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SHORT COMMUNICATION

Encapsulation of eugenyl acetate in PHBV using SEDS technique and in vitro release evaluation

Raquel A. Loss[1,2] · Gabriela N. Pereira[1] · Daiane L. Boschetto[1] · Gean S. P. Aguiar[1] · Juliana R. Machado[1] · Lorenzo M. P. C. Chaves[1] · Maria J. A. Silva[1] · Débora Oliveira[1] · J. Vladimir Oliveira[1]

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Abstract Eugenyl acetate obtained via enzymatic esterification using Lipozyme TL IM enzyme was encapsulated in biopolymer poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) through solution-enhanced dispersion by supercritical fluids (SEDS). Produced particles were characterized by SEM and confocal microscopy techniques and in addition in vitro release assays were performed in isopropanol and ethyl acetate. Experimental micronization conditions comprised 8 and 10 MPa, 308 and 313 K and eugenyl acetate concentration ranging from 5 to 20 mg mL^{-1}, keeping PHBV concentration constant (20 mg mL^{-1} in dichloromethane). The maximum encapsulation efficiency was 58.0 % for 5 mg $mL^{-1}$of eugenyl acetate at 8 MPa and 308 K. The morphology of the encapsulated particles for most of the trials was spherical, with particle size ranging from 0.061 to 0.276 µm. Regarding the release in ethyl acetate and isopropanol solvents the higher the affinity of the encapsulated ester of these solvents, the faster the release was observed. These results demonstrate the importance of essential clove oil esterification reaction and encapsulation of the ester by SEDS method so that this encapsulated ester can be used in different industrial applications.

Keywords Eugenyl acetate · SEDS · Encapsulation · Release

Introduction

Eugenyl acetate is an ester that can be used as antimicrobial agent (Silva et al. 2015; Chiaradia et al. 2012), anticancerous (Carrasco et al. 2008), acaricide (Pasay et al. 2010), growth inhibitor of *Aedes aegypti* mosquito that transmits dengue (Pandey et al. 2013), prevent teeth decalcification (Marya et al. 2012) and may be employed as additive in biofuels (Kadarohman et al. 2012). Besides, eugenyl acetate forms part of the European Union database and is authorized for use in food. However, this compound is hardly soluble in water, which hinders its direct use in foods and beverages.

An alternative to enable the utilization of eugenyl acetate would be to obtain micro or nanoparticles of this compound, since they can increase the dissolution of poorly water soluble materials (Bahrami and Ranjbarian 2007).

Obtaining micro and nanoparticles can be performed by many conventional encapsulation techniques such as spray drying, freeze drying, solute recrystallization and interfacial polymerization. However, these techniques demonstrate serious drawbacks, like excessive use of solvent, heat degradation of solute, chemical changes, and difficulty in controlling the particle size (Priamo et al. 2011; Aguiar et al. 2016).

In recent years, several researchers have demonstrated that supercritical fluids, especially supercritical carbon dioxide, are useful as anti-solvents to modify material properties such as particle size, size distribution and morphology. The application of supercritical fluids shows interesting advantages like the possibility of eliminating or reducing the use of organic solvents, the efficient

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✉ J. Vladimir Oliveira
jose.vladimir@ufsc.br

[1] Department of Chemical and Food Engineering, UFSC, Florianópolis, SC, Brazil

[2] Universidade Estadual do Estado de Mato Grosso, Barra do Bugres, MT 78390-000, Brazil

separation of the solvent and anti-solvent of the particles after precipitation, hence allowing obtaining solvent-free products (Franceschi et al. 2008a, 2009; Priamo et al. 2011; Boschetto et al. 2013; Machado Jr et al. 2016; Aguiar et al. 2016). Besides, the use of supercritical carbon dioxide as an anti-solvent in the SEDS technique allows working at near-ambient temperature, avoiding degradation of thermosensitive compounds (Cocero et al. 2009).

Thus, since there are no articles in the literature about encapsulation eugenyl acetate either by using supercritical technology or even conventional techniques, in this study, the eugenyl acetate was encapsulated in PHBV by the technique of solution-enhanced dispersion by supercritical fluids (SEDS), with later evaluation of solute release kinetics in ethyl acetate and isopropanol. It is important to highlight that this work is part of comprehensive project whose main aim is to build a database with information of relevant components encapsulated in biopolymers using supercritical fluid technique.

Materials and methods

Materials

Eugenyl acetate was produced by enzymatic synthesis as observed earlier (Silva et al. 2015). Eugenyl acetate was obtained by the acetylation of acetic anhydride with clove essential oil in solvent free system, using 5 wt% of Lipozyme TL IM as catalyst, eugenol/acetic anhydride molar ratio of 1:5 at 70 °C and 150 rpm. After synthesis, the ester was purified in a rotary evaporator under reduced pressure.

Dichloromethane was purchased from Macron Chemicals (USA, 99.5 % purity), ethyl acetate (9.5 % purity) and isopropanol (99.5 % purity) were from LAFAN (Brazil) while carbon dioxide (99.9 % in liquid phase) was supplied by White Martins S.A. (Brazil). Poly(3-hydroxybutirate-co-hydroxyvalerate) (PHBV), with molecular mass (Mw) of 196,000 and poly dispersity index of 1.85 was kindly supplied by PHB Industrial S.A. (Brazil) and was subjected to further purification by dissolving in chloroform (99.5 %) and re-precipitation in *n*-heptane (99.5 %), both purchased from VETEC (Brazil).

Experimental apparatus and procedure of precipitation

The experimental apparatus used in this work for the precipitation of eugenyl acetate with PHBV was the same employed in a previous work by this group and a more detailed description of the apparatus and experimental procedure can be found in Priamo et al. (2011).

For the encapsulation of eugenyl acetate, the experimental conditions used were based on the work of Boschetto et al. (2014): solution flow rate of 1 mL min^{-1}, anti-solvent flow rate of 20 mL min^{-1}, PHBV concentration fixed at 20 mg mL^{-1} in the organic solution. Dichloromethane was used as solvent and CO_2 was used as anti-solvent by SEDS technique.

To evaluate the eugenyl acetate encapsulation by the SEDS technique, assays varying the temperature, pressure and mass of the eugenyl acetate were conducted, where the temperature ranged from 308 to 313 K, pressure from 8 to 10 MPa and weight ester used ranged from 5 to 20 mg mL^{-1}. For comparison, precipitation of pure PHBV was also performed at 10 MPa and 313 K, solution flow rate of 1 mL min^{-1}, anti-solvent flow rate of 20 mL min^{-1}, and PHBV concentration at 20 mg mL^{-1} in the organic solution (Franceschi et al. 2008a, b).

Particle characterization

The shape and particle morphology of the precipitated particles were analyzed by a scanning electron microscope (JEOL JSM-6390LV, United States). The particle size and size distribution was determined using the software Meter Size (version 1.1). From the calculated average particle size (X) and the variation coefficient (VC) were used as statistical tools to express the variability of the data.

Encapsulation efficiency

This parameter was determined as described earlier (Franceschi et al. 2008a, b). A sample of eugenyl acetate and PHBV was weighed and was dissolved in dichloromethane and the solution was analyzed using UV–VIS spectrophotometer (FEMTO, model 800XI, Brazil), with an absorbance at 320 nm. The encapsulation efficiency (EE %) of eugenyl acetate in each run were evaluated by comparison of the results with a standard curve using equation below (Boschetto et al. 2014):

$$\mathrm{EE}\,[\%] = \frac{\text{Mass of eugenyl acetate encapsulated}}{\text{Theoritical loading percentage of eugenyl acetate encapsulated}} \times 100$$

where the theoretical loading percentage of eugenyl acetate encapsulated was the ratio between the mass of eugenyl acetate and the total mass of eugenyl acetate and PHBV used in the precipitation experiments. It was assumed that the ratio between eugenyl acetate and PHBV remained constant after the precipitation.

In vitro release experiments

For the in vitro tests, procedure described earlier was used (Priamo et al. 2011). The encapsulated particles were evaluated in two pure solvents: isopropanol and ethyl acetate, to obtain the release behavior. Encapsulated sample (approximately 0.01 mg mL^{-1}) was placed in contact with solvents (ethyl acetate and isopropanol). The encapsulated esther was dissolved and the solution solvent and esther was incubated in an orbital shaker (Nova Etica, model 501/1D) with a controlled temperature (301 K). All tests were performed in 100 mL Erlenmeyers flasks, covered with plastic wrap to prevent solvent evaporation.

At scheduled time intervals, 2.0 mL were collected from the solution and immediately replenished with pure solvent to maintain the original volume. The samples were filtered and analyzed at 320 nm to determine the concentration of eugenyl acetate released.

Confocal fluorescence microscopy

This technique was used to identify the presence and shape of the eugenyl acetate and it were encapsulated and experimental procedure were reported earlier (Poszwa et al. 2016).

Results and discussion

Co-precipitation of eugenyl acetate and PHBV

The particles obtained in the encapsulation of eugenyl acetate using SEDS technique were characterized by scanning electronic microscopy, with micrographs depicted in Fig. 1. This figure shows that the morphology of eugenyl acetate encapsulated particles for most of the trials was of spherical type. Some experiments resulted in agglomerates

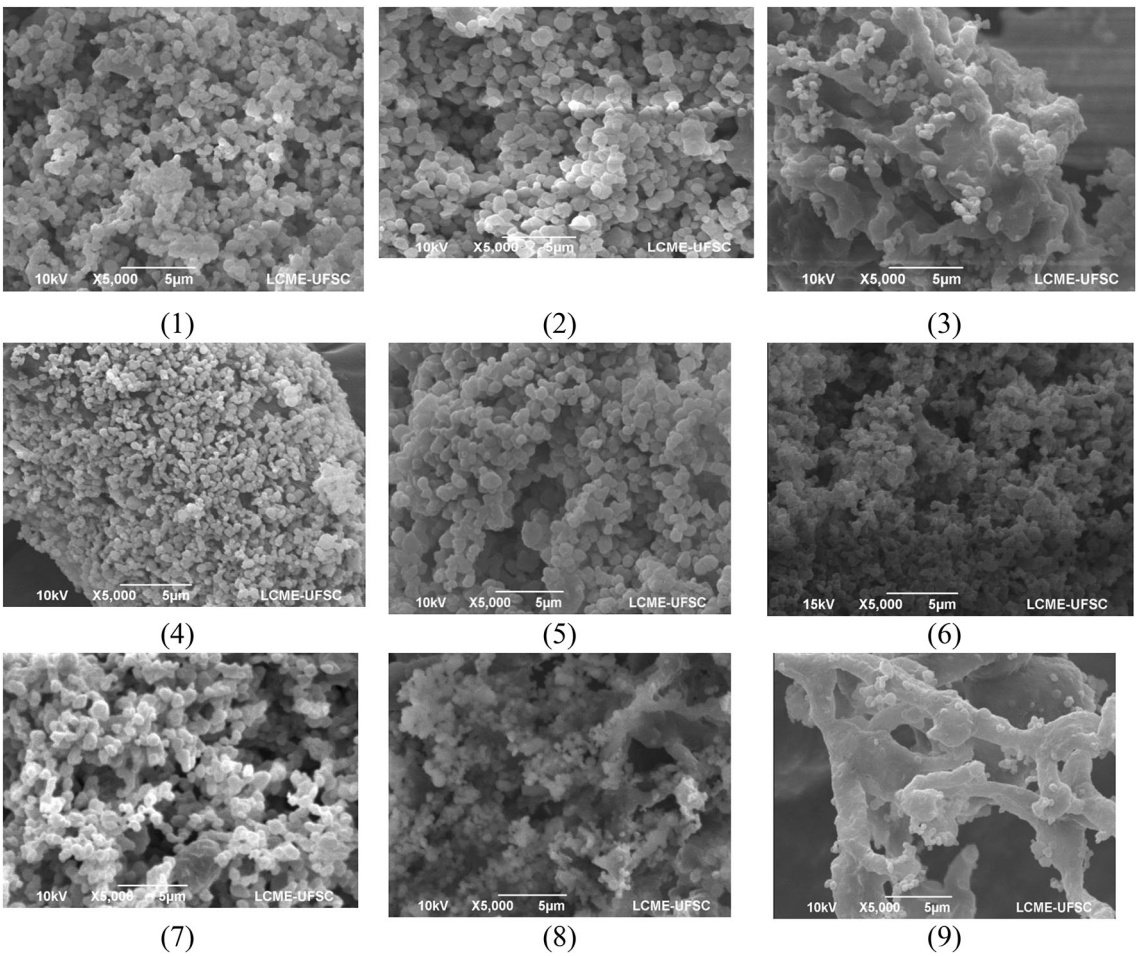

Fig. 1 Morphology of the particles obtained in the encapsulation assays **1**–**8** and precipitation of pure PHBV (run **9**). Experimental conditions: **1** and **2** performed at pressure of 8 MPa, 308 and 313 K, respectively, in concentrations of 20 mg mL^{-1} of PHBV and 20 mg mL^{-1} of eugenyl acetate, **3** 10 MPa, 308 K and at the concentrations of 20 mg mL^{-1} of PHBV and 20 mg $mL^{-1}$of eugenyl acetate, **4** and **5** 8 MPa, 308 and 315 K, respectively, and concentrations of 20 mg mL^{-1} of PHBV and 10 mg mL^{-1} of eugenyl acetate, **6** and **7** 8 MPa, 308 and 313 K, respectively at concentrations of 20 mg mL^{-1} of PHBV and 5 mg mL^{-1} of eugenyl acetate, **8** 10 MPa and 308 K at concentrations of 20 mg mL^{-1} of PHBV and 5 mg mL^{-1} of eugenyl acetate and **9** micronized pure polymer

Table 1 Results of mean particle size (X), variation coefficient (VC) and encapsulation efficiency (EE) of eugenyl acetate in PHBV by SEDS technique

Run	Eugenyl acetate to PHBV mass ratio	Pressure (MPa)	Temperature (K)	X (μm)	VC (%)	EE (%)
1	20:20	8	308	0.098 ± 0.04	37.8	28.5
2	20:20	8	313	0.145 ± 0.05	31.3	25.3
3	20:20	10	308	0.105 ± 0.03	29.0	16.3
4	10:20	8	308	0.061 ± 0.02	40.3	38.8
5	10:20	8	313	0.149 ± 0.02	19.2	38.9
6	5:20	8	308	0.170 ± 0.08	47.0	58.0
7	5:20	8	313	0.276 ± 0.14	53.1	46.5
8	5:20	10	308	0.107 ± 0.02	21.9	38.0

and some particles were somewhat irregular, however the spherical morphology is evident from the assays (1), (2), (4), (5) and (7), which was generally considered of great interest for industrial applications, such as their use in controlled release systems (Reverchon et al. 2008).

It is possible to observe a degree of similarity between the micrograph of micronized pure polymer, without eugenyl acetate extract, run 9, and the micrograph of co-precipitated particles of run 3. This similarity may be due to the low incorporation of eugenyl acetate in the biopolymer under the experimental conditions used in the run (10 MPa and 308 K), which resulted in an almost pure polymer. This behavior can be further evidenced by the lowest encapsulation efficiency obtained for this experiment, as can be seen in Table 1 that shows the experimental conditions adopted in this work together with the results of mean particle size (X), variation coefficient (VC) and encapsulation efficiency of eugenyl acetate in PHBV by SEDS technique.

From these results it can be seen that, for all assays, small particle sizes were obtained, ranging from 0.061 μm (run 4) to 0.276 μm (run 7). The particle size obtained here was smaller or similar to those reported by Boschetto et al. (2014), Machado et al. (2014), Priamo et al. (2010) and Franceschi et al. (2008a) for the precipitation of pure PHBV, 0.20–0.55 μm, 0.13–0.26 μm, 0.7–1.1 μm and 0.28 μm–0.57 μm, respectively. Azeredo (2005) and Matté and Rosa (2013) affirmed that nanoparticle size should be smaller than 0.2 μm and microparticle from 0.5 to 5000 μm. Thus, particles produced in this study were in a size range that can be considered as nanoparticles.

Concerning particle size distribution it can be seen that the lowest variation coefficient was observed in experiment (5) with 19.15 % at 40 °C and 8 MPa, 20 mg mL^{-1} of PHBV and 10 mg mL^{-1} of eugenyl acetate, while the highest value was 53.1 % for the experiment (7). Most of variation coefficients were lower than those found by Franceschi et al. (2008a, 2009) for encapsulated β-carotene in PHBV by the same technique.

Regarding the encapsulation efficiency, PHBV concentrations for all experimental conditions were kept constant at 20 mg mL^{-1} only varying the concentrations of eugenyl acetate. Table 1 shows that the highest encapsulation efficiency was 58.0 % for the assay 6 (8 MPa and 308 K), which corresponds to the lowest concentration of eugenyl acetate (5 mg mL^{-1}). Such maximum encapsulation efficiency was higher than that obtained by Machado et al. (2014), 48.25 %, in the co-precipitation of astaxanthin in PHBV using SEDS technique.

It was also possible to check the influence of eugenyl acetate concentration comparing assays 1, 4 and 6, for which the same temperature and pressure conditions (308 K and 8 MPa) were adopted. When lower concentrations of eugenyl acetate were used (assay 6), an increase of encapsulation efficiency occurred, which was similar to the results obtained by Boschetto et al. (2014) and Franceschi et al. (2008a), using the same technique in the encapsulation of bixin and β-carotene, respectively, and by Franceschi et al. (2008b) for the micronization of theophylline.

According to Cosijns et al. (2009) an increase in temperature can promote greater interaction between solvent and the extract, hence increasing extract solubility in solvent, thereby reducing the encapsulation efficiency. This phenomenon can be observed comparing assays 1 and 2, 4 and 5 and 6 and 7, for which solute concentration and pressure were kept constant for each pair of experiments, allowing evaluation of the influence of temperature. Besides, when observing the particle size in relation to processing temperature, it can be concluded that the higher the temperature, the bigger the particle size for all assays at the same concentration and pressure, varying the temperature from 308 to 313 K (1 and 2), (4 and 5) and (6 and 7). This behavior was also observed by Aguiar et al. (2016) in trans-resveratrol micronization by SEDS technique.

Regarding processing pressure, it was experimentally observed that lower encapsulation efficiency occurred at higher pressures, which may be explained in terms of solvent solubility increase in the anti-solvent (carbon

dioxide), a behavior also noted by Varona et al. (2010) for lavender essential oil encapsulation using the PGSS technique. Chen et al. (2007) affirmed that an increase in pressure enhanced the dispersion of the solution into carbon dioxide, hence reducing the particle size and increasing the mass transfer. In this work, this fact can be observed comparing runs 6 and 8, both at 308 K and solute concentration of 5 mg mL^{-1}, an increase in pressure from 8 to 10 MPa. However, such behavior was not verified for assays 1 and 3, but can be explained by the investigations conducted by Cardoso et al. (2009) and Miguel et al. (2006). These authors showed that an increase in pressure led to an increment of solute solubility in the mixture, reducing the supersaturation and nucleation, leading to a particle growth, also explaining the case of particles size rise over the concentration for these same tests.

In vitro release behavior of encapsulated eugenyl acetate in PHBV

For this purpose, runs 5, 6, 7 and 8 were employed due to higher encapsulation efficiencies exhibited. Figure 2 shows the time evolution of release percentage in ethyl acetate (a) and in isopropanol (b) for the assays 5, 6, 7 and 8, from 0 to 40 min.

For the in vitro release experiments in ethyl acetate, a very fast delivery was noticed in the first minutes for the encapsulated particles of eugenyl acetate in PHBV, which may be due to the high affinity of sample-solvent. It can be also observed in 30 min a release peak of the encapsulated active principle for all samples in ethyl acetate, with the major release occurred for sample 8 with 99.15 % release, then sample 7 with 99 06 %, sample 6 with 94.47 % and finally sample 5 with 75.21 %. Priamo et al. (2011) investigated the in vitro release of encapsulated β-carotene in PHBV in the same solvent and obtained a maximum release at 10 min, thus showing higher affinity of encapsulated β-carotene in PHBV samples in the dissolution medium as compared to eugenyl acetate in this work.

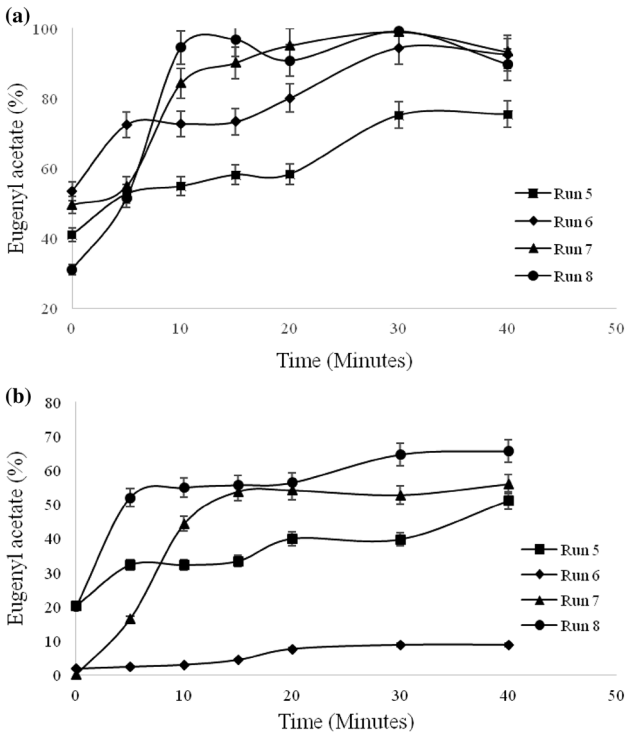

Fig. 2 Time-evolution of in vitro release of encapsulated eugenyl acetate in PHBV subjected to **a** ethyl acetate and **b** isopropanol at 301 K and 80 rpm

The initial release in isopropanol revealed to be much lower when compared to that observed in ethyl acetate, which can probably be explained in terms of sample affinity with the solvent. The release peak was lower than that shown for ethyl acetate and occurred at different times for samples 5, 6 and 7 in 40 min and 15 min for sample 8.

Food industries are seeking for innovative technologies to protect flavors and aromas, but with rapid release during consumption of the final product, which makes release tests relevant in attempt to elucidate how fast it occurs and the consequent mechanism involved (Azeredo 2005).

Confocal fluorescence microscopy

Observation of images obtained by confocal fluorescence microscopy (Fig. 3, shown as supplementary material) demonstrates the encapsulation of eugenyl acetate in PHBV and pure PHBV. The images were obtained in bright-field, which highlighted only the fluorescence of the compound, since PHBV had no fluorescence (Fig. 3b - supplementary material). To get the images blue color filters were used, and analysis must be done quickly because eugenyl acetate emitted light rapidly and soon ceased. A similar result was obtained by Carvalho (2009), which observed through confocal microscopy the presence of encapsulated oregano oil by the spray dryer technique, noting that the oil was not inside the capsules, but connected to the matrix due the affinity of oil by the encapsulating agent (gelatin).

Another aspect of great importance in this analysis was the experimental evidence that eugenyl acetate presented the characteristics of fluorescence, a fact previously not shown in literature. According to Rangel and Merçon (2012), it was difficult to predict the photoluminescence characteristic of a molecule, but certain general characteristics of the structure verified the favoring efficient radioactive deactivation. Most of the compounds exhibiting fluorescence were aromatic, and some aliphatic, alicyclic or carbonylated structures with highly conjugated double bonds that may show fluorescence that can be explained by the fact that aromatics have transition levels ($\pi \rightarrow \pi$) of low power.

Conclusion

Maximum encapsulation efficiency was 58.0 %, and spherical particles were noted for most of the experiments performed, where the average particle size ranged between 0.061 and 0.276 μm. It was also observed a greater affinity of encapsulated particles in ethyl acetate medium compared to isopropanol. Interestingly eugenyl acetate exhibited characteristics of fluorescence.

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