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DEVELOPMENT AND STABILITY OF A NANOSTRUCTURED LIPID CARRIER LOADED WITH QUERCETIN INCORPORATED IN A GEL FOR TRANSDERMAL USE

DESENVOLVIMENTO E ESTABILIDADE DE UM CARREADOR LIPÍDICO NANOESTRUTURADO CARREGADO COM QUERCETINA INCORPORADO EM GEL PARA USO TRANSDÉRMICO

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ABSTRACT

Quercetin is a flavonoid with antioxidant and anti-inflammatory potential. However, it has low skin permeation and stability. One way to improve this is to incorporate it into nanocarriers. This work aimed to develop and evaluate the stability of a nanostructured lipidic carrier with quercetin (NLC-Q) under different storage conditions for 30th days and its incorporation in the gel for 60th days. The NLC-Q were developed by the high shear rate method, and the physicochemical stability was performed at temperatures of 5, 25 and 40 ^{o}C on days 0, 7, 15, 22 and 30. The gel containing NLC-Q had its stability evaluated on days 1, 7, 15, 30, 45 and 60. The three environments' size, polydispersity index (PdI), zeta potential (ZP), content (%), and pH are within the recommended parameters. Unlike the climatic chamber, where NLC-Q increased size, PdI and quercetin content (%) were reduced throughout the experiment. In the gel with NLC-Q, pH, viscosity, spreadability, size and PdI are within recommendations found in the literature. The NLC-Q kept at room temperature showed good stability throughout the study.

Keywords: lipidic nanoparticle; skin permeation; physiotherapy; lipidic nanocarrier gel

RESUMO

A quercetina é um flavonoide com potencial antioxidante e anti-inflamatório. Porém, apresenta baixa permeação cutânea e estabilidade. Uma forma de melhorar isto é incorporá-la em nanocarreadores. Este trabalho objetivou desenvolver e avaliar a estabilidade de um carreador lipídico nanoestruturado com quercetina (CLN-Q) em diferentes condições de armazenamento por 30 dias assim como sua incorporação em gel por 60 dias. Os CLN-Q foram desenvolvidos por método de alta taxa de cisalhamento, e a estabilidade físico-química foi realizada nas temperaturas de 5, 25 e 40 ^{o}C nos dias 0, 7, 15, 22 e 30. O gel contendo CLN-Q teve sua estabilidade avaliada nos dias 1, 7, 15, 30, 45 e 60. O diâmetro médio da partícula (DMP), índice de polidispersão (IP), potencial zeta, teor (%) e pH das três condições estão dentro dos parâmetros encontrados na literatura. Diferente da câmara climática, onde aumentou o DMP das CLN-Q e reduziu o IP e o teor (%) de quercetina ao longo do experimento. No gel com CLN-Q, o pH, a viscosidade, a espalhabilidade,

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DMP, o IP e a concentração estão dentro dos parâmetros encontrados na literatura. Os CLN-Q mantidos na temperatura ambiente apresentaram boa estabilidade ao longo do estudo.

Palavras-chave: *nanopartícula lipídica; permeação cutânea; fisioterapia; gel nanocarreador lipídico.*

INTRODUCTION

Quercetin is a flavonoid that has attracted attention from many researchers due to its health benefits, which include: protection to DNA damage, antioxidant, anti-inflammatory, hepatoprotective and antitumor activity (LESJAK *et al*., 2018; REN *et al*., 2017; HAO *et al*., 2017; LEE *et al*., 2016; BOSE; MICHNIAK-KOHN, 2013). However, because of its low bioavailability, poor absorption, poor water solubility, structural instability in the face of temperature, pH, presence of metal ions that generate its oxidation and degradation, and the extensive first-pass metabolism, makes its incorporation into formulations is difficult (LESJAK *et al*., 2018; WANG *et al*., 2016)one of the most well-known flavonoids, has been included in human diet for a long history. The use of quercetin has been widely associated with a great number of health benefits, including antioxidant, anti-inflammatory, antiviral and anticancer as well as the function to ease some cardiovascular diseases (i.e., heart disease, hypertension, and high blood cholesterol. One way to improve this is to incorporate quercetin in nanostructures, favoring extended release, improving its stability and controlling the rate of first-pass metabolism, and allowing it to be administered by the routes: intravenous, dermal, inhalation and gastric (GUPTA *et al*., 2015).

Lipidic nanoparticles are an excellent alternative for drug delivery systems, especially those with lipophilic character (BEHBAHANI *et al*., 2017). Second-generation nanostructured lipid carriers (NLC) are composed of solid and liquid lipids forming an imperfect crystalline structure, supporting higher drug loading and physical stability (SAUPE; GORDON; RADES, 2006; MUSSOI, 2019). NLCs can increase the apparent solubility, control the release rate, and improve the bioavailability of encapsulated compounds, and are used in cosmetics, oral and dermal pharmaceuticals, and functional foods (BABAZADEH; GHANBARZADEH; HAMISHEHKAR, 2017). Previous research by Pivetta *et al*., (2019), Huang *et al.* (2017) and Bose; Michniak-Kohn (2013) encapsulated quercetin in NLCs for cutaneous use and demonstrated that these are well tolerated by the skin, including natural lipids with biological properties, presenting a good option for topical application.

The skin has been historically employed for topical and systemic administration of actives. Compared to the oral route of administration, the transdermal route had advantages, as it avoids drug degradation by gastric juice action, avoids first-pass metabolism by the liver and allows controlled drug release (ZHAI *et al*., 2014). However, the impermeable nature of the skin is the major complicating factor for topical drug administration. This renders the therapeutic effect of conventional dosages ineffective, which has led to the development of new drug carrier systems (NAGULA AND

WAIRKAR, 2019). Among the most commonly used forms for topical administration are creams and gels, with gels presenting as advantages the refreshment and retention at the administration site, avoiding the run-off of the formulation (SARAF, 2010).

Gels are materials of elastic or rigid nature, presented in a semi-solid dispersed form, composed of an external solvent phase of hydrophobic or hydrophilic nature, immobilized within the spaces of a three-dimensional polymeric structure (REHMAN AND ZULFCAR, 2013). Gels called emulgels are a combination of a gel with a water-in-oil (W/O) or oil-in-water (O/W) and are characterized by delivering hydrophobic drugs (RAHMANI-NEISHABOOR, 2012). With the introduction of nanotechnology in emulsion-based gels, a reduction in particle diameter has occurred, allowing for increased penetration of the drug through the skin and an increase in its stability (AZEN *et al*., 2012; NAGULA AND WAIRKAR, 2019).

In this context, this study aimed was to produce a nanostructured lipid carried loaded with quercetin (NLC-Q) incorporated into a gel and to evaluate its stability at room temperature (25 ºC), at refrigeration temperature (5 °C) and in a climatic chamber (40 °C).

MATERIALS

Ester, stearic and palmitic acid with glycerol (Imwitor 900 K®, kindly donated by PIC Química, São Paulo, Brazil), triglycerides of capric-caprylic acid (Crodamol GTCC®, Alpha Química, Brazil), sorbitan monostearate (SPAN® 60) and polysorbate 80 (Tween® 80, Sigma-Aldrich), quercetin (Pharma Nostra, lot VQ-1512 -270515.1183), ultrapure water (MilliQ®), methanol and acetonitrile HPLC grade (Merck® Germany - Lot: L631830212); hydrochloric acid (Synth - Brazil - Lot 197257), acetic acid (Synth - Brazil - Lot 197979); ethanol (Química Moderna® - Brazil - Lot: 00171); 0.45 µm regenerated cellulose syringe filter (Sartorius Stedim® - Germany - Lot: 17765); 0.45 µm regenerated cellulose membrane (Unifil - Lote: 6912-1211). Carbopol 2020® ETD polymer (Lubrizol - Brazil); Germall™ 115 (Ashland - Brazil); and Triethanolamine (Adcos professional - Brazil).

METHODS

DEVELOPMENT OF NANOSTRUCTURED LIPID CARRIER CONTAINING QUERCETIN (NLC-Q)

The NLC-Q was developed in the Nanotechnology Laboratory of the Franciscan University, according to the methodology of MUSSOI (2019). The high shear rate method used an Ultraturrax® (T-18, IKA® Brazil) to produce the NLC-Qs. Three lots (L1, L2, L3) of 100 mL each were produced

according to the composition described in Table 1. The formulations were analyzed in triplicate and characterized for size, polydispersity index (PdI), zeta potential (ZP), content (%) and pH.

Table 1 - Composition of nanostructured lipid carriers loaded with quercetin (NLC-Q).

Formulation	
Organic phase	
Inwitor 900 (g)	0.8
Crodamol® (g)	4.2
Span 60 (g)	1.0
Quercetin (g)	0.1
Aqueous phase	
Tween 80 (g)	2.0
MilliQ Water (mL)	92
Total volume (mL)	100

Source: Author's creation

The reagents of each phase were weighed separately using an analytical balance (AUW 220D, Shimadzu, Japan), except quercetin that was weighed separately. Afterwards, they were placed in a water bath between 65 and 68 °C, under magnetic stirring (RH basic, IKA® Brazil) for 10 minutes. Quercetin was then added to the organic phase and allowed to stir for another 5 minutes, and the aqueous phase was poured over the organic phase and stirred for 10 minutes. The NLC-Q was taken to the Ultraturrax® (T-18, IKA® Brazil) at 18000 rpm for 30 min, and the formulation was cooled to room temperature and packaged in 100 mL amber flasks (MUSSOI, 2019).

CHARACTERIZATION AND STABILITY OF NLC-Q.

The size and the polydispersity index (PdI) were determined by dynamic light scattering in the Zetasizer® (Malvern USA) at a fixed scattering angle of 90° at 25 °C. The samples were diluted 500 times (v/v) in MilliQ® water and filtered on a 0.45 µm regenerated cellulose filter using a syringe (Sartorius Stedim® - Germany - Lot: 17765), with the results expressed as mean ± standard deviation, in nanometers for size, and the PdI is an admensional parameter. The zeta potential (ZP) was performed by electrophoresis in the Zetasizer® (Malvern USA), in which the samples were diluted 500 times (v/v) in 10 mM sodium chloride and filtered through a 0.45 µm regenerated cellulose filter using a syringe (Sartorius Stedim® - Germany - Lot: 17765), and the results were expresses as mean ± standard deviation, in millivolts (mV).

Quantification of the active (%) was performed by high-performance liquid chromatography (HPLC) using a Shimadzu Prominence chromatograph (Tokyo, Japan) equipped with a DGU-20A 5R degasser LC-20AT pump, CBM-20A system controller, SIL-20A HT autosampler, SPD-M20A detector and a CTO-20AC column oven. Phenomenex® Gemini C18 column (250 x 4.6 mm, 5 µm),

mobile phase (acetonitrile: acidified water with acetic acid to pH 2.8 at 40:60 (v/v)) was used, with a flow rate of 1.3 mL/min at 35 °C, injection volume of 20 µL, detection at 370 nm (MUSSOI, 2019). Nanoparticle suspensions were subjected to ultrafiltration-centrifugation (400 µL), using Microcron® - Millipore 10.000 Å filter and centrifuged at 3.000 rpm for 10 minutes (Novatécnica® refrigerated microcentrifuge, model Nt 805 Brazil). The amount of quercetin not incorporated into NLC was determined in the ultrafiltrate. The amount of quercetin associated to the NLC was determined by the content (%), which corresponds to the division of the active content (total) and the concentration of the active in the continuous phase (free, ultrafiltered) by the active content (total), multiplied by 100.

The pH was determined in a pH meter (Ultra Basic, UB-10, USA) previously calibrated in Milli-Q® water pH 6.5 to 7. The measurements were performed in triplicate, and the results are expressed as mean ± standard deviation.

DEVELOPMENT OF THE GEL CONTAINING NLC-Q

Three batches of 100 g each of Gel-NLC-Q (G1, G2, G3) prepared according to Melo, Domingues and Lima (2018) with modifications were formulated. 0.4 g of carbopol® 2020 (0.4%), 0.3 g of imidazolidylurea® (germall) (0.3%) and 0.25 g of triethanolamine® (0.25%) were weighed on an analytical balance (AUW 220D, Shimadzu, Japan). The carbopol 2020 and 100 mL of NLC-Q were slowly added to a porcelain crucible and mixed manually with a pistol. Then triethanolamine was added and homogenized, and finally germall was added. A white gel was produced for use as a negative control. After preparation, centrifugation tests were performed before inserting the material in the stability study, in which three samples of the NLC-Q gel (8 g) were submitted to the centrifugation test in test tubes (centrifuge model TDL 80-2B, China) for 30 min at 3000 rpm. This test causes an increase in the force of gravity, enhancing the mobility of the particles, which can cause phase separation, sediment or supernatant formation and coalescence. Any sign of instability indicates the need for product reformulation (MELO, DOMINGUES END LIMA, 2018). After this evaluation, the samples were then exposed to the conditions of the stability study, and the gels were divided into three double-walled polypropylene packages with a capacity of 60 g and conditioned in the different temperatures (room 25 ºC, refrigerator 5 °C and climate chamber 40 °C) for the stability study.

STABILITY OF THE GEL-NLC-Q FORMULATION

The stability study followed the stability study guide for cosmetic products (ANVISA 2004). The GEL-NLC-Q formulations were exposed to conditions: room temperature (25 ºC), refrigeration (5 °C) and climatic chamber (40 °C), and characterized for size, PdI, quercetin concentration and pH (methods already described) on days 0, 7th, 15th, 22th and 30th.

The gel containing NLC-Q was evaluated according to the accelerated stability study, under the conditions shown in Table 2, evaluated on days 1th, 7th, 15th, 30th, 45th and 60th.

Table 2 - Storage conditions, considering temperature and relative humidity.

Condition	Temperature (°C)	Relative Humidity (%)
Room Temperature	25	50 ± 5
Refrigerator	5	62 ± 5
Climate Chamber	40	60 ± 5

Source: Created by the author: The relative humidity is expressed in (mean ± SD).

At each time of the gel samples containing NLC-Q in the stability conditions, samples were collected, and the organoleptic characteristics, viscosity, spreadability, size, PdI, concentration and pH were evaluated.

The organoleptic characteristics (appearance, color and odor) of gel formulations were classified according to the Stability Guide for Cosmetic Products (ANVISA, 2004). The appearance is classified as normal without change (N), slightly separated (LS), slightly precipitated (LP), and slightly turbid (LT), and the color and odor in normal without change (N), slightly modified (LM), modified (M) and intensely modified (IM).

The viscosity was determined in a digital rotational viscometer (Brookfield model RVDV - I+, USA), with spindle 4, using 6 speeds (0.3, 0.6, 1.5, 3.0, 6.0 and 12 rpm) applied to samples stored at room temperature (25 °C) after 60 days of storage, and the values were read after 1 minute of deformation.

The spreadability of the Gel-NLC-Q was performed according to Knorst (1991), using a 20 x 20 cm glass plate on millimeter paper where the sample is applied through a mold plate with a 1.2 cm central hole, which is then removed. Over the sample were added 10 x 10 cm thickness, previously weighed, and the horizontal and vertical diameter (mm) is read 1 min after adding the plate. The spreadability is calculated as follows: $Ei = [(d^2). \pi] /4$ where, Ei (mm^2), the spreadability of the sample is achieved as a function of added weights of glass plates on the sample that diffuse to an average diameter (mm). The analysis was performed in triplicate, obtaining the mean ± standard deviation (SD).

The evaluation of size, PdI were performed as already described for the characterization of NLC-Q previously, being the samples of Gel-CNL-Q weighed (0.002021 g), diluted 500 times (v/v) in 10 mL of MilliQ® water and filtered through a 0.45 µm filter.

The concentration of quercetin in Gel-NLC-Q was determined by HPLC (Shimadzu, Japan), 0.2044 g of the sample was weighed on an AUW 220D analytical balance (Shimadzu, Japan). 4 mL of HPLC grade acetonitrile was added and sonicated (USC-1600, Unique, Brazil) for 10 min. After that, 0.48 mL of water pH 2.8 and 0.72 mL of HPLC grade acetonitrile were added to complete 10 mL. The solution was kept for 10 min in the vortex at speed 5, followed by 10 min in the ultrasound and

8 min in the Centribio centrifuge (model: TDL80-2B) at 3000 rpm, filtered through 0.45 µm filters and stored in vials.

The pH was measured directly on the gel, in triplicate, in a pH meter (Ultra Basic, UB-10, USA), previously calibrated in Milli-Q water, pH 6.5 to 7.0, and the result was expressed as mean ± SD.

STATISTICAL ANALYSIS

Data were evaluated by one-way and two-way analysis of variance (ANOVA) for repeated measures (group and time), followed by Bonferroni *post hoc*. They are presented as mean ± standard deviation. A α 5% ($p < 0.05$) error rate was considered significant.

RESULTS AND DISCUSSION

CHARACTERIZATION AND STABILITY OF NLC-Q

The raw data of the physicochemical variables of the NLC-Q evaluated over 30 days (0, 7th, 15th, 22nd and 30th day) are presented in Table 3.

Table 3 - NLC-Q characterization along 30 days of storage in different temperature: room (25 °C), refrigerated (5 °C) and climate chamber (40 ºC).

Variables	Time Days	Room Temperature (25 ºC)	Refrigerated Temperature (5 °C)	Climate chamber temperature (40 °C)	2 way Anova P Value	
					Time	Temperature
Size (nm)	**0**	103.26 ± 1.32	103.26 ± 1.32	103.26 ± 1.32	**p < 0.001**	**p < 0.001**
	7th	106.12 ± 2.26 *	107.38 ± 1.40 *	108.79 ± 1.52 *†‡		
	15th	106.76 ± 2.61	106.99 ± 1.62 *	108.30 ± 1.23 *		
	22nd	103.43 ± 1.35 *	104.82 ± 0.96	108.94 ± 1.67 *†‡		
	30th	105.70 ± 1.41 *	105.70 ± 1.41 *	106.99 ± 3.40 *†‡		
PdI	**0**	0.167 ± 0.010	0.167 ± 0.010	0.167 ± 0.010	**p< 0.001**	**p < 0.001**
	7th	0.168 ± 0.016	0.151± 0.010	0.157 ± 0.007		
	15th	0.173 ± 0.017	0.170 ± 0.018	0.170 ± 0.018		
	22nd	0.172 ± 0.018	0.274 ± 0.315	0.130 ± 0.016 *†‡		
	30th	0.165 ± 0.024	0.162 ± 0.014	0.133 ± 0.023 *†‡		
ZP (mV)	**0**	-6.78 ± 1.25	-6.78 ± 1.25	-6.78 ± 1.25	**p < 0.001**	**p = 0.439**
	7th	-8.79 ± 1.46	-9.02 ± 2.83 *	-8.77 ± 3.24		
	15th	-11.30 ± 0.30 *	-9.71 ± 1.87 *	-9.44 ± 2.36 *		
	22nd	-8.75 ± 1.43	-13.69 ± 1.74 *	-10.75 ± 2.22 *		
	30th	-9.94 ± 0.98 *	-10.00 ± 1.02 *	-11.04 ± 0.58 *		
Content (%)	**0**	97.51 ± 0.70	97.51 ± 0.70	97.51 ± 0.70	**p < 0.001**	**p < 0.001**
	7th	93.76 ± 2.58 *	91.62 ± 0.52 *†	70.36 ± 0.19 *†‡		
	15th	91.05 ± 0.37 *	90.67 ± 0.33 *	64.52 ± 0.04 *†‡		
	22nd	90.82 ± 0.24 *	91.83 ± 1.58 *	55.05 ± 0.13 *†‡		
	30th	90.90 ± 0.18 *	86.73 ± 0.11 *†	48.78 ± 0.09 *†‡		

Data presented in mean ± SD Size: mean particle diameter; PdI: Polydispersity Index ZP: zeta potential; quercetin content (%). (*) $p < 0.05$ vs day 0, † $p < 0.05$ vs Room temperature (25 ºC), ‡ $p < 0.05$ vs refrigerator temperature (5 ºC).

Over time, at room temperature, there was an increase ($p < 0.001$) in size on the 7th MD: 2.9 nm, on the 15th MD: 3.5 nm and on the 30th MD: 2.4 nm compared to day 0, representing respectively, 2.7%, 3.3% and 2.3%. In the cooler, there was also an increase ($p < 0.001$) in size on the 7th MD: 4.1 nm, on the 15th, MD: 3.7 nm and on the 30th MD: 2.7 nm, relative to day 0, which respectively corresponded to 3.9%, 3.5% and 2.6%. In the climatic chamber, this increase over day 0, ($p < 0.001$) in size occurred on all days evaluated and were respectively 5.5%, MD: 2.7 nm, on the 7th, 4.8%, MD: 5.0 nm, on the 15th, 5.4%, MD: 5.6 nm on the 22nd and 13.3%, MD: 13.7 nm on the 30th day.

The size of room and refrigerator temperatures were similar during the study. However, the climatic chamber increased ($p < 0.001$) size relative to room and refrigerator temperatures on days 7, 22 and 30. These increases correspond to 2.7%, MD: 2.7 nm on day 7, 5%, MD: 5.5 nm on day 22 and 9.7% MD: 11.3 nm on day 30 for the room temperature. For storage at refrigerator temperature, this increase was 3.8% MD: 4.1 nm on day 22 and 9.4% MD: 11 nm on the 30th day.

Particle size is an indicator of NLC stability, and it is advocated that these may undergo a small variation in their size during storage (KHOSA; REDDI; SAHA, 2018). The investigation of long-term stability in the change of the nanoparticle size brings information about the physical ability of the formulation. NLC size can be changed by several factors, such as the amount of surfactant, the structure of lipids, the amount and type of incorporated drug the choice of preparation method, equipment, temperature, number of cycles in homogenization, lyophilization (ÜNER, 2015).

In the present study, the preparation method of NLC-Q and the formulation components were not changed. It was observed that the 40 ºC temperature of the climatic chamber increased the size compared to the other temperatures of the other storage forms tested. These results are in agreement with previous studies with NLC-Q (OBEIDAT *et al*., 2010), (HUANG *et al*., 2017), (PIVETTA *et al*., 2019), (MUSSOI, 2019), (BOSE; MICHNIAK-KOHN, 2013) (PINHEIRO *et al*., 2020). Obeidat *et al*. (2010) formulated an NLC with quercetin and observed its stability at room temperature for 30 days and found no differences in particle size during this period. Huang *et al*. (2017) also observed an NLC-Q at 25 °C for 90 days, and the results showed increases in size on the 30th day (92.8 ± 0.8), 60 (94.2 ± 0.6) and 90th (95.7 ± 0.5) days compared to day 1 (89.2 ± 0.2). Mussoi (2019) observed the size of an NLC-Q for 30 days at room temperature (133.2 nm), refrigeration (133.4 nm) and oven (141.9 nm). In this work, the size of NLC-Q in the room (105.05 ± 1.60), in refrigeration (105.68 ± 1.92) and climatic chamber (107.25 ± 2.63) suffered a small variation during storage (data Table 3), but maintain their stability because these values are below the reference values in the literature. NLC should be between 10 to 1000 nm and for cutaneous use; these particles should be between 40 and 800 nm, as these measurements allow for easy passage through barriers, greater uptake into cells, and rapid action (KHOSA; REDDI; SAHA, 2018). This is reinforced by other studies with NLC-Q, which observed size variations over time (PIVETTA *et al*., 2019; BOSE, MICHNIAK-KOHN, 2013; MUSSOI, 2019). Pivetta *et al*. (2019) observed for 100 days the mean

and standard deviation (MPD), which stood at 130 nm and concluded that there was no difference in this period. Bose, Michniak-Kohn (2013) observed NLC for 30 days, at a temperature between 2 to 8 °C and, the size was 281.9 nm for day 1 and 294.6 nm for day 30. (MUSSOI, 2019) observed for 30 days an NLC-Q at room temperature, refrigerator and oven and obtained sizes of 133.2, 133.4 and 149.9 nm, respectively. Another study observed NLC-Q for 90 days at room temperature for use in Alzheimer' disease and found that on day 1 the size was 170 nm, and at the end, it was 250 nm (PINHEIRO *et al*., 2020). The results of this study show size values below those found in other studies in the three storage environments, demonstrating that they have good stability.

PdI was reduced ($p < 0.001$) only in the climatic chamber on the 22nd day (0.130 ± 0.016 and the 30th (0.133 ± 0.023) compared the day 0 (0.167 ± 0.010), this reduction was 29% (MD: -0.038) and 26% (DM: -0.035) respectively. The different forms of storage showed differences ($p < 0.001$), where the climatic chamber reduced the PdI relative to room temperature and the refrigerator on day 22 and day 30. This reduction in room temperature was 33% (MD: -0.04) and 24% (MD: -0.03). For the refrigerator, this decrease was 25% (MD: -0.03) and 22% (MD: -0.03).

The PdI is a way to evaluate the physical stability of nanosuspensions over time. Tamjidi *et al*., (2013) reported that recommended values are between 0.1 to 0.25 (KHOSA; REDDI AND SAHA, 2018). In the present study, mean results throughout the experiment in storage at room temperature (0.169 ± 0.001), refrigerator (0.184 ± 0.003) and climatic chamber (0.151 ± 0.025) are in the recommended range (Table 3). The PdI measures the particle diameter distribution of the sample, and when it is above 0.5, it indicates a wide diameter distribution of the formulation, which suggests a large polydispersity (KHOSA; REDDI AND SAHA, 2018). Therefore, the presented results demonstrate homogeneity of the samples under the evaluated conditions. A previous study followed the stability of an NLC-Q for 90 days at a temperature of 25 °C. At the end of the experiment, the PdI was 0.253 (± 0.01) (HUANG *et al*., 2017). The PdI of an NLC-Q was evaluated for 90 days at room temperature, and the results showed a lower PdI of 0,2 (PINHEIRO *et al*., 2020). Another study evaluated the PdI of an NLC-Q for topical use at day 1, 8 weeks and 14 weeks at a temperature between 2 to 8 °C (refrigerator); the PdIs were 0.306, 0.310 and 0.315 respectively (BOSE; MICHNIAK-KOHN, 2013). Pivetta *et al*., (2019) formulated an NLC-Q for topical use and observed its stability for 100 days, and at the end of that period, the PdI was 0.260. The authors considered a low polydispersity index showing very stability (PIVETTA *et al*., 2019). Mussoi (2019) evaluated an NLC-Q at room temperature, refrigeration and oven for 30 days, had a PdI of 0.208, 0.185 and 0.182, respectively. The divergence in the results of these studies Huang *et al*. (2017), Bose and Michniak-Kohn (2013), Pivetta *et al*. (2019) and Mussoi (2019) are due to different formulations and mainly to different follow-up times.

The different temperatures modified the zeta potential variable throughout the experiment ($p < 0.05$). Room temperature decreased ($p < 0.05$) the ZP by 66% (MD: -4.5 mV) on day 15 and 45% (MD: -3.1 mV) on day 30 compared to day 0. Refrigeration decreased this variable on all the

days evaluated, respectively 33% on day 7 (MD: -2.2 mV), 43% on day 15 (MD: -2.3 mV), 101% on day 22 (MD: -6.9 mV), and 47% on day 30 (MD: -3.2 mV). As for the climate chamber this decrease ($p < 0.05$) compared to the day (0) evaluation, was 39% (MD: -2.6, mV) on day 15, 58% (MD: -4 mV) 22nd, and 62% (MD: -4.2 mV) 30th day.

The different storage forms of NLC-Q particles did not interfere ($p = 0.433$) in the ZP throughout the experiment. This parameter evaluates the repulsion of NLC-Q particles and refers to the non-aggregation and electrostatic stability of the nanodispersion, and a ZP of at least ± 30 mV is required (TAMJIDI *et al*., 2013) (KHOSA; REDDI AND SAHA, 2018). The ZP is also closely related to the particle surface morphology of the suspension stability, pH, ionic strength, and types of ions present (CZAJKOWSKA-KOŚNIK; SZEKALSKA AND WINNICKA, 2019). The mean values throughout the present study of ZP in storage at room temperature -9.1 ± 2.2 mV, refrigerator -10.1 ± 1.3 mV and climatic chamber -9.7 ± 1.9 mV demonstrate that these were below the reference value during the experiment (data Table 3).

The obtained ZP values are related to Tween 80® in the NLC formulation, which is a non-ionic surfactant and causes a steric repulsion on stabilization. ZP values less than 10 mV are considered neutral (KUMAR *et al*., 2016). Studies have reported that the use of Tween 80® causes a steric stabilization of NLC with low ZP values (ADITYA *et al*., 2014), -17 mV and (KUMAR *et al*., 2016), - 8.9 mV. Another study evaluated the influence of Tween 80® concentration on the ZP of NLC, and concluded that concentrations of 2.5% of this surfactant leave the ZP above -18 mV (PARK *et al*., 2018). The study by MUSSOI (2019) used 2% Tween 80® in NLC-Q and the ZP was at -5.90 mV in the room, -5.77 mV in the refrigerator and -5.72 mV in the oven, a result very close to that found in the present study. The surfactant poloxamer 188 (non-ionic) in NLC-Q obtained ZP of -13 mV at the end of 90 days at room temperature (PIVETTA *et al*., 2019). NLC-Q at a temperature between 2 to 8 °C and the initial value was -36.57 mV, and after 14 weeks, it was -36,88 mV (BOSE; MICHNIAK-KOHN, 2013).

A reduction ($p < 0.05$) was observed in the NLC-Q content about the day 0 evaluations. At room temperature, there was a reduction in the averages in the quercetin content on days 7, 15, 22 and 30, and they are respectively -3.7%, -6.5%, -6.7%, -6.6%. In the refrigerator temperature, this reduction on day 7 was -5.8%, day 15 -6.8%, day 22 -5.7%, and day 30 -10.8%. In the climatic chamber, on day 7, the reduction was -27.1%, on day 15 -33%, on day 22 -42.5%, and on day 30 -48.7%.

The different forms of storage ($p < 0.001$) also modified the content throughout the study. About room temperature, the percentage of NLC-Q content compared to refrigeration on the 7th day was -2.1% and at 30 ºC was -4.2%. As for the climatic chamber, about room temperature, was respectively at day 7 of -23.4%, at day 15 of -26.5%, at day 22 of -35.7% and at day 30 of -48.8%. The content of NLC-Q in the climatic chamber, compared to refrigeration, was lower throughout the study being at day 7 -21.6%, at day 15 -26.3%, at day 22 -36.8% and at day 30 -37.9%. The average

percentage of the initial (0) NLC-Q content for the three different storage temperatures was 97.7% (± 0.7) of the total solution. A previous study with NLC-Q, which evaluated 2 hours after its production, found the content of 91% (±4) (ADITYA *et al*., 2014), demonstrating the present research's encapsulation efficiency.

The entrapment efficiency determines the ability to transport and release the drug, and NLCs have imperfect lipid and solid fillers in their structure and show higher encapsulation efficiency than solid lipid nanoparticles (NLS) (KHOSA, REDDI & SAHA 2018). In the present study, at the end of 30 days, these values corresponded to the storage of room temperature 90.9 ± 0.2, for the refrigerator 86.7 (± 0.1) and for the climatic chamber 48.8 (± 0.1), respectively showed a reduction of -6.6%, -10.7% and -48.7%. NLCs can undergo the recrystallization process, which causes the nanoparticle to grow faster over time, decreasing the entrapment of the active, resulting in its expulsion during storage time (MONTEIRO *et al*., 2017; PIVETTA *et al*., 2019). The content and stability directly relate to the recrystallization rate (POONIA *et al*., 2016). This may have occurred in the present study, particularly with NLC-Q stored in the climate chamber (40 ºC).

The storage under room temperature and refrigeration are corroborated by previous studies (HUANG *et al*., 2017) (PIVETTA *et al*., 2019) (GUO *et al*., 2012). The stability of NLC-Q (the 0.1% and 0.2% quercetin formulation) at 25 °C temperature evaluated for 90 days demonstrated that at the end of the period, the values found were 92.8% (±0.4) and 92.5% (±0.5) respectively (HUANG *et al*., 2017). These results are reinforced by previous studies, which followed 100 days the formulation and obtained 97.4% (±1.9) (PIVETTA *et al*., 2019) and 89.95% (GUO *et al*., 2012) compared to the beginning of the experiment. In the study of Mussoi (2019), the content was 93.50% in the room, 97.80% in the refrigerator and 69.28% in the oven, with the highest loss in the oven as well as in the present study.

In the present study there was an increase in the size of the NLC-Q, this being greater in the climate chamber storage (40 ºC), as well as a reduction of approximately half of the quercetin content at the end of 30 days. The release mechanisms of encapsulated actives may change according to temperature, pH, permeability, biodegradation (ASSIS *et al*., 2012). The type of lipid used in the formulation alters the kinetics of drug release and the rate of degradation, with short-chain triglycerides being degraded faster than those and long-chain (OLBRICH; KAYSER; MU, 2002). A medium-chain triglyceride, Crodamol®, was used in the formulation of NLC-Q in the present study. Thus, the loss of quercetin at the climate chamber temperature (40 °C) at the end of 30 days found in this research may have been influenced by the temperature and the triglyceride used in the formulation.

The pH results at the beginning (room: 5.46 ± 0.03; refrigeration: 5.46 ± 0.05; climatic chamber: 5.45 ± 0.08) and at the end of the experiment for the different temperatures studied (room: 5.55 ± 0.1; refrigeration: 5.44 ± 0.08; climatic chamber: 5.26 ± 0.02) are within the pH range compatible with the skin, being safe for application. Preservation of the skin's pH is important to maintain its function,

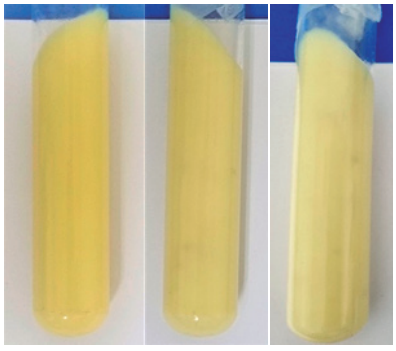
and the physiological range is between 5.4 and 5.9. Drugs outside this range have irritating effects and should be excluded from preparations for dermal or transdermal use (CZAJKOWSKA-KOŚNIK; SZEKALSKA; WINNICKA, 2019).

CHARACTERIZATION AND STABILITY OF GEL-NLC-Q

Centrifugation test

Three batches of gel (G1, G2, G3) were submitted to the centrifugation test for 30 min at 3000 rpm, 12 h after their productions. There was no phase separation, sediment formation, supernatant or coalescence as shown in Figure 1. These results demonstrate the stability of the sample (MELO, DOMINGUES AND LIMA, 2018).

Figure 1 - NLC-Q gel after centrifugation test, stored at room temperature 25 °C (G1), refrigerated at 5 °C (G2) and climate chamber 40 °C (G3).



G1 G2 G3

Source: Created by the author

Organoleptic evaluation

Table 4 shows the results of the organoleptic characteristics of the Gel-NLC-Q. At room temperature, the appearance was “slightly separated” from day 45 onwards. The color was slightly modified from day 15 and modified on day 60. The odor was slightly modified from day 30. In the refrigeration (5 °C) aspect, color and odor did not change throughout the study. In the climatic chamber, only the odor changed, to slightly modified on the 7th day and modified starting on the 45th day. The refrigeration (5°C) proved to have the best stability, followed by the climatic chamber (40 °C) and room temperature (25 °C).

Table 4 - Organoleptic behavior of Gel-NLC-Q during 60th days conditioned in the environment, refrigerator (5 ± 2 °C) and climatic chamber (40 ± 2 °C).

Days	Room (25 ºC)			Refrigeration (5 ºC)			C. Chamber (40 ºC)		
	Asp.	Color	Odor	Asp.	Color	Odor	Asp.	Color	Odor
1	N	N	N	N	N	N	N	N	N
7	N	N	N	N	N	N	N	N	LM
15	N	LM	N	N	N	N	N	N	LM
30	N	LM	LM	N	N	N	N	N	LM
45	LS	LM	LM	N	N	N	N	N	M
60	LS	M	LM	N	N	N	N	N	M

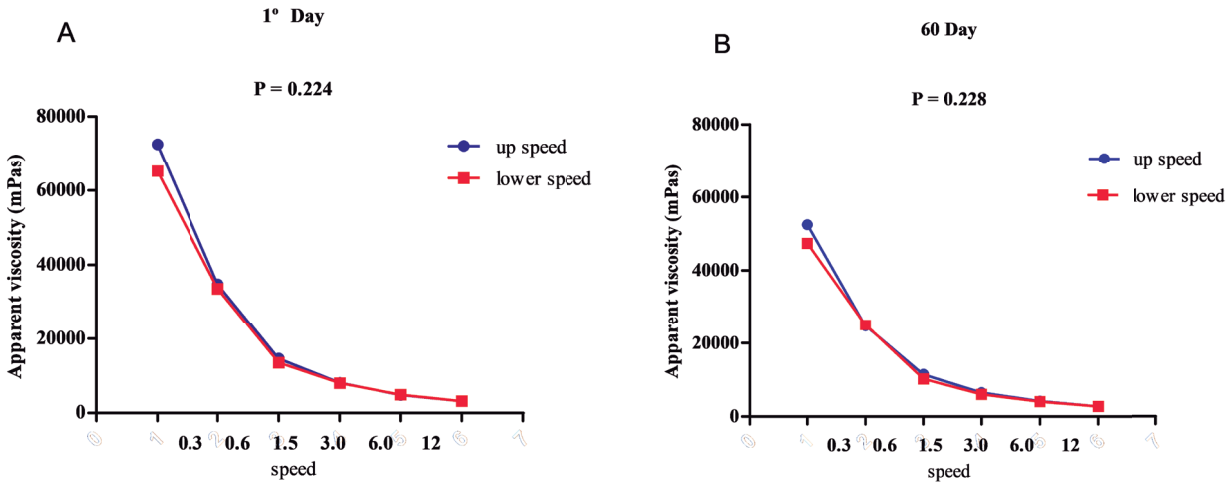
Source: Created by the author.

Legend: Room temperature (25 ºC); Refrigerator (5ºC); C. Climatic Chamber (40 °C). Temperature of the Climatic Chamber. Aspect: Normal without alteration (N), slightly separated (LS), slightly precipitated (LP), slightly turbid (LT). Color: Normal without alteration (N), slightly modified (LM), modified (M), intensely modified (IM). Odor: Normal without alteration (N), slightly modified (LM), modified (M), intensely modified (IM). Source: Stability Guide for Cosmetic Products (ANVISA, 2004).

Evaluation of apparent viscosity

Viscosity was evaluated only at room temperature, and the data are shown in Figure 2. Viscosity measures the system's resistance when subjected to a force and is a parameter that determines whether a product has proper fluidity or consistency influencing its stability (MELO, DOMINGUES and LIMA, 2018).

Figure 2 - Comparison of the viscosities at room temperature of the Gel-NLC-Q, on the 1st day fig. A and on the 60th day fig. B.



Source: Created by the author.

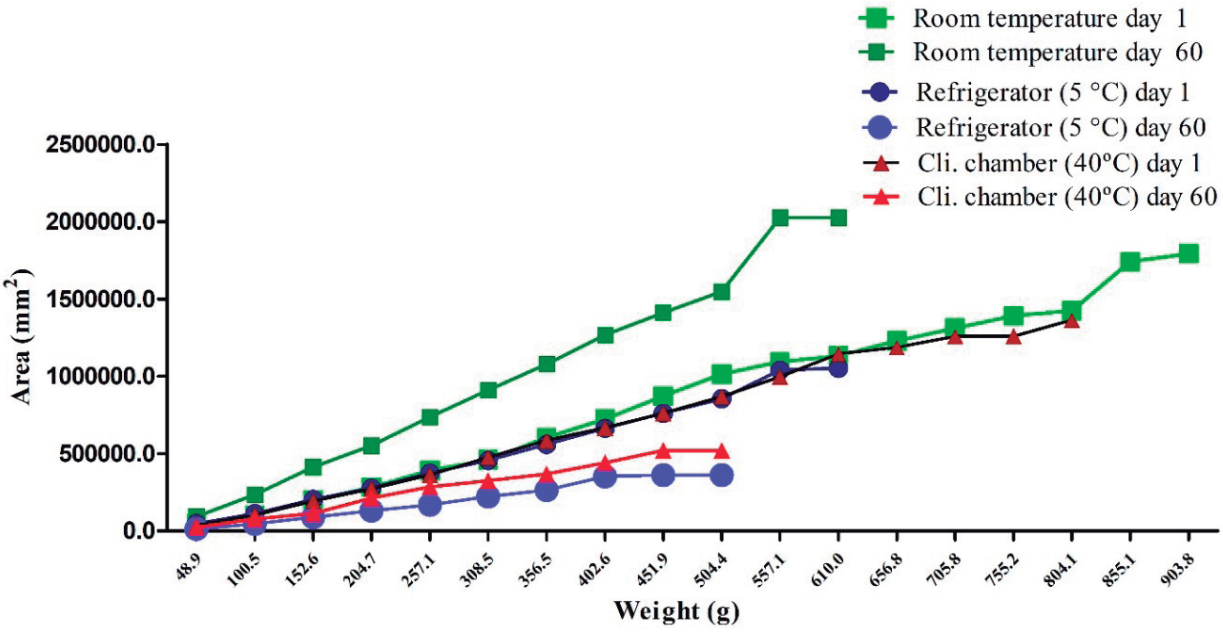
In the present study, the viscosity of Gel-NLC-Q at room temperature on 1st and 60th day, showed no changes during the period (p = 0.119 and p = 0.103, respectively) evaluated by the viscosity test. The results demonstrate a pseudoplastic non-Newtonian behavior, in which increasing shear

stress decreased the viscosity and are in agreement with previous studies (GOKHALE, MAHAJAN E SURANA, 2019) (PIVETTA *et al*., 2019). Corroborating with the results of this research, a previous study by Pivetta *et al*. (2019) demonstrated that Gel-NLC-Q exposed for 60 days in the environment maintained its viscosity similar to the initial one, with no change in the behavior of the curve. Evaluation of the viscosity of microemulsion gel with diclofenac performed by Shewaiter *et al*. (2021) and with resveratrol gel by Nemen (2011) demonstrated that increasing the shear rate decreased the viscosity, conferring a pseudoplastic non-Newtonian behavior. The pseudoplastic behavior brings advantages to pharmaceutical formulations in terms of applicability and handling on the skin surface because upon rubbing, and the viscosity decreases, making it easier to spread (SHEWAITER *et al*., 2021; NEMEN, 2011).

SPREADABILITY EVALUATION

The results of the spreadability are shown in Figure 3. Over time the spreadability decreased at all storage temperatures. In ambient temperature, on day 1 the spreadability reached its maximum at 855.1 g and on day 60 at 504.4 g. In refrigeration (5 °C), the 1st day had a spreadability to plate weight 557.1 g, compared to the 60th day, which spread to weight 551.9 g. In the climatic chamber, the 1st day had its greatest spreadability to the weight 804.1 g of the plate, compared to the 60th day that spread to the weight 451.9 g of the plate.

Figure 3 - Area (mm^2) of the spreadability of the Gel-NLC-Q gel in relation to the plate weight (g) at room, refrigeration (5 °C) and climatic chamber (40 °C) temperatures throughout the experiment.



Source: Created by the author.

The spreadability reflects the efficacy of the gel because the larger the area of coverage, the greater the bioavailability and absorption of the formulation (SHEWAITER *et al*., 2021). The spreadability of the climate chamber (40 °C) was lower at the end of the 60th day compared to the other forms of storage. The temperature elevation leads to water loss from the gel, making it thicker and thus decreasing its spreadability (ANDRADE (2017).

Gel-NLC-Q CHARACTERIZATION AS TO SIZE, PdI, CONCENTRATION AND pH

Table 5 presents the results of size, polydispersity index (PdI), quercetin concentration and pH at different storage temperatures for 60 days of the Gel-NLC-Q throughout the experiment.

Table 5 - Characterization of Gel-NLC-Q gel over 60 days storage at different temperatures: room temperature (25 °C), under refrigeration (5 °C) and climatic chamber (40 ºC).

Variables	Time Days	Room (25 ºC)	Refrigeration (5 °C)	C. Chamber (40 °C)	2-way Anova P Value	
					Time	Temperature
Size (nm)	1st	124.35 ± 13.37	123.32 ± 6.58	132.08 ± 16.27	p < 0.001	p < 0.001
	7th	121.74 ± 3.06	125.62 ± 7.25	126.48 ± 7.35		
	15th	125.65 ± 8.46	139.35 ± 21.13	126.02 ± 5.43		
	30th	126.26 ± 7.45	126.80 ± 6.12	118.62 ± 7.26		
	45th	132.91 ± 5.36	127.62 ± 1.08	136.48 ± 3.86		
	60th	136.47 ± 2.43	139.37 ± 8.14 *	137.3 ± 11.38		
PdI	1st	0.194 ± 0.047	0.199 ± 0.014	0.198 ± 0.042	p < 0.028	p < 0.001
	7th	0.158 ± 0.024	0,180 ± 0.038	0.183 ± 0.018		
	15th	0.206 ± 0.028	0.240 ± 0.041 *	0.189 ± 0.021 ‡		
	30th	0.213 ± 0.032	0.243 ± 0.018 *	0.172 ± 0.017 †‡		
	45th	0.186 ± 0.031	0.170 ± 0.010	0,176 ± 0.033		
	60th	0.192 ± 0.023	0.180 ± 0,033	0.154 ± 0.034 *		
Concentration µg/mL	1st	4.86 ± 1.11	6.69 ± 0.94	6.29 ± 0.92	0.691	
	7th	8.26 ± 1.51	9.95 ± 2.78	11.19 ± 0.81		
	15th	9.89 ± 1.31	12.66 ± 1.57	11.61 ± 1.18		
	30th	7.17 ± 1.70	9.43 ± 1.17	11.60 ± 1.16		
	45th	9.16 ± 0.45	8.98 ± 1.43	7.93 ± 0.86		
	60th	3.95 ± 1.46	3.52 ± 0.77	4.09 ± 0.52		
pH	1st	5.04	5,30	4.98	p = 0.099	
	7th	4.99	5,12	5.01		
	15th	5.01	5,08	4.99		
	30th	5.01	4.96	4.97		
	45th	5.07	4.98	4.98		
	60th	5	5.01	5.95		

Legend: Data presented as mean and standard deviation (±).
* p < 0.05 vs Day first. † p < 0.05 vs Room temperature; ‡ p < 0.05 vs Refrigerator.

The size at room temperature, refrigeration and climatic chamber (40 °C) were 128.61 (± 4.00), 130.25 (± 6.71) and 129.50 (± 4.52) nm, respectively. This measurement showed no differences between storage temperatures but changed over time in the study. At room temperature and in the

climatic chamber, the size did not change from day 1. However, in the refrigerator (5 °C), this diameter increased by 13% (MD: 16.1 nm, $p < 0.001$) compared to day 1 of the experiments. Only the samples conditioned in the refrigerator showed an increase in size on day 60 compared to the beginning of the study but within the recommended values. The size of nanoparticles should stay below 200 nm, as these measurements are recommended for topical applications due to their greater penetration into the skin (GOKHALE, MAHAJAN AND SURANA, 2019) (KAUR AND AJITHA, 2019). The CNL-Q Gel developed in the present research are found below this size in all storage conditions.

The PdI showed differences in conditioning ($p = 0.028$) and time ($p < 0.001$) throughout the study. Regarding room temperature, samples conditioned in the climatic chamber decreased the PdI by 19% on day 30, but the confidence interval did not confirm these results (MD: -0.040). The samples stored in the refrigerator, compared to the climatic chamber, were higher in the evaluations performed on the 15th and 30th days. These values were respectively 21% (MD: -0.051, $p < 0.01$) and 29% (MD: -0.071, $p < 0.001$).

Relative to day 1 (time: $p < 0.001$), room temperature did not modify the PdI throughout the days evaluated. The chiller was modified on day 15 compared to day 1, but this change was not confirmed by the confidence interval (MD: -0.009, $p < 0.05$). However, on day 30 the chiller increased the PdI by 22% (MD: 0.043, $p < 0.001$) compared to day 1. On the other hand, the climatic chamber decreased the PdI by 23% (MD: -0.044, $p < 0.001$) on day 60 compared to day 1. The PdI of room temperature, refrigeration and climatic chamber at the end 60 days of storage were 0.191 ± 0.008, 0.202 ± 0.031 and 0.179 ± 0.010, respectively, even though there were some variations among the temperatures. The best PdI found throughout the experiment was in the climatic chamber. The PdI's particle diameter distribution is preferable, with values below 0.1 up to 0.25, indicating a premiere diameter distribution (KHOSA, REDDI E SAHA, 2018).

QUANTIFICATION OF QUERCETIN FROM THE GEL FORMULATION CONTAINING NLC-Q

Table 5 shows the results of quercetin concentration in µg/mL throughout the experiment, in the different storage forms, with no differences in quercetin concentration contained in the gels at different temperatures.

It is possible to observe that, because it is a lipid nanoparticle in a gel, the active is released in a slow and sustained manner over time, with an increase in concentrations being observed at 15 days and a subsequent reduction by the end of the exposure period at the different temperatures, possibly due to its degradation. In NLC, drug release kinetics and lipid degradation rate depend on the type of lipid used, and short-chain triglycerides are degraded faster than long-chain ones

(KHOSA *et al*., 2018). In the present study, a medium-chain and a long chain triglyceride were used. Perhaps this is why quercetin had a sustained release over the 60 days.

pH EVALUATION

The pH of the NLC-Q Gel did not change throughout the study (Table 5). Topical gels should be compatible with the skin's pH to avoid causing irritation or allergies (GOKHALE *et al.*, 2019).

At the end of the 60 days, the pH of the environment, the refrigeration and the climatic chamber agree with the pH of the skin, cited in the literature, which is 4.0 to 5.6 (PHAD *et al*., 2018). These results show a slightly acidic pH and are close to the pH of 5.81 (±0.03) found in a previous study with quercetin gel in nanoemulsion (GOKHALE *et al*., 2019).

CONCLUSION

Although the variables size, PdI, ZP, content (%) and pH showed significant differences, they agree with other findings within the parameters recommended in the literature. The nanostructured lipid carrier with quercetin (NLC-Q) showed the best stability during the 30 days at ambient (25 °C) and refrigeration (5 °C) temperatures. The storage at 40 °C in the climatic chamber caused a great loss of quercetin from the nanostructured lipidic carrier, destabilizing this structure, and its storage at this temperature is not recommended.

As for the organoleptic characteristics of the nanostructured lipid carrier gel with quercetin, the best storage is in refrigeration, which showed no changes at the end of 60 days. The viscosity measured at room temperature showed no differences between the 1st and the 60th day, showing a non-Newtonian behavior. The spreadability showed a better behavior at room temperature at the end of the experiment.

The size, PdI, ZP, concentration and pH variables at room temperature, refrigeration and climatic chamber showed stability at the end of 60 days. The climatic chamber showed decreased PdI about the ambient and refrigeration, which is preferable for this variable. However, the best storage temperature was room temperature (25 °C) for the above variables.

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FIGURE CAPTION

Figure 1 - Gel-NLC-Q after centrifugation test, stored at room temperature 25 °C (G1), in refrigeration 5 ºC (G2) and climatic chamber 40 °C (G3).

Figure 2 - Comparison of room temperature viscosities of Gel-NLC-Q at day 1 fig A and at day 60 fig. B
Source: Built by the author

Figure 3 - Spreadability area (mm2) of the Gel-NLC-Q in relation to the weight of the plate (g) room temperature, refrigeration (5 ºC) and climatic chamber (40 °C) temperatures throughout the experiment.
Source: Built by the author

TABLE LIST

Table 1 - Composition of nanostructured lipid carriers loaded with quercetin (NLC-Q).

Table 2 - Storage conditions, considering temperature and relative humidity.
Source: Created by the author

Table 3 - NLC-Q characterization along 30 days of storage in different temperature: room (25 °C), refrigerated (5 °C) and climate chamber (40 ºC).
Legend: Data presented in mean and standard deviation MPD: mean particle diameter; PI: Polydispersity Index ZP: zeta potential; quercetin content (%). $p < 0.05$ vs day 0, † $P < 0.05$ vs Room temperature (25 ºC), ‡ $p < 0.05$ vs refrigerator temperature (5 ºC).

Table 4 - Organoleptic behavior of Gel-NLC-Q during 60 days conditioned in the environment, refrigerator (5 ± 2 °C) and climatic chamber (40 ± 2 °C).
Source: Built by the author.
Legend: Room temperature (25 ºC); Refrigerator (5ºC); C. Climatic Chamber (40 °C). Temperature of the Climatic Chamber. Aspect: Normal without alteration (N), slightly separated (LS), slightly precipitated (LP), slightly turbid (LT). Color: Normal without alteration (N), slightly modified (LM), modified (M), intensely modified (IM). Odor: Normal without alteration (N), slightly modified (LM), modified (M), intensely modified (IM). Source: Stability Guide for Cosmetic Products (ANVISA, 2004).

Table 5 - Characterization of Gel-NLC-Q over 60 days storage at different temperatures: room temperature (25 °C), under refrigeration (5 °C) and climatic chamber (40 ºC). Legend: Data presented as mean and standard deviation (±). ***** $P < 0.05$ vs Day first. † $P < 0.05$ vs Room temperature; ‡ $P < 0.05$ vs Refrigerator.