SELECTIVE REMOVAL OF FAT FROM ACID WHEY DURING WHEY PROTEIN CONCENTRATE MANUFACTURE

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"The heavens declare the glory of God

The skies proclaim the work of his hands."

Psalm 19:1(NIV Bible)

..... as does all creation, great and *small*.

ABSTRACT

The purpose of this study was to develop a low cost technology for selective removal of lipids from acid whey during whey protein concentrate manufacture. Attention was focused on gaining a better understanding of the structure and composition of the lipids in whey and ultrafiltration retentate. The effects of varying dilutions, pH, salt concentration, temperature and holding time on the flocculation of lipids in the whey and retentate were investigated.

The composition and structure of lipids in acid whey and retentate were determined by ultracentrifugation, compositional analysis, integrated light scattering and confocal scanning laser microscopy (CLSM) techniques. Acid whey contained ~ 0.034% lipids. The size of the milk fat globules (MFG) in whey varied from 0.1 and 10 μm, with the majority of the globules < 1 μm in diameter. The retentate contained ~ 0.36% lipids. The size of the MFG in the retentate ranged between 0.1 and 20 μm, generally larger than the MFG in the acid whey.

Investigation into the removal of lipids from acid whey revealed that flocculation of MFG in the acid whey occurred at temperatures between 40 and 50 °C and at pH values from 5.8 to 7.0. It was observed that under these conditions, high-density lipid containing flocculent/precipitates was formed, which subsequently sedimented upon centrifugation (at 1126 *g* for 10 min). The MFG removed in the flocculent/precipitate appears to be either part of a calcium-MFG complex or MFG entrapped by precipitation of calcium precipitate.

Examination of the effects of physiochemical factors on the flocculation of MFG in between the retentate revealed that flocculation occurred upon dilution and at pH values between 4.5 and 4.7. It was found that at increasing dilutions, there was an increase in the removal of MFG and in the retention of protein in the supernatant. At retentate dilution of 1:6, the majority of the MFG was removed and a majority of protein was retained in the supernatant.

Flocculation of MFG in the diluted retentate was influenced by ionic strength (at Low pH values) of the system. This flocculation is thought to result from the hydrophobic association of proteins of MFGM, aggregates of serum proteins, lipoprotein complexes or individual denatured serum proteins.

Low fat whey protein concentrate powder (WPC) was produced on a pilot-scale plant using the process conditions determined at the laboratory scale to remove MFG from acid whey retentate. The resulting product contained ~ 1% fat, considerably less than the normal commercial WPC. On a dry basis the protein content was ~ 96% as compared to ~ 85% in the commercial WPC. Examination of the functionality of the low fat WPC revealed the heat-induced gels formed from 15% WPC were more elastic, had better water holding capacity, and were more "gelatinous" in nature. Their gelation properties were markedly superior to the commercial WPCs currently manufactured.

Based on the results of this study, recommendations are made on possible areas of process improvement and development opportunities.

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CHAPTER 1

INTRODUCTION

Whey is a by-product of cheese or casein manufacture. Cheese whey is produced after casein is separated from milk by the addition of the rennet enzyme in the initial stages of cheese manufacture. Acid whey is produced after casein is separated from milk by addition of mineral acid in the manufacture of casein. The whey streams are concentrated using ultrafiltration and evaporation, and then spray dried to produce whey protein concentrate powders (WPCs). These products are used as ingredients in many applications by the food industry. The ability of these products to form heat-induced gels makes them an attractive functional product for use as a gelling agent in many food applications.

Cheese WPC is produced in a much larger quantities by the New Zealand dairy industry but is sold at a lower price because the functional properties, especially gelling ability, of the WPC is inferior to that of the acid WPC. Recent research (Havea, 1998) showed that it may be possible to manipulate the manufacturing conditions to improve the functionality of cheese WPC, and possibly match the gelling abilities with that of acid WPC. It is forecasted that cheese WPC would compete with the acid WPC in the market place. This presents a threat to the acid WPC manufacturer. If the cheese WPC could provide the same functional properties as the acid WPC, then it would probably be sold at a lower price than that of acid WPC because of the larger quantities available. If this happened, the acid WPC would be phased out of the market.

It is therefore desirable that development work be conducted into the improvement of the acid WPC properties and thus its position in the world market. One possibility is to try to develop a new product from acid WPC that is similar to whey protein isolate (WPI), using a low cost technology. WPI is a relatively new whey protein product

produced by the New Zealand dairy industry. This product has a high content of protein (~ 94%) and a low fat content (< 1%), and is largely used in the nutritional and sports formulations. Current work indicates that WPIs are superior than the WPCs from a view point of lipid and protein content, protein solubility, foam expansion and stability, absence of protein denaturation and aggregation, and flavour rating (Morr and Foegeding, 1990). Compared to WPIs, the poor flavour and relatively high lactose, lipid and mineral content of WPCs represent a serious problem that undoubtedly limits their acceptance and use by the food industry, especially in nutritional applications. WPI is sold at a very high price in the world market. However, production of WPI involves very high cost technologies, such as micro-filtration and ion exchange.

In recent years, a greater understanding of the relationship between the basic physiochemical and functional properties of whey proteins as influenced by compositional and processing factors has been gained. Considerable effort has been focused on understanding and improving the functional properties of whey proteins. Significant improvements in the functional properties of whey products have been attributed to the lower proportion of non-protein components such as lipids, lactose and minerals. The development and production of a WPC with similar composition properties to those of WPI products, given proper handling and pretreatment of the whey prior to ultrafiltration (UF) and diafiltration (DF) processing and drying, may result in a lower cost (comparative to WPI), highly functional and nutritional protein product. The required reduction in minerals and lactose are achievable using the current ultrafiltration and diafiltration technologies. However, currently a process for reducing the lipid content from whey is not well established.

It is extremely difficult to remove all residual lipid from whey protein products (Kilara, 1994), because this material seems to be small in size and probably associated with proteins. Extensive studies have been carried out on the removal of lipid from both cheese and acid whey (Attebury, 1971; Hobman, 1992; Breslau *et al.*, 1975; Fauquant *et al.*, 1985*a* & *b*; Maubois *et al.*, 1987; Kim, *et al.*, 1989; Daufin *et al.* 1993; Hwang and Damodaran, 1994, 1995; Karleskind *et al.*, 1995*a*).

Investigations have successfully applied various pretreatments to produce acid whey protein concentrates with reduced lipid content and improved functional properties (Fauquant *et al.*, 1985*a* & *b*). However the majority of the work was conducted on cheese whey. Moreover, there are problems translating the results into industrial scale production of low fat WPC. To successfully remove the lipid material from acid whey during manufacture, a greater understanding of this material is required.

The purpose of this study was to develop a low cost technology for selective removal of lipids from acid whey during whey protein concentrate manufacture. This was achieved through the development of an understanding of the structure and composition of the lipids in whey and conducting an exploration into methods to induce flocculation of lipid material in whey. This study also sought to gain information on the effects of varying pH, temperature and salt concentration on the flocculation of lipid material. Knowledge gained throughout this study was used in the development of a process to remove lipids from whey and to produce a low fat WPC on a pilot-scale plant.

CHAPTER 2

LITERATURE RESEARCH

2.1 Introduction

The food industry requires low-cost, functional, and nutritionally excellent proteins for use in processed food. There are several factors that currently limit the use of whey protein products in this role. These include, the economics of producing whey proteins with low lactose and mineral content, flavour stability of WPC's during storage and processing, and poor functionality, such as foaming, emulsification and gelation properties. The root cause of these problems can be attributed to the lipid present in the whey (Hwang and Damodaran, 1994). Acid whey usually contains about 0.03% lipids (Mulvihill and Donovan, 1987). Subsequent processing in the production of whey protein concentrate (WPC), including ultrafiltration (UF), evaporation and drying, result in the concentration of the lipid component to final concentrations between ~ 3 and 7% (Morr and Ha, 1993).

Whey protein concentrate (WPC) and whey protein isolate (WPI) are two products derived from whey that can provide an excellent source of high quality functional and nutritional protein. WPI usually has a lipid content of between 0.39 and 0.67%, which is significantly reduced compared to that of WPC, which has a usual lipid content of between ~ 3 and 7% (Morr and Ha, 1993). Further, the protein content is higher in the WPI products than it is in the WPC products. It is well know that WPI has a much greater potential for use as a functional food ingredient than WPC (Morr and Foegeding, 1990). The better solubility, gelling, foaming and flavour properties of WPI were attributed to the lower proportion of nonprotein components such as lipids, lactose and minerals. It was found by de Boer *et al.* (1977) that the oxidation of lipids predominately phospholipids during storage and processing impaired the flavour stability of WPC and WPC-containing food products. The

presence of lipids was also shown to adversely affect the emulsifying and foaming properties of the whey proteins (Morr, 1985).

Methods are currently in use to remove the nonprotein components of the whey produced in the manufacture of casein and cheese whey. The economics of the removal of the lipids, lactose and minerals components is however quite costly, especially for the removal of the lipid fraction that remains in the whey. UF processing is widely used in the New Zealand dairy industry in the manufacture of WPC in removal of minerals and lactose components of whey. Currently, the removal of the lipid fraction is not commonly undertaken in the manufacture of WPC in the New Zealand. Recent studies conducted have shown that lipid fraction can be removed from cheese whey (Hwang and Damodaran, 1994; Karleskind *et al.*, 1995; Rinn *et al.*, 1990; Hwang and Damodaran, 1995). However, very little work has been carried out on the removal of the lipid fraction from acid whey in the manufacture of WPC.

The aim of this study is to develop a low cost technology for selective removal of lipids from acid whey during whey protein concentrate manufacture. To do this it is important to gain an understanding of the nature of lipids and content in milk and whey, the changes the lipids are subjected to during processing, possible methods for the commercial separation of the lipids from whey, and possible methods for quantifying lipids. This review will endeavour to cover these areas so that a reasonable level of understanding may be attained.

2.2 Milk

2.2.1 Composition of milk and milk lipids

The composition of milk from cows is affected by the stage of lactation, diet, breed as well as other factors. For this reason an average composition of milk from cows in New Zealand is given is Table 1. The main components of bovine milk are milk fat, protein, lactose, minerals and water. Bovine milk usually contains between 35 and 50 g/L milk fat, which are present as triacylglycerols, free fatty acids, phospholipids, monoacylglycerols and diacylglycerols. Milk usually contains between 30 and

36 g/L of protein, which is present in two forms, casein and whey (Swaisgood, 1996). Lactose, the predominant carbohydrate, is present between 4.6 and 4.9 g/L in bovine milk (Swaisgood, 1996). The mineral component of bovine milk remains relatively constant at ~ 7 g/L, which includes a wide range of components (Corbin and Whittier, 1965).

Table 2.1 Average composition of New Zealand bovine milk [a]

Composition	Quantity (g/L)
Milk fat	50
Protein	34
- Casein	27
- Whey protein	7
Lactose	47
Minerals [b]	7
Water	860

[a] From Creamer & MacGibbon (1996)
[b] From Jensen *et al.* (1991)

The lipid fraction of milk is composed mainly of triaclglycerols (triglycerides), with minor contributions from phospholipids, diacylglycerides, cholesterol (a sterol), monoacylglycerols and cholesterol esters (sterols) (Table 2.2). General compositions of the lipid fraction of bovine milk have recently been reviewed by Jensen *et al.* (1991), Banks (1991) and Christie (1995). The values presented by these authors reflect variation consistent with variations resulting from different stages in lactation and diet of the cow (Creamer and MacGibbon, 1996). The majority of the lipids in bovine milk are associated with the milk fat globules (MFG).

Table 2.2 Composition of individual simple lipids and total phospholipids in bovine milk [a]

Lipid class	Content (%)
Triacylglycerols	97.5
Phospholipids	0.60
Diacylglycerols	0.36
Cholesterol	0.31
Monoacylglycerols	0.027
Cholesterol esters	Trace

[a] From Kurtz (1974)

The protein fraction of milk consists of two main types, casein and whey protein. Milk proteins can be readily separated into these two fractions, both containing several different types of protein. The casein fraction, consisting of α_s-caseins, β-casein, κ-casein and γ-caseins, makes up the majority of the protein, between 75 and 88% of the total protein in cow's milk. The whey fraction consists mainly of β-lactoglobulin, α-lactalbumin and proteose-peptones with a small quantity of blood proteins and makes up the smaller fraction of the protein between 15 and 20%.

2.2.2 Milk fat globule (MFG)

Bovine milk usually contains between 35 and 50 g/L milk fat and most of this exists as fat globules suspended in the aqueous phase of milk. The fat globules in bovine milk are primarily made up of a core, mainly triacylglycerols, a membrane made of a layer of some cytoplasmic proteins adsorbed prior to excretion, and an outer covering of plasma. The milk fat globules in bovine milk generally fall in and around the range from 0.2 to 10 μm in diameter (Walstra, 1969). Light scattering by these large globules is responsible for the 'creamy' appearance of whole milk (Swaisgood, 1996).

The majority of the MFG is bovine milk are small (< 1 μm diameter). Although these MFG account for 80% or more of the total number of globules, they contain less than 10% of the total volume of milk fat. Globules between 1 and 8 μm in diameter contain 90% or more of the total volume of milk fat. Droplets larger than 8

μm are few in number but account for 1-3% of the fat volume of milk (Keenan and Dylewski, 1995).

The milk fat globule contains mostly triacylglycerols and to a lesser extent other non-polar substances such as cholesterol, esters and retinyl esters (Jensen *et al.*, 1991). It is generally accepted that 95% or more of the fat in milk is present in the milk fat globules (MFG). Approximately 99% of the fat/lipid in the globules are triacylglycerols, the very small remaining fraction consists of free fatty acids, unsaponifiable lipids and phospholipids (Table 2.3). It must noted when dealing with bovine milk that the fat consists of numerous components and the composition of fat differs between globules. Thus, the physical properties of the fats may vary with variation of fatty acids chain length, number and position of double bonds and their configuration (cis or trans isomers).

Table 2.3 Lipids of milk fat globules and membranes [a]

Lipids	Fat globule	Fat globule membrane
Triacylglycerols	98.6	61.7
Free fatty acids	0.30	6.7
Unsaponifiable	0.28	0.89
Phospholipids	0.26	22.1
Diacylglycerols	-	8.9
Monoacylglycerols	-	Traces

[a] Modified from Jensen *et al.* (1991)

The fat globules are coated with a thin protective layer, commonly known as the milk fat globular membrane (MFGM). This membrane acts as a natural emulsifying agent enabling the fat to remain dispersed throughout the aqueous phase of milk, preventing to some extent flocculation and coalescence. The membrane consists of a complex mixture of proteins, high melting triglycerides, cholesterols, enzymes and other minor components (Walstra and Jenness, 1984; Morr & Ha, 1993). The total mass of fat globules that is accounted for by membrane material has not been determined with certainty. Keenan and Dylewski (1995) roughly estimate from available data that membrane associated materials comprise from 2 to more than 6% of the mass of the

globules. The MFGM in whole milk consists of ~ 46% protein and ~ 48% lipid. Other components which include bound water, vitamin A, cartenoids and minerals are present in small concentrations (King, 1955).

The protein in the MFGM is small in quantity compared to the mass of the total MFG, however it plays an important role in maintaining the integrity of the membrane (McPherson and Kitchen, 1983). Most of the membrane proteins are highly specific and their compositions about as intricate as the plasma proteins. There are at least ten different species with molecular weights ranging from 50,000 to 150,000. They are predominantly glycoproteins, notable for their highly hydrophobic regions which bind to the lipids of the membrane.

The lipid composition of the MFGM in whole milk is shown in Table 2.3. The lipid component of the MFGM is composed of ~ 62% high melting triacylglycerols, ~ 22% phospholipids, ~ 9% diacylglycerols, ~ 7% free fatty acids and small quantities of unsaponifiable lipids and monoacylglycerols (Jensen *et al.*, 1991). This membrane coat, contains nearly 70% of the phospholipid and 85% of the cholesterol in milk (Swasigood, 1996).

McPherson and Kitchen (1983) proposed a model for the MFGM structure, which is shown in Figure 2.1. The main body of the membrane is composed of phospholipids, cholesterol, and cholesterol esters in which are embedded various polypeptides. Xanthine oxidase appeared to be located just below the outer surface, while the enzyme 5'-nucleotidase was on the outer surface of the membrane. The major protein component was shown to be polypeptides (16,15 and 3). Between the main body of the membrane and the lipid core is a layer of high melting triacylglycerols.

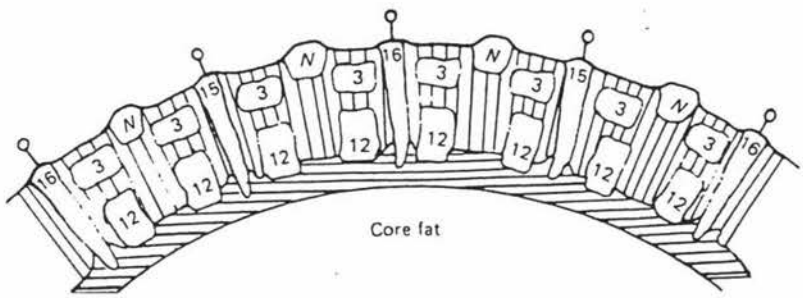


Figure 2.1 Proposed model for the bovine milk fat globule membrane. Polypeptides are numbered using the nomenclature where carbohydrates side chains are shown as small circles attached to extension from polypeptides 15 and 16, the polypeptide labelled N is 5'-nucleotidase, horizontal shading indicates the high-melting triglyceride regions, and vertical shading indicates the main body of the membrane, comprising phospholipids, cholesterol, cholesterol esters and other triglycerides (McPherson and Kitchen, 1983).

The fat globules in milk carry an electric charge on their surface (King, 1955) so they migrate to an electric field. At higher pH values this charge is negative and at lower pH values it is positive. The isoelectric point for the milk fat globules should be found within the range of pH 4.1 to 4.5.

Creaming in unhomogenised milk occurs much more rapidly than would be predicted from the size of the individual fat globules. This increased creaming rate is due to the clustering of globules caused by the interaction of membrane proteins with immunoglobulin (IgM) and protein in the skim milk membrane vesicles (Fennema, 1996). The large clusters rise more rapidly than single globules. Flocculation of MFG will therefore increase the ease with which separation of the MFG can be performed.

2.2.3 Effect of processing on milk fat globules (MFG)

Processes that the milk undergoes significantly change the structure of the components in milk. Changes to the structure and composition of the MFG occur during agitation, cooling, heating, and casein making (acidification) processes, before the whey is finally produced.

2.2.3.1 Agitation and shear force

Agitation of the milk or application of shear occurs during processing steps such as pumping, clarification or heat exchange. The application of low shear applied between 10 and 40 °C removes some phospholipids and might slightly damage the MFG. The slightly damaged globules will tend to coalesce, forming clumps that increase the proportion of larger fat globules. The initial cooling and agitation of raw milk that might occur after milking causes deformation of the globules, tearing of membrane with loss of liquid fat, and some degree of adsorption of casein micelles onto the globules (Jensen *et al.*, 1991). More intense agitation that applies a high shear can have a significant effect. At high shear applied above 40 °C the globules may be disrupted resulting in the globule to break up (Mulder and Walstra, 1974). MFGM is also released from solidified MFG in cold milk by high-shear processing such as mixing and pumping (Morr and Ha, 1993).

Generally, when the membrane of the original milk fat globule is damaged, surfactant proteins from the plasma phase quickly gather at the fat-plasma interface forming a new membrane. The way that the protein reaches the interface is different for different degrees of agitation. If the agitation in slight the protein will reach the interface by diffusion, where the smallest molecules diffuse the fastest and are therefore preferentially adsorbed. If the agitation is great, such as in homogenisation or pumping then the protein arrives via convection, and larger particles are adsorbed preferentially (Christie, 1995). More intense agitation results in thicker adsorbed layers that contain relatively less serum protein.

2.2.3.2 Cooling

It has been observed that at low temperatures, part of the immunoglobulins of the milk serum are adsorbed onto the fat globules. This may amount to 0.5 g protein/100g fat globules or even more. Upon cooling some lipoproteins in the milk plasma also tend to adsorb onto the fat globule (Mulder and Walstra, 1974).

2.2.3.3 Heat treatment

The main reason that milk is treated with heat is in order to aid its preservation, by killing micro-organisms, inactivating enzymes and changing the chemical composition. Heat treatment of milk causes several changes (Mulder and Walstra, 1974). These include changes to several membrane proteins that are susceptible to heat denaturation and association of serum proteins to the MFGM.

Heating causes changes in the MFGM proteins, which include some denaturation. Membrane proteins will denature above 70 °C, and reactive groups may be exposed. Heat treatment releases sulphydryl compounds, which include hydrogen sulphide, from the milk. A large proportion of this is derived from the membrane (Badings, 1969). The cysteine residues in the membrane proteins are considered to be responsible for the release of the sulphydryl compounds and seem to be more reactive than the bulk of cysteine in the milk plasma. As a result of this, disulphide interchange reactions may occur (van Boekel and Walstra, 1989).

During heating serum proteins are deposited onto the fat globules (Fink and Kessler, 1985). Investigation by Dalgleish and Banks (1991) revealed that the serum proteins, β-lactoglobulin and α-lactalbumin bound to the milk fat during heating. This adsorption was accompanied by an increased amount of protein associated with the milk fat. It is currently thought that the MFGM proteins may catalyse whey protein denaturation, with denaturation and binding occurring at one site. Dalgleish and Banks (1991) suggested that whey proteins are incorporated into the MFGM during heating displacing some polypeptides from the membrane into the plasma. Jensen *et al.* (1991) states that apart from the protein denaturation, that heating by itself did not affect the integrity of the membrane. However, heat is normally accompanied by vigorous agitation that may cause coalescence of the fat globules and some disruption of the MFGM, particularly at higher temperatures.

2.2.3.4 Acidification

Acidification of the milk occurs in the casein making process. The acidification of the milk causes changes in the membranes, primarily precipitation of casein onto the

fat globules. The MFG separated to the whey (after acidification) may contain residual casein as part of the MFGM. It should be noted that the pH has a distinct influence on the properties of the natural membrane with respect to stability to coalescence (Mulder and Walstra, 1974).

2.2.4 Free fat

The term 'free fat' has been used in a number of articles on the effect of processing on the milk fat globule. As soon as the uncovered milk fat comes into contact with the milk plasma, proteins adsorb onto the surface and therefore covering it (Christie, 1995). It is therefore thought that uncovered fat will not normally occur in liquid milk products.

2.3 Whey

Whey is the by-product of cheese or casein manufacture. There are two different types of whey: sweet and acid whey. These are a result of different methods of removing the casein from milk. The sweet whey is produced when the casein is separated from milk by the addition of rennet. Acid whey is produced after the removal of casein by acidification of milk either by the addition of mineral acids or by the production of lactic acid by starter bacteria. The whey is then removed from the precipitated casein for further processing.

2.3.1 Composition of whey

The composition of the different types of whey is dependent on the source of the milk and the processing method used to achieve removal of the casein proteins from the milk. This may include the different processes for different types of cheese or casein. The general compositions of several different types of whey are compared in Table 2.4. Most notably, the milk fat content of acid whey is higher than cheese whey at 0.3 and 0.2 g/L respectively.

Whey is considered to be a dilute liquid containing lactose, protein, minerals and traces of fat. Whey usually contains approximately only 60 to 70 g/L total solids. The lactose makes up between 44 and 53 g/L of the total whey, which accounts for more than 70% of the total solids. The protein content varies between 6.2 and 6.6 g/L for different types of whey, with a slightly higher content in the sweet wheys. A marked different exists in the content of salts with roughly 2 g/L higher content in acid whey. A significant difference in pH is also noted with cheese whey exhibiting a higher pH than acid whey.

Table 2.4 Weighed seasonal averages of composition and pH of wheys from Rennet, Lactic and Mineral acid casein and Cheddar cheese manufacture. [a]

Components	Composition (g/L)			
	Sweet whey		Acid whey	
	Rennet Casein	Cheddar Cheese	Mineral Acid Casein	Lactic Casein
Total solids	66.0	67	63.0	64.0
Protein (N*6.38)	6.2	6.2	5.8	5.8
Lactose	52.3	52.4	46.9	44.3
Milk fat	0.2	0.2	0.3	0.3
Minerals (as ash)	5.0	5.2	7.9	7.5
Calcium	0.5	0.4	1.4	1.6
Sodium	0.53	0.50	0.50	0.51
Potassium	1.45	1.50	1.40	1.40
pH	6.4	5.9	4.7	4.6

[a] Adapted from Mulvihill and Donovan (1987)
TN = total nitrogen; NPN = non-protein nitrogen

2.3.2 Processing of whey

The components of whey can be isolated by different processes to produce various products. With the emphasis on the protein content of the whey, processes have been focused on producing economically a product that is high in functional protein. Various protein products are produced, whey protein concentrates (WPCs), whey protein isolates (WPIs) and fractionated proteins, such as β-lactoglobulin, α-lactalbumin (Mulvihill, 1992). The whey protein products of most commercial importance are WPC (up to 82% protein) and WPI (95% protein) (Havea, 1998). These products are mainly used in food for their functional attributes, such as gelling, foaming and nutritional attributes.

In recent years, manufacture of lactose and mineral products from whey has gained commercial significance. Commercial production of lactose is conducted using either crystallisation or precipitation methods. The lactose is manufactured from either whey or de-proteinated milk serum from both sweet and acid whey (Hobman, 1984).

The commercial manufacture of lactose has been reviewed by Zadow (1984) and Harper (1992). Commercial production of mineral products from whey, most significantly calcium, is conducted mostly by mineral precipitation. In the New Zealand dairy industry, calcium phosphate is precipitated from de-proteinated milk serum, and dried. The mineral powder is sold as a dairy ingredient, being used to increase the levels of calcium in consumer products.

2.3.3 Production of WPC

Sweet or acid whey derived from cheese or casein production can be used in manufacturing WPC. During the manufacture of these products a quality must be maintained so that the WPC has consistent composition and functional properties. During processing of sweet or acid whey, it is necessary to keep to a minimum the number of psychrotropic and thermoduric microorganisms. The simplified schematic flow diagram of the process commonly used in industry in the manufacture of sweet and acid whey is shown in Figure 2.2. The process can be grouped into three broad categories; (1) pretreatment, (2) ultrafiltration/diafiltration, and (3) concentration and drying.

Whey pretreatment (1)

To obtain product of acceptable uniformity and storage stability certain pretreatments of the 'raw' whey are necessary. There is a range of pretreatment processes that are carried out to change the composition or functional properties, or improve and reduce fouling during UF. Clarification may be employed to remove fine insoluble particles that have the potential to foul membrane and reduce flux during UF. Centrifugal clarification to remove particulate matter such as casein fines or cheese curd, fat and microorganisms is widely practised commercially (Hobman, 1992).

Following clarification, whey is usually preheated and pasteurised. In contrast, cheese whey is pasteurised whereas acid whey is stored at ~ 54 °C, which is sufficient to inhibit the growth of microorganisms (Havea, 1998). Acid whey is generally not pasteurised because such heat treatment at the natural pH of whey (~ pH 4.6) can result in protein denaturation.

Whey can also be preconcentrated prior to UF. The advantages of this for WPC production include reduction of whey transport costs, storage volumes and energy consumption, the possibility of improved fat separation, increased total solids and a reduction in the quantity of water to be removed during evaporation and drying (Nielsen, 1988).

Ultrafiltration (UF) and diafiltration (DF) (2)

Ultrafiltration (UF) is a pressure driven filtration process in which porous membranes are used to separate components of a solid-liquid mixture on the basis of size and shape and, in some instances, charge (Hobman, 1992). The pore dimensions are typically in the range from 1 to 100 nm nominal diameter, although commercially available UF membranes usually have an effective diameter of between 2 and 10 nm (Beaton and Steadly, 1982).

When a pressure gradient is applied across the membrane, the liquid is forced to flow through the pores to the low-pressure side, transporting with it any components that are smaller than the size of the membrane pores. In this process, the high molecular weight (retentate) and the low molecular weight (permeate) fractions are separated. The permeate obtained from the UF consists of water, lactose, minerals, vitamins and amino acids, whereas the retentate stream contains higher molecular weight material (protein and fat) water, and small quantity of the low molecular weight matter. WPC, high in protein, can be produced in a multi-phase process by the addition of diafiltration (DF). DF is a process by which the retentate is diluted with water and further ultrafiltrated. This allows further removal of low molecular weight material from the retentate (Havea, 1998).

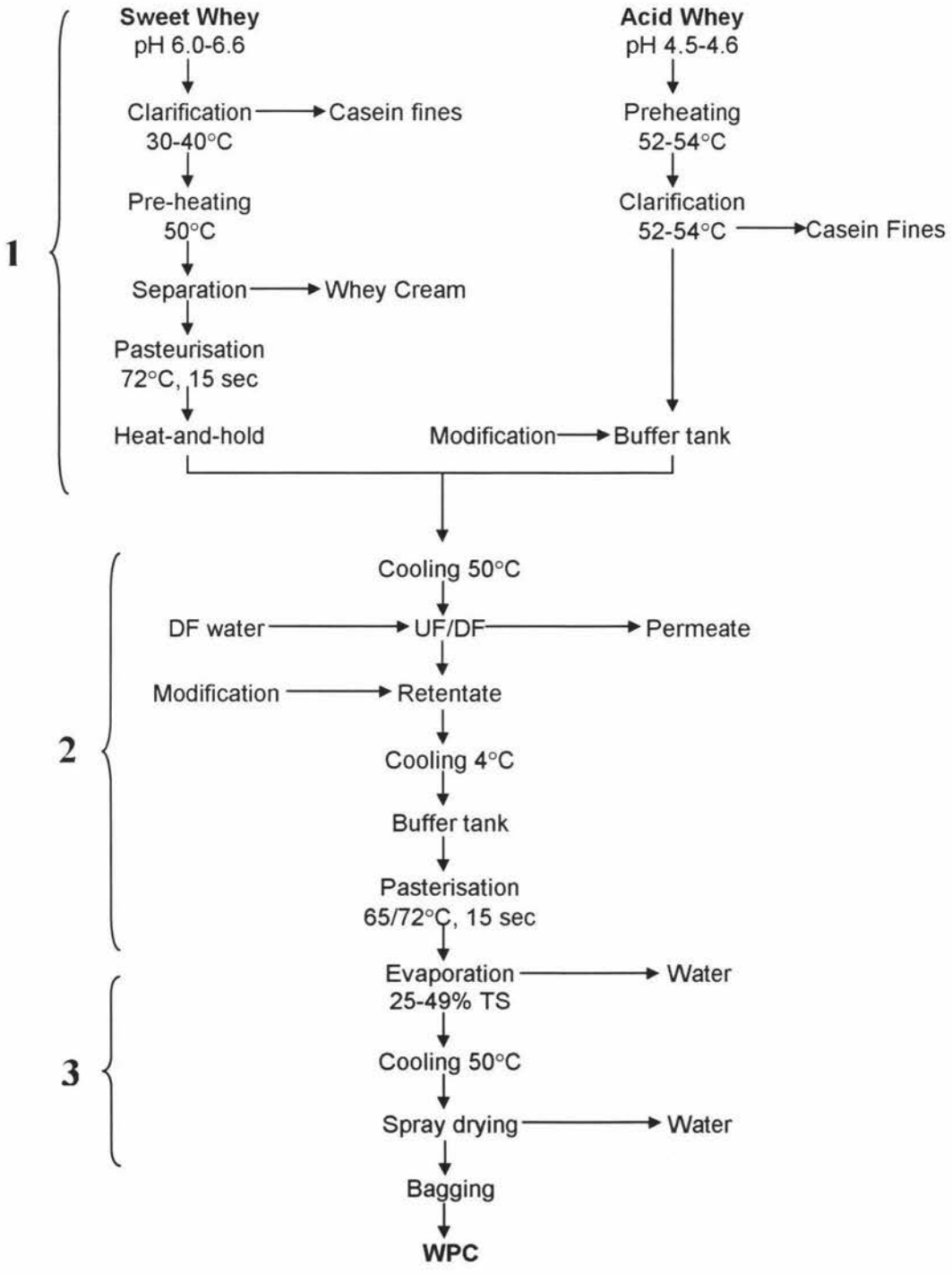


Figure 2.2. Simplified schematic flow diagram for the production of WPC. The three broad categories that the processing steps may be grouped together are (1) pretreatment, (2) ultrafiltration/diafiltration, and (3) concentration and drying (adapted from Nielsen, 1988)

UF of whey is commonly carried out at ~ 50 °C. At this temperature acceptable flux levels and problems with membrane fouling, growth of microorganisms and thermal denaturation of protein are avoided. Operation at lower temperatures (e.g. 10 °C) is used by some manufactures in New Zealand (Havea, 1998). The major disadvantage of such low temperatures is the reduced fluxes achieved and the operation may be more susceptible to microbiological contamination (Nielsen, 1988).

Diafiltration (DF) is employed to produce WPC with protein contents > 65%. The quality of water used in DF is of particular important to avoid fouling. Purified water (demineralised, evaporator condensate or reverse osmosis permeate) is commonly used. Typically, the flow rate of DF water to each stage ranges from 40 to 95% of the flow rate from the stage (Hobman, 1992).

The retentate from the UF plant may need to be cooled (to ~ 4 °C) and stored until sufficient volume has been accumulated before drying can commence. Pasteurisation of the retentate using heat treatment of 66-72 °C for 15 s may also be necessary to reduce the number of bacteria (Hobman, 1992).

Concentration and drying (3)

Retentate may be concentrated before drying to minimise the cost of water removal and improve the physical properties of the powder. The use of specially designed, high-vacuum, low-boiling temperature (e.g. 50 °C) falling film evaporator enables effective concentration of the retentate (Nielsen, 1988), while avoiding thermal denaturation of proteins. Recent improvements in the design of UF equipment enable retentates of high total solids to be produced and in some cases can eliminate the need for evaporation.

Drying of the retentate is usually performed using a spray drier fitted with nozzle atomisation. Typically, the inlet and outlet air temperatures used are between 160 and 180 °C, and less than 80 °C, respectively (Hobman, 1992).

2.3.4 General lipid composition and concentration in whey protein concentrate (WPC)

The composition of WPCs, although reflecting the source of the raw whey, can be largely influenced by the processing to which it is subjected. There is a considerable focus on producing WPCs that have high protein content as they are marketed on their protein content.

Commercially available WPCs contain between 35 and 85% protein. If they are added to foods on a dry basis, there will be large differences in the functionality due to the increased protein content. The WPCs containing 35-55% protein are used mostly for animal feed manufacture. Speciality WPC and WPI products that contain > 70% protein are used extensively as functional and nutritional ingredients in medical, pharmaceutical and human food products such as infant formula, health food and drinks, high gel product applications and frozen foods (Morr and Ha, 1993). The average composition of eight commercial WPCs is shown in Table 2.5 (Morr & Foegeding, 1990).

Table 2.5 Chemical composition of commercial WPCs (%) [a]

Components	**Mean ± S.D.**
Moisture	5.31 ± 0.66
Protein	73.8 ± 1.64
Lactose	3.92 ± 1.20
Total Lipids	5.00 ± 1.27
Phospholipids	1.28 ± 0.23
Ash	4.28 ± 1.29
Sodium	1.04 ± 0.65
Potassium	0.25 ± 0.17
Calcium	0.46 ± 0.27
Magnesium	0.09 ± 0.12
Phosphorus	0.44 ± 0.35

[a] Adapted from Morr & Foegeding (1990), n=8.

2.3.4.1 Total Lipids

Commercial WPC manufactured by UF membrane processing generally contains 5 to 6.4% total lipids (Morr, 1989), despite attempts by producers to remove as much of the lipid fraction as possible from whey. Results reported by Morr and Foegeding found the majority of the commercial WPCs tested to contain between ~ 4 and 6% lipids. The lipid found in WPC does not have the same composition as the bulk of the lipid in milk, but is greatly enriched in phospholipids and MFGM material (Houlihan and Hirst, 1987). The membrane material usually consists of high-molecular-weight proteins complexed with phospholipid, cholesterol, and triglycerides that are know as phospholipoprotein or lipoprotein complexes. Another source of MFGM is significant numbers of small MFG recovered in the whey. In total the WPC contains about 3% of the MFGM material (Houlihan and Goddard, 1991). This material consists of about 65% protein, 7% triglyceride and 30% phospholipid.

Overall, the lipid fraction of WPC consists of 48 to 53% triglycerides, 6 to 15% diglycerides, 7 to 15% free fatty acids and 27 to 28% phospholipids (Morr and Ha, 1993). Further details of each of the lipid components is given below.

2.3.4.2 Triacylglycerols

The triacylglycerols represent between 48 and 53% of milk lipids in whey. The triacylglycerol molecule has a glycerol backbone with a fatty acid molecule attached at three positions of the molecule. The triacylgcerols of milk fat are made up of a wide variety of fatty acids, different combinations and positional distributions making the various triglycerides. These vary in concentration depending on a range of factors, such as the stage of lactation and the diet of the cow (Creamer and MacGibbon, 1996).

The complex mixture of triacylglycerols is responsible in the most part for the rheological properties of milk fat (Jensen *et al.*, 1991), including the milk fat's broad melting range. Triglycerides are also invariably accompanied by small amounts of

di- and monoacylglycerides, free cholesterol and cholesterol esters, unesterified fatty acids and phospholipids.

2.3.4.3 Phospholipids

Phospholipids are usually found at a concentration between 27 and 28% of the lipid fraction in whey. The phospholipids are mainly found in the milk fat globule membrane and other membrane material. Their role in the MFGM is as emulsifiers and MFG stabilisers. They also bind cations and possibly interact with digestive enzymes (Jensen *et al.*, 1991). Because of their location in the MFGM and polyunsaturated fatty acids content, they are very susceptible to oxidation.

Kamath and Morr (1997) using methodology outlined by Stahl (1965) found Swiss cheese whey to contain phosphatidylcholine, phosphatidylethanolamine and sphingomyelin with trace amounts of lysophosphatidylcholine. It was suggested that the phospholipids recovered from the Swiss cheese whey, may have been contributed by milk fat globules that were small enough that they were not removed during centrifugation and separation in cheese processing. Alternatively, the phospholipids could be contained in membrane fragments from larger milk fat globules.

Results gained by Vaghela and Kilara (1995*a*) differed slightly to those found by Kamath and Morr (1997). The most significant proportion of phospholipid were phosphatidylinositol, phosphatidylcholine, and sphingomyelin with smaller amounts of phosphatidylethanolamine, phosphatidylserine and cerebrosides.

2.3.4.4 *Fatty Acids*

Fatty acids usually make 7 to 15% of the lipids in whey, much lower than the content of phospholipids. The residual lipid content and the type of free fatty acids present can be very detrimental to the functionality of the WPC. In particular the foaming properties are adversely affected by the unsaturated fatty acids (de Wit *et al.*, 1986).

Only thirteen fatty acids are present at concentrations exceeding 1% (w/w). These are the 4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 15:0, 16:0, 18:0, 14:1, 16:1, 18:1 and 18:2 fatty acids (Kamath and Morr, 1997).

2.3.4.5 Minor simple lipid constituents

A number of lipid- and fat-soluble compounds have been found in milk. These compounds that exist at relatively small amounts include glycerolipids, sterols, hydrocarbons, lipid-soluble vitamins, prostoglandins, carnitine, acylcarnitines and flavour compounds (Christie, 1995).

2.4 Removal of fat from whey

The need for low cost, functional, and nutritionally excellent proteins for use in processed food has lead the way for a lot of research to be conducted in the area of whey delipidisation. Early work by Breslau *et al.* (1975) looked at the production of a crystal clear bland tasting protein solution from cheese whey. In this process the pH was adjusted to a level of 4.6 with an acid either organic or inorganic before ultrafiltration to 4% protein and diafiltration to varying extents. The pH of the retentate was then adjusted to a level between 4.5 and 4.6 and after standing, a white floc formed and settled, leaving clear supernatant. The loss of turbidity of the permeate when the floc formed indicated that macromolecules and colloidal material such as traces of casein and tiny fat globules had been removed. Breslau *et al.* (1975) work was not specifically focused on the removal of fat from the whey, however more recent work has assumed that the clarity of the supernatant is directly related to its lipid content.

Attebury (1971) conducted work that involved the combination of the addition of divalent metal ions, adjustment of pH, and heating of the whey, to remove the lipids. This work was extended by Fauquant *et al.* (1985*a* & *b*) and Maubois *et al.* (1987) using calcium ions. Downard (1988) following this work conducted an investigation into the removal of lipids from acid whey. The results suggested the levels of calcium, the pH and the temperature were all significant in the results gained. It was assumed in this work that the optical density of the whey was directly relational to the

quantity of lipids in the whey. This was not proved, and further doubt can be cast upon this assumption with the correlation that the total solids in the supernatant had with the optical density. The time of heating did not appear to have a significant effect on the reduction of the optical density.

More recently combinations of chemicals, salts, temperature and pH have been experimented with to delipidise whey. The use of saponin and bile salts altering concentration, pH, temperature and NaCl (Hwang and Damodaran, 1994), Chitosan, temperature and pH (Hwang and Damodaran, 1995) and calcium salts, temperature and pH (Daufin *et al.*, 1993; Kim *et al.*, 1989) have been tried.

Clarification of whey using chitosan has been shown to be successful by Hwang and Damodaran (1995). Most of the lipids in the whey were attributed to fragments of the MFGM that remained dispersed in a stable colloidal form. Chitosan is a natural polyglucosamine polymer derived from chitin and displays polycationic character at acidic pH. The work done by Hwang and Damodaran (1995) used sweet cheese whey (pH 6.2) and varying the pH, temperature, and the concentrations of chitosan, whey and NaCl. The optimal conditions for the clarification of whey was found to be at a pH of 4.4-4.6 with 0.01-0.016% chitosan at 25 °C. It was found that the addition of NaCl to whey treated with chitosan increased the turbidity, probably due to the formation of a stable colloidal form with the MFGM precipitate. It was suggested that because there was no increase in turbidity when NaCl was added to the untreated whey, that the increase in the turbidity of the treated whey must have been due to a MFGM-chitosan complex. Treating unconcentrated, 2-fold and 4-fold concentrated whey indicated that the most efficient removal of lipids was achieved when whey was in an unconcentrated state. The treatment with chitosan removed more than 92% of the lipid content originally present.

Hwang and Damodaran (1994) carried out delipidisation of cheese whey using saponin and bile salts. Their work involved altering the concentration of the saponin and bile salts, pH, temperature, and NaCl concentration. Rational behind the approach was, if hydrophobicity of the membrane fragments could be increased by

attaching hydrophobic molecules on to the surface of the membrane, above a critical hydrophobicity/hydrophilicity ratio membrane particles should overcome the electrostatic repulsive forces and should spontaneously flocculate via hydrophobic interactions. Results indicated that separation occurs best below a pH of 5, at a temperature of 20°C with 0.05% saponin and 0.01% bile salts. The resulting complex was easily remove by centrifugation. The resulting whey contained less than 0.15% fat on dry basis.

Kim *et al.* (1989) described work by Maubois *et al.* (1987) on a whey clarification process. The process involved the use of a combination of calcium, temperature and pH to remove most of the lipid fraction. Calcium chloride was added to a final concentration of 1.2 g/L Ca to cheese whey at 2 °C, and the pH was adjusted to 7.3 with a solution of NaOH. The whey is then rapidly heated to 50 °C, held for 8 minutes and centrifuged to remove the insoluble precipitate.

The use of calcium salts such as calcium chloride and calcium phosphate in the removal of lipids from whey in combination with centrifugation or microfiltration or ultrafiltration seems to be the most widely used method. Most of the current work has however been focussed on the delipidisation of sweet cheese whey or Swiss cheese whey with very little work examining acid casein whey (mineral or lactic). It is important to note that there is a significant difference in the calcium content between cheese and acid whey. Cheese whey has an initial calcium content of 0.4-0.5 g/L which is increased to 1.2 g/L upon addition of $CaCl_2$ in the removal of lipids from whey. Acid casein has an initial concentration of 1.4-1.6 g/L of calcium. This could possibly allow for the precipitation of a calcium-lipid complex under certain conditions.

2.5 Characterisation of lipids in whey

In the study of whey or whey protein concentrate (WPC), it is necessary to achieve total extraction of these components without chemically damaging them. The extraction of lipids from WPC or whey is difficult due to: (1) low levels of total lipids, (2) high protein content, which interferes during phase separation of solvents by producing a stable emulsion, and (3) presence of stable lipid-protein complexes (Theodet and Gamdemer, 1990). It will be important to succeed in completing a quantitative extraction of the lipids without causing damage to the lipids and the lipoproteins.

It should be noted that the preparation of the samples may influence the resulting lipid extracted. Rehydration of dry samples is required before a quantitative extraction can be conducted. This is due to the underestimation of lipid content of a product when direct extraction is conducted. It should not however be necessary to rehydrate the sample fully. Tests conducted by Theodet and Gamdemer (1990) established that the addition of 20 ml of water to 10 g of lyophilisate was satisfactory for the majority of testing. Rehydration was performed by agitating the mixture for approximately 2 hours with a magnetic agitator.

2.5.1 Fractionation of lipids in whey

Kamath and Morr (1997) used a procedure to recover and fractionate residual whey lipids by centrifugation. This involved the separation of the whey into three different fractions, a low-density lipid fraction (LDLF), a medium-density lipid fraction (MDLF) and a high-density lipid fraction (HDLF). The method by which this was done is displayed by Figure 2.3. The method of separation could be useful in characterising the lipid content of the whey.

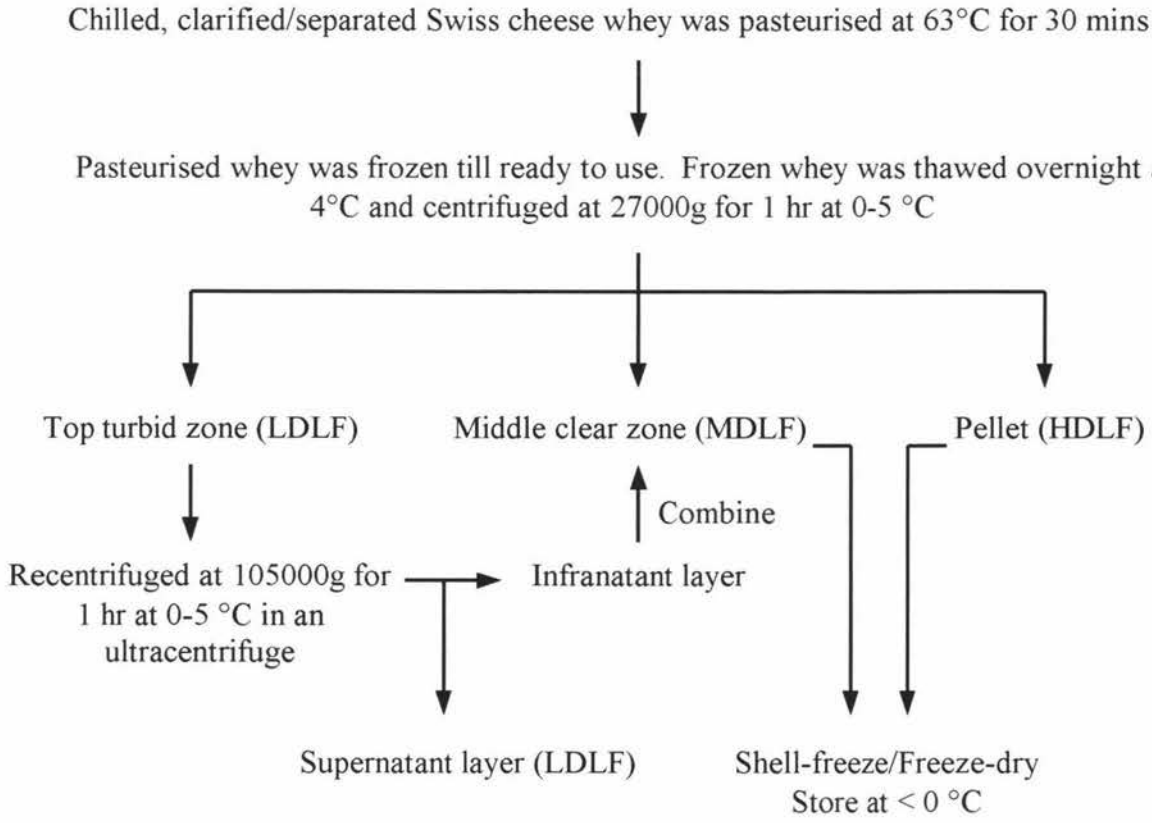


Figure 2.3 Fractionation of residual whey lipids (Kamath and Morr, 1997)

2.5.2 Quantitative determination of lipids in whey

Until recently very little is known about the character of lipids in whey and their derivatives. The majority of the information available relates to the lipid content of the milk and milk products. However, to successfully eliminate these lipids from the whey it has been necessary to gain more knowledge about the character of lipids contained in the whey. Early work was focused on clarification of whey and the development of processes to eliminate lipids from whey to allow for the whey proteins to be fractionated (Fauquant *et al.*, 1985*a* & *b*). Only recent work by Theodet and Gamdemer (1990), Vaghela and Kilara (1996, 1995*a* & *b*) and de Wit (1984) have provided more insight into the character of the lipids in whey in relation to their removal from whey. These include methodologies by which the total lipids, total phospholipid, triacylglycerol, total free fatty acid and diglycerol fractions can be determined.

To characterise the lipids in whey it is necessary to extract them without damaging them. It is also necessary to achieve total extraction of the lipids to successfully characterise and quantify the lipids. It is therefore necessary to extract the lipids by a method that meets these criteria. The methods can be classed into two groups, (1) Standardised reference methods, and (2) Cold extraction methods.

2.5.2.1 Standardised Reference Methods

Standardised reference methods for the extraction of lipids in dairy products (Schmidt Bonzynski Ratzlaff Method (SBR) and Roese-Gottlieb). These methods are used for the extraction and quantification of the lipids in dairy products. They use a base or acid to dissociate the lipid-protein complexes. After this hydrolysis, the lipids are extracted with a mixture of diethyl oxide and petroleum ether after the addition of ethanol. There are no further steps to purify the lipid extract to eliminate non-lipid material.

A comparison of lipid extraction methods conducted by Theodet and Gamdemer (1990) found the SBR method to produce results similar to other methods examined. It was however noted that the SBR method slightly overestimates the lipid content in samples, also noted by other authors (Walstra and Mulder, 1962). These excessive values were explained by the presence of non-lipid contaminants in the lipid fraction. The application of heat and the presence of an acid or base is likely to lead to oxidation and hydrolysis of the lipids, particularly unsaturated fatty acids and phospholipids.

Heat is applied to evaporate the solvents used in both the extractions. This should not however lead to oxidation during the Roese-Gottlieb method and therefore only the SBR should not be used to extract lipids required for further analysis.

2.5.2.2 Cold Extraction Methods

These methods use a binary mixture made up of a good polar solvent (chloroform, hexane) for lipids and a more polar solvent (methanol, isopropanol), which makes it

possible to break the lipid-protein bond. This extraction is carried out in the cold state (Theodet and Gamdemer, 1990).

Different cold methods of extraction are based on binary solvents such as chloroform-methanol (Folch *et al.*, 1957), dichloromethane-methanol (Hara and Radin, 1978) and hexane-isopropanol (Marmer and Maxwell, 1981). Modifications to these methods have been proposed by various authors to adapt them to a dairy context (Timmen and Dimick, 1972; Clark *et al.*, 1982; Maxwell *et al.*, 1986; Wolff and Castera-Rossignol, 1987).

The lipid extraction method used by Folch *et al.* (1957) involved the use of chloroform/ methanol (2:1 by volume) solvent mixture. The extract was purified by washing the organic phase with a saline solution (0.73%). The method used by Folch *et al.* (1957) as altered by Clark *et al.* (1982) involved the use of chloroform-methanol (2:1 by volume) solvent and the separation in a separating funnel. The alteration made to decantation and re-suspension avoided the mechanical homogenisation that formed a stable suspension as was seen in the original method used by Folch *et al.* (1957). The disadvantage in using this technique is the duration of 2-3 days for the decanting procedure to be completed.

The extraction method used by Wolff and Castera-Rossignol (1987) involved the extraction of the lipids using hexane-isopropanol (3:2 by volume). The removal of the contaminants was conducted using a dry column (celite 545/Na_2SO_4) to filter the lipid extract. This technique was quite rapid and only took 2 hours.

Theodet and Gamdemer (1990) conducted a comparison of lipid extraction methods. Their results suggest that the best method for quantitative analysis of lipids in whey is the method given by Clark *et al.* (1982). This method enabled a good evaluation of lipid content and enables complete extraction of the phospholipids without significant oxidation of the polyunsaturated fatty acids. The duration of the extraction process is however quite lengthy and time consuming.

Theodet and Gamdemer (1990) also concluded that rapid methods, the Roese-Gottlieb and that used by Wolff and Castera-Rossignol (1987) may constitute as an acceptable compromise. The Roese-Gottlieb provides a correct evaluation of the lipid content of dairy products and the extraction of phospholipids may be regarded as satisfactory, since there has been no evidence of discrimination between the principle categories of phospholipids. The extraction however did appear to cause slight damage to the polyunsaturated fatty acids of the phospholipids. The method used by Wolff and Castera-Rossignol (1987) was shown to be a good alternative to the Roese- Gottlieb when separating the phospholipids into classes. The Wolff and Castera-Rossignol method should not however be used to determine the total lipid content of a sample due to the excessive values produced.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Clarified acid whey and ultrafiltration retentate was obtained fresh from the New Zealand Dairy Group, Edgecumbe site. Upon arrival sodium azide was added to a concentration of 0.02% and the whey and retentate were chilled to below 5°C. All chemicals used were of analytical grade.

3.2 Compositional analyses

The protein concentration of samples was estimated by determining the total nitrogen content by the Kjeldahl method (AOAC, 1995), using a Kjeltec 1026 system (Tecator, Sweden). The total protein contents were calculated from the nitrogen percentage by multiplying by factor of 6.38. The moisture content of solid sediment samples was determined by using a modified AOAC method (1984). Triplicate samples were weighed (~ 1 g) into separate aluminium dishes and dried at 70 °C under vacuum for 5 hr, cooled in a desiccator for 2 hr and reweighed. Total solids of treated whey supernatants were also determined using a modified AOAC method (1984). The samples (~ 10 g) were heated on a steam bath until the majority of the water was evaporated before drying in the vacuum oven. Total ash contents were determined using a procedure adapted from AOAC (1995). Duplicates or triplicates of sediment (~ 1 g) and supernatant (~ 10 g) were weighed accurately into crucibles, charred over a bunsen burner, ignited in an electric muffle furnace at 550°C for 5 hr, cooled in a desiccator, and reweighed. Total lipid content was measured using the Roese-Gottlieb method (AOAC, 1995). The lactose content of the powder products was determined using the gravimetric method (AOAC, 1995). The calcium content was determined by a quantitative method. Powder samples of 0.4 g were added to 5 ml of 0.01M EDTA, 1 ml of 0.05M magnesium sulphate and 2 ml of 8M sodium hydroxide solution. Allowed to stand for 30 min, Patton and Reeders indicator was added until a distinct blue colour was observed. This was titrated immediately with

0.010 M calcium chloride to the end point where a pink colour was observed. The calcium concentration was calculated using the equation shown in [1]

$$\% \text{ Calcium} = \frac{V1 - V2}{10} \quad [1]$$

where: V1 = ml of EDTA solution used
V2 = ml of calcium chloride solution used

3.3 Recovery and fractionation of whey and retentate lipids

Samples (20 ml) of whey were centrifuged for 60 min at 90,000 *g* (20 ± 2 °C) in the Beckman L2-65B ultracentrifuge equipped with a Type 50.2Ti, 26° fixed-angle rotor (Beckman Instruments Inc., California). Three layers were observed similar to those observed by Kamath and Morr (1997). The top layer was turbid, referred to as the low-density lipid-containing fraction (LDLF), the middle layer was clear, referred to as clear, the medium-density lipid-containing fraction (MDLF), the bottom layer was a "gelatinous pellet" on the bottom of the tube, referred to as the high-density lipid-containing fraction (HDLF). The LDLF was withdrawn from the top of the centrifuged whey sample with a Pasteur pipette. The ultracentrifuge tube was inverted to remove the MDLF; after draining the HDLF was scraped off with a spatula. Whey fraction weights were determined by weight difference before and after each fraction was removed.

Samples of whey and retentate and their fractions were sealed in plastic bags and frozen at –30 °C. The top face of the plastic was removed from the frozen samples before they were freeze dried at 50 °C at 0.01 mbar gauge, using a Cuddon freeze drier (Blenheim, NZ).

Lipid extraction from the freeze dried whey and retentate and their fractions, was conducted at the New Zealand Dairy Research Institute. Freeze-dried samples (~ 2 g) were extracted using the accelerated solvent extractor, ASE-200 (Dionex Corp, Sunnyvale, CA) at 80 °C/1500 psi. The solvent used was a mixture of petroleum ether (40-60 °C B.P.): dichloromethane: methanol in volume ratio of 2:3:3. The

solvent was evaporated using a heat block and the weight of crude fat was recorded. The fat was separated from non-lipid material by back-extraction with 2 × 25 ml of hexane:chloroform (1:1) and the true fat determined by weight loss.

3.4 Size distribution of fat globules

A Malvern MasterSizer E (Model MAE5000, Malvern Instruments Ltd., Malvern, U.K.) with a size range of 0.1 to 80 μm was used to determine the size distribution of the fat globules in the whey fractions. Several drops of each of the samples were added to distilled water to achieve the target obscuration of 20%. The particle sizes were analysed with the presentation mode 2NAD polydispersed model.

In this method, a low power beam is diffracted by the fat globules in the solution and the diffracted light is collected over a range of scattering angles by a series of semicircular photo-electric diodes. The sizes of fat globules are divided into 22 classes across the sub-micron range of light diffracted at each angle using Lozenz-Mie theory. For the calculation of size distribution, the refractive index of the medium in which the particles are dispersed is required.

3.5 Microstructure of fat globules (Confocal Scanning Laser Microscopy)

The size and shape characteristics of milk fat globules were determined by examination using Confocal Scanning Laser Microscopy (CSLM) operated in fluorescent mode as described by Lucey *et al.* (1998). The dyes Nile Blue (hydrogen sulphate, BDH Chemicals Ltd., England) and Fast Green FCF (Merck, Darmstadt, Germany) were used to stain the fat globules and protein matrix respectively. A few grains of each dye were added and gently mix with the each of the samples. A drop of each sample was placed on a microscope slide, covered with a cover slip and then viewed under the CSLM. Duplicate samples were examined on a Leica TCS 4D confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a 100× oil immersion objective (type: Plan Apochromatic with a numerical aperture of 1.4). The CSLM had an air-cooled Ar/Kr laser which was used at wavelengths of 488 nm and 568 nm, for Nile Blue and Fast Green FCF respectively. If no reference is made to dye or wavelength, it should be assumed that the sample has been stained with Nile

Blue and viewed at 488 nm. Each sample was prepared on two separate occasions. Multiple fields were viewed and typical fields were acquired and stored as TIFF (format) files. The brightness/contrast of these files was adjusted using Paint Shop ProTM Version 3.12 software package (Jasc Software, Inc., Eden Prairie, Minnesota, USA)

3.6 Spectrophotometry

The absorbance at 500 nm (A_{500}), determined with the Pharmacia Ultraspec 2 spectrophotometer, was used to measure the degree of fat removal from the whey. The A_{500} of triplicate supernatant samples of treated whey (after centrifugation at 1126 *g* for 10 min) were measured. Turbid samples were agitated immediately before measurement. The absorbance of the supernatant was also measured at 280 and 320 nm (A_{280} and A_{320} respectively) in triplicate. The resulting absorbance at 280 nm was subtracted from that at 320 nm to give an approximation of the protein content ($A_{280-320}$). Samples that gave absorbances > 1 were further diluted. Absorbances from the diluted sub-samples were multiplied by their dilution factor for comparison to the absorbance of other dilution treatments.

3.7 WPC functionality

3.7.1 Gel sample preparation

WPC solution (150 g/kg) was prepared by dissolving appropriate quantities of WPC powder in demineralised water followed by stirring using a magnetic stirrer and then adjusting the pH to 6.8 with 1 M NaOH. The resulting samples were left to stand for 30 minutes at room temperature (~ 20 °C). The rehydrated WPC was transferred to flexible tubing (35 mm inside diameter × 200 mm long) and tied off at both ends. The samples were heated at 75 °C for 1 hr in a temperature controlled water bath and then cooled with tap water for 30 min (~ 15 °C). The gel were refrigerated (~ 4 °C) overnight (~ 18 hrs). The gels were carefully removed from the tubing and cut into 15 mm lengths before measurements of gel strength and syneresis were taken.

3.7.2 Gel strength

The first yield point (or inflection point) of the gels segments (cylindrical shape, 35 mm in diameter and 15 mm in height) was measured with a Fudoh rheometer NRM-2002J (Fudoh Kogyo Co., Ltd, Tokyo, Japan) using a flat plunger 3 mm in diameter (surface area = 0.071 cm^2). Gel samples were placed on a platform raised upward at 6 cm/min until the first yield of the plunger. Breaking strength (grams) was measured using a force compression curve at the point that gave maximum force. The data was plotted with the Rheo Plotter FR-31 and analysed manually. Nine separate breaking strength measurements were taken from three different gel segments, and the average calculated. The gel strength was calculated using equation [2] shown below.

$$\text{Gel strength } (g/cm^2) = \frac{\text{Average breaking strength (g)}}{\text{Area of probe } (cm^2)} \qquad [2]$$

3.7.3 Gel syneresis

The water-holding capacity of the gels was measured in terms of water released from the gels onto filter paper. Preweighed, triplicate gel segments (cylindrical shape, 35 mm in diameter and 15 mm in height) were laid flat between wire mesh, with filter paper on the outer surfaces (3 sheets of number 2, 125 mm circles; Toyo Roshi Kaisha, Ltd. Japan). A glass plate laid across the segments and filter set-up, and a 1 kg weight was applied for 10 min at room temperature (~ 20 °C). The gel segments were reweighed and the syneresis (% water lost) was calculated.

CHAPTER 4

CHARACTERISATION AND SEPARATION OF LIPIDS FROM ACID WHEY

4.1 Introduction

The functionality of whey protein products is influenced by many factors, including their composition. Generally, WPC contains ~ 3 to 7% lipids. Several studies have shown that the presence of lipids has adverse effects on the functionality of whey proteins. Morr (1992) reported that the presence of high concentrations of residual lipids in the whey retentate is detrimental to some aspects of functionality of the resulting WPC. Rinn *et al.* (1990) showed that reduced lipid content in WPC resulted in a highly functional product in terms of the solubility, foam expansion, emulsifying activity and gelation. Morr and Foegeding (1990) attributed better solubility, gelling, foaming and flavour properties to specialised whey products with lower proportion of non-protein components, such as lipids, lactose and minerals. The presence of lipids was also reported to have detrimental effects on the flavour stability. de Boer *et al.* (1977) found that the oxidation of lipids, predominantly phospholipids, during storage and processing impaired the flavour stability of WPC and WPC-containing food products.

To improve the functionality of WPC, it is desirable to remove the non-protein components during manufacture (Morr & Foegeding, 1990). Removal of the lipids from whey during WPC manufacture requires a thorough understanding of the physical, chemical and microstructural properties of this material. The aim of this chapter was to gain a greater understanding of the physical and microstructural characteristics of residual milk fat globules (MFG) in whey and the factors that may assist their removal during WPC manufacture.

4.1.1 Characterisation of lipids in acid whey

Acid whey was separated by ultracentrifugation into three fractions; the low-density lipid-containing fraction (LDLF), the medium-density lipid-containing fraction (MDLF), and the high-density lipid-containing fraction (HDLF). The weights and lipid content of these fractions were determined as described in Section 3.3. The size distribution of milk fat globules in the three fractions (LDLF, MDLF and HDLF), acid whey (AW), whole milk (WM) and homogenised milk (HM) were examined using the Malvern MasterSizer (Section 3.4). Samples of LDLF, MDLF, HDLF and AW were also examined using confocal microscopy (Section 3.5).

4.1.2 Lipid content

The lipid content and weight fraction of AW, LDLF, MDLF and HDLF are shown in Table 4.1. The MDLF (82.7%) had the highest weight fraction of the fractionated whey after centrifugation compared to the LDLF (15.6%) and the HDLF (1.7%). The lipid content was determined as a percentage of total solids (% TS). As the total solids of each of the fractions did not vary greatly the concentration of the dried fraction may be compared. The HDLF contained the highest concentration of lipids (~ 15%) relative to the LDLF (~ 1%) and MDLF (~ 0.2%).

Table 4.1 Weight (wet weight) and lipid composition (percent of dry weight) of acid whey fractions.

Sample	**Weight fraction [a] (%)**	**Lipids concentration (% TS)**
Acid whey (AW)	100.0	0.56
Low density lipid containing fraction (LDLF)	15.6	1.01
Medium density lipid containing fraction (MDLF)	82.7	0.24
High density lipid containing fraction (HDLF)	1.7	15.05

[a] N = 3

The concentration of lipids in the LDLF (~ 1.0%) was higher than whey (~ 0.6%). This may be attributed to some of the MFG having significantly lower density relative to whey. The HDLF however had a significantly higher concentration of lipids (~ 15%) compared to the LDLF and the MDLF. The lipids in the HDLF can be considered more dense than the remaining components of whey (~ 98% by weight).

The LDLF and MDLF, although low in concentration, contained respectively ~ 25% and ~ 32%, of the lipids in whey. This is attributed to the larger volumes of these fractions compared to the HDLF. No significant concentration of lipids was achieved in the whey with the application of 90,000 *g* for 60 mins. Even under significant centrifugal force the separation of lipids under normal conditions from whey is difficult.

The HDLF was found to have a high concentration of lipids (~ 42%) despite its small volume. This indicates a segment of lipid material with a higher density than the majority of the components of whey. The density of normal milk fat at 20 °C is 0.93 g/mL (Webb and Johnson, 1965) and thus, it is expected that milk fat would rise, being separated into the LDLF. Association of milk fat with other components of whey, in the form of the milk fat globule membrane (MFGM), increases the density of the lipid fraction. The effect of the MFGM on the overall density of the globule is determined by the quantity and composition of the membrane components. Denatured proteins, residual casein and lipoproteins associated with the MFGM are the most likely components of whey that would increase the density of the MFG sufficiently so that it is sedimented (at 90,000 *g*).

Some of the lipids recovered in the high-density fraction are likely to be lipoprotein in nature. In part this is due to apparent density values greater than the density of skim milk of 1.035 g/mL (Sherbon, 1988). Also the phospholipids are likely to be associated with relatively dense protein components, being commonly found in high concentrations in the MFGM, where typically 50% of the phospholipids reside (Brunner, 1974). Thus, phospholipids are likely to be in higher concentrations in the HDLF and therefore contribute significantly its high relatively lipid content. This

was confirmed by Kamath and Morr (1997) who determined that the lipid component of the HDLF (after centrifugation for 1 hr at 27,000 *g* and 4 °C) of Swiss cheese whey contained 36% phospholipids.

4.1.3 Size distribution of fat globules

The weighted volume mean diameter (d_{43}) of the MFG in WM was more than twice that of the MFG in HM and AW, and more than four times that of the MFG in LDLF (Table 4.2). This result suggests that homogenisation of milk results in a significant (~ 66% on a volume basis) reduction of the average MFG size. It is interesting to note that the MFG in AW had a slightly larger d_{43} than that in HM. Skim milk used in production of AW was subjected to a separation that removed the larger MFG, leaving smaller MFG of similar sizes to that in HM. The d_{43} of the MFG in LDLF was almost half that of the MFG in AW indicating that the larger particles present in AW had been separated from those present in LDLF. These larger particles were probably casein fines or lipids that associated with higher molecular weight material and had been incorporated in the HDLF. The d_{43} value of the HDLF was more than three times the size of whole milk and nine times that of AW. This supports the view of sedimentation of larger or higher molecular weight particles during the ultracentrifugation process.

Table 4.2 Volume mean diameter (d_{43}) of MFG in dairy samples as determined by Malvern MasterSizer E

Sample	**Volume mean diameter, d_{43} (μm)**
Whole Milk (WM)	2.77
Homogenised Milk (HM)	0.94
Acid whey (AW)	0.99
Low density lipid containing fraction (LDLF)	0.57
Medium density lipid containing fraction (MDLF)	N/D
High density lipid containing fraction (HDLF)	6.55

The MFG in WM exhibited a bimodal size distribution, ~ 75% of the particles were between 1 and 10 μm and ~ 25% between 0.1 and 1 μm (Figure 4.1*a*). The MFG in HM had a monomodal size distribution ranging from 0.1 to 4 μm. For AW approximately ~ 80% of the MFG were within the range of 0.1 to 1 μm, with the rest residing in the range of 1 to 10 μm. The MFG in LDLF and MDLF had similar size distributions to those in AW, with the majority (~ 90%) of MFG < 1 μm, and the rest residing in the range of 1 to 10 μm (Figure 4.1*b*). In contrast, the HDLF exhibited a bimodal distribution with two separate populations, ~ 35% of the particles were between 0 and 0.7 μm and ~ 65% were between 2 and 30 μm. It should be noted that the distribution observed in Figure 4.1 is in terms of % volume.

Comparing the MFG size distribution in AW (Figure 4.1*a*) with those of the whey fractions (Figure 4.1*b*), the majority of the smaller particles (< 2 μm) have been separated into the LDLF and the MDLF, while the large particles (> 2 μm) have been separated into the HDLF. This again suggests that the larger particles were denser than those that were found in the LDLF and MDLF and therefore were probably casein fines or lipids associated with higher molecular weight material.

Little work has been conducted on the size distribution of MFG in whey. A study by Kamath *et al.* (1998) examined MFG in Swiss cheese whey (SCW) and LDLF, using the Coulter LS130 Particle Size Analyser. The size distribution of the acid whey and fractions examined in this work were quite different from those found by Kamath *et al.* (1998). Both the SCW and SCW LDLF had polymodal size distributions while the AW and AW LDLF had bimodal distributions. These differences can be attributed to the significantly different processes and manufactured product (i.e. Swiss cheese and mineral acid casein).

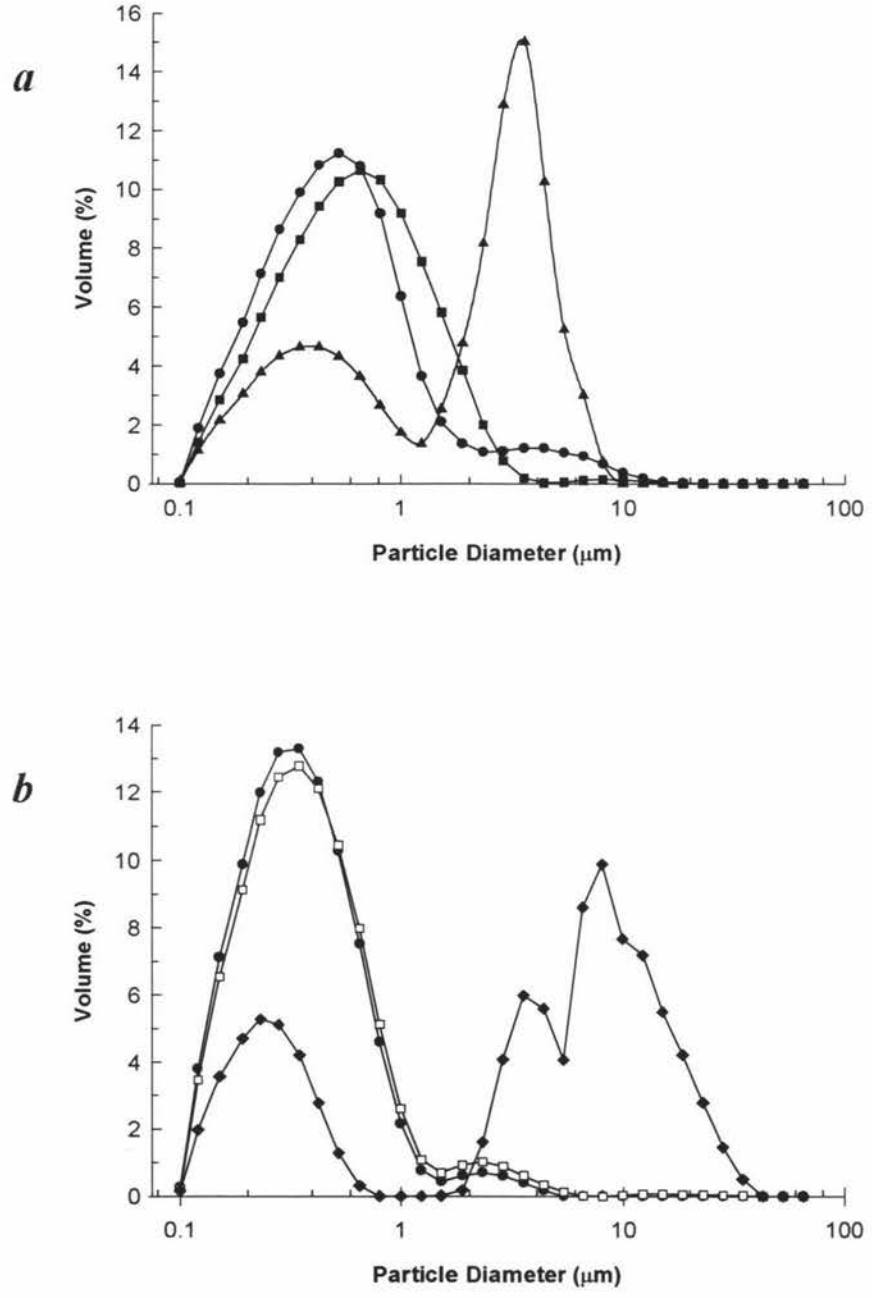


Figure 4.1 MFG size distribution in (*a*) whole milk (▲), homogenised milk (■), and acid whey (●); and (*b*) low density lipid fraction (□), medium density lipid fraction (●) and high density lipid fraction (◆) as determined by the Malvern MasterSizer E.

4.1.4 Microstructure of fat globules by confocal scanning laser microscopy(CSLM)

Samples of whey and whey fractions were prepared and stained appropriately as described in Section 3.5, and viewed under the CSLM. Samples of AW and LDLF, which were viewed showed that the majority of the MFG were small (< 1 μm) with only a few larger ones (> 1 μm) (Figure 4.2*a* & *b*). It also appears that some of the MFG in the LDLF were present in clusters of up to 5 μm (Figure 4.2*c*). The HDLF contained MFG of similar size distribution to AW, the majority of the MFG were small (< 1 μm) with a few larger ones (1 to 3 μm) (Figure 4.2*d*). The MFG in MDLF were observed to be relatively small in comparison to the other fractions where most were < 0.5 μm diameter (results not shown). On the other hand, the HDLF, which was stained with fast green to stain proteins, and viewed at 568nm was observed to contain areas of high protein concentration, similar in size to the MFG present (Figure 4.2*e*).

The clusters of MFG present in the LDLF (Figure 4.2*c*) were not observed in the acid whey and therefore, were probably formed during ultra-centrifugation (90,000 *g* for 60 mins at 20 °C). In contrast to the size distribution results of the HDLF (Figure 4.1*b*), the confocal micrographs did not show many MFG > 3 μm. It must therefore be assumed that the majority of the particles > 3 μm in the HDLF were aggregates of protein or other non-fat material and not MFG.

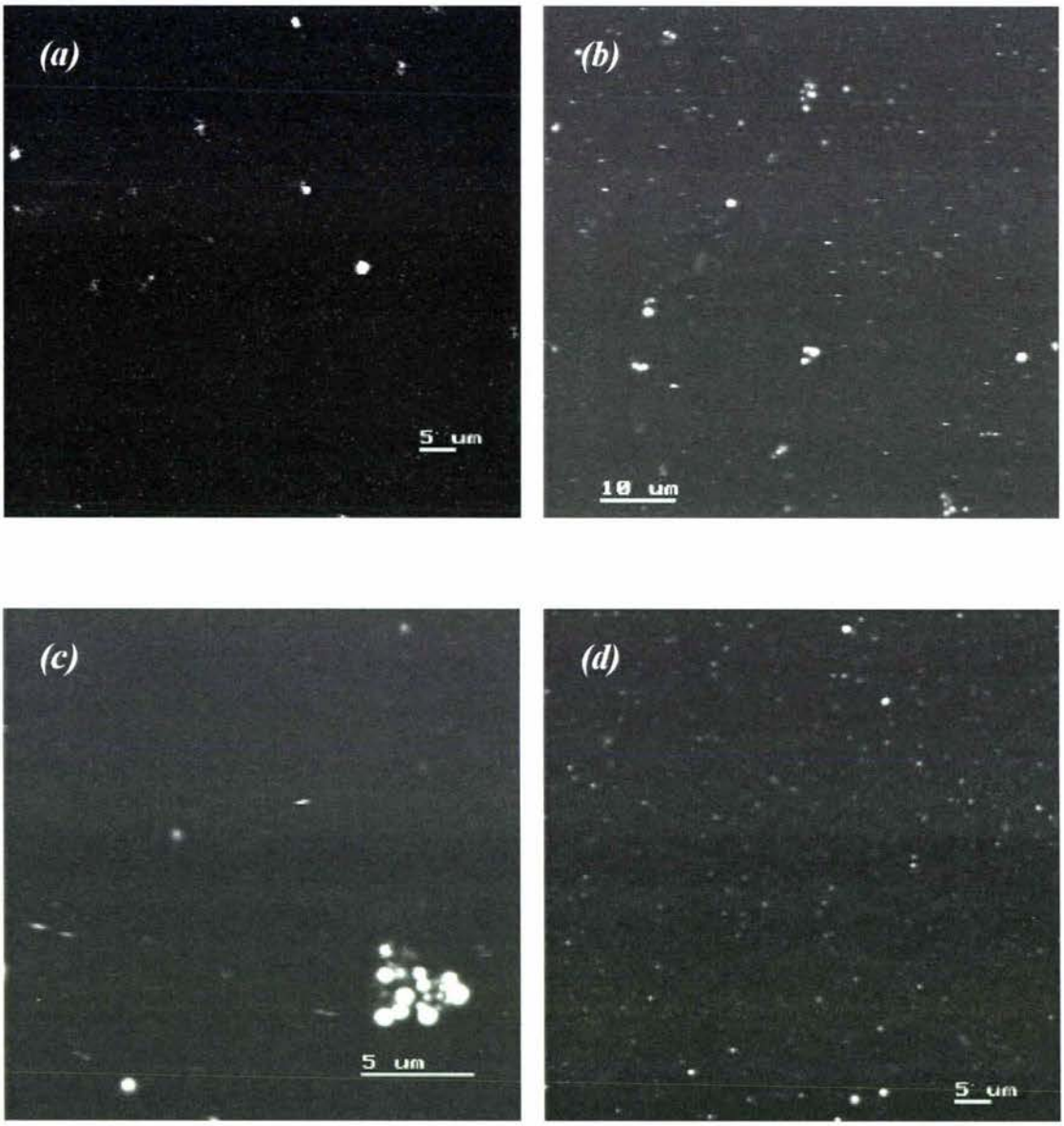


Figure 4.2 Confocal micrograph of acid whey (*a*), low density lipid fraction, LDLF (*b*), LDLF at magnification × 20,000 (*c*), High density lipid fraction, HDLF (*d*). If not stated otherwise magnification was × 10,000, and the sample was dyed with Nile blue and viewed at 488 nm.

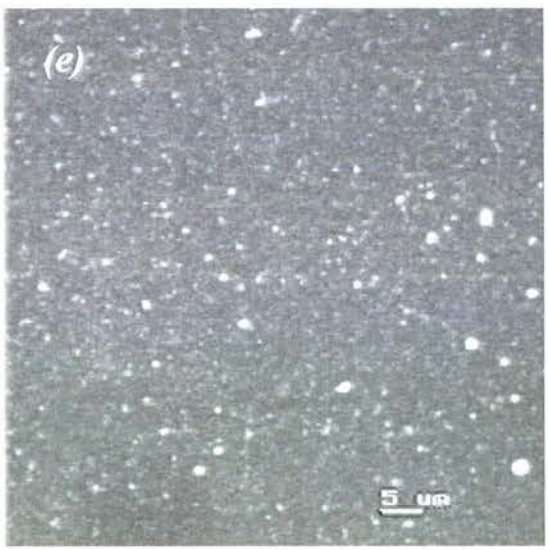


Figure 4.2 Continued. Confocal micrograph of the high density lipid fraction, HDLF, stained with Fast Green, viewed at 568nm and × 10,000 magnification (*e*).

4.1.5 Discussion

A centrifugal method was used to separate acid whey into three fractions. Analysis was conducted on lipid components of the acid whey and acid whey fractions using quantitative, size determination and CSLM techniques. The whey contained ~ 0.56% lipids based on the total solids. Fractionation of acid whey resulted in an increased concentration of lipids in the HDLF and LDLF and a decrease of concentration in the MDLF.

The composition and structure of MFG in whole milk and the effect of processing on the MFG and the MFGM is described in Chapter 2. This study has shown that the MFG in whey vary in size between 0 and 10 μm. The majority (~ 90%) of the MFG in acid whey were < 1μm in diameter. Fractionation of acid whey indicated that the fractions containing the lower density components, LDLF and MDLF, contained smaller particles than the HDLF. It was suggested that the larger particles in the HDLF were aggregates of protein or other non-fat material and not entirely MFG. Observation of the MFG using CLSM suggested that the MFG were surrounded with a layer of protein or protein membrane.

The overall density of the MFG is significantly influenced by the quantity and composition of the membrane components. The HDLF was observed to contain a significant quantity of lipids, suggesting that a portion of MFG had a relatively high density. During the processing of milk and whey, increased density of the MFG may occur through association of high-density components with the MFG. Cho (1996) observed increased amounts of casein proteins associated with the membrane when milk samples were heated for 5 to 10 min at temperatures ranging from 70 to 85 °C. It was suggested that casein proteins were absorbed onto exposed sites of the fat globule surface which resulted from the loss of high molecular weight polypeptides and lipid material from the MFGM. It was also observed that a temperatures from 70 to 85 °C, significant amounts of β-lactoglobulin became associated with the fat globule membrane (Dalgleish and Banks, 1991; Cho, 1996; Corredig and Dalgleish, 1996). As a result of these associations, it is likely that the densities of the MFG will vary. The range of temperatures investigated was consistent with those normally used in the processing of milk and whey, in steps such as pasteurisation.

The density of the MFG is also influenced by their size, and as a result, the surface area to volume ratio. Smaller MFG have a larger surface area to volume ratio while larger MFG have a smaller surface area to volume ratio. MFG with larger surface area to volume ratio are more likely to have an overall higher density due to protein interacting with the surface of the fat globule.

In conclusion, acid whey contains ~ 0.56% lipids based on the total solids, the most significant part of which is in the form of milk fat globules (MFG). The majority of the MFG were small (< 1μm diameter). The MFG also range in density, which probably results from the association of varying quantities of proteins with the MFGM.

4.2 Removal of lipids from acid whey

The effects of pH and temperature on the efficiency of fat removal from whey were determined using centrifugation and spectrophotometry. The pH of the AW was adjusted to 5.00, 5.25, 5.50, 5.75, 6.00, 6.25, 6.50, 6.75, 7.00, 7.25, 7.50, 7.75 and 8.00 using 1N NaOH. The samples (40 ml) were then incubated at 20, 30, 40 and 50 °C for 1 hour using a thermostatically controlled water bath. At the end of the incubation period, the samples were centrifuged at 1126 g for 10 minutes using a laboratory clinical centrifuge (IEC International, model UV, Boston, Mass., USA). The centrifugation resulted in the formation of two fractions, a clear supernatant and a sediment. The weight of the sediment was determined. The absorbance of the supernatant at 500 nm (A_{500}) was used to estimate the concentration of fat globules and the degree of fat removal from the whey (Section 3.6). The absorbance of the supernatant was also measured at 280 and 320 nm (A_{280} and A_{320} respectively). The resulting absorbance at 280 nm was subtracted from that at 320 nm to give an approximation of the protein content ($A_{280-320}$).

The turbidity of whey is mainly due to light scattering caused by micrometer-sized membrane fragments or MFG (Hwang and Damodaran, 1994). Determination of turbidity can therefore be used to estimate the concentration of MFG, measured by absorbance of visible light at 500nm (A_{500}). The solubility and lack of size of other components; lactose, protein and minerals, result in very little effect on A_{500}. Investigations into removal of lipids from whey have used A_{500} as a quick and relatively good comparison of MFG concentration between samples (Fauquant *et al.*, 1985*a* & *b*; Kim *et al.*, 1989; Rinn *et al.*, 1990; Hwang and Damodaran, 1994, 1995).

4.2.1 Effect of pH and temperature on separation of MFG.

During incubation at different temperatures, precipitates were formed in the samples. During centrifugation, a proportion of these precipitates became sedimented, leaving a clear supernatant. The absorbance at 500 nm (A_{500}) of the supernatant indicated the degree to which MFG were sedimented from the treated samples. The results (Figure 4.3) showed that temperature had no effect on A_{500} between pH 4.6 and 5.8. However, between pH 5.8 and 7.0, the A_{500} of the supernatant increased at 20 and 30

°C, while at 40 and 50 °C, A_{500} decreased markedly which resulted in an essentially clear supernatant. The decreased A_{500} or lack of turbidity strongly suggests the removal of the majority of the MFG from whey. These results also suggest that at 20 and 30 °C, the increase in pH beyond 5.8 resulted in the formation of very small particles that were not recovered during centrifugation but remained in suspension and gave an increase in A_{500} (Figure 4.4). However, at 40 and 50 °C, the particles were probably large and/or dense enough that they were effectively removed during centrifugation, hence the decreased A_{500} of the supernatant at pH 5.8 to pH 7. Beyond pH 7, the particles were fine, similar to those formed at 20 and 30 °C, so they were not removed during centrifugation, hence the increase in A_{500} beyond pH 7 (Figure 4.3).

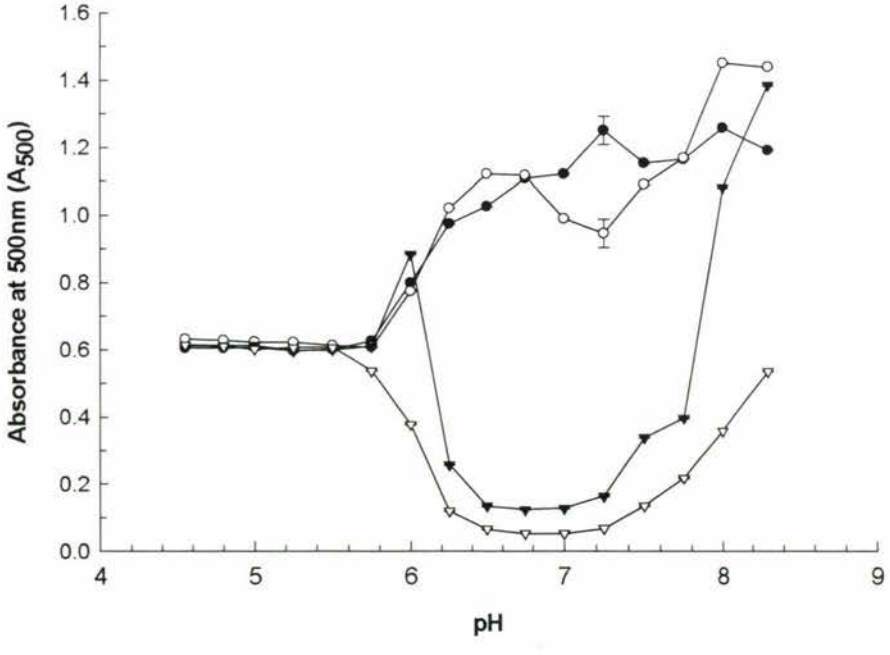


Figure 4.3 Effect of pH on the absorbance at 500nm (A_{500}) of the supernatant obtained from centrifugation of whey samples. The whey samples were adjusted to different pH values and then incubated at temperatures of 20 °C (●), 30 °C (O), 40 °C (▼) and 50 °C (▽). Each point is an average of triplicate measurements. Error bars indicate the variation between results.

4.2.2 Effect of pH and temperature on amount of sediment

Figure 4.4 shows the effect of pH on the weight of sediment recovered from whey at different temperatures, after centrifugation (1126 *g* for 10 min). The results showed that temperature had no effect on the sediment weight between pH 4.6 and 5.8. However at temperatures of 20, 30, 40 and 50 °C, the sediment weights increased between pH 5.8 and 7.4. At the lower temperatures of 20 and 30 °C, the increase in sediment weight (2.2 and 3.3 g/100ml of whey) was significantly smaller than at the higher temperatures of 40 and 50 °C (6.5 and 6.6 g/100ml of whey). The temperature of the whey therefore has a considerable impact on the separation of the particles which are likely to contain MFG, removing the most MFG at temperatures > 40 °C.

These results are closely related to those observed in Section 4.2.1. The lower temperatures (20 and 30 °C) resulted in an increase in A_{500}, while the higher temperatures (40 and 50 °C) resulted in a decrease A_{500} above pH 5.8 (Figure 4.3). The slight increase in sediment at 20 and 30 °C suggests that some of the particles

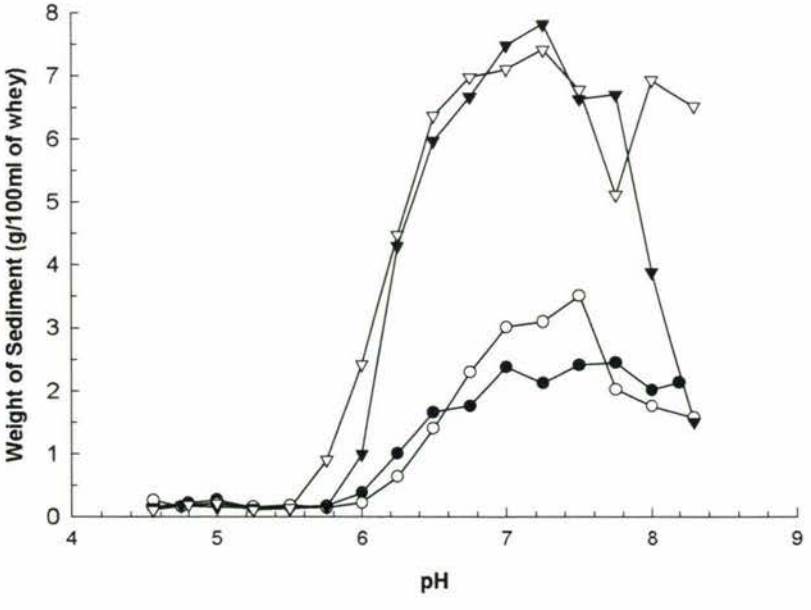


Figure 4.4 Effect of pH on the sediment weight of the supernatant obtained from centrifugation of whey samples. The whey samples were adjust to different pH values and then incubated at temperatures of 20 °C (●), 30 °C (○), 40 °C (▼) and 50 °C (▽). Each point is an average of triplicate measurements.

formed were of sufficient density that they sedimented upon centrifugation while some remained in suspension, hence increasing the A_{500} values. The increased sedimentation at 40 and 50 °C indicates that the precipitate formed was consistently denser than that formed at 20 and 30 °C, so that it was separated from the bulk of the whey during centrifugation (1126 *g* for 10 min). The significantly higher quantities of sediment recovered at 40 and 50 °C (Figure 4.4) combined with a significant decrease in the A_{500} of the supernatant (Figure 4.3) suggesting the removal of the majority of the MFG.

4.2.3 Composition of supernatant and sediment

The results presented in Figure 4.3 show two vastly different trends as the pH increased from 5.8 to 7.0; an increase in A_{500} between 20 and 30 °C, and a decrease in A_{500} between 40 and 50 °C. These trends are reflected in the sediment weights (Figure 4.4). To investigate the mechanism involved in this behaviour, further analysis of the two resulting fractions after centrifugation was conducted. Samples of whey supernatant and sediment were produced at pH 4.6 and 20 °C, and pH 6.8 and 20 °C, and pH 6.8 and 50 °C (Section 4.2). The total solids, protein and ash content (mineral content) of supernatant and sediment samples (Section 3.2) are shown in Table 4.3 and Table 4.4 respectively. Other components of whey not quantified were lactose and lipids. It should be noted that the units used to describe the composition of the supernatant and sediment are different. The composition of the supernatant is presented as g/100g of supernatant, whereas composition of the sediment is presented as g/100g of whey.

Table 4.3 Variation in the total solids, protein and ash content of the supernatant as affected by pH and temperature.

	Composition (g/100g of supernatant)		
Sample	**Total Solid** [a]	**Protein** [a]	**Ash** [a]
Whey	5.39	0.77	0.77
pH 4.6 at 20 °C	5.44	0.73	0.76
pH 6.8 at 20 °C	5.27	0.71	0.87
pH 6.8 at 50 °C	5.11	0.70	0.67

[a] N = 3

At pH 6.8 increased holding temperature resulted in a significant decrease in total solids and ash content in the supernatant. Increased holding temperature at pH 6.8 was also observed to remove the majority of the turbidity (Figure 4.3). Possible interaction between MFG and part of the mineral component may have been responsible for the flocculation and subsequent sedimentation of these components.

The ash content of the supernatant increased at 20 °C with increased pH. The addition of NaOH during pH alteration probably accounts for the increased mineral content (as measured by ash) in the supernatant at pH 6.8.

It should be noted that the protein composition of the supernatant does not directly relate to the total quantity of protein recovered from each treatment, as varying quantities of supernatant were recovered from each treatment. The quantity of protein sedimented should enable a better assessment of protein loss at different treatments (Table 4.4). The general trend however indicates that protein recovered in the supernatant decreases at higher pH and temperature values.

Table 4.4 Variation in the total solids, protein and ash content of the sediment as affected by pH and temperature.

	Sediment Composition (g/100ml of whey)			
Sample	**Sediment weight [a]**	**Total solids [a]**	**Protein [a]**	**Ash [a]**
pH 4.6 at 20 °C	0.23	0.04	0.004	0.018
pH 6.8 at 20 °C	1.51	0.19	0.032	0.084
pH 6.8 at 50 °C	5.64	0.64	0.136	0.217

[a] N = 3

The data in Table 4.4 is presented as a proportion of the original whey before treatment and separation, to allow for a better comparison of the quantity of components sedimented.

Sediment weights increased significantly as pH was increased from 4.6 to 6.8 and holding temperature increased from 20 to 50 °C (Table 4.4). The increase in sediment weight was reflected in the increased quantities of protein and minerals (measured as ash) sedimented. The A_{500} of the supernatant decreased significantly at pH 6.8 and 50 °C (Section 4.2.1), strongly suggesting the sedimentation of the majority of the MFG. The corresponding increase in protein and ash content of the sediment indicates a probable interaction between the protein associated with the MFG, a segment of the milk minerals and possible serum proteins.

4.2.4 Effect of pH re-adjustment on the turbidity of the supernatant

Whey samples produced in Section 4.2 were adjusted to pH values ranging from 5.0 to 8.0, held at 20 or 50 °C for 1hr and then centrifuged at 1126 *g* for 10 minutes. Sub-samples of each supernatant were taken and the pH adjusted to 4.5 using 1N HCl. The absorbances at 500nm (A_{500}) of the original supernatants and supernatants adjusted to pH 4.5 were measured. The temperatures of 20 and 50 °C were used to represent the two entirely different trends observed in Section 4.2.

The A_{500} of supernatants between pH 4.6 and 5.8 at both temperatures were largely unaffected when their pH values were adjusted to 4.5 (Figure 4.5*a* & *b*). However,

re- adjustment of pH to 4.5 of supernatant samples between pH 5.8 and 8.2 treated at 20 °C, resulted in decreased A_{500} (Figure 4.5*b*). These absorbances differed markedly from A_{500} of the supernatant at the original pH (Figure 4.5*a*). The A_{500} of the supernatant at 50 °C was unaffected by the re-adjustment of pH to 4.5 between the original pH values of 4.6 and 7.2. However, above pH of 7.2 the A_{500} of the supernatant decreased slightly (Figure 4.5*b*) as opposed to the sharp increase observed at the original pH (Figure 4.5*a*). The change between the original pH and pH 4.5 did not result in a significant change in the composition of the supernatant (only the addition of 1N HCl to change the pH). Thus, the only change within the system involved the structure of the components present in the supernatant.

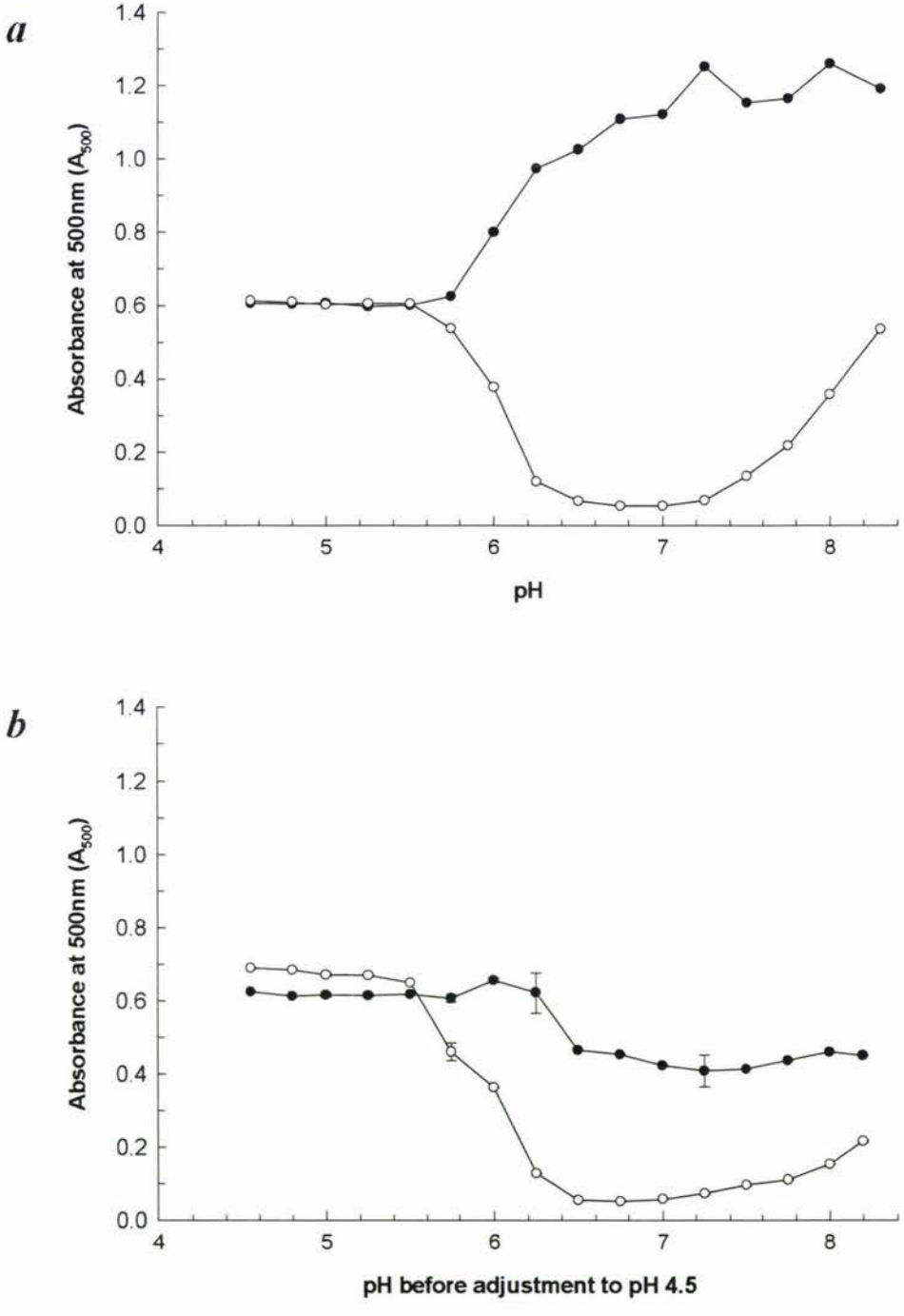


Figure 4.5 Effect of pH on the absorbance at 500nm (A_{500}) of the supernatants at (*a*) original pH, and (*b*) pH re-adjusted to 4.5. The whey samples were adjusted to various pH values, incubated at temperatures of 20 °C (●) and 50 °C (O) and centrifuged at 1126 *g* for 10 min. Aliquots of the supernatants were re-adjusted to pH 4.5. Each point is an average of triplicate measurements. Error bars indicate the variation between results. Error bars are present when the error is larger than the data

4.2.5 Discussion

The effects of pH and temperature on the efficiency of fat removal from whey were determined using centrifugation and spectrophotometry. The results clearly showed that at certain pH values and temperatures, it was possible to cause significant sedimentation of particles which resulted in a large reduction in turbidity of the supernatant, hence the concentration of MFG (Figure 4.3). Under conditions where the sedimentation of suspended material, which includes MFG, was most effective, the amount of minerals and protein in the sediment also increased.

MFG separation from whey

The results clearly indicated that at temperatures between 40 and 50 °C and pH values between 5.8 and 7.0, the formation of lipid containing sediments was favourable. The resulting supernatant was essentially a clear liquid, indicating that the majority of the MFG had been removed. Fauquant *et al.* (1985*a*) reported that a rapid increase in pH (~ 7.0) and temperature (40-50 °C) of acid whey resulted in the simultaneous precipitation of calcium phosphate and lipoproteins. This suggests that at the temperatures and pH conditions used in this study (40-50 °C, pH 5.8-7.0), precipitation of calcium phosphate induced interactions between proteins and the lipid material. The lipid material present in whey, and probably that sedimented, is thought to include MFG and lipoprotein complexes (Section 4.1.2, 4.1.5). It is not clear, however, whether this interaction involves the formation of calcium-MFG complexes or the lipoprotein/MFG material simply entrapped within the calcium phosphate precipitate.

Acid whey is produced from casein manufacture where milk is acidified to pH 4.6. This process releases all the calcium and phosphate from the casein micelles into the acid whey. Hence acid whey is always supersaturated with respect to concentration of calcium phosphate, and so is very sensitive to changes in environment.

The behaviour of the calcium and phosphate in milk varies depending on the composition and conditions of its environment. Calcium phosphate precipitation may be induced by heat treatment and changes in pH. Brule *et al.* (1978) showed that

calcium phosphate precipitation can be induced in sweet whey permeate by heating between 60 and 90 °C (initial pH of 6.7). Visser *et al.* (1986) showed that an amorphous calcium phosphate formation started between 35 and 40 °C upon heating of a synthetic milk ultrafiltrate at pH 6.8. The quantity of calcium phosphate precipitated in acid whey has also been observed to be highly dependent on pH (Fauquant *et al.*, 1985*a*). They showed that, in samples progressively heated to temperatures of 40, 45 and 50 °C, more calcium phosphate precipitated as pH increased from 6.0 to 7.0. Furthermore Brule *et al.* (1978) observed that in milk permeates heated for 30 min at 80 °C, calcium phosphate precipitation increased with increasing pH (from 5.8 to 6.6). Work conducted by Pouliot *et al.* (1991) with sweet whey permeate at 50 °C found that pH values between 6.5 and 8.0 were favourable for the formation of "insoluble" calcium phosphates, such as tricalcium and octacalcium phosphate. The results of the current study are consistent with the finding of these investigations.

Possible association of MFG or lipoprotein complexes with calcium phosphate may have resulted in their coprecipitation at pH values between 5.8 and 7.0 (Figure 4.3). Milk proteins are known to develop a higher affinity for calcium and phosphate at increased pH values (Holt 1982). Interactions between proteins contained in the MFGM or lipoproteins complexes and the destablised calcium and phosphate, may result in the formation of "insoluble" calcium phosphate on the MFG and lipoprotein complexes. The coprecipitation may also be a result of lipoprotein/MFG material simply entrapped within the calcium phosphate precipitate. The high density precipitate was sedimented at an increased rate using centrifugation.

Protein loss

The results showed that the quantity of protein in the supernatant decreased by ~ 9% from original protein content (Table 4.3) when the pH was altered to 6.8 and then treated at 50 °C for 60 min. Smaller quantities of protein were lost at the lower pH values and temperatures. The increase in quantity of sedimented protein contributed to the increased weight of sediment. These proteins may either be associated with the

MFG or present as denatured proteins or aggregates suspended in solution. It is well known that denaturation of the serum proteins would not occur at treatment temperatures of 40 and 50 °C. It is more likely that denaturation of whey proteins took place during previous processes, involving heat treatment e.g. milk pasteurisation.

Most whey proteins are sensitive to heat denaturation and become less soluble if milk is heated (Walstra & Jenness, 1984). The extent of heat-induced denaturation and aggregation of protein during WPC manufacture is affected by other factors, such as pH and ionic strength (de Wit & de Boer, 1975). Heating of whey protein solutions at pHs close to the protein isoelectric points (pH 4.3-5.0) generally result in a measurable reduction in protein solubility (Morr *et al.*, 1985). It has been reported that during heating, denatured serum proteins either associate with the MFGM via disulphide bonding, or absorb onto the fat globule surface (Dalgleish & Banks, 1991; Houlihan *et al.*, 1992; Kim & Jimenez-Flores, 1995; Corredig & Dalgleish, 1996, 1998). Denaturation of protein and subsequent bonding onto the milkfat globular membrane (MFGM) may explain the high-density characteristics of the MFG, contributing to part of the protein sedimented when whey is treated at 40-50 °C with pH 5.8-7.0. It should be noted that this mechanism implies a possible yield loss in the commercial process of manufacturing whey protein concentrate.

In conclusion, the MFG were removed from acid whey at pH values between 5.8 and 7.0 and temperatures ≥ 40 °C, where interaction between the MFG and precipitating calcium phosphate resulted in a coprecipitation. At temperatures below 40 °C, a fine calcium phosphate precipitate formed but was not separated when centrifuged (1126 *g* for 10 min). It was not clear, whether the separation of the MFG involved the formation of calcium-MFG complexes or simply the entrapment of the lipoprotein/MFG material within the calcium phosphate precipitate. A significant quantity of protein was also sedimented under the same conditions that lead to the sedimentation of MFG. This protein was probably associated with the MFG, phospholipoprotein complexes and aggregates of denatured protein.

CHAPTER 5

THE CHARACTERISATION AND SEPARATION OF LIPIDS FROM UF RETENTATE OBTAINED FROM ACID WHEY

5.1 Introduction

It has been previously determined that the presence of lipids has an adverse effect on the functionality of whey proteins (Morr, 1992; Rinn *et al.*, 1990; Morr and Foegeding, 1990; de Boer *et al.*, 1977). Results presented in Chapter 4 showed that the majority of the lipid component of acid whey could be removed by alteration of pH and temperature. Changes in pH and composition of whey during this process were thought to be detrimental to the products for subsequent processing. During the commercial ultrafiltration and diafiltration (UF/DF) process, permeate containing water, lactose and minerals is separated from retentate containing the protein and lipid components of the whey. Further processing of the permeate is conducted to remove the calcium from the permeate, which when dried is sold as a calcium rich ingredient (called Alamin), that is primarily used to fortify consumer food products. Investigations have indicated that at conditions where the MFG are removed from the whey (pH 5.8 to 7.0 and 40 to 50 °C), calcium phosphate is destabilised and most probably sedimented with the MFG. Utilisation of this sediment in the production of Alamin will most probably result in a significantly increased lipid content, beyond the acceptable concentration defined in the product specifications. From a manufacturers viewpoint this is not acceptable.

Acid whey retentate is an intermediate product in the manufacture of whey protein concentrate (WPC) powder. At this stage the whey has undergone UF/DF steps to remove the majority of lactose and minerals and a substantial quantity of water. Before the retentate is subjected to pH alteration and drying, there is an opportunity to remove the residual lipids. To date, no investigations have been carried out into the removal of lipids from acid whey retentate.

Improvement in the functionality of WPC can be achieved through the removal of residual lipid fraction from acid whey retentate. The removal of lipids from the retentate requires a thorough understanding of the microstructural properties of the residual milk fat globules (MFG) and the influence of environmental factors on MFG properties. Hence, the microstructural characteristics of MFG in retentate and factors that may assist their removal from the retentate were investigated.

5.2 Characterisation of milk fat globules in acid whey retentate

Acid whey retentate was separated by ultracentrifugation into three fractions: the retentate low-density lipid-containing fraction (RLDLF), the retentate medium-density lipid-containing fraction (RMDLF) and the retentate high-density lipid-containing fraction (RHDLF). The weights and lipid content of these fractions were determined as described in Section 3.2.1 and Section 3.2.2. The size and distribution of milk fat globules in the three retentate fractions (RLDLF, RMDLF and RHDLF) and original retentate was determined using the Malvern MasterSizer (Section 3.2.3). Samples of RLDLF, RMDLF, RHDLF and retentate were also examined using confocal scanning laser microscopy (Section 3.2.4).

5.2.1 Lipid content

The lipid content and weight fraction of retentate, RLDLF, RMDLF and RHDLF are shown in Table 5.1. The RMDLF had the highest weight fraction (89.0%) after centrifugation compared to the RHDLF (9.7%) and RLDLF (1.3%). As expected, the RLDLF contained the highest proportion of lipids as a percentage of total solids (% TS) (~ 15%) followed by RHDLF (~ 6%) and RMDLF (~ 3%).

Table 5.1 Lipid content of fractions of retentate

Sample	Weight fraction [a] (%)	Total lipids (% TS)
Retentate	100.0	6.42
Retentate low density lipid containing fraction (RLDLF)	1.3	14.51
Retentate medium density lipid containing fraction (RMDLF)	89.0	3.26
Retentate high density lipid containing fraction (RHDLF)	9.7	5.58

[a] N = 2

5.2.2 Size distribution of fat globules

The Malvern MasterSizer produces an analysis of particle size in 22 classes between 0.1 to 80 μm. Retentate contains water, protein, lactose, minerals and fat. Of these components, the fat in the form of MFG is thought to be the only component that resides in the size range from 0.1 to 80 μm. Therefore it was considered that the result obtained by the Malvern MasterSizer give a direct size distribution of MFG in the retentate.

The weighted volume mean diameter (d_{43}) of MFG in retentate was much greater than that of acid whey (AW) (Table 5.2), suggesting there was an increase in particle size during ultrafiltration. As expected, the average fat globule size of the fractions followed the order; RHDLF > RMDLF > RLDLF. Again, as with fractionation of acid whey, this supports the notion of sedimentation of larger or higher molecular weight particles in the ultra-centrifugation process.

Table 5.2 Weighted volume mean diameter (d_{43}) of MFG in retentate and retentate fractions as determined by Malvern MasterSizer E.

Sample	Volume mean diameter, d_{43} (μm)
Acid whey (AW)	0.99
Retentate	3.47
RLDLF	1.53
RMDLF	2.79
RHDLF	4.04

The MFG in acid whey (AW) exhibited an essentially monomodal size distribution with the majority of the particles, ~ 94% between 0 and 2 μm, while the retentate shows a distinct bimodal distribution, ~ 37% between 0 and 1 μm and ~ 63% between 2 and 20 μm (Figure 5.1*a*). The appearance of the second population of MFG (between 2 and 20 μm) suggests that some MFG aggregate or coalesce to form larger particles during the UF/DF process.

All of the fractions of retentate (RLDLF, RMDLF and RHDLF) exhibited bimodal distributions (Figure 5.1*b*). The MFG in RLDLF ranged from 0 to 10 μm in diameter. The majority (~ 70%) of the MFG in RLDLF had diameters within the range of 0 to 1.5 μm, with the rest residing in the range of 1.5 to 10 μm. The MFG in RMDLF and RHDLF had similar distributions where two distinct separate populations were observed. About 43% of MFG in RMDLF had diameters between 0.1 and 0.7 μm, with the rest residing in the range of 2 to 10 μm (Figure 5.1*b*). A similar size distribution was exhibited in the RHDLF where, ~ 46% of MFG had diameters between 0 and 1.0 μm and ~ 54% had diameters between 1.5 and 10 μm. It should be noted that distribution observed in Figure 5.1 is in terms of % volume.

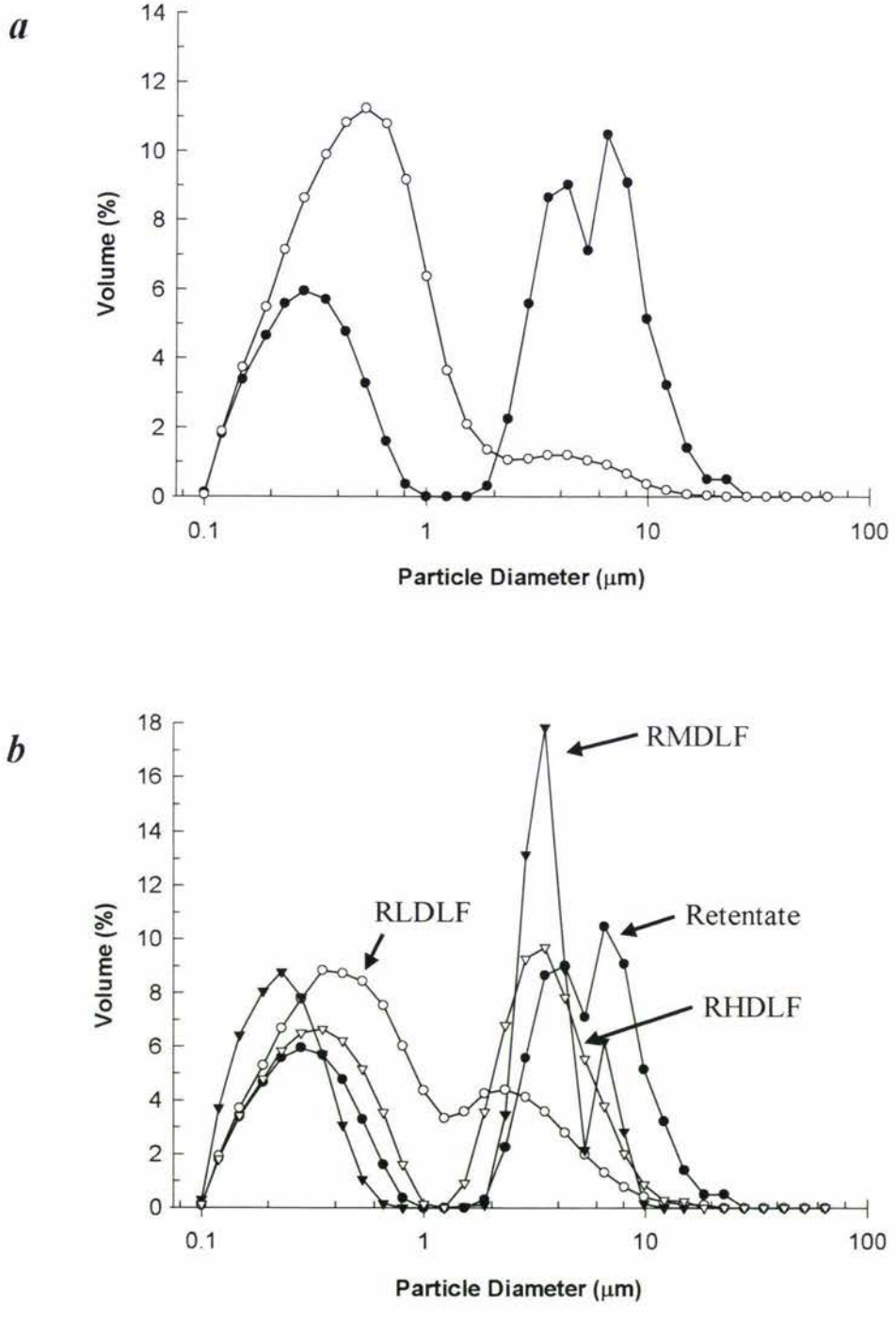


Figure 5.1 MFG size distribution in (*a*) acid whey (O) and retentate (●), and (*b*) retentate (●), retentate low density lipid fraction (O), retentate medium density lipid fraction (▼) and retentate high density lipid fraction (▽), as determined by the Malvern MasterSizer E.

5.2.3 Confocal Microscopy

Samples of retentate and retentate fractions were examined using Confocal Scanning Laser Microscopy (CSLM), as described in Section 3.2.4.

The confocal micrographs of retentate showed many small MFG (< 1 μm) and a number of much larger MFG (between 2 and 5 μm) (Figure 5.2*a*). This is not consistent with the results shown in Figure 5.1*a*. The size distribution of the MFG determined using the Malvern Mastersizer (MM) showed a significant population between 2 and 20 μm (Figure 5.1*a*). No MFG were observed with the CSLM > 5 μm diameter.

The micrograph of the RLDLF (Figure 5.2*b*) showed a highly increased concentration of MFG, ranging in size from 0.1 to 7 μm. These results are mostly consistent with those observed in Figure 5.1*b*.

Clusters up to 8 μm in diameter of MFG were observed in the RLDLF at a higher magnification (× 20,000) (Figure 5.2*c*) but no individual MFG > 5 μm diameter were observed. The presence of clusters may account for MFG in the RLDLF measured by the Malvern Mastersizer > 5 μm (Figure 5.1*b*). Since the clusters of MFG present in the RLDLF were not observed in the retentate, they were probably formed during ultra-centrifugation (90,000 *g* for 60 mins at 20 °C).

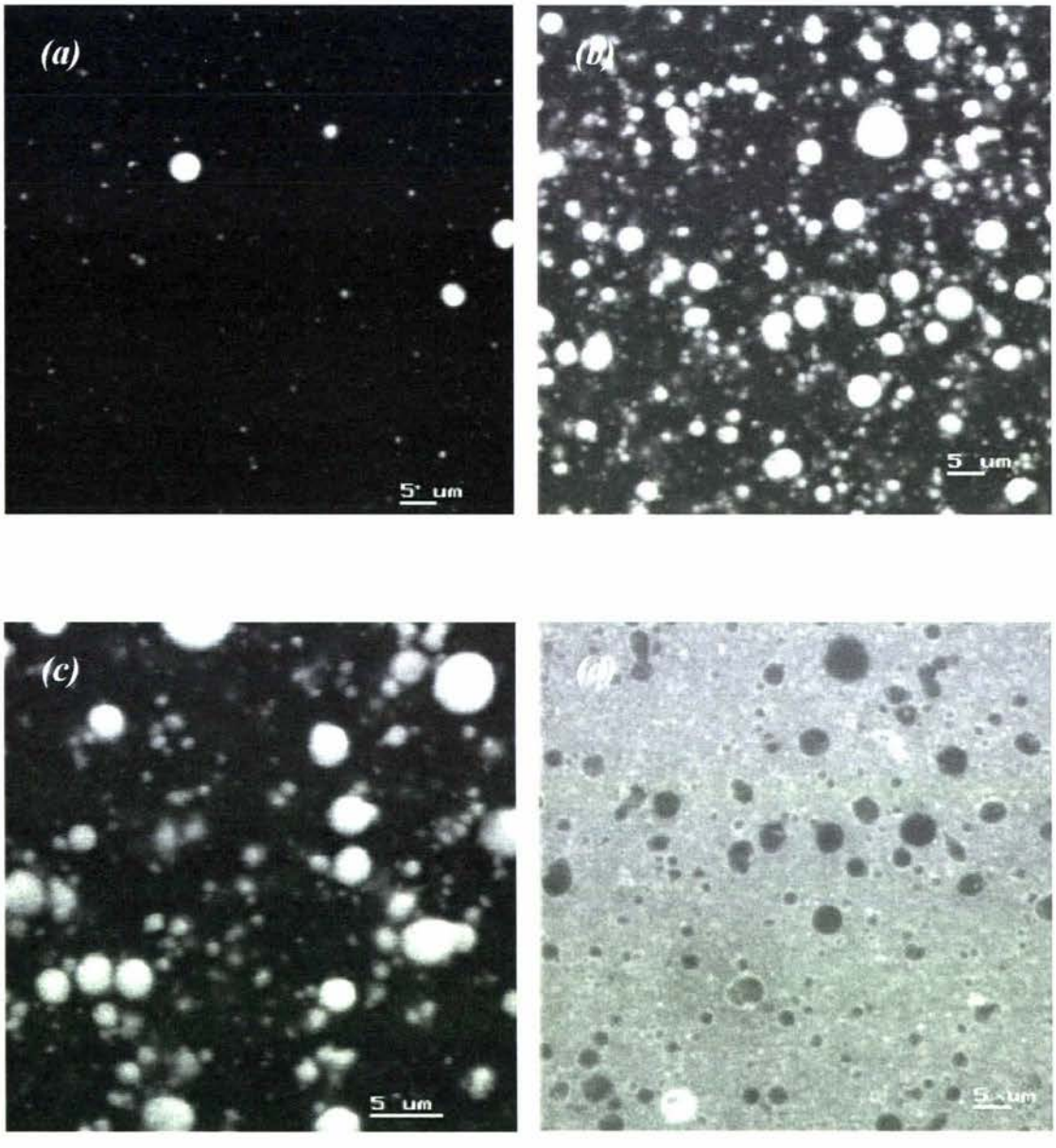


Figure 5.2 Confocal micrograph of retentate and retentate fraction separated by ultracentrifugation at 90, 000 *g* for 60 min (~ 20 °C). Retentate (*a*), retentate low density lipid-fraction (RLDLF) (*b*), RLDLF, magnification × 20,000 (*c*), RLDLF dyed with Fast green and viewed at 568 nm (*d*). If not stated otherwise, magnification was × 10,000, and the sample was dyed with Nile blue and viewed at 488 nm.

The micrograph of RLDLF stained with Fast green and viewed at 568 nm (× 10,000 magnification) revealed that some protein was present on the surface of the majority of the MFG (Figure 5.2*d*). Darker oval areas represent the lipid of the MFG surrounded by the "halos" of fluorescing stained protein. A few MFG fluoresced totally: these were possibly whole MFG which were not dissected by the focal plane, but positioned slightly below, resulting in a view of the protein coated surface of the MFG. Alternatively, these fully fluorescing particles could be protein aggregates.

The micrographs showed the majority of the MFG contained in RHDLF were small (< 1 μm) whereas the majority of the MFG in RMDLF were observed to be even smaller (< 0.5 μm diameter) (results not shown). Particle sizes determined using the Malvern MasterSizer (Section 5.2.2) indicated a significant population of particles between 2 and 20 μm in both the RMDLF and RHDLF. If it is assumed that only MFG reside from 0.1 to 80 μm, these results are inconsistent with those obtained using the CLSM. It must therefore be assumed that the majority of the particles > 1 μm in the RHDLF and RMDLF are aggregates of MFG (Section 5.3.1).

5.2.4 Discussion

In this section, a centrifugal method was used to separate acid whey retentate into three fractions. Analysis was conducted on lipid components of the retentate and retentate fractions using quantitative size determination and CSLM techniques.

The results showed that the forces up to 90,000 *g* did not adequately separate the majority of the MFG from the retentate. Although containing the smallest concentration of lipids, the most significant proportion of the total lipids was contained in the RMDLF, due to its large volume. The RLDLF, which had the highest concentration of lipids, only represented 1.3% of the weight fraction. Thus, either the density of the majority of MFG does not greatly differ from the bulk of retentate, or the majority of the MFG can not be readily separated from the bulk of the retentate due to physiochemical factors such as viscosity.

The fat in the form of MFG was observed to range in size from 0.1 to 20 μm. Surprisingly, the RLDLF contained a smaller proportion of large MFG, as determined by the Malvern MasterSizer. It might be expected that large MFG would have lower overall densities than the smaller MFG, due to the smaller surface to volume ratio. It is suspected that some of the larger MFG in each of the fractions measured by the Malvern MasterSizer might be aggregates of MFG. Aggregates of the MFG may have formed during measurement, upon dilution of the retentate.

A very clear difference in size was observed between the MFG in the whey and retentate. The increase in MFG size probably takes place in the UF/DF step (Section 5.2.2). The mechanism of this size change is not obvious from the work conducted. Most, if not all, of the MFG were observed to have a protein membrane at the lipid/aqueous interface (Section 5.3.2). Coalescence of MFG is unlikely to occur in the bulk of the liquid retentate during the UF/DF process. However, it is possible that interactions between the membrane components adhered to the MFG could occur, resulting in MFG flocculation.

5.3 MFG separation from UF retentate

Investigation into the removal of MFG from whey was carried out in Chapter 4. Results showed that the majority of the MFG could be removed from whey by pH alteration (between pH 5.8 and 7.0) and a temperature treatment of between 40 and 50 °C (for 1 hr). The flocculent formed was separated by centrifugation (at 1126 g for 10 min). The composition of retentate is significantly different from that of whey. Retentate has greatly reduced quantities of lactose, minerals and water. The MFG are concentrated during the UF/DF process as other components are selectively removed.

5.3.1 Preliminary experiments

Separation of the MFG from the retentate was investigated using the conditions determined in Chapter 4. The pH of the retentate was adjusted to 6.8, held at 50 °C for 1 hr, and then centrifuged at 1126 *g* for 10 min (Section 3.2.5).

The sample resulting after centrifugation showed no apparent change in turbidity, although a small amount of sediment was observed. The lack of change in turbidity indicated that little or no separation of MFG had occurred. Increased total solids from whey to retentate resulted in a significant increase in the viscosity, which adversely affected the separation of MFG.

Retentate was diluted with distilled water to the same protein concentration as that in the acid whey. The protein contents of the whey and the original retentate were 0.77% and 19.3% respectively. Retentate samples were therefore diluted, 1 part retentate to 24 parts distilled water (1:24). The different treatments of the diluted retentate are shown in Figure 5.3. The pH values of samples were adjusted from pH 4.5 to 6.8 using 1N NaOH. Samples were then incubated at 20 or 50 °C for 1 hr and subsequently centrifuged at 1126 *g* for 10 min (Section 4.3). As the diluted retentate is low in calcium as compared with acid whey, $CaCl_2$ (1.2 g/L) was added to one sample prior to pH adjustment. These experimental conditions were chosen because previous research has shown the adequate reduction in lipid content in cheese whey

was achieved at 1.2 g/L $CaCl_2$ at pH 7.3 heated at 50 °C for 8 min (Maubois *et al.*, 1987).

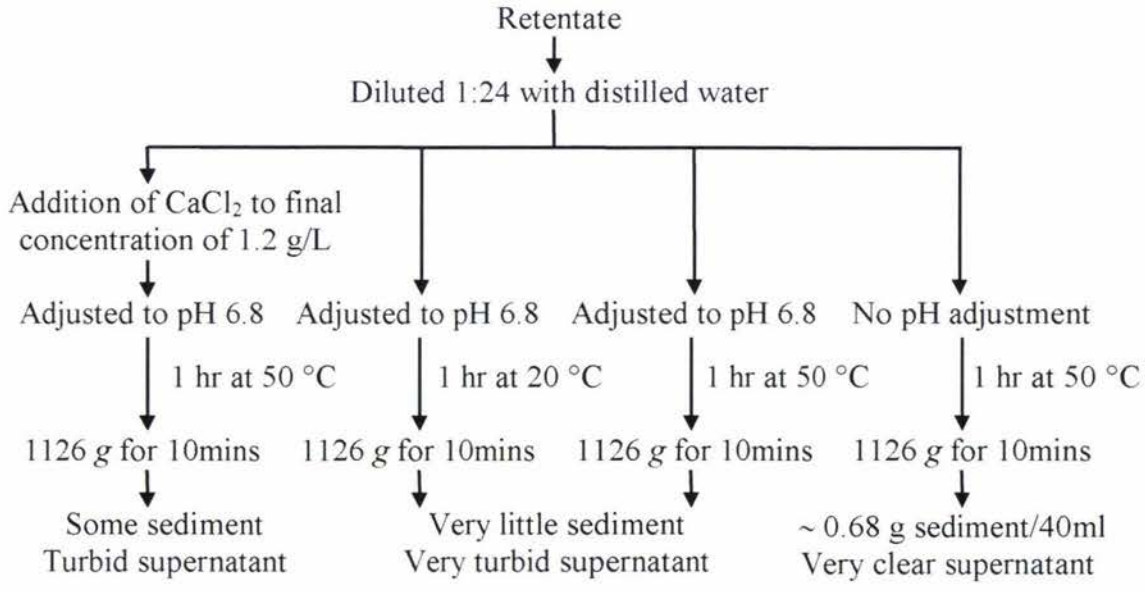


Figure 5.3 Experimental procedure and observed results of the effect of selected pH and temperature on the separation of MFG from retentate.

Dilution of retentate with distilled water, calcium addition and pH adjustment to 6.8 resulted in some reduction in the turbidity of the supernatant with a moderate amount of sediment. This suggests a possible removal of only some MFG from the retentate. The diluted retentate, adjusted to pH 6.8 at both 20 and 50 °C showed very little sedimentation and no significant decrease in the turbidity of the supernatant. However, the supernatant of the control diluted retentate, where the pH was not adjusted, was almost clear. This lack of tubidity indicates the removal of a significant quantity of MFG and the associated lipids. Dilution of the retentate provided an environment in which flocculation of MFG occurs. This type of flocculation (at pH ~ 4.5) is different to that observed in whey between pH 6.5 and7.5 in Chapter 4. To gain a greater understanding of how the MFG can be removed, it will be important to determine the effect of dilution, pH, ionic strength, temperature and time on the flocculation and separation of MFG from diluted retentate.

5.3.2 Effect of dilution on separation of MFG

Dilutions of acid whey retentate of 1:2, 1:3, 1:5, 1:10 and 1:20 were made with distilled water. Samples (40 ml) of the diluted retentate were taken and heated at 50 °C for 1 hr using a thermostatically controlled water bath. At the end of the incubation period, the samples were centrifuged at 1126 g for 10 minutes using a laboratory clinical centrifuge (IEC International, model UV, Boston, Mass., USA) (Figure 5.4). The supernatant was poured off and the tubes inverted and drained on paper towels. The absorbance at 500nm (A_{500}), 320nm (A_{320}), and 280nm (A_{280}) of the original diluted retentate and supernatant was measured (Section 3.6). Samples that gave absorbances > 1 were further diluted. Absorbances from the diluted sub-samples were multiplied by their dilution factor for comparison to the absorbance of other dilution treatments.

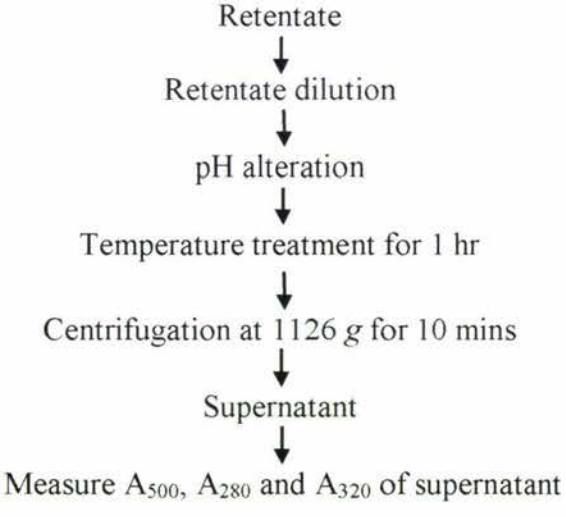


Figure 5.4 General experimental procedure for determination of the effect of selected variables on the flocculation and separation of MFG from retentate.

Turbidity of supernatant

Figure 5.5 shows the effect of dilution on the absorbance at 500 nm (A_{500}). As the extent of dilution increased, there was a decrease in the A_{500} of both the original diluted retentate and the resulting supernatant after centrifugation. (It should be noted that the dilution factor decreases as the extent of dilution increases. For example dilution factors of 0.1 and 0.5 correspond to dilutions of 1:10 and 1:2

respectively). The A_{500} of the supernatant was very low at dilution factors of ≤ 0.2. This relatively small absorbance or lack of turbidity of the supernatants (Figure 5.6) is assumed to represent an absence of the majority of MFG (Chapter 4).

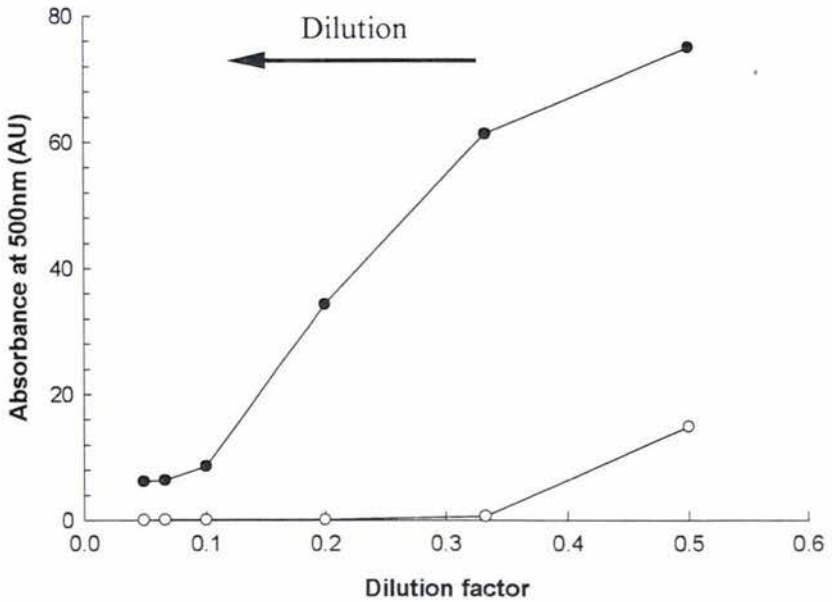


Figure 5.5 Effect of retentate dilution on absorbance at 500nm (A_{500}) for the original sample (●), and supernatant (O) obtained after centrifugation at 1126 *g* for 10 mins. Diluted retentates were incubated at 50 °C for 1 hr before centrifugation. Samples were further diluted with water to keep absorbances < 1. The resulting absorbances were multiplied by their dilution for comparison to the absorbance of other dilution treatments and designated Arbitary Units (AU).

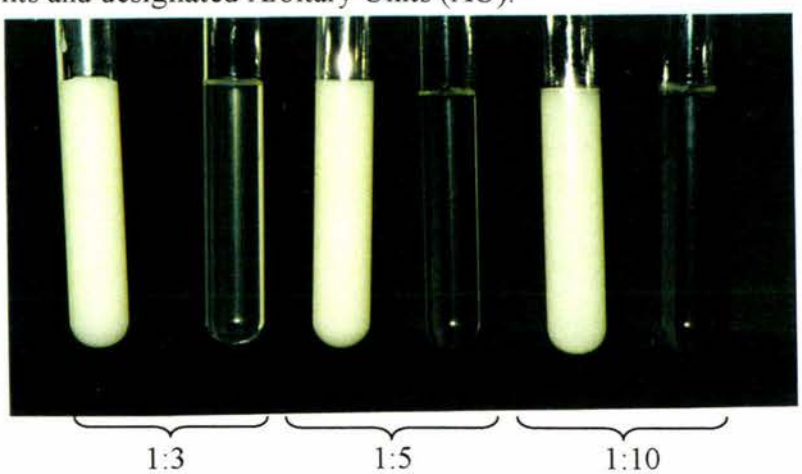


Figure 5.6 Photograph of the diluted retentate before centrifugation and the supernatants of the treated samples at dilutions 1:3, 1:5 and 1:10. The solutions were centrifuged at 1126 *g* for 10 min.

It was interesting to note that the difference in A_{500} between the original diluted retentate and the corresponding supernatant increased as the dilution decreased. Therefore, increased quantities of MFG were removed as the dilution decreased. However, it should be noted that there was less residual MFG material in the supernatant at higher dilutions.

In a commercial process, the optimal removal of MFG (lipids) from the retentate is required with maximum efficiency. Water added to dilute the retentate in this process must be removed later before a dry WPC is produced. It is therefore important from a commercial viewpoint to minimise the quantity of water added, and therefore removed. Adequate MFG removal occurs at retentate dilutions > 1:3.

Protein content of supernatants

Since the aromatic side chains in proteins are known to absorb ultraviolet light at 280 nm (Walstra and Jenness, 1984), A_{280} measurements were used to make comparisons between samples with different protein contents. Absorbance at 320 nm (A_{320}) was subtracted from A_{280} to remove the effect of particles that contribute to turbidity. A_{280} is not an absolute measurement of protein content but an indication of the relative protein content of different samples.

The protein concentration of the original diluted retentate and the supernatant is shown in Figure 5.7. The protein content increased as the dilution decreased in both the original diluted retentate and the supernatant. At dilution factors ≤ 0.10 the difference in protein concentration of the original diluted retentate and the supernatant did not appear to be significant. However, at dilution factors > 0.10, an increased difference in protein content was observed with decreased dilution. At dilution factors of 0.2, 0.33 and 0.5, the supernatant had a lower protein content compared to the original diluted retentate (by 32%, 31% and 38% respectively), indicating that at lower dilutions significant quantities of protein were sedimented.

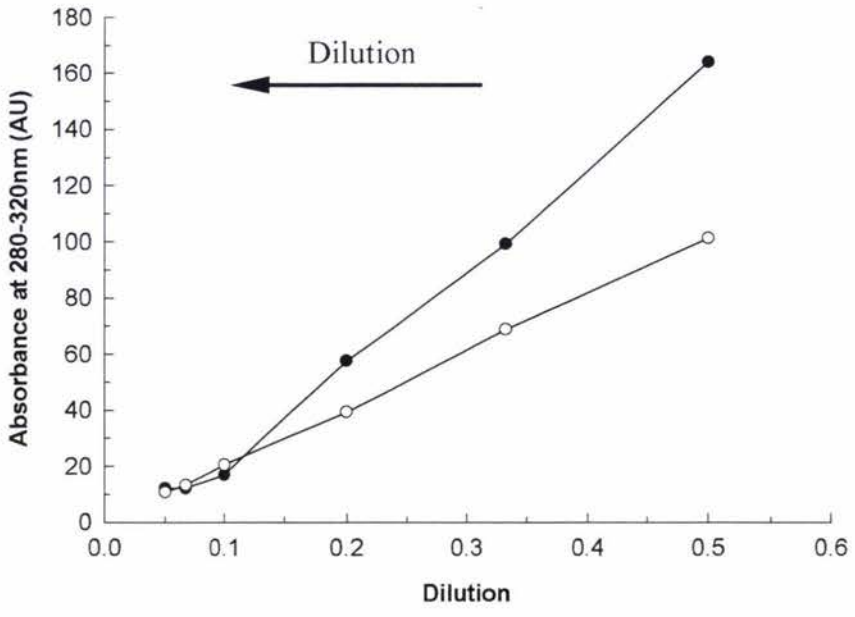


Figure 5.7 Effect of retentate dilution on absorbance at 280-320nm ($A_{280-320}$) for the original dilute retentate (●), and supernatant (O) obtained after centrifugation at 1126 *g* for 10 mins. Diluted retentates were incubated at 50°C for 1 hr before centrifugation. Samples were further diluted to gain absorbances < 1. The measured absorbances were multiplied by their dilution for comparison to the absorbance of other dilution treatments. AU = Arbitary units.

Overall, when determining the appropriate level of dilution in further work, factors that should be investigated are the A_{500} and the protein content. These relate to the removal of MFG, and retention of protein in the supernatant. It is important that MFG be removed while the protein retention is maximised, providing an acceptable yield. The level of dilution should be kept to a minimum therefore maximising the cost effectiveness of the process. Dilutions between 1:10 and 1:3 were felt to balance the areas of consideration most adequately. Dilutions within this range were used in subsequent investigations to determine the effect of other factors on the separation of MFG.

5.3.3 Effect of dilution and pH on MFG separation

Retentate was diluted to 1:3, 1:5 and 1:10 with distilled water, and aliquots of the diluted retentates were adjusted to pH 3.0, 3.5, 4.0, 4.5, 4.6 (natural pH of acid

whey), 5.0, 5.5 and 6.0 using 1N NaOH (> pH 4.6) or 1N HCl (< pH 4.6). Samples were treated (held at 50 °C for 1 hr then centrifuged) and the A_{500}, A_{280} and A_{320} of the supernatants were measured (Section 5.3.2).

Turbidity of the supernatant

The A_{500} of the supernatants obtained from retentates diluted to different extents and adjusted to various pH values is shown in Figure 5.8. At all dilutions, A_{500} decreased as the pH increased from pH 3.0 to 4.6, but with further increase in pH, the values of A_{500} increased. From these results it appears that the removal of MFG was most effective at pH 4.6, regardless of the dilution. This finding is supported by the observation that the supernatant of the samples at pH values of 4.6 and 5.0 were very clear (Figure 5.9). At higher and lower pH values, increasing A_{500} indicates a less effective removal of MFG from the dilute retentate.

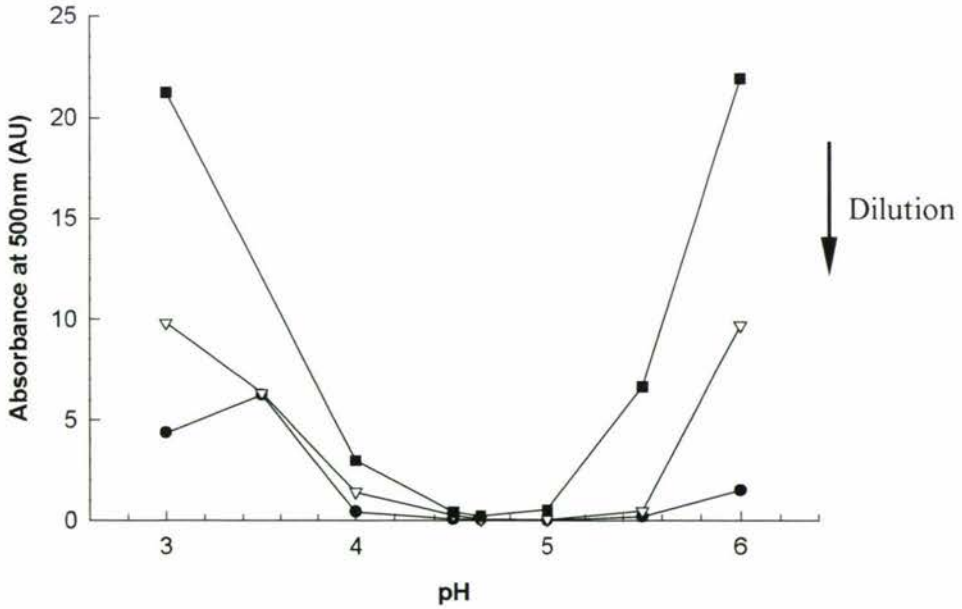


Figure 5.8 Effect of pH on the absorbance at 500nm (A_{500}) of the supernatant. Retentate was diluted to 1:3 (■), 1:5 (▽), and 1:10 (●) and held at 50 °C for 1 hr before centrifugation (1126 *g* for 10 mins). AU = Arbitary units.

Changes in A_{500} were greater at lower dilutions (larger concentrations) over pH range 3.0 to 4.6. This indicates that significantly more MFG are removed at lower dilutions due to their higher original concentration.

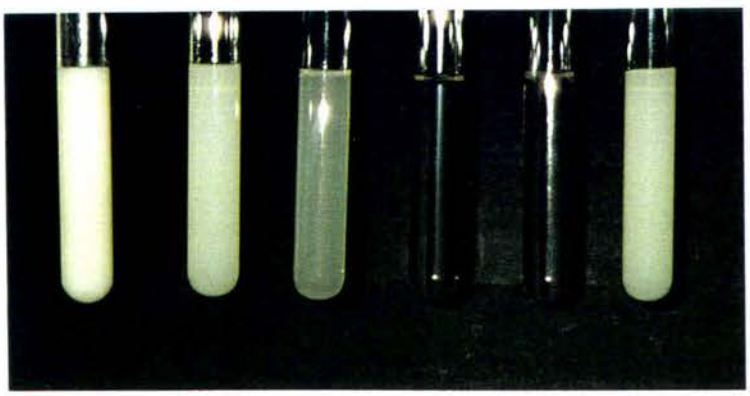


Figure 5.9 Photograph of (left to right) retentate at 1:5 dilution and supernatants at pH 3.0, 4.0, 4.6, 5.0 and 6.0. The solutions were centrifuged at 1126 *g* for 10 min.

Protein content of the supernatant

The protein content, as estimated by $A_{280-320}$, of the supernatant initially decreased as pH increased from 3.0 to 4.5 and then increased as pH from pH 4.6 to 6.0 (Figure 5.10). This trend is similar to that observed in Figure 5.8 with A_{500}. The trend is more evident at lower dilutions (e.g. 1:3 dilution).

Overall, removal of MFG was most effective at pH 4.6, regardless of dilution. At pH values lower or higher than 4.6, the quantity of MFG removed decreased. These results clearly indicate that further investigation should focus on flocculation and separation of MFG at pH 4.6. Although there was some protein loss at dilution 1:5, it was considered to be acceptable.

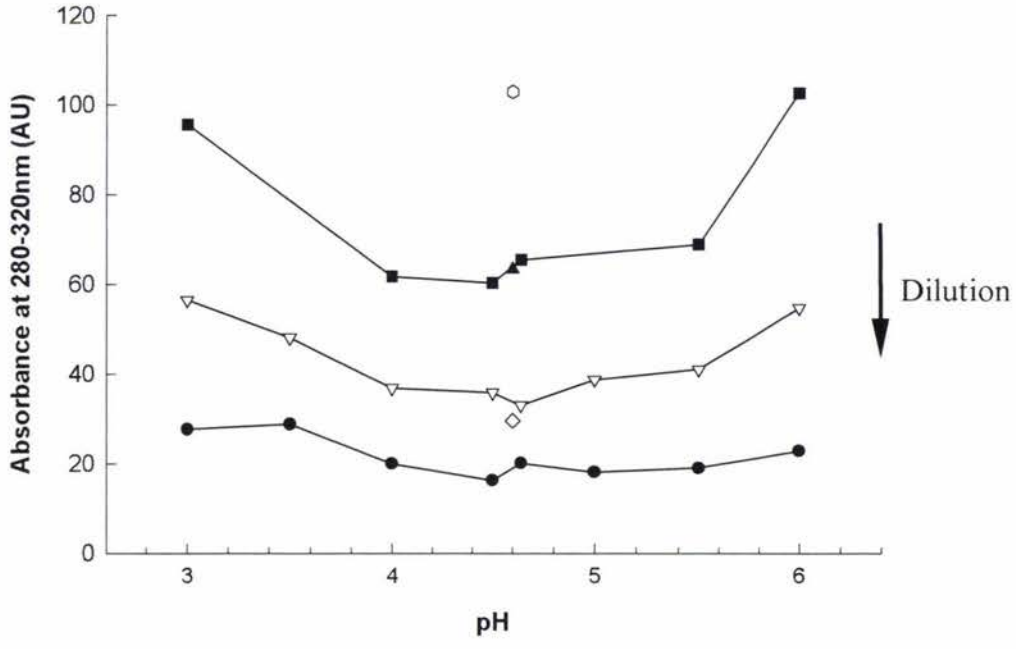


Figure 5.10 Effect of pH on the absorbance at 280-320nm ($A_{280-320}$) of the supernatant. Retentate solutions diluted at 1:3 (■), 1:5 (▽), and 1:10 (●) subsequent to centrifugation (1126 *g* for 10 mins). Retentate dilutions at 1:3 (O), 1:5 (▲), and 1:10 (◇) prior to centrifugation. AU = Arbitary units.

5.3.4 Effect of NaCl addition on MFG separation

Dilutions of acid whey retentate of 1:4, 1:5 and 1:6 were made with distilled water. To each diluted retentate, 1N NaCl was added to give concentrations of 0.00, 0.11, 0.21 and 0.32 g/L of NaCl. The samples were treated and the A_{500} of the supernatant determined (Section 5.3.2). Total protein contents of the supernatants were also determined using the Kjeldahl method (Section 3.2.1).

Turbidity of the supernantant

At all dilutions studied, the A_{500} values increased gradually with increase in NaCl from 0 to 0.35 g/L (Figure 5.11, 5.12). The effect appears to be more pronounced at lower dilutions. It should however be noted that the extent of MFG removal at the different dilutions might prove to be similar at lower concentrations of NaCl. The absorbance of the supernatants, if diluted to the same dilution (i.e. 1:6), may be very similar.

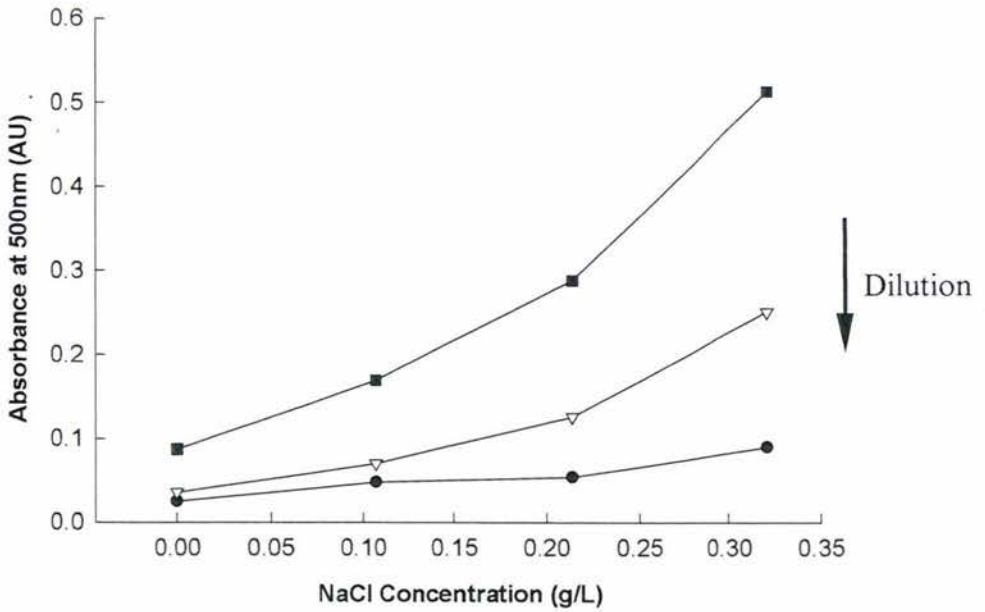


Figure 5.11 Effect of NaCl addition to dilute retentates on absorbance at 500nm (A_{500}) of supernatant. Retentates diluted at 1:4 (■), 1:5 (▽) and 1:6 (●) held at 50 °C for 60 min before centrifugation (1126 *g* for 10 mins). AU = Arbitary units.

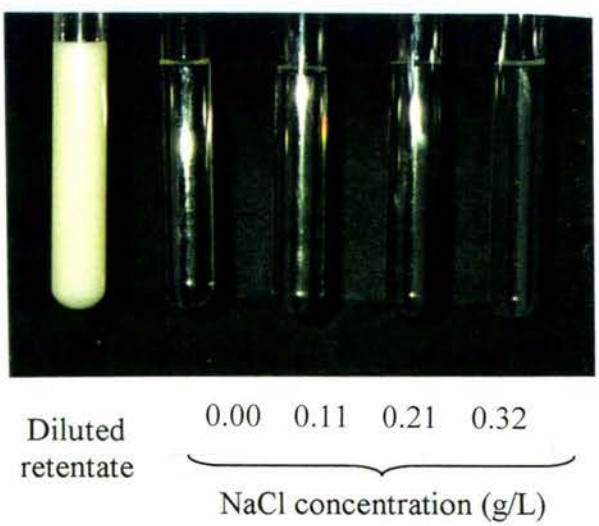


Figure 5.12 Photograph of diluted retentate (1:6 dilution) and supernatants with 0, 0.11, 0.21, 0.32 g/L of NaCl. The solutions were centrifuged at 1126 *g* for 10 min.

Protein content of the supernatant

Concentrations of NaCl below 0.1 g/L had little or no effect on the protein content of the supernatant. As the concentration increased from 0.1 to 0.3 g/L there was an apparent increase in the protein content in the supernatant at retentate dilutions of 1:4 and 1:5 (retentate:water). However, at 1:6 dilution the protein content decreased slightly (Figure 5.13).

The increase in protein content with increasing NaCl concentration suggests increased solubility of protein with increasing ionic strength. de Wit and van Kessel (1996) observed the solubility of whey proteins to increase with increasing ionic strength (by the addition of NaCl). Polis *et al.* (1950) determined that β-lactoglobulin at the isoelectric point was 10 times more soluble in a 0.2 M NaCl solution.

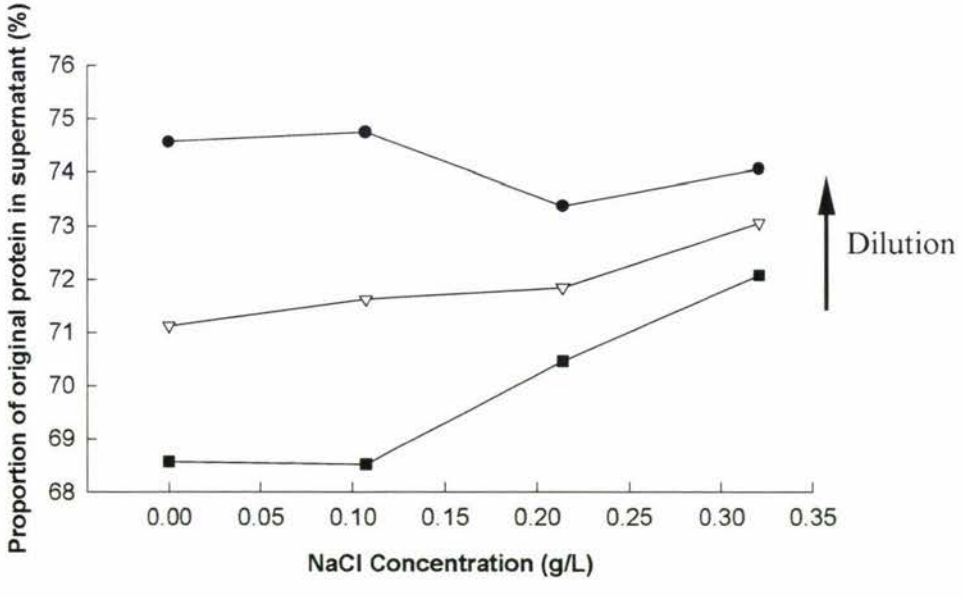


Figure 5.13 Effect of NaCl addition to dilute retentates on absorbance at 500nm (A_{500}) of supernatant. Retentates diluted at 1:4 (■), 1:5 (▽) and 1:6 (●) held at 50 °C for 60 min before centrifugation (1126 *g* for 10 mins).

The general trend of increasing protein content with ionic strength (Figure 5.13) corresponds to an increased A_{500} and therefore a decreased removal of MFG from the samples at constant dilutions (Figure 5.13). Increased retention of MFG in the

supernatant probably resulted from the increased solubility of protein associated with MFG.

Overall, 1:6 dilution of retentate with no added NaCl was the most effective procedure for removing the MFG and retaining the protein.

5.3.5 Effect of temperature and time

The retentate was diluted (1:6) and samples (300ml) were held at 20 or 50 °C, using thermostatically controlled water baths. Aliquots (40 ml) of these samples were taken at 0, 5, 10, 15, 30 and 60 minutes and centrifuged at 1126 *g* for 10 minutes. The absorbance at 500nm (A_{500}) of the resultant supernatants were measured (Section 3.6). The total protein of each supernatant was also determined using the Kjeldahl method (Section 3.2.1).

Turbidity of the supernatant

The data presented in Figure 5.13 shows that neither temperature nor holding time had a significant effect on the A_{500} values of the supernatant (Figure 5.14). The MFG content of the supernatants was therefore not significantly affected by the different holding times at 20 and 50 °C.

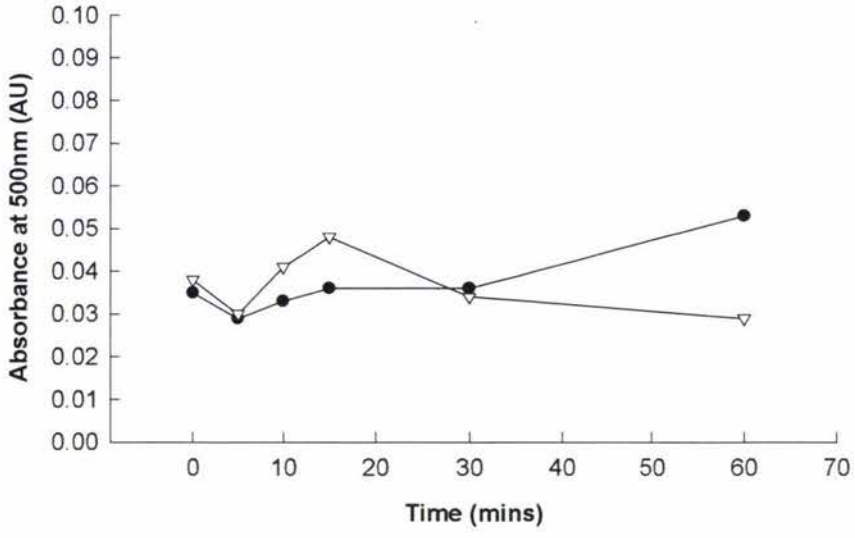


Figure 5.14 Effect of temperature and time on the absorbance at 500nm (A_{500}) of the supernatant. Retentate was diluted (1:6) and held at temperatures 20 °C (●) and 50 °C (▽) for 10 to 60 mins before centrifugation at 1126 *g* for 10 mins. AU = Arbitary units.

Protein content of the supernatant

The temperature and holding time had a slight effect on the proportion of original protein in the supernatant (Figure 5.15). Decreases of ~ 4% at 50°C and ~ 2% at 20 °C were observed after 60 min. An initial holding period up to 15 min did not

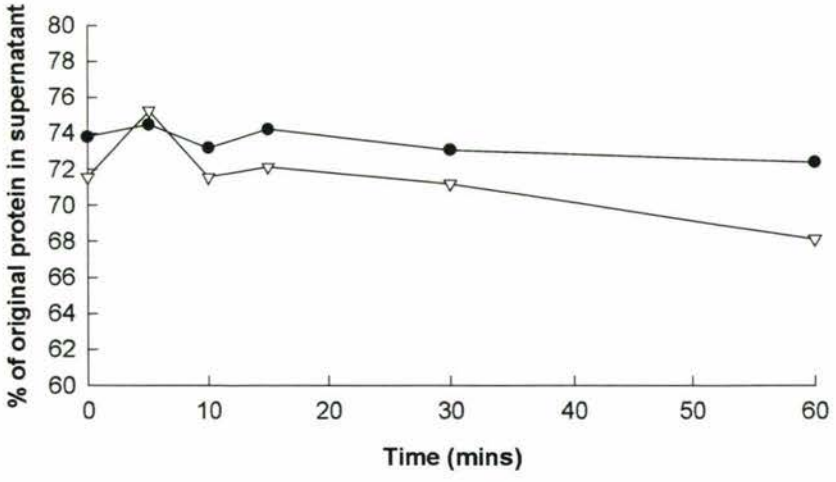


Figure 5.15 Effect of temperature and time on the proportion of the original protein in the supernatant. Retentate was dilute 1:6 and held at temperatures 20 °C (●) and 50 °C (▽) for 10 to 60 min before centrifugation at 1126 *g* for 10 mins.

affect the protein content of the supernatants, but a slight loss of protein from the supernatant occurred after 15 min at both temperatures. It should be noted that the lower holding temperature of 20 °C resulted in a slightly higher protein content over the entire time range

In a commercial process, holding a product for a period of time significantly affects the amount of product processed and the initial cost of setting up the manufacturing facility. It was therefore important to ascertain whether a holding period was required in the process currently under development. The present results showed that holding the diluted retentate for 60 min resulted in a small decrease of protein content of ~ 4% at 50 °C and ~ 2% at 20 °C. The holding period had no significant effect on the removal of MFG. Thus, treatment of diluted retentate does not require a holding time to effectively remove MFG. Therefore, this process can be run continuously in a commercial production.

5.4 Possible mechanisms of aggregation

It seems that under certain conditions (1:6 dilution of retentate with water at pH 4.6), fat globules undergo flocculation, forming large and dense aggregates that tend to sediment. The behaviour of the MFG is significantly influenced by the milk fat globule membrane (MFGM) and associated proteins. Hence it is crucial to determine the nature and role of the MFGM in aggregation and sedimentation of MFG.

Retentate was diluted to 1:6 with either distilled water or SDS/EDTA solution (2.0% SDS: 0.002% EDTA). Both samples (40 ml) were prepared in triplicate and held at 50 °C for 1 hr using thermostatically controlled water baths. The samples were then centrifuged at 1126 *g* for 10 minutes (Figure 5.16). Observations were recorded and the different phases that were present after centrifugation were examined using CLSM (Section 3.2.4).

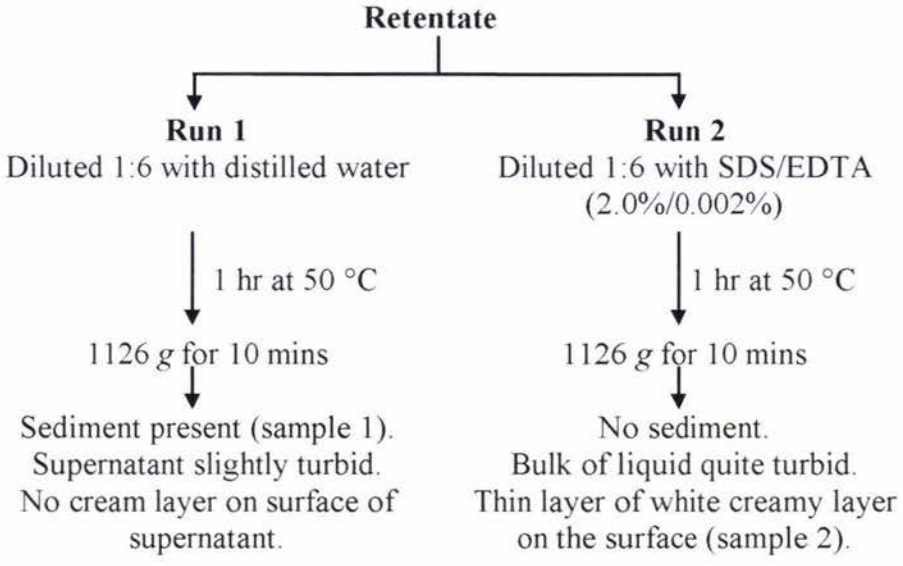


Figure 5.16 Experimental procedure and observed results of the effect of SDS on the mode of separation of MFG from retentate.

Sodium dodecylsulphate (SDS) strongly binds to proteins, mainly through hydrophobic interactions (Grappin and Ribadeau-Dumas, 1992). The resulting action of the SDS on the MFG in the diluted retentate is the removal of proteins from the MFGM. EDTA binds to calcium and causes casein micelles to disintegrate (Andrews, 1992), enabling the suspension of casein proteins removed from the MFG.

The observations suggest that the samples diluted with distilled water and SDS/EDTA solution (2.0%/0.002%) had different mechanisms of MFG separation. Firstly, part of the lipid fraction of the retentate sample, diluted with SDS/EDTA solution, rose to the surface during centrifugation, whereas the majority of the lipid fraction in retentate, diluted with distilled water, sedimented. Furthermore, no sediment was observed in the retentate diluted with SDS/EDTA. The removal of the majority of the protein components of the MFGM by SDS appeared to result in a significant change in the density characteristics of the MFG. This suggests that the protein associated with the MFGM, and that involved in the flocculation of the MFG, are responsible for the high density characteristics of the MFG observed throughout this chapter. With the removal of a significant proportion of the protein from the MFG, the globule would become less dense.

Secondly, the separation of the lipid component from the retentate diluted with water was more effective than the retentate diluted with the SDS/EDTA solution. The presence of significant turbidity in the supernatant, of retentate diluted with the SDS/EDTA solution, suggests that a segment of the MFG was not separated from the bulk of the liquid during centrifugation. The aggregation of the MFG in retentate when diluted with water seems to involve the majority of the MFG. The removal of protein from the MFG results in a less effective separation on dilution. This indicates the mechanism of aggregation, during the dilution of retenate with water, is dependent on the protein present in the MFGM.

Samples of diluted retentate, diluted retentate sediment (sample 1), and the lipid fraction from retentate diluted with SDS/EDTA (sample 2), were examined using the confocal microscope (Figure 5.17). MFG in retentate diluted with distilled water formed what seemed to be loose aggregates (Figure 5.17*a*). Viewed under a light microscope the core of the aggregate seemed to be mostly stable. However, a few individual MFG were observed to break off from the surface of MFG aggregates with the movement of the continuous phase due to pressure differences between the cover slip and the microscope slide (results not shown). After centrifugation (at 1126 *g*) the MFG viewed with CLSM showed aggregates present in the sediment (Figure 5.17*b*). The brightness of the globules decreases as the plane moved through the sample. Examined closely it is possible to see the three-dimensional strands and complexes of the MFG aggregates. These aggregates are more closely packed than those observed in diluted retentate.

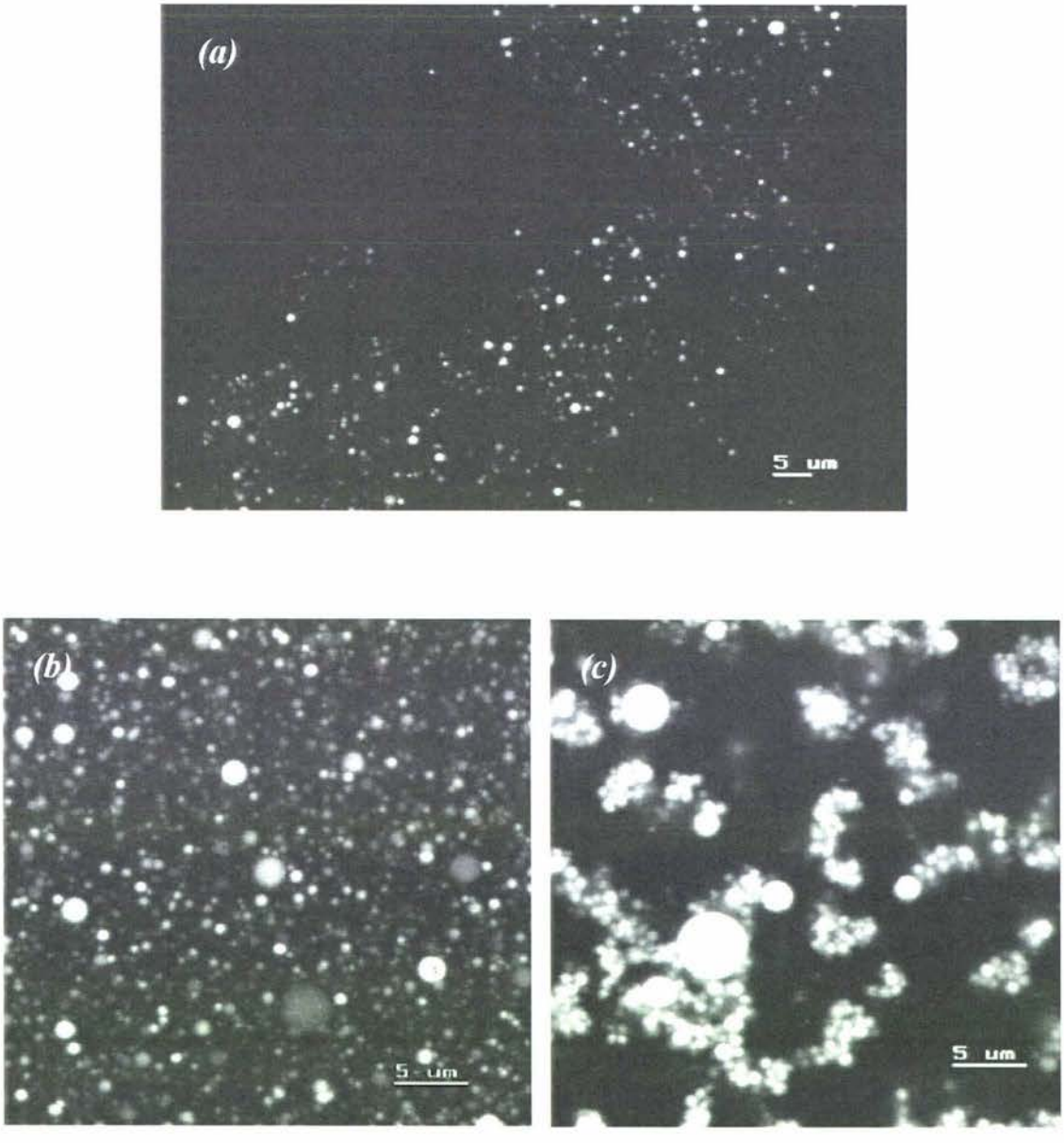


Figure 5.17 Confocal micrograph of retentate diluted to 1:6 with distilled (*a*), sediment of diluted retentate after centrifugation at 1126 *g* for 10 min (*b*), cream of retentate diluted with SDS/EDTA solution (2.0%/0.002%) after centrifugation at 1126 *g* for 10 min (*c*). If not stated otherwise magnification was × 10,000, and the sample was dyed with Nile blue and viewed at 488 nm.

The lipid fraction of the retentate diluted with SDS/EDTA solution (sample 2) consisted of tightly packed aggregates of MFG (Figure 5.17*c*). The MFG present in the lipid fraction appeared to be at the upper end of the size range present in retentate. This observation supports the suggestion that the smaller MFG were not separated from the bulk of the liquid, resulting in turbidity of the bulk of the liquid subsequent to centrifugation. In retentates diluted with SDS/EDTA, the MFG most probably underwent creaming (during centrifugation).

5.5 Discussion

In this section, the effects of dilution, pH, ionic strength, temperature and time on the effectiveness of fat (MFG) removal from the retentate were determined using centrifugation, spectrophotometry and quantitative analysis. The results clearly showed that, like whey, at certain levels of dilution, pH, ionic strength, temperature and time, it is possible to significantly decrease the A_{500} of the supernatant, hence the concentration of MFG (Figure 5.5, 5.8 & 5.11). The effects of different variables were investigated with respect to minimising the A_{500} and maximising the protein retained in the liquid fraction. The levels or variables determined in this study to; (1) remove adequate quantities of MFG, (2) minimise protein loss from the supernatant, and (3) keep the quantity of water added to a minimum, are shown in Table 5.3.

Table 5.3 Variables and appropriate levels to achieve optimal separation of MFG from retentate and retention of protein.

Variable	Level
Dilution	1:6
pH alteration	none
NaCl concentration	0.0 g/L
Holding time	0 min
Holding temperature	20 °C

Flocculation of MFG

It is well known that the lipid phase of the milk system is stabilised, in part, by a protective layer, consisting mainly of proteins, forming a membrane on the fat

globule surface (Mulder and Walstra, 1974). MFG have not been observed to naturally flocculate in milk (van Boekel and Folkerts, 1991) or in whey (Chapter 4) in the absence of sufficient heat or shear force. However, the MFG were observed to readily flocculate in retentate upon dilution with distilled water. The flocculent was separated from the bulk of the solution upon centrifugation (1126 *g* for 10 min). The mechanism of flocculation must be directly associated with the physiochemical changes that occurred during the UF/DF and dilution processes. Changes that occurred in the system were; (1) the majority of the mineral and lactose components were removed during the UF/DF process, and (2) the retentate was diluted with demineralised water. Furthermore the loss of protein into the sediment (Figure 5.13, 5.15) during removal of the MFG suggests possible interaction between the protein and the milk fat globule.

Heat denaturation of protein, occurring in preheating or pasteurisation, significantly affects the protein-protein interactions. Initial heat denaturation of whey proteins occurs at 78 °C (β-lactoglobulin), 62 °C (α-lactalbumin), 72 °C (Immunoglobulin) and 64 °C (Bovine serum albumin) (de Wit and Klarenbeek, 1984). When the protein molecules are surrounded by water, the polar regions interact via hydrogen bonding and electrostatic interactions with water whereas the non-polar residues are strongly 'tucked' and hidden within the tertiary structure of the molecules. During the denaturation process, the hydrophobic residues are exposed resulting in strong interactions between non-polar residues of denatured molecules and formation of hydrophobically-associated aggregates (Howell, 1992).

The milk fat globule membrane (MFGM) in whey has been shown (Houlihan and Goddard, 1991; Patel *et al.*, 1990) to contain a significant amount of protein (~ 65% of membrane). Dalgleish and Banks (1991) report that at temperatures above 70 °C significant amounts of protein, especially β-lactoglobulin, become associated with the MFG. It has been proposed that the presence of whey proteins at the interface of fat globules, after treatment at 60-65 °C, results in either, disulphide bonding with outer proteins (the original MFGM) or, physical absorption of whey proteins directly to the fat-water interface (Corredig and Dalgleish, 1996).

Formation of strong interactions between denatured protein and proteins associated with MFG or MFGM fragments may explain the formation of a high density flocculent which sediments, removing the majority of the MFG from the dilute retentate. The partially or fully denatured proteins contained in the MFGM have the ability to interact with other denatured proteins via hydrophobic interactions, whether part of another MFGM, aggregate of serum proteins, lipoprotein complex or individual denatured serum proteins. In heated milk systems, denatured whey protein associates with casein micelles, or interacts with whey proteins that are adsorbed to the surface of the fat globules (Singh *et al.*, 1996). Havea (1998) suggests that the same interactions take place in WPC manufacture, resulting in formation of dense fat-protein complexes which sediment on centrifugation (45,000 *g* for 2 hr at 20°C). It should be noted however that the aggregates in the later study were only separated after the application of a significant amount of force for an extended period of time (i.e. 45,000 *g* for 2 hr).

Flocculation of the MFG and denatured protein into very large aggregates occurred upon dilution of the retentate. It is likely that the destabilisation of denaturated whey proteins is responsible for the aggregation. The salt content in milk/whey is widely known to have a significant effect on the behaviour of the whey proteins. It is proposed that the destablisation of the protein resulted from the reduction in concentration of minerals occurring during the UF/DF process. Upon dilution of the retentate, the ionic strength within the system greatly decreases. In a previous study the removal of salts, at pH values in the isoelectric region, from WPC resulted in a significant decrease in the stability of the serum proteins (Varunsatian *et al.*, 1983). The denatured proteins hydrophobically interact to form insoluble marco-aggregates containing aggregates of denatured protein, MFG and lipoprotein complexes. Due to the high protein content of these aggregates their density is significantly greater than the bulk of the diluted retentate. These aggregates were effectively separated using a centrifugal force of 1126 *g* for 10 min (Chapter 5).

The current study showed that the most effective removal of MFG was achieved at pH 4.6. It should be noted that at pH values around that isoelectric point, whey

proteins are the least soluble (Hambling *et al.*, 1992). Significant variation in the pH from pH 4.6 resulted in a marked decrease in MFG removal (Section 5.3.3). The formation of the insoluble macro-aggregates is therefore dependent on pH being close to the isoelectric in conjunction with low ionic strength within the system.

The ionic strength of the diluted retentate system influences that retention of MFG during separation. Depending on the protein and the environmental conditions, denaturation may be confined to a segment of the protein (Brendts, 1967). Increased ionic strength may result in an increased solubility of the segments of the proteins associated with the MFGM. The stability of the MFG may therefore be increased resulting in increased quantities of MFG stabilised in suspension. Thus, the quantity of MFG in the supernatant increases resulting in increased turbidity. Although increased protein retention in the supernatant will improve the yield of this process, the resulting high A_{500} and consequent increase of MFG content is not desirable.

Protein loss

Separation of MFG from diluted retentate (Figure 5.13, 5.15) showed that a significant quantity of protein was lost from the samples. The percentage protein recovered in the supernatant, ranged from ~ 75 to 69% for dilutions from 1:6 to 1:4, representing a loss of between 25 and 31% of the original protein content of retentate. The original protein content of the membrane fraction was determined by Houlihan and Goddard (1991) to be less than 3% of the total protein in WPC. There is a significant difference between the protein lost during dilution and the protein determined to be originally associated with the MFG (between 22 and 28%). It is proposed that these proteins were in the form of protein aggregates, lipoprotein complexes, denatured serum protein, or individual proteins, destabilised by the low ionic strength, which then interacted with proteins in the MFGM. The inclusion of this significant quantity of protein in the formation of the flocculent, formed at low ionic strength, results in relatively dense aggregates. These aggregates subsequently readily sediment during centrifugation (1126 *g* for 10 min) and also sediment without centrifugation after a short period of time.

Conclusions

In conclusion, MFG are removed from retentate upon dilution, at pH values close to 4.6 (upon centrifugation at 1126 *g* for 10 min). The majority of the MFG were removed at dilutions greater than 1:3. At increasing dilutions, small increases in removal of MFG occurred (up to dilution 1:10), however, significantly higher quantities of protein were recovered. The addition of NaCl generally resulted in a decrease in the removal of MFG and an increase in protein retention. The effect of holding temperature and holding time were determined to only have a small effect of the removal of MFG and retention of protein.

Association of partially or fully denatured proteins contained in the MFGM may occur between other denatured proteins, whether part of another MFGM, aggregate of serum proteins, lipoprotein complex or individual denatured serum proteins. It is proposed that the low ionic strength of dilute retentate at pH 4.6 results in hydrophobic interactions between denatured proteins to form insoluble marco-aggregates containing aggregates of denatured protein, MFG and lipoprotein complexes. The resulting marco-aggregate may be easily separated from the majority of the protein contained in the supernatant, due to their relatively high density.

CHAPTER 6

PILOT-SCALE PLANT PRODUCTION OF A LOW FAT WHEY PROTEIN CONCENTRATE POWDER

6.1 Introduction

The manufacture of WPC involves a number of steps including thermal processing, ultrafiltration, evaporation and spray drying (Sherwin and Foegeding, 1997). The effect of dilution of retentate on the turbidity and protein content of whey was shown in Chapter 5. However, the effect of commercial processing on the final composition and functionality of the low fat whey protein concentrate (WPC) needs to be determined. Production of the low fat WPC using a pilot-scale plant should result in a product very similar to full-scale manufacture, allowing the composition and functionality to be characterised.

The aim of this chapter was to manufacture on a pilot-scale plant a low fat whey protein concentrate using the procedure developed in Chapter 5. The success of the production of this product will be determined by comparing its composition, in particular the fat content, to commercial WPC and WPI.

6.2 Low fat WPC production process

The process variables and the process flow for the production of low fat WPC on a pilot-scale plant should be defined.

6.2.1 Process variables

The process variables and levels were determined in laboratory scale experiments (Chapter 5). In scaling-up the process for pilot plant trials, alterations were made to the levels of some variables to improve the perceived cost effectiveness of the

process. The most significant alterations were made to (1) the dilution of the retentate and (2) the temperature of the diluted retentate.

Dilution of retentate was shown to significantly affect the removal of the MFG and the retention of protein (Section 5.3.2). Results showed that retentate at 1:6 dilution, when centrifuged, gave good removal of MFG and retention of protein in the supernatant fraction (Section 5.3.4). However, the removal of water from retentate using ultrafiltration is costly. Work conducted in Section 5.3.4 (Figure 5.13) showed that ~ 3% protein was lost when the retentate dilution was decreased from 1:6 to 1:5 (with no added NaCl) and the resulting change in the MFG removal was only small (Figure 5.11). Thus, for the process to be more cost effective, 1:5 dilution of retentate was investigated in the pilot plant trial.

The temperature of the diluted retentate was shown to affect the retention of protein in the supernatant. Results showed that diluted retentate held at 20 °C prior to centrifugation, as compared to 50 °C, had slightly higher quantity of protein retained in the supernatant (Section 5.3.5). However, it is well known that the alteration of product temperature can be costly. Currently UF/DF processing is carried out at 50 °C. Further UF processing (at 50 °C) is required after the diluted retentate has been centrifuged to remove the water added during dilution. The most cost-effective way of achieving the addition and subsequent removal of water is by keeping a constant temperature of 50 °C. This may be achieved by diluting the retentate with 50 °C demineralised water. It was felt that the costs incurred in cooling of the retentate subsequent to primary UF, and heating of the supernatant prior to secondary UF would far outweigh the cost of the loss of protein. Demineralised water at 50 °C is currently supplied to the diafiltration loops and added during the filtration process.

The pH of the diluted retentate is 4.6 and this will remain unchanged because the best separation of MFG occurs at pH. In addition, the diluted retentate will be held for a short time as possible, to prevent further loss of protein. The current pilot plant set up

does not allow inline dilution of retentate, therefore the period between dilution and clarification can only be kept to a minimum.

6.2.2 Process flow outline

The simplified process flow diagram of the proposed process is shown in Figure 6.1. Samples of retentate obtained from the main UF process were designated as primary retentate (1° retentate) while those obtained subsequent to UF using the pilot plant were designated as secondary retentate (2° retentate).

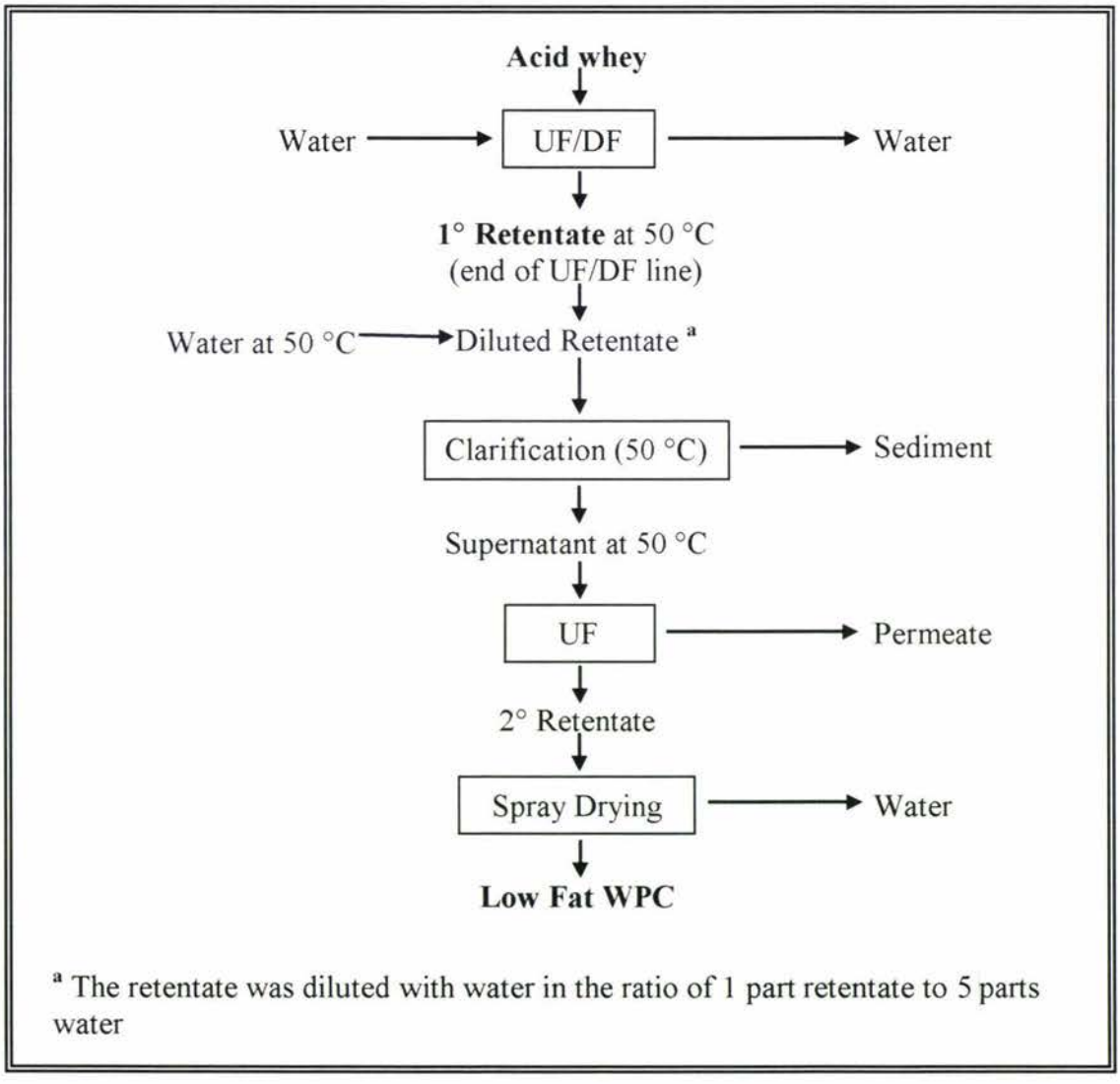


Figure 6.1 Simplified process flow diagram for the pilot plant production of low fat WPC.

6.3 Production of low fat WPC

Production of low fat WPC followed the process flow shown in Figure 6.1. Retentate at 50 °C was obtained at the end of the ultrafiltration line (1° retentate) from the Anchor Products site, Edgecumbe. 200L of retentate at 50 °C was mixed with 800L of 50 °C demineralised water in a reactor vessel with a capacity of 1000L. The vessel's agitator remained at 18 Hz throughout the separation. Diluted retentate was pumped at ~ 800 L/hr through a nozzle bowl clarifier (model KNA3, Westfalia Separator, Germany) running at 1415 rpm. The clarifier was run with two 0.5 mm diameter nozzles, and two blanks nozzles. The supernatant from the clarifier was pumped into a holding tank before the secondary ultrafiltration step. Two separate runs of 1000L each were conducted to get the required quantity of supernatant to run the UF plant. The supernatant was then ultrafiltrated (at 47 °C) though one of four ICI polyethersulfone membranes (supplied by Niro Ltd., New Zealand). The resulting 2° retentate was reheated to 40 °C in a scraped surface heat exchanger (model CH-4310, Fryma, Rheinfelden, Switzerland) and dried in an atomizing spray dryer (30 kg water/hr) (Niro, Copenhagen, Denmark) run in multi-stage drying (MSD) mode.

Samples of 1° retentate, diluted retentate, supernatant and sediment ex-clarifier, 2° retentate and the final low fat WPC were taken during production. The composition of each of these samples was determined (Table 6.1, 6.2 and 6.3). Total solids, protein, fat, minerals (ash), lactose and calcium were determined as outlined in Section 3.2.1. The absorbance at 500nm of triplicate supernatant samples were determined as described in Section 3.2.5.

6.3.1 Composition of dilute retentate and its fractions after clarification

Dilute retentate was clarified in two runs (1000L each), using a nozzle bowl clarifier, to produce enough supernatant to run the UF pilot plant. Clarification of the dilute retentate resulted in two fractions, the supernatant (lipid-depleted fraction), and sediment (lipid-rich fraction). The composition of the main components of the original diluted retentate, the supernatant, and the sediment was determined. The

supernatant was not tested for fat content due to the lack of accuracy of the test method at fat levels lower than 0.05%. The composition of the diluted retentate, supernatant and sediment is presented in two forms; (1) percentage of the total sample weight (Table 6.1), and (2) percentage of the solid content (Table 6.2).

The total solids content of 1° retentate was ~ 22%. The diluted 1° retentate had a total solids content of ~ 5.8%, indicating between a 1:4 and 1:5 dilution. The total solids content of the resulting supernatant decreased to ~ 4.6%, whereas the sediment increased to ~ 10.7%. Similarly the protein concentration decreased in the supernatant (~ 4.0%) and increased in the sediment (~ 8.3%). The mineral concentration of the supernatant was similar to the diluted retentate (~ 0.06%) while a slight increase was observed in the sediment (0.10%). The quantity of lactose sedimented was slightly higher than in the diluted retentate. The fat concentration of the sediment was more than five-times higher than the diluted retentate (~ 1.7% and ~ 0.3% respectively). Although the fat concentration of the supernatant was not determined, the marked increase in concentration of the sediment indicated a significant decrease in concentration of fat in the supernatant.

Table 6.1 Composition of diluted 1° retentate and the supernatant and sediment produced by clarification.

Component	**% Composition (g/100g sample)**		
	Diluted 1° retentate [a]	**Supernatant** [a]	**Sediment** [a]
Protein	4.87 ± 0.03	4.04 ± 0.10	8.32 ± 0.37
Fat	0.31 ± 0.00	N/D	1.67 ± 0.19
Minerals (ash)	0.06 ± 0.00	0.06 ± 0.01	0.10 ± 0.02
Lactose [b]	0.54	N/D	0.63
Total solids	5.78 ± 0.03	4.59 ± 0.11	10.72 ± 0.50

[a] N = 2. The errors indicate the difference between the two values
[b] The lactose content was determined by the difference of the sum of the components and the total solids.
N/D – Not determined

Table 6.2 Composition of diluted 1° retentate and the supernatant and sediment produced by clarification of diluted retentate.

Component	% Composition of total solids (g/100g TS)		
	Diluted 1° retentate [a]	**Supernatant [a]**	**Sediment [a]**
Protein	84.17 ± 0.01	88.01 ± 0.06	77.58 ± 0.22
Fat	5.37 ± 0.03	N/D	15.53 ± 1.05
Minerals (ash)	1.04 ± 0.01	1.31 ± 0.19	0.65 ± 0.33
Lactose [b]	9.42	N/D	6.24
Total solids	100	100	100

[a] N=2. The errors indicate the difference between the two values
[b] The lactose content was determined by the difference of the sum of the components and the total solids.
N/D – Not determined

The difference in total solids content of the supernatant and the sediment makes it difficult to compare the composition of the two fractions. The compositions of diluted 1° retentate, supernatant and sediment are presented as percentages of the total solids in Table 6.2. This enables a direct comparison of composition between components. Comparatively, the supernatant has a higher proportion of protein (~ 88%) than either the diluted retentate (~ 84%) or the sediment (~ 78%). During clarification, three times the quantity of fat in the diluted retentate was separated into the sediment. The higher proportion of protein separated into the supernatant during clarification, together with the increased proportion of fat in the sediment, again suggests significant decreases in the proportion of fat in the supernatant.

The supernatant was only slightly turbid, much less turbid than the diluted retentate while the sediment was observed to be opaque with a creamy consistency. These observations support the results in Table 6.2 and suggests that the concentration of fat decreased in the supernatant.

The A_{500} of the supernatant produced during the clarification of diluted retentate ranged between 1.06 and 1.17. These A_{500} values were significantly higher than those measurements at dilutions, 1:5 (0.036) and 1:4 (0.087), observed in Chapter 5

(Figure 5.7). Thus, the separation of MFG from the diluted retentate was not as effective in the pilot plant trials as in earlier laboratory scale experiments. The floc formed in diluted retentate was observed to be quite fragile (Section 5.3.5). The aggregated MFG may have been affected by the high shear present at the surface of the clarifier disks which may have resulted in breakdown of MFG aggregates and subsequently the release of some MFG back into the continuous phase.

6.3.2 Composition of the 1° and 2° retentates

Supernatant collected from the clarification process was ultrafiltrated to remove the low molecular weight components, such as water, lactose and salts. The compositions of 1° and 2° retentate are shown in Table 6.3. Comparison between the 1° and 2° retentate was conducted using dry basis composition (g/100g TS), as the amount of water present depends on the processing conditions during UF, which may be varied.

During clarification and UF processes significant decreases in the fat (~ 5.4 to 0.8 g/100g TS) and mineral content (~ 1 to 0.3 g/100g TS) were observed (Table 6.3). The majority of the fat was most likely removed in the clarification process, and the lactose and minerals were most probably removed during subsequent UF. The removal of non-protein components such as the fat, lactose and mineral components resulted in increased proportion of protein (from ~ 84 to 95% g/100g TS), based on the total solids content in the 2° retentate.

Table 6.3 Composition of 1° Retentate (before dilution) and 2° Retentate (ex-clarifier and ultrafiltration)

Component	Composition			
	1° Retentate		2° Retentate	
	g/100g sample	g/100g TS	g/100g sample	g/100g TS
Total solids	25.92	-	21.64	-
Protein	21.82	84.18	20.52	94.82
Fat	1.40	5.40	0.17	0.79
Minerals (ash)	0.28	1.08	0.07	0.32

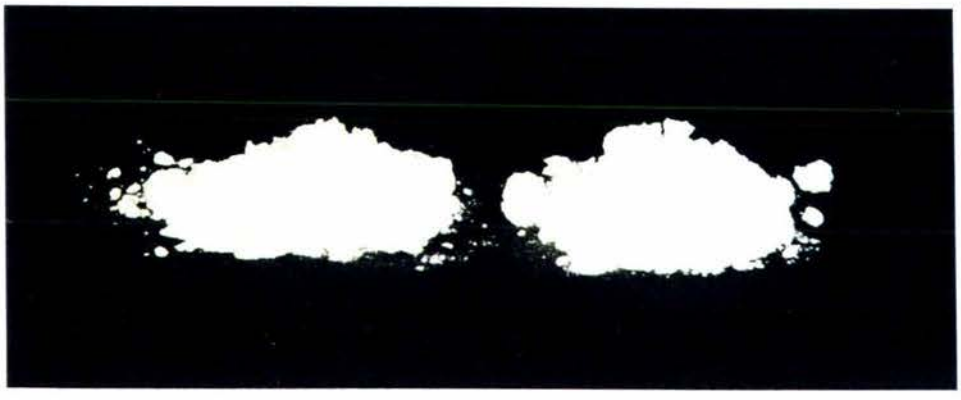
6.3.3 Composition of low fat WPC

The compositions of low fat WPC, a commercial acid WPC and WPI are shown in Table 6.4. The low fat WPC powder contained ~ 1% fat as compared to ~ 5.5% fat in commercial WPC (Table 6.4) and was noticeably whiter in colour (Figure 6.2). The low fat WPC also contained an increased proportion of protein (~ 12%), and decreased proportion of lactose (~ 1%), minerals (~ 2%) and calcium (~ 0.1%), compared to normal WPC. The high proportion of protein in the low fat WPC reflected the decreased content of other components.

Table 6.4 Composition of low fat WPC, commercial acid WPC and WPI.

Component.	% Composition of dry weight (g/100g TS)		
	Commercial WPC [a]	Low fat WPC [c]	WPI [bc]
Protein	84.5	96.4 ± 0.5	94.5 ± 1.80
Fat (Total lipids)	5.5	1.13 ± 0.01	0.59 ± 0.14
Lactose	< 4.22	1.14 ± 0.05	0.46 ± 0.02
Minerals (ash)	~ 2.60	0.59 ± 0.05	1.89 ± 0.33
- Calcium	0.17	0.07 ± 0.01	0.23 ± 0.03
Total [d]	**~ 96.82**	**99.33**	**97.67**

[a] From Havea (1998)
[b] Adapted from Morr & Foegeding (1990). WPI products produced by ion exchange adsorption.
[c] N = 2
[d] Estimation of the total quantities, the average values were summed (except calcium)



a *b*

Figure 6.2 Photographs of normal acid WPC (*a*) and low fat WPC (*b*) produced on industrial and pilot-scale plant, respectively.

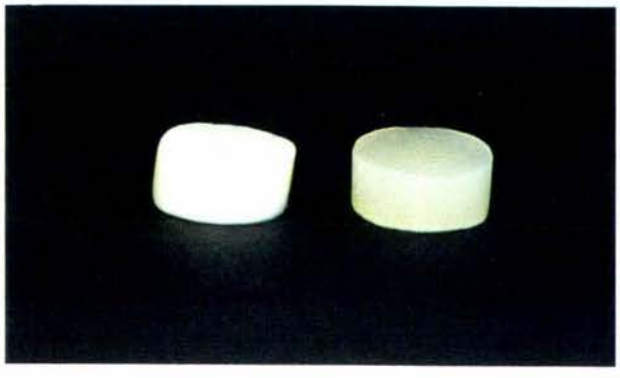
The compositions of low fat WPC and WPI are very similar (Table 6.4). Composition of WPI was obtained from products produced using ion absorption technology, similar to that used in New Zealand. The low fat WPC contains ~ 96% protein on a dry basis, which is comparable to WPI products that contain between ~ 94 and 95% (Morr & Foegeding, 1990). Concentrations of lactose and fat were respectively ~ 0.7% and ~ 0.5% higher in the low fat WPC than in WPI. However, the mineral concentration was lower in low fat WPC compared to average concentration in WPI.

6.3.4 Functionality of low fat WPC

The ability of whey protein concentrates (WPC) to form heat-induced gels that immobilise large amounts of water in food systems is one of their most important functional properties (Hermansson and Akesson, 1975; Morr and Ha, 1993). Heat induced gelation is an important criterion for selecting a commercial WPC for bakery, confectionery, structured meat and seafood applications (Mei *et al.*, 1996). Thus, the aspects of the functionality that were investigated were the gel strength, with respect to penetration, and the syneresis of the gel. Commercial WPC and low fat WPC solutions, at a concentration of 150 g/kg and pH 6.8 (Section 3.2.6), were used to prepare gel cylinders for measurement of gel strength (Section 3.7.2) and gel syneresis (Section 3.7.3).

Gel strength

Figure 6.2 shows a photograph of cylindrical WPC gel samples formed by heating commercial WPC and low fat WPC at 75 °C (for 60 min). The WPC gel was less transparent, harder and more brittle than the low fat WPC gel, whereas the low fat WPC gel was more elastic, more "gelatinous" in nature (Figure 6.2).



a ***b***

Figure 6.3 Photographs of commercial acid WPC (*a*) and low fat WPC (*b*) gel cylinders formed by heating 150 g/kg WPC solutions (pH 6.8) at 75 °C for 60 min.

Segments of gel cylinders were used to find the first yield point using a Fudoh rheometer. When the samples were penetrated and the first yield point occurred, the breaking strength of the gel was determined from the force-compression curve produced (Section 3.7.2). The low fat WPC gel had higher gel strength (1688 g/cm^2) than the control WPC gel, which broke under a smaller force (1415 g/cm^2) (Table 6.4). The low fat WPC gel appeared to be more elastic while the control WPC gel was more brittle. These results suggest that the removal of the fat from the WPC will enable a more extensive protein-protein matrix resulting in a stronger gel.

Table 6.4 Properties of WPC gels formed with control WPC or low fat WPC [a]

Functional aspects	Control WPC [b]	Low fat WPC [c]
Gel strength (g/cm^2)	1415	1688
Syneresis (%)	2.61	1.53

[a] WPC solutions (150 g/kg, pH 6.8) were heated in 35 mm diameter plastic tubes at 75 °C for 60 min, kept at 4 °C overnight, cut into 15 mm lengths and then used for the gel strength and syneresis tests.
[b] N = 9
[c] N = 3

The greater gel strength observed in low fat WPC compared to the control WPC is consistent with other studies (Sternberg *et al.*, 1976; Karleskind *et al.*, 1995*b*) which reported a decreased gel strength with increased fat content. Sternberg *et al.* (1976) reported that residual lipids inhibit the gel forming properties of WPC. Morr (1992) postulated that non-polar lipids may inhibit the ability of protein molecules to form a continuous gel structure by competing for hydrophobic sites on unfolding protein molecules. Work conducted by Karleskind *et al.* (1995*b*) supported this theory, suggesting that the presence of lipids interferes in the formation of the gel matrix. Therefore, the removal of non-polar lipids increases the ability of the protein molecules to form continuous gel structures resulting in the formation of stronger gels.

A distinction should be made between non-polar and polar lipids. Unlike non-polar lipids, polar lipids might enhance the gelling properties. Mei *et al.* (1996) suggested that polar lipids such as phospholipids promote gel cohesiveness by functioning as emulsifiers with in the continuous phase of the gel structure. The quantity of milk fat globule membrane (MFGM) was shown to affect the gel strength of WPC (Joseph and Mangino, 1988*b*). In this study, addition of 2 to 6 g of MFGM added to a 10 g/kg solution of β-lactoglobulin, in simulated milk ultrafiltrate, pH 6.7, (heated at 90 °C for 15 min) was shown to increase the strength of the gel produced. It is well known that phospholipids are present at higher concentrations in the MFGM at the core of the fat globule.

Gel syneresis

The syneresis of gels (150 g/kg) made from control WPC and low fat WPC were measured as described in Section 3.7.3. The syneresis of low fat WPC gels (1.53%) was lower than that of control WPC gels (2.61%) (Table 6.4) which suggests that the reduced fat WPC has an increased water binding capability compared to commercial acid WPC. The increased water binding capability of the low fat WPC gel suggests that a gel structure with narrower or more uniform pores was produced. The frequency of formation of interaction bonds between protein molecules must be sufficient to provide a three-dimensional protein structure capable of physically retaining the solvent. Less interference in the formation of the gel matrix by lipid material, resulting in improved protein-protein interactions, was probably responsible for the improvement in this aspect of the functionality.

Only two aspects of functionality of whey proteins were investigated in this study; gel strength and gel syneresis (at WPC concentrations of 150 g/kg). Some other important functional properties of whey proteins are viscosity, emulsification, foaming, solubility and adsorption at air-water and oil-water interfaces. It would be interesting to investigate other functional properties of the low fat WPC, commercial acid WPC and WPI. Also, further investigation into the functional performance of low fat WPC should be conducted to determine opportunities for commercial use.

6.4 Discussion

In this chapter, a pilot plant was used to manufacture low fat WPC using the process shown in Figure 6.1. The low fat WPC contained ~ 1% fat as compared to 5.5% fat in commercial acid WPC (on a dry basis). The low fat WPC also contained lower levels of lactose, total minerals and calcium, and a higher level of protein, compared to normal WPC. The low fat WPC had a similar concentration of protein to WPI, and only slightly higher fat concentration. The gel strength and water holding capacity of low fat WPC were higher than those of commercial WPC. The gel produced with low fat WPC was also observed to be more elastic and transparent.

Removal of fat during clarification

Decreased removal of MFG during separation was observed in the pilot plant trials compared to the laboratory scale experiments (Section 6.3.1). The decreased effectiveness of the separation of MFG suggests an increased lipid content of the supernatant. MFG aggregates formed during the dilution of retentate are not robust and may be disrupted by high shear force present at the surface of the clarifier disks. A more effective separation of the aggregated MFG, and therefore the lipids, might be achieved using a more 'gentle' separation, such as the use of a decanter or hydro-cyclone. Increased clarity and decreased lipid content of the low fat WPC may lead to the use in nutritional, sports formulations, and other WPI applications.

Removal of mineral and lactose components during ultrafiltration

The low fat WPC showed a significant decrease in the content of minerals and lactose compared to acid WPC produced commercially (Table 6.4). The difference in mineral content was also apparent between 1° and 2° retentate (Table 6.3). As removal of minerals and lactose appeared to be minor during clarification (Table 6.1) the majority of the removal of these components occurred during the UF process subsequent to clarification. The difference between the mineral contents of commercial WPC and low fat WPC products could be largely attributed to the secondary ultrafiltration step performed in the production low fat WPC.

Functionality of low fat WPC

As indicated by work conducted in this present study (Section 6.3.4), reducing the lipid content in WPC increases the strength and water holding capacity of gels formed. The production of a low fat WPC with > 94% protein and a fat content of ~ 1% presents a highly competitive alternative to WPI, manufactured using ion exchange or microfiltration technology, whether on a compositional or functional basis.

Mineral composition is well known to have a significant effect on the functional properties of whey protein products. Many studies have addressed the effects of mineral concentration on WPC and WPI functionality (Schmidt *et al.*, 1978;

Mulvihill & Kinsella, 1988; Kuhn & Foegeding, 1991*a, b*; Tang *et al.*, 1993; Kinekawa *et al.*, 1998). In the current study, mineral and calcium concentrations in low fat WPC were determined to be significantly lower than those found in acid WPC and WPI (Table 6.4). Previous work has shown that the maximum hardness of WPI gels occurred with the addition of $CaCl_2$ or NaCl (Schmidt *et al.*, 1978; Mulvihill & Kinsella, 1988; Kuhn & Foegeding, 1991*a*). Further improvements in the functionality of the low fat WPC may be achieved by the increase of mineral concentration, especially that of calcium.

The proximate compositions of the low fat WPC and WPI were determined to be fairly similar (Table 6.4). However, the process developed in the current work to produce a low fat WPC, differs greatly from the current WPI manufacturing process. Small differences in the composition of components have the potential to significantly alter the behaviour of the low fat WPC in different environments. WPI products are generally manufactured using the stirred-bed ion exchange adsorption process. The pH of the whey is adjusted to provide the proper charge on the protein molecule, it is passed through the ion exchange to absorb the protein molecule, and deproteinised whey is eluted from the reactor. The pH is readjusted to desorb the proteins, and the desorbed proteins are eluted from the ion exchange, concentrated by UF, and spray dried (Morr and Ha, 1993). It is probable, that some of the components lost from whey in the ion exchange are retained in the ultrafiltration process. Also, some of the more subtle differences in composition of the low fat WPC and WPI may result in differences in functionality. Thus, the functionality of the low fat WPC should not be assumed to be the same as WPI, and a more detailed evaluation of functional properties should take place in subsequent work.

Utilisation of lipid fraction separated upon clarification

The lipids found in whey protein concentrates do not have the same composition as the bulk lipid of milk, but are greatly enriched in phospholipids and milk fat globule membrane material (Houlihan and Hirst, 1987). Joseph and Mangino (1988*a, b*) demonstrated that there is milk fat globule membrane material associated with the residual phospholipid fraction of whey protein concentrates. The majority of this material

was separated into the sediment fraction in the current process. Therefore, the sediment is likely to be a rich source of phospholipid material. Further, the removal of lipids from these MFGM and associated lipoproteins was reported to increase their foam-suppressing properties (Joseph and Mangino, 1988*a*). Currently, phospholipids products are manufactured at the Edgecumbe site and the sediment fraction may be a good source for the production of these products. Further work should investigate the composition and state of the phospholipids present in the sediment fraction, and explore its functional properties.

CHAPTER 7

OVERALL CONCLUSIONS AND RECOMMENDATIONS

7.1 Study approach

The purpose of this research was to develop a process to remove the residual lipids from acid whey in order to produce a low-fat whey protein concentrate (WPC). The approach taken in this study involved (a) the determination of physical and microstructural characteristics of residual milk fat globules (MFG) in whey or UF retentate, and (b) determination of the effect of parameters such as, pH, temperature, holding time, dilution, presence of calcium and ionic strength, on flocculation of the MFG. This approach enabled us to improve our understanding of the mechanism by which flocculation of MFG occurs in the development of the process to produce low fat WPC.

7.2 Experimental Methodology

An investigation was conducted into the flocculation and separation of MFG from whey (Chapter 4). The first part of this investigation provided information on the physical and microstructural characteristics of residual milk fat globules (MFG) in whey. This work involved the fractionation of whey, compositional analysis, and examination of MFG using the Malvern MasterSizer and confocal scanning laser microscopy. The second part examined the effects of various parameters on the flocculation of MFG in the acid whey UF retentate, using spectrophotometery and confocal scanning laser microscopy.

A further investigation was undertaken to remove the MFG from retentate, an intermediary product in the production of WPC (Chapter 5). The first part of this investigation involved the determination of the physical and microstructural properties of the residual lipid in retentate. The second part involved investigation of

the effects of dilution, pH, ionic strength, and holding temperature and time on the flocculation of MFG in retentate.

A pilot plant trial was carried out to produce low fat WPC using the process parameter and levels determined in the investigations outlined above (Chapter 6). The composition of several intermediate products and the final product, and some aspects of the functional behaviour of the low fat WPC produced were determined.

7.3 Microstructural properties of fat in acid whey and its retentate

The microstructural properties of whey and retentate were investigated in Chapters 4 and 5, respectively. Both whey and retentate were separated into three fractions (LDLF, MDLF and HDLF) at high centrifugal force. The lipid content and the physical and microstructural characteristics of the MFG were determined for the whey and retentate fractions.

Acid whey contained ~ 0.56% lipids on a dry basis, and had a total solids content of ~ 6%. The size of the MFG in whey varied from 0.1 and 10 μm, the majority (~ 90%) were < 1 μm in diameter. The HDLF contained the highest concentration of lipids of the fractions, followed by the MDLF and then the LDLF. The majority of the smaller MFG in whey were separated into the MDLF and HDLF, while the denser MFG were sedimented into the HDLF. It is likely that the density characteristics of the MFG were altered by association of denatured protein with either the surface of the MFG or lipoprotein complexes (MFGM fragments). The larger particles that sedimented were probably not only MFG, but also casein fines and lipids associated with protein.

Retentate contained ~ 1.4% lipids and ~ 26% total solids. The highest concentration of lipids was found in the RLDLF (the smallest fraction). However, the overall greatest quantity of lipid was contained in the RMDLF, suggesting that the density of the MFG is similar to the bulk of the retentate. The size of the MFG in the retentate as determined by the Malvern MasterSizer (MM) ranged between 0.1 and 20 μm, generally larger than the MFG in the acid whey. However, no MFG greater than 5 μm diameter were observed by confocal microscopy. It suggested that some of the

larger particles measured by the Malvern MasterSizer in the RMDLF and RLDLF, might be aggregates of MFG. Aggregation of the MFG may have occurred during measurement, upon dilution of the retentate in the MasterSizer.

7.4 Factors affecting the flocculation of MFG in the acid whey

Results indicated that flocculation of MFG from the whey occurred at temperatures between 40 and 50 °C and at pH values from 5.8 to 7.0 (Chapter 4). It was observed that under these conditions high-density lipid containing flocculent/precipitates were formed, which subsequently sedimented upon centrifugation (at 1126 *g* for 10 min). It was proposed that the MFG removed in the flocculent/precipitate were either part of a calcium-MFG complex or a MFG/lipoprotein complex entrapped by aggregation of mineral salts.

7.5 Factors affecting the flocculation of MFG in the retentate

Factors affecting the separation of MFG from retentate and the retention of protein in the retentate during this separation process were investigated in Chapter 5. These included calcium content, dilution, pH, ionic strength, temperature and time. The factors which had the most significant were dilution, pH and ionic strength.

7.5.1 Dilution

Dilution of the retentate had a major effect on the separation of the MFG. It was found that as dilution increased (from 1:2 to 1:20) there was an increase in the removal of MFG, and in the retention of protein in the supernatant. The effectiveness with which MFG were removed from the retentate increased as the dilution was increased up to 1:5 dilution. At dilutions above this value the turbidity of the supernatant did not change significantly. The retentate dilution of 1:6 was found to remove the majority of the MFG and retain a significant proportion of protein in the supernatant. Further dilution resulted in a small increase in protein retention (up to the limit of 1:10 dilution). This suggests that the solubility of protein increases with increasing dilution.

7.5.2 pH

The pH of the diluted retentate was found to significantly affect removal of the MFG. MFG were most effectively removed from the diluted retentate at the natural pH (pH ~ 4.5). This trend was observed regardless of the dilution of the retentate. It was also determined that the most significant loss of protein occurred at pH ~ 4.5 (Figure 5.9). However this loss is minimised by increasing dilution up to a ratio of 1:10. The decrease in protein content of the supernatant is very likely related to the flocculation and subsequent sedimentation of the MFG.

7.5.3 Ionic strength

Experimental work conducted in Chapter 5 showed that the removal of MFG and the retention of protein in the retentate varied at different concentrations of NaCl (different ionic strengths) and different dilutions. The removal of MFG and the retention of protein at the higher dilution (1:6) were only slightly affected by the ionic strength. However, at the lower dilutions of retentate (1:5 and 1:4), increased ionic strength resulted in a decreased MFG removal and increased protein retention. It was proposed that increased protein stability (and therefore solubility) at higher ionic strengths prevented protein-protein interactions that may have occurred at lower ionic strengths. This increased solubility of the protein components of the MFGM which occurs at higher ionic strengths will influence the stability of the MFG. This in turn will result in increased quantities of MFG being stabilised in suspension. Subsequently, a higher quantity of MFG and protein are contained in the supernatant after treatment.

7.5.4 Temperature and time of heat treatment

Results showed that holding temperature (20 or 50 °C) and time (0-60 min) did not significantly affect the removal of MFG or protein retention in the supernatant. However, higher temperature and longer times resulted in slightly higher losses of protein from the supernatant.

7.6 Proposed mechanism of aggregation of MFG in the retentate

The current work has determined that MFG in acid whey retentate aggregate upon dilution at pH values within the isoelectric range. The mechanism by which this aggregation occurs is discussed in Chapter 5 and is summarised below.

The low ionic strength of the dilute retentate system is most likely responsible for the flocculation of MFG. At low ionic strength the stability of the serum proteins significantly decreases, increasing the susceptibility of denaturated proteins to form protein-protein interactions. Partially or fully denatured proteins on the surface of the MFG are thought to interact with other denatured proteins which are destabilised at low ionic strength and low pH. The denatured MFGM proteins probably associate hydrophobically with proteins of another MFGM, or aggregates of serum proteins, or lipoprotein complexes or individual denatured serum proteins. The high level of protein in the macro-aggregates result in the high density that may be separated with minimal force (1126 *g* for 10 mins).

7.7 Production of low fat WPC

Low fat WPC was produced in a pilot plant trial (Chapter 6) using the experimental conditions determined to remove MFG from acid whey retentate (Chapter 5). The product resulting from this trial contained ~ 1% fat, considerably less than commercial WPC, and was significantly whiter in colour. The gel formed from 15% WPC solution heated at 75 °C was more elastic, had better water holding capability, and was more "gelatinous" in nature. This low fat WPC has exhibited functional properties which appear to be markedly superior to the commercial WPCs currently manufactured. Further improvements in these aspects of functionality suggest that other areas of functionality may also have improved. Removal of more fat from the low fat WPC may result in further improvements in functionality. It is hoped that the removal of the majority of the MFG from WPC will produce a product that results in a transparent gel.

7.8 Further studies

Based on the results presented in the current study, the following areas are recommended for further investigation. These recommendations may be conducted at one of two levels: (1) at a fundamental level, (2) at a more practical level within the dairy industry.

Method of separation of lipid fraction from diluted retentate

A further investigation should be carried out into the optimisation of the separation method of the lipid fraction from the diluted retentate. Using a benchtop centrifuge the separation of MFG from the dilute retentate was observed to leave a clear supernatant. During pilot plant trials, removal of MFG using a clarifier was less effective. Investigation into the use of the separation techniques that either results in, a 'gentler' separation, or increased residence time, may result in a more effective separation of MFG. Utilisation of a decanter or hydrocyclone to separate the MFG aggregates from the dilute retentate, or the decreased flow rate of the separation process, may give the desired result. This work should be conducted on a pilot-scale plant within the dairy industry as continuous flow conditions can not easily be conducted in the laboratory.

Process optimisation

Depending on the relative costs of water removal after dilution and loss of protein, the dilution may be altered to increase cost effectiveness of the process. Where the cost of removing water is lower comparative to the cost of lost protein, the dilution can be increased. Conversely, where the water removal incurs a higher cost comparative to the loss of protein, the dilution may be decreased. It should be noted that the extent to which the dilution may be varied is limited by the required final concentration of fat in the WPC. It is therefore recommended that an optimisation of this process be carried with respect to process costs and final WPC composition. This should involve a cost analysis of the separation and UF processes. Further, it is

recommended that the upper limit of retentate dilution should be 1 part retentate to 10 parts demineralised water.

Utilisation of lipid fraction separated during clarification

Compositional analyses should be conducted on the lipid rich fraction produced in the clarification stage of removing the fat from the dilute retentate. It is likely that the sedimented fraction is rich in phospholipids and may be a good source of phospholipids for products currently manufactured at the Edgecumbe site. The utilisation of the lipid fraction of the whey, in the production of phospholipid products could result in a valuable product. The functional properties of this fraction should be explored.

Low fat WPC functionality

The functional aspects of the low fat WPC relative to WPC and WPI should be investigated in greater depth. The areas of functionality that are of interest include solubility, gelation properties, foaming expansion and stability, emulsification and acid stability. The effect of the physical and chemical factors on these functional properties should also be investigated in aqueous solutions as well as in model food systems. Of particular interest is the effect of salt concentration on the gelling properties of the low fat WPC.

Flavour stability

The stability of flavour in some WPC products has been poor due to oxidation of the fat. The significant decrease in the quantity of fat in the low fat WPC may result in a product with greater flavour stability. The stability of the flavour in the low fat WPC should be investigated as this might lead to this product being more attractive to food manufacturers. Shelf life testing could either be conducted within the industry using sensory techniques as an indicator of fat oxidation, or at a fundamental level where the level of fat oxidation would measured quantitatively.

REFERENCES

Andrews, A. T. (1992). Indigenous Enzymes in Milk - Phosphatases. In *Advanced Dairy Chemistry - 1: Proteins*, ed. P. F. Fox. Elsevier Applied Science, London, pp 310-320.

AOAC (1984). *Official Methods of Analysis*. 14th edn. Association of Official Analytical Chemists, Arlington, Virginia.

AOAC (1995). *Official Methods of Analysis of AOAC International*. 16th edn., AOAC International, Garthersburg, Maryland, USA.

Attebury, J. M. (1971). Removing lipid material from whey. U.S., Pat 3,560,219.

Badings, H. T. (1969). Simple method for reducing H_2S and Cu contents of pasteurized cream and butter. *Voedingsmiddelon Technologie*, **3** (50), 1207-1210.

Banks, W. (1991). Milk lipids. In *Utilization of Milkfat*, International Dairy Federation , Brussels, pp 3-6.

Beaton, N. C. and Steadly, H. (1982). Industrial ultrafiltration. In *Recent Developments in Separation Science*, Vol. 7, ed. N. N. Li. CRC Press, Boca Raton, pp 1-29.

Brendts, J. F. (1967). Heat Effects in Proteins and Enzymes. In *Thermobiology*, ed. A. N. Rose. Academic Press, New York, pp 25-72.

Breslau, B. R., Goulet, J., and Cross, R. A. (1975). Production of a crystal clear, bland tasting, protein solution from cheese whey. *Cultured Dairy Products Journal*, **10**, 13-14.

Brule, G., Real Del Sol, E., Fauquant, J., and Fiaud, C. (1978). Mineral salt stability in aqueous phase of milk: Influence of heat treatments. *Journal of Dairy Science*, **61**, 1225-1232.

Brunner, J. R. (1974). Physical equilibria in milk: the lipid phase. In *Fundamentals of Dairy Chemistry*, 2nd edn., ed. B. H. Webb, A. H. Johnson, and J. A. Alford. AVI Publishing Co. Inc., Westport, Connecticut, USA, pp 474-602.

Cho, Y.-H. (1996). The effect of heat on the composition of the milk fat globule membrane. Diploma in Technology thesis, Massey University, Palmerston North.

Christie, W. W. (1995). Composition and Structure of Milk Lipids. In *Advanced Dairy Chemistry - 2: Lipids*, 2nd edn., ed. P.F. Fox. Chapman and Hall, London, pp 1-36.

Clark, R. M., Ferris, A. M., Fey, M., Brown, P. B., Hundrieser, K. E., and Jensen, R. G. (1982). Changes in the lipids of human milk from 2 to 16 weeks pospartum. *Journal of Pediatric Gastroenterology and Nutrition*, **1** (3), 311-315.

Corbin, E. A. and Whittier, E. O. (1965). Composition of milk. In *Fundamentals of Dairy Chemistry*, ed. Webb, B. H. and Johnson, A. H. AVI Publishing Company, Inc., Westport, Connecticut, pp 1-36.

Corredig, M. and Dalgleish, D. (1996). Effect of different heat treatments on the strong binding interactions between whey proteins and milk fat globules in whole milk. *Journal of Dairy Research*, **63**, 441-449.

Corredig, M. and Dalgleish, D. (1998). Effect of heating of cream on the properties of milk fat globule membrane isolates. *Journal of Agricultural Food Chemistry*, **46**, 2533-2540.

Creamer, L. K. and MacGibbon, A. K. H. (1996). Some recent advances in the basic chemistry of milk proteins and lipids. *International Dairy Journal.* **6** (6), 539-568.

Dalgliesh, D. G. and Banks, J. M. (1991). The formation of complexes between serum proteins and fat globules during heating of whole milk. *Milchwissenschaft*, **46**, 75-70.

Daufin, G., Michel, F., Labbe, J. P., Quemerais, A., and Grangeon, A. (1993). Ultrafiltration of defatted whey: Improving performance by limiting membrane fouling. *Journal of Dairy Research*, **60**, 79-88.

de Boer, R., de Wit, J. N., and Hiddink, J. (1977). Processing of whey by means of membranes and some applications of whey protein concentrate. *Journal of the Society of Dairy Technology*, **30** (2), 112-120.

de Wit, J. N. (1984). Functional properties of whey proteins in food systems. *Netherlands Milk and Dairy Journal* , (38), 71-89.

de Wit, J. N. and de Boer, R. (1975). Ultrafiltration of cheese whey and some functional properties of the resulting whey protein concentrates. *Netherlands Milk and Dairy Journal*, **29**, 198-211.

de Wit, J.N. and Klarenbeek, G. (1984). Effect of various heat treatment on structure and solubility of whey protein. *Journal of Dairy Science*, **67**, 2701-2710.

de Wit, J. N., Klarenbeek, G., and Adamse, M. (1986). Evaluation of functional properties of whey protein concentrates and whey protein isolates. 2. Effects of processing history and composition. *Netherlands Milk and Dairy Journal*, **40**, 41-56.

de Wit, J. N. and van Kessel, T. (1996). Effects of ionic strength on the solubility of whey protein products. A colloid chemical approach. *Food Hydrocolloids*, **10** (2), 143-149.

Downard, C. J. (1988). Removal of lipid material from whey. Diploma in Science and Technology, Massey University, Palmerston North.

Fauquant, J., Pierre, A., and Brule, G. (1985*a*). Clarification of acid whey from a casein factory. *La Technique Laitiere*, **1003**, 37-41.

Fauquant, J., Vieco, E., Brule, G., and Maubois, J.-L. (1985*b*). Clarification of sweet wheys by thermocalcic aggregation of residual fatty material. *Lait*, **65**, 1-20.

Fennema, O. R. (1996). *Food Chemistry*. 3rd edn. Marcel Dekker Inc., New York.

Fink, A. and Kessler, H.G. (1985). Changes in the fat globule membrane produced by heating. *Milchwissenschaft*, **40** (5), 261-264.

Folch, J., Less, M., and Sloane Stanley, G. H. (1957). A simple method for isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry*, **226**, 497.

Grappin, R. and Ribadeau-Dumas, B. (1992). Analytical Methods for Milk Proteins. In *Advanced Dairy Chemistry - 1: Proteins* , ed. P. F. Fox. Elsevier Applied Science, London, pp 1-62.

Hambling, S. G., McAlpine, A. S., and L. Sawyer. (1992). β-Lactoglobulin. In *Advanced Dairy Chemistry - 1: Proteins*, ed. P. F. Fox. Elsevier Applies Science, London, pp 141-190.

Hara, A. and Radin, S. (1978). Lipid extraction of tissues with a low-toxicity solvent. *Analytical Biochemistry*, **90**, 420-426.

Harper, W. J. (1992). Lactose and Lactose Derivatives. In *Whey and Lactose Processing*, ed J. G. Zadow. Elservier Applied Science, London, pp 317-360.

Havea, P. (1998). Studies on Heat-Induced Interactions and Gelation of Whey Proteins. Ph.D. thesis, Massey University, Palmerston North.

Hermansson, A.-M. and Akesson, C. (1975). Functional properties of added proteins correlated with properties of meat systems. *Effect of Salt on Water-Binding Properties of Model Meat Systems*, **40**, 603.

Hobman, P. G. (1984). Review of processes and products for utilisation of lactose in deproteinated milk serum. *Journal of Dairy Science*, **67**, 2630-2653.

Hobman, P. G. (1992). Ultrafiltration and Manufacture of Whey Protein Concentrates. In *Whey and Lactose Processing*, ed. J. G. Zadow. Elsevier Applied Science, London, pp 195-230.

Holt, C. (1985). The milk salts: Their secretion, concentrations and physical chemistry. In *Developments in Dairy Chemistry - 1: Lactose and minor constituents*, ed. P. F. Fox. Elsevier Applied Science, London, pp 143-181.

Holulihan, A. V. and Hirst, P. A. (1987). *Estimation of milk membrane fraction from whey protein concentrates*. Paper presented at the Australia, New Zealand, United States Collaborative Conference of Whey Protein Utilisation. Columbus, Ohio, 17-19 June.

Houlihan, A. V. and Goddard, P. A. (1991). Analysis of a milk membrane fraction isolated from whey protein concentrates. *The Australian Journal of Dairy Technology*, **Nov**, 77-81.

Houlihan, A. V., Goddard, P. A., Kitchen, B. J., and Masters, C. J. (1992). Changes in structure of the bovine milk fat globules membrane on heating whole milk. *Journal of Dairy Research*, **59**, 321-329.

Howell, N. K. (1992). Protein-Protein Interactions. In *Developments in Food Proteins*, Vol. 7, Elsevier Applied Science, London, pp 231-270.

Hwang, D. C. and Damodaran, S. (1994). Selective precipitation of fat globule membranes of cheese whey by saponin and bile salt. *Journal of Agricultural & Food Chemistry*, **42**, 1872-1878.

Hwang, D. C. and Damodaran, S. (1995). Selective precipitation and removal of lipids from cheese whey using chitosan. *Journal of Agricultural & Food Chemistry*, **43** (1), 33-37.

Jensen, R. G., Ferris, A. M., and Lammi-Keepe, C. J. (1991). The composition of milk fat. *Journal of Dairy Science*, **74**, 3228-3243.

Joseph, M. S. B. and Mangino, M. E. (1988*a*). Contribution of milk fat globule membrane proteins to the effective hydrophobicity of whey protein concentrates. *Australian Journal of Dairy Technology*, **43** (1), 6-8.

Joseph, M. S. B. and Mangino, M. E. (1988*b*). The effects of milk fat globule membrane protein on the foaming and gelation properties of β-lactoglobulin solutions and whey protein concentrates. *Australian Journal of Dairy Technology*. **43** (1), 9-11.

Kamath, S. R and Morr, C. V. (1997). Recovery and composition of Swiss cheese whey lipid-containing fractions. *Journal of Agricultural & Food Chemistry*, **45** (11), 4271-4274.

Kamath, S. R., Morr, C. V., and Schenz, T. (1998). Laser light scattering and microscopic properties of milk fat globules in Swiss cheese whey low density lipid-containing fraction. *Lebensmittel-Wissenschaft & Technologie.* **31** (3), 274-278.

Karleskind, D., Laye, I., Mei, F. I., and Morr, C. V. (1995*a*). Chemical pretreatment and microfiltration for making delipidized whey protein concentrate. *Journal of Food Science.* **60** (2), 221-226.

Karleskind, D., Laye, I., Mei, F. -I., and Morr, C. V. (1995*b*). Gelation properies of lipid-reduced, and calcium-reduced whey protein concentrates. *Journal of Food Science*, **60** (4), 731-741.

Keenan, T. W. and Dylewski, D. P. (1995). Intracellular origin of milk lipid globules and the nature and structure of milk lipid globule membrane. In *Advanced Dairy Chemistry - Volume 2 - Lipids*, 2nd edn., ed. P.F. Fox. Chapman and Hall, London.

Kilara, A. (1994). Whey Protein Functionality. In *Protein Functionality in Food Systems*, Ed. N.S. Hettiarachahy and G. R. Ziegler. Marcel Dekker, Inc., New York, pp 325-355.

Kim, S.-H. and Jimenez-Flores, R. (1995). Heat-induced interactions between the proteins of milk fat globule membrane and skim milk. *Journal of Dairy Science*, **78**, 24-35.

Kim, S.-H., Morr, C. V., Seo, A., and Surak, J. G. (1989). Effect of whey pretreatment on composition and functional properties of whey protein concentrate. *Journal of Food Science*, **54** (1), 25-29.

Kinekawa, Y.-I., Fuyuki, T., and Kitabatake, N. (1998). Effects of salts on the properties of sols and gels prepared from whey protein isolate and process whey protein. *Journal of Dairy Science*, **81**, 1532-1544.

King, N. (1955) *The milk fat globule membrane and some associated phenomena.* Commonwealth Agricultural Bureaux, Farnham Royal, England .

Kitchen, B. J. (1974). A comparison of the properties of membrane isolated from bovine skim milk and cream. *Biochimica et Biophysica Acta*, **356**, 257-269.

Kostygov, L. and Rogov, I. (1966). Stimulation of fat separation by means of a uniform electric field of alternating current. *XVII International Dairy Congress* Harausgeber.

Kuhn, P. R. and Foegeding, E. A. (1991*a*). Mineral salts effect on whey protein gelation. *Journal of Agricultral and Food Chemistry*, **39** (6), 1013-1016.

Kuhn, P. R. and Foegeding, E. A. (1991*b*). Factors influencing whey protein gel rheology: Dialysis and calcium chelation. *Journal of Agricultral and Food Chemistry*, **56**, 789-791.

Kurtz, F. E. (1974). The Lipids of Milk: Composition and Properties. In *Fundamentals of Dairy Chemistry*, 2nd edn., ed. B.H. Webb, A.H. Johnson, and J.A. Alford. Avi Publishing Co., Westport CT, pp 125-219.

Lucey, J. A., Munro, P. A., and Singh, H. (1998). Rheological properties and microstructure of acid milk gels as affected by fat content and heat treatment. *Journal of Food Science*, **63** (4), 660-664.

Marmer, W. N. and Maxwell, R. J. (1981). Dry column method for the quantitative extraction and simultaneous class separation of lipids from muscle tissue. *Lipids*, **16** (5), 365-371.

Maubois, M.-L., Pierre, A., Fauquant, J., and Piot, M. (1987). Industrial fractionation of main whey proteins. *Bulletin of the International Dairy Federation*, **212**, 154-189.

Maxwell, R. J., Mondimore, D., and Tobias, J. (1986). Rapid method for the quantitative extraction and simultaneous class separation of milk lipids. *Journal of Dairy Science*, (69), 321-325.

McPherson, A. V. and Kitchen, B. J. (1983). Reviews of the progress of dairy science: The bovine milk fat globule- it's formation, composition, structure and behaviour in milk and dairy products. *Journal of Dairy Research*, **50**, 107-133.

Mei, F. I., Laye, I., Karleskind, D., and Morr, C. V. (1996). Gelation of calcium-reduced and lipid-reduced whey protein concentrates as affected by total and ionic mineral concentrations. *Journal of Food Science*. **61** (5), 899-905.

Morr, C. V. (1985). Composition, physicochemical and functional properties of reference whey protein concentrates. *Journal of Food Science*, **50**, 1406-1411.

Morr, C. V. (1989). Whey Protein: Manufacture. In *Developments in Dairy Chemistry, Vol. 4*, ed. P. F. Fox. Elsevier Applied Science, New York, pp pp245.

Morr, C. V. (1992). Improving the texture and functionality of whey protein concentrate. *Food Technology*. **46** (1), 110-113.

Morr, C. V. and Foegeding, E. A. (1990). Composition and functionality of commercial whey and milk protein concentrates and isolates: A status report. *Food Technology*, **44** (4), 100-112.

Morr, C. V., German, B., Kinsella, J. E., Regenstein, J. M., Bureni, J. P. van, Kilara, A., Lewis, B. A., and Mangino, M. E. (1985). A collaborative study to develop a standardised food protein solubility procedure. *Journal of Food Science*, **50** (6), 4715-4718.

Morr, C. V. and Ha, E. Y. W. (1993). Whey protein concentrates and isolates: Processing and functional properties. *Critical Reviews in Food Science and Nutrition*, **33** (6), 431-476.

Mulder, H. and Walstra, P. (eds). (1974) *The milk fat globule - emulsion science as applied to milk products and comparable foods.* Centre for Agricultural Publishing and Documentation (PUDOC), Wageningen.

Mulvihill, D. M. (1992). Production, functional properties and utilisation of milk protein products. In *Advanced Dairy Chemistry, Proteins*, Vol. 1, ed. P. E. Fox. Elsvier Science Publishers Ltd., London, pp 369-404.

Mulvihill, D. M. and Donovan, M. (1987). Whey proteins and their thermal denaturation - a review. *Irish Journal of Food Science and Technology*, **11**, 43-75.

Mulvihill, D. M. and Kinsella, J. E. (1988). Gelation of β-lactoglobulin: effects of sodium chloride and calcium chloride on the rheological and structural properties of gels. *Journal of Food Science*, **53**, 231-236.

Nielsen, P. S. (1988) *Membrane Filtration for Whey Protein Concentrate, Marketing Bulletin*. APV Pasilas AC, Aarhus, Denmark.

Patel, M. T., Kilara, A., Huffman, L. M., Hewitt, S. A., and Houlihan, A. V. (1990). Studies on whey protein concentrates. I. Compositional and thermal properties. *Journal of Dairy Science.* **73** (6), 1439-1449.

Polis, P. D., Schmuckler, H. W., Custer, J. H., and McMeekin, T. L. (1950). Isolation of an electrophoretically homogeneous crystalline component of β-lactoglobulin. *Journal of American Chemist Society* , **72**, 4965-4968.

Pouloit, Y., Landry, J., and Glasson, J. (1991). Induction of calcuim phosphate precipitate in sweet whey permeate. *Lait*, **71**, 313-320.

Rinn, J. C., Morr, C. V., Seo, A. , and Surak, J. G. (1990). Evaluation of nine semi-pilot scale whey pretreatment modifications for producing whey protein concentrate. *Journal of Food Science*, **55** (2), 510-515.

Schmidt, R. H., Illingworth, B. L., Ahmed, E. M., and Richter, R. L. (1978). The effect of dialysis on heat-induced gelation of whey protein concentrate. *Journal of Food Process and Preservation*, **2**, 111-121.

Sherbon, J. W. (1988). Physical properties of milk. In *Fundamentals of Dairy Chemistry*, 3rd edn., ed. N. P. Wong. Van Nostrand Reinhold Co. Ltd., Wokingham, UK, pp 409-460.

Sherwin, C. P. and Foegeding, E. A. (1997). The effects of $CaCl_2$ on aggregation of whey proteins. *Milchwissenschaft*, **52**, 93-96.

Singh, H., Sharma, R., Taylor, M. W. and Creamer, L. K. (1996). Heat-induced aggregation and dissociation of protein and fat particles in recombined milk. *Netherlands Milk and Dairy Journal*, **50**, 140-166.

Stahl, E. (1965) *Thin-Layer Chromatography. A Laboratory Hand-book*. Academic Press, New York.

Sternberg, M., Chiang, J. P., and Eberts, N. J. (1976). Cheese whey protein isolated with polyacrylic acid. *Journal of Agricultural and Food Chemistry*, **59**, 1042-1050.

Swaisgood, H. E. (1996). Characteristics of Milk. In *Food Chemistry*, 3rd edn., ed. O. R. Fennema. Marcel Dekker Inc., New York, pp 841-878.

Tang, Q., McCarthy, O. J., and Munro, P. A. (1993). Oscillatory rheological study of the gelation mechanism of whey protein concentrate solutions: effect of physicochemical variables on gel formation. *Journal of Dairy Research*, **60**, 543-555.

Theodet, C. and G. Gandemer. (1990). Comparison of 5 methods for lipid extraction from whey and its byproducts. *Lait* , **71** (1), 41-54.

Timmen, H. and Dimick, P.S. (1972). Structure and synthesis of milk fat. Characterisation of the major hydroxy compounds in milk lipids. *Journal of Dairy Science*, **55**, 919-925.

Vaghela, M. N. and Kilara, A. (1995*a*). Quantitative analysis of phospholipids from whey protein concentrates by high-performance liquid chromatography with a narrow-bore column and an evaporative light-scattering detector. *Journal of the American Oil Chemists Society*, **72** (6), 729-733.

Vaghela, M. N. and Kilara, A. (1995*b*). A rapid method for extraction of total lipids from whey protein concentrates and separation of lipid classes with solid phase extraction. *Journal of the American Oil Chemists Society*, **72** (10), 1117-1121.

Vaghela, M. N. and Kilara, A. (1996). Lipid composition of whey protein concentrates manufactured commercially and in the laboratory. *Journal of Dairy Science*, **79** (7), 1172-1183.

van Boekel, M. A. J. S. and Folkerts, T. (1991). Effect of heat treatment on the stability of milk fat globules. *Milchwissenschaft*, **46** (12), 758-764.

van Boekel, M. A. J. S. and Walstra, P. (1989). Physical changes in the fat globules in unhomogenized and homogenized milk. *International Dairy Federation*, Bulletin No. **238**, 13-16.

Varunsatian, S., Watanabe, K., Hayakawa, S., and Nakamura, R. (1983). Effects of Ca^{++}, Mg^{++} and Na^{+} on heat aggregation of whey protein concentrates. *Journal of Food Science*, **48**, 42-46, 70.

Visser, J., Minihan, A., Smits, P., Tjan, S. B., and Heertji, I. (1986). Effects of pH and temperature on the milk salt system. *Netherland Milk and Dairy Journal*, **40**, 351-368.

Walstra, P. (1969). Studies on milk fat dispersion. 2. The globule size distribution of cows milk. *Netherland Milk Dairy Journal*, **23**, 99-110.

Walstra, P. and Jenness, R. (1984) *Dairy Chemistry and Physics*. John Wiley & Sons, New York.

Walstra, P. and Mulder, H. (1962). Gravimeteric method for the determination of the fat content of milk and milk products: The Rose-Gottlieb method. *Netherlands Milk and Dairy Journal*, (16), 172-184.

Webb, B. H. and Johnson, A. H. (1965) *Fundamentals of Dairy Chemistry*. The AVI Publishing Company, Inc., Westport, Connecticut.

Wolff, R. L. and Castera-Rossignol, A. F. M. (1987). Mise au point d'une methode d'extraction de la matiere grasse de fromage de type emmental. *Revue Francaise Des Corps Gras*, **34**, 123-132.

Zadow, J. G. (1984). Lactose: properties and uses. *Journal of Agricultural and Food Chemistry*, **67**, 2654-2679.