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Authors: C. Gimenez-Rota, I. Palazzo, MR. Scognamiglio, A. Mainar, E. Reverchon, G. Della Porta



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β -Carotene, α -Tocopherol and Rosmarinic Acid encapsulated within PLA/PLGA microcarriers by Supercritical Emulsion Extraction: encapsulation efficiency, drugs shelf-life and antioxidant activity

Gimenez-Rota C.^{1,2}, Palazzo I.¹, Scognamiglio MR.², Mainar A.²,

Reverchon E.¹, Della Porta G.^{1,3*}

¹ Supercritical Fluids Lab., Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II, 84084 Fisciano-SA, Italy.

² Group of Applied Thermodynamics and Surfaces (GATHERS), I3A Aragón Institute of Engineering Research, Universidad de Zaragoza, C/ PedroCerbuna12, 50009 Zaragoza, Spain.

³ Traslational Medicine Lab., Department of Medicine and Surgery, University of Salerno, Via S. Allende, 84081 Baronissi-SA, Italy.

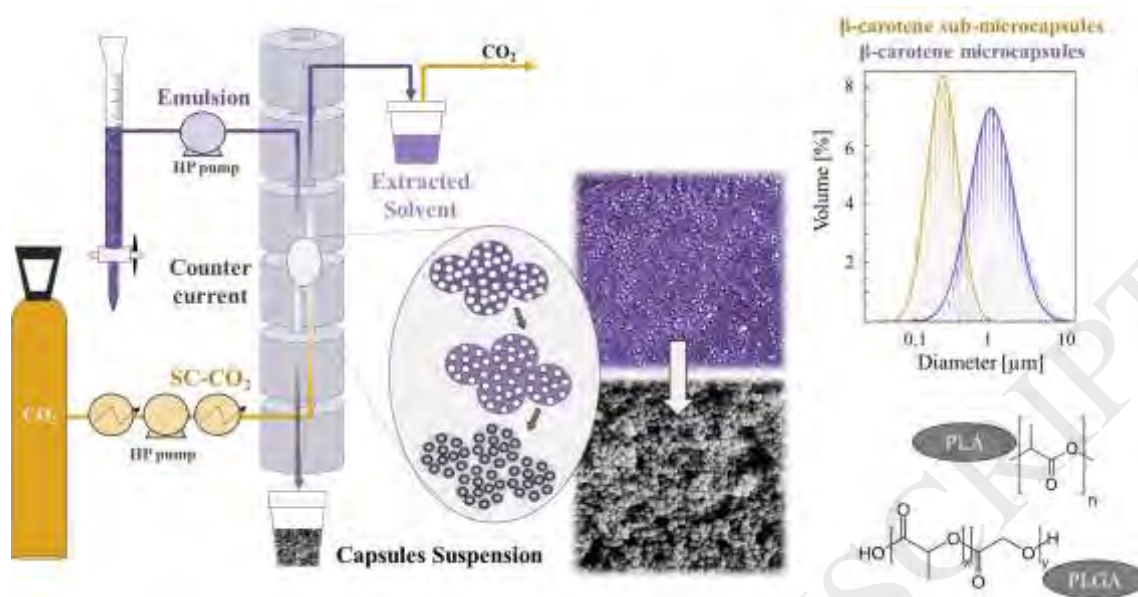
Keywords: Supercritical Emulsion Extraction, micro and capsules, antioxidant activity, functional excipient, shelf-life.

Chemical compounds studied in this article

β -carotene (PubChem: 5280489), α -tocopherol (PubChem: 14985), Rosmarinic acid (PubChem: 5281792).

() Corresponding Author: Della Porta Giovanna, prof.
Department of Medicine and Surgery "Scuola Medica Salernitana", University of Salerno, Via S. Allende, Baronissi (SA) 84084, Italy. e-mail: gdellaporta@unisa.it;
tel: +39089964104; fax: +39089964057.*

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ABSTRACT

β -Carotene (β -CA) is largely used antioxidant with a high sensibility to oxidation when in contact with light and temperature. To improve its shelf life and develop a nutraceutical formulation it was encapsulated into *poly-lactic-co-glycolic acid* (PLGA) and *poly-lactic acid* (PLA) carriers using Supercritical Emulsion Extraction (SEE). α -Tocopherol (α -TOC) and Rosmarinic Acid (RA) were proposed as excipients to improve product shelf-life. Different emulsion formulation conditions, such as compositions and mixing rate were used; whereas, SEE operating conditions in the counter-current tower were fixed at 80 bar and 38 °C with an L/G ratio of 0.1. In these conditions, PLA and PLGA carriers with sizes ranges between $1.5 \pm 0.5 \mu\text{m}$ and $0.3 \pm 0.1 \mu\text{m}$ were fabricated with Encapsulation Efficiencies (EEs) between 50-80%. The co-encapsulation of α -TOC with β -CA gained to prolonged drug shelf life; whereas, RA co-encapsulation was not successfully and, in some cases, also reduced β -CA-EE. The poor loading

experienced, in the case of RA, was probably due to its high solubility into the high-pressure mixture of carbon dioxide and organic solvent formed during the emulsion extraction. This behaviour was defined “*co-extraction effect*” and may limit the application of SEE technology in the encapsulation of molecules soluble in the mixture obtained during oily phase extraction. Shelf-life studies were performed after UV irradiation for ten days and after carriers storage for 2 years at 4°C. The better performance in terms of shelf life was observed for PLGA capsules loaded with β -CA/ α -TOC; whereas, PLA formulation with β -CA/ α -TOC showed a superior antioxidant activity with an half minimal Inhibitory Concentration (IC_{50}) against the free radical DPPH of 1.8 mg/mL of carriers.

1. INTRODUCTION

β -Carotene (β -CA) is widely used in the food, cosmetic and pharmaceutical industries, as natural photo protector colorant and antioxidant [1]. β -CA has a high free radical scavenging and antioxidant activity due to their multiple conjugated double bonds; moreover, it is the major precursor of vitamin A [2,3]. Others biological effects of β -CA are: decrease of cancer risks enhancing immune responses [1], stimulation of gap-junctional communication [4], protection from arterial disease [5] and induction of a light barrier avoiding cell damaging [6]. Concerning food applications, its inclusion in food matrix allows to obtain the *so-called* functional or nutraceutical food; moreover, carotenoids are also used as colorants to recover the colour lost during food processing and storage [7].

β -CA molecule is highly hydrophobic and difficult to disperse in water [2]. For this reason, to improve the bioavailability and enhance its water dispersion, it is often micronized [2,8] or co-precipitated with protein [3]. β -CA is also very susceptible to degradation under temperature, light and oxygen [9,10]; clinical studies revealed that its degradation products are highly reactive and can shift properties from antioxidant to pro-oxidant [11]. In order to prevent molecule degradation, different polymers were studied as carrier for β -CA encapsulation such as, tapioca starch, maltodextrin or oleoresin using spray drying technology [12–14], or casein using solid lipid nanoparticles (SLNs) protocol and galactan by evaporation technology [9,15]. The co-encapsulation with other antioxidant excipients, such as α -tocopherol (α -TOC) and ascorbic acid can also provide a better protective effects on oxidation [16].

Poly-lactic acid (PLA) and *poly-lactic-co-glycolic acid* (PLGA) are biocompatible and biodegradable polymers which have recently been the subject of extensive investigation

[17-18,19-20]. α -Tocopherol (α -TOC), also known as vitamin E, is a fat-soluble highly potent antioxidant, abundant in vegetable oils or wheat germs; it is widely used by the pharmaceutical, cosmetic and food industries because of its clinical and preservative applications [21-22]. Rosmarinic Acid (RA) is a polyphenol with important biological activities, such as anti-inflammatory, antiagregant and antioxidant [23]. β -CA and carotenoids loading into PLA and PLGA biopolymers have been already proposed by different fabrication technologies for micro-capsules preparation [10]. The emulsification-evaporation method [24-27], spontaneous emulsification solvent diffusion method (SESD) [18], nanoprecipitation method [2,28] are all widely used in preparing PLA/PLGA micro-carriers of various mean sizes. Each of these methods employs a similar first step, where β -CA solution is emulsified in a water solution to form an *oil-in-water* dispersion (*o-w*). If appropriate, the molecule may also be dispersed as a solid powder in an organic polymer solution, or co-dissolved in a common solvent with the polymer. The solution or dispersion is then processed according to one of the aforementioned methods. During the solid carriers formation using emulsification-evaporation and precipitation approaches, organic solvents such as dichloromethane and chloroform are usually employed. To meet the requirement for the food and pharmaceutical use, residual solvents should be completely removed from fabricated micro systems [29].

Supercritical Emulsion Extraction (SEE) technique has been proposed to encapsulate several drugs and molecules [30-31]. In the process, the dense gas is used to extract the oily phase from a pre-formulated emulsion and to fabricate solvent free micro and nano-carriers [32-34]. The use of dense gas, such as Supercritical Carbon Dioxide (SC-CO₂) is an alternative to almost all the conventional processes because it is possible to work

at near-ambient temperatures, avoiding the degradation of thermolabile substances; SC-CO₂ also provides an inert medium suitable for processing oxidable substances.

In this paper, the β -CA encapsulation into *poly-lactide* (PLA) and *poly-L-lactide-co-glycolide* (PLGA) is proposed using SEE technique with the aim of improving its shelf-life and preserving its antioxidant activity. The co-encapsulation with other natural antioxidants, such as α -TOC and RA is also described to improve micro-capsules shelf-life and antioxidant activity. The possibility of fabricate carriers with different sizes will be also explored and the related encapsulation efficiency and antioxidant activity will be tested. Products shelf-life will be monitored by UV exposure for 10 days and after storage for 2 years. The combination of β -CA with other antioxidants will be also tested in order to understand the formulations stability and their biological activity and to demonstrate the versatility of the SEE technology in producing complex formulation for several applications.

2. MATERIALS AND METHODS

2.1 Materials

CO₂ (99.9%, Morlando Group, Naples, Italy), chloroform anhydrous (CL), methanol (ME), acetone (AC), ethanol (ET), acetonitrile (ACN), acetic acid (ACE), ethyl acetate (EA) all of purity 99.9% were supplied from Carlo Erba Reagents (Milan, Italy). Water (HPLC grade), glycerol (GLY, purity 99%, Aldrich Chemical Co.), sorbitan monolaurate (Span 20 Sigma-Aldrich), Tween 80 (Sigma-Aldrich), β -carotene (β -CA, Aldrich Chemical Co), α -Tocopherol (α -TOC, Sigma-Aldrich), Rosmarinic acid (RA, 96% Sigma-Aldrich), *poly-lactic acid* (PLA, MW: 60000 g/mol, Resomer RG 708H Boehringer Ingelheim), *poly-lactic-co-glycolic acid* (PLGA, 75:25 MW: 20000 g/mol,

Resomer RG 752S, Boehringer), 1,1-diphenyl-2-picrylhydrazine (DPPH, 97% Sigma-Aldrich), were used as received.

2.2 Emulsions Preparation with water as external phase

To encapsulate β -CA, α -TOC and RA, several double $o_1/o_2/w$ emulsions were formulated with a composition ratio of 4:16:80 w/w/w. The internal o_1 phase contained the active principles solved into CL with 0.06% Span 20; the second oily phase, o_2 , was formed by EA with a given amount of biopolymer dissolved (0.4 to 0.8 g of PLA or PLGA). For emulsions formulation, the inner phase o_1 , was mixed with the second oily phase o_2 to form the o_1/o_2 emulsion by ultrasonication (mod. S-450D, Branson Ultrasonics Corporation, Danbury, CT, USA). The o_1/o_2 emulsion was, then, immediately added into a known amount of EA-saturated aqueous Tween 80 solution (0.6%, w/w of Tween) with the high-speed stirrer (model L4RT, Silverson Machines Ltd., Waterside, Chesham Bucks, United Kingdom) for a time ranges between 4-6 min and at with stirring ranges of 2800-3600 rpm. Other procedures such as direct solvent mixture preparation (CL: EA ratio 4:16) did not worked properly and β -CA precipitation was observed; indeed, the surfactant (Span 20) added in CL oily phase improved its mixing with the EA oily phase assuring: (i) an excellent solubilisation of all the antioxidants in the o_1 phase; (ii) the good solubilisation of the biopolymers in the second o_2 phase.

Different formulations of RA alone into 1g of PLA were also prepared. In this case $w_1/o_1/w_2$ emulsions were formulated with a ratio of 1:19:80 w/w/w. ET plus 0.06% w/w of PVA was used as solvent of the internal w_1 phase; o_2 phase contained 1 g of PLA into EA; w_2 external phase was of EA-saturated aqueous Tween 80 solution (0.6% w/w).

2.3 Emulsions Preparation with water-glycerol as external phase

Some runs were performed fixing the external water phase composition at 80% (w/w) glycerol and 20% (w/w) distilled water plus 0.6% w/w of Tween 80; in this condition we used AC as solvent for the oily phase. The overall ratio *o/w* was always maintained at 20/80 for all the emulsion prepared and PLGA amount in the oily phase was fixed at 1 g, while the β -C concentration was fixed at 6 mg/g; the use of AC allowed the solubilization of both compounds (β -CA/PLGA) in the oily phase. AC was not used to form emulsion with water because of their mutual solubilisation in all compositions, which prevent the correct emulsion formulation.

2.4 Supercritical emulsion extraction (SEE)

All emulsions formulated were processed through SEE in a continuous experimental laboratory apparatus, as better described elsewhere [34-37]. Briefly, the apparatus consists of a stainless steel packed column with an internal diameter of 13 mm, in which carbon dioxide is fed from the bottom of the column using a high-pressure diaphragm pump, and emulsions are delivered from the top of the column using a high pressure piston pump. The column is formed by three AISI 316 stainless steel cylindrical sections of 30 cm height, connected by four cross-unions and is filled with stainless steel packing elements of 4 mm nominal size with 1889 m^{-1} specific surface and 0.94 of voidage (0.16 inch Pro-Pak, Scientific Development Company, State College, PA, USA). The apparatus is thermally insulated by ceramic cloths and its temperature is controlled by six controllers (TC1-TC6, Gordon J/Series 93, Watlow, Milan, Italy) at different heights of the column. Oily phase solvent is recovered in a separator located downstream the top of the column, in which the pressure is regulated by a backpressure valve (V5, 26-1700 Series, Tescom, Selmsdorf, Germany). A rotameter (mod. N5-2500, ASA, Sesto San Giovanni, Italy) located at the exit of the separator measures the CO_2 flow rate delivered. A schematic representation of the SEE layout is also reported in

Figure 1a. The operating pressure and temperature were fixed at 80 bar and 37°C. SC-CO₂ flow rate was 1.4 kg/h and emulsion flow rate 0.14 kg/h maintaining a ratio between liquid and SC-CO₂ (L/G) of 0.1. At this experimental conditions, all the organic solvents used to obtain the oily phase of the emulsions showed a large miscibility in SC-CO₂ [38-42]. A mean recovery efficiency of 80% was measured in each run. The lost material was due to the shutdown protocol, which avoid the recovering the last washing fractions because they may give less accurate data on particle size, due to the not steady state conditions operation during the final operation of washing. The biopolymer suspensions were collected at the bottom of the column. They were washed with distilled water by 2 serial centrifugations for 20 min at 6500 rpm at 4°C and then, recovered on a membrane filter (porosity 0.2 µm). The recovery of the micro-carriers from water-glycerol external phase required multiple dilution in pure water before the filtering step to recover the carriers. .

2.4 Droplets and Microspheres morphology & size distributions

The droplets formed in the emulsion were observed using an optical microscope (mod. BX 50 Olympus, Tokyo, Japan) equipped with a phase contrast condenser. A Field Emission-Scanning Electron Microscope (FE-SEM mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany) was used to study the morphology of the produced capsules. Samples were coated with gold (layer thickness 250Å) using a sputter coater (mod.108 A, Agar Scientific, Stansted, UK). *Droplets Size Distributions* (DSD) and *Particles Size Distributions* (PSD) were measured by dynamic light scattering (DLS, mod. Mastersizer S, Malvern Instruments Ltd., Worcesterstershire, UK) immediately after the preparation of the emulsion and the suspension, using 1 mL of each sample. The distributions proposed in this paper are the mean of 10 measurements. The

shrinkage factor percentage (SF%), defined as the ratio between the particle mean size and droplets mean size percentage [34] was also measured for each run.

2.6 Solvent residue analysis

Solvents (EA, AC, CL, ET,) contents in the water suspensions at the exit of the column was analysed, to monitor the efficiency of solvent removal by SC-CO₂ extraction. The solvent residue was measured using a head space sampler (mod. 50 Scan, Hewlett & Packard, Palo Alto, CA, USA) coupled to a gas chromatograph interfaced with a flame ionization detector (GC-FID, mod. 6890 Agilent Series, Agilent Technologies Inc., Wilmington, DE). Organic solvents were separated using a fused-silica capillary column 30 m length, 0.25 mm internal diameter, 0.25 µm film thickness (mod. DB-1, J&W, Folsom, CA, USA). GC conditions were: oven temperature at 40°C for 8 min. The injector was maintained at 180°C (split mode, ratio 1:1) and Helium was used as the carrier gas (7 mL/min). Head space conditions were: equilibration time 60 min at 100°C, pressurization time 2 min, loop fill time 1 min. Head space samples were prepared in 10 mL vials filled with 3 mL of suspension. Analyses were performed on each sample in three replicates.

2.7 Drug loading

For the loading quantification, a known mass (approximately 15 mg) of recovered capsules were dissolved in 0.5 mL of AC in order to break the biopolymer and solubilize the encapsulated molecules. Then 3.5 mL of ET were added to precipitated the biopolymer. The suspension was then centrifuged for 30 min at 6500 rpm at 4°C and the supernatant was collected for concentration measurements. The HPLC-PDA (mod. 1100 and 1200 series; Agilent LC system) was equipped with a Waters Spherisorb ODS-2 (Ø 5 µm 150x4.6 mm) column. The mobile phase used was 80:20

ME:WATER acidified 0.1% ACE; the flow rate was of 1 mL/min. The injection volume was 20 μ L in every test and the detection wavelength of: 450 nm for β -CA, 292 nm for α -TOC and 330 nm for RA. A calibration curve was built for each compound. The encapsulation efficiency percentage (EE%) was calculated as amount (mass) measured/amount (mass) loaded x100.

2.8 Shelf life study

The assays were performed on capsules containing β -CA and β -CA with α -TOC. In the first case, by quantifying the remaining β -CA in the capsules compared with the pure compound after exposed to UV radiation ($\lambda=254$ nm) for 1 hour a day for 10 days. In the second case, by measuring the remaining encapsulated β -CA after 2 years of storage at 4°C in the dark. In both shelf life studies, samples were solved in AC and the amount of active β -CA was measured at 450 nm using a spectrophotometer UV-vis (mod. Cary 5000, Agilent LC system).

2.9 Antioxidant activity

The 2,2-diphenyl-1-picryl-hydrazil (DPPH) radical scavenging activity was determined [36]. Firstly, the calibration curve was performed monitoring the antioxidant activity of pure β -CA. 1.5 mL of β -CA solution were added to 1.5 mL of 0.1 mM DPPH both dissolved in mixture of AC:ET (1:7) and then kept in the dark for 18 hours at room temperature. The biopolymer capsules were solved in 0.5 mL AC first and then 3.5 mL of ET was added; the mixture was centrifuged at 6500 rpm for 30 min. 3 different concentrations of the supernatant were prepared. Finally, 1.5 ml of these solutions were added to 1.5mL of 0.1 mM DPPH. The antioxidant activity was measured 18 hour at 517nm. The DPPH inhibition percentage (%) was defined as in eq. (1):

$$DPPH \text{ inhibition } \% = 100 \times (Abs \text{ DPPH} - Abs \text{ sample}) / Abs \text{ DPPH} \quad (1)$$

where *Abs sample* is the absorbance measured for the sample solution with DPPH after the reaction and *Abs DPPH* is the absorbance of DPPH solution. The theoretical inhibition percentage was calculated for each concentration of capsules and a theoretical inhibition curve was built for each product or for their mixtures (measured by simple mixing the two or three components). Theoretical half minimal Inhibitory Concentration (IC_{50}) was compared with the IC_{50} obtained experimentally.

3. RESULTS AND DISCUSSION

Samples of water suspension recovered at the bottom of the column showed solvent residue values between 50-100 ppm for EA and AC; no other solvents were found in all the formulations processed. Repeatability of about 90% was always observed in all the experiment described. Indeed, the relatively high amount of emulsion pumped for each run (100 mL) and the steady state conditions set before the emulsion processing by feeding external water phase for a proper time, provided a good simulation of a continuous operating system.

SEE-C encapsulation of β -CA and α -TOC in PLA and PLGA carriers

Different emulsion formulations were explored in order to assure the fabrication of microcarriers with different sizes. All the emulsion formulations, carrier size, and antioxidant loading are summarized in **Table 1**. In the case of PLA, 400 mg of biopolymer were always solubilized in the oily phase, obtaining micro-carriers of $1.4 \pm 0.5 \mu\text{m}$ with 68% of EE. When α -TOC was included in the formulation (ratio 9:1, β -CA: α -TOC) along with β -CA, the microcapsules mean diameter did not changed and the EE was of 72%. The same formulation with 400 mg of biopolymer in the oily phase was also tested for PLGA; in this case, microcarriers with mean size of $1.5 \pm 0.6 \mu\text{m}$ were obtained but with a poor EE of only 22%; the use of α -TOC in this formulation did

not strongly improved the EE. When, the amount of PLGA in the oily phase of emulsion was increased up to 800 mg, microcarriers of $2.0 \pm 0.6 \mu\text{m}$ were obtained with an improved EE of 58%. A shrinkage was always observed between the droplets and the solid particles in all experiments performed by SEE [36]. Nevertheless, in the case of emulsion formulations with PLA the SF was almost always of 24%; whereas, for PLGA a reduced shrinkage factor was observed of 37% and 10%, respectively when 400 to 800 mg of biopolymer were solubilized in the oily phase.

In order to reduce the droplet mean sizes and, therefore, to fabricate smaller carriers, the rotation per minute of the emulsifier was increased during emulsion preparation [37]. For both PLA (400 mg load in oily phase) and PLGA (800 mg load in oily phase), the droplets mean sizes were reduced to $1.2 \pm 0.1 \mu\text{m}$ and $1.2 \pm 0.1 \mu\text{m}$, respectively, and the resulting carriers mean diameters were of $0.3 \pm 0.1 \mu\text{m}$ in both cases, respectively, after SEE processing. Both EE data were measured at 62%. **Figures 1b-c** illustrated the PSDs of the carriers fabricated using both PLA and PLGA, biopolymers. The emulsion optical microscope images and the SEM images of the produced microsystems are illustrated in **Figure 2a-d and 3a-d**. Spherical shaped devices were always fabricated.

The improved EE% observed in the PLGA carriers when the amount of polymer was doubled in the oily phase may be due to the extremely low molecular weight of PLGA used in this work. Indeed, it was of 20000 g/mol for PLGA, so 3 times lower with respect the one of PLA that was 60000 g/mol; as a consequence, we supposed that the dynamic viscosity of the two oily phase solutions could be extremely different, when the same amount of biopolymer was dissolved in it. The large difference in oily phase dynamic behaviour may strongly influence the capability of the oily phase in holding the antioxidant molecules during the emulsion preparation (sonication and stirring phases) and, therefore, it may explain the necessity of a double amount of PLGA, in the

oily phase, to assure better encapsulation efficiency. However, further investigation involving the experimental measurement of dynamic viscosity for the oily phase solutions in relation of the amount of polymer solubilized within and its molecular weight should provide a better understanding of the described behaviour.

3.2 RA encapsulation into PLA and PLGA

Several runs were performed to test the RA encapsulation into PLA and PLGA systems; then, it was co-encapsulated with β -CA and α -TOC (ratio 8:1:1; β -CA: α -TOC:RA). The first set of experiments used a $w_1/o/w_2$ double emulsion where RA was solved in the aqueous internal phase formed by ET and water (w_1). The proportion of ET/WATER in the internal water phase was varied together with RA concentrations. All the conditions explored are described in **Table 2**. In all cases, very low EE were experienced ranging between 1.3% to 3.9%. Further investigations were performed in order to modify the overall system compositions and gain a good RA encapsulation; indeed, emulsion type $o_1/o_2/w$ using β -CA and α -TOC (with Span20 0.06% w/w), with both PLA and PLGA were tested and carriers with a mean diameter of 0.5 ± 0.1 μm for PLA and PLGA, respectively were obtained with an RA-EE% always no higher than 5%. Additionally, in the case of PLA processing, the inclusion of RA in the emulsion formulation, caused a further reduction of β -CA-EE% from 62% to 35%. When the co-encapsulation was performed into PLGA carriers, RA-EE was of 12% and β -CA-EE was maintained at 53%. The described data seemed in contrast with the ones reported by others authors that indicated RA-EE of 60-78% in *poly-capro-lactone* and *carboxy-methyl cellulose*, respectively; however, these results were obtained by using conventional evaporation/extraction processes [38-39]. Taking into account the SEE operative conditions used of 80 bar and 37°C, the low RA-EE cannot be due to its solubilisation in SC-CO₂ because higher P and T values are required to properly solubilized it [39].

However, considering the presence of the high pressure mixtures formed in the column during the oily phase extraction, the RA solubilisation can still occur. Indeed, in the literature, it is largely reported that mixtures of organic solvent such as EA and CL with CO₂, lead to expanded liquid formation, at the P and T conditions used for SEE processing [40-42]; this mixtures can extract the RA before or meanwhile the biopolymer is hardened. A better understanding of the behaviour observed can be possible with an accurate and deeper knowledge of the behaviour of the complex mixture CO₂/EA/RA, (ET and CL may be neglected due to the very low amount in the emulsion formulation) at high pressure. Indeed, we hypothesized that the complex composition system, formed at the P and T conditions tested, has a miscibility hole very sharp or not large enough to prevent the molecule co-extraction in every conditions explored. This behaviour, described as “*co-extraction effect*” may limit the application of SEE technology in the encapsulation of compounds with high solubility in the high-pressure mixture formed during the oily phase extraction. On the other hand, different molecular affinity between RA and the two biopolymers tested, may explain the higher encapsulation rate observed in the case of PLGA, which showed more polar functional groups with respected to PLA.

3.3 SEE processing of emulsions with glycerol-water phase

Some runs were performed fixing the water phase composition at GLY:WATER of 80:20 with 0.6% (w/w) of Tween 80; the overall ratio *o/w* was always maintained at 20/80 for all the emulsion prepared. Using GLY in the water phase, AC can be used as solvent of the oily phase, due to the immiscibility hole described in the ternary diagrams reported in **Figure 4b**. PLGA amount in the oily phase was fixed at 1 g, while the β -C loading was fixed at 30 mg/g. From the optical image of the emulsion droplets in **Figure 4a** it is possible to observe the presence of crystals of β -CA in the oily saturated

phase. For this reason, the emulsion can be more correctly described as *solid-oil-water s/o/w*. In **Figure 4c-d** a SEM image of the fabricated carriers is also reported with the PSD that showed a mean size of 4.4 ± 0.7 μm ; encapsulation rate of 82% has been also reported. SEE pressure and temperature conditions used, were of 80 bar and 40°C with an L/G ratio of 0.3; however, difficulties in SEE process managing have to be reported because of the high viscosity of the GLY-WATER phase that caused severe column blockage, preventing a good plant operation. For this reason this emulsion formulation was considered not good, even if excellent EE was monitored.

3.4 Shelf life study

Shelf life studies were proposed. Firstly, it was quantified the remaining β -CA into all PLA and PLGA capsules containing β -CA and β -CA+ α -TOC after its exposure to the light; the control test with pure β -CA was also performed. Results are illustrated in **Figure 5a**. The 83% of pure β -CA degrades after 3 days of UV exposure, while only the 21.7% and 16.8%, respectively, of the encapsulated drug degraded when loaded in PLA and PLGA capsules. After 10 days of UV exposure, pure β -CA was completely degraded; whereas, 4% and 2.6% of drug was still monitored, when encapsulated into PLA and PLGA, respectively. If β -CA was co-encapsulated with α -TOC, the remaining active drug was of 22% and 30% in PLA and PLGA, respectively after 10 days. This result is in agreement with several authors which reported the beneficial combination of α -TOC presence to prevent degradation of the carotenoid [43-45]. Indeed, the co-encapsulation of β -CA with α -TOC has been reported to block the oxygen radicals chain reaction, prolonging its shelf life in colloidal lipid particles of fat-in-water dispersions [46-47]. In the second shelf life study, the remaining β -CA was quantified after 2 years of storage in the dark at 4°C when co-encapsulated with α -TOC in both

PLA and PLGA capsules. As we can see from **Figure 5b**, only the 53% of the active degraded in PLA and the 63% one when encapsulated in PLGA. Both studies confirmed a better protection against oxidation for β -CA and good performances of the carriers fabricated by SEE; the best carrier formulation seemed to be the ones with β -CA/ α -TOC in PLGA.

3.5 Antioxidant activity

In order to measure the functionality of the micro capsules, their antioxidant activity was assayed against DPPH radical. According to β -CA calibration curve and the loading in each carriers tested, the theoretical antioxidant activity of micro capsules was measured and compared. The antioxidant activity of PLA and PLGA capsules was first monitored after 2 years of storage, as illustrated in **Figure 6a**. For PLA formulation, the activity measured was lower than expected ($IC_{50\text{expected}}$ 3.2 mg/mL vs $IC_{50\text{measured}}$ 6.7mg/mL) because of a higher β -CA degradation in these carriers; whereas for PLGA carriers the performance was better, confirming the lower degradation of β -CA in the system after two years with an activity maintained at IC_{50} 2.3 mg/mL. From **Figure 6a**, although the theoretical antioxidant activity of PLA capsules seemed higher than the ones of PLGA capsules, it is only due to the higher loading of β -CA in the PLA system. This difference could be also a consequence of a higher content of the co-encapsulated α -TOC (26.78% into PLA vs 52.97% into PLGA). Looking at the real activity, and in accordance with the shelf life results, PLGA seemed to be the more protecting biopolymer, preserving the β -CA activity along the time.

PLA and PLGA antioxidant activity was also measured immediately after their formulation for both PLA and PLGA systems. β -CA+ α -TOC theoretical IC_{50} according to their loading (6.2 mg/g and 3.1 mg/g, respectively) was of 2.7 mg/mL and 5.6

mg/mL; however, the measured antioxidant activity was always higher and of 1.8 mg/mL and 3 mg/mL, respectively, as reported (in percentage) in **Figure 6b**. When RA was included in the formulation, a higher theoretical IC₅₀ was expected, and 6.3 mg/mL and 4.2 mg/mL for PLA and PLGA carriers was measured; however, their antioxidant activity was lower than the equivalent PLA and PLGA carriers without RA because of the overall reduction of β -CA loading. Nevertheless, as described in **Figure 6c**, the measured activity is higher than the expected, when carriers with the co-encapsulation of the three bio-actives are tested. This is probably due to the combination of the three actives (β -CA+ α -TOC+RA) even if they are entrapped at an overall lower concentration. β -CA/ α -TOC/PLA capsules showed a superior antioxidant activity with an IC₅₀ against the free radical DPPH of 1.8 mg/mL.

CONCLUSIONS AND PERSPECTIVES

The Supercritical Emulsion Extraction (SEE-C) allowed the formulation of β -CA/PLA and β -CA/PLGA carriers with a wide range of sizes and with good EEs. The fabricated microsystem demonstrated to provide drug protection from UV radiation and along two years of storage, improving drug shelf life. α -TOC co-encapsulations gives an extra protection to β -CA degradation in both oxidizing conditions; whereas, encapsulation within PLGA gave better β -CA preservation and an improved shelf-life. A SEE limitation was detected when RA was processed to be encapsulated; indeed a lack of EE in the case of molecules extremely soluble in the high-pressure mixture formed during the oily phase extraction may be described in that case. More data on oily phases dynamic viscosity and high pressure behaviours of complex mixture will improve SEE technology managing and may provide deeper understanding of process performances.

It is also worth of note that the emulsions formulated using AC as a solvent of the oily phase gave the best results in terms of EE, but required the presence of an external water phase formed by GLY/Water, which was difficult to manage within the high pressure packed column and, therefore, this solution was discarded. AC was able to dissolve great amount of β -CA and biopolymer and several authors reported nano-emulsions obtained using AC and water [49-50]; however, AC completely miscibility with water prevented the formation of stable emulsion that can be properly processed by our SEE layout. Indeed, when AC oily phase (loaded with polymer) was mixed with water (ratio 20:80), the resulted emulsion was always unstable and polymer precipitation/aggregation was observed in all cases, even if surfactant was added in both phases.

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FIGURE CAPTIONS

Figure 1. Schematic representation of SEE apparatus layout with description of some emulsions used **(a)**. Particle Size Distributions (PSDs) of PLA and PLGA carriers **(b-c)**; the possibility of varying carriers size modifying the droplet sizes in emulsion was demonstrated for both biopolymers.

Figure 2. Optical microscope images of droplets in emulsions **(a, c)** and SEM images of PLA/ β -CA/ α -TOC carriers fabricated after emulsion processing by SEE technology **(b, d)**. Carriers with mean size of $0.3 \pm 0.1 \mu\text{m}$ with antioxidant loading of 6.2 mg/g **(b)** and of $1.5 \pm 0.5 \mu\text{m}$ with an antioxidant loading of 6.8 mg/g **(d)** were obtained. In order to reduce droplets and, therefore, particle size, higher value of rpm were used in emulsion formulation.

Figure 3. Optical microscope images of droplets in emulsions **(a, c)** and SEM images of PLGA/ β -CA/ α -TOC carriers fabricated after emulsion processing by SEE technology **(b, d)**. Carriers with a mean size of $0.5 \pm 0.1 \mu\text{m}$ with antioxidant loading of 2.7 mg/g **(b)** and of $2.0 \pm 0.6 \mu\text{m}$ with antioxidant loading 2.6 mg/g **(d)** were obtained. In order to reduce droplets and, therefore, particle size, higher value of rpm were used in emulsion formulation.

Figure 4. Optical microscope images of the emulsions **(a)** and SEM images of the related micro-capsules **(b)** produced by SEE using an *o-w* emulsions (20/80) AC:GLY/WATER. The emulsion composition is represented in the ternary diagram **(c)**. Particle Size Distribution (PSD) of PLGA micro-capsules fabricated is also reported **(d)**; mean size of $4.3 \mu\text{m}$ with β -CA loading of 30 mg/g was obtained.

Figure 5. Shelf-life studies results. Pure β -CA and encapsulated β -CA degradation tendencies along time after its exposure to UV radiation (UV length 259 nm) for one hour during ten days **(a)**; encapsulated β -CA degradation tendencies along two years after its storage at 4 °C in the dark **(b)**.

Figure 6. Antioxidant activity theoretical vs real. PLA and PLGA carriers loaded with β -CA and α -TOC, after 2 years **(a)**; loaded with β -CA and α -TOC, just after their fabrication **(b)**; loaded with β -CA, α -TOC and RA, just after their fabrication **(c)**.

ACCEPTED MANUSCRIPT

Table 1. Different emulsion formulations tested by SEE-C: antioxidants loaded in the o_1 phase and biopolymer loaded in the o_2 phase; mean sizes of the obtained droplets and particles with standard deviation. Encapsulation Efficiency (EE, %) and overall Antioxidant Loading (AL, mg/g). Ratio β -CA: α -TOC was of 9:1; Ratio β -CA: α -TOC:RA was of 8:1:1.

Polymer in o_2 (g)	Antioxidant in o_1	Droplets size (μm)	Carriers size (μm)	EE (%)	AL (mg/g)
PLA					
0.4	β -CA	2.1 \pm 0.5	1.4 \pm 0.5	68	6.8
0.4	β -CA/ α -TOC	2.1 \pm 0.5	1.4 \pm 0.5	72	5.0
0.4*	β -CA/ α -TOC	1.2 \pm 0.1	0.3 \pm 0.1	62	6.2
0.4	β -CA/ α -TOC RA	1.4 \pm 0.2	0.5 \pm 0.1	35	3.5
PLGA					
0.4	β -CA	2.4 \pm 0.6	1.5 \pm 0.6	22	2.1
0.8	β -CA/ α -TOC	2.1 \pm 0.6	2.0 \pm 0.6	58	2.6
0.8*	β -CA/ α -TOC	1.2 \pm 0.1	0.3 \pm 0.1	62	3.1
0.8	β -CA/ α -TOC RA	1.3 \pm 0.2	0.5 \pm 0.1	53	2.7
1**	β -CA/ α -TOC	7.8 \pm 0.9	4.3 \pm 0.7	82	25

* Higher value of rpm were used in emulsion formulation to reduce droplets size;

**AC:GLY/WATER emulsion.

Table 2. Different emulsion formulations processed to RA encapsulation; oily phase was formed of EA with 1 g of PLA; the external water phase was always formed by saturated water with 0.6% w/w of Tween 80.

RA loaded (mg)	Composition of w_1 (1 mL)	Composition of o_2 (19 mL)	EE (%)
20	20:80 ET:Water	PLA	3.8
100	ET	PLA	2.1
37	20:80 ET:Water	PLA	1.3
20	30:70 ET:Water	PLA	3.5
10	Water	PLA	3.5
150	--	PLA	-
150	--	PLGA	-

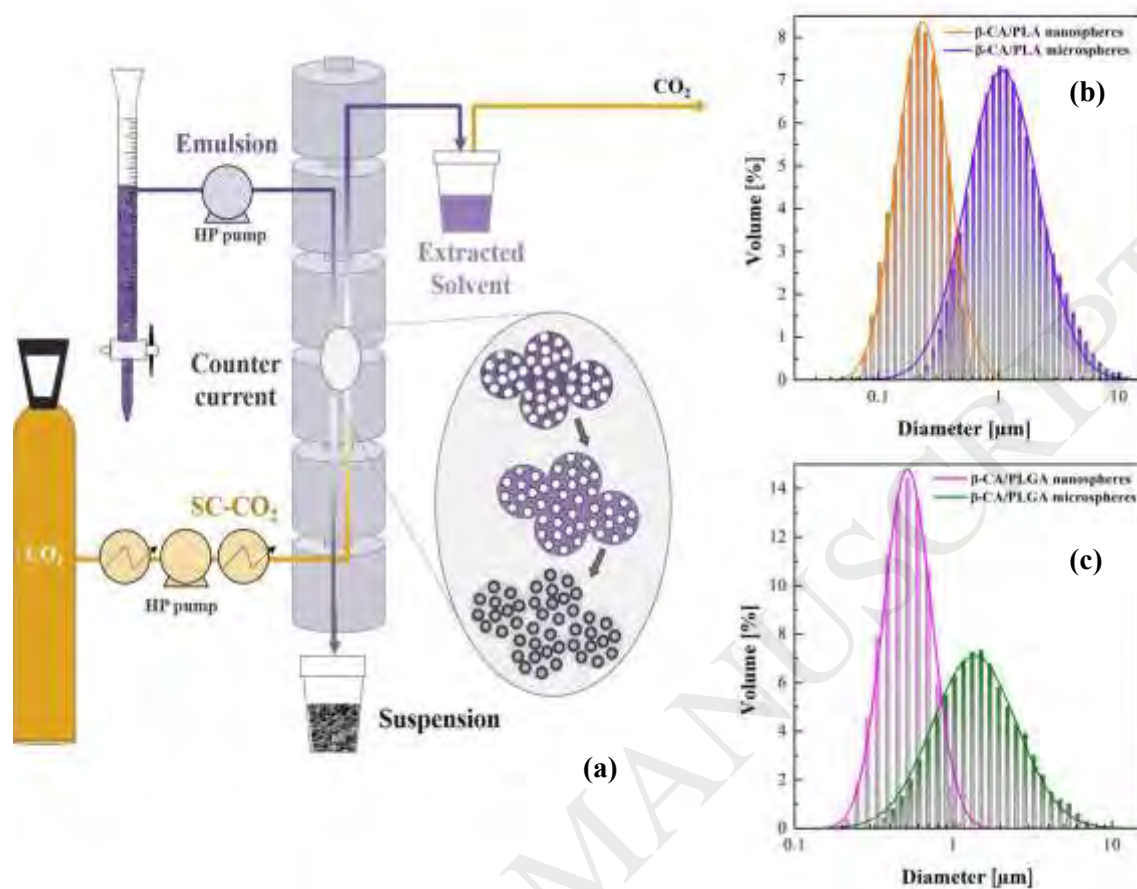


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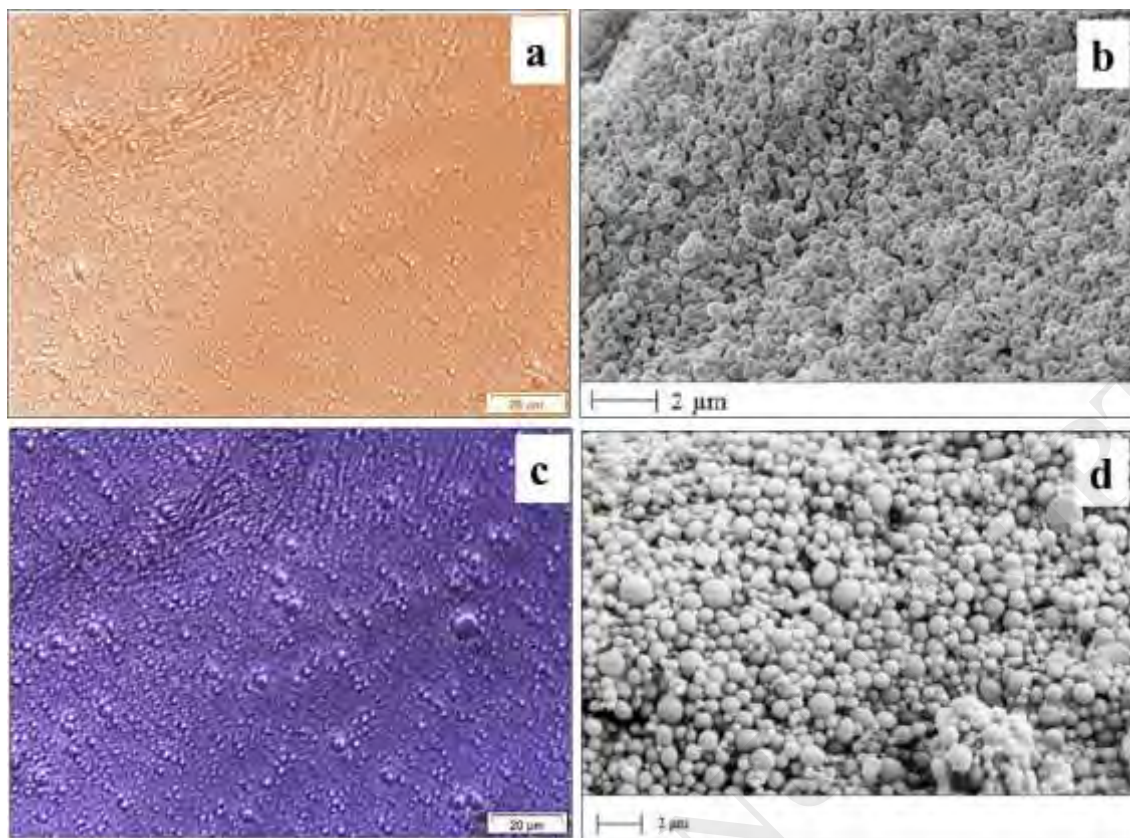


Figure 2. Optical microscope images of droplets in emulsions (**a, c**) and SEM images of PLA/ β -CA/ α -TOC carriers fabricated after emulsion processing by SEE technology (**b, d**). Carriers with mean size of $0.3 \pm 0.1 \mu\text{m}$ with antioxidant loading of 6.2 mg/g (**b**) and of $1.5 \pm 0.5 \mu\text{m}$ with an antioxidant loading of 6.8 mg/g (**d**) were obtained. In order to reduce droplets and, therefore, particle size, higher value of rpm were used in emulsion formulation.

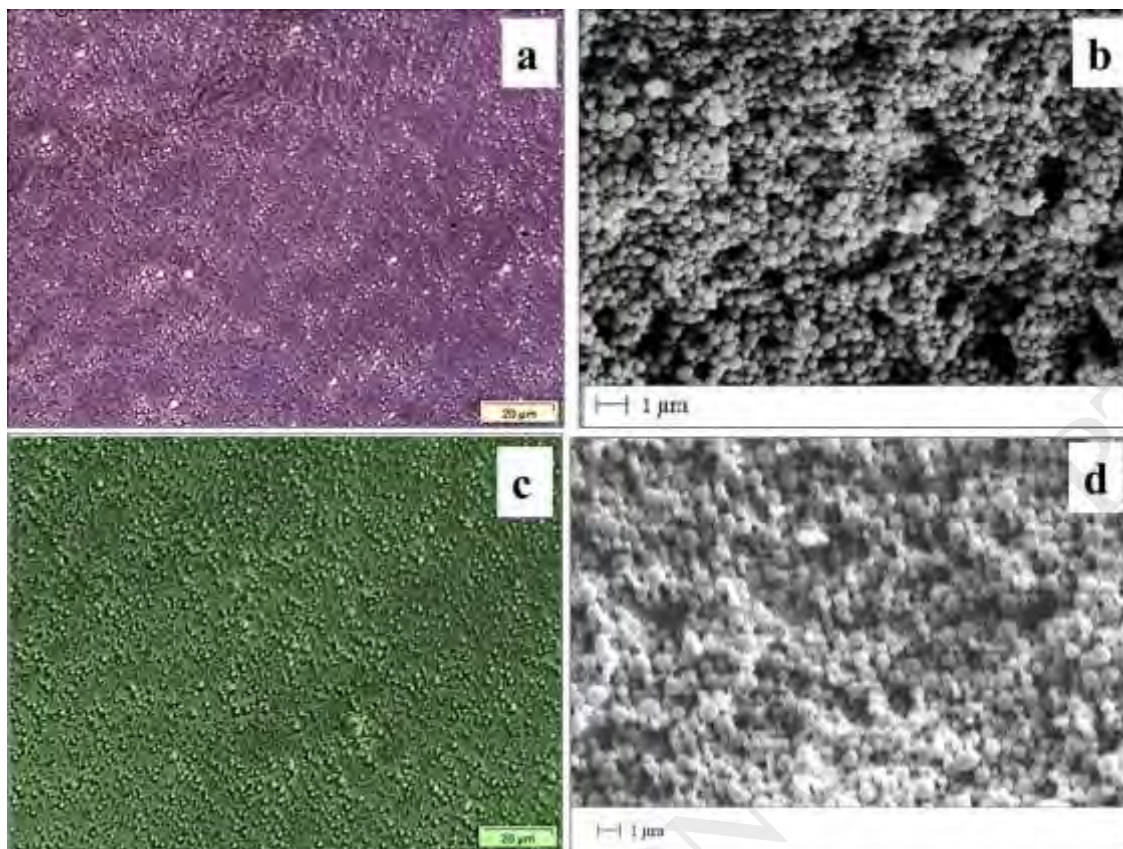


Figure 3. Optical microscope images of droplets in emulsions (**a**, **c**) and SEM images of PLGA/ β -CA/ α -TOC carriers fabricated after emulsion processing by SEE technology (**b**, **d**). Carriers with a mean size of $0.5 \pm 0.1 \mu\text{m}$ with antioxidant loading of 2.7 mg/g (**b**) and of $2.0 \pm 0.6 \mu\text{m}$ with antioxidant loading 2.6 mg/g (**d**) were obtained. In order to reduce droplets and, therefore, particle size, higher value of rpm were used in emulsion formulation.

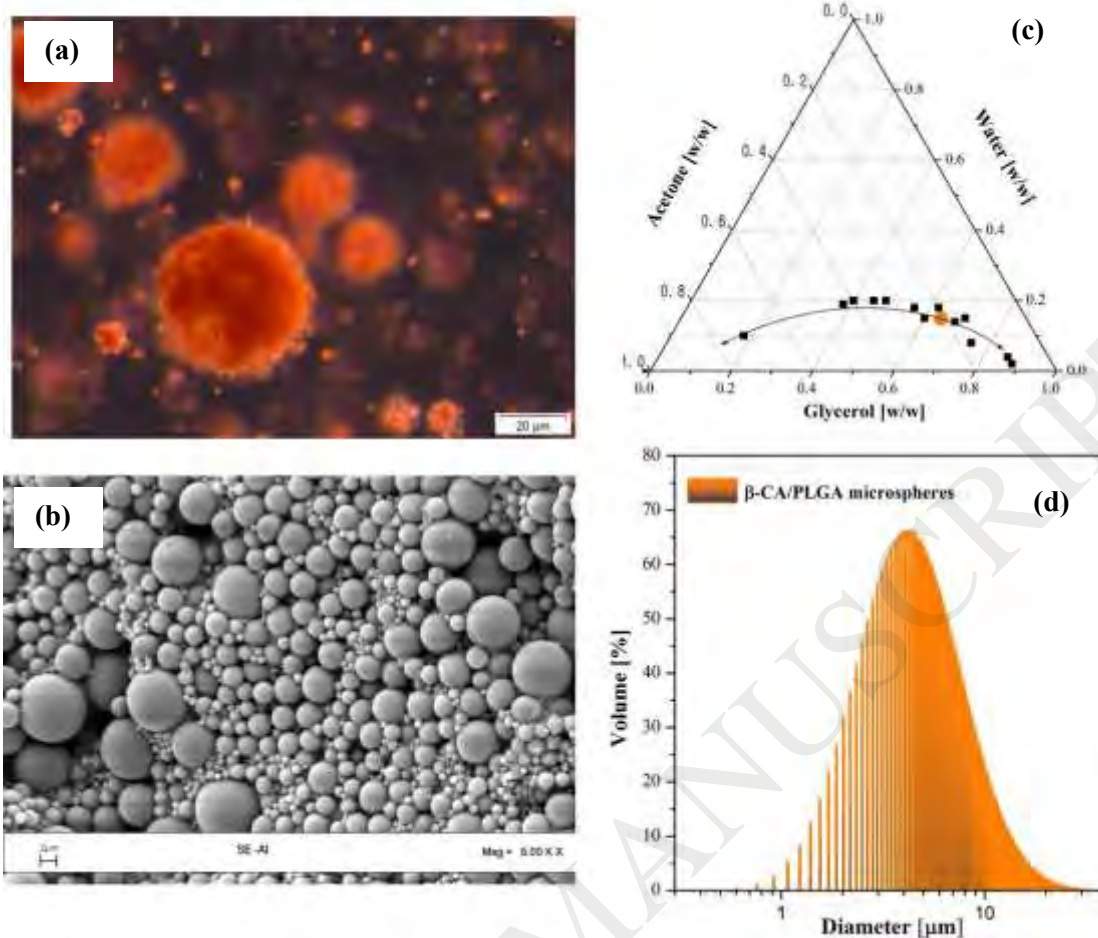


Figure 4. Optical microscope images of the emulsions (a) and SEM images of the related microcapsules (b) produced by SEE using an *o-w* emulsions (20/80) AC:GLY/WATER. The emulsion composition is represented in the ternary diagram (c). Particle Size Distribution (PSD) of PLGA microcapsules is also reported (d); a mean size of $4.3 \pm 0.7 \mu\text{m}$ with $\beta\text{-CA}$ loading of 25 mg/g was obtained.

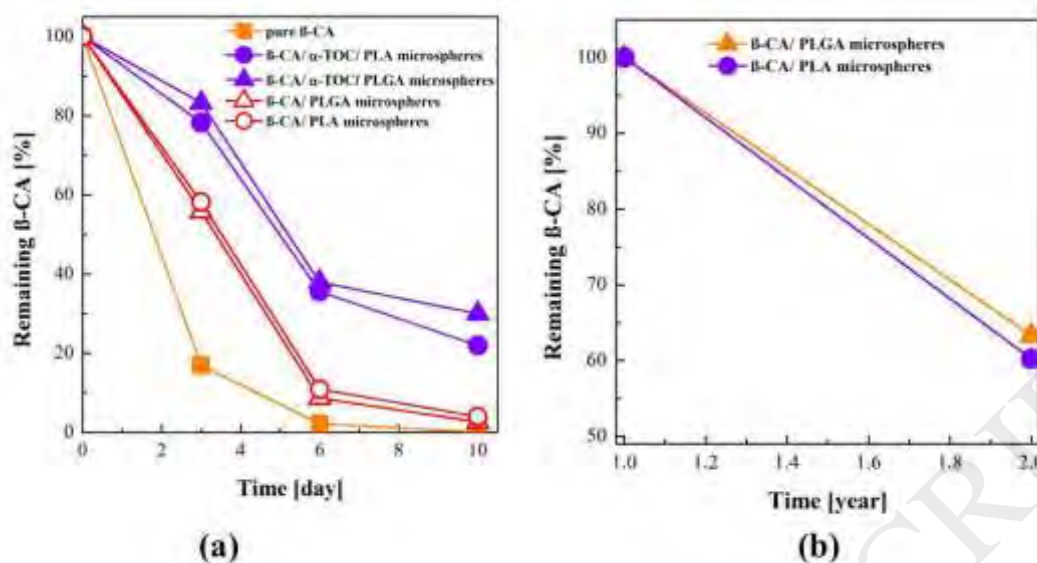


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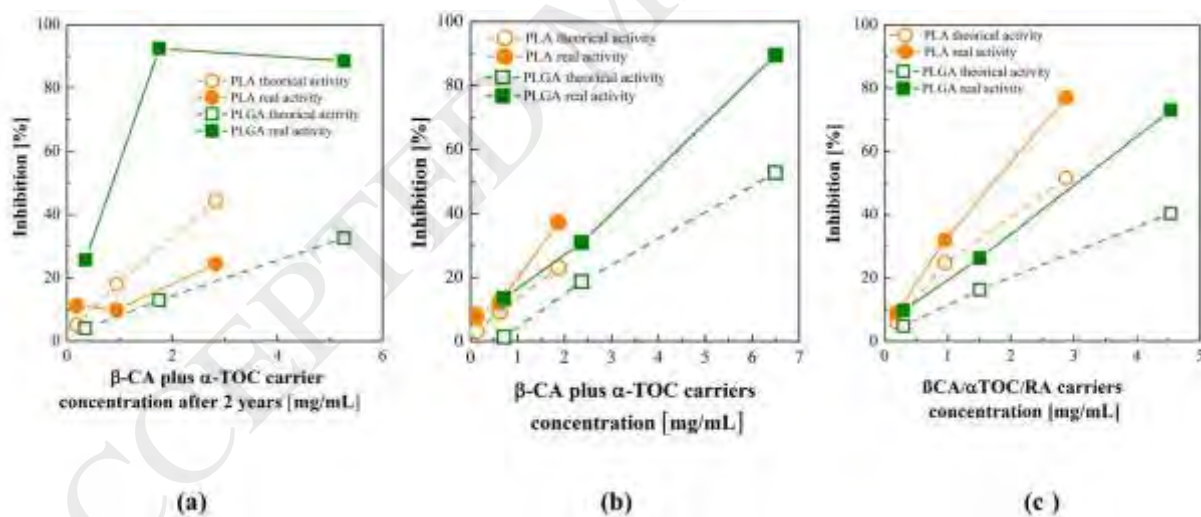


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