Influence of soy protein's structural modifications on their microencapsulation properties: α-tocopherol microparticles preparation

Alla Nesterenko[a,b], Isabelle Alric[a,b], Françoise Silvestre[a,b], Vanessa Durrieu[a,b]

[a] *Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle), F-31030 Toulouse, France*

[b] *INRA, UMR 1010 CAI, F-31030 Toulouse, France*

Corresponding author email: Vanessa.Durrieu@ensiacet.fr

phone number : +33 (0)5 34 32 35 58

fax : +33 (0)5 34 32 35 98

ABSTRACT

Enzymatic and chemical modifications of soy protein isolate (SPI) were studied in order to improve SPI properties for their use as wall material for α-tocopherol microencapsulation by spray-drying. The structural modifications of SPI by enzymatic hydrolysis and/or N-acylation were carried out in aqueous media without any use of organic solvent neither surfactant. Emulsions from aqueous solutions of native or modified SPI and hydrophobic α-tocopherol, were prepared and spray-dried to produce α-tocopherol microparticles. The effect of protein modifications and the influence of the core/shell ratio on both emulsions and microparticles properties were characterised. The obtained results demonstrated that oil-in-water emulsions prepared with modified proteins had lower droplet size (0.5-0.9 µm) and viscosity (3.6-14.8 mPa·s) compared to those prepared with native proteins (1.1 µm and 15.0 mPa·s respectively). Efficiency of oil retention decreased after protein hydrolysis from 79.7 to 38.9%, but the grafting of hydrophobic chain by acylation improved efficiency of α-tocopherol retention up to 94.8%. Moreover, higher emulsion viscosity, particle size and process efficiency were observed with the increase of α-tocopherol amount.

Keywords : Soy protein isolate, Acylation, Hydrolysis, α-Tocopherol, Microencapsulation, Spray-drying

1. Introduction

Vegetable proteins, being relatively word cheap, non-toxic, biocompatible and biodegradable biopolymers, are actually becoming a realistic alternative to animal proteins and even synthetic polymers for some specific applications (Baniel, Caer, Colas, & Gueguen, 1992; Chel-Guerrero, Perez-Flores, Betancur-Ancona, & Davila-Ortiz, 2002; Gennadios & Weller, 1990; Mateos-Aparicio, Redondo Cuenca, Villanueva-Suárez, & Zapata-Revilla, 2008; Vliet, Martin, & Bos, 2002). Their use as wall material for active components microencapsulation reflects this actual tendency, particularly in nutritional, pharmaceutical and cosmetic fields (Gharsallaoui et al., 2010; Nori, Favaro-Trindade, Alencar, Thomazini, & Balieiro, 2010; Patel, Heussen, Hazekamp, Dorst, & Velikov, 2012; Rascon, Beristain, Garcie, & Salgado, 2011; R. Wang, Tian, & Chen, 2011). The water solubility and amphiphilic properties, the ability to self-associate and interact with variety of substances, the high molecular weight and molecular chain flexibility of vegetable proteins give them excellent surfactant properties for emulsification. These proteins are thus very suitable for

microencapsulation techniques requiring preliminary emulsions such as spray-drying, coacervation and solvent evaporation. Finally, due to their facility for adhesion and good film formation, their resistance toward oils or organic solvents and gas barrier properties (De Graaf, Harmsen, Vereijken, & Monikes, 2001), proteins are really good wall forming product.

Among vegetable proteins, soy protein isolate (SPI) has been extensively studied for numerous applications. As protein from legume seeds, it has a potential role as substrate for the development of delivery systems due to its functional properties and high nutritional value (Pereira et al., 2009). Indeed, its good gelling, emulsifying, fat-absorbing and water binding properties (Caillard, Remondetto, & Subirade, 2009; Hua, Cui, Wang, Mine, & Poysa, 2005; Nunes, Batista, Raymundo, Alves, & Sousa, 2003), make it very relevant on a microencapsulation point of view. For spray-drying microencapsulation, the selection of encapsulant agents is based on their interfacial functionality, good solubility and low viscosity at high solid content (Kim, Morr, & Schenz, 1996). Soy protein possesses good solubility in water at pH higher than 9 and is able to form a dried matrix around dispersed compounds in dehydration processes, which entraps them inside the matrix and protect them from air oxidation and UV degradation (Charve & Reineccius, 2009).

Microencapsulation of oil or water soluble substances with SPI was particularly investigated using spray-drying (Augustin, Sanguansri, & Bode, 2006; Charve & Reineccius, 2009; Kim et al., 1996; Ortiz, Mauri, Monterrey-Quintero, & Trindade, 2009; Rascon et al., 2011; Rusli, Sanguansri, & Augustin, 2006; Yu, Wang, Yao, & Liu, 2007), simple (Gan, Cheng, & Easa, 2008) or complex coacervation (Lazko, Popineau, & Legrand, 2004; Mendanha et al., 2009; Nori et al., 2010) and gelation (Chen & Subirade, 2009) techniques. Soy protein is generally used as individual coating material, but also can be mixed with polysaccharides such as maltodextrin, pectin, etc. (Augustin et al., 2006; Favaro-Trindade, Santana, Monterrey-Quintero, Trindade, & Netto, 2010; Mendanha et al., 2009; Nori et al., 2010; Yu et al., 2007). In case of hydrophobic core, microencapsulation using SPI or SPI/polysaccharide blends, oil-in-water emulsion stabilization before spray-drying is often effectively carried out by high pressure homogenization.

The continuous need for multi-functional materials has required to develop different modification techniques (chemical, enzymatic or physico-chemical) to enhance and diversify protein functionalities, more appropriate to current microencapsulation techniques (Magdassi, 1996). Protein hydrolyzates have been shown to have various interesting properties such as higher solubility, foaming and emulsifying properties compared to the native proteins (Kong, Guo, Cao, & Zhang, 2008). Acylation was used to enhance protein surface hydrophobicity,

solubility, emulsifying and foaming properties, offering a range of possibilities for the development of new applications (Yin, Tang, Wena, Yang, & Yuan, 2010). Indeed, attachment of hydrocarbon chains may modify the charge and structural properties of water-soluble proteins, increasing their hydrophobicity, and enhancing the amphiphilic characteristics. Thus, protein affinity with oil should be reinforced, inducing an improved microencapsulation efficiency for hydrophobic active core (Lazko et al., 2004).

This study was conducted to investigate the effect of soy protein isolates modifications on their microencapsulation properties, compared to native protein. Proteinic chains of SPI were modified by enzymatic hydrolysis and/or *N*-acylation. Acylation reaction was carried out with three different hydrophobic groups (8, 12 and 16 carbon atoms). Modified and native proteins were then used for α-tocopherol microencapsulation by spray-drying technique. In the context of green chemistry, all the modifications and preparations were carried out without use of organic solvent and chemical catalyst, that was appropriated to food industry (Aider, 2010; Vilkhu, Mawson, Simons, & Bates D., 2008). The effects of proteins chemical modifications on emulsions properties, process yield, efficiency of oil retention, microparticles morphology and size distributions were examined. Finally, the soy protein/α-tocopherol ratio was also varied in order to examine its influence on emulsions and microparticles properties.

2. Materials and methods

2.1. Materials

Soy protein isolate, 90% pure, was purchased from Lustrel Laboratoires SAS (Saint Jean de Vedas, France). All other chemicals were of analytical grade. α-Tocopherol, alcalase (protease from Bacillus licheniformis), amino acid standard, NaOH, HCl 37%, octanoyl chloride, dodecanoyl chloride, hexadecanoyl chloride and cyclohexane (HPLC grade) were purchased from Sigma (Saint-Quentin Fallavier, France).

2.2. SPI characterisations

Soy protein isolate was analysed for proximate composition – moisture, lipid, ash and protein contents. The moisture content and the ash content were determined by heating a

sample of the air-dried material in an oven at 105 and 550-600°C respectively to constant weight (AOAC, 1995). The protein content was determined by Kjeldahl method (N×6.25) (Helrich, 1990; McGeehan & Naylor, 1988) and lipid content by conventional Soxhlet extraction in cyclohexane during 7 hours. These analyses were performed in triplicate.

Amino acid profile of SPI was identified after total acid hydrolysis. Initially, 1 g of SPI was hydrolyzed with 10 mL of 5.37 N HCl at 105°C for 24 hours under a nitrogen atmosphere in a sealed tube. Then liquid phase was removed by evaporation and the sample was dissolved in 10 mL of buffer (pH 2.2), filtered through a 0.45 μm PTFE membrane filter and analysed by using the Biochrom 30 amino acid analyzer (Serlabo Techonogies, Entraigues sur la Sorgue, France). All determinations were carried out in triplicate.

Protein solubility profile was determined by the following method (Zheng, Yang, Tnag, Li, & Ahmad, 2008). Protein samples were mixed with deionized water in the ratio of 5% w/w and the pH of the mixture was adjusted to 1.0-13.0 with 4 M NaOH or 4 M HCl. Protein solubility was determined at room temperature and at 70°C. Due to the low solubility at room temperature, the temperature of 70 °C was chosen in order to obtain an improved solubility without protein denaturation, as the major globulin fraction of soy protein is still resistant to thermic denaturation in aqueous solution at 65-73°C (Sun & Arntfield, 2012). All suspensions with different pH values were stirred for 1 hour at both temperatures and centrifuged at 10000 g for 15 min (Sigma Laborzentrifugen, Osterode, Germany). Protein content in the supernatant was determined by Kjeldahl method. Protein solubility (S%, w/w) was calculated using the formula:

S (%) = protein content in the supernatant/total protein content in solution×100 (1)

2.3. SPI Modifications

2.3.1. Enzymatic hydrolysis

The enzymatic hydrolysis was carried out according to the method described in literature (Kong et al., 2008). The controlled hydrolysis conditions were: time, temperature, pH, enzyme/protein ratio (0.002 w/w) protein concentration (5% w/w). SPI in water solution was prepared and incubated in a bath at 50°C for 10 min. When the protein solution reached 50°C, enzyme solution with the activity of 2.4 U/g was added. The pH of solution was maintained constantly at 7.0 by adding a 4M NaOH solution during 15 minutes of reaction. The reaction was ended by adjusting the pH of the solution to 4.5 with a 4M HCl solution. After the reaction period, the mixture was cooled, adjusted to pH 7.0 and heated at 95°C for

10 min to inactivate enzyme. Then the mixture was freeze-dried using a Cryo-Rivoire equipment at 20 Pa (Cryonext, Saint Gely du Fesc, France) and stored at 4°C (M1) or kept for the acylation (M2, M3).

The degree of hydrolysis (DH) was analysed by the method using o-phthaldialdehyde (OPA) (Church, Swaisgood, Porter, & Catignani, 1983; Goodno, Swaisgood, & Catignani, 1981). OPA reacts with primary amino groups i.e. N-terminal and lysine residues of proteins forming a chromophore with UV-absorbance optimum at 340 nm with UV Spectometer (UV-1800, Shimadzu, Kyoto, Japan). Non hydrolyzed, totally hydrolyzed (acid hydrolysis at 105°C during 24 h) and partially hydrolyzed proteins were analysed by OPA method, and DH was calculated using following equation:

$$DH\ (\%) = (N_h\text{-}N_0)/(N_t\text{-}N_0)\times 100 \quad (2)$$

where N_h – molar quantity of amino groups per gram of partially hydrolyzed protein, N_0 – molar quantity of amino groups per gram of non hydrolyzed protein, N_t – molar quantity of amino groups per gram of totally hydrolyzed protein. Analyses were carried out in triplicate.

2.3.2. N-acylation

N-acylation reactions were carried out with SPI or SPI hydrolyzates (started in the same medium after hydrolysis and enzyme deactivation), using octanoyl chloride (C8), dodecanoyl chloride (C12) or hexadecanoyl chloride (C16), following Schotten-Baumann reaction (Rondel, Alric, Mouloungui, Blanco, & Silvestre, 2009). SPI solution (5% w/w, pH 10) was prepared in deionized water and the fatty acid chloride was slowly added to this mixture. The molar ratios fatty acid chloride/NH_2 of protein used for reaction were 0.5 and 1. Depending on these two ratios the protein samples obtained after acylation with C8 (C12, C16) were called M4 and M5 (M6 and M7, M8 and M9) respectively. The solution was stirred during 30 min at room temperature, then 180 min at 50°C. During acylation, pH was maintained constantly at 10.0 by addition of a 4M NaOH solution. Then the mixture was freeze-dried and the powder was stored at 4°C.

The degree of acylation (DA) was determined by OPA-method according to the same protocol as degree of hydrolysis. DA was calculated as:

$$DA\ (\%) = (n\text{-}n_a)/n \times 100 \quad (3)$$

where n - number of amino groups from non hydrolyzed or partially hydrolyzed protein, n_a - number of amino groups from protein after acylation. Analyses were carried out in triplicate.

2.4. Emulsion preparation

Oil-in-water emulsions were prepared according to the following procedure: 8% w/w protein solution based on intact or modified SPI with pH adjusted to 10.5 was heated to 70°C for 120 minutes under constant mechanical stirring up to maximal protein solubilisation. α-Tocopherol (T) was preheated to 70°C and quickly added to the protein solution under mechanical stirring at 500 tr/min for obtaining o/w pre-emulsion in which the ratio SPI/α-tocopherol was 2/1 (11.5% w/w of total solids). To obtain a stable and uniform emulsion of α-tocopherol in SPI solutions, homogenization was carried out at 50 MPa with double circulation through homogenizator (APV Systems, Albertslund, Denmark). The first circulation is known to disintegrate and disperse particles throughout the fluid, whereas the second would to break down the newly formed or re-agglomerated clusters of droplets after the first homogenization to enhance the emulsions quality (Yu et al., 2007). Proportions wall/core of 1/1 and 1/2 (14.8% w/w and 20.7% w/w of total solids) were also tested using SPI as a wall material to obtain the emulsions M0* and M0** or SPI acylated with C12 wall material to obtain the emulsions M6* and M6** respectively.

2.5. Emulsion characterisations

2.5.1. Emulsion droplet size measurements

Oil droplets size average and distribution in emulsions were measured by laser diffraction instrument (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK). To avoid multiple scattering effects, emulsions were diluted 100 times with deionized water before measurements. A relative refractive index n_{oil}/n_{water}=1.12 (n_{oil}=1.49, n_{water}=1.33) was used for the calculation of particle size distributions, assuming that all droplets were spherical in shape. The volume particle diameter (D_{43} or D_v) was calculated as mean of three readings per sample.

2.5.2. Emulsion morphology

To complete the information obtained by laser granulometry, emulsions were visualised using an Eclipse E600 optical microscope (Nikon, Sendai, Japan), connected to a digital video camera (DXM1200, Nikon, Sendai, Japan) at magnication of 1000×.

2.5.3. Emulsion viscosity measurements

Emulsion viscosity before and after homogenization was determined at 20°C and shear stress variation between 0 and 1 N/m^2 during 3 min, using a Rheometer CSL100 (Carri-Med LTD, Dorking, UK) with cone-plate geometry of 6 mm diameter and 0.035 rad angle. All emulsions were characterized as Newtonian fluids.

2.6. Spray-dried microparticles preparation

Freshly homogenized emulsions were spray-dried in a Mini Spray Dryer B-290 (Büchi, Flawil, Switzerland) at the following process conditions, as follows: inlet air temperature at 120±4°C and outlet at 75±4°C, drying air flow rate of 470 L/h, liquid feed flow rate of 0.33 L/h and aspiration of 100%. Microparticles were collected from the container, closed hermetically in an opaque packaging and stored at 4°C. The yield of spray-drying was defined as follows:

$$\text{Spray-drying yield (\%)} = M_P/M_{SPI+T}\times 100 \quad (4)$$

where M_P – the mass of collected powder and M_{SPI+T} – the initial mass of solid content added in emulsion including soy protein and α-tocopherol.

2.7. Microparticles characterisations

2.7.1. Moisture content

Moisture content of all the obtained powders was determined with infrared moisture balance (Sartorius, Goettingen, Germany) by drying at 105°C to constant weight.

2.7.2. Retention efficiency (RE)

Efficiency of α-tocopherol retention after microencapsulation was calculated as the percent ratio of estimated active agent (α-tocopherol) content of obtained particles or obtained powder (T_{exp}) on theoretical active agent content (T_{theo}):

$$RE\ (\%) = T_{exp}/T_{theo}\times 100 \quad (5)$$

To determine the amount of α-tocopherol in the prepared microspheres, UV/VIS spectroscopy was used (Faria, Mignone, Montenegro, Mercadante, & Borsarelli, 2010). Initially, a calibration curve absorbance vs. concentration was prepared with the T dissolved in

cyclohexane (previously, protein insolubility in this solvent was verified) and analysed at λ_{max} of 298 nm. After, 5 mg of microspheres containing α-tocopherol to be determined were broken in mortar and dissolved in 10 mL of cyclohexane. The solution was stirred 10 min and filtered through a 0.2 μm PTFE membrane filter. The absorbance of the solution was measured using a UV/VIS absorbance spectrometer at 298 nm. This procedure was carried out in triplicate.

Loading efficiency (LE) is the α-tocopherol content per 100 g of powder. This parameter was calculated as:

$$LE\ (\%) = T_{theo} \times RE \quad (6)$$

2.7.3. Microparticles size distribution

Particle size distribution in dry powder was determined by the scattering pattern of a transverse laser light using the equipment Scirocco 2000 (Malvern Instruments, Worcestershire, UK), that determined the particle mean diameters ranging from 0.2 to 2000 μm. The used refractive index is 1.52, pressure of air of dispersion – 4 Bars, degree of vibration – 70%. The volume particle diameter (D_{43} or D_v) was calculated as mean of three measurements per sample.

2.7.4. Microparticles microstructure

Morphology of the microparticles was examined by scanning electron microscopy (SEM). The particles were deposited on conductive double-faced adhesive tape and sputter-coated with silver. In order to examine the inner structure of prepared microparticles, the powder was froze in liquid nitrogen and broke in mortar. SEM observations were performed with a LEO435VP scanning electron microscope (LEO Electron microscopy Ltd., Cambridge, UK) operated at 8 kV.

2.7.5. Thermogravimetric analysis (TGA)

TGA was carried out using ATG/DSC Q600 from TA Instruments (New Castle, US) at a linear heating rate of 10°C/min. The weight of all samples was kept within 9-10 mg in a platinum pan. The temperature range was from 20 to 1100°C. Thermal stability of wall material (SPI), active core (T) and prepared microparticles (M0) was represented by TG curves.

2.8. Statistical analysis

The experimental data was statistically analyzed using Minitab 16 software (State College, USA). A one way analysis of variances (ANOVA) was performed to determine significant differences ($P<0.05$) among the samples. Tukey's test was adopted as the multiple comparison procedure.

3. Results and discussion

3.1. SPI characterization

According to obtained results, the wall material, SPI, was predominantly constituted of proteins (82.3% or 89.5% in dry matter), but also presented 1.6% of lipids, 5% of ahs and 8% of moisture. The carbohydrate content was calculated as the difference between 100% and the combined percentage of crude protein, lipid, ash and moisture. Amino acids composition of hydrolyzed SPI showed that this protein was principally made up of 16 amino acids (Fig. 1), that is characteristic for soy protein (George & Lumen, 1991; Zamora, 2005).

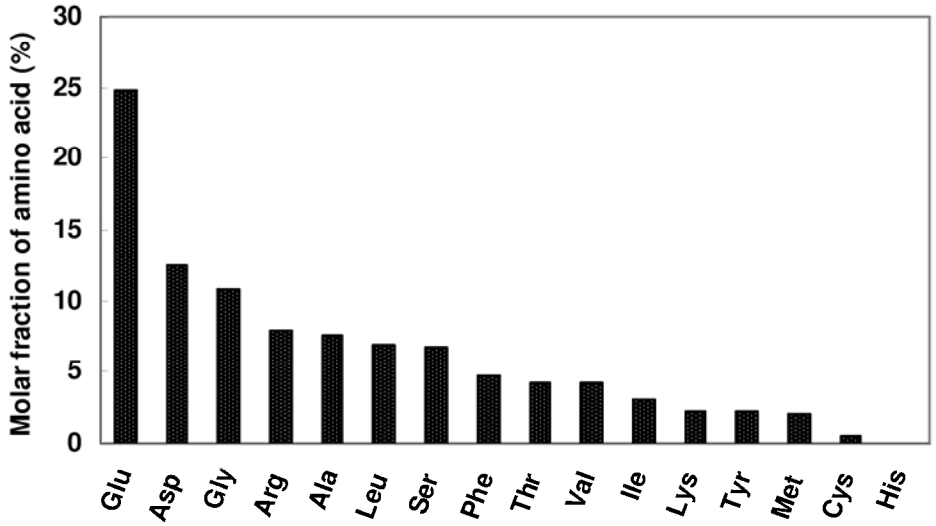

Fig. 1. Amino acid composition of soy protein isolate.

The solubility profiles for the SPI as a function of pH at two different temperatures are presented in Fig. 2. The presence of two amino acids, glutamic acid and aspartic acid, in greatest concentrations explains the low isoelectic point 4.7 of protein.

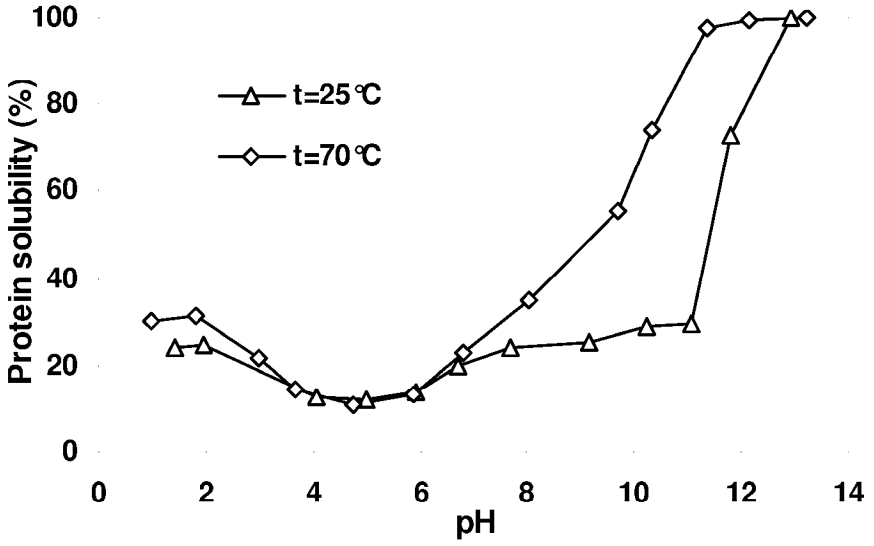

Fig. 2. Effect of pH and temperature on the solubility profile of SPI.

These observations are typical of soybean proteins (Deng et al., 2006; Solina, Baumgartner, Johnson, & Whitfield, 2005). The results in Fig. 2 show that solubility of SPI at alkaline pH was significantly higher at 70°C than solubility at room temperature. The protein higher solubility at alkaline pH values can be explained by protein functionality which is closely related to the conformational state, influenced by processing conditions (temperature, pH, solvent) (Harwalkar & Ma, 1991) and affected by the equilibrium between protein-solvent and protein-protein interactions (Mo, Zhong, Wang, & Sun, 2006). Due to their high amount in aspartic and glutamic acids, proteins have negative net charges (COO^-) on their surfaces at alkaline pH. The repulsion between polymeric chains increase and the protein-protein interactions decrease, which favours the protein-water interactions and explains the maximum of SPI solubility at pH higher than 10 (Fennema, 1993). The energy brought by heating has also a positive effect on the protein solubilisation, increasing the fraction of soluble SPI in solution. A good solubility of wall material before spray-drying is essential to enhance microencapsulation efficiency (Gouin, 2004). For this reason all the SPI/T emulsions were prepared with the soy protein solubilised at pH 10.5 at the temperature of 70°C.

3.2. Emulsion preparation and characterisation

Emulsion preparation is the first step involved in the process of microencapsulation by spray-drying. Emulsion parameters have significant influence on microparticles properties. For example, different authors showed that retention of active compounds during spray-drying could be enhanced by reducing the mean emulsion droplet diameter of dispersed components during emulsification (Mongenot, Charrier, & Chalier, 2000; Sheu & Rosenberg, 1995; Soottitantawat, Yoshii, Furuta, Ohkawara, & Linko, 2003). Accordingly, high-pressure homogenization has already been widely used in emulsion preparation for microencapsulation (Augustin et al., 2006; Kim et al., 1996; Rascon et al., 2011; Rusli et al., 2006; Yu et al., 2007). Indeed, in addition to an evident emulsion stability improvement, the authors (Rusli et al., 2006; Yu et al., 2007) noted that the high pressure homogenization decreases slightly the emulsion viscosity of and the oil droplet average size.

To control the good dispersion of oil in protein solution, emulsions stability and uniformity of droplet size, preparations were analysed by light scattering and observed by optical microscope at 1 hour, 1 day and 1 week after homogenization (Fig. 3 and Fig. 4). The volume average diameter (D_v) of emulsion remained 0.95±0.1 μm. No significant change in oil droplet size in time was observed. Emulsions were remained stable and displayed bimodal distributions indicating the presence of small portion of coalesced particles.

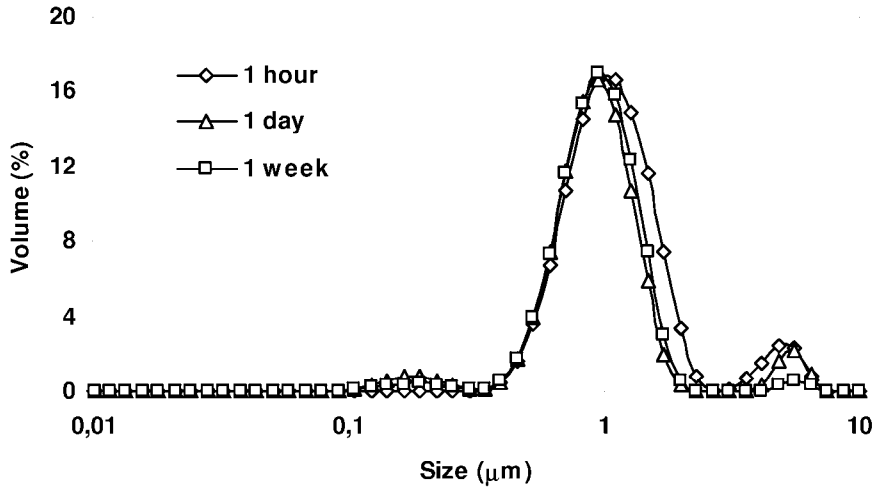

Fig. 3. Droplet size distribution of SPI/α-tocopherol emulsion (M0) 1 hour, 1 day and 1 week after high pressure homogenization at 50 MPa. The oil/protein ratio of the emulsion was 1/2.

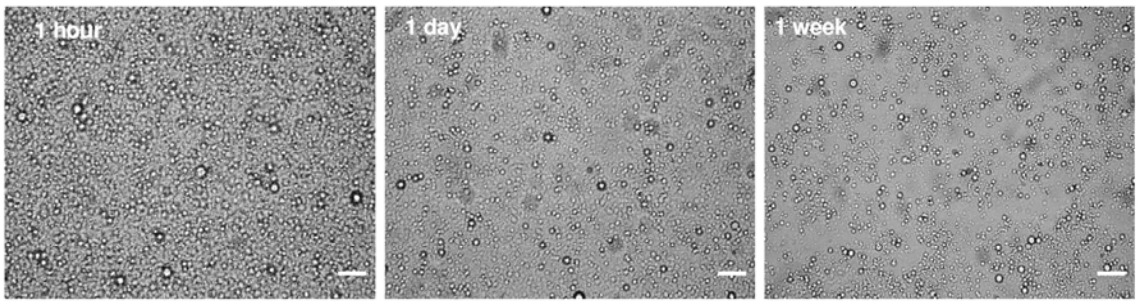

Fig. 4. Optical micrographs of SPI/α-tocopherol emulsion (M0) 1 hour, 1 day and 1 week after high pressure homogenization at 50 MPa. Scale bar – 10 μm.

Emulsion viscosity is an important parameter affecting mainly the size of microparticles and the thickness of their wall (Risch & Reineccius, 1988; Rosenberg, Kopelman, & Talmon, 1990). With viscosity increase, the residence time of emulsion in spray-dryer was higher and the droplets formation is more difficult. Thus, solvent evaporation rate is changing as well as droplet and microparticle size (Lefebvre, 1989). The rheological properties were studied to determine the influence of homogenization pressure to the viscosity of the emulsions. Viscosity of emulsion M0 before homogenization was about 44 mPa·s and decreased significantly after homogenization to 15 mPa·s. That could be due to breaking of intermolecular hydrogen bonds and Van der Waals attractive forces between the proteins and water molecules during homogenization. Under giveng conditions soy proteins were not completely soluble and homogenization pressure caused the interruptions of all intermolecular bonds between protein chains and so affected the decrease of viscosity. Furthermore, the mechanical energy transfer to fluid particles under high pressure involves the microfluidization process, generating microstreams with increased velocity (Ciron, Gee, Kelly, & Auty, 2010; Tunick, Hekken, Cooke, Smith, & Malin, 2000) and formation of fine emulsion with reduced viscosity.

After stabilisation, emulsion M0 was spray-dried at inlet temperature of 120°C and the obtained powder of microparticles was collected for following analysis.

3.3. Thermal analysis

Fig. 5 shows the TG mass loss curves of the soy protein isolate (SPI), α-tocopherol (T) and SPI/T microparticles (M0). The mass loss of α-tocopherol of 100% with complete

decomposition occurred in the range of 220-380°C. No degradation of α-tocopherol was observed before 200°C. These results confirm that this core material would not been affected by spray-drying temperature of 120°C. Moreover, during spray-drying process the particles temperature reached approximately 70°C due to cooling through evaporating water.

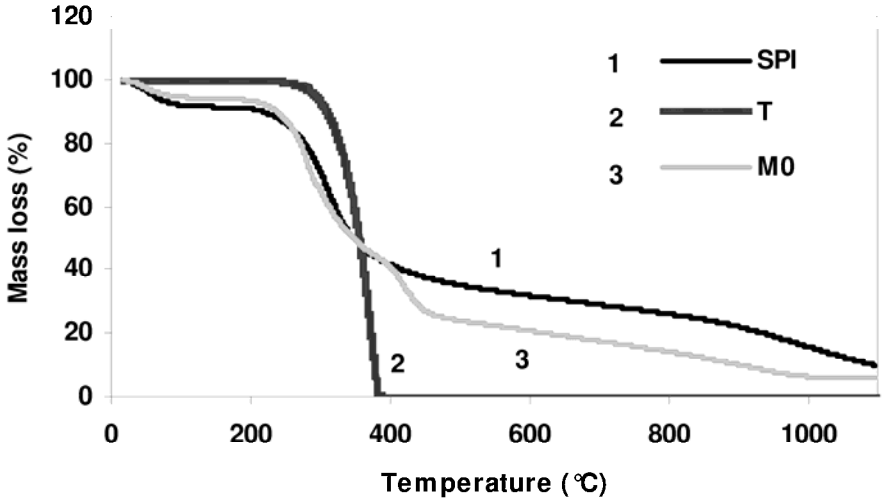

Fig. 5. TG curves of SPI, T and SPI/T (M0) microparticles.

Small mass loss at 100°C is related to residual water molecules presented in SPI and M0. The visible decrease of M0 TG curve after 400°C compared to SPI curve is attributed to evaporation of α-tocopherol situated in microparticles. Soy protein degradation was observed at temperatures higher than 200°C with maximal mass loss (more than 50%) between 200-400°C. The same thermal behaviour was described by other authors for the soy proteins (Guerrero, Retegi, Gabilondo, & Caba, 2010; Schmidt, Giacomelli, & Soldi, 2005; H. Wang, Jiang, & Fu, 2007).

3.4. SPI modifications

Protein hydrolyzates have shown various interesting properties such as solubility, foaming and emulsifying properties compared to the intact proteins (Kong et al., 2008). Enzymatic hydrolysis resulted in decrease of protein molecular mass with less secondary structure, and in improvement of protein's functional properties and solubility. This method also permits the producing of more uniform products. Hydrolysis is very promising way for development of functional and nutritious ingredients. In our work, soy protein hydrolyzate

having a hydrolysis degree of 4% (acylated or not) was prepared and studied as a wall material for the α-tocopherol microencapsulation.

Soy proteins acylation is also known to affect their physico-chemical properties, particularly their emulsifying one (Matemu, Kayahara, Murasawa, Katayama, & Nakamura, 2011). Indeed, attachment of hydrocarbon chains modifies the charge and structural properties of water-soluble soy proteins, increasing their hydrophobicity, thus improving their affinity for hydrophobic substrates such as α-tocopherol. The acylation rate of SPI and hydrolyzed SPI was determined as described previously. Table 1 shows the degree of these modifications.

Table 1. Modification degree of soy proteins.

Sample[A]	NH_2/fatty acid (n/n)	DH[B] (%)	DA[C] (%)
M1	-	4±1	-
M2	1/0.5	4±1	31±1.8
M3	1/1	4±1	61±2.5
M4	1/0.5	-	25±1.2
M5	1/1	-	31±2.3
M6	1/0.5	-	32±0.8
M7	1/1	-	38±1.3
M8	1/0.5	-	29±0.8
M9	1/1	-	31±1.8

[A] M1: hydrolysed soy proteins; M2, M3: hydrolysed and acylated with C12 soy proteins; M4, M5: acylated with C8 soy proteins; M6, M7: acylated with C12 soy proteins; M8, M9: acylated with C16 soy proteins;
[B] DH: degree of hydrolysis determined by OPA method;
[C] DA: degree of acylation determined by OPA method.

In case of protein's NH_2/fatty acid ratio of 1/0.5, the protein (native or hydrolyzed) chain length and the hydrocarbon chain length had no significant influence on acylation degree (varying between 25 and 31%). The variation in the number of free amino groups accessible to interact with fatty acid induced by protein hydrolysis influenced significantly the acylation degree. For the ratio protein's NH_2/fatty acid of 1/1, the acylation degree of hydrolyzed SPI was 61% (M3) and that of native SPI was 31-38% (M5, M7 and M9).

Concerning acylated SPI, the acylation degrees were similar whatever the protein's NH_2/fatty acid ratio. The only difference between these samples (M4 and M5, M6 and M7, M8 and M9 respectively) was the residual fatty acid salt rate. This rate was about 0.18-0.19 mmol per gram of protein for M4, M6 and M8, and 0.34-0.37 mmol per gram of protein for

M5, M7 and M9. As these fatty acid salts have surfactant properties, they were maintained in the mixtures to eventually contribute to emulsion stabilisation.

3.5. Influence of SPI modifications on emulsions properties

Modified SPI/T emulsion characterisations are presented in Table 2. These emulsions were prepared with high pressure homogenization to ensure an effective dispersion of oil in protein solution, to limit droplets coalescence and to produce stable emulsions as shown in Fig. 6. The homogenization step did not involve significant changes in emulsion viscosity for almost of the samples, and in any case, obtained viscosities were all suitable for following spray-drying process.

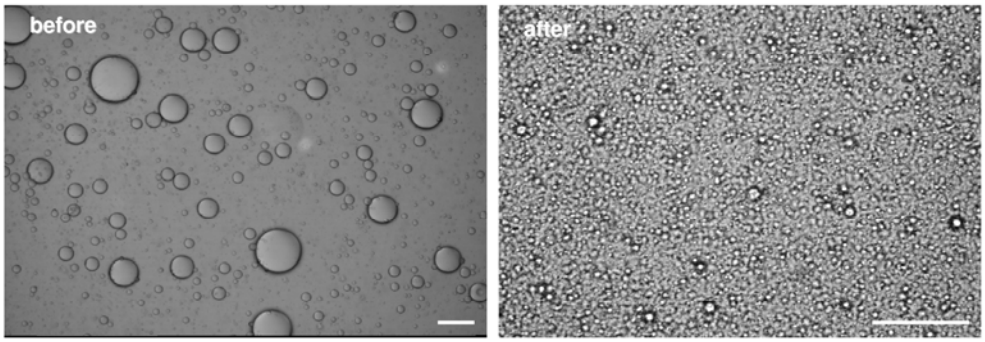

Fig. 6. Optical micrographs of SPI/T emulsion (M0) before and after high pressure homogenization at 50 MPa. Scale bar – 25 μm.

The results in table 2 showed that emulsion viscosity was affected by protein modifications. The decrease in emulsion viscosity after protein hydrolysis and acylation was observed. Protein molecular chain length reduction due to hydrolysis provided the fall of emulsion viscosity for the samples M1, M2 and M3. This can be connected to the relation between polymer molecular weight and its viscosity, usually described using the Mark-Houwink-Sakaruda equation. The same dependence of molecular weight and viscosity was also found in literature for other biopolymers (Avaltroni, Bouquerand, & Normand, 2004; Burkus & Temelli, 2003). During acylation reaction of proteins, the acid chloride reacted preferentially with the protein amine functions, because of their higher reactivity in aqueous media than the carboxylic function one. This resulted in the formation of amphiphilic macromolecules, having a polar moiety formed by the numerous carboxyl groups (hydrophilic part) and a non-polar carbon chain (hydrophobic part). This improved amphiphilic character

of protein and the addition of a small amount of surfactant, due to the residual carboxyl ions, explain the observed decrease of emulsion viscosity with protein acylation, compared with the intact protein.

Table 2. Properties of SPI/T emulsions prepared with native or modified protein.

Sample[A]	Emulsion droplet size, D_v (μm)	Emulsion viscosity before homogenization (mPa·s)	Emulsion viscosity after homogenization (mPa·s)
M0	1.1±0.02	44.0±1.30	15.0±0.02
M1	0.5±0.03	3.7±0.05	3.6±0.01
M2	0.6±0.05	3.5±0.10	4.5±0.01
M3	0.6±0.05	5.2±0.13	7.4±0.01
M4	0.7±0.04	16.8±0.70	8.6±0.01
M5	0.8±0.03	15.0±0.52	8.0±0.01
M6	0.7±0.04	8.9±0.09	8.0±0.02
M7	0.8±0.04	13.5±0.12	14.5±0.01
M8	0.9±0.02	6.4±0.05	10.2±0.01
M9	0.9±0.04	14.0±0.14	14.8±0.02

[A] M1: hydrolysed soy proteins; M2, M3: hydrolysed and acylated with C12 soy proteins; M4, M5: acylated with C8 soy proteins; M6, M7: acylated with C12 soy proteins; M8, M9: acylated with C16 soy proteins.

The oil droplet size in emulsions prepared with modified SPI and T stabilized by high-pressure homogenization were examined by laser diffraction. We observed a decrease of average droplet diameter with protein acylation, due to higher surfactant properties as previously explained. In the same way, protein hydrolysis and thus protein molecular weight diminution also involved lower droplet size. Nevertheless, the emulsion droplet size distribution curves (Fig. 7) demonstrated the narrow size distribution from 0.5 μm for emulsion with hydrolyzed SPI/T to 0.7-0.9 μm for emulsion with acylated SPI/T, and the bimodal character, previously observed with SPI/T emulsion (Fig. 3).

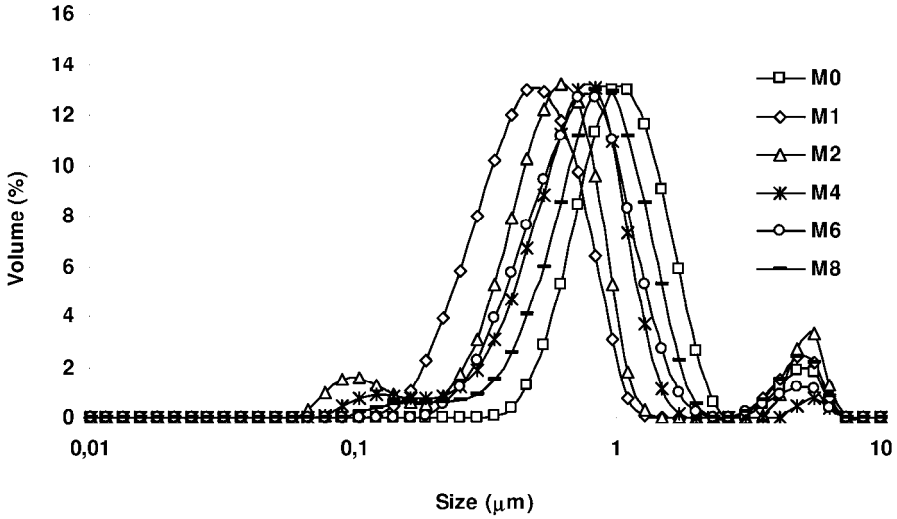

Fig. 7. Droplet size distributions of native (M0), hydrolysed (M1), hydrolysed and acylated (M2), acylated with C8 (M4), C12 (M6) or C16 (M8) SPI/T emulsions 1 hour after high pressure homogenization at 50 MPa. The oil/protein ratio of the emulsion was 1/2.

Moreover, the mean diameter values are significantly lower than those obtained in several studies dealing with native vegetable proteins as wall material for spray-drying microencapsulation (Kim et al., 1996; Rascon et al., 2011; Walstra, 1975). Small emulsion droplet average sizes prevent their coalescence during the spray-drying process and make emulsions more stable (Rascon et al., 2011). Thus, protein hydrolysis and acylation seemed to be efficient modifications for improving emulsion stability and properties.

3.6. Influence of SPI modifications on microparticles properties

The efficiency of oil retention, process yield, microparticles size and morphology were determined for spray-dried powders prepared from native SPI, native SPI/T and modified SPI (hydrolyzed and/or acylated)/T systems (Table 3). The yield of drying processes for all the emulsions were about 62±4 % whereas SPI solution (8% w/w) was spray-dried in the same conditions with a process yield of 83%. This undesirable decrease of spray-drying yield was mainly due to the addition of the hydrophobic and viscous oil – α-tocopherol to protein solution, resulting in a stickier liquid phase, which involves a higher microparticles accumulation inside the drying chamber. This was corroborated by the characterisation of samples M0*, M0**; M6* and M6 as discussed below. Nevertheless, the yields obtained in

our study were relatively high compared to those obtained literature, amounting to 30-60% (Johansen, Merkle, & Gander, 2000; Su et al., 2008).

Table 3. Characteristics of SPI and SPI/T powders, produced by spray-drying, and efficiency of this process.

Sample[A]	Spray-drying yield (%)	RE[B] (%)	LE[C] (%)	Moisture content (%)	Particle size (μm)
SPI	83	-	-	4.8±0.7	5.5±0.1
M0	65	79.7±1.0^{d}	26.3±0.3^{d}	5.5±0.4^{ab}	9.3±0.1^{a}
M1	57	38.9±2.4^{e}	12.8±0.8^{e}	6.5±0.9^{ab}	6.3±0.1^{e}
M2	64	87.4±3.9^{bc}	28.8±1.3^{bc}	5.8±0.5^{ab}	8.0±0.2^{bc}
M3	62	88.9±3.5^{b}	29.3±1.3^{b}	5.3±0.6^{ab}	6.9±0.3^{de}
M4	63	83.0±1.4^{cd}	27.4±0.5^{cd}	6.6±0.4^{a}	7.6±0.2^{bc}
M5	68	90.9±1.1^{ab}	30.0±0.4^{ab}	4.9±0.5^{b}	8.0±0.2^{bc}
M6	62	94.8±2.2^{a}	31.3±0.7^{a}	5.8±0.3^{ab}	7.7±0.2^{bc}
M7	59	88.1±2.6^{bc}	29.1±0.9^{bc}	5.3±0.2^{ab}	7.4±0.1^{cd}
M8	60	87.9±1.2^{bc}	29.0±0.4^{bc}	5.8±0.5^{ab}	8.2±0.3^{b}
M9	62	87.3±1.9^{bc}	28.9±0.6^{bc}	5.5±0.8^{ab}	8.9±0.3^{a}

[a-e] Different letters in the same column indicate a statistically difference between the mean values ($P<0.05$)
[A] M1: hydrolysed soy proteins; M2, M3: hydrolysed and acylated with C12 soy proteins; M4, M5: acylated with C8 soy proteins; M6, M7: acylated with C12 soy proteins; M8, M9: acylated with C16 soy proteins;
[B] RE: retention efficiency determined by UV spectroscopy;
[C] LE: loading efficiency or α-tocopherol content per 100 g of powder.

Representative retention efficiency data presented in Table 3 indicate the effect of the wall material modifications. According to these results, significant differences in active core retention efficiency were observed between native and modified soy proteins. The lowest efficiency level (38.9%) was obtained with wall system consisting of hydrolyzed soy proteins (M1). This can be explained by the insufficient chain length of wall material to produce a sufficiently strong structural matrix to encapsulate the α-tocopherol. Proteinic chains accumulate at the air/water interface of emulsion droplets during drying and thus represent the surface of formed powder particles (Faldt & Bergenstahl, 1996). Nevertheless, increasing of hydrophobic protein character with acylation significantly improved the RE of spray-dried powders (87.4 and 88.9% for the samples M2 and M3 respectively), even exceeding the RE obtained with native SPI (79.7% for M0). These results confirmed those obtained for acylated

SPI/T microparticles, and showed the significant contribution of grafting a hydrophobic portion on SPI chains affinity with oil and on the RE. Indeed, for acylated SPI, whatever the alkyl chain length (C8, C12, C16), the RE values were all higher than the RE obtained with non modified SPI/T microparticles. The highest RE for T encapsulation (94.8%) was observed with using soy protein acylated with dodecanoyl chloride as a wall material (M6). This significant RE improvement is mainly explained by the increase of hydrophobic character of SPI (and hydrolyzed SPI) after attachment of alkyl chains, resulting in an increased surface hydrophobicity of SPI and enhanced the amphiphilic characteristics of proteins.

Moisture content of spray-dried emulsions varied from 4.8 to 6.6% and no significant difference was detected between samples based on native and modified proteins. In spite of significantly different mean diameters of the obtained particles varied from 6.3 to 9.3 μm (Table 3), these results are coherent within the range expected for microspheres produced by spray-drying comprised between a few micrometers and a several hundred micrometers (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). It was observed that particle average diameters were relatively lower for modified SPI/T microparticles. These results can be related to the lower emulsion droplet average size and viscosity, as commented previously (Rascon et al., 2011).

3.7. Influence of wall/core ratio on emulsion and microparticles properties

For specific applications, particularly nutritional and pharmaceutical ones, different content of active material in microparticles could be needed (Elizondo et al., 2011). With the purpose to increase the T proportion in the microparticles, SPI/T (or acylated SPI/T) ratios of 1/1 and 1/2 were tested. Data of Table 4 indicate the effect of the active material concentration on emulsions and microparticles properties. A significant increase in emulsion viscosity after increasing core/wall ratio, particularly for acylated SPI microparticles, was observed. In literature similar results were obtained (Rusli et al., 2006) and authors gave two explanations for this phenomenon. The first one is the larger particle size and coalescence in the former emulsion. But, in our case, emulsion droplet size was not affected by the core/shell ratio and no coalescence was observed. The other explanation is the increase in total solid content due to the increase in T concentration. This explanation corroborates our results, as the protein concentration was kept constant (8% w/w) in our emulsions, while the T

concentration was increased. Moreover as T viscosity was higher than that of 8% SPI solution, its addition resulted in emulsion viscosity increase.

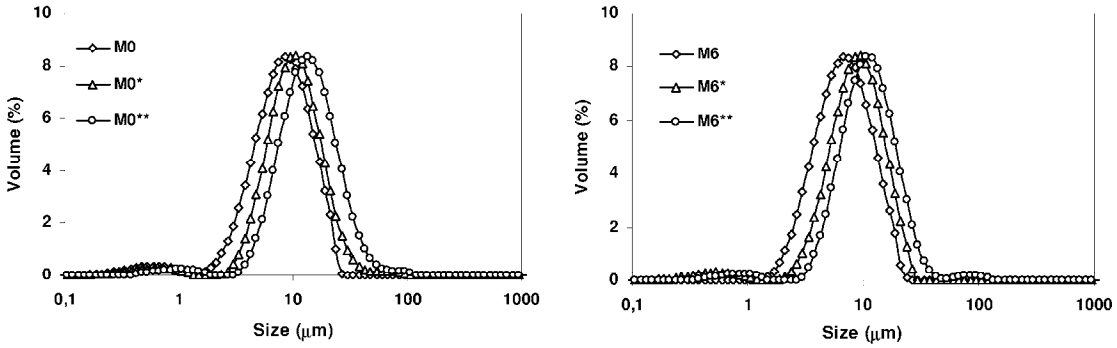

Fig. 8. Particle size distributions of spray-dried SPI/T emulsions (M0) or acylated SPI/T emulsions (M6) with core/wall ratio of 1/2, 1/1 (M0* and M6*) and 2/1 (M0** and M6**).

Increasing the core/wall ratio from 1/2 to 2/1 resulted in a slow decrease in spray-drying yield from 65 to 51% for SPI based emulsion and from 62 to 52% for acylated SPI based emulsion. This effect was reflected by the presence of high quantity of oil in emulsion and the most important clamminess of powders during spray-drying process due to availability of surface oil. For the same reasons, and because of the emulsion viscosity increase, increasing T amount in emulsions resulted in microparticles agglomeration and growth of their medium size from 9.3 to 15.9 μm for SPI/T dried emulsions and from 7.7 to 12.9 μm for acylated SPI/T dried emulsions (Table 4, Fig. 8). Concerning RE, except for formulations M0 and M6**, no significant differences were observed between the other samples. Nevertheless, increasing the T concentration in emulsions involved higher efficiency levels, both for SPI/T and acylated SPI/T microparticles. This put in evidence the suitability of SPI (acylated or not) as wall material for spray-drying as microencapsulation process, even for high loaded microparticles.

Table 4. Properties of SPI/T (M0) and acylated SPI/T (M3) emulsions and powders with different wall/core ratio.

Sample[A]	M0	M0*	M0**	M6	M6*	M6**
Ratio core/wall	1/2	1/1	2/1	1/2	1/1	2/1
Emulsion droplet size, D_v (μm)	1.1±0.02	1.0±0.04	1.1±0.06	0.7±0.04	0.8±0.03	0.8±0.02
Emulsion viscosity after homogenization (mPa·s)	15.0±0.02	18.2±0.03	19.4±0.02	8.0±0.02	13.5±0.02	19.3±0.01
Spray-drying yield (%)	65	55	51	62	60	52
RE[B] (%)	79.7±1.0^{c}	92.6±3.1^{b}	96.4±2.4^{b}	94.8±2.2^{b}	95.1±4.3^{b}	107.8±4.5^{a}
LE[C] (%)	26.3±0.6^{e}	46.6±1.5^{c}	63.6±1.5^{b}	31.3±0.7^{d}	47.6±2.2^{c}	71.9±3^{a}
Moisture content (%)	5.5±0.4^{a}	5.0±0.4^{a}	3.5±0.3^{b}	5.8±0.3^{a}	3.8±0.4^{b}	3.5±0.2^{b}
Particle size (μm)	9.3±0.1^{c}	12.0±0.3^{b}	15.9±0.3^{a}	7.7±0.2^{d}	9.5±0.2^{c}	12.9±0.2^{b}

[a-e] Different letters in the same line indicate a statistically difference between the mean values ($P<0.05$)

[A] M0, M0*, M0**: samples prepared with non modified soy proteins; M6, M6*, M6**: samples prepared with acylated with C12 soy proteins;

[B] RE: retention efficiency determined by UV spectroscopy;

[C] LE: loading efficiency or α-tocopherol content per 100 g of powder.

SEM micrographs in Fig. 9 showed that microparticles SPI/T obtained by spray-drying were surrounded by a continuous shell. The powder consisted of spherical particles, having diameter 2 to 14 μm, rounded shape and wrinkled surface without fissures, cracks or disruptions. This structure revealed the good film-forming property of SPI. The internal structure of obtained microparticles showed the porous nature of the soy protein matrix where α-tocopherol was located. With increasing of active core ratio, particles morphology becomes more complex, combining two structures into one: microcapsule, where core is presented as reservoir in wall film, and microsphere, where core is dispersed in wall porous matrix.

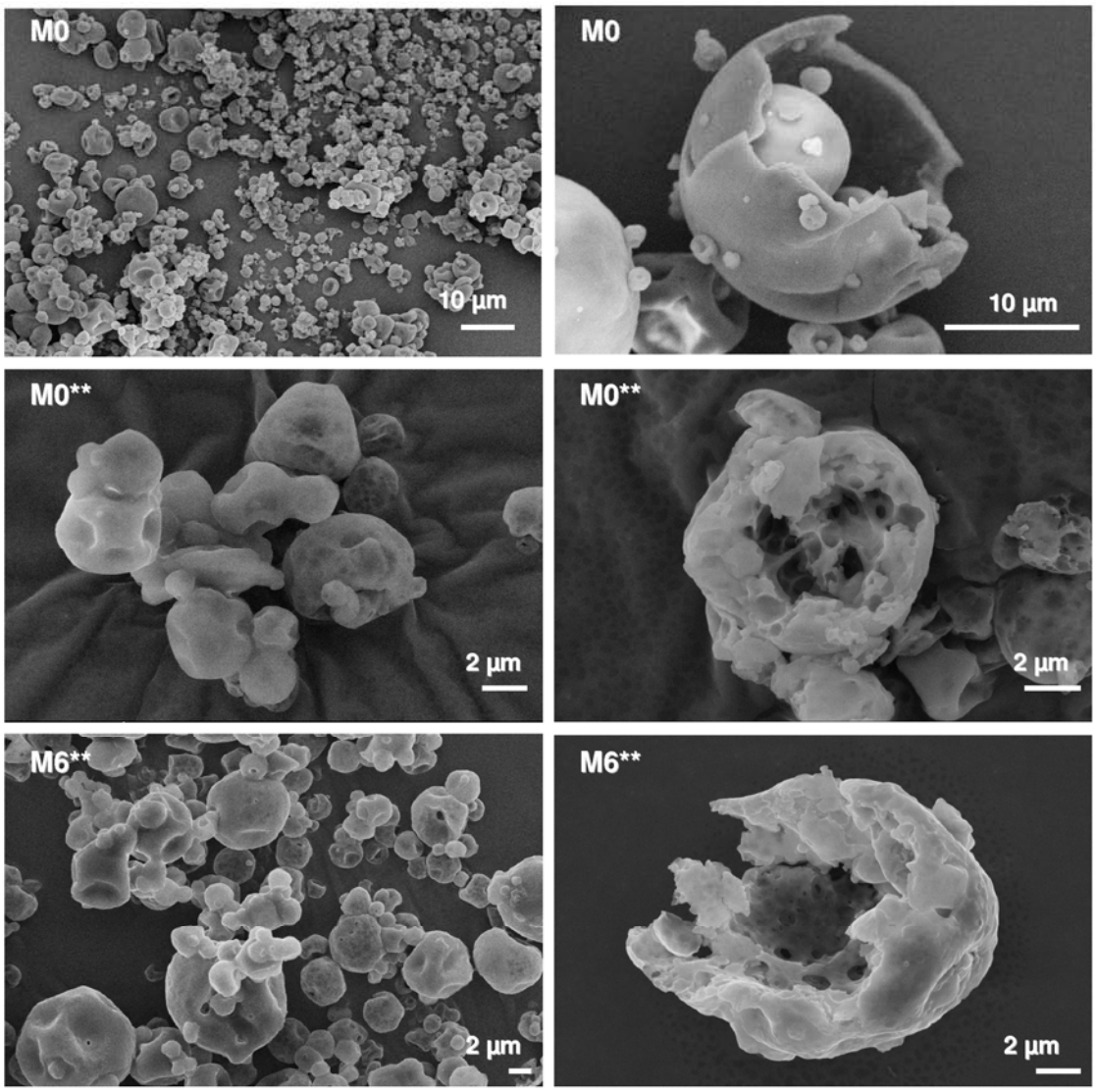

Fig. 9. Scanning electron micrographs of spray dried M0, M0** and M6** emulsions (external and internal structures).

4. Conclusions

This study developed the use of native and functionalized soybean proteins in microencapsulation of α-tocopherol by spray-drying method. Enzymatic hydrolysis and/or N-acylation were studied in order to improve some physico-chemical properties of soy protein and make them more relevant as encapsulant agents.

Emulsions droplet average size and emulsion viscosity decreased with soy protein modifications. Emulsion viscosity fall and more efficient oil dispersion (resulting in smaller

droplets) can be explained by the presence of shorter proteinic chains after hydrolysis or by improved protein active surface properties after acylation.

Low retention efficiency of 38.9% was observed for hydrolyzed protein based microparticles, probably due to insufficient chain length of hydrolyzed proteins to produce a structural matrix strong enough for α-tocopherol efficient encapsulation. Nevertheless, acylation of these hydrolyzates, increasing their affinity with the hydrophobic α-tocopherol, allowed to reserve this phenomenon, and to obtain microparticles with retention efficiency better than those from native proteins (87.4% and 88.9% for hydrolyzed and acylated proteins based microparticles, and 79.7% for native proteins ones). Acylation of native proteins confirmed this result, involving a significant increase of oil retention efficiency up to 94.8% for C12 acylated proteins based microparticles.

Moreover, this improved retention efficiency after protein acylation was observed for different α-tocopherol ratios, demonstrating that soy proteins and acylated soy proteins are relevant encapsulant agents for hydrophobic active materials, even for high loaded microparticles (loading efficiency up to 71.9% was obtained with C12 acylated proteins based microparticles).

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