APPLICATION OF SUPERCRITICAL ANTI-SOLVENT (SAS) PROCESS TO PRODUCE COMMINUTED PHOSPHOLIPIDS AS LIPOSOME PRECURSORS

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ABSTRACT

This study deals with liposome formation from raw soy lecithin. Two procedures were used: the first one provides aqueous dispersions of liposomes obtained through the hydration of micronized lecithin produced by a dense carbon dioxide (CO_2) method called supercritical anti-solvent (SAS) process. The second, called Bangham method, enables liposome formation through the dispersion in water of lecithin which has been previously dissolved in a solvent and submitted to solvent evaporation. One of the purposes of this work is to determine the influence of experimental parameters (pressure, CO_2/solvent molar ratio and lecithin concentration) on the characteristics of micronized lecithin and liposomes formed with the SAS method. The Bangham method was adopted to compare the characteristics of liposomes (residual solvent level of processed lecithin; size and encapsulation efficiency of liposomes) prepared by a conventional method with those of liposomes prepared by a dense gas method (SAS method). Thus, several operating conditions were tested at 308 K with a phospholipid organic solution flow rate of 22.8 $mL.h^{-1}$ and ethyl alcohol as a solvent: pressure varying from 9 to 13 MPa, phospholipid organic solution composition from 15 to 25 wt% and CO_2/solvent molar ratio from 50 to 100. Liposomes with diameters between 0.1 and 200 µm were formed. According to the results, the CO_2/solvent molar ratio has the most significant effect on the micronization. To comminute phospholipids, CO_2/solvent molar ratio must be higher than 60. Unlike pressure, phospholipid organic solution concentration greatly influences the micronization: higher lecithin concentration leads to bigger particles. As concerns the second step of liposome formation consisting in the hydration of the micronized lecithin, it appears that the variation of lecithin powder characteristics have small effect on liposome size distribution. Finally, size distribution and encapsulation efficiency of liposomes were studied according to the method used for their production. SAS method appears to be the more suitable one to produce liposomes.

I - INTRODUCTION

Liposomes are phospholipid vesicles. Phospholipids are non-ionic surfactants and in the presence of water, their hydrophilic heads tend to face water and their hydrophobic tails are forced to stick together, forming spherical vesicles with one or more phospholipidic bilayers, i.e. liposomes. Phospholipids from soy lecithin are used for that purpose. Because they are non-toxic and biodegradable, liposomes serve as convenient delivery vehicles for cosmetic formulations or for biologically active compounds [1]. Lastly, the use of liposomes as DNA delivery vectors for transfection in gene therapy turns out to be the main hope of liposome medical use [2, 3]. DNA vaccination with liposomes is also under way [4, 5]. Given the

widespread interest in the use of liposomes and since conventional methods use large quantities of toxic organic solvents, environmentally-friendly and pharmaceutically acceptable procedures are required. The residual organic solvent level in the end-product has to be taken into account to assess liposome quality as well as the size, the encapsulation efficiency and the stability.

A wide variety of conventional methods exists to produce liposomes, including the Bangham method, the detergent depletion method, the ether/ethanol injection method, the reverse phase evaporation method and the emulsion method [6, 7]. These methods suffer from lots of drawbacks and especially the use of organic solvents which need to be removed at the end of the process. On the contrary, dense gas methods and especially supercritical carbon dioxide (CO_2) methods provide clean and effective alternatives to conventional methods: supercritical CO_2 acts as a solvent or an anti-solvent to process phospholipids.

The aim of this study is to compare two methods to produce liposomes from raw lecithin. The first one involved the micronization of an organic solution of soy lecithin (ethanol) with supercritical carbon dioxide (called SAS method). Then, hydration of the comminuted phospholipids under stirring is achieved to form liposomes. In the second method, soy lecithin is first dissolved in ethanol; next the solvent is eliminated by evaporation. Hydration of the resulting thin lecithin layer (also under stirring) leads to the formation of liposomes. The former is a dense gas method (supercritical CO_2 method) and the latter is a conventional method. Previous works have already described the micronization of soy lecithin by SAS process [8, 9, 10]. The study presented here is more complete since the formation of the liposomes after the micronization has been systematically carried out and the characteristics of the resulting liposomes have been discussed.

The methods were compared according to the residual solvent level of processed lecithin; size and encapsulation efficiency of liposomes. Concerning the SAS method, the influence of experimental parameters (pressure, CO_2/solvent molar ratio and lecithin concentration) on micronized lecithin and liposomes characteristics was studied. Several characterizations were undergone such as scanning electron microscopy to observe the particles morphology; infrared spectroscopy to evaluate the amount of residual solvent (ethanol); laser diffraction to work out liposome diameters and fluorescent spectrophotometer to determine the encapsulation efficiency of liposomes.

II - MATERIALS AND METHODS

II.1. Chemicals

Soy lecithin S75 (71% phosphatidylcholine) was purchased from LIPOID (Ludwigshafen, Germany). Analytical grade analysis ethyl alcohol was obtained from Sigma Aldrich (St Louis, MO). Instrument grade carbon dioxide (purity of 99.7%) from Air Liquide Méditerranée (Vitrolles, France) was used. Cholesterol, 4',5'-bis[N,Nbis(carboxymethyl) aminomethyl]fluorescein (calcein), Cobalt(II) chloride hexahydrate and non-ionic surfactant Triton X-100 (octylphenol polyethoxylated) were purchased from Sigma Aldrich (St Louis, MO) and used to assess the encapsulation efficiency of liposomes. Double distilled and deionized water was used throughout the experiments.

II.2. Preparation of liposomes

Our study is focused on the semicontinuous supercritical antisolvent precipitation process, called SAS. The SAS process is used to comminute lecithin, i.e. to produce divided phospholipids. A second step is performed to achieve liposome formation and consists in the hydration of the microparticles under stirring. As a reference for our experiments, the conventional Bangham method [11, 12, 13] is also used to prepare liposomes and the method is described later.

II.2.1. Liposome preparation by the SAS method

The experimental set-up for SAS process was described elsewhere [9, 10]. Briefly, the SAS process involved the co-currently spraying of a solution composed of the dissoluted solute (soy lecithin), cholesterol (cholesterol/lecithin weight ratio=0.49) and the organic solvent (absolute ethyl alcohol) through a capillary (127 µm I.D.; Chrompack, Les Ulis, France) into a continuous supercritical phase. The supercritical fluid used was CO_2. The latter was used as an anti-solvent for the solute but as a solvent with respect to the organic solvent. The simultaneous dissolution of the supercritical fluid in the liquid phase and the evaporation of the organic solvent in the supercritical phase lead to the supersaturation of the solute into the liquid phase and then its precipitation. For all experiments, the solution was sprayed in the vessel once the steady state (constant CO_2/solvent molar ratio in the vessel) was reached [14]. All experiments were carried out at 308 K, with a liquid flow rate of 22.8 $mL.h^{-1}$ and ethyl alcohol as co-solvent. Moreover, for each experiment, 2 grams of lecithin were precipitated in the high pressure vessel. Liquid CO_2 was pumped with a high-pressure pump (Dosapro Milton Roy, Pont-Saint-Pierre, France) and the organic solution was injected in the high pressure vessel with a high pressure liquid pump Gilson 307 (Villiers le Bel, France). After the injection phase, a washing step was carried out in order to completely remove the residual solvent. The washing step was performed given that the residence time distribution of the solvent during washing is similar to the residence time distribution of a plug flow reactor [14]. At the end of the process, after depressurization, small samples of microparticles were collected and immediately submitted to characterisation. Then, the hydration step was achieved as described in part II.2.3.

Table 1 summarizes the experimental conditions; in each case the liquid solution flow rate was 22.8 $mL.h^{-1}$ and temperature was 308 K.

Table 1 : Experimental domain for SAS experiments

Run	*P* / MPa	CO_2/solvent molar ratio	Solute concentration / wt %
1	**9**	60	15
2	**10**	60	15
3	**11**	60	15
4	**13**	60	15
5	9	**50**	15
6	9	**60**	15
7	9	**70**	15
8	9	**80**	15
9	9	**100**	15
10	9	60	**15**
11	9	60	**20**
12	9	60	**25**

II.2.2. Liposome preparation by the Bangham method

Phospholipids were dissolved in absolute ethyl alcohol. The solvent was removed by evaporation (323 K) with a rotary evaporator 4000eco (Heidolph Laborota). After a while, a film was formed at the surface of the evaporator flask. Then, the hydration step was achieved as described in part II.2.3.

II.2.3. Hydration of microparticles and encapsulation of calcein

Calcein solution was used both to hydrate the phospholipid microparticles and form liposomes; and to achieve encapsulation (calcein as fluorescent marker).

Calcein solution (0.0622 $g.mL^{-1}$) was added in the vessel or the evaporator flask. Then, stirring was performed using a high speed mixer Ultraturax T25 (Ika Labortechnik, Staufen, Germany) at 11 000 $tr.min^{-1}$ during 10 min. According to Oku et al. [15], the volume of calcein solution was determined given that the weight ratio calcein/lecithin should be equal to 0.008.

II.3. Characterization

II.3.1. Dry microparticle powders produced by the SAS method

Yield

Micronization yield was calculated as the ratio between lecithin recovered in the vessel and lecithin effectively injected in the vessel during the SAS process. *Particles size and morphology*

Size and morphology of microparticles were observed using a Hitachi S-3000 (Hitachi, Japan) Scanning Electron Microscope (SEM). Each sample was prepared with a SC7620 Sputter Coater (Quorum Technologies, England) which deposits a 2 nm layer of gold and palladium in order to optimize the image resolution.

Control of residual solvent

Micronised lecithin produced by the SAS method were compared to unprocessed lecithin samples and lecithin samples which were previously loaded with known amount of absolute ethanol. InfraRed Spectroscopy was used to compare the samples. Two characteristics absorption bands were chosenat 1735 cm^{-1} and 1058 cm^{-1}. The first one is representative of the ester group and the second one of the carboxyle group. For each sample, the height ratio of the absorption bands $h_{1058cm\text{-}1}/h_{1735cm\text{-}1}$ was calculated. This ratio was taken into account to assess the residual solvent level.

II.3.2. Hydrated particles or liposomes

Liposomes size distribution

Particle size distribution was measured by laser diffraction using Malvern Mastersizer S (laser 663 nm) (Malvern Instrument S.A., Orsay, France). The instrument covers a particle size range between 50 nm – 3 mm. All measurements were carried out triplicate.

Encapsulation efficiency

The aim was to determine the fraction of encapsulated calcein in liposomes. The method used the ability of non-permeant Co^{2+} to completely quench the fluorescence of calcein through the formation of a Co-calcein complex, as described by Kendall and MacDonald [16]. The procedure was first experimented by Oku et al. [15]. Briefly, the liposomal suspension was first diluted 200-fold in distilled water, and fluorescence intensity was measured before (F_{Total}) and after the addition of $CoCl_2$ ($F_{Encapsulated}$) and Triton X-100 (F_{Totalq}).

As Co^{2+} is non-permeant, it forms a complex only with non-entrapped calcein and $F_{encapsulated}$ refers to the fluorescence contribution of encapsulated calcein.

Triton X-100 is a non-ionic detergent. Interactions between Triton X-100 and liposomal membranes have been clearly established by Hertz and Barenholz [17]. First of all, they found that the effect of Triton X-100 is time dependant. Then, they showed that when treated with Triton X-100, most of the lipid bilayers were damaged and seemed to have fused. The product of this fusion was a complex network of lipid layers. Concerning the release of encapsulated marker (glucose), Hertz and Barenholz [17] noticed that several milligrams of Triton X-100 yield to the release of 90% of an encapsulated marker.

F_{Total} typically represented less than 5% of corresponding F_{Total} [18]. The encapsulation efficiency was then calculated as: 100 x ($F_{Encapsulated} - F_{totalq}$)/($F_{Total} - F_{totalq}$) [15]. Fluorescent measurements were carried out with a Perkin Elmer Luminescence spectrometer LS50 (Perkin Elmer, Courtaboeuf, France). Synchronous excitation emission mode was used from 400 to 600 nm (excitation slit: 5; emission slit: 10; wavelength interval: 23). All measurements were carried out triplicate. From the procedure described by Oku et al. [15], several fits need to be done. First of all, a 80 mL flask of diluted liposomal suspension was used. Samples for analysis were taken from this flask. After the determination of F_{Total}, 200 µL of $CoCl_2$ (10 mM) was added and the mixture was stirred with a glass mixer. Time for reaction between calcein and Co^{2+} was estimated at 15 min. Thus, measurements of $F_{Encapsulated}$ were performed 15 min after introducing the $CoCl_2$ solution. Lastly, approximately 3 mL of Triton X-100 was introduced in the flask and vigorous stirring (with a glass mixer) was needed to dilute Triton X-100. Investigations showed that measurement of F_{Totalq} needs to be done.

III - RESULTS

III.1. Micronization results and yields

SAS process produces agglomerated spherical micro-particles of lecithin. Themicronized lecithin was light yellow-white and localised in the bottom of the vessel, as shown in Figure 1. The production yield was between 75 and 85%. In the next part of this study, characteristics of lecithin powders (particles size and morphology) were determined and compared according to operating variables.

Figure 1 : Photographs of micronized lecithin at the bottom of the vessel

III.2. Effect of operating conditions on the micronized lecithin powder

III.2.1. Influence of precipitation pressure

Figure 2 shows the SEM micrographs of the micronized phospholipid powders achieved at 9, 10, 11 and 13 MPa (runs 1, 2, 3 and 4 respectively). Particles are always spherical and agglomerated, with mean diameters varying from 5 to 50 µm. However, for the lower precipitation pressure, particles seam to fuse together in large agglomerates. This phenomenon was not observed for higher pressure experiments. In these cases, particles were less agglomerated. In this study, only a quite narrow range of pressures was tested and results are not meaningful. Further trials have to be carried out in a wider range of pressures.

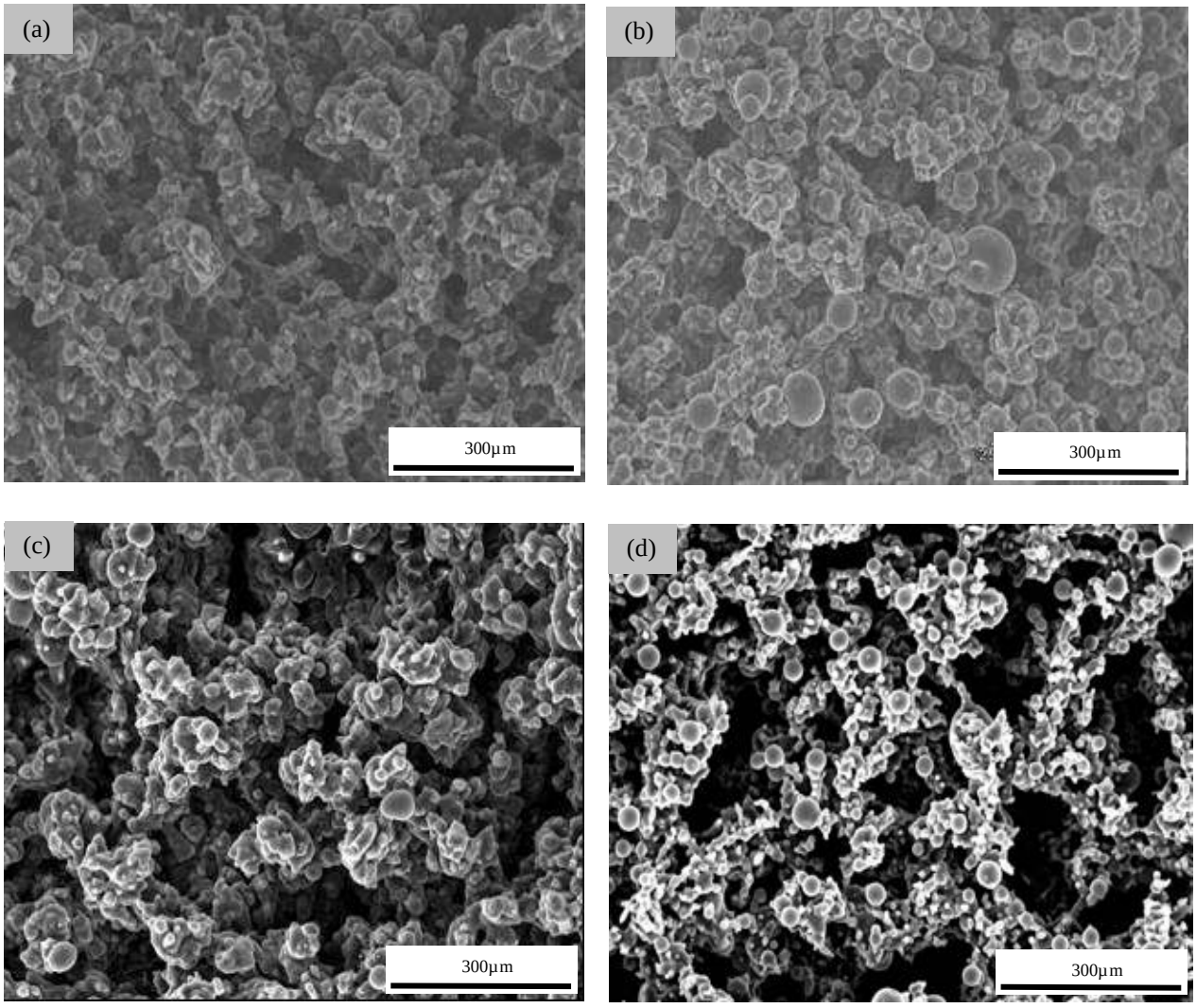

Figure 2 : SEM micrographs of precipitated lecithin prepared at different pressures: (a) 9 MPa (run 1). (b) 10 MPa (run 2). (c) 11 MPa (run 3). (d) 13 MPa (run 4).

Concerning data reported in the literature, Kunastitchai et al. [19] (temperature range from 304 to 333 K; and pressure range from 8.5 to 10.5 MPa) observed small effects of pressure on the micronization yield (yield determined by weighing the microparticles recovered in the precipitation chamber and calculating the percentage of yield with respect to the initial amount added into the process system). They concluded that the lower operating conditions are more favourable from an energetic point of view.

Contrary to the results of Kunastitchai et al. [19] and ours, Li et al. [20] (temperature range from 303 to 313 K; pressure range from 8-12 MPa) found that the higher the pressure, the larger the particles size of micronized powder.

To conclude on ours results, in the range of pressure studied, particles size and morphology are quite the same. Thus, to an energetic point of view, the lower pressure (9 MPa) was more favourable than the other trials.

III.2.2. Influence of CO_2/solvent molar ratio

First of all, to set the CO_2/solvent molar ratio variation range, trial and error experimentations were conducted. Below 50, the CO_2/solvent molar ratio did not allow to micronized lecithin and a gel like mixture was formed at the bottom of the vessel.

According to Figure 3, nearly spherical particles constituted of aggregates and with a characteristic size about 20 µm were formed in every run. For run 5 (Figure 3.a.), particles were less divided compared with runs 6, 7, 8 and 9. Thus, CO_2/solvent molar ratio of run 6 (60) was defined as the threshold value for the effective micronization of lecithin in the high pressure vessel.

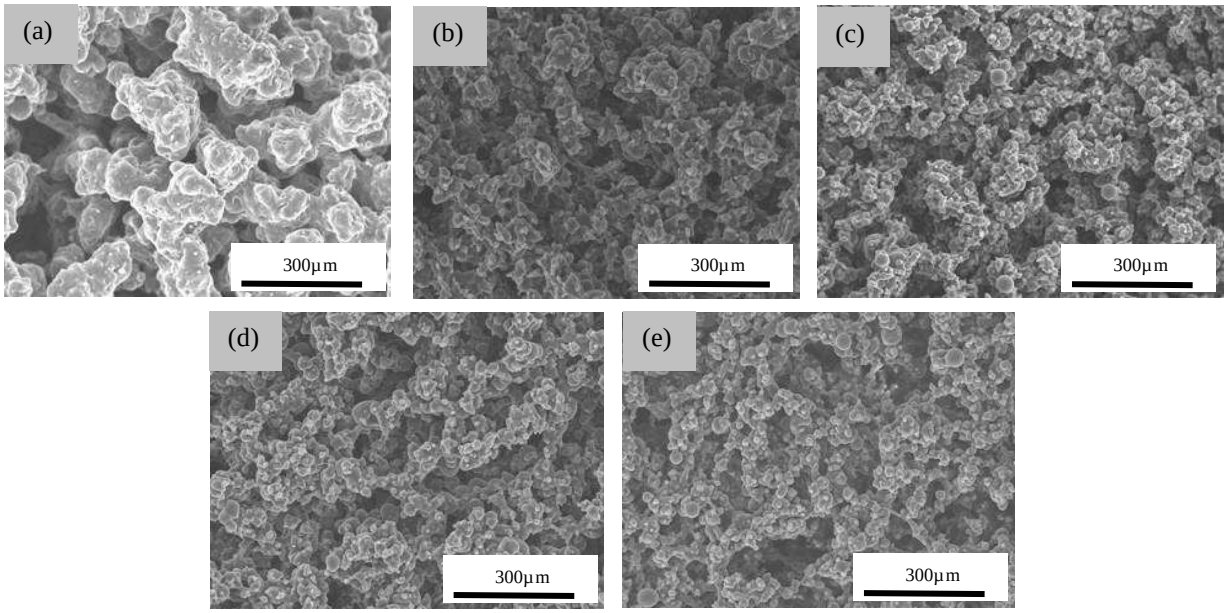

Figure 3 : SEM micrographs of precipitated lecithin processed at different CO_2/solvent molar ratio: (a) 50 (run 5). (b) 60 (run 6). (c) 70 (run 7). (d) 80 (run 8). (e) 100 (run 9).

II.2.3. Influence of solute concentration

According to Figure 4, solute concentration appears to play a major role on the particules size: 20 µm for a solute concentration of 15wt% (run 10); 50 µm for a solute concentration of 20wt% (run 11); and 60 µm for a solute concentration of 25wt% (run 12). Thus, increasing solute concentration leads to bigger solid particles. Moreover, it appears that higher concentrated solution leads to more distinct spherical objects. The increase of particles size with the increase of solute concentration can be explained in terms of nucleation and growth processes. According to Reverchon et al. [21], when diluted solutions are injected, saturation and precipitation of solute is reached very late during the droplet expansion process; therefore, nucleation is the prevailing mechanism and thus smaller particles are formed. When concentrated solutions are used, the precipitation of the solute is obtained early during the expansion process and the growth process is the prevailing mechanism thus producing larger particles.

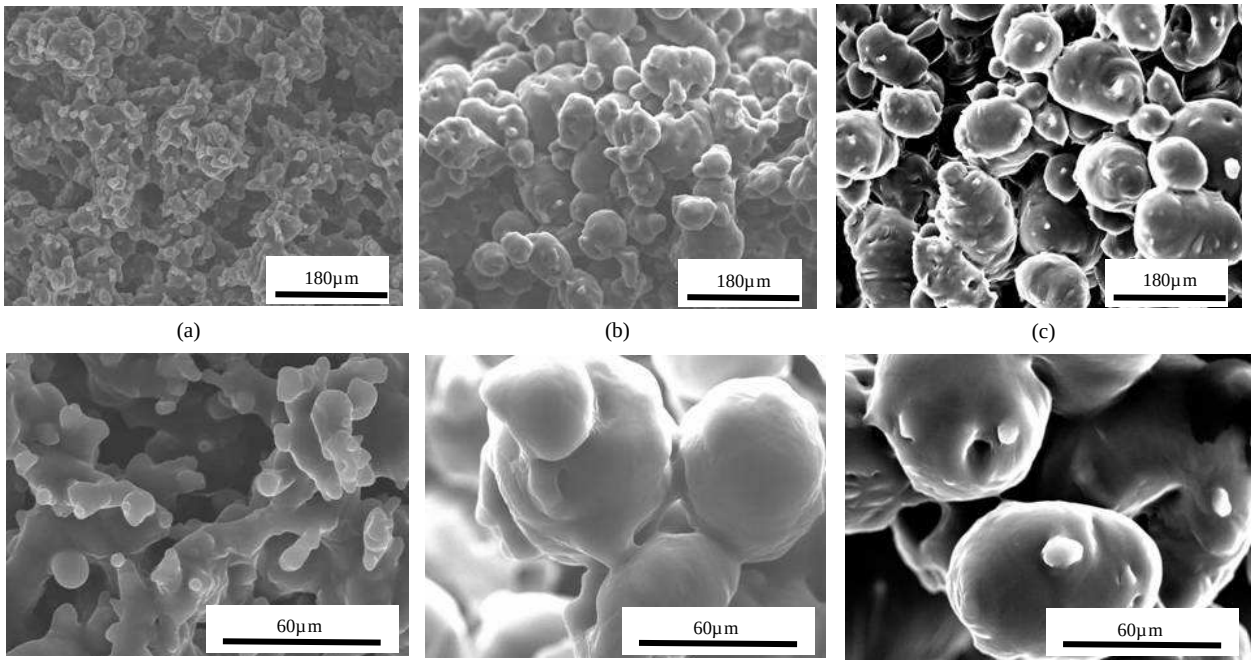

Figure 4 : SEM micrographs of precipitated lecithin processed at different lecithin concentrations: (a) 15wt% (run 10). (b) 20wt% (run 11). (c) 25wt% (run 12).

III.3. Control of residual solvent

Figure 5 presents the spectrums of several samples and the two characteristic absorption bands used for calculation.

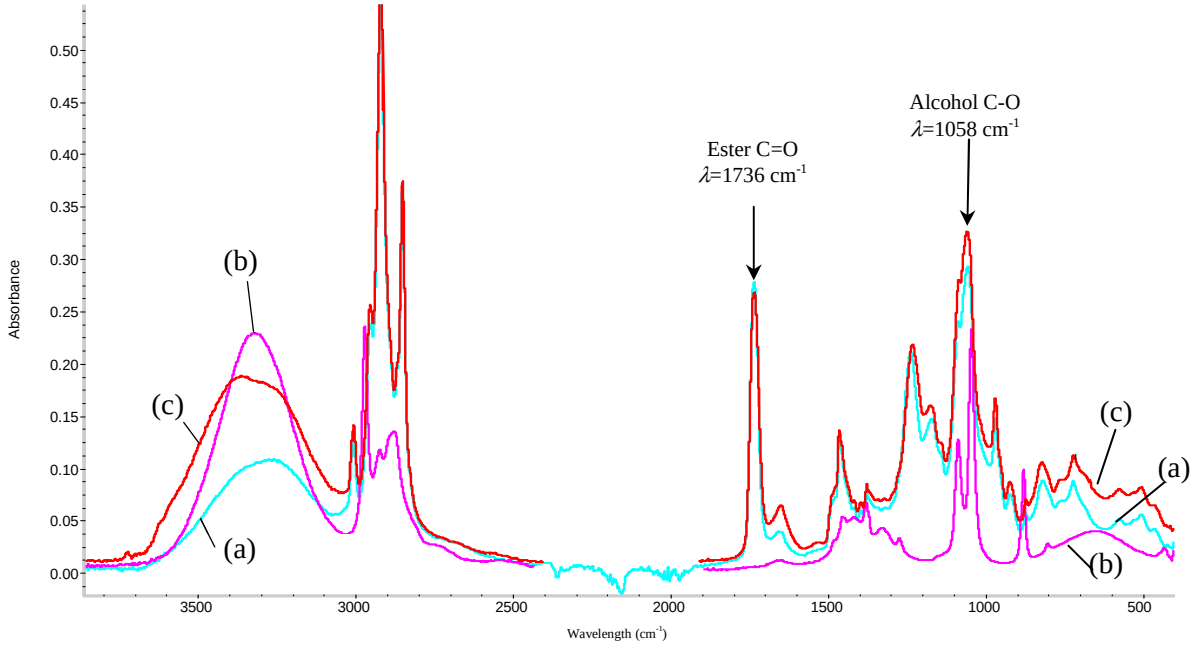

Figure 5 : InfraRed spectrums of respectively : (a) unprocessed lecithin S75. (b) absolute ethanol. (c) micronized lecithin with the SAS process.

Table 2 presents the values of $h_{1058cm-1}/h_{1735cm-1}$ for samples with known amount of absolute ethanol. Values of $h_{1058cm-1}/h_{1735cm-1}$ range between 0.76 and 1.1; and for the conventional method, the value of $h_{1058cm-1}/h_{1735cm-1}$ is about 1.2. Thus, the quantity of solvent

for samples processed with the SAS method is below 10wt%; for the conventional technique, the remaining quantity of solvent in the final product is higher than 10wt%. These results account for the effectiveness of the washing step of the SAS process.

Table 2 : Values of heights for the characteristic absorption bands and value of the height ratio (according to InfraRed spectrums) for several samples

Sample	$h_{1058cm-1}$	$h_{1735cm-1}$	$h_{1058cm-1}/h_{1735cm-1}$
Unprocessed lecithin S75	0.258	0.267	0.966
Lecithin loaded with 1wt% of absolute ethanol	0.237	0.236	1.004
Lecithin loaded with 5.5wt% of absolute ethanol	0.264	0.254	1.039
Lecithin loaded with 10wt% of absolute ethanol	0.243	0.222	1.095

III.4. Liposomes characteristics

III.4.1. Liposomes size distribution

The variation of micronized particles characteristics (due to micronization under different operating conditions) does not have any influence on the liposomes size distribution.

According to Figure 6 and 7, the average polydispersity of the liposome samples ranged from 0.1 to 1000 µm. Most of the size distribution curves are bimodal or trimodal. Therefore, only some curves are represented to give the trend. Size measurements reported below are conducted the same day than the liposomes production.

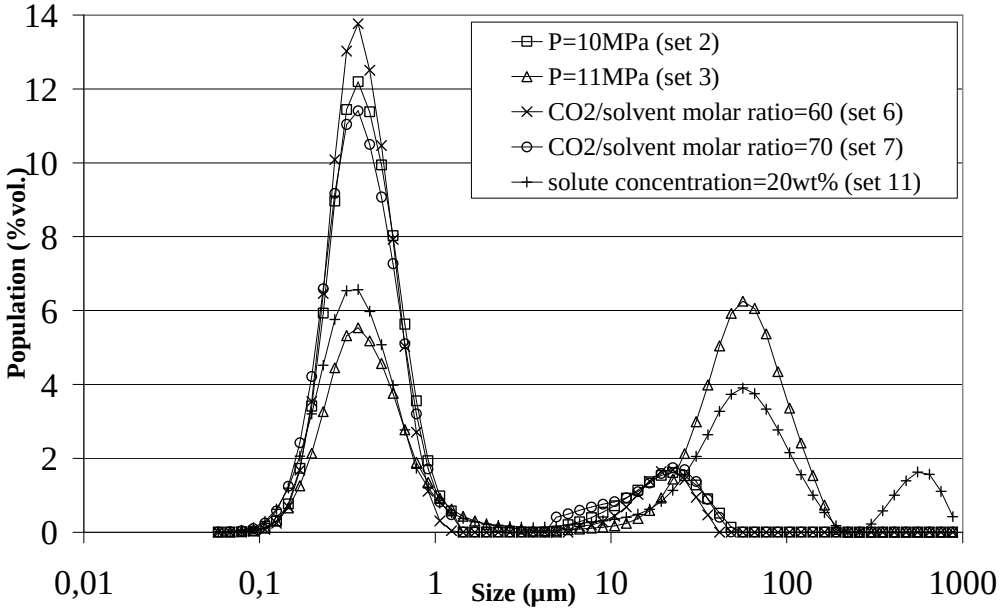

Figure 6: Population of liposomes (%vol.) versus liposome size (µm) respectively for run 2, 3, 6, 7 and 11

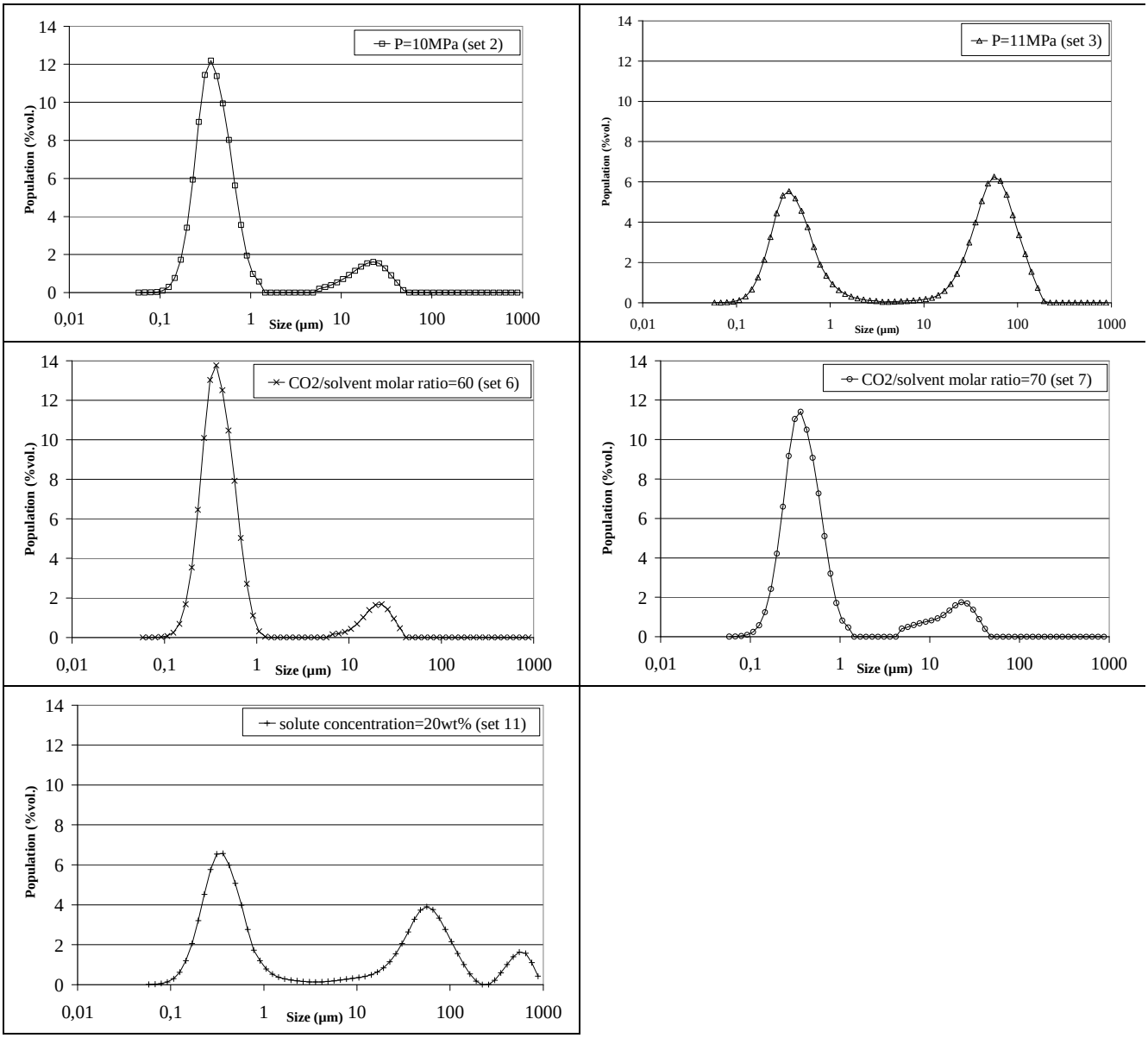

Figure 7 : Population of liposomes (%vol.) versus liposome size (µm) respectively for srun 2, 3, 6, 7 and 11

Figure 8 shows the size distribution of liposomes produced with the conventional method. The trend seams to be similar to the size distribution of liposomes produced with the SAS process. However, a main population appears at 1000 µm. This result is encouraging: SAS process enables to produce smaller liposomes than the conventional technique.

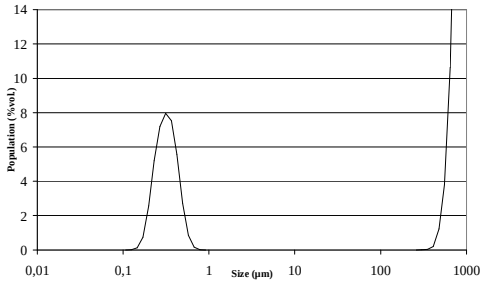

Figure 8 : Liposomes population (%vol.) versus liposomes size (µm) for the conventional technique

III.4.2. Liposomes encapsulation efficiency

As for liposomes size distribution, the influence of micronized lecithin characteristics was not meaningful for encapsulation efficiency. Analyses showed that the encapsulation efficiency of liposomes produced with the SAS process was about 10 and 20% (run 1 to set 12) compared with 20% for liposomes produced with the conventional technique.

IV - CONCLUSION

This study has shown that the SAS method can be considered as an efficient and environmentally-friendly process to form liposomes. Residual solvent level of the processed lecithin and size distribution of liposomes correspond to commonly expected values. Encapsulation efficiencies of the two methods are equal. To conclude, the use of supercritical CO_2 to produce liposomes is a promising way.

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