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1 Optimization of the freeze-drying process for microemulsion systems.

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1 **Abstract:** The aim of this work was to develop a freeze-dried microemulsion using Design of
2 Experiment and loaded with Amphotericin B, which is quite unstable in aqueous media, as a
3 drug model. Different types of cryoprotectants were studied. The microemulsion containing
4 Maltose at 5% (w/w), frozen at the temperature of $-80\text{ }^{\circ}\text{C}$, and performed with a 24h of
5 freeze-drying time yielded the best results. The freeze-drying process reduced the
6 microemulsion droplet size and don't change the AmB content. Therefore, microemulsions
7 containing Maltose are suitable for drug incorporation and the freeze-drying was able to
8 enhance the drug stability in the system.

9 **Keywords:** Amphotericin B; statistical experimental design; lyophilization; dispersed system.

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1 **1. Introduction**

2 Colloidal drug delivery systems are become more attractive in pharmaceutical technology
3 because they are able to control drug release and to improve bioavailability^[1]. Microemulsions
4 (MEs), optically transparent systems with low viscosity, are thermodynamically stable
5 dispersions of two immiscible liquids, stabilized by an interfacial film of a surfactant, usually in
6 combination with a co-surfactant^[2]. They have shown several advantages over conventional
7 formulations, including enhanced drug solubility, good thermodynamic stability, an increased
8 surface area improving absorption and enhancement of transdermal passage^[3]. These
9 properties give MEs potential applications in the food, cosmetic and pharmaceutical industries
10 when solubilization of lipophilic or hydrophilic ingredients is necessary^[4]. In pharmaceuticals,
11 MEs are used as vehicles to deliver a number of drugs due to their thermodynamic stability,
12 simple preparation and good appearance^[2].

13 Amphotericin B (AmB) is the drug of choice for the treatment in immunodeficient patients
14 affected by systemic fungal infections^[5]. However, due to the high incidence of adverse drug
15 reactions, i.e. cardiotoxicity and hepatotoxicity of conventional formulations^[6], AmB has been
16 incorporated into a number of colloidal drug delivery systems, including MEs, in order to
17 improve its therapeutic index^[7, 8].

18 Generally, pharmaceutical MEs are produced with water as the continuous phase, which
19 carries some risks such as microbiological contamination, degradation by hydrolysis and loss of
20 pharmacological activity of the drug. A possible solution to these problems consists of the use
21 of freeze-drying process, also known as lyophilization. Freeze-dried products have good
22 stability and are easy to transport and store^[9]. However, the freeze-drying process for MEs has
23 rarely been studied in pharmaceutical technology due to the difficulties that arise during
24 freezing of colloidal systems. Lyophilization consists in removing water by sublimation
25 through three steps: freezing, primary drying and secondary drying^[10]. During this process

1 stress may be generated that could destabilize the colloidal structure of the MEs, in particular
2 the freezing stage, during which the crystallization of ice may produce mechanical stress.
3 However, cryoprotectants (CP) can be used in order to protect these systems against damage
4 ^[11]. Among the compounds that can exert cryoprotective effects, carbohydrates are interesting
5 because are chemically innocuous and can be easily vitrified during freezing, supporting their
6 use as CPs in the freeze-drying process ^[8].

7 The freeze-drying process has been used for several years in the pharmaceutical and
8 biotechnology industries, leading to products destined for various administration routes such as
9 parenteral, oral, nasal or pulmonary. However, the freeze-drying of colloidal systems is delicate
10 due to the tendency of the droplets to interact and due to the damage caused by the freezing
11 process, which can increase the droplet size and destabilize the system ^[9, 12]. In fact, there are
12 very few reports in the literature of the successful freeze-drying of colloidal systems such as
13 ME ^[12, 13].

14 The quality of the final freeze-dried product can be influenced by several factors relating to
15 the formulation, to the container, to the equipment and to the freeze-drying process ^[9].
16 Therefore, the process efficiency must be optimized through adjustment of these factors.
17 Indeed, process monitoring is crucial in order to obtain a product with the desired quality. It is
18 necessary to select suitable freeze-drying parameters, evaluate the effects of these parameters
19 and their possible interactions with the entire process. Therefore, Design of Experiment (DOE)
20 has been used for the optimization, modeling and characterization of the freeze-drying process
21 of some pharmaceutical products that are not colloidal systems ^[14].

22 DOE is a statistical approach used to determine the influence of several independents
23 variables on the dependent variable of the process. The optimal design allows the time and cost
24 of the experimentation to be reduced, as well as improving the process yield ^[15]. Therefore, this
25 method is used much more often than the One Factor at a Time Method, which is

1 time-consuming and expensive because it requires a large number of experiments and does not
2 examine interactions between the variables ^[16]. The response surface methodology is a
3 technique of DOE that combines mathematics and statistics to analyze the relative significance
4 of different parameters, finding the optimal working conditions, by combining a small number
5 of variables, resulting in fewer experiments ^[17].

6 The aim of this work was to develop a freeze-dried ME product as a model to design
7 freeze-dried ME systems containing drugs and, thereby, improve their stability. Therefore,
8 AmB was used as a model drug incorporated within the ME. A DOE approach was used in
9 order to establish the ideal CP content and the technical procedure to produce freeze-dried
10 colloidal systems. Important parameters, which can influence the droplet size of this system,
11 such as concentration and type of CP (Mannitol (MN), Glucose (GC), Lactose (LT) Sorbitol
12 (ST) and Maltose (MT)), freezing temperature and freeze-drying time were also evaluated.

13 **2. Materials and Methods**

14 **2.1 Materials**

15 Miglyol[®] 812 was obtained from CONDEA Chemie GmbH (Hamburg, Germany), Lipoid[®]
16 S100 was purchased from LIPOID GMBH (Ludwigshafen, Germany), Tween[®] 80, Mannitol
17 (MN), Glucose (GC), Lactose (LT) Sorbitol (ST) and Maltose (MT) were obtained from Sigma
18 Aldrich Inc (St. Louis, USA). The Na₂HPO₄ and the NaHPO₄, used to produce phosphate buffer
19 pH 7.4, were purchased from Vetec Química Fina Ltda (Rio de Janeiro, Brazil). Ultra-pure
20 water obtained by a Milli Q water purification system (Merck Millipore, Massachusetts,
21 U.S.A.) was used along the experiments.

22 **2.2 ME preparation**

23 The MEs were prepared by mixing 68% (w/w) of phosphate buffer pH 7.4, 14.7% (w/w) of
24 Tween[®] 80, 6.3% (w/w) of Lipoid[®] S100 and 11% (w/w) of Miglyol[®] 812 using magnetic

1 stirring, following by three cycles of sonication (40 watts power output for 1.5 min) using a
2 Vibra Cell 75041 (Bioblock scientific) and ultrasound bath (120 watts power for 3 min) using a
3 USC-1800A (Unique). The MN, MT, GC and LT were added to the MEs prior to the
4 freeze-drying process for the DOE study.

5 **2.3 Sample characterization**

6 *2.3.1 Macroscopic aspect, pH evaluation, isotropy and conductivity analysis*

7 The color and homogeneity of the freeze-dried products (cake) were evaluated with the
8 naked eye. The isotropy of the samples was evaluated by polarized light microscopy using an
9 Olympus BX4 (Olympus Corporation, Tokyo, Japan) apparatus. The electrical conductivity
10 was measured using a DM-32 conductivity meter (Digicrom Analytical, SP, Brazil), with a cell
11 constant of 0.11 cm^{-1} . The pH values were measured by a PG-2000 pHmeter (GEHAKA, SP,
12 Brazil). All analyses were performed in triplicate at $25 \pm 2 \text{ }^\circ\text{C}$.

13 *2.3.2 Dynamic Light Scattering (DLS)*

14 In order to evaluate the influence of the different CP types and concentration on the ME
15 droplet size before the DOE study, MT, MN, GC, LT and ST, **as a powder**, were **added (and**
16 **dissolved)** to the MEs at 5% **(w/w)**, 10% **(w/w)**, 15% **(w/w)** and 20% **(w/w)** and the droplet size
17 of all preparations was analyzed. The samples were previously diluted with water in a ratio of
18 1:20 and their droplet size distribution was evaluated using a DLS, ZetaPlus (Brookhaven
19 Instruments Corporation, NY, USA). Previous experiments revealed that this dilution process
20 has no influence on the DLS measurements.

21 *2.3.3 Differential Scanning Calorimetry (DSC)*

22 In order to characterize the type of system (water-in-oil, bicontinuous or oil-in-water) ^[18],
23 the thermal behavior of the ME and its components were analyzed by DSC using a DSC-60

1 Shimadzu (Shimadzu Scientific Instruments, Kyoto, Japan). The samples (5-15 mg) were
2 weighed into aluminum pans. The thermal analyses were performed by a cooling step on a
3 TAC-60i system (Shimadzu Scientific Instruments, Kyoto, Japan) at a predetermined rate from
4 25 °C to -40 °C. After 10 min at -40 °C, the samples were heated at 5 °C/min to 110 °C. A flow
5 of nitrogen atmosphere of 50 ml/min was used.

6 *2.4 DOE methodology for freeze-drying conditions*

7 In order to determine the optimum parameters for the freeze-drying process, the DOE
8 methodology was performed for ME systems for which no changes in droplet size were
9 observed after CP incorporation. The freezing temperature (x_1), the CP type and concentrations
10 (x_2), and the freeze-drying time (x_3) were chosen as independent variables. The ME droplet size
11 after freeze-drying process, on the other hand, was chosen as the dependent output response
12 variable. The experimental levels of independent variables for ME droplet size after
13 freeze-drying are given in Table 1.

14 **Table 1.**

15

16 The levels of the variables, the CP concentration and the freeze-drying time were chosen in
17 accordance with the values commonly found on the literature. On the other hand, the freezing
18 temperature used was selected according to the temperature attained by the use of liquid
19 nitrogen (-196 °C – the minimum temperature), the temperature produced by a regular freezer
20 (Frost Free 260 (Brastemp, São Paulo, Brazil)) (-20 °C – the maximum temperature) and the
21 temperature produced by a ultra-freezer Glacier NU-9438 ULT Freezer (NuAire, Inc.,
22 Minnesota, U.S.A.) (-80 °C – which is the DOE replicated point). A two-level three-factor
23 full-factorial design with three replicated points leading to 8 experimental randomizations runs
24 was performed for each CP (Table 2).

1 **Table 2.**

2 Freeze-drying was performed using a Christ Alpha 1-2 LD freeze-dryer (Martin Christ
3 Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). After the freeze-drying
4 process, the MEs were reconstituted by adding the same amount of water as that lost during the
5 process, followed by vortex mixing and one cycle of sonication (40 watts power output for 1.5
6 min), after which the droplet size was measured by DLS.

7 The effect of the studied variables was graphically and statistically interpreted using the
8 Statistic software version 7.0 (StatSoft Inc., Oklahoma, USA). Based on the statistical results,
9 the parameters that would produce a dry powder ME after freeze-drying and that would present
10 the smallest droplet size values after reconstitution were determined. Thereafter, the ME
11 systems that showed the best aspect and concentration of CP, and the best freezing temperature
12 and the freeze-drying time, was produced and thermodynamically characterized for their pH,
13 conductivity and droplet size before and after the freeze-drying process. The optimized
14 formulation was then used for further studies, including drug loading.

15 ***2.5 Incorporation of AmB to the ME system***

16 AmB was incorporated, as a model drug, into the optimal ME system determined by the
17 DOE methodology. The aim was to observe the efficiency of the freeze-drying process on the
18 production of dried ME systems containing drugs. The production of a dried powder for this
19 system was verified and pH, conductivity and droplet size before and after freeze-drying were
20 measured. Furthermore, the influence of drug loading on the thermodynamic stability of the ME
21 was evaluated.

22 The incorporation of AmB was performed by adding the drug to the ME to a final
23 concentration of 2×10^{-3} M, under continuous stirring. In order to improve the AmB
24 dissolution, the pH of the ME was increased ($\text{pH} \geq 10.0$) by the addition of sodium hydroxide

1 solution (NaOH 1 N). Afterwards, the pH was reduced using hydrochloric acid solution (HCl 1
2 N) to physiological pH (pH 7.4) [7].

3 **2.6 Spectrophotometric analysis**

4 A spectrophotometric assay was performed to analyze and compare the amount of AmB
5 before and after the freeze-drying process, and to evaluate its stability. The experiment was
6 carried out using a Biochrom Libra S32 UV-VIS spectrophotometer (Biochrom US,
7 Massachusetts, USA), at the wavelength of 405 nm, by measuring the absorbances of the
8 reference solution (methanol) and of the ME with and without AmB. The assay was performed
9 in triplicate. The ME samples were first diluted with DMSO: methanol (1:9) at the ratio of 1:10
10 and, then, 50 μ L of this solution was diluted with 20 ml of methanol. The drug loading
11 efficiency was calculated by comparing the AmB concentration before (AmBb) and after
12 (AmBa) centrifugation (Centrifuge 5410, Eppendorf, Hamburg, Germany), as shown in Eq. (1).
13 The method was performed in triplicate by centrifuging 1 ml of ME with and without AmB at
14 14,000 g for 20 min to precipitate any insoluble content. Moreover, an aliquot of 100 μ L of the
15 supernatant of each sample was used to evaluate the AmB content as previously described.

$$16 \quad \text{Loading efficiency} = 100 \times \frac{AmBa}{AmBb} \quad (1)$$

17 **2.7 Statistical Analysis**

18 The statistical tests used in the experiments were the Analysis of Variance (ANOVA)
19 performed to analyze the statistical significance between 3 groups, followed by the Dunnet
20 post-test, to compare with the control or the Bonferroni to compare all samples. Finally, a
21 paired t-test was used in case of 2 unpaired groups. p values less than 0.05 ($p < 0.05$) were
22 considered to indicate significance.

23 **3. Results and Discussion**

1 *3.1 Characterization of the ME system before adding the CP*

2 *3.1.1. Macroscopic aspect, pH evaluation, isotropy and conductivity analysis*

3 The ME, a homogeneous and clear yellowish product that appeared dark under
4 cross-polarized light microscopy (no birefringence), was classified as an isotropic system. Its
5 pH value was 7.1 ± 0.09 , which is considered physiologically acceptable.

6 In general, MEs can be divided into 3 types: water-in-oil (W/O), bicontinuous, and
7 oil-in-water (O/W)^[19]. A correlation between ME structure and electrical conductivity has been
8 demonstrated and can be used as a tool to assess its properties^[20]. The electrical conductivity of
9 the studied ME was $832 \pm 22 \mu\text{S cm}^{-1}$. It is well known that O/W MEs have conductivity values
10 similar to the aqueous phase^[21, 22]. This high value is probably due to the large volume
11 percentage of water in the system, from which can be inferred that the ME is of the O/W type^{[23,}
12 ^{24]}.

13 *3.1.2. DSC analysis of ME components*

14 The thermal behavior of the ME and its individual components (phosphate buffer solution
15 alone, oil phase and surfactants) was evaluated by DSC analysis (Figure 1A and Figure 1B).
16 Figure 1A shows thermal events associated with the ME: a large exothermic peak at $-13.54 \text{ }^\circ\text{C}$
17 during the cooling curve, which was due to water freezing, following by an endothermic peak at
18 $4.26 \text{ }^\circ\text{C}$ during the heating curve explained by the melting of water. A second, broad
19 endothermic peak for ME at a temperature between $50 \text{ }^\circ\text{C}$ and $100 \text{ }^\circ\text{C}$ with an enthalpy value of
20 -1.57 kJ/g probably represents water loss. It was also possible to observe small exothermic
21 peaks for Tween[®] 80 and Miglyol[®] 812, following by only one endothermic peak for Miglyol[®]
22 812.

23 **Figure 1.**

1 Since it represents the freezing of water during the cooling curve, the position of the
2 exothermic peak may indicate the state of water in the ME. In fact, the strong interaction among
3 water and surfactant molecules compared to the weaker interactions among the water molecules
4 themselves or water molecules and phosphate buffer may induce a large change in the
5 temperature at which the water freezes ^[25]. The exothermic peak on Figure 1A indicates the
6 freezing of water with less molecular interaction with surfactant molecules, since the onset
7 freezing temperature of ME is approximately, at -18 °C, which is near to that of phosphate
8 buffer solution (-19.09 °C). Moreover, no peaks characteristic of Lipoid or Tween[®] 80 were
9 observed in the ME thermal profile, which suggests not only a strong interaction between the
10 surfactants and other components of the system, but also that they could be located at the
11 internal phase or at the interface of the ME droplet and not in contact with the continuous phase.

12 Therefore, the results of DSC measurements lead to the conclusion that water is the
13 continuous phase ^[3], thus the ME is of the O/W type, confirming the results of the electrical
14 conductivity.

15 *3.1.3. Droplet size evaluation after addition of CP*

16 Based on the hypothesis that ME stability could be modified by the type and concentration
17 of the CP used, while, on the other hand, the CP could affect both the droplet size distribution
18 and the freeze-drying process, the influence of different CP types and concentrations in the ME
19 systems were evaluated.

20 Macroscopically, all the ME systems, with and without CP, were clear, isotropic and
21 homogeneous. On the other hand, those containing LT and the MN presented a turbid aspect
22 when used at 15% (w/w) and at 20% (w/w), respectively, showing that these additives are not
23 very soluble in the ME system.

24 The influence of CP addition on the droplet size can be observed in Figure 2. MEs showed
25 an average diameter of 22 nm that do not change with the addition of MT, MN, GC and LT

1 ($p>0.05$). However, in the case of ST, the addition of even a small amount caused an increase in
2 the ME droplet size (28 nm) and with the highest concentration it reached 62 nm. Those
3 variations, compared with the ME without CP, were statistically significant ($p<0.05$).

4 **Figure 2.**

5 Therefore ST was excluded from the DOE, while the other CPs: MN, MT, GC and LT, at
6 concentrations from 5% (w/w) to 20% (w/w) may be used to protect MEs during the freezing
7 and drying steps required for the freeze-drying process, since they did not cause significant
8 changes in the ME droplet size.

9 **3.2 DOE for freeze-drying parameters**

10 It has known that many factors, mainly related to the process, can influence the quality of
11 the freeze-dried product. On this study three of them were evaluated: the freezing temperature,
12 the freeze-drying time and the cryoprotectant concentration. **The analysis of** the freezing
13 temperature is important because small and numerous ice crystals are formed using fast
14 freezing whereas larger and less numerous crystals are produced when applying slow freezing.
15 Such phenomena influence the further freeze-drying steps, since diffusivity is related to the
16 pore size (the movement of water vapor are facilitated in large ice crystals). On the other hand,
17 the freeze-drying time also influences on the final product. Indeed, if the time is not sufficient,
18 the water would not evaporate properly, and the product will collapse. Furthermore, the
19 cryoprotectant type and concentration is a key factor to be evaluated since these components
20 could avoid the droplet aggregation or fusion during the freezing. Also, it could avoid phase
21 transition of hydrated phospholipid during the drying steps. Therefore, a suitable amount and
22 type of cryoprotectant is mandatory to stabilize the system ^[12].

23 Among the samples submitted to the freeze-drying process, those containing MT and LT
24 produced dry powders. Disaccharides, the class to which these CPs belong, are more effective

1 freeze-drying protectants than other types of molecule because they are able to form an
2 amorphous sugar glass ^[9].

3 Although the ME-LT at the lower level (5% (w/w)) produced a powder, it did not appear to
4 be completely dry. This is probably because the concentration of CP was not high enough to
5 completely stabilize the ME ^[26]. On the other hand, all MEs containing MT produced dry
6 powders, showing the high capacity of this CP to stabilize freeze-dried MEs.

7 The use of the monosaccharide GC resulted in freeze-dried cakes, which might be
8 generated due to the primary drying temperature, during the freeze-drying process, being above
9 the glass transition temperature (T_g) (transition temperature range in which an amorphous
10 sample in the glassy state change to a rubbery state or the reverse ^[27]) of the MEs containing
11 GC, causing the collapse of the product ^[28].

12 Only the ME containing MN at 20% (w/w), frozen at the highest level (-196 °C), yielded
13 dry powders after freeze-drying, probably because only this MN concentration was sufficient to
14 stabilize the system while the rapid cooling inhibited the MN crystallization ^[29]. However, at
15 this concentration, this CP was not soluble in the system and after a period of time at room
16 temperature the dry powder changed to a rubbery state, revealing that MN was not the best
17 choice as CP. The MEs containing MN at the other DOE levels (5% (w/w) and 12.5% (w/w))
18 did not produce powders, probably due to the tendency of MN to partly crystallize into MN
19 hemi-hydrate. This could release water on conversion to the anhydrous crystal form and,
20 thereby, cause phase separation in the cryo-concentrated portion of the frozen samples,
21 resulting in the loss of CP activity and destabilization of the system ^[16, 30].

22 Formulations in which a dry powder was generated, ME containing MN 20% (w/w) and all
23 samples of MEs containing MT and LT, were reconstituted with the same amount of water as
24 that lost during the freeze-drying process and presented a Winsor IV ME system. Moreover, the
25 droplet sizes of these samples were evaluated. Also, a Pareto's Chart, to investigate the

1 standardized effect of the independent variables and their interaction with the ME droplet size,
2 was constructed. The results for MEs containing MT showed that the “square freezing” is the
3 most important factor influencing the droplet size. The negative coefficients for the square
4 freezing variables indicated an unfavorable or antagonistic effect on the droplet size. Therefore,
5 the closer to the lower limit (-1 level) of the square freezing temperature (-20 °C), larger the
6 droplet sizes. In other words, the higher the freezing temperatures, the larger the ME droplet
7 sizes (Figure 3a) ^[31].

8 Freezing is the stage at which most of the water is separated from the solutes to form ice
9 and concentrated solution ^[10, 32]. In the specific case of MEs, the “concentrated solution” is
10 composed of oil, surfactants and buffer salts. Increasing the concentration may enhance the
11 interaction between the droplets, leading to their aggregation or fusion and increasing the
12 droplet sizes ^[26].

13 The Pareto’s Chart analysis of the MEs containing LT (Figure 3b) showed positive
14 coefficients, for model components (x_2 , x_3 , x_2x_3 , x_1x_3), and a synergistic or favorable effect on
15 the droplet size. Additionally, the CP concentration was the most significant variable for droplet
16 size variation ^[31].

17 **Figure 3.**

18 Date et al. also observed that the droplet size of MEs increased with CP concentration ^[33].
19 Additionally, Abdelwahed et al. demonstrated that certain amount of CP was necessary to
20 effectively preserve the droplet size and ensure a maximum stabilization of nanoparticulate
21 systems ^[9].

22 An empirical second order polynomial equation was developed for the response variable,
23 droplet size, in terms of the three independent variables. This equation for MEs containing MT
24 is expressed by the Eq. (1), and for MEs containing LT by the Eq. (2) in which y_i ($i=1, 2$)

1 represents the response variable (droplet size). The coded values of the test variables are
2 represented by x_1 (freezing), x_2 (CP concentration) and x_3 (freeze-drying time), respectively.

3 $y_1 = 23.4 + 6.11x_1^2$ Eq. (1)

4 $y_2 = 30.93 + 9.86x_2 + 4.19x_3 + 2.26x_1x_2 + 3.19x_1x_3 + 4.16x_2x_3$ Eq. (2)

5 The statistical significance, evaluated using the Fisher's F-test and ANOVA, showed,
6 based on probability values inferior than 0.05, that it was indicated for the quadratic term of
7 freezing (x_1^2) in MEs containing MT. On the other hand, MEs containing LT presented
8 statistical significance for concentration (x_2), freeze-drying (x_3), interaction between them
9 (x_2x_3), and interaction between freezing and freeze-drying (x_1x_3). Furthermore, the ANOVA
10 results (Table 3) for both MEs showed that the calculated F value was found to be greater than
11 the tabulated F value at the CP 5% (w/w) level, indicating that the treatment combinations are
12 significant. Moreover, concerning the lack of fit and pure error, the calculated $F < \text{tabulated } F$
13 demonstrated that the model was predictive^[17]. The value of the coefficient of determination
14 for MEs containing MT and LT were, respectively, $R^2 = 0.9236$ and $R^2 = 0.9120$, indicating that
15 92.36% and 91.2% of the response variability could be explained by the previously discussed
16 models.

17 **Table 3.**

18
19 The effect of CP concentration (for ME with MT) at the lowest level of freeze-drying time
20 showed that the lowest droplet size was observed using the lowest CP level (5% (w/w) of MT),
21 regardless of the freezing temperature. However, at 48 h of freeze-drying time, the droplet size
22 varies depending on the freezing temperature. In fact, freezing at -196 °C generates smaller ME
23 droplet size when 5% (w/w) of MT was used, while for high freezing temperatures (-20 °C) a
24 20% (w/w) of MT was necessary to achieve the same result. It was also observed that the

1 droplet size increased with the CP concentration when the rapid freezing process (-196 °C) was
2 used.

3 The analysis of the marginal means of ME containing LT showed that for both the high and
4 the low level of freeze-drying times (48 h and 24 h) the ME droplet size increased with
5 increasing CP concentration, regardless of the freezing temperature. Nevertheless, at the
6 minimum freeze-drying time level (20 h) the freezing temperature of -20 °C produced a larger
7 droplet size than the one at -196 °C. Otherwise, the freeze-drying time of 48 h led to a lower
8 droplet size at this longer freezing time. Both marginal levels of freezing temperature showed
9 decreased droplet size with decreasing CP concentration.

10 The relationship between the dependent and independent variables was further elucidated
11 by the analysis of response surface diagrams, which was able to evaluate the relative
12 significance of all factors involved in the process (Figure 4). Throughout this analysis it was
13 possible to determine the optimum operational conditions for the system and the process^[34]. **In**
14 **this study, the constant value of the remaining variables were maintained at the level 0 (central**
15 **point)**. It was observed that the ME droplet size decreased with the reduction on the
16 freeze-drying time and MT concentration in a linear way. However, when freezing time and
17 other independent variables were compared, no direct linear relationship among them was
18 observed. Nevertheless, it was possible to identify the smaller droplet size values of ME-MT at
19 - 80 °C of freezing temperature (Figure 4a and Figure 4b).

20 The response surface analysis revealed that the droplet size increased with increasing LT
21 concentration and freeze-drying time. In fact, the smallest droplet size was found at the central
22 point of freezing temperature (Figure 4c and Figure 4d).

23 **Figure 4.**

24 Therefore, the best parameters for producing small droplets for freeze-dried MEs using
25 either MT or LT as CP were a concentration of 5% (w/w), a freezing temperature of -80 °C and

1 a freeze-drying time of 24 h. Since at this concentration ME-LT become rubbery few minutes
2 after the freeze-drying process and at a higher concentration the CP could not be dissolved in
3 the system, MT was chosen as the best CP for this ME.

4 **3.3 Characterization of the ME before and after freeze-drying**

5 The general physicochemical characteristics of the ME-MT at 5% (w/w) showed some
6 changes before and after the freeze drying process. Before freeze drying, the ME-MT presented
7 a droplet size of $48.13 \text{ nm} \pm 1.57$, a pH value of 7.23 ± 0.04 and a conductivity value of 749.1
8 $\mu\text{S cm}^{-1} \pm 25.65$. After the freeze drying process and subsequent dispersion with water, its
9 macroscopic appearance remained homogeneous and a clear yellowish product was obtained.
10 However its pH, conductivity values and droplet size were slightly different (Table 4).

11 **Table 4.**

12 The pH value decreased, probably due to the crystallization of the buffer salts during the
13 freezing drying process^[10]. However, even at this pH (6.86), the ME-MT is physiologically
14 acceptable.

15 The reduction on the droplet size of the ME-MT might be due to the co-surfactant role that
16 CP could play. The short poly-hydroxyl chain-alcohol could be adsorbed and intercalated into
17 the interfacial film of the ME after the freeze-drying process and reconstitution, thereby,
18 decreasing its surface tension^[35, 36]. However, at this droplet size (20.13 nm) the sample
19 remains within the ME droplet size range of 10 – 100 nm^[37]. Furthermore, the droplet size
20 result after freeze-drying corroborates experimentally with the Eq. (1) previously obtained from
21 the DOE, since replacing the used coded value ($y_1 = 23.4 + 6.11x_1^2$) a droplet size close to
22 23.4 nm, which were within the range obtained in the experiment (16.83 nm – 23.43 nm), was
23 expected.

1 The decrease of conductivity just after CP addition and before the freeze drying process
2 was probably due to its protective mechanism that enhances the viscosity of the system due to
3 the interaction between the hydroxyl from the CP and the water molecules. In fact, it is well
4 known that the crystallization of ice can be suppressed by the increase on the viscosity of the
5 medium, which limits the mechanical damage ^[26]. Therefore, a decrease in the amount of ions
6 can induce a reduction in the conductivity value of the ME-MT. On the other hand, the increase
7 of this conductivity value in the reconstituted ME-MT **may be due to the presence of maltose in**
8 **aqueous media, or due to the H⁺ from hydrolysis of maltose.**

9 The protective mechanism of the CPs may be also explained by (i) the formation of an
10 eutectic in the presence of water, due the multi-hydroxyl compounds, that leads to the formation
11 of amorphous or imperfect ice crystalloids; (ii) the maintenance of the spatial orientation and
12 distance between the droplets when the ice sublimates during the freeze-drying process,
13 preventing the formation of aggregates ^[38]; and (iii) the amorphous glass formation into which
14 the cryo-concentrated solution may vitrify at its T_g. The immobilization of the droplets within a
15 glassy matrix of CP can prevent their aggregation and protect them from the mechanical stress
16 of ice crystals ^[28].

17 ***3.4 Incorporation and freeze-drying of ME containing AmB***

18 The incorporation of AmB into the ME generated a product with a homogeneous and clear
19 yellowish aspect. The addition of MT at 5% (w/w) did not cause any significant changes in the
20 ME appearance neither in the droplet size (from 57.4 ± 4.1 nm to 54.1 ± 2.4 nm). However, the
21 freeze-drying process (24 h of freezing at - 80 °C and freeze-drying for 24 h) altered the
22 AmB-ME formulation, although a dry powder was produced. In fact, the reconstituted
23 AmB-ME, which was made by adding the same amount of water as that lost during the
24 freeze-drying process and using a probe sonicator with an iced bath for fast reconstitution,
25 showed changes in its electrical conductivity, pH and droplet size (Table 5). The

1 aforementioned reasons for changes in unloaded ME-MT formulations can also explain such
2 changes in the AmB-ME product.

3 **Table 5.**

4 The addition of AmB increased the ME droplet size and the conductivity value (Table 4
5 and Table 5). The diameter of the droplet size was changed, probably, due to the AmB
6 organization in the oil-water interface, increasing the volume of the droplets ^[39]. In fact, the
7 ionization of functional groups on AmB (carboxyl and/or amino groups) as well as the NaOH
8 and HCl added to solubilize and recover the neutral pH, may increase the amount of ions in the
9 system, thereby, increasing the electrical conductivity ^[40]. The increase in the droplet size may
10 be explained by the partitioning of AmB molecules into the oil phase since it is insoluble in
11 water, increasing the oily phase volume. Furthermore, due its amphiphilic properties, the
12 molecule might be located in the system interface, also increasing the droplet size ^[41].

13 The **UV-Vis spectrophotometric** method showed to be robust, efficient and sensitive. Thus,
14 the linear equation ($y = 1.2565x - 0.0257$ and $R^2 = 0.9992$) could fit more than 99% of the
15 experimental data. Therefore, the methodology may be confidently used for quantitative
16 analysis of AmB. The AmB content of the ME remained unchanged after the freeze-drying
17 process ($p > 0.05$). Therefore, MT at 5% (**w/w**) was effective in preventing ME diameter changes
18 and loss of AmB.

19 Although the AmB loading efficiency (encapsulation of the drug) was low (24.4%), the
20 freeze-drying process did not change the concentration of entrapped drug ($p > 0.05$). Therefore,
21 no loss of drug occurred during the process. **Despite adding a set amount of drug per mL of**
22 **microemulsion, this result represents the amount of AmB that remained soluble on the system**
23 **after centrifugation. Thus, about 75.6 % of the drug added to the system was not properly**
24 **incorporated.**

1 MT was an efficient CP avoiding drug loss, probably as a result of its ability to form
2 hydrogen bonds to phospholipid head groups and replacing the water by reducing the gel to
3 liquid crystalline phase transition that occurs when the dried phospholipids are rehydrated. In
4 this way, the MT did not lead to inhomogeneous rearrangement of phospholipids that could
5 result in particle aggregation and loss of incorporated drug into the aqueous medium ^[9].

6

7 **4. Conclusions**

8 Research and development of freeze-dried microemulsion systems has been a challenge
9 for several research teams. In fact, several parameters may affect the system stability during the
10 freeze-drying process. However, this work revealed that DOE can be a successful tool allowing
11 determining the optimal freeze-drying conditions to achieve this task. By taking in account the
12 type and concentration of CP, the freeze-drying time and freezing time temperature on the
13 DOE, and using the droplet size as variable of response, a ME dried product can be produced
14 using a low number of experiments. MT at 5% (w/w) as CP, - 80 °C of freezing time
15 temperature and 24 h of freeze-drying time were the best parameters to produce a good aspect
16 brick of freeze-drying ME. The incorporation of AmB did not dramatically change the ME
17 physicochemical properties. Moreover, freeze-dried AmB-ME can become a new antifungal
18 and antiprotozoal product to treat not only systemic candidiasis, but also leishmaniasis
19 infection.

20

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23

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5

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Figure captions

- 1
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- 3 **Figure 1:** DSC thermograms of ME and its components: (A) (---) Phosphate Buffer; (Black
4 continuous line) Microemulsion; (B) (.....) Lipoid[®] S100; (Blue continuous line)
5 Miglyol[®] 812; (- - -) Tween[®] 80.
- 6 **Figure 2:** Influence of the type and concentration of CP on the droplet size of the ME
7 formulations before the freeze-drying process.
- 8 **Figure 3:** Pareto Chart with Linear (L) and Quadratic (Q) standardized effects of the factors on
9 the (A) ME containing MT droplet size and (B) ME containing LT droplet size.
- 10 **Figure 4:** Response surface plot showing the effects of the mutual interactions between two
11 independent variables on the droplet size. The effect of the freezing temperature and
12 CP concentration on the MEs containing MT (a) and the effect of the freezing time
13 and CP concentration on MEs containing LT (c). The effect of the freezing
14 temperature and freeze-drying time on the MEs containing MT (b) and MEs
15 containing LT (d). The constant value of the remaining variables was maintained at
16 the level 0 corresponding at the central point.
17

1

List of tables

2

Table 1: Experimental levels of independent variables of the DOE for droplet size

3

evaluation of ME after freeze-drying process.

Independent variable		Level		
i	X_i	-1	Replicated (0)	+1
1	Freezing temperature (°C)	- 20	- 80	-196
2	CP concentration (%w/w)	5	12.5	20
3	Freeze- drying time (h)	24	36	48

4

1 **Table 2:** DOE for the freeze-drying process of MEs for each CP type.

Samples	Freeze-drying temperature	CP concentration	Freeze – drying time
1	- 1	- 1	- 1
2	+ 1	- 1	- 1
3	- 1	+ 1	- 1
4	+ 1	+ 1	- 1
5	- 1	- 1	+ 1
6	+ 1	- 1	+ 1
7	- 1	+ 1	+ 1
8	+ 1	+ 1	+ 1
9	0	0	0
10	0	0	0
11	0	0	0

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1 **Table 3:** Statistical Analysis (ANOVA) of results of DOE for ME-MT and ME-LT
 2 samples.

Source	ME – MT				ME –LT			
	Mean square	Calculated F	Tabulated F	R ²	Mean square	Calculated F	Tabulated F	R ²
Regression	81.518	9.9861	5.117	0.9236	25.315	12.5735	5.050	0.9120
Residue	8.163				19.908			
Lack of fit	9.755	3.7666	19.35		31.284	11.0027	19.16	
Pure Error	2.590				2.843			
Total	15.498				135.111			

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1 **Table 4:** Physicochemical characterization of the ME-MT before and after the
 2 freeze-drying process (FDP).

Samples	Appearance	pH	Electrical conductivity ($\mu\text{S cm}^{-1}$)	Droplet size (nm)
ME + MT at 5% (w/w) before FDP	Homogeneous and clear yellowish	7.23 ± 0.04	749.1 ± 25.65	48.13 ± 1.57
ME + MT at 5% (w/w) after FDP	Homogeneous and clear yellowish	$6.86 \pm 0.01^*$	$876.5 \pm 14.80^*$	$20.13 \pm 3.30^*$

3 Average values \pm SD

4 $*p < 0.05$ compared with the same sample before freeze-drying.

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1 **Table 5:** Physicochemical characterization of ME with AmB containing 5% (w/w) of
 2 MT before and after freeze-drying process.

Samples	Appearance	pH	Electrical conductivity ($\mu\text{S cm}^{-1}$)	Droplet size (nm)	AmB concentration (M)
Before freeze-drying	Homogeneous and clear yellowish	7.59 \pm 0.10	1583 \pm 39.55	54.07 \pm 2.35	1.97 x 10 ⁻³ \pm 0.03
After freeze-drying	Homogeneous and clear yellowish	7.4 \pm 0.11*	1639 \pm 53.75*	32.30 \pm 4.20*	1.94 x 10 ⁻³ \pm 0.02**

3 Average values \pm SD reported

4 *p < 0.05 compared with the same sample before freeze-drying process.

5 **p > 0.05 compared with the same sample before freeze-drying process.