Universidad de Valladolid

ESCUELA DE INGENIERÍAS INDUSTRIALES

DEPARTAMENTO DE INGENIERÍA QUÍMICA Y TECNOLOGÍA DEL MEDIO AMBIENTE

TESIS DOCTORAL:

STUDIES OF PROCESS INTENSIFICATION FOR THE DEVELOPMENT OF HYDROPHILIC AND HYDROPHOBIC β-CAROTENE FORMULATIONS

Presentada por Esther de Paz Barragán para optar al grado de doctor por la Universidad de Valladolid

Dirigida por:

Dr.- Ing. Ángel Martín Martínez
Prof. María José Cocero Alonso

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ESTUDIOS PARA LA INTENSIFICACIÓN DEL PROCESO PARA EL DESARROLLO DE FORMULACIONES DE β-CAROTENO HIDROFÍLICAS E HIDROFÓBICAS

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Memoria para optar al grado de Doctor,

con **Mención Doctor Internacional**,

presentada por la Ingeniera Química:

Esther de Paz Barragán

Siendo los tutores en la **Universidad de Valladolid**:

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Valladolid, Mayo de 2013

UNIVERSIDAD DE VALLADOLID

ESCUELA DE INGENIERÍAS INDUSTRIALES

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La presente tesis doctoral queda registrada en el folio número _____ del correspondiente libro de registro número _____

Valladolid, a _____ de_____ de 2013

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Certifican que:

ESTHER DE PAZ BARRAGÁN ha realizado bajo su dirección el trabajo *"Studies of Process Intensification for the development of hydrophilic and hydrophobic β-carotene formulations"*, en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

Valladolid, a _____ de_____ de 2013

Fdo. Ángel Martín Martínez Fdo. María José Cocero Alonso

Reunido el tribunal que ha juzgado la Tesis Doctoral titulada *"Studies of Process Intensification for the development of hydrophilic and hydrophobic β-carotene formulations"* presentada por la Ingeniera Química Esther de Paz Barragán y en cumplimiento con lo establecido por el Real Decreto 99/2011 de 28 de enero de 2011 acuerda conceder por_____ la calificación de _____.

Valladolid, a _____ de_____ de 2013

PRESIDENTE SECRETARIO

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A mi hermana, Ana

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Abstract

Studies of process intensification for the development of the hydrophilic and hydrophobic β-carotene formulations

Nowadays the food market demands functional foods and healthy products. While the use of synthetic chemical compounds in food products is considered negatively by consumers, natural additives provide the final product with a healthy value.

Carotenoids are widely used as natural additives in food products. One of the most common, abundant and used carotenoid is β-carotene. Besides its excellent colorant properties, β-carotene acts as precursor of retinol and retinoic acid which have an important role in human health as vitamin A precursor and as cellular regulatory signal, respectively. However, the application of β-carotene as a natural colorant in food and nutraceutical products requires an appropriate formulation in order to protect the active compound against oxidation and degradation processes, and to overcome the low bioavailability due to the low solubility of β -carotene in aqueous media. Because of this, **the aim of this PhD Thesis** is the design of different β-carotene formulations for application as a natural colorant, developing efficient and innovative technologies for the production of these formulations. Biodegradable polymers have been used in all formulations: starches modified with the group n-octenyl succinic (OSA), poly-caprolactones and soybean lecithin.

The first processing technology developed in this thesis consists of putting into contact a hot, pressurized molecular solution of the carotenoid in a hydrophilic solvent with a cold aqueous solution of carrier material, using a mixing chamber. The process is designed in order to approximate the time scales of mixing to the time scales of particle nucleation, Thus, β-carotene precipitation, due to antisolvent and thermal shock, occurs in a fraction of second time scale, enabling to produce a highly homogeneous product with high efficiency. The reduced process volume, due to these short processing time scales, as well as the increased throughput of the process due to the possibility of operating it continuously, leads to a considerable process intensification. In **CHAPTER 1**, this process is developed studying the formulation of β-carotene by precipitation from pressurized ethyl acetate-on-water emulsions using modified OSA-starch refined from waxy maize as carrier material. Results showed that it is possible to obtain a formulation of β-carotene with a high encapsulation efficiency of β-carotene (over 70%) and with a particle size in the range of 300-600 nm. Different process parameters with strong influence on product properties were researched, such as concentration of surfactant (which was varied from 37 to 367 gL^{-1}) and organic-water ratio (from 0.6 until 1.3 mL/mL). Results obtained in this chapter revealed that high concentrations of modified starch were required (over 100 gL^{-1}) in order to obtain a high percentage of encapsulated β-carotene and high emulsion stability. Regarding the organic-water ratio, it was shown that the best results were obtained with low ratios (between 0.65 and 0.73 mL/mL).

When the organic-water ratio was increased, the particle sizes increased as well, observing a drastic increase at high organic-water ratios (over 0.85).

In the following chapters, different alternative configurations of this process are analyzed. In **CHAPTER 2**, the use of ethanol as water-miscible hydrophilic solvent instead of ethyl acetate, which is only partially water-miscible, is analyzed. Results obtained using ethanol as organic solvent revealed that it is possible to obtain a formulation of β-carotene with maximum encapsulation efficiency of 30%-40% and micellar particle sizes in the range of 120 – 550 nm. As in experiments with ethyl acetate, results showed an increase in the particle size when the organic-water ratio was increased. Compared with the results using ethyl acetate, lower encapsulation efficiencies were obtained in experiments with ethanol , resulting in poorer product properties.

Continuing with the analysis of the process, in **CHAPTER 3**, the effect of using four different OSA-starches for developing the formulation of β-carotene by precipitation from pressurized ethyl acetate-on-water emulsions was investigated. The same process parameters as in previous studies were considered: concentration of surfactant varied from 37 to 367 gL^{-1} and organic-water ratio modified from 0.6 until 1.2 mL/mL. An OSA-starch derived from waxy maize blend with dried glucose syrup was not suitable for encapsulating β-carotene due to the fact the maximum encapsulation efficiency achieved was less than 30% with particle sizes of approximately 1 μm. For the rest of OSA-starches, a minimum concentration of surfactant of 100 gL^{-1} was required to obtain high encapsulation efficiencies. As in results discussed in chapter 1, when the concentration of surfactant increased, the encapsulation efficiency increased as well, and at high organic-water ratios, a drastic increase in the particle size was observed, independently of the OSA-starch used. Regarding the particle size, particle sizes in the suspension of 350-760 nm were obtained. With respect to the effect of the organic-water ratio, the encapsulation efficiency was kept constant between 30-45% and particle sizes below 500 nm, when OSA-dextrin derived from waxy maize and OSA-dextrin derived from tapioca were used. Comparing these results from those shown in chapter 1, results obtained with OSA-starch refined from waxy maize presented better results, achieving maximum encapsulation efficiencies of 70-80 % with particle sizes in the nanometer range.

Finally, the performance of this novel high-pressure emulsion processing technique was compared to that of the emulsion evaporation process commonly used in most industrial applications. This comparison is presented in **CHAPTER 4**, using ethyl acetate as organic solvent and OSA-starch refined from waxy maize, according to the best results obtained in the previous chapters. In addition to the conventional high-shear emulsification process, chapter 4 presents the results obtained by ultrasound emulsification, completing in both cases the

processing with a last step of organic solvent removal. Different process parameters were studied by ultrasound emulsification, such as organic-water ratio (varying from 0.275 until 0.73 mL/mL), time of application of ultrasounds (from 6 until 65 minutes), amplitude (from 20 to 100 μm) and duty cicle (from 0.5 until 1). The best results were obtained with low organic-water ratios, concretely 0.275, and using 100 μm amplitude with a duty cycle of 1.0, achieving particle sizes lower than 200 nm and encapsulation efficiencies of 30%. In comparison, results obtained by high-shear emulsification showed particle sizes in the same range (less than 240 nm), but much lower encapsulation efficiencies (below 8%), probably due to the harsher conditions during emulsification. Comparing with the results obtained with the high pressure emulsion technique described in chapters 1-3, particle sizes obtained by this technique were higher (in the range of 400 nm), indicating that ultrasound emulsification induces a more efficient mixing than the mixing chamber employed in the high pressure emulsion technique. On the contrary, the much higher encapsulation efficiencies obtained by the high pressure process (70 - 80%) indicate that the very short processing times required by this process has a direct effect on a better preservation of the structure of the emulsion template during the precipitation, and therefore on the efficiency of the incorporation of β-carotene into the carrier.

The second part of the thesis deals with the development of supercritical fluid-based technologies for the production of β-carotene formulations. In particular the application of the PGSS (Particles from Gas Saturated Solutions) process was studied. Two different types of formulation were developed: one based on liposome-forming phospholipids, as an alternative water-soluble formulation, and the second one based on the water-insoluble, slow degrading polymer polycaprolactone, as a product aimed for a protection against degradation and a slow release of the active material.

As the first step for the development of the PGSS process, in **CHAPTER 5**, the solid-liquid-gas equilibrium in poly-(ε-caprolactone) + CO_2 systems at high pressure (from 0.1 to 25 MPa) was determined in order to get a detailed knowledge of the phase behavior of the polymer and supercritical fluid mixtures. Experiments were carried out with three polycaprolactones with different molecular weights (4000, 10000 and 25000 $gmol^{-1}$). The SLG equilibrium was developed by visual determination of the first melting point using a high pressure optical cell. The SLG equilibrium lines show a maximum temperature at low pressures (between 0.5 MPa and 1.6 MPa) and a minimum temperature at moderate pressures (between 8 MPa and 10 MPa, depending on the used polycaprolactone). The maximum reduction of melting temperature was between 12.5 and 16.0 K depending on the molecular weight of the polycaprolactone. Also, a thermodynamic model based on the PC-SAFT equation of state was

developed to describe experimental data, and model correctly predicts the solubility of CO_2 in the molten polymer as well as the melting temperature at pressures below the pressure of the minimum melting point, but it was not able to describe the variation of melting temperature at higher pressures. Results obtained in this work were useful for carrying out the investigation presented in chapter 6.

In **CHAPTER 6**, the formulation of β-carotene with poly-(ε-caprolactones) by Particles from Gas Saturated Solutions (PGSS) process was developed. The effect of different process parameters on particle size and on β-carotene content were studied, including β-carotene:polymer molar ratio (0.13, 0.16 and 0.25), time of homogenization in the mixing chamber (60, 120 and 240 minutes), temperature (50 and 70 ºC) and pressure (11 and 15 MPa). Two different polycaprolactones were used, obtaining particles with a particle size in the range of 270-650 µm with a β-carotene content of up to 340 ppm when polycaprolactone with a molecular weight of 10000 g mol^{-1} was used while using polycaprolactone with 4000 g mol^{-1}, particle size was reduced to 110-130 µm. The molar ratio has an important influence on particle size, obtaining an increase in particle size when molar ratio was increased as well. Also bigger particles were obtained when pressure and temperature inside the mixing chamber were 15 MPa and 50 ºC, respectively compared with the other two values selected (11 MPa and 70 ºC). Regarding the β-carotene content, low loadings were obtained, and the highest β-carotene concentrations (306-336 ppm) were obtained at high pressures and temperatures (15 MPa and 70 ºC) and with short homogenization times, concretely 60 minutes. This research was done at Instituto de Biologia Experimental e Tecnológica (IBET), Oeiras (Portugal).

In **CHAPTER 7**, the formulation of β-carotene with soybean lecithin by PGSS-drying was investigated. Results showed that it was possible to obtain dry particles with a particle size in the range of 10-500 µm constituted by agglomerated spheres with encapsulation efficiencies of β-carotene up to 60%, which can be hydrated forming β-carotene-loaded liposomes with sizes ranging between 1-5 µm. By this rehydration process, large particles corresponding to non-encapsulated β-carotene were also obtained. The main process parameters, which were studied their effect on the product characteristics, were the pre-expansion temperature (100-130ºC), pre-expansion pressure (8-10 MPa), gas to product ratio (21-27-32) and concentration of soybean lecithin (55-62-72 gL^{-1}). Regarding the particle size, smaller particle sizes were obtained when pre-expansion pressure or concentration of lecithin were increased, or when pre-expansion temperature and GPR (Gas to Product Ratio) were decreased. With respect to the encapsulation efficiency, it was increased from 30% until 60% when the pre-expansion temperature was increased in the selected range of temperatures (100-130ºC).

In order to obtain basic properties for the analysis of the performance of the encapsulation process by PGSS, in **CHAPTER 8**, the solubility of β-carotene in three different poly-(ε-caprolactones) with different molecular weights (4000, 10000 and 25000 $gmol^{-1}$) in colloidal state was studied. The determination of the solubility of β-carotene was carried out by two different processes: equilibration-impregnation and equilibration-de-supersaturation. The maximum β-carotene contents achieved by equilibration impregnation process were between 87 and 191 ppm depending on the molecular weight of the polycaprolactone. These results corroborated results obtained in chapter 6, since the concentration of β-carotene obtained by PGSS process agree well with the order of magnitude of the saturation of β-carotene in the polymer. Regarding the equilibration de-supersaturation process, β-carotene concentrations were considerably higher than those obtained in impregnation experiments, obtaining a maximum concentration of 8800 ppm when polycaprolactone with the highest molecular weight was used. Results showed in this chapter justify the experimental results obtained in PGSS experiments, revealing that polycaprolactones are not suitable for carrying out a formulation of β-carotene due to the low affinity between β-carotene and the polymer. This research was done at ETH Zürich - Swiss Federal Institute of Technology, Zurich (Switzerland).

Introduction and Aims

Studies of process intensification for the development of the hydrophilic and hydrophobic β-carotene formulations

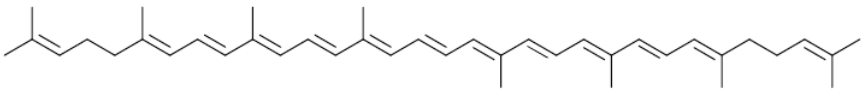
Carotenoids

The use of synthetic food colorants that only have a cosmetic value and are associated with detrimental effects for health has always been target of complains of the food industry consumers. Nowadays the food market demands functional foods and healthy products. While the use of chemical products is negatively considered, natural additives provide the final product with the added value of their healthy properties.

Carotenoids can be a valuable natural additive for food products. One of the most common, abundant and used carotenoid is β-carotene. Besides its excellent colorant properties, β-carotene has been exhaustively employed as precursor of retinol and retinoic acid which have an important role in human health as vitamin A precursor and as cellular regulatory signal, respectively. Since animals and humans cannot produce carotenoids in their organisms, they need to acquire them from their food sources.

Carotenoids are compounds constituted by eight isoprene units joined in a head to tail pattern. Most of them have 40 carbon atoms and present a chemical structure similar to the one presented in Figure 1.

Isoprene group



C_{40} Carotenoids (8 Isoprene groups). Lycopene.

Figure 1. Carotenoid typical chemical structure.

Carotenoids can be classified in two different ways, by their chemical structure or by their functionality. They can be classified by their structure as Carotenes when elements are carbon and hydrogen like β-carotene or lycopene or they can be considered as Xantophylls when they also present oxygen in their structure as lutein or zeoxanthin. Their functionality in plants determines whether they are primary or secondary carotenoids. Primary carotenoids are necessary in the photosynthetic process like β-carotene, lutein or neoxanthin (Matea et. al. 2009). They are structural and functional components of the cellular photosynthetic apparatus and are usually localized in the thylakoid membrane which is an internal vesicle of chroloplast in which reactions of light caption of photosynthesis and phosphorylation take place. Hence,

they are essential for survival (Guedes et al. 2011). On the other hand, secondary carotenoids are not directly involved in the survival of the plant like α-carotene, capsanthin or lycopene.

There are more than 600 different carotenoids in nature, distributed among higher plants, bacteria, fungi and some animals. Most of them can be found in higher plants, especially in their leaves, flowers and fruits. Also, some specific carotenoids are present in algae, being flucoxanthin the most important of them. In addition, photosynthetic related bacteria contain carotenoids with some different structural groups. Fungi contain mainly mono and bi cyclic carotenoids like canthaxanthin, and finally some carotenoids are also presented in animals like yellow or red coloured birds, fishes like salmon and marine invertebrates that mainly have astaxanthin related carotenoids (Matea et. al. 2009). Some of the most common carotenoids are presented in figure 2.

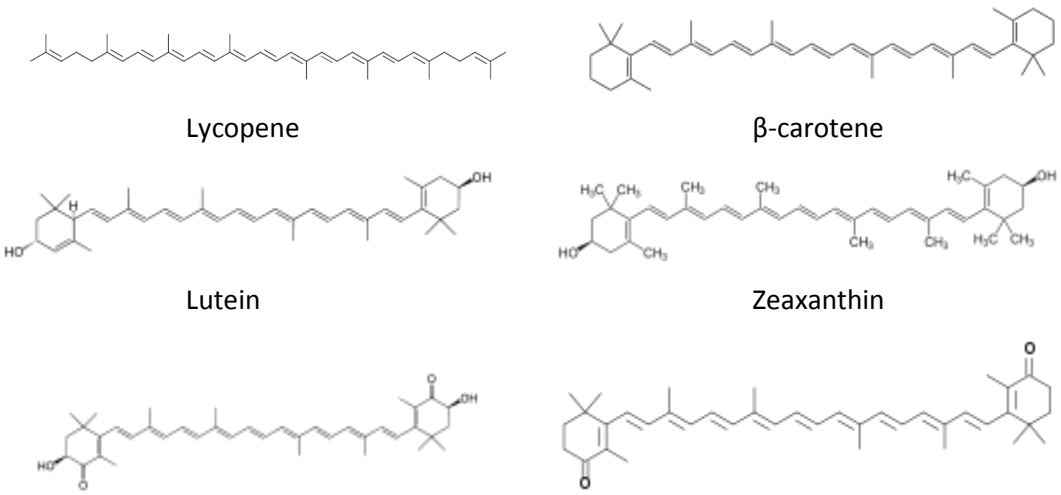


Figure 2. Chemical structure of the most common carotenoids.

Application of carotenoids in food products

Functional ingredients such as carotenoids, fatty acids, natural antioxidants and numerous other compounds, are being extensively used on a great variety of food products (Moraru et al. 2003). Carotenoids are some of the most common pigments in nature, being the most abundant β-carotene, lycopene, lutein and zeaxanthin. The main roles of carotenoids in human diet are as precursors of Vitamin A and as antioxidants. Among the carotenoids, β-carotene has the highest pro-vitamin A, and is therefore a strong candidate for incorporation into functional foods (Boon et al. 2010).

When carotenoids are consumed in sufficient levels, they have been claimed to have biological activities that may reduce the risk of certain chronic diseases, such cancers, cardiovascular disease, age-related macular degeneration and cataracts (Qian et al. 2012a). Since they are

authorized food ingredients, carotenoids are widely used in the food, cosmetic and pharmaceutical industries as natural colorants.

For many industrial applications, a mixture of the carotenoid with a biopolymer is used. Covering carotenoids with polymers provides protection against oxidation and degradation processes (Martin et al. 2007). Moreover, the high hydrophobicity of carotenoids makes them insoluble in aqueous systems, and therefore they have a poor intake in the body. To improve their dispersability in water, coloring strength potential and also to increase their bioavailability during gastro-intestinal passage, carotenoid crystals must be formulated (Ribeiro et al. 2008). For the use of carotenoids as natural colorants, it is important to obtain an appropriate colour intensity of the formulation which depends on the properties of the particles, including a restricted particle size and a controlled crystallinity.

Formulation of β-carotene

There are several methods of precipitation and condensation for the production of organic nanoparticles in aqueous media (Horn & Rieger, 2001). Recently, there has been great interest in utilizing nanoemulsions to encapsulate bioactive components for applications in food and beverage products (McClements & Rao, 2011). Oil-in-water nanoemulsions consists of small lipid droplets ($r < 100$ nm) dispersed within an aqueous continuous phase. Nanoemulsions are thermodynamically unstable systems that tend to break down over the time (Qian et al. 2012b). Nanoemulsions containing β-carotene have been studied by several authors.

Qian et al. (2012b) showed that β-carotene can be effectively encapsulated within food-grade nanoemulsions stabilized by globular proteins (β-lactoglobulin) or non-ionic surfactants using a high pressure microfluidiser, demonstrating that β-carotene encapsulated within protein-coated lipid droplets was more stable to chemical degradation than that encapsulated within non-ionic surfactant (Tween 20)-coated droplets. Also they showed that β-carotene degradation, by storage at elevated temperatures, could be effectively retarded by adding water-soluble (EDTA or ascorbic acid) or oil-soluble antioxidants (Vitamin E acetate or Coenzyme Q10) to the nanoemulsions (Qian et al. 2012c). The same authors (Qian et al. 2012a) carried out the same study using different kinds of carrier lipids coated by non-ionic surfactants. They examined the impact of carrier oil type on the bioaccessibility of β-carotene encapsulated within nanoemulsion-based delivery.

Mao et al. (2009-2010), investigated the characteristics of β-carotene nanoemulsions prepared by high pressure homogenization using two large molecule emulsifiers (octenyl succinate starch and whey protein isolate) and two small molecule emulsifiers (tween 20 and decaglycerol monolaurate) obtaining a fine size distribution.

The same technique was used by Liang et al. (2013) who investigated the physicochemical and biological properties of β-carotene nanoemulsions stabilized by modified starches, considering the particle size, β-carotene retention and in vitro digestion. During 30 days of storage at different conditions, the main diameters of the emulsion systems were increased by 30-85%. The retention of β-carotene in nanoemulsions was significantly higher compared to that of the β-carotene dispersed in bulk oil. After in vitro digestion, the bioaccessibility of β-carotene was increased from 3.1% to 35.6%.

High pressure homogenization was also used by Yuan et al. (2008a) to study the characterization of β-carotene oil-in-water nanoemulsions using series of polyoxythylene sorbitan esters of fatty acids (Tween 20, 40, 60 and 80) as emulsifiers. The influence of the emulsifier type and concentration, as well as the homogenization conditions of pressure, temperature and cycle on the physicochemical properties of the nanoemulsions was examined. They obtained mean diameters of the dispersed particles containing β-carotene in the range of 132-184 nm, and the smallest particle sizes and narrowest particle size distributions were obtained using Tween 20. The same authors (Yuan et al. (2008b)) also investigated the optimization of conditions for the preparation of β-carotene nanoemulsions using response surface methodology. They predicted that the optimum conditions were a homogenization pressure of 129 MPa, a homogenization temperature of 47 ºC, a β-carotene concentration of 0.82 % and an emulsifier concentration of 8.2 %.

Tan and Nakajima (2005a) investigated the preparation of β-carotene nanodispersions by a process based on an emulsification-evaporation technique which consists in producing the oil-in-water emulsion by high-pressure homogenization, and removal of organic solvent from the sample by rotary evaporation. Hexane was used as organic phase and the aqueous phase contained Tween 20. With this technique, they were capable of producing β-carotene nanodispersions with particle sizes below 100 nm. This technique was also used by the same authors (Tan and Nakajima, 2005b) for producing β-carotene nanodispersions using six different polyglycerol esters of fatty acids (PGE) as nonionic emulsifiers in order to observe their effect on the physicochemical properties and stability of β-carotene nanoparticles. They demonstrated that an increase in the degree of glycerol polymerization in the PGE led to dispersions with smaller particles and higher stabilities, obtaining a mean diameter ranged from 85 to 132 nm.

The same technique was used by Chu et al. (2007a) for the preparation of protein-stabilized β-carotene nanodispersions. Sodium caseinate was the most effective emulsifier among selected proteins in preparing the nanodispersion, with a monomodal β-carotene particle size distribution and a 17nm mean particle size.

Focusing in results obtained by Tan and Nakajima (2005a), Silva et al. (2011) replaced the high-pressure homogenization by conventional homogenization which consists in homogenizing the sample using an Ultra-Turrax homogenizer, and removing hexane from the emulsion by rotary evaporation. They confirmed that it is possible to prepare emulsions of β-carotene and obtain structures in the nano range, without using high-pressure homogenization, obtaining droplets in the range of 100 nm. They observed that β-carotene nanoemulsions showed a good physical stability in terms of size distribution, but they were chemically unstable during storage.

Ribeiro et al. (2008) studied the production of β-carotene-loaded nanodispersions containing poly(D,L-lactic acid) and poly(D,L-lactic coglycolic acid) by solvent displacement method. An additional evaporation process was needed to remove the organic solvent (acetone) by rotary evaporation obtaining nanodispersions with narrow droplet size distributions.

Yin et al. (2009) used the solvent displacement technique for studying the characteristics of β-carotene nanodispersions prepared with different emulsifiers, being these sodium caseinate, Tween 20, decaglycerol monolaurate and sucrose fatty acid ester. The main particle size of the nanodispersions ranged from 30 to 206 nm, depending on the emulsifier used. Sodium caseinate-stabilized nanodispersions had the largest particle size, while those prepared with Tween 20 had the smallest. The results showed that the emulsifiers not only influenced the mean particle size of the nanodispersions but also the chemical stability of encapsulated β-carotene.

On the other hand, Wang et al. (2012) studied the main factors which govern the bioaccessibility of β-carotene incorporated into dispersions, using an in vitro model preparing the β-carotene dispersions by high pressure homogenization or combining emulsification and evaporation. They demonstrated that the concentration of β-carotene, bile extract and pancreatic lipase, pH and the particle size of the dispersions (ranged from 45 to 18300 nm) significantly affected the transfer of β-carotene from dispersions to micelles.

Cao-Hoang et al. (2011) investigated the oxidation of β-carotene from synthetic and natural origins after dispersion in Tween micelles or poly lactic acid particles preparing the incorporation of β-carotene into Tween 80 micelles by conventional homogenization (Ultra-Turrax) and removing the organic solvent by rotary evaporation.

Supercritical fluid processes

The application of supercritical fluids for particles precipitation has been an active field of research and innovation during the past two decades (Jung, & Perrut, 2001; Shariati, & Peters, 2003; Martín, & Cocero, 2008). The main motivation for this is the possibility of exploiting the peculiar properties of supercritical fluids, and in particular of supercritical carbon dioxide, the

most used supercritical fluid for precipitation processes (Cocero et al. 2009). Supercritical carbon dioxide is non-toxic and can be easily and completely removed from the solid product as a gas simply by depressurization, a very convenient property for applications concerning products for human consumption as pharmaceuticals or food additives in which to avoid the contamination of the product by toxic solvents is an important issue (Martín & Cocero, 2008).

Several authors have studied the applications of supercritical fluid technology to the precipitation of β-carotene. Cocero and Ferrero (2002) researched the precipitation of β-carotene from ethyl acetate and dichloromethane solutions using carbon dioxide as antisolvent by GAS process. They obtained crystals smaller than 1 μm with specific operating conditions of temperature and pressure of 298 K and 5.8 MPa, respectively. Cardoso et al. (2009) used the supercritical antisolvent (SAS) technique for carrying out the micronisation of β-carotene using tetrahydrofuran as solvent and CO_2 as antisolvent, with the objective of increasing its bioavailability and facilitating its dispersion in oil and emulsion formulations as a result of its smaller particle size (mean particle diameters around 100 μm).

He et al. (2006) developed the supercritical antisolvent micronization of natural carotene by the SEDS (solution-enhanced dispersion) process through prefilming atomization obtaining small droplets and subsequently fine natural carotene particles (with main sizes between 0.4 and 5 μm) with a narrow particle size distribution.

Frasceschi et al. (2009) also investigated the precipitation of β-carotene microparticles by SEDS technique, obtaining a mean particle size varying from 3.2 to 96.8 μm with morphology of β-carotene microparticles changing from plate-like to leaf-like particles. However, Yin et al. (2011) developed a novel technique called SEDS-EM which combines the traditional SEDS technique with ultrasonication from the supercritical antiosolvent with enhanced mass transfer precipitation technique. For this technique, the β-carotene solution is premixed with the supercritical CO_2 in a coaxial nozzle and then, it is sprayed onto a surface vibrating at an ultrasonic frequency. The solution jet is dispersed by the coaxial nozzle and further atomized into very small droplets by the ultrasonic vibrating surface. The resultant β-carotene particles decreased in size from the micrometer range (2-5μm) by SEDS to the nanometer range (20-205 nm) by SEDS-EM.

On the other hand, the formulation of β-carotene with biopolymers using supercritical fluids has also been investigated. Martín et al. (2007) studied the co-precipitation of β-carotene with polyethylene glycol using supercritical carbon dioxide as antisolvent. The results indicated that the concentration ratio had a very important influence over the morphology of the particles. Franceschi et al. (2008) studied the coprecipitation of β-carotene and PHBV from SEDS technique obtaining an encapsulation efficiency of approximately 80%. Priamo et al. (2010)

used the same technique for the encapsulation of β-carotene in the same biopolymer, obtaining a maximum encapsulation percentage of 55%. The same authors also investigated the characteristics of the in vitro release of β-carotene encapsulated in PHBV (Priamo et al. 2011). He et al. (2007) performed the co-precipitation of natural carotene and polyethyleneglycol using dichloromethane as organic solvent and CO_2 as antisolvent and reported values of percentage of encapsulation up to 50%. And finally, Mattea et al. (2009) studied the formulation of β-carotene by supercritical antisolvent precipitation from a dichloromethane in water emulsion (SFEE), using as main surfactants the OSA surfactant and a blend of Tween 20 and Span 20. The final product was formed by particles with a mean size below 400 nm in suspension in an aqueous media. The same technique was used by Santos et al. (2012) obtaining suspensions of β-carotene with a particle size of 345 nm and encapsulation efficiency between 60-89%.

Recently, Reverchon and co-workers (Campardelli et al. 2012) have used an innovative technique for the production of stable aqueous nanodispersion of β-carotene named Supercritical Assisted Injection in Liquid Antisolvent (SAILA). An expanded liquid solution was formed using SC-CO_2 and an organic solvent in which β-carotene is also solubilized (acetone). Then, the expanded ternary solution is depressurized directly into water (in which Tween 80 was added) where the solute is not soluble and the organic solvent is miscible; therefore, the water based solution works as a liquid antisolvent. Operating in this manner, nanoparticles in the range of 50-150 nm were produced varying the process conditions.

All this literature review about formulation of β-carotene is summarized in Table 1.

Table 1. Literature review of formulation of β-carotene.

Researcher	Formulation method	Stabilizer/Surfactant	Oil phase/solvent	Process technique	PS / EE
Qian et al. (2012b)	(o/w) nanoemulsions	β-lactoglobulin Tween 20	Orange oil	High Pressure Homogenization (HPH)	PS: 158 nm
Qian et al. (2012c)		β-lactoglobulin or Tween 20 Addition water soluble: EDTA and ascorbic acid Addition oil-soluble: Vitamin E acetate or Coenzyme Q10	Corn oil		PS: 180 nm
Qian et al. (2012a)		Tween 20	Corn oil (long chain triglyceride) Miglyol 812 (medium chain triglyceride) Orange oil		PS: 140 - 170 nm
Mao et al. (2009-2010)		Tween 20 Decaglycerol monolaurate (DML) Octenyl succinate starch Whey protein isolate Blend of Tween 20 and whey protein isolate	Sunflower oil	High Pressure Homogenization (HPH)	PS: 115 - 300 nm
				Microfluidization	PS: 120 - 370 nm
Liang et al. (2013)		OSA-modified starches: HI-CAP 100, CAPSUL, CAPSUL-TA	Medium chain triacylglycerol	High Pressure Homogenization (HPH)	PS: 150 nm
Yuan et al. (2008a)		Tween 20 Tween 40 Tween 60 Tween 80	Medium chain triglyceride		PS: 132-184 nm
Yuan et al. (2008b)		Tween 20	Medium chain triglyceride	HPH (Response surface methodology)	PS: 120 - 170 nm
Silva et al. (2011)		Tween 20	Hexane	High energy emulsification-evaporation	PS: 9 - 280 nm

Table 1. Continuation 1. Literature review of formulation of β-carotene.

Researcher	Formulation method	Stabilizer/Surfactant	Oil phase/solvent	Process technique	PS / EE
Tan & Nakajima (2005)	Nanodispersions	Tween 20	Hexane	Emulsification (HPH)-evaporation	PS: 100 nm
Tan & Nakajima (2005)		Polyglycerol esters of fatty acids			PS: 85 - 132 nm
Chu et al. (2007)		Protein			PS: 17 nm
Ribeiro et al. (2008)		Poly(D,L-lactic acid) Poly(D,L-lactic coglycolic acid)	Acetone	Solvent Displacement Method	PS: 100 nm
Chu et al. (2007b)		Protein			PS: 171 nm
Yin et al. (2009)		Sodium caseinate Tween 20 Decaglycerol monolaurate Sucrose fatty acid ester			PS: 30 - 206 nm
Wang et al. (2012)	Dispersions	Decaglycerol monolaurate	Soybean oil	HPH or combining emulsification-evaporation	PS: 45 nm - 18.3 µm
Cao-Hoang et al. (2011)		Tween 80 or poly lactic acid	Chloroform	Conventional homogenization	PS: 150 nm

Table 1. Continuation 2. Literature review of formulation of β-carotene.

Researcher	Formulation method	Stabilizer/Surfactant	Oil phase/solvent	Process technique	PS / EE
Cocero & Ferrero (2002)	SCF precipitation		Ethyl acetate, dichloromethane	GAS	PS: < 1µm
Cardoso et al. (2009)			Tetrahydrofuran	SAS	PS: 100 µm
He et al. (2006)			Dichloromethane	SEDS-PA	PS: 0.4 - 5 µm
Franceschi et al. (2009)				SEDS	PS: 3.2 - 96.8 µm
Yin et al. (2011)				SEDS-EM	PS: 20 - 205 nm
Martín et al. (2007)	SCF co-precipitation	Polyethylene glycol		SAS	
Franceschi et al. (2008)		Poly(3-hydroxybutirate-co-hydroxyvalerate) (PHBV)		SEDS	PS: 3.8 - 246.8 µm EE: 80%
Priamo et al. (2010)		Poly(3-hydroxybutirate-co-hydroxyvalerate) (PHBV)		SEDS	EE: 55%
He et al. (2007)		Poly(ethylene glycol) (PEG)		SEDS-PA	PS: 1 -6 µm EE: 50%
Mattea et al. (2009)	SCF co-formulation	OSA surfactant Blend of Tween 20 and Span 20		SFEE	PS: 400 nm
Santos et al. (2012)		n-octenyl succinic anhydride (OSA)-modified starch		SFEE	PS: 345 nm
Campardelli et al. (2012)		Tween 80	Acetone	SAILA	PS: 50 - 150 nm

Outlook

From this summarized literature review, it can be concluded that the precipitation and formulation of β-carotene has been extensively investigated by several authors due to its high interest as natural colorant and antioxidant in food industry. Conventional techniques such as emulsification-evaporation or solvent displacement method have been used for this purpose. Nanoemulsions and nanodispersions obtained by high pressure homogenization technique have been extensively studied due the favourable properties of the final product in terms of stability and particle size. The use of supercritical fluid technologies might also be a good alternative for conventional processes thanks to the possibility to operate at mild conditions avoiding degradation. Moreover, due to the high solubility of organic solvents in supercritical carbon dioxide, these solvents can be efficiently removed from the product by treatment with the supercritical fluid. However, the main limitation in many applications based in supercritical fluid technology is the particle size, obtaining in most cases particles over 1 μm. Using supercritical fluid technology, particles in the nanometer range have been obtained in aqueous dispersions, using methods such as SFEE, SEDS-EM and SAILA.

On the other hand, the organic solvents, which have been used for developing the formulation of β-carotene, have been commonly dichloromethane, chloroform and hexane which are highly toxic solvents not recommendable for food applications. Due to this, there is a necessity of developing novel technologies improving the conventional processes to carry out the formulation of β-carotene using other organic solvents with low toxicity. Avoiding the degradation of the carotenoid due to exposure to high temperatures and long processing times is also necessary. Additionally, it is important to ensure the reproducibility and predictability of product properties, and to intensify the process increasing the production capacity and making it continuous if possible.

Objectives

The main **AIM OF THIS WORK** is to *develop different β-carotene formulations (hydrophilic and hydrophobic), in order to use them as natural colorant, and to implement novel, intensified techniques capable of producing these formulations with good controllability and predictability of product properties, as well as high throughput.*

With the general aim already presented, the thesis was organized with the following partial objectives:

- Study of different water-soluble β-carotene formulations using n-octenyl succinic anhydride (OSA)-modified starch as carrier material.

- Design and study of a novel pressurized emulsion process.
  - Characterization of final product and optimization of process parameters (such as concentration of surfactant or organic-water ratio) in order to achieve a formulation with stability over time, high encapsulation efficiency and particle size in the nanometer range which are required for its use as natural colorant.
  - Study of the antioxidant properties of the formulated product.
  - Study of effect of using different (OSA)-modified starches and different organic solvents.
- Comparison with products obtained by conventional emulsification - solvent evaporation techniques, using both usual high-shear emulsification and novel ultrasound emulsification. Study of different process parameters: influence of organic-water ratio, time of application of ultrasounds, amplitude and duty cicle on the encapsulation efficiency and on the particle size.

- Development of different β-carotene formulations based on supercritical fluid technologies.

  - Study of formulation of β-carotene based on liposome-forming phospholipids as an alternative water-soluble formulation.
    - Development of this formulation by PGSS-Drying obtaining solid particles which can be re-hydrated forming liposomes. Effect of the operating variables (pre-expansion temperature and pressure, gas to product ratio (GPR) and concentration of soybean lecithin). Characterization of the obtained particles.
  - Study of the phase behaviour of different polycaprolactones and supercritical fluid mixtures in order to get a detailed knowledge for carrying out the formulation with this polymer.
  - Development of a hydrophobic formulation of β-carotene with polycaprolactones by PGSS. Effect of the operating variables (molar ratio of compounds, time of mixture homogenization, pre-expansion temperature and pressure).
  - Study of the solubility of β-carotene in polycaprolactones by equilibration-impregnation and equilibration-de-supersaturation processes, in order to

obtain basic properties for the performance of the encapsulation process by PGSS.

In order to achieve these objectives, this work has been structured in 8 chapters in which challenges, objectives and partial objectives are collected. The main contents of each chapter are described below.

In CHAPTER 1, **"Formulation of β-carotene by precipitation from pressurized ethyl acetate-on-water emulsions for application as natural colorant"**, the formulation of β-carotene with modified OSA- starch using pressurized ethyl acetate emulsions with a final step of organic solvent removal, was investigated. It was demonstrated that OSA-starch was a good surfactant, obtaining stable aqueous suspensions of β-carotene. Several process parameters were studied, such as the influence of the concentration of surfactant and the organic-water ratio on the encapsulation efficiency and on the particle size. Also, these process variables were optimized in order to reach a good formulation of β-carotene. With this technique, it was possible to obtain stable suspensions of β-carotene with high encapsulation efficiencies and small particle size (below 600nm).

In chapters 2 and 3, this line of investigation was continued following with the use of the same technique for carrying out the formulation of β-carotene. Concretely, in CHAPTER 2, **"Water – soluble formulation of β-carotene in OSA-starch micelles by high-pressure, high temperature antisolvent precipitation from pressurized ethanol emulsions"**, the formulation of β-carotene with modified OSA- starch using ethanol as organic solvent was investigated. Also, a comparison between results obtained in Chapter 1 (using ethyl acetate as organic solvent) and those obtained with ethanol was presented. The influence of the organic-water ratio was studied in order to observe its influence on product characteristics. Results shown in this chapter revealed that it was possible to obtain a formulation of β-carotene using a non-toxic solvent. However, the encapsulation efficiencies were lower than those achieved with ethyl acetate. In CHAPTER 3, **"Formulation of of β-carotene by precipitation from pressurized ethyl acetate-on-water emulsions: Effect of different OSA-starches"**, the influence of using four different OSA-starches as carrier materials on product characteristics was studied. The investigation included the effect of the concentration of surfactant and the organic-water ratio for each OSA-starch, on the encapsulation efficiency and on the particle size. A similar trend of results was observed with all of them, but however there was a strong difference among them regarding the obtained results, being one of them not suitable for carrying out the

encapsulation of β-carotene due to the low encapsulation efficiency and high particle size achieved, independently of the concentration of surfactant and organic-water ratio selected.

In CHAPTER 4, "**Production of water-soluble β-carotene formulations: comparison between ultrasound emulsification and emulsion evaporation, and precipitation from a pressurized emulsion"**, the formulation of β-carotene with OSA-starch was developed by ultrasound emulsification with a posterior step of organic solvent removal. Different process parameters, such as organic-water ratio, time of application of ultrasounds, amplitude and duty cicle were studied in order to observe their influence on final product characteristics. Furthermore, this study includes a comparison of the results obtained among the different techniques for preparing the emulsion of β-carotene including, in all of them, a final step of organic solvent removal: ultrasound emulsification, high-shear emulsification and precipitation from a pressurized emulsion technique.

Besides the water-soluble formulations prepared by precipitation from a pressurized emulsion, alternative formulations consisting of an encapsulation in the slow-degrading polymer polycaprolactone were developed, employing a Particles from Gas Saturated Solutions (PGSS) technique. For this purpose, in CHAPTER 5, **"Determination of phase equilibrium (solid-liquid-gas) in poly-(ε-caprolactone) – carbon dioxide systems"**, the solid-liquid-gas equilibrium of polycaprolactone-carbon dioxide systems (using three different polycaprolactones with different molecular weights) was measured by visual determination of the first melting point using a high pressure optical cell. This study was developed in order to get a detailed knowledge of the phase behavior of the polymer and supercritical fluid mixtures due to the fact that this information is necessary to carry out the precipitation techniques using supercritical fluids and optimize the operation conditions. Taking into account results obtained in this chapter, in CHAPTER 6, **"Formulation of β-carotene with poly-(ε-caprolactone) by PGSS process"**, the formulation of β-carotene with two different polycaprolactones (with different molecular weights), was carried out by Particles from Gas Saturated Solutions process, obtaining solid particles with a particle size over 100 μm. The influence of several process parameters on particle size and β-carotene content was studied, including pressure, temperature, time of contact between CO_2 and polymer melt for mixture homogenization and molar ratio β-carotene:polymer.

As an alternative water-soluble formulation, in CHAPTER 7, **"Formulation of β-carotene with soybean lecithin by PGSS (Particles from Gas Saturated Solutions)-drying"**, the formulation of β-carotene in liposome-forming soybean lecithn was developed by PGSS-Drying obtaining dry particles (10-500 μm) which can be hydrated forming β-carotene-loaded liposomes between 1-

5 μm. The main process parameters were studied being these the pre-expansion temperature and pressure, gas to product ratio (GPR) and concentration of soybean lecithin.

Finally, in CHAPTER 8, **"Solubility of β-carotene in different poly-(ε-caprolactones) in colloidal state"**, the solubility of β-carotene in three different poly-(ε-caprolactones) with different molecular weights was studied. The determination of the solubility of β-carotene was carried out by two different processes: equilibration-impregnation and equilibration-de-supersaturation. In both processes, there was a previous step in which the organic solvent presented in the emulsion (dichloromethane) was extracted by SFEE (Supercritical Fluid Extraction from Emulsions) obtaining an aqueous suspension of polycaprolactone particles for developing the equilibration-impregnation experiments and an aqueous suspension of polycaprolactone- β-carotene particles for carrying out the equilibration-de-supersaturation experiments.

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Chapter 1

Formulation of β-carotene by precipitation from pressurized ethyl acetate-on-water emulsions for application as natural colorant

Formulation of β-carotene by precipitation from pressurized ethyl acetate-on-water emulsions for application as natural colorant[1]

Abstract

β-carotene formulations are very attractive as natural colorants as they provide additional value to the product due to their antioxidant and provitamin activities. Application of β-carotene as colorants in beverages requires an appropriate formulation in order to stabilize the particles of β-carotene in suspension and provide the desired color. This work presents a study of the formulation of β-carotene by precipitation from a pressurized ethyl acetate-in-water emulsion using modified OSA-starch refined from waxy maize as carrier material. Formulations of β-carotene with a high encapsulation efficiency of β-carotene (over 65% in most cases and as high as 90% with specific conditions), high antioxidant activity and a micellar particle size in the range of 300 - 600 nm were obtained. The concentration of modified starch and the organic solvent-water flow ratio were the process parameters with most influence on product properties.

Keywords: β-carotene, colorant, antioxidant, nano suspension, OSA-starch, emulsion.

[1] Food Hydrocolloids 26 (2012), 17-27

1. INTRODUCTION

Carotenoids are some of the most common pigments in nature, the most abundant being β-carotene, lycopene, lutein and zeaxanthin. The mail roles of carotenoids in human diet are as precursors of Vitamin A and as antioxidants. Since they are authorized food ingredients, carotenoids are widely used in the food, cosmetic and pharmaceutical industries as natural colorants. For many industrial applications, a mixture of carotenoids with biopolymers is used. Covering carotenoids with polymers provides protection against oxidation and degradation processes (Martín, et al. 2007). Moreover, the high hydrophobicity of carotenoids makes them insoluble in aqueous systems, and therefore they have a poor intake in the body. To improve their dispersibility in water, coloring strength potential and also to increase their bioavailability during gastro-intestinal passage, carotenoid crystals must be formulated (Ribeiro et al. 2008).

Food colorants have always been target of complains of the food industry consumers. Nowadays the food market demands functional foods and healthy products, using natural additives which provide the final product with a healthy added value (Mattea et al. 2009a). For the use of carotenoids as natural colorants, a formulation of the active compound is required with a determined particle size. It is important to obtain an appropriate colour intensity of the formulation which depends on the properties of particles.

There are several methods of precipitation and condensation for the production of organic nanoparticles in aqueous media (Horn and Rieger, 2001). Tan and Nakajima (2005) investigated the preparation of β-carotene nanodispersions by a process based on an emulsification-evaporation technique. Ribeiro et al. (2008) studied the production of β-carotene-loaded nanodispersions containing poly(D,L-lactic acid) and poly(D,L-lactic coglycolic acid) by solvent displacement method. Also, Chu et al. (2007) demonstrated the preparation of protein-stabilized β-carotene nanodispersions by the same method. Yuan et al. (2008a) used the response surface methodology to optimise the conditions for preparing β-carotene nanoemulsions. Yuan et al. (2008b) also studied the production of oil-in water nanoemulsions of β-carotene by high pressure homogenization. Yin et al. (2009) studied the characteristics of β-carotene nanodispersions prepared with different emulsifiers using a solvent displacement technique. This work demonstrated the importance of emulsifier in determining the characteristics and stability of β-carotene nanodispersions. Silva et al. (2011) prepared nanoemulsions of β-carotene using a high-energy emulsification-evaporation technique. Results showed that it was possible to obtain dispersions at a nanoscale size range. Cao-Hoang

et al. (2011) investigated the oxidation of β-carotene from synthetic and natural origins after dispersion in Tween micelles or poly lactic acid particles. The application of supercritical fluid technology for the precipitation of carotenoids has also been abundantly studied (Cocero & Ferrero, 2002; Cardoso et al. 2009; He et al. 2006; Martín et al. 2007; Franceschi et al. 2008; Franceschi et al. 2009), obtaining particle sizes in the micron range in most cases. For applications of β-carotene as colorant in a suspension it would be desirable to obtain particle sizes in the sub-micron or nanometer range (Mattea et al. 2009b).

This work presents a study of the formulation of β-carotene using a modified n-octenyl succinate (OSA) starch refined from waxy maize as carrier material for applications as natural colorant. Formulations have been prepared with a process based in the formation of an oil-in-water emulsion with pressurized fluids. The aim in the conception of this process is to improve over the conventional emulsion evaporation process, accelerating the mass transfer kinetics to the time scales of the precipitation processes. Such an intensification of the process may allow for an enhanced control over the precipitation to be obtained, at the same time that the exposition of the product to detrimental high-temperature conditions is reduced. Ethyl acetate has been chosen as organic solvent because it is a Generally Recognized as Safe (GRAS) solvent with low toxicity (Lethal Dose LD_{50} in rats: 11.3 g/kg, Riemenschneider and Bolt, 2005), and it can be safely used as a flavouring agent with concentrations in final products up to 1400 ppm, and the solubility of β-carotene in this solvent is relatively high. Três et al. (2007) have studied the effect of temperature on the solubility of β-carotene in organic solvents under ambient pressure. For example, the solubility of β-carotene in ethyl acetate varies from 0.680 to 15.180 mg mL^{-1} in a temperature range from 10 to 60ºC respectively. Using chlorinated solvents such as dichloromethane, the solubility is higher: at 10ºC, the solubility is 4.767 mg mL^{-1} and at 35ºC, the solubility of β-carotene is 22.160 mg mL^{-1}, but dichloromethane is a highly toxic solvent not recommendable for food applications. The influence of main process parameters has been studied, including the effect of the concentration of the modified-starch dissolution, the flows of organic solvent, suspension of β-carotene and dissolution of modified-starch and also the organic-water ratio. Product properties analyzed include particle size and morphology, encapsulation efficiency, cis/trans β-carotene composition, antioxidant activity and colour parameters.

2. MATERIALS AND METHODS

2.1. Materials

Crystalline β-carotene with a minimum purity of 99% was provided by Vitatene (León, Spain). Ethyl Acetate with a purity of 99.5% was purchased from Panreac Química (Barcelona, Spain). Modified OSA-starch refined from waxy maize was kindly provided by National Starch Group (Hamburg, Germany).

2.2. Equipment

Figure 1 presents the schematic flow diagram of the experimental apparatus. It consists of three small storages at ambient pressure, corresponding to the feed of pure organic solvent, β-carotene suspension in the same organic solvent and the aqueous solution of the modified OSA-starch. The installation also counts with two piston pumps GILSON 305 (maximum flow rate: 25 mL min^{-1}; flow control with an a accuracy of 0.1 mL·min^{-1}) used to feed the aqueous dissolution of modified starch and the β-carotene suspension (pumps P-3 and P-2, respectively) and a piston pump JASCO PU-2080 plus (maximum flow rate: 10 mL min^{-1}; flow control with an a accuracy of 0.1 ml·min^{-1}) used to feed the pure organic solvent (pump P-1). The stream of the organic solvent is preheated in a chromatographic oven (KNK-2000-C series GAS CHROMATOGRAPH) to a temperature of about 165ºC in order to reach the specified operation temperature after the mixing with the β-carotene suspension (typically 145ºC). All streams are pressurized with the pumps in order to keep them in the liquid phase at this temperature (typical operating pressure: 6.0 - 6.5 MPa). The suspension of β-carotene is pumped at ambient temperature. Then it is mixed with the hot organic solvent stream using the T-mixer M1 and shortly afterwards with the cold aqueous solution of surfactant using the T-mixer M2, in order to reduce the contact time of β-carotene particles with the hot organic solvent and avoid the isomerization and degradation of the product. The estimated residence time between the two T-mixers is approximately 0.5-2 s, depending on the flow rates. With the contact of the suspension of β-carotene particles with the stream of hot organic solvent, a complete dissolution of β-carotene is achieved, because the solubility of β-carotene increases when temperature is increased (Três et al. 2007). The contact of the hot solution of β-carotene with the aqueous solution in mixer M-2 causes the emulsification of the organic solvent and the precipitation of β-carotene by a combined antisolvent and cooling effect. Then, the effluent (emulsion) is collected. The organic solvent is removed from the sample using a rotary

evaporator (BÜCHI 011-BÜCHI 461 Water Bath), thus producing a suspension of β-carotene nanoparticles in water stabilized with the surfactant. This suspension can be further processed removing water to produce a dry powder. In this work, this has been done by spray-drying using a MOBILE MINOR^{TM} TYPE MM-BASIC-PSR equipment.

The installation also counts with pressure and temperature sensors. Temperature is measured in the injection point of all streams and in intermediate points between the mixers, as shown in Figure 1. Pt-100 thermo-resistances with an accuracy of 0.1ºC have been used to measure temperature. Pressure has been determined using membrane pressure meters (DESIN TPR-18/V2, accuracy 0.01 MPa). In a typical experiment, the operation temperature and pressure during the process oscillated among ±5 ºC and ±0.5 MPa, respectively.

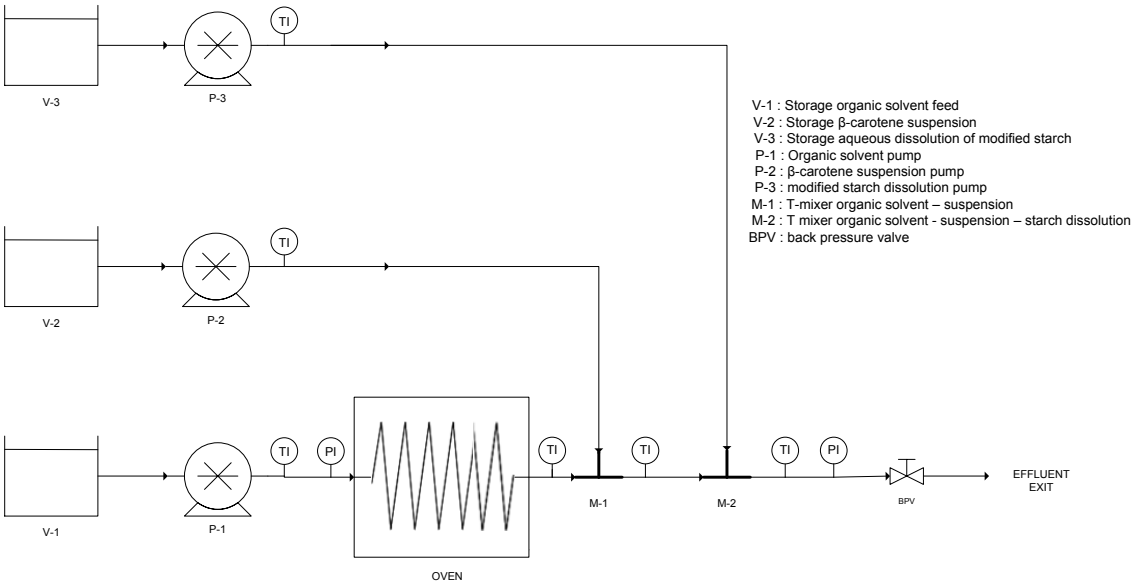


Fig.1. Schematic flow diagram of the experimental apparatus employed for β-carotene formulation.

2.3. Experimental procedure

A typical experiment starts with the preparation of the dissolution of modified starch and the β-carotene suspension in ethyl acetate. Two concentrations of β-carotene suspension were used: 34 g L^{-1} and 50 g L^{-1}. The concentration of the dissolution of modified starch was varied from 37 g L^{-1} to 367 g L^{-1}. Then, the flows used in the experiment were fixed. The flows of the organic solvent and β-carotene suspension were varied from 2.2 to 10 mL min^{-1} and the flow of

the dissolution of modified starch were varied from 9 to 24.6 mL min^{-1}. Regarding the ratio of organic-water (flows of organic solvent and β-carotene suspension/flow of the dissolution of modified starch), it was modified between the values 0.5 and 1.3. After that, pumps were switched on and the pressure in the system was fixed (6.0-6.5 MPa). The oven was switched on to heat the organic solvent until the desired temperature (140-150ºC). When the required temperature was reached in mixer M-1, the organic solvents used until this moment were replaced with the β-carotene suspension (in continuous agitation because a homogeneous suspension is needed) and with the dissolution of the modified starch. In a typical experiment, a volume of 50 mL of suspension containing 2.5 g of β-carotene was processed. Afterwards, the organic solvent of the obtained emulsion was removed from the sample using a rotary evaporator, thus obtaining an aqueous sample free from organic solvent. The last step in this process was to evaporate the water. For doing this, the sample was introduced in a spray-dryer. The air temperature in the spray drying system was 160ºC, the exit temperature varied between 85ºC and 91ºC and the feed flow velocity was approximately 1 L h^{-1}. The powder finally obtained was introduced in a closed container covered with aluminium paper in order to protect the sample from the effect of light and oxygen, and it was stored in a cold camera (T = 6ºC) to avoid the effect of temperature. In Table 1, a summary of the process conditions in all experiments performed is shown.

Table 1. Summary of operating conditions.

	$C_{\beta\text{-carotene}}$ ($g \cdot L^{-1}$)	C_{starch} ($g \cdot L^{-1}$)	$F_{organic}$ ($mL \cdot min^{-1}$)	$F_{\beta\text{-carotene}}$ ($mL \cdot min^{-1}$)	F_{starch} ($mL \cdot min^{-1}$)	$r_{organic\text{-}water}$	$T_{operation}$ (ºC)	$P_{operation}$ (MPa)
E 1	34	37	2.2	2.2	9	0.49	145 - 153	6 - 6.5
E 2	34	37	2.2	2.2	9	0.49	138 - 145	6 - 6.5
E 3	34	46	5	2.2	9	0.80	145 - 155	6 - 6.5
E 4	34	46	5	2.2	9	0.80	142 - 145	6 - 6.5
E 5	34	61	5	2.2	9	0.80	140 - 145	6 - 6.5
E 6	34	61	5	2.2	9	0.80	143 - 145	6 - 6.5
E 7	34	92	5	2.2	9	0.80	140 - 145	6 - 6.5
E 8	34	92	5	2.2	9	0.80	140 - 145	6 - 6.5
E 9	34	183	5	2.2	9	0.80	144 - 148	6 - 6.5
E 10	34	183	5	2.2	9	0.80	140 - 145	6 - 6.5
E 11	34	229	5	2.2	9	0.80	140 - 145	6 - 6.5
E 12	34	229	5	2.2	9	0.80	140 - 145	6 - 6.5
E 13	34	306	5	2.2	9	0.80	140 - 145	6 - 6.5
E 14	34	306	5	2.2	9	0.80	140 - 145	6 - 6.5
E 15	34	367	5	2.2	9	0.80	137 - 145	6 - 6.5
E 16	34	367	5	2.2	9	0.80	142 - 145	6 - 6.5
E 17	50	229	7	5	16.5	0.73	141 - 145	6 - 6.5
E 18	50	229	7	5	16.5	0.73	145 - 150	6 - 6.5
E 19	50	367	10	10	15	1.33	145 - 152	6 - 6.5
E 20	50	367	10	10	15	1.33	140 - 145	6 - 6.5
E 21	50	367	8	10	15	1.20	137 - 145	6 - 6.5
E 22	50	367	9	6	20	0.75	139 - 145	6 - 6.5
E 23	60	367	9	7	20	0.80	139 - 148	6 - 6.5
E 24	60	367	8	8	20	0.80	132 - 141	6 - 6.5
E 25	50	367	7	5	16.5	0.73	136 - 145	6 - 6.5
E 26	50	367	9	8	20	0.85	140 - 147	6 - 6.5
E 27	50	367	9	9	20	0.90	136 - 145	6 - 6.5
E 28	50	367	7	7	20	0.70	130 - 137	6 - 6.5
E 29	50	367	7	6	16.5	0.79	140 - 150	6 - 6.5
E 30	50	367	7	6	19.3	0.67	133 - 140	6 - 6.5
E 31	50	367	8	6	16.5	0.85	142 - 147	6 - 6.5
E 32	50	367	8	6	19.3	0.73	144 - 150	6 - 6.5
E 33	50	367	7	6	17.9	0.73	134 - 145	6 - 6.5
E 34	50	367	9	6	20.6	0.73	144 - 150	6 - 6.5
E 35	50	367	9	6	18.6	0.81	145 - 152	6 - 6.5
E 36	50	367	9	4.5	16.8	0.80	144 - 149	6 - 6.5
E 37	50	367	8	7	16.5	0.91	132 - 142	6 - 6.5
E 38	50	367	8	7	20.6	0.73	132 - 142	6 - 6.5
E 39	50	367	8	7	18.7	0.80	138 - 147	6 - 6.5
E 40	50	367	7	5	14.1	0.85	134 - 145	6 - 6.5
E 41	50	367	7	5	15	0.80	137 - 147	6 - 6.5
E 42	50	367	9	7	16.5	0.97	140 - 148	6 - 6.5
E 43	50	367	9	7	22	0.73	137 - 147	6 - 6.5
E 44	50	367	9	6	25	0.60	143 - 157	6 - 6.5
E 45	50	367	9	6	23	0.65	142 - 150	6 - 6.5
E 46	50	367	9	7	24.6	0.65	135 - 148	6 - 6.5
E 47	50	367	7	5	20	0.60	135 - 145	6 - 6.5
E 48	50	367	7	5	18.4	0.65	136 - 150	6 - 6.5
E 49	50	367	8	6	23.3	0.60	140 - 150	6 - 6.5
E 50	50	367	8	6	21.5	0.65	138 - 150	6 - 6.5

2.4. Product characterization

2.4.1. Weight of dry product

The dry weight of the sample was determined using a Halogen Moisture Analyzer model Ohaus MB35 set at 160º to remove water. This weight included crystalline, non encapsulated β-carotene, encapsulated β-carotene and modified starch.

2.4.2. Percentage of encapsulated β-carotene

The sample was analysed by a UV/VIS spectrophotometer model Agilent 8453. The wavelength selected was 456 nm. The absorbance determined with this method is proportional to the amount of β-carotene dispersed in solution. The particles of crystalline β-carotene not stabilized in the suspension do not contribute to the absorbance determined with this method. The ratio of this concentration, corresponding to the amount of β-carotene stabilized in the suspension, to the total β-carotene concentration in the product, is reported in this work as percentage of encapsulated β-carotene.

2.4.3. Particle size

The particle size analysis was carried out by laser diffraction (model Beckman Coulter LS230) and photo correlation scattering (model Malverm Zetasizer 1000 HS).

2.4.4. Trans / Cis β-carotene HPLC profile

Crystalline β-carotene used as feed material contained 95%wt of trans β-carotene and 5%wt of cis β-carotene, but some isomerization took place during the high temperature steps of the process. The degree of isomerisation was determined using a Waters Alliance 2695 HPLC chromatograph with PDA detector 2996 equipped with a Vydac 218TP54 5 µm (4.6×250) column. The mobile phase was methanol + 1% of tetrahydrofuran stabilized with 50 ppm of ascorbic acid. The temperature of the column was 30ºC. The solvent for the samples was ethanol/tetrahydrofuran stabilized with 50 ppm of ascorbic acid.

2.4.5. Colour

The colour of the obtained nano-suspensions was characterized by a UV/Vis spectrophotometer model Agilent 8453 operated in a wide range of wavelength.

2.4.6. Microscopy

Pictures of the particles collected after spray drying process were taken by means of a scanning electron microscope (SEM) model JEOL JSM-820. Samples were gold-sputtered before observation.

2.4.7. Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry profiles were obtained using a differential scanning calorimeter DSC 822e Mettler Toledo SAE.

2.4.8. Oxygen radical absorbance capacity (ORAC)

ORAC assay was used to evaluate the antioxidant capacity of the samples towards peroxyl radicals. The assay was carried out following a modified method (Serra et al. 2011) as described by Ou et al. (2001), using a microplate fluorescent reader (FL800 Bio-Tek Instruments, Winooski, VT, USA). This essay measured the ability of the antioxidant species present in the sample to inhibit the oxidation of disodium fluorescein (FL) catalysed by peroxyl radicals generated from α, α´-Azodiisobutyramidine Dihydrochloride porum (AAPH). The composition of the reaction mixture was $2 \cdot 10^{-7}$ mM FL, 0.0414 $g \cdot mL^{-1}$ AAPH (prepared in phosphate buffer solution (PBS), pH 7.4) and an appropriately diluted sample (0.002g ml^{-1}). The reaction was started by the addition of AAPH to the mixture at 37ºC. Fluorescence was measured and recorded every 1 minute for a period of 30 minutes. PBS was used as a blank and 50, 40, 20, 10 and 5 µM Trolox solutions were used as control standards. The blank and the control standards were analysed in triplicate and the samples were analysed six fold. Final ORAC values were calculated using a correlation equation between the Trolox concentration and the net area under the FL decay curve. All data were expressed as micromoles of Trolox equivalents antioxidant capacity per gram of particle (µmol TEAC g^{-1} particle).

2.4.9. Hydroxyl radical adverting capacity (HORAC)

HORAC assay was used to characterize the antioxidant capacity of the samples evaluating hydroxyl radical prevention capacity using fluorescein (FL) as the probe. The hydroxyl radical is generated by a Co(II)-mediated Fenton-like reaction. The HORAC assay was based on a previously reported method (Ou et al., 2002), modified (Serra et al., 2010) for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA). The reader was used with fluorescence filters for an excitation wavelength of 485 ± 20 nm and an emission

wavelength of 530 ± 25 nm, and the plate reader was controlled by software Gen5. The composition of the mixture was 4×10^{-6} mM FL, 0.55M H_2O_2, a cobalt solution which was prepared dissolving 3.92 mg of cobalt (II) fluoride tetrahydrate and 5 mg of picolinic acid in 5 mL of distilled water and an appropriately diluted sample (0.02 g ml^{-1}). Fluorescence was measured and recorded every 1 minute for a period of 30 minutes. PBS was used as a blank and 600, 400, 300, 200 and 100 μM caffeic acid solutions were used as control standards. Caffeic acid was used as a standard as it provides a wider linear range as compared to gallic acid (Dubost et al., 2007). The blank and the control standards were analysed in triplicate and the samples were analysed six fold. Final HORAC values were calculated using a regression equation between the caffeic acid concentration and the net area under the FL decay curve. Data were expressed as micromoles of caffeic acid equivalents antioxidant capacity per gram of particle (μmol CAEAC g^{-1} particle).

3. RESULTS AND DISCUSSION

Table 1 shows a summary of the process conditions in all experiments performed, while Table 2 presents the main experimental results obtained in each experiment. As shown in Table 1, the main process parameters were changed in order to analyse the influence of this parameters on product characteristics, including:

- Concentration of β-carotene in the initial suspension $C_{\beta\text{-carotene}}$ (g L^{-1}).
- Concentration of OSA-starch in the initial aqueous solution C_{starch} (g L^{-1}).
- Flowrates of organic solvent $F_{organic}$, β-carotene suspension $F_{\beta\text{-carotene}}$ and surfactant solution F_{starch} (mL min^{-1}).

Several parameters were carried out in duplicate to test the reproducibility of the results. In most cases, very similar results have been obtained in duplicate experiments demonstrating the reproducibility of results (e.g. experiments 1-2, 3-4, 7-8 etc.), with few exceptions (experiments 5-6 and 15-16). The most common operating problem found in experiments was the blockage of pumps.

Table 2. Summary of experimental results

	% Dry product	% β-carotene In sample	% encapsulated β-carotene	% trans / cis β-carotene	UV/VIS spectrum peaks	Particle Size (mean peak) (nm)
E 1	3.0	7.6	20.2	91.7 / 8.3	451 / 480 / 515	265
E 2	3,5	7.1	22.4	93.1 / 6.9	451 / 478 / 516	273
E 3	4,5	4.8	46.0	82.8 / 17.2	451 / 480 / 520	203
E 4	4.0	3.6	41.9	77.8 / 22.2	451 / 479 / 517	238
E 5	5.8	3.0	49.5	80.4 / 19.6	451 / 478 / 517	209
E 6	5.1	3.2	68.2	80.0 / 20.0	447 / 476 / 514	246
E 7	8.3	1.7	45.9	71.5 / 28.5	443 / 512	265
E 8	9.8	1.8	57.8	72.4 / 27.6	393	399
E 9	18.1	1.0	63.3	75.8 / 24.2	410	395
E 10	18.5	1.0	63.1	76.5 / 23.5	415	504
E 11	18.0	0.8	58.6	77.3 / 22.7	424 / 436 / 509	406
E 12	18.3	1.0	64.6	77.0 / 23.0	294 / 439 / 510	371
E 13	23.0	0.7	65.0	76.5 / 23.5	294 / 442 / 512	415
E 14	24.7	0.7	58.0	76.7 / 23.3	294 / 440 / 511	541
E 15	32.2	0.5	91.7	75.2 / 24.8	294 / 441 / 511	600
E 16	26.6	0.9	51.9	74.4 / 25.6	440,5	432
E 17	17.0	3.9	54.7	71.4 / 28.6	441	915
E 18	18.4	5.7	40.3	69.4 / 30.6	438	999
E 19	26.0	6.4	61.1	49.8 / 50.2	444 / 472 / 514	2900
E 20	24.7	6.0	62.0	61.4 / 38.6	440	30000
E 21	27.2	4.9	74.6	65.6 / 34.4	441 / 512	3360
E 22	26.4	1.9	73.9	81.8 / 18.2	444 / 512	470
E 23	26.9	2.2	63.1	70.0 / 30.0	445 / 471 / 512	422
E 24	26.6	2.1	67.3	82.9 / 17.1	443 / 471 / 513	400
E 25	26.6	2.3	74.3	81.3 / 18.7	443 / 512	367
E 26	26.5	2.3	97.0	73.2 / 26.8	442 / 510	748
E 27	25.2	3.0	77.3	70.6 / 29.4	442 / 510	470
E 28	26.5	2.6	72.6	81.9 / 18.1	445 / 473 / 514	505
E 29	27.6	1.8	95.1	80.5 / 19.5	445 / 473 / 514	1072
E 30	26.9	1.8	63.1	80.5 / 19.5	445 / 473 / 514	1012
E 31	27.3	2.1	73.7	72.7 / 27.3	445 / 474 / 515	1300
E 32	26.3	2.8	58.0	65.2 / 34.8	442 / 513	604
E 33	27.5	2.7	58.7	65.5 / 34.5	443 / 513	800
E 34	28.7	2.1	70.5	67.7 / 32.3	447 / 475 / 515	462
E 35	27.4	2.6	94.0	69.4 / 30.6	449 / 477 / 517	850
E 36	27.1	2.2	70.8	72.1 / 27.9	446 / 474 / 515	908
E 37	27.3	3.1	79.0	71.8 / 28.2	450 / 477 / 517	2013
E 38	25.2	3.6	71.5	75.7 / 24.3	449 / 476 / 516	1126
E 39	27.9	3.6	50.7	64.3 / 35.7	446 / 473 / 514	754
E 40	28.7	2.5	54.2	83.2 / 16.8	449 / 478 / 516	484
E 41	28.0	2.1	52.9	74.6 / 25.4	449 / 478 / 518	4736
E 42	26.7	4.2	40.7	67.4 / 32.6	448 / 475 / 516	622
E 43	25.7	3.7	43.9	73.5 / 26.5	450 / 475 / 515	548
E 44	27.4	2.4	76.0	68.7 / 31.3	450 / 478 / 517	431
E 45	27.3	2.4	69.9	68.9 / 31.1	450 / 476 / 517	417
E 46	24.5	4.6	32.7	69.5 / 30.5	450 / 476 / 516	464
E 47	26.8.	2.5	59.8	67.5 / 32.5	450 / 477 / 517	394
E 48	27.4	2.1	74.8	73.6 / 26.4	451 / 478 / 517	421
E 49	28.0	2.5	82.0	70.2 / 29.8	447 / 475 / 516	440
E 50	26.4	2.5	92.7	67.6 / 32.4	450 / 477 / 517	404

3.1 Influence of the concentration of surfactant

The effect of the concentration of the modified starch dissolution has been studied with experiments E1-E16. As shown in Table 1, all the process parameters were kept constant in these experiments except the concentration of the modified-starch dissolution, which was varied from 37 g L^{-1} to 367 g L^{-1}. Figures 2 and 3 present the influence of the concentration of surfactant on the percentage of encapsulated β-carotene and micellar particle size.

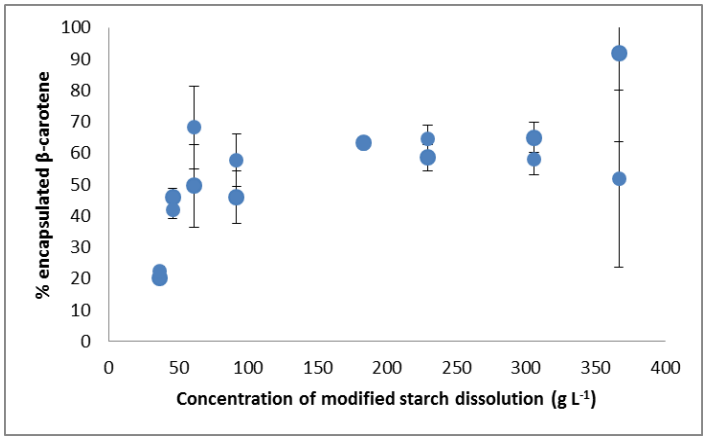


Fig.2. Effect of the concentration of modified starch dissolution on the percentage of encapsulated β-carotene.

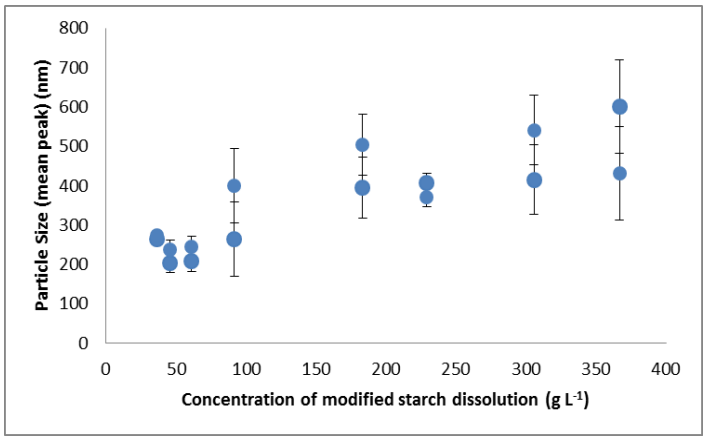


Fig.3. Effect of the concentration of modified starch dissolution on the micellar particle size.

Analysing the results presented in Table 2 and Figure 2, experiments E1 and E2 show the worst results in relation with the percentage of encapsulated β-carotene, with an encapsulation efficiency of 20% - 22%, meaning that the remaining 80% of β-carotene dosed precipitated in crystal form and was not stabilized inside the micelles of the emulsion. This result is due to the low concentration of surfactant used in these two experiments (37 g L^{-1}). As shown in Figure 2,

when the concentration of modified starch dissolution was increased, the percentage of encapsulated β-carotene was also higher. With regard to the micellar particle size, the mean sizes obtained ranged from 200 nm to 600 nm. Figure 3 shows that the micellar particle size was higher when the concentration of modified starch was increased. Although an increase in the micellar size is in general disadvantageous for the stability of the suspension, it must be taken into account that the use of high concentrations of starch allows to encapsulate a higher percentage β-carotene and to obtain a better emulsion stability. Moreover, particles with a size in the range of 1 μm may provide higher colour intensity than particles in the sub-micrometer range (Horn and Rieger, 2001).

The same conclusion can be obtained by comparing experiments E17 and E18 (with a surfactant concentration of 229 g L^{-1}) and experiment E25 (with a higher surfactant concentration of 367 g L^{-1} and equal values of the remaining process parameters).

3.2 Influence of the ratio between organic solvent flow rate and aqueous solution flow rate

The effect of the organic-water ratio (flow of organic solvent plus flow of β-carotene suspension divided by flow of modified starch dissolution) has been studied with experiments E17-E50. Figures 4 and 5 show the variation of the micellar particle size and the percentage of encapsulated β-carotene with the organic-water ratio. In Figure 4, the ratio was varied maintaining a constant flow of organic solvent + β-carotene suspension and using variable flows of surfactant solution, while in Figure 5 the flow of surfactant solution was approximately constant and the flows of organic solutions were changed.

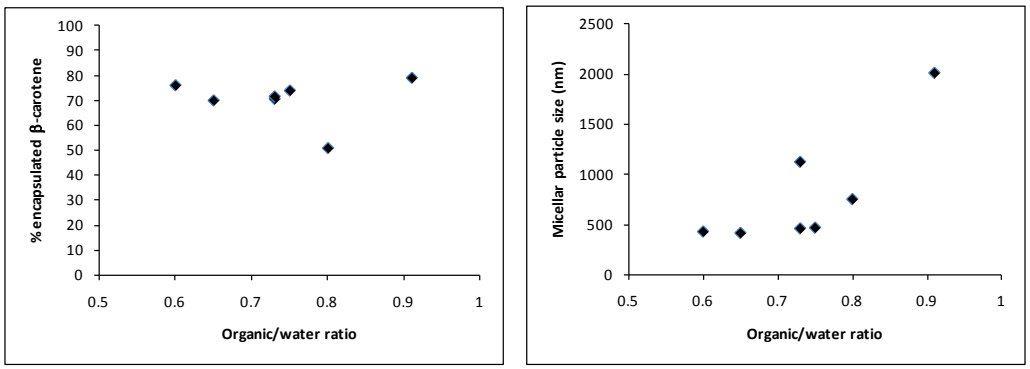


Fig.4. Effect of the organic/water ratio in the percentage of encapsulated β-carotene and in the micellar particle size, with a constant total flow rate of organic solvent + β-carotene suspension of 15 mL min^{-1} and variable flow rates of surfactant solution ranging from 16.5 mL min^{-1} to 25 mL min^{-1}.

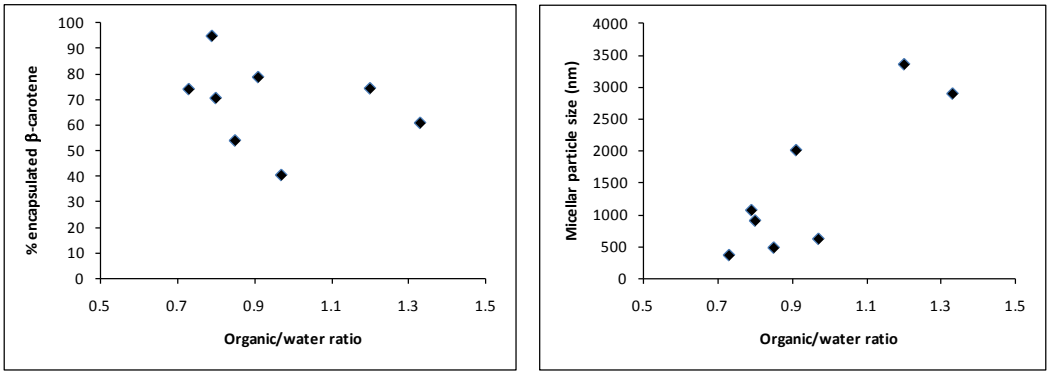


Fig.5. Effect of the organic/water ratio in the percentage of encapsulated β-carotene and in the micellar particle size, with an approximately constant total flow rate of surfactant solution of 15 mL min^{-1} - 16.5 mL min^{-1} and variable flow rates of organic solvent + β-carotene suspension ranging from 12 mL min^{-1} to 20 mL min^{-1}.

As presented in Figures 4 and 5, the organic/water ratio has a strong influence on the micellar particle size, and as this ratio is increased, the micellar size increases as well. This can be due to the formation of an emulsion with a larger droplet size after the mixing step M-2, which results in the formation of bigger particles inside the droplets of the emulsion. On the other hand, the encapsulation efficiency does not show a clear variation with the organic/water ratio. In Figure 4, it can be seen that a nearly constant encapsulation efficiency of 70% - 80% was obtained when the flow rate of surfactant solution was changed with a constant total flowrate of organic solutions, while the results of encapsulation efficiency presented in Figure 5 are more scattered, but do not show a clear trend of variation.

Figure 6 presents the influence of the organic/water ratio with specified constant flow rates of both organic solvent and β-carotene suspension instead of a constant total flow as in Figure 4. The conclusions obtained from Figure 6 are similar to the previous results presented in Figure 4 and 5: when the organic/water ratio is increased, the micellar particle size increases, and the encapsulation efficiency either no varies or shows a small reduction, probably due to the addition of more organic solvent with a fixed amount of starch surfactant and the formation of bigger emulsion droplets. Thus it can be concluded that the added flow rates of organic solvent and β-carotene solution is a more relevant parameter for the analysis of results that these two flow rates considered independently.

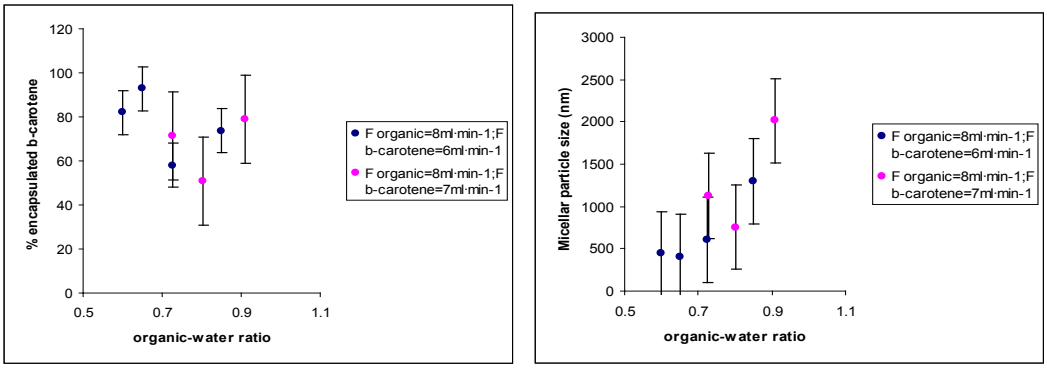


Fig. 6. Effect of organic-water ratio in the percentage of encapsulated β-carotene and in the micellar particle size with constant flow rates of organic solvent and β-carotene solution (F1 and F2, respectively) and variable flow rate of surfactant solution.

3.3. Influence of the total flow rates

The effect of the flow rate of modified starch dissolution in the percentage of encapsulated β-carotene and micellar particle size keeping constant the organic-water ratio can be observed in Figure 7. In the experiments presented in this figure, a constant organic-water ratio was kept. In Figure 7 a) and b) the organic-water ratio is 0.73, while in Figure 7 c) and d), the organic-water ratio is 0.65.

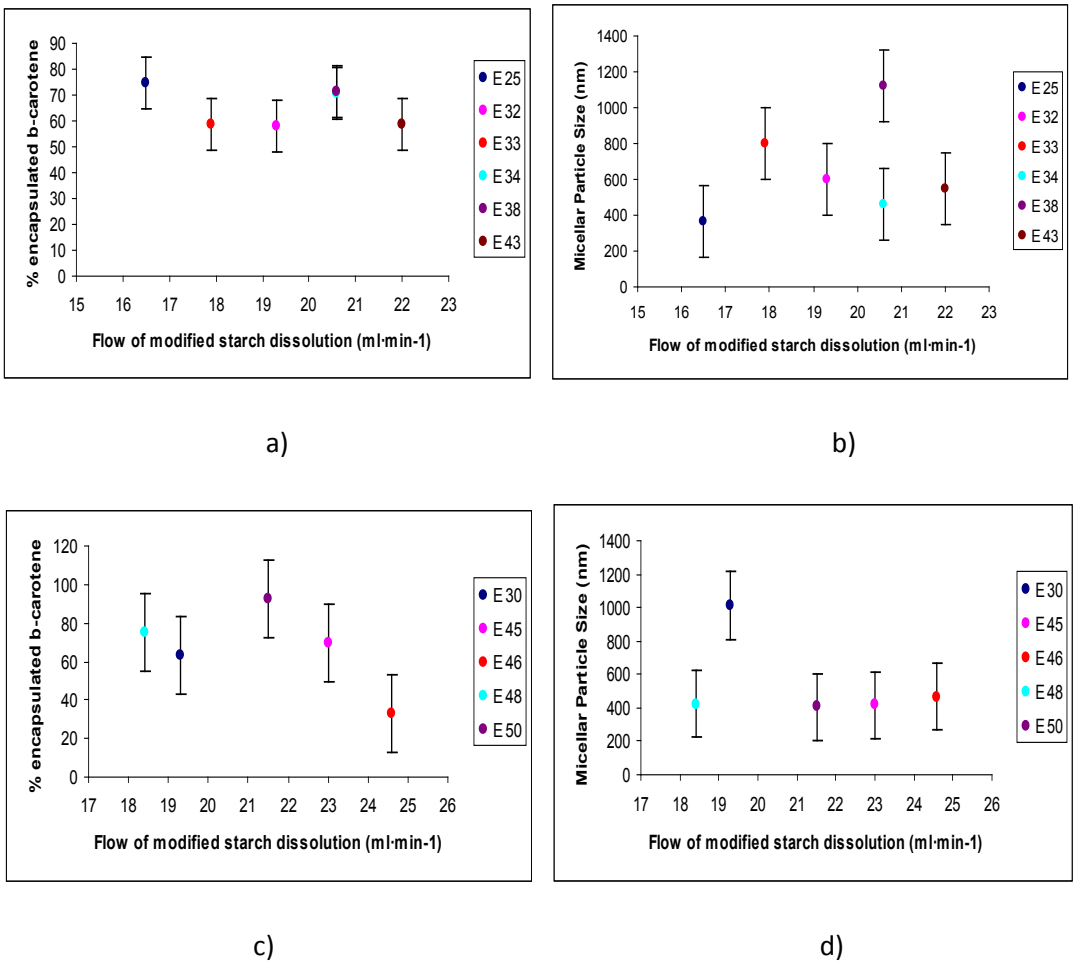


Fig. 7. Effect of the modified starch dissolution in the percentage of encapsulated β-carotene and micellar particle size. a) and b) Organic-water ratio = 0.73, c) and d) organic-water ratio = 0.65.

As presented in Figure 7 a), using an organic-water flow ratio of 0.73, good results were obtained in relation with the percentage of encapsulated β-carotene, which ranged from 60% to 75%. In the case of an organic-water ratio of 0.65 (Fig.7 c), high encapsulation efficiencies were obtained (between 63-93%) with the exception of one experiment (E46) which presented a lower percentage of 33%. This may indicate that the efficiency of encapsulation decreases when high aqueous solution flow rates are used, although due to the high dispersion of results presented in Figure 7 (a) and (c) a definitive conclusion cannot be reached.

Regarding the micellar particle size, presented in Figures 7 (b) and 7 (d), with a constant organic-water ratio there was not a clear trend of variation of micellar size with the flow rate. Micellar sizes obtained with an organic-water ratio of 0.65 were between 400 and 800 nm, while sizes of about 400 nm were obtained in experiments with an organic-water ratio of 0.73, with the exception of two experiments (E30 and E38), in which particles were bigger than 1

μm. This result is in agreement with the conclusions obtained in section 3.2 which indicate that smaller micellar sizes were obtained when the organic-water ratio was reduced.

As presented in Tables 1 and 2, there are other groups of experiments in which the organic-water ratio was kept constant in values of 0.60, 0.80 and 0.85, which present similar results as the cases discussed in Fig. 7. As a general conclusion, the results discussed in this section indicate that the properties of the product do not depend significantly on the total flow rate. Indeed, the ratio between organic and water flow rates appear as a more important parameter for controlling product characteristics than individual flow rates.

3.4 Cis-trans composition and antioxidant activity

Analysing the trans / cis β-carotene HPLC profile, it can be seen that isomerisation from trans to cis β-carotene has occurred in a percentage ranging from 15% to 50% with the exception of experiments E1 and E2 in which the isomerization was lower. It is expected that the isomerization is due to the exposition of the product to a high temperature in part of the process, although a clear relationship between process conditions and degree of isomerization has not been found.

Figures 8 and 9 show the results achieved by ORAC and HORAC antioxidant assays with respect to the percentage of β-carotene in the sample and the percentage of the encapsulated β-carotene. The results are expressed as micromoles of Trolox equivalents per gram of particle in the case of the ORAC assays. For HORAC assays, results are expressed as micromoles of caffeic acid equivalents per gram of particle. As it can be seen in Figure 8, ORAC ranged from 9.3 to 13.7 μmol Trolox g^{-1} and in Figure 9, HORAC ranged from 7.3 to 11.8 μmol caffeic acid g^{-1}. The ORAC correlates directly with the amount of β-carotene present in the sample with the exception of 4 experiments (E23-E24-E25-E34). As it can be observed in Figure 8, when the amount of β-carotene in the sample increases, the ORAC value is higher. However, a clear variation is not observed with the percentage of encapsulated β-carotene. In contrast, the antioxidant activity by HORAC assays varies both with the percentage of β-carotene in the sample and with the percentage of encapsulated β-carotene: as it can be seen in Figure 9, when the percentage of β-carotene in the sample is higher, the HORAC antioxidant activity increases. The same phenomenon can be observed with the percentage of encapsulated β-carotene.

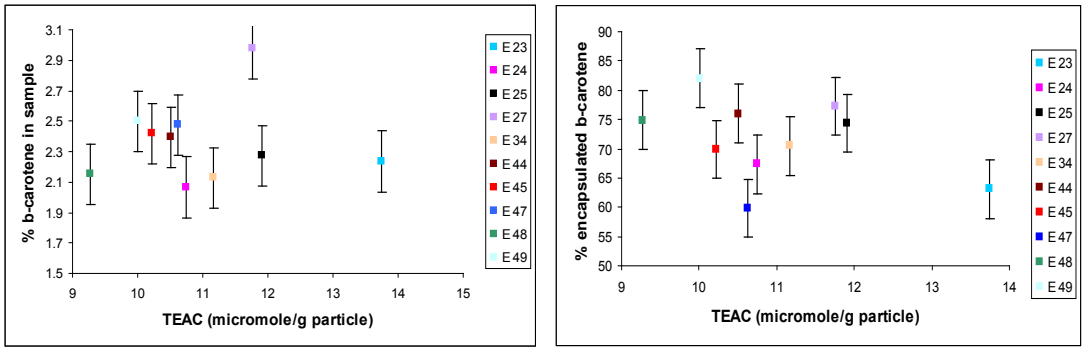


Fig.8. Antioxidant activity results by ORAC assays as a function of the total concentration of β-carotene in the sample and the concentration of encapsulated β-carotene in the sample.

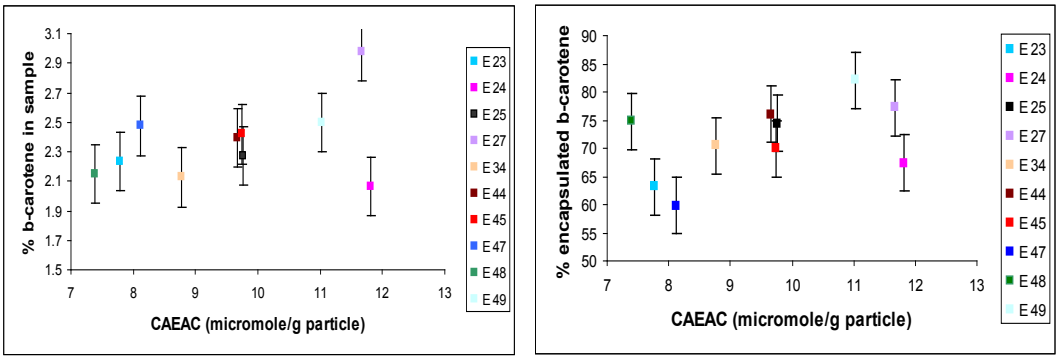
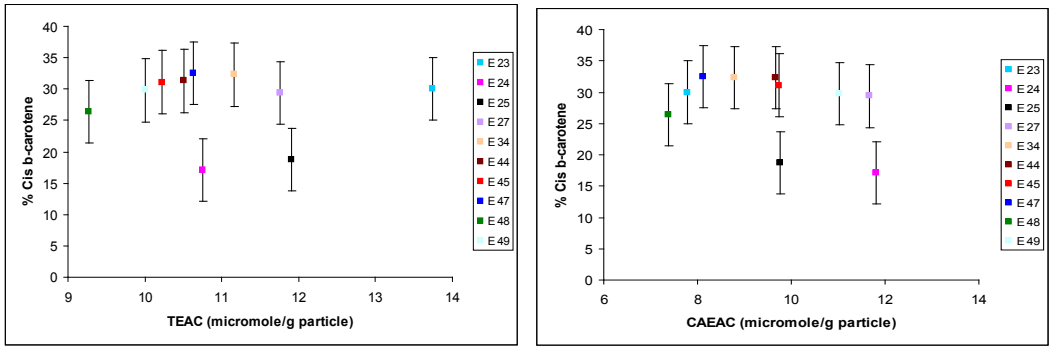


Fig.9. Antioxidant activity results by HORAC assays as a function of the total concentration of β-carotene in the sample and the concentration of encapsulated β-carotene in the sample.

Figure 10 presents the results obtained by ORAC and HORAC antioxidant assays with respect to the percentage of cis-β-carotene of the samples. As shown in Figure 10 (a), the results obtained with the ORAC assay presents an increase of the antioxidant activity when the percentage of cis-β-carotene increases. The same result is obtained with the HORAC assays as presented in Figure 10 (b). Levin and Mokady (1994) have studied the antioxidant activity of 9-cis compared to all-trans β-carotene and the obtained results suggested that 9-cis β-carotene has a higher antioxidant potency than that of the all-trans isomer.



a) b)

Fig. 10. Influence of the fraction of cis-β-carotene in product in the antioxidant activity results obtained a) by ORAC assays, b) by HORAC assays.

3.5. Characterization of product morphology and colour parameters

In Figure 11, the results of the DSC analysis of experiment E48 is shown. As it can be seen in the figure, two peaks are present which belong to the modified starch (at T_1 = 87 ºC and T_2 = 225 ºC) and another peak much more smaller corresponding to β-carotene (at T = 160ºC), due to the fact the percentage of β-carotene in the sample is lower than the percentage of the modified starch. The melting temperature of β-carotene observed in the product is smaller than the melting temperature of crystalline β-carotene (180-182ºC), which may indicate a certain amorphous fraction in the particles of β-carotene associated to the reduction of particle size. DSC analysis of particles obtained in all other experiments were very similar.

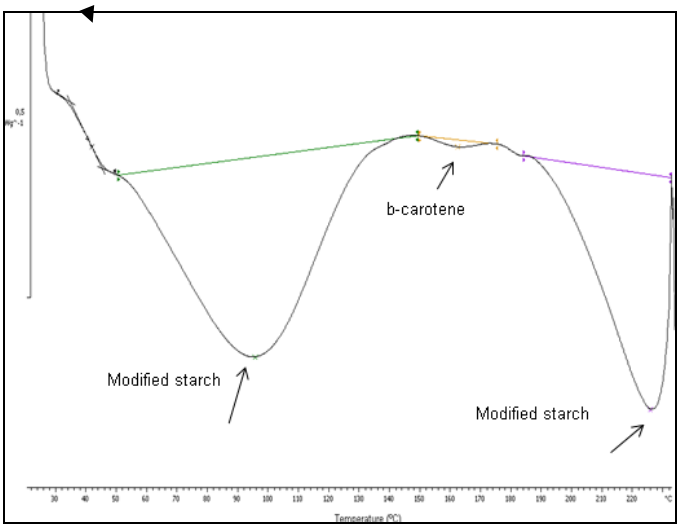
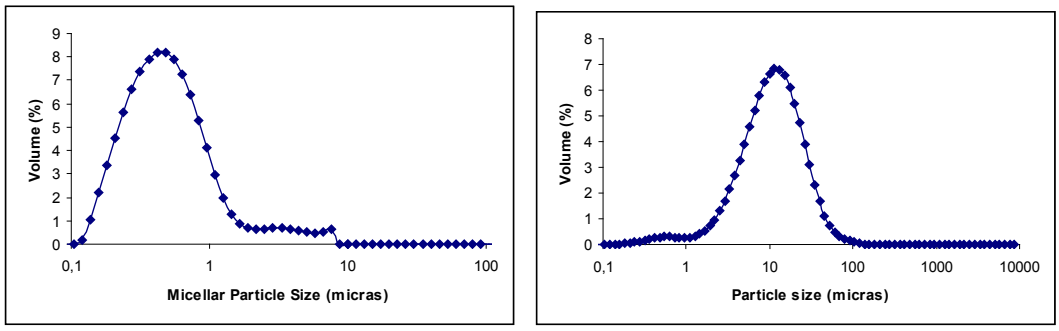


Fig. 11. DSC analysis of dry powder obtained with experiment E 48 (β-carotene concentration: 50 g L^{-1}, OSA starch concentration: 367 g L^{-1}, organic-water flow ratio: 0.65).

In Figure 12, the particle size distribution of the micellar particle size and the particles collected after spray drying process of the experiment E 45 is shown. As it can be seen in Figure 12, in this experiment the average micellar particle size was around 500nm and the average particles collected after spray drying process is 12 µm. In this case the average micellar particle size of the 10% of the particles ($d_{0.1}$) is less than 231nm and the average micellar particle size of the 90% of the particles ($d_{0.9}$) is less than 1.327 µm. In the case of the particles collected after spray drying process, $d_{0.1}$ is less than 3.580 µm and $d_{0.9}$ is less than 30.456 µm. The span of particle size distributions obtained in other experiments was in general similar to these results.



a) b)

Fig. 12. Particle size distribution of the experiment E45 (β-carotene concentration: 50 g L^{-1}, OSA starch concentration: 367 g L^{-1}, organic-water flow ratio: 0.65).
a) Micellar Particle Size, b) spray-dryed powder particle size.

In Figure 13, SEM micrographs of the particles collected after spray drying process are shown (experiments E24, E44, E48 and E50). After spray drying process, a powder is obtained which is formed by particles of modified starch containing the nano particles of β-carotene produced with the emulsion precipitation process. Because of this, the size of these particles is higher than micellar particle size.

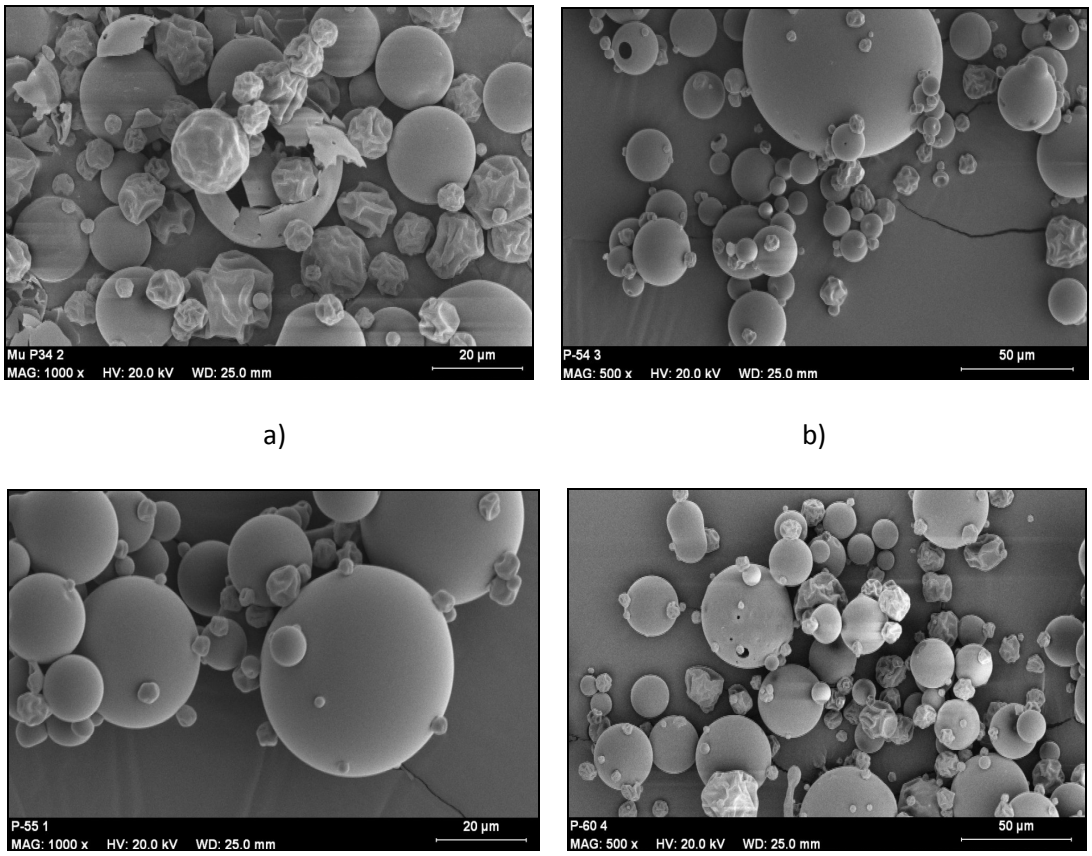
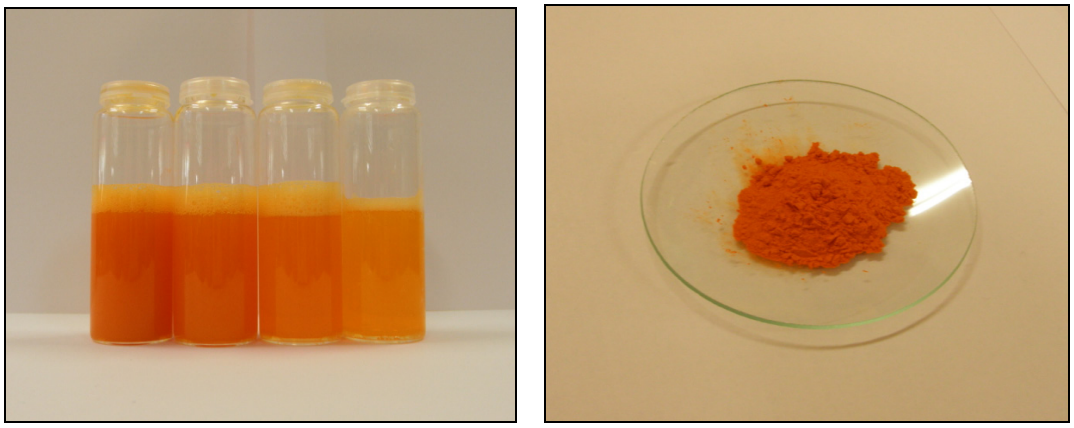


Figure 13. SEM micrographs of particles obtained after spray-drying, a) Experiment E24 (β-carotene concentration: 60 g L^{-1}, OSA-starch concentration: 367 g L^{-1}, organic-water flow ratio: 0.8, b) Experiment E44 (β-carotene concentration: 50 g L^{-1}, OSA-starch concentration: 367 g L^{-1}, organic-water flow ratio: 0.6, c) Experiment E45 (β-carotene concentration: 50 g L^{-1}, OSA-starch concentration: 367 g L^{-1}, organic-water flow ratio: 0.65, d) Experiment E50 (β-carotene concentration: 50 g L^{-1}, OSA-starch concentration: 367 g L^{-1}, organic-water flow ratio: 0.65).

Regarding the UV/VIS spectra, it can be seen in Table 2 that three peaks were observed in all samples which represent a typical orange colour (the wavelength of these peaks belongs to the range of yellow and red colour). According to Horn and Rieger (2001), the colour of the suspension can be varied from orange to red by modification of the crystalline structure of the particles. However, suspensions with red colour could not be obtained in this work.

In Figure 14, pictures of encapsulated β-carotene dispersions in water and powder collected after spray drying process are shown, using four different concentrations: a total concentration (β-carotene + OSA-starch) of 0.01, 0.005, 0.0025 and 0.00125 g·mL^{-1}, with corresponding β-carotene concentrations of 298, 149, 74.5 and 37.5 ppm, respectively.



a) b)

Figure 14. a) Encapsulated β-carotene dispersions in water with β-carotene concentrations of 298, 149, 47.5 and 37.5 ppm (left to right) b) Powder collected after spray drying process.

4. CONCLUSIONS

The formulation of β-carotene with OSA-starch using pressurized ethyl acetate emulsions was investigated in this work. The results showed that it is possible to obtain a formulation of β-carotene with a micellar particle size in the range of 300-600 nm and with a high percentage of encapsulated β-carotene (over 70%). It was demonstrated that high concentrations of modified starch (over 100 g L^{-1}) were required to obtain a high percentage of encapsulated β-carotene and high emulsion stability. With regard to the organic-water ratio (mL min^{-1} of organic solvent plus mL min^{-1} of β-carotene suspension divided by mL min^{-1} of modified-starch dissolution), it was shown that the best results were obtained with low ratios (in the range between 0.65 and 0.73). Using high organic-water ratios (over 0.85), a drastic increase in the micellar size was observed. A significant isomerisation from trans to cis β-carotene, ranging from 15% to 50%, was observed as a consequence of the exposition of the product to high temperatures. With regard to the UV/Vis spectra, in almost all experiments three peaks were observed which represent a typical orange color in samples. ORAC and HORAC assays show that the formulations retain high antioxidant activities.

Acknowledgements

Authors thank Diputación de Valladolid and Junta de Castilla y León (project GR11) and the financial support from Conselho de Reitores das Universidades Portuguesas and the Spanish Ministerio de Ciencia e Innovación (PT2009-0173) through the Integrated Actions Portugal–Spain, as well as National Starch Group (Hamburg, Germany) for providing materials used in

this work. E. de Paz performed this work with a FPI-UVa research fellowship. Á Martín thanks the Spanish Ministry of Science and Innovation for a *Ramón y Cajal* research fellowship.

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Chapter 2

Water-soluble formulation of β-carotene in OSA-starch micelles by high-pressure, high temperature antisolvent precipitation from pressurized ethanol solutions

Water-soluble formulation of β-carotene in OSA-starch micelles by high-pressure, high temperature antisolvent precipitation from pressurized ethanol solutions

Abstract

β-carotene is one of the most common pigments in nature. β-carotene formulations can provide protection of the active compound against oxidation and degradation processes, and can facilitate the dispersion and stabilization of this hydrophobic compound in aqueous media. Such formulations are very attractive as natural colorants because they add value to the product due to their antioxidant and pro-vitamin activities. This work presents a study of the formulation of β-carotene by precipitation from a pressurized ethanol-in-water solution using modified OSA-starch refined from waxy maize as carrier material. This study allowed to obtain formulations of β-carotene with encapsulation efficiencies up to 60% and with micellar particle sizes down to 120 nm. Particle size drastically increased when the concentration of β-carotene in the initial suspension was increased, reaching values above 10 μm with concentrations of 10 g/L. Particle size was also increased when the organic-water ratio was increased. In successful experiments, the particle size of aqueous β-carotene suspensions was well preserved after 4 months of storage. A comparison between the results using ethyl acetate instead of ethanol as organic solvent is also presented. Experiments with ethanol resulted on a lower encapsulation efficiency than experiments with ethyl acetate, and in order to achieve equivalent particle sizes, the concentrations of β-carotene in experiments with ethanol had to be reduced more than 10-fold with respect to experiments with ethyl acetate, thus decreasing the throughput of the process and increasing the amount of organic solvent needed.

Keywords: β-carotene, colorant, emulsion, OSA-starch, suspension.

1. INTRODUCTION

Carotenoids are some of the most common pigments in nature, being the most abundant β-carotene, lycopene, lutein and zeaxanthin. The main roles of carotenoids in human diet are as precursors of vitamin A and as antioxidants [1]. Among the carotenoids, β-carotene has the highest pro-vitamin A activity, and therefore it is a strong candidate for incorporation into functional foods [2]. Researchers have shown that carotenoids can be beneficial for human health disorders such as cardiovascular disease, macular degeneration or cataracts [3]. Since carotenoids are authorized food ingredients, carotenoids are widely used in the food, cosmetic and pharmaceutical industries as natural colorants [1]. However, carotenoids are highly prone to chemical degradation during food processing and storage, due to the effects of chemical, mechanical and thermal stresses [4-5]. On the other hand, carotenoids are insoluble in water due to their high hydrophobicity. This makes it difficult to disperse them in water, as required, for example, for application as natural colorant in beverages. In order to avoid the degradation of carotenoids and also to improve their dispersibility in water, coloring strength potential and to increase their bioavailability during gastro-intestinal passage, an adequate formulation of carotenoids is required [6-7-8]. It is thus necessary to develop effective delivery systems to improve the bioavailability and stability of carotenoids in foods, and, particularly, in beverages [9, 3].

Recently, there has been great interest in utilizing nanoemulsions to encapsulate bioactive components for applications in food and beverage products [10]. Oil-in-water nanoemulsions consist of small lipid droplets ($r < 100$ nm) dispersed within an aqueous continuous phase. Nanoemulsions are thermodynamically unstable systems that tend to breakdown over the time [5]. Nanoemulsions containing β-carotene have been studied by several authors. Qian et al. (2012) [5] showed that β-carotene can be effectively encapsulated within food-grade nanoemulsions stabilized by globular proteins or non-ionic surfactants using a high pressure microfluidiser. The same authors [11] carried out a similar study considering different kinds of carrier lipids coated by non-ionic surfactants. Mao et al. (2009-2010) [4, 9] investigated the characteristics of β-carotene nanoemulsions prepared by high pressure homogenization using two large molecule emulsifiers (octenyl succinate starch and whey protein isolate) and two small molecule emulsifiers (tween 20 and decaglycerol monolaurate) obtaining a fine size distribution. The same technique was used by Yuan et al. (2008) [12] to study the production of oil-in-water nanoemulsions of β-carotene. Silva et al. (2010) [3] presented the use of high-energy emulsification-evaporation technique to produce oil-in-water nanoemulsions of β-carotene. On the other hand, Tan and Nakajima (2005) [13] investigated the preparation of β-carotene nanodispersions by a process based on an emulsification-evaporation technique.

Ribeiro et al. (2008) [14] studied the production of β-carotene-loaded nanodispersions containing poly(D,L-lactic acid) and poly(D,L-lactic coglycolic acid) by a solvent displacement method. The same technique was used by Yin et al. (2009) [15] to study the characteristics of β-carotene nanodispersions prepared with different emulsifiers.

In a previous study [7], authors developed a method for the encapsulation of β-carotene in n-octenyl succinate (OSA) starch refined from waxy maize, based on the production and processing of ethyl acetate-on-water emulsions at high temperature and pressure. This study showed that, by intensification of the dissolution, emulsification and precipitation processes, stable suspensions with micellar particle sizes down to 400 nm and encapsulation efficiencies up to 80% could be obtained with high throughput and negligible β-carotene degradation. This study continues with the development of the process, analyzing the possibilities for modifying the process in order to use ethanol as organic phase instead of ethyl acetate. Compared with ethyl acetate, ethanol has the advantage of a lower toxicity and a wide applicability in the food industry. However, since ethanol is completely miscible with water, the mechanism of particle formation is changed, since the possibilities for controlling particle size through the emulsification process are lost. In order to evaluate the importance of this variation in mechanisms, in this work an analysis of the influence of process conditions on particle size and encapsulation efficiency using ethanol as organic solvent is presented and compared with the results obtained using ethyl acetate-on-water pressurized emulsions.

2. MATERIALS AND METHODS

2.1. Materials

Crystalline β-carotene with a minimum purity of 99%, manufactured using a fermentation process, was handed over by DSM (León, Spain). Ethanol with a purity of 96% was purchased from Panreac Química (Barcelona, Spain). Modified OSA-starch refined from waxy maize was kindly provided by National Starch Group (Hamburg, Germany).

2.2. Equipment

Fig. 1 presents the schematic flow diagram of the experimental apparatus which was used by the same authors in a previous work [7]. A detailed description of the equipment can be found in the previous work.

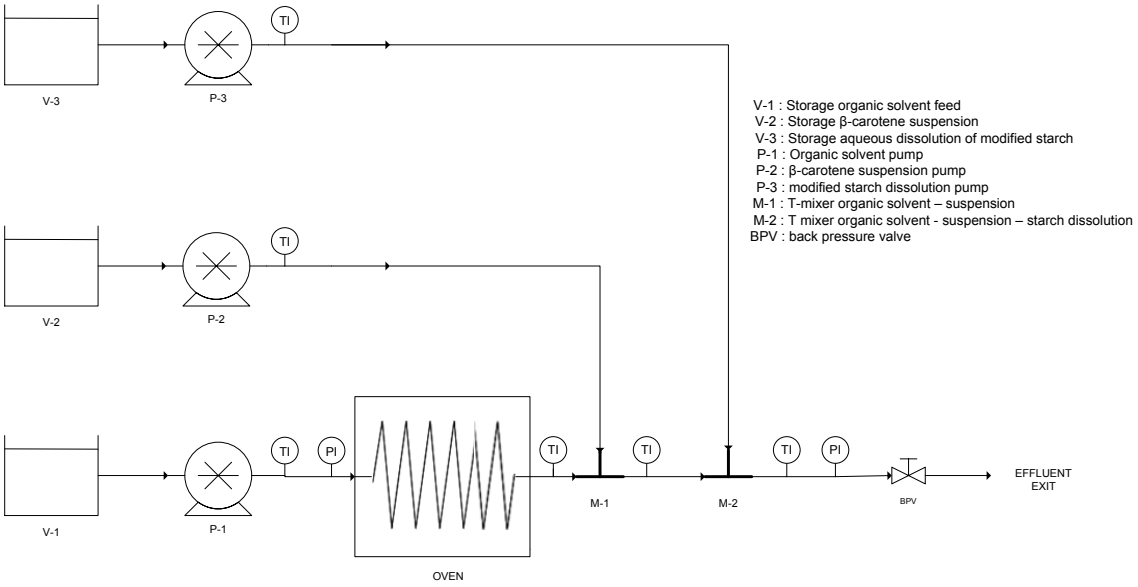

Figure 1. Schematic flow diagram of the experimental apparatus employed for β-carotene formulation.

The process consists of three feeds at ambient temperature: pure organic solvent (ethanol in this case), β-carotene suspension in the same organic solvent, and an aqueous solution of the modified OSA-starch. The stream of pure ethanol is preheated in a chromatographic oven in order to reach the desired operation temperature after mixing with β-carotene suspension, which typically is 145ºC. In order to maintain all streams in the liquid state, they are pressurized above 5 MPa. The suspension of β-carotene is pumped and then mixed with the hot ethanol stream using a T-mixer (M1). This allows a total dissolution of β-carotene because the solubility of β-carotene increases when temperature is increased [16], thus allowing to increase process throughput as well. Shortly afterwards this hot, pressurized solution is mixed with the ambient-temperature aqueous solution of modified OSA-starch using the second T-mixer of the equipment (M2). Due to this, the contact time between β-carotene and hot solvent is reduced to less than 2 seconds, and the isomerization and degradation of the product is avoided as well. Moreover, the mixing in M2 causes the precipitation of β-carotene dissolved in ethanol by a combined antisolvent and cooling effect. After that, the effluent stream is depressurized and the suspension of β-carotene particles is collected. Finally, ethanol is removed from the solution using a rotary evaporator, producing a suspension of β-carotene particles in water stabilized with the modified OSA-starch.

Table 1 shows a summary of experimental conditions considered in this work. The concentration of β-carotene in the suspension ranged from 1 g/L to 10 g/L, and the concentration of OSA-starch in the solution was correspondingly varied from 7.3 g/L to 73 g/L.

With this, a constant OSA-starch / β-carotene ratio of 7.3 g/g was maintained, which was determined as the optimum value for the stability of the suspension in the previous work using ethyl acetate as organic solvent [7]. It must be noted that the concentrations of β-carotene and OSA starch considered in this work were considerably lower than the concentrations used in the previous study with ethyl acetate (which were up to 50 g/L of β-carotene and 367 g/L of OSA-starch [7]). This is due to the lower solubility of β-carotene in ethanol compared to the solubility in ethyl acetate. Indeed, at 20ºC and ambient pressure, the solubility of β-carotene in ethyl acetate is 0.627 mg/mL and in ethanol is 0.360 mg/mL [16]. The flow rates of ethanol and β-carotene suspension were varied from 5 to 10 mL/min and the flow of surfactant dissolution was varied from 17.9 to 25 mL/min.

Table 1. Summary of operating conditions.

	$C_{\beta\text{-carotene}}$	C_{starch}	$F_{ethanol}$	$F_{\beta\text{-carotene}}$	F_{starch}	$r_{organic\text{-}water}$	$T_{operation}$	$P_{operation}$
	(g L^{-1})	(g L^{-1})	(mL min^{-1})	(mL min^{-1})	(mL min^{-1})		(ºC)	(MPa)
E1	10	73.3	9	6	20	0.75	135 - 143	5 - 5.2
E2	10	73.4	9	6	25	0.6	137 - 144	5 - 5.2
E3	10	73.4	8	6	16.5	0.85	135 - 142	5 - 5.2
E4	5	36.7	9	6	20	0.75	145 - 155	5 - 5.5
E5	5	36.7	9	6	25	0.6	145 - 153	5 - 5.5
E6	5	36.7	8	6	16.5	0.85	145 - 160	5 - 5.5
E7	2.5	18.3	9	6	20	0.75	142 - 152	5 - 5.5
E8	2.5	18.3	9	6	25	0.6	135 - 149	5 - 5.5
E9	2.5	18.3	8	6	16.5	0.85	147 - 160	5 - 5.5
E10	1.5	11	9	6	20	0.75	148 - 162	5 - 5.5
E11	1.5	11	9	6	25	0.6	152 - 164	5 - 5.5
E12	1	7.3	9	6	20	0.75	140 - 155	5 - 5.5
E13	1	7.3	9	6	25	0.6	140 - 151	5 - 5.5
E14	1	7.3	8	6	16.5	0.85	138 - 150	5 - 5.5

2.3 Product characterization

2.3.1. Weight of dry product

The sample was dried and its dry weight was measured by a Halogen Moisture analyzer model Ohaus MB35 set at 160ºC to remove the water. This measurement includes the weight of crystalline β-carotene (which is the non-encapsulated β-carotene), encapsulated β-carotene and modified starch (carrier).

2.3.2. Percentage of encapsulated β-carotene

The sample was analysed by a UV/Vis spectrophotometer model Agilent 8453. The wavelength was set at 456 nm. As it was explained by the same authors in the previous work [7], the absorbance determined with this method is proportional to the amount of β-carotene dispersed in solution which corresponds to the encapsulated β-carotene. The percentage of encapsulated β-carotene is reported as the ratio between the concentration of β-carotene dispersed in solution and total β-carotene concentration in the product.

2.3.3. Particle size

The particle size analysis was carried out by laser diffraction (model Beckman Coulter LS 13 320).

2.3.4. Trans/cis β-carotene HPLC profile

Crystalline β-carotene used as feed material contained 95%wt of trans β-carotene and 5%wt of cis β-carotene. However, some isomerization from trans to cis β-carotene took place during the high temperature steps of the process. The degree of isomerization was determined using a Waters Alliance 2695 HPLC chromatograph with PDA detector 2996 equipped with a Vydac 218TP54 5 μm (4.6 × 250) column. The mobile phase was methanol + 1% of tetrahydrofuran stabilized with 50 ppm of ascorbic acid. The temperature of the column was 30ºC. The solvent for the samples was ethanol/tetrahydrofuran stabilized with 50 ppm of ascorbic acid.

2.3.5. Colour

The colour of the obtained aqueous dispersions of β-carotene and modified starch was characterized by UV/Vis spectrophotometer model Agilent 8453 operated in a wide range of wavelengths.

3. RESULTS AND DISCUSSION

Table 2 presents a summary of the main experimental results obtained. These results include the concentration of β-carotene in the product, the efficiency of encapsulation of β-carotene, isomer composition, the main characteristics of the colour contours and the micellar particle size.

Table 2. Summary of experimental results.

	% Dry product	% β-Carotene in sample	% Encapsulated β-Carotene	% Trans/cis β-Carotene	UV/Vis spectrum peaks (nm)	Particle size (d_{50}) (μm)
E1	6.34	3.5	41.1	94.10 / 5.90	276 / 493 / 533	13.57
E2	6.96	2.41	57.2	92.82 / 7.18	281 / 486 / 525	12.85
E3	5.85	3.26	43.6	89.29 / 10.71	276 / 559 / 584	15.95
E4	3.11	4.4	32.3	84.96 / 15.04	491 / 532.5	0.516
E5	4.66	3.81	37.3	84.24 / 15.76	456 / 482 / 522.5	0.142
E6	3.76	5.88	24.0	64.14 / 35.86	490.5	0.144
E7	2.51	5.13	34.9	75.17 / 24.83	471.5	0.127
E8	1.79	3.69	31.7	89.31 / 10.69	276 / 424 / 445	0.130
E9	-	6.04	21.1	-	-	0.155
E10	1.6	6.26	38.0	75.76 / 24.24	276 / 344	0.133
E11	0.91	3.94	27.6	83.12 / 16.88	276 / 447 / 474	0.121
E12	0.59	5.83	37.0	58.86 / 41.14	280 / 381 / 410	0.129
E13	1.08	6.26	38.2	81.00 / 19.00	282 / 445 / 513	0.123
E14	1.03	6.04	30.5	59.53 / 40.47	281 / 412 / 436	0.144

3.1. Influence of β-carotene concentration

Figure 2 presents the variation of the micellar particle size and the encapsulation efficiency with the concentration of β-carotene in the suspension at different organic / water ratios.

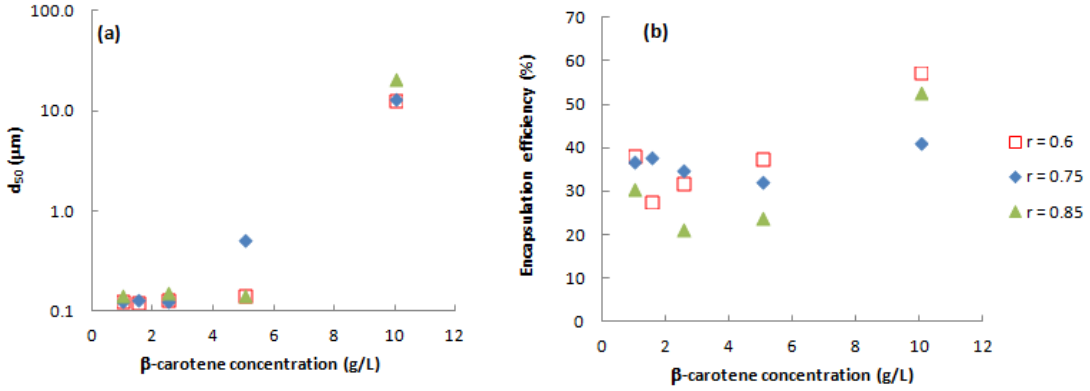

Figure 2. Variation of micellar particle size (a) and encapsulation efficiency (b) with the concentration of β-carotene in the initial suspension.

As shown in Figure 2 (a), the concentration of β-carotene had a strong influence on particle size. Bigger particle sizes were obtained when the concentration of β-carotene was increased. In particular, particle sizes above 10 μm were obtained in all experiments carried out with an initial β-carotene concentration of 10 g/L, which are excessive for the stabilization of particles

for application as natural colorant. This result indicates that this concentration might be too high in order to obtain the total dissolution of β-carotene in the first T-mixer (M-1). Thus a homogeneous suspension and a complete recrystallization of β-carotene in the second T-mixer (M-2) cannot be achieved. On the contrary, when the concentration of β-carotene was reduced below 5 g/L, in general particle sizes in the sub-micrometer to nanometer range were obtained (120 nm to 500 nm).

Regarding the encapsulation efficiency, nearly constant values were observed with all β-carotene concentrations tested, with a small increase in the efficiency observed in experiments carried out with 10 g/L of β-carotene with respect to the experiments with lower concentrations. As presented in Figure 2 (b), encapsulation efficiencies in the range 30% - 40% were obtained in most cases.

3.2. Influence of the organic - water ratio

Figure 3 presents the variation of the micellar particle size and the encapsulation efficiency with the organic - water ratio.

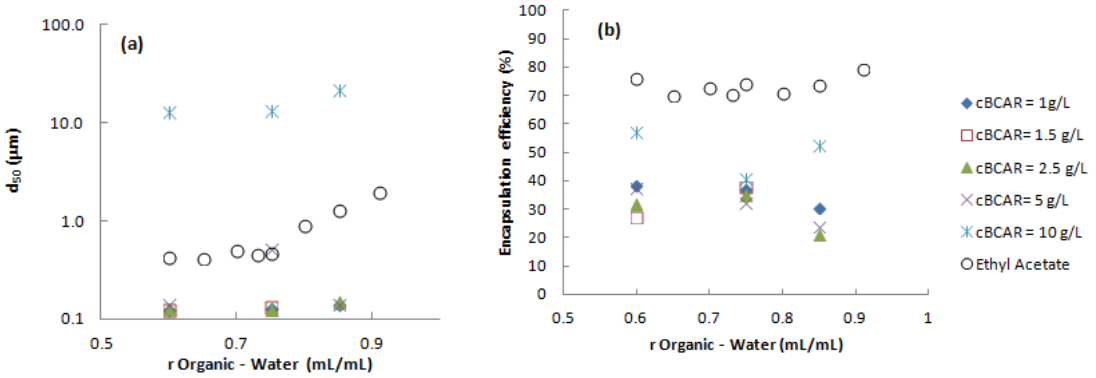

Figure 3. Variation of micellar particle size (a) and encapsulation efficiency (b) with the organic-water ratio. Results obtained in a previous work using ethyl acetate instead of ethanol as organic solvent are also presented [7].

As shown in Figure 3, the organic-water ratio has a strong influence on particle size. In particular, an increase of micellar particle size was observed when the organic-water ratio was increased to 0.85 mL/mL. The encapsulation efficiency did not show a clear trend of variation with the organic-water ratios, with most experiments showing efficiencies in the range 30% - 40% as previously discussed.

3.3 Comparison with the results of precipitation from a pressurized ethyl acetate-on-water emulsion

Figure 3 also presents the results obtained in a previous study using ethyl acetate as organic solvent [7]. As shown in this figure, the trends of variation of particle size and encapsulation efficiency with the organic - water ratio obtained with both solvents are equivalent: particle size increases at higher organic-water ratios, while approximately constant encapsulation efficiencies are obtained. However, considerable differences between the values of particle size and efficiency obtained with the two solvents can be observed.

In particular, the encapsulation efficiencies obtained using ethanol as organic solvent (30% - 40%) are clearly lower than those achieved using ethyl acetate (70%-80%). This can be a consequence of the different miscibilities of these solvents with water. Ethanol is completely miscible with water, while water and ethyl acetate show a relatively low miscibility (8.7 % wt ethyl acetate/wt water at 20ºC). The immiscibility of ethyl acetate and water enables to form an emulsion upon mixing of the organic and aqueous solutions in the second T-mixer M2. This emulsion, consisting in β-carotene dissolved in the droplets of dispersed organic phase, which are surrounded by OSA-starch surfactant, acts as a template for the final product, consisting of β-carotene particles encapsulated in micelles of OSA-starch surfactant. The lack of this emulsion template in experiments carried out with ethanol may be the reason for the lower encapsulation efficiency obtained using this solvent.

Regarding the particle size, as shown in Figure 3, approximately equivalent values of 100 nm - 500 nm were obtained in successful experiments using either ethyl acetate or ethanol. However, due to the lower solubility of β-carotene in ethanol compared to ethyl acetate, the maximum β-carotene concentrations that could be processed using ethanol were at least 10 times lower than concentrations in experiments with ethyl acetate. This implies a reduction of the throughput of the process, associated with the production of a more diluted product stream, as well as an increase of the amount of organic solvents needed.

3.4. Evaluation of the stability of aqueous β-carotene suspensions

In order to evaluate the stability of the dispersions, the micellar particle size of aqueous solutions was measured after four months of storage. During this period, the solutions were stored in a cold camera at temperatures below 6ºC and protected from light. Results are reported in Table 3. As presented in this table, particle size was well preserved in most samples after the storage period of four months. This result indicates that the dispersions presented a good stability during this period. The main exception are samples obtained with

the lowest β-carotene concentration of 1 g/L, and experiments with the highest organic-water ratio of 0.85 mL/mL, which showed a drastic increase of particle size at the end of the storage period.

Table 3. Evolution of micellar particle size of aqueous dispersions after four months of storage

	Initial particle size (d_{50}) (μm)	Particle size after 4 months (d_{50}) (μm)
E4	0.516	0.43
E5	0.142	0.21
E6	0.144	17.11
E7	0.127	0.25
E8	0.13	0.23
E9	0.155	14.76
E10	0.133	0.49
E11	0.121	0.30
E12	0.129	45.57
E13	0.123	0.23
E14	0.144	74.89

Moreover, Figure 4 presents the particle size distributions obtained in experiments E5 and E7 directly after the preparation of the suspension and after four months of storage. As presented in Table 3 and Figure 4, the mean particle sizes only showed a slight increase after the storage period. Indeed, the micellar particle size (d_{50}) corresponding to this peak just after the preparation of the samples was similar in both cases, concretely 0.142 and 0.127 μm for experiments E5 and E7, respectively. After samples were stored during four months in dark and in a cold camera, micellar particle sizes increased until 0.207 and 0.248 μm, respectively. Analyzing the shape of the size distributions, shown in Figure 4, the initial distributions are bimodal, with a main peak in the nanometer - sub-micrometer range, corresponding to β-carotene particles that were successfully prepared and encapsulated, and another peak at sizes near 1 μm corresponding to larger particles that were not successfully encapsulated. As presented in Figure 4, the main peak of the size distribution shows little variation after the storage period, with a modest increase in the mean size of this fraction of particles. However, after storage the peaks above 1 μm are displaced to considerably higher particle sizes, indicating that these bigger particles are becoming more agglomerated and destabilized out of the suspension due to their bigger size.

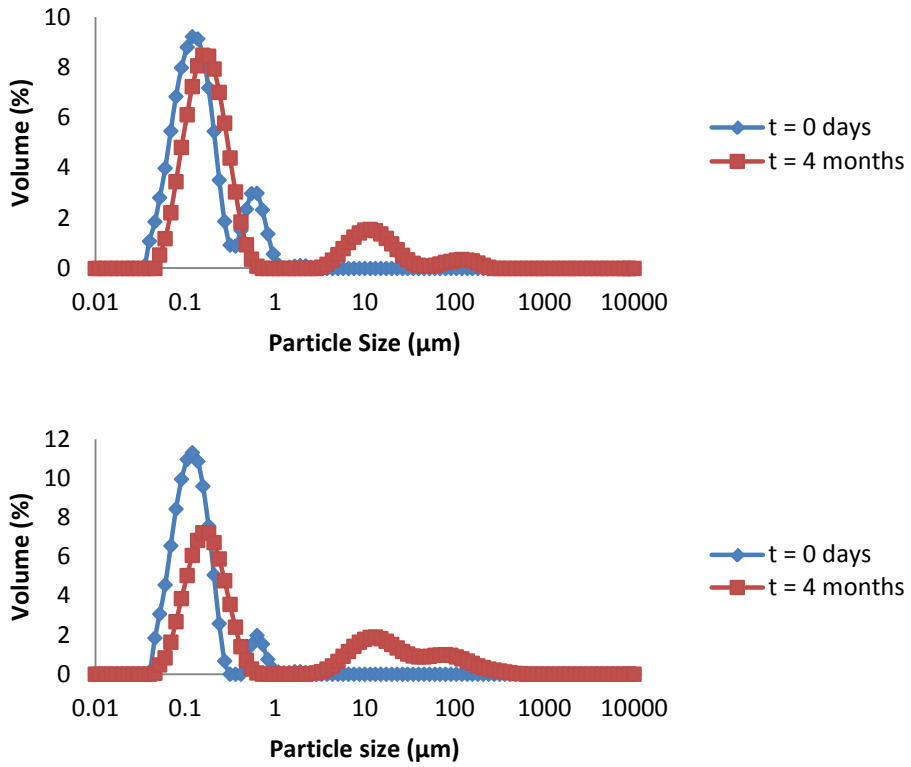

Figure 4. Particle size distributions of experiments E5 (above) and E7 (below) measured directly after preparation of the suspension and after four months of storage

3.5. Colour

Table 2 presents the main peaks observed in the UV/Vis spectra of the samples. In general, samples with particle sizes above 1 μm presented a very low colour intensity and did not present the typical spectrum with three well defined peaks near 290 nm, 450 nm and 510 nm, indicating that these samples were not suitable for application as natural colorant. Samples with lower particle size presented a UV/Vis spectra corresponding with a characteristic orange color of the suspensions. Figure 5 presents a photograph of aqueous dispersions of the formulations with different β-carotene concentrations. As shown in this figure, homogeneous dispersions ranging from very turbid to more transparent products could be obtained modifying the concentration of β-carotene added to the solution.

Figure 5. Encapsulated β-carotene dispersions in water with β-carotene concentrations of 64.1, 12.8, 6.4 and 1.3 ppm (left to right).

3.6. *Trans/cis β-carotene HPLC profile*

As previously explained, the crystalline β-carotene used as feed material contains 95%wt of trans β-carotene and 5%wt of cis β-carotene. However, due to the high-temperature steps of the process (T mixer M-1, at 145ºC), some isomerization took place. In table 2, it can be observed that in general, the degree of isomerization ranged from 6 to 25%, with the exception of three experiments which presented a percentage between 36% and 41% as consequence of achieving a higher temperature (over 150ºC) in T mixer M1.

4. CONCLUSIONS

The formulation of β-carotene with OSA-starch using pressurized ethanol-on-water solutions was studied in this work. From the results obtained in this work, it is possible to obtain a formulation of β-carotene with maximum encapsulation efficiency of 30% - 40% and micellar particle sizes in the range of 120 - 550 nm, using β-carotene concentrations between 1 to 5 g/L. These particle sizes were well preserved after four months of storage, although in this period a selective agglomeration and destabilization of the fraction of non-encapsulated particles with larger initial sizes was observed. Experiments with a higher β-carotene concentration of 10 g/L led to an unsuccessful precipitation and encapsulation, producing particle sizes above 10 μm. The influence of the organic-water ratio in the encapsulation efficiency and in the micellar particle size was also studied. Results showed an increase in the micellar particle size when the organic-water ratio was increased as well. The same effect was observed in a previous work by the same authors using ethyl acetate as organic solvent [7].

However, comparing the results obtained in this work using ethanol as organic solvent with previous results using ethyl acetate, lower encapsulation efficiencies were obtained in experiments with ethanol, resulting in poorer product properties. This may be due to the lack of an emulsion template during the precipitation and encapsulation of particles, that can be formed using water-immiscible ethyl acetate, but not with ethanol. Moreover, due to the lower solubility of β-carotene in ethanol compared to ethyl acetate, β-carotene concentrations in experiments with ethanol had to be reduced more than 10-fold with respect to the concentrations used with ethyl acetate in order to achieve equivalent particle sizes.

Acknowledgements

Authors thank DSM-León (Spain) for providing materials used in this work. E. de Paz performed this work with an FPI-UVa research fellowship. Á. Martín thanks the Spanish Ministry of Economy and Competitiveness for Ramón y Cajal research fellowship. This research work was partially funded through project ENE2011-24547 of the Spanish Ministry of Economy and Competitiveness.

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Chapter 3

Formulation of β-carotene by precipitation from pressurized ethyl acetate-on-water emulsions for application as natural colorant: Effect of different modified OSA-starches

Formulation of β-carotene by precipitation from pressurized ethyl acetate-on-water emulsions for application as natural colorant: Effect of different modified OSA-starches

Abstract

β-carotene is one of the most common pigments in nature. The application of β-carotene as natural colorant in food and nutraceutical products requires an appropriate formulation in order to protect the active compound from degradation and overcome the low bioavailability due to a low solubilility in aqueous media. This work presents a study of the formulation of β-carotene using four different modified n-octenyl succinate (OSA) starches as carrier materials by precipitation from a pressurized ethyl acetate-in-water emulsion. Results showed that an OSA-starch derived from waxy maize blend with dried glucose syrup (OSA-1) was not suitable for encapsulating β-carotene due to the low encapsulating efficiencies achieved (below 30%) and high micellar particle sizes. With OSA-2 and OSA-3 (OSA-dextrin derived from waxy maize and OSA- dextrin derived from tapioca, respectively) formulations of β-carotene with particle sizes in the range of 350-760 nm and encapsulation efficiencies between 30-45 % were obtained. However, experiments with OSA-4 (OSA-starch refined from waxy maize) presented better results, achieving maximum encapsulation efficiencies of 70-80% with particle sizes in the sub-micrometer range.

Keywords: β-carotene, colorant, OSA-starch, suspension, emulsion.

1.INTRODUCTION

Carotenoids are some of the most common pigments in nature, being the most abundant β-carotene, lycopene, lutein and zeaxanthin. Carotenoids can be found in vegetables, fruits, leaves, fish and other sea products [1]. Since carotenoids are authorized food ingredients, they are widely used in the food, cosmetic and pharmaceutical industries as natural colorants. When they are consumed in sufficient levels, they have been claimed to have biological activities that may reduce the risk of certain chronic diseases, such as cardiovascular disease, age-related macular degeneration and cataracts [2]. In particular β-carotene has a high pro-vitamin A activity and a strong antioxidant activity, and therefore it is a strong candidate for incorporation into functional foods [3]. On the other hand, carotenoids are prone to degradation process in presence of oxygen, temperature or light. Moreover, they are insoluble in water which reduces their bioavailability and makes it difficult to produce stable dispersions in water-based beverages. In order to avoid the degradation of carotenoids, to improve their dispersability in water and to increase their bioavailability during gastro-intestinal passage, a suitable formulation of carotenoids is required [4, 5, 6].

There are several methods of precipitation and condensation for the production of organic nanoparticles in aqueous media [4, 7]. Tan and Nakajima [8, 9] investigated the preparation of β-carotene nanodispersions by a process based on an emulsification-evaporation technique. The same technique was used by Chu et al. [10] for the preparation of protein-stabilized β-carotene nanodispersions. Silva et al. [11] prepared nanoemulsions of β-carotene by high energy emulsification-evaporation technique obtaining dispersions at a nanoscale size range. Ribeiro et al. [5] studied the production of β-carotene-loaded nanodispersions containing poly(D,L-lactic acid) and poly(D,L-lactic coglycolic acid) by solvent displacement method. Yin et al. [12] used the same technique for studying the characteristics of β-carotene nanodispersions prepared with different emulsifiers.

On the other hand, nanoemulsions have a great interest to encapsulate bioactive components for application in food and beverage products [13]. Several authors have studied nanoemulsions containing β-carotene. Qian et al. [14, 15] stabilized nanoemulsions with globular proteins and with different kind of carrier lipids coated by non-ionic surfactants using a high pressure microfluidizer. Mao et al. [16, 17] investigated the characteristics of β-carotene nanoemulsions prepared by high pressure homogenization using two large molecule emulsifiers (octenyl succinate starch and whey protein isolate) and two small molecule emulsifiers (tween 20 and decaglycerol monolaurate) obtaining a fine size distribution. This technique was also used by Yuan et al. [18] to study the characterization of β-carotene oil-in-

water nanoemulsions using series of polyoxythylene sorbitan esters of fatty acid as emulsifiers. Neves et al. [19] studied the formation of emulsions of a β-carotene-rich palm oil. And recently, Liang et al. [20] used the same technique for developing oil-in-water nanoemulsions of β-carotene stabilized by food-grade modified starches.

Modified starches are food-grade biopolymeric emulsifiers which provide high stability against oil droplet coalescence caused by the change of pH, ionic strength and temperature [21]. Due to their branched structures, modified starches are prone to stabilize emulsions mainly through steric repulsion [22]. Because of this, modified starches are widely used for preparing emulsions in foods and beverages [23]. Numerous reports have indicated that n-octenyl succinic anhydride (OSA)-modified starches are suitable for the encapsulation of flavours, clouds, vitamins and spices [24, 25, 26, 27, 28].

In this work, a study of the formulation of β-carotene using four different modified n-octenyl succinate (OSA) starches as carrier materials is presented. Formulations have been developed by a novel process which consists in the formation of a pressurized ethyl acetate-in-water emulsion. This process, based on an intensified emulsification and particle formation through turbulent mixing of streams, allows reducing the time scale of the particle formation processes to less than 1 second, thus promoting product homogeneity and increasing throughput. The influence of the concentration of the four different OSA-starches and the effect of the organic-water ratio on the emulsification and particle formation were investigated.

2. MATERIALS AND METHODS

2.1. Materials

Crystalline β-carotene with a minimum purity of 99% was manufactured by DSM-León (Spain) using a fermentation process. Ethyl acetate with a purity of 99.5% was purchased from Panreac Química (Barcelona, Spain). The following n-octenyl succinic anhydre (OSA)-modified starches were used as emulsifier agents: OSA-starch derived from waxy maize blend with dried glucose syrup (OSA-1), OSA-Dextrin derived from waxy maize (OSA-2), OSA- Dextrin derived from tapioca (OSA-3) and OSA-starch derived from waxy maize (OSA-4). (OSA)-modified starches were kindly provided by National Starch Group (Hamburg, Germany).

2.2. Equipment

Fig. 1 presents the schematic flow diagram of the experimental apparatus which was used by the same authors in a previous work [6]. A detailed description of the equipment used can be found in the previous work.

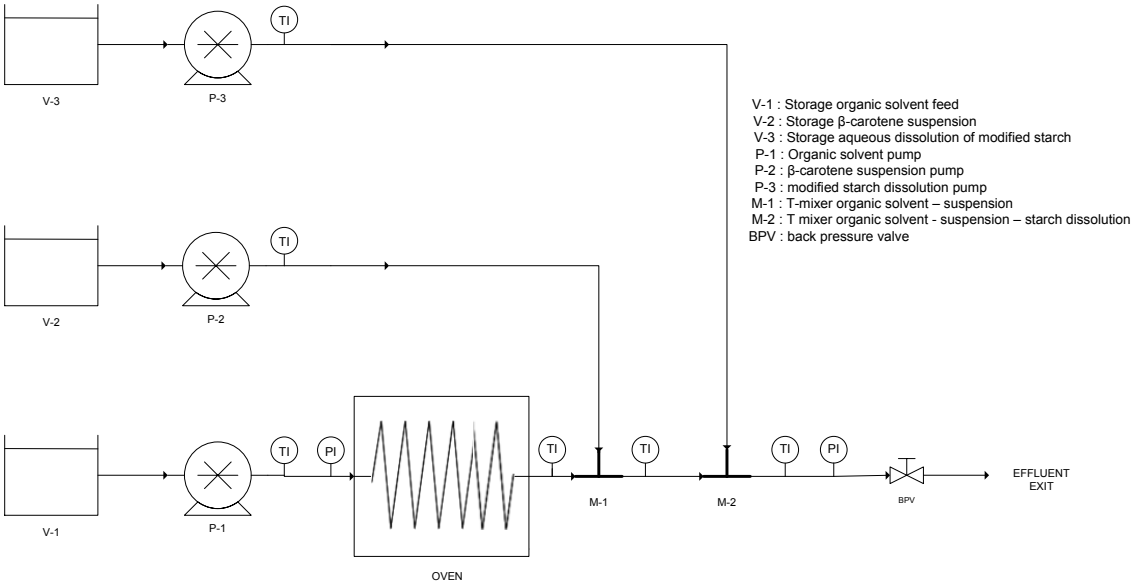

Fig.1. Schematic flow diagram of the experimental apparatus employed for β-carotene formulation.

2.3. Fundamentals of the process

The technique of β-carotene precipitation from a pressurized emulsion consists in a first step of total dissolution of β-carotene in hot and pressurized organic solvent (in this case, ethyl acetate). By increasing temperature, the solubility of β-carotene in ethyl acetate is increased (i.e., the solubility of β-carotene in ethyl acetate at 10ºC is 0.680 mg/mL which is increased to 15.18 mg/mL when temperature is increased to 60ºC [29]). This enables increasing the production capacity of the process, as well as reducing the amount of organic solvents needed. In this work, a temperature of 145ºC was used [6], keeping constant pressure between 6.0-6.5 MPa in order to maintain ethyl acetate in liquid state. In order to reduce the exposure of β-carotene to high temperatures, the dissolution is achieved by mixing a suspension of β-carotene particles in pressurized ethyl acetate at ambient temperature, with a stream of preheated and pressurized organic solvent, using a T-mixer (M-1 in Figure 1).

The second step of this process is the mixing (in T-mixer M-2) of this hot dissolution of β-carotene in ethyl acetate with an aqueous solution of OSA-starch surfactant at ambient temperature. This causes the formation of an ethyl acetate-on-water emulsion, as well as the precipitation of β-carotene due to the drastic reduction of β-carotene solubility in ethyl acetate because temperature is reduced considerably and due to the water antisolvent effect. During this process, the emulsion acts as a template for the particle formation process, enabling a precise control over particle properties.

The last step is the removal of the organic solvent from the formed emulsion by vacuum evaporation, in order to obtain an aqueous dispersion of β-carotene particles stabilized by the OSA-starch surfactant.

2.4. Experimental procedure

A typical experiment started with the preparation of the dissolution of modified starch and the β-carotene suspension in ethyl acetate. After that, pumps were switched on and the pressure in the system was fixed (6.0-6.5 MPa). The oven was switched on to heat the organic solvent until the desired temperature (140-150ºC). When the required temperature was reached in mixer M-1, the pure organic solvents used until this moment were replaced with the β-carotene suspension (in continuous agitation because a homogeneous suspension is needed) and with the dissolution of the modified starch. In all experiments, a volume of 50 mL of suspension containing 2.5 g of β-carotene was processed. Afterwards, the organic solvent of the obtained emulsion was removed from the sample using a rotary evaporator, thus obtaining an aqueous sample free from organic solvent.

Table 1 shows a summary of the experimental conditions considered in experiments reported in this work. According to results obtained in a previous study [6], a constant concentration of β-carotene suspension of 50 g L^{-1} was selected in all experiments. In a first series of experiments (experiments E1-E32), the concentration of the dissolution of modified starch was varied from 37 g L^{-1} to 367 g L^{-1}, using four different OSA-starches as described in section 2.1. The flows of organic solvent, β-carotene suspension and dissolution of modified starch were kept constant at 9, 6 and 20.6 mL min^{-1}, respectively, being the organic-water ratio (flows of organic solvent and β-carotene suspension/flow of the dissolution of modified starch) 0.73 mL/mL. In a second series of experiments (experiments E33-E49), the flows of the organic solvent, β-carotene suspension and dissolution of modified starch were varied in the range between 7-9 mL min^{-1}, 5-10 mL min^{-1} and 15-25 mL min^{-1}, respectively. This allowed varying the organic-water ratio from 0.6 mL/mL to 1.2 mL/mL.

Table 1. Summary of operating conditions.

	Starch	$C_{\beta\text{-carotene}}$ (g L^{-1})	C_{starch} (g L^{-1})	$F_{ethyl\ acetate}$ (mL min^{-1})	$F_{\beta\text{-carotene}}$ (mL min^{-1})	F_{starch} (mL min^{-1})	$r_{organic\text{-}water}$	$T_{operation}$ (ºC)	$P_{operation}$ (MPa)
E 1	OSA-1	50	37	9	6	20.6	0.73	144 - 150	6 - 6.5
E 2	OSA-1	50	46	9	6	20.6	0.73	143 - 149	6 - 6.5
E 3	OSA-1	50	61	9	6	20.6	0.73	142 - 148	6 - 6.5
E 4	OSA-1	50	92	9	6	20.6	0.73	143 - 148	6 - 6.5
E 5	OSA-1	50	183	9	6	20.6	0.73	144 - 149	6 - 6.5
E 6	OSA-1	50	229	9	6	20.6	0.73	146 - 149	6 - 6.5
E 7	OSA-1	50	306	9	6	20.6	0.73	143 - 148	6 - 6.5
E 8	OSA-1	50	367	9	6	20.6	0.73	144 - 149	6 - 6.5
E 9	OSA-2	50	37	9	6	20.6	0.73	142 - 148	6 - 6.5
E 10	OSA-2	50	46	9	6	20.6	0.73	141 - 150	6 - 6.5
E 11	OSA-2	50	61	9	6	20.6	0.73	139 - 145	6 - 6.5
E 12	OSA-2	50	92	9	6	20.6	0.73	144 - 150	6 - 6.5
E 13	OSA-2	50	183	9	6	20.6	0.73	144 - 149	6 - 6.5
E 14	OSA-2	50	229	9	6	20.6	0.73	138 - 147	6 - 6.5
E 15	OSA-2	50	306	9	6	20.6	0.73	140 - 148	6 - 6.5
E 16	OSA-2	50	367	9	6	20.6	0.73	144 - 150	6 - 6.5
E 17	OSA-3	50	37	9	6	20.6	0.73	145 - 150	6 - 6.5
E 18	OSA-3	50	46	9	6	20.6	0.73	145 - 151	6 - 6.5
E 19	OSA-3	50	61	9	6	20.6	0.73	142 - 147	6 - 6.5
E 20	OSA-3	50	92	9	6	20.6	0.73	141 - 148	6 - 6.5
E 21	OSA-3	50	183	9	6	20.6	0.73	143 - 149	6 - 6.5
E 22	OSA-3	50	229	9	6	20.6	0.73	140 - 148	6 - 6.5
E 23	OSA-3	50	306	9	6	20.6	0.73	139 - 146	6 - 6.5
E 24	OSA-3	50	367	9	6	20.6	0.73	140 - 145	6 - 6.5
E 25	OSA-4	50	37	9	6	20.6	0.73	139 - 145	6 - 6.5
E 26	OSA-4	50	46	9	6	20.6	0.73	142 - 148	6 - 6.5
E 27	OSA-4	50	61	9	6	20.6	0.73	143 - 148	6 - 6.5
E 28	OSA-4	50	92	9	6	20.6	0.73	141 - 147	6 - 6.5
E 29	OSA-4	50	183	9	6	20.6	0.73	144 - 150	6 - 6.5
E 30	OSA-4	50	229	9	6	20.6	0.73	142 - 148	6 - 6.5
E 31	OSA-4	50	306	9	6	20.6	0.73	144 - 147	6 - 6.5
E 32	OSA-4	50	367	9	6	20.6	0.73	144 - 150	6 - 6.5
E 33	OSA-2	50	367	9	6	25	0.60	134 - 145	6 - 6.5
E 34	OSA-2	50	367	9	6	23	0.65	145 - 150	6 - 6.5
E 35	OSA-2	50	367	8	7	20.6	0.73	145 - 152	6 - 6.5
E 36	OSA-2	50	367	8	7	18.7	0.80	142 - 148	6 - 6.5
E 37	OSA-2	50	367	7	5	16.5	0.73	145 - 150	6 - 6.5
E 38	OSA-2	50	367	7	6	16.5	0.79	140 - 150	6 - 6.5
E 39	OSA-2	50	367	9	7	16.5	0.97	147 - 152	6 - 6.5
E 40	OSA-2	50	367	8	10	15	1.20	143 - 150	6 - 6.5
E 41	OSA-3	50	367	9	6	25	0.60	145 - 152	6 - 6.5
E 42	OSA-3	50	367	9	6	23	0.65	147 - 153	6 - 6.5
E 43	OSA-3	50	367	8	7	20.6	0.73	136 - 142	6 - 6.5
E 44	OSA-3	50	367	8	7	18.7	0.80	145 - 152	6 - 6.5
E 45	OSA-3	50	367	8	7	16.5	0.91	144 - 150	6 - 6.5
E 46	OSA-3	50	367	7	5	16.5	0.73	142 - 148	6 - 6.5
E 47	OSA-3	50	367	7	6	16.5	0.79	145 - 150	6 - 6.5
E 48	OSA-3	50	367	9	7	16.5	0.97	138 - 145	6 - 6.5
E 49	OSA-3	50	367	8	10	15	1.20	135 - 142	6 - 6.5

2.5. Product characterization

2.5.1. Weight of dry product

The sample was dried and its dry weight was measured by a Halogen Moisture analyzer model Ohaus MB35 set at 160ºC to remove the water. This dry weight includes crystalline β-carotene (which is the non-encapsulated β-carotene), encapsulated β-carotene and modified starch (carrier).

2.5.2. Percentage of encapsulated β-carotene

The sample was analysed by a UV/Vis spectrophotometer model Agilent 8453. The wavelength was selected at 456 nm. As explained in a previous work [6], the absorbance determined with this method is proportional to the amount of β-carotene dispersed in solution which corresponds to the encapsulated β-carotene. The percentage of encapsulated β-carotene is reported as the ratio between the concentration of β-carotene dispersed in solution and total β-carotene concentration in the product.

2.5.3. Particle size

The particle size analysis was carried out by laser diffraction (model Beckman Coulter LS 13 320).

2.5.4. Trans/cis β-carotene HPLC profile

Crystalline β-carotene was used as feed material containing 95%wt of trans β-carotene and 5%wt of cis β-carotene. However, some isomerization from trans to cis β-carotene took place during the high temperature steps of the process. The degree of isomerization was determined using a Waters Alliance 2695 HPLC chromatograph with PDA detector 2996 equipped with a Vydac 218TP54 5 µm (4.6 × 250) column. The mobile phase was methanol + 1% of tetrahydrofuran stabilized with 50 ppm of ascorbic acid. The temperature of the column was 30ºC. The solvent for the samples was ethanol/tetrahydrofuran stabilized with 50 ppm of ascorbic acid.

2.5.5. Colour

The colour of the obtained aqueous dispersions of β-carotene and modified starch was characterized by UV/Vis spectrophotometer model Agilent 8453 operated in a wide range of wavelengths.

3. RESULTS AND DISCUSSION

3.1. Influence of the concentration of surfactant

Table 2 presents a summary of the main experimental results. As described in Section 2.4, the effect of the concentration of the surfactant dissolution using four different OSA-starches was studied with experiments E1-E32, observing the effect of the concentration of OSA-1 dissolution with experiments E1-E8, the effect of OSA-2 with E9-E16, the effect of OSA-3 with E17-E24 and the effect of OSA-4 with E25-E32. As shown in Table 1, all process parameters were kept constant in these experiments with the exception of the concentration of modified starch dissolution which was varied from 37 to 367 g L^{-1} in order to observe the influence of this parameter on product characteristics.

Table 2. Summary of experimental results.

	% Dry product	% β-carotene in sample	% Encapsulated β-carotene	% Trans/cis β-carotene	UV/Vis spectrum peaks	Particle size D50 (nm)
E 1	3.4	17.2	3.9	51.9 / 48.1	442 / 466 / 490	76890
E 2	4.8	12.3	8.2	80.5 / 19.5	449 / 475 / 514	135900
E 3	5.6	10.9	5.2	94.7 / 5.3	484 / 489 / 531	926
E 4	8.3	8.7	4.5	84.4 / 15.6	484 / 490 / 534	798
E 5	14.9	3.6	20.4	84.2 / 15.8	533	846
E 6	17.7	2.6	29.8	83.9 / 16.1	484 / 490 / 533	552
E 7	22.7	2.5	22.6	82.6 / 17.4	288 / 484 / 525	676
E 8	25.5	3.0	21.0	72.6 / 27.4	430 / 465	10880
E 9	3.8	17.9	18.9	86.5 / 13.5	292 / 485 / 525	355
E 10	4.7	16.5	24.5	82.8 / 17.2	454 / 481 / 520	760
E 11	5.7	10.0	22.7	83.4 / 16.6	454 / 480 / 519	362
E 12	9.4	8.3	35.6	76.6 / 23.4	449 / 477 / 516	513
E 13	15.8	3.2	43.3	90.1 / 9.9	450 / 477 / 516	607
E 14	19.2	3.6	49.8	86.4 / 13.6	451 / 478 / 517	0
E 15	24.1	2.9	49.3	68.1 / 31.9	289 / 443 / 633	633
E 16	27.4	2.6	42.7	86.5 / 13.5	450 / 477 / 517	767
E 17	3.5	15.9	27.4	83.7 / 16.3	292 / 485 / 524	350
E 18	4.5	13.5	24.7	85.1 / 14.9	282 / 485 / 522	439
E 19	5.6	10.3	24.4	89.8 / 10.2	456 / 483 / 520	348
E 20	8.4	7.0	30.7	82.3 / 17.7	452 / 479 / 517	207
E 21	13.8	3.1	55.9	90.7 / 9.3	447 / 474 / 513	344
E 22	18.1	3.5	63.4	87.20/ 12.8	448 / 476 / 515	721
E 23	20.9	3.0	41.1	91.0 / 9.0	450 / 476 / 515	356
E 24	24.3	2.4	54.4	78.3 / 21.7	448 / 474 / 514	353
E 25	3.8	18.3	25.7	93.6 / 6.4	484 / 488 / 526	361
E 26	4.9	14.5	28.3	92.0 / 8.0	455 / 483 / 524	355
E 27	6.1	11.1	44.5	93.2 / 6.8	451 / 479 / 518	358
E 28	8.6	8.5	35.9	83.5 / 16.5	451 / 480 / 519	349
E 29	15.4	4.3	39.4	84.5 / 15.5	451 / 480 / 519	358
E 30	18.9	3.6	60.3	83.0 / 17.0	452 / 479 / 520	550
E 31	22.7	2.8	54.4	81.3 / 18.7	297 / 441	366
E 32	28.7	2.1	70.5	67.7 / 32.3	447 / 475 / 515	462
E 33	24.1	2.2	32.3	84.1 / 15.9	290 / 660	248
E 34	25.3	2.3	38.8	83.0 / 17.0	285 / 660	239
E 35	24.5	3.0	42.3	79.7 / 20.3	289 / 440 / 659	193
E 36	24.5	3.3	39.3	79.7 / 20.3	289 / 440 / 462	137
E 37	24.0	2.4	35.0	71.7 / 28.3	284 / 359 / 438	1708
E 38	23.4	2.9	30.9	68.7 / 31.3	284 / 438	8566
E 39	23.6	3.6	23.6	74.3 / 25.7	284 / 438	4456
E 40	23.8	5.0	66.5	69.4 / 30.6	184 / 440 / 659	25230
E 41	25.6	2.7	44.9	57.0 / 43.0	449 / 476 / 515	650
E 42	23.5	2.5	31.0	72.9 / 27.1	287 / 435	382
E 43	24.7	3.1	27.0	73.7 / 26.3	450 / 474 / 514	352
E 44	23.8	3.4	27.0	63.0 / 37.0	288 / 415	363
E 45	25.8	3.7	27.9	47.1 / 52.9	289 / 437 / 509	375
E 46	23.4	3.5	22.2	70.9 / 29.1	287 / 435	363
E 47	25.7	3.7	25.7	63.5 / 36.5	445 / 472 / 513	364
E 48	25.1	3.8	19.9	68.3 / 31.7	286 / 434	372
E 49	24.8	5.8	33.2	52.2 / 47.8	286 / 433	56000

Figure 2 presents a comparative of the results obtained with the four different OSA-starches with different concentrations.

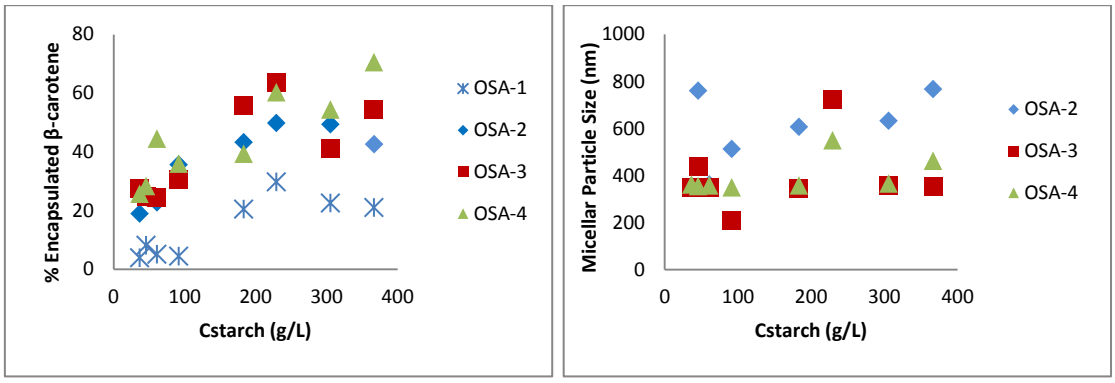

Fig. 2. Comparative of the four different modified OSA-starches on the encapsulation efficiency and micellar particle size.

As shown in figure 2, the same trend of data was obtained independently of which OSA-starch was used, observing an increase in the encapsulation efficiency when the concentration of surfactant was increased. A minimum concentration of surfactant of 100 g L^{-1} was required in order to obtain high percentages of encapsulated β-carotene and stable aqueous suspensions of β-carotene, independently of which OSA-starch was used. At concentrations lower than 100 g L^{-1}, maximum encapsulation efficiencies between 20 and 40% were achieved depending on the OSA-starch used. On the contrary, at concentrations over 100 g L^{-1}, the percentage of encapsulated β-carotene increased, reaching maximum values between 30 and 70%. Specifically, efficiencies observed were 30% with OSA-1 (waxy maize blend with dried glucose syrup), 50% with OSA-2 (OSA-dextrin derived from waxy maize), 63% with OSA-3 (OSA- dextrin derived from tapioca) and 70% with OSA-4 (OSA-starch derived from waxy maize).

Figure 2 also shows the micellar particle sizes observed. With OSA-1, the obtained sizes ranged from more than 100 µm when the concentration of surfactant was below 60 g L^{-1}, to sizes in the range of 1 µm at higher concentrations (results not presented in figure 2 for clarity). This result, combined with the low encapsulation efficiencies obtained with this surfactant, allows concluding that OSA-1 is not suitable for β-carotene encapsulation. With the other three OSA-starches, particle sizes in the sub-micrometer range were achieved, being concretely in the range of 350-760 nm with OSA-2, 350-440 nm in the case of OSA-3 and 350-550 nm in the case of OSA-4. These results did not show a clear trend of variation with the concentration of surfactant.

3.2. Influence of the ratio between organic solvent flow rate and aqueous solution flow rate

The effect of the organic-water ratio (flow of organic solvent plus flow of β-carotene suspension divided by flow of modified-starch dissolution) was studied with experiments E33-E49. Figures 3 and 4 show the variation of the encapsulation efficiency and micellar particle size with the organic-water ratio observed in these experiments. Experiments with OSA-1 were not carried out due to the fact that, as described in section 3.1, it was demonstrated that OSA-1 was not a suitable surfactant to develop the formulation of β-carotene as consequence of the low encapsulation efficiencies and high micellar particle sizes achieved. With regard to OSA-4, the same authors studied the effect of the organic-water ratio with this surfactant in a previous work [6] and results reported in this work are included in figures 3 and 4 for comparison purposes. In figure 3, the organic-water ratio was varied keeping constant the flow rate of organic solvent + β-carotene suspension and using variable flow rates of surfactant solution, while in figure 4 the flow of surfactant solution was approximately constant and the flow rate of organic solvent + β-carotene suspension was modified instead.

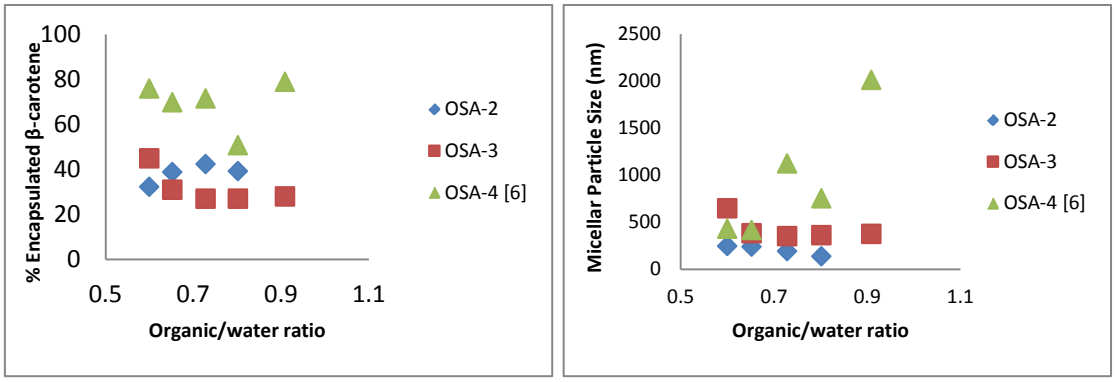

Fig.3. Effect of the organic/water ratio in the percentage of encapsulated β-carotene and in the micellar particle size, with a constant total flow rate of organic solvent + β-carotene suspension of 15 mL min^{-1} and variable flow rates of surfactant solution ranging from 16.5 to 25 mL min^{-1}.

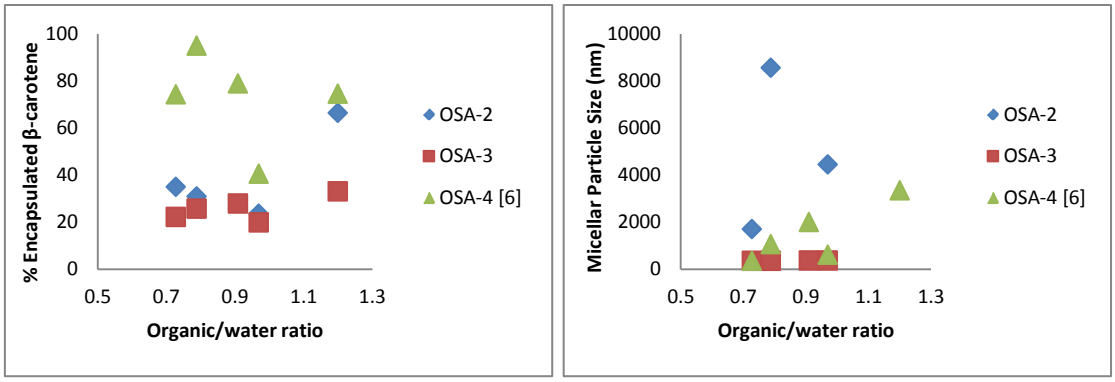

Fig.4. Effect of the organic-water ratio in the percentage of encapsulated β-carotene and in the micellar particle size, with an approximately constant total flow rate of surfactant solution of 15-16.5 mL min^{-1} and variable flow rates of organic solvent+ β-carotene suspension ranging from 12 to 18 mL min^{-1}.

As presented in figure 3, the encapsulation efficiency shows constant values independently of the OSA-starch used, obtaining a small increase in the percentage of encapsulated β-carotene with OSA-2 and a small decrease with OSA-3 when the organic water ratio was increased. Regarding the micellar particle size, constant particle sizes (less than 250 nm) were obtained when OSA-2 was used. With OSA-3, a small decrease in particle size was observed when the organic-water ratio was increased, obtaining micellar particle size in the range of 375-650 nm. However, the organic-water ratio had a strong influence on particle size when OSA-4 was used, obtaining a drastic increase in the micellar particle size when the organic-water ratio increased as well.

This effect can also be observed in figure 4, displaying experiments where the organic-water ratio was modified by variation of the flow of organic solution (for clarity, some results were not presented in the figure because the micellar particle sizes obtained at the highest organic-water ratios were too high, see experiments E39, E40 and E49 in table 2). With respect with the encapsulation efficiency, in figure 4 is shown that the percentages of encapsulated β-carotene were approximately constant independently of the OSA-starch used, achieving higher encapsulation efficiencies with OSA-4 (75-95%).

As conclusion, the best results were obtained using OSA starch-4, refined from waxy maize. Experiments with this starch yielded high encapsulation efficiencies (70-80%) and particle sizes in the sub-micrometer range at low organic-water ratios (between 0.65 and 0.73) These product characteristics indicate that it is possible to obtain a stable aqueous suspension of β-carotene with this surfactant, as reported in a previous work [6].

Results obtained in this work corroborate results from literature. Liang et al. [20] developed the formation of oil-in-water nanoemulsions stabilized by the same modified starches used in this work, using a high-pressure homogenization technique. Results obtained by these authors revealed that the use of a modified starch with high dispersed molecular density led to a higher retention of β-carotene in nanoemulsions, being OSA-4 the modified starch with the highest dispersed molecular density, specifically 28 g/mol nm^3 (compared to 12.7 and 20.1 g/mol nm^3 for OSA-2 and OSA-3, respectively). Also, the same authors claimed that modified starches with higher dispersed molecular density would give a thicker and denser layer over the oil droplets, which would further increase the mean diameter, improve the chemical stability and lower bioacesibility. This increase in the mean diameter was observed in results presented in this work, which showed micellar particle sizes with OSA-4 in the range of 350-550 nm at low organic-water ratios, that drastically increased to more than 2 μm when the ratio was increased, while micellar particle sizes were below 350 nm were obtained when OSA-2 and OSA-3 were used in equivalent conditions (Figure 3).

3.3. Cis-trans composition and colour of samples

As consequence of the exposition of the β-carotene a high temperatures during the process, some isomerization from trans to cis β-carotene took place. Analysing the trans/cis β-carotene HPLC profile, it can be seen that isomerization from trans to cis β-carotene has occurred in a range of 10-50%, with the exception of few experiments in which the isomerization was lower. Regarding the colour of the obtained aqueous dispersions of β-carotene and modified starch, it was characterized by UV/Vis spectra in a wide range of wavelength (250-700 nm). In table 2, it can be seen the peaks which were observed in all samples. In most cases three peaks were observed which represent a typical orange colour (450/478/515) characteristic of carotenoids, which correspond to the range of yellow and red colour.

4. CONCLUSIONS

The influence of four different modified starches for developing the formulation of β-carotene by precipitation from pressurized ethyl acetate-on-water emulsions was investigated in this work. Results showed that an OSA-starch derived from waxy maize blend with dried glucose syrup (OSA-1) was not suitable for encapsulating β-carotene due to the low encapsulating efficiencies achieved (below 30%) and high micellar particle sizes (from 1 μm to more than 100 μm). With the rest of OSA-starches, a minimum concentration of surfactant of 100 g L^{-1} was required to obtain high encapsulation efficiencies. Two process parameters were studied:

concentration of surfactant and organic-water ratio, observing that an increase in the concentration of surfactant produced an increase in the encapsulation efficiency as well, and at high organic-water ratios, a drastic increase in the particle size was observed, independently of the OSA-starch used. With respect to the particle size, particle sizes in the aqueous suspension of β-carotene in the range of 350-760 nm were obtained. Regarding to the effect of the organic-water ratio, the encapsulation efficiency was kept constant between 30-45% and particle sizes below 500 nm, when OSA-2 and OSA-3 were used. Experiments carried out with OSA-4 presented better results, achieving maximum encapsulation efficiencies of 70-80% with particle sizes in the sub-micrometer range.

Acknowledgements

Authors thank DSM-León (Spain) for providing materials used in this work. E. de Paz performed this work with an FPI-UVa research fellowship. Á. Martín thanks the Spanish Ministry of Economy and Competitiveness for Ramón y Cajal research fellowship. This research work was partially funded through project ENE2011-24547 of the Spanish Ministry of Economy and Competitiveness.

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Chapter 4

Production of water-soluble β-carotene formulations: comparison between ultrasound emulsification and emulsion evaporation, and precipitation from a pressurized emulsion

Production of water-soluble β-carotene formulations: comparison between ultrasound emulsification and emulsion evaporation, and precipitation from a pressurized emulsion[1]

Abstract

β-carotene is one of the most common pigments in nature. β-carotene can easily suffer process of degradation in presence of light, heat and oxygen. β-carotene formulations provide protection of the active compound and overcome the low bioavailability due to the low solubility in aqueous media. The use of β-carotene as natural colorant requires an appropriate formulation in order to stabilize the particles of β-carotene in suspension and provide the desired colour. This work presents a study of the production water-soluble β-carotene formulations using OSA-starch as carrier material preparing the emulsions by different techniques: ultrasound emulsification, high-shear emulsification and precipitation from a pressurized emulsion. Formulations of β-carotene with encapsulating efficiencies of 30% and a micellar particle size less than 200 nm were obtained by ultrasound emulsification. Different parameters were investigated as time of application of ultrasound, amplitude, duty cycle and organic-water ratio. By high-shear emulsification, lower encapsulation efficiencies were obtained (below 8%) with micellar particle sizes in a similar range (less than 240 nm). And regarding the precipitation from a pressurized emulsion, the encapsulation efficiencies achieved were much higher (70-80%), but micellar particle sizes increased to 400 nm.

Keywords: β-carotene, colorant, OSA-starch, formulation, ultrasound, emulsification.

[1] Industrial & Engineering Chemistry Research (Submitted)

1. INTRODUCTION

Carotenoids are natural compounds whose application as nutraceutical presents a great interest. Carotenoids are natural pigments that are present in fruits, vegetables, eggs and seafood, among other sources. They can be used as yellow, orange or red colorants in food products, substituting synthetic colorants that are not well accepted by consumers. Some carotenoids are also precursors of aromatic compounds (β-ionone, β-damascenone, safranal, etc.) that can contribute to the organoleptic properties of a food. Moreover, many carotenoids show important biological functions, as antioxidants, vitamin A precursors, etc. Due to this, natural colorants based on carotenoids, and particularly on β-carotene, are being increasingly used by the food industry.

The application of β-carotene in food products faces some problems. β-carotene is a very reactive compound that can be degraded by the action of oxygen, moderate temperatures or light. Moreover, β-carotene is insoluble in water. Due to this, appropriate formulations must be used, that can contribute to protect β-carotene against degradation, and, in the case of water-based products such as beverages and juices, allow dispersing and stabilizing the compound in aqueous media. Different types of formulations have been developed, which are in general based on the reduction of β-carotene particle size to the sub-micrometer or nanometer range, and the use of encapsulation and surfactant materials suitable for food applications (Horn and Rieger, 2001).

Formulations based on emulsions have been particularly successful, since the emulsion can provide a template that allow controlling and reducing the particle size, and facilitate the encapsulation of β-carotene particles in the surfactant material used to form the emulsion. Oil-in-water emulsions with an edible oil as disperse phase, or even β-carotene solutions or dispersions in the oil, can be directly used to add the compound to fat-rich food products (e.g., butter). For example, Yuan et al. used a high pressure homogenization technique to prepare β-carotene nanoemulsions using sunflower oil as disperse phase and different polisorbate (Tween^{TM}) surfactants (Yuan et al. 2008). They formed emulsions with average droplet sizes of 150 - 200 nm with good physical stability, although β-carotene suffered chemical degradation after 28 days of storage. In the case of food products not based on fats, the oil phase of the emulsion must be removed from the formulation. This is frequently done using a volatile organic solvent as disperse phase of the emulsion (e.g. hexane), and removing this solvent by vacuum evaporation. For example, Tan and Nakajima formed β-carotene nanodispersions using an emulsion evaporation technique, employing a conventional homogenizer and a high

pressure microfluidizer to prepare emulsions with hexane as disperse phase and Tween-20 as surfactant (Tan and Nakajima, 2005). These authors obtained particles of 60 - 140 nm which retained up to 40% of their initial β-carotene content after twelve weeks of storage. Particle size and chemical stability depended on the organic/water ratio in the initial emulsion. Similarly, Silva et al. (Silva et al. 2011) used a high-energy emulsification technique to prepare β-carotene nanoemulsions, also using hexane as disperse organic phase and Tween 20 as surfactant. These authors observed a good physical stability of emulsions, but a chemical degradation of β-carotene which resulted in a variation of the color of the nanodispersion. Chu et al. (2007) encapsulated β-carotene in proteins by high pressure emulsification and emulsion evaporation. The best results were obtained with sodium caseinate, reaching particle sizes as small as 17 nm with high β-potentials that indicate high stability against aggregation processes. Besides emulsion-evaporation techniques, other emulsion-template methods can be used in order to produce β-carotene nanodispersions in water. Santos et al. applied a supercritical CO_2 extraction of emulsions to produce dispersions of β-carotene and lycopene in water, with a particle size in the range of 300 nm and a carotenoid encapsulation efficiency of 50% (Santos et al. 2012). De Paz et al. (2012) applied a high pressure antisolvent precipitation from an emulsion in order to encapsulate β-carotene in n-octenyl succinate (OSA)-modified starch, and tested the colorant and antioxidant properties of the product.

The objective of this work is to study the production of β-carotene nanodispersions by an emulsion evaporation method, focusing on the variation of the micellar particle size and the efficiency of encapsulation of β-carotene with process conditions. In contrast with previous works, in this work an ultrasound emulsification technique has been used to produce the emulsions. Ultrasounds are increasingly used in the food industry (Patist and Bates, 2008), and particularly for emulsification processes, in which they often show a better performance than shear-based methods (Abismaïl et al. 1999). In this work, the results obtained by ultrasound emulsification are compared with the results of a conventional high shear rate emulsification. Moreover, food-grade OSA-starches have been used as surfactant materials, and compared to Tween 20, which as previously described has been frequently used to formulate β-carotene. Moreover, ethyl acetate has been used as disperse organic solvent, instead of hexane, that was the solvent used in most previous works (Tan and Nakajima, 2005; Silva et al. 2011; Chu et al. 2007), because it has been considered that in a food application it is preferable to use ethyl acetate, which has a low toxicity and is Generally Recognized as Safe (GRAS), instead a more toxic solvent such as hexane. Indeed, according to the European Commission directives, ethyl acetate is included in the list of solvents that are accepted for any use in the food industry if the process complies with good manufacturing practices (European Commision, DIRECTIVE

2009/32/EC). In USA - FDA regulations, ethyl acetate is classified as a class 3 substance, or "solvent with low toxic potential", with a concentration limit of 5 000 ppm, while hexane is considered a class 2 solvent, "solvent to be limited", with a concentration limit in food products of 290 ppm (F.D.A. 2012). Finally, in this work the results obtained by the emulsion evaporation technique have been compared with the results of an antisolvent precipitation from a pressurized emulsion (De Paz et al. 2012).

2. MATERIALS AND METHODS

2.1 Materials

Crystalline β-carotene with a minimum purity of 99%, manufactured using a fermentation process, was kindly provided by DSM - León (Spain). Ethyl Acetate with a purity of 99.5% and n-hexane with a purity of 95% were purchased from Panreac Química (Barcelona, Spain). Modified n-octenyl succinic anhydride OSA-starch refined from waxy maize was kindly provided by National Starch Group (Hamburg, Germany). Tween®20 was purchased from Sigma-Aldrich.

2.2 Ultrasound/high shear emulsification and emulsion evaporation

An ultrasound emulsifier (UP400S Ultrasonic Processor, Hielscher, Germany), or a high-shear rotor-stator machine (IKA Ultra-Turrax® LABOR PILOT 2000/4) were used to prepare emulsions. A Rotavapor (BÜCHI 011-BÜCHI 461 Water Bath) was used to eliminate the organic solvent in emulsion evaporation experiments. A dissolution of β-carotene in ethyl acetate (β-carotene concentration: 5 g/L) and a dissolution of OSA-starch in de-ionized water (starch concentration: 36 g/L) were prepared. Both dissolutions were heated to 55ºC under stirring to ensure complete dissolution of the compounds. The two dissolutions were mixed, using different proportions of organic and aqueous solutions resulting in organic/water volume ratios ranging from 0.275 mL organic/mL water to 0.73 mL/mL. Then, the mixture was emulsified using the ultrasound equipment. Different parameters of the emulsification were varied, including: the apparatus used (ultrasound or high-shear Ultra-Turrax), and in ultrasound emulsification experiments, the time emulsification, the ultrasound pulse amplitude and the ultrasound duty cycle (i.e., the fraction of time that ultrasound is being generated). After the emulsification, the organic solvent was eliminated by vacuum evaporation at 60ºC, thus producing a suspension of β-carotene particles in water. Suspensions were stored under 5ºC and protected from light and oxygen until characterization

assays. Table 1 presents a summary of experimental conditions tested in emulsion evaporation experiments.

Table 1. Emulsion evaporation experiments. Summary of operating conditions and experimental results

Experiment	$r_{Organic\text{-}Water}$	Emulsification time (min)	Ultrasound amplitude (μm)	Duty cycle	% Encapsulated β-carotene	Particle size (d_{50}) (μm)
1	0.73	15	80	1	7	92.32
2	0.73	8	100	1	8.2	99.59
3	0.73	6	100	1	7.9	31.67
4	0.73	10	100	1	11.4	50.19
5	0.73	12	100	1	11.5	22.64
6	0.73	20	100	1	13.1	0.80
7	0.73	25	100	1	11.4	5.10
8	0.73	35	100	1	12.7	0.83
9	0.73	45	100	1	12	0.25
10	0.73	55	100	1	13.7	8.61
11	0.73	65	100	1	17.9	5.69
12	0.6	65	100	1	11.2	12.15
13	0.5	65	100	1	24.3	5.83
14	0.4	65	100	1	31.2	0.60
15	0.275	65	100	1	24.2	0.14
16	0.275	14	100	1	26.6	0.18
17	0.275	14	20	1	9.6	356.57
18	0.275	14	40	1	15	198.25
19	0.275	14	60	1	19.4	20.05
20	0.275	14	80	1	17.9	37.04
21	0.275	14	90	1	18	11.66
22	0.275	14	100	0.5	30	41.08
23	0.275	14	100	0.7	29.9	18.90
24	0.275	14	100	0.9	42.1	0.17

2.3 Precipitation from a pressurized emulsion

The technique of β-carotene precipitation from a pressurized ethyl acetate-on-water emulsion consists of the following steps:

1) Dissolution of β-carotene in hot, pressurized ethyl acetate. Increasing temperature allows dissolving a higher concentration of β-carotene (i.e., the solubility of β-carotene in ethyl acetate at 10ºC is 0.680 g/mL, and the solubility increases to 15.18 g/mL when temperature is

increased to 60ºC (Três et al. 2007). In this work, a temperature of 145ºC was used, maintaining pressure above 60 bar in order to maintain ethyl acetate in the liquid state.

2) Mixing of this dissolution with an aqueous solution of OSA-starch at ambient temperature. With this, two effects occur simultaneously: an ethyl acetate-on-water emulsion is formed, and β-carotene precipitation occurs due to the drastic reduction of β-carotene solubility in ethyl acetate when temperature is reduced, and due to the antisolvent effect of water.

3) Elimination of the organic solvent by vacuum evaporation.

This 3-step procedure must be carried out minimizing the exposure of β-carotene to high temperatures that can cause degradation of this compound. This is done using the installation presented in Figure 1. It consists of three small storages at ambient pressure, corresponding to the feed of pure ethyl acetate, β-carotene suspension in ethyl acetate and the aqueous solution of modified OSA-starch. The installation also counts with two piston pumps GILSON 305 (maximum flow rate: 25 mL/min; flow rate control with an a accuracy of 0.1 mL/min) used to feed the aqueous dissolution of modified starch and the β-carotene suspension (pumps P-3 and P-2, respectively) and a piston pump JASCO PU-2080 plus (maximum flow rate: 10 mL/min; flow control with an a accuracy of 0.1 ml/min) used to feed the pure organic solvent (pump P-1). The stream of the organic solvent is preheated in a chromatographic oven (KNK-2000-C series GAS CHROMATOGRAPH) to a temperature of about 165ºC in order to reach the specified operation temperature after the mixing with the β-carotene suspension (145 ºC). All streams are pressurized with the pumps in order to keep them in the liquid phase at this temperature, at 60 - 65 bar. The suspension of β-carotene is pumped at ambient temperature. Then it is mixed with the hot organic solvent stream using the T-mixer M1 and shortly afterwards with the cold aqueous solution of surfactant using the T-mixer M2, in order to reduce the contact time of β-carotene particles with the hot organic solvent and avoid the isomerization and degradation of the product. The estimated residence time between the two T-mixers is approximately 0.5 s - 2 s, depending on the flow rates. The contact of the hot solution of β-carotene with the aqueous solution in mixer M-2 causes the emulsification of the organic solvent and the precipitation of β-carotene by a combined antisolvent and cooling effect.

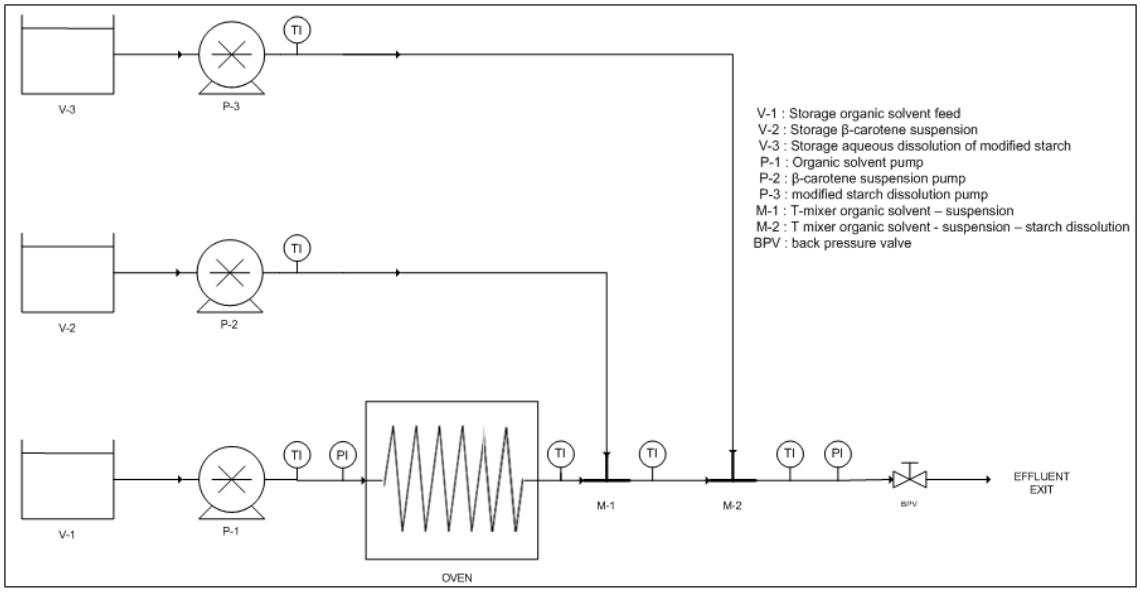

Figure 1. Schematic flow diagram of the experimental apparatus employed for β-carotene formulation by precipitation from pressurized emulsions.

As described in a previous work (De Paz et al. 2012), the performance of this system strongly depends on the organic-water ratio. Table 2 presents a summary of experimental results obtained with this method, varying the organic-water ratio. Fixed concentrations of β-carotene in ethyl acetate of 50 g/L, and OSA-starch in the aqueous solution of 367 g/L were used in these experiments. It can be seen that these concentrations are ten times higher than the concentrations used in emulsion evaporation experiments described in section 2.2, due to the increase in solubility when temperature is increased. However, the same β-carotene-OSA starch ratios were used with both techniques.

Table 2. Precipitation from pressurized emulsions. Summary of operating conditions and experimental results (De Paz et al. 2012).

Experiment	$r_{Organic\text{-}Water}$	% Encapsulated β-carotene	Particle size (d_{50}) (μm)
1	0.6	76.0	0.431
2	0.65	69.9	0.417
3	0.7	72.6	0.505
4	0.73	70.5	0.462
5	0.75	73.9	0.47
6	0.8	70.8	0.908
8	0.85	73.7	1.3
9	0.91	79.0	2.013
11	1.2	62.0	3.36

2.4 Product characterization

Micellar particle size analysis was carried out by Dynamic Light Scattering (DLS) equipment model Malvern Mastersizer 2000.

The fraction of encapsulated β-carotene was determined by spectrophotometry. The sample was analyzed by a UV/VIS spectrophotometer model UV-2550 SHIMADZU. The wavelength selected was 456 nm. The absorbance determined with this method is proportional to the amount of β-carotene dispersed in solution. The ratio of this concentration, corresponding to the amount of β-carotene stabilized in the suspension, to the total β-carotene concentration in the product, is reported in this work as percentage of encapsulated β-carotene.

The colour of the obtained nano-suspensions was characterized using a UV/VIS spectrophotometer model UV-2550 SHIMADZU operated in a wide range of wavelength.

3. RESULTS AND DISCUSSION

3.1 Formulation by ultrasound emulsification followed by emulsion evaporation. Influence of emulsification conditions

With experiments 2-11 of Table 1, the variation of micellar size and encapsulation efficiency with the time of application of ultrasounds was studied, maintaining the remaining process parameters in constant conditions ($r_{Organic\text{-}Water}$ = 0.73, Amplitude = 100%, duty cycle = 1.0). The results are presented in Figure 2. It can be seen that the encapsulation efficiency increased and the micellar particle size decreased when the time of application of ultrasounds was increased from 6 min to 20 min. When ultrasounds were applied for more than 14 min, approximately constant values of encapsulation efficiency of 12-13%, and micellar particle size of 1 - 5 μm, were obtained. This result indicates that a complete emulsification was achieved after application of ultrasounds during 20 min, and a longer application of ultrasounds did not improve the emulsification and the final results.

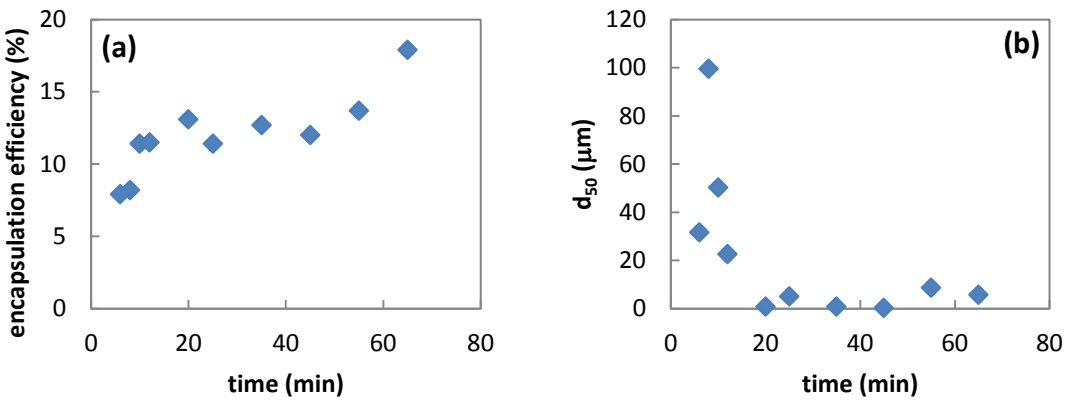

Figure 2: Influence of the time of application of ultrasounds on the encapsulation efficiency (a) and the micellar particle size (b)

With experiments 11-15 of Table 1, the influence of the ratio between the volume of organic phase and aqueous phase, $r_{Organic\text{-}Water}$, was studied with constant values of the remaining parameters (emulsification time: 65 min, Amplitude = 100%, duty cycle = 1.0). Since the concentrations of β-carotene and OSA-starch in the solutions were maintained constant, as the parameter $r_{Organic\text{-}Water}$ was reduced from 0.73 to 0.275, the proportion between OSA-starch and β-carotene was increased from 9.6 g OSA-starch/g β-carotene to 26.2 g OSA-starch/g β-carotene.

As presented in Figure 3, when the ratio was reduced from $r_{Organic\text{-}Water}$ = 0.73 to $r_{Organic\text{-}Water}$ = 0.275, the encapsulation efficiency was increased from 18% to 24%, and the micellar particle size was considerably reduced from 6 µm to 140 nm. These variations of results can be correlated with the reduction of emulsion droplet size and the increase of emulsion stability when the fraction of organic solvent in the emulsion is reduced and the amount of surfactant is increased (Patist and Bates, 2008). Tan and Nakajima (2005) also observed an increase in particle size, resulting in a lower protection of β-carotene against degradation, when the ratio between organic solvent and aqueous phase was increased.

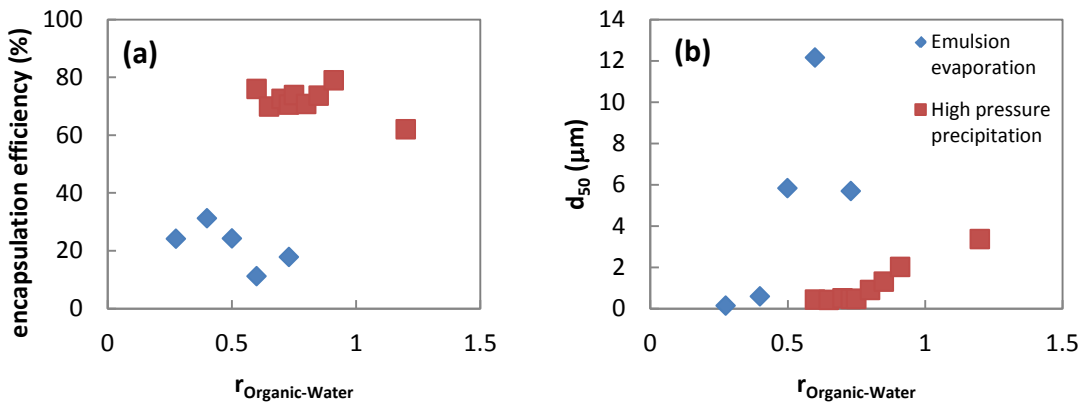

Figure 3: Influence of the ratio between the volume of organic phase and the volume of aqueous phase $r_{Organic\text{-}Water}$ on the encapsulation efficiency (a) and the micellar particle size (b) obtained by emulsion evaporation (♦) and by precipitation from a pressurized emulsion (■) (De Paz et al. 2012).

Experiments 16-21 show the influence of ultrasound amplitude with constant values of the remaining process parameters (emulsification time: 14 min, $r_{Organic\text{-}water}$ = 0.275, duty cycle = 1.0). As presented in Figure 4, micellar particle size decreased when the amplitude was increased, while the encapsulation efficiency increased. Both results indicate a more efficient emulsion formation when the ultrasound amplitude is increased, as it is normally observed in ultrasound emulsification processes (Patist and Bates, 2008).

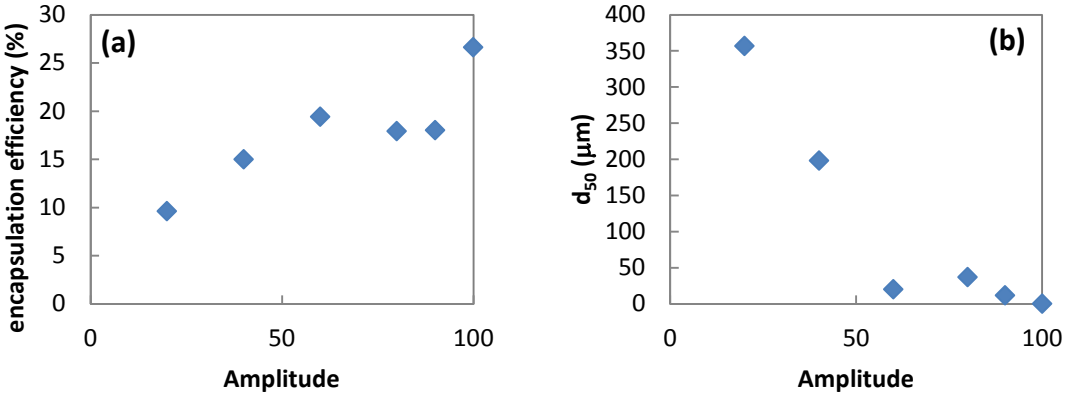

Figure 4: Influence of the ultrasound amplitude on the encapsulation efficiency (a) and the micellar particle size (b)

Finally, with experiments 22-24 the influence of the duty cycle was analyzed (emulsification time: 14 min, $r_{Organic\text{-}water}$ = 0.275, amplitude = 100%). As presented in Figure 5, the micellar size

decreased when the duty cycle was increased, while the encapsulation efficiency remained approximately constant.

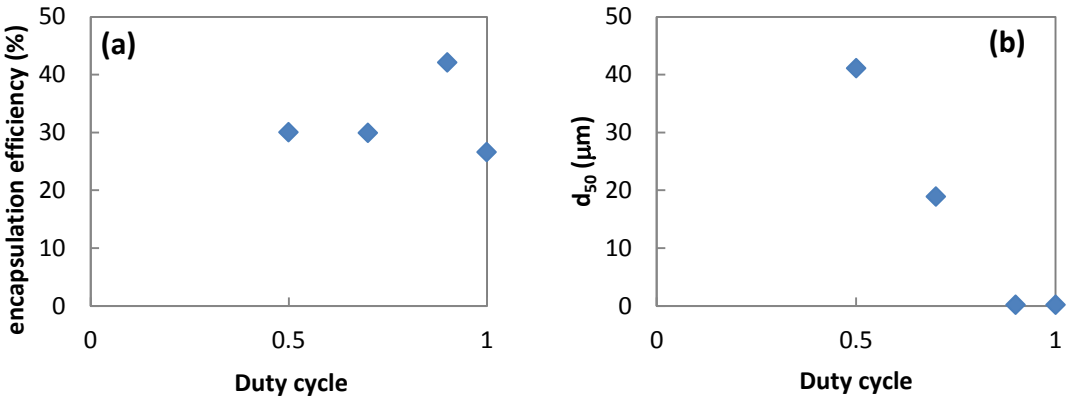

Figure 5: Influence of the duty cycle on the encapsulation efficiency (a) and the micellar particle size (b)

In summary, the best results were obtained with low organic-water ratios ($r_{Organic\text{-}water}$ = 0.275), and using 100% amplitude with a duty cycle of 1.0. A complete emulsification was achieved after 14 min, and results were not improved with longer times of application of ultrasounds. With these conditions, micellar particle sizes of less than 200 nm, with encapsulation efficiencies of 30%, were achieved.

3.2 Application of Tween 20 as encapsulation material

The formulation of β-carotene has also been carried out using Tween 20 as surfactant in order to check if the preparation of β-carotene emulsions by ultrasound technique with this surfactant is better than using OSA-starch as encapsulation material. Tan and Nakajima (2005) developed β-carotene nanodispersions using Tween 20 as surfactant and hexane as dispersed phase preparing emulsions by a conventional homogenizer and a high pressure microfluidizer. In this work, the same conditions reported by Tan and Nakajima (2005) have been used in order to prepare the emulsions by ultrasound technique, using the same concentrations (0.3% w/w of β-carotene in hexane and 0.5% w/w of Tween 20 in de-ionized water) and the same organic-water volume ratios (1:9 and 2:8). The time of application of ultrasounds, amplitude and duty cycle were kept constant in both experiments, being 14 min, 100% and 1.0, respectively.

Results showed that nearly 100% encapsulation efficiencies were obtained just after the preparation of the samples. Regarding the micellar particle size (d_{50}), in both cases was similar,

concretely 6.81 µm when the organic-water ratio was 1:9 and 6.46 µm when the ratio was 2:8. Particle size distribution was bimodal in both cases, as it is presented in Figure 6. As it is shown in figure 6, two different groups of particles are presented in samples; one with a micellar particle size below 10µm and another with a micellar particle size between 10 and 100 µm. On the other hand, the concentration of β-carotene in samples was measured after ten days and after two months of storage at 4ºC. It must be taken into account that there was a loss of β-carotene. In the case of 1:9 ratio, the encapsulation efficiency was 64.7% after ten days and 19.8% after two months. When the organic-water ratio was 2:8, the loss of β-carotene was lower. The encapsulation efficiency was 79.4 % after 10 days and 49.1% after two months. Tan and Nakajima (2005) also observed a loss of β-carotene during storage, retaining up to 40% of their initial β-carotene content after twelve weeks. It is necessary to enhance that Mao et al. (2009), studied the effect of small and large molecule emulsifiers on the characteristics of β-carotene nanoemulsions by high pressure homogenization. They concluded that nanoemulsions stabilized with Tween 20 had smaller droplet size but relatively poorly stability, compared with the ones stabilized with OSA-starch. The results obtained in this work using Tween 20 as surfactant corroborate results from literature due to the loss of β-carotene during storage. However, it is necessary to enhance that the encapsulation efficiency using Tween 20 is much higher than using OSA-starch, achieving maximum values of nearly 100% and 30%, respectively, so it means that the preparation of emulsions by ultrasounds technique is suitable. On the other hand, the micellar particle size was much lower when OSA-starch was used (200 nm).

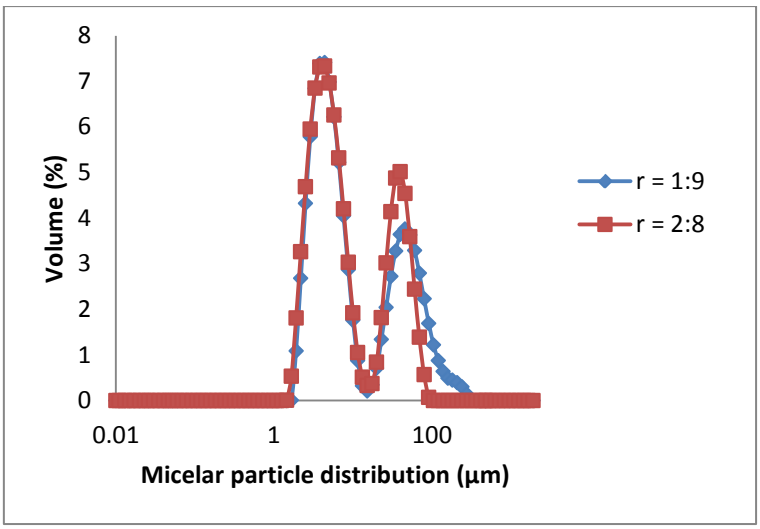

Figure 6: Particle Size Distribution of samples using tween 20 as carrier material.

3.3 Comparison between ultrasound and high-shear emulsification

The encapsulation of β-carotene in OSA-starch has also been carried out by high-shear emulsification. Four different experiments at four different organic-water volume ratios were carried out in order to compare with experiments by ultrasound emulsification. The selected organic-water ratios were between 0.275 and 0.73, in the same range that experiments prepared by ultrasound emulsification. In figure 7, it can be seen the effect of the organic-water ratio on the encapsulation efficiency and on the micellar particle size, showing also the comparison between ultrasound and high-shear emulsification.

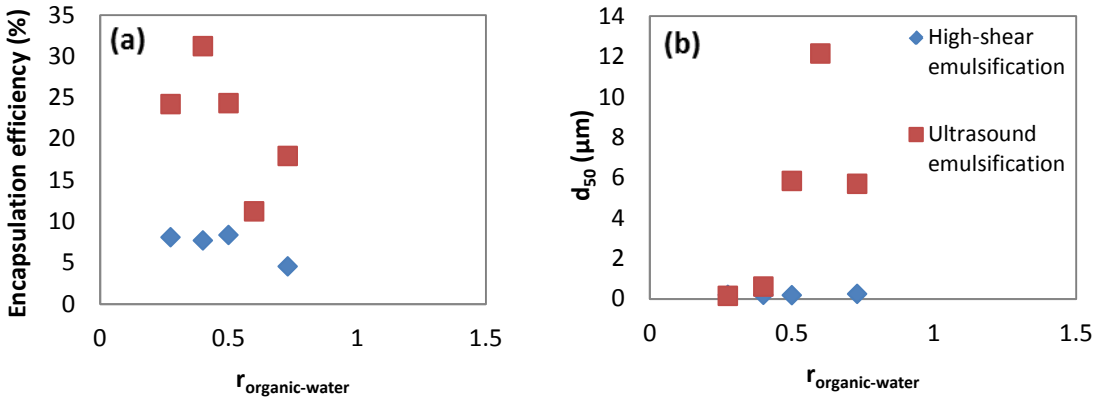

Figure 7: Influence of the ratio between the volume of organic phase and the volume of aqueous phase $r_{Organic\text{-}Water}$ on the encapsulation efficiency (a) and the micellar particle size (b) obtained by high-shear emulsification (♦) and by ultrasound emulsification (■).

As it is presented in figure 7, the encapsulation efficiency remains approximately constant between 4.6 and 8.0% with experiments carried out by high-shear emulsification. However, comparing these results with ultrasound emulsification ones, it is necessary to enhance that better results were obtained by ultrasound technique, achieving maximum values of 31%, depending on the selected organic-water ratio. Regarding the micellar particle size, it is kept constant in all experiments carried out by high-shear emulsification, in the range of nanometers (183-236 nm). However, the organic-water ratio has a strong influence on the micellar particle size with experiments by ultrasound emulsification as it was described in section 3.1. A comparative of particle size distribution of an experiment carried out by ultrasound technique and another by high-shear emulsification can be seen in figure 8. For both techniques, similar particle size distributions were obtained in all experiments. As it is shown in this figure, samples present a mean peak in the range of nanometers and on the

other hand, there are other peaks with a larger particle size in samples obtained by both techniques. As conclusion, results obtained by high-shear emulsification present a very low encapsulation efficiency but with a small micellar particle size, probably as a consequence of the more aggresive conditions during high-shear emulsification compared to ultrasound emulsification.

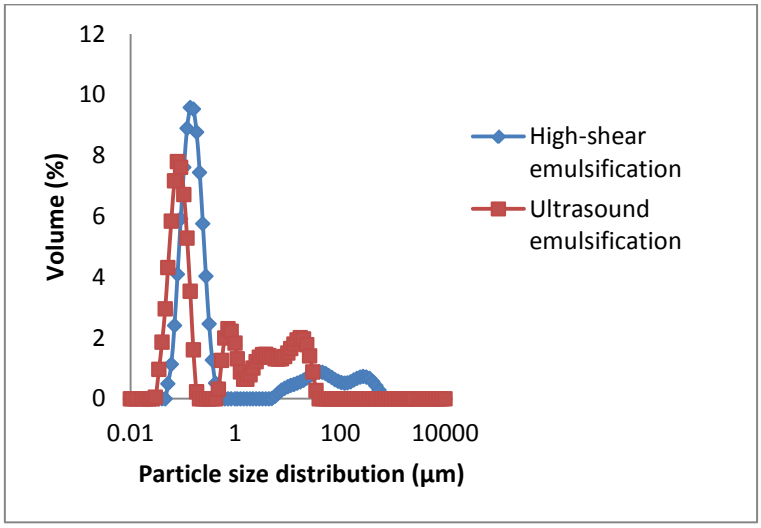

Figure 8: Comparative of particle size distribution of samples obtained by high-shear emulsification and ultrasound emulsification

3.4 Comparison between emulsion evaporation and precipitation from a pressurized emulsion

The encapsulation of β-carotene in OSA-starch has also been carried out using the high pressure emulsion technique presented in section 2.3. As described in a previous work (Yuan et al. 2008), the ratio between the volume of organic solution and the volume of aqueous solution is a main parameter that determines the performance of this process.

Figure 3 presents the variation of the micellar particle size and encapsulation efficiency as a function of the organic-aqueous ratio $r_{Organic\text{-}Water}$. Results obtained by emulsion evaporation and by precipitation from a pressurized emulsion are presented in this Figure. As discussed in Section 2, since the solubility of β-carotene in ethyl acetate increases when temperature is increased, the concentrations of β-carotene and OSA-starch in the organic and aqueous solutions employed in experiments of precipitation from a pressurized emulsion (β-carotene in ethyl acetate: 50 g/L, OSA-starch in water: 367 g/L) are ten times higher than the concentrations used in emulsion evaporation experiments (β-carotene in ethyl acetate: 5 g/L, OSA-starch in water: 36 g/L), but the proportion between the concentrations of β-carotene

and OSA-starch in the organic and aqueous solutions was the same in both series of experiments.

In Figure 3, it can be seen that the same trends of variation were obtained with both techniques: as the ratio $r_{Organic\text{-}Water}$ was increased, the micellar particle size increased, while the encapsulation efficiency decreased at high ratios. As previously discussed, these variations can be related to the efficiency of the emulsification, as more stable emulsions with a smaller droplet size can be obtained when the ratio between the volumes of organic phase and aqueous phase is reduced.

Even though the trends of variation of results with the parameter $r_{Organic\text{-}Water}$ are equivalent, in Figure 3 it can be seen that the quantitative results obtained with each technique are very different. The encapsulation efficiencies achieved by precipitation from a pressurized emulsion (70 - 80%) are much higher than the efficiencies obtained by emulsion evaporation (20 - 30%). Regarding the micellar particle size, the smallest micellar size achieved by precipitation from a pressurized emulsion (aprox. 400 nm) is bigger than the sizes obtained by emulsion evaporation (100 - 200 nm). However, by precipitation from a pressurized emulsion, sub-micron micellar particle sizes of 400-500 nm can be achieved with $r_{Organic\text{-}Water}$ ratios up to 0.75 (corresponding to 9.8 g OSA-starch/g β-carotene), while with the emulsion evaporation techniques, sub-micron sizes are only obtained when this ratio is reduced below $r_{Organic\text{-}Water}$ = 0.4 (corresponding to 18 g OSA-starch/g β-carotene).

Comparing the results obtained with the two techniques, it can be concluded that, with respect to the formation of emulsions with a small droplet size, and the consequent encapsulation of β-carotene with a small micellar size, the ultrasound emulsification technique is more efficient than the T-mixer used in high pressure emulsion precipitation experiments. On the other hand, the encapsulation efficiencies obtained by the high pressure emulsion technique are considerably higher. This is probably due to a partial destabilization of the emulsion during the long processing times required for emulsion evaporation, which are reduced to fractions of seconds using the high pressure precipitation technique. Moreover, the high pressure emulsion technique allows reducing the amount of organic solvents required, due to the increase of β-carotene solubility with temperature, as well as using less encapsulation material, due to the possibility of operating with a higher organic/water ratio.

4. CONCLUSIONS

Water-soluble β-carotene formulations using OSA-starch as carrier material were studied in this work preparing the emulsions by different techniques: ultrasound emulsification, high-shear emulsification and precipitation from a pressurized emulsion (De Paz et al. 2012). These

techniques were followed by the evaporation of the organic solvent in order to obtain water-soluble formulations. Results from these different techniques were compared. By ultrasound emulsification, the effect of the time of application of ultrasound, amplitude, cycle and organic-water ratio was studied. The best results were obtained with low organic-water ratios, concretely 0.275, and using 100 μm amplitude with a duty cycle of 1.0, achieving values of micellar particle sizes less than 200 nm and encapsulation efficiencies of 30%. Using high-shear emulsification, similar micellar particle sizes were obtained (183-236 nm) but with low encapsulation efficiencies (below 8%). However, comparing with results from precipitation from a pressurized emulsion, the encapsulation efficiencies achieved were much higher (70-80%) than efficiencies obtained by ultrasound emulsification. Regarding the micellar particle size, results showed micellar particle sizes of approximately 400 nm.

Acknowledges

Authors thank DSM - León (Spain) for providing materials used in this work. E. de Paz performed this work with an FPI-UVa research fellowship. Á. Martín thanks the Spanish Ministry of Economy and Competitiveness for Ramón y Cajal research fellowship.

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Chapter 5

Determination of phase equilibrium (solid-liquid-gas) in poly-(ε-caprolactone) – carbon dioxide systems

Determination of phase equilibrium (solid-liquid-gas) in poly-(ε-caprolactone) – carbon dioxide systems[1]

Abstract

Solid-Liquid-Gas equilibrium of poly-(ε-caprolactone) – carbon dioxide systems at high pressure has been determined by visual determination of the first melting point using a static apparatus. Measurements have been performed with poly-(ε-caprolactones) of three different molecular weights: 4 000 g/mol, 10 000 g/mol and 25 000 g/mol. The determined SLG equilibrium lines show a temperature maximum at low pressures (0.5 Pa < P < 1.6 MPa) and a temperature minimum at moderate pressures (8 MPa < P < 10 MPa). The maximum reduction of melting temperature ranges from 12.5 K to 16.0 K depending on the molecular weight of the polymer. A thermodynamic model based on the PC-SAFT equation of state has been developed to describe experimental data.

Keywords: poly-(ε-caprolactone), carbon dioxide, SLG equilibrium, melting point.

[1]Journal of Chemical Engineering Data 55 (2010), 2781 - 2785

1.INTRODUCTION

Controlled release formulations are often prepared by encapsulation of the active compound in a natural or biodegradable polymer. Polymeric nanocarriers can show several desirable properties including controlled release of the active compound, long longevity in the body or even specific targeting to certain disease sites[1]. Polyesters such as poly(lactic acid) (PLA) or poly(glycolic acid) (PLG) have been widely employed to prepare carriers for controlled release of drugs and proteins. These polymers degrade by bulk hydrolysis of ester bonds, and their rate of degradation depends on several parameters such as chemical structure, crystallinity, hydrophobicity and molecular weight[2]. The successful use of these polymers in pharmaceutical applications has led to the evaluation of other aliphatic polyesters such as poly(ε-caprolactone) (PCL).

Poly(ε-caprolactone) is a synthetic biocompatible semi-crystalline polymer. PCL exhibits a low glass transition temperature (from -60 ºC to -10 ºC, depending on its molecular weight) which imparts a rubbery characteristic to the material, which results in high permeability. This property has been exploited for delivery of low molecular weight drugs such as steroids and vaccines[3]. PCL has been also used as platform for high molecular weight substances as for example proteins where protection against degradation provided by PCL has been proved to be better than the protection achieved with other polyesters[4,5].

Precipitation processes based on the use of supercritical fluids, and in particular of supercritical carbon dioxide have been successfully applied to produce polymeric carriers. Different techniques have been developed, including Rapid Expansion of Supercritical Solutions (RESS), Supercritical Anti Solvent (SAS) and Particles from Gas Saturated Solutions (PGSS), which present significant advantages over other precipitation techniques including the possibility to process the material in an inert and non-toxic medium and an enhanced control over particle size and morphology[6]. In order to design these processes and optimize the operating conditions a detailed knowledge of the phase behavior of polymer + supercritical fluid mixtures is required.

It is well known that supercritical carbon dioxide interacts with polymers acting as a plasticizing and swelling agent, and reduces significantly the glass transition and melting temperatures of the polymer[7]. Knowledge of Solid-Liquid-Gas (SLG) phase behavior of polymer + CO_2 mixtures is therefore very important for developing formulation processes because it determines the

conditions in which solid particles can actually be produced, as well as near-melting conditions which may be favorable for impregnation processes.

Several articles deal with the determination of the phase behavior of PCL + CO_2 mixtures. Cotugno et al.[8] reported the solubility of near-critical CO_2 in PCL (molecular weight: 80 000 g mol^{-1}, melting temperature: 58ºC) at high temperature (from 70 to 85ºC). Domingo et al.[9] measured the solubility of a polymer blend (PMMA/PCL) in supercritical CO_2 without and with cosolvents (acetone, dichloromethane and ethanol). Rodríguez-Rojo et al.[10] determined the solubility of PCL with average molecular weight 1 000 g mol^{-1} in CO_2 using ethanol as co-solvent. Leeke et al.[11] measured the solubility of supercritical CO_2 in the polymer in the temperature range from 40 to 60ºC with pressures up to 20 MPa. Lian et al.[12] determined the SLG phase behavior of PCL with average mol weight 4 000 g mol^{-1} with CO_2.

In this work the SLG phase behavior of PCL and CO_2 at high pressure (P = 0.1 MPa to 25 MPa) is reported. Measurements have been carried out using PCL with three different molecular weights: 4 000 g mol^{-1}, 10 000 g mol^{-1} and 25 000 g mol^{-1}. Furthermore, a thermodynamic model of the phase behavior of CO_2 + PCL mixtures based on the PC-SAFT equation of state[13] is presented.

2. EXPERIMENTAL SECTION

2.1. Materials

Carbon dioxide (purity: 99.5%) was provided by S. E. Carburos Metálicos S. A. (Spain). Poly-(ε-caprolactones) were kindly supplied by Solvay Caprolactones (Solvay Interox Ltd, United Kingdom). Three different commercial poly-(ε-caprolactones) were used: CAPA 2043D, CAPA 6100 and CAPA 6250. Physical properties of PCLs used in this work are summarized in Table 1. All products were used as received.

Table 1. Physical properties of poly-(ε-caprolactones) used in this work[a]

PCL	MW (g /mol)	T_m (ºC)
CAPA 2403D	4 000	50 – 60
CAPA 6100	10 000	58 – 60
CAPA 6250	25 000	60 – 70

[a]Data provided by the supplier.

2.2. Equipment

A schematic diagram of the equipment used for melting point determinations is shown in Figure 1. The main part of the equipment is an optical cell (SITEC 740.2120) with an inner volume of 25 mL. Maximum operating conditions inside the cell are P = 50 MPa and T = 473 K. The cell has two opposite sapphire windows. Through one of them, a cold light lamp is situated to allow the visualization of the different processes taking place inside the cell aided by a CCD camcorder (Eurotechnica Ingenieurbüro GMBH) connected to a personal computer. The internal temperature of the cell is controlled by a PID temperature controller (OMRON E5GN) acting over an electrical jacket and measuring the temperature with a J type thermocouple (SITEC 770.5131-22, accuracy 0.1 K). Pressure is measured with a membrane pressure meter (DESIN TPR-18/V2, accuracy 0.01 MPa). The system also has a manual pressure generator (SITEC 750.1060) which adjusted the system volume and a diaphragm pump (Dosapro).

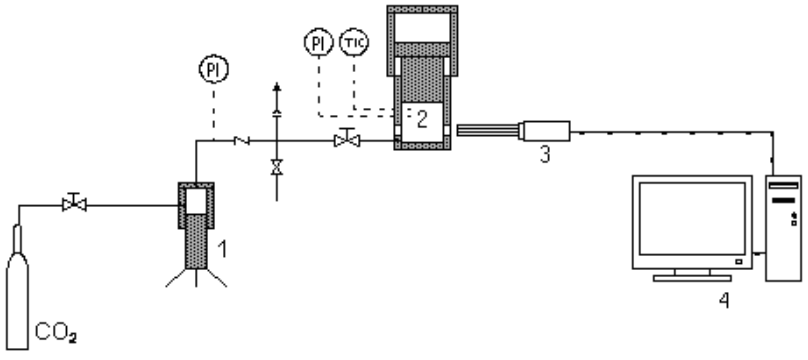

Figure 1. Schematic diagram of the measurement equipment. 1: Manual pressure generator; 2: High pressure optical cell; 3: CCD camera system; 4: personal computer.

2.3. Method

The melting point of PCL with CO_2 at high pressure was measured by visually determining the temperature at which melting started at a certain fixed pressure, according to the first melting point method[12]. For doing so, a small amount of PCL was put in a glass vial and introduced in the optical cell. Afterwards the cell was flushed with CO_2 to remove air, and the cell was filled with CO_2 and pressurized up to desired operating pressure by using the diaphragm pump. Then the cell was gradually heated with the electrical jacket until melting of the PCL sample was observed with the CCD camcorder. After each temperature variation, temperature and pressure were maintained constant during a minimum of 30 min to ensure system equilibration. During the heating process, pressure was maintained constant within ±0.1 MPa by manipulation of the manual pressure generator. Preliminary experiments showed that melting temperatures visually determined with this method were reproducible within ±0.5 K.

3. THERMODYNAMIC MODEL

The PC-SAFT Equation of State considers molecules to be constituted by chains of freely jointed spherical segments. This equation was developed in terms of the residual Helmholtz free energy a^{res}, which can be calculated as the sum of three contributions, as presented in Eq. (1):

$$a^{res} = a^{hc} + a^{disp} + a^{ass} \quad (1)$$

In this equation, a^{hc} accounts for the repulsion of the chain-like molecule, using the hard-chain expression derived by Chapman et al.[14], a^{disp} accounts for the dispersion forces due to attraction between temporarily induced dipoles, and a^{ass} accounts for the association between molecules, described by the association term of the original SAFT Equation of State[15]. Full details about the calculation of these contributions to the residual Helmholtz energy were provided by Gross and Sadowski[13] and by Chapman et al.[14].

Within this framework, non-associating molecules are described by three parameters: the number of segments per chain m, the segment diameter σ, and the depth of the pair potential ε/k_b. Conventional mixing rules can be used for the parameters σ and ε/k_b:

$$\sigma_{ij} = \frac{1}{2}\left(\sigma_i + \sigma_j\right) \quad (2)$$

$$\varepsilon_{ij} = \left(\varepsilon_i \cdot \varepsilon_j\right)^{0.5} \cdot \left(1 - k_{ij}\right) \quad (3)$$

Where k_b and k_{ij} are Boltzmann´s constant and binary interaction parameter, respectively.
For calculation of SLG equilibria, this equation of state has to be applied to solve the condition of equality of fugacity of each component in each phase:

$$f_i^S = f_i^L = f_i^G \quad (4)$$

As most equations of state, PC-SAFT can only be used to calculate the fugacity of fluid phases, and not of solid phases. As an approximation, the fugacity of the solid phase can be calculated as a function of the fugacity of a reference, subcooled liquid f^{SCL} with the following equation, which is strictly valid in the triple point of the substance[16]:

$$f_i^S\left(T,P\right) = f_i^{SCL}\left(T,P\right) \cdot \exp\left[\frac{\left(v_2^S - v_2^{SCL}\right)\left(P - P^0\right)}{RT} + \frac{\Delta h^{fus}}{RT^0}\left(1 - \frac{T^0}{T}\right)\right] \quad (5)$$

where T^0 and Δh^{fus} are the melting temperature and heat of fusion of the PCL at the triple point pressure P^0 which can be approximated with good accuracy by the corresponding values at normal conditions. v_2^S and v_2^{SCL} are the molar volumes of solid and sub-cooled liquid polymer, respectively.

4. RESULTS AND DISCUSSION

Experimental results obtained in this work are presented in Tables 2, 3 and 4 and in Figures 2, 3 and 4. It can be seen that the melting point curves obtained with the three investigated PCLs have a similar shape: at low pressures, there is a certain increase of melting temperature when pressure is increased. In the case of CAPA 2403D melting temperature increases as much as 3.5 K, while in the case of the other two PCLs the extent of this increase is of 1 K. For CAPA 2043D, the maximum melting temperature is observed at 1.6 MPa. In the case of CAPA 6100 and CAPA 6250, this temperature is observed at 0.8 and 0.5 MPa, respectively. In the pressure range 1 MPa < P < 8 MPa melting temperature strongly decreases with pressures with a nearly linear variation. At

higher pressures melting temperature either shows no variation or increases slightly when pressure is increased. The maximum melting temperature depression is 12.5 K in the case of CAPA 2403D, 15.5 K in the case of CAPA 6100 and 16.0 K in the case of CAPA 6250.

Table 2. Experimental Solid-Liquid-Gas phase equilibrium results of CAPA 2403D (molecular weight: 4 000 g mol^{-1}) + CO_2 mixtures[a]

P /MPa	T /K	P /MPa	T /K
0.1	327.0	12.1	315.0
0.1	326.0	12.6	313.5
1.0	327.5	13.1	315.0
1.2	330.0	14.0	314.0
1.6	330.5	15.2	314.0
1.9	326.5	16.5	315.0
2.1	326.0	18.9	315.0
3.0	324.0	20.1	314.0
4.2	322.0	21.1	315.0
5.3	320.5	22.3	314.0
6.2	320.5	23.4	315.0
7.4	317.0	24.2	315.5
8.4	314.5	25.6	315.0
9.3	314.5	27.1	315.0
10.4	315.0		

[a]Experimental uncertainties: ±0.5 K (temperature), ±0.1 MPa (pressure)

Table 3. Experimental Solid-Liquid-Gas phase equilibrium results of CAPA 6100 (molecular weight: 10 000 g mol^{-1}) + CO_2 mixtures[a]

P /MPa	T /K	P /MPa	T /K
0.1	331.0	9.3	313.0
0.1	331.0	10.1	314.0
0.6	332.0	10.6	315.0
0.9	332.0	10.6	315.0
1.1	331.5	10.6	315.0
2.0	330.5	11.6	316.0
2.5	330.0	12.5	314.0
3.6	328.0	14.1	315.0
5.0	323.0	15.3	316.0
5.3	325.5	16.2	316.0
6.3	321.0	17.1	315.0
7.4	318.5	19.3	315.0
8.3	316.0	20.1	316.0
8.3	316.0	21.6	316.0
8.9	314.0	22.8	315.5
9.1	314.0	25.1	316.0

[a]Experimental uncertainties: ±0.5 K (temperature), ±0.1 MPa (pressure)

Table 4. Experimental Solid-Liquid-Gas phase equilibrium results of CAPA 6250 (molecular weight: 25 000 g mol^{-1}) + CO_2 mixtures[a]

P /MPa	T /K	P /MPa	T /K
0.1	335.0	6.1	321.0
0.1	334.0	7.1	318.5
0.6	336.0	8.3	318.5
0.8	335.5	9.4	319.0
1.5	334.0	10.2	318.0
2.2	332.5	11.2	319.5
3.1	330.0	12.1	318.5
4.6	326.5	13.1	319.0
5.2	325.5	14.1	320.5

[a]Experimental uncertainties: ±0.5 K (temperature), ±0.1 MPa (pressure)

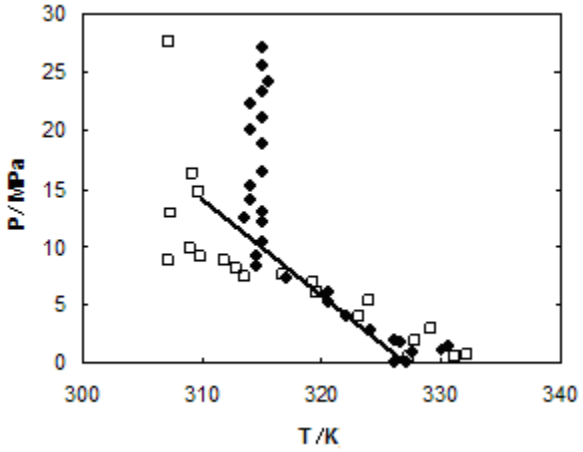

Figure 2. SLG equilibrium of PCL – CO_2 systems: results with PCL with molecular weight 4 000 g mol^{-1} (CAPA 2403D). Symbols: (◆) Experimental results. (□ Literature data[8]). Continuous line, model results.

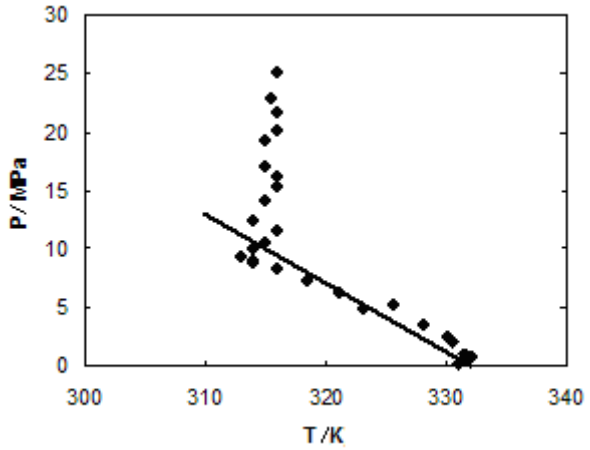

Figure 3. SLG equilibrium of PCL – CO_2 systems: results with PCL with molecular weight 10 000 g mol^{-1} (CAPA 6100). Symbols, Experimental results; continuous line: model results.

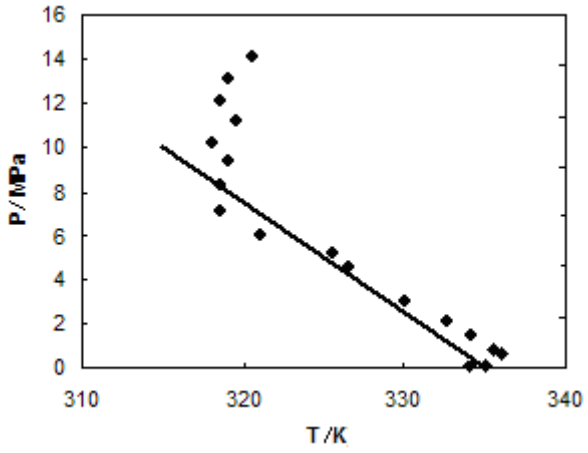

Figure 4. SLG equilibrium of PCL – CO_2 systems: results with PCL with molecular weight 25 000 g mol^{-1} (CAPA 6250). Symbols, experimental results; continuous line: model results.

Lian et al.[12] also determined the SLG phase equilibria of a poly-(ε-caprolactone) with average molecular weight of 4 000 g mol^{-1}, equivalent to that of CAPA 2043D. For comparison purposes, in Figure 2 the results of these authors are represented together with results obtained in this work. It can be seen that the results of Lian et al. present the same shape of data obtained in this work. Both data sets report the same melting temperature at ambient conditions (327 K), a similar

maximum melting temperature (T = 330.5 K at P = 1.6 MPa in this work, and T = 332.2 K at P = 0.7 MPa in the data set of Lian et al.), and the slope of the *T* vs. *P* curve in the region of moderate pressures (1 MPa – 8 MPa) is also similar in both data sets. However, significant differences can be observed between the two data sets in the region of high pressure, because Lian et al. observed a minimum melting temperature of about 310 K, which is 4.5 K lower than that observed in this work. It is worth mentioning that the PCLs used in the two works were obtained from different suppliers: Solvay Policaprolactones in this work and Dow Chemicals in the work of Lian et al. It is therefore possible that these products have differences in properties such as the exact value of the mean molecular weight or the polydispersity index which could justify the observed differences in the melting behavior.

A similar shape of the SLG equilibrium lines with maximum and minimum melting temperatures was observed by Weidner et al. for the system carbon dioxide + polyethylene glycol[17]. The minimum melting temperature phenomenon can be easily explained by considering the balanced effects of the dissolution of CO_2 into the polymer, which tends to reduce the melting temperature, and hydrostatic pressure, which tends to increase this temperature. Indeed, the solubility of CO_2 in most molten polymers can be very high and at moderate pressures it increases rapidly and almost linearly when pressure is increased. Over a certain pressure, the solubility of CO_2 increases by a very small extent even if large pressure increases are applied, resulting in an almost vertical *P* vs. x_{CO2} equilibrium line[11]. Therefore it can be argued that at moderate pressures the effect of CO_2 solubilization prevails, hence the important reduction of melting temperature in this pressure range, while at high pressures the solubility of CO_2 in the molten polymer increases by a small extent when pressure is increased. Therefore the hydrostatic pressure effect prevails. However, the maximum melting temperature phenomenon cannot be easily explained by thermodynamic considerations. In a detailed thermodynamic analysis, de Loos[18] concluded that in a binary system such behavior is possible only if the solubility of CO_2 in the solid phase is higher than in the liquid phase. Nevertheless de Loos pointed out that such behavior is very rare, as in most cases a negligible solubility of CO_2 in the solid is expected at low pressures.

On the other hand, Lian et al.[12], who also observed a maximum melting temperature in CO_2 + PCL systems, attributed this phenomenon to lamellar thickening of PCL due to the enhanced polymer-chain mobility in the CO_2-exposed amorphous regions. This explanation was based on the experimental observations of Siheh and Yang[19], who observed this phenomenon in PCL exposed to CO_2 at high pressure by application of Differential Scanning Calorimetry (DSC) and Small-Angle X-

ray Scattering (SAXS). However, it must be pointed out that according to Siheh and Yang[19], these modifications in the crystalline structure of PCL only took place when the polymer was exposed to CO_2 at moderately high pressures (P > 3.6 MPa). A similar behavior has been observed with other polymers: López-Periago et al.[20] found that CO_2 could cause a solvent-induced crystallization in poly lactic acid exposed to CO_2 at temperatures below the glass transition temperature. This crystallization resulted in an increase of the melting temperature and melting enthalpy, but high CO_2 pressure was required (10 MPa < P < 20 MPa).

Another possible explanation of the maximum melting temperature phenomenon arises from the fact that it has only been observed with polymers (e.g. PEG by Weidner et al.[17] or PCL by Lian et al.[12] and in this work). Indeed, SLG measurements with other substances such as tristearin, tripalmitin[18], tetradecanid acid, hexadecanoic acid or 1-hexadecanol[21] do not show a temperature maximum. It is possible that the maximum melting point phenomenon is a consequence of PCL + CO_2 mixtures not being binary systems due to the polydispersity of the polymer. CO_2 may be able to selectively extract low molecular weight oligomers from the polymer which act as plasticizing agents, thus causing an increase in the melting point.

A thermodynamic model of the phase equilibrium of (PCL + CO_2) mixtures based on the PC-SAFT EoS[13] has been developed in this work. Pure component parameters required by this model are presented in Table 5. The parameters of CO_2 have been taken from the literature[13] while the parameters of PCL have been estimated with the group contribution method developed by Tihic et al.[22]. As shown in this table, parameter *m* of PCL depends on the molecular weight of the polymer, which enables using this set of parameters with PCLs of different molecular weights.

Table 5. PC-SAFT EoS pure component properties considered in this work

	m	**σ/Å**	**ε/k_b/K**	**ρ /kg m^{-3}**	**ΔH_{fus} /J g^{-1}**
CO_2	2.0729	2.7852	169.21		
PCL	0.0396 · MW	3.3908	235.67	1140	76.9

As previously described, in order to apply the PC-SAFT EoS to mixtures the binary interaction parameter must be calculated. The interaction parameter between CO_2 and PCL has been calculated by correlation of vapor-liquid equilibrium data reported by Cotugno et al.[8] in the temperature range 343 K < T < 358 K. The correlation has been performed by minimization of the

Average Absolute Pressure Deviation AAPD between experimental and calculated bubble point pressures, according to the objective function presented in Eq. (6). With this procedure, the following expression has been obtained for the binary interaction coefficient between CO_2 and PCL: $k_{12} = 177.966/T - 0.4558$. With this parameterization, the maximum deviation between experimental LV bubble point pressures and PC-SAFT calculations is AAPD = 6.8%.

$$AAPD = \frac{100}{n_{\exp}} \sum_{i}^{n\exp} \frac{\left| P^{calc} - P^{\exp} \right|}{P^{\exp}} \qquad (6)$$

Density and heat of fusion data of PCLs required for SLG equilibrium calculations with Eq. (5) were taken from the literature[8, 19] and are listed in Table 5. Results of equilibrium calculations with the PC-SAFT equation are shown in Figures 2, 3 and 4 together with experimental results. It can be seen that the model correctly describes the decrease of melting temperature with increasing pressure in the region of moderate pressures (P < 10 MPa). It can be therefore used to estimate the melting temperature of PCLs of different molecular weights in this pressure range, which is of interest for several supercritical precipitation techniques such as SAS or PGSS. However, the model fails to predict the minimum melting point temperature and the increase of melting point with pressure at high pressures. This is probably due to the simplicity of the model used to represent the solid phase (Eq. 5). A similar limitation of this model was found in the modeling of SLG equilibrium of the system polyethylene glycol + CO_2[23].

5. CONCLUSIONS

The solid-liquid-gas equilibrium in poly-(ε-caprolactone) + CO_2 systems has been determined. Measurements have been carried out with PCLs of three different molecular weights: 4 000 g mol^{-1}, 10 000 g mol^{-1} and 25 000 g mol^{-1}. The experimentally determined SLG equilibrium curves show both a temperature maximum at low pressures (0.5 MPa < P < 1.6 MPa) and a temperature minimum at moderate pressures (8 MPa < P < 10 MPa). The maximum reduction in melting temperature is 12.5 K in the case of PCL 4 000 g mol^{-1} , 15.5 K in the case PCL 10 000 g mol^{-1} and 16.0 K in the case of PCL 25 000 g mol^{-1}**.** A thermodynamic model based on the PC-SAFT equation of state has been developed to describe experimental data. The model correctly predicts the solubility of CO_2 in the molten polymer as well as the melting temperature at pressures below the pressure of the minimum melting temperature, but it is not able to describe the variation of melting temperature at higher pressures.

Acknowledgements

The authors thank Solvay Caprolactones for providing the poly-(ε-caprolactones) used in this work and Junta de Castilla y León (Spain) for funding this research (project GR-11). A. Martín thanks the Alexander von Humboldt foundation (Germany) for a postdoctoral research grant.

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Chapter 6

Formulation of β-carotene with poly-(ε-caprolactones) by PGSS process

Formulation of β-carotene with poly-(ε-caprolactones) by PGSS process[1]

Abstract

β-carotene is one of the most common pigments in nature. β-carotene formulations provide protection against oxidation and degradation processes. They are very attractive as natural colorants because they add value to the product due to their antioxidant and pro-vitamin activities. This work presents a study of the formulation of β-carotene with poly-(ε-caprolactone) by Particles from Gas Saturated Solutions (PGSS) process. Particle sizes in the range of 270 - 650 μm with a β-carotene content of up to 340 ppm were obtained using polycaprolactone with a molecular weight of 10 000 $g \cdot mol^{-1}$, while using a polycaprolactone with a molecular weight of 4 000 $g \cdot mol^{-1}$ the particle size was reduced to 110 - 130 μm. The influence of several process parameters on particle size and β-carotene content was studied, including pressure, temperature, time of contact between CO_2 and polymer melt for mixture homogenization, and molar ratio β-carotene : polymer.

Keywords: Particles from gas saturated solutions PGSS, β-carotene, polycaprolactone, supercritical carbon dioxide, mixture homogenization.

[1]Powder Technology 217 (2012), 77-83

1. INTRODUCTION

The food market demands functional foods and healthy products, using natural additives which provide the final product with a healthy added value [1]. Functional ingredients such as carotenoids, fatty acids, natural antioxidants and numerous other compounds, are being extensively used on a great variety of food products [2]. Carotenoids are some of the most common pigments in nature, the most abundant being β-carotene, lycopene, lutein and zeaxanthin. The main roles of carotenoids in human diet are as precursors of Vitamin A and as antioxidants. It has been suggested that carotenoids can be beneficial for human health disorders such as cardiovascular disease, macular degeneration or cataracts [3]. For many industrial applications, a mixture of the carotenoid with a polymer is used. Covering carotenoids with polymers provides protection against oxidation and degradation processes [4]. For the use of carotenoids as natural colorants, a formulation of the active compound is required with a restricted particle size. It is important to obtain an appropriate colour intensity of the formulation which depends on the properties of particles [5].

The application of supercritical fluids as an alternative to the conventional precipitation processes has been an active field of research and innovation during the past two decades [6-8]. The main motivation for this is the possibility of exploiting the peculiar properties of supercritical fluids, and in particular of supercritical carbon dioxide, the most used supercritical fluid for precipitation processes [9]. In particular, the applications of supercritical fluid technology to the precipitation of β-carotene have been intensively studied [1]. Cocero and Ferrero [10] researched the precipitation of β-carotene from ethyl acetate and dichloromethane solutions using carbon dioxide as antisolvent by a batch Gas Anti-Solvent (GAS) process. Cardoso et al. [11] investigated the Supercritical Anti-Solvent (SAS) micronization of β-carotene using tetrahydrofuran as solvent because of the high solvent properties for β-carotene. He et al. [12] studied the micronization of natural carotene by SEDS (Supercritical-Enhanced Dispersion of Solutions) process through prefilming atomization. Franceschi et al. [13] also used SEDS technique for the precipitation of β-carotene. The formulation of β-carotene with biopolymers using supercritical fluids has also been investigated. Martín et al. [4] studied the co-precipitation of β-carotene with polyethylene glycol using supercritical carbon dioxide as antisolvent. The results indicated that the concentration ratio had a very important influence over the morphology of the particles. Franceschi et al. [13] investigated the coprecipitation of β-carotene and PHBV from solution-enhanced dispersion technique and Priamo et al. [14] also researched the precipitation and

encapsulation of β-carotene with the same process, obtaining a maximum value of encapsulation efficiency of 55%. Mattea et al. [15] studied the formulation of β-carotene by supercritical antisolvent precipitation from a dichloromethane in water emulsion obtaining a suspension of sub-micron carotene particles.

The Particles from Gas Saturated Solutions (PGSS) process consists of saturating a solute, either dissolved in a liquid solvent or melted itself, with carbon dioxide in supercritical conditions (temperature > 31ºC, pressure > 7.4 MPa). A gas-saturated solution is thus formed which is subsequently expanded to atmospheric conditions through an atomization nozzle. During the expansion, carbon dioxide is suddenly vaporized and intensely cooled down by Joule-Thomson effect, thus providing the driving force for the solidification of the solute [8]. Weidner et al [16] studied the precipitation of polyethyleneglycols with a high molar weight (up to 35 000 g·mol^{-1}) by PGSS obtaining a relatively small particle size (150 to 400 μm) and regular spherical particles. This technique has been widely used for the encapsulation of different active compounds. Sampaio de Sousa et al. [17] investigated the feasibility of glyceryl monosterate as carrier using caffeine as active substance and also carried out the lipidic formulation of chalcone by PGSS [18]. This process was also used by García-González et al. [19] to study the production of controlled drug delivery systems based on solid lipid particles. Vezzú et al. [20] also used this technique to research the production of lipid microparticles containing bioactive molecules functionalized with PEG. Varona et al. [21] investigated the encapsulation of lavandin essential oil with biopolymers.

Poly-(ε-caprolactone) (PCL) is a synthetic biocompatible semicrystalline polymer. PCL exhibits a low glass transition temperature (from -60 to -10 ºC, depending on its molecular weight) which imparts a rubbery characteristic to the material, resulting in high permeability for small molecules. This propertie has been exploited for the delivery of different active compounds such as steroids and vaccines [22, 23]. PCL are water-insoluble polymers which shows a very slow degradation rate in the digestive system, and particularly in the stomach. They are therefore suitable encapsulation agents if a slow delivery of the active compound, or a targeted delivery to the intestinal tract is intended.

In a previous work of the authors [24], the melting point of three polycaprolactones with different molecular weights (4 000, 10 000 and 25 000 g·mol^{-1}) was determined under carbon dioxide pressure. Results showed a minimum in melting temperature ot moderate pressures (8-10 MPa). The maximum reduction of melting temperature ranged from 12.5 to 16.0 K, depending on the molecular weight of the polycaprolactone. According to this study, the physical properties of CO_2 + polycaprolactone mixtures are adequate for a successful PGSS

micronization. This work presents the encapsulation of β-carotene with two different polycaprolactones (CAPA 2403D and CAPA 6100) as carrier materials by PGSS process. As far as authors know, the PGSS micronization of an active compound using polycaprolactone as carrier material has not been previously described in the literature.

2. MATERIALS AND METHODS

2.1. Materials

Crystalline β-carotene with a minimum purity of 99% was manufactured by Vitatene SA (León, Spain) using a fermentation process. Poly-(ε-caprolactones) (PCLs) were kindly supplied by Solvay Caprolactones (Solvay Interox Ltd., United Kingdom). Two different commercial poly-(ε-caprolactones) were used: CAPA 2403D and CAPA 6100. Physical properties of PCLs used in this work are summarized in Table 1. Carbon dioxide (purity: 99.5%) was provided by Air Liquide (Portugal). All products were used as received.

Table 1. Physical properties of poly-(ε-caprolactones) used in this work (data provided by the supplier).

PCL	Molecular Weight ($g\ mol^{-1}$)	Melting Temperature (ºC)	Viscosity at 100ºC (mPa·s)
CAPA 2403D	4000	50 to 60	1670
CAPA 6100	10000	58 to 60	9300

2.2. PGSS Equipment

Figure 1 presents the schematic flow diagram of the experimental apparatus described previously by Sampaio de Sousa et al. [17].

CO_2 is fed by a piston pump (Haskel model MCPV-71) to the high pressure mixing chamber where it is mixed with crystalline β-carotene and polycaprolactone by a mechanical stirrer. The internal temperature of this stirred vessel is controlled by a PID temperature controller (WatlowSTB3J2J1) acting over an electrical jacket and measuring the temperature with a J type thermocouple (accuracy 0.1 K). The system is depressurized by opening a valve (V-3) and atomized through a 600 μm nozzle (Teejet TGSS, Spraying Systems Co.) into a collector where particles obtained are recovered.

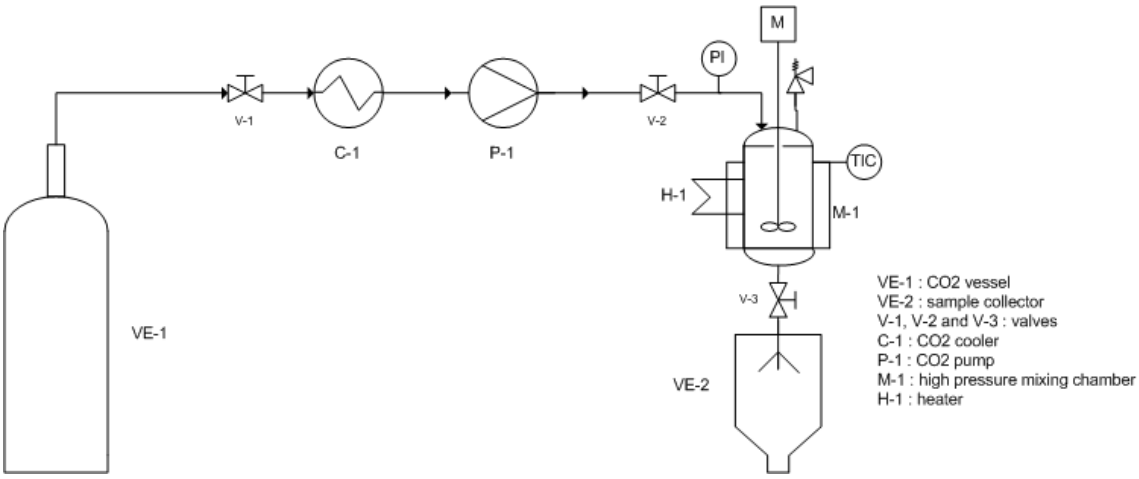


Fig.1. Schematic flow diagram of the experimental apparatus employed for the formulation of β-carotene with poly-(ε-caprolactones) by PGSS process.

2.3 View cell experiments

An essential requisite for a successful PGSS precipitation is to obtain a homogeneous mixture of the polymer, active compound and CO_2 in the mixing chamber before the depressurization. Before starting the PGSS assays, preliminary experiments for the characterization of the mixing between polymer, β-carotene and CO_2 were carried out using a stirred high pressure view cell. These experiments consisted in loading the cell with a mixture of polycaprolactone and β-carotene, setting a pressure of 11 MPa as in PGSS experiments, and gradually increasing temperature while observing the evolution of the mixture through the windows of the cell. Upon reaching the melting temperature of polycaprolacone, a homogeneous melt was obtained, with a uniform red colour indicating that β-carotene particles were either dissolved in the melt or evenly dispersed in it. This result indicates that a homogeneous mixture of the active compound and the polymer is obtained inside the high pressure mixing chamber of the PGSS equipment, as required for application of the PGSS process.

2.4. PGSS Experimental procedure

A typical experiment with the PGSS process began with the preparation of the mixture of the active compound (crystalline β-carotene) and the carrier (polycaprolactone), which was introduced in the high pressure mixing chamber. This mixture was prepared with three different molar ratios (moles of β-carotene: moles of polycaprolactone), being specifically 1:4, 1:6 and 1:8 (corresponding mass compositions; 1.3 wt.%, 0.9 wt.% and 0.7 wt.% in the case of CAPA 6100, and 3.2 wt.%, 2.2 wt.% and 1.7 wt.% in the case of CAPA 2403D). Experiments were carried out using two polycaprolactones with different molecular weights: CAPA 6100

(molecular weight: 10 000 g mol^{-1}) and CAPA 2403D (molecular weight: 4 000 g mol^{-1}). CO_2 was then pumped to the high pressure mixing chamber until the desired pressure was achieved. The operating pressure and temperature in the mixing chamber were chosen according to the results of a previous study of the solid-liquid-gas equilibrium of CO_2 + polycaprolactone mixtures [24], which showed that the highest melting point depression induced by CO_2 could be observed in the pressure range 8 - 10 MPa, and that both polycaprolactones used in this work were melted at a temperature higher than 45ºC when CO_2 pressure was higher than 10 MPa. According to these results, two pressures and two temperatures were used, being 11 and 15 MPa and 50 and 70 ºC, respectively. Once that the operating pressure and temperature in the mixing chamber were stable, the mechanical mixing was switched on during a specified time in order to obtain a gas-saturated homogeneous mixture. Three different times of the mixture homogenization were tested: 60, 120 and 240 minutes. Finally, the system was depressurized by opening the valve between the mixing chamber and the spray nozzle, and the particles obtained were recovered in a collector. A summary of all process conditions tested is presented in Table 2.

Table 2. Summary of operating conditions

	Poly-ε-caprolactone	Molar ratio carotene/PCL	Temperature (ºC)	Pressure (MPa)	Time mixture homogenization (minutes)
E 1	CAPA 6100	0.25	70	15	60
E 2	CAPA 6100	0.25	70	15	120
E 3	CAPA 6100	0.16	70	15	60
E 4	CAPA 6100	0.16	70	15	120
E 5	CAPA 6100	0.13	70	15	60
E 6	CAPA 6100	0.25	50	15	60
E 7	CAPA 6100	0.25	70	11	60
E 8	CAPA 6100	0.13	70	15	120
E 9	CAPA 6100	0.16	50	15	60
E 10	CAPA 6100	0.16	70	11	60
E 11	CAPA 6100	0.13	50	15	60
E 12	CAPA 6100	0.13	70	11	60
E 13	CAPA 6100	0.25	70	15	240
E 14	CAPA 6100	0.13	70	15	240
E 15	CAPA 2403D	0.25	70	15	60
E 16	CAPA 2403D	0.16	70	15	60
E 17	CAPA 2403D	0.13	70	15	60

2.5. Product characterization

2.5.1. Yield of collected particles

The yield of the collected particles by PGSS process was determined by weighting as the amount of the obtained particles in the sample collector divided by the amount of mass introduced in the mixing chamber.

2.5.2. β-carotene content

The sample dissolved in dichloromethane was analysed by UV/VIS spectrophotometer model HITACHI U-2000. The wavelength selected was 456 nm. The absorbance determined with this method is proportional to the amount of β-carotene in the dissolution and therefore allows to calculate the concentration of β-carotene in the obtained particles.

2.5.3. Particle size

The particle size analysis was carried out by a Dynamic Light Scattering (DLS) equipment model Malvern Mastersizer 2000.

2.5.4. Microscopy

Pictures of the particles collected by PGSS process were taken by means of a scanning electron microscope (SEM) model JEOL JSM-820. Samples were gold-sputtered before observation.

2.5.5. Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry profiles were obtained using a differential scanning calorimeter DSC 822e Mettler Toledo SAE (heating rate: 10ºC/min, temperature range from 25 ºC to 100 ºC).

3. RESULTS AND DISCUSSION

Table 2 shows a summary of the process conditions in all experiments performed. The main process parameters were changed in order to analyse the influence of these parameters on product characteristics, being these parameters the molar ratio (moles of β-carotene: moles of polycaprolactone), time of mixture homogenization before the expansion, and temperature and pressure inside the mixing chamber. Table 3 presents the main experimental results

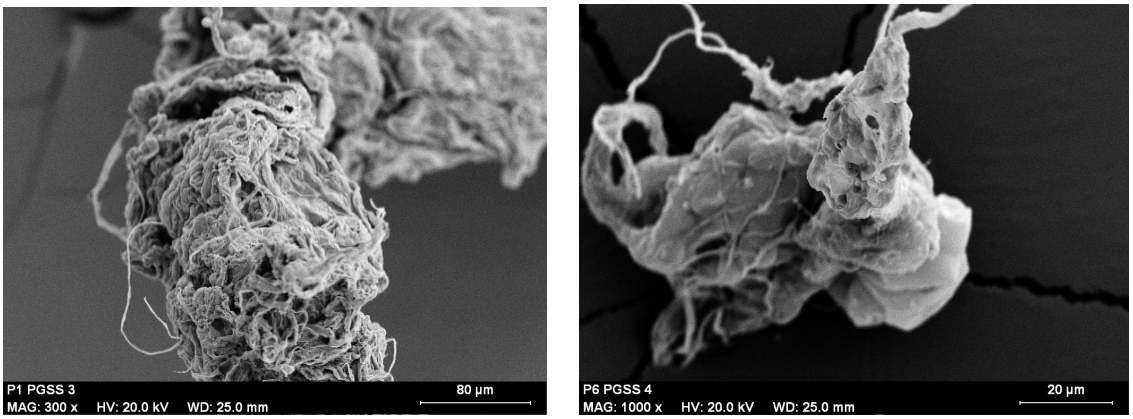
obtained in each experiment, including the yield of the collected particles with respect to the amount of material introduced in the mixing chamber, β-carotene content and particle size.

Table 3. Summary of experimental results.

	Yield of collected particles (%)	β-carotene content (ppm)	Particle size (μm)
E 1	28	336	578
E 2	15	227	591
E 3	15	306	501
E 4	16	35	613
E 5	21	57	467
E 6	8	183	606
E 7	23	161	467
E 8	19	27	523
E 9	19	158	652
E 10	27	10	394
E 11	5	124	510
E 12	33	17	276
E 13	19	49	525
E 14	18	37	431
E 15	32	89	111
E 16	35	94	120
E 17	44	32	132

3.1 Precipitation experiments with CAPA 6100 (MW: 10 000 g mol^{-1})

Experiments 1 to 14 in Table 2 were performed using CAPA 6100 as carrier material. Figure 2 shows SEM micrographs of particles obtained in some of these experiments. The material observed in the SEM micrographs is the polymer used as encapsulating agent, while crystalline particles which could correspond to segregated, crystalline β-carotene were not observed in any of the SEM micrographs taken. The product collected in every experiment presented the same morphology, consisting of sheet or sphere-like particles attached and agglomerated by large filaments of polymer. This morphology is also reflected in the Particle Size Distribution (PSD) measurements presented in Figure 3. In general, PSDs of CAPA 6100 particles were bimodal, with a peak located around 100 μm and a second peak near 1 mm, corresponding to the individual particles and the larger agglomerates, respectively. The relative size of each peak varied between experiments. In some cases, like experiment 10, the peak of 100 μm was clearly visible, while in other cases, like experiment 6, it only appeared as a shoulder in the peak of 1 mm. The naked-eye appearance of the product was of spongy, agglomerated particles with yellow to orange colour due to their content in β-carotene.



a) b)

Fig.2. SEM micrographs of the particles obtained by PGSS process using CAPA 6100, a) Experiment 1, b) Experiment 6

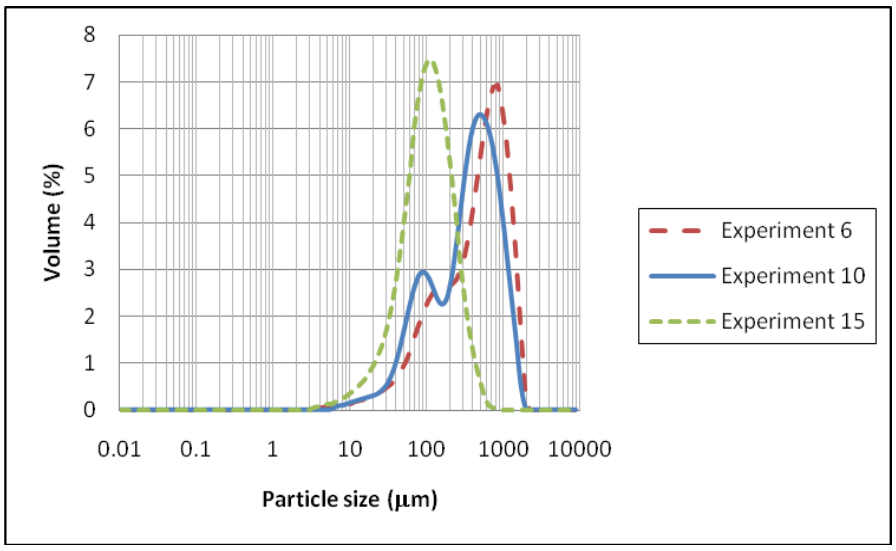


Fig. 3. Particle Size Distribution obtained in experiments 6 and 10, performed with CAPA 6100, and in experiment 15, performed with CAPA 2403D

As shown in Table 3, the yield of collected particles (mass of product collected/mass of product introduced in the mixing chamber) was low in all experiments, indicating a loss of product and possibly of fine particles, and did not show any clear trend of variation with process conditions. Due to the discontinuous operation procedure, a significant fraction of the initial polymer/β-carotene mixture remained in the mixing vessel which could not be sprayed into the precipitation vessel once that the pressure in the mixing vessel had decreased, contributing to the low precipitation yields observed. Moreover, nozzle obstructions occurred in several experiments with CAPA 6100 which may have cause instability in the process and further

reductions of the amount of product that could be sprayed from the mixing vessel into the precipitation vessel. Nevertheless, the amount of sample that could be recovered after every experiment ranged from 1 g to 5 g, which ensures that the results reported are representative of the properties of the product obtained.

3.2. Influence of the molar ratio

As shown in Table 2, the influence of the molar ratio (β-carotene/polycaprolactone) has been studied testing three levels of this variable: 0.13, 0.16 and 0.25, maintaining a constant temperature and pressure inside the mixing chamber of 70 ºC and 15 MPa, respectively. Figure 4 presents the influence of the molar ratio on the particle size and on the β-carotene content, for three different values of the time of homogenization in the mixing chamber (60, 120 and 240 minutes).

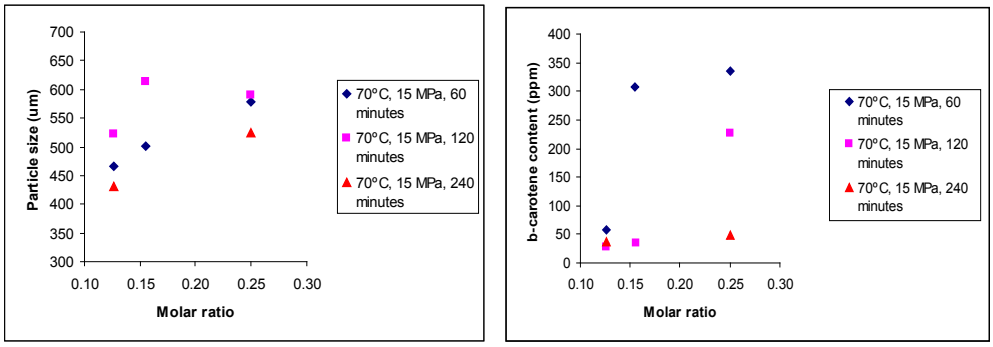


Fig.4. Effect of the molar ratio on the particle size and β-carotene content with different times of homogenization.

As presented in Figure 4, the molar ratio had a strong influence on the particle size, and as this molar ratio was increased, the particle size increased as well independently of the time of mixture homogenization used. Furthermore, as the molar ratio was increased, the size of the peak corresponding to the individual particles in the PSD decreased with respect to the size of the peak corresponding to agglomerates, and the position of both peaks was displaced to larger values of particle size, indicating that bigger and more agglomerated particles were produced. With regard to the β-carotene content in the product, as expected it increased when the molar ratio was increased independently of the time of mixture homogenization employed, reaching values of up to 336 ppm with the highest molar ratio tested.

3.3. Influence of the time of mixture homogenization

As shown in Table 2 and Figure 4, the effect of the time of mixture homogenization was studied carrying out experiments with three different mixing times (60, 120 and 240 minutes) at constant mixing temperature and pressure of 70ºC and 15 MPa, respectively.

Analysing the results presented in Figure 4, it can be observed that the smallest particle sizes were obtained with the longest mixing time (240 min), although an increase in particle size is observed when mixing time is increased from 60 min to 120 min. This result indicates that a longer mixing time allows to obtain a more homogeneous and stable mixture with a higher saturation with carbon dioxide, which improves the performance of the process and allows to produce smaller particles. Regarding the β-carotene content in the sample, it was considerably increased when the homogenization time was decreased.

3.4. Influence of the temperature

The influence of the temperature inside of the high pressure mixing chamber has been studied carrying out experiments at temperatures of 50ºC and 70 ºC with a constant pressure of 15 MPa and a constant time of homogenization of 60 minutes. Figure 5 presents the variation of particle size and β-carotene content as a function of the temperature in the mixing chamber and the molar ratio between β-carotene and polycaprolactone.

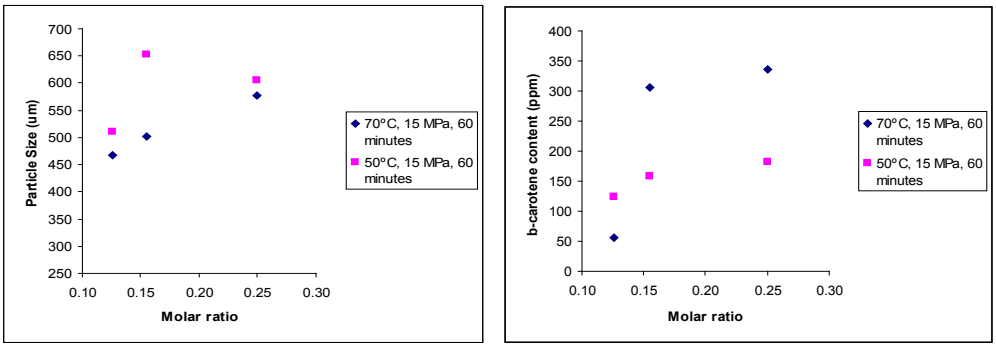


Fig.5. Effect of the temperature in the high pressure mixing chamber on the particle size and on the β-carotene content.

As it can be observed in Figure 5, the temperature inside the high pressure mixing chamber had a comparatively smaller influence on the particle size with respect to other process parameters. In general, higher particles sizes were obtained when the temperature used was the lowest (50 ºC), although the differences between particle sizes obtained at the two

temperatures tested are small in most cases. In a previous work [24], the first melting point of different polycaprolactones under CO_2 pressure was investigated. It was obtained that the first melting point of CAPA 6100 at 15 MPa was 43ºC. The operation with a mixing chamber temperature of 50ºC, relatively close to the melting point of the polycaprolactone, may have caused a higher melt viscosity and a correspondingly worse mixture homogenization than the operation at 70ºC. Moreover, the reduction of the viscosity of the melt when temperature is increased can also favour the atomization during the expansion through the nozzle, leading to the production of smaller droplets of polymer which upon solidification form smaller particles. Regarding the amount of β-carotene in the sample, higher β-carotene contents were obtained when the temperature inside the high pressure mixing chamber was 70ºC. This result may be also due to the reduction in the viscosity of the melt at higher temperatures, which may allow to achieve a better homogenization between polymer and β-carotene.

3.5. Influence of pressure

In order to investigate the effect of the pressure inside the mixing chamber, experiments with pressures of 11 and 15 MPa were carried out, keeping constant the temperature and time of homogenization (70ºC and 60 minutes respectively). The results obtained with respect to the effect of the pressure on the particle size and on the β-carotene content are presented in Figure 6.

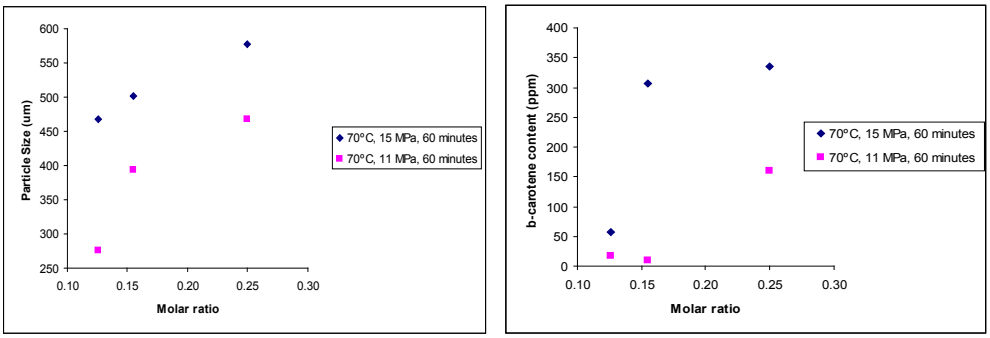


Fig. 6. Effect of the pressure inside the mixing chamber on the particle size and on the β-carotene content.

Analysing the results presented in Figure 6, it can be seen that the pressure inside the mixing chamber had a strong influence on the particle size, and lower particle sizes were obtained operating with 11 MPa than in experiments carried out at 15 MPa. As in previous cases, the particle size increased when the molar ratio β-carotene/polycaprolactone was increased.

Regarding the amount of β-carotene in the sample, there were drastic variations in this content when pressure was varied. As it can be seen in figure 6, at the highest pressure, higher concentrations of β-carotene were obtained.

3.6. Precipitation experiments with CAPA 2403D (MW: 4 000 g mol^{-1})

Experiments E15-E17 of Table 2 and 3 were carried out using CAPA 2403D as carrier material instead of CAPA 6100. As reported in Table 1, CAPA 2403D has a lower molecular weight than CAPA 6100, which results in a slightly lower melting temperature and a lower melt viscosity.

Figure 7 presents the variation of particle size of particles of CAPA 2403D as a function of the molar ratio β-carotene:polycaprolactone, compared to results obtained with CAPA 6100 in the same conditions of temperature (70ºC), pressure (15 MPa) and time of homogenization (60 minutes).

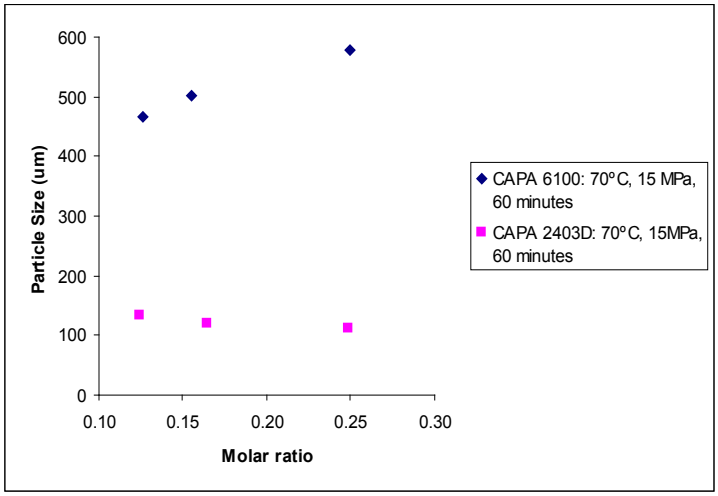


Fig. 7. Effect of the molar ratio. Comparison between CAPA 6100 and CAPA 2403D.

As it can be observed in Figure 7, drastic differences between the particle sizes obtained using both polycaprolactones were observed. The particle sizes obtained using CAPA 2403D were in the range 110 μm - 130 μm, which are considerably smaller than the particle sizes achieved with CAPA 6100 in the same conditions. Furthermore, as presented in Figure 3, the PSDs of particles of CAPA 2403D were unimodal. The SEM micrographs of these particles, presented in Figure 8, did not show the thin fibres that formed the agglomerates in the case of CAPA 6100 particles (Figure 2). In agreement with the results of PSD measurements and SEM micrographs, the product obtained with CAPA 2403D was a free-flowing powder.

The melting temperatures of both polycaprolactones in contact with CO_2 at a pressure of 15 MPa, as determined in a previous work [24], are very similar: 41ºC in the case of CAPA 2403D, with a melting point reduction of 13ºC with respect to atmospheric conditions, and 43ºC for CAPA 6100, which corresponds to a reduction of 15ºC in the melting point. Therefore no differences in the results are expected as a consequence of this property. Another important parameter for the PGSS process is the amount of CO_2 dissolved in the melt, which upon depressurization causes the solidification of particles by Joule-Thomson effect. The solubility of CO_2 in the polycaprolactones has not been determined experimentally. However, in a previous work [24], a PC-SAFT thermodynamic model was developed to describe the solid-liquid-gas phase behaviour of polycaprolactone + CO_2 mixtures, and this model can be used to estimate the solubility of CO_2 in the melted polymers. According to this model, the estimated solubility of CO_2 in melted CAPA 6100 at 15 MPa and 70ºC is 2.7 wt%, while a higher CO_2 solubility of 5.3 wt% is estimated for CAPA 2403D. This estimation agrees with the observed differences in particle size, because a higher CO_2 content can cause a more intense Joule Thomson cooling effect, promoting the formation of smaller particles. The differences between the results obtained with the two polycaprolactones can also be related to the differences in the viscosity of the two melted polymers, reported in Table 1. Since CAPA 2403D is less viscous than CAPA 6100, the atomization of CAPA 2403D during the expansion in the nozzle can be more effective, producing smaller droplets and therefore leading to the formation of smaller particles, as observed in the experiments with this polymer.

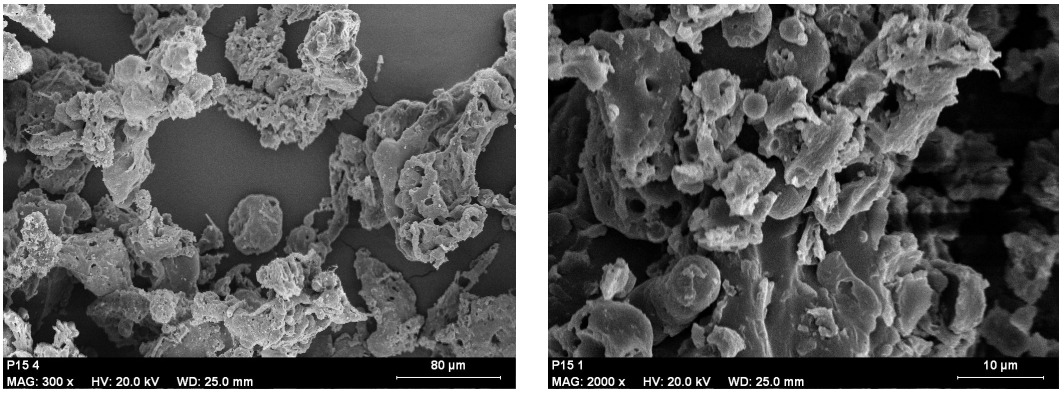
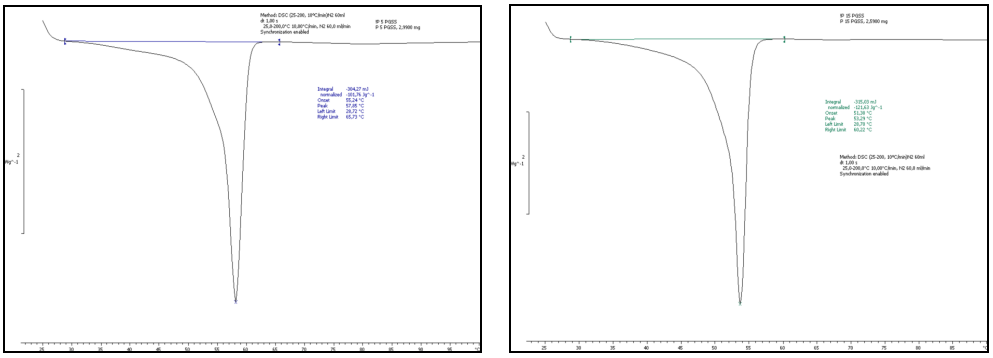


Fig.8. SEM micrographs of the particles obtained by PGSS process of CAPA 2403D (Experiment 15).

3.7. Differential Scanning Calorimetry Assays

DSC analyses of particles obtained in all experiments only showed an endothermic peak, either at 58 ºC in the experiments with CAPA 6100, corresponding to the melting temperature of this

polymer reported in Table 1, or at 54 ºC in the case of experiments with CAPA 2403D, also in agreement with the melting temperature of this polymer. Only minor differences in the heat of fusion of the polymer were observed between unprocessed CAPA 2403D and PGSS-processed samples. These results indicate that no variations in the crystalline structure of the polymer have occurred as a consequence of the PGSS processing. On the other hand, the DCS analysis do not show any peaks corresponding to crystalline β-carotene, which should be observed at 180 ºC, even if DSC analysis were conducted until a temperature of 200 ºC. The melting peak was also not observed in physical mixtures of crystalline β-carotene and polymer, even with β-carotene concentrations as high as 25000 ppm. This result indicates that β-carotene is solubilized in the polymer melt, as observed in the experiments with the view cell described in Section 2.3.



a) b)

Fig. 9. DSC analysis, a) Experiment E5, b) Experiment E15.

4. CONCLUSIONS

The formulation of β-carotene with poly-(ε-caprolactones) by PGSS process was investigated in this work. The particles obtained had a particle size in the range of 270-650 µm using CAPA 6100 (molecular weight: 10 000 g mol^{-1}), and a size of 110-130 µm when CAPA 2403D was used (molecular weight: 4 000 g mol^{-1}). The observed differences in particle sizes can be related to the different solubility of CO_2 in the melted polymers, and to differences in the viscosity of the respective polymer melts. Melted CAPA 2403D presents a lower viscosity than CAPA 6100, making the atomization of CAPA 2403D more effective and allowing smaller particle sizes to be obtained. Moreover, estimations with a PC-SAFT thermodynamic model reveal that the solubility of CO_2 in CAPA 2403D could be higher than in CAPA 6100. With a higher CO_2 content,

the Joule-Thompson cooling effect, which is the driving force for particle formation by PGSS process, becomes more intense, promoting the formation of smaller particles.

It was shown that several process parameters had an important influence on particle size. With regard to the molar ratio, when it was increased the particle size increased as well. Temperature and pressure inside the mixing chamber were other important factors. Bigger particles were obtained when the pressure and temperature inside the mixing chamber were 15 MPa and 50ºC, respectively, compared to the results obtained at 70ºC and 11 MPa. Regarding the β-carotene content, it was low in all cases, but varied considerably with process parameters. As expected, when the molar ratio was increased, the β-carotene content increased as well. The highest β-carotene concentrations (306 - 336 ppm) were obtained at high pressures and temperatures (70ºC and 15 MPa, respectively) and with short homogenization times (60 minutes).

Acknowledgements

Authors thank Vitatene S.A. for providing technical support as well as the crystalline β-carotene used in this work, and Solvay Caprolactones (United Kingdom) for providing the polymers used in experiments. Authors acknowledge the financial support from Conselho de Reitores das Universidades Portuguesas and the Spanish Ministerio de Ciencia e Innovación (PT2009-0173) through the Integrated Actions Portugal–Spain. E. de Paz performed this work with a *FPI-UVa* predoctoral fellowship. A. Martín acknowledges the Spanish Ministry of Science and Innovation for a *Ramón y Cajal* research fellowship.

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Chapter 7

Formulation of β-carotene with soybean lecithin by PGSS (Particles from Gas Saturated Solutions)-drying

Formulation of β-carotene with soybean lecithin by PGSS (Particles from Gas Saturated Solutions) – drying[1]

Abstract

The application of β-carotene as a natural colorant in food and nutraceutical products requires an appropriate formulation in order to protect the active compound from degradation and overcome the low bioavailabity due to a low solubility in aqueous media. Liposomes as carriers for β-carotene can enhance their release to the organism and improve their preservation. In this work, β-carotene has been encapsulated in soybean lecithin using the novel PGSS (Particles from Gas Saturated Solutions) - drying technique. An experimental study of the influence of the main process parameters (pressure, temperature, gas to product ratio and concentration of carrier material) has been carried out. Dry particles of 10-500 μm, constituted by fused spherical particles of less than 10 μm, have been obtained, with β-carotene encapsulation efficiencies up to 60%. By hydration of these particles, β-carotene-loaded multillamelar liposomes of 1 - 5 μm have been obtained.

Keywords: PGSS-Drying, β-carotene, soybean lecithin, supercritical carbon dioxide, liposome, emulsion

[1]The Journal of Supercritical Fluids 72 (2012) 125-133.

1.INTRODUCTION

Some carotenoids such as β-carotene are authorized food ingredients that can be used as natural colorants, with the added value of their biological activity as antioxidants or vitamin precursors. For such applications, carotenoids must be appropriately formulated. Since carotenoids are prone to degradation processes by the action of oxygen, temperature or light, they must be formulated with protecting compounds and/or encapsulated in carrier materials. Furthermore, carotenoids are highly hydrophobic, a property that reduces their bioavailability and makes it difficult to produce stable dispersions in water-based beverages. In order to overcome these limitations, several formulation processes have been developed in order to protect carotenoids from degradation, improve their bioavailability, and produce stable dispersions in water [1, 2].

Liposomes are nanocarriers that are formed spontaneously by self-assembly of phospholipids in water, forming vesicles consisting of an aqueous medium surrounded by a lipid membrane. Liposomes can encapsulate hydrophilic substances in their inner cavity as well as hydrophobic compounds inside the lipid bi-layer. Liposomes can contribute to protect the encapsulated compounds from degradation processes, and they can provide an enhanced release of these compounds due to their capacity to bind to intestinal mucosa and deliver compounds through the membrane of cells [3, 4].

It has been shown that the encapsulation of β-carotene in liposomes can substantially reduce the degradation of β-carotene in aqueous media [5]. β-carotene loaded liposomes have been produced by high-sear homogenization techniques, with encapsulation efficiencies up to 40% [6]. It has been found that the presence of encapsulated β-carotene modify the viscoelastic properties of liposome solutions, reducing their shear-thinning behaviour, which indicates that β-carotene is stored in the lipid bi-layer and interacts with the end caps of the micelles [7].

Supercritical fluids, and particularly supercritical carbon dioxide, are a convenient medium for the production of liposomes loaded with bioactive compounds. Carbon dioxide is a non-toxic solvent and is completely released from the product as a gas upon depressurization to atmospheric conditions. Supercritical carbon dioxide provides an intrinsically inert environment which prevents degradation processes by oxygen. Furthermore, due to the high solubility of organic solvents in supercritical carbon dioxide, these solvents can be efficiently removed from the product by treatment with the supercritical fluid. For these reasons, supercritical carbon dioxide has been widely used for the processing of carotenoids [8] and the production of liposomes [9].

The PGSS (Particles From Gas Saturated Solutions) - drying process is a precipitation technique based on the use of supercritical carbon dioxide as propellant and drying medium [10]. This technique can be used to produce particles from aqueous solutions. The PGSS-drying is an alternative to processes such as spray-drying and freeze-drying. The main advantages of the PGSS-drying method over these conventional techniques are a more efficient atomization, due to the rapid release and expansion of the gas from the solution during depressurization from supercritical to ambient conditions, and the possibility to dry the material with lower temperatures in the spray tower (40-80ºC), which limit the exposition of the bioactive material to detrimental high temperature conditions, and make it possible to use carrier materials that cannot be processed by conventional spray-drying because their melting temperature is too low (as, for example, polyethylene glycols [11], or, in the case of this study, soybean lecithin, whose melting temperature is lower than 80ºC). In a previous work, the PGSS-drying technique was used to encapsulate lavandin essential oil in liposome-forming lecithin [12].

The objective of this work is to study the encapsulation of β-carotene in lecithin by PGSS-drying technique. The influence of the main process parameters on product characteristics (particle size and morphology, encapsulation efficiency) has been studied. Furthermore, the reconstitution of liposomes by hydration of the dry particles obtained by PGSS-drying has been tested.

2. MATERIALS AND METHODS

2.1. Materials

Crystalline β-carotene with a minimum purity of 99% was manufactured by Vitatene SA (León, Spain) using a fermentation process. Soybean lecithin (97% phospholipids) was obtained from Glama-Sot (SOTYA, Madrid, Spain). Carbon dioxide was provided by Carburos Metálicos (Barcelona, Spain). Dichloromethane (purity > 99.8%) was purchased from Panreac Química (Barcelona, Spain).

2.2. Preparation of organic-on-water β-carotene emulsions and aqueous β-carotene suspensions

A dissolution of lecithin in water was prepared at 50ºC with continuous agitation using a magnetic stirrer. The selected concentration of soybean lecithin in water was 62 g/L. However, a few experiments were carried out with concentrations of 55 and 72 g/L in order to observe

the effect of this parameter. Afterwards, 0.13 g of β-carotene was dissolved in 20 mL of dichloromethane and then added to 400 mL of the dissolution of lecithin. The mixture was stirred during 5 minutes in order to obtain a macroemulsion. After that, the macroemulsion was fed into a rotor-stator machine (IKA LABOR PILOT 2000/4, Staufen, Germany) whose capacity is 200 mL and the macroemulsion was processed during 2 minutes for fine emulsification. Finally, the dichloromethane was removed from the emulsion using a rotary evaporator (BÜCHI 011-BÜCHI 461 Water Bath), thus obtaining an aqueous dispersion of β-carotene particles free from organic solvent.

2.3 Precipitation of lecithin by PGSS-drying

The aqueous suspensions of β-carotene were processed using the PGSS-drying technique in order to remove water and precipitate the dissolved lecithin over the preformed β-carotene particles. Figure 1 presents the schematic flow diagram of the PGSS-drying apparatus. This system was previously described by Varona et al. [12].

The PGSS-drying technique consists on saturating an aqueous solution with carbon dioxide using a static mixer operating at high pressure (above the critical pressure of CO_2, 7.38 MPa), and then expanding this gas-saturated solution down to atmospheric pressure through a nozzle. The depressurization causes a sudden vaporization of the dissolved CO_2 as well as a volumetric expansion of the gas bubbles. Both effects contribute to improve the atomization of the sprayed solution forming small droplets, thus enhancing the subsequent drying and allowing to produce correspondingly small particles [13]. In order to achieve a complete drying of particles, the conditions in the spray tower (particularly, temperature and gas-to-product ratio, GPR) must be fixed in order to operate above the dew line of the carbon dioxide - water system [11, 13].

In the experimental apparatus shown in Figure 1, CO_2 was fed by a membrane pump (Milton Roy, maximum flow rate 15 kg CO_2/h) and preheated using electrical resistances before introducing it into the static mixer, where it was mixed with the aqueous dispersion of β-carotene and soybean lecithin at high pressure and temperature. The flow rate of CO_2 was determined with a Coriolis flow meter (Sensor MICRO Motion Elite CMF010 NB, Transmitter MICRO Motion Elite RFT91) with an accuracy of ±0.01 kg/h, and temperature after the electrical preheater was measured with a Pt100 thermoresistance with an accuracy of ±0.1 K and controlled using a PID controller. On the other hand, the aqueous dispersion of β-carotene was pumped to the static mixer using a GILSON 305 piston pump (maximum flow rate: 25 mL/min, flow control with an accuracy of 0.1 mL/min). Temperature before and after the static

mixer was measured using Pt100 thermoresistances, and pressure in the static mixer was measured with a DESIN TPR-10 digital pressure meter (DESIN Instruments, Spain, accuracy of ±0.05 MPa). Expansion of the gas-saturated solution into the spraying tower at atmospheric pressure was accomplished using a capillary nozzle (Spraying Systems, nozzle diameter 500 μm). Temperature in the spray tower was also determined with a Pt100 probe. Particles were collected using a metallic cloth filter (pore diameter 1 - 5 μm) attached inside the spray tower.

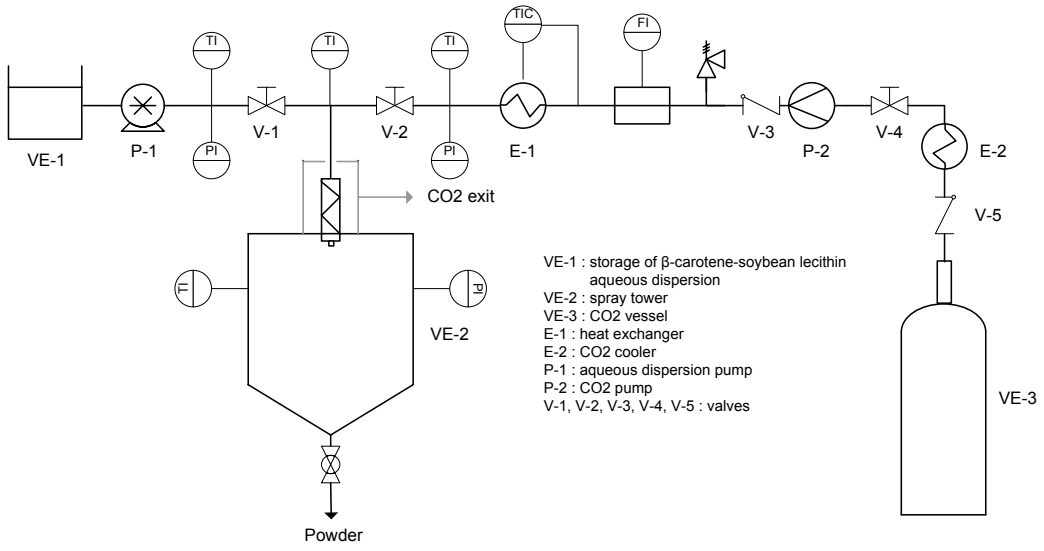

Figure 1. Flow diagram of the PGSS-drying equipment.

A typical experiment with the PGSS-drying process began with the preheating of the system until a desired temperature in the static mixer. This temperature was varied from 100 to 130ºC in order to observe the effect of the pre-expansion temperature on powder characteristics. When the temperature was achieved, CO_2 was pumped and the system was pressurized until a selected pressure. This pressure in the static mixer (pre-expansion pressure) was modified from 8 to 10 MPa (varying the CO_2 flowrate and therefore the pressure drop in the nozzle) in order to study its influence on powder characteristics. Then the suspension pump was started and the suspension flow rate was adjusted in order to obtain the desired gas to product ratio (GPR). This parameter was varied between 21 and 37 g/g. The experiment was run during a time of 60 min (volume of suspension processed: 1600 mL) in order to ensure steady state conditions. After this, the system was depressurized and particles were collected from the filter in the spray tower and stored in a refrigerator (temperature < 5ºC) for subsequent analyses. Table 1 presents a summary of tested process conditions.

Table 1. Summary of operating conditions.

	T_{mixer} (K)	$P_{pre\text{-}exp}$ (MPa)	GPR	$C_{soybean\ lecithin}$ (g/L)
E 1	102	8.1	27	62
E 2	111	8.3	27	62
E 3	121	8.9	27	62
E 4	115	10.2	27	62
E 5	123	8.2	27	62
E 6	112	8.9	27	62
E 7	132	9.0	27	62
E 8	110	10.0	27	62
E 9	104	10.0	27	62
E 10	121	8.8	27	62
E 11	116	10.0	27	72
E 12	115	10.1	27	55
E 13	114	10.1	32	62
E 14	113	10.3	21	62

2.4. Product characterization

2.4.1. Yield of collected particles

The yield of the particles collected by PGSS-drying process was determined by weighing as the amount of the obtained powder particles in the spray tower divided by the amount of solids in the dispersion pumped to the system (grams of soybean lecithin plus grams of β-carotene).

2.4.2. Percentage of encapsulated β-carotene

A sample of the obtained powder was dissolved in water and analysed by a UV/VIS spectrophotometer model HITACHI U-2000. The wavelength selected was 456 nm. The absorbance determined with this method is proportional to the amount of β-carotene dispersed in solution. The ratio of this concentration, corresponding to the amount of β-carotene stabilized in the solution, to the total β-carotene concentration in the product, is reported in this work as percentage of encapsulated β-carotene.

2.4.3. Particle size and particle size distribution (PSD)

The particle size analysis was carried out by a Dynamic Light Scattering (DLS) equipment model Malvern Mastersizer 2000.

2.4.4. Microscopy

Micrographs of the particles collected by PGSS process were taken by means of an environmental scanning electron microscope (ESEM) model FEI-Quanta 200 FEG. Particles were coated with iron before observation and analyzed with an accelerating voltage of 10 kV and magnification ratios ranging from 100x to 2000x. Hydrated liposomes were observed using an optical microscope (Leica DM400 B, Wetzlar, Germany).

3. RESULTS AND DISCUSSION

Table 2 presents the main experimental results obtained in each experiment, including the yield of the collected particles in the spray tower, the fraction of encapsulated β-carotene, dry particle size, and size of hydrated liposomes. It can be observed that the typical results obtained were dry particles with a size of 10 - 500 μm with an encapsulation efficiency of 30-60%, that after hydration produce liposomes of 1 - 6 μm. The low yields of particle collection (20-50%) are associated with experimental difficulties related to the recollection of particles from the metallic cloth filter rather than with intrinsic limitations of the process.

Table 2. Summary of experimental results.

	Yield of collected particles (%)	% encapsulated β-carotene	Mean particle size (μm)	Liposome particle size (μm)
E 1	37.6	34.4	20.8	6.1
E 2	34.3	42.9	408.8	3.0
E 3	19.2	56.6	124.7	2.2
E 4	34.3	49.1	39.1	2.8
E 5	34.5	44.5	59.3	1.8
E 6	29.4	29.0	57.1	1.3
E 7	44.9	49.1	203.4	1.4
E 8	38.4	39.9	13.7	2.0
E 9	36.5	40.0	22.6	1.1
E 10	34.9	44.5	13.4	1.9
E 11	31.3	47.7	24.1	1.9
E 12	23.0	35.5	448.9	1.7
E 13	40.8	58.7	87.4	1.9
E 14	35.7	51.8	26.3	0.9

3.1 Emulsification and emulsion evaporation

Figure 2 present the size distributions of the emulsion and the aqueous dispersion of particles obtained after vacuum evaporation of the organic solvent from the emulsion. As shown in this figure, an emulsion with an average droplet size ($d_{0.5}$) of 11.4 μm is obtained. As shown in Figure 2, the mean size is preserved after the formation of the aqueous dispersion of particles by vacuum evaporation, although a more pronounced peak in the particle size range near 1 μm is observed after the vacuum evaporation.

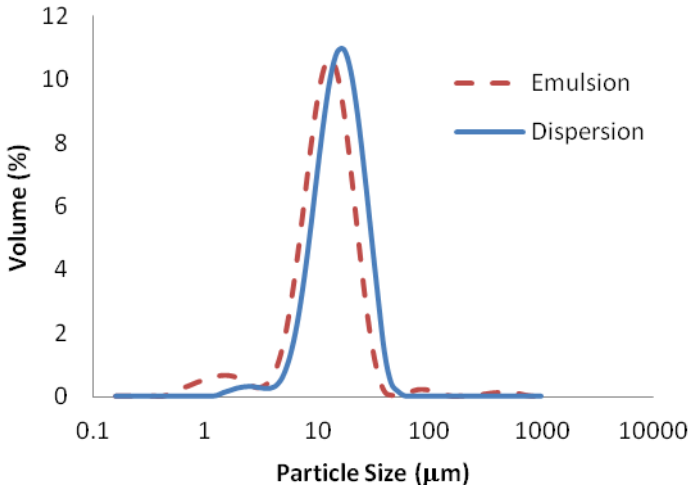

Figure 2. Particle size distributions of o/w emulsion and β-carotene dispersion.

Figure 3 presents micrographs of the suspension obtained after vacuum evaporation of the organic solvent. As shown in this figure, two types of structures can be observed. On the other hand, agglomerates of particles are observed are observed encapsulated inside vesicles. The main size of these micelles, measured by image analysis of the micrographs, is 13.4 μm. Therefore they correspond to the main peak of the particle size distribution presented in figure 2. The agglomerates of particles are mostly constituted by smaller particles of β-carotene of less than 1 μm, although some larger β-carotene crystals can also be observed (see, for example, figure 3a). These structures probably result from the preservation of the emulsion template during the solvent evaporation. On the other hand, some isolated particles, as well as smaller liposomes, are also observed (see figure 3d). The main size of these smaller liposomes obtained by image analysis is 1.3 μm, therefore corresponding to the secondary peak of the particle size distribution (figure 2).

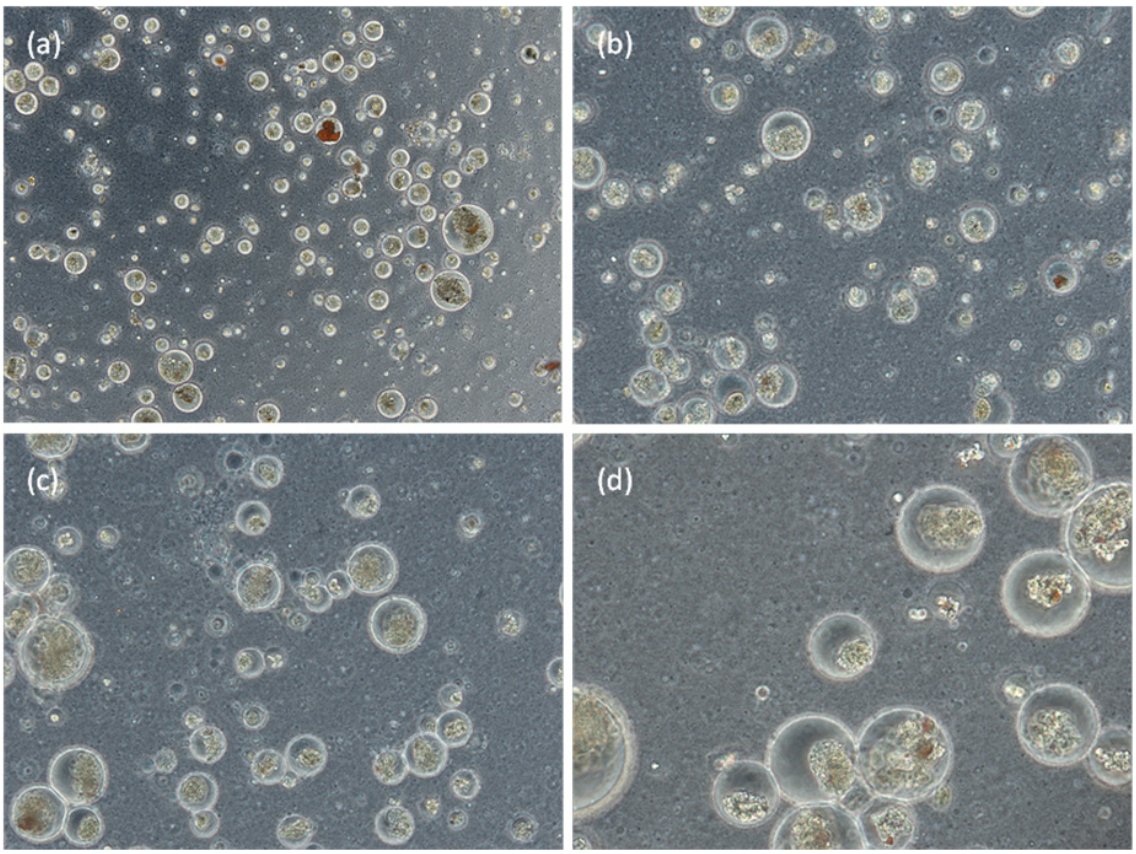

Figure 3. Micrographs of suspension after vacuum evaporation of the organic solvent, obtained with an optical microscope and magnification ratios of 10x (a), 20x (b and c) and 40x (d).

3.2 Particle size and morphology

Figure 4 presents the variation of the mean particle size with different process parameters (pre-expansion temperature, pre-expansion pressure, GPR and concentration of lecithin). As presented in this figure, smaller particle sizes were obtained when pre-expansion pressure or concentration of lecithin were increased, or when pre-expansion temperature and GPR were decreased.

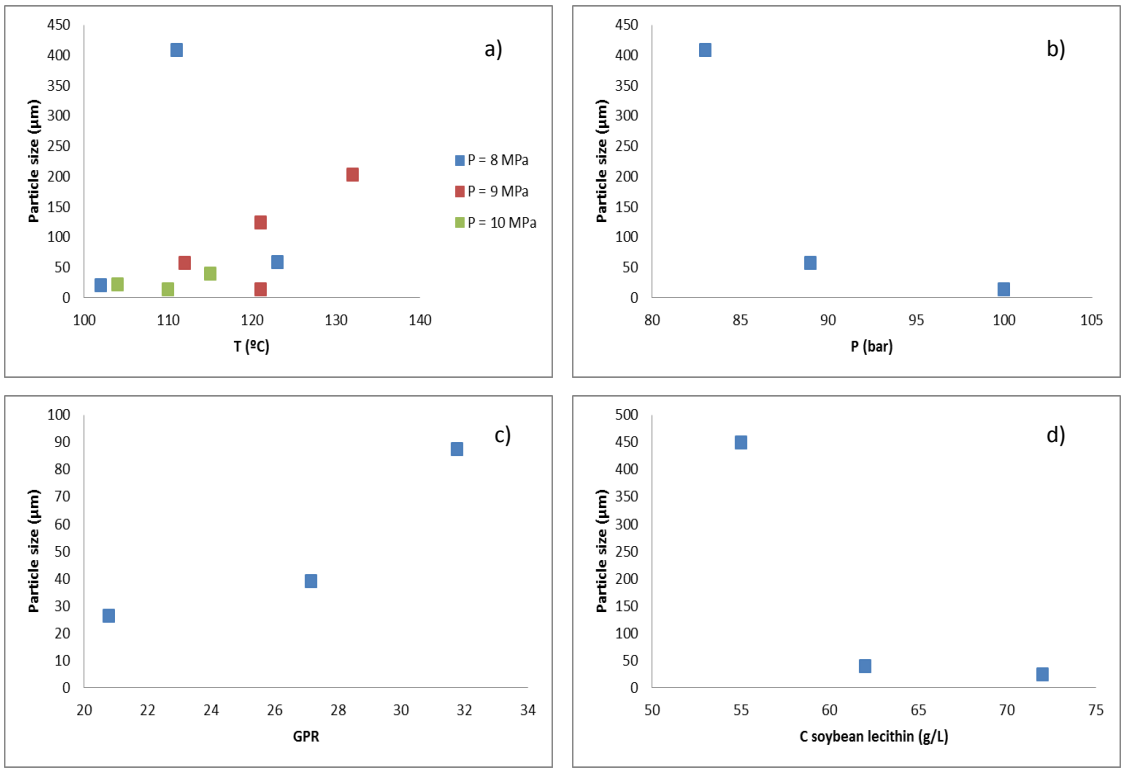

Figure 4. Variation of particle size with: pre-expansion temperature (gas-to-product ratio: 27kg/kg, concentration of soybean lecithin: 62 g/L, pre-expansion pressure: 8, 9 and 10 MPa) (a), pre-expansion pressure (gas-to-product ratio: 27 kg/kg, concentration of soybean lecithin: 62 g/L, pre-expansion temperature: 115ºC) (b), gas-to-product ratio (concentration of soybean lecithin: 62 g/L, pre-expansion temperature: 115ºC, pre-expansion pressure: 10 MPa) (c) and concentration of soybean lecithin (gas-to-product ratio: 27 kg/kg, pre-expansion temperature: 115 ºC, pre-expansion pressure: 10 MPa (d).

As previously discussed, the main mechanism for the atomization of the solution and the subsequent formation of particles is the saturation of the solution with carbon dioxide, and the sudden vaporization and expansion of the gas during the depressurization in the nozzle. In a previous work, an analysis of experimental results of micronization of polyethylene glycol by PGSS-drying showed a relationship between the saturation concentration of carbon dioxide in the solution and particle size [11, 12], indicating that if the concentration of carbon dioxide in the gas-saturated solution is increased, the atomization is more effective leading to the production of smaller particles.

Figure 5 presents the estimated solutility of carbon dioxide in water in the range of process conditions considered in this work, expressed as the molar fraction of carbon dioxide in the mixture (x_{CO2}). Results presented in this figure have been calculated with the PC-SAFT equation

of state [14]. The parameters required to apply the PC-SAFT equation to this system were obtained and validated in a previous work [15].

As shown in Figure 5, within the range of process conditions considered in this work, the solubility of carbon dioxide in water increases when pressure is increased at constant temperature, and decreases when temperature is increased at constant pressure. Furthermore, pre-expansion pressure and temperature fix the thermodynamic conditions during the expansion path in the nozzle. Figure 6 present the calculated temperature after the expansion, obtained with the PC-SAFT equation assuming an isoenthalpic expansion according to the method described in a previous work [13]. With this information, the volume expansion of carbon dioxide can be calculated with the equation of state. This information is presented in Figure 7 as the ratio of gas specific volumes (kg/m^3) after and before the expansion (V post expansion/V pre expansion). As presented in this figure, the expansion ratio considerably increases when pre-expansion pressure is increased, and slightly decreases when pre-expansion temperature is increased.

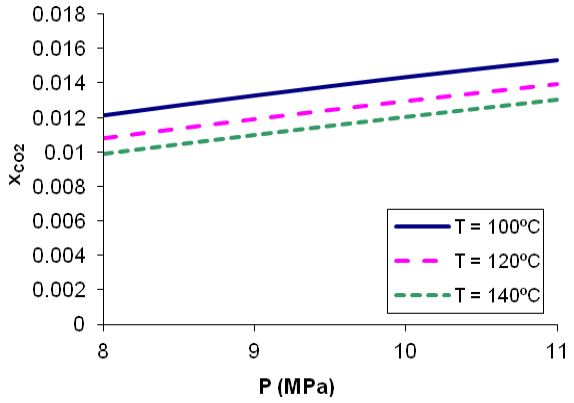

Figure 5. Variation of the solubility of carbon dioxide (molar fraction, x_{CO2}) in water as a function of pre-expansion pressure and temperature. Results calculated with the PC-SAFT equation of state.

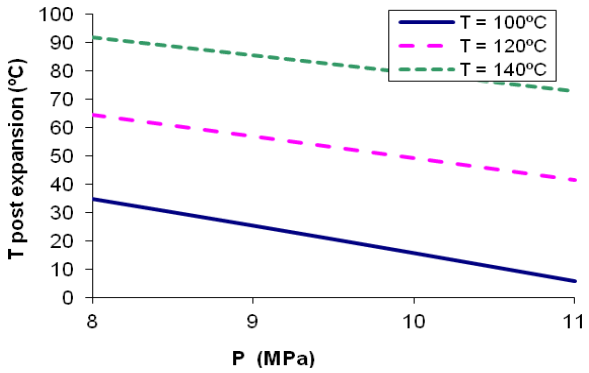

Figure 6. Variation of the post expansion temperature as a function of pre-expansion pressure and temperature. Results calculated with the PC-SAFT equation of state.

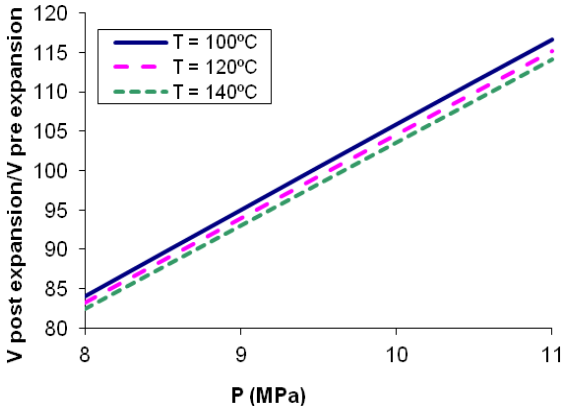

Figure 7. Variation of the volume expansion ratio of carbon dioxide (V post-expansion / V pre-expansion) as a function of pre-expansion pressure and temperature. Results calculated with the PC-SAFT equation of state.

The atomization can be expected to be more effective when the amount of carbon dioxide dissolved in the solution as well as the volumetric expansion ratio are increased. As shown in Figures 5 and 7, both parameters increase when pre-expansion pressure is increased and pre-expansion temperature is decreased, which as shown in Figure 4 correspond to the conditions which allowed producing smaller particles. Therefore this variation in results can be justified by an improvement of the atomization. Similar results were observed in the micronization of polyethylene glycol by PGSS-drying [11], while in experiments of encapsulation of lavandin essential oil in lecithin by PGSS-drying particle size also decreased when pressure was increased, but no variation or a small reduction of particle size was observed when pre-expansion temperature was increased [12].

With respect to the influence of the gas-to-product ratio (GPR), the trend observed in this work (increase of particle size with higher GPR) does not agree with previous results, which indicate a reduction of particle size with higher GPR [11,12]. Variations of GPR cause several effects: increasing the GPR ratio allows to accelerate the mass transfer in the static mixer, thus increasing the amount of CO_2 dissolved and improving the atomization [12]. On the other hand, with a higher GPR, more water is extracted by CO_2 in the static mixer, producing a more concentrated solution, which therefore is more viscous and more difficult to atomize. Indeed, according to model results presented in a previous work [12], it can be estimated that in the experimental conditions of this work up to 70% of water in the solution is extracted by CO_2 in the static mixer. Nevertheless, it must be pointed out that the range of GPR explored in this work is narrow compared to previous studies (from 21 to 32 g/g in this work, compared to 10 - 80 g/g in ref. [11] and 10 - 40 g/g in ref.[12]).

Finally, increasing the concentration of lecithin has several contraposed effects on the process: as a fatty substance, lecithin can act as a co-solvent for carbon dioxide, thus increasing the solubility of CO_2 in the solution and enhancing the atomization, while higher concentrations of lecithin increase the viscosity of the solution and make it more difficult to atomize. While in this work a reduction of particle size was observed with higer concentrations of lecithin, the opposite trend was observed in a previous work of encapsulation of lavandin oil in lecithin [12]. These contradictory results may be justified by the different operating pressures applied: 10 MPa in this work compared to 7 MPa in the previous reference. With an increased pressure, the effect of enhancement of CO_2 solubility may become dominant over the influence of the increased viscosity, thus allowing to produce smaller particles.

Figure 8 presents ESEM micrographs of particles obtained in several experiments. Micrographs a, b and c show different views of particles obtained in experiment 3 of Table 1. It can be seen that particles are constituted by spheres of about 10 μm which are highly agglomerated and fused together. The same morphology are observed in all experiments, as for example in experiment 5 (micrograph d and close-up view in micrograph e). Even in experiments in which the mean particle size was much higher the same basic morphology was observed. For example, micrograph f shows particles obtained in experiment 12. As reported in Table 2 and observed in this micrograph, a mean particle size over 400 μm was obtained in this experiment, but particles are still constituted by agglomerates of partially fused spheres. Compared to this result, in the previous work of encapsulation of lavandin oil in lecithin [12],

the same basic morphology of 10 μm spheres was obtained, but with a much lower degree of agglomeration.

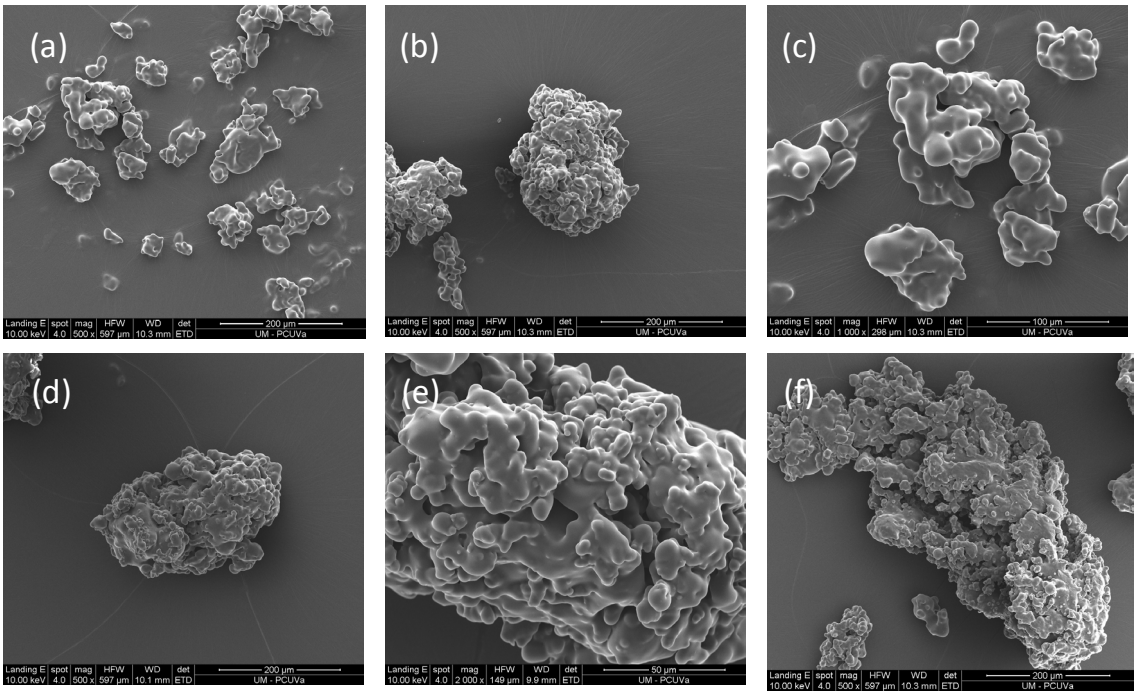

Figure 8. ESEM microcraphs of particles obtained in several experiments: experiment 3 of Table 1, magnification ratio 500x (a); experiment 3, magnification ratio 500x (b); experiment 3, magnification ratio 1000x (c); experiment 5, magnification ratio 500x (d); experiment 5, magnification ratio 2000x (e); experiment 12, magnification ratio 500x.

3.3 Encapsulation efficiency

Figure 9 show the variation of the encapsulation efficiency of β-carotene in lecithin with process conditions. As shown in this figure, the encapsulation efficiency increased when the pre-expansion temperature was increased. The encapsulation efficiency also increased when the concentration of lecithin was increased. This result showed no clear trend of variation with the remaining process parameters.

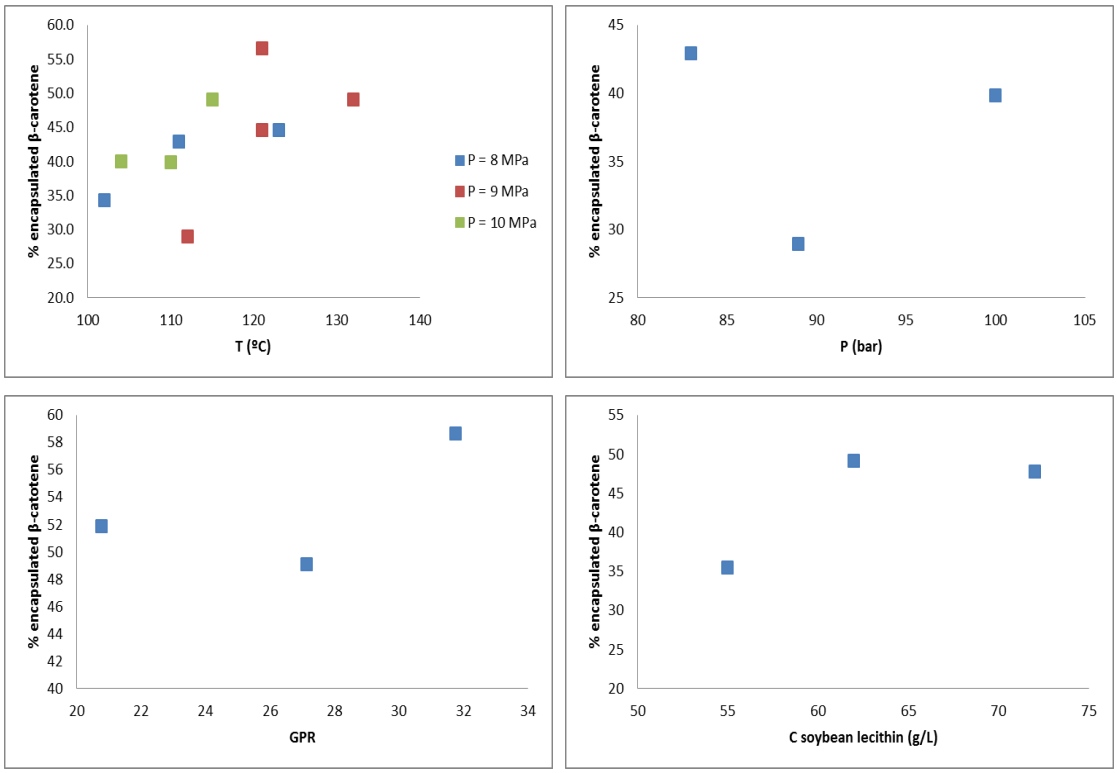

Figure 9. Variation of the efficiency of encapsulation of β-carotene in lecithin with pre-expansion temperature (gas-to-product ratio: 27kg/kg, concentration of soybean lecithin: 62 g/L, pre-expansion pressure: 8, 9 and 10 MPa) (a), pre-expansion pressure (gas-to-product ratio: 27 kg/kg, concentration of soybean lecithin: 62 g/L, pre-expansion temperature: 115 ºC (b) gas-to-product ratio (concentration of soybean lecithin: 62 g/L, pre-expansion temperature: 115 ºC, pre-expansion pressure: 10 MPa (c) and concentration of soybean lecithin (gas-to-product ratio: 27 kg/kg, pre-expansion temperature: 115 ºC, pre-expansion pressure: 10 MPa (d).

In a previous work of encapsulation of lavandin essential oil by PGSS-drying, an increase of encapsulation efficiency was also observed when the pre-expansion temperature was increased [12], although the characteristics of the encapsulated materials in the two cases are very different (liquid oil droplets in the previous study compared to solid particles in this work). Nevertheless, it can be considered that, in both cases, a basic requirement for a high encasulation efficiency is a good dispersion of the material to be encapsulated (particles or oil droplets) within the carrier matrix in the static mixer. If the pre-expansion temperature is increased, more water is extracted in the static mixer because the solubility of water in CO_2 increases with temperature [15]. According to model results [13], in the conditions tested in this work the fraction of water extracted in the static mixer increases from 40% with a pre-

expansion temperature of 100ºC, to 70% with a pre-expansion temperature of 120ºC. With a concentrated solution of lecithin already formed in the static mixer, particles or oil droplets can more easily be surrounded by a shell of carrier material that can be mantained upon drying in the spray tower, leading to the prodution of microcapsules and an increase of the encapsulation efficiency.

With respect to the variation of the encapsulation efficiency with the remaining process parameters, in the encapsulation of lavandin oil these variations were associated with loss of efficiency due to a partial extraction of the oil by supercritical carbon dioxide (i.e., reduction of encapsulation efficiency when pressure or GPR were increased since more oil was extracted with these conditions [12]). This effect of the process conditions is not observed in this work since the solubility of β-carotene in CO_2 in the conditions of this study is very low [16].

3.4 Rehydration of liposomes

Figure 10 presents microscopic images of rehydrated liposomes. Images obtained with low magnification ratios (e.g., Figure 10 a) allow to clearly identify large crystalline particles of β-carotene, which are not stabilized inside the liposomes and correspond to the fraction of non-encapsulated β-carotene reported in Table 2. In contrast with these large particles, areas with diffuse orange-red color, which are located in the borders of vesicles, probably correspond to smaller particles of β-carotene which are encapsulated in the hydrophobic region inside the lipid bilayer of liposomes (see Figure 10 a and b). In all experiments, formed liposomes show a certain dispersion of size, with average sizes between 1 and 5 μm (Figure 10 c). Enlargements of the images show that liposomes present multilamellar structures (Figure 10 d). In contrast with the results obtained directly after vacuum evaporation of the organic solvent from the emulsion (Figure 3), in the rehydrated pruduct large agglomerates of particles encapsulated in the inner aqueous cavity of the vesicles were not observed. Similar sizes and structures of liposomes were observed in a previous work of encapsulation of lavandin oil in lecithin [12].

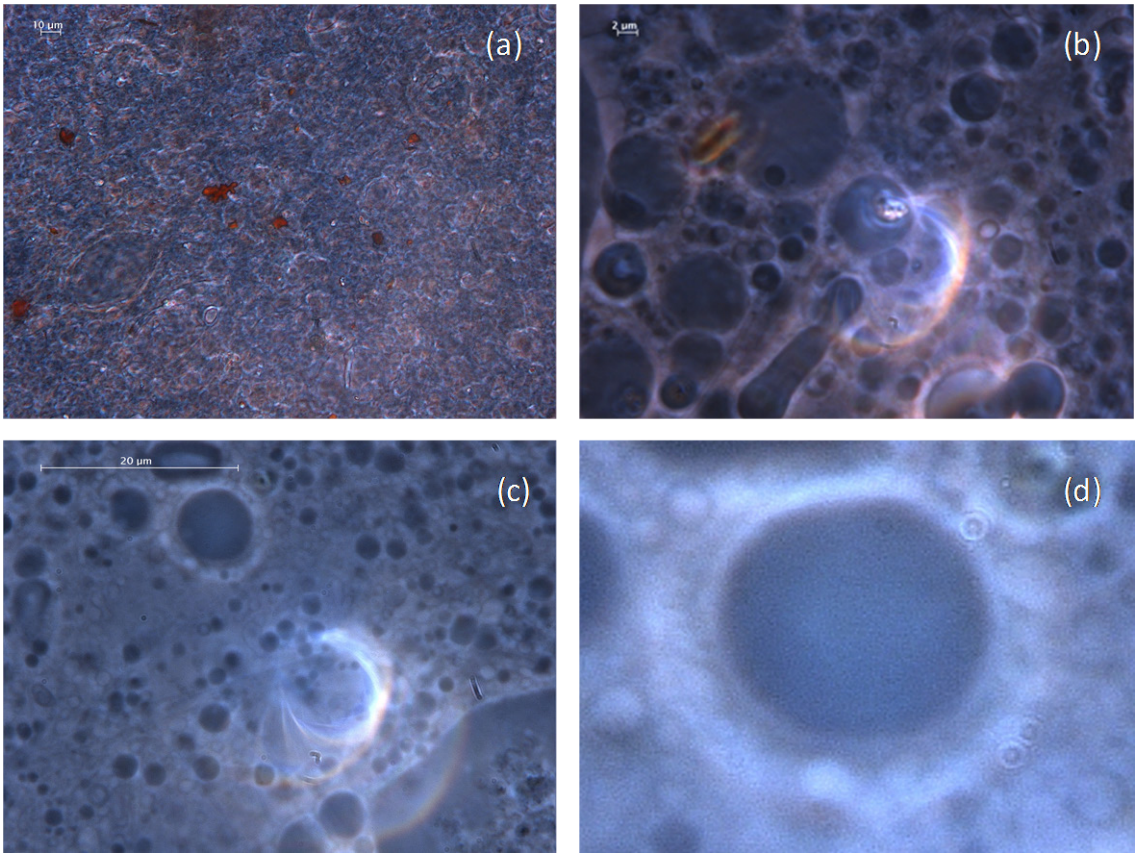

Figure 10. Microscopic images of rehydrated liposomes: experiment 1 (a); experiment 2 (b); experiment 10 (c); enlargement of a liposome (d).

Figure 11 presents pictures of dry particles and rehydrated solutions. While particles show the typical orange colour of β-carotene, suspensions show a homogeneous yellow colour. This can also be appreciated in the absorbance spectrum obtained by UV/VIS spectrophotometry, presented in Figure 12. As shown in this figure, the spectrum presents a high absorbance in the range 400 - 450 nm, with absorption peaks at 435 nm and 456 nm, characteristic of a yellow tonality, but shows a small absorbance in the range of 500 - 550 nm which would be required for a orange - red colour. The colour of β-carotene suspensions depend on the morphology of the particles, with a displacement from red colour in suspensions of crystalline particles, through orange, to yellow for suspensions of amorphous particles [1]. Thus, the colour observed is consistent with the morphology of the rehydrated product, in which stabilized crystalline particles of β-carotene were not observed. In comparison, by an alternative technique of precipitation from pressurized ethyl acetate - on - water emulsions, which allowed to produce crystalline particles of β-carotene encapsulated in starch, the rehydrated

suspensions showed significative absorbance peaks near 515 nm, resulting in orange-red colour [2].

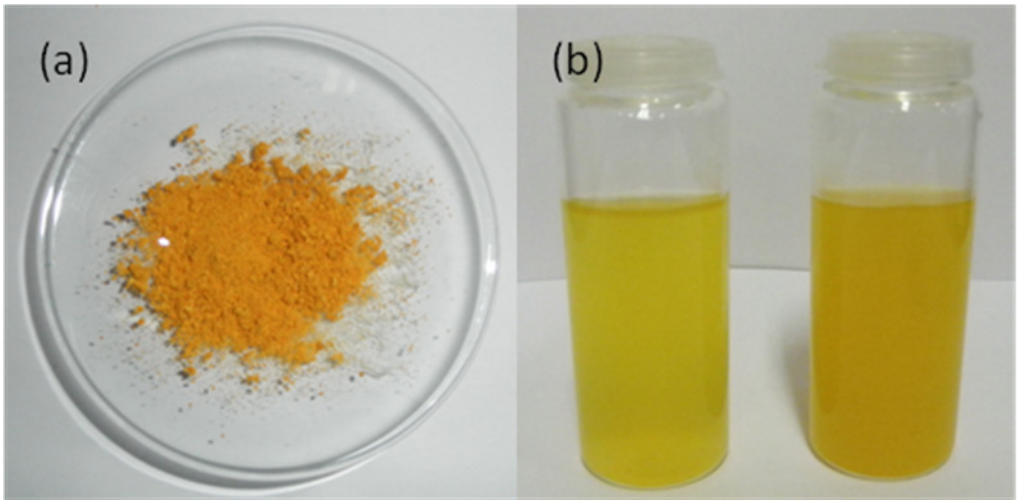

Figure 11. Picture of dry particles (a) and rehidrated solutions with a concentration of particles of 2.4 g/L (estimated β-carotene concentration: 6 ppm) (left) and 6 g/L (estimated β-carotene concentration: 15 ppm) (right) (b). (For interpretation of the references to color in the text, the reader is referred to web version of the article).

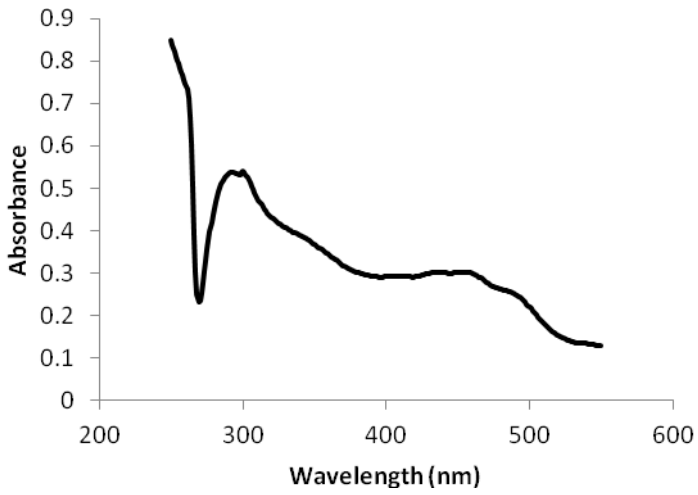

Figure 12. UV/VIS absorbance spectrum of rehydrated particles.

4. CONCLUSIONS

β-carotene has been encapsulated in soybean lecithin by PGSS-drying, obtaining particles of 10 - 500 μm constituted by agglomerated spheres, with encapsulation efficiencies of β-carotene up to 60%. The influence of process variables on particle size can be correlated with the atomization process, which is enhanced when the amount of carbon dioxide dissolved in the solution and when the volumetric expansion ratio in the nozzle are increased. With respect to the encapsulation efficiency, a strong influence of temperature has been observed, which was also shown in previous works with different active compounds. This influence can be related to the formation of a solution more concentrated in carrier material in the static mixer. By rehydration, multilamellar liposomes as well as large particles corresponding to the non-encapsulated β-carotene were obtained.

Acknowledgements

Authors thank VITATENE S.A. (León, Spain) for providing technical support and materials used in this work. Authors acknowledge the financial support from the Spanish Ministry of Economy and Competitiveness (PT2009-0173). E. de Paz performed this work with an FPI-UVa research fellowship. Á Martín thanks the Spanish Ministry of Economy and Competitiveness for a Ramón y Cajal research fellowship.

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Chapter 8

Solubility of β-carotene in different poly-(ε-caprolactones) in colloidal state

Solubility of β-carotene in different poly-(ε-caprolactones) in colloidal state

Abstract

β-carotene is one of the most common pigments in nature. In food applications, β-carotene formulations are very attractive because they add value to the product due to their antioxidant and pro-vitamin activities. On the other hand, miscibility and solubility data of the active compound in the carrier material are relevant for the design and development of optimum formulations. This work presents a study of the solubility of β-carotene in poly-(ε-caprolactones) with different molecular weights (between 4000 and 25000 g mol^{-1}) in colloidal state in the range of temperature between 10ºC and 50ºC. The determination of the solubility of β-carotene was carried out by two different processes: equilibration-impregnation and equilibration-de-supersaturation. Results obtained by impregnation showed that β-carotene content impregnated into the PCLs increased gradually when the temperature was increased, obtaining maximum β-carotene contents between 87 and 191 ppm depending on the molecular weight of the polycaprolactone. However, results obtained by de-supersaturation experiments showed β-carotene contents considerably higher than those achieved in impregnation experiments, obtaining a maximum β-carotene content of 8800 ppm when polycaprolactone with the highest molecular weight was used. It is due to the slow release of entrapped β-carotene from PCL particles, so the equilibration by de-supersaturation approach is not suitable for the determination of equilibrium concentrations of β-carotene in PCLs.

Keywords: β-carotene, poly-(ε-caprolactones), supercritical extraction of emulsions, carbon dioxide, impregnation, de-supersaturation.

1. INTRODUCTION

Carotenoids are some of the most common pigments in nature, being the most abundant β-carotene, lycopene, lutein and zeaxanthin. The main roles of carotenoids in human diet are as precursors of vitamin A and as antioxidants [1]. Carotenoids can be found in vegetables, fruits, leaves, fish and other sea products. When they are consumed in sufficient levels, they have been claimed to have biological activities that may reduce the risk of certain chronic diseases, such as cardiovascular disease, age-related macular degeneration and cataracts [2]. Their utilization as nutraceutical ingredients within foods is currently limited because of their poor solubility in water, high melting point, chemical instability and low bioavailability [3].

For many industrial applications, a mixture of the carotenoid with a polymer is used. Covering carotenoids with polymers provides protection against oxidation and degradation process [1]. Moreover, due to the low solubility in water, for the use of carotenoids as natural colorants in beverages, a formulation of the active compound is required with a restricted particle size and with an appropriate colour intensity which depends on the properties of particles [4]. There are several techniques for carrying out the entrapment of active compounds into polymeric particles, including conventional methods such as spray drying, o/w emulsion solvent extraction/evaporation or w/o/w emulsion solvent evaporation technique [5].

The application of supercritical fluids for particle formation has been an active field of research and innovation during the past two decades [6-8]. The main motivation for this is the possibility of using the peculiar properties of supercritical fluids, and particularly of supercritical carbon dioxide [9]. In particular, the Supercritical Fluid Extraction of Emulsions (SFEE) technique has been successful in the formulation of β-carotene using a dichloromethane in water emulsion obtaining a suspension of sub-micron carotene particles [10] and also in the formulation of micro- and nanoparticles of water insoluble pharmaceutical polymers such as poly(lactic-co-glycolic) acid (PLGA) [11].

Poly-(ε-caprolactone) (PCL) is a synthetic biocompatible semi-crystalline polymer. PCL exhibits a low glass transition temperature (from -60 to -10 ºC depending on its molecular weight). The rubbery characteristics of PCL results in high permeability which has been exploited for delivery of low molecular weight drugs such as steroids and vaccines [5, 12]. Due to its slow degradation, PCL is ideally suitable for long-term delivery. This has led to its application in the preparation of different delivery systems in the form of microspheres, nanospheres and implants. Various categories of drugs have been encapsulated in PCL for targeted drug delivery and for controlled drug release [5]. PCLs are insoluble in water and degrade slowly, making

them suitable as encapsulating agents if a slow delivery of the active compound is required, or a targeted delivery to the intestinal tract is intended [13].

On the other hand, miscibility and solubility data of the active compound and polymer are relevant for the design and development of optimum formulations. However, there is still a lack of such data, as well as of robust and reliable experimental methods to generate such information in an accurate and efficient manner. An important step is the determination of the concentration of active compound present in the polymer phase and if the equilibrium was achieved with the active compound in the crystalline state (i.e. the solubility of the drug in the polymeric matrix) [14]. Kluge et al. [11, 14] demonstrated that solvent-free PLGA nanoparticles obtained by supercritical fluid extraction of emulsions (SFEE) may be used to investigate the equilibration of PLGA with crystalline Ketoprofen.

Following this investigation line, in this work the solubility of β-carotene in poly-(ε-caprolactones) with different molecular weights has been analyzed. For this purpose, the experimental procedure presented by Kluge et al. [11, 14] has been used, consisting of processing the polymeric nanoparticles with SFEE process and carrying out the equilibration in a thermal shaking bath.

2. MATERIALS AND METHODS

2.1. Materials

Crystalline β-carotene with a minimum purity of 99% was manufactured by Vitatene (León, Spain) using a fermentation process. Poly-(ε-caprolactones) (PCLs) were kindly supplied by Perstorp UK Limited (United Kingdom). Three different commercial poly-(ε-caprolactones) were used: CAPA 2403D, CAPA 6100 and CAPA 6250. Physical properties of PCLs used in this work are summarized in Table 1. Dichloromethane and tetrahydrofuran, with a minimum purity of 98.0% and 99.5% respectively, and poly(vinyl alcohol) 4-88 (PVA) were purchased from Sigma-Aldrich (Buchs, Switzerland). Carbon dioxide (purity: 99.9%) was provided by PanGas (Schlieren, Switzerland). All products were used as received.

Table 1. Physical properties of poly-(ε-caprolactones) used in this work (data provided by the supplier).

PCL	Molecular Weight ($g \cdot mol^{-1}$)	Melting Temperature (ºC)
CAPA 2403D	4000	50 - 60
CAPA 6100	10000	58 - 60
CAPA 6250	25000	60 - 70

2.2. Preparation of the emulsion

For the impregnation process, which is described in section 2.5, the poly-(ε-caprolactones) were dissolved at 5 wt.% in dichloromethane. For the equilibration-de-supersaturation process, which is described in section 2.6, a predetermined amount of β-carotene was added to the dichloromethane solution of polycaprolactone which was at 5 wt.%, keeping constant a ratio β-carotene: PCL in weight of 1:30. A 1 wt.% solution of PVA was prepared in water saturated with dichloromethane. The organic and aqueous solutions were mixed at a proportion 20:80 wt.%, respectively, to form an oil-in-water (o/w) emulsion upon ultrasonication with a Branson Sonifier 450. For each 100 mL of emulsion, a sonication time of 2 minutes was applied with a duty cycle of 50% and an amplitude of 20% while the emulsion was being cooled on ice.

2.3. Supercritical fluid extraction from emulsions (SFEE) process and procedure

Directly after the preparation of the emulsion, the organic solvent was extracted from the emulsion using supercritical carbon dioxide by SFEE process. A scheme of the experimental setup used for the SFEE experiments as well as a detailed description of the process can be found in a previous study [11]. The operating temperature and pressure inside the reactor were selected at 45ºC and 8 MPa, respectively. The formation of the particles of polymer takes place by the solvent extraction from the emulsion droplets and these particles remain suspended in the continuous water phase.

A typical experiment of SFEE started with the filling of CO_2 in the reactor. Then, the feed of the streams was started and backpressure regulators were are adjusted. When the conditions of operating pressure and temperature were achieved (8 MPa and 45ºC), water was pumped to the reactor until steady state was reached. Afterwards, the emulsion was fed to the reactor for carrying out the extraction of the organic solvent from the emulsion. When the desired amount of emulsion had been processed, the line was purged with water and after that, CO_2

feed was stopped. The suspension of particles in the aqueous media was collected, and experiment finished with the depressurization of the reactor.

2.4. Preparation of β-carotene crystals

β-carotene crystals were used for carrying out the impregnation process and for the de-supersaturation process, by adding them to the particles obtained by SFEE, in order to improve the separation of β-carotene from colloidal suspension for further analysis. A ratio β-carotene: PCL in weight of 1:30 was used in the impregnation experiments. β-carotene was dissolved in tetrahydrofuran and then, the solution was stirred at 400 rpm. Afterwards water was added slowly. This solution was kept under stirring during two hours, and then the solution was stored during one day, period during which the recrystallization of β-carotene took place. In order to collect β-carotene crystals and remove the organic solvent, the solution was centrifuged at 3750 rpm (Beckman Coulter Allegra^{TM} X-12R). After that, the crystals were resuspended in water with PVA (1 wt.%) and the suspension was centrifuged again at the same conditions. The supernatant was removed from the solution and the crystals were added to the suspension of PCL in water which was previously processed by SFEE.

2.5. Equilibration-impregnation experiments

Experiments for equilibration by impregnation were carried out in a thermostated shaking water bath (GFL 1086). As previously described, each PCL (CAPA 2403D, CAPA 6100 and CAPA 6250), was processed previously by supercritical fluid extraction of emulsions obtaining a suspension of pure PCL nanoparticles in water. After that, each suspension of the different PCLs was mixed with β-carotene crystals keeping constant the weight ratio of 1:30 (β-carotene: PCL) in all experiments. Each suspension (mixture of PCL and β-carotene crystals) was prepared in duplicate to corroborate the results. For carrying out the equilibration process, the mixed suspensions were introduced in the shaking bath and kept at a constant temperature. Firstly, the selected temperature in the shaking bath was 10ºC during 1 day and after this time, a sample was taken to analyse the β-carotene content in each PCL through impregnation. Afterwards, the temperature in the bath was gradually increased to 20, 30, 40 and 50 ºC. Samples were taken after 1 day of equilibration at each temperature.

2.6. Equilibration-de-supersaturation experiments

Equilibration-de-supersaturation experiments were carried out in the same thermostated shaking water bath used for impregnation experiments. Previously, the organic solvent of the β-carotene and PCL emulsion (keeping constant the ratio β-carotene: PCL in weight of 1:30) was extracted by SFEE process, obtaining a co-formulation of β-carotene and PCL, in the form of suspended particles, which was introduced in the shaking bath. Three equilibration-de-supersaturation experiments were carried out, each one with a different PCL (CAPA 2403D, CAPA 6100 and CAPA 6250), without adding extra β-carotene crystals. Another three experiments were carried out adding β-carotene crystals in order to observe if there were differences in the equilibration among the experiments. For equilibration, the particles suspensions were put in the shaking water bath and kept at a constant temperature. Firstly, the selected temperature in the shaking bath was 20ºC during 4 days and after this time, a sample was taken to analyse the β-carotene content in each PCL. Then, the temperature in the bath was gradually decreased to 15 and 10ºC. Samples were taken after 4 days of equilibration at each temperature.

2.7. β-carotene retention in PCL- β-carotene suspensions

Analysis of β-carotene retention of PCL- β-carotene suspensions was carried out measuring the β-carotene content over time of storage by UV-Vis spectroscopy (Beckman Coulter, DU520). Three PCL- β-carotene suspensions were processed, each one with a different PCL, being concretely CAPA 2403D, CAPA 6100 and CAPA 6250). These suspensions were prepared as those which were used in de-supersaturation experiments, preparing an oil-in-water (o/w) emulsion upon ultrasonication as it is described in section 2.2 and then, extracting the organic solvent by SFEE process, obtaining a co-formulation of β-carotene and PCL. These suspensions were storage in dark at room temperature. Samples were taken to analyse the β-carotene content after 0, 7, 14, 21, 28 and 60 days of storage. Sample preparation for determining the β-carotene content after specific times of storage was the same which was used in impregnation and de-supersaturation experiments, as described in section 2.8.1. The method for determining the β-carotene content by UV-Vis spectroscopy is described in section 2.8.4.

2.8. Product characterization

2.8.1. Sample preparation

For each temperature, a sample of equilibrated PCL particles was taken. Firstly, the sample was filtered by vacuum to isolate the β-carotene crystals from the suspension of particles. The β-carotene crystals were recycled to the equilibration experiment. After that, the filtered suspension was centrifuged at ambient temperature during 20 minutes at a rotation speed of 20000 rpm (Beckman Coulter$_{TM}$, Avanti^{TM} Centrifuge J-25). The particle-free supernatant was removed from the sample and finally, the particles of β-carotene and PCL were resuspended in pure water and freeze-dried for further analysis (FlexiDry, FTS Systems). The analysis of β-carotene content by UV-Vis spectroscopy is described in section 2.8.4.

2.8.2. SEM micrographs

In order to assess the morphology of the particles, SEM micrographs were obtained from dried samples sputter-coated with about 5 nm platinum using a Zeiss Gemini 5 1530 FEG scanning electron microscope.

2.8.3. Differential scanning calorimetry (DSC)

Differential scanning calorimetry profiles and crystallinity fraction of polycaprolactones particles were obtained by a differential scanning calorimeter DSC 822e Mettler Toledo. Samples (ca. 4-5 mg) were heated from 0 to 200ºC with a heating rate of 10ºC/min under N_2 atmosphere using non-hermetically sealed (pinholed) aluminium pans.

2.8.4. β-carotene content by UV spectroscopy

The β-carotene content in the polycaprolactone particles was determined by UV spectroscopy (Beckman Coulter, DU520). The wavelength selected was 456 nm. Freeze-dried samples were dissolved in a mixture of dichloromethane and dimethylsulfoxide in a ratio of 9:5 wt.%.

2.8.5. Particle size analysis

Particle size of the emulsions and PCL- β-carotene suspensions was determined by laser difraction in a HELOS-BR by Sympactec GmbH (Germany) using a wet dispersion unit SUCELL and an optical module for measuring particles in the range from 0.1 to 35 micron.

3. RESULTS AND DISCUSSION

3.1. Equilibration-impregnation experiments

As it is explained in Section 2.2, emulsions with three different PCLs were prepared and then the organic solvent was extracted from the emulsion using supercritical carbon dioxide by SFEE process. Then, the obtained suspension was mixed with β-carotene crystals and was introduced in a thermostated shaking bath for carrying out the equilibration-impregnation process, as described in Section 2.5.

Regarding the results of the SFEE processing of PCL, figure 1 presents the influence of the molecular weight of the three different PCLs used (CAPA 6250, CAPA 6100 and CAPA 2403D) on the particle size distribution of the initial emulsion and the final particle suspension prepared by SFEE.

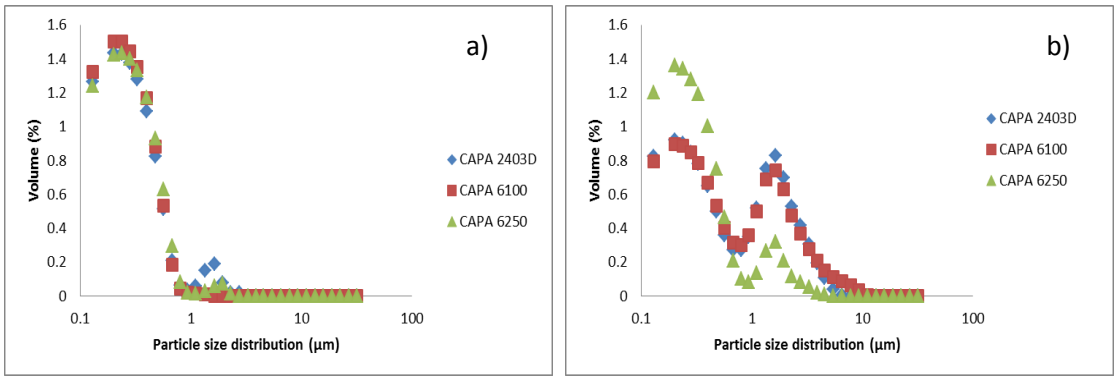

Figure 1: Influence of the molecular weight of the PCLs on the particle size distribution a) of the emulsions (before SFEE process), b) of the suspensions (after SFEE process).

As shown in figure 1, particle size of emulsion droplets was in the range d_{50} = 0.230-0.240 μm. Particles obtained after SFEE processing showed a larger mean particle size and a bimodal distribution, with a first peak in the range of 0.2 μm, equivalent to emulsion droplet size, and a second peak around 2 μm.

Particle size distribution in the original emulsion was similar for the three different PCLs. The only remarkable difference was a small secondary peak around 2 μm in the emulsion produced with CAPA 2403D. Therefore, the molecular weight had not an important effect on the emulsification process. However, after SFEE processing, the particle size distributions of the suspensions of particles of CAPA 2403D and CAPA 6100 were very similar, but in the case of

the suspension of CAPA 6250, there was a smaller proportion of particles bigger than 1 μm than in the case of the other two PCLs.

PCL particles obtained by SFEE processing were used in impregnation experiments. In figure 2, results of β-carotene content in the three different PCLs (CAPA 2403D, CAPA 6100 and CAPA 6250) obtained by equilibration-impregnation process are shown.

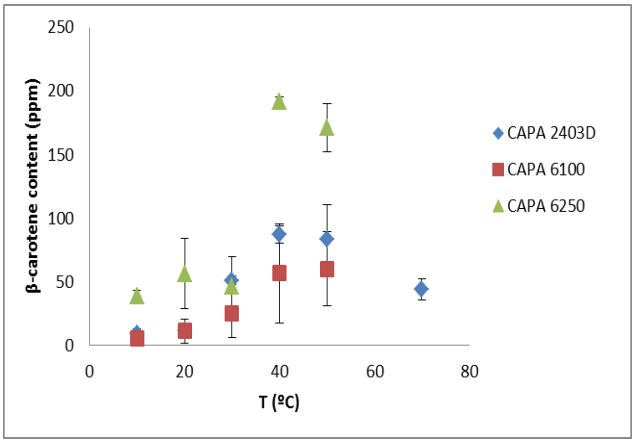

Figure 2. β-carotene content in the three PCLs after equilibration by impregnation at different temperatures.

As it can be observed in figure 2, the β-carotene content impregnated into the PCLs increased gradually when the temperature was increased. The maximum β-carotene contents achieved by equilibration-impregnation experiments were 87, 60 and 191 ppm for CAPA 2403D, CAPA 6100 and CAPA 6250, respectively. In the case of CAPA 2403 particles, an additional impregnation experiment was carried out at 70ºC. In Figure 2, it can be seen that the concentration of impregnated β-carotene at this temperature was considerably lower than the concentrations at lower temperatures. This may be due to a partial degradation of β-carotene due to the exposure of the material to 70ºC during a prolonged period of 24 h. The small increases or even reductions observed in β-carotene content when temperature was increased from 40ºC to 50ºC probably are due to the same reason.

Comparing the results obtained with the three different polycaprolactones, it can be observed that very similar results were obtained using CAPA 2403D and CAPA 6100, while the concentrations of β-carotene achieved using CAPA 6250 were considerably higher. In addition to possible differences in the equilibrium behaviour with CAPA 6250 (which has a considerably higher molecular weight than the other two PCLs), this result may be related to the different particle sizes obtained by SFEE processing of the polymers, because the smaller and more

homogeneous particle sizes obtained with CAPA 6250 may have favoured the impregnation process by providing a higher specific surface area with shorter diffusion lengths.

On the other hand, results obtained in equilibration-impregnation experiments agree with previous results by the same authors. In a previous work [13], authors studied the encapsulation of β-carotene in CAPA 2403D and CAPA 6100 by precipitation using a PGSS (Particles from Gas Saturated Solutions) process, obtaining a maximum β-carotene content in the particles of 94 ppm using CAPA 2403D, and 336 ppm using CAPA 6100. Comparing with the results obtained in impregnation experiments in this work (60 and 87 ppm, respectively), the β-carotene concentrations obtained by PGSS process agree well with the order of magnitude of the saturation concentration of β-carotene in the polymer, with an excess concentration above the saturation concentration due to the physical entrapment of some non-impregnated β-carotene particles in PGSS experiments.

3.2. Equilibration-de-supersaturation experiments

As described in Section 2.2, emulsions with crystalline β-carotene and different PCLs were prepared and then the organic solvent was extracted from the emulsion using supercritical carbon dioxide by SFEE process. The obtained suspension, with polymer particles supersaturated with β-carotene, was analyzed in de-supersaturation experiments. De-supersaturation kinetics are known to be significantly slower as compared to those of impregnation [14]. Because of this, mixtures were sampled after an equilibration time of 96 h, compared to only 24 h in saturation experiments. All three PCLs were tested in experiments carried out both mixing the obtained suspension with β-carotene crystals, and without adding extra β-carotene crystals. In all cases, samples were introduced in a thermostated shaking bath for carrying out the equilibration-de-supersaturation process, as explained in Section 2.6.

Figure 3 presents the influence of the molecular weight of the three different PCLs used on the particle size distribution of the emulsion droplet and on the obtained suspensions (after SFEE process) prepared for carrying out the de-supersaturation process.

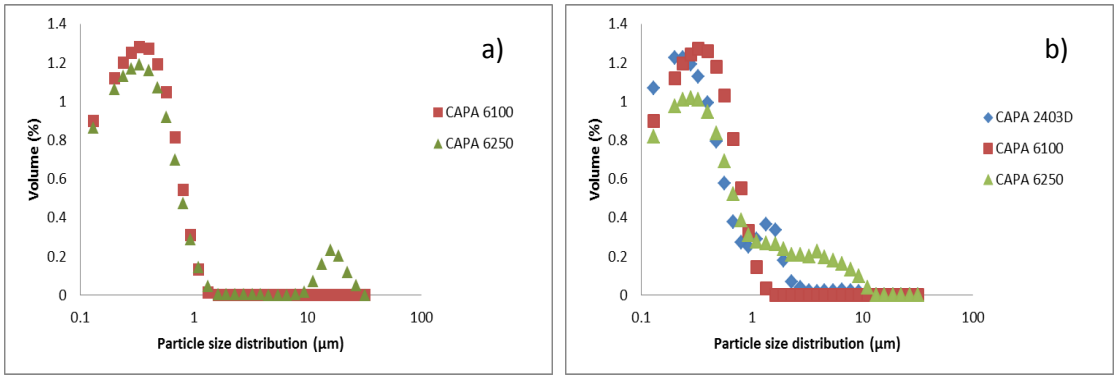

Figure 3. Influence of the molecular weight of the PCLs on the particle size distribution a) of the emulsions, b) of the suspensions (after SFEE process).

As shown in figure 3, the particle size distribution of the emulsion droplet and of the suspension of β-carotene with the three PCLs were similar, obtaining a d_{50} of 0.30 - 0.32 µm. These results were also similar to the mean particle sizes obtained in SFEE experiments without addition of β-carotene, described in the previous Section 3.1. As in the SFEE experiments without β-carotene crystals previously described, particle size distributions obtained after SFEE using CAPA 2403D and CAPA 6250 present a bimodal particle size distribution, while the particle size distribution using CAPA 6100 was monomodal.

Figure 4 presents the results of β-carotene content in the three different PCLs (CAPA 2403D, CAPA 6100 and CAPA 6250) obtained by equilibration-de-supersaturation experiments. Two series of experiments were carried out: one with addition of extra β-carotene crystals to the suspension, and a second one without addition of extra β-carotene. Experiments were initiated at a temperature of 20ºC instead of 50ºC, because samples could be suffering a degradation process as consequence of high temperature. After 4 days of equilibration, temperature was decreased to 15ºC, and after 4 additional days to 10ºC. Finally, at a temperature of 10ºC the equilibration period was extended to 10 days.

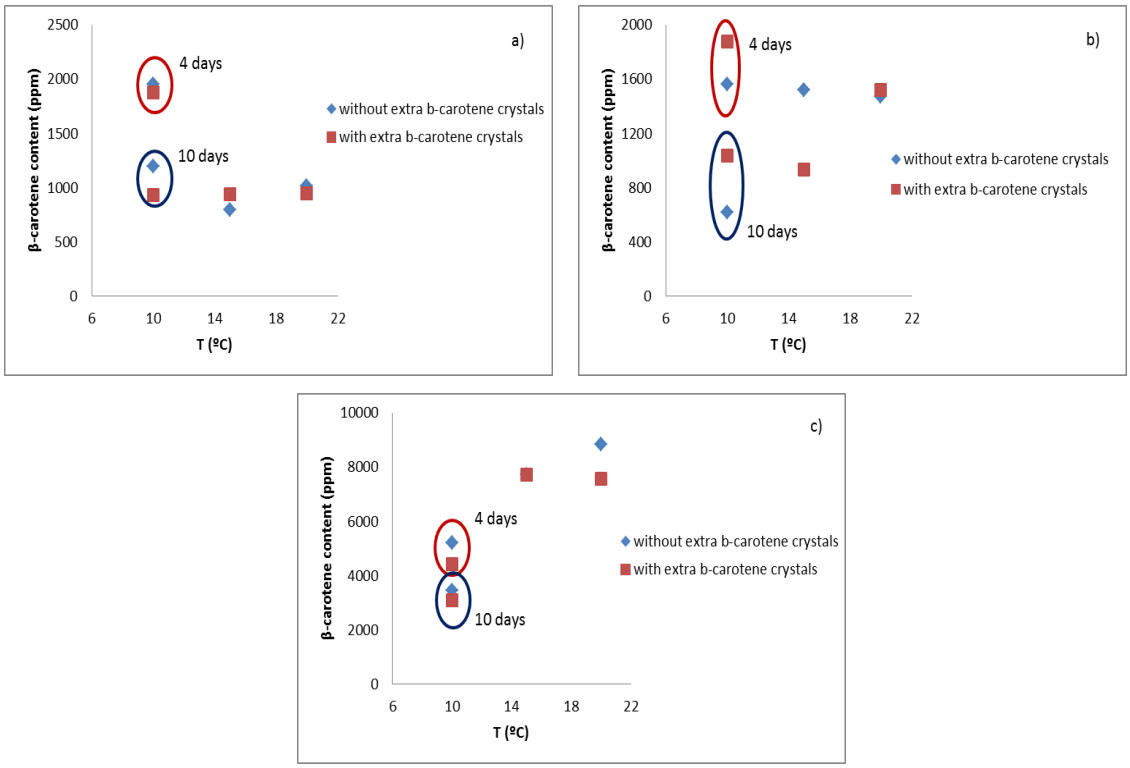

Figure 4. β-carotene content in the three PCLs after equilibration by de-supersaturation experiments: a) CAPA 2403D, b) CAPA 6100 and c) CAPA 6250.

Figure 4 show sthat, as expected from the results of impregnation experiments, as temperature was reduced, β-carotene concentration decreased as well. Comparing the results obtained with the three PCLs, once again the β-carotene concentrations observed in CAPA 2403D and CAPA 6100 were similar, while a significantly higher concentration was observed in CAPA 6250. Compared with the results without addition of crystals, smaller concentrations were observed in experiments with addition of extra β-carotene crystals, probably as a consequence of an Ostwald rippening effect promoted by the added crystals.

However, comparing the values of concentration of β-carotene in de-supersaturation experiments with those obtained from impregnation experiments, very high discrepancies are evident. Indeed, β-carotene concentrations in de-supersaturation experiments rise to 2000 - 8000 ppm, compared to just 50 - 200 ppm in impregnation experiments. Moreover, as shown in Figure 4, after 10 days of equilibriation a significant reduction in β-carotene concentration with respect to the results after 4 days of equilibration was observed, indicating that after this period the system was not equilibrated yet.

This results indicate that the diffusion of non-equilibrated β-carotene from inside the particles of PCL is very slow. As previously discussed, the properties of PCLs as carrier materials make them suitable for applications in which a slow delivery of the encapsulated active compound is

intended, a property that is manifested in the results of de-supersaturation experiments presented in Figure 4.

The application of an equilibration period longer than 10 days has not been considered, since at such long times a significant degradation of β-carotene can occur, interfering with the results. Thus, it must be concluded that, due to the slow release of entrapped β-carotene from PCL particles, the equilibration by de-supersaturation approach is not suitable for the determination of equilibrium concentrations of β-carotene in PCLs.

3.3. β-carotene retention in PCL-β-carotene suspensions

For carrying out the measurement of β-carotene retention of three different aqueous PCL- β-carotene suspensions processed by SFEE (each one with a determined PCL, namely: CAPA 2403D, CAPA 6100 and CAPA 6250), β-carotene content was analysed after 0, 7, 14, 21, 28 and 60 days of storage in dark at room temperature. Sample preparation for determining the β-carotene content was the same which was used for analysing the concentration of β-carotene in impregnation and de-supersaturation experiments. In figure 5, results of the loss of β-carotene content after different exposition times at dark light are presented.

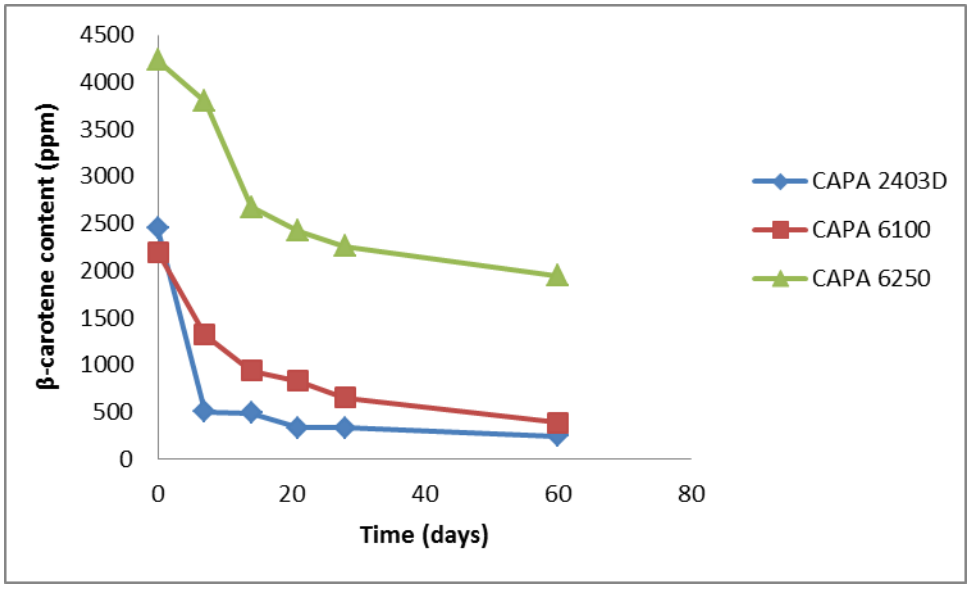

Figure 5. Stability of aqueous PCL-β-carotene suspensions.

Figure 5 clearly shows that β-carotene was degraded during storage time. As shown in figure, there was a drastic decrease in the β-carotene content when CAPA 2403D and CAPA 6100 were used in the first seven days of exposure. Then, the concentration of β-carotene in the suspensions decreased slightly achieving minimum values of 244 and 380 ppm after 60 days of

exposure when CAPA 2403D and CAPA 6100 were used, respectively. Regarding the suspension of β-carotene with CAPA 6250, β-carotene content also decreased considerably during the first 14 days of storage. Then, as when other PCLs were used, the concentration of β-carotene decreased more slowly, reaching a minimum value of 1945 ppm at 60 days of exposure, which is much higher than minimum values achieved at the same time of exposure with the other PCLs. It is necessary to enhance that the β-carotene content was considerably higher when CAPA 6250 was used, as it was observed as well in impregnation and de-supersaturation experiments, which could be indicate that CAPA 6250 would be more suitable than the other two PCLs tested for developing the formulation of β-carotene.

3.4. Characterization of product morphology: SEM and DSC

In figure 6, SEM micrographs of the three different polycaprolactones particles studied in this work (CAPA 2403D, CAPA 6100 and CAPA 6250) and the co-formulation particles of the different polycaprolactones with β-carotene, processed by SFEE and then freeze-dried, are presented. As it can be observed, polycaprolactone particles and co-formulate particles exhibit in all cases a very similar morphology and particle size, independently of which polycaprolactone was used, observing spheres with a size in the order of 200 nm with a rough surface, in agreement with the results of particle size distribution measurements previously reported.

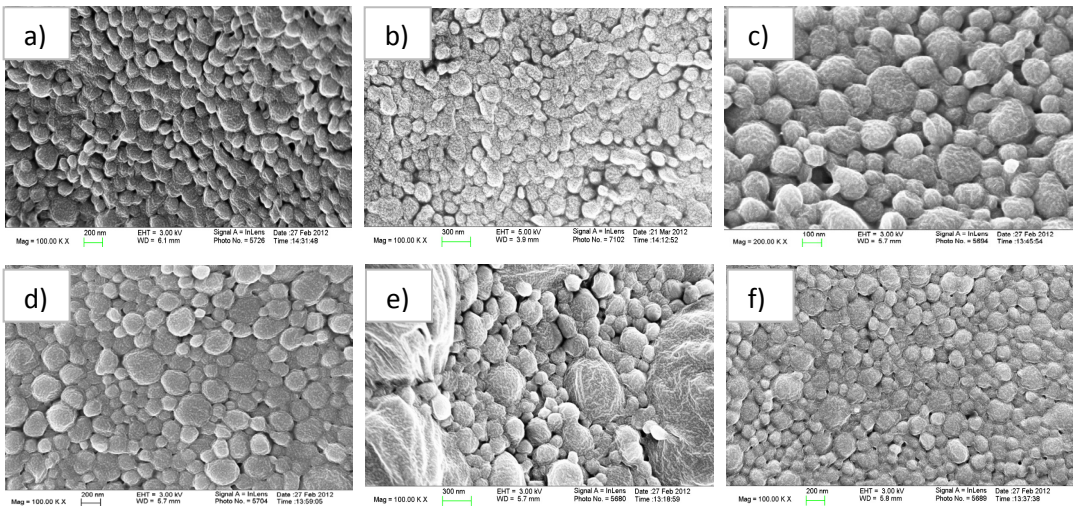

Figure 6. SEM micrographs of particles processed by SFEE: a) CAPA 2403D, b) CAPA 2403D- β-carotene, c) CAPA 6100, d) CAPA 6100-β-carotene, e) CAPA 6250, f) CAPA 6250- β-carotene.

The thermal properties of the formulated polymers were determined by DSC analysis. Figure 8 presents a comparison of DSC profiles between different samples obtained with CAPA 2403D, specifically: pure PCL, processed by SFEE and one sample after impregnation process at 50ºC. DSC analysis revealed that the melting temperatures of different pure PCLs were 62.6ºC, 65.3ºC and 66ºC for CAPA 2403D, CAPA 6100 and CAPA 6250, respectively. The cristallinity is determined according to equation (1):

$$X\,(\%) = \frac{\Delta H_m}{\Delta H_{100\%}} \cdot 100 \qquad (1)$$

Where ΔH_m is the melting enthalpy of the sample calculated from DSC measurements and $\Delta H_{100\%}$ is the entalphy of melting per gram of a pure crystal of PCL which has been taken to be equal to 135.3 J g^{-1}, according to data available in the literature [15].

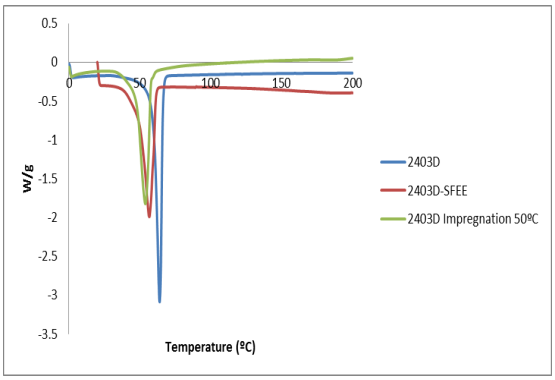

Figure 8. DSC profiles: comparison among pure CAPA 2403D, CAPA 2403D after SFEE and CAPA 2403D after impregnation process at 50ºC.

From DSC analysis, the obtained percentages of crystallinity of the three different pure polycaprolactones were 63.9%, 52.9% and 53.3 % for pure CAPA 2403D, CAPA 6100 and CAPA 6250, respectively. The melting point of PCLs after SFEE process was slightly smaller, being 56ºC, 58.9ºC and 56.9ºC for CAPA 2403D, CAPA 6100 and CAPA 6250, respectively. On the other hand, DSC analysis revealed that the percentage of crystallinity of the polymer after processing of samples was similar to the percentage of crystallinity of pure polymer. For example in the case of CAPA 2403D, results from DSC profiles (which are shown in figure 8) revealed that the percentage of crystallinity after SFEE process was 65.5 % and 62.7 % after impregnation process at 50ºC. These values are similar to the pure CAPA 2403D crystallinity.

The same occurred with the other PCLs. It might be concluded saying that there were no changes in crystallinity after processing PCLs.

4. CONCLUSIONS

The solubility of β-carotene in three poly-ε-caprolactones with different molecular weights (4000, 10000 and 25000 g mol^{-1}) in colloidal state has been studied in the range of temperature between 10ºC and 50ºC. Two different processes were used to carry out the determination of the solubility of β-carotene: equilibration-impregnation and equilibration-de-supersaturation, both developed in a thermal shaking bath. By equilibration-impregnation process, the β-carotene content impregnated into the PCLs increased gradually when the temperature was increased, obtaining maximum β-carotene contents between 87 and 191 ppm depending on the molecular weight of the polycaprolactone. Regarding the equilibration-de-supersaturation experiments, the obtained β-carotene concentrations were considerably higher than those achieved in impregnation experiments, obtaining a maximum β-carotene content of 8800 ppm when polycaprolactone with the highest molecular weight was used (CAPA 6250). Moreover, results showed that in de-supersaturation the system was not equilibrated even after 10 days, indicating that this procedure is not suitable for determining the equilbrium concentration of β-carotene in PCLs. The loss of β-carotene content of PCL- β-carotene suspensions during different exposition times at dark light was determined, obtaining a drastic decrease in β-carotene content after 7 days of exposition time when CAPA 2403D and CAPA 6100 were used and after 14 days of exposure when CAPA 6250 was used. DSC analysis revealed that there were not changes in the crystalinity of polycaprolactones after processing them by SFEE and after the impregnation process of them with β-carotene.

Acknowledgements

Authors thank DSM - León (Spain) for providing materials used in this work. E. de Paz performed this work with an FPI-UVa research fellowship. Á. Martín thanks the Spanish Ministry of Economy and Competitiveness for Ramón y Cajal research fellowship. Soraya Rodríguez-Rojo acknowledges the Spanish Ministry of Education for her postdoctoral grant. This research has been partially funded through project ENE2011-24547 of the Spanish Ministry of Economy and Competitiveness.

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Conclusions

This work is a contribution for the development of different β-carotene formulations for application as natural colorant carrying out efficient and innovative technologies. The main conclusions of this work are summarized below:

NOVEL PRESSURIZED EMULSION PROCESS

- The precipitation from pressurized emulsions process is an efficient and innovative method for developing the formulation of β-carotene with OSA-starch refined from waxy maize using ethyl acetate as organic solvent. It was demonstrated that OSA-starch was a good surfactant agent, that allowed obtaining stable aqueous suspensions of β-carotene with high percentages of encapsulated β-carotene (over 70%) and a micellar particle size in the range of 300-600nm.

- The parameters with stronger influence on product properties are the surfactant concentration (varying from 37 to 367 gL^{-1}) and organic-water ratio (from 0.6 until 1.3 mL/mL). Results revealed that high concentrations of modified starch (over 100 gL^{-1}) and low organic-water ratios (in the range between 0.65 and 0.73 mL/mL) were required in order to obtain a high percentage of encapsulated β-carotene and high emulsion stability.

- The optimization of the different process parameters allowed developing different alternative configurations of the process:
    - The study of the use of ethanol as water-miscible solvent revealed that it is possible to obtain a formulation of β-carotene with maximum encapsulation efficiency of 30%-40% and with a particle size in the range of 120-550 nm, using β-carotene concentrations between 1 to 5 g/L, obtaining the best results with low ratios (0.65-0.73 mL/mL). Thus, compared with results using ethyl acetate as organic solvent, lower encapsulation efficiencies were obtained with ethanol, resulting in poorer product properties.
    - The study of using four different OSA-starches for developing the formulation of β-carotene allowed to conclude that an OSA-starch derived from waxy maize blend with dried glucose syrup was not suitable for obtaining stable aqueous suspensions of β-carotene due to the low encapsulation efficiency achieved (less than 30%) , with particle sizes of approximately 1 μm. With the

other OSA-starches, encapsulation efficiencies between 30-45 % and particle sizes below 500 nm were obtained. However, results obtained with OSA-starch refined from waxy maize were better, presenting higher encapsulation efficiencies (70-80%) with particle size in the nanometer range.

ULTRASOUND EMULSIFICATION PROCESS

- Ultrasound emulsification technique is an efficient process for the formation of β-carotene emulsions with OSA-starch refined from waxy maize. The optimization of the main process parameters such as time of application of ultrasound, amplitude, duty cycle and organic water ratio, allowed obtaining aqueous suspensions of β-carotene with encapsulation efficiencies of 30% and a micellar particle size lower than 200nm.

- Compared with ultrasound emulsification, high-shear rate emulsification produced particles with similar sizes, lower than 240 nm, but the encapsulation efficiency was much worse, below 8 %. Precipitation from pressurized emulsion process allows obtaining much higher encapsulation efficiencies (70-80 %), but micellar particle sizes increase to 400 nm.

SUPERCRITICAL FLUID PROCESSES

- The study of the solid-liquid-gas equilibrium of polycaprolactone-carbon dioxide systems (considering three polycaprolactones with different molecular weights) by visual determination of the first melting point revealed that SLG equilibrium curves show a temperature maximum at low pressures (0.5 – 1.6 MPa) and a temperature minimum at moderate pressures (8 - 10 MPa) depending on the molecular weight of polycaprolactones. This information is necessary to carry out the precipitation techniques using supercritical fluids, and to optimize the operation conditions.

- PGSS (Particles from Gas Saturated Solutions) was used for the development of a hydrophobic β-carotene formulation with different polycaprolactones, obtaining solid particles with a particle size over 100 μm and a β-carotene content of up to 340 ppm. The process parameters which have an important influence on particle size were the molar ratio β-carotene:polymer, pressure, temperature and time of contact between

CO_2 and polymer melt for mixture homogenization. The highest β-carotene concentrations (306-336 ppm) were obtained at high pressures (15 MPa) and temperatures (70°C) and short homogenization times (60 minutes).

- The study of the solubility of β-carotene in different polycaprolactones in colloidal state show that the maximum β-carotene contents achieved by equilibration-impregnation process were in the range of 87-191 ppm depending on the molecular weight of the polycaprolactone. These results corroborate results obtained by PGSS process, as the concentration of β-carotene obtained by PGSS process agree well with the order of magnitude of the saturation concentrtion of β-carotene in the polymer. On the contrary, results by equilibration-de-supersaturation process show β-carotene contents considerably higher (between 1000 - 8800 ppm depending the molecular weight of the polycaprolactone), possibly due to the slow release of entrapped β-carotene from slow-degrading polycaprolactone particles, so the equilibration by de-supersaturation approach is not suitable for the determination of equilibrium concentrations of β-carotene in polycaprolactones.

- PGSS (Particles from Gas Saturated Solutions) -Drying process is an suitable technique for the development of an alternative water soluble formulation of β-carotene in liposome-forming soybean lecithin. Dry particles with sizes in the range of 10-500 μm and encapsulation efficiencies of β-carotene up to 60 % were obtained. These particles can be rehydrated forming β-carotene-loaded multillamelar liposomes with sizes in the range of 1-5 μm. The process parameters with strong influence on product characteristics are pre-expansion temperature and pressure, gas to product ratio and concentration of soybean lecithin.

Resumen

Estudios para la intensificación del proceso para el desarrollo de formulaciones de β–caroteno hidrofílicas e hidrofóbicas

1. INTRODUCCIÓN

El uso de colorantes alimentarios sintéticos se asocia con efectos perjudiciales para la salud, siendo objetivo de quejas por parte de los consumidores de la industria alimentaria. Actualmente, el mercado de los alimentos demanda alimentos funcionales y productos saludables. Mientras el uso de productos químicos se considera de una forma negativa, los aditivos naturales proporcionan un producto final con el valor añadido de sus propiedades saludables.

Los carotenoides pueden ser un aditivo natural valioso para los productos alimentarios. β-caroteno es uno de los carotenoides más comunes, abundantes y empleados. Además de sus excelentes propiedades como colorante, el β-caroteno se ha empleado exhaustivamente como precursor del retinol y ácido retinoico, que tienen un papel importante en la salud humana como precursor de la vitamina A y como señal reguladora celular. Los animales y humanos no pueden producir carotenoides en sus organismos, de este modo ellos necesitan adquirir los carotenoides a partir de las fuentes de alimentación. La estructura del β-caroteno se muestra en la figura 1.

Figura 1. Estructura del β-caroteno

Ingredientes funcionales como los carotenoides, ácidos grasos, antioxidantes naturales y otros numerosos compuestos, están siendo empleados exhaustivamente en una gran variedad de productos alimentarios. Los carotenoides son algunos de los pigmentos más comunes en la naturaleza, siendo los más abundantes el β-caroteno, licopeno, luteína y zeaxantina. Las principales labores de los carotenoides en la dieta humana son como precursores de vitamina A y como antioxidantes. Entre los carotenoides, el β-caroteno tiene la provitamina A más elevada, por lo tanto, es un elemento fuerte para la incorporación en los alimentos funcionales. Cuando los carotenoides se consumen en unos niveles suficientes, se ha afirmado que tienen actividades biológicas que podrían reducir el riesgo de ciertas enfermedades crónicas, como cáncer, enfermedades cardiovasculares, degeneración macular relacionada con la edad y cataratas. Desde el momento en que los carotenoides han sido autorizados como ingredientes alimentarios, los carotenoides se han empleado extensamente en la industria alimentaria, cosmética y farmacéutica como colorantes naturales.

Para muchas aplicaciones industriales, se emplea una mezcla del carotenoide con un biopolímero. El recubrimiento de carotenoides con polímeros proporciona protección contra

los procesos de degradación y de oxidación. Además, la hidrofobicidad alta de los carotenoides los hace insolubles en sistemas acuosos, y por lo tanto, tienen una peor absorción en el cuerpo. Para mejorar su dispersabilidad en agua, el potencial de la fuerza colorante y también para incrementar su biodisponibilidad durante el tránsito gastrointestinal, los carotenoides se deben formular. Para el uso de los carotenoides como colorantes naturales, es importante obtener una intensidad de color apropiada de la formulación que depende de las propiedades de las partículas, incluyendo un tamaño de partícula limitado y una cristalinidad controlada.

Varios autores han investigado la precipitación y formulación de β-caroteno debido al gran interés que tiene como colorante natural y antioxidante en la industria alimentaria. Para este objetivo se emplean técnicas convencionales como emulsificación-evaporación o el método de desplazamiento de disolvente orgánico. Las nanoemulsiones y nanodispersiones que se obtienen mediante la técnica de homogenización a alta presión se han estudiado exhaustivamente debido a las propiedades favorables que tiene el producto final en términos de estabilidad y tamaño de partícula. El empleo de tecnologías con fluidos supercríticos podría ser una buena alternativa para los procesos convencionales gracias a la posibilidad de poder operar en condiciones no muy severas evitando la degradación del producto. Además, debido a la alta solubilidad de los disolventes orgánicos en dióxido de carbono supercrítico, estos disolventes se pueden eliminar del producto de una forma eficiente mediante el tratamiento con el fluido supercrítico. Sin embargo, la principal limitación en muchas aplicaciones basadas en la tecnología de fluidos supercríticos es el tamaño de partícula, obteniendo en la mayoría de los casos, partículas mayores de 1 μm.

Por otro lado, los disolventes orgánicos que se emplean para el desarrollo de la formulación de β-caroteno han sido diclorometano, cloroformo y hexano, siendo disolventes con una toxicidad elevada no recomendados para aplicaciones alimentarias. Debido a esto, hay una necesidad de desarrollar tecnologías innovadoras que mejoren los procesos convencionales para llevar a cabo la formulación de β-caroteno empleando otros disolventes orgánicos con menos toxicidad. También es necesario evitar la degradación del carotenoide debido a la exposición del mismo a temperaturas altas y tiempos de procesado largos. Por último, también es importante asegurar la reproducibilidad y predictibilidad de las propiedades del producto e intensificar el proceso incrementando la capacidad de producción y haciendo un proceso continuo si es posible. Por todo ello, en este trabajo se han estudiado y desarrollado distintos procesos para llevar a cabo la formulación de β-caroteno.

2. OBJETIVOS

El objetivo principal de esta tesis doctoral es el desarrollo de diferentes formulaciones de β-caroteno (hidrofílicas e hidrofóbicas) para su uso como colorante natural e implementar técnicas innovadoras y de intensificación capaces de producir estas formulaciones con una buena controlabilidad y predictibilidad de las propiedades del producto así como un alto rendimiento. Para cumplir este objetivo se han planteado los siguientes objetivos parciales:

- Estudio de distintas formulaciones de β-caroteno solubles en agua empleando almidón modificado con el grupo n-octenil anhídrido succínico (OSA) como agente encapsulante.
    - Diseño y estudio de un proceso innovador de emulsión presurizada.
        - Caracterización del producto final y optimización de los parámetros de proceso (como concentración de surfactante o relación orgánico-agua) para obtener una formulación estable en el tiempo, una eficiencia de encapsulación alta y un tamaño de partícula en el rango de los nanómetros, que se requiere para su uso como colorante natural.
        - Estudio de las propiedades antioxidantes del producto formulado.
        - Estudio del efecto de distintos almidones modificados (OSA) y distintos disolventes orgánicos.
    - Comparativa con los productos obtenidos con las técnicas convencionales de emulsificación-evaporación de disolvente orgánico mediante emulsificación por cizalladura y emulsificación con ultrasonidos. Estudio del efecto de los distintos parámetros de proceso en la eficiencia de encapsulación y en el tamaño de partícula: influencia de la relación orgánico-agua, tiempo de aplicación de ultrasonidos, amplitud de oscilación y ciclo.
- Desarrollo de distintas formulaciones de β-caroteno basadas en tecnologías con fluido supercrítico.
    - Estudio de la formulación de β-caroteno basada en fosfolípidos formando liposomas como una alternativa de formulación soluble en agua.
        - Desarrollo de esta formulación mediante secado PGSS (partículas a partir de disoluciones saturadas de gas) obteniendo partículas sólidas que se pueden rehidratar formando liposomas. Efecto de las variables de operación (temperatura y presión antes de la expansión, relación

de gas/disolución (GPR) y concentración de lecitina de soja). Caracterización de las partículas obtenidas.

- Estudio del comportamiento de fase de mezclas de distintas policaprolactonas y fluido supercrítico para obtener un conocimiento detallado y poder desarrollar la formulación con este polímero.
- Desarrollo de una formulación de β-caroteno hidrofóbica con policaprolactonas mediante PGSS. Efecto de las variables de operación (relación molar de los compuestos, tiempo de homogenización de la mezcla, temperatura y presión antes de la expansión).
- Estudio de la solubilidad de β-caroteno en policaprolactonas mediante procesos de equilibrio-impregnación y equilibrio des-saturación para la obtención de las propiedades básicas para el desarrollo del proceso de encapsulación por PGSS.

3. RESULTADOS Y DISCUSIÓN

CAPÍTULOS 1-2-3

FORMULACIÓN DE β-CAROTENO MEDIANTE PRECIPITACIÓN A PARTIR DE EMULSIONES PRESURIZADAS

La técnica de precipitación a partir de emulsiones presurizadas para llevar a cabo la formulación de β-caroteno se estudia en los tres primeros capítulos. Este proceso consiste en poner en contacto una disolución de β-caroteno a elevada temperatura y presurizada en un disolvente orgánico, con una disolución acuosa de agente encapsulante (almidón modificado) a temperatura ambiente, empleando un mezclador tipo T. El proceso se diseñó para la aproximación de los tiempos de mezclado a escala de tiempos de nucleación de partícula. De este modo, debido a los efectos antidisolvente y termal, la precipitación de β-caroteno tiene lugar en una escala de tiempo de una fracción de segundo, permitiendo la obtención de un producto altamente homogéneo con una eficiencia elevada. El estudio de esta técnica y la optimización de los parámetros de proceso se desarrollan en el capítulo 1. En el capítulo 2, se estudia el efecto de emplear etanol como alternativa al primer disolvente orgánico seleccionado, acetato de etilo. Y por último, en el capítulo 3 se realiza un estudio del efecto de emplear cuatro almidones distintos para la formulación de β-caroteno.

CAPÍTULO 1

FORMULACIÓN DE β-CAROTENO MEDIANTE PRECIPITACIÓN A PARTIR DE EMULSIONES DE ACETATO DE ETILO-AGUA PRESURIZADAS PARA APLICACIÓN COMO COLORANTE NATURAL

El objetivo de este capítulo es el estudio de la técnica de precipitación a partir de emulsiones presurizadas (anteriormente descrita) y la optimización de los parámetros de proceso para llevar a cabo la formulación de β-caroteno empleando almidón modificado con el grupo n-octenil anhídrido succínico (OSA) refinado a partir de maíz ceroso, como agente encapsulante.

Los parámetros de proceso con mayor influencia en las propiedades del producto fueron la concentración de almidón modificado (variando desde 37 hasta 367 g L^{-1}) y la relación orgánico-agua (desde 0.6 hasta 1.3 mL/mL).

La representación del efecto de la concentración de almidón en la eficiencia de encapsulación del β-caroteno puede verse en la figura 2, de la cual puede extraerse que es necesario emplear

concentraciones altas de almidón modificado (por encima de 100 g L^{-1}) para la obtención de un porcentaje de β-caroteno encapsulado alto y una estabilidad de la emulsión elevada.

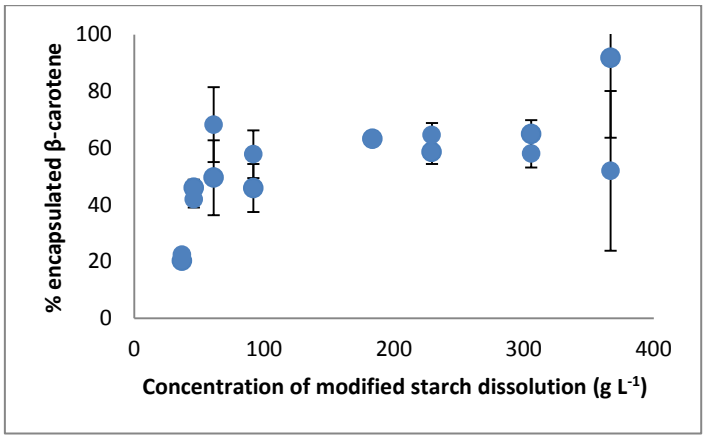
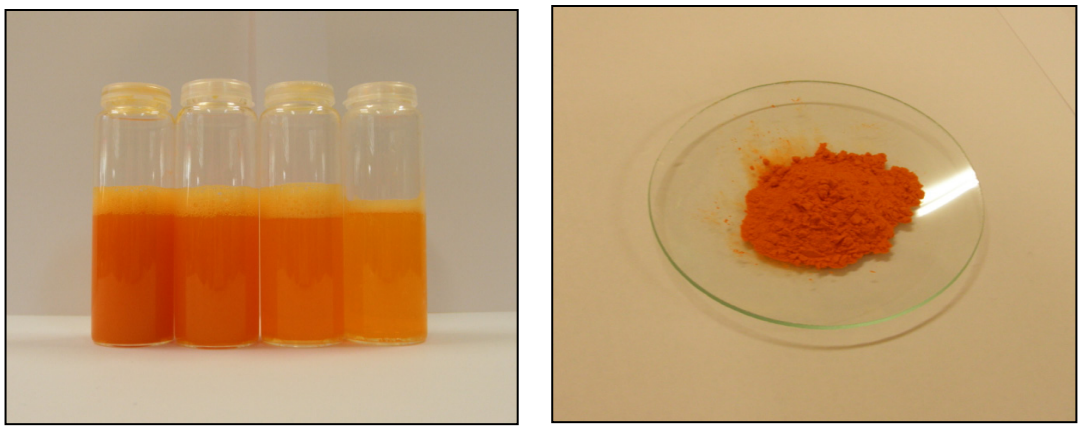


Figura 2. Efecto de la concentración de la disolución de almidón modificado en el porcentaje de β-caroteno encapsulado.

Con respecto a la relación orgánico-agua, los mejores resultados se alcanzaron con relaciones bajas (entre 0.65 y 0.73). Cuando se emplearon relaciones de orgánico-agua más altas que 0.85, se produjo un incremento drástico en el tamaño de partícula micelar.

Por otro lado, como consecuencia de la exposición del producto a elevadas temperaturas durante el proceso, se produjo una isomerización importante del trans al cis β-caroteno, concretamente entre un 15-50 %. El espectro UV/VIS muestra que, en casi todos los experimentos, se observan tres picos que corresponden al típico color anaranjado. Por último, los análisis de las propiedades antioxidantes demuestran que las formulaciones de β-caroteno retienen actividades antioxidantes elevadas.

En la figura 3, se puede observar la intensidad de color de las dispersiones acuosas de β-caroteno encapsulado a varias concentraciones así como las partículas secas obtenidas después de haber realizado el proceso de secado por spray.



a) *b)*

Figura 3. a) Dispersiones acuosas de β-caroteno encapsulado con concentraciones de β-caroteno de 298, 149, 47.5 y 37.5 ppm (de izquierda a derecha), b) partículas obtenidas después del proceso de secado por spray.

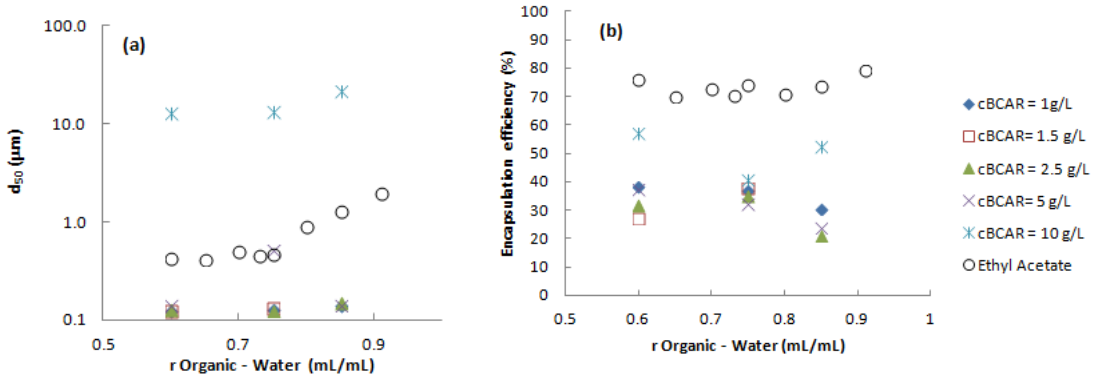
De este capítulo se pudo concluir que es posible obtener una formulación de β-caroteno con una eficiencia de encapsulación de β-caroteno elevada (por encima del 70%) y con un tamaño de partícula en el rango de 300-600 nm.

Los resultados obtenidos en este capítulo se emplearon como base para el capítulo 2, en el que se empleó la misma técnica para la formulación de β-caroteno con almidón modificado empleando etanol como disolvente orgánico.

CAPÍTULO 2

FORMULACIÓN DE β-CAROTENO EN MICELAS DE ALMIDÓN MODIFICADO (OSA) SOLUBLES EN AGUA MEDIANTE PROCESO A ALTA PRESIÓN Y TEMPERATURA, CON PRECIPITACIÓN POR EFECTO ANTIDISOLVENTE A PARTIR DE DISOLUCIONES DE ETANOL PRESURIZADAS

El objetivo de este capítulo es el estudio de la formulación de β-caroteno con almidón modificado con el grupo OSA refinado a partir de maíz ceroso mediante precipitación a partir de disoluciones de etanol presurizadas, así como el estudio de los parámetros de proceso con mayor influencia en las características del producto: la relación orgánico- agua y la concentración de β-caroteno en etanol. Se varió la relación orgánico-agua entre 0.6 y 0.85 mL/mL y la concentración de β-caroteno entre 1 y 10 g L^{-1}. En la figura 4 se recoge la variación del tamaño de partícula micelar y la eficiencia de encapsulación con la relación orgánico-agua.



a) *b)*

Figura 4. Variación del tamaño de partícula micelar (a) y eficiencia de encapsulación (b)with the organic –water ratio. Representación de los resultados que se han obtenido en el trabajo previo empleando acetato de etilo en lugar de etanol como disolvente orgánico (capítulo 1).

Este estudio permitió obtener formulaciones de β-caroteno con eficiencias de encapsulación máximas de 30%-40% y con tamaños de partícula micelar menores de 550 nm cuando se emplearon concentraciones de β-caroteno entre 1 – 5 g L^{-1}, como se puede observar en la figura 4. Estos tamaños de partícula se preservaron después de cuatro meses de almacenamiento, aunque en este período de tiempo se observa una desestabilización y aglomeración de las partículas que no fueron encapsuladas con tamaños de partícula iniciales más grandes. Los experimentos con concentraciones de β-caroteno de 10 g L^{-1} conllevan a una mala precipitación y encapsulación, produciendo tamaños de partícula mayores de 10 µm.

El efecto de la relación orgánico-agua se estudió en la eficiencia de encapsulación y en el tamaño de partícula micelar. Los resultados obtenidos indicaron que un aumento en la relación orgánico-agua producía un incremento en el tamaño de partícula, lo cual también se observó en el trabajo previo (capítulo 1) cuando se empleó acetato de etilo como disolvente orgánico. Comparando los resultados obtenidos en este trabajo empleando etanol como disolvente orgánico con los resultados previos con acetato de etilo, se obtuvieron eficiencias de encapsulación más bajas en los experimentos con etanol, evidenciándose propiedades del producto con peor calidad. Esto puede ser debido a que no se forma una emulsión durante la precipitación y encapsulación de las partículas, que se forma empleando acetato de etilo que es inmiscible con el agua, pero no con el etanol.

De este capítulo se pudo concluir que es posible obtener una formulación de β-caroteno con una eficiencia máxima de encapsulación de 30-40% con tamaños de partícula micelar en el rango de 120-550 nm, cuando se emplean concentraciones de β-caroteno entre 1 y 5 g L^{-1}.

CAPÍTULO 3

FORMULACIÓN DE β-CAROTENO MEDIANTE PRECIPITACIÓN A PARTIR DE EMULSIONES DE ACETATO DE ETILO-AGUA PRESURIZADAS PARA APLICACIÓN COMO COLORANTE NATURAL: EFECTO DE DIFERENTES ALMIDONES MODIFICADOS CON EL GRUPO (OSA)

El objetivo de este capítulo es el estudio de la influencia de cuatro almidones modificados con el grupo grupo n-octenil anhídrido succínico (OSA) para el desarrollo de la formulación de β-caroteno mediante precipitación a partir de emulsiones de acetato de etilo-agua, así como el estudio de los parámetros de proceso con mayor influencia en las características del producto: concentración de almidón modificado y relación orgánico-agua. Los cuatro almidones modificados que se han empleado han sido: OSA-1 (almidón modificado derivado de maíz ceroso combinado con jarabe de glucosa deshidratado), OSA-2 (dextrina modificada derivada de maíz ceroso), OSA-3 (dextrina modificada derivada de tapioca) y OSA-4 (almidón modificado refinado a partir de maíz ceroso). Se varió la concentración de almidón modificado entre 37 y 367 g L^{-1} y la relación orgánico-agua entre 0.6 y 1.20 mL/mL. En la figura 5, se muestra una comparativa de los cuatro almidones modificados que se han empleado, observando el efecto de la concentración de almidón modificado en la eficiencia de encapsulación del β-caroteno y en el tamaño de partícula micelar.

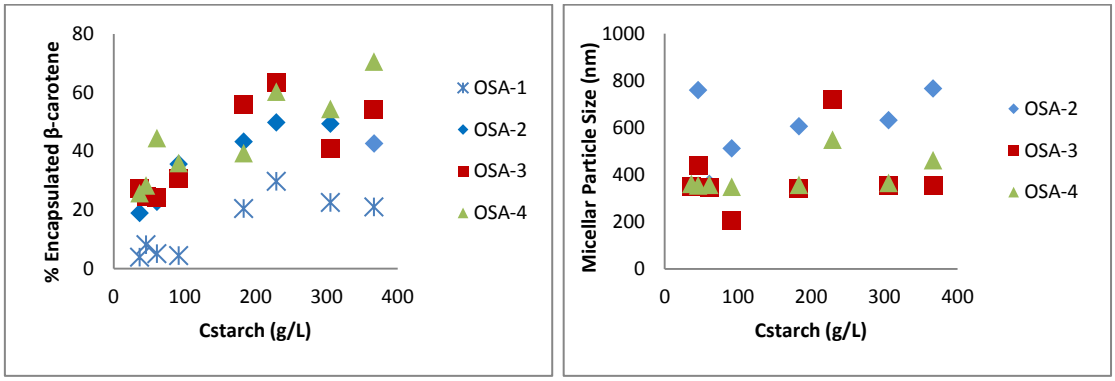


Figura 5. Comparativa de los cuatro almidones modificados con grupo OSA en la eficiencia de encapsulación y en el tamaño de partícula micelar.

La representación de la concentración de almidón frente a la eficiencia de encapsulación mostró que es necesaria una concentración mínima de surfactante de 100 g L^{-1} para poder obtener eficiencias de encapsulación altas. Se comprobó que cuando la concentración de almidón modificado aumenta, también se incrementa la eficiencia de encapsulación, como

puede verse en la figura 5. Los resultados obtenidos con OSA-1 (almidón modificado derivado de maíz ceroso combinado con jarabe de glucosa deshidratado) indicaron que este almidón no era adecuado para la encapsulación de β-caroteno debido a que se obtuvieron eficiencias de encapsulación bajas (menores del 30%) y tamaños de partícula micelar elevados (desde 1 μm hasta más de 100 μm).

Con respecto al efecto de la relación orgánico-agua, cuando se emplean relaciones orgánico-agua altas, se observa un incremento drástico en el tamaño de partícula micelar, independientemente del almidón modificado empleado. En relación al tamaño de partícula, las suspensiones acuosas de β-caroteno que se obtienen presentan un tamaño de partícula en el rango de 350-760 nm. Cuando se emplearon OSA-2 y OSA-3, las eficiencias de encapsulación permanecieron constantes entre 30-45% y se obtuvieron tamaños de partícula menores de 500 nm. Sin embargo, con OSA-4 se obtuvieron mejores resultados, alcanzando eficiencias de encapsulación máximas de 70-80% con tamaños de partícula menores de 1 μm.

De este capítulo se pudo concluir que OSA-1 no es adecuado para llevar a cabo la formulación de β-caroteno mediante precipitación a partir de emulsiones presurizas debido a que se obtuvieron eficiencias de encapsulación bajas y tamaños de partícula elevados. Sin embargo, el almidón modificado OSA-4 presenta las mejores eficiencias de encapsulación (70-80%) con tamaños de partícula micelar menores de 1 μm.

CAPÍTULO 4

PRODUCCIÓN DE FORMULACIONES DE β-CAROTENO SOLUBLES EN AGUA: COMPARACIÓN ENTRE EMULSIFICACIÓN POR ULTRASONIDOS Y EVAPORACIÓN DE LA EMULSIÓN, Y PRECIPITACIÓN A PARTIR DE UNA EMULSIÓN PRESURIZADA

El objetivo de este capítulo es el estudio de distintas formulaciones de β-caroteno solubles en agua empleando almidón modificado con el grupo OSA refinado a partir de maíz ceroso como agente encapsulante, preparando las emulsiones con distintas técnicas: emulsificación por ultrasonidos, emulsificación por cizalladura y precipitación a partir de emulsiones presurizadas. Para la emulsificación por ultrasonidos se empleó un sonicador de 400W (UP400S, Hielscher) con un sonotrodo de 22 mm fabricado en titanio. Se estudiaron como variables de operación el tiempo de aplicación de ultrasonidos (entre 6 y 65 minutos), amplitud de oscilación del sonotrodo (20-40-60-80-90-100 μm), ciclo (0.5-0.7-0.9-1.0) y relación orgánico-agua (entre 0.275 y 0.73).

Los mejores resultados se obtuvieron con relaciones orgánico-agua bajas, concretamente 0.275 y empleando 100 µm de amplitud con un ciclo de 1.0, alcanzando valores de tamaño de partícula micelar menores de 200 nm y eficiencias de encapsulación del 30%. Mediante la técnica de emulsificación por cizalladura se obtuvieron tamaños de partícula muy similares (183-236 nm) pero con eficiencias de encapsulación bajas (menores de 8%).

En la figura 6 se representa el efecto de la relación orgánico-agua en la eficiencia de encapsulación del β-caroteno y en el tamaño de partícula de los resultados obtenidos mediante emulsificación por ultrasonidos (con una etapa posterior de evaporación del disolvente orgánico para obtener la dispersión acuosa de β-caroteno) y los resultados obtenidos mediante precipitación a partir de emulsiones presurizadas (capítulo 1).

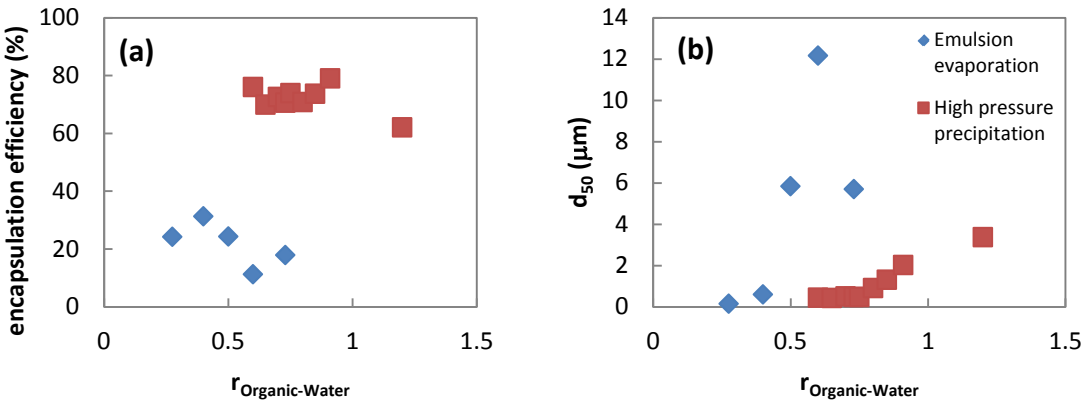


Figura 6. Influencia de la relación orgánico-agua en la eficiencia de encapsulación (a) y en el tamaño de partícula (b) obtenidos mediante evaporación de la emulsión (♦) y mediante precipitación a partir de emulsiones presurizadas (■) (Capítulo 1).

La representación en la figura 6 del efecto de la relación orgánico-agua en la eficiencia de encapsulación y en el tamaño de partícula revela que cuando la relación orgánico-agua se incrementa, el tamaño de partícula micelar también aumenta. A elevadas relaciones orgánico-agua, la eficiencia de encapsulación disminuye. Como se puede ver en la figura 6, las eficiencias de encapsulación que se obtuvieron mediante precipitación a partir de emulsiones presurizadas (70%-80%) son mucho más altas que las que se obtuvieron por emulsificación por ultrasonidos con una etapa posterior de evaporación del disolvente orgánico (20%-30%). Con respecto a los tamaños de partícula micelar, se obtuvieron tamaños más grandes con la tecnología de precipitación a partir de emulsiones presurizadas (aproximadamente 400nm) que mediante emulsificación por ultrasonidos (100-200 nm).

De este capítulo se pudo concluir que es posible obtener una formulación de β-caroteno con una eficiencia máxima de encapsulación del 30% con tamaños de partícula micelar menores de 200nm mediante emulsificación por ultrasonidos. Sin embargo, se obtuvieron eficiencias de encapsulación más altas mediante precipitación a partir de emulsiones presurizadas, probablemente debido a una desestabilización parcial de la emulsión durante los tiempos de procesado largos requeridos para el método de emulsificación por ultrasonidos, que son reducidos a fracciones de segundo empleando la técnica de emulsiones presurizadas.

CAPÍTULO 5

DETERMINACIÓN DEL EQUILIBRIO DE FASE (SÓLIDO-LÍQUIDO-GAS) EN SISTEMAS DE POLI-(ε-CAPROLACTONA)-DIÓXIDO DE CARBONO

El objetivo de este capítulo es el estudio del comportamiento de fase de mezclas de tres policaprolactonas distintas y fluido supercrítico obteniendo un conocimiento detallado para llevar a cabo las técnicas de precipitación empleando fluidos supercríticos y para optimizar las condiciones de operación. Los experimentos se han llevado a cabo con tres policaprolactonas de distintas masas moleculares: 4000 g mol^{-1}, 10000 g mol^{-1} y 25000 g mol^{-1}. Para realizar este estudio se empleó el método de determinación visual del primer punto de fusión de las policaprolactonas (temperatura a la que el polímero empieza a fundir a una determinada presión) mediante una celda óptica de alta presión. Se varió la presión entre 0.1 y 25 MPa para las tres policaprolactones. En la figura 7 se representa el equilibrio sólido-líquido-gas de los sistemas de policaprolactona-dióxido de carbono, para las tres policaprolactonas, así como los resultados que se obtuvieron mediante el modelo termodinámico basado en la ecuación de estado PC-SAFT que se ha empleado para describir los datos experimentales.

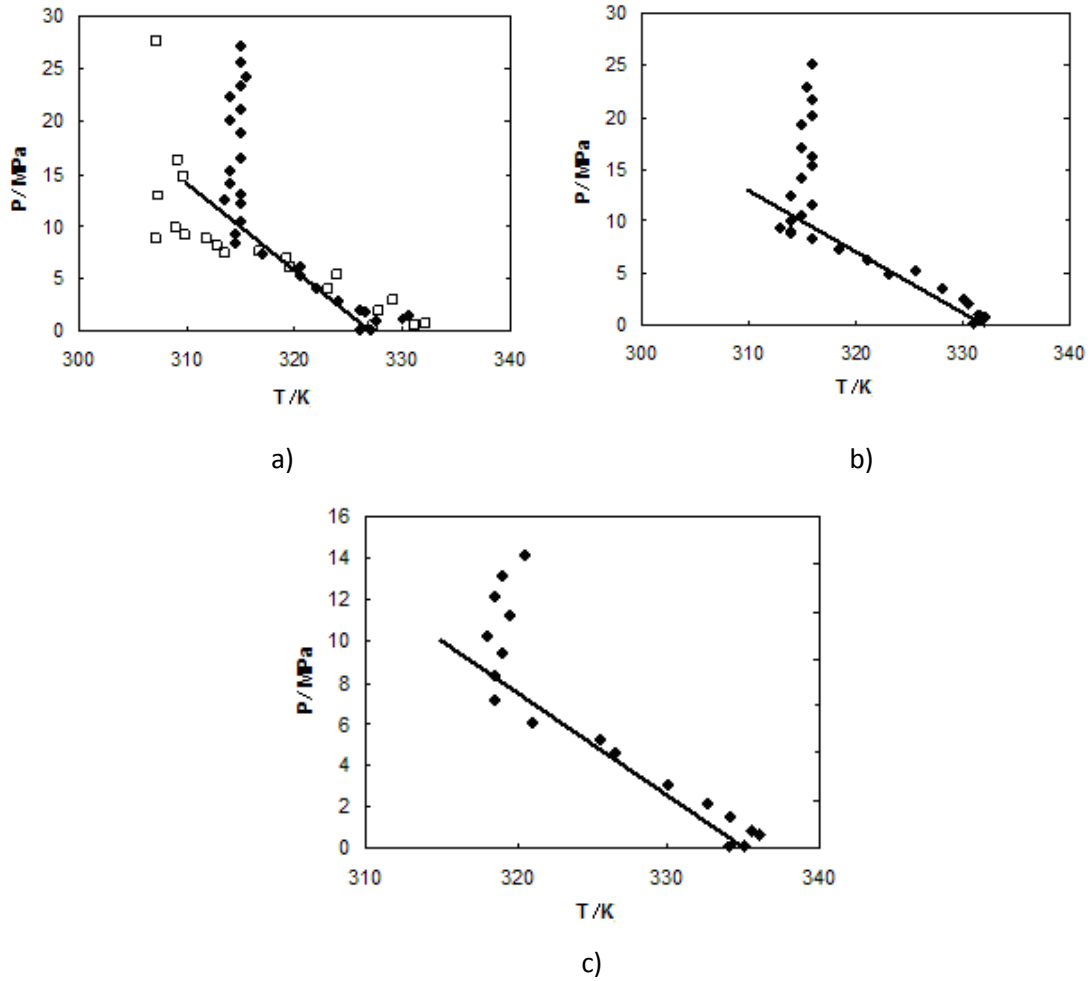


Figura 7. Equilibrio sólido-líquido-gas de los sistemas de policaprolactonas-CO_2, a) resultados con policaprolactona de masa molecular 4000 g mol^{-1}, b) resultados con policaprolactona de masa molecular 10000 g mol^{-1}, c) resultados con policaprolactona de masa molecular 25000 g mol^{-1}.

Las curvas de equilibrio sólido-liquido-gas determinadas experimentalmente muestran una temperatura máxima a presiones bajas (entre 0.5 y 1.6 MPa) y una temperatura mínima a presiones moderadas (entre 8 y 10 MPa), dependiendo de la masa molecular de las policaprolactonas. La máxima reducción en la temperatura de fusión es de 12.5 K para el caso de la policaprolactona de 4000 g mol^{-1}, 15.5 K para la policaprolactona de 10000 g mol^{-1} y 16.0 K para la policaprolactona de 25000 g mol^{-1}. El modelo termodinámico que se basa en la ecuación de estado PC-SAFT que se ha desarrollado para describir los datos experimentales, predice correctamente la solubilidad del CO_2 en el polímero fundido así como la temperatura de fusión a presiones más bajas que la presión a la que se consigue la temperatura mínima de fusión. Sin embargo, con este modelo no es posible describir la variación de la temperatura de fusión a presiones más altas.

De este capítulo se pudo concluir que las curvas de equilibrio sólido-líquido-gas de los sistemas de policaprolactona-dióxido de carbono presentan una temperatura máxima a bajas presiones (0.5-1.6 MPa) y una temperatura mínima a presiones moderadas (8-10 MPa).

Los resultados obtenidos en este capítulo se emplearon como base para el capítulo 6, en el que es necesario tener un conocimiento detallado del equilibrio SLG de los sistemas de policaprolactonas-dióxido de carbono para poder llevar a cabo las técnicas de precipitación con este polímero empleando fluidos supercríticos y para optimizar las condiciones de operación.

CAPÍTULO 6

FORMULACIÓN DE β-CAROTENO CON POLI-(ε-CAPROLACTONAS) MEDIANTE PROCESO PGSS

El proceso PGSS consiste en saturar un soluto, ya sea disolviéndolo en un disolvente líquido o fundirlo, con dióxido de carbono en condiciones supercríticas (temperatura > 31ºC, presión > 7.4 MPa). Se forma una disolución saturada de gas que posteriormente se expande a condiciones atmosféricas a través de una boquilla de atomización. Durante la expansión, el dióxido de carbono se vaporiza rápidamente y se enfría intensamente a causa del efecto Joule-Thomson, produciéndose la solidificación del soluto.

El objetivo de este capítulo es el estudio de la formulación de β-caroteno con policaprolactonas mediante el proceso PGSS (partículas a partir de disoluciones saturadas de gas), así como el estudio del efecto de los parámetros del proceso en las características del producto. Se estudiaron la influencia de varios parámetros de proceso en el tamaño de partícula y en el contenido de β-caroteno, incluyendo presión en el recipiente de mezclado (11 y 15 MPa), temperatura (50 y 70 ºC), tiempo de contacto entre CO_2 y el polímero fundido para la homogeneización de la mezcla (60-120-240 minutes) y relación molar β-caroteno:polímero (0.13-0.16-0.25). Con respecto al efecto de la relación molar, cuándo ésta se incrementa, el tamaño de partícula también aumenta. La temperatura y la presión en el recipiente de mezclado también son parámetros importantes del proceso. Cuando la presión y la temperatura en el interior del recipiente de mezclado fueron 15 MPa y 50 ºC, se obtuvieron partículas más grandes, comparado con los resultados obtenidos a 70 ºC y 11 MPa. En relación al contenido de β-caroteno, fue bajo en todos los casos, pero varió según los parámetros de proceso seleccionados. Como se esperaba, cuando la relación molar aumenta, también aumenta el contenido de β-caroteno. Las concentraciones de β-caroteno más altas (306-336

ppm) se obtuvieron a presiones y temperaturas elevadas (70 ºC y 15 MPa, respectivamente) y con tiempos de homogeneización cortos (60 minutes).

En la figura 8 se muestra la variación del tamaño de partícula de las partículas de CAPA 2403D (4000 g mol^{-1}) como una función de la relación molar de β-caroteno:policaprolactona, comparando con los resultados obtenidos con CAPA 6100 (10000 g mol^{-1}) en las mismas condiciones de temperatura (70 ºC), presión (15 MPa) y tiempo de homogeneización (60 minutes).

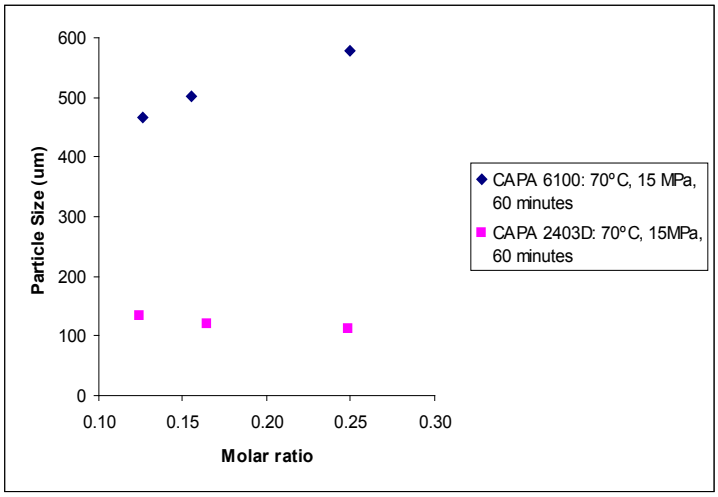


Figura 8. Efecto de la relación molar. Comparación entre CAPA 6100 (10000 g mol^{-1}) y CAPA 2403D (4000 g mol^{-1}).

En la representación en la figura 8 se puede observar que una gran diferencia en el tamaño de partícula cuando se emplean ambas policaprolactonas. Los tamaños de partícula obtenidos con CAPA 2403D fueron del orden de 110-130 µm, que son considerablemente más pequeños que los que se obtuvieron con CAPA 6100 en las mismas condiciones. Esto podría ser debido a la solubilidad distinta del CO_2 en los polímeros fundidos y también a las posibles diferencias en la viscosidad de los respectivos polímeros fundidos. La policaprolactona CAPA 2403D fundida presenta una viscosidad más baja que la CAPA 6100, siendo la atomización de CAPA 2403D más efectiva y permitiendo la obtención de partículas con tamaño más pequeño.

De este capítulo se pudo concluir que se ha llevado a cabo un estudio de la formulación de β-caroteno con policaprolactonas mediante el proceso PGSS, obteniendo partículas con un tamaño en el rango de 270-650 µm con un contenido máximo de β-caroteno de 340 ppm

cuando se emplea CAPA 6100, mientras que cuando se emplea CAPA 2403D, el tamaño de partícula se redujo a 110-130 µm.

CAPÍTULO 7

FORMULACIÓN DE β-CAROTENO CON LECITINA DE SOJA MEDIANTE SECADO-PGSS (PARTÍCULAS A PARTIR DE DISOLUCIONES SATURADAS DE GAS)

El secado-PGSS es una técnica de precipitación que se emplea para producir partículas a partir de disoluciones acuosas. Las principales ventajas del secado-PGSS sobre las técnicas convencionales (secado spray y liofilización) son una atomización más eficiente debido a la liberación rápida y expansión del gas a partir de la disolución durante la despresurización desde condiciones supercríticas a condiciones ambiente, y la posibilidad de secar el producto con temperaturas más bajas en la torre spray (40-80ºC).

El objetivo de este capítulo es el estudio de la formulación de β-caroteno con lecitina de soja mediante secado-PGSS, así como el estudio de la influencia de los parámetros principales del proceso. Se estudiaron la influencia de varios parámetros de proceso en la eficiencia de encapsulación de β-caroteno y en el tamaño de partícula, incluyendo la presión antes de la expansión (8-9-10 MPa), temperatura en el mezclador (variando entre 100ºC y 130ºC), relación gas/disolución (21-27-32) y concentración de lecitina de soja (55-62-72 g L^{-1}).

En la figura 9 se muestra la variación del tamaño de partícula y de la eficiencia de encapsulación con la temperatura antes de la expansión.

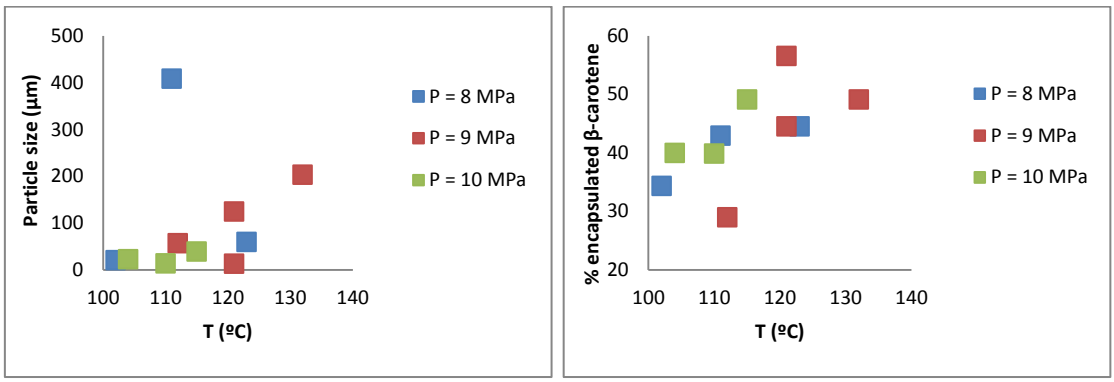


Figura 9. Efecto de la temperatura antes de la expansión en el tamaño de partícula y en la eficiencia de encapsulación.

La representación en la figura 9 revela que se obtienen tamaños de partícula más pequeños cuando la presión antes de la expansión aumenta y cuando la temperatura en el mezclador aumenta. Con respecto a la eficiencia de encapsulación, el porcentaje β-caroteno encapsulado aumenta cuando la temperatura en el mezclador se incrementa.
El resto de parámetros de proceso también tienen influencia en el tamaño de partícula y en la eficiencia de encapsulación, obteniendo tamaños de partícula más pequeños cuando la concentración de lecitina aumenta o cuando la relación gas/disolución disminuye. Por otro lado, la eficiencia de encapsulación aumenta cuando la concentración de lecitina de soja aumenta.

De este capítulo se pudo concluir que es posible obtener una formulación de β-caroteno mediante secado-PGSS obteniendo partículas secas en el rango de 10-500 μm y con eficiencias de encapsulación de β-caroteno de hasta el 60%. Estas partículas se pueden rehidratar formando de este modo liposomas multilamelares con β-caroteno, con tamaños en el rango de 1-5 μm.

CAPÍTULO 8

SOLUBILIDAD DE β-CAROTENO EN DIFERENTES POLI-(ε-CAPROLACTONAS) EN ESTADO COLOIDAL

El objetivo de este capítulo es el estudio de la solubilidad de β-caroteno en policaprolactonas con diferentes masas moleculares (CAPA 2403D = 4000 g mol^{-1}, CAPA 6100 = 10000 g mol^{-1} y CAPA 6250 = 25000 g mol^{-1}) en estado coloidal en un rango de temperatura entre 10ºC y 50ºC. La determinación de la solubilidad de β-caroteno se llevó a cabo mediante dos procesos diferentes: equilibrio-impregnación y equilibrio des-saturación, ambos llevados a cabo en un baño termal agitado.
Los resultados obtenidos mediante el proceso equilibrio-impregnación se muestran en la figura 10, observándose un incremento gradual en el contenido de β-caroteno impregnado en las policaprolactonas cuando la temperatura se incrementó, obteniendo contenidos máximos de β-caroteno entre 87 y 191 ppm dependiendo de la masa molecular de la policaprolactona.

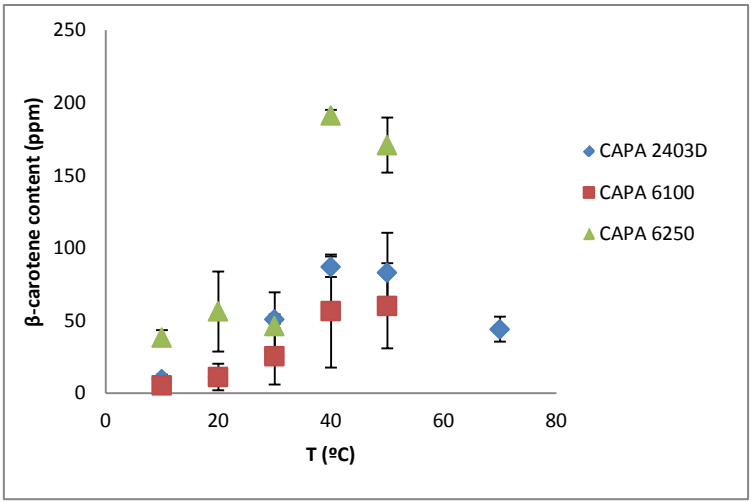


Figura 10. Contenido de β-caroteno en las tres policaprolactonas después del equilibrio por impregnación a diferentes temperaturas.

Con respecto a los experimentos de equilibrio des-saturación, las concentraciones de β-caroteno que se obtuvieron fueron mucho mayores que las obtenidas en los experimentos de impregnación, obteniendo un contenido de β-caroteno máximo de 8800 ppm cuando se empleó la policaprolactona de mayor masa molecular (CAPA 6250). Esto puede ser debido a la liberación lenta del β-caroteno a partir de las partículas de policaprolactona por su lenta degradación, confirmando que el proceso de equilibrio mediante des-saturación no es adecuado para la determinación de las concentraciones de equilibrio de β-caroteno en las policaprolactonas.

Por otro lado, se observó la pérdida de contenido de β-caroteno de las suspensiones de policaprolactona- β-caroteno durante distintos tiempos de exposición en la oscuridad, obteniendo un descenso drástico en el contenido de β-caroteno después de 7 días de exposición cuando se emplearon las policaprolactonas CAPA 2403D y CAPA 6100 y después de 14 días de exposición cuando se empleó CAPA 6250.

De este capítulo se pudo concluir que es posible la determinación de la solubilidad de β-caroteno mediante el proceso de equilibrio-impregnación, obteniendo contenidos máximos de β-caroteno entre 87 y 191ppm dependiendo de la masa molecular de la policaprolactona. Estos resultados corroboran los resultados obtenidos en el capítulo 6, obteniendo concentraciones de β-caroteno del mismo orden de magnitud.

4. CONCLUSIONES

Este trabajo es una contribución para el desarrollo de diferentes formulaciones de β-caroteno para su aplicación como colorante natural mediante tecnologías eficientes e innovadoras. A continuación se presentan las conclusiones más relevantes del presente trabajo de tesis doctoral:

PROCESO INNOVADOR DE EMULSIÓN PRESURIZADA

- La precipitación a partir del proceso emulsiones presurizadas es un método eficiente e innovador para desarrollar la formulación de β-caroteno con almidón modificado con el grupo OSA (n-(octenil) anhídrido succínico) empleando acetato de etilo como disolvente orgánico. Se ha demostrado que el almidón modificado fue un buen agente surfactante, permitiendo obtener suspensiones acuosas de β-caroteno estables con elevados porcentajes de β-caroteno encapsulado (por encima de 70%) y un tamaño de partícula micelar en el rango de 300-600 nm.
- Los parámetros con mayor influencia en las propiedades del producto son la concentración de surfactante (variando desde 37 hasta 367 g L^{-1}) y la relación orgánico-agua (desde 0.6 hasta 1.3 mL/mL). Los resultados han revelado que es necesario emplear concentraciones altas de almidón modificado (por encima de 100 g L^{-1}) y relaciones de orgánico-agua bajas (en el rango entre 0.65 y 0.73 mL/mL) para poder obtener un porcentaje de β-caroteno encapsulado alto y una estabilidad de la emulsión elevada.
- La optimización de los distintos parámetros del proceso permitieron desarrollar diferentes configuraciones alternativas del proceso:
    - El estudio del empleo de etanol como disolvente orgánico miscible en agua reveló es posible obtener una formulación de β-caroteno con una eficiencia de encapsulación máxima de 60% y con un tamaño de partícula en el rango de 1-23 μm, obteniendo los mejores resultados con relaciones orgánico-agua bajas (0.65-0.73 mL/mL). Cuando se emplearon concentraciones de β-caroteno muy bajas (1-5 g L^{-1}), los tamaños de partícula micelar disminuyeron por debajo de 560 nm pero las eficiencias de encapsulación máximas que se obtuvieron fueron del 38%. Por consiguiente, comparado con los resultados cuando se emplea acetato de etilo como disolvente orgánico, se obtuvieron eficiencias de encapsulación más bajas y tamaños de partícula más altos cuando se empleó etanol, evidenciándose propiedades del producto con peor calidad.

- El estudio del empleo de cuatro almidones modificados diferentes para el desarrollo de la formulación de β-caroteno permitió comprobar que un almidón modificado con el grupo OSA (n-(octenil) anhídrido succínico) derivado de maíz ceroso combinado con jarabe de glucosa deshidratado no es adecuado para la obtención de suspensiones acuosas de β-caroteno estables, debido a que se alcanzaron eficiencias de encapsulación bajas (menores de 30%), con tamaños de partícula de 1 μm, aproximadamente. Con los otros almidones modificados se alcanzaron eficiencias de encapsulación entre 30-45% y tamaños de partícula menores de 500 nm. Sin embargo, se obtuvieron mejores resultados con el almidón modificado refinado a partir de maíz ceroso, presentando eficiencias de encapsulación más altas (70-80%) con tamaños de partícula en el rango de los nanómetros.

PROCESO DE EMULSIFICACIÓN POR ULTRASONIDOS

- La técnica de emulsificación por ultrasonidos es un proceso eficiente para la formación de emulsiones de β-caroteno con almidón modificado con el grupo OSA (n-(octenil) anhídrido succínico) refinado a partir de maíz ceroso. La optimización de los parámetros de proceso más relevantes como tiempo de aplicación de ultrasonidos, amplitud de oscilación, ciclo y relación orgánico-agua, permitió la obtención de suspensiones acuosas de β-caroteno con eficiencias de encapsulación del 30% y con tamaños de partícula micelar menores de 200 nm.
- Comparado con emulsificación por ultrasonidos, la emulsificación por cizalladura produjo partículas con tamaños similares (menores de 240 nm), pero la eficiencia de encapsulación obtenida fue mucho más baja (8%). El proceso de precipitación a partir de emulsiones presurizadas permite obtener eficiencias de encapsulación mucho más elevadas (70-80%), pero con tamaños de partícula micelar mayores, 400nm.

PROCESOS CON FLUIDOS SUPERCRÍTICOS

- El estudio del equilibrio solido-líquido-gas de sistemas de policaprolactona-dióxido de carbono (considerando tres policaprolactonas con distintas masas moleculares) mediante determinación visual del primer punto de fusión reveló que las curvas de equilibrio sólido-líquido-gas muestran una temperatura máxima a presiones bajas (0.5-1.6 MPa) y una temperatura mínima a presiones moderadas (8-10 MPa) dependiendo

de la masa molecular de las policaprolactonas. Esta información es necesaria para llevar a cabo las técnicas de precipitación empleando fluidos supercríticos y para optimizar las condiciones de operación.

- La técnica PGSS (partículas a partir de disoluciones saturadas de gas) se empleó para el desarrollo de una formulación de β-caroteno hidrofóbica con distintas policaprolactonas, obteniendo partículas sólidas con un tamaño de partícula por encima de las 100 μm y un contenido de β-caroteno de hasta 340 ppm. Los parámetros del proceso con una influencia importante en el tamaño de partícula fueron la relación molar β-caroteno:polímero, presión, temperatura y tiempo de contacto entre el CO_2 y el polímero fundido para la homogeneización de la mezcla. Las concentraciones más altas de β-caroteno (306-336 ppm) se obtuvieron a elevadas presiones (15 MPa) y temperaturas (70ºC) y tiempos de homogeneización cortos (60 minutes).
- El estudio de la solubilidad de β-caroteno en distintas policaprolactonas en estado coloidal mediante el proceso de equilibrio-impregnación muestra que se alcanzan contenidos máximos de β-caroteno en el rango de 87-191 ppm dependiendo de la masa molecular de la policaprolactona. Estos resultados corroboran los resultados obtenidos mediante el proceso PGSS, ya que la concentración de β-caroteno que se obtiene por el proceso PGSS es del mismo orden de magnitud que la concentración de saturación de β-caroteno en el polímero. Por el contrario, los resultados que se obtienen mediante el proceso de equilibrio des-saturación muestran contenidos de β-caroteno considerablemente más altos (entre 1000 – 8800 ppm dependiendo de la masa molecular de la policaprolactona), posiblemente debido a la liberación lenta del β-caroteno a partir de las partículas de policaprolactona por su lenta degradación. De este modo, el equilibrio por des-saturación no es adecuado para la determinación de las concentraciones de equilibrio de β-caroteno en policaprolactonas.
- El proceso de secado-PGSS es una técnica adecuada para el desarrollo de una formulación soluble en agua de β-caroteno en lecitina de soja formando liposomas. Mediante este proceso, se han obtenido partículas secas en el rango de 10-500 μm y eficiencias de encapsulación de β-caroteno de hasta el 60%. Estas partículas se pueden rehidratar formando de este modo liposomas multilamelares con β-caroteno, con tamaños en el rango de 1-5 μm. Los parámetros de proceso con mayor influencia en las características del producto son la temperatura y la presión antes de la expansión, relación de gas/disolución (GPR) y concentración de lecitina de soja.

ACKNOWLEDGEMENTS

En primer lugar, quiero agradecer a mis directores de tesis, María José Cocero y Ángel Martín, la confianza depositada en mí desde el primer día y la ayuda recibida en todo momento a lo largo de todos estos años.

I would like to acknowledge Prof.Dr. Marco Mazzotti, head of the group of Separations Processes Laboratory (Swiss Federal Institute of Technology Zurich, ETH Zurich), for the possibility to carry out a research collaboration in his group. I would also like to acknowledge Dr. Johannes Kluge for his support and kindness. And finally, I would specially like to thank the members of this group, for the great reception and pleasant work environment.

I am very grateful to Dr. Catarina M.M. Duarte for giving me the possibility of doing a research collaboration in her group and for her guidance and advice during my stay. This collaboration could not be possible without the financial support from Conselho de Reitores das Universidades Portuguesas and the Spanish Ministerio de Ciencia e Innovación (PT2009-0173) through the Integrated Actions Portugal–Spain.

Me gustaría agradecer al programa de becas FPI UVa (Formación de personal investigador de la Universidad de Valladolid) por la financiación recibida.

Tengo que agradecer también a Javier Herreras, María Largo, Elvira Mateos y Alejandro Bartolomé por su aportación a esta tesis mediante trabajos de investigación. Ha sido muy agradable trabajar con todos ellos.

A todos mis compañeros del Departamento, a la DDC (las chicas…) y a la FAF (los chicos…). De una forma especial quiero nombrar a Pablo, ya que ha sido mi compañero a lo largo de todos estos años de trabajo y con el que compartí la famosa y gran aventura de la “cárcel”, a Sali por su ayuda en todo momento, a Danilo por ayudarme siempre con las Gilson…, al Chatín (alias Don Perfecto) por ayudarme con los problemas típicos de última hora de formatos, pdf y demás, y a Cris por ser mi amiga y escucharme en todo momento.
Por otro lado, tengo que mencionar al grupo de los “pinchines”, que gracias a ellos la última etapa de la tesis ha sido muy amena… (descubriendo garitos “todo guapos”

especialmente el “Gao” y cerrando we rock….) ayudándome a desconectar un poco de tanto redactar… Quiero nombrar de una forma muy especial a Mariana, que gracias al congreso de Lyon se ha convertido en una gran amiga (¡las agachadizas de largo pico y rápido vuelo!).

A ti LUISMI, simplemente gracias por estar ahí y por hacerme pasar cada día momentos inolvidables (lul, qué chica qué chica, 2 rombos, los globitos, la cobra y la mangostilla, “pon un fresno en tu vida”, reina “mora”, tommy, el puente y el príncipe, el abuelo y la abuela, city girl….).

Sin duda, a mis padres, a mi hermana y a David, por haberme animado a tomar la decisión de hacer un doctorado, por estar siempre ahí, por el apoyo y la confianza que me han dado en todos estos años y también por aguantar mis lloreras, que no han sido pocas. Por último, a mis sobrinos, Nicolás y Daniela, por darme tanta alegría y felicidad.

ABOUT THE AUTHOR

Esther de Paz Barragán (Santa María del Páramo (León), 1984) started the studies of Chemical Engineer at the University of Valladolid in 2002. In the academic course of 2007-2008, she spent 6 months at the Budapest University of Technology and Economics (Budapest, Hungary) for the development of the Final Project, in the frame of the Erasmus Program. This stay was in the SFE Group (Supercritical Fluid Extraction Research Group) working on the "Resolution of menthol with O-O´-2R, 3R-Dibenzoyl –Tartaric acid in pressurized carbon dioxide". So, those 6 months were the first contact with the supercritical fluids research.

At the return from Budapest, she graduated (April, 2008), and afterwards, she started to work in a company as quality technician (León, Spain). Few months later, in November 2008, she gave up her job in order to start a PhD in the High Pressure Processes Group of the Department of Chemical Engineering (University of Valladolid). Her PhD was focused in the formulation of β-carotene for application as natural colorant. In 2010, she was in the Instituto de Biologia Experimental e Technológica, IBET (Oeiras, Portugal), doing a stay during two months. And in 2011, she was at Swiss Federal Institute of Technology Zurich, ETH Zurich (Zurich, Switzerland) during three months of stay.

The formulation of β-carotene carried out by precipitation from pressurized emulsions, ultrasound emulsification and supercritical fluid process are the main topics which have been investigated by the author.

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- **De Paz, E.**, Martín, Á., Cocero, M.J. Formulation of β-carotene with soybean lecithin by PGSS (Particles from Gas Saturated Solutions)- drying. Journal of Supercritical Fluids, 2012, 72, 125-133.
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