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Supercritical Assisted Atomization for the stabilization of active compounds

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ABSTRACT

Nowadays, application of several technologies in processing active natural compounds found in vegetables, fruits and plant extracts receives great interest due to their several pharmacological and nutraceutical properties. These active substances can lead to innovative therapeutic agents for cancer and promote human health without recognizable side effects; however, they have very low solubility in water and, consequently, poor bioavailability, a high tendency to crystallize and a certain instability in presence of light, heat and oxygen atmosphere. Amorphous formulations have great potential to overcome these drawbacks and, in this work, Supercritical Assisted Atomization (SAA) is proposed to produce stable microparticles with controlled size and distribution loaded with active principles of natural origin, starting from water/ethanol and ethanol solutions. Macromolecules such as polysaccharides, cyclodextrins and polymers are used as carriers due to their physical-chemical properties and physiological acceptance. The carriers proposed in this work are dextran (DEX), hydroxypropyl-β-cyclodextrin (HPβCD) and polyvinylpyrrolidone (PVP); they are highly water soluble, crystal growth inhibitor, approved by Food & Drug Administration (FDA) and moreover they can protect the bioactivity of natural compounds. Several active principles (curcumin, beta-carotene, propolis, luteolin and saffron extracts) have been selected as model compounds. Examples of the results of this application are the production of microparticles of HPβCD and PVP loaded with propolis, and DEX and PVP loaded with luteolin. Different particle morphologies have been obtained; the mean particle sizes for all studied systems are decreased to approximately one hundredth of its original value, whereas the dissolution rates are highly increased. Free radical scavenging assay results on SAA powders indicates that antioxidant power of these natural compounds is preserved after the process.

INTRODUCTION

In the last decades, researches have been increasingly focused on the production of formulations based on natural compounds found in vegetables and plant extracts with pharmacological and/or nutraceutical characteristics. They can have indeed antioxidant, antiseptic, antibiotic and antitumoral properties. These compounds of natural origin can lead to new, innovative therapeutic agents, since they may benefit in cancer prevention and promote human health without recognizable side effects (pharmaceutical target) [1-3]. Furthermore, there has been a significant interest in incorporating these kind of compounds in functional food and beverage products to benefit human health and wellness through the diet (nutraceutical target). Hence, the boundary between functional food/nutraceuticals and pharmaceutical ingredients is continuously narrowing.

Most of the active compounds is classified as poorly water-soluble, due to their lipidic nature, and, consequently, shows a poor bioavailability, that represents a great limitation to their use both as therapeutic and nutraceutical agent. A low solubility in aqueous media, indeed, induces a limited absorption; the improvement of their bioavailability is one of the most challenging target of new formulation development process, especially for oral delivery system. Furthermore, the principles with antioxidant properties have high instability in presence of light, heat and oxygen; hence, they

may be affected by physical and chemical degradation during processing, transport or preparation [1-3].
Various formulation strategies have been widely investigated to improve the dissolution rates of these compounds; among these, the reduction of their particles size (PS) can improve their bioavailability because the exposed surface in contact with aqueous medium increases. However, these substances often show great tendency to fast crystallization and agglomeration that make them difficult to be processed. A crystalline structure, generally shows slower dissolution rate compared to disordered amorphous form. Both reduction of size and change in amorphous structure make the active compounds more sensitive to degradation. The incorporation of these bioactive compounds in carrier matrices represents a suitable method to overcome all of these drawbacks problems [4, 5]. The encapsulation of functional components allows their easy incorporation into food systems; then, it may protect them from the environment; finally, the delivery systems may release faster the active substances in a particular site of action [6]. In these fields, formulations based on microspheres formed by a solid dispersion of an active compound in a carrier matrix have been demonstrated to be very promising [4, 5]. Conventional techniques like spray-drying, emulsion/solvent evaporation, jet milling show several disadvantages, such as: wide particle size distributions, high temperatures with possible degradation of active compounds, large use of heavy and toxic solvent and high solvent residues in final product. Therefore, several supercritical fluids (SCF) based techniques have been proposed to overcome some of these drawbacks. Particularly, Supercritical Assisted Atomization (SAA) has been used to micronize pure compounds [7-9]; but, also for the production of composite microparticles formed by a polymeric carrier in which a drug can be homogeneously dispersed [10-16].
Hence, the aim of this work is to identify carriers able to stabilize active natural compounds producing composite systems using SAA technique. The role of the carrier in influencing size distribution, morphology, solid state, but especially in inhibiting recrystallization and enhancing bioavailability, and in protecting antioxidant activity (in the case of nutraceutical food purposes) has been investigated. In this work, different kinds of carrier have been considered: polyvinylpyrrolidone (PVP), hydroxypropyl-β-cyclodextrin (HPβCD) and dextran (DEX), that are a synthetic polymer, a cyclodextrin and a polysaccharide respectively. All of these carriers are water-soluble, biocompatible, biodegradable, accepted by FDA (Food and Drug Administration) and able to inhibit the crystallization of active molecules [17-20]. Different principles, such as curcumin (CUR, a curcuminoid), luteolin (LUT, a flavon), beta-carotene (BC, a caratenoid), extract of propolis (EP, a natural resinous mixture) and saffron petals (a spice derived from the flower of Crocus sativus) have been used as active compound examples.

APPARATUS, MATERIALS AND METHODS

SAA laboratory apparatus consisted of two high-pressure pumps delivering the liquid solution and liquid CO_2 to a saturator. The saturator is a high pressure vessel (25 cm^3) loaded with stainless steel perforated saddles thus assuring the formation of an expanded liquid. The solution obtained is sprayed through a thin wall (80 µm ID) injection nozzle into the precipitator (3 dm^3) operating at atmospheric pressure. A controlled flow of N_2 is taken from a cylinder, heated and sent to the precipitator to assist liquid droplet evaporation. A stainless steel filter located at the bottom of the precipitator allows powder collection and the gaseous stream can flow out. SAA apparatus layout and further details on the experimental procedures were published elsewhere [21].
Carbon dioxide (CO_2; purity 99.9%) was purchased from Morland Group and Nitrogen (N_2; purity 99.9%) from SON. The carriers used in this work are: polyvinylpyrrolidone (PVP, Mw: 10,000, Fluka); hydroxypropyl-β-cyclodextrin (HPβCD, Acros Organic); dextran from *Leuconostoc mesenteroides* (DEX, MW 43,000, Sigma Aldrich); the solvent used is ethanol (99.9%, Sigma Aldrich). The active compounds studied are: Luteolin (LUT, Epitech Group Res Labs), β-carotene

(BC, Sigma Aldrich) and an ethanolic extract of propolis (EP) from a Chilean beekeeper, saffron petals from Magnano in Riviera. Gallic Acid and Folin-Ciocalteu reagent were supplied from Merck Millipore; 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich.

Particle size distribution

The morphology of SAA powders was observed by a field emission-scanning electron microscope (FESEM, mod. LEO 1525, Carl Zeiss). Particle size distribution (PSD) of the microparticles were measured from FESEM photomicrographs using the Sigma Scan Pro Software (release 5.0). Histograms representing the PSD were fitted using Microcal Origin Software (release 8.0).

Antioxidant activity

Total phenolic content of the samples was determined with the Folin Ciocalteau method [22]. SAA sample was dissolved in ethanol (70% v/v), mixed with distillated water, the Folin Ciocalteau reagent and a carbonate solution and, after 30 min under dark, the absorbance was carried out in a Tecan microplate reader at 760 nm. Calibration was performed using Gallic Acid as reference compound and the results were expressed in mg of Gallic Acid Equivalents (GAE)/g of powder. DPPH radical scavenging activity of powder was measured according to the method described by Brand-Williams et al. [23]. SAA sample was dissolved in ethanol (70% v/v) and mixed with an ethanolic solution (70% v/v) of DPPH (20 $mg*dm^{-3}$) and left under dark for 30 minutes in a 96-well microplate. The determination of the absorbance was carried out in a Tecan microplate reader at 515 nm. Results were expressed as the half maximum inhibitory concentration of DPPH radicals (IC_{50}), in $\mu g*cm^{-3}$.

Loading and dissolution tests

The amount of active compound loaded in the carrier was determined by UV/vis spectrophotometer (model Cary 50, Varian, Palo Alto, CA). Loading efficiencies were calculated as the ratio of the effective over the theoretical content of active compound. For dissolution tests, samples containing an equivalent amount of active compound (5 ppm) were weighted, placed in a dialysis sack and incubated in 400 mL of phosphate buffer saline (PBS) at pH 7.4, stirred at 200 rpm and 37°C.

RESULTS AND DISCUSSION

Active compounds alone have been processed by SAA, such as CUR [14], BC, EP [11]and LUT [15], in the attempt of reducing controlling principle particle size and crystallization. In all cases, no particles or irregular, partly recrystallized particles were obtained and SAA tests were considered unsuccessful [14-16]. These results have evidenced a difficulty in processing these active compounds. The production of a homogeneous dispersion of the active principle in a carrier matrix in forms of dried particles can be a suitable option to entrap the compounds during the atomization process and to stabilize amorphous form against crystallization [24]. Some interesting carriers, such as polymer, cyclodextrin and polysaccharide [24], have shown to be good candidates to produce coprecipitates by SAA technique [10, 14-16]. Table 1 reports some coprecipitation experiments performed by SAA, using PVP, DEX and HPβCD as carrier.

Table 1. SAA process conditions and parameters explored for each active compound/solvent system.

	PVP		DEX		HPβCD	
	Conditions	**Explored**	**Conditions**	**Explored**	**Conditions**	**Explored**
LUT	ethanol P_s=95-98 bar T_s=T_p=80°C [15]	R (1/2÷1/8)	water/ethanol (70/30 v/v) P_s=92-95 bar T_s=85°C, T_p=100°C	R (1/2÷1/8) C (5-20 mg/mL)	-	-
EP	water/ethanol [11] (30/70 v/v) P_s=90 bar T_s=80°C, T_p=90°C	C (30÷60 mg/mL) R (1/2÷1/8)	-		water/ethanol (30/70 v/v) P_s=90 bar T_s=80°C, T_p=90°C	C (30÷60 mg/mL) R (1/2÷1/8)
BC	ethanol P_s=85 bar T_s=T_p=80°C	C (2÷10 mg/mL) R (1/4÷1/20)	-	-	-	-
CUR	ethanol P_s=93-99 bar T_s=T_p=80°C [14]	R (1/2÷1/8)	-	-	-	-

P_s: saturator pressure; T_s: saturator temperature; T_p: precipitation temperature;
C: total solute concentration; R=active principle/carrier weight ratios.

Examples of coprecipitated particles are shown in Figure 1, reporting FESEM images. Figure 1a, 1c, 1e and 1f show PVP coprecipitated with LUT, EP, CUR and BC respectively; figure 1b e 1d show coprecipitates of DEX-LUT and HPβCD-EP, respectively.

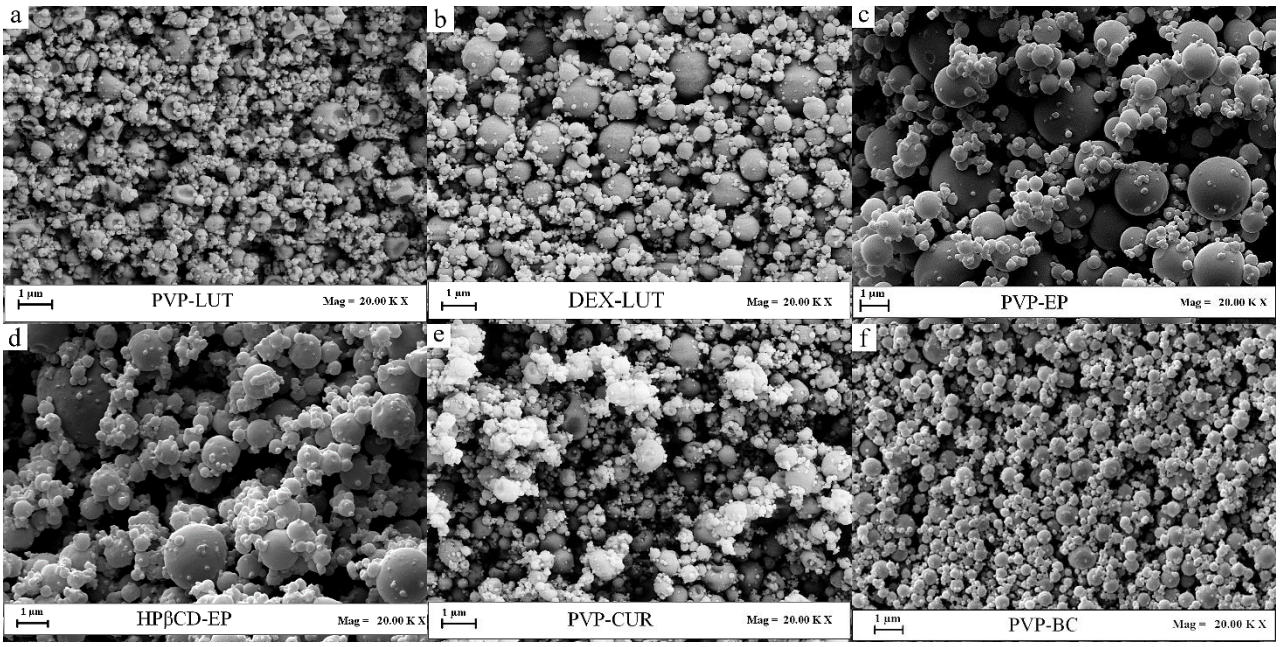

Figure 1. FESEM images of SAA particles of: a) PVP-LUT (R=1/6); b) DEX-LUT (R=1/6); c) PVP-EP (R=1/5); d) HPβCD-EP (R=1/5); e) PVP-CUR (R=1/8); f) PVP-BC (R=1/20).

PVP showed to be a good carrier for the inhibition of LUT crystallization, indeed spherical and/or collapsed particles (Figure 1a) were produced by SAA, due probably to the fragility of the solid structure [15]. PVP has also been effective for entrapping active compounds not easy to process, such as CUR, BC and EP. PVP-EP and PVP-BC spherical particles were obtained (Figure 1c and 1f), whereas PVP-CUR particles (Figure 1e) show a slightly collapsed morphology [14].

Using DEX as carrier to stabilize and process LUT (Figure 1b), more regular particles were formed compared to PVP-LUT ones. For the stabilization of EP, when HPβCD was used as carrier, at high

R (R= 1/3 and 1/2, image not reported) coalescing particles were obtained, whereas, when R was decreased down to 1/5 (Figure 1d) and 1/8, well defined spherical particles were produced. Furthermore, at the same operative conditions, lower amounts of PVP compared to HPβCD are necessary to obtain regular particles.
Looking at the overall results, submicrometric particles were always produced and R play an important role on PSDs., as summarized in Table 2, where PSD values (in terms of particle volume) expressed as mean diameter d_{10}, d_{50} and d_{90}, indicating the diameters at 10^{th}, 50^{th} and 90^{th} percentiles, are reported.

Table 2. PSDs data in terms of particles volume.

	PVP+LUT (R=1/6)	**DEX+LUT (R=1/6)**	**PVP+EP (R=1/5)**	**HPβCD+EP (R=1/5)**	**PVP+CUR (R=1/8)**	**PVP+βC (R=1/6)**
d_{10} (µm)	0.31	0.20	0.54	0.50	0.24	0.22
d_{50} (µm)	0.61	0.23	1.48	1.16	0.54	0.49
d_{90} (µm)	0.98	0.63	2.65	2.09	0.94	0.83

Solid state
DSC and X-Ray analyses were performed on unprocessed materials and on SAA processed active principle-carrier, to evaluate the potential changes in the thermal and the crystallization behavior of the active compound. Examples of diffractograms related to pure compounds and SAA coprecipitates are reported in Figure 2.

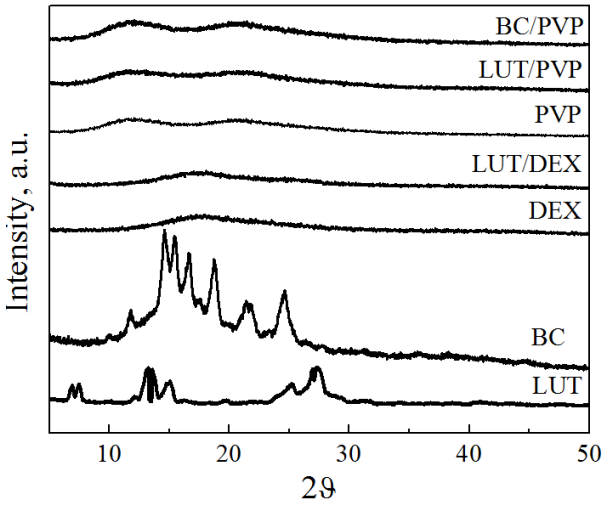

Figure 2. Diffractograms of pure compounds and SAA coprecipitates.

Assuming that PVP, DEX and HPβCD are all amorphous carrier, the active compounds selected have a spectrum typical of crystalline form. SAA coprecipitates, independently from the carriers used, have an amorphous structure: this might be explained by the homogeneous dispersion of the active principle as nanometric or amorphous objects in carrier matrix obtained in all cases studied.

FTIR analyses (images not reported) have revealed that LUT, for its chemical structure, has created some hydrogen bonds with the two carriers used (PVP and DEX). For the other systems investigated, no interactions were found: this shows that the ability to create new interactions does not depend on the carrier but on the active compound.

Improvement of bioavailability

The loading efficiency of the active principle in SAA coprecipitates and dissolution behavior are the major evidence of a successful coprecipitation. Table 3 reports some results obtained for SAA coprecipitates.

Table 3. Loading efficiences and improvement of dissolution time.

Sample	Loading efficiency (%)	Dissolution time (h)	Improvement compared to physical mixture
PVP+CUR (R=1/8)	100	≈ 5	≈ 5 times
DEX+LUT (R=1/6)	100	≈ 4	≈ 18 times
PVP+LUT (R=1/4)	99	≈ 8	≈ 9 times
PVP+BC (R=1/6)	75	≈ 19	≈ 22 times

As reported, SAA coprecipitates show high loading efficiency, up to 100% in most of the systems selected. To verify the improvement of bioavailability, release behaviour was investigated for each system, following the recommendation of US Pharmacopeia. All coprecipitates show a faster release than untreated physical mixture related to an improvement of dissolution rate of the principles tested. The results obtained confirm that the dissolution rate of SAA produced microparticles, and hence the bioavailability, is influenced by several factors, like reduction of particle size, morphology and solid state (including chemical interactions between principle and carrier).

Protection of bioactivity

The antioxidant activity of coprecipitates obtained by SAA was studied for the system PVP-EP and HPβCD-EP, and PVP-saffron extracts that have nutraceutical applications.

Folin-Ciocalteau method results show that SAA particles have a total polyphenol content up to 100 and 96% for HPβCD coprecipitates and for PVP coprecipitates, respectively. Hence, the entrapment of polyphenols in the two carriers used for these investigations is effective; these data confirm that the coprecipitation process obtained by SAA leads to the production of polyphenol-rich particles.

DPPH assay was used to measure the ability of the particles produced to act as free radical scavengers after coprecipitation process. The homogeneous dispersion of EP in the two matrices selected is able to preserve the activity of the starting materials, indeed, the IC_{50} values of free radical scavenging activity ranged between 56.6 and 17.2 μg/mL for HPβCD and 67.2 and 17.3 μg/mL for PVP; whereas, the IC_{50} of EP alone is 17.1 μg/mL. A lower IC_{50} means a higher free radical scavenging activity of the compounds. Therefore, the coprecipitation of EP-carrier and the size reduction may allow to maintain a good quality of the scavenging activity of unprocessed EP [25]. These results may be a remarkable added value for the production of nutraceutical and functional products.

Using ethanol and a water:ethanol solution (41:59 v/v) as extraction solvent, a yellow and a brown extracts were obtained with a scavenging activity (on a sample at total concentration of 1000 ppm) of 60% and 93%, respectively. After two months, these antioxidant capacities were reduced down to 49% and 60% respectively due to the degradation of the active compounds because of light, oxygen and heat. Producing SAA coprecipitates based on the extracted compounds from saffron petals and PVP, spherical particles with entrapment efficiencies up to 100% were produced. The powders were very stable and, after 2 months, scavenging activities were practically confirmed.

CONCLUSION

The production of amorphous particles based on a homogeneous dispersion of an active principle in a carrier matrix produced by SAA is very promising both in pharmaceutical and nutraceutical field. SAA technique is efficient for the processability of difficult compound, for the enhancement of dissolution rate of poorly water-soluble ingredients and for the protection of their antioxidant properties against light, heat and oxygen. Furthermore, carriers such as PVP, DEX and HPβCD are able to improve the handling of the product and simplify its storage.

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