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CRYOPROTECTION OF EMULSIONS IN FREEZE-DRYING: FREEZING PROCESS ANALYSIS

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ABSTRACT

We report the effects of different freeze/thawing processes on oil-in-water emulsions. The influence of the oil phase concentration, the cooling rate and the addition of carbohydrates as cryoprotectors were investigated.
It appears that a slow cooling rate and the addition of cryoprotectants which do not crystallize during freeze/thawing have the best stabilizing effects. These stabilizing effects were attributed to the amorphous state of the cryoprotectants and the lower mechanical stress due to the slow cooling rate. Heat treatment before thawing causes crystallization and decreases the stabilizing effects.
These results show the importance of crystalline state of additives used as cryoprotectants during freeze/thawing.

INTRODUCTION

Different studies have already shown the critical effect of freezing on diverse biological systems (1, 2, 3).
The aim of freeze-drying of pharmaceutical preparations is to preserve the stability of the substance of interest. When the material is complex i.e. (emulsions, colloidal systems), experience has shown that freeze-drying without additives and at inappropriate cooling rates can lead unsatisfactory results (4, 12).
In this work we have studied several parameters which could influence the continuous aqueous phase behavior in dispersed systems during freeze/thawing. For this study we needed a simple characterization system, thus we used the classical oil-in-water emulsion as a model.
Previous studies have already demonstrated the pertinence of the use of such a model to investigate the oil droplet size and the morphological differences which appear under different freezing conditions (5). This model avoids the osmotic effect involved in cryoprotection of vesicular systems, since only changes in the continuous aqueous phase and oil droplet size distribution are involved.
To determine the relative advantage of various cooling rates and cryoprotective additives on oil droplet stability and aqueous phase behavior, we have used size distribution analysis and rheological study before and after freeze/thawing.

We have established a correlation between the influence of the initial oil phase concentration, the addition of cryoprotective agents, the cooling rate and the stability of the emulsion.

MATERIALS AND METHODS

Materials

The BRIJ®72 (Polyoxethylene (2) stearyl ether) and BRIJ®76 (Polyoxyethylene (6) stearyl ether) commercial grade used as surfactant agents were obtained from I.C.I. (France S.A.). Light paraffin oil, used as the dispersed oil phase and D(-) mannitol, D(+) sucrose, D(+) glucose, chosen as cryoprotective agents were, obtained from Sigma (St. Louis, MO).

Preparation of emulsions for freezing

We prepared the most stable emulsion possible in order to be able to evaluate the destabilization induced by the freezing conditions. The general formula used is shown below:

Light paraffin oil	5%; 10%; 20%; 50%
Brij 72	1,12%
Brij 76	1,38%
Cryoprotective agent	2,5%; 5% 10%
Demineralized water	QSP 100%

Surfactants were mixed with the oil phase at 70°C. The aqueous solution containing the cryoprotective agent was then slowly added at the same temperature to the oil phase. The primary emulsion obtained was stirred with a Micro-Vortex at 700 RPM for 10 min, then progressively cooled to reach 20°C in 30 minutes at the same stirring speed.
Emulsion size distribution was determined with a Coulter Counter (Model TA II; Coultronic France S.A.). For each sample, 50000 particles are counted and distributed into 16 channels in a range of diameters from 1.5 to 50 μm. The measured oil droplet size distribution was mathematically analysed in order to obtain the average size distribution, which is represented by the volumic fraction of particles which are counted in each counter channel.

Cooling rate study

We used an isolating bath containing different volumes of ethanol in order to modify the cooling rate. The cooling rate was controlled by using a cryogenic probe with a fixed heat capacity plunged in the ethanol bath. The freezing device is illustrated in Figure 1. The emulsions were immersed in the bath and cooled to reach -30°C, which is the lowest temperature allowed by the system. This method allowed us to select 3 differents cooling modes.
Firstly, a direct immersion of the sample in a 2.5L ethanol volume already cooled at -30°C, corresponded to a cooling rate of -5°C/min, the fast mode in our conditions.
Secondly, an "intermediate cooling" mode of -2°C/min was obtained by using 2.5L of ethanol progressively cooled from 0 to -30°C.
Finally, a "slow cooling" mode of -0.5°C/min was obtained with 7L of ethanol cooled under the same conditions as above.

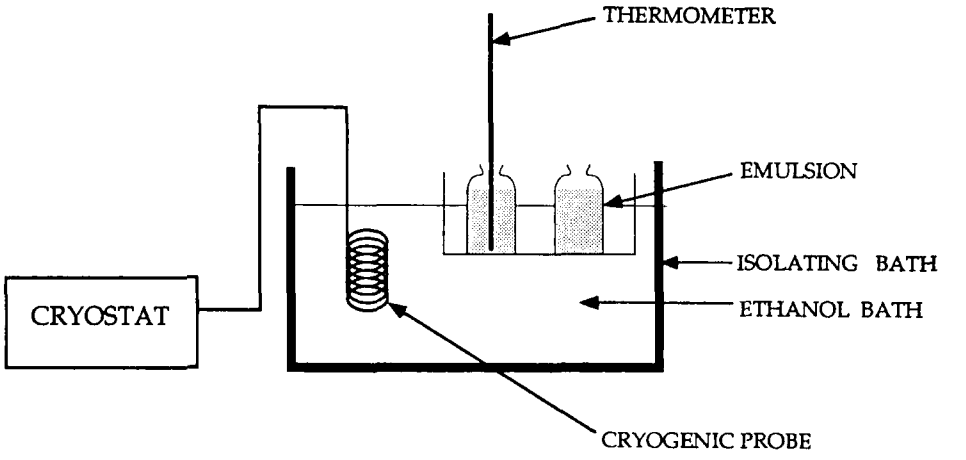

FIGURE 1
Freezing device

Rheological analysis

The rheological properties of the emulsions were determined with a Carrimed CSL 100 (RHEO France) control stress rheometer. A plan-cone geometry with 2 degrees of angle and 6 cm of diameter was used. We chose an experimental protocol which gave a uniform shearing stress within the sample (linear variation of the shear stress at 20°C).
For each sample, a constraint cycle was applied as follows a rising curve was obtained over in 2 minutes from 0 to 10 N/m^2 , then a plateau was held for 1 minute at the constant constraint of 10 N/m^2 followed by a decreasing curve from 10 to 0 N/m^2 in 2 minutes.

RESULTS AND DISCUSSION

Influence of the oil phase concentration

The analysis of oil droplet size distribution of 2 differents emulsions containing 5% and 50% of oil, just after the preparation in absence of any carbohydrates is shown in Figure 2 before and after the freeze/thaw cycle.

Different size distributions were observed for the two emulsions. The size distribution of 5% oil concentration emulsion was confined toward the smaller sizes. For the different oil concentrations used, Table 1 presents the results expressed as the percentage of increase of oil droplet average size after 4 hours of freezing at the different cooling rates with respect to the initial size.

An increase of the droplet size was seen in all cases. It is interesting to note that the emulsion containing 5% oil and the fast cooling rate give the largest increase.

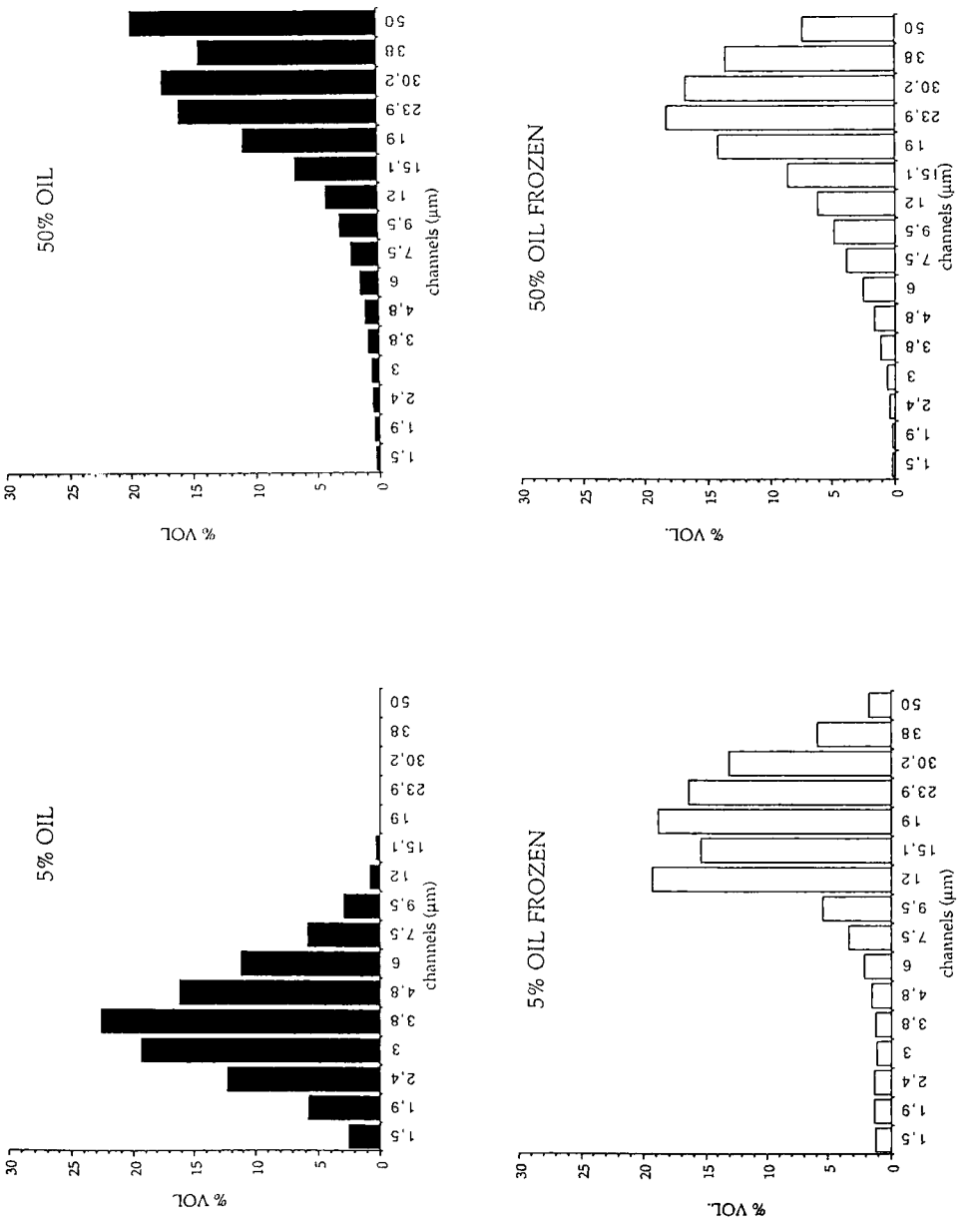

FIGURE 2
Size distribution of 5% and 50% oil emulsion before and after a freeze/thaw cycle

TABLE 1
Percentage increase in oil droplet average size after freeze/thawing.

Cooling rate	5% oil emulsion	10% oil emulsion	20% oil emulsion	50% oil emulsion
-0,5°C/mn	229,3%	149,5%	105,7%	105,3%
-2°C/mn	265,3%	205,7%	113,4%	113,4%
-5°C/mn	294,9%	215,1%	147,7%	124,7%

Previous studies (5, 6, 7) have shown that for different cooling rates, the average size of the emulsified droplets or liposomes after a freeze/thaw cycle reached a stable value and did not vary even after numerous freeze/thaw cycles. It was observed that for each cooling rate and oil concentration, the system adjusted to a preferential size of the oil droplets. An explanation of these results could be given by the fact that when freezing occurs, the ice crystal topology obtained gives rise to a preferential size of oil droplet with regard to the greatest stability of the emulsion.
We have seen that in the case of the 5% oil concentration emulsion, the size distribution of the oil droplets was confined toward the smaller sizes. For this emulsion many particles whose size is very different from the preferential one will tend to coalesce. In contrast, in the case of the 50% oil, the droplet size distribution is confined to the preferential size.
Thus we chose the 5% oil concentration emulsion for subsequent studies because it allowed us to observe the most important parameter in the structural evolution which could occur during the freezing process.

Cryoprotective agents influence

A preliminary screening study to evaluate the cryoprotective effect of various carbohydrates: glucose, mannitol, sucrose at different concentrations 2.5; 5 and 10% w/w was performed. Figure 3 summarizes the results of the size distribution of the emulsified droplets before and after a freeze/thawing cycle at the intermediate cooling rate of -2°C/mn.

In every case, an increase of the average size of the droplets was observed. The increase of the cryoprotector concentration gave rise to a greater stabilizing effect, a result which is in accord with Crowe's for the cryoprotection of liposomes (6). It seems that mannitol possesses a poor protecting effect with respect to other carbohydrates, which is related to its ability to crystallize during freezing (8, 9).
Although none of the sugar offered protection against an increase in apparent droplet size, glucose and sucrose at 10% gave the smallest increment of the droplet size. Consequently glucose and sucrose at 10% were chosen as reference for emulsion's cryoprotector to study the influence of the cooling rate, and to try to explain their cryoprotective effect.

Influence of the cooling rate

Emulsions with or without 10% of glucose or sucrose were frozen at 3 differents cooling rates: -0.5°C/min; -2°C/min and -5°C/min. The results are illustrated in Figure 4

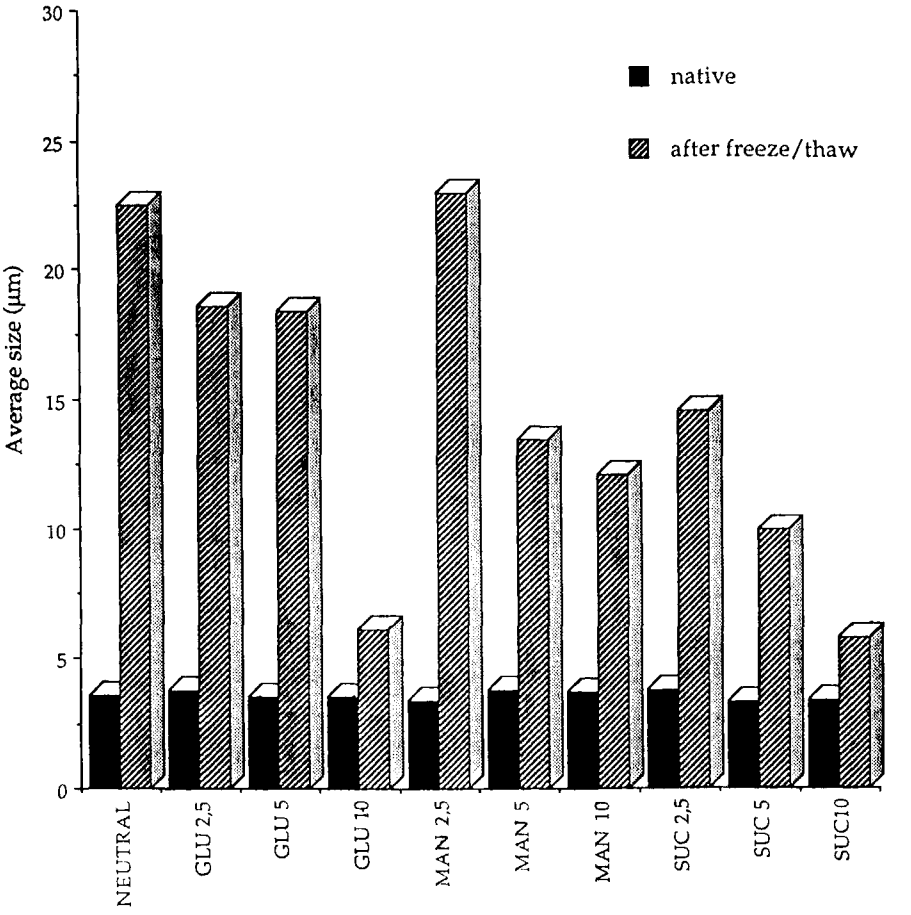

FIGURE 3
Average size of emulsion oil droplets after 4 hours of freezing for a screening of cryoprotectants (emulsion without cryoprotectants is called "neutral")

An increase of the cooling rate always provoked an increase of the droplet sizes, but this effect was clearly reduced by the presence of the cryoprotective agents. One possible explanation of this result could be the relationship which exist between the continuous aqueous phase structure, oil droplets and the freezing rate. For a slow cooling rate, the continuous aqueous phase evolves slowly, allowing the oil droplets to become organized between the first ice crystals which progressively appear and therefore to a lower destabilizing effect. In the case of a fast cooling rate, numerous ice crystals appear rapidly, so the oil droplets cannot reorganize to resist the freezing mechanical stress and thus collapse, increasing the average size after thawing.

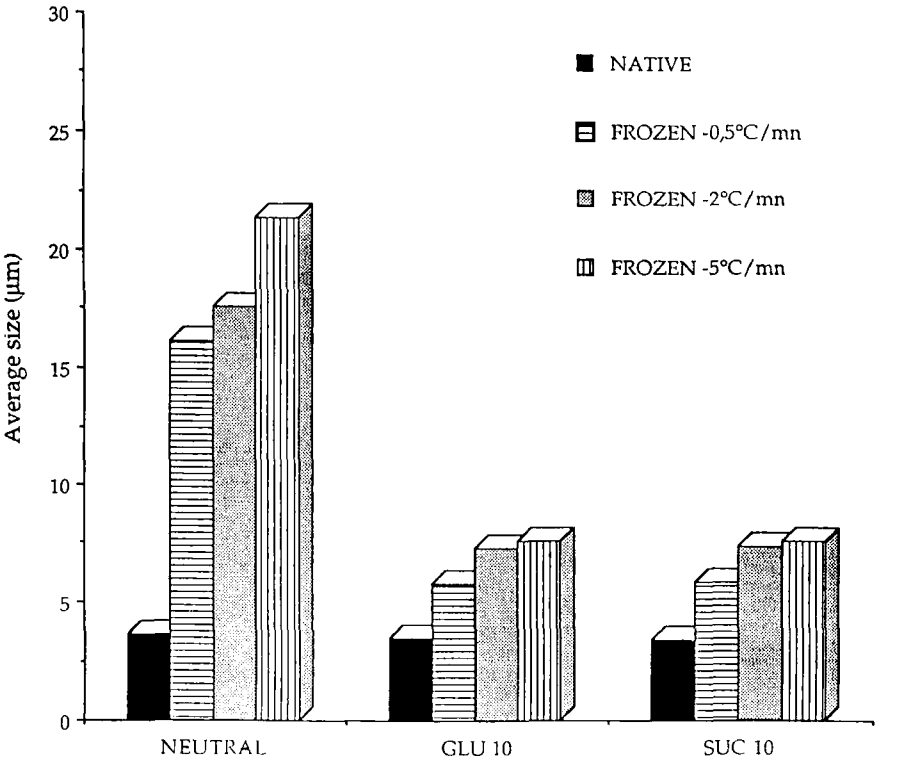

FIGURE 4
Average size evolution of oil droplets in regard to the cooling rates

It is well known that glucose and sucrose, which do not crystallize but promote the formation of an amorphous matrix containing unfrozen water, present a particular transition temperature called Tg' (respectively -43°C and -32°C for glucose and sucrose), below which the matrix is definitively in a solid glassy state. When the conservation temperature is above Tg' and below the melting temperature, the matrix is in a rubbery state referred to as a high viscosity fluid state containing unfrozen water surrounding ice crystal. During freezing, this high viscosity slows down the diffusion of water molecules i.e. the rate of ice crystal appearance (10, 11), a phenomenon which is highly favourable for oil droplet stability.
These results show that the variation of the emulsified droplets size is correlated with the crystallization of the aqueous phase during freezing. The cryoprotective effect of the studied sugars towards oil droplet stability has been clearly demonstrated to be mediated by modification of the aqueous structure during the freezing process, as already reported by different authors.

Influence of time conservation

In order to evaluate the conservation of frozen samples, we have compared the increment of droplet size of frozen emulsions (-2°C/min), kept at -25°C for 4 hours, 1 week and 1 month. The results are shown in Figure 5

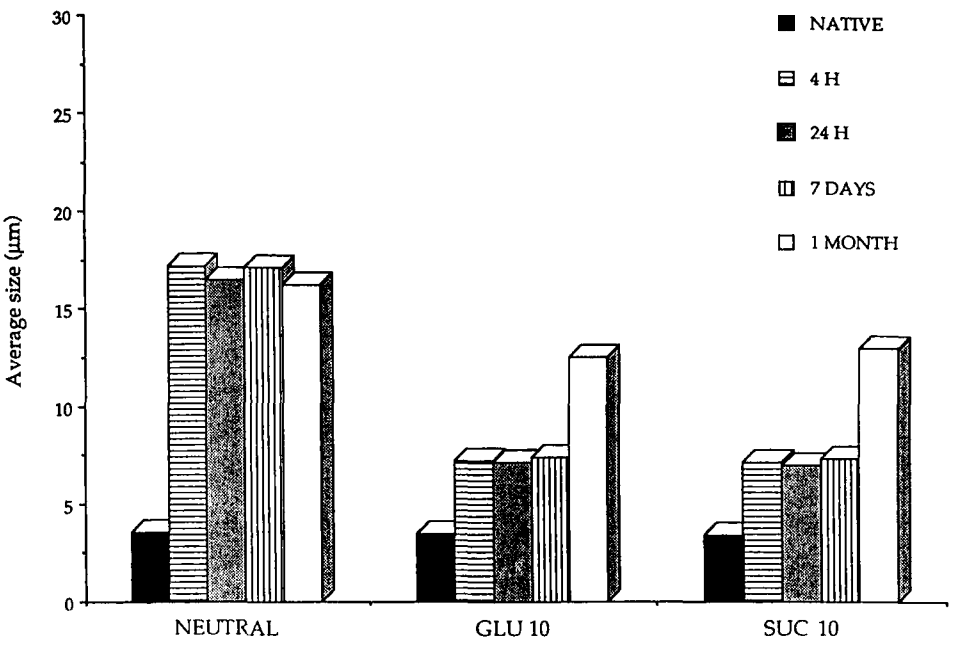

FIGURE 5
Average size evolution of oil droplets in regard to the time conservation at -25°C

In the absence of cryoprotective agents, an increase of the emulsified droplet size rapidly occurred during freezing, as already shown, but there was no further for the longest conservation time.
On the other hand, for the first seven days of conservation, the emulsified droplets seem to be well protected in the presence of cryoprotective agents; however thereafter an increase in droplet size occurred. When the conservation temperature is -25°C, above the Tg' of both carbohydrates, unfrozen water molecules could progressively diffuse and recrystallize(10). So, after one month of conservation in those conditions, this recrystallization could gives rises to coalescence and hence to an increase of the size of the oil droplets.
This observation is in good accordance with the hypothesis of cryoprotectants effect on the crystalline structure.

Rheological analysis

Rheological analysis is a classical and informative way to characterize dispersed liquid systems such as emulsions. The rheological characterization can also reveal the evolution of emulsion structure after a freeze/thaw cycle. We have performed rheograms of our emulsions, which exhibit a shear thinning behavior with a threshold constraint and without thixotropy, as shown in Figure 6 for the native emulsion.

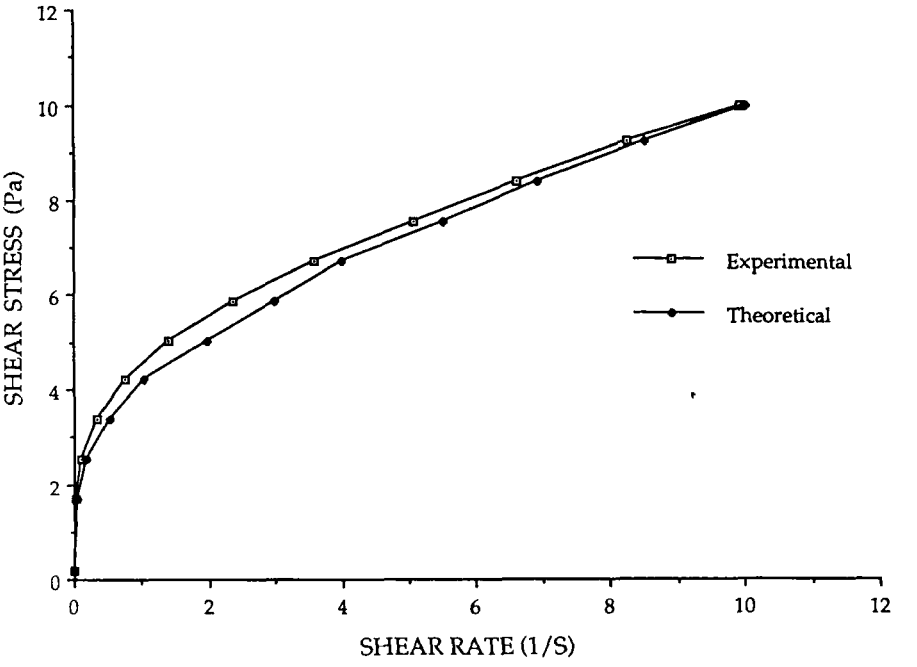

FIGURE 6
Rheogram of 5% oil concentration emulsion before freezing

The rheograms were mathematically treated by Herschel-Bulkley's (13) rheological equation with 3 parameters:

$$\tau = \tau_c + K.\dot{\varepsilon}^n$$

where τ is the shearing constraint, τ_c the threshold constraint, $\dot{\varepsilon}$ the shearing gradient, n a pseudoplastic index and K the apparent viscosity coefficient. The same shear thinning behavior was observed for frozen/thawed emulsions as for the native one but with different parameter values, especially for the apparent viscosity coefficient.

Figure 7 represents the variations of K at $10N/m^2$ constraint of the emulsion after a freeze/thaw cycle performed at -0.5°C/min and -5°C/min cooling rate in comparison with the native emulsion viscosity coefficient.

An increase of K is observed for the systems containing cryoprotective agents. This result seems to be in contradiction with the droplet size evolution described above.

An explanation of this apparent contradiction can be obtained by taking into account Mooney's rheological (14) equation:

$$\text{Ln } \eta = \frac{\alpha.\phi}{1-\lambda.\phi}$$

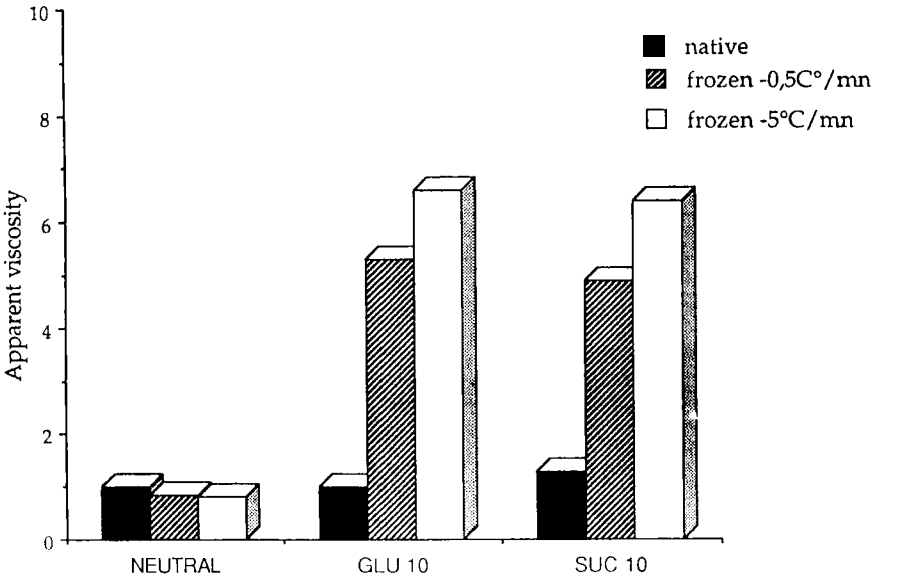

FIGURE 7
Variation of apparent viscosity according to the cooling rates

where η is the system viscosity, α the shape factor, ϕ the droplet volume fraction and λ the size distribution coefficient. Although in the Herschel-Bulkley's equation K cannot be assimilated to a newtonian viscosity, its value can be related to the apparent viscosity of the system.

For the system without cryoprotective agents, we have already seen that the size distribution is confined towards the smaller sizes before freezing, and becomes centered around the preferential size after freeze/thaw but remains narrow. There is a slight modification of λ, so the apparent viscosity is not modified. On the other hand, in the presence of cryoprotective agents, a population of small oil droplets which are not destabilized during freezing is still present (see Figure 8), giving rise to an . increase of the size dispersion λ, leading to an increase of apparent viscosity. This increase is not caused by the increase of droplet size, but by the augmentation of the size distribution coefficient λ.

CONCLUSION

The use of a simple model like an emulsion whose structure is very sensitive to freezing conditions, and combined with suitable physico-chemical characterizations techniques such as size distribution and rheological studies have allowed us to specify the predominant role of the freezing process on oil droplets size stability.

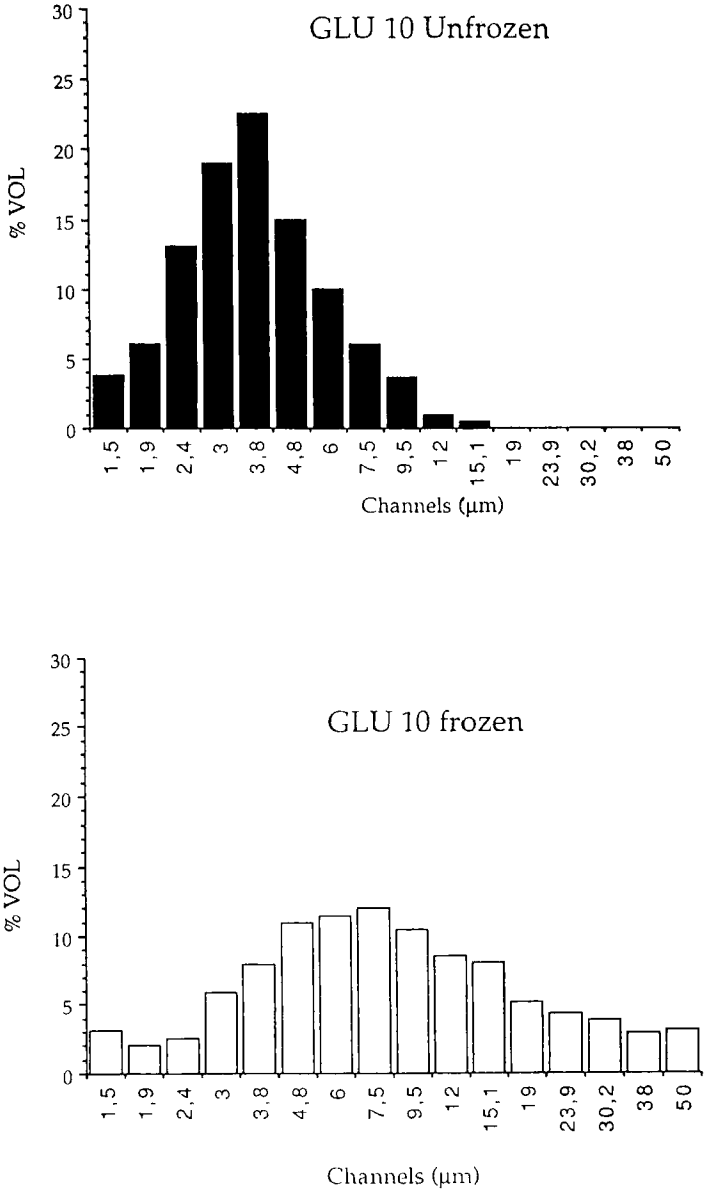

FIGURE 8
Size distribution of an emulsion containing 10% glucose before and after freeze/thaw cycle

Slower cooling combined with addition of glucose and sucrose as cryoprotective agents gave rise to the greatest protection of the emulsion. This stabilizing effect most probably depends on the formation of a high viscosity fluid state during freezing which slows down the diffusion of water molecules through the system, i.e. the crystallization of water. The results obtained have also shown that the presence of cryoprotective agents permits a greater stability of the emulsions when kept at -25°C for seven days, a period which is largely sufficient for the freeze-drying process. However, for a long conservation time (one month) above the critical transition temperature Tg' of the amorphous matrix, recrystallization progressively occurs leading to an increase in the average size of the oil droplets.

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