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Lipid digestion, micelle formation and carotenoid bioaccessibility kinetics: Influence of emulsion droplet size

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ABSTRACT

Carotenoid-enriched oil-in-water emulsions with different droplet sizes (small: d_{43} 0.72 μm; medium: d_{43} 1.9 μm; large: d_{43} 15.1 μm) were subjected to simulated gastrointestinal conditions. The kinetics of lipolysis, micelle formation and carotenoid bioaccessibility were monitored during the intestinal phase. The rates of all three processes increased with decreasing droplet size. The large droplet size emulsion contained undigested oil at the end of digestion, whereas an almost complete hydrolysis was observed for the other two emulsions. The sub-micron emulsion presented a higher conversion of MAGs to FFAs during digestion, which led to a higher concentration of FFAs in the mixed micelles. The incorporation of carotenoids into mixed micelles occurred faster and reached a higher final value for the small droplet size emulsion, leading to final carotenoids bioaccessibility values of around 70%. This work provides valuable information for developing *in silico* models to simulate the lipid digestibility and carotenoid bioaccessibility.

1. Introduction

Carotenoids are lipophilic natural pigments synthesized and bioaccumulated in subcellular organelles of plants (chromoplasts), and are responsible for their orange, yellow or red colour (Fraser & Bramley, 2004). Carotenoids exert pro-vitamin A activity in the human body and present antioxidant activity, the latter due to their ability of scavenging free radicals (Halliwell, 1996). Moreover, the consumption of carotenoid-rich foods has been associated with a reduced risk of cardiovascular diseases and certain types of cancer (Rao & Rao, 2007). However, the bioaccessibility of carotenoids is typically very low due to their high level of bio-encapsulation in plant tissues and their poor solubility in the aqueous intestinal juices (Dimitrov et al., 1988). In this context, three main steps are required for optimal absorption of carotenoids in the human body: (*i*) the release from the food matrix; (*ii*) incorporation into an oil phase (during food processing or during digestion) and (*iii*) a complete digestion of the oil phase where carotenoids are solubilized in.

First, effective strategies for the disruption of plant cellular structures, *e.g.* mechanical or plant endogenous enzymes, have shown to increase the *in vitro* bioaccessibility of carotenoids (Palmero, Colle, et al., 2016; Palmero, Panozzo, et al., 2016). Second, the presence of a lipid source during digestion seems crucial to significantly enhance the carotenoid bioaccessibility (Lemmens et al., 2014; Unlu, Bohn, Clinton, & Schwartz, 2005). In fact, carotenoids are lipid soluble compounds, thus they follow the same absorptive pathways as other dietary lipids (Furr & Clark, 1997). The hydrolysis of lipids during digestion leads to the appearance of lipid digestion products (monoacylglycerols and free fatty acids) that, with bile salts, contribute to the formation of mixed micelles. In turn, mixed micelles encapsulate lipophilic bioactive compounds, such as carotenoids, thus becoming available for uptake in the epithelial cells of the small intestine. The transfer of carotenoids towards the lipid matrix during digestion therefore enhances the carotenoid bioaccessibility and highly depends on the polarity of the carotenoids (Palmero, Panozzo, Simatupang, Hendrickx, & Van Loey, 2014). In this context, incorporation of carotenoids from the food matrix to an oil phase prior to digestion (e.g. during processing, via high-pressure homogenization), has been proven a successful strategy to create food products with high carotenoid bioaccessibility. Processing here creates a carotenoid-rich emulsified lipid phase (Mutsokoti, Panozzo, Musabe, Van Loey, & Hendrickx, 2015) by removing the natural barriers that could hinder the transfer of carotenoids to the lipid phase during digestion. Third, properties of the emulsified lipid phase may largely determine the behaviour of the emulsion droplets during their passage

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though the gastrointestinal tract (McClements, Decker, & Park, 2009). Depending on the emulsion characteristics, such as oil composition and interfacial composition or oil droplet size, the interaction of lipids with intestinal lipase may vary, thus influencing the oil digestibility and in turn micelle formation and the associated carotenoid bioaccessibility.

In recent years, a number of studies have been conducted on emulsion-based delivery systems of lipophilic bioactive compounds (Qian, Decker, Xiao, & McClements, 2012b; Zhang et al., 2016). They are fabricated by emulsifying a lipid core that acts as a carrier of the lipophilic active compounds into an aqueous phase. Emulsion droplet size has been reported to directly impact lipid digestion and carotenoid bioaccessibility (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013a). Emulsions with smaller droplet sizes present a higher active surface area, and therefore more binding sites for intestinal lipases to adsorb thus leading to a faster and higher lipid digestibility, which is strongly correlated with a higher carotenoid bioaccessibility. However, these studies have been carried out in model emulsified systems with lipid carriers enriched with synthetic lipophilic compounds. Furthermore, these delivery systems are intended to be used as supplementary ingredients for food fortification. A rationalization of naturally occurring carotenoids in foods could be achieved by obtaining targeted *in situ* emulsified lipids loaded with carotenoids from the food matrix with specific characteristics. Moreover, there is a lack of information about the kinetics of lipid hydrolysis occurring in the intestine and its relationship with the formation rate of mixed micelles and the subsequent incorporation of carotenoids in the micelle fraction. A full comprehensive understanding of the above mentioned sequential processes would allow the design of *in situ* generated emulsified delivery systems of naturally occurring lipophilic compounds, such as carotenoids, for optimal oral bioavailability.

Therefore, the aim of the present work was to study the influence of the emulsion droplet size on the lipid digestion rate, micelle formation and composition and associated carotenoid bioaccessibility under *in vitro* gastrointestinal conditions, thereby focusing on the kinetics of all three processes. For this purpose, a carotenoid-enriched oil phase was obtained from carrot puree treated by high-pressure homogenization. The carotenoid-rich oil fraction was used to produce emulsions with different droplet sizes. Moreover, their behaviour during *in vitro* digestion was assessed in terms of particle size distribution, electrical charge (ζ-potential) and microstructure.

2. Material and methods

2.1. Materials

Carrots (*Daucus carota* cv. *Nerac*) were purchased from a local grocery store and stored at 4 °C until use. Corn oil was bought from a local supermarket. All chemicals and reagents were from Sigma Aldrich, except for NaCl, HCl, urea, anhydrous sodium sulphate and ethanol (from VWR); $CaCl_2{\cdot}2H_20$, NH_4Cl and $MgCl_2$ (from Merck); hexane, sulphuric acid and acetone (from Chem Lab); and $NaHCO_3$ (from Fisher Scientific); heptane (from Fluka); KCl (from MP Biomedicals) and diethylether (from Riedel-De Haën). All chemicals and reagents were of analytical grade.

2.2. Methods

2.2.1. Oil enrichment

Carotenoids from vegetable-based food products are reported to be more bioaccessible when they are previously solubilized in a lipid phase reducing their level of bioencapsulation (Mutsokoti et al., 2015). Corn oil has been shown to produce a wide range of particle sizes when fabricating emulsions by high pressure homogenization (Salvia-Trujillo et al., 2013a). Therefore, corn oil was enriched with carotenoids from carrot puree. First, carrots were washed, peeled and cut in dices of 1 × 1 cm. Carrot pieces were blended with 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. Afterwards, carrot puree and corn oil (15% w/v) were mixed (ultra turrax, Waring Commercial, Torrington, CT, USA) at 12,500 rpm for 10 min. Then, 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 corn 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. The α and β-carotene concentration of the enriched corn oil were 75.6 ± 1.3 and 204.6 ± 5.1 μg/g oil, respectively.

2.2.2. Emulsion formation

Emulsions with different droplet sizes were obtained by different treatments. A coarse emulsion was obtained by mixing 5% (w/w) enriched corn oil with 0.5% (w/w) Tween 80 as surfactant and 94.5% (w/w) Milli-Q water with a high shear mixer (Silverson L5M-A, Silverson Machines, Inc., Massachusetts, USA) operating at 400 rpm for 5 min. The coarse emulsion was used as such and further referred as large emulsion (LE). Part of the coarse emulsion was processed with a high-pressure homogenizer (Stansted Fluid Power, Pressure cell homogenizer, U.K.) one cycle at 20 MPa to obtain an emulsion with medium droplet size (ME), or at 200 MPa to obtain an emulsion with small droplets (SE). LE, ME and SE had a volume-weighted average droplet diameter (d_{43}) of 15.1 ± 1.1, 1.93 ± 0.09 and 0.72 ± 0.13 μm, determined by laser diffraction as described below.

2.2.3. In vitro digestion

A simulation of gastrointestinal conditions, consisting on an *in vitro* gastric and an intestinal phase, based on an international consensus method (Minekus et al., 2014) was carried out in order to investigate the behaviour of emulsions under *in vitro* digestion conditions. *In vitro* digestion experiments were conducted under subdued light conditions.

Gastric phase: in a brown falcon tube, a 5 ml aliquot of emulsion was mixed with 5 ml Milli-Q water, 7.5 ml of Simulated Gastric Fluid (SGF), 5 μl of $CaCl_2$ (0.3 M) and HCl (2 M) to reach pH 3. Afterwards 1.6 ml of pepsin solution previously dissolved in SGF was added to achieve 2000 U/ml. The total volume of the gastric phase was 20 ml. Falcon tubes were flushed with N_2 for 10 s and incubated at 37 °C for 2 h in an end-over-end rotator.

Small intestine phase: after the gastric phase, 11 ml of Simulated Intestinal Fluid (SIF) were added to the falcon tubes. Subsequently, 2.5 ml of bile solution (160 mM) and 40 μl of $CaCl_2$ (0.3 M) dissolved in SIF were added. Milli-Q water and NaOH (1 M) were added to bring the pH of the samples up to 7. Finally, 5 ml of a "pancreatic solution" consisting of 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) were added. The final lipase activity in the "pancreatic solution" was 2000 U/ml. The final volume of the intestinal phase was 40 ml. The tubes were flushed with N_2 for 10 s and incubated in an end-over-end rotator at 37 °C. During the small intestinal phase a kinetic study was conducted. For this, 6 individual falcon tubes were prepared to determine the lipid digestion and carotenoid bioaccessibility at 6 time points (7.5, 15, 30, 45, 60 and 120 min after the addition of "pancreatin solution"). To stop