Development of W/O emulsion for encapsulation of “Pitanga” (*Eugenia uniflora* L.) leaf hydroethanolic extract: droplet size, physical stability and rheology

Larissa TESSARO¹, Milena MARTELLI-TOSI¹, Paulo José do Amaral SOBRAL¹²*  

1 Introduction

The “Pitangueira” tree (*Eugenia uniflora* L.) can be found in South America, Southern Asia and Africa (Arai et al., 1999). Its fruits, called “Pitanga” or the Brazilian cherry, are edible and eaten fresh or in marmalade (Rattmann et al., 2012). The leaves of these trees are also well known and used in folk medicine to assist in the treatment of several diseases (Arai et al., 1999; Consolini & Sarubbio, 2002; Rattmann et al., 2012).

Recently, several researchers have demonstrated the high antioxidant activity of the “Pitanga” leaf’s extracts (Garmus et al., 2014; Lorenzo et al., 2018; Schumacher et al., 2015; Vargas et al., 2016, 2019). These bioactivities are due to the presence of some phenolic acids and flavonoids (e.g. gallic acid, elagic acid and myricitrin) (Bezerra et al., 2018; de Oliveira et al., 2018) and terpenoids (i.e. germacrene D and trans-caryophyllene) (Garmus et al., 2014). Nevertheless, these active compounds are very sensitive to environmental conditions, such as light and oxygen, and their storage can be an important challenge.

The encapsulation of PLHE in a W/O emulsion by adding this product in the water phase can be an approach to guarantee its stability (Ali & Akhtar, 2014, Kaimainen et al., 2015, Tepsongkroh et al., 2015, Liu et al., 2018, Rahpeyma & Sekhavatizadeh, 2020). A predominantly hydrophobic emulsifier, such as Polyglycerol polyricinoleate (PGPR), is necessary to produce a W/O emulsion (Fraj et al., 2017). The PGPR has been widely used in researches on W/O emulsions because it is an effective hydrophobic emulsifier, and has been used in several similar studies (Márquez et al., 2010, Ushikubo & Cunha, 2014, Tepsongkroh et al., 2015, Dridi et al., 2016, Matos et al., 2018, Velderrain-Rodriguez et al., 2019). It has been also widely used in food industries to stabilize W/O emulsions such as margarines, butter, salad dressing and chocolate (Okuro et al., 2019).

W/O emulsions containing plant extracts in the W inner phase can be applied in different industries, such as foods, pharmaceuticals and cosmetics. In the food industry, for example, W/O emulsions containing plant extracts are widely used to produce functional oily foods with a lower total fat content and desirable texture (i.e. dairy products) (Rahpeyma & Sekhavatizadeh, 2020), to increase the oxidative stability of the dispersing oil phase (Liu et al., 2018), and to protect and control the release of active aqueous compounds (Mohammadi et al., 2016; Rabelo et al., 2018). In the pharmaceutical and cosmetic industries, W/O emulsions containing plant extracts can be used to produce topical products for the treatment of diseases (Rasul et al., 2011) or pro-age (Huma et al., 2020), respectively.

The aim of this study was to encapsulate PLHE in a W/O emulsion and analyze the effects of emulsifier concentrations and phase ratios on its droplet size and physical stability. Some physical properties, including viscosity, of W/O emulsion with the more stable formulation, were also studied. To the best of our knowledge, no works on PHLE encapsulation in an W/O emulsion has been previously published.
2 Materials and methods

2.1 Material

“Pitanga” (Eugenia uniflora L.) leaves were collected in Pirassununga (SP), Brazil. Grinsted Polyglycerol Polyricinoleate (PGPR®) was donated by DuPont (São Paulo, Brazil).

2.2 Production of “pitanga” (Eugenia uniflora L.) leaf hydroethanolic extract (PLHE)

“Pitanga” leaves were washed and dried in an air circulating oven (MA035/5, Marconi, Brazil) at 42 ºC/72 h, then they were ground and sifted in a 48-mesh sieve. The leaf powder was dispersed (1g/10 mL) in a 60% ethanol solution and treated by ultrasound at 40 kHz (Odontobras, 1440 DA, Brazil) at 25 ºC/40 min. Subsequently, this dispersion was treated in a magnetic heater (Gehaka, AA-2050, Brazil) at 80 ºC for 30 min and then filtered using filter paper (Whatman n°1). This extract was evaporated under vacuum (Tecnal, TE-211, Brazil) at 40 ºC/4 h and then water was removed by freeze-drying (Heto, model FD 1.0-60, Germany) for 5 days and stored in the dark at 4 ºC (Vargas et al., 2019). The freeze-dried powder was re-suspended in distilled water (1 g/10 mL water) prior to the production of W/O emulsions.

2.3 Production of water-in-oil (W/O) emulsions

The W/O emulsion was prepared using PLHE in the aqueous phase (10, 20 and 30% w/w) and soybean oil containing hydrophobic emulsifier PGPR (3 and 5 g/100 g oil) as the oil phase (90, 80 and 70% w/w). Briefly, the W/O emulsion was prepared by dropwise addition of PLHE in the oil phase under homogenization at 15,000 rpm for 5 minutes (Labotechnik, IKA T25, Germany). Then, this coarse W/O emulsion was homogenized through power ultrasound (Branson Ultrasonics, Sonifier® SFX550, USA) operating at 20 kHz with an amplitude of 30% during 3 cycles/30 s (Leong et al., 2018), both preparations were in ice bath. These emulsions were produced in triplicate and characterized just after their production.

2.4 Characterization of the emulsions

All emulsions characterizations were done in triplicate, at least.

Droplet size distributions and zeta-potential measurements

Prior to these analyses, the W/O emulsion samples were diluted 100x in chloroform, according to Souilem et al. (2014). Droplet size distributions and Zeta-potential of the W/O emulsions were determined by dynamic light scattering (Malvern Instruments, Zeta Sizer Nano ZS, UK) at 25 ºC.

The droplet size distributions and the surface-based average droplet size of the W/O emulsions were calculated using ZetaSizer Nano software (Dammak & Sobral, 2018; Matos et al., 2014). The Sauter size (D_{3,2}) and span were calculated using the Equations 1 and 2, respectively, where d_i is the droplet size and n_i the surface frequency of droplets with size d_i.

\[
D_{3,2} = \frac{\sum n_id_i^3}{\sum n_id_i^2}
\]

(1)

\[
\text{span} = \frac{D_{90,90} - D_{10,10}}{D_{90,90}}
\]

(2)

When bimodal droplet size distribution occurred, D_{3,2} and span values were calculated for each peak using Equations 1 and 2, respectively, where \(D_{v,10}, D_{v,50}, D_{v,90}\) corresponded to the surface-based size at 10, 50 and 90% of cumulative surface for each peak (Dammak & Sobral, 2017, 2018).

2.5 Analysis of the physical stability

The physical stability of W/O emulsions was studied using a multi-sample analytical photocentrifuge (L.U.M. GmbH, LUMiSizer, Germany) with the following parameters: 1.8 mL of sample, 2,32 5x g, time 3,600 s, time interval 10 s, at 20, 40 and 60 ºC (Dammak & Sobral, 2017, 2018). The instability Index (ii) was calculated as the ratio of the height of the creamy layer in relation of the height of the initial emulsion.

2.6 Confocal laser scanning microscopy (CLSM)

The morphology of the droplets of the W/O emulsion with the more stable formulation was qualitatively analyzed using a confocal laser scanning microscope (Leica Microsystems GmbH, SP5, Germany), with objective of 63x (1.4 aperture and oil immersion). The W/O emulsion was prepared with rhodamine B solution (0.1% m/v) added in the PLHE. Then, the W/O was diluted 200x in chloroform, and the rhodamine B was excited with HeNe laser at 543 nm and the emitted light was recorded between 570 to 640 nm. These analyses were made in the Multi-User Laboratory for Confocal Microscopy – LMMC of FCMRP-USP.

2.7 Physical properties

The W/O emulsion that had the more stable formulation was qualitatively analyzed using a confocal laser scanning microscope (Leica Microsystems GmbH, SP5, Germany), with objective of 63x (1.4 aperture and oil immersion). The W/O emulsion was prepared with rhodamine B solution (0.1% m/v) added in the PLHE. Then, the W/O was diluted 200x in chloroform, and the rhodamine B was excited with HeNe laser at 543 nm and the emitted light was recorded between 570 to 640 nm. These analyses were made in the Multi-User Laboratory for Confocal Microscopy – LMMC of FCMRP-USP.
the knowledge of the critical micellar concentration would be necessary.

For the purpose of comparison, the \( D_{3,2} \) of W/O emulsions was calculated, and it ranged from 0.3 to 4.5 µm (Table 1), which is in the range of those found in the literature (Table 2). The increase in PGPR concentration from 3 to 5 g/100 g of oil resulted in a decrease (\( p < 0.05 \)) in the \( D_{3,2} \) for the W/O emulsions with 10/90 (4.5 to 0.4 µm) and 30/70 (1.5 to 0.9 µm) W/O ratios. Similarly, Márquez et al. (2010) observed that the increase of the PGPR concentration from 0.2 to 1% (in whole emulsion) in 20:80 W/O emulsions provoked a reduction in \( D_{3,2} \) from 2.9 to 1.3 µm (Table 2), as in this work (Table 1).
These behaviors were observed by Tepsongkroh et al. (2015), who reported similar behavior for W/O produced with different concentrations of PGPR emulsifier. Furthermore, Matos et al. (2018) compared the $D_{32}$ values of W/O emulsions with different emulsifiers (PGPR, Span 80, Plurol oleique and PeccoI) and observed that the W/O emulsion containing PGPR had the smallest $D_{32}$ value (Table 2), confirming that PGPR was a more effective emulsifier in the production and stabilization of W/O emulsions. Nevertheless, for the W/O emulsion with 20/80 ratio, the PGPR concentration did not affect ($p>0.05$) the $D_{32}$ values (Table 1). A possible explanation is that the presence of some bioactive compound present in PLHE extracts could act as W/O stabilizer considering that these emulsions can be applied to thermal processed foods. This temperature range was interesting because these emulsions will guarantee, thus, that the PLHE will be well dispersed into biopolymer matrix.

Further, the W/O ratios also affected ($p < 0.05$) the $D_{32}$ values (Table 1). Considering that the expected behavior must be similar to that observed by Katsouli et al. (2017), even if working with nanoeumulsions (NE): increasing W phase content in W/O NE led to higher droplet sizes; it can be considered that for emulsions containing 3 g PGPR/100 g oil, the $D_{32}$ increased from 0.25 to 1.53 μm when the W phase content increased from 20 to 30% (Table 1). The higher value (4.52 μm) for 10/90 emulsion containing 3 g PGPR/100 g oil was due to the insufficient stabilization of droplets, as explained above. For emulsions containing 5 g PGPR/100 g oil, overall, the $D_{32}$ increased with the W phase content and the higher value (0.85 μm) was observed in the 30/70 W/O emulsion (Table 1).

But, Ushikubo & Cunha (2014), studying the effect of two W/O ratios on emulsions produced with PGPR as emulsifier, didn’t find such effect ($D_{43}(30:70) = 1.15 \mu m \approx D_{43}(60:40) = 1.19 \mu m$). And, curiously, Okuro et al. (2019) produced a W/O emulsion with very high-water phase ratio (75:25) and emulsified by PGPR, and determined $D_{43}(2.1 \mu m)$, in the same range of this work (Table 1), which means that the W/O ratio could not have so important effect on droplet size. Similar results were observed by Gomes et al. (2016), also working with 75:25 W/O emulsion and with Gallic acid solution as W phase. By the way, these authors determined higher $D_{43}$ values when using pure water as W phase.

Matos et al. (2018) determined $D_{40.5} = 0.66 \mu m$ for W/O emulsions produced with 20% (v/v) W phase (water/ethanol) containing trans-Resveratrol, and 80% of miglyol 812 containing 5% of PGPR, as O phase (Table 2). This value was similar to those determined in this study (Table 1).

**Physical stability of W/O emulsions**

The physical stability of W/O emulsions was studied at 20, 40 and 60 °C. This temperature range was interesting because these emulsions can be applied to thermal processed foods. For instance, it can be used to produce active films loaded with encapsulated PLHE in such emulsions, and film processing imply the preparation of film-forming solution in temperature above the room temperature (Tessaro et al. 2021). The good stability of these emulsions will guarantee, thus, that the PLHE will be well dispersed into biopolymer matrix.
The curves of the W/O emulsion's light transmission profiles, as determined by the change in laser transmission in a layer (33-35 mm) of W/O emulsion, were basically straight horizontal lines (Figure 3) and always demonstrated a low transmission (<10%) for all studied temperatures. This behavior was a consequence of the opacity in the whole sample, which was due to a uniform droplet distribution throughout the sample layer inside the cuvette (Figure 3), not destroyed by the high gravity force during analysis, confirming the high stability of these emulsions (Dammak & Sobral, 2017).

The instability Index (ii), calculated from the spectra presented in the Figure 3, has an arbitrary scale ranging from 0 (more stable) to 1 (less stable) (Figure 4). Regardless of temperature and the emulsion's formulation, the ii ranged from 0.009 to 0.063 (ii<<0.1), meaning that these systems were highly stable. Overall, iIs were correlated with $D_{3,2}$, but it was significant only for ii determined at 60 °C: $ii = 7.6 \times 10^{-3} D_{3,2} + 0.026$, $R^2 = 0.81$. Moreover, the high stability of W/O emulsions can be also associated to two factors: the viscosity of the lipid phase (and consequently, the high viscosity of the whole emulsion, as can be seen in 3.1.4) and the use of PGPR as emulsifier (Dickinson, 2011; Ilić et al., 2017; Tepsongkroh et al., 2015). According to Gomes et al. (2016), the good stability of the emulsions with PGPR could be attributed to the attachment of PGPR hydrophobic chains in the branched structure of the oil.

Ushikubo & Cunha (2014) studied the stability of W/O emulsions produced with different oils and emulsifiers and observed that emulsions produced with PGPR and soybean oil were the most stable. On the other side, Rabelo et al. (2018) studied the stability of nanoemulsions with different W/O ratio and Açaí extract (AE) concentrations by measuring its creaming index, and observed that samples with higher AE concentrations were able to stabilize the nanoemulsions systems, especially for 30:70 W/O. They explained these results considering that AE is rich in amino acids, such as methionine, threonine, and lysine; hence, they might play a role in stabilizing the nanoemulsions.

Although no significant differences were observed between emulsions containing 3 and 5 g PGPR/100 g oil for 20/80 W/O

![Figure 3](image.jpg) Profiles transmission of W/O emulsions determined using an analytical centrifuge (2.325 x g, time 3600 s) at 20, 40 and 60 °C (photo of cuvettes with samples after analysis were attached in figures). Red-green lines are the light transmission profiles measured during analysis for each time.

![Figure 4](image.jpg) Instability index* of the W/O emulsions with different W/O ratios, at 20, 40 and 60 °C. *Different lowercase letters at the same concentration of emulsifiers and different uppercase letters in same ratio indicate significant differences between averages according to Tukey test (p < 0.05).
condition at 20 and 40 °C, a significant difference (p<0.05) was observed at 60 °C: 3 g PGPR/100 g oil produced a more stable 20/80 W/O emulsion (Figure 4). And, this can be important for eventual application of this emulsion on thermal processed food, for instance. This W/O emulsion showed il of 0.009 (20 °C), 0.016 (40 °C) and 0.024 (60 °C) (Figure 4). Therefore, the emulsion with a 20/80 W/O ratio and 3 g PGPR/100 g oil was chosen as the most stable formulation because they presented the well distributed droplet size (unimodal), the lowest D₃,₂ (0.25 ± 0.02 mm), and the highest physical stability at 60 °C.

Confocal laser scanning microscopy (CLSM)

The micrographs obtained using the CLSM allowed observation of a drop of PLHE dispersed in the oil phase (Figure 5). Due to the limited sensitivity of the microscope, it was not possible to observe the smallest drops of PLHE. The red color at the PLHE-oil interface corresponds to the excitation of rhodamine B present in the PLHE. This showed that the active compounds present in PLHE, predominantly polyphenols, tended to remain at the PLHE-oil interface after the process of emulsification.

In fact, it is known that some antioxidant compounds, such as polyphenols, can remain at the water-oil interface of emulsions, interacting with both phases contributing to the reduction of the interfacial tension between them (Katsouli et al., 2017; Velderrain-Rodriguez et al., 2019). That is: the PLHE may have some contribution to the high stability of the W/O emulsions produced. Gomes et al. (2016) determined lower sedimentation index (higher stability) for emulsions prepared with Gallic acid solution as W phase compared with this result for emulsion with pure water, as W phase. According to these authors, the Gallic acid could increase emulsions stability in two ways: a) changing the polarity of the phases leading to increased solubility between them or b) penetrating the interfacial film as a surfactant causing increased flexibility of the interface. The result presented in the Figure 5 corroborates with this last explanation (b).

Some physical properties of W/O emulsion with the more stable formulation

The best W/O emulsion presented a density equal to 0.937 ± 0.001 g/cm³, lower than that of water due to the presence of the oil phase. Considering the soybean oil density (ρₒ = ~ 0.92 g/cm³) (De Almeida et al., 2013), and the water density (ρₖ = 0.999 g/cm³), the emulsion density (ρₑ) can be calculated using the principle of volume additivity (1/ρₑ = Wₒ/ρₒ + Wₖ/ρₖ) as 0.935 g/cm³, very close of the experimental value. The contribution of PGPR and PLHE can be neglected because its concentrations in the whole emulsion were very low.

The pH of this emulsion was 5.12 ± 0.01, due to the presence of some acids in the PLHE whose pH is 4.12 ± 0.02. An acidic pH (4.2-5.5) in W/O emulsion containing Moringa leaves extract in W phase at various storage conditions was also observed by Ali & Akhtar (2014). Similar values were determined by Huma et al. (2020). The pH value determined in this work is acceptable for its use as a food additive or even in the pharmaceutical and cosmetic industries, since human skin has a pH between 4.5 and 6.0, and cosmetics must have a pH in this range (Ali & Akhtar, 2014).

The refractive index of this emulsion was 1.640 ± 0.004, which is typical of a mixture with phases with different refractive indices values (water = 1.333; soybean oil = 1.467) (Chanthrapornchai et al., 2001), as in the case of emulsions, which consequently appear to be opaque (Figure 1). This result is in accordance with the low light transmittance through the sample, as can be observed in Figure 3.

Regarding the emulsion viscosity, it was observed that best W/O emulsion behaves as a Newtonian fluid (Figure 6). Its viscosity was calculated (R² > 0.99) as 104.6 ± 0.6 mPa.s. This value was higher than that of pure water (~1 mPa.s) and even that of soybean oil (~60 mPa.s) at 25 °C (Ilić et al., 2017), because the droplets contributed to increasing the viscosity

![Figure 5. Confocal laser scanning micrographs of the W/O emulsion with the more stable formulation and stained with Rhodamine B. Source: own authorship.](image)

![Figure 6. Flow curve of the W/O emulsions, at 25 °C.](image)
of the system, according to the Krieger Dougherty relation (Shrestha et al., 2011). Tepsongkroh et al. (2015) observed that the increase of PGPR concentration, from 2 to 8%, increased the viscosity of 20:80 W/O emulsions from ~90 to ~116 mPa.s, that is, in the range of that determined in this work.

Nevertheless, a non-Newtonian behavior has been observed by Ushikubo & Cunha (2014), working with 30:70 W/O emulsions prepared with soybean oil and PGPR, and water, and by Rahpeyma & Sekhavatizadeh (2020), working with 10:90 W/O emulsions prepared with canola oil with GMS, and water containing green coffee extract. Okuro et al. (2019) also observed a non-Newtonian behavior working on 75:25 W/O emulsions emulsified by PGPR.

The high viscosity found in this work also could have contributed to the high stability of W/O emulsion (McClements, 2004), and could be interesting for application of this emulsion in dressing sauces, for example, even if this product must be thermal treated until 60 °C because it is very stable in this condition.

4 Conclusions

This study showed that it is feasible to encapsulate the PLHE in a stable W/O emulsion system using PGPR as emulsifier and ultrasound equipment to homogenize. The W/O emulsions showed small and very homogeneous droplet sizes and a very high physical stability and Newtonian behavior. Overall, these properties were affected by the concentrations of the emulsifier (PGPR) and phase ratios. In addition, the analyzed physical properties of the more stable primary emulsion have shown that it can have potential applications in the food, pharmaceutical and cosmetic industry.

Acknowledgements

The authors acknowledge the São Paulo State Research Foundation (FAPESP) for the grants (2013/07914-8 and 2016/18788-1) and the Brazilian National Council for Scientific and Technological Development (CNPq) for the Research fellowship of Paulo J.A. Sobral (30.0799/2013-6). This study was financed in part by the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil” (CAPES) - Finance Code 001 (PhD fellowships of Larissa Tessaro).

References


Garmus, T. T., Paviani, L. C., Queiroga, C. L., Magalhães, P. M., & Cabral, F. A. (2014). Extraction of phenolic compounds from pitanga (Eugenia uniflora L.) leaves by sequential extraction in fixed bed extractor using supercritical CO2, ethanol and water as solvents. The Journal of Supercritical Fluids, 86, 4-14. http://dx.doi.org/10.1016/j.jsuf.2013.11.014.


