

SUPERCritical ANTISOLVENT (SAS) CO-PRECIPIATION OF ETHYL CELLULOSE AND NATURAL EXTRACTS IN SC-CO₂

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The objective of this work has been the co-precipitation of the coating material Ethyl Cellulose (EC) and two natural extracts (IDOKI SCF Technologies), R1 and P1, using the Supercritical Antisolvent (SAS) precipitation technique with supercritical carbon dioxide (SC-CO₂), in order to obtain active microparticles. EC is a hydrophobic material widely used for controlled delivery systems, sustained release, drug packaging, long-acting formulations, taste masking or coatings. R1 and P1 natural extracts are characterized for their antioxidant/antimicrobial properties. For that purpose, a Thar SAS-50 vessel connected to a Thar R100 supercritical fluids system has been used to study different parameters: pressure, EC:extract, flow rate or solution concentration. The morphology and size of the microparticles were analyzed by scanning electron microscopy and the co-precipitation rate was quantified by S-L extraction and further chromatographic analysis. Agglomerated active EC microparticles (1-4µm) have been precipitated. AZTI-Tecnalia has evaluated their antimicrobial properties and GAIKER has checked their antioxidant activity.

Keywords: supercritical antisolvent, ethylcellulose, natural extracts, microparticles, food packaging.

1. INTRODUCTION

Microencapsulation is the process in which tiny particles or droplets are surrounded by a coating or embedded in a homogeneous or heterogeneous matrix. In the food field, microencapsulation is especially used for improving the thermal and chemical stability of interesting natural compounds, to promote their easier handling when processing, for controlled release or taste/odour masking [1,2]. In addition, food industry is considering consumers claim for chemical additives substitution with natural extracts in food ingredients and packaging coatings or devices [3-4].

Different ways of microencapsulation have been described, such as emulsion evaporation, spray drying, freeze-drying, extrusion, coacervation or complexation [5-9]. However, conventional encapsulation techniques require large amounts of organic solvents, surfactants, other additives and need further processing of the products [10]. For those reasons alternative methods for encapsulation or co-precipitation of interesting compounds have been

investigated. In that way, the interest in supercritical fluids has increased recently, since with this technology it is possible to produce small particles with a narrow size distribution using mild and inert conditions [11].

The aim of this work has been the design of a SAS process for encapsulation or co-precipitation of two natural active extracts and the coating agent ethylcellulose (EC), which is a hydrophobic material widely used for controlled delivery systems, sustained release, drug packaging, long-acting formulations, taste masking or coatings [12]. Natural extracts, R1 and P1, were provided by IDOKI SCF Technologies and were selected as active compounds due to their antioxidant and/or antimicrobial properties, which could be taken advantage for food packaging applications leading to chemical additives replacement.

2. MATERIALS AND METHODS

2.1. Materials

Ethyl Cellulose (EC), ethoxyl content 48%, viscosity 4cP, 5% in toluene/ethanol, (CAS: 9004-57-3) and DPPH-2,2-Diphenyl-1-picrylhydrazyl, free radical (CAS:1898-66-4) were purchased from Sigma-Aldrich. Natural extracts, R1 and P1, were provided by IDOKI SCF Technologies. CO₂ SCF (purity: 99.998%) was supplied by Air Liquide-Spain. Acetone HPLC (CAS: 67-64-1, purity: 99.8%) and n-Hexane 95% HPLC (CAS:110-54-3) were purchased from LAB-SCAN analytical sciences. Methanol for liquid chromatography (CAS:67-56-1, purity GC_≥99.8%) was purchased from Merck. The reference materials, α -pinene (CAS:7785-26-4), Eucalyptol (CAS:470-82-6) and (-)-Camphor (CAS: 464-48-2), were of grade Puriss. p.a., terpene standards for GC_>99.0%, Sigma-Aldrich, and were purchased from Fluka. *Listeria innocua* CEC 910 brain for microbiological analysis. All chemicals were used as received, without further treatment.

2.2. Methods

Several methodologies were adapted and optimized for microparticles characterization, precipitation and validation of their antioxidant/antimicrobial properties.

2.2.1. Gas Chromatography

Gas chromatography (GC) was used for R1/P1 natural extracts detection and quantification: R1 was analysed by GC-FID [13], whereas P1 was quantified by GC-MS. Firstly, SAS co-precipitated microparticles were washed with n-hexane by 2 consecutive S-L extractions in order to extract the active compounds placed in the outside part of the microparticles. After those S-L extractions, the remaining microparticles were totally dissolved in acetone, what led to the complete extraction of the active compounds embedded in the ethylcellulose matrix. These solutions were analysed by GC, and characteristic peaks were compared to the calibration curve obtained with the reference materials to calculate the actual natural extracts concentration in the EC matrix.

2.2.2. Scanning Electron Microscopy (SEM)

A scanning electron microscope (model: EVO 50, ZEISS) was employed to determine EC+R1 and EC+P1 microparticles size and morphology after SAS co-precipitation in SC-CO₂. This technique was optimized by using high vacuum, different detectors (secondary electron detector-SE or retrodispersed electron detector-BSC) and sample preparation with sputtering (applying a thin Au-Pd layer on the sample) to enhance their conductivity for SEM image quality improvement.

2.2.3. Supercritical Antisolvent (SAS) co-precipitation

The supercritical antisolvent (SAS) technique, employing SC-CO₂ as antisolvent, was used for co-precipitation of EC and natural extracts. The SCF Thar R100 System of GAIKER with an auxiliary Thar SAS-50 vessel (V=500ml) was used for experimental. This technique consists in feeding a solution (containing the coating agent EC and the natural extract dissolved in acetone) with a HPLC pump through a stainless steel nozzle (200µm) into the SAS precipitation vessel, where supercritical conditions have been previously reached. When the solution contacts the supercritical medium, the CO₂ acts as antisolvent and originates solution supersaturation. At that moment, the coating agent EC and the natural extract precipitate as microparticles (co-precipitation) or microcapsules (encapsulation). The organic solvent is removed by the continuous SC-CO₂ current up to a separator, and the microparticles remain in the SAS-50 vessel, fig.1. The system is depressurized and the microparticles are collected from the vessel.

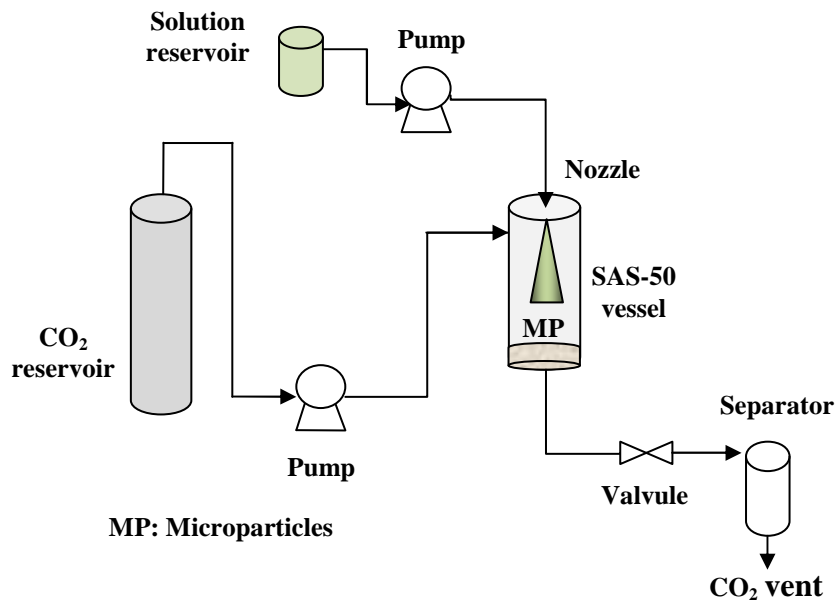


Figure 1: Scheme of SAS method (Supercritical Antisolvent).

2.2.4. Antioxidant activity validation

The antioxidant activity of the obtained microparticles was assessed according to their scavenging ability on the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical [14]. Each product sample (0.2-10mg/ml, 4ml in MeOH) was mixed with 1ml DPPH methanolic solution ($A=0,9$, $\lambda=517\text{nm}$), shaken and left to stand in the dark for 30 minutes for reaction. Afterwards, the absorbance was measured at 517 nm against a blank, employing a UV-1800 SHIMADZU spectrophotometer. The scavenging activity on DPPH was calculated as follows (1):

$$\text{Scavenging ability (\%)} = \frac{(\Delta\text{Absorbance}_{517\text{ control}} - \Delta\text{Absorbance}_{517\text{ sample}})}{\Delta\text{Absorbance}_{517\text{ control}}} \cdot 100 \quad (1)$$

In this study antioxidant activity was measured for a concentration range (0.2-10mg/ml) and the natural extracts R1 and P1 (IDOKI) were used as reference materials as they showed high antioxidant activity. A value of %PI (DPPH) $\geq 90\%$ was fixed to establish what concentration of each product was necessary to provide a high antioxidant activity.

2.2.5. Antimicrobial activity validation

Three methodologies were used by AZTI-Tecnalia, with the aim of determine the Minimum Inhibitory Concentration value (MIC) of both products, EC+R1 and EC+P1, using a *Listeria innocua* CEC 910 strain: (a) Broth microdilution method; (b) Broth dilution method with shaking; (c) Agar diffusion method.

3. RESULTS

3.1. SAS co-precipitation process optimization

In order to establish the most suitable supercritical conditions for SAS co-precipitation of EC and R1 natural extract (IDOKI), an experimental matrix was firstly designed with the statistical Minitab 16 software. This matrix, which consisted of 16 assays, was obtained considering 3 variable parameters (Q_{solution} , EC:R1 and EC weight), and keeping some fixed parameters: $P=80\text{bar}$, $T=35^\circ\text{C}$, $Q_{\text{CO}_2}=20\text{g/min}$, nozzle: $200\ \mu\text{m}$, tables 1 and 2. Every test led to EC+R1 agglomerated microparticles of 1-4 μm . In general, it could be observed that particle size increased with Q_{solution} , and that the amount of R1 extract co-precipitated with EC was low (<4%), being no significant difference in R1 content (1-4%) among all the samples.

The SAS process for co-precipitation of EC and the natural extract P1 (IDOKI) was studied taking on account the most significant parameters previously obtained in experimental for EC+R1. For that purpose, 8 tests were carried out changing 3 parameters (Q_{solution} , EC:P1 and P) and maintaining other parameters constant: $T=35^\circ\text{C}$, $Q_{\text{CO}_2}=20\text{g/min}$, nozzle: $200\ \mu\text{m}$ and EC=0.5g. Similarly to EC+R1, all the EC+P1 samples were constituted by agglomerated microparticles. Whereas EC+R1 microparticles showed homogeneous particle size (1-3 μm), EC+P1 microparticles size was more heterogeneous, and even particles with nanometric size (<1 μm) could be found.

3.2. Statistical study

In both cases, conclusions predicted in the experimentation were assessed by statistical data treatment with Minitab 16 software, in order to confirm which of the studied parameters were the most influential in the designed SAS co-precipitation process in SC-CO₂. In the case of EC+R1 co-precipitation, Qsolution, EC:R1 and EC weight did not show any influence in particle size. By contrast, R1 content depended on solution flow (ml/min) and EC weight. The statistical study for SAS co-precipitation of EC and P1 extract showed that pressure and EC:P1 were significant parameters in the process, but in comparison, EC:P1 showed a greater influence.

3.3. Product validation

Once the most suitable supercritical conditions were established for SAS co-precipitation of EC and each natural extract, R1 and P1 (IDOKI), approximately 1g of each product was precipitated using the Thar SAS-50 vessel of GAIKER, fig. 2, with the aim of obtaining enough product quantity for carrying out the characterization and validation tests. According to SAS co-precipitation rate, EC recovery was higher in the co-precipitation of EC and P1, 65%, whereas for R1 extract only 51% of the fed EC was recovered after the process, table 4.

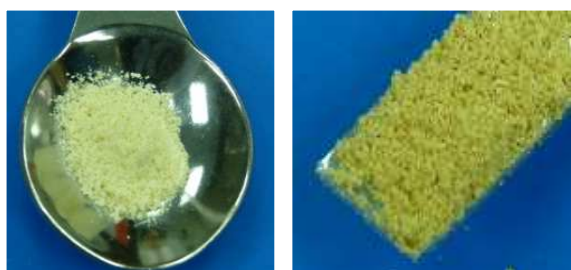
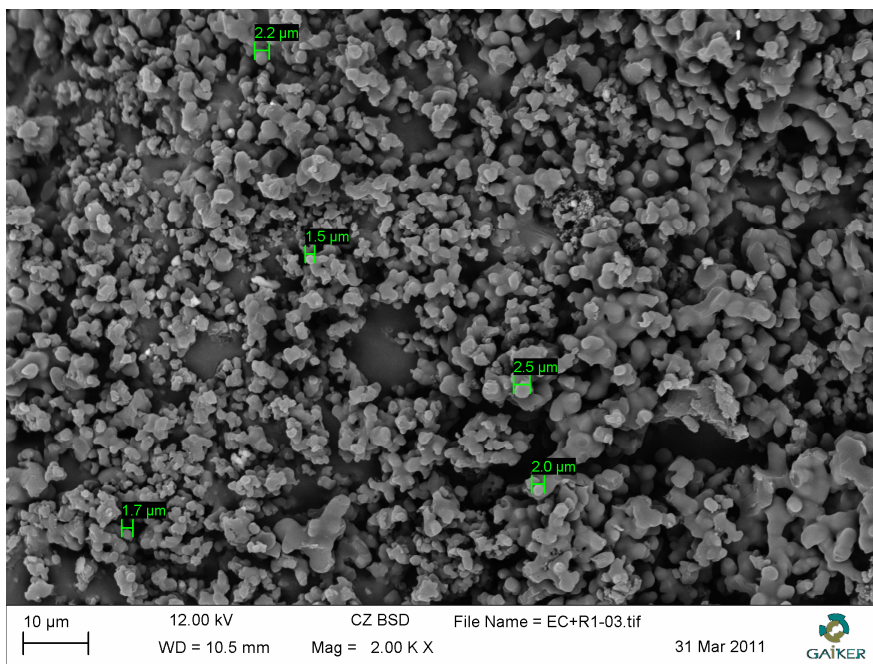


Figure 2: Products obtained in the SAS co-precipitation process. EC+R1 (left) and EC+P1 (right).

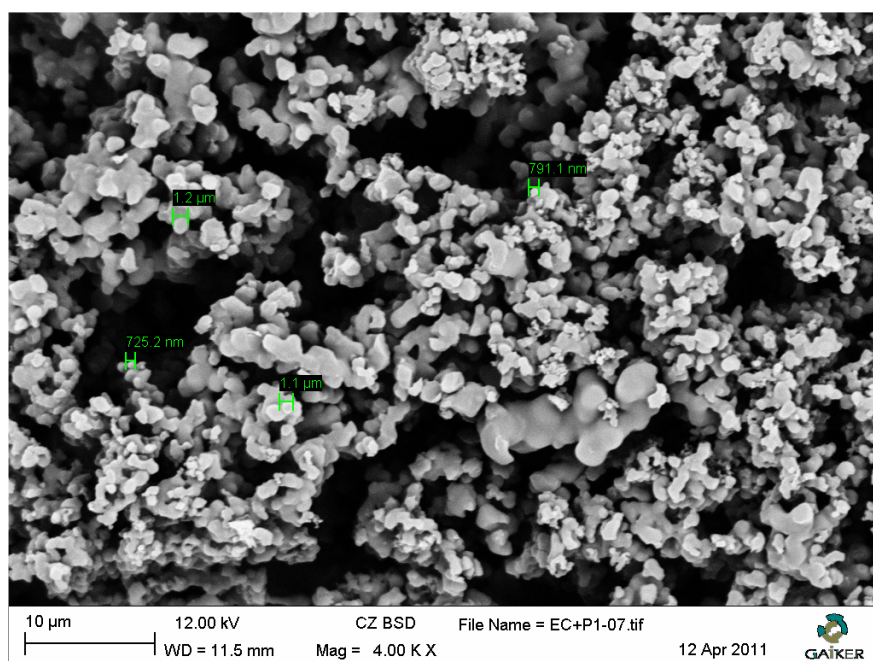
EC+R1 and EC+P1 were validated in order to assess that the characteristic properties of the original natural extracts R1 and P1 (IDOKI) were maintained after the designed process. With that aim, both products underwent different tests with regard to the following properties: (a) microparticles size, particle size distribution (PSD) and morphology (SEM); (b) %Natural extract (GC); (c) Antioxidant activity (%PI DPPH>90%), and (d) antimicrobial activity (3 methods tested by AZTI-Tecnalia). The results are shown in table 1.

Table 1: Validation results for EC active microparticles (MP).

Product	%EC recovery	Particle size (µm)	PSD (µm) (90%MP)	%Extract	Antioxidant Activity	Antimicrobial Activity
EC+R1	51	1-3	1-2.5	3% R1	≥1mg/ml	No evidence
EC+P1	65	<1-3	0.8-2.4	8% P1	≥0.1mg/ml	No evidence



(a)



(b)

Figure 3: SEM image for (a) EC+R1, and (b) EC+P1.

As can be observed both products were formed by agglomerated microparticles with a mean size of 1-3 μ m, fig. 3, however EC+P1 showed no normal particle size distribution. EC+R1 and EC+P1 microparticles maintained the antioxidant properties of the natural extracts (IDOKI) after SAS co-precipitation in SC-CO₂. Antimicrobial activity could not be assessed

due to the insolubility of EC in the growth media, leading to no interaction between the active extract and the microorganisms.

4. CONCLUSIONS

Supercritical antisolvent (SAS) process using SC-CO₂ is a suitable method for coprecipitation of ethylcellulose and natural extracts, R1 and P1 (IDOKI), leading to active microparticles formation. GAIKER studied and optimized different parameters for designing the process, and as result, agglomerated active EC microparticles were obtained with 50-65% recovery. EC+R1 microparticles had 3% R1 content and showed homogeneous particle size distribution with mean size of 1-2.5µm, whereas EC+P1 microparticles showed more extract concentration (8% P1) and had heterogeneous particle size (0.8-2.4µm). Both products kept the antioxidant activity of the natural extracts after the process. AZTI-Tecnalia evaluated their antimicrobial activity; however, this property could not be assessed due to the insolubility of EC in the growth media. The main advantages of these active products are: (a) their easier handling; (b) they maintain the antioxidant activity of the natural extracts; and (c) the SAS coprecipitation designed process leads to extracts odour masking which could provide new applications for R1 and P1 extracts (IDOKI).

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REFERENCES:

- [1] A. Gharsallaouri, G. Roudant, O. Chamblin, A. Voilley, R. Saurel; “Applications of spray-drying in microencapsulation of food ingredients: An overview“, *Food Research International* 40, **2007**, p. 1107.
- [2] Costescu, C; Hadaruga, NG; Iadaruga, DI; Rivis, A; Ardelean, A; Lupea, AX; “Bionanomaterials: Synthesis, physico-chemical and multivariate analyses of the dicotyledonatae and pinatae essential oil/beta-cyclodextrin nanoparticles”, *Revista de Chimie*, Vol. 59 (7), **2008**, p. 739.
- [3] EP 1 477 519 A1. Antioxidant active varnish.
- [4] European Patent Application EP 1 657 181 A1: Antimicrobial packaging based on the use of natural extracts and the process to obtain this packaging, **2006**.
- [5] B. Voncina, et al., Encapsulation of Rosemary Oil in Ethylcellulose Microcapsules, *Textile and Polymer Journal*, Vol. 1, No. 1, **2009**, p. 13.
- [6] W.E. Bangs and G.A. Reineccius; “Characterization of Selected Materials for Lemon Oil Encapsulation by Spray Drying”; *Journal of Food Science*, Vol. 55, No. 5, **1995**, p. 1356.
- [7] Luciana P. Fernandes, W.P Oliveira, J. Sztatisz, I.M. Szilágyi and Cs. Novák; “Solid state studies on molecular inclusions of *Lippia Sidoides* essential oil obtained by spray drying”, *Journal of thermal analysis and calorimetry*, Vol. 95, 3, **2009**, p. 855.

- [8] B.CMM Ribeiro, M.L. Beirao-da-Costa, M Moldao-Martins; "Origanum virens L. Flavor Encapsulation in a Spray dried Starch Matrix"; 2nd Mercosur Congress on Chemical Engineering.
- [9] Ronald B. Pegg, F. Shahidi; "Encapsulación y liberación controlada en la conservación de alimentos" Book: MANUAL DE CONSERVACIÓN DE LOS ALIMENTOS. Autor: M. Shafiur Rahman, Cap. 21, **2003**, p. 649.
- [10] Y. Wang, R. Pfeffer, and Rajesh Dave; "Polymer Encapsulation of Fine Particles by a Supercritical Antisolvent Process" AIChE Journal, Vol. 51, No. 2, **2005**, p. 440.
- [11] Elvassore, N., Parton, T., Bertucco, A., Di Noto, V., "Kinetics of particle formation in the gas antisolvent precipitation process" AIChE J. 49, **2003**, p. 859.
- [12] Ana Rita C. Duarte, et al., Preparation of controlled release microspheres using supercritical fluid technology for delivery of anti-inflammatory drugs. Int. J. of Pharmaceutics, 308, **2006**, p. 168.
- [13] Norma UNE 84-225-92. Aceites esenciales. Cromatografía en fase gaseosa. Método general para columnas capilares. **1992**.
- [14] Teresa C.P. Dinis, Vítor M.C. Madeira and Leonor M- Almeida, Action of Phenolic Derivatives (Acetaminophen, Salicylate, and 5-Aminosalicylate) as Inhibitors of Membrane Lipid Peroxidation and as Peroxyl Radical Scavengers. Archives of Biochemistry and Biophysics, Vol. 315, No. 1, **1994**, p. 161.