# Characterization of a Double Emulsion System (oil-in-water-in-oil emulsion) with Low Solid Fats: Microstructure

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**ABSTRACT:** A double emulsion system [oil-in-water-in-oil (O/W/O)] with 16.3% (w/w) water and 83% (w/w) oil was prepared and stabilized using a novel method of mixing two oil-in-water (O/W) emulsions together. The first emulsion consisted of 85% (w/w) liquid canola oil, 14.4% (w/w) water, 0.5% (w/w) sodium caseinate, and 0.1% (w/w) lecithin and the second emulsion contained 73% (w/w) canola oil, 8% (w/w) palm–cotton stearin (50:50), 0.2% (w/w) lecithin, 18.2% (w/w) water, and 0.6% (w/w) sodium caseinate. Mixing the two emulsions (50:50) by weight produced a product with 79% (w/w) liquid canola oil and 4% (w/w) palm–cotton stearin. The two O/W emulsions were prepared separately at 50°C, mixed together at 45°C for 2–5 min, and then supercooled in a –5°C ice/salt bath while mixing at low shear rates (2,000–3,000 rpm). Under supercooling conditions the fat globules in the second emulsion (containing liquid oil and stearin) began to break down as a result of fat crystal growth and shearing action and release plastic fat. During this stage, the continuous aqueous phase underwent a phase transition and the emulsion viscosity dropped from 37,000–50,000 to 250 cP. The released plastic fat continued to harden as the temperature dropped and stabilized the first O/W emulsion (containing only liquid oil). The low shear rate mixing was stopped when the temperature dropped below 15°C and before the O/W/O emulsion hardens. Microstructural analysis of the first emulsion before and after supercooling showed essentially intact fat globules. The microstructure of the second emulsion before supercooling showed the same intact globules as the first emulsion, but after supercooling, an amorphous mass with only a few intact globules was seen. By mixing the two emulsions together and supercooling, a stable O/W/O emulsion was formed with plastic fat as the continuous phase and the first O/W emulsion as the dispersed phase.

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**KEY WORDS:** Double emulsion, microstructure, O/W emulsion, O/W/O emulsion, palm–cotton stearin, supercooling.

An oil-in-water-in-oil (O/W/O) emulsion is a double emulsion system in which the dispersed phase is an oil-in-water (O/W) emulsion, and the continuous phase is oil or plastic fat (1). Because of its limited application in food products, comparatively little work has been done on the O/W/O emulsion microstructure, although some low-fat spreads do have similar structures (2,3). More work has been reported on emulsions with two-liquid phases plus a solid phase at the O/W interface (three-phase emulsion) (4). These emulsions are sometimes referred to as Pickering emulsions and have been documented for almost a century (5–8). In these emulsions solid particles adsorb at the oil–water interface and stabilize the emulsions against coalescence.

The stabilization of an O/W/O emulsion is not straightforward because the hydrophobic moieties of the water-phase emulsifier in the O/W emulsion will be positioned on the oil droplet surface with the polar moieties positioned in the water phase surrounding the oil droplets (9). To form an O/W/O emulsion, the stabilized O/W droplet must be surrounded by oil, which will require the stabilization of a second W/O interface with an additional oil emulsifier. Moreover, since the continuous phase in an O/W emulsion is water, the O/W emulsion cannot be easily dispersed in oil or plastic fat due to differences in their surface tension, wettability, and polarity. A method has been developed to disperse an O/W emulsion in plastic fat and stabilize the resulting O/W/O emulsion.

Although the microstructures of emulsions and fat-based products have been discussed by many authors (10–12), the O/W/O emulsions with their unique structure merit investigation. The objectives of this study were to prepare and characterize a stable O/W/O emulsion (having plastic fat properties) by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal scanning laser microscopy.

## EXPERIMENTAL PROCEDURES

*Materials*. Sodium caseinate [Alanate 180; New Zealand Milk Products Inc., Santa Rosa, CA; 92.7% protein (N × 6.38), 3.5% ash, 4.2% moisture, 0.8% fat, 0.1% lactose, and <0.01% antibiotics, based on the product specification (Cat. no. 13C180)] was the water phase emulsifier. Cotton stearin; refined, bleached, and deodorized canola oil; and soy lecithin were obtained from Acatris, Inc. (Oakville, Ontario, Canada). Palm stearin was obtained from CanAmera Foods (Toronto, Canada). Fluorol Yellow 088 (Solvent Green 4) was purchased from Sigma Co. (Oakville, Ontario, Canada).

*Methods*. Two different O/W emulsions (**A** and **B**) were used to prepare the O/W/O emulsion. The basic formulations for emulsions **A** and **B** are given in Table 1. Emulsion **A** contained liquid oil in the fat phase, and emulsion **B** contained liquid oil and stearin.

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**TABLE 1**
**Composition of Emulsion A and B in O/W/O Emulsion**

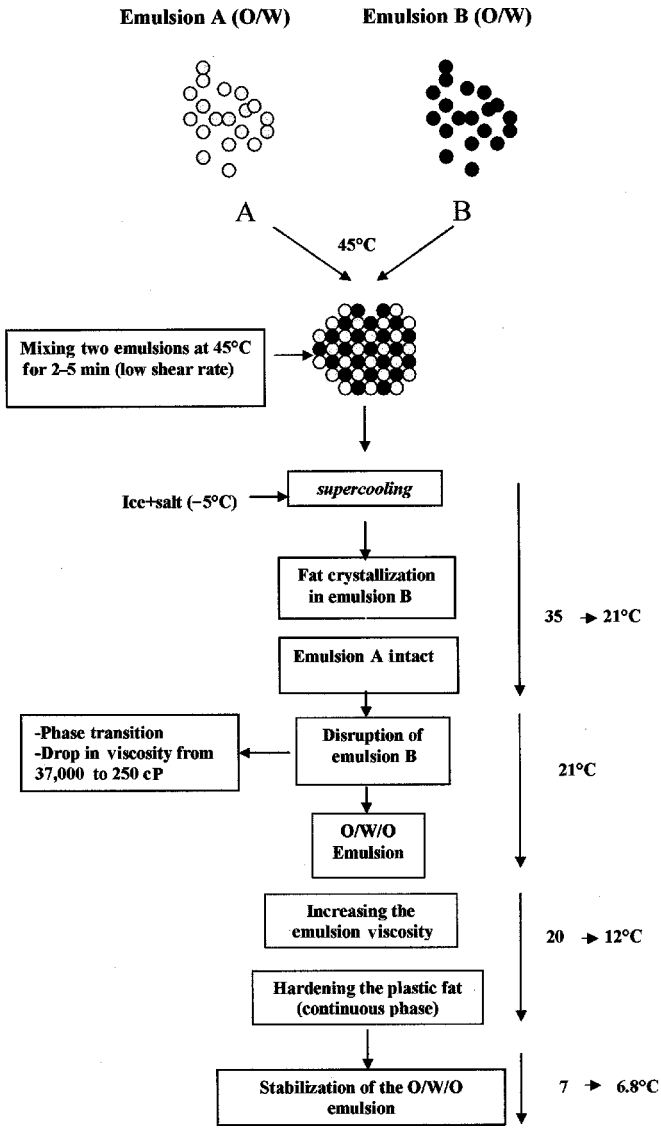
| Ingredients | Emulsion **A** (%) | Emulsion **B** (%) | O/W/O[a] emulsion (%) |
|---|---|---|---|
| Water | 14.4 | 18.2 | 16.3 |
| Liquid canola oil | 85 | 73 | 79 |
| Sodium caseinate | 0.5 | 0.6 | 0.55 |
| Palm–cotton stearin | — | 8 | 4 |
| Lecithin | 0.1 | 0.2 | 0.15 |
| Total | 100 | 100 | 100 |

[a]O/W/O, oil-in-water-in-oil.

*(i) Emulsion A*. Emulsion **A** is an O/W emulsion containing sodium caseinate, water, and canola oil (no added solid fat). One hundred grams of emulsion **A** was prepared by mixing sodium caseinate (0.5 g) and water (14.4 g) at 50°C in a 150-mL beaker. In a separate container a mixture of liquid canola oil (85 g) and lecithin (0.1 g) was heated to 50°C and then poured into the aqueous phase at a rate of approximately 20 mL/min, while homogenizing with a Tissumizer (Tekmar, Cincinnati, OH) at 20,000 rpm. During the addition of the oil, the emulsion viscosity increased dramatically to 37,000–50,000 cP, forming a creamy emulsion that was stable for at least 24 h at RT. This emulsion was kept at 45°C for 10 min while emulsion **B** was prepared.

*(ii) Emulsion B*. The procedure to make 100 g of emulsion **B** was similar to that of emulsion **A**. The aqueous phase, consisting of 18.2 g water and 0.6 g sodium caseinate, was mixed at 50°C in a 150-mL beaker and blended with a Tissumizer (Tekmar) at 20,000 rpm. The oil phase consisted of 73 g canola oil, 8 g palm–cotton stearin (50:50) and 0.2 g lecithin. The stearin was stirred into the liquid oil at 70°C until no visible solids remained in the mixture, then cooled to 50°C just prior to homogenization with the aqueous phase. The oil phase was poured into the aqueous phase at a rate of approximately 20 mL/min, while homogenizing with a Tissumizer (20,000 rpm). The temperature was maintained at 45–50°C during homogenization to prevent the crystallization of stearins.

*(iii) Preparation of O/W/O emulsion*. Emulsions **A** and **B** were mixed (100 g each) at 45°C in a 400-mL beaker with a Tissumizer set at 6,000 rpm for 2–5 min to completely disperse the oil droplets from the two emulsions into one uniform mixture (Scheme 1). The emulsion mixture was placed into a water bath containing ice and salt (–5°C) to produce supercooling conditions while mixing under a low shear rate (3,000 rpm). As the sample temperature dropped below the stearin/oil mixture crystallization point, fat crystals started to form in emulsion **B** droplets and disrupt the globules. At this stage, the viscosity of the mixture dropped sharply from 37,000–50,000 to 250 cP, indicating the occurrence of a phase transition. During the phase transition, the continuous aqueous phase was inverted from water to oil. In an O/W emulsion, the aqueous phase is conductive, but the conductivity will drop if a phase inversion occurs or the emulsion system breaks down (13–15). The electrical conductivity was measured during the mixing of the two O/W emulsions, and it dropped from 240 to 0.1 mA at the point where the phase inversion occurred. As the sample continued to cool, the released fat continued to crystallize and form a plastic fat matrix that surrounded and stabilized the emulsion **A** globules.




**SCHEME 1**

*Cryo-SEM*. A Hitachi S-570 Scanning Electron Microscope (SEM) equipped with an EM Scope SP-2000 A cryo preparation system (Ashford, Kent, United Kingdom) was used to investigate the microstructure of the emulsions. Approximately 0.1 g of emulsions **A** and **B** at two time intervals (immediately after preparation, and after holding for 15 min at –5°C) and 0.1 g of the O/W/O emulsion (within 30 min after preparation) were mounted on brass sample holders containing two holes and drawn into the liquid nitrogen. The frozen samples were freeze-fractured at less than –130°C and sublimated at –80°C for 50 min, then sputter-coated with 20 nm of gold in the cryo unit and examined at –130 to –170°C. The samples were examined at an accelerating voltage of 10 kV, and the micrographs were recorded on Quartz PCI software (Vancouver, Canada).

*Transmission electron microscope (TEM)*. A Balzer 360 M freeze-etching instrument and a Phillips EM 300 Transmission Electron Microscope were used to study the globule interface. Emulsion **A** (20 µL) was placed on the gold freeze etch planchet and immediately frozen by inserting it into liquid propane at −196°C. Samples were fractured at −100°C and coated with platinum (20 nm) and carbon (20 nm) for 7 s. The organic compounds were oxidized and removed from the replica at room temperature (20°C) by using $H_2SO_4$ and Javex (sodium hypochlorite), respectively, then washed with water and transferred onto a 400-mesh copper grid for TEM analysis.

*Confocal scanning laser microscopy*. A Leica microscope (model DMR BE; Mannheim, Germany) equipped with a Leica TCS 4D confocal system, krypton-argon laser, and 100× Plan-Apo lens was used to study the O/W/O emulsion. The excitation wavelength was 488 nm, and the emission barrier filter was adjusted at 515 nm. The O/W/O emulsion was prepared as described above, using a liquid oil stained with 0.01% Fluorol Yellow 088. Approximately 0.1 g of the emulsion (1 h after preparation) was introduced onto the microscopic slide at 20°C, covered with a cover slip, and visualized by Sony CCD digital images.

*Emulsion particle size*. A Mastersizer X (Malvern Instrument, Malvern, United Kingdom) with a small sample presentation unit equipped with the computer NEC PowerMate SX/16I and printer Okidata microline 320 was used to measure the particle size of emulsion **A**. Immediately after preparation, approximately one drop of the emulsion was added to the bath chamber and mixed with Millipore water and circulated at 20°C. The particle size was measured at 2.4 nm beam length, and volume distribution and pattern were calculated by computer between 1 and 100 µm.

*Viscosity*. The viscosity of emulsions (**A**, **B**, and O/W/O) was determined by the method of Konstance and Strange (16) immediately after preparation and when phase transition occurred. The apparent viscosity of the emulsion was measured with a Brookfield viscometer (Model RVF), using a shear rate of 10 and spindle number 6.

*Dropping point*. A Mettler FP83 automatic dropping point apparatus equipped with a Mettler FP-80 control processor was used for measuring the dropping point of emulsions **A**, **B**, O/W/O, and four commercial margarines with similar hardness. Samples were held in the sample holder at –22°C for at least 90 min prior to measurement, then adjusted to 5°C below the expected dropping point and heated at 1°C/min as recommended by the manufacturer. Each dropping point was replicated twice.

## RESULTS AND DISCUSSION

An O/W/O emulsion with 79% liquid oil and 4% hard fraction was prepared by mixing two O/W emulsions (**A** and **B**) together. The main factor stabilizing the O/W/O emulsion was the insertion of fat crystals (in the form of plastic fat from disrupted emulsion **B** globules) between the emulsion **A** globules.

*Emulsion **A***. Emulsion **A** holds about 54% of the liquid oil in the final O/W/O emulsion and must remain intact if a stable O/W/O emulsion is to be made. Emulsion **A** is inherently unstable owing to its high liquid oil content, but it showed good emulsion stability because of its high viscosity. As more liquid oil is incorporated, the number of globules per unit volume increases, which promotes protein–protein interactions at the interface and as a consequence increases emulsion viscosity. The fat globule size, physicochemical characteristics of the membrane, and the concentration of globules are also important factors affecting the stability of the O/W emulsion.

Figure 1 is a cryo-SEM micrograph of emulsion **A** immediately after preparation, showing the densely packed globules. Particles between 0.1 and 13 µm make up 55% of the total globule number, whereas particles between 13 and 52 µm make up the rest. This wide particle size distribution is common with the Tissumizer. A closer examination of the cryo-SEM micrograph shows that the oil droplets (dark areas) have a protein–water coat (light areas), indicating that the aqueous phase surrounds the fat globules (Fig. 1A). The globules are so dense that in some areas they stick together, flocculate, and prevent free movement. Some globules also show the early stage of coalescence, which causes emulsion instability. Figure 1B is a cryo-SEM micrograph of emulsion **A** after 15 min in an ice/salt bath (–5°C). The globules show little evidence of disruption or loss of emulsion stability. The stability of emulsion **A** globules depends on the formation of a strong membrane at the water–oil interface. Figure 2A is a freeze-etch TEM micrograph of emulsion **A** immediately after preparation. The micrograph shows what appears to be the start of coalescence of two globules, but a closer examination of the boundary (Fig. 2B) indicates that a thin layer (membrane) separates the two oil phases and that the globules are flocculated. The strong membrane produced by sodium caseinate and bound water around emulsion **A** globules stabilizes the emulsion and overcomes the destabilizing effects of flocculation. The liquid oil droplets have an amorphous structure and are surrounded by a thin aqueous continuous phase.

*Emulsion **B***. The microstructure of emulsion **B** was also studied by cryo-SEM. The micrograph of the emulsion immediately after preparation shows intact globules that are very similar in appearance to emulsion **A** (Fig. 3A). Although emulsion **B** was produced in the same manner as emulsion **A**, it was less stable than emulsion **A**. Emulsion **B** held about 46% of the total liquid oil and 100% of the hard fraction (stearin). Emulsion **B** when prepared at 50°C had physical properties similar to emulsion **A**, with high viscosity (45,000–50,000 cP) and strong conductivity (240 mA) indicating the formation of an O/W emulsion. To investigate the behavior of emulsion **B** under supercooling conditions, the emulsion was cooled to –5°C while stirring at a low shear rate (2,000–3,000 rpm). As the emulsion temperature dropped and fat crystals started to form, some of the globule membranes were pierced by the growing fat crystals. Other studies showed similar effects of fat crystals on the instability of O/W emulsions prepared with tristearate and paraffin (17,18). The

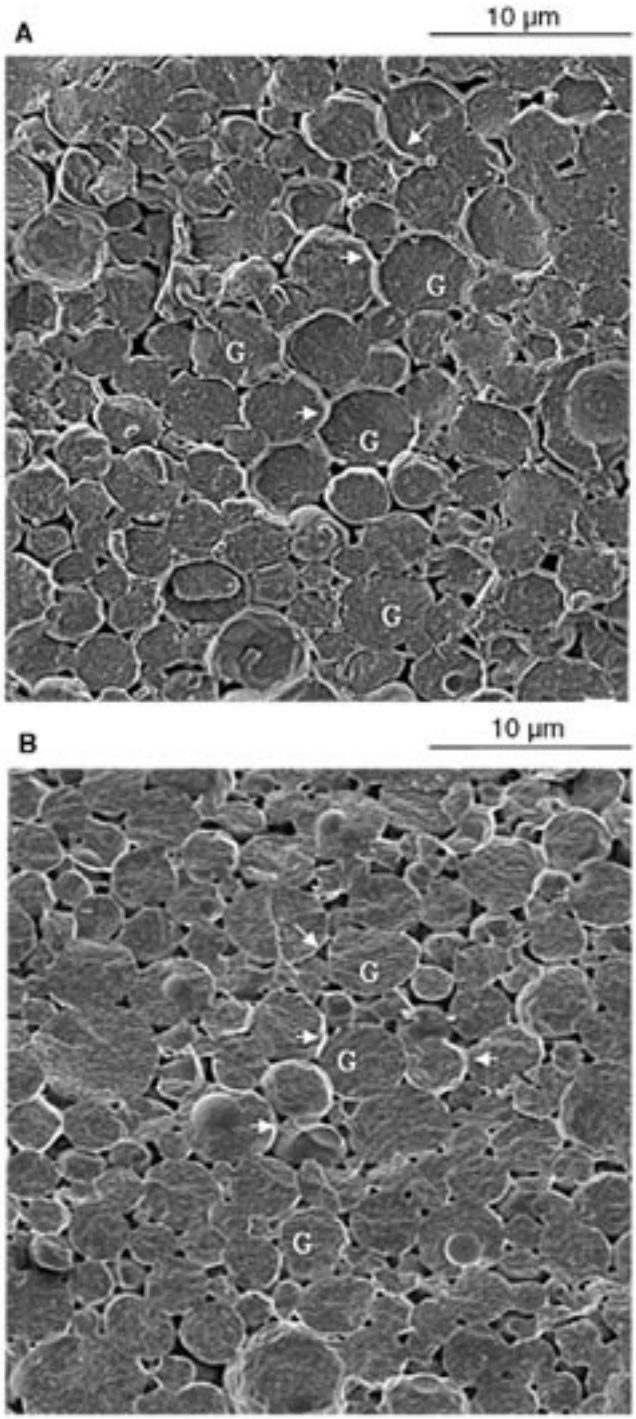



**FIG. 1.** Cryo-scanning electron microscopy (cryo-SEM) micrographs of emulsion **A** (A) immediately after preparation and (B) supercooled in ice/salt bath (–5°C) and held for 15 min. G, globules. Arrows point to interface.

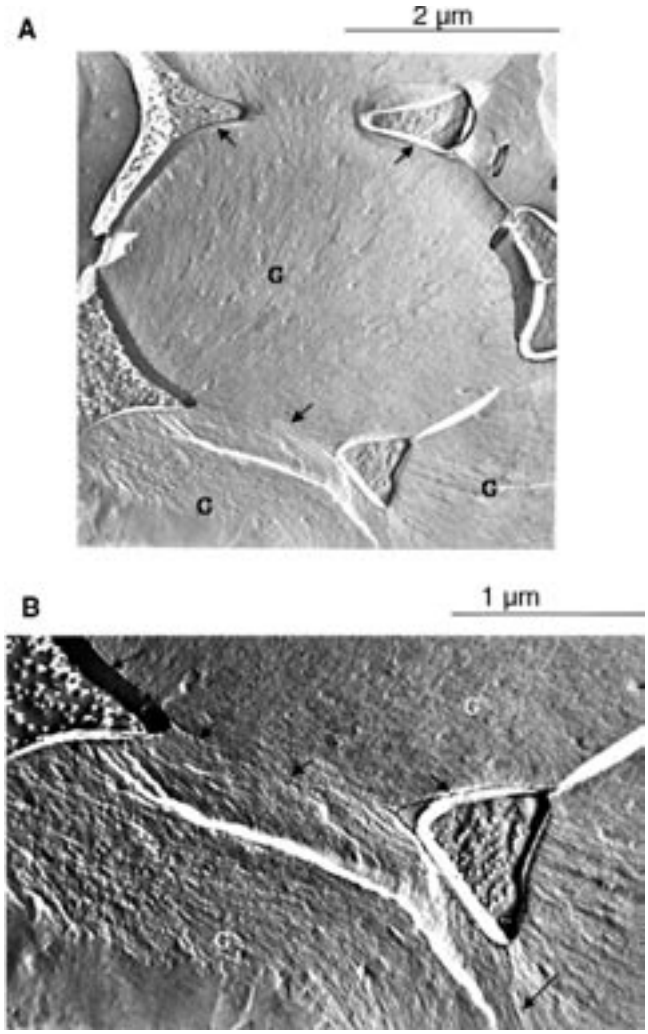



**FIG. 2.** Transmission electron microscopy micrographs of emulsion **A** globules. (A) Densely packed globules with sporadic areas containing the aqueous phase. (B) Close-up of contact area showing the presence of a thin membrane that prevents coalescence.

low shearing action also promoted globule disruption owing to the abrasive action of fat crystals on the globule membrane. The microstructure of emulsion **B** when supercooled and held for 15 min at –5°C showed dramatic changes in structure with most globules disrupted and the plastic fat hardened into an amorphous mass (Fig. 3B). To form a stable O/W/O emulsion, all the globules in emulsion **B** should be disrupted during supercooling. It was also important during the initial preparation of emulsion **B** that the temperature be kept at 45–50°C to prevent crystallization of the hard fraction in the oil/fat blend and to delay disruption of the globules.

In fat-based products, in which the fat crystal network is the main structure to stabilize the system, dropping point can be used to determine the temperatures where the fat crystal network collapses and the system loses integrity. In this study the dropping point was determined on emulsions **A** and **B**. During the preparation of the samples for dropping point analysis, the emulsions were cooled to –22°C and held for 90 min, which is a more rigorous cooling treatment than supercooling to –5°C and would be expected to disrupt emulsion **B**. If during this cooling procedure emulsion **B** globules are disrupted, plastic fat will be released, and if emulsion **A** globules are disrupted, liquid oil will be released. The dropping point of emulsion **B** was 46.4°C, which is close to the m.p. of free plastic fat and indicates disruption and release of plastic

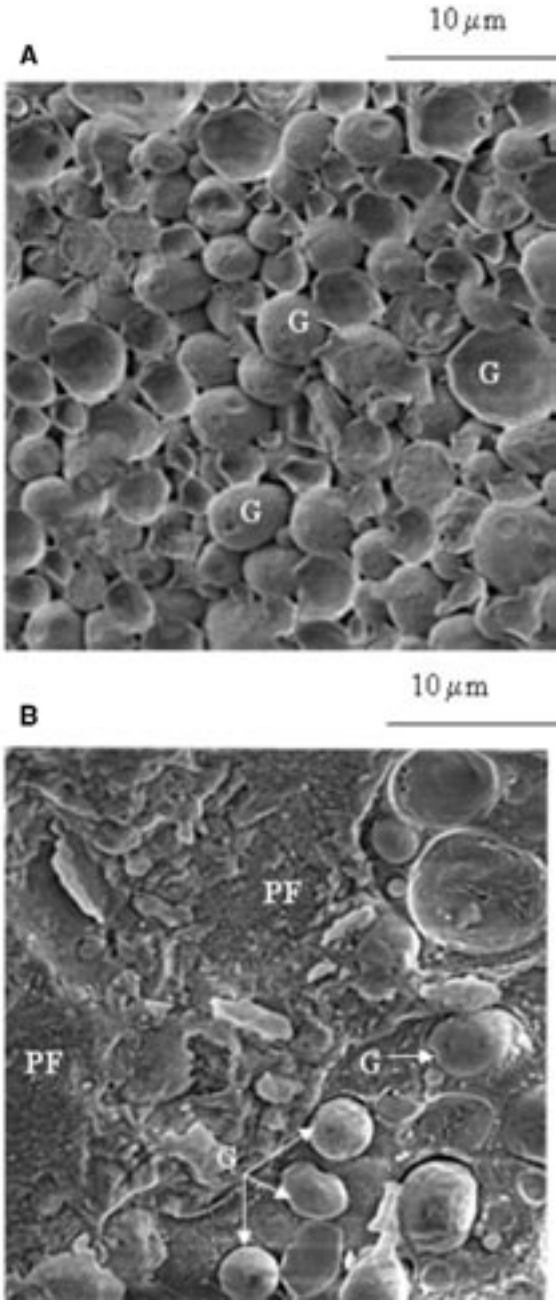



**FIG. 3.** Cryo-SEM micrographs of emulsion **B** (A) immediately after preparation at 40–45°C and (B) supercooled in ice/salt bath (–5°C) and held for 15 min. PF, plastic fat; for other abbreviations see Figure 1.

fat during the cooling procedure. Since emulsion **A** globules contain only liquid oil, the dropping point will be a measure of emulsion stability and not the m.p. of fats. The measured dropping point of emulsion **A** was 77.2°C, which indicates that the emulsion was intact (no free liquid oil) and that a high temperature was required to break the emulsion and cause the sample to flow from the sample holder. The stability of canola oil emulsions to low temperatures (–20°C with no crystal formation) was shown by Harada and Yokomizo (19). The dropping point of emulsion **B** was similar to the O/W/O emulsion

**TABLE 2**
**Dropping Points of Emulsions A, B, and Some Selected O/W/O[a] Emulsions and Margarines from a Local Market**

| Sample | Dropping point (°C) |
|---|---|
| Emulsion **A** | 77.2 ± 0.2 |
| Emulsion **B** | 46.4 ± 0.1 |
| O/W/O emulsion with 8% added solid | 46.0 ± 0.15 |
| O/W/O emulsion with 6% added solid | 43.6 ± 0.1 |
| O/W/O emulsion with 4% added solid | 40.8 ± 0.1 |
| Blue Bonnet™ (margarine #1) | 35.2 ± 0.1 |
| President's Choice™ (margarine #2) | 37.5 ± 0.1 |
| Lactancia™ (margarine #3) | 32.8 ± 0.1 |
| Master Choice™ (margarine #4) | 38.4 ± 0.1 |

[a]For abbreviation see Table 1.

and to the four commercial margarines (Table 2); with these emulsions the dropping points are a measure of the m.p. of the continuous plastic fat phase.

*O/W/O emulsion*. The O/W/O emulsion was produced by mixing the two O/W emulsions together, supercooling the mixture to disrupt emulsion **B** globules, and allowing the plastic fat from emulsion **B** to spread between the emulsion **A** globules. Emulsion **B** carries the hard fraction, and upon disruption it surrounds emulsion **A** globules. The images obtained from the O/W/O emulsion by cryo-SEM show an amorphous background of plastic fat with globules embedded throughout (Fig. 4). The presence of liquid oil and solids in the form of plastic fat in the O/W/O emulsion resulted in a cryo-SEM micrograph with a pasty appearance. The background shows no evidence of needle-form crystals, only amorphous plastic fat. The micrograph of the nonfractured sample of O/W/O emulsion at low magnification shows irregular-shaped globules distributed in plastic fat and, in some areas, a cluster of emulsion **A** globules (Fig. 4A). The globules show irregular structures since they are not floating in the aqueous or oil phase but are embedded in a plastic fat. The fractured sample shows individual globules from emulsion **A** surrounded by the continuous plastic fat from emulsion **B** (Figs. 4B,C).

The plastic fat released from emulsion **B** resulted in a phase transition and changed the continuous phase from water to oil (Scheme 1). Immediately after the phase transition, the viscosity of the emulsion mixture dropped sharply, but as crystallization proceeded, the viscosity gradually increased until the plastic fat solidified and stabilized the O/W/O emulsion. When the O/W/O emulsion has hardened and stabilized, mixing of the O/W/O emulsion should be stopped to prevent damage to emulsion **A** globules. If mixing were to continue, liquid oil would be released due to the disruption of emulsion **A** globules.

The structure of the O/W/O emulsion was studied by confocal scanning laser microscopy. Emulsions **A** and **B** have the same continuous phase (i.e., water) and therefore can be easily mixed together. After mixing the two emulsions together and supercooling, the plastic fat from emulsion **B** should be evenly distributed between emulsion **A** globules. Figure 5A shows emulsion **A** globules distributed in plastic fat with small areas of cluster formation. These results are possible only when both emulsions remain intact during the initial

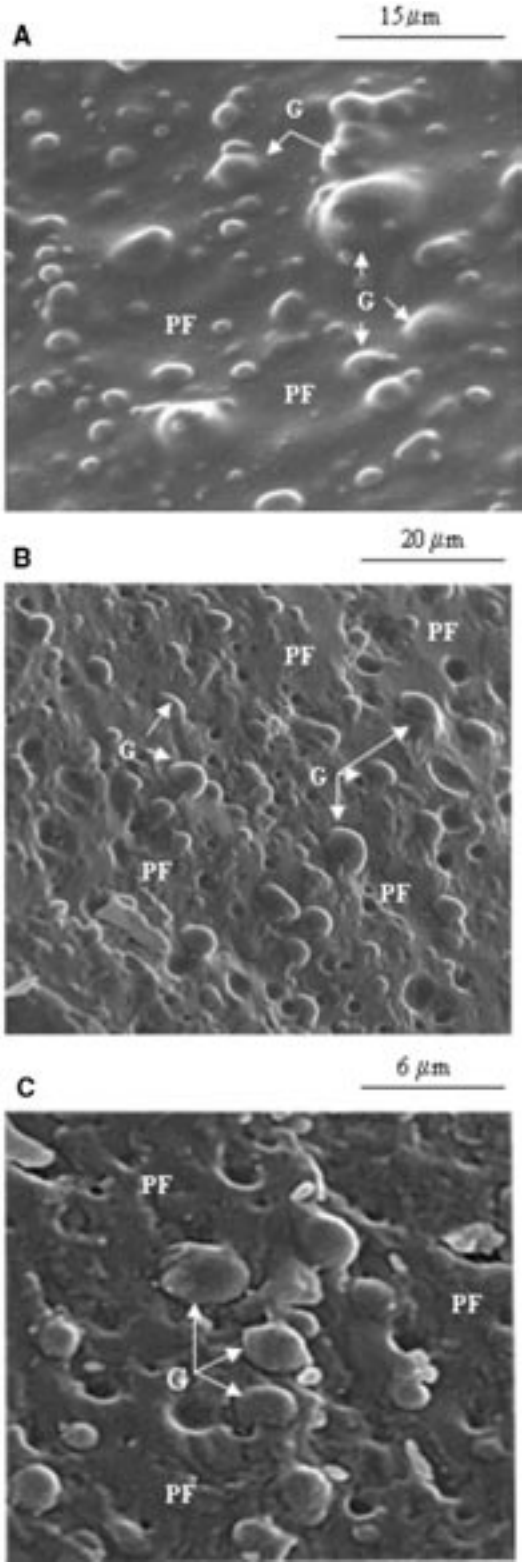



**FIG. 4.** Cryo-SEM micrographs of the oil-in-water-in-oil (O/W/O) emulsion. (A) Surface of the O/W/O emulsion (nonfractured) showing amorphous PF surrounding the oil globules; (B) freeze-fractured O/W/O emulsion showing distribution of globules inside the emulsion; (C) higher magnification of freeze-fractured emulsion. Arrows point to depressions left by globules. For other abbreviations see Figures 1 and 3.

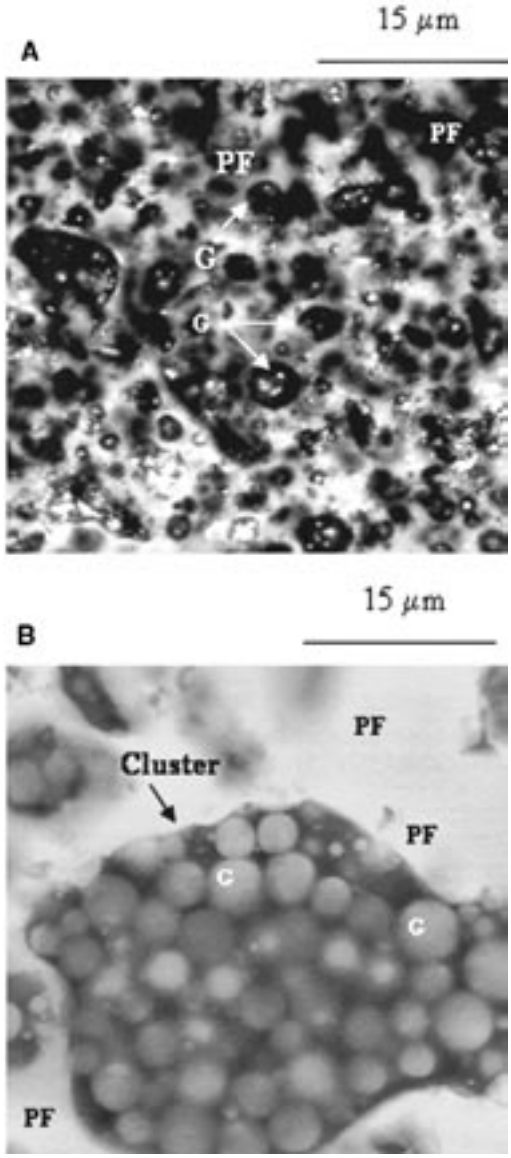



**FIG. 5.** Confocal scanning laser microscopy micrographs of the O/W/O emulsion. (A) Typical distribution of fat globules with pockets of small clusters formed by the incomplete mixing of emulsions **A** and **B**; (B) large cluster of emulsion **A** globules surrounded by PF. The fat in emulsions **A** and **B** was stained with 0.01% Fluorol Yellow 088 before mixing. For abbreviations see Figures 3 and 4.

mixing procedure. If emulsion **B** globules disrupt prior to mixing with emulsion **A**, the released plastic fat will surround groups of emulsion **A** globules and form clusters. Under these conditions, a uniform distribution of globules will not be possible, and these cluster-type structures will predominate (Fig. 5B). It is hypothesized that these clusters will produce a less stable O/W/O emulsion.

To further show that the intact globules are predominantly from emulsion **A** and not **B**, the oil used in emulsion **A** was stained with Fluorol Yellow 088 and mixed with unstained emulsion **B**. The O/W/O emulsion was prepared as described previously and viewed by confocal scanning laser microscopy. The images showed a plastic fat in the background

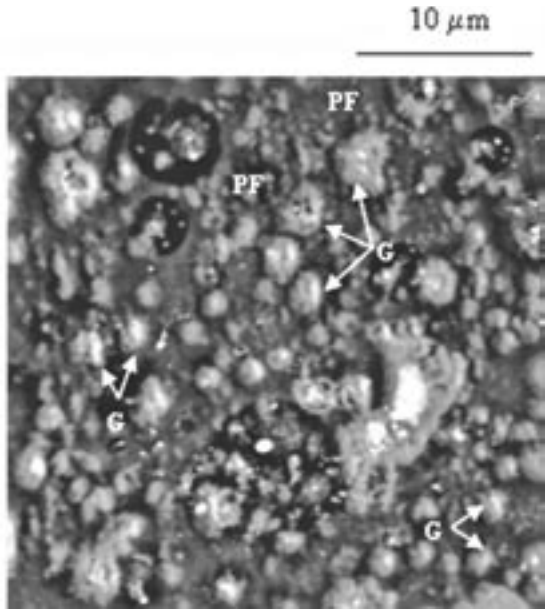



**FIG. 6.** Confocal scanning laser microscopy micrographs of the O/W/O emulsion. Emulsion **A** was stained with 0.01% Fluorol Yellow 088, and emulsion **B** was not stained before mixing. Arrows: Globules from emulsion **A**. Dark areas surrounding the globules are PF. For abbreviations see Figures 3 and 4.

with stained globules from emulsion **A** (Fig. 6). The dark areas in Figure 6 represent the plastic fat and crystals around the globules. The intact globules are lighter due to emission of fluorescent light, indicating the presence of stained oil. The confocal microstructure confirms the presence of intact globules from emulsion **A** in the final O/W/O emulsion.

In this study a double emulsion system (O/W/O) was formed and stabilized with only 4% added palm and cotton stearins by mixing two O/W emulsions together followed by supercooling. The stability of the double emulsion was achieved when plastic fat was inserted between emulsion **A** globules. Microstructure analysis was shown to be a complementary method to follow structural changes during the formation of the double emulsion. Cryo-SEM, TEM and confocal micrographs of the O/W and double emulsions support the hypothesis that plastic fat can be inserted between the emulsion **A** globules *via* emulsion **B** and form a stable O/W/O emulsion.

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