CHAPTER 3. THE EFFECT OF MILKFAT FRACTION MELTING PROPERTIES ON CHEMICAL PROPERTIES OF 20% REFORMULATED CREAM

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(ABSTRACT)

Chemical characteristics of components obtained at two separation temperatures (49^{o}C and 55^{o}C) and 20% milkfat creams were analyzed. Butter-derived aqueous phase and sweet buttermilk components had higher ($p \leq 0.01$) amounts of lipid, cholesterol, and protein than skim component. Skim component, however, had a higher ($p \leq 0.01$) amount of protein than butter-derived aqueous phase. Commercially produced butter-derived aqueous phase had a lower ($p \leq 0.05$) lipid content than butter-derived aqueous phase obtained in the pilot plant, resulting in lower ($p \leq 0.01$) amounts of cholesterol and phospholipid. Sweet buttermilk and butter-derived aqueous phase had higher amounts of individual fatty acids and more unsaturated fatty acids than skim. Creams formulated with skim had lower ($p \leq 0.01$) amounts of phospholipid than natural creams and creams comprised of sweet buttermilk and butter-derived aqueous phase. In comparison to natural creams, creams formulated from skim were lower ($p \leq 0.01$) in fat content, but had a higher ($p \leq 0.01$) protein content. Creams formulated with sweet buttermilk and butter-derived aqueous phase had higher ($p \leq 0.01$) amounts of cholesterol than natural creams and creams consisting of skim component. Creams formulated with medium-melt butteroils had lower amounts of cholesterol ($p \leq 0.01$) than creams having emulsified low-melt butteroil. In most instances, creams formulated with medium-melt fractionated butteroil had higher levels of long chain saturated fatty acids while creams with low-melt butteroil had higher degrees of unsaturated fatty acids. Natural creams and creams formulated from sweet buttermilk and butter-derived aqueous phase had significantly higher ($p \leq 0.05$) amounts of phospholipid occurring in the membrane material than skim milk formulations.

Introduction. The dairy industry has made recent advances in modifying the chemical and physical attributes of milkfat. Modified milkfat has replaced traditional milkfat in various dairy and food systems. Modified milkfat, having some different nutritional and functional attributes than natural milkfat, still requires emulsification in many food systems. The challenges of emulsification can be overcome by combining altered milkfat with surface active agents into reformulated dairy products such as creams. The final dairy product should have chemical and physical attributes comparable to natural dairy products.

Milk-derived components such as skim milk, sweet buttermilk, butter-derived aqueous phase, whey proteins, casein dispersions, and purified milkfat globule membrane suspensions have proven to successfully emulsify butteroil (Smith and Dairiki, 1975; Oortwijn et al., 1977; Oortwijn and Walstra, 1979; Kanno, 1989; Kanno et al., 1991; Rosenberg and Lee, 1993; Oehlmann et al., 1994; Tomas et al., 1994; Elling et al., 1996). Skim milk is a rich source of whey and casein proteins whereas sweet buttermilk and butter-derived aqueous phase are also abundant in phospholipids from milkfat globule membrane fractions (Elling et al., 1996).

The milkfat globule membrane is comprised of proteins, phospholipids, glycoproteins, triglycerides, cholesterol, and enzymes (McPherson and Kitchen, 1983). Approximately half of the phospholipids occurring in milk are associated with the milkfat globule membrane (McPherson and Kitchen, 1983). The neutral lipid content of the milkfat globule membrane is approximately 83-88% triglycerides, 5-14% diglycerides, and 1-5% free fatty acids (Fox and McSweeney, 1998). High amounts of diglycerides occurring in the milkfat globule membrane can be attributed to the membrane's capacity as an emulsifier.

Processing steps such as homogenization and pasteurization, although essential in preserving the stability and safety of dairy products, alter the milkfat globule membrane. During homogenization, the native milkfat globule membrane is disrupted. The concentration of the original milkfat globule membrane fails to completely cover the increased surface area of the fat globules caused by homogenization (Cano-Ruiz and Richter, 1997). As a result, skim milk proteins become adsorbed on the surface of lipid globules.

In the proper ratio, skim milk can be combined with milkfat to yield an oil-in-water emulsion (Elling et al., 1996). The presence of flexible, unstructured casein proteins and structured globular whey proteins make skim milk a fairly good emulsifying agent. Several noteworthy structural features of casein contribute to increased emulsifying capacity. Although casein proteins have a rather high percent of apolar amino acids, the presence of polar phosphate groups counteract their effect, thus increasing solubility in foods (Fox and McSweeney, 1998). Of equal importance, features of casein proteins such as open structures, high apolar amino acid residues, and uneven amino acid

distribution result in adsorbance at oil-water interfaces. Additionally, lack of secondary and tertiary structures due to the presence of proline residues result in high stability to heat denaturation (Fox and McSweeney, 1998). Whey proteins are characterized as possessing compact globular structures, resulting in structural restriction and stabilization (Kinsella and Whitehead,1989). Whey protein denaturation is characterized by unfolding of its compact, globular structure. The compact, organized structure of whey proteins influences various functional characteristics, most importantly functionality during heat processing. At temperatures above 65^{o}C, whey proteins readily denature (Kinsella, 1984). During heat processing, whey proteins engage in thiol/disulphide interchanges (de Wit, 1981). Thermal unfolding of whey proteins, particularly β-lactoglobulin, have demonstrated an increase in emulsifying characteristics, however, prolonged denaturation decreases activity at the interface (Leman and Kinsella, 1989). The emulsification of anhydrous butteroil with skim milk and proteins has been accomplished (Oortwijn et al., 1977; Oortwijn and Walstra, 1979; Oortwijn and Walstra, 1982; Melsen and Walstra, 1989). Oortwijn et al. (1977) emulsified milkfat with skim milk, whey, and casein dispersions. Electron microscopic evidence proved that emulsions were characteristic of the oil-in-water type. Also, casein and whey proteins interacted at the fat-lipid interface (Oortwijn et al., 1977). Oortwijn and Walstra (1979) discovered that protein load (mg/m^2) for milkfat emulsified by casein dispersion, skim milk, and whey dispersion was 20, 10, and 2.5 mg/m^2.

In comparison to skim milk, buttermilk and butter-derived aqueous phase have higher phospholipid contents as well as casein and whey proteins (Elling et al., 1996). Oehlmann et al. (1994) and Elling et al. (1996) incorporated reduced cholesterol butteroils into emulsion systems consisting of sweet buttermilk, butter-derived aqueous phase, skim milk, or a combination of these particular components. Oehlmann et al. (1994) found that protein and phospholipid compositions of 30% milkfat reformulated creams were comparable to components used in each reformulation. Electron microscopy, however, revealed that emulsions were water-in-oil as opposed to desired oil-in-water emulsions. Elling et al. (1996) found that emulsifying reduced cholesterol butteroil (20%) with sweet buttermilk (70%) and butter-derived aqueous phase (10%) yielded oil-in-water emulsions. The fact that Elling et al. (1996) observed oil-in-water characteristics of natural and reformulated creams may be related to fat content. Oehlmann et al. (1994) and Elling et al. (1996) standardized natural and reformulated creams to final milkfat contents of 30% and 20%, respectively. For Oehlmann et al. (1994), the fat content of creams may have been rather high, resulting in insufficient amounts of surface active agents for proper emulsification. However, fat globules occurring in creams processed by Elling et al. (1996) were sufficiently adsorbed by proteins and milkfat globule membrane fragments. Elling et al. (1996) also discovered that creams formulated with sweet buttermilk or sweet buttermilk and butter-derived aqueous phase had protein and phospholipid contents similar to control creams. Differences in creams processed by Oehlmann et al. (1996) and Elling et al. (1996) can also be attributed to processing parameters such as homogenization and use of components for reformulation. Unlike Oehlmann et al. (1996), Elling et al. (1996) did not use milk lipid globule membrane suspensions as an emulsifying agent. Cream homogenization was accomplished by either ultrasound dispersal or two-stage

homogenization at 140.6 and 35.1 kg/cm^2 on stage 1 and 2 valves or 10.2/3.4 MPa or 13.6/3.4 Mpa by Oehlmann et al. (1994) and Elling et al. (1996), respectively.

Elling et al. (1996) determined that reformulated creams consisting of buttermilk or buttermilk/aqueous phase exhibited protein and phospholipid composition most similar to natural homogenized cream. Formulation did not have a significant effect ($p>0.05$) on the total fat and total protein contents of the creams. When analyzing milkfat globule surface characteristics, Elling et al. (1996) found that isolated milkfat globule surface material in creams consisting of sweet buttermilk alone and sweet buttermilk and butter-derived aqueous were comparable to control formulations. Milkfat globule surface material in skim milk formulated creams was significantly higher ($p \leq 0.05$) than other reformulated and natural creams. Cream formulated with skim milk and homogenized at 13.6/3.4 MPa had significantly higher ($p \leq 0.05$) protein associated with the milkfat surface material than all creams consisting of only buttermilk as a reformulating component and creams consisting of buttermilk and butter-derived aqueous phase homogenized at 10.2/3.4 MPa. Overall, control creams and creams formulated from either sweet buttermilk alone or sweet buttermilk and butter-derived aqueous phase had significantly ($p < 0.05$) higher amounts of phospholipid in association with milkfat surface material.

Specifically, the objectives of this research were to evaluate:

1). the influence of emulsifying formulations (skim milk, sweet buttermilk, and butter-derived aqueous phase) and modified butteroil fractions (low-melt and medium-melt) on the chemical characteristics of 20% milkfat reformulated creams as compared to natural creams;

2). the influence of separation temperature (49^{o}C and 55^{o}C) on the chemical composition of milk-derived components (skim, sweet buttermilk, butter-derived aqueous phase), reformulated creams (low-melt and medium-melt butteroil only), and natural creams.

3). the differences in chemical composition of milk-derived components (sweet buttermilk and butter-derived aqueous phase) obtained from pilot plant operations as compared to a commercial operation.

Materials and Methods. Separation of Cream and Skim. Raw milk was obtained from the Virginia Tech dairy farm and separated into 30-35% milkfat cream and skim milk by a pilot plant separator (Elecrem separator, model 1G, 6400 rpm, Bonanza Industries, Inc., Calgary, Alberta). Separation occurred at two temperatures, 49^{o}C and 55^{o}C and the skim milk from each separation retained for subsequent use as a component for reformulation of creams. The modified Babcock procedure (Marshall, 1993) was used to determine the percent milkfat of the separated cream. Creams, obtained at 49^{o}C and 55^{o}C separation, were standardized to 30-33% milkfat using skim milk obtained from the appropriate separation. Each cream was vat pasteurized at 68.3^o C for 30 min. Each cream was subsequently cooled to13^{o}C in an ice bath. Portions of cream from each separation were used as controls and held until the day of reformulation.

Preparation of Components (Buttermilk and Butter-Derived Aqueous Phase)**.** Components (sweet buttermilk, butter-derived aqueous phase) were obtained as described by Elling et al. (1996). Creams (30-33% milkfat from separations at 49°C and 55°C) was tempered to 13-14°C. Sweet buttermilk and butter were obtained by mechanical churning (Gem Dandy Standard Electric Churn, Bonanza Industries, Inc., Calgary, Alberta) of cream. Sweet buttermilk was separated from butter granules by pouring it through cheesecloth and by pressing excess buttermilk from butter granules with a stainless steel spoon. Excess buttermilk was separated by pressing the butter granules with a stainless steel spoon. Sweet buttermilk, obtained from creams, was retained at 3.3°C for further processing and subsequent use as a component for reformulation of creams.
Sweet buttermilk was obtained from a commercial processor (Grasslands Dairy Products, Inc., Greenwood, WI) for chemical comparison to sweet buttermilk obtained from creams. Commercial buttermilk was obtained from churning 38.5% milkfat cream. Cream for churning was preheated to 85°C and subsequently pasteurized at 87.2-87.8°C. Following pasteurization, the cream was cooled to 6.1°C by a heat exchange plate. Prior to churning, the cream was stored in a tank for 6-8 hours. Churning occurred at 10.6° C.

Butter-derived aqueous phase was obtained from a commercial processor (Grasslands Dairy Products, Inc., Greenwood, WI) for the use as a component in cream reformulation. Cream (38.5% milkfat) used to obtain aqueous phase was pasteurized at 85.6° C. Following pasteurization the cream went through the 300 separator at 62.8° C and 80 separator at 93.3° C. After separation, the serum was recovered.

Butter-derived aqueous phase was produced in the pilot plant to make chemical comparisons to commercially produced butter-derived aqueous phase. Butter-derived aqueous phase was obtained by melting, accompanied by occasional stirring, the butter obtained from churning at 55-60°C in a water bath. The melted butter was refrigerated at 3°C for approximately 30 min to separate butteroil and aqueous phase. Aqueous phase from butter was collected with a 25 ml pipette and refrigerated at 3°C overnight. The following day, solidified lipid was removed from the aqueous phase by filtering through cheesecloth. The pilot plant processed aqueous phase was vat pasteurized at 62.8° C for 30 min. The volume of aqueous phase obtained from the pilot plant operation was not adequate for pasteurization in the tubular heat exchanger.

Processing of Components. Each component (skim:49°C and 55°C separations; sweet buttermilk:49°C and 55°C separations and, commercially obtained butter-derived aqueous phase) used for reformulation was pasteurized in a tubular heat exchanger (Microthermics UHT/HTST Lab 25-HV, Microthermics, Inc., Raleigh, NC) at 71.7°C for 15 seconds. Components were cooled to 21.5°C by the HTST pasteurization unit and subsequently stored at 3.3°C for 24 hours prior to cream reformulation.

Characterization of Low-melt and Medium-melt Fractionated Butteroils. Low-melt and medium-melt fractionated butteroils were obtained from anhydrous milkfat utilizing the Tirtiaux fractionation procedure at the Wisconsin Center for Dairy Research (University of Wisconsin, Madison). Low-melt fraction butteroil had a dropping point between 10-

25°C whereas medium-melt fraction butteroil had a dropping point between 25-35°C (Kaylegian, 1998). Percent solid fat was zero at approximately 25°C and 30°C for low-melt and medium melt fractions, respectively. Both butteroils were described as having butter-like flavors and medium-yellow coloration. Fatty acid profiles of low-melt and medium-melt butteroils are provided (Table 3-1; Kaylegian, 1998).

Table 3-1. Fatty acid analysis (wt. %) of low-melt and medium-melt fractionated butteroils

	Fatty acid (wt. %)										
Butteroil Fraction	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2
Low-melt	4.6	2.3	2.2	3.8	3.7	9.4	19.6	1.8	8.6	33.1	2.1
Medium-melt	6.3	2.7	1.4	3.9	3.1	8.5	29.3	3.7	9.6	23.5	3.0

Cream Reformulation. Creams, (20% milkfat) were formulated from low-melt and medium-melt fractionated butteroils. Butteroils were placed in a water bath and melted at 45-50°C prior to reformulation. To obtain a homogenous mixture, each cream was stirred with a stainless steel spoon. Before homogenization, each mixture was pre-heated to 55°C. Each cream was homogenized in a two-stage homogenizer (APV Gaulin, Inc., Model 15MR, Everett, MA) at 13.6/3.4 MPa, in the first and second stages respectively. A Braun hand mixer was used to achieve uniformity before each cream was passed through the homogenizer. Following homogenization, each cream was cooled to 29.4° C in an ice bath and then pasteurized at 77.8° C for 15 sec in a tubular heat exchanger (Microthermics UHT/HTST Lab 25-HV, Microthermics, Inc., Raleigh, NC). The following formulations (Table 3-2) were used to process natural and reformulated creams:

Table 3-2. Description of 20% natural and reformulated creams processed from components obtained at two different separation temperatures (49°C and 55°C) and butteroils having different melting range characteristics (low and medium-melt)

Reformulated Cream Description	Separation Temperature (°C)	Formulation % (kg)	Butteroil % (kg)
80% Skim + 20% low-melt butteroil	49°C	80% Skim (3.2)	20% Low-melt (0.8)
80% Skim + 20% low-melt butteroil	55°C	80% Skim (3.2)	20% Low-melt (0.8)
80% Skim + 20% medium-melt butteroil	55°C	80% Skim (3.2)	20% Medium-melt (0.8)
70% buttermilk+ 20% low-melt butteroil + 10% aqueous phase	49°C	70% Buttermilk (2.8) 10% aqueous phase (0.4)	20% Low-melt (0.8)
70% buttermilk + 20% low-melt butteroil + 10% aqueous phase	55°C	70% Buttermilk (2.8) 10% aqueous phase (0.4)	20% Low-melt (0.8)
70% buttermilk + 20% low-melt butteroil + 10% aqueous phase	55°C	70% Buttermilk (2.8) 10% aqueous phase (0.4)	20% Medium-melt (0.8)
20% Natural Cream	49°C	Control	None
20% Natural Cream	55°C	Control	None

Fat, Protein, Cholesterol, and Phospholipid Determination of Components and Creams. Determination of fat, protein, phospholipid, and cholesterol content of pasteurized components (skim, buttermilk, and butter-derived aqueous phase), commercially obtained components, and creams were made. Protein content was analyzed by the DC Bio Rad assay (Bio Rad Laboratories, Hercules, CA). The modified Babcock procedure (Marshall, 1993) was used to measure fat content of reformulated and cream controls. Lipids were extracted (Bligh and Dyer, 1959) to determine total lipid content of components. Analysis of phospholipid content required lipid extraction as outlined by Folch et al. (1957) for creams and by Bligh and Dyer (1959) for components. Phospholipids in the extract were separated using a silicic acid column (Rouser et al., 1967). A quantitative analysis of the amount of phosphorous in the phospholipid extract was made using a spectrophotometric method (Rouser et al., 1966). The value obtained from the analysis was multiplied by a factor of 25 to convert from phosphorous content to phospholipid content (Anderson et al., 1977). Individual phospholipids from components and creams were separated using thin layer chromatography (Heape et al., 1985). Prior to conducting thin layer chromatography, lipid extraction of from components was achieved using the Bligh and Dyer lipid extraction (1959) and Folch lipid extraction for creams (1956) and phospholipids were extracted using silicic column assay. Cholesterol content was determined by spectrophotometric methods (Stadtman, 1957).

Transmission Electron Microscopy of Creams. Transmission electron microscopy was used to visually examine the relationship of butteroil to milkfat globule membrane fragments and milk proteins (Elling et al., 1996). Electron microscopy of reformulated and control creams was performed on only one replication. Electron micrographs were examined and compared by counting the number of globules and determinng the average globule diameter (μm). To determine fat glouble size, the diameters of lipid globules in electron micrographs with final magnifications of 7200, 9600, 11,600, and 14,400 times were measured.

Creams were prepared for electron microscopy as described by Elling et al. (1996). Two ml of each sample was gently mixed in approximately 8 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7) at room temperature for 1 hr. The cream samples were centrifuged at a low speed to float lipid globules, which then were immobilized in a 2% agarose solution. The agarose-lipid mixture was minced into 1 mm^3 pieces and washed three times with 0.1 M cacodylate buffer (pH 7). A 1% osmium tetroxide solution was used to fix the minced samples and distilled water was used to wash them three times. The fixed samples were dehydrated in a series of ethanol solutions (30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, 100% three times, 50% ethanol/50% acetone, 100% acetone three times) and embedded in Epon 812. Treatment in Epon consisted of soaking in Epon 812/50% acetone for at least 3 h, then soaking in 75% epon 812/25% acetone for 6-8 h, and soaking in 100% Epon 812 at room temperature for 3 h. Thin sections of the specimens were examined and photographed by a JEOL 100 CX-II transmission electron microscope.

Total Fatty Acid Profiles of Components and Creams. Total fatty acid profiles were determined by the methods described in Supelco Bulletin 856. Fatty acid profiles were done in triplicate and reported as an average on one replication. Fatty acid profiles for components and creams were determined by gas chromatography after extraction of fatty acids from 10 ml of fresh sample. An internal standard (C17:0) was added (150 µl) to fresh cream before extraction of lipids.

Lipids were extracted by adding 10 ml of sample, 10 ml of ethanol, 3 mL of 28% ammonium hydroxide, 25 ml petroleum ether, and 25 ml of diethyl ether to a separatory funnel. The mixture was shaken for approximately 5 min and allowed to sit undisturbed for 20 min. The bottom phase was drained off, the ether phase was removed and dried under nitrogen. Approximately 3 ml of 0.5N NaOH in methanol was added to the sample which was heated on a steam bath for 15 min. Five milliliters of water and 2N HCl was added until the pH was equal to 2. Fatty acid methyl esters were extracted with 5 ml of petroleum ether and 5 ml of diethyl ether.

Extract volumes of 0.2-0.3 µl were directly injected into an HP5890A gas chromatography system (Hewlett-Packard Co., Avondale, PA) fitted with a Nukol™ (Supelco, Inc., Bellafonte, PA) fused silica capillary column (15m, 0.53 i.d.) and a flame ionization detector. Helium, the carrier gas, had a flow rate of 20 ml/min. Oven temperature was approximately 110°C for 2 min after injection. Temperature was increased 8°C per min to a final temperature of 200°C for and held close for 18 min. Free fatty acid standards (C4:0, C6:0, C10:0, C12:0, C14:0, C16:0, C17:0, C18:0, C18:1, C18:2) (Supelco, Inc) and were mixed in diethyl ether to give a standard solution having 2357 µg/ml of each free fatty acid. Free fatty acid quantification was determined as described by Woo and Lindsay (1980). Response factors were determined by the following equation with respect to the internal standard C17:0: Response factor of free fatty acid = (wt. FFA/area internal std.)/(wt. internal std./area FFA). Free fatty acids in component and cream extracts were quantified using the following equation: µg/ml of free fatty acid = (response factor)(area of FFA)(wt. of internal standard)/(area internal std.).

Determination of Types of Proteins Associated with Lipid Globules. Electrophoretic separation of proteins in natural and reformulated creams was conducted according to Laemmli (1970). SDS-PAGE was conducted on one replication. Creams were vortexed and 1.5 ml of each cream was mixed with 1.5 ml of 2M sucrose. The solution was overlaid with 2 ml of 10 mM Tris buffer adjusted to pH 7 and centrifuged (30 min, 2°C, 40,000 rpm) in a Beckman L5-50B Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The floating cream plug was collected and 400 µl of 3X solubilization buffer (4.5 gm Trizma base+12 gm SDS+30 ml β-mercaptoethanol+60 ml glycerol+120 mg Bromophenol blue dissolved in distilled water, adjusted to pH 6.8, and brought to a final volume of 200 ml with distilled water). The sample and buffer were heated in a boiling water bath for approximately 5 min, cooled to room temperature, and centrifuged (5 min at 14,000 rpm) in an Eppendorf 5415 microcentrifuge (Brinkmann Instruments, Inc.,

Westbury, NY 11590). The subnatant was collected for protein separation by SDS-PAGE.

Components used in formulating creams were mixed 1:1 with 3X solubilization buffer, heated in a boiling water bath for approximately 5 min, and centrifuged at 14,000 rpm for 2 min. Five microliter aliquots of subnatant from each cream and supernatant from each component were separated by SDS-Page, using 8-20% Tris-glycine precast gradient gels (1.5 mm thickness). Five microliters of standard consisting of MLGM proteins were used as a reference. A Novex Xcell II™ Mini Cell electrophoresis unit (Novex™, San Diego, CA) was filled with 1X running buffer (10X = 29 gm Trizma base+144 gm glycine+10 gm SDS dissolved in distilled water, adjusted to pH 8.3 and brought to a final volume of 1 L) (1 part buffer+9 parts distilled water =1X solution). Protein separation was achieved at 125v for 90 min. Gels were placed in a tray containing a staining solution (0.05 gm Coomassie brilliant blue + 800 ml methanol + 140 ml acetic acid brought to 2 L volume with distilled water). Gels were destained in two steps (first in 400 ml ethanol + 70 ml acetic acid brought to a 1 L volume with distilled water and second in 10% acetic acid in water and photographed.

Analysis of Milkfat Surface Material. The amount of phospholipid and protein adsorbed on the surface of the milkfat globule was analyzed as described by Elling et al. (1996). Creams were centrifuged (60 min, 2^{o}C, 175,000 x g) in a Beckman L2-65B Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). Each cream was separated into two components, a lipid rich cream plug and a skim phase. The cream plug contained the components of the milkfat globule surface material and lipid associated protein. The components of the milkfat surface material were released from the milkfat into the cream plug by two cycles of slow freezing and thawing. Collection was made by centrifugation (60 min, 2^{o}C, 175,00 x g) in a Beckman L2-65B Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The pellet was lyophilized in a freeze drier (Freezemobile 12 SL, Virtis Co., Inc., Gardiner, NY) with drying chamber (10-MR-SM Vacuum Stoppering and Manifold Drying Chamber, Virtis Co., Inc., Gardiner, NY). The pellets were ground into powder form with a mortar and pestle. Lipid extraction on the pellet was conducted (Bligh and Dyer, 1959). The amount of protein on the milkfat surface was determined by the DC Bio Rad Protein Assay. Gel electrophoresis (Laemmili, 1970) was used to determine the types of protein surrounding milkfat globules. Phospholipids were obtained by lipid extraction (Bligh and Dyer, 1959) and phosphorous content was determined by a spectrophotometric method (Rouser et al., 1966). The value was multiplied by a factor of 25 to convert to phospholipid content (Anderson et al., 1977). Enzymatic activity of phosphodiesterase I was determined as described by Brown et al. (1976).

Statistical analyses. The study was replicated three times. Testing of the chemical analyses was done in duplicate. An augmented randomized block design was used for analysis of data generated from fat, protein, phospholipid, cholesterol, and amount of milkfat globule surface material. The effects of formulation, separation temperature in obtaining components, and melting point characteristics of butteroil were tested using a

complete randomized block design. For contrasts in formulation, skim milk creams were tested against buttermilk creams, buttermilk creams against natural creams, and natural creams against skim milk creams. Within treatments without regard to melting point characteristics, the cream set processed from components obtained at 49^{o}C were compared to the 2 creams manufactured from components obtained at 55^{o}C. Within reformulated cream treatments, the two creams consisting of low-melt fraction butteroil, one consisting of components obtained at 49^{o}C and the other processed from 55^{o}C components, were statistically compared to medium-melt butteroil creams processed from 55^{o}C components. A p-value of 0.01 was used to determine if formulation, separation temperature in obtaining components, or melting point characteristics had a significant effect on chemical characteristics of natural and reformulated creams. A p-value of 0.01 was used as opposed to a p-value equal to 0.05 to minimize Type 1 Error. With a p-value of 0.01, the chance of making at least one Type 1 Error is 16% (0.01 x 8 contrasts x 2) whereas a p-value of 0.05 yields a maximum 80% chance [(0.05 x 8 contrasts x 2) x 100] of committing at least one Type 1 Error. Statistical analyses were conducted on SAS (Cary, NC).

Results and Discussion. Skim milk, sweet buttermilk, and butter-derived aqueous phase were used as components in cream reformulation due to emulsifying capacity. Elling et al. (1996) found that reformulated creams consisting of 80% skim milk+20% butteroil and 70% sweet buttermilk+20% butteroil+10% butter-derived aqueous phase had optimal emulsion stability.

Component Composition and Potential Emulsifying Characteristics. Understanding the composition of components is valuable in developing an understanding of the emulsion characteristics, functionality, and stability of reformulated creams. Chemical analysis of components helped to determine chemical composition and nature of surface material associated with lipid globules in both reformulated and control creams. Fat, protein, cholesterol, and phospholipid analyses were completed on skim, sweet buttermilk, and butter-derived aqueous phase components obtained in the pilot plant by separation of milk at 49^{o}C and 55^{o}C as well as commercially obtained sweet buttermilk and aqueous phase (Table 3-3).

The skim component had significantly lower ($p \leq 0.01$) fat, cholesterol, and phospholipid content than the other components and a higher protein content than the butter-derived aqueous phase and commercially produced sweet buttermilk. Sweet buttermilk and butter-derived aqueous phase had higher fat contents because both were derived from churning cream (30% milkfat). Butter-derived aqueous phase component was significantly higher ($p \leq 0.05$) in lipid content than sweet buttermilk. The cream churning process yielded two products, sweet buttermilk and butter. Sweet buttermilk is associated with the aqueous phase of cream whereas butter-derived aqueous phase is associated with the aqueous portion of butter. No significant ($p > 0.01$) differences in lipid content existed between sweet buttermilk processed in the pilot plant for reformulation and Grasslands buttermilk. Pilot plant generated butter-derived aqueous associated with the aqueous portion of butter. No significant ($p > 0.01$) differences in lipid content existed between sweet buttermilk processed in the pilot plant for reformulation and Grasslands buttermilk. Pilot plant generated butter-derived aqueous

Table 3-3. Chemical composition[1] (% lipid, protein, cholesterol, and phospholipid) of pilot plant produced components (skim, Sweet buttermilk, and butter-derived aqueous) obtained at two different separation temperatures (49°C and 55°C) and commercially processed components (sweet buttermilk and butter-derived aqueous phase).

	Separation Temperature (°C)	Lipid (%)	Protein (mg/g)	Cholesterol (mg/g)	Phospholipid (mg/g)
Skim	49	0.205	29.65	0.087	0.155
Skim	55	0.210	30.19	0.079	0.156
Buttermilk	49	0.682	27.78	0.147	1.013
Buttermilk	55	0.690	28.93	0.167	1.004
Commercial Buttermilk		0.753	24.46	0.175	1.393
Aqueous Phase	49	1.464	22.43	0.345	5.015
Aqueous Phase	55	1.538	26.12	0.390	4.634
Commercial Aqueous Phase		0.786	11.67	0.135	0.741
Standard Error		0.100	1.145	0.007	0.220
CONTRASTS					
Skim 49°C * Skim 55°C		0.9703	0.7425	0.3616	0.9980
Buttermilk 49°C * Buttermilk 55°C		0.9524	0.4916	0.0562	0.9793
AP 49°C * AP 55°C		0.6121	0.0387	* 0.0003	0.2429
Pilot Plant Buttermilk * Commercial Buttermilk		0.5965	* 0.0149	0.0453	0.1777
Pilot Plant AP * Commercial AP		* 0.0003	* 0.0001	* 0.0001	* 0.0001
Skim * Buttermilk		* 0.0004	0.1927	* 0.0001	* 0.0019
Skim* AP		* 0.0001	* 0.0002	* 0.0001	* 0.0001
Buttermilk * AP		* 0.0001	* 0.0031	* 0.0001	* 0.0001

[1]Values are means and standard errors for 3 replications.

[2] AP = aqueous phase

*predetermined p-value = 0.01 used for determining statistical significance

phase, obtained from melting butter, had a higher fat content. Commercial butter-derived aqueous phase was lower ($p \geq 0.01$) than aqueous phase produced in the pilot plant, suggesting the separation of milkfat from the butter-derived aqueous phase in the pilot plant was not as efficient as the commercial operation. For all components, separation temperature used in obtaining components did not have a significant ($p > 0.05$) effect on percent lipid.

Protein content of pilot plant processed skim and buttermilk components was significantly higher ($p \leq 0.01$) than pilot plant processed butter-derived aqueous phase. Both commercially obtained sweet buttermilk and butter-derived aqueous phase had significantly lower ($p \leq 0.01$) amounts of protein than sweet buttermilk and butter-derived aqueous phase processed in the pilot plant.

Gel electrophoresis indicated that casein was the predominant protein in components, regardless of separation temperature and source of processing (Figure 3-1). Whey proteins α-lactalbumin and β-lactoglobulin also were present. Distinct bands of proteins associated with the milkfat globule membrane such as butyrophilin and xanthine oxidase were also present for all components, with more distinct bands associated with all sweet buttermilk components. Butter-derived aqueous phase obtained from a commercial source had more distinct bands of milkfat globule membrane proteins than butter-derived aqueous phase produced in the pilot plant.

Elling et al. (1996) also determined fat (%) and protein (mg/g) content for skim, buttermilk, and butter-derived aqueous phase. Elling et al. (1996) discovered that sweet buttermilk and butter-derived aqueous phase had significantly higher ($p \leq 0.05$) fat contents than skim milk whereas butter-derived aqueous phase had significantly higher ($p \leq 0.05$) amounts of protein in comparison to skim milk and sweet buttermilk. Approximately $1.50 \pm 0.36\%$, $1.13 \pm 0.15\%$, and $0.02 \pm 0.00\%$ total fat were found in the butter-derived aqueous phase, buttermilk, and skim milk, respectively (Elling et al., 1996). Butter-derived aqueous phase, buttermilk, and skim milk were found to have 31.75 ± 0.89, 27.00 ± 0.84, and 28.01 ± 0.68 mg protein/g of lipid, respectively (Elling et al., 1996).

Cholesterol content was determined for all components. Butter-derived aqueous phase had significantly ($p \leq 0.01$) higher amounts of cholesterol than sweet buttermilk and skim components. Of the components, skim component had the lowest amount of cholesterol ($p \leq 0.01$), even having significantly less ($p \leq 0.01$) cholesterol than sweet buttermilk. These results are in accord with the findings of Elling et al. (1996). Elling et al. (1996) found that 0.31 ± 0.02, 0.14 ± 0.0, and 0.05 ± 0.00 mg/g cholesterol occurred in butter-derived aqueous phase, sweet buttermilk, and skim milk, respectively. Higher amounts of cholesterol are expected to be associated with sweet buttermilk and butter-derived aqueous phase since cholesterol is concentrated in the milkfat globule membrane. The effect of separation temperature on cholesterol content was significant ($p \leq 0.01$) for butter-derived aqueous phase, with butter-derived aqueous phase obtained from $55^{o}C$ separation having significantly higher ($p \leq 0.01$) amounts of cholesterol. In comparison

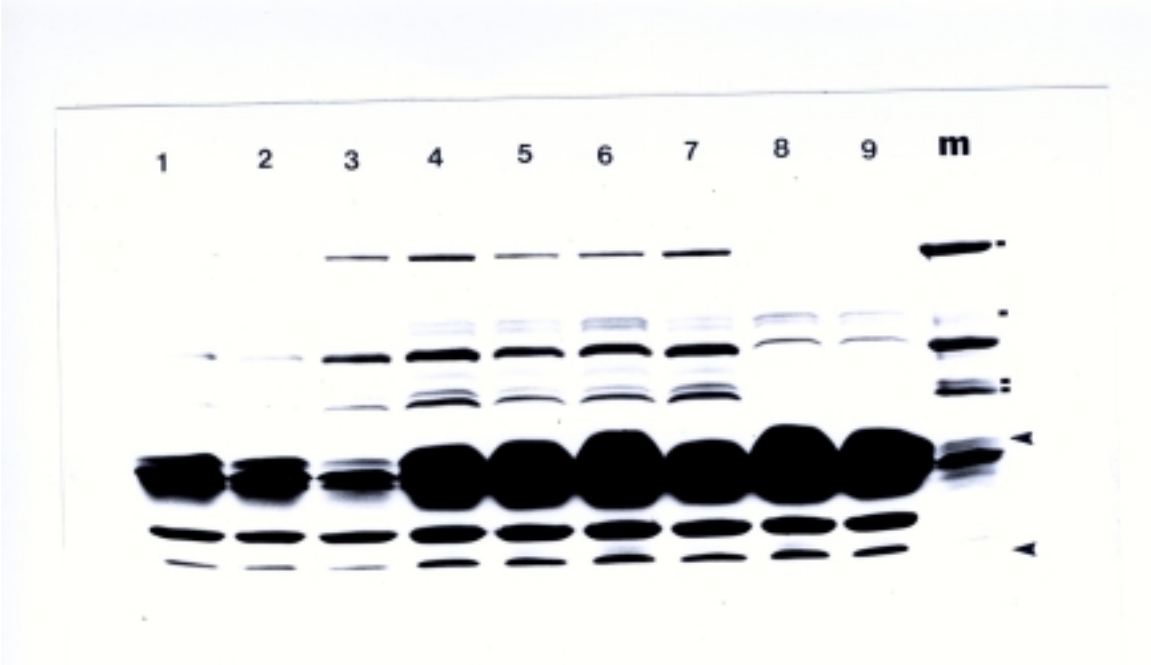

Figure 3-1. Polypeptide profiles of pilot plant produced components obtained at two different separation temperatures (49°C and 55°C) and commercially obtained components (1, 49°C butter-derived aqueous phase) (2, 55°C butter-derived aqueous phase) (3, commercial butter-derived aqueous phase) (4, commercial sweet buttermilk) (5, 49°C sweet buttermilk) (6, 55°C sweet buttermilk) (7, unpasteurized commercial sweet buttermilk) (8, 49°C skim component) (9, 55°C skim component) (m, milkfat globule membrane extract) (dashes from top to bottom: xanthine oxidase, CD36, butyrophilin, and glycoprotein B) (arrowheads from top to bottom: casein and whey proteins, β-lactoglobulin and α-lactalbumin, respectively)

of commercial components to pilot plant processed components, commercially processed butter-derived aqueous phase had significantly lower ($p \leq 0.01$) amounts of cholesterol than pilot plant processed butter-derived aqueous phase. Lipid content of commercial butter-derived aqueous phase was lower than that of pilot plant processed butter-derived aqueous phase, thus contributing to a lower amount of cholesterol.

The fat content of the components also influenced the amount of phospholipid found in each component. The phospholipid content serves as an indication of the presence of milkfat globule membrane fragments in the components. Butter-derived aqueous phase had significantly higher ($p \leq 0.01$) amounts of phospholipid than skim and sweet buttermilk components (Tables 3-3 and B-1). Sweet buttermilk had significantly higher ($p \leq 0.01$) phospholipid content than skim component. Elling et al. (1996) also reported that butter-derived aqueous phase had significantly ($p \leq 0.05$) higher amounts of phospholipid than skim and sweet buttermilk components. Elling et al. (1996), however, did not find significant ($p > 0.01$) differences in phospholipid content between skim and sweet buttermilk components. In most instances, slightly higher amounts of phopholipid were found in components than those used by Elling et al. (1996). Phospholipid content did not differ significantly ($p > 0.01$) between pilot plant processed sweet buttermilk and commercially manufactured buttermilk. Pilot plant processed butter-derived aqueous phase, however, had a significantly higher ($p \leq 0.01$) phospholipid content than butter-derived aqueous phase produced at the commercial operation. As with cholesterol, the particular observation of pilot plant butter-derived aqueous phase having a higher phospholipid content than commercially processed butter-derived aqueous phase can be attributed to the higher lipid content associated with pilot plant processed butter-derived aqueous phase.

Analysis of individual phospholipids indicated that sphingomyelin, phosphatidyl choline, and phosphatidyl ethanolamine were the most distinct bands of phospholipids occurring in components (Figure 3-2). Less distinct bands of phosphatidyl serine and phosphatidyl inisitol were observed in components (Figure 3-2). The results were expected since sphingomyelin, phosphatidyl choline, and phosphatidyl ethanolamine are the predominant phospholipids occurring in milk (Deeth, 1997). In regard to different components, butter-derived aqueous phase and sweet buttermilk appeared to have more distinct bands of individual phospholipids than skim. This fact can be attributed to the higher amounts of phospholipid, which remain as fragments of the milkfat globule membrane after churning, in both sweet buttermilk and butter-derived aqueous phase.

Determination of the types and amounts of fatty acids associated with components was determined by gas chromatography on one replication. Higher levels of unsaturated fatty acids such as oleic acid and linoleic acid were detected in buttermilk and butter-derived aqueous phase components than skim component (Table 3-4). Longer chained saturated fatty acids were associated with skim component, the most abundant being palmitic acid. Very similar amounts of the fatty acids myristic, palmitic, stearic, and oleic acid occurred in skim obtained at 49^{o}C and 55^{o}C separations. Amounts of individual fatty acids for sweet buttermilk obtained at 49^{o}C and 55^{o}C had very little variation, however commercial buttermilk had lesser amounts of fatty acids. Palmitic acid was the

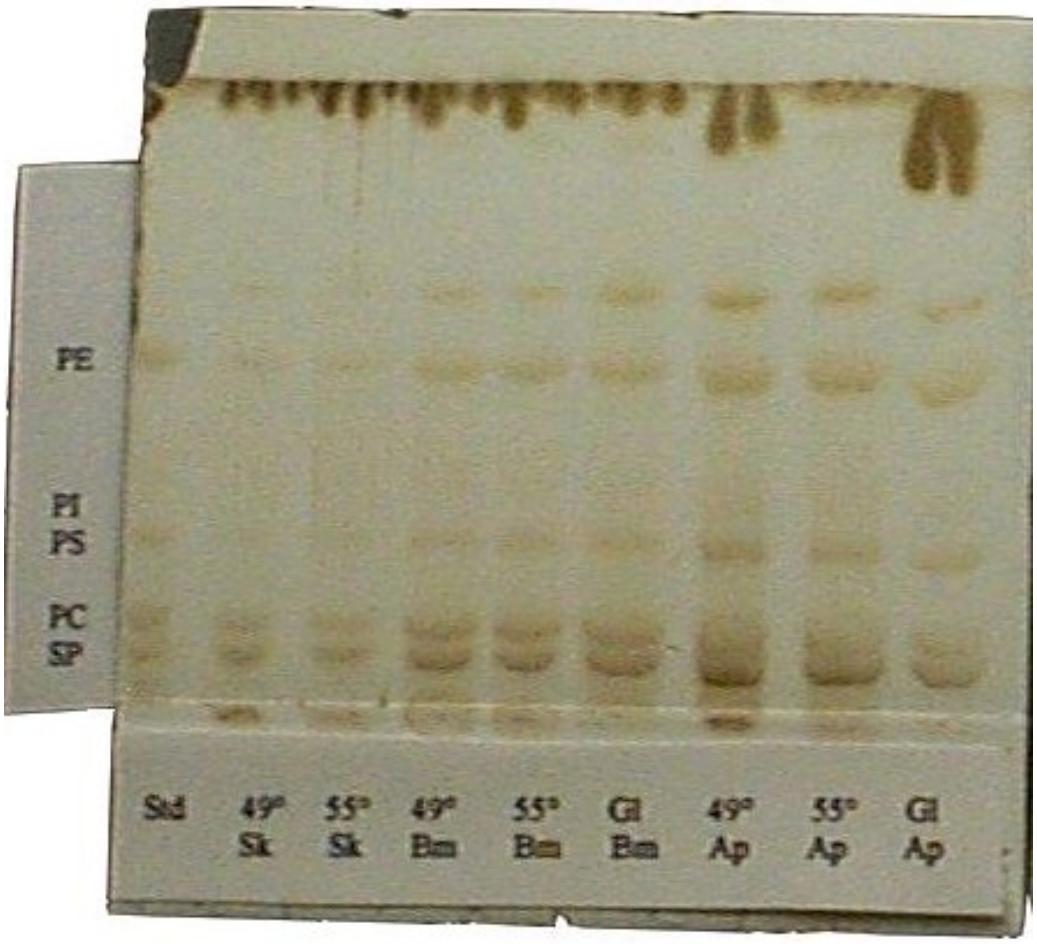

Figure 3-2. Thin layer chromatographs of individual phospholipids sphingomyelin, phosphatidyl choline, phosphatidyl serine, phosphatidyl inisitol, and phosphatidyl ethanolamine) for pilot plant processed components obtained at two separation temperatures (49oC and 55oC) and commercially obtained components (Gl Bm = commercial buttermilk) (Gl Ap = commercial butter-derived aqueous phase).

Table 3-4. Fatty acid analysis[1] of components used for cream reformulation obtained at two different separation temperatures (49°C and 55°C).

	Fatty acid amount (μg/ml)										
Component	C4:0	C6:0	C8:0	C10:0	C12:0	C:14:0	C16:0	C16:1	C18:0	C18:1	C18:2
49°C Skim						0.73	1.65		0.47	0.78	
55°C Skim						0.71	1.51		0.42	0.74	
49°C BM	1.43	3.14	1.87	3.05	2.65	6.04	13.7	1.02	3.31	8.22	1.05
55°C BM	1.77	3.59	4.60	3.51	2.98	6.62	15.2	1.14	3.91	9.31	1.08
Com. BM	0.62	1.38	0.90	1.95	1.34	3.00	6.80	0.58	1.92	4.44	0.66
49°C AP	3.20	4.14	2.99	3.79	3.87	12.02	28.49		10.58	13.56	
55°C AP	3.06	3.94	2.59	3.29	3.69	11.35	28.69		11.43	11.29	
Com. AP	4.89	6.40	2.95	5.60	6.32	12.48	31.69	2.98	10.53	23.79	3.32

[1]Means from triplicate measurements from one replication
[2]BM = buttermilk; Com. = commercial; AP = aqueous phase

predominant saturated fatty acid occurring in buttermilk and high levels of the unsaturated fatty acid oleic acid were also present. Caproic and capric acids were the short chain fatty acids in highest concentration in buttermilk. In comparison to butter-derived aqueous phases obtained by 49^{o}C and 55^{o}C separations, commercial butter-derived aqueous phase had higher amounts of short chained, saturated fatty acids. All sources of butter-derived aqueous phase had relatively abundant amounts of oleic acid. For all components, high levels of palmitic acid and oleic acid are expected since both contribute 20-25% and 30-38% of total fatty acid content, respectively (German and Dillard, 1998).

Skim, sweet buttermilk, and butter-derived aqueous phase, if used in sufficient amounts, can be utilized as components in emulsification of butteroil (Elling et al., 1996). The emulsifying capacity of skim, sweet buttermilk, and butter-derived aqueous phase can be attributed to the presence of surface active molecules such as proteins and phospholipids, which maintain emulsion stability at the oil/water interface. Skim is a rich of proteins such as whey and casein. These particular proteins also are associated with sweet buttermilk and butter-derived aqueous phase. The latter also are abundant in milkfat globule membrane fragments, which result from the churning process.

Emulsifying ability of casein is attributed to factors such as open structures, high levels of apolar amino acids, and uneven pattern of amino acids (Fox and McSweeney). As a result, casein proteins adsorb readily at oil-water interfaces. Whey proteins also play an important role in emulsification. However, emulsifying capacity is somewhat limited in comparison to casein proteins, due to susceptibility to heat denaturation and structural restrictions. Skim also contains a considerable amount of phospholipids, approximately 17-30%, indicating that some membrane material is associated with skim (Fox and McSweeney, 1998). Sweet buttermilk and butter-derived aqueous phase typically have some native milkfat globule membrane components such as phospholipids and proteins. These particular molecules remain as fragments after the native milkfat globule membrane has been disrupted by the churning process. Approximately 50% of all phospholipids in cream are associated with the milkfat globule membrane (McPherson and Kitchen, 1983). As a lipid group, phospholipids prove to be excellent emulsifying agents due to its amphiphillic nature, having affinity for both polar and apolar regions. The major milkfat globule membrane protein is butyrophilin, a highly hydrophobic protein which is strongly attracted to lipids.

Visual Characterization of Natural and Reformulated Creams. Transmission electron microscopy was used to visualize emulsion characteristics of 20% milkfat reformulated and natural creams. In previous studies butteroil was recombined into dairy systems consisting of components such as skim milk, sweet buttermilk, and butter-derived aqueous phase (Oehlmann et al., 1994; Elling et al., 1996). Electron microscopy was used to determine emulsion characteristics and the relationship of fat globules to membrane material. Oehlmann et al. (1994) found that water-in-oil emulsions (30% milkfat) were produced upon emulsification of butteroil by milk lipid globule membrane,

sweet buttermilk, or sweet buttermilk + butter-derived aqueous phase. However, Elling et al. (1996) found that 20% milkfat reformulations consisting of skim, sweet buttermilk, butter-derived aqueous phase components and reduced cholesterol butteroil were characteristic of oil-in-water emulsions in dairy creams after determining an appropriate ratio of components and butteroil needed for optimum emulsification.

Regardless of formulation, milkfat melting point characteristics, and separation temperature used in obtaining components, all reformulated and natural creams were characterized as oil-in-water emulsions (Figures 3-3 and 3-4). Lipid globules were roughly spherical in shape. Membrane material fragments and caseins were observed on the lipid surface. Average globule diameter ranged from 0.49 μm to 0.87μm, but the globule size range within formulations was very wide (Table 3-5). Overall, globule sizes ranged between 0.09-6.94 μm (Table 3-5). Creams processed from low-melt fraction butteroil and skim component had many small globules and were relatively uniform in size (Table 3-5; Figure 3-3). In contrast, creams formulated with medium-melt butteroil, sweet buttermilk, and butter-derived aqueous phase had fat globules with a large average diameter with high standard deviations (Table 3-5; Figure 3-4).

Natural and reformulated creams displayed fat globule clustering, resulting from the homogenization process. However, clustering in most reformulated creams was characterized by the presence of smaller globules surrounded by one or two large globules. In natural creams, clustering mainly involved globules which had similar diameters. Fat globule clustering is a phenomenon resulting from homogenization (Walstra and Jenness, 1984). During homogenization, the native milkfat globule membrane is fragmented. However, proteins from the serum phase adsorb to fat globules. Clustering results when at least two fat globules share surface material. Elling et al. (1996) also reported fat globule clustering in control and reformulated creams.

Chemical Characterization of Natural and Reformulated Creams. The implications of formulation, separation temperature in obtaining components, and butteroil melting range characteristics were considered in making chemical comparisons of natural and reformulated creams. In determining the chemical composition of natural and reformulated creams, separation temperature in obtaining components, butteroil melting range characteristics, nor formulation had a significant effect on the percent fat content of creams (Table 3-6). Percent lipid was determined using both the Babcock method and extraction methods outlined by Folch et al. (1957). Percent values from the Babcock method were reported since this particular method was used in standardization of natural and reformulated creams. Slight differences in lipid content between the two methods were observed. Also, lipid loss tends to occur with extraction procedures, such as the Folch lipid extraction method (Folch et al., 1957), since the lipid material undergoes a series of extractions and is constantly transferred to different types of tubes. This may explain the difference in fat content observed by the two methods.