MICRO AND NANOPARTICLES PRECIPITATION OF PURE AND ENCAPSULATED β-CAROTENE IN PHBV FROM SEDS TECHNIQUE USING SUPERCRITICAL CO_2

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Abstract: The objective of this work was to investigate the application of the Solution Enhanced Dispersion by Supercritical fluids (SEDS) technique to the precipitation of pure β-carotene and copolymer poly(3-hydroxybutirate-co-hydroxyvalerate) (PHBV), and encapsulation tests of the solute in the biopolymer. Dichloromethane was used as organic solvent. The following parameters were investigated in the precipitation of pure β-carotene: pressure (8.0 and 12.0 MPa), anti-solvent flow rate (20 and 40 mL/min) and concentration of β-carotene in the organic solution (4.0 and 8.0 mg/mL). For the pure polymer, the same values of pressure and anti-solvent flow rate used for β-carotene were adopted, and the concentration of polymer in the solution was modified to 10 and 40 mg/mL. In the co-precipitation tests, the ratio of β-carotene to polymer in the organic solution were 1:2, 1:3 and 1:4 (w/w), the pressure was maintained at 8.0 MPa and the anti-solvent flow ratio was fixed to 40 mL/min. In all precipitation experiments temperature, solution flow rate and capillary internal diameter were fixed at 313 K, 1 mL/min and 100 μm, respectively. For β-carotene, the results showed that the mean particle size varied from 3.8 μm to 344 μm, and an increase in pressure and solution concentration led to an enhancement in mean particle size and particle size distribution of β-carotene precipitated, while the anti-solvent flow rate did not influence the particle size within the range investigated. The morphology of β-carotene was modified from plate-like to leaf-like particles, as verified by SEM micrographs. In the PHBV precipitation, the SEM micrographs showed that for all experimental conditions the morphology of polymer was different from the unprocessed material. The precipitated polymer presented a quasi-spherical shape with interconnected particles in the submicrometric range, while the unprocessed was made of films and great blocks. Depending on the value of the parameters investigated, the mean particle size of precipitated PHBV ranged from 278 to 570 nm. An increase in pressure and anti-solvent flow rate led to an increase the mean particle size and particle size distribution of precipitated PHBV, while an increase in solution concentration led to a slight decrease in mean particle size and particle size distribution. The co-precipitation experiments showed that the best ratio investigated of β-carotene to PHBV in the solution was 1:3 (w/w) that presented encapsulation percentage around 80% of β-carotene in PHBV. Fluid phase behavior of ternary systems of CO_2 + dichloromethane + β-carotene and CO_2 + dichloromethane + PHBV was also investigated with the aim of elucidating the region of the phase diagram in which the precipitation occurs. The temperature range was from 303 to 343 K, with CO_2 compositions ranging between 43 and 95 mol% for β-carotene and between 15 and 95 mol% for PHBV. Vapor-liquid and also solid-vapor-liquid phase transitions were observed in the phase equilibrium study. The presence of β-carotene or PHBV in the ternary mixture had a little influence on the fluid phase behavior of the systems. Results obtained demonstrated that precipitation occurred in a single supercritical fluid phase.

1. Introduction

Incorporation of bioactive compounds such as vitamins, probiotics, bioactive peptides and antioxidants into polymeric matrixes with application in food systems provide a simple way to develop novel functional foods that may have physiological benefits or reduce the risks of diseases[1].

Carotenoids are considered nutraceutical compounds and are the most common group of pigments in nature. In spite of existing more than 600 different carotenoids, only 40 of them are present in foods and 20 in the body. The most abundant are β-carotene, lycopene, lutein and zeaxanthin. The main role of these compounds in human diet are to act as vitamin A precursor and antioxidant.[2] The presence of small amounts of carotenoids in foods can help to prevent the fast oxidation of foods constituents due to the singlet oxygen quenching activity.

Due to the large amount of the carbon-carbon double bound, factors such as heath, light and acids cause isomerization of the *trans* carotenoid form that is the more stable in nature than the *cis* carotenoid form, promoting a loss of color and provitamin activity. Distinct from synthetic carotenoids, the natural ones are more easily oxidized[3] and their oxidation products have little or almost any pigmentation, provitamin-A activity and singlet oxygen quenching activity[4]. In this sense, it may be important to promote the protection of this compound, which can be attained through their encapsulation in polymeric systems.

There is a range of polymers that can be employed to encapsulate bioactive compounds, due to their biocompatibility and biodegradability. Several biopolymers were already used for encapsulate pharmaceutical compounds[5], proteins[6] and carotenoids[2].

Polyhydoxyalkanoates (PHA) are polyesters produced by microorganisms under unbalanced growth conditions. PHA is generally biodegradable, with good biocompatibility, making them attractive as encapsulating agent[7]. The most common type of PHAs is poly (3-hydroxibutirate) (PHB). However, PHB is stiff and brittle thus restricting its range of applications. On the other hand, PHB copolymers with 3-hydroxyvalerate (PHBV) are less stiff, tougher and crystalline[8]. The use of PHBV had an increase in applications in the biomedical field due to the fact that it is possible to prepare an appropriate controlled drug delivery system that gradually degrades in the body[7]. Thus, the copolymer PHBV can be used as encapsulation media for the protection and controlled delivery of carotenoids in foods.

The use of supercritical or near critical fluids as solvents or anti-solvents in the particles precipitation/encapsulation was shown for several searchers as useful into modification of material properties such as particle size, size distribution and morphology. Other advantages of these techniques are the efficient separation of the solvent and anti-solvent of the particles after precipitation. It is possible then to avoid organic solvent residue in the final product and give an advantageous reutilization of solvent and antisolvent[9].

In this context, the objective of this work was to investigate the effect of processing parameters (pressure, flow rate of solution and anti-solvent, initial concentration of the solid in solution and mass ratio between β-carotene and PHBV) on the precipitation of pure β-carotene and PHBV using the SEDS technique and focusing on particle size, particle size distribution, morphology and encapsulation efficiency. The fluid phase behavior of the systems solvent/solutes/anti-solvent was also investigated to select the proper operating point in the phase diagrams. The size and morphology of precipitated powders were characterized by scanning electronic microscopy (SEM) and encapsulation efficiency was verified by UV-spectrophotometry.

2. Experimental

2.1. Materials

All-trans-β-carotene, with a purity of 95 % was purchased from Sigma-Aldrich, (USA). Dichloromethane (DCM - 99.5 %) was supplied by Merck (Germany), carbon dioxide (99.9 % in liquid phase) was supplied by White Martins S.A., and the co-polymer Poly(3-hydroxybutirate-co-hydroxyvalerate) (PHBV) was kindly supplied by the PHB Industrial S/A company.

2.2. High-Pressure Phase Equilibrium Apparatus and Experimental Procedure

Fluid phase equilibrium experiments were conducted employing the static synthetic method in a high-pressure variable-volume view cell. A detailed description of the experimental apparatus and procedure can be found elsewhere[10].

2.2. Precipitation Apparatus and Procedure

All precipitation experiments were carried out in an apparatus based on the SEDS technique. A detailed description of the experimental apparatus can be found elsewere[11]. Briefly, the experimental procedure started with CO_2 filling the precipitation chamber up to the desired pressure. The anti-solvent flow rate was controlled by setting valves before and after the precipitation chamber, and was continuously monitored by the syringe pump. When the temperature, pressure and antisolvent flow rate were stabilized, the organic solution was added to precipitation chamber. The pressure for solution spray in the precipitator was controlled by a back-pressure manipulation and monitored by the liquid pump. The solution volume added to the chamber was 20 mL, which was sufficient to have the necessary amount of precipitated powder for analysis. After the specified volume of solution has been delivered, the solution flow rate was stopped and CO_2 continued to flow in order to dry the precipitated particles inside the precipitation chamber; in this step, the total amount of CO_2 used was 800 mL. After the drying particles step, the anti-solvent flow rate was stopped and the precipitation chamber was slowly depressurized to atmospheric pressure. The precipitated particles were collected and stored at appropriate conditions for subsequent analysis and characterization.

All runs of β-carotene precipitation are summarized in Table 1. For the PHBV precipitation, the same parameters and conditions of β-carotene precipitation were employed, but the concentration of PHBV in organic solution was modified for a range of 10 to 40 mg/mL, while for β-carotene this range was 4 to 8 mg/mL. For the encapsulation tests, the condition selected was the one that gave smaller particle size for β-carotene and bigger particle size for PHBV. The parameters investigated were Pressure (P), solution concentration (SC) and anti-solvent flow rate (F).

Table 1 - Experimental conditions for β-carotene precipitation by the SEDS technique.

Run	SC ($mg.mL^{-1}$)	P (bar)	F (mL/min)	Run	SC ($mg.mL^{-1}$)	P (bar)	F (mL/min)
1	4	80	20	7	4	120	40
2	8	80	20	8	8	120	40
3	4	120	20	9	6	100	30
4	8	120	20	10	6	100	30
5	4	80	40	11	6	100	30
6	8	80	40				

The effect of the variables was determined using the commercial software Statistica® 6.0. Response for the variable effects was particle size (PS). Results were obtained by measuring the length and width of about 200 particles for each experimental condition using the software Size Meter version 1.1.

2.2. Encapsulation percentage

The encapsulation percentage was verified by UV-spectrophotometry. Initially, it was taken a certain sample of co-precipitated β-carotene/PHBV particles. It was assumed that in this sample the β-carotene/PHBV mass ratio was the same of that initially present in the organic solution before precipitation. This sample was weighed and washed for three times with ethanol for five minutes under sonication to remove carotene not encapsulated. After this, the suspension was filtered and dried in a chamber under controlled temperature (50°C) and vacuum for 24h. After this time, β-carotene was

then extracted of the resulting powder and their absorbance was measured in a UV-spectrophotometer and compared with a standard curve of concentration versus absorbance. The encapsulation percentage was then verified by the ratio between the mass of β-carotene present in the initial sample and the one obtained through spectrophotometric analysis.

3. Results and discussion

The precipitation of pure β-carotene and PHBV was carried out by varying three experimental parameters: precipitation pressure, solute concentration in the organic solution and anti-solvent flow rate.

In the β-carotene precipitation experiments, the smaller particle size was found in run 6, with a mean particle size of 3.8 μm, and run 3 afforded the biggest mean particle size of 344.6 μm. In the PHBV precipitation, run 7 led to the smallest particle size (278 nm) while run 6 presented the biggest particle size (570 nm). For the encapsulation experiments, run 6 was chosen because presented the smaller particle size in relation to β-carotene and bigger size regarding PHBV. In these experiments, the mass ratio between β-carotene and PHBV were 1:2, 1:3 and 1:4.

The statistical analysis showed, with a confidence level of 95% ($p<0.05$), that of the investigated parameters in the pure β-carotene and PHBV precipitation (pressure, solution concentration and anti-solvent flow rate) only pressure and solution concentration presented a significant effect in relation to particle size. Figure 1 shows SEM micrographs of different experimental conditions of β-carotene and PHBV precipitation and mixture co-precipitation.

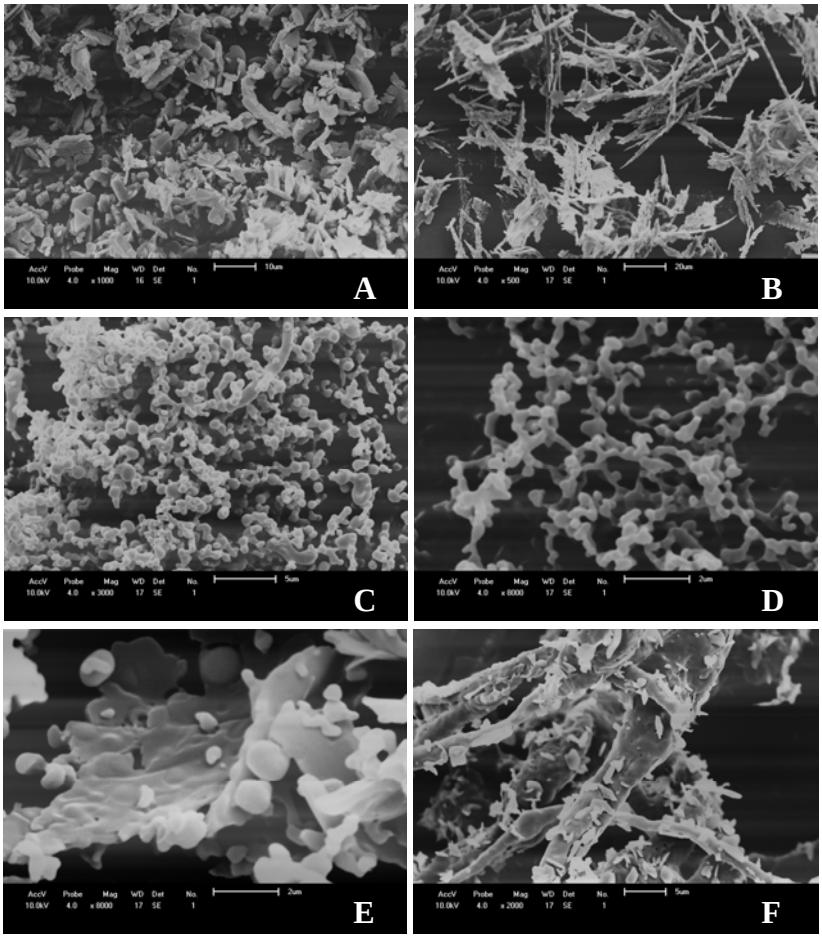

Figure 1 – SEM micrographs of pure β-carotene: (A) run 6, (B) run 2; pure PHBV: (C) run 6, (D) run 7; and encapsulation tests: (E) 1:3 w/w β-carotene/PHBV, (F)1:4 w/w β-carotene/PHBV.

3.1. Fluid phase behavior

Fluid phase behavior of ternary systems of CO_2 + dichloromethane + β-carotene and CO_2 + dichloromethane + PHBV was investigated with the aim of elucidating the region of the phase diagram in which the precipitation occurs. The temperature range was from 303 to 343 K, with CO_2 compositions ranging between 43 and 95 mol% for β-carotene and between 15 and 95 mol% for PHBV. The organic solvent/solute ratio was kept constant to the same values of the precipitation in all experiments of fluid phase behavior. Vapor-liquid and also solid-vapor-liquid phase transitions were observed in the phase equilibrium study. The presence of β-carotene or PHBV in the ternary mixture had a negligible influence on the fluid phase behavior of the systems. In this sense, it was considered only the fluid phase behavior data of the binary system CO_2 + dichloromethane presented in literature[12]. According to these data, the precipitation experiments carried out in this work were performed in a single supercritical phase.

3.2. Pressure effect

An increase in precipitation pressure causes an pronounced increase on mean particle size of β-carotene of 3.8 to 344.6 μm. The more pronounced increase in particle size was observed by varying the pressure with the solution concentration and anti-solvent flow rate in their smaller values. To occur an elevated nucleation rate, it is necessary that the solution concentration be close to the saturation and to have anti-solvent in the flow quite enough to a fast extraction of organic solvent in the solution generating a high supersaturation. As the solution concentration and anti-solvent flow rate are low in these experimental conditions, the supersaturarion and consequently the nucleation rate are low, generating larger particles. In relation to the density, in the lower pressure the CO_2 density is lower, consequently their solubility decrease regarding the organic solvent. In this case, the rates of mass transfer are reduced, resulting in smaller nucleation rates and bigger particles when compared to the larger pressure where the CO_2 density is greater.

Regarding the PHBV precipitation, the increase in precipitation pressure, in a general way, cause a decrease in mean particle size of the precipitated powder. When the pressure was enhanced maintaining the solution concentration and anti-solvent flow rate in the lowest values, the mean particle size decreased from 487 nm to 329 nm. When the solution concentration and anti-solvent flow rate were raised to the highest values, the pressure increase generated a slight decrease in mean particle size of PHBV from 570 nm to 563 nm. These results are in agreement with literature[13] that point outs a decrease in particle size with increasing pressure. In all experiments of PHBV precipitation the particles presented a spherical shape with interconnected particles, as can be seen in Figs. 1C and 1D.

3.3. Solution concentration effect

When the β-carotene concentration in organic solution increased from 4.0 to 8.0 mg/mL, the particle size was decreased. There were also modifications on morphology of precipitated particles, that was changed from plate to leaf, as can be seen in Figs. 1A and 1B. The same Figs. still show a certain adhesion, mainly in Fig. 2A, where the solution concentration is high. According to literature[14] an increase in solution initial concentration can lead to a solute re-condensation during the precipitation. Since the condensation is directly proportional to solute concentration, the more concentrated is the solution, the higher is the solute re-condensation, promoting an increase in size of precipitated particles.

Regarding PHBV precipitation, increasing the solution concentration resulted in an increase in mean particle size and agglomeration. When the pressure and anti-solvent flow rate were maintained in their smallest values, a raise in solution concentration led to an increase in mean particle size of precipitated PHBV from 487 nm to 505 nm and, when the solution concentration was increased maintaining the pressure and anti-solvent flow rate on their highest values, there was a pronounced increase in mean particle size from 278 nm to 563 nm. The increase in particle size when the solution concentration is increased is characteristic in polymer solutions. Concentrated polymeric solutions

have a high viscosity, hindering the mass transfer between the anti-solvent and the solution drops generated in the spray formation into the precipitation chamber, leading to an increase in mean particle size and particle adhesion.

3.5. Encapsulation percentage

For the encapsulation experiments, the values of pressure and anti-solvent flow rate were adopted the ones of run 6 of precipitation of pure β-carotene and PHBV. The mass ratio carotene/polymer was 1:2, 1:3 and 1:4. The results showed that for the mass ratio of 1:2, the encapsulated carotene was around 5%, indicating that the amount of polymer is not sufficient to encapsulate the carotene. For the mass ratio of 1:3 the encapsulation percentage was close to 80%, indicating that probably the β-carotene was precipitated earlier and the polymer later, using the carotene precipitated particles to seeds, recovering them. For the mass ratio of 1:4, the β-carotene particles remained adsorbed on the polymer surface, indicating that due to the high polymer concentration into solution, this has firstly precipitated and the β-carotene has precipitated later and has adhered into polymer surface.

4. Conclusion

Experimental results of β-carotene and PHBV precipitation showed a strong influence of precipitation pressure and solution concentration on particle size and morphology of the precipitated powder. In a general sense, for β-carotene, increasing the pressure increase the particle size and an increase in solution concentration led to a decrease in particle size. For the PHBV precipitation, the opposite effect was verified, an increase in pressure led to a decrease in particle size and an increase in solution concentration led to an increase also in particle size.

The encapsulation experiments revealed that the best mass ratio between β-carotene and PHBV in the organic solution, in the investigated range, was 1:3, presenting an encapsulation percentage of approximately 80%.

For the fluid phase behavior, the results showed that the presence of the solutes in the system did not influence the fluid phase behavior of the binary system solvent + anti-solvent, then allowing to consider only the fluid phase behavior of the binary system. Yet, in the pressure and temperature conditions used in the precipitation experiments the system is in a single supercritical phase.

5. References

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