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Comparative study on lipid digestion and carotenoid bioaccessibility of emulsions, nanoemulsions and vegetable-based *in situ* emulsions

Salvia-Trujillo, L.^{1*}; Verkempinck, S.H.E¹; Zhang, X.¹; Van Loey, A.M.¹; Grauwet, T.¹; Hendrickx, M.E.¹

¹ Laboratory of Food Technology, Leuven Food Science and Nutrition Research Centre (LFoRCe), Department of Microbial and Molecular Systems (M²S), KU Leuven, Kasteelpark Arenberg 22, PB 2457, 3001, Leuven, Belgium.

Author's e-mails

Verkempinck, S.H.E.: sarah.verkempinck@kuleuven.be

Zhang, X.: xiaoxi.zhang@student.kuleuven.be

Van Loey, A.M.: ann.vanloey@kuleuven.be

Grauwet, T.: tara.grauwet@kuleuven.be

Hendrickx, M.E.: marceg.hendrickx@kuleuven.be

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*author whom correspondence should be addressed: laura.salviatrujillo@kuleuven.be

Permanent e-mail address of the corresponding author (due to change of institution):

lsalvia@tecal.udl.cat

Abstract

Emulsions (d_{43} 0.484-0.897 μm) and nanoemulsions (d_{43} 0.279-0.226 μm) based on lipid fractions enriched with carotenoids from different sources were produced by increasing the amount of a non-ionic surfactant (Tween 80) at surfactant-to-oil ratio (SOR) 0.1 or 2, respectively. Alternatively, carrot purée *in situ* emulsions were fabricated by processing carrot purée with a non-enriched oil fraction and Tween 80 (SOR 0.1). Emulsions, nanoemulsions and *in situ* emulsion were subjected to *in vitro* digestion conditions with focus on the kinetics of lipid digestion, micelle formation and carotenoid bioaccessibility during the small intestinal phase. Nanoemulsions produced with a high amount of surfactant showed an almost complete lipid hydrolysis inhibition probably due to hindered lipase adsorption at oil-water interfaces. Emulsions presented a gradual lipid digestion during the intestinal phase and subsequent incorporation of lipid digestion products into mixed micelles. Interestingly, the *in situ* emulsion presented a similar lipid digestion trend than emulsions, yet a lower concentration of lipid digestion products incorporated into mixed micelles, which resulted in a lower carotenoid bioaccessibility. Overall, this study highlights the potential of using nanoemulsions to modulate lipid digestion. Moreover, it also indicates possible interactions between food components and lipid digestion products during the formation of mixed micelles and its implications on the bioaccessibility of lipophilic bioactives such as carotenoids.

Keywords: Lipid; digestion; nanoemulsions; emulsions; carotenoids; bioaccessibility

1 Introduction

Proper absorption of dietary fats has a major impact on human nutrition and is related to an optimal absorption metabolism of fat-soluble vitamins (Bauer, Jakob, & Mosenthin, 2005). Lipids, mainly composed of triglycerides (TAG) are enzymatically digested by lipases and hydrolyzed into lipid digestion products, such as monoacylglycerols (MAG) and free fatty acids (FFA). These lipid digestion products contribute to the formation of mixed micelles where lipid soluble compounds are solubilized and further absorbed by the small intestine epithelium cells (Furr & Clark, 1997; Mu & Høy, 2004). In recent years, efforts have been put on developing new edible delivery systems for the optimization of the absorption of healthy dietary lipids and lipophilic bioactive compounds. In this sense, nanoemulsions have been described as possible carriers of health-related dietary lipids or lipophilic bioactives with substantial benefits over conventional emulsions (Salvia-Trujillo, Soliva-Fortuny, Rojas-Grau, McClements, & Martín-Belloso, 2017).

Nanoemulsions, due to their small particle size ($d < 200$ nm) present a higher active surface area, thus being potentially digested at higher rate in comparison with emulsions with a larger droplet size. In fact, recent studies revealed that the smaller the particle size, the higher the lipid digestion rate, which results in a higher bioaccessibility of lipophilic bioactives (Salvia-Trujillo, Verkempinck, et al., 2017; Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013). Nanoemulsions can typically be produced by high-energy or low-energy approaches. In case of low-energy approaches, a high concentration of surfactant is required to spontaneously emulsify the oil phase (Komaiko & McClements, 2016). Nonetheless, high-energy approaches might also require the use of a high surfactant concentration necessary to cover the surface of the newly created oil droplets (Silva, Cerqueira, & Vicente, 2015). In this regard, the surfactant concentration used for the

fabrication of nanoemulsions might have an impact on their behavior during the digestive tract. In fact, recent findings described that non-ionic surfactants, such as Tweens, may displace lipases from oil/water interfaces and therefore affect lipid digestion in emulsions (Yao et al., 2013, 2018).

Additionally, emulsion-based delivery systems loaded with lipophilic bioactive compounds with small droplet size have shown to highly improve their bioaccessibility. For instance, bioaccessibility of carotenoids in vegetables and their derived products is extremely low (< 5%) due to their high level of entrapment in chromoplast structures and cells creating multiple barriers (Lemmens et al., 2014). However, process-induced transfer of carotenoids from vegetable matrices into an oil phase has shown to greatly enhance the bioaccessibility of carotenoids under *in vitro* gastrointestinal conditions, up to values above 50 % (Palmero et al., 2016). In this regard, high-pressure homogenization treatments have proven to effectively disrupt the cell wall of vegetable tissues and further solubilize the carotenoid fraction into a lipid phase (Mutsokoti, Panozzo, Musabe, Van Loey, & Hendrickx, 2015). Nevertheless, the physicochemical characteristics of emulsified lipids are known to highly impact the oil digestion, and subsequently the bioaccessibility of lipophilic bioactive compounds that are solubilized within the oil droplets (Salvia-Trujillo, Soliva-Fortuny, et al., 2017). Thus, the addition of surfactant into oil-vegetable blends would allow to simultaneously form an *in situ* emulsified system with carotene-loaded oil droplets dispersed within vegetable-based food products.

In this context, the aim of the present work was to study (i) the influence of the surfactant concentration to fabricate nanoemulsions and its subsequent impact on their *in vitro* digestive behavior and (ii) the food matrix effect on the formation and digestion of emulsions within vegetable-based food products.

In a first step, the influence of the surfactant (Tween 80) concentration on the formation of emulsions or nanoemulsions with corn oil olive oil enriched with carotenoids from different sources (from carrot purée or synthetic β -carotene) was studied in relation to their behavior during *in vitro* digestive conditions with regard to lipid hydrolysis, micelle formation and carotenoid bioaccessibility kinetics. In a second step, this study aimed at producing *in situ* generated emulsions within carrot purée by high pressure homogenization, investigate its fate during *in vitro* digestion and compare it with carotenoid-rich model oil-in-water (o/w) emulsions in order to elucidate the food matrix effect on lipid digestibility and carotenoid bioaccessibility of emulsion-based systems.

2 Material and methods

2.1 Materials

Corn oil (CO) and olive oil (OO) were bought from a local supermarket. Orange carrots (*Daucus carota* cv. Nerac) were purchased at a local grocery store and stored at 4 °C until use. All chemicals and reagents were from Sigma Aldrich, except for KCl, $\text{MgCl}_2(\text{H}_2\text{O})_6$, NaOH, heptane, methanol, methyl-tert-butyl-ether and ethyl acetate (Acros Organics); NaHCO_3 , NaCl, H_2SO_4 , ethanol, acetone and trimethylamine (Fisher Scientific); HCl, diethylether and iso-propanol (VWR); acetone (Carlo Erba); $\text{CaCl}_2(\text{H}_2\text{O})_2$ (Chem Lab); KH_2PO_4 (UCB) and lipid standards (Larodan). Synthetic β -carotene had a purity above 97 % (Sigma Aldrich). All chemicals and reagents were of analytical grade.

2.2 Methods

2.2.1 Oil enrichment

The oil fraction of emulsions and nanoemulsions consisted on corn oil or olive oil enriched with carotenoids from carrot purée or enriched with crystalline synthetic β -carotene. For the enrichment of OO or CO with carotenoids from carrot purée, the procedure described in a previously published paper was followed (Salvia-Trujillo, Verkempinck, et al., 2017). Briefly, carrots were washed, peeled, cut into dices and blended with a Milli-Q water (1:1) ratio with a food blender (Model 7011G, Waring Commercial,) at low speed for 20 s and high speed for 40 s to obtain a puree. Subsequently, a high pressure homogenization (Panda 2 K, Gea Niro Soavi, Mechelen, Belgium) for one cycle at 100 MPa was conducted in order to further disrupt the vegetable matrix and release the carotenoid-rich chromoplast fraction. Afterwards, carrot puree and olive or corn oil (15% w/v) were mixed (ultra turrax, Waring Commercial, Torrington, CT, USA) at 12,500 rpm for 10 min. The pre-emulsified oil-carrot puree blend was passed once through a high-pressure homogenizer at 100 MPa (Pony NS2006L, GEA, NH, USA). After homogenization, in order to break the emulsion, the oil-carrot puree blend was freeze-thawed at -40 °C and 25 °C, and ultracentrifuged (Optima XPN-80, Beckman Coulter, IN, USA) at 165,000 g for 15 min at 4 °C. The carotenoid-rich upper oil layer was collected and stored at -80 °C until use.

Alternatively, in order to produce the oil enriched with synthetic carotenoids, crystalline β -carotene was dissolved into CO. The oil was heated up to 50 °C and the powder β -carotene was added and stirred with a magnetic stirrer for 10 min, followed by sonication for 5 min. This procedure was repeated three times in order to achieve the complete dissolution of the β -carotene crystals. Afterwards the enriched oil fraction was stored at -80 °C until use.

The α and β -carotene concentration of the carrot-enriched olive oil (CE-OO) were 89.9 ± 1.6 and 204.7 ± 3.0 , while for the corn oil (CE-CO) were 68.6 ± 1.6 and 187.2 ± 2.4 $\mu\text{g/g}$ oil, respectively. The β -carotene concentration of the synthetic β -carotene-enriched corn oil (SCE-CO) was 192.7 ± 22.6 $\mu\text{g/g}$ oil.

2.2.2 Emulsion and nanoemulsion formation

Emulsions and nanoemulsions were produced by formulation with different concentrations of surfactant. Emulsions were formulated with a surfactant-to-oil ratio (SOR) of 0.1 while nanoemulsions were formulated with an SOR of 2. First, a coarse emulsion was obtained by mixing 5% (w/w) enriched oil with 0.5% or 10% (w/w) Tween 80 as surfactant for emulsions or nanoemulsions, respectively, and Milli-Q water with a high shear mixer (Silverson L5M-A, Silverson Machines, Inc., Massachusetts, USA) operating at 4000 rpm for 5 min. Afterwards, coarse emulsions were immediately processed with a high pressure homogenizer (Stansted Fluid Power, Pressure cell homogenizer, U.K.) for 3 cycles at 100 MPa. Emulsions and nanoemulsions were stored in the cooling room (4°C) before use. The initial average particle size of emulsions ranged between 0.484 and 0.897 μm , while the one for nanoemulsions ranged between 0.226 and 0.279 μm .

2.2.3 *In situ* vegetable-based emulsion formation

In order to mimic the formation of emulsions within vegetable-based products, an *in situ* emulsion was formulated using the same procedure as described for the formation of emulsions substituting the aqueous phase by a carrot purée. Firstly, a carrot purée was produced as described above. Then, an *in situ* emulsion was formulated with 5% (w/w) non-enriched OO, 0.5% (w/w) Tween 80 as a surfactant (SOR 0.1) and 94.5% (w/w) carrot purée. A prior pre-emulsification treatment was conducted with a high shear mixer (Silverson L5M-

A, Silverson Machines, Inc., Massachusetts, USA) operating at 4000 rpm for 5 min. To obtain the *in situ* carrot-purée emulsion, the mixture was treated by high-pressure homogenization (Panda 2 K, Gea Niro Soavi, Mechelen, Belgium) for 3 cycles at 100 MPa.

2.2.4 *In vitro* digestion

A simulation of gastrointestinal conditions, consisting on a gastric and intestinal phase, based on an international consensus method (Minekus et al., 2014) was carried out in order to investigate the behavior of emulsions, nanoemulsions and the *in situ* emulsion under *in vitro* digestion conditions. *In vitro* digestion experiments were conducted under subdued light conditions. Both after simulation of stomach and small intestine, emulsions were characterized in terms of particle size distribution and interfacial electrical charge (ζ -potential). Moreover, during the small intestinal phase, the kinetics of lipid digestion were followed determining the decrease of triacylglycerols (TAG) and subsequent release of monoacylglycerols (MAG) and free fatty acids (FFA), and the bioaccessibility of carotenoids measuring the concentration of carotenoids incorporated in mixed micelles.

To simulate the gastric phase, a 5 mL aliquot of emulsion, nanoemulsion or *in situ* emulsion was placed in a brown falcon tube and mixed with 5 mL Milli-Q water, 7.5 mL of Simulated Gastric Fluid (SGF), 5 μ L of CaCl_2 (0.3M) and HCl (2M) to reach pH 3. Afterwards 1.6 mL of pepsin solution previously dissolved in SGF was added to achieve 2000 U/mL in the final chyme. The total volume of the gastric phase was 20 mL. Tubes containing the chyme were flushed for 10 s with N_2 and incubated with an end-over-end rotator at 37 °C for 2 h. For the small intestinal phase, 11 mL of Simulated Intestinal Fluid (SIF) were added to the chyme. The rest of the solutions were dissolved in SIF. Subsequently, 2.5 mL of bile solution (160 mM), 40 μ L of CaCl_2 (0.3M), Milli-Q water and NaOH (1M) to bring the pH up to 7 were added. Finally, 5 mL of a so called “pancreatic solution” consisting on a mixture of

pancreatic lipase (lipase from porcine pancreas type II, batch #SLBN3801V, Sigma Aldrich) and pancreatin (pancreatin from porcine pancreas, batch #SLBJ8147V, Sigma Aldrich) was added. The final lipase activity in the “pancreatic solution” was 1600 U/mL of pancreatic solution, thus the final lipase activity in the digest was 200 U/mL of digest. The final volume of the digest was 40 mL. The tubes were flushed with N₂ for 10 s and incubated in an end-over-end rotator at 37 °C for 2 hours. During the small intestinal phase a kinetic study was conducted in order to determine the lipid digestion and carotenoid bioaccessibility at several time moments. For this, 6 individual tubes were prepared for each time moment, being 7.5, 15, 30, 45, 60 and 120 min from the addition of the pancreatin solution. To stop the digestion, the digests were transferred into glass tubes and heat-shocked at 85 °C for 5 min after which they were placed in an iced-water bath. For each time moment, the micelle fraction was harvested by ultracentrifugation (165,000 g; 1 h and 5 min; 4 °C) (Optima XPN-80, Beckman Coulter, IN, USA).

2.2.5 Particle physicochemical characteristics

Particle size: The particle size distribution of initial samples and after gastric and intestinal phases were measured by laser diffraction (Beckman Coulter Inc., LS 13 320, FL, USA). A few drops of the sample were poured into a stirring tank, filled with deionized water. The sample was pumped into the measurement cell wherein the laser light (wavelength main illumination source: 750 nm; wavelengths halogen light for Polarization Intensity Differential Scattering (PIDS): 450 nm, 600 nm, 900 nm) is scattered by the particles. Additionally, the average particle size was reported as the volume-weighted mean diameter (d_{43}) in μm .

Particle electrical charge: the ζ -potential was measured by phase-analysis light scattering (Zetasizer NanoZS, Malvern Instruments, Worcestershire, UK). Samples were diluted 1:10 with either Milli-Q water, SGF or SIF for the initial emulsions and after the gastric and

intestinal phases, respectively. Then, they were placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles.

Microstructure: the microstructure of the samples and after gastric and intestinal conditions was studied with an optical microscope (Olympus BX-41) equipped with an Olympus XC-50 digital camera (Olympus, Opticel Co. Ltd., Tokyo, Japan).

2.2.6 Lipid analysis

Lipid digestion was followed determining the TAG hydrolysis and the subsequent release of MAG and FFA. Lipid digestion products of the digests as well as the corresponding micelle fractions obtained at the different small intestine times were analyzed. A 1-mL aliquot of the digest or micelle fraction was mixed with 2 mL of ethanol, 3 mL of diethylether:heptane (1:1) and 0.2 mL of sulphuric acid (2.5 M), vortexed for 2 min and further centrifuged (Sigma 4-16KS, Sigma, Germany) at 500 g for 5 min at 20 °C. Afterwards, the top layer was collected in a 5 mL volumetric flask. The bottom layer was mixed with 1 mL of diethylether:heptane (1:1) and the process was repeated. The organic layer was added to the previous one, the volume was brought up to 5 mL and was filtered (Chromafil PET filters, 0.20 µm pore size, 25 mm diameter). The lipid extract was kept at -80 °C until analysis.

The analysis and quantification of the digestion products was carried out based on a method proposed previously (Graeve & Janssen, 2009) with some modifications. An HPLC system (Agilent Technologies 1200 Series, Diegem, Belgium) was equipped with a silica column (5µm x 150 x 4,6 mm ID, Spherisorb, Waters) and protected with a guard column in an oven (5310, VWR, Hitachi Ltd., Tokyo, Japan) at 40 °C. The separation of the lipid products was performed using a quaternary gradient consisting on isooctane (solvent A), actone:ethyl acetate (2:1 v/v) containing 70 mM acetic acid (solvent B), propan-2-ol:water (85:15 v/v)

containing 7.5 mM acetic acid and 7.5 mM triethylamine (solvent C), and propan-2-ol (solvent D). Coupled to the HPLC system, an evaporative light scattering detector (ELSD) (Agilent Technologies 1260 Infinity, Diegem, Belgium) was used to detect the different lipid species. TAGs, MAGs and FFAs were quantified using calibration curves of trilinolein, monolinolein and linoleic acid standards, respectively (Larodan Fine Chemicals AB, Malmö, Sweden).

Based on the analytical results of MAGs and FFAs, the amount of glycerol (GLY) released at each time moment was calculated based on the following stoichiometric reactions (Moquin & Temelli, 2008):

Moreover, from the analytical results of TAG, MAG, FFA and calculated GLY at each time moment, the total amount of TAG moles present at the beginning of the intestinal phase could be calculated from the following expression:

$$TAG_0 = TAG_t + MAG_t + GLY_t \quad \text{Eq. 1}$$

where TAG_0 is the number of TAG moles at time 0, TAG_t , MAG_t and GLY_t are the number of TAG, MAG and GLY moles at time t. The amount of undigested TAG at each time moment was calculated based on the amount of lipid digestion products at each time moment, and expressed as percentage of undigested TAG (%):

$$TAG_t(\%) = \frac{(MAG_t + FFA_t + GLY_t)}{TAG_0} \times 100 \quad \text{Eq. 2}$$

where TAG_t is the percentage of undigested TAG at each time moment; MAG_t , FFA_t and GLY_t are the concentration of MAG, FFA and GLY (mg/mL of emulsion) at each time moment; and TAG_0 is the initial amount of TAG (mg/mL of emulsion) at the beginning of the intestinal phase as calculated by Eq 1.

The molecular weight of trilinolein (879.38 g/mol), monolinolein (354.2 g/mol), linoleic acid (280.45 g/mol) and glycerol (92.09 g/mol) was taken into account for the above mentioned calculations. The lipid hydrolysis was reported in percentage of undigested TAG (%) while the formation of lipid digestion products in the total digest and micelle fraction during the small intestinal phase was expressed in concentration (mg/mL emulsion).

2.2.7 Carotenoid analysis and bioaccessibility

The carotenoid extraction and analysis was carried out based on a previously reported method (Sadler, Davis, & Dezman, 1990) with minor changes. Briefly, aliquots of 2; 5 or 10 mL of initial samples, digest or micelles respectively were mixed with 1 g of NaCl and 25 mL of hexane:acetone:ethanol (50:25:25) containing 0.1% butylated hydroxytoluene and stirred during 20 min at 4 °C in the dark. Afterwards, 7.5 mL of Milli-Q water was added and stirred for 10 min. The mixture was transferred into a glass tube and the upper organic layer was collected and filtered (Chromafil PET filters, 0.20 µm pore size, 25 mm diameter) and analyzed for carotenoid content by RP-HPLC-DAD. The HPLC system (Agilent Technologies 1200 Series, Diegem, Belgium) used was equipped with a C30 column (3 µm × 150 mm × 4.6 mm, YMC Europe, Dinslaken, Germany) coupled with a guard column. The column was kept at 25 °C. The separation was performed with a gradient elution with Milli-Q water (solvent A), methanol (solvent B) and methyl-t-butyl-ether (solvent C). For the quantification α and β-carotene HPLC-DAD peak responses were measured at 450 nm, and the peak area was compared with a calibration curve with purified standards (CaroteNature, Lupsingen, Switzerland).

Carotenoid bioaccessibility (BA) is reported as the percentage fraction of carotenoids incorporated in the micelles versus the concentration in the initial emulsions (BA, %).

2.2.8 Statistical analysis and kinetic modeling

Statistical analysis and modelling of data was carried out with JMP statistical software (JMP 12, SAS Institute Inc.). Physicochemical characteristics (droplet size and ζ -potential) were determined in duplicate for each individual sample. An analysis of variance was carried out and the Student's *t* test was run to determine significant differences at a 5% significance level ($p < 0.05$).

The lipid digestion and carotenoid bioaccessibility kinetics during the small intestinal phase, were modeled using empirical models assuming a time independent rate constant (experiment was carried out at a constant temperature) and parameters were estimated by non-linear regression. The changes in undigested TAG (Eq. 3) and subsequent release of MAG, FFA and GLY in the digest as well as in the micellar fraction was modeled by a fractional conversion model (Eq. 4). Likewise, the carotenoid BA values were modeled by a fractional conversion model (Eq. 4). The concentration of TAG in the digest at the start of the intestinal phase was taken as the concentration of TAG in the initial emulsion. The initial concentration of MAG or FFA in the digest or micelle fraction as well as the carotenoid BA at the start of the intestinal phase was assumed to be 0.

$$C = C_f + (C_0 - C_f)e^{-kt} \quad \text{Eq. 3}$$

$$C = C_f(1 - e^{-kt}) \quad \text{Eq. 4}$$

Where *C* is the parameter value at digestion time *t* (min); *C*₀ is the initial parameter value at time 0 (start of the intestinal phase), that in the case of TAG is equal to 100 %; *C*_{*f*} is the estimated asymptotic parameter value when digestion time (*t*) is ∞, which represents final concentration in the case of MAG, FFA and GLY (mg/mL of emulsion) and percentage (%) in the case of carotenoid bioaccessibility; and *k* is the estimated reaction rate constant (min⁻¹). For the release of MAG, FFAs and GLY. For all the modeled curves, the fit of the model was

assessed by calculating R^2 and R^2 adjusted and visually analyzing the residue plots. Significant differences between the estimated parameters were determined by calculating the confidence intervals (95%).

3 Results and discussion

Emulsions produced with an SOR of 0.1 and nanoemulsions with an SOR of 2 were subjected to simulated gastrointestinal conditions and analyzed in terms of physicochemical characteristics at the end of the gastric and intestinal phases, by determining their droplet size, droplet size distribution, microstructure and ζ -potential. In the small intestinal phase, a kinetic study was conducted in order to obtain the lipid digestion, micelle formation and carotenoids bioaccessibility kinetics. Additionally, the *in situ* carrot purée emulsion was analyzed similarly and compared to o/w emulsions.

3.1 Physicochemical changes during *in vitro* digestion

3.1.1 Particle size, particle size distribution and microstructure

Emulsions and nanoemulsions

The initial particle size of emulsions and nanoemulsions produced by increasing the amount of surfactant used, differed significantly. Emulsions presented an average droplet size (d_{43}) ranging from 0.484 to 0.897 μm depending on the oil type and carotenoid source, while nanoemulsions had a d_{43} ranging from 0.279 to 0.226 μm (Table 1). The droplet size distribution of emulsions showed intensity peaks of particles above 1 μm , while nanoemulsions presented a monomodal particle size distribution with an intensity peak of particles below 1 μm (Figure 1). In fact, differences in particle size between emulsions and nanoemulsions were also detected by microscopy images (Figure 3). Emulsions presented

clearly a larger particle size in comparison with nanoemulsions, the latter ones were almost undetectable by the optical microscope due to their small droplet size.

After *in vitro* gastric conditions, emulsions and nanoemulsions remained stable in terms of particle size and particle size distribution (Table 1 and Figure 1), without significant changes in comparison with the initial samples as could also be concluded from the microscopy images (Figure 3). Emulsions stabilized with non-ionic surfactants, such as Tween 80, have shown high stability under gastric conditions (low pH) (Golding et al., 2011). Nonetheless, after the simulation of the small intestinal phase, emulsions and nanoemulsions presented a different behavior. Emulsions exhibited a large increase in the average particle size (Table 1) and a multimodal particle size distribution (Figure 1), with intensity peaks corresponding to small particles (between 0.1 and 1 μm) (Figure 1A and B) probably due to the presence of mixed micelles or other vesicles, and with large particles (larger than 10 μm), corresponding to larger colloidal structures or undigested coalesced oil droplets. However, nanoemulsions' digest presented almost no changes with respect to the chyme, with the appearance of small minor larger peaks (above 10 μm) in the case of CE-OO (Figure 1 D) or CE-CO (Figure 1 E) nanoemulsions. These results suggest beforehand that oil droplet digestion hardly occurred in the case of nanoemulsions regardless of the oil type or the carotenoid enrichment source. This was confirmed by the results of lipid digestion as discussed in the following sections.

In situ emulsion

The initial particle size of the *in situ* carrot purée emulsion was 32 μm (Figure 2 A) with a bimodal particle size distribution (Figure 2 B). A main intensity peak around 35 μm was detected, which can be attributed, on the one hand to vegetable tissue cell fragments (Moelants et al., 2013) and on the other hand, to large oil droplets. In fact, other authors have

found similar particle sizes in carrot purées after being processed by high-pressure homogenization (Mutsokoti et al., 2015). Also, large oil bodies loaded with carotenoids were detected within the food matrix of *in situ* carrot purée emulsion (Figure 3). Additionally, a minor intensity peak around 0.5 μm was detected (Figure 2B), which might be attributed to the presence of smaller fiber structures or to oil droplets of similar size of the o/w emulsions. As a matter of fact, the initial microstructure of the *in situ* carrot purée emulsion consisted on small and large fibrous-like vegetable cell fragments together with carotenoid-loaded oil droplets with a yellowish appearance of heterogeneous size (Figure 3). The particle size and particle size distribution of the *in situ* emulsion remained stable under gastric conditions, and experienced a dramatic change after small intestinal conditions. The appearance of a multimodal particle size distribution (Figure 2 B) might be attributed to the digestion of larger oil bodies, which were no longer present after simulated intestinal conditions. In the end, amorphous-shaped chromoplast structures, small carotenoid-loaded undigested oil bodies and fibrous cell fragments remained present at the end of the intestinal phase (Figure 3).

3.1.2 Particle ζ -potential

Emulsions and nanoemulsions

The ζ -potential of emulsions, nanoemulsions (Table 1) and *in situ* emulsions (Figure 2 C) was measured initially and after gastric and intestinal conditions in order to study the electrical properties of the emulsion-based systems during *in vitro* digestion as an indication of their stability and potential interactions with other compounds present in the digestive system. The ζ -potential of the initial emulsions and nanoemulsions ranged between -45 and -23 mV, which indicates the anionic nature of the oil droplets for all the studied samples. In this study Tween 80 is used as a non-ionic surfactant, thus the negative charge of the oil droplets might be attributed to intrinsic oil physicochemical characteristics. In fact, it is

reported that in the absence of any surfactant, lipids present a highly negative electrical charge, which has been attributed to either the orientation of OH^- ions from the aqueous phase in the vicinity of the oil droplets or the adsorption of HCO_3^- and CO_3^{2-} ions due to dissolved CO_2 from the atmosphere into the aqueous phase (Marinova et al., 1996). Moreover, we observed that nanoemulsions formed with a higher amount of surfactant presented a less negative charge than their respective emulsions formulated with a lower surfactant concentration. This is in agreement with the experiments of Marinova et al. (1996), where they observed a decrease in the negative charge of oil droplets after the addition of non-ionic surfactants. Additionally, we observed that the type of oil used significantly influenced the ζ -potential of the oil droplet surface, being more negative in those emulsions containing OO in comparison to CO. Namely, CE-OO emulsion had a ζ -potential of -45 mV while for the corresponding CE-CO it was -31 mV.

On the one hand, after subjecting emulsions and nanoemulsions to gastric conditions the ζ -potential became less negative for all the studied samples to almost neutral values ranging between -2.5 and -2.1 mV. This is due to the measurement of the ζ -potential at low pH conditions (pH 3) in the gastric phase. On the other hand, after small intestinal conditions, the ζ -potential of emulsions and nanoemulsions was significantly different, being more negative in the case of emulsions in comparison with nanoemulsions. In fact, the ζ -potential of emulsions' digest ranged between -31.7 and -34.1 mV while in the case of nanoemulsions' digest ranged between -5.6 and -6.8 mV. This is probably related to their different lipid digestibility under small intestinal conditions. After TAG digestion, the release of anionic FFA contributes predominantly to the overall negative charge of digested emulsions (Singh, Ye, & Horne, 2009). These results are highly related to the differences in lipolysis reaction of

both emulsions and nanoemulsions as discussed later, which indicates that nanoemulsions released a lower amount of FFA after small intestinal conditions in comparison to emulsions.

In situ emulsion

The initial ζ -potential of the *in situ* carrot-based emulsions was highly negative, with a value of -55 mV (Figure 2 C). The strong negative charge of the *in situ* emulsion might be attributed to the presence of solubilized pectin from the vegetable cell wall. In fact, the anionic nature of pectin is related to the ionization of non-methylesterified galacturonic acid units from the pectin molecule, which may lead to negative charges at a pH above the pK_a of pectin (≈ 3.5) (Kohn, 1987). After gastric and intestinal phases, the *in situ* emulsion presented a similar behavior to the one of the o/w emulsions, being almost neutral after gastric conditions and becoming more negative after small intestinal conditions. In this sense, the ζ -potential of the *in situ* emulsion was -27.7 mV while it was -31.7 mV for the corresponding emulsion containing OO, which might indicate a slightly lower release of anionic FFA in the case of the *in situ* emulsion.

3.2 Lipid digestion and micelle formation

The lipolysis reaction of the emulsions, nanoemulsions and *in situ* emulsion was followed by determining the decrease in undigested TAG and subsequent production of MAG, FFA and GLY in the digested samples along the small intestinal phase. Additionally, the simultaneous mixed micelle formation was characterized in terms of MAG and FFA incorporation. To this end, the SCE-CO emulsions (SOR 0.1) or nanoemulsions (SOR 2) were selected as representative samples with different surfactant concentration and droplet size. The lipid hydrolysis reaction and micelle formation kinetics in the *in situ* emulsion was also determined in order to study the digestive behavior of complex emulsion-based systems in comparison with model o/w emulsions.

Emulsions and nanoemulsions

The lipolysis reaction rate was dramatically different for emulsions produced with a low surfactant concentration (SOR 0.1) (Figure 4 A) in comparison with nanoemulsions produced with a high surfactant concentration (SOR 2) (Figure 4 C). For emulsions, a gradual decrease in the amount of undigested TAG was observed during the intestinal phase, reaching complete TAG digestion at the end of the *in vitro* small intestine (Figure 4 A). This was simultaneously accompanied by a gradual increase in lipid digestion species, namely MAG, FFA and GLY, until a plateau phase was reached (Figure 4 A). The concentration of FFA release during digestion in emulsions was higher in comparison to the one of MAG. In this regard, the concentration of FFA at the end of the intestinal phase was 38 mg/mL of emulsion, while the final MAG concentration was 4.7 mg/mL of emulsion. These results suggest that the release of FFA due to hydrolysis occurs faster in comparison to the MAG release. In fact, based on the calculations of the stoichiometric reactions and mass balance during digestion, we detected the hydrolysis of three FFA units from part of the TAG molecules rather than the release of MAG and two FFA. Actually, previous results suggest that the complete hydrolysis of TAG into three FFA molecules occurs during *in vitro* lipid digestion by pancreatic lipases (Salvia-Trujillo, Verkempinck, et al., 2017). Moreover, lipid digestion products, were immediately incorporated into mixed micelles. Actually, the FFA and MAG micellarization kinetics followed the same trend than their release in the digest (Figure 4 B). The average percentage of incorporation of MAG and FFA released in the digest into the micellar fraction was 100 % and 84 %, respectively. Actually, the estimated rate constants (k-value) of the MAG and FFA release in the digest were 0.492 ± 0.495 and 0.060 ± 0.007 (min^{-1}) respectively, and in the micellar fraction were 0.212 ± 0.028 and 0.048 ± 0.007 (min^{-1}) respectively. This is especially relevant in the case of MAG digestion, as they became

entrapped in mixed micelles immediately after being released, thus not being accessible for further lipase activity.

Nanoemulsions, despite their smaller particle size than emulsions, presented almost negligible lipid digestion under intestinal conditions (Figure 4 C). In fact, TAG largely remained undigested at the end of the intestinal phase, with values of undigested TAG above 99 %. Subsequently, the release of lipid digestion products was extremely limited, with only the detection of minor amounts of FFA, ranging around 0.2 mg/mL of emulsion, while MAG remained undetectable during the intestinal phase. Hence, the micellar phase of the digested nanoemulsions presented a very low concentration of FFA (Figure 4 D). Nonetheless, during micelle formation, a gradual incorporation of FFA was observed until reaching a plateau phase. However, the absolute amount of FFA reached at the end of the intestinal phase was around 0.13 mg/mL of emulsion. These results suggest that in nanoemulsions (as prepared in this study), lipid digestion was almost completely hindered, evidencing that pancreatic lipases did not access the substrate. This is probably due to the high amount of surfactant (SOR 2) used in the present work to produce nano-sized oil droplets. In fact, it has been previously reported that surfactants with high interfacial activity are able to prevent adsorption of lipases at the o/w interfaces (Reis et al., 2008; Yao et al., 2013). Thus, it may be hypothesized that the excess of Tween 80 may hinder the lipase adsorption at the o/w interface.

In situ emulsion

The lipolysis reaction in the *in situ* carrot purée emulsions (Figure 4 E) followed a similar trend as the model o/w emulsion produced with the same amount of surfactant (SOR 0.1) (Figure 4 A). However, the overall lipolysis was slightly slower and to a lower extent in the *in situ* emulsion in comparison with the o/w emulsion, as shown by the estimated kinetic parameters (Table 2). On the one hand, the TAG digestion rate constant of the *in situ*

emulsion was 0.071 ± 0.011 (min^{-1}) while it was 0.083 ± 0.008 (min^{-1}) for the emulsion. Moreover, the final predicted value of undigested TAG at the end of the intestinal phase was 3.1 ± 2.3 and 18.9 ± 18.9 % in the emulsion and *in situ* emulsion, respectively. On the other hand, there were no differences in the FFA generation rate constants (k-values; min^{-1}) or final concentrations of FFA at the end of the intestinal phase (C_f ; mg/mL emulsion) between the emulsion and the *in situ* emulsion (Table 2). Nonetheless, differences between the species of lipid digestion products generated during the digestion of the emulsion or the *in situ* emulsion could be detected. In fact, MAG were not produced during the digestion of the *in situ* emulsion, thus indicating that TAG were completely hydrolyzed.

With regard to the micelle formation, a gradual increase of the concentration of FFA in the micellar fraction was observed until a plateau was reached (Figure 4 F). However, a much lower proportion of the generated FFA in the digest of the *in situ* emulsion were incorporated into the micellar fraction in comparison with the emulsion. In this sense, an average of 35 % of the FFA generated in the digest of the *in situ* emulsion were embodied into mixed micelles, while for the emulsion it was 84 %. This evidences that despite that the lipolysis reaction of oil droplets was not dramatically affected by the food matrix components, the migration of lipid digestion products into forming mixed micelles was. In fact, the influence of dietary fibers, such as pectin as main component of soluble biopolymers in carrots, may potentially alter the lipid digestion by a number of physicochemical mechanisms (Lairon, Play, & Jourdeuil-Rahmani, 2007): (i) via interaction of fibers and pancreatic lipases reducing enzyme activity (O'Connor, Sun, Smith, & Melton, 2003); (ii) adsorption of pectin at the oil/water interface preventing lipase to reach the substrate (Mun, Decker, Park, Weiss, & McClements, 2006); (iii) binding of fibers with bile salts thus preventing the removal of lipid digestion products from the oil droplet surface (Thongngam & McClements, 2005); and (iv)

increased viscosity of the aqueous phase thus potentially lowering the transport of molecules from the lipid droplet surfaces. In this regard, the present work, to the best of our knowledge, is the first one to evidence that food matrix components may not affect digestion of emulsified lipids *per se* but may hinder the absorption of lipid digestion products and subsequently lipophilic bioactive compounds by hindering the migration of lipid digestion molecules into mixed micelles.

3.3 Carotenoid bioaccessibility

In this section, the α - and β -carotene BA kinetics of emulsions (SOR 0.1) and nanoemulsions (SOR 2) containing carotenoid-rich lipid fractions enriched from different sources, namely carrot purée or synthetic β -carotene, during the small intestinal phase is discussed (Figure 5). Additionally, the carotenoid BA kinetics of the *in situ* carrot purée emulsion was also evaluated and compared to the one of the o/w emulsion (Figure 5).

Emulsions and nanoemulsions

The carotenoid BA kinetics for all the studied samples followed a gradual increase during the small intestinal phase until a plateau value was reached (Figure 5). Firstly, the BA kinetics significantly differed between emulsions and nanoemulsions regardless the carotenoid enrichment source, and was highly linked to their respective lipolysis reaction and subsequent micelle formation (Figure 4 A, B, C and D). In this regard, emulsions, produced with low amounts of surfactant (SOR 0.1) presented a higher α - and β -carotene BA in comparison with nanoemulsions produced with high amounts of surfactant (SOR 2). This was evidenced by the final BA values of emulsions or nanoemulsions at the end of the intestinal phase predicted by the fractional conversion model (Table 3). While the final values of α - and β -carotene BA in emulsions ranged between 63.4 – 67.4 and 64.3 – 73.9 %, respectively, it was between 12.9 – 14.2 and 8.4 – 14.4 % in the case of nanoemulsions. The higher carotenoid BA observed for

emulsions could be related to the much higher lipolysis observed in those systems as opposed to the one of the nanoemulsions. The poor lipolysis of nanoemulsions led to carotenoid-loaded lipid droplets remaining undigested, thus explaining their low carotenoid BA. Moreover, the carotenoid BA was influenced by the type of oil and also carotenoid enrichment source. On the one hand, emulsions formulated with OO reached higher both α - and β -carotene BA at the end of the intestinal phase in comparison with emulsions formulated with CO. This is in agreement with previous studies *in vivo*, where they observed a higher lycopene and astaxanthin BA in emulsions formulated with OO in comparison with CO (Clark, Yao, She, & Furr, 2000). OO (C18:1) is mainly composed of oleic acid, which is monounsaturated (MUFA), while CO is mainly composed of linoleic acid (C18:2), which is polyunsaturated (PUFA). In this regard, PUFA are more prone to oxidation in comparison to MUFA, thus might induce oxidative damage to carotenoids. On the other hand, emulsions formulated with CO, showed a different behavior depending on the source of carotenoid enrichment. In fact, emulsions CO enriched with synthetic β -carotene showed a faster carotene micellarization in comparison with CO enriched with carotenoids from carrot purée, yet reached similar final values at the end of the intestinal phase, which was evidenced by the higher predicted k-values and similar final BA values (Table 3). This might be due to a competition between α - and β -carotene micellarization in the case of CE-CO, while for SCE-CO there is only the presence of β -carotene. Moreover, other lipophilic components from the vegetable matrix, such as those constituting the chromoplast fraction (Vishnevetsky, Ovadis, & Vainstein, 1999) might have also been transferred into the oil phase and therefore compete with carotenoids for their incorporation within mixed micelles.

In situ emulsion

The α - and β -carotene BA kinetics of the *in situ* emulsion (Figure 5) followed a similar trend than its lipid digestibility and micelle formation kinetics (Figure 4 E, F), thus showing a gradual increase at early time moments of the intestinal phase, reaching a plateau phase. The carotenoid BA kinetics of the *in situ* emulsion was slower and up to a lower extent in comparison with the respective simple o/w emulsion formulated with OO and an SOR of 0.1. In this sense, the estimated k-value (min^{-1}) and C_f (%) of the CE-OO SOR 0.1 emulsion were 0.102 min^{-1} and 73 % while for the *in situ* emulsion was 0.027 min^{-1} and 46 %. This could be related to the lower concentration of lipid digestion products incorporated in mixed micelles in the *in situ* emulsion in comparison with the o/w emulsion, as discussed in the previous section (Figure 4). In fact, it is known that the amount of free fatty acids incorporated in the micellar fraction determines their solubilization capacity of lipophilic species, such as carotenoids (Huo, Ferruzzi, Schwartz, & Failla, 2007; Salvia-Trujillo, Verkempinck, et al., 2017).

4 Conclusions

The current work evidences that nanoemulsions when formulated with high concentrations of surfactant may present low lipid digestibility. Consequently, carotenoids remained entrapped in the core of undigested oil droplets, thus showing low bioaccessibility. This might open possibilities to modulate lipid digestion by formulation of emulsion-based systems, but also highlights the possible disadvantages of using high surfactant concentrations for producing nanoemulsions as carriers of lipophilic bioactive compounds.

Interestingly, carrot purée *in situ* emulsion was produced and presented a high lipolysis rate and extent, similar to the one of a model o/w emulsion. However, it showed a lower incorporation of lipid digestion products into mixed micelles, probably due to the interaction

with the components of the vegetable food matrix. Nevertheless, despite having a lower carotenoid bioaccessibility than o/w emulsions, the *in situ* emulsion it had a high carotenoid micellarization ($\approx 50\%$) after intestinal conditions. Overall, this study arises the possible drawbacks of using high amounts of surfactant to produce nanoemulsions presenting almost a complete lipolysis inhibition. Nonetheless, the production of vegetable-based *in situ* emulsions might be a good strategy to enhance carotenoid bioaccessibility.

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Declaration of interests

The authors of the present work declare no conflict of interests.

References

- Bauer, E., Jakob, S., & Mosenthin, R. (2005). Principles of physiology of lipid digestion. *Asian-Australasian Journal of Animal Sciences*, 18(2), 282–295.
- Clark, R. M., Yao, L. L., She, L., & Furr, H. C. (2000). A comparison of lycopene and astaxanthin absorption from corn oil and olive oil emulsions. *Lipids*, 35(7), 803–806.
- Furr, H. C., & Clark, R. M. (1997). Intestinal absorption and tissue distribution of carotenoids. *Journal of Nutritional Biochemistry*, 8(7), 364–377.
- Golding, M., Wooster, T. J., Day, L., Xu, M., Lundin, L., Keogh, J., & Clifton, P. (2011). Impact of gastric structuring on the lipolysis of emulsified lipids. *Soft Matter*, 7(7).
- Graeve, M., & Janssen, D. (2009). Improved separation and quantification of neutral and polar lipid classes by HPLC–ELSD using a monolithic silica phase: Application to exceptional marine lipids. *Journal of Chromatography B*, 877(20–21), 1815–1819.
- Huo, T., Ferruzzi, M. G., Schwartz, S. J., & Failla, M. L. (2007). Impact of fatty acyl composition and quantity of triglycerides on bioaccessibility of dietary carotenoids. *Journal of Agricultural and Food Chemistry*, 55(22), 8950–8957.

- <https://doi.org/10.1021/jf071687a>
- Kohn, R. (1987). Binding of divalent cations to oligomeric fragments of pectin. *Carbohydrate Research*, 160, 343–353.
- Komaiko, J. S., & McClements, D. J. (2016). Formation of Food-Grade Nanoemulsions Using Low-Energy Preparation Methods: A Review of Available Methods. *Comprehensive Reviews in Food Science and Food Safety*, 15(2), 331–352.
- Lairon, D., Play, B., & Jourdeuil-Rahmani, D. (2007). Digestible and indigestible carbohydrates: interactions with postprandial lipid metabolism. *Journal of Nutritional Biochemistry*, 18(4), 217–227.
- Lemmens, L., Colle, I., Van Buggenhout, S., Palmero, P., Van Loey, A., & Hendrickx, M. (2014). Carotenoid bioaccessibility in fruit- and vegetable-based food products as affected by product (micro) structural characteristics and the presence of lipids: A review. *Trends in Food Science & Technology*, 38(2), 125–135.
- Marinova, K. G., Alargova, R. G., Denkov, N. D., Velez, O. D., Petsev, D. N., Ivanov, I. B., & Borwankar, R. P. (1996). Charging of oil-water interfaces due to spontaneous adsorption of hydroxyl ions. *Langmuir*, 12(8), 2045–2051.
- Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., ... Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *Food & Function*, 5(6), 1113–1124.
- Moelants, K. R. N., Cardinaels, R., Jolie, R. P., Verrijssen, T. A. J., Van Buggenhout, S., Van Loey, A. M., ... Hendrickx, M. E. (2013). Rheology of Concentrated Tomato-Derived Suspensions: Effects of Particle Characteristics. *Food and Bioprocess Technology*, 1–17.
- Moquin, P. H. L., & Temelli, F. (2008). Kinetic modeling of hydrolysis of canola oil in supercritical media. *Journal of Supercritical Fluids*, 45(1), 94–101.
- Mu, H., & Høy, C.-E. (2004). The digestion of dietary triacylglycerols. *Progress in Lipid Research*, 43(2), 105–133.
- Mun, S., Decker, E. A., Park, Y., Weiss, J., & McClements, D. J. (2006). Influence of interfacial composition on in vitro digestibility of emulsified lipids: Potential mechanism for chitosan's ability to inhibit fat digestion. *Food Biophysics*, 1(1), 21–29.
- Mutsokoti, L., Panozzo, A., Musabe, E. T., Van Loey, A., & Hendrickx, M. (2015). Carotenoid transfer to oil upon high pressure homogenisation of tomato and carrot based matrices. *Journal of Functional Foods*, 19, 775–785.
- O'Connor, C. J., Sun, D., Smith, B. G., & Melton, L. D. (2003). Effect of soluble dietary fibers on lipase-catalyzed hydrolysis of tributyrin. *Journal of Food Science*, 68(3), 1093–1099.
- Palmero, P., Panozzo, A., Colle, I., Chigwedere, C., Hendrickx, M., & Van Loey, A. (2016). Role of structural barriers for carotenoid bioaccessibility upon high pressure homogenization. *Food Chemistry*, 199, 423–432.
- Reis, P. M., Raab, T. W., Chuat, J. Y., Leser, M. E., Miller, R., Watzke, H. J., & Holmberg, K. (2008). Influence of Surfactants on Lipase Fat Digestion in a Model Gastro-intestinal System. *Food Biophysics*, 3(4), 370–381.
- Sadler, G., Davis, J., & Dezman, D. (1990). Rapid extraction of lycopene and beta-carotene from reconstituted tomato paste and pink grapefruit homogenates. *Journal of Food Science*, 55(5), 1460–1461.
- Salvia-Trujillo, L., Qian, C., Martín-Belloso, O., & McClements, D. J. (2013). Influence of particle size on lipid digestion and β -carotene bioaccessibility in emulsions and nanoemulsions. *Food Chemistry*, 141(2).
- Salvia-Trujillo, L., Soliva-Fortuny, R., Rojas-Grau, M. A., McClements, D. J., & Martín-

- Belloso, O. (2017). Edible Nanoemulsions as Carriers of Active Ingredients: A Review. *Annual Review of Food Science and Technology*, Vol 8, 8, 439–466.
- Salvia-Trujillo, L., Verkempinck, S. H. E., Sun, L., Van Loey, A. M., Grauwet, T., & Hendrickx, M. E. (2017). Lipid digestion, micelle formation and carotenoid bioaccessibility kinetics: Influence of emulsion droplet size. *Food Chemistry*, 229, 653–662.
- Silva, H. D., Cerqueira, M. A., & Vicente, A. A. (2015). Influence of surfactant and processing conditions in the stability of oil-in-water nanoemulsions. *Journal of Food Engineering*, 167, 89–98.
- Singh, H., Ye, A., & Horne, D. (2009). Structuring food emulsions in the gastrointestinal tract to modify lipid digestion. *Progress in Lipid Research*, 48(2), 92–100.
- Thongngam, M., & McClements, D. J. (2005). Isothermal titration calorimetry study of the interactions between chitosan and a bile salt (sodium taurocholate). *Food Hydrocolloids*, 19(5), 813–819.
- Vishnevetsky, M., Ovadis, M., & Vainstein, A. (1999). Carotenoid sequestration in plants: the role of carotenoid-associated proteins. *Trends in Plant Science*, 4(6), 232–235.
- Yao, X., Nie, K., Chen, Y., Jiang, F., Kuang, Y., Yan, H., ... Phillips, G. O. (2018). The influence of non-ionic surfactant on lipid digestion of gum Arabic stabilized oil-in-water emulsion. *Food Hydrocolloids*, 74, 78–86.
- Yao, X., Wang, N., Fang, Y., Phillips, G. O., Jiang, F., Hu, J., ... Tian, D. (2013). Impact of surfactants on the lipase digestibility of gum arabic-stabilized O/W emulsions. *Food Hydrocolloids*, 33(2), 393–401.

Figure Captions

Figure 1. Volume-weighted particle size distribution of emulsions (SOR 0.1) (A, B and C) or nanoemulsions (SOR 2) (D, E and F) containing carrot-enriched olive oil (A and D), corn oil (B and E) or synthetic carotenoid-enriched corn oil (C and F).

Figure 2. Volume-weighted average particle size (A), particle size distribution (B) and ζ -potential (C) of *in situ* emulsions (5% olive oil and 0.5% Tween 80 in carrot purée). Different capital letters indicate significant differences between simulated gastrointestinal phases.

Figure 3. Microscopy images of the carrot-enriched olive oil (CE-OO) emulsion formulated with a surfactant-to-oil ratio (SOR) of 0.1 or 2 or the *in situ* emulsion (5% olive oil and 0.5% Tween 80 in carrot purée) after gastric and intestinal simulated conditions. Scale bar represents 50 μm .

Figure 4. Lipid digestion (A, C and E) and micelle formation (B, D and F) kinetics of synthetic carotenoid-enriched corn oil emulsions formulated with a surfactant-to-oil ratio (SOR) of 0.1 (A, B) or 2 (C, D), and *in situ* emulsions at an SOR 0.1 (5% olive oil and 0.5% Tween 80 in carrot purée) during small intestinal conditions. **Symbols represent experimental data of MAG ($\circ$), FFA (Δ), GLY ($\times$) and TAG ($\blacksquare$).** Lines represent predicted data by a fractional conversion model. TAG: triacylglycerides; MAG: monoacylglycerides; FFA: free fatty acids; GLY: glycerol.

Figure 5. α -carotene (A) and β -carotene (B) bioaccessibility (%) kinetics of carrot-enriched corn oil (CE-CO), olive oil (CE-OO), or synthetic carotene-enriched corn oil (SCE-CO) emulsions (SOR 0.1), nanoemulsions (SOR 2) or carrot-purée olive oil *in situ* emulsion (SOR 0.1) during small intestinal conditions. **Symbols represent experimental data of α - and β -carotene bioaccessibility of emulsions (full symbols) and nanoemulsions (empty symbols).** Lines represent predicted data by a fractional conversion model, being CE-CO the dotted lines, CE-OO the full lines, SCE-CO the dashed lines and *in situ* emulsion the dotted-dashed line, respectively.

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

Highlights

- Emulsions with sub-micron particle size had fast and complete *in vitro* lipolysis
- Nanoemulsions were produced with high surfactant concentration
- Nanoemulsions presented an inhibited *in vitro* lipolysis
- *In situ* emulsions within vegetable-based purées had a complete lipolysis
- Carotenoid bioaccessibility was determined by lipid digestion products in micelles

Emulsions loaded with carotenoids

Nanoemulsions loaded with carotenoids

In situ emulsions within carrot purée

Table 1. Volume weighed average particle diameter (d_{43} in μm) and ζ -potential (mV) of emulsions (SOR 0.1) or nanoemulsions (SOR 2) containing carrot-enriched olive oil (CE-OO) or corn oil (CE-CO), or synthetic carotenoid-enriched corn oil (SCE-CO).

	Gastrointestinal phase	d_{43} (μm)	ζ -potential (mV)
CE-OO SOR 0.1	Initial	0.787 ± 0.001 ^{Ba}	-45.58 ± 0.57 ^{Aa}
	Gastric	0.675 ± 0.057 ^{Ba}	-2.57 ± 0.77 ^{Ca}
	Intestinal	33.018 ± 16.629 ^{Aa}	-31.73 ± 1.56 ^{Ba}
CE-OO SOR 2	Initial	0.279 ± 0.001 ^{Bb}	-29.75 ± 3.88 ^{Ab}
	Gastric	0.291 ± 0.016 ^{Bb}	-2.74 ± 0.76 ^{Ca}
	Intestinal	4.437 ± 0.001 ^{Ab}	-6.54 ± 0.271 ^{Bb}
CE-CO SOR 0.1	Initial	0.897 ± 0.159 ^{Ba}	-31.30 ± 0.73 ^{Aa}
	Gastric	0.595 ± 0.015 ^{Ba}	-2.45 ± 0.91 ^{Ba}
	Intestinal	59.688 ± 7.660 ^{Aa}	-34.13 ± 2.09 ^{Aa}
CE-CO SOR 2	Initial	0.249 ± 0.028 ^{Bb}	-22.95 ± 0.43 ^{Ab}
	Gastric	0.265 ± 0.001 ^{Bb}	-2.81 ± 0.99 ^{Ca}
	Intestinal	6.860 ± 2.053 ^{Ab}	-5.66 ± 0.55 ^{Bb}
SCE-CO SOR 0.1	Initial	0.484 ± 0.001 ^{Ba}	-29.03 ± 1.93 ^{Ba}
	Gastric	0.466 ± 0.001 ^{Ba}	-2.27 ± 0.48 ^{Ca}
	Intestinal	1.038 ± 0.047 ^{Aa}	-33.28 ± 1.28 ^{Aa}
SCE-CO SOR 2	Initial	0.226 ± 0.007 ^{Ab}	-23.23 ± 2.22 ^{Ab}
	Gastric	0.221 ± 0.004 ^{Ab}	-2.17 ± 0.59 ^{Ca}
	Intestinal	0.231 ± 0.005 ^{Ab}	-6.88 ± 0.97 ^{Bb}

Different capital letters indicate significant differences between simulated gastrointestinal phases of the same sample. Different lower case letters indicate significant differences between the two SOR's of samples in the same gastrointestinal phase.

Table 2. Estimated parameters k-value (min^{-1}) and C_f (% of undigested TAG or mg.mL^{-1} of lipid digestion products) of the fractional conversion model for lipid digestion kinetics during the small intestinal phase and micelle formation

	k	C_f	R^2	R^2 adjusted
TAG - digest				
SCE-CO SOR 0.1	0.083 ± 0.008^a	3.1 ± 2.3^a	0.991	0.954
SCE-CO SOR 2	-	-	-	-
In situ SOR 0.1	0.071 ± 0.011^a	18.9 ± 3.4^b	0.977	0.889
MAG - digest				
SCE-CO SOR 0.1	0.492 ± 0.495	4.6 ± 0.1	0.967	0.837
SCE-CO SOR 2	-	-	-	-
In situ SOR 0.1	-	-	-	-
FFA - digest				
SCE-CO SOR 0.1	0.060 ± 0.007^a	36.2 ± 1.2^a	0.986	0.932
SCE-CO SOR 2	-	-	-	-
In situ SOR 0.1	0.061 ± 0.010^a	39.25 ± 1.9^a	0.974	0.873
GLY - digest				
SCE-CO SOR 0.1	0.040 ± 0.005^a	3.2 ± 0.15^a	0.982	0.931
SCE-CO SOR 2	-	-	-	-
In situ SOR 0.1	0.056 ± 0.011^a	4.24 ± 0.3^a	0.972	0.861
MAG - micelles				
SCE-CO SOR 0.1	0.212 ± 0.028	4.9 ± 0.1	0.989	0.949
SCE-CO SOR 2	-	-	-	-
In situ SOR 0.1	-	-	-	-
FFA - micelles				
SCE-CO SOR 0.1	0.048 ± 0.007^a	32.6 ± 1.5^b	0.940	0.703
SCE-CO SOR 2	-	-	-	-
In situ SOR 0.1	0.040 ± 0.005^a	15.7 ± 0.7^a	0.906	0.532

TAG: triacylglycerol; MAG: monoacylglycerol; FFA: free fatty acids; GLY; glycerol.

Carrot-enriched corn oil (CE-CO), olive oil (CE-OO), or synthetic carotene-enriched corn oil (SCE-CO) emulsions (SOR 0.1) or nanoemulsions (SOR 2).

Different lower case letter indicate significant differences between each kinetic estimated parameter of each individual lipid specie of the emulsions with different SOR.

Table 3. Estimated parameters k-value (min^{-1}) and C_f (final bioaccessibility value in %) of the fractional conversion model for α - and β -carotene bioaccessibility during the small intestinal phase

α -carotene	k	C_f	R^2	R^2 adjusted
CE-OO SOR 0.1	0.099 ± 0.014^b	67.4 ± 2.2^a	0.979	0.899
CE-OO SOR 2	0.104 ± 0.045^{ab}	12.9 ± 1.3^b	0.829	0.149
CE-CO SOR 0.1	0.036 ± 0.13^{ab}	63.4 ± 8.5^a	0.885	0.426
CE-CO SOR 2	0.084 ± 0.033^{ab}	14.2 ± 1.4^b	0.851	0.256
SCE-CO SOR 0.1	-	-	-	-
SCE-CO SOR 2	-	-	-	-
In situ SOR 0.1	0.027 ± 0.004^a	57.5 ± 4.3^a	0.976	0.883
β -carotene	k	C_f	R^2	R^2 adjusted
CE-OO SOR 0.1	0.102 ± 0.009^b	73.9 ± 1.6^c	0.991	0.955
CE-OO SOR 2	0.131 ± 0.056^{ab}	12.67 ± 1.1^a	0.848	0.240
CE-CO SOR 0.1	0.027 ± 0.011^a	65.9 ± 11.9^{bc}	0.865	0.324
CE-CO SOR 2	0.025 ± 0.007^a	14.4 ± 2.0^a	0.928	0.640
SCE-CO SOR 0.1	0.104 ± 0.023^{ab}	64.3 ± 3.4^{bc}	0.946	0.734
SCE-CO SOR 2	0.331 ± 0.236^{ab}	8.4 ± 0.5^a	0.903	0.513
In situ SOR 0.1	0.027 ± 0.005^a	46.5 ± 3.9^b	0.971	0.853

Carrot-enriched olive oil (CE-OO), corn oil (CE-CO), or synthetic carotene-enriched corn oil (SCE-CO) emulsions (SOR 0.1) or nanoemulsions (SOR 2).

Different lower case letter indicate significant differences between the α - or β -carotene bioaccessibility kinetic estimated parameter of the different samples.