In vitro and *in vivo* study of fucoxanthin bioavailability from nanoemulsion-based delivery systems: Impact of lipid carrier type

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Abstract

The impact of carrier oil type on the functionality of a lipophilic nutraceutical (fucoxanthin) encapsulated within nanoemulsions was investigated. Three carrier oils were investigated: long chain triacylglycerols (LCT); medium chain triacylglycerols (MCT); and indigestible oil (orange/mineral oil). Nanoemulsions containing LCT and MCT were completely digested under simulated gastrointestinal conditions, whereas those containing indigestible oil were not digested. Fucoxanthin solubility in mixed micelles formed by *in vitro* digestion decreased in the following order: LCT > MCT > indigestible oil. Animal feeding studies revealed that fucoxanthin was absorbed into the intestinal epithelial cells in the same order as observed for the *in vitro* solubility. However, the concentration of fucoxanthin in the serum of the rats was similar for all carrier oils. The present work highlights the importance of contrasting *in vitro* and *in vivo* experiments to assess the biological fate of functional ingredients incorporated in emulsion-based delivery systems.

Keywords: lipid digestibility; fucoxanthin; *in vitro* digestion; nanoemulsions; *in vivo* bioavailability; nutraceuticals; rats

Chemical compounds studied in this work: Fucoxanthin (PubChem CID: 5281239); Fucoxanthinol (PubChem CID: 11273547); Amarouciaxanthin A (PubChem CID: 16061220); Triheptadecanoin (PubChem CID: 3625612)

1. Introduction

The isolation, purification, and utilization of functional ingredients derived from algae is gaining importance due to their bioactivity, sustainability, and low cultivation costs (Holdt & Kraan, 2011; Ugwu, Aoyagi, & Uchiyama, 2008). Fucoxanthin is a carotenoid belonging to the hydroxylated xanthophyll class that has an unusual allenic bond in the 5,6-monoepoxide (Kotake-Nara et al., 2001). It is found in specific types of brown algae where it is involved in the photosynthesis reaction (Maeda, Tsukui, Sashima, Hosokawa, & Miyashita, 2008; Takaichi, 2011). Fucoxanthin is responsible for the brown colour of this type of algae due to the fact that it selectively absorbs light in the visible region. It has also been reported to have antioxidant and anti-inflammatory properties (Peng, Yuan, Wu, & Wang, 2011; Sachindra et al., 2007) as well as anti-cancer activity (Kumar, Hosokawa, & Miyashita, 2013; Moghadamtousi et al., 2014). Moreover, recent studies have reported anti-obesity and anti-diabetic properties associated with the consumption of fucoxanthin (Awang et al., 2014; Maeda, Hosokawa, Sashima, Murakami-Funayama, & Miyashita, 2009). Fucoxanthin therefore has considerable potential as a nutraceutical ingredient that could be incorporated into functional food and beverage products. However, as with other carotenoids, there are a number of challenges that limit its potential application in foods, such as its low water-solubility, high melting point, and chemical instability (Hii, Choong, Woo, & Wong, 2010; Muthuirulappan & Francis, 2013).

Food grade nanoemulsions, which consist of small lipid droplets (< 100 nm) dispersed in water, may be a suitable strategy for incorporating carotenoids into foods and of enhancing their bioactivity profiles (McClements, 2011; McClements & Xiao, 2012). Indeed, it has been reported that the administration of lipophilic bioactive compounds within a lipid matrix favors their incorporation into mixed micelles and therefore enhances their bioavailability in the gastrointestinal tract (Donhowe & Kong, 2014). When the lipase hydrolyses the triacylglycerols, the free fatty acids released form mixed micelles that solubilize and transport the lipophilic bioactive compounds (Yonekura & Nagao, 2007). Nevertheless, the micelle solubilization of lipophilic active compounds in the small intestine is driven by the intrinsic characteristics of the lipid nanoparticles that they are included in. For example, studies have shown that carotenoid (β -carotene) bioaccessibility increases with decreasing droplet size in emulsion-based delivery systems, which was attributed to faster and more complete digestion of the lipid phase leading to greater release and higher solubilization (Salvia-Trujillo, Qian,

Martín-Belloso, & McClements, 2013a). In addition, the nature of the lipid phase used to prepare the nanoemulsions has also been shown to have a major impact on the bioaccessibility of lipophilic bioactive compounds. Specifically, the triacylglycerol chain length determines the micelle structure and size and in turn the bioactive compounds bioaccessibility in the gastrointestinal tract (Huo, Ferruzzi, Schwartz, & Failla, 2007). The use of long chain triacylglycerol oils for the formation of nanoemulsions led to a higher β -carotene bioaccessibility compared to medium or short chain triglycerides (C. Qian, Decker, Xiao, & McClements, 2012; Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013b). Consequently, the selection of an appropriate lipid carrier and particle size is critical for designing emulsion-based delivery systems with optimized biological activities.

In vitro methods that simulate gastrointestinal tract (GIT) conditions are often used to assess the potential gastrointestinal fate of emulsion-based delivery systems (Fatouros & Mullertz, 2008; Hur, Lim, Decker, & McClements, 2011; McClements & Li, 2010a; Minekus et al., 2014). *In vitro* methods are particularly useful to rapidly and cheaply screen delivery systems with different characteristics, and therefore avoid the use of time-consuming, costly, and ethically challenging animal studies. Moreover, *in vitro* experiments enable one to identify critical physicochemical processes that may influence the performance of delivery systems under gastrointestinal conditions, such as the integrity, interactions, and release characteristics of colloidal particles in different regions of the GIT. However, *in vitro* models cannot mimic the complex physicochemical and physiological processes occurring in the digestive tracts of animals or humans (G. Y. Park et al., 2007). To obtain more reliable and accurate information about the potential biological activity of functional ingredients encapsulated within delivery systems it is therefore important to compare the results of *in vitro* models with those of *in vivo* studies (Lee et al., 2008; Ostrowski & Baczek, 2010; Porter et al., 2004).

The purpose of the current work was therefore to study the influence of lipid carrier type on the biological activity of fucoxanthin-loaded nanoemulsions. Corn oil was used as an example of a digestible long chain triacylglycerol (LCT), Mygliol was used as an example of a digestible medium chain triacylglycerol (MCT), and a mixture of orange oil and mineral oil (80:20) was used as an example of a non-digestible oil. The results obtained using a well-established *in vitro* gastrointestinal model were compared with those obtained using an *in vivo* animal (rats) feeding model. Ultimately, the goal of this work was to provide important

insights into the major factors influencing the design of nanoemulsion-based delivery systems to improve the oral bioavailability of lipophilic nutraceuticals.

2. Material and methods

2.1. Materials

Corn oil (long chain triacylglycerol, LCT) was purchased from a local supermarket. Miglyol 812 (medium chain triacylglycerol, MCT) was purchased from SASOL (Houston, TX, USA). Orange flavor oil was obtained from The Chemistry Store (Cayce, SC, USA). Mineral oil, Tween 80, monobasic and dibasic phosphates, and Nile Red dye were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pepsin, bile salts and lipase were also obtained from Sigma. Fucoxanthin enriched MCT (1% w/w fucoxanthin) oil was bought from Restore Labs Co, (Gangneung, Korea). Triheptadecanoin (C_{17:0}) and tridecenoic acid (C_{13:0}) were purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). All aqueous solutions were prepared using purified water from a Mili-Q filtration system.

2.2. Methods

2.2.1. Nanoemulsion formation

A lipid phase was prepared by mixing a 10% (v/v) of fucoxanthin-enriched MCT oil with 90% (v/v) of carrier oil (LCT, MCT, or non-digestible oil). The non-digestible oil consisted of a mixture of orange oil and mineral oil (80:20 v/v). Mineral oil was used as a ripening inhibitor to prevent Ostwald ripening from occurring in the nanoemulsion containing orange oil. Heptadecanoic (C_{17:0}) acid at 0.1% (w/w) was dissolved in the lipid phase by stirring until complete dissolution was achieved. We included heptadecanoic (C_{17:0}) acid in the nanoemulsions as a model fatty acid since it is not normally found in the animal's body, and therefore an increase in its concentration in small intestine tissues is a measure of its absorption. The final fucoxanthin concentration in the initial nanoemulsions was 0.1% (w/w). The aqueous phase used to prepare the emulsions consisted of phosphate buffered saline (PBS) and 3% (w/w) surfactant (Tween 80). Coarse emulsions were obtained by blending 30 % (w/w) of the oil phase and 70 % (w/w) of the aqueous phase using a high-shear mixer at 20,000 rpm for 2 min. The three different coarse emulsions were then immediately passed

three times through a microfluidizer (model M110-P, Microfluidics, Newton, MA, USA) working at 12,000 psi to form nanoemulsions.

2.2.2. Nanoemulsion characterization

The particle size distribution of the initial nanoemulsions was measured by dynamic light scattering (DLS) (Zetasizer NanoZS, Malvern Instruments Ltd, Worcestershire, UK) at a wavelength of 633 nm and temperature of 25 °C using a backscatter detector (173 °). The droplet size was reported as Z-average diameter (nm). On the other hand, the particle size distribution of the samples exposed to simulated gastrointestinal conditions was measured by static light scattering (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) due to the relatively large particles formed. Samples were diluted in 10 mM phosphate buffer (pH 7.0) to avoid multiple scattering effects, and then stirred in the dispersion unit of the instrument at a speed of 1250 rpm to ensure they were homogeneous prior to analysis. The particle size was reported as either the surface-weighted mean diameter (d_{32}) or volume-weighted mean diameter (d_{43}) in μm (McClements, 2005).

The ζ -potential of the particles was measured by phase-analysis light scattering (Zetasizer NanoZS, Malvern Instruments, Worcestershire, UK). Samples were diluted 1:10 with 10 mM phosphate buffer solution (pH 7.0) and then placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles.

In this study, all samples were diluted in the same buffer solution so that their particle characteristics could be compared under similar conditions.

2.2.3. *In vitro* digestion

An *in vitro* gastrointestinal tract (GIT) model consisting of mouth, gastric and intestinal phases were used to simulate the biological fate of ingested samples. Nanoemulsions were diluted so that they had an initial oil concentration of 2% (w/w) prior to passing through the GIT model.

Mouth phase: Simulated saliva fluid (SSF), containing mucin and various salts, was prepared according to a previous study (Sarkar, Goh, & Singh, 2009). A 10 mL-aliquot of the emulsions was mixed with 10 mL of SSF, so that the final mixture contained 1% (w/w) oil. The pH of the mixture was adjusted to 6.8 and it was incubated at 37 °C for 10 min with continuous agitation at 100 rpm.

Gastric phase: Simulated gastric fluid (SGF) was prepared using a method reported previously (Sarkar, Goh, Singh, & Singh, 2009) by dissolving 2 g of NaCl and 7 mL of HCl (37%) in 1 L of water and adjusting the pH to 1.2 using 1.0 M HCl. The “bolus” sample from the mouth phase was mixed with SGF at a 50:50 volume ratio so that the final mixture contained 0.5% (w/w) oil. The pH of the sample was adjusted to 2.5 using NaOH (1M) and incubated at 37° C for 2 hours with continuous agitation at 100 rpm.

Small intestinal phase: A pH-stat (Metrohm, 147, Riverview, FL, USA) was used to simulate the conditions in the small intestinal phase of the GIT (McClements & Li, 2010b). An aliquot (30 mL) of sample from the gastric phase was placed in a temperature-controlled (37 °C) chamber and the pH was set at 7.0 using NaOH solution. Then, 4 mL of bile extract (46.87 mg/mL) and 1 mL of calcium chloride (110 mg/mL) solutions dissolved in phosphate buffer were added to the sample and the pH was adjusted to 7.0 if necessary. Afterwards, 2.5 mL of freshly prepared lipase suspension (24 mg/mL) dissolved in phosphate buffer was incorporated into the mixture. The pH of the mixture was monitored and the volume of 0.1 M NaOH (mL) necessary to neutralize the free fatty acids (FFA) released from the lipid digestion (*i.e.*, to keep pH at 7.0) was recorded during 2 h. The amount of free fatty acids released was calculated from the titration curves as described previously (Li & McClements, 2010).

2.2.4. *In vivo* studies

The bioavailability of triglycerides and fucoxanthin were determined using a rat model. Three male and 7 female Sprague Dawley rats (250-300g) were purchased from Charles River Laboratories (Wilmington, MA, USA). Rats were divided into 3 groups with 1 male and 2-3 females per group. Rats were housed in individual stainless steel cages and maintained under a 12 h Light/Dark cycle and temperature and humidity controlled environment. After a week of adaptation, rats were fasted overnight and then a total 5 ml of emulsion, divided into five doses given at 30 min intervals, was administered into a rat’s stomach by gavage. 30 minutes after the final administration, the rats were sacrificed by CO₂ gas asphyxiation and cardiac puncture was conducted to collect blood samples. Blood samples were put on ice and allowed to clot for 30 minutes. Then the blood samples were centrifuged for 20 minutes at 800 g at 4°C and the serum was collected. Serum was stored at -80 °C until analysis. The small intestine was cut into 3 parts (upper part, middle part and lower part), the small intestine

content was collected and then the small intestine was washed with PBS 5 times. The upper part, middle part, and lower part of the small intestine were kept at -80 °C until analysis.

2.2.5. Heptadecanoic analysis

Total lipids from the small intestines and serum were extracted and methylated prior to fatty acid analysis using gas chromatography (Folch, Lees, & Stanley, 1957; Y. Park & Pariza, 1998). A gas chromatography instrument (GC-17A, Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector was used to determine fatty acids methyl esters. A fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 mm film thickness) was used (DB-23, Agilent Technologies, Wilmington, DE, USA) and the oven temperature was programmed to be held at 150 °C for 4 min, increased by 4 °C/min to 250 °C, and then held for 40 min. Tridecenoic acid was used as an internal standard, and absorbed heptadecanoic acid was calculated relative to the tissue weight.

2.2.6. Fucoxanthin analysis

Fucoxanthin is known to be hydrolyzed into two metabolites, fucoxanthinol and amarouciaxanthin A in mice and HepG2 cells (Sugawara, Baskaran, Tsuzuki, & Nagao, 2002). It is suggested that prior to absorption in the intestine, fucoxanthin is hydrolyzed into fucoxanthinol in the GIT, which is later converted to amarouciaxanthin A in the liver, both of which along with, fucoxanthin have similar UV absorption max and intensity at 445-450 nm. Thus we quantified fucoxanthinol and amarouciaxanthin A content in the tissue samples using fucoxanthin as a standard at 450 nm.

Fucoxanthin standard solution (0.25, 0.5, 1, 2.5, 5 µg /mL) was prepared by dissolving fucoxanthin in methanol. Fucoxanthin and its metabolites were extracted from tissue, serum, and *in vitro* digestion samples. The small intestine samples (150 mg) were homogenized in a dark room with 2 mL chloroform/ methanol (2:1 v/v). Serum (280 µL) or *in vitro* digestion samples (500 µL) were vortexed for 1 min with 2 ml chloroform/ methanol (2:1 v/v). Then 1 mL of 4% KCl was added, mixed for 1 min, and centrifuged. The chloroform layer was dried under nitrogen, solubilized in 1 ml methanol, and subjected to high performance liquid chromatography (HPLC) after filtration.

Fucoxanthin and its metabolites were determined using HPLC (Fung, Hamid, & Lu, 2013; Kim et al., 2012; Kim, Shang, & Um, 2011). An Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a G1310B iso pump, a G1329B auto-

sampler, a G4208A controller and a G1314F UV detector was used. The samples and standards were separated on a C₁₈ reverse phase column (Eclipse Plus C₁₈ 3.5 µm 100 x 4.6 mm, Agilent) with 85% acetonitrile in methanol (v/v) at a flow rate of 1 ml/min and integrated with Astra 6 software (Wayatt technology, Santa Barbara, CA, USA). Fucoxanthin was detected at 450 nm.

2.2.7. Confocal fluorescence microscopy

Confocal fluorescence microscopy images were taken to determine destabilization phenomena in nanoemulsions during the *in vitro* digestion and also to assess the nanoemulsion microstructure in the rat's gastrointestinal tract (stomach, upper, middle and lower small intestine). Samples were dyed with Nile red (a fat-soluble fluorescent dye) that was previously dissolved at 0.1% (w/v) in ethanol. An air-cooled argon ion laser Model IMA1010 BOS (Melles Griot, Carlsbad, CA, USA) was used to excite Nile red at 488 nm. A Nikon Confocal Microscope (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, USA) with a 60× oil immersion objective lens was used to capture the confocal images. The resulting fluorescent spectra of Nile red were detected in the 515 nm channel equipped with a narrow pass filter (HQ 515/30 m) with a pinhole size of 150 µm. The images generated had a size of 512 × 512 pixels, with a pixel size of 414 nm, and a pixel dwell time of 61.44 µs. All images were taken and processed using the instrument software program (EZ- CS1 version 3.8, Nikon, Melville, NY, USA).

2.2.8. Statistical analysis

In vitro experiments were carried out in duplicate, and the results were expressed as the mean and the standard deviation. A statistical analysis software program (JMP 8, SAS Institute Inc.) was used to perform the analysis of variance. The Tukey-Kramer HSD test was run to determine significant differences at a 5% significance level ($p < 0.05$). The data from *in vivo* experiments were analyzed with the Statistical Analysis System (SAS Institute, Cary, NC, USA). Significant differences between treatments were determined using the GLM procedure. Significant differences were defined at $P < 0.05$.

3. Results and discussion

3.1. Initial nanoemulsions

The particle size distribution and mean particle diameter of the nanoemulsions containing fucoxanthin were measured immediately after they were produced by high-pressure homogenization. The mean droplet diameter of the nanoemulsions formulated with LCT, MCT or indigestible oil was 202.3 ± 0.6 , 201.0 ± 1.4 and 249 ± 39 nm, respectively. It was observed that the droplet size distribution was narrower in the MCT nanoemulsion compared to LCT nanoemulsion. This phenomenon has been observed in other studies where it was attributed to differences in the viscosities of the lipid phases (Cheng Qian & McClements, 2011). The nanoemulsion containing a blend of orange oil and mineral oil (80:20) had a slightly larger droplet size, and appeared to contain a small population of larger particles around 8 μ m. This suggests that there may have been some growth of the oil droplets due to Ostwald ripening or coalescence. Nanoemulsions containing pure flavor oils are known to be highly unstable to droplet growth promoted by Ostwald ripening due to their relatively high water-solubility (Chang & McClements, 2014; Rao & McClements, 2011; Wooster, Golding, & Sanguansri, 2008). The addition of a highly hydrophobic oil phase, such as the mineral oil used in this study, helps prevent droplet growth due to this mechanism (Chang, McLandsborough, & McClements, 2012). However, it appears that some droplet growth still occurred in the emulsions.

3.2. *In vitro* studies

3.2.1 Physicochemical changes during *in vitro* digestion

In this section, we examined the effect of carrier type (LCT, MCT or indigestible oil) on the physicochemical and microstructural changes of fucoxanthin-enriched nanoemulsions during passage through a simulated gastrointestinal tract. The mean particle diameter of the nanoemulsions was mainly affected by the exposure to the different gastrointestinal conditions (mouth, stomach and small intestine) whereas the oil type had a minor effect on the oil droplet size in each *in vitro* digestion phase (**Table 1**). Orange oil nanoemulsions presented larger particle sizes in the mouth compared to the MCT or LCT nanoemulsions whereas MCT nanoemulsions showed larger particle sizes after the small intestine phase. However, the oil droplet size increased during the passage through the different *in vitro* gastrointestinal conditions regardless of the oil type used in the formulation of the

288 nanoemulsions. For instance, the initial droplet diameter of the nanoemulsions containing
289 indigestible oil was around 250 nm, which increased to 10.8, 8.2 and 17.1 μm after exposure
290 to the mouth, stomach and small intestine phases, respectively. Other authors have also
291 described droplet aggregation in nanoemulsions subjected to *in vitro* digestion, and have
292 reported that the extent of aggregation depended on the oil type (C. Qian et al., 2012). Within
293 the mouth phase, the oil droplets might undergo aggregation through bridging and depletion
294 flocculation due to the presence of polymeric mucin molecules in the simulated saliva
295 (Vingerhoeds, Blijdenstein, Zoet, & van Aken, 2005). Within the stomach phase, oil droplets
296 might undergo destabilization due to changes in electrostatic interactions associated with
297 alterations in the pH and ionic strength. The largest increase in particle size was observed
298 after exposure to simulated small intestine conditions. In the small intestine phase, the lipase-
299 induced hydrolysis of triacylglycerol molecules generates lipid digestion products, such as
300 micelles, vesicles and other colloidal structures (Mu & Hoy, 2004), which may contribute to
301 the light scattering signal used to measure the particle size. In addition, there may be non-
302 digested lipid droplets and insoluble calcium-fatty acid soaps that can also contribute to the
303 overall signal.

304 Changes in the microstructure of the nanoemulsions during passage through the
305 simulated GIT were also studied using confocal fluorescence microscopy (**Figure 1**). The
306 images of the initial nanoemulsions are not shown because the individual oil droplets were
307 too small to be observed by optical microscopy. Instead, there appeared to be a uniform
308 distribution of oil droplets throughout the confocal images. However, the presence of
309 flocculated droplets in the mouth was clearly visible in the three different nanoemulsions,
310 which confirms the results obtained by light scattering. In the confocal images the largest
311 particles were observed in the mouth phase (**Figure 2**), but with the light scattering technique
312 the largest particles were detected in the small intestine phase (**Table 1**). This difference
313 might be related to the fact that the samples were diluted and stirred before being analyzed
314 using the light scattering technique, which may have led to some breakdown of flocs. This
315 observation highlights the need to use several techniques to study changes in the
316 microstructure of delivery systems under simulated gastrointestinal conditions.

317 We found significant differences in the particle charges (ζ -potentials) of the fucoxanthin
318 nanoemulsions depending on the GIT phase and oil carrier type (**Table 1**). The initial
319 nanoemulsions had a slightly negative electrical charge (-2.3 and -3.5 mV) due to the

adsorption of non-ionic surfactant (Tween 80) molecules to the oil-water interface. Even though Tween 80 is a non-ionic surfactant, it is known that can give negative charge to oil droplets due to the presence of anionic impurities such as free fatty acids (McClements, 2005). The ζ -potential of fucoxanthin nanoemulsions became gradually more negative after passing through the *in vitro* digestion phases regardless of oil type. In the mouth phase, the adsorption of anionic molecules, such as mucin, to the droplet surfaces led to an increase in the negative charge on the oil droplets (-11.9 and -18.7 mV). The most pronounced increase in the negative charge on the particles in the fucoxanthin nanoemulsions was observed after exposure to simulated small intestine conditions, with the ζ -potential reaching values as negative as -44 mV. The strong anionic nature of free fatty acids released after lipolysis of oil as well as the presence of bile salts and phospholipids from small intestinal fluids causes this increase in negative charge (Singh, Ye, & Horne, 2009). Moreover, there were significant differences in the ζ -potentials of the nanoemulsions depending on the fatty acid chain length. Thus, the longer the chain of the fatty acids the more negative the electrical charge was in the small intestine phases, which has also been reported in other studies (Salvia-Trujillo et al., 2013b). This behavior is attributed to the ability of medium chain fatty acids (from MCT) to rapidly migrate from the surface of the oil droplets into the surrounding aqueous phase, whereas the long chain fatty acids (from LCT) tend to accumulate at the oil-water interface (Ahmed, Li, McClements, & Xiao, 2012; Pouton & Porter, 2008). Therefore, long chain free fatty acids may contribute a higher negative charge to the droplet surfaces than medium chain ones.

3.2.2 Fucoxanthin nanoemulsion digestibility

The *in vitro* digestibility of the lipid phase under simulated small intestine conditions was monitored using a pH-stat method for the fucoxanthin nanoemulsions formulated with indigestible oil, MCT, or LCT. Then the volume of alkaline solution necessary to neutralize the drop of pH caused by the lipolysis reaction during the *in vitro* digestion time was monitored and the release of FFAs from the triglyceride molecules was calculated (**Figure 2**). The behaviour of fucoxanthin nanoemulsions under simulated small intestine conditions depended strongly on carrier oil type. As expected, for the indigestible oil, we observed only a small initial release of FFAs (2%), which remained constant during the *in vitro* digestion. The oil phase of this nanoemulsion is a mixture of orange oil and mineral oil (80:20), which are both non-digestible oils, and so no FFA release would be expected. This small amount of

FFAs released in this system probably arise from the MCT in which the fucoxanthin was initially dissolved.

The fucoxanthin nanoemulsions formulated with MCT or LCT showed a rapid release of FFAs up to values above 80% after the first 5 minutes of the *in vitro* small intestine phase, with no significant differences between them. Then, the amount of FFAs kept increasing at a slower rate until values around 110 % were reached after 2 hours of *in vitro* digestion. The fact that the calculated FFA release was greater than 100% might have been due to some conversion of monoacylglycerols into glycerol and FFAs (Carey, Small, & Bliss, 1983), which was not considered in the FFA release calculations.

We measured the concentration of fucoxanthin present in the mixed micelle fraction formed after exposure of the samples to the simulated small intestine phase (**Figure 3**). This value was assumed to represent the amount of bioactive compound solubilized in the intestinal fluids that was available for absorption by the GIT (Carbonell-Capella, Buniowska, Barba, Esteve, & Frigola, 2014). The concentration of fucoxanthin solubilized in the micelle fraction followed the following order: LCT > MCT > indigestible oil. The relatively low value observed for the indigestible oil was attributed to the fact that some of the fucoxanthin remained trapped within the oil phase, and that there were fewer mixed micelles available to solubilize the fucoxanthin. In the case of MCT and LCT, there was rapid digestion of the lipid droplets leading to rapid liberation of the FFAs, which in turn would lead to the formation of mixed micelles capable of solubilizing the fucoxanthin. These results are in agreement with previous studies confirming the lower bioaccessibility of lipophilic active compounds incorporated in non-digestible oils (C. Qian et al., 2012). Moreover, even though there were no significant differences between the fucoxanthin concentration in the micelle fraction of MCT and LCT nanoemulsions, the amount of solubilized fucoxanthin was slightly higher in LCT nanoemulsions compared to MCT nanoemulsions. **In this sense, other authors have reported a high *in vitro* cellular uptake of lutein incorporated in emulsions (Frede et al., 2014).** Previous studies have also reported a higher bioaccessibility for other types of carotenoids encapsulated in nanoemulsions when LCT was used as a carrier oil compared to MCT (Salvia-Trujillo et al., 2013b). The origin of this effect is that the long chain fatty acids from LCT form micelles and vesicles containing hydrophobic regions with dimensions capable of incorporating long non-polar molecules (such as carotenoids).

3.3. *In vivo* studies

In this set of experiments, the same fucoxanthin nanoemulsions were used to test the influence of carrier oil type on the bioavailability of fucoxanthin using an animal model so that we could make an *in vitro* versus *in vivo* comparison on similar systems.

3.3.1 Microstructural changes of nanoemulsions in the gastrointestinal tract

The microstructure of the fucoxanthin nanoemulsions in different regions of the gastrointestinal tracts (stomach and upper, middle and lower small intestine) of the rats were studied by analyzing the gut content *post mortem* using confocal microscopy (**Figure 4**). A drastic increase in the oil droplet size in the fucoxanthin nanoemulsions was observed in the stomach of the rats regardless of oil type. Nevertheless, MCT nanoemulsions had slightly larger oil droplets than the other nanoemulsions. The initial oil droplet size of the nanoemulsions was < 250 nm, whereas in the stomach of the rats it was around 20 μm . These results are in agreement with the light scattering data obtained after the *in vitro* digestion (**Table 1**), which also indicated a significant destabilization of the nanoemulsions after passing through the gastrointestinal tract.

The fucoxanthin nanoemulsions exhibited different behaviors in the small intestine of the rats depending on the carrier oil used. There appeared to be very few oil droplets present in the nanoemulsions formulated using the indigestible oil remained throughout the small intestine. However, there was evidence that some small oil droplets persisted into the lower small intestine. It is possible that some of the orange oil was actually absorbed within the rat gastrointestinal tract (even though it was not digested), and that the remaining oil droplets were comprised of mineral oil (which should not be absorbed). Orange oil contains much smaller and more polar molecules than mineral oil, and therefore it is possible for them to travel across the epithelium cells. The nanoemulsions formulated with MCT or LCT appeared to be progressively digested as they passed through the small intestine. There appeared to be large lipid-rich clusters in the upper and middle small intestine phase. These clusters probably consisted of oil droplets and other lipid-rich particles (such as micelles and vesicles) trapped within the digestive bolus. In the lower small intestine, the nanoemulsions formulated with MCT and LCT appeared to be fully digested, as there were no oil droplets visible in the confocal images (**Figure 4**). The confocal images suggest that nanoemulsions containing MCT were digested more rapidly in the small intestine than those containing LCT, because the lipid-rich clusters (red regions) present in this system were smaller and

disappeared faster. These results are in agreement with other authors who have reported a slower *in vitro* and *in vivo* digestibility of long triglyceride oils compared to medium or short chain ones (Dahan & Hoffman, 2007; Sek, Porter, Kaukonen, & Charman, 2002). MCT may digest more quickly because medium chain fatty acids leave the fat droplet surfaces more rapidly after their formation, thereby allowing the lipase to work more effectively. Some studies have shown that appreciable MCT hydrolysis occurs in the stomach, which leads to faster gastric transit and more efficient absorption (Bach, Ingenbleek, & Frey, 1996). To the best of our knowledge, the current work is the first to report differences in MCT and LCT digestibility in the gastrointestinal tract of an animal model (rats) using confocal microscopy.

3.3.2 Heptadecanoic acid bioavailability

Triheptadecanoin (TG form of heptadecanoates) was incorporated into the three oil phases used to fabricate the nanoemulsions so as to establish the influence of oil type on the bioaccessibility of a model fatty acid. Heptadecanoic acid is an odd-carbon fatty acid (C₁₇) that is naturally present at very low levels in animal tissues, and which can therefore be used as a fatty acid marker for absorption studies. The concentration of heptadecanoic acid was measured in the small intestine tissue and serum collected from the rats after administration of the various nanoemulsion treatments.

We observed significant amounts of heptadecanoic acid in the small intestine tissue after exposure to the nanoemulsions (**Figure 5**), which suggested that some of this fatty acid had been absorbed by the epithelium cells. The concentration of fatty acid adsorbed increased the further one moved along the small intestine, *i.e.*, lower > middle > upper. This trend may have occurred for a number of reasons. First, the fatty acids absorbed in the upper small intestine might have already been incorporated into chylomicrons and been removed from the intestinal tissue. Second, there may have been faster absorption of the fatty acids in the lower regions of the small intestine. Third, there may have been a higher amount of fatty acid incorporated into the mixed micelles in the lower regions of the small intestine. The type of carrier oil used did not have a major influence on the absorption of heptadecanoic acid by the small intestine cells, showing no significant differences among the nanoemulsions formulated from indigestible oil, MCT or LCT. Differences in the metabolism of rats within the same group as well as the limited number of animals used in each group (3-4 rats) led to a large variability of results in rats administered the same type of nanoemulsions, thus making difficult the establishment of statistically-based differences among carrier oils. Nonetheless,

in the upper and medium small intestine we observed a slightly higher heptadecanoic acid concentration in rats fed with LCT and MCT nanoemulsions compared to the indigestible oil. Surprisingly, rats had the same heptadecanoic acid concentration in the lower small intestine regardless of oil type. This result suggests that fatty acids incorporated in non-digestible oils can either move out from the oil phase and be accumulated in the intestinal epithelium cells or that the small droplets themselves might be absorbed.

Despite the fact that the heptadecanoic acid concentration in the tissue samples was practically the same for all three types of nanoemulsion, its concentration in the blood stream was significantly higher in the nanoemulsions formulated from the digestible oils (MCT or LCT) than those from the indigestible oil (orange/mineral oil) (**Table 2**). This fact might be due to the fact that there was a higher concentration of free fatty acids available to form chylomicrons in the MCT and LCT samples, which are responsible for the absorption and transport of lipophilic active compounds into the blood system (Iqbal & Hussain, 2009).

3.3.3 Fucoxanthin bioavailability

Fucoxanthin was also incorporated into the three oil phases used to fabricate the nanoemulsions so as to establish the influence of oil type on the bioaccessibility of a model hydrophobic nutraceutical. The concentration of fucoxanthin was measured in the small intestine tissue and serum collected from the rats after administration of the nanoemulsions.

Fucoxanthin absorption by the intestinal epithelium cells followed a similar trend to that of heptadecanoic acid, being significantly higher in the lower small intestine than in the upper small intestine (**Figure 6**). Moreover, the fucoxanthin concentration in the lower small intestine clearly depended on carrier oil type, decreasing in the following order: LCT > MCT > indigestible oil. Nevertheless, the amount of fucoxanthin measured in the serum was fairly similar for all three carrier oil types being between 1 and 1.5 µg/ml (**Table 2**). The amount of fucoxanthin in the blood stream was significantly higher for the LCT nanoemulsions, but this effect was relatively small. There are a number of potential reasons for these observations. It has been reported that fucoxanthin is typically transported into the blood circulation by being solubilized in mixed micelles, absorbed by epithelium cells, and then packaged into chylomicrons that move into the systemic circulation (Peng et al., 2011; Sugawara et al., 2002). Consequently, one would expect that co-ingestion of a carrier oil that contained long chain fatty acids (such as LCT) would enhance the amount of fucoxanthin present in the serum because this should stimulate chylomicron production. On the other hand, co-ingestion

of a carrier oil containing medium chain fatty acids (such as MCT) or an indigestible oil should not stimulate chylomicron production (Shiau, 1990), and would therefore be expected to lead to a lower concentration of fucoxanthin in the blood. Instead, one would expect that bioactives would be transported *via* the portal vein, rather than the lymphatic system, for these types of carrier oil.

Having said this, the dependence of portal or lymphatic transport on the chain length of the fatty acid is unclear as there are reports of lymphatic transport of medium chain fatty acids and of portal transport of long chain fatty acids (McDonald, Saunders, Weidman, & Fisher, 1980; McDonald & Weidman, 1987). In addition, there is contradictory evidence of the influence of fatty acid chain length on the bioavailability of lipophilic bioactives. In a recent study, an appreciable enhancement in the absorption of a hydrophobic drug (anethol trithione) was reported when lipid-based delivery systems were formulated with LCT rather than MCT (Han et al., 2009). Conversely, other authors have reported no differences between MCT and LCT on the *in vitro* and *in vivo* bioavailability of certain hydrophobic drugs (Grove, Pedersen, Nielsen, & Mullertz, 2005; You, Ling, Qu, & Bistran, 2008).

We hypothesize a number of possible reasons for the fact that we observed an appreciable effect of carrier oil type on the concentration of fucoxanthin in the small intestine tissues (**Figure 6**), but not in the serum (**Table 2**). First, there may have been insufficient time between administering the nanoemulsions and measuring the serum levels of fucoxanthin. It is possible that the fucoxanthin was stored within oil bodies in the epithelium cells, and was only slowly incorporated into chylomicrons and released into the blood. Second, it is possible that fucoxanthin could be absorbed by the epithelium cells and transported into the blood, even in the absence of a carrier oil containing appreciable amounts of long chain free fatty acids. For example, the indigestible oil droplets containing fucoxanthin may have been directly absorbed by the epithelium cells. Third, the presence of the oil phases may have impact other physicochemical phenomenon that impact the overall bioavailability of the fucoxanthin, *e.g.*, solubilization in intestinal fluids, transport across the mucus layer, or cell membrane permeability. Clearly, further studies are required to identify the precise mechanisms involved.

4. Conclusions

The present work showed that carrier oil type has an appreciable impact on the bioavailability of fucoxanthin encapsulated within nanoemulsion-based delivery systems. *In vitro* experiments showed that medium and long chain triacylglycerols were both fully digested at an equal rate, whereas a mixture of orange oil and mineral oil was not. The nature of the carrier oil decreased the solubility of fucoxanthin in the mixed micelles formed in the *in vitro* GIT model in the following order: LCT > MCT > indigestible oil. Animal studies showed that a similar trend was observed for the concentration of fucoxanthin present in the small intestine epithelium cells (LCT > MCT > indigestible oil). However, the concentration of fucoxanthin in the serum of the rats was fairly similar regardless of the oil type used in the formulation of the nanoemulsions. These results suggest that there may be some mechanism that limits the transport of fucoxanthin from the epithelium cells into the blood. The fact that the serum levels did not depend strongly on carrier oil type, suggests that the ability of long chain fatty acids to form chylomicrons did not play a major role. Further work is clearly needed to establish the physicochemical or biochemical mechanisms responsible for this phenomenon. The information obtained in this study should facilitate the formulation of nanoemulsion-based delivery systems containing fucoxanthin for the optimal design of functional foods.

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Table captions

Table 1. Droplet diameter (d_{43} , μm) and ζ -potential (mV) of fucoxanthin nanoemulsions formulated with different oil carrier type (orange oil, MCT or LCT) during the *in vitro* digestion phases (Initial, mouth, stomach and small intestine).

Table 2. Influence of oil types on concentration of heptadecanoic acid or fucoxanthin in serum ($\mu\text{g/mL}$ serum) of rats after feeding. Data points represent means ($n = 4$) $\pm$ standard deviations.

Figure captions

Figure 1. Confocal images of the fucoxanthin nanoemulsions formulated with different oil carriers (Orange oil, MCT or LCT) as passed through the *in vitro* mouth, stomach and small intestine phases.

Figure 2. Calculated free fatty acids released (%) from the orange oil, MCT or LCT nanoemulsions containing fucoxanthin over time during the course of the *in vitro* small intestine stage.

Figure 3. Influence of the oil carrier type (orange oil, MCT, LCT) on the fucoxanthin concentration (ppm) of the micelle fraction after the *in vitro* small intestine phase

Figure 4. Confocal images of the gastrointestinal content in different regions of the digestive system (stomach, upper small intestine, middle small intestine and lower small intestine) of rats fed with fucoxanthin nanoemulsions formulated with different oil carriers (orange oil, MCT or LCT).

Figure 5. Influence of oil types on concentration of heptadecanoic acid in Upper, Middle, and Lower small intestine of rats small intestines after feeding. Data points represent means ($n = 3-4$) $\pm$ standard deviations. Different capital letters (A, B) mean statistical differences in

the heptadecanoic acid concentration in Upper, Middle, and Lower of rats small intestines in samples of a given emulsion type (i.e., the effect of intestinal region). Different lower case letters (a, b) mean statistical differences in the heptadecanoic acid concentration in particular small intestine regions in samples with different emulsion types (i.e., the effect of different kinds of emulsions).

Figure 6. Influence of oil type on concentration of fucoxanthin and its metabolites in Upper and Lower small intestine of rats small intestines after feeding. Data points represent means ($n = 3-4 \pm$ standard deviations). Different capital letters (A, B) mean statistical differences in the heptadecanoic acid concentration in Upper and Lower of rats small intestines in samples of a given emulsion type (i.e., the effect of intestinal region). Different lower case letters (a, b) mean statistical differences in the fucoxanthin concentration in particular small intestine regions in samples with different emulsion types (i.e., the effect of different kinds of emulsions).

Table 1

Table 1

	Particle diameter			ζ-potential		
	Orange oil	MCT	LCT	Orange oil	MCT	LCT
Initial	0.78 ± 0.08 ^{Ca}	0.161 ± 0.006 ^{Cc}	0.26 ± 0.06 ^{Bb}	-2.3 ± 0.9 ^{Aa}	-3.3 ± 0.8 ^{Aa}	-3.5 ± 0.7 ^{Aa}
Mouth	10.8 ± 0.6 ^{ABa}	3.8 ± 0.5 ^{Bb}	3.3 ± 0.2 ^{ABb}	-12 ± 1 ^{Ba}	-15.7 ± 0.6 ^{Bb}	-19 ± 2 ^{Bc}
Stomach	5.1 ± 0.5 ^{Ba}	3.5 ± 0.4 ^{Ba}	2.9 ± 0.3 ^{ABa}	-14.3 ± 0.6 ^{Ba}	-19 ± 1 ^{Cb}	-21 ± 2 ^{Bc}
Intestine	17.1 ± 0.5 ^{Ab}	30 ± 7 ^{Aa}	4.0 ± 0.9 ^{Ac}	-26 ± 4 ^{Ca}	-27 ± 1 ^{Da}	-44 ± 2 ^{Cb}

Different capital letters represent significant differences ($p < 0.05$) between *in vitro* digestion phases given an oil carrier type (orange oil, MCT or LCT). Different lower case letters represent significant differences ($p < 0.05$) between oil type carriers in a given *in vitro* digestion phase (initial, mouth, stomach or small intestine).

Table 2

	Heptadecanoic acid	Fucoxanthin
Orange oil	5.1 ± 0.9 ^a	1.23 ± 0.09 ^{ab}
MCT	8.6 ± 1.7 ^b	1.13 ± 0.05 ^b
LCT	8.6 ± 1.3 ^b	1.52 ± 1.14 ^a

Different lower case letters (a, b) mean statistical differences in the heptadecanoic acid or fucoxanthin concentration in serum with different emulsion types (i.e., the effect of different kinds of emulsions)

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Figure 5

Figure 5

Figure 6

Figure 6