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Methods of incorporation of D-limonene microparticles in edible films

Métodos de incorporação de micropartículas de D-limoneno em filmes comestíveis

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Abstract

In the present work, D-limonene was microencapsulated by ionic gelation using alginate and calcium ion and applied in the formulation of gelatin and chitosan biodegradable edible films. Three methods of incorporation of the microparticles were evaluated in the film-forming solution: magnetic stirring, high speed stirring (Ultra-turrax), and sonication. The morphology of the microparticles and films were evaluated by optical microscopy and scanning electron microscopy (SEM). The efficiency of encapsulation (EE), and mean diameter of the microparticles, and mechanical properties and water vapor permeability (WVP) of the films were also determined. The D-limonene microparticles showed a spherical shape with a mean diameter of 134.6 μm, and EE of 83.95 %. The incorporation of the microparticles increased tensile strength, Young's modulus, and WVP, and it reduced the elongation at break of the films. However, among the incorporation methods there was no significant difference in the evaluated properties, suggesting that simple method such as magnetic stirring was sufficient to disperse the microparticles in the filmogenic solution.

Keywords: Biopolymer. Essential oil. Ionic gelation. Optical microscopy. Microencapsulation.

Resumo

No presente trabalho, D-limoneno foi microencapsulado por gelificação iônica utilizando alginato e íons cálcio e foi aplicado na formulação de filmes comestíveis biodegradáveis de gelatina e quitosana. Foram avaliados três métodos de incorporação das micropartículas na solução filmogênica: agitação magnética, agitação de alta velocidade (Ultra-turrax) e sonicação. A morfologia das micropartículas e dos filmes foram avaliados por microscopia óptica e microscopia eletrônica de varredura (MEV). Além disso, nas micropartículas determinou-se a eficiência de encapsulação (EE) e o diâmetro médio e nos filmes as propriedades mecânicas e a permeabilidade ao vapor de água (PVA). As micropartículas de D-limoneno apresentaram formato esférico, diâmetro médio de 134,6 μm e EE de 83,95%. A incorporação de micropartículas elevou a resistência à tração, o módulo de Young e a PVA e reduziu a elongação na ruptura dos filmes. Porém, considerando os métodos de incorporação estudados, não houve diferença significativa, sugerindo que um simples método como a agitação magnética foi suficiente para dispersar as micropartículas na solução filmogênica.

Palavras-chave: Biopolímero. Óleo essencial. Gelificação iônica. Microscopia óptica. Microencapsulação.

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Introduction

Biopolymers, such as proteins and polysaccharides, have been applied in the production of edible coatings and films, because they are non-toxic, biocompatible and biodegradable, and they are derived from renewable sources. In this case, biodegradable films produced by a blend of chitosan and gelatin are being widely studied for application in several foods, because they have good mechanical properties and oxygen barrier. In addition, they are good carriers of bioactive substances (BONILLA; SOBRAL, 2016; HOSSEINI *et al*., 2015; PEREDA *et al*., 2011).

The improvements in the functional properties of gelatin/chitosan films is due to the formation of a polyelectrolyte complex through electrostatic interactions that occur between the positive charged amine groups of the chitosan and the negative charged carboxyl groups present in the side chains of the gelatin (YIN *et al*., 2005). Thus, the formation range of the biopolymer complexes depends on the degree of polymers ionization and, therefore, on the pH of the medium (WEINBRECK; MINOR; DE KRUIF, 2004). The complexation of B type gelatin and chitosan should be performed at pH between 5.5 and 6.5, because above this value the deprotonation of amido groups present in the chain of chitosan may occur, and this makes it insoluble (PRATA; GROSSO, 2015).

The essential oils are natural substances which function is to protect plants and they can be used as natural additives in foods, because they are recognized as GRAS (Generally Recognized as Safe) (RUIZ-NAVAJAS *et al*., 2013). D-limonene (4-isopropenyl-1-methylcyclohexene) is a monoterpene that is usually found in citrus peels such as in lemon and orange peels. It is used as a flavouring in food and as an additive in the cosmetic industry (SUN, 2007). It is also notable for showing antimicrobial activity (ZAHI; LIANG; YUAN, 2015), antioxidant capacity (ROBERTO *et al*., 2010), and chemopreventive and anticarcinogenic properties (CROWELL; GOULD, 1994).

The direct incorporation of essential oils into foods is limited because of their strong taste and flavour. Because they are volatile, they can be lost during food processing. Thus, inclusion in biodegradable films would represent an interesting alternative, because it would allow for the acquisition of active materials that could help extend the shelf-life and add value to the food (ATARÉS; CHIRALT, 2016). Another form of incorporation of essential oils into films could be in the encapsulated form. The advantage is that the oil would be protected from drastic conditions such as light, oxygen, and heat (RUIZ-NAVAJAS *et al*., 2013). This would prevent its degradation or volatilization during film processing. Furthermore, microencapsulation may be a method to slow down the process of releasing the essential oils from the film into the medium, allowing the activity to be maintained for a longer time (GÓMEZ-ESTACA *et al*., 2014).

Among the techniques of microencapsulation, spray drying and lyophilization are widely employed to microencapsulate D-limonene (BURGOS-DÍAZ *et al*., 2018; ORDOÑEZ; HERRERA, 2014; PARAMITA; FURUTA; YOSHII, 2010). However, the particles obtained are hygroscopic and their application in the production of films by casting is difficult, because in this method there is a step of solubilization of the polymers in solvent, which is usually water. With this, the particle would be solubilized and the bioactive compound would be released during the film's production.

In this context, the D-limonene particles obtained by the ionic gelation technique are interesting and feasible alternatives to be used in the production of these types of films. These particles are produced from dripping an anionic polymer solution containing the isolated or emulsified nutrients on an ionic solution to form a complex that has on average 98% moisture (BEAULIEU *et al*., 2002). Thus, both the water of the film and the particles can be removed in the drying step.

The effect of incorporation of microparticles on the functional properties of biodegradable films has been widely reported (CRIZEL *et al*., 2017; DAMMAK *et al*., 2017; KIM *et al*., 2013; MARTÍNEZ-ORTIZ *et al*., 2017; MEDEIROS *et al*., 2019; PAGLIONE *et al*., 2019). However, the methods of microparticle incorporation in these films are seldom discussed.

The aim of this work was to evaluate different methods of incorporation (magnetic stirrer, sonication, and high-speed stirring) of D-limonene microparticles obtained by ionic gelation in biodegradable films produced by casting.

In addition, this work proposes a new technique for evaluating the morphology of the particles present in the film. For this reason, we have chosen to work with films of gelatin and chitosan that are transparent.

Material and Methods

Material

The films were produced using low molar mass chitosan (Sigma Aldrich, USA) with an 85% degree of deacetylation, type B bovin gelatin (Gelita do Brasil, Brazil) with Bloom 250, and glycerol (Dinâmica, Brazil). To obtain the microparticles, sodium alginate (FMC, Brazil, a viscosity of between 400 and 600 mPas, 1%, 20 °C) with a high content of guluronic acid, calcium chloride (Synth, Brazil), and D-limonene (FMC, Brazil) were used.

Production of microparticles of D-limonene for ionic gelation

The microparticles were produced by ionic gelation according previous report (MEDEIROS *et al*., 2019; PAGLIONE *et al*., 2019). Initially, an emulsion containing sodium alginate (1.25%, w/v), D-limonene (ratio of 2:1 in relation to sodium alginate mass), and oleoresin paprika (2% in relation to D-limonene mass) was prepared. The solution was emulsified in an Ultra-turrax (IKA, T18 model, EUA) at 15.000 rpm for 3 min.

The emulsion prepared was atomized over a 2% calcium chloride solution (w/v) with continuous agitation using a double fluid atomizer (Labmaq, Brazil) with a 7 mm diameter. The processing conditions were as follows: emulsion flow rate was 0.03 L/min, air flow rate was 15 L/min, air pressure was 0.125 kgf/cm^2, and spray height (distance between spray nozzle and the sodium chloride solution) was 23 cm. After atomization, we cured the microparticles and kept them under constant stirring in a calcium chloride solution for 15 minutes. After curing, the microparticles were washed several times with water in a 53 μm aperture steel sieve to remove non-complexed calcium chloride.

Characterization of D-limonene microparticles

The encapsulation efficiency test was performed in triplicate using steam distillation with the aid of a Clevenger, as described previous (MEDEIROS et al., 2019; PAGLIONE et al., 2019).

The moisture content of the microparticles was determined by gravimetric method in an oven at 60 °C for 24 h.

The mean diameter (D_{50}) of the wet microparticles were determined by light scattering (Horiba, model LV950, Japan) using distilled water as the dispersing medium.

The morphology of the wet microparticles was visualized in a trinocular optical microscope (Motic, model BA210, China) coupled with a digital camera (Motic, model Moticam +5, China) with image capture using a 10x magnification objective.

In addition, the morphology of the dried microparticles (lyophilized) was evaluated by scanning electron microscopy (Philips, model FEI Quanta 200 Japan). The particles were covered with gold in a Sputter Coater (BAL-TEC, model SCD-050, Balzers, Liechtenstein) and visualized in a scanning electron microscope at an acceleration power of 20 kV. The magnification of observation was 800 x.

Production of gelatin and chitosan films with d-limonene microcapsules

The films were produced by the casting technique (BONILLA; SOBRAL, 2016; CARVALHO *et al*., 2019), where chitosan and gelatin solutions were prepared separately. Chitosan 2% (w/v) was dissolved in in 1% (v/v) acetic acid solution under magnetic stirring at 45 °C for 1 hour, and the pH of the solution was 4.76. Gelatin solution (4%, w/v) with glycerol addition (1 g / 100 ml of gelatin solution) was prepared under magnetic stirring at 55 °C for 35 min, and the pH of the solution was 5.75. To obtain the films, the gelatin and chitosan solutions were mixed in the ratio of 1: 1 (w/w) and 25.4% (w/w) of wet particle was added to the mass of the film-forming solution. This concentration was chosen so that the film would contain 2.5% of essential oil, because according to preliminary tests, this quantity in the films presented antimicrobial and antioxidant activity. Three methods of incorporating the microparticles into the film-forming solution were investigated: (a) magnetic stirring for 10 minutes (MF); (b) high speed stirring (Ultra-turrax) at 10.000 rpm for 4 min (FT) and (c) sonication with 50% power for 2 min, pulse on 20 seconds and pulse off 15 seconds (FS). For all formulations, 50 g of film-forming solution was poured into acrylic plates and dried at 25 °C and 45% RH overnight.

Characterization of films with D-limonene microparticles

The tensile test was performed in a texturometer and the properties determined were tensile strength (MPa), elongation at break (%), and Young's modulus (MPa) according the American Society for Testing and Material (ASTM, 2002). Ten specimens of each formulation were prepared.

The water vapor permeability of the films was determined by a gravimetric method according to the American Society for Testing and Material (ASTM, 2000). The assay was performed in triplicate.

Microstructure analysis of the films was performed using a scanning electron microscope. The samples were previously dried in a desiccator containing silica gel for 14 days. After this period, they were fractured in liquid nitrogen and fixed on stubs with carbon ribbons. The samples were covered with gold in a sputter coater and then the surface and fracture area were visualized in a scanning electron microscope (Philips, model FEI Quanta 200 Japan) at an acceleration power of 20 kV. The magnitude of observation was 400x for the fracture area and 200x for the surface.

The morphology of the films was also performed using an optical microscope (Olympus, model BX4, Tokyo, Japan) with a 10x objective lens. Images were acquired with a digital camera (Olympus, model Q-color 3, Tokyo, Japan) using an optical fiber light source (Olympus, Optical Light Source with Olympus 9095 Fibre Optical Cable, Tokyo, Japan) that focused directly on the film sample (SILVÉRIO *et al*., 2018). This artifact allowed us to obtain images with a three-dimensional depth effect and with better results than conventional optical microscopy.

Statistical analysis

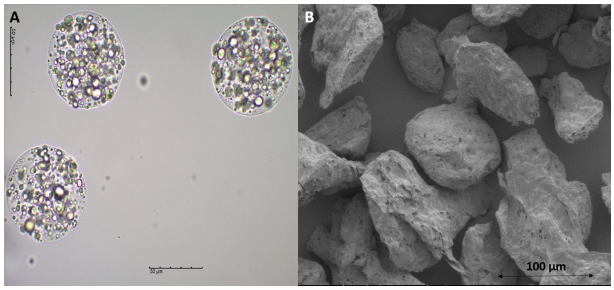
The results of mechanical properties and WVP of the films were evaluated by analysis of variance (ANOVA) and the means of the treatments were compared by the Tukey test at the 5% significance level ($p < 0.05$) using Software Statistica10 (Statsoft, Tulsa, OK, USA).

Results and Discussion

Morphology, mean diameter and efficiency of encapsulation of the D-limonene microparticles

The microencapsulation of D-limonene was successfully performed using the ionic gelation technique with sodium alginate as a wall material and calcium chloride as a crosslinking agent. Figure 1-A shows the morphology and internal structure of the moist microcapsules (approxima-tely 98% moisture), which were presented in spherical format and it was possible to verify well-distributed oil droplets in the sodium alginate matrix.

Figure 1 – Optical microscopy images (A) and scanning electron microscopy (B) of D-limonene microparticles.



Source: The authors.

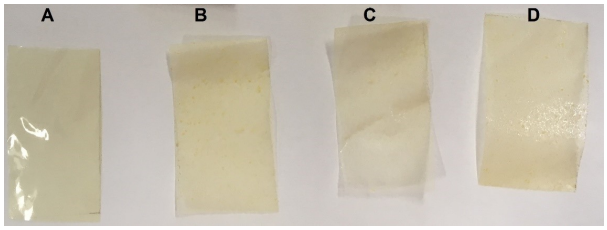
After lyophilization, the microparticle morphology was evaluated by SEM (Figure 1-B) and it was observed that the spherical shape of the microcapsule was not maintained and the surface was rough. Similar behaviour was reported by other authors (AGUILAR *et al*., 2015; TELLO *et al*., 2015) in alginate and pectin microparticles produced by ionic gelation and adsorbed with proteins (ovalbumin and whey protein).

The mean diameter (D_{50}) of the microparticles was $134.6 \pm 2.8\ \mu$m and the values are in agreement with those reported by other authors (AGUILAR *et al*., 2015; BENAVIDES *et al*., 2016; NOGUEIRA; PRATA; GROSSO, 2017; TELLO *et al*., 2015) in alginate microparticles produced by ionic gelation. EE was 83.9% and also in agreement with other studies about microparticles produced by ionic gelation (AGUILAR *et al*., 2015; NOGUEIRA; PRATA; GROSSO, 2017; TELLO *et al*., 2015).

Morphology of films added of the D-limonene microparticles by different methods

The images of the control films, FS, FT, and FM are arranged in Figure 2.

Figure 2 – Gelatin and chitosan films with of D-limonene microparticles added with different methods. (A) FC control; (B) FS microparticles incorporated by sonication; (C) FT microparticles incorporated by high speed stirring; (D) FM microparticles incorporated by magnetic stirring.

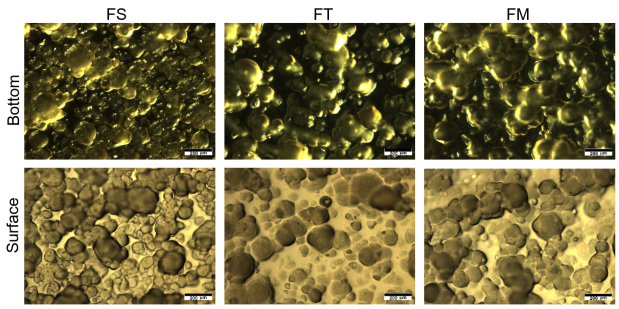


Source: The authors.

The films exhibited, Figure 2, good manuseability and were easily removed from the acrylic plates after drying. The control film was transparent and bright, and its surface was smooth. The films with D-limonene microparticles presented a rough surface and yellowish colour due to the dispersion of the microparticles in the films. The surface that was in contact with the bottom of the acrylic plate was less rough and it is possible that the particles have migrated to the surface during the drying of the films, because they have a lower density than the film-forming solution. The FS, FT, and FM films showed no difference in appearance.

Since the surface roughness of the films was different, optical microscopy images (Figure 3) were obtained by focusing the light source on the surface and also at the bottom of the film. Regardless of the method of incorporation, it was possible to see that microparticles of different sizes were dispersed in the film matrix in the form of aggregates, possibly because of the high amount of microparticles added.

Figure 3 – Surface and bottom optical microscopy of gelatin and chitosan films with D-limonene microparticles incorporated with different methods.

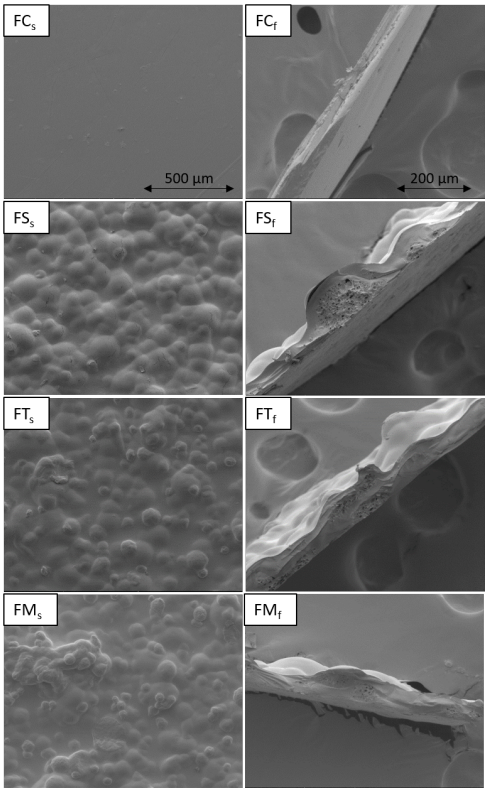


Source: The authors.

The advantage of optical microscopy is that it is possible to realistically view the presence of dispersed microparticles in the film matrix and also their shape (morphology). It is different from SEM because the analysis is simpler and it does not require several steps of sample preparation, such as gold covering, for example. Thus, in this work we propose a new technique to evaluate the morphology of films containing microparticles by using optical microscopy.

Figure 4 shows the surface and fracture SEM images of the films. The surface of the FC film was smooth and homogeneous, indicating good interaction between gelatin and chitosan. The films FM, FS, and FT presented a rough surface due to the presence of microparticles, corroborating with the images obtained by optical microscopy. In the fracture images, the presence of the microparticles is confirmed and they have a porous structure due to the essential oil of D-limonene being dispersed in the internal matrix.

Figure 4 – Surface and fracture scanning electron microscopy of the gelatin and chitosan films with D-limonene microparticles incorporated with different methods (subindex s- means surface images 200x magnification and f- means fracture images 400x magnification).



Source: The authors.

Mechanical properties and WVP of films incorporated with D-limonene microparticles by different methods

The thickness of the films ranged from 117.3 to 199.7 μm, and the addition of D-limonene microparticles, regardless of the method, significantly increased the values. The average thickness of the films FM, FT, and FS approximate the average diameter of the microparticles, as presented previously.

The results of thickness, WVP and mechanical properties of the gelatin and chitosan films with D-limonene microparticles added by different methods are shown in Table 1.

Regarding WVP, in general, the incorporation of microparticles increased the values significantly, independent of the dispersion method studied.

Table 1 – Mechanical properties and WVP of gelatin and chitosan films incorporated with D-limonene microparticles by different methods.

Film	WVP x 10^{11} (g/m.Pa.s)	YM (MPa)	T (MPa)	ε (%)
Control	$6.03^{c}\pm 0.69$	$253.8^{b}\pm 29.1$	$16.9^{b}\pm 2.7$	$58.3^{a}\pm 8.9$
FM	$10.6^{a}\pm 0.09$	$416.4^{a}\pm 48.6$	$20.5^{a}\pm 1.7$	$16.7^{c}\pm 1.6$
FT	$8.39^{b}\pm 0.09$	$414.2^{a}\pm 32.3$	$20.4^{a}\pm 1.0$	$14.5^{c}\pm 1.9$
FS	$9.52^{a,b}\pm 0.06$	$391.6^{a}\pm 28.5$	$19.6^{a}\pm 1.6$	$23.4^{b}\pm 2.6$

WVP = water vapor permeability;
YM = Young's modulus;
T = tensile strength;
ε = elongation at break.
^{a,b,c} Equal letters in the column did not present significant difference by the Tukey test ($p > 0.05$).

Source: The authors.

This may have been due to the lack of chemical interaction between the film matrix (gelatin and chitosan) and the microparticles, resulting in empty spaces (pores and cracks or the presence of irregular structures represented by the particles) that allowed the passage of water vapor. In addition, the amount of microparticles added in the film was high and they formed aggregates as observed by the microscopy images, and this may also have contributed to the increase of empty spaces. The lower WVP value of the control film suggests that chitosan and gelatin presented good interaction, providing a cohesive and homogeneous matrix.

For the mechanical properties, the control film showed higher elongation (E) and lower tensile strength (T) and Young's modulus (MY) than other films. The increase in T value in the FM, FT, and FS samples could be explained by a reinforcing effect from the addition of microparticles which can affect the stress distribution in the films. This was observed in chayotextle starch films with vitamin C microcapsules incorporated and produced by spray drying (MARTÍNEZ-ORTIZ *et al*., 2017). Dammak *et al*. (2017) found that the addition of chitosan and rutin microparticles in gelatin films reduced the value of T, MY, and ε. In the soy protein films the oregano essential oil microparticles increased T and decreased ε values due the reinforcement effect of the microparticles (PAGLIONE *et al*., 2019). Finally, Kim *et al*. (2013) reported that there was no change in mechanical properties when cinnamon oil microcapsules produced by spray drying in low-density polyethylene films were added.

Considering the method of incorporation of the microparticles in the film, no significant difference was observed in the mechanical properties. This suggests that a simple method such as magnetic stirring was enough to disperse the microparticles in the film-forming solution.

Conclusion

In this work it was possible to produce gelatin and chitosan films with D-limonene microparticles. The incorporation of the microparticles provided more resistant films, but there was an increase in WVP. Among the dispersion methods (magnetic stirring, homogenization with an Ultra-turrax, and sonication) of the microparticles in the film-forming solution did not interfere with the mechanical properties and the WVP.

Microencapsulation is a promising technique that enables the application of essential oils in biodegradable films. However, further investigations about the incorporation of different concentrations of particles (wet and dry) in film, evaluation of the antimicrobial and antioxidant activity, and a study of the diffusion characteristics of the D-limonene from the particle to film and subsequently from the film to different release media are necessary.

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