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

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ABSTRACT

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

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1. INTRODUCTION

Fortifying foods and beverages with lipophilic compounds is a challenge that the food industry is facing in order to develop functional foods and satisfy consumers' demand for healthier products. Lipophilic compounds, such as carotenoids, play biological functions in our body and provide multiple health benefits [1]. However, the majority of food matrices are mainly composed of water, which makes the incorporation of these compounds difficult because of its low water solubility. In addition, external factors such as exposition to light and high temperatures can contribute to lipophilic compounds degradation during food manufacturing and storage [2,3]. Nanoemulsions can be used to encapsulate lipophilic compounds, which would not only make the incorporation of these compounds within aqueous environments possible but would also prevent and/or delay their degradation as well as maintain their functionality and bioactivity. Adding emulsifiers to nanoemulsions helps on their formation and stabilisation as they are surface-active compounds adsorbed at the oil-water interface of the droplets. According to the structure and properties of each emulsifier, they will act and deposit at the interface of the droplets differently, thereby defining the properties of nanoemulsions. At the same time, susceptibility of nanoemulsions to undergo physical and chemical changes when they are subjected to an *in vitro* gastrointestinal tract (GIT) are related to the properties of the emulsifier covering the droplets. During the *in vitro* GIT, the pH of the different digestion phases, digestive fluids and enzymes added are responsible for the constant changes suffered at the interface of droplets. These changes can affect the physicochemical stability of nanoemulsions, promoting or preventing destabilisation processes, such as flocculation or coalescence. During the last years, some studies have investigated the stability of nanoemulsions along the GIT and the processes taking place

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within it [4–9]. Besides that, few studies have focused their investigation on how the emulsifier concentration can affect the free fatty acids release during the GIT intestinal phase [10–12]. To the best of our knowledge, only one study has determined the digestive stability of β-carotene-enriched nanoemulsions containing different concentrations of a particular emulsifier (L-α-phosphatidylcholine) [13]. Conducting studies using emulsifiers with different structures (biosurfactants, phospholipids, biopolymers, colloidal particles), properties (low and high mass), and from various sources (synthetic and natural) to understand in which way the composition of nanoemulsions influence on the lipid digestion processes, would provide knowledge to design effective nanoemulsions as targeted delivery systems for lipophilic compounds.

Therefore, the goal of this study was to determine how different emulsifiers (Tween 20, lecithin, sodium caseinate, sucrose palmitate) and concentrations (2-8%) would impact on the stability (particle size and zeta potential) of nanoemulsions as they passed through a simulated *in vitro* GIT. The lipid digestibility and bioaccessibility of β-carotene-enriched nanoemulsions was also evaluated.

2. MATERIAL AND METHODS

2.1. Materials

Tween 20, pepsin from porcine gastric, pancreatin from porcine pancreatin, sodium phosphate monobasic and β-carotene were from Sigma Aldrich. Lecithin and sucrose palmitate were obtained from Alfa Aesar. Sodium caseinate, magnesium chloride hexahydrate, potassium phosphate monobasic were from Acros Organics. Hydrochloric acid (HCl) and sodium chlorure (NaCl) were from Poch S.A. Bile, sodium azide, calcium chloride dehydrate, and chloroform were obtained from Fisher. Sodium hydroxide

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(0.25N) and potassium chloride were from Panreac. Corn oil (Koipe Asua) was purchased from a local market. Milli-Q water was used to prepare all nanoemulsions.

2.2. Methods

2.2.1. Preparation of nanoemulsions

Primary oil-in-water emulsions were prepared by mixing 4% (w/w) of the lipid phase (corn oil enriched with 0.5% of β-carotene) with 96% (w/w) of the aqueous phase containing the emulsifier (Tween 20, lecithin, sodium caseinate, sucrose palmitate) at different concentrations (2%, 4% and 8% w/w). Both phases were mixed with an ultraturrax (Janke & Kunkel, Staufen, Germany) at 9.500 rpm for 2 minutes. Once the primary oil-in-water emulsion was formed, it was passed until 5 times through a microfluidizer (Microfluidics M-110P) equipped with a 75 µm ceramic interaction chamber (F20Y) at a pressure of 30,000 psi during all the treatment. Apparent viscosity of nanoemulsions using a viscometer (SV-10, A&D Company,Tokyo, Japan) vibrating at 30 Hz was determined (Table 1).

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

2.2.2. *In vitro* digestion

Nanoemulsions were subjected to a simulated *in vitro* gastrointestinal tract (GIT) digestion, which takes into account a stomach and an intestinal phase. This procedure was adapted from an standardised method [14]. For the stomach phase, each nanoemulsion was mixed with simulated gastric fluids (SGF) containing pepsin (2000

U/mL in the final mixture), $CaCl_2(H_20)$ (0.3M), milli-Q water and HCl (1M), and were incubated (Incubator OPAQ, OVAN, Barcelona, Spain) during 2h at 37ºC with a continuous agitation (100 rpm). Following that, the sample from the stomach phase was placed in a water bath (37 ºC) to simulate the intestinal phase using a pH-stat (Metrohm USA Inc., Riverview, FL, USA), and simulated intestinal fluids (SIF) (0.150 M NaCl and 0.01 M $CaCl_2$), bile extract (54 mg/mL) and pancreatin (75 mg/mL) were added to the sample. During the intesintal phase, conversion of triacylglycerols and diacylglycerols from the oil present in nanoemulsions into free fatty acids (FFA) is produced due to the lipase action. The release of these FFA was the cause of the pH reduction of the samples, which was mainted at a pH of 7 by adding NaOH (0.25 M) during 2h. The total volume of NaOH spent to keep pH at 7 during the intestinal phase was used to calculate the lipid digestibility of nanoemulsions thereby obtaining the total FFA release, using equation 1.

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

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

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

2.2.3. Determination of physicochemical properties

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The physicochemical properties of nanoemulsions before and during the different phases of the simulated *in vitro* GIT (stomach and intestine) were determined in terms of particle size and zeta potential.

2.2.3.1. Particle size

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

2.2.3.2. Optical microscopy

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

2.2.3.3. Zeta potential

The zeta potential was determined using a Zetasizer (Malvern Instruments Ltd Worcerstershire, UK). Previously to the measurement, nanoemulsions were diluted 1/10 within milli-Q water, simulated gastric fluids or simulated intestinal fluids, depending on

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the digestion phase analysed. Samples were equilibrated inside the equipment during 60 seconds.

2.2.4. Determination of β-carotene bioaccessibility

The fraction obtained after the intestinal phase was centrifuged (AVANTI J-25, Beckman Instruments Inc., Fullerton, CA, USA) at 4000 rpm for 40 minutes at 4ºC [15]. The upper part of the centrifuged liquid was collected and considered the micelle fraction, in which the mixed micelles formed during the *in vitro* digestion containing the solubilised β-carotene were present. In some samples, a layer of oil could be observed on top of the liquid, which was dismissed since it was non-digested during the *in vitro* digestion. The concentration of β-carotene in nanoemulsions and in micelle fraction was determined using a previously reported method [16]. The absorbance was measured spectrophotometrically (CECIL CE 2021; Cecil Instruments Ltd., Cambridge, UK) at 450 nm, using chloroform as a blank. Lastly, the β-carotene bioaccessibility was calculated using equation 2.

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

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

2.2.5. Statistical analysis

The analysis of variance (ANOVA) was conducted using a Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md, USA). *In vitro* digestions were

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performed in duplicate for each nanoemulsion prepared. Particle size and zeta potential were analysed in triplicate for each nanoemulsion and the data was reported as the mean with standard deviation. The least significant difference (LSD) test was carried to determine significant differences ($p \leq 0.05$) among nanoemulsions containing different emulsifiers and concentrations at a 5% significance level.

3. RESULTS AND DISCUSSION

3.1. Stability of nanoemulsions during *in vitro* digestion

Particle size. Increasing the emulsifier concentration from 2% to 8% resulted in a significant reduction in particle size of Tween 20- (from 0.35 to 0.30 µm), lecithin- (from 0.36 to 0.25 µm) and sodium caseinate- (from 0.62 to 0.47 µm) stabilised nanoemulsions (Figure 1A, 1B and 1C). In general, evidence of tiny droplets formation when using these three emulsifiers could be observed in microscope images. However, nanoemulsions stabilised at high concentrations of sodium caseinate showed some droplet aggregation (Figure 2). Thus, low mass emulsifiers such as Tween 20 and lecithin, were more effective at producing small sizes than sodium caseinate, which is known to be a high mass emulsifier with a complex and large molecular structure. In addition, low mass emulsifiers have the ability to adsorb quickly at the droplets surface during the formation of nanoemulsions, preventing the re-coalescence [17]. On the other hand, particle size of sucrose palmitate-stabilised nanoemulsions remarkably raised from 0.29 to 4.73 µm when increasing emulsifier concentration from 2% to 8% (Figure 1D). Results showed that adding to nanoemulsions 4% of sucrose palmitate was enough to cover all the surface of the formed droplets, assuming that a maximum number of tiny droplets were reached and that the particle size could not be reduced any further. Microscope

images confirmed that adding high concentrations (8%) could have led to non-absorbed molecules in the media, generating attractive forces between droplets and resulting in droplet-droplet interactions (depletion flocculation) (Figure 2).

After the stomach GIT phase, particle size of Tween 20- and lecithin-stabilised nanoemulsions remained unchanged irrespective of emulsifier concentration (Figure 1A and 1B). It is known that some non-ionic emulsifiers, such as Tween 20, are quite stable to droplet aggregation beyond low pH [19]. In particular, its high stability under acidic conditions is attributed to the polyoxyethylene head group, which produces steric repulsion between droplets. Besides that, lecithin contains a mixture of phospholipids, being phosphatidylcholine its major component. The formation of lamellar structures at the oil-water interface in which two layers of phosphatidylcholine were deposited [20,21], might have provided stability to nanoemulsions against simulated gastric fluids (SGF) and to a drastic reduction of pH during stomach phase. Nanoemulsions stabilised with 2% of sodium caseinate presented a sharp increase of particle size from 0.62 μm (undigested) to 3.74 μm (after stomach phase), while those stabilised with higher concentrations (4% and 8%) were more resistant under gastric conditions (Figure 1C). It is known that the emulsifying behaviour of sodium caseinate is associated to β-casein, which has 50 hydrophilic amino acid residues projected to the aqueous phase as tails (external layer) and 159 hydrophobic residues attached to the droplets surface forming trains (inner layer) [22]. The layer thickness around the oil droplets is attributed to the high mass properties of the protein and to the steric stabilisation of β-casein, which is mainly provided by the phosphoserine residues present in the protein. However, phosphoserine residues can suffer conformational changes when calcium ions are present, thereby resulting in a reduction of the layer thickness covering the droplets and

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stabilisation of nanoemulsions [23]. In this study, the low stability of sodium caseinate-stabilised nanoemulsions at 2% under acidic conditions, suggested the formation of a thinner layer around droplets compared to nanoemulsions containing a higher amount of sodium caseinate. The addition of gastric fluids together with the presence of pepsin, made the nanoemulsion stabilised with 2% of sodium caseinate more prone to underwent flocculation processes, as the hydrolysis of the sodium caseinate layer occurred. Microscope images confirmed that flocculated droplets were detected after stomach phase (Figure 3). On the contrary, particle size of sodium caseinate-stabilised nanoemulsions at 4 and 8% slightly changed after stomach phase, owing to the formation of a dense and thick layer around the droplets due to the β-casein deposition and the phosphoserine residues present. Sucrose palmitate-stabilised nanoemulsions presented a notable increase of particle size after stomach GIT (Figure 1D) phase and evidence of large particle aggregation could also be observed in microscope images (Figure 3). This enhancement might be related to the interaction between pepsin and sucrose esters and/or that the sucrose head would have been inverted, resulting into flocculation and/or coalescence of droplets due to the low pH (2.5) [24]. In addition, Rao & McClements, (2011)[25] studied the stability of sucrose monopalmitate nanoemulsions at different pH and reported that when the pH is below the pk_a of the carboxylic acid group from the palmitic acid (4.9), the acid loses its charge [26], being the droplets more prone to aggregate as attractive forces are more dominant than the repulsive ones.

After the GIT intestinal phase and regardless of the emulsifier concentration used, nanoemulsions suffered a steep increase in their particle size, except for those stabilised with sucrose palmitate. In the latter case, flocculated droplets formed during the stomach phase due to the drastic change of pH could have been re-dispersed during the

intestinal phase [27]. On the other hand, large values of particle size obtained for the rest of nanoemulsions could be attributed to various reasons. Firstly, partial or total displacement of emulsifier molecules from the droplet surfaces when free fatty acids were produced, together with their hydrolysis by intestinal enzymes, would have resulted in a single emulsifier molecule attached to the surface of more than one droplet, provoking aggregation phenomena (bridging flocculation)[28]. Secondly, deposition of free fatty acids at the oil droplets interface as well as the presence of partially digested lipid droplets could have led to coalescence processes. Finally, all type of particles such as mixed micelles, vesicles and insoluble calcium complexes are formed during the lipid digestion [29], which would have contributed on the particle size increase. Different particle size behaviour was observed in nanoemulsions stabilised with Tween 20, lecithin and sodium caseinate after the GIT intestinal phase. Tween 20- and sodium caseinate-stabilised nanoemulsions presented a greater particle size as emulsifier concentration increased, reaching values of around 3.50 µm in all samples (Figure 1A and 1C). The high amount of emulsifier added initially, would have formed complex aggregates and/or particles with other digestion components, boosting even further destabilisation processes during the intestinal phase. An opposite trend was observed for lecithin-stabilised nanoemulsions, decreasing the particle size until 0.48 µm with the addition of 8% of lecithin (Figure 1B), which might be related to the triglycerides hydrolysis from lipid droplets.

Simultaneous and complex physicochemical processes occurring during lipid digestion such as interactions and associations between all the particles present in that moment and the formation of new species [30], could have influenced on the particle size of nanoemulsions. In this sense, microscope images of nanoemulsions after intestinal

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phase showed the presence of two types of particles: some of them had an irregular shape and others were so small that could barely be observed in microscope images (Figure 3). When analysing particle size of nanoemulsions with light scattering technique (such as Mastersizer) and microscope images, complementary information could be obtained. Using both approaches is of great importance when determining nanoemulsions particle size characteristics.

Zeta potential. All nanoemulsions presented negative values of zeta potential irrespective of the emulsifier nature and concentration. The most negative values were exhibited by lecithin-stabilised nanoemulsions, with values around -80 mV. The phosphate groups from the different types of phospholipids contained in the lecithin were the reason for the elevated negative charge [31]. Even though Tween 20 and sucrose palmitate are non-ionic emulsifiers, the zeta potential of undigested nanoemulsions stabilised with both emulsifiers were negative, being around -24 mV and -49 mV, respectively and for all emulsifier concentrations (Figure 4A and 4D). One argument to explain these results would be the adsorption of OH^- species from the aqueous phase to the interface of the oil droplets. Alternatively, cationic species of the oil [32], the presence of residual non-esterified fatty acids in the sucrose ester [33] or impurities (palmitic acid) [34] in the case of sucrose palmitate, could have been the cause of the negative charges. The zeta potential values of sodium caseinate-stabilised nanoemulsions ranged between -40 and -48 mV, being slightly less negative as emulsifier concentration increased. Adding sodium caseinate to nanoemulsions might have decreased their pH until near the isoelectric point of caseinate (4.5). In this situation, there were a sufficient number of amino groups of caseinate positively charged that would have increased the zeta potential from -48 to -39 mV (Figure 4C).

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The zeta potential of all nanoemulsions became less negative after the stomach phase with values between -24.2 and -1.5 mV regardless of the emulsifier nature and concentration. The simulated gastric fluids used in this work containing free ions and the acidic pH during the stomach phase, would have attenuated the charges of the nanoemulsion droplets (electrostatic screening effect) [18]. It is interesting to mention that the negative zeta potential (-9.70 mV) obtained for sodium caseinate-stabilised nanoemulsions at 2%, confirmed that some of the sodium caseinate covering the droplets was partially displaced from the interface during the stomach GIT phase. Nanoemulsions with higher concentrations of sodium caseinate presented values of zeta potential slightly more negative (around -11.75 mV), suggesting that the interface of the droplets lightly changed. It should be noted that whether the pH of stomach phase (2.5) is below the isoelectric point of the proteins, a positive zeta potential is expected to be obtained. In this case, it was assumed that changes on the interface of the droplets consisting on the displacement of the sodium caseinate covering the droplets, and the absorption of negatively charged particles would have been the reason of negative values. Meanwhile, zeta potential of all nanoemulsions became slightly negative after being subjected to intestinal conditions, reaching values similar to those of undigested nanoemulsions. The production of different particles in the process of lipid digestion (undigested lipid droplets, vesicles or micelles), as well as the presence of digestion components (bile and pancreatin, calcium) [35], could have had an impact on the zeta potential of nanoemulsions after the intestinal phase (Figure 4). Simultaneous processes taking place during lipid digestion (enzyme hydrolysis, formation of new species, interactions between components, among others) result in a constant changing of the interfacial properties of nanoemulsion droplets, affecting on their electric charge. In

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addition, the neutral pH during intestinal phase similar to undigested nanoemulsions might be another reason why similar values of zeta potential were obtained.

3.2. Oil digestibility of nanoemulsions

Different free fatty acids profiles were observed depending on the emulsifier nature and concentration used to elaborate nanoemulsions. In general, there was a quick increase of the free fatty acids release during the first 10-15 min of the intestinal phase for all the nanoemulsions. Then, there was a period of time where the free fatty acids released remained constant until the end of the intestinal phase (Figure 5). Free fatty acids (FFA) are produced during the hydrolysis of the triacylglycerides from lipid droplets by the lipase present in the pancreatin. These FFA are considered to be surface-active components, which can be deposited and accumulated at the surface of the droplets as they are being produced [36,37]. In this situation, new FFA are not generated because droplets interface is collapsed by the FFA already produced, inhibiting the deposition and action of the pancreatin at the interface of the droplets. As an exception, nanoemulsions stabilised with Tween 20 at concentrations ≥ 4%, presented an interval period at the beginning of the intestinal phase (40-60 minutes), where no free fatty acids were produced (Figure 5A). Presumably, it was the time needed for the bile to be placed on the surface of the droplets and displace the Tween 20. Other studies have also observed an interval period without production of free fatty acids for nanoemulsions stabilised with lower concentrations of Tween 20 (0.6% and 1%) [28,37].

Despite observing different behaviours during the lipid digestion among nanoemulsions and independently of the large values of particle size observed for certain nanoemulsions after stomach phase (section 3.1), no significant differences in the total

amount of free fatty acids were observed at the end of the intestinal GIT phase. Particle size of nanoemulsions entering within the intestinal phase is supposed to be an important factor that influences on the digestibility of nanoemulsions [16]. However, our results suggested that the great particle size of these nanoemulsions before entering the intestine was due to droplet flocculation, meaning that weak bonds linked the droplets together. As nanoemulsions were exposed to intestinal conditions, these bonds were broken down resulting in a re-dispersion of the droplets which could have facilitated the digestion of lipids.

It is interesting to remark that nanoemulsions stabilised with 8% of lecithin presented a higher lipid digestibility (100%) compared with those nanoemulsions stabilised with lower concentrations (around 73% of lipid digestibility). In this sense, Yang Decker, Xiao, & McClements (2015)[38] suggested that the addition of phospholipids in the simulated intestinal fluids (36 mg) increased the final extent of lipid digestion, since phospholipids may facilitate the ability of lipase to interact with the emulsified triglycerides.

3.3. Bioaccessibility of β-carotene-enriched nanoemulsions

Based on the obtained results, not only the emulsifier nature but also their concentration present within nanoemulsions had an impact on β-carotene bioaccessibility (Figure 6). For Tween 20-stabilised nanoemulsions, β-carotene bioaccessibility was around 16%, without significant differences regardless of the emulsifier concentration. On the contrary, for sodium caseinate-stabilised and sucrose palmitate-stabilised nanoemulsions, the β-carotene bioaccessibility significantly decreased when high concentrations of emulsifier (8%) were added. Considering that the quantity of free fatty acids released during the lipid digestion was similar among

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nanoemulsions prepared with these emulsifiers, different hypothesis were suggested in order to explain this tendency. Firstly, not all the free fatty acids produced might have participated in the formation of mixed micelles, since some interactions with the digestion products may have occurred. Secondly, mixed micelles formed during the digestion may not have incorporated and solubilised the β-carotene, assuming that if β-carotene is not present within the mixed micelles, is not considered absorbable. Thirdly, interactions between the β-carotene itself and/or mixed micelles containing β-carotene with the emulsifier could have occurred. Indeed, it is known that proteins can form complexes with carotenoids through hydrophobic interactions [39], as well as promote aggregation and precipitation of mixed micelles [5]. Lecithin-stabilised nanoemulsions presented a greater β-carotene bioaccessibility as emulsifier concentration increased, raising from 9.9% to 23.5%. These results agreed upon the lipid digestibility results (section 3.2): the high number of free fatty acids produced for nanoemulsions with 8% of lecithin could lead to the formation of a large number of mixed micelles, enhancing not only the lipid digestibility but also β-carotene bioaccessibility. Mixed micelles are comprised of bile salts, phospholipids from the bile and pancreatic juices as well as lipid digestion products from the action of lipases, such as free fatty acids [40,41]. Thus, lecithin, a nontoxic emulsifier generally recognized as safe (GRAS) that predominantly contains phospholipids, could have contributed to these particles formation as well as increase their solubilisation capacity [38,42].

4. CONCLUSIONS

Results obtained in this work indicated that β-carotene-enriched nanoemulsions presented different initial physicochemical properties and behaviours along the GIT

owing to the emulsifier nature and concentration. Despite this fact, nanoemulsions presented similar lipid digestibility results, with the exception to those elaborated with lecithin at 8%. In turn, these latter nanoemulsions showed the highest β-carotene bioaccessibility after being digested through an *in vitro* GIT. This work revealed that using lecithin could be a good option when designing nanoemulsions as delivery systems of lipophilic compounds. Further investigation with *in vivo* studies (animal and human) are required to elucidate the importance of nanoemulsions composition relationship with digestive processes and bioaccessibility of encapsulated compounds.

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Table 1. Viscosity of nanoemulsions stabilised with different emulsifiers.

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

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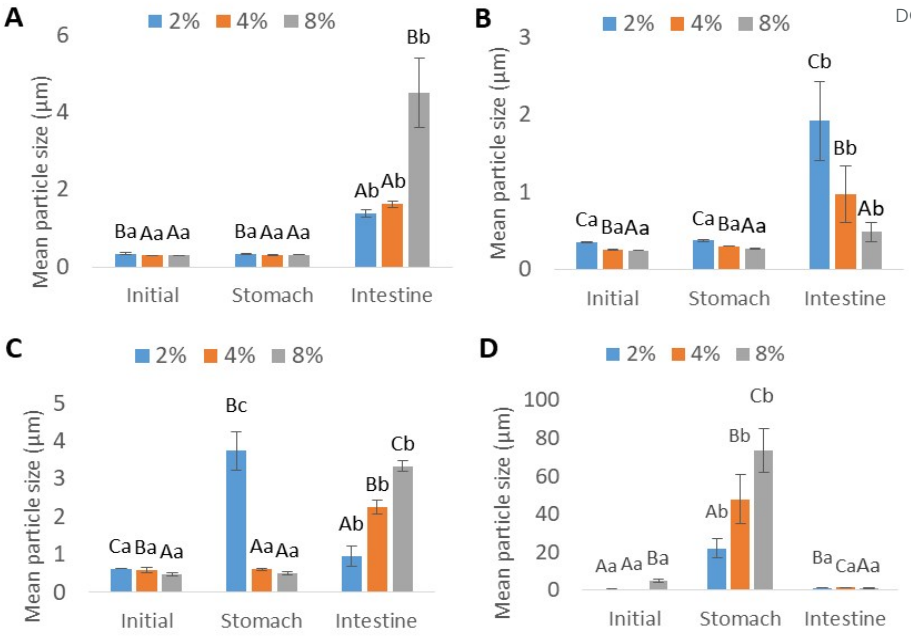

Figure 1. Particle size (µm) of β-carotene enriched nanoemulsions stabilised with Tween 20 (A), Lecithin (B), Sodium Caseinate (C), or Sucrose palmitate (D) at different concentrations (2%, 4% and 8%) initially and during *in vitro* digestion phases (stomach, intestine). Different capital letters indicate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

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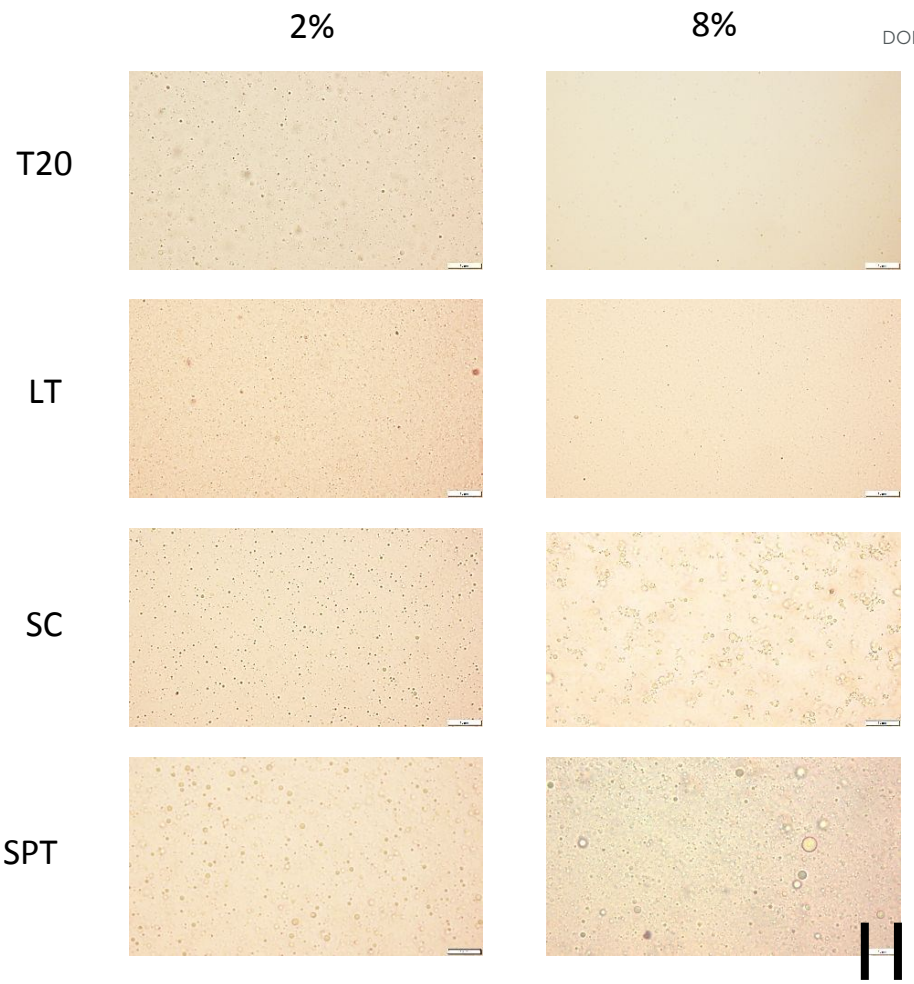

Figure 2. Images of β-carotene enriched nanoemulsions stabilised with different emulsifiers (T20: Tween 20; LT: lecithin; SC: sodium caseinate; SPT: sucrose palmitate) at two concentrations (2% and 8%). Scales bar are 10µm long.

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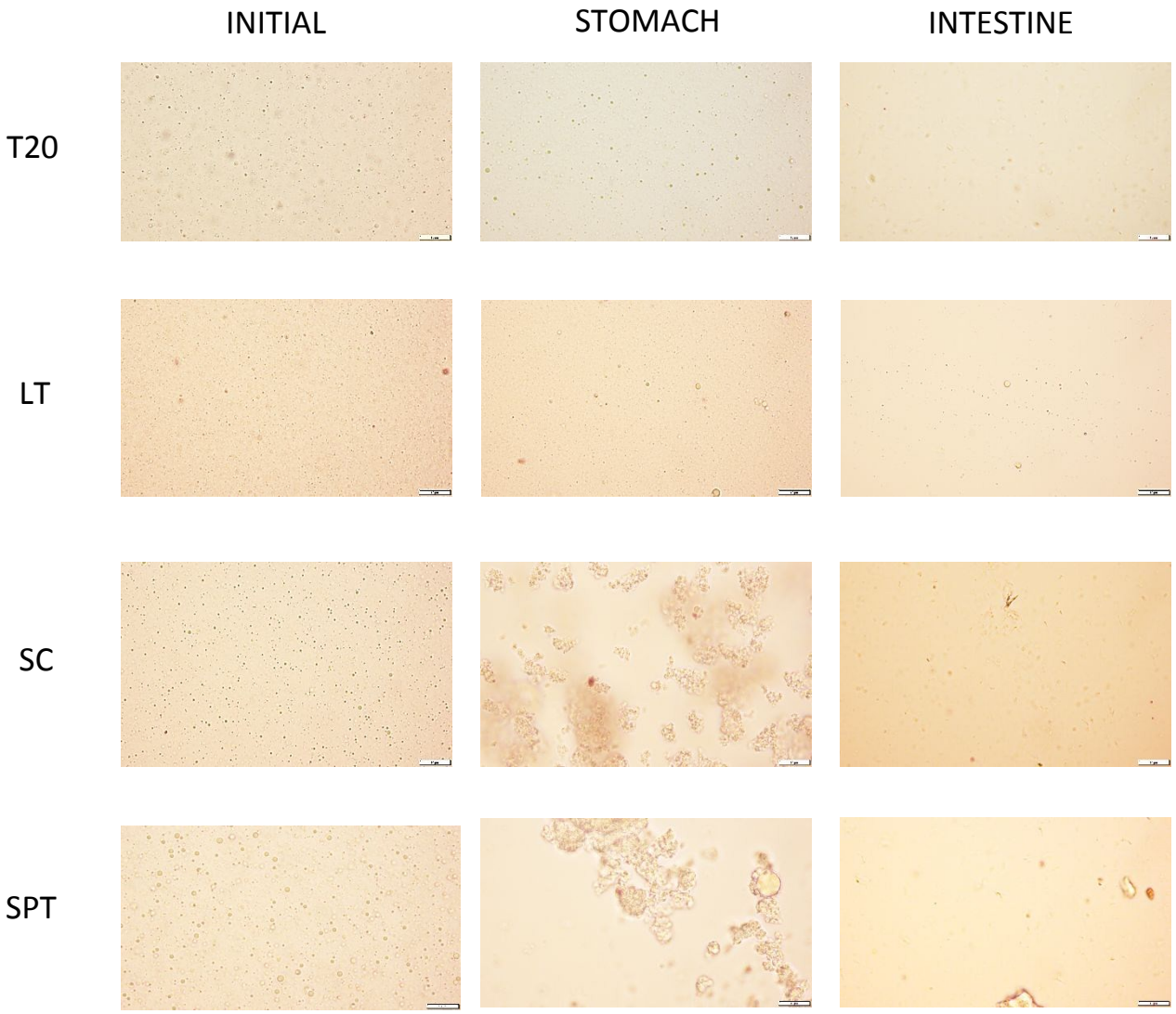

Figure 3. Representative microscope images of β-carotene enriched nanoemulsions stabilised with different emulsifiers (T20: Tween 20; LT: lecithin; SC: sodium caseinate; SPT: sucrose palmitate) at 2% along the different phases (stomach and intestine) of the *in vitro* gastrointestinal tract (GIT). Scales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

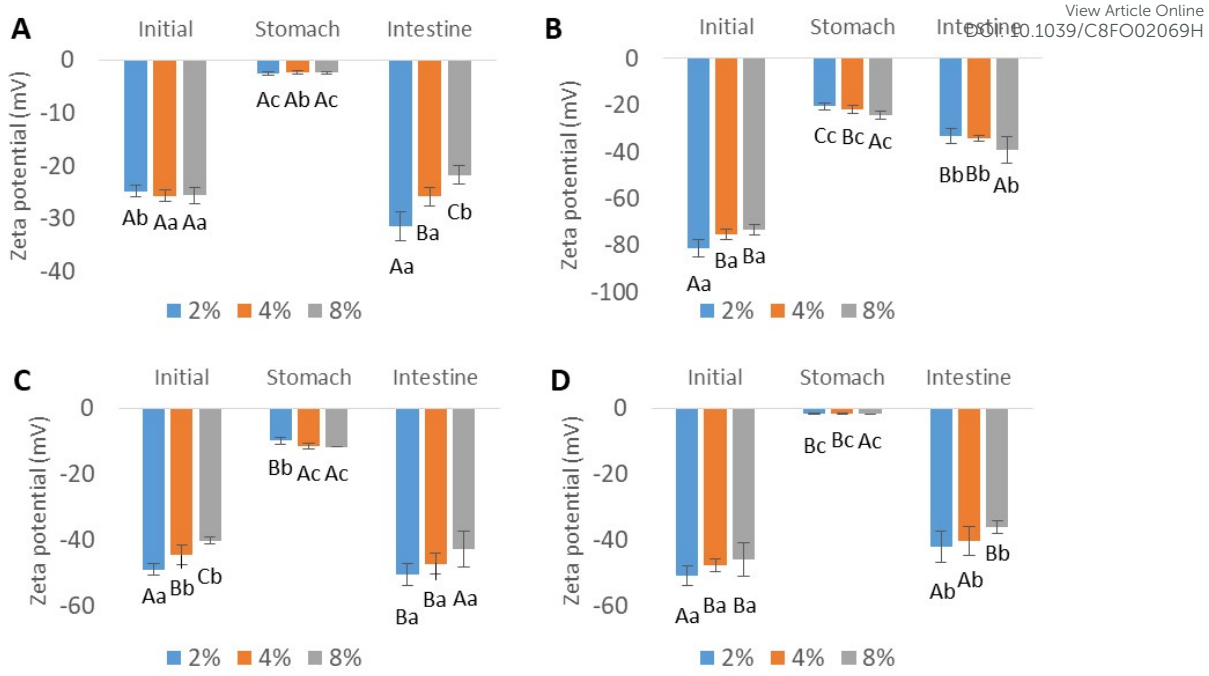

Figure 4. Changes in zeta-potential (mV) of β-carotene enriched nanoemulsions stabilised with Tween 20 (A), Lecithin (B), Sodium Caseinate (C), or Sucrose palmitate (D) at different concentrations (2%, 4% and 8%) initially and during *in vitro* digestion phases (stomach, intestine). Different capital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

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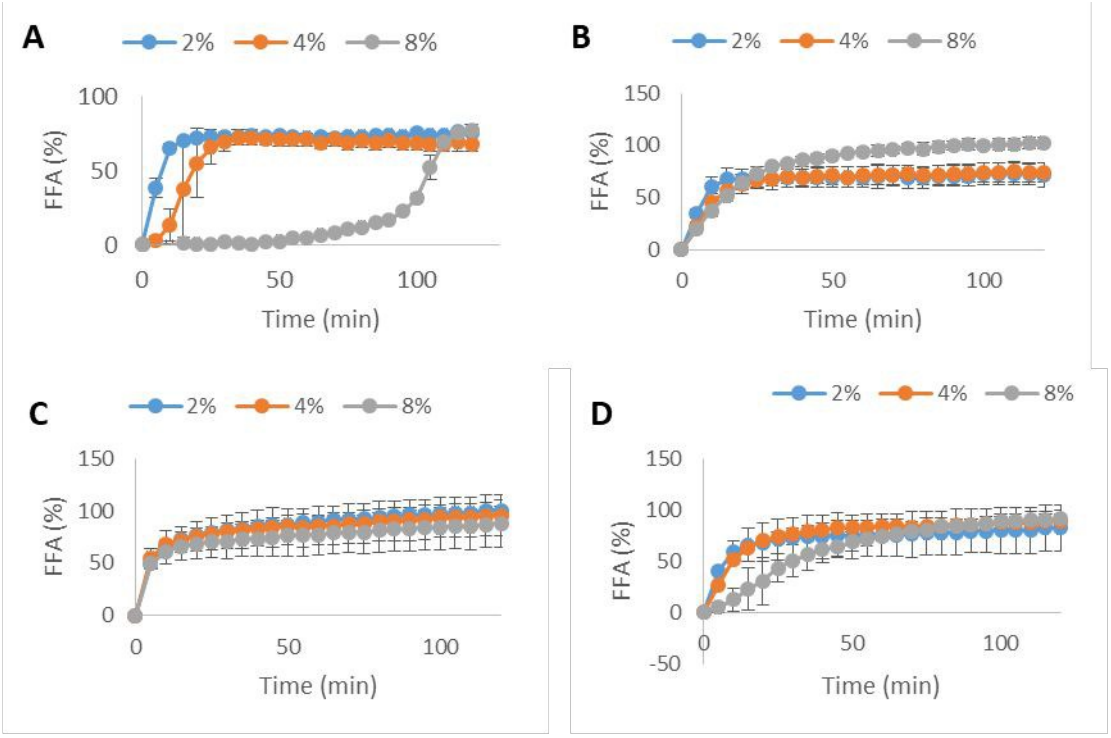

Figure 5. Influence of emulsifier nature (Tween 20 (A), Lecithin (B), Sodium Caseinate (C), Sucrose palmitate (D)) and concentration (2%, 4% and 8%) on the free fatty acids (FFA) release from β-carotene-enriched nanoemulsions during the intestinal phase.

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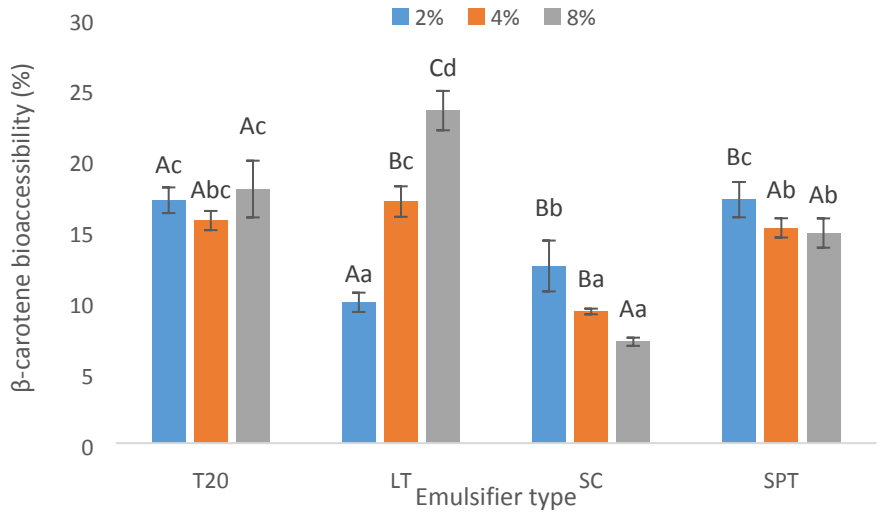

Figure 6. Influence of emulsifier nature (T20: Tween 20; LT: lecithin; SC: sodium caseinate; SPT: sucrose palmitate) and concentration (2%, 4% and 8%) on the bioaccessibility of β-carotene enriched nanoemulsions. Different capital letters indicate significant differences ($p<0.05$) of β-carotene bioaccessibilty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

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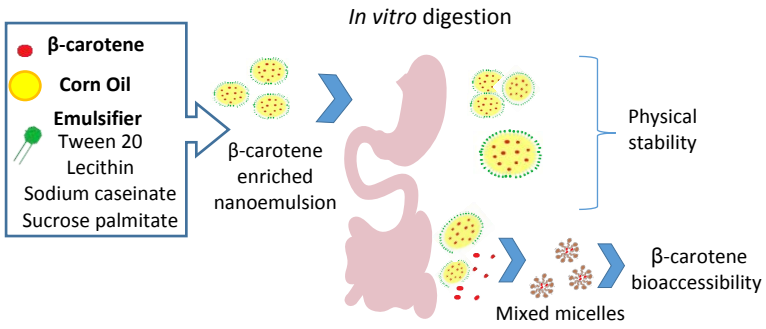

This study reveals the importance of the emulsifier nature and concentration used to elaborate nanoemulsions as targeted delivery systems for β-carotene.