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

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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.

1 Introduction

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

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

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

drawbacks related to its physico-chemical properties.

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

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

Liposomes are microscopic vesicles in which an aqueous volume is entirely enclosed by a membrane. They are usually made of natural, biodegradable, non-toxic and non-immunogenic lipid molecules ^[19]. In aqueous media, the phospholipids can slowly become oxidized or hydrolyzed ^[20] and this could induce liposomes membrane destabilization and leakage of the encapsulated drug. Freeze-drying liposomes increased their shelf-life ^[21]; however during freeze-drying the disruption of liposome membrane 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).

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

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

The encapsulation efficiency was calculated using the following equation:

$$EE_{CD}(\%) = \frac{m \text{ Euc}_{exp}}{m \text{ Euc}_i} \times 100 \quad (1)$$

where $m \text{ Euc}_{exp}$ is the mass of Euc experimentally determined by HPLC in the inclusion complex solution and $m \text{ Euc}_i$ is the mass of Euc initially used to prepare the inclusion complex.

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

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

2.4 Preparation of liposomes by ethanol injection method

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

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

2.5 Freeze-drying

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

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

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, polydispersity index and zeta potential values of liposome batches ^[12].

2.6.2 Morphological characterization by transmission electron microscopy

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

2.6.3 Determination of encapsulation efficiency and loading rate of Euc

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

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

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

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

The LR of Euc 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 Euc-DCL is the initial mass of Euc used to prepare CD/Euc inclusion complex. For Euc-CL, m_{initial} is the initial mass of Euc added to the organic phase during liposome preparation. m_{reconstituted liposomes} is the mass of Euc totally found in the liposomal suspension after freeze-drying and reconstitution of liposomes.

2.7 HPLC method

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

added to 200 µL of eugenol (1 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 eugenol and Euc were 4.3 and 7.3 min, respectively.

2.8 Fluorescence anisotropy measurements

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

2.9 Release of Euc

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

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

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

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

2.10 Loading capacity determination

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

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

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

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

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

3.2 Liposomes characterization before and after freeze-drying

3.2.1 Size, polydispersity index and zeta potential values

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

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

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

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

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

3.2.2 Morphology

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

3.2.3 Encapsulation efficiency

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

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

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

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

3.2.4 Fluorescence anisotropy

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

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

3.3 Release studies

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

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

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

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

3.4 Determination of loading capacity of Euc

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

4 Conclusion

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

Conflicts of interest

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

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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 ± 10.16
10	71.51 ± 6.95
25	92.78 ± 5.37
50	98.66 ± 6.20
100	99.08 ± 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				After freeze-drying		
	Size (nm)	pdI	Zeta (mV)	Dispersing of the pellet with	Size (nm)	pdI	Zeta (mV)
Blank-CL	179 ± 9	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*
Euc-CL	180 ± 4	0.09 ± 0.02	-8.8 ± 3.5	CD 25 mM	182 ± 9	0.22 ± 0.08	-14.8 ± 4.5*
				CD 50 mM	175 ± 3	0.15 ± 0.04	-8.9 ± 2.1
Blank-DCL	196 ± 2	0.17 ± 0.01	-2.4 ± 2.5	CD 50 mM	193 ± 4	0.14 ± 0.03	-14.0 ± 1.7
Euc-DCL	201 ± 9	0.20 ± 0.00	-8.8 ± 5.6	CD 50 mM	208 ± 21	0.21 ± 0.03	-10.1 ± 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