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First study on the release of a natural antimicrobial agent, estragole, from freeze-dried delivery systems based on cyclodextrins and liposomes

Riham Gharib^{a,b,c}, Souha Haydar^a, Catherine Charcosset^b, Sophie Fourmentin^c, Hélène Greige-Gerges^{a*}

^aBioactive Molecules Research Laboratory, Faculty of Sciences, Doctoral School of Sciences and Technologies, Lebanese University, Lebanon.

^bLaboratoire d'Automatique et de Génie des Procédés, Université Claude Bernard Lyon 1, UMR 5007, CNRS, CPE, 43 bd du 11 Novembre, 691622 Villeurbanne Cedex, France

^cUnité de Chimie Environnementale et Interactions sur le Vivant (UCEIV), EA 4492 SFR Condorcet FR CNRS 3417, Université du Littoral-Côte d'Opale, 59140 Dunkerque, France.

*Corresponding author: Hélène Greige-Gerges, Professor, Faculty of Sciences, Section II, Bioactive Molecules Research Laboratory, Lebanese University, B.P. 90656, Jdaidet El-Matn, Lebanon. Tel: 961-3 341011. E-mail: greigegeorges@yahoo.com; hgreige@ul.edu.lb

E-mail address: hgreige@ul.edu.lb (H. Greige-Gerges); lamotte@univ-littoral.fr (S. Fourmentin); charcosset@lagep.univ-lyon1.fr (C. Charcosset); shaydar@ul.edu.lb (S. Haydar) and gharib_riham@hotmail.com (R. Gharib).

Abstract

Estragole is considered as a promising candidate for food preservation due to its antioxidant and antimicrobial properties. However, its sensitivity to light and oxygen, its volatility and hydrophobicity prevent its large application. In order to increase the stability of estragole, conventional liposomes (CL) and drug-in-cyclodextrin-in-liposomes (DCL) were prepared by the ethanol-injection method using Phospholipon 90H in combination with cholesterol and then freeze-dried using hydroxypropyl- β -cyclodextrin (HP- β -CD) as a cryoprotectant. Fresh and reconstituted vesicles demonstrated nanometric vesicles size, spherical shape and negative surface charge. Moreover, all suspensions were homogeneous. Compared to CL, DCL improved estragole encapsulation efficiency, and conserved the same loading rate during freeze-drying. Freeze-dried CL and DCL retained estragole more efficiently compared to the reconstituted ones, as evidenced by release study performed by multiple headspace extraction. Finally, we demonstrated, for the first time, that HP- β -CD did not affect the membrane fluidity of fresh and reconstituted Phospholipon 90H/cholesterol CL and DCL.

Keywords: conventional liposomes; drug-in-cyclodextrin-in-liposomes; estragole; freeze-drying; hydroxypropyl- β -cyclodextrin.

1 Introduction

Estragole (Est), 4-methoxyallylbenzene, is a natural phenylpropene found in many aromatic plants. It is a constituent of tarragon (60-75%), basil (2-43%), star anise (5-6%), and fennel (5-20%) essential oils [1]. Estragole is Generally Recognized As Safe (GRAS) and approved by the Expert Panel of the Flavor and Extract Manufacturer's Association (FEMA, USA) that concluded that the dietary intake of estragole from the consumption of spices do not pose a health risk [2]. Estragole has a taste described as spice, cinnamon, mouth tingle, fresh, peppery and woody. It is used for the modification of spice flavors and seasonings for condiments and meats as well as in heavy fruit, root beer and anise-type flavors [2] as well in teas to prevent flatulence and spasms. Moreover, estragole exhibits myorelaxant, anticonvulsant, anesthetic, bradycardic, vasoactive and antioxidative properties [2-4]

Estragole proved an antifungal activity against *Aspergillus niger*, *A. flavus*, *Trichoderma viride*, *Candida albicans*, *C. utilis*, *C. tropicalis*, *Cryptococcus neoformans*, *Trichophyton mucoides*, *T. tonsurans*, and *Blastoschizomyces capitatus* [6,7]. In addition, estragole presented fairly significant zones of inhibition against Gram-positive bacteria [8].

Widespread application of estragole is limited by its volatility and poor water solubility. Moreover, external factors such as temperature, light, and accessibility to atmospheric oxygen affect the stability of estragole [9]. For example, the photooxidation of estragole produced 4-methoxybenzaldehyde (anisaldehyde) and 4-methoxybenzene acetaldehyde [10]. Attempts were made to increase the stability of essential oil containing estragole through encapsulation of basil oil in gelatin microcapsules [11, 12], in nano- and micro-emulsion [12, 14] or in cyclodextrins (CD) [15-17]. CDs increased the solubility and

photostability of estragole (3-12 and 6-18 fold, respectively, depending on CD type) and also maintained its bioactivity.

Liposomes are viewed as attractive novel materials by the food and medical industries. Interest in liposome has increased due to their reported functional properties, including their efficient encapsulation capacity, biocompatibility with food constituents and low cost of raw materials used for manufacturing [18-21]. However, the main obstacle to the use of liposomes is oxidation of lipids during storage. The presence of water may favor bacterial growth and promote phospholipid degradation. Therefore, a way to stabilize them is to use freeze-drying process. Unfortunately, lyophilization itself can alter the liposome membrane bilayer. Therefore, it must be conducted according to a defined protocol and with added excipients that preserve the bilayer structure [22].

The type of phospholipid and the presence of cholesterol in the bilayer affect the stability of liposome structure during lyophilization in addition to the cryoprotector [23]. Cholesterol is often included in liposome composition because it can modulate the physicochemical properties of the lipid bilayer by disordering the gel state of the lipid bilayer or ordering the liquid state [24]. Lyophilization of liposomes without addition of cryoprotectants provokes their coalescence and aggregation. It has been demonstrated that hydroxypropyl- β -cyclodextrin (HP- β -CD) maintains the physical and chemical stability of conventional liposomes made from Phospholipon 90H during freeze-drying. Moreover, the presence of HP- β -CD in the aqueous phase of DCL system showed a protective effect during freeze-drying [25].

We recently studied the encapsulation of essential oil components in conventional liposomes and in drug-in-CD-in-liposome (DCL). This system, combining the relative

advantages of both carriers [26], improved the encapsulation of essential oil components such as *trans*-anethole [27, 28] and eugenol [29] and reduced drug release compared to conventional liposomes [28].

In the present study, different CL and DCL formulations containing estragole were prepared by ethanol injection method using hydrogenated phospholipid in presence of cholesterol. These liposomes formulations were characterized before and after freeze-drying. The size, polydispersity index, zeta potential of liposomes formulations were determined as well as the morphology using transmission electron microscopy. The loading rate of estragole was determined using HPLC. The stability of vesicles was assessed after 6 months of storage at 4°C in powder form and the release of estragole from the inclusion complex, CL and DCL was studied by multiple headspace extraction at 60°C. The volatility of estragole and the fluidity of the membrane may affect the estragole encapsulation. Hence, the Henry's law constant of estragole was determined at 30 and 60°C, and the fluorescence anisotropy of 1,6-diphenylhexatriene (DPH) inserted in the lipid membranes of CL and DCL was studied for the first time before and after freeze-drying at 25 and 37°C.

2 Materials and methods

2.1 Materials

Hydrogenated phospholipon 90H (90% soybean phosphatidylcholine, 4% lysophosphatidylcholine, 2% triglycerides, 2% water, 0.5% ethanol, 1% iodine) was supplied by Lipoid GmbH (Ludwigshafen, Germany). HP- β -CD-oral grade (MS=0.85) was obtained from Roquette (Lestrem, France), thymol, absolute ethanol, cholesterol and

methanol-HPLC grade were from Sigma-Aldrich (Germany) and estragole was purchased from Sigma-Aldrich (China). Water was purified on a Milli-Q system obtained from a Millipore® synergy system (Millipore, Billerica, Massachusetts, USA).

2.2 Preparation of HP-β-CD/Est inclusion complex

7.44 mg of estragole was added to 5 mL HP-β-CD solutions (0, 10, 25, 50, 75 and 100 mM). The mixtures were shaken at 25 °C for 24 h then filtered through a 0.45 μm membrane filter. The concentrations of estragole in the filtrates were determined by the HPLC method described below.

The complexation efficiency was calculated using the following equation:

$$CE_{CD}(\%) = \frac{m_{Est_{exp}}}{m_{Est_i}} \times 100 \quad (1)$$

where $m_{Est_{exp}}$ is the mass of estragole experimentally determined by HPLC in the inclusion complex solution and m_{Est_i} is the mass of estragole initially used to prepare the inclusion complex [30].

2.3 Preparation of HP-β-CD/Est inclusion complex for DCL preparations

HP-β-CD was dissolved in ultrapure water and the required amount of estragole was added to obtain HP-β-CD:Est molar ratio of 75:10 and 100:10; the concentration of HP-β-CD were of 111 and 148 mg/mL, respectively. The mixture was stirred (120 rpm for 24 h at 25 °C), filtered through a 0.45 μm membrane, and then used in DCL preparations.

2.4 Preparation of liposomes by ethanol injection method

The liposomes were prepared by the ethanol injection method. Phospholipon 90H (10 mg/mL) and cholesterol (5 mg/mL) were dissolved in absolute ethanol. The resulting organic phase (10 mL) was then injected, using a syringe pump (Fortuna optima, GmbH-Germany), into the aqueous phase (20 mL) at 55°C (temperature above the transition temperature of Phospholipon 90H) under magnetic stirring at 400 rpm. Spontaneous liposome formation occurred as soon as the ethanolic solution was in contact with the aqueous phase. The liposomal suspension was then left for 15 min at 25 °C under stirring (400 rpm). Finally the ethanol was removed by rotary evaporation (BÜCHI Rotavapor R-100) under reduced pressure at 40 °C. Four batches were prepared: 1) blank-CL; 2) blank-DCL, where HP- β -CD was dissolved in the aqueous phase at concentrations of 75 (blank-DCL 75) and 100 mM (blank-DCL 100); 3) Est-loaded liposomes (Est-CL), where estragole was added in the organic phase at a concentration of 2.5 mg/mL; 4) HP- β -CD/Est inclusion complex-loaded liposomes (Est-DCL), where the HP- β -CD/Est solution prepared previously (Est-DCL 75 and Est-DCL 100 correspond to DCL preparation using HP- β -CD:Est molar ratio of 75:10 and 100:10, respectively). Each batch was prepared in triplicate and underwent characterization as described below.

2.5 Freeze-drying

Freshly prepared blank-CL, blank-DCL, Est-CL, Est DCL 75 and Est DCL 100 (5 mL) underwent ultracentrifugation at 170000 g for 1 h at 4 °C. The supernatant was discarded and the pellet was reconstituted in 2 mL of water or HP- β -CD solution (25 and 50 mM) for CL or in 2 mL of HP- β -CD solution of 75 or 100 mM, for Est-DCL 75 and Est-

DCL100, respectively. The liposomes suspensions and the inclusion complex HP- β -CD/Est molar ratio of 75:10 and 100:10 were freeze-dried according to the protocol described in our previous study [25]. Finally, the vials were removed from the freeze-dryer, closed with rubber caps and stored at 4 °C. The lyophilized liposomes were then reconstituted with ultra-pure water to its original volume (5 mL) prior to characterization and further analysis.

2.6 Liposome characterization

2.6.1 Dynamic Light Scattering Analysis

Malvern Zetasizer Nanoseries (Zetasizer Nano ZS; Malvern Instruments Ltd, France) was used to determine the mean size of liposome batches, its range of detection is between 30 nm and 10 μ m. All batches were diluted 10-fold with ultrapure water. The particle size distribution data were collected using the DTS (nano) software (version 5.10) provided with the instrument. The polydispersity index (PDI), which gives an indication of the width of particle size distribution, ranges from 0 (monodispersed) to 1 (very broad distribution). Data were expressed as the mean \pm standard deviation. Zeta potential was calculated by Smoluchowski's equation from the electrophoretic mobility of liposomes. All measurements were carried out at 25 °C after 3 min of equilibration and performed in triplicate.

2.6.2 Morphological characterization by transmission electron microscopy

Blank-DCL 75, blank-DCL 100, Est-DCL 75 and Est-DCL 100 before and after freeze-

drying were imaged with transmission electron microscope (TEM) (CM 120; Philips, Eindhoven, Netherlands) operating at an accelerating voltage of 120 kV. A drop of liposomes was placed onto a carbon-coated copper grid for 3 min; the excess suspension was removed with a filter paper, leaving a thin liquid film stretched over the pores. Negative staining using a sodium silicotungstate solution (1%) was directly made on the deposit over 30 s. The excess sodium silicotungstate solution was then removed with a filter paper and the stained samples were examined.

2.6.3 Determination of encapsulation efficiency and loading rate for estragole in liposomes

The EE of estragole into liposomes was determined upon HPLC analysis of free and total estragole present in the liposome suspensions. Aliquots were removed from the liposomal suspension to determine the total estragole concentration ($[Est]_{Total}$). The liposomal suspension underwent ultracentrifugation (OptimaTM Ultracentrifuge, Beckman Coulter, USA) at 170000g for 1 h at 4 °C. Aliquots were removed from the supernatant to determine the concentration of free Est ($[Est]_F$). EE of Est was calculated as follows:

$$EE (\%) = \frac{[Est]_{Tot} - [Est]_F}{[Est]_{Tot}} \times 100 \quad (2)$$

where $[Est]_{Tot}$ and $[Est]_F$ corresponds to the concentration of total and free estragole, determined by the HPLC method described below, respectively.

The LR of estragole was calculated by the following equation:

$$LR = \frac{m_{liposomal\ suspension} - m_{filtrate}}{m_{Initial}} \times 100 \quad (3)$$

The LR after freeze-drying was calculated using the following equation:

$$LR_{fd} = \frac{m_{reconstituted\ liposomes}}{m_{Initial}} \times 100 \quad (4)$$

where $m_{initial}$ for Est-DCL is the initial mass of estragole used to prepare CD/Est inclusion complex. For Est-CL, $m_{initial}$ is the initial mass of estragole added to the organic phase during liposome preparation. $m_{reconstituted\ liposomes}$ is the mass of estragole found in the aqueous phase after freeze-drying and reconstitution of liposome.

2.7 HPLC assay of estragole

Stock standard solutions of estragole (1 mg/mL) and of the internal standard, thymol (1 mg/mL) were prepared in methanol. Aliquots were removed from the estragole stock solution and diluted in methanol to obtain final concentrations of estragole ranging from 1 to 250 µg/mL. The diluted solution of thymol (100 µg/mL) was prepared in methanol. Two hundred µL of each filtrate (aqueous solution of HP-β-CD/ Est inclusion complex) were added to 200 µL of thymol (100 µg/mL) and 400 µL of methanol. The samples were sonicated for 10 min at room temperature. The samples were analyzed by HPLC (Agilent Technologie 1200 series) using an analytical column C18 15 cm x 4.6 mm, 5 µm, (Agilent Technologies). The mobile phase was a mixture of methanol and water (70/30). The flow rate was fixed at 1 mL/min and the detection was set at 206 nm.

The retention times of thymol (internal standard) and estragole were 5.7 and 8.4 min, respectively. Calibration curves were constructed by plotting the estragole/thymol area ratio against the concentration of estragole in µg/mL. The linear relationships were evaluated by regression analysis with the least squares method and the correlation

coefficient ranged from 0.996 to 0.999.

2.8 Fluorescence anisotropy measurements

To evaluate the effect of estragole, HP- β -CD and freeze-drying on the membrane fluidity of vesicles composed of phospholipon 90H:cholesterol, steady state fluorescence polarization technique using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) was used. DPH was dissolved in tetrahydrofuran (4.30×10^{-3} mol/L) then an aliquot was added to the vesicles suspension (blank-CL, blank-DCL 75, blank-DCL 100, Est-CL, Est-DCL 75 and Est-DCL 100) to obtain DPH:phospholipid molar ratio of 1:500. The mixture was incubated overnight in the dark to allow the intercalation of the DPH molecules into the lipid bilayer. The final concentration of THF did not exceed 0.01% and showed no effect on the fluorescence anisotropy [31]. The experiments were performed at 25 and 37 °C. The probe was excited with a manual polarizer accessory (Cary Eclipse Manual Polarizer, Agilent Technologies; Malaysia) using vertically polarized light at 360 nm. The emission intensities were measured at 450 nm at both parallel and perpendicular to the polarization vector of the exciting light yielding components I_{VV} and I_{VH} , respectively. The Cary Eclipse Bio Software, delivered from the spectrophotometer, gives the fluorescence anisotropy (R), which is defined as

$$R = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (5)$$

where I_{\parallel} and I_{\perp} are the fluorescence intensities of the light emitted with its polarization plane parallel (\parallel) and perpendiculaire (\perp) to that of the exciting beam.

2.9 Release study

Multiple headspace extraction (MHE) is a dynamic gas extraction carried out stepwise [32]. At each step, equilibrium conditions are established in the vial between the sample and its gas phase. The vials containing free estragole, Est-CL, Est-DCL 75, Est-DCL 100 or the inclusion complex (HP- β -CD/Est molar ratio of 75:10 and 100:10) were submitted to 24 successive gas extractions at constant interval (8 min) in the same operating conditions. At each extraction, 1 ml of the vapor from the headspace of the solution was withdrawn from the vial using a gas-tight syringe and injected in the chromatographic column via a transfer line. All measurements were conducted with an Agilent G1888 headspace sampler coupled to a Perkin Elmer Autosystem XL gas chromatography equipped with a flame ionization detector and a DB624 column. Headspace operating conditions were as follows: oven temperature of 60°C, transfer line temperature of 250°C and nitrogen as carrier vector. GC conditions were as follows: column temperature of 160°C and GC cycle of 8 min.

2.9.1 Release of estragole

After 6 months of storage of CL and DCL at 4°C, the release of estragole from freeze-dried CL and DCL as well as from reconstituted powders was studied by MHE coupled to gas chromatography. Est-CL, Est-DCL 75 or Est-DCL 100 in powder form (10 mg), Est-CL, Est-DCL 75, Est-DCL 100, HP- β -CD/Est 75:10, HP- β -CD/Est 100:10 inclusion complex solutions and estragole (0.5 mg/mL) were placed in 22 ml sealed vials. After equilibrium, the release of estragole at time t was calculated as follows:

$$\text{Percentage of remaining Est} = \frac{A_t}{A_0} \times 100 \quad (6)$$

Where, A_t and A_0 corresponds to the area of the chromatographic peak of estragole at time t and at the first extraction, respectively. The plot of $\ln (A_t/A_0)$ as a function of time followed a first-order release kinetics. The release rate constant K (min^{-1}) was calculated using the following equation:

$$\ln (A_t/A_0) = -Kt \quad (7)$$

2.9.2 Loading capacity determination

The amount of estragole in the inclusion complex HP- β -CD/Est, CL and DCL was determined after 6 months of storage at 4°C in powder form using MHE. The concentration of the analyte in the gas phase of the vial decreases at each extraction step. Thus, the sum of the amounts of the analyte removed in the individual extractions will be equal to the total amount of analyte present in the original sample [32].

A linear regression was constructed for the standard and the samples using the following equation:

$$\ln A_t = -q(t-1) + \ln A_1 \quad (8)$$

where A_1 is the area of the chromatographic peak of estragole at the first extraction; A_t is the peak area of estragole at the extraction t and $-q$ is the slope of the regression curve that illustrates $\ln A_t$ as a function of $(t - 1)$.

Q is a constant parameter calculated as follows:

$$Q = e^{-q} \quad (9)$$

The sum of peak areas corresponding to the total amount of estragole present in the sample or in the standard are obtained as follows:

289
$$\Sigma A_t = \frac{A_1}{1-Q} (10)$$

290 The amount of estragole in the IC is calculated using the following relation:

291
$$Est_{exp}(mg) = \frac{\Sigma A_{sample}}{\Sigma A_{standard}} \times Std (mg) (11)$$

292 where ΣA_{sample} and $\Sigma A_{standard}$ are the sum of peak areas of estragole corresponding to the
 293 sample and the external standard, respectively; Std is the amount of the standard (0.005
 294 mg).

295 Loading capacity (LC m_{Est}/m_{powder}) of estragole is expressed as mg of encapsulated
 296 estragole per gram of the powder of the delivery system.

297
$$LC (m_{Est}/m_{powder}) = \frac{Est_{exp} (mg)}{mass\ of\ powder\ (mg)} (12)$$

298

299 **2.10 Determination of Henry's law constant of estragole**

300

301 The Henry's law constant (H_C) is the vapor-liquid partition coefficient expressed as
 302 followed [32]:

$$H_C = \frac{C_G}{C_L} (13)$$

303 where C_G is the concentration of estragole in the vapor phase and C_L , the concentration of
 304 estragole in the aqueous phase.

305 The value of H_C for estragole was determined experimentally by static headspace–gas
 306 chromatography (SH-GC) at two temperatures (30 and 60°C). The GC column
 307 temperature was set at 160 °C. The headspace settings were as follows: an oven
 308 temperature of 30 or 60 °C and an equilibrium time of 30 min. The transfer line
 309 temperature was set at 250 °C.

For the determination of H_C in water, several headspace vials were prepared containing different amounts of water (0.5; 0.6; 1, 2, 3 and 5 mL) to which the same amount of estragole was added (10 μ L from a standard solution of 10000 ppm). Using essentially the phase ratio variation method as described by Kolb and Ettre [32], the values of H_C were determined by the relationship between the reciprocal chromatographic peak areas and the vapor–liquid volumetric ratio (V_G/V_L , with V_G and V_L being the vapor volume and the liquid volume, respectively):

$$\frac{1}{AV_L} = \frac{1}{\alpha} \frac{V_G}{V_L} + \frac{1}{\alpha H_C} \quad (14)$$

where A is the peak area, α is a constant incorporating several parameters and H_C is the vapor-liquid partition coefficient, directly calculated from the ratio of the slope and intercept of the fit of the experimental data obtained after allowing a vapor-liquid equilibrium time of 30 min in the oven.

2.11 Statistical analysis

Statistical analysis was performed using the Student's t-test. P values equal or less than 0.05 were considered statistically significant.

3 Results and discussion

3.1 HP- β -CD/estragole inclusion complex

The HP- β -CD concentration able to solubilize the initial amount of estragole (7.44 mg) was determined; a complete solubilization of this mass leads to a solution containing

estragole (10 mM). Figure 1 shows the variation of estragole concentration in CD/Est inclusion complex solution at various HP- β -CD concentrations. In the absence of CD, the concentration of estragole was 17.73 ± 2.82 $\mu\text{g/ml}$. As we can see, a CD concentration of 100 mM was necessary to solubilize 7.44 mg of estragole. Table 1 shows the complexation efficiency of estragole at various HP- β -CD concentrations. The CE_{CD} values were calculated using equation 1. The CE_{CD} value was 34% at HP- β -CD:Est molar ratio of 10:10, then CE_{CD} values increased with HP- β -CD concentration, which is in agreement with Kfoury et al. [16]. The CE_{CD} values of estragole at HP- β -CD:Est molar ratio of 75:10 and 100:10 were 91 and 100%, respectively (Table 1). Consequently, the HP- β -CD:Est molar ratios of 75:10 and 100:10 were used to prepare DCL batches. We should mention that CDs at high concentration can perturb the liposomes membrane properties and extract lipid components [33]. For that, we did not use a concentration of HP- β -CD above 100 mM as our aim was to develop the DCL system loading estragole. Hence, the limitation of CD concentration and the solubilization of estragole were mainly considered in the selection of CD:Est molar ratio.

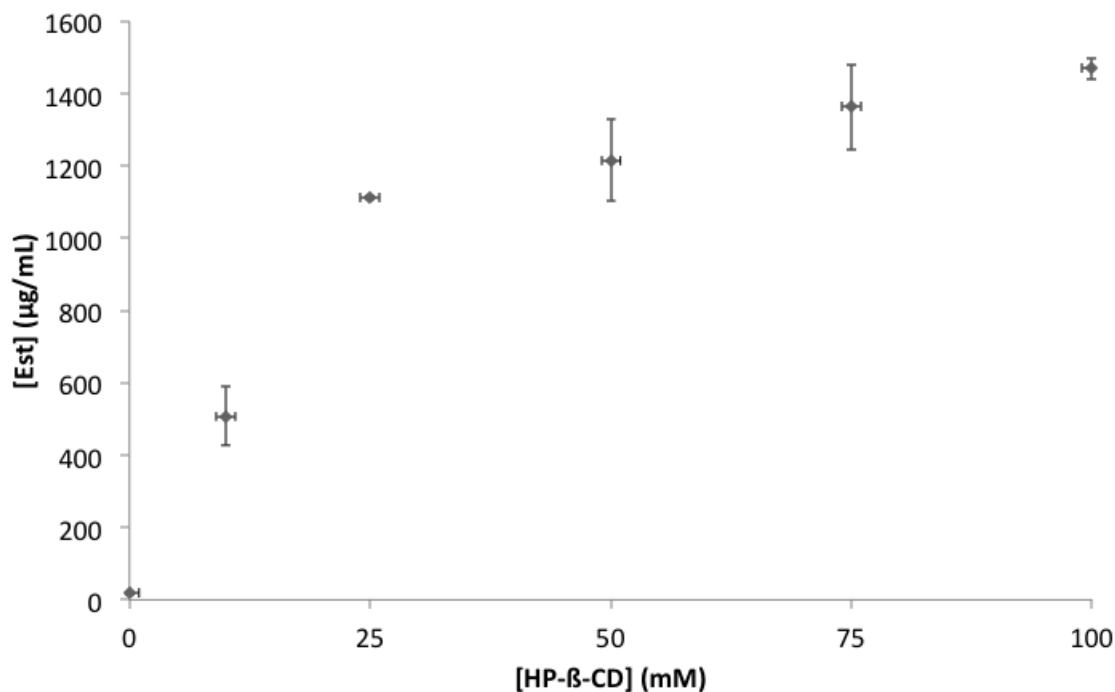


Figure 1: The variation of the concentration of estragole (µg/ml) with CD concentration.

Table 1: Complexation efficiency of estragole in HP-β-CD as a function of CD concentration

| HP-β-CD :Est molar ratio | CE _{CD} (%) |
|--------------------------|----------------------|
| 10:10 | 34.12 ± 5.41 |
| 25:10 | 74.78 ± 0.29 |
| 50:10 | 81.65 ± 21.75 |
| 75:10 | 91.56 ± 7.97 |
| 100:10 | 98.76 ± 2.32 |

3.2 Mean size, pDI, zeta potential values of liposomes before and after freeze-drying

The fresh and reconstituted liposome suspensions were characterized and compared in terms of mean particle size, PdI and zeta potential values before and after freeze-drying, respectively and the results are listed in Table 2.

Table 2: Size, polydispersity index, zeta potential values for fresh and reconstituted Est-CL and Est-DCL from Phospholipon 90H before and after freeze-drying. The values obtained after lyophilization were compared to those before lyophilization and * indicates a value of $P < 0.05$ statistically significant.

| | Before freeze-drying | | | | After freeze-drying | | |
|---------------|----------------------|-------------|--------------|-------------------------------|---------------------|--------------|-------------|
| | Size (nm) | pdI | Zeta (mV) | Dispersion of the pellet with | Size (nm) | pdI | Zeta (mV) |
| Blank-CL | 179 ± 10 | 0.08 ± 0.01 | -9.1 ± 6.3 | CD 25 mM | 169 ± 8 | 0.22 ± 0.04 | -7.4 ± 1.7 |
| | | | | CD 50 mM | 180 ± 17 | 0.15 ± 0.02* | -5.0 ± 4.0* |
| Est-CL | 176 ± 2 | 0.10 ± 0.2 | -9.0 ± 0.9 | CD 25 mM | 173 ± 0.3 | 0.14 ± 0.01* | -6.4 ± 0.2 |
| | | | | CD 50 mM | 165 ± 9 | 0.14 ± 0.01* | -2.4 ± 1.6* |
| Blank-DCL 75 | 205 ± 3 | 0.23 ± 0.00 | -10.7 ± 1.4 | Water | 201 ± 2 | 0.16 ± 0.00* | -3.8 ± 0.2* |
| | | | | CD 75 mM | 198 ± 3 | 0.12 ± 0.00 | -12.2 ± 3.8 |
| Est-DCL 75 | 191 ± 13 | 0.28 ± 0.07 | -6.8 ± 6.2 | CD 75 mM | 187 ± 3 | 0.14 ± 0.07 | -9.2 ± 3.7 |
| Blank-DCL 100 | 210 ± 17 | 0.31 ± 0.01 | -15.8 ± 10.3 | Water | 192 ± 6.9 | 0.16 ± 0.03 | -8.2 ± 0.1 |
| | | | | CD 100 mM | 197 ± 16 | 0.21 ± 0.07 | -10.1 ± 3.8 |
| Est-DCL 100 | 188 ± 19 | 0.39 ± 0.13 | -12.3 ± 2.2 | CD 100 mM | 209 ± 16 | 0.14 ± 0.00* | -11.7 ± 4.5 |

Before freeze-drying, the analysis of particle size showed no significant difference between the various formulations, since Est-CLs and Est-DCLs had similar range of particle size of (176-210 nm). Thus the presence of HP-β-CD or estragole did not affect the liposome size. These results are in accordance with the literature [25, 28, 34, 35]. Besides, PdI values for all liposome formulations were less than 0.4, suggesting that liposomes suspensions were homogenous (Table 2). The presence of HP-β-CD or estragole did not affect the zeta potential values, which are negative ranging between (-6;

-15 mV).

In a previous work, we evaluated the effect of hydrogenation and composition of soybean phospholipids on the characteristics and the stability of blank-CL and blank-DCL during freeze-drying [25]. The results showed that HP- β -CD protect hydrogenated liposomes (Phospholipon 90H:cholesterol or Phospholipon 80H:cholesterol formulation) during freeze-drying. Furthermore, dispersing the pellet of CL in HP- β -CD solution of (25 or 50 mM), and dispersing the pellet of DCL in water or in HP- β -CD solution (at the same concentration present in the internal aqueous phase of liposome) protect the structures during freeze-drying. Here we applied the conditions to Est-CL and Est-DCL formulations for freeze-drying.

As can be seen in Table 2, after freeze-drying the size, pDI and zeta potential values of liposomes were maintained since no changes were observed for particle analysis before and after freeze drying. These results demonstrate a successful freeze-drying of estragole loaded Phospholipon 90H liposomes.

3.3 Encapsulation efficiency and loading rate values of estragole in liposomes

The encapsulation efficiency (EE) and the loading rate (LR) values of estragole loaded CL and DCL before and after freeze-drying were determined according to equations (2-4) and the results are listed in Table 3.

Table 3: Encapsulation efficiency of Est for fresh CL and DCL suspensions and loading rate of Est in fresh and reconstituted CL and DCL before and after freeze-drying.

| | Before freeze-drying Fresh liposomes | | After freeze-drying Reconstituted liposomes |
|-------------|---|---------------|--|
| | EE (%) | LR (%) | LR_{fd} (%) |
| Est-CL | ND* | ND* | ND* |
| Est-DCL 75 | 8.47 ± 1.21 | 1.58 ± 0.25 | 1.30 ± 0.78 |
| Est-DCL 100 | 11.36 ± 2.31 | 3.25 ± 0.68 | 2.52 ± 1.15 |

ND: not detected

Before freeze-drying, the estragole concentration in CL formulations was under limit of estragole detection by HPLC method, suggesting that Phospholipon 90H-CL were not able to encapsulate estragole by ethanol injection method. The preparation of liposomes requires heating at 55°C, above the main transition temperature of Phospholipon 90H, which could induce the loss of volatile estragole during preparation. This is in line with H_C value of estragole as it increased with temperature. The DCL carrier system improved the estragole encapsulation. Indeed, the EE values of estragole were 8.47 ± 1.21 and 11.36 ± 2.31 % for Est-DCL 75 and Est-DCL 100 respectively and the LR values of estragole were 1.58 ± 0.25 and 3.25 ± 0.68 % for Est-DCL 75 and Est-DCL 100 respectively. In term of LR, DCL represents a better encapsulation system than CL for numerous drugs including volatile ones such as *trans*-anethole [28] and non-volatile compound like betamethasone [36]; celcoxib [37], curcumin [38] and indomethacin [39]. Besides, the amount of estragole in CD/drug inclusion complex solutions increased upon increasing the HP-β-CD:Est molar ratio from 75:10 to 100:10. Same results were observed by Maestrelli et al., [40], where the EE values of ketoprofen increased with

increasing the concentration of HP- β -CD:ketoprofen inclusion complex when encapsulated in the aqueous phase of liposomes.

After freeze-drying, the LR of estragole in Est-DCL 75 and Est-DCL 100 were quasi maintained (Table 3).

3.4 Morphology

The TEM images showed the formation of nanometer-sized vesicles (Figure 2). The images in Figure 2 (A-D) and (A'-D') are examples of blank-DCL 75, blank-DCL 100; Est-DCL 75 and Est-DCL 100 obtained before and after freeze drying, respectively. The TEM results are in agreement with the dynamic light scattering results since nanometric sized vesicles appeared in the images. The vesicles are oligolamellar and spherical in shape. CLs and DCLs loading estragole present similar structures to *trans*-anethole and eugenol loaded CL and DCL [25, 29].

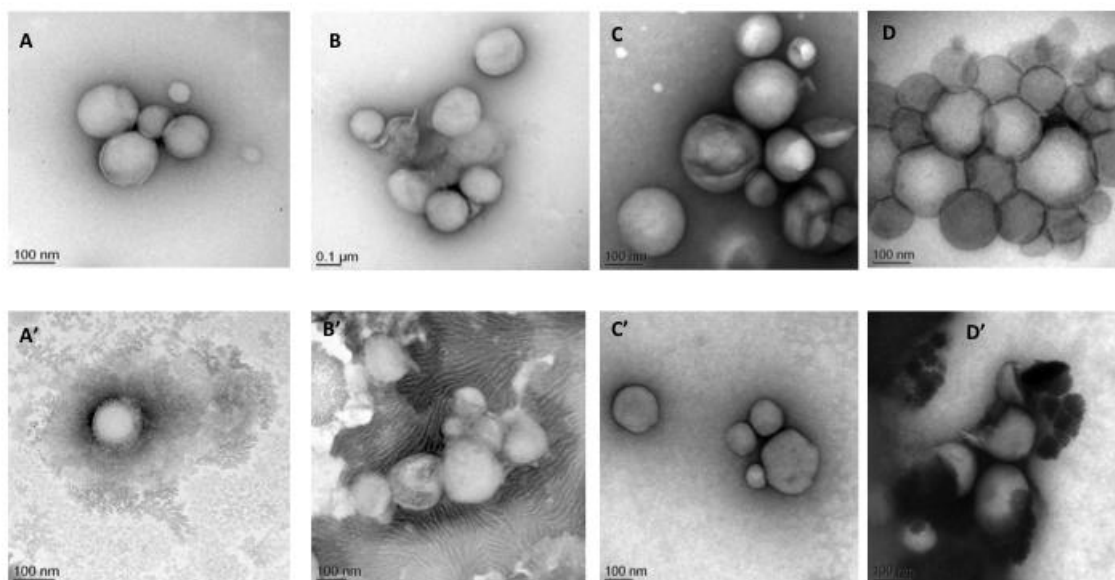


Figure 2: TEM images of Phospholipon 90H liposomes before freeze-drying: blank-DCL 75 (A); blank-DCL 100 (B); Est-DCL 75 (C) and Est-DCL 100 (D) and after freeze drying: blank-DCL 75 (A'); blank-DCL 100 (B'); Est-DCL 75 (C') and Est-DCL 100 (D').

3.5 Release studies

The release of volatile drugs from the samples (aqueous solution of a drug, delivery systems incorporating a drug in liquid or powder form) can be tracked using MHE mode. The procedure is well described by Kolb & Ettre [32]. It consists in carrying out a sampling sequence for the same sample. There is equilibrium between the sample and the gas phase in the vial. At every extraction the headspace determined the concentration of estragole in the gas phase. As the extractions proceed, the amount of the volatile guest present in the headspace decreases to zero, as do the areas of the corresponding chromatographic peaks. The sum of the areas obtained will therefore be equal to the amount of guest present in the sample. The MHE therefore allows the illustration of the release profile of the volatile product.

The release studies of estragole from freeze-dried HP- β -CD/Est inclusion complex, Est-CL and Est-DCL stored at 4°C for 6 months, were performed by the MHE method. The estragole peak areas of the external standard (0.5 ppm of estragole in water), inclusion complex HP- β -CD:Est (75:10 and 100:10 molar ratios in water) and estragole loaded liposomes (CL and DCL in powder or reconstituted in water) are illustrated in.

The amount of estragole determined from powder samples (Est-CL, Est-DCL 75 and Est-DCL 100, appeared in the insert of figure 3) was very low suggesting either high retention of Est or weak presence of Est in all powder samples. For this reason, the powders were dissolved in 10 ml water and placed in sealed vials and analyzed by MHE.

The results showed that the estragole peak area increased for the reconstituted samples when compared to freeze-dried samples, meaning that the powder liposomes exhibit a higher retention of estragole than their aqueous dispersed forms. Compared to Est-CL, the encapsulation of estragole in lipid vesicles was improved with Est-DCL 75 and Est-DCL 100 while the HP- β -CD inclusion complex showed the higher retention of estragole (Figure 3). Reconstituted batches were then considered for the release study and determination of the LC $m_{\text{Est}}/m_{\text{powder}}$ of estragole.

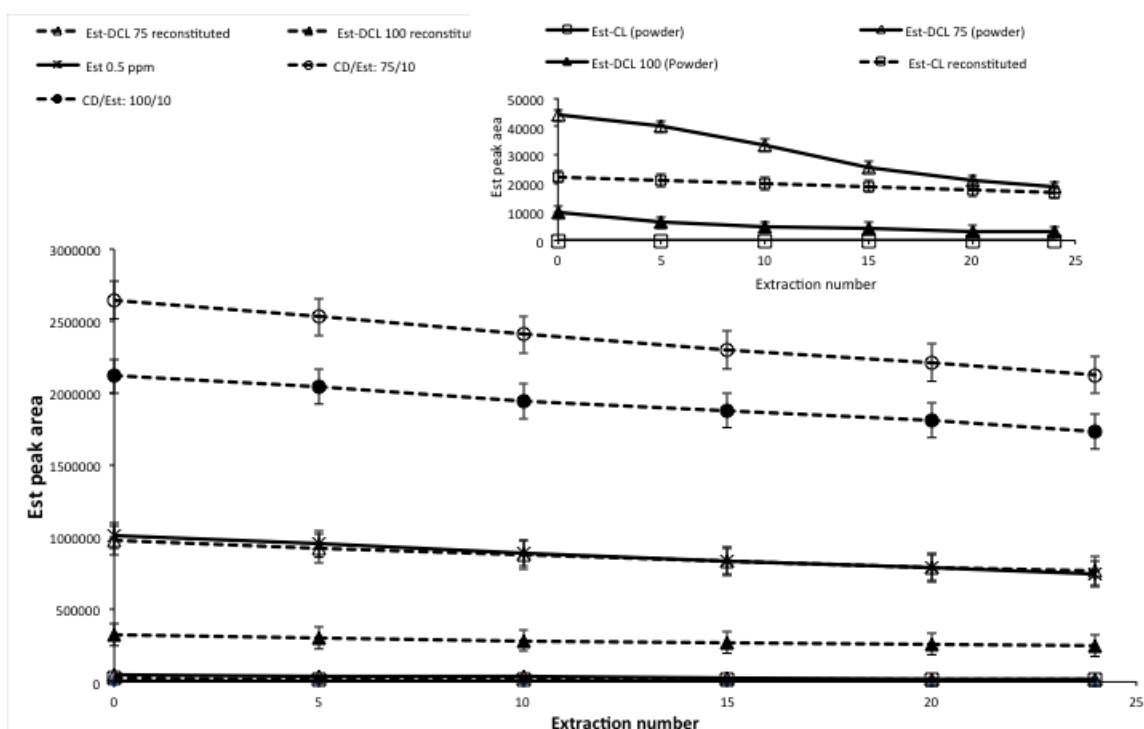
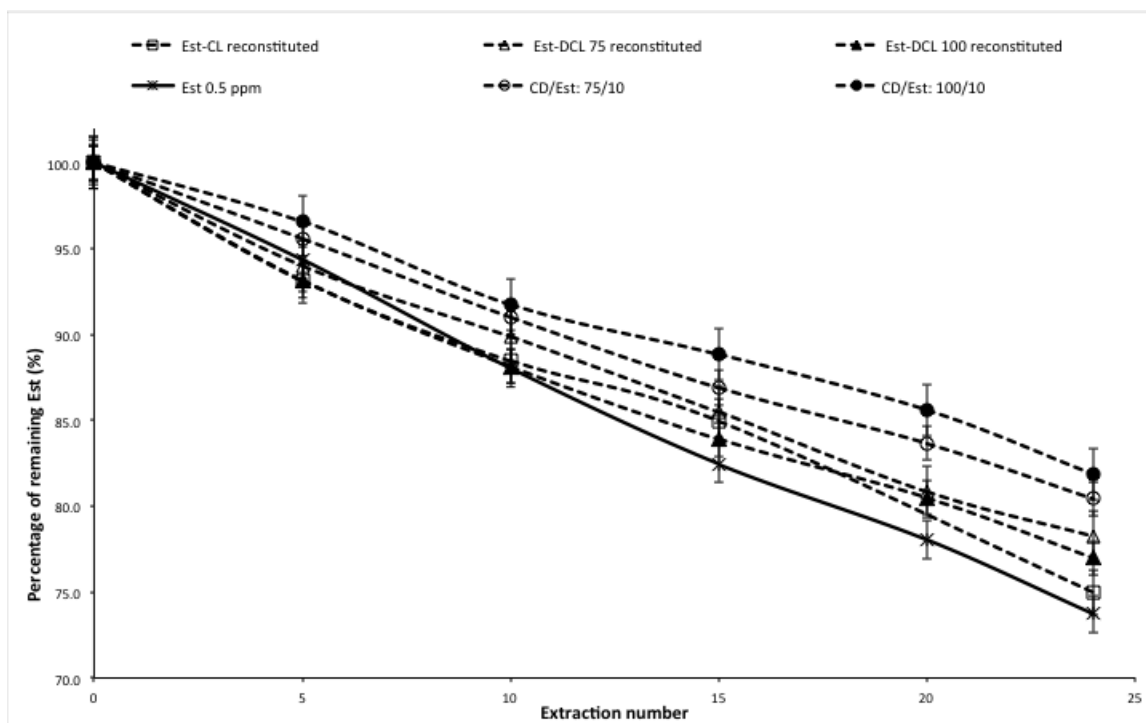


Figure 3: The estragole peak areas of the external standard (0.5 ppm of Est in water), inclusion complex HP- β -CD:Est (75:10 and 100:10 molar ratios in water) and Est loaded liposomes (CL and DCL in powder or reconstituted in water). The insert represents the peak areas for CL and DCL in powder form and CL reconstituted in water.

Figure 4 resumes the percentage of remaining estragole from the reference solution (0.5 ppm of estragole in water), HP- β -CD inclusion complex (75:10 and 100:10 molar ratio in water) and liposomes (CL and DCL reconstituted in water). The remaining percentage of

475 estragole in the inclusion complex, Est-CL and Est-DCL suspensions was higher than
 476 that of the reference solution. After 24 extractions (24 h), the remaining percentage of Est
 477 was 81, 80, 78, 77 and 75% for HP- β -CD:Est 100:10, HP- β -CD:Est 75:10, Est-DCL 75,
 478 Est-DCL 100 and Est-CL, respectively.



479

480 Figure 4: The percentage of remaining Est from Est external standard (0.5 ppm of estragole in
 481 water), inclusion complex HP- β -CD:Est (75:10 and 100:10 molar ratios in water) and estragole
 482 loaded liposomes (CL and DCL reconstituted in water).

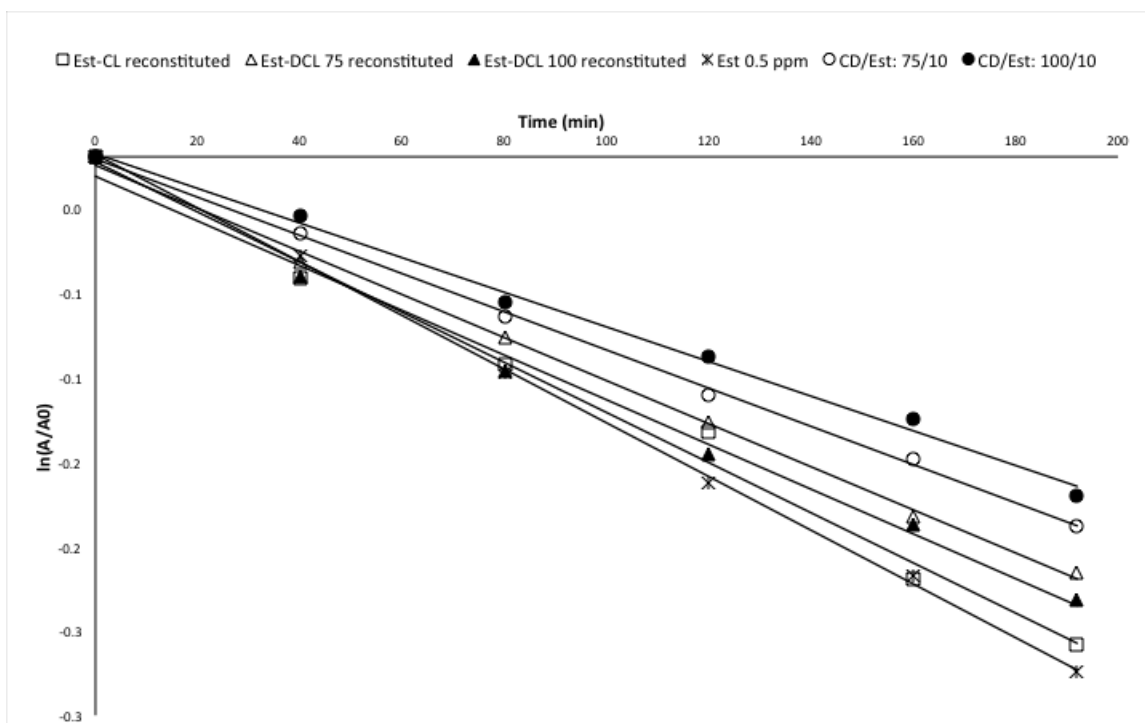


Figure 5 : The plot of $\ln (A_t/A_0)$ as a function of time for Est external standard (0.5 ppm of estragole in water), inclusion complex HP- β -CD:Est (75:10 and 100:10 molar ratios in water) and estragole loaded liposomes (CL and DCL reconstituted in water).

The release rate constant was calculated according to equation 7 and the values are presented in Table 4. The retention of estragole was improved by 1.6, 1.4, 1.3 and 1.2 times for HP- β -CD/Est 100:10, HP- β -CD/Est 75:10, reconstituted Est-DCL 75 and reconstituted Est-DCL 100 compared to free estragole (0.5ppm).

Table 4: Release rate constants of free estragole, HP- β -CD/Est inclusion complex, Est-CL and Est-DCL formulations

| | Release rate constant K (min^{-1}) |
|----------------------------|--|
| Est 0.5 ppm | 0.013 |
| HP- β -CD/Est 100:10 | 0.008 |
| HP- β -CD/Est 75:10 | 0.009 |
| Est DCL 75 reconstituted | 0.010 |

| | |
|---------------------------|-------|
| Est DCL 100 reconstituted | 0.011 |
| Est-CL reconstituted | 0.012 |

The incorporation of a volatile drug from a delivery system and its release could be affected by its volatility. For that, the vapor-liquid partition coefficient (H_C) of estragole was determined using HS-GC at 30 and 60°C. The H_C value of estragole was 0.03 at 30°C and increased to 0.07 at 60°C. Hence, the volatility of estragole increased with temperature. Thus, the HP- β -CD/Est inclusion complex, CL or DCL improved the retention of estragole and controlled its release. These results were in agreement with Kfoury et al., [41] who showed that β -CD derivatives improved the complexation in CDs of essential oils components such as α -pinene, mycrene, limonene, *p*-cymene and terpinene.

3.6 Determination of loading capacity of estragole ($m_{\text{Est}}/m_{\text{powder}}$)

The amount of estragole in the inclusion complex obtained at both molar ratios (HP- β -CD:Est 75:10 and 100:10) and in the reconstituted liposomes was quantified by SH-GC after 6 months of storage at 4°C. The LC values expressed as $m_{\text{Est}}/m_{\text{powder}}$ and calculated using equation 12, are listed in Table 5. The LC value of estragole in Est-CL was 0.01 μg of Est/mg of powder. The CL formulations contain small amount of estragole, which was not detected by HPLC. The encapsulation of estragole in DCL system was improved with LC values of 0.6 and 0.2 μg of Est/mg of powder for Est-DCL 75 and Est-DCL 100, respectively. However, the LC values of estragole in the inclusion complex HP- β -CD/Est 75:10 and 100:10 were 10.8 and 9.3 μg of Est/mg of powder, respectively. These values

were higher than those of Est-DCL 75 and Est-DCL 100. These results suggest that DCL system improve the encapsulation of estragole compared to CL. The inclusion complex HP- β -CD/Est showed the best ability to retain high amount of estragole. Among the three systems, CD presents the higher LC followed by Est-DCL and Est-CL.

Table 5: Loading capacity $m_{\text{Est}}/m_{\text{powder}}$ in HP- β -CD/Est inclusion complex and in Est-CL and Est-DCL carrier systems after 6 months of storage at 4°C.

| | LC (μg of Est/mg of powder) |
|----------------------------|---|
| HP- β -CD:Est 75:10 | 10.8 |
| HP- β -CD:Est 100:10 | 9.3 |
| Est-CL | 0.01 |
| Est-DCL 75 | 0.6 |
| Est-DCL 100 | 0.2 |

3.7 Fluorescence anisotropy

The fluorescence anisotropy measurements of DPH in liposomes give information on membrane fluidity of liposomes membrane [42]. The DPH anisotropy values obtained at 25 and 37°C for blank-CL, blank-DCL 75, blank-DCL 100, Est-CL, Est-DCL 75 and Est-DCL 100 before and after freeze-drying are presented in Table 6.

Table 6: DPH fluorescence anisotropy measurements for blank-CL, blank-DCL, Est-CL and Est-DCL made from Phospholipon 90H and cholesterol at 25 and 37°C before and after freeze-drying.

| | Before freeze-drying | | After freeze-drying | |
|---------------|----------------------|-----------------|---------------------|-----------------|
| | 25°C | 37°C | 25°C | 37°C |
| Blank-CL | 0.22 \pm 0.01 | 0.17 \pm 0.00 | n.d. | n.d. |
| Blank-DCL 75 | 0.21 \pm 0.00 | 0.17 \pm 0.00 | 0.21 \pm 0.00 | 0.17 \pm 0.00 |
| Blank-DCL 100 | 0.20 \pm 0.00 | 0.18 \pm 0.01 | 0.20 \pm 0.00 | 0.18 \pm 0.00 |
| Est-CL | 0.22 \pm 0.01 | 0.18 \pm 0.01 | n.d. | n.d. |
| Est-DCL 75 | 0.22 \pm 0.01 | 0.18 \pm 0.01 | 0.22 \pm 0.01 | 0.18 \pm 0.01 |
| Est-DCL 100 | 0.21 \pm 0.00 | 0.18 \pm 0.01 | 0.21 \pm 0.01 | 0.18 \pm 0.01 |

Values are expressed as the means of three repetitions \pm SD; n.d.: not determined

- $P < 0.05$ compared to those before freeze-drying

To the best of our knowledge, this is the first example of DPH fluorescence anisotropy measurements conducted on freeze-dried DCLs. As expected, an increase of temperature leads to an increase of membrane fluidity.

Compared to blank-CL, the presence of estragole or the inclusion complex HP- β -CD/Est (75:10 and 100:10 molar ratio) did not affect the DPH anisotropy values at 25 and 37°C.

The results obtained for Est-CL has been expected since a small amount of estragole was encapsulated in CL, which could not affect the membrane fluidity. Gharib, et al., [43] demonstrated that the presence of estragole in DPPC membrane, at molar ratios Est:DPPC largely higher than those obtained in this study, reduced the DPH anisotropy values at 28, 41 and 50°C in a concentration-dependent manner.

Besides, dispersing the pellet of CL and DCLs with HP- β -CD solution before freeze drying did not affect the membrane fluidity, since the DPH anisotropy values remain unchanged after freeze-drying for all batches (Table 6). These results proved that HP- β -CD could be used as membrane protectant during freeze-drying for CL and for DCL system made from saturated phospholipids.

4 Conclusion

The characteristics of hydrogenated-liposomes (size, pDI, zeta potential and LR of estragole) were maintained after freeze-drying using HP- β -CD as cryoprotectant for CL and DCL systems. Moreover, HP- β -CD did not affect the fluidity of phospholipon

90H/cholesterol. In addition, the presence of estragole within HP- β -CD, CL or DCL reduced its volatility and allowed its controlled release. The HP- β -CD:Est inclusion complex and Est-DCL system showed high retention of estragole and displayed a good stability. These results suggest that freeze-dried CD inclusion complexes and DCL systems could be considered as efficient carriers for essential oil components retention, preservation, storage and controlled release of estragole.

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