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

3  
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21

22 **Abstract**

23 Estragole is considered as a promising candidate for food preservation due to its  
24 antioxidant and antimicrobial properties. However, its sensitivity to light and oxygen, its  
25 volatility and hydrophobicity prevent its large application. In order to increase the  
26 stability of estragole, conventional liposomes (CL) and drug-in-cyclodextrin-in-  
27 liposomes (DCL) were prepared by the ethanol-injection method using Phospholipon  
28 90H in combination with cholesterol and then freeze-dried using hydroxypropyl- $\beta$ -  
29 cyclodextrin (HP- $\beta$ -CD) as a cryoprotectant. Fresh and reconstituted vesicles  
30 demonstrated nanometric vesicles size, spherical shape and negative surface charge.  
31 Moreover, all suspensions were homogeneous. Compared to CL, DCL improved  
32 estragole encapsulation efficiency, and conserved the same loading rate during freeze-  
33 drying. Freeze-dried CL and DCL retained estragole more efficiently compared to the  
34 reconstituted ones, as evidenced by release study performed by multiple headspace  
35 extraction. Finally, we demonstrated, for the first time, that HP- $\beta$ -CD did not affect the  
36 membrane fluidity of fresh and reconstituted Phospholipon 90H/cholesterol CL and DCL.  
37

38 **Keywords:** conventional liposomes; drug-in-cyclodextrin-in-liposomes; estragole;  
39 freeze-drying; hydroxypropyl- $\beta$ -cyclodextrin.

## 40 **1 Introduction**

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

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

56 Widespread application of estragole is limited by its volatility and poor water solubility.  
57 Moreover, external factors such as temperature, light, and accessibility to atmospheric  
58 oxygen affect the stability of estragole [9]. For example, the photooxidation of estragole  
59 produced 4-methoxybenzaldehyde (anisaldehyde) and 4-methoxybenzene acetaldehyde  
60 [10]. Attempts were made to increase the stability of essential oil containing estragole  
61 through encapsulation of basil oil in gelatin microcapsules [11, 12], in nano- and micro-  
62 emulsion [12, 14] or in cyclodextrins (CD) [15-17]. CDs increased the solubility and

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

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

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

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

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

89 In the present study, different CL and DCL formulations containing estragole were  
90 prepared by ethanol injection method using hydrogenated phospholipid in presence of  
91 cholesterol. These liposomes formulations were characterized before and after freeze-  
92 drying. The size, polydispersity index, zeta potential of liposomes formulations were  
93 determined as well as the morphology using transmission electron microscopy. The  
94 loading rate of estragole was determined using HPLC. The stability of vesicles was  
95 assessed after 6 months of storage at 4°C in powder form and the release of estragole  
96 from the inclusion complex, CL and DCL was studied by multiple headspace extraction  
97 at 60°C. The volatility of estragole and the fluidity of the membrane may affect the  
98 estragole encapsulation. Hence, the Henry's law constant of estragole was determined at  
99 30 and 60°C, and the fluorescence anisotropy of 1,6-diphenylhexatriene (DPH) inserted  
100 in the lipid membranes of CL and DCL was studied for the first time before and after  
101 freeze-drying at 25 and 37°C.

## 102 **2 Materials and methods**

### 103 **2.1 Materials**

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

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

111

## 112 **2.2 Preparation of HP-β-CD/Est inclusion complex**

113

114 7.44 mg of estragole was added to 5 mL HP-β-CD solutions (0, 10, 25, 50, 75 and 100  
115 mM). The mixtures were shaken at 25 °C for 24 h then filtered through a 0.45 μm  
116 membrane filter. The concentrations of estragole in the filtrates were determined by the  
117 HPLC method described below.

118

119 The complexation efficiency was calculated using the following equation:

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

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

123

## 124 **2.3 Preparation of HP-β-CD/Est inclusion complex for DCL preparations**

125

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

130

## 131 **2.4 Preparation of liposomes by ethanol injection method**

132

133 The liposomes were prepared by the ethanol injection method. Phospholipon 90H (10  
134 mg/mL) and cholesterol (5 mg/mL) were dissolved in absolute ethanol. The resulting  
135 organic phase (10 mL) was then injected, using a syringe pump (Fortuna optima, GmbH-  
136 Germany), into the aqueous phase (20 mL) at 55°C (temperature above the transition  
137 temperature of Phospholipon 90H) under magnetic stirring at 400 rpm. Spontaneous  
138 liposome formation occurred as soon as the ethanolic solution was in contact with the  
139 aqueous phase. The liposomal suspension was then left for 15 min at 25 °C under stirring  
140 (400 rpm). Finally the ethanol was removed by rotary evaporation (BÜCHI Rotavapor R-  
141 100) under reduced pressure at 40 °C. Four batches were prepared: 1) blank-CL; 2)  
142 blank-DCL, where HP-β-CD was dissolved in the aqueous phase at concentrations of 75  
143 (blank-DCL 75) and 100 mM (blank-DCL 100); 3) Est-loaded liposomes (Est-CL), where  
144 estragole was added in the organic phase at a concentration of 2.5 mg/mL; 4) HP-β-  
145 CD/Est inclusion complex-loaded liposomes (Est-DCL), where the HP-β-CD/Est solution  
146 prepared previously (Est-DCL 75 and Est-DCL 100 correspond to DCL preparation using  
147 HP-β-CD:Est molar ratio of 75:10 and 100:10, respectively). Each batch was prepared in  
148 triplicate and underwent characterization as described below.

149

## 150 **2.5 Freeze-drying**

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

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

161

## 162 **2.6 Liposome characterization**

### 163 **2.6.1 Dynamic Light Scattering Analysis**

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

174

### 175 **2.6.2 Morphological characterization by transmission electron microscopy**

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

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

### 184 **2.6.3 Determination of encapsulation efficiency and loading rate for estragole in** 185 **liposomes**

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

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

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

194

195 The LR of estragole was calculated by the following equation:

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

197

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

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

199

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

204

## 205 **2.7 HPLC assay of estragole**

206

207 Stock standard solutions of estragole (1 mg/mL) and of the internal standard, thymol (1  
208 mg/mL) were prepared in methanol. Aliquots were removed from the estragole stock  
209 solution and diluted in methanol to obtain final concentrations of estragole ranging from  
210 1 to 250  $\mu\text{g/mL}$ . The diluted solution of thymol (100  $\mu\text{g/mL}$ ) was prepared in methanol.  
211 Two hundred  $\mu\text{L}$  of each filtrate (aqueous solution of HP- $\beta$ -CD/ Est inclusion complex)  
212 were added to 200  $\mu\text{L}$  of thymol (100  $\mu\text{g/mL}$ ) and 400  $\mu\text{L}$  of methanol. The samples  
213 were sonicated for 10 min at room temperature. The samples were analyzed by HPLC  
214 (Agilent Technologie 1200 series) using an analytical column C18 15 cm x 4.6 mm, 5  
215  $\mu\text{m}$ , (Agilent Technologies). The mobile phase was a mixture of methanol and water  
216 (70/30). The flow rate was fixed at 1 mL/min and the detection was set at 206 nm.

217 The retention times of thymol (internal standard) and estragole were 5.7 and 8.4 min,  
218 respectively. Calibration curves were constructed by plotting the estragole/thymol area  
219 ratio against the concentration of estragole in  $\mu\text{g/mL}$ . The linear relationships were  
220 evaluated by regression analysis with the least squares method and the correlation

221 coefficient ranged from 0.996 to 0.999.

222

## 223 **2.8 Fluorescence anisotropy measurements**

224

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

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

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

242

## 243 **2.9 Release study**

244  
245 Multiple headspace extraction (MHE) is a dynamic gas extraction carried out stepwise  
246 [32]. At each step, equilibrium conditions are established in the vial between the sample  
247 and its gas phase. The vials containing free estragole, Est-CL, Est-DCL 75, Est-DCL 100  
248 or the inclusion complex (HP- $\beta$ -CD/Est molar ratio of 75:10 and 100:10) were submitted  
249 to 24 successive gas extractions at constant interval (8 min) in the same operating  
250 conditions. At each extraction, 1 ml of the vapor from the headspace of the solution was  
251 withdrawn from the vial using a gas-tight syringe and injected in the chromatographic  
252 column via a transfer line. All measurements were conducted with an Agilent G1888  
253 headspace sampler coupled to a Perkin Elmer Autosystem XL gas chromatography  
254 equipped with a flame ionization detector and a DB624 column. Headspace operating  
255 conditions were as follows: oven temperature of 60°C, transfer line temperature of 250°C  
256 and nitrogen as carrier vector. GC conditions were as follows: column temperature of  
257 160°C and GC cycle of 8 min.

258

### 259 **2.9.1 Release of estragole**

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

266 
$$\text{Percentage of remaining Est} = \frac{A_t}{A_0} \times 100 \quad (6)$$

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

271 
$$\ln (A_t/A_0) = -Kt \quad (7)$$

## 272 **2.9.2 Loading capacity determination**

273 The amount of estragole in the inclusion complex HP- $\beta$ -CD/Est, CL and DCL was  
274 determined after 6 months of storage at  $4^\circ\text{C}$  in powder form using MHE. The  
275 concentration of the analyte in the gas phase of the vial decreases at each extraction step.  
276 Thus, the sum of the amounts of the analyte removed in the individual extractions will be  
277 equal to the total amount of analyte present in the original sample [32].

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

280 
$$\ln A_t = -q(t-1) + \ln A_1 \quad (8)$$

281  
282 where  $A_1$  is the area of the chromatographic peak of estragole at the first extraction;  $A_t$  is  
283 the peak area of estragole at the extraction  $t$  and  $-q$  is the slope of the regression curve  
284 that illustrates  $\ln A_t$  as a function of  $(t - 1)$ .

285  $Q$  is a constant parameter calculated as follows:

286 
$$Q = e^{-q} \quad (9)$$

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

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

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

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

292 where  $\sum A_{sample}$  and  $\sum A_{standard}$  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 
$$LC (m_{Est}/m_{powder}) = \frac{Est_{exp} (mg)}{mass\ of\ powder (mg)} \quad (12)$$

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]:

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

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.

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

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

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

321

## 322 **2.11 Statistical analysis**

323

324 Statistical analysis was performed using the Student's *t*-test. *P* values equal or less than

325 0.05 were considered statistically significant.

326

## 327 **3 Results and discussion**

### 328 **3.1 HP- $\beta$ -CD/estragole inclusion complex**

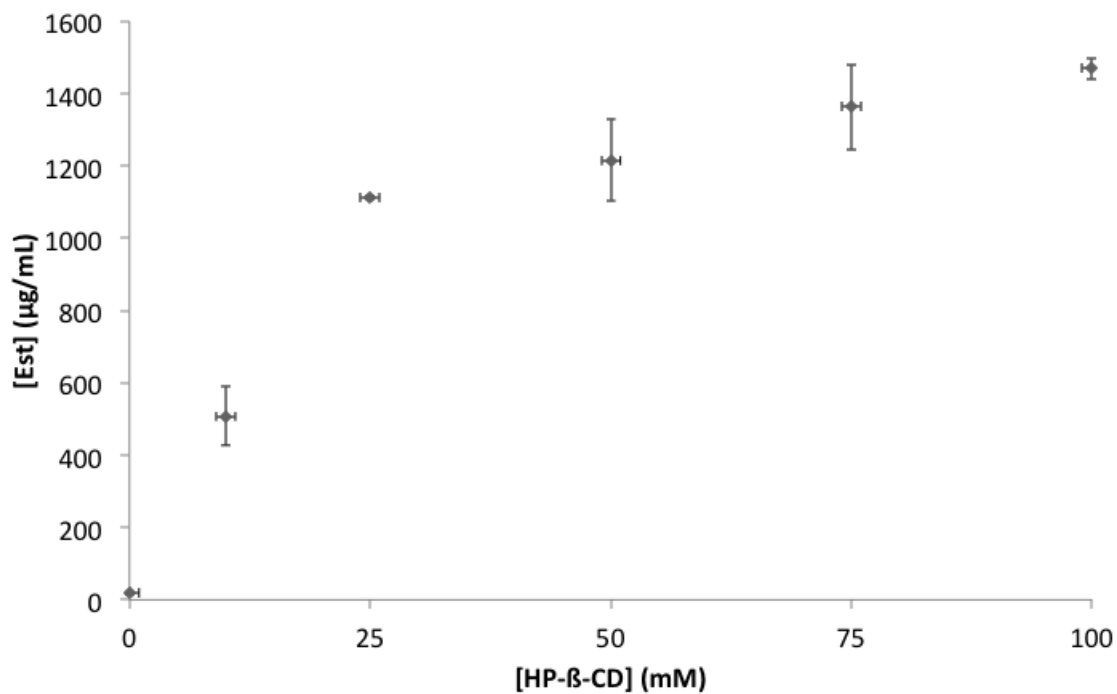
329 The HP- $\beta$ -CD concentration able to solubilize the initial amount of estragole (7.44 mg)

330 was determined; a complete solubilization of this mass leads to a solution containing

331 estragole (10 mM). Figure 1 shows the variation of estragole concentration in CD/Est  
332 inclusion complex solution at various HP- $\beta$ -CD concentrations. In the absence of CD, the  
333 concentration of estragole was  $17.73 \pm 2.82$   $\mu\text{g/ml}$ . As we can see, a CD concentration of  
334 100 mM was necessary to solubilize 7.44 mg of estragole. Table 1 shows the  
335 complexation efficiency of estragole at various HP- $\beta$ -CD concentrations. The  $\text{CE}_{\text{CD}}$   
336 values were calculated using equation 1. The  $\text{CE}_{\text{CD}}$  value was 34% at HP- $\beta$ -CD:Est molar  
337 ratio of 10:10, then  $\text{CE}_{\text{CD}}$  values increased with HP- $\beta$ -CD concentration, which is in  
338 agreement with Kfoury et al. [16]. The  $\text{CE}_{\text{CD}}$  values of estragole at HP- $\beta$ -CD:Est molar  
339 ratio of 75:10 and 100:10 were 91 and 100%, respectively (Table 1). Consequently, the  
340 HP- $\beta$ -CD:Est molar ratios of 75:10 and 100:10 were used to prepare DCL batches. We  
341 should mention that CDs at high concentration can perturb the liposomes membrane  
342 properties and extract lipid components [33]. For that, we did not use a concentration of  
343 HP- $\beta$ -CD above 100 mM as our aim was to develop the DCL system loading estragole.  
344 Hence, the limitation of CD concentration and the solubilization of estragole were mainly  
345 considered in the selection of CD:Est molar ratio.

346

347



348

349 Figure 1: The variation of the concentration of estragole ( $\mu\text{g/ml}$ ) with CD concentration.

350

351

352

353 Table 1: Complexation efficiency of estragole in HP- $\beta$ -CD as a function of CD concentration

354

HP- $\beta$ -CD :Est molar ratio	CE <sub>CD</sub> (%)
10:10	34.12 $\pm$ 5.41
25:10	74.78 $\pm$ 0.29
50:10	81.65 $\pm$ 21.75
75:10	91.56 $\pm$ 7.97
100:10	98.76 $\pm$ 2.32

355

356

### 357 **3.2 Mean size, pDI, zeta potential values of liposomes before and after** 358 **freeze-drying**

359

360 The fresh and reconstituted liposome suspensions were characterized and compared in

361 terms of mean particle size, PdI and zeta potential values before and after freeze-drying,

362 respectively and the results are listed in Table 2.

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

367

	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

368

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

376 -15 mV).

377

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

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

391

### 392 **3.3 Encapsulation efficiency and loading rate values of estragole in** 393 **liposomes**

394

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

398

399 Table 3: Encapsulation efficiency of Est for fresh CL and DCL suspensions and loading rate of  
400 Est in fresh and reconstituted CL and DCL before and after freeze-drying.  
401

	Before freeze-drying Fresh liposomes		After freeze-drying Reconstituted liposomes
	EE (%)	LR (%)	LR <sub>fd</sub> (%)
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

402 ND: not detected

403

404 Before freeze-drying, the estragole concentration in CL formulations was under limit of  
405 estragole detection by HPLC method, suggesting that Phospholipon 90H-CL were not  
406 able to encapsulate estragole by ethanol injection method. The preparation of liposomes  
407 requires heating at 55°C, above the main transition temperature of Phospholipon 90H,  
408 which could induce the loss of volatile estragole during preparation. This is in line with  
409 H<sub>C</sub> value of estragole as it increased with temperature. The DCL carrier system improved  
410 the estragole encapsulation. Indeed, the EE values of estragole were 8.47 ± 1.21 and  
411 11.36 ± 2.31 % for Est-DCL 75 and Est-DCL 100 respectively and the LR values of  
412 estragole were 1.58 ± 0.25 and 3.25 ± 0.68 % for Est-DCL 75 and Est-DCL 100  
413 respectively. In term of LR, DCL represents a better encapsulation system than CL for  
414 numerous drugs including volatile ones such as *trans*-anethole [28] and non-volatile  
415 compound like betamethasone [36]; celcoxib [37], curcumin [38] and indomethacin [39].  
416 Besides, the amount of estragole in CD/drug inclusion complex solutions increased upon  
417 increasing the HP-β-CD:Est molar ratio from 75:10 to 100:10. Same results were  
418 observed by Maestrelli et al., [40], where the EE values of ketoprofen increased with

419 increasing the concentration of HP- $\beta$ -CD:ketoprofen inclusion complex when  
420 encapsulated in the aqueous phase of liposomes.

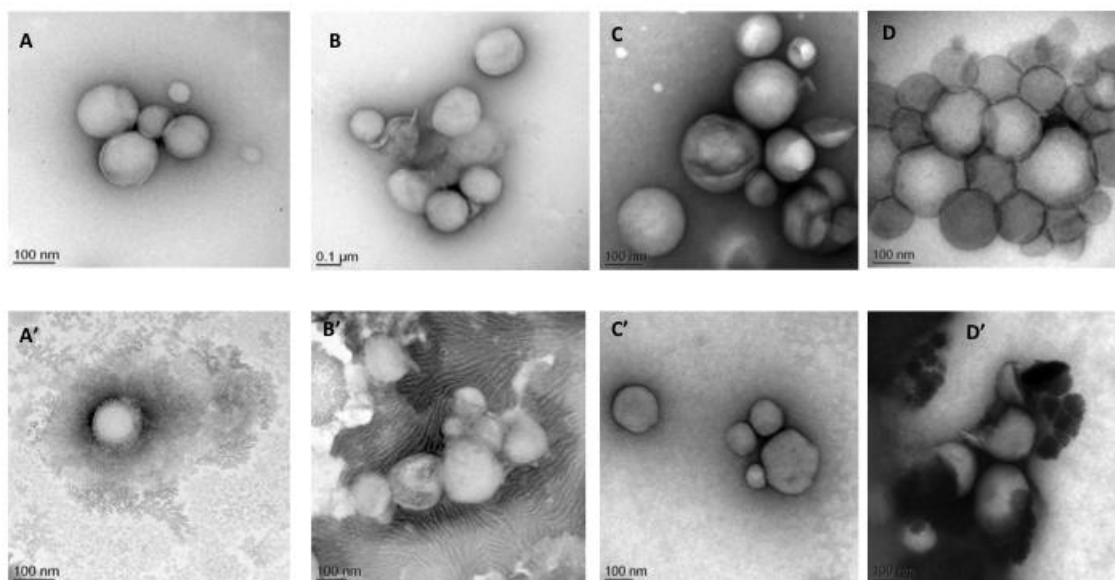
421 After freeze-drying, the LR of estragole in Est-DCL 75 and Est-DCL 100 were quasi  
422 maintained (Table 3).

423

### 424 3.4 Morphology

425

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



433

434 Figure 2: TEM images of Phospholipon 90H liposomes before freeze-drying: blank-DCL 75 (A);  
435 blank-DCL 100 (B); Est-DCL 75 (C) and Est-DCL 100 (D) and after freeze drying: blank-DCL  
436 75 (A'); blank-DCL 100 (B'); Est-DCL 75 (C') and Est-DCL 100 (D').  
437

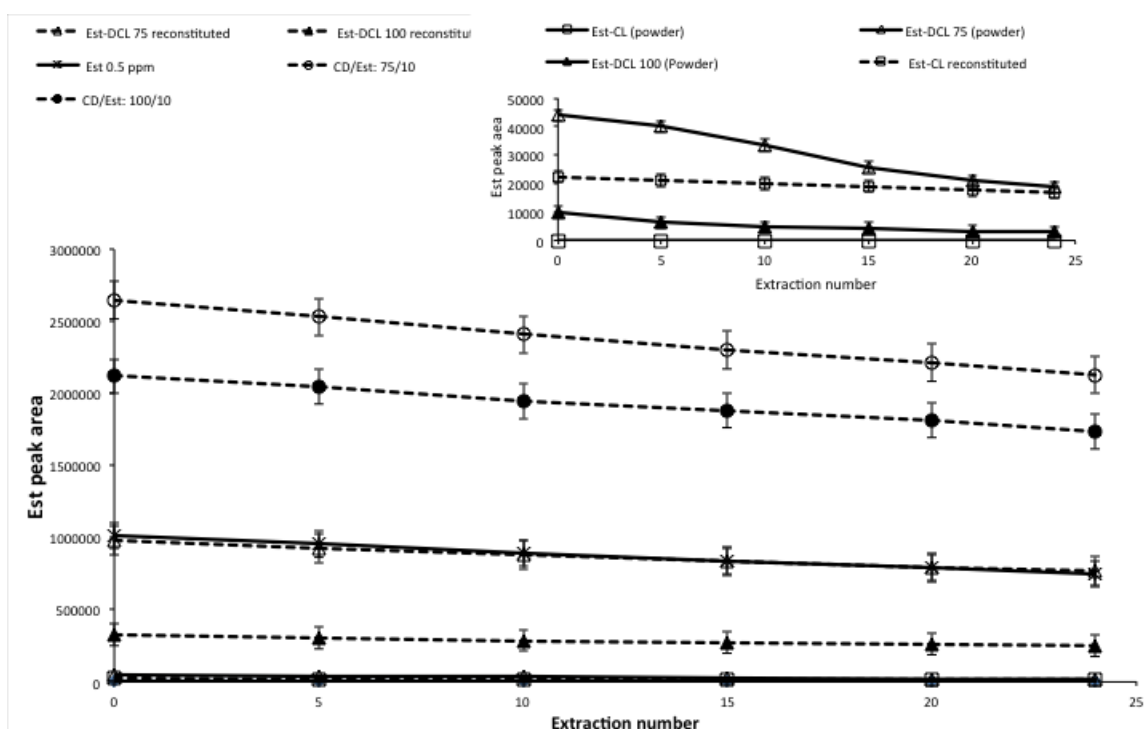
### 438 **3.5 Release studies**

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

449  
450 The release studies of estragole from freeze-dried HP- $\beta$ -CD/Est inclusion complex, Est-  
451 CL and Est-DCL stored at 4°C for 6 months, were performed by the MHE method. The  
452 estragole peak areas of the external standard (0.5 ppm of estragole in water), inclusion  
453 complex HP- $\beta$ -CD:Est (75:10 and 100:10 molar ratios in water) and estragole loaded  
454 liposomes (CL and DCL in powder or reconstituted in water) are illustrated in.

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

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



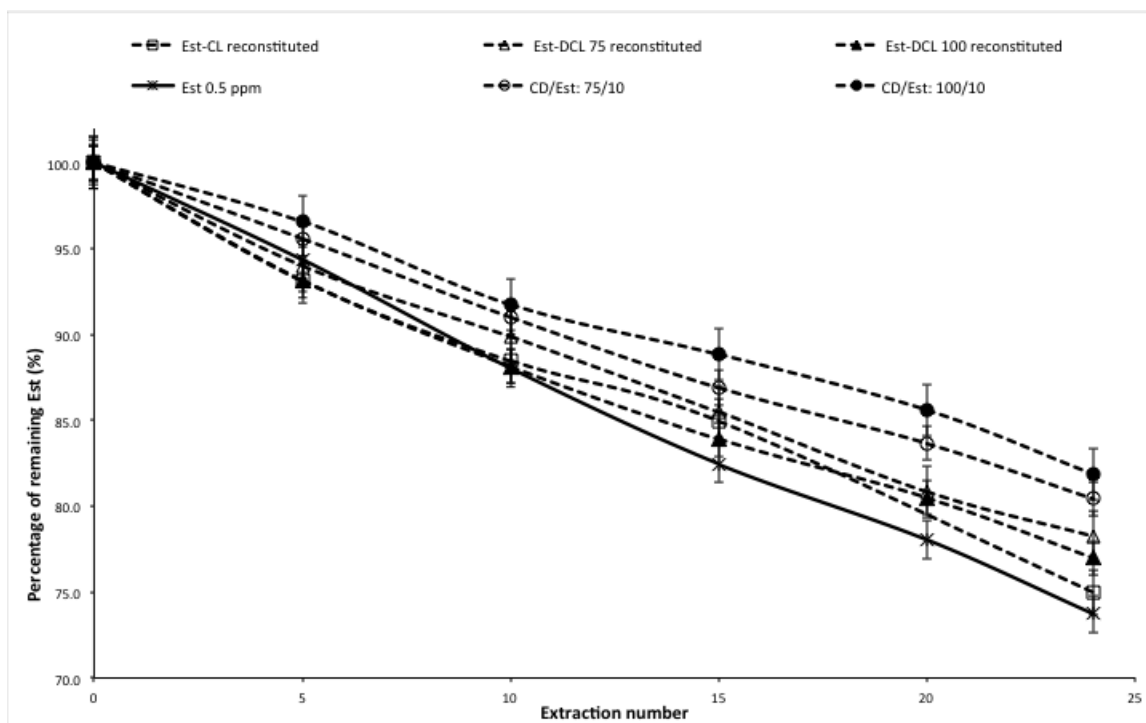
466

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

471

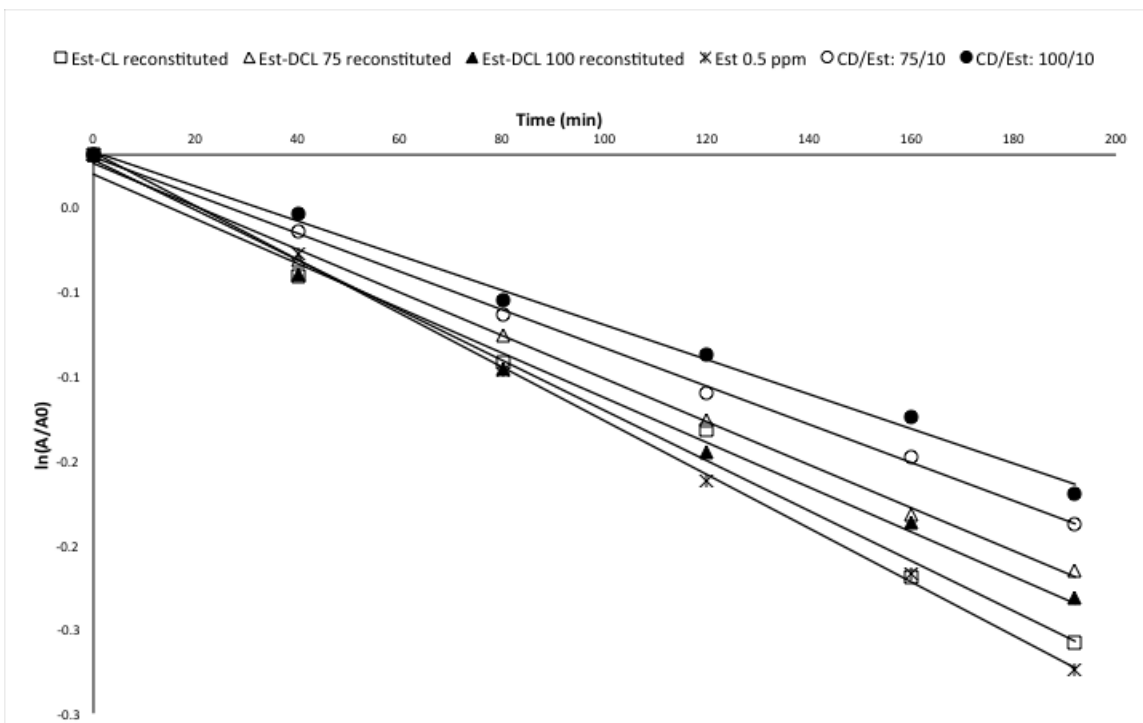
472 Figure 4 resumes the percentage of remaining estragole from the reference solution (0.5  
 473 ppm of estragole in water), HP- $\beta$ -CD inclusion complex (75:10 and 100:10 molar ratio in  
 474 water) and liposomes (CL and DCL reconstituted in water). The remaining percentage of

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- $\beta$ -CD:Est 100:10, HP- $\beta$ -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- $\beta$ -CD:Est (75:10 and 100:10 molar ratios in water) and estragole  
482 loaded liposomes (CL and DCL reconstituted in water).



483  
 484 Figure 5 : The plot of  $\ln (A_t/A_0)$  as a function of time for Est external standard (0.5 ppm of  
 485 estragole in water), inclusion complex HP- $\beta$ -CD:Est (75:10 and 100:10 molar ratios in water) and  
 486 estragole loaded liposomes (CL and DCL reconstituted in water).

487  
 488 The release rate constant was calculated according to equation 7 and the values are  
 489 presented in Table 4. The retention of estragole was improved by 1.6, 1.4, 1.3 and 1.2  
 490 times for HP- $\beta$ -CD/Est 100:10, HP- $\beta$ -CD/Est 75:10, reconstituted Est-DCL 75 and  
 491 reconstituted Est-DCL 100 compared to free estragole (0.5ppm).

492

493 Table 4: Release rate constants of free estragole, HP- $\beta$ -CD/Est inclusion complex, Est-CL and  
 494 Est-DCL formulations

495

496

	<b>Release rate constant K (min<sup>-1</sup>)</b>
Est 0.5 ppm	0.013
HP- $\beta$ -CD/Est 100:10	0.008
HP- $\beta$ -CD/Est 75:10	0.009
Est DCL 75 reconstituted	0.010

Est DCL 100 reconstituted	0.011
Est-CL reconstituted	0.012

497

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

507

### 508 **3.6 Determination of loading capacity of estragole ( $m_{Est}/m_{powder}$ )**

509 The amount of estragole in the inclusion complex obtained at both molar ratios (HP- $\beta$ -  
 510 CD:Est 75:10 and 100:10) and in the reconstituted liposomes was quantified by SH-GC  
 511 after 6 months of storage at 4°C. The LC values expressed as  $m_{Est}/m_{powder}$  and calculated  
 512 using equation 12, are listed in Table 5. The LC value of estragole in Est-CL was 0.01  $\mu$ g  
 513 of Est/mg of powder. The CL formulations contain small amount of estragole, which was  
 514 not detected by HPLC. The encapsulation of estragole in DCL system was improved with  
 515 LC values of 0.6 and 0.2  $\mu$ g of Est/mg of powder for Est-DCL 75 and Est-DCL 100,  
 516 respectively. However, the LC values of estragole in the inclusion complex HP- $\beta$ -CD/Est  
 517 75:10 and 100:10 were 10.8 and 9.3  $\mu$ g of Est/mg of powder, respectively. These values

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

522

523 Table 5: Loading capacity  $m_{\text{Est}}/m_{\text{powder}}$  in HP-β-CD/Est inclusion complex and in Est-CL and  
 524 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

525

### 526 3.7 Fluorescence anisotropy

527 The fluorescence anisotropy measurements of DPH in liposomes give information on  
 528 membrane fluidity of liposomes membrane [42]. The DPH anisotropy values obtained at  
 529 25 and 37°C for blank-CL, blank-DCL 75, blank-DCL 100, Est-CL, Est-DCL 75 and Est-  
 530 DCL 100 before and after freeze-drying are presented in Table 6.

531 Table 6: DPH fluorescence anisotropy measurements for blank-CL, blank-DCL, Est-CL and Est-  
 532 DCL made from Phospholipon 90H and cholesterol at 25 and 37°C before and after freeze-drying.  
 533

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

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

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

536

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

540 Compared to blank-CL, the presence of estragole or the inclusion complex HP- $\beta$ -CD/Est  
541 (75:10 and 100:10 molar ratio) did not affect the DPH anisotropy values at 25 and 37°C.

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

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

552

## 553 **4 Conclusion**

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

557 90H/cholesterol. In addition, the presence of estragole within HP- $\beta$ -CD, CL or DCL  
558 reduced its volatility and allowed its controlled release. The HP- $\beta$ -CD:Est inclusion  
559 complex and Est-DCL system showed high retention of estragole and displayed a good  
560 stability. These results suggest that freeze-dried CD inclusion complexes and DCL  
561 systems could be considered as efficient carriers for essential oil components retention,  
562 preservation, storage and controlled release of estragole.

563

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