Original Article

Effect of high-pressure homogenization preparation on mean globule size and large-diameter tail of oil-in-water injectable emulsions

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Abstract

The effect of different high pressure homogenization energy input parameters on mean diameter droplet size (MDS) and droplets with > 5 μm of lipid injectable emulsions were evaluated. All emulsions were prepared at different water bath temperatures or at different rotation speeds and rotor-stator system times, and using different homogenization pressures and numbers of high-pressure system recirculations. The MDS and polydispersity index (PI) value of the emulsions were determined using the dynamic light scattering (DLS) method, and large-diameter tail assessments were performed using the light-obscuration/single particle optical sensing (LO/SPOS) method. Using 1000 bar homogenization pressure and seven recirculations, the energy input parameters related to the rotor-stator system will not have an effect on the final particle size results. When rotor-stator system energy input parameters are fixed, homogenization pressure and recirculation will affect mean particle size and large diameter droplet. Particle size will decrease with increasing homogenization pressure from 400 bar to 1300 bar when homogenization recirculation is fixed; when the homogenization pressure is fixed at 1000 bar, the particle size of both MDS and percent of fat droplets exceeding 5 μm (PFAT5) will decrease with increasing homogenization recirculations, MDS dropped to 173 nm after five cycles and maintained this level, volume-weighted PFAT5 will drop to 0.038% after three cycles, so the “plateau” of MDS will come up later than that of PFAT5, and the optimal particle size is produced when both of them remained at plateau. Excess homogenization recirculation such as nine times under the 1000 bar may lead to PFAT5 increase to 0.060% rather than a decrease; therefore, the high-pressure homogenization procedure is the key factor affecting the particle size distribution of emulsions. Varying storage conditions (4–25 °C) also influenced particle size, especially the PFAT5.

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1. Introduction

An emulsion is defined as “a system comprised of two immiscible liquids, one of which is dispersed as droplet (the dispersed or internal phase) throughout the other (the continuous or external phase)” [1]. An oil-in-water emulsion contains small oil droplets dispersed within a watery continuous phase, with each oil droplet surrounded by a protective coating of emulsifier molecules [2]. Clinically, lipid emulsions are mostly administrated by intravenous injection, and are commonly used for patients who require parenteral nutrition as a source of essential fatty acids. Lipid emulsions have recently been used as drug delivery vehicles for poorly soluble drugs. The quality control indicators of lipid-injectable emulsions include appearance, particle size and size distribution, pH value, Zeta potential and lipid oxidation. Particle size plays a key role in many emulsion properties such as appearance, rheology, color, texture, stability, and shelf-life [3]; various factors such as the formulation design including the selection of oil phase, water phase and emulsifier, and even the container of emulsions [4] may have an effect on particle size and size distribution. To produce emulsions with fine particle size and narrow size distribution, either a large amount of energy or surfactant or the combination of both is required. There are two methods to produce emulsions: low-energy emulsification and high-energy emulsification. Low-energy emulsification methods, such as the phase inversion temperature technique, involve transitional inversion induced by changing factors that affect the hydrophilic lipophilic balance (HLB) of the system [5,6]. This method has several limitations, including requiring a large amount of surfactants during preparation, and it cannot be used for large-scale industrial production [1]. High-energy emulsification utilizes mechanical devices such as high-pressure homogenizers, microfluidizers, and sonication methods, which can generate intense disruptive forces that break up the oil and water phases and lead to the formation of tiny oil droplets [7]. High-energy emulsification methods are applicable to industrial operations because there is flexible control of emulsion droplet size distributions, and the energy needed can generally be achieved in high-pressure systems [1]. In laboratory studies and most emulsion preparations, it is more efficient and convenient to produce emulsions in two steps: (1) conversion of separate oil and water phases into a coarse emulsion usually through rotor-stator devices; and (2) the final fine emulsion is then prepared using high-pressure systems.

Mean droplet size and size distribution are two significant parameters during lipid injectable emulsion preparation procedures [8,9]. In addition, fat droplets with diameters larger than 5 μm in injectable emulsions should also be taken into account because they are associated with emulsion stability and safety. When the volume-weighted percent of fat droplets exceeding 5 μm (PFAT5) exceed 0.4%, phase separation is visually observed [10]; however, a large PFAT5 can also cause safety problems, such as emboli in the lungs and abnormal liver function [11–13]. The United States Pharmacopeia (USP) emphasizes that “these two regions of the globule size distribution (MDS and PFAT5) must be controlled within specified limits” [14]. The USP has proposed specific globule size distribution limits in Chapter 729 [14]. It recommends two methods for particle size determination: Method I and Method II. Method I involves light-scattering techniques that are used for determination of the mean droplet size in lipid injectable emulsions, and MDS should not exceed 500 nm. Method II is a light obscuration (LO) or light extinction (LE) method that is used to determine the extent of the large-diameter droplet tail (> 5 μm) in lipid-injectable emulsions, and PFAT5 should not exceed 0.05% of the total lipid component.

MDS tends to decrease with increasing homogenization pressure and recirculation, but interestingly, higher pressures with longer emulsification times may lead to overprocessing, which may result in increased MDS [1,2,15–17]. However, the influence of overprocessing on PFAT5 has not been evaluated, because researchers have previously focused on the behavior of mean particle size rather than large droplet size. The aim of this study is to systematically examine the influence of the rotor-stator system, homogenization pressure, homogenization cycles, interval place time and different storage conditions on MDS and PFAT5, and to determine the key factor that affects MDS and PFAT5 during the emulsion preparation procedures.

2. Materials and methods

2.1. Materials

Soybean oil (long chain triglyceride; LCT) and medium chain triglyceride (MCT) were supplied by Zhonghang Pharmaceutical Company (LCT: Lot No. 13060105-2-01; MCT: Lot No. 120901-2-01, Tieling, China). Cholesterol was purchased from Nanjing Xinbai Pharmacy (Lot No. 121201, Nanjing, China). Glycerol was purchased from Jiahe Biotechnology Company (Lot No. 131207, Shantou, China). Poloxamer 188 was purchased from BASF Company (Lot No. WPCH530B, Germany). Soybean lecithin was purchased from Toshisun Biology & Technology Corporation (Lot No. 560400-2130332-01, Lipid GmbH, Germany). Hydrochloric acid was supplied by Beijing Chemical Works (Lot No. 20140316, Beijing, China). Double distilled water was used for the preparation of all solutions.

2.2. Preparation of the emulsions

Our laboratory had developed a novel Cremophor-free, autoclave stable, intravenous emulsion for paclitaxel, a paclitaxel–cholesterol complex was used as the drug carrier to improve the solubility of paclitaxel in the oil phase of emulsions [18]. Based on the existing ingredients and produce process of paclitaxel emulsions, the blank paclitaxel emulsions (without drug) would become the test object. At first, the cholesterol was dissolved in 20% MCT/LCT (mean chain triglyceride/soybean oil = 1:1 by mass) at 60 °C. Next, the aqueous phase consisting of soybean lecithin, poloxamer 188, and glycerol was uniformly dispersed at 60 °C in a water bath. Then, the coarse emulsion was prepared at 60 °C with high shear mixing using a Fluko homogenizer FA25 model (Fluko Equipment Shanghai Co. Ltd., China) by rapidly adding the oil phase to the aqueous phase at 10,000 rpm. The high shear
mixing process was carried out for 8 minutes at 19,000 rpm and the final emulsion was obtained by high-pressure homogenization using homogenization equipment (APV-2000, Germany) at 1000 bar for seven cycles. The temperature of the whole homogenization process was maintained below 40 °C in a cycle ice-water bath. The pH value was adjusted to 4.5 with 0.1M hydrochloric acid and the emulsion was finally transferred to vials and autoclaved using autoclave (ZDX-35SBI, Shen’an Medical Instruments, Shanghai) at 115 °C for 30 minutes.

2.3. Study design

Batches of emulsions were prepared using the same ingredients and different technological parameters. Coarse emulsions were prepared at different temperatures (25 °C, 40 °C, and 60 °C). The oil was homogenized to the aqueous phase using an Ultra Turrax mixer. In this process, different coarse emulsions were prepared using varying the rotation speeds (from 13,000 rpm to 19,000 rpm) or rotation times (from 4 minutes to 8 minutes). A batch of coarse emulsion (water bath temperature: 60 °C, rotation speed: 19,000 rpm, rotation time: 8 minutes) was placed for different times (30–120 minutes) to simulate the interruption incidentally during emulsion preparation, and then passed through a high-pressure homogenizer. Parts of coarse emulsions were homogenized using seven cycles but under different pressures in the range of 400–1300 bar, and other parts were homogenized under 1000 bar using different homogenization recirculations, from one to nine times. The homogenizing chamber was cooled using a cooling jacket containing cold water at approximately 5 °C, to slow the rise in temperature. The pH of each emulsion was adjusted to 4.5 using 0.1M HCl. After nitrogen purging, emulsions were aliquoted into several chambers. Each emulsion was carefully homogenized using an Ultra Turrax model. In this process, different factors that may affect the particle size were investigated during emulsion preparation, and then passed through a high-pressure homogenizer. Parts of coarse emulsions were homogenized using seven cycles but under different pressures in the range of 400–1300 bar, and other parts were homogenized under 1000 bar using different homogenization recirculations, from one to nine times. The homogenizing chamber was cooled using a cooling jacket containing cold water at approximately 5 °C, to slow the rise in temperature. The pH of each emulsion was adjusted to 4.5 using 0.1M HCl. After nitrogen purging, emulsions were aliquoted into several cylindrical glass tubes (volume, 10 mL; internal diameter, 20 mm; height, 40 mm) and sterilization at 115 °C for 30 minutes using an autoclave. Each emulsion was carefully collected and stored at 4 °C before particle size analysis. All factors that may affect the particle size were investigated during emulsion preparation. The emulsions were prepared as described in Table 1.

The particle size (MDS and PFAT) of most emulsions were analyzed after storage at 4 °C for 1 night. One batch of the emulsion (Lot No. 20140522-K), which was prepared using the standard technological parameters described earlier, was divided into several bottles. Some bottles were exposed to either the ambient environment or to high temperature (60 °C) for at least 10 days, and particle size was measured every 5 days. Other emulsion aliquots were stored at 4 °C, 15 °C, or 25 °C for at least 2 months, and particle size was measured for each month.

2.4. Analytical methods

The MDS and polydispersity index (PI) value of the emulsions were determined using the dynamic light scattering (DLS) method. The PI is an important parameter that can reflect the state of emulsion particle size distribution; a smaller PI value indicates a more concentrated particle size distribution. Usually both the MDS and PI are required to reflect the mean emulsion particle size. The diffractometer model used was the NiComp 380ZLS submicron particle sizer and the Zeta potential analyzer subsystem (Particle Sizing Systems, Santa Barbara, CA, USA) was equipped with 635 nm red laser diode and high-gain photomultiplier (PMT) detector, which detected sizes ranging from 0.5 nm to 6 μm. The emulsion was diluted to about 1/500 with distilled water in the diffractometer cell, stirred, and the diluted emulsion was measured for at least 5 minutes. Measurements were repeated twice for each sample. The diluted sample particle dispersed in solvent because the suspended particles move randomly as a result of brownian motion. A monochromatic coherent laser beam irradiated the suspension, the time scale of the fluctuation of scattering light intensity is dependent on the size of the particle. A small particle will “jitter” about in the solution relatively rapidly, resulting in a rapidly fluctuating intensity signal; by contrast, large particles will diffuse more slowly, resulting in a more slowly varying intensity. A fluctuating intensity signal is collected by the PMT detector at a certain angle, such as 90°, and it can “transport” the fluctuating intensity signal to the digital correlation processor. The diffusion coefficient D can then be obtained. From D, we can calculate the particle radius R using the well-known Stokes–Einstein relation:

$$D = k T/6\pi \eta R$$

where k is Boltzmann constant (1.38 × 10^{-16} erg K^{-1}), T is the temperature (K = °C + 273) and η is the shear viscosity of the solvent (e.g., η is the shear × 10^{-2} poise for water at 20 °C).

The large-diameter tail assessments were performed using the light-obscuration/single particle optical sensing (LO/SPOS) method. The AccuSizer 780APS automatic particle sizer (Particle Sizing Systems, Santa Barbara, CA, USA), equipped with an automatic dilution system and connected to a LE400-05 sensor in extinction mode that was previously calibrated using polystyrene latex spheres, was used to detect the large-diameter tails. The emulsion samples were removed from each container using an automatic bottle sampler and transferred to the dilution system. The applied dilution factor was set according to the oil concentration of each product to achieve an acceptable level of cumulative particle counts, which was approximately one third of the coincidence limit of the sensor (9000 particles/mL). Samples were run at least in triplicate and the mean value is reported. The calculations and technique for employing a particle counter to assess the number of large oil droplets present in a lipid emulsion have been described elsewhere [19,20]. Normalization of the data requires converting the results for each size channel to its equivalent spherical volume (ESV) using the following formula:

$$ESV = (\pi \times D^3)/6$$

where D is the diameter in centimeters of each size channel and ESV is expressed in cubic centimeters (cm^3). The number of particles is then multiplied by the ESV yielding a calculated total spherical volume (TSV) for a given channel of data:
The percentage of the fat concentration in the injectable emulsion formulations that exists as enlarged lipid globules is then calculated using the following formula:

\[
\%\text{Fat} = \frac{100 \times \frac{\text{TSV} (\text{cm}^3) \times \text{density (g/mL}) \times \text{dilution factor}}{\text{sample volume} (\text{cm}^3) \times \text{fat composition}}}{\text{density (g/mL}}} \tag{3}
\]

where the density of LCT/MCT is equal to 0.93 g/mL. The PFAT\text{5} value was calculated from data for the size distribution of globules > 5 \(\mu\text{m}\).

### 3. Results and discussion

The emulsion energy input parameters included water bath temperature, rotation speed, and time, which were related to coarse emulsion preparation, and homogenization pressure and recirculation, which were related to homogenization procedures. These parameters were chosen based on a previous study: rotation speed, exposure time, gap distance, and disk design where energy input parameters belonging to rotor-stator systems, and homogenization pressure, recirculation, and nozzle design belonged to the high-pressure systems [1]. Because the shearing head and homogenization machine are fixed, the gap distance, and the disk design and nozzle design are constant, the remaining parameters represent the key steps in whole emulsion preparation. The influence of different coarse emulsion and homogenization emulsion times on particle size was taken into consideration because, during emulsions preparation, coarse emulsions could not be homogenized or emulsions could not be nitrogen sealed in time. This was inevitable because of equipment failure, power failure, and other emergencies. Prepared emulsions presented as a milky liquid with a white color appearance, and the MDS, PI, and PFAT\text{5} were examined in the current study. The results for the coarse emulsion preparation process are presented in Table 2. Altering any of the three parameters (water bath temperature, rotation speed, and rotation time) resulted in a PFAT\text{5} value that ranged from 0.035% to 0.042%, a MDS that ranged from 170 nm to 180 nm, and a PI that fluctuated from 0.1 to 0.2. Changing the energy input parameters related to coarse emulsion seems to have almost no influence on MDS and the PFAT\text{5} value. We can ensure that altering the process of making coarse emulsions will not affect the MDS and PFAT\text{5} value, because there was a large decrease in the particle size when the coarse emulsions were passed through the high-pressure homogenization under certain pressures.

To investigate the influence of exposure to ambient temperatures for a period of time on coarse emulsion particle size (MDS and PFAT\text{5}), a batch of coarse emulsion was exposed to air at ambient temperature (with the cap cover in place to prevent contamination of the emulsion) for 30 minutes, 60 minutes, or 120 minutes, and then homogenized in chronological order. The results are presented in Table 2. The PFAT\text{5} value was close to 0.038%, within a fluctuation range of 0.04%. The MDS values were approximately 170 nm and the PI values were approximately 0.1. Many factors (such as determinative error) were taken into account, and there was almost no influence on MDS and the PFAT\text{5} value when the coarse emulsion was incubated at ambient temperature for 120 minutes before homogenization. The coarse emulsion was incubated

### Table 1 — Energy input parameters of 20% MCT/LCT injectable emulsions.

| Lot no. | Water bath temperature (°C) | Rotation speed (rpm) | Rotation time (min) | Time 1\textsuperscript{a} (min) | Pressure (bar) | Cycles | Time 2\textsuperscript{b} (min) |
|---------|-----------------------------|----------------------|---------------------|-----------------------------|---------------|--------|-----------------------------|
| 20140527-1 | 25                          | 19,000              | 8                   | 0                           | 1000          | 7      | 0                           |
| 20140527-J | 40                          | 19,000              | 8                   | 0                           | 1000          | 7      | 0                           |
| 20140522-K | 60                          | 19,000              | 8                   | 0                           | 1000          | 7      | 0                           |
| 20140519-7  | 60                          | 16,000              | 8                   | 0                           | 1000          | 7      | 0                           |
| 20140527-L  | 60                          | 13,000              | 8                   | 0                           | 1000          | 7      | 0                           |
| 20140522-M  | 60                          | 19,000              | 6                   | 0                           | 1000          | 7      | 0                           |
| 20140522-N  | 60                          | 19,000              | 4                   | 0                           | 1000          | 7      | 0                           |
| 20140519-30t | 60                          | 19,000              | 8                   | 30                          | 1000          | 7      | 0                           |
| 20140519-60t | 60                          | 19,000              | 8                   | 60                          | 1000          | 7      | 0                           |
| 20140519-120t | 60                         | 19,000              | 8                   | 120                         | 1000          | 7      | 0                           |
| 20140522-Q  | 60                          | 19,000              | 8                   | 0                           | 1300          | 7      | 0                           |
| 20140522-P  | 60                          | 19,000              | 8                   | 0                           | 700           | 7      | 0                           |
| 20140522-O  | 60                          | 19,000              | 8                   | 0                           | 400           | 7      | 0                           |
| 20140519-1  | 60                          | 16,000              | 8                   | 0                           | 1000          | 3      | 0                           |
| 20140519-3  | 60                          | 16,000              | 8                   | 0                           | 1000          | 5      | 0                           |
| 20140519-5  | 60                          | 16,000              | 8                   | 0                           | 1000          | 9      | 0                           |
| 20140519-9  | 60                          | 16,000              | 8                   | 0                           | 1000          | 7      | 30                          |
| 20140527-R30 | 60                         | 19,000              | 8                   | 0                           | 1000          | 7      | 60                          |
| 20140527-R60 | 60                         | 19,000              | 8                   | 0                           | 1000          | 7      | 120                         |
| 20140527-R120 | 60                        | 19,000              | 8                   | 0                           | 1000          | 7      | 120                         |

\textsuperscript{a} Time 1: the delayed time of coarse emulsion for homogenization.

\textsuperscript{b} Time 2: the delayed time of homogenization emulsions for autoclaved.
for different lengths of time, then passed for seven cycles under 1000 bar pressure. The MDS and PFAT₅ values are presented in Fig. 1. The mean droplet diameter decreased from 2373 nm when rotation ended to 168.7 nm after homogenization, 2373 nm after 30-minute incubation to 174.3 nm after homogenization, 1600 nm after 60-minute incubation to 177.9 nm after homogenization, and 1526 nm after 120-minute incubation to 166.5 nm after homogenization. The PFAT₅ value before homogenization was as high as 117%. It was 117% after 30-minute incubation, 109% after 60-minute incubation, and 96% after 120-minute incubation, and this PFAT₅ value decreased to 0.038%, 0.037%, 0.034%, and 0.042%, respectively, after homogenization. These results indicate that homogenization is particularly efficient at forming small droplet sizes in emulsions, and droplet disruption in rotor-stator systems is generally less efficient than high-pressure devices. Because the dispersing zones of rotor-stator systems usually have larger volumes, at constant energy density and volume flow rate, the mean power density in rotor-stator systems is lower than that in the nozzles of high-pressure devices [21].

No matter how coarse the emulsion is, when it was passed through the same homogenization recirculation under the same homogenization pressures, the same fine levels in both PFAT₅ and MDS can be attained. However, the rotor-stator system is still important because it can efficiently convert separate oil and water phases into macroemulsions with a fairly large droplet size to alleviate the wear and tear on the homogenizer. The Ultra Turrax model was widely used during emulsions preparation because it is simple to operate and it can produce a good dispersing effect at a low shear force (Table 2, Fig. 1).

During the high-pressure homogenization, emulsions were produced using a fixed number of seven homogenization cycles and the homogenization pressure was varied from 400 bar to 1300 bar. The MDS and PFAT₅ values are presented in Table 3. The PFAT₅ value of emulsions under 400 bar homogenization pressure was as high as 0.157%, which was almost three times the large diameter limit of 0.05%, and the mean droplet size increased to approximately 300 nm. The results showed that low pressure cannot produce a fine emulsion, and that MDS and the PFAT₅ value decrease with increasing homogenization pressure, whereas the value of PI remained at approximately the same level from 400 bar to 1300 bar. The results showed that increasing the homogenization pressure is an effective way to reduce the particle size for both MDS and PFAT₅, and the value of PI may be dependent on homogenization recirculation rather than homogenization pressure. The MDS and PFAT₅ value of the emulsions that were produced under 1000 bar homogenization pressure but using different numbers of homogenization recirculations (from 1 to 9) is presented in Table 3. When the results of a previous study are included, the PFAT₅ value of coarse emulsions was 117%. For the emulsion that was passed for one cycle under 1000 bar, the PFAT₅ decreased to 0.129%, which illustrated that high-pressure homogenization is particularly efficient at breaking large droplets into small ones. However, 0.129% was still above the PFAT₅ standard of 0.05%, and homogenizing only one cycle at very high pressure was not enough to form emulsions with an appropriate particle size; multiple recirculations were required to make fine emulsions. Results showed that after three cycles, the coarse emulsions PFAT₅ value was 0.039%. Increasing the recirculation times from five to nine

| Variables                      | MDS (nm) | PI       | PFAT₅% Average | SD   |
|-------------------------------|----------|----------|----------------|------|
| Water bath temperature (°C)   |          |          |                |      |
| 25                            | 176.7    | 0.139    | 0.036          | 0.003|
| 40                            | 173.2    | 0.123    | 0.031          | 0.002|
| 60                            | 179.4    | 0.150    | 0.038          | 0.002|
| Rotation speed (rpm)          |          |          |                |      |
| 13,000                        | 174.1    | 0.113    | 0.042          | 0.001|
| 16,000                        | 168.7    | 0.116    | 0.038          | 0.001|
| 19,000                        | 179.4    | 0.150    | 0.038          | 0.002|
| Rotation time (min)           |          |          |                |      |
| 4                             | 170.1    | 0.129    | 0.037          | 0.004|
| 6                             | 184.5    | 0.167    | 0.035          | 0.002|
| 8                             | 179.4    | 0.150    | 0.038          | 0.002|
| Coarse emulsions placed time (min) |      |          |                |      |
| 0                             | 168.7    | 0.116    | 0.038          | 0.001|
| 30                            | 174.3    | 0.092    | 0.037          | 0.003|
| 60                            | 177.9    | 0.124    | 0.034          | 0.001|
| 120                           | 166.5    | 0.102    | 0.042          | 0.001|

MDS = mean diameter droplet size; PFAT₅% = percent of fat droplets exceeding 5 μm; PI = polydispersity index; SD = standard deviation.
The particle size results of changing technical parameters increased. MDS and PFAT₅ results indicated three key messages: (1) the value of MDS and PFAT₅ decreases as the number of recirculations increases, and now the overprocessing may influence the overprocessing phenomenon through measuring the MDS value, and now the overprocessing may influence the PFAT₅ value. It needs to be stressed that the phenomenon of increased MDS that results from overprocessing was not a certainty, and there is a certain size below which MDS cannot be caused by poor stabilization of the newly formed droplets and is also a result of overprocessing [1]. In this research, the MDS did not increase with repeated emulsification. The value fluctuated around 170 nm, and this value was smaller than that of the commercially available emulsions, because poloxamer was added to our emulsions. This may also explain why there was no overprocessing after repeated homogenization recirculations.

Table 3 – The particle size results of changing technical parameters that were related to the process of homogenization.

| Variables                        | MDS (nm) | PI     | PFAT₅% | Average | SD  |
|----------------------------------|----------|--------|--------|---------|-----|
| Homogenization pressure (bar)    |          |        |        |         |     |
| 400                              | 284.2    | 0.146  | 0.157  | 0.015   |     |
| 700                              | 196.7    | 0.127  | 0.045  | 0.005   |     |
| 1000                             | 179.4    | 0.150  | 0.038  | 0.002   |     |
| 1300                             | 162.4    | 0.154  | 0.025  | 0.000   |     |
| Homogenization recirculation < 1000 bar |          |        |        |         |     |
| 1                                | 270.3    | 0.151  | 0.129  | 0.016   |     |
| 3                                | 216.3    | 0.160  | 0.039  | 0.007   |     |
| 5                                | 173.4    | 0.112  | 0.039  | 0.003   |     |
| 7                                | 168.7    | 0.116  | 0.038  | 0.001   |     |
| 9                                | 173.2    | 0.093  | 0.027  | 0.000   |     |
| Homogenization recirculation < 700 bar |          |        |        |         |     |
| 1                                | 280.3    | 0.181  | 0.181  | 0.004   |     |
| 3                                | 243.1    | 0.151  | 0.043  | 0.004   |     |
| 5                                | 213.3    | 0.135  | 0.021  | 0.003   |     |
| 7                                | 204.0    | 0.116  | 0.039  | 0.002   |     |
| 9                                | 201.1    | 0.100  | 0.038  | 0.001   |     |
| Homogenization emulsions placed time (min) |          |        |        |         |     |
| 0                                | 179.4    | 0.150  | 0.038  | 0.002   |     |
| 30                               | 172.5    | 0.109  | 0.030  | 0.002   |     |
| 60                               | 172.0    | 0.106  | 0.037  | 0.006   |     |
| 120                              | 171.3    | 0.119  | 0.038  | 0.002   |     |

MDS = mean diameter droplet size; PFAT₅% = percent of fat droplets exceeding 5 μm; PI = polydispersity index; SD = standard deviation.

a The value of some bottles is 0.060%.
b The value of some bottles is 0.083%.

Interestingly, in the process of particle size measurement, results from the same batch of emulsions may be different between different aliquots. The PFAT₅ value of some emulsion aliquots, which was produced under 1000 bar within nine cycles, increased quickly to 0.060%. When the same preparation process was repeated, the phenomenon stayed the same. This situation was also present in the emulsion that was produced under 700 bar within nine cycles; the PFAT₅ of some aliquots increased to 0.083%, whereas the situation was not present in the emulsion that was produced using other, lower homogenization pressures. Commercially available emulsions also exhibit the phenomenon that large droplet size in the same batch of emulsions may be different between different aliquots. This suggests that too many homogenization recirculations may increase the PFAT₅ value rather than decrease the PFAT₅ value under high pressure. As the homogenization pressure increases, an increased PFAT₅ value produced by repeated recirculation will become more and more common. This situation likely occurred as a result of ultra-high pressures that did not seem to benefit emulsification efficiency because of high recoalescence rates [1,2,15–17]. The emulsions droplet size is the result of equilibrium between the droplet breakup and recoalescence [1]. Between new droplet formation and its subsequent encounter with surrounding droplets, surfactants adsorb onto the created interface to prevent its recoalescence. If the timescale of surfactant absorption is longer than the timescale of collision, the fresh interface will not be completely covered and this will lead to recoalescence. This means that although the energy input during emulsification has been increased, the obtained emulsions have a larger droplet size rather than the expected smaller sizes. This phenomenon is called overprocessing [1]. Previous research demonstrated the overprocessing phenomenon through measuring the MDS value, and now the overprocessing may influence the PFAT₅ value. It needs to be stressed that the phenomenon of increased MDS that results from overprocessing was not a certainty, and there is a certain size below which MDS cannot be reduced with repeated emulsification. An increase in MDS can be caused by poor stabilization of the newly formed droplets and is also a result of overprocessing [1]. In this research, the MDS did not increase with repeated emulsification. The value fluctuated around 170 nm, and this value was smaller than that of the commercially available emulsions, because poloxamer was added to our emulsions. This may also explain why there was no overprocessing after repeated homogenization recirculations.

To determine whether sterilization in time is vital to emulsion particle size, three batches of emulsions were homogenized and then sterilized after a delay of 30 minutes, 60 minutes, or 120 minutes. The particle size results are presented in Table 3. There was almost no difference in the particle size (MDS and PFAT₅) of emulsions compared with the particle size of the emulsion that was autoclaved immediately after homogenization. The results indicate that the emulsion particle size after homogenization is stable within 120 minutes when it is exposed to the ambient environment without sterilization (Table 3).

The storage instructions for the commercially available emulsions are to refrigerate below 25°C but not to freeze. Each
The particle size results of the emulsions that were exposed to air and high temperature.

| Sample | Exposed to air | High temperature 60°C |
|--------|----------------|-----------------------|
|        | MDS (nm) | P.I. | PFAT₅% | Average | SD | MDS (nm) | P.I. | PFAT₅% | Average | SD |
| 0 d    | 179.4    | 0.150 | 0.038 | 0.002 | 178.4 | 0.148 | 0.038 | 0.002 |
| 5 d    | 167.7    | 0.131 | 0.047 | 0.003 | 169.9 | 0.113 | 0.038 | 0.002 |
| 10 d   | 178.7    | 0.114 | 0.043 | 0.002 | 172.3 | 0.124 | 0.052 | 0.002 |

MDS = mean diameter droplet size; PFAT₅% = percent of fat droplets exceeding 5 μm; P.I. = polydispersity index; SD = standard deviation.

Table 5 – The particle size results of the emulsions that were stored under different temperatures.

| Sample | 4°C Storage | 15°C Storage | 25°C Storage |
|--------|-------------|--------------|--------------|
|        | MDS (nm) | P.I. | PFAT₅% | Average | SD | MDS (nm) | P.I. | PFAT₅% | Average | SD | MDS (nm) | P.I. | PFAT₅% | Average | SD |
| 1 mo   | 179.4    | 0.121 | 0.030 | 0.002 | 170.5 | 0.114 | 0.032 | 0.001 | 175.3 | 0.121 | 0.101 | 0.001 |
| 2 mo   | 177.4    | 0.130 | 0.034 | 0.001 | 174.0 | 0.099 | 0.038 | 0.002 | 178.2 | 0.123 | 0.093 | 0.002 |

MDS = mean diameter droplet size; PFAT₅% = percent of fat droplets exceeding 5 μm; P.I. = polydispersity index; SD = standard deviation.
Conflicts of interest

The authors declare that there are no conflicts of interest.

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