemulsions the same amount of pectin was adsorbed on the interface. Therefore, in the nanoemulsion with 2% of pectin, a high amount of this biopolymer was remaining in the aqueous phase.

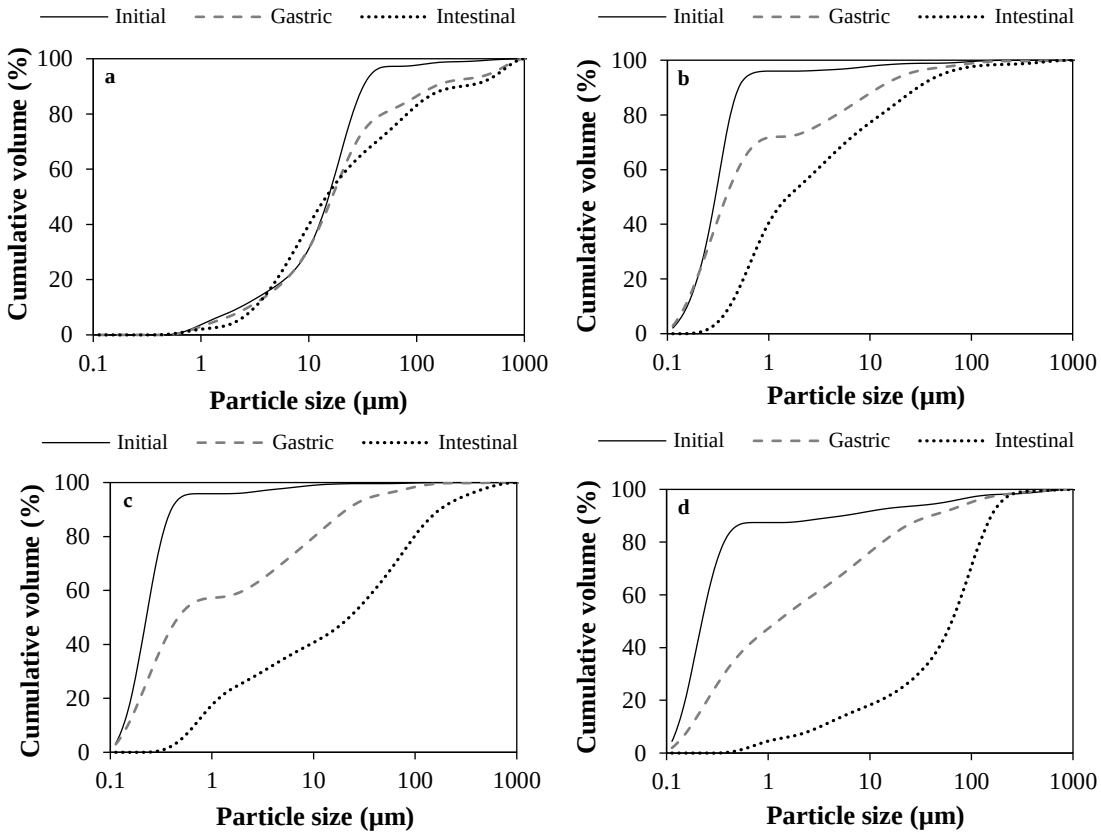

Figure 1. Particle size distribution of coarse emulsion and nanoemulsions with different pectin concentrations (0%, 1%, and 2%) at different phases of the *in vitro* digestion. (a) Coarse emulsion without pectin; (b) Nanoemulsion without pectin; (c) Nanoemulsion with 1% of pectin; (d) Nanoemulsion with 2% of pectin.

Table 1. Particle size, viscosity, ζ-potential, and β-carotene bioaccessibility of coarse emulsion and nanoemulsions with different pectin concentrations (0%, 1%, and 2%).

	CE0 [1]	**NE0** [2]	**NE1** [3]	**NE2** [4]
Particle diameter d_{32} (nm)	7300 ± 307 ^{B}	313.4 ± 20.2 ^{Ab}	242.6 ± 10.9 ^{a}	247.1 ± 24.6 ^{a}
Particle diameter d_{90} (nm)	25464 ± 1401 ^{B}	540.7 ± 59.7 ^{Ab}	448.4 ± 21.4 ^{a}	439.3 ±23.3 ^{a}
Viscosity (mPa·s)	1.31 ± 0.02 ^{A}	1.23 ± 0.05 ^{Aa}	6.51 ± 0.10 ^{b}	19.77 ± 0.26 ^{c}
ζ-potential (mV)	-32.1 ± 1.9 ^{A}	-20.9 ± 2.1 ^{Ba}	-7.6 ± 0.2 ^{b}	-7.4 ± 0.4 ^{b}
β-carotene bioaccessibility (%)	20.9 ± 1.4 ^{A}	25.0 ± 2.4 ^{Ba}	29.5 ± 1.7 ^{b}	36.9 ± 2.2 ^{c}

[1] CE0: coarse emulsion without pectin. [2] NE0: nanoemulsion without pectin. [3] NE1: nanoemulsion with 1% of pectin. [4] NE2: nanoemulsion with 2% of pectin. Values are expressed as mean ± standard deviation. Different capital letters indicate significant ($p < 0.05$) differences between the coarse emulsion and nanoemulsion without pectin. Different lowercase letters indicate significant differences ($p < 0.05$) between nanoemulsions with different pectin concentrations.

3.2. Stability

Initially, the coarse emulsion presented a higher backscattering value at the top zone compared with nanoemulsions as a consequence of the creaming formation in a few minutes after their preparation (Figure 2a). Moreover, in this emulsion, phase separation was observed after 2 days since the oil migrated to the top of the tube, increasing the backscattering at this zone and decreasing it in the middle zone (Figure 2a, b). In contrast, all nanoemulsions exhibited lower variations in backscattering, being the nanoemulsion without pectin the one with least variations (< 4%) (Figure 2). Therefore, this nanoemulsion can be considered stable since only variations greater than 10%, either as a positive or as a negative in the graphical scale of backscattering, are considered an indicator of instability (Celia et al., 2009). Although both coarse emulsion and nanoemulsion are thermodynamically unstable systems, the small particle size of the nanoemulsion could prevent instability phenomena as sedimentation or creaming since the Brownian motion, and consequently the diffusion rate, are greater than the sedimentation or creaming rate induced by the gravity (Solans et al., 2005).

The creaming phenomenon was detected in pectin nanoemulsions after 2 days of storage. An increase of 26.8% and 18.5% of the backscattering at the top zone was observed when pectin was added at 1% and 2%, respectively (Figure 2a). Moreover, these nanoemulsions presented an increase of the backscattering in the middle zone after 35 days of storage, more pronounced when the polymer was added at 2% (Figure 2b). This increase in the middle zone is suggested to be a consequence of the reversible flocculation phenomenon rather than coalescence of the droplets since the light scattering measurements did not show an increase of the particle size (Figure 3).

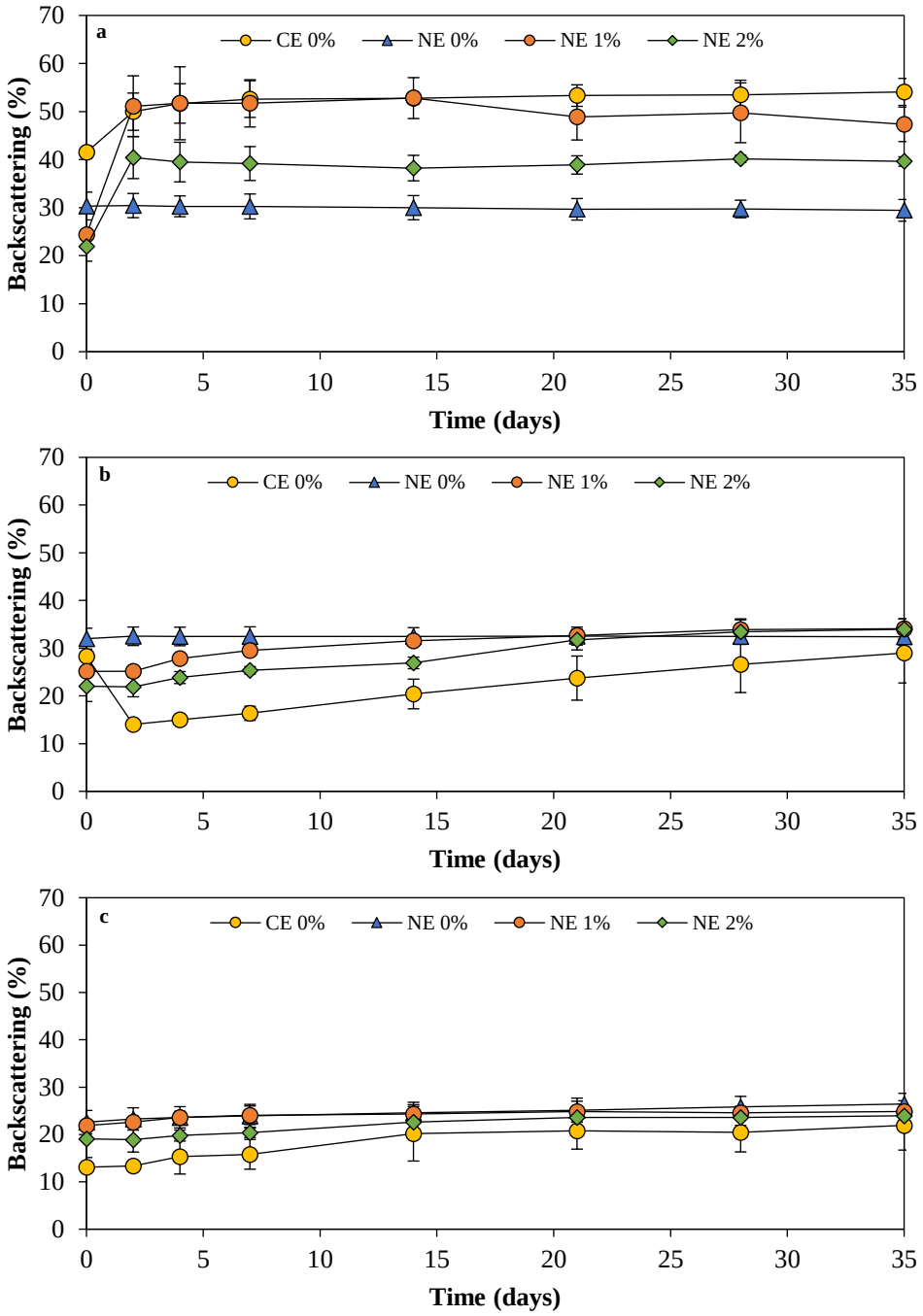

Figure 2. Variation of backscattering values in coarse emulsion and nanoemulsions with different pectin concentrations (0%, 1%, and 2%) at different zones of the test tube during 35 days at 4 ºC. (a) Top zone; (b) Middle zone; (c) Bottom zone. CE 0, coarse emulsion without pectin; NE 0 nanoemulsion without pectin; NE 1, nanoemulsion with 1% of pectin; NE 2, nanoemulsion with 2% of pectin.

Other authors also observed flocculation of droplets, and consequently, the appearance of creaming when citrus pectin was added at concentrations of ≥ 0.02% in emulsion-

based systems (Beysseriat et al., 2006; Celus et al., 2018). When pectin is used at low concentrations, the repulsive interactions between droplets are sufficiently large to overcome the attractive interactions, but there is a critical concentration of the polymer over which the attraction is sufficiently strong to promote flocculation of droplets, and thereby, creaming occurs (Espinal-Ruiz et al., 2014; McClements, 2000). As previously mentioned, we suggest that the part of pectin that has not been adsorbed at the interface is remaining at the aqueous phase, creating an osmotic imbalance, which promoted the depletion flocculation phenomena (Verkempinck et al., 2018a). Moreover, the higher amount of flocculation observed in the nanoemulsion with 2% of pectin compared with those with 1%, may be due to the higher amount of biopolymer remaining at the continuous phase.

3.3. β-carotene degradation

During the storage, the β-carotene content was reduced by 50% in the coarse emulsion, whereas in the nanoemulsion without pectin, the reduction was about 14% (Figure 4). Our results are similar to those reported by other authors who observed degradation of about 14-25% in nanoemulsions formulated with small molecule surfactants, after 4 weeks of storage at 4 ºC (Tan & Nakajima, 2005; Yuan et al., 2008). The reduction of the particle size could increase carotenoid degradation because of a higher surface area exposed to oxidation (Qian et al., 2012b; Tan & Nakajima, 2005). However, in our study, a rapid phase separation was observed in the coarse emulsion. This phenomenon resulted in a loss of the emulsion structure, which could promote the β-carotene oxidation because it was more exposed to the environment. The addition of pectin reduced the compound degradation during the 35 days of storage at 4 ºC irrespective of the concentration used (Figure 4). At day 35, the nanoemulsion without pectin showed a β-carotene content of 85.3% ± 3.2%, while nanoemulsions with 1% and 2% of pectin presented values of 97.0% ± 4.4% and 94.9% ± 2.6%, respectively. The presence of pectin in the aqueous phase or at the interface may physically hinder the ability of pro-oxidants to interact with β-carotene within the oil droplets by providing a steric barrier (Xu et al., 2012). Moreover, pectin has been reported to be effective inhibiting lipid oxidation due to their free radical scavenging activity and their iron-binding capacity (Chen et al., 2010). Although pectin nanoemulsions have been observed to present

flocculation and creaming (Section 3.2), we suggest that the flocculation phenomenon could be acting as a protective effect by entrapping the compound in the flocs produced by pectin.

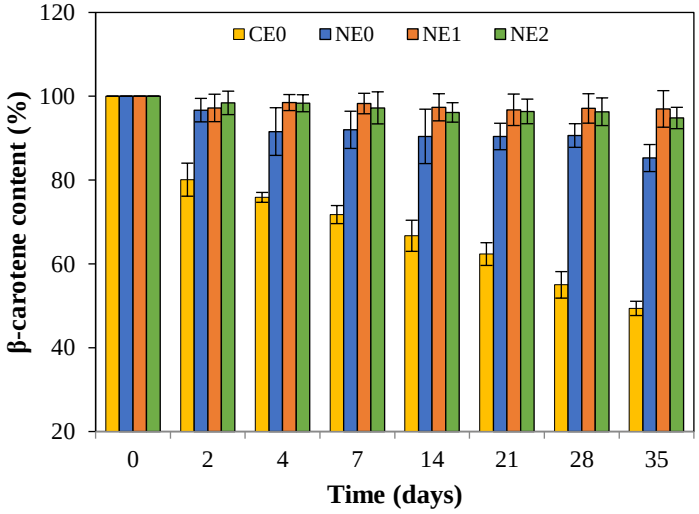

Figure 4. β-carotene content on coarse emulsion and nanoemulsions with different pectin concentrations (0%, 1%, and 2%) during 35 days at 4 ºC. CE0, coarse emulsion without pectin; NE0 nanoemulsion without pectin; NE1, nanoemulsion with 1% of pectin; NE2, nanoemulsion with 2% of pectin.

3.4. Gastrointestinal behaviour of the emulsions

3.4.1. Particle size

In the gastric phase, no significant changes in the mean particle size were observed between coarse emulsion and nanoemulsion without pectin although some changes in particle size distribution were detected (Figures 1 and 5). In both cases, the particle size distribution became more polydisperse, meaning that some large particles were formed (Figure 1). This fact can also be observed in the microscope images of these emulsions in the gastric phase (Figure 6). Few changes on the mean particle size during the gastric digestion were also reported by other authors in oil-in-water emulsions formulated with small surfactants as Tween 20 or Tween 80 (Gasa-Falcon et al., 2019; Verkempinck et al., 2018c). This type of surfactants has been reported to provide greater stability under gastric conditions when compared with others like lecithin or proteins, which have shown to be less effective against flocculation under gastric conditions (Chang & McClements, 2016; van Aken et al., 2011). In nanoemulsions with pectin, the mean

particle size increased during the gastric phase, more noticeably when the polymer was added at 2% (Figure 5). Moreover, the particle size distribution became more polydisperse, similar to the nanoemulsion without pectin, because of an increase of large particles (Figure 1). The flocculation and coalescence phenomena, which can be observed in the microscope images of pectin nanoemulsions in the gastric phase (Figure 6), could be the reason for the increased particle size at this stage. In that sense, the pectin remaining in the continuous phase of these nanoemulsions may be promoting the formation of aggregates containing both oil droplets and pectin molecules (Verkempinck et al., 2018b). Moreover, in the microscope images of the nanoemulsion with 2% of pectin (Figure 6), it can be observed that pectin could have induced the formation of gel-like pectin, namely Ca^{+2}-crosslinkings with the available calcium ions in the stomach juice, which are clustering oil droplets (Verrijssen al., 2015).

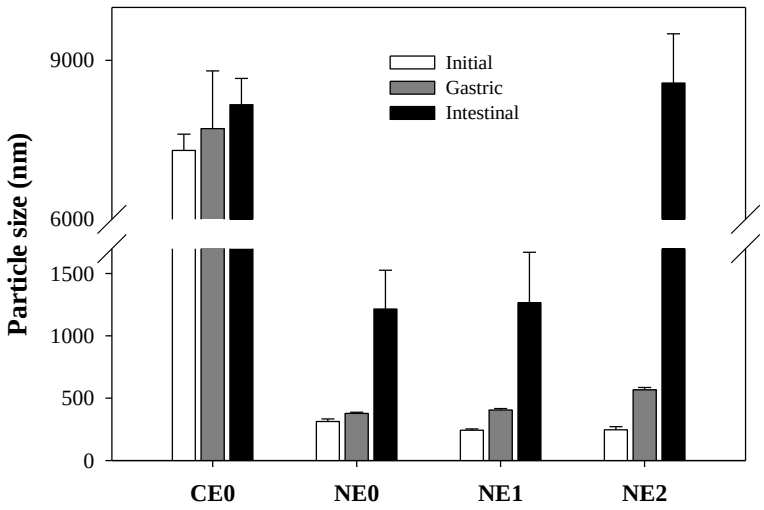

Figure 5. Mean particle size values (d_{32}) of coarse emulsion and nanoemulsions with different pectin concentrations (0%, 1%, and 2%) at different phases of the *in vitro* digestion. CE0, coarse emulsion without pectin; NE0, nanoemulsion without pectin; NE1, nanoemulsion with 1% of pectin; NE2, nanoemulsion with 2% of pectin.

During the intestinal phase, the mean particle size increased substantially in the nanoemulsion without pectin, while in the coarse emulsion, no significant variation was detected with respect to the gastric phase (Figure 5). The particle size distribution graphs also showed such an increase in the particle size in the nanoemulsion without pectin (Figure 1). During the lipid digestion, the oil droplets are digested by the intestinal

enzymes and the mixed micelles are formed. In that sense, the increase of particle size at this stage could be related to the formation of the mixed micelles, the presence of some other molecules produced in the intestinal digestion or undigested oil droplets. Otherwise, during the intestinal phase, droplet coalescence can also occur, since the release of surface-active products generated during the lipid digestion can displace the interface stabilizers so they are ineffectively preventing the coalescence of oil droplets (Mao & Miao, 2015; Mun et al., 2007). In nanoemulsions with pectin, larger particle sizes were observed compared with those without the biopolymer, especially in the nanoemulsion with 2% of the pectin (Figures 1 and 5). This fact is confirmed by the microscope images of nanoemulsions with pectin, in which larger particles can be observed (Figure 6).

3.4.2. Electrical charge

During the gastric phase, the ζ-potential becomes less negative in both coarse emulsion and nanoemulsion without pectin (Figure 7). The electrostatic screening effects resulting from the interactions between oil-water surfaces and ions present at this stage (Ca^{2+}, K^{+}, and H^{+}) could produce a decrease in the negative charge (Verkempinck et al., 2018b; Zhang et al., 2015). Otherwise, the electrical charge measurements of nanoemulsions with pectin showed that there were little changes under gastric conditions (Figure 5). The initial pH of these nanoemulsions was ≈ 3, so when they were subjected to the gastric phase, there was no important variation on the pH (2.5). Thus, as well as in the initial systems, the majority of carboxyl groups of pectin were protonated at these conditions, so they cannot interact with the ions present at this stage.

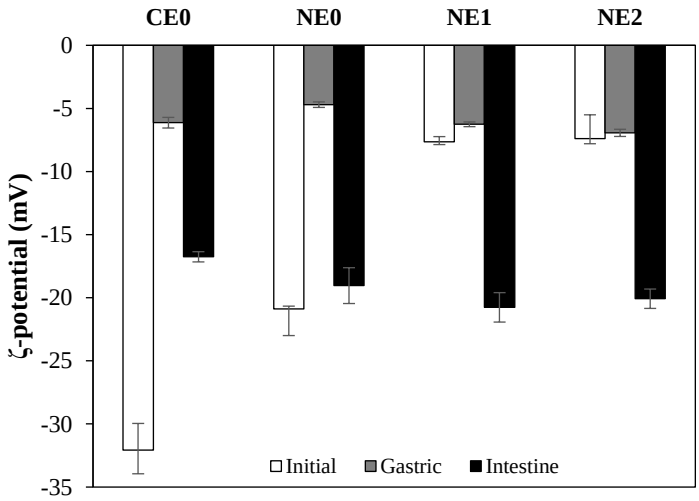

Figure 7. ζ-potential values of coarse emulsion and nanoemulsions with different pectin concentrations (0%, 1%, and 2%) at different phases of the *in vitro* digestion. CE0, coarse emulsion without pectin; NE0, nanoemulsion without pectin; NE1, nanoemulsion with 1% of pectin; NE2, nanoemulsion with 2% of pectin.

The ζ-potential of emulsions had a relatively high negative charge under simulated intestinal conditions, being more negative in nanoemulsions compared with coarse emulsion (Figure 7). The general increase in the electrical charge (in absolute terms) at this stage could be caused due to the presence of some negatively charged molecules resulting from the intestinal digestion process as bile salts, lipase, or phospholipids that compete with surface-active particles initially present at the droplet surface (McClements & Xiao, 2012; Mun et al., 2007; Singh et al., 2009). No significant differences were observed between the electrical charge of nanoemulsions with and without pectin at this stage, as reported previously (Verkempinck et al., 2018b). At neutral pH of the intestine, both the pectin molecule and the lipid droplets have high negative charges, so there could exist a high electrostatic repulsion between them, which may inhibit the pectin adsorption on the interface (Simo et al., 2012). Moreover, it is also possible that the high impact of the anionic species resulting from intestinal digestion on the electrical charge was covering up the effect of pectin (Chang & McClements, 2016), making it difficult to be observed in the electrical charge measurements.

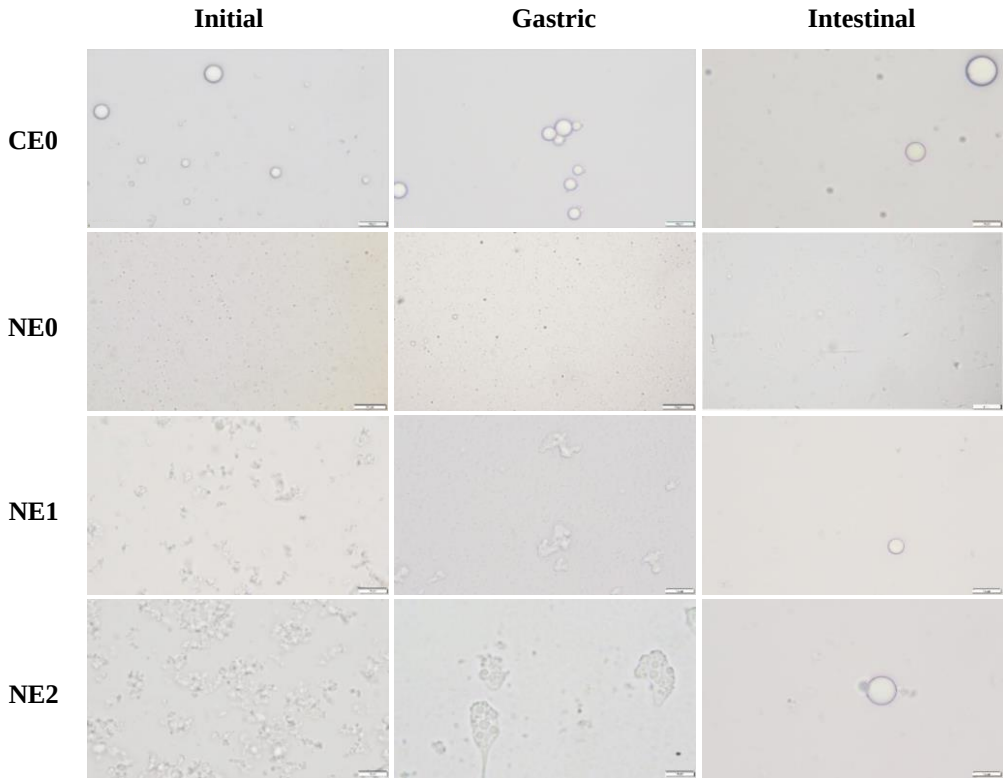

Figure 6. Images of coarse emulsion and nanoemulsions with different pectin concentrations (0%, 1%, and 2%) at different phases of the *in vitro* digestion. CE0, coarse emulsion without pectin; NE0, nanoemulsion without pectin; NE1, nanoemulsion with 1% of pectin; NE2, nanoemulsion with 2% of pectin. Scale bars were 10 μm long.

3.4.3. Oil digestibility

As it can be seen in Figure 8, all emulsions presented the same oil digestibility (about 80%) after the 2 h of the intestinal digestion and the lipid digestion profile presented a similar trend. The amount of FFAs increased rapidly at the beginning of the lipid digestion but slightly at longer digestion times. This suggests that the lipids initially present in the oil droplets were rapidly converted into monoacylglycerols and FFAs. Similar profiles have been previously observed when corn oil has been used in the formulation of emulsions and nanoemulsions (Qian et al., 2012a; Rao et al., 2013; Zhang et al., 2015). But looking at the results more specifically, in our study, the initial digestion rate in the coarse emulsion was slower in comparison to all nanoemulsions (Figure 8). In fact, the FFAs liberated in the intestinal phase after 5 min were ≈ 60% in nanoemulsions and ≈ 40% in the course emulsion. These differences could be attributed to the particle size of emulsions at the end of the gastric phase. In nanoemulsions, the small particle sizes increase the lipid surface area exposed to lipase activity, enhancing

the FFAs release during the intestinal digestion (Gasa-Falcon et al., 2017; Li et al., 2011; Salvia-Trujillo et al., 2013). Moreover, the access to lipase could be easier in nanoemulsions because they present a thinner layer at the interface due to the higher surface area and same surfactant concentration. This fact could provide less steric hindrance, improving lipase access to oil (Yi et al., 2014). The results of this study show that the presence of pectin did not have a significant impact on the rate of lipid digestion of these systems, similar to other published results (Simo et al., 2012). As it was previously mentioned, pectin may not be adsorbed at the intestinal pH (pH 7), whereby not affecting the lipolysis process. Other anionic biopolymers as fucoidan had also shown no effect on the lipid digestibility when they are used in combination with small molecule surfactants (Chang & McClements, 2016).

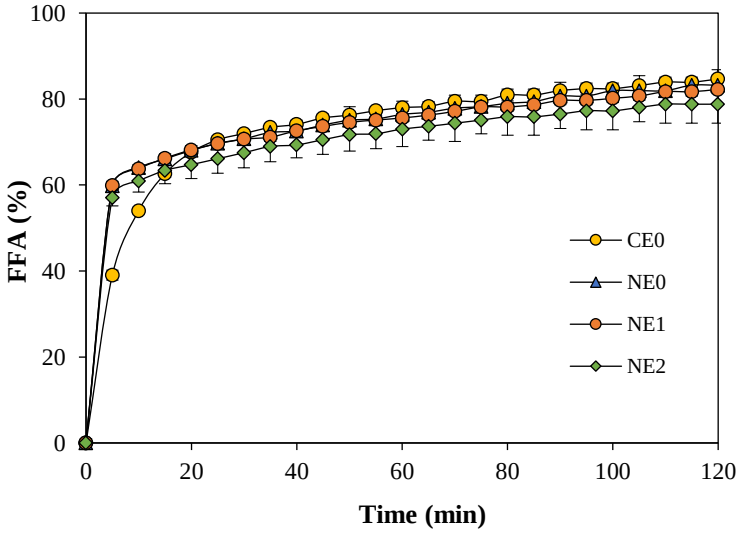

Figure 8. Free fatty acid (FFA) release during the intestinal phase of coarse emulsion and nanoemulsions with different pectin concentrations (0%, 1%, and 2%). CE0, coarse emulsion without pectin; NE0, nanoemulsion without pectin; NE1, nanoemulsion with 1% of pectin; NE2, nanoemulsion with 2% of pectin.

3.4.4. β-carotene bioaccessibility

The bioaccessibility of a lipophilic compound can be defined as the fraction of the substance which is solubilized within the gastrointestinal intestinal fluids in a suitable form for absorption (McClements, 2018). β-carotene bioaccessibility increased from 20.9% ± 1.4% in coarse emulsion to 25.0% ± 2.4% in the nanoemulsion without pectin (Table 1). Generally, smaller particle size, i.e., higher surface area, improves the oil

digestibility and transfer of β-carotene to micelles (Yi et al., 2014). Nevertheless, in our study, no differences were detected between the digestibility of the nanoemulsion and the coarse emulsion. In that sense, although both emulsions presented the same FFAs at the end of the lipid digestion, the faster initial FFAs release in nanoemulsions could have favored the incorporation of β-carotene to the mixed micelles.

The pectin addition affected the bioaccessibility of β-carotene. The higher the pectin concentration, the greater the bioaccessibility, reaching maximum values of 36.9% ± 2.2% in nanoemulsions with 2% of pectin. A similar effect has been observed using mandarin fiber, rich in pectin, on the bioaccessibility of β-carotene nanoemulsions at concentrations up to 1% (Gasa-Falcon et al., 2017). In our study, we hypothesize that the flocculation phenomenon in the gastric phase and the formation of pectin gels induced by the presence of pectin in the aqueous phase could act as a steric barrier, reducing the compound degradation during its pass through the gastrointestinal tract and enhancing the β-carotene bioaccessibility. Indeed, the highest bioaccessibility observed in the nanoemulsion with 2% of pectin must be due to the greater flocculation and formation of gels in the gastric phase, as a consequence of a higher amount of pectin present in the aqueous phase. Therefore, although all nanoemulsions exhibited the same amount of free fatty acids at the end of the intestinal digestion, it seems that other factors influenced the bioaccessibility of β-carotene. Some authors have noted that lipid availability for mixed micelle formation is not always correlated with the bioaccessibility of β-carotene (Verrijssen et al., 2016). In this regard, factors such as the composition of active molecules in the micelle appear to have also a significant effect.

4. Conclusions

The results show that by adding pectin at 2% in nanoemulsions, the lowest β-carotene degradation and the highest bioaccessibility were observed. These results could be related to the ability of this biopolymer to protect the compound by providing a steric barrier and due to its free radical scavenging activity. The reduction in particle significantly increased the stability of the systems, which was probably the cause of the decreased β-carotene degradation observed in the nanoemulsions during the storage. Moreover, lipid digestion was faster in the nanoemulsions, which could produce a faster incorporation of β-carotene in the mixed micelles and, thus, higher bioaccessibility

values compared to the coarse emulsion. However, further *in vivo* investigations are needed to better understand the digestive behavior of pectin nanoemulsions containing β-carotene and to assess the encapsulated compound bioavailability.

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Chapter II

Enhancing the gastrointestinal stability of curcumin by using sodium alginate-based nanoemulsions containing natural emulsifiers

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Abstract

Curcumin presents interesting biological activities but low chemical stability, so it has been incorporated into different emulsion-based systems in order to increase its bioaccessibility. Many strategies are being investigated to increase the stability of these systems. Among them, the use of polysaccharides has been seen to highly improve the emulsion stability but also to modulate their digestibility and the release of the encapsulated compounds. However, the effect of these polysaccharides on nanoemulsions depends on the presence of other components. Then, this work aimed to study the effect of alginate addition at different concentrations (0 - 1.5%) on the gastrointestinal fate and stability of curcumin-loaded nanoemulsions formulated using soybean lecithin or whey protein as emulsifiers. Results showed that, in the absence of polysaccharides, whey protein was more effective than lecithin in preventing curcumin degradation during digestion and its use also provided greater lipid digestibility and higher curcumin bioaccessibility. The addition of alginate, especially at ≥ 1%, greatly prevented curcumin degradation during digestion up to 23% and improved the stability of nanoemulsions over time. However, it reduced lipid digestibility and curcumin bioaccessibility. Our results provide relevant information on the use of alginate on different emulsifier-based nanoemulsions to act as carriers of curcumin.

Keywords: curcumin, nanoemulsions, bioaccessibility, stability, sodium alginate

1. Introduction

Curcumin, chemically known as diferuloylmethane, is a low molecular weight (368.37 $g \cdot mol^{-1}$) polyphenol found in the rhizome of the *Curcuma longa* plant (turmeric). In its structure, it presents two aryl rings that contain *ortho*-methoxy phenolic groups symmetrically linked to a β-diketone moiety (Araiza-Calahorra et al., 2018; Liu et al., 2020). This compound has been shown to target multiple signalling molecules and presents antioxidant, anti-inflammatory, antibacterial and anticarcinogenic properties (Anand et al., 2007; Hewlings & Kalman, 2017; Peng et al., 2018). However, its incorporation in food matrices is difficult due to its poor water solubility and low chemical stability (Zheng et al., 2017). Furthermore, curcumin is highly unstable at the physiological pH (Wang et al., 1997), so it can be easily degraded in the intestinal tract. Oil-in-water emulsion-based systems appear to be feasible curcumin carriers due to the lipophilic nature of the compound. Indeed, many authors have studied the incorporation of curcumin into different encapsulation systems (Ahmed et al., 2012; Artiga-Artigas et al., 2018; Lu et al., 2019; Ma et al., 2017; Zheng et al., 2017; Zhu et al., 2021). Among them, nanoemulsions are interesting options since they present higher physical stability against gravitational processes compared to conventional emulsions and enhanced functionality due to the increased surface area of droplets (Silva et al., 2012). These systems have been seen to prevent curcumin degradation over time and increase its stability during gastrointestinal digestion, as well as enhance its bioaccessibility (Zheng et al., 2017, 2019). However, recent studies have highlighted that the nature of the emulsifier, the type and the amount of carrier oil and emulsifier–curcumin interactions are key factors that influence curcumin delivery in nanoemulsions (Jiang et al., 2020; Pinheiro et al., 2013).

Emulsifiers are needed to obtain stable nanoemulsions and can have a significant impact on their properties and digestibility, as well as on the bioaccessibility of bioactive compounds (Araiza-Calahorra et al., 2018; Gasa-Falcon et al., 2019; Pinheiro et al., 2013). Among the possible emulsifiers to be used, naturals seem to be more suitable than synthetics because of general public concern about environmental issues and the demand for label-friendly food products. In this regard, soybean lecithin (SBL) and whey protein isolate (WPI) are natural emulsifiers with different chemical compositions previously

used in the food industry. Lecithin is a lipid-based emulsifier that can be obtained from natural sources like soybean, sunflower or egg yolk and is able to stabilize emulsions forming a multilamellar shell around the droplets, acting as a mechanical barrier (Klang & Valenta, 2011). This emulsifier has been shown to provide good electrostatic stabilization due to its strong negative electrical charge in a wide range of pH (McClements et al., 2017; Ozturk et al., 2014; Zheng et al., 2021). Moreover, natural lecithin is biodegradable and among the safest emulsifying agents, being the incidence of allergic reactions to lecithin is very rare (Klang & Valenta, 2011; Lin et al., 2009). On the other hand, WPI is a protein-based emulsifier that consists of β-lactoglobulin and α-lactalbumin together with other minor proteins such as bovine serum albumin, lactoferrin and immunoglobulins (Li et al., 2013). This emulsifier can form a dense layer at the surface of the droplets, providing stability to emulsion-based systems for flocculation and coalescence (Dickinson, 2001; Li et al., 2013). Moreover, this emulsifier has been used to formulate nanoemulsions containing bioactive compounds such as β-carotene, shown to be highly effective in the prevention of compound degradation and providing high stability over time (Bai et al., 2016; Jo & Kwon, 2014; Mao et al., 2010).

Polysaccharides of different natures have been used in the formulation of emulsion-based systems. For instance, they have been used as solid particle stabilizers in Pickering emulsions, showing to be promising ingredients to replace synthetic ingredients (Lu et al., 2019; Shah et al., 2016; Zhu et al., 2021). In addition, the incorporation of polysaccharides in other systems, such as nanoemulsions, has been seen to increase their physical stability by reducing the movement of droplets (Artiga-Artigas et al., 2017; Richa & Choudhury, 2020; Sabet et al., 2020). According to these studies, the addition of polysaccharides seems to be an interesting strategy for producing systems with enhanced stability. However, from a nutritional point of view, the digestibility of emulsion-based delivery systems can be affected by the type and concentration of polysaccharides added. According to previous literature, the effect of the polysaccharides depends on the emulsifier nature used to formulate the emulsions. Indeed, 0.2% fucoidan has been shown to increase lipid digestibility in caseinate emulsions but had no effect on Tween 20 or lecithin emulsions (Chang & McClements, 2016). In the same way, other authors have observed an increased lipid digestibility by adding pectin in lecithin emulsions but a reduced digestibility when it was added to Tween 20 emulsions

(Verkempinck et al., 2018a). Based on these findings, the interactions that take place on the interface between the emulsifier and the polysaccharide can influence the digestibility of nanoemulsions. Previous authors have reported that the presence of polysaccharides, such as mandarin fibre or chitosan, in nanoemulsions can have an impact on the bioaccessibility of β-carotene or vitamin D, although controversial results were obtained (Gasa-Falcon et al., 2017; Tan et al., 2020).

Among the polysaccharides, the interest in the use of sodium alginate to encapsulate bioactive compounds has increased in the past two decades due to its biocompatibility, biodegradability and environmental sensitivity properties (Wang et al., 2019). Moreover, this polysaccharide has been seen to be extremely effective in enhancing the stability of nanoemulsions (Artiga-Artigas et al., 2017; Pallandre et al., 2007). However, there is a lack of knowledge on how the incorporation of sodium alginate in oil-in-water nanoemulsions may affect their digestibility and the bioaccessibility of the lipophilic encapsulated compound. Thus, the aim of the study was to investigate the effect of sodium alginate addition on the physical properties, stability and the digestibility of curcumin-loaded nanoemulsions formulated using different natural emulsifiers (soybean lecithin or whey protein isolate) and focus on their gastrointestinal stability and bioaccessibility of curcumin.

2. Materials and methods

2.1. Materials

Curcumin, pepsin (from porcine gastric mucosa), pancreatin (from porcine pancreas), and bile extract (porcine) were obtained from Sigma-Aldrich, INC (St. Louis, MO, USA). Corn oil (Koipesol Asua, Deoleo, Spain) was purchased from a local supermarket. Soybean lecithin (SBL) was acquired from Alfa Aesar (Thermo Fisher Scientific, Waltham, WA, USA). Whey protein isolate (WPI) was kindly provided by El Pastoret de la Segarra, S.L. (Spain). Sodium alginate (MANUCOL® DH) was provided by FMC Biopolymers Ltd. (Scotland, UK). Ultrapure water obtained from a milli-Q filtration system was used to prepare all solutions.

2.2 Methods

2.2.1. Nanoemulsion preparation

To obtain the lipid phase, curcumin was solubilized in corn oil (1 $mg \cdot g^{-1}$) by stirring for 20 min at 60 °C and sonicating for 20 min. To formulate the aqueous phase, sodium alginate (0, 0.5, 1 or 1.5 % w/w) was added into ultrapure water and stirred overnight at room temperature (≈ 20 °C). Then, SBL or WPI was added (5% w/w), and the mixture was stirred. To formulate the coarse emulsion, the lipid phase (5% w/w) and the aqueous phase (95% w/w) were mixed using an Ultra-Turrax at 11000 rpm for 2 min (Janke & Kundel, Staufen, Germany). Finally, to obtain the nanoemulsion, the coarse emulsion was treated with a UP400S sonifier (Hielscher Ultrasound Technology, Teltow, Germany) of 400 W nominal power and a frequency of 24 kHz equipped with a 22 mm sonotrode for 3 min at 100% of amplitude.

2.2.2. Physicochemical characterization

The particle size and particle size distribution of nanoemulsions were measured using a Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). Samples were diluted in ultrapure water and stirred in the dispersion unit with a constant speed of 1800 rpm. The mean particle size was expressed as surface area mean diameter (d_{32}) in micrometers (μm), fixing a refractive index of the corn oil of 1.473 and 1.333 for water.

The ζ-potential was measured by phase analysis light scattering (PALS) using a Zetasizer laser diffractometer (NanoZS Malvern Instruments Ltd. Worcestershire, UK). Prior to the analysis, nanoemulsions were diluted (1:100) in ultrapure water and placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles. The results were reported in millivolts (mV).

To study the encapsulation efficiency (EE), 10 mL of nanoemulsion was placed inside a dialysis tubing cellulose membrane of 43 mm × 27 mm (Sigma-Aldrich, Darmstadt, Germany). Then, the membrane was inserted into a centrifuge tube containing 20 mL of ethanol and centrifuged at 2000 rpm for 10 min. Finally, the non-encapsulated curcumin content was quantified using a UV-visible spectrophotometer (CECIL CE 2021; Cecil Instruments Ltd., Cambridge, UK) at 425 nm. The encapsulation efficiency (%) was

calculated using Equation (1) (Xing et al., 2004):

$$EE\ (\%) = \frac{\text{Total amount of curcumin - Free curcumin}}{\text{Total amount of curcumin}} \times 100 \quad (1)$$

where the total amount of curcumin is the initial concentration added in nanoemulsions, and the free curcumin is the concentration of the compound that was not loaded in nanoemulsions.

The apparent viscosity of nanoemulsions was determined using an SV-10 vibro-viscometer (A&D Company, Tokyo, Japan), which produces a vibration of 30 Hz and a constant amplitude of 0.4 mm at controlled room temperature. The results were expressed in mPa·s.

2.2.3. Stability of nanoemulsions

Stability of emulsions was studied using an optical scan analyzer Turbiscan MA 2000 (Formulaction, Toulouse, France), which can measure the static stability of samples without destruction and detect the cause of instability (flocculation, coalescence, sedimentation or creaming) by the multiple light scattering technique. A sample of 7 mL was introduced into a glass cylindrical cell and analyzed by a light beam emitted in near-infrared wavelength, which scanned vertically from the bottom to the top of the sample cell. Two synchronous optical sensors receive light backscattered by the sample (45° from the incident radiation). In this study, the variation of backscattering (BS) during 21 days at 4 °C was studied to assess the stability of the nanoemulsions over time.

2.2.4. Curcuminoid extraction from nanoemulsions and quantification

To extract curcumin from nanoemulsions, a previously reported method with some modifications was used (Lu et al., 2019). First, aliquots of 250 µL of the sample were mixed with 1000 µL of ethyl acetate and vortexed for 1 min and centrifuged at 9000 rpm for 10 min at 4 °C. Afterwards, the upper organic layer was collected and evaporated under N_2 and stored at −40 °C. The quantification of curcumin was carried out using an HPLC system equipped with a 600 Controller and a diode array detector (Waters, Milford, MA) following a previously described method with some modifications (Setthacheewakul et al., 2010). Before injecting the samples into the HPLC, they were

reconstituted with 1 mL of methanol and filtered. Curcumin was identified using a reverse-phase C18 Spherisorb® ODS2 (5 µm) stainless steel column (4.6 mm × 250 mm) at room temperature. The separation was performed with a linear gradient elution 2% aqueous acetic acid (solvent A) and acetonitrile (solvent B). Detection was by UV spectroscopy at a wavelength of 425 nm, and the flow rate was 1 mL/min. UV-Vis spectral data and their retention times were used to determine the curcumin present in the vials being quantified by comparing them with an external curcumin standard.

2.2.5. In vitro gastrointestinal digestion

To simulate the human digestion process, an *in vitro* gastrointestinal digestion based on an international consensus method (Brodkorb et al., 2019) was used.

The protocol included both gastric and small intestinal phases. The mouth phase was not performed since nanoemulsions were liquid. To perform the gastric phase, 20 mL of nanoemulsion was mixed 1:1 with simulated gastric fluids containing pepsin (2000 U/mL) and 10 µL of a $CaCl_2$ solution (0.3 M). Then, the pH was adjusted to 3 using HCl (1 M), and the mixture was placed into an incubator at 37 °C for 2 h while shaking at 100 rpm. To simulate the intestinal phase, a pH-stat device was used. Once the gastric phase was completed, the gastric sample was placed in a 37 °C water bath and mixed 1:1 with simulated intestinal fluids containing 10 mM bile solution. Then, the pH was adjusted to 7 with NaOH (1 M), and pancreatin and lipase enzymes were added to the final mixture to achieve 100 U/mL of trypsin and 2000 U/mL pancreatic lipase. The pH of the sample was maintained at 7 by adding NaOH (0.25 M) constantly for 2 h. According to previous authors (Li & Mcclements, 2010), the FFA (%) was determined using Equation (2):

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

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

2.2.6. Curcumin degradation

To study the degradation of curcumin along the simulated gastrointestinal tract, 1 mL of

digesta was collected at specific digestion times (0, 30, 60, 90, 120, 150, 180, 210 and 240 min). Then curcumin was extracted and quantified as described in Section 2.2.4 to obtain curcumin content values. Finally, the curcumin content in the aliquots collected at different digestion times was obtained according to Equation (3):

$$\text{Curcumin content (\%)} = \frac{C_{digesta}}{C_{initial}} \times 100 \quad (3)$$

where $C_{digesta}$ is the curcumin concentration (mg/mL) in the digesta and $C_{initial}$ is the initial curcumin concentration (mg/mL) in the nanoemulsion.

2.2.7. Bioaccessibility

To study the curcumin bioaccessibility, after the digestion process, the digested nanoemulsions were centrifuged at 9000 rpm for 30 min at 4 °C to obtain the micellar fraction. Then, an extraction and quantification of the curcumin content was performed both in the initial and micellar fraction following the method described in Section 2.2.4. Finally, curcumin bioaccessibility was calculated according to Equation (4):

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

where $C_{micelle}$ is the curcumin concentration (mg/mL) in the micellar fraction and $C_{initial}$ is the initial curcumin concentration (mg/mL) in the nanoemulsion.

2.2.8. Optical microscopy

Images of curcumin-loaded nanoemulsions were obtained using an optical microscope (Olympus BX41, Olympus America Inc., Melville, NY, USA) with a 100x objective lens. The images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus).

2.2.9. Statistical analysis

All experiments were assayed in duplicate and three repetitions of each analysis were carried out on each parameter in order to obtain mean values. Analysis of the variance (ANOVA) was performed to compare treatments. Least significant difference (LSD) test

was employed to determine differences between means. The confidence interval was set at 0.95 and all results were analyzed using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, MD, USA).

3. Results and discussion

3.1. Initial systems

Particle size and distribution. After the microfluidization process, SBL nanoemulsion without sodium alginate exhibited a smaller particle size (343.8 ± 9.7 nm) with a less polydisperse distribution than WPI nanoemulsion without the polysaccharide (462.4 ± 14.7 nm) (Table 1 and Figure 1). In addition, SBL reduced the interfacial tension (up to 14.66 mN/m) to a higher extent than WPI (up to 20.52 mN/m). The same trend was previously observed by other authors (Flores-Andrade et al., 2021) and indicates that the higher surface activity of SBL may have facilitated the formation of small droplets rather than WPI.

The addition of sodium alginate at any concentration significantly increased the particle size of WPI nanoemulsions due to the aggregation of droplets (Figure 2); the most noteworthy changes were observed when using 1% and 1.5% sodium alginate concentrations (Table 1). The particle size distribution changed from monomodal to bimodal when the polysaccharide was added to the WPI nanoemulsion, irrespective of the concentration used, leading to an increased polydispersity index. Conversely, sodium alginate addition had little effect on SBL nanoemulsions (Figure 1 and Table 1). As observed in the microscope images, all the obtained nanoemulsions exhibited flocculation when sodium alginate was added, regardless of the type of emulsifier and sodium alginate concentration (Figure 2). However, in SBL nanoemulsions, the mean particle size was not affected by the flocculation of droplets because aggregates were disrupted during the particle size measurements, as previously observed by other authors (Chang & McClements, 2016). The aggregation of droplets observed in SBL and WPI nanoemulsions containing sodium alginate may be a consequence of non-adsorbed polymer molecules that were present in the aqueous phase surrounding the lipid droplets (Dickinson et al., 1997; McClements, 2015).

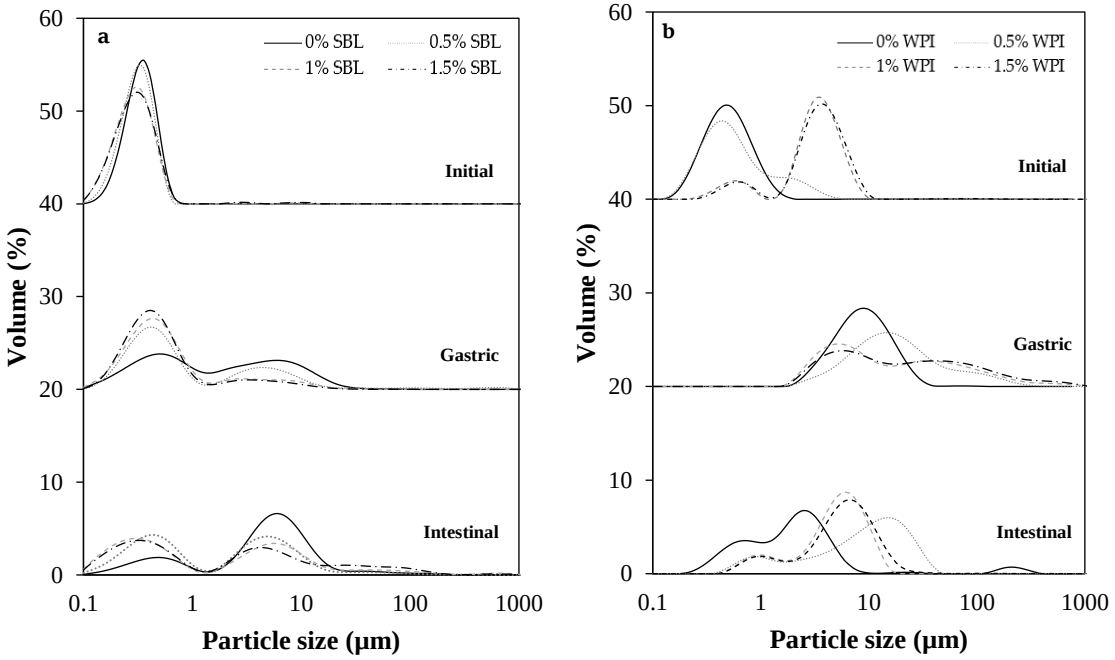

Figure 1. Particle size distribution of curcumin-loaded nanoemulsions stabilized with (a) soybean lecithin (SBL) or (b) whey protein isolate nanoemulsions (WPI) and different sodium alginate concentrations (0, 0.5, 1, and 1.5%) at different phases of *in vitro* digestion.

ζ-potential. Among non-added polysaccharide nanoemulsions formulated with SBL showed a more negative ζ-potential than WPI (Table 1). In SBL nanoemulsions, the negative charges conferred by the phosphate groups of phospholipids that were mostly negatively charged, except phosphatidylethanolamine and phosphatidylcholine, which were zwitterionic and neutral molecules at the pH of the nanoemulsions (Artiga-Artigas et al., 2018). WPI presented a negative charge at the neutral pH of the nanoemulsions since the carboxyl groups were negatively charged ($–COO^–$) and the amino groups were neutral ($–NH_2$) at a pH above the isoelectric point of the protein (≈ 5) (Kulmyrzaev et al., 2000; Zhang et al., 2016).

When adding sodium alginate, ζ-potential values of SBL and WPI nanoemulsions became more negative (Table 1). In both emulsifier-type nanoemulsions, the higher the polysaccharide concentration, the more negative the electrical charge was, up to values of −77.8 ± 4.3 mV and −57.7 ± 1.4 mV in SBL and WPI nanoemulsion with 1.5% polysaccharide, respectively. Sodium alginate has an anionic character due to the carboxylate and hydroxyl groups present on its molecule, so when it is adsorbed at the interface, it confers a negative charge (Artiga-Artigas et al., 2018). Indeed, flocculation observed in the microscope images (Figure 2) proved that a layer of sodium alginate was

surrounding the droplets. This phenomenon may be a consequence of non-adsorbed sodium alginate molecules forming a network structure surrounding the oil droplets.

Table 1. Initial characterization of soybean lecithin and whey protein isolate curcumin-loaded nanoemulsions containing different sodium alginate concentrations.

Emulsifier Type	Alginate (%)	d_{32} (μm)	ζ-Potential (mV)	Encapsulation Efficiency (%)	Viscosity (mPa·s)
Soybean lecithin	*0*	0.344 ± 0.01 ^{c}	−47.1 ± 3.3 ^{c}	97.0 ± 0.8 ^{ab}	1.59 ± 0.13 ^{a}
	0.5	0.308 ± 0.02 ^{b}	−65.7 ± 2.6 ^{b}	97.1 ± 1.0 ^{ab}	10.28 ± 0.79 ^{b}
	1	0.288 ± 0.02 ^{a}	−76.6 ± 3.6 ^{a}	97.5 ± 0.2 ^{b}	41.01 ± 4.76 ^{c}
	1.5	0.288 ± 0.02 ^{a}	−77.8 ± 4.3 ^{a}	96.7 ± 0.3 ^{a}	110.78 ± 3.79 ^{d}
Whey protein isolate	*0*	0.487 ± 0.01 ^{a}	−37.3 ± 2.5 ^{d}	95.1 ± 0.2 ^{a}	1.44 ± 0.03 ^{a}
	0.5	0.499 ± 0.03 ^{b}	−43.9 ± 2.3 ^{c}	95.2 ± 0.7 ^{a}	18.72 ± 0.30 ^{b}
	1	2.080 ± 0.11 ^{c}	−52.1 ± 1.1 ^{b}	95.6 ± 1.1 ^{a}	57.53 ± 1.16 ^{c}
	1.5	2.251 ± 0.27 ^{d}	−57.7 ± 1.4 ^{a}	95.7 ± 0.2 ^{a}	118.33 ± 3.56 ^{d}

Values are expressed as mean ± standard deviation. Different capital letters indicate significant differences ($p < 0.05$) between nanoemulsions with different sodium alginate concentration.

Encapsulation efficiency. When sodium alginate was not present in the formulations, SBL nanoemulsion showed a slightly higher encapsulation efficiency (≈ 97%) than WPI nanoemulsion (≈ 95%) (Table 1). The phenolic hydroxyl group of curcumin can form H-bonds with the phosphate ion of lecithin so that the curcumin is surrounded by the two long aliphatic chains of lecithin, which may be favouring the retention of the compound within the droplets (Artiga-Artigas et al., 2018; Kumar et al., 2008). In contrast, WPI presented a less negative electrical charge than SBL, which indicates that it probably presented fewer hydroxyl groups that can be bound with curcumin. Moreover, since SBL presents a lower molecular weight and reduces more efficiently interfacial tension than WPI, the retention of curcumin would be higher in SBL nanoemulsion due to the more compact structure in the interface.

The addition of sodium alginate did not affect the encapsulation efficiency of the nanoemulsions regardless of the surfactant used. In fact, in previous studies, no significant differences were detected when increasing the concentration of sodium alginate, as in the case of gelatine-sodium alginate nanocapsules (Heckert Bastos et al., 2020).

Viscosity. Values of viscosity in nanoemulsions without sodium alginate were low and similar using SBL or WPI, both around 1.5 mPa·s (Table 1). The addition of sodium alginate noticeably increased the viscosity of nanoemulsions at any concentration, as expected. The higher the polysaccharide concentration, the higher the viscosity of the nanoemulsions was, up to a maximum of ≈ 118 mPa·s and ≈ 111 mPa·s in WPI and SBL nanoemulsions, respectively, containing 1.5% polysaccharide. In general, polysaccharides such as sodium alginate are used as thickening agents because they can increase the viscosity of the aqueous phase, hindering the movement of the droplets and, thereby, increasing the emulsion stability (Shao et al., 2020). At intermediate polysaccharide concentrations (0.5-1%), SBL nanoemulsions presented a significantly lower viscosity than those formulated with WPI (Table 1). This fact may be attributed to the higher hydrodynamic diameter that WPI nanoemulsions presented in comparison to those with SBL (Table 1 and Figure 2), resulting in a higher viscosity (Phillips & Williams, 2009). Nevertheless, this difference was not observed when using the highest polysaccharide concentration (1.5%). In fact, in previous studies, similar viscosity values were observed when adding sodium alginate in nanoemulsions containing other emulsifiers such as Tween 20 (Artiga-Artigas et al., 2017). This indicates that the viscosity of sodium-alginate-added nanoemulsions is governed by the concentration of the polysaccharide used, while the nature of the emulsifier used has little impact on the system viscosity.

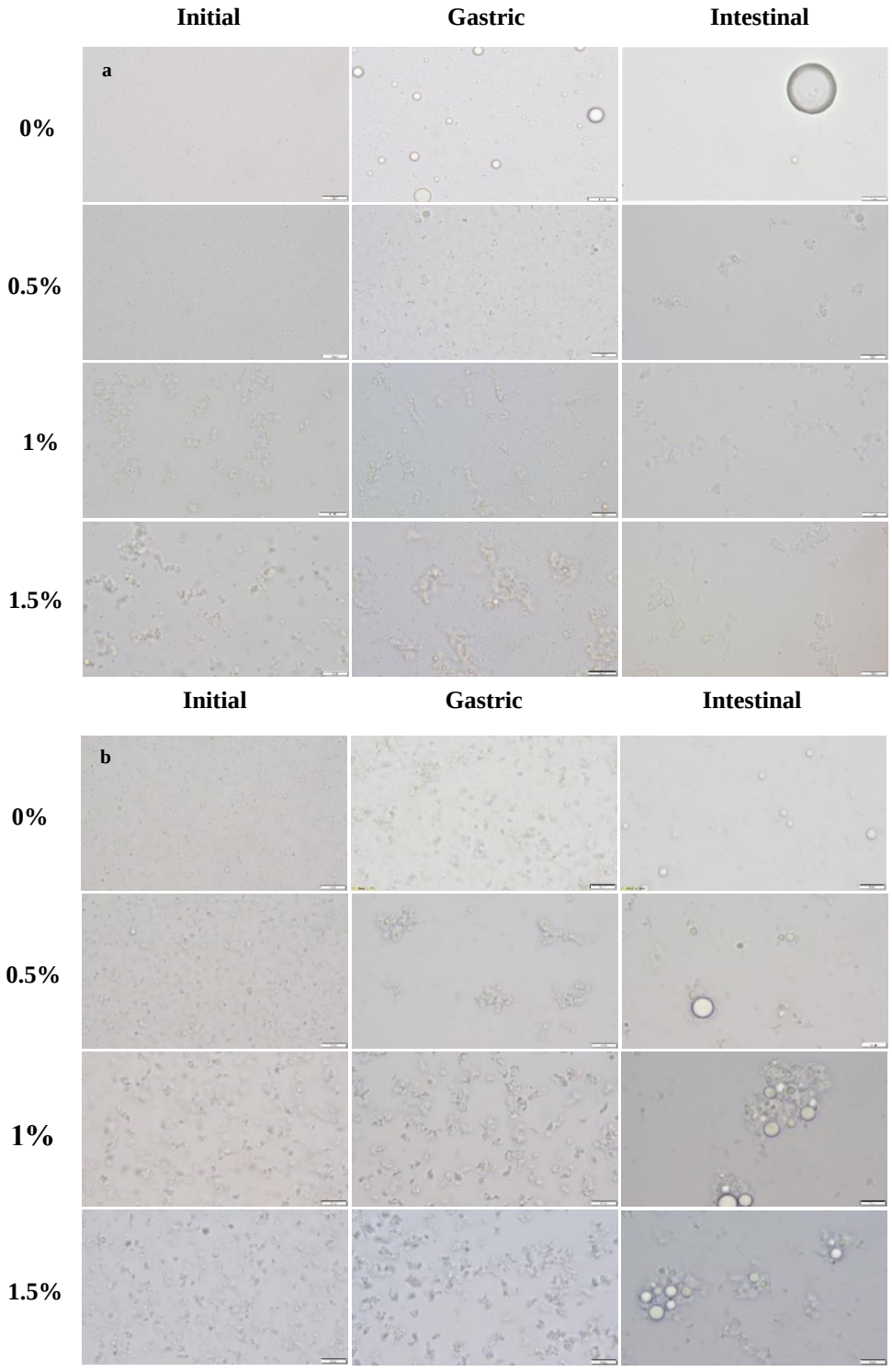

Figure 2. Microscope images of curcumin-loaded nanoemulsions stabilized with (a) soybean lecithin (SBL) or (b) whey protein isolate (WPI) and different sodium alginate concentrations (0, 0.5, 1 and 1.5%) at different phases of *in vitro* digestion. Scale bars were 10 μm long.

Stability. Both SBL and WPI nanoemulsions showed an increase in backscattering in the upper part of the test tube and a decrease in the lower part over time (Figure 3). This indicates that the droplets have migrated to the top, promoting creaming. However, it should be noted that this phenomenon was observed earlier and to a greater extent in WPI nanoemulsion (from day 3) than in SBL nanoemulsion (from day 6). Creaming is a common destabilisation phenomenon observed in nanoemulsions and can be the result of flocculation or coalescence (Artiga-Artigas et al., 2018; Xu et al., 2012). In our study, WPI nanoemulsion presented a less negative charge than that with SBL (Table 1), so the repulsion between droplets may be lower in the nanoemulsion formulated with WPI rather than with SBL. Therefore, in WPI, nanoemulsion droplets may be more prone to aggregation and, consequently, to migrate to the top producing creaming. The addition of sodium alginate increased the stability of WPI nanoemulsion over time, regardless of the concentration used. In this sense, although flocculation was observed in these nanoemulsions (Figure 2b), their high viscosity may have decreased the movement of droplets, preventing the creaming (Salvia-Trujillo et al., 2016). In contrast, the addition of polysaccharides enhanced the stability of SBL nanoemulsion when used at concentrations of ≥ 1% (Figure 3). As mentioned in the previous section, the viscosity of SBL nanoemulsions containing 0.5% sodium alginate was lower than that with WPI and the same polysaccharide concentration (Table 1). Therefore, it seems that the viscosity of SBL nanoemulsion containing 0.5% polysaccharide was not high enough to prevent the movement of the droplets.

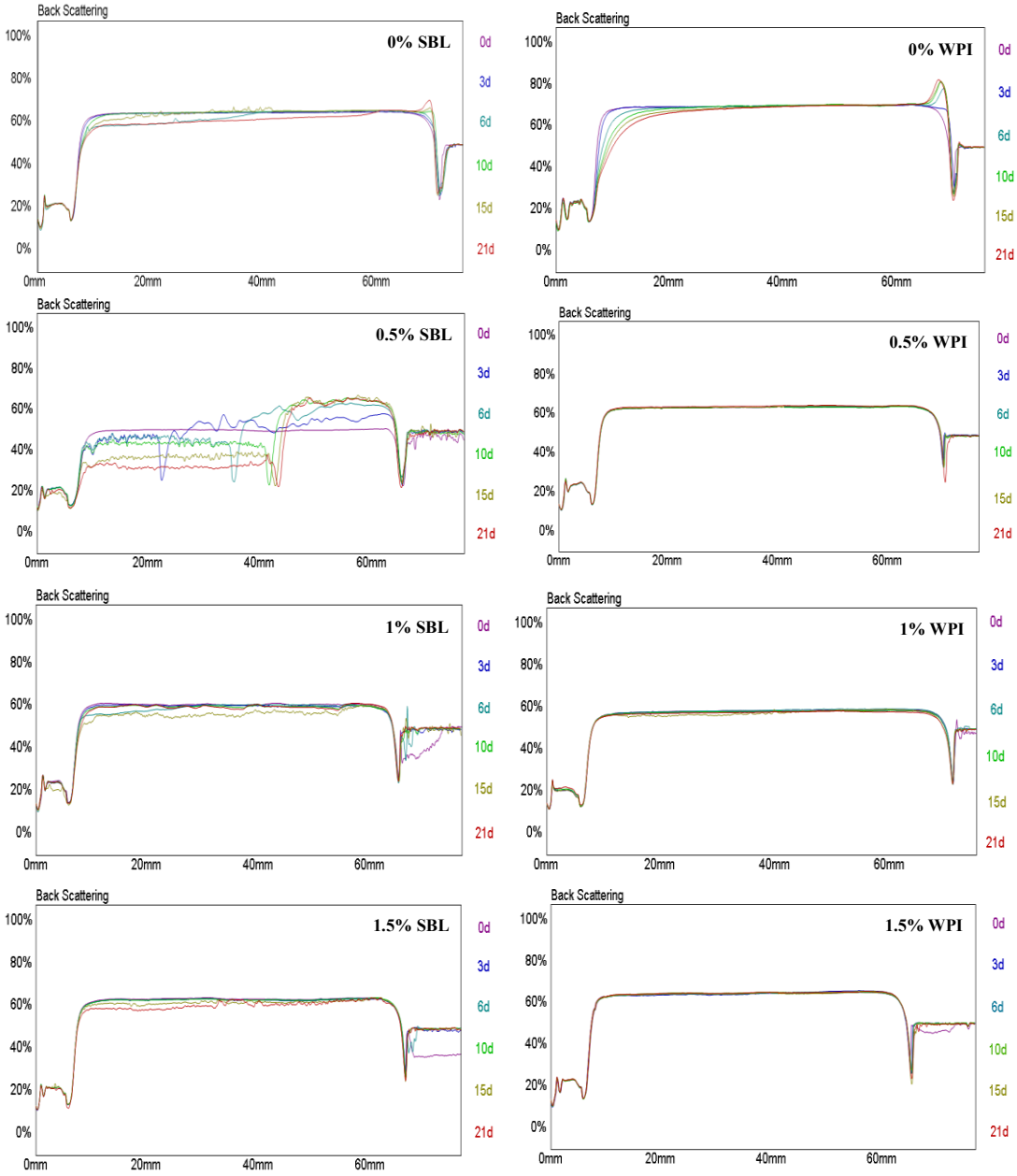

Figure 3. Variations on the backscattering profile of curcumin-loaded nanoemulsions stabilized with soybean lecithin (SBL) or whey protein isolate (WPI) and different sodium alginate concentrations (0, 0.5, 1 and 1.5%) during 21 days at 4 ºC.

3.2. Gastrointestinal *in vitro* digestion

3.2.1. Physicochemical changes during in vitro digestion

Gastric phase. Among non-added polysaccharide nanoemulsions, those formulated using WPI showed a significantly larger particle size in the gastric stage than with SBL (Figure 4). As confirmed in the microscope images, WPI nanoemulsion presented flocculation, while SBL nanoemulsion showed coalescence (Figure 2). Droplets in WPI nanoemulsion became closer due to the WPI proteolysis induced by pepsin during gastric digestion, which may have weakened the viscoelastic interfacial layer (Mantovani et al., 2013). Moreover, the net electrical charge of WPI nanoemulsion was reduced up to 0.55 ± 1.08 mV (Figure 5b) due to the protonation of the amino group ($-NH_2$) of WPI at a pH under its pI (5). Such a loss of electric repulsion between the droplets may have favoured the flocculation (Figure 3b). In SBL nanoemulsion, the proximity of the acidic conditions to the pK_a (≈ 1.5) of the phospholipid reduced the electrostatic repulsion between the droplets, which may favour the coalescence (Ogawa et al., 2004).

WPI nanoemulsions containing sodium alginate at ≥ 1% concentration presented larger particle sizes in the gastric phase than those without the polysaccharide (Figure 4b). This could be attributed to the formation of WPI-sodium alginate electrostatic complexes due to the attractive forces generated between the negatively charged polysaccharide and the positively charged domains of the protein (Li et al., 2021). SBL nanoemulsions containing sodium alginate showed a less noteworthy particle size increase than the nanoemulsion without the polysaccharide and presented flocculation at all sodium alginate concentrations (Figure 3a and 4b). In these nanoemulsions, since SBL is a high surface-active emulsifier, non-adsorbed polysaccharide molecules may remain in the aqueous phase, promoting depletion flocculation. In this regard, the presence of sodium alginate molecules in the aqueous phase would promote an increase in the attractive forces between droplets due to an osmotic process associated with the exclusion of polysaccharide molecules from a narrow region surrounding the droplets (Fioramonti et al., 2015; Guzey & McClements, 2006).

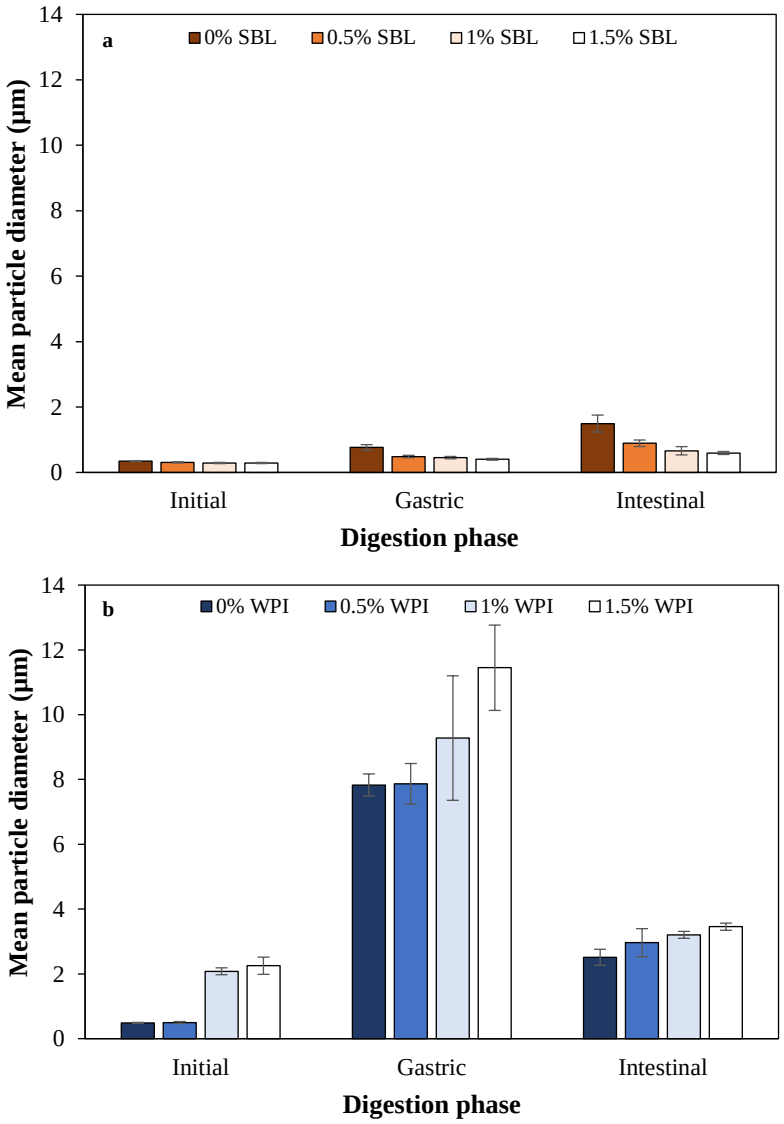

Figure 4. Particle size (µm) of curcumin-loaded nanoemulsions stabilized with (a) soybean lecithin (SBL) or (b) whey protein isolate (WPI) and different sodium alginate concentrations (0, 0.5, 1 and 1.5%) at different phases of *in vitro* digestion.

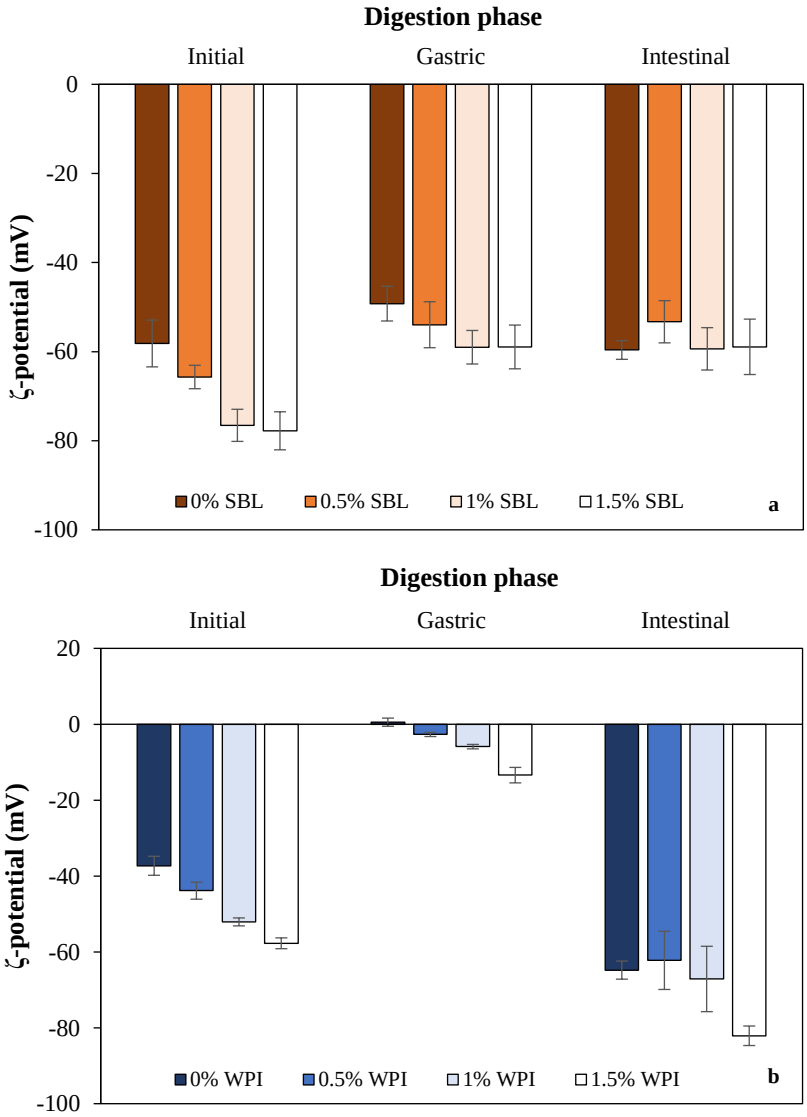

Figure 5. ζ-potential of curcumin-loaded nanoemulsions stabilized with (a) soybean lecithin (SBL) or (b) whey protein isolate (WPI) and different sodium alginate concentrations (0, 0.5, 1 and 1.5%) at different phases of *in vitro* digestion.

Intestinal phase. At pH 7, WPI and SBL nanoemulsions without sodium alginate exhibited a similar and highly negative electrical charge (Figure 5). On the one hand, the electrical charge of the SBL nanoemulsion was quite similar to that of the gastric phase. On the other hand, WPI nanoemulsion experienced a noteworthy change in the electrical charge from the gastric to the intestine stage because at pH 7, the carboxyl groups ($-COO^-$) of WPI became negatively charged, and the amino groups ($-NH_2$) became neutral (Kulmyrzaev et al., 2000). As previously reported, this promoted the redispersion of droplets that were aggregated in the gastric stage due to electrostatic stabilization (Mantovani et al., 2013). Therefore, in our study, droplets in WPI nanoemulsion may be

redispersed when entering the intestinal phase, presenting small particle sizes at early times of intestinal digestion. Conversely, droplets in SBL nanoemulsion could not be redispersed since coalescence is an irreversible phenomenon (Chang & McClements, 2016; Gonçalves et al., 2021). However, at the end of the intestinal phase, coalescence was also detected in WPI nanoemulsion, as shown in microscope images (Figure 3b). Coalescence occurred during intestinal digestion due to the proteolysis of WPI by pancreatic trypsin, which resulted in small peptides that were unable to stabilize the oil droplets (Chen et al., 2020). All WPI nanoemulsions containing sodium alginate presented a larger particle size than those without the polysaccharide at the end of the intestinal phase (Figure 4b). As can be observed in the microscope images, in WPI nanoemulsions with sodium alginate, the coalesced droplets became aggregated in clusters that increased the particle size (Figure 3b). Contrarily, all SBL nanoemulsions containing sodium alginate presented a smaller particle size than those without the polysaccharide (Figure 3a). In polysaccharide-added nanoemulsions, the flocculation rather than coalescence in the gastric phase could have favoured the dispersion of aggregated droplets when entering the intestinal phase. However, as observed in WPI nanoemulsions, during intestinal digestion, aggregation of droplets occurred as a consequence of lipid digestion.

3.2.2. Lipid digestibility

During the first minutes of intestinal digestion, WPI nanoemulsion showed a faster rate of FFA release than SBL nanoemulsion in the absence of sodium alginate (Figure 6). WPI nanoemulsion may present a higher surface area during the first minutes due to the redispersion of aggregated droplets in the gastric phase (Section .2.1). In these nanoemulsions, higher incorporation of lipase molecules at the oil–water interface may occur, bringing lipase into direct contact with the emulsified lipid at early times of lipid digestion (Li, Hu, & McClements, 2011; Reis et al., 2009). Conversely, in SBL nanoemulsions, lipase could not be easily attached to the interface of droplets since SBL present a high surface activity and, in these nanoemulsions, droplets presented a lower surface area, thus showing a slow initial FFA release rate. However, at the end of intestinal digestion, SBL and WPI nanoemulsions without sodium alginate presented the same lipid digestibility, which was about 65%.

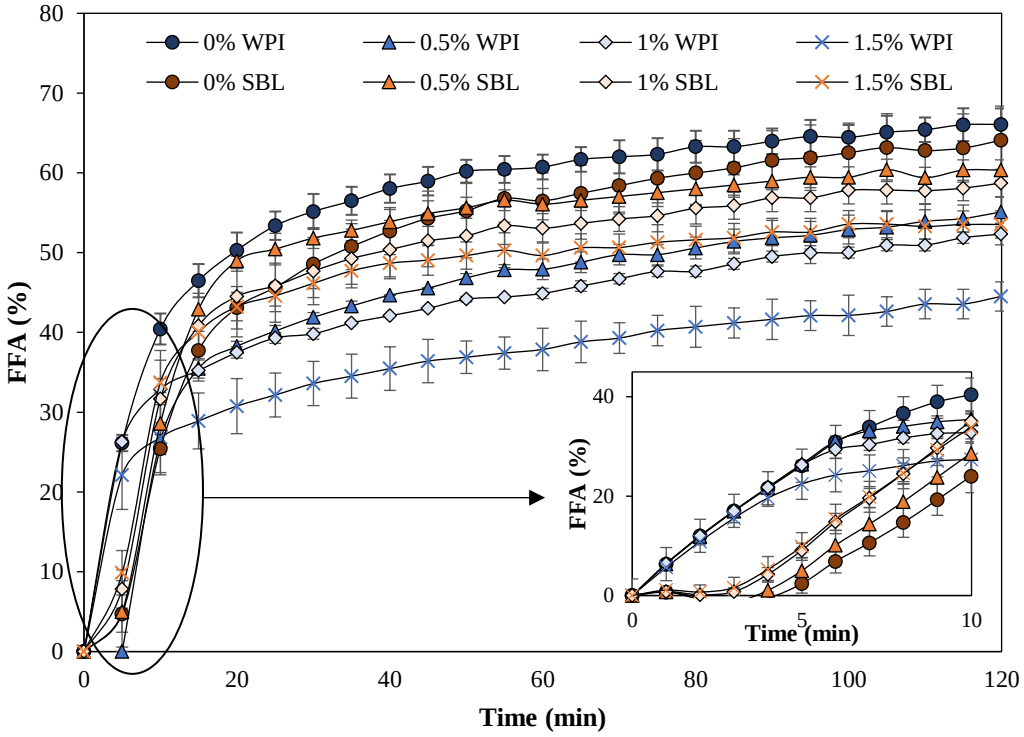

Figure 6. Free fatty acids (FFA) release during the intestinal *in vitro* digestion of curcumin-loaded nanoemulsions stabilized with lecithin (SBL) or whey protein isolate (WPI) and different sodium alginate concentrations (0, 0.5, 1 and 1.5%).

The addition of sodium alginate reduced the lipid digestibility in all nanoemulsions (Figure 6), which is in accordance with previous works (Li et al., 2011; Qin et al., 2016). The increased viscosity of nanoemulsions containing the polysaccharide (Figure 7) could have slowed down the molecular diffusion, reducing the mobility of the species involved in intestinal digestion (Dima & Dima, 2020; Espinal-Ruiz et al., 2014; Velderrain-Rodríguez et al., 2019). Moreover, the anionic alginate molecules could strongly bind to cationic calcium ions, which could slow down digestion by preventing the removal of FFA from the surfaces by calcium or by restricting lipase access due to the formation of gels (Li et al., 2011). The initial tendency of WPI and SBL nanoemulsions containing sodium alginate was similar to that observed in nanoemulsions without the polysaccharide. However, at longer times of lipid digestion, the FFA release continued increasing in SBL nanoemulsions with sodium alginate, while it was stabilized in WPI nanoemulsions containing the polysaccharide (Figure 6). At the end of lipid digestion, WPI nanoemulsions containing sodium alginate showed reduced values in comparison to SBL nanoemulsions at the same polysaccharide concentration. As an example, the digestibility of WPI nanoemulsion with 1.5% sodium alginate was 46.4 ± 3.5%, whereas

it was 53.6 ± 1.7% in that formulated with SBL at the same polysaccharide concentration. The lower lipid digestibility of WPI nanoemulsions containing sodium alginate could be related to the larger particle size of droplets in the intestinal phase (Figure 1 and 4). As previously mentioned, the coalesced oil droplets in these nanoemulsions became aggregated in clusters of large size. Therefore, these nanoemulsions presented a lower surface area, which reduced the access of lipase to the lipid substrate and, thereby, the lipid digestibility at the end of intestinal digestion (Salvia-Trujillo et al., 2013).

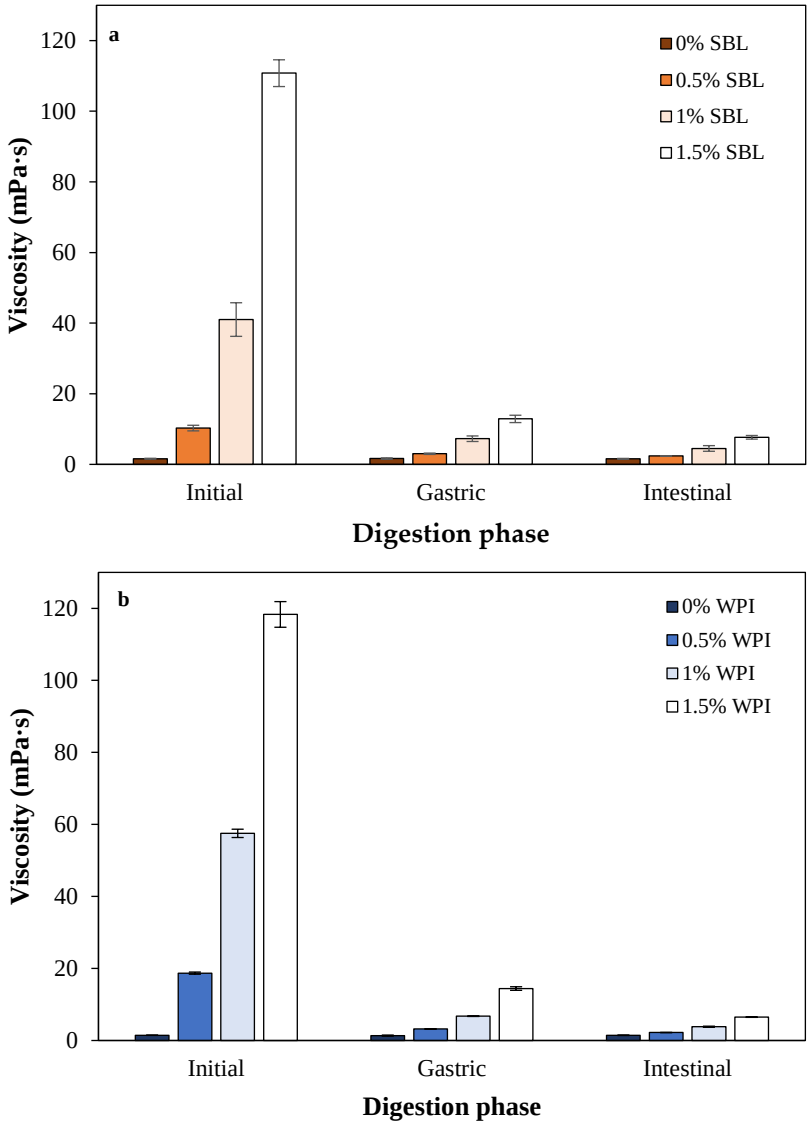

Figure 7. Viscosity of curcumin-loaded nanoemulsions stabilized with (a) soybean lecithin (SBL) or (b) whey protein isolate (WPI) and different sodium alginate concentrations (0, 0.5, 1 and 1.5%) at different phases of *in vitro* digestion.

3.2.3. Curcumin degradation during digestion

During gastric digestion, the degradation of curcumin in nanoemulsions without sodium alginate was lower using WPI (≈13%) than SBL (≈18%) emulsifiers (Figure 8). β-lactoglobulin, which is one of the major components of WPI, has been shown to form complexes with curcumin that protect the compound from degradation during the gastric stage (Sneharani et al., 2010). Peptides resulting from the WPI hydrolysis induced by pepsin are also reported to present antioxidant properties (Korhonen & Pihlanto, 2006; Madureira et al., 2010), which could contribute to reducing curcumin oxidation.

The addition of sodium alginate at the different concentrations used greatly reduced the degradation of curcumin in both SBL and WPI nanoemulsions. Specifically, the lowest degradation (between 0-4%) was observed in WPI nanoemulsion containing 1.5% polysaccharide, which also presented the highest viscosity (Figure 7). The high viscosity proffered by alginate, and the egg-box model gel that this polysaccharide produced due to the presence of Ca^{2+} ions in the gastric fluids (Cao et al., 2020), may have reduced the diffusion rate of oxidative molecules that can promote curcumin degradation. Moreover, since the pH during the gastric phase was below the pK_a of sodium alginate (≈ 3.4), the carboxylic acid groups were non-ionized, thus decreasing the repulsion of the negative charges and causing less polymer chain expansion and low swelling (Agüero et al., 2017). Therefore, curcumin released from oil droplets may be entrapped in the alginate network, which avoids compound degradation.

The degradation in the intestinal phase was much higher than that observed during the gastric phase (Figure 8). During intestinal digestion, lipid droplets were digested, leading to the loss of the system structure and the release of the encapsulated compound. This resulted in the degradation of curcumin due to the physiological pH of the intestinal fluids (Wang et al., 1997). Among nanoemulsions without sodium alginate, WPI nanoemulsions prevented curcumin degradation to a higher extent than that formulated with SBL (Figure 8). The peptides resulting from the WPI hydrolysis during the gastric phase may still be proffering protection to curcumin against degradation due to their antioxidant activity. At the end of the intestinal digestion, WPI nanoemulsion showed a similar curcumin degradation to that formulated with SBL, ≈ 28% and ≈ 33%, respectively. These values are quite similar to those obtained in previous studies with

other emulsifiers, such as quillaja saponin (Zheng et al., 2019).

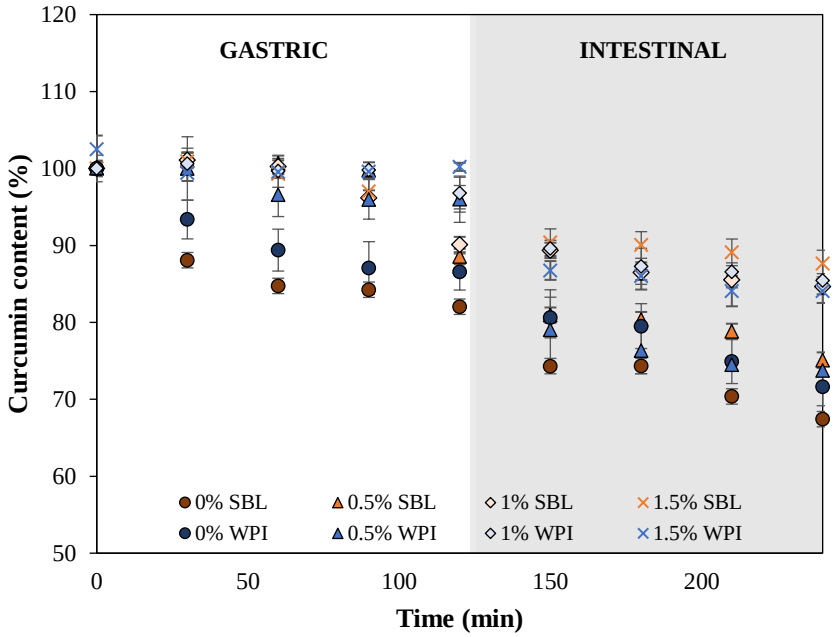

Figure 8. Curcumin content of nanoemulsions stabilized with lecithin (SBL) or whey protein isolate (WPI) and different sodium alginate concentrations (0, 0.5, 1 and 1.5%) during the gastrointestinal *in vitro* digestion.

High sodium alginate concentrations continued to prevent curcumin degradation during intestinal digestion (Figure 8). At the end of this stage, the degradation of curcumin was the lowest (15%) in nanoemulsions with high alginate concentrations, while it was up to 32% in nanoemulsions without the polysaccharide. However, it should be noted that curcumin degradation in nanoemulsions containing sodium alginate was higher during intestinal digestion rather than during gastric digestion (Figure 8). Carboxylic acid groups of alginate molecules became ionized at the pH of the intestine, which resulted in a polymer chain expansion and more matrix swelling (Agüero et al., 2017; Apoorva et al., 2020). Therefore, curcumin could be easily released and degraded when exposed to the aqueous media.

3.3.3. Curcumin bioaccessibility

Curcumin bioaccessibility from nanoemulsions without sodium alginate was significantly higher using WPI than with SBL, 67.5 ± 1.8% and 58.8 ± 1.8%, respectively (Figure 9). In our study, the higher bioaccessibility observed in WPI nanoemulsions

compared to those with SBL could be attributed to the less curcumin degradation observed in nanoemulsions formulated with the protein emulsifier (Figure 8). Therefore, although both nanoemulsions presented the same lipid digestibility at the end of intestinal digestion (Figure 6), the curcumin content available to be incorporated into the mixed micelles was higher in WPI nanoemulsion than in those with SBL. Previous works reported similar values using whey protein isolate (76%) or lecithin (72%) but also using other emulsifiers such as quillaja saponin (74-79%) (Gonçalves et al., 2021; Zheng et al., 2018; Zou et al., 2015). This indicates that the emulsifier nature has little effect on the bioaccessibility of curcumin.

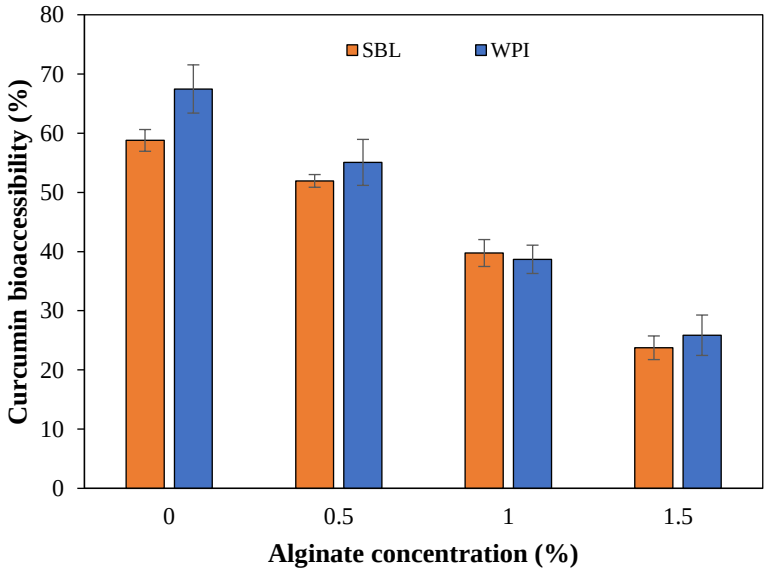

Figure 9. Curcumin bioaccessibility of nanoemulsions stabilized with soybean lecithin (SBL) or whey protein isolate (WPI) and different sodium alginate concentrations (0, 0.5, 1 and 1.5%).

The presence of sodium alginate noticeably decreased the curcumin bioaccessibility in SBL and WPI nanoemulsions, irrespective of the emulsifier type used. As can be observed in Figure 9, the higher the polysaccharide concentration, the lower the curcumin bioaccessibility. In fact, the bioaccessibility was reduced by 15%, 35% and 60% by adding 0.5%, 1% and 1.5% sodium alginate, respectively. The reduced curcumin bioaccessibility that presented sodium alginate nanoemulsions in comparison to non-added sodium alginate nanoemulsions can be related to the digestibility results. In polysaccharide-added nanoemulsions, the reduced digestibility promoted the presence of more undigested oil at the end of the intestinal digestion that can be entrapping curcumin

(Chen et al., 2018; Zhang et al., 2016).

At the same sodium alginate concentration, SBL and WPI nanoemulsions showed the same curcumin bioaccessibility (Figure 9), although the digestibility of WPI nanoemulsions was lower (Figure 6). Based on the findings of previous works, it could be expected that WPI nanoemulsions presented reduced bioaccessibility due to their low digestibility. However, as mentioned in the previous Section 3.2.3, WPI nanoemulsions were more effective in preventing curcumin degradation during the gastric stage (Figure 8). Therefore, WPI nanoemulsions containing sodium alginate presented the highest curcumin concentration during intestinal digestion. As a result, although polysaccharide nanoemulsions containing WPI as an emulsifier presented less lipid digestibility than those with SBL, both emulsifier-type nanoemulsions presented the same curcumin bioaccessibility.

4. Conclusions

This study has shown that both lipidic and protein emulsifiers can be used to produce stable nanoemulsions with a high curcumin encapsulation efficiency (≈ 96%). The addition of sodium alginate influenced the physical properties of nanoemulsions and increased their stability over time at concentrations of 1-1.5%. During gastrointestinal digestion, WPI better-prevented curcumin degradation than SBL, which resulted in a higher curcumin bioaccessibility. The addition of sodium alginate greatly reduced curcumin degradation during digestion, especially when used with the protein emulsifier during the gastric phase as a consequence of WPI-polysaccharide complex formation. However, the presence of the polysaccharide reduced the digestibility of nanoemulsions and, thereby, curcumin bioaccessibility. Sodium alginate seems to be a promising stabilizer for curcumin nanoemulsions when used at 1-1.5%, greatly preventing curcumin degradation during gastrointestinal digestion. However, a 1% concentration seems to be the most suitable, as it reduces curcumin bioaccessibility to a lesser extent.

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–original draft, J.T.-R.; Writing—review & editing, G.O.-O., I.O.-S. and O.M.-B. All authors have read and agreed to the published version of the manuscript.

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Chapter III

Enhancing *in vivo* retinol bioavailability by incorporating β-carotene from alga *Dunaliella salina* into nanoemulsions containing natural-based emulsifiers

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Abstract

The use of microalgae as a source of bioactive compounds has gained interest since they present advantages *vs* higher plants. Among them, *Dunaliella salina* is one of the best sources of natural β-carotene, which is the precursor of vitamin A. However, β-carotene shows reduced oral bioavailability due to its chemical degradation and poor absorption. The work aimed to evaluate the influence of the emulsifier and oil concentration on the digestive stability of *Dunaliella Salina*-based nanoemulsions and study their influence on the digestibility and the β-carotene bioaccessibility. In addition, the effect of the emulsifier nature on the absorption of β-carotene and its conversion to retinol *in vivo* was also investigated. Results showed that the coalescence observed in soybean lecithin nanoemulsion during the gastrointestinal digestion reduced the digestibility and β-carotene bioaccessibility. In contrast, whey protein nanoemulsion that showed aggregation in the gastric phase could be redispersed in the intestinal phase facilitating the digestibility and bioaccessibility of the compound. *In vivo* results confirmed that whey protein nanoemulsion increased the bioavailability of retinol to a higher extent (C_{max} 685 ng/mL) than soybean lecithin nanoemulsion (C_{max} 394 ng/mL), because of an enhanced β-carotene absorption.

Keywords: β-carotene, retinol, bioaccessibility, bioavailability, nanoemulsion, algae

1. Introduction

In the last years, the use of microalgae as a source of bioactive compounds has been widely investigated. These unicellular organisms have simple growth requirements and can sustainably generate lipids, proteins, carbohydrates and bioactive compounds, making them a promising environmentally friendly alternative to current products (Buono et al., 2014). Among them, *Dunaliella salina* is one of the best sources of natural β-carotene, up to 14% of its total dry weight (Harvey & Ben-Amotz, 2020). Moreover, this microalga accumulates the 9-cis β-carotene isomer to a higher extent than vegetables and fruits. Specifically, in *Dunaliella* species, it can be 50% of the total β-carotene content (Delman et al., 2021). This isomer has been shown to have high antioxidant activity (Levin et al., 1997) and beneficial effects on atherosclerosis, fatty liver, and diabetes mellitus in animal models (Harari et al., 2008, 2013, 2020), while less beneficial effects have been observed for the all-trans β-carotene (Clarke & Armitage, 2002; Hennekens et al., 1996). Moreover, once β-carotene is absorbed, it can be converted to retinol, which is the active form of vitamin A (Harrison, 2012). Several studies have shown that population in developing countries has a vitamin A deficiency, but this problem also concerns developed countries, which often have a suboptimal intake (Borel & Desmarchelier, 2017; Troesch et al., 2012). In that sense, previous works have reported that two-thirds of dietary β-carotene was either inaccessible for absorption or excreted and only a half of the amount absorbed was converted to retinol (Thurnham, 2007).

To overcome these challenges and increase its bioavailability, encapsulation of β-carotene in nanoemulsion-based delivery systems seems to be a promising strategy. The use of this systems has shown to increase the stability of the encapsulated compound, control its release and improve the *in vitro* bioaccessibility (McClements, 2018). Some advantages have been observed when reducing the particle size of emulsions, thus formulating nanoemulsions, which present not only high stability to particle aggregation and gravitational separation, but also enhanced compound bioaccessibility and bioavailability (Fan et al., 2017; Li et al., 2013; Soukoulis & Bohn, 2017).

Some of the most effective currently used emulsifiers used in the formulation of emulsions and food production, in general, are synthetic (McClements et al., 2017).

However, a recent study has related that the administration of synthetic emulsifiers such as Tween 40 could be associated with some health issues such as liver damage (Jimenez-Escobar et al., 2020). Other studies also reported that synthetic emulsifiers such as polyoxyethylene (40) stearate or Tween 80 could cause intestinal barrier dysfunction by inhibiting the activity of P-glycoprotein, which plays an important role in intestinal permeability (Zhang et al., 2003; Zhu et al., 2009). Therefore, the interest in the use of natural emulsifiers as an alternative to those synthetic to produce food nanoemulsions is increasing. Among them, soybean lecithin (SBL) and whey protein isolate (WPI) have been shown to decrease the interfacial tension to a great extent and produce nanoemulsions with good stability over time (Artiga-Artigas et al., 2018; Gasa-Falcon et al., 2019; Infantes-Garcia et al., 2021).

Some researchers have studied how the interfacial composition can affect the emulsion stability and the *in vitro* release and bioaccessibility of encapsulated compounds. The findings of these studies have revealed that the election of the emulsifier can highly affect the microstructural changes during *in vitro* digestion and the release and bioaccessibility of the encapsulated compound (Chang & McClements, 2016; Gasa-Falcon et al., 2019; Lv et al., 2019). *In vitro* digestion methods and cell cultures are useful to mimic the gastrointestinal digestion process and the absorption of the encapsulated bioactive compounds. However, *in vivo* studies are essential for understanding the complex processes that take place in the gastrointestinal tract and elucidating the bioavailability of bioactive compounds enclosed in nanoemulsions. Previous authors have investigated the administration of *Dunaliella Salina* in the diet, reporting that it can enhance or maintain the activity of hepatic enzymes that are involved in combating reactive oxygen species (ROS) (Honda et al., 2021; Murthy et al., 2005). Nevertheless, there are no studies on the *in vivo* gastrointestinal stability and the bioavailability of the provitamin A carotenoids from algae enclosed in nanoemulsions.

For that reason, the aim of this study was to study the influence of two different natural-based emulsifiers (soybean lecithin and whey protein isolate) and oil concentration (10% or 30%) on the *in vitro* gastrointestinal stability and bioaccessibility of carotenoid-rich *Dunaliella Salina* nanoemulsions. Moreover, the effect of the emulsifier nature on the

β-carotene bioavailability and conversion to retinol in rats after the oral administration of nanoemulsions was also analyzed.

2. Materials and methods

2.1. Materials

Freeze-dried alga *Dunaliella salina* was kindly provided by Monzón Biotech (Spain). Pepsin (from porcine gastric mucosa), pancreatin (from porcine pancreas), bile extract (bovine), lipase (from porcine pancreas) and Nile red were obtained from Sigma-Aldrich, INC (St. Louis, MO). Corn oil (Koipesol Asua, Deoleo, Spain) was purchased from a local supermarket. Soybean lecithin (SBL) was acquired from Alfa Aesar (Thermo Fisher Scientific, Massachusetts, USA). Whey protein isolate (WPI) was kindly provided by El Pastoret de la Segarra (Spain). Ultrapure water obtained from a milli-Q filtration system was used to prepare of all solutions.

2.2. Methods

2.2.1. Preparation of β-carotene enriched oil

To obtain the β-carotene enriched oil, alga *Dunaliella salina* was mixed with corn oil at 65 ºC (0.4 g alga/mL oil) and vortexed at 3000 rpm for 1 min. Then, the mixture was treated with an Ultra-Turrax (IKA, Staufen, Germany) at 17500 rpm for 2 min and submerged in a sonication bath for 5 min without discarding the precipitate. This process was repeated twice to ensure cell disruption. Finally, the mixture was centrifuged at 9000 rpm for 15 min and the upper part was collected as the oil phase. The final concentration was 20 mg β-carotene/g oil.

2.2.2. Nanoemulsion preparation

To obtain the aqueous phase, SBL or WPI were added into ultrapure water and stirred 4 h at room temperature. Then, the enriched β-carotene oil was added to the mixture and homogenized using an Ultra-Turrax (IKA, Staufen, Germany) at 11000 rpm for 2 min. Finally, nanoemulsions were obtained by passing coarse emulsions through a microfluidizer (LM10, Microfluidics, USA) at 130 MPa for 5 cycles. In this study, the

effect of the oil concentration in nanoemulsion was investigated, using two different concentrations (10% or 30%). The oil concentrations chosen in this study are due to the need to have a sufficient dose of bioactive compound to be able to perform *in vivo* studies. However, the emulsifier was used at a 0.4 surfactant-oil ratio (SOR) in all nanoemulsions. Thus, four different nanoemulsions were studied: SBL10% (soybean lecithin nanoemulsion with 10% oil concentration), SBL30% (soybean lecithin nanoemulsion with 30% oil concentration), WPI10% (whey protein isolate nanoemulsion with 10% oil concentration), and WPI30% (whey protein isolate nanoemulsion with 30% oil concentration).

2.2.3. Nanoemulsion characterization

Particle size. The particle size of nanoemulsions was measured using a Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). Samples were diluted in ultrapure water and stirred in the dispersion unit with a constant speed of 1800 rpm. The mean particle size was expressed as surface area mean diameter (d_{32}) in micrometres (μm), fixing a refractive index of the corn oil of 1.473 and 1.333 for water.

ζ-potential. The ζ-potential was measured by phase-analysis light scattering (PALS) using Zetasizer laser diffractometer (NanoZS Malvern Instruments Ltd. Worcestershire, UK). Prior to the analysis, nanoemulsions were diluted (1:100) in ultrapure water. Gastric and intestinal samples were diluted maintaining its pH at 3 and 7, respectively. Then, the diluted samples were placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles. The results were reported in millivolts (mV).

Viscosity. Apparent viscosity of nanoemulsions was determined using a SV-10 vibro-viscometer (A&D Company, Tokyo, Japan), which produces a vibration of 30 Hz and a constant amplitude of 0.4 mm at controlled room temperature. The results were expressed in mPa·s.

Optical microscopy. Nanoemulsions were dyed with Nile Red, previously dissolved at 0.1% (w/v) in ethanol. Then, micrographs of nanoemulsions were obtained using fluorescence with an optical microscope (Olympus BX41, Olympus, Göttingen,

Germany) with a 100x objective lens. The images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus Göttingen, Germany).

2.2.4. In vitro gastrointestinal digestion

To simulate the human digestion process, an *in vitro* gastrointestinal tract digestion based on an international consensus method (Brodkorb et al., 2019; Minekus et al., 2014) with some modifications was used.

The protocol included both gastric and small intestinal phases. The mouth phase was not performed since nanoemulsions were liquid. To perform the gastric phase, 20 mL of nanoemulsion was mixed 1:1 with simulated gastric fluids (SGF) containing pepsin (2000 U/mL) and 10 μL of a $CaCl_2$ solution (0.3 M). Then, the pH was adjusted to 3 using HCl (1M) and the mixture was placed into an incubator at 37 °C for 2 h while shaking at 100 rpm. To simulate the intestinal phase, a pH-stat device was used. Once the gastric phase was completed, an aliquot of 20 mL of gastric sample was placed in a 37 °C water bath and mixed 1:1 with simulated intestinal fluids (SIF) containing 10 mM bile solution. Then, the pH was adjusted to 7 with NaOH (1 M) and pancreatin and lipase enzymes were added to the final mixture to achieve 100 U/mL of trypsin and 2000 U/mL pancreatic lipase. The pH of the sample was maintained to 7 by adding NaOH (0.25 M) constantly for 2 h. The final volume of NaOH (0.25 M) was recorded and used to calculate the amount of free fatty acids (FFA) released during intestinal digestion. The FFA (%) was determined according to Eq. (1):

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

where V_{NaOH} is NaOH volume (L) used during the intestinal digestion, C_{NaOH} is NaOH molarity (0.25 mol/L), M_{oil} is corn oil molecular weight (800 g/mol) and m_{oil} is the weight of the oil phase present in the intestinal digestion phase (g).

2.2.5. In vitro bioaccessibility

To study the β-carotene bioaccessibility, after the digestion process, digested nanoemulsions were centrifuged at 4000 rpm during 40 min at 4 ºC to obtain the micellar

fraction. Then, an extraction and quantification of the β-carotene content was performed in both the initial nanoemulsion and micellar fraction following the method described in section 2.2.7. Finally, β-carotene bioaccessibility was calculated according to Eq. (2):

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

where $C_{micelle}$ is the β-carotene concentration (mg/mL) in the micellar fraction and $C_{initial}$ is the initial β-carotene concentration (mg/mL) in the nanoemulsion.

2.2.6 In vivo bioavailability

Female Sprague Dawley rats weighing 200-250 g were used for the *in vivo* bioavailability study of β-carotene encapsulated in different carriers. The animal procedures were conducted in accordance with EU Directive 2010/63/EU guidelines for animal experiments and approved by the Animal Ethics Committee of Universitat de Lleida. The animals were randomly divided into three groups. In the first (n=5) and second group (n=5) rats were fed with the 30% SBL nanoemulsion and 30% WPI nanoemulsion, respectively. The third group (n=5) was used as a control and rats were fed with an aqueous β-carotene suspension. All the animals were fed with a dose of 60 mg of β-carotene/kg body weight, irrespective of the vehicle used. Rats were housed at constant temperature (20 ± 0.5 °C) and humidity (50 ± 10%) and a 12:12 h day/night cycle. Animals were fasted for 12 h before the experiment with free access to water. The animals were fed with a single dose of one of the different β-carotene nanoemulsions or a β-carotene suspension. Blood samples were taken at 0, 30, 60, 120, 240, and 480 min after administration from the tail vein. Plasma was immediately separated by centrifuging the blood samples at 4000 rpm for 10 min at 4 ºC and stored at -80 ºC. Then, retinol and β-carotene content were extracted and quantified in plasma samples as described in 2.2.7.

The pharmacokinetic analysis was performed using a non-compartmental design. The area under the drug concentration *versus* time curve from 0 to 8 hours ($AUC_{0–8\ h}$) was calculated using the trapezoidal rule. The maximum plasma concentration of β-carotene and retinol (C_{max}) and the time to reach maximum plasma concentration (T_{max}) were directly obtained from plasma analyses.

2.2.7. Carotenoid extraction and quantification

Nanoemulsions and micellar samples. β-carotene extraction was performed according to a previously reported method with some modifications (Zhou et al., 2018). Aliquots of 1 mL of sample (nanoemulsion or micellar) were mixed with 2 mL of ethanol/hexane (2:3 *v/v*) and vortexed at 1500 rpm for 1 min. Then, the mixture was centrifuged at 4000 rpm for 5 min at 4 ºC, and the supernatant was collected. This process was performed three times to ensure the total carotenoid extraction. Afterwards, the collected upper organic layers were collected and evaporated under N_2 and stored at -40 ºC. The quantification of β-carotene was carried out using an HPLC system equipped with a 600 Controller and a diode array detector (Waters, Milford, MA) following the method described by Vallverdú-Queralt et al. (2013). Before injecting the samples into the HPLC, they were reconstituted with 1 mL of MTBE and filtered. β-carotene was identified using a C30 column 250 x 4.6 mm i.d, 5 µm (Bischoff Chromatography, Leonberg, Germany) at room temperature. The injection volume was 20 µL and the flow rate was 1 mL/min. Mobile phases consisted of water (A), methanol (B) and MTBE (C). Separation was carried out in 23 min under the following conditions: 0 min, 70% B; 10 min, 20% B; 20 min, 6% B; 21 min, 6% B; 23 min, 70% B. Water was kept constant at 4% throughout the analysis. The column was equilibrated for 10 min prior to each analysis. A β-carotene standard was used to identify analytes by retention times and ultraviolet–visible (UV–vis) spectra. The HPLC–UV chromatograms were acquired, selecting the 450 nm wavelength; afterwards, the UV–vis spectra were recorded in the range 350–550 nm.

Plasma samples. Retinol and β-carotene extraction in plasma samples was performed according to a previously reported method with some modifications (Fan, et al., 2017). Aliquots of 150 µL of plasma were mixed with the internal standard trans-β-apo-8′-carotenal and 600 mL of ethanol/hexane (1:2 v/v) containing 0.1% BHT. Then, the mixture was vortexed at 1000 rpm for 1 min, centrifuged at 9000 rpm for 5 min at 4 ºC, and the organic fraction was collected. This process was performed 3 times to ensure the total carotenoid extraction. Afterwards, the collected upper organic layers were evaporated under N_2 and stored at -80 ºC. The quantification of β-carotene and retinol from plasma samples was performed using a Acquity UPLC with a photodiode array

detector from Waters (Milford, MA, USA) equipped with a binary solvent delivery system. The analysis was performed using a reverse-phase C18 column (ACQUITY UPLC® BEH 1.7µm 150 x 2.1 mm) kept at constant temperature (32 ºC). The volume injected was 7.5 µL and the mobile phase consisted of a gradient of (A) acetonitrile: methanol (70:30 v/v) and (B) water: acetonitrile (95:5 v/v). The flow rate was 1 mL/min. The gradient profile of the mobile phase was set at 90% A and increased linearly to 100% A over 5.5 min with a 6.5-min hold, after which the mobile phase was changed back to 90% A over 2 min and the held for 2 min. β-carotene was detected at 450 nm and retinol at 325 nm.

2.2.8. Statistical analysis

All experiments were assayed in duplicate and three repetitions of each analysis were carried out on each parameter in order to obtain mean values. Analysis of the variance (ANOVA) was performed to compare treatments. Least significant difference (LSD) test was employed to determine differences between means. The confidence interval was set at 0.95 and all results were analyzed using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, MD, USA).

3. Results and discussion

3.1. Initial emulsion characterization

Particle size. Initially, WPI nanoemulsions showed a lower mean particle size (Table 1) with a more polydisperse distribution than SBL nanoemulsions (Figure 1). These results indicate that WPI was more effective than SBL to obtain small oil droplets by microfluidization, which was also observed in previous studies (Chang & McClements, 2016). Low molecular weight surfactants such as SBL can decrease the interfacial tension to a higher extent than proteins such as WPI due to its orientation and configuration at the interface. However, high molecular surfactants such as WPI are more effective in the formation of a viscoelastic film that surrounds oil droplets (Fuentes et al., 2021), which can prevent the recoalescence of the droplets. In this study, it seems that preventing droplet coalescence was more determinant to achieve small particle sizes than the capacity to quickly reduce the interfacial tension. Additionally, as can be

observed in the microscopy images (Figure 2), all nanoemulsions formulated in this study presented small dispersed oil droplets, meaning that they did not present any instability phenomenon. By increasing the oil content from 10% to 30%, the mean particle size decreased and the particle size distribution became more polydisperse in SBL nanoemulsions. The higher viscosity of SBL30% compared to SBL10% may have increased the shear disruptive stresses favouring droplet fragmentation (Qian & McClements, 2011). Conversely, the mean particle size of WPI nanoemulsions increased when the oil content was augmented from 10% to 30%. Moreover, the particle size distribution became slightly more polydisperse, although the particle size value of WPI30% was still lower than that of the SBL30% nanoemulsion.

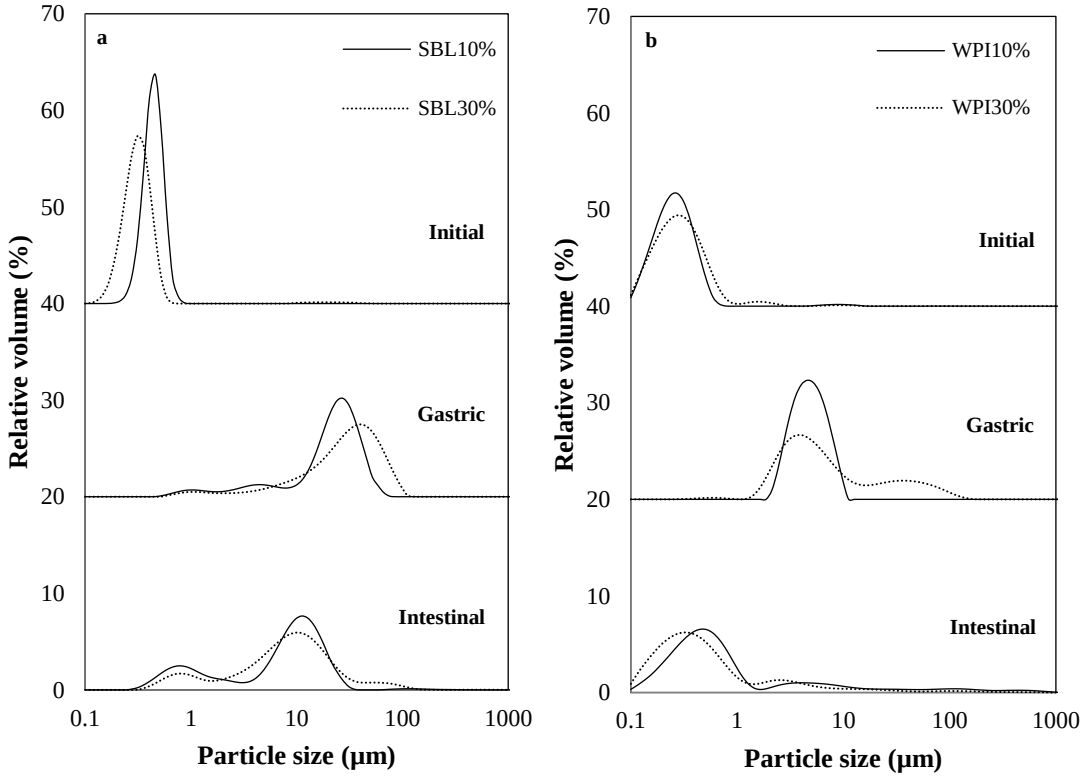

Figure 1. Particle size distribution of soybean lecithin (SBL) nanoemulsions (a) and whey protein isolate (WPI) nanoemulsions (b) at different oil concentrations (10% and 30%) during the different phases of the *in vitro* gastrointestinal digestion.

Table 1. Physical properties of soybean lecithin (SBL) and whey protein isolate (WPI) β-carotene-loaded nanoemulsions at different oil concentrations (10% and 30%).

	Particle size (μm)	**ζ-potential (mV)**	**Viscosity (mPa·s)**
SBL10%	0.455 ± 0.004 ^{Bb}	-49.63 ± 2.96 ^{Aa}	1.66 ± 0.16 ^{Aa}
SBL30%	0.317 ± 0.005 ^{Ba}	-49.86 ± 2.14 ^{Aa}	37.32 ± 5.10 ^{Ab}
WPI10%	0.250 ± 0.010 ^{Aa}	-36.93 ± 1.14 ^{Ba}	2.07 ± 0.13 ^{Ba}
WPI30%	0.269 ± 0.006 ^{Ab}	-35.30 ± 3.06 ^{Ba}	38.57 ± 4.09 ^{Ab}

Values are expressed as mean ± standard deviation. Different capital letters indicate significant differences ($p < 0.05$) among nanoemulsions with different emulsifier and same oil concentration. Different lowercase letters indicate significant differences ($p < 0.05$) among nanoemulsions with different oil concentration and same emulsifier.

ζ-potential. In general, SBL nanoemulsions showed a more negative electrical charge than WPI nanoemulsions, being around -50 mV and -36 mV, respectively (Table 1). These results are in accordance with previous findings and indicate that SBL provided a higher electrostatic stabilization than WPI. At the pH of the nanoemulsions (≈ 6) the phosphate groups from the majority of phospholipids of SBL were negatively charged (Artiga-Artigas et al., 2018), conferring a highly negative electrical charge. In the case of WPI, the negative electrical charges were proffered by the amino groups that were not protonated at a pH above the pI of the protein (≈ 5).

By increasing the oil content from 10% to 30% no changes were detected in the electrical charge of nanoemulsions. In this sense, it should be noted that all nanoemulsions in this study had an equal surfactant-oil ratio (SOR), meaning that the concentration of emulsifier molecules in the interface of droplets was similar in all emulsions, irrespective of the oil concentration.

Viscosity. At 10% oil concentration, WPI nanoemulsion showed a slightly higher viscosity than SBL nanoemulsion, which could be related to the differences in particle size between these nanoemulsions. The smaller droplet size in WPI10% nanoemulsion increased the mean separation distance between the droplets in comparison to SBL10%. This increased separation distance could produce an increase in hydrodynamic interactions between the droplets and, consequently, a higher viscosity (Pal, 2000). However, no differences were observed between the viscosity of SBL and WPI nanoemulsions containing a 30% oil concentration, probably because the difference in the particle size between these nanoemulsions was less noteworthy (Table 1).

Nanoemulsions with 30% oil content presented a noticeably higher viscosity (37 mPa·s) than nanoemulsions with 10% oil content (1.7-2 mPa·s) (Table 1). Higher viscosities have been previously observed when the oil content has been increased in emulsions, which may be a consequence of the high packing state of droplets in these emulsions (Artiga-Artigas et al., 2019).

3.2. *In vitro* gastrointestinal digestion

3.2.1. Physicochemical changes during in vitro digestion

Gastric phase. As can be observed in the microscope images, coalescence occurred in SBL nanoemulsions (Figure 2), irrespective of the oil concentration used. This phenomenon has been previously observed in nanoemulsions containing lecithin as emulsifier but different oils such as fish or soybean oil (Chang & McClements, 2016; Park et al., 2018). Although, initially, these nanoemulsions presented a highly negative electrical charge due to the neutral pH of the systems (section 3.1), the electrostatic repulsion was reduced into the gastric phase (Figure 3a) due to the proximity of acidic conditions to the lecithin pK_a (around 1.5), leading droplets became closer and causing coalescence (Mantovani et al., 2013). Mean particle size values confirmed the noticeable increase of particle size in these nanoemulsions (up to ≈ 12 μm), especially in SBL30% (Figure 3b). Conversely, flocculation in WPI nanoemulsions was observed at the gastric stage (Figure 2), which also produced a particle size increase respect to the initial systems (from ≈ 0.26 to ≈ 5 μm) and a more polydisperse particle size distribution (Figures 1 and 3b). This instability phenomenon, usually observed in WPI nanoemulsions in the gastric stage, may be due to the proteolysis of the proteinaceous interfacial layer by pepsin (Mantovani et al., 2013; Park et al., 2018). Moreover, the negative electrical charge was also reduced compared to the initial systems (from ≈ -36 mV to ≈ 13 mV) as a consequence of the protonation of the amino groups of WPI at a pH below the pI, which may have also contributed to the destabilization. The electrical charge of these nanoemulsions became slightly positive during the gastric stage (Figure 3a), so the electrostatic repulsion was dominated by the van der Waals attraction and droplets became aggregated.

By increasing the oil concentration from 10% to 30% in SBL nanoemulsions coalescence was favoured (Figure 2) and a higher particle size (Figure 3b) with more polydisperse distribution was detected (Figure 1). In WPI nanoemulsions, more flocculation was observed increasing the oil concentration and they became more polydisperse. Nevertheless, the mean particle size of WPI nanoemulsions was similar regardless of the oil concentration used (Figure 3b).

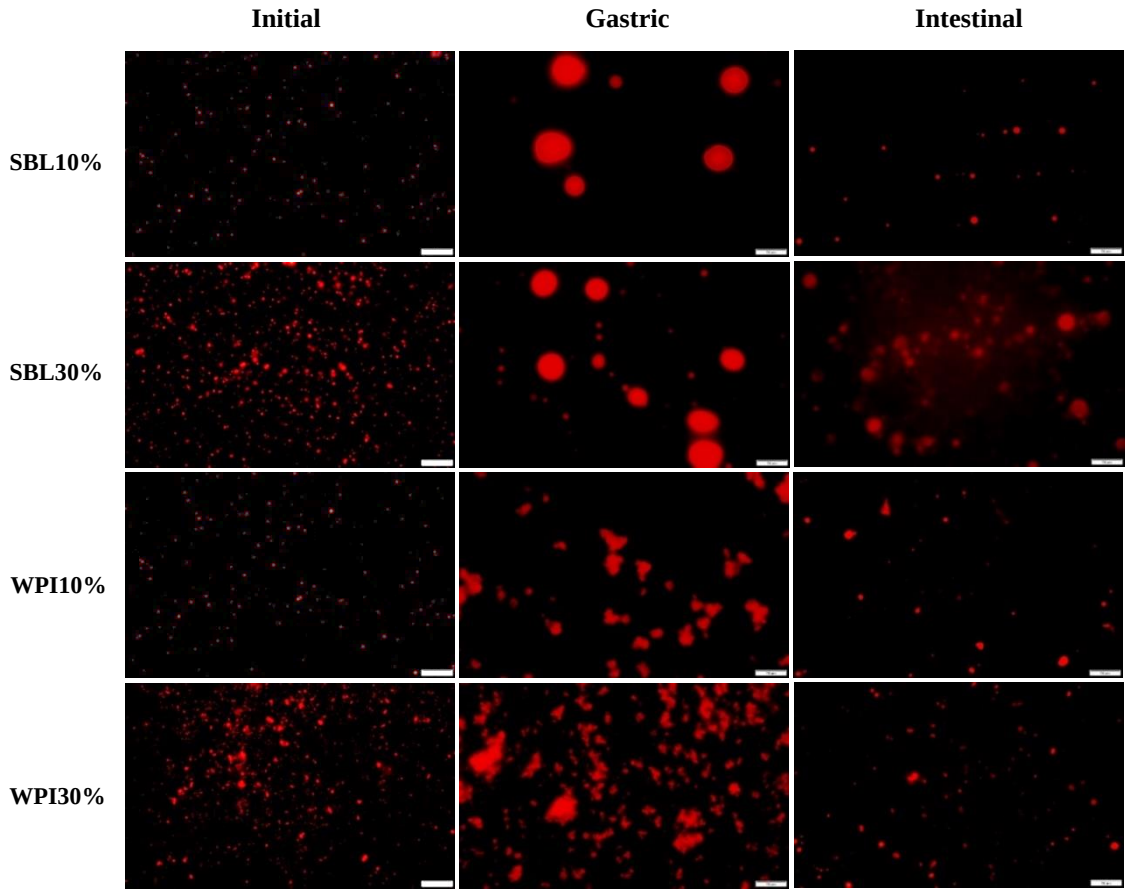

Figure 2. Microscope images of soybean lecithin (SBL) and whey protein isolate (WPI) β-carotene-loaded nanoemulsions at different oil concentrations (10 % and 30 %) during the different phases of the *in vitro* gastrointestinal digestion. Scale bars are 10 μm long.

Intestinal phase. The electrical charge of all nanoemulsions became negative and similar among them at this stage (Figure 3a). According to other authors (Mantovani et al., 2013), WPI aggregated droplets of the gastric phase would be redispersed due to an electrostatic stabilization promoted by the change of pH from 3 to 7 when entering the intestinal stage. Conversely, this was not possible in SBL nanoemulsions since coalescence is an irreversible phenomenon. A general particle size reduction was

observed during intestinal digestion (Figure 3b) probably due to the conversion of oil droplets to free fatty acids and monoacylglycerols by lipase (Chang & McClements, 2016). At the end of the intestinal digestion, the mean particle size of SBL nanoemulsions was higher than that of WPI nanoemulsions at this stage, being ≈ 3 μm and ≈ 0.4 μm, respectively.

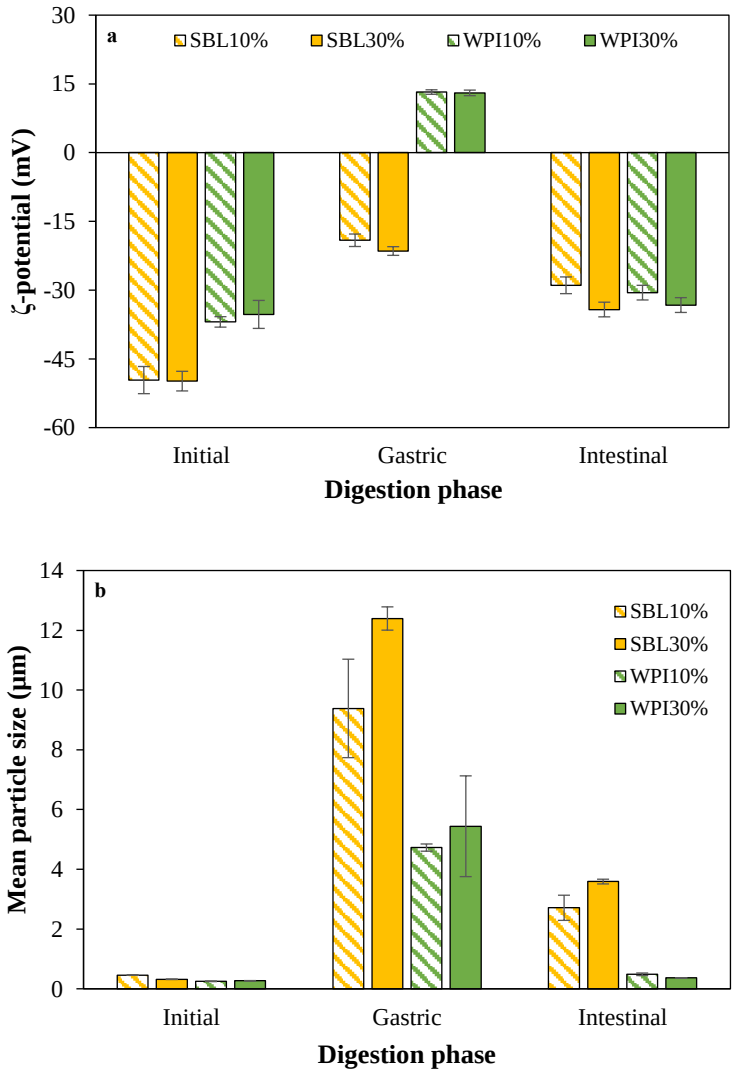

Figure 3. ζ-potential (a) and mean particle size (b) of soybean lecithin (SBL) and whey protein isolate (WPI) β-carotene-loaded nanoemulsions at different oil concentrations (10 % and 30 %) during the different phases of the *in vitro* gastrointestinal digestion.

SBL nanoemulsions with 30% oil concentration presented a higher mean particle size than that with 10%; the same trend that was observed at the gastric stage. The high oil concentration of SBL30% nanoemulsion may have presented a higher non-digested oil content at the end of the intestinal digestion than SBL10%, which may have favoured

the coalescence of non-digested oil droplets. Conversely, the mean particle sizes of WPI10% and WPI30% were small and similar, which may be attributed to the redispersion of aggregated droplets in the intestinal phase.

3.2.2. Lipid digestibility

At a 10% oil concentration, WPI showed a noteworthy higher lipid digestibility than SBL, being about 60% and 40%, respectively (Figure 4). This fact may be attributed to the redispersion of aggregated droplets in WPI nanoemulsions when entering the intestinal phase, which may have favoured lipid digestion due to the increased surface area of oil droplets (Salvia-Trujillo et al., 2013). Moreover, WPI is less surface-active than SBL since it reduces the interfacial tension to a lower extent and it is partially digested by the pepsin during the gastric stage forming a weak interfacial layer. Therefore, it may be easily displaced from the interface for other surface-active molecules present in the intestine such as bile salts and lipase, as also observed when comparing the lipid digestibility of WPI with other emulsifiers such as quillaja saponin (Lv et al., 2019).

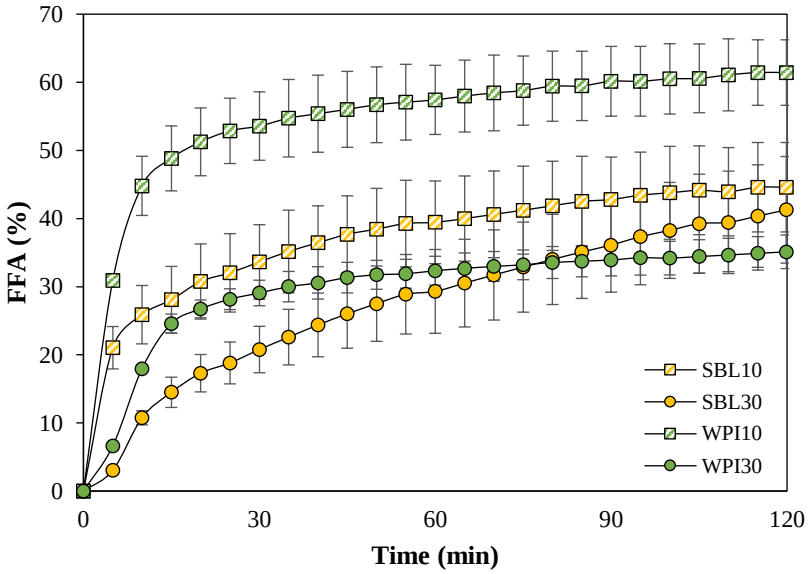

Figure 4. Free fatty acid (FFA) release during the *in vitro* intestinal lipid digestion of soybean lecithin (SBL) and whey protein isolate (WPI) β-carotene-loaded nanoemulsions at different oil concentrations (10% and 30%).

Nanoemulsions with a 30% oil concentration showed a lower and slower FFA release than nanoemulsions containing 10% of oil (Figure 4). The higher viscosity of nanoemulsions containing 30% oil compared with those with 10% (Figure 5) could have reduced the intestinal enzyme diffusion process, thereby, decreasing the rate and extent of lipolysis (Espinal-Ruiz et al., 2014). Additionally, the high oil content of nanoemulsions with 30% oil concentration may have hindered the activity of the lipases. The ratio lipase-to-fat and calcium-to-FFA decreased as the oil concentration increased, reducing the rate of lipolysis and increasing FFA accumulation on the droplet interface (Li et al., 2011). The reduction of digestibility was more noteworthy in WPI nanoemulsions rather than SBL nanoemulsions. This fact may be attributed to the higher viscosity of WPI30% at this stage in comparison to SBL30% (Figure 5) which may have reduced the intestinal enzyme diffusion to a higher extent, therefore reducing the digestibility. At the end of the intestinal digestion, both SBL30% and WPI30% showed the same digestibility (≈ 38%), although WPI30% nanoemulsion showed a higher initial FFA release rate than SBL30% during the first 30 min. As it was also observed in nanoemulsions containing 10% oil, these differences at early stages of the lipolysis among 30% oil nanoemulsions can be related to the particle size of these nanoemulsions when entering the intestinal phase.

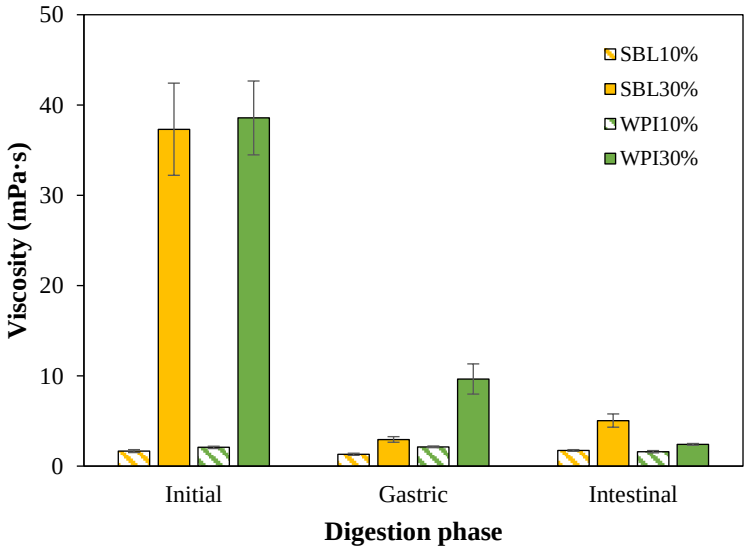

Figure 5. Viscosity of soybean lecithin (SBL) and whey protein isolate (WPI) β-carotene-loaded nanoemulsions at different oil concentrations (10 % and 30 %) during the different phases of the *in vitro* gastrointestinal digestion.

3.2.3. In vitro bioaccessibility

Nanoemulsions formulated with WPI showed a noticeable higher β-carotene bioaccessibility than those formulated with SBL (Figure 6). Specifically, WPI10% presented the highest β-carotene bioaccessibility (≈ 64%), a value which is in accordance with previous studies (Fan et al., 2017; Zhou et al., 2018). The highest bioaccessibility of WPI10% could be related to the high digestibility that presented this nanoemulsion (Figure 4). However, WPI30% presented the same digestibility as SBL30% but a higher bioaccessibility (Figure 6). Previous studies also did not observe a strong correlation between the final level of lipid digestion and compound bioaccessibility using WPI and other emulsifiers such as quillaja saponin or gum arabic (Lv et al., 2019). It seems that the bioaccessibility of lipophilic bioactive compounds is not only influenced by lipolysis but also by other factors such as the degradation of the compound during gastrointestinal digestion. Milk proteins have shown to offer great protection from chemical degradation due to their high free radical scavenging and iron chelation properties (Chen et al., 2018). Specifically, whey proteins may provide high protection against β-carotene degradation during gastrointestinal digestion since, after peptic and trypsin digestion, whey proteins are hydrolysed into peptides with increased antioxidant capacity (Embiriekah et al., 2018). Additionally, β-carotene can be bound by β-lactoglobulin presenting a high affinity to the internal cavity (Mensi et al., 2013), which suggests that WPI could play a transporter role for some carotenoids.

β-carotene bioaccessibility was reduced by increasing the oil concentration from 10% to 30% (Figure 6). In WPI nanoemulsions this fact could be attributed to the higher lipid digestibility of WPI10% in comparison to WPI30%, which may have increased the content of β-carotene incorporated in the mixed micelles. In SBL nanoemulsions, such a decrease in bioaccessibility when increasing the oil concentration could be related to the higher amount of emulsifier present in 30% oil nanoemulsion compared to that with 10% oil. Previous works have reported that the low β-carotene bioaccessibility observed using lysolecithin as emulsifier may be due to the precipitation of β-carotene (Tan et al., 2020). Therefore, in our study it seems that augmenting the amount of SBL in nanoemulsion with 30% of oil could have increased the sedimentation of β-carotene, reducing its bioaccessibility in comparison to nanoemulsion with 10% of oil.

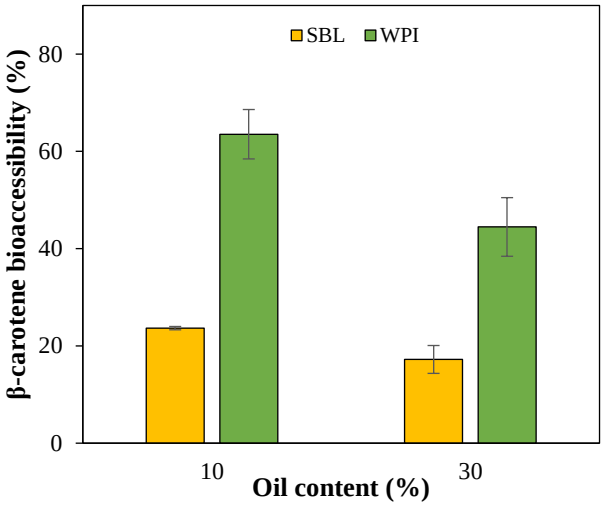

Figure 6. β-carotene bioaccessibility of soybean lecithin (SBL) and whey protein isolate (WPI) β-carotene-loaded nanoemulsions at different oil concentrations (10 % and 30 %).

3.3. In vivo bioavailability

The impact of delivery-system type on the *in vivo* β-carotene and retinol bioavailability was investigated by measuring the β-carotene and retinol plasma profiles after the oral administration of the different β-carotene vehicles (WPI30% nanoemulsion, SBL30% nanoemulsion or control suspension). To perform the *in vivo* study, nanoemulsions with the higher β-carotene content were used to ensure the maximum administration dose. Moreover, some important pharmacokinetic parameters such as C_{max}, T_{max} and $AUC_{0\text{-}8h}$ were studied (Table 2).

As observed in Figure 7a, all plasma samples contained a higher content of retinol than β-carotene. Once β-carotene is absorbed in the intestinal mucosa, it is converted to retinal by β-C 15,15′ oxygenase 1 (BCO1), and the retinal is then reduced to retinol by a retinal reductase (Paik et al., 2001). Moreover, by using nanoemulsions the levels of retinol in rat plasma were greatly improved after the oral administration compared to the control suspension. These results show that, by encapsulating β-carotene in nanoemulsions, the bioavailability of retinol, which is the active form of vitamin A, improved. In fact, the retinol C_{max} of the suspension, SBL30% nanoemulsion and WPI30% nanoemulsion was 164.37 ± 61.78, 394.08 ± 59.83 and 684.58 ± 121.50 $ng \cdot mL^{-1}$, respectively. However, some β-carotene was present in the plasma after the oral administration. As observed in human studies, not all of the β-carotene is converted

to retinol in the intestine, but some part is absorbed as β-carotene form (Blomstrand & Werner, 1967). By using nanoemulsions, the retinol $AUC_{0\text{-}8h}$ was increased by more than 2-fold compared to suspension (Table 2). These results are in accordance with previous studies that have reported a higher bioavailability of β-carotene and also other compounds such as curcumin by encapsulating them in emulsion-based delivery systems (Fan et al., 2017; Nazari-Vanani et al., 2017; Vecchione et al., 2016). On the one hand, such an enhanced bioavailability can be related to the presence of lipid in emulsion-based delivery systems. Indeed, lipids have been reported to stimulate the secretion of bile salts and micelle production, resulting in an increased amount of carotenoids solubilized within micelles. Moreover, lipids may enhance carotenoid absorption by promoting chylomicron secretion, thereby increasing carotenoid secretion out of enterocytes and preventing the intracellular accumulation of the bioactive compound (Boonlao et al., 2022).

Table 2. Pharmacokinetic parameters of retinol and β-carotene in rat plasma after the oral administration of whey protein nanoemulsion (WPI30%), soybean lecithin nanoemulsion (SBL30%) or suspension.

	Delivery system	**C_{max} ($ng \cdot mL^{-1}$)**	**T_{max} (h)**	**$AUC_{0\text{-}8h}$ ($ng \cdot h \cdot mL^{-1}$)**
Retinol	*Suspension*	164.37 ± 61.78	0.5	1087.44
	SBL30%	394.08 ± 59.83	4	2335.02
	WPI30%	684.58 ± 121.50	4	3050.93
β-carotene	*Suspension*	38.70 ± 4.34	2	277.04
	SBL30%	81.52 ± 40.31	4	544.19
	WPI30%	35.25 ± 21.13	4	186.33

Data of C_{max} are expressed as mean ± standard deviation. $AUC_{0\text{-}8h}$ = area under the plasma concentration–time curve from 0 to 8 h; C_{max} = peak concentration; T_{max} = time to reach peak concentration.

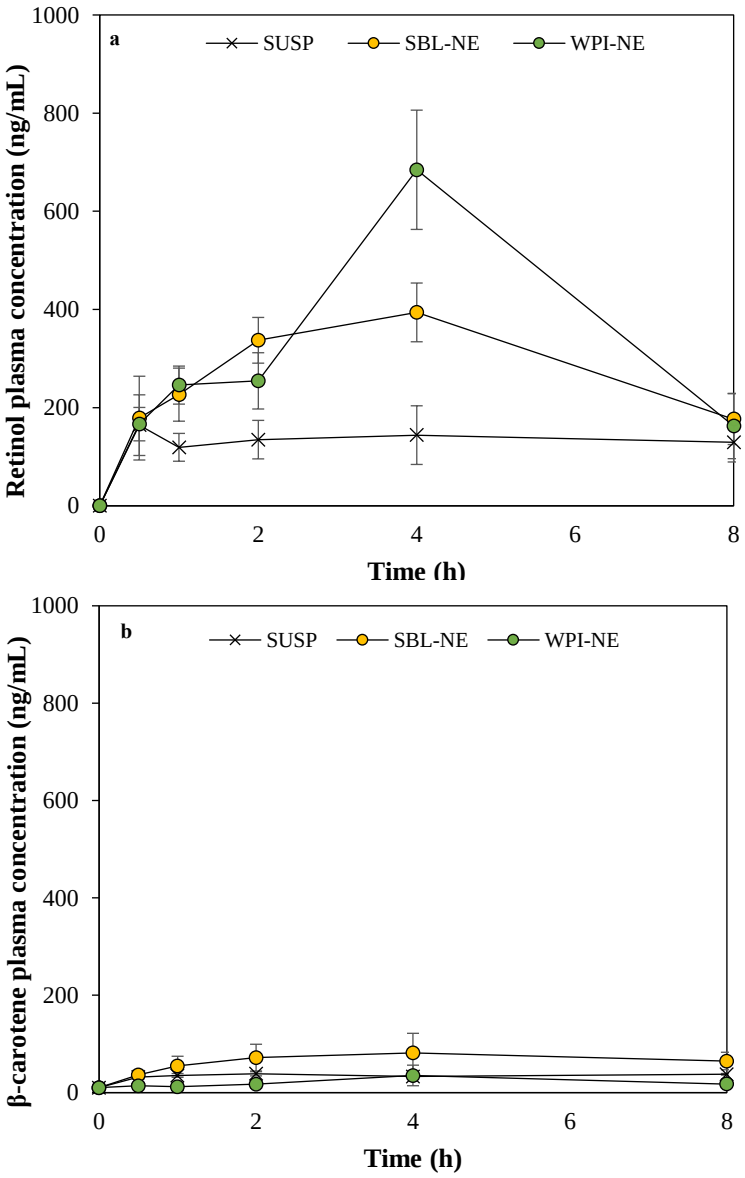

Figure 7. Retinol (a) and β-carotene (b) concentrations in rat plasma during 8 h after a single-dose administration of suspension, soybean lecithin (SBL) or whey protein isolate (WPI) β-carotene-loaded nanoemulsion with 30% oil concentration.

On the other hand, according to previous works, the presence of emulsifiers in nanoemulsions could have reduced the interfacial tension and increased the membrane fluidity enhancing the absorption of the bioactive compound in the intestinal cells (Pan-On et al., 2022).

The T_{max} value was smaller for suspension (0.5 h) than for nanoemulsions (4 h), which indicates that the β-carotene absorption was faster using the suspension (Shargel et al., 2012). The presence of oil in nanoemulsions retarded the compound absorption since it

had to be released from oil droplets and incorporated into mixed micelles before being absorbed. Therefore, C_{max} value was more rapidly achieved in the suspension than in nanoemulsions due to the faster β-carotene absorption, but the values were lower due to the poor absorption in the absence of lipid (Table 2).

Among the nanoemulsions tested, the highest retinol bioavailability was observed in WPI30% nanoemulsion, which is in accordance with the results of β-carotene bioaccessibility observed in this nanoemulsion (Figure 6). As suggested by our *in vitro* results, a higher content of potentially absorbable β-carotene may be present in WPI nanoemulsions than in SBL nanoemulsions, thereby, increasing the bioavailability of the compound. The use of milk proteins has been shown to enhance the bioactive compound bioavailability in nanoemulsions (Niu et al., 2020). The mechanisms are not completely understood, but it is suggested that proteins could participate in the formation of mixed micelles and increase drug absorption. In fact, previous studies have reported the high affinity of β-lactoglobulin for retinol (Mensi et al., 2013). However, by using SBL30% nanoemulsion a higher β-carotene $AUC_{0\text{-}8h}$ than WPI30% nanoemulsion was observed, which were 544.19 $ng \cdot h \cdot mL^{-1}$ and 186.33 $ng \cdot h \cdot mL^{-1}$, respectively. In that sense, we hypothesize that the lower β-carotene values obtained using WPI in comparison to SBL could be a consequence of the faster conversion of β-carotene to retinol using WPI. In addition, the T_{max} of either retinol or β-carotene was the same using both nanoemulsions (Table 2), which indicates that the rate of β-carotene absorption was the same using both emulsifiers.

4. Conclusions

The results obtained in this work showed that the emulsifier used has a strong impact on the nanoemulsion properties, being WPI more adequate to achieve small-particle nanoemulsions than SBL when using high oil concentrations (10-30%). In contrast, the oil concentration seems to have less effect on the nanoemulsions particle size and electrical charge, although it showed a strong impact on the system viscosity.

During the gastric digestion, although both emulsifier-type nanoemulsions showed instability phenomena, in the case of WPI it was reversible flocculation that could be dispersed when entering the intestinal stage. This fact promoted a higher initial digestion

rate in WPI nanoemulsions than in those with SBL. In addition, using WPI greater bioaccessibility values were obtained, which may be related to the protection against the degradation that antioxidant peptides produced during the peptic and tryptic hydrolysis of the protein emulsifier proffered to the compound.

Moreover, *in vivo* bioavailability data obtained in this study is in accordance with the *in vitro* observations. Both WPI and SBL nanoemulsions showed higher retinol levels in plasma than the control suspension after their oral administration. However, WPI nanoemulsion increased the retinol bioavailability to a higher extent than SBL nanoemulsion, which may be related to the higher β-carotene bioaccessibility in the gut. Therefore, WPI seems to be a promising natural emulsifier to increase the bioavailability of retinol.

Declarations of interest

The authors report no declarations of interest.

Acknowledgments

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Chapter IV

Effect of the emulsifier used in *Dunaliella salina*-based nanoemulsions formulation on the β-carotene absorption and metabolism in rats

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Abstract

Microalgae such as *Dunaliella salina* are a potential sustainable source of natural β-carotene due to their fast growth and high adaptability to environmental conditions. This work aimed to evaluate the effect of the incorporation of β-carotene from this alga into different emulsifier-type nanoemulsions (soybean lecithin [SBL], whey protein isolate [WPI], sodium caseinate [SDC]) on its absorption, metabolization and biodistribution in rats. Nanoemulsions formulated with different emulsifiers at 8% concentration were obtained by 5 cycles of microfluidization at 130 mPa, then exposed to an *in vitro* digestion or orally administered to rats. Feeding rats with nanoemulsions improved β-carotene uptake compared to control suspension, especially using SDC and WPI as emulsifiers. A greater presence of β-carotene and retinol in the intestine, plasma and liver was observed, being the liver the tissue that showed the highest accumulation. This fact could be a consequence of the smaller droplets that protein-nanoemulsions presented compared to that with SBL in the intestine of rats, which promoted faster digestibility and higher β-carotene bioaccessibility (35-50% more) according to the *in vitro* observations. Nanoemulsions, especially those formulated with protein emulsifiers, are effective systems for increasing β-carotene absorption, as well as retinol concentration in different rat tissues.

Keywords: β-carotene, bioavailability, *Dunaliella Salina,* microalgae, retinol

1. Introduction

Microalgae are photosynthetic microorganisms with enormous potential to be used as a source of bioactive compounds in functional food production due to their rapid growth under a wide range of environmental conditions (Domínguez, 2013). Moreover, these organisms present some advantages compared with higher plants, such as faster growth, higher yield, and shorter cultivation time, which make them an interesting source of bioactive compounds such as astaxanthin, fucoxanthin, β-carotene, omega-3 fatty acids and polyphenols (Barkia et al., 2019; Gammone et al., 2015; Goiris et al., 2012; Haimeur et al., 2012; Wang et al., 2022). Among them, *Dunaliella salina* is one of the best sources of natural β-carotene, up to 14% of its total dry weight (Harvey & Ben-Amotz, 2020), presenting the 50% of total β-carotene as a 9-cis isomer. Previous studies have reported that the administration of this β-carotene isomer presented a higher tissue accumulation, antioxidant and biological effects than (all-*E*)-β-carotene (Ben-Amotz & Levy, 1996; Relevy et al., 2015). However, despite the higher biological value that β-carotene presents, it is easily degraded due to its highly unsaturated structure (Liu et al., 2016; Zuidam & Nedović, 2010). The incorporation of lipophilic bioactive compounds into oil-in-water nanoemulsions has been shown to be effective to protect them from degradation, facilitate their incorporation in aqueous matrices, and increase their absorption (Chávez-Zamudio et al., 2017; Gasa-Falcon et al., 2019; Lu et al., 2018; Sessa et al., 2014). These systems that consist of a lipid phase dispersed into an aqueous phase usually need an emulsifier for their stabilization, which can be classified according to their nature into proteins, phospholipids, polysaccharides or others.

The election of emulsifier has been shown to highly affect the physicochemical properties of nanoemulsions; moreover *in vitro* studies revealed that it also affects the digestibility and the encapsulated compound bioaccessibility (Gasa-Falcon et al., 2019; Lv et al., 2019; Park et al., 2018). However, *in vivo* studies are required to elucidate the impact of emulsifiers on the oral bioavailability of β-carotene. Since the current consumption trend is towards natural products, the emulsifiers used in this study were different natural ingredients: soybean lecithin (SBL), whey protein (WPI) and sodium caseinate (SDC) (Dammak et al., 2020; McClements & Gumus, 2016; Ozturk & McClements, 2016). SBL has been chosen as a phospholipid-based emulsifier, while

WPI and SDC have been chosen as protein-based emulsifiers.

Previous authors have studied the *in vivo* bioavailability of synthetic β-carotene enclosed in emulsions or nanoemulsions. These studies have concluded that emulsion-based delivery systems can enhance the bioavailability of the compound, and that by reducing the particle size, the absorption and metabolism of β-carotene can be improved (Chen et al., 2021; Yu & Huang, 2012). Nevertheless, as far as we know, there are no studies reporting the bioavailability of β-carotene from a raw material such as alga *D. salina* enclosed in nanoemulsions.

Administration of *Dunaliella* algae species to mice has been shown to be effective in inhibiting atherogenesis and fatty liver formation, showing that the activity was 9-cis dependent (Harari et al., 2008). Other authors reported that carotenoids obtained from an algal source have a higher antihepatotoxic effect in rats compared with synthetic β-carotene and with β-carotene alone extracted from a natural source (Murthy et al., 2005). Moreover, *D. Salina* revealed a significant antifibrotic effect in induced-fibrosis rats via ameliorating the elevation of liver enzymes, inflammatory mediators and fibrotic markers (El-Baz et al., 2020). In addition, β-carotene is transformed into retinol (vitamin A) in the intestine and other organs, which is an essential vitamin with numerous biological functions (Harrison, 2012a)

The study of the biodistribution and accumulation of bioactive compounds in the body is essential, since the biological effectiveness of bioactive compounds is determined by their storage sites. Moreover, the microstructural changes that nanoemulsions undergo in the different parts of the *in vivo* gastrointestinal tract can be of high interest to better understand how nanoemulsion stability is related to the bioavailability of the compound. Therefore, the aim of this work was to study the impact of the emulsifier (SBL, WPI, and SDC) on the *in vitro* and *in vivo* gastrointestinal stability and digestibility of nanoemulsions. In addition, the oral bioavailability and biodistribution of β-carotene extracted from microalgae *D. salina* and its metabolite (retinol) in rats was evaluated.

2. Materials and methods

2.1. Materials

Freeze-dried alga *D. salina* was kindly provided by Monzón Biotech (Huesca, Spain). Pepsin (porcine), pancreatin (porcine), bile extract (bovine), lipase (porcine), Nile red and SDC were obtained from Sigma-Aldrich (St. Louis, MO). Corn oil (Koipesol Asua, Deoleo, Spain) was purchased from a local supermarket. SBL was acquired from Alfa Aesar (Thermo Fisher Scientific, Massachusetts, USA). WPI was kindly provided by El Pastoret de la Segarra, S.L. (Spain).

2.2 Methods

2.2.1. Preparation of β-carotene enriched oil and β-carotene suspension

To obtain the β-carotene enriched oil, alga *D. salina* was mixed with corn oil at 65 ºC (0.4 g alga·mL^{-1} oil) and vortexed at 3000 rpm for 1 min. The mixture was treated with an Ultra-Turrax at 17500 rpm for 2 min (IKA, Staufen, Germany) and submerged in a sonication bath for 5 min. Finally, the mixture was centrifuged at 9000 rpm for 15 min and the upper part was collected as the oil phase. The final concentration was 20 mg β-carotene/g oil, quantified by HPLC using the methodology described in Subsection 2.2.6. To obtain the β-carotene suspension, the alga *D. salina* was mixed with water at 65 ºC (0.4 g alga·mL^{-1} oil) and vortexed at 3000 rpm for 1 min. The mixture was treated with an Ultra-Turrax at 17500 rpm for 2 min (IKA, Staufen, Germany) and submerged in a sonication bath for 5 min.

2.2.2. Nanoemulsion preparation

To obtain the aqueous phase, 8% w/w of SBL, WPI or SDC were added into ultrapure water and stirred for 4 h at room temperature. Then, 20% w/w of enriched β-carotene oil and 80% w/w of aqueous phase were mixed and homogenized using an Ultra-Turrax (IKA, Staufen, Germany) at 11000 rpm for 2 min. Finally, nanoemulsions were obtained by passing coarse emulsions through a microfluidizer (LM10, Microfluidics, USA) at 130 MPa for 5 cycles. All formulated nanoemulsions presented a pH ≈ 6.

2.2.3. Nanoemulsion characterization

Particle size. The particle size of nanoemulsions was measured using a Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). Samples were diluted in ultrapure water and stirred in the dispersion unit with a constant speed of 1800 rpm. The mean particle size was expressed as surface area mean diameter (d_{32}) in micrometres (μm), fixing a refractive index of the corn oil of 1.473 and 1.333 for water.

ζ-potential. The ζ-potential was measured by phase-analysis light scattering (PALS) using Zetasizer laser diffractometer (NanoZS Malvern Instruments Ltd. Worcestershire, UK). Prior to the analysis, nanoemulsions were diluted (1:100) in ultrapure water. Gastric and intestinal samples were diluted maintaining its pH at 3 and 7, respectively. Then, the diluted samples were placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles. The results were reported in millivolts (mV).

Viscosity. Apparent viscosity of nanoemulsions was determined using a SV-10 vibro-viscometer (A&D Company, Tokyo, Japan), which produces a vibration of 30 Hz and a constant amplitude of 0.4 mm at controlled room temperature. The results were expressed in mPa·s.

2.2.4. In vitro study

2.2.4.1. Gastrointestinal digestion

To simulate the human digestion process, an *in vitro* gastrointestinal tract (GIT) digestion based on an international consensus method (Brodkorb et al., 2019; Minekus et al., 2014) with some modifications was used.

To perform the gastric phase, 20 mL of nanoemulsion was mixed 1:1 with simulated gastric fluids containing pepsin (2000 U/mL) and 10 μL of a $CaCl_2$ solution (0.3 M). Then, the pH was adjusted to 3 using HCl (1M) and the mixture was placed into an incubator at 37 °C for 2 h while shaking at 100 rpm. To simulate the intestinal phase, a pH-stat device was used. Once the gastric phase was completed, an aliquot of 20 mL of gastric sample was placed in a 37 °C water bath and mixed 1:1 with simulated intestinal

fluids containing 10 mM bile solution. Then, the pH was adjusted to 7 with NaOH (1 M) and pancreatin and lipase enzymes were added to the final mixture to achieve 100 U mL^{-1} of trypsin and 2000 U/mL pancreatic lipase. The pH of the sample was maintained to 7 by adding NaOH (0.25 M) constantly for 2 h. The free fatty acids (FFA) release was determined according to Eq. (1):

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

where V_{NaOH} is NaOH volume (L) used during the intestinal digestion, C_{NaOH} is NaOH molarity (0.25 $mol \cdot L^{-1}$), M_{oil} is corn oil molecular weight (800 $g \cdot mol^{-1}$) and m_{oil} is the weight of the oil phase present in the intestinal digestion phase (g).

2.2.4.2. In vitro bioaccessibility

Digested nanoemulsions were centrifuged at 4000 rpm during 40 min at 4 ºC to obtain the micellar fraction. Then, an extraction and quantification of the β-carotene content was performed in both the initial nanoemulsion and micellar fraction following the method described in section 2.2.6. Finally, β-carotene bioaccessibility was calculated according to Eq. (2):

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

where $C_{micelle}$ is the β-carotene concentration ($mg \cdot mL^{-1}$) in the micellar fraction and $C_{initial}$ is the initial β-carotene concentration ($mg \cdot mL^{-1}$) in the nanoemulsion.

2.2.5 In vivo study

2.2.5.1. Animals and study design

Female Sprague Dawley rats weighing 200-250 g were used for the *in vivo* study. The animal procedures were conducted in accordance with EU Directive 2010/63/EU for animal experiments and approved by the Animal Ethics Committee of Universitat de Lleida (CEEA 01-04/18). After an acclimatization period, animals were randomly divided into four groups (n=5) (suspension, SBL nanoemulsion, WPI nanoemulsion and SDC nanoemulsion). Rats were fasted for 12 h before the experiment with free access

to water. Rats were anesthetised with isoflurane and blood samples were taken by cardiac puncture. Plasma was immediately separated by centrifuging the blood samples at 4000 rpm for 10 min at 4 ºC and stored at -80 ºC. Rats were sacrificed by exsanguination and the stomach, duodenum, jejunum, ileum and colon were removed and their content was collected and immediately characterized by microscopy. Then, the mentioned tissues, liver, kidney, white adipose tissue (mesenteric) and brown adipose tissue (cervical) were collected, rinsed with phosphate buffered saline, weighted and stored at -80 ºC.

2.2.5.2. Dosage information

Rats were orally administered at a volume of 20 $mL \cdot kg^{-1}$ with a feeding needle. All animals were fed with a dose of 60 mg of β-carotene per kilogram body weight, irrespective of the vehicle used, which is only achievable through supplements in humans. Control suspension consisted on the β-carotene from alga diluted in water. Sample administration was conducted in five times with 30 min intervals. Rats were sacrificed thirty minutes after the last administration.

2.2.6. Carotenoid extraction and quantification

Nanoemulsions and micellar samples. β-carotene extraction was performed according to a previously reported method with some modifications (Zhou et al., 2018). Aliquots of 1 mL of sample were mixed with 2 mL of ethanol/hexane (2:3 v/v), vortexed at 1500 rpm for 1 min, and centrifuged at 4000 rpm for 5 min at 4 ºC. Afterwards, the collected upper organic layers were collected and evaporated under N_2 and stored at -40 ºC. The quantification of β-carotene was carried out using an HPLC system equipped with a 600 Controller and a diode array detector (Waters, Milford, MA) following the method described by Vallverdú-Queralt et al. (2013). β-carotene was identified using C30 column 250 x 4.6 mm i.d, 5 µm (Bischoff Chromatography, Leonberg, Germany) at room temperature. The injection volume was 20 µL and the flow rate was 1 $mL \cdot min^{-1}$. Separation was carried out in 23 min under the following conditions: 0 min, 70% B; 10 min, 20% B; 20 min, 6% B; 21 min, 6% B; 23 min, 70% B. Water was kept constant at 4%. A β-carotene standard was used to identify analytes by retention times and ultraviolet–visible spectra. The HPLC–UV chromatograms were acquired, selecting the 450 nm wavelength.

Plasma and tissue samples. Retinol and β-carotene extraction was performed in a dark room to avoid compound degradation, following a previously reported method with some modifications (Fan, et al., 2017). For plasma samples, 150 µL were mixed with the internal standard trans-β-apo-8′-carotenal and 600 µL of ethanol/hexane (1:2 v/v) containing 0.1% BHT. Then, the mixture was vortexed at 1000 rpm for 1 min, centrifuged at 9000 rpm for 5 min at 4 ºC, and the organic fraction was collected. Afterwards, the collected upper organic layers were collected and evaporated under N_2 and stored at -80 ºC. For tissue samples, 250 mg of tissue was mixed with 600 µL of milli-Q water and homogenized using an Ultra-Turrax at 9000 rpm for 1 min. After that, tissue homogenates were mixed with the internal standard trans-β-apo-8′-carotenal and 1000 µL of ethanol/hexane (1:2 v/v) containing 0.1% BHT, and the same procedure as for plasma samples was followed. The quantification of β-carotene and retinol from plasma and tissue samples was performed using a Acquity UPLC with a photodiode array detector from Waters (Milford, MA, USA) equipped with a binary solvent delivery system. The analysis was performed using a reverse-phase C_{18} column (ACQUITY UPLC® BEH 1.7µm 150 x 2.1 mm) kept at 32 ºC. The volume injected was 7.5 µL and the mobile phase consisted of a gradient of (A) acetonitrile: methanol (70:30 v/v) and (B) water: acetonitrile (95:5 v/v). The flow rate was 1 $mL \cdot min^{-1}$. The gradient profile of the mobile phase was set at 90% A and increased linearly to 100% A over 5.5 min with a 6.5-min hold, after which the mobile phase was changed back to 90% A over 2 min and the held for 2 min. β-carotene was detected at 450 nm and retinol at 325 nm.

2.2.7. Fluorescence optical microscopy

Initial nanoemulsions, nanoemulsions at the different phases of *in vitro* digestion, and nanoemulsions from the different gastrointestinal tract of rats were collected and dyed with Nile Red, previously dissolved at 0.1% (w/v) in ethanol. Then, micrographs were obtained using fluorescence with an optical microscope (Olympus BX41, Olympus, Göttingen, Germany) with a 100x objective lens. The images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus Göttingen, Germany).

2.2.8. Statistical analysis

All experiments were assayed in duplicate and three repetitions of each analysis were carried out on each parameter in order to obtain mean values. Analysis of the variance (ANOVA) was performed to compare treatments. Least significant difference (LSD) test was employed to determine differences between means. The confidence interval was set at 0.95 and all results were analyzed using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, MD, USA).

3. Results and discussion

3.1. Characteristics of nanoemulsions

The electrical charge of all nanoemulsions was negative being about – 48 mV in both SBL and SDC nanoemulsions and about -35 mV in that with WPI (Table 1). The negative electrical charge of the phospholipid (SBL) nanoemulsion was due to the negatively charged phosphate groups at the pH of the nanoemulsions (≈ 6) (Artiga-Artigas et al., 2018). In protein-based nanoemulsions (those containing WPI or SDC) the negative ζ-potential was because the pH was above the pI of the proteins (≈ 5), so the ionizable groups were deprotonated and the net charge was negative (Hu et al., 2003; Surh et al., 2006). The smaller mean particle size values of protein-based nanoemulsions (≈ 0.250 μm) in comparison to that with the phospholipid (≈ 0.380 μm), may be attributed to the higher capacity of high weight emulsifiers (such as WPI or SDC) to form a viscoelastic film surrounding the droplets than low weight emulsifiers like SBL (Fuentes et al., 2021). As can be observed in the microscope images (Figure 1), protein-based nanoemulsions presented aggregated droplets and a polydisperse distribution (Figure 2). In contrast, nanoemulsion formulated using the phospholipid showed bigger but well dispersed droplets than protein-based nanoemulsions, and therefore, a less polydisperse distribution (Figure 2).

Table 1. Characteristics of soybean lecithin (SBL), whey protein isolate (WPI) and sodium caseinate (SDC) β-carotene-loaded nanoemulsions during the different stages of the gastrointestinal *in vitro* digestion.

	Emulsifier	**Particle size (μm)**	**ζ-potential (mV)**	**Viscosity (mPa·s)**
Initial	*SBL*	0.377 ± 0.015 ^{Ac}	- 49.90 ± 2.71 ^{Aa}	3.96 ± 0.35 ^{Ba}
	WPI	0.262 ± 0.003 ^{Ab}	-35.07 ± 0.90 ^{Ac}	7.28 ± 1.97 ^{Ca}
	SDC	0.232 ± 0.009 ^{Aa}	- 47.21 ± 1.39 ^{Ab}	50.0 ± 6.9 ^{Bb}
Gastric	*SBL*	11.62 ± 0.64 ^{Cb}	-21.07 ± 1.48 ^{Ca}	2.88 ± 0.67 ^{Aa}
	WPI	1.70 ± 0.31 ^{Ba}	13.43 ± 0.77 ^{Cb}	4.55 ± 1.20 ^{Bb}
	SDC	12.48 ± 0.53 ^{Cc}	14.90 ± 1.30 ^{Cc}	5.62 ± 0.20 ^{Ac}
Intestinal	*SBL*	3.10 ± 0.15 ^{Bc}	-33.25 ± 1.97 ^{Ba}	2.56 ± 0.32 ^{Ab}
	WPI	0.39 ± 0.02 ^{Aa}	-33.41 ± 1.79 ^{Ba}	1.75 ± 0.04 ^{Aa}
	SDC	0.66 ± 0.06 ^{Bb}	-27.73 ± 1.11 ^{Bb}	1.54 ± 0.02 ^{Aa}

Values are expressed as mean ± standard deviation. Different capital letters indicate significant differences ($p<0.05$) between different digestion phases of the same nanoemulsion. Different lowercase letters indicate significant differences ($p<0.05$) between nanoemulsions with different emulsifier.

Flocculation observed in protein-based nanoemulsions may be a consequence of the strong hydrophobic attraction generated between protein-coated droplets promoted by the increased surface hydrophobicity caused by the conformational changes that proteins suffer when they are adsorbed at the interface (McClements & Gumus, 2016). Phospholipid-based nanoemulsion showed the lowest viscosity, followed by that formulated using WPI, being about 4 and 7 mPa·s, respectively. SDC nanoemulsion showed a significantly higher viscosity than the other nanoemulsions, which was about 50 mPa·s. Previous authors have also reported a relatively higher viscosity using SDC rather than other emulsifiers like SBL (Gasa-Falcon et al., 2019). Such a high viscosity obtained using sodium caseinate may be a consequence of the formation of star-like aggregates that jam in dense aqueous suspensions and lead to a sharp increase of the viscosity (Pitkowski et al., 2008).

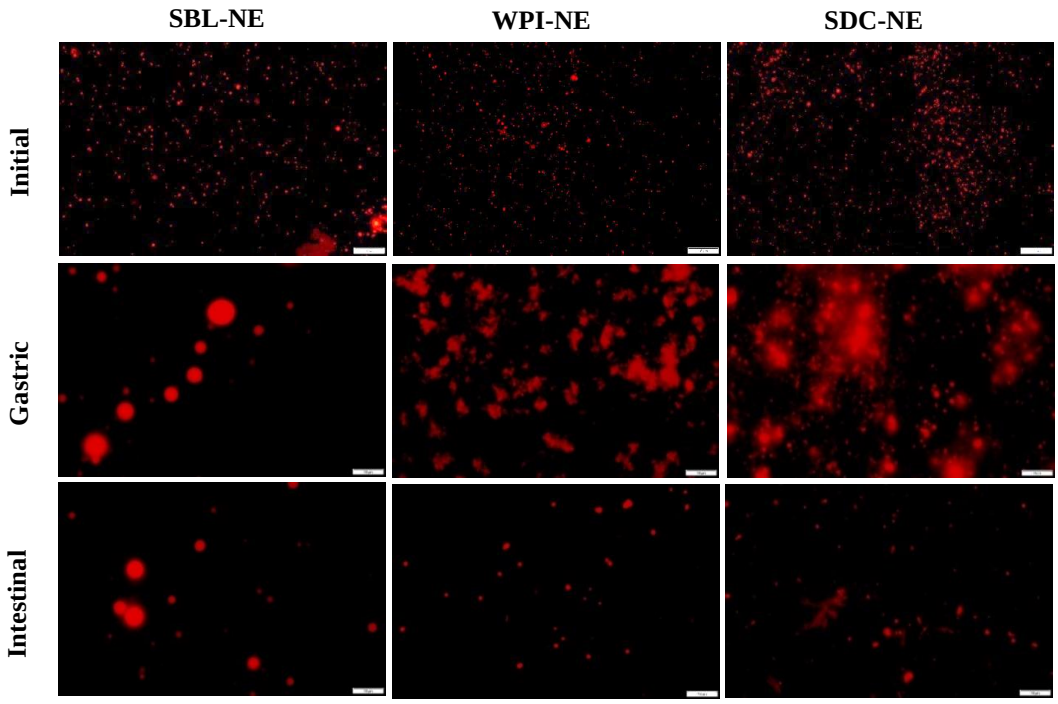

Figure 1. Micrographs of soybean lecithin (SBL), whey protein isolate (WPI) and sodium caseinate (SDC) β-carotene nanoemulsions during the different phases of the *in vitro* gastrointestinal digestion. Scale bars are 10 μm long.

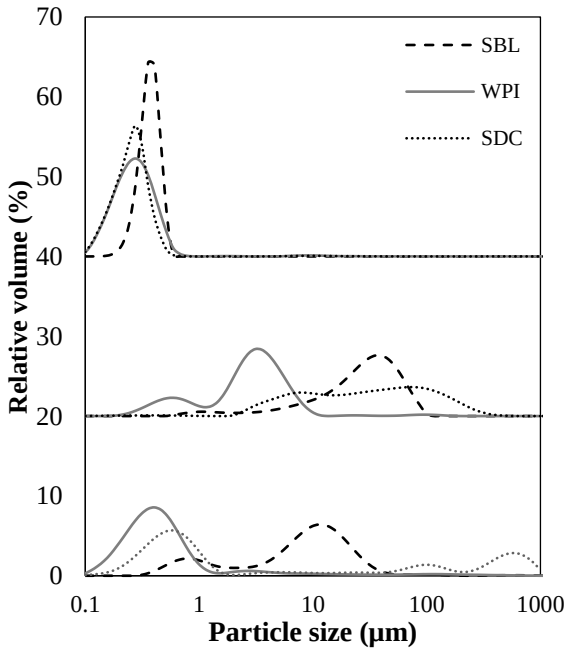

Figure 2. Particle size distribution of soybean lecithin (SBL), whey protein isolate (WPI) and sodium caseinate (SDC) β-carotene nanoemulsions.

3.2. *In vitro* gastrointestinal digestion of β-carotene nanoemulsions

Nanoemulsions formulated with a protein emulsifier (WPI or SDC) presented a higher initial rate of FFA release than that formulated with a phospholipid (SBL), although no differences were observed in the final extent (≈ 43%) (Figure 3). These results evidenced that the election of emulsifier has an impact on the digestibility of nanoemulsions and are in agreement with previous works (Mun et al., 2007). The differences in the initial rate of FFA release observed between protein- and phospholipid-based nanoemulsions may be attributed to the particle size presented during gastrointestinal digestion. When entering the intestine, protein-based nanoemulsions presented small particle sizes due to the redispersion of the gastric aggregates as a consequence of the electrostatic stabilization (Mantovani et al., 2013) (Table 1 and Figure 1). Such small particle sizes increased the surface area of lipid exposed to lipase and facilitate lipid digestion (Salvia-Trujillo et al., 2013). Conversely, the phospholipid-based nanoemulsion presented a higher particle size due to the coalescence phenomenon (Figure 1), and therefore, a lower surface area and reduced access of lipase to the lipid substrate. Moreover, protein-based emulsifiers presented a lower surface activity than the phospholipid, so they were more likely to be partially digested by pepsin. Thus, protein nanoemulsions may present a relatively weak interfacial layer and facilitate absorption of bile salts and or/lipase (Lv et al., 2019).

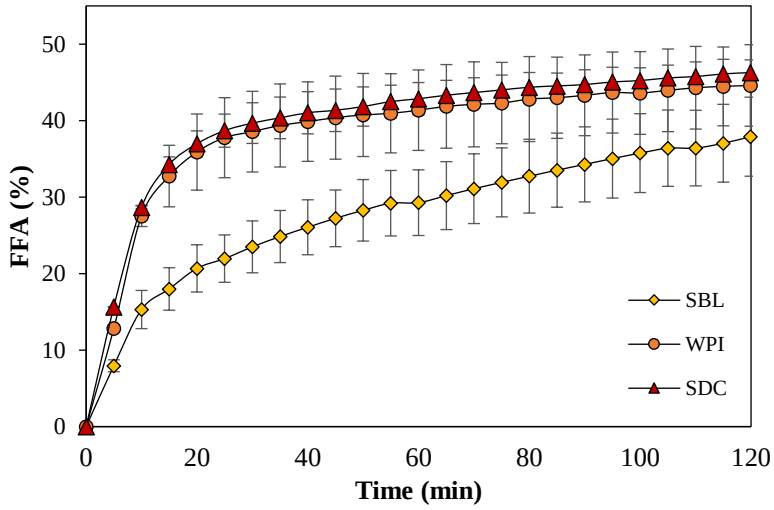

Figure 3. *In vitro* lipid digestibility of soybean lecithin (SBL), whey protein isolate (WPI) and sodium caseinate (SDC) β-carotene nanoemulsions.

The highest β-carotene bioaccessibility was found in WPI nanoemulsion (≈ 68.5%), followed by SDC nanoemulsion (≈ 55.5%), and SBL nanoemulsion (≈ 20%) (Figure 4). These results highlight that the emulsifier nature significantly impacted the bioaccessibility of β-carotene, although all nanoemulsions presented the same final lipid digestibility (Figure 3), as observed previously by other authors (Gasa-Falcon et al., 2019). Moreover, the results obtained in this work evidenced that the incorporation of β-carotene into nanoemulsions can greatly enhance its bioaccessibility. Non-encapsulated β-carotene has been reported to present a very low stability under gastrointestinal conditions, being mostly degraded during its pass through the gastrointestinal tract (Blanquet-Diot et al., 2009).

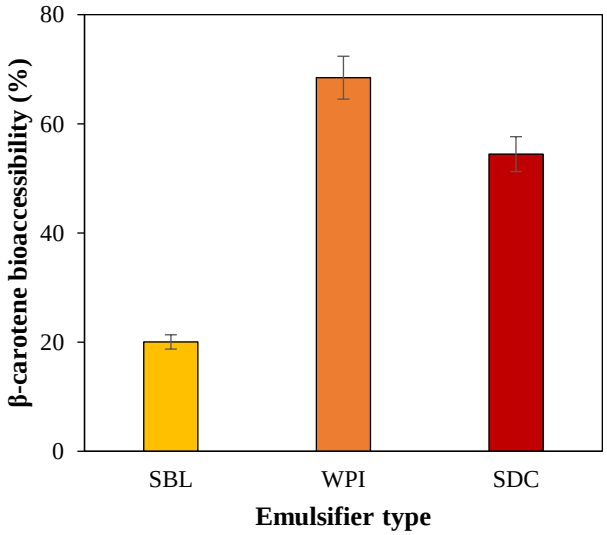

Figure 4. *In vitro* β-carotene bioaccessibility of soybean lecithin (SBL), whey protein isolate (WPI) and sodium caseinate (SDC) nanoemulsions.

The higher β-carotene bioaccessibility achieved using WPI or SDC compared to SBL could be a consequence of the antioxidant activity provided by the peptides resulting from the protein digestion (Embiriekah et al., 2018). In fact, previous studies have also reported a high β-carotene bioaccessibility using WPI as emulsifier of nanoemulsions (Fan et al., 2017). Moreover, the higher β-carotene bioaccessibility of WPI compared to SDC nanoemulsions despite the same FFA release profile (Figure 3 and 4) could be due that WPI can better inhibit lipid oxidation than SDC (Qiu et al., 2015). The affinity for iron of phosphate groups of casein molecules is greater and stronger than that of carboxylate groups present in other proteins (Sugiarto et al., 2009). Thus, SDC can

promote lipid oxidation due to the chelating properties and electrostatic interactions of adsorbed casein molecules that favour the positioning of transition metal ions on the surfaces (Villiere et al., 2005). In contrast, WPI has a more compact structure and a lack of phosphate groups, which could limit the ability to bind iron, avoid oxidation (Guzun-Cojocaru et al., 2011) and prevent lipid oxidation by inactivating peroxyl radicals (Osborn & Akoh, 2004; Tong et al., 2000). Finally, the markedly reduced bioaccessibility observed using the phospholipid-based nanoemulsion may be attributed to the low capacity of the phospholipid emulsifier to inhibit oxidation. In addition, previous authors have observed reduced β-carotene bioaccessibility by using phospholipid-based emulsifiers such as soy lysolecithin due to the sedimentation of the compound during the centrifugation (Tan et al., 2020).

3.3. *In vivo* bioavailability

3.3.1. Microstructural changes in rat digesta

The microstructure of the SBL, SDC and WPI nanoemulsions in the digesta of the different regions of the gastrointestinal tracts (stomach, duodenum, jejunum, ileum and colon) of the rats were studied by analysing the digesta of the different regions post-mortem, using an optical microscopy (Figure 5). Microscope images of digesta from rats fed with the suspension were also taken as a control.

In the gastric digesta, protein-based nanoemulsions presented aggregation of droplets, while phospholipid-based nanoemulsion showed droplet coalescence (Figure 5). Other *in vivo* studies also showed flocculation in the stomach of rats using nanoemulsions containing SDC as emulsifier (Yao et al., 2021). In addition, these observations are consistent with the *in vitro* results from the present study (Figure 1). These instability phenomena may be promoted by the reduction in the ζ-potential of nanoemulsions (Table 1).

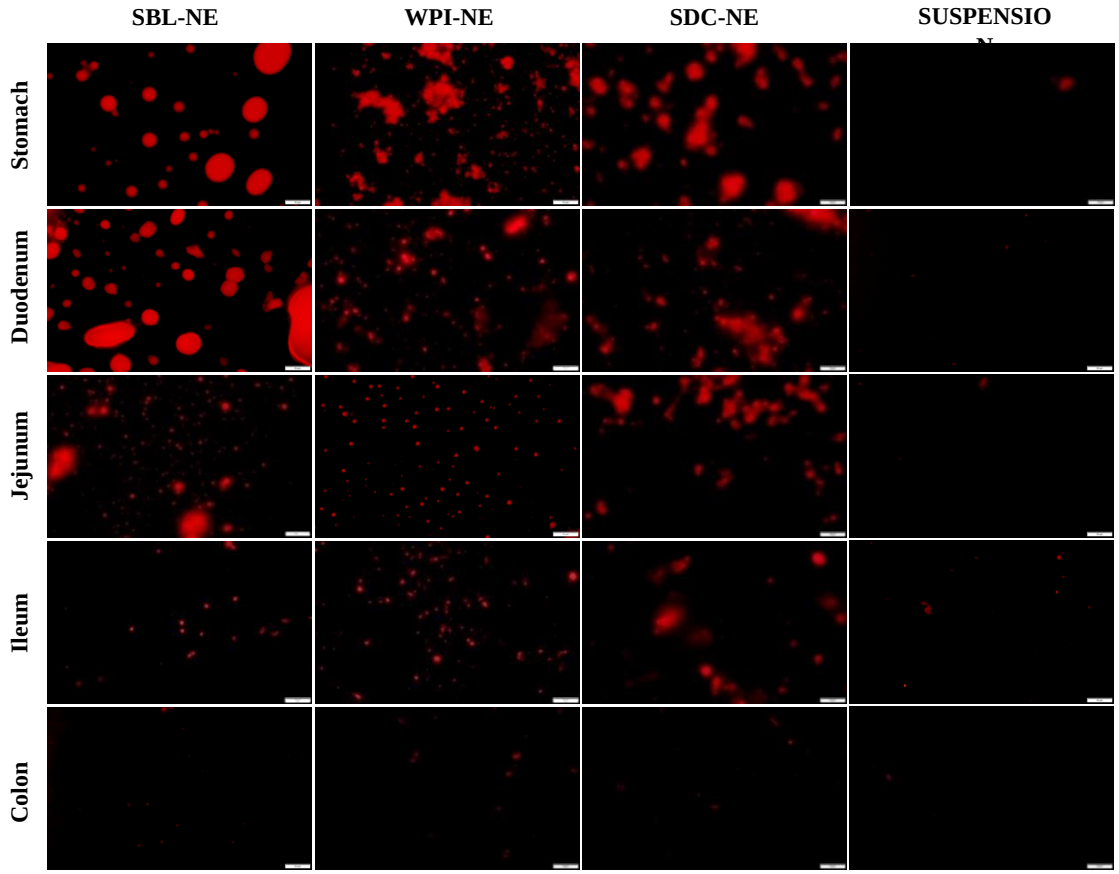

Figure 5. Micrographs of suspension, soybean lecithin (SBL), whey protein isolate (WPI) and sodium caseinate (SDC) β-carotene nanoemulsions at the different parts of the gastrointestinal tract of the rats after oral administration. Scale bars are 10 μm long.

Duodenum digesta microstructure of rats was also different depending on the emulsifier used. Microscope images showed that nanoemulsion containing the phospholipid presented higher particle sizes than those formulated using proteins. Similarly, to *in vitro* observations, aggregated droplets of protein-based nanoemulsions were dispersed in the duodenum due to the electrostatic stabilization promoted by an increase of the ζ-potential (Table 1 and Figure 1). In fact, previous authors (Yao et al., 2021) observed the same effect in rats fed with nanoemulsions containing SDC as emulsifier at different oil concentrations. In contrast, the particle size of phospholipid-based nanoemulsion increased as a result of droplet coalescence. As the nanoemulsions progressed throughout the distal parts of the small intestine, fewer and smaller oil droplets were observed (Figure 5). This indicates that oil droplets were successfully digested and absorbed through the enterocytes of the intestinal mucosa by being incorporated into the mixed micelles (Goncalves et al., 2015). Even so, in the jejunum digesta of rats fed with

phospholipid-based nanoemulsion were still larger droplets than in those of rats fed with protein-based nanoemulsions (Figure 5), which may be attributed to the presence of lipid droplets, micelles and vesicles (Yao et al., 2021). It seems that nanoemulsion containing the phospholipid was digested more slowly than protein nanoemulsions, as can be seen in Figure 3. At the end of the gastrointestinal digestion, very few lipid droplets were observed in the colon of rats, irrespective of the emulsifier used. This indicates that all nanoemulsions were successfully digested and lipids were absorbed.

3.3.2. Retinol and β-carotene concentration in rat plasma and tissues

β-carotene is absorbed in the intestinal cells (enterocytes) mainly via passive diffusion or through facilitated transport via scavenger receptor class B member 1 (SR-BI) and possibly other lipid transporters, such as CD36 (Rodriguez-Concepcion et al., 2018). As can be observed in Table 2, the β-carotene concentration in the duodenum was greatly increased when the bioactive compound was enclosed in nanoemulsion (≈ 1289-2000 $ng \cdot g^{-1}$) rather than administered in suspension (≈ 125 $ng \cdot g^{-1}$). The presence of oil in nanoemulsions could have enhanced the absorption of β-carotene, since the co-ingestion of carotenoids with lipids has been reported to enhance its absorption (Brown et al., 2004; Goltz et al., 2012). Moreover, according to previous works, the presence of emulsifiers could have reduced the interfacial tension and increased the membrane fluidity enhancing the absorption of the bioactive compounds in the intestinal cells (Pan-On et al., 2022). Higher absorption was detected in rats fed with protein-based nanoemulsions than those fed with phospholipid-based nanoemulsion. Indeed, these results are in accordance with our *in vitro* observations, in which the bioaccessibility was higher using WPI or SDC rather than SBL (Figure 4). As mentioned in the previous section, this fact can be attributed to the higher capacity of protein emulsifiers than phospholipids to prevent β-carotene degradation during the gastrointestinal tract and increase the bioaccessibility of the compound. In fact, some authors have reported that the presence of proteins such as WPI or SDC can increase the solubilisation and transition of β-carotene into the mixed micelles (Iddir et al., 2021, 2022).

Table 2. β-carotene concentration in different tissue samples and plasma of rats after oral administration of β-carotene suspension (SUSP), soybean lecithin β-carotene-loaded nanoemulsion (SBL-NE), whey protein isolate β-carotene-loaded nanoemulsion (WPI-NE) or sodium caseinate β-carotene-loaded nanoemulsion (SDC-NE).

	β-carotene (ng/g tissue o mL plasma)			
	SUSP	**SBL-NE**	**SDC-NE**	**WPI-NE**
Duodenum	125.2 ± 69.0 ^{a}	1289.0 ± 565.5 ^{b}	1903.9 ± 759.0 ^{bc}	2088.6 ± 621.9 ^{c}
Jejunum	1724.5 ± 1599.6 ^{a}	6742.6 ± 1924.6 ^{ab}	7175.9 ± 5061.8 ^{b}	14200.4 ± 4453.0 ^{c}
Ileum	1599.6 ± 1829.1 ^{a}	1737.0 ± 784.2 ^{a}	3404.9 ± 293.6 ^{b}	8381.5 ± 1390.3 ^{c}
Colon	105.7 ± 80.8 ^{ab}	148.5 ± 92.9 ^{ab}	182.2 ± 36.9 ^{ab}	96.7 ± 15.6 ^{a}
Liver	64.1 ± 43.8 ^{a}	1853.2 ± 292.1 ^{b}	1684.7 ± 636.4 ^{b}	1760.7 ± 408.4 ^{b}
Kidney	ND	ND	ND	ND
White adipose	ND	ND	ND	ND
Brown adipose	ND	ND	ND	ND
Plasma	42.9 ± 29.9 ^{a}	1410.0 ± 344.9 ^{b}	1259.7 ± 224.4 ^{b}	1201.0 ± 108.4 ^{b}

Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p<0.05$) between vehicles.

Once absorbed in the intestine, β-carotene is mainly cleaved to retinol and other metabolites by the β-carotene-15,15'-oxygenase (BCO1) but also through β-carotene-9',10'-oxygenase (BCO2) enzyme in the intestinal cells, where rats present the highest activity of both enzymes (Harrison, 2012). In fact, this could be the reason why β-carotene could have not been quantified in some tissues such as the kidney, white adipose and brown adipose tissue of rats (Table 2). However, although rodents are very efficient in cleavage of β-carotene to retinol in the intestine, it is possible to detect circulating β-carotene when it is administered in supraphysiological amounts (Van Vliet, 1996), as is the case of the present study, where β-carotene was detected in plasma and liver tissues (Table 2), being the latter one of the most important sites of β-carotene accumulation (Shete & Quadro, 2013).

Table 3 shows that the orally administered β-carotene was conversed to retinol and was found mainly in the latter form in rat tissues. Higher retinol concentrations in rats administered with nanoemulsions than in those fed with the suspension were observed (Table 3), as a consequence of the greater β-carotene absorption using nanoemulsions rather than the suspension (Table 2). Again, differences were observed depending on the emulsifier used, being SDC and WPI nanoemulsions the systems that promoted the

highest retinol concentrations, especially in the duodenum, liver and kidney of rats (Table 3). This fact can be attributed to the presence of higher levels of β-carotene in the intestinal cells of rats fed with SDC or WPI nanoemulsions rather than with SBL nanoemulsion (Table 2). However, although adipose tissue is an important site of β-carotene accumulation in humans, it was not detected in rats in this study (Table 2). In fact, previous studies have also failed to detect this compound in rat adipose tissue after administration, even after chronic feeding (Lakshman et al., 1989; Parker, 1988). In contrast, retinol was quantified in the adipose tissue of rats, although no differences were detected among vehicles (Table 3). The presence of retinol in rat adipocytes could be related to the presence of the retinol-binding protein (RBP) in these cells.

Table 3. Retinol concentration in different tissue samples and plasma of rats after oral administration of β-carotene suspension (SUSP), soybean lecithin β-carotene-loaded nanoemulsion (SBL-NE), whey protein isolate β-carotene-loaded nanoemulsion (WPI-NE) or sodium caseinate β-carotene-loaded nanoemulsion (SDC-NE).

	Retinol (ng/g tissue o mL plasma)			
	SUSP	**SBL-NE**	**SDC-NE**	**WPI-NE**
Duodenum	147.8 ± 43.4 ^{a}	123.8 ± 48.7 ^{a}	250.6 ± 70.8 ^{b}	238.6 ± 45.2 ^{b}
Jejunum	58.7 ± 54.7 ^{a}	204.0 ± 73.7 ^{b}	248.7 ± 65.3 ^{b}	312.5 ± 169.3 ^{b}
Ileum	217.5 ± 104.2 ^{a}	280.1 ± 91.9 ^{a}	262.8 ± 85.1 ^{a}	263.3 ± 77.5 ^{a}
Colon	56.3 ± 17.4 ^{a}	62.4 ± 27.6 ^{a}	87.2 ± 27.8 ^{a}	51.4 ± 42.4 ^{a}
Liver	745.8 ± 514.3 ^{a}	1825.0 ± 355.7 ^{b}	1962.3 ± 690.9 ^{b}	3253.5 ± 645.2 ^{c}
Kidney	634.3 ± 294.3 ^{a}	821.4 ± 324.7 ^{a}	1242.8 ± 305.8 ^{b}	2324.0 ± 507.2 ^{c}
White adipose	212.8 ± 22.9 ^{ab}	225.1 ± 48.8 ^{b}	123.8 ± 36.4 ^{a}	189.5 ± 82.7 ^{ab}
Brown adipose	214.1 ± 93.0 ^{a}	189.3 ± 51.7 ^{a}	234.6 ± 54.2 ^{a}	249.7 ± 78.9 ^{a}
Plasma	104.5 ± 49.9 ^{a}	232.7 ± 33.3 ^{b}	369.5 ± 62.0 ^{c}	473.2 ± 23.0 ^{d}

Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$) between vehicles.

Interestingly, although retinol and β-carotene concentrations in the liver and plasma were higher using nanoemulsions than the suspension, they showed different trends depending on the vehicle used. Feeding the rats with the suspension, retinol concentrations were higher than those of β-carotene in these tissues. In contrast, using nanoemulsions, a higher concentration of β-carotene rather than retinol was observed in the plasma of rats and similar values were detected in the liver (Tables 2 and 3). This suggests that the metabolism of β-carotene could be different when enclosed in

nanoemulsions than in suspension. Although β-carotene is usually cleaved in the intestine, it can also follow another pathway. Intact β-carotene can escape the intestinal cleavage, be incorporated into chylomicrons and released by exocytosis to the lymphatic system for delivery to the bloodstream and eventually directed to the liver (Rodriguez-Concepcion et al., 2018). Therefore, it seems that by using the suspension, the β-carotene is more likely to be cleaved into retinol in the intestinal cells, whereas the use of nanoemulsions appears to promote the lymphatic pathway, therefore, showing high levels of intact β-carotene in the plasma and liver (Table 2). Once β-carotene arrives in the liver, it can be cleaved into retinol since this organ also contains BCO1 and BCO2 enzymes, although the activity that they present in this tissue is reduced compared to that in the intestinal tissue (Harrison, 2009). In fact, the liver was the tissue that presented the highest retinol accumulation, followed by the kidney (Table 3).

4. Concluding remarks

All studied emulsifier-type nanoemulsions showed to be effective to encapsulate and protect β-carotene extracted from alga *D. salina*. Feeding rats with nanoemulsions increased the intestinal absorption of β-carotene, increasing the concentration of the compound in the enterocytes compared to the suspension. Moreover, the retinol concentration was higher using nanoemulsions than the suspension, both in the intestine and in the liver, kidney and plasma. Among the nanoemulsions studied, those formulated with protein emulsifiers (WPI or SDC), promoted a higher concentration of β-carotene in the intestine of rats rather than that with the phospholipid (SBL). Indeed, by administering nanoemulsions formulated with WPI as emulsifier, the highest concentration of retinol was detected in plasma, duodenum, liver, and kidney. The low efficiency of SBL to enhance β-carotene absorption could be related to the coalescence phenomena observed in the stomach of rats and the reduced β-carotene bioaccessibility that revealed *in vitro* experiments.

Therefore, protein-based nanoemulsions, especially those with WPI, appear to be promising encapsulation systems to increase the absorption of β-carotene from alga *D. salina* and enhance the bioavailability and biodistribution of β-carotene and retinol in rats. Nevertheless, more studies should be done to better understand the specific mechanisms involved in the β-carotene metabolism when ingested enclosed in

nanoemulsions, as well as to do further steps to scale the results obtained up to humans.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

J.T.R. conducted the *in vitro* and *in vivo* experiments. All authors contributed to the design of the study, interpretation of the data and edition and revision of this manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter V

Encapsulation and controlled release of phycocyanin during the *in vitro* digestion using polysaccharide-added double emulsions ($W_1/O/W_2$)

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Abstract

Phycocyanin could be a potential substitute for synthetic food coloring due to its health-related properties. However, this protein is easily degraded by light, temperature or pH. Double emulsions have proven to be good systems to encapsulate and protect hydrophilic compounds such as phycocyanin. Moreover, the addition of polysaccharides to the outer aqueous phase (W_2) of these emulsions seems to improve their stability, although the impact on their digestive fate needs to be further studied. The aim of this study was to evaluate the effect of increasing the viscosity of W_2 by adding arabic gum, pectin or sodium alginate at different concentrations (0%, 0.5%, 1%, 1.5% or 2%) on the physical properties and digestibility of emulsions, and on phycocyanin bioaccessibility. Results showed an efficient phycocyanin encapsulation in the inner aqueous phase (W_1) of the emulsions containing sodium alginate and pectin at 1.5–2%. During the digestion, the use of all polysaccharides at 2% prevented the phycocyanin release into the gastric phase, its degradation during the intestinal phase and improved lipid digestibility. However, sodium alginate showed more noticeable effects, followed by pectin and arabic gum. Therefore, sodium alginate at concentrations 1.5-2% seems to be the most suitable polysaccharide for improving encapsulation efficiency, lipid digestibility and protecting phycocyanin during the gastrointestinal digestion.

Keywords: polysaccharides, $W_1/O/W_2$ emulsions, phycocyanin, bioaccessibility, digestibility

1. Introduction

Currently, interest in microalgae is growing, as they are a promising source of high valuable bioactive compounds. One of these microalgae is *Arthrospira* (*Spirulina*), which is rich in phycocyanin (PC), a phycobiliprotein that possesses antioxidant, anti-inflammatory, hepatoprotective and neuroprotective properties (Hussein et al., 2015; Romay et al., 2005). In addition, PC has shown to be a good natural food colouring in various food products such as yoghurts, biscuits or ice creams due to its intense blue colour, being useful to replace synthetic food colouring (Campos Assumpção de Amarante et al., 2020; El Baky et al., 2015; Kaur et al., 2019; Mohammadi-Gouraji et al., 2019). Nevertheless, PC is prone to suffer denaturation and/or proteolysis, especially due to external factors, such as light, temperature and pH, which limits its use in the food industry (Chaiklahan et al., 2012; Jespersen et al., 2005; Martelli et al., 2014). Moreover, as PC is unstable at pH values under 5.5 and above 6 (Chaiklahan et al., 2012), it could be easily degraded during the gastrointestinal digestion. In fact, protein denaturalization has been observed at low pH (Silva et al., 2009).

Double emulsions ($W_1/O/W_2$) have been used to incorporate hydrophilic compounds such as bioactive peptides (Giroux et al., 2016), vitamin C (Kheynoor et al., 2018), chlorophyllin (Artiga-Artigas et al., 2019) or anthocyanins (de Almeida Paula et al., 2018; Teixé-Roig et al., 2018) showing high encapsulation efficiencies (≥ 90%) and good protection against the degradation caused by external factors such as light or temperature. Besides, these systems could also be useful in preventing the degradation of bioactive compounds during the gastrointestinal digestion process and may offer a targeted release of the encapsulated compound in the small intestine (Giroux et al., 2013; Mao & Miao, 2015; Velderrain-Rodríguez, et al., 2019). In that sense, the incorporation of PC in $W_1/O/W_2$ emulsions seems to be a promising strategy to prevent its degradation during the digestion process and increase its bioaccessibility, in order to improve its health benefits.

In general, polysaccharides can act as stabilizing agents in $W_1/O/W_2$ emulsions by increasing the viscosity of the W_2 (Benna-Zayani et al., 2008) and/or acting as emulsifiers, due to their surface activity (Dickinson, 2009). Polysaccharides may also influence the lipid digestion due to either the increased viscosity in the intestine, which

can alter mass transport, or the formation of protective coatings around lipid droplets that inhibit the lipase access and binding species that play a key role in digestion (bile salts, phospholipids, enzymes or calcium), among others (Dongowski, 2007; Espinal-Ruiz et al., 2014; Fabek et al., 2014; Kristensen & Jensen, 2011; Li & McClements, 2014; Tokle et al., 2010). However, the ability of polysaccharides to affect the digestion process may be different depending on their molecular and physicochemical properties (Mudgil & Barak, 2013). In this regard, polysaccharides that provide low viscosity seems to not interfere with the digestibility of emulsions (Gasa-Falcon et al., 2017). Conversely, the presence of high-viscous polysaccharides in the W_2 phase of $W_1/O/W_2$ emulsions showed to slow down the lipolysis reaction rate at the beginning of the intestinal digestion, since the high micro-viscosity around the oil droplets may retard the movement of lipases at the interface (Teixé-Roig et al., 2018; Velderrain-Rodríguez, Salvia-Trujillo, et al., 2019). Then, based on these premises and in order to further investigate the effect of the viscosity of the W_2 phase of double emulsions on their digestive stability, three different polysaccharides conferring different levels of viscosity were studied (arabic gum, pectin and sodium alginate). Arabic gum has been chosen as it provides low viscosities, pectin intermediate viscosities and alginate high viscosities. In addition, according to our previous studies, the addition of polysaccharides could ameliorate the bioaccessibility of the hydrophilic bioactive compounds encapsulated in $W_1/O/W_2$ emulsions (Teixé-Roig et al., 2018). Moreover, as far as we know, there are no studies that report the encapsulation of PC in $W_1/O/W_2$ emulsions to control its release into the gastrointestinal tract. Therefore, this study aims to evaluate the effect of increasing the viscosity of the W_2 phase of $W_1/O/W_2$ emulsions by incorporating gum arabic, pectin or sodium alginate at different concentrations (0, 0.5, 1, 1.5 or 2% w/w), on the physical properties of the emulsions and the gastrointestinal fate of PC during an *in vitro* digestion process.

2. Materials and methods

2.1. Materials

PC extract from *Athrosphira* (*Spirulina*) platensis algae was provided by GNT (Dervy, United Kingdom), polyglycerol polyricinoleate (PGPR) from castor oil by Danisco (DuPont, EEUU) and lecithin from Alfa Aesar ThermoFisher (Karlsruhe, Germany).

Sodium caseinate, arabic gum (with a molecular weight of 250 g/mol and a protein content of 2-2.5%) and food-grade high methoxyl citrus pectin (with a degree of methylesterification from 67 to 71% and a molecular weight of 150 g/mol) were obtained from Acros organics (Geel, Belgium). Sodium alginate (MANUCOL® DH with a molecular weight of 680 g/mol) was acquired from FMC Biopolymer Ltd (Scotland, U.K.) and corn oil was purchased from a local supermarket. Pepsin from porcine gastric mucosa (77160), pancreatin from porcine pancreas (P1625) and bovine bile extract (B3883) were acquired from Sigma Aldrich (St. Louis, MO). Ultrapure water was obtained from milli-Q filtration system.

2.2 Methods

2.2.1. Formation of $W_1/O/W_2$ emulsions

$W_1/O/W_2$ emulsions were prepared using a two-step emulsification process previously described (Teixé-Roig et al., 2018), with minor modifications.

Firstly, a primary W_1/O emulsion was formulated with 30% of inner aqueous phase and 70% oil phase (O). The W_1 contained the PC extract (1.5% w/w), the hydrophilic surfactant sodium caseinate (0.5% w/w) and a NaCl 0.1M solution (28% w/w), whereas, the oil phase (O) contained the hydrophobic surfactant polyglycerol polyricinoleate (PGPR) (2% w/w) and corn oil (68% w/w). Sodium caseinate was added due to the observed synergistic effect between them and PGPR in producing a more viscoelastic adsorbed layer that could prevent the release of the encapsulated compound from the internal aqueous phase to the external aqueous phase (Su et al., 2006). Using these emulsifiers, we obtained water inner droplets with a droplet size of about 300 nm (data not shown). Both phases were homogenized using a high-speed homogenizer (Ultra Turrax homogenizer, Janke & Kundel, Staufen, Germany) at 8000 rpm for 2 min and sonicated using a P400S Hielscher sonicer (Hielscher Ultrasound Technology, Teltow, Germany) for 50 s at a frequency of 24 kHz and 35% amplitude.

The W_2 was prepared by adding lecithin with an HLB of 8 (2% w/w) to a NaCl 0.1 M pH 7 solution that contained different polysaccharides (arabic gum, pectin and sodium alginate) at different concentrations (0, 0.5, 1, 1.5 and 2%). Then, the $W_1/O/W_2$ emulsion

was formed by the dispersion of the W_1/O emulsion to the W_2 phase, at a ratio of 20/80 (w/w), which was homogenized (8000 rpm for 2 min) and sonicated for 30 s at a frequency of 24 kHz and 30% amplitude. After the preparation, the pH of all double emulsions was adjusted to 7.

2.2.2. Characterization of $W_1/O/W_2$ emulsions

The viscosity of emulsions was determined using a SV-10 vibro-viscometer (A&D Company, Tokyo, Japan), which produces a vibration of 30 Hz and constant amplitude (0.4 mm) at a controlled room temperature. The results were expressed in millipascal·seconds (mPa·s).

The particle size diameter of oil droplets (μm) and the particle size distribution were measured using static light scattering technique (Mastersizer 3000, Malvern Instruments Ltd., Worcestershire, UK). The particle size diameter (μm) was measured as the volume mean diameter (d_{43}).

The ζ-potential of $W_1/O/W_2$ emulsions was measured by phase-analysis light scattering (PALS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcerstershire, UK) to determine the apparent surface charge at the interface of the droplets. The $W_1/O/W_2$ emulsions were diluted (1:100) in milli-Q water and placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles. Otherwise, ζ-potential of the polysaccharides studied in this work (sodium alginate, pectin and arabic gum) was also measured at pH 3 and pH 7, which were the pH of the gastric and intestinal phase of the *in vitro* digestion. Polysaccharides were dissolved in aqueous solutions, which were subsequently adjusted to pH 3 and pH 7. The results were expressed in millivolts (mV).

Images of $W_1/O/W_2$ emulsions were obtained using an optical microscope (Olympus BX41, Olympus America Inc, Melville, NY, USA). Images were captured using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus).

The encapsulation efficiency (EE) of PC was determined according to a previously reported method (O'Regan & Mulvihill, 2010). First, aliquots of 10 mL of $W_1/O/W_2$ emulsions were centrifuged at 18566 g for 10 min at 4 ºC. Then, the W_2 phase containing

the non-encapsulated PC was collected and the PC content of the W_2 was quantified as described in section 2.1.3. Then, using the PC content in W_2, and the PC content in the total emulsion (previously quantified using the methodology described in section 2.1.3), the EE was determined according to Eq. (1):

$$EE\ (\%) = \frac{\text{Total PC-Free PC}}{\text{Total PC}} \times 100 \quad (1)$$

where Total PC and Free PC were the PC concentration in the double emulsion and the amount of PC determined in the W_2, respectively. Additionally, it should be noted that it is important to avoid PC degradation during the EE determination since the EE values may be overestimated.

2.2.3. Phycocyanin quantification

To PC extraction and quantification were performed using a method described by Chen et al. (2010) with some modifications. Firstly, 1 mL of sample was mixed with 4 mL of sodium phosphate buffer (pH 7) and the mixture was centrifuged at 12429 g for 15 min at 4 ºC. Then, the aqueous solution was filtered. Subsequently, the absorbance of the solution was measured at 615 and 625 nm using a spectrophotometer (V-670, Jasco Corporation, Tokyo, Japan), and the PC content was estimated according to Eq. (2):

$$PC\ (g/L) = \frac{A_{615} - 0{,}474 \times A_{652}}{5{,}34} \quad (2)$$

where A_{615} and A_{652} are the absorbance readings at 615 and 652 nm, respectively.

2.2.4. In vitro digestion of $W_1/O/W_2$ emulsions

The *in vitro* digestion of $W_1/O/W_2$ emulsions was performed based on an international consensus method (Minekus et al., 2014). Firstly, 20 mL of the emulsion was mixed with 18.2 mL of simulated gastric fluid (SGF) containing pepsin (2000 U/mL in the final mixture), 10 µL of a CaCl2 solution (0.3 M) and HCl (1M) to reach a pH of 3. Finally, milli-Q water was added to reach a final volume of 40 mL. The mixture was placed into an incubator OPAQ (OVAN, Barcelona, Spain) for 2 h at 37 °C with a constant agitation of 100 rpm. Once the gastric phase was completed, 30 mL of gastric sample were placed in a water bath (37 ºC) to simulate the intestinal phase using a pH-stat (Metrohm USA,

Riverview, FL, USA). Then, a bile solution (10mM) and a salt solution (NaCl 0.150 mM and $CaCl_2$ 0.01 mM) were added to the sample and the pH was adjusted to 7 with NaOH (1 M). Finally, a pancreatin solution (100 U/mL) was added to the mixture. The pH of the sample was maintained to 7 by adding NaOH (0.25 M) during 2 h. The amount of free fatty acids (FFA) released during the intestinal phase was calculated from the total volume of NaOH (0.25 M) added at the end of the digestion using the Eq (3):

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

where V_{NaOH} is the final NaOH volume (L) which is used during *in vitro* digestion to compensate the FFA, C_{NaOH} is NaOH molarity (0.25 M), M_{oil} is corn oil molecular weight (800 $g \cdot mol^{-1}$), and m_{oil} was the corn oil total weight present in the double emulsion (g).

2.2.5. Phycocyanin bioaccessibility during in vitro digestion

During the *in vitro* digestion process, aliquots of 2 mL were collected at different time intervals, 0, 15, 30, 60, 90, 120, 150, 180 and 240 min, in order to study the PC bioaccessibility. The samples collected during the digestion were centrifuged at 1690 g for 40 min at 4 ºC to obtain the micellar fraction. After the centrifugation, the aqueous phase was collected and the PC content was determined as described in section 2.1.3. Finally, the PC bioaccessibility was calculated according to Eq (4):

$$\text{Bioaccessibility of PC (\%)} = \frac{C_{micellar}}{C_{emulsion}} \times 100 \quad (4)$$

where $C_{micellar}$ is the PC concentration (mg/mL) of micellar samples and $C_{emulsion}$ is the PC concentration (mg/mL) of the initial $W_1/O/W_2$ emulsions.

2.2.6 Statistical analysis

All experiments were assayed in triplicate and three repetitions of each analysis were carried out in order to obtain mean values. The results were expressed as the mean ± standard deviation. The results were analyzed using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md) with a confidence interval set at 0.95.

The analysis of variance (ANOVA) was applied to compare treatments. Moreover, the least significant difference (LSD) was performed to determine the significant differences ($p \leq 0.05$) between the means.

3. Results and discussion

3.1. Characterization of $W_1/O/W_2$ emulsions

3.1.1. Viscosity

Initially, the viscosity of the $W_1/O/W_2$ emulsion without polysaccharides was 1.85 ± 0.04 mPa·s. The addition of polysaccharides to the W_2 phase of $W_1/O/W_2$ emulsions increased the viscosity of the emulsions, although the magnitude varied depending on the type of polysaccharide used (Table 1). The highest viscosities were observed adding sodium alginate, reaching the maximum value at 2% (196.67 ± 8.01 mPa·s). At this concentration, the viscosity of pectin-added emulsions was 37.17 ± 1.51 mPa·s, lower than those of sodium alginate but higher than the obtained with arabic gum (3.08 ± 0.16 mPa·s). In general, polysaccharides are added to emulsion-based systems due to its thickening properties that could reduce instability phenomena such as creaming by modifying the rheology of the continuous phase (Phillips & Williams, 2009). However, the impact of the polysaccharides on the emulsion viscosity depends on their molecular characteristics. In general, more extended molecules present a larger hydrodynamic diameter, meaning that they can entrain large quantities of water than more compact molecules. Furthermore, the apparent surface electrical charge of the molecules can affect their hydrodynamic diameter since the charged groups on their backbones can repel each other resulting in a more extended molecule (Bai et al., 2017; Phillips & Williams, 2009). In that way, polysaccharides used in this study exhibited different negative electrical charges at pH 7, being about -50 mV in the case of sodium alginate, -33 mV in the case of pectin, and about -28 mV in the case of arabic gum (Table 2). Thus, sodium alginate, which is the most negatively charged at pH 7, may present a large hydrodynamic diameter and could entrain more water, resulting in more viscous solutions than pectin and arabic gum, which are less charged at this pH. In fact, other authors have observed a noticeable increase in viscosity when adding sodium alginate in emulsions (Artiga-Artigas et al., 2019; Yang et al., 2012), and low viscosities when

using arabic gum, even at high concentrations (5-10%) (Mehrnia et al., 2017).

Table 1. Physical properties of $W_1/O/W_2$ emulsions containing different polysaccharides at variable concentrations.

Polysaccharide		Viscosity (mPa·s)	Size (μm)	ζ -Potential (mV)	Encapsulation efficiency (%)
Arabic gum	*0%*	1.85 ± 0.04 ^{Aa}	4.21 ± 0.26 ^{Ab}	-59.91 ± 2.42 ^{Aa}	82.36 ± 3.73 ^{Abc}
	0.5%	1.71 ± 0.01 ^{Aa}	4.30 ± 0.33 ^{Cb}	-55.68 ± 1.12 ^{Bb}	83.87 ± 4.28 ^{Ac}
	1%	2.10 ± 0.03 ^{Ab}	3.87 ± 0.27 ^{Bb}	-50.38 ± 1.17 ^{Bc}	70.45 ± 1.93 ^{Aa}
	1.5%	2.68 ± 0.24 ^{Ac}	3.76 ± 0.26 ^{Bb}	-49.03 ± 2.55 ^{Bc}	68.95 ± 2.61 ^{Aa}
	2%	3.08 ± 0.16 ^{Ad}	3.36 ± 0.14 ^{Aa}	-51.79 ± 2.2 ^{Bc}	78.71 ± 1.78 ^{Ab}
Pectin	*0%*	1.85 ± 0.04 ^{Aa}	4.21 ± 0.26 ^{Ad}	-59.91 ± 2.42 ^{Aa}	82.36 ± 3.73 ^{Aa}
	0.5%	5.71 ± 0.17 ^{Bb}	3.17 ±0.17 ^{Aab}	-51.21 ± 2.35 ^{Cbc}	93.93 ± 0.3 ^{Bc}
	1%	11.97 ± 0.05 ^{Bc}	3.37 ± 0.16 ^{Abc}	-49.28 ± 1.96 ^{Bc}	93.90 ± 3.73 ^{Bc}
	1.5%	19.10 ± 1.13 ^{Bd}	3.03 ± 0.15 ^{Aa}	-52.62 ± 1.5 ^{Bb}	94.77 ± 1.77 ^{Bc}
	2%	37.17 ± 1.51 ^{Be}	3.52 ± 0.24 ^{Abc}	-52.81 ± 2.25 ^{Bb}	90.86 ± 0.41 ^{Bb}
Sodium Alginate	*0%*	1.85 ± 0.04 ^{Aa}	4.21 ± 0.26 ^{Ab}	-59.91 ± 2.42 ^{Ab}	82.36 ± 3.73 ^{Aa}
	0.5%	11.55 ± 3.42 ^{Cb}	3.78 ± 0.17 ^{Ba}	-61.82 ± 2.47 ^{Aab}	90.71 ± 2.94 ^{Bb}
	1%	44.17 ± 2.50 ^{Cc}	3.57 ± 0.16 ^{Ba}	-65.55 ±2.11 ^{Aa}	93.1 ± 1.66 ^{Bbc}
	1.5%	116.67 ± 13.2 ^{Cd}	4.14 ± 0.04 ^{Cb}	-67.15 ± 4.27 ^{Aa}	95.58 ± 2.9 ^{Bc}
	2%	196.67 ± 8.01 ^{Ce}	4.31 ± 0.24 ^{Bb}	-68.24 ± 4.9 ^{Aa}	95.26 ± 2.05 ^{Cc}

Values are expressed as mean ± standard deviation. Different capital letters indicate significant differences ($p<0.05$) between emulsions with different polysaccharides at the same concentration. Different lowercase letters indicate significant differences ($p<0.05$) between emulsions with different concentrations of a particular polysaccharide.

Table 2. Electrical charge (mV) of the polysaccharides studied at different pH values.

Polysaccharide	pH 3	pH 7
Arabic Gum	-13.21 ± 0.61 ^{B}	-27.48 ± 1.21 ^{C}
Citrus pectin	-13.65 ± 0.74 ^{B}	-32.68 ± 1.58 ^{B}
Sodium Alginate	-40.29 ± 2.70 ^{A}	-49.54 ± 3.61 ^{A}

Values are expressed as mean ± standard deviation. Different capital letters indicate significant differences ($p<0.05$) between polysaccharides at a same pH value.

3.1.2. Particle size and size distribution

In general, the particle size values were about 3-4 μm, being the $W_1/O/W_2$ emulsion without polysaccharides those with the highest values (Table 1). Additionally, all $W_1/O/W_2$ emulsions presented a bimodal distribution (Figure 2), which has been widely observed by other authors (Artiga-Artigas et al., 2019; Díaz-Ruiz et al., 2020; Matos et al., 2018; Velderrain-Rodríguez et al., 2019). Indeed, for all emulsions, a main peak was observed from 0.8 to 10 μm, related to the presence of oil droplets containing the inner water phase. A smaller peak ranging between 0.2 and 0.8 μm was also detected, probably as a consequence of the formation of PGPR micelles (Matos et al., 2015).

In general, the addition of polysaccharides in the W_2 phase of $W_1/O/W_2$ emulsions reduced the particle size (Table 1), as it can be observed in the microscope images (Figure 1). The smallest particles were detected adding pectin, especially at 1.5% concentration (3.03 ± 0.15 μm). Otherwise, sodium alginate was effective in reducing the particle size at 0.5% and 1% concentrations, reaching minimum values of 3.57 ± 0.16 μm in emulsions with 1% concentration. The effect of arabic gum was smaller and only the addition of arabic gum at 2% reduced significantly the particle size of emulsions (Table 1). On the one hand, it was hypothesized that in polysaccharide-added double emulsions, lecithin covered the majority of the interface as it has a higher interfacial activity (it reduced the interfacial tension up to a 14.66 mN/m) than sodium alginate (21.6 mN/m), pectin (23.56 mN/m) and arabic gum (21.17 mN/m). However, as observed in previous works with Tween 20 and pectin, the presence of polysaccharides may have created a competitional effect for the interface, whereby lecithin moved slightly faster to the interface, leading to smaller particle sizes (Verkempinck et al., 2018). On the other hand, the increased viscosity of the W_2 phase of polysaccharide-added emulsions could have increased the shear disruptive forces favouring the reduction of the particle size (Qian & McClements, 2011). Moreover, it seems that intermediate viscosities were the most effective in the particle size reduction (Table 2). Otherwise, the high viscosity of emulsions with ≥ 1.5% sodium alginate, probably hindered the particle size reduction during the homogenization and sonication processes.

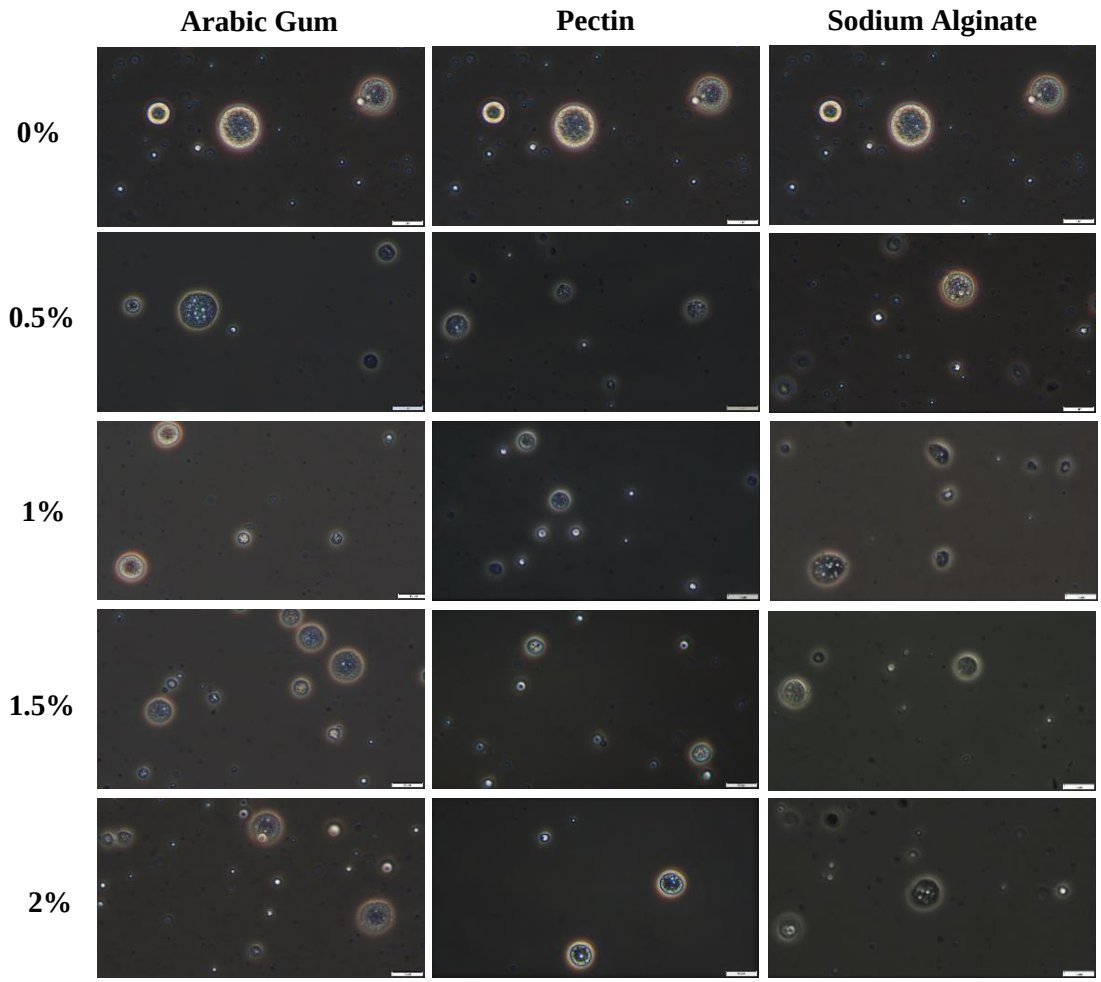

Figure 1. Optical microscope images of $W_1/O/W_2$ emulsions containing different polysaccharides at variable concentrations. Scale bars were 10 μm long.

3.1.3. ζ-potential

All $W_1/O/W_2$ emulsions presented negative ζ-potential values, being about - 60 mV for the emulsion without polysaccharides (Table 1). The addition of sodium alginate at ≥ 1% produced a more negative ζ-potential in comparison to the emulsion without polysaccharides. Conversely, the ζ-potential became less negative with the addition of pectin and arabic gum in the emulsions (Table 1). In this regard, the emulsifier used to stabilize de O/W_2 interface was lecithin, a zwitterionic polar lipid that, at the pH of the emulsions (≈ 7), has shown to present a negative charge due to their negatively charged phospholipids (Artiga-Artigas et al., 2018; Chang & McClements, 2016). At pH 7, pectin, sodium alginate and arabic gum also present a negative electrical charge (Table 2). As it was mentioned before, lecithin covered the majority of the interface as it has a

higher interfacial activity. However, the presence of polysaccharide molecules at the interface is confirmed by the changes in the electrical charge compared to no-added polysaccharide emulsions (Table 1). Otherwise, the ζ-potential no longer changed at concentrations above 1%, probably because no more sodium alginate, pectin or gum arabic molecules could be adsorbed at the interface. Therefore, most of the polysaccharide molecules would remain in the W_2 phase of the emulsions. Besides, in the case of sodium alginate, the ζ-potential becomes more negative as this polysaccharide has a high negative charge and contributes to increasing the negativity of the charge at the interface. On the other hand, pectin and arabic gum have a less negative charge than lecithin at this pH, so that, once adsorbed at the interface, the molecules of these polysaccharides contribute to reducing the negativity of the apparent electrical charge of the interface.

3.1.4. Encapsulation efficiency of PC

As it can be observed in Table 1, the encapsulation efficiency (EE) of the $W_1/O/W_2$ emulsion without polysaccharides was 82.36 ± 3.73% (Table 1), which increased by the addition of pectin and sodium alginate, being the 1.5 and 2% alginate emulsions those with the highest EE values (≈ 95%). On the one hand, the decreased EE in emulsions containing only lecithin as a stabilizer of the O/W_2 interface may be due the notorious lipophilicity of this emulsifier, which would have diffused through the oil film, causing competition with PGPR and sodium caseinate at the W_1/O interface. This fact could have promoted the destabilization of the emulsion (Michaut et al., 2004) and consequent loss of PC from W_1 to W_2 due to the coalescence between the W_1 droplets or between the W_1 and the globule interface (Ficheux et al., 1998). On the other hand, the increased of EE in pectin- and alginate-added emulsions could be related to a pronounced increase in their viscosity. In this sense, these emulsions could present a higher stability at the O/W_2 interface that would prevent droplet diffusion from W_1 to W_2. Alginate, pectin and other high-viscose polysaccharides (Chan et al., 2011; Matos et al., 2014; Teixé-Roig et al., 2018), have been reported to enhance the EE of $W_1/O/W_2$ emulsions when they have been added in the W_2 aqueous phase, which indicates that the increased viscosity of W_2 plays a significant role in the EE of $W_1/O/W_2$ emulsions. Conversely, adding arabic gum, the EE did not improve, which could be related to the low increase

of viscosity observed in these emulsions. These results are consistent with other studies, in which no improvement in EE was observed by increasing the concentration of arabic gum (Su et al., 2008).

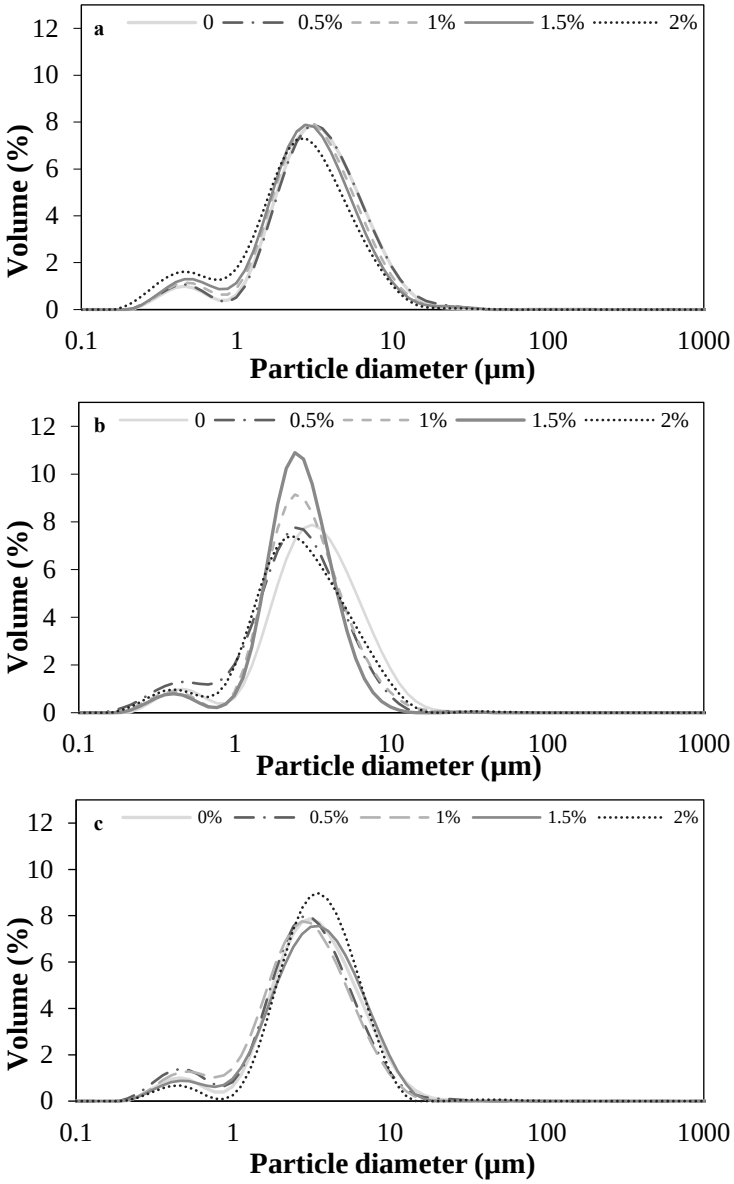

Figure 2. Droplet size distribution of $W_1/O/W_2$ emulsions containing different polysaccharides at variable concentrations. Arabic gum (A); Pectin (B); Sodium alginate (C).

3.2. Gastrointestinal digestion of $W_1/O/W_2$ emulsions

3.2.1. Lipid digestibility of emulsions

The *in vitro* lipid digestion of $W_1/O/W_2$ emulsions was evaluated in terms of FFA release (%) during the intestinal phase (Figure 3).

All $W_1/O/W_2$ emulsions showed a gradual release of FFA during the first 60 minutes of intestinal digestion. However, from this moment, the FFA release in emulsions containing ≥ 1.5% sodium alginate and 2% pectin continued increasing, whereas in the other emulsions it was stabilized. The increased FFA release in emulsions with higher viscosities (≥ 1.5% sodium alginate and 2% pectin) from minute 60 to 90 of intestinal digestion suggests that the viscosity plays an important role in the digestibility of double emulsions. In these emulsions, once the lecithin was displaced from the interface by the action of lipase, the oil droplets may present higher stability in the intestinal medium towards coalescence due to the high viscosity of W_2. Therefore, these emulsions probably presented lower particle sizes that may facilitate the activity of intestinal enzymes by the increased surface area (Gasa-Falcon et al., 2017; Li et al., 2011). However, during the last 30 min, FFA release did not further increase, indicating that the oil droplets had probably already lost their stability and increased their particle size by coalescence (Figure 5). In fact, at the end of intestinal digestion, these emulsions presented a similar particle size as emulsions with lower lipid digestibility (Figure 4). Moreover, at the end of the intestinal phase, $W_1/O/W_2$ emulsions containing 2% pectin or ≥ 1.5% sodium alginate showed a higher digestion extent (≈ 50%) than the emulsion without polysaccharides (≈ 30%). In contrast, emulsions containing arabic gum, ≤ 1% sodium alginate or ≤ 1.5% pectin in the W_2 phase showed a similar final FFA release to the emulsion without polysaccharides. This polysaccharide may not provide stabilization to droplets against coalescence once lecithin was displaced from the interface; therefore, due to the coalescence of droplets, the lipase had difficulties in adhering and digesting the lipid fraction.

Furthermore, it should be noted that low release of FFA was observed during the first 60 minutes of intestinal digestion in emulsions containing pectin and sodium alginate at high concentrations. This could be due to a reduced intestinal enzyme diffusion process

as a consequence of the high viscosity around the oil droplets and/or because polysaccharide molecules may adsorb at the O/W_2 interface, delaying lipase adsorption (Dima & Dima, 2020; Espinal-Ruiz et al., 2014; Teixé-Roig et al., 2018; Velderrain-Rodríguez et al., 2019). Conversely, this phenomenon was not observed using arabic gum because the low viscosity provided by this polysaccharide did not alter the diffusion process of intestinal enzymes (Figure 6).

In summary, it seems that sodium alginate and pectin at high concentrations (≥ 1.5% and 2%, respectively), presented a positive effect on the lipid digestibility of double emulsions. Conversely, the presence of these polysaccharides at low concentrations or of Arabic gum had no effect on the lipolysis.

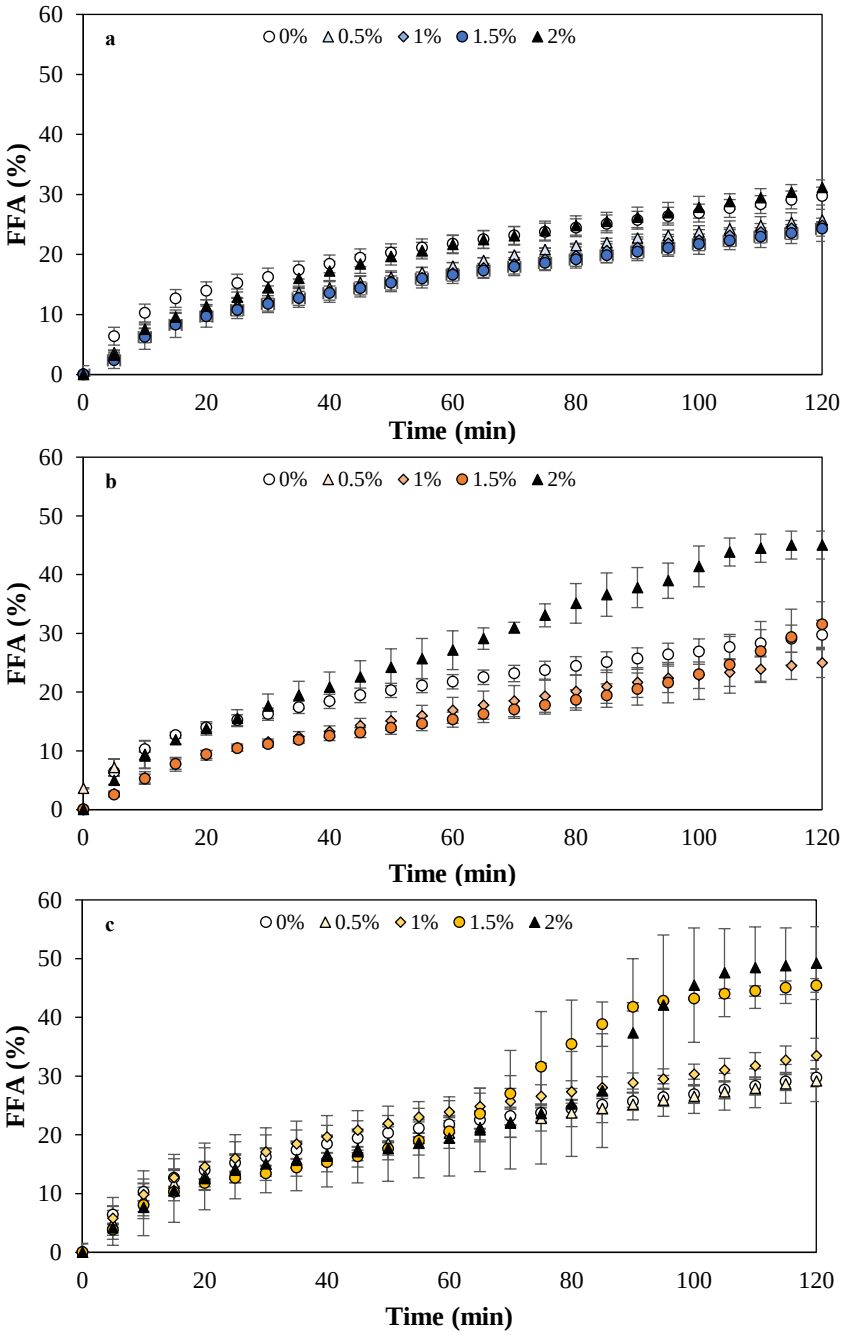

Figure 3. Free fatty acids release (FFA%) of $W_1/O/W_2$ emulsions containing different polysaccharides at variable concentrations. Arabic gum (a); Pectin (b); Sodium alginate (c).

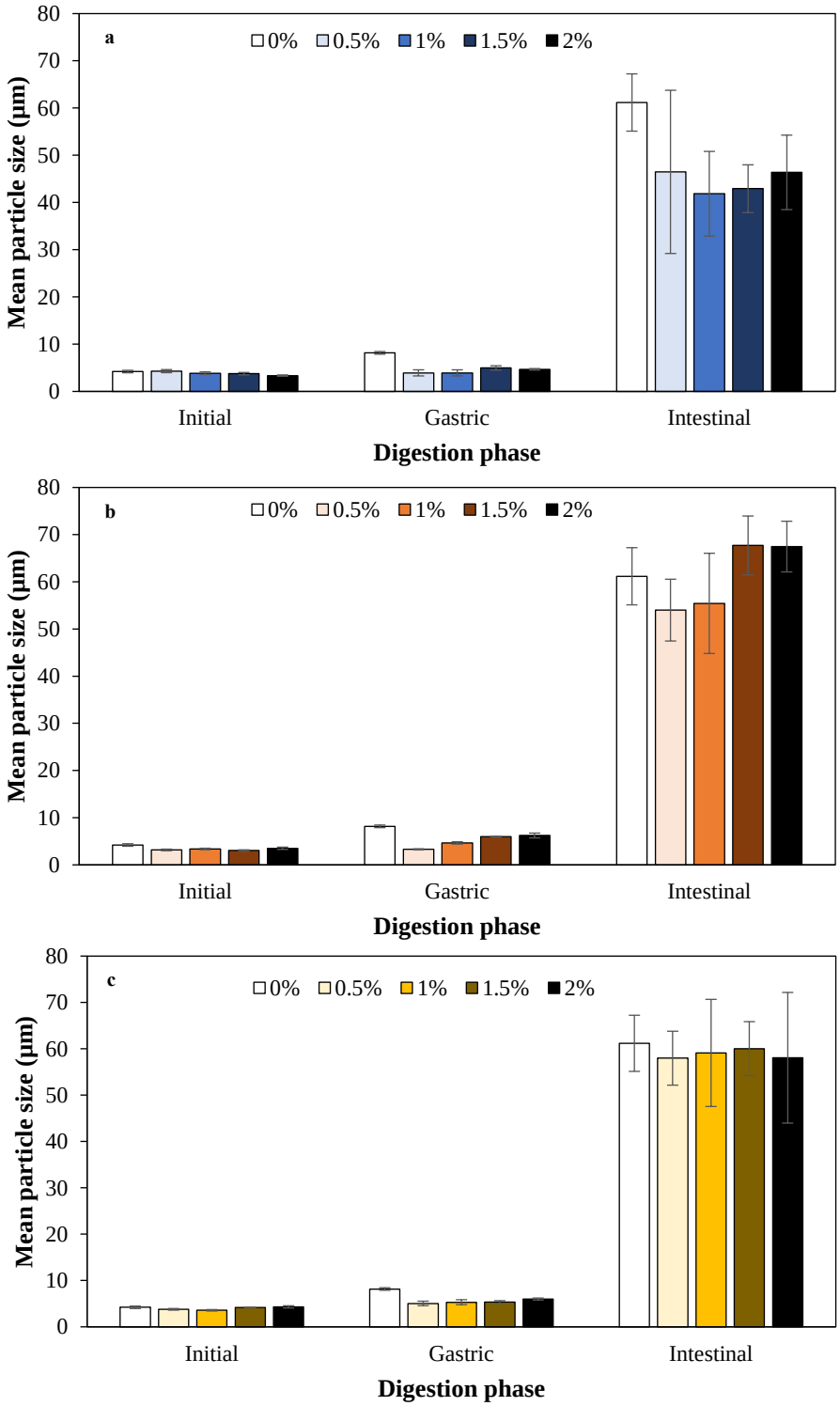

Figure 4. Droplet size of $W_1/O/W_2$ emulsions containing different polysaccharides at variable concentrations during the *in vitro* gastrointestinal digestion. Arabic gum (a); Pectin (b); Sodium alginate (c).

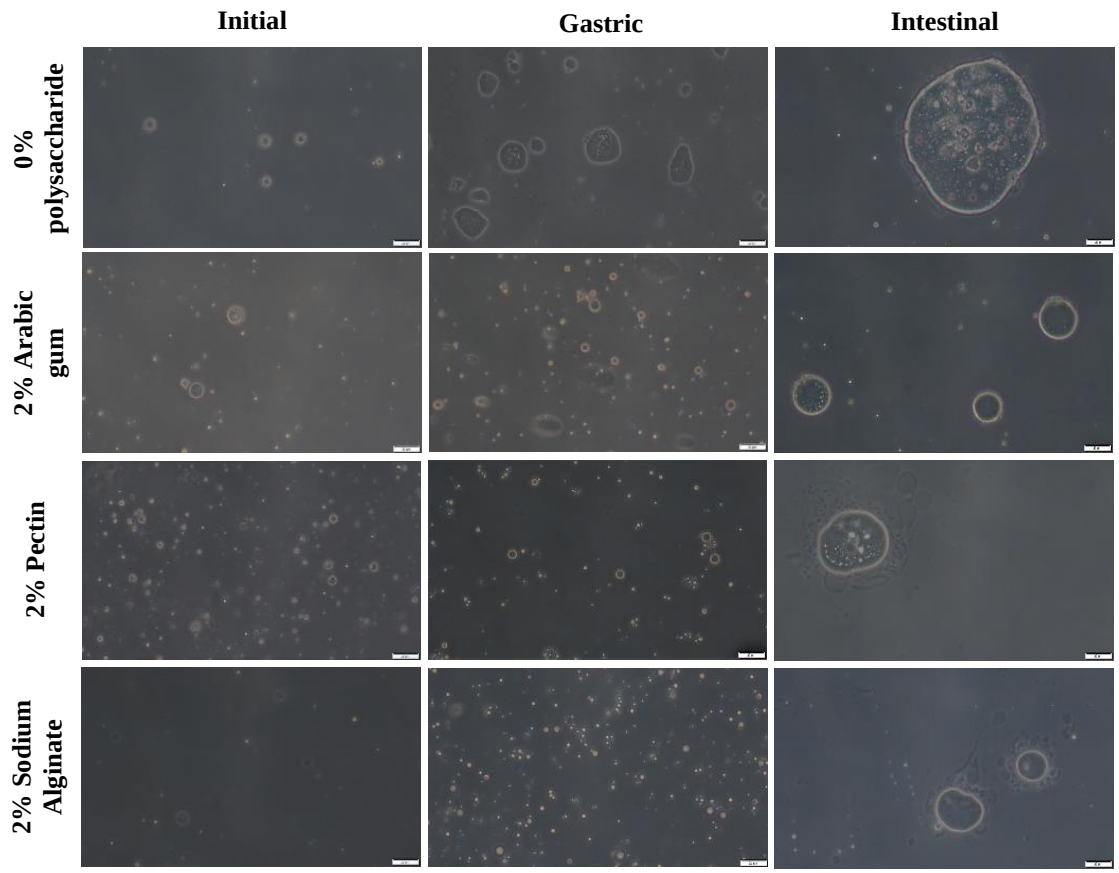

Figure 5. Optical microscopy images of $W_1/O/W_2$ emulsions containing different polysaccharides (Arabic gum, pectin and sodium alginate) at 2% or without polysaccharides during the *in vitro* gastrointestinal digestion. Scale bars were 20 μm long.

3.2.2. Bioaccessibility of PC during in vitro digestion

The bioaccessibility of PC during *in vitro* digestion at different time intervals (0, 30, 45, 60, 90, 120, 150, 180 and 240 min) is depicted in Fig. 6. The results showed that PC bioaccessibility gradually increased during the gastric phase, meaning that part of the encapsulated compound was being released from W_1 to W_2 during this stage (Fig. 6a, c and e). However, the lowest PC bioaccessibility values at the end of the gastric phase were observed in emulsions containing 2% and 1.5% sodium alginate, which were also those that presented the highest viscosity values (Fig. 6e). The high viscosity and the presence of polysaccharides at the O/W_2 interface probably reduced the diffusion phenomena through the oil film, thereby, decreasing the PC release (Herzi et al., 2014; Pays et al., 2001). Nevertheless, pectin at ≥ 1.5% and arabic gum at 2% concentration also inhibited PC release, although to a lower extent than sodium alginate (Fig. 6a, c and e).

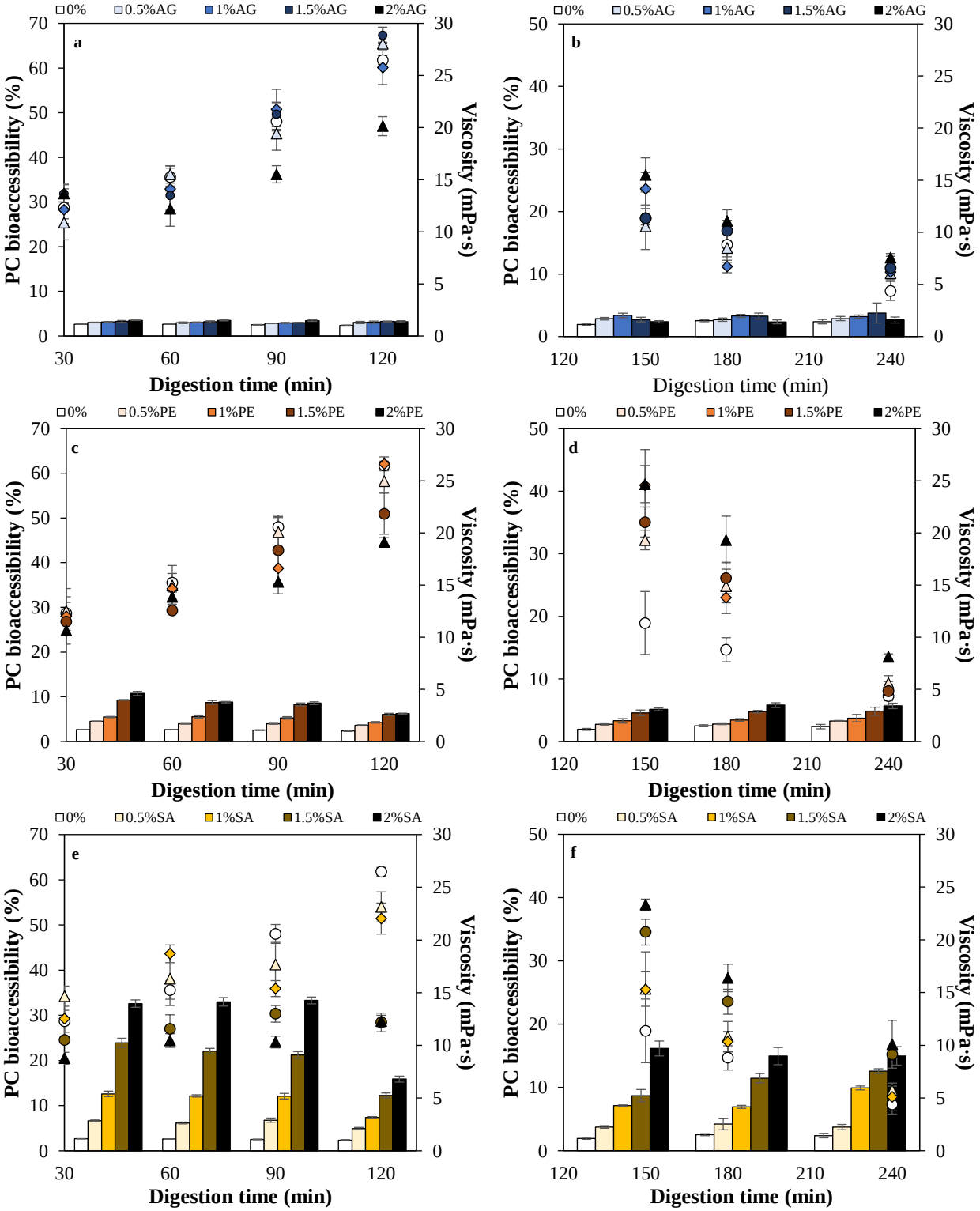

Figure 6. Phycocyanin bioaccessibility (markers) and emulsion viscosity (bars) during the *in vitro* gastrointestinal digestion of $W_1/O/W_2$ emulsions containing different polysaccharides at variable concentrations. Gastric digestion Arabic gum (a); intestinal digestion Arabic gum (b); gastric digestion pectin (c); intestinal digestion pectin (d); gastric digestion sodium alginate (e); intestinal digestion sodium alginate (f).

These emulsions presented a lower viscosity than sodium alginate emulsions and similar values between them, meaning that other factors were affecting the PC release on these emulsions. The higher presence of pectin and arabic gum molecules on the interface or around it could be preventing the PC release. In that sense, high methoxyl pectin has shown to present a high compact organization on the interface due to the charged carboxylic groups (Verkempinck et al., 2018). This fact may create less inter-molecular repulsive forces and reduce the PC release due to the less spaces at the interface. Moreover, all polysaccharides presented a negative charge at pH 3, being about - 40 mV in the case of sodium alginate and about - 13 mV in the case of pectin and arabic gum (Table 2). Thus, polysaccharides are able to bind with PC (positively charged) through electrostatic interactions, offering protection to the compound and preventing the degradation of its blue chromophores, once it is liberated from the W_1.

During the intestinal digestion, the oil phase of the emulsion was digested leading to the release of PC from W_1 into the intestinal fluids to be absorbed. However, a decrease in PC bioaccessibility is observed in Fig. 6b, d and f a phenomenon that could be related to the pH- dependent disposition of PC, which is easily degraded at pH 7. In this sense, the hexameric arrangement of this protein or its dissociation to a trimeric form determines its susceptibility to denaturation, being more resistant to denaturation in the hexameric form. At the pH 7 of the intestinal phase, PC is arranged mostly in trimeric form, which makes it susceptible to degradation (Adams et al., 1979; Chaiklahan et al., 2012). Contrarily, at a more acidic pH (5.5-6) PC is arranged mainly in a hexameric form, which is less susceptible to denaturation. However, emulsions containing 2% or 1.5% sodium alginate showed the highest bioaccessibility values (16.82 ± 3.77% and 15.20 ± 0.54%, respectively) at the end of the intestinal digestion, followed by 2% pectin (13.56 ± 0.46%) and 2% arabic gum emulsions (12.64 ± 1.51%) (Figure 6B, D and F). Bioaccessibility of sodium alginate and pectin emulsions could be related to lipolysis, since emulsions that presented the highest lipid digestibility (Figure 3), achieved the highest PC bioaccessibility in the intestinal stage. In less digested emulsions, PC bioaccessibility was lower probably because PC may remain in the W_1 phase entrapped in the non-digested oil. Moreover, the high viscosity and the presence of alginate gels in the intestinal phase due to the differences in pH and ion concentrations with respect to the gastric phase (Dima & Dima, 2020) may have prevented the PC degradation at this

stage. The high viscosity probably reduced the diffusion rate of pro-oxidant molecules through the intestinal fluids, thereby reducing the contact between this molecules and PC. Nevertheless, although 2% arabic gum emulsion showed a higher PC bioaccessibility than that with 0% polysaccharide, no differences were observed in the digestibility of those emulsions. Therefore, it is suggested that arabic gum may interact with PC, and increasing its bioaccessibility by preventing the degradation of the compound due to its antioxidant properties (Ali & EL Said, 2020).

4. Conclusions

The $W_1/O/W_2$ emulsions formulated in this study are effective systems for PC encapsulation, showing a high encapsulation efficiency that improves with the addition of pectin or alginate but not with the addition of gum arabic. Moreover, the addition of polysaccharides to the W_2 phase of emulsions improved the retention of PC during the gastric phase and increased the PC bioaccessibility at the intestinal phase, especially when used at high concentrations (2%). Nevertheless, the effectiveness of polysaccharides decreased in the following order: sodium alginate > pectin > arabic gum. These results could be related to (1) the increased viscosity as well as to the ability of polysaccharides to prevent the diffusion of W_1 droplets to the W_2 due to a better stabilization of O/W_2 interface (2) the increased oil digestibility of polysaccharide-added emulsions, especially those containing pectin and sodium alginate at high concentrations (2% and ≥ 1.5%, respectively). Then, among the polysaccharides studied, sodium alginate seems to be the most suitable to improve the digestibility of $W_1/O/W_2$ emulsions and protect PC during the gastrointestinal digestion.

Acknowledgements

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Chapter VI

Highly-concentrated emulsions to increase curcumin bioavailability and biodistribution in rats

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In preparation

Abstract

Clinical studies have proved the interesting health-related effects of curcumin, but it has low chemical stability and little bioavailability. In this sense, emulsions have shown to be effective encapsulation systems for the delivery of bioactive compounds, but highly-concentrated emulsions seem to be especially promising due to the high amount of compound that they can enclose. Therefore, the aim of this work was to study the pharmacokinetics and biodistribution of curcumin when enclosed in optimized highly-concentrated emulsions at 2 h or 4 h post-oral administration to rats. The most stable highly-concentrated emulsion was that with 50% of oil and obtained by high-shear homogenization followed by a microfluidization process, which contained a curcumin concentration of 15 mg/g. After orally administered, the pharmacokinetic study revealed that curcumin glucuronide was the main compound present in plasma (AUC_{0-t} = 1556.3 $ng \cdot h \cdot ml^{-1}$), especially at 2-4 h post-administration. Moreover, curcuminoid bioavailability was increased by 10.6-fold when rats were fed with the highly-concentrated curcumin emulsion rather than with a control suspension. The biodistribution study showed an improved curcumin absorption by using the highly-concentrated emulsion versus the suspension, being the highest concentration found at 2 h post-administration. Moreover, rats fed with the highly-concentrated emulsion showed the highest accumulation of curcuminoids in the liver (≈129 ng curcumin/g tissue) and brown adipose tissue (≈ 193 ng curcumin/g tissue). Thus, highly-concentrated emulsions appear to be feasible to increase the bioavailability of curcumin and its presence in organs where the compound may play a relevant role in the prevention of metabolic disorders.

1. Introduction

Curcumin, a polyphenol extracted from the rhizomes of *Curcuma longa*, have been showb to be effective against several chronic diseases and targets diverse molecular pathways without any associated toxicity or resistance (Prasad et al., 2014). Specifically, this lipophilic compound presents antioxidant and anti-inflammatory properties that provide multiple health benefits (Hewlings & Kalman, 2017; Pulido-Moran et al., 2016). Despite its numerous applications, curcumin has molecular instability, poor solubility in water, rapid conjugation to hydrophilic molecules (like glucuronic acid and sulphate) in the liver with biliary excretion and poor enteral absorption, which limit its utility as a health-promoting agent (Anand et al., 2007). To overcome these challenges, delivery systems such as nanoparticles, liposomes, micelles, and phospholipid complexes have been developed, showing to prevent the degradation in the gastrointestinal tract and to increase the permeation in the small intestine, increasing curcumin bioavailability (Hu et al., 2017; Stohs et al., 2020). Among emulsion-based delivery systems, highly-concentrated emulsions (> 40% oil phase w/w) have been studied since they can allow the oral administration of high doses of bioactive compounds and present an improved shelf-life of chemically labile products such as curcumin due to the high lipid content (Artiga-Artigas et al., 2019; Luo et al., 2017).

The potential of emulsion-based delivery systems to increase the bioavailability of curcumin has been widely investigated. As an example, by using an emulsion stabilized with a protein-polysaccharide conjugate (bovine serum albumin-dextran), the bioavailability of curcumin increased by 4.8-fold compared to a curcumin suspension (Wang et al., 2016). Other authors observed that a self-nanoemulsifying drug delivery system containing 50% ethyl oleate, 40% Tween 80 and 10% PEG 600 led to the maximum concentration in plasma of curcumin, which was 3.95-times higher than when using a curcumin suspension (Nazari-Vanani et al., 2017). Nanoemulsions have been also studied as curcumin carriers, showing a 3-fold increase in curcumin bioavailability with respect to a curcumin suspension when formulated with lecithin as surfactant (Vecchione et al., 2016). Moreover, these authors reported a higher increase (5.7-fold) when coating nanoemulsions with chitosan due to its mucoadhesive properties. Additionally, Takahashi et al. (2009) observed a higher bioavailability and faster

absorption by encapsulating curcumin into liposomes rather than using free curcumin. However, to the best of our knowledge, there are no studies investigating the use of highly-concentrated emulsions to increase the *in vivo* bioavailability of lipophilic compounds such as curcumin.

Most *in vivo* studies investigating the use of emulsion-based delivery systems to increase the absorption and bioavailability of curcumin are based on pharmacokinetic studies. Curcumin delivered orally undergoes extensive metabolism reduction and conjugation, which results in the presence of so many different metabolites. Recent studies have revealed that some reductive metabolites such us tetrahydrocurcumin may present therapeutic properties, although any of them have shown more biological effect than curcumin in its native form (Pandey et al., 2020). In contrast, metabolites resulting from conjugation like curcumin glucuronide, have shown very reduced effects (Choudhury et al., 2015; Kunihiro et al., 2019; Shoji et al., 2014). Nevertheless, most *in vivo* pharmacokinetic studies have used enzymatic hydrolysis of plasma prior to analysis, reporting the results as total curcumin, and not determining the part of the compound present as curcumin glucuronide (Stohs et al., 2020). Furthermore, few studies have investigated the effect of curcumin incorporation into emulsion-based delivery systems on the biodistribution of the compound. Knowing the target organs of curcumin and the form in which this compound is present when administered orally enclosed in emulsions is of great importance. This provides more information on which organs this compound can act and exert beneficial effects, providing insights into the different diseases in which these curcumin systems can improve treatment or prevention.

Therefore, the aim of this work was, firstly, to obtain a stable highly-concentrated emulsion containing a curcuminoid extract. Secondly, to study the effect of the encapsulation of curcumin in a highly-concentrated emulsion on the *in vivo* absorption of the different curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin), as well as their metabolization into curcumin glucuronide and the biodistribution of the different forms (free or conjugated) in the organs.

2. Materials and Methods

2.1. Material

Powdered curcumin extract (mixture of ≥ 78% curcumin, 15% demethoxycurcumin, and 1% bisdemethoxycurcumin) with a purity of ≥ 98% was purchased from Acros Organics. As a lipid phase MCT oil (Miglyol, Oxi-med expres) (99.9% of purity) was used. Soybean lecithin was acquired from Alfa Aesar (Thermo Fisher Scientific, Massachusetts, USA). Curcumin, demethoxycurcumin, and bisdemethoxycurcumin standards were obtained from Sigma Aldrich (St. Louis, MO). Curcumin glucuronide was acquired from Toronto Research Chemicals (Toronto, Canada). Ultrapure water obtained from a milli-Q filtration system was used to the preparation of all solutions.

2.2. Methods

2.2.1. Preparation of highly-concentrated emulsions

To obtain the lipid phase, curcumin was solubilized in MCT oil (30 $mg \cdot g^{-1}$) by stirring during 20 min at 60 ºC and sonicating for 20 min. Different concentrations of oil were tested during the optimization process (10-50% w/w). To obtain the aqueous phase, soybean lecithin (which was used as emulsifier) was added in ultrapure water and stirred during 4 h. Different surfactant-oil-ratio (SOR) were tested (0.05, 0.1, and 0.2), thereby, varying the concentration of soybean lecithin in the emulsions.

Two different methodologies were tested to formulate highly concentrated emulsions (HC-emulsions). In the first methodology only a high-shear homogenization (HSH) was used. The lipid phase (10 or 50% w/w) and the aqueous phase were mixed and homogenized at 11000 rpm for 2 min using an Ultra-Turrax (Janke & Kundel, Staufen, Germany). The second methodology included a HSH followed by a microfluidization process (MF). For the MF process, emulsions were passed through a microfluidizer (M-110P, Microfluidics, USA) at 100 MPa for 5 cycles.

2.2.2. Characterization of highly-concentrated emulsions

Particle size. The particle size of HC-emulsions was measured using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK). Samples were diluted in ultrapure water and stirred in the dispersion unit with a constant speed of 1800 rpm. The mean particle size was expressed as surface area mean diameter (d_{32}) in nanometers (nm), fixing a refractive index of the MCT oil of 1.45 and 1.333 for water.

ζ-potential. The ζ-potential was measured by phase-analysis light scattering (PALS) using Zetasizer laser diffractometer (NanoZS Malvern Instruments Ltd Worcestershire, UK). Prior to the analysis, HC-emulsions were diluted (1:100) in ultrapure water. Then, the diluted samples were placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles. The results were reported in millivolts (mV).

Fluorescence microscopy. HC-emulsions were dyed with Nile Red, previously dissolved at 0.1% (w/v) in ethanol. Then, micrographs of HC-emulsions were obtained using fluorescence with an optical microscope (Olympus BX41, Olympus, Göttingen, Germany) with a 100x objective lens. The images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus Göttingen, Germany).

Stability. The stability of HC-emulsions was studied using an optical scan analyzer Turbiscan MA 2000 (Formulaction, Toulouse, France), which is a non-destructive method that can measure the static stability of samples and detect the cause of instability (flocculation, coalescence, sedimentation, or creaming) by the multiple light scattering technique. A sample of 7 mL was introduced into a glass cylindrical cell and analyzed by a light beam emitted in near infrared wavelength, which scanned vertically from the bottom to the top of the sample cell. Two synchronous optical sensors received light backscattered by the sample (45º from the incident radiation). In this study, the backscattering was measured during 15 days at 4 ºC to assess the stability of HC-emulsions over time. The backscattering was analyzed at three different zones of the test tube (top, middle, and bottom) in order to study different instability phenomena throughout the tube such as creaming at the top, flocculation, or coalescence in the

middle and sedimentation at the bottom.

2.2.3. In vivo studies

2.2.3.1. Animal experiments

Female Sprague Dawley rats weighing 200-250 g were used for the *in vivo* studies. The animal procedures were conducted in accordance with EU Directive 2010/63/EU for animal experiments and approved by the Animal Ethics Committee of Universitat de Lleida (CEEA 01-04/18).

Pharmacokinetic study. For the pharmacokinetic study the animals were randomly divided into two groups (fed with a HC-emulsion or suspension) containing five rats each group. After an acclimatation period, rats were fasted for 12 h before the experiment with free access to water. The animals were administered with curcumin HC-emulsion or curcumin suspension, both containing a dose of 150 mg of curcumin/kg body weight. Blood samples were taken at 0, 30, 60, 120, 240, and 480 min after administration from the tail vein. Plasma was immediately separated by centrifuging the blood samples at 4000 rpm for 10 min at 4 ºC and stored at -80 ºC. Then, curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin glucuronide were extracted and quantified in plasma samples as described in 2.2.3.2. The pharmacokinetic analysis was performed for formulations using a non-compartmental design. The area under the drug concentration versus time curve from zero to 8 hours ($AUC_{0–8\ h}$) was calculated using the trapezoidal rule. The maximum plasma concentration of curcumin (C_{max}) and the time to reach maximum plasma concentration (T_{max}) were directly obtained from plasma analyses.

Biodistribution study. Rats were randomly divided in 2 groups; one group was fed with a curcumin HC-emulsion (n=8) and the other group was fed with a curcumin suspension (n=8). Rats were fasted for 12 h before the experiment with free access to water. The animals were administered a dose of 150 mg of curcumin/kg body weight, irrespective of the curcumin vehicle used. Rats were sacrificed at 2 h or 4 h post-administration and tissue samples were collected (kidney, liver, duodenum, jejunum, ileum, colon, brown adipose tissue and white adipose tissue). Tissue samples were washed with phosphate

buffered saline (PBS), dried on a filter paper, weighted, and stored at -80 ºC. Curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin glucuronide were extracted and quantified in tissue samples as described in 2.2.3.2.

2.2.3.2. Curcuminoid extraction and quantification in plasma and tissues

Extraction from plasma. Curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin glucuronide extraction in plasma samples was performed according to a previously reported method with some modifications (Li et al., 2011). Aliquots of 150 µL of plasma were mixed with the internal standard (Honokiol) and 600 mL of acetonitrile and vortexed at 1800 rpm for 1 min. Then, the mixture was submerged intro an ultrasonic bath for 30 sec, centrifuged at 9000 rpm for 10 min at 4 ºC, and the organic fraction was collected. Afterwards, the collected upper organic layers were evaporated under N_2 and stored at -80 ºC until quantification.

Extraction from tissues. Curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin glucuronide extraction in tissue samples was performed according to a previously reported method with some modifications (Chirio et al., 2019). Initially, tissue samples were mixed with milli-Q water and homogenized with an Ultra-Turrax at 9000 rpm for 1 min to obtain the tissue homogenates. Then internal standard (Honokiol) and 1 mL of acetonitrile were added to tissue homogenates and the mixture was vortexed at 2000 rpm for 1 min. Then, the mixture was centrifuged at 9000 rpm for 5 min at 4 ºC, and the organic fraction was collected. Afterwards, the collected upper organic layers evaporated under N_2 and stored at -80 ºC until quantification.

Curcuminoids quantification. The quantification of curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin glucuronide was performed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI–MS/MS) using the method described by Liu et al. (2018), with some modifications. To perform the analyses an ACQUITY UPLC binary (Waters, Milford, MA, USA) coupled to a Xevo TQS (triple quadrupole) (Waters, Milford, MA, USA) was used. Chromatographic separation was performed on a 150 mm × 2.1 mm i.d., 1.6 µm CORTECS Phenyl Column (Waters, Milford, MA, USA) with mobile phase A [acetonitrile containing 0.1% (v/v) formic acid] and mobile phase B [98% water and 2% acetonitrile containing 0.1%

(v/v) formic acid]. The injection volume was 2.5 µL and the flow rate was 0.4 mL/min. Separation was carried out in 5.5 min under the following conditions: 0 min, 60% B; 4 min, 25% B; 4.1 min, 60%B; 5.5 min, 06% B. The column was equilibrated for 10 min prior to each analysis. The column temperature was maintained at 40 ºC. The MS operated in electrospray ionization (ESI) in negative mode and nitrogen was used as the source gas in all cases.

2.2.4. Statistical analysis

All experiments were assayed in duplicate and three repetitions of each analysis were carried out on each parameter in order to obtain mean values. Analysis of the variance (ANOVA) was performed to compare treatments. Least significant difference (LSD) test was employed to determine differences between means. The confidence interval was set at 0.95 and all results were analyzed using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md).

3. Results and discussion

3.1. Highly-concentrated emulsion optimization

3.1.1. Effect of emulsification method and oil content

Particle size. Among emulsions obtained using HSH, those containing 50% oil presented a lower particle size than those with 10% oil, being ≈ 4.9 µm and ≈ 3.5 µm, respectively (Table 1). This fact could be attributed to the increased packing state of droplets caused by the increased oil concentration, which helps to prevent droplet re-coalescence, resulting in a smaller particle size (Artiga-Artigas et al., 2019). Additionally, other authors have also reported that by increasing the oil concentration, the stress forces during the emulsification process were higher, thereby reducing the particle size of droplets (Briceño et al., 2001). When a MF process was applied to emulsions, those with 50% oil showed a reduced particle size (0.53 µm), which can be considered nanoemulsions. In contrast, those with 10% of oil, presented a higher particle size (0.72 µm).

The particle size of emulsions obtained with HSH+MF were lower than those obtained just with HSH. Such a reduction can be attributed to the inertial forces in turbulent flow along with cavitation that promotes droplet disruption during the microfluidization process. The flow stream by high pressure through microchannels toward an impingement area creates a shearing action, which can provide fine emulsions (Maa & Hsu, 1999; Schultz et al., 2004). Furthermore, the particle size distribution became less polydisperse after the MF process (Figure 1), which has been also observed by other authors studying highly-concentrated emulsions (Artiga-Artigas et al., 2019).

Viscosity. By increasing the oil concentration, the viscosity of emulsions was greatly increased, regardless of the treatment applied (Table 1). Such an increase may be a consequence of the reduced particle size that increased the number of dispersed phase particles in the emulsion causing a high packing state of the droplets as well as the interactions between them (Brinkman, 1952; Krieger & Dougherty, 1959). Indeed, this was confirmed by the microscope images (Figure 1), in which more spherical and packed oil droplets can be observed in the emulsions formulated with 50% rather than 10% oil. Emulsions obtained by HSH+MF presented a higher viscosity than those obtained using HSH (Table 1) which can be attributed to the reduced particle size of the formers.

Table 1. Particle size, ζ-potential and viscosity of emulsions containing 10% or 50% of oil phase formulated using high-shear homogenization (HSH) or high-shear homogenization and microfluidization (HSH+MF).

Emulsion	Particle size (μm)	ζ-potential (mV)	Viscosity (mPa·s)
10% HSH	4.87 ± 0.18 ^{Bb}	-61.32 ± 1.04 ^{Ab}	1.50 ± 0.05 ^{Aa}
50% HSH	3.52 ± 0.06 ^{Ba}	-68.47 ± 2.97 ^{Aa}	26.4 ± 0.32 ^{Ab}
10% HSH+MF	0.72 ± 0.01 ^{Ab}	-60.73 ± 0.93 ^{Ab}	5.04 ± 0.20 ^{Ba}
50% HSH+MF	0.53 ± 0.06 ^{Aa}	-62.96 ± 2.03 ^{Ba}	27.87 ± 0.32 ^{Bb}

Values are expressed as mean ± standard deviation. Different capital letters indicate significant differences ($p<0.05$) between emulsions with different formulation methodology and same oil concentration. Different lowercase letters indicate significant differences ($p<0.05$) between emulsions with different oil concentration and same formulation methodology.

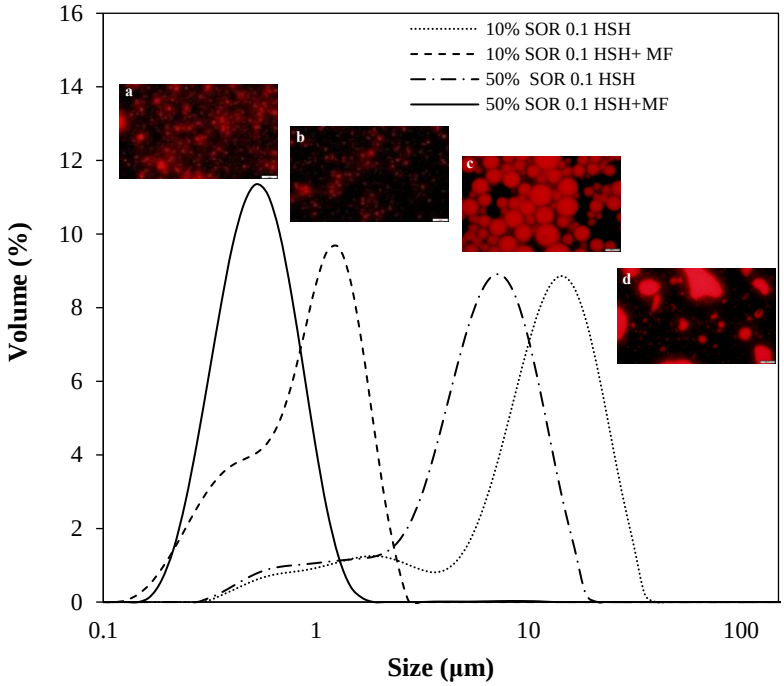

Figure 1. Particle size distribution and microscope images of emulsions containing 10% or 50% of oil phase formulated using high-shear homogenization (HSH) or high-shear homogenization and microfluidization (HSH+MF). (a) Microscope image of HC 50% SOR 0.1 HSH+MF; (b) Microscope image of HC 10% SOR 0.1 HSH+MF; (c) Microscope image of HC 50% SOR 0.1 HSH; (d) Microscope image of HC 10% SOR 0.1 HSH. Scale bars are 10 µm long.

ζ-potential. The electrical charge of all emulsions was negative and values ranged from -61 mV to -68 mV (Table 1). The presence of lecithin molecules at the interface could be the reason of these values, since the majority of their phospholipids were negatively charged at pH 7 (Zhang et al., 2012). However, emulsions with 50% oil presented a more negative value than emulsions with 10% oil (Table 1). The smaller particle size of 50% oil compared to 10% oil, provided a higher surface area in the emulsions. Therefore, emulsions containing 50% oil may present more negatively charged molecules in the interface than those with 10%, as observed using other surfactants such as Tween 80 (Artiga-Artigas et al., 2019).

3.1.2. Effect of surfactant-oil-ratio (SOR)

In view of the results presented in the previous section, for the following study of the effect of SOR (0.05, 0.1 and 0.2) on the stabilisation of HC-emulsions, those obtained by HSH+MF containing 50% oil were used.

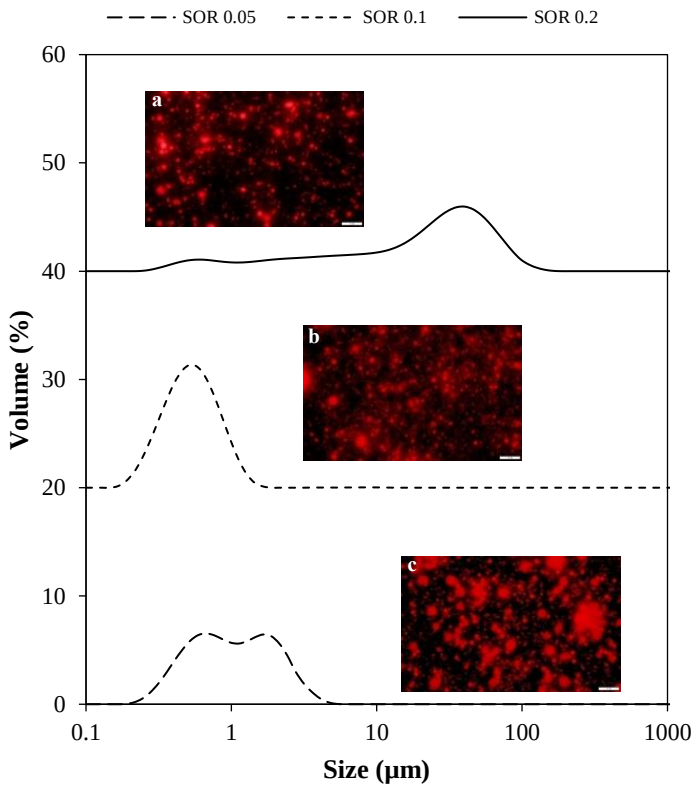

Figure 2. Particle size distribution and microscope images of highly-concentrated (HC) emulsions containing 50% of oil phase and different surfactant-oil-ratio (SOR). (a) Microscope image of HC SOR 0.05; (b) Microscope image of HC SOR 0.1; (c) Microscope image of HC SOR 0.2. Scale bars are 10 µm long.

Particle size. The smallest particle sizes were observed when using 0.1 SOR (≈ 0.53 µm), followed by HC-emulsions containing 0.05 SOR (≈ 0.84 µm) and 0.2 SOR (≈ 3.73 µm) (Table 2). Moreover, the particle size polydispersion increased in the same order: 0.1 SOR < 0.05 SOR < 0.2 SOR (Figure 2). This indicates that 0.1 SOR, which is a 5% of lecithin in an HC-emulsion containing 50% of oil, was the most suitable concentration of surfactant to obtain small particle sizes. It is well known that a higher concentration of surfactant can cover a greater surface area (Luo et al., 2017). However, it is also known that an excess of surfactant can cause the aggregation of oil droplets (An et al., 2014; Dalvi & Dave, 2009). Therefore, it seems that, in the studied HC-emulsions, 0.05 SOR (2.5% soybean lecithin) was not enough to completely cover the total oil surface. Conversely, at 0.2 SOR (10% soybean lecithin), an excess of surfactant that was promoting flocculation of lipid droplets was confirmed in the microscope images (Figure 2).

Table 2. Mean particle size and viscosity of emulsions containing 50% of oil phase and different surfactant-oil-ratio (SOR).

SOR	Particle size (µm)	ζ-potential (mV)	Viscosity (mPa·s)
0.05	0.84 ± 0.14 [b]	-52.83 ± 1.27 [c]	7.89 ± 0.11 [a]
0.1	0.53 ± 0.06 [a]	-62.96 ± 2.03 [b]	27.87 ± 0.32 [b]
0.2	3.73 ± 0.19 [c]	-68.50 ± 2.51 [a]	52.10 ±1.22 [c]

Values are expressed as mean ± standard deviation. Different lowercase letters indicate significant differences ($p<0.05$) between emulsions with different oil concentration and same formulation methodology.

Viscosity. The viscosity of HC-emulsions was increased when the SOR was augmented, being the HC-emulsion with 0.2 SOR those with the highest viscosity (52.10 ± 1.22 mPa·s) (Table 2). It has been previously stated that the particle size governs the viscosity of HC-emulsions (Artiga-Artigas et al., 2019). In our study, this trend was observed in HC-emulsions with 0.05 and 0.1 SOR, being the later the one with the smallest particle size (0.53 ± 0.06 µm) and the highest viscosity (27.87 ± 0.32 mPa·s). Nevertheless, this was not observed in the HC-emulsion with a 0.2 SOR, which presented the highest viscosity although containing the droplets with the highest size and most polydisperse particle size distribution. In this case, the high viscosity may be a consequence of the aggregation of the droplets rather than the particle size.

ζ-potential. As observed in Table 2, the HC-emulsion with 0.2 SOR presented the most negative ζ-potential (-68.50 ± 2.51 mV), followed by those with 0.1 SOR (-62.96 ± 2.03 mV) and 0.05 SOR (-52.83 ± 1.27 mV). When increasing the SOR, the number of lecithin molecules was also increased for the same oil content, so there were more negative charged molecules that could be positioned in the interface contributing to the negative ζ-potential.

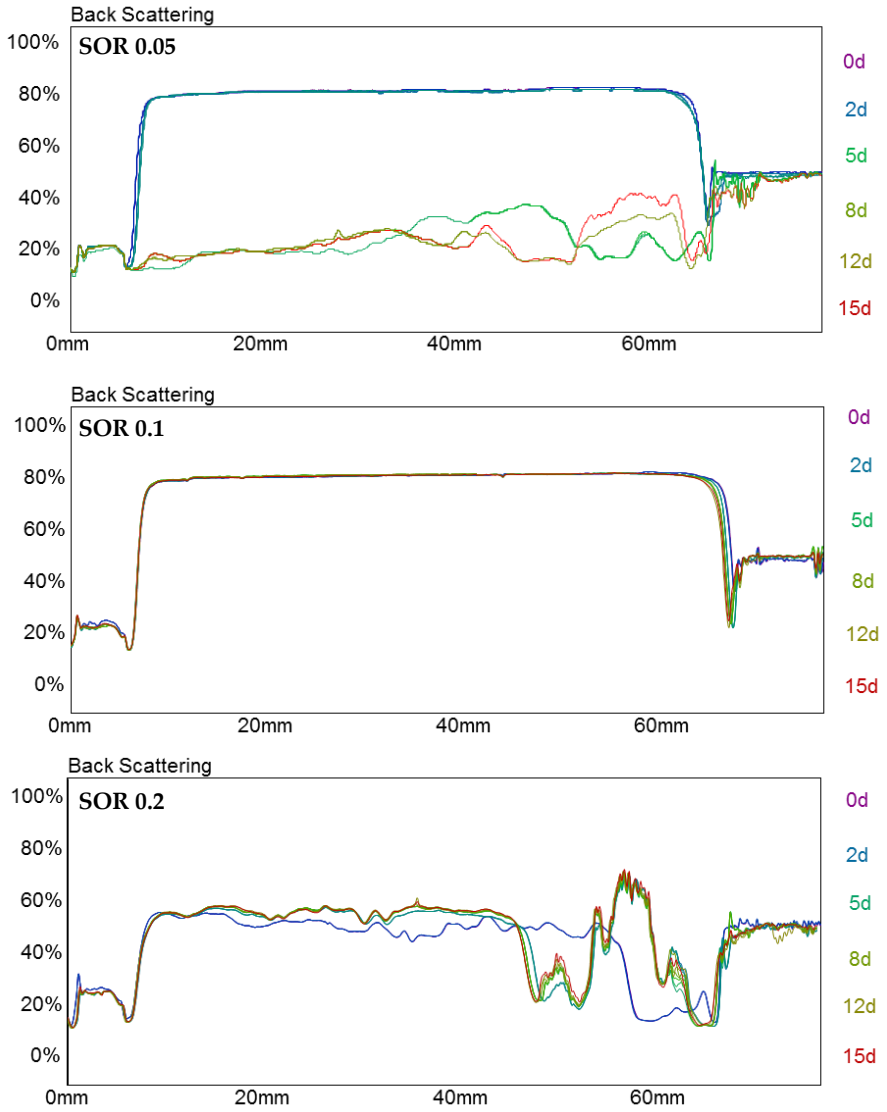

Figure 3. Backscattering profile of curcumin emulsions containing 50% of lipid phase and a different surfactant-oil-ratio (SOR 0.05, SOR 0.1, and SOR 0.2) during 15 days at 4 ºC.

Stability. HC-emulsions with 0.1 SOR showed the highest stability over the 15 days of storage at 4 ºC, presenting no instability phenomena (Figure 3). In this HC-emulsion, it seems that the repulsive interactions generated by lecithin were strong enough to overcome any attractive interactions (van der Waals) between the droplets (Hunter, 2001; Luo et al., 2017). In contrast, both 0.05 and 0.2 SOR HC-emulsions were unstable, being the emulsion with 0.2 SOR the less stable. Indeed, the latter was immediately destabilized after its preparation, which could be attributed to an excess of surfactant that promoted the aggregation of droplets in the emulsion facilitating the destabilization of the system (Figure 1). In that sense, the rates of Ostwald ripening and coalescence in

emulsions have been reported to be higher in the presence of excess surfactant due to micelle-mediated surfactant transport of oil between droplets (Klang & Valenta, 2011; Tadros et al., 2004). In contrast, 0.05 SOR emulsion was stable for two days, and started to present sedimentation from day 5 (Figure 3). In this case, the instability phenomena may be caused by the lack of surfactant to completely cover the surface area of droplets. Therefore, since 0.1 was the most suitable SOR for HC-emulsions containing 50% oil, the following *in vivo* studies were performed using this emulsion.

3.2. *In vivo* studies

3.2.1. Pharmacokinetic study

Rats were administered with a HC-emulsion containing 50% of oil and at 0.1 SOR prepared using HSH+MF (50HC-emulsion) or a control suspension. After the oral administration of the curcuminoid carriers, the plasma of 50HC-emulsion-fed rats presented higher concentrations of all curcuminoids compared to those of suspension-fed rats (Figure 4).

Among the studied curcuminoids, curcumin glucuronide exhibited the highest concentration in rat plasma, being the concentration in 50HC-emulsion-fed rats higher than those of suspension-fed rats (Figure 4). Indeed, the $AUC_{0\text{-}8h}$ of curcumin glucuronide was > 100-fold higher than the $AUC_{0\text{-}8h}$ of the free forms (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) in the case of 50HC-emulsion, and up to 10-fold higher in the case of suspension (Table 3). In that sense, it is suggested that when curcumin is orally administered to rodents, it undergoes intestinal metabolism, as well as rapid first-pass metabolism and excretion in bile (Sharma et al., 2005). In fact, previous studies in rats have reported that curcumin undergoes O-conjugation to curcumin glucuronide and curcumin sulphate, as well as reduction to tetrahydrocurcumin, hexahydrocurcumin and others (Asai & Miyazawa, 2000; Ireson et al., 2001). However, according to previous works, among the metabolites formed, curcumin glucuronide is the major one found in the plasma after oral administration of curcumin to rats, while the free forms are normally negligible (Ozawa et al., 2017; Peng et al., 2018). Unlike curcumin, curcumin conjugates are water soluble, which may explain why a substantial proportion of curcumin in the general circulation exists as

conjugates (Toden & Goel, 2017). Conjugation with glucuronic acid is considered a fundamental mechanism in nature for detoxifying and eliminating lipophilic chemicals from the organism, being the resultant glucuronides less biologically or chemically reactive than their corresponding aglycones (Ritter, 2000). In our study, the concentration of free forms was also low in the plasma of rats after the oral administration through the different carriers (Figure 4).

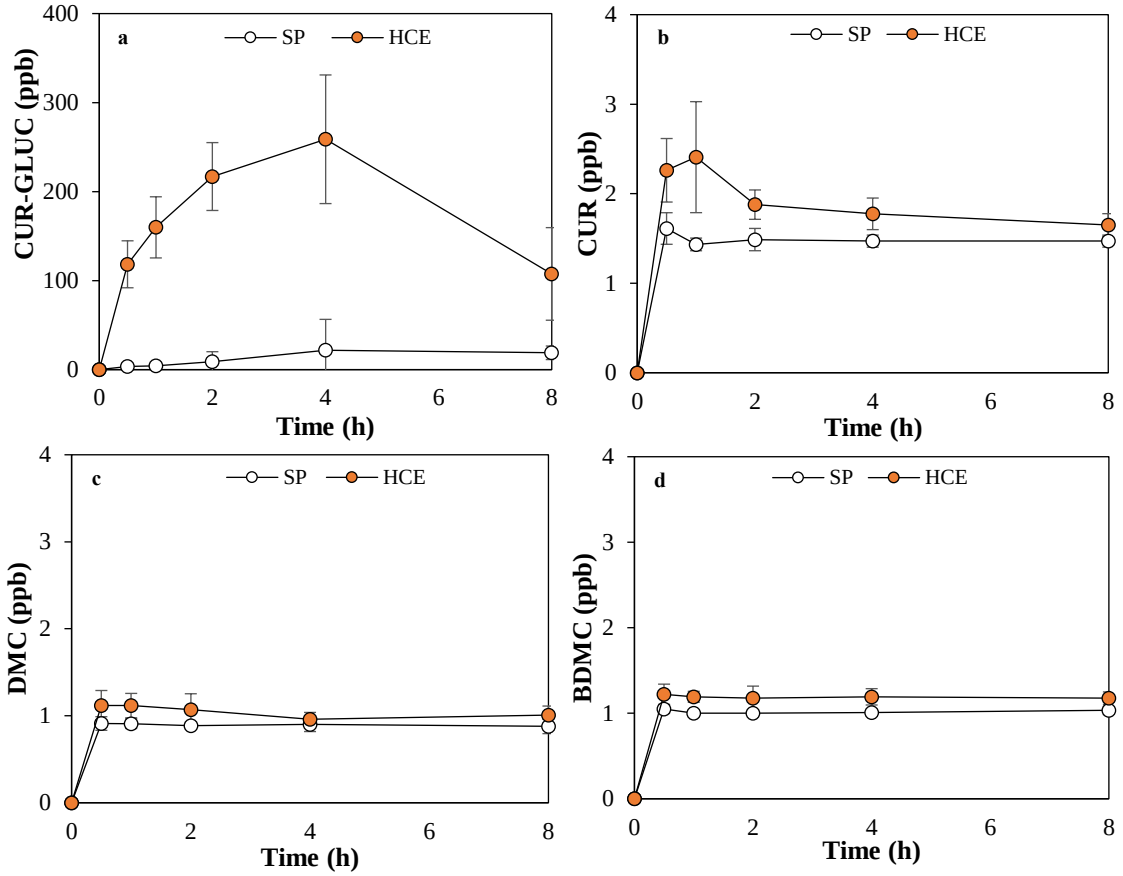

Figure 4. Plasma-concentration times profile of (a) curcumin glucuronide (CUR-GLUC), (b) curcumin (CUR), (c) demethoxycurcumin (DMC) and (d) bisdemethoxycurcumin (BDMC) after oral administration of a single-dose curcumin highly-concentrated emulsion (HCE) or curcumin suspension (SP).

Moreover, the curcumin concentration in plasma (≈ 12-15 ng·h·ml^{-1}) was higher compared to the concentration of demethoxycurcumin and bisdemethoxycurcumin (≈ 7-9 ng·h·ml^{-1}), irrespective of the carrier used. This fact can be attributed to the composition of the administered curcuminoid extract, which contained a higher amount of curcumin (≥ 78%) than demethoxycurcumin (15%) or bisdemethoxycurcumin (1%).

The concentration in plasma of all studied compounds was higher in 50HC-emulsion-fed rats than in suspension-fed rats (Figure 4 and Table 3). Indeed, the $AUC_{0\text{-}8h}$ for curcumin glucuronide was 1556.32 ± 210.44 $ng \cdot h \cdot ml^{-1}$ in rats fed with the 50HC-emulsion, while it was 121.52 ± 137.39 $ng \cdot h \cdot ml^{-1}$ in those fed with the control suspension. However, the differences in the other studied compounds (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) were not as noticeable. As an example, the $AUC_{0\text{-}8h}$ for curcumin was 14.47 ± 0.78 $ng \cdot h \cdot ml^{-1}$ and ≈ 11.52 ± 0.14 $ng \cdot h \cdot ml^{-1}$ in rats fed with the 50HC-emulsion or the control suspension, respectively. Nevertheless, the overall curcuminoid bioavailability was greatly improved (by 10.6-fold) when the compound was enclosed in the 50HC-emulsion rather than in suspension. Values similar to those obtained in our work have been reported by other authors using other curcumin emulsion-based delivery systems. For instance, rice bran protein nanoparticles or organogel-based emulsions showed to improve the oral bioavailability of curcumin by approximately 9.2 or 9.5-fold compared to free curcumin (Liu et al., 2018; Yu & Huang, 2012).

Table 3. Pharmacokinetic parameters of curcumin glucuronide (CUR-GLUC), curcumin (CUR), demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) after oral administration of a single-dose highly-concentrated curcumin emulsion (HCE) or curcumin suspension (SP).

Carrier	Parameters	CUR-GLUC	CUR	DMC	BDMC
HCE	C_{max} $(ng \cdot ml^{-1})$	256.66 ± 68.91 [b]	2.43 ± 0.65 [a]	1.15 ± 0.14 [a]	1.22 ± 0.12 [a]
	T_{max} (h)	4	1	0.5	0.5
	$AUC_{0\text{-}t}$ $(ng \cdot h \cdot ml^{-1})$	1556.32 ± 210.44 [b]	14.47 ± 0.78 [b]	7.88 ± 0.47 [b]	9.19 ± 0.39 [b]
SP	C_{max} $(ng \cdot ml^{-1})$	21.76 ± 34.69 [a]	1.61 ± 0.18 [a]	0.91 ± 0.08 [a]	1.05 ± 0.05 [a]
	T_{max} (h)	4	0.5	0.5	0.5
	$AUC_{0\text{-}t}$ $(ng \cdot h \cdot ml^{-1})$	121.52 ± 137.93 [a]	11.52 ± 0.14 [a]	6.91 ± 0.24 [a]	7.92 ± 0.16 [a]

Data of C_{max} and $AUC_{0\text{-}t}$ are expressed as mean ± standard deviation. $AUC_{0\text{-}t}$ = area under the plasma concentration–time curve; C_{max} = peak concentration; T_{max} = time to reach peak concentration. Different letters (a, b) indicate significant differences ($p < 0.05$) among carriers.

The T_{max} of curcumin was lower using the suspension (0.5 h) than the 50HC-emulsion (1 h) (Table 3). The presence of high quantities of oil (50%) of our HC-emulsion could have retarded the gastric emptying and, therefore, the curcumin absorption. In contrast, the T_{max} of demethoxycurcumin and bisdemethoxycurcumin was 0.5 h for both curcumin vehicles (Table 3), although the concentration in plasma of these compounds was mostly

constant along the studied time (Figure 4). The T_{max} of curcumin glucuronide was 4 h, irrespective of the carrier used (Table 4). This value is higher than that observed for the free forms and may be a consequence of curcumin metabolization. Indeed, a recent *in vivo* study with Caco-2 cell reported that the percentage of phase II metabolites (which includes curcumin glucuronide) increased from ≈ 10% at 0.5 h to > 60% at 4 h post-administration of different emulsions (Luo et al., 2022)

3.2.2. Biodistribution study

The concentration of curcuminoids in the different tissues of rats was quantified at 2 h and 4 h after the oral administration of the curcumin carriers (50HC-emulsion or suspension).

In general, the highest concentration of curcuminoids was observed in the intestinal tissues (Table 4), especially of curcumin, which presented concentrations up to ≈ 841 ng/g tissue. In contrast, the concentration of curcuminoids in non-intestinal tissues (liver, kidney, white and brown adipose tissue) was lower (up to ≈193 ng/g tissue). These observations are in accordance with previous works that have also found the major concentration of curcumin in the intestine after an oral administration of curcumin in combination with a treated soybean lecithin in rats, followed by liver and kidney (Marczylo et al., 2009).

Regarding the intestinal tract, curcumin showed the highest concentration (up to ≈ 841 ng/g), followed by demethoxycurcumin (up to ≈ 233 ng/g), bisdemethoxycurcumin (up to ≈ 48 ng/g) (Table 4), which was expected due to the curcuminoid composition (≥ 78% curcumin, 12% demethoxycurcumin, and 1% bisdemethoxycurcumin).

Curcumin glucuronide was also present in the intestine of rats, especially in the ileum of 50HC-emulsion-fed rats at 4h post-administration (≈ 121 ng/g), which may indicate that some metabolization has occurred there. Previous works have reported the presence UDP-glucuronosyltransferases in the intestinal mucosa of rats, which is the enzyme responsible for curcumin glucuronidation (Asai & Miyazawa, 2000).

The concentration of all curcuminoids was higher in the intestine of rats fed with 50HC-emulsion rather than in those fed with the suspension, especially in the duodenum and

jejunum (Table 4). In that sense, the presence of lecithin could have increased the curcumin absorption by reducing the interfacial surface tension and increasing cell membrane fluidity and the penetration of curcumin through the epithelial cells (Pan-On et al., 2022). In contrast, the low concentration observed using the suspension may be a consequence of the inadequate absorption and avid metabolism of free curcumin that rodents present (Ireson et al., 2002).

As can be observed in Table 4, although curcumin glucuronide was the major metabolite observed in plasma analysis (section 3.2.1), the concentration of this metabolite in the intestinal tissues of rats was low. Indeed, in our work, curcumin glucuronide has been quantified in plasma, intestinal mucosa and kidney (Table 4). These results are in accordance with previous works, which also detected this metabolite in plasma and intestinal mucosa of rats after the oral administration of curcumin with phosphatidylcholine (Marczylo et al., 2007) and can be a consequence of the fast metabolism of curcumin.

The concentration of curcumin, demethoxycurcumin and bisdemethoxycurcumin concentrations in the intestinal tissues was higher at 2 h rather than at 4 h post-administration (Table 4). This indicates that the absorption of the free forms was higher at early times (2 h) and decreased at longer times (4 h). In the same way, previous works reported the highest curcumin concentration at 1 h post-administration, which decreased at 3 h (Suresh & Srinivasan, 2010). Moreover, at 4 h post-administration, suspension-fed rats presented a higher curcumin concentration in the colon than 50HC-emulsion-fed rats. This may indicate that, by using the suspension, more compound was non-adsorbed and arrived at the colon to be excreted. The concentration of curcumin glucuronide in the duodenum and ileum of rats was the same at 2 h and 4 h post-administration. However, a higher concentration was observed in the ileum of 50HC-emulsion-fed rats at 4 h than at 2 h, and no compound was detected in the colon.

Among the non-intestinal tissues, the liver and brown adipose tissue were those that presented the highest curcuminoid concentrations, especially at 2 h post-administration (Table 4). The liver has been seen to be an important accumulation site of curcumin even after being orally or intravenously administered to rodents (Arozal et al., 2019; Ryu et al., 2006; Wei et al., 2014). In our work, curcumin was the main compound present in

the liver of rats, followed by demethoxycurcumin, bisdemethoxycurcumin, and finally, curcumin glucuronide, which was non detected in this organ. Although the liver is an important glucuronidation site in humans, previous works have postulated that most of the orally administered curcuminoids are conjugated to glucuronides in the intestinal mucosa of rats (Asai & Miyazawa, 2000). In fact, previous authors have investigated the capacity of rat hepatocytes to generate different curcumin metabolites, reporting that glucuronides are generated only in small amounts in the liver, whereas they are abundant in plasma after oral administration (Ireson et al., 2001). These authors stated that rat liver reduces curcumin to hexahydrocurcumin and to hexahydrocurcuminol, whereas conjugation of curcumin is only a minor hepatic biotransformation route.

Interestingly, no differences were observed between the concentration of curcumin present in the kidney of suspension-fed rats and 50HC-emulsion-fed rats, although curcumin absorption was much higher in the case of 50HCemulsion-fed rats (Table 4). This may indicate that, in suspension-fed rats, the rate of curcumin excreted with respect to absorbed curcumin was greater than in the case of 50HC-emulsion-fed rats. Additionally, the kidney was the unique non-intestinal tissue in which curcumin glucuronide was detected (Table 4), probably because is the main organ related to its excretion from the organism.

In white and brown adipose tissues, free form curcuminoids were also detected (Table 4), meaning that these compounds can reach these tissues in the active form, which present more therapeutic properties (Pandey et al., 2020). However, the values were higher in the brown adipose tissue (192.94 ± 11.69 ng curcumin/g tissue) rather than in the withe adipose tissue (9.43 ± 4.49 ng curcumin/g tissue). Moreover, in the case of brown adipose tissue, rats fed with the 50HC-emulsion presented a higher curcuminoid accumulation rather than rats fed with the suspension. Previous authors have stated that the presence of curcumin in brown and white adipose can play a relevant role in the prevention of obesity and its related metabolic disorders. It has been seen that curcumin can increase the expression of uncoupling protein 1 (UPC1) in the brown adipose tissue, which has been related to an increased energy expenditure. Moreover, it can also decrease inflammation in white adipose tissue (Santos et al., 2023; Song et al., 2018; Zhu et al., 2021).

Table 4. Curcumin (CUR), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) and curcumin glucuronide (CUR-GLUC) concentration in different rat tissues at 2 h or 4 h after the oral administration of curcumin suspension (SP) or curcumin highly-concentrated emulsions (HCE).

	Vehicle	Time (h)	Concentration in tissue (ng/g tissue)							
			Duodenum	*Jejunum*	*Ileum*	*Colon*	*Liver*	*Kidney*	*Brown adipose*	*White adipose*
CUR	*HCE*	*2*	562.36 ± 168.27 ^{Bb}	587.61 ± 140.85 ^{Bb}	840.69 ± 116.24 ^{Bb}	132.15 ± 117.73 ^{Ab}	129.35 ± 52.07 ^{Ab}	13.87 ± 5.77 ^{Aa}	192.94 ± 11.69 ^{Bb}	9.43 ± 4.49 ^{Aa}
		4	229.19 ± 128.9 ^{Ab}	212.79 ± 116.37 ^{Ab}	545.48 ± 207.90 ^{Ab}	444.58 ± 153.30 ^{Ba}	18.61 ± 23.41 ^{Aa}	10.75 ± 2.46 ^{Aa}	44.70 ± 24.75 ^{Aa}	17.91 ± 6.87 ^{Ab}
	SP	*2*	27.66 ± 11.79 ^{Aa}	68.83 ± 49.11 ^{Aa}	481.27 ± 110.61 ^{Ba}	12.00 ± 9.57 ^{Aa}	21.86 ± 16.27 ^{Aa}	23.03 ± 10.29 ^{Aa}	39.42 ± 31.34 ^{Aa}	10.66 ± 3.98 ^{Aa}
		4	20.15 ± 15.34 ^{Aa}	29.34 ± 19.20 ^{Aa}	200.92 ± 89.47 ^{Aa}	482.16 ± 277.71 ^{Ba}	10.62 ± 10.56 ^{Aa}	16.16 ± 10.04 ^{Aa}	30.22 ± 6.74 ^{Aa}	6.22 ± 2.35 ^{Aa}
DMC	*HCE*	*2*	136.02 ± 37.35 ^{Bb}	190.63 ± 67.90 ^{Bb}	232.85 ± 89.39 ^{Aa}	36.52 ± 29.19 ^{Aa}	20.54 ± 9.01 ^{Ab}	3.32 ± 2.06 ^{Aa}	103.79 ± 51.37 ^{Bb}	4.91 ± 0.98 ^{Aa}
		4	39.57 ± 23.70 ^{Ab}	46.37 ± 32.39 ^{Ab}	174.90 ± 47.83 ^{Aa}	123.20 ± 57.76 ^{Ba}	10.24 ± 4.96 ^{Aa}	1.99 ± 1.25 ^{Aa}	28.32 ± 21.77 ^{Aa}	5.77 ± 1.13 ^{Aa}
	SP	*2*	7.11 ± 3.29 ^{Aa}	17.43 ± 11.13 ^{Aa}	158.55 ± 51.09 ^{Aa}	10.01 ± 3.79 ^{Aa}	10.45 ± 2.49 ^{Aa}	5.22 ± 4.54 ^{Aa}	14.70 ± 4.78 ^{Aa}	6.17 ± 1.31 ^{Aa}
		4	7.74 ± 4.74 ^{Aa}	12.01 ± 6.28 ^{Aa}	90.74 ± 50.87 ^{Aa}	188.48 ± 39.25 ^{Ba}	8.49 ± 1.05 ^{Aa}	3.76 ± 1.38 ^{Ab}	14.45 ± 9.33 ^{Aa}	5.64 ± 1.11 ^{Aa}
BDMC	*HCE*	*2*	32.66 ± 11.29 ^{Ab}	47.10 ± 17.24 ^{Bb}	47.79 ± 18.46 ^{Aa}	10.19 ± 6.98 ^{Ab}	7.10 ± 2.15 ^{Bb}	2.57 ± 1.14 ^{Aa}	12.47 ± 2.07 ^{Bb}	3.33 ± 0.42 ^{Aa}
		4	12.28 ± 9.79 ^{Ab}	4.37 ± 8.61 ^{Ab}	19.61 ± 10.79 ^{Aa}	22.01 ± 8.85 ^{Aa}	3.87 ± 0.61 ^{Aa}	1.87 ± 1.25 ^{Aa}	3.88 ± 1.27 ^{Aa}	3.18 ± 1.62 ^{Aa}
	SP	*2*	0.47 ± 1.42 ^{Aa}	0.71 ± 1.39 ^{Aa}	51.63 ± 14.37 ^{Aa}	3.80 ± 0.56 ^{Aa}	4.28 ± 0.78 ^{Aa}	6.93 ± 1.91 ^{Ab}	4.09 ± 2.12 ^{Aa}	3.94 ± 0.85 ^{Aa}
		4	0.35 ± 0.75 ^{Aa}	0.15 ± 0.31 ^{Aa}	41.91 ± 18.15 ^{Ab}	26.36 ± 9.61 ^{Ba}	4.29 ± 0.99 ^{Aa}	3.91 ± 1.85 ^{Aa}	5.78 ± 1.40 ^{Aa}	4.64 ± 1.67 ^{Aa}
CUR-GLUC	*HCE*	*2*	7.45 ± 1.36 ^{Aa}	14.21 ± 5.79 ^{Aa}	60.83 ± 24.65 ^{Aa}	ND	ND	11.79 ± 2.40 ^{Ba}	ND	ND
		4	7.02 ± 0.64 ^{Ab}	13.33 ± 4.18 ^{Aa}	120.93 ± 48.28 ^{Ab}	ND	ND	ND ^{Aa}	ND	ND
	SP	*2*	6.06 ± 2.89 ^{Ba}	8.91 ± 0.80 ^{Aa}	44.52 ± 20.40 ^{Aa}	ND	ND	11.00 ± 1.81 ^{Ba}	ND	ND
		4	ND ^{Aa}	8.92 ± 1.35 ^{Aa}	33.82 ± 13.93 ^{Aa}	ND	ND	ND ^{Aa}	ND	ND

Values are expressed as mean ± standard deviation. Different capital letters (A, B) indicate significant differences ($p < 0.05$) among curcumin carriers at a same time. Different lowercase letters (a, b) indicate significant differences ($p < 0.05$) among same carrier and different time.

4. Conclusions

Stable highly-concentrated emulsions were formulated using MCT oil and lecithin at a surfactant-oil ratio of 0.1. After orally administered to rats, the pharmacokinetic study revealed that curcumin in plasma was mainly found in the glucuronide form, being 4 h the time of maximum concentration. Moreover, the encapsulation of curcuminoids in a highly-concentrated emulsion improved their bioavailability by 10.6-fold compared to the suspension. The biodistribution study exposed that the presence of curcumin, as well as demethoxycurcumin and bisdemethoxycurcumin in the small intestine of rats was higher in emulsion-fed rats rather than in suspension-fed rats, indicating an improved absorption by the intestinal cells. Furthermore, enclosing curcumin in highly-concentrated emulsions increased its accumulation in free form in the liver and brown adipose tissue, which could have relevant implications in the prevention or treatment of metabolic diseases. The increased absorption by using the emulsion may be a consequence of (1) the protection that emulsions provide against degradation during its pass through the gastrointestinal tract, and (2) the capacity of surfactants such as lecithin to improve the membrane fluidity and the penetration of curcumin through the epithelial cells. This work highlights the potential of highly-concentrated emulsions to increase the bioavailability of curcumin and increase its accumulation in the organs in the free form, which presents the most biological activity.

Keywords: Curcumin, highly-concentrated emulsions, bioavailability, biodistribution.

Author Contributions

Conceptualization, J.T, G.O, M.A, I.O and O.M.; methodology, J.T, G.O, M.A, I.O and O.M.; formal analysis, J.T and M.A.; investigation, J.T and M.A.; resources, O.M.; writing—original draft preparation, J.T and G.O ; writing—review and editing, J.T, G.O, M.A, I.O and O.M.; visualization, J.T, G.O, M.A, I.O and O.M.; supervision, G.O, I.O and O.M.; project administration, O.M.; funding acquisition, O.M. “All authors have read and agreed to the published version of the manuscript.”

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Conflicts of Interest

The authors declare no conflict of interest.

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Chapter VII

In vivo oral bioavailability and biodistribution of curcuminoids encapsulated in solid- and liquid- lipid nanoparticles as affected by the type of oil

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In preparation

Abstract

Curcumin is a highly lipophilic compound with multiple biological functions, but it is easily degraded and has low bioavailability. Encapsulation in emulsion-based systems has been shown to significantly increase the bioavailability of this compound. However, *in vivo* studies about how the lipid state and its composition can modify the functionality of these systems are lacking. Therefore, this study aimed to investigate the effect of the lipid state and lipid type of nanoparticles on curcuminoid absorption and biodistribution. The studied nanoparticles were: CO-LLN (corn oil liquid lipid nanoparticles), MG-LLN (miglyol liquid lipid nanoparticles), HPO-SLN (hydrogenated palm oil solid lipid nanoparticles), and CN-SLN (coconut oil solid lipid nanoparticles). After the oral administration of nanoemulsions (< 200 nm), the study on microstructural changes during digestion showed that those containing MCT were digested more rapidly than those formulated with LCT. Moreover, HPO-SLN presented a slowed digestion rate due to the high melting point of HPO. The presence of curcuminoids in the rat intestines was as follows: CO-LLN > HPO-SLN = MG-LLN = CN-SLN. Moreover, CO-LLN presented the highest concentrations of curcumin glucuronide in plasma (1613 ng/mL) and of free curcumin in brown adipose tissue (up to 1402 ng/g). Therefore, not only the lipid type, but also the lipid state can influence the absorption and biodistribution of curcuminoids, being CO-LLN those with more potential to be used as curcuminoid-carriers.

Keywords: bioavailability, curcumin, nanoparticles, lipid state, lipid type

1. Introduction

Curcumin is a highly lipophilic polyphenolic compound present in the rhizomes of Curcuma longa (turmeric). Numerous studies have revealed its multiple beneficial biological activities, including antioxidant, anti-inflammatory and anti-cancer activities (Strimpakos & Sharma, 2008). However, this compound presents molecular instability, poor solubility in water, rapid conjugation to hydrophilic molecules (like glucuronic acid and sulphate) and poor enteral absorption, which limit its utility as a health-promoting agent (Anand et al., 2007). To overcome these challenges, many authors have investigated the encapsulation of curcumin in various emulsion-based systems. Several *in vivo* studies have revealed that, through the incorporation of this compound in emulsion-based delivery systems, such as emulsions or nanoemulsions its bioavailability increased considerably (Hu et al., 2012; Liu et al., 2017; Lu et al., 2018; Nazari-Vanani et al., 2017). In addition, it has been seen that there are factors, such as the composition of the lipid used in the formulation of these systems and its state of crystallization (solid or liquid), that could affect the properties of the emulsions. According to previous works, emulsions with a solid lipid state can better protect the encapsulated lipophilic bioactive compounds by reducing diffusion processes between the aqueous and the lipid core (Nik et al., 2012; Salvia-Trujillo et al., 2019). Nanoemulsions with solid nanoparticles seem to increase the control over the release and stability of the encapsulated bioactives since by controlling the physical state of the lipid matrix the mobility of the encapsulated compounds can be modulated (Weiss et al., 2008). Indeed, investigations using *in vitro* gastrointestinal digestion revealed that not only the lipid crystallization state but also the lipid type had a significant impact on the lipid digestibility and compound bioaccessibility (de Abreu-Martins et al., 2020). Many *in vitro* studies have stated that the fatty acid chain length and the unsaturation degree affects the rate and extent of lipid digestibility, as well as the bioaccessibility of the encapsulated lipophilic compounds such as curcumin since they can alter the composition and structural characteristics of the mixed micelles formed (Jiang & Charcosset, 2022; Majeed et al., 2016; Zhang et al., 2015). In addition, a recently published study using excipient nanoemulsions revealed that the trans-enterocyte transport of curcumin in Caco-2 cells was highly dependent on the lipid type used to formulate the systems (Luo et al., 2022).

However, there is a lack of *in vivo* studies reporting the effect of lipid composition and crystallization state of nanoemulsions on curcuminoid absorption and organ biodistribution. Therefore, this study aimed to investigate the effect of the lipid composition (MCT or LCT) and the lipid crystallization state (liquid or solid) of nanoparticles enclosing curcumin on the absorption, metabolization and biodistribution of the encapsulated compound in rats.

2. Materials and methods

2.1. Materials

Curcumin was purchased from Acros Organics (Thermo Fischer Scientific, India). Miglyol® 812 N was obtained from IOI Oleochemical GmbH (Hamburg, Germany). Hydrogenated palm oil was acquired from Mystic Moments (Hants, UK). Corn oil was purchased from local market (Spain) and coconut oil from HMF Food Production (GmbH & Co. KG, Germany). Tween 80 (Polyoxyethylenesorbitan monoesterate) was used as a food-grade hydrophilic emulsifier from Lab Scharlab (Sentmenat, Spain). The nanoemulsions were prepared using ultrapure milli-Q water supplied by a Millipore filtration system (18.2 mΩ.cm, Merck Millipore, Madrid, Spain).

2.2. Methods

2.2.1. Formation of nanoemulsions containing solid- or liquid- lipid nanoparticles

To obtain the lipid phase, curcumin (6% w/w) was dispersed in the different carrier lipids: miglyol (MG), corn oil (CO), coconut oil (CN), or hydrogenated palm oil (HPO), while magnetically stirring at 450 rpm for 30 min at 60 °C. Following this, 15 minutes of sonication were used to ensure complete dissolution. To prepare the aqueous phase, 2.5% w/w of Tween 80 in milli-Q water were stirred at 60 °C. Afterwards, both the lipid phase (21% w/w) and aqueous phase (79% w/w) were mixed and homogenized using an Ultra-Turrax (IKA, Staufen, Germany) at 7600 rpm for 3 min. Then, nanoparticles were obtained by passing the coarse emulsions through a microfluidizer (LM10, Microfluidics, USA) at 100 MPa for 3 cycles. Finally, the temperature of nanoparticles was reduced to 4 °C for 2 h in order to allow fat recrystallization. As a result, 4 different nanoemulsions were obtained: CO-LLN (corn oil liquid lipid nanoparticles), MG-LLN

(miglyol liquid lipid nanoparticles), HPO-SLN (hydrogenated palm oil solid lipid nanoparticles), and CN-SLN (coconut oil solid lipid nanoparticles).

2.2.2. Nanoparticle characterization

Particle size. The particle size of LLNs and SLNs was measured using a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd., Worcestershire, U.K.) operating at 633 nm, 25 °C, and equipped with a backscatter detector (173°). Nanoemulsions were characterized by average droplet diameter (nm) and intensity (%). Prior to measurement, samples were diluted in milli-Q water at a ratio of 1:100.

ζ-potential. The ζ-potential was measured by phase-analysis light scattering (PALS) using Zetasizer laser diffractometer (Malvern Instruments Ltd Worcestershire, UK). Prior to the analysis, liquid or solid lipid nanoparticles were diluted (1:100) in ultrapure water. The results were reported in millivolts (mV).

Physical stability. Stability of nanoemulsions was studied using an optical scan analyzer Turbiscan MA 2000 (Formulaction, Toulouse, France), which can measure the static stability of samples without destruction and detect the cause of instability (flocculation, coalescence, sedimentation or creaming) by the multiple light scattering technique. A sample of 7 mL was introduced into a glass cylindrical cell and analyzed by a light beam emitted in near infrared wavelength, which scanned vertically from the bottom to the top the sample cell. Two synchronous optical sensors receive light backscattered by the sample (45° from the incident radiation). In this study, a monitoring period of 18 h was conducted to determine physical stability.

2.2.3. In vivo study

2.2.3.1. Animals and study design

Female Sprague Dawley rats weighing 200-250 g were used for the *in vivo* study. The animal procedures were conducted in accordance with EU Directive 2010/63/EU for animal experiments and approved by the Animal Ethics Committee of Universitat de Lleida (CEEA 01-04/18). After an acclimatization period, animals were randomly divided into four groups (n=4) (CO-LLN, MG-LLN, HPO-SLN, CN-SLN). Rats were

fasted for 12 h before the experiment with free access to water. Rats were anesthetised with isoflurane and blood samples were taken by cardiac puncture. Plasma was immediately separated by centrifuging the blood samples at 4000 rpm for 10 min at 4 ºC and stored at -80 ºC. Rats were sacrificed by exsanguination and the stomach, duodenum, jejunum, ileum and colon were removed and their content was collected and immediately characterized by microscopy. Then, the mentioned tissues, liver, kidney, white adipose tissue (mesenteric) and brown adipose tissue (cervical) were collected, rinsed with phosphate buffered saline, weighted and stored at -80 ºC.

2.2.3.2. Dosage information

Rats were orally administered at a volume of 20 mL/kg with a feeding needle. All animals were fed with a dose of 250 mg of curcumin/kg body weight, irrespective of the vehicle used, which is only achievable through supplements in humans. Sample administration was conducted in five times with 30 min intervals. Rats were sacrificed 30 min after the last dose administration.

2.2.3.3. Curcuminoid extraction and quantification in plasma and tissues

Extration from plasma. Curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin glucuronide extraction in plasma samples was performed according to a previously reported method with some modifications (Li et al., 2011). Aliquots of 150 µL of plasma were mixed with the internal standard (Honokiol) and 600 mL of acetonitrile and vortexed at 1800 rpm for 1 min. Then, the mixture was submerged intro an ultrasonic bath for 30 sec, centrifuged at 9000 rpm for 10 min at 4 ºC, and the organic fraction was collected. Afterwards, the collected upper organic layers were evaporated under N_2 and stored at -80 ºC until quantification.

Extraction from tissues. Curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin glucuronide extraction in tissue samples was performed according to a previously reported method with some modifications (Chirio et al., 2019). Initially, tissue samples were mixed with milli-Q water and homogenized with an Ultra-Turrax at 9000 rpm for 1 min to obtain the tissue homogenates. Then internal standard (Honokiol) and 1 mL of acetonitrile were added to tissue homogenates and the mixture was vortexed

at 2000 rpm for 1 min. Then, the mixture was centrifuged at 9000 rpm for 5 min at 4 ºC, and the organic fraction was collected. Afterwards, the collected upper organic layers evaporated under N_2 and stored at -80 ºC until quantification.

Curcuminoids quantification. The quantification of curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin glucuronide was performed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI–MS/MS) using the method described by Liu et al. (2018), with some modifications. To perform the analyses an ACQUITY UPLC binary (Waters, Milford, MA, USA) coupled to a Xevo TQS (triple quadrupole) (Waters, Milford, MA, USA) was used. Chromatographic separation was performed on a 150 mm × 2.1 mm i.d., 1.6 µm CORTECS Phenyl Column (Waters, Milford, MA, USA) with mobile phase A [acetonitrile containing 0.1% (v/v) formic acid] and mobile phase B [98% water and 2% acetonitrile containing 0.1% (v/v) formic acid]. The injection volume was 2.5 µL and the flow rate was 0.4 mL/min. Separation was carried out in 5.5 min under the following conditions: 0 min, 60% B; 4 min, 25% B; 4.1 min, 60% B; 5.5 min, 06% B. The column was equilibrated for 10 min prior to each analysis. The column temperature was maintained at 40 ºC. The MS operated in electrospray ionization (ESI) in negative mode and nitrogen was used as the source gas in all cases.

2.2.4. Fluorescence optical microscopy

Digesta from the different parts of the gastrointestinal tract of rats was collected and dyed with Nile Red, previously dissolved at 0.1% (w/v) in ethanol, to observe SLNs and LLNs. Then, micrographs were obtained using fluorescence with an optical microscope (Olympus BX41, Olympus, Göttingen, Germany) with a 100x objective lens. The images were obtained using a digital camera (Olympus DP74) and processed with the software CellSens (Olympus Göttingen, Germany).

2.2.5. Statistical analysis

All experiments were assayed in duplicate and three repetitions of every analysis were carried out on each parameter in order to obtain mean values. Analysis of the variance (ANOVA) was performed to compare treatments. Least significant difference (LSD) test

was employed to determine differences between means. The confidence interval was set at 0.95 and all results were analyzed using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md).

3. Results and discussion

3.1. Characterization of solid lipid (SLN) and liquid lipid (LLN) nanoparticles

Particle size. Nanoemulsions containing liquid nanoparticles (CO-LLN or MG-LLN) had significantly smaller initial particle sizes (≈ 158-169 nm) than those with solid nanoparticles (HPO-SLN or CN-SLN) (≈ 174-181 nm). CO and MG lipids have a reduced viscosity at room temperature, so less energy was required to disrupt the oil droplets, resulting in smaller droplets (de Abreu-Martins et al., 2020). Nanoemulsions with CN-SLN also presented smaller sizes than those with HPO-SLN particles probably because CN is mainly composed of lauric acid, which confers high polarity and low dipole interactions. These characteristics result in a reduced viscosity and a very low contact angle, which may facilitate droplet disruption during nanoemulsification. Moreover, the straight chain of lauric acid can also facilitate particle size reduction (Sarheed et al., 2020). Moreover, nanoemulsions with MG-LLN also showed smaller droplet sizes than those with CO-LLN. As stated by Sarheed et al. (2020), shorter oil chains allow oil molecules to penetrate the interfacial film, resulting in small particles.

ζ-potential. Nanoemulsions with MG-LLN, CO-LLN and CN-SLN had ζ-potential values of -27.9 ± 8.1 mV, -27 ± 7.4 mV, and -20.1 ± 1.4 mV, respectively (Table 1). The surfactant used in this study, Tween 80, is a non-ionic emulsifier that, at certain pH levels, causes oil droplets to have a negative charge (de Abreu-Martins et al., 2020). Furthermore, oils and surfactants preferentially absorb negative hydroxyl ions from the aqueous phase (Salvia-Trujillo et al., 2013a). The ζ-potential of nanoemulsions with CN-SLN was slightly but not significantly ($p < 0.05$) less negative than that of CO-LLN and MG-LLN, which was consistent with previous findings (de Abreu-Martins et al., 2020). Due to its high saturated fat content, CN has a higher melting point (25.5 °C) than CO (-16 °C) and MG (6 °C). Due to this, the particles had semisolid or solid properties at room temperature, which resulted in larger particle sizes upon nanoemulsification (Table 1). Those large nanoparticles presented a low surface area that was probably fully coated

with surfactant molecules. Interfaces that are effectively covered with non-ionic Tween 80 will have a reduced ζ-potential. According to Sarheed et al. (2020), as surfactant concentrations exceed a critical value, OH-groups can be suddenly expelled from the O/W interface, reducing zeta potential. Systems with HPO-SLN had a positive zeta potential value of 26.3 ± 2.19 mV which is caused by hydrogen ions at the O/W interface.

Table 1. Physical properties, and stability of nanoemulsions nanoemulsions containing containing liquid lipid (LLN) or solid lipid (SLN) nanoparticles containing different lipid types: corn oil (CO), miglyol (MG), palm oil (HPO) or coconut oil (CN).

Lipid state	Lipid type	Zeta potential (mv)	Droplet size (nm)	Sedimentation thickness (mm)	Instability rate × 10^3 (%BS·h^{-1})
LLN	*CO*	-27.00 ± 7.49^b	169.40 ± 0.85^c	0.43	-2.18
LLN	*MG*	-27.93 ± 8.11^b	157.53 ± 1.16^d	1.49	-4.93
SLN	*HPO*	26.30 ± 2.19^a	180.73 ± 0.02^a	0.79	-1.81
SLN	*CN*	-20.13 ± 1.45^b	174.33 ± 0.01^b	0.88	-2.53

Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$) among curcumin nanoparticles. CO: corn oil. MG: miglyol. HPO: hydrogenated palm oil. CN: coconut oil.

Stability. For all nanoemulsions, the BS in the middle zone of the test tube decreased approximately by 0.1%, indicating high colloidal stability (Figure 1) which indicates that the surfactant used was able to stabilize the interfacial area. However, the BS continued to decrease during the 18-hour period, indicating particle aggregation. Except for the nanoemulsion containing MG-LLN, BS spectra in the middle zone of the test tube were parallel to straight lines, indicating: (i) gradients of particle concentration along the vial are constant, and (ii) variations are dominated by particle aggregation. For nanoemulsions with MG-LLN, the inclination of the backscattering spectra indicates a concentration gradient of particles, caused by higher sedimentation. Accordingly, the higher sediment thickness of those with MG-LLN can be identified by a hill with a diameter of about 1.5 mm at the bottom of the vial (Figure 1A and Table 1).

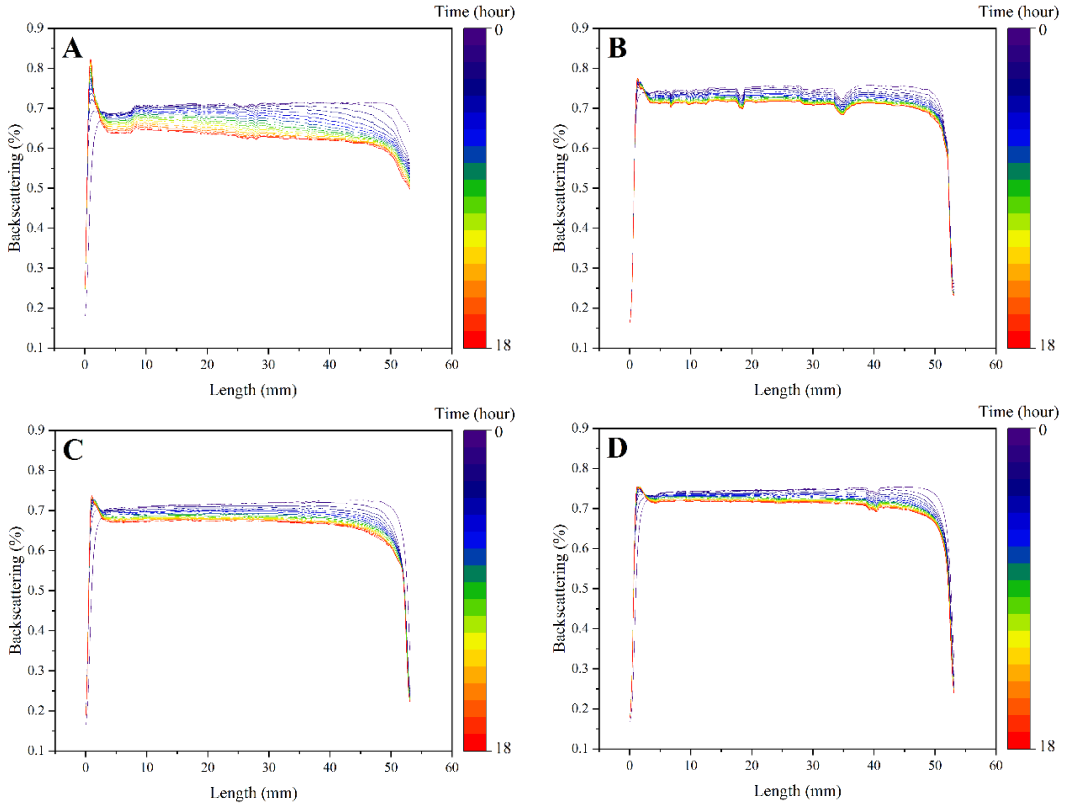

Figure 1. Backscattering profiles of nanoemulsions containing containing liquid lipid (LLN) or solid lipid (SLN) nanoparticles containing different lipid types: corn oil (CO), miglyol (MG), palm oil (HPO) or coconut oil (CN) scanned every 1 hour for 18 hours. (A) MG-LLN; (B) CO-LLN; (C) CN-SLN; (D) HPO-SLN.

For a better understanding of colloidal changes, the backscattering variation at a length of 25 mm was also illustrated (Figure 2). Nanoemulsions with MG-LLN showed the highest instability, followed by those with CN-SLN, CO-LLN, and HPO-SLN, respectively. Since excess of Tween 80 effectively coated the O/W interfaces, the difference in stability cannot be explained by recoalescence. In a nanoemulsification process, droplet breakup depends on the shear rate, surfactant type/concentration, and relative viscosities of the dispersed and continuous phases. All nanoemulsions undergo the same shear and surfactant type/concentration, so the only remaining possibility is a difference in the viscosity of the four oil phases. MG has a lower viscosity (30 mPa·s) than CO (53.7 mPa·s), CN (55 mPa·s) and HPO (solid at room temperature). As a result, MG-LLN has a lower differential viscosity than other LLN and SLN, accelerating sedimentation according to Stokes' law.

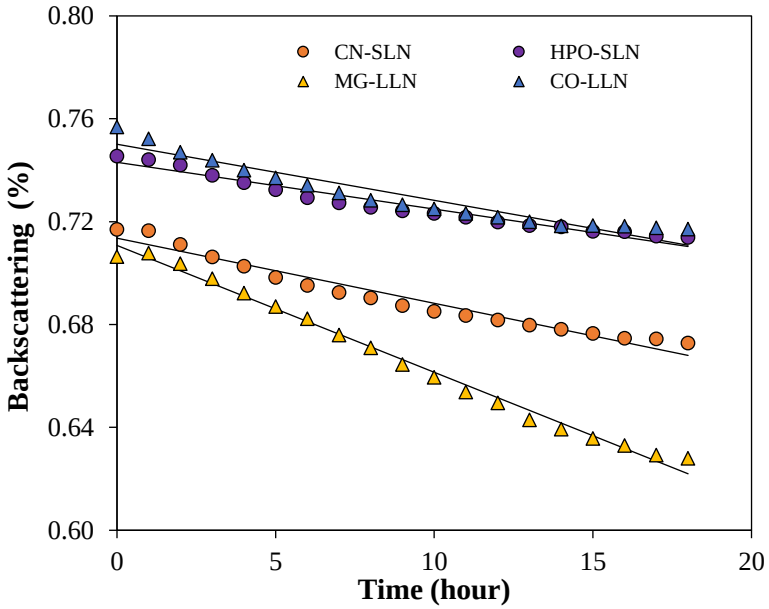

Figure 2. Backscattering profiles of nanoemulsions containing containing liquid lipid (LLN) or solid lipid (SLN) nanoparticles containing different lipid types: corn oil (CO), miglyol (MG), palm oil (HPO) or coconut oil (CN) at a vial length of 25 mm. Symbols represent experimental data points, and solid lines.

3.2. *In vivo* study

3.2.1. Microstructural changes in rat digesta

The changes in the microstructure of the studied LLN (CO-LLN and MG-LLN) and SLN (HPO-SLN and CN-SLN) were studied by obtaining the digesta from the different parts of the gastrointestinal tract of the rats (stomach, duodenum, jejunum, ileum and colon) and analysing them using a fluorescence optical microscopy.

As observed in Figure 3, all nanoparticles suffered a noticeable increase in the particle size in the stomach. Indeed, the initial particle size of nanoemulsions was about 170 nm (Table 1), while in the stomach of rats, the particles were > 1 μm, irrespective of the lipid state and lipid type. However, dispersed particles were observed in the gastric medium, meaning that they were stable in the gastric environment. This fact can be a consequence of the emulsifier selected to formulate the nanoemulsions, which was Tween 80. Small molecule surfactants such as Tween 80 have been seen to be acid stable, maintaining better the microstructure stability than others such as sucrose palmitate (Marciani et al., 2006; Verkempinck et al., 2018).

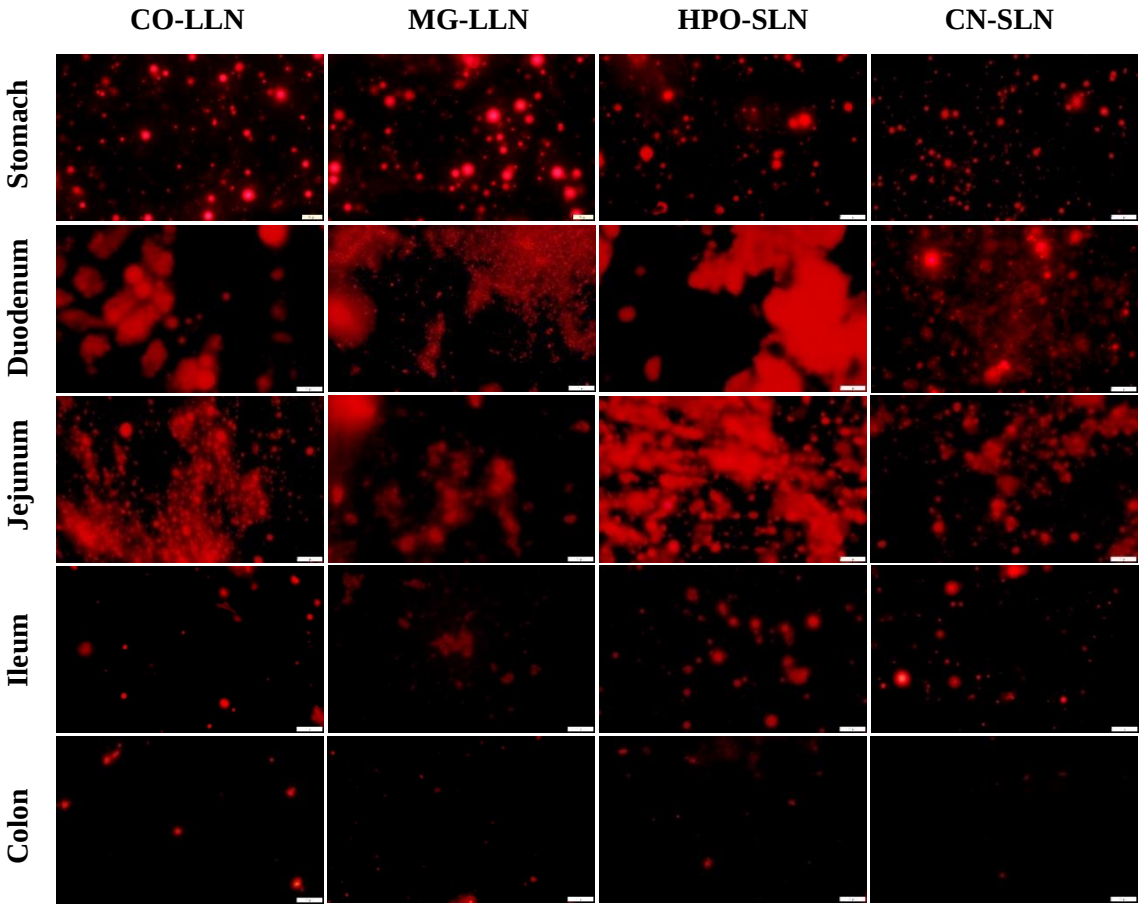

Figure 3. Micrographs of nanemulsions containing solid lipid nanoparticles (SLN) or liquid lipid nanoparticles (LLN) containing different lipid types: corn oil (CO), miglyol (MG), palm oil (HPO) or coconut oil (CN) at the different parts of the gastrointestinal tract of rats after oral administration. Scale bars are 10 μm long.

Differences were observed in the intestinal digesta obtained from rats fed with different nanoemulsions, especially in the duodenum and jejunum (Figure 3). Digesta from rats fed with nanoemulsions containing LCT nanoparticles (CO or HPO) presented more lipid content in the duodenum and jejunum and with a higher particle size than those fed with nanoemulsions containing MCT nanoparticles (MG or CN). This suggests that nanoparticles containing MCT were digested faster than those with LCT. This trend has been observed in other emulsion-based delivery systems and can be a consequence of the capacity of medium-chain fatty acids to migrate to the surrounding aqueous phase, where they are readily dispersible, while long-chain fatty acids tend to accumulate at the oil-water interface inhibiting lipase activity (Ahmed et al., 2012; Li et al., 2011; Salvia-Trujillo et al., 2013b). The initial lipid state (liquid or solid) did not have a strong effect on the lipid digestibility of nanoemulsions formulated with MCT (MG or CN), which

can be related to the melting point of these lipids that were 6 ºC for miglyol and 25 ºC for coconut oil. Therefore, it seems that nanoemulsions containing CN-SLN melted during its pass during the gastrointestinal tract of rats (which presents a temperature of ≈ 37 ºC). Conversely, differences were observed in the lipid digestion of LCT-nanoparticles with a different initial lipid state (CO-LLN or HPO-SLN). As observed in the microscope images (Figure 3), the digesta of rats fed with nanoemulsions containing CO-LLN presented less lipid content in the duodenum and jejunum than the digesta of rats fed with those containing HPO-SLN, meaning that CO-LLN were digested faster than HPO-SLN. In this sense, it should be noted that the melting point of HPO (35 ºC) was very close to the temperature of the rat intestine, meaning that HPO-SLN could present a semi-solid lipid state in the gastrointestinal system of rats, which may be slowing the digestion rate. Rats fed with nanoemulsions containing HPO-SLN presented the highest lipid content in the ileum, which confirm the slowed digestion. Indeed, previous authors have observed that the lipid digestion of SLN decreased when the percentage of HPO in a blended MCT:HPO lipid phase increased, detecting the lowest digestion rate and extent at 100% HPO (de Abreu-Martins et al., 2020). Nevertheless, digesta obtained from the colon of rats presented small lipid content, regardless of the nanoparticle type administered. This confirms that all the administered nanoemulsions were totally digested during their past through the intestine.

3.2.2. Curcuminoid concentration in rat plasma and tissues

After the oral administration of different nanoemulsions studied, the highest curcuminoid concentrations (curcumin, demethoxycurcumin and bisdemethoxycurcumin) were found in the intestine of rats (Table 2). Moreover, rats fed with nanoemulsions containing CO-LLN exhibited the highest content of curcuminoids in the small intestine (≈ 10990 ng/g tissue). However, no significant differences were observed in the intestinal curcuminoid concentration of rats fed with nanoemulsions containing HPO-SLN, MG-LLN, or CN-SLN (4300-7500 ng/g tissue). In general, long-chain fatty acids resulting from the digestion of oils such as CO or HPO have been reported to form mixed micelles more easily than medium-chain fatty acids (Jiang et al., 2020). Moreover, the mixed micelles formed by long-chain fatty acids have a better solubilization capacity for lipophilic compounds such as curcumin than medium-chain

fatty acids because of the large dimensions of the hydrophobic core (Lin et al., 2022). However, the reduced curcuminoid concentration by using HPO-SLN in this work may be related to the slowed digestion rate of these systems. In addition, the concentration of curcuminoids in tissues decreased in the following order curcumin > demethoxycurcumin > bisdemethoxycurcumin (Table 2). This trend was expected as curcumin was the major component of the mixture of curcuminoids orally administered to rats (≥ 78%), followed by demethoxycurcumin (15%) and finally, bisdemethoxycurcumin (1%).

Curcumin glucuronide was also found in the ileum of rats in a concentration ranging from 56 to 267 ng/g tissue (Table 2). According to previous authors, glucuronidation of curcumin occurs in the intestine of rats due to the presence of the UDP-glucuronosyltransferases in the intestinal mucosa (Ireson et al., 2001). Moreover, among rats, those fed with nanoemulsions containing CO-LLN presented the highest curcumin glucuronide concentration in the ileum (≈ 267 ng/g tissue), which may be a consequence of the highest absorption of curcumin detected using this type of nanoparticles. Nevertheless, the highest concentrations of curcumin glucuronide were detected in the plasma of rats, especially in the plasma of CO-LLN-fed rats (1612.63 ± 362.14 ng/mL). In contrast, the concentrations of free form curcuminoids in the plasma of rats were very low. Indeed, the same trend has been observed by previous authors investigating the bioavailability and metabolism of curcumin (Ozawa et al., 2017; Peng et al., 2018). Curcumin glucuronide was also present at small concentrations in other tissues such as liver, kidney, brown adipose tissue and white adipose tissue, presenting concentrations of about 10 - 25 ng/g tissue. However, these organs contained a higher concentration of free form curcumin (up to 1400 ng/g tissue) than curcumin glucuronide (Table 2). This information is of high relevance since some studies have revealed that free-from curcumin exhibits the highest therapeutic effects, while curcumin glucuronide shows reduced effect (Choudhury et al., 2015; Pandey et al., 2020; Shoji et al., 2014). Many authors have stated that lipophilic compounds enclosed in nanoparticles can be delivered into the lymphatic system by being incorporated in chylomicrons in the enterocytes (Makwana et al., 2015). Therefore, the encapsulated compounds, especially those highly lipophilic such as curcumin, can avoid hepatic first-pass metabolism (Paliwal et al., 2009; Porter et al., 2007; Trevaskis et al., 2015). This could explain the low

concentrations of curcuminoids found in the liver of the rats in our study and the presence of free forms in different organs. Among the non-intestinal tissues, the highest free-from curcumin concentrations were detected in brown adipose tissue, especially in rats fed with nanoemulsions containing CO-LLN (Table 2). Values of curcumin ranged from 460 to 1400 ng/g, while the concentrations in the white adipose tissue, liver and kidney were about 20 - 40 ng/g. The high presence of curcumin in brown adipose tissue is of high relevance, since previous works have demonstrated that the presence of curcumin in this tissue can play a relevant role in the prevention of obesity and its related metabolic disorders (Song et al., 2018; Zhu et al., 2021).

Table 2. Curcumin (CUR), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) and curcumin glucuronide (CUR GLUC) concentration in different rat tissues at after the oral administration of nanoemulsions containing liquid-lipid nanoparticles (LLN) or solid-lipid nanoparticles (SLN) and different lipids: corn oil (CO), miglyol (MG), hydrogenated palm oil (HPO), or coconut oil (CN).

Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$) among curcumin nanoparticles.

	Lipid state	**Lipid type**	**Concentration in tissue (ng/g tissue or mL plasma)**								
			Duodenum	***Jejunum***	***Ileum***	***Colon***	***Liver***	***Kidney***	***Brown adipose***	***White adipose***	***Plasma***
CUR	*LLN*	*CO*	3840.81 ± 1384.72 ^{b}	1168.84 ± 309.33 ^{b}	2960.02 ± 375.67 ^{b}	311.97 ± 190.37 ^{b}	23.67 ± 6.30 ^{a}	18.03 ± 2.26 ^{a}	1401.95 ± 467.54 ^{b}	20.18 ± 11.53 ^{a}	6.79 ± 1.85 ^{a}
		MG	1091.78 ± 252.78 ^{a}	645.06 ± 175.05 ^{a}	2309.49 ± 639.94 ^{b}	49.50 ± 36.41 ^{a}	30.38 ± 6.75 ^{a}	20.18 ± 2.38 ^{ab}	529.18 ± 248.62 ^{a}	43.23 ± 6.20 ^{b}	6.97 ± 2.32 ^{a}
	SLN	*HPO*	2057.27 ± 807.71 ^{a}	1160.06 ± 363.59 ^{b}	2390.10 ± 738.26 ^{b}	74.71 ± 22.75 ^{a}	22.72 ± 3.16 ^{a}	21.86 ± 2.57 ^{b}	460.69 ± 139.40 ^{a}	35.48 ± 15.18 ^{ab}	5.76 ± 2.80 ^{a}
		CN	1249.42 ± 361.39 ^{a}	600.81 ± 139.91 ^{a}	1315.48 ± 482.65 ^{a}	30.01 ± 13.44 ^{a}	19.42 ± 3.90 ^{a}	19.96 ± 2.75 ^{ab}	535.45 ± 242.51 ^{a}	31.16 ± 13.39 ^{ab}	7.60 ± 2.40 ^{a}
DMC	*LLN*	*CO*	1025.20 ± 364.88 ^{c}	276.27 ± 67.53 ^{b}	829.85 ± 82.63 ^{c}	109.88 ± 34.83 ^{b}	17.70 ± 7.25 ^{b}	31.10 ± 17.63 ^{b}	314.45 ± 352.29 ^{a}	11.82 ± 2.95 ^{a}	2.55 ± 1.05 ^{a}
		MG	349.64 ± 124.89 ^{a}	153.20 ± 32.12 ^{a}	514.26 ± 215.69 ^{ab}	19.51 ± 16.27 ^{a}	13.23 ± 1.77 ^{ab}	21.42 ± 4.43 ^{a}	127.48 ± 15.08 ^{a}	16.93 ± 1.47 ^{b}	2.14 ± 0.76 ^{a}
	SLN	*HPO*	640.35 ± 274.57 ^{b}	229.72 ± 60.01 ^{b}	561.35 ± 207.54 ^{b}	17.25 ± 7.55 ^{a}	11.98 ± 3.60 ^{a}	24.88 ± 2.41 ^{ab}	92.71 ± 22.57 ^{a}	14.21 ± 5.21 ^{ab}	2.96 ± 1.78 ^{a}
		CN	355.58 ± 72.65 ^{a}	129.42 ± 43.65 ^{a}	360.26 ± 136.11 ^{a}	4.99 ± 3.19 ^{a}	12.35 ± 3.34 ^{a}	26.12 ± 4.23 ^{ab}	196.97 ± 100.61 ^{a}	15.25 ± 2.68 ^{ab}	3.38 ± 1.14 ^{a}
BDMC	*LLN*	*CO*	208.26 ± 70.45 ^{b}	81.05 ± 20.63 ^{b}	160.24 ± 12.42 ^{b}	18.41 ± 6.80 ^{b}	10.34 ± 1.54 ^{a}	18.39 ± 2.16 ^{b}	128.21 ± 86.31 ^{b}	13.01 ± 6.59 ^{a}	1.16 ± 0.53 ^{a}
		MG	123.25 ± 46.79 ^{a}	58.69 ± 10.16 ^{ab}	95.14 ± 44.79 ^{a}	8.58 ± 3.94 ^{a}	9.79 ± 1.08 ^{a}	15.58 ± 1.72 ^{a}	18.44 ± 6.20 ^{a}	11.13 ± 4.81 ^{a}	1.15 ± 0.74 ^{a}
	SLN	*HPO*	134.89 ± 101.81 ^{ab}	110.69 ± 43.71 ^{c}	156.39 ± 44.79 ^{b}	6.56 ± 1.78 ^{a}	9.10 ± 2.29 ^{a}	19.43 ± 2.10 ^{bc}	19.57 ± 3.17 ^{a}	10.86 ± 2.09 ^{a}	1.31 ± 0.79 ^{a}
		CN	137.63 ± 33.67 ^{ab}	37.67 ± 15.02 ^{a}	80.30 ± 28.34 ^{a}	4.64 ± 1.35 ^{a}	9.40 ± 2.74 ^{a}	21.11 ± 3.38 ^{c}	35.85 ± 25.69 ^{ab}	11.03 ± 2.45 ^{a}	2.05 ± 1.00 ^{a}
CUR GLUC	*LLN*	*CO*	ND	ND	266.92 ± 144.62 ^{c}	18.31 ± 7.51 ^{b}	11.86 ± 1.82 ^{a}	17.47 ± 1.51 ^{b}	21.81 ± 5.87 ^{b}	14.35 ± 7.42 ^{a}	1612.63 ± 362.14 ^{b}
		MG	ND	ND	172.19 ± 113.61 ^{bc}	13.73 ± 1.22 ^{a}	11.99 ± 1.62 ^{a}	14.16 ± 1.99 ^{a}	12.57 ± 3.06 ^{a}	16.53 ± 2.73 ^{a}	870.52 ± 266.74 ^{a}
	SLN	*HPO*	ND	ND	55.59 ± 11.96 ^{a}	11.35 ± 2.87 ^{a}	10.66 ± 3.32 ^{a}	18.02 ± 2.60 ^{b}	20.80 ± 1.53 ^{ab}	19.58 ± 3.58 ^{a}	1003.77 ± 286.47 ^{ab}
		CN	ND	ND	63.56 ± 23.83 ^{ab}	13.08 ± 2.91 ^{a}	10.78 ± 1.99 ^{a}	19.35 ± 3.43 ^{b}	18.23 ± 3.49 ^{ab}	24.20 ± 7.41 ^{a}	906.54 ± 297.06 ^{ab}

4. Conclusions

Stable nanoemulsion with small-sized nanoparticles (< 200 nm) have been obtained using different lipids (CO, MG, HPO, and CO). The lipid state (solid or liquid) had little effect on the initial properties of nanoemulsions, but those formulated using LCT presented higher physical stability than those containing MCT. After orally administered to rats, nanoemulsions containing MCT nanoparticles showed a faster digestibility than those with LCT. Moreover, HPO-SLN presented a slowed digestion rate due to the high melting point of HPO, near the body temperature (37 ºC). Rats fed with nanoemulsions containing CO-LLN presented the highest absorption of curcuminoids, followed by those with HPO-SLN, MG-LLN, and CN-SLN. These results indicate that although LCT-based systems are known to better increase the absorption of curcuminoids, the lipid state of LCT-based nanoparticles plays a relevant role in curcuminoid absorption. Moreover, rats fed with nanoemulsions containing CO-LLN presented the highest glucuronide concentrations in plasma and the highest accumulation of curcumin free form, which has more biological activity, in the brown adipose tissue. The results obtained in this work provide relevant information on the design of curcumin-loaded nanoparticles to increase curcuminoid bioavailability and be used in the prevention of diseases such as obesity.

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GENERAL DISCUSSION

GENERAL DISCUSSION

In this doctoral thesis, the properties and functionality of different emulsion-based delivery systems were studied. In this regard, the discussion of the results obtained is divided into four sections according to the type of system studied. Section I focuses on oil-in-water (O/W) nanoemulsions. Section II includes the discussion about double water-in-oil-in-water emulsions ($W_1/O/W_2$). Section III discusses about highly-concentrated emulsions. Finally, section IV focuses on nanoparticles. Moreover, a general overview of the benefits and limitations of emulsion-based delivery systems is included at the end of the section.

SECTION I: O/W NANOEMULSIONS

1.1. Impact of the emulsifier used on the properties and functionality of nanoemulsions

Emulsifiers are important molecules in the formulation of nanoemulsions since they contribute to the formation and stabilization of nanoemulsions. These molecules present an amphiphilic structure, containing both lipophilic and hydrophilic groups. Emulsifiers are adsorbed at the oil-water interface of nanoemulsions, so they are supposed to modify the properties of these systems and also their functionality. In this regard, the influence of different emulsifiers (soybean lecithin, whey protein isolate and sodium caseinate) on the properties and functionality of nanoemulsions containing β-carotene or curcumin was assessed in chapters II, III and IV.

1.1.1. Effect of the emulsifier used on the physicochemical properties of nanoemulsions

Curcumin-loaded nanoemulsion formulated with soybean lecithin showed smaller particle sizes (343.8 ± 9.7 nm) in comparison to that formulated with whey protein (462.4 ± 14.7 nm) (chapter II). These differences were attributed to the higher capacity of soybean lecithin to reduce the interfacial tension in the oil-water interface (up to 14.66 mN/m) rather than whey protein (up to 20.52 mN/m). Conversely, in β-carotene-loaded nanoemulsions (chapter III and IV), smaller particle sizes were observed by using protein-based emulsifiers (whey protein or sodium caseinate) rather than lecithin.

The different emulsifier-type trends observed between curcumin-loaded nanoemulsions and β-carotene-loaded nanoemulsions can be related to different factors. First, the concentration of oil in these emulsions was different, being so much lower in those with curcumin (5%) than in those with β-carotene (10-30%). In this regard, it seems that the efficiency of the emulsifier to obtain small particle sizes depends on the concentration of oil present in nanoemulsions. In nanoemulsions containing high amounts of oil, the high capacity of protein emulsifiers to avoid recoalescence due to the formation of a viscoelastic film surrounding lipid droplets (Fuentes et al., 2021), was more determinant to obtain small particle sizes than the capacity of lecithin to quickly reduce the interfacial tension. Second, the surfactant-oil-ratio (SOR) in curcumin-loaded nanoemulsions was 1, while it was 0.4 in β-carotene-loaded nanoemulsions. In that sense, at a low SOR (0.4) nanoemulsions containing protein emulsifiers presented smaller particle sizes than those with the phospholipid. However, at a high SOR (1), this trend was reversed. Previous authors have observed that a phospholipid-based emulsifier was more efficient than Tween to reduce particle sizes at low a SOR (0.1-0.5), but that the trend was reversed at a high SOR (1) (Komaiko et al., 2015). In this regard, it seems that phospholipid-based emulsifiers are highly effective at low SOR, but not at high SOR. In contrast, protein-based or synthetic emulsifiers seem to be more effective than phospholipids-based at high SOR. Third, curcumin-loaded nanoemulsions were formulated using ultrasounds, while those with β-carotene were obtained by microfluidization.

In this doctoral thesis, all studied nanoemulsions presented negative ζ-potential values, irrespective of the emulsifier used. The ζ-potential of emulsions can be related to the physical system stability. Emulsions presenting ζ-potential values more positive than +30 mV or more negative than -30 mV are normally considered to be electrically stable, since it is assumed that repulsive forces are strong enough between droplets (Gref & Couvreur, 2006; Preetz et al., 2010). The emulsifier type showed to influence the electrical charge of nanoemulsions. Indeed, nanoemulsions containing whey protein as emulsifier showed a less negative value (≈ -36 mV) than those formulated using sodium caseinate (≈ -47 mV) or soybean lecithin (≈ -55 mV). The highest negative values observed in soybean lecithin nanoemulsion were conferred by the phosphate groups that were highly negatively charged at the neutral pH of the nanoemulsions (Artiga-Artigas et al., 2018). Otherwise, in whey protein and sodium caseinate nanoemulsions, the

negative ζ-potential was due to the fact that their pH was above the pI of the proteins (≈ 5), so the ionizable groups were deprotonated and the net charge was negative (Hu et al., 2003; Surh et al., 2006). Moreover, nanoemulsions formulated using soybean lecithin showed better physical stability than those formulated using whey protein (chapter II). Although both emulsifier-type nanoemulsions presented creaming, this phenomenon appeared earlier in nanoemulsions containing whey protein (from day 3) than in those with soybean lecithin (from day 6). In that sense, the higher stability observed in lecithin nanoemulsion can be related to its more negative ζ-potential value, in comparison to the whey protein nanoemulsion that presented a less negative ζ-potential. In whey protein nanoemulsion, the repulsion among droplets may be lower, resulting in the flocculation or coalescence of droplets, phenomena that usually ends with the appearance of creaming (Artiga-Artigas et al., 2018; Xu et al., 2012).

The highest viscosity was observed in nanoemulsion formulated with sodium caseinate (50 mPa·s) which was so much higher than those nanoemulsions containing soybean lecithin or whey protein (chapter IV). Such a high viscosity of sodium caseinate nanoemulsions was attributed to the aggregation of droplets that presented this system (Pitkowski et al., 2008). In general, no noticeable differences were observed between the viscosity of nanoemulsions containing soybean lecithin or whey protein as emulsifiers. However, in some experiments, nanoemulsions with soybean lecithin presented a slightly lower viscosity than those with whey protein, as is the case of β-carotene-loaded nanoemulsions containing 10% or 20% of oil. Therefore, it seems that the effect that the emulsifier type had on the viscosity of the samples depends on the oil content, which will be discussed in section 1.3.

1.1.2. Effect of the emulsifier used on the in vitro lipid digestibility of nanoemulsions

The *in vitro* lipid digestibility in emulsion-based systems refers to the study of the hydrolysis of lipid droplets, which produce free fatty acids, diacylglycerols and monoacylglycerols. In this doctoral thesis, all studied nanoemulsions formulated using whey protein as emulsifier exhibited a faster free fatty acid release than those formulated using soybean lecithin. Indeed, after 5 min of digestion, curcumin nanoemulsions (chapter II) with whey protein presented 26% of free fatty acids released, while in those with soybean lecithin it was 2.3%. In the same way, the lipid digestibility in β-carotene

nanoemulsion containing 20% oil (chapter IV) at 5 minutes was higher for protein nanoemulsions (12-15%) rather than for that formulated using the phospholipid (8%). After 10 minutes of digestion, protein nanoemulsion still showed a higher digestibility (30%) than that with soybean lecithin (15%). On the one hand, such a faster digestibility during the first minutes of intestinal digestion in protein nanoemulsion may be related to their particle size when entering the intestine. Protein-based nanoemulsions (those with whey protein or sodium caseinate) presented smaller particle sizes when entering the simulated intestinal phase than those with soybean lecithin. Such a difference was a consequence of the instability phenomena observed in the emulsion during gastric digestion. Protein-based nanoemulsions were prone to flocculation in the stomach due to the proteolysis and/or the reduced electrostatic repulsion that presented those emulsions in that stage by the protonation of the amino groups at the pH 3 of the stomach (Mantovani et al., 2013; Park et al., 2018). However, the droplets in these nanoemulsions were redispersed when entering the intestinal phase due to the electrostatic stabilization of these systems at the pH of the simulated intestine (7). In contrast, in phospholipid-based nanoemulsions, irreversible coalescence of droplets occurred during gastric and intestinal digestion reducing the access of lipase to the substrate. On the other hand, protein-based emulsifiers presented a lower surface activity than the phospholipid and they were more likely to be partially digested by pepsin. Thus, protein nanoemulsions may present a relatively weak interfacial layer and facilitate the absorption of bile salts and or/lipase (Lv et al., 2019). At the end of the intestinal digestion, both protein and phospholipid-based nanoemulsions presented the same free fatty acid release extent in most of the experiments. Nanoemulsions containing 5%, 20% or 30% oil showed a final digestibility of 65%, 43% and 38%, respectively. In contrast, in chapter III, nanoemulsions containing 10% oil the final digestibility was higher in whey protein (60%) nanoemulsions than in those containing soybean lecithin (40%). Therefore, not only the emulsifier used, but other parameters such as the oil content or the concentration of emulsifier can play an important role in the digestibility of nanoemulsions.

1.1.3. Effect of the emulsifier used on the in vitro bioaccessibility of the encapsulated compound in nanoemulsions

When lipid droplets are digested, the lipophilic bioactive encapsulated compounds are

released and incorporated into mixed micelles, from where they can be absorbed in the small intestine. The term bioaccessibility can be defined as the fraction of the compound released from the delivery vehicle that is present within the gastrointestinal fluids in a form suitable for absorption (Marze, 2017; McClements, 2018).

Nanoemulsions formulated with whey protein as emulsifier presented the highest curcumin (70%) and β-carotene (65-70%) *in vitro* bioaccessibility, irrespective of the oil concentration (chapters II, III, and IV). In contrast, the bioaccessibility of curcumin and β-carotene enclosed in nanoemulsions formulated using soybean lecithin was relatively lower, being ≈ 60% and ≈ 20%, respectively. Such a high bioaccessibility obtained by using whey protein may be related to the high free radical scavenging and iron chelation properties of milk proteins that offer great protection from chemical degradation (Chen et al., 2018). Specifically, whey proteins may provide high protection against β-carotene degradation during gastrointestinal digestion. After peptic and trypsin digestion, whey proteins are hydrolysed into peptides with increased antioxidant capacity (Embiriekah et al., 2018). These results were confirmed by the quantification of curcumin degradation during *in vitro* gastrointestinal digestion (chapter II). In this study, curcumin presented less degradation during digestion when enclosed in nanoemulsions with whey protein (28%) than in those with soybean lecithin (33%). In contrast, the lower bioaccessibility observed using the phospholipid-based nanoemulsions may be attributed to the low capacity of the phospholipid emulsifier to inhibit oxidation during digestion.

Sodium caseinate was also studied as emulsifier of nanoemulsions containing β-carotene (chapter IV). In this experiment, the bioaccessibility of β-carotene enclosed in nanoemulsions formulated using whey protein was the highest (68%), followed by sodium caseinate (55.5%) and soybean lecithin (20%). This study revealed that although protein emulsifiers seem to be more effective in increasing β-carotene bioaccessibility than phospholipids, not all proteins are equally effective in preventing compound oxidation. Such a difference between the two studied proteins (whey protein and sodium caseinate) may be related to the structure of both proteins. Sodium caseinate is less effective in preventing oxidation due to the chelating properties and electrostatic interactions of adsorbed casein molecules that favour the positioning of transition metal ions on the surfaces (Villiere et al., 2005). In contrast, whey protein is more efficient

preventing oxidation due to its ability to inactivate peroxyl radicals (Osborn & Akoh, 2004; Tong et al., 2000). Moreover, it has a more compact structure and a lack of phosphate groups, which could limit the ability to bind iron, avoiding oxidation (Guzun-Cojocaru et al., 2011).

1.1.4. Effect of the emulsifier used on the in vivo bioavailability of the compound encapsulated in nanoemulsions

To study the bioavailability, metabolism and biodistribution of lipophilic compounds in the organism, *in vivo* studies are required. In this doctoral thesis, bioavailability studies were performed using Sprague-Dawley rats as an animal model. The impact of the emulsifier type on the bioavailability of β-carotene is reported in chapters III and IV. In chapter III, bioavailability results based on the pharmacokinetics of the compound after its oral administration were discussed. In chapter IV, the focus was on the metabolism and biodistribution of the compound and its main metabolite (retinol) in the different organs.

During the intestinal digestion of the emulsion-based delivery systems, β-carotene is released from the lipid droplets and incorporated into the mixed micelles to be absorbed in the small intestine. After, the mixed micelles that can penetrate the mucus layer, arrive at the brush borders of the epithelium cells, where they are broken down and release the encapsulated compounds into the enterocyte (Sabet et al., 2021). In chapter III, the pharmacokinetic study revealed that the main compound present in the plasma of rats after the oral administration of nanoemulsions and the control suspension (non-encapsulated compound) was retinol, which is the active form of vitamin A. Once β-carotene is absorbed in the intestinal mucosa, it is converted to retinal by β-carotene-15,15' oxygenase 1 (BCO1), and the retinal is then reduced to retinol by a retinal reductase (Paik et al., 2001). Moreover, all studied nanoemulsion-delivery systems increased the bioavailability of retinol when compared to the suspension. These results evidenced that, by encapsulating β-carotene in nanoemulsions, the bioavailability of vitamin A improved. Moreover, the area under the plasma concentration-time curve (AUC) of retinol, which indicates the total content of compound present in the plasma of rats during the studied period, was much higher in whey protein nanoemulsion-fed rats (3051 $ng \cdot h \cdot mL^{-1}$) rather than in soybean lecithin nanoemulsion-fed rats (2335

$ng \cdot h \cdot mL^{-1}$). These results can be related to the *in vitro* observations since whey protein nanoemulsions (≈ 17%) exhibited a greater β-carotene bioaccessibility under simulated intestinal conditions rather than those with soybean lecithin (≈ 45%). The use of milk proteins has been shown to enhance the bioactive compound bioavailability in nanoemulsions (Niu et al., 2020). The mechanisms are not completely understood, but it is suggested that proteins could participate in the formation of mixed micelles and increase drug absorption. In fact, previous studies have reported the high affinity of β-lactoglobulin for retinol (Mensi et al., 2013)

The biodistribution study revealed that the β-carotene absorption in the intestine was greatly increased when the bioactive compound was enclosed in a nanoemulsion system rather than administered in suspension (chapter IV). The presence of oil in nanoemulsions could have enhanced the absorption of β-carotene since the co-ingestion of carotenoids with lipids has been reported to enhance its absorption (Brown et al., 2004; Goltz et al., 2012). Moreover, according to previous works, the presence of emulsifiers could have reduced the interfacial tension and increased the membrane fluidity enhancing the absorption of the bioactive compounds in the intestinal cells (Pan-On et al., 2022). Higher absorption was detected in rats fed with protein-based nanoemulsions than in those fed with a phospholipid-based nanoemulsion. Indeed, these results are in accordance with our *in vitro* observations, in which the bioaccessibility was higher using whey protein or sodium caseinate rather than soybean lecithin.

Orally administered β-carotene was conversed to retinol and was found mainly in the latter form in rat tissues. Moreover, emulsion-fed rats presented higher retinol concentrations than suspension-fed rats as a consequence of the greater β-carotene absorption by using nanoemulsions. Again, differences were observed depending on the emulsifier used, being whey protein and sodium caseinate nanoemulsions the systems that promoted the highest retinol concentrations, especially in the duodenum (up to 250 ng/g tissue), liver (up to 3253 ng/g tissue) and kidney (up to 2324 ng/g tissue) of rats. This fact can be attributed to the presence of higher levels of β-carotene in the intestinal cells of rats fed with protein nanoemulsions rather than with the nanoemulsion containing soybean lecithin. However, although adipose tissue is an important site of β-carotene accumulation in humans, it was not detected in rats in this study. In fact,

previous studies have also failed to detect this compound in rat adipose tissue after administration, even after chronic feeding (Lakshman et al., 1989; Parker, 1988). In contrast, retinol was quantified in the adipose tissue of rats, although no differences were detected among rats fed with different β-carotene carriers (≈ 200 ng/g tissue). Indeed, previous authors reported that adipose tissue plays an important role in retinol storage and retinol-binding protein (RBP) synthesis (Zovich et al., 1992).

Interestingly, the liver and plasma of suspension-fed rats presented a lower concentration of β-carotene (64 ng/g and 43 ng/mL, respectively) rather than retinol (746 ng/g and 104 ng/mL, respectively). In contrast, the plasma of nanoemulsion-fed rats presented a higher concentration of β-carotene (≈ 1300 ng/mL) rather than retinol (≈ 400 ng/mL) and similar values were detected in the liver. This suggests that the metabolism of β-carotene could be different when orally administered enclosed in nanoemulsions than in suspension. Although β-carotene is usually cleaved into retinol in the intestine, it can also follow another pathway. Intact β-carotene can escape the intestinal cleavage by being incorporated into chylomicrons and released by exocytosis to the lymphatic system for delivery to the bloodstream and eventually directed to the liver (Rodriguez-Concepcion et al., 2018). Therefore, it seems that by using the suspension, β-carotene is more likely to be cleaved into retinol in the intestinal cells, whereas the use of nanoemulsions appears to promote the lymphatic pathway, therefore, showing high levels of intact β-carotene in the plasma and liver.

1.2. Impact of polysaccharide addition on the properties and functionality of nanoemulsions

Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharides units bound by glycosidic bonds (Guo et al., 2017). These molecules can present different structural properties, which will determine their functional properties. Usually, polysaccharides are added to nanoemulsions in order to enhance their stability. These molecules are normally dissolved in the aqueous phase acting as thickening agents. Nevertheless, some polysaccharides can adsorb at the oil-water interface. Therefore, the presence of polysaccharides can modify the physical properties of nanoemulsions, but also their stability during digestion, lipid digestibility or compound bioaccessibility. In this doctoral thesis, the influence of different

polysaccharides on the properties and functionality of nanoemulsions were assessed in chapters I and II. In chapter I, the effect of citrus pectin at different concentrations (0, 1 and 2%) was studied on β-carotene-loaded nanoemulsions formulated using Tween 20 as emulsifier. In chapter II the effect of sodium alginate addition was investigated on curcumin-loaded nanoemulsions formulated using different emulsifiers: soybean lecithin or whey protein.

1.2.1. Effect of the polysaccharide addition on the physicochemical properties of nanoemulsions

Citrus pectin at different concentrations (0, 1, 2%) was added into nanoemulsions formulated using Tween 20 as the main emulsifier (chapter I). The addition of pectin reduced the particle size of nanoemulsions when compared to the control nanoemulsion (without pectin). Such a reduction is suggested to be a result of a competitive effect between Tween 20 and pectin since both present affinity for the interface. Previous authors have also reported this phenomenon, reporting that Tween 20, which has a lower molecular weight than pectin, may move faster to the interface, leading to small particle sizes (Verkempinck et al., 2018). In addition, by adding the polysaccharide, the viscosity of nanoemulsions was increased, up to 19.77 mPa·s in nanoemulsions containing 2% pectin. In this regard, the increased viscosity of pectin-nanoemulsions could have enhanced the particle size reduction by increasing the disruptive shear stresses (Qian & McClements, 2011).

The addition of sodium alginate at different concentrations (0, 0.5, 1, 1.5%) into nanoemulsions containing soybean lecithin or whey protein as emulsifiers was studied in chapter II. The obtained results showed that, when adding the polysaccharide into a nanoemulsion containing soybean lecithin, the particle size was reduced. As observed using pectin, such a reduction can be produced by the increased viscosity that enhanced the shear disruptive forces during the homogenization process. Indeed, the addition of sodium alginate to nanoemulsions noticeably increased the viscosity of the systems, up to 110 mPa·s when using 1.5% polysaccharide. In contrast, the particle size of nanoemulsion containing whey protein was increased when sodium alginate was added, being the higher the polysaccharide concentration, the higher the particle size. In this nanoemulsion, although the viscosity was increased it presented extensive flocculation

when the polysaccharide was added, which resulted in high particle sizes. The aggregation of droplets observed in whey protein nanoemulsions may be a consequence of non-adsorbed polymer molecules that were present in the aqueous phase surrounding the lipid droplets (Dickinson et al., 1997; McClements, 2015).

The ζ-potential of nanoemulsions containing Tween 20 became less negative when pectin was added to the systems (chapter I). Control nanoemulsion presented a ζ-potential of -21 mV, while those containing pectin presented a value of about -7.5 mV, irrespective of the polysaccharide concentration used. The negative electrical charge observed in Tween 20 nanoemulsions without pectin can be a consequence of a preferential absorption of OH- species from water to the oil-water interface (McClements, 2005; Mun et al., 2007).

These results can be related to those of the physical stability study, which revealed a decrease in the physical stability when pectin was added to nanoemulsions. The incorporation of pectin promoted the creaming phenomenon, which was observed since day 2. Previous authors have observed the flocculation of droplets, and consequently, the appearance of creaming when citrus pectin was added at concentrations of ≥ 0.02% in emulsion-based systems (Beysseriat et al., 2006; Celus et al., 2018). It seems that when pectin is added at low concentrations, the repulsive interactions between droplets are sufficiently large to overcome the attractive interactions, but there is a critical concentration of the polymer over which the attraction is sufficiently strong to promote flocculation of droplets, and thereby, creaming occurs (McClements, 2000; Schmidt et al., 2015).

Conversely, the ζ-potential of nanoemulsions became more negative when sodium alginate was added to nanoemulsions containing soybean lecithin or whey protein as emulsifiers (chapter II). Moreover, in both emulsifier-type nanoemulsions, the higher the polysaccharide concentration, the more negative the ζ-potential was. This fact was mainly attributed to the flocculation phenomenon that was observed in the microscope observations. Flocculation on these nanoemulsions may be a consequence of non-adsorbed sodium alginate molecules forming a network structure surrounding the oil droplets. Therefore, since sodium alginate has an anionic character due to the carboxylate and hydroxyl groups present in its molecule (Artiga-Artigas et al., 2018),

the presence of this layer contributed to the negative electrical charge of nanoemulsions. As in chapter I, ζ-potential values can be related to the physical stability of the nanoemulsions. In that sense, the addition of sodium alginate increased the stability of nanoemulsion over time, especially at a 1.5% concentration. In this sense, although flocculation was observed in these nanoemulsions, their high viscosity may have decreased the movement of droplets, preventing the formation of creaming (Salvia-Trujillo et al., 2016).

1.2.2. Effect of the polysaccharide addition on the in vitro lipid digestibility of nanoemulsions

The presence of pectin did not affect the digestibility of nanoemulsions containing Tween 20 as emulsifier (chapter I). Indeed, nanoemulsions containing 0, 1 or 2% of pectin did not show differences in the free fatty acid release profile, nor on the final extent, which was about 80%. At pH 7 of the intestine, pectin may not be adsorbed at the interface but would be remaining in the aqueous phase. Therefore, the polysaccharide may not be interfering with the lipolysis process. In fact, other anionic biopolymers such as fucoidan had also shown no effect on lipid digestibility when they are used in combination with small molecule surfactants (Chang & McClements, 2016).

The presence of sodium alginate reduced the digestibility of nanoemulsions containing whey protein or soybean lecithin as emulsifiers (chapter II). The increase in the viscosity of the nanoemulsions by adding sodium alginate could have slowed molecular diffusion, reducing the mobility of species involved in intestinal digestion (Dima & Dima, 2020; Espinal-Ruiz et al., 2014; Velderrain-Rodríguez et al., 2019). Moreover, the anionic alginate molecules could strongly bind to cationic calcium ions, thereby preventing the removal of free fatty acids from the surfaces by calcium and slowing lipid digestion down digestion (Li et al., 2011). However, the reduction was more pronounced in nanoemulsions containing whey protein (20% less digestibility using 1.5% alginate) than in those containing soybean lecithin (10% less digestibility using 1.5% alginate). This difference could be related to the phenomena observed during gastric digestion which determined the particle size of the droplets as they entered the intestinal phase. On the one hand, protein nanoemulsions showed aggregation, due to the formation of electrostatic complexes between whey protein and sodium alginate (Li et al., 2021). As

a result, when they entered the intestinal environment, the aggregated droplets presented a large size and small surface area, reducing lipase access to the lipid substrate and, therefore, the overall lipid digestibility (Salvia-Trujillo et al., 2013). On the other hand, soybean lecithin nanoemulsions did not show aggregation during the gastric phase as both the emulsifier and alginate were negatively charged at stomach pH (3). As a result, the soybean lecithin nanoemulsions had smaller particle sizes, increasing the surface area available for intestinal enzymes.

1.2.3. Effect of the polysaccharide addition on the in vitro bioaccessibility of the encapsulated compound in nanoemulsions

The addition of pectin into nanoemulsions formulated using Tween 20 as emulsifier increased the bioaccessibility of β-carotene, which was about 21% without pectin. Moreover, the higher the pectin concentration, the greater the bioaccessibility, reaching maximum values of about 37% in nanoemulsions with 2% pectin. It should be noted that all nanoemulsions in this study exhibited the same amount of free fatty acids at the end of the intestinal digestion, so it seems that other factors influenced the bioaccessibility of β-carotene. Some authors have noted that lipid availability for mixed micelle formation is not always correlated with the bioaccessibility of β-carotene (Verrijssen et al., 2016). In this regard, in this doctoral thesis, the flocculation of droplets could have acted as a steric barrier, reducing the compound degradation during its pass through the gastrointestinal tract and enhancing the β-carotene bioaccessibility. Indeed, the highest bioaccessibility observed in the nanoemulsion with 2% of pectin must be due to the greater flocculation and formation of gels in the gastric phase, as a consequence of a higher amount of pectin present in the aqueous phase.

The addition of sodium alginate reduced the bioaccessibility of curcumin, regardless of the emulsifier used. In these nanoemulsions, the higher the polysaccharide concentration, the lower the curcumin bioaccessibility was. This fact may be related to the lipolysis results, which followed the same trend. Therefore, in these nanoemulsions, since the lipid digestibility was noticeably reduced when adding the polysaccharide, part of curcumin could be entrapped in the non-digested lipid fraction. In fact, the same phenomenon was been also reported by previous authors using other delivery systems enclosing curcumin (Zhang et al., 2016) or quercetin (Chen et al., 2018).

1.3. Impact of oil concentration on the properties and functionality of nanoemulsions

In chapter III, nanoemulsions enclosing β-carotene from alga *Dunaliella Salina* were formulated using 10 or 30% of oil to study the impact of oil concentration on their properties and functionality. Moreover, the study was performed using two different emulsifiers, whey protein or soybean lecithin. Since the effect of the emulsifier has been analyzed in previous sections, in the present section the discussion will focus on the impact of the oil concentration.

1.3.1. Effect of the oil concentration on the physicochemical properties of nanoemulsions

The viscosity was directly related to the oil concentration, being higher in nanoemulsions with 30% oil content (37 mPa·s) than in nanoemulsions with 10% oil content (1.7-2 mPa·s). This increased viscosity may be a consequence of the high packing state of droplets in these emulsions when increasing the oil concentration, as stated by previous authors (Artiga-Artigas et al., 2019). Moreover, the oil concentration had a different effect on nanoemulsions, depending on the emulsifier used. In the case of soybean lecithin nanoemulsions, by increasing the oil content from 10% to 30%, the mean particle size decreased and the particle size distribution became more polydisperse. The higher viscosity of the nanoemulsion containing 30% of oil compared to those with 10% may have increased the shear disruptive stresses favouring droplet fragmentation (Qian & McClements, 2011). Conversely, the mean particle size of whey protein nanoemulsions increased when the oil content was augmented from 10% to 30%. Such an increase in nanoemulsions formulated with whey protein could be a consequence of the flocculation of droplets. By increasing the volume of oil and emulsifier, droplets became closer and aggregation was favoured, leading to aggregates of high size. In contrast, the electrical charge of emulsions was not affected by the oil content. The ζ-potential values, mainly provided by the presence of an emulsifier, were the same for all oil concentrations due to the equal surfactant-oil ratio.

1.3.2. Effect of the oil concentration on the in vitro lipid digestibility of nanoemulsions

The effect of oil content on lipid digestibility was different depending on the emulsifier used. In whey protein nanoemulsions, the free fatty acids release was slower in nanoemulsions containing 30% oil concentration rather than in those with 10%. Moreover, the final extent of free fatty acids release was about 61% in nanoemulsions with 10% of oil, while in those with 30%, oil it was about 35%. The lower digestibility observed by increasing the oil concentration may be related to the viscosity of the nanoemulsions, which increased from 2.14 mPa·s in 10% oil nanoemulsions to 9.7 mPa·s in those with 30%. In this sense, the high viscosity of nanoemulsions containing 30% of oil may have reduced the intestinal enzyme diffusion process, thereby, decreasing the lipolysis (Espinal-Ruiz et al., 2014). Moreover, the ratio of lipase-to-fat and calcium-to-free-fatty-acid decreased as the oil concentration increased, reducing the rate of lipolysis and increasing the accumulation of free fatty acids on the interface of droplets (Li et al., 2011). In contrast, although soybean lecithin nanoemulsions were digested faster when containing 10% oil than 30% oil, both nanoemulsions presented the same final extent of free fatty acids release (≈ 43%). In these nanoemulsions, the difference in viscosity was not as higher as in protein nanoemulsions when entering the intestinal phase. Indeed, values of viscosity in 10% oil nanoemulsion and 30% oil nanoemulsions were about 1.31 mPa·s and 2.96 mPa·s, respectively.

1.3.3. Effect of the oil concentration on the in vitro bioaccessibility of the encapsulated compound in nanoemulsions

The β-carotene bioaccessibility was reduced by increasing the oil concentration from 10% to 30%, regardless of the emulsifier used. On the one hand, the reduction in whey protein nanoemulsions can be related to the digestibility results. Whey protein nanoemulsion containing 30% of oil presented a lower digestibility and a lower bioaccessibility than that with 10% of oil. Therefore, in more digested nanoemulsions (10% oil nanoemulsions), a higher amount of compound was released and incorporated into the mixed micelles. On the other hand, the decrease in soybean lecithin nanoemulsions when increasing the oil content can be related to the higher amount of emulsifier present in 30% oil nanoemulsion compared to that with 10% oil. Previous

works have reported that the low β-carotene bioaccessibility observed using lysolecithin as emulsifier may be due to the precipitation of β-carotene (Tan et al., 2020). Therefore, in the present study, it seems that increasing the amount of soybean lecithin in nanoemulsion with 30% of oil could have increased the sedimentation of β-carotene, reducing its bioaccessibility in comparison to nanoemulsion with 10% of oil.

SECTION II: $W_1/O/W_2$ DOUBLE EMULSIONS

2.1. Impact of the emulsifier used on the properties and functionality of double emulsions

Water-in-oil-in-water double emulsions enclosing anthocyanins were formulated using polyglycerol polyricinoleate (PGPR) as emulsifier in the inner interface (W_1/O), but two different emulsifiers in the outer interface (O/W_2). Specifically, the emulsifiers compared were soybean lecithin as a natural phospholipid emulsifier and Tween 20 as a non-ionic synthetic emulsifier (Annex I).

2.1.1. Effect of the emulsifier used on the physicochemical properties of double emulsions

Double emulsion formulated using Tween 20 exhibited smaller particle sizes (≈ 4.4 μm) than that formulated with soybean lecithin (≈ 5.7 μm). Small molecule surfactants such as Tween 20 have been reported to move quickly to the interface leading to the formation of small sizes. Moreover, the hydrophilic-lipophilic balance (HLB) of Tween 20 (16.7) is higher than that of lecithin (9). In that sense, since Tween 20 presents greater hydrophilicity than lecithin, it could wrap and stabilize the particles in an O/W nanoemulsion more efficiently, thus resulting in small particles (Tan & Nakajima, 2005; Yuan et al., 2008).

The electrical charge of double emulsion formulated using soybean lecithin was more negative (about -65.6 mV) than those of nanoemulsion containing Tween 20 (about -54.8). The more negative charge observed using the natural emulsifier was provided by the negatively charged phospholipids of lecithin at the pH of the emulsions (4.8). In fact, these phospholipids can provide a high negative ζ-potential in a wide range of pH (Bot et al., 2021). On the opposite, the negative charges of emulsion containing Tween 20, a non-ionic emulsifier, may be a consequence of the presence of free fatty acid impurities in the surfactant or oil or due to the preferential absorption of OH- from water to the droplet surfaces (Mun et al., 2007; Uluata et al., 2015). Nevertheless, both emulsifier-type nanoemulsions exhibited a notable negative electrical ζ-potential, which indicates that the repulsion among droplets was relatively high.

2.1.2. Effect of the emulsifier used on the stability over time of double emulsions

In this study, the stability over time of double emulsions was monitored for 21 days at 4 ºC. The stability was evaluated through the changes in the backscattering profile of double emulsions, as well as the changes observed in parameters such as the particle size or the electrical charge over time. The study on the backscattering profile revealed that droplets of both emulsifier-type emulsions were moving from the bottom to the top of the sample over time, leading to the appearance of creaming. Typically, the creaming phenomenon is preceded by the appearance of previous instability phenomena such as aggregation or coalescence of droplets. In this regard, when droplets lose their individual integrity and became aggregated or coalesced, they are more likely to migrate to the top of the sample causing creaming (Robins, 2000). In our work, this phenomenon was clearly reflected in the backscattering profile of soybean lecithin double emulsions, which presented a decrease in the backscattered light in the central part of the sample. This fact indicates that the size of droplets was becoming greater over time. Furthermore, particle size measurements confirmed that droplet coalescence was occurring in this system due to the increase in the particle size from 5.7 μm on day 0 to 24.4 μm on day 21. On the other hand, in the double emulsions containing Tween 20 this phenomenon was less visible in the backscatter profile and no variation in particle size was observed during the 21 days of the experiment. As suggested by Chang & McClements (2016), the interfacial coating formed by soybean lecithin seems to be less resistant to coalescence than that formed by other emulsifiers.

2.1.3. Effect of the emulsifier used on the in vitro lipid digestibility of double emulsions and bioaccessibility of the encapsulated compound

The emulsifier used in the formulation of double emulsions had a strong impact on lipid digestibility. Tween 20 double emulsions exhibited a fast release of free fatty acids during the first 30 minutes of intestinal digestion, followed by a more gradual release. The final digestibility of this double emulsion was about 43%. In contrast, double emulsions formulated using soybean lecithin exhibited a slower free fatty acid release during all the digestion, presenting a 23% of digestibility at the end of the lipid digestion. As observed in our experiment, also previous authors have reported a higher lipid digestibility when the interface was covered by Tween 20 rather than lecithin (Chang &

McClements, 2016). At high concentrations of the phospholipid-based emulsifier, the excess surfactant molecules may compete with bile salts and lipase at the oil-water interface, thus inhibiting the digestion of lipids. Additionally, the particle size of emulsions when entering the intestine has been shown to be a relevant parameter that affects lipolysis (Salvia-Trujillo et al., 2013). In this sense, lecithin-coated droplets have been shown to be prone to coalescence under gastric conditions while Tween 20-coated droplets have been reported to be highly acid-stable. Therefore, the high particle size of soybean lecithin emulsions when entering the intestine may have decreased the surface area available for intestinal enzymes. In contrast, Tween 20 emulsion may present a higher surface area due to the small particle sizes in the intestine, as also observed by other authors using Tween (Zhang et al., 2015). However, the bioaccessibility of anthocyanins was the same for the two double emulsifier-type emulsions (≈ 23%). Thus, despite their lower digestibility, the lecithin emulsions did not show reduced bioaccessibility. In this regard, it should be noted that, although the final digestibility of the lecithin emulsions was lower than that of the Tween emulsions, the rate of the former was slower than that of the latter. The slow lipid digestion observed in the lecithin emulsions reduced the time in which the anthocyanins were free in the intestinal environment, where can be easily degraded due to the basic pH (Ekici et al., 2014; Kirca et al., 2007).

2.2. Impact of polysaccharide addition on the properties and functionality of double emulsions

In this doctoral thesis, polysaccharides were added into the outer water phase (W_2) of water-in-oil-in-water ($W_1/O/W_2$) double emulsions. In chapter V, three polysaccharides (pectin, sodium alginate and Arabic gum) at different concentrations (0, 0.5, 1, 1.5 and 2%) were added in the W_2 of double emulsions enclosing phycocyanin. Additionally, the incorporation of sodium carboxymethyl cellulose (CMCNa) was studied in double emulsions enclosing anthocyanins and formulated using two different emulsifiers: Tween 20 or soybean lecithin (Annex I).

2.2.1. Effect of the polysaccharide addition on the physicochemical properties of double emulsions

In general, the particle size of double emulsions was reduced when the polysaccharides were added to the W_2. However, the type of polysaccharide and the concentration used were determinants. By using pectin, the smallest particle sizes were detected (≈ 3 μm), which were achieved when incorporating 1.5% polysaccharide (chapter V). However, when using Arabic gum, 2% concentration was needed to achieve the smallest droplets (≈ 3.4 μm). Regarding the use of sodium alginate, the smallest particle sizes (≈ 3.6 μm) were achieved by using a 1% polysaccharide. In addition, the particle size of soybean lecithin double emulsions decreased from 4.4 μm to 3.4 μm when CMCNa was incorporated (Annex I). Some of the studied polysaccharides (pectin and Arabic gum) presented a relevant interfacial activity, being able to be adsorbed in the interface and contribute to the emulsion stabilisation and particle size reduction. However, some others such as CMCNa or sodium alginate presented reduced interfacial activity. Therefore, the reduction observed in the particle size of these double emulsions may be mainly attributed to the changes in viscosity when adding the polysaccharide. In that sense, by increasing the viscosity of emulsions to an intermediate viscosity (up to 20 mPa·s), the shear disruptive forces were also increased, favouring the reduction of particle size (Qian & McClements, 2011). However, when using pectin or sodium alginate at the highest concentrations the particle size was not decreased. This fact may be related to the extremely high viscosity of these nanoemulsions (up to 196.7 mPa·s in double emulsion containing 2% alginate), which could have hindered the particle size reduction during the homogenization and sonication processes.

The electrical charge became more negative when incorporating pectin, sodium alginate and Arabic gum into soybean lecithin double emulsions (chapter V). Additionally, the same effect was detected when CMCNa was added in double emulsions containing soybean lecithin, which indicates that some polysaccharide molecules were adsorbed in the interface (Annex I). These results can be related to the interfacial activity of the different components (emulsifier and polysaccharides) of the emulsions which usually determines their presence on the interface. In that sense, in all these double emulsions, the emulsifier (soybean lecithin) was supposed to cover the majority of the interface

since it presents a high interfacial activity (reducing the interfacial tension to 14.66 mN/m). But also, all the studied polysaccharides have shown to present interfacial activity. In fact, pectin, sodium alginate and Arabic gum have been shown to reduce the interfacial tension to 23.56 mN/m, 21.6 mN/m and 21.17 mN/m, respectively. Therefore, these polysaccharides may be also present on the interface contributing to the ζ-potential of the droplets. In contrast, in double emulsions with Tween 20 the ζ-potential did not change when CMCNa was incorporated into the formulation. Tween 20 is a small surfactant molecule with a high interfacial activity, while CMCNa presents a very reduced interfacial activity. Therefore, in Tween 20 double emulsions, the non-ionic emulsifier would have rapidly covered the interface, leading the CMCNa molecules to remain dispersed in the aqueous phase, thereby not contributing to the electrical charge on the droplet surface.

In general, the encapsulation efficiency of double emulsions improved by the presence of polysaccharides on the W_2. In chapter V, the double emulsion without polysaccharide presented 82.36% encapsulation efficiency, while those with pectin or sodium alginate presented up to 94.77% and 95.58%, respectively. In the same way, the addition of CMCNa to double emulsions increased encapsulation efficiency by 11.8% in those containing soybean lecithin, and 17.7% in those containing Tween 20 (Annex I). Conversely, the addition of Arabic gum did not affect the encapsulation efficiency of double emulsions. The obtained results can be related to the viscosity, being those emulsions with higher viscosities those that presented a higher encapsulation efficiency. In this regard, the presence of polysaccharides in the W_2 increased the stability at the O/W_2 interface and the viscosity of the W_2 phase, which prevented the diffusion of droplets from W_1 to W_2 (Heidari et al., 2022).

2.2.2. Effect of the polysaccharide addition on the stability over time of double emulsions

The addition of CMCNa in double emulsions increased their stability over time, irrespective of the emulsifier used (Annex I). The backscattering observations revealed no instability phenomena when CMCNa was added into soybean lecithin double emulsions. These results were confirmed by particle size measurements, in which no changes were detected during the days of the study. In Tween 20 double emulsions, the

addition of polysaccharide also has a positive effect on their stability, decreasing the creaming phenomena. The particle size of this emulsion decreased from 3.4 μm on day 0 to 2.8 μm on day 21. However, the backscattering profile indicated a particle size increase in the static analysis. This means that probably droplets were becoming aggregated during the time, but the unions among droplets were weak enough to be redispersed during the agitation performed during the particle size measurements. According to previous authors, small particle sizes tend to aggregate when they are numerous at a given phase ratio and more susceptible to Brownian motion, which would result in a greater probability of droplet collision (McClements, 2015).

2.2.3. Effect of the polysaccharide addition on the in vitro lipid digestibility of double emulsions

The addition of polysaccharides to the W_2 of double emulsions has a different effect on the lipid digestibility depending on the emulsifier, the type of polysaccharide used and the concentration of polysaccharide added.

By adding 2% citrus pectin or 1.5% - 2% sodium alginate the lipid digestibility was increased by 15.3% and 19.5%, respectively (chapter V). Interestingly, these double emulsions were the ones that presented the highest viscosities, so these results suggest that the viscosity of the emulsions plays an important role in lipolysis. In that sense, it is supposed that once the emulsifier (soybean lecithin) was displaced from the interface by the action of lipase, these emulsions presented lower particle sizes that facilitated the activity of intestinal enzymes by the increased surface area (Gasa-Falcon et al., 2017; Li, Hu, & McClements, 2011). The oil droplets presented higher stability in the intestinal medium towards coalescence due to the high viscosity of W_2. In contrast, the addition of Arabic gum, ≤ 1.5% pectin, or ≤ 1% alginate did not affect the lipid digestibility of double emulsions, showing similar digestibility values as the control emulsion without polysaccharide (≈ 30%). In these double emulsions, the polysaccharide may not provide stabilization to droplets against coalescence once lecithin was displaced from the interface; therefore, due to the coalescence of droplets, the lipase had difficulties in adhering and digesting.

The addition of CMCNa in the W_2 of double emulsions containing Tween 20 as

emulsifier showed to slow down the release of free fatty acids (Annex I). This digestion behaviour may be related to the time taken for surface-active components from the bile extract to displace the polysaccharide from the oil droplet surface and thereby facilitate lipase adsorption and activity (Mun et al., 2007). However, at the end of the intestinal digestion phase, this emulsion showed no differences in the final digestibility (≈ 40%) compared to the control emulsion without the polysaccharide. Conversely, in double emulsions formulated using soybean lecithin as emulsifier, the addition of CMCNa did not affect the free fatty acids release during the first 40 min. However, after the first 50 min, there was an increased free fatty acid release in emulsions with CMCNa, while in those without polysaccharides the release, it was lower. As a result, the final extent of free fatty acids released was significantly higher in CMCNa emulsions than in those without the polysaccharide. This fact can be related to the ability of CMCNa to inhibit aggregation of the outer droplets (Pays et al., 2001; Schuch et al., 2015). Therefore, once the polysaccharide was released, the oil droplets may have been smaller in CMCNa emulsions than in those without the polysaccharide, facilitating lipid digestion.

2.2.4. Effect of the polysaccharide addition on the in vitro bioaccessibility of the compound enclosed in double emulsions

During the intestinal digestion of $W_1/O/W_2$ emulsions, the oil phase of double emulsions is digested, releasing the encapsulated compound from W_1 into the intestinal fluids to be absorbed.

The presence of alginate, pectin or Arabic gum in the W_2 of double emulsions enhanced the bioaccessibility of encapsulated phycocyanin (chapter V). In the same way, the addition of CMCNa in the W_2 of double emulsions formulated with the same emulsifier (soybean lecithin) also improved the bioaccessibility of anthocyanins, compared with the control emulsion without polysaccharides (Annex I). In contrast, in Tween 20 double emulsions the addition of CMCNa in the W_2 did not affect the anthocyanin bioaccessibility. On the one hand, these observations can be related to the lipid digestibility of these emulsions. Soybean lecithin double emulsions with added pectin, alginate, or CMCNa presented a higher digestibility than the emulsions with the same emulsifier and without the polysaccharide. In this regard, higher bioaccessibility was obtained in more digested emulsions since a higher amount of compound could be

liberated from W_1 enclosed in the lipid droplets.

Conversely, in the case of Arabic gum, the enhanced phycocyanin bioaccessibility cannot be related to the digestibility, since the presence of this polysaccharide did not enhance the lipolysis. It is suggested that Arabic gum may interact with phycocyanin, and increase its bioaccessibility by preventing the degradation of the compound due to its antioxidant properties (Ali & EL Said, 2020). The presence of polysaccharides could have prevented compound degradation during digestion due to the high viscosity and the presence of gels in the intestinal phase (Dima & Dima, 2020). The high viscosity may have reduced the diffusion rate of pro-oxidant molecules through the intestinal fluids, thereby reducing the contact between these molecules and bioactive compounds.

SECTION III: HIGHLY-CONCENTRATED EMULSIONS

In chapter VI, highly-concentrated emulsions were investigated as delivery systems for curcumin. First, an optimization process of the emulsification method, oil content and surfactant-oil-ratio (SOR) of the system was performed. After, the highly-concentrated emulsion presenting the best properties and higher stability over time was used for further experiments. The *in vivo* experiments included a pharmacokinetic study of curcumin encapsulated in a highly-concentrated emulsion *vs* the non-encapsulated compound, as well as the study of curcumin absorption, metabolism and biodistribution.

3.1. Optimization of highly-concentrated emulsions: emulsification method, oil concentration and surfactant-oil-ratio (SOR).

Emulsions formulated using high-shear homogenization (HSH) or high-shear homogenization followed by microfluidization (HSH+MF) were studied at two different oil concentrations (10% or 50%). Emulsions formulated using HSH+MF presented smaller particle sizes (0.5-0.7 μm) than those formulated using only HSH (3-5 μm), irrespective of the oil concentration used. Such a reduction observed when adding MF to the formulation process can be attributed to the inertial forces in turbulent flow along with cavitation that promotes droplet disruption during the microfluidization process. The flow stream by high pressure through microchannels toward an impingement area creates a shearing action, which can provide fine emulsions (Maa & Hsu, 1999; Schultz et al., 2004). Moreover, by increasing the oil concentration from 10% to 50% the oil droplets became smaller. Indeed, emulsions formulated using HSH or HSH+MF that contained 10% of oil, presented a particle size of 4.9 μm and 0.72 μm, respectively, while those with 50% of oil presented a particle size of 3.5 μm and 0.53 μm, respectively. This fact could be attributed due to the increased packing state of droplets caused by augmenting the oil concentration, which contributes to preventing droplet re-coalescence rendering a smaller particle size (Artiga-Artigas et al., 2019). In addition, the increased packing state of droplets caused by augmenting the oil concentration resulted in an increased viscosity of the emulsions.

To continue with the optimization, the emulsion containing 50% oil and formulated using HSH+MF was selected due to its small particle size and low polydisperse distribution. In the next step, different SOR were tested (0.05, 0.1 and 0.2) to evaluate the effect of the emulsifier-oil concentration ratio on the system properties and stability. Results revealed that when increasing the SOR from 0.05 to 0.1, the particle size decreased (from 0.84 μm to 0.53 μm) and became less polydisperse. This could be explained because of the high amount of emulsifier at the same oil concentration, which can cover a greater surface area (Luo et al., 2017). However, when the SOR was further increased to 0.2, the particle size noticeably increased (up to 3.7 μm) and the distribution became more polydisperse. It is suggested that the emulsion with a 0.2 SOR, presented an excess of emulsifier that was promoting the flocculation of lipid droplets, as confirmed in the microscope images.

By increasing the SOR, the viscosity of the emulsions increased and the electrical charge became more negative. The stability study revealed that the emulsion containing 0.1 SOR was the most stable, presenting no instability phenomena during the 15 days of study at 4 ºC. In this emulsion, the repulsive interactions generated by lecithin were strong enough to overcome any attractive interactions (van der Waals) between the droplets (Hunter, 2001; Luo et al., 2017). In contrast, both 0.05 and 0.2 SOR emulsions were unstable, being the emulsion with 0.2 SOR that with the lowest stability. The lack of enough emulsifier to completely cover the droplet surface may be the cause of the instability of the emulsion with 0.05 SOR. Conversely, the instability of emulsions with 0.2 SOR could be attributed to an excess of emulsifier. In these emulsions, the rates of Ostwald ripening and coalescence may have been higher due to the micelle-mediated transport of oil favoured by the excess of emulsifier (Klang & Valenta, 2011; Tadros et al., 2004).

3.2. Pharmacokinetic study of curcumin enclosed in highly-concentrated emulsions or in suspension

The most suitable emulsion selected to perform further *in vivo* experiments was that with 50% oil and 0.1 SOR. Curcumin enclosed in the selected highly concentrated emulsion or in non-encapsulated form (suspension) was orally administered to Sprague Dawley rats. The dose of administered curcumin was 150 mg/kg body weight, irrespective of the

carrier used. The plasma of rats was collected at different time points post-administration (0, 0.5, 1, 2, 4, and 8 h) to study the pharmacokinetics of curcumin.

The curcuminoid concentration in the plasma of emulsion-fed rats (≈ 1588 ng/mL) was higher than in that of suspension-fed rats (≈ 148 ng/mL). The obtained plasma-concentration time curves of all fed rats showed that curcumin was mainly present as curcumin glucuronide in plasma, regardless of the carrier administered. In contrast, curcumin, demethoxycurcumin and bisdemethoxycurcumin were present in small concentrations. These results are in accordance with previous works that have also observed mainly curcumin glucuronide in the plasma of rats or mice orally administered with curcumin (Asai & Miyazawa, 2000; Harigae et al., 2016; Zhongfa et al., 2012). When curcumin is orally administered to rodents, it is suggested to undergo intestinal metabolism and when the compound is absorbed, it undergoes rapid first-pass metabolism and excretion in bile (Sharma et al., 2005). In fact, previous studies in rats have reported that curcumin undergoes O-conjugation to curcumin glucuronide and curcumin sulphate as well as reduction to tetrahydrocurcumin, hexahydrocurcumin and others (Asai & Miyazawa, 2000; Ireson et al., 2001). However, according to previous works, among the metabolites formed, curcumin glucuronide is the major one found in the plasma after oral administration of curcumin to rats, while the free forms are normally negligible (Ozawa et al., 2017; Peng et al., 2018).

In our study, the area under the curve of curcumin glucuronide was about 1556.3 ng·h·ml-1 in emulsion-fed rats and 121.52 $ng \cdot h \cdot ml^{-1}$ in suspension-fed rats. The obtained results revealed that by enclosing curcumin in highly-concentrated emulsions, its bioavailability was increased by 10.6-fold when compared to the administration of the non-encapsulated compound. This value is quite higher than that obtained by previous authors using lower doses (100 mg/kg body weight), who observed a 1.9-fold increase in curcumin bioavailability by using nanoemulsions rather than the suspension (Lu et al., 2018). Values similar to those obtained in our work have been reported by other authors using different curcumin carriers. As an example, organogel-based emulsions showed to improve the oral bioavailability of curcumin by approximately 9-fold compared to free curcumin (Yu & Huang, 2012). However, by using these carriers, authors administered a dose of 240 mg/kg body weight, a value higher than those used

in our work (150 mg/kg body weight).

The highest concentration of curcumin in plasma was found earlier using the suspension (0.5 h) than the HC-emulsion (1 h) (Table 3). The presence of high quantities of oil (50%) of our highly-concentrated emulsion could have retarded the gastric emptying and, therefore, the curcumin absorption. Indeed, previous authors using emulsions with lower oil content (10%), observed the highest concetration at 15 min post-administration (Vecchione et al., 2016). In contrast, the highest concentration of demethoxycurcumin and bisdemethoxycurcumin was found at 0.5 h for both curcumin vehicles, although the concentration in plasma of these compounds was very low and mostly constant along the studied time. The maximum concentration of curcumin glucuronide was observed at 4 h post-administration, irrespective of the carrier used. This value is higher than the one observed for the free forms and may be a consequence of curcumin metabolization. Indeed, a recent study reported that after exposing curcumin emulsions to Caco-2 cells, the percentage of conjugated curcumin metabolites (which includes curcumin glucuronide) increased from ≈10% at 0.5 h to > 60% at 4 h post-administration of different emulsions (Luo et al., 2022).

3.3. Metabolism and biodistribution of curcumin enclosed in highly-concentrated emulsions or in suspension

To study the metabolism and biodistribution of curcumin, the concentration of curcuminoids in the different rat tissues was quantified at 2 h or 4 h after the oral administration of the curcumin carriers (highly-concentrated emulsion or suspension).

In this work, after feeding rats with curcumin, the highest concentration of curcuminoids was observed in the intestinal tissues, where the compound is mainly absorbed. In fact, the highest concentration was observed in the ileum of rats, which presented values up to 840 ng/g tissue in emulsion-fed rats and up to 481 ng/g tissue in suspension-fed rats. These results are in accordance with previous works, which have also found the major concentration of curcumin in the intestine after an oral administration of curcumin in rats, followed by the liver and kidney (Marczylo et al., 2009). The concentration of curcuminoids in the intestine decreased in the following order: curcumin > demethoxycurcumin > bisdemethoxycurcumin > curcumin glucuronide. This trend was

expected as the mixture of curcuminoids administered to rats presented the curcuminoids in different percentages (≥ 78% curcumin, 15% demethoxycurcumin, and 1% bisdemethoxycurcumin). Moreover, rats fed with the emulsion presented a high curcuminoid concentration in the intestines than rats fed with the suspension, meaning that by using the emulsion, the curcumin absorption was increased. On the one hand, the higher concentration achieved by using the emulsions rather than the suspension may be attributed to the high protection against degradation provided by the emulsion rather than the suspension. On the other hand, the presence of oil in emulsions could have enhanced the absorption of curcumin, since the co-ingestion of lipophilic compounds such as curcumin with lipids has been reported to enhance its absorption (Brown et al., 2004; Goltz et al., 2012; McClements & Xiao, 2012). Moreover, the emulsifier (soybean lecithin) could be increasing curcumin absorption by reducing the interfacial surface tension and increasing cell membrane fluidity and the penetration of curcumin through the epithelial cells (Pan-On et al., 2022). In contrast, the low concentration observed using the suspension may be a consequence of the degradation of curcumin during its pass through the gastrointestinal tract, the inadequate absorption and the avid metabolism of curcumin that rodents present (Ireson et al., 2002).

The concentration of curcuminoids in the intestinal tissues was higher at 2 h (1991 ng/mg in emulsion-fed rats and 987 ng/mg in suspension fed-rats) rather than at 4 h post-administration (578 ng/mg in emulsion-fed rats and 250 ng/mg in suspension-fed rats), meaning that curcumin absorption was higher at early times (2 h) and decreased at longer times (4 h). Among the non-intestinal tissues, the liver and brown adipose tissue were those that presented the highest curcuminoid concentrations, especially at 2 h after administration. The liver has been seen to be an important accumulation site of curcumin even after being orally or intravenously administered to rodents (Arozal et al., 2019; Ryu et al., 2006; Wei et al., 2014). In our work, curcumin was the main compound present in the liver of rats, followed by demethoxycurcumin, bisdemethoxycurcumin, and finally, curcumin glucuronide, which was non detected in this organ. Previous works have postulated that most of the orally administered curcuminoids are conjugated to glucuronides in the intestinal mucosa (Asai & Miyazawa, 2000). Moreover, investigations on the capacity of rat hepatocytes to generate different curcumin metabolites concluded that glucuronides are generated only in small amounts in the liver,

whereas they are abundant in plasma after oral administration (Ireson et al., 2001). This investigation also revealed that rat liver reduces curcumin to hexahydrocurcumin and hexahydrocurcuminol, whereas conjugation of curcumin is only a minor hepatic biotransformation route. In our work, it should be noted that although curcumin glucuronide was the major metabolite observed in plasma analysis, the concentration of this metabolite in the intestinal tissues of rats was low. Indeed, in our work, curcumin glucuronide has just been quantified in plasma, intestinal mucosa and kidney. These results are in accordance with previous works, which also detected this metabolite in plasma and intestinal mucosa of rats after the oral administration of curcumin with phosphatidylcholine (Marczylo et al., 2007). In that sense, many authors have stated that when enclosed in nanoparticles, highly-lipophilic compounds like curcumin can avoid first-pass metabolism by being delivered into the lymphatic system through chylomicrons (Paliwak et al., 2009; Porter et al., 2007; Trevaskis et al., 2015).

Interestingly, no differences were observed between the concentration of curcumin in the kidney of suspension-fed-rats and emulsion-fed rats, although curcumin absorption was much higher in the case of emulsion-fed rats. This may indicate that in suspension-fed rats, the rate of curcumin excreted with respect to absorbed curcumin was higher than in the case of emulsion-fed rats. Additionally, the kidney was the unique non-intestinal tissue in which curcumin glucuronide was detected, probably because is the main organ related to its excretion from the organism. In white and brown adipose tissues different curcuminoids were also detected, meaning these tissues can accumulate these compounds. However, the values were relatively higher in the brown adipose tissue (≈ 193 ng curcumin/g tissue) rather than in the white adipose tissue (≈ 9 ng curcumin/g tissue). Moreover, in the case of brown adipose tissue, rats fed with the emulsions presented a higher curcuminoid accumulation rather than rats fed with the suspension. This information is of high relevance, since previous authors have stated that the presence of curcumin in brown and white adipose can play a relevant role in the prevention of obesity and its related metabolic disorders (Santos et al., 2023; Song et al., 2018; Zhu et al., 2021).

SECTION IV: SOLID vs LIQUID NANOPARTICLES

In chapter VII, nanoemulsions containing solid or liquid lipid nanoparticles and different oil types were investigated as delivery systems for curcumin. The effect of the lipid state (solid or liquid) and the lipid type (MCT or LCT) on the properties and digestion of nanoemulsions, as well as on the absorption, and biodistribution of curcumin were investigated. The studied nanoparticles were: CO-LLN (corn oil liquid lipid nanoparticles), MG-LLN (miglyol liquid lipid nanoparticles), HPO-SLN (hydrogenated palm oil solid lipid nanoparticles), and CN-SLN (coconut oil solid lipid nanoparticles).

4.1. Effect of the lipid state (solid or lipid) and lipid type (MCT or LCT) on the physical properties of nanoemulsions

Nanoemulsions containing liquid lipid nanoparticles (CO-LLN or MG-LLN) presented smaller particle sizes than those containing solid lipid nanoparticles (HPO-SLN or CN-SLN). This fact can be attributed to the reduced viscosity at room temperature of the lipids used to formulate LLN (corn oil and miglyol) (de Abreu-Martins et al., 2020). Therefore, less energy was required to disrupt the oil droplets in LLN than in SLN-based nanoemulsions, resulting in smaller droplets of the former. Moreover, differences were detected depending on the lipid type used, being MCT-based nanoparticles those with the smallest particles. Indeed, the highest particle size (≈ 180 nm) was observed in HPO-SLN, while the lowest values (≈ 158 nm) were observed in MG-LLN. As stated by Sarheed et al. (2020), lipids with shorter oil chains such as MCT allow oil molecules to penetrate the interfacial film, resulting in smaller particles. The electrical charge was negative and similar for all nanoemulsions (- 20 to -27 mV), except for those containing hydrogenated HPO-SLN (26 mV). The emulsifier used in the formulation of all nanoparticles was Tween 80, which has been reported to give droplets a negative charge, depending on the pH.

4.2. Effect of the lipid state (solid or lipid) and lipid type (MCT or LCT) on the *in vivo* gastrointestinal stability of nanoemulsions

After being orally administered to rats, all nanoemulsions suffered a noticeable increase in the particle size in the stomach. Indeed, the initial particle size was about 170 nm,

while in the stomach of rats, the particles were > 1 μm, irrespective of the lipid state (solid or liquid) and lipid type (MCT or LCT). However, dispersed particles were observed in the gastric medium, meaning that they were stable in the gastric environment. This fact can be a consequence of the emulsifier selected to formulate the nanoemulsions, which was Tween 80. Small molecule surfactants such as Tween 80 have been seen to be acid stable, maintaining better the microstructure stability than others such as sucrose palmitate (Marciani et al., 2006; Verkempinck et al., 2018).

Differences were observed in the intestinal digesta obtained from rats fed with nanoemulsions containing different nanoparticles, especially in the duodenum and jejunum. Digesta from rats fed with nanoemulsions containing LCT nanoparticles (corn oil or palm oil) presented more lipid content in the duodenum and jejunum and with a higher particle size than those with MCT nanoparticles (miglyol or coconut oil). This suggests that MCT nanoparticles were digested faster than those with LCT. This trend has been observed in other emulsion-based delivery systems and can be a consequence of the capacity of medium-chain fatty acids to migrate to the surrounding aqueous phase, where they are readily dispersible, while long-chain fatty acids tend to accumulate at the oil-water interface inhibiting lipase activity (Ahmed et al., 2012; Li et al., 2011; Salvia-Trujillo et al., 2013b). The initial lipid state (liquid or solid) did not have a strong effect on the lipid digestibility of nanoemulsions formulated with MCT oils (miglyol or coconut), which can be related to the melting point of these lipids. In this regard, the melting points were 6 ºC for miglyol and 25 ºC for coconut oil. Therefore, it seems that CN-SLN melted during its pass during the gastrointestinal tract of rats (which presents a temperature of ≈ 37 ºC), not affecting the lipolysis process. Conversely, differences were observed in the lipid digestion of nanoemulsions containing LCT nanoparticles (corn oil or hydrogenated palm oil) with a different initial lipid state (liquid or solid). Indeed, the digesta of rats fed with nanoemulsions containing CO-LLN presented less lipid content in the duodenum and jejunum than the digesta of rats fed with nanoemulsions containing HPO-SLN, meaning that the former was digested faster than the latter. In this sense, it should be noted that the melting point of hydrogenated palm oil (35 ºC) was very close to the temperature of the rat intestine (37 ºC), meaning that SLN with that oil could present a semi-solid lipid state in the gastrointestinal system of rats, which may be retarding the lipid digestion. Rats fed with nanoemulsions containing

HPO-SLN presented the highest lipid content in the ileum, which confirm the slowed digestion of these systems. Indeed, previous authors have observed that the lipid digestion of SLN decreased when the percentage of HPO in a blended MCT:HPO lipid phase was increased, detecting the lowest digestion rate and extent at 100% hydrogenated palm oil (de Abreu-Martins et al., 2020). Nevertheless, digesta obtained from the colon of rats presented small lipid content, regardless of the nanoparticle type administered. This confirms that all the administered nanoemulsions were totally digested during their past through the intestine.

4.3. Effect of the lipid state (solid or lipid) and lipid type (MCT or LCT) on the *in vivo* bioavailability of the encapsulated compound

After the oral administration of the different curcumin-loaded nanoemulsions (all with a dose of 250 mg curcumin/kg rat), the highest curcuminoid concentrations were found in the intestine of rats. Rats fed with the nanoemulsions with CO-LLN exhibited the highest content of curcuminoids in the intestine (≈ 10990 ng/g tissue). However, no significant differences were observed in the intestinal curcuminoid concentration of rats fed with nanoemulsions containing HPO-SLN, MG-LLN, or CN-SLN (4300- 7500 ng/g tissue). In general, long-chain fatty acids resulting from the digestion of oils such as CO or HPO have been reported to form mixed micelles more easily than medium-chain fatty acids (Jiang et al., 2020). Moreover, the mixed micelles formed by long-chain fatty acids have a better solubilization capacity for lipophilic compounds such as curcumin than medium-chain fatty acids because of the large dimensions of the hydrophobic core (Lin et al., 2022). However, the reduced curcuminoid concentration by using HPO-SLN in this work may be related to the slowed digestion rate of these systems. In addition, the concentration of curcuminoids in tissues decreased in the following order curcumin > demethoxycurcumin > bisdemethoxycurcumin (Table 2). This trend was expected as curcumin was the major component of the mixture of curcuminoids orally administered to rats (≥ 78%), followed by demethoxycurcumin (15%) and finally, bisdemethoxycurcumin (1%).

Curcumin glucuronide was found in the intestine of rats, meaning that glucuronidation of curcumin was occurring in this tissue. It has been previously reported that rats present UDP-glucuronosyltransferases in the intestinal mucosa which are responsible for the

glucuronidation of substances such as curcumin (Ireson et al., 2001). Moreover, rats fed with nanoemulsions containing CO-LLN presented the highest content (≈ 267 ng/g tissue), which may be a consequence of the highest absorption of curcumin detected using these nanoparticles. Nevertheless, the highest concentrations of curcumin glucuronide were detected in the plasma of rats, especially in the plasma of rats fed with nanoemulsions with CO-LLN (≈ 1613 ng/mL). In contrast, the concentrations of free form curcuminoids in the plasma of rats were very low. Indeed, the same trend has been observed by previous authors investigating the bioavailability and metabolism of curcumin (Ozawa et al., 2017; Peng et al., 2018).

Curcumin glucuronide was also present at small concentrations in other tissues such as liver, kidney, brown adipose tissue and white adipose tissue, presenting concentrations of about 10 - 25 ng/g tissue. However, these organs contained a higher concentration of free form curcumin (up to 1400 ng/g tissue) than curcumin glucuronide. This information is of high relevance since, according to previous studies, free-from curcumin exhibits the highest therapeutic effects, while curcumin glucuronide exhibits reduced effect (Choudhury et al., 2015; Pandey et al., 2020; Shoji et al., 2014). Many authors have stated that lipophilic compounds enclosed in nanoparticles can be delivered into the lymphatic system by being incorporated in chylomicrons in the enterocytes (Makwana et al., 2015). Therefore, the encapsulated compounds, especially those highly lipophilic such as curcumin can avoid hepatic first-pass metabolism (Paliwal et al., 2009; Porter et al., 2007; Trevaskis et al., 2015). This could explain the low concentrations of curcuminoids found in the liver of rats in this study and the presence of free forms in different organs. Among the non-intestinal tissues, the highest free-from curcumin concentrations were detected in brown adipose tissue, especially in rats fed with CO-LLN. Indeed, values of curcumin ranged from 460 to 1400 ng/g in brown adipose tissue, while the concentrations in the white adipose tissue, liver and kidney were about 20 - 40 ng/g. The high concentrations achieved in the brown adipose tissue highlight the potential of the studied nanoparticles to be used in the prevention of obesity and its related metabolic diseases.

BENEFITS AND LIMITATIONS OF EMULSION-BASED DELIVERY SYSTEMS

In light of the results obtained in this doctoral thesis, emulsion-based delivery systems have proven to be efficient in encapsulating bioactive compounds of lipophilic and hydrophilic nature. However, the different studied systems differ in their stability and show different levels of protection for bioactive compounds, so the advantages and disadvantages of their use must be assessed in each case.

In this doctoral thesis, lipophilic compounds have been incorporated into conventional emulsions, nanoemulsions, highly-concentrated emulsions and solid-lipid nanoparticles. Conventional emulsions have shown to be easy-to-prepare systems and are useful to increase the bioaccessibility of encapsulated compounds. However, these emulsion-based delivery systems present some limitations. First, they show reduced physical stability (up to 2 days) than others such as nanoemulsions (up to 35 days). Second, conventional emulsions have shown to provide lower chemical stability during storage (50% compound degradation during 35 days of storage) and reduced bioaccessibility (up to 20%), compared with nanoemulsions. Therefore, nanoemulsions seem to be more promising systems than conventional emulsions to encapsulate lipophilic bioactive compounds.

Nanoemulsions have been shown to provide high encapsulation efficiencies (up to 97%), physical stability (up to 35 days), compound stability (15% compound degradation during 35 days of storage) and *in vitro* bioaccessibility (up to 68%) due to their reduced particle size. In addition, it has been observed that, by controlling the composition of these systems, their functionality can be tailored for specific applications. As an example, by adding polysaccharides to the aqueous phase of nanoemulsions, the lipid digestibility, as well as the compound release and bioaccessibility can be controlled. Besides, *in vivo* studies performed in this doctoral thesis have demonstrated that nanoemulsions are highly effective in enhancing the bioavailability of lipophilic compounds, as well as increasing their concentration in tissues where they can have a therapeutic effect.

According to the literature, solid-lipid nanoparticles were designed to overcome some

limitations detected in other systems such as nanoemulsions. Some studies have shown that these emulsion-based delivery systems provide higher chemical stability to encapsulated compounds and offer a more controlled release due to their structured solid matrix. Nevertheless, the results obtained in this doctoral thesis indicate that these systems are less effective than nanoemulsions to increase the bioavailability and biodistribution of lipophilic compounds. Specifically, curcumin encapsulated in liquid long-chain oil-based nanoparticles (corn oil) showed a higher bioavailability (1.5-fold higher) than enclosed in solid long-chain oil-based nanoparticles (palm oil).

Experiments performed in this thesis using highly-concentrated emulsions (containing soybean lecithin as emulsifier) have also demonstrated that these systems increase the bioavailability of lipophilic compounds. Indeed, curcumin presented a 10-fold higher bioavailability when enclosed in highly-concentrated emulsions rather than when unencapsulated. However, nanoemulsions containing the same oil type but Tween 80 as emulsifier were more efficient in increasing the bioavailability of curcumin than the studied highly-concentrated emulsions. It should be noted that the administration of emulsion was different in these two studies, being single-dose in the case of the highly-concentrated emulsions and multiple-dose in the case of the Tween 80 nanoemulsions. This indicates that the differences observed between these two systems could also be attributed to the way the emulsions were administered, and not only to the type of emulsifier used. In this sense, it seems that the administration type can be a limiting factor, with the multiple-dose administration in lower volumes being the most suitable option.

Double emulsions have been tested as encapsulation systems of hydrophilic compounds in this doctoral thesis. The results obtained proved that these emulsions are effective systems for encapsulating compounds such as anthocyanins and phycocyanin, providing encapsulation efficiencies of over 90%. In addition, these systems offer some advantages over simple emulsions, such as the possibility to formulate fat-reduced products when incorporated into food matrices or the encapsulation of two different compounds within the same system (of special interest for compounds with a synergetic effect). However, these systems are highly susceptible to breakdown during storage. In the experiments performed in the present doctoral thesis, it has been seen that this limitation can be

overcome by incorporating polysaccharides in the external aqueous phase of the systems. By using this strategy, not only the physical stability of these systems can be enhanced, but also the stability of the encapsulated compounds during digestion and their bioaccessibility. Even so, the in vitro bioaccessibilities obtained using these systems were relatively low (up to 16.8% in the case of phycocyanin and up to 31.1% in the case of anthocyanins). In fact, due to the low bioaccessibility observed *in vitro* and the low dose of bioactive compound that can be incorporated by using double emulsions, these systems could not be tested *in vivo* in this doctoral thesis.

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CONCLUSIONS

The results obtained in this doctoral thesis have contributed to elucidating how the composition of emulsion-based delivery systems affects their properties, stability and functionality. The main findings obtained are organized in four sections depending on the type of system used:

Section I: O/W nanoemulsions

- The emulsifier choice affected the physical properties of nanoemulsions, being those with protein-based emulsifiers the ones with smaller particle size and less electrical charge. The addition of pectin increased the viscosity of nanoemulsions and reduced their particle size, electrical charge and stability over time. In contrast, the addition of alginate increased the electrical charge, viscosity and stability of nanoemulsions over time.
- The instability phenomena observed during gastrointestinal digestion were dependent on the emulsifier used. Nanoemulsions containing protein-based emulsifiers (whey protein and sodium caseinate) presented aggregated droplets in the stomach, but they could be redispersed in the intestine according to the *in vitro* and *in vivo* observations. In contrast, in the phospholipid-based nanoemulsion (containing soybean lecithin) coalescence of droplets was detected. The addition of pectin or alginate into nanoemulsions promoted the flocculation of droplets during *in vitro* digestion but prevented their coalescence.
- The instability phenomena that nanoemulsions underwent during digestion influenced the digestibility of the lipid fraction. The effect of the emulsifier on digestibility depended on the concentration of oil. Moreover, the presence of alginate decreased the lipid digestibility of nanoemulsions due to its high viscosity and its ability to bind calcium ions.
- The bioaccessibility of curcumin and β-carotene was greater in nanoemulsions containing protein emulsifiers than in that with the phospholipid, which was related to the greater protection provided by the proteins against the degradation of the compound during digestion. In addition, by increasing the oil concentration in nanoemulsions, β-carotene bioaccessibility was reduced as a consequence of a reduced lipid digestibility. The addition of pectin increased β-carotene bioaccessibility due to an increased protection of the compound against degradation,

while alginate reduced curcumin bioaccessibility as a consequence of the reduced lipid digestibility.

- Nanoemulsions were efficient systems to enhance the oral bioavailability of β-carotene and retinol compared to the control suspension in rats. Moreover, nanoemulsions containing protein-based emulsifiers were more efficient than those with a phospholipid-based emulsifier in increasing the absorption of β-carotene.
- *In vivo* studies revealed that once absorbed, β-carotene was cleaved to retinol (the active form of vitamin A), the form in which it was mostly found in the different organs studied. Moreover, the liver was the organ with more retinol accumulation, especially in rats fed with nanoemulsions containing whey protein as emulsifier.

Section II: $W_1/O/W_2$ double emulsions

- Double emulsions formulated with Tween 20 showed smaller particle sizes and higher stability over time than those with soybean lecithin. The addition of polysaccharides (carboxymethyl cellulose, Arabic gum, alginate) in the external aqueous phase of double emulsions reduced the particle size, increased the encapsulation efficiency, and improved the physical stability of the systems over time.
- The *in vitro* lipid digestibility of double emulsions was higher using Tween 20 than soybean lecithin due to the higher stability provided by the former under gastric conditions against coalescence. The addition of polysaccharides that significantly increased the system viscosity (2% pectin, ≥ 1.5 alginate, or 1% carboxymethyl cellulose) enhanced the lipid digestibility of soybean lecithin double emulsions by increasing the stability of droplets in the intestinal medium.
- The emulsifier type did not affect the bioaccessibility of anthocyanins enclosed in double emulsions. In contrast, the addition of polysaccharides in the external aqueous phase increased the bioaccessibility of the encapsulated compound (anthocyanins or phycocyanin) in lecithin-containing emulsions due to (1) increased protection against degradation of the compound during digestion and/or (2) enhanced lipid digestibility.

Section III: highly-concentrated emulsions

- Highly-concentrated emulsions were obtained using 50% oil and 0.1 surfactant-oil-ratio (soybean lecithin), which showed no instability phenomena during 15 days.
- The formulated highly-concentrated emulsions showed to increase by 10.6-fold the *in vivo* oral bioavailability of curcuminoids compared to the control suspension.
- Curcumin was mainly present in the glucuronide form in plasma, which presented the highest concentrations at 2 h - 4 h post-administration.
- Curcumin was mainly found in its free form, which presents the most biological activity, in the studied organs. In addition, the highest concentrations were observed in brown adipose tissue, where curcumin can play an important role in the prevention of diseases such as obesity.

Section IV: solid and liquid lipid nanoparticles

- Nanoemulsions containing liquid lipid nanoparticles (corn oil or miglyol) presented smaller particle sizes than those containing solid lipid nanoparticles (hydrogenated palm oil or coconut oil) due to the lower viscosity of the formers. Moreover, the type of oil has also an impact on the initial particle size, with MCT-nanoparticles (miglyol or coconut oil) presenting the smallest particle sizes.
- The lipid digestibility of nanoemulsions was faster when they contained MCT-nanoparticles than LCT-nanoparticles, due to the greater ability of MCT to migrate and disperse in the surrounding aqueous medium. Among the nanoemulsions containing solid lipid nanoparticles, the lipid digestibility was only slowed in those formulated using hydrogenated palm oil, as the high melting point of the lipid allowed nanoparticles to retain a semi-solid state at body temperature.
- Nanoemulsions containing LCT liquid lipid nanoparticles (corn oil) increased the *in vivo* curcuminoid bioavailability to a higher extent than those with MCT. However, when the LCT was solid (hydrogenated palm oil) it presented the same absorption as the MCT-based nanoparticles.
- After the oral administration of nanoemulsions to rats, curcumin was mainly found in the glucuronidated form in plasma. However, it was found in its free from in most organs, especially in the brown adipose tissue that presented the highest concentrations.

FUTURE RESEARCH

The results obtained in this doctoral thesis have shown that the composition of emulsion-based delivery systems strongly affects the stability of the systems and the bioavailability of encapsulated bioactive compounds. Nonetheless, further investigation needs still to be done to cover remaining gaps of knowledge. Therefore, in view of the results obtained during this doctoral thesis, the following research is proposed:

- Incorporate the studied emulsion-delivery systems into food matrices in order to assess their efficiency as delivery systems and study the possible interactions with the components of food products.

- Validate in humans the studies carried out with animal models in this doctoral thesis to obtain more information on the functionality of these systems.

- Focus on the research of new sustainable sources of natural ingredients to formulate these systems, especially those of plant origin.

- Conduct studies investigating consumer acceptance of food products containing emulsion-based delivery systems.

ANNEXES

Annex I

The effect of sodium carboxymethylcellulose on the stability and bioaccessibility of anthocyanin water-in-oil-in-water emulsions

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Abstract

Water-in-oil-in-water ($W_1/O/W_2$) emulsions provide protective encapsulation to plant bioactive compounds in food matrix and under gastrointestinal conditions. However, the stability of the emulsions during the storage is crucial for their use in the food industry. Hence, the aim of this study was to enhance the stability and bioaccessibility of $W_1/O/W_2$ emulsions containing anthocyanins with the use of sodium carboxymethylcellulose (CMCNa). The emulsions were prepared by ultrasound technology, adding polyglycerol polyricinoleate (PGPR) in the inner aqueous phase of emulsions, and lecithin and Tween 20 in the outer aqueous phase. The systems were physicochemical characterized over the time and their behavior under simulated gastrointestinal conditions was investigated. Our results showed high encapsulation efficiencies above 90% and an increase in bioaccessibility with the use of CMCNa. Moreover, the polymer addition slowed down the free fatty acid release and increased the oil digestibility of lecithin-stabilized emulsions. These latter emulsions presented the highest bioaccessibility (31.08 ± 1.73%), the more negative values of ζ-potential and no variations on the particle size and the backscattering profile over the time, thus being the most stable emulsions. These results provide useful information for the design of anthocyanin emulsion-based delivery systems to guarantee their functionality in food matrices as well as through the gastrointestinal tract.

Keywords: water-in-oil-in-water emulsion, ultrasound technology, anthocyanins, sodium carboxymethylcellulose, stability, bioaccessibility

1. Introduction

Anthocyanins are flavonoids belonging to an important group of water-soluble blue, red, purple and orange natural pigments of different fruits and vegetables. Nutraceuticals containing anthocyanins purified from bilberry (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*), have been used in clinical studies demonstrating major health benefits such as the modulation of several pro-inflammatory mediators in healthy adults (Karlsen et al., 2007), the improvement of the lipoprotein profile (HDL-cholesterol/LDL-cholesterol) in dyslipidemic patients (Qin et al., 2009), the enhancement of the antioxidant capacity and the prevention of insulin resistance in subjects with type 2 diabetes (Li et al., 2015). However, the use of these nutraceuticals has not been exploited for their application in food so far. The use of anthocyanin extracts as replacers of synthetic colorants in food is limited due to their low stability during processing and storage of food products. The color and stability of anthocyanins can be affected by physical and chemical factors such temperature, pH, light, chemical structure, concentration, presence of copigments, metallic ions, enzymes, oxygen, ascorbic acid, sugar, among others (Cavalcanti et al., 2011; Sari et al., 2012; Shipp & Abdel-Aal, 2010). Therefore, new encapsulating delivery systems for anthocyanins are needed for further food applications.

Water-in-oil-in-water ($W_1/O/W_2$) double emulsions consist of a W_1 phase dispersed as small water particles in a lipid phase, which separates the internal water phase from the external water phase (W_2) (Qi et al., 2011). Currently, $W_1/O/W_2$ emulsions are of great importance in the food industry because they allow to prepare reduced-fat emulsion products compared to conventional oil-in-water emulsions (O/W) maintaining a similar in-mouth perceived texture and flavor. They have also the capacity to encapsulate and protect both lipophilic and hydrophilic compounds, which are isolated from the surrounding aqueous environment, and control its release during the digestion process (Aditya et al., 2015b; Kaimainen et al., 2015; Matos et al., 2014; Muschiolik & Dickinson, 2017). However, the stabilization of these systems and further application in food is still a challenge.

Emulsifiers or surfactants are surface-active amphiphilic molecules often selected based on their ability to enhance physical stability of emulsions. Polyglycerol polyricinoleate

(PGPR) is the most commonly used hydrophobic emulsifier in food-based $W_1/O/W_2$ emulsions to promote and stabilize the inner phase (W_1/O) (Aditya et al., 2015a; Aditya et al., 2015b; Frank et al., 2012; Kaimainen et al., 2015). In contrast, lecithin and Tween 20 were added to the outer phase (O/W_2) in order to study their influence in the stabilization of the inner phase into the outer phase. Tween 20 is a synthetic hydrophilic emulsifier used in the aqueous phase of $W_1/O/W_2$ double emulsions (Frank et al., 2011), whereas lecithin, a mixture of different glycerophospholipids, can also be adsorbed to oil-water interface acting as a natural emulsifier (Klang & Valenta, 2011; Pichot et al., 2013). The use of different biopolymers as thickening and gelling agents has shown to improve encapsulation efficiency and stability of $W_1/O/W_2$ double emulsions (Benna-Zayani et al., 2008; Dickinson, 2011; Su et al., 2008). Sodium carboxymethylcellulose (CMCNa) is proved to be a suitable emulsifier for $W_1/O/W_2$ double emulsions and it is commonly chosen as a stabilizing agent for its low cost (Matos et al., 2014; Schuch et al., 2015). The aim of this study was to encapsulate and stabilize anthocyanins in double emulsions for its further application in food. We compared the influence of using different stabilizers in the outer phase of the $W_1/O/W_2$ double emulsions, a natural emulsifier (lecithin) compared to a synthetic emulsifier (Tween 20) and the addition of a biopolymer (sodium carboxymethylcellulose) on size, zeta potential, encapsulation efficiency, free fatty acid release, bioaccessibility and stability of the systems during 21 days of the storage at 4 ºC.

2. Materials and methods

2.1. Materials

Anthocyanin extract (Medox ®, Biolink Group AS, Sandnes, Norway), glycerol, polyglycerol polyricinoleate (PGPR) (Danisco, DuPont, EEUU), Tween 20 (Scharlab, Sentmenat, Spain), lecithin Alfa Aesar ThermoFisher (Kandel, Germany), sodium carboxymethylcellulose (CMCNa) (Sigma-Aldrich, Inc. St. Louis, MO), sodium acetate (Scharlab, Sentmenat, Spain), potassium chloride (Panreac AppliChem ITW Reagents, Barcelona, Spain) and corn oil was purchased from a local supermarket. Ultrapure water obtained from a milli-Q filtration system was used to prepare emulsions and reagents of the experiment.

2.2. Methods

The $W_1/O/W_2$ emulsions were prepared using a unique inner emulsifier (PGPR) at a final concentration in the emulsion of 1.25% w/w. Tween 20 and lecithin emulsions at a concentration of 1.5% w/w were formulated to compare the effect of the two different outer surfactants. Moreover, to investigate the effect of CMCNa on the systems, Tween 20 and lecithin emulsions were stabilized with 1% w/w and without the biopolymer.

2.2.1. Preparation of water-in-oil-in-water ($W_1/O/W_2$) double emulsions

Firstly, the anthocyanins extract was diluted 1:20 in a NaCl 0.1M solution, by agitation (10 min), centrifugation (15 min at 14000 rpm) and filtration.

For the formulation of the double emulsions, a two-step emulsification method described by Aditya et al. (2015a) with some modifications was used. To obtain the primary W1/O emulsion, the two phases were prepared separately. The W_1 phase, comprising of 22 % of the extract solution and 3 % of glycerol, and the oil phase, consisting of 70 % of corn oil and 5 % of PGPR, were heated at 60 ºC for 15 min, separately. Subsequently, the two phases were mixed and homogenized using a high-speed homogenizer (Ultra-Turrax, Janke & Kundel, Staufen, Germany) at 6000 rpm for 8 min. Lastly, a sonication of the primary W_1/O emulsion was performed using a P400S Hielscher sonicer (Hielscher Ultrasound Technology, Teltow, Germany) for 180 s at a frequency of 24 kHz and amplitude of 40%.

The secondary water phase (W_2) was prepared adding the surfactant (lecithin or Tween 20) on the NaCl 0.1M solution and heated at 60 ºC for 30 min. In the emulsions with biopolymer, CMCNa was also added into the W_2 phase. Finally, W_1/O and W_2 were mixed at a ratio of 25:75, homogenized using the high-speed homogenizer at 6000 rpm for 4 minutes and sonicated for 90 s at a frequency of 24 kHz and amplitude of 30%.

2.2.2. Quantification of anthocyanins in emulsions

The anthocyanin content was determined by the spectrophotometric pH-differential method in which two buffer systems were used, potassium chloride (0.025 M pH 1) and sodium acetate (0.4 M pH 4.5) following Turfan et al. (2011) method. Briefly, samples

were diluted 1:3 with each buffer and the absorbance of the mixtures at pH 1 and 4.5 was then measured with a UV-visible-NIR spectrophotometer (V-670, Jasco Corporation, Tokio, Japan) at 515 and 700 nm against a blank of methanol. The anthocyanin content (mg/L) was calculated according to equation (1) and expressed as equivalents of cyanidin-3-glucoside.

$$TA = \frac{\left[(A_{515} - A_{700})_{pH\ 1.0} - (A_{515} - A_{700})_{pH\ 4.5}\right] \times MW \times DF \times 1000}{\varepsilon \times L} \quad (1)$$

where MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF is the dilution factor, L is the pathlength in cm and ε is the molar extinction coefficient for cyanidin-3-glucoside (26900 L · mol-1 · cm-1).

2.2.3. Determination of encapsulation efficiency (EE)

To determine the encapsulation efficiency of the anthocyanins, a previously described method by Aditya et al. (2015b) with some slight modifications was used. A portion of 10 mL of the emulsions were centrifuged at 4500 rpm for 10 min at 4 ºC. Then, the W_2 phase at the bottom of the tube, containing free anthocyanins, was collected, and centrifuged at 7500 rpm for 15 min at 4 ºC, prior a dilution at 1:4 with methanol. Finally, the amount of free anthocyanins was determined using the differential pH method described in the previous section (2.2.1.).

Based on the content of free anthocyanins obtained according to the equation (1), the encapsulation efficiency was calculated according to the equation (2):

$$EE\ (\%) = \frac{Total\ anthocyanins - Free\ anthocyanins}{Total\ anthocyanins} \cdot 100 \quad (2)$$

where the total anthocyanins is the amount of the compound added to the inner aqueous phase in the preparation of the emulsions. The total anthocyanins value was determined by reference to a calibration curve of the extract diluted in NaCl 0.1 M.

2.2.4. Emulsion characterization

The mean droplet diameter (nm) and the particle size distribution were measured using static light scattering technique (Mastersizer 2000, Malvern Instruments Ltd,

Worcestershire, UK). Emulsions were previously diluted in ultrapure water and stirred at 1800 rpm. The particle size diameter (μm) was reported as the volume mean diameter (d43).

The electrical charge (ζ-potential) was measured by phase-analysis light scattering (PALS) using a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd Worcerstershire, UK) to determine the surface charge (mV) at the droplet interface. Emulsions were previously diluted in ultrapure water at a dilution ratio of 1/2000 sample-solvent and placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles. The results were expressed in millivolts (mV).

Apparent viscosity of emulsions was determined using a SV-10 vibro-viscometer (A&D Company, Tokyo, Japan), which produces a vibration of 30 Hz and constant amplitude of 0.4 mm at a controlled room temperature. The results were expressed in mPa·s.

The optical properties of the emulsions were measured using a colorimeter (Konica Minolta CR-400, Konica Minolta Sensing, Inc, Osaka, Japan). The equipment was set up for an illuminant D65 and 10º observer angle. CIE L* (lightness), a* (green- red) and b* (blue- yellow) values were reported.

2.2.5. Confocal scanning laser microscopy

Micrographs of the $W_1/O/W_2$ double emulsions were obtained with a confocal scanning laser microscopy (CSLM) (Olympus FV1000 Spectral Confocal Microscope Olympus, Melville, NY). Samples of W1/O/W2 emulsions were stained with Nile red (fluorescent lipid dye) and examined with a 100X magnifications lens and a laser with an excitation line of 559 nm.

2.2.6. In vitro digestion

The *in vitro* digestion method was performed according to Minekus et al. (2014). The protocol included both gastric and small intestinal phases. Briefly, 20 mL of the sample were mixed with 18.2 mL of simulated gastric fluid (SGF) containing pepsin (2000 U/mL), 0.4 mL HCl solution (1 M) and 10 μL of a CaCl2 solution (0.3 M). Finally, 1.39 mL of Milli Q water was added to reach a final volume of 40 mL. The mixture was

placed into an incubator at 37 °C for 2 h while shaking gently. In order to carry out the intestinal phase, a pH-stat device was used. Once the gastric phase was completed, an aliquot of 30 mL of gastric sample were placed in a 37 °C water bath. Then, 3.5 mL of bile solution (54 mg/mL) and 1.5 mL of salt solution (NaCl 0.150 mM and CaCl2 0.01 mM) were added and the pH was adjusted to 7 with NaOH (1 M). Finally, 2.5 mL of pancreatin solution (75 mg/mL) was incorporated to the mixture. The pH of the sample was maintained to 7 by adding NaOH (0.25 M) constantly for 2 h. The final volume of NaOH (0.25 M) was recorded and used to calculate the amount of free fatty acids (FFA) released during the intestinal phase. The % FFA was determined according to equation (3):

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

where V_{NaOH} was the final volume of NaOH consumed during the *in vitro* digestion, M_{NaOH} was the molarity of NaOH (0.25 M), M_{oil} was the molarity of the oil used in the emulsion (800 g · mol-1) and moil was the mass of oil that was digested during the intestinal phase of the digestion (2.63 g).

2.2.7. Anthocyanin bioaccessibility

Anthocyanin extraction was carried out following the method proposed by Gómez-Plaza et al. (2008) with some modifications. An aliquot of 10 mL of digested sample was centrifuged at 11000 rpm for 15 min at 4 °C. The micelle phase was discarded and the aqueous phase was recovered. Then, 1 mL of the aqueous phase was mixed with 3 mL of methanol. The mixture was vortexed and centrifuged at 9000 rpm for 15 min at 4 °C. Aqueous phase was recovered and pellet was discarded.

The anthocyanin content (mg/L) was determined by the spectrophotometric pH-differential method described by Turfan et al. (2011), as it was described previously (section 2.2.1.). The results were expressed as equivalents of cyanidin-3-glucoside.

The bioaccessibility was obtained according the equation (4):

$$\text{Bioaccessibility (\%)} = \frac{C_{\text{digestion}}}{C_{\text{emulsion}}} \text{ x } 100 \quad (4)$$

where $C_{digestion}$ is the anthocyanin content of digested samples and $C_{emulsion}$ is the anthocyanin content in the initial emulsion.

2.2.8. Stability by Turbiscan

Stability of emulsions was studied using a vertical scan analyzer Turbiscan MA 2000 (Formulaction, Toulouse, France) during 21 days of storage at 4 ºC. A sample of 7 mL were introduced into a glass cylindrical cell and analyzed by a light beam emitted in near infrared (800 nm) wavelength which scanned vertically, from bottom to top, the sample cell. Two synchronous optical sensors receive respectively light transmitted through the sample (180° from the incident light), and light backscattered by the sample (45° from the incident radiation). In this study, the variation of backscattering (BS) during 21 days at 4 ºC was studied to assess the stability of emulsions over the time.

2.2.9. Statistical analyses

All the experiments were assayed in duplicate and three analyses were carried for each sample. The results were analyzed using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md), being the confidence interval set at 0.95. Analysis of the variance (ANOVA) was performed to compare treatments. In addition, multiple rang tests were carried out to determine significant differences between the obtained averages. Least significant difference (LSD) test was employed to determine differences between means.

3. Results and Discussion

3.1. Characterization of initial $W_1/O/W_2$ emulsions

Encapsulation efficiency. High values of encapsulation efficiency ranged from 74.84 ± 1.53% in Tween 20-stabilized $W_1/O/W_2$ double emulsions to 92.57 ± 0.20% in the same emulsions stabilized with CMCNa (Table 1). Aditya et al. (2015a) managed to

encapsulate catechin and curcumin in double emulsions with similar encapsulation efficiencies, around 90-95%. In our study, a significant increase of 15% and 25% in lecithin- and Tween 20-stabilized $W_1/O/W_2$ double emulsions, respectively, was observed with the addition of CMCNa. Similar behaviour was observed in the study of Matos et al. (2014), who reported higher encapsulation efficiency as the concentration of CMCNa increased (0 - 0.5% w/v) in the W_2 outer phase of Tween 20-stabilized $W_1/O/W_2$ double emulsions. CMCNa may affect the interactions between Tween 20 or lecithin molecules adsorbed at the outer interface. Due to the low interfacial activity of CMCNa, its presence in the W_2 outer phase could provide a steric effect, physical barrier for particle interactions, playing a significant role in the stabilization of the outer interface (Matos et al., 2014; Schuch et al., 2015). Different hydrocolloids added to the W_2 outer phase have increased encapsulation efficiency. Values higher than 90% were observed with the addition of 10% of a modified gum arabic (Acacia (sen) SUPER GUM^{TM}) to the W_2 outer phase of $W_1/O/W_2$ double emulsions (Su et al., 2008). The encapsulation efficiencies of $W_1/O/W_2$ double emulsions stabilized by CMCNa have shown much higher encapsulation efficiencies than the emulsions stabilized by beet pectin or gum Arabic (Schuch et al., 2015). In literature, a greater retention of active material is related to a reduction of emulsion droplet size, which generally represents an increased stability (Schuch et al., 2015; Schuch et al., 2013). However, our results obtained for encapsulation efficiency could not be related to the droplet size. In accordance with our results, Carneiro et al. (2013) attributed the differences observed in the encapsulation efficiency of flaxseed oil microencapsulated by spray drying to the wall materials used, with different interfacial properties, rather than the droplet size.

Particle size and size distribution. The particle size of the $W_1/O/W_2$ double emulsions are shown in Table 1. The values were in the 3.36 ± 0.69 and 8.39 ± 0.83 μm range. Similar particle sizes (5.05 ± 0.16 μm to 8.28 ± 0.33 μm) were reported in CMCNa Tween 20-stabilized $W_1/O/W_2$ double emulsions containing trans-resveratrol at different biopolymer concentrations, from 0 to 0.5% w/v (Matos et al., 2014). In our study, emulsions formulated with Tween 20 presented lower particle size compared to those formulated with lecithin ($p < 0.05$), which could be explained by the hydrophilic-lipophilic balance (HLB) of the emulsifiers. According to literature, the higher the HLB values the smaller the particles in an O/W emulsion (Yuan et al., 2008). The HLB values

of Tween 20 are 16.7 but the HLB of lecithin are rarely higher than 9, commonly ranging between 2 and 7. Thus, the lowest droplet size observed in Tween 20 emulsions could be explained because of its high HLB value.

Table 1. Initial encapsulation efficiency (EE), particle size, ζ-potential, viscosity, color and pH of $W_1/O/W_2$ double emulsions.

	Lecithin [1]	**Tween 20 [2]**	**CMCNa Lecithin [3]**	**CMCNa Tween 20 [4]**
EE (%)	78.38 ± 3.87 ^{A}	74.84 ± 1.53 ^{A}	90.14 ± 1.57 ^{B}	92.57 ± 0.20 ^{B}
Particle size (μm)	5.66 ± 0.70 ^{C}	4.39 ± 0.55 ^{B}	8.39 ± 0.83 ^{D}	3.36 ± 0.69 ^{A}
ζ-potential (mV)	-65.58 ± 4.81 ^{B}	-54.83 ± 2.36 ^{C}	-88.06 ± 4.51 ^{A}	-56.3 ± 4.00 ^{C}
Viscosity (mPa·s)	2.16 ± 0.08 ^{A}	2.02 ± 0.06 ^{A}	277.67 ± 38.41 ^{B}	257.50 ± 27.69 ^{B}
Color (a*)	14.50 ± 0.65 ^{C}	11.57 ± 0.59 ^{B}	12.71 ± 0.75 ^{B}	5.24 ± 1.52 ^{A}
pH	4.83 ± 0.07 ^{B}	3.37 ± 0.37 ^{A}	5.49 ± 0.06 ^{C}	5.38 ± 0.03 ^{C}

Values are expressed as mean ± standard deviation. Different letters within the same line indicate significant differences ($p < 0.05$) among emulsions. [1] Lecithin: lecithin-stabilized emulsions. [2] Tween 20: Tween 20-stabilized emulsions. [3] CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions. [4] CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions.

Particle size was significantly affected by the addition of CMCNa in $W_1/O/W_2$ emulsions, although the effect varied depending on the surfactant. These differences between Tween 20 and lecithin could be attributed to changes in the initial pH before the addition of CMCNa. In the case of lecithin emulsions (pH ≈ 5), the addition of the biopolymer increased the particle size. In contrast, in the case of Tween 20 (pH ≈ 3), the addition of CMCNa decreased the particle size. It has been also reported a particle size increase of casein micelles in a mixture with carboxymethylcellulose at pH 5 in comparison with that in absence of the hydrocolloid. The increase was attributed to the adsorption of carboxymethylcellulose onto casein micelles, which led to effectively larger particles (Du et al., 2009). Based on this latter study, at low pH, the decrease of particle size with the addition of CMCNa in Tween 20-stabilized $W_1/O/W_2$ double emulsions could be due to the presence of adsorbed surfactant that might hinder the adsorption of arriving CMCNa chains due to the electrostatic repulsion. Therefore, interactions between CMCNa and the emulsifiers onto the oil surface are suggested to be pH dependent. Apart from these interactions, Matos et al. (2014) proposed that the addition of CMCNa in the outer W_2 phase of Tween 20-stabilized $W_1/O/W_2$ double emulsions could be associated to its capacity to reduce the interfacial tension, which

would also explain the decrease of the mean diameter values in our study.

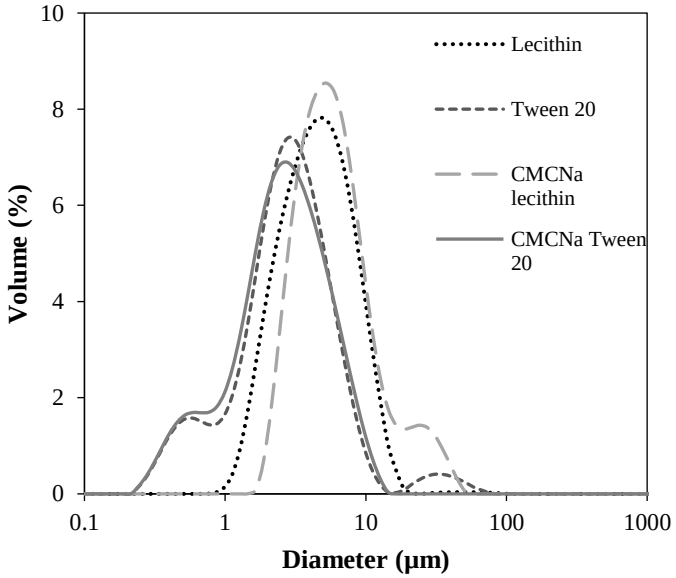

Figure 1. Droplet size distribution of the different $W_1/O/W_2$ double emulsions. Lecithin, lecithin-stabilized emulsions; Tween 20, Tween 20-stabilized emulsions; CMCNa lecithin, sodium carboxymethylcellulose and lecithin-stabilized emulsions; CMCNa Tween 20, sodium carboxymethylcellulose and Tween 20- stabilized emulsions.

Figure 1 shows the droplet size distribution of $W_1/O/W_2$ emulsions. Lecithin-stabilized double emulsions presented a well-defined monomodal particle size distribution, ranging from 1 to 20 µm. In contrast, Tween 20- stabilized $W_1/O/W_2$ double emulsions showed a bimodal distribution, with small particles in the range from 0.2 to 12 µm and large particles from 12 to 80 µm. The polydispersity of Tween 20-stabilized double emulsions decreased with the addition of CMCNa and small particles from 0.2 to 12 µm were shown. However, the values increased with the addition of the biopolymer in lecithin emulsions and large particles up to 50 µm were detected. A bimodal distribution has been typically observed in $W_1/O/W_2$ emulsions formulated with PGPR as lipophilic emulsifier (Hemar et al., 2010; Su et al., 2006), but a monomodal distribution was observed by Cofrades et al. (2013) when sodium caseinate and whey protein concentrate were used as hydrophilic emulsifiers. In other studies, $W_1/O/W_2$ emulsions stabilized with different hydrocolloids, such as propylene glycol alginate, gellan, carragenan, pectin methylcellulose, gum Arabic, xanthan and others, have shown to exhibit high polydispersity, similar to our double emulsions with CMCNa (Huang et al., 2001).

Confocal scanning laser microscopy (CSLM). Oil fat globules containing small droplets inside (inner phase) can be clearly identified in Figure 2. CSLM images have proved the formation of double emulsions in other studies (Aditya et al., 2015a). These authors confirmed that a secondary emulsification does not result in the destruction of the primary water-in oil emulsion. The addition of CMCNa increased the oil droplet size in lecithin-stabilized emulsions (Figure 2a and c), whereas, smaller droplets were observed for emulsions with the addition of CMCNa in Tween 20-stabilized $W_1/O/W2$ double emulsions (Figure 2b and d). These results are confirmed by the particle size measurements (Table 1). The confocal images also presented some aggregation of the oil droplets of Tween 20 emulsions, not detected in lecithin emulsions.

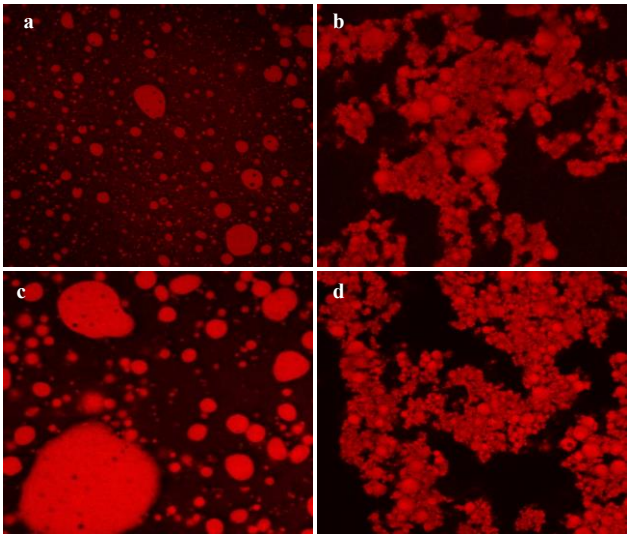

Figure 2. Confocal laser scanning microscopy images of the different W1/O/W2 double emulsions. Lecithin, lecithin- stabilized emulsions (a); Tween 20, Tween 20-stabilized emulsions (b); CMCNa lecithin, sodium carboxymethylcellulose and lecithin-stabilized emulsions (c); CMCNa Tween 20, sodium carboxymethylcellulose and Tween 20-stabilized emulsions (d).

ζ-potential. In Table 1 are shown the electrical charges of the $W_1/O/W_2$ double emulsions. The values were negative and oscillated between -54.83 ± 2.36 mV and -88.06 ± 4.51 mV. Initially, ζ-potential values of lecithin-stabilized emulsions were more negative than those of Tween 20 emulsions ($p < 0.05$) (Table 1). Although Tween 20 is a non-ionic emulsifier, negative charges were observed in this study. It could be explained because of the presence of free fatty acid impurities in the surfactant or oil, or due to the preferential absorption of OH^- from water to the droplet surfaces (Hu et al., 2012; Uluata et al., 2015). The droplet surface charge of lecithin-stabilized emulsions is

usually in the negative range due the presence of negatively charged phospholipids. This natural emulsifier, mainly consisted of phosphatidyl choline, is a zwitterionic polar lipid that has previously been reported to give emulsion droplets a negative charge (Chang & McClements, 2016; Hu et al., 2012; Uluata et al., 2015). The addition of CMCNa in lecithin-stabilized emulsions could have increased significantly the electrostatic repulsion between oil droplets, causing a decrease in ζ-potential from -65.58 ± 4.81 to -88.06 ± 4.51. Similar effect was observed in acidified milk drinks, where the addition of carboxymethylcellulose to casein micelles also increased the absolute magnitude of ζ-potential, contributing to the stability of the system by electrostatic repulsion (Due et al., 2009). In comparison with other biopolymers, CMCNa or gum arabic hydrocolloids provided more negative charges than whey protein isolate in O/W emulsions (Berendsen et al., 2014).

Viscosity. Apparent viscosity values of $W_1/O/W_2$ double emulsions ranged from 2.02 ± 0.06 to 277.67 ± 38.41 mPa·s. No differences on viscosity were observed between surfactants, but as it can be shown in Table 1, there was a significant increase of the viscosity in the emulsions when the biopolymer was added. The addition of CMCNa, as well as other polymers, in $W_1/O/W_2$ emulsions causes a considerably increase of viscosity (Benna-Zayani et al., 2008; Dickinson, 2011; Matos et al., 2014), and permit to achieve a rheological control of the continuous phase acting as thickening agents (Dickinson, 2009). Such effect may improve the stability of the $W_1/O/W_2$ emulsion. Moreover, an increase of the viscosity has been reported to help reducing the particle size of double emulsions (Li et al., 2012), which has also been observed in this study in Tween 20-stabilized emulsions (Table 1).

Color and pH. Color intensity was measured by a* values, which ranged from 5.24 ± 1.52 in CMCNa Tween 20-stabilized $W_1/O/W_2$ double emulsions to 14.50 ± 0.65 in lecithin-stabilized $W_1/O/W_2$ double emulsions (Table 1). The a* values in emulsions stabilized with lecithin were higher than those of Tween 20-stabilized emulsions ($p < 0.05$), which indicated that lecithin-stabilized emulsions were more red than those with Tween 20. When CMCNa was added, a* values decreased and thus, emulsions became less red. It is generally accepted that the color, intensity and stability of anthocyanins changed significantly in the pH 1 - 12 range. pH values affected their stability, being

highly stable at very acidic pH < 3 and low stable at the slightly acid to neutral pH values of most foods (Cabrita et al., 2000). In the present study, pH values ranged from 3.37 ± 0.37 in Tween 20-stabilized emulsions to 5.49 ± 0.06 in CMCNa lecithin-stabilized $W_1/O/W_2$ double emulsions. Although CMCNa addition increased significantly the emulsion pH, especially in lecithin-stabilized $W_1/O/W_2$ double emulsions, the compound encapsulation could overcome the stability-related limitations of anthocyanin utilization in food.

3.2. Oil digestibility and anthocyanin bioaccessibility

The lipid digestion profile was significantly different among lecithin- and Tween 20-stabilized emulsions. The rate and extent of lipid digestion of emulsions stabilized with lecithin was significantly lower than those where Tween 20 was used as surfactant (Figure 3). A rapid FFA release up to 30% was observed after 30 min of the *in vitro* duodenal digestion in Tween 20-stabilized $W_1/O/W_2$ double emulsions, followed by a more gradual increase up to 42.68% at the end of the 2 h duodenal digestion. In contrast, in lecithin-stabilized $W_1/O/W_2$ double emulsions the initial increase was much smaller, and the final total FFA released was the lowest (23.44%), probably due to the difficulty for lipase to act. The low lipolysis that extends up to 30% in some studies could be explained either by a possible accumulation of fatty acids in the O/W-interfaces or by an inhibitory effect of the emulsifiers (Frank et al., 2012). In addition, the *in vitro* digestibility of lipid droplets by pancreatic lipase can vary as a function of the type and the amount of the emulsifier used (Mun et al., 2007; Nik et al., 2011; Yao et al., 2013). Mun et al. (2007) reported an improved digestibility of whey protein isolate WPI-stabilized emulsions compared to lecithin- and Tween 20-stabilized emulsions. Chang and McClements (2016) also showed a faster rate of lipid digestion for Tween 80 than for lecithin. Several studies indeed reported an increase of the droplet size during the digestion, usually due to droplet aggregation observed by optical microscopy (Mun et al., 2007; Nik et al., 2011; Salvia-Trujillo et al., 2013). Chang and McClements (2016) related the initial lipid digestion rate of lecithin-stabilized fish oil-in-water emulsions to the moderate amount of droplet aggregation observed in these emulsions prior to the addition of lipase. Although they also observed large dense aggregates in Tween 80-stabilized emulsions at the beginning of the small intestine phase, in this case, they

suggested that these aggregates consisted of lipid droplets that were only weakly held together by attractive forces, and were therefore easily disrupted during lipid digestion. Kaimainen et al. (2015) suggested that not only the aggregation of the outer droplets may inhibit the lipase activity, but also the high concentration of lecithin, with phospholipids, could compete with bile salts and lipase at the oil-water interface, thus inhibiting the digestion of lipids.

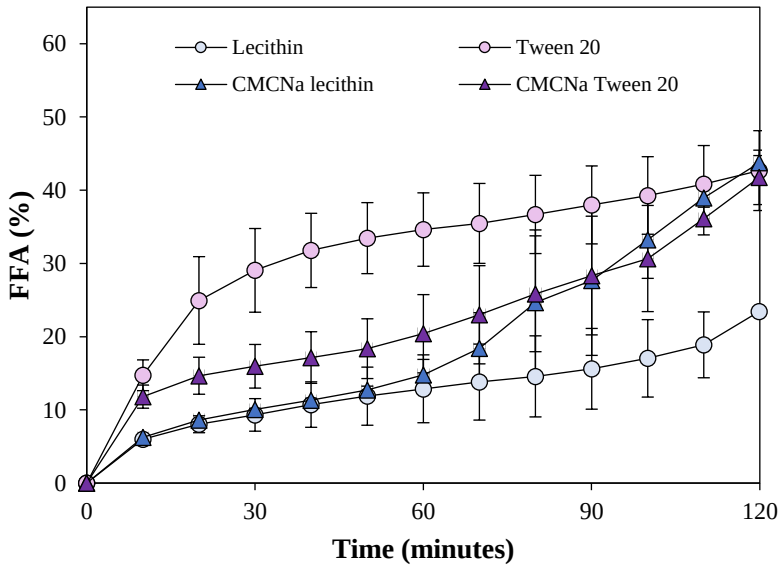

Figure 3. Free fatty acid (FFA%) release during the intestinal phase of the different $W_1/O/W_2$ double emulsions. Lecithin, lecithin-stabilized emulsions; Tween 20, Tween 20-stabilized emulsions; CMCNa lecithin, sodium carboxymethylcellulose and lecithin-stabilized emulsions; CMCNa Tween 20, sodium carboxymethylcellulose and Tween 20- stabilized emulsions.

A different lipid digestion profile was observed with the addition of CMCNa in either lecithin- or Tween 20-stabilized $W_1/O/W_2$ double emulsions. In Tween 20 emulsions, the addition of CMCNa slowed down the release of FFA, but, at the end of the intestinal digestion phase, no differences were observed in the total amount released. In the lecithin-stabilized $W_1/O/W_2$ double emulsions, the addition of CMCNa had no effect initially, but a rapid increase was observed after 50 min, being the final amount of FFA released significantly higher than in the emulsions without polymer. CMCNa has been reported to inhibit aggregation of the outer droplets (Pays et al., 2002; Schuch et al., 2015). Therefore, although the addition of CMCNa slowed down the initial digestion rate, a remarkable FFA release is observed at the end of the lipid digestion, being the total FFA release of ≈ 40%. This digestion behavior may be related to the time take for

surface-active components from the bile extract to displace the biopolymer from the oil droplet surface and thereby facilitate lipase adsorption and activity (Mun et al., 2007).

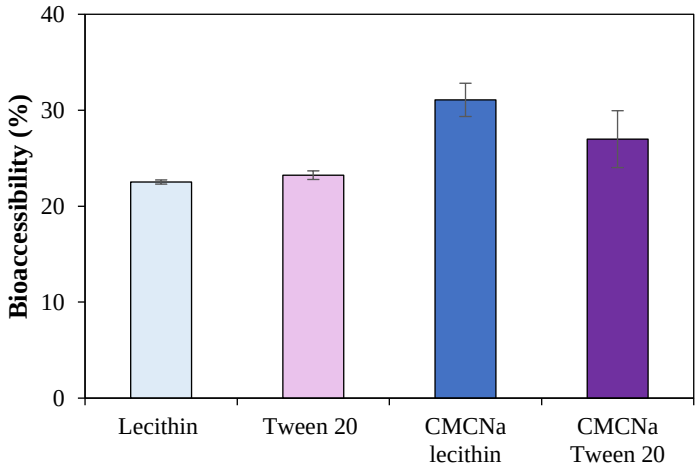

Figure 4. Anthocyanin bioaccessibility (%) of the different $W_1/O/W_2$ double emulsions. Lecithin, lecithin-stabilized emulsions; Tween 20, Tween 20-stabilized emulsions; CMCNa lecithin, sodium carboxymethylcellulose and lecithin-stabilized emulsions; CMCNa Tween 20, sodium carboxymethylcellulose and Tween 20-stabilized emulsions. Different capital letters indicate significant differences ($p < 0.05$) among emulsions.

The bioaccessibility of an aqueous solution of the anthocyanin extract used in this study was about 2% (data not shown). It has been reported a low bioaccessibility of anthocyanin extracts, which may be due to their notoriously low stability into simulated intestinal fluids. This is one of the reasons because anthocyanins isolated from plant cells are easily degraded during their passage throughout the human digestive system and they are poorly absorbed and rapidly excreted (Frank et al., 2012). Hence, the systemic bioavailability of anthocyanins is estimated to be 0.26-1.8% in animal studies (Borges et al., 2007; Ichiyanagi et al. 2006; Koli et al., 2010). In our study, the bioaccessibility of anthocyanin-loaded emulsions increased substantially in comparison with the aqueous solution of the anthocyanin extract. The values ranged from 22.52 ± 0.21% in lecithin-stabilized $W_1/O/W_2$ double emulsions and 31.08 ± 1.73% CMCNa lecithin-stabilized $W_1/O/W_2$ double emulsions (Figure 4). No differences between surfactants were observed, but the addition of CMCNa showed a positive effect on the bioaccessibility, only significant in lecithin emulsions, probably because of the stabilization effect of the biopolymer, as it has been previously mentioned. The highest bioaccessibility observed in CMCNa lecithin emulsions could be attributed to the fact

that the oil droplets are more stable in the simulated gastrointestinal fluids and the release of FFA in these samples is more progressive, extending the anthocyanin protection and avoiding its degradation (Figure 3).

3.3. Changes of $W_1/O/W_2$ emulsion properties over time

The emulsions were stored at 4 ºC in darkness and changes in the emulsion properties were monitored for 21 days. Table 2 shows the encapsulation efficiencies over the time for all the emulsions studied. The amount of anthocyanins enclosed of the total compound initially encapsulated in lecithin and Tween 20-stabilized double emulsions was ≈ 70-75% after 2 weeks and ≈ 65% after 21 days. Similar retention has been reported in $W_1/O/W_2$ emulsions containing trans-resveratrol over the time (Hemar et al., 2010; Matos et al., 2014).

Initial encapsulation efficiency was maintained during the storage with the addition of CMCNa, which could be attributed to the capacity of biopolymers to stabilize the outer droplets of double emulsions, preventing creaming and coalescence phenomena (Benna-Zayani et al., 2008; Dickinson, 2011; Matos et al., 2014). An emulsion has a good stability when the initial encapsulation efficiency is around 90-95%, and about 70-80% after a few weeks of storage (Dickinson, 2011; O'Regan & Mulvihill, 2010). Hence, the emulsions formulated in this study present a good stability.

Table 2. Encapsulation efficiency of the different $W_1/O/W_2$ double emulsions during 21 days at 4 ºC.

Time (days)	Emulsions			
	Lecithin [1]	Tween 20 [2]	CMCNa lecithin [3]	CMCNa Tween 20 [4]
0	78.38 ± 3.87 ^{Ab}	74.84 ± 1.53 ^{Ab}	90.14 ± 1.57 ^{Ba}	92.57 ± 0.20 ^{Bab}
2	72.87 ± 2.77 ^{Aab}	74.20 ± 0.91 ^{Ab}	90.14 ± 0.59 ^{Ba}	90.14 ± 0.59 ^{Ba}
4	76.54 ± 1.77 ^{Ab}	74.41 ± 0.26 ^{Ab}	90.84 ± 0.25 ^{Ba}	91.84 ± 2.30 ^{Bab}
7	74.31 ± 2.98 ^{Ab}	75.53 ± 0.45 ^{Ab}	89.16 ± 2.40 ^{Ba}	95.49 ± 0.00 ^{Bb}
14	76.17 ± 3.20 ^{Ab}	69.71 ± 4.79 ^{Aab}	89.60 ±0.22 ^{Ba}	92.55 ± 1.75 ^{Bab}
21	66.11 ± 1.12 ^{Aa}	66.26 ± 3.56 ^{Aa}	91.29 ± 2.86 ^{Ba}	92.29 ± 2.57 ^{Bab}

Values are expressed as mean ± standard deviation. Different capital letters within the same line indicate significant differences ($p < 0.05$) among emulsions in a day. Different lowercase letters within the same column indicate significant differences ($p < 0.05$) among emulsions throughout the storage time. [1] Lecithin: lecithin-stabilized emulsions. [2] Tween 20: Tween 20-stabilized emulsions. [3] CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions. [4] CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions.

The droplet size of double emulsions stabilized with Tween 20 did not change during 21 days of storage. Contrary, the particle size increased in the emulsions formulated with lecithin after 14 days of storage, which may be due to droplet coalescence, as it was confirmed by Turbiscan measurements (Figures 5 and 7). Chang and McClements (2016) suggested that the interfacial coating formed by lecithin molecules was less resistance to coalescence than that formed by the other emulsifiers. With the addition of CMCNa in double emulsions, no important droplet size variations were shown over the time. Similar observations have been reported by Schuch et al. (2015), who suggested CMCNa and gum arabic as a suitable alternative for stabilizing the outer drops in double emulsions by hydrophilic emulsifiers.

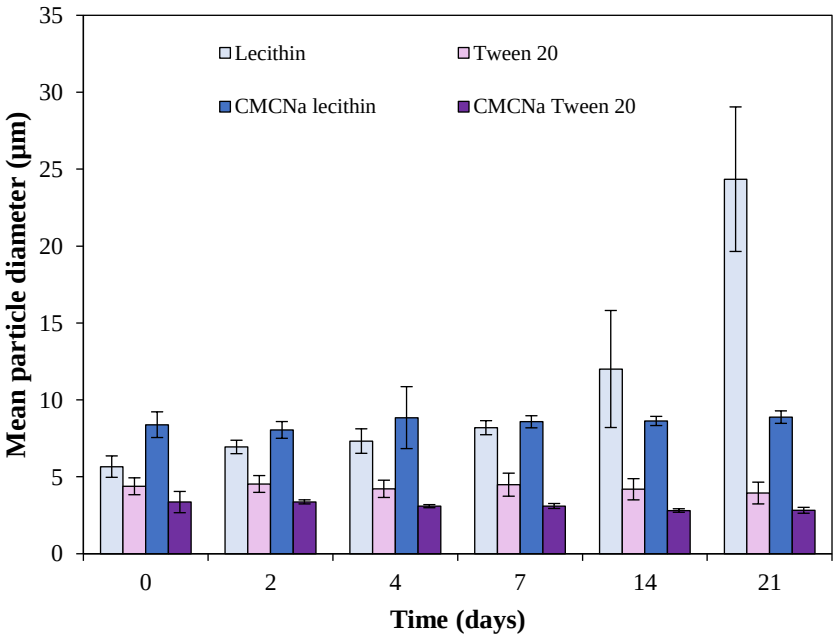

Figure 5. Mean particle size of the different $W_1/O/W_2$ double emulsions during 21 days at 4 °C. Lecithin, lecithin-stabilized emulsions; Tween 20, Tween 20-stabilized emulsions; CMCNa lecithin, sodium carboxymethylcellulose and lecithin-stabilized emulsions; CMCNa Tween 20, sodium carboxymethylcellulose and Tween 20-stabilized emulsions.

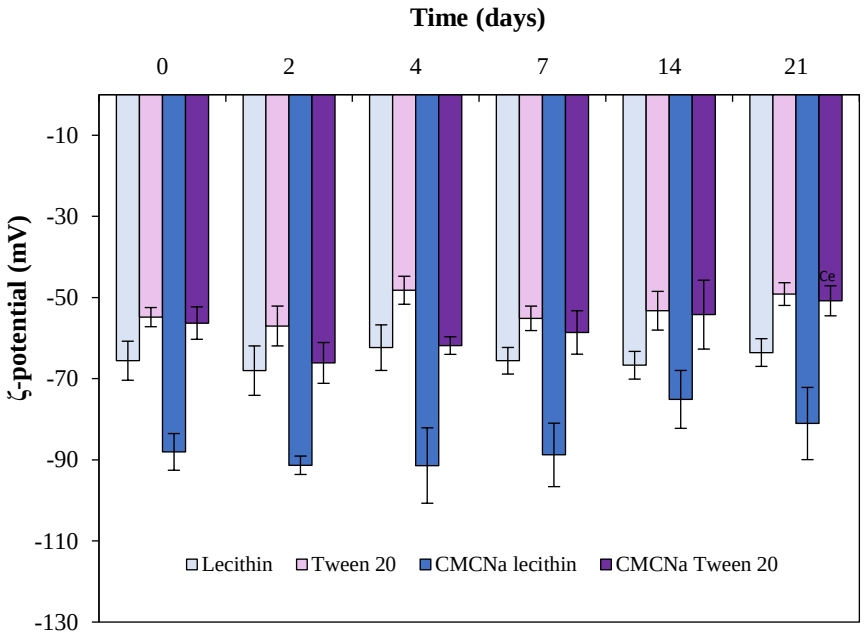

Figure 6. ζ-potential of the different $W_1/O/W_2$ double emulsions during 21 days at 4 °C. Lecithin, lecithin-stabilized emulsions; Tween 20, Tween 20-stabilized emulsions; CMCNa lecithin, sodium carboxymethylcellulose and lecithin-stabilized emulsions; CMCNa Tween 20, sodium carboxymethylcellulose and Tween 20-stabilized emulsions.

No noteworthy variation of ζ-potential values in emulsions was observed over the 21 days of storage at 4 ºC (Figure 6). Values greater than 25 mV (absolute values) are considered generally stable, and the higher the ζ-potential, the more stable the emulsion is, since the predominance of repulsion forces will avoid the flocculation phenomenon (Lamba et al., 2015). Considering that the values obtained in this study are relatively high in absolute magnitude, we can assume that stable $W_1/O/W_2$ emulsions have been formulated, especially in the case of emulsions formulated with lecithin and CMCNa, with very negative values lower than -80 mV. The stability is suggested to be attributed to the interfacial composition of emulsions and the electrostatic repulsion between oil droplets over the time. In comparison with other biopolymers, it has been reported that CMCNa provided more negative charge in O/W emulsions than whey protein isolate (Berendsen et al. 2014).

Table 3. Apparent viscosity of the different $W_1/O/W_2$ double emulsions during 21 days at 4 ºC

Time (days)	Emulsions			
	Lecithin [1]	Tween 20 [2]	CMCNa lecithin [3]	CMCNa Tween 20 [4]
0	2.16 ± 0.08 ^{Aa}	2.02 ± 0.06 ^{Aa}	277.67 ± 38.41 ^{Bab}	257.50 ± 27.69 ^{Bab}
2	2.87 ± 0.07 ^{Ab}	2.32 ± 0.20 ^{Ab}	236.50 ± 8.92 ^{Ba}	248.17 ± 19.23 ^{Bab}
4	2.95 ± 0.17 ^{Ab}	2.67 ± 0.45 ^{Ac}	286.50 ± 36.64 ^{Bab}	273.50 ± 13.37 ^{Bab}
7	3.27 ± 0.48 ^{Abc}	2.17 ± 0.10 ^{Aab}	301.50 ± 36.77 ^{Bb}	277.83 ± 29.79 ^{Bb}
14	3.58 ± 0.41 ^{Ac}	2.64 ± 0.31 ^{Ac}	304.67 ± 69.92 ^{Cb}	234.17 ± 21.63 ^{Ba}
21	5.78 ± 0.67 ^{Ad}	2.05 ± 0.09 ^{Aab}	309.33 ± 82.58 ^{Bb}	250.83 ± 63.68 ^{Bab}

Values are expressed as mean ± standard deviation. Different capital letters within the same line indicate significant differences ($p < 0.05$) among emulsions in a day. Different lowercase letters within the same column indicate significant differences ($p < 0.05$) among emulsions throughout the storage time. [1] Lecithin: lecithin-stabilized emulsions. [2] Tween 20: Tween 20-stabilized emulsions. [3] CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions. [4] CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions.

Viscosity values remained stable in Tween 20-stabilized double emulsions over the time. In contrast, an increase of viscosity was observed in the emulsions formulated with lecithin without biopolymer after 2 days and throughout the storage time (Table 3). According to our results, the addition of biopolymer prevented a viscosity increase. The rise of viscosity during the storage time could be associated with the droplet aggregation or flocculation (Regan & Mulvihill, 2009). Thus, the CMCNa addition could prevent these instability issues. The use of polysaccharides in the outer phase of double emulsions, as thickening and gelling agents, could prevent creaming and coalescence. Scleroglucan as well as mixtures of xanthan and locust bean solutions exhibited relatively low values of yield stress and plastic viscosity, which could better maintain the large droplets suspended in the external aqueous phase and avoid the breakdown of multiple droplets (Benna-Zayani et al., 2008).

3.4. Stability of $W_1/O/W_2$ emulsions by Turbiscan

The backscattering (BS) profiles obtained in a Turbiscan apparatus of $W_1/O/W_2$ emulsions were shown in Figure 7. The profiles were analysed in three different zones of the test tube (bottom, middle and top) during 21 days of storage in order to detect different instability phenomena in the emulsions. The oil droplets are susceptible to creaming, flocculation, coalescence, and Ostwald ripening, meanwhile the inner droplets are susceptible to flocculation, coalescence and Ostwald ripening process (McClements et al., 2009). No important variations in the sample mean that the formulation is stable.

A variation greater than 10% either as a positive or as a negative of BS value could be an indicator of instability (Celia et al., 2009). Maximum variations of BS were detected in lecithin and Tween 20-stabilized $W_1/O/W_2$ double emulsions at the bottom of the test tube (Figure 7A and B). In both cases, the destabilization process involves creaming, since the BS of the emulsions decreased in the bottom of the test tube because droplet concentration is reduced (clarification), whereas it increased on the top due to an increase of droplet concentration (creaming), as it was reported by Wulff-Pérez et al. (2009) and Wang et al. (2017). In addition, lecithin-stabilized emulsions presented a decrease of BS at the middle of the test tube (Figure 7A), suggesting that coalescence of the oil droplets could have led to an increase of particle size over time, as it was mentioned.

In emulsions stabilized with CMCNa, low backscattering variation was observed (Figure 7C and D), especially in those formulated with lecithin. In CMCNa lecithin-stabilized emulsions, backscattering variation over time was very low in all the sample (< 10%), indicating no significant changes in droplet size and high stability for 21 days. Such as long-term stability has been related to the biopolymer capacity to increase the viscosity of the external aqueous phase (Dickinson, 2011). Nevertheless, the addition of CMCNa in Tween 20-stabilized double emulsions extended the stability of the systems, although a remarkable decline of the BS was observed after 14 days (Figure 7D), suggesting flocculation of droplets as the particle size did not change (section 3.3). It could be explained because of the tendency of small particles to aggregate when they are numerous at a given phase ratio and more susceptible to the influence of Brownian motion, which would result in a greater probability of collision (McClements, 2016). The presence of biopolymer acting as a thickening agent reduced creaming by increasing the viscosity of the continuous phase.

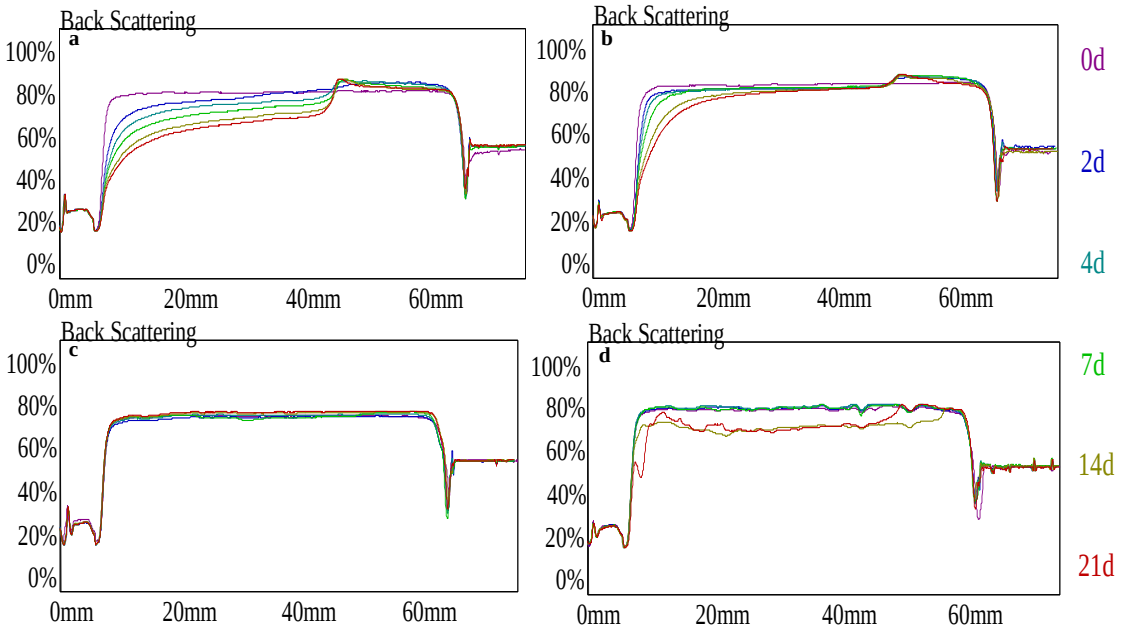

Figure 7. Variation of backscattering (BS) of the different $W_1/O/W_2$ double emulsions during 21 days at 4 °C. Lecithin, lecithin-stabilized emulsions (a); Tween 20, Tween 20-stabilized emulsions (a); CMCNa lecithin, sodium carboxymethylcellulose and lecithin-stabilized emulsions (c); CMCNa Tween 20, sodium carboxymethylcellulose and Tween 20- stabilized emulsions (d).

4. Conclusions

CMCNa-stabilized $W_1/O/W_2$ emulsions were formulated using a two-step emulsification method. The addition of CMCNa with low interfacial activity increased the encapsulation efficiency and provided higher protection to anthocyanins throughout the simulated digestion. CMCNa lecithin-stabilized emulsions showed the highest anthocyanin bioaccessibility, probably because the compound remained more time encapsulated during the lipid digestion, and were the most stable systems over the time. The combination of lecithin and CMCNa in the external aqueous phase inhibited aggregation of the outer droplets during the storage. Therefore, our results have shown that double emulsions are appropriate systems for the encapsulation of anthocyanins and for their controlled release through the gastrointestinal tract. Moreover, the effect of CMCNa in our study seems to be more beneficial with the use of lecithin rather than Tween 20.

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