Evaluation of Nanostructured Lipid Carriers Produced with Interesterified Buriti Oil

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SUMMARY

Research background. Extracted from the pulp of an Amazonian fruit, buriti oil is rich in micronutrients with antioxidant properties and has high biological value. The few studies available indicate that this oil could be used in a wide range of applications; however, there are no studies that work on the improvement in the characteristics of this oil for commercial application. The enzymatic interesterification is one of the tools available to improve the properties of oils and fats and our recent studies demonstrated that the lipase could specifically act on buriti oil to produce structured lipids rich in oleic acid, while preserving most of minority compounds present in this oil. Still looking for ways to expand applicability of this raw oil, in this recent work, we are interested in studying how is the behavior of this structured oil in nanostructured lipid carriers (NLCs).

Experimental approach. Samples were stored at 4 and 25 °C for 30 days and their physicochemical properties were evaluated.

Results and conclusions. The results showed that the interesterification formed more unsaturated triacylglycerols (TAGs) and NLCs prepared with interesterified buriti oil presented small droplets than NLCs with original buriti oil. Particles remained stable throughout the storage period and NLCs exhibited complex polymorphism with the presence of three crystalline forms. The ORAC value was approximately 23 % higher in nanolipid carries with structured lipids in comparison with the nano lipid carriers with original buriti oil, and the FRAP value, 16 % higher, demonstrating the influence of interesterification on the antioxidant activity of nanocarriers. Thus, NLCs prepared with interesterified
buriti oil exhibit smaller droplets, high stability and antioxidant capacity and have potential for nutritional and biological applications.

**Novelty and scientific contribution.** This research showed that interesterification positively influenced the physicochemical properties of NLCs, producing oils rich in oleic acid, high stability and antioxidant capacity. Therefore, it may be interesting to use these nanocarriers to obtain efficient carrier systems for future applications.

**Key words:** buriti oil, enzymatic interesterification, structured lipids, nanocarrier, droplet size, antioxidant activity

**INTRODUCTION**

The Amazon region has suitable climatic conditions for a large number of underexploited native and exotic palm trees with interest to the agricultural industry, which can offer a future income source for local people. Buriti palm tree is an example that has high ecological, cultural and economic value, due mainly to its fruits that have valuable oil for the industries. It is estimated that the average annual production of pulp is approximately 0.79 t/ha and oil is 17.0 kg/ha (1). Extraction by cold pressing yields about 45 kg of buriti oil from 1000 kg of ripe fruits, which is considered as an Amazonian resource for cosmetic, food, polymeric and pharmaceutical purposes (2,3). It is commonly used by the local population as healing, sunscreen, for the treatment of burns, for the prevention of skin aging, and acts as anti-inflammatory and antibiotic (3). Buriti oil is rich in micronutrients with antioxidant properties and has high biological value. It contains a high concentration of monounsaturated fatty acids and the content is very similar to olive oil; oleic acid is the major component of buriti oil, followed by palmitic acid (4). In addition, the oil is rich in minor compounds, such as carotenoids, tocopherols, and sterols (5). Buriti oil is also a source of phenolic compounds (3), which are present in concentrations greater than those commonly found in other vegetable oils. The few studies available indicate that these oils are rich in bioactive compounds and can be used in a wide range of applications.

However, there are no studies that evaluate the improvement in the characteristics of these oils in order to expand their application. The enzymatic interesterification is one of the tools available to improve the properties of these oils and fats. Recent studies from our research group have shown that enzymatic interesterification can improve the biological characteristics of buriti oil expanding its potential for applications in the development of cosmetic, pharmaceutical, and food products with functional and medicinal effects. In the study by Speranza et al. (6), the Amazonian buriti oil and murumuru fat were subjected to enzymatic interesterification using two lipases in three different
enzyme systems. The three enzyme systems were able to catalyze the reaction, but the enzymes showed different specificities, producing interesterified lipids with different properties. Speranza et al. (7) evaluated the antimicrobial potential of emulsions formulated with interesterified Amazonian oils. The results suggested that the interesterification of these oils may be responsible for changes in the physicochemical characteristics of the emulsions, producing droplets with smaller size and greater antimicrobial activity.

In this recent work, we are interested in studying how is the behavior of this structured buriti oil in nanostructured lipid carriers. In recent years, several companies have developed foods and beverages with nutraceutical and functional properties, based on the use of special oils; stimulating the emergence of novelcarrier systems for lipids with active compounds. For instance, nanostructured lipid carriers (NLCs) have been used successfully applied in several areas to improve the solubility, bioavailability, and stability of active compounds. NLCs might be an appropriate alternative to deliver structured buriti oil on foods and cosmetics as a functional ingredient and as vehicles or delivery systems for other lipophilic compounds, such as drugs, nutraceuticals, flavorings, antioxidants, and antimicrobial agents.

NLCs are composed of a mixture of solid lipids and liquid lipids. The purpose of these formulations is to produce particles in which the oil phase is incorporated into the core of a solid lipid phase. NLCs have high carrying capacity and provide controlled release of the active compound (8). Thus, NLCs can enhance encapsulation efficiency, active compound binding, and physical stability and may be a valuable option to improve the chemical stability, bioavailability, and controlled release of lipophilic compounds in functional foods. NLCs are able to immobilize active compounds within the solid lipid matrix, protecting them from degradation (9) by acting as a physical barrier against aqueous phase components.

For all that, we aimed to study the viability of producing NLCs with the structured buriti oil, and the characteristics of these nanoparticles formed with this special oil for future application as delivery systems in food and pharmaceutical industries.

MATERIALS AND METHODS

Materials

Crude buriti oil was purchased from Beraca Sabará (São Paulo, SP, Brazil). Compritol 888 ATO (glyceryl behenate, a mixture of mono, di, and triacylglycerols from behenic acid) was bought from Brasquim (Porto Alegre, RS, Brazil). Immobilized *Thermomyces lanuginosus* lipase (Lipozyme TL-IM) was obtained from Novozymes Latin America Ltda (Araucária, PR, Brazil). All other reagents and solvents were of analytical grade.
Enzymatic interesterification

Enzymatic interesterification of buriti oil was performed in an orbital-shaking water bath, model TE-0532 (Tecnal, Piracicaba, SP, Brazil), for 6 h or 24 h at 150 rpm and 40°C using 10 g of buriti oil and 2.5 % (m/m) Lipozyme TL-IM (6). After the reaction was completed, the interesterified oil was immediately filtered through a 0.45 μm membrane. The filtrate was purified with 96% ethanol at 40°C to remove free fatty acids (FFA). A stream of pure nitrogen was passed through the reaction mixture to prevent fat oxidation and samples were stored in a freezer at −18°C.

Structured oil characterization

Fatty acid composition of buriti oil

The fatty acid composition of buriti oil was determined by gas chromatography (Shimadzu), model GCMS-QP2010S, coupled to an FID flame ionization detector according to AOCS method Ce 1-62 (10). Fatty acids methyl esters were separated according to Hartman and Lago's method (11) on Agilent DB - 23 capillary column (50 % cyanopropyl - methylpolysiloxane), dimensions 60 m, φ int: 0.25 mm, 0.25 μm film. The following oven temperature schedule was employed: 110 ° C-5min, 110 ° C-215 ° C (5 ° C/min), 215 ° C – 24 min; detector temperature: 280 ° C; 250 ° C injector temperature; column flow: 1.0 mL/min; linear velocity: 24 cm/s; entrainment gas: Helium; 1:50 split split; volume injected: 1.0 μL. The molar fatty acid composition was calculated using the reference standard for the mixture of methyl fatty acid esters (FAME) obtained from Sigma-Aldrich (Supelco 37 Component FAME mix, Darmstadt, Germany). Fatty acid methyl esters were quantified using relative peak areas. Analyses were carried out in duplicate, and the mean and standard deviation of each sample were calculated (12).

Lipid class analysis

Lipid class (triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), and free fatty acids (FFA)) analysis of buriti oil and structured buriti oil was performed by high-performance size exclusion chromatography (HPSEC). The samples were diluted to 1:100 in tetrahydrofuran and analyzed (20 μL) in a liquid chromatograph equipped with a Rheodyne 7725i injector and a Waters 510 pump (Waters Associates, Milford, Mass., USA) with two Ultrastyragel columns (100 and 500 Å) (Waters Associates, Milford, Mass., USA), each with 25 cm x 0.77 cm id, packed with a styrene–divinylbenzene copolymer (10 mm internal diameter), connected in series, and a refractive index detector (Hewlett Packard, CA, USA). Conditions: mobile phase: tetrahydrofuran (HPLC grade), flow: 1 mL/min, injection volume: 20.0 μL (13). The classes of
compounds were identified by comparing the elution times with triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG) and free fatty acids (FFA) standards and analysis was performed in duplicate.

TAG composition

The TAG composition analysis of buriti oil was analyzed by reversed-phase HPLC, following the method described by Holcapek et al. (14) and adapted by Carvalho et al. (15). The TAG profile in samples was determined based on the chromatographic peaks' profile and retention times in soybean oil, that was used as a reference sample (16). Raw material and the identification of these peaks was a tentative based on the expected elution order (considering that retention is increased, and on the samples FAS compositions, that were used as a reference. TAG composition of the samples was determined by internal normalization (10). Analysis was carried out in duplicate.

Determination of tocopherols

The levels of α, β, γ, and δ-tocopherols were determined according to AOCS method Ce 8-89 (10). Samples were diluted in hexane PA at a concentration of 0.1mg / mL and then injected in UHPLC Ultimate® 3000 liquid chromatography (Dionex, United States) with a Perkin Elmer Series 200a fluorescence detector (290 nm excitation and 330 nm emission). The microparticulate column of silica was 250 mm long, 4 mm internal diameter, with each particle measuring approximately 5 microns and the mobile phase was composed of hexane grade HPLC (99 %) and isopropanol (1 %).

The qualitative composition of tocopherols was determined by comparison of peak retention times with those of tocopherol standards (α-, β-, γ-, and δ-tocopherols), and the quantitative composition was determined by normalizing the area under the curve. Analysis was performed in triplicate, and results are expressed in milligrams of tocopherol per kilogram of oil. The conversion to α-tocopherol equivalent unit (α-TE) was obtained through coefficient 1 for α-tocoferal, 0.5 for β-tocopherol, 0.1 for γ-tocopherol and 0.03 for δ-tocopherol, according to Darnet et al. (17).

Determination of β-carotene

β-Carotene content was determined spectrophotometrically, according to the method of França et al. (18). An aliquot of 0.1 g of oil was diluted in 25 mL of a 7:3 vol./vol. solvent mixture of n-hexane and acetone P.A. (>99 %, Merck, Darmstadt, Germany) and absorbance was read at 453 nm using a UV-Vis Agilent (Agilent, Waldbronn, Germany) spectrophotometer. Carotenoids in the extracts were calculated in terms of β-carotene, using a standard absorbance curve calibrated with β-carotene (>99 %, Merck, Darmstadt, Germany). The standard curve was prepared with 0.1 g of β-carotene
diluted up to 100 mL with the 7:3 vol./vol. solvent mixture of n-hexane and acetone. Aliquots were taken from this solution and diluted to five different concentrations. Absorbance was read at 453 nm. Results are given as μg of β-carotene per g of buriti oil. The analysis was performed in triplicate.

Phenolic compound determination

Phenolic compounds were determined by the Folin–Ciocalteu method, according to Hrnčirik and Fritsche (19). This method is based on the reduction of phosphomolybdic and phosphotungstic acids by phenolic hydroxyls, which produces a blue color. Phenolic compounds were extracted from the oil with a solution of water/methanol 60:40 (by volume). Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to suitable aliquots of the extracts. After 3 min, a sodium carbonate solution (35 %) was added to the mixture, which was diluted with water to a final volume of 1000 μL. Absorbance was measured after 2 h at a wavelength of 725 nm on a Shimadzu spectrophotometer UV-1800 (Kyoto, Japan). The blank was composed of all the constituents of the reaction except that the phenolic solution was substituted by distilled water. A calibration curve was prepared using a standard solution of gallic acid (Sigma-Aldrich) at concentrations of 0.01–0.1 μg/mL. Analysis was performed in triplicate, and results are expressed as micrograms of gallic acid equivalents per gram of buriti oil.

NLC preparation

NLCs were prepared according to the method of Müller et al. (20) and Averina et al. (21). NLC aqueous dispersions containing 10 % (m/m) lipid phase were prepared by hot high-pressure homogenization. The lipid phase, composed of a 1:1 (m/m) mixture of solid lipids (Compritol 888 ATO) and buriti oil (non-interesterified, interesterified for 6 h, or interesterified for 24 h), was previously heated to 85°C. The aqueous phase, composed of 1.2 % Tween-80 emulsifier in deionized water, was also previously heated to 85°C. Then, the lipid phase was added to the aqueous phase under continuous stirring using an Ultra-Turrax T25 (IKA/Staufen, Germany), and the mixture was kept at 85°C and 7000 rpm for 5 min. The pre-emulsion was subsequently homogenized in a high-pressure homogenizer (GEA-Niro-Soavi, Parma, Italy) at 85°C and 8 x 10⁴ kPa for 3 cycles. The oil-in-water emulsion was cooled to room temperature to promote the recrystallization of the lipid phase, and thus NLCs were formed (22). NLCs prepared with non-interesterified buriti oil (NLCBO), NLCs prepared with buriti oil that was on interesterification reaction for 6 h (NLCBO6h), and NLCs prepared with buriti oil that was on interesterification reaction for 24 h (NLCBO24h) were stored at refrigeration temperature (4°C) and room (25°C) temperature.
NLC characterization

Droplet size measurement

The droplet size distribution of NLCs was measured using dynamic light scattering (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) (23). Results are presented as Z-average particle diameters. The refractive indexes of the dispersed and continuous phases used in these calculations were 1.46 and 1.33, respectively. Analyses were performed in triplicate with all samples: NLC_{BO}, NLC_{BO6h}, and NLC_{BO24h}; after 1, 15, and 30 days of storage at refrigeration temperature (4 °C) and room (25 °C) temperature.

Zeta potential measurement

Zeta potential (caused by electric charges around droplets) can provide information about interfacial properties. Zeta potential was determined using Zetasizer Nano-ZS equipment (Malvern Instruments, Malvern, UK) and samples were diluted (1:100) in distilled water (24). Analyses were performed in triplicate with NLC_{BO}, NLC_{BO6h}, and NLC_{BO24h} after 1, 15, and 30 days of storage at refrigeration temperature (4 °C) and room (25 °C) temperature.

Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) was carried out using a TCS SP5 II microscope (Leica Microsystems, Heidelberg, Germany). The oil phase of NLCs (100 μL) was dyed with 10 μL of Nile Red solution (1 mg Nile Red/mL ethanol). Samples were mounted on glass slides and examined under 10× and 63× magnification at an excitation wavelength of 543 nm and an emission wavelength of 605 nm. Autofluorescence was analyzed using the built-in software LAS lite (Zeiss Inc., Toronto, ON, Canada) to evaluate the morphology of fat crystals (25). Analyses were performed with NLC_{BO}, NLC_{BO6h}, and NLC_{BO24h} after 1, 15, and 30 days of storage at room (25 °C) temperature.

Polymorphism analysis

The polymorphic forms of fat crystals were investigated by X-ray diffraction, according to AOCS method Cj 2-95 (10). Analyses were performed using a PW 1710 Philips diffractometer (PANalytical, Almelo, The Netherlands) with Bragg–Brentano geometry (0:2θ) and Cu-Kα radiation (λ=1.54056 Å) at 40 kV and 30 mA. Step sizes of 0.02° (2θ scale) and an acquisition time of 2s were used in the scanning range of 5–40° (2θ scale). Polymorphic forms were identified by the characteristic short spacings of each crystal. Analyses were performed with NLC_{BO} and NLC_{BO24h} after 30 and 120 days of storage at room (25 °C) temperature.
Antioxidant activity (ORAC) assay

Oxygen radical absorbance capacity (ORAC) assay was performed in a 96-well plate (TPP Techno PlasticProducts AG, Trasadingen, Switzerland), using fluorescein (Sigma-Aldrich) as a fluorescent probe, according to the procedures of Dávalos et al. (26). The measurements were performed on a fluorometer (FLUOstar OPTIMA – BMG Labtech, Germany) with fluorescence filters at 485 nm excitation and 520 nm emission. The reaction, performed at 37 °C, was started by thermal decomposition of 2,2’ azobis(2-methylpropionamidine) (AAPH; Sigma-Aldrich, Steinheim, Germany) in a 75 mM-phosphate buffer (pH=7.4). ORAC values were calculated using the difference between the area under the fluorescein decay curve of the sample and the blank (net area under curve, AUC). Standard curves were constructed using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich) at concentrations between 30 and 1500 μmol/mL. Regression equations between net AUC and antioxidant concentration were calculated. Final values are expressed as micromol Trolox equivalents (TE) per milliliter of dry matter and analyses were performed in triplicate with NLC_{BO} and NLC_{BO24h}.

Ferric-reducing antioxidant power (FRAP) assay

FRAP assay was carried out according to Benzie and Strain (27). In the dark environment, aliquots of 30 μL of standard or blank samples were added to 90 μL of distilled water and 900 μL of the FRAP reagent (2.25 mL of 0.3 M acetate buffer (Riedel-de Haen, Germany) pH 3.6, 225 μL of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine, Fluka Chemicals, Switzerland) in 40 mM HCl (BDH), and 225 μL of 20 mM ferric chloride solution (BDH)). The mixture was vortexed and 200 μL aliquots were transferred to a clear 96-well microplate. Absorbance reading was performed on a fluorometer (FLUOstar OPTIMA – BMG Labtech, Germany) at 595 nm at 37 °C for 30 minutes of reaction. To achieve the standard curve, Trolox® solutions were prepared, at concentrations between 15 and 1500 μmol/mL. Results are expressed as micromol Trolox equivalents (TE) per milliliter of dry matter and analyses were performed in triplicate with NLC_{BO} and NLC_{BO24h}.

Statistical analysis

Results are presented as mean±standard deviation of replicates. Results were compared by analysis of variance (ANOVA) using Statistica version 7.0 (Statsoft Inc., USA) (28), and p<0.05 was considered to denote significant differences between results.
RESULTS AND DISCUSSION

Buriti oil characterization

Before the interesterification reaction, buriti oil was analyzed for fatty acid composition and the interesterification was evaluated through triacylglycerol composition and lipid classes. The complete characterization was published in our earlier report (12). Nevertheless, in this paper, we summarize the most relevant data for the present discussion.

Fatty acid composition analysis showed that the tested buriti oil is rich in oleic acid (C18:1) (74.2 %) and palmitic acid (C16:0) (19.8 %). The main types of triacylglycerols (TAG) in buriti oil were POO (38.9 %) and OOO (32.7 %). After interesterification, there was a reduction in the levels of POO (32.7 %) and an increase in OOO (36.8 %). These modifications affect the functionality of the oil and reduce the melting range, altering lubrication properties, mechanical performance, structure, and nutritional properties (29). Lipid class analysis showed that TAG structure was preserved after 24 h of interesterification. The 24 h interesterification reaction promoted a small increase (from 6.7 to 9.2 %) in diacylglycerols (DAG) concentration but no formation of monoacylglycerols (MAG) or free fatty acids (FFA).

Buriti oil contained four tocopherol isomers (α, β, γ, and δ) and the β isomer (83.62 ± 1.82 mg/kg) was predominant in this oil. After 24 h of interesterification, there was a significant loss (60.42 ± 0.92 mg/kg) of this isomer. In a study by Darnet et al. (30), the most abundant fraction was also β-tocopherol (57 mg/kg). The four tocopherol isomers were also detected in previous work performed by our research group (3), but at different levels. Differences in the concentration of tocopherol isomers may be caused by the refining conditions of the oil or by the soil and climate conditions under which buriti palm trees are cultivated. In contrast to the observed in relation to tocopherol content, carotenoids were not affected by interesterification; that is, β-carotene concentration in structured buriti oil did not differ from that of the crude oil.

Regarding total phenolics, buriti oil exhibited value (292.31 ± 6.81 µg/g) close to those found in other vegetable oils. After interesterification, total phenolic concentration increased (329.75 ± 10.15 µg/g), which was not an expected result. Speranza et al. (12) suggest that this result was a consequence of improved solubilization of structured lipids in the reaction medium. However, further investigations are needed at this point.

Droplet size

NLCs have been shown to be carriers with great ability to disperse bioactive compounds. The addition of solid lipids to emulsions improves stability and allows greater control of the release of bioactive molecules (21).
In this study, NLCs prepared with interesterified buriti oil presented smaller droplets, especially NLC\textsubscript{BO24h} sample, which had droplets that were 46% smaller than those of NLC\textsubscript{BO} (Table 1). This is a very interesting result, for the same process conditions, we obtained smaller nanocarriers with the structured oil. The TAG modifications from the biotransformation reaction may have influenced on the particles sizes. The interesterification formed more unsaturated TAG (OOO), we believe, exerting a positive effect on NLC droplet size, even after the addition of Compritol 888 ATO to the system. Nanocarriers prepared with non-interesterified oils have larger droplets and are less stable than those prepared with interesterified oils, as their components have different properties (such as viscosity and melting temperature). Interesterification can produce single-phase lipids that do not undergo phase separation, which results in more stable emulsions (31,32). Furthermore, interesterification can reduce the types of TAGs initially present in the starting oil, thereby forming a lipid phase with a narrower range of polymorphic forms, greater capacity for interaction with other components, and more organized packing (32).

Storage time and temperature did not influence droplet size or stability. The properties of lipid nanoparticles are typically affected by many factors, including type and concentration of lipids and surfactants and viscosity of the lipid phase. It has been reported that particle size increases as the viscosity of the lipid phase increases (33) and that NLCs have smaller droplets when oil is added to the system, as liquid fats reduce lipid phase viscosity and surface tension. In contrast, increased surfactant concentration decreases particle size (34), because surfactants facilitate the fragmentation of nanoparticles during homogenization, reducing surface tension at the interface between solid and liquid phases.

In a study by Walker, Decker & Mcclements (35), nanoemulsions were prepared using spontaneous emulsification with 10% total oil phase (50 wt % fish oil and 50 wt % lemon oil) and different surfactant/oil. These nanoparticles were maintained at 5, 20 and 37 °C for 14 days to assess their physical stability. The surfactant concentration also had a large impact on the average particle diameter of the emulsions: as the surfactant/oil ratio increased, the mean particle diameter decreased. In addition, the mean particle size did not depend strongly on storage time or temperature, as occurred in our study. The fact that there was no significant change in the average particle size of the nanoemulsions after being maintained at 5, 20 and 37 °C for 14 days suggests that they were stable against flocculation, coalescence and maturation of Ostwald.

Yang et al. (36) investigated the effect of different liquid carrier oils on the crystallization and aggregation behavior of tristearin NLC dispersions. The results demonstrated that NLC suspension stability was strongly affected by the type and amount of the carrier oil. Unsaturated fatty acids and short chain fatty acids result in lower melting points, affecting the stability of NLC dispersions. These
results were confirmed by using olive oil (melting point of −6 °C) and palm oil (melting point of 35 °C) for the production of nanoparticles. Olive oil is rich in oleic acid, as is buriti oil; this property allowed for a much more stable dispersion than that obtained with palm oil.

Zeta potential of droplets

Zeta potential is a parameter used to evaluate the stability and characteristics of particles. It indicates the electric charge on the surface of nanoparticles as well as the electrostatic repulsion between them. Table 2 shows the zeta potential of NLCs prepared with buriti oil and structured buriti oil after 1, 15, and 30 days of storage at 4 °C or 25 °C.

A minimum zeta-potential value of ±30 mV is required to prevent coalescence of droplets (37). Interesterification of buriti oil influenced the interfacial properties of droplets under the evaluated conditions. However, although NLCBO initially had higher zeta-potential values than NLCBO6h and NLCBO24h, differences among the zeta-potential values of NLCs became non-significant with storage time, indicating that interesterification helped maintain stability over time. The higher value of zeta potential, positive or negative, the lower the probability of the droplets coalescing and better their stability (38). Thus, the physical stability of the nanostructured lipid carriers was maintained over time.

All dispersions exhibited negative zeta-potential values, showing that repulsion forces predominated over attraction forces. This property is desirable in NLCs, as it prevents the formation of aggregates and is an indicator of particle stability. NLCs, solid lipid nanoparticles, and lipid emulsions also had a negative charge in a study by Fang et al. (39). The negative charge was caused by the anionic fractions of the lipophilic emulsifier (soybean phosphatidylcholine) and the fatty acid glycerides in the lipid core of Compritol and Precirol.

The stability of lipid nanoparticles is affected by the type and concentration of lipids. Niculae et al. (22) used different lipid matrices to prepare NLCs. Carnauba oil was added to the carrier system initially prepared with pomegranate seed oil only, and the zeta potential increased from −21.7 to −30.9 mV. The components of carnauba oil might have disrupted the surfactant shell, causing a rearrangement of the surface charge and a consequent change in zeta potential. In addition, the authors found that when oil concentration decreased, the physical stability of nanocarriers slightly increased. In a study by How, Rasedee and Abbasalipourkabir (40) three different formulations of NLC were prepared, containing olive oil/hydrogenated palm oil (HPO) proportions of 1:9, 2:8 and 3:7 and Polysorbate 80. These authors observed that increase in the oil to HPO proportion had caused increase in the zeta potential of the NLC, while increase in surfactant content decreased particle size.

Surfactants also play an important role in the stabilization of NLC lipid particles. Tween-80, the surfactant used in this study, is a non-ionic surfactant that provides steric repulsion, through which
the semi-solid particles of NLCs are stabilized. If the amount of surfactant is not sufficient, some lipid particles might be uncovered, which can lead to flocculation, aggregation, and gelling (41). In a study by Niculae et al. (42), lipid nanoparticles prepared with Tween-80 as the main surfactant had better physical stability than those prepared with Tween-20, showing the importance of surfactants for nanoparticle stability. Thus, interesterification of buriti oil might have allowed for a better interaction between emulsifier and oil, providing greater stability.

Morphology

As storage temperature did not affect particle stability, microscopic analysis was not performed with samples stored under refrigeration. The morphology of NLCs after 1, 15, and 30 days of storage at room temperature are depicted in Fig. 1.

The confocal microscopy images (Fig. 1) show individual droplets with a spherical shape. In general, NLCs had a relatively wide droplet size range and droplets were polydisperse, without uniformity. The distribution of NLCBO droplets was more homogeneous up to 15 days of storage, but droplets were more agglomerated after 30 days in comparison with those of other NLCs. NLCBO6h showed a similar behavior, but droplet aggregation was lower than that of NLCBO after 30 days of storage. In contrast, NLCBO24h had a more heterogeneous structure, exhibiting droplet aggregation throughout the storage period (43). Wegmüller et al. (44) reported the occurrence of particle agglomerates and fat crystals in the microcapsules developed in their study. Agglomeration might have occurred because microparticles were not completely solidified when cooled. The authors observed that small particles showed fewer fat crystals on the surface than larger ones, as small particles require less time to be totally solidified.

Different NLC morphologies are observed when using different microscopes, and these morphological discrepancies might be attributed to the type of lipid and surfactant or the method used to develop NLCs. Many studies suggest that incorporation of bioactive compounds into a solid lipid matrix can improve controlled release, charge, and physical and chemical stability by disrupting the crystal packing structure (20).

Polymorphism

TAGs occur in three crystalline forms, α, β’, and β, which differ in the arrangement of fatty acids. X-ray diffraction analysis was performed to investigate the arrangement of fatty acid chains and identify the polymorphism of crystals by determining the dimensions of the crystal unit and sub-cells. Polymorphs diffract X-rays at different angles as a result of their different geometric configurations,
and diffraction at wide angles correspond to short spacings (distances between parallel acyl groups in TAG) of sub-cells, which allows the characterization of different polymorphs in fats (45).

The short spacings and polymorphic forms of NLC_{BO} and NLC_{BO24h} are presented in Table 3. NLCs presented a complex polymorphism, as a result of β’ fusion and solid lipid recrystallization. This behavior can be explained by the nano-size of NLC particles, which cause slower transition to a stable state, and may also be related to the presence of DAG above 5 % in crude and interesterified buriti oil (12), which is known to favor the formation of β’ polymorph. In addition, interesterification reduced TAG melting range, forming more unsaturated fatty acids and causing changes to crystal morphology and polymorphic forms. TAGs usually crystallize first in α and β’ forms even though β form is more stable. Factors such as cooling rate, heat of crystallization, level of agitation, and formulation can affect the number and type of crystals. However, if fats are complex mixtures of TAGs, different polymorphic forms and liquid oil can coexist at a certain temperature (46).

Solid lipids are predominantly found in the stable β-modification. When incorporated into lipid nanoparticles, the lipid transforms into the less-ordered, metastable β’-modification, causing a distortion in the crystalline structure (47). Previous study demonstrated that the incorporation of lipids in NLCs leads to slower polymorphic transition and lower polydispersity index (48). In addition, NLC crystallization, melting temperatures, and polymorphic content have been shown to be strongly dependent on the amount of oil. Jenning et al. (49) prepared NLCs with glyceryl behenate (Compritol 888 ATO) as the solid lipid and caprylic/capric triglycerides (Miglyol 812) as the liquid lipid. NLC dispersions were most stable in the absence of oil and at high concentrations of oil. Other studies showed that the addition of oil to solid lipid nanoparticles did not disrupt the structure of lipid crystals, contrary to the expected (48,50). Given the contradictory results, the effect of oil type on NLC polymorphic behavior and morphology is still poorly understood (20).

Antioxidant activity

The ORAC assay measures the ability of an antioxidant to sequester free radicals through the donation of hydrogen atoms. Pulido et al. (51) described the FRAP method as an alternative to determine the ferric reducing capacity of biological fluids and aqueous solutions of pure compounds. These two assays are of great importance because they indicate the antioxidant action in foods and physiological systems.

The results of the ORAC and FRAP assays are presented in Table 4.

In both assays, antioxidant activity was highest in NLC_{BO24h}. The ORAC value was approximately 23 % higher in NLC_{BO24h} in comparison with NLC_{BO}, and the FRAP value, 16 % higher, demonstrating the influence of interesterification on the antioxidant activity of nanocarriers. Although
there was a significant loss of tocopherols after 24 h of interesterification, the concentration of β-carotene was not affected and the concentrations of OOO-TAG (12.5 %) and phenolic compounds increased, which could explain the increase in antioxidant activity (12). In addition, it can be considered that larger particles, such as NLC_{BO}, tend to generate imperfect, amorphous crystals and can expel bioactive compounds, which justifies a lower antioxidant capacity of these samples (52).

Haeiwa et al. (53) reported that unsaturated fatty acids, particularly oleic acid, increase the intracellular levels of lipid peroxidation products, indicating that oleic acid can promote adaptive response and protect cells against oxidative stress-related injury. Buriti oil contains considerable amounts of carotenoids, tocopherols, and phenolic compounds, which give it an important antioxidant power, favoring its own conservation and contributing to the treatment of oxidative stress-related diseases (30).

In a previous study carried out by our research group, the antioxidant capacity of the samples was also higher for the structured lipids produced with Lipozyme TL-IM and enzyme from Rhizopus sp., respectively, followed by pure buriti oil. This result indicates that enzymatic interesterification significantly increased the antioxidant capacity of the oil, independently of the content of minor compounds (54). In a study by Poyato et al. (55), the antioxidant capacity, measured by the L-ORAC (lipophilic radical absorption capacity of oxygen) assay, of linseed oil emulsions (8315.4 μmol Trolox equivalents/100 g oil) was higher than those of NLCs developed in the present study, whereas the antioxidant capacity of olive oil emulsions (1978.9 μmol Trolox equivalents/100 g oil) was similar to those of NLCs. These data suggest that the antioxidant capacity of emulsions differs according to oil type and antioxidant compound.

The antioxidant activity of bioactive compounds is associated with the inhibition of free-radical chain initiation by oxygen binding or chelation of catalytic metal ions to retard oxidation and peroxide decomposition, prevent continuous hydrogen abstraction, and protect DNA, proteins, and lipids against oxidative damage (3). The results of this study suggest that nanocarriers prepared with interesterified buriti oil can be used in several applications for the improvement of health, including cosmetic formulations, food products, and pharmaceuticals.

CONCLUSIONS

Interesterification reaction time significantly influenced particle size and interfacial properties on the process of NLC production. NLCs prepared with interesterified buriti oil formed more unsaturated TAGs and presented small droplets than NLC_{BO}. Although interesterification of buriti oil influenced the interfacial properties of droplets under the evaluated conditions, particles remained stable throughout the storage period.
Besides that, NLCs presented a complex polymorphism with the presence of three crystalline forms and NLCs containing structured buriti oil had higher antioxidant capacity by ORAC and FRAP assays than NLCs without structured lipids.

This research showed that interesterification positively influenced the physicochemical properties of NLCs, producing oils rich in oleic acid, high stability and antioxidant capacity. Therefore, it may be interesting to use these nanocarriers to obtain efficient carrier systems for future applications.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Table 1. Droplet size (nm) of nanostructured lipid carriers after 1, 15, and 30 days of storage at refrigeration (4 °C) and room (25 °C) temperatures.

<table>
<thead>
<tr>
<th>Temperature/°C</th>
<th>t/day</th>
<th>(d_\text{32}/\text{nm}) NLC\textsubscript{BO}</th>
<th>NLC\textsubscript{BO6h}</th>
<th>NLC\textsubscript{BO24h}</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1</td>
<td>(717±0.01)b</td>
<td>(524±0.01)c</td>
<td>(401±0.01)d</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>(729±0.01)\textsuperscript{ab}</td>
<td>(537±0.01)c</td>
<td>(393±0.01)d</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>(729±0.03)\textsuperscript{ab}</td>
<td>(538±0.01)c</td>
<td>(406±0.01)d</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>(748±0.01)a</td>
<td>(532±0.01)c</td>
<td>(393±0.01)d</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>(737±0.01)\textsuperscript{ab}</td>
<td>(530±0.01)c</td>
<td>(405±0.01)d</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>(744±0.01)a</td>
<td>(529±0.01)c</td>
<td>(402±0.01)d</td>
</tr>
</tbody>
</table>

NLC\textsubscript{BO}: Nanostructured lipid carrier prepared with non-interesterified buriti oil; NLC\textsubscript{BO6h}: nanostructured lipid carrier prepared with buriti oil interesterified for 6 h; NLC\textsubscript{BO24h}: nanostructured lipid carrier prepared with buriti oil interesterified for 24 h. \textsuperscript{a,b,c,d} Values (mean) followed by different letters differ significantly (\(p \leq 0.05\)) by Tukey’s test.
Table 2. Zeta potential of nanostructured lipid carriers after 1, 15, and 30 days of storage at refrigeration (4 °C) and room (25 °C) temperatures.

<table>
<thead>
<tr>
<th>Temperature/°C</th>
<th>t/day</th>
<th>NLC&lt;sub&gt;BO&lt;/sub&gt;</th>
<th>NLC&lt;sub&gt;BO6h&lt;/sub&gt;</th>
<th>NLC&lt;sub&gt;BO24h&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1</td>
<td>(−26.1±0.67)&lt;sup&gt;def&lt;/sup&gt;</td>
<td>(−24.8±0.61)&lt;sup&gt;abcde&lt;/sup&gt;</td>
<td>(−23.1±0.62)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>(−26.0±0.25)&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>(−25.6±0.29)&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>(−24.9±0.15)&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>(−26.2±0.50)&lt;sup&gt;def&lt;/sup&gt;</td>
<td>(−26.0±0.21)&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>(−24.0±0.59)&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>(−28.1±1.02)&lt;sup&gt;ig&lt;/sup&gt;</td>
<td>(−24.5±0.31)&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>(−23.5±0.64)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>(−26.7±1.28)&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>(−24.6±0.90)&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>(−24.7±0.29)&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>(−24.8±0.60)&lt;sup&gt;abcdef&lt;/sup&gt;</td>
<td>(−29.3±0.90)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>(−24.6±0.86)&lt;sup&gt;abcdef&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NLC<sub>BO</sub>: Nanostructured lipid carrier prepared with non-interesterified buriti oil; NLC<sub>BO6h</sub>: nanostructured lipid carrier prepared with buriti oil interesterified for 6 h; NLC<sub>BO24h</sub>: nanostructured lipid carrier prepared with buriti oil interesterified for 24 h. Values (mean ± SD) followed by different letters differ significantly (p≤0.05) by Tukey’s test.

Table 3. Short spacings (distance between parallel acyl groups in the TAG) and polymorphic (crystalline) forms of Buriti oil, Compritol 888 ATO, NLC<sub>BO</sub> and NLC<sub>BO24h</sub> after 30 and 120 days of storage at 25°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>t/day</th>
<th>Short spacings (Å)</th>
<th>Polymorphic form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buriti Oil</td>
<td>4.38&lt;sup&gt;(3)&lt;/sup&gt;</td>
<td>4.19&lt;sup&gt;(3)&lt;/sup&gt;</td>
<td>3.92&lt;sup&gt;(3)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compritol 888 ATO</td>
<td></td>
<td>4.25&lt;sup&gt;(4)&lt;/sup&gt;</td>
<td>3.82&lt;sup&gt;(1)&lt;/sup&gt;</td>
</tr>
<tr>
<td>NLC&lt;sub&gt;BO&lt;/sub&gt;</td>
<td>30</td>
<td>4.68&lt;sup&gt;(3)&lt;/sup&gt;</td>
<td>4.45&lt;sup&gt;(3)&lt;/sup&gt;</td>
</tr>
<tr>
<td>NLC&lt;sub&gt;BO24h&lt;/sub&gt;</td>
<td>30</td>
<td>4.55&lt;sup&gt;(3)&lt;/sup&gt;</td>
<td>4.32&lt;sup&gt;(3)&lt;/sup&gt;</td>
</tr>
<tr>
<td>NLC&lt;sub&gt;BO&lt;/sub&gt;</td>
<td>120</td>
<td>4.67&lt;sup&gt;(3)&lt;/sup&gt;</td>
<td>4.46&lt;sup&gt;(3)&lt;/sup&gt;</td>
</tr>
<tr>
<td>NLC&lt;sub&gt;BO24h&lt;/sub&gt;</td>
<td>120</td>
<td>4.63&lt;sup&gt;(3)&lt;/sup&gt;</td>
<td>4.41&lt;sup&gt;(3)&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NLC<sub>BO</sub>: Nanostructured lipid carrier prepared with non-interesterified buriti oil; NLC<sub>BO24h</sub>: nanostructured lipid carrier prepared with buriti oil interesterified for 24 h. (1) weak; (2) medium; (3) strong; (4) very strong.
Table 4. ORAC value, linearity (area under curve vs. concentration), and FRAP value of NLCBo and NLCBo24h.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ORAC assay</th>
<th>FRAP assay</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ORAC TE/(μmol/mL)</td>
<td>γ(sample)/(mg/mL)</td>
<td>Linearity</td>
</tr>
<tr>
<td>NLCBo</td>
<td>(1223.21±38.838)²</td>
<td>0.2–0.05</td>
<td>47.41</td>
</tr>
<tr>
<td>NLCBo24h</td>
<td>(1592.40±94.877)²</td>
<td>0.2–0.05</td>
<td>53.52</td>
</tr>
</tbody>
</table>

NLCBo: Nanostructured lipid carrier prepared with non-interesterified buriti oil; NLCBo24h: nanostructured lipid carrier prepared with buriti oil interesterified for 24 h. Results are expressed as mean ± SD (n = 3). ² Values followed by different letters are significantly different at p < 0.05.

Fig. 1. Influence of oil type on the morphology of nanostructured lipid carriers (NLCs). The oil phase is stained red, and the scale bar is 25 μm. NLCBo: Nanostructured lipid carrier prepared with non-interesterified buriti oil; NLCBo6h: nanostructured lipid carrier prepared with buriti oil interesterified for 6 h; NLCBo24h: nanostructured lipid carrier prepared with buriti oil interesterified for 24 h.