Nanoemulsion containing caffeine for cellulite treatment: characterization and *in vitro* evaluation

Thamires Batello Freire[1]*, **Michelli Ferrera Dario**[1], **Otávio Gil Mendes**[2], **Andressa Costa de Oliveira**[1], **Alberto Vetore Neto**[1], **Dalva Lucia Araújo de Faria**[2], **Vânia Rodrigues Leite e Silva**[3], **André Rolim Baby**[1], **Maria Valéria Robles Velasco**[1]

[1]*Department of Pharmacy, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil, [2]Department of Fundamental Chemistry, Chemistry Institute, University of São Paulo, São Paulo, Brazil, [3]Department of Exact and Earth Sciences, Institute of Environmental Sciences, Chemistry and Pharmaceuticals São Paulo, Federal University of São Paulo, São Paulo, Brazil*

The Ginoide Hydrolipodystrophy (GHLD), commonly known as cellulite, occurs in 80-90% of the female population after the puberty period and comes from a metabolic modification in the cutaneous adipose tissue. Caffeine has been used in topical formulations due to its lipolytic action. We studied a nanoemulsion (**F3**) containing caffeine with two surfactants (*oleth-3* and *oleth-20*) by emulsification method by phase inversion temperature inversion (PIT). The polydispersion indices (PDI) showed the reduced deviation of 0.1. The mean droplet size was ~ 40 nm. The evaluated constant of Ostwald, in the refrigerator condition was the most favorable during the stability test. In the In Raman spectroscopy assay, the caffeine bands found in **F3** were compatible with those found in the caffeine solution (1337, 652.5 and 558.2 cm^{-1}). There was no interaction of caffeine anhydrous with other ingredients in nanoemulsion. In the *in vitro* safety assay the result of 1.4 ranked the **F3** as slightly irritating. In the natural membrane, cutaneous permeation test (human skin) permeate concentrations did not exceed the saturation concentration of the PBS buffer (48.96 µg/3 mL). The caffeine solution and **F3** permeated statistically equal, but the nanoemulsion visually and sensorially improved the caffeine precipitation.

Keywords: Cellulite. Caffeine. Gynoid hydrolipodystrophy. Nanoemulsion. Permeation.

INTRODUCTION

Gynoid Hydrolipodystrophy (GHLD), a modification of the cutaneous relief due to morphological, histochemical, biochemical and ultrastructural change in the skin, occurs around 80 to 90% of the female population after puberty, appearing in the pelvic, lower limb and abdomen regions (Santos *et al*., 2011).

The dermoepidermal changes, with an increase in the size and number of adipocytes (hypertrophy and hyperplasia, respectively), are responsible for the skin alteration resulting in the orange peel appearance. These changes become more pronounced due to the fixation of the septa to the dense fibrous connective tissue covering the blood vessels, nerves, bones and muscles (deep fascia or muscular fascia). In addition, these septa are fixed with the reticular dermis, linking both (Guirro, Guirro, 2007). Considering the most accepted theories for the pathophysiology of GHLD, researchers tend to develop topical cosmetic formulations containing active ingredients with a microvascular, lymphatic flow stimulatory and lipolytic effects (Ramalho, Curvelo, 2006). Among these ingredients are the methylxanthines (caffeine, theophylline, aminophylline), retinol, phosphatidylcholine, L-carnitine, ginkgo biloba, methyl nicotinate, menthol, camphor and silicon (Krupek, Costa, 2013).

Caffeine, the most widely used methylxanthine, acts on the metabolism of adipocytes and promotes the inhibition of the phosphodiesterase enzyme (Krupek, Costa, 2013; Bertin *et al*., 2001), which causes an increase in the cyclic adenosine monophosphate (cAMP). This mechanism results in lysis of triacylglycerol in fatty acid plus glycerol (lipolysis) (Rawlings, 2006; Ribeiro, 2010). The efficacy of caffeine was previously revealed

***Correspondence:** T. B. Freire. Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo. Av. Prof. Lineu Prestes, 580, Bl-13/15, 05508-900, São Paulo – SP, Brazil. Fax: 55 11 3815-4418. Phone: 55 11 3091-3623

by reducing 17% of adipocyte diameter in an animal model (albino/Wistar rats) using an oil-in-water emulsion containing 4% w/w caffeine (Velasco *et al*., 2008).

Furthermore, caffeine is considered a model of hydrophilic skin-permeation substance in animal and human membranes, but it tends to precipitate and may lead to the formation of clumps of difficult redispersion depending on the vehicle used (Herman, Herman, 2013; Fernandes *et al*., 2015). Thus, an innovative formulation, which can address this drawback of the conventional formulation, will allow improving the sensorial hindered by the precipitation of the caffeine.

According Yukuyama *et al*. (2016), the superior property of a nanoemulsion compared to a macroemulsions is explained by the following characteristics: small droplet size ensures uniform distribution on the skin, large surface area, modified release, better film formation on the skin, high stability, pleasant aesthetic character and skin feel.

The formulation can be classified as nanoemulsion because of the droplet size. However, the ranges vary, according to the authors, between 20-500 nm (Mei *et al*., 2011), (50-500 nm) (Tadros *et al*., 2004) and from 20 to 200 nm (Izquierdo *et al*., 2004). Also, the droplet size influences its appearance droplet size influences its appearance since formulation can be transparent or translucent (50-200 nm) or milky (up to 500 nm) (Izquierdo *et al*., 2002).

The key factor for obtaining a successful nanoemulsion is the selection of most suitable process and components, which ensure the desired properties of the final obtained nanodroplets (Yukuyama *et al*., 2016). Among the methods mentioned in the scientific literature, the Phase Inversion Temperature (PIT) of the emulsions uses low energy, it has low cost and it is simple to execute (Shinoda, Saito, 1968; Mei *et al*., 2011; Dario *et al*., 2016). These characteristics qualified it for this research.

Several methods are employed in the preparation; this research used low energy methods. This method provides inversion phase by variations in the volume fraction or temperature variations known as Phase Inversion Temperature (PIT) emulsification (Shinoda, Saito, 1968).

Thus, the purpose of the present work was to develop a nanoemulsion containing caffeine (1% w/v) with two surfactants (*oleth-3* and *oleth-20* using a low energy process, the Phase Inversion Temperature, and its physical-chemical characterization and *in vitro* efficacy evaluation.

MATERIAL AND METHODS

Caffeine Anhydrous (Henrifarma) of 101.0% purity, caffeine anhydrous (Sigma Aldrich) analytical

reference standard of purity of 100%, sodium chloride (Pharma Special), capric/caprylic triglyceride (Mapric), phenoxyethanol (and) parabens (Phenonip®, Pharma Special) were purchased. Oleth-3 and oleth-20 were kindly donated by Croda.

Preparation of nanoemulsion containing caffeine

The nanoemulsions were developed by the sub-PIT method. About 100 g of each formulation were prepared by varying the proportion of the two nonionic surfactants necessary for the formation of the nanoemulsion (oleth-20 and oleth-3), keeping the sum of their proportion in the formulation (11.2% w/w), with final EHL of 11.1 (Table I) (Dario *et al.*, 2016; Mei *et al*., 2011; Roger, Cabane, Olsson, 2010). The proportion of caffeine (1.0% w/w) was chosen according to the literature (Sintov, Greenberg, 2014). This proportion is according with the Brazilian law of cosmetic products (Brazil, 2002; Shakeel, Ramadan, 2010; Fotticchia *et al*., 2014). The value of pH was corrected for 5.7.

The components of the formulations were mixed and heated under magnetic stirring (approximately 350 rpm) until reaching 80.0 ± 5.0 °C , the temperature at which the minimum turbidity was observed and below the PIT (Mei *et al*., 2011). Subsequently, the formulation was rapidly cooled in an ice bath to ~ 40.0 ºC.

Normal Stability Assay (NSA)

The stability of the nanoemulsion was tested by simultaneously maintaining separate samples in the oven (45.0 ± 2.0 °C), in the refrigerator (5.0 ± 2.0 °C) and at room temperature (25.0 ± 2.0 °C) for 90 days (Dario *et al.,* 2016; Brazil, 2004). Possible changes in organoleptic properties (appearance, color, and odor). The pH was determined with a Quimis® pHmeter. The droplet mean diameter and the polydispersity index were determined by Dynamic Light Scattering (DLS) at an angle of 90 °C at 25 °C. The zeta potential was determined at a field strength of 20 V/cm. Both analyses was performed in a Malvern ZetaSizer Nano ZS90 (Kaszuba, 2015).

Ostwald ripening constant

From the data obtained in NSA test, the Ostwald ripening constant was calculated according to the methodology described by Dario *et al.* (2016).

Nanoemulsion Raman Spectroscopy

A spectrometer (Renishaw in Via Reflex), equipped

TABLE I - Qualitative and quantitative composition (% w/w) of the preparations (**1** to **6**)

Ingredients*	F1	F2	F3	F4	F5	F6
1. Caprylic/capric triglyceride	5.0					
2. Oleth-3	9.4	6.7	5.6	0.6	6.2	4.2
3. Oleth-20	1.8	4.5	5.6	10.6	5.0	7.0
4. Sodium chloride				2.0		
5. (phenoxyethanol (and) methylparaben (and) ethylparaben (and) butylparaben (and) propylparaben (and) isobutylparaben)				0.5		
6. caffeine				1.0		
7. acqua qsp				100.0		

qsp: sufficient quantity to; * INCI Name: International Nomenclature of Cosmetic Ingredient

with a thermally cooled and coupled camera (CCD) (Renishaw 600 x 400 pixels) was coupled to a microscope (Leica, model: DM2500M); the laser line at 785 nm (diode laser, Renishaw) was focused on the sample by an objective (Leica) with a 50x magnification (numerical aperture of 0.75). Samples were placed on a carrier for liquid samples and spectra were recorded in the 400-1800 cm^{-1} range with the laser power at approximately 10 mW in the sample. Anhydrous caffeine spectra and caffeine solutions were compared at several values of pH in order to verify the possible protonation, as well as the comparison of this active ingredient with other organic ingredients of the nanoemulsion (Gil, 2015).

Entrapment Efficiency (EE%)

The caffeine encapsulation efficiency in the nanoemulsion droplets was determined by the difference between the total caffeine concentration and the free caffeine in the nanoemulsion. The quantification method of caffeine was previously validated by High-Performance Liquid Chromatography (HPLC). To calculate the free caffeine concentration, 300 μl of the nanoemulsion was added to a 10 kDa filtration unit (Amicon; Millipore, USA) and centrifuged at 21,400 g for 30 min at 25.0 ± 1.0 °C. The caffeine in the supernatant was quantified and the encapsulation efficiency was calculated according to the *Equation 1* (Oliveira *et al.*, 2016).

$$EE\% = \frac{C(T) - C(F)}{C(F)} \times 100 \qquad (1)$$

where: EE: Entrapment Efficiency; C(T): total concentration of caffeine; C(F): concentration of free caffeine in the formulation

In vitro safety evaluation

The irritant potential was determined by the *in vitro* HET-CAM (Hen's Egg Test - Chorioallantoic Membrane) method. Fertile White Leghorn hen's eggs were kept in an incubator for 9 days at 37 °C and 65% relative humidity. After the incubation period, the chorioallantoic membrane (CAM) was exposed and hydrated with 0.9% NaCl solution. On undamaged CAM, 300.0 μl of **F3** were applied (triplicate). The same aliquot of 1.0% sodium dodecyl sulfate (SDS) solution was applied as a positive control. The vascular events (hemorrhage, lysis or coagulation) were recorded for 300 s and the ocular irritation index was calculated (Kalweit *et al.*, 1990; National Institute of Environmental Health Sciences, 2006).

Skin permeation

The *in vitro* permeation of caffeine was performed in two-compartment vertical diffusion cells (Franz cells) as described in United States Pharmacopeia, NF31 (USP, 2013) and Pharmacopeia Forum (2008) for semi-solid and transdermal products with modifications.

Human skin was taken from abdominal plastic surgery, cleaned and the subcutaneous fat was removed. The experiment was approved by the Ethics Committee of Faculty of Pharmacy/University of São Paulo, process number 516508.15.0.3001.0067. The skin was frozen at -5.0 °C and used one week later. After being rehydrated with Phosphate Buffered Saline (PBS), skin integrity was evaluated by transepidermal water loss (TEWL) method. This procedure is an approval or disapproval criterion for the skin (Gioia, Celleno, 2002; Paepe, Roseeuw, Rogiers, 2002).

Caffeine quantification method was previously validated according to the literature (ICH, 2005; USP, 2013; Ribani *et al.*, 2004). A 150 µl (10 mg/mL) aliquot of **F3** was applied to the skin surface. PBS was used as receiving fluid, according to the Brazilian Pharmacopeia 5th ed (2010) and placed in a heated bath at 32.0 ºC, under constant agitation. The solubility of the substances in the receiving fluid was checked previously to the experiment. Samples were collected at times: t0, t1, t2, t3, t4, t22 and t24 h.

Epidermis and dermis were separated by the technique of immersion in water at 60 ºC for 1minute and 30 seconds, followed by mechanical separation of them with the aid of surgical tweezers. Afterwards, they were stored in glass vials with 10 mL of the buffer solution and subjected to mixing at 3000 rpm for 2 minutes, 24 hours of rest and 20 minutes of sonication (Junyaprasert *et al.*, 2009).

The concentration of caffeine was identified and quantified in 4 different skin regions: Skin Surface by Swab 2 (**SP**), Epidermis (**E**), Dermis (**D**) and receiving fluid (**FR**) by HPLC.

The concentration of caffeine accumulated in the skin was plotted as a function of time (t). The apparent permeability coefficient (Papp) was calculated using *Equation 2* (Sintov, Greenberg, 2014).

$$Papp = \frac{\Delta Q}{\Delta t} \times \frac{1}{A} \times \frac{1}{C_0} \qquad (2)$$

where: Papp: apparent permeability coefficient, ΔQ: accumulated caffeine concentration, Δt: time variation in hours, A: diffusion area in the donor compartment and C_0: initial concentration of caffeine in the nanoemulsion added in the donor compartment.

Statistical analysis

Statistical analysis was performed using Minitab software (Version 17), by one-way ANOVA followed by the Tukey test or 2-sample Student's T-Test. Differences were considered statistically significant at a value of $p < 0.05$.

RESULTS AND DISCUSSION

Caffeine nanoemulsion preparation

After the emulsion preparation, the **F3** formulation with EHL of 11.1, with the more translucent aspect, was selected. This characteristic indicated a probable nanometric droplet size due to the high light spreadability

(Sarker, 2005). As this formulation presented PIT at 83.5 ºC, the formulation was heated at ~ 80.0 °C, the clearing-boundary temperature (T_{cb}) when formulation became opalescent and slightly bluish (Mei *et al.*, 2011).

The other formulations (**F1, F2, F4** to **F6**) were not stable and/or more translucent than the **F3** that was choseniced, because it occurred phase separation and very opalescent appearance, so they were not recorded to the others analyses.

Obtaining formulations by the sub-PIT method was accelerated by the addition of salts that provide the salting-out effect. In this work, the sodium chloride (NaCl) was added in order to obtain the salting-out effect and, consequently, reducing T_{cb} and avoid providing more energy to the system (Mei *et al.*, 2011). The conductivity in an O/W emulsion is due to the presence of electrolytes in the aqueous phase (Zhang, Michniak-kon, 2011). During the PIT emulsification process, at the PIT temperature occurs the inversion of phases, i.e, the oil phase becomes the continuous phase and the conductivity drops abruptly. Thus, the electrical conductivity of the formulations is an indicator of phase inversion. As soon as the PIT temperature was achieved, the formulation must be cooled quickly, preferably in an ice bath. This process inverted the phases again, yielding an emulsion with reduced droplet diameter, that is, an O/W nanoemulsion (Mei *et al.*, 2011).

By the exposed, the measurement of the conductivity, as well as the determination of PIT, became commonly used methods to characterize nanoemulsions. The temperature that initiated the phase inversion in **F3** was 82.0 ºC and at 85.0 ºC the lowest conductivity value occurred. Following, the formulation was cooled in an ice bath, the conductivity presented values near the initials, indicating the return of the state of nanoemulsion O/W. This conductivity profile was similar to the one found in the literature. Due to their thermodynamic instability, emulsions tend to reduce their free energy and interfacial area through some degradation process, such as cremation/sedimentation, aggregation, flocculation, coalescence and Ostwald ripening (Mei *et al.*, 2011).

Characterization of nanoemulsion

By the graphs of intensity distribution, number and volume of **F3**, it could be concluded that the preparation method used (sub-PIT) was effective in obtaining monodisperse nanoemulsion. Nanoemulsion **F3** presented a mean droplet diameter equal to ~ 40 nm. The polydispersity index (PDI) was lower than 0.3 adequate according to the literature (Kaszuba, 2015; Dario *et al.*, 2016; Anton, Vandamme, 2008).

Normal Stability Assessment

During the NSA, **F3** did not have the organoleptic properties (appearance, color and odor) changed during the 90 days of the test, when stored at 25.0 ± 20 °C (room temperature) and 5.0 ± 2.0 °C (refrigerator). However, at 45.0 ± 2.0 °C, the formulation became opaque whitish and presented phase separation before 7 days because the high-temperature condition usually leads to greater instability in the formulations (Baby *et al*., 2007; Pianovski *et al*., 2008; Dario *et al*., 2016). For this reason, the data of size, polydispersity and zeta were not registered for this condition. These results indicated that high temperatures storage conditions should be avoided.

The pH value is an important parameter for monitoring the stability of emulsions as changes in this value indicate the occurrence of chemical reactions that may compromise the quality of the final product and its shelf-life. Emulsions formulated with vegetable oils, such as capric/caprylic acid triglycerides, may have their pH value decrease due to the hydrolysis of the esters of fatty acids which generate free fatty acids (Martini, 2005). In addition, the final pH of the nanoemulsion may exert an influence on the extent of ionization of the interface components.

The pH values of **F3** stored at 25.0 ± 2.0 °C and 5.0 ± 2.0 °C decreased over the 90 days of the assay (pH was 5.7 in t_0). The results in the period between t_0-t_7 days presupposed time to stabilize the pH value, probably because the nanoemulsions are thermodynamically unstable (Dario *et al*., 2016). In the interval of t_7 to t_{60} days the pH value was stable and close to the value 5.0 in both temperature conditions (room and refrigerator), Figure 1. The variation observed initially was within the range of physiological pH (4.0 – 6.0) (Pires, Pscheidt, 2017).

It is important to mention that the tendency to aggregation and sedimentation of dispersed nanoparticles, as a function of time, can be monitored by determining changes in particle size distribution (Schaffazick *et al*., 2003). Therefore, it is important to monitor particle size over time and during the NSA.

The PDI value remained constant at 0.2 at the refrigerator temperature (5.0 ± 2.0 °C) while at room temperature condition (25.0 ± 2.0 °C) this parameter dropped to 0.1 and remained constant, and it was considered adequate for both storage conditions (Nemen, Lemos-Senna, 2011; Anton, Vandamme, 2009; Kaszuba, 2015).

At 5.0 ± 2.0 °C the mean droplet diameter increased 21.7% after 90 days, while this parameter increased 50,8% at 25.0 ± 2.0 °C, which indicates that low
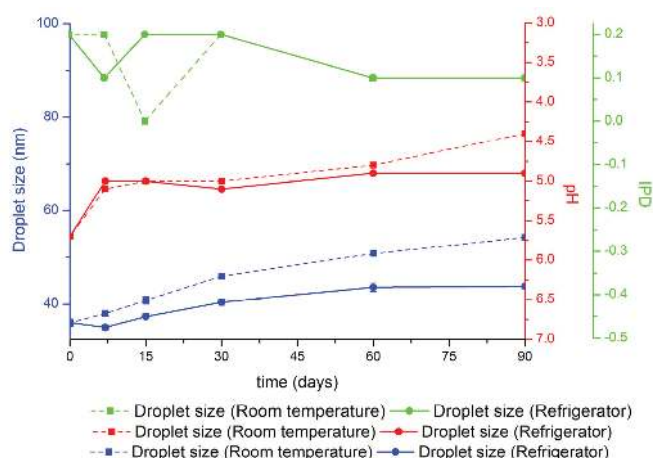

FIGURE 1 - Nanoemulsion **F3:** Droplet size, Polydispersion index (PDI) and pH value in the Normal Stability Assessment at 25.0 ± 2.0 °C (room temperature) and 5.0 ± 2.0 °C (refrigerator).

temperature (refrigerator) is the best condition of storage for this nanoemulsion. Due to the physical instability, this parameter was not assessed for the formulation stored at 45.0 ± 2.0 °C.

A likely explanation for emulsion instability is the maturation of Ostwald, known as Ostwald ripening. This is related to the instability rate of the dispersed phase component and can be described by using certain parameters, which are known as solubility coefficient, dispersed phase diffusion and interfacial tension between the droplets and the continuous phase. That is, the monomers of the surfactants below the critical micelle concentration (CMC) can migrate to other micelles increasing the size of existing micelles. However, concentrations above CMC do not have as much effect on stability once since the highest concentration of possible micelles was formed, stabilizing the medium (Rinaldo *et al*., 2012). The formation of an emulsion is related to its oil phase content and composition, especially the surfactant/oil phase ratio which determines the size of the emulsion droplets (Mei *et al.,* 2011).

According to Martini (2005), the zeta potential was defined as the potential difference between the ions strongly attached to the particle surface and an uncharged neutral region of the solution, with a significant difference in viscoelasticity when compared to the solution adjacent to the droplets. When the zeta potential is 30 mV (in modulus) or larger, the repulsive force of the double layer is greater than van der Waals' attractive force, thus avoiding possible flocculation. A high positive or negative value of the zeta potential is important for the physicochemical stability of the emulsion since the repulsive forces between the droplets

prevent flocculation. Changes in the ionic atmosphere, such as that one caused by the addition of electrolytes, ionic surfactants and changes in the pH of the solution, can directly affect the charge of the droplet.

The zeta potential of **F3** was, initially, -3.9 mV at pH 5.7, due to the non-ionic surfactant used in the nanoemulsion. The probable explanation is due to the theory of the influence of the hydrogen bonds, and the negative surface charge was derived from the specific adsorption of hydroxyl ions directly from the formation of hydrogen bonds between hydroxyl ions and water molecules in the boundary layer. They also mentioned that the presence of fatty acids assists in the negative charge of the droplet (Mei *et al.*, 2011). Once **F3** has an ingredient with triacylglycerols in its composition, the negative interface attracts ions H$^+$, these protonate the fatty acids and attenuate the negative charge resulting in a low value of zeta potential.

On the other hand, non-ionic surfactants, such as those used in **F3** nanoemulsion, provide steric stability in the dispersed phase, delaying Ostwald ripening and coalescence (Kourniatis *et al.*, 2010). The hydrophilic groups derived from the nonionic surfactants attract the water around them giving rise to a protective barrier that prevents coagulation, that justifies the stability of **F3** (Oliveira, Mei, 2009).

Ostwald ripening constant

Emulsions and nanoemulsions are thermo-dynamically unstable systems, thus tending to destabilize during storage by various mechanisms, such as gravitational separation, flocculation, coalescence and Ostwald maturation. Emulsified systems should inhibit these mechanisms and provide enough kinetic stability to maintain the product's validity (Piorkowski, Mcclements, 2014).

At 25.0 ± 2.0 ºC there was a slightly better linear correlation for the parameter ω_3 (R^2 = 0.984) in relation to the parameter ω_2 (R^2 = 0.969), suggesting that the diffusion of oil molecules was the first mechanism responsible for increasing the diameter of the drop in this temperature condition. The values of the Ostwald ripening constant in the room temperature condition (25.0 ± 2.0 °C) ($\omega_2 = 3.8 \times 10^{-5}$ nm^2 s^{-1} and $\omega_3 = 0.01043$ nm^3 s^{-1}) were lower than the value mentioned by Wooster, Golding and Sanduansri (2008) ($\omega_3 = 0.0483$ nm^3 s^{-1}). Dario *et al.* (2016) obtained the best correlation for $\omega_3 = 2.5 \times 10^{-6}$ nm^3 s^{-1}, probably due to the addition of cetyltrimethylammonium chloride, a cationic surfactant to a similar formulation which improved the stability.

The values of the Ostwald ripening constant for 5.0 ± 2.0 °C condition ($\omega_2 = 6.2 \times 10^{-6}$ nm^2 s^{-1} and $\omega_3 = 0.0001$ nm^3 s^{-1}) were lower than those observed for at 25.0 ± 2.0 °C, which indicates that the storage at low temperatures (refrigerator condition) improves nanoemulsion stability. This phenomenon is observed because higher temperatures allow the oil to become more soluble in the aqueous phase promoting instability system (Dario *et al.*, 2016).

Raman spectroscopy

The Raman spectra provide information about the structure of the vibrational transitions of molecules, assigning a wave number and specific bands, which can be called the ''fingerprint'' of the substances (Andreev *et al.*, 2001), which is based on the results obtained in the literature.

Pavel *et al.* (2003) labeled some specific structural characteristics of the anhydrous caffeine molecule. The N^7 methylated atom in the ν (CN) stretching occurs at 1408 cm^{-1}, numbered in the molecular structure of caffeine as N^7-C^{14}. The draw ν (CN) at C^2-N^3 occurs at 1284 cm^{-1}; the ν (CN) stretch at C^8-N^7 occurs at 1554 cm^{-1}; The C^{14}-N^7-C^8 deformation (CNC) occurs at 556 cm^{-1}; The δ deformation (HC = N) in C^8-N^9 occurs at 1073 cm^{-1}. In addition, the least specific molecular assignments were made for the modes ν (CN, C = C) and ν (CN) at 1600 e 1360 cm^{-1}, respectively (Figure 2) (Pavel *et al.*, 2003).
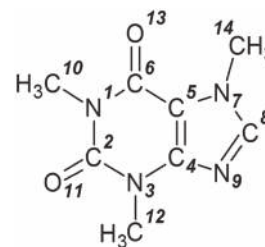

FIGURE 2 - Chemical structural of caffeine (Chemsketch software version 11, 2007; Tagliari *et al.*, 2012).

Raman spectra of anhydrous caffeine are present in Figure 3, and values have been reported in the range of 400-1800 cm^{-1}. The wave numbers of the bands are presented in the Table II and the vibrational assignments are provided for caffeine from data published by Pavel *et al.* (2003) and Matas *et al.* (1998). Edwards, Munshi and Anstis (2005) mentioned that water is easily lost from the crystalline mesh of caffeine and significant changes are observed in the stretches ν (C=O) and in the region of the vibrational structure by the decrease of the intermolar attraction CH… OH$_2$ (Edwards, Munshi, Anstis, 2005).

TABLE II - Raman spectrum and attributions for hydrated and anhydrous caffeine

Hydrated caffeine pH 7	Caffeine anhydrous	Attribution
1643(m)	1657(m)	v(C=O)out of phase + $_v$(C=C)
1337(s)	1328(s)	v(ring imidazole)
1296(m)	1284(m)	v(C-N) + ρ(CH$_3$)
651.5(m)	642.9(m)	δ(OCN) + δ(anel imidazole pirimidina)
556.2(s)	555.4(s)	δ(anel pirimidina) + δ(CNC) + ρ(CH$_3$)

Legend: (v)=stretch, (δ)=angular deformation; (m)=medium, (s)=strong, (ρ)=translational mode, (Edwards, Munshi, Anstis, 2005; Pavel et al., 2003).

The difference in the position of the bands was used to show in which phase the caffeine is mostly found. The caffeine spectrum was compared in different pH values (2.0; 5.0; 5.5; 6.0; 6.5 and 7.0) in order to verify the possible displacement of bands, which would suggest its protonation. This fact did not occur in the experimental conditions, which indicated that caffeine had no influence on the pH values evaluated.

The caffeine bands found in the **F3** nanoemulsion were compatible with those found in the caffeine solution (bands 1337, 652.5 and 558.2 cm^{-1}), probably due to their hydrophilicity and as it is in the hydrated form in the nanoemulsion (Figure 3). Caffeine has a hydrophilic character with log P = -0.07 and pka = 8.3 (Tagliari *et al.*, 2012; Sintov, Greenberg, 2014; Abd *et al.*, 2016).

The difference in the anhydrous caffeine compared to the solution was expressed by more intense bands displaced to the lowest wave number, Figure 3. The assignment of bands is in Table II. Displacements greater than 2 cm^{-1} are indicative of interactions (Gil, 2015). Thus, according to Table II, there was an interaction of the caffeine with the aqueous phase, probably due to its hydrophilic nature, except for the assignment corresponding to the δ (pyrimidine ring) + δ (CNC) + ρ (CH3) that has a lipophilic character.

Entrapment Efficiency (EE%)

The obtaining method and formulation composition provided a low entrapment efficiency (4.8%), probably due to the hydrophilicity of caffeine (log P = -0,07) (Tagliari *et al.*, 2012; Sintov; Greenberg, 2014; Abd *et al.*, 2016). This result indicated that caffeine was mostly present in the continuous phase. As Hernández-Marín *et al.* (2016) achieved the encapsulation efficiency > 80% in caffeine multiples emulsions, water in oil nanoemulsion should be more efficient in order to improve the encapsulation efficiency (Hernández-marín *et al.*, 2016).

Eye irritation potential (HET-CAM)

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (2010) advocated that the results of HET-CAM test are valid only if the positive controls were classified as strongly irritating by the eye Irritation Index (I.I.). In this study, the positive control was considered highly irritating because presented eye I.I. = 1.4. Hemorrhage was present only in one sample of the triplicate and no coagulation or lysis was observed (Figure 4).
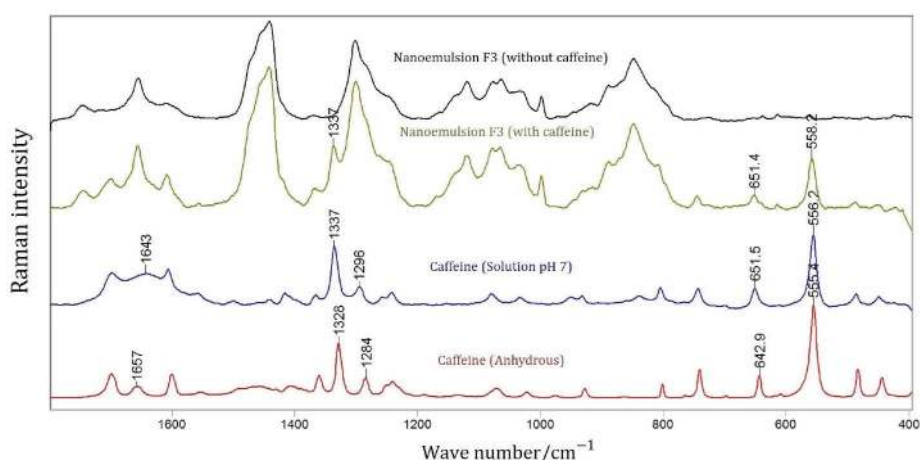

FIGURE 3 – Comparison of the Raman spectrum bands of caffeine in solution and anhydrous with the nanoemulsion **F3** in the presence or absence of the same.
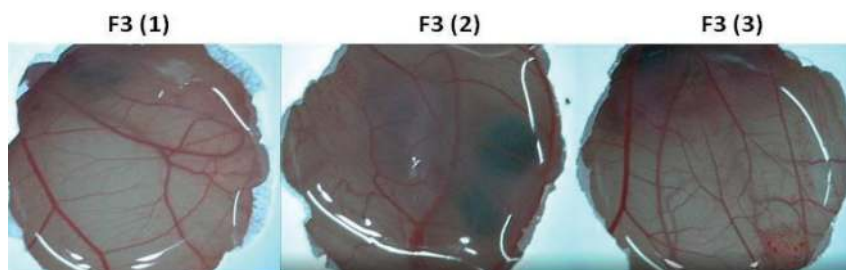
FIGURE 4 - Image of chorioallantoic membranes (CAM) after 300 s contact with nanoemulsion F3 (in triplicate).

Skin permeation assay

Challenging the hypothesis that the nanoemulsion may improve the cutaneous permeation, diffusion cells also called Franz cells (Smith, Haigh, 1989) were used.

The solubility of caffeine in PBS was evaluated preliminary with a value that is lower (0.02 mg/mL), relative to water solubility, than Budavari *et al.* (1996) (21.74 mg/mL). The soluble caffeine value in the saturated PBS buffer was 0.09%, considering 21.74 mg/mL as 100% solubility for caffeine in aqueous unsaturated solution.

Before the test, the Transepidermal Water Loss (TEWL) of skin was evaluated in order to determine its integrity and was 12.63 $g/m^2/h$ (n = 3), which is within the range of acceptable TEWL of skin (between: 6.11-47.67 $g/m^2/h$) (Gardien *et al.*, 2016). The area of diffusion was 2.26 cm^2 and the mean thickness of the skin was 3.67 mm. The volume occupied by the receiving fluid was 3.0 mL.

In vitro permeation study indicated that caffeine incorporated in **F3** and the caffeine solution permeated equally statistically, with p-Value > 0.05 (α = 0.05). Caffeine solution presented higher apparent permeability coefficient than **F3**, 0.0024 cm/h and 0.00005016 cm/h, respectively. These results suggested that the saturation of caffeine in the donor compartment favored the skin's permeation, although the permeated values were statistically equal. The other compartments presented the concentrations presented in Table III.

Sintov and Greenberg (2014) mentioned the low apparent permeability coefficient for the human membrane relative to other animal models such as pig.

Swabs were used to remove the excess of formulation from the skin. Thus, higher concentrations of caffeine were expected to be quantified in this location. The condition of caffeine bound in the O/W emulsified system did not help its cutaneous permeation, however there was the visual benefit of aspect of formulation F3 with low caffeine precipitation.

CONCLUSIONS

The sub-PIT formulation methodology was effective in obtaining a monodispersed nanoemulsion with reduced average droplet diameter (~ 40 nm) with a narrow distribution (PDI < 0.3). The best formulation was achieved using surfactant mixture HLB equal to 11.1. Caffeine-loaded nanoemulsion (**F3**) was found to be very stable when stored under low (5.0 ± 2.0 °C) temperature condition. Raman spectra assay corroborated the low caffeine entrapment efficiency (4.8%) and the *in vitro* permeation assay results, permeation assay results, suggesting that caffeine remained in the continuous phase (aqueous) probably due to the hydrophilicity of caffeine in the O/W nanoemulsion system. However,

TABLE III - *In vitro* permeation profile in human skin of nanoemulsion with caffeine 1% w/v and caffeine solution in water (1% w/v) after 24 h assay

Location	Experimental concentration (µg/mL) ± SD		Student's T-Test
	F3	Sol. CAF 1%	p-value
Swab 1	55.072± 41.93	38.5± 32.38	0.626
Swab 2	36.58± 27.51	22.8± 5.01	0.187
Epidermis	0.43± 0.38	1.03± 1.52	0.573
Dermis	5.45± 1.76	20.55± 17.84	0.277

Legend: **F3** = nanoemulsion containing caffeine (1% w/w); **SD:** standard deviation; **SWAB:** cotton swab; **Sol. CAF 1%:** caffeine solution 1% w/v.

despite these results, the caffeine-loaded nanoemulsion presented visually and sensorially better than the solution, once solubility was improved by the surfactants added to the formulation. Thus, the developed nanoemulsion may not be a viable alternative to enhance skin permeation of hydrophilic active ingredients like caffeine, but may enhance its solubility and stability in cosmetic formulation.

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