Relation between *in vitro* lipid digestion and β -carotene bioaccessibility in β -carotene-enriched emulsions with different concentrations of L- α -phosphatidylcholine

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Abstract

In view of consumer health, it is desirable to promote the bioaccessibility of lipid-soluble compounds like carotenoids, while limiting the lipid intake. The objective of this work was to examine the relation between *in vitro* lipid digestion and β -carotene bioaccessibility of carrot-based model food emulsions containing water, 5% olive oil enriched with β -carotene (from carrots) and different concentrations (1-2-3-4%) of L- α -phosphatidylcholine (PHC), as an emulsifier. The lipid digestion (hydrolysis of triacylglycerols (TAGS) and incorporation of free fatty acids (FFAs) and monoacylglycerols (MAGs) in the micelles) and the β -carotene bioaccessibility (incorporation of β -carotene in the micelles) were studied after an *in vitro* digestion procedure wherein the stomach phase was mimicked for 2.0 h (37 °C) and the small intestinal phase was mimicked for 1.0 h, 1.5 h and 2.0 h (37 °C) (both end-over-end rotations). As a consequence, not only the influence of the emulsifier concentration, but also the influence of the duration in the small intestinal phase was investigated in this study. The oil droplet size distributions of the emulsions at different stages of digestion were shown to be dependent on the phosphatidylcholine concentration, but independent on the duration in the small intestinal phase (1.0 h – 2.0 h). Furthermore, all TAGs were already hydrolysed into FFAs and MAGs after 1.0 h small intestinal phase and the incorporation of FFAs and MAGs into micelles seemed to reach a maximum for all emulsions (approximately 26.5%), independent on the phosphatidylcholine concentration and thus on the particle size distributions. Finally, the β -carotene bioaccessibility increased with increasing phosphatidylcholine concentration, ranging from 33.2% to 79.8% for a 1% and 4% PHC emulsion respectively. No significant differences in β -carotene bioaccessibility were however noticed for the different durations in the small intestinal phase tested. In conclusion, a higher phosphatidylcholine concentration in emulsions leads to higher β -carotene bioaccessibility while the incorporation of lipids into micelles did not increase.

1 Introduction

Consumers are becoming more and more aware of the importance of a balanced and healthy diet. This balanced and healthy diet includes besides fruit and vegetables, containing carotenoids, also a low amount of fat or oil. Carotenoids (e.g. β -carotene) are a group of lipid-soluble bioactive compounds which seem to have a positive impact on human health, because of their antioxidant capacity or immune system enhancing properties. In addition, carotenoids such as β -carotene act as provitamin A (Dutta, Chaudhuri, & Chakraborty, 2005; Fernández-García et al., 2012). Mammals however need to obtain carotenoids from their diet, hence a good level of absorption or bioavailability from the food is desirable. The bioavailability is the fraction of the ingested (micro)nutrient that is available for utilization in physiological functions and for storage in the body. It is partially determined by its bioaccessibility, which is in case of a lipid soluble (micro)nutrient, the fraction of the ingested (micro)nutrient that is incorporated into micelles and thus becomes available for absorption in the body (Hedren, Diaz, & Svanberg, 2002; Palafox-Carlos, Ayala-Zavala, & Gonzalez-Aguilar, 2011). Bioavailability is dependent on several factors, grouped in the word “SLAMENGHI”. The letters of “SLAMENGHI” stand for Species of carotenoids, molecular Linkage, Amount of carotenoids consumed in a meal, Matrix in which the carotenoid is incorporated, Effectors of absorption and bioconversion, Nutrient status of the host, Genetic factors, Host-related factors and Interactions (Castenmiller & West, 1998). Because carotenoids are lipophilic, the presence of lipids constitute an effector of absorption (E) (Castenmiller & West, 1998; Hedren et al., 2002). In this context, it has already been demonstrated that the absorption of carotenoids increases by adding lipids to the diet, hence a minimum amount of lipids is needed for the carotenoid absorption (Castenmiller & West, 1998). Over-consumption of lipids on the other hand can lead to obesity and increasing risks of cardiovascular diseases (Simopoulos, 1999; McClements & Decker, 2009).

To understand and control the absorption of lipophilic micronutrients, knowledge about lipid digestion is needed. Lipid digestion can be divided into two steps, (i) the hydrolysis and (ii)

micellization of the dietary lipids. Specifically, triacylglycerols (TAGs) (like triolein, mostly present in olive oil) are hydrolyzed into diacylglycerols (DAGs), monoacylglycerols (MAGs) and free fatty acids (FFAs), after which the MAGs and FFAs can be incorporated into micelles. Micelle formation is essential for the absorption in the enterocytes. Micelles contain, besides MAGs and FFAs, bile salts, phospholipids and lipid-soluble compounds, like β -carotene (Hofmann & Borgstrom, 1962; Yonekura & Nagao, 2007). Hence the ingestion and hydrolysis of lipids (resulting in MAGs and FFAs) along with carotenoids is thought to be essential for the absorption of carotenoids (van het Hof, West, Weststrate, & Hautvast, 2000). The rate of hydrolysis by lipase can depend on different factors. The oil droplet size for instance influences the area of lipid surface available to lipase. Moreover, the composition of the droplet surfaces and the rheological properties of the surrounding medium are important as well. Very small oil droplets covered with surfactants which are strongly bound to the oil droplets in a viscous medium will be hydrolysed slower by lipase than larger oil droplets covered with less bound surfactants in a low viscous medium for example (McClements & Decker, 2009). Finally, the rate of micelle formation can be affected by the surfactant type and concentration, the viscosity of the surrounding medium and the amount of hydrolysed products amongst others (McClements & Decker, 2009).

In view of consumer health, it is desirable to promote the bioaccessibility of lipid-soluble compounds like carotenoids, while on the other hand the lipid intake should be reduced. As a consequence, knowledge on the link between lipid digestion and β -carotene bioaccessibility is important and can be used to design food systems in which these properties are controlled. Specifically to understand this link, simple model emulsions with a low amount of lipids and different properties, like different oil droplet size (distributions) can be used as study object. The oil droplet size (distribution) seems in turn to influence the activity of lipase (Armand et al., 1996; Mun, Decker, & McClements, 2007) and is expected to influence the lipid digestion and β -carotene bioaccessibility therefore. To prepare emulsions with different oil droplet size distributions, different concentrations of emulsifiers can be utilised because the emulsifier concentration is expected to influence the oil droplet size distribution

(Mun et al., 2007; Hur, Decker, & McClements, 2009). As emulsifier, a natural, harmless and useful surfactant, phosphatidylcholine, was chosen. Phosphatidylcholine is the major membrane phospholipid in mammalian cells, critical for the membrane structure and function (Gennis, 1989; Venturoli, Sperotto, Kranenburg, & Smit, 2006). Besides this, phosphatidylcholine is an important source of choline, an essential nutrient, in the body, which is needed for vital functions like the synthesis of cell signaling molecules and neurotransmitters involved in muscle control and memory (Zeisel, 2006; Sanders & Zeisel, 2007)

In the present work, the influence of the L- α -phosphatidylcholine concentration on the *in vitro* lipid digestion and β -carotene bioaccessibility in low fat emulsion systems was investigated. To investigate lipid digestion, lipid extraction and quantification was optimized and implemented, based on a method of Helbig et al. (2012). Furthermore, the particle size distributions of the digests at different stages of digestion as well as the influence of the duration in the small intestinal phase on lipid digestion and β -carotene bioaccessibility were investigated. In this way, more information about lipase activity, micelle formation and β -carotene incorporation could be obtained. Finally, the link between the micellar lipid incorporation and the β -carotene bioaccessibility was evaluated.

2 Materials and methods

2.1 Materials

Fresh carrots (*Daucus carota* cv. Nerac) were purchased in a local shop in Belgium and stored at 4 °C. Olive oil (extra virgin) was kindly donated by Vandemoortele (Ghent, Belgium). All chemicals and reagents were from Sigma Aldrich, except for NaCl, HCl, urea, anhydrous sodium sulphate and ethanol (from VWR); CaCl₂·2H₂O, NH₄Cl and MgCl₂ (from Merck); hexane, sulphuric acid and acetone (from Chem Lab); glucose and NaHCO₃ (from Fisher Scientific); heptane (from Fluka); KCl (from MP Biomedicals) and diethylether (from Riedel-De Haën). All chemicals and reagents were of analytical grade. The phosphatidylcholine Assay Kit is from Cayman Chemical Company.

2.2 Preparation of β -carotene enriched oil-in-water emulsions

Olive oil enriched with β -carotene (from carrots) was prepared according to the procedure described by Verrijssen et al. (2014). Emulsions were prepared by blending (10 min, ultra turax (Waring Commercial, Torrington, CT, USA)) 5% (w/w) of this enriched oil with demineralized water, in which 1, 2, 3 or 4% (w/w) L- α -phosphatidylcholine (PHC) was dissolved. After blending, the emulsions were homogenized (Stansted Fluid Power, Pressure cell homogeniser, U.K.) at 100 MPa using a single cycle. The pH was adjusted to 6.0 using a (1 M) sodium hydroxide solution. The different emulsions are further indicated as "1% PHC emulsion", "2% PHC emulsion", "3% PHC emulsion" and "4% PHC emulsion". Emulsions were prepared in duplicate. Each emulsion was independently submitted to the *in vitro* digestion procedure.

2.3 *In vitro* digestion of β -carotene enriched emulsions

2.3.1 *Digestion model*

Digests were passed through a two-step static *in vitro* digestion model based on the method described by Versantvoort et al. (2005). The first step in the digestion method is the simulation of the stomach phase by addition of 12 ml stomach juice (pH 1.3), which contains mainly electrolytes, bovine serum albumine, pepsin and mucin (Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005) to 6 g emulsion and rotating this mixture 2.0 h end-over-end (40 rpm) at 37°C. After the stomach phase, the intestinal phase is simulated by mixing (end-over-end, 40 rpm) 12 ml duodenal juice, 6 ml bile juice and 2 ml 1 M bicarbonate with the emulsion and stomach juice (37°C). The duration of the small intestinal phase was 1.0 h, 1.5 h and 2.0 h (further indicated as "SIP 1.0 h", "SIP 1.5 h" and "SIP 2.0 h" respectively). To minimize the influence of light and oxygen, the samples were kept in the dark during the whole digestion procedure and the headspace of the tubes was flushed with nitrogen prior to each incubation step.

2.3.2 *Particle size distribution during digestion*

The particle size and particle size distribution of the samples were measured by laser diffraction (Beckman Coulter Inc, LS 13 320, Miami, Florida) during digestion, i.e. before digestion, after the stomach phase (2.0 h) and after the small intestinal phase (SIP 1.0 h, SIP 1.5 h and SIP 2.0 h). The sample was poured into a stirred tank, filled with deionized water, and 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 particles in the sample. All analyses were carried out in duplicate.

Besides laser diffraction, microscopy pictures were taken to visualize the microstructure of the samples. This was done using a light microscope (Olympus BX-41) equipped with an Olympus XC-50 digital camera (Olympus, Opticel Co. Ltd., Tokyo, Japan).

2.3.3 Lipid digestion

The amount of lipids was measured before digestion (in the initial emulsions), after the small intestinal phase (SIP 1.0 h, SIP 1.5 h and SIP 2.0 h) and in the micelle fraction (collected after the small intestinal phase). As a consequence, not only the release of FFAs and MAG from TAG, but also their incorporation in the micelle fraction could be investigated. The micelle fraction was in this respect collected after the small intestinal phase (SIP 1.0 h, SIP 1.5 h and SIP 2.0 h) by ultracentrifugation (165,000 g, 1 h and 5 min, 4 °C). To identify and quantify the amount of TAG, DAG (diacylglycerol), MAG and FFA in the different types of samples, a procedure was implemented and optimised based on the procedure of Helbig et al. (2012). Basically, some modifications to the extraction step and the quantification step were applied. In the first step of this procedure, the extraction step, 2 ml sample was taken and poured into an extraction mix, containing 2 ml ethanol, 3 ml diethylether-heptane (DEE-Hep, 1:1 v/v) and 0.2 ml 2.5 M sulphuric acid. This mixture was vortexed for 2 min after which it was mixed (end-over-end) for 30 min and centrifuged for 5 min at 500 g (20 °C). The upper layer was collected in a new tube. 1 ml of DEE-Hep was added to the lower layer and the mixture was vortexed (1 min), mixed end-over-end (15 min) and centrifuged again. The upper layer was again collected and added to the previous upper layer. 100 mg anhydrous sodium

171 sulphate and an internal standard (50 µl trilaurin (0.2 g / 5 ml DEE-Hep) for the quantification of TAGs
172 and 100 µl methyl heptadecanoate (0.04 g / 5 ml DEE-Hep) for the quantification of MAGs and FFAs)
173 were added to the collection of the two upper layers, after which the whole volume was brought to 5
174 ml with DEE-Hep. The type and concentration of internal standard were chosen so to not interfere
175 with the compounds present in the sample and to have an abundance comparable to the lipids in the
176 sample.

177 The second step was the identification and quantification of the compounds with the GC-MS. In the
178 GC (Agilent Technologies, 6890N Network GC System, United States) with column VF-ht, UltiMetal
179 (Varian BV, Middelburg, Netherlands), 0.2 µl sample was injected splitless. The oven temperature
180 was programmed at 80 °C (1 min) and increased with 6 °C.min⁻¹ to 400 °C (2 min). For the detection
181 of the compounds, a MS-system (Agilent Technologies, 5973 inert Mass Selective Detector, United
182 States), has been used. Standard curves were used to quantify the different compounds based on the
183 amount internal standard added.

184 The $\frac{L_M}{L_{SIP}}$ -value was calculated which is the the amount of lipids (FFAs and MAGs) in the micelle
185 fraction (after digestion) per g initial emulsion (L_M) relatively to the amount of lipids (FFAs and MAGs)
186 after the small intestinal phase (SIP) per g initial emulsion (L_{SIP}) . Both values were calculated using
187 the standard curves. Because the L_{SIP} -value was the same for all samples (results not shown) and no
188 DAG and TAG were available in the digested samples, the $\frac{L_M}{L_{SIP}}$ -value is representative for the
189 incorporation of lipids in the micelles.

190 2.3.4 In vitro β -carotene bioaccessibility

191 The *in vitro* β -carotene bioaccessibility ($\frac{B}{C}$) was calculated as the amount of β -carotene in the
192 micelles after digestion per g initial emulsion (B) relative to the initial amount of β -carotene in the
193 digest per g initial emulsion (C) according to the procedure described by Verrijssen et al. (2014). This
194 value thus represents the incorporation of β -carotene into the micelles. All analyses were carried out
195 in triplicate.

2.3.5 Phosphatidylcholine concentration

The phosphatidylcholine concentration was quantified before digestion, after the small intestinal phase and in the micellar fraction using the Phosphatidylcholine Assay Kit (Cayman Chemical Company, Michigan, USA). In this kit, the quantification is done by using phospholipase D, choline oxidase and peroxidase to hydrolyze and color the phosphatidylcholine present. The absorbance of this color, a blue dye, is measured with a plate reader at 600 nm. By using a standard curve, plotting the absorbance in function of the concentration phosphatidylcholine (from 0 to 150 mg phosphatidylcholine per dl sample), the concentration could be calculated. Dilutions of the samples were made to not exceed the concentrations of the standard curve.

2.3.6 Statistical analysis

Differences in mean $\frac{L_M}{L_{SIP}}$ -value and β -carotene bioaccessibility were analyzed using one-way anova and the Tukey's Studentized Rand Post-hoc Test (Statistical Software Package SAS, version 9.2., Cary, N.C., U.S.A.). The level of significance was 95% ($P < 0.05$).

3 Results and discussion

3.1 Particle size distribution of emulsions during *in vitro* digestion

3.1.1 Initial emulsions

The particle size distributions of the different initial emulsions are shown in Fig. 1. It can be concluded that increasing the phosphatidylcholine concentration results in a decrease of oil droplet size. The median decreases namely from $1.20 \pm 0.08 \mu\text{m}$ to $0.84 \pm 0.01 \mu\text{m}$, $0.61 \pm 0.05 \mu\text{m}$ and $0.46 \pm 0.12 \mu\text{m}$ for a 1%, 2%, 3% and 4% PHC emulsion respectively. Moreover, the width of the oil droplet size distributions decreased with increasing phosphatidylcholine concentration. Mun et al. (2007) and Hur et al. (2009) found as well that oil droplet size and size distribution decrease with an increase in the emulsifier concentration (tested with lecithine and Tween 20). It has to be noted however that differences in oil droplet size and size distribution are relatively small probably due to the high pressure homogenization step during the preparation of the emulsions.

In Fig. 2, microscopic images confirm the trends found in the particle size distributions of the different emulsions (Fig. 1). Although (high pressure) homogenization is applied, some large oil droplets still exist in the 1% PHC emulsion and the emulsion is more heterogeneous compared to the 4% PHC emulsion (Figs. 2a and b). Probably some oil droplet coalescence appeared immediately after applying the high pressure (short-term stability) because the interfacial layer (between oil and water) was not fully occupied with phosphatidylcholine. The emulsion is however stable and does not changed in time (long-term stability) (McKenna, 2003). Fig. 2b shows that the 4% PHC emulsion contains small oil droplets only and is more homogeneous.

3.1.2 *Digested emulsions*

The particle size distribution of the four emulsions was also examined at different stages of digestion. To understand the digestion, next to the particle sizes of the initial emulsions, also the particle sizes after the stomach phase and after the small intestinal phase are important. The particle size after the stomach phase determines the surface area where lipase will interact whereas the particle size distribution resulting after the small intestinal phase can give some information on the presence of aggregated material and/or remaining oil droplets. In addition, the influence of the phosphatidylcholine concentration on the particle size distribution during digestion is of interest.

In Fig. 3, the particle size distributions before digestion, after the stomach phase and after the small intestinal phase (SIP 1.0 h, SIP 1.5 h and SIP 2.0 h) are given for the 1% PHC emulsion (Fig. 3a) and the 4% PHC emulsion (Fig. 3b). Microscopic images of oil droplets after the stomach phase are given for the 1% PHC emulsion and the 4% PHC emulsion in Figs. 4a and b respectively. The results on the particle size distributions of all emulsions showed an increase in particle size after the stomach phase compared to the initial size (before digestion), except for the 4% PHC emulsion (Fig. 3b) (results of 2% and 3% PHC emulsion not shown). Oil droplet clustering (seen in the microscopic picture, Fig. 4a) due to mucin present in the stomach phase can be the reason for this increase in particle size in the 1%, 2% and 3% PHC emulsion. Mucin, a highly glycosylated protein, can promote oil droplet

flocculation which may be due to a bridging or depletion mechanism (Vingerhoeds, Blijdenstein, Zoet, & van Aken, 2005; Singh & Sarkar, 2011). It can be hypothesized that the high phosphatidylcholine concentration in the 4% PHC emulsion hinders this oil droplet clustering (Fig. 4b) by interacting with mucin or by protecting the oil droplets from mucin. At the low pH of the stomach phase, repulsion may occur between the positive charge of phosphatidylcholine and the positively charged mucins. Differences in particle size distribution after the stomach phase, and so before small intestinal phase, resulting in differences in oil-water-interfaces, are expected to influence the lipase activity (McClements & Decker, 2009).

Although the duration in the small intestinal phase does not seem to influence the particle size (Figs. 3a and b), a remarkable difference has been noted between the different emulsions. By increasing the phosphatidylcholine concentration, the particle size increased after the small intestinal phase. This might be explained as follow. By digesting the oil droplets in the small intestinal phase and/or by adding other surfactant molecules (present in the small intestinal juice), phosphatidylcholine might come into the aqueous phase of the sample. The higher the phosphatidylcholine concentration, the more phosphatidylcholine will be present in this aqueous phase. Phosphatidylcholine is however an amphiphilic molecule with hydrophobic and hydrophilic groups. For this reason, in a bulk aqueous phase, phosphatidylcholine may form aggregates with proteins, bile acids or hydrolysis products (Mun et al., 2007; Hur et al., 2009) with their hydrophobic tail in the core and the hydrophilic head to the surrounding aqueous phase. Salvia-Trujillo et al. (2013a) found however that the size of micelles (containing bile acids, hydrolysis products and other fat-soluble compounds) is between 1-1000 nm. The interaction between phosphatidylcholine and proteins may explain the larger particles measured by laser diffraction (size around 30 μ m) (Fig. 3b).

3.2 Lipid digestion

In the initial emulsions, three kind of TAGs were detected, i.e. glycerol bound with three C18-fatty acids (mostly oleic acid, linoleic acid, stearic acid or vaccenic acid), glycerol bound with two C18-fatty

acids and one C16-fatty acid (mostly palmitic acid or palmitoleic acid) and glycerol bound with two C16-fatty acids and one C18-fatty acid (results not shown). These results are in agreement with the fatty acid (FA) composition of the olive oil (extra virgin) as determined by Vandermoortele (Ghent, Belgium) (78.5% oleic acid, 10.6% palmitic acid, 5.0% linoleic acid, 3.7% stearic acid, 1.0% palmitoleic acid and other fatty acids < 1.0%).

After the stomach phase, the same lipid composition was found compared to the initial composition (results not shown), which is explained by the fact that no hydrolyzing enzymes (such as gastric lipase) were added in the stomach phase so the composition of lipids did not change.

After the small intestinal phase (1.0 h, 1.5 h and 2.0 h) on the other hand, all TAGs were hydrolyzed to MAGs and FFAs. By comparing the composition of the FFAs in the TAGs of the initial emulsions and the FFAs in the small intestinal phase with the FFAs incorporated in the micelles, it was noticed that palmitic acid (hexadecanoic acid, C16), a saturated fatty acid, was not incorporated into the micelles. In this context, Karupaiah and Sundram (2007) found that long chain saturated FFAs are poorly absorbed because they would form a kind of soap in the small intestinal phase due to their strong electrostatic interactions with cationic multivalent mineral ions (for example calcium or magnesium, present in digestion juices) and due to their high melting point. Because of the controversy about saturated fatty acids in a diet due to their hypercholesterolaemic-atherogenic potential (Connor et al., 1986; Ulbricht & Southgate, 1991), it is thus an important finding that saturated acids, such as palmitic acid, might be less incorporated into micelles.

Fig. 5 shows that there is no significant difference between the incorporation of lipids into the micelles (indicated as the $\frac{L_M}{L_{SIP}}$ -value), nor for the different phosphatidylcholine concentrations in the emulsions nor between the different durations in the small intestinal phase. After 1.0 h SIP already all TAGs were hydrolysed into MAGs and FFAs (L_{SIP} -value was always the same and no DAGs or TAGs were present in the small intestinal phase) and incorporation of lipids in the micelles reached an equilibrium (L_M -value is also always the same, for all durations and for all different emulsions). It

seems that a maximum (of approximately 26.5%) has been reached and that the particle size is not the (main) influencing factor. It should be noted that on the one hand the differences in initial particle size between the different emulsions were rather small in this experiment which may have caused the apparent lack of influence of oil droplet size, although larger differences in particle size were noted between the different emulsions after the stomach phase (Fig. 4). And on the other hand, that measuring particle sizes and particle size distributions in systems, containing e.g. oil droplets, micelles, non-digested fat and enzymes is complex.

3.3 *In vitro* β -carotene bioaccessibility

From Fig. 6, it can be observed that the β -carotene bioaccessibility increases with increasing phosphatidylcholine concentration, going from an average $\frac{B}{C}$ -value of 33.2% (1% PHC emulsion) to 79.8% (4% PHC emulsion). No significant differences in β -carotene bioaccessibility were however noticed for different durations in the small intestinal phase. From the results of the oil droplet size distributions (at different stages of digestion) (Figs. 1 and 3), it was expected that the 4% PHC emulsion would result in a higher β -carotene bioaccessibility because smaller oil droplet size, observed after the stomach phase in the 4% PHC emulsion, can improve the lipase-activity due to their higher surface area compared to larger oil droplets (Armand et al., 1996; Salvia-Trujillo, Qian, Martin-Belloso, & McClements, 2013b). Improved lipase-activity in turn leads to more hydrolysis products (MAGs and FFAs) which can improve micelle-formation. Finally, those micelles can incorporate β -carotene hence making this lipophilic micronutrient accessible for absorption in the small intestinal phase. Increasing the formation of micelles is thus supposed to increase the β -carotene bioaccessibility (Salvia-Trujillo et al., 2013b). However, if the oil droplet size would be the (main) explanation of the increased β -carotene bioaccessibility of the 4% PHC emulsion, it would be expected that there was a difference between the different durations in the small intestinal phase. Specifically, the longer lipase can interact with the dietary lipids, the more hydrolysis products could be released and the more micelles could be formed. Because no significant difference in β -carotene

321 bioaccessibility with different durations in the small intestinal phase was noticed, it seems that an
322 equilibrium of the incorporation of β -carotene in the micelles is reached for each emulsion but that
323 this equilibrium depends on the phosphatidylcholine concentration. It can be hypothesized that
324 the 1.0 h incubation time in the small intestinal phase was already sufficiently long to allow all TAGs
325 present in the initial oil to be digested, irrespective of the oil droplet size. As oil droplet size can not
326 explain the β -carotene bioaccessibility observations, other hypotheses need to be put forward.
327 Measuring the phosphatidylcholine concentration in the micellar fraction resulted into the
328 knowledge that increasing the initial phosphatidylcholine concentration leads to an increasing
329 concentration in the micellar fraction (results not shown). This leads to the hypothesis that a higher
330 phosphatidylcholine concentration in the micelles enhances the incorporation of β -carotene due to
331 solubilisation of β -carotene or that phosphatidylcholine, as a surfactant, improves micelle formation
332 (Akoh & Min, 2002). In this context, Nishimukai and Hara (2004) observed that the absorption of
333 lycopene (another lipid-soluble nutrient) increased by adding phosphatidylcholine to soy oil.
334 Furthermore, Pugnali *et al.* (2004) and Mackie and Wilde (2005) found that there can be a
335 competitive adsorption between surface active molecules present in the sample (for example
336 phosphatidylcholine, hydrolysis products or bile acids in this study) which might cause some
337 displacement on the lipid interfaces. The displacement is dependent on the surface activities of the
338 different molecules on the one hand but also on the (relative) concentrations on the other hand. The
339 component with the highest surface activity and concentration will dominate the interface. Also the
340 micellar electric charge or micelle pH can differ, depending on the molecules present in the micelle,
341 which can differ the solubility efficiency (Goncalves *et al.*, 2013). These factors can cause that in this
342 case, the interface of the 1% PHC emulsion can be different than this of the interface of the 2,3 and
343 4% PHC emulsions, which can explain the different β -carotene incorporation into micelles. This
344 observation of different interfaces which may lead to different types of particles is confirmed by the
345 particle size distributions (Fig. 3), where not only differences between 1% PHC emulsion and 4% PHC
346 emulsion were shown in particle size but also in particle populations. Whereas the 4% PHC emulsion

contains two different particle population after the small intestinal phase, the 1% PHC emulsion contains six populations.

A first hypothesis on the link between lipid digestion and β -carotene bioaccessibility is that the same amount of micelles is formed for all emulsions, thus independent on the phosphatidylcholine concentration. As a result, the lipid incorporation reached a maximum. On the other hand, the maximal incorporation of β -carotene in the micelles is not reached yet or is dependent on the phosphatidylcholine concentration present in the micelles. As phosphatidylcholine might solubilise β -carotene, a higher amount of this emulsifier in the micelles, leads to a higher level of β -carotene incorporation in the micelles whereas the incorporation of lipids is not affected. Another hypothesis is that more micelles are formed in the emulsions with more phosphatidylcholine and that some micelles do not contain MAGs or FFAs so that phosphatidylcholine is used as surfactant in those micelles. The extra micelles formed with only phosphatidylcholine can incorporate β -carotene as well, which can explain the higher β -carotene bioaccessibility in emulsions with a higher phosphatidylcholine concentration (Fig. 6). Finally, because increasing the bile acids concentration did not lead to an increase of lipid incorporation (results not shown), it seems that the maximum lipid incorporation into micelles is due to the properties of the micelles, such as the pH, micellar electric charge (zeta-potential) or surface active compounds. These factors will change by incorporating compounds thereby affecting the incorporation of other compounds in the micelles (McClements, Decker, & Park, 2008; Goncalves et al., 2013).

4 Conclusion

This research investigated if it is possible to promote the β -carotene bioaccessibility in food systems containing the same amount of lipids by influencing the lipid digestion and if there is a clear link between lipid digestion and β -carotene bioaccessibility. The results show that a higher phosphatidylcholine concentration in emulsions leads to a higher β -carotene bioaccessibility whereas the incorporation of lipids into micelles did not increase compared with emulsions with a lower

amount of phosphatidylcholine. It has to be noted however that maximal lipid incorporation into micelles seems to be reached so that this can be the reason that no clear link can be found between the lipid digestion and the β -carotene bioaccessibility. The next step in this research can be to study emulsions where the maximum lipid incorporation into micelles is not reached and to investigate if in those emulsions a link between β -carotene bioaccessibility and lipid digestion exists.

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Fig. 1: Oil droplet size distribution of 1% PHC emulsion ($\diamond$), 2% PHC emulsion ($\blacksquare$), 3% PHC emulsion ($\blacktriangle$) and 4% PHC emulsion ($\times$).

Fig. 2: Representative microscopic images of oil droplets in (a) a 1% PHC emulsion and (b) a 4% PHC emulsion.

(a)

Fig. 3: Particle size distributions of the 1% PHC emulsion (A) and the 4% PHC emulsion (B) before digestion (◆), after stomach phase (□) and after small intestinal phase (SIP 1.0 h: △, SIP 1.5 h: ● and SIP 2.0 h: ×).

Fig. 4: Representative microscopic images of oil droplets in (a) a 1% PHC emulsion and (b) a 4% PHC emulsion after the stomach phase.

Fig. 5: Percentage $\frac{L_M}{L_{SIP}}$ -values (%) of the different emulsions after 1.0 h SIP (■), 1.5 h SIP (■) and 2.0 h SIP (■), with L_M = the amount of lipids in the micelles per g emulsion and L_{SIP} = the amount of lipids after the small intestinal phase (SIP) per g emulsion. Significant differences (Tukey test, $P < 0.05$) are indicated with letters.

Fig. 6: Percentage *in vitro* β -carotene bioaccessibility (%) for the different PHC emulsions (calculated as the absolute β -carotene bioaccessibility (B) divided by the initial amount of β -carotene (C) in the emulsion) (mean $\pm$ standard deviation) measured after 1.0 h SIP (■), 1.5 h SIP (■) and 2.0 h SIP (■). Significant differences (Tukey test, $P < 0.05$) are indicated with different letters (α , β , γ).