Measurements of Size Distribution and Density of a Pharmaceutical Fat Emulsion, Using Field-Programmed Sedimentation Field-Flow Fractionation (SdFFF)

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Purpose. The main goal was to establish that sedimentation field-flow fractionation (SdFFF), operated with power based field programming, is effective in the characterization of a commercial emulsion, Medialipide®. This emulsion is used clinically for total parenteral nutrition and it is consisted of a mixture of long-chain triglycerides (LCT, soybean oil) with medium-chain triglycerides (MCT) emulsified by phospholipids. ***Methods.*** Different field programming methods were used in the analysis to establish the limits of applicability of the technique. ***Results.*** Identical size distribution profiles were obtained under various conditions of the analysis. The density of the droplets was determined by collecting fractions from the SdFFF eluting bands, and analyzing them by photon correlation spectroscopy. The value of density of the oil droplets was changed in the SdFFF data, until best agreement with the PCS values was achieved. The value of density corresponding to the best agreement was considered as the oil density, and it was closed to the weighted average value between soybean and MCT oils. ***Conclusions.*** Field programming extends the capabilities of sedimentation field-flow fractionation in handling and characterizing complex and delicate samples as Medialipide®.

KEY WORDS: sedimentation field-flow fractionation; field programming; fat emulsions; submicron; MCT; long-chain; medium-chain triglyceride; particle size distribution.

INTRODUCTION

Fat Emulsions

Fat o/w emulsions, i.e., phospholipid stabilized vegetable oil emulsions, are used for intravenous fat supply to patients and/or for carrying drugs (1, 2). Conventional formulations are consisted of long-chain triglycerides (LCT), the most common being soybean oil. During recent years new types of emulsions based on medium-chain triglycerides (MCT) were introduced as well, and appeared to be superior energy sources compared to LCT fat emulsions in many cases.

Emulsions in general are inherently unstable systems that tend to cream (settle) and coalesce (3). Both processes depend on physical properties, such as interfacial surface tension, ζ potential, density and viscosity of the two liquid phases (4-7). The mean size of the oil droplets and their size distribution can be indicative to the tendency of the emulsion to undergo destabilization processes. The mean size and size distribution of oil droplets of emulsions of pharmaceutical interest determine also the fate of the droplets in the living body.

The Technique—Field-Flow Fractionation

Sedimentation field-flow fractionation (SdFFF) has proven capable of separation and characterization of emulsions (7-10). Sedimentation is one mode in a group of separative field-flow fractionation (FFF) techniques that employ ultra-thin channels as the separation duct. The sedimentation mode combines sedimentation field with flow. The ultra-thin separation channel is integrated around the perimeter of a centrifuge rotor, so that the sedimentation field is applied across its two walls, an inner and outer wall. A flowing fluid carries the sample along this channel and the sedimentation field is perpendicular to the flow axis. The field forces the sample's components towards one wall, the accumulation wall. If the particles' density is higher than the mobile fluid, they accumulate at the outer wall of the channel, whereas if their density is lower, such as oil in water (o/w) fat emulsions, the particles accumulate at the inner channel wall. The accumulation of the sample particles near the wall affects their retention, and is related to their physical properties. In the case of sedimentation FFF it is the effective mass, or particle diameter and density. The rigorous relationship between the particle diameter and its retention enables the determination of size distribution by sedimentation field-flow fractionation.

The fat emulsion that was studied here, Medialipide®, consists of a 50:50 (w/w) physical mixture of two oils, medium chain triglycerides (MCT) and long chain triglycerides (LCT, soybean oil), emulsified by phospholipids. The emulsion is used for intravenous nutrition. Frequently the population of oil droplets in such emulsions can be polydisperse in size so that it would be advantageous to use field programming, in which the decrease of field strength with time gradually reduces the retention of the sample components. The gradient of field in the present work is based on a power function (11). Field programming extends the sensitivity of detection when relatively large particles are present in the sample along with small particles. Although SdFFF is a separative technique, the system, fluid carrier and channel, comprise a relatively gentle environment that does not inflict extreme risks of shear stress. Therefore, almost no changes of the original sample components were usually observed at various conditions of the analysis (9). Density of oil droplets was already measured before by combining SdFFF to photon correlation spectroscopy (PCS) using a constant sedimentation field (8). We demonstrated here that the same principles can be applied using power programming in SdFFF.

EXPERIMENTAL

Materials

The mobile fluid in the SdFFF system was made up of

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2.25% (w/v) glycerin in double distilled water with 0.0125% (w/v) sodium azide added as bactericide (refractive index 1.33 close to water's). The fluid was filtered through a 0.2 μm filter before use. Density of the solution was determined by pycnometer as 1.005 g/ml.

The emulsion, 20% Medialipide®, was a product of Laboratoires Bruneau (Boulogne, France) lot No. 3243A81, expiration date: June, 15, 1995. The emulsion consisted of 20% (w/v) of 50:50 (w/w) mixture of medium-chain and long-chain triglycerides. Density of the MCT oil, reported in the literature is 0.945 g/ml (4), and density of the LCT (soybean oil) is 0.917 g/ml. The average of the difference in density between the oil and the carrier fluid ($\Delta\rho$) would then be approximately 0.074 g/ml.

Another commercial emulsion that was used in this study for comparison was Intralipid 20% (w/w), a soybean fat emulsion, marketed by Kabi Vitrum (Sweden) date of expiration 1.1.93.

Instrumentation

A basic unit of particle and colloid fractionator, SedFFF model S101, equipped with a data station and control of RPM from FFFractionation Inc. (Salt-Lake City, Utah), capable of data acquisition and processing, was used for the fractionation. A 880-PU HPLC pump (Jasco, Japan) and a UV detector model LC-85B from Perkin Elmer (Norwalk, Connecticut, USA), detecting at 260 nm, completed the fully operating sedimentation field-flow fractionation system. Channel dimensions were 2 cm in breadth, 0.0254 cm in thickness and 90 cm in length. Radius of the rotor was 15.1 cm. Void volume, measured using various small molecular weight substances, was 4.6 ml. Stop-flow duration was 40 min in all the experiments, except for the operation with a constant field where it was 80 min.

Fractions were collected by a Pharmacia Frac-100 fractions collector (Bromma, Sweden). Size analysis of the fractions collected from the FFF instrument was done using the submicron particle analyzer Coulter model N4SD. Details of the operation of the SdFFF system as well as the measurements by photon correlation spectrometer are given in our previous publication (9).

RESULTS AND DISCUSSION

Principle of the Determination

The basic mechanism of the normal mode of retention in SdFFF has been described numerous times (7, 8, 11-14), therefore, only a short explanation will be given here for clarification.

The clouds of droplets, which are formed under the influence of the sedimentation field, move downstream at a velocity proportional to their mean thickness. The more compressed to the wall, the slower they move. Droplets of different sizes form clouds of different thickness, which move downstream at different velocities, thus separation takes place. The raw experimental data appears in the form of the detector response as a function of the elution time of sample components, i.e., the fractogram. The currently available SdFFF instruments manipulate the raw data automatically to obtain size distribution profile. The experimental retention times are converted to the respective retention parameters, λ, [the exact relationship is described in many earlier FFF publications (7, 8, 11-14)] and to diameters. The following parameters are given to the instrument: $\Delta\rho$, the difference in density between the suspending fluid and the sample components, flow rate, the void volume and w the channel thickness. The relative mass for each diameter is calculated according to the procedure described by Yang et al. (12). An output of the instrument, a report of droplet size distribution is shown in Figure 1.

Various Conditions of Field Decay

The details of operation under power field-decay are described in refs. (9-11), therefore, only a short explanation will be given here. In the power-based field programming the initial field strength S_0 is held constant for a period of time t_1 (time-lag). Subsequently, field strength is decreased over a period of time until it reaches a pre-chosen constant value (~1 g in this study). After t_1 has elapsed (at $t > t_1$) the field decays according to the expression:

$$S(t) = S_0 \left(\frac{t_1 - t_a}{t - t_a} \right)^p \qquad (1)$$

where S(t) is the field strength at time t, p is the variable of the power program and $t_a = -p\, t_1$ (11). In sedimentation FFF p = 8 is typically used to attain a uniform fractionating power. Comparison of the field strength as function of time,

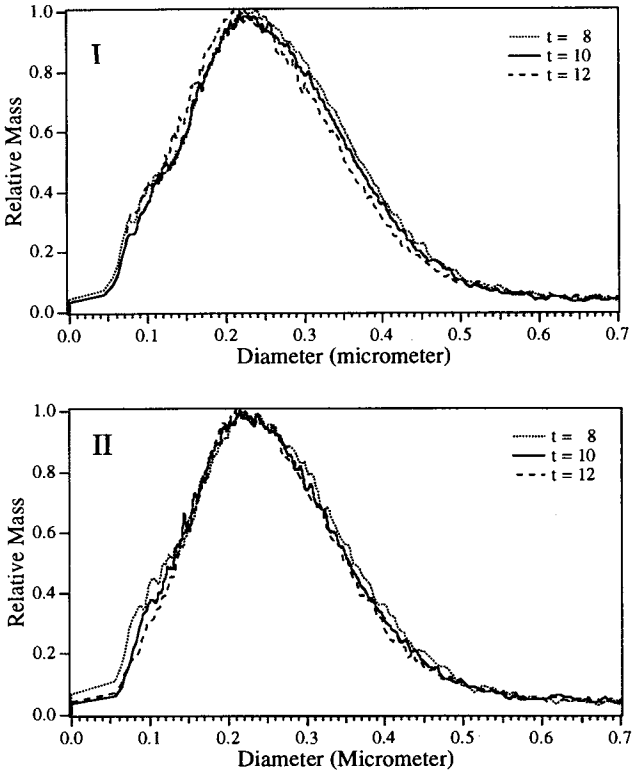

Fig. 1. Size distribution of the oil droplets of Medialipide® using three different rates of field-decay: **Program A.** $t_1 = 8$ min ($t_a = -64$ min); **Program B.** $t_1 = 10$ min ($t_a = -80$ min); **Program C.** $t_1 = 12$ min ($t_a = -96$ min). Initial field was 380 g and final field was approximately 1 g, flow rate = **I.** 1.5 ml/min, **II.** 2 ml/min.

calculated both from the RPM value given by the instrument at any moment in time, with the value calculated from Eqn 1, can be used as a test for the performance of the system (9). Comparison between the two calculations under various conditions showed that the SdFFF system used in this study performed very well.

The resolving power of the system is characterized by the fractionating power, F_d, the resolution between two close lying particles ($\delta t_r / 4\sigma_t$) divided by their relative diameter differences.

$$F_d = \frac{\delta t_R / 4\sigma_t}{\delta d / d} \quad (2)$$

t_R is the retention time, σ_t is the standard deviation in retention time for particles of diameter d, and δd is the difference in diameter between the two close lying particles. In power programming F_d is uniform over a wide diameters range when $p = 3n - 1$ (n = the exponent in the relation between d and the retention parameter, λ) (11). Equation 44 in ref. 11 fully describes the fractionating power F_d. In the presently operating SdFFF system when $n = 3$ and $p = 8$ it can be reduced to the following equation, provided that steric effects are neglected:

$$F_d = 0.171 \frac{(Dt^0)^{1/2}}{w} \left(\frac{1}{\lambda_0}\right)^{1/6} \left(\frac{t_l - t_a}{t^0}\right)^{4/3} \quad (3)$$

where D is the diffusion coefficient which can be calculated from the Stokes-Einstein equation using the measured d), t^0 is the void time, λ_0 is the retention parameter under the initial field.

Three different programs of the sedimentation field were tested for the analysis of Medialipide® at 1.5 and 2 ml/min: **Program A**: $t_l = 8$ min ($t_a = -64$ min); **Program B**: $t_l = 10$ min ($t_a = -80$ min); **Program C**: $t_l = 12$ min ($t_a = -96$ min). The rest of the experimental parameters, such as the initial (380 g) and final (1 g) field strengths, stop-flow duration, and rate of data acquisition, were identical in all three cases. The initial value of $\Delta\rho$ used for the calculation of these profiles by the instrument was 0.074 (see experimental section). The F_d values at the various conditions, calculated using Eqn 3, are specified in Table 1. The profiles of size distribution obtained by Program A, B, C at flow rates 1.5 and 2 ml/min nearly overlapped under the different rates of field decay. Similar results were reported earlier using other emulsions (9, 10).

Table 1. Values of F_d Used in the Analysis of Medialipide®. Three Different Programs of the Sedimentation Field Were Used at 1.5 and 2 ml/min: **Program A**: $t_l = 8$ min ($t_a = -64$); **Program B**: $t_l = 10$ min ($t_a = -80$); **Program C**: $t_l = 12$ min ($t_a = -96$). The Initial and Final Field Strengths Were 380 g and 1 g Respectively, Stop-Flow Duration was 40 min and $\Delta\rho$ Was 0.075 g/ml

	1.5 ml/min	2.0 ml/min
Program A	1.85	2.35
Program B	2.49	3.16
Program C	3.17	4.03

Measurement of Density of Oil Droplets

The composition of the oil from which the droplets are formed during the emulsification was a 50:50 (w/w) mixture of MCT and LCT (soybean). The preliminary value of $\Delta\rho$ (the difference in density between the continuous carrier fluid and the oil droplets) that was given to the SdFFF instrument for the calculations of diameters was 0.074 g/ml, an approximation of the average $\Delta\rho$ value of the two oils. Nevertheless, it was necessary to measure the density of the oil droplets.

It was assumed in this approach that the density of the oil droplets was constant over the entire range of diameters above the limit of applicability of the currently used SdFFF instrument. The assumption was based on findings by Westesen (6), who showed that the majority of the emulsion droplets in a model intravenous emulsion (Intralipid) have an ideal structure, i.e., an oil core covered by an emulsifier mono-layer. It was also shown in this work that the excess of emulsifier (phospholipid) is arranged in vesicles. We assumed here that the phospholipid mono-layer did not contribute significantly to the droplets' density, because liposomes (phospholipid vesicles) are neutral buoyancy colloids.

Methods for the determination of particle density from retention measurements at several carrier densities were described by Kirkland (13). In these methods a series of measurements of $1/\lambda$ as a function of the density of the fluid carrier gave a straight line with an intercept equal to the particle density. A method that requires changes in the carrier fluid' density might be problematic when emulsions are concerned. The oil droplets are stabilized by phospholipids and the composition of the continuous aqueous phase, in which they are suspended, should be controlled very carefully. Addition of various density-changing substances might affect the size characteristics of the original sample and introduce artifacts in the analysis.

A different approach to the measurement of density of the oil droplets was applied in the present work, based on the combination of photon correlation spectroscopy with SdFFF. The approach was first introduced by Caldwell, using a constant field (8). Fractions from the eluting SdFFF band were collected and characterized by photon correlation spectroscopy (PCS). The PCS diameters were then used to correct the value of $\Delta\rho$ that was used in the calculations of the corresponding SdFFF diameter.

The PCS instrument measures intensity-weighted size distribution, which is displayed as a histogram (the relative intensity of scattered light for each size). The intensity distribution can be converted by the PCS instrument to weight distribution (the relative weight of droplets of each size in the sample) using a known refractive index of the particles and the carrier fluid, and Mie equation. The histograms are analyzed for the mean size and standard deviation that are reported by the instrument.

The values given by the PCS were considered to be absolute values of diameters and the SdFFF diameters were compared to them, since the PCS measurement was not density dependent. The accuracy of the PCS measurement was considered high due to the relatively monodisperse population in the fractions from the FFF. The precision of the mea-

surements, however, was relatively low in the fractions that were taken away from the peak maximum, since the concentration of the oil droplets in these fractions gave rise to a sample count at the lower end of the PCS range of measurements.

The value of the SdFFF diameters for each fraction was taken from the mid-point of the two minute fractions. Each fraction contained 3 ml effluent (at 1.5 ml/min), or 4 ml effluent (at 2 ml/min). A set of diameters was given by the data system of the SdFFF instrument for the various fractions. These values were recalculated by changing the values of $\Delta\rho$ in steps of 0.005 g/ml, over the range of $\Delta\rho = 0.05$ to 0.1 g/ml, until they coincided with the values obtained from the PCS. The relation between d and $\Delta\rho$ is:

$$d^3 = \frac{6\Delta m}{\pi\Delta\rho} \tag{4}$$

The values of d_{FFF} in the fractions were multiplied by the factor:

$$d_{new} = d_{FFF}\left(\frac{\Delta\rho}{\Delta\rho_{new}}\right)^{1/3} \tag{5}$$

to accommodate the change in density. The various sets of SdFFF (d_{FFF}) diameters are shown in Figures 2 I - III (1.5 ml/min) and 3 I - III (2 ml/min). The Figures show the superposition of the average diameters in the collected fractions obtained by the two methods, photon correlation spectroscopy and sedimentation field-flow fractionation. The standard deviations of the PCS diameters are shown as error bars.

A considerable deviation of the PCS diameters from the SdFFF diameters was noticed around the void peak of the fractogram. These points were not included in the calculation of the SdFFF diameters because they were probably fractions containing phospholipid vesicles, which are suspected to be omnipresent in emulsions emulsified by phospholipids (6). In spite of their relatively large diameters ($\approx$ 200 nm) such vesicles would be poorly retained due to their neutral buoyancy. The actual composition of the fractions collected near the void SdFFF peak remains to be determined in future works.

The RMS values between the various sets of the SdFFF diameters (d_{FFF} in Figures 2, 3) and sets of PCS diameters [by intensity ($PCS_{int.}$ in Fig. 2, 3) and by weight ($PCS_{wt.}$ in Fig. 2, 3)] were calculated as the following:

$$\text{RMS} = \sqrt{\sum_{1}^{n} \frac{(d_{FFF} - d_{PCS})^2}{n}} \tag{6}$$

The best RMS values between the SdFFF diameters and the PCS diameters both by intensity and by weight were obtained for $\Delta\rho = 0.075$ g/ml (d_{FFF} *chosen* in Fig. 2, 3). The value $\Delta\rho = 0.075$ g/ml was finally used for the determination of size distribution of the emulsion under the various conditions. The final size distribution profiles calculated using this value are shown in Figure 1. The density of the 50:50 (w/w) mixed medium-chain and long-chain triglycerides oil is therefore 0.93 g/ml.

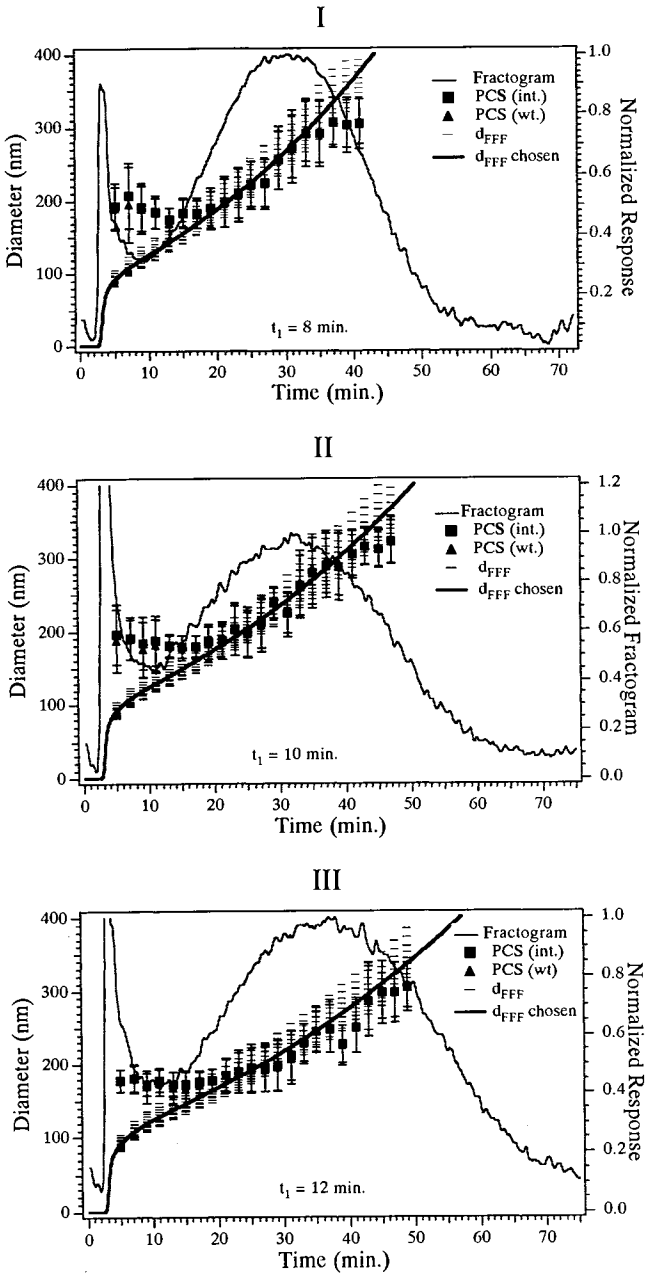

Fig. 2. Average diameter of droplets in the fractions, collected from the SdFFF instrument during elution, measured by SdFFF and by PCS, *int.* - from the intensity distribution and *wt.* - from the weight distribution. **Program A; II. Program B; III. Program C** as specified in Figure 1, flow rate was 1.5 ml/min.

Limits of Applicability

The agreement between PCS diameters and SdFFF diameters was best (RMS ~ 1.5%) when relatively high fractionating powers were used, i.e., $F_d \geq 2$ (see Table 1). The poorest agreement, especially at the trailing edge, was obtained at $F_d \approx 1.8$ (t_1 = 8 min and flow rate = 1.5 ml/min). It seems from work done so far on the analysis of emulsions by power programming SdFFF that deviations between the SdFFF diameters and the PCS diameters at the trailing edge of the profile were observed when $F_d \approx 1.5$ when S_0 = 108 g (9), and $F_d \approx 1.8$ when S_0 = 380 g in this work and a

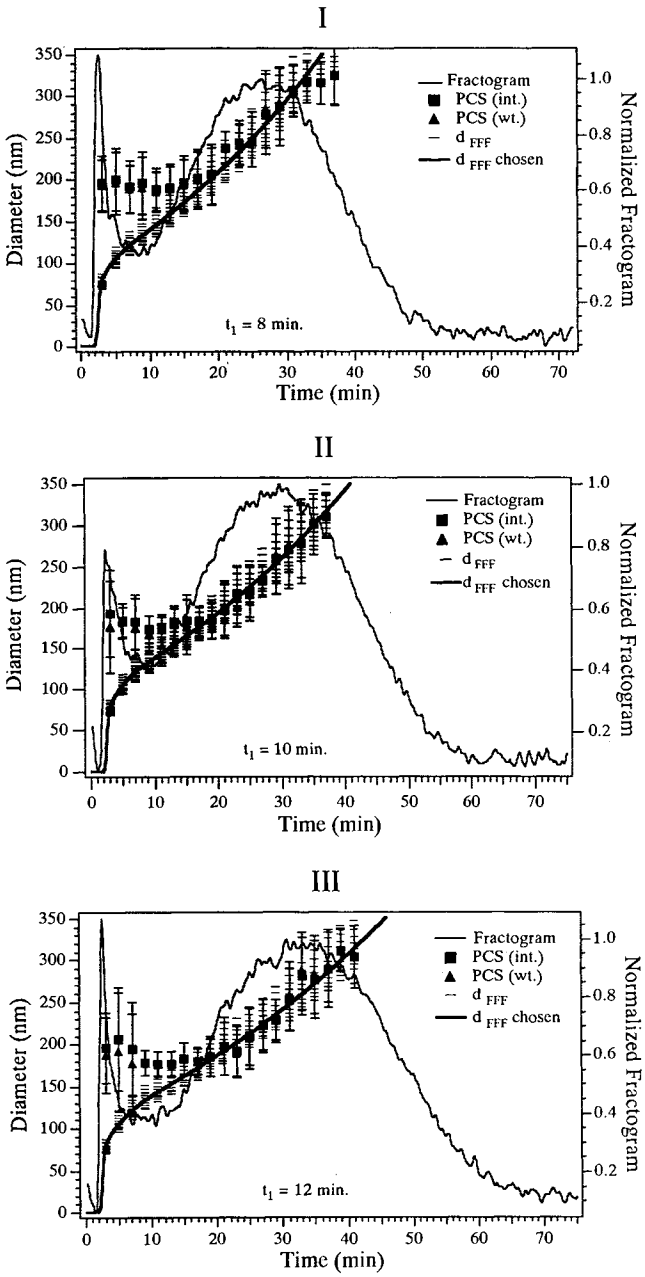

Fig. 3. Average diameter of droplets in the fractions, collected from the SdFFF instrument during elution, measured by SdFFF and by PCS, *int.* - from the intensity distribution and *wt.* - from the weight distribution. I. Program A; II. Program B; III. Program C as specified in Figure 1, flow rate was 2 ml/min.

previous one (10). The overestimation of the diameters by SdFFF under these conditions may result from non sufficient relaxation during field decay (secondary relaxation). The theory of power programming suggests that it is preferable to operate the system at higher fractionating powers to obtain more accurate results (11).

The agreement between the d_{mean} of the droplets in the collected fractions determined in the intensity and weight modes by the PCS instrument indicates that light scattering distortions are relatively small in this emulsion, therefore, the SdFFF detector signal is also not expected to be distorted significantly.

Domination of Polydispersity on the SdFFF Profile

The width of the eluting band of droplets is used to characterize the size distribution. The detector response as a function of time (fractogram), describes a wide spread band for the fat emulsion. As a rule, the sample zone is dispersed as a result of migration along the channel length L under the influence of the perpendicular field. The extent of dispersion is related to the *plate height,* H, or *height equivalent to a theoretical plate,* HETP. For uniform channels $H = \sigma^2/ L$ (σ^2 is the variance of the profile).

The accurate measurement of the dispersion is complex, *but it is necessary to understand it, in order to obtain an accurate size distribution profile.* There are two major contributions to the broadening of the SdFFF peak, non-equilibrium effects and sample polydispersity, as expressed in the following equation (14):

$$H = \frac{3\pi\eta d w^2 \langle v \rangle 24\lambda^3}{kT} + 9L\left(\frac{\sigma_d}{d_p}\right)^2 \qquad (7)$$

where η is the viscosity of the fluid carrier, $\langle v \rangle$ is the average velocity of the flowing streams, and σ_d/ d_p is the polydispersity. A typical apparent σ_d/ d_p value for Medialipide® was 0.41. The contribution of non-equilibrium effects constitutes the first term in the equation, and is highly sensitive to the retention parameter λ. Peak dispersion can be measured at several flow velocities to obtain H as a function of $\langle v \rangle$. The polydispersity of the sample components can be sorted out from the intercept of this curve using Eqn 7. A constant field (750 RPM, 95 g) was applied at two different flow rates, 0.5 and 2 ml/min. The two profiles obtained from these two runs overlapped each other, and a fit to Gaussian showed $\leq$ 2% difference between their σ values. Therefore, it was assumed that polydispersity dominated the profile of Medialipide®.

A supporting evidence to the domination of polydispersity in the emulsion peak came from the characterization of fractions, collected from both sides of the center of gravity of the eluting SdFFF band, by photon correlation spectroscopy (PCS). If the FFF band reflects the actual distribution of sizes, the SdFFF diameters in the collected fractions would agree with the PCS diameters not just near the maximum of the peak, but also on both its sides. When the oil droplets are driven away from the center of gravity of the band due to non-equilibrium and/or diffusive effects and not due to differences in their size, the SdFFF diameters are smaller than the PCS diameters in the fractions preceding the peak maximum and larger than them in the fractions succeeding it. The good agreement between SdFFF and PCS on both sides of the peak maximum, as shown in Figures 3 II and III, indicates that polydispersity is dominant in the SdFFF profile of the Medialipide®. The deviations at the trailing edge of the profile, which were discussed in section II, occur at $F_d \leq 3$ where nonsufficient relaxation during field decay may have caused overestimated diameters of the larger droplets. In addition, the deviations near the void peak, which were also discussed in section II, were not taken into account in this consideration because they are suspected to contain phospholipid vesicles in addition to oil droplets.

Comparison to the Non-Fractionated Sample

The results from the SdFFF instrument after a fit to Gaussian distribution were d_{mean} = 0.246 μm, and σ / d_{mean} = 0.35 using a constant field, and d_{mean} = 0.241 μm and σ / d_{mean} = 0.40, using power field programming (Programs A, B and C at flow rate 2 ml/min).

Occasionally a size distribution profile of the original sample of Medialipide® was measured by photon correlation spectroscopy. The average diameter was around 0.230-0.250 micrometer, close to the result given by the SdFFF system, however, there was relatively high variability of the mean size and the size distribution between measurements. Narrow, high or bimodal distributions were reported on various occasions, given identical input. It seems that the polydispersity of the non-fractionated sample gave false results of size distribution profile by the photon correlation spectroscopy instrument. Similar behavior was observed with Intralipid (9), which is also relatively polydisperse, but not with a MCT emulsion (10), which is much less polydisperse.

In contrast to the PCS measurements, the reproducibility of the measurements of average diameter as well as of profiles of size distribution obtained by SdFFF are typically very high, even on relatively high polydisperse samples (9). Analysis of the samples was repeated numerous times, and identical size distribution profile was obtained in each case.

Comparison to Intralipid

The profile of size distribution of the Medialipide® was compared to a previously characterized soybean based commercial fat emulsion, Intralipid (9). The two emulsions (20% w/v oil) were analyzed at the same conditions: the initial field was 380 g (1500 RPM), flow rate was 2 ml/min, final field was 1 g, and initial time lag was t_1 = 8 min. The fractograms are shown in Figure 4 I and the corresponding profiles of size distributions are shown in Figure 4 II. The profiles were fitted to Gaussian and the results of mean diameter and the peak broadening are given in Table 2. The commercial emulsions were rather polydisperse in size, and the mean droplet size was higher in the LCT emulsion. It is ill-advised to compare the two emulsions based just on the composition of oil, since the method of preparation is different, leading to different mean diameters. Nevertheless, it was noteworthy that the polydispersity was relatively high and was almost identical in the two emulsions.

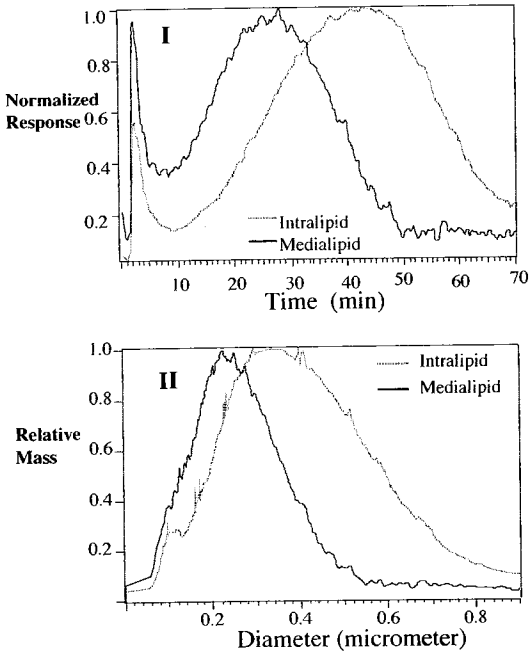

Fig. 4. Characterization of two sub-micrometer o/w fat emulsions, Intralipid and Medialipide. I. Fractograms; II. Droplets size distribution. Conditions: Flow rate = 2 ml/min, initial field strength = 380 g (1500 RPM), final field strength = 1 g, t_1 = 8 min (t_a = −64).

Table 2. The Mean Diameters and Standard Deviation of the Fit to Gaussian of the Profiles of Size Distribution of the Two Fat Emulsions (Medialipide® and Intralipid) Shown in Figure 4

	d_{mean}	σ	σ/d_{mean}
Medialipide®	0.247	0.103	0.42
Intralipid	0.377	0.155	0.41

CONCLUSION

When characterizing an unknown sample of sub-micrometer emulsion by SdFFF, the analysis requires a previous knowledge of the difference in density between the oil droplets and the suspending solvent, Δρ, and the geometrical parameters of the operating system. When the exact value of oil density is not known, such as the case of mixed oil emulsions, an initial guess of Δρ can be given to the SdFFF data system and a preliminary profile of size distribution can be calculated. The exact value of Δρ can be then recalculated by combining SdFFF with photon correlation spectroscopy (PCS). The calculations of the corrected SdFFF diameters should be then reiterated to obtain the final profile of size distribution of the mixed oil's emulsion.

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