



HAL
open science

Retention of Eucalyptol, a Natural Volatile Insecticide, in Delivery Systems Based on Hydroxypropyl- β -Cyclodextrin and Liposomes

Riham Gharib, Jouda Mediouni Ben Jemâa, Catherine Charcosset, Sophie
Fourmentin, Helene Greige-Gerges

► **To cite this version:**

Riham Gharib, Jouda Mediouni Ben Jemâa, Catherine Charcosset, Sophie Fourmentin, Helene Greige-Gerges. Retention of Eucalyptol, a Natural Volatile Insecticide, in Delivery Systems Based on Hydroxypropyl- β -Cyclodextrin and Liposomes. *European Journal of Lipid Science and Technology*, 2020, 122 (5), pp.1900402. 10.1002/ejlt.201900402 . hal-03033940

HAL Id: hal-03033940

<https://hal.science/hal-03033940>

Submitted on 1 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Retention of eucalyptol, a natural volatile insecticide, in delivery systems based on hydroxypropyl- β -cyclodextrin and liposomes

Riham Gharib^{1,2,3}, Jouda Mediouni Ben Jemâa⁴, Catherine Charcosset,² Sophie Fourmentin³, H  l  ne Greige-Gerges^{1*}

¹Bioactive Molecules Research Laboratory, Faculty of Sciences, Doctoral School of Sciences and Technologies, Lebanese University, Lebanon.

²Laboratoire d'Automatique et de G  nie des Proc  d  s, Universit   Claude Bernard Lyon 1, UMR 5007, CNRS, CPE, 43 bd du 11 Novembre, 691622 Villeurbanne Cedex, France

³Unit   de Chimie Environnementale et Interactions sur le Vivant (UCEIV), EA 4492 SFR Condorcet FR CNRS 3417, Universit   du Littoral-C  te d'Opale, 59140 Dunkerque, France.

⁴Laboratory of Biotechnology Applied to Agriculture, National Agricultural Research Institute of Tunisia (INRAT), University of Carthage, Tunisia

*Corresponding author: H  l  ne 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

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

Abbreviations: conventional liposome (CL); cyclodextrin (CD); 1,6-diphenylhexatriene (DPH); encapsulation efficiency (EE); eucalyptol (Euc); drug-in-cyclodextrin-in-liposome (DCL); hydroxypropyl- β -cyclodextrin (HP- β -CD); loading capacity (LC);

loading rate (LR); multiple head extraction (MHE); transmission electron microscope (TEM).

1 **Abstract**

2 Eucalyptol (Euc) is a natural monoterpene with insecticide effects. Being highly volatile
3 and sensitive to ambient conditions, its encapsulation would enlarge its application. Euc-
4 loaded conventional liposomes (CL), cyclodextrin/drug inclusion complex and drug-in-
5 cyclodextrin-in-liposomes (DCL) were prepared to protect Euc from degradation, reduce
6 its evaporation and provide its controlled release. The liposomal suspension was freeze-
7 dried using hydroxypropyl- β -cyclodextrin (HP- β -CD) as cryoprotectant. The liposomes
8 were characterized before and after freeze-drying. The effect of Euc on the fluidity of
9 liposomal membrane was also examined. A release study of Euc from delivery systems,
10 in powder and reconstituted forms, was performed by multiple head extraction at 60 °C
11 after 6 months of storage at 4 °C. CL and DCL suspensions were homogeneous, showed
12 nanometric vesicles size, spherical shape and negative surface charge before and after
13 freeze-drying. Moreover, HP- β -CD did not affect the fluidity of liposomes. CL
14 formulations presented a weak encapsulation for Euc. The loading capacity of eucalyptol
15 in DCL was 38 times higher than that in CL formulation. In addition, freeze-dried DCL
16 and HP- β -CD/Euc inclusion complex showed a higher retention of eucalyptol than CL
17 delivery system.

18 Both carrier systems HP- β -CD/Euc and Euc-loaded DCL decreased Euc evaporation and
19 improved its retention.

20 **1 Introduction**

21 Eucalyptol (Euc) or 1,8-cineole, is a natural monoterpene. It is a major compound of
22 many plant essential oils and is mainly extracted from *Eucalyptus globulus* and
23 *Rosmarinus officinalis* essential oils ^[1].

24 Batish et al. ^[2] pointed out that 1,8-cineole supports the pesticidal activity of *Eucalyptus*
25 oils. Similarly, Scriven and Meloan ^[3] reported that cineole act as a natural repellent
26 against *Periplaneta americana*. Moreover, Corbet et al., ^[4] proved that cineole is a
27 mosquito larvicide. Additionally, Euc is also used as a fumigant agent against insect-pest
28 of stored grains ^[5]. Considering the repellent index, it was reported that *Eucalyptos*
29 *kruseana* oil showed the highest repellency as compared to other essential oils ^[6]. In
30 human, Euc has been used to treat lung inflammation and respiratory diseases and is
31 found as active principle in a commercial medicinal product “Soledum[®] Kapseln”
32 contained pure isolated monoterpene 1,8-cineol ^[1], and it has been demonstrated that Euc
33 showed neuroprotective effects in an ischemic stroke model ^[7].

34 To our knowledge there is no data concerning the stability study of eucalyptol under light
35 and at various temperatures. The product is recommended to be stored at 4°C and in a
36 dark bottle. Generally, chemical reactions are accelerated with increasing heat. Some
37 essential oil components including eucalyptol are known to easily convert into each other
38 by oxidation, isomerization, cyclization, or dehydrogenation reactions, triggered either
39 enzymatically or chemically ^[8]. The chemical oxidation of Euc has been carried out by
40 strong oxidizing reagents to give oxo compounds (3-oxo-1,8-cineole, 5-oxo-1,8-cineole
41 and 3,5-dioxo-1,8-cineole) ^[9]. The sites of oxidation are the carbon 3 and/or 5. The
42 encapsulation of Euc can be considered as a potential solution to overcome the

43 drawbacks related to its physico-chemical properties.

44 Based on the drug-in-cyclodextrin-in-liposomes (DCL) delivery system proposed by
45 McCormack & Gregoriadis ^[10], we developed many formulations loading essential oil
46 compounds to protect them from degradation, to achieve their controlled release, and to
47 facilitate their handling through preparation in freeze-dried forms ^[11, 12, 13]. The DCL
48 system, combining the relative advantages of both carriers, improved the encapsulation of
49 many bioactive compounds such as *trans*-anethole ^[11], eugenol ^[14], estragole ^[13],
50 quercetin ^[15], nerolidol ^[16], and reduced their release compared to conventional liposomes
51 ^[11, 13].

52 Cyclodextrins (CDs) are cyclic oligosaccharides consisted of glucopyranose units. They
53 are non-hygroscopic substances, homogeneous and crystalline ^[17]. CDs have a form of a
54 truncated cone with an internal hydrophobic cavity and an external hydrophilic surface.
55 This particular structure confers to CDs the ability to form inclusion complexes with a
56 large number of hydrophobic guest molecules ^[18]. Among numerous CDs,
57 hydroxypropyl- β -cyclodextrin (HP- β -CD) was shown to be very effective for Euc
58 encapsulation ^[18].

59 Liposomes are microscopic vesicles in which an aqueous volume is entirely enclosed by
60 a membrane. They are usually made of natural, biodegradable, non-toxic and non-
61 immunogenic lipid molecules ^[19]. In aqueous media, the phospholipids can slowly
62 become oxidized or hydrolyzed ^[20] and this could induce liposomes membrane
63 destabilization and leakage of the encapsulated drug. Freeze-drying liposomes increased
64 their shelf-life ^[21]; however during freeze-drying the disruption of liposome membrane
65 may occur leading to vesicles aggregation ^[20]; for that cryoprotecting agents such as HP-

66 β -CD are generally used to stabilize liposome structures during freeze-drying ^[12].
67 In this study, ethanol injection method was applied using hydrogenated soybean
68 phospholipid and cholesterol to prepare conventional liposomes (CL) and DCL. These
69 liposomes were characterized for their size, polydispersity index, zeta potential and
70 morphology before and after freeze-drying. To obtain reproducible and efficient
71 insecticidal effects, the homogeneity and the stability of liposome formulations should be
72 ensured. For that, the particle size distribution value of the batches was determined. Zeta
73 potential is a parameter used to evaluate the physical stability of liposomal formulations.
74 It characterizes the particles surface charge and gives an indication about repulsive forces
75 between particles, thus allowing predicting stability of colloidal dispersions ^[22]. The
76 loading rate of Euc was determined after its HPLC analysis. The release of Euc from the
77 inclusion complex, CL and DCL was studied by multiple headspace extraction at 60°C.
78 Hence, the fluorescence anisotropy of 1,6-diphenylhexatriene (DPH) inserted in the lipid
79 membranes of CLs and DCLs was studied before and after freeze-drying at 25 and 37°C.

80 **2 Materials and methods**

81 **2.1 Materials**

82 Hydrogenated phospholipon 90H (90% soybean phosphatidylcholine, 4%
83 lysophosphatidylcholine, 2% triglycerides, 2% water, 0.5% ethanol, 1% iodine) was
84 supplied by Lipoid GmbH (Ludwigshafen, Germany). HP- β -CD-oral grade (MS=0.85)
85 was obtained from Roquette (Lestrem, France), eugenol, absolute ethanol, cholesterol and
86 methanol-HPLC grade were from Sigma-Aldrich (Germany) and Euc was purchased
87 from Sigma-Aldrich (USA). Water was purified on a Milli-Q system obtained from a
88 Millipore® synergy system (Millipore, Billerica, Massachusetts, USA).

89 **2.2 Preparation of HP-β-CD/Euc inclusion complex**

90 Excess amount of Euc (7.712 mg) was added to 5 mL HP-β-CD solutions (0, 10, 25, 50,
91 and 100 mM). The mixtures were shaken at 25 °C for 24 h then filtered through a 0.45
92 μm membrane filter. The HPLC method described below was used to determine the
93 concentrations of Euc in the filtrates.

94 The encapsulation efficiency was calculated using the following equation:

$$EE_{CD}(\%) = \frac{m Euc_{exp}}{m Euc_i} \times 100 \quad (1)$$

95 where $m Euc_{exp}$ is the mass of Euc experimentally determined by HPLC in the inclusion
96 complex solution and $m Euc_i$ is the mass of Euc initially used to prepare the inclusion
97 complex.

98 **2.3 Preparation of HP-β-CD/Euc inclusion complex for DCL preparations**

99 HP-β-CD (50 mM, 74 mg/mL) was dissolved in ultrapure water and the required amount
100 of Euc was added to obtain HP-β-CD:Euc molar ratio of 5:1. HP-β-CD/Euc inclusion
101 complex was prepared as described above and the solution was used for DCL
102 preparations.

103 **2.4 Preparation of liposomes by ethanol injection method**

104 The liposomes were prepared by the ethanol injection method using Phospholipon 90H
105 (10 mg/mL) and cholesterol (5 mg/mL) according to the protocol described previously
106 ^[11]. Different formulations were prepared: 1) blank-CL; 2) blank-DCL, where HP-β-CD
107 was dissolved in the aqueous phase at a concentration of 50 mM; 3) Euc-loaded
108 liposomes (Euc-CL), where Euc was added in the organic phase at a concentration of 2.5
109 mg/mL; 4) HP-β-CD/Euc inclusion complex-loaded liposomes (Euc-DCL), where HP-β-
110 CD/Euc inclusion complex obtained at molar ratio of 5:1 was used as an aqueous phase.

111 Each batch was prepared in triplicate and underwent characterization as described below.

112 **2.5 Freeze-drying**

113 Freshly prepared liposomes (5 mL) were freeze-dried according to the protocol described
114 in our previous study ^[12], where HP- β -CD was used as a cryoprotector agent at
115 concentrations of 25 and 50 mM for CL and 50 mM for DCL formulations. The obtained
116 powders were then reconstituted with ultra-pure water to the original volume (5 mL)
117 prior to characterization and further analysis.

118 HP- β -CD/Euc inclusion complex was also freeze-dried and studied for its ability to retain
119 Euc as described below.

120 **2.6 Liposome characterization**

121 **2.6.1 Dynamic Light Scattering Analysis**

122 Malvern Zetasizer Nanoseries (Zetasizer Nano ZS; Malvern Instruments Ltd, France) was
123 used to determine the mean size, polydispersity index and zeta potential values of
124 liposome batches ^[12].

125 **2.6.2 Morphological characterization by transmission electron microscopy**

126 Blank-DCL and Euc-DCL before and after freeze-drying were imaged with transmission
127 electron microscope (TEM) (CM 120; Philips, Eindhoven, Netherlands) according to the
128 protocol described by Gharib et al ^[11].

129 **2.6.3 Determination of encapsulation efficiency and loading rate of Euc**

130 The EE of Euc into liposomes was calculated after determination of the concentrations of
131 free and total Euc present in the liposome suspensions by HPLC. Aliquots were removed
132 from the liposomal suspension to determine the total Euc concentration ($[Euc]_{Tot}$). The

133 liposomal suspension underwent ultracentrifugation (Optima™ Ultracentrifuge, Beckman
134 Coulter, USA) at 170000g for 1 h at 4 °C. Aliquots were removed from the supernatant to
135 determine the concentration of free Euc ([Euc]_F). The EE of Euc was calculated as
136 follows:

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

137 where [Euc]_{Tot} and [Euc]_F corresponds to the concentration of total and free Euc,
138 respectively.

139 The LR of Euc was calculated by the following equation:

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

141

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

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

143 where $m_{Initial}$ for Euc-DCL is the initial mass of Euc used to prepare CD/Euc inclusion
144 complex. For Euc-CL, $m_{initial}$ is the initial mass of Euc added to the organic phase during
145 liposome preparation. $m_{reconstituted\ liposomes}$ is the mass of Euc totally found in the liposomal
146 suspension after freeze-drying and reconstitution of liposomes.

147 **2.7 HPLC method**

148 The concentration of Euc was determined by HPLC. Stock standard solutions of Euc (1
149 mg/mL) and of the internal standard, eugenol (1 mg/mL) were prepared in methanol.
150 Aliquots were removed from the Euc stock solution and diluted in methanol to obtain
151 final concentrations of Euc ranging from 100 to 1000 µg/mL. The diluted solution of
152 eugenol (1 µg/mL) was prepared in methanol. Two hundred µL of each filtrate were

153 added to 200 μL of eugenol (1 $\mu\text{g}/\text{mL}$) and 400 μL of methanol. The samples were
154 sonicated for 10 min at room temperature. The samples were analyzed by HPLC (Agilent
155 Technologie 1200 series) using an analytical column C18 15 cm x 4.6 mm, 5 μm ,
156 (Agilent Technologies). The mobile phase was a mixture of methanol and water (70/30).
157 The flow rate was fixed at 1 mL/min and the detection was set at 206 nm.
158 The retention times of eugenol and Euc were 4.3 and 7.3 min, respectively.

159 **2.8 Fluorescence anisotropy measurements**

160 The effect of Euc, HP- β -CD and freeze-drying process on the membrane fluidity of
161 vesicles (blank-CL, blank-DCL, Euc-CL, Euc-DCL), the steady state fluorescence
162 polarization measurements were determined using a Cary Eclipse Fluorescence
163 Spectrophotometer (Agilent Technologies). DPH was dissolved in tetrahydrofuran (4.30
164 10^{-3} mol/L) then an aliquot was added to the vesicles suspension to obtain
165 DPH:phospholipid molar ratio of 1:500. The measurements were conducted following the
166 protocol described previously [23].

167 **2.9 Release of Euc**

168 Multiple headspace extraction (MHE) coupled to gas chromatography was used to study
169 the release of Euc form freeze-dried CL and DCL as well as from reconstituted vesicles
170 after 6 months of storage at 4°C. Freeze-dried powders of Euc-CL, Euc-DCL (10 mg),
171 reconstituted Euc-CL, Euc-DCL in 10 mL water, inclusion complex solution prepared at
172 a molar ratio HP- β -CD/Euc of 5:1, and Euc dissolved in water (0.2 mg/mL) were placed
173 in 22 ml sealed vials. The protocol described in a previous study [13] was used.
174 After equilibrium, the release of Euc at time t was calculated as follows:

175
$$\text{Percentage of remaining Euc} = \frac{A_t}{A_1} \times 100 \quad (6)$$

176 Where, A_t and A_1 corresponds to the area of the chromatographic peak of Euc at time t
177 and at the first extraction, respectively. The plot of $\ln (A_t/A_1)$ as a function of time
178 followed a first-order release kinetics. The release rate constant K was calculated using
179 the following equation:

$$180 \quad \ln (A_t/A_1) = -Kt + b \quad (7)$$

181 **2.10 Loading capacity determination**

182 The amount of Euc in freeze-dried forms of CL and DCL was determined after 6 months
183 of storage at 4°C using MHE. The concentration of Euc was determined according to the
184 protocol developed by Kolb & Etre [24] and used in our previous studies [13].

185 Loading capacity (LC) of Euc is expressed as mg of encapsulated Euc per gram of
186 liposomes.

$$187 \quad LC = \frac{Euc_{exp} (mg)}{masse \text{ of liposomes } (mg)} \quad (8)$$

188 **2.11 Statistical analysis**

189 Statistical analysis was performed using the Student's t-test. P values equal or less than
190 0.05 were considered statistically significant.

191 **3 Results and discussion**

192 **3.1 Determination of the optimal HP- β -CD/Euc inclusion complex**

193 Table 1 presents the encapsulation efficiency of Euc using various HP- β -CD
194 concentrations. EE_{CD} values were calculated using equation 1. It was found that a HP- β -
195 CD concentration of 50 mM was necessary to dissolve the total amount of Euc. Same
196 result was found for anethole, another essential oil component [12]. HP- β -CD:Euc of 5:1
197 molar ratio was used subsequently to prepare DCL formulations.

198 **3.2 Liposomes characterization before and after freeze-drying**

199 **3.2.1 Size, polydispersity index and zeta potential values**

200 CL and DCL liposomal suspension were prepared by the ethanol injection method using
201 hydrogenated phospholipids (Phospholipon 90H) and cholesterol. Table 2 summarizes
202 the size, polydispersity index and zeta potential values of vesicles before and after freeze-
203 drying.

204 Before freeze-drying, the size of blank-DCL, Euc-CL and Euc-DCL showed no
205 significant difference compared to blank-CL, since similar range of size (179 to 201 nm)
206 was obtained. These results demonstrated that the presence of HP- β -CD and/or Euc did
207 not affect the liposome particle size. Same results were reported for encapsulation of
208 anethole ^[11, 12], estragole ^[13] and clove essential oil ^[14] in liposomes composed of
209 hydrogenated phospholipids. HP- β -CD and essential oil components are able to affect the
210 size of liposomes composed of unsaturated phospholipids ^[25]. Besides, polydispersity
211 index values for all batches were less than 0.2, suggesting that liposomes suspensions
212 were homogenous (Table 2). The zeta potential values for the various formulations were
213 negative ranging between (-2 to -9 mV) suggesting that the presence of HP- β -CD or Euc
214 did not affect the zeta potential values.

215 In a previous work, we optimized the freeze-drying of hydrogenated liposomes using HP-
216 β -CD as cryoprotectant for CL and DCLs ^[12]. The results showed that dispersing the
217 pellet of CL in HP- β -CD solution (25 or 50 mM), that of DCL in water or in HP- β -CD
218 solution (10 to 100 mM) protect the structures during freeze-drying. For DCL pellet, HP-
219 β -CD solution was used at the same CD concentration present in the internal aqueous
220 phase of vesicles. The Euc-DCL formulation was prepared using HP- β -CD/Euc molar

221 ratio of 5:1 where HP- β -CD (50 mM) and Euc (10 mM) were used to prepare the
222 inclusion complex. Thus HP- β -CD (50 mM) was used as a cryoprotector for DCL
223 formulations.

224 After freeze-drying, the size, pDI and zeta potential values were maintained proving that
225 the freeze-drying process was well optimized and could be used for a large number of
226 bioactive compounds loaded CL or DCL (Table 2).

227 **3.2.2 Morphology**

228 The TEM images of phospholipon 90H liposomes obtained before and after freeze-drying
229 showed the formation of nanometer-sized vesicles. Figure 1 (A–B) and (C–D) presented
230 the images of blank-DCL and Euc-DCL obtained before and after freeze-drying,
231 respectively. The vesicles appeared to be oligolamellar and spherical in shape. Similar
232 morphology was found for DCLs loading anethole ^[12, 26], estragole ^[13] and eugenol ^[14].
233 The sizes of the vesicles were well below those determined by DLS technique. In this
234 work, the number of TEM images taken for each formulation was not enough to
235 determine the mean particle size for each batch; moreover some factors may affect the
236 results obtained by DLS such as temperature and the viscosity of the sample.

237 **3.2.3 Encapsulation efficiency**

238 The encapsulation efficiency (EE) and the loading rate (LR) of Euc-loaded liposomes
239 (CL and DCL) before and after freeze-drying were calculated using equations (2-4) and
240 the values are listed in Table 3. The concentration of Euc before freeze-drying in Euc-CL
241 was not detected by HPLC analysis. Same results were obtained for estragole ^[13]. The
242 Henry's law constant, previously determined by static headspace–gas chromatography at
243 30°C were of 0.01 and 0.03 for Euc and estragole, respectively. It has been reported that

244 these molecules exhibited higher volatility compared to other essential oil compounds;
245 the Henry's law constant for isoeugenol, pulegone, terpineol, and thymol at 30°C were of
246 0.005, 0.005, 0.001 and 0.003 respectively. The Henry's law constant was increased to
247 0.05, 0.07, 0.008, 0.009, 0.003 and 0.009 for Euc, estargole, isoeugenol, pulegone,
248 terpineol, and thymol respectively upon increasing the temperature from 30 to 60 °C [25].
249 The chromatogram obtained for eucalyptol at 60°C did not shown any additional peak or
250 presence of a degradation product during the time of the analysis (8 min). The
251 volatilization of Euc during the preparation of liposomes at 55°C can explain the dramatic
252 loss of this volatile agent.

253 The Euc-DCL system improved the encapsulation of Euc with EE value $78.24 \pm 2.33\%$,
254 and the LR value was $8.59 \pm 1.61\%$. Hence, DCL improved the encapsulation of different
255 drugs including volatile ones like *trans*-anethole [12], estragole [13]. Some non-volatile
256 compounds like betamethasone [27], celcoxib [28] and curcumin [29] were also better
257 encapsulated in DCL compared to conventional liposomes.

258 The LR of Euc significantly decreased after freeze-drying to 0.81 ± 0.22 for Euc-DCL
259 (Table 3). Also, a remarkable anethole loss from DCL was obtained during freeze-drying
260 process [12]. The vacuum pressure of 100 µbar provokes probably anethole and Euc loss.
261 For that, further experiments should be performed to optimize the freeze-drying of DCL
262 loading highly volatile compounds.

263 **3.2.4 Fluorescence anisotropy**

264 Table 4 showed the DPH anisotropy values obtained at 25 and 37 °C for blank-CL, blank-
265 DCL, Euc-CL and Euc-DCL before and after freeze-drying.

266 The membrane fluidity increased with the temperature in accordance with literature ^[23].
267 Compared to blank-CL, the presence of Euc or the inclusion complex HP- β -CD/Euc did
268 not affect the DPH anisotropy values at 25 and 37 °C. The result obtained for Euc-CL has
269 been expected since Euc was not detected in Euc-loaded CL by HPLC. Gharib, et al ^[30]
270 demonstrated that the presence of monoterpenes (pulegone, terpineol and eucalyptol) at
271 high concentration of monoterpene to phospholipid molar ratios (10%) decreased in a
272 concentration dependent manner the anisotropy value of DPPC membrane at 28, 41 and
273 50 °C. Besides, the presence of HP- β -CD as cryoprotectant did not affect the membrane
274 fluidity, since no changes of the DPH anisotropy values were observed before and after
275 freeze-drying for all batches (Table 4).

276 **3.3 Release studies**

277 The Euc release experiments from freeze-dried HP- β -CD/Euc inclusion complex, Euc-CL
278 and Euc-DCL were conducted at 60°C using the MHE method after 6 months of storage
279 at 4°C. Figure 2 showed the Euc peak areas of the external standard (0.2 ppm of Euc in
280 water), inclusion complex HP- β -CD/Euc, Euc-CL and Euc-DCL (in powder or
281 reconstituted in water). The amount of Euc in powders (CL and DCL) was very low
282 compared to reconstituted liposomes suggesting the high retention of Euc in powder
283 samples. Thus, the powders were dissolved in 10 mL water and placed in sealed vials and
284 analyzed by MHE. Compared to CL, the encapsulation of Euc was better in DCL, while
285 the inclusion complex showed the higher ability to retain Euc (Figure 2). Reconstituted
286 batches were then considered for the release study and for the determination of the LC
287 $m_{\text{Euc}}/m_{\text{powder}}$ of Euc.

288 The remaining percentage of Euc as free drug or when encapsulated in lipid vesicles are

289 illustrated in Figure 3. Surprisingly, the remaining percentage of Euc was lower in CL
290 (58.5%) than in free Euc (78.2%). Thus, CL prepared in our conditions failed to be an
291 effective delivery system for Euc. For the other formulations, the remaining percentage of
292 Euc increased in the order: free Euc (78.2%) > Euc-DCL (82.5%) > HP- β -CD:Euc
293 (85%). Thus the Euc of eucalyptol in DCL system and HP- β -CD:Euc inclusion complex
294 improve its release compared to free Euc.

295 The release rate constant was calculated according to equation 7 and the values are listed
296 in Table 5. The retention of Euc was improved by 1.6 and 1.3 times for HP- β -CD/Euc
297 inclusion complex and Euc-DCL compared to free Euc (0.2 ppm).

298 **3.4 Determination of loading capacity of Euc**

299 The LC values of Euc in HP- β -CD/Euc inclusion complex, Euc-CL and Euc-DCL, after 6
300 months of storage at 4°C, were determined using equation 8 and presented in Table 6. As
301 expected, a small amount of Euc was obtained in Euc-CL with LC value 0.0155 μ g of
302 Euc/mg of powder, since Euc was not detected by HPLC method. However, Hammoud et
303 al ^[25] studied the encapsulation of Euc in liposomes composed of unsaturated soybean
304 phospholipid (Lipoid S100) and the results showed that CL loading Euc had a low
305 loading capacity value of Euc (1.1 μ g of drug/mg of total organic components). The
306 encapsulation of Euc in DCL system was 38 times greater than CL formulations with LC
307 values of 0.6 μ g of Euc/mg of powder (Table 6). However, the higher LC value of Euc
308 was obtained with HP- β -CD/Euc inclusion complex with 4.5 μ g of Euc/mg of powder.
309 These results suggest that DCL system improve the encapsulation of Euc compared to
310 CL. The inclusion complex HP- β -CD/Euc showed the best ability to retain high amount
311 of Euc.

312 **4 Conclusion**

313 In this paper, we demonstrated a successful freeze-drying of Euc loaded Phospholipon
314 90H liposomes where the characteristics of hydrogenated-liposomes (size, pDI, zeta
315 potential and morphology) were maintained after freeze-drying using HP- β -CD as
316 cryoprotectant for CL and DCL systems. HP- β -CD/Euc and Euc-DCL controlled drug
317 release and displayed a good stability after 6 months of storage at 4 °C in powder form.
318 These results revealed that freeze-dried CD/drug inclusion complex and DCL reduced the
319 volatilization of Euc and improved its retention. This work suggests that the encapsulated
320 Euc could be considered as a suitable approach for the insecticide application.

321 **Conflicts of interest**

322 We confirm that there are no known conflicts of interest associated with this publication.

323 **Acknowledgements**

324 The research was supported by the Research Funding Program at the Lebanese University
325 (2018-2020) and the “Agence Universitaire de la Francophonie”, projet PCSI (2018-
326 2020).

References

- [1]. U. R. Juergens, *Drug Res.* **2014**, 64, 638.
- [2]. D. R. Batish, H. P. Singh, N. Setia, S. Kaur, R. K. Kohli, *Z. Naturforsch C. J. Biosci.* **2006**, 61, 465.
- [3]. R. Scriven, C. E. Meloan, *Ohio J. Sci.* **1984**, 84, 85.
- [4]. S. A. Corbet, G. W. Danahar, V. King, C. L. Chalmers, C. F. Tiley, *Entom. Exp. App.* **1995**, 75, 229.
- [5]. J. Mediouni Ben Jemâa, S. Haouel, M. Bouaziz, M. L. Khouja, *J. Stored Prod. Res.* **2012**, 48, 61.
- [6]. A. K. Dhakad, V. V. Pandey, S. Beg, J. M. Rawat, A. Singh, *J. Sci. Food Agric.* **2018**, 98, 833.
- [7]. P. R. Lima, T. S. de Melo, K. M. Carvalho, I. B. de Oliveira, B. R. Arruda, G. A. de Castro Brito, V. S. Rao, F. A. Santos, *Life Sci.* **2013**, 92, 1195.
- [8]. C. Turek, F. C. Stintzing, *Comp. Rev. Food Sci. Food Safety* **2013**, 12, 40.
- [9]. R. Azerard, *ChemPlusChem.* **2014**, doi.org/10.1002/cplu.201300422 .
- [10]. B. McCormack, G. Gregoriadis, *Int. J. Pharm.* **1994**, 112, 249.
- [11]. R. Gharib, L. Auezova, C. Charcosset, H. Greige-Gerges, *Food Chem.* **2017**, 218, 365.
- [12]. R. Gharib, H. Greige-Gerges, S. Fourmentin, C. Charcosset, *Food Chem.* **2018**, 267, 67.
- [13]. R. Gharib, S. Haydar, C. Charcosset, S. Fourmentin, H. Greige-Gerges, *J. Drug Del. Sci. Tech.* **2019**, 52, 794.

- [14]. C. Sebaaly, C. Charcosset, S. Stainmesse, H. Fessi, H. Greige-gerges, *Carbohydr. Polym.* **2016**, 138, 75.
- [15]. J. Azzi, A. Jraij, L. Auezova, S. Fourmentin, H. Greige-Gerges, *Food Hydrocoll.* **2018**, 81, 328.
- [16]. J. Azzi, L. Auezova, P. E. Danjou, S. Fourmentin, H. Greige-Gerges, *Food Chem.* **2018**, 255, 399.
- [17]. J. Szejtli, *Pure Appl. Chem.* **2004**, 76, 1825.
- [18]. M. Kfoury, L. Auezova, S. Fourmentin, H. Greige-Gerges, *J. Incl. Phenom. Macro. Chem.* **2014**, 80, 51.
- [19]. H. Anwekar, S. Patel, A. K. Singhai, *Int. J. Pharm. Life Sci.* **2011**, 2, 945.
- [20]. C. Chen, D. Han, C. Cai, X. Tang, *J. Control. Release* **2010**, 142, 299.
- [21]. A. Wieber, T. Selzer, J. Kreuter, *Europ. J. Pharm. Biopharm.* **2012**, 80, 358.
- [22]. M. M. Domingues, P. S. Santiago, M. A. Castanho, N. C. Santos, *J Pept Sci.* **2008**, 14, 394.
- [23]. R. Abboud, C. Charcosset, H. Greige-Gerges, *J. Membr. Biol.* **2016**, 249, 327.
- [24]. B. Kolb, L. S. Ettre, *Static Headspace–Gas Chromatography: Theory and Practice (Second edition)*, New Jersey: John Wiley & Sons, Inc. Hoboken **2006**
- [25]. Z. Hammoud, R. Gharib, S. Fourmentin, A. Elaissari, H. Greige-Gerges, *Int. J. Pharm.* **2019**, 561, 1610.
- [26]. R. Gharib, H. Greige-Gerges, A. Jraij, L. Auezova, C. Charcosset, *Carbohydr. Polym.* **2016**, 154, 276.

- [27]. G. Piel, M. Piette, V. Barillaro, D. Castagne, B. Evrard, L. Delattre, *Int. J. Pharm.* **2006**, 312, 75.
- [28]. S. K. Jain, Y. Gupta, A. Jain, M. Bhola, *Drug Deliv.* **2007**, 14, 327.
- [29]. S. S. Dhule, P. Penfornis, T. Frazier, R. Walker, J. Feldman, G. Tan, J. He, A. Alb, V. John, R. Pochampally, *Nanomedicine* **2012**, 8, 440.
- [30]. R. Gharib, L. Auezova, C. Charcosset, H. Greige- Gerges, *J. Iran. Chem. Soc.* **2018** , 15, 75.

Figures

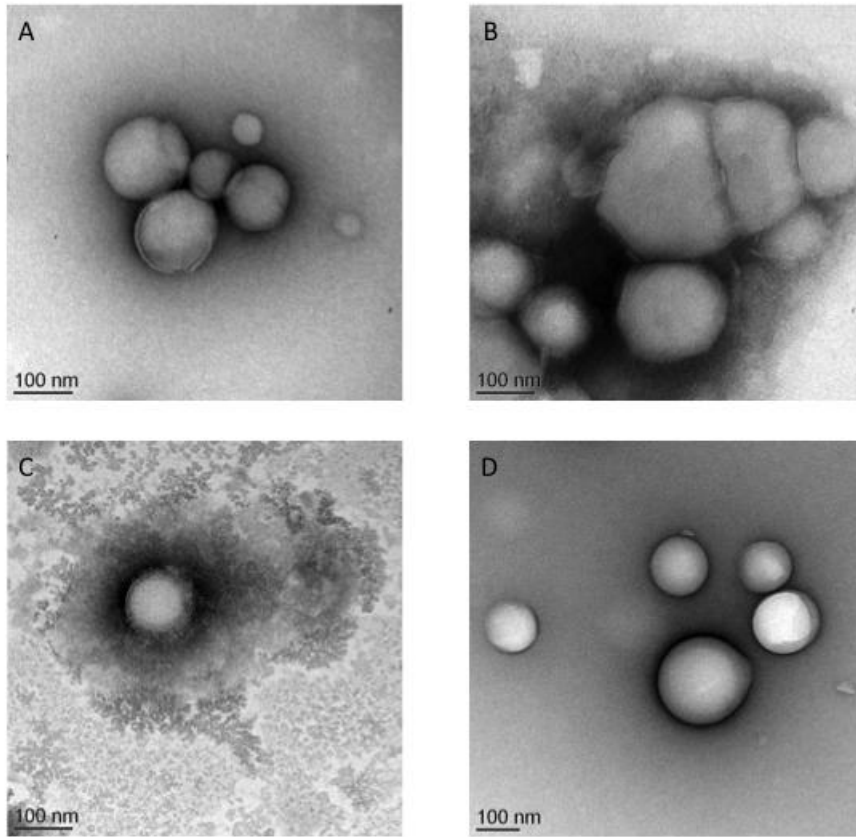


Figure 1: TEM images of Phospholipon 90H liposomes before freeze-drying: blank-DCL 50 (A) and Euc-DCL 50 (B) and after freeze-drying: blank-DCL 50 (C); and Euc-DCL 50 (D).

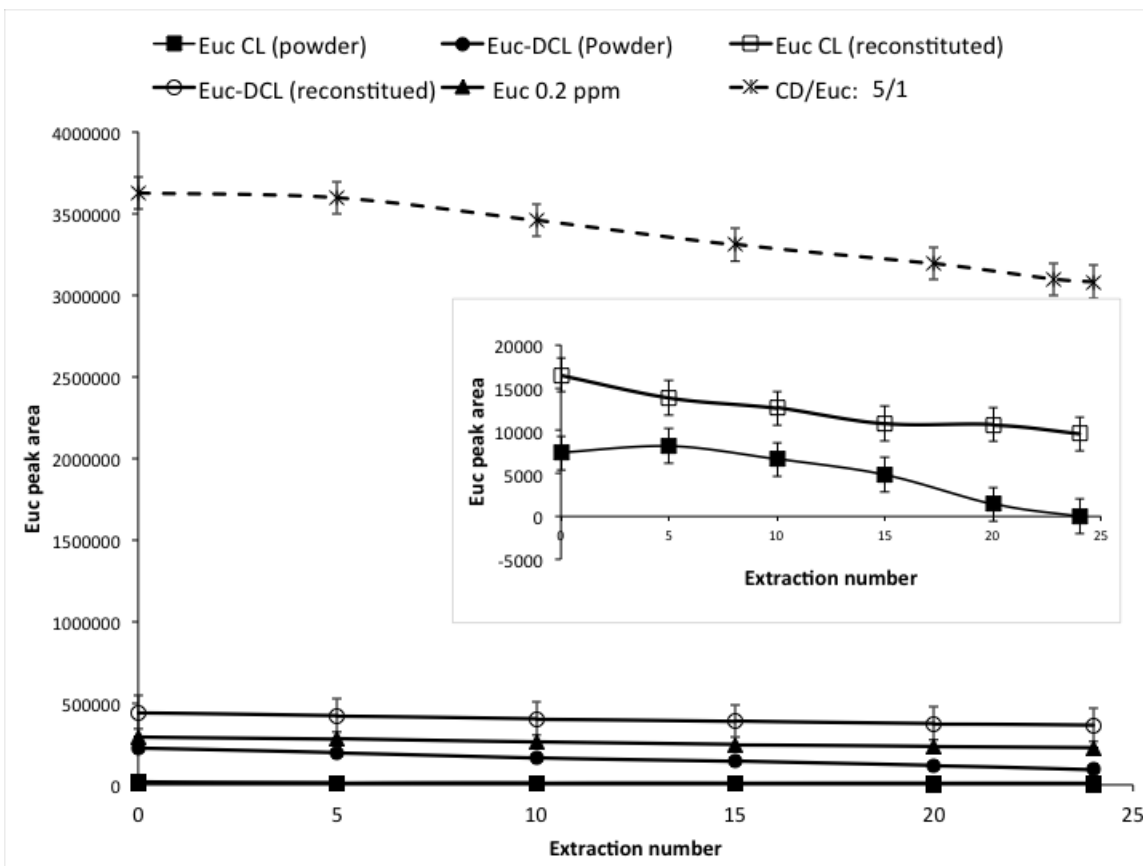


Figure 2: The Euc peak areas of the external standard (0.2 ppm of Euc in water), inclusion complex HP- β -CD:Euc (5:1 molar ratio in water) and Euc-CL and Euc-DCL (in powder or reconstituted in water). The insert represents the peak areas for CL in powder form and reconstituted in water.

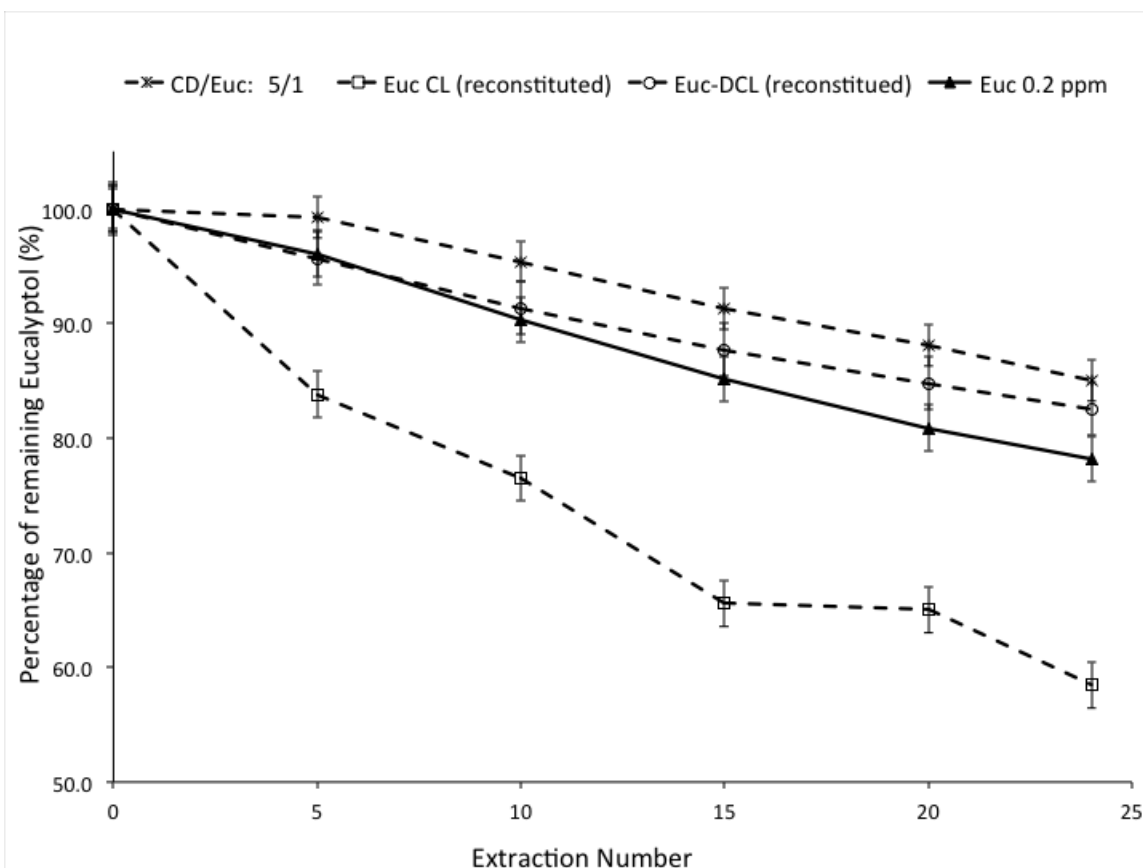


Figure 3: The percentage of remaining Euc from Euc external standard (0.2 ppm of eucalyptol in water), HP- β -CD:Euc inclusion complex and Euc-CL and DCL (reconstituted).

Table 1: The encapsulation efficiency of Euc in HP- β -CD/Euc inclusion complex using different HP- β -CD concentrations.

[HP- β -CD] mM	EE _{CD} (%)
0	72.72 \pm 10.16
10	71.51 \pm 6.95
25	92.78 \pm 5.37
50	98.66 \pm 6.20
100	99.08 \pm 2.2

Table 2: Size, polydispersity index, zeta potential values for fresh and reconstituted Euc-CL and Euc-DCL from Phospholipon 90H before and after freeze-drying.

The values obtained after lyophilization were compared to those before lyophilization.

	Before freeze-drying			Dispersing of the pellet with	After freeze-drying		
	Size (nm)	pdI	Zeta (mV)		Size (nm)	pdI	Zeta (mV)
Blank-CL	179 \pm 9	0.08 \pm 0.01	-9.1 \pm 6.3	CD 25 mM	169 \pm 8	0.22 \pm 0.04	-7.4 \pm 1.7
				CD 50 mM	180 \pm 17	0.15 \pm 0.02*	-5.0 \pm 4.0*
Euc-CL	180 \pm 4	0.09 \pm 0.02	-8.8 \pm 3.5	CD 25 mM	182 \pm 9	0.22 \pm 0.08	-14.8 \pm 4.5*
				CD 50 mM	175 \pm 3	0.15 \pm 0.04	-8.9 \pm 2.1
Blank-DCL	196 \pm 2	0.17 \pm 0.01	-2.4 \pm 2.5	CD 50 mM	193 \pm 4	0.14 \pm 0.03	-14.0 \pm 1.7
Euc-DCL	201 \pm 9	0.20 \pm 0.00	-8.8 \pm 5.6	CD 50 mM	208 \pm 21	0.21 \pm 0.03	-10.1 \pm 5.3

* indicates a value of P < 0.05 statistically significant.

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

	Before freeze-drying		After freeze-drying
	Fresh liposomes		Reconstituted liposomes
	EE (%)	LR _I (%)	LR _{fd} (%)
Euc-CL	ND	ND	ND
Euc-DCL 50	78.24 ± 2.33	8.59 ± 1.61	0.81 ± 0.22*

ND: not detected

- indicates a value of $P < 0.05$ statistically significant.

Table 4: DPH fluorescence anisotropy measurements for blank-CL, blank-DCL, Euc-CL and Euc-DCL made from Phospholipon 90H and cholesterol at 25 and 37°C before and after freeze-drying.

	Before lyophilization		After lyophilization	
	25°C	37°C	25°C	37°C
Blank-CL	0.22 ± 0.0	0.17 ± 0.0	n.d.	n.d.
Blank DCL	0.21 ± 0.0	0.17 ± 0.0	0.22 ± 0.00	0.17 ± 0
Euc-CL	0.22 ± 0.0	0.17 ± 0.0	n.d.	n.d.
Euc-DCL	0.21 ± 0.0	0.18 ± 0.0	0.22 ± 0.01	0.17 ± 0

Values are expressed as the means of three repetitions ± SD; n.d.: not determined
 $P < 0.05$ compared to those before freeze-drying

Table 5: Release rate constants of free Euc, HP- β -CD/Euc inclusion complex, Euc-CL and Euc-DCL formulations

	Release rate constant K (min⁻¹)
Euc 0.2 ppm	0.011
Euc-CL reconstituted	0.021
Euc-DCL reconstituted	0.008
HP- β -CD:Euc complex	0.007

Table 6: Loading capacity of Euc in HP- β -CD/Euc inclusion complex and in Euc-CL and Euc-DCL carrier systems after 6 months of storage at 4°C.

	LC (μg of Euc/mg of powder)
HP- β -CD:Euc	4.5
Euc-CL	0.0155
Euc-DCL	0.6