PM15

Production of β-carotene/PVP coprecipitates using Supercritical Assisted Atomization

Alessia Di Capua[a], Renata Adami[a], Emanuela Cosenza[a], Vincent Jalaber[a], Christelle Crampon[b], Elisabeth Badens[b], Ernesto Reverchon[a]

[a] Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II 132, 84084 Fisciano (SA), Italy
[b] Aix Marseille Univ, CNRS, Centrale Marseille, M2P2, Marseille, France
* radami@unisa.it

ABSTRACT
The application of β-carotene (BC), a bioactive compound with high antioxidant activity, in various food systems, nutraceutical and pharmaceutical products is limited because of its poor solubility in water, low bioavailability, lipophilic and crystalline nature and instability in presence of light, heat and oxygen. Incorporation of BC in polymeric matrices represents a suitable method to stabilize and protect it from environmental conditions. Therefore, Supercritical Assisted Atomization (SAA) was used to produce a particulate system, consisting of a biocompatible, biodegradable, and water-soluble polymer (polyvinylpyrrolidone, PVP) loaded with BC. The coprecipitation experiments of BC with PVP were performed using ethanol as solvent. SAA produced spherical, well-separated and regular microspheres with mean diameters ranging between 0.42 and 0.84 µm. Produced powders were characterized by X-Ray diffractometry, calorimetry: these analyses showed that the microspheres were amorphous and that BC was intimately mixed with the polymer. UV-vis analyses revealed BC content up to 92 % in SAA powders. Preliminary BC dissolution tests in a phosphate buffered saline solution (PBS) showed that SAA powders dissolve faster than physical mixture.

INTRODUCTION
Carotenoids are the most common natural pigments used in food industry; they can be classified in Carotenes, if their chemical structure are only based on carbon and hydrogen, and Xantophylls, if they have also oxygen in their structure [1]. The most common and abundant carotene in nature is β-carotene (BC), that is a strongly red orange-colored pigment, mostly present in carrots, apricot, tomato, paprika, broccoli and asparagus [2]. BC is the major carotenoid in human diet, blood and tissue, with several biological activities, high antioxidant activity, high pro-vitamin A activity. Clinical studies have demonstrated that BC may reduce the risk of certain chronic diseases, such as lung cancer, cardiovascular and heart disease and may protect against cutaneous photodamage [2-3]. BC is a poorly water soluble compound with low bioavailability, crystalline nature and instability in presence of light, heat and oxygen [1-3]; these disadvantageous features limit the application of BC in various food systems, nutraceutical products. In order to enhance BC bioavailability and stability, coprecipitation with a water-soluble carrier can be a promising approach to stabilize and prevent oxidation of the bioactive compound and to produce a functional product [4]. Different traditional techniques have been proposed for this purpose: spray drying, homogeneization, freeze drying, emulsion/solvent evaporation [5-8]. These traditional processes show some disadvantages, such as excessive use of organic solvent with the consequent high residual solvent concentration in the product, thermal degradation, difficulty in controlling particle size distribution. Supercritical fluids (SCF) based techniques have been frequently suggested to overcome these limitations [9].

The application of SCF techniques to the extraction of BC from vegetables and microalgae [10], to the precipitation of BC alone [11, 12] and to the production of composite systems based on BC and biopolymers have been investigated [13]. For example, Mattea et al. [14] obtained three different BC crystal forms depending on the initial concentration in dichloromethane and on the type of injector, using Supercritical Antisolvent process. Franceschi et al. [15] proposed the precipitation of BC with poly(3-hydroxybutirate-co-hydroxyvalerate) from dichloromethane using SEDS technique, obtaining irregular and connected particles, with loading efficiencies lower than 35%. However, few researchers have focused their attention on the enhancement of BC bioavailability and on the study of the antioxidant properties of the composite products after process.

Supercritical Assisted Atomization (SAA) is one of the most efficient SCF based process to produce controlled micro and submicroparticles of pure ingredients [16] but also composite systems (coprecipitates) formed by a carrier in which an active principle is uniformly dispersed [17, 18]. In this work, a biocompatible, no toxic, FDA (*Food and Drug Administration*) approved [19] and water-soluble polymer, poly(vinylpirrolidone) (PVP), was used as carrier to produce coprecipitated microparticles of BC-PVP, in order to reduce BC crystallization tendency and produce regular particles, to prevent BC degradation during storage and enhance its bioavailability.

Hence, this study has investigated the effect of varying BC/PVP weight ratios towards solid state morphology, particles size distribution, BC content in the formulations, antioxidant activity after SAA process and dissolution properties to assess SAA efficiency on the production of stable and BC-rich powders.

MATERIALS AND METHODS

Polyvinylpyrrolidone (PVP, Mw: 10,000, Fluka, Milan, Italy), β-carotene (BC, purity >93%, Sigma Aldrich (cod. C9750); ethanol (99.9% Sigma Aldrich Chemical Co., Milan, Italy), carbon dioxide (CO_2, 99.9% Morland Group, Naples, Italy) and nitrogen (N_2, 99% SOL, Milan, Italy). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO).

SAA apparatus

A schematic representation of SAA process layout is published elsewhere [16-18]. It mainly consists of two feed lines, used to deliver compressed carbon dioxide and the liquid solution (in which one or more solid solutes are dissolved) to a saturator, through two high pressure pumps (mod. 305, Gilson). The saturator is a high pressure vessel (50 cm^3 internal volume) loaded with stainless steel perforated saddles to maximize the contact surface between the liquid solution and CO_2. The solubilisation of CO_2 in the solution forms an expanded liquid that is sprayed into the precipitator (3 dm^3 internal volume) using a nozzle with an internal diameter of 80 μm. A controlled flow of N_2 is taken from a cylinder, heated in an electric heat exchanger (mod. CBEN 24G6, Watlow) and sent to the precipitator to promote droplets evaporation. The saturator and the precipitator are electrically heated using thin band heaters (Watlow, mod. STB3EA10). At the bottom of the precipitator, a stainless steel filter (pore size: 0.1 μm) allows powder collection, whereas the gaseous flow of CO_2, N_2 and solvent is sent to a condenser. [16-18].

Analytical methods

The morphology of SAA particles was observed by a Field Emission-Scanning Electron Microscope (FESEM, mod. LEO 1525, Carl Zeiss). Particle size (PS) and particle size distribution (PSD) were measured from FESEM photomicrographs using Sigma Scan Pro Software (release 5.0). Approximately 800 particles were measured for each PSD calculation. Histograms, representing the PSD, were fitted using Microcal Origin Software (release 8.0, Microcal Software) and converted in volumetric distributions. Solid state of particles was studied by X-Ray diffractometry and calorimetry. X-ray diffractograms were obtained using a diffractometer (XRPD, model D8 Discover; Bruker) with following conditions: Ni-filtered Cu Ka radiation, 2Θ angle ranging from 2 to 60° with a step size of 0.022°, a scan rate of 0.2°s/step and λ= 1.54 A. Microparticles thermal behavior was determined by Differential Scanning Calorimetry (DSC, model TC11, Mettler Toledo). The sample

was placed in aluminum pan and heated from 30 to 200°C at a scanning rate of 10°C/min in N_2 atmosphere.

BC loading and powder dissolution studies were performed using an UV/vis spectrophotometer (model Cary 50, Varian, Palo Alto, CA). Absorbances (read at wavelength 450 nm) related to coprecipitates dissolved in ethanol were converted into BC concentration, using a calibration curve. Loading efficiencies were calculated as the ratio of the effective over the theoretical content. For dissolution tests, samples containing an equivalent amount of BC (5 ppm) for each R were weighted, placed in a dialysis sack (cut off = 3500 Da) and incubated in 400 mL of phosphate buffer saline (PBS) at pH 7.4, stirred at 200 rpm and 37°C.

Antioxidant activity

The DPPH radical scavenging activity of each powder sample was measured using the stable radical DPPH, according to the method described by Brand-Williams et al. [20] with minor modifications. SAA samples were dissolved in ethanol (50 ppm); 1 mL of this solution was placed in 3 mL of 10^{-4} M of an ethanolic solution of DPPH and left under dark for 16 hours at 25°C. Absorbance at 517 nm was determined using an UV/vis spectrophotometer (model Cary 50, Varian, Palo Alto, CA). The results were expressed in terms of scavenging activity (SA, %), calculated according to the equation:

$$SA = 100 * (1 - \frac{A_S}{A_c})$$

where A_s is the absorbance of the sample solution, A_c is the absorbance of the control (solution of DPPH).

RESULTS AND DISCUSSION

BC coprecipitation experiments were performed using PVP, selected for its capability to act as crystallization inhibitor of active principles [19] and ethanol as solvent. SAA operative conditions were chosen on the basis of previous SAA experiences [16]. Temperature and pressure conditions in the saturator were fixed at 80°C and 85 bar, *gas to liquid ratio* (mass based, defined as GLR) was 1.8 and precipitation chamber temperature was 80°C. BC concentration in ethanol was fixed at 0.5 mg/mL, whereas PVP concentration was varied between 2 and 10 mg/mL, to study the effect of BC/PVP weight ratio (R, w/w) on the morphology and PSDs.

Produced powders were homogeneously orange, that is a compromise between the color of PVP (white) and of BC (red). Examples of FESEM photomicrographs reported in Figure 1 show that spherical microparticles with a regular morphology were produced for each R.
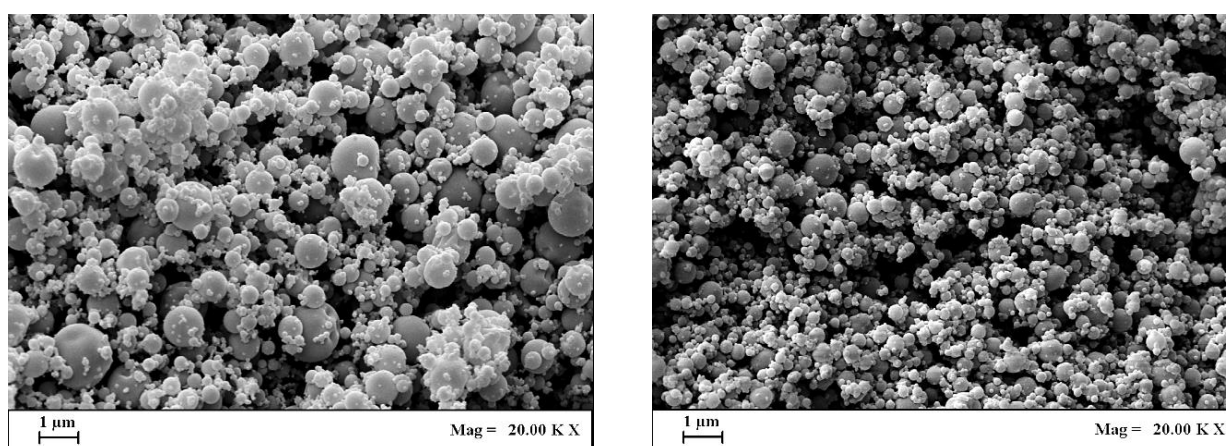

Figure 1. FESEM photomicrographs of BC-PVP microparticles
at BC concentrations of 0.5 mg/mL: left) R=1/20, right) R=1/4.

Looking at particle size distributions, FESEM images analyses showed that polymer concentration can play an important role in particle size: smaller particles have been obtained with lower PVP concentration (when R increases) and, hence, with a decrease of total concentration in starting

suspension. A graphical comparison among the volumetric cumulative PSDs of BC-PVP microspheres are reported in Figure 2.
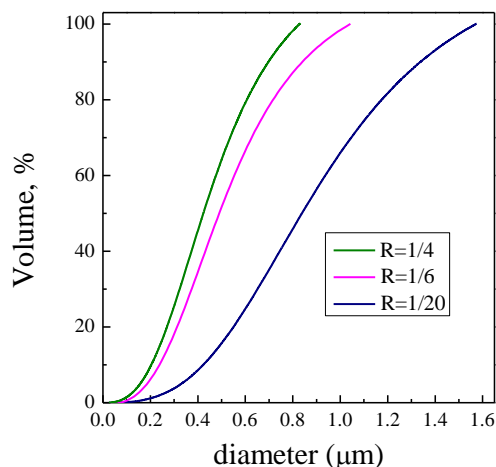

Figure 2. PSDs of BC-PVP at different R.
Calculations in terms of volumetric cumulative percentages.

Solid characterization of BC-PVP coprecipitates
Using DSC analyses (Figure 3a), the changes in the thermal behavior of the active principle and polymer in the coprecipitates have been studied. Raw BC shows a broad endothermic peak ranging between 50 and 100°C, due to the loss of water, and a second endothermic peak at about 170°C, due to the melting of crystalline structure. Unprocessed PVP shows only the removal of water (50-130°C), whereas the physical mixture (at R=1/6) shows both the polymer and BC peaks. All SAA coprecipitates show no melting peak of BC and microspheres are in an amorphous state. X-Ray analyses (Figure 3b) confirmed these results: the diffractograms related to coprecipitated powders show a complete amorphous solid state, with no characteristic BC peaks.
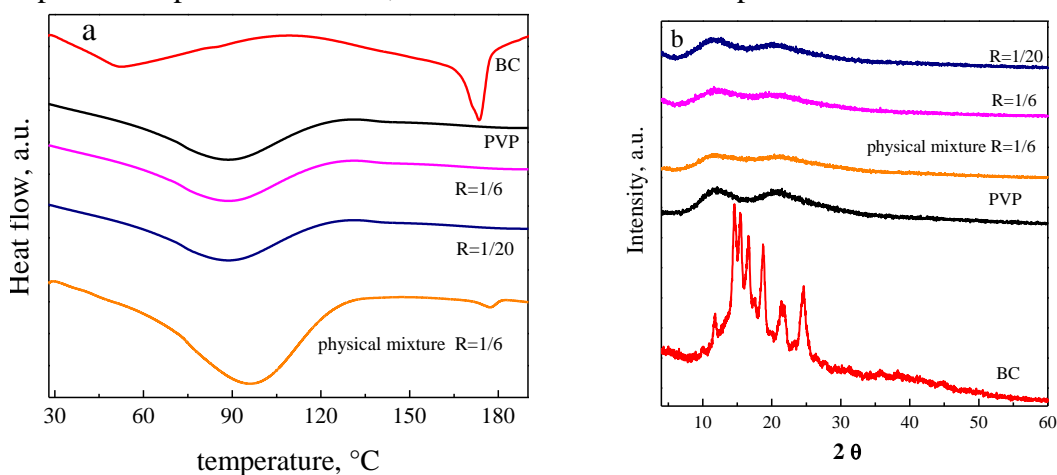

Figure 3. DSC analyses (a) and X-Ray (b) of BC/PVP: comparison among raw materials and SAA coprecipitated microspheres.

Loading efficiencies and dissolution tests
BC entrapment in SAA particles for each R weight ratio was measured by UV-vis spectrophotometer to verify SAA efficiency in BC-PVP coprecipitation. The loading efficiency values (content of BC in SAA powder vs its amount in the starting suspension) ranged between 73% and 92%, as summarized in Table 1.

Table 1. BC loading efficiencies and scavenging activity (DPPH method) in SAA particles

R	Theoretical loading (%)	Effective loading (%)	Loading efficiency (%)	SA (%)

1/20	4.7	3.4	72.3	20.8
1/6	14.3	10.7	75	11.3
1/4	20	18.5	92.6	10.5

R= BC/PVP w/w

Preliminary dissolution tests were performed simulating conditions of the small intestine. The dissolution profiles related to the physical mixture and SAA coprecipitate at R=1/6 are compared in Figure 4.
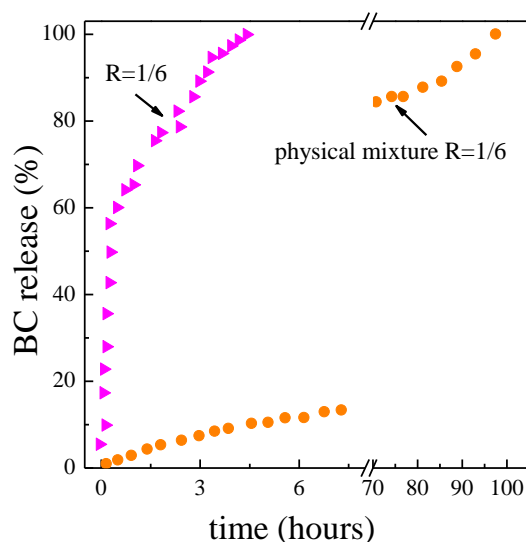

Figure 4. Release profiles of BC in PBS at 37°c and pH 7.4.

The physical mixture (R=1/6) of the raw materials dissolved completely in 4.2 days, whereas SAA coprecipitates show a faster release: for example, the coprecipitate at R=1/6 shows a total dissolution time of about 4.5 hours, that is about 22 times faster than the one of the physical mixture. As a general comment, the dissolution behavior of coprecipitates is affected by several parameters: interactions between compounds, particle size, solid state [17-18]. In case of BC-PVP coprecipitates, both the size of particles and polymer solubility in water affect the improvement of the dissolution rate compared to the physical mixture.

Antioxidant activity
Antioxidant activity of untreated BC was measured using the DPPH assay to measure the capability of the particles to act as free radical scavengers. The results in Table 1 indicate that SAA coprecipitates show a good preservation of BC scavenging activity (SA) with values between 20.8% and 10.5%. A higher SA means a higher free radical scavenging activity of the compounds. The antioxidant activity of SAA coprecipitates increases with R decrease (in correspondence of higher PVP concentration). The best result was obtained for sample produced at R=1/20 (SA=20.8%) when the largest diameters were obtained, as demonstrated by Reksamunandar et al. [21]. These results suggest that the coprecipitation of BC-PVP prevent the degradation of the active principle and allow to protect BC during storage keeping its antioxidant activity: this fact could be an interesting added value for the production of nutraceutical and functional products.

All SAA formulations showed a higher antioxidant power compared to the untreated BC. This is an unexpected result that might be due to several aspects. Researchers have demonstrated that reduced size of a pure active principle can improve free radical scavenging activity, due to the enhanced dissolution rate of the compound [22, 23].

Summarizing, the micronization and coprecipitation of BC-PVP allow to enhance the dissolution rate of BC and to increase hydrogen donors to quench DPPH radical, and, consequently, to preserve the antioxidant activity.

CONCLUSION

SAA technique is demonstrated very efficient in the entrapment (up to 92%) of BC in a polymeric hydrosoluble matrix as PVP. The proposed results suggested that the composite microspheres produced can be used to stabilize and protect BC and to improve its bioavailability. Using a BC/PVP weight ratio of R=1/6, indeed, an improvement of BC dissolution rate up to 22 times with respect to the physical mixture was obtained. The high bioavailability and antioxidant power make these particles rich in bioactive compounds very interesting as nutraceutical, pharmaceutical and functional products.

REFERENCES

[1] FIEDOR, J., BURDA, K., Potential Role of Carotenoids as Antioxidants in Human Health and Disease, Nutrients, Vol. 6, 2014, p. 466.

[2] GUL, K., TAK, A., SINGH, A.K., SINGH, P., YOUSUF, B., WANI, A.A., Chemistry, encapsulation, and health benefits of β-carotene-A review, Cogent Food & Agriculture, Vol. 1, 2015, p. 1.

[3] GORALCZYK, R., Beta-carotene and lung cancer in smokers: review of hypotheses and status of research, Nutrition and Cancer, Vol. 61, 2009, p. 767.

[4] CHAMPAGNE, C.P., FUSTIER, P., Microencapsulation for the improved delivery of bioactive compounds into foods, Current Opinion in Biotechnology, Vol. 18, 2007, p. 184.

[5] YUAN, Y., GAO, Y., ZHAO, J., MAO, L., Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions, Food Research International, Vol. 41 , 2008, p. 61.

[6] LOKSUWAN, J., Characteristics of microencapsulated β-carotene formed by spray drying with modified tapioca starch, native tapioca starch and maltodextrin, Food Hydrocolloids, Vol. 21, 2007, p. 928.

[7] CORONEL-AGUILERA, C.P., SAN MARTÍN-GONZÁLEZ, M.F., Encapsulation of spray dried β-carotene emulsion by fluidized bed coating technology, LWT-Food Science and technology, Vol. 62, 2015, p. 187.

[8] CHU, B.S., ICHIKAWA, S., KANAFUSA, S., NAKAJIMA, M., Preparation and characterization of beta-carotene nanodispersions prepared by solvent displacement technique, Journal of Agricultural and Food Chemistry, Vol. 55, 2007, p. 6754.

[9] GIROTRA, P., SINGH, S.K., NAGPAL, K., Supercritical fluid technology: a promising approach in pharmaceutical research, Pharmaceutical Development and Technology, Vol. 18, 2013, p. 22.

[10] MOUAHID, A., CRAMPON, C., AMINE TOUDJI, S.A., BADENS, E., Effects of high water content and drying pre-treatment on supercritical CO_2 extraction from *Dunaliella salina* microalgae: Experiments and modeling, Journal of Supercritical Fluids, Vol. 116, 2016, p. 271.

[11] MATTEA, F., MARTÍN, Á., MATIAS-GAGO, A., COCERO, M.J., Supercritical antisolvent precipitation from an emulsion: β-carotene nanoparticle formation, Journal of Supercritical Fluids, Vol. 51, 2009, p. 238.

[12] FRANCESCHI, E., DE CESARO, A.M., FERREIRA, S.R.S., OLIVEIRA, J.V., Precipitation of β-carotene microparticles from SEDS technique using supercritical CO_2, Journal of Food Engineering, Vol. 95, 2009, p. 656.

[13] JANISZEWSKA-TURAK, E., Carotenoids microencapsulation by spray drying method and supercritical micronization, Food Research International, Vol. 99, 2017, p. 891.

[14] MATTEA, F., MARTÍN, Á., COCERO, M.J., Carotenoid processing with supercritical fluids, Journal of Food Engineering, Vol. 93, 2009, p. 255.

[15] FRANCESCHI, E., DE CEZARO, A., FERREIRA, S.R.S., KUNITA, M.H., MUNIZ, E.C., RUBIRA, A.F., OLIVEIRA, J.V., Co-Precipitation of Beta-Carotene and Bio-Polymer Using Supercritical Carbon Dioxide as Antisolvent, The Open Chemical Engineering Journal, Vol. 4, 2010, p. 11.

[16] LIPAROTI, S., ADAMI, R., REVERCHON, E., Supercritical Assisted Atomization: effect of operative conditions on PVP microparticles size and morphology, Journal of Supercritical Fluids, Vol. 97, 2015, p. 31.
[17] ADAMI, R., DI CAPUA, A., REVERCHON, E., Supercritical Assisted Atomization for the production of curcumin-biopolymer microspheres, Powder Technology, Vol. 305, 2017, p. 455.
[18] DI CAPUA, A., ADAMI, R., REVERCHON, E., Production of Luteolin/Biopolymer Microspheres by Supercritical Assisted Atomization, Industrial & Engineering Chemistry Research, Vol. 56, 2017, p. 4334.
[19] WANG, B., WANG, D., ZHAO, S., HUANG, X., ZHANG, J., LV, Y., LIU, X., LV, G., MA, X., Evaluate the ability of PVP to inhibit crystallization of amorphous solid dispersions by density functional theory and experimental verify, European Journal of Pharmaceutical Sciences, Vol. 96, 2017, p. 45.
[20] BRAND-WILLIAMS, W., CUVELIER, M.E., BERSET, C., Use of a free radical method to evaluate antioxidant activity, LWT-Food Science and technology, Vol. 28, 1995, p. 25.
[21] REKSAMUNANDAR, R.P., EDIKRESNHA, D., MUNIR, M.M., DAMAYANTI, S., KHAIRURRIJAL, Encapsulation of β-carotene in poly(vinylpyrrolidone) (PVP) by electrospinning technique, Procedia Engineering, Vol. 170, 2017, p. 19.
[22] KAKRAN, M., SAHOO, N.G., TAN, I.L., LI, L., Preparation of nanoparticles of poorly water-soluble antioxidant curcumin by antisolvent precipitation methods, Journal of Nanoparticle Research, Vol. 14, 2012, p. 1.
[23] MENESES, M.A., CAPUTO, G., SCOGNAMIGLIO, M., REVERCHON, E., ADAMI, R., Antioxidant phenolic compounds recovery from Mangifera indica L. by-products by supercritical antisolvent extraction, Journal of Food Engineering, Vol. 163, 2015, p. 45.