

TABLE II

Amounts of Neutral Oil Recovered from 2-g. Samples of Soybean Oil by Different Volumes of Wash Chloroform. Silicic Acid: 25 g.

Wash chloroform in ml.	Neutral oil in percentages
100.....	91.0
150.....	92.9
200.....	93.0
250.....	93.1
300.....	93.1
500.....	93.1

Recovered by chromatographic method, 93.1%.

the determination of neutral oil is proposed. Shake 2 to 3 g. of crude oil, 25 g. of silicic acid, and 50 ml. of chloroform in a 125-ml. Erlenmeyer flask for 10

TABLE III

Neutral Oil Content of Cottonseed and Corn Oils

Oil	Wt. of oil in grams	Percentages of neutral oil
Corn.....	2.0043	89.9
Corn.....	2.5125	89.8
Corn.....	3.1238	89.8

Recovered by chromatographic method, 89.8%.

Cottonseed.....	2.6279	95.0
Cottonseed.....	2.6279	94.9
Cottonseed.....	3.2587	95.0

Recovered by chromatographic method, 95.0%.

min. Filter through a sintered glass funnel under vacuum, washing the silicic acid with five 50-ml. lots of chloroform. Combine the filtrate and washings, and evaporate the chloroform on a water bath. Heat to constant weight at 105°C. in a vacuum oven or in an atmosphere of nitrogen. The percentage of neutral oil equals the weight of the extracted oil over the sample weight times 100.

The phospholipid contents of the crude oils were determined by washing the silicic acid residues with methyl alcohol, followed by evaporation of the alcohol and weighing. The results were as follows: soybean oil, 6.25%; corn oil, 5.83%; and cottonseed oil, 3.25%.

The method outlined for neutral oil does not require learning special or complicated techniques or the use of special apparatus. The chloroform, used as a solvent, is nonflammable, and its vapors are only moderately toxic. The results check well with the conventional chromatographic method.

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## Factors Affecting Oil Particle Size in the Freezing and Thawing of Fat Emulsions<sup>1, 2</sup>

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EMULSIONS OF VEGETABLE OILS, for example, cottonseed, have considerable potential use as high caloric materials for intravenous alimentation. Such emulsions usually contain 10-15% of oil finely dispersed in an isotonic solution of dextrose, with emulsifying agents present to promote and maintain dispersion. Lipomul-I.V. is such a product, as reported by Meyer *et al.* (3).

One of the critical requirements of these emulsions is that the size of the dispersed oil particles be sufficiently small and remain so on prolonged storage. Meyer and co-workers report the size of the oil particles of Lipomul-I.V. to be predominantly 0.5 to 1 micron in diameter, with not more than 0.02% of the particles larger than 1.5 microns. The size distribution of the oil particles is maintained during storage of the emulsion at 5°C. However if Lipomul-I.V. and emulsions similar to Lipomul-I.V. either inadvertently or deliberately are allowed to freeze, many of the dispersed oil particles after thawing of the emulsion can be observed to have increased in diameter to 7

microns or more. Emulsions containing particles of this size are no longer suitable for use in intravenous alimentation.

In an investigation of the mechanism whereby freezing broke an emulsion of a benzene-carbon tetrachloride mixture in water, Rochow and Mason (4) determined that, as ice crystals form, they are separated from droplets of the internal phase by a membrane. This membrane thins and breaks, and the destruction of the membrane, rather than mere freezing of the continuous phase, is reported to be essential to coalescence of the oil droplets on thawing. Berkman and Egloff (2) explain the breaking of an emulsion by freezing as a withdrawal of free water from the films between droplets which are in contact, and coalescence of the oil droplets with thawing of the ice.

The present investigation was for the purpose of determining whether the increase in the size of some of the oil particles, such as generally occurs when emulsions of the type mentioned are frozen and thawed, could be prevented or minimized. The effects of control of the rates of freezing and thawing of emulsions, type and melting point of oils constituting the oil phase, concentration of the oil phase, and two systems of emulsifying agents were investigated.

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### Experimental

**Materials.** Emulsions of cottonseed and safflower oils, prepared with emulsifying systems of two types, were used. One of the cottonseed oil emulsions used was the commercial product Lipomul-I.V. This emulsion contained, on whole-weight basis, 15% of a refined, bleached, winterized, and deodorized cottonseed oil; 1.2% of fractionated or purified soya phosphatides; 0.3% of Pluronic F68 (polyoxyethylene-propylene, Wyandotte Chemical Company); and isotonic dextrose solution (3). This same emulsifying system was used in the laboratory preparation of emulsions of safflower oil and of a refined, bleached, and deodorized cottonseed oil which was solvent-fractionated to remove approximately 40% of the more saturated glycerides. These latter two emulsions provided products in which the melting points of the respective oil phases were lower than that of Lipomul-I.V. The capillary melting-points of these oils were the cottonseed oil used in Lipomul-I.V.,  $-1^{\circ}\text{C}.$ ; safflower,  $-12^{\circ}\text{C}.$ ; and solvent-fractionated cottonseed,  $-5^{\circ}\text{C}.$

A second emulsifying system was used to prepare emulsions of the same cottonseed oil as that in Lipomul-I.V., the safflower oil, and the solvent-fractionated cottonseed oil. In addition, emulsions of a refined, bleached, and deodorized cottonseed oil, melting point  $10^{\circ}\text{C}.$ , and a hydrogenated cottonseed oil, melting point  $49^{\circ}\text{C}.$ , were prepared. The emulsifying system used in the preparation of these emulsions consisted of 1.25% Drumulse 536R (polyglycerol ester, E. F. Drew and Company), 0.2% TEM 4T (acetylated tartaric acid ester of monoglycerides, Hachmeister Inc.), and 0.2% Pluronic F68. The oil content of these emulsions was 15%, and the aqueous phase was isotonic dextrose solution.

**Preparation of Emulsions.** The laboratory method of high pressure homogenization by which the emulsions were prepared has been described elsewhere (5). Each emulsion was sterilized so that its physical treatment was identical with that of emulsions prepared commercially for intravenous use. The size of the dispersed oil particles in the laboratory-prepared emulsions was the same as described for Lipomul-I.V.

**Methods of Freezing and Thawing.** Preliminary to the major portion of this investigation, a thin film of Lipomul-I.V. was frozen and thawed under microscopic observation to determine the general characteristics of the phenomenon involved, as described by Rochow and Mason (4). This microscopic observation of the freezing and thawing of the emulsion was accomplished by using a hollow glass cell mounted on the stage of a microscope. A film of the emulsion was applied to the top surface of the cell, covered with a cover glass, and observed by use of an oil immersion lens (total magnification  $950\times$ ). Carbon dioxide flowing through the cell cooled the emulsion to its freezing temperature. By reducing the flow of  $\text{CO}_2$ , the emulsion was thawed. This freezing-thawing cycle could be repeated as desired.

The major portion of this investigation was concerned with the freezing and thawing of 500-ml. bottles of the emulsions, which are the usual volume of commercial emulsions packaged for intravenous use. For a moderate rate of freezing the bottles of emulsions were kept over-night in a refrigerator at  $-5^{\circ}\text{C}.$  A dry ice-acetone ( $-60^{\circ}\text{C}.$ ) was used for fast freezing, which could be accomplished within 5 min. Thawing was allowed to proceed at three different

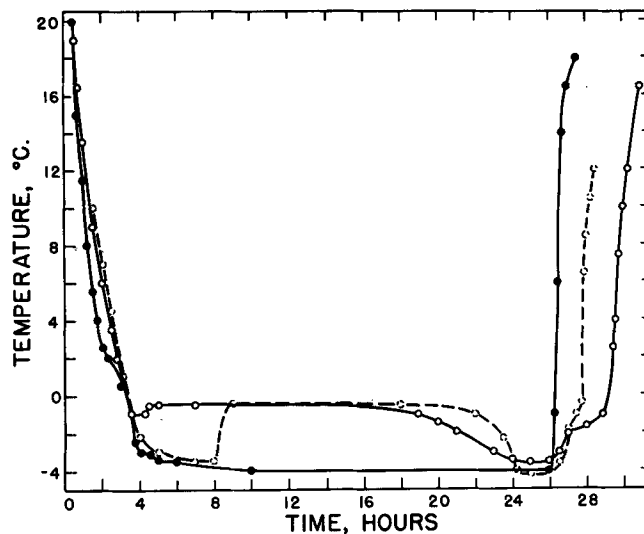


Fig. 1. Cooling and heating curves of Lipomul-I.V. (open circles), isotonic dextrose (broken lines), and winterized cottonseed oil (filled circles).

rates: fast thawing by placing a bottle of frozen emulsion in a water bath maintained at  $75-80^{\circ}\text{C}.$ ; moderate thawing in air at  $26^{\circ}\text{C}.$  (room temperature); and slow thawing by packing in crushed ice, which was allowed to melt gradually. The average times required for thawing at these temperatures were 0.25 hr., 5 hrs., and 20 hrs., respectively. There was no agitation of the contents of the bottles of emulsions during freezing or thawing, except for that induced by gentle swirling. Mechanical stirring was not employed, as it ordinarily would not be done in the use of the product.

The temperature of each emulsion was recorded continuously during a freezing-thawing cycle by means of a temperature recorder equipped with double thermocouples. One thermocouple was placed centrally in the bottle of emulsion, and a second thermocouple was placed next to the wall of the bottle. There was good agreement between these respective temperatures. This investigation was not concerned with determinations of the specific heats of various emulsions involved in freezing and thawing. Temperature-time measurements were obtained merely for the purpose of ascertaining the influence of the rate of freezing and thawing of the emulsions on the particle size of the dispersed oil phase.

The size of the particles of the dispersed oil phase in the various emulsions was determined by means of a microscope equipped with a calibrated micrometer scale and by use of an oil-immersion lens. Five milliliters of emulsion were diluted to 25 ml., and a drop was placed on a slide, covered, and observed. At least five fields in different parts of the slide were observed, and the average distribution of larger particles of dispersed oil per field was calculated. For an emulsion to be considered as undamaged by freezing, or of acceptable particle size, the diameter of the oil droplets was required to be less than 7 microns. The numbers of oil droplets less than 1 micron in diameter were not recorded.

Photomicrographs of emulsions were obtained in some instances. Brownian movement in an emulsion being photographed was stopped by mixing with a small amount of Carbopol 934 gel (Goodrich Chemical Company).

### Results and Discussion

*Freezing-Thawing of Emulsion Film.* As the temperature of a film of the Lipomul-I.V. emulsion was lowered by means of the CO<sub>2</sub> cell, Brownian movement of the oil particles slowed, and stopped when the aqueous phase solidified. Solidification was practically instantaneous. A maze of "blind canals" was formed, in which the oil phase was seen as a yellow liquid, separated from the water phase. This is in accordance with the observations of Rochow and Mason (4). Upon thawing, complete reconstitution of the emulsion occurred. There was no observable growth in size of the oil particles after the freezing-thawing cycle. Repetition of freezing-thawing cycles of the same film of emulsion for six to eight times resulted in a gradual increase in size of the dispersed oil particles to above 7 microns, probably because of the weakening of the membrane separating the two phases.

*Freezing-Thawing of Bottled Emulsion.* The solid-liquid phase relationships during a typical freezing and thawing cycle of a full bottle of Lipomul-I.V. emulsion and of the individual oil and aqueous phases of such an emulsion were determined. Bottles of Lipomul-I.V., winterized cottonseed oil containing 1.2% soya phosphatide, and 5% aqueous solution of dextrose containing 0.3% Pluronic F68, respectively, were placed in a refrigerator at -5°C., periodically swirled, and allowed to remain in the refrigerator over-night. These materials were then thawed at room temperature, while being swirled. Time-temperature data for each of the three materials were obtained during the respective freezing-thawing cycles and are plotted in Figure 1.

The cooling curves of the dextrose solution and of the emulsion were essentially identical until a temperature slightly below 0°C. was reached. The similarity of these cooling curves might be expected, because of the quantity of dextrose solution which constituted the aqueous phase of the emulsion. Solidification of the emulsion began prior to the beginning of solidification of the dextrose solution. The latter solution supercooled to a considerable extent. Some solidification of the winterized cottonseed oil sample began at about 2°C., and further solidification was evident at -3°C. These are two of the transition points of cottonseed oil which have been reported (1).

During the period of several hours in which the emulsion and the dextrose solution were freezing, the temperature of both remained constant. Upon completion of freezing, the temperature of these materials decreased to that of the environment.

When the samples were removed from the refrigerator and thawed at room temperature, the emulsion slowly melted over a period of 4-5 hrs. whereas the samples of oil and dextrose solution melted at a more rapid rate. Visually the oil sample was entirely liquid at about 8°C. while the emulsion appeared to be solid at that temperature. Photomicrographs of the emulsion before and after freezing and thawing are given in Figure 2, to illustrate typical effects of freezing and thawing on the size of oil particles. Figure 2A is the original emulsion, and Figure 2B is the same emulsion after freezing and thawing. The increase in size of some of the oil particles is obvious.

*Effect of Rate of Freezing and Thawing of Lipomul-I.V.* Lipomul-I.V. was frozen and thawed at different rates to determine the effect of these rates on

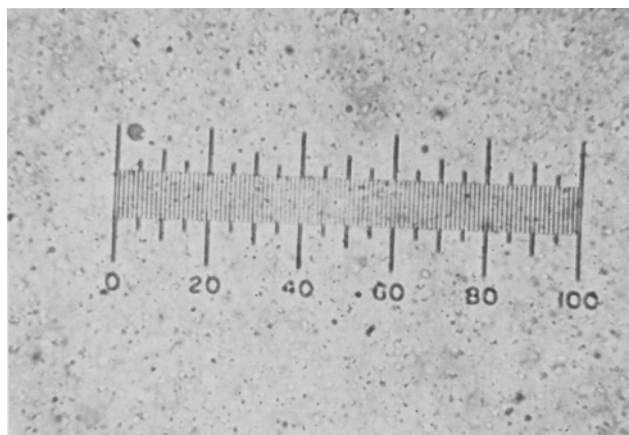


FIG. 2. A—Lipomul-I.V. prior to slow freezing.

the size of the oil particles. Duplicate bottles of the emulsion were placed in a refrigerator at -5°C. overnight for slow freezing. Other bottles of the same emulsion were frozen rapidly in dry ice-acetone. Emulsions frozen under these two conditions, respectively, were then thawed in such manner as to provide samples thawed at slow, moderate, and rapid rates, respectively, as previously described. The observed oil particle sizes and their distribution per microscopic field are given in Table I.

TABLE I  
Average Distribution of Dispersed Oil Particles Larger than One Micron in Lipomul-I.V. Frozen and Thawed at Various Rates

Rate of freezing	Size of oil particles, microns	Average number of oil particles		
		Rate of thawing		
		Rapid	Moderate	Slow
Slow.....	1-2	5	4	7
	3-6	3	2	5
	>7	2	1	0
Rapid.....	1-2	7	8	10
	3-6	5	3	5
	>7	3	5	0

Oil particles in excess of 7 microns in diameter were observed at the rapid and moderate rates of thaw, irrespective of rate of freezing. There seemed to be more of these larger oil particles present following rapid freezing and moderate thawing than under

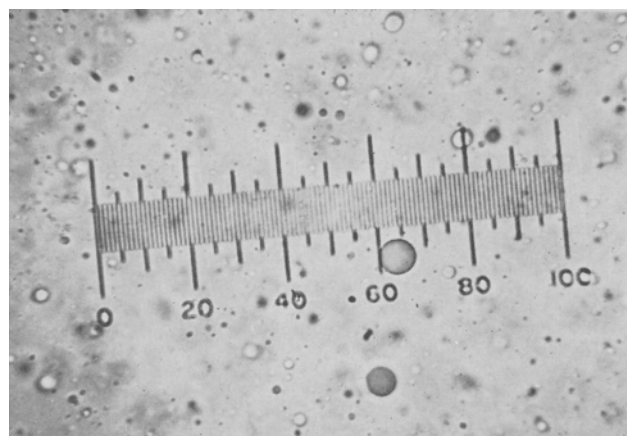


FIG. 2. B—Lipomul-I.V. after slow freezing and thawing at moderate rate.

other conditions. At the slow rate of thawing after either rate of freezing, there were no oil particles 7 microns or greater in diameter. The distribution of oil particles 1-6 microns in diameter appeared to have been somewhat greater after rapid freezing than after slow freezing, at all rates of thawing.

Apparently the rate of thawing of frozen Lipomul-I.V. was more critical with respect to an increase in size of the oil particles than was the rate of freezing of this emulsion.

*Effect of Melting Point of the Oil Phase.* For determination of the effect of melting point of the oil phase of an emulsion on the size of the oil particles after freezing and thawing, emulsions were prepared with the solvent-fractionated cottonseed and safflower oils. The melting points of these oils were lower by 4°C. and 11°C., respectively, than the melting point of the cottonseed oil in Lipomul-I.V. The emulsifier system was the same as that in Lipomul-I.V. Each emulsion in duplicate was frozen at the slow rate, then thawed at each of the slow, moderate, and rapid rates. Microscopic observations of sizes and distribution of the oil particles are given in Table II.

TABLE II

Average Distribution of Dispersed Oil Particles Larger than One Micron in Emulsions Prepared with Soya Phosphatide-Pluronic F68 Frozen at Slow Rate and Thawed at Various Rates

Oil phase of emulsion	Size of oil particles, microns	Average number of oil particles		
		Rate of thawing		
		Rapid	Moderate	Slow
Solvent-fractionated cottonseed, m.p. -5°C.	1-2	3	4	3
	3-6	2	5	4
	>7	0	0	0
Safflower, m.p. -12°C.	1-2	3	4	5
	3-6	3	2	3
	>7	0	0	0

Whereas frozen Lipomul-I.V. required a slow rate of thawing to prevent oil particles of 7 microns or higher in size, emulsions of the fractionated cottonseed oil and the safflower oil, with the same emulsifier system as Lipomul-I.V., could be thawed at either of the three rates with no apparent increase in size of the oil particles. The microscopic appearance of these emulsions at all rates of freezing and thawing were almost identical.

It would appear that the melting point of the oil phase of an emulsion is one of the contributing factors to maintaining acceptable size of the dispersed oil particles in emulsions which have been frozen and thawed.

*Effect of Emulsifiers.* Emulsions of cottonseed and safflower oils were prepared with the Drumulse 536R-TEM 4T-Pluronic F68 emulsifying system, for comparison after freezing and thawing with emulsions of the same oils prepared with the phosphatide-Pluronic F68 system. These oils were the winterized cottonseed, the solvent-fractionated cottonseed, and the safflower. Emulsions of the nonwinterized cottonseed and hydrogenated cottonseed oils also were prepared, with the Drumulse 536R-TEM 4T-Pluronic F68 system. These emulsions were frozen at the slow rate, then thawed under the three conditions as described. The sizes of the oil particles of the emulsions after thawing are given in Table III.

The winterized cottonseed oil emulsion had no oil particles of 7 microns or over in size at the moderate rate of thawing. At both the rapid and slow rates of

TABLE III  
Average Distribution of Dispersed Oil Particles Larger than One Micron in Emulsions Prepared with Drumulse 536R-TEM 4T-Pluronic F68 Frozen at Slow Rate and Thawed at Various Rates

Oil phase of emulsion	Size of oil particles, microns	Average number of oil particles		
		Rate of thawing		
		Rapid	Moderate	Slow
Safflower, m.p. -12°C.	1-2	6	5	5
	3-6	3	3	4
	>7	0	0	0
Solvent-fractionated cottonseed, m.p. -5°C.	1-2	4	6	6
	3-6	2	4	5
	>7	1	0	0
Winterized cottonseed (as in Lipomul-I.V.), m.p. -1°C.	1-2	10	8	15
	3-6	6	4	10
	>7	1	0	7 <sup>a</sup>
Nonwinterized cottonseed, m.p. 10°C.	1-2	10	12	20
	3-6	8	15	10
	>7	4	5	14
Hydrogenated cottonseed, m.p. 49°C.	.....	broken, watery layer, very grainy, thick upper layer		

<sup>a</sup> Clustered.

thawing however, oil particles over 7 microns in size were observed. The influence of the three rates of thawing of this emulsion was more apparent upon examination after storage for an interval of three days at room temperature. This second examination revealed a perceptible thickening and creaming in the slowly thawed product but no change in the size of the oil droplets in the emulsions thawed at the rapid and moderate rates.

The results obtained in this instance are opposed to those obtained with Lipomul-I.V., which contained the same winterized cottonseed oil but different emulsifying agents. Apparently the different solubilities of the two emulsifying systems in the same cottonseed oil, and possibly the dehydration and rehydration effects of these systems on the membrane between the oil and water phases, were contributing factors to the results obtained.

The safflower oil emulsion (Table III) appeared to have equally good oil particle size at all rates of thawing. There appeared to be a slightly greater number of oil particles in the 1 to 2- and 3 to 6-micron size ranges in this emulsion as compared with the same oil emulsified with the phosphatide system, but in no instance were oil particles of 7 microns observed.

The solvent-fractionated cottonseed oil emulsion contained an average of 1 oil particle per microscope field over 7 microns in size at the rapid rate of thawing, but the oil particles were of acceptable size at the other rates of thawing. Similar to the effects observed with the safflower oil emulsion, this cottonseed oil emulsion prepared with the Drumulse 536R system of emulsifiers had a slightly greater number of oil particles in the respective size ranges than did the emulsion with the phosphatide system and was inferior to the latter product at the rapid rate of thawing.

Results obtained with the nonwinterized and hydrogenated cottonseed oils are given in Table III. Emulsions of these oils were not acceptable in the size of the oil particles at any rate of thawing. The emulsion of hydrogenated oil, in which the melting point of the oil phase was relatively high, was visibly broken at all rates of thawing. The nonwinterized oil emulsion, while not visibly broken, had oil particles of large size at all rates of thawing. A comparison of the dispersed oil particles in the four cottonseed oil emulsions (Table III) indicates that, at any particular

rate of thawing, the number of larger oil particles is less with a progressively lower melting-point of the respective oils. The melting point of the oil phase, and possibly solubility of the emulsifiers in the oil phase or crystal inhibition, apparently were factors in the freezing and thawing stability of emulsions prepared with the Drumulse 536R-TEM 4T-Pluronic F68 system, as with the phosphatide-Pluronic F68 system.

*Effect of Repeated Freezing-Thawing.* The winterized cottonseed and safflower oil emulsions prepared with the Drumulse 536R-TEM 4T-Pluronic F68 system (Table III) had approximately the same distribution of oil particles after freezing and thawing at the moderate rate. These emulsions were again frozen and thawed in the same manner for three complete cycles and again were compared. The winterized cottonseed oil emulsion contained particles of oil which approached 7 microns in diameter. The safflower oil emulsion had no apparent increase in the size of the oil particles. It would appear therefore that emulsions of oils of low melting-point, such as safflower, are able to withstand repeated freezing-thawing cycles to a greater extent than emulsions of oils, such as the winterized cottonseed oil, with the same emulsifier system.

*Effect of Oil Content.* An emulsion containing 30% of winterized cottonseed oil was prepared with the Drumulse 536R-TEM 4T-Pluronic F68 system. This emulsion in duplicate was frozen at the slow rate, then thawed under the three conditions previously used. At each rate of thaw, particle size was not acceptable, and all emulsions creamed on thawing. High oil concentration did not appear to prevent an increase in oil particle size of a frozen emulsion.

### Summary

A film of Lipomul-I.V. can be frozen and thawed repeatedly with no apparent increase in the size of the dispersed oil particles. The lack of increase in oil particle size of such a film most probably is caused by the extreme rapidity of heat transfer in this small quantity of emulsion. There probably is not sufficient time for the separating membrane between the water and oil phases to weaken and break. Therefore the oil particles do not increase in size and coalesce as they do in larger volumes of emulsion.

The rate at which a full, frozen bottle of Lipomul-I.V. was thawed appeared to be more important than the rate of freezing in preventing an increase in oil particle size. Frozen Lipomul-I.V. was acceptable in particle size only when thawed over a period of 20 hrs., irrespective of the rate of freezing. Such a period of thaw, in an environment of slowly melting ice, possibly allowed the water phase of the emulsion to liquefy prior to complete melting of the oil phase, thereby preventing extensive coalescence of the oil droplets.

The melting point of the oil phase of an emulsion, and possibly the variation in solubility of the emulsifier system in the particular oil phase, apparently have an effect on the oil particle size stability of an emulsion of 15% oil content, which is subjected to freezing and thawing. The optimum thawing rate of a frozen emulsion varies with the melting point of the oil, provided the melting point of the oil is lower than the melting point of water, and with the type of emulsifying system used. Cottonseed oils with melting points of 10° and 49°C., respectively, had increases in particle size with three different rates of thaw. Oils with low melting-points are less affected by the type of emulsifier system than those with higher melting points, probably because of the solubility of the emulsifier system and the solid-liquid relationships existing in the oil phase at various temperatures.

Safflower oil emulsions are acceptable in particle size with at least two different emulsifier systems after freezing and thawing under conditions of slow, moderate, and rapid rates. Emulsions can be made with fractionated cottonseed oil, with either a phosphatide or nonphosphatide emulsifier system, not only to equal in particle size emulsions made with safflower oil but to thaw in the same manner as safflower oil.

An oil content of 30% in an emulsion did not prevent an increase in oil particle size after a freezing-thawing cycle. Such an emulsion creamed on thawing.

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