Pequi Oil Microencapsulation by Complex Coacervation using Gelatin-Cashew Gum

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Abstract

New functional foods and beverages can be developed using bioactive compounds present in pequi oil. Complex coacervation is an encapsulation method used for preserving bioactive molecules, especially those that are hydrophobic or sensitive to high temperatures. The objective of this work was to produce and characterize pequi oil microparticles using cashew gum/gelatin matrix (CG/GE) through complex coacervation. Gum Arabic (GA) was also studied in comparison with CG. The coacervation process was performed without pequi oil to determine the ideal proportions of the matrix components, followed by the embedding of the oil in the microparticles for evaluation. Satisfactory microparticles were produced at pH 4.5 in the weight ratios of CG/GE = 2:1 and GA/GE = 1:3. Pequi oil release was greater in acidic pH, especially at pH 2 for the CG/GE matrix. The encapsulation efficiency for CG/GE and GA/GE was 72.53% (±4.80) and 82.77% (±6.09), respectively. The results showed that the CG/GE combination seemed very promising as an encapsulation matrix, especially for food applications involving pH values higher than 3.

Keywords: Anacardium occidentale; Coacervate; Encapsulation; Gelatin; Caryocar coriaceum; Polysaccharides

1 Introduction

Microencapsulation is often used in powder technology to describe the process of forming an amorphous polymeric coating around a core to control mass transfer and provide protection in the dry state from interactions with its environment, thereby minimizing changes in colour, aroma and flavour and enabling controlled release (Gouin, 2004).

The particles derived from encapsulation methods often display different physical shapes and structures that influence the release profile and storage stability of the core material (Aguilera,

2005; Re, 2006). In the present study, microencapsulation was achieved through the complex coacervation method, which entails the preparation of a mixture of polyelectrolytes of opposite charges in an aqueous solution, resulting in separate layers; the dense layer is rich in polymers (coacervate) and the dilute phase is depleted in polymers (Black et al., 2014). This method is also recommended for the microencapsulation of hydrophobic materials and/or substances sensitive to high temperatures (Alvim & Ferreira Grosso, 2010).

Pequi tree oil is a seed oil with a peculiar odour and aroma that is distinct and noticeable. The consumption of this oil has attracted attention due to its potential health benefits. It is rich in oleic acid (Lopes et al., 2008) and widely used in foods because of its high content of vitamins (de Paula-Ju, H. Rocha, Donatti, Fadel-Picheth & Weffort-Santos, 2006) and carotenoids (Azevedo-Meleiro & Rodriguez-Amaya, 2004). The presence of these compounds provides skin protection by preventing free radical formation and, therefore, slowing down the aging process (Pianovski et al., 2008). Other benefits associated with the use of this oil include treatment for gastric ulcers (Quirino et al., 2009), anti-inflammatory property, and cutaneous wound-healing support (Batista et al., 2010). In view of its beneficial effects, it is highly desirable to preserve bioactive compounds in pequi oil through microencapsulation in order to develop functional food and drinks for the market.

Gums are among the biopolymers most commonly used as wall materials in microencapsulation methods (Jafari, Mahdavi-Khazaei & Hemmati-Kakhki, 2016; Khoshakhlagh, Koocheki, Mohebbi & Allafchian, 2017; Revuelta, Chacon Villalba, Navarro, Guida & Castro, 2016). Gum Arabic is widely used, despite its cost and occasional supply problems related to climatic, economic and political problems in the African region that produces it (Andrade, Carvalho & Takeiti, 2013). Complex coacervation with gum Arabic and gelatin has been extensively investigated (Anvari & Chung, 2016; Habibi, Keramat, Hojjatoleslamy & Tamjidi, 2017; Lv, Yang, Li, Zhang & Abbas, 2014); however, studies involving cashew gum-gelatin using this method are recent and

relatively sparse in the literature (Comunian et al., 2016; de Souza et al., 2018; Gomez-Estaca, Comunian, Montero, Ferro-Furtado & Favaro-Trindade, 2016). Cashew gum is considered an alternative to gum Arabic, although it is not yet a commercial product (Rodrigues, 2004). Cashew gum is an anionic polysaccharide and has low viscosity in water. The negative charge on cashew gum in aqueous solution makes it possible to interact with positively charged polymers. Earlier studies in this laboratory have demonstrated cashew gum as an efficient encapsulating matrix (da Silva et al., 2018; de Oliveira, Paula & de Paula, 2014; Gomez-Estaca et al., 2016; Rodrigues & Grosso, 2008). In this work, pequi oil microparticles were produced using cashew gum/gelatin matrix through complex coacervation. Moreover, the particles were characterized by microscopy, encapsulation efficiency (superficial and total oil), yield and oil release, and particle size.

2 Materials and Methods

2.1 Materials

Cashew gum (CG) was collected from *Anacardium occidentale* L. plants from Embrapa Tropical Agroindustry Experimental Field in Ceará - Brazil (coordinates: 4˚11'26.62" S and 38˚29'50.78" W). Gum Arabic (GA) was purchased from JB Química Indústria e Comercio Ltda and the gelatin (GE) 225H type B was provided by Rousselot®. The pequi oil (*Caryocar coriaceum* Wittm.) was purchased from a local provider in Ceará, Brazil (coordinates: 07˚18'19" S and 39˚18'08" W). All reagents used were analytical grade.

2.2 Cashew Gum Isolation

The isolation of cashew tree polysaccharide exudate was carried out according to Torquato et al. (2004), with some modifications. A 500 g exudate sample was ground by a knife mill and solubilized in water in the proportion of 300:1 (g/L). After solubilization, the sample was filtered, centrifuged at 15,303 x g for 10 min at 25°C and precipitated in 1:3 ethanol (v/v) for 24

h at 10°C. The precipitate was dried in an air-circulating oven at 60°C for 24h and was ground afterwards. The moisture of the polysaccharide isolated was 12.58 ± 0.43 %.

2.3 Microparticles without pequi oil

Suspensions of CG 1% (w/v), GA 1% (w/v) and gelatin 1% (w/v) were prepared at pH 4, 4.5 and 5, respectively, for zeta potential analysis. From the data, the proportions of each polymer and the ideal pHs for the formation of the microparticles (CG/GE and GA/GE) were established in accordance with Prata and Grosso (2015).

The procedure used for the production of microparticles was as follows. The gelatin and gum Arabic solutions were prepared separately followed by homogenisation in an Ultra-Turrax homogeniser at 10,000 rpm for 3 min at room temperature. 100 mL of each suspension was homogenised in this way at room temperature. Then 400 mL of distilled water was added and homogenised in the Ultra-Turrax at 10,000 rpm for 3 min at room temperature. The pH was adjusted with hydrochloric acid (2M) to 4.0, 4.5 and 5.0, and the solutions were refrigerated (8 ± 2 ^{o}C) overnight for precipitation of the particles. Subsequently, excess water was eliminated and coacervate suspensions were obtained for the analyses.

Zeta potential analysis

Coacervate suspensions were lightly homogenised and the zeta potential was determined with a Zetasizer Nano ZS 3000 dynamic light scattering instrument (Malvern Instruments, UK), operating with a laser light at a wavelength of 633 nm.

Spectrophotometric analysis

The coacervate formation was indirectly analyzed by the reading of suspension absorbance using a spectrophotometer (Cary 50 Conc, Varian) before and after cooling (8 ± 2 ^{o}C) at the wavelength of 200 nm. Only the supernatant from each sample was used for the analysis (da Silva et al., 2018).

Coacervate yield

Coacervates were centrifuged at 15,303x g for 10 min at 25 ^{o}C and dried in a drying oven at 105 ^{o}C until constant weight was achieved. The yield was calculated through the relationship between the initial dry mass used and the final mass obtained from the formula R = 100 m$_f$ / m$_0$, where R is the percent yield, mf is the final dry mass and m$_0$ the initial mass (gum mass + gelatin mass) (Huang, Sun, Xiao & Yang, 2012).

2.4 Microparticles with pequi oil

Emulsions were prepared with pequi oil in gelatin dispersion at 10,000 rpm for 5 min at room temperature. The GA solution was then slowly added to the gelatin-stabilized emulsion to a final aqueous volume of 200 mL, using the same procedure adopted for evaluation of the coacervation process presented before without pequi oil (Section 2.3). Five levels of pequi oil were tested: 0.5g, 0.75g, 1g, 2g and 2.5g. Coacervates were characterised for their yield, oil release property, encapsulation efficiency, and by microscopy.

Characterisation by optical microscopy

The microscopic slides were previously sterilised with 70% ethyl alcohol. A drop of the coacervate suspension and a cover slip were placed on each slide. Optical micrographs were recorded on a Zeiss optical microscope coupled with a digital image acquisition system through a CCD camera.

Particle size determination

Microparticle size was determined with the use of the Malvern 3000 Zetasizer Nano ZS laser light scattering instrument (Malvern Instruments, UK). Precipitated coacervates were suspended in isopropyl alcohol in the proportion of 0.5 g to 25 mL of alcohol. The volume moment mean diameter D$_{4,3}$ was measured, which represents the diameter of a sphere with the same average volume in the sample and the Span which gives the information on the homogeneity of the

size distribution of the particles (Hosseini et al., 2013).

Yield analysis

The yield was calculated from the formula R = 100 m_f/m_0 as previously described (Section 2.3.3), in which R is the percent yield, m_f is the final dry mass and m_0 the initial mass (gum mass + gelatin mass + oil mass) (Huang et al., 2012).

Encapsulation efficiency

The suspensions of microcapsules were previously frozen in an ultra-freezer (Liotop FV500, Liobras) and put in the freeze dryer for 72 h (Liotop, model K1005) for determination of surface and total oil. The moisture content (%) was calculated on a wet-weight basis. The encapsulation efficiency (EE) was determined by Equation 1:

$$EE(\%) = \frac{TO - SO}{TO} \times 100 \qquad (1)$$

Where TO is the amount of the total oil and SO is the amount of the surface oil.

Determination of total oil (TO): Total oil extraction was performed according to the Bligh-Dyer method with modifications (Checci, 2003). Thus, 0.1 g of the freeze-dried microcapsules were weighed and resuspended in 10 mL chloroform, 20 mL methanol and 8 mL distilled water. The mixture was homogenized in a magnetic stirrer for 30 minutes; then, 10 mL chloroform and 10 mL of 1.5 % sodium sulphate solution were added and stirred for another 2 min. The material was transferred to a separating funnel and allowed to stand until complete phase separation. From the organic phase, approximately 15 mL was collected. About 1 g of sodium sulphate was added to the organic phase and then filtered. A 1 mL portion was transferred to a 10-mL volumetric flask, and made to volume with hexane. The amount of oil in the microparticles was calculated by using an appropriate calibration curve of free oil in hexane obtained on a spectrophotometer at 450 nm wavelength. Each batch of samples was measured in triplicate.

Determination of surface oil (SO): The amount of oil present on the surface of the freeze-dried microcapsules was evaluated spectrophotometrically, according to a method proposed by Higuita (2013) with modifications. About 0.1 g microcapsules was resuspended in 10 mL of hexane in a test tube and stirred on a vortex shaker for approximately 1 min. The amount of oil in the microparticles was calculated by using an appropriate calibration curve of free oil in hexane obtained on a spectrophotometer at 450 nm wavelength. Each batch of samples was measured in triplicate.

2.5 Oil release

Pequi oil release at different pH ranges was evaluated using the methodology described by Comunian et al. (2016) with some modifications. After microparticle formation, the pH was adjusted from 2.0 to 9.0 in 100 mL of coacervate suspension, which was kept under agitation with a magnetic stirrer for 2 min. Hexane was added in a ratio of 2:1 (v/v), agitated for 1 min, and centrifuged at 15.303x g for 10 min at 25ºC . The organic phase containing the hexane was isolated and rotoevaporated for the determination of free pequi oil present. The amount of oil mass was measured in the pre-weighed boiling flask.

2.6 Statistical analysis

A completely randomized design was used in this work and the results represent the means of three replicates. The means were compared through Student's t-test. The statistical analysis was performed with Statistica 13.0 software.

3 Results and Discussions

3.1 Microparticles without pequi oil

In an acid medium, gelatin is positively charged (below the isoelectric point) and attracted to

the negatively charged cashew gum to form co-acervate droplets. Thus, the proportions of the encapsulating matrices were determined based on an electrical equivalence study performed through an electrical charge analysis (zeta potential) of each biopolymer in solution at pH 4.0, 4.5 and 5.0. The zeta potential values of the polysaccharides and protein in the pH range at room temperature are shown in Table 1.

The optimum condition for coacervate formation is reached at a pH where the associated biopolymers are electrically equivalent in terms of opposing charges (Comunian et al., 2016; de Kruif, Weinbreck & de Vries, 2004; Schmitt & Turgeon, 2011). In order to reach this electrical neutrality, the proportions found for the CG/GE treatments were 2:1 at pH 4.5 and 1:1 at pH 5.0; for the GA/GE treatment, they were 1:4 at pH 4.0 and 1:5 at pH 4.5. However, in an attempt performed with 1:5 ratio at pH 4.5, the GA/GE suspension after refrigeration (8º C ±2) formed a very viscous gel due to the high gelatin concentration, making it impossible to form the coacervate droplets. For this reason, the ratio was reduced to 1:3 at pH 4.5. Thus, the formation of the polyelectrolyte complex depends on the degree of polymer ionization, and, therefore, on the pH. In addition, the polymer stoichiometry, structural parameter (conformation and chain length), and other parameters such as ionic strength, temperature and nature of the reactants may also exert an influence on the complex formation and stability (Kim et al., 2016; Siow & Ong, 2013). Thus, although the electrical equivalence of the complexes has been deduced for the polymers separately, the zeta potential corresponding to each complex formed was also analyzed, as shown in Table 2.

G/GE, at the ratio of 2:1 and pH 4.5, showed a zeta potential value close to zero, demonstrating electrical equivalence between biopolymers that is conducive to coacervate formation. The combination of the two biopolymers in the proportion of 2.5:1 at pH 4.0 was previously tested by Comunian et al. (2016) to encapsulate Echium oil, although a prospective study of the best proportion at a different pH range has not yet been reported. At pH 4.5, GE acquires a positive charge and forms coacervates with anionic polysaccharides, as does GA. As the GE used is

type B (acidic) and its isoelectric point lies in the range of 4.7 to 5.3, the pH selected to encapsulate pequi oil was in accordance with those reported in the literature (Azeredo, 2008).

The absorbance of the CG/GE and GA/GE suspensions before and after the refrigeration process (8 ± 2 ºC) was monitored, so that the absorbance variation could be related to the coacervate precipitation. The results of the absorbance variation for the CG/GE and GA/GE treatments are shown in Figure 1.

A greater absorbance variation was found in the CG/GE complex at ratio 2:1 and pH 4.5, indicating a higher level of coacervate precipitation when compared to 1:1 at pH 5.0. For the GA/GE treatment, the ratio of 1:4 at pH 4.0 possibly presented a negative variation, indicating that, after refrigeration (8 ± 2 ºC), the suspension became cloudier than before, probably because of the formation of a very dense gel. The negative absorbance variation value reflected the poor coacervate precipitation in this condition. However, for the ratio of 1:3 at pH 4.5, it displayed a considerable variation in absorbance and, consequently, more significant coacervate precipitation.

The conditions determined for each CG/GE and GA/GE complex were also evaluated regarding yield at a pH range. Yield is important because it is related to the production cost of the encapsulation process. The result found was in agreement with spectrophotometric and zeta potential data, where the treatments for 2:1 CG/GE at pH 4.5 and 1:3 GA/GE at pH 4.5 were found to be the most appropriate for coacervate formation (Figure 2).

3.2 Microparticles with pequi oil

Pequi oil microparticles were produced using five levels of oil (0.5 g, 0.75 g, 1 g, 2 g and 2.5 g) while adhering to the experimental settings of 2:1 CG/GE at pH 4.5 and 1:3 GA/GE at pH 4.5. Microparticles formed and precipitated were subjected to yield and particle size analysis by laser diffraction.

The yield based on dry-weight of the microparticles formed is shown in Figure 3. Through this analysis it was possible to establish a rela-

Table 1: Zeta potential values of cashew gum (CG), gum Arabic (GA) and gelatin (GE) in the pH range from 4.0 to 5 at room temperature.

pH	CG (1% w/v)	GA (1% w/v)	GE (1% w/v)
	Zeta potential (mV)		
4.0	3.11	-16.40	3.65
4.5	-2.09	-21.07	4.24
5.0	-1.55	-21.80	1.02

Table 2: Zeta potential values of coacervate suspensions without pequi oil at different pH values after cooling (8 ^{o}C) overnight for precipitation of the particles. The measurements were conducted at room temperature.

Treatment	Ratio (w/w) and pH	Zeta potential (mV)*
CG/GE	2:1 pH 4.5	-0.25 $\pm$ 0.47[a]
	1:1 pH 5.0	-2.96 $\pm$ 0.70[b]
GA/GE	1:4 pH 4.0	7.25 $\pm$ 2.33[c]
	1:3 pH 4.5	1.34 $\pm$ 0.41[d]

*All mean zeta potentials differed significantly ($p \geq 0.05$).
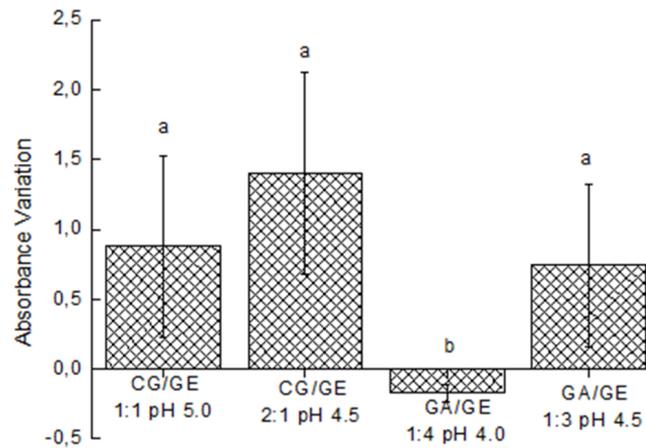
Figure 1: The variation of absorbance (at 200 nm) before and after coacervate refrigeration (8 $\pm$ 2 ^{o}C) with the use of cashew gum/gelatin (CG/GE) at 1:1 ratio/pH 5 and 2:1 ratio/pH 4.5, and gum Arabic/gelatin (GA/GE) at 1:4 ratio/pH 4 and 1:3 ratio/pH 4.5. Bars: standard deviations (n = 3)
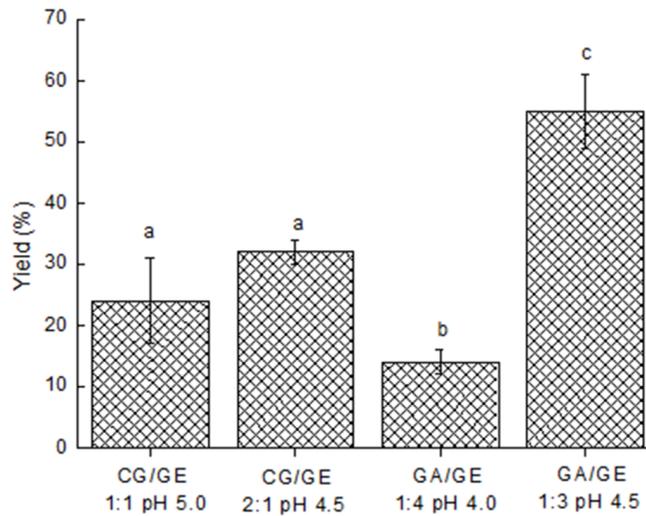
Figure 2: Coacervate yields of cashew gum/gelatin (CG/GE) at 1:1 ratio/pH 5.0 and 2:1 ratio/pH 4.5, and gum Arabic/gelatin (GA/GE) at 1:4 ratio/pH 4.0 and 1:3 ratio/pH 4.5 after drying in an oven at 105 ºC. Bars: standard deviations (n = 3)

tionship between the matrix components and the core and coacervate formation, thereby avoiding material waste and ensuring optimal conditions for encapsulation.

Higher yields of CG/GE particles were found at the lower oil levels. According to de Conto, Grosso and Gonçalves (2013), the higher the wall material concentration and the lower the oil concentration, the higher the yield. However, GA/GE had a higher proportion of wall material in the coacervation process, and we obtained a higher yield when a larger quantity of oil was used. Therefore, in the ensuing work, the use of 1g and 2.5g of pequi oil was selected, respectively, for the treatments of 2:1 CG/GE at pH 4.5 and 1:3 GA/GE at pH 4.5. The 2:1 GC/GE and 1:3 GA/GE particles had a moisture content of 4.7 ± 0.50% and 5.54 ± 0.16%, respectively. These were the largest quantities of oil among those tested with each treatment that had good yield values. Yield results show that GA/GE matrix was more promising than CG/GE in pequi oil encapsulation. However, cashew gum is a reasonable alternative to gum Arabic especially for use in cashew-producing areas in the world.

The size distribution of the particles obtained by laser diffraction analysis displayed a unimodal distribution for the CG/GE and GA/GE treatments. The average particle size values are shown in Figure 4. The $D_{4,3}$ particles were micrometric in size, with GA/GE (4311.85 ± 1428.32 nm) smaller than CG/GE particles (8216.72 ± 1853.32 nm). The Span value found was 0.45 ± 0.04 and 0.23 ± 0.03 for GA/GE and CG/GE respectively, indicating a small dispersity of the particles. Optical microscopy analysis also confirmed the smaller size of GA/GE particles (Figure 5). It is known that the particle size distribution of emulsion droplets is affected by many factors relating to biopolymers and pH, e.g., the addition rate of the acidifying agent for anionic polysaccharides and the rate of formation of gelatin coacervates (Jyothi et al., 2010). In this work the greater amount of gelatin employed in the GA/GE complex probably promoted a better emulsification of the solution and resulted in smaller particles.
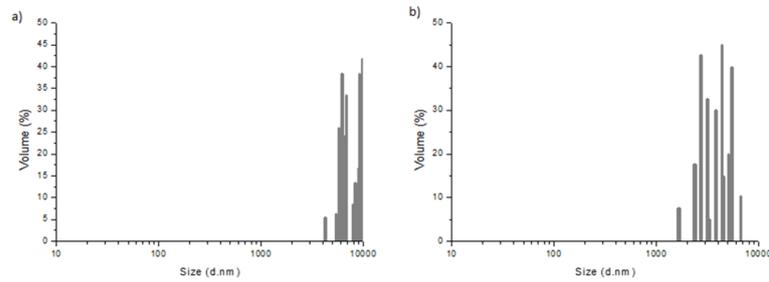
Figure 3: Microparticle yields with 0.5g, 0.75g, 1g, 2g and 2.5g of pequi oil in cashew gum/gelatin matrices (CG/GE) at ratio 2:1/pH 4.5 and gum Arabic/gelatin (GA/GE) at ratio 1:3/pH 4.5 after drying in an oven at 105 ^{o}C. Bars: standard deviations (n = 3)
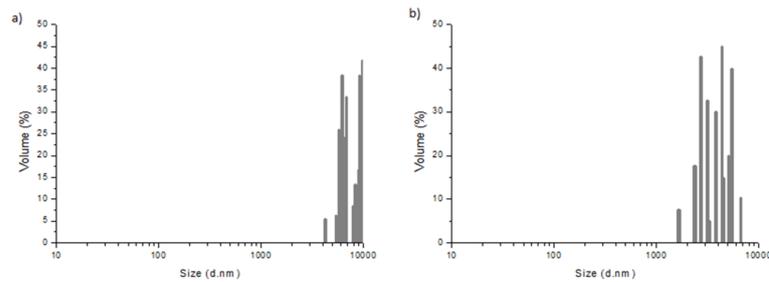

Figure 4: Size of microparticles of (a) cashew gum/gelatin (CG/GE) at ratio 2:1/pH 4.5 with 1g of pequi oil, and (b) gum Arabic/gelatin (GA/GE) at ratio 1:3/pH 4.5 with 2.5g of pequi oil. The measurements were conducted at room temperature.
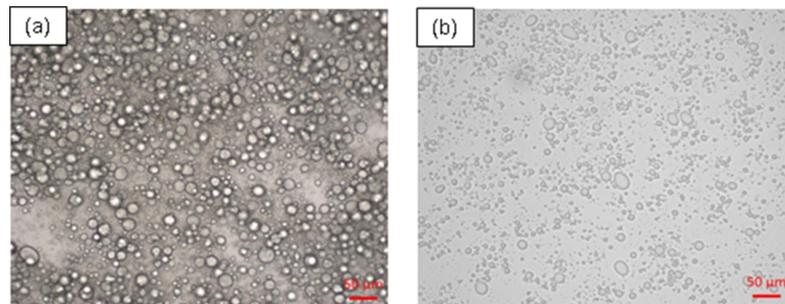

Figure 5: Images obtained by optical microscopy of (a) cashew gum/gelatin (CG/GE) microparticles with 1g of pequi oil, and (b) gum Arabic/gelatin (GA/GE) with 2.5g of pequi oil. The measurements were conducted at room temperature.
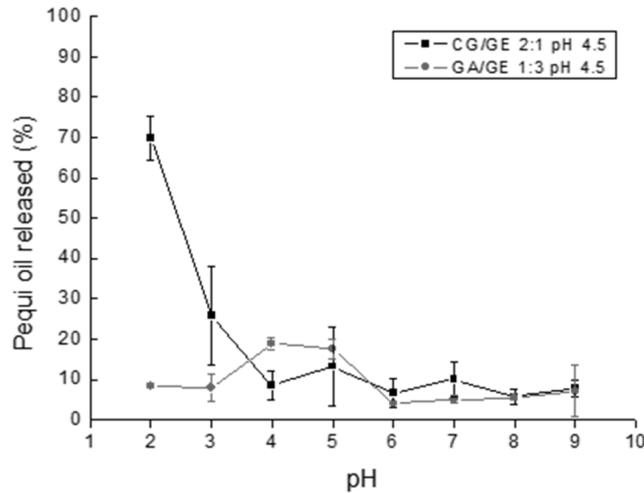
Figure 6: Pequi oil release from cashew gum/gelatin (CG/GE) microparticles at 2:1 ratio/pH 4.5 with 1g of pequi oil, and gum Arabic/gelatin (GA/GE) at 1:3 ratio/pH 4.5 with 2.5g of pequi oil, in the pH range of 2.0 to 9.0 at room temperature. Bars: standard deviations (n = 3)

Encapsulation efficiency

Encapsulation efficiency (EE) reflects the amount of oil trapped inside the particles. GC/GE and GA/GE showed EE of 72.53% ($\pm$4.80) of 82.77% ($\pm$6.09), respectively. Oil retention within the particles is affected by the encapsulation method and the nature of the biopolymers, as well as the particle wall thickness. Thus, a larger amount of wall material may favour oil retention and consequently optimize the encapsulation efficiency (Tang & Li, 2013). In this work, the experimental parameters for polysaccharide-protein complexation were experimentally determined that aimed at the best conditions for coacervate formation, and therefore showed different proportions that also resulted in statistically different EE (p <5%). Similar EE are found in literature using gum Arabic and gelatin for oil encapsulation, although there are different sources, suppliers and viscoelastic properties of gelatin. Liu, Low and T. Nickerson (2010) reported flaxseed oil EE of 84% using type-A gelatin from porcine skin and gum Arabic 1:1 at a constant total biopolymer concentration (2% w/v) and core-to-wall ratio (50:50). Gonçalves, Grosso, Rabelo, Hubinger and Prata (2018) worked with type-B gelatin from bovine skin and gum Arabic 1:1 for thyme essential oil encapsulation and found EE of 90%. Marfil, Paulo, Alvim and Nicoletti (2018) foundan EE of 83.5% for palm oil encapsulation using a 2% concentration of wall material (gelatin type-B from bovine skin and gum Arabic) and 42.8 : 57:2 ratio between core and wall material.

Oil release

The release of the core material is facilitated by certain conditions, such as pH alteration, mechanical stress, temperature, time and osmotic force, among others. This kind of study may be useful for future commercial applications in order to avoid unwanted release and thus contribute to preserving the integrity of the active compound. In this study we evaluated the oil release in the pH range from 2 to 9.

The 2:1 CG/GE treatment presented a high oil release with acidic pH, practically reaching a re-

lease of 70% at pH 2.0. This release decreased until pH reached 4.0, and then showed slight oscillations until pH reached 9.0. It can be affirmed that the pH range in which these microparticles released the most oil was between 2.0 and 3.0. This behaviour is visualized in Figure 6. Since the complex coacervation process is strongly influenced by pH variations, it is likely that in more acidic pH ranges, in which there should be a large CG/GE oil release, the electrostatic interactions that depend on the degree of biopolymer ionization have been undone, leading to a greater oil release. On the other hand, 1:3 GA/GE treatment exhibited low release in the pH range evaluated in this study (<20%). According to Shaddel et al. (2018) the best potential food items for enrichment purposes using gum Arabic and gelatin wall material and black raspberry core seem to be the ones with the pH values between 3.0 and 5.0. Siow and Ong (2013) evaluated garlic oil release from gelatin and gum Arabic particles and found about 90% release during incubation at 37 °C in pepsin solution (pH 2) for 3h. The same authors demonstrated that the oil release from microparticles is slower in cross-linked particles.

4 Conclusion

Pequi oil was successfully incorporated during the formation of CG/GE coacervates. Food applications for these microparticles involving a pH above 3 are recommended because of the greater oil release at acidic pH. It is clear that cashew gum has great potential as an encapsulation matrix, although it is possible to encapsulate a larger amount of pequi oil using GA/GE. Since cashew gum is a by-product of the cashew industry and currently has little commercial value, the possibility of using cashew gum for encapsulation should be a welcome development for the industry.

Acknowledgements

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