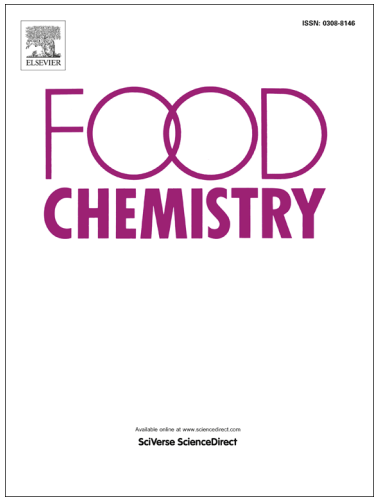

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Lipid nanoparticles with fats or oils containing β-carotene: storage stability and *in vitro* digestibility kinetics

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Abstract

The aim of this work was to study the formation of lipid nanoparticles (LNPs) with low (corn and olive oil) or high temperature melting lipids (cocoa butter and hydrogenated coconut oil). Moreover, their β-carotene stability and *in vitro* digestibility kinetics was evaluated. Submicron LNPs (d_{43} ≈ 570 -780 nm) were stabilized with Tween 80 at a surfactant-to-oil ratio (SOR) of 0.1. It was reduced below 200 nm at an SOR of 1. The β-carotene retention at 25 °C was not related to the lipid type but rather to the particle size, being lower in samples with smaller particle sizes. Cocoa butter LNPs presented an equally complete digestion as corn oil LNPs and a high β-carotene bioaccessibility, which was related to the high degree of micellarization of monoacylglycerols. This work evidences the potential of LNPs to protect lipophilic bioactive compounds with a high digestibility and bioaccessibility.

Keywords: Lipid; digestion; β-carotene; solid lipid nanoparticles; bioaccessibility

1 Introduction

The digestion and absorption of dietary lipids in the human gastrointestinal tract is being intensively studied in recent years due to its numerous implications on the human nutrition. Lipid digestion and its kinetics are related to the (*i*) satiation and subsequent energy regulation; (*ii*) absorption of essential fatty acids and (*iii*) uptake of lipophilic bioactive compounds (Reis, Holmberg, Watzke, Leser, & Miller, 2009). Lipolysis occurs mainly in the small intestine by pancreatic lipases, where triacylglycerols (TAG) are hydrolyzed and the subsequent release of lipid digestion products, being monoacylglycerols (MAG) and free fatty acids (FFA), takes place (Mu & Høy, 2004). Consequently, lipid digestion products together with bile salts are incorporated in mixed micelles, which are dispersed in the aqueous intestinal juices and are able to penetrate the small intestine epithelium cells. Moreover, mixed micelles may incorporate lipophilic bioactive compounds in their inner core, thus enabling their bioaccessibility (Marze, 2015). It has been recently observed that the kinetics of the lipolysis reaction, subsequent incorporation of lipid digestion products into mixed micelles and the further micellarization of lipophilic bioactive compounds are interrelated stepwise reactions that are determined by the characteristics of the emulsified systems. For instance, the smaller the emulsion droplet size, the higher the interfacial area and this may result in a faster lipid hydrolysis (Salvia-Trujillo et al., 2017). Moreover, the colloidal stability of emulsions as affected by the emulsifier type used is also of crucial importance to determine their lipolysis kinetics (Mun, Decker, & McClements, 2007; Verkempinck, Salvia-Trujillo, Moens, Charleer, et al., 2018). Nevertheless, also the lipid state, whether liquid (oil) or solid (fat), may have an impact on lipid digestion (Golding et al., 2011). Therefore the control of the lipid emulsion physicochemical characteristics arises as a potential strategy for its targeted digestion dynamics.

Solid Lipid Nanoparticles (SLNPs) are colloidal systems of solid fat dispersed in an aqueous phase that might be suitable for the encapsulation, protection and delivery of lipophilic bioactive compounds (Muller, Mader, & Gohla, 2000). Typically, SLNPs are produced by homogenization at elevated temperature, i.e. heating a solid fat above its melting temperature with lipophilic bioactive compounds solubilized therein, and posterior homogenization with an aqueous surfactant solution. Afterwards, this hot nanoemulsion is cooled down in order to induce the recrystallization of the lipid droplets (Weiss et al., 2008). The solid lipid state may reduce diffusion processes between the aqueous phase and the lipid core, thus better protecting the encapsulated lipophilic bioactive compounds (Amir Malaki Nik, Langmaid, & Wright, 2012). Most of the studies conducted on SLNPs for the protection of lipophilic bioactive compounds are conducted with saturated monoacid triacylglycerols, such as tripalmitin or tristearin. However, they render highly compacted crystalline conformations with reduced space to accommodate bioactive compounds in the lipid core, thus causing its expulsion and resulting in a faster degradation (Pan, Tikekar, & Nitin, 2016). Moreover, they have very high melting temperatures, typically above 60 °C, thus they remain in solid state during their passage through intestinal conditions. This may lead to a slow lipid digestion due to a limited access of lipases to their substrate (Bonnaire et al., 2008; A M Nik, Langmaid, & Wright, 2012), and in turn to a longer retention of lipophilic bioactive compounds in the undigested lipid core, which may result in a low bioaccessibility. Therefore, the formulation of SLNPs with commercial high melting temperature lipids, such as hydrogenated coconut oil or cocoa butter, may be of interest for a more relevant scale-up to industrial level. Vegetable solid fats consist of a mixture of several triacylglycerols and they present melting points around or just below the human body temperature (≈ 37 °C). This may allow, on one hand, to fabricate SLNPs with a less compact crystalline structure, thus forming

fat crystals large enough to accommodate lipophilic bioactive compounds in the lipid inner core, resulting in a high storage stability. And on the other hand, they may be present in liquid state after their ingestion, which may imply a fast digestion and leading to a potentially high biaccessibility of lipophilic bioactive compounds.

Hence, the aim of the present work was to study and compare the physicochemical characteristics of lipid nanoparticles (LNPs) formulated with liquid oils (corn or olive oil) or with high melting temperature lipids (cocoa butter and hydrogenated coconut oil) by cold or hot homogenization respectively, obtained at varying surfactant-to-oil ratios (SOR). Afterwards, the protection of β-carotene encapsulated within LNPs formulated with different lipids during storage (at 25 °C) was evaluated. Finally, their behavior during *in vitro* digestion conditions was studied by conducting a kinetic study in the intestinal phase in order to monitor the lipolysis dynamics in representative systems of LNPs. Moreover, the kinetics of micelle formation and subsequent β-carotene micellarization was quantified.

2 Material and methods

2.1 Materials

Corn and olive oil were bought from a local supermarket and stored at room temperature in dark conditions until use. Cocoa butter was kindly donated by Barry Callebaut AG (Zurich, Switzerland), while fully hydrogenated coconut oil was donated by Vandemoortele (Gent, Belgium) and were kept at 4˚C in the dark until use. In the case of lipids with high melting temperature, the solid fat content (SFC) profiles in percentage of lipids in solid state (%) at different temperatures determined by nuclear magnetic resonance (NMR) is provided (supplementary material, Figure 1). The results for hydrogenated coconut oil were provided by the supplier while the results for cocoa butter were the ones reported by Geary & Hartel

(2017). Synthetic β-carotene was obtained from Sigma Aldrich (purity > 97.0 %). All other chemicals and reagents were obtained from Sigma Aldrich, except for NaCl, HCl, urea, anhydrous sodium sulphate and ethanol (from VWR); $CaCl_2.2H_20$, 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 LNPs formation

The LNPs formation experiments were conducted with non-enriched lipid fractions. For the production of LNPs with liquid oils, a conventional cold homogenization procedure was followed while for LNPs with high melting lipids a hot homogenization technique was conducted. Liquid oils, were mixed with Milli-Q water and Tween 80 as non-ionic surfactant. The oil was kept at 5 % (*w/w*) and the surfactant concentration varied at different SOR, being 0.1, 0.25, 0.5, 0.75, 1 and 2, corresponding to final surfactant concentrations of 0.5, 1.25, 2.5, 3.75, 5 and 10 % (*w/w*). Then coarse emulsions were produced by blending the oil, surfactant and the water phase with a high shear mixer (Silverson L5M-A, Silverson Machines, Inc. Massachusetts, USA) at 4000 rpm for 5 min. Coarse emulsions were immediately processed with a high pressure homogenization (Stansted Fluid Power, Pressure cell homogenizer, U.K.) using 3 cycles at 100 MPa to obtain LNPs. LNPs with liquid oils were equilibrated at room temperature and analyzed.

For LNPs with high melting temperature lipids, the same procedure was followed yet using mild temperatures during homogenization to maintain the lipids in liquid form. Coconut oil and cocoa butter were melted at 55 °C and the Tween 80 and Milli-Q water were kept at

the same temperature. After high pressure homogenization, samples were cooled in an iced water bath and further stored at 4 °C for at least 3 hours to allow recrystallization, and subsequently equilibrated at room temperature to be further analyzed.

2.2.2 Lipid enrichment with β-carotene

The lipid phase of liquid oils and high melting temperature lipids was enriched with 0.05 % (*w/w*) of β-carotene. Lipids were firstly heated to 50 °C, then crystalline β-carotene powder was added and hereafter the mixture was stirred for 10 minutes in the absence of light and sonicated for 1 minute. The process of hot stirring and sonication was repeated until a complete dissolution of β-carotene crystals was observed. The β-carotene enriched lipid phases were kept at -80 °C until use. LNPs enriched with β-carotene were obtained using the method described in the previous section.

2.2.3 β-carotene stability studies

LNPs enriched with β-carotene and formulated at two different SOR (0.1 and 1) were prepared, and aliquots of 5-mL were placed in small glass tubes to ensure minimum head space. Glass tubes were flushed with nitrogen gas and stored in the dark at 25 °C for 48 days. Two individual tubes were taken each day for analysis of lipid droplet size and β-carotene concentration. The β-carotene concentration was determined as described in section 2.2.7. Results were expressed as percentage of β-carotene retention at each day of analysis with respect to the initial concentration of each sample immediately after their fabrication. The production of the LNPs with high melting temperature lipids by the hot homogenization procedure decreased the β-carotene concentration between 7 and 14 % respect the concentration in the LNPs with liquid oils produced by the conventional cold homogenization procedure.

2.2.4 *In vitro* digestion

A simulation of gastrointestinal conditions, consisting of a gastric and intestinal phase, using the international consensus method (Minekus et al., 2014) with some modifications (Salvia-Trujillo et al., 2017), was carried out. *In vitro* digestion experiments were conducted under subdued light conditions. After simulation of stomach and small intestine, LNPs were characterized in terms of particle size distribution and interfacial electrical charge. Moreover, during the small intestinal phase, the kinetics of lipid digestion was analyzed determining the decrease of TAG and subsequent release of MAG and FFA (diacylglycerols were below the detection limit). Additionally, the micellarization of such lipid digestion products, and the bioaccessibility measuring the β-carotene concentration incorporated in mixed micelles was determined.

To simulate the gastric phase, a 5 mL aliquot of LNPs was placed in a brown falcon tube and mixed with 5 mL Milli-Q water, 7.5 mL of Simulated Gastric Fluid (SGF), 5 μL of $CaCl_2$ (0.3M) and HCl (2M) to reach pH 3. Subsequently, 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 ºC for 2 h. For the small intestinal phase, 11 mL of Simulated Intestinal Fluid (SIF) was added to the chyme. The rest of the solutions were dissolved in SIF. Subsequently, 2.5 mL of bile solution (160 mM), 40 μL of $CaCl_2$ (0.3M), Milli-Q water and NaOH (1M) to bring the pH up to 7 were added. Finally, 5 mL of "pancreatic solution" was added, which consisted on a mixture of pancreatic lipase (lipase from porcine pancreas type II, batch #SLBN3801V, Sigma Aldrich), pancreatin (pancreatin from porcine pancreas, batch #SLBJ8147V, Sigma Aldrich), pyrogallol and alfa-tocopherol. 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 (Verkempinck, Salvia-Trujillo, Moens, Carrillo, et al., 2018). The final volume of the digest was 40 mL. The tubes were flushed with N_2 for 10 s and incubated in an end-over-end rotator at 37 ºC for 2 hours. During the small intestinal phase a kinetic study was conducted in order to determine the lipid digestion, micelle formation and β-carotene 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 moment of the pancreatin solution addition. To stop digestion reaction, the digests were transferred into glass tubes and heat-shocked at 85 ºC for 10 min after which they were placed in an iced-water bath. The β-carotene retention after the complete *in vitro* digestion procedure was calculated with regards the initial concentration in the respective LNPs, which was above 80 % in all the studied samples. For each time moment, the micelle fraction was separated by ultracentrifugation (165,000 g; 1 h and 5 min; 4 °C) (Optima XPN-80, Beckman Coulter, IN, USA). After the ultracentrifugation, the pellet at the bottom and undigested lipids at the top of the centrifuge tube were discarded and the middle clear aqueous phase was collected as the micellar fraction (Porter & Saunders, 1971).

2.2.5 Particle physicochemical characteristics

The physicochemical characteristics, being particle size, ζ-potential and microstructure of LNPs were determined immediately after production, during the storage stability study and during *in vitro* digestion.

Particle size: The particle size distribution was measured by static light scattering (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 is scattered by the particles. The particle size was calculated with the Mie Theory taking into account a refractive index of 1.470, 1.475, 1.460 and 1.450 for olive

oil, corn oil, coconut oil and cocoa butter respectively. The average particle size was reported as the volume-weighed 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 LNPs and after the gastric and intestinal phases, respectively.

Microstructure: The microstructure of the samples before and after the gastric and intestinal digestion conditions was studied with an optical microscope (Olympus BX-41) equipped with an Olympus XC-50 digital camera (Olympus, Opticel Co. Ltd., Tokyo, Japan). Initial samples prior to being submitted to the *in vitro* digestion procedure were observed at 25 °C, while chyme and digest samples were immediately analyzed after each digestive phase in order to observe the samples at physiologically relevant conditions.

2.2.6 Lipid analysis

Lipid digestion was evaluated by determining the kinetics of TAG hydrolysis and the subsequent release of MAG and FFA. The different lipid species of the digests as well as the corresponding micelle fractions obtained at the different digestion times were extracted. 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 ºC. 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 ºC until analysis.

The analysis and quantification of the lipid digestion products was carried out based on a method previously proposed (Graeve & Janssen, 2009). An HPLC system (Agilent Technologies 1200 Series, Diegem, Belgium) was equipped with a silica column (5µm x 150 x 4,6 mm ID, Sperisorb, Waters) and protected with a guard column in an oven (5310, VWR, Hitachi Ltd., Tokyo, Japan) at 40 ºC. 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 1290 Infinity, Agilent Technologies, Diegem, Belgium) was used to detect the different lipid species. TAG, MAG and FFA 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 MAG and FFA, the amount of glycerol (GLY) released at each time moment was calculated using the following stoichiometric reactions (Moquin & Temelli, 2008):

1 TAG + 2 H_2O→ 1 MAG+ 2 FFA

1 MAG + 1 H_2O → 1 GLY+ 1 FFA

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 β-carotene analysis and bio-accessibility

The β-carotene extraction and analysis was carried out based on a previously reported method (Colle, Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010) with minor modifications. Briefly, aliquots of 2, 5 or 10 mL of initial LNPs, digest or micelles 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 by RP-HPLC-DAD. The HPLC system (Agilent Technolgies 1200 Series, Diegem, Belgium) used was equipped with a C_{30} column (3 μm × 150 mm × 4.6 mm, YMC Europe, Dinslaken, Germany) coupled with a guard column at 25

°C. Elution was done using a linear gradient with starting conditions of 4% of reagent grade water (A), 81% methanol (B) and 15% methyl-tert-butyl-ether (C). Final conditions of the elution method were 4% A, 41% B and 55% C (run time: 17 min). For the quantification of β-carotene, HPLC-DAD peak responses were measured at 450 nm, and the peak area was compared with a calibration curve of purified standards (CaroteNature, Lupsingen, Switzerland).

β-carotene bioaccessibility (BA) was reported as the percentage fraction of β-carotene incorporated in the micelles versus the concentration in the initial systems (BA, %).

2.2.8 Statistical analysis and kinetic modeling

Statistical analysis and data modelling was carried out with JMP software (JMP 12, SAS Institute Inc.). Physicochemical characteristics (droplet size and ζ-potential) were determined in duplicate for each sample, and samples were fabricated in duplicate. 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 β-carotene BA kinetics during the small intestinal phase, were modelled using empirical models 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 modelled by a fractional conversion model (Eq. 4). Likewise, the time evolution of β-carotene BA was modelled 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 LNPs. The initial concentration of MAG or FFA in the digest or micelle fraction as well as the β-carotene BA at the start of the intestinal phase was assumed to be 0.

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

$$C = C_f(1 - e^{-kt})$$ 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 concentrations 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 all the modelled 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

3.1 LNPs formation and characterization

The influence of the surfactant amount (SOR) on the lipid particle size (Figure 1 A and C) and ζ-potential (Figure 1 B and D) was studied depending on the lipid state (liquid or solid). LNPs with corn or olive oil as liquid lipids (Figure 1 A and B) or LNPs with cocoa butter or fully hydrogenated coconut oil as high melting temperature lipids (Figure 1 C and D) were produced. A minimum amount of surfactant molecules is necessary to cover the interfacial area of the newly created lipid droplets during homogenization (C Qian & McClements, 2011). Thus, the SOR was increased from 0.1 up to 2 in order to evaluate the formation of particles in the submicron range. The particle size of LNPs significantly decreased by increasing the SOR used for all the lipid types and states (Figure 1 A and C). At a minimum SOR of 0.1, the formation of submicron nanoparticles was achieved, with particles ranging from 570 and 780 nm depending on the lipid type. In the case of LNPs with liquid oils, corn oil LNPs exhibited a more pronounced decrease of the lipid particle size with

increasing surfactant concentrations in comparison with olive oil. Corn oil LNPs showed a minimum average particle size of 120 nm at an SOR of 1, without a further reduction when the SOR was increased up to 2. On the contrary, olive oil LNPs presented a minimum average particle size of 280 nm when the SOR increased to 2. The differences between corn and olive in the production of particles in the nanometer range might be due to the fact that olive oil presents a higher viscosity than corn oil (Santos, Santos, & Souza, 2005). In fact, the dispersed phase viscosity is described to reduce the oil droplet breakup during homogenization (Qian & McClements, 2011). On the other hand, LNPs with high melting temperature lipids presented initially a particle size around 570 nm when using an SOR of 0.1, and decreased down to particles sizes around 150 nm when the SOR was increased up to 2.

The ζ-potential of the lipid nanoparticles depended on the type of the lipid. In general, all samples studied presented a negative interfacial charge despite the use of a nonionic surfactant. This could be attributed to a number of reasons. On the one hand, it could be due to the presence of anionic free fatty acid impurities from the oil or the surfactant. On the other hand, it might be caused by the orientation of OH^- ions from the aqueous phase in the vicinity of the oil droplets (Vácha et al., 2011) or the adsorption of HCO_3^- andCO_3^{2-} ions due to dissolved CO_2 from the atmosphere into the aqueous phase (Marinova et al., 1996). LNPs formulated with corn oil or olive oil presented a ζ-potential between -25 and -28 mV, without significant differences between them. Moreover, it was not affected by the increase in the surfactant concentration. LNPs with high melting temperature lipids presented slightly more negative ζ-potential in comparison with LNPs with liquid oils. In this regard, coconut oil LNPs presented a ζ-potential of -33 mV while for cocoa butter LNPs was -39 mV when formulated at an SOR of 0.1. Additionally, it was observed that the ζ-potential of LNPs with

high melting temperature lipids became less negative at increasing surfactant concentrations. In fact, other authors have reported the same behavior, given that surfactants might counteract the negative charge of the lipid particle surface (Schubert & Müller-Goymann, 2005).

3.2 β-carotene stability during storage

A comparison between the storage stability of β-carotene in nanoparticles formulated with a liquid lipid (corn oil) or high melting temperature lipids (fully hydrogenated coconut oil or cocoa butter) was conducted through a kinetic study at 25 °C for 48 days. Moreover, the additional effect of the lipid particle size on β-carotene stability was evaluated in LNPs with larger particles (SOR 0.1) or smaller particles (SOR 1) (Figure 2). The lipids selection for this set of experiments was based on those lipid types that rendered smaller particles sizes when increasing the SOR (section 3.1).

In general, the β-carotene stability during storage differed significantly between different lipid types. However, it was rather determined by the lipid particle size than by the state of the lipids used for the formulation of LNPs. Lipid particles produced at a low SOR (0.1) presented d_{43} values between 500-700 nm, while particles produced at a high SOR (1) had d_{43} values between 120-260 nm (Figure 1 AC). In the case of larger particles (Figure 2A), LNPs formulated with corn oil or with cocoa butter showed the highest β-carotene stability, retaining around 70% of the initial β-carotene after 48 days. On the contrary, coconut oil LNPs experienced a quick drop of the β-carotene concentration reaching to levels around 15% β-carotene retention at the end of the storage period. The differences between the two types of high melting temperature lipids might be attributed to a number of reasons. Firstly, cocoa butter is reported to have a 55 % Solid Fat Content (SFC) at 25 °C while hydrogenated coconut oil has a 35% SFC (supplementary material Figure 1), thus the latter can potentially

protect β-carotene through a diminished diffusion between the lipid core and the aqueous phase (Weiss et al., 2008). Secondly, coconut oil contains fatty acids with medium chain length, being mainly lauric acid (C12:0), while cocoa butter contains longer chain fatty acids, being mainly palmitic (C16:0) and stearic (C18:0). In this regard, it is reported that medium chain tryglycerides lead to the formation of more compact crystalline structures in comparison to long chain tryglycerides, which form more polymorphic crystalline structures (Rao, Sankar, Sambaiah, & Lokesh, 2001). Hence, coconut oil LNPs might present less available space for the bioactive in the lipid core in comparison to cocoa butter, thus leading to the expulsion of the β-carotene from the lipid core into the aqueous phase, promoting its degradation (Helgason et al., 2009). In fact, this effect has been consistently observed in most of the research articles studying the retention of β-carotene within solid LNPs (Amir Malaki Nik et al., 2012; Cheng Qian, Decker, Xiao, & McClements, 2013). Thirdly, it has been reported that cocoa butter naturally contains significant amounts of tocopherols (Lipp & Anklam, 1998), which have antioxidant activity thus possibly protecting β-carotene from degradation. Similarly, tocopherols are typically added in commercial oils to avoid oxidation during storage, and might have been responsible for the high β-carotene retention observed in corn oil LNPs at an SOR of 0.1.

For LNPs produced with a higher SOR (i.e. SOR of 1), as a result presenting a smaller particle size (section 3.1), a significant decrease in the β-carotene stability was observed (Figure 2B) in comparison with their respective lipid nanoparticles of larger size (Figure 2A). Specifically, corn oil LNPs presented a dramatic decrease in β-carotene stability after a reduction of their particle size, with a β-carotene retention of around 10 % at the end of the storage period in case of SOR 1. The same trend was observed in cocoa butter LNPs with smaller particle size (SOR 1), which presented a lower β-carotene retention in comparison

with their respective LNPs with a lower SOR (0.1). The higher β-carotene degradation observed in lipid nanoparticles of smaller particle size might be attributed to a number of reasons. First, the higher interfacial area created due to the reduction in particle size maximizes the area exposed to the aqueous phase and may cause the subsequent increase in the oxidation processes (Boon, McClements, Weiss, & Decker, 2010). Second, there is a particle-size dependency in the melting behavior of lipid nanoparticles. In fact, solid lipid nanoparticles with smaller particle size (150-200 nm) present lower melting temperature in comparison with their respective bulk lipids or with larger particles, which has been attributed to the ultrastructure of the triglyceride nanoparticles (Bunjes, 2011). This implies that LNPs from high melting temperature lipids of smaller particle size might contain higher amount of lipids in molten state in comparison with larger particles. And third, the higher surfactant used to produce LNPs with high melting temperature lipids of smaller particle size might have additionally decreased the compactness of the LNPs (Bunjes, Koch, & Westesen, 2003), thus contributing to the lower retention of β-carotene during storage. Nonetheless, given that the high melting temperature lipids used in the current study present relatively low melting temperatures (≈ 30 °C) compared to other solid fats typically used to produce solid LNPs, such as tripalmitin or tristearin, which have melting temperatures above 40 °C, probably only limited amounts of solid fats remained within the LNPs. Therefore, the differences observed between LNPs formulated with high melting temperature lipids and liquid oils might be due to their differences in composition and the presence of antioxidants.

3.3 Physicochemical changes during *in vitro* digestion

Corn oil and cocoa butter LNPs formulated at a lower SOR (0.1) exhibited the highest β-carotene retention during storage thus presenting a high potential as carriers of β-carotene for product formulation. Therefore, they were selected to study their behavior during *in vitro*

digestive conditions. The changes in particle size (Figure 5A), particle size distribution (Figure 3), microstructure (Figure 4) and ζ-potential (Figure 5B) of LNPs were evaluated in order to study their colloidal stability during digestion. The *in vitro* digestion experiments were conducted at 37 °C, temperature at which LNPs formulated with high melting temperature lipids might be partially or fully melted in liquid state, and the measurement of the physicochemical characteristics was conducted immediately after each digestive phase.

Initially, corn oil and cocoa butter LNPs presented a particle sizes of 890 nm and 480 nm respectively (Figure 5 A). Moreover, corn oil LNPs showed a wider particle size distribution (Figure 3A) as compared to cocoa butter LNPs, the latter presenting a bimodal particle size distribution of two narrow peaks around 300 nm and 1.3 µm. This suggests that corn oil LNPs presented a higher polydispersity in the particle size distribution, while cocoa butter LNPs were mostly smaller in size yet with the presence of a small amount of agglomerated particles. After simulated gastric conditions, both LNPs remained stable and presented similar respective initial particle sizes (Figure 3 B), particle size distribution (Figure 5B) and microstructure (Figure 4). This indicates that both systems were stable under low pH conditions of the gastric phase. The colloidal stability of emulsion-based systems during gastrointestinal conditions is of great importance when studying lipid digestion kinetics, since the particle size and therefore the surface area of lipid dispersions when they enter the small intestine phase may determine the digestion dynamics (Golding et al., 2011). In this regard, non-ionic small molecule surfactants, such as Tween 80, have been reported to provide a high stability in o/w emulsions during gastric conditions (Verkempinck, Salvia-Trujillo, Moens, Charleer, et al., 2018), thus rendering emulsions with an unchanged particle size at the beginning of the small intestinal phase. After simulated intestinal conditions, the particle size distribution of corn oil and cocoa butter LNPs changed dramatically depending on the type of

lipid. Corn oil LNPs presented a multimodal particle size distribution, with the presence of large particles above 10 µm, and smaller particles of intensity peaks around 200 nm (Figure 3C). The larger particles might be attributed to coalesced undigested oil droplets or to the formation of colloidal structures from lipid digestion products, while smaller particles might be due to the presence of mixed micelles. On the other hand, cocoa butter LNPs presented a bimodal particle size distribution, with a main intensity peak around 10 µm and a minor intensity peak around 100 nm. The main large peak might be attributed to the formation of crystalline structures from the released free fatty acids after digestion, since FFA from the digestion were not efficiently incorporated in the micelle fraction (section 3.5) and stearic acid, which is the main fatty acid from cocoa butter, is highly insoluble in water (Jarray, Gerbaud, & Hemati, 2016). In this regard, thin crystals were visualized with the microscopy analysis of the cocoa butter LNPs after small intestinal conditions (Figure 4), even at the temperature at which the microscopy analysis was conducted (37 °C).

The ζ-potential of initial corn oil and cocoa butter LNPs was negative, being -29 and -42 mV respectively (Figure 5B). It became less negative up to almost neutral values after simulated gastric conditions, without significant differences between LNPs. This is probably due to the low pH conditions during the gastric phase (pH 3), which may induce a lower ionization degree of the surface active molecules at the oil-water interface. Nonetheless, after simulated small intestine conditions, corn oil LNPs and cocoa butter LNPs presented a negative ζ-potential, with values of -33 and -28 mV respectively. The anionic nature of the digests was probably due to the presence of anionic FFA released after digestion.

3.4 Lipid digestion kinetics

The lipolysis of corn oil LNPs (Figure 6A) and cocoa butter LNPs (Figure 6D) during the simulated intestinal phase was evaluated in a kinetic study. TAG hydrolysis and the subsequent release of multiple lipid digestion products was monitored at several time moments along the intestinal phase. Experimental data were modelled with fractional conversion models and kinetic parameters were estimated (supplementary material, Table 2). This allowed a mechanistic comprehension of the lipase activity as affected by the lipid type, and to quantitatively describe the digestion reaction rate and extent.

In general, both emulsion-based systems presented a similar lipid digestion trend, with a gradual decrease in the TAG concentration, leading to a complete digestion at the end of the intestinal phase (120 min). Simultaneously, a gradual increase in the concentration of MAG, FFA and GLY was detected from the beginning of the intestinal phase, until reaching a plateau after approximately 60 min (Figure 6 A, D). However, slight differences could be detected in the lipolysis kinetics between corn oil and cocoa butter LNPs (supplementary material, Table 2). In this regard, the reaction rate constant (*k*-value) of TAG hydrolysis was slightly lower for cocoa butter LNPs (0.089 min^{-1}) than for corn oil LNPs (0.156 min^{-1}), as well as the FFA release which showed a higher rate constant value for corn oil LNPs (0.118 min^{-1}) in comparison with cocoa butter LNPs (0.066 min^{-1}). Moreover, differences could be detected in the release of MAG between the two LNPs. Similarly to the release of FFA, corn oil LNPs showed a higher rate constant for MAG release (0.567 min^{-1}) compared to cocoa butter LNPs (0.097 min^{-1}). Nevertheless, cocoa butter LNPs presented a significantly higher concentration of MAG at the end of the intestinal phase (C_f) in comparison with corn oil LNPs. It has been reported that lipid droplets in solid state might present a slower digestion than lipids in liquid form due to difficulties of lipase to adsorb at the oil/water interface

(Bonnaire et al., 2008; A M Nik et al., 2012). However, the lack of strong differences between lipolysis kinetics between both emulsified systems studied in the present work can be explained by that cocoa butter LNPs is probably present in liquid form at the temperature of the *in vitro* digestion experiment (37 °C), being completely melted at a temperature above 35 °C (supplementary material, Figure 1). Thus, the slight differences observed in lipolysis reaction might be rather due to the differences in TAG composition between corn oil and cocoa butter. In this sense, corn oil mainly contains linoleic acid (C18:2) being a long chain polyunsaturated fatty acid, while cocoa butter contains mainly stearic acid (C18:0) and palmitic acid (C16:0), which are saturated fatty acids. In fact, it has been reported that the digestibility of fatty acids is reduced in TAG containing saturated fatty acids in comparison with unsaturated fatty acids, which has been attributed to the formation of mineral soaps in the intestinal juices or due to insoluble acylglycerols (Livesey, 2000).

3.5 Micelle formation kinetics

Simultaneously with lipid digestion, the micelle formation kinetics were evaluated during the small intestinal phase by obtaining the micellar fraction from the digests of corn oil and cocoa butter LNPs at several time moments and determining the concentration of the micellarized MAG and FFA (Figure 6 B, E). Experimental data was modelled with a fractional conversion model and the kinetic parameters were estimated (supplementary material, Table 2). The micellarization of lipid digestion products (MAG and FFA) from the two types of LNPs followed the same trend as the one observed in the lipid digestion kinetics, presenting a gradual increase at early time moments until reaching a plateau phase. Nonetheless, the micellarization kinetics of lipid digestion product formation were significantly different between corn oil and cocoa butter LNPs. In this regard, the

micellarization of FFA from corn oil LNPs showed a higher rate constant and a higher final value when compared to cocoa butter LNPs. The FFA micellarization rate constant (*k*-value) and final concentration at the plateau phase (C_f) for corn oil LNPs was 0.135 min^{-1} and 24.5 mg/mL of emulsion, while for cocoa butter LNPs was 0.035 min^{-1} and 16.6 mg/mL of emulsion. Moreover, there was a large difference in the ratio of micellarized FFA to FFA present in the digest when comparing corn oil and cocoa butter LNPs. While for corn oil LNPs there was an average FFA micellarization ratio of 88.8 %, in cocoa butter LNPs it was 38.8 %. This evidences that FFA present in cocoa butter (mainly palmitic and stearic acids) were released during digestion but were not as efficiently incorporated into mixed micelles as FFA released from corn oil (mainly linoleic acid). This might be related to the fact that pure FFA have a higher melting temperature in comparison with their respective emulsified TAG, and therefore might present poor solubility in mixed micelles. In fact, it has been recently reported that the solubility of fatty acids in mixed micelles is determined by their chain length (Phan et al., 2015). In this sense, when the solubility limit of a certain fatty acid in mixed micelles is exceeded, crystallization might occur. In our case, the reported melting temperature for pure palmitic and stearic acid (present in cocoa butter) are 62.5 °C and 69.4 °C respectively, which are above the physiological temperature (Eckert, Dasgupta, Selge, & Ay, 2016), while for linoleic acid is -5.21 °C (Eckert, Dasgupta, Selge, & Ay, 2017). Overall, our results suggest that fatty acids from cocoa butter might present a lower solubility in mixed micelles in comparison with fatty acids from corn oil, which might be related to either their saturation degree or chain length.

However, the opposite behavior was observed for MAG micellarization kinetics. In this case, cocoa butter LNPs presented a higher final estimated concentration of MAG (C_f) in the micelle fraction than corn oil LNPs, being 10.1 and 7.5 mg/mL of emulsion respectively.

Despite this, both emulsified systems presented a similarly high MAG micellarization rate, which averaged between 90 and 100 %. This suggests that MAG molecules remained in a non-crystalline form after being released regardless their composition in fatty acids thus being efficiently incorporated into mixed micelles.

3.6 β-carotene bioaccessibility

The incorporation of β-carotene into mixed micelles was evaluated simultaneously with the kinetics of lipid digestion and micelle formation in order to establish the relationship between these processes. The β-carotene BA experimental values were modelled with a fractional conversion model (supplementary material, Table 3). β-carotene BA followed a gradual increase at early time moments of the intestinal phase, until it reached a plateau phase (Figure 6C, F). In general, both corn and cocoa butter LNPs presented a similar β-carotene BA trend, reaching to similar β-carotene BA values. Nonetheless, corn oil LNPs showed a slightly higher β-carotene micellarization rate constant (*k*-values) in comparison with cocoa butter LNPs, being 0.104 and 0.064 min^{-1}, respectively. In fact, we observed very similar reaction rate constants of the FFA release and FFA micellarization and β-carotene BA, both for corn oil or cocoa butter LNPs respectively (supplementary material Table 2 and 3). This suggests that β-carotene was immediately accommodated into micellar structures after their formation. On the other hand, the final BA at the end of the intestinal phase was somewhat higher for cocoa butter LNPs in comparison with corn oil LNPs. In this regard, final BA values at the plateau phase (C_f) were slightly higher for cocoa butter than corn oil LNPs, being 81.9 and 64. 3%, respectively (supplementary material, Table 3).

For the micellarization of lipophilic bioactive compounds, a number of steps are needed. First, lipid digestion must occur in order that β-carotene does not remain entrapped in

undigested lipid droplets (Verkempinck, Salvia-Trujillo, Moens, Charleer, et al., 2018). Secondly, lipid digestion products may be incorporated into mixed micelles in order to increase their solubilization capacity of lipophilic bioactive compounds. Actually, a strong relationship has been established between the lipid digestion products and the carotenoid concentration in the micelle fraction, which evidences the micelle formation (Mutsokoti et al., 2017; Salvia-Trujillo et al., 2017). However, in the present work, cocoa butter LNPs had a much lower FFA concentration in mixed micelles in comparison with corn oil LNPs, yet the concentration of MAG incorporated into mixed micelles was higher for cocoa butter LNPs than in corn oil LNPs. Therefore, the higher β-carotene BA observed in cocoa butter LNPs might be attributed either to the type of FFA present in mixed micelles resulting from cocoa butter LNPs digestion or to the type of lipid species. In this sense, cocoa butter mainly contains saturated fatty acids, which have been reported to render higher carotenoid BA values in comparison with mono- or poly-unsaturated fatty acids (Gleize et al., 2013). This might be related to the lower polarity of saturated fatty acids in comparison with unsaturated fatty acids, thus being able to solubilize in a higher extent lipophilic bioactive compounds. Nonetheless, also the higher amount of MAG present after digestion of cocoa butter LNPs compared to corn oil LNPs might have contributed to their higher β-carotene micellarization capacity. This evidences that the lipophilic compounds solubilization capacity of mixed micelles does not only depend on the concentration of lipid digestion products but also on the lipid species and their molecular structure.

4 Conclusions

This work demonstrates the possibility to form submicron lipid nanoparticles with edible oils as carriers of β-carotene with high retention during storage as well as lipid digestibility. In contrast with the initial hypothesis, no direct relation could be established between the state of the lipids used to form lipid nanoparticles and the encapsulated β-carotene stability. We observed that the β-carotene stability was rather determined by the lipid type and its lipid particle size. LNPs formulated with corn oil or cocoa butter with a SOR of 0.1, with particle sizes of 700 and 570 nm respectively, presented the highest β-carotene retention. A decrease in the lipid nanoparticles particle size below 200 nm caused a significant decrease in the β-carotene stability, being attributed to an increase in the surface area. *In vitro* digestion studies revealed that lipid particles formulated with high melting temperature lipids (cocoa butter, molten during digestion) were fully digestible under intestinal conditions, being similar to the digestion kinetics of lipid particles formulated with a liquid oil (corn oil). Nonetheless, cocoa butter LNPs showed a lower degree of micellarization of FFA in comparison with corn oil LNPs. Both emulsion-based systems, with liquid or solid lipids, presented high β-carotene BA, which could be linked with a high micellarization of monoacylglycerols regardless of their composition. Overall, this study shows the potential to selectively design lipid nanoparticles with controlled physiochemical characteristics for the encapsulation of lipophilic bioactive compounds with high digestibility and bioaccessibility.

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Figure 1

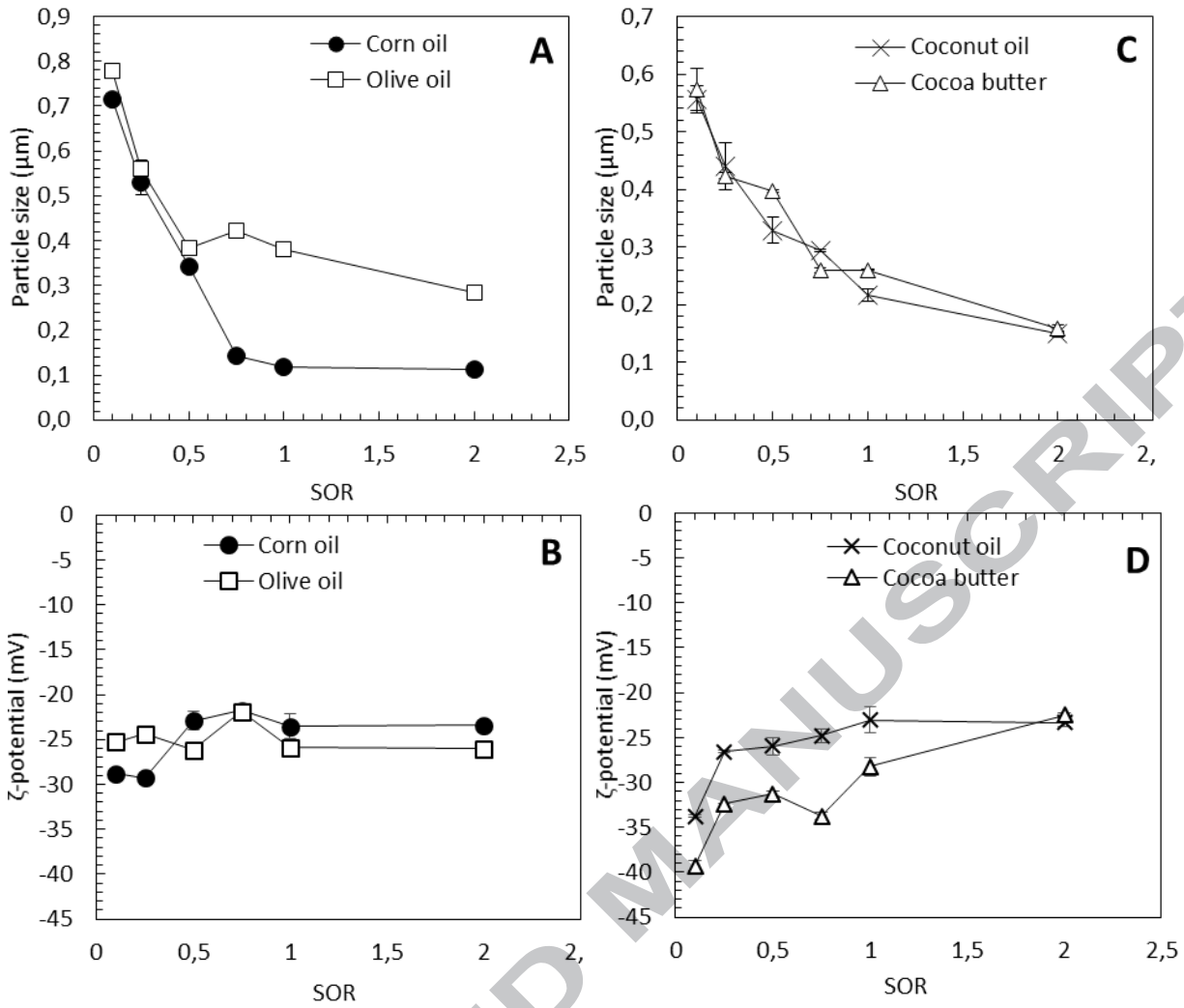

Figure 1. Particle size (d_{43}) (A, C) and ζ-zeta potential (B, D) of lipid nanoparticles with liquid oils (corn oil or olive oil) or lipid nanoparticles with high melting temperature lipids (coconut oil or cocoa butter) as affected by the surfactant-to-oil ratio (SOR). Symbols are connected with lines for visual guidance and do not represent any modelling of experimental data.

Figure 2

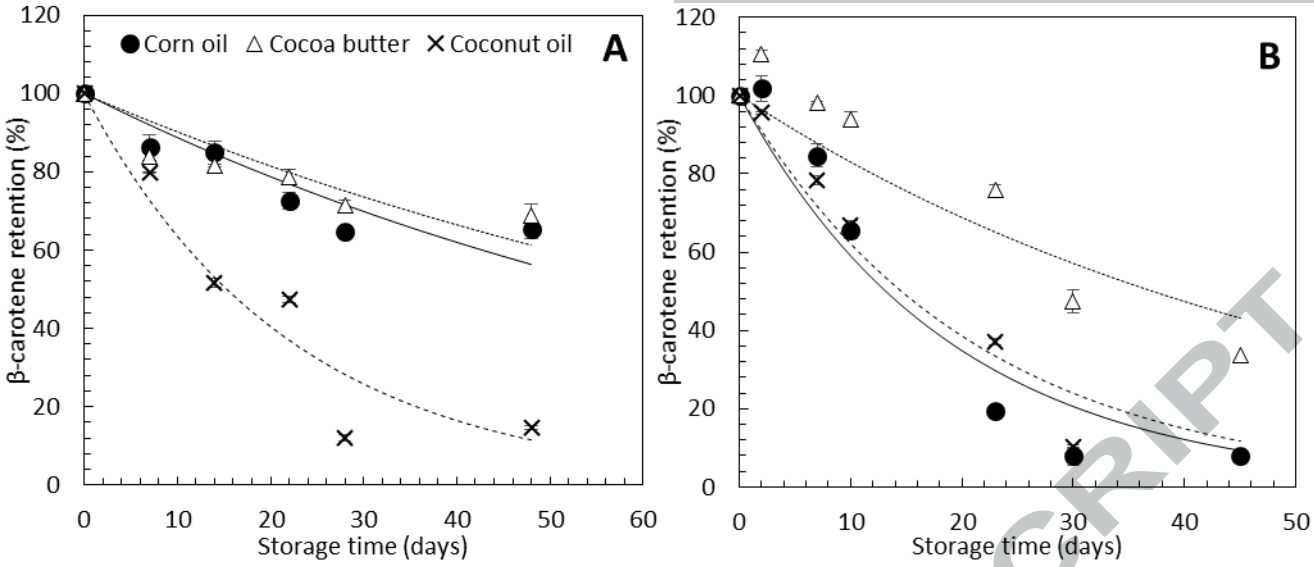

Figure 2. β-carotene retention (%) in lipid nanoparticles of corn oil or lipid nanoparticles of cocoa butter or coconut oil fabricated with a surfactant-to-oil ratio of 0.1 (A) or 1 (B) during storage time (days) at 25 °C. The symbols represent the experiment values, while the lines represent the predicted values by an exponential model of the corn oil (solid line), cocoa butter (dotted line) or coconut oil (dashed line).

Figure 3

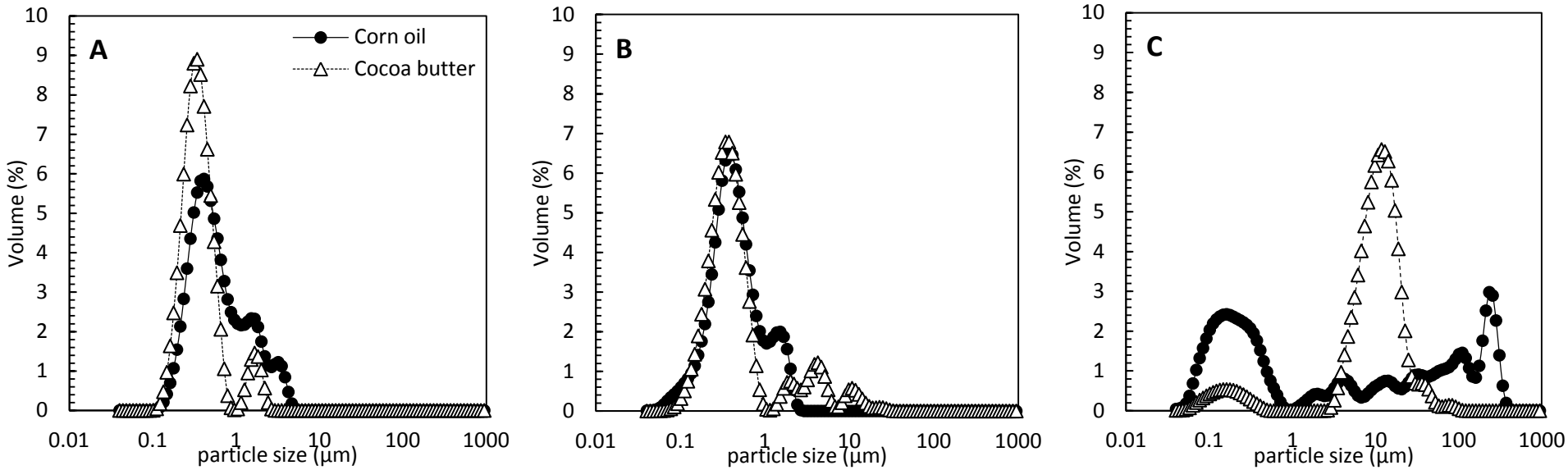

Figure 3 Particle size distribution of corn oil lipid nanoparticles and cocoa butter lipid nanoparticles (A) initially, (B) after the gastric phase and (C) after the intestinal phase

Figure 4

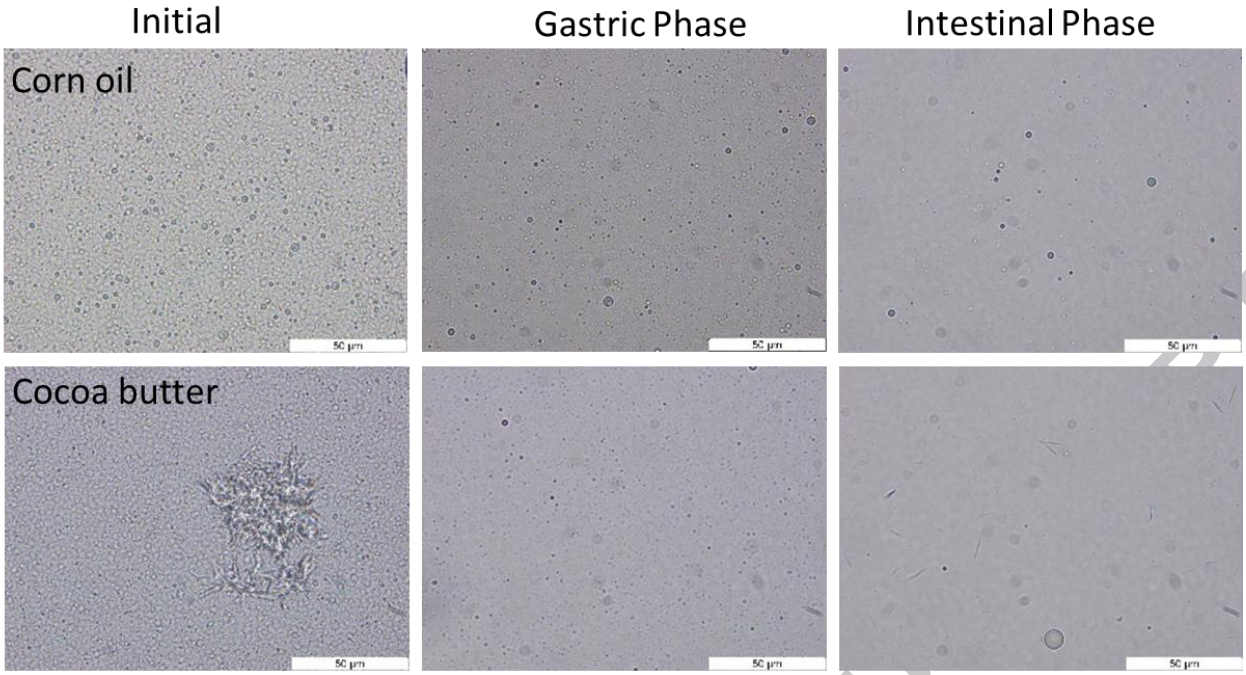

Figure 4. Microscopy images of corn oil lipid nanoparticles and cocoa butter lipid nanoparticles during *in vitro* digestion. Scale bar is 50 µm.

Figure 5

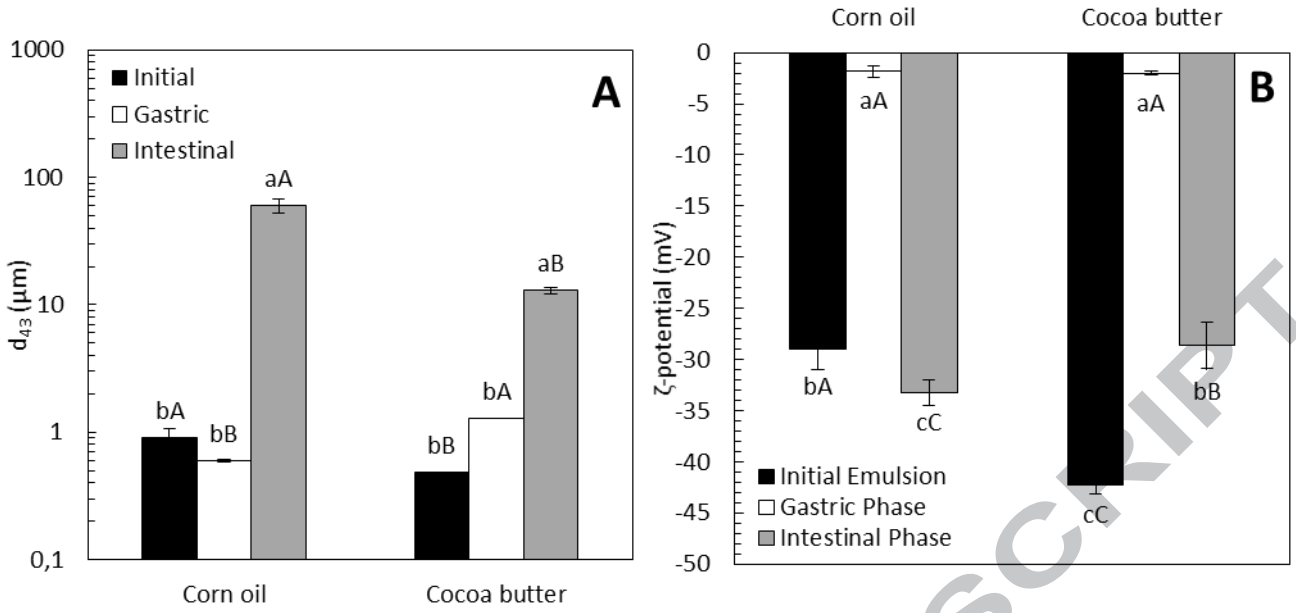

Figure 5. Average (A) particle size (d_{43}) and (B) ζ-potential (B) of corn oil lipid nanoparticles and cocoa butter lipid nanoparticles during simulated gastrointestinal conditions. Different lower case letters mean significant differences between different gastrointestinal phases of the same sample. Different upper case letters mean significant differences between samples in the same gastrointestinal phase.

Figure 6

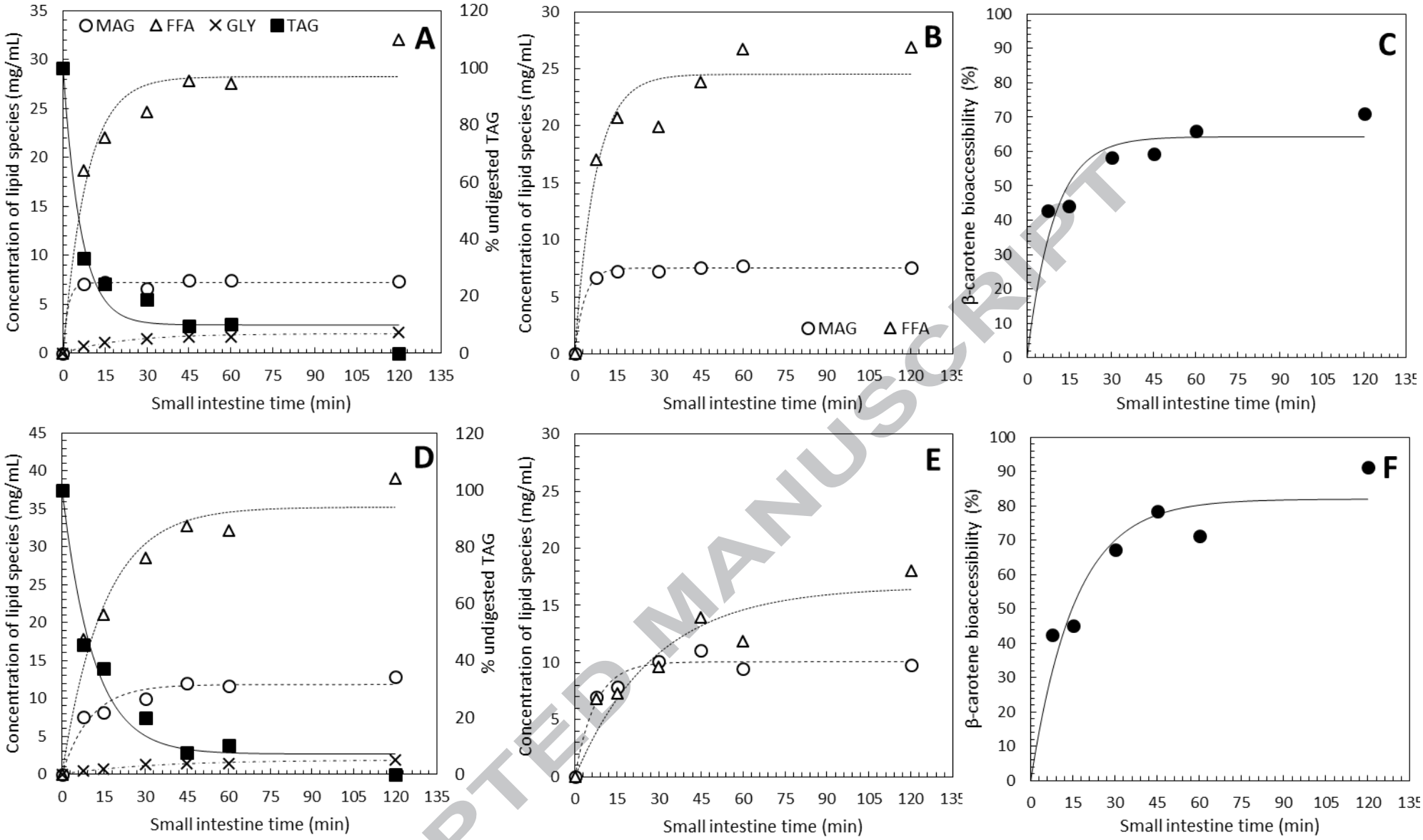

Figure 6. Lipid digestion represented as TAG hydrolysis (% of undigested TAG) and release of MAG, FFA and GLY (mg/mL) (A, D), micelle formation (mg/mL) (B, E) and β-carotene bioaccessibility (%) kinetics during small intestine time (C, F) in corn oil lipid nanoparticles (A, B, C) and cocoa butter lipid nanoparticles (D, E, F). TAG: triacylglycerols; MAG: monoacylglycerols; FFA: free fatty acids; and GLY: glycerol.

Highlights

- Lipid nanoparticles (LNPs) were used as carriers of β-carotene
- β-carotene storage stability at 25 ℃ was affected by oil droplet size
- Cocoa butter lipid nanoparticles were fully digested during *in vitro* conditions
- Corn oil and cocoa butter LNPs presented equally high bioaccessibility