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Physico-chemical stability and *in vitro* digestibility of beta-carotene-loaded lipid nanoparticles of cupuacu butter (*Theobroma grandiflorum*) produced by the phase inversion temperature (PIT) method

Graziela V.L. Gomes [a], Mirella R. Sola [a], Luís F.P. Marostegan [a], Camila G. Jange [a], Camila P.S. Cazado [a], Ana C. Pinheiro [b], António A. Vicente [b], Samantha C. Pinho [a, *]

[a] Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo (USP), Pirassununga, SP, Brazil
[b] CEB, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal

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ABSTRACT

Beta-carotene is a carotenoid with a wide spectrum of biological activities (e.g., anti-cancer, anti-hypertensive, and anti-inflammatory). However, because of its extremely high hydrophobicity, it is difficult to incorporate in food formulations and its bioavailability is fairly low. Lipid-based encapsulation colloidal systems such as lipid nanoparticles can help overcome these issues. In this study, beta-carotene-loaded lipid nanoparticles were produced by the phase inversion temperature (PIT) method from 10% cupuacu butter and 20% surfactant (Cremophor RH40 and Span 80). The inversion temperature of the nanoparticles was 74 °C and their average diameter was 35 nm. After 100 days of storage, 85% of the initial amount of beta-carotene remained in the nanoparticles; alpha-tocopherol was found to be essential for carotenoid preservation. Comparison of the results of *in vitro* digestion between static and dynamic systems was performed, and the characteristics of each digestion system led to diverse results in terms of average particle size and beta-carotene bioaccessibility. Although the static system was much simpler than the dynamic system, it could not provide reliable data of the digestibility of the lipid nanoparticles. The bioaccessibility of beta-carotene in the static system was 92%, very similar to the results found in the literature; by comparison, the dynamic system revealed a beta-carotene bioaccessibility of nearly 20%. Despite this discrepancy, the highly realistic conditions of digestion simulated by the dynamic *in vitro* system indicate that the results of this system are more reliable than those obtained from the simplified static system applied in this research.

1. Introduction

Incorporation of antioxidant bioactives into food products has developed into a major field in the food industry. Carotenoids are among the most promising antioxidant bioactives that can be incorporated into food; however, these compounds are prone to degradation during storage and processing (Dias et al., 2015). They are also lipophilic, which reduces their bioaccessibility (Porter et al., 2007) and restricts their incorporation into water-based matrices. These drawbacks may be overcome by designing lipid-based water-dispersible microencapsulation systems. Nanoemulsions, which present high physico-chemical stability during storage and a translucent or even transparent appearance because of their very small emulsion droplets (smaller than 100 nm) (Izquierdo et al., 2004), are an example of such systems. When nanoemulsions are produced with lipids that are in the solid state at room temperature, they are called solid lipid nanoparticles (Müller et al., 2000). By controlling the physical state of the lipid matrix, control of the mobility of bioactives within these structures may be achieved (Mehnert and Mäder, 2001) and minimization or prevention of some problems such as bioactive expulsion, low bioactive loading, and physical instability (Müller et al., 2002; Wissing et al., 2004) may be possible. The use of lipid mixtures to form nanoparticle cores allows the formation of imperfect crystals, amorphous lipid cores and can minimize or avoid expulsion of the bioactive to the external regions of the particle, thereby protecting them from the action of oxidant agents (Weiss et al., 2008; Tikekar et al., 2011). Vegetable butters, as cupuacu, are excellent raw materials for

* Corresponding author.
E-mail address: samantha@usp.br (S.C. Pinho).

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producing solid lipid nanoparticles because these butters are composed of mixtures of different triglycerides.

Solid lipid nanoparticles, similar to nanoemulsions, are thermodynamically unstable and require energy input for formation. This energy can be supplied by high-energy methods, or the chemical energy of components stored in the system (Solé et al., 2006). High-energy methods involve the use of specific devices, such as high-shear stirrers, high pressure homogenizers, microfluidizers, and ultrasound generators (McClements and Rao, 2011), to produce intense disruptive forces. A disadvantage of this approach is that the smaller the droplet size, the higher the surfactant and/or energy requirements of the system. By contrast, low-energy emulsification methods employ the intrinsic physicochemical properties of the system, as changes in the spontaneous curvature of the surfactant, to produce nanoemulsions (Fernandez et al., 2004; Solans and Solè, 2012). Furthermore, low-energy methods generally allow production of much smaller droplet sizes than those obtained through high-energy processes (Anton and Vandamme, 2009).

The phase inversion temperature (PIT) method is a low-energy method based on the particular ability of polyethoxylated (PEO) nonionic surfactants to become dehydrated with heating, thereby altering their hydrophilic–lipophilic balance. In the PIT method, the surfactant is hydrophilic at low temperatures but becomes lipophilic with increasing temperature because of dehydration of the polyoxyethylene chains. Then, at a specific temperature (also called the PIT temperature, T_{PIT}), the emulsion is inverted. If the system is quickly cooled, the surfactant becomes hydrophilic once more and very small droplets can be produced (Anton and Vandamme, 2009). This method can also be used to produce solid lipid nanoparticles (Montenegro et al., 2011a; Montenegro et al., 2011b).

To investigate the bioavailability of encapsulated bioactives in lipid nanoparticles, *in vitro* static and dynamic digestion models have been developed to investigate the physicochemical processes associated with digestion (Pinheiro et al., 2013). Dynamic *in vitro* models have attempted to simulate digestion in continuous mode with multiple compartments; the mechanical events (e.g., peristaltic movements) of digestion have also been studied (Reis et al., 2008). One of the dynamic *in vitro* digestion simulation systems is the TNO intestinal model, a multicompartment dynamic system controlled by computational systems that simulate the *in vivo* conditions and kinetic events in the human gastrointestinal tract. These events include pH changes, temperature, peristaltic movements, juices secretion, and absorption of digestion products by the intestinal mucosa (McCallister, 2010).

The present research work aimed to produce beta-carotene loaded solid lipid nanoparticles from cupuacu (*Theobroma grandiflorum*) butter via the PIT method. The physicochemical stability of the nanoparticles was evaluated over a storage period of 120 days and under different stress conditions (i.e., heat, ionic strength, and sucrose), and a comparison of their static and dynamic *in vitro* digestibilities was carried out.

2. Materials and methods

2.1. Materials

Nanoemulsions were produced using cupuacu butter (*T. grandiflorum*) (Jacy Fragrâncias, Santa Bárbara D'Oeste, SP, Brazil). Beta-carotene and alpha-tocopherol and span 80 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cremophor RH40 (40PEG hydroxylated castor oil) was obtained from BASF (Ludwigshafen, Germany). All of the reagents used in the *in vitro* digestibility experiments were purchased from Sigma-Aldrich (St Louis, MO, USA) and of reagent grade. Ultrapure water (from a Millipore system, Millipore, Billerica, MA, USA) was used throughout the experiments. All other chemicals used were reagent grade.

2.2. Production of the lipid nanoparticles by the PIT method

The nanoemulsions formulations contained, in 100 g of nanoemulsions: 80 g of deionized water, 12 g of Cremophor RH 40, 8 g of Span 80, and 10 g of cupuacu butter. A hot dispersion of the surfactants was dispersed in melted cupuacu butter through mechanical stirring at 500 rpm. The emulsion was then submitted to two heating and cooling cycles. Beta-carotene (0.6 g) and alpha-tocopherol (when present, 0.3 g) were added to the mixture in the second heating cycle. Curves of conductivity (obtained using a Inolab 740, WTW, Weilheim, Germany) versus temperature were plotted, and the T_{PIT} was determined from the average temperature between the onset of decrease in conductivity and the minimum temperature reached by the system after phase inversion. The system was heated to 80 °C (by placing the beckers with the mixture of surfactants and cupuacu butter in a thermostatized bath) and cooled to 20 °C (by placing the dispersions in jacketed vessels cooled by water at 2 °C). The cooling and heating rates were 10 and 6 °C/min, respectively.

2.3. Determination of average particle size, particle size distribution, and polydispersity

The average hydrodynamic diameter, particle size distribution and polydispersity (PDI) of the nanoparticles were determined using dynamic light scattering (quasi-elastic light scattering) ZetaPlus equipment (Brookhaven Instruments Company, Holtsville, NY, USA) at 25 °C. The samples were diluted with ultra-purified water to prevent multiple light scattering. Data analyses were performed using the software included with the system (90Plus/BiMAS).

2.4. Quantification of beta-carotene

The quantification of beta-carotene in the lipid nanoparticles was carried out according to Cornacchia and Roos (2011a). The sample of lipid nanoparticles was diluted with deionized water (100×). Two mililiters of this dilution was mixed with 1.5 mL of ethanol and 1 mL of methanol saturated with KOH. After vortexing, the mixture was heated to 45 °C for 30 min. Beta-carotene was extracted after washing the total volume of this vortexed mixture (4.5 mL) three times with 2 mL of n-hexane containing 0.1% (w/v) butylated hydroxytoluene. The organic solvent was added to the emulsion, after which the mixture was stirred and then left to stand for 10 min. The organic layer, which contained beta-carotene, was carefully removed and its absorbance was obtained spectrophotometrically (Libra 22S, Biochrom, Cambridge, UK) at 450 nm.

2.5. Quantification of alpha-tocopherol

Alpha-tocopherol was extracted from the lipid nanoparticles using the same protocol described for beta-carotene extraction (Section 2.3). However, after emulsion destabilization and phase demixing, the top layer, which contained alpha-tocopherol, was analyzed by spectrofluorometry (LS55, Perkin Elmer, Waltham, MA, USA) at excitation and emission wavelengths of 290 and 327 nm, respectively (Relkin and Shukat, 2012).