

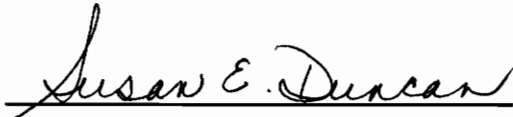
REENVELOPMENT OF NATIVE AND PROCESSED BUTTEROILS
INTO GLOBULES RESEMBLING MILK LIPID GLOBULES
IN FUNCTIONAL PROPERTIES

by

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Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree
MASTER OF SCIENCE
in
Food Science and Technology

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June, 1992

Blacksburg, Virginia

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ABSTRACT

A method of reencapsulating native and reduced-cholesterol butteroil within natural membrane material and proteins of milk such that milkfat globule size and function resembled native cream as closely as possible was developed and evaluated. By replacing the native butteroil with reduced-cholesterol butteroil, an approximate cholesterol reduction of 80% was possible.

Milkfat globule membrane material and proteins associated with the lipid phase were evaluated indirectly by measuring light scattering and phosphodiesterase activity in centrifuged reformulated raw cream. Functional stability of cream was measured by evaluating emulsion stability. Optimum conditions for reformulation using ultrasound included ratios of 1:1:1 (butteroil:buttermilk/aqueous phase:skim milk), emulsification time of 2.0 minutes for a 15 ml sample, and emulsification temperature of 42°C. Optimized conditions were used in scale-up reformulation of 750 ml samples replacing ultrasound with homogenization. Treatments included both native butteroil and reduced-cholesterol reformulated creams and unwashed natural cream as the control. Reformulation conditions included phase ratios of 1:1:1 (butteroil: buttermilk/aqueous phase:skim milk), homogenization temperature of 42°C, two-stage homogenization pressures of 500 psig/2000 psig, and product

passed through the homogenizer twice. Much more membrane material and proteins became reenvolved into the cream phase with the homogenization process than with ultrasound.

Stability of raw homogenized creams over 4 days at 2°C was evaluated by light scattering, phosphodiesterase, and emulsion stability. Overall, there were significant differences among treatments for light scattering and phosphodiesterase assays but not for emulsion stability. Significant differences among days within treatments indicated that the raw creams did change significantly over the time of storage. No differences were found among replications within treatments. Churn time, as a measure of functional stability, was measured on the first day after reformulation. No differences among treatments were observed.

Practical applications of this reenvolvement method may include the use of butteroil in a wider range of reformulated dairy products including fluid milk, ice creams, and cheese products. Development of any full-fat reduced-cholesterol product will require a reformulation step unless cholesterol is directly removed from the food.

ACKNOWLEDGMENTS

I would like express my sincere appreciation to my major professor and advisor, Dr. Susan E. Duncan, for her insight and support throughout my Master's coursework and research.

I would also like to thank Dr. Thomas W. Keenan for having enough confidence in me to place me on the grant in the first place, for the use of his lab and equipment, and for preparing and explaining the electron microscopy samples. Thanks are extended to Dr. Kent K. Stewart for encouraging me to go to graduate school and for his guidance of statistical analyses of my data.

My gratitude is extended to the National Dairy Promotion and Research Board for providing financial support to sponsor this research during the past year.

Special recognition and thanks are given to Walter Hartman for assisting me during cream processing, and to Janet Webster for preparation of electron microscopy samples.

DEDICATION

This manuscript is dedicated to my parents, Les and Anna Marie Oehlmann, for their moral and monetary support throughout my years at Virginia Tech, without which I could not have gone on to graduate school in a field I truly enjoy. Thank you for teaching me not only the value of an education, but also of the satisfaction which comes from learning.

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I. INTRODUCTION AND OBJECTIVES

Reformulation of milk lipid globules involves reenvelopment of butteroil droplets by milk fat globule membrane (MFGM) alone or with available proteins in the milk serum. MFGM is a complex bilayer membrane which forms as globules are secreted from the apical region of lactating cells and enveloped by plasma membrane material (Patton and Keenan, 1975). After envelopment, polar lipids may realign themselves according to hydrophilic/hydrophobic forces and some of the membrane lipids may become dissolved into the lipid core. Over time, some of the bilayer's outer interface between globule and aqueous phase may be lost and replaced by milk proteins which adsorb onto the globule surface and maintain stability of the globule. This can be induced by homogenization, which reduces globule size and increases the surface area the MFGM needs to cover. Gaps in membrane material are filled by adsorbed proteins from the serum. Both MFGM and serum proteins act to stabilize the emulsion of milk lipid in aqueous milk serum (Mulder and Walstra, 1974).

Mechanical stress from homogenization has been found to affect the amount of membrane material retained by milk lipid globules (Keenan et al., 1983b). Changes in lipid composition, membrane-associated enzymes, and overall

morphology of the membrane were documented. However, it was concluded that a large fraction of the original MFGM remained associated with the lipid globules, though it was probably spread over a larger surface area.

Homogenization speed, emulsifying time and temperature, concentrations of milk fat and MFGM, pH, and post-emulsification storage temperature may all affect emulsion properties and must be optimized to successfully reformulate a stable and commercially useful reformulated cream (Kanno, 1989). If such research were to prove successful, the butteroil phase could potentially be removed from the MFGM and aqueous phases, treated to reduce its cholesterol content, and reencapsulated with membrane. Reformulation techniques could be applied not only to reduced-cholesterol dairy products but also to products where it may be useful to remove and fractionate butteroil in order to obtain desired melting or baking qualities.

Milk fat (butteroil) has been fractionated using supercritical fluid extraction (SFE) with supercritical carbon dioxide (SC-CO₂) as the solvent/carrier gas, short-path molecular distillation, and steam stripping. Fractions separated according to melting point, molecular weight, volatility, and intermolecular attraction of constituent triglycerides (Arul et al., 1987). These fractionation

methods can concentrate cholesterol into certain fractions, providing an avenue of reducing total cholesterol in the remaining butteroil (Kankare and Antila, 1989).

SFE has been used to remove cholesterol from milk fat in a fairly exclusive manner. Using SC-CO₂ and an ascending pressure profile extraction, it was possible to separate 90% of the cholesterol from butteroil (Bradley, 1989). Short-path molecular distillation consists of evaporation of molecules, mostly fat-soluble vitamins, sterols, and volatiles, from natural oils into a vacuum. This is an effective process for fractionating butteroil into liquid, intermediate, and solid fractions, and for reducing cholesterol in the oils (Arul et al., 1988b). Another cholesterol-reducing treatment, steam-stripping, is a distillation method of removing cholesterol from butteroil. The OmegaSource Corporation (Burnsville, Minnesota) uses high-pressure steam to distill off cholesterol-rich fractions of butteroil from preheated anhydrous milkfat. The cholesterol-reduced milkfat contains only about 5% of the cholesterol originally present.

Although the health community still remains divided over the health implications of dietary cholesterol, the public seems to have reached its own conclusions (Best, 1988). According to a survey published in the Journal of

the American Medical Association (Schucker, 1987), 72% of adults polled (n=4000) believed that reducing blood cholesterol would have a major beneficial effect on heart disease, and more than 60% cited diet as the key method for reducing blood cholesterol. Twenty-three percent said they had already made diet changes to reduce their blood cholesterol. Consumers now have the option to substitute low-fat or skim milk products for whole milk products to reduce the amount of fat and cholesterol in their diets. However, consumption patterns indicate that consumers prefer low-fat to skim milk products. The dairy manufacturers' answer to this consumer preference may be to add cholesterol-reduced milk fat to skim milk to provide the mouth-feel and flavor of low-fat milk products, yet have the cholesterol content of a skim product (Schroder and Baer, 1990). At any rate, the potential market for low-cholesterol foods has been estimated to be 25 billion dollars (Best, 1988). This could be lucrative for the dairy industry once practical methods of reducing cholesterol are designed and perfected, and stability of low-cholesterol products can be assured.

Experimentation is needed to examine feasibility of reencapsulating native and reduced-cholesterol butteroil with natural cream components. Reformulation should mimic the natural system of membrane surrounding lipid globules

both structurally and functionally. The primary objective of this research was to combine MFGM reformulation techniques and a cholesterol-reduction method to develop a commercially feasible modified cream with stability and utility characteristics of native cream. Specific objectives included 1) optimization of phase ratios (butteroil:buttermilk/aqueous phase:skim milk) to obtain a product similar to homogenized cream; 2) development of redispersal time and temperature conditions to create fat globules with diameters in the range of <1 - 8 μm ; 3) modification of phosphodiesterase and light scattering assays to assess reencapsulation of milk fat globule by MFGM and serum proteins; 4) utilization of reformulation techniques with reduced-cholesterol butteroil; and 5) application of optimum conditions for reformulation of both native butteroil and reduced-cholesterol butteroil using a pilot plant homogenizer.

II. REVIEW OF LITERATURE

A. Milk and Milk Fat Globule Membrane (MFGM)

Bovine Milk and Milk Fat Composition

Bovine milk contains five primary constituents: water (87%), lactose (4.6%), fat (3.9%), proteins (3.5%), and minerals (0.7%), as well as trace amounts of vitamins. Fat content of milk varies from 2.5% - 6.0% depending on the breed of cattle; almost all occurs as small lipid globules <1-8 um diameter encapsulated by membrane material. Globule size also is affected by breed and is related directly to fat content of milk; cows producing milk with higher fat content usually produce larger globules instead of more globules (Walstra, 1983). Lipids in milk are distributed primarily in globules but also in membrane material and skim phase.

Total milk lipid is composed of triacylglycerol (97-98%), mono- and diacylglycerols (0.30 - 0.63%), free fatty acids (0.10 - 0.44%), free sterols which are primarily cholesterol (0.22 - 0.41%), phospholipids (0.2 - 1.0%), and trace amounts of hydrocarbons and sterol esters (Jenness, 1988). Cholesterol in the core lipid makes up about 75% of the total cholesterol in milk, the rest is contained in MFGM (10%) and membranes in the skim milk phase (15%) (Mulder and

Walstra, 1974). Total cholesterol content is 14-15.2 mg/100 g whole milk (Feeley, 1972; Mulder and Walstra, 1974). Sterol esters are concentrated in the serum phase, where they account for 0.9 - 3.3% of the nonphosphatide lipid (Huang and Kuksis, 1967).

Milk Fat Globule Membrane Composition

The greatest difference between native and reformulated dairy products is in the fat globules emulsified into the aqueous phase. Though size and fat composition of globules are important with respect to properties of products obtained, properties of surface layers acquired during reformulation are also important (Oortwijn et al., 1977). The unique properties of milk fat globule membrane and its constituents have been studied extensively to understand the characteristics of the surface layers created under different reformulation mechanisms and the effects on functional properties of the lipid globules.

Native MFGM is a membrane about 10 nm thick which encapsulates milkfat to permit a dispersion of fat into aqueous skim phase, or milk serum, producing an emulsion. MFGM is predominantly lipid (47% - 49% dry weight basis) and protein (41 - 43% dry weight); but these account for only 1-2% and 1% of total milk lipid and protein, respectively. MFGM lipid is 55-69% nonphosphatide lipid (including

glycerides) and 3-30% phospholipid (Mulder and Walstra, 1974). Triglycerides in the MFGM have a higher average degree of saturation and a higher average melting point range than those of the lipid core and are called high-melting triglycerides. Unsaturated fatty acids or glycerides are not readily incorporated into the MFGM (Huang and Kuksis, 1967). Other membrane constituents include protein- and lipid-bound sialic acids (63 nmol/mg protein), hexoses (0.6 umol/mg protein), hexosamines (0.3 umol/mg protein), free sterols and sterol esters, enzymes, and minerals generally found in milk. Eighty percent of total sterol in MFGM occurs in free form, 20% occurs in esterified form. Cholesterol and cholesterol ester are the major sterols in MFGM; other sterols, lanosterol and dihydrolanosterol, are insignificant in comparison (Patton and Keenan, 1975). Free sterols constitute up to 4% (dry weight) of membrane whereas the lipid core of the globule is only 0.16-0.3% free sterol. This does not indicate that there is a higher total amount of cholesterol in MFGM than in the fat globule, only that there is a higher concentration. The enzymes are involved in various degradation reactions once they are released from the MFGM. Primary enzymes of the MFGM include alkaline phosphatase, acid phosphatase, 5'-nucleotidase, phosphodiesterase I, and xanthine oxidase (Dapper et al., 1987).

Milk Fat Globule Formation

A complete understanding of milk synthesis and encapsulation with MFGM has not been possible because of the complexity of biosynthesis and of the bilayer membrane. Lactating mammary tissue contains structural units called alveoli. An alveolus is a spherical arrangement of lactating cells surrounding a lumen which serves as a receptacle for milk secreted by cells. The collective lumina drain into a duct system which carries the milk to outlet(s) at the skin surface (Patton and Keenan, 1975). It has been theorized that milk fat droplets form and grow near granular endoplasmic reticulum (ER); the ER may possibly provide a nucleation site for initiation of lipid droplet formation as well as stabilizing lipid globules in the cytosol. A basis for such theories is the enzymatic machinery contained in the ER which has the ability to incorporate fatty acids into triglycerides and phospholipids. Fatty acids utilized by the ER are equally derived from two sources, existing blood lipids and de novo synthesis in mammary tissue (Patton and Keenan, 1975).

The pathway by which triglycerides are formed is thought to be one involving monoglyceride as substrate. Monoglycerides and fatty acids released by lipase in the intestinal lumen are transported into mucosal cells where they are resynthesized into triglycerides. Fatty acids

involved in this synthesis must be previously activated by ATP and Coenzyme A (Patton, 1973). Synthesized triglycerides are maintained in a liquid state through two devices. The first is incorporation of short chain fatty acids into triglycerides, which induces a low melting point. The second is conversion of saturated fatty acid to unsaturated fatty acid which has a lower melting point than the saturated form; the unsaturated fatty acid is then used in triglyceride synthesis (Patton, 1973).

Growing liquid fat droplets gradually migrate from the interior to the apical or secretory region of the cell. The exact mechanism for migration is not known but several factors may be involved in the process. The apical region contains fewer organelles and therefore has more open space for occupation by fat droplets and secretory vesicles. Also, the shape of cells in the alveolus is affected by the presence or absence of milk in the lumen (Patton and Keenan, 1975). Cells are flattened by pressure of milk in a full lumen, and elongated into the lumen when it is empty. The elongation of cells draws fat droplets into the apical plasma membrane (Jeffers, 1935). Developing fat globules within the cell do not have a limiting membrane until they leave the cell and become subsequently surrounded by plasma membrane, as documented by electron microscopy (Patton and Trams, 1971).

The mechanism for attraction of fat droplets to apical plasma membrane has not been fully determined either; however, it is likely that the attraction is due, primarily, to London - van der Waals forces between the two predominantly-lipid surfaces (Patton and Fowkes, 1967). Binding of globules to the inner apical membrane surface is dependent on properties of the inner protein coat of the plasma membrane (Keenan and Dylewski, 1991). Properties such as acylation of proteins with long-chain fatty acids could enhance hydrophobic attraction to lipid droplets (Keenan et al., 1982).

Secretion of the fat globule most likely occurs through progressive envelopment of the droplet by plasma membrane until it is pinched off and released into the lumen. This theory is supported by the many similarities between the MFGM and plasma membrane, such as phospholipid distribution and fatty acid composition of individual phospholipids and cholesterol esters (Keenan et al., 1970; Patton and Keenan, 1975). The first documentation of globule secretion was carried out by Bargmann and Knoop (1959) and Bargmann, Fleischauer, and Knoop (1961) using electron microscopy. Patton and Keenan (1975) also have documented secretion of a rat milk fat globule using electron photomicrography.

There are many difficulties involved in assessing the secretion mechanism by examining composition and

distribution of membrane components. There are three principal surfaces in the secreted milk fat globule: the surface it has inside the cell before secretion and the inner and outer surface of the plasma membrane which surrounds the native globule surface. This coat material is not entirely plasma membrane, other surface-active molecules such as phospholipids, cholesterol, and proteins are present on the inner surface of the membrane. Therefore, isolation of the milk fat globule core lipid is problematic due to the high variety of membrane components which must be excluded (Patton, 1973).

Variability in observed composition of MFGM may be explained by age of the membrane and globule. Structure of the MFGM varies considerably depending upon amount of time passed since globule secretion. Wooding (1974) has suggested that, as milk fat globule is being released from secretory tissue, it consists of a continuous unit membrane with a thin layer of cytoplasmic material between the fat globule and unit membrane. This membrane may be derived partly from secretory cell plasma membrane and partly from Golgi vesicles. The globules appear to lose a large portion of this initial MFGM by a process in which the outer surface pinches off into small vesicles surrounded by unit membrane. This leaves the globule bound only by secondary MFGM which is not visible in electron microscopy photographs until the

initial MFGM layer begins to lift off. A consequence of the rapid breakdown of initial MFGM is that it is not possible to isolate bovine milk fat globules with more than 30-40% of their surface occupied by initial MFGM, since a considerable amount of initial membrane has been released into the skim milk phase. Isolation of membrane material from skim phase is possible through ultracentrifugation of skim milk and appears as a fluffy layer on the surface of the casein pellet (Wooding, 1974). The theory behind this loss of initial membrane is that stability of the milk fat globules of expressed milk is not dependent upon a continuous unit membrane such as plasma membrane, but rather on a secondary membrane formed from part of that initial MFGM. Initially the membrane may be more complex to allow secretion of the milk fat globule without great loss of cytoplasmic constituents and to maintain integrity of the secretory cell during and after secretion (Wooding, 1971). Occasionally, cytoplasmic crescents are caught between the lipid globule and its surrounding membrane during secretion; this occurs in about 1-5% of secreted milk fat globules. This cytoplasmic material may contain cytoplasmic organelles which contribute enzymes detected in association with milk fat globules (Wooding et al., 1970).

Replenishment of plasma membrane material lost during envelopment of milk fat globules leaving the cell is

accomplished when secretory vesicle membrane fuses with apical plasma membrane during exocytosis. This theory is supported by enzymological similarity of secretory vesicle enriched fractions to Golgi apparatus and apical plasma membrane-derived MFGM (Keenan et al., 1979).

Disagreement as to the extent to which membrane proteins rearrange within the lipid bilayer after milk fat secretion and the resulting stability of the milk fat globule has indicated that complete understanding of MFGM organization has not yet been achieved (Wooding, 1971). Recent morphological evidence shows that the MFGM has a typical bilayer structure with a layer of proteinaceous coat material along the inner interface between the membrane and lipid core. Intramembranous particles are aggregated, which leaves large areas of membrane surfaces depleted of these particles. This suggests membrane constituents forming these particles are rearranged in plasma membrane regions which envelop fat globules. Carbohydrates are asymmetrically distributed with respect to the bilayer but uniformly distributed over the outer surface of the membrane. MFGM proteins also display asymmetric orientation, some occurring on the inner surface and others on the outer.

Effects of Homogenization/Ultrasound on MFGM

Native MFGM is disrupted during homogenization, a mechanical process which produces a more complete and stable emulsion of milkfat into the skim phase. Milk fat globules are reduced in size from the average 4 μm to less than 1 μm and the surface area of globules is increased by 4-10 times (Keenan et al., 1983b). Keenan et al. (1983b) noted a net increase in amount of milk protein associated with lipid globules and a corresponding decrease in amount of protein in the skim phase. Fewer lipid globules could be recovered by flotation as more remained dispersed in the serum. A loss of phospholipid and cholesterol in the lipid phase corresponding to loss of total lipid was attributed to loss of free membrane material and formation of protein-triglyceride aggregates. It was concluded that homogenization served to spread MFGM material over a larger surface area.

Polytron homogenization has been used to induce isolated MFGM to reencapsulate pure butteroil. Optimum conditions for reconstitution of milk fat globules in this manner was accomplished by homogenization at 19000 rpm for an emulsifying time of 1 minute at 45°C. The concentration of milk fat was 25% with MFGM concentration of 2% at a pH of 4.0, and a post-emulsification storage temperature of

<30°C (Kanno, 1989). Kanno et al. (1991) described the physiochemical properties of MFGM which allowed globules to maintain their integrity and rendered them compatible with the serum phase. These physiochemical properties, examined by varying emulsifying time, pH, and milkfat and MFGM concentration, included viscosity, adsorbed protein, and globule size distribution.

Milk Quality Changes Resulting from Disruption of MFGM

Agitation from excessive pumping or stirring, or homogenization causes loss of MFGM, especially if air becomes incorporated into the milk. Disruption of the membrane can cause release of alkaline phosphatase into the skim phase. Destabilization of the membrane also increases susceptibility of core lipid to lipolytic attack, releasing free fatty acids that can produce rancid flavors. Xanthine oxidase, an enzyme abundant in MFGM, can catalyze a reaction producing superoxide, which causes lipid peroxidation and consequent oxidative rancidity. Activity of xanthine oxidase is further increased if heat denaturation of milk proteins has occurred (McPherson and Kitchen, 1983).

Extreme heat treatment such as ultra-high temperature (UHT) pasteurization may cause sulfhydryl compounds to form from precursors in MFGM. Residues such as cysteine are much

more reactive in MFGM than in serum even though there are significantly fewer residues (McPherson and Kitchen, 1983).

As stated previously, homogenization decreases fat globule size and increases total surface area. Membrane material does not completely cover the increased surface area and skim milk proteins adsorb to the exposed globule surfaces. Creaming of whole milk is retarded because globules have decreased affinity for each other, as their surfaces are more heterogeneous. Homogenized milk is whiter, with a bland flavor. Milk is generally less heat-stable and more susceptible to light-induced deterioration after homogenization (McPherson and Kitchen, 1983).

Freezing and thawing slowly causes a nearly complete dissociation of MFGM from lipid globules. Rapid freezing and thawing produces a less complete separation. Temperature fluctuations can occur in processing and destabilize the emulsion, causing oiling-off, or coalescence of lipid (McPherson and Kitchen, 1983).

Methods of Monitoring MFGM Structure and Composition

Monitoring changes in MFGM that occur during synthesis and storage or from mechanical agitation requires assessment of many components and, therefore, several methods are usually used simultaneously. Structure of the lactating cell and inner organelles can be documented using electron

microscopy. Enzyme analyses of the skim milk fraction have been used to determine amount of MFGM released into serum. Composition of MFGM may differ depending on the method used to prepare/isolate the membrane, which explains variability reported above for membrane components (Mulder and Walstra, 1974; Patton and Keenan, 1975). A simple and rapid procedure has been developed by Kanno and Kim (1990). Membrane fragments released from bovine milk fat globules were recovered as MFGM by acidification at pH 4.8 and centrifugation. Keenan et al. (1983b) induced release of membrane material from washed lipid globules by one cycle of freezing and thawing, then centrifugation. Wooding (1974) isolated membrane material from skim milk through ultracentrifugation to obtain a casein pellet with a layer of loosely packed membrane material on the pellet surface.

Providing relative information about components within the membrane provides a basis for understanding changes in composition. Measuring total solids, total lipids, proteins, sterols, neutral lipids and phospholipids, and enzymes requires different methods and provides certain key pieces of information for understanding the complex membrane. Methods used to identify and/or quantitate constituents of the MFGM are conducted on isolated MFGM material. Recovery of intact membrane material with exclusion of core lipid material has not always been

achieved, explaining the high variability in percent lipids associated with the membrane.

Total solids provides information on dry weight percent and water content of the membrane. Together, proteins and lipids account for over 90% of dry weight of MFGM (Patton and Keenan, 1975). A standard method of determining total solids is given in "Official Methods of Analysis of the Association of Official Analytical Chemists," (Helrich, 1990, #925.23).

Since milk fat globules are >95% triglyceride, the amount of this lipid included in the membrane preparation can influence greatly analytical results for protein content of the membrane. By assaying total lipids (Folch et al., 1957) and comparing the value to published values (Patton and Keenan, 1975), contamination of the membrane fraction by core fat can be assessed. Also, since lipid constituents usually are measured as percent of total lipids, obviously total lipids must be assayed before any constituent values may be obtained.

Proteins and glycoproteins are major components of MFGM, and early analyses were conducted by Lowry et al. (1951) to electrophoretically characterize MFGM proteins. Later, these methods of analysis were modified (Shimizu et al., 1976; Peterson, 1977; Markwell et al., 1978) to improve the methodology when it was found that treatment with sodium

dodecyl sulfate and 2-mercaptoethanol would allow more than 98% of the MFGM protein to be solubilized. Electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels has greatly expanded the number of polypeptides known to occur in the MFGM (Anderson et al., 1974; Patton and Keenan, 1975).

Sterols, which constitute a significant proportion of membrane lipids, are principally cholesterol and cholesterol esters. In addition to the basic cholesterol content of the membrane, the cholesterol-to-phospholipid ratio also is used to compare membranes and intracellular endomembranes from various tissues. Cholesterol may be determined colorimetrically with an o-phthalaldehyde reagent (Rudel and Morris, 1973; Bachman et al., 1976), or as a component of the neutral lipid fraction using thin-layer chromatography (Bligh and Dyer, 1959). Other neutral lipids identified by this method include free fatty acids, triglycerides, and cholesterol esters.

MFGM contains about 60% of the total milk phospholipids, the remainder is found in the serum phase. Major phospholipids of the globule membrane are sphingomyelin and phosphatides of choline, ethanolamine, inositol, and serine. Phospholipid distribution, or amount of specific phospholipids present, does not differ among whole milk, globule membrane, and milk serum (Keenan et al.,

1988). Phospholipid distribution, like cholesterol-to-phospholipid ratio, is used to compare MFGM with other membranes such as plasma membrane, which are similar. A spectrophotometric assay for measuring total lipid phosphorus was developed by Bartlett (1959). A combination of lipid extraction (Bligh and Dyer, 1959) and sample preparation (Kates, 1972) are used when separating and identifying individual phospholipids with thin layer chromatography.

While several enzymes with high specific activity have been detected in lipid globule membrane, few have been purified and characterized. Xanthine oxidase, which has been linked to the NADH- and NADPH-cytochrome c reductase activity and a cytochrome-linked redox system in MFGM, has been extensively studied (Jarasch et al., 1977; Bruder et al., 1982). An enzyme which is normally associated with lysosomes, acid phosphatase, is present in high specific activities in MFGM and is thought to be a true constituent of primary MFGM (Anderson and Cawston, 1975). Alkaline phosphatase is unusually stable in the presence of sodium dodecyl sulfate which allows its detection in SDS-polyacrylamide gels (Mather and Keenan, 1974). The glycoprotein 5'-nucleotidase may actually be a mixture of two distinct enzymes (Huang and Keenan, 1972a). Adenosine triphosphatase activity of globule membranes is stimulated

by K^+ and Mg^{2+} (Huang and Keenan, 1972b). Phosphodiesterase I activity can be easily measured photometrically using the artificial substrate p-nitrophenyl-5'-thymidylate (Brown et al., 1976). These last three enzymes have high specific activities in the MFGM, but are characteristically found in plasma membranes and are sometimes used as marker enzymes for plasma membrane (Keenan et al., 1988).

B. Cholesterol

Function of Cholesterol in MFGM

Cholesterol is an important structural component of animal cell membranes, in particular of the myelin sheaths and plasma membranes, and including the MFGM (Schlimme, 1990). It is also a precursor of bile acids, steroid hormones, and vitamin D (Sabine, 1977). Cholesterol is present both in core lipid and membrane fractions of milk; lipid phosphorus (phospholipids), however, are present almost exclusively as components of biological membranes (Mulder and Walstra, 1974). Bachman and Wilcox (1976) found that lipid phosphorus averaged 19.0 ug/ml and was equally distributed between cream and skim phases.

An electron microscopic method designed for detection of cholesterol in milkfat has been developed based on

incubation of cream from raw milk with filipin (a polyene antibiotic), which has specific affinity for cholesterol. The specific binding of cholesterol with filipin creates a complex that is observable by freeze fracture electron microscopy and enables localization information (Martin, 1989). Using this method, Martin (1989) found that cholesterol was localized within the membrane and the triglyceride core of milkfat globules. Higher organization of cholesterol was seen within MFGM than in the triglyceride core. The configuration of localized cholesterol was similar to those reported for plasma membranes.

Though reducing the fat content of a food may reduce the cholesterol content as well, it is not usually a proportionate reduction. Isolated butteroil (about 100% fat) contains 3-3.4 mg cholesterol/g fat, whole milk (3.5% fat) contains about 4 mg cholesterol/g fat, whereas skim milk (0.1% fat) contains about 30 mg/g fat (Feeley et al. 1972; Schlimme, 1990). The cholesterol in skim milk and other very low-fat foods is most likely derived from membrane material (Bachman and Wilcox, 1976).

Dietary/Health Concerns about Cholesterol

The dairy industry, being part of the broader industry of animal-based products, came under scrutiny in the 1980's as consumers became more aware of nutritional components in

their food, particularly fat and cholesterol, and the reputed causal relationship of these components to heart disease. This caused a problem for the dairy industry because, traditionally, milk fat has always commanded the highest economic value of all milk constituents, and amount of fat present in milk usually served as the basis for paying the milk producer. The value of fat depends, however, on several factors, including the type of dairy product being produced (Smith, 1973). Fortunately, the dairy industry had research and development capabilities to respond quickly to changing consumer demands for low-fat, low-calorie, and low-cholesterol products. Defatted milk provides consumers with 2%, 1%, and skim milk, and manufacture of lower fat versions of sour cream, yogurt, cheese, and even frozen dairy products solved the demand for low-fat, low-calorie, and low-cholesterol dairy foods (Best, 1989). An interesting controversy exists, however, in that consumers still prefer the sensory characteristics of higher-fat foods, as long as they are not informed of the fat content of the product. Information may influence purchase of the product, but eventually liking is determined by sensory attributes (Light et al., 1992). As consumers often negatively associate fat with cholesterol, reduced-cholesterol butteroil may find its niche in the market of full-fat foods.

While the dairy industry successfully responded to consumer concerns about fat and cholesterol, consumers still harbor distrust for many dairy products, based on false impressions. The butter industry has lost 50% of its market in the past 25 years, largely attributed to cheaper vegetable oil substitutes and anti-cholesterol propaganda which causes consumers to have a basic mistrust of the food industry and a fear of animal fats and cholesterol (Bradley, 1989). The consumption of milkfat in the total diet is usually falsely perceived by consumers to be higher than it actually is. In the average U.S. diet, milkfat provides only 10-15% of dietary fat and approximately 12% of dietary cholesterol (O'Donnell, 1989). Even so, cholesterol remains a critical obstacle to the acceptance of butter and butter-based products for many consumers, even though no correlation has been found between coronary heart disease mortality rates and butterfat consumption (Yudkin, 1957; Coates, 1983; Berthier, 1990), or between fat consumption and plasma total cholesterol values (Salmela, 1990). Genetics, not dietary fat, plays the most important role in determining serum cholesterol levels in humans (Stehbens, 1989). Individuals vary greatly in their response to dietary cholesterol (Ahrens, 1982); in healthy people, there is a control mechanism which balances cholesterol synthesis and intake so a relatively constant cholesterol level is

maintained (Glaeser and Keane, 1992). As with other potentially unhealthy foods or behaviors, many people would prefer to take precautionary measures to maintain their health rather than feel at risk of heart disease. Dietary modification to reduce cholesterol and saturated fat intake has been emphasized by the popular press as a means of reducing blood cholesterol levels and as precaution against coronary heart disease (Schucker et al., 1987). The media are flooded with negative information about dietary cholesterol and cardiovascular diseases and this health concern has contributed to the decline of fat-containing dairy products such as butter, cream, cheese, and full cream milk. In many countries, their official guidelines even recommend reduced cholesterol intake for all people. Therefore, there is a compelling reason for the dairy industry to offer low-cholesterol products (Versteeg, 1991).

Methods of Reducing Cholesterol in Butteroil

New technology is emerging which will help reduce the cholesterol content of food. Supercritical fluid extraction, short-path molecular distillation, and steam-stripping methods each have potential in reducing cholesterol and/or fractionating milkfat. Supercritical fluid extraction (SFE) uses very dense gas (gas above its critical value) to extract soluble components from a

mixture. The high density of the gas provides liquid-like solvent powers, but the low viscosity of dense gas allows faster mass transport characteristics relative to liquid. By varying the density of the gas, the solubility of compounds being dissolved into the gas can be altered. SFE is actually a simultaneous distillation and extraction process which does not require organic solvents (Arul et al., 1987).

Cholesterol has been removed from butteroil on a pilot-plant SFE system (Bradley, 1989) and milkfat has been fractionated into several fractions with unique properties. Transfer of this technology to manufacturing of low-cholesterol butteroil has not yet occurred. Its limited use may be attributed to high capital costs for plant start-up and operation, lack of understanding of theories behind the process, and an absence of engineering data for complete scale-up technology (Rizvi et al., 1986).

Many of the desired properties of milkfat can be selected by 'dry fractionation' of the whole milkfat. The process is designated as dry if no chemicals, soaps, or solvents are used. Only milk heating, cooling, and filtration are involved in order to preserve the flavor and nature of milkfat (Versteeg, 1991). Short-path molecular distillation provides an excellent method of obtaining fractions from butteroil with distinctive chemical and

physical properties. Because melting point and degree of saturation (solid fat content) are important criteria in selection of fats and oils in food-fat applications, fractionation of milk fat should increase utilization of milk fat in those applications (Arul et al., 1988b). The fractionation process consists of evaporation of molecules, mostly vitamins A and D, sterols, and volatiles, from natural oils into a vacuum. Arul et al. (1988b) have shown that this is an effective process for fractionating butteroil into liquid (MP = 12 C, ave. molecular weight = 622), intermediate (MP = 21 C, ave. molecular weight = 674), and solid (MP = 39 C, ave. molecular weight = 783) fractions. Though the melting point of cholesterol is 148.2°C, the highest incidence of cholesterol (78%) was in the liquid fraction. The intermediate fraction contained 18% of total cholesterol, and the solid fraction contained 4%. This and other results led to the suggestion that cholesterol has high affinity for short- and medium-chain triglycerides and some affinity for long-chain unsaturated triglycerides (Arul et al, 1988a). Unfortunately, this concentration of cholesterol in the soft fraction of butterfat is not desirable from a nutritional standpoint, because soft butterfat is nutritionally very valuable (Schlimme, 1990). Flavors are also preferentially

fractionated into the low-melting fraction, making it particularly useful for applications where high flavor intensity is desirable (Urbach, 1991). With respect to concentration of fatty acids by fractionation, fatty acid composition of the fractions fall within the range of normal variation in milkfat, even though the softening points of the fractions fall outside the range for normal milkfat (Versteeg, 1991).

Steam-stripping is a distillation method which can remove cholesterol from milk. The OmegaSource Corporation (Burnsville, Minnesota) uses high-pressure steam to distill off cholesterol-rich butteroil from preheated raw milk. The operating conditions involve a much higher pressure than that of molecular distillation so that steam is maintained. The distillate fraction may be reduced to 1%, as opposed to 20% in molecular distillation. This fraction contains cholesterol, butyric acid, low-molecular weight fatty acids, phospholipids, β -carotene, and α -tocopherol (vitamin E). As α -tocopherol is a natural antioxidant, removal of it and other volatiles decreases the stability of the treated butteroil (Massie, 1992). However, the cholesterol-reduced milkfat product does contain only 5 to 7% of its original cholesterol content, that is, less than 25 mg per 100 g of fat (Schroder and Baer, 1990). Processing steps for steam stripping are as follows (US #4,996,072, #4,804,555): an

anhydrous butteroil having a native cholesterol and odiferous material level is preheated to a temperature of 400-550° F (204-288°C), then flash-vaporized to achieve a par-treated liquid oil phase and a loaded vaporous phase, and maintained at 400-550° F. The par-treated oil is stripped as a thin film on an evaporative surface at pressure of 1-7 mm Hg, with a counter-current of steam which strips off cholesterol and other volatiles. When cholesterol removal is effectively complete, loaded vapor is removed and clean oil is cooled to below 100° F. This reduced-cholesterol product is currently being used in cholesterol reduced 2% milk, which is being marketed in 12 states. Expansion into butter-based spreads and structured cheeses is planned for the near future. The process also is applicable to deodorization and removal of cholesterol from fish oils (Massie, 1992).

Cholesterol also may be extracted from butteroil by mixing fat with aqueous β-cyclodextrin solution, forming inclusion complexes between the carbohydrates and cholesterol, which are removed by the addition of water and subsequent separation. Supposedly, in a continuous process, it would be possible to remove up to 80% of the cholesterol (Versteeg, 1991). This process has already been implemented in the industry and it is claimed that cholesterol is

selectively removed without the loss of fat-soluble vitamins and flavor compounds (Glaeser and Keane, 1992).

Enzymatic conversion of cholesterol to biologically inactive yet non-toxic and non-reabsorbable products has been suggested and partially verified physiologically. Cholesterol reductase can convert cholesterol to coprosterol, which is marginally reabsorbed by the body if absorbed at all. This cholesterol-elimination process has been found implemented by intestinal bacteria. Also conceivable is the reduction of cholesterol to 7-dehydrocholesterol by 7-dehydrocholesterol reductase, then conversion photochemically to precalciferol and vitamin D₃. Cholesterol may be oxidatively modified by cholesterol oxidase; however, the products are toxic to humans (Schlimme, 1990).

Lack of affordable enzymes and the need for further nutritional studies of the various conversion products makes enzymatic conversion commercially unfeasible in the short term. Long term prospects are good because it is likely that milkfat flavor will not be affected and the technology can be applied in both milkfat and milk products (Versteeg, 1991).

C. Cream and Other Dairy Products

Processing and Stability of Cream

Cream is a high-fat dairy product with a high concentration of cholesterol associated with proportionally large amounts of MFGM and core lipid. Cream production is basically a concentration of the fat in milk by centrifugal separation. For regulatory purposes, cream is normally specified according to milkfat content. Depending upon the desired use for the cream it may be separated from whole milk to achieve 10-80% or higher fat content. Standardized commercial cream products include half-cream (10-18% milkfat); pasteurized, sterilized, and ultra-high temperature (UHT) cream (>18% fat); whipping cream (>28% fat); heavy whipping cream (>35% fat); cream for butter production (40% fat); and double cream (>45% fat). Standards are also listed for stabilizers, thickeners, and modifiers which may be added to cream (Towler, 1982; Towler, 1986).

Compositional variations of cream give a variety of physical states, and different processes may modify the properties of cream. Separation temperatures affect both separating efficiency, or total amount of milkfat removed from milk, and amount of free milkfat, or fat not enveloped in membrane, in the cream. Maximum separation (efficiency)

of milkfat from skim occurs at about 50°C; both higher and lower temperatures decrease the percentage of fat removed. Free fat is more likely to occur at higher temperatures when it is in a liquid state than at lower temperatures when it is more solid. However, free fat formation is reduced when lipid is in either a completely solid or liquid form (Towler, 1986). Milk fat is liquid above 40°C and completely solid below -40°C. Between those extremes it is a mixture of fat crystals in oil (Jensen and Clark, 1988). The critical intermediate solidity range is 20-40°C. Free fat formation also can be reduced by eliminating incorporation of air during handling and separation (Towler, 1986).

Cream separators operate as modified centrifuges, separating by both gravity and centrifugal force. They are usually composed of a stacked series of identical conical discs and may or may not be hermetically sealed, self-desludging, or modified to clean-in-place. Whole milk enters the bottom of the discs and flows upward through holes in the discs. Skim milk eventually flows outward and downward and is collected from the perimeter of the rotating discs. Cream flows inward and upward and is collected from the center of the discs. Efficiency of separation can be achieved with bowl speeds of 4000-5000 rpm, though it is

important to maintain constant speed to insure uniform skimming. Set spacing of discs and constant feed rate of whole milk are also critical in producing a consistently uniform cream. Separation of a cream containing about 40% fat and skim milk containing 0.04-0.06% fat is considered good separating efficiency. The remaining fat in skim milk is attributed to milk fat globules smaller than 1 μm (Towler, 1986).

Churning of cream converts an emulsion of fat-in-water to a water-in-fat emulsion. Both mechanical shear and incorporation of air play a part in damaging the MFGM. The released fat agglomerates until the emulsion is broken and phase inversion occurs (Mortensen, 1983). Reports on optimum conditions for churning cream into butter vary, (Hupping, 1986; Wilbey, 1986; Morr and Richter, 1988) but, generally, cream containing 30-40% milkfat is chilled at or below optimum churning temperature for 12-20 hours or overnight. Optimum churning temperatures vary according to the season due to the higher melting milkfats in winter milk and lower melting milkfats in summer milk. A consistent product should be produced if the cream is cooled overnight at 6-8°C, or at least below 10°C. Before churning, the cream is warmed to 12-14°C, although lower temperatures may be used for summer creams and higher temperatures for winter

creams (Wilbey, 1986; Huppig, 1986).

Raw cream should be churned within 4 days or the cream will be too rancid to produce an acceptable butter (Huppig, 1986). Rancid cream foams excessively and may take up to 5 times longer to churn (Deeth and Fitz-Gerald, 1983). Rancidity is caused by naturally occurring lipase enzyme which catalyzes hydrolysis of esters from emulsified glycerides at an oil-water interface. This hydrolysis frees fatty acids, some of which, at proper concentrations, produce off-flavors and odors. The presence of lipase in separated whole milk occurs almost exclusively in the skim milk where 90% of it is bound to casein micelles and 10% is in the aqueous phase. A small amount of lipase does separate with the cream fraction. All methods of agitation increase rate of lipolysis and consequent rancidity through increasing the surface area of the lipid (Weihrauch, 1988). Homogenization of raw milk, when conducted at temperatures between 37.7°C and 54.4°C, may induce rancidity in a matter of minutes. Homogenization time and pressure also affect directly the amount of lipolysis (Schwartz, 1974). Lipases can be inactivated by standard pasteurization techniques, or heating at 80°C for 20 seconds (Weihrauch, 1988).

A convenient form of cream is full cream milk powder (FCMP), which contains 26-29% milkfat. The powder is formed

by spray-drying an oil-in-water emulsion usually containing emulsifying and stabilizing agents to give the dried product its desired functional properties. FCMP and other high fat powders may be used to contribute to another product's flavour, viscosity, or texture, and it is considered a very convenient form for handling milk fat and incorporating it into foods as a shortening (Munns, 1991). Foods containing such powders include bakery products confectionery; dry mixes of ice cream, cakes, soups, and sauces; and canned products containing cream.

Reduced-Cholesterol Dairy Products

The most efficient methods of cholesterol removal from milkfat have been discussed, but the majority of those methods involve treating isolated butteroil. A reconstitution step for dispersion of the treated butteroil into an aqueous phase and to introduce the fat back into the dairy food is inevitable. The demand for reduced cholesterol butteroil and dairy products places a burden on the dairy industry to develop new products that are stable and have similar functional properties to those of the original products. Several "light" butters are already being marketed in Canada and Europe. "Pure and Simple," marketed in Canada by Ault Foods Ltd., Canada, contains 52% less fat and 46% less cholesterol than regular butter.

"Diat-Butter Pro Herz" is produced by the Hoche Company, Germany, and claims to contain 70% less cholesterol than butter. A cholesterol-free butter is produced by Entremont, France, and is available in both full-fat and reduced-fat versions (Schlimme, 1990). Reduced-cholesterol novelty ice cream products have been test marketed in some areas, and reduced-cholesterol cheeses are currently being developed (Massey, 1992).

In development of these products, the company must consider how the reformulation effects contribute to or detract from the product's acceptability. If a cream product such as whipped cream were to be made from reduced-cholesterol cream, for example, several characteristics would be desired in the final product. Whipped cream is a multiphase network of air bubbles, serum, liquid oil, and solid fat. Therefore, the reduced-cholesterol product should have finely distributed air bubbles, sufficient milkfat (30-45%) to allow all air bubbles to be surrounded by fat, presence of a high proportion of crystallized fat in order to bind as much butteroil as possible after ageing, and prevention of the formation of too much butteroil by whipping at 2-4°C and storage at <10°C (Towler, 1982). Other factors to consider are pasteurization, which appears to have little effect on whipping properties, and

homogenization, which increases whip time and produces a softer whip due to smaller and more stable fat globules. Additives such as sodium tetraphosphate and triethanolamine have been found to improve whipping properties of cream such as whipping time, increase in volume, firmness of whip, and leakage of serum. Milk derivatives such as buttermilk, buttermilk extracts, and whey powder have been used to improve whipping properties. Xanthan gum, carrageenan, and sodium alginate have been found to have a desirable effect in reduction of separation and serum leakage during cream storage (Towler, 1982). These additives, used in various combination, may provide enough stability to a reduced-cholesterol whipped cream so that it could be successfully produced and marketed as one of the new reduced-cholesterol dairy products.

III. MATERIALS AND METHODS

A. Optimum Reformulation of Cream Using Ultrasound

Separation of Cream Phases

Milk of 3.8% average fat content was obtained from the Virginia Tech dairy farm. Cream (34-35% fat) was obtained by separation of prewarmed (24-27°C) raw milk using a pilot plant separator (Elecrem separator, model 1G, 6400 rpm, Bonanza Industries, Inc., Calgary, Alberta). Skim was retained for use during reformulation. Cream was washed twice by replacing separated skim milk with an equal weight of tap water of the same temperature and separating the mixture to remove milk serum proteins while retaining MFGM (Mather and Keenan, 1975). Cream was separated at room temperature rather than at cooler temperatures to resuspend the cream more completely and prevent loss of MFGM proteins (Mather and Keenan, 1975). Washing tends to increase the volume of cream, so care must be taken to maintain a constant water temperature throughout the separation process and to accurately weigh the water used for washing. Percent fat in the cream was determined by Babcock method (Richardson, 1985). Fat content of the washed cream was about 25-30%, reduced by 10-11% compared to original cream.

Washed cream was cooled to an optimum churning temperature of 13-14°C (Wilbey, 1986). Approximately 473 ml (16 oz) of cream were placed into a blender (Waring Commercial Blendor 700, model no. 33BL79, on-off only) attached to a lower-speed regulator (Powerstat 716, Superior Electric Co., Bristol, Conn.). Cream was mixed for 3.0 min at Level 40, stopped to scrape lipid clusters from blender sides, and mixed at Level 60 with stirring as necessary until the butter had amassed into a large ball. Butter, pressed free of buttermilk, was saved for further clarification into butteroil. Remaining buttermilk was filtered through cheesecloth to remove butter particles, then stored for use during reformulation.

Butter was heated with occasional stirring to 40°C. Melted butter was centrifuged at 4000 rpm and 10°C for 10 min and chilled (Beckman refrigerated centrifuge, model #J-6B) until the butteroil had solidified. The butteroil was removed with a spatula and saved, and the aqueous phase was added to the buttermilk phase in all trials except the first preliminary ultrasound experimentation. All phases (skim, butteroil, buttermilk/ aqueous phase) were stored at approximately 10°C. The skim phase was a primary source of serum proteins, and both skim and buttermilk/aqueous phases contained MFGM material for reencapsulation of butteroil.

Reformulation of Cream through Ultrasound

Butteroil was heated in a 45-50°C water bath until completely liquid. Phases were combined in desired ratio(s) (5 ml aliquots) of butteroil, buttermilk/aqueous phase, and skim milk into 30 ml plastic centrifuge tubes. Samples were heated in the water bath until the mixtures reached the desired temperature. Each sample was treated with ultrasound at Level 5 or 1/2 full power (Sonifier Cell Disruptor, model no. W140, Heat Systems-Ultrasonics, Inc., Plainview, Long Island, NY) for the desired time. Desired temperatures and times are described in the following section. Samples were then centrifuged for 5 min at 4000 rpm, and visual examination and subsequent tests were completed.

Samples were visually examined for separation of phases and free oil on sample surface, and measured for emulsion stability (ES). Evaluation of light scattering at 600 nm (Spectronic 1001, Milton Roy Co., Rochester, NY) was completed to determine the amounts of insoluble particulated in the serum phase as compared to amounts in buttermilk/aqueous phase and skim phase, and in this manner estimate the proteins and MFGM in the cream plug. Phosphodiesterase I, a marker enzyme, was measured to estimate the amount of MFGM in the serum phase as compared

to buttermilk/aqueous phase and skim phase and therefore estimate the amount of membrane which had reassociated with milkfat globules (Brown et al., 1976) Electron microscopy was conducted to assess the nature and size of reformulated globules.

Optimization of Cream Reformulation Using Simplex-Vtm

Preliminary work on unwashed cream was conducted to optimize conditions for reformulation of milk fat globules into cream before any modified butteroil was used. The parameters optimized using ultrasound were phase ratio, treatment time, and treatment temperature, in that order. Once one parameter had been determined, it was used as a standard condition during determination of the next parameter. During this preliminary testing, aqueous phase and buttermilk were added as separate phases.

The phase ratios used were 1:1:1 (butteroil (bo): buttermilk (bm):skimmilk (sm)), 1:1:1 (bo:bm:aqueous phase (ap)), 1:1:1:2 (bo:bm:ap:sm), and 1:1:2 (bo:bm:sm). Other conditions included: heating milk to 24-27°C before separation, heating samples to 37-39°C before 4 minutes of ultrasound treatment, and centrifugation for 5 min at 4000 rpm. Phosphodiesterase activity and visual examination were used to evaluate reformulated samples.

More extensive testing using unwashed cream was completed using a wider range for time and temperature of ultrasound treatment. Butteroil:buttermilk:aqueous phase were mixed in 1:1:1 aliquots, respectively, and treated with ultrasound for 1.5, 2.0, 2.5, and 3.0 minutes. Emulsification temperatures used were 37, 38, 39, and 40°C, milk was heated before separation, and centrifugation conditions were as before. Phosphodiesterase activity and visual examination were used to evaluate reformulated samples.

In an attempt to minimize the variability observed in phosphodiesterase activity among samples, washed cream was used for all subsequent reformulation processed.

To more methodically approach an optimum reformulation, a computer optimization program was used to define the optimum conditions for reformulation of milk fat globules in washed cream. The computer optimization program Simplex-V™ (Statistical Programs, Houston, TX), minimized the number of samples needed to determine optimum conditions for reformulation through ultrasound. This program, based on sequential simplex algorithms, takes experimental results and chooses sets of conditions that have a high probability of producing generally improved results for continued experimentation. A simplex is a geometric figure having a

number of vertexes, the number dependent on number of variables and/or number of samples being evaluated by the program. Simplex-Vtm has the ability to assess interactive effects of several experimental variables without the tedious process of changing and optimizing each variable sequentially (Statistical Programs, 1987). Parameters of the program which were chosen for ultrasound optimization include a variable-size simplex with user-defined starting simplex; optimization by maximization; four reformulation factors buttermilk (ml), skim milk (ml), treatment temperature (°C), and treatment time (min); and three multiple responses of light scattering, phosphodiesterase activity, and emulsion stability.

Percent butteroil was maintained at 33% when phase ratios were optimized; only amounts of skim milk and buttermilk/aqueous phase were varied. Aqueous and buttermilk phases were combined as one phase for all subsequent reformulation processes. The phase ratios used were 1:1:1 (butteroil: buttermilk/aqueous phase:skim milk), 1:0:2 (bo:bm/ap:sm), 1:2:0 (bo:bm/ap:sm), and 1:1.5:0.5 (bo:bm/ap:sm). Treatment temperatures varied in increments of 1 degree from 38-43°C, and ultrasound treatment times varied in increments of 0.5 min from 1.5-2.5 min. Temperature of milk during separation was standardized at

24°C, and reformulated samples were always centrifuged for 5 min at 4,000 rpm and 10°C prior to evaluation. Samples produced with different phase ratios at different ultrasound temperatures and times were produce in duplicate and results of light scattering, phosphodiesterase activity, and emulsion stability assays were entered into the optimization program. A set of variable parameters were computed as the optimum conditions, based on that data. This procedure was repeated 8 times using 4 different samples in each set to insure the output parameters were indeed optimum for all data sets combined.

Native and reduced-cholesterol cream samples were manufactured by ultrasound under the optimum conditions and using native or reduced-cholesterol butteroils as the appropriate lipid phase. Optimum conditions were phase ratio of 1:1:1 (bo:bm/ap:sm), ultrasound treatment time of 2 min, and emulsification temperature of 42°C. Four 15 ml samples each of native butteroil and reduced-cholesterol cream samples were produced and evaluated according to emulsion stability, light scattering of the serum and phosphodiesterase activity in the serum. Analysis of variance (SAS, 1986) was conducted to determine variance among replications and among treatments. Differences among means were determined by least square means.

by least square means.

B. Application of Optimized Parameters to Reformulation of Cream Using Homogenization

Separation of Cream Phases for Homogenization

Washed cream was obtained as previously described and cooled to optimum churning temperature of 13-14°C (Wilbey, 1986). Approximately 473 ml (16 oz) of cream was placed into a blender (Oster brand Galaxie, model #848-31M) and mixed at lowest speed until phase inversion occurred and butter amassed into a large ball. Butter and buttermilk phases were treated as before, storing buttermilk for reformulation and continuing separation of butter as previously described. Skim milk obtained from the initial cream separation was retained for use in the reformulation process.

The refrigerated centrifuge (Sorvall Superspeed model #RC2-B, DuPont Biomedical Products Division, Wilmington, DE) was operated at 4000 rpm and 0-2°C for 15 min or until butteroil layer had solidified, the only deviation from the previous method.

Reformulation of Cream

Butteroil was heated in a 45-50°C water bath until completely liquid. Butteroil, buttermilk/aqueous phase, and skim milk were combined (500 ml aliquots) in ratio(s) found to produce optimum results during the previous reformulations using ultrasound (1:1:1). The mixtures were heated to the optimum temperature, 42°C, and homogenized twice in a pilot-plant size two-stage homogenizer (model no. 15MR, APV Gaulin, Inc., Everett, Mass.; min. sample size = 1 pt, max. pressure = 8000 psig) at 500 psig in the first stage and 2000 psig in the second stage (2500 psig total). Five 10ml aliquots were removed from each homogenized sample and placed into 15 ml conical glass centrifuge tubes for evaluation of the emulsion over four days at 2°C. The remainder of each sample was stored overnight at 6-8°C. Churn time was determined for each cream as an indication of cream stability.

C. Reduction of Cholesterol in Butteroil

Reduced-cholesterol butteroil (OmegaSource Corp., Burnsville, MN) obtained by steam-stripping was used in place of native butteroil for reformulation of a reduced-cholesterol cream.

D. Assessment of Cream Reformulation

Measurement of Membrane Material Remaining in Serum Phase (Phosphodiesterase I Assay)

The phosphodiesterase assay was adapted from a procedure developed by Brown et al. (1976). It was conducted only on the serum phase of the centrifuged cream and not on the entire cream sample due to interference from fat globules. It must not be assumed that this is a direct method of assessing the amount of membrane material associated with lipid separated into the cream plug of the sample. It provides only a general method of comparing the membrane originally present to that which has not been incorporated into the cream plug. Serum (0.1 ml) removed from centrifuged cream samples was centrifuged in microcentrifuge tubes for 5 minutes at 4000 rpm to insure all cream particles were separated from the serum. A 10 μ l aliquot of sample was added to the reaction mixture composed of 0.19 ml deionized water and 0.80 ml substrate; the reaction mixture was then vortexed. The reaction was halted after 15 min with addition of 2 ml 0.2 N NaOH, and the mixture was vortexed. Absorbance of the 1:300 dilution was measured at 400 nm (Spectronic 1001, Milton-Roy Co., Rochester, NY). A blank contained 0.2 ml deionized water, 0.8 ml substrate, and 2.0 ml 0.2 N NaOH (molecular weight of

sodium hydroxide = 40 g/mole). The substrate was 2 mM p-nitrophenyl-5'-thymidylate (molecular weight of p-nitrophenyl-5'-thymidylate = 465 g/mole) in 0.1 M glycine buffer (molecular weight of glycine = 75.1 g/mole), titrated to pH 9.6. Sensitivity of this assay was 5.900 absorbance units/umole p-nitrophenol (p-NP) for the standard curve. Limit of detection was 0.0017 umole of p-NP, and linear range was 0.0017 - 0.254 umole of p-NP.

Measurement of Insoluble Particulates Remaining in Serum Phase (Light Scattering Assay)

This procedure was suggested by committee members Thomas W. Keenan and Kent K. Stewart based on prior experience. Information applicable to this assay had been presented by Sherbon (1988). It is used for estimating and comparing the amount of insoluble particulates (including MFGM and serum proteins) added to samples with the amount which did not reassociate with lipid during treatment. This assay is an indirect method, and it is not a measure of the protein which has been incorporated into the cream during treatment. Serum (20 ul), as obtained for the phosphodiesterase assay, was added to 1.98 ml of 0.25 M sucrose solution (molecular weight of sucrose = 342.2 g/mole). Tubes were vortexed and absorbance of the 1:100 dilution was read at 600 nm.

Emulsion Stability

The emulsion stability of cream samples was measured as described by Kanno (1989) and was determined by measuring the height of the cream layer at its lowest point (since a fixed-angle centrifuge head was used), and the height of the serum (or bottom of cream) at its lowest point. The equation for emulsion stability is $A-B/A$, where A is the measurement from the bottom to the top of the entire emulsion, and B is the measurement from the bottom of the emulsion to the top of the serum phase. From this equation it can be seen that the emulsion stability value is not a percent, but a less sensitive value which is dependent upon the quantity of liquid and the size of the container.

Electron Microscopy of Cream Samples

Samples were prepared using the methodology of Dylewski et al. (1984). A cream sample was placed in 2% buffered gluteraldehyde and held for a minimum of 2 hours at refrigeration temperature. Gluteraldehyde was removed and the sample rinsed by placing it in buffer and changing the buffer 3 times over a 30 min period at refrigeration temperature. The sample was then fixed in agar. A one percent osmium tetroxide solution was added and the sample was held 12 - 18 hours at 4°C or 1 hour at room temperature.

The osmium tetroxide was removed, and the sample was rinsed 3 times as described above. The contrast in samples was increased by saturating with aqueous uranyl acetate solution (0.5%) for 1-4 hours, then rinsing 3 times as previously described.

Samples were dehydrated through a gradient of acetone and water solutions, allowing 10 min in each solution at room temperature, in a progression from 25% acetone to 50% acetone to 70% acetone to 90% acetone to 95% acetone, and finally to 100% acetone which was changed 3 times during 1 hour.

Fixation was begun by adding an equal volume of plastic to the sample in the final 100% acetone and allowing it to stand for 1 hour at room temperature, yielding a plastic content of 50%. Another equal volume of plastic was added and sample was allowed to stand 1 hour, bringing the plastic content to 75%. The solution was then drained off, and pure plastic was added and allowed to stand for 1 - 3 hours. The solution was drained again, pure plastic was added and allowed to stand another 1 - 3 hours at room temperature or overnight in a refrigerator. Oven-dried Beem capsules were filled with pure plastic and specimens were dropped in. Capsules were held at 70°C for 8 hours.

E. Evaluation of Cream Stability

Churn Time

Homogenized unwashed cream, washed cream, and native and reduced-cholesterol reformulated creams were chilled overnight at 6°C. Aliquots of 500 ml were poured into 1.25 L blender jars, acclimated to 13°C, and mixed at the lowest speed (Oster brand blender, "Osterizer Galaxie," dual range 14, service# 848-31M). Churn time was determined by recording the time from when mixing began until butter granules clearly agglomerated into a large cluster and separated from the liquid phase. Analysis of variance (SAS, 1986) was conducted to observe differences among replications and among treatments. Least squares mean was used to determine differences in means.

Storage Stability

Ten ml aliquots of native and reduced-cholesterol reformulated creams and unwashed homogenized cream were stored at 2°C, and one of each was evaluated each day for 5 days for phosphodiesterase activity, light scattering, and emulsion stability. Day 0 was the first day after homogenization and the same day as churn time evaluation. Each tested sample was centrifuged at 3400 rpm and room

temperature for 15 minutes, and chilled at -8°C for 5 minutes to solidify the cream plug for measurement of emulsion stability. The lowest ends of top and bottom of cream layer were marked on tubes as cream plugs in older samples tended to turn in the tubes. Serum was obtained for phosphodiesterase activity and light scattering by withdrawing the cream plug to one side with a spatula to allow removal of 1-2 ml serum from the bottom of each tube using Pasteur pipettes. Serum was recentrifuged for 5 minutes at 3400 rpm before removing aliquots for light scattering or phosphodiesterase assays. Analysis of variance (SAS, 1986) was conducted to determine differences among replications, among treatments, and among days within treatments. Means were compared using least squares mean.

IV. RESULTS AND DISCUSSION

A. Reformulation of Cream Using Ultrasound

Preliminary Trials

After treatment by ultrasound, centrifugation of cream samples at 4000 rpm produced two layers in the cream. The upper layer was a condensed cream plug containing most of the lipid, and the bottom layer was a thin liquid, labeled "serum" throughout this manuscript, containing proteins and membrane material that had not recombined into the cream.

Although results stated below are based upon measured absorbances to determine phosphodiesterase activity and light scattering of the samples, it must be kept in mind that these assays were indirect; they were conducted on the aqueous phase, or serum, which separated from the cream plug upon centrifugation. This serum was part of the treated cream, but it had dissociated from the lipid portion of the cream by the gravitational and centrifugal forces associated with centrifugation. Therefore these assays were conducted to compare the membrane material and other particulates, including proteins, contained in the cream serum with what was originally provided by the buttermilk and skim milk, in order to assess the amount of those constituents which remained associated with the lipid phase.

Results from visual examination and phosphodiesterase activity in preliminary ultrasound trials with unwashed cream indicated that the best phase ratios under these conditions were 1:1:1 (bo:bm:ap) and 1:1:2 (bo:bm:sm). The ratio 1:1:1 (bo:bm:ap) was ultimately selected as the best ratio for continued optimization of unwashed cream reformulation for several reasons. The aqueous phase seemed more effective in providing membrane material for reencapsulating lipid globules since at least twice as much skim milk was required to provide the same visual results. Buttermilk and aqueous phases were found to contain about the same amount of membrane material, but the skim milk contained considerably less as indicated by phosphodiesterase activity. Composition of the original cream was most similar to 1:1:1 (bo:bm:ap) since the lipid content of the original separated cream was about 33%.

Unwashed cream samples emulsified for 2.0 min by ultrasound at 39°C seemed to yield a stable product based on the reduced amount of free butteroil (oiling-off) on the cream-plug surface and the lower phosphodiesterase activity in serum after centrifugation of the cream. Reformulations produced at other temperatures ranging from 37-40°C and treatment times of 1.5-3.0 min did not appear as stable.

The reassociation of membrane material with lipid

globules was only about 3% as determined by comparing average phosphodiesterase activity in the buttermilk and the aqueous phase used for reformulation to phosphodiesterase activity in the serum after centrifugation of the cream. The average phosphodiesterase assay absorbance of serum at 400 nm was 0.933 (1:300 dilution), while absorbance of the cream at 400 nm was 0.963. Standard deviation for phosphodiesterase activity among the 5 samples tested was 13%. Stability of the emulsion, as indicated by visual examination, was attributed to milk proteins contained in the buttermilk and aqueous phase, which probably coated the surface of the globules instead of allowing a true reencapsulation of lipid with MFGM. Further research was conducted using washed cream as a starting material to attempt to minimize variability among samples by providing a limited amount of serum proteins, primarily those present in skim milk, to each sample.

Optimization

Computer optimization of phase ratios was based on three assay methods, light scattering, phosphodiesterase activity, and emulsion stability, in order to provide a broader quantitative base for defining optimum parameters and to reduce the number of samples produced by ultrasound that were necessary. The phase ratio providing optimum

reformulation was 1:1:1 (bo:bm:sm), as compared to preliminary indications that 1:1:1 (bo:bm:ap) would be best. Optimum emulsification temperature was 42-43°C, higher than the 39°C previously noted as optimum; and optimum emulsification time was 2.0 min as previously determined. The temperature of the sample did not change during emulsification using ultrasound. The composition of the original cream was most similar to 1:1:1 (bo:bm:sm), assuming that the lipid content of the original cream was about 33%.

A sample of the computer feedback of all optimized conditions is presented in Table 1. Experimental results were input as vertexes (labeled I - 1) and a combined proportional value (response) was assigned by the computer based on the minimum and maximum parameters of the variables. The sixth vertex (R - 0) provided the computer-generated optimum for the input set of vertexes. In general, absorbances for light scattering and phosphodiesterase activity increased as variables were increased or decreased from optimum conditions. Emulsion stability decreased, though less dramatically than light scattering and phosphodiesterase values, when emulsification temperatures were lowered or when emulsification time was varied from the optimum.

Table 1. Final Simplex Output Generated by Simplex-Vtm for

Optimization of Reformulation through Ultrasound

VERTEX	Buttermilk 5 ml aliquot	Skim Milk 5 ml aliquot	Temperature °C	Time min	RESPONSE ¹
1) I - 1 ²	1.0	1.0	40.0	2.5	33.48
2) I - 1 ²	1.0	1.0	39.0	1.5	31.43
3) I - 1 ²	0.0	2.0	41.0	2.0	25.94
4) I - 1 ²	0.0	2.0	40.0	2.0	22.62
5) I - 1 ²	0.0	2.0	38.0	2.0	13.54
6) R - 0 ³	1.0	1.0	42.0	2.0	0.00

¹ Computer generated combination of light scattering, phosphodiesterase, and emulsion stability values based on assay max and min parameters

² Ultrasound trials input into Simplex-Vtm

³ Computer generated optimum vertex based on results of ultrasound trials

In comparison, work completed by Kanno (1989) utilizing a Polytron homogenizer to reencapsulate milkfat with pure MFGM indicated optimum emulsion stability was obtained with a MFGM content of 2% and milkfat content of 25%, and homogenization time and temperature of 1 min at 19,000 rpm and 45°C, respectively. In that study (Kanno, 1989), emulsion stability decreased after 1 min, supporting the theory that a long emulsification time causes irreversible reduction of interfacial area as well as incorporation of small air bubbles which induce coalescence of fat globules. Although the highest emulsion stability values were obtained at 45°C, slightly lower temperatures (>38°C) allowed the emulsion to be more stable after centrifugation. A milkfat optimum of 25% instead of 35% or higher was probably found because there was not enough membrane material to sufficiently cover the complete globule surface.

In a naturally occurring product, other milk proteins would associate with the globule, filling in spaces where MFGM was lacking. Since Kanno (1989) used purified MFGM in buffer to reencapsulate the milkfat, there were no other proteins available. By using buttermilk and skim milk obtained during separation of the cream, both MFGM and milk proteins were abundantly available to envelop fat globules.

Replication

Reproducibility of the emulsion produced under the determined optimum conditions was determined in 4 replications for cream manufactured from native and steam-stripped butteroil obtained from the OmegaSource Company (Burnsville, MN). Results of these replications are presented in Table 2. Light scattering is an indication of the amount of protein remaining in the serum as compared to that in the average of the buttermilk/aqueous and skim phases. When native butteroil and reduced-cholesterol butteroil cream samples were compared by the light scattering of the serum phase, the native butteroil serum (Ave = 0.071 ± 0.0053) had approximately 42% less than the average of buttermilk/aqueous and skim phases (Ave = 0.122), and the reduced-cholesterol serum (Ave = 0.064 ± 0.0026) had about 47% less protein than the initial components. Therefore, by indirect assessment, 42% and 47% of protein in native and reduced-cholesterol reformulations respectively was incorporated and stabilized into the cream plug of the centrifutged emulsion. When comparing the same samples according to phosphodiesterase remaining in the serum, native butteroil serum (Ave = 0.409 ± 0.0159) had an average absorbance decrease of 5% from the average of buttermilk/aqueous and skim phases (Ave = 0.431) and the reduced-cholesterol serum (Ave = 0.400 ± 0.0122) had an

Table 2. Comparison of Creams Reformulated (bo:bm:sm) with Ultrasound Containing Native or Reduced-Cholesterol Butteroil Using Light Scattering, Phosphodiesterase, and Emulsion Stability Assays

Assay	Treatment			
	Native Butteroil		Reduced-Cholesterol Butteroil	
	Ave ¹	± Std Dev	Ave ¹	± Std Dev
Light Scattering ²	0.071	± 0.0026	0.064	± 0.0053
Phosphodiesterase ³	0.409	± 0.0159	0.400	± 0.0122
Emulsion Stability ⁴	2.590	± 0.0180	2.509	± 0.1073

¹ Means (n=4) within rows are not significantly different by t-test (p≥0.05)

² Absorbance = 600 nm

³ Absorbance = 400 nm

⁴ Emulsion stability = Height of cream sample - (height of serum / height of cream sample)

average decrease of 7%. Phosphodiesterase is considered a marker enzyme for MFGM (Patton and Keenan, 1975), suggesting that slightly more membrane was incorporated into the cream in the reduced-cholesterol reformulation. The reduced-cholesterol samples had about 4.6% higher emulsion stability than the native butteroil samples. However, no significant differences were observed ($p \geq 0.05$) for absorbances of light scattering or phosphodiesterase activity in the serum or for emulsion stability of native or reduced-cholesterol cream. When interpreting all the results as a whole, the reduced-cholesterol butteroil produced a cream emulsion with slightly more protein and more membrane material incorporated than the emulsion containing nativebutteroil, and as a possible consequence, had higher emulsion stability. This may be due to the higher purity of the steam-stripped butteroil or its slightly higher melting point (both due to lack of other volatile compounds). Higher purity of the butteroil could induce a more uniform emulsion, whereas higher melting point could decrease the tendency of liquid globules to reassociate after disruption through ultrasound. Statistically, however, the cream reformulated with native butteroil was not different from the cream containing reduced-cholesterol butteroil. No electron microscopy work was completed on ultrasound reformulations.

The reformulated native and reduced-cholesterol cream

products did not appear or perform similar to natural cream and were easily separated into their components with gravitational and centrifugal force. Though the end product was not identical to raw cream, optimized parameters for these products were determined under the conditions established and were later applied to creams reformulated by homogenization with greater success.

B. Homogenization Results

Preliminary Trials

All homogenization samples contained 250 ml each of butteroil (native or reduced-cholesterol), buttermilk/ aqueous phase, and skim milk. Both washed unhomogenized and unwashed homogenized creams were run as controls. Unwashed homogenized cream served as the true control to which the reformulated samples were compared because it was the raw commercial product this reformulation research was attempting to imitate. Several preliminary runs served to perfect techniques, to standardize the performance of the separator and homogenizer, and to provide a comparison of the native butteroil and reduced-cholesterol butteroil reformulations. Light scattering and phosphodiesterase assays as well as emulsion stability were used to assess the reformulation. During these trials there were variations in the performance of the

separator - the fat content was too low (25% milkfat), then too high (52% milkfat). Aging substrate for the phosphodiesterase assay also made application of those results marginally useful. In the native butteroil samples, about 23% of the original membrane material and 74% of the total protein became reformulated into the cream phase, based on comparisons of absorbance values for light scattering and phosphodiesterase in the serum of reformulated centrifuged creams and the source skim and buttermilk/aqueous phases. Approximately 27% of the membrane material and 82% of the total protein became reformulated into the cream manufactured from reduced-cholesterol butteroil using these same methods of assessment. These results compared very favorably to ultrasound studies in which only 5% of membrane material and 42% of protein were incorporated into native butteroil reformulations, and only 7% membrane material and 47% protein were incorporated into reduced-cholesterol reformulations. The mechanical shearing during homogenization was much more effective than emulsification by ultrasound in reducing milk fat to small globules and disrupting membrane and protein material so they could reassociate with the globules.

Churn Times

Churn time indicates stability of the cream emulsion. A homogenized cream would require a longer churn time than an

unhomogenized cream because fat globules are disrupted into smaller globules with greater total surface area to which surface-active material from the serum are adsorbed. Smaller fat globules are more stable to coalescence, as the new proteinaceous surface material prevents globules from recombining (Walstra, 1983). Results from churning homogenized cream and reformulated samples are presented in Table 3. There were no significant differences found among the homogenized samples ($p \geq 0.05$), but it was noted that the variation among trials was high. Variation may be attributed to small batch size and pilot-plant equipment performance. Even when samples were shaken vigorously before homogenization, some oil separated from the emulsion before homogenization was complete. This oil, if crystallized at the oil-water interface, may have promoted coalescence of fat particles upon churning in some of the reformulated samples (Walstra, 1983).

Storage Studies

Reformulated and natural creams were stored at 2°C for 5 days and phosphodiesterase activity, light scattering, and emulsion stability were measured daily for each treatment.

Overall, there were significant differences among treatments for light scattering and phosphodiesterase assays

Table 3. Means¹ and Standard Deviation of Churn Time (min) of Homogenized Natural Cream and Homogenized Cream Reformulated with Native and Reduced-Cholesterol Butteroil

Treatment								
Homogenized ²			Native ³			Reduced ⁴		
Ave	±	Std Dev	Ave	±	Std Dev	Ave	±	Std Dev
5	±	0.4	6	±	1.0	5	±	1.1

¹ n = 5; p ≤ 0.05

² Unwashed homogenized cream

³ Cream reformulated with native butteroil from washed cream

⁴ Cream reformulated with reduced-cholesterol butteroil from washed cream

but not for emulsion stability. All assays indicated significant differences among days within treatments, so the raw creams did change significantly over 4 days of storage. No differences were found among replications within treatments (Appendices 1,2,3,4,5,6).

Absorbances for light scattering in separated serum differed among treatments ($p \geq 0.05$), and among days within treatments (Table 4). Absorbances for light scattering of separated serum from creams reformulated with native and reduced-cholesterol butteroil were not statistically different, but both differed from that from the homogenized cream, particularly at the beginning and the end of the storage study. Variation was greatest for the reduced-cholesterol product (22%) but was relatively high for the homogenized and native butteroil treatments as well (8 and 16.7% respectively). Changes occurring over time are summarized in Figure 1. Over time, the absorbance values for light scattering of serum from treatments began to approach that of skim milk phase (Figure 2), which had remained relatively stable during the same period. For all treatments, the overall trend of increasing particulates in the serum phase after centrifugation was probably caused by weakening of the cream emulsion and loss of protein and MFGM from the surface of fat globules.

Table 4. Means (n=5) and Standard Deviations of Light Scattering Assay (A=600 nm) in Serum of Centrifuged Homogenized Natural Cream and Homogenized Creams Reformulated with Native or Reduced-Cholesterol Butteroil over 4 Days of Storage at 2°C.

Day	Treatment								
	Homogenized ¹			Native ²			Reduced ³		
	Mean	±	StdDev	Mean	±	StdDev	Mean	±	StdDev
0	0.051 ^c	±	0.0063	0.031 ^a	±	0.0040	0.033 ^a	±	0.0184
1	0.054 ^{cd}	±	0.0035	0.040 ^{abc}	±	0.0046	0.029 ^a	±	0.0065
2	0.043 ^{abc}	±	0.0023	0.040 ^{abc}	±	0.0063	0.035 ^{ab}	±	0.0147
3	0.034 ^{ab}	±	0.0056	0.051 ^c	±	0.0192	0.035 ^{ab}	±	0.0123
4	0.069 ^d	±	0.0285	0.051 ^c	±	0.0193	0.050 ^{bc}	±	0.0051

¹Homogenized unwashed natural cream

²Cream reformulated with native butteroil

³Cream reformulated with reduced-cholesterol butteroil, (OmegaSource, Inc.)

^{a, b, c, d} Means (n=5) within a row or column with different superscripts are different (p≤0.05)

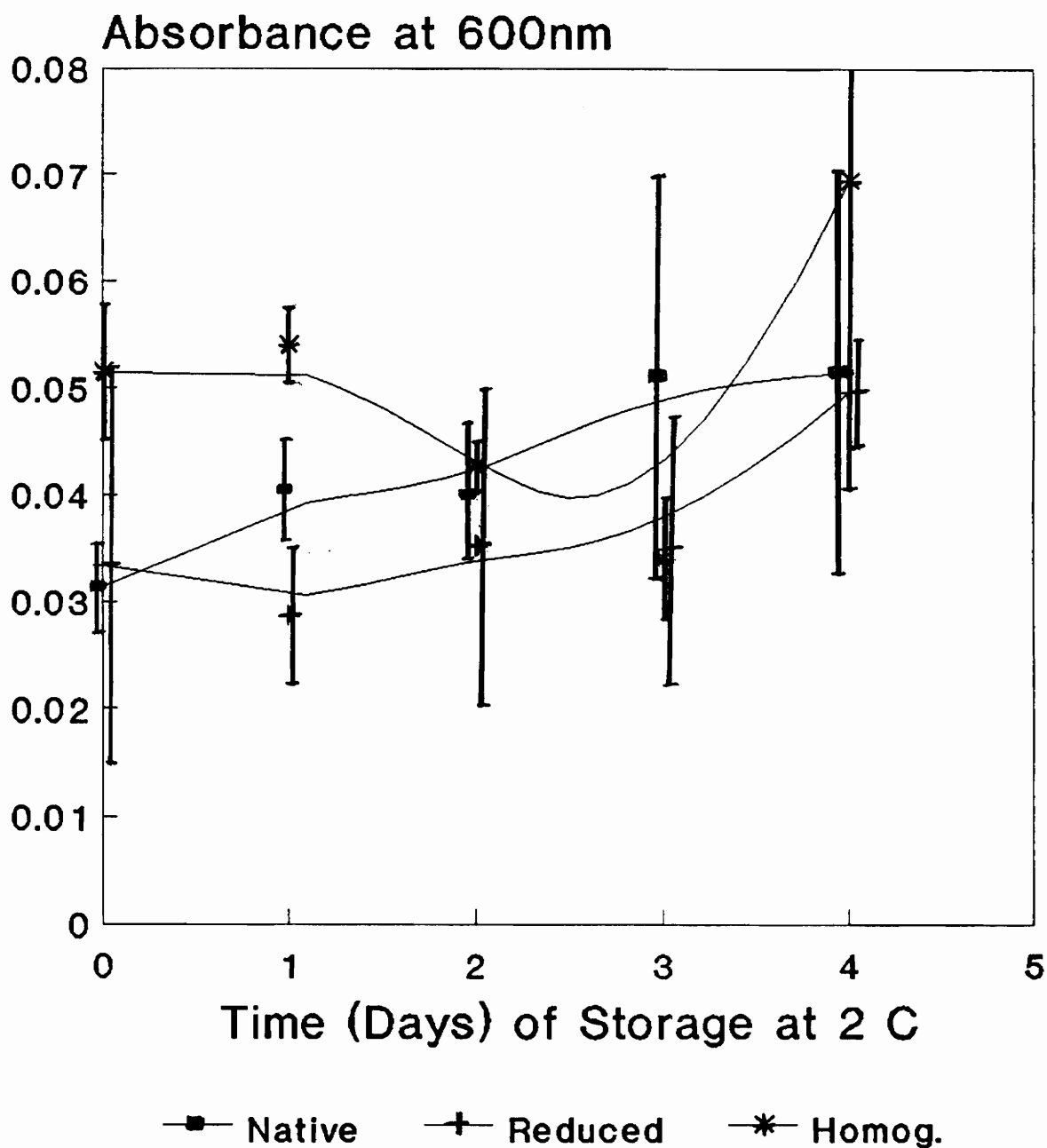


Figure 1. Means and standard deviations (n=5) and general trends of light scattering assay ($\lambda=600\text{nm}$) in serum of centrifuged homogenized natural cream and homogenized creams reformulated with native or reduced-cholesterol butteroil over 4 days of storage at 2°C .

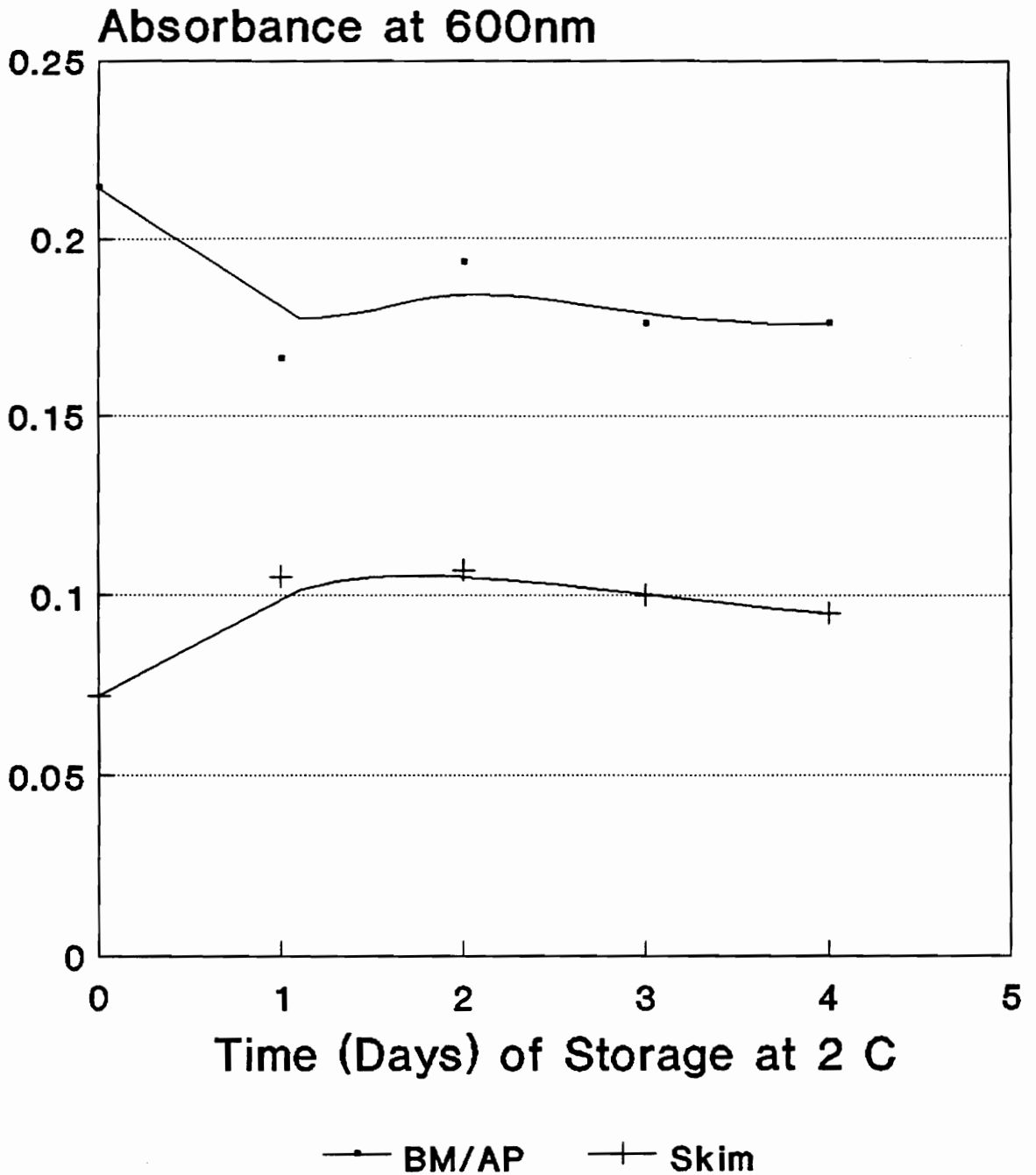


Figure 2. Means (n=5) and general trend of light scattering assay ($\lambda=600\text{nm}$) for buttermilk/aqueous phase and skim milk over 4 days of storage at 2°C .

Higher light scattering values in serum from centrifuged homogenized cream were indicative of a more complete homogenization. Successful homogenization of natural cream produces very small, dense milk fat globules which tend to remain in serum instead of separating as cream during centrifugal flotation. The decrease in serum particulates during 2 and 3 days of storage probably indicates the maximum stability of the emulsion during that time, after which the cream matrix began to break down. This assay was not particularly useful for describing changes happening in the creams over time because of the high variability produced, especially when the aging emulsion began to break down. Particles of the cream emulsion were very difficult to exclude from samples even after recentrifugation of serum, and caused drastic increases in light scattering if accidentally included with serum.

Differences among treatments and among days within treatments ($p \leq 0.05$) were evident from the absorbance values for phosphodiesterase in the serum of the reformulated and natural homogenized cream samples. Serum from both reformulated creams had similar phosphodiesterase absorbance values, but the values for the homogenized control were different. Similarities and differences among treatments and days can be seen in Table 5. Large variation existed

Table 5. Means (n=5) and Standard Deviations of Phosphodiesterase Assay (A=400nm) from Serum of Centrifuged Homogenized Natural Cream and Homogenized Creams Reformulated with Native and Reduced-Cholesterol Butteroil over 4 Days of Storage at 2°C.

Day	Treatment					
	Homogenized ¹		Native ²		Reduced ³	
	Mean	± StdDev	Mean	± StdDev	Mean	± StdDev
0	1.130 ^e	± 0.0670	0.571 ^d	± 0.0618	0.573 ^d	± 0.1083
1	0.986 ^{ef}	± 0.0554	0.472 ^c	± 0.0687	0.435 ^{bc}	± 0.0470
2	0.925 ^c	± 0.0290	0.453 ^{bc}	± 0.0181	0.453 ^{bc}	± 0.0451
3	1.063 ^{fg}	± 0.0391	0.407 ^{abc}	± 0.1095	0.374 ^{ab}	± 0.0779
4	1.0790 ^{fg}	± 0.1084	0.3656 ^{ab}	± 0.0945	0.3380 ^a	± 0.0767

¹Homogenized unwashed natural cream

²Cream reformulated with native butteroil

³Cream reformulated with reduced-cholesterol butteroil, (OmegaSource, Inc.)

^{a, b, c, d} Means (n=5) within a row or column with different superscripts are different (p≤0.05).

among replications indicating variable success in stabilizing the lipid with MFGM. Changes occurring over time are summarized in Figure 3, which indicates a fairly constant decrease in phosphodiesterase activity in the serum of the reformulated treatments but a variable response in the serum of the homogenized cream. Figure 3 also demonstrates the similarity of the native butteroil and reduced-cholesterol butteroil reformulations by homogenization. Just as with the light scattering assay, the phosphodiesterase activity found in the serum of the homogenized cream was much higher than in the serum of either reformulated cream. This was another indication of the effective homogenization in the standard; that is, the homogenized standard contained many more small globules dispersed in the serum than the reformulated creams. In comparison to changes in phosphodiesterase activity in buttermilk/aqueous phase and skim milk over time presented in Figure 4, serum of cream reformulated with native butteroil (mean slope = 0.035) and serum of cream reformulated with reduced-cholesterol butteroil (mean slope = 0.054) exhibited a decrease in phosphodiesterase activity similar to that in skim milk (mean slope = 0.058). The buttermilk/aqueous phase exhibited a much more dramatic decrease in phosphodiesterase activity over time (mean slope = 0.156). This may be attributed to the higher original

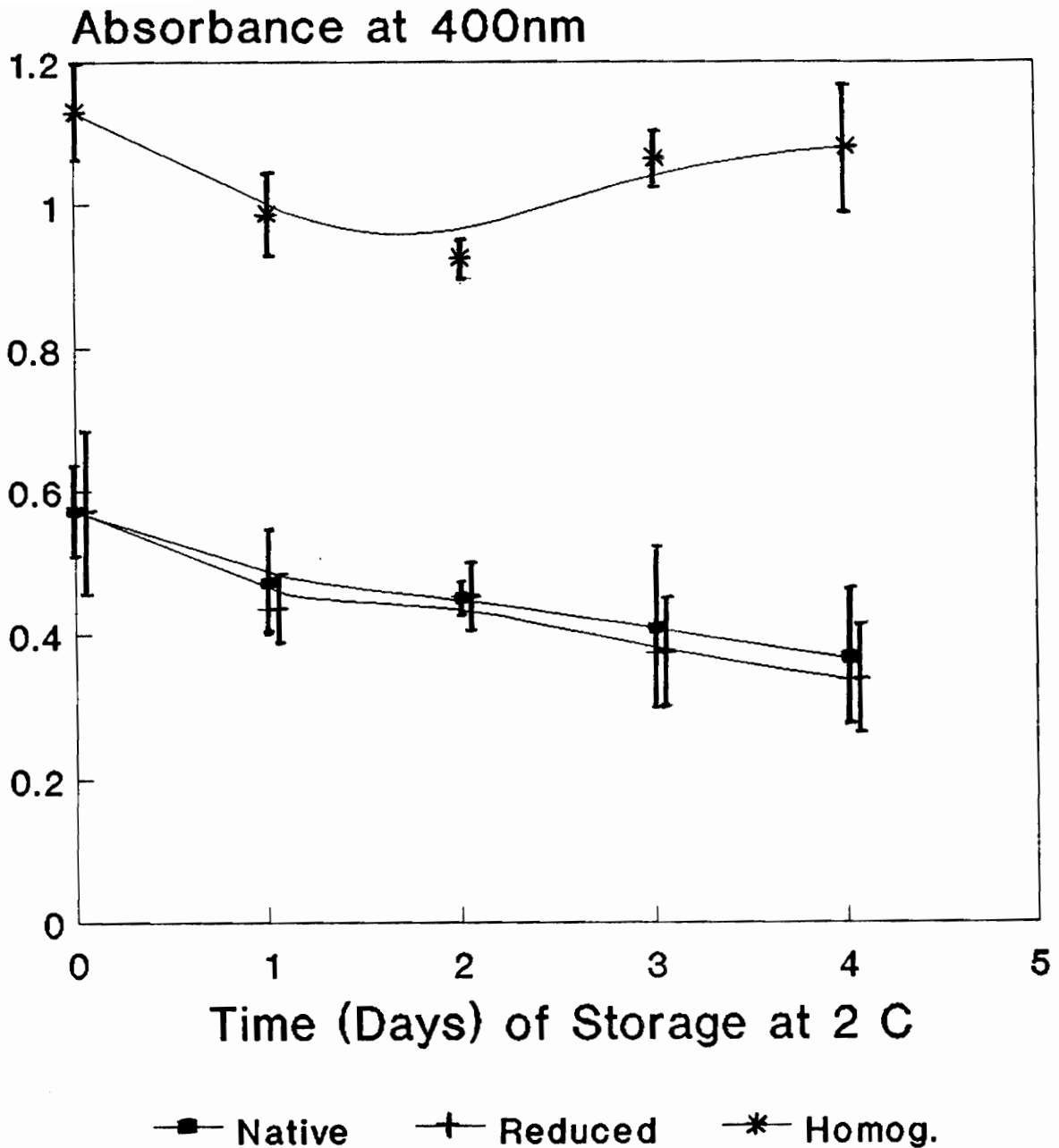


Figure 3. Means and standard deviation (n=5) and general trends of phosphodiesterase assay ($\lambda=400\text{nm}$) in serum of centrifuged homogenized natural cream and homogenized creams reformulated with native or reduced-cholesterol butteroil over 4 days of storage at 2°C.

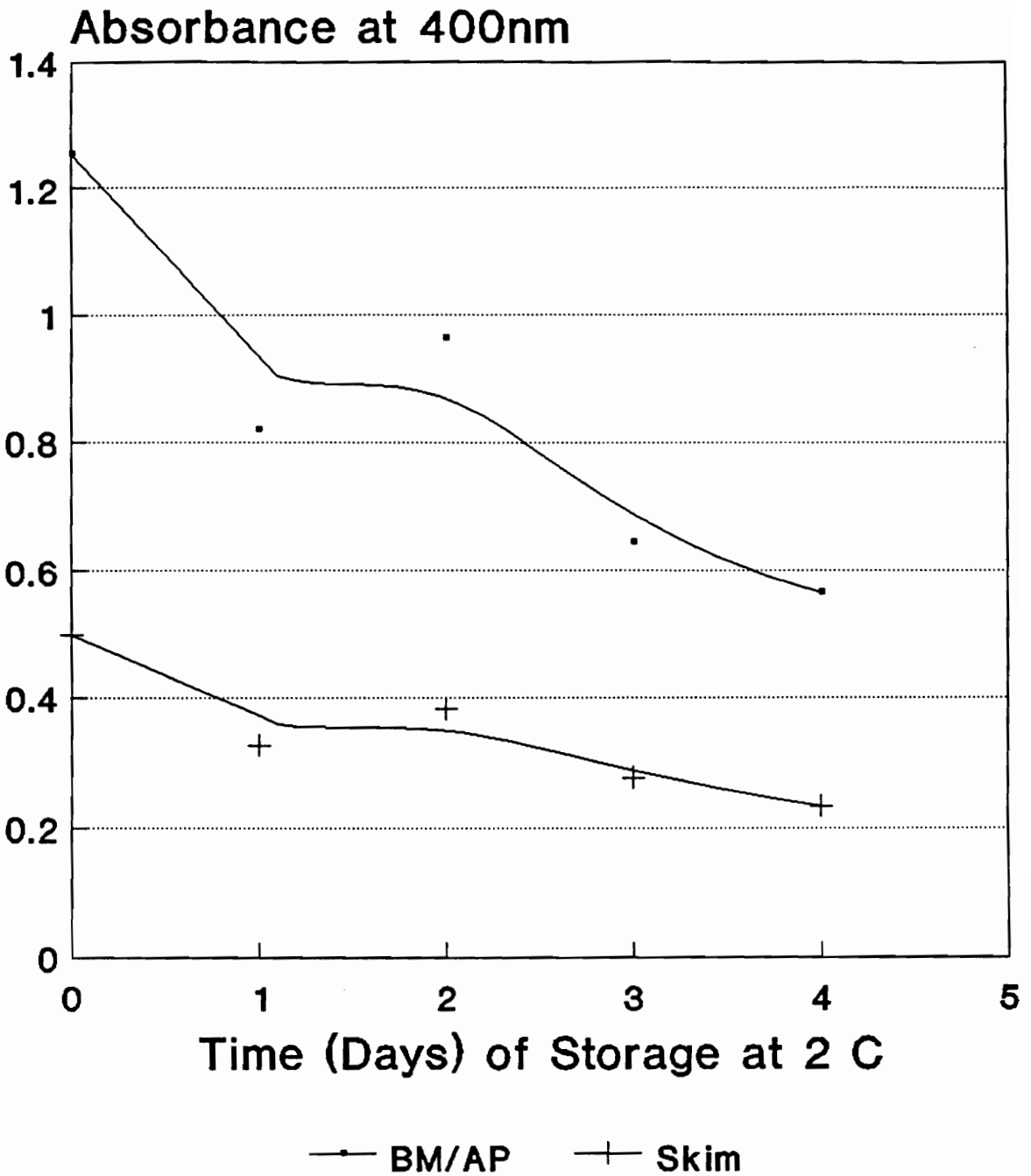


Figure 4. Means and (n=5) and general trend of phosphodiesterase assay ($\lambda=400\text{nm}$) for buttermilk/aqueous phase and skim milk over 4 days of storage at 2°C.

concentration of membrane material in the buttermilk/aqueous phase, although that does not explain the apparent decline in enzyme activity. It is possible that the membrane, mechanically disrupted during churning, did not restabilize and lost enzymatic activity at a higher rate. Although the reformulated samples were also subjected to mechanical disruption, some membrane material was stabilized by association with milk fat globules along with proteins derived from skim milk. Phosphodiesterase activity in the serum of homogenized cream decreased at first, probably during crystallization and stabilization of the lipid globules, then increased as the cream aged, possibly from breakdown of the cream matrix and consequent leaching of proteins into the serum.

The emulsion stability assay indicated overall differences occurred among days within treatments, but that treatments did not differ. Further analysis of treatment similarities indicated reformulated creams were not statistically different from each other in emulsion stability, but the reduced-cholesterol reformulation differed from the homogenized cream; those similarities and differences can be seen in Table 6. Emulsion stability had greater variability among replications of reduced-cholesterol cream than among replications of native butteroil cream or homogenized natural cream, which may be

Table 6. Means (n=5) and Standard Deviations of Emulsion Stability Assay for Homogenized Natural Cream and Homogenized Creams Reformulated with Native or Reduced-cholesterol Butteroil over 4 Days of Storage at 2°C.

Day	Treatment					
	Homogenized ¹		Native ²		Reduced ³	
	Mean ±	StdDev	Mean ±	StdDev	Mean ±	StdDev
0	6.77 ^d ±	0.184	6.51 ^{ab} ±	0.118	6.49 ^a ±	0.237
1	6.69 ^{cd} ±	0.074	6.63 ^{abcd} ±	0.098	6.68 ^{cd} ±	0.075
2	6.71 ^{cd} ±	0.052	6.76 ^d ±	0.006	6.56 ^{abc} ±	0.062
3	6.63 ^{abcd} ±	0.089	6.75 ^d ±	0.055	6.66 ^{cd} ±	0.221
4	6.720 ^d ±	0.0765	6.660 ^{bcd} ±	0.1030	6.718 ^d ±	0.2124

¹Homogenized unwashed natural cream

²Cream reformulated with native butteroil

³Cream reformulated with reduced-cholesterol butteroil, (OmegaSource, Inc.)

^{a, b, c, d} Means (n=5) within a row or column with different superscripts are different (p≤0.05).

due to age of the reduced-cholesterol butteroil when these experiments were performed. Since the butteroil was commercially steam-stripped, it lacked stabilizing components such as the antioxidant α -tocopherol, and had probably become somewhat unstable from freezing and thawing during reformulation trials. Overall, variation in emulsion stability results was quite high. Changes in emulsion stability for native, reduced-cholesterol, and natural homogenized creams over time are presented in Figure 5. Emulsion stability tended to increase slightly over time, possibly due to crystallization of fat and consequent stabilization of the cream phase.

The emulsion stability assay seemed to produce less variation among replications than the light scattering assay, but did not distinguish different samples as effectively as the phosphodiesterase assay. Using this assay only cream samples may be compared; comparisons to cream components such as buttermilk/aqueous phase and skim milk can not be made.

All storage study results indicated a change in the products over time. Since the creams were not pasteurized, their shelf-lives were quite short and rancid odors were detectable after the first day of storage. The two reformulated creams definitely performed differently than the homogenized standard cream, but stability of the

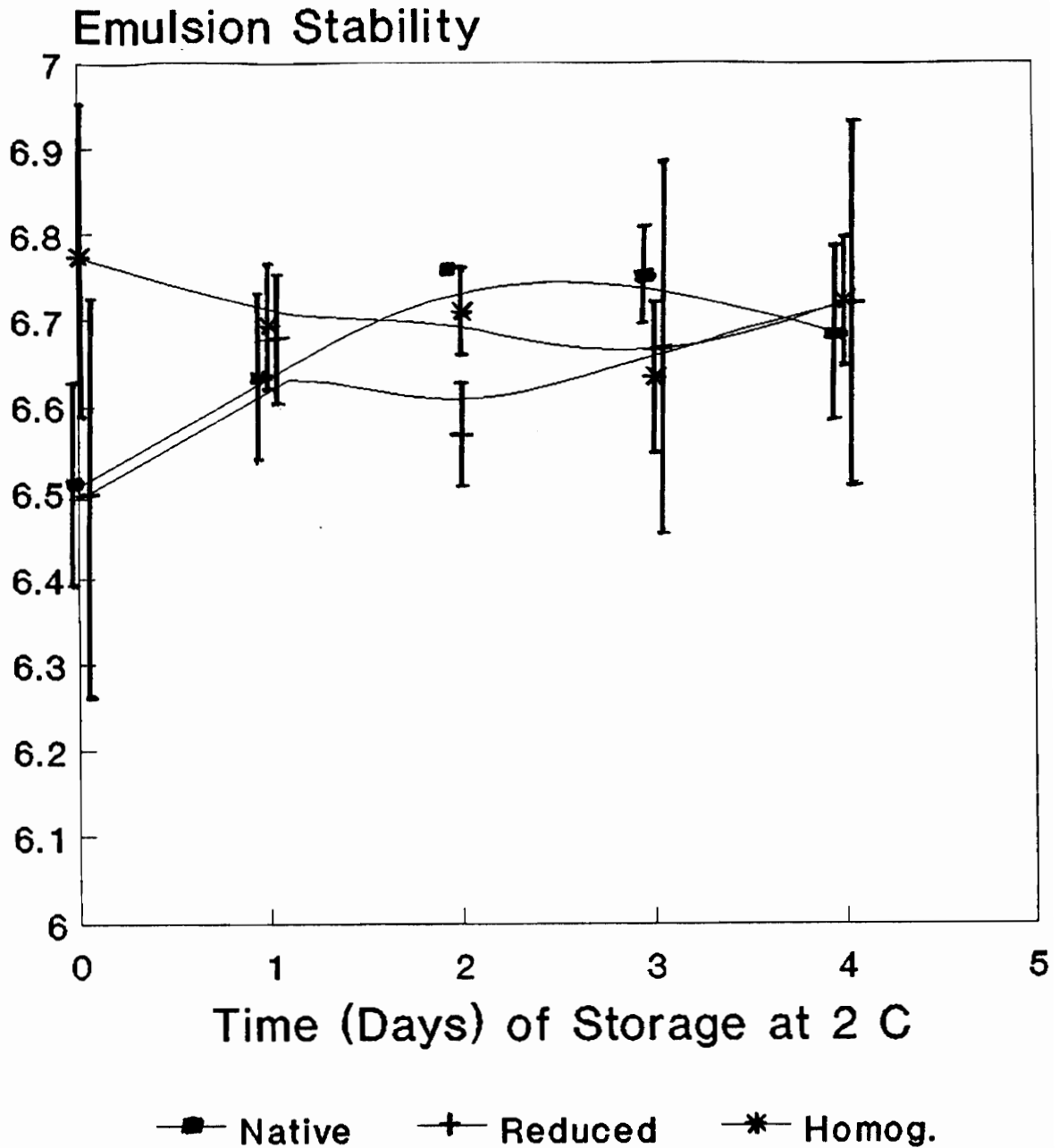


Figure 5. Means and standard deviations (n=5) and general trends of emulsion stability assay for homogenized natural cream and homogenized creams reformulated with native or reduced-cholesterol butteroil over 4 days of storage at 2°C.

lipid phase in each reformulated sample was comparable to the natural homogenized cream. Reduced-cholesterol butteroil did not perform differently than native butteroil in any aspect of the study. Reformulation through homogenization is certainly more uniform and successful than reformulation by ultrasound; however, reformulation does not produce a cream that is the same as homogenized cream based on assays used for the evaluation.

Electron Microscopy Results

Electron microscopy was conducted on samples of raw unhomogenized cream and homogenized creams reformulated with native and reduced-cholesterol butteroils. Figure 6 shows the relatively uniform size and shape of natural milk fat globules (darker areas) dispersed in the serum phase of cream (white areas). Globules ranged in size from 1.1-5.0 μm , which is about average for bovine milk fat globules (Mulder and Walstra, 1974). Figure 7 provides closer examination of the membrane material and serum proteins surrounding the globule. This enveloped globule is the desired result of reformulation by homogenization. Globule size is about 0.3 μm . Figures 8 and 9 are representative samples of reformulated creams containing native and reduced-cholesterol butteroils, respectively. Both photographs indicate a water-in-oil emulsion, a

discontinuous mixture of serum and butteroil. A distinct interface is present between the two phases, but the physical composition of this interface is not known. The reformulated cream containing native butteroil seemed to have a more complete separation of serum and butteroil. The reformulated cream containing reduced-cholesterol butteroil (Figure 9) had some butteroil dispersed into the serum phase, though not in globule form, and some serum retained in the oil phase. This may be explained by the melting point of the steam-stripped butteroil, which is slightly higher than that of native butteroil due to its purity and lack of volatiles (Massey, 1992). At the same homogenization temperature, 42°C, the reduced-cholesterol butteroil may be less liquid and tend to solidify faster upon refrigeration, becoming solid before those dispersed portions could reassociate with their respective phases. The objective to develop reformulated creams with fat globules in the range of <1-8 um by optimization of treatment parameters and homogenization was not achieved, as the emulsions present in the creams reformulated with native and reduced-cholesterol butteroil were non-symmetrical serum-in-oil emulsions, not the characteristic round globules of butteroil enveloped in membrane material.

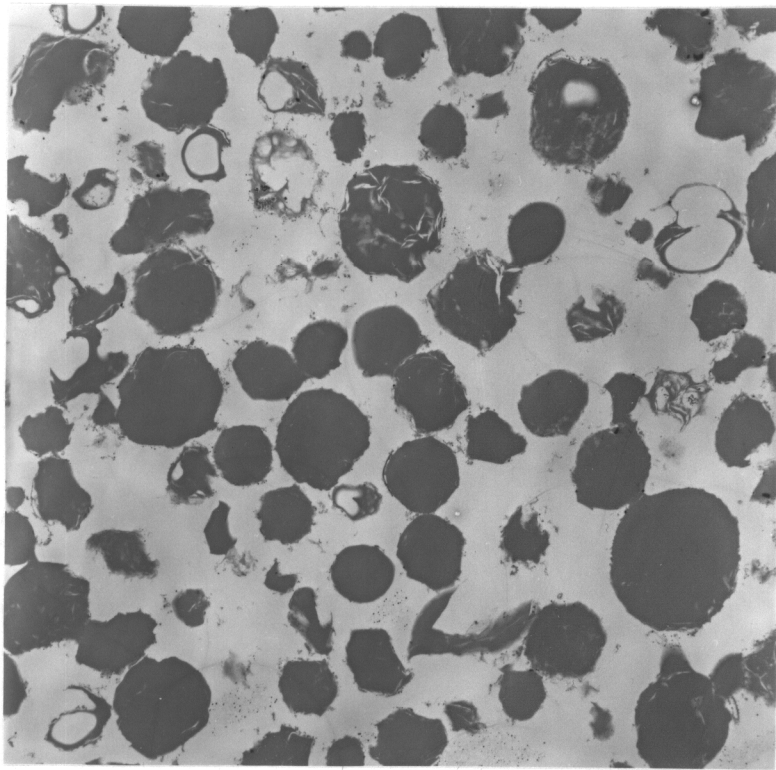


Figure 6. Electron micrograph of natural milk fat globules from raw unhomogenized cream. Magnification = 3300 X.

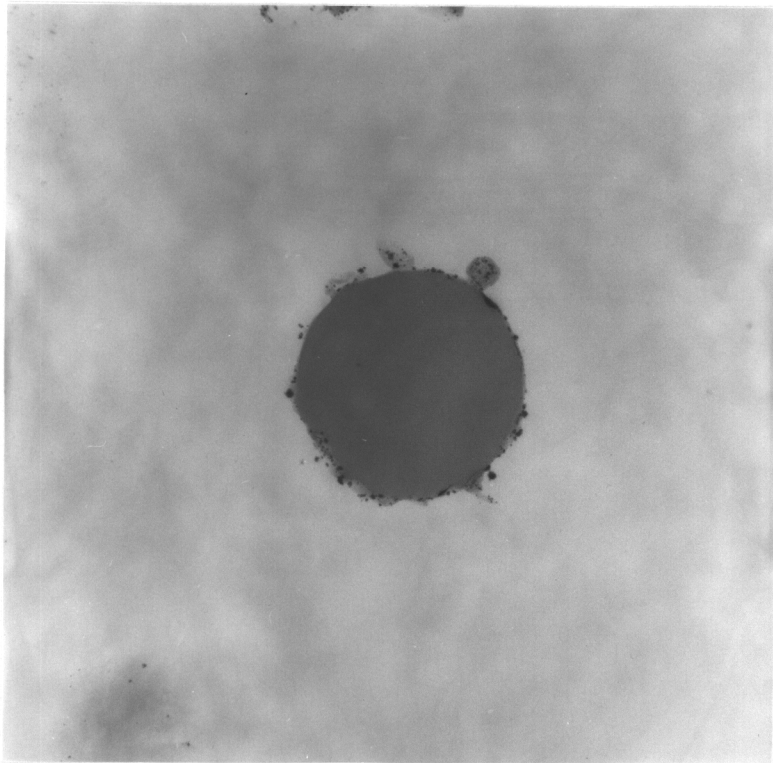


Figure 7. Electron micrograph of a single natural milk fat globule isolated from raw unhomogenized cream. Magnification = 32100 X.

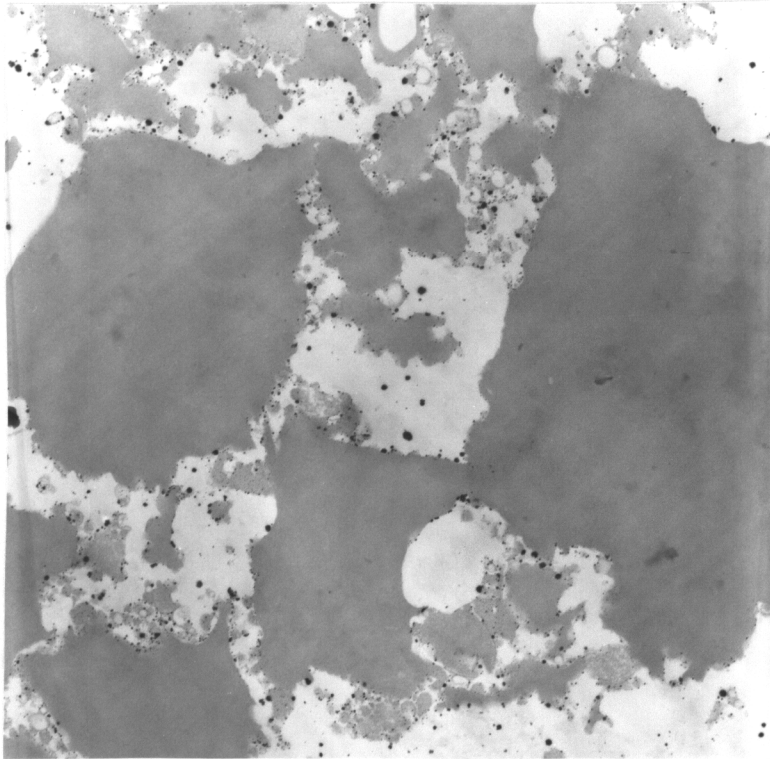


Figure 8. Electron micrograph of cream reformulated by homogenization which contains native butteroil. Magnification = 15500 X.

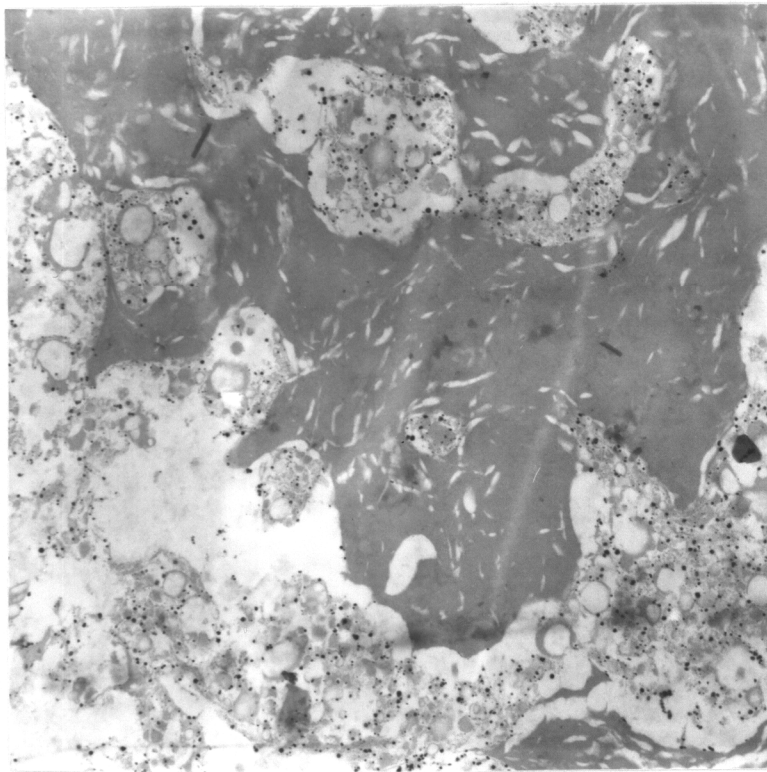


Figure 9. Electron micrograph of cream reformulated by homogenization which contains reduced-cholesterol butteroil. Magnification = 12900 X.

V. CONCLUSIONS

This research was designed to combine reformulation methods with a method of removing or reducing cholesterol in butteroil to produce a commercially practical modified cream structurally and functionally similar to natural cream.

In general, a procedure of cream reformulation was optimized such that cream with very limited separation of oil and serum phases and good stability characteristics was manufactured. The reformulation did not successfully redevelop milkfat with membrane material to reform milkfat globules. However, the raw reformulated emulsions seemed to remain fairly stable over time, which may be as important as redevelopment of the globules with membrane material. Further evaluation of the exact nature of the cream plug after centrifugation, particularly what proteins are present and in what ratios, and examination of the interface material between the oil and water phases of the emulsion is the next phase of this research. Research using pasteurized creams and homogenization to determine the effects of pasteurization on extended storage stability of the reformulated products is important in the commercial application of this reformulation process. Incorporation of reformulated creams into food products such as whipped cream, spray-dried cream powder, ice cream or cheese would

allow analysis of their performance in processed foods and of properties they may impart to these foods. This research could help the dairy industry provide consumers with cholesterol-free full-fat dairy products which are not currently available to the public, and provide a much-needed use for the excess butterfat currently being produced by the dairy industry. It could also aid development of more intricate procedures for reenvelopment of milk fat with natural components found in milk, MFGM and serum proteins.

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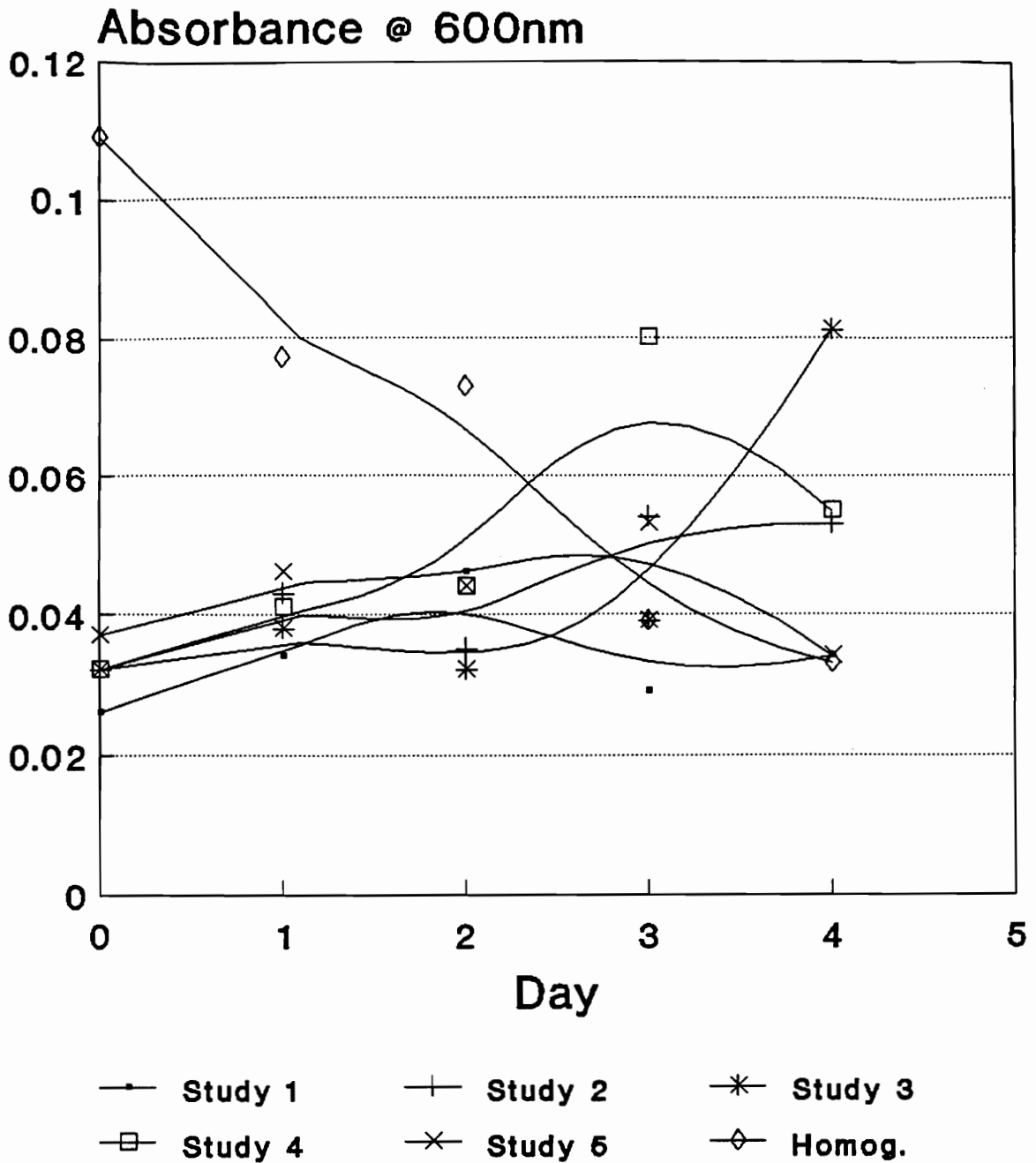
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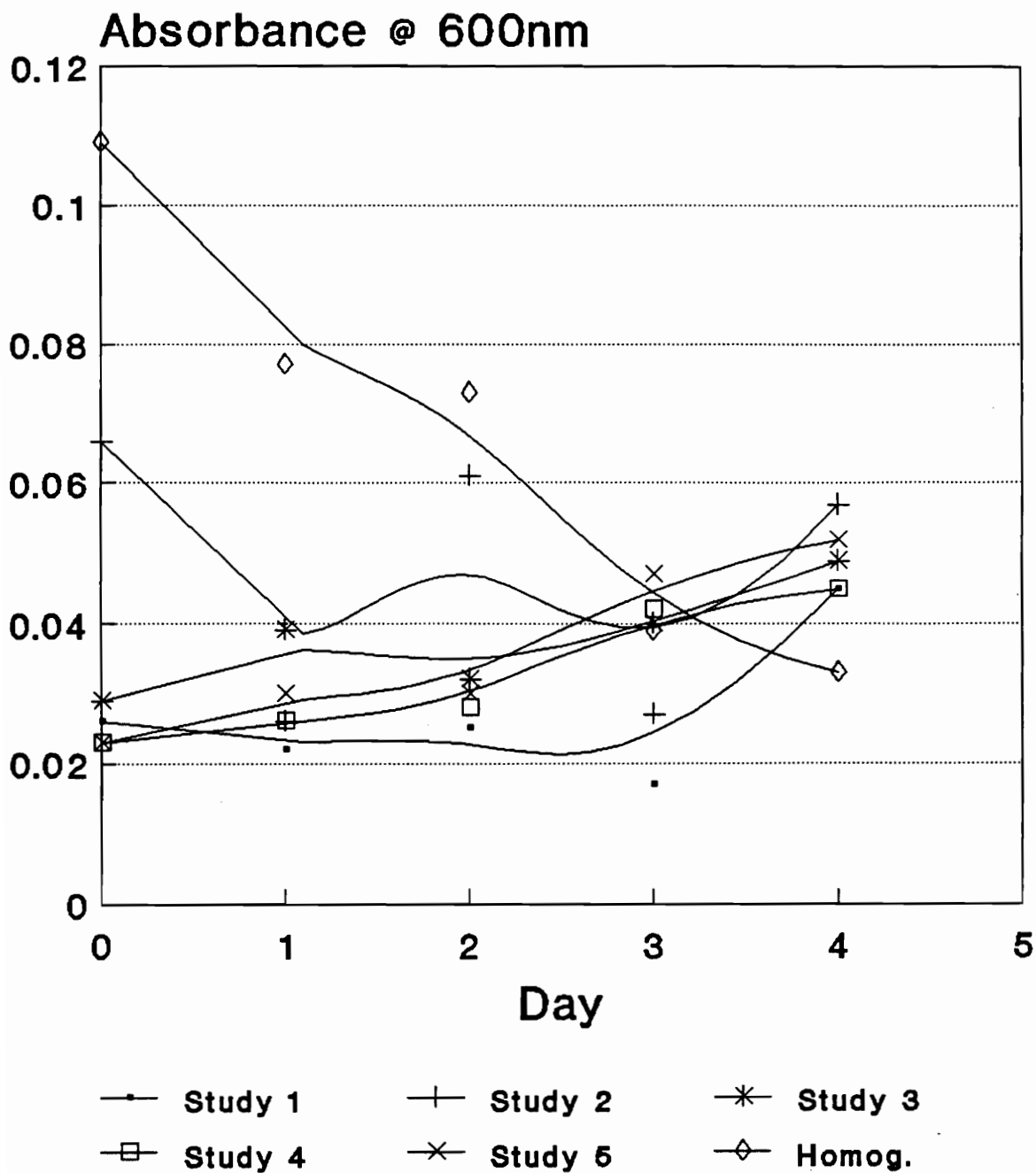
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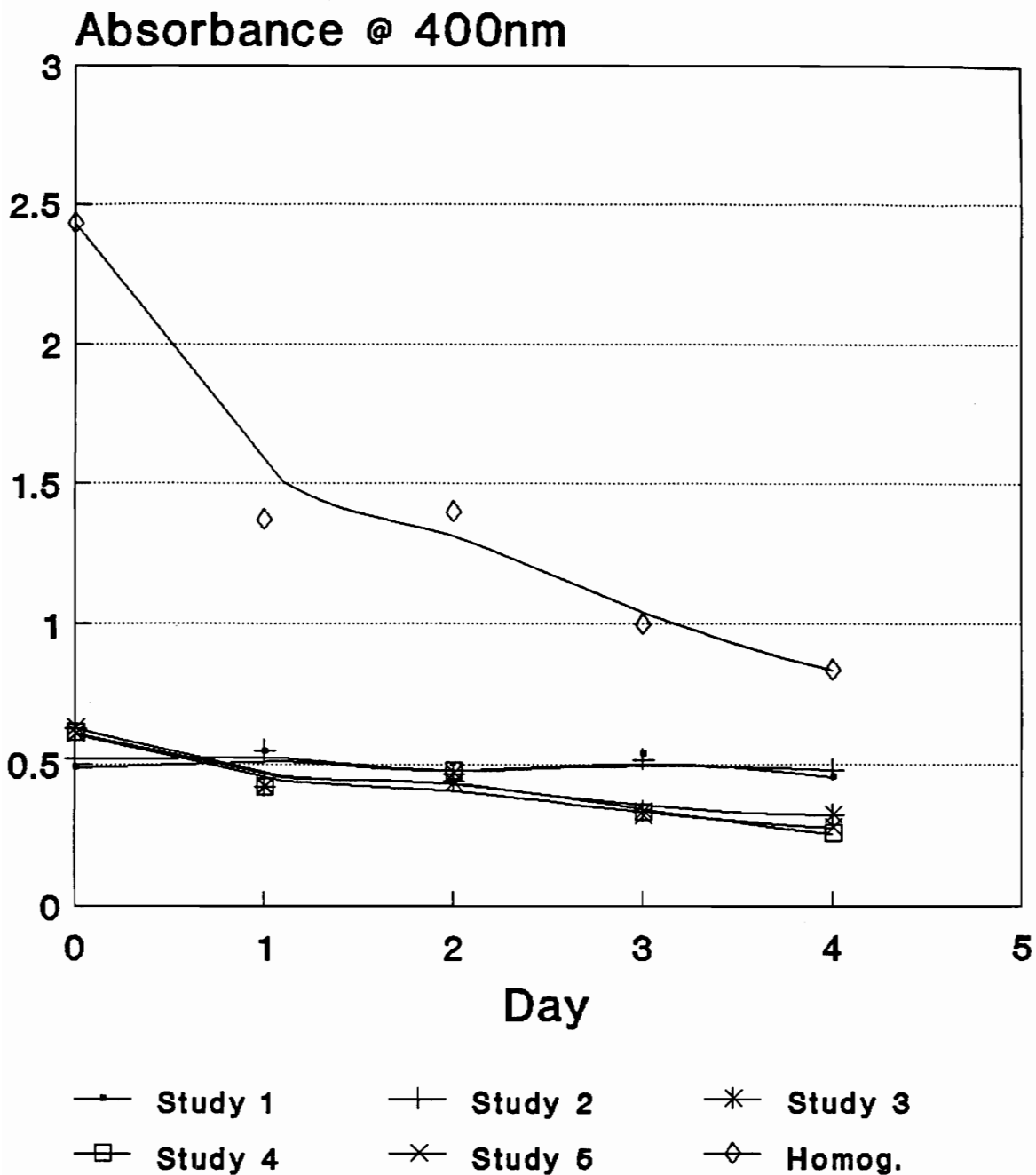
VII. APPENDIX



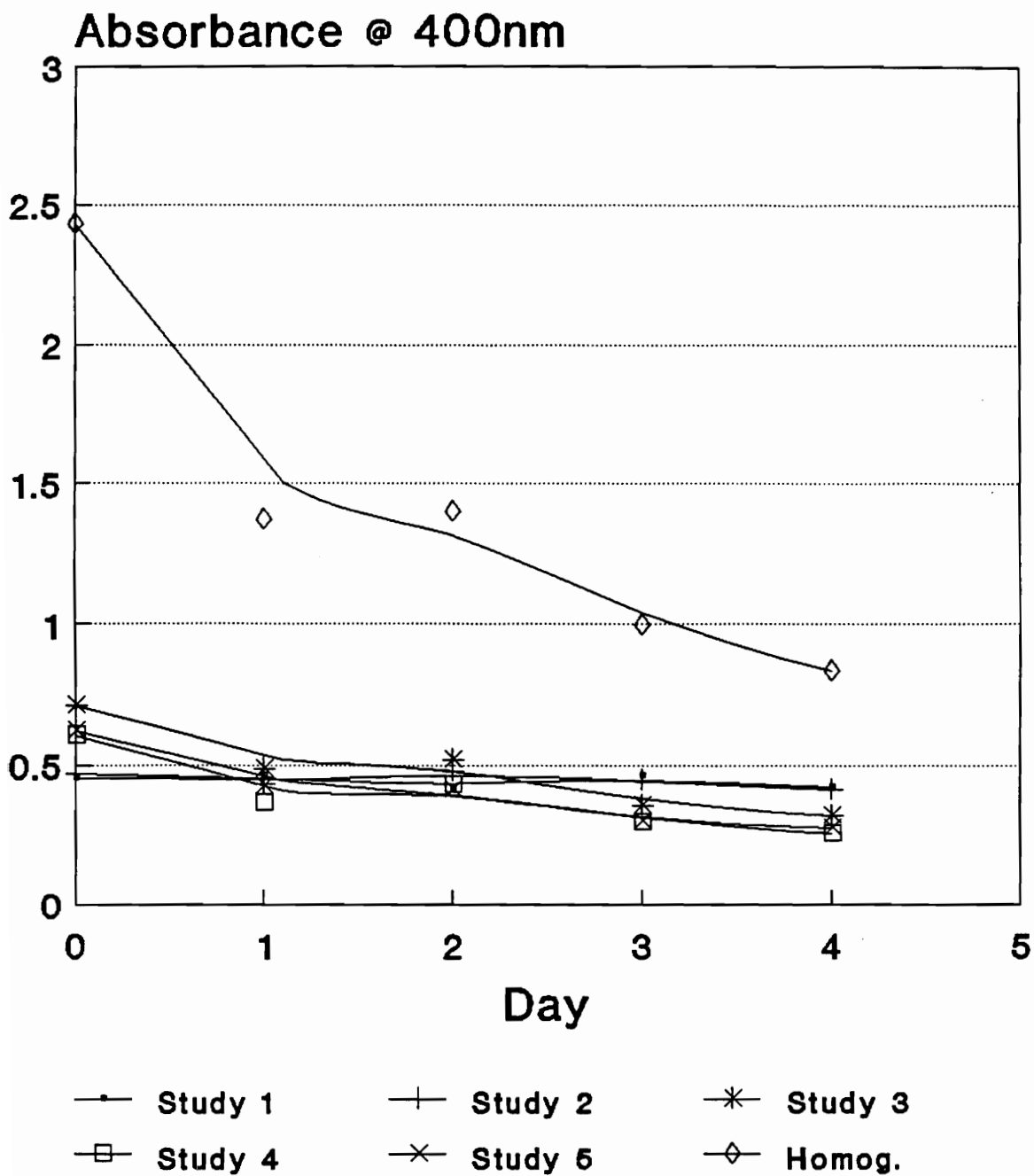
Appendix 1. Values and general trends for replications of light scattering assay for cream reformulated with native butteroil and means (n=5) and trend of homogenized natural cream over 4 days of storage at 2°C.



Appendix 2. Values and general trends for replications of light scattering assay for cream reformulated with reduced-cholesterol butteroil and means (n=5) and trend of homogenized natural cream over 4 days of storage at 2°C.

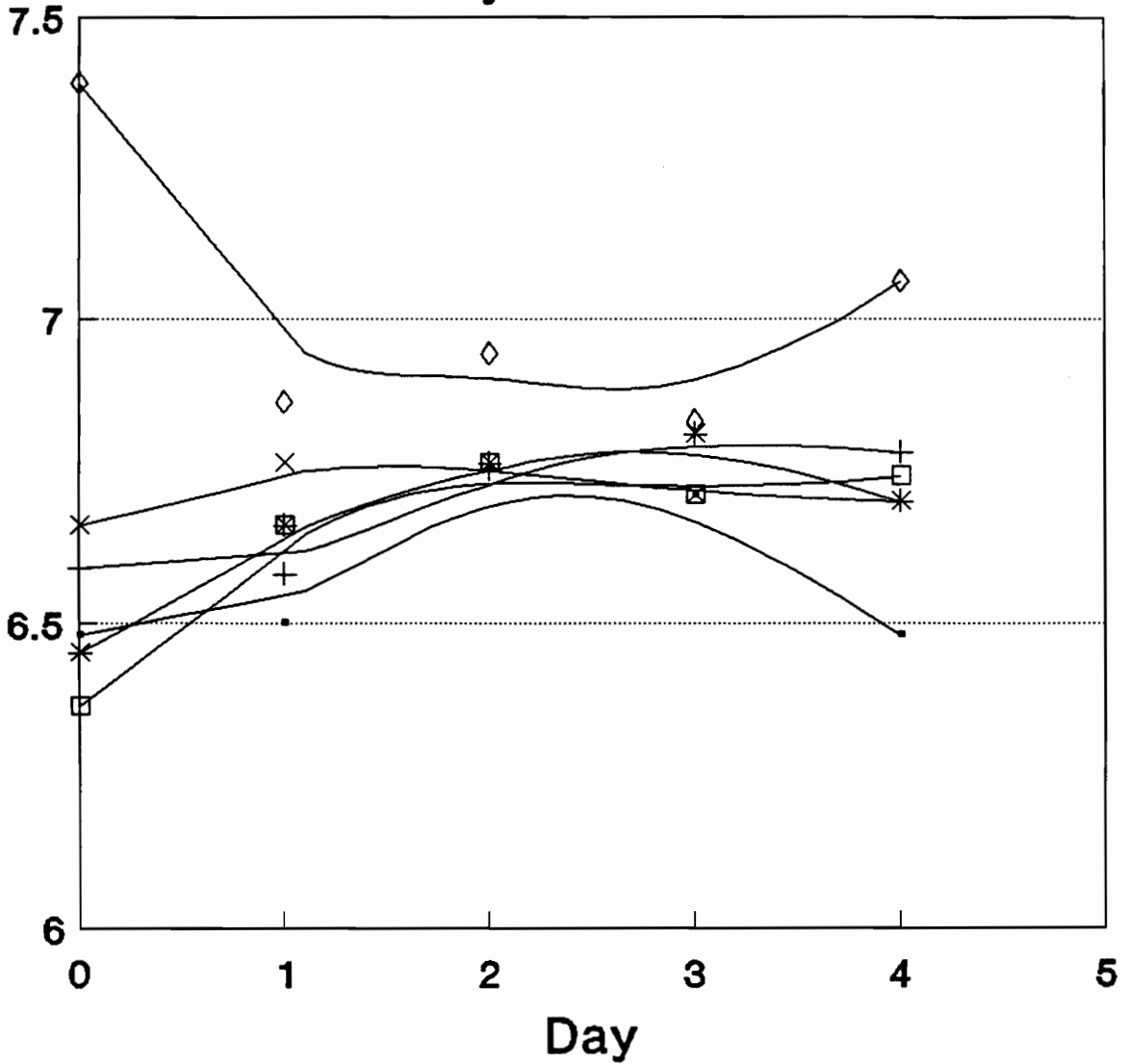


Appendix 3. Values and general trends for replications of phosphodiesterase assay for cream reformulated with native butteroil and means (n=5) and trend of homogenized natural cream over 4 days of storage at 2°C.



Appendix 4. Values and general trends for replications of phosphodiesterase assay for cream reformulated with reduced-cholesterol butteroil and means (n=5) and trend of homogenized natural cream over 4 days of storage at 2°C.

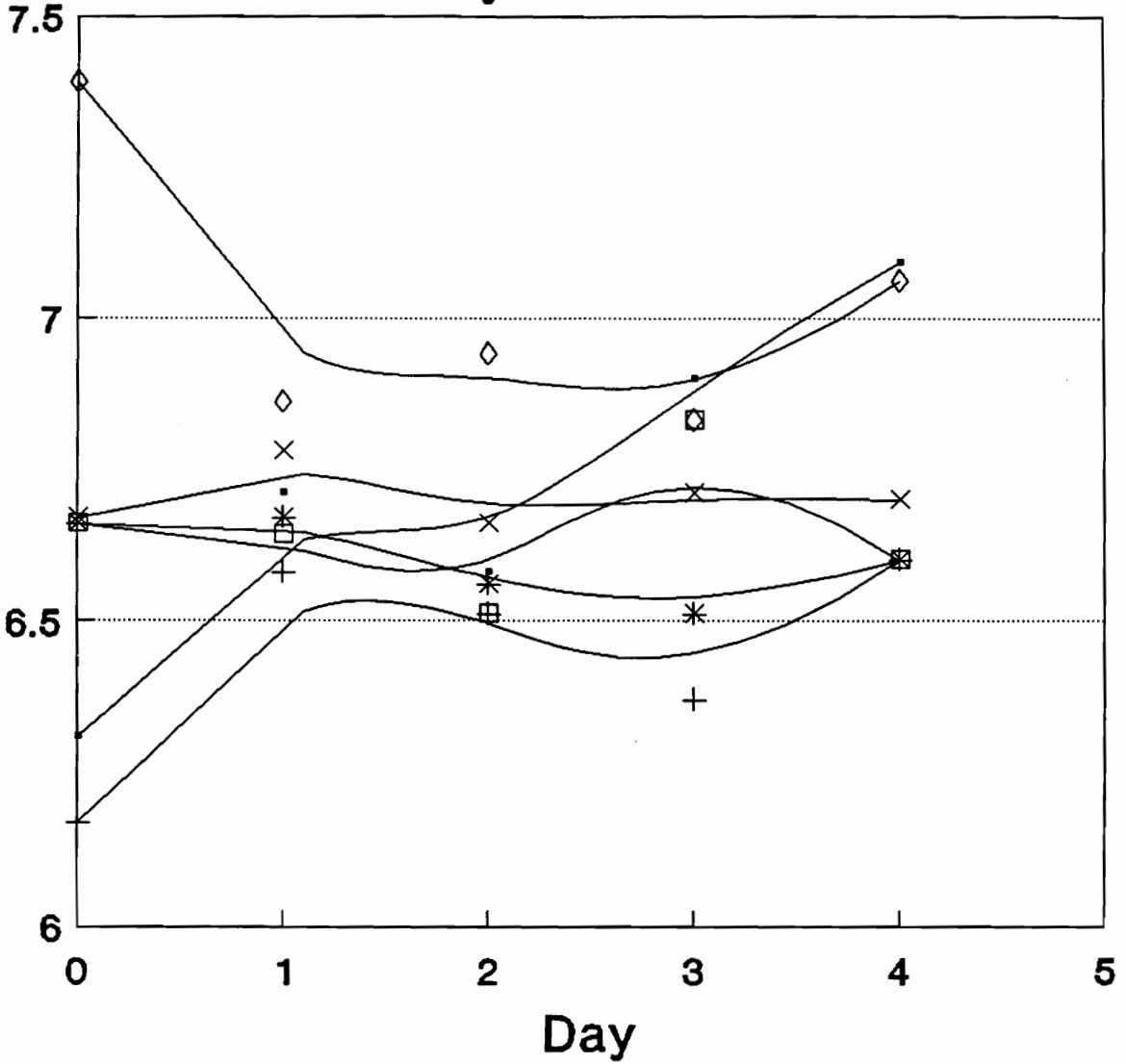
Emulsion stability



- Study 1 + Study 2 * Study 3
- Study 4 x Study 5 ◇ Homog.

Appendix 5. Values and general trends for replications of emulsion stability assay for cream reformulated with native butteroil and means (n=5) and trend of homogenized natural cream over 4 days of storage at 2°C.

Emulsion stability



- Study 1 —+— Study 2 —*— Study 3
- Study 4 —×— Study 5 —◇— Homog.

Appendix 6. Values and general trends for replications of emulsion stability assay for cream reformulated with reduced-cholesterol butteroil and means (n=5) and trend of homogenized natural cream over 4 days of storage at 2°C.

VIII. VITAE

Shelley M. Oehlmann was born in Parkersburg, WV on June 16, 1968. She graduated from Parkersburg High School in June, 1986, and entered Virginia Tech in the fall of that year. She graduated from Virginia Tech in May, 1990, with a Bachelor's degree in Biochemistry and Nutrition with a Chemistry minor. In January of 1990, she also began her graduate studies in the Department of Food Science and Technology at Virginia Tech under the direction of Dr. Susan Duncan. She received her Master of Science degree in Food Science and Technology in June, 1992. In August she will begin a career in product development with the Pilgrim's Pride Corporation in Mount Pleasant, Texas.