

to my parents

Promotor Prof. Dr. Ir. Paul Van der Meeren
Faculty of Bioscience Engineering
Department of Applied Analytical and Physical Chemistry
Particle and Interfacial Technology Group

Dean Prof. Dr. Ir. Guido Van Huylbroeck

Rector Prof. Dr. Anne De Paepe

Kasinos Marios

**EFFECT OF POLAR LIPIDS ON HEAT INDUCED
CHANGES IN CONCENTRATED MILK SYSTEMS**

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied
Biological Sciences

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List of abbreviations and symbols

ADPH	Adipophilin
ANOVA	Analysis Of Variance
BSA	Bovine Serum Albumin
BTN	Butyrophilin
C14-LPC	1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine
C16-LPC	1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine
C18-LPC	1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine
CAC	Critical Aggregation Concentration
CD	Circular Dichroism
CD36	Cluster of Differentiation 36
CMC	Critical Micelle Concentration
COSY	Correlation Spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill Sequence
CRP	Cream Residue Powder
Cys	Cysteine
D	Diffusion coefficient
$d_{3,2}$	Sauter mean diameter of oil droplets
$d_{4,3}$	Volume-weighted average diameter of oil droplets
DMPA	1,2-dimyristoyl-sn-glycero-3-phosphate
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMPG	1,2-dimyristoyl-sn-glycero-3-phosphoglycerol
DPPA	1,2-dipalmitoyl-sn-glycero-3-phosphate
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
DPPG	1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol

EDTA	Ethylenediaminetetraacetic acid
ELSD	Evaporative Light Scattering Detector
EM	Evaporated Milk
g	Gram
G*	Complex modulus
Gang	Ganglioside
GluCer	Glucosylceramide
GPC	Glycerophosphorylcholine
GPE	Glycerophosphorylethanolamine
GPI	Glycerophosphorylinositol
GPL	Glycerophosphorylic compounds
HCT	Heat Coagulation Time
HLB	Hydrophilic Lipophilic Balance
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Chromatography
K	Consistency coefficient
kDa	Kilodalton
kg	Kilogram
LacCer	Lactosylceramide
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
MALDI-TOF MS	Matrix-Assisted Laser Desorption and Ionization Time-of-Flight Mass Spectroscopy
MFGM	Milk Fat Globule Membrane
min	Minute

mL	Milliliter
mPa.s	Millipascal.second
MUC1	Mucin 1
MW	Molecular Weight
n	Flow behavior index
nM	Nanomolar
NOE	Nuclear Overhauser Effect
O/W	Oil in Water emulsions
³¹ P-NMR	Phosphorus Nuclear Magnetic Resonance
Pa	Pascal
Pa.s	Pascal.second
PA	Phosphatidic acid
PAGE	Polyacrylamide Gel Electrophoresis
PAS-6/7	Lactadherin
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PP3	Proteose Peptone 3
ppm	Parts per million
PS	Phosphatidylserine
RCM	Recombined Concentrated Milk
REM	Recombined Evaporated Milk
RPM	Rotations Per Minute
Sec	Seconds
SBP	Sweet Buttermilk Powder

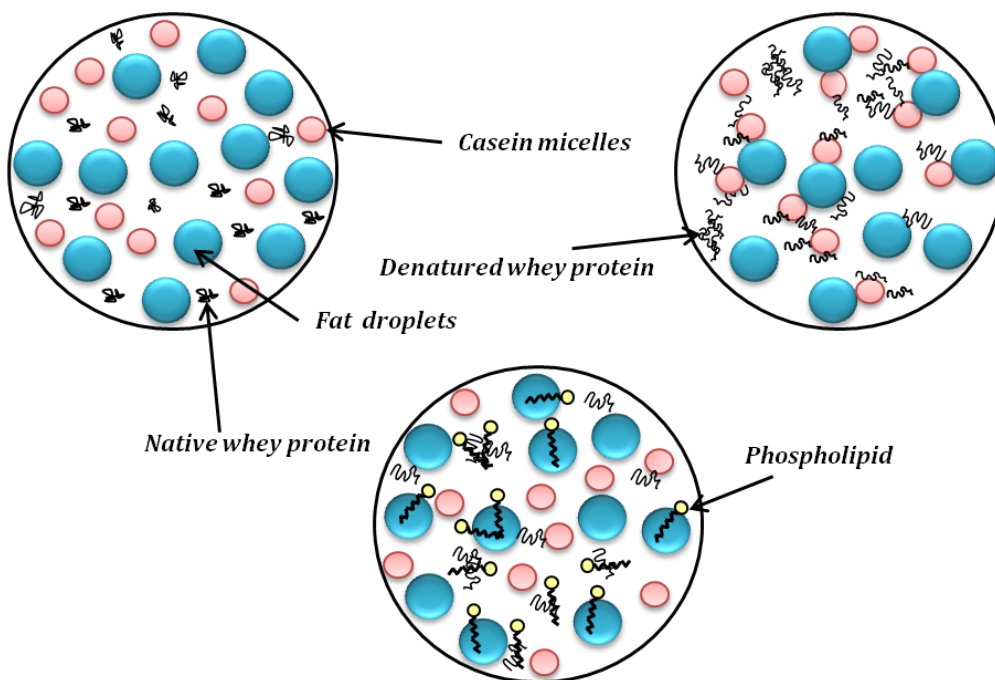
SDS	Sodium Dodecyl Sulfate
SMP	Skim Milk Powder
STD	Saturation Transfer Difference
$t_{1/2}$	Half-life time
T_m	Phase transition temperature
TOCSY	Total Correlation spectroscopy
u	Unknown compound
UHT	Ultra High Temperature
W/O	Water in Oil emulsion
WPI	Whey Protein Isolate
XO	Xanthin Oxidase
Γ	Protein load
γ	Shear rate
θ	Ellipticity
μ	Apparent viscosity
μm	Micrometer
ρ	Density
τ	Shear stress

Chapter 1

GENERAL INTRODUCTION

Whey protein containing emulsion

Heat coagulation



Phospholipid stabilized emulsion

Chapter cover image: Schematic representation of the heat-induced protein-protein and phospholipid-protein interactions in O/W emulsions.

1.1. Introduction

The term milk refers to the white opaque fluid secreted by female mammals for suckling their neonates. Its utilization dates back to antiquity, as it was considered one of the most precious trade commodities which through the millennia has been championed as the perfect food. Nowadays, milk is consumed either directly or as raw material for the production of numerous dairy products including cheese, butter, ice cream and yogurt. Whereas milk constitutes a principal part in human's diet due to its high content in essential food components, such as amino acids, lipids, sugar, minerals and vitamins, its richness makes it a fertile medium for the growth of microorganisms, which either spoil the product or present a risk of food poisoning or food borne diseases. In addition to being a potential source of pathogenic bacteria, raw milk is also susceptible to spoilage by the action of naturally occurring enzymes that can contribute to undesirable changes during storage (Harwalkar, 1982; Kelly, Datta, & Deeth, 2005). To overcome this hindrance, a range of preservation techniques and strategies have evolved over the centuries with the most commonly used method being the application of heat or the conversion of milk into other products which are more stable (Euston, 1997). Today, the most important heat treatments in dairy industry can be classified into five groups: thermization (15 sec, 63-65°C) for killing spoilage microorganisms, low pasteurization (20 sec, 74°C) for inactivating pathogenic microorganisms, high pasteurization (20 sec, 85°C) for killing all microorganisms but not spores, sterilization (30 min, 110°C) and UHT-ultra high temperature (5 sec, 140°C) for destroying spores (de Jong, 2008; Kelly et al., 2005; O'Connell & Fox, 2003).

1.2. Milk composition

From a colloidal chemical point of view, milk can be described as an oil-in-water (O/W) emulsion, where the fat globules are dispersed into an aqueous continuous phase including proteins, salts and lactose (Tran Le et al., 2007; Van der Meeren, El-Bakry, Neiryneck, & Noppe, 2005). Due to its predominance in the alimentary chain but moreover due its complexity as a biological material, milk has been one of the most examined food products from early years.

Bovine milk, which is considered as the most frequently consumed type of milk, consists of about 4.5 % fat, 3.5 % protein, 4.8 % lactose and 0.7 % minerals (Euston, 1997; Wong,

Camirand & Pavlath, 1996). The milk of any particular species varies with the individuality of the animal, the breed, the health condition (mastitis and other diseases), the nutritional status, the stage of lactation, the age and the interval between milkings (Brans, Schroën, van der Sman & Boom, 2004; Walstra, Geurts, Noomen, Jellema & van Boekel, 1999).

1.2.1. Milk fat

According to Fox and McSweeney (1998), the importance of milk fat emerges from the fact that is a valuable source of essential fatty acids and fat soluble vitamins (A, D, E, K), as well as due to its contribution to the flavor and rheological properties of dairy products. Triglycerides represent more than 95 % of the total lipids in the milk of most species, whereas the rest is comprised of very small proportions of diglycerides, monoglycerides, phospholipids, cholesterol, free sterols and free acids (Garton, 1963; Patton & Keenan, 1975). Milk triglycerides are dispersed in the aqueous phase in the form of spherical droplets or globules whose size in native milk emulsions varies considerably, ranging from less than 1 to about 10 μm , with an average of about 4 μm (Jensen, 2002; Singh, 2006). Their structure is responsible for the melting point, crystallization behavior and rheological properties of milk fat.

During secretion, the fat globules in milk are enveloped in a membranous covering of a thickness of approximately 9 nm, known as milk fat globule membrane (MFGM) (Dowben, Bunner, & Philpott, 1967). Its origin, composition and organization have been the main subject of several literature reports. Like other biological membranes, MFGM consists of a complex mixture of membrane-specific proteins, mainly glycoproteins, polar lipids (phospho- and sphingolipids), enzymes and neutral lipids (triglycerides, diglycerides, monoglycerides, cholesterol and its esters), arranged in a trilayer configuration (figure 1.1) (Dewettinck et al., 2008; Fox & McSweeney, 1998; Jensen & Clark, 1995; Keena et al., 1970). This composition ascribes to the membrane natural emulsifying and surface active properties, preventing flocculation and coalescence of fat globules in milk and protecting them from enzymatic degradation (Fong et al., 2007; Singh, 2006).

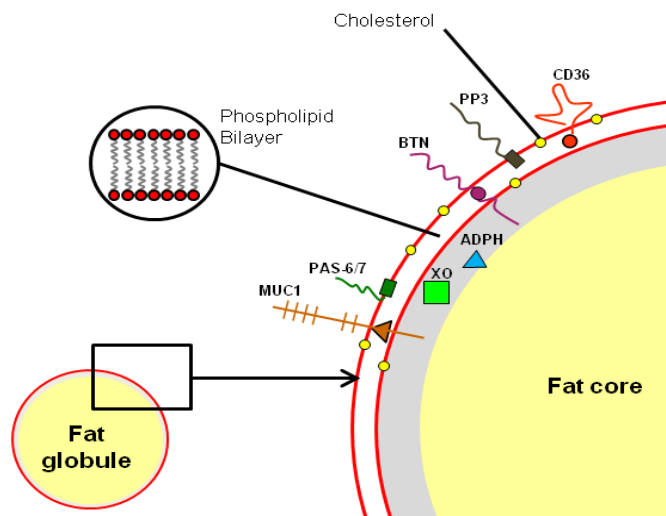


Figure 1.1. Schematic representation of the native milk fat globule membrane, adapted from Dewettinck et al. (2008) and Evers (2004).

MFGM proteins:

PAS-6/7: lactadherin

PP3: protease peptone 3

CD36: cluster of

differentiation 36

XO: xanthin oxidase

ADPH: adipophilin

MUC1: mucin 1

BTN: butyrophilin

Protein and phospholipids together account for over 90 % of the membrane dry weight, but the relative proportions of lipids and proteins may vary widely. Even though milk contains as little as 2 g/L of MFGM material, it has gained a precious attention in recent years due to the technological and emulsifying functionalities as well as the health-beneficial properties of the whole membrane or the separate lipid and protein components (Fong et al., 2007; Mather, 2000). More precisely, the latter were demonstrated to be vitamin binders, to have an inhibitory effect for cancer cell growth or for *Helicobacter pylori*, and to bear a potential protective role against Alzheimer's disease, rotavirus infection, or multiple sclerosis (Boddaert et al., 2007; Guggenmos et al., 2004; Hancock et al., 2002; Kvistgaard et al., 2004; Mana, 2004; Spitsberg, 2005; Wang, Hirno, Willén, & Wadström, 2001). These unique properties of MFGM have led to research into developing technologies for the isolation and separation of MFGM material from milk, and subsequently be incorporated into food emulsions, and could potentially result in new functional foods and nutraceuticals. MFGM can be isolated from cream, buttermilk or whole milk.

1.2.1.1. Polar lipids in milk

Phospholipids are low molecular weight surfactants (**surface active agents**) with a pronounced amphiphilic nature due to the coexistence of a hydrophilic head group and a hydrophobic tail. They can be mainly classified into two categories: glycerophospholipids and sphingolipids (figure 1.2). The former are molecules built on a glycerol backbone to which two fatty acids are esterified in the sn-1 and sn-2 positions.

The third hydroxyl group is linked with a phosphate group where different organic residues can be attached (choline, ethanolamine, inositol and serine). Sphingolipids are molecules derived from sphingosine, a long chain C12-22 aliphatic amine containing either two or three hydroxyl groups. In case where the amino group is linked with a fatty acid, a ceramide is formed. On this ceramide unit, an organophosphate group can be bound to form a sphingophospholipid (e.g. phosphocholine in the case of sphingomyelin, SM) or a saccharide to form the sphingoglycolipids (Dewettinck et al., 2008; Yang, Yu et al., 2004). The most important kinds of phospholipids present in MFGM are the zwitterionic phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which are the most abundant glycerophospholipids, while smaller amounts of the negatively charged phosphatidylserine (PS) and phosphatidylinositol (PI) are also present. In addition, glucosylceramide (GlucCer), lactosylceramide (LacCer) and gangliosides (Gang) are present in trace amounts (Avalli & Contarini, 2005).

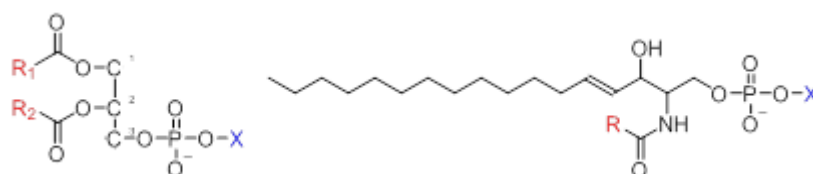


Figure 1.2. General structure of phospholipids: Glycerophospholipid (left) and Sphingolipid (right). R stands for fatty acids and X for the polar head group (e.g. choline or serine).

The composition of the major neutral and polar lipids of MFGM (table 1.1) is well studied and has been extensively reviewed (Jensen, 2002; Keenan & Dylewski, 1995; Singh, 2006). Severe mechanical processing of milk, including homogenization, result to the disruption and release of MFGM material from the fat globules to the serum phase (Huang & Kuksis, 1967; Lilbaek, et al. 2007; Michalski et al., 2002; Patton & Keenan, 1971).

Despite the fact that phospholipids is not the major group in the total milk lipids, they play a significant role affecting numerous functions including protection against food-borne gastrointestinal infections, memory, inhibition of colon cancer, cholesterol trafficking and homeostasis and age related diseases (McDaniel, Maier & Einstein, 2003; Noh & Koo, 2004; Parodi, 1997; Spitsberg, 2005; Sprong, Hulstein & van der Meer, 2002). Moreover, polar lipids are extensively used for their functionality and emulsifying qualities in several food systems. Their combined hydrophilic and

hydrophobic nature constitutes a key parameter for stabilizing the suspension of milk fat in the aqueous environment of milk (Deeth, 1997).

Table 1.1. Lipid composition of MFGM, adapted from Keenan and Dylewski (1995).

Constituent class	Total lipids (%)
Triacylglycerols	62
Diacylglycerols	9
Monoacylglycerols	Traces
Sterols	0.2-2
Free fatty acids	0.6-6
Phospholipids	26-31
Constituent class	Phospholipids (%)
Sphingomyelin	22
Phosphatidylcholine	36
Phosphatidylethanolamine	27
Phosphatidylinositol	11
Phosphatidylserine	4
Lysophosphatidylcholine	2

In the bulk medium (water or oil) phospholipids are present as monomers (figure 1.3), whereas above a certain concentration they start forming aggregates which are characterized by different structures. The limit concentration is known as Critical Aggregation Concentration (CAC) above which all additional phospholipid molecules added to the system are used to form association structures, such as micelles or bilayers (Weschayanwiwat et al., 2005). For practical purposes, phospholipids and surfactants in general can be categorized into two groups, according to their Hydrophilic:Lipophilic Balance (HLB value). Surfactants with high HLB values are used for stabilizing O/W emulsions whereas W/O emulsions are stabilized by surfactants characterized by a lower HLB value (Dickinson, 1993).

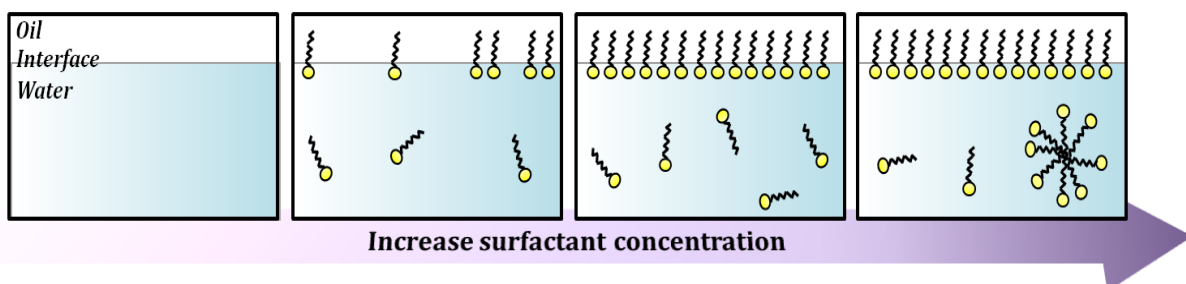


Figure 1.3. Association of surfactant molecules upon increasing the incorporated concentration in an O/W emulsion.

1.2.2. Milk proteins

Milk proteins are traditionally classified in two major groups: the casein and the whey protein fraction. The former accounts for approximately 80 % of the total milk protein and due to its importance in dairy chemistry, it has been studied to a significant extent throughout the years. The casein fraction is primarily found as large colloidal particles of a diameter in the range of 50-600 nm, called casein micelles, with a molecular mass from 10^6 to 10^9 Da, which are able to scatter light and this attributes to the white color in skim milk (Fox & McSweeney, 1998). They are composed of four individual gene product components, denoted α_{s1} -, α_{s2} -, β - and κ -casein, which differ in primary structure (De ruif et al., 2012; Mezzenga & Fischer, 2013) and they are highly porous hydrated with about 4 g of water/g of casein (Dupont et al., 2011). The individual casein molecules and the casein aggregates have multiple hydrophobic regions, regions of high negative charge, some regions of positive charge and yet other regions that can be very susceptible to proteinases. It is the unique combination of these characteristics that makes the caseins such valuable food ingredients (Creamer & MacGibbon, 1996).

Despite the fact that caseins represent one of the most widely investigated cases of food protein, their exact structure has been the center of discuss during the past decades, which has not yet been conclusively solved. Several models of casein micelle structure have been proposed, from which the submicelle model is the most enduring (figure 1.4). This model was elaborated by Schmidt (1982) and Walstra (1990, 1999) who described casein micelles as structures composed of smaller proteinaceous subunits, the submicelles, held together by calcium phosphate clusters (figure 1.4, a, b). According to Walstra (1999), submicelles may differ in composition and they can be classified into two main groups: one primarily consisting of α_s - and β -caseins, and the other of α_s - and κ -caseins. The ones containing the κ -caseins, are located on the exterior of the micelle structure, where the C-terminal molecular part of κ -casein molecules is predominately present as a hairy layer protruding to the outside of the micelle core providing them stability against flocculation (figure 1.4, b).

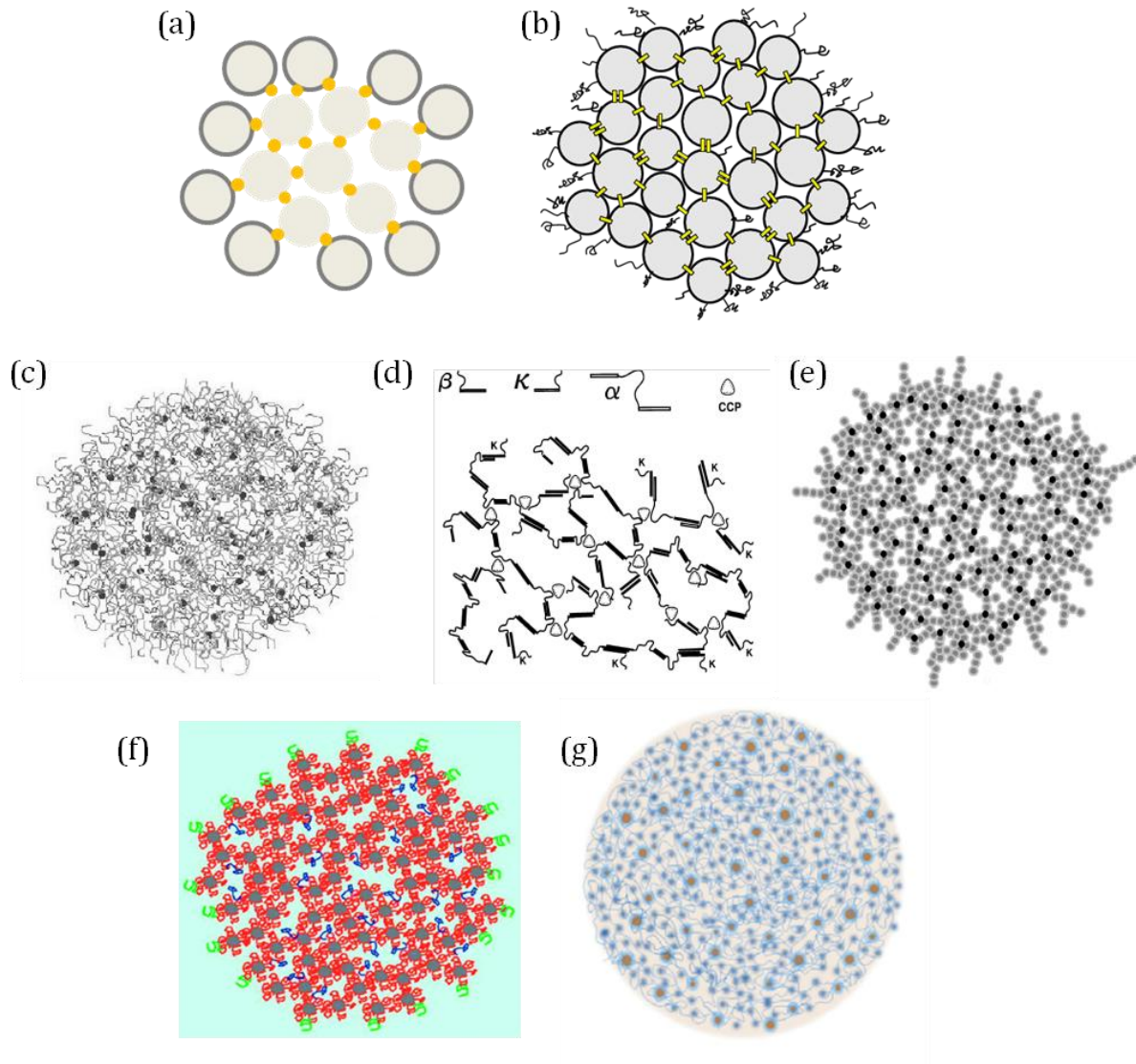


Figure 1.4. Schematic representation of casein micelle models proposed by Schmidt (a), Walstra (b), Holt (c), Horne (d), McMahon and Oommen (e), Dalglish (f) and De Kruif et al. (g).

This model of submicelles held together by calcium phosphate clusters was afterwards evolved into a new model proposed by Holt (1992). He proposed that the casein micelles are formed by unfolded casein chains (figure 1.4, c) binding the calcium phosphate clusters, similar to a microgel cross-linked by weak interactions (i.e. hydrophobic interactions, hydrogen bonding, ion bonding, and electrostatic interactions). This model is known as the nanocluster model, based on the fact that the calcium phosphate nanoclusters are acting as the cement holding the unfolded protein chains together. The model from Holt was further improved by Horne (1998) who proposed the dual binding model (figure 1.4, d) in which binding occurring between the hydrophobic regions of α - and β -casein is sufficient to hold together the structure, with the κ -casein chains acting as termination units sitting in the external parts of micelles. Later studies based on high-

resolution transmission electron microscopy micrographs, McMahon and Oommen (2008), proposed an interlocking lattice model of the casein micelle supramolecule, in which the calcium phosphate nanoclusters act as supramolecular bridges for the casein micelles, leading to a fractal network in which casein molecules and water are highly segregated (figure 1.4, e). In a recent review Dalgleish (2011), pointed out that the coexistence of structural heterogeneities with a high water content in the casein micelles are compatible with an internal structure of casein micelles rich in water channels (figure 1.4, f). De Kruif et al. (2012), showed that the internal structure of the casein micelles is homogeneous and is most likely formed by unfolded protein chains cross-linked by calcium phosphate nanoclusters, results which seem to be in line with the model proposed by Holt (figure 1.4, g).

The whey protein fraction, soluble in the aqueous phase of milk, represents the remaining 20 % of milk proteins which are primarily present as a mixture of β -lactoglobulin (β -lg), α -lactalbumin (α -lac), bovine serum albumin (BSA) and immunoglobulins. Due to their important biological (nutritional value, digestibility, amino acid pattern, sensory characteristics), and physico-chemical functional properties, whey proteins are widely used as food ingredients and they have been studied more extensively than any other food protein (Dissanayake & Vasiljevic, 2009). Emulsifying, foaming, solubility, mineral binding, gelation, taste and color contribution, constitute only some of the very important attributes of whey proteins which are utilized in plentiful food systems, such as bakery, confectionary, infant foods, meat and fish products and nutritional foods.

Whey proteins and caseins are differentiated from each other by their physical and chemical properties. Whereas caseins have a random molecular structure and precipitate in acidic conditions as well as in the presence of polyvalent salts, whey proteins have a compact globular structure and high solubility due to a large proportion of surface hydrophilic residues (Dissanayake & Vasiljevic, 2009).

Within the whey proteins, β -lg, member of the protein family of lipocalins, constitutes a small globular protein of 162 amino acids and a monomeric molecular mass of 18.3 kDa which represents more than 50 % of the total whey protein fraction in bovine milk (Verheul et al., 1998). At room temperature and at physiological pH, β -lg (figure 1.5) is

predominantly present as a dimer whose globular structure results from the disulphide bridges formed between the cysteine residues Cys66-Cys160 and Cys106-Cys119, whereas a free sulphhydryl group (-SH group) exists at position Cys121 (Galani & Apenten, 1999; Hansted et al., 2011; Mercadante et al., 2012; Verheul et al., 1998). Its secondary structure involves one α -helix, eight anti-parallel β -sheets that constitute about 50 % of the protein's secondary structure, and some random coils (De Wit, 2001; Galani & Apenten, 1999; Kontopidis et al., 2004; Monaco et al., 1987; Moro et al., 2011; Viseu et al., 2004).

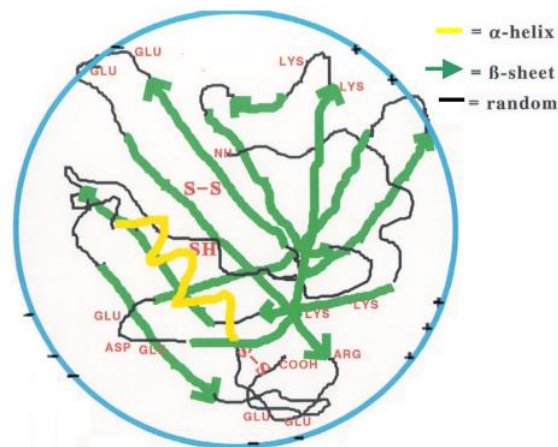


Figure 1.5. Structure of β -lactoglobulin from De Wit (2001).

The protein is shaped in such a way which forms a conical calyx or cavity, inside which a hydrophobic ligand may be located (Lefèvre & Subirade, 2000; Wu, Perez, Puyol, & Sawyer, 1999). Due to this structure, β -lg is characterized by the ability of binding a range of different amphiphilic or hydrophobic molecules, such as surfactants, phospholipids, fatty acids, alkanes and retinol (Lefèvre & Subirade, 2001; Sawyer et al., 1985; Zhang & Keiderling, 2006). α -lac, whose molecular weight is about 14.2 kDa, is stabilized via four disulphide bonds and does not contain a free thiol group (Schokker, Singh, & Creamer, 2000).

1.3. Effect of processing on milk constituents

Several stages during milk processing have been suggested to modify the functional properties of the principal components and especially milk proteins, thus affecting the structure and properties of the final product (Nicorescu et al., 2008). High-pressure treatment (Kielczewska et al., 2006; Lopez-Fandiño et al., 1996), shear rate, enzymatic hydrolysis (Chobert, Bertrand-Harb, & Nicolas, 1988; Turgeon, Gauthier, & Paquin,

1991) and most importantly, heating at mild or severe temperatures (Gulzar et al., 2011; Rattray & Jelen, 1997; Singh, 2004; Verheul et al., 1999), constitute some of the techniques which target to the production of a final product which preserves its nutritional value and it also satisfies the needs of the consumer. In addition, variations in processing conditions including pH, treatment temperature and rate of heating as well as ionic strength can strongly influence the outcome (Chen et al., 2000).

1.3.1. Effect of heat treatment

The idea of preserving milk by thermal processing dates back to 1856 when Gail Borden patented a method for heating and condensing milk under vacuum, followed by addition of sugar for preservation (Holsinger, Rajkowski, & Stabel, 1997; Singh, 2004). During the First World War, condensed milk became one of the most important dairy products, due to its easy transport and long shelf-life (Singh, 2004). However, several problems emerged including milk gelation or coagulation during heat treatment. This problem was controlled by examining the raw milk, with the intention to understand and justify the factors influencing the heat stability of milk. Webb (1928) suggested that for every milk there is an optimum pH, the attainment of which will result in maximum heat stability for the system involved. The effect of heating prior to evaporation (forewarming), which is now a standard commercial practice for the manufacture of evaporated milk, was studied in detail by Webb and Bell (1942), who showed that it may lead to increased heat stability. Rose (1961) revealed the relevance of pH upon heating milk, whereas a decade after, the impact of β -lg and κ -casein on heat stability started to be unraveled. Important changes which occur in milk upon severe heating were listed by Fox (1981), including pH decrease, Maillard browning, casein modification, changes in micellar structure and denaturation of whey proteins and interaction with casein micelles.

In spite of the extraordinary work which has been accomplished over the years, some aspects of the mechanism of heat coagulation have not been completely explained at a molecular level yet.

1.3.1.1. Effect of heat treatment on milk proteins

Heat treatment, which is considered to be the most commonly used and perhaps inevitable method in food processing, has been shown to affect the structure and functionality of milk proteins and particularly the whey fraction. The effect of heating on the conformational and chemical properties of milk proteins, usually called denaturation, has been extensively examined in the literature (Nicorescu et al., 2008; Raikos, 2010). Caseins in micellar form are considered to be very thermostable due to the low levels of secondary and tertiary structures. However, some researchers noted that drastic heat treatment could possibly lead to their complete coagulation (Howat & Wright, 1934). Furthermore, heating in the range of 80-150°C induces modifications in caseins, including (1) dephosphorylation, (2) proteolysis, (3) covalent bond formation, and (4) changes in casein micellar structure (Robin, Turgeon & Paquin, 1993).

- (1) Belec and Jenness (1962) mentioned that the dephosphorylation of casein in milk occurred within one hour at heating temperatures ranging from 110 to 140°C. Before that, casein dephosphorylation upon heating was extensively examined by Howat and Wright (1934), who suggested that after five hours of continuous heating at 120°C, a sodium caseinate solution has been completely dephosphorylated; dephosphorylated casein is less able to bind calcium and is more heat labile. In a subsequent experimental study, the same authors proposed that casein heat coagulation and dephosphorylation are two correlated reactions: for each rise in temperature of 10°C, the reaction velocity of dephosphorylation and coagulation increases three-fold (Howat & Wright, 1936).
- (2) Alais, Kiger and Jollès (1967) examined the behavior of caseins during heating, focusing on the degradation of cow κ -casein. They found that when isolated κ -casein was heated for 20 min at 120°C, a peptide was formed which was similar to the caseino-glycopeptide, a peptide released by the action of rennin on casein. Similarly, Hindle and Wheelock (1970) reported the appearance of glycopeptides (i.e. peptides which contain carbohydrate moieties) at temperatures as low as 50°C and found that there may be a production of glycopeptides during storage of heat-sterilized milk or milk products over a long period.
- (3) During heat treatment, reactions may take place between reactive side chains of some amino acids, such as cysteine or lysine, and other amino acid residues, carbohydrates, or lipids. The browning which occurs upon heating milk at

elevated temperatures is a consequence of the Maillard reaction, whereby lactose and lysine are the reactants (Tran Le, 2010).

- (4) Heating milk at high temperatures causes changes in micellar structure and especially an increase in casein micelle size. Carroll, Thompson and Melnychyn (1971) proposed that this phenomenon is a result of the interaction of heat denatured whey protein and other serum components with the micelle surface, as well as due to the increase in calcium content on the surface of the casein micelle which certainly leads to calcium bridging among micelles with a concomitant increase in micelle size.

Despite of its significance in food manufacturing, the whey protein fraction faces a major obstacle restricting its application and this is due to its instability upon heating at elevated temperatures. Upon heating above 60°C, globular proteins, and especially β -lg, undergo conformational changes and partially unfold. This phenomenon is known as denaturation during which previously hidden hydrophobic groups, as well as the free thiol group are exposed (Croguennec, O'Kennedy, & Mehra, 2004; Iametti, Gregori, Vecchio, & Bonomi, 1996; Verheul et al., 1998). Denaturation decreases protein solubility compared to native protein, and leads to aggregation of whey proteins with themselves or with caseins (Pelegrine & Gasparetto, 2005). These alterations at molecular level may have an impact on protein functionality which is sometimes required but other times can be disadvantageous (Raikos, 2010).

1.3.1.2. Heat-induced whey protein-casein interactions

Upon treating milk at elevated temperatures, numerous reactions may occur, including extensive denaturation and aggregation of whey proteins and complex formation between whey proteins, caseins and fat globules (Corredig & Dalgleish, 1999; Jeurink & De Kruif, 1993; Nielsen, Singh, & Latham, 1996; Verheul et al., 1998). According to Rüegg, Moor and Blanc (1977), immunoglobulins and serum albumin are the most easily denatured, β -lg is intermediate, whereas α -lac is the most heat stable. The kinetics of protein denaturation and aggregation are highly influenced by several factors, including treatment temperature, ionic strength, pH and rate of heating (Boye et al., 1995; Chen et al., 2000).

As stated by Gough and Jenness (1962), thermal denaturation of β -lg proceeds through several steps. At room temperature and at conditions found in milk (pH, salt concentration), β -lg predominantly exists as a dimer (Gough & Jenness, 1962; Verheul et al., 1999). As the temperature increases, dimer dissociation into monomers takes place, and finally the monomers start to unfold at temperatures higher than 65°C (Nielsen et al., 1996).

The main reactions during heat treatment of milk are believed to be (1) the complex formation between β -lg/ κ -casein, (2) the interaction between denatured β -lg molecules with themselves, (3) the complex formation between β -lg aggregates/ κ -casein and (4) the interaction between β -lg/ α -lac and subsequently with κ -casein (Corredig & Dalgleish, 1996, 1999; Guyomarc'h, Law & Dalgleish, 2003; Kessler & Beyer, 1991; Oldfield, Singh, Taylor & Pearce, 2000).

According to previous research (Dalgleish, Mourik, & Corredig, 1997; Dalgleish, Senaratne & Francois, 1997; Elfagm & Wheelock, 1978), β -lg, or β -lg together with α -lac molecules might interact directly with each other during the initial stages of heating, resulting in the formation of soluble-primary aggregates, mainly through hydrophobic and disulfide bonding, and subsequently this product forms complexes with κ -casein on the surface of the casein micelle. Some other studies (Anema, 2007) mentioned that κ -casein dissociates from the micelles during heating milk at high temperatures and this evidence suggests that formation of the heat-induced whey protein/ κ -casein serum complexes is somewhat related to dissociation of κ -casein. Therefore, the question arises as to whether the κ -casein dissociation takes place before or after its interaction with the denatured β -lg, or with the primary heat-induced whey protein aggregates (figure 1.6).

Hydrophobic interactions may have a significant part within the aggregates, but the role of disulfide bonds in both the initial aggregation period and in attaching large aggregates in later stages seems to be major. Denaturation of β -lg during heating, leads to the exposure of the previously buried hydrophobic groups as well as of the free thiol group at Cys121. The exposed thiol group may now induce thiol-disulfide interactions resulting into the formation of disulfide linked aggregates (Anema & Li, 2003a; Croguennec et al., 2003; Jang & Swaisgood, 1990; Sawyer, 1969; Schokker et al., 2000).

The contribution of thiol-disulfide exchange reactions in the heat-induced aggregation of milk proteins has been extensively investigated. Using a combination of SDS-PAGE with matrix-assisted laser desorption and ionization-time-of-flight mass spectroscopy (MALDI-TOF MS), Surroca, Haverkamp, & Heck (2002), showed that aggregation of β -lg upon heating at 68.5°C, is driven by thiol-disulfide bond formation between Cys121-Cys66, Cys160-Cys160 and Cys121-Cys160.

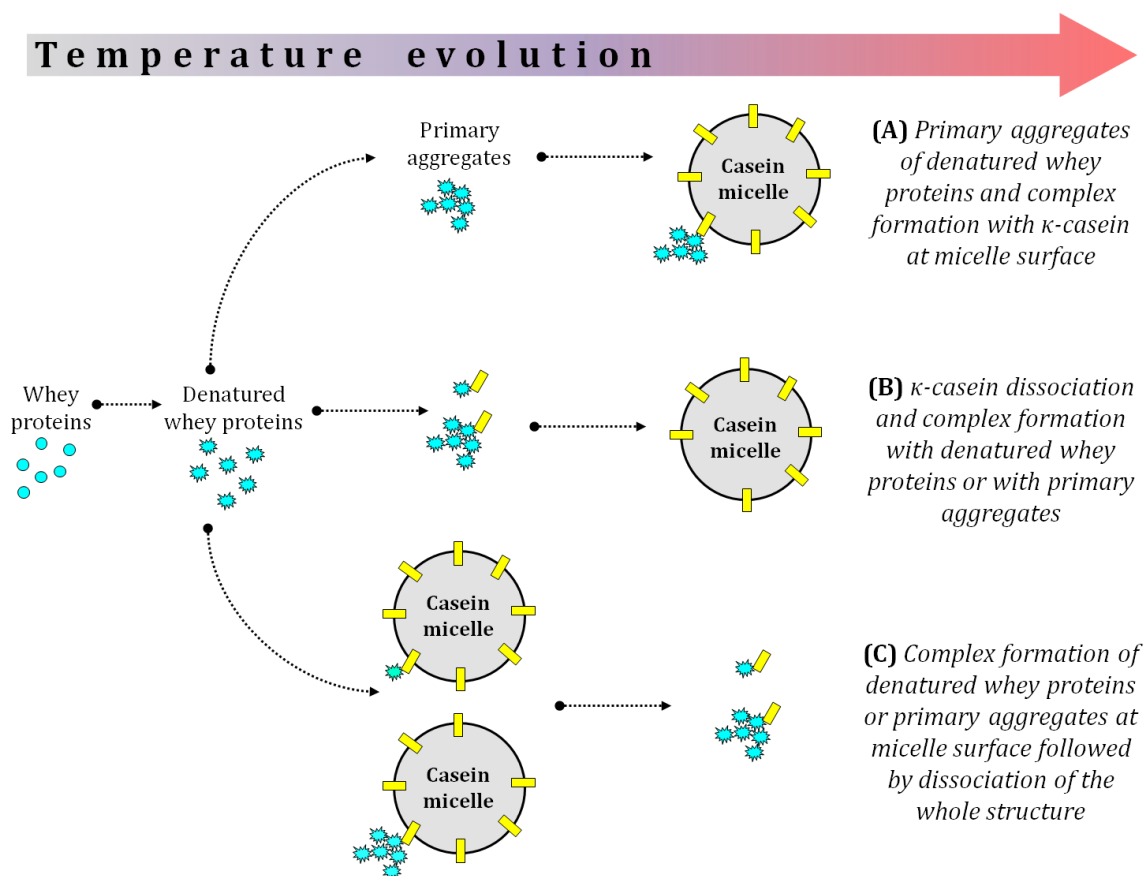


Figure 1.6. Schematic representation of possible mechanisms for formation of the heat-induced whey protein/ κ -casein complexes in heated skim milk (adapted from Donato & Guyomarc'h, 2009).

Interestingly, Henry, Mollé, Morgan, Fauquant & Bouhallab (2002) identified that a disulfide bond between the Cys160 of β -lg and Cys88 of casein was formed upon heating casein micelles and β -lg isolated from goat's milk. Livney & Dalgleish (2004) found that Cys119 and 121 of β -lg were involved in bond formation with all the other possible cysteines of both β -lg and κ -casein. In addition, the authors mentioned that Cys106 was not found to bond to κ -casein, a fact which suggests that it remains buried in the core of the protein during heating. Evidence demonstrated that α -lac is also involved in the formation of whey protein/ κ -casein complexes (Noh & Richardson, 1989), whereby

disulphide bond formation between β -lg and α -lac upon heating, has been shown to particularly involve Cys111, 120, 61 and 6 of α -lac (Livney, Verespej & Dalgleish, 2003).

1.3.2. Effect of milk composition

The composition of the milk system prior to heating constitutes a critical parameter regarding the formation and type of complexes. Several studies related to the effect of different concentrations of protein, non-protein soluble components and lactose on the whey protein denaturation in skim milk have been conducted. Anema (2000) and Anema, Lee & Klostermeyer (2006) found that β -lg and α -lac denaturation was increased by increasing the protein concentration, whereas increasing the concentration of non-protein soluble components resulted into a delayed protein denaturation, which was ascribed to the increased lactose content in the milk. Similarly, Law & Leaver (1997) examined the effect of protein concentration on the whey protein thermal denaturation and concluded that denaturation of both β -lg and α -lac was increased when the milk protein concentration was doubled and the milk was treated at 80°C. In addition, Boye et al. (1995) mentioned that for a certain kind of protein, a critical concentration is required for the formation of a gel and also, the type of gel formed at a given temperature fluctuates with protein concentration. According to Corredig & Dalgleish (1996 and 1999), the original amount of β -lg and α -lac present in milk will eventually affect the composition of the final micelle-bound complex. More precisely, when α -lac was added to skim milk prior to treatment at 80°C, so that the concentrations of β -lg and α -lac were comparable, the proteins had the same reaction kinetics and similar amounts were found associated with the casein micelle. When β -lg was added, the amount of β -lg/ κ -casein reached a plateau value not significantly different from that of control milk. The findings proposed that only a particular number of binding sites were available for the interaction between β -lg and casein micelles, whereas the amount of α -lac associated with the micelles was proportional to its concentration in milk. The casein/whey protein ratio might also play an important role in the heat-induced aggregation of milk proteins. Upon investigating the thermal aggregation in model systems with a varying casein/whey protein concentration, Beaulieu, Pouliot & Pouliot (1999) confirmed that higher whey protein concentrations led to the formation of new particles when more whey protein and/or less casein were present. Surprisingly, when isolated κ -casein was added in milk, only little effect was noticed in the size or the amount of soluble

complexes formed upon heating of milk (Donato et al., 2007). However, the presence of caseins, other than κ -casein, such as α_s - or β -casein, demonstrated a protective role toward whey protein heat-induced aggregation, even at acidic pH values (O'Kennedy & Mounsey, 2006).

1.3.3. Effect of pH

Heat stability of milk refers to its ability to endure high processing temperatures without visible coagulation or gelation, whereas the heat coagulation time (HCT) is defined as the heating time required for visible coagulation (Singh, 2004; Van der Meeren et al., 2005). HCT is influenced by numerous factors, from which pH seems to play a critical role. The effect of temperature and pH on the association behavior between whey proteins and casein micelles was widely investigated over the decades by numerous researchers (Anema & Li, 2003b; Beaulieu et al., 1999; Pelegrine & Gasparetto, 2005; Rattray & Jelen, 1997; Singh & Fox, 1985, 1986; Smits & van Brouwershaven, 1980). All studies concluded that the pH at which milk is heated plays a determinant role for the extent of casein dissociation from the micelle, as well as for the whey protein association with the micelle.

Smits and van Brouwershaven (1980) showed that upon treating model milk systems at 90°C for 20 min, 83 % of β -lg was associated with the micelles at pH 5.8, and this decreased to 76 % at pH 6.3, 44 % at pH 6.8, and 24 % at pH 7.3. A very helpful schematic representation of whey protein-casein micelle association occurring in milk, was proposed by Vasbinder & De Kruif (2003), as a function of the pH and during heating at 80°C for 10 min (figure 1.7).

They suggested that heating at pH 6.55 lead to a rather uniform coverage of casein micelles by whey proteins, whereas at higher pH values, only a small fraction of the whey proteins is involved in the coating, while the rest is present in the bulk as large whey protein aggregates. When the pH decreases below 6.55, a more inhomogeneous whey protein coating of the casein micelles takes place and hardly any soluble aggregates are formed

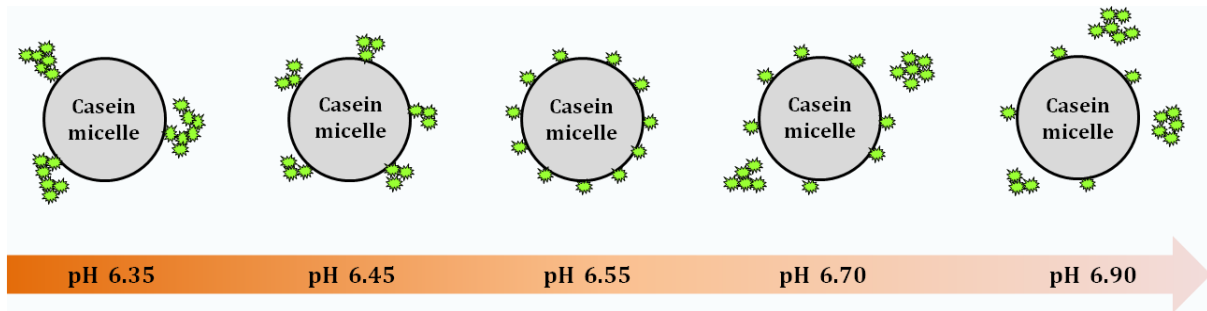


Figure 1.7. Schematic illustration of heat-induced interactions between casein micelles and whey proteins in milk during heating at 80°C for 10 min, at varying pH values. Green spheres represent denatured whey proteins and grey spheres the casein micelles. Whey proteins are either present in aggregates or covalently associated with the casein micelle. Native whey proteins are not shown in the scheme (adapted from Vasbinder & de Kruif, 2003).

After processing skim milk at temperatures in the range 75-90°C and at pH values of 6.8, 6.2 and 5.8, Corredig and Dalgleish (1996) concluded that a faster reaction of whey proteins with casein micelles was found at lower pH and higher temperatures. More precisely, the authors reported that when milk was treated at its natural pH (6.8), both β -lg and α -lac appeared to interact similarly with casein micelles, at temperature up to 85°C. At lower pH (6.2), the reaction took place within a shorter period due to faster heat-induced interactions, whereas when the pH value was decreased to 5.8, visible coagulation of milk occurred after a few min of treatment, at all of the temperatures employed.

Anema and Li (2003) monitored the changes in casein micelle size together with the level of whey protein denaturation and of protein interaction, upon treating milk at temperatures from 80 to 100°C, while adjusting the pH between 6.5 and 6.7. They concluded that the size of casein micelles was remarkably affected by the pH and heating; whereas at high pH values (6.7), the size of casein micelles remained almost intact, when the pH decreased to 6.5, the size was markedly increased (up to 30-35 nm). The authors related the size changes to the increased interaction of the denatured whey proteins with the casein micelles. They also mentioned that the changes in pH did not affect the rate of whey protein denaturation, but only the association behavior of the denatured whey proteins with the casein micelles: up to 70 % of the total denatured whey protein associated with casein micelles at pH 6.5, whereas at higher pH (6.7), the associated amount was decreased to 30 %.

Similarly, Oldfield et al. (2000) investigated the interaction of whey proteins with casein micelles upon regulating the pH of milk samples to 6.48, 6.60 and 6.83, and heating at different temperature-time profiles. The researchers observed that upon heating at 90°C, approximately 90 % of the whey protein associated with the casein micelles at pH 6.48, whereas at pH 6.60 and 6.83, the amount was decreased to 80 and 60 %, respectively. Oldfield et al. (2000) and Anema and Li (2000) proposed that the decreased association of whey proteins with the casein micelles at high pH values was due to the partial dissociation of κ -casein from the micelles. As the disulfide bonds of κ -casein are placed near the boundary between the para- κ -casein region (associated with the micelle core) and the glycomacropeptide (the flexible hair), the denatured whey protein is required to penetrate through the entire hairy layer in order to interact with the disulfide bond of κ -casein. As the pH is increased, the micelle surface charge and, especially, the charge on the hairs will increase. This will cause the hairs to extend further from the micelle surface and hence may reduce the tendency of interactions between denatured whey proteins and casein micelles. However, at higher pH values the propensity for serum phase aggregation reactions may occur, either among the denatured whey proteins or even between the denatured whey proteins and the low levels of serum phase κ -casein (Anema & Li, 2003).

1.3.4. Effect of homogenization

Homogenization constitutes an important mechanical process, widely used in food industry, aiming to the production of emulsions, where the disperse phase is uniformly distributed throughout the continuous phase. This process is crucial in defining the stability of the formed emulsions, as well as their rheological properties, consistency, color or even taste of the final product (Stang et al., 2001).

During homogenization of milk, the average fat droplet diameter decreases to less than 1 μm , whereas the milk fat surface area increases by 4 to 10 times. This causes association of surface active compounds, present in the serum (mainly caseins plus whey proteins), with the fat globule surface after homogenization, since the MFGM is damaged and insufficient to cover the newly formed fat surfaces (Cano-Ruiz & Richter, 1997; Keenan et al., 1983; Michalski et al., 2002). Several equipments are available for this purpose, including dynamic high pressure homogenizers and microfluidizers, performing at varying pressures and temperatures.

Conventional homogenization was patented by Auguste Gaulin in 1899 and today it has been extensively adopted by the dairy industry. Homogenization is usually performed at 60°C using a two-stage homogenization process, in which the first stage decreases the size of fat droplets, whereas the second stage disrupts clusters that may be formed into individual fat globules (Zamora et al., 2007). The newly formed membrane after homogenization constitutes an important parameter for the stability of the emulsions upon heat treatment. It has been previously shown that homogenization decreases the heat stability of milk and therefore, the manufacturer has to confront a critical dilemma between products that are heat stable, but have the tendency to cream due to low pressure or no homogenization, and those that are physically stable but upon heating may undergo coagulation (Deysner et al., 1929; Kielczewska et al., 2006; Webb & Holm, 1928).

McCrae & Muir (1991) evaluated the effect of homogenization on the heat coagulation time of recombined milk, upon heating at 140°C. Their results showed that by intensifying the severity of homogenization, from 6.9 to 34.5 MPa, a progressive decrease of the heat stability of milk was induced. However, when the whey proteins were removed from the system prior to homogenization, the effect was moderated and this was explained by two possible mechanisms: (1) the presence of serum proteins had a detrimental effect on the heat stability due to their adsorption on the surface of fat globules and/or (2) the presence of whey proteins led to their interaction with the surface adsorbed caseins during heating, hence inducing coagulation. However, when the whey proteins were re-incorporated into the system after homogenization, this resulted into a similar heat coagulation time to the one observed without removing the whey proteins from the system. Therefore, it is more likely that the second mechanism is responsible for promoting heat coagulation upon heat treatment of recombined milk and that the fat surface area rather than the casein load affected these interfacial protein-protein interactions during heating.

1.4. Improving the heat stability of milk emulsions: surface active agents

Due to their important technological and functional properties, surfactants have been extensively used in numerous applications in many domains including food processing, cosmetics, paints and pharmaceuticals. The main purpose of utilizing these molecular

substances in food and particularly in dairy industry is to assist the emulsification process.

Low molecular weight surfactants are characterized by their ability to migrate rapidly to the interface, forming a membrane which surrounds the fat droplets and thus preventing coalescence and flocculation (McSweeney, Healy & Mulvihill, 2008). Lecithin is a well-known low molecular weight emulsifier available as a natural component of both animal and vegetable products, with the most common commercial source being soy beans and egg yolk (Dickinson, 1993; Yamamoto & Araki, 1997). It is composed of a mixture of phospholipids, of which the most abundant species are phosphatidylcholine and phosphatidylethanolamine (Dickinson & Yamamoto, 1996).

Previous research studies demonstrated that lecithin can influence the heat stability of whey protein containing emulsions by displacing proteins or interacting with proteins bound on the interface and/or by interacting with free proteins in solution, including β -lg (Crujisen, 1996; Hardy et al., 1985; McCrae, 1999; McSweeney, Healy, & Mulvihill, 2008; Tran Le et al., 2007). However, the effects have been described either as positive or negative, depending on the type and amount of added lecithin (Yamamoto & Araki, 1997). The effect of soya lecithin on the heat stability of concentrated milk was studied by Hardy et al. (1985b). The authors found that upon lecithin addition (0.2 % w/w), the heat coagulation time of homogenized evaporated milk was increased by approximately 30 min. Similarly, McCrae & Muir (1992) showed that incorporation of soya lecithin at a varying concentration range is capable of improving the heat stability, over a wide pH range. However, the beneficial effect of lecithin was lost as the concentration reached a threshold value. When McSweeney et al. (2008) studied the effect of lecithin on the heat stability of a model infant formula emulsion, they noticed an upward shift of the heat coagulation time, upon heating at 140°C, and by increasing the concentration of the added lecithin. More precisely, at pH 6.6, the heat coagulation time was increased from 5 min, to approximately 6, 17, 22, 24 and 25 min, upon increasing the concentration of lecithin from 0, to 1, 2, 3, 4, and 5 g/L, respectively. In addition, the researchers performed experiments to determine the particle size distribution of emulsions containing the same range of lecithin concentration. The results revealed that after heating at 140°C for 10 min, not obvious changes were noticed.

However, as the heating continued, the emulsion developed larger aggregates, which was not the case when the emulsions were treated in the presence of lecithin (figure 1.8). The authors attributed the beneficial effect of lecithin to the protein displacement from the interface of emulsion droplets by surfactants and/or changes in protein conformation at the interface or in the serum phase due to interactions between proteins and surfactants.

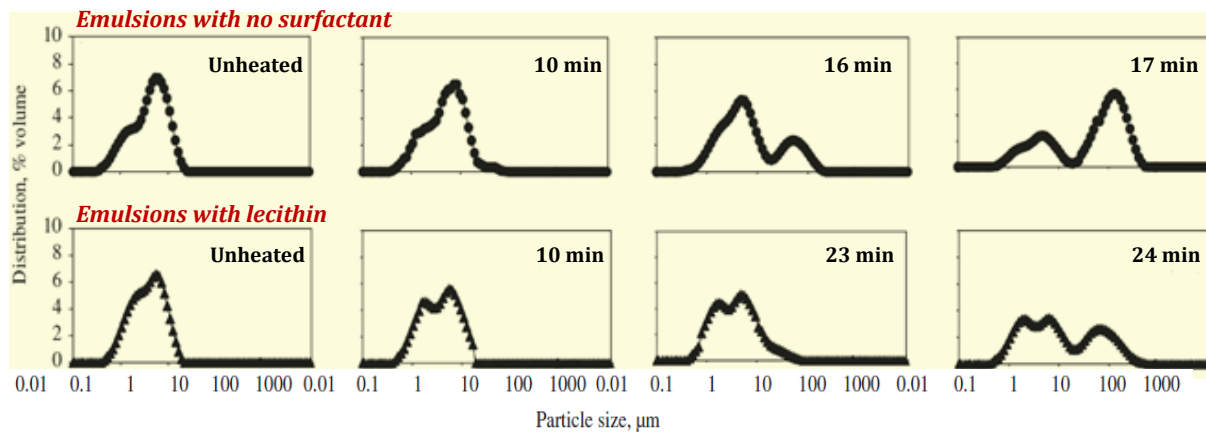


Figure 1.8. Particle size distributions of emulsion with no added surfactant and emulsion containing 5 g/L added lecithin, heated at 140°C for the time indicated (McSweeney et al., 2008).

Cabezas et al. (2011), McCrae (1999), Sünder, Scherze, & Muschiolik (2001), Tran Le et al. (2007) and Van der Meeren et al. (2005) described the enhanced heat-stabilizing effects of hydrolyzed over native lecithin, after its inclusion in O/W emulsions. More precisely, Tran Le et al. (2007) demonstrated that the effect of hydrolyzed soybean lecithin in decreasing heat-induced whey protein-casein interactions was significant, whereas the effect of non-hydrolyzed lecithin was almost negligible. Similarly, particle size analysis performed by Sünder, Scherze and Muschiolik (2001) and Cabezas et al. (2011), as well as heat stability evaluation by McCrae (1999), found hydrolyzed lecithins to be most effective, whereas Jost, Dannenberg and Rosset (1989) mentioned that hydrolyzed lecithin incorporation, before or during emulsification, reduced the strength of a heat-set whey protein emulsion gel.

Apart from lecithin, phospholipid enriched dairy by-products, such as buttermilk, have also been applied and exploited for their heat-stabilizing properties. Buttermilk is the liquid product obtained during churning of cream in the butter making process. It contains all the water-soluble components of cream such as milk proteins, lactose and minerals (Sodini et al., 2006; Vanderghem et al., 2010). Buttermilk has been used as

animal feed or has been dried to be incorporated in bakery products due to its impact in flavor (Vanderghem et al., 2010). However, through the years it acquired a considerable potentiality because of its high content in MFGM material and therefore in phospholipids (Rombaut, Dejonckheere, & Dewettinck, 2007). The MFGM is fractured during churning and migrates into the buttermilk portion. In a quite similar way, a MFGM-enriched side stream is also obtained during butter oil production by mechanical treatment to induce phase separation, and centrifugation of concentrated dairy cream. In this case too, the MFGM becomes displaced to the aqueous phase due to the mechanical stress.

The mechanisms by which surface active species can improve the heat stability of dairy emulsions have been considerably examined by several researchers during the past decades. Dickinson et al. (2007) explained some possible mechanisms by which low molecular weight surfactants have an impact on the stability of dairy emulsions, by (1) decreasing the surface tension, thus resulting in the formation of small fat droplets during homogenization, (2) displacing protein from the fat globule interface, that may otherwise be available for bridging flocculation, (3) interacting with interfacial protein, leading to a thicker and stronger adsorbed layer and (4) increasing the viscosity of the aqueous phase through the formation of self-bodying structures.

Low-molecular weight surfactants and emulsifiers are usually more surface active as compared to proteins, and will consequently compete for the interfacial area (Wilde et al., 2004). Competitive adsorption between many surface-active components at the interface controls the composition of the stabilizing layer at the emulsion droplet interface (Dickinson & Gelin, 1992). Over time, surfactants may partly or completely displace adsorbed protein from the droplet interface, depending on the type and concentration of the incorporated surfactant.

The competitive displacement of proteins by surfactants from the interface has been experimentally identified. The studies of Heertje et al. (1996) on the protein displacement by phospholipid emulsifiers showed that at very low concentrations of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (0.1 g DMPC per kg), partial displacement of the protein was already noticed, whereas 1,2-dipalmitoyl-3-phosphatidylethanolamine (DPPE) resulted in complete protein displacement from the interface at even lower concentrations (50 mg DPPE per kg).

Using 50 wt. % O/W emulsions, de Feijter, Benjamins & Tamboer (1987) found protein displacement by surfactants. Their results showed that the influence was hardly affected by the type of the proteins which were studied (β -lg and β -casein), whereas it was strongly affected by the type and the concentration of the surfactant employed.

Similarly, Courthaudon, Dickinson & Dalgleish (1991) and Courthaudon, Dickinson, Matsumura & Clark (1991), showed that the type of surfactant constitutes a key parameter in protein displacement, and particularly they proved that water soluble surfactants, such as Tween 20, are more effective in displacing proteins from the interface. In addition, the authors mentioned that the competitive adsorption of β -casein and surfactants is effectively dependent on whether the surfactant is present before or after emulsification. More specifically, their experimental analysis showed that the addition of water-soluble nonionic surfactants after emulsification leads to complete protein displacement, whereas upon addition before homogenization, there was only a partial displacement.

Apart from competing with proteins for place at the surface, emulsifiers can also interact with proteins which are situated not only at the interface but in the serum phase as well. According to McSweeney et al. (2008) and Agboola et al. (1998), the heat stability of milk is achieved through a combination of protein displacement of adsorbed protein by surfactant and formation of protein/surfactant complexes.

Tran Le et al. (2007) explained that phospholipids constitute a stabilizing factor of the hydrophobic as well as the free thiol groups of denatured proteins and therefore there is less possibility of interaction with hydrophobic and/or thiol groups of neighboring denatured whey protein molecules. Van der Meeren et al. (2005) described that the addition of phospholipids reduced the tendency of whey protein denaturation and/or interaction with casein micelles. They proved that the denaturation temperature of whey proteins was shifted from approximately 76 to 81°C, upon utilization of hydrolyzed lecithin.

The heat stabilizing effect of phospholipids against whey protein aggregation is dependent on the phospholipid molecular structure (Kristensen et al., 1997). Goddard & Ananthapadmanabhan (1993) mentioned that anionic surfactants interact strongly with proteins giving rise to protein-surfactant complexes, which could induce the unfolding of

proteins, whereas cationic surfactants exhibit a lower tendency to interact with proteins. On the other hand, non-ionic surfactants generally bind very weakly. This can be ascribed to their lower critical micelle concentration, as well as the absence of electrostatic interaction with the proteins and hence making the formation of micelles a more favorable procedure than binding to proteins.

According to Jones (1992) and Oakes (1974), three consecutive steps possibly take place upon raising the concentration of anionic surfactant: 'specific binding' at low concentration, 'non-cooperative binding' at higher concentration and 'cooperative binding' at even higher concentration (figure 1.9). The first area, during which the lowest surfactant concentrations are used, is associated to the binding to specific binding sites on the protein, and the interactions are mostly electrostatic. The second area is related to a plateau which is associated to non-cooperative interactions. Finally, the third area corresponds to a substantial increase in binding due to cooperative ligand interactions. The unfolding of the protein is believed to take place in this region.

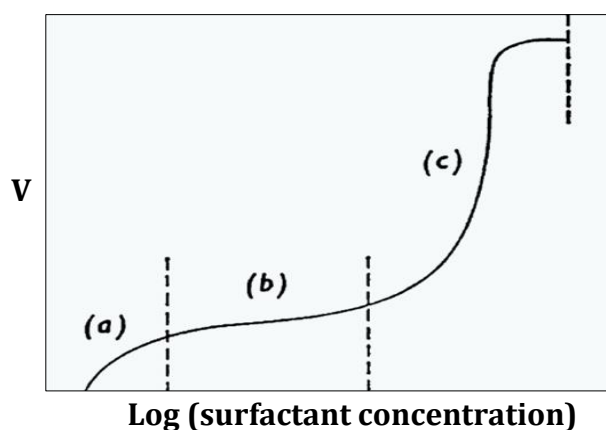


Figure 1.9. Schematic plot of the number of bound anionic surfactant molecules (V) to a water-soluble protein as a function of the logarithm of the concentration of the surfactant. Region (a): specific binding, region (b): non-cooperative binding, region (c): cooperative binding and saturation (adapted from Jones, 1975).

When the binding sites become saturated, there is another plateau which demonstrates that there is no further surfactant binding on the protein and that micelle formation occurs as more surfactant is added.

Lu et al. (2005) showed that high concentrations of the anionic decyl sulphonate $C_{10}SO_3$ had a significant impact on the BSA secondary structure. On the contrary, when the

surfactant was utilized at low concentrations, the binding with the protein was characterized as specific and caused only an insignificant expansion of the protein.

Similarly, Chen & Dickinson (1998), Dickinson & Hong (1997) and Giroux & Britten (2004) described analytically the influence of sodium dodecyl sulfate (SDS) addition, an anionic surfactant, on the solubility of β -lg in water, as well as on the rheology of β -lg-containing emulsions. A significant increase in solubility was observed upon addition at low concentrations since SDS binds strongly to specific binding sites. During this stage, the anionic surfactant causes an increase of the net negative charge of the protein (Chen & Dickinson, 1998; Dickinson & Hong, 1997), providing a stabilizing effect. Upon further SDS addition, the beginning of a co-operative SDS/ β -lg binding occurs, stimulating an extensive unfolding of the protein molecules. As a result, the protein's solubility was declined.

In addition, Gelamo & Tabak (2000) classified surfactants in two groups according to their influence on BSA structure: anionic on the one hand and either cationic or zwitterionic on the other hand. Their results indicated that the protein structure upon the addition of zwitterionic surfactants remained almost intact, whereas it was affected upon the addition of the anionic SDS. Correspondingly, Lefèvre & Subirade (2000, 2001) verified that whey protein denaturation takes place due to the presence of certain phospholipids. More precisely, they concluded that conformational modifications of β -lg did not occur in the presence of the zwitterionic 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), as opposed to the anionic 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG)

The above indications imply that both the polar head group of the phospholipids and their fatty acid composition (which determines their T_m) are key parameters for modulating the phospholipid-induced denaturation process.

1.5. Objectives and outline of the thesis

Heat treatment constitutes one of the most principal unit operations in dairy processing, aiming the manufacture of a stable product with a prolonged shelf life. However, elevated temperatures affect a number of physicochemical attributes of the final product, including viscosity increase, which can sometimes be an unwanted

characteristic for the consumer. The increased viscosity, accompanied with the increased particle size of heated dairy products is a result of a phenomenon known as heat coagulation which is mainly based on the heat-induced interactions between whey proteins and casein micelles. During the past years, several studies have been conducted in order to find answers and solutions to overcome this obstacle. Numerous researchers have reached to the conclusion that the incorporation of surface active molecules such as phospholipids can be the key for coping with this undesirable occurrence. The objective of this thesis is to firstly clarify which are the parameters that induce the initiation of heat coagulation in whey protein containing emulsions and solutions, and furthermore to examine the effect of the incorporation of several phospholipid sources prior to heating at elevated temperatures. Therefore, two different models in this dissertation were used. A similar product to evaporated milk (i.e. the product obtained after the removal of about 60 % of milk's water, and such contains at least 6.5 % milk fat and 16.5 % milk solids-non-fat) was obtained by mixing 6.5 % of a vegetable oil (sunflower oil) and 16.5 % high-heat skim milk powder (SMP) in water, which is referred to as recombined concentrated milk (RCM). High-heat SMP was selected with the purpose to mimic industrial practice, based on the fact that is less sensitive towards heating as compared to the low-heat SMP. In most of the cases, the ingredients were mixed in such concentrations that the amount of the total solids remained constant at 23 %. Additionally, and based on the fact that heat-induced coagulation largely depends on the whey protein fraction of milk, a second system was utilized containing whey protein isolate in the presence of various types of phospholipids.

The dissertation is divided in three parts. The first part mainly focuses on the optimization of a testing procedure, whereas the second part describes the attempts for the prevention or minimization of heat coagulation, by the utilization of phospholipid containing ingredients. Finally, the third part of the thesis outlines the interactions which take place between phospholipids and proteins, before and after heating, which lead to the limitation or prevention of heat coagulation. In addition, the last part analyzes the different effects that phospholipids can exert on heat coagulation, based on the different attributes of their molecular structure.

Chapter 2 and 3 constitute an introductory part of this dissertation, aiming to a better understanding of heat coagulation and the parameters which are detrimental for the

initiation of this phenomenon. Therefore, heating temperature, heating time, homogenization pressure and emulsion protein content are only some of the parameters which are investigated by performing viscosity, particle size distribution and protein recovery measurements. In addition, chapter 2 points toward the development and optimization of a method which allows the assessment of heat coagulation of recombined concentrated milk emulsions.

Chapter 4 focuses on the effect of phospholipid enriched dairy by-products, originating from the production of butter oil from dairy cream. Heat induced effects were derived from both viscosity and particle size analyses, whereas protein surface load determinations were performed to obtain a better understanding of the effect of the MFGM-enriched dairy products on protein interactions in the recombined concentrated milk.

Chapter 5 describes the effect of the addition of different types of sunflower lecithin on the heat stability of recombined concentrated milk emulsions. The effect of lecithin was tested before and after its hydrolysis for various time intervals and the evaluation was conducted by performing viscosity, particle size distribution, protein recovery and rheological measurements.

The decisive role of the phospholipid molecular structure on the heat stability of whey protein containing emulsions is described in chapter 6 which constitutes an in-depth examination of aggregation phenomena occurring in recombined concentrated milk emulsions, upon addition of phospholipids which are distinguished by their different structure.

In chapter 7, the effect of phospholipids and of their molecular structure was investigated in more detail. Hereby, a variety of both zwitterionic and anionic phospholipids with different chain length and thus phase transition temperature (T_m) were investigated, whereby protein-phospholipid mixtures were incubated both above and below the phospholipid phase transition temperature. The aim of this chapter is to clarify whether interactions between phospholipids and whey proteins take place, as well as the kind of the interaction and the protein modification which occurs as a result of this interaction. A schematic overview of the structure of the dissertation is depicted in figure 1.10.

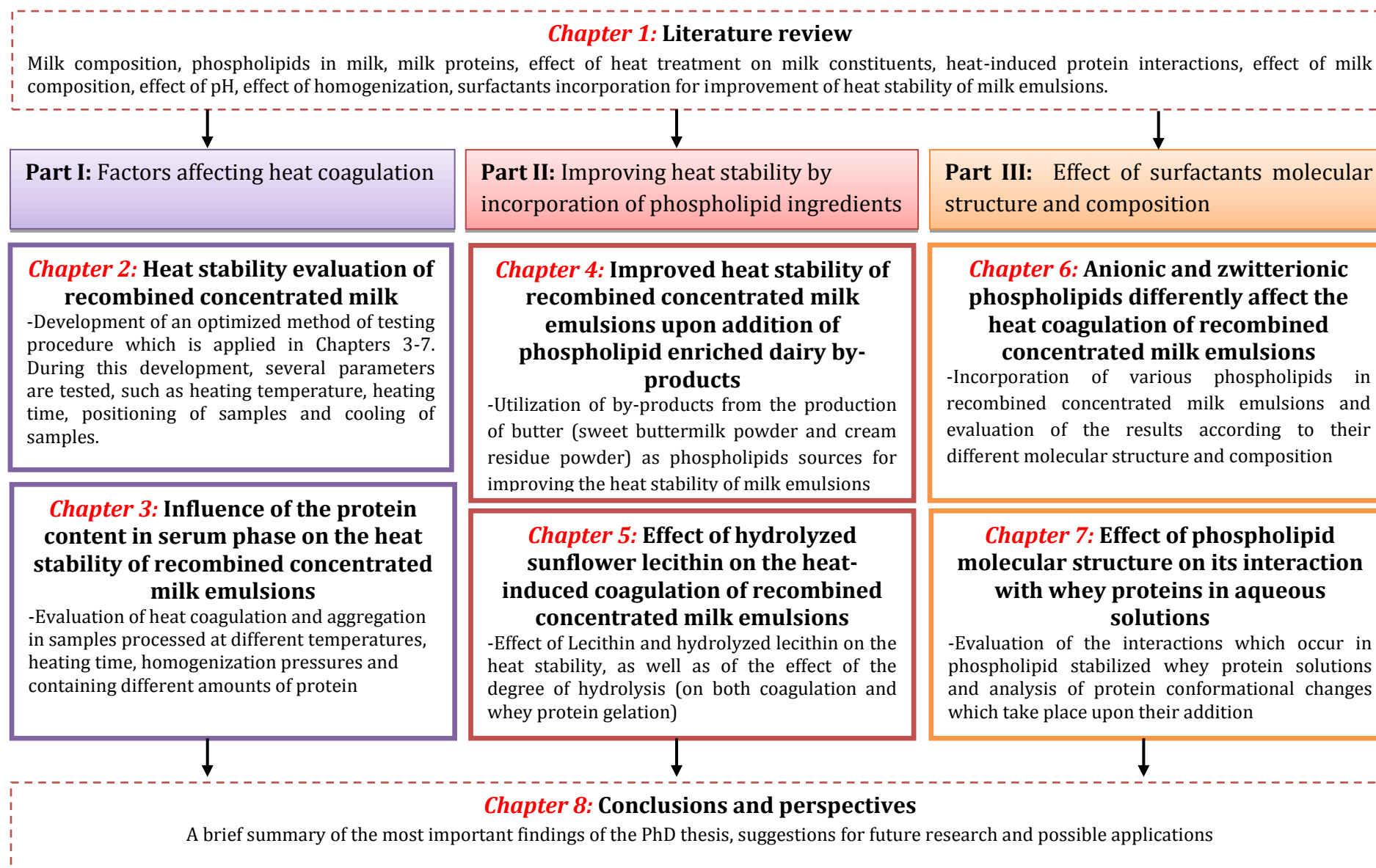
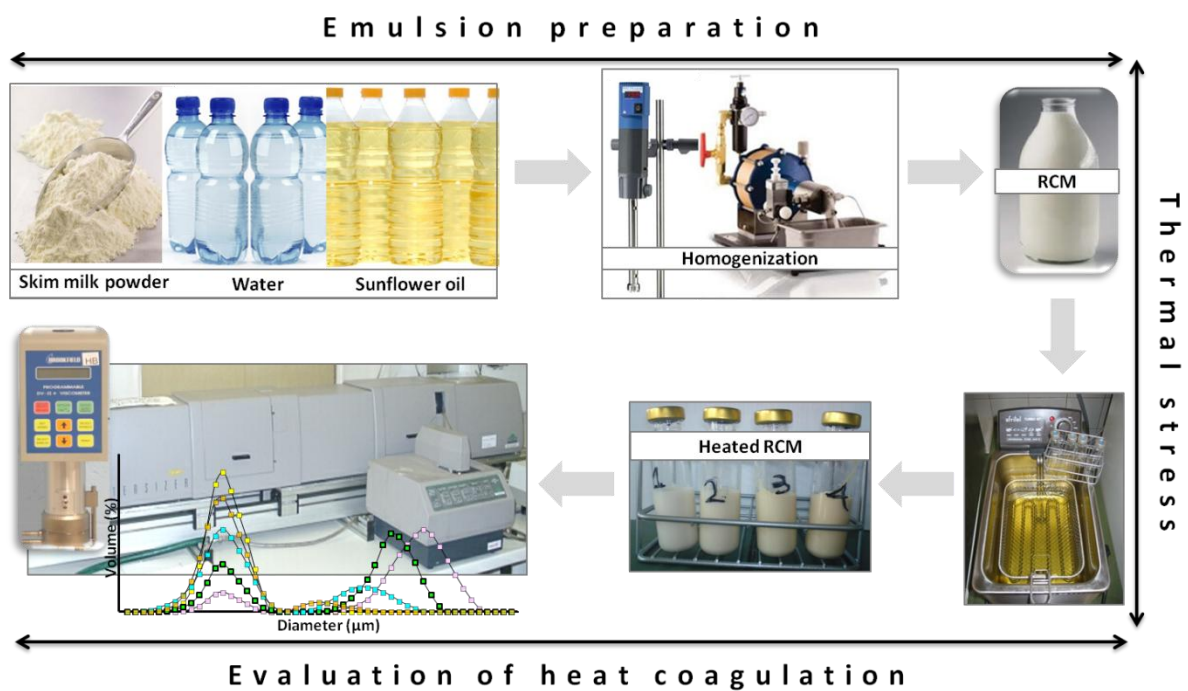


Figure 1.10. Schematic structure of the dissertation.

Chapter 2

METHOD DEVELOPMENT FOR HEAT STABILITY EVALUATION TEST OF RECOMBINED CONCENTRATED MILK



This chapter is redrafted from: Kasinos M., Karbakhsh, R. R. and Van der Meeren P. (2014), International Journal of Dairy Technology, In press

Chapter cover image: Methodology which was applied for the preparation of recombined concentrated milk emulsions, the application of heat stress and finally the evaluation of heat stability by means of viscosity and particle size distribution analysis.

Abstract

To quantify the stability towards heat coagulation, an objective test method was developed. The combined use of emulsion preparation by microfluidisation and heat stress by immersion of capped samples in an oil bath enabled small-volume heat stability evaluation of milk formulations. From experimental data, it became obvious that a heating period of about 9 min was necessary for the samples to acquire the requested temperature (i.e. 121°C). Similarly, both viscosity and particle size analysis showed an increased aggregation tendency when samples were heated for longer than 10 min, whereby their positioning and the cooling medium seemed to have no significant effect.

2.1. Introduction

Whereas milk has been proclaimed as nature's perfect food due to its high content in essential food nutrients, including amino acids, lipids, sugar, minerals and vitamins, its richness makes this product hazardous for consumption in case of microbial growth, which either spoil the product or present a risk of food poisoning or food borne diseases. Therefore, a range of different preservation techniques and strategies have been proposed and evolved through the decades, with the most frequently used method being the application of heat. Whereas heat treatment is considered to be the most commonly used and perhaps inevitable method in dairy industry, it seems to trigger the initiation of important physicochemical modifications, especially when applied at high temperatures. These modifications include denaturation of the globular whey protein fraction, and consequently the formation of aggregates between the unfolded whey proteins with themselves and/or with the casein micelles and the fat globules (Corredig & Dalgleish, 1999; Jeurnink & De Kruif, 1993; Nielsen, Singh & Latham, 1996; Oldfield et al., 2000; Tran Le et al., 2007). This phenomenon which is known as heat coagulation, has been extensively studied in order to analyze and explain the possible mechanisms which result to a final product which is distinguished by its elevated viscosity and particle size (Havea, Carr & Creamer, 2004; Jiménez-Flores, Ye & Singh, 2005; Kasinos et al., 2014; Kasinos, Tran Le & Van der Meeren, 2014; Van der Meeren et al., 2005).

The heat-induced whey protein denaturation and particularly the hydrophobic associations which take place between the denatured whey proteins seem to play a determinant role (Anema & Li, 2003; Corredig & Dalgleish, 1996; Galani & Apenten, 1999; Hoffmann & Van Mil, 1997; Nielsen et al., 1996; Noh, Richardson & Creamer, 1989). Despite the significant contribution of the hydrophobic interactions in the heat-induced aggregate formation, disulfide bonding is also of high importance. More precisely, heat-induced exposure of the thiol group in β -lg may induce thiol-disulfide interactions causing the formation of disulfide linked aggregates and subsequently complex formation with κ -casein on the surface of the casein micelles. Some other studies mentioned that κ -casein possibly dissociates from the micelles surface upon heating milk at elevated temperatures and this would result into the formation of whey protein/ κ -casein complexes in the serum phase (Anema, 2007).

Heat stability of milk is defined as the resistance to coagulate upon heating at high temperatures. During the past decades, several tests have been applied to evaluate the heat stability of dairy emulsions. These tests can be subdivided in subjective and objective methods. Whereas the former include the heating of a sample until a particular change is observed, the latter consist of a predefined heating protocol after which some parameters such as viscosity and particle size are determined, that are linked to heat-induced changes in milk.

A method development of a heat stability evaluation test for recombined concentrated milk was conducted, including viscosity as well as particle size analysis, following a heat treatment under well defined conditions. Consequently, a number of different experiments were performed, including alterations in heating time, sample volume and sample positioning in the oil bath, as well as variations in the temperature of the heating and the cooling medium.

2.2. *Materials and methods*

2.2.1. *Materials*

High-heat skim milk powder (SMP) was obtained from FrieslandCampina (Deventer, The Netherlands). According to the manufacturer's specifications, the SMP contained 37.3 % (w/w) protein, 0.5 % (w/w) fat and 0.15 % (w/w) phospholipids. The high oleic sunflower oil (Hozol, Contined, The Netherlands) contained maximum 0.05 % free fatty acid as oleic. Its melting point is at 0°C and hence the oil remains clear even after heating for 10 h at 4°C.

2.2.2. *Emulsion preparation*

Recombined concentrated milk emulsions (RCM) were prepared containing 16.5 % (w/w) SMP and 6.5 % (w/w) oil. The milk powder was diluted into 0.02 % NaN₃ (Acros Organics, Geel, Belgium) aqueous solution in order to prevent any potential microbial contamination, whereas Hozol oil was added as the dispersed phase. The samples were pre-homogenized by using IKA Ultra-Turrax TV45 (Janke & Kunkel, Staufen, Germany) for 1 min and afterwards were homogenized by using Microfluidiser 110S (Microfluidics Corporation, Newton, MA, USA), with its heat exchange coil immersed in a water bath. The temperature of the latter was set at 55°C. The samples were finally microfluidized at

a compressed air pressure of 2 bar, corresponding to a liquid pressure of about 280 bar for 2 min. By using Microfluidiser 110S, samples of only 20 ml can be processed. After emulsification, the RCM emulsions were allowed to cool to room temperature by their exposition in the air. The pH which was determined after homogenization (HI 4222, Hanna instruments, Temse, Belgium) was 6.5 for all RCM emulsions.

2.2.3. Heat treatment

Prior to heat treatment, RCM samples of 10 ml volume were transferred into 20 ml headspace vials (75.5 x 22.5 mm, 1st hydrolytic class, Grace, Deerfield, IL, USA), placed in a metallic twelve-place holder and positioned in a temperature-controlled oil bath (Fritel turbo SF®, 5 L capacity). The level of the oil inside the bath was sufficient to have the content of the glass tubes completely immersed.

The temperature inside the oil bath was kept uniform by stirring the oil throughout the heating process by an IKA RW20 stirrer with a 5 bladed metallic propeller stirrer of 5 cm diameter (Janke & Kunkel, Staufen, Germany). The stirrer was placed in the left corner in the oil bath. To investigate the temperature evolution during heating, a needle-like thermocouple (type J) connected to an electronic digital thermometer (Agilent 34970A, Diegm, Belgium) was pierced through the silicone cover, measuring the exact temperature inside the sample every 30 sec. The temperature of the oil bath was also checked by using two thermometers which were placed in the upper right and lower left corner, respectively. The samples were heated for different time intervals (0-20 min) whereas after heat treatment, they were allowed to cool either in air or tap water. The analysis of samples took place when the temperature inside the samples reached the room temperature.

2.2.4. Viscosity measurements

A Programmable LV-DV-II+ Viscometer (Brookfield, Stoughton, MA, USA), at speeds ranging from 0-200 rpm (rotations per minute) was used to measure the viscosity of samples before and after heating. All measurements were performed at room temperature. Spindle 21 (with an 8 ml small volume adapter) has been used to measure the viscosity of samples which retained their liquid structure, whereas spindle 34 (immersed directly into the glass vials) has been used to measure the viscosity of samples which obtained a gel-like structure. The conversion of rpm into shear rates (s^{-1})

is performed by multiplying the value of the rotational speed with the shear rate coefficients, which are 0.93 and 0.38 s⁻¹ for spindle 21 and 34, respectively. The experimental data fitted very well to a power law relation:

$$\tau = K \cdot \gamma^n \quad (1)$$

As the apparent viscosity μ is obtained as the ratio of shear stress τ (in Pa) to shear rate γ (in s⁻¹), the power law equation can be rewritten as:

$$\mu = K \cdot \gamma^{n-1} \quad (2)$$

whereby K represents the consistency coefficient and that is the viscosity in mPa.s at a shear rate of 1 s⁻¹, and n represents the flow behavior index. Based on the value of the latter, fluids can be classified in three categories: Newtonian fluids when n=1, pseudoplastic when n<1 and dilatants when n>1.

2.2.5. Particle size distribution

The particle size distribution of the RCM samples was determined with a Mastersizer S long bed (version 2.15, Malvern Instruments Ltd, Malvern, UK) equipped with a 300RF lens and a MSX-17 wet sample dispersion unit. Sample was added dropwise until an obscuration of about 10 % was obtained. For the results calculation, the presentation code incorporated a continuous phase refractive index of 1.33 and a real and imaginary dispersed phase refractive index of 1.4600 and 0.0100, respectively. For the particle size distribution determination, the polydisperse model was chosen in the Malvern Mastersizer software.

2.3. Results and discussion

2.3.1. Temperature evolution during heating

Thermal treatment constitutes a common step in processing and preservation of dairy products, during which important physicochemical modifications may arise, including changes in the milk proteins' structure and functionality and particularly the whey fraction. At temperatures higher than 70°C, whey protein denaturation takes place, whereas a complete unfolding of their globular configuration seems to occur upon

heating at temperatures of 90°C and above (Chen et al., 2005; Corredig & Dalgleish, 1996; De Wit & Klarenbeek, 1984; De Wit, 2009; Singh, 2004; Tran Le et al., 2007).

The rate of heating along with the temperature, represent two of the most fundamental factors affecting the heat stability of milk emulsions. It has been previously reported that there is an increased occurrence of aggregation by raising both the temperature and the time of heating (Boye et al., 1995; Corredig & Dalgleish, 1999; Kasinos et al., 2014; Tran Le et al., 2007). A 'heat stability evaluation test' was developed in order to quantify the stability of RCM emulsions containing 23 % total milk solids (16.5 % solids non-fat and 6.5 % fat). For that reason, experiments were designed to investigate the evolution of temperature inside RCM samples upon heating in an oil bath at 121°C for 20 min. In addition, the influence of the heating time and heating temperature on the coagulation tendency was evaluated, by means of both viscosity and particle size measurements. As high-heat SMP contains denatured whey proteins and whey protein aggregates due to the preceded heat treatment, heat coagulation of RCM appears to be related to the continuation of whey protein aggregation by further increasing the temperature and the time of heating. Our speculation seems to be in accordance with the model proposed by Remondetto and Subirade (2003) who studied the structural changes of β -lg by using Fourier transform-infrared spectroscopy and rheological methods. Their work showed that the aggregates formed during the preheating treatment constituted the structural basis of further aggregation.

During sterilization, milk samples acquire the desired temperature after a certain time (*heating time*), during which the temperature gradually evolves and eventually reaches a plateau. On a first step, in our research study, an evaluation of the heating process was constructed, to ensure that the temperature of the RCM emulsions was maintained at 121°C for a certain period of heating. Therefore, a thermocouple has been used enabling temperature determination in the interior of the samples, whilst the temperature in the oil bath was set at 121°C. Since the glass vials were hermetically sealed with a silicone cap, the thermocouple was pierced and placed in the center of the treated sample.

In figure 2.1, the time-temperature profile inside a RCM sample of 10 ml, during heating in a temperature-controlled oil bath at 121°C, is illustrated. As it can be observed, the temperature was rapidly increased within the first 5 to 6 min after the sample was immersed inside the oil bath.

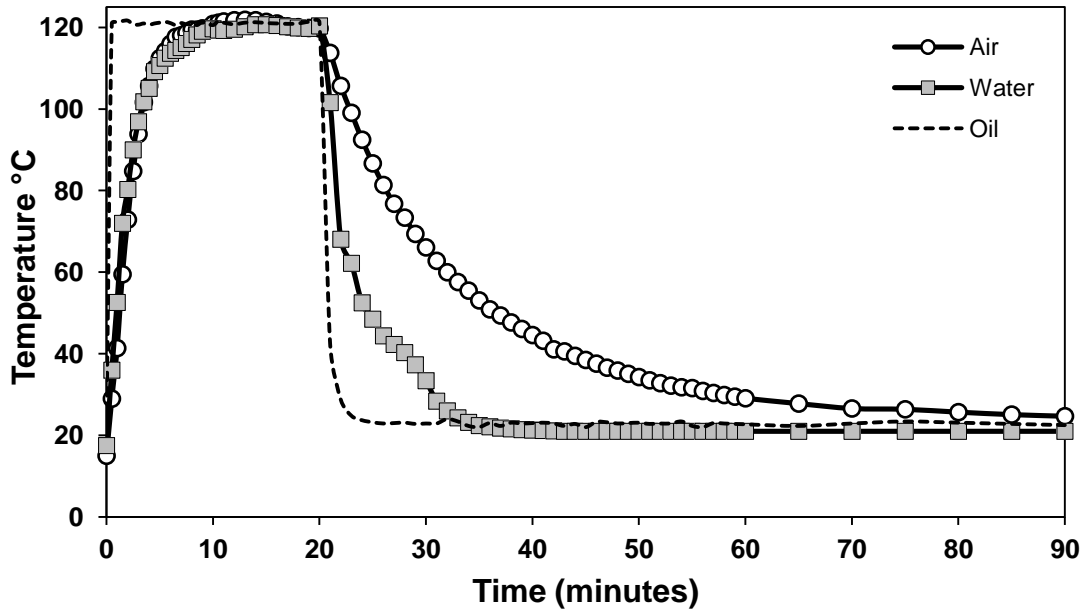


Figure 2.1. Time-temperature profile of recombined concentrated milk emulsion samples upon heating in a temperature-controlled oil bath at 121°C for 20 min, and cooling in air or tap water. The dotted line represents the temperature recorded by the sensor when put directly inside the oil bath during the first 20 min and in air afterwards.

However, a period of approximately 9 to 10 min was required for RCM emulsions to reach 121°C. Subsequently, the temperature remained constant until the 20th min (*holding time*), whereas about 70 min elapsed for cooling down and reaching room temperature after exposing to air. In the case where tap water was used as cooling medium, only 13 min were needed for attaining room temperature. In addition, it should also be mentioned that when the viscosity was measured (data not shown), no important changes were noticed between the samples which were cooled in air or water, which leads to the conclusion that the cooling medium does not play a crucial role on the aggregation tendency.

Fitting the temperature profile to a first order kinetic model, a time constant (τ) of 2.47 min was obtained for the heating phase, whereas for the cooling phase it was 11.74 and 4.38 min, when air and tap water were used as the cooling medium, respectively. The time constants give information related to the time representing the speed with which the samples can respond to the temperature changes. Therefore, during heating, samples changed temperature within a shorter period of time as compared to cooling and especially when air was used as the cooling medium.

The corresponding heat transfer coefficient (k), which gives information related to the amount of heat which passes through the system in a unit time, was 0.53 min⁻¹ for the

heating phase, whereas for the cooling phase it was 0.22 min^{-1} when cooling in stationary tap water and only 0.07 min^{-1} upon cooling in air. The latter is due to the much less effective heat transfer to a gaseous as compared to a liquid medium.

Consequently, when the aggregation tendency upon sterilization has to be assessed for a specific heating period, milk samples need to remain inside the oil bath for an additional time of approximately 9 min, to guarantee that the temperature in the interior is raised to 121°C . When the time-temperature profiles of 10 and 15 ml of samples were recorded and compared, after 15 min of heating (data not shown), no significant differences ($P > 0.05$) were noticed at a 95 % level of confidence. The heat transfer coefficient was $0.55 \pm 0.04 \text{ min}^{-1}$ and $0.52 \pm 0.01 \text{ min}^{-1}$, for sample volumes of 10 and 15 ml, respectively, a fact which denotes that the temperature evolution inside the sample only depends on the temperature of the heating medium. In addition, it should be indicated that a RCM sample of 20 ml volume is better to be avoided as the limited headspace was frequently insufficient to resist the increase in pressure upon heating, leading to deformation of the septum and sample loss.

2.3.2. Effect of sample positioning in the temperature-controlled oil bath

Individual RCM samples were placed at different positions on a twelve-placed metallic holder, while their temperature was determined during heating for 15 min at 121°C . Figure 2.2 depicts the time-temperature profile of RCM emulsions, accompanied with a schematic drawing portraying the positioning of four samples placed at different positions: one close to the stirrer (position 2), two in the middle of the rack (positions 6 and 7) and one far from the stirrer (position 12).

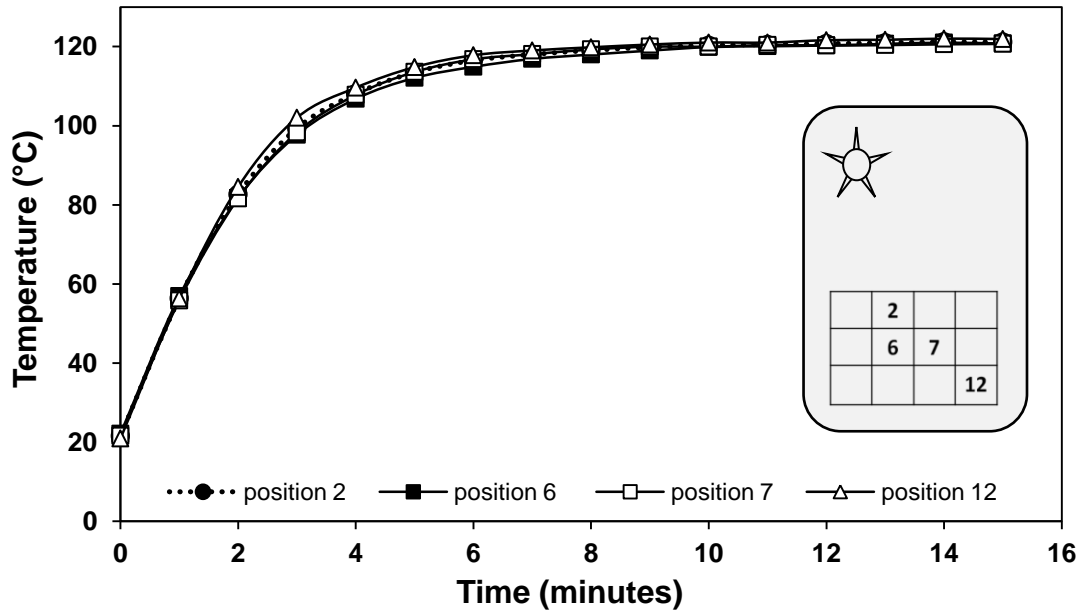


Figure 2.2. Time-temperature profile of recombined concentrated milk emulsions, placed on different positions on a metallic rack, upon heating in a temperature-controlled oil bath at 121°C. The schematic drawing describes the positioning of 4 different samples and of the stirrer inside the oil bath.

The recorded profiles for all four different samples were comparable ($P > 0.05$) at a 95 % level of confidence. The calculated heat transfer coefficients (k) for samples placed in positions 2, 6, 7 and 12 were 0.49, 0.47, 0.48 and 0.50 min^{-1} , respectively. Consequently, sample positioning cannot be considered as an influential parameter for future experiments, since temperature was proved to be homogeneously distributed inside the heating apparatus throughout the entire heating procedure.

In addition, the influence of the heat capacity of the oil in the bath was recorded, upon insertion of 12 tubes containing 10 ml of recombined concentrated milk samples. The results showed that the oil temperature dropped 2°C (i.e. approximately 0.17°C per glass vial) which was recovered within 1.5 min. Therefore, the oil temperature was maintained at 123°C until the moment the samples were placed in the oil bath, whereas afterwards it was held constant at 121°C.

2.3.3. Effect of temperature evolution on viscosity and particle size distribution

The recombined concentrated milk samples retained their liquid structure upon the above described heat treatment, whereby a slight increase in viscosity was observed.

Figure 2.3 illustrates the changes in consistency coefficient of recombined concentrated milk emulsions, upon heating at 121°C for 15 min.

Overall, the consistency coefficient did not show remarkable variations during the first 9 min of heating and this is confirmed in figure 2.1, from which it is revealed that the samples acquired the sterilization temperature only 9 min after their immersion in the oil bath. As expected, the consistency coefficient gradually increased upon increasing the time of heating. This observation points toward aggregation which becomes more intense as the time of sterilization increases. Kiesecker and Aitken (1988) obtained similar observations upon treating recombined milk containing 26 % total solids (18 % solids non-fat and 8 % fat) at different time/temperature conditions. They noticed a gradual viscosity increase when increasing the temperature and/or the time of heating. Similar observations are withdrawn from figure 2.4, which shows the volume-weighted average diameter $d_{4,3}$ of recombined concentrated milk emulsions, upon heating for 15 min at 121°C.

A limited increase of the particle diameter was observed during the first 10 min of heating, corresponding to 0.34 μm for the unheated sample and 0.43 μm for the sample which was heated for 10 min. On the other hand, the increase in the average diameter became more pronounced after 12 min, which can be attributed to aggregation. The differential and cumulative volume-weighted particle size distribution, shown in figure 2.5, confirm the results mentioned above; a minor aggregate peak in the range of 1-5 μm was evident upon heating for 10 min, which was gradually shifted toward 1-8 and 1-11 μm when the heating time increased to 11 and 15 min, respectively.

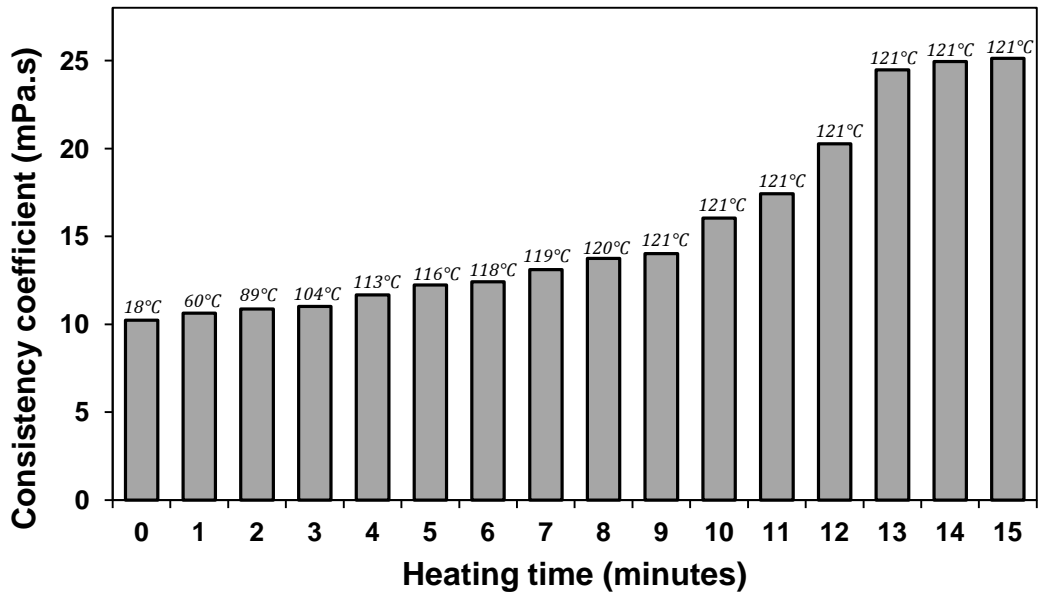


Figure 2.3. Consistency coefficient (mPa.s) of recombined concentrated milk emulsions as a function of time during heating for 15 min at 121°C. The temperature at the top of each column indicates the temperature inside the sample.

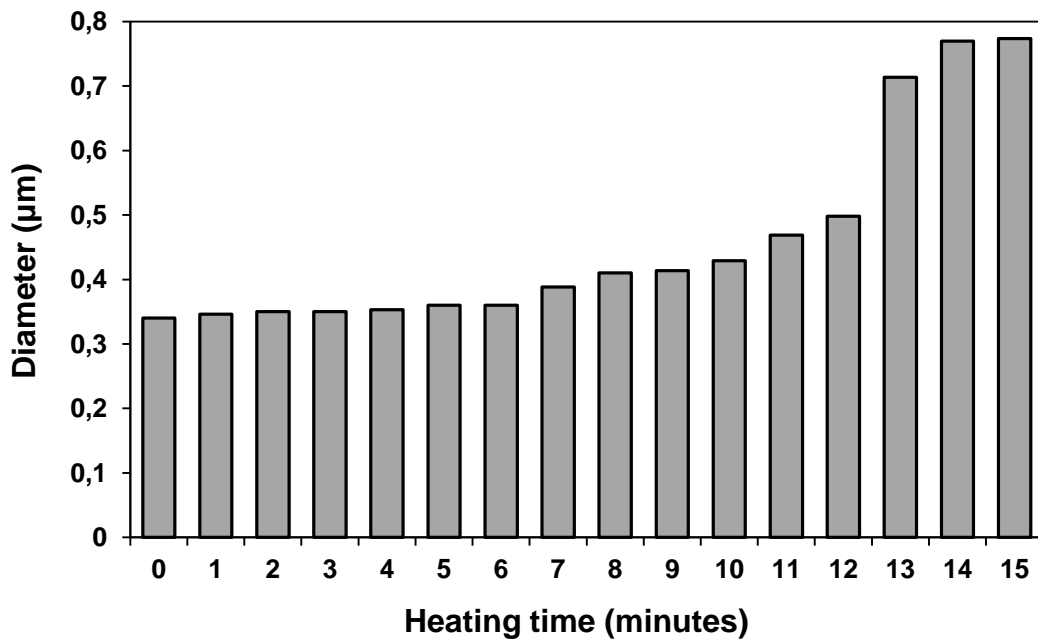


Figure 2.4. Volume-weighted average diameter $d_{4,3}$ (μm) of recombined concentrated milk emulsions as a function of time during heating for 15 min at 121°C.

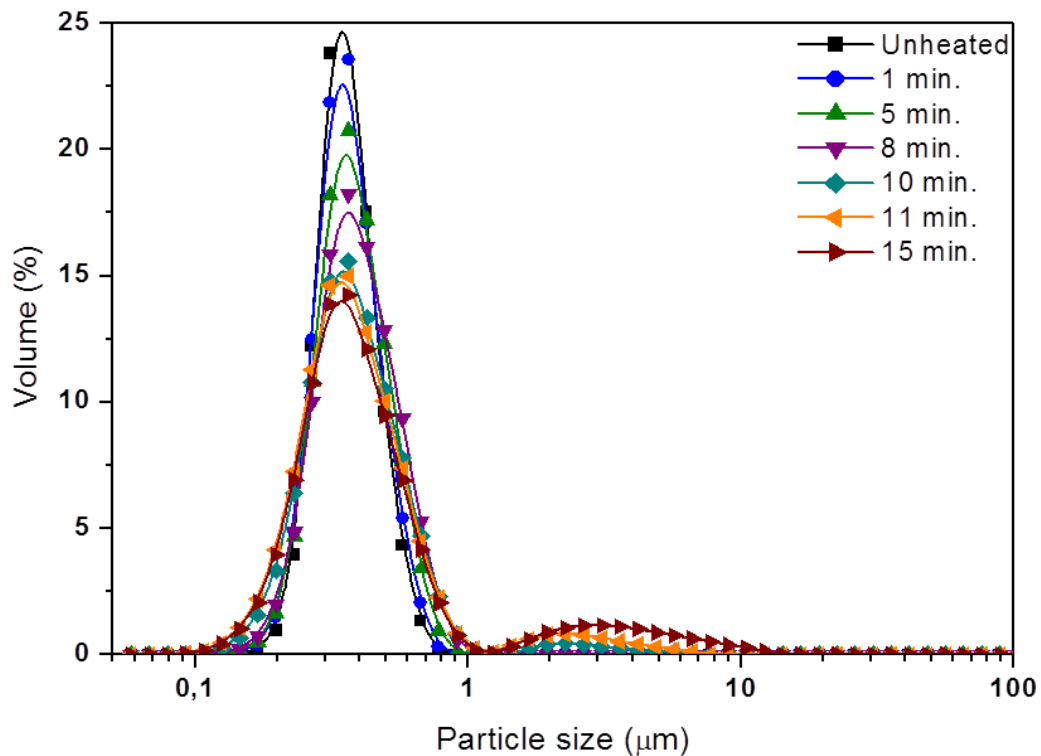


Figure 2.5. Differential and cumulative volume-weighted particle size distribution (in μm) of recombined concentrated milk emulsions as a function of time during heating for 15 min at 121°C .

Figure 2.6 reveals that the average diameter and the consistency coefficient were highly correlated: whereas both variables were only slightly affected during the first 10 min, it becomes clear that after 10 min there is an obvious increase.

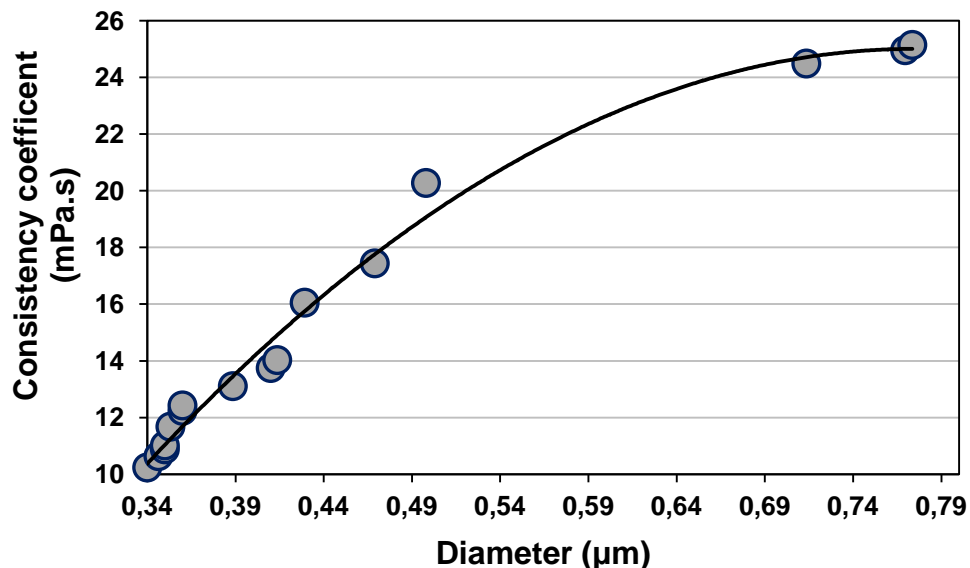


Figure 2.6. Consistency coefficient (mPa.s) as a function of the volume-weighted average diameter of recombined milk emulsions after heating for 15 min at 121°C . Each point represents a min of heating.

2.3.4. Effect of temperature on viscosity

In order to choose the temperature at which the experiments for aggregation tendency evaluation will take place, recombined concentrated milk emulsions were treated at three different heating temperatures: 116, 121 and 126°C, for 20 min. As it is portrayed in figure 2.7, viscosity remained almost unchanged upon heating at 116°C: samples maintained their liquid structure, due to the absence of pronounced heat coagulation ($n = 0.812$ as compared to the flow behavior index of the unheated samples which was 0.835).

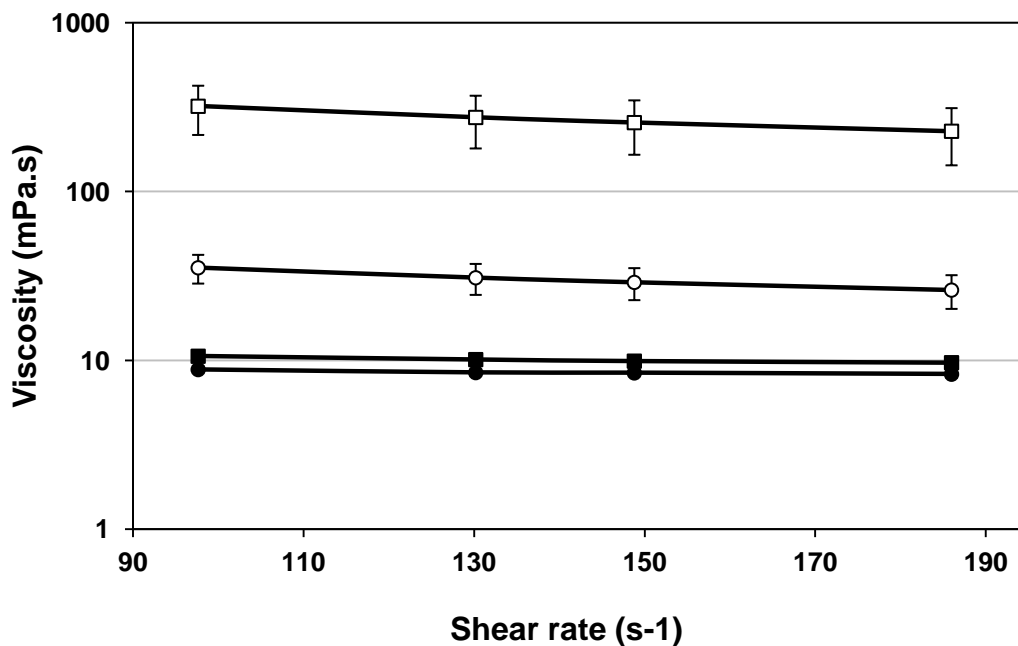


Figure 2.7. Viscosity (mPa.s) of recombined concentrated milk emulsions ($n=3$) before (●) and after heating for 20 min at 116 (■), 121 (○) and 126°C (□).

On the other hand, upon heating at 126°C, samples were characterized by a solid or gelled structure ($n = 0.462$) which is attributed to pronounced aggregates formation. In this case, where aggregation occurs in advanced levels, the measurements concerning stability evaluation become problematic due to the solidified structure of the samples.

Finally, upon heat treatment at 121°C, aggregate formation was also noticed but only at moderate levels ($n = 0.559$). The less coagulated structure of the samples allows their easy handling regarding the performance of experiments such as particle size and viscosity analyses and consequently, 121°C was chosen as the temperature at which the samples will be treated in future experiments. Williams D'Ath and Zisu (2008) suggested that different aggregation processes are involved at different time-temperature

combinations resulting to the creation of discretely differently sized populations of aggregates.

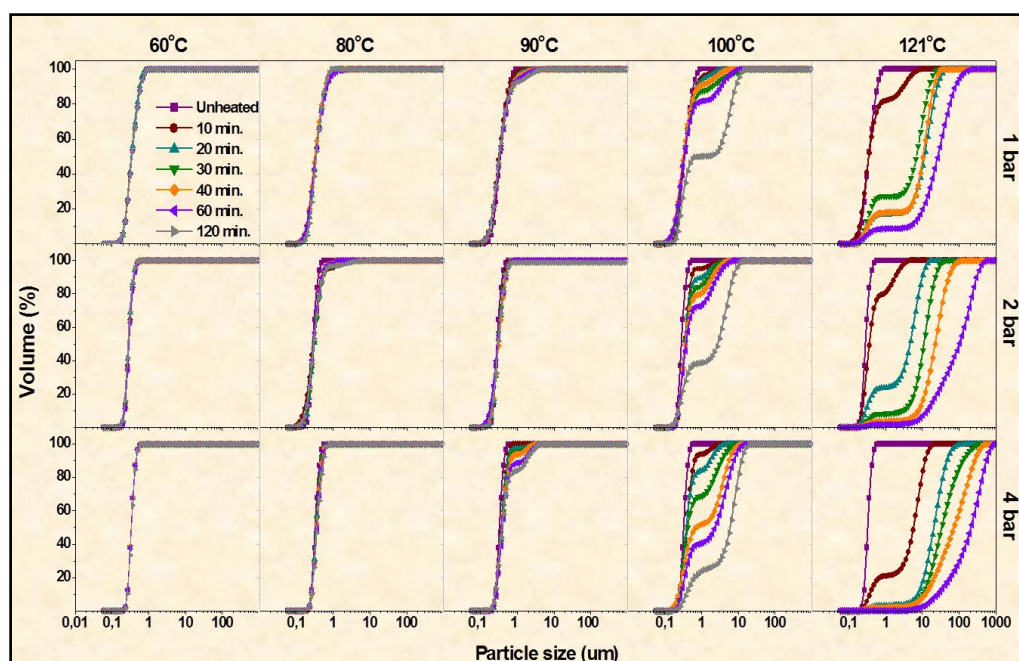
2.4. Conclusions

Combining emulsion preparation by microfluidisation with heat treatment in capped flasks and subsequent viscosity and/or particle size analysis, the heat stability of recombined concentrated milk samples of only 20 ml could be evaluated. The method is ideally suited to test highly purified ingredient components.

Time-temperature profiles of recombined concentrated milk emulsions revealed that in the experimental set-up used a period of approximately 9 min is required for the samples to attain the sterilization temperature. Moreover, when the effect of sample position inside the oil bath was investigated, no significant changes were noticed related to the temperature evolution inside the samples.

Chapter 3

INFLUENCE OF THE PROTEIN CONTENT IN THE SERUM PHASE ON THE HEAT STABILITY OF RECOMBINED CONCENTRATED MILK EMULSIONS



Chapter cover image: Cumulative volume-weighted particle size distribution of recombined concentrated milk emulsions containing 23 % SMP and 6.5 % oil, after heating at 121 °C in a temperature-controlled oil bath.

Abstract

In dairy industry, heat treatment is by far the most commonly used technique that provides stability, and hence an extended shelf-life to the final product. For that reason, this study was carried out aiming to a more coherent understanding of the heat-induced modifications which take place in recombined concentrated dairy emulsions upon changing formulation parameters during processing. The effect of composition by ranging the SMP at constant oil content and by varying the SMP content at fixed total solids, was checked. Heat coagulation was evaluated by means of viscosity and particle size measurements, as well as protein load. Firstly, the effect of different protein to oil ratios was examined, which showed that by increasing the oil and decreasing the protein content, a gradual increase of heat coagulation was observed. In addition, this work indicated an onset of heat coagulation of concentrated milk emulsions containing 23 % of skim milk powder and 6.5 % oil after heating at 80°C, whereas the effect was much more enhanced by raising both the temperature up to sterilization conditions and the period of heating. In the case of samples consisting of 12 % skim milk powder and 6.5 % oil, heat coagulation was not very obvious at temperatures lower than 121°C. In addition, homogenization pressure was proven to be a parameter to be reckoned: whereas microfluidisation at 1 or 2 bar of driving air pressure yielded more heat stable emulsions, 4 bar resulted into an enhanced heat-induced coagulation tendency.

3.1. Introduction

Due to its high nutritional value and its major importance in human's diet, milk represents one of the most investigated food products over the centuries. From a colloidal point of view, milk can be described as an oil-in-water emulsion where the fat droplets are dispersed in an aqueous solution containing both casein micelles and whey proteins, which are differentiated from each other by their physical and chemical properties (Euston, 1997; Tran Le et al., 2007; Van der Meeren, El-Bakry, Neiryck & Noppe, 2005).

Heat treatment, which is considered to be the most commonly used method in food processing, has been shown to affect the structure and functionality of milk proteins and mainly the whey fraction. Upon milk processing at elevated temperatures, numerous reactions may occur, including extensive denaturation and aggregation of whey proteins and complex formation between unfolded whey proteins with themselves and/or with caseins micelles and fat globules (Corredig & Dalgleish, 1999; Jeurink & De Kruif, 1993; Kasinos et al., 2013; Nicorescu et al., 2008; Nielsen, Singh & Latham, 1996; Raikos, 2010; Singh, 2004; Verheul, Pedersen, Roefs & De Kruif, 1998). Considering the important role attributed to the whey protein in the serum phase, it was our purpose to check the effect of this characteristic on the heat stability of RCM emulsions. Hereby, the serum whey protein content was not only varied by changing the amount of skim milk powder added, but also by changing the homogenization pressure: as higher pressures promote smaller droplets with a larger interfacial area, less residual (whey) protein is expected in the serum phase.

Hence, the aim of this contribution was to investigate the effect of some of the most critical parameters throughout the preparation process of recombined concentrated milk (RCM) emulsions. These parameters involve emulsion protein and fat content, heating temperature and time, as well as homogenization pressure. During the manufacture of RCM emulsions, the latter parameter is of special interest as it contributes to the size reduction of fat globules and hence prevents creaming upon long shelf-storage (Lopez-Fandiño, Carrascosa & Olano, 1996; McCrae, Hirst, Law & Muir, 1994; McCrae & Muir, 1991; Thiebaud, Dumay, Picart, Guiraud & Cheftel, 2003; Walstra, 1983).

3.2. Materials and methods

3.2.1. Materials

For preparing RCM emulsions, high-heat skim milk powder (SMP) was obtained from FrieslandCampina (Deventer, the Netherlands) and high oleic sunflower oil (Hozol, Contined, The Netherlands). Their specifications are described in section 2.2.1.

3.2.2. Emulsion preparation

Evaporated milk (EM) is defined as the product obtained after the removal of about 60 % of milk's water, and such contains at least 6.5 % milk fat and 16.5 % milk solids-non-fat (minimum 23 % w/w of total milk solids). A similar product may be obtained by mixing 6.5 % of a vegetable oil (such as sunflower) and 16.5 % SMP in water, which is referred to as recombined concentrated milk (RCM).

At a first step, and in order to check the effect of the amount of SMP on the heat stability of RCM emulsions, samples were prepared containing 23.0 and 12.0 % (w/w) SMP, corresponding to 8.6 and 4.5 g of protein, whereas 6.5 % (w/w) of Hozol oil was used as the dispersed phase. Using these emulsions, the effect of different homogenization pressures and heating temperatures and times was evaluated. In all cases, milk powder was diluted into 0.02 % NaN_3 aqueous solution to prevent microbial contamination. All samples were pre-homogenized by an IKA Ultra-Turrax TV45 (Janke & Kunkel, Staufen, Germany) for about 1 min and homogenized by a Microfluidiser 110S (Microfluidics Corporation, Newton, MA, USA) having its coil immersed in a water bath at 55°C. The samples were microfluidized for 2 min at a compressed air pressure of 2 bar, corresponding to a liquid pressure of about 280 bar, whereas in the case where the effect of homogenization pressure was tested, the samples were microfluidized at a compressed air pressure of 1, 2 and 4 bar, corresponding to a liquid pressure of about 140, 280 and 560 bar, respectively. The pH of the emulsions measured after homogenization was 6.5.

In a second series of experiments and to examine the effect of the emulsion composition on the heat stability of RCM emulsions, samples were prepared containing different protein to oil ratio, as described in table 3.1, keeping constant the amount of total solids to 23 % w/w.

Table 3.1. Compositions of oil/water emulsions with constant amount of 23 % (w/w) total solids.

	Emulsion type					
	A & B	C	D	E	F	G
Milk solids-non-fat (% w/w)	23.0	21.0	19.0	16.5	14.0	12.0
Fat (% w/w)	0.0	2.0	4.0	6.5	9.0	11.0

3.2.3. Heat treatment

Prior to heat treatment, 10 ml of each sample was transferred into 20 ml headspace vials (75.5 x 22.5 mm, Grace, Deerfield, IL, USA) which were placed in a twelve-placed metallic rack and positioned in a temperature-controlled oil bath (Fritel turbo SF®, 5 L capacity) to be heated at 121°C for 10-120 min. To evaluate their stability, the emulsions were also heated at lower temperatures (80-100°C) in a water bath, for 10-120 min.

3.2.4. Viscosity measurements

To assess the tendency of heat-induced coagulation, viscosity measurements of RCM emulsions were executed before and after heat treatment as described in section 2.2.4.

The experimental data fitted very well to a power law relation:

$$\tau = K \cdot \gamma^n \quad (1)$$

As the apparent viscosity μ is obtained as the ratio of shear stress τ (in Pa) to shear rate γ (in s⁻¹), the power law equation can be rewritten as:

$$\mu = K \cdot \gamma^{n-1} \quad (2)$$

where K represents the consistency coefficient and n represents the flow behavior index.

3.2.5. Particle size distribution

The particle size distribution of RCM emulsions was examined by using a Mastersizer S long bed (version 2.15, Malvern Instruments Ltd, Malvern, UK) as explained in section 2.2.5.

3.2.6. Protein load

The protein load (i.e. the amount of protein per m² of oil-water interface) of RCM samples was determined before and after heating. Emulsions prepared as described in table 3.1 were heated at 121°C for 20 min, whereas to check the effect of different heating temperatures and times, samples containing 23 % of SMP and 6.5 % of oil, were heated for 10, 20, 30, 40 and 60 min at 100°C and for 10, 15 and 20 min at 121°C, respectively.

The cream layer was separated by a Beckman L7-55 ultra speed centrifuge using the SW 40 Ti rotor (Pasadena, CA, USA), at 284.061 g for 120 min and at 20°C. Sucrose (VWR International, Leuven, Belgium) was added as a density increasing agent (Kasinos, Tran Le & Van der Meeren, 2014; Van der Meeren et al., 2005), whereby 2.5 g sucrose was dissolved in 5 g of emulsion. Subsequently, 5 g of the sugar-containing emulsion was transferred into the Beckman polyallomer centrifuge tubes (13 x 51 mm, Beckman Coulter, Pasadena, CA, USA). After centrifugation, the cream layer was carefully removed, whereas the remaining serum and sediment phase were mixed thoroughly. The protein content of the mixture was determined by the Kjeldahl method using a conversion factor of 6.38. The protein recovery is calculated as the ratio of the amount of protein recovered in the continuous phase (upon centrifugation) to the total protein content of the emulsion.

It should be mentioned that protein load Γ (mg/m²) and protein recovery (in %) are related by the use of equation (3) :

$$\Gamma = \frac{(1-\text{recovery}) \times C_{\text{SMP}} \times C_{\text{prot}}}{\text{SSA} \times C_{\text{oil}}} \quad (3)$$

whereby C_{SMP} and C_{oil} represent the concentration of SMP and hozol oil in the recombined concentrated milk emulsions respectively, C_{prot} represents the protein content of SMP and SSA stands for the specific surface area (in m²/kg oil) of the oil droplets (equation 4).

$$\text{SSA} = \frac{6}{(d_{3,2})_{\text{preheat}} \times \rho_{\text{oil}}} \quad (4)$$

$d_{3,2}$ represents the Sauter mean diameter of the oil droplets in the sample before heating, whereas ρ_{oil} represents the oil density (915 kg/m^3).

3.3. Results and discussion

The effect of temperature and heating time on the heat stability of RCM emulsions was evaluated. Two different lots of oil-in-water emulsions were prepared containing 12.0 and 23.0 % of SMP and 6.5 % Hozol oil, and heated up to 121°C for different time intervals. As heat coagulation relies on the whey protein mediated formation of a three-dimensional aggregated structure, a better heat stability was expected at the lower protein content, provided that enough of protein was present to stabilize the oil-water interface. A visual examination after cooling down the samples, confirmed an obvious destabilization of the emulsions containing 23 % SMP, which were heated at temperatures higher than 90°C for 10 min or longer. On the other hand, heating at 80°C resulted in emulsions with a liquid-like appearance, comparable to the ones which remained unheated. In case of emulsions containing 12 % SMP and 6.5 % oil, a visible change was noticeable only in cases where samples were heated at 121°C . To acquire more detailed information, viscosity as well as particle diameter measurements were performed.

As shown in figure 3.1, a gradual increase of viscosity was detected upon heating samples containing 23 % SMP and 6.5 % oil, prepared at varying homogenization pressures, whereby the increase evolved upon rising both temperature and heating time. On the other hand, when these samples were heated at 80°C , the viscosity ranged from 7.5 to 12.7 mPa.s (determined at a shear rate of 186.0 s^{-1}), whereas the unheated sample had viscosities ranging from 7.2 to 9.7 mPa.s. Hereby, the higher values were obtained at the lowest homogenization pressure. Whereas it becomes evident from figure 3.1 that samples containing 23 % SMP and 6.5 % oil were characterized by increased viscosity at temperatures of 90°C and above, in case of 12 % SMP and 6.5 % oil (figure 3.2), the viscosity remained almost constant at temperatures up to 100°C , and for that reason the curves representing heat treatment of 90 and 100°C , overlap for all three homogenization pressures. For this specific case, it is thought that whey protein aggregation is not sufficiently pronounced to be detectable via the viscosity measurements.

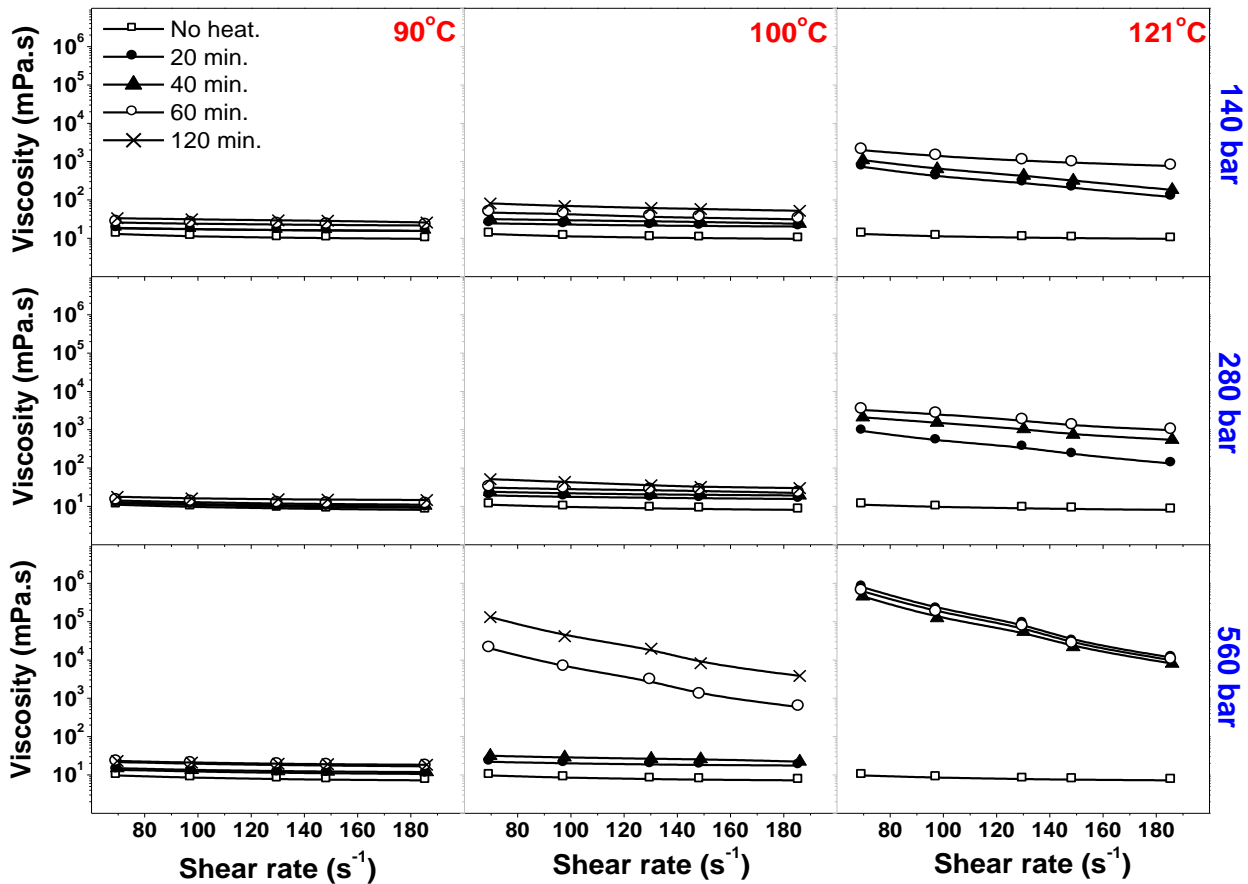


Figure 3.1. Viscosity (mPa.s) of RCM emulsions containing 23 % SMP and 6.5 % Hozol oil, as a function of shear rate (s^{-1}). Samples were homogenized at 140, 280 and 560 bar and the viscosity was measured after heating at 90, 100 and 121°C for 0 (\square), 20 (\bullet), 40 (\blacktriangle), 60 (\circ) and 120 (\times) min.

Similarly, figure 3.3, which depicts the average particle diameter upon heating, reveals that there were no significant changes in the diameter of samples containing 12 % SMP and 6.5 % oil, treated at temperatures below 121°C, even after heating for 120 min. On the other hand, samples with the same SMP concentration which remained for more than 40 min at 121°C, demonstrated a severe increase in both viscosity and volume weighted average diameter. The increased viscosity and average diameter of milk emulsions is attributed to a complicated phenomenon known as heat coagulation. Several authors (Jeurnink & De Kruif, 1993; Nielsen et al., 1996; Verheul et al., 1998), described that the whey protein changes which take place during this phenomenon include the dissociation of dimers into monomers together with intermolecular interaction consisting of hydrophobic bonding and thiol-disulphide exchange. However, as high-heat SMP already contains denatured whey proteins and protein aggregates, we

speculate that these aggregates they constitute the structural basis of further aggregation and gelation upon additional heating at elevated temperatures.

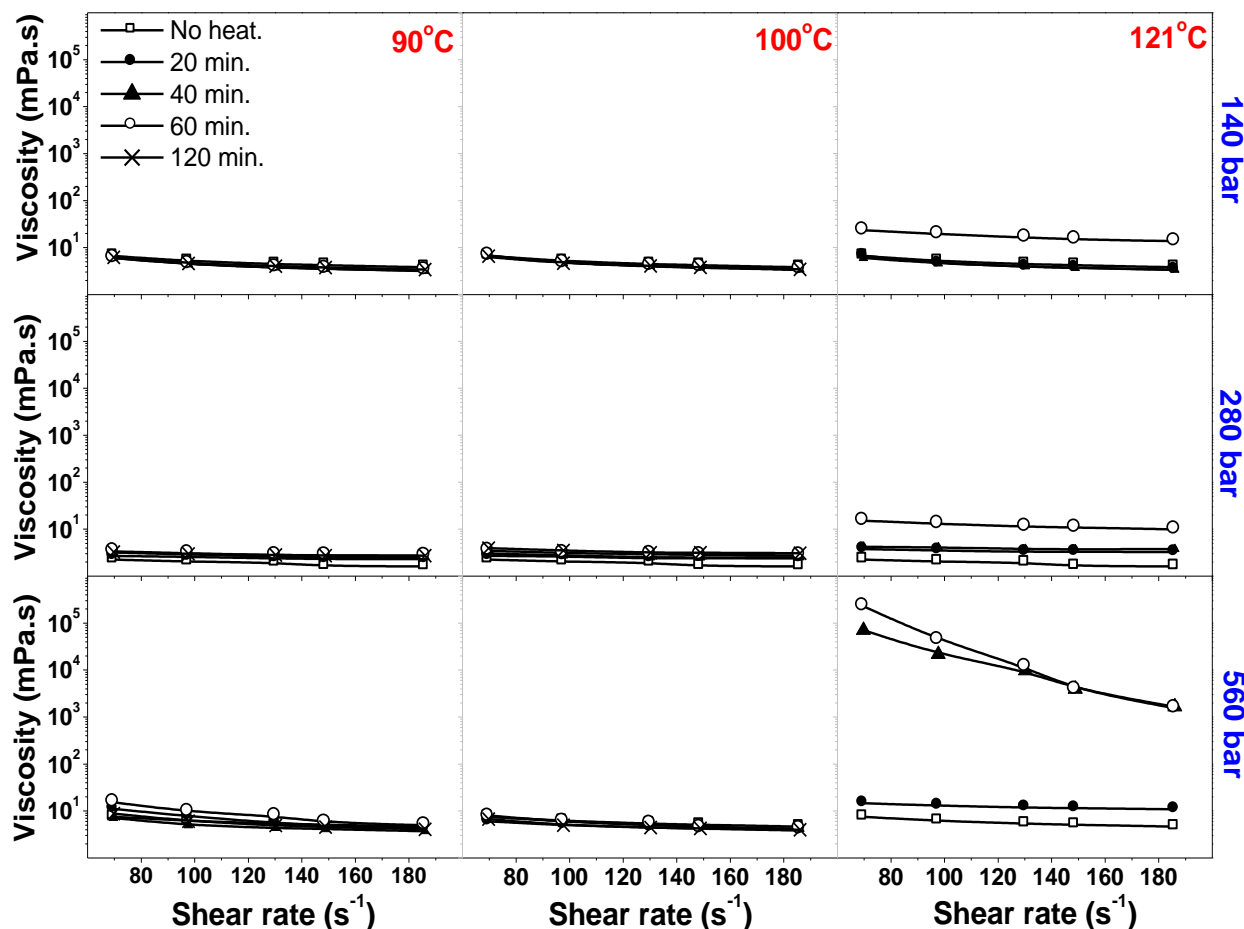


Figure 3.2. Viscosity (mPa.s) of RCM emulsions containing 12 % SMP and 6.5 % Hozol oil, as a function of shear rate (s⁻¹). Samples were homogenized at 140, 280 and 560 bar and the viscosity was measured after heating at 90, 100 and 121°C for 0 (□), 20 (●), 40 (▲), 60 (○) and 120 (×) min.

Our results are in agreement with other research studies which refer to whey protein denaturation in the region of 70°C and above (Chen et al., 2005; de Wit, 2009). Homogenization of milk constitutes a necessary process in dairy industry in order to ensure a stable emulsion by preventing creaming during long shelf-storage. Stability after homogenization is acquired due to the fine distribution of the dispersed phase throughout the continuous phase. The size of the fat globules is reduced and an increased milk fat surface area is achieved (Cano-Ruiz & Richter, 1997; Kielczewska, Kruk, Czerniewicz & Haponiuk, 2006; Thiebaud et al., 2003). Starting from raw milk, the native milk fat globule membrane (MFGM) is insufficient to fully cover the increased fat surface area during homogenization and hence adsorption of new material from the milk serum at the oil-water interface takes place.

The newly formed interfacial layer consists of native MFGM together with adsorbed proteins, with caseins being the dominant group (Cano-Ruiz & Richter, 1997).

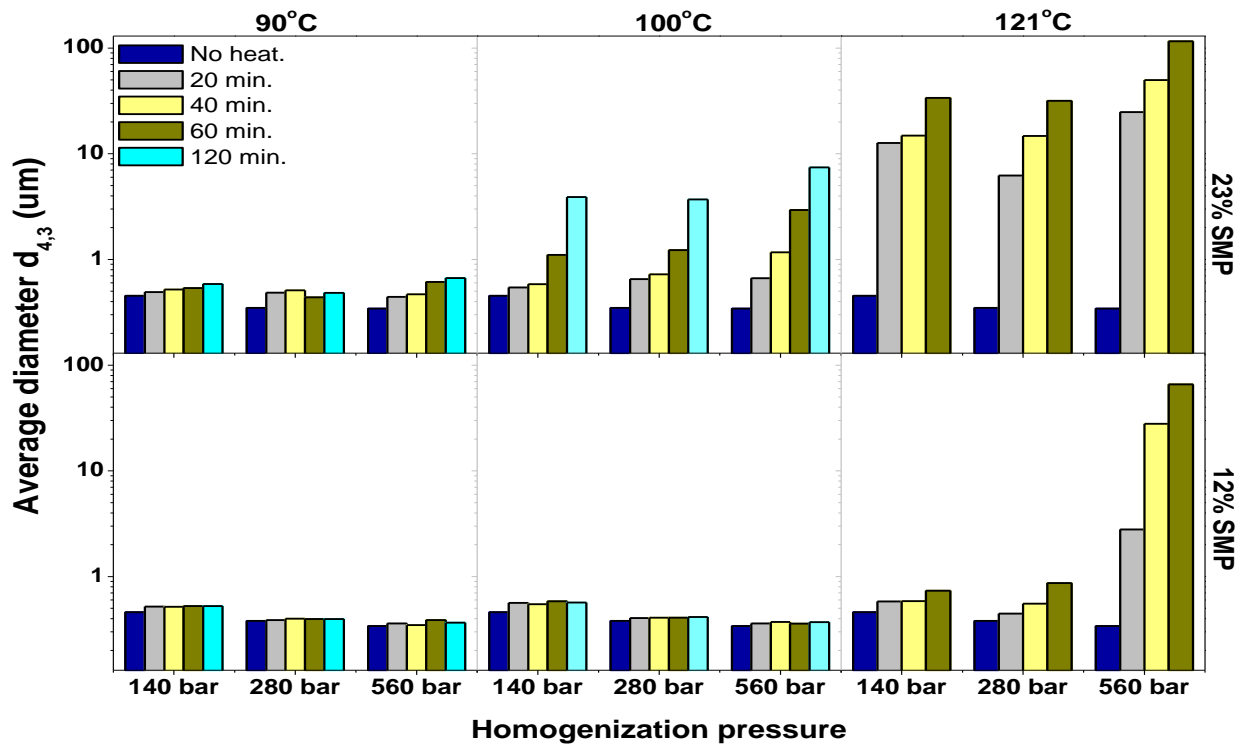


Figure 3.3. Volume-weighted average particle diameter ($d_{4,3}$) of RCM emulsions containing 23 and 12 % SMP, as well as 6.5 % oil, as a function of heating time, heating temperature and homogenization pressure.

It is more likely that heat-induced interactions of whey proteins with surface-adsorbed casein by the well known β -lactoglobulin- κ -casein interaction promote heat coagulation (Cano-Ruiz & Richter, 1997; McCrae et al., 1994). For recombined dairy emulsions, the interfacial layer is presumed to have similar properties irrespective of homogenization pressure. As less residual serum protein is left, a better heat stability might be expected at higher homogenization pressure. However, according to the von Smoluchowski collision theory, the increased number of emulsion droplets and hence smaller interdroplet distance seem to favor more pronounced 3-D aggregate formation for recombined milk processed at higher homogenization pressure.

From figure 3.3, it becomes clear that the average particle diameter of unheated samples decreased by increasing the homogenization pressure. The same observation can be obtained by inspecting the viscosity values of unheated samples containing 23 % SMP and 6.5 % oil; the viscosity gradually decreased from 9.7 to 8.1 and finally to 7.2 mPa.s for 140, 280 and 560 bar, respectively. However, upon heating, a different behavior was observed. The highest pressure (i.e. 560 bar) was proven to be the least effective in

terms of heat stability. More specifically, viscosity and average particle diameter were shifted towards higher values upon increasing the heating temperature, the heating time as well as the homogenization pressure. Comparable researches conducted previously referred to a reduced heat stability of milk with increasing pressure in the range 3.5-34.6 MPa (Jang & Swaisgood, 1990). This shift denotes an elevated degree of aggregation and thus decreased heat stability. The mechanism by which heat destabilization is favored due to homogenization is not yet fully understood but one would expect that the changed interfacial properties upon homogenization are involved (Anema & Li, 2003b).

For the sake of completeness it should be mentioned that statistical analysis (one-way Anova) which was performed in the case of samples containing 12 % SMP and 6.5 % oil, did not reveal any significant difference ($P > 0.05$) when the particle diameter was compared as a function of the homogenization pressure and the heating period, at a 95 % level of confidence. However, a significant difference was only observed ($P \leq 0.05$) upon comparison of particle diameter and temperature, a fact which denotes that temperature constitutes the detrimental parameter for heat-induced aggregation, in the case of samples with lower whey protein content in the serum phase. On the other hand, upon increasing the protein content (23 % SMP) more factors can initiate aggregate formation. More precisely, statistical analysis showed that aside from temperature, heating time ($P \leq 0.05$) can largely induce significant differences in the volume weighted average diameter.

Protein load alterations of RCM emulsions are displayed in table 3.2, both before and after heating at 100 and 121°C, at various time intervals. As it was expected, upon increasing the temperature and the heating time, the calculated protein load increases. The increase of protein load follows logically from the well-known interaction of denatured whey proteins with κ -casein upon heating which gives rise to additional protein deposition onto the oil-water interface. However, as high-heat SMP already contains denatured serum proteins and protein aggregates, the increase in protein load seems to be related with further increase of aggregation by increasing both the temperature and the time of heating. In other words, the higher the temperature and the heating period, the larger the degree of the heat-induced aggregation of the denatured whey proteins with κ -casein and fat droplets, as well as the degree of complexation between the incipient protein aggregates.

Table 3.2. Protein load of RCM ($n=3$) emulsions prepared by microfluidisation at a liquid pressure of 560 bar containing 23 % SMP and 6.5 % oil, as a function of heating time and temperature.

T (°C)	Heating time (min)	Protein recovery (%)	Protein load (mg/m²)
20	0	63.3	24.2±0.8
100	10	44.5	36.7±1.6
	20	32.1	45.0±1.4
	30	26.8	48.4±0.7
	40	26.5	48.6±1.8
	60	21.6	51.8±2.9
121	10	15.4	55.9±0.6
	15	15.5	56.0±2.1
	20	14.8	56.4±3.3

On a next step of this experimental study, and in order to investigate the effect of different emulsion formulations (table 3.1) with constant total solids content (i.e. 23 % w/w), the volume-weighted average diameter and the particle size distribution of emulsions was measured, following emulsification and heating at 121°C for 20 min. From figure 3.1 it becomes obvious that the average diameter was gradually increased upon increasing the oil content and decreasing the amount of solids-non-fat, compared to the unheated oil-free sample which was containing 23 % SMP and it was used as a reference. It is worth to mention that comparing the particle size of all samples prior to heating (data not shown), no remarkable changes were noticed upon changing the SMP:oil ratio.

Similarly, the particle size measurements clearly show that there is a monomodal differential distribution in the case of sample containing 23 % SMP and no oil, both before and after heating, whereas upon increasing the oil concentration, a gradual shift toward larger particle sizes was obtained and the curves became bimodal. Finally, upon measurement of the sample with the highest oil concentration, a curve which was

shifted to particle sizes between 10 and 1000 μm was obtained, a fact which can be ascribed to the heat-induced additional protein deposition on the oil/water interface in the cases where the fat content was increased.

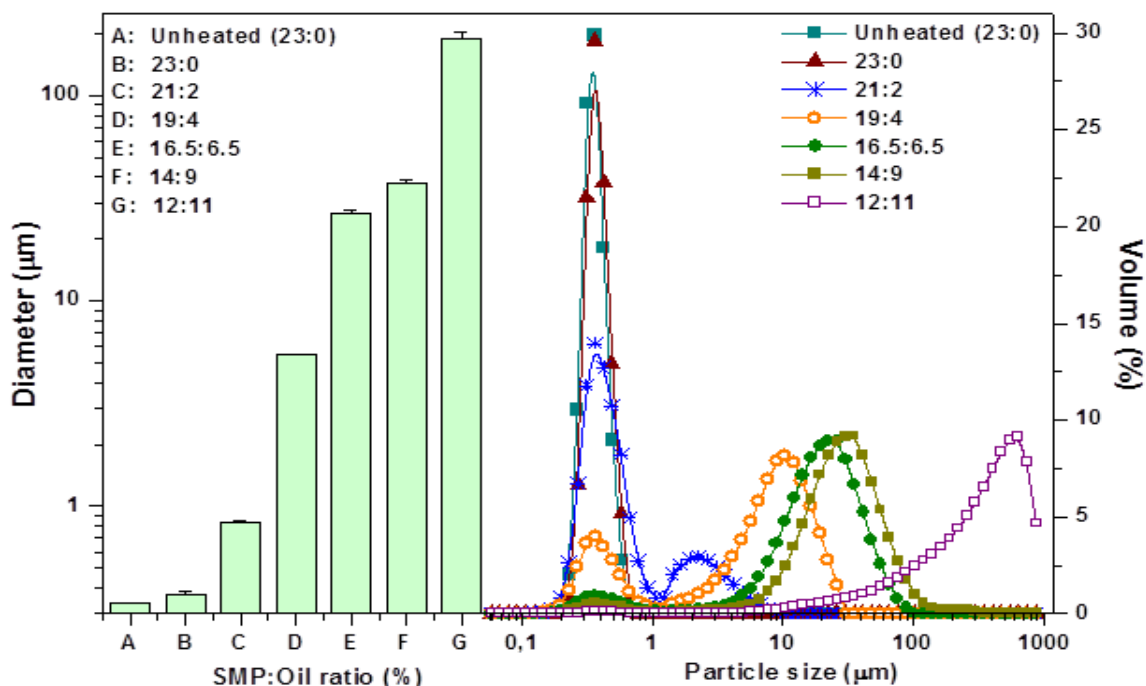


Figure 3.1. Volume-weighted average diameter ($d_{4,3}$) and differential particle size distribution of emulsions with a varying SMP:Oil ratio after heating for 20 min at 121°C.

Similarly, when the protein recovery was calculated (figure 3.2), a decrease of the residual protein in the serum phase was noticed upon increasing the oil and decreasing the protein content.

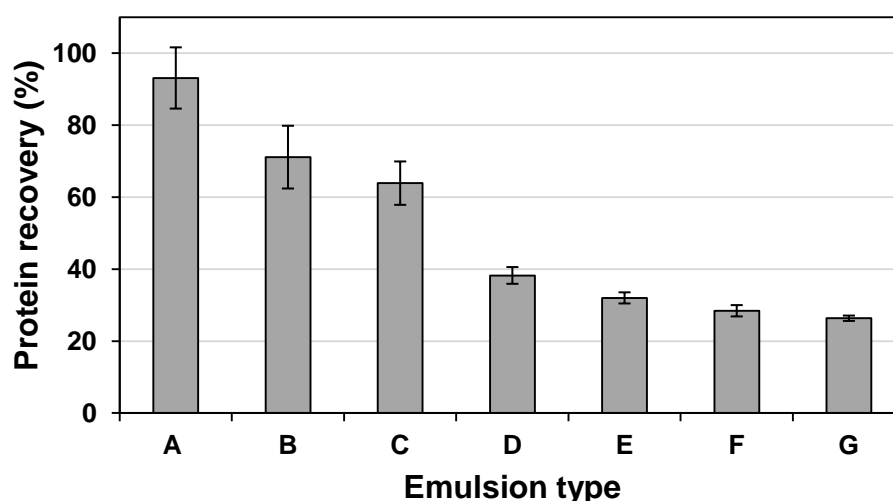


Figure 3.2. Protein recovery ($n=5$) of emulsions containing different SMP:oil ratio after heating for 20 min at 121°C. Description of samples A-G is given in table 3.1.

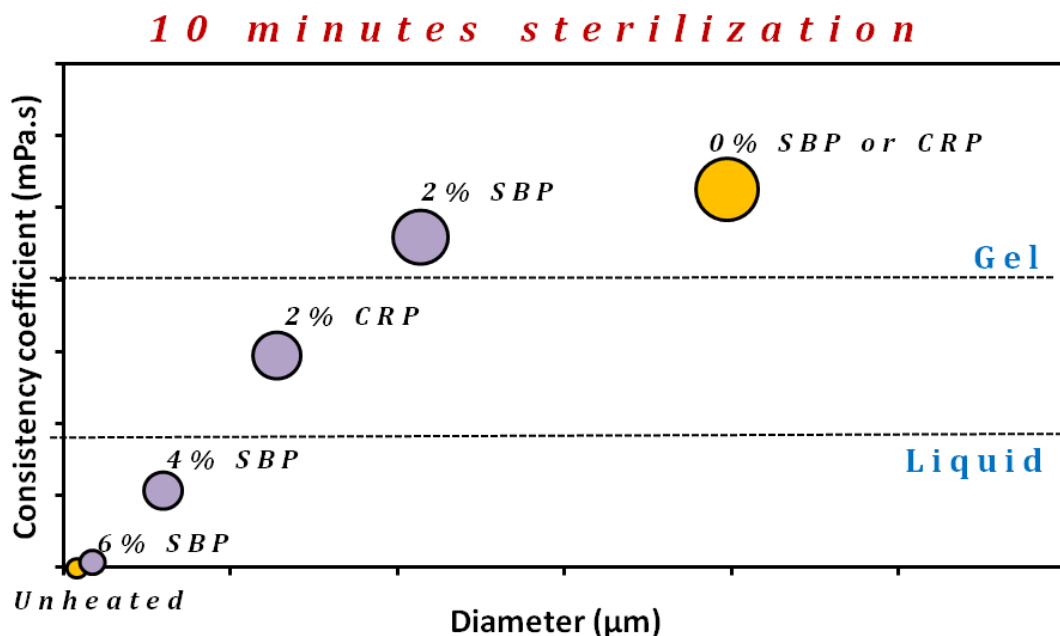
3.4. Conclusions

Viscosity and particle size measurements revealed that the protein content together with the homogenization pressure and the heating temperature play a crucial role in determining the heat stability of recombined concentrated milk emulsions. Heat treatment at 80°C, for oil-in-water emulsions containing 23 % SMP and 6.5 % sunflower oil, resulted in the onset of heat coagulation and hence increased viscosity and average diameter whereas more elevated temperatures caused the formation of a solid-like structure. In the case of 12 % SMP and 6.5 % oil, the effect of temperature and heating time was less pronounced due the lower content of protein.

Homogenization pressure was also proved to have a fundamental role in the heat stability of recombined concentrated milk emulsions. Whereas the average particle diameter of the unheated samples was decreased upon increasing the homogenization pressure, upon heating, the highest pressure (i.e. 560 bar) was proven to be the least effective in improving the heat stability. Increased particle number concentration is more decisive than reduced residual (whey) protein content in continuous phase. Finally, protein load determination indicated that sterilization gave rise to a significant increase of the amount of protein attached to the oil-water interface. In cases where the particle size and average diameter of emulsions with constant total milk solids, but with a varying SMP to oil ratio was determined, it was proven that upon heating, emulsions with higher fat content resulted in an increased amount of protein associated at the oil/water interface.

Chapter 4

IMPROVED HEAT STABILITY OF RECOMBINED CONCENTRATED MILK EMULSIONS UPON ADDITION OF POLAR LIPID ENRICHED DAIRY BY-PRODUCTS



This chapter is redrafted from: Kasinos M., Tran Le T. and Van der Meeren P. (2014), *Food Hydrocolloids*, 34, 112-118.

Chapter cover image: Consistency coefficient as a function of the volume-weighted average diameter of recombined concentrated milk samples containing different concentrations of phospholipids enriched dairy by-products, after heating at 121°C for 10 min.

Abstract

Over the last decades, milk fat globule membrane (MFGM) fragments have gained a considerable attention for their beneficial technological and nutritional properties due to the presence of proteins and phospholipids. During butter production, the MFGM is ruptured and a great amount of membrane material migrates to the aqueous fraction, known as buttermilk. Its high phospholipid concentration attributes a very interesting functionality to buttermilk.

As it has been shown before that phospholipid addition may improve the heat stability of concentrated dairy emulsions during sterilization, the effect of two phospholipid enriched dairy by-products on the heat stability of recombined concentrated milk emulsions was investigated. To that end, a cream residue powder (CRP) originating from the production of butter oil from dairy cream, as well as a sweet buttermilk powder (SBP) have been used to reduce the undesirable changes taking place during intense heating of concentrated milk. Samples were prepared containing CRP or SBP in different concentrations (0-6 %) and were heated for multiple time intervals at sterilization conditions (121°C). Both phospholipid enriched dairy by-products could largely reduce the pronounced viscosity increase as well as the increase in particle size observed upon intensive thermal treatment. Whereas the stabilizing effect of both products was directly proportional to their concentration, still the effect of CRP was more pronounced as compared to SBP: the addition of the maximum concentration (6 %) of both products resulted in a similar particle size distribution and viscosity as compared to the original emulsion before heating, while a lower concentration of CRP (4 %) also had a significant heat stabilizing effect. Whereas the difference in effectiveness could be probably related to the phospholipid content of both dairy ingredients, still it has to be kept in mind that these two ingredients not only differed in this aspect. Determination of the protein load revealed that phospholipid enriched dairy by-products reduced the increase in surface protein load upon sterilization, which points toward a reduced heat-induced interaction between the dairy proteins. Overall, our experiments revealed that phospholipid enriched dairy by-products have interesting functional properties and largely improve the heat stability of recombined concentrated milk emulsions. For the two products considered, their effect seemed to be related to their phospholipid content.

4.1. Introduction

Buttermilk is the liquid product obtained during churning of cream in the butter making process. It contains all the water-soluble components of cream such as the milk proteins, lactose and minerals (Sodini, Morin, Olabi & Jiménez-Flores, 2006; Vanderghem et al., 2010). Buttermilk has been used as animal feed or has been dried to be incorporated in bakery products due to its positive impact on flavor (Vanderghem et al., 2010). However, over the years it gained a considerable potentiality because of its high content in milk fat globule membrane (MFGM) material, a real biological membrane, composed mainly of polar lipids and unique membrane specific proteins. (Corredig & Dalgleish, 1997; Phan et al., 2013; Rombaut et al., 2007; Singh, 2006). The MFGM is disrupted during churning and migrates into the buttermilk portion. In a quite similar way, a MFGM-enriched side stream is also obtained during butter oil production by mechanical treatment to induce phase separation, and centrifugation of concentrated dairy cream. In this case too, the MFGM becomes displaced to the aqueous phase due to the mechanical stress. The phospholipid fraction of the MFGM consists of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) (Keenan & Dylewski, 1995; Patton & Keenan, 1975). Due to their enhanced biological activity and their positive effects on health, phospholipids have been extensively used for several medicinal studies (Britten, Lamothe & Robitaille, 2008), e.g. related to cell growth and development, prevention of Alzheimer's disease (Spitsberg, 2005), or protection against bacterial toxins and infections (Sprong, Hulstein & Van der Meer, 2002). Apart from phospholipids, and based on the isolation method chosen and the sample history, protein accounts for 25-60 % of the mass of the membrane material (Singh, 2006). Major MFGM proteins such as Mucin 1, Xanthine Dehydrogenase/Oxidase, Adipophilin, Butyrophilin, CD36 and PAS 6/7, have been purified and characterized (Dewettinck et al., 2008; Mather, 2000).

MFGM acts in milk as a natural emulsifying agent that protects the fat globules from coalescence. It is found in significant amounts in several dairy products including cream, butter, buttermilk, butteroil and cheese, and due to its unique functional properties and health benefits, led to extensive research and development of methods to isolate, purify and apply the MFGM material in different food emulsions (Corredig & Dalgleish, 1998; Elling et al., 1996; Phan, Le, Van der Meeren, & Dewettinck, 2014; Wong & Kitts, 2003).

Heat treatment is a very important unit operation during milk processing. However, upon heating at elevated temperatures, heat coagulation takes place as a result of whey protein denaturation (Journink & De Kruif, 1993; Singh, 2004), whereby heat induced whey protein interactions seem to play a crucial role. More precisely, at temperatures higher than 70°C, β -lg undergoes several conformational changes, making the hydrophobic residues as well as the free sulfhydryl group accessible (Galani & Apenten, 1999; Hansted, Wejse, Bertelsen & Otzen, 2011; Tran Le et al., 2011). The addition of phospholipids has been proved to protect milk against heat coagulation upon severe heating (Chen & Dickinson, 1998; Chen, Dickinson, Langton & Hermansson, 2000; McCrae & Muir, 1992; Tran Le et al., 2007). Hereby, it either displaces the protein from or it interacts with the protein at the interface (Brown, Carroll, Pfeffer & Sampugna, 1983; Kristensen, Nylander, Paulsson & Carlsson, 1997; McCrae, 1999).

In this contribution, the effect of two MFGM enriched and hence phospholipid enriched dairy by-products on the heat stability of recombined concentrated milk was evaluated. Heat induced effects were derived from both viscosity and particle size analyses, whereas protein surface load determinations were performed to obtain a better understanding of the effect of the MFGM-enriched dairy products on protein interactions in the recombined concentrated milk.

4.2. Materials and methods

4.2.1. Materials

High-heat skimmed milk powder (SMP), sweet buttermilk powder (SBP), and cream residue powder (CRP), also known as butteroil, were obtained from FrieslandCampina (Deventer, The Netherlands). According to the manufacturer, the SMP contained 37.3 % (w/w) protein, 0.5 % (w/w) fat and 0.15 % (w/w) phospholipids. SBP contained 32.0 % (w/w) protein, 9.0 % (w/w) fat and 2.9 % (w/w) phospholipids, whereas the CRP sample contained 30.2 % (w/w) protein, 15.0 % (w/w) fat and 6.4 % (w/w) phospholipids.

The high oleic sunflower oil (Hozol, Contined, Bennekom, The Netherlands) contained maximum 0.05 % free fatty acid as oleic. Its melting point is at 0°C and hence the oil remains clear even after 10 hours storage at 4°C.

4.2.2. Emulsion preparation

RCM samples were prepared containing 16.5 % (w/w) SMP and 6.5 % (w/w) oil. SBP or CRP were added in concentrations ranging from 0 to 6 %. Hereby, the composition of the samples was adjusted to ensure that they all contained the same amount of protein and fat (table 4.1).

Milk powders were diluted into 0.02 % NaN_3 (Acros Organics, Geel, Belgium) aqueous solution aiming to prevent microbial contamination, whereas Hozol oil was added as the dispersed phase.

Table 4.1. Calculated composition of recombined milk model systems with partial replacement of SMP by 2.0 %, 4.0 % and 6.0 % (w/w) sweet butter milk powder and cream residue powder, respectively, in order to have the same oil and protein content as in an emulsion containing 6.5 % (w/w) Hozol oil and 16.5 % (w/w) high heat skimmed milk powder.

Components	Composition (% w/w)						
	Reference	+Sweet Buttermilk Powder (%)			+Cream Residue Powder (%)		
	0.0	2.0	4.0	6.0	2.0	4.0	6.0
Skim milk powder	16.5	14.7	13.0	11.2	15.1	13.7	12.3
Hozol oil	6.5	6.3	6.1	5.9	6.1	5.8	5.4
NaN_3 aqueous solution	77.0	77.0	76.9	76.8	76.8	76.6	76.3
Phospholipid content	0.02	0.08	0.14	0.19	0.15	0.28	0.40

The samples were pre-homogenized by an IKA Ultra-Turrax TV45 (Janke & Kunkel, Staufen, Germany) for about 1 min and homogenized by a Microfluidiser 110S (Microfluidics Corporation, Newton, MA, USA) having its heat exchange coil immersed in a water bath at 55°C. The samples were microfluidized at a compressed air pressure of 2 bar, corresponding to a liquid pressure of about 280 bar for 2 min. The pH of the samples was determined (HI 4222, Hanna instruments) after homogenization, and was approximately 6.52 for all samples.

4.2.3. Heat treatment

Prior to heat treatment, a volume of 10 ml of each sample was transferred into 20 ml headspace vials (75.5 x 22.5 mm, Grace, Deerfield, IL, USA) which were clipped on a platform and positioned in a temperature-controlled oil bath (Fritel turbo SF®, 5L capacity) in order to be heated at 121°C for a time range of 0-15 min.

In order to optimize the heating process, preliminary measurements were performed including samples in the absence of phospholipid-enriched dairy products. During heating, the temperature inside the sample was measured by using thermocouples (type J) connected with an electronic digital thermometer (Agilent 34970A). According to the recorded temperature profiles, it took about 9 min before the whole contents reached a temperature of 121°C. Hence, in order to evaluate the influence of sterilization temperature on RCM samples with and without phospholipids, the heating time was considered from the time that the temperature was homogeneous inside the sample. Therefore, the maximum heating time of 15 min at 121°C actually involved submersion of the glass vials for 24 min, i.e. 9 min equilibration to reach 121°C and 15 min holding at 121°C.

4.2.4. Viscosity measurements

To assess the tendency of heat-induced coagulation, viscosity measurements of RCM emulsions were executed before and after heat treatment as described in section 2.2.4.

The experimental data fitted very well to a power law relation:

$$\tau = K \cdot \dot{\gamma}^n \quad (1)$$

As the apparent viscosity μ is obtained as the ratio of shear stress τ (in Pa) to shear rate $\dot{\gamma}$ (in s⁻¹), the power law equation can be rewritten as:

$$\mu = K \cdot \dot{\gamma}^{n-1} \quad (2)$$

where K represents the consistency coefficient and n represents the flow behavior index.

4.2.5. Particle size distribution

The particle size distribution of RCM emulsions was examined by using a Mastersizer S long bed (version 2.15, Malvern Instruments Ltd, Malvern, UK) as explained in section 2.2.5.

4.2.6. Protein load

The protein load was calculated from the ratio of protein to fat content in the cream phase before and after heating. The cream phase was separated by a Sorvall RC 6+ Centrifuge (Milford, MA, USA) at 22,090 x g for 90 min at room temperature. Sucrose (VWR International, Leuven, Belgium) was used as a density increasing agent (Van der Meeren et al., 2005); samples of 40 mL were prepared and heated for 0, 2, 6 and 10 min at 121°C. Sucrose (20 g) was added directly into the emulsions which were subsequently stirred vigorously until its complete dissolution. Finally, 40 mL of the final samples were transferred in the centrifuge tubes (Beckman, Brea, CA, USA). After centrifugation, the cream layer was carefully separated to determine protein and fat content by the Kjeldahl and Rose-Gottlieb methods, respectively.

4.3. Results and discussion

4.3.1. Effect on viscosity and particle size distribution

The heat stabilizing effect of sweet buttermilk and cream residue powder on recombined concentrated milk emulsions was evaluated, upon heating at sterilization conditions and at various time intervals. For that reason, SMP was partially replaced by 0, 2, 4 or 6 % phospholipid enriched dairy by-products. A visual observation after cooling down the samples confirmed an obvious destabilization of the emulsions containing the lowest concentrations of SBP or CRP and heated for an extensive period. On the other hand, samples containing 6 % of both powders retained their liquid-like appearance, even after heating for 15 min at 121°C.

To collect more detailed information, viscosity and particle size distribution measurements were performed. Figures 4.1 and 4.2 illustrate the changes in viscosity and particle size distribution of recombined concentrated milk emulsions upon addition of either SBP or CRP at different quantities. Both dairy by-products were highly effective to largely prevent the heat-induced increase in viscosity and particle size distribution. Whereas the viscosity of the reference sample was highly affected after heating for 2 min, the addition of 6 % SBP resulted in samples with viscosity properties similar to the emulsion which was not subjected to any heat treatment.

By increasing the time of heating up to 15 min, the viscosity was significantly increased and only the maximum concentration of SBP (6 %) was capable to maintain the viscosity

at relatively low levels. On the other hand, absence of SBP resulted in the formation of a solid-like structure, which provides evidence of heat coagulation. The calculated consistency coefficient depicted in table 4.2, shows a gradual decline upon increasing SBP content, for each heating time.

Table 4.2. Consistency coefficient (in mPa.s) of recombined concentrated milk samples without and with partial replacement by 2.0 %, 4.0 % and 6.0 % (w/w) sweet butter milk powder and cream residue powder, respectively, as a function of heating time at 121°C.

Heating time (min.)	Consistency coefficient (mPa.s)						
	Reference 0.0	+Sweet Buttermilk Powder (%)			+Cream Residue Powder (%)		
		2.0	4.0	6.0	2.0	4.0	6.0
0	7						
2	2657	2552	505	45	260	28	26
6	3847	2654	606	43	2844	30	27
10	5253	4600	1081	89	2964	102	29
15	6200	5966	2864	733	3655	134	64

As the slope of the logarithm of the apparent viscosity as a function of shear rate corresponds to one diminished by the flow behavior index, it is obvious from figure 4.1 that the flow behavior of the unheated sample was near-to-newtonian ($n=0.836$), whereas the behavior became increasingly more pseudoplastic upon heat coagulation. More precisely, upon heating samples containing 6 % SBP, the flow behavior index (n) values were 0.766, 0.661, 0.450 and 0.421 upon 2, 6, 10 and 15 min sterilization, respectively. A further analysis of the data obtained at different heating times and with different SBP levels, revealed an exponential relationship between the consistency coefficient and the flow behavior index (n) with a determination coefficient of 0.75 (i.e. a correlation coefficient of 0.87).

Samples containing CRP revealed results with a similar trend compared to SBP: the addition of CRP had a pronounced effect on the prevention of the heat induced increase of viscosity. Whereas, 2 % of CRP, as in the case of SBP, led to the formation of samples with strong aggregation attributes upon heating for 15 min at 121°C, 4 % CRP was sufficient to keep the viscosity to levels comparable to the unheated milk emulsions: the viscosity upon heating for 15 min at 121°C diminished from 377 (0 % CRP) to 33 (4 % CRP) and 19 mPa.s (6 % CRP) at a shear rate of 56 s⁻¹. The decreased consistency

coefficient of recombined concentrated milk samples upon CRP addition, heated from 2-15 min, is shown in table 4.2. From figure 4.1 it becomes obvious that the viscosity of the samples increased upon increasing the heating time. However, the addition of the maximum concentration (6 %) of both dairy by-products resulted in viscosity curves which follow similar pattern as compared to the ones without heating (flow behavior index more close to unity).

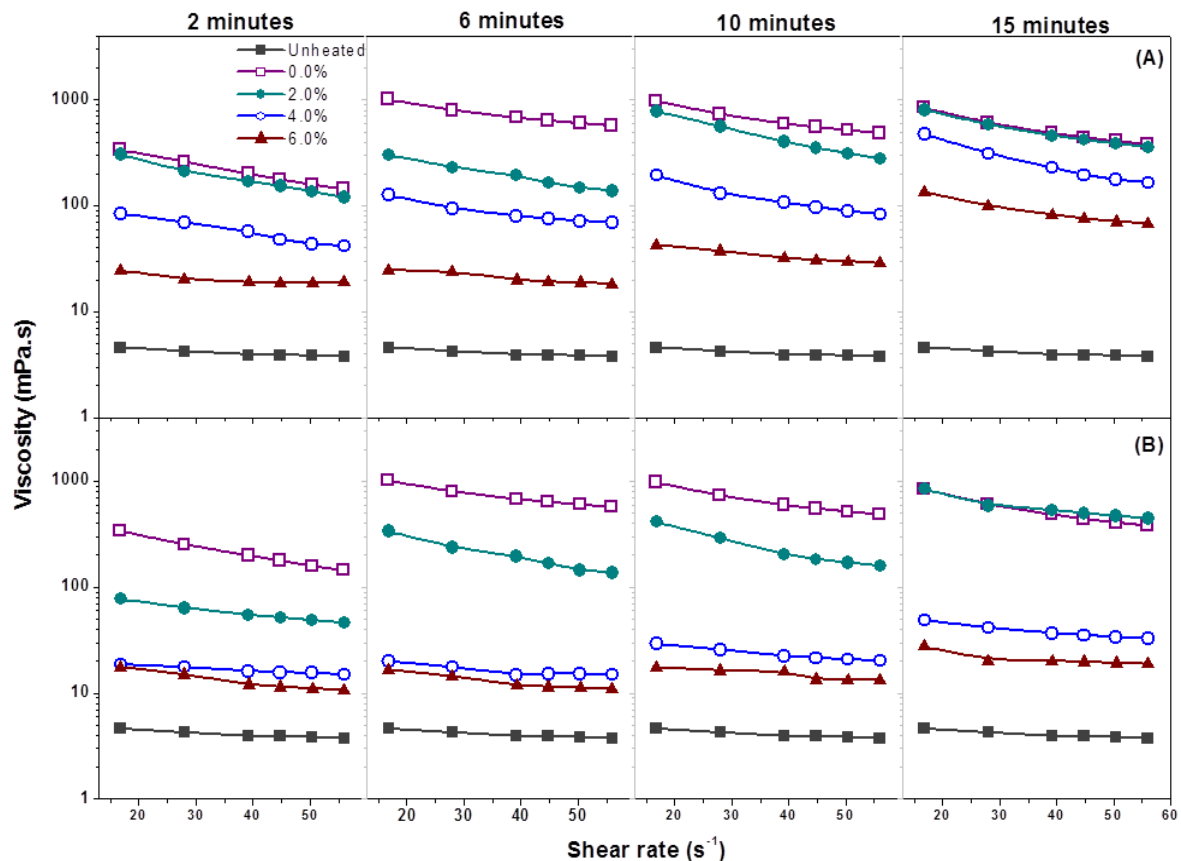


Figure 4.1. Apparent viscosity of recombined concentrated milk emulsions containing (\square) 0.0 %, (\bullet) 2.0 %, (\circ) 4.0 % and (\blacktriangle) 6.0 % (w/w) sweet buttermilk powder (A) and cream residue powder (B) as a function of shear rate (s^{-1}). Viscosity was measured after heating for 2, 6, 10 and 15 min at 121°C.

However, the effect of CRP was much larger compared to SBP: lower concentrations of CRP (4 %) also had a significant heat stabilizing effect. The differential volume-weighted particle size distributions shown in figure 4.2 confirm the results mentioned above related to the addition of SBP and CRP to recombined concentrated milk emulsions.

The effect of MFGM-enriched dairy products on the particle size distribution of the heated samples was clearly demonstrated by the significant shift of the differential curves toward smaller particle size when increasing the concentration of both SBP and CRP.

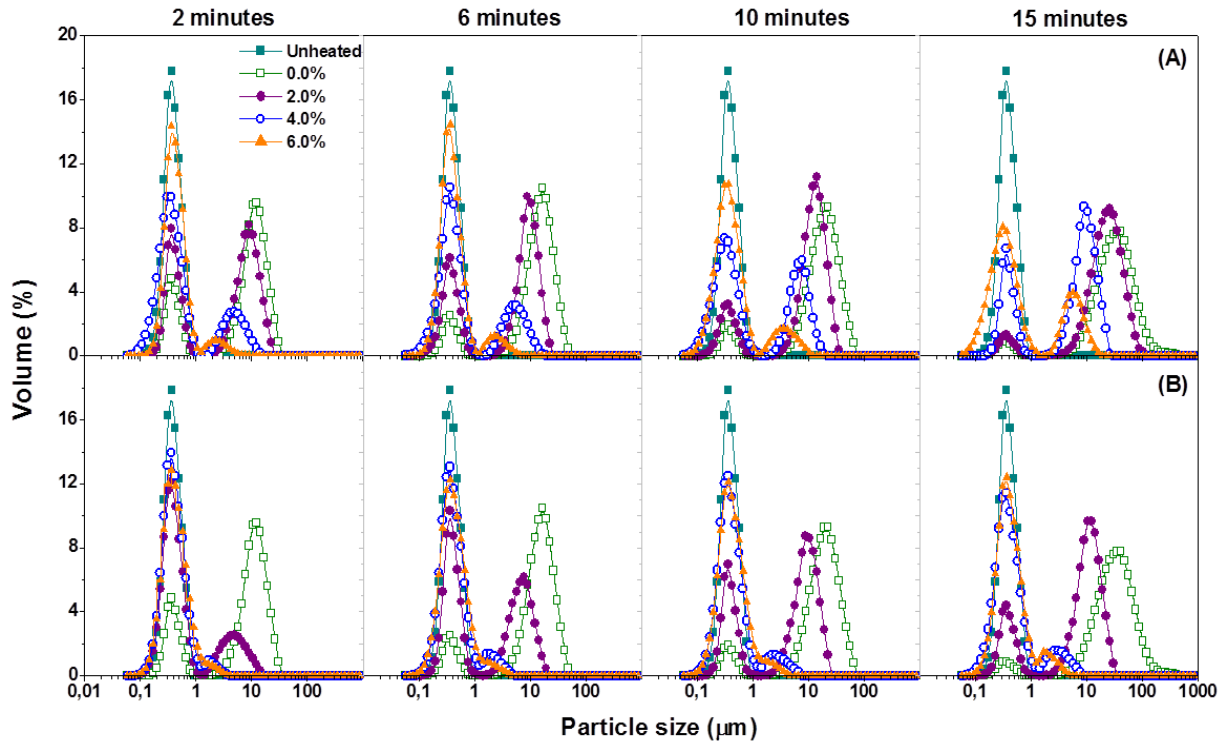


Figure 4.2. Differential volume-weighted particle diameter distribution (in μm) of recombined concentrated milk emulsions containing (\square) 0.0 %, (\bullet) 2.0 %, (\circ) 4.0 % and (\blacktriangle) 6.0 % (w/w) sweet buttermilk powder (A) and cream residue powder (B) as a function of heating time at 121°C.

When no or low concentration (2 and 4 %) of SBP was used, there was an increase of particle size upon heating due to agglomeration and this was even more pronounced when the heating time was raised (6-15 min). It is obvious that when the highest amount of SBP (6 %) was added, a remarkable decrease of the aggregation tendency was observed. However, when CRP was used as phospholipid source, 4 % was enough to decrease the heat coagulation phenomenon to insignificant levels.

The stronger effect of CRP is in line with its (almost double) phospholipid concentration: CRP contained 6.4 % of phospholipids, as opposed to SBP with a total phospholipid content of only 2.9 %.

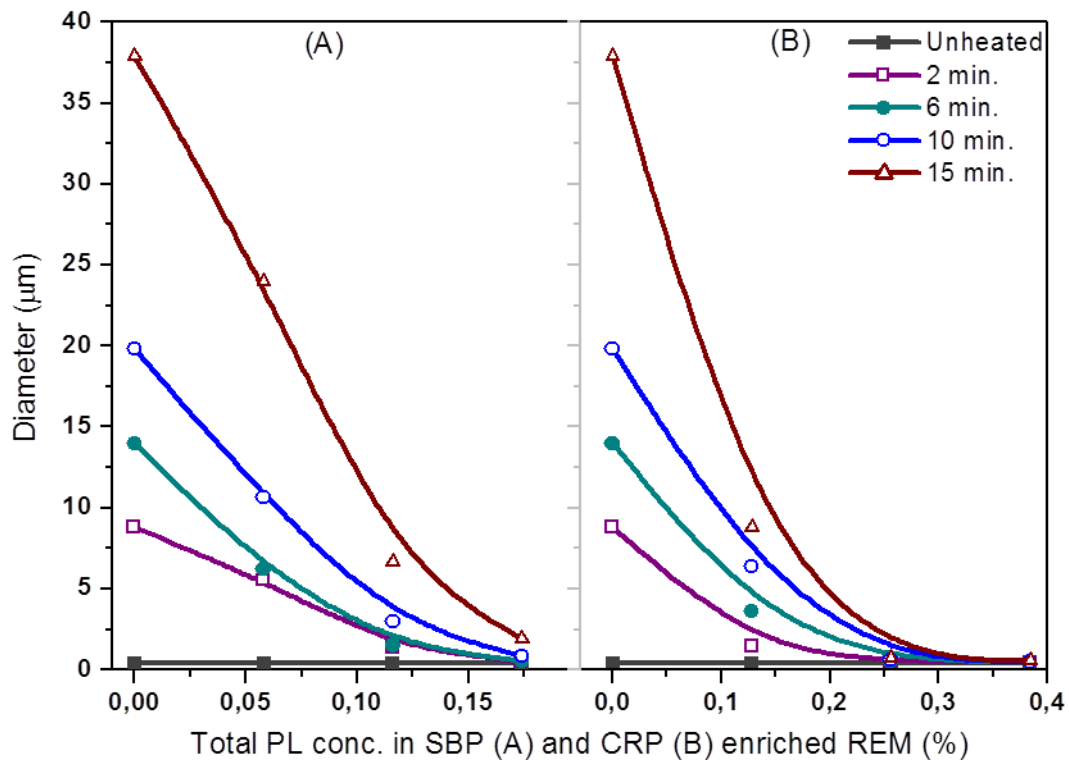


Figure 4.3. Volume-weighted average diameter (in μm) of recombined concentrated milk emulsions containing (A) sweet buttermilk powder and (B) cream residue powder as a function of the total phospholipid concentration. The diameter was measured after heating for (\square) 2, (\bullet) 6, (\circ) 10 and (\blacktriangle) 15 min at 121°C .

To enable comparison, the total phospholipid content of all samples was calculated based on the composition of SMP, SBP and CRP (table 4.1). When the volume weighted average diameter $d_{4,3}$ was plotted as a function of the total phospholipid concentration in both SBP and CRP stabilized emulsions (figure 4.3), a much more similar effect was observed for both MFGM enriched products.

For the sake of completeness, it has to be mentioned that CRP and SBP do not only differ in phospholipid content, and hence other factors may contribute to the observed differences as well.

These observations are in line with the decreased droplet size in a coffee cream simulant upon addition of highly hydrolyzed soybean lecithin, as observed by Van der Meeren et al. (2005). Kristensen, Nylander, Paulsson and Carlsson (1997) concluded from DSC measurements that the interaction between β -lg and phospholipids leads to a stabilization of the protein against thermal unfolding. Hence the effect of the phospholipid enriched dairy by-products is assumed to result (at least partly) from phospholipid-whey protein interactions, hereby reducing interactions between whey

proteins as well as between whey proteins and casein micelles. An alternative explanation might be the displacement of proteins from the interface (McCrae, 1999) resulting in less protein adsorption and hence in a thinner adsorbed layer. For the sake of clarification, it should be mentioned that the reduced heat-induced aggregation of RCM in the presence of phospholipids, seems to be mostly correlated with the interactions which take place between phospholipids and whey protein aggregates, rather than individual denatured whey proteins, since high-heat SMP has been produced by heating at high temperatures and therefore the serum proteins have already undergone denaturation. For that reason, the beneficial effect of phospholipids seems to be related to the prevention of further aggregation and hence coagulation, by further increasing the heating time and temperature.

4.3.2. Protein load

In order to elucidate the physicochemical changes taking place upon sterilization of recombined concentrated milk, the protein load was determined from the protein and fat content of the cream layer of the recombined concentrated milk emulsions obtained by centrifugation of the samples (before and after heating at sterilization temperatures) both in the absence and presence of SBP. Overall, the protein load in the cream layer increased significantly by rising the heating time: a paired two samples t-test for means, showed a significant difference between samples that were not heated and those heated for 2 min ($P \leq 0.05$), with a 95 % level of confidence, whereas even more significant differences were found between the unheated samples and those heated for 6 min ($P \leq 0.01$) and 10 min ($P \leq 0.01$), respectively. On the other hand, this increasing effect was partly prevented by increasing the concentration of SBP. This effect was most obvious after sterilization for 15 min, where the protein load decreased from approximately 25 to 11 mg/m², after addition of 6 % SBP. More precisely, the protein load after 2 and 6 min of heating, increased to almost 8.5 mg/m², whereas 10 min of heating resulted in a protein load of almost 11 mg/m², as compared to the protein load of the unheated sample, in the absence of SBP, which was approximately 7 mg/m².

The decreased protein load upon heating in the presence of SBP is in line with the heat stabilizing properties of phospholipids. Van der Meeren et al. (2005) observed a similar effect upon addition of hydrolyzed soybean lecithin to a coffee cream simulant. Crujisen

(1996) also found a doubling of the protein surface load of caseinate-stabilized emulsions upon sterilization, in the absence of lecithin, whereas no increase was observed upon crude soybean lecithin addition.

The effect of MFGM-enriched material on the protein load could be probably ascribed to the prevention of interfacial protein interactions upon heating, rather than toward interfacial protein displacement. On the other hand, some limited protein displacement cannot be ruled out, considering the slight reduction in protein load in unheated samples upon partial replacement of SMP by SBP (figure 4.4).

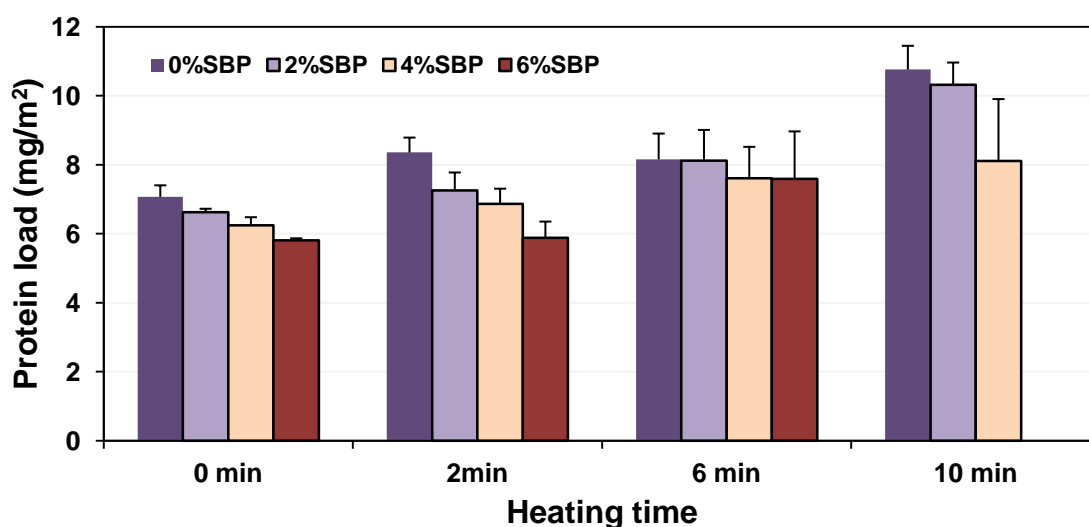


Figure 4.4. Protein load (in mg/m^2) of recombinated concentrated milk emulsions ($n=2$) containing 0, 2, 4 and 6 % of SBP and heated for 0, 2, 6 and 10 min at 121°C .

4.4. Conclusions

Viscosity and particle size distribution measurements revealed that MFGM-enriched and hence phospholipid enriched dairy by-products, such as Sweet Buttermilk Powder and Cream Residue Powder, largely improve the heat stability of recombinated concentrated milk. For this type of milk products heat treatment at sterilization conditions (121°C) resulted in the formation of a solid-gel structure, in the case of samples containing no phospholipids, which is believed to be due to further whey protein aggregation as well as whey protein-casein interaction.

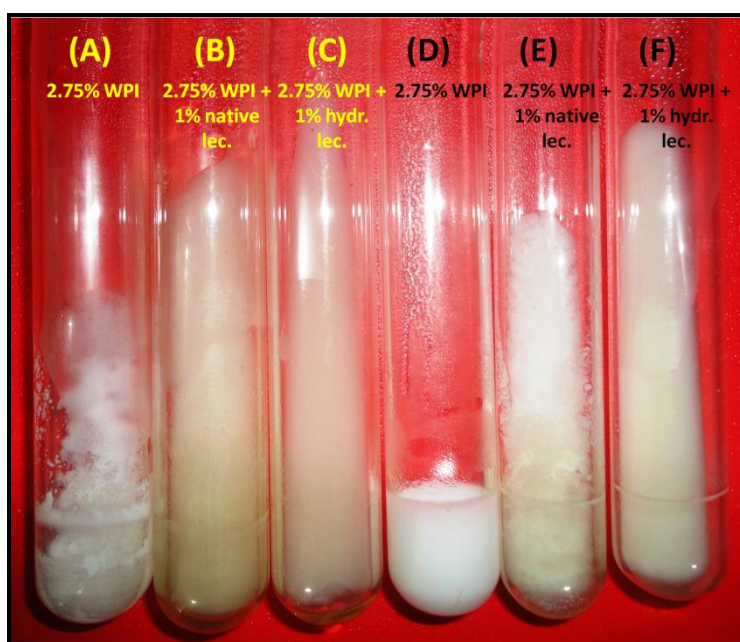
The addition of both SBP and CRP exercised a heat stabilizing effect, largely preventing the heat-induced increase in viscosity and particle size. The effect of both dairy by-products was proportional to the added concentration. As the observed differences in

viscosity and particle size distribution could be related to the different phospholipid content of the two ingredients, their heat-stabilizing effect was thought to be due to the beneficial effect of the phospholipids originating from the MFGM that they contained, although other factors could also play a role. MFGM-specific proteins are also characterized by an amphiphilic nature and acting as surface-active compounds, and hence heat-stabilizing effect could also be influenced by the presence of these components. Finally, MFGM-enriched dairy products gradually reduced the heat-induced increase in protein surface load, which points toward the decreased occurrence of heat-induced protein-protein interactions that lead to particle aggregation and concomitant viscosity increase.

In conclusion, phospholipid-enriched dairy by-products are very promising ingredients for recombined milk emulsions that are subjected to an intense heat treatment because they enable heat stabilization without exogenous ingredients, such as soybean lecithins or alternative surfactants.

Chapter 5

EFFECT OF HYDROLYZED SUNFLOWER LECITHIN ON THE HEAT-INDUCED COAGULATION OF RECOMBINED CONCENTRATED MILK EMULSIONS



This chapter is redrafted from: Kasinos M., Goñi M. L., Nguyen M. T., Sabatino P., Martins J.C., Dewettinck K. and Van der Meeren P. (2014), International Dairy Journal, 38, 187-194

Chapter cover image: Slanted tubes containing 2.75 % whey protein isolate (A) and (D), a mixture of 2.75 % whey protein isolate with 1 % native lecithin (B) and (E), and a mixture of 2.75 % whey protein isolate with 1 % lecithin hydrolyzed for 20 min (C) and (F), after incubation for 1 and 2 min at 80°C.

Abstract

In an attempt to decrease the degree of heat-induced intermolecular protein interactions in recombined concentrated milk, the effect of sunflower lecithin, a natural source of phospholipids, was examined. The obtained results proved that lecithin supplemented milk emulsions demonstrated a less pronounced increase in viscosity as well as particle size, upon severe heating.

Additionally, enzymatic hydrolysis was performed using a phospholipase A1. Phosphorus Nuclear Magnetic Resonance (³¹P-NMR) and High Performance Liquid Chromatography (HPLC) revealed that the lysophospholipid content reached a maximum after 20 min of hydrolysis and gradually declined as the hydrolysis reaction duration was extended.

This work showed that sunflower lecithin largely improves the heat stability of whey protein containing solutions and emulsions due to its high phospholipid content. As the phospholipid molecular structure is important, it follows that the heat stability of whey protein containing products may be optimized by appropriate processing, such as enzymatic hydrolysis of intact lecithins.

5.1. Introduction

From a physico-chemical point of view, milk is considered as a natural oil-in-water (O/W) emulsion, where fat droplets are dispersed in an aqueous continuous phase. Whereas the interface of the fat globules in raw milk is covered by the so-called milk fat globule membrane (MFGM), the interface in homogenized and recombined milk is mainly covered by the main milk proteins, i.e. casein micelle particles and whey proteins.

To obtain a microbiologically stable product, intense heating is mostly applied. A number of studies have shown that upon heating β -lactoglobulin, the major globular protein of the whey fraction, its dimeric configuration dissociates into monomeric. In addition, structural rearrangements occur making accessible the hydrophobic residues and the thiol group, which were previously enclosed in the globular structure; they can therefore be involved into aggregates formation via thiol-disulphide exchange reactions with κ -casein, as well as with other denatured whey protein molecules (Anema & Li, 2003; Pelegrine & Gasparetto, 2005; Verheul et al., 1998).

During heating of milk, several reactions take place, including denaturation of whey proteins and complex formation between denatured whey proteins, casein micelles and fat droplets, giving rise to a phenomenon known as heat coagulation (Corredig & Dalgleish, 1996; Dissanayake & Vasiljevic, 2009; Jeurink & De Kruif, 1993; Kasinos, Tran Le & Van der Meeren, 2014; Oldfield, Singh, Taylor & Pearce, 2000; Singh, 2004; Verheul, Pedersen, Roefs & De Kruif, 1998).

Previous research demonstrated that lecithin, a natural low molecular weight surfactant composed of a mixture of various phospholipids, can influence the heat stability of whey protein containing emulsions (Crujisen, 1996; Hardy, Sweetsur, West & Muir, 1985a; McSweeney, Healy & Mulvihill, 2008; Tran Le et al., 2007). However, the effects have been described either as positive or negative, depending on the type and amount of added lecithin (Yamamoto & Araki, 1997) and consequently, an in-depth analysis is required.

Cabezas, Madoery, Diehl and Tomás (2011), McCrae (1999), Tran Le et al. (2011) and Van der Meeren, El-Bakry, Neiryneck & Noppe (2005), described the improved heat-stabilizing effects of hydrolyzed over native lecithin, after its incorporation in O/W

emulsions. In addition, Tran Le et al. (2007) demonstrated that the effect of hydrolyzed soybean lecithin in decreasing heat-induced whey protein-casein interactions was significant, whereas the effect of non-hydrolyzed lecithin was almost negligible. Similarly, particle size analysis performed by Sünder, Scherze and Muschiolik (2001) and Cabezas et al. (2011), as well as heat stability evaluation by McCrae (1999), found hydrolyzed lecithins to be most effective, whereas Jost, Dannenberg and Rosset (1989) mentioned that hydrolyzed lecithin incorporation, before or during emulsification, reduced the strength of a heat-set whey protein emulsion gel.

The aim of this contribution was to study the effect of sunflower lecithin, a byproduct from the degumming process of crude sunflower seed oils (Pan, Tomás & Añón, 2002), after its incorporation prior to emulsification, on the heat induced coagulation of recombined concentrated milk emulsions, subjected to intense heating. The main justification for sunflower lecithin utilization emerges from its classification as a non-GMO product and it can therefore be considered as an attractive replacement for soybean lecithin.

In addition, intact sunflower lecithin was enzymatically treated by Lecitase® Ultra, a protein-engineered phospholipase A1 (Mishra, Kumaraguru, Sheelu & Fadnavis, 2009). The enzymatic treatment was carried out in order to establish the potential of hydrolyzed lecithin to further reduce the coagulation tendency of concentrated milk. Finally, hydrolysis of lecithin was performed for varying time intervals, in order to determine the significance of hydrolysis on heat stability.

5.2. *Materials and methods*

5.2.1. *Materials*

High-heat skimmed milk powder (SMP) was obtained from FrieslandCampina (Deventer, The Netherlands). According to the manufacturer, the SMP contained 37.3 % (w/w) protein, 0.5 % (w/w) fat and 0.15 % (w/w) phospholipids. Whey protein isolate (WPI) was obtained from Davisco Foods International, Inc. (BiPro®, Le Sueur, MN, USA). Kjeldahl analysis revealed that this WPI contained 92.6 % of protein, whereas polyacrylamide gel electrophoresis (PAGE) indicated that approximately 85 % of the

total protein consisted of β -lactoglobulin. Besides, the WPI contained 1.6 % ash (by incineration at 525°C), 5.0 % moisture (from weight loss at 102°C) and 0.8 % fat.

Sunflower lecithin was obtained from Oleaginoso Moreno (Bahia Blanca, Argentina). According to the manufacturer, this lecithin contains 66.9 % acetone insoluble matter, 0.5 % moisture and 32 % oil.

Lecitase® Ultra was purchased from Novozymes (Bagsværd, Denmark). This is a protein-engineered phospholipase A1 containing 6.5 % protein and is a carboxylic ester hydrolase from *Thermomyces lanuginosus*/*Fusarium oxysporum* produced by the submerged fermentation of a genetically modified *Aspergillus oryzae* (Mishra et al., 2009).

The high oleic sunflower oil (Hozol, Contined, Bennekom, The Netherlands) contained maximum 0.05 % free fatty acid as oleic. Its melting point is at 0°C and hence the oil remains clear even after 10 h storage at 4°C.

Ca-imidazole buffer containing 20 mM imidazole, 5 mM CaCl₂.H₂O, 30 mM NaCl and 1.5 mM NaN₃ was prepared according to Anema (1997). Its pH was adjusted to 6.55 with 1N HCl. This buffer was selected to have a pH and calcium activity that resembled those of cow's milk.

5.2.2. Enzymatic hydrolysis

Non-hydrolyzed sunflower lecithin dispersion was prepared by adding 4 % (w/w) lecithin in distilled water and was stirred with a magnetic stirrer at 50°C, until homogeneous. HCl (0.1N) was used in order to adjust the pH of the reaction medium to 5.0. The mixture was transferred into a waterbath to control and maintain the temperature at 50°C and subsequently, 0.05 % (v/v) Lecitase® Ultra was added to the dispersion. As free fatty acids are released upon enzymatic hydrolysis, NaOH addition is required to keep pH constant. For that purpose, an automatic titrator, Dosimat 765 (Metrohm, Herisau, Switzerland), was used to measure pH and add the required volume of NaOH. This method involves the continuous titration of the fatty acids released during enzymatic hydrolysis of the phospholipids.

The samples remained in the water bath for 10, 20, 30, 40 and 60 min. To stop the reaction, the mixtures were subsequently incubated at 100°C for 5 min, in order to inactivate the enzyme.

5.2.3. NMR measurement

Quantitative ^{31}P -NMR was used to analyze non-hydrolyzed and hydrolyzed sunflower lecithin. Samples were prepared with distilled water and D_2O (99.8 % AtomD, Armar Chemicals, Gottingen, Switzerland) at a 1:1 ratio, containing 20 mg/mL of either non-hydrolyzed or hydrolyzed sunflower lecithin, 10 % (w/w) sodium deoxycholate (Sigma-Aldrich, Steinheim, Germany), 2 mM Na-EDTA (Acros Organic, Geel, Belgium) and 1 mM Glyphosate (Sigma-Aldrich) as internal standards for quantification. All samples were mixed using a Sonifier 250/450 (Branson Ultrasonics, Danbury, CT, USA), as well as Shaker SM-30 (Edmund Bühler, Hechingen, Germany) at 40°C alternately, whereas the pH was adjusted to 8.0 with NaOH before measurements. ^{31}P NMR spectra were performed on a Bruker Avance 500 (Rheinstetten, Germany) operating at a ^{31}P frequency of 202.46 MHz, equipped with a BBI 5 mm probe. Inverse gate proton decoupling was used for suppression of Nuclear Overhauser Effect (NOE). Measurements were performed at 40°C and the temperature was controlled to within $\pm 0.01^\circ\text{C}$ with a Eurotherm 3000 VT (Ashburn, VA, USA) digital controller. The following instrument settings were used: 65180 data points, 90° excitation pulse, number of scans of 256 with a 12.25 s relaxation delay time and a decay acquisition time of 3.22 s. Phospholipon 90G, a highly purified soy bean lecithin containing at least 90 % PC (Rhône-Poulenc, Köln, Germany) and pure lysophospholipids standards (NOF Corporation, Tokyo, Japan) were used for peak identification.

5.2.4. HPLC analysis

Phospholipids analysis was carried out with a Shimadzu HPLC System (Tokyo, Japan) with an evaporative light scattering detector (ELSD, Alltech-3300, Alltech Associates Inc., Lokeren, Belgium). The analysis procedure was performed according to the method described by Nguyen et al. (2014). All analyses were executed in duplicate.

5.2.5. Emulsion preparation

Recombined concentrated milk emulsions were prepared using 16.5 % (w/w) SMP, 6.5 % (w/w) oil, as well as 0.0 - 0.3 % of (hydrolyzed) sunflower lecithin. The lecithins were first added into 0.02 % NaN_3 (Acros Organics, Geel, Belgium) aqueous solution, to prevent microbial contamination, and the solutions were subsequently stirred over night until a homogeneous dispersion was obtained. Finally, SMP powder and high oleic

sunflower oil were added and the emulsions were pre-homogenized by IKA Ultra-Turrax TV-45 (Janke & Kunkel, Staufen, Germany) for about 1 min, followed by homogenization at 55°C using a Microfluidiser 110S (Microfluidics Corporation, Newton, MA, USA). The samples were microfluidized at a compressed air pressure of 4 bar corresponding to a liquid pressure of 560 bar for 2 min. After microfluidisation, the emulsions were left to cool down to room temperature. The pH of all prepared emulsions was 6.6.

5.2.6. Heat treatment

Prior to heat treatment, a volume of 10 mL of each emulsion was transferred into 20 mL vials (75.5 x 22.5 mm, Grace, Deerfield, IL, USA) which were placed in a temperature controlled oil bath (Fritel turbo SF®, 5 L capacity) and heated for 0-35 min at 121°C. After heat treatment, the samples were placed under tap water to allow rapid cooling.

5.2.7. Viscosity measurements

To assess the tendency of heat-induced coagulation, viscosity measurements of RCM emulsions were executed before and after heat treatment as described in section 2.2.4.

The experimental data fitted very well to a power law relation:

$$\tau = K \cdot \gamma^n \quad (1)$$

As the apparent viscosity μ is obtained as the ratio of shear stress τ (in Pa) to shear rate γ (in s⁻¹), the power law equation can be rewritten as:

$$\mu = K \cdot \gamma^{n-1} \quad (2)$$

where K represents the consistency coefficient and n represents the flow behavior index.

5.2.8. Particle size distribution

The particle size distribution of RCM emulsions was examined by using a Mastersizer S long bed (version 2.15, Malvern Instruments Ltd, Malvern, UK) as explained in section 2.2.5.

5.2.9. Oscillation rheological measurements

An AR2000 Rheometer (TA instruments, Zellik, Belgium) equipped with a 28 mm conical concentric cylinder measuring system was used in all experiments. An amount of 20 mL of a mixture of equal volumes of WPI (5.5 %) and intact or hydrolyzed lecithin (2 %) solution in Ca-imidazole buffer was poured gently into the cup and covered by aluminum foil to prevent water evaporation during the experiment. Gels were formed by heating the samples from 20 to 80°C at a rate of 2°C min⁻¹, holding at 80°C for 15 min and cooling to 20°C at a rate of 2°C min⁻¹. Measurements were taken at a frequency of 1 Hz and at a maximum strain of 0.002.

5.2.10. Residual protein content

1 mL of 5.5 % (w/v) WPI stock solution was mixed with 1.0 mL of 2 % (w/v) of intact or hydrolyzed sunflower lecithin, all prepared in Ca-imidazole buffer. The samples were prepared in glass tubes and they were subsequently heated for 1 and 2 min in a water bath at 80°C and finally cooled with tap water to room temperature. All heated samples were centrifuged for 20 min at 2900 g using a 1-15P microcentrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany).

The residual protein content present in the supernatant was determined based on the colorimetric method of Schacterle and Pollac (1973) by spectrophotometry using a Ultrospec® 1000 UV-spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden) at a wavelength of 650 nm.

5.3. Results and discussion

5.3.1. Effect of intact sunflower lecithin on heat stability

The impact of lecithin on the heat-induced coagulation was investigated, by heating recombined concentrated milk emulsions for 10-25 min, at 121°C. Therefore, non-hydrolyzed sunflower lecithin was incorporated at varying concentrations and the changes in viscosity and particle size distribution are depicted in table 5.1 and figure 5.1, respectively.

Overall, intact sunflower lecithin was effective in reducing the heat-induced increase in both viscosity and average particle size. Whereas the viscosity of the reference sample (0.0 % lecithin) was strongly affected by increasing the heating time, the addition of 0.3

% intact sunflower lecithin resulted in samples with viscosity properties almost similar to the unheated emulsion. A final product characterized by a gelled or solid-like structure was obtained in the absence of phospholipids, after heating for 25 min at 121°C, which provides an indication of heat coagulation. In such cases, visible clots were evident in the heating vials.

Table 5.1. Calculated consistency coefficient (in mPa.s) of recombined concentrated milk emulsions containing 0.0, 0.1, 0.2 and 0.3 % intact sunflower lecithin, as a function of heating time at 121°C.

Consistency coefficient (mPa.s)				
Heating time (min)	Intact sunflower lecithin (%)			
	0.0	0.1	0.2	0.3
0	11			
10	28	24	22	21
15	49	26	21	18
20	164	41	37	19
25	1092	267	56	31

On the other hand, the consistency coefficient of heated emulsions, as described in table 5.1, is characterized by a gradual decrease upon increasing the incorporated amount of intact lecithin. In addition, the flow behavior of the unheated sample was closer to Newtonian ($n = 0.827$), whereas the behavior became increasingly more pseudoplastic upon heating. A further analysis of the data obtained at different heating times and with different intact sunflower lecithin levels revealed an exponential relationship between the consistency coefficient and the flow behavior index (n) with a determination coefficient of 0.67 (i.e. a correlation coefficient of 0.82). Similarly, Hardy, Sweetser, West, and Muir (1985), observed that 0.2 % (w/w) lecithin addition had a stabilizing effect on homogenized concentrated milk emulsions, by increasing the heat coagulation time by approximately 30 min, after heating at 120°C.

The differential particle size distributions shown in figure 5.1 confirm the results discussed above. The effect of phospholipids was clearly demonstrated by the fact that the emergence of particles larger than 1 μm was largely limited when a larger amount of lecithin was incorporated.

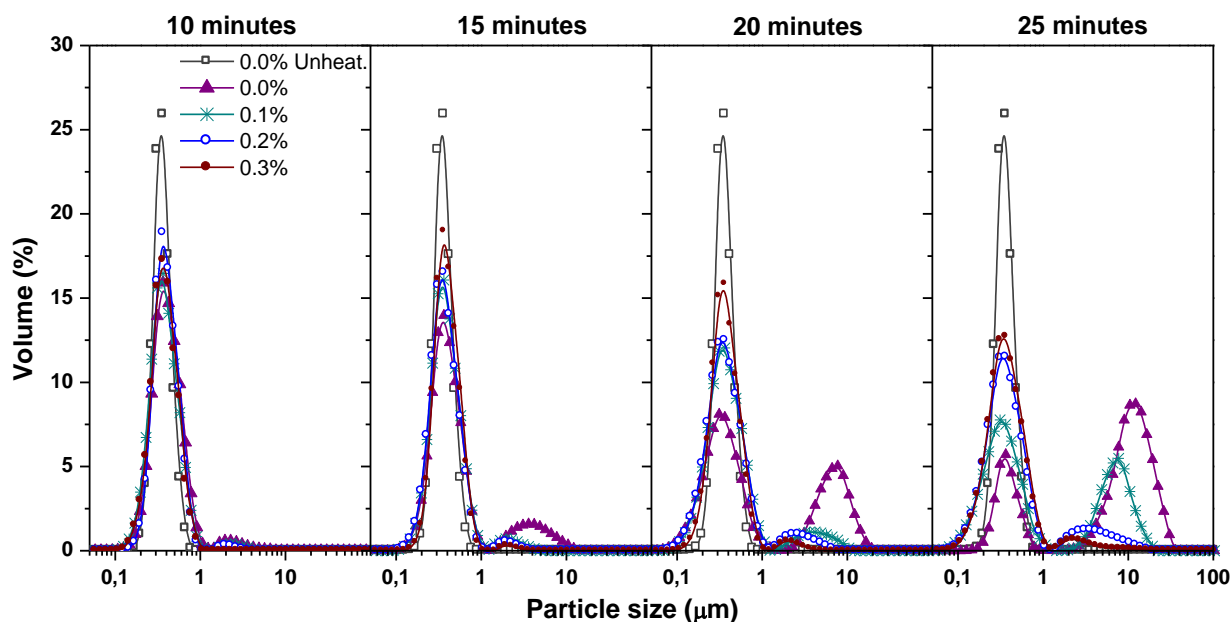


Figure 5.1. Differential volume-weighted particle size distribution (μm) of recombined concentrated milk emulsions containing (\blacktriangle) 0.0 %, ($*$) 0.1 %, (\circ) 0.2 % and (\bullet) 0.3 % (w/w) intact sunflower lecithin as a function of heating time at 121°C.

From figure 5.1, it becomes clear that the onset of aggregation took place during heating for 10 min, as a small peak in the range 1-5 μm was evident which is due to emulsion droplet aggregate formation. The volume weighted average diameter $d_{4,3}$ was markedly increased from 0.34 (unheated sample) to 0.47, 0.95, 2.29 and 7.39 μm when recombined concentrated milk was heated for 10, 15, 20 and 25 min, respectively, in the absence of lecithin. On the contrary, upon incorporation of 0.3 % lecithin, these values were drastically reduced to 0.36, 0.40, 0.48 and 0.52 μm , indicating that the emulsions were more resistant toward heat-induced coagulation. These observations are in line with the increased heat stability of a model infant formula emulsion in the presence of lecithin, as observed by McSweeney, Healy and Mulvihill (2008). They found that no significant changes took place in the control sample (no lecithin) upon heating for 10 min, whereas after 18 min, the system coagulated. On the other hand, emulsions containing 5 g/L lecithin were more stable and coagulation only occurred after 25 min (McSweeney, Healy and Mulvihill, 2008). The authors ascribed the improved heat

stability, in the presence of lecithin, to the formation of protein-phospholipid complexes. Similarly, Crujisen (1996) observed that the addition of crude soybean lecithin in caseinate-stabilized emulsions was beneficial with regard to heat stability, when it was added prior to homogenization: the heat coagulation time increased from 15 to 65 min, upon heat treatment at 120°C. However, the heat stabilizing effect of intact sunflower lecithin as reflected by both viscosity and particle size distribution is in contrast with the results of Tran Le et al. (2007) who reported that adding intact soybean lecithin had an insignificant effect on the average diameter of particles present in mixtures of casein micelles and WPI, after heating for 15 min at 80°C.

5.3.2. Enzymatic hydrolysis of intact sunflower lecithin and its impact on the heat stability

Due to indications of improved efficacy of lysolecithin as compared to intact lecithin, hydrolyzed lecithin has been utilized in several studies throughout the decades, in order to investigate its heat-stabilizing effect on whey protein-containing emulsions (Eric Dickinson & Yamamoto, 1996; C.H. McCrae, 1999; Sünder et al., 2001; Tran Le et al., 2007, 2011a; Van der Meeren et al., 2005). Nevertheless, no information is available on the efficacy of hydrolyzed lecithin as a function of degree of hydrolysis. Therefore, intact sunflower lecithin was subjected to enzymatic hydrolysis, using Lecitase® Ultra, a protein-engineered phospholipase A1 (with a molecular mass of 35 KDa) which possesses both phospholipase A1 and lipase activity (Mishra et al., 2009).

The enzymatic hydrolysis was performed for 0, 10, 20, 30, 40 and 60 min. The hydrolyzed samples were subsequently analyzed by ³¹P-NMR to examine alterations in phospholipid composition. Table 5.2 confirms that the phospholipid content was dramatically changed by hydrolysis. As expected, the phospholipid concentration was reduced from 0.43 to 0.08, by increasing hydrolysis time from 0 to 60 min, respectively. On the other hand, an increase of the lysophospholipid content was observed, after 10 min of enzymatic hydrolysis. Interestingly, the amount of the lysophospholipids did not increase continuously throughout the hydrolysis reaction, but it reached an optimum concentration after 20 min, whereas afterwards it started to decline gradually.

The kinetics of the enzymatic hydrolysis were examined following a total reaction time of 360 min, which was determined to be a first order reaction. The half-life time ($t_{1/2}$), based on the amount of NaOH needed to keep the pH constant, gave a value of 36.8 min. This indicates that within 40 min, half of the total amount of fatty acids was liberated due to hydrolysis, whereas afterwards the rate of reaction gradually decreased.

Table 5.2. Phospholipid and lysophospholipid concentration of sunflower lecithin before and after enzymatic hydrolysis, expressed in mmol g⁻¹ lecithin, as determined by ³¹P-NMR.

Hydrolysis time (min)	Concentration (mmol g ⁻¹ lecithin)	
	Phospholipid	Lysophospholipid
0	0.43	0.07
10	0.20	0.25
20	0.12	0.29
30	0.10	0.27
40	0.08	0.22
60	0.08	0.23

The ³¹P-NMR spectrum of intact sunflower lecithin shows the characteristic resonances of phospholipids (figure 5.2). In contrast, 20 min of hydrolysis led to a general loss of the phospholipids along with a significant increase of the lysophospholipids analogues, derived as a result of the hydrolytic cleavage of a fatty acid. Ongoing hydrolysis (60 min) resulted in cleavage of the second fatty acid residue, eventually yielding molecules of the respective glycerophosphorylic (GPL) compounds.

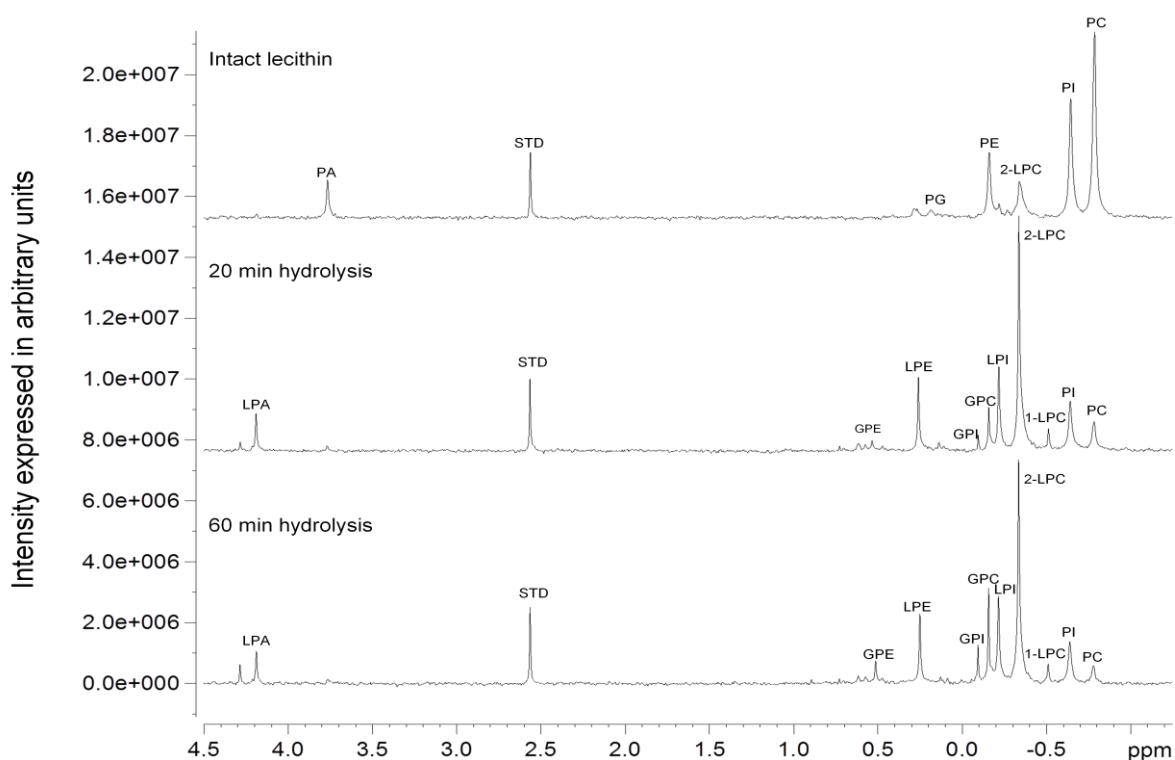


Figure 5.2. ^{31}P -NMR spectra of intact sunflower lecithin and lecithin hydrolysates following 20 and 60 min hydrolysis, showing resolution of phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidic acid (LPA), lysophosphatidylinositol (LPI), glycerophosphorylcholine (GPC), glycerophosphorylinositol (GPI), glycerophosphorylethanolamine (GPE) and the internal standard (STD).

Similarly, figure 5.3 illustrates the changes in phospholipid composition of intact sunflower lecithin, before and after its hydrolysis for 20 and 60 min, as it was analyzed by HPLC. Upon hydrolysis, a decrease of phosphatidylcholine (PC) content is evident, accompanied by a major increase of its hydrolyzed form LPC. From the data obtained by the analysis, the area of LPC increased from 2.3×10^5 to 1.5×10^6 after 20 min of hydrolysis, whereas it decreased to 9.8×10^5 upon continuation of the reaction. It is also worth mentioning that the ratio of PC over LPC decreased from 32.0, in the case of intact sunflower lecithin, to 0.54 and 0.26, after 20 and 60 min of hydrolysis, respectively.

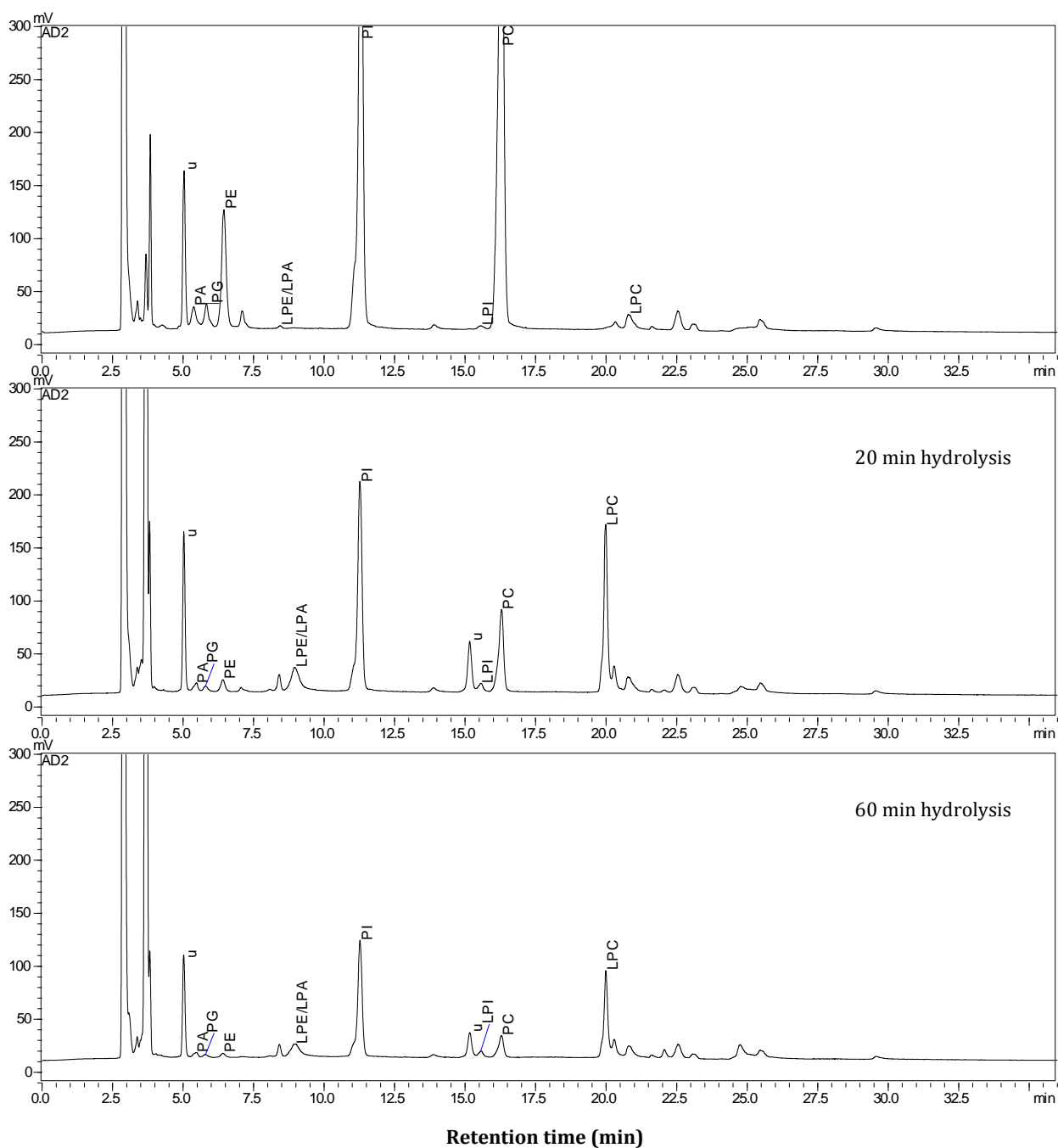


Figure 5.3. Examples of chromatograms obtained by HPLC-ELSD analysis of intact sunflower lecithin, before and after its enzymatic hydrolysis for 20 and 60 min, showing the separation of phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), lysophosphatidylethanolamine (LPE), lysophosphatidic acid (LPA), lysophosphatidylinositol (LPI) and lysophosphatidylcholine (LPC), with also some unknown compounds (u).

Figure 5.4 indicates that the degree of enzymatic hydrolysis plays a pivotal role in the effect of lecithin on the heat-induced increases in viscosity of recombined concentrated milk emulsions. The time of heat treatment, as compared to the previous experiments, was increased from 25 to 35 min due to the fact that at less intense heating, the intact sunflower lecithin has such a pronounced stabilizing effect that hardly any improvement can be seen.

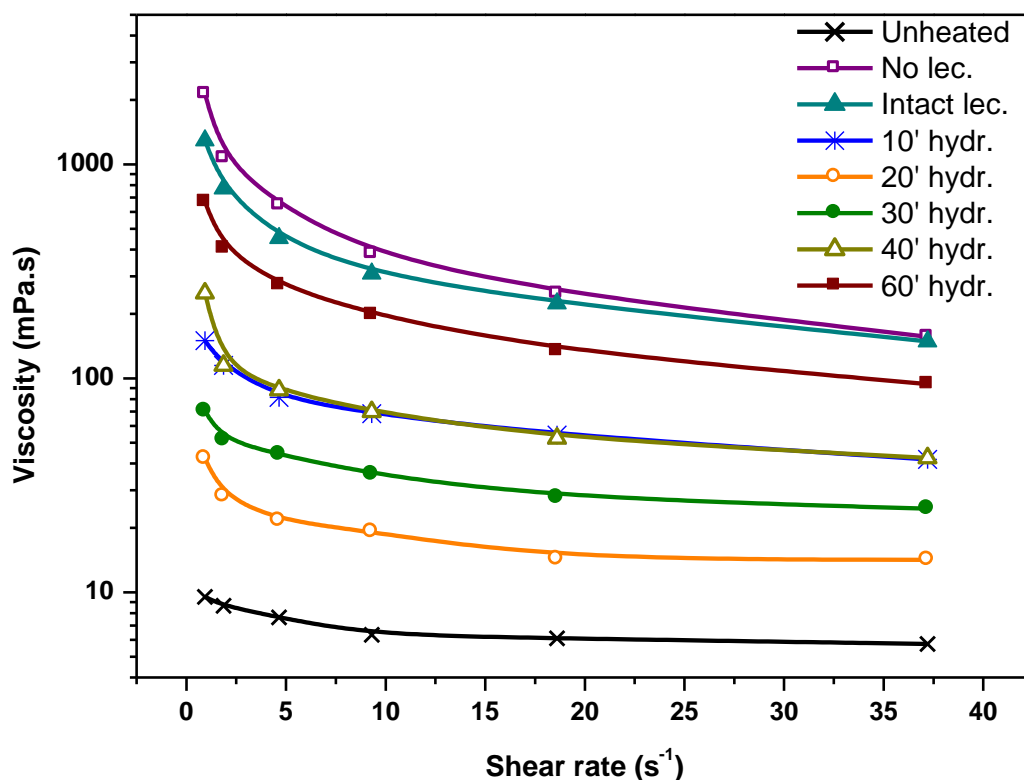


Figure 5.4. Apparent viscosity of recombined concentrated milk emulsions containing 0.0 % (□) and 0.3 % (▲) intact sunflower lecithin, as well as 0.3 % hydrolyzed lecithin after hydrolysis for 10 (*), 20 (○), 30 (●), 40 (Δ) and 60 (■) min. Viscosity was measured after heating for 35 min at 121°C.

Whereas 0.3 % intact sunflower lecithin did not show a remarkable heat stabilizing effect, a fact which can be ascribed to the very intense heating conditions, the addition of 0.3 % hydrolyzed lecithin treated with Lecitase® Ultra for 20 min, appeared to be the most effective. For the sake of completeness, it has to be mentioned that addition of 0.3 % lecithin, hydrolyzed for 10, 30, 40 and 60 min, also had a very pronounced stabilizing effects as compared to the emulsion without lecithin. In the absence of phospholipids, the consistency coefficient was 1771 mPa.s, whereas upon addition of 0.3 % intact sunflower lecithin, it was decreased to 1099 mPa.s. The corresponding consistency

coefficient in the presence of 0.3 % hydrolyzed lecithin was 143, 37, 66, 190 and 606 mPa.s, after 10, 20, 30, 40 and 60 min of enzymatic hydrolysis, respectively.

The cumulative volume-weighted particle size distributions shown in figure 5.5, confirm the results mentioned above. It is evident that upon heat treatment for 35 min and in the absence of lecithin, a significant increase of the particle size was noticed due to further aggregation.

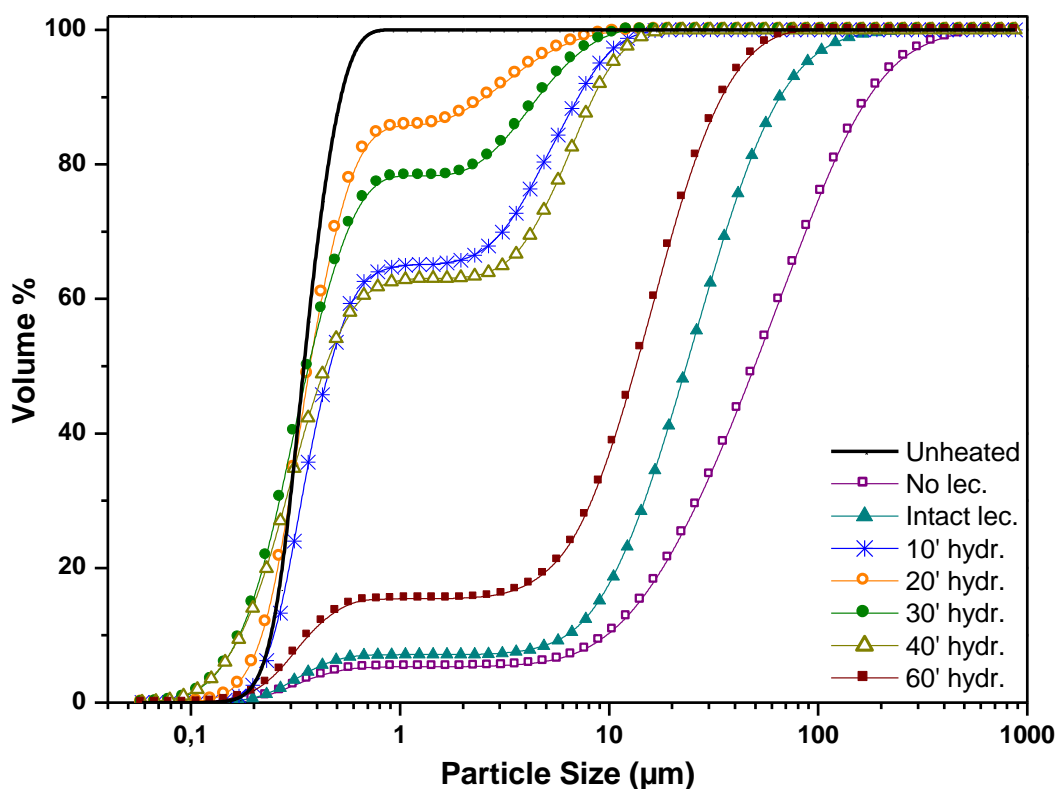


Figure 5.5. Cumulative volume-weighted particle size distribution (μm) of recombined concentrated milk emulsions containing 0.0 % (\square) and 0.3 % (\blacktriangle) intact sunflower lecithin, as well as 0.3 % hydrolyzed lecithin after hydrolysis for 10 (*), 20 (\circ), 30 (\bullet), 40 (Δ) and 60 (\blacksquare) min. Size distribution was measured after heating for 35 min at 121°C.

This phenomenon could be attributed to the complex formation between the denatured whey proteins, casein micelles and fat droplets (Oldfield, Singh, Taylor & Pearce, 2000; Singh, 2004). Incorporation of intact sunflower lecithin shifted the particle size distribution toward smaller size, an indication of a more heat-resistant emulsion, whereas adding lecithin which has been hydrolyzed for 20 min, resulted in emulsions with limited coagulation, even after heating under such severe conditions. In line with previous NMR experiments, the heat-stabilizing properties of the lecithins are thought to result from their interaction with whey proteins, whereby lysophospholipids are

apparently more effective in preventing protein interactions than dialkyl phospholipids. On the other hand, a gradual increase of particle size was observed when 40 and 60 min hydrolyzed lecithin was added. The volume weighted average diameter $d_{4,3}$ of the reference (unheated) sample was $0.36 \pm 0.01 \mu\text{m}$, which was raised to $76.3 \pm 13.4 \mu\text{m}$ after 35 min of heating. Intact sunflower lecithin resulted in the formation of emulsions characterized by a $d_{4,3}$ of $31.4 \pm 0.81 \mu\text{m}$, whereas the addition of hydrolyzed lecithin could diminish the average diameter to 2.31 ± 0.01 , 0.88 ± 0.04 , 1.32 ± 0.05 , 2.77 ± 0.21 and $16.3 \pm 0.16 \mu\text{m}$ when enzymatic hydrolysis of sunflower lecithin took place for 10, 20, 30, 40 and 60 min, respectively.

To explain in more detail the effect of the degree of hydrolysis on the heat stability of whey protein-containing emulsions, a series of experiments was performed using mixtures of WPI with lecithin, which was enzymatically hydrolyzed for different time intervals. The mixtures were prepared in imidazole buffer and were subsequently heated for either 1 or 2 min at 80°C and afterwards were cooled down by tap water. After centrifugation for 20 min at 2900 g, the residual protein content of the serum phase was determined. Figure 5.6 illustrates that the WPI recovery in the serum phase was significantly reduced, especially after 2 min of incubation at 80°C .

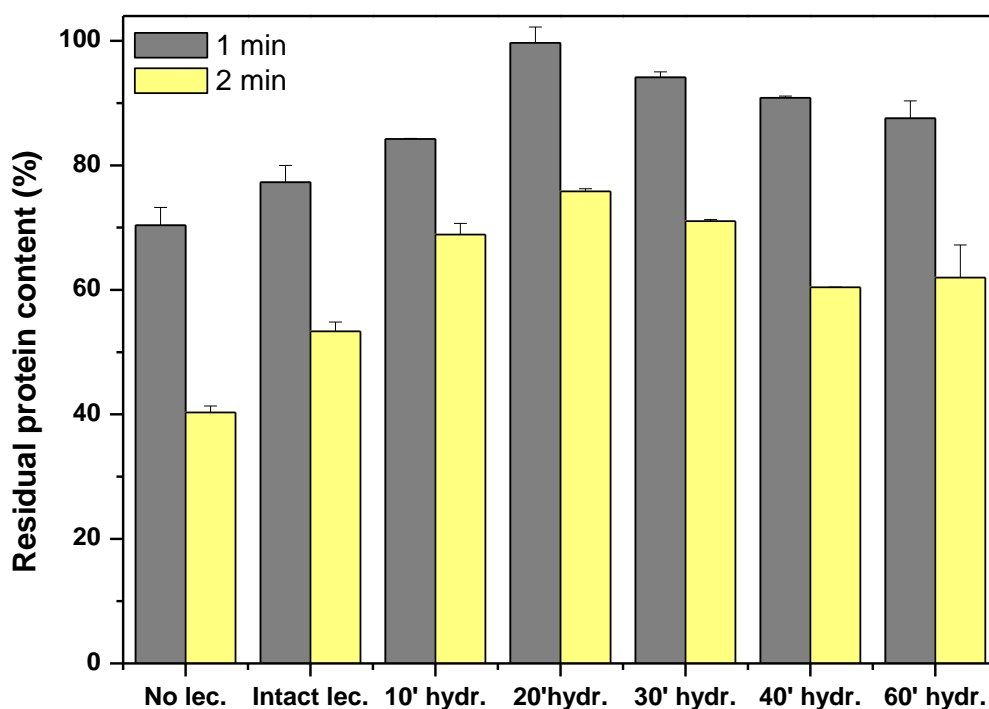


Figure 5.6. Residual protein content (%) of mixtures containing 2.75 % (w/v) whey protein isolate and 1 % (w/v) lecithin, before and after hydrolysis for 0, 10, 20, 30, 40 and 60 min. The residual protein content was determined after heating at 80°C for 1 (grey bars) and 2 (yellow bars) min.

In the presence of 1 % lecithin, enzymatically treated for 10 min, the protein recovery was increased to approximately 80 %, whereas mixtures containing 1 % lecithin hydrolyzed for 20 min were hardly affected after 1 min of incubation. On the other hand, lecithin addition, hydrolyzed for extended period (30-60 min), did not show the same positive results. These observations provide an additional verification that hydrolyzed lecithin constitutes a valuable tool for improving heat stability of whey protein mixtures, and most importantly, it makes clear that the degree of hydrolysis can largely enhance or decline this outcome. Similarly, Tran Le et al. (2011) showed that upon heating WPI solutions, in the presence of various surfactants, including hydrolyzed soybean lecithin, the aggregation intensity was less pronounced and this was ascribed to the complex formation of whey proteins and phospholipids. However, the authors concluded that the beneficial heat stabilizing effect of hydrolyzed lecithin was improved as the percentage of hydrolysis was increased, and therefore, the main effect was related to its degree of hydrolysis. This is in contradiction with our observations, as we proved that there is an optimum degree of hydrolysis, above which the heat stabilizing effect of hydrolyzed lecithin decreases.

Finally, with the purpose of showing the difference in effectiveness between intact and hydrolyzed lecithin on the heat stability of WPI solutions, oscillatory rheology measurements were executed, including the incorporation of intact and hydrolyzed sunflower lecithin. The latter was enzymatically treated for 20 min with Lecitase® Ultra. Oscillatory rheology has been previously used to investigate the effect of surfactants on heat-induced gelation of whey protein (Chen & Dickinson, 1998; Chen, Dickinson, Langton & Hermansson, 2000; Dickinson & Yamamoto, 1996; Tran Le et al., 2011). Figure 5.7 depicts the changes in rheological properties of a 2.75 % WPI solution in the presence of 1 % of either intact or hydrolyzed sunflower lecithin. The gelation curves show that hydrolyzed lecithin reduced significantly the complex modulus G^* , both in the heating and cooling period. Similarly, Tran Le et al. (2007) described that hydrolyzed lecithin was very effective in decreasing the average diameter of mixtures of casein micelles and whey proteins after heating for 15 min at 80°C.

Furthermore, from figure 5.7 it also becomes evident that intact lecithin reduced the strength of the WPI gel, postulating that the whey protein solutions were more resistant toward heat-induced coagulation; however, the impact was not as profound as in the case of hydrolyzed lecithin.

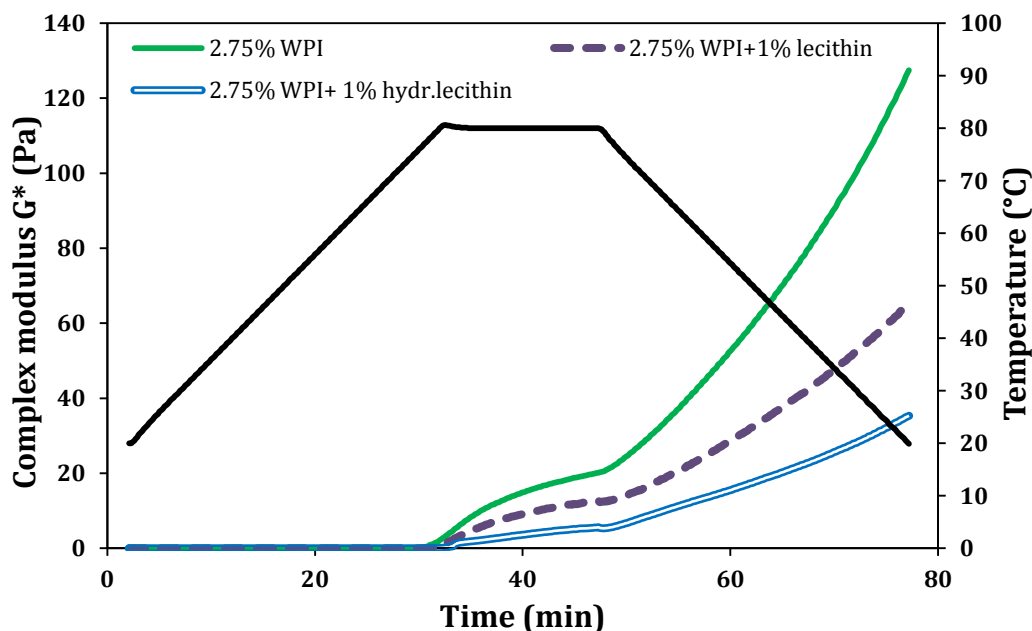


Figure 5.7. Complex modulus G^* of a 2.75 % (w/v) whey protein isolate solution containing 1 % of lecithin, before and after its hydrolysis for 20 min. The grey line represents the temperature profile.

The above observations provide a strong indication that the heat-stabilizing properties of whey protein containing emulsions and solutions seemed to be largely influenced by the type of lecithin used. Kristensen, Nylander, Paulsson and Carlsson (1997) and similarly Kasinos et al. (2013), concluded that the interaction between phospholipids and β -lactoglobulin is dependent on the type of phospholipid polar head group as well as on the fatty acid composition. Hydrolysis of sunflower lecithin further enhanced the heat-stabilizing properties of recombined concentrated milk emulsions. This effect was, however, strongly dependent on the degree of the hydrolysis reaction, where an optimum hydrolysis time of 20 min was established. In other words, the lysophospholipid content seems to play a determinant role on the heat-induced aggregation phenomena, and the maximum content was attained after 20 min of enzymatic hydrolysis. The effect of hydrolyzed lecithin is thought to result from binding of phospholipids to the denatured whey proteins and/or protein aggregates, which inhibits further interaction with casein micelles or other denatured whey proteins and

protein aggregates, and hence prevents or postpones the occurrence of additional heat-induced aggregation phenomena.

5.4. Conclusions

Heat coagulation experiments revealed that both intact and hydrolyzed sunflower lecithin resulted in reduced coagulation tendency, which may be ascribed to complex formation between phospholipids and the denatured whey proteins and protein aggregates which are present in high-heat SMP.

Firstly, the incorporation of intact sunflower lecithin in RCM emulsions upon heating at 121°C up to 25 min, demonstrated a remarkable decrease in coagulation tendency with the effect directly proportional to the added concentration. This seems to be related with the increase in polar lipids content and therefore it is speculated that a higher degree of interactions takes place between the amphiphilic molecules and the denatured whey proteins and/or the whey protein aggregates, resulting in a less pronounced development of further aggregation and hence coagulation. Average particle size measurements revealed that the addition of the maximum concentration of intact sunflower lecithin (0.3 %) resulted in emulsions with similar size characteristics as the emulsions which remained unheated.

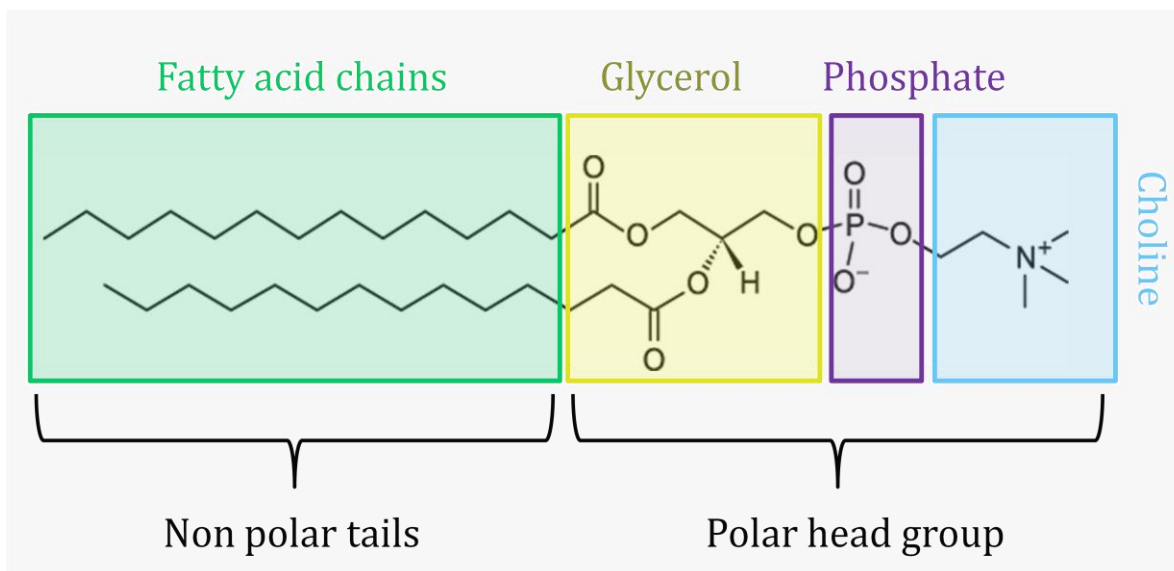
Upon increasing the heating time to 35 min and comparing intact and hydrolyzed sunflower lecithin, the latter seemed more effective in improving the heat stability of both RCM emulsions and WPI solutions, which could be explained by its higher lysophospholipid content and/or by the liberation of free fatty acids.

In addition, enzymatic hydrolysis of intact sunflower lecithin for different time intervals showed that the degree of hydrolysis can play a decisive role on the heat stability of whey protein containing emulsions and solutions: an optimum hydrolysis time of 20 min was found, above which the beneficial effect of hydrolyzed lecithin addition was reduced. As the experimental heat stability seemed to correlate well with the lysophospholipid content as obtained by P-NMR, the initial beneficial effect of enzymatic hydrolysis was thought to result from the conversion of dialkyl phospholipids to more effective monoalkyl lysophospholipids, whereas the subsequent deteriorating effect could be ascribed to the further conversion of amphiphilic lysophospholipids to

hydrophilic glycerophosphate compounds. However, the possible beneficial effect of the fatty acid produced during hydrolysis cannot be excluded.

Chapter 6

ANIONIC AND ZWITTERIONIC PHOSPHOLIPIDS DIFFERENTLY AFFECT THE HEAT COAGULATION OF RECOMBINED CONCENTRATED MILK EMULSIONS



This chapter is redrafted from: Kasinos M., Mukarukundo F., De Beuf K. and Van der Meeren P. (2014), International Dairy Journal, 39, 131-138.

Chapter cover image: Schematic representation of a phospholipid's (1,2-dimyristoyl-sn-glycero-3-phosphocholine-DMPC) molecular structure.

Abstract

Heat-induced modifications were investigated upon heating recombined concentrated milk emulsions in the absence and presence of zwitterionic lysophospholipids or zwitterionic and anionic dialkylphospholipids of varying alkyl chain length. Among the lysophospholipids, 1-stearoyl-2-hydroxy-sn-3-phosphocholine (C18-LPC) demonstrated the greatest heat-stabilizing effect, whereas 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (C14-LPC) resulted in increased viscosity and particle size as compared to its longer analogue. The protein recovery results showed that additional protein deposition at the oil-water interface was partly prevented upon lysophospholipids addition, especially by the ones characterized by a longer alkyl chain. The dialkylphospholipids exhibited a remarkable heat-stabilizing effect upon addition at relatively low concentrations. As in the case of lysophospholipids, the longest alkyl chain phospholipids provided evidence of increased heat stability compared to 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC). Moreover, anionic PG was characterized by an optimum concentration above which the heat-stabilizing effect was weakened, whereas in the case of zwitterionic PC, the beneficial effect increased continuously with added concentration.

6.1. Introduction

Due to its high content in essential food components, milk owns a principal place in the diet of humans. However, its richness makes milk a fertile substance for microorganisms, which may constitute a potential risk of food poisoning or food borne diseases. To overcome this risk, a number of preservation techniques have been developed over the centuries, with the most commonly used method being the application of heat. Whereas milk processing at temperatures higher than 60°C does not have an impact on the heat stable caseins, the whey protein fraction, and especially β -lactoglobulin (β -lg), undergoes denaturation, during which the globular structure unfolds and the hydrophobic groups as well as the free thiol group are exposed (Corredig & Dalgleish, 1999; Croguennec, O’Kennedy, & Mehra, 2004; Iametti, Gregori, Vecchio, & Bonomi, 1996; Jeurink & De Kruif, 1993; Nielsen, Singh, & Latham, 1996; Verheul, Pedersen, Roefs, & De Kruif, 1998; Verheul et al., 1998).

According to previous research (Dalgleish, Mourik, & Corredig, 1997; Dalgleish, Senaratne, & Francois, 1997; Elfagm & Wheelock, 1978), β -lg, or β -lg together with α -lactalbumin (α -lac) molecules may interact directly with each other during the initial stages of heating, resulting in aggregate formation, mainly via hydrophobic interactions. Despite the significant contribution of the hydrophobic interactions in the heat-induced aggregate formation, disulfide bonding is also of high importance. More precisely, heat-induced exposure of the thiol group in β -lg may induce thiol-disulfide interactions causing the formation of disulfide linked aggregates (Anema & Li, 2003a; Croguennec et al., 2003; Jang & Swaisgood, 1990; Sawyer, 1969; Schokker et al., 2000) and complex formation with κ -casein on the surface of the casein micelles (Anema & Li, 2003a; Corredig & Dalgleish, 1996; Croguennec et al., 2003; Guyomarc’h, Law, & Dalgleish, 2003; Jang & Swaisgood, 1990; Kessler & Beyer, 1991; Oldfield, Singh, Taylor, & Pearce, 2000; Sawyer, 1969; Schokker et al., 2000). Other studies showed that κ -casein may dissociate from the micelles during heating milk at high temperatures which may result into the formation of whey protein/ κ -casein complexes in the serum phase (Anema, 2007). Hereby, the question arises as to whether the κ -casein dissociation from the micelles takes place before or after its interaction with the denatured whey proteins and/or whey protein aggregates.

These changes give rise to a final product which is characterized by increased viscosity and particle size (Havea et al., 2004; Van der Meeren et al., 2005), which is usually undesirable for the consumer. During the past decades, numerous studies have shown that the utilization of surface active molecules, such as phospholipids, may protect milk against heat coagulation upon severe heating (Chen & Dickinson, 1998; Chen, Dickinson, Langton, & Hermansson, 2000; Hardy, Sweetsur, West, & Muir, 1985; Ikeda & Foegeding, 1999; Kasinos, Goñi, et al., 2014; McCrae, 1999; McSweeney, Healy, & Mulvihill, 2008; Pan, Tomás, & Añón, 2002; Sünder, Scherze, & Muschiolik, 2001; Tran Le et al., 2007; Yamamoto & Araki, 1997). Their advantageous effect is ascribed to their ability to either displace the protein from, or interact with the protein at, the emulsion droplet interface (Brown, Carroll, Pfeffer & Sampugna, 1983; Kristensen, Nylander, Paulsson & Carlsson, 1997; McCrae, 1999). However, the type and the molecular structure of the phospholipids employed significantly affect the extent of whey protein aggregation, and consequently heat coagulation (Euston, Finnigan, & Hirst, 2000; Giroux & Britten, 2004; Kristensen et al., 1997; Robb, 1981).

Goddard and Ananthapadmanabhan (1993) mentioned that anionic surfactants interact strongly with proteins resulting in the formation of protein-surfactant complexes, which could induce the unfolding of proteins, whereas cationic surfactants are characterized by a lower tendency to interact. On the other hand, non-ionic surfactants bind very weakly. This can be ascribed to the lower critical micelle concentration of the latter, as well as the absence of electrostatic relevance, having as a consequence the formation of micelles. In addition, Gelamo and Tabak (2000) classified surfactants in two groups according to their influence on the bovine serum albumin (BSA) structure: anionic on the one hand and either cationic or zwitterionic on the other. Their results indicated that the protein structure upon addition of the zwitterionic surfactants remained almost intact, whereas it was strongly affected upon addition of the anionic sodium dodecyl sulfate (SDS).

In our recent study (Kasinos et al., 2013), the effect of anionic and zwitterionic phospholipids on the secondary structure of β -lg, was investigated by circular dichroism analysis. The results indicated that upon incubation of whey protein isolate in the presence of the anionic phospholipid 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) and at room temperature conditions, major structural modifications were

observed. On the other hand, when the zwitterionic phospholipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (C14-LPC) were tested at the same conditions, no protein conformational changes were detected. This suggests an absence of association between zwitterionic phospholipids and whey protein and/or their inability to cause whey protein denaturation.

The aim of this study was to examine the significance of the phospholipid molecular structure upon their application into concentrated milk emulsions. For that purpose, different kinds of purified phospholipids were incorporated in recombined concentrated milk emulsions, with the aim of better understanding and gathering of information which will be helpful in deciding which kind of phospholipid is more appropriate to be utilized in dairy products, in cases where increased heat stability is necessary.

For that reason, the effect of three monoalkyl phospholipids (lysophospholipids) on the heat stability of concentrated dairy protein-stabilized emulsions was compared, in order to examine the effect of the fatty alkyl chain length. Furthermore, the influence of anionic dialkyl phospholipids (phosphatidylglycerol) addition was compared to zwitterionic dialkyl phospholipids (phosphatidylcholine) addition, in order to provide evidence of the role of the polar head group in preventing heat-induced aggregation phenomena. The effect of heating was evaluated by performing particle size and rheological measurements, as well as by determining the residual amount of protein in the continuous phase upon removal of the dispersed phase by centrifugation.

6.2. Materials and methods

6.2.1. Materials

High-heat skimmed milk powder (SMP) was obtained from FrieslandCampina (Deventer, The Netherlands). Based on the manufacturer's specifications, the SMP contained 37.3 % protein, 0.5 % fat and 0.15 % phospholipids. The high oleic sunflower oil (Hozol, Contined, Bennekom, The Netherlands) contained maximum 0.05 % free fatty acids as oleic acid. Its melting point is at 0°C and hence the oil remains clear, even after 10 h at 4°C.

Three different lysophosphatidylcholine (LPC) products were purchased from NOF Corporation (Tokyo, Japan): 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (C14-

LPC, MW = 468 g mol⁻¹, CMC = 0.036 mM), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (C16-LPC, MW = 496 g mol⁻¹, CMC = 0.0032 mM) and 1-stearoyl-2-hydroxy-sn-3-phosphocholine (C18-LPC, MW = 533 g mol⁻¹, CMC = 0.0004 mM), for which the purity was over 99 %. In addition, four dialkylphospholipids were also obtained from NOF Corporation from which: 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG, MW = 689 g mol⁻¹, CMC = 0.011 mM, T_m = 23°C) and 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG, MW = 745 g mol⁻¹, T_m = 41°C) are anionic whereas 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, MW = 678 g mol⁻¹, CMC = 6 nM, T_m = 23°C) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, MW = 734 g mol⁻¹, CMC = 0.46 nM, T_m = 41°C) are zwitterionic.

6.2.2. Preparation of SMP emulsions

To study the effect of phospholipids on the heat stability of recombined concentrated milk emulsions, samples containing 16.5 % (w/w) SMP and 6.5 % (w/w) high oleic sunflower oil were prepared in 0.02 % NaN₃ (Acros Organics, Geel, Belgium) aqueous solution. The latter was used in order to avoid any potential microbial growth. All samples were pre-homogenized by an IKA Ultra-Turrax TV45 (Janke & Kunkel, Staufen, Germany) for 1 min and homogenized by a Microfluidiser 110S (Microfluidics Corporation, Newton, MA, USA), with its coil being immersed in a water bath at 55°C. Phospholipids addition took place prior to homogenization following pre-homogenization, in dry form and at concentrations ranging from 0.0 - 0.2 % (w/w). The samples were microfluidized at a compressed air pressure of 2 bar, corresponding to a liquid pressure of about 280 bar, for about 4 min. After microfluidisation, the emulsions were allowed to cool to room temperature. The pH of all prepared samples was 6.6.

6.2.3. Heat treatment

Heat treatment of recombined concentrated milk emulsions, as well as of phospholipid enriched concentrated milk emulsions, was carried out as described in Kasinos, Tran Le and Van der Meeren (2014). The 10 mL samples were heated at 121°C for 20 min. As previous experiments revealed that approximately 9 min is needed to ensure that the samples approach the oil bath temperature, samples were immersed 29 min. After heat treatment, the samples were cooled in tap water.

6.2.4. Viscosity measurements

In order to evaluate the degree of heat coagulation and to clarify the effect of various phospholipids, viscosity measurements of recombined concentrated milk emulsions were performed before and after heating, as described in section 2.2.4.

The experimental data fitted very well to a power law relation:

$$\tau = K \cdot \gamma^n \quad (1)$$

As the apparent viscosity μ is obtained as the ratio of shear stress τ (in Pa) to shear rate γ (in s^{-1}), the power law equation can be rewritten as:

$$\mu = K \cdot \gamma^{n-1} \quad (2)$$

where K represents the consistency coefficient and n represents the flow behavior index.

6.2.5. Particle size distribution

The particle size distribution of recombined concentrated milk samples was determined with a Mastersizer S long bed (version 2.15, Malvern Instruments Ltd, Malvern, UK) as described in section 2.2.5.

6.2.6. Protein recovery

Protein recovery is defined as the amount of protein which was recovered from the continuous phase of the recombined concentrated milk after removal of the dispersed phase by centrifugation, relative to the amount of protein present in the aqueous phase before oil addition. As all emulsions contain 16.5 % SMP (with 37.3 % protein content), it follows that the latter corresponds to 6.15 g per 100 g of recombined concentrated milk. The protein content in the serum phase of the recombined concentrated milk samples was determined before and after heating at 121°C for 20 min. The cream layer was separated by a Beckman Coulter L7-55 Ultracentrifuge using the SW 40 Ti rotor (Pasadena, CA, USA) at 284,061 x g for 120 min at room temperature. Sucrose (VWR International, Leuven, Belgium) was added as a density increasing agent (Kasinos, Tran Le & Van der Meeren, 2014; Van der Meeren et al., 2005). To that end, 2.5 g sucrose was added directly to 5 g of emulsions and 5 g of the sugar-supplemented samples was transferred into the polyallomer centrifuge tubes (13 x 51 mm, Beckman Coulter, Pasadena, CA, USA). After centrifugation, the cream layer was carefully removed,

whereas the remaining serum (containing non-adsorbed whey proteins as well as part of the non-adsorbed casein micelles) and sediment phase (containing the remaining part of the non-adsorbed casein micelles) were mixed thoroughly. The protein content P (in g) of 0.5 g of this mixture was determined by Kjeldahl analysis, using a conversion factor of 6.38.

It should be mentioned that protein recovery and protein load Γ (in mg/m²) are related by the use of equation (3) :

$$\Gamma = \frac{(1-\text{recovery}) \times C_{\text{SMP}} \times C_{\text{prot}}}{\text{SSA} \times C_{\text{oil}}} \quad (3)$$

whereby C_{SMP} and C_{oil} represent the concentration of SMP and hozol oil in the recombined concentrated milk emulsions (16.5 % w/w and 6.5% w/w, respectively), C_{prot} represents the protein content of SMP (37.3 %) and SSA stands for the specific surface area (in m²/kg oil) of the oil droplets (equation 5).

$$\text{SSA} = \frac{6}{(d_{3,2})_{\text{preheat}} \times \rho_{\text{oil}}} \quad (4)$$

$d_{3,2}$ represents the Sauter mean diameter of the oil droplets in the sample before heating, whereas ρ_{oil} represents the oil density (915 kg/m³). From equation (3), it becomes evident that the amount of protein associated per m² of fat droplet surface area becomes larger as the protein recovery obtained upon centrifugation and separation of the cream layer is smaller:

$$\Gamma = (1-\text{recovery})/\text{SSA} \times 946846 \text{ (in mg.m}^{-2}\text{)} \quad (5)$$

6.2.7. Statistical analysis

R software for statistical computing, version 3.0.1 (R Development Core Team, 2010), was used for all statistical analyses. The effect of both lysophosphatidylcholine and dialkylphospholipids on the protein recovery in recombined concentrated milk emulsions was statistically assessed using one-way analysis of variance at separate concentrations (type I error rate $\alpha=0.05$). Because the assumptions of normality were violated, a permutation-based ANOVA approach was taken to obtain correct p-values.

The add-on package 'lmPerm' (Wheeler, 2010) was used for this purpose. Pairwise differences between three types of lysophosphatidylcholine (first analysis) and between four types of dialkylphospholipids (second analysis) were assessed in a post-hoc test. Tukey's Honest Significant Difference method was used to control the overall type I error rate across multiple comparisons (Westfall & Young, 1993)

6.3. Results and discussion

6.3.1. Effect of lysophosphatidylcholine on recombined concentrated milk emulsions

The heat-stabilizing effect of three different types of lysophosphatidylcholine (C14-LPC, C16-LPC and C18-LPC) on recombined concentrated milk emulsions was evaluated upon heating at 121°C for 20 min. Inspection of the heated samples after cooling down at room temperature indicated a noticeable destabilization in the emulsions which did not contain lysophospholipids, which were characterized by a solid-like and dense structure. However, samples containing 0.2 % of LPC resembled the samples which were not subjected to any heating, retaining their liquid-like appearance.

To gather more detailed information, particle size distribution and viscosity measurements were performed, both before and after heat treatment. The particle size distribution prior to heating was mono-modal, whereas after heating a bi-modal distribution was obtained. Table 6.1 presents the changes in the volume-weighted average diameter ($d_{4,3}$) of recombined concentrated milk emulsions, both in the absence and presence of lysophospholipids. As is clearly shown in table 6.1, the average diameter of all emulsions prior to heating was highly similar, a fact which proves that the addition of lysophosphatidylcholine did not have any significant effect on the emulsification process.

Considering the heated samples, the heat-stabilizing effect was proportional to the concentration of LPC added for all three LPC species: the higher the concentration, the smaller the volume-weighted average particle diameter. However, the more pronounced stabilizing effect of the C16-LPC and especially C18-LPC is evident. On the other hand, in the case of C14-LPC addition, the effect was less significant. Interestingly, the average diameter decreased to $7.2 \pm 0.1 \mu\text{m}$ upon addition of the maximum concentration (i.e., 0.2 %) in the case of C14-LPC, whereas a similar average diameter value ($7.3 \pm 0.3 \mu\text{m}$) was obtained upon addition of only 0.03 % of the longest C18-LPC.

Table 6.1. Volume-weighted average diameter (μm) of recombined concentrated milk emulsions, as a function concentration of 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (C14-LPC), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (C16-LPC) and 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (C18-LPC), before and after heating at 121°C for 20 min. The variability indicators represent the standard deviation from (n=3) repeated experiments.

Concentration (%)	C14-LPC		C16-LPC		C18-LPC	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
0.00	0.38±0.0	25.0±2.9	0.38±0.0	25.0±2.9	0.38±0.0	25.0±2.9
0.03	0.39±0.0	25.0±2.0	0.39±0.0	20.0±1.3	0.38±0.0	7.3±0.3
0.05	0.39±0.0	22.0±0.2	0.39±0.0	15.0±0.9	0.37±0.0	5.8±0.1
0.10	0.38±0.0	20.0±1.2	0.38±0.0	8.1±0.7	0.38±0.0	2.9±0.2
0.20	0.37±0.0	7.2±0.1	0.39±0.0	1.5±0.1	0.39±0.0	1.1±0.0

Figure 6.1 illustrates the changes in consistency coefficient of recombined concentrated milk samples upon addition of C14-, C16- or C18-LPC at concentrations varying from 0.0 - 0.2 %. An increase in viscosity was detected upon heat treatment of samples in the absence of LPC. More precisely, the consistency coefficient of the unheated sample was 9.5 mPa.s, while it increased significantly up to 3.3 Pa.s, after heating for 20 min at 121°C. Whereas the protein fraction of high-heat SMP consists of a mixture of casein micelles with denatured whey proteins and whey protein aggregates attached to them and of aggregates of whey proteins, further aggregation by additional and prolonged heating is expected to be the reason for such prominent alterations (Pelegri & Gasparetto, 2005; Remondetto & Subirade, 2003).

As no significant differences were noticed in the viscosity behavior of the unheated emulsions, the consistency coefficient of the unheated sample, which was used as a reference in figure 6.1, was the average of all the unheated samples. On the contrary, when all three LPC species were tested, they were verified to be highly effective to largely prevent the heat-induced increase in both viscosity and particle size. However, the results revealed that the heat-stabilizing effect of the LPC species was alkyl chain length-dependent. More precisely, the longer chain palmitoyl (C16-LPC) and stearoyl (C18-LPC) lysophospholipids showed a more pronounced heat-stabilizing effect compared to the shorter chain myristoyl (C14-LPC) lysophospholipid. C16- and C18-

LPC, included at 0.2 %, was sufficient to maintain the viscosity at the desired levels, whereas C14-LPC did not deliver the same advantageous results compared to its longer analogues (figure 6.1).

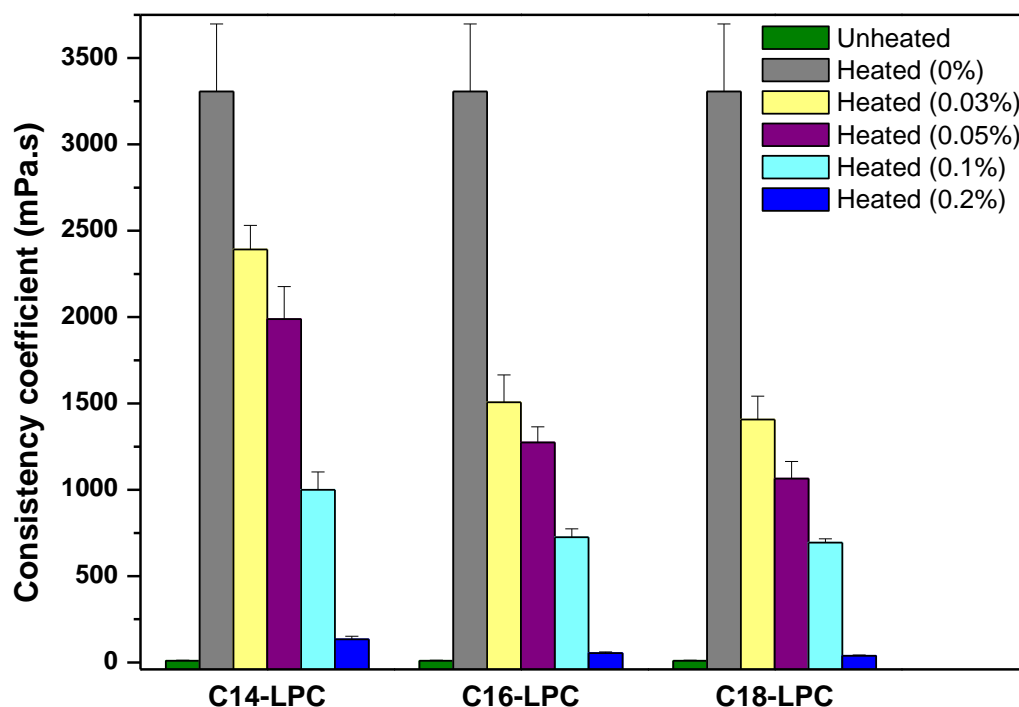


Figure 6.1. Consistency coefficient (in mPa.s) of unheated as well as heated recombined concentrated milk emulsions containing 0.00 %, 0.03 %, 0.05 %, 0.10 % and 0.20 % (w/v) 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (C14-LPC), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (C16-LPC) and 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (C18-LPC), respectively. Viscosity was measured at room temperature either before or after heating at 121°C for 20 min. The unheated value is the average of all unheated samples.

Our results are in line with Tran Le et al. (2011). Their results also revealed a beneficial heat-stabilizing effect of C14-, C16- and C18-LPC. However, in their contribution stearoyl LPC proved to be the least effective, which was attributed to its lower CMC value. The above findings may possibly be explained by the increased hydrophobicity and hence surface activity of the longest (i.e. stearoyl) LPC resulting in a higher extent of interaction with the hydrophobic residues of the denatured whey proteins upon heating and hence preventing the continuation of protein-protein interactions and protein aggregation, and providing better heat stability, as compared to the shorter analogues, which are more hydrophilic, as can be seen from their larger CMC values.

6.3.2. Effect of dialkylphospholipids on recombined concentrated milk emulsions

The effect of two anionic dialkyl-phospholipids (DMPG and DPPG) as well as of two zwitterionic dialkyl-phospholipids (DMPC and DPPC) was tested by carrying out viscosity and particle size distribution measurements, upon severe heating. The objective of this sequence of experiments was to detect and explain possible variations in the behavior of the incorporated phospholipids on the heat stability of recombined concentrated milk emulsions, based on their different polar head chemistry and hydrocarbon chain length. Figure 6.2 portrays the changes in consistency coefficient as a function of the type and concentration of the added dialkyl phospholipid, whereas figure 6.3 shows how the volume-weighted average particle diameter ($d_{4,3}$) was altered upon phospholipid addition.

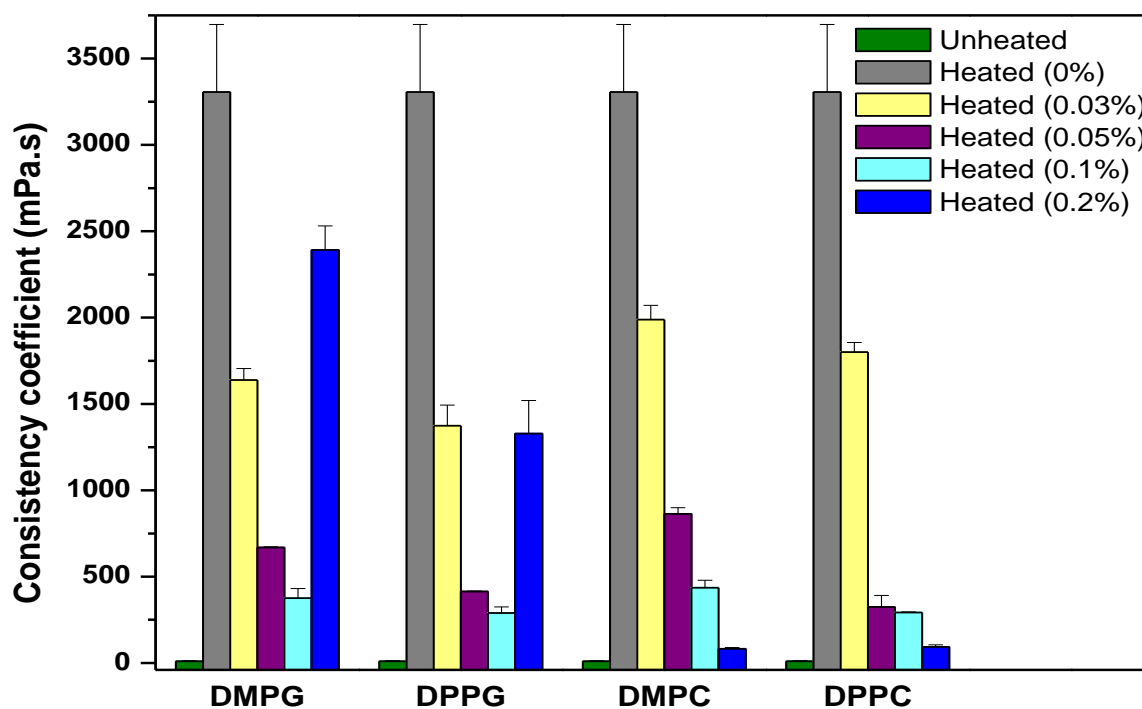


Figure 6.2. Consistency coefficient (in mPa.s) of unheated as well as heated recombined concentrated milk emulsions containing 0.00 %, 0.03 %, 0.05 %, 0.10 % and 0.20 % (w/v) 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), respectively. Viscosity was measured at room temperature either before or after heating at 121°C for 20 min. The unheated value is the average of all unheated samples.

Because of insignificant changes in both viscosity and particle size distribution between the unheated samples, an average value of consistency coefficient and particle diameter was calculated based on the corresponding values of all the unheated samples. This average was used as a reference in figure 6.2 and figure 6.3. A major increase in viscosity and particle size was noticed upon heating at 121°C for 20 min, whereas dialkyl phospholipid supplementation resulted in a considerable decrease of the aggregation tendency.

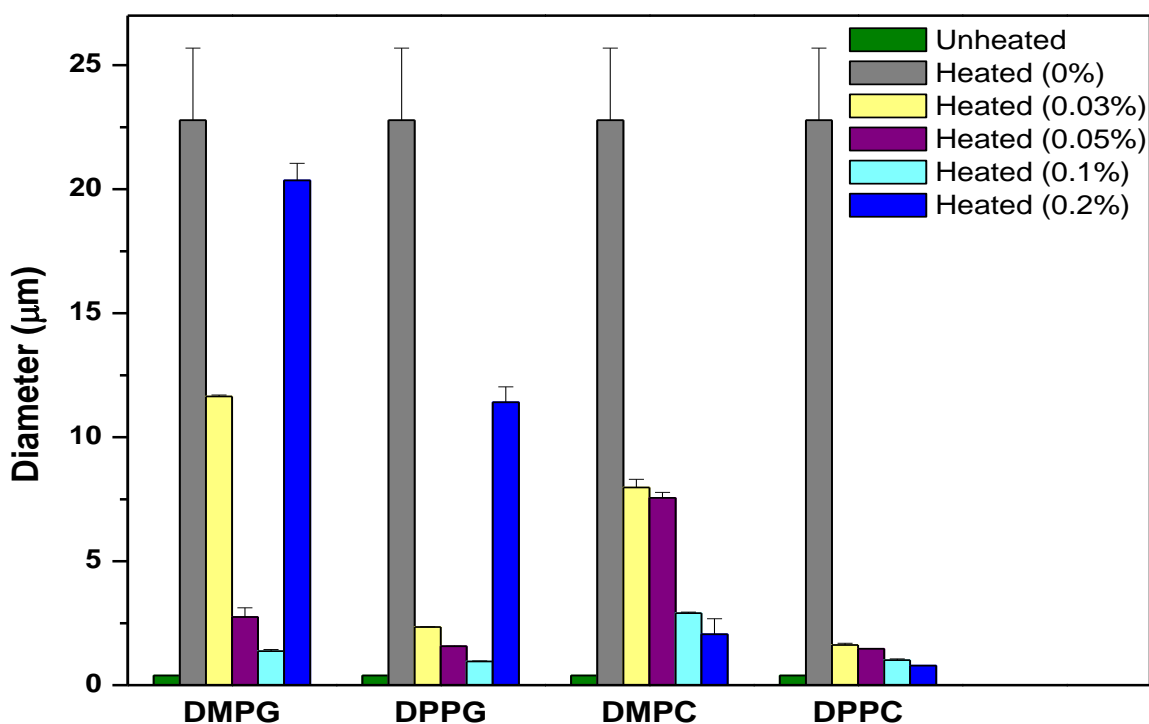


Figure 6.3. Volume-weighted average particle diameter $d_{4,3}$ (in μm) of unheated as well as heated recombined concentrated milk emulsions, as a function of phospholipid type and concentration (%), after heating at 121°C for 20 min. The variability indicators represent the standard deviation from ($n=3$) repeated experiments. The unheated value is the average of all unheated samples.

These observations are in line with the decreased droplet size in a coffee cream simulant upon addition of highly hydrolyzed lecithin, as observed by Van der Meeren et al. (2005). Similarly, Kasinos et al. (2014) proved that phospholipid incorporation in recombined concentrated milk emulsions has a great heat-stabilizing effect, as the average particle diameter was drastically reduced upon addition of 0.3 % of sunflower lecithin.

When the longer alkyl chain phospholipids DPPC and DPPG were used, even at relatively low concentrations (0.03 %), a significant decrease in the aggregation tendency was

observed, with the volume average particle diameters decreasing from $22.8 \pm 2.9 \mu\text{m}$ to 1.6 ± 0.1 and $2.3 \pm 0.0 \mu\text{m}$, respectively, whereas the corresponding values for DMPC and DMPG were 7.9 ± 0.3 and $11.6 \pm 0.1 \mu\text{m}$. The more efficient heat-stabilizing effect of the longest alkyl chain surfactants is presumed to be associated with the increased hydrophobicity and hence the larger degree of interaction with the whey protein hydrophobic residues which are already exposed due to the heat induced denaturation.

Both consistency coefficient and volume weighted average particle diameter decreased upon increasing the concentration of the applied phospholipids as depicted in figures 6.2 and 6.3, respectively. Upon addition of 0.1 % of all dialkyl phospholipids and especially DPPC and DPPG, the aggregation tendency was prevented, resulting in recombined milk emulsions with a liquid structure. However, in the case of the two anionic (PG) phospholipids, the beneficial effect was lost upon further increase of the added concentration (i.e. 0.2 %), giving rise to a significant increase of both viscosity and particle size, a fact which confirms the importance of the phospholipid polar head group on the heat coagulation of recombined concentrated milk emulsions. McCrae and Muir (1992) compared the heat-stabilizing effect of two different types of lecithin, egg and soya lecithin, on recombined milk upon addition at varying concentrations. Their results showed that soya lecithin, which contains greater amounts of anionic phospholipids as compared to egg lecithin, resulted into increased heat stability with increasing the added concentration. However, the beneficial effect was lost as the concentration surpassed an optimum value and the heat stability was decreased. In order to explain the effect of anionic surfactants in whey protein containing solutions, Chen and Dickinson (1998), Dickinson and Hong (1997) and Giroux and Britten (2004) investigated the influence of sodium dodecyl sulfate (SDS) addition on the solubility of β -lg in water, as well as on the rheology of β -lg-containing emulsions. A significant increase in solubility was observed upon SDS inclusion at low concentrations, due to the strong binding of SDS to specific binding sites of the whey protein. According to their explanation, SDS causes an increase of the net negative charge of the protein providing a stabilizing effect. Upon increasing SDS concentration, the beginning of a co-operative SDS/ β -lg binding takes place, inciting an extensive unfolding of the protein molecules and resulting in a decrease in protein solubility. Correspondingly, Lefèvre and Subirade (2000, 2001) verified that whey protein denaturation takes place in the presence of

certain phospholipids. More precisely, they concluded that conformational modifications of β -lg did not occur in the presence of the zwitterionic DMPC and DPPC, as opposed to the anionic DMPG.

Observing the results in figure 6.3, and based on the fact that denatured proteins still contain residual secondary structure (Matsuo et al., 2007), we can speculate that when the anionic phospholipids DMPG and DPPG are used at concentrations lower than 0.1 %, 'specific binding' takes place. During this stage, electrostatic interactions resulting to the increase of the net negative charge of the protein take place, protecting the protein molecules or the protein aggregates from further aggregation due to the increased electrostatic repulsion (Dickinson & Hong, 1997). In addition, hydrophobic interactions between the alkyl chain of the anionic phospholipid and the non-polar protein regions are also observed (Giroux & Britten, 2004). As the concentration of the added anionic phospholipid increases, all the specific binding sites of the proteins become occupied, resulting to the complete unfolding of the protein and the extensive protein aggregation.

Considering the composition of the emulsions, this hypothesis seems to be realistic. In fact, the β -lg content of the concentrated milk samples is estimated to be 45 μ mol per 100 g of emulsion, based on their SMP content, the protein content of the SMP and the β -lg content of milk protein. Considering the molar mass, it follows that 0.031 % w/w of DMPG can be bound by specific binding with the protein, assuming a 1/1 molar ratio, and hence a much larger anionic phospholipid concentration is needed for cooperative binding. According to this binding mechanism, the heat treated emulsion at DMPG concentrations higher than 0.1 % might contain more intensively aggregated unfolded protein as compared to the emulsions containing lower concentrations.

6.3.3. Protein recovery and surface load

In order to explain the modifications which take place during heating at elevated temperatures in recombined concentrated milk, the protein recovery in the continuous phase following emulsification and heating was determined. The cream layer of all samples was very carefully removed after high-speed centrifugation, whereas the serum phase (containing dissolved whey proteins as well as casein micelles) was thoroughly mixed with the sediment (containing casein micelles). From the protein recovery, the protein surface load can be calculated. Based on the average Sauter mean diameter of all

unheated samples (0.32 μm), the specific surface area was calculated to be $2.10^4 \text{ m}^2/\text{kg}$ Hozol.

McCrae (1999) mentioned that protein load can discriminate (at least partly) between 2 mechanisms: lecithin association with the free protein in solution and therefore influencing heat-induced free protein/bound protein interactions, and/or displacement of or interaction with interface associated protein. The protein recovery is depicted in tables 6.2 and 6.3, for samples in the presence and absence of mono- and dialkyl-phospholipids, respectively, as well as before and after heating. The results from both tables show that the protein content in the serum phase of the unheated samples did not change significantly upon increasing the concentration of the added phospholipids. As statistical analysis (one-way ANOVA) did not show any significant effect ($P \leq 0.05$) of dialkyl- and lysophospholipids addition in the unheated samples when tested with a 95 % level of confidence, it can be concluded that protein displacement did not take place at the low phospholipid to protein ratios used. This seems in contradiction to the observations by Courthaudon, Dickinson and Dalgleish (1991) who described that the addition of a water soluble nonionic surfactant to a β -casein stabilized emulsion leads to complete protein displacement from the interface. However, these authors focused on samples with a large molar excess of surfactant to protein; they mention a complete displacement at a Tween 20 to protein molar ratio of 17:1.

From the overall average value of the protein recovery in the unheated samples (87.2 ± 0.7 %), the average protein surface load in the unheated samples can be calculated: the value obtained was $5.9 \pm 0.4 \text{ mg}/\text{m}^2$, which is comparable to the literature (Cho, Lucey & Singh, 1999). On the other hand, the protein recovery in the serum phase was largely decreased upon heating for 20 min at 121°C , denoting heat-induced additional protein deposition on the O/W interface. Table 6.2 shows a gradual increase in protein content in the serum phase of all heated samples by increasing the incorporated lysophospholipid concentration. Hence, phospholipid addition minimizes the additional protein deposition during severe heating.

Table 6.2. Protein recovery (%) in the serum phase of recombined concentrated milk emulsions containing 0.00, 0.03, 0.05, 0.10 and 0.20 % of 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (C14-LPC), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (C16-LPC) and 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (C18-LPC), after heating for 20 min at 121°C. The variability indicators represent the standard deviation from (n=3) repeated experiments. The values of the heated samples with the same superscript, in each row, are not significantly different from each other based on a 95 % confidence level.

Concentration (%)	C14-LPC		C16-LPC		C18-LPC	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
0.00	88.7±0.9	37.2±0.4 ^a	86.8±0.1	38.9±4.5 ^a	88.6±0.0	37.7±0.1 ^a
0.03	87.2±1.7	38.7±2.7 ^a	84.1±1.3	48.6±6.4 ^{a,b}	85.7±2.5	51.7±2.5 ^b
0.05	87.2±1.5	41.2±0.6 ^a	84.6±2.2	50.8±1.5 ^b	87.3±0.5	52.4±2.1 ^b
0.10	86.5±2.3	43.1±1.0 ^a	87.9±1.3	52.6±0.7 ^{a,b}	88.1±4.2	58.9±1.5 ^b
0.20	87.2±2.6	51.2±6.7 ^a	82.7±2.3	57.4±0.3 ^{a,b}	88.4±5.5	69.0±2.6 ^b

However, these results were highly dependent on the length of the lysophospholipid hydrocarbon chain. More specifically, the most pronounced effect on the prevention of the heat-induced additional protein deposition was obtained upon addition of the longest C18-LPC. Interestingly, and similarly to the case of the volume weighted average particle diameter (table 6.1), only 0.03 % of the C18-LPC was needed to obtain 50 % of protein recovery, whereas in the case of the shortest C14-LPC, the maximum concentration (i.e. 0.2 %) was necessary to attain the same amount of recovery. Moreover, it should be mentioned that statistical analysis showed that there were significant differences ($P \leq 0.05$) in protein recovery at all concentrations between the longest monoalkyl phospholipids C16 and C18 and the shortest C14, a fact which proves their better capacity in preventing heat-induced changes.

Table 6.3 reveals that an overall increase of the protein recovery in the heated samples was observed upon addition of both anionic and zwitterionic phospholipids. When zwitterionic phospholipids were utilized, the protein recovery was increased with increasing concentration, whereas in the case of the anionic DMPG and DPPG, the effect was positive at lower concentrations, but after further increasing (above 0.1 %), the protein recovery was decreased. Statistical analysis showed that significant differences ($P \leq 0.05$) were observed when 0.03 % of dialkylphospholipids was added in recombined concentrated milk emulsions: The protein recovery obtained upon DPPG

addition was significantly higher as compared to the zwitterionic DMPC and DPPC, as well as to its shorter analogue DMPG, a fact which denotes the remarkable heat-stabilizing effect of the longer chain anionic phospholipids at relatively low concentrations. Upon addition of higher concentrations (0.05 %), significant differences were only observed between DPPG and DMPC, indicating that at higher concentrations, phospholipids with shorter alkyl chains (DMPG) as well as zwitterionic phospholipids (DPPC) can be very beneficial in exhibiting heat-stabilizing characteristics. Finally, upon supplementation of the highest concentrations (0.2 %), significant differences were found between the zwitterionic DPPC and DMPG.

As it can be also noticed from table 6.3, the anionic phospholipids lost their beneficial influence in improving the heat stability of recombined concentrated milk emulsions upon exceeding a threshold concentration.

Table 6.3. Protein recovery (%) in the serum phase of recombined concentrated milk emulsions containing 0.00, 0.03, 0.05, 0.10, 0.20 % of 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), after heating for 20 min at 121°C. The variability indicators represent the standard deviation from (n=3) repeated experiments. The values of the heated samples with the same superscript, in each row, are not significantly different from each other based on a 95 % confidence level.

Conc. (%)	DMPG		DPPG		DMPC		DPPC	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
0.00	87.1±1.4	44.9±4.4 ^a	89.4±0.2	57.9±1.4 ^a	87.0±0.4	49.8±3.1 ^a	89.5±2.0	48.4±1.8 ^a
0.03	89.1±4.4	50.5±1.3 ^a	88.4±3.5	70.6±0.7 ^b	86.6±3.3	52.0±1.3 ^a	84.9±2.5	53.7±0.7 ^a
0.05	88.3±3.9	58.1±2.1 ^{a,b}	88.4±0.5	73.1±5.1 ^a	84.6±2.8	56.0±0.2 ^b	87.0±4.0	62.5±0.4 ^{a,b}
0.10	89.2±0.7	61.3±0.8 ^a	89.9±0.9	75.3±2.9 ^b	85.0±3.6	64.2±1.9 ^a	89.0±1.6	66.1±0.9 ^{a,b}
0.20	84.3±3.8	41.7±0.1 ^a	85.1±1.8	54.1±0.4 ^{a,b}	87.4±6.7	68.4±0.8 ^b	87.0±1.2	79.2±4.8 ^b

The effect of dialkylphospholipids on the protein recovery could be attributed to their hydrophobic interaction (Kasinos et al., 2013) with the exposed hydrophobic residues of the unfolded whey proteins and hence preventing further heat-induced aggregation.

The overall outcome emerging from the protein recovery data indicates that heat coagulation largely depends on additional protein deposition on the surface of the

emulsion droplets upon heating, which seems to induce emulsion droplet aggregation, and subsequently provoking a significant increase in both viscosity and particle size.

6.4. Conclusions

Processing of RCM emulsions at high temperatures gave rise to the formation of a gel-like structure, which provides evidence of heat coagulation. Taking into account that high-heat SMP was treated at high temperatures during its manufacture, and hence it contains protein aggregates consisting of denatured whey proteins and whey protein aggregates attached to casein micelles, it is speculated that heat coagulation of RCM is a result of further aggregation and complexation of the already existing aggregates, which constitute the structural units for gelation upon further heating. Viscosity and particle size distribution measurements revealed that the addition of either lyso- or dialkylphospholipids can increase the heat stability of milk emulsions to a great extent. The results clearly indicate that the stability of RCM emulsions upon heating importantly depends on the type and concentration of the added phospholipid.

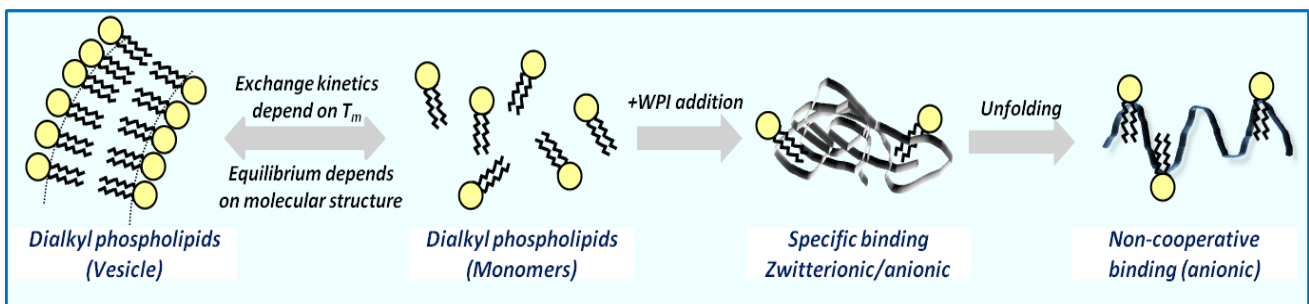
When the effect of the alkyl chain length was investigated, the more pronounced heat-stabilizing effect of the longest C16-LPC and especially C18-LPC was evident, whereas in the case of C14-LPC, the effect was less significant. The same observations were noticed when the longer dialkylphospholipids DPPG and DPPC, were compared to their shorter analogues DPPG and DMPC, respectively. The better heat-stabilizing effect of the longer alkyl chain polar lipids could be attributed to their increased hydrophobicity and hence surface activity. A higher extent of interaction with the hydrophobic residues of the denatured whey proteins and/or protein aggregates upon heating can be speculated, hence preventing further aggregation and providing better heat stability, as compared to the shorter analogues, which are more hydrophilic as can be deduced from their larger CMC values.

Whereas the heat-stabilizing effect of the zwitterionic phospholipids was directly proportional to the added amount, the influence of the anionic phospholipids was greatly dependent on the concentration. At low concentrations, the heat-stability of RCM emulsions was drastically improved upon heating at elevated temperatures, whereas upon further increase of the added concentration, the beneficial effect was lost. This particular behavior of the anionic polar lipids is believed to be attributed to their

binding with specific binding sites of whey proteins, leading to an overall increase of the net negative charge, and hence to enhanced electrostatic repulsion between the protein molecules. Furthermore, hydrophobic interactions between the surfactant tails and the hydrophobic residues of the whey proteins take place, preventing further complexation of whey protein aggregates. Upon higher concentrations, all binding sites become saturated, the protein becomes completely unfolded and further aggregation takes place.

Chapter 7

EFFECT OF PHOSPHOLIPID MOLECULAR STRUCTURE ON ITS INTERACTION WITH WHEY PROTEINS IN AQUEOUS SOLUTION



This chapter is redrafted from: Kasinos M., Sabatino P., Vanloo B., Gevaert K., Martins, J.C. and Van der Meeren P. (2013), *Food Hydrocolloids*, 32, 312-321.

Chapter cover image: Schematic representation of the effect of phospholipid molecular structure on its interaction with whey proteins.

Abstract

Because most dairy processes require heat treatment to ensure microbiological safety during storage, dairy industry has invested a lot of effort to acquire knowledge related to all the changes that may take place during heating. More precisely, measures have to be taken to avoid undesired changes that can badly affect the quality of the final product. Whey protein denaturation is one of the major changes that take place upon sterilization. The purpose of the present study was to investigate and compare the degree and type of interaction that occurs between whey protein isolate and different kinds of dialkyl phospholipids, as a function of their molecular structure, upon heating at different temperatures. Circular Dichroism (CD) experiments were performed to assess the extent of secondary structural changes of β -lactoglobulin upon incubation in the presence of both anionic and zwitterionic phospholipids. The far-UV spectra of the former revealed a significant change in ellipticities in the region between 208 and 222 nm, which represents the α -helical content of peptides and proteins upon incubation in the presence of anionic phospholipids at temperatures above their phase transition temperature. On the other hand, these changes were not evident upon incubation with phospholipids in the gel state or when applying zwitterionic phospholipids. Nuclear Magnetic Resonance (NMR) has been used to investigate the whey protein-surfactant interaction into more detail. The chemical shift of the surfactant methyl groups towards a more hydrophobic region in the NMR spectra revealed a hydrophobic whey protein-phospholipid interaction. This finding was further supported by Saturation Transfer Difference-NMR (STD-NMR) measurements. The NMR experiments also revealed that the lack of protein structural reorganization upon incubation in the presence of zwitterionic (lyso)-phospholipids could not be ascribed to a lack of interaction, but was caused by the fact that the interaction did not affect the protein's structure.

Overall, this work indicated that phospholipids can modify the secondary structure of whey proteins (i.e. when using anionic phospholipids in the liquid crystalline state) due to hydrophobic interactions. In addition, the results prove that the heat stability of whey protein containing products may be optimized by appropriate selection of the phospholipid composition.

7.1. Introduction

Bovine milk contains two major groups of proteins: the casein and the whey protein fraction. The latter, due to its important functional properties, is increasingly used as food ingredient. Within the whey proteins, β -lactoglobulin (β -lg) is the major component, which constitutes more than 50 % of the total whey protein in bovine milk (Verheul, Pedersen, Roefs, & De Kruif, 1998). It is a small globular protein (162 amino acid residues) with a molecular mass of 18.3 kDa. At room temperature and in concentrations as in bovine milk, the protein is predominately present as a dimer in aqueous solutions at a pH between 5.5 and 7.5 (Mercadante et al., 2012; Verheul et al., 1998). Its secondary structure mainly consists of β -sheets (> 50 %), little of α helices and some random coils (Monaco et al., 1987). Whey proteins are also described as the 'heat labile' fraction of milk protein, since upon raising the temperature above 70°C, they undergo numerous conformational changes. These changes are thought to play an essential role in heat-induced coagulation (Jeurnink & De Kruif, 1993; Singh, 2004; Tran Le et al., 2011a).

Surface active species such as phospholipids have been shown to have an important effect on the heat stability of milk protein stabilized emulsions. Through extensive research it has been shown that polar lipids can interact with proteins and alter their molecular structures as well as their physico-chemical properties (Dickinson & Hong, 1997; Giroux & Britten, 2004; Tran Le et al., 2007). The degree of modification depends on various factors such as temperature, ionic strength, protein concentration, and polar lipid characteristics.

During the last decades it has been reported that anionic surfactants, such as SDS, bind strongly with β -lg causing the formation of protein-surfactant complexes inducing the unfolding of proteins, whereas non-ionic surfactants bind very weakly (Goddard & Ananthapadmanabhan, 1993; Lefèvre & Subirade, 2001; Liu, Shang, Jiang, Dong, & Wang, 2006; Lu et al., 2005; Lu, Cao, Lai, & Xiao, 2006; Moore, Puvvada, & Blankschtein, 2003). Therefore, the resulting secondary structure of the protein depends on the nature of the surfactant binding to the protein (Takeda, Shigeta, & Aoki, 1987).

The aim of this contribution is to provide insight into the interaction between β -lg and various phospholipids. Whereas Circular Dichroism (CD) reveals information related to

the protein secondary structure, the use of high-resolution diffusion- and STD-NMR enables to study the interaction mechanism between the protein and the phospholipids in more depth. Using this combination of techniques enabled to evaluate whether the absence of protein structural rearrangements was either due to the absence of interactions or due to the fact that the interactions did not have any effect on the protein's structure.

In order to investigate the effect of phospholipid molecular properties, a variety of both zwitterionic and anionic phospholipids with different chain length and hence phase transition temperature (T_m) were investigated, whereby protein-phospholipid mixtures were incubated both above and below the phospholipid phase transition temperature.

7.2. Materials and methods

7.2.1. Materials

Whey protein isolate (WPI) was obtained from Davisco Foods International, Inc. (BiPro®, Le Sueur, MN, USA). Kjeldahl analysis revealed that this WPI contained 92.6 % of protein, whereas polyacrylamide gel electrophoresis (PAGE) indicated that approximately 85 % of the total protein consisted of β -lactoglobulin. Besides, the WPI contained 1.6 % ash (by incineration at 525°C), 5.0 % moisture (from weight loss at 102°C) and 0.8 % fat.

Six dialkyl-phospholipids were obtained from NOF Corporation (Tokyo, Japan). Whereas 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA) and 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA) are anionic, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) are zwitterionic. The 1-myristoyl-sn-glycero-3-phosphocholine (C14-LPC) monoalkyl phospholipid was also obtained from NOF Corporation (Tokyo, Japan). According to the manufacturer, the phase transition temperature (T_m) of DMPG and DMPC is 23°C, for DPPG and DPPC it is 41°C, whereas for DMPA and DPPA it is 50 and 67°C, respectively.

7.2.2. Sample preparation

Samples containing 5 mg/ml of DMPG, DMPC, DPPG, DPPC, DMPA and DPPA were prepared in distilled water. In addition, WPI containing samples were prepared

containing 5mg/ml of surfactant and 10 mg/ml of WPI. Phospholipid solutions were sonicated by using a Sonifier 250 (Branson, Canada) tip sonicator. The 13 mm tip was immersed in 10 ml of vesicular dispersion, approximately two thirds of the sample height. The power monitor indicated 20 %. In the beginning and after every two min of sonication, the samples were left to rest in a water bath at 55°C during one min. A 50% duty cycle was selected to prevent heating of the sample. For CD samples, the sonication time was always 2 min, whereas for NMR the sonication time of the vesicular dispersions was always 10 min in total. After sonication, the samples were cooled down to room temperature and mixed with protein.

7.2.3. Circular dichroism measurements

Circular Dichroism is a technique which is based on the differential absorption of the left and right circularly polarized light (LCP and RCP), absorbed by an optically active molecule. The molar circular dichroism is wavelength dependent but also depends on the molecules conformation, which can be a function of concentration, temperature, and chemical environment. If, after the passage through the sample being analyzed, the LCP and RCP components are not absorbed or absorbed to equal amounts, their recombination will generate radiation polarized in the original plane. However, if they are absorbed to different extents, the resulting radiation will be no longer linearly but elliptically polarized, and the resultant will trace out an ellipse (Kelly, Jess, & Price, 2005). The CD is expressed in degrees of ellipticity, θ (equation 1), which is defined as the tangent of the ratio of the minor to major elliptical axis:

$$\theta = \tan^{-1}(b/a) \quad (1)$$

where b and a are the minor and major axes of the resulting ellipse, respectively.

In general, CD can be used in applications where protein-protein interactions are required to be determined or, protein-DNA, protein-ligand and DNA-ligand interactions.

The CD spectra were obtained on a Jasco J-710 (Easton, MD, USA) spectrometer. β -lg secondary structure without or in the presence of phospholipids was followed in the wavelength range of the peptide bond absorption, in the far-UV (200 - 260 nm) region. For each sample, nine scans were performed using a scan rate of 50 nm/min and

response time of 0.5 s, whereas the sensitivity was 20 mdegrees. For CD measurements, the concentrations were 40 times lower as compared to NMR. Hence, the CD samples contained 250 μg of WPI/ml, as well as 125 μg of phospholipid/ml and they were injected into a 1 mm path length cuvette. In order to evaluate the effect of temperature, each sample was incubated at room temperature as well as at 45 and 90°C in a water bath for one hour. The results were expressed as mean residue ellipticity. From the CD results, the amount of the α -helical content was calculated according to Chen, Yang, & Martinez (1972). To enable a statistical comparison of the spectra, the ratio of the molar ellipticities at a wavelength of 208 and 220 nm was considered.

7.2.4. NMR measurements

Samples containing 5 mg/ml of DMPG, DMPC, DPPC, DPPG as well as C14-LPC were prepared in 5 mM sodium acetate trihydrate (MW=136.08 g/mol; Normapur, Lutterworth, UK) in D₂O (99.8 % AtomD, Armar Chemicals, Döttingen, Switzerland). In addition, whey protein containing samples were prepared containing 5 mg/ml of phospholipid, 10 mg/ml of whey protein isolate and 5 mM sodium acetate trihydrate in D₂O. Sodium acetate was used as internal standard. The phospholipid-protein mixtures were incubated in a water bath above the phospholipid transition phase temperature, for 1 hour: DMPG and DMPC were incubated at 25°C, whereas DPPG and DPPC were treated at 50°C.

All the NMR experiments were performed on a Bruker Avance II spectrometer operating at a ¹H frequency of 700.13 MHz and equipped with a 5 mm ¹H TXI-Z gradient probe with a maximum gradient strength of 57.7 G/cm. Measurements were performed at both 25 and 50°C and the temperature was controlled to within $\pm 0.01^\circ\text{C}$ with a Eurotherm 3000 VT digital controller.

Diffusion coefficients were measured by pfg-NMR with convection compensated double stimulated echo experiments (Jerschow & Müller, 1997) using monopolar smoothed rectangular shaped gradient pulses and a modified phase cycle to minimise phase distortions due to unwanted gradient echo's (Connell et al., 2009). The echo-decay of the resonance intensity obtained with the double stimulated echo sequence obeys equation 1, from which it is clear that the diffusion coefficient D is derived from the echo-decay as a function of the parameter k.

$$I = I_0 \exp[-D(\gamma G \delta s)^2 \Delta'] \quad (1)$$

$$I = I_0 \exp[-D \cdot k]$$

where I is the echo intensity with gradient; I_0 is the echo intensity at zero gradient; D is the diffusion coefficient; g the gyromagnetic ratio; G the maximum gradient amplitude; δ the duration of the gradient pulse and Δ' is the diffusion delay corrected for the finite gradient pulse duration ($\Delta' = \Delta - 0.6021 \cdot \delta$). The gradient shape factor s was set to 0.9 to account for the smoothed rectangular gradient shape used here. The determination of the diffusion coefficient was based on the fitting of a mono-exponential curve to the echo decay of the peak intensity of the selected resonances (Alper & Gelb, 1990).

T_2 relaxation experiments were carried out using the Carr-Purcell-Meiboom-Gill (CPMG) sequence $[90^\circ - (\tau - 180^\circ - \tau)_n]$, where the echo time τ is 1 ms.

STD measurements were obtained using the standard pseudo2D version of the STD NMR pulse sequence (stddiff) wherein on and off resonance spectra are recorded in an interleaved manner (Szczygiel, Timmermans, Fritzingler, & Martins, 2009). Following optimization of the off-resonance frequency, all STD spectra were recorded with on and off-resonance frequency of 8 and 300 ppm, respectively. The saturation was performed via a cascade of 720° Gaussian pulses with a length of 50 ms and an interpulse delay of 1 ms. The total duration of the saturation sequence was varied from 0.01-5 s to obtain the STD intensity buildup. To allow complete relaxation of both receptor and ligand, a relaxation delay of 20 s was used throughout. For negative controls, we acquired STD spectra of each surfactant without the protein, using the same experimental setup. No STD response was noted in any of the control experiments.

7.3. Results and discussion

7.3.1. Circular dichroism

7.3.1.1. Circular dichroism spectra of WPI

The unfolding of native whey proteins and especially β -lg, either by heat or by addition of specific substances, is the main issue of numerous scientific studies. CD comprises a very helpful method to characterize the secondary structure of globular proteins, such as β -lg, due to the peptide bond absorption in the far-UV region (Chen et al., 2005; Kelly

& Price, 1997; Viseu, Carvalho, & Costa, 2004). Here, CD was used in order to provide structural information about WPI at different experimental conditions.

The CD spectrum of the WPI at room temperature in the far-UV region (figure 7.1) was comparable to literature data for native β -lg. The band is characterized by a negative dichroic peak with a minimum in the 215 nm region, which is typical for a protein with a β -sheet configuration (Brown, Carroll, Pfeffer, & Sampugna, 1983; Chen et al., 2005; De Jongh, Gröneveld, & De Groot, 2001; Liu et al., 2006; Su & Jirgensons, 1977; Zhang & Keiderling, 2006).

Moreover, figure 7.1 depicts how the far-UV spectrum of WPI changes as a function of temperature. Clearly, no significant changes were observed upon incubation for one hour at 45°C: the spectrum remained almost identical as in the case of incubation at room temperature, with the symmetrical deep minimum in the region around 215 nm. However, significant conformational changes were noted when the WPI solutions were incubated for one hour at 90°C. These data indicated that there was an increase of the percentage of the calculated α -helical content of the WPI upon heating at 90°C (25 %) compared to that at room temperature and 45°C (19 %).

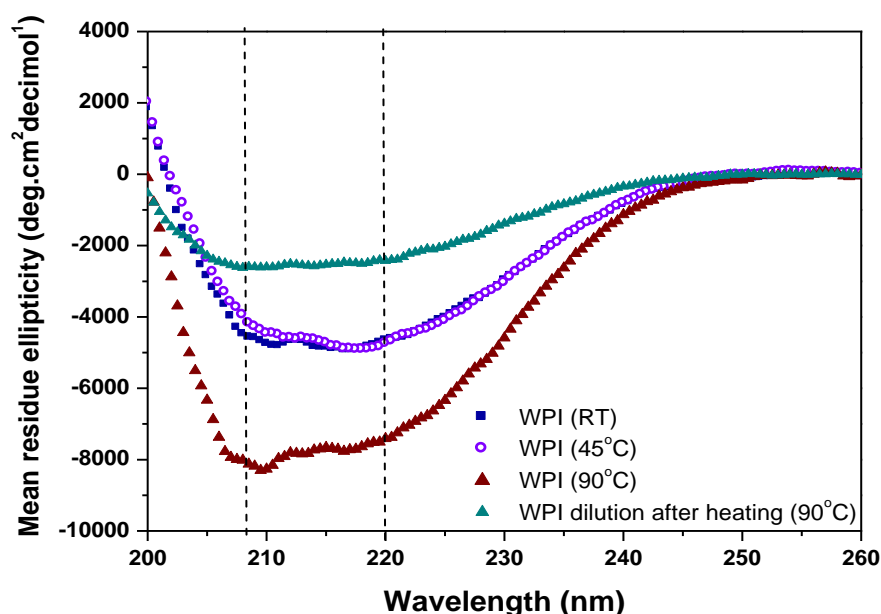


Figure 7.1. Far-UV circular dichroic spectra of 250 μ g/ml whey protein isolate in water at (■) room temperature and after incubation for one hour at (●) 45 and 90°C when dilution took place before (▲) and (□) after heating.

These observations are in line with Wada, Fujita, & Kitabatake (2006), Kim, Cornec, & Narsimhan (2005) and Moro, Báez, Busti, Ballerini, & Delorenzi (2011), who also found that significant changes on a structural level occurred upon rising the temperature above 80°C, whereas no or minimal conformational changes were noticed after heating at temperatures between 50 and 60°C (Chen et al., 2005). It is indeed well known that β -lg undergoes thermal unfolding at temperatures in the region of 70°C and above (De Wit, 2009). These conformational changes at elevated temperatures include the dissociation of dimers into monomers together with intermolecular interactions consisting of hydrophobic bonding and thiol-disulphide exchange (De Wit, 2009; Hansted et al., 2011; Tran Le et al., 2011) with consequent aggregation phenomena. Bhattacharjee et al. (2005) found that this phenomenon is accompanied by a loss of β -sheet structure and an increase in α -helical and random coil content. Likewise, De Jongh et al. (2001) mentioned that after incubation of β -lg at 70°C, there was an irreversible loss of 32 % of the β -strand, which was increasing with increasing temperature. In figure 7.1, a shift of the dichroic peak by approximately 7 nm was noticed after heating at 90°C whereby the ellipticity values in the vicinity of 208 nm showed a major increase of the negative extreme. From the spectra shown in figure 7.1, it is obvious that the ratio of the molar ellipticities at 208 and 220 nm can be used as a good indicator for structural changes: statistical analysis of the data obtained for WPI in the absence of phospholipids (table 7.1) indicated no significant difference between 25 and 45°C, whereas the data obtained upon incubation at 90°C were significantly larger.

In addition, figure 7.1 shows the difference in dichroic spectra of WPI, obtained upon different experimental conditions. When the protein samples were heated at 90°C, in their original concentration (10 mg/ml) and diluted 40 times after heating, the CD spectrum was similar in shape but much less intense as compared to the spectrum obtained from the protein solution that was 40 times diluted before heating. When dilution took place prior to heating, aggregation was largely prevented. On the other hand, heating a concentrated protein solution promoted protein aggregation and precipitation and hence only a smaller amount of denatured whey protein could be detected.

Table 7.1. Calculated mean residue ellipticity $[\theta]_{208}/[\theta]_{220}$ ratio as well as standard deviation of WPI in the presence of phospholipids, after incubation for one hour at 25, 45 and 90°C. The last column indicates the phase transition temperature (T_m) of the phospholipid used.

Phospholipid	Incubation temperature			T_m (°C)
	25°C	45°C	90°C	
-	0.96 ± 0.00	0.92 ± 0.03	1.08 ± 0.02	-
DMPG	1.07 ± 0.03***	1.09 ± 0.03***	1.11 ± 0.02	23
DPPG	1.01 ± 0.03	1.09 ± 0.02***	1.11 ± 0.01	41
DMPA	0.98 ± 0.00	1.08 ± 0.01***	1.06 ± 0.05	50
DPPA	0.97 ± 0.03	0.94 ± 0.01	1.11 ± 0.02	67
DMPC	0.95 ± 0.02	0.95 ± 0.01	1.20 ± 0.01***	23
DPPC	0.96 ± 0.02	0.95 ± 0.05	1.15 ± 0.05	41
C14-LPC	0.92 ± 0.03	0.98 ± 0.11	1.13 ± 0.01	-

*** significantly different as compared to WPI in the absence of phospholipids at 99 % confidence level

7.3.1.2. *Circular dichroism spectra in the presence of phospholipids*

CD measurements were performed to monitor phospholipid-whey protein interactions by studying the structural changes in WPI induced upon polar lipid addition. The measurements were executed in the far-UV region and initially at room temperature. Different phospholipids were considered, aiming to a better understanding of the effect on the secondary structure of WPI. The range of phospholipids has been selected in order to compare their effect on the secondary structure of WPI based on their fatty acyl composition (and hence T_m), the number of the acyl chains as well as the polar head group.

The results revealed that whey protein denaturation can possibly take place at room temperature, i.e. at conditions much below the whey protein denaturation temperature, in the presence of certain phospholipids.

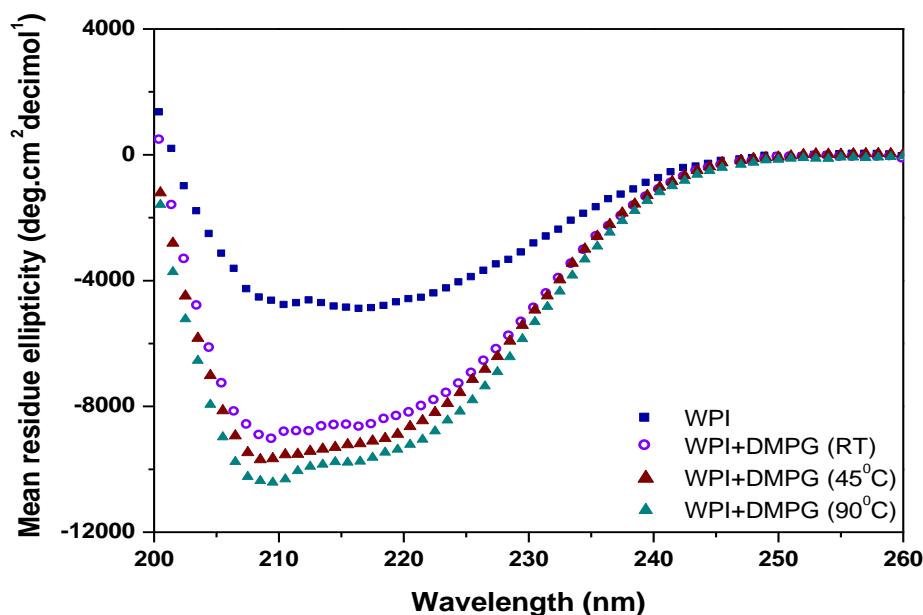


Figure 7.2. Far-UV dichroic spectra of WPI in the absence (■) as well as in the presence of anionic DMPG after incubation for one hour (□) at room temperature at (●) 45 and 90°C (○).

Table 7.1 shows the calculated ratios derived from the mean residue ellipticity values at 208 ($[\theta]_{208}$) and 220 ($[\theta]_{220}$) nm wavelengths. An obvious increase of the ratio was obtained after the addition of the anionic DMPG at room temperature (figure 7.2), whereas the rest of phospholipids retained the ratio in comparable levels as WPI.

Statistical analysis (two-way analysis of variance) showed a significant increase of the ratio upon incubation in the presence of the anionic DMPG at room temperature, whereas all other phospholipids studied had no significant effect. More precisely, the $[\theta]_{208}/[\theta]_{220}$ ratio increased from 0.96 to 1.07 ± 0.03 in the case of DMPG at 25°C, implying a typical manifestation of protein denaturation. In fact, the calculated content of α -helix increased from 19 to 27 % when DMPG was examined, whereas in the presence of DPPG, DMPA and DPPA the amount remained unchanged. Based on infrared spectroscopy, Lefèvre and Subirade (2001) also found conformational changes of β -lg upon interaction with DMPG bilayers. In their study, however, a total concentration of 10 % (w/v) was used with a protein to lipid ratio of 1:10 w/w. Similarly, Liu et al. (2006) also found a structural reorganization of β -lg induced by DMPG by using CD as well as fluorescence. However, also these authors mainly focused on samples with a large excess of phospholipids relative to protein. The prominent influence of DMPG can be explained by its shorter fatty acyl chain length and consequently its lower T_m compared to the other anionic phospholipids. Hence, DMPG vesicles are in the liquid crystalline state at room temperature, and DMPG molecules are thus much more

dynamic as compared to DPPG, DMPA or DPPA molecules, which are in the more rigid gel state at room temperature. Besides, DMPG has a higher critical micelle concentration (CMC) and hence more monomers are available for binding to the protein as compared to its DPPG analogue. Hence, the observations upon incubation at room temperature point to the large impact of the phospholipid's physicochemical state: only anionic phospholipids in the liquid crystalline state introduce β -lg denaturation, as also observed for micelle-forming anionic surfactants, whereas anionic phospholipids in the gel state do not have this effect.

On the other hand, WPI in the presence of the zwitterionic DMPC (figure 7.3), DPPC and C14-LPC (spectra not shown) revealed a very similar spectrum at room temperature as WPI in the absence of phospholipids, with about 20 % of α -helical content, indicating no significant effect of DMPC on the whey protein configuration. Additionally, their 208/220 ellipticity ratios did not show any significant difference as compared to WPI (table 7.1).

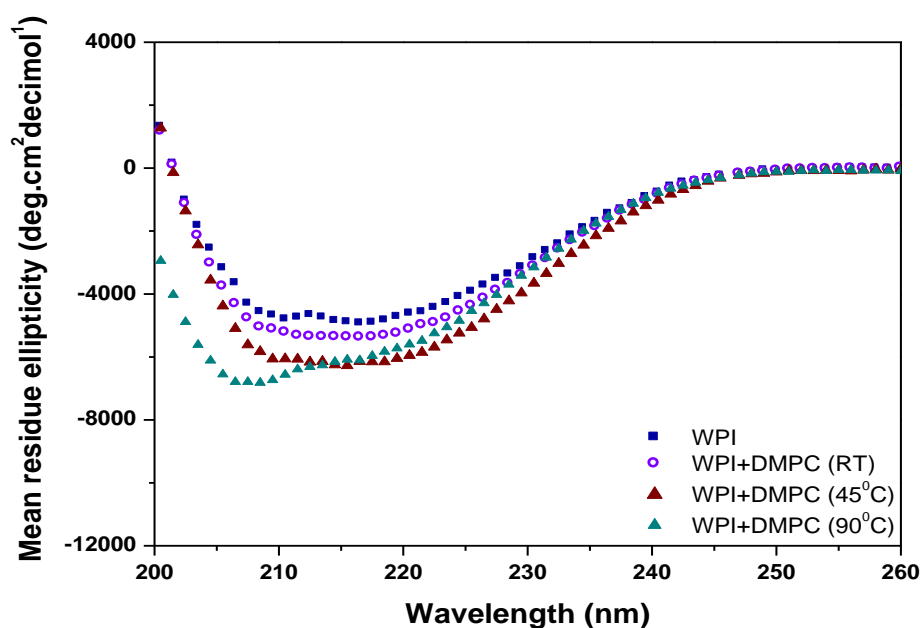


Figure 7.3. Far-UV dichroic spectra of WPI in the absence (■) as well as in the presence of zwitterionic DMPC after incubation for one hour (□) at room temperature at (●) 45 and (○) at 90°C.

Hence, these zwitterionic (lyso)-phospholipids either do not bind to the protein or the binding does not induce significant structural rearrangements. Lefèvre and Subirade (2000) also found that the secondary structure of β -lg was not affected in the presence of DMPC and DPPC (as opposed to DMPG), from which they concluded that no

interaction occurs between PC and β -lg. From this different effect of zwitterionic and anionic phospholipids, Zhang and Keiderling (2006) concluded that hydrophobic interactions must have a minor role.

Goddard & Ananthapadmanabhan (1993) mentioned that anionic surfactants interact strongly with proteins giving rise to the formation of protein-surfactant complexes, which could induce the unfolding of proteins, whereas non-ionic surfactants generally bind very weakly. According to Jones (1992) and Oakes (1974), three consecutive steps probably occur upon increasing the concentration of anionic surfactant: 'specific binding' at low concentration, 'non-cooperative binding' at higher concentration and 'cooperative binding' at even higher concentration. Our results revealed that the addition of high concentrations of anionic phospholipids resulted in a non-cooperative or cooperative binding to WPI inducing its unfolding. This is in accord with the effect of the anionic decyl sulphonate $C_{10}SO_3$ on the BSA structure: according to Lu et al. (2005), high concentrations caused an obvious modification of the BSA secondary organization. Similarly, Gelamo & Tabak (2000) classified surfactants in two groups according to their impact on BSA structure: anionic on the one hand and either cationic or zwitterionic on the other hand. Their results indicated that the protein structure upon the addition of zwitterionic surfactants remained almost intact, whereas it was affected upon the addition of the anionic SDS.

Apparently, the behavior of dialkyl phospholipids is in line with the reported behavior of mono alkyl surfactants upon incubation at temperatures above their T_m , whereas no effect was observed at temperatures below T_m both for anionic and zwitterionic dialkyl phospholipids. The above indications suggest that both the polar head group of the phospholipids and their fatty acid composition (which determines their T_m) are decisive factors for modulating the phospholipid-induced denaturation process.

In order to further sustain the proposed effect of T_m , samples of WPI together with phospholipids were incubated for one hour at 45°C, prior to the execution of CD measurements in the far-UV region in a second series of experiments. In fact, both DPPG and DPPC were in the gel state at room temperature but were transformed to the liquid crystalline state upon incubation at 45°C. A structural effect on the WPI configuration was observed (table 7.1) in the presence of the anionic phospholipids: DMPG (figure

7.2), DPPG and DMPA, as illustrated by the significant increase of the $[\theta]_{208}/[\theta]_{220}$ ratio, indicating a loss of protein β -sheet structure.

Hence, whereas DPPG and DMPA had no considerable effect at room temperature, they clearly interacted with the WPI upon incubation at 45°C. Upon heating, the monomer-vesicle exchange kinetics are largely improved around the T_m and hence more monomers are available to interact with the whey proteins. For the sake of completeness, it should be mentioned that the same significant differences were found when the statistical analysis was performed with a 95 % level of confidence. Both zwitterionic lipids, DMPC (figure 7.3) and DPPC (data not shown), had no significant structural effect even above their T_m . This is attributed to their low CMC and/or the absence of electrostatic interactions between proteins and zwitterionic phospholipids, which makes the formation of phospholipid aggregates (such as vesicles) a more favorable process than binding to proteins (Goddard & Ananthapadmanabhan, 1993). According to the manufacturer, DMPC and DPPC have a CMC of 6.10^{-6} and $4.6.10^{-7}$ mM, as compared to DMPG with a CMC of 0.011 mM.

Finally, the effect of phospholipids was evaluated after incubation for one hour at 90°C which is above the whey protein denaturation temperature to check whether some phospholipids might prevent heat-induced denaturation. Table 7.1 indicates very similar values (but larger than at 25 and 45°C), irrespective of the absence or presence of phospholipids. Hence, these data point to the thermal unfolding of whey protein at such high temperature conditions in all samples considered.

7.3.2. Whey protein-phospholipid interaction by NMR measurement

In order to have a clearer judgment of the whey protein-surfactant interaction, ^1H and STD-NMR spectroscopy were used. The aim of this series of experiments was to clarify the kind of interaction which takes place upon phospholipid addition, with special focus on the effect of phospholipid molecular structure. Hereby, five different phospholipids were used. In a first step, DMPG and DPPG were selected in order to be compared to their zwitterionic analogues DMPC and DPPC. All four surfactants were subjected to NMR measurements above their phase transition temperature. In addition, the zwitterionic lysophospholipid C14-LPC, was tested.

Figure 7.4 (A) shows the ^1H -NMR spectra of the whey protein isolate at 25°C. The sharp resonances present belong to residual HDO (around 4.8 ppm) and to sodium acetate (around 2.0 ppm). All the other broad resonances observed are due to the protein protons.

Figure 7.4 (B) displays the spectra of the anionic phospholipid DMPG alone and in the presence of whey protein as well as the chemical structure of the surfactant. The surfactant concentration was 5 mg/ml corresponding to 7.3 mM which is much higher than the reported CMC (0.011 mM). The spectra were recorded at 25°C i.e. above the transition temperature of the phospholipid of 23°C.

The spectrum of the DMPG alone nicely shows the characteristic resonances of the surfactant. The signal at 0.90 ppm is related to the terminal methyl group. The intense resonance at 1.35 ppm belongs to the methylene groups of the long alkyl chain. The two resonances at 1.65 and 2.38 ppm are due to the methylene groups in β and α positions to the carboxyl group, respectively. The peak at 4.8 ppm corresponds to residual HDO. The signals between 3.5 and 5.4 ppm were assigned using a series of 2D-NMR experiments including COSY, TOCSY ^1H - ^{13}C HSQC as well as 1D ^{13}C (APT) experiments (data not shown). From this, the signals at 4.04 and 5.35 ppm were assigned to the methylene and methyne protons close to the ester groups, respectively.

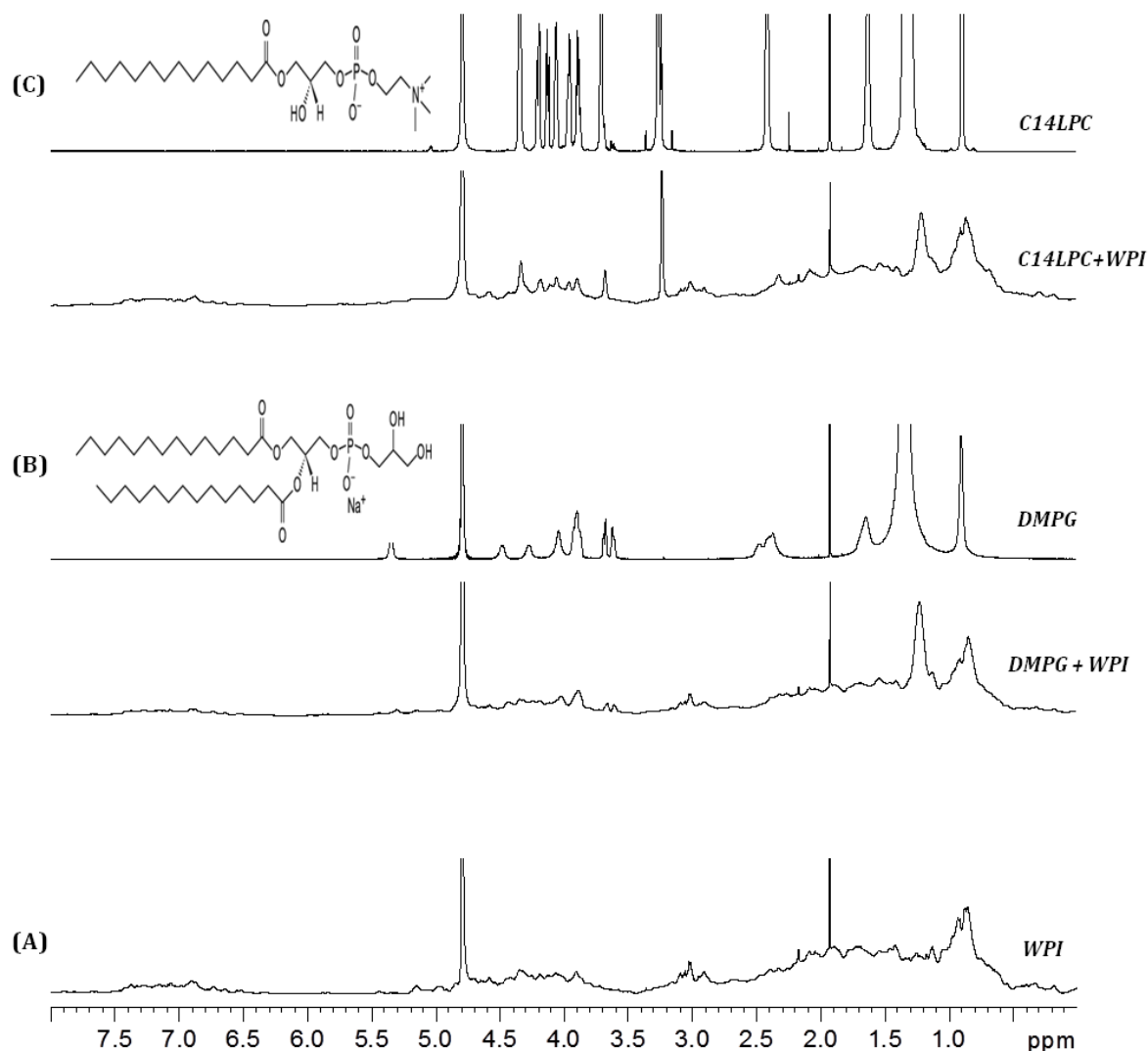


Figure 7.4. ^1H -NMR spectra for WPI at 25°C (A), as well as free DMPG and a mixture of DMPG and WPI at 25°C (B) and C14-LPC and a mixture of WPI and C14-LPC at 25°C (C).

Comparing the ^1H spectra of DMPG alone and in the presence of whey protein, the terminal methyl group as well as the α and β methylene groups are no longer discernible in the spectrum with protein because of the overlap with protein resonances. The other phospholipid signals are slightly broader in the presence of protein as confirmed by the values of the width at half height summarized in table 7.2. Moreover, the signals are reduced in intensity due to the presence of protein. These effects are mainly caused by more efficient (faster) transverse relaxation corresponding to shorter T_2 relaxation times (table 7.2). It is known from literature that the T_2 times are shortened by (non covalent) interactions that limit the degree of freedom (mobility) of the bound species.

Hence, the peak broadening indicates a lower mobility of surfactant that can be ascribed to binding to the proteins.

Table 7.2. Line width, chemical shift and T_2 relaxation times of DMPG alone and in the presence of protein. Measurements were performed at 25°C.

DMPG	Line Width (Hz)		Chemical shift (ppm)		T_2 (ms)	
	DMPG	DMPG/BiPro	DMPG	DMPG/BiPro	DMPG	DMPG/BiPro
Proton resonances						
(CH ₂) _n	50.8	61.8	1.35	1.24	9.6	6.6
CH ₂ OCOR	23.1	33.6	4.04	4.04	22.6	11.9
CHOCOR	23.3	35.0	5.35	5.31	25.8	19.9

Although a general loss of information is evident in the ¹H spectrum of DMPG with whey protein, the resonance related to the long alkyl chain of the surfactant is still clearly discernible. This peak, however, is shifted towards smaller chemical shift values (upfield shift) if compared to the same signal recorded for the DMPG alone (table 7.2). Since the chemical shift depends on the chemical environment of the proton that is giving rise to a specific peak, the shift noticed points to a shift to a less polar environment that can be attributed to adsorption of the phospholipid aliphatic chain to more hydrophobic protein sites. On the other hand, the NMR contributions of the polar head group (in the 3.5 to 5.4 ppm region) are much less shifted by protein addition (table 7.2), which indicates that DMPG-whey protein interaction is primarily based on hydrophobic interaction. Both observations, based on line width and chemical shift analysis, suggest the idea that DMPG strongly interacts with whey protein by means of hydrophobic interactions.

To further confirm that the interaction between phospholipid and protein is due to hydrophobic interaction, saturation-transfer difference (STD) NMR spectroscopy measurements were performed (Mayer & Meyer, 1999). According to our knowledge our study is the first to use this technique to further unravel protein-lipid interaction. An STD measurement consists of two ¹H-NMR experiments: on and off-resonance. The on-resonance ¹H spectrum is acquired irradiating the receptor (in this case the protein) with a soft Gaussian shaped pulse at a frequency at least 700 Hz away from the closest ligand signal (DMPG). As the most shifted resonance visible in the DMPG-whey protein

spectrum, is centred at 5.35 ppm, irradiation at 8 ppm was considered to be a good choice. Irradiating at this value, only the (protein) receptor will be directly saturated. However, if the ligand binds to the receptor, the saturation will be transferred to it and then into solution (upon dissociation of the ligand). Hence, the resonances of those ligand protons that interact with the receptor show a decrease in intensity while the other proton resonances remain unvaried. In the off-resonance experiment, the irradiation frequency is set to a value far away from any other ligand or receptor signal (300 ppm). In this condition, the STD experiment results in a normal ^1H -spectrum similar to that shown in figure 7.4 (B).

The difference between off and on-resonance spectra yields an NMR spectrum where only the signals of the protons attenuated by the saturation are visible. The intensity of these signals in the difference spectrum response increases as a function of the irradiation (saturation) time. In our study the saturation time was varied from 0.01 up to 5 seconds. From figure 7.5, where the STD response, $I_{\text{STD}} = [(I_0 - I_{\text{sat}})/I_0]$, is plotted as a function of the saturation time, it is evident that the terminal methyl group as well as the long alkyl chain show a bigger STD response while the methyn proton closer to the head of the surfactant is more weakly affected by the saturation transfer and its STD response is largely reduced.

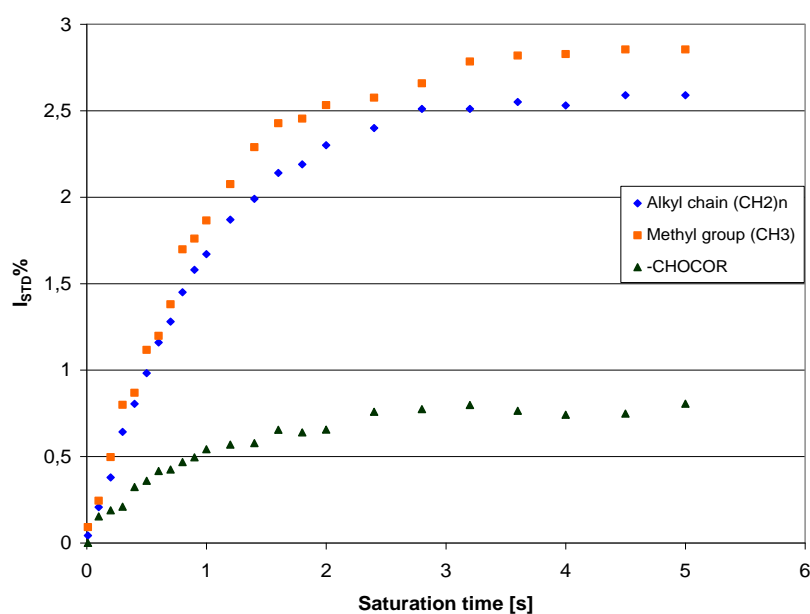


Figure 7.5. Saturation-transfer difference (STD) response of different DMPG protons upon mixing with WPI at a 1:2 ratio, as a function of the saturation time.

In addition, the alpha and beta methylene groups showed a very similar STD response as the terminal group, while the acetate did not show any response at all which indicates that it does not interact at all with the protein.

These observations further sustain the idea that the ligand interacts preferably with the receptor via the alkyl chain, which points towards an adsorption process driven by hydrophobic interactions and are hence not in agreement with Zhang and Keiderling (2006) who stated that hydrophobic interactions must have a minor role in β -lg DMPG interaction. Since the STD response is evaluated by integration, its reliability strictly depends on the signal to noise ratio. Moreover, in the vicinity of the water peak, proton resonances are affected by phase distortions and, hence, an accurate integration is not possible. For these reasons only the STD responses for the most intense peaks are shown in figure 7.5.

Finally, pulsed field gradient (pfg)-NMR measurements were used to quantify the surfactant interaction with WPI. Hereby, the observed diffusion coefficient of the surfactant in the presence of proteins (D_{obs}) is the weighted average of non-bound surfactant aggregates (with diffusion coefficient D_{ves}) and protein bound molecules with the same diffusion coefficient as the protein D_{pro} , the latter being not significantly influenced by the presence of the surfactant. In fact, the protein alone had a diffusion coefficient of $[0.57 \pm 0.01]10^{-10} \text{ m}^2/\text{s}$ while in the presence of DMPG a value of $[0.54 \pm 0.01]10^{-10} \text{ m}^2\text{s}^{-1}$ was found. These values were obtained following the echo decay of the protein proton signal centered at 3.02 ppm because no DMPG contributions were present in this region. These values clearly show that the protein did not interact with phospholipid vesicles, as claimed by Lefèvre and Subirade (2000, 2001) and Zhang and Keiderling (2006). Hence, the interaction mechanism between proteins and phospholipids clearly depends on the (relative) concentrations used: whereas Lefèvre and Subirade (2000, 2001) selected a β -lg to lipid ratio of 1:10 (w/w) with an overall concentration of 10 % (w/v), we used a whey protein to lipid ratio of 2:1 (w/w) and an overall concentration of maximum 1.5 % (w/v). Hence, in our contribution there is an interaction of phospholipid molecules with whey proteins, rather than an interaction of protein molecules with phospholipid vesicles as described by Lefèvre and Subirade (2000, 2001).

If the bound fraction is represented by p , the weighted average may be calculated according to equation (2) (Stilbs, Arvidson, & Lindblom, 1984):

$$D_{obs} = p \cdot D_{pro} + (1-p)D_{ves} \quad (2)$$

from which:

$$p = \frac{D_{obs} - D_{ves}}{D_{pro} - D_{ves}} \quad (3)$$

Hence, the protein bound fraction follows from experimental values of the diffusion coefficient of surfactants in the absence (D_{ves}) and presence (D_{obs}) of protein, as well as from the diffusion coefficient of the protein (D_{pro}). DMPG diffusion coefficients (free and observed) have been evaluated analyzing the echo decay of the resonances centered at 3.67 and 3.61 ppm. These two signals were chosen because they do not overlap with protein resonances and, hence, the exponential decay observed is only due to the phospholipid diffusion behavior.

The diffusion coefficients of phospholipid alone (D_{ves}), phospholipid in protein (D_{obs}) and protein (D_{pro}) were: $D_{ves} = (0.27 \pm 0.00) \cdot 10^{-10} \text{ m}^2/\text{s}$, $D_{obs} = (0.35 \pm 0.01) \cdot 10^{-10} \text{ m}^2/\text{s}$ and $D_{pro} = (0.57 \pm 0.01) \cdot 10^{-10} \text{ m}^2/\text{s}$, from which the bound fraction was calculated to be $(27 \pm 4) \%$. Based on the molar mass of DMPG and β -lactoglobulin, this bound fraction corresponds on average to 4.6 DMPG molecules per β -lactoglobulin monomer (assuming that all WPI protein would be β -lactoglobulin).

The ^1H -NMR spectrum for DPPG, an anionic phospholipid with longer fatty acyl chains as compared to DMPG, appeared to be broad at 25°C and therefore, no useful information could be obtained. The pronounced line width broadening is mainly due to the higher transition temperature of the surfactant (i.e. 41°C). As a result, DPPG is in a “frozen state” characterized by a low mobility and hence short T_2 relaxation times at 25°C.

To overcome this problem, additional measurements were carried out at 50°C (data not shown). At this temperature the more dynamic state of the surfactant is characterized by longer T_2 relaxation times, enabling to achieve sharper resonances in the ^1H spectrum. Also in this case, a considerable shift towards smaller chemical shift values is observed

for the long fatty acyl chain methylene groups (from 1.60 to 1.52 ppm) and for the terminal methyl group, whereas a much weaker effect was found for the hydrophilic head group protons. As explained before, a peak shift towards smaller values indicates a hydrophobic interaction between the anionic surfactant and the whey protein.

The diffusion coefficients (at 50°C) of free DPPG (D_{ves}), DPPG in the presence of protein (D_{obs}) and protein (D_{pro}) were $D_{ves} = (0.45 \pm 0.01) \cdot 10^{-10} \text{ m}^2/\text{s}$, $D_{obs} = (0.57 \pm 0.01) \cdot 10^{-10} \text{ m}^2/\text{s}$ and $D_{pro} = (1.03 \pm 0.01) \cdot 10^{-10} \text{ m}^2/\text{s}$, from which the bound fraction is calculated to be $(21 \pm 4) \%$. Based on the molar mass of DPPG and β -lg, this bound fraction corresponds to about 3.3 DPPG molecules per β -lg monomer (assuming that all WPI protein would be β -lg).

Figure 7.6 (B) shows the chemical structure of the zwitterionic phospholipid DPPC as well as its ^1H spectrum at 50°C, i.e. above its phase transition temperature. The latter was also recorded for DMPC at 25°C (data not shown).

The resonances for DPPC and DMPC in these NMR spectra are slightly broader as compared to DMPG and DPPG. The line broadening in these two cases can be ascribed to the bigger size of the liposomes formed by the zwitterionic surfactants. For the anionic phospholipids (DMPG and DPPG), the vesicle size is limited by electrostatic repulsion between the charged molecules and hence between the lamellar bilayers, leading to the formation of smaller, less multilamellar aggregates. The zwitterionic phospholipids, on the other hand, are electrically neutral and the multilamellar liposomes formed result in a bigger size, characterized, as consequence, by shorter T_2 relaxation times and hence broader signals. The NMR spectrum of DPPC in the presence of whey protein is also reported. The latter, contrary to what was observed for DMPG and DPPG, does not show any significant variation in chemical shift nor line broadening effect.

Moreover, the DPPC diffusion coefficient, estimated following the echo decay of the methyl protons of the quaternary ammonium group (around 3.5 ppm), is similar both in the presence and absence of protein. From these observations, it can be concluded that no interactions between the dialkyl zwitterionic phospholipid and the whey protein can be detected by NMR. As molecular interaction requires that individual phospholipid molecules travel through the aqueous phase, the lack of interaction is a logical consequence of the extremely slow spontaneous lipid exchange between neutrally

charged membranes with time scales of days, as reported by Pownall, Bick and Massey (1991).

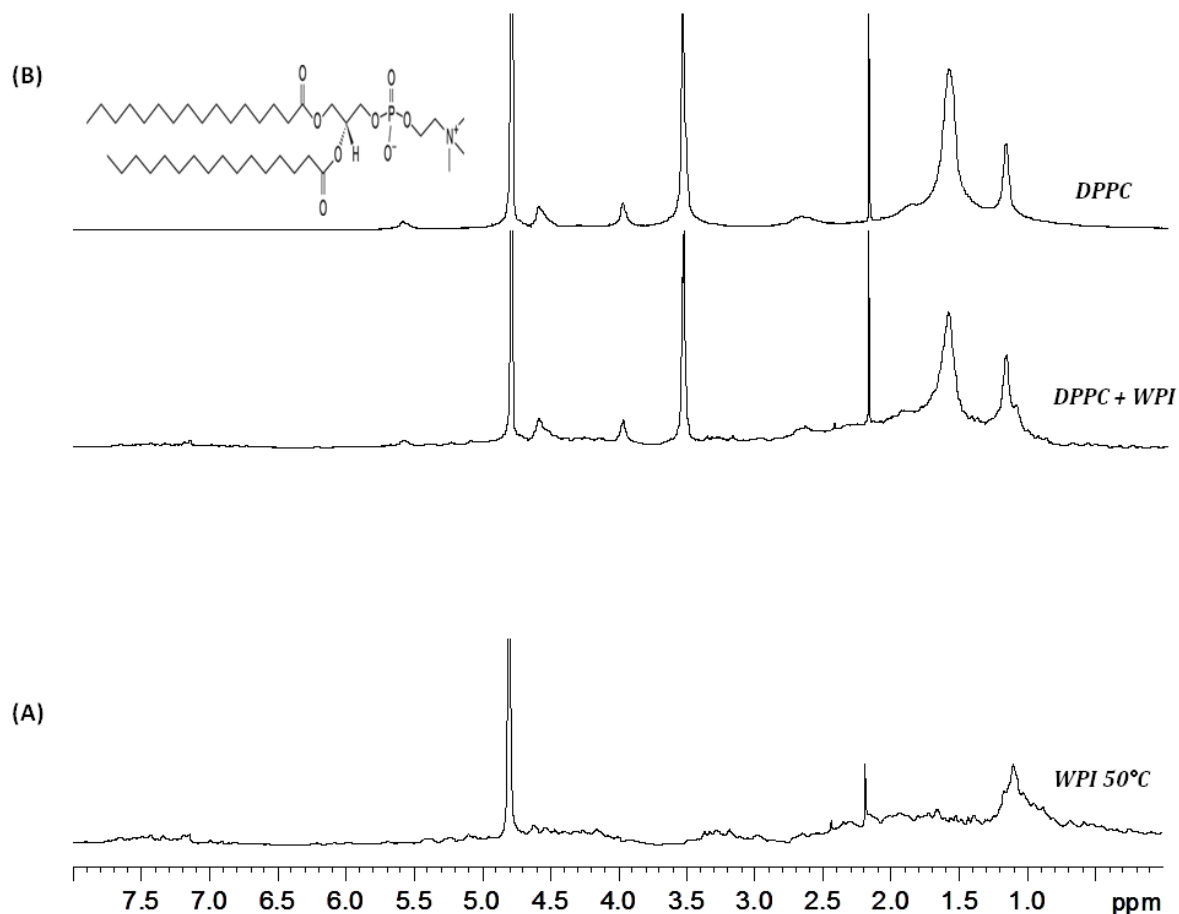


Figure 7.6. ¹H-NMR spectra for WPI at 50°C (A), as well as free DPPC and a mixture of DPPC and WPI at 50°C (B).

Charged phospholipids above their phase transition temperature, on the other hand, have a much faster intermembrane exchange, as shown by De Cuyper, Joniau and Dangreau (1983).

Finally the chemical structure of the zwitterionic mono-alkyl C14-LPC and its NMR spectra obtained at 25°C are presented in figure 7.4 (C). The surfactant concentration used (i.e. 5 mg/ml) corresponded to 10.7 mM which is much higher than its CMC. The peak at 0.90 ppm is related to the last methyl group of the alkyl chain, whereas the peak at 1.32 ppm belongs to the long fatty acyl chain of the surfactant. The less pronounced peaks at 1.63 ppm and 2.42 ppm belong to the methylene groups in β and in α position to the carboxyl group, respectively. In addition, the peak at 3.27 ppm corresponds to the

three methyl groups bound to the nitrogen, whereas the one at 3.71 ppm comes from the methylene group which is directly bound to the nitrogen atom. The quintet around 3.90 ppm is due to the proton which is present on the chiral carbon. The other 4 resonances in the range 3.96-4.21 ppm are due to diastereotopic protons directly bound to the chiral centre. Finally, the peak at 4.34 ppm belongs to the methylene group located to the side of the polar head and directly bound to the phosphate group. In table 7.3, the chemical shift values for both free C14-LPC and C14-LPC in the presence of WPI are reported.

Table 7.3. Chemical shift values (ppm) of myristoyl LPC protons in the absence and presence of WPI. Measurements were performed at 25°C.

	α	β	$(\text{CH}_2)_n$	CH_3	1	2	$\text{N}^+(\text{CH}_3)_3$
Free C14-LPC	2.42	1.63	1.32	0.90	3.71	4.34	3.27
C14-LPC + WPI	2.33	-	1.22	-	3.68	4.34	3.24

From this table, it is obvious that the shift differences between free C14-LPC and C14-LPC in the presence of WPI are very small or nonexistent for all protons belonging to the polar head group of the lysophospholipid. On the other hand, a considerable upfield shift is observed for both the resonances of the methylene groups in β and α position to the carboxyl group as well as the long acyl chain resonance. Since only the aliphatic part of the surfactant feels a change to a less polar environment, it may be concluded that hydrophobic interactions between whey protein and lysophospholipids take place.

Hence, whereas no adsorption could be detected of dialkyl PC, its monoalkyl equivalent clearly interacted with the whey protein. According to our opinion, the difference may be explained from the difference in the critical micelle concentration (CMC) or critical association concentration (CAC). Hence, the concentration of monomeric molecules is much larger for the mono- as compared to the dialkyl PC. As the CD spectrum of the WPI did not change upon LPC addition (table 7.1), it follows that the lack of conformational rearrangements in the presence of zwitterionic phospholipids cannot be directly linked to a lack of interaction. Combining the CD and NMR data for zwitterionic lysophospholipid and the anionic phospholipids, it becomes obvious that it is not the interaction between globular proteins and phospholipids that is controlled by

electrostatics, but rather the resulting structural rearrangement: only upon interaction with anionic phospholipids a significant transition from β -sheet to α -helix occurs.

Diffusion experiments performed on lysophospholipid samples showed that the surfactant has a similar diffusion coefficient both in the presence and absence of WPI and these are similar to the diffusion coefficient of the protein alone [$(D_{mic}=0.54\pm 0.01)$, $(D_{obs}=0.46\pm 0.01)$, $(D_{pro}=0.57\pm 0.01)$]. 10^{-10} m²/s]. Since the diffusion coefficients are related to the hydrodynamic radius of the diffusing species, by means of the Stokes-Einstein relation, it follows that the dimensions of the diffusing units of proteins and surfactants must be of the same order of magnitude, which follows logically from the fact that the surfactant's CMC is largely surpassed. Indeed, micelles at low concentration are spherical aggregates whose radius is of the order of magnitude of some nm, which is quite similar to the dimensions of globular proteins. For this reason, it is not possible to evaluate the fraction of lysophospholipid bound to the protein from diffusion data.

7.4. Conclusions

Circular dichroism has an important role in providing structural information about protein unfolding. Far-UV spectra of whey protein isolate confirmed that its secondary structure undergoes denaturation upon heating at high temperatures, whereas at 45°C, no particular changes were observed. These changes are accompanied by an increase of the α -helical content at the expense of β -sheet structure. As it is known that not only an increased temperature but also the addition of surfactants may result in unfolding of β -lactoglobulin, the effect of anionic (DMPG, DPPG, DMPA, and DPPA) as well as zwitterionic phospholipids (DMPC, DPPC and C14-LPC) was examined.

As DMPG already showed a significant unfolding effect at room temperature, whereas DMPA, DPPA and DPPG only revealed a downward shift of the ellipticity at 45°C, it was concluded that whey protein-phospholipid interaction was highly favored at temperatures above the phospholipid's phase transition temperature T_m . On the other hand, the zwitterionic DMPC, DPPC and C14-LPC did not induce any significant whey protein conformational changes at both room temperature and 45°C, which indicates that zwitterionic phospholipids either do not interact with whey proteins or at least do not induce structural modifications upon sorption. Changes in ellipticity were only noticed after incubation at 90°C which, however, was due to thermal unfolding of

protein. The above observations indicate that the polar head group of phospholipids largely affects its interaction with globular proteins.

The interaction between the above mentioned phospholipids and whey proteins was also studied into more detail by high resolution NMR measurements. As the fatty acyl chain resonances in anionic phospholipids (DMPG and DPPG) as well as in myristoyl LPC, shifted to a less polar environment upon addition of whey protein, whereas the hydrophilic parts were hardly affected, it was concluded that the protein-phospholipid interaction occurred mainly by hydrophobic interaction. This hypothesis was further sustained by STD-NMR experiments. Comparison of the CD and NMR data obtained in the presence of myristoyl LPC clearly indicated that the lack of protein structural reorganization could not be ascribed to a lack of interaction with this monoalkyl zwitterionic phospholipid. On the other hand, the NMR spectra of zwitterionic dialkyl phospholipids were not affected by whey protein addition, which indicates that these phospholipids prefer interaction with other phospholipids rather than with whey proteins. In addition, diffusion NMR clearly showed that in the experimental conditions used, phospholipid molecules could interact with proteins, whereas protein binding to phospholipid vesicles did not occur. From the largely different effect of different phospholipids, it follows that the functional properties of phospholipid mixtures as present in lecithins, strongly depend on their phospholipid composition, which opens interesting perspectives for optimization.

Chapter 8

CONCLUSIONS AND FURTHER PERSPECTIVES

8.1. Conclusions

Heat treatment probably constitutes the most important step during processing of dairy products, aiming to the manufacture of a microbiologically stable product which is safe for the consumer but it is also characterized by an extended shelf life. However, due to its complexity as a colloidal dispersion, when (concentrated) milk is subjected to elevated temperatures, numerous physicochemical modifications take place, which can sometimes result into the rejection of the final product by the consumer. The increased viscosity of the high temperature-treated dairy products is a consequence of a phenomenon known as heat coagulation which is the result of multiple processes, including heat-induced interactions between whey proteins and casein micelles. During the past years, several studies have been carried out trying to find solutions to overcome this complicated occurrence, whereby some researchers demonstrated that the incorporation of surface active molecules can reduce the heat-induced increase in viscosity.

The aim of this dissertation, which was divided in three main parts, was initially to explain the parameters that induce the initiation of heat coagulation in whey protein containing emulsions and solutions, and furthermore to examine the effect of the incorporation of polar lipids from various sources, prior to heating at high temperatures.

For that reason, two different model systems were used. In most of the experimental studies, a RCM model containing a total amount of 23 % of solids was examined, from which 16.5 % was high-heat SMP and 6.5 % sunflower oil. The reason of utilizing high-heat SMP was mainly to imitate industrial practice. In addition, and based on the fact that heat-induced coagulation is largely affected by the whey protein fraction of milk, whey protein isolate solutions were investigated during treatment at high temperatures and in the presence of different kinds of purified phospholipids.

In the first experimental part, a method was developed for the heat stability evaluation of small-volume RCM emulsions. The method was ideally suited to test highly purified ingredient components. During this development, several parameters were tested, such as temperature, heating time, as well as positioning and cooling of samples. In order to choose the temperature at which the experiments for aggregation tendency evaluation would be performed, RCM emulsions were treated at three different heating temperatures: 116, 121 and 126°C, for 20 min. Upon heating at 116°C, the viscosity

remained almost unchanged: samples maintained their liquid structure, due to the absence of pronounced heat coagulation. On the other hand, upon heating at 126°C, samples were characterized by a solid or gelled structure and this was attributed to pronounced aggregates formation. Finally, upon heat treatment at 121°C, aggregates formation was also noticed but only at moderate levels. The reduced tendency of heat-induced coagulation of the samples, allowed their easy handling regarding the performance of experiments such as particle size and viscosity analyses and, consequently, 121°C was chosen as the temperature at which the samples were treated in all further experiments. In a subsequent step of this experimental part, when the effect of sample positioning inside the oil bath, as well as the effect of the cooling medium (air versus tap water) was tested, no significant changes were noticed related to the tendency of aggregation of RCM emulsions. The results from the time-temperature profiles showed that in the experimental set-up which was used, a period of approximately 9 min was required for the samples to reach the target temperature (i.e. 121°C).

In the second experimental part, the effect of phospholipid containing ingredients on the heat stability of RCM samples as well as WPI solutions was investigated with the purpose to examine to which extent polar lipids, such as phospholipids can influence the coagulation tendency.

Cream residue powder (CRP), originating from the production of butter oil from dairy cream, as well as sweet buttermilk powder (SBP) have been utilized. The addition of both CRP and SBP in concentrations up to 6 %, demonstrated a heat-stabilizing effect by largely preventing the heat-induced increase in both viscosity and particle size in RCM emulsions. The effect of both dairy by-products was proportional to the added concentration. As the observed differences in viscosity and particle size distribution could be related to the different phospholipid content of the two ingredients, their heat-stabilizing effect was thought to be due to the beneficial effect of the phospholipids originating from the milk fat globule membrane (MFGM) that they contained. However, the possible beneficial effect of the membrane proteins cannot be excluded. Finally, the addition of MFGM-enriched dairy products gradually reduced the heat-induced increase in protein surface load, which points toward the decreased occurrence of further heat-induced protein-protein interactions and/or further protein aggregation which would

lead to increased coagulation and concomitant viscosity increase. Therefore, MFGM-enriched dairy by-products represent very promising ingredients which can be used in cases where recombined milk emulsions are subjected to intense heating, as they allow heat stabilization without exogenous ingredients, such as soybean lecithins or alternative surfactants.

Furthermore, the effect of addition of different types of sunflower lecithin, a natural source of phospholipids which is obtained as a by-product from the degumming process of crude sunflower seed oils, on the heat stability of RCM emulsions was investigated. The effect of lecithin was tested before and after its enzymatic hydrolysis by Lecitase® Ultra, a protein-engineered phospholipase A1, for various time intervals. Heat coagulation experiments revealed that both intact and hydrolyzed sunflower lecithin resulted in reduced coagulation tendency, which may be ascribed to the complexation between denatured whey proteins and/or protein aggregates with phospholipids. Firstly, the incorporation of intact sunflower lecithin in RCM emulsions upon heating at 121°C up to 25 min, demonstrated a remarkable decrease in coagulation tendency with the effect to be directly proportional to the added concentration. Particle size measurements revealed that the addition of 0.3 % of lecithin resulted in emulsions with similar size characteristics upon heating as the emulsions which remained unheated. Upon increasing the heating time to 35 min and comparing intact and hydrolyzed lecithin, the latter seemed more effective in improving the heat stability of RCM emulsions and whey protein isolate (WPI) solutions. In addition, enzymatic hydrolysis of intact sunflower lecithin for different time intervals showed that the degree of hydrolysis can play a decisive role on the heat stability of whey protein containing emulsions and solutions: an optimum hydrolysis time of 20 min was found in the set-up used, above which the beneficial effect of hydrolyzed lecithin addition was reduced. This was very well observed by performing both viscosity and particle size measurements of RCM emulsions, whereby an enhanced coagulation tendency was noticed in samples which contained lecithin hydrolyzed for 30, 40 or 60 min. Similar results were obtained when the residual soluble protein content of WPI solutions was checked, in the presence of sunflower lecithin which was enzymatically treated for different time intervals: whereas the protein solubility in WPI solutions with lecithin hydrolyzed for 20 min almost reached 100 %, upon further hydrolysis, lecithin's beneficial effect was lost.

In the third experimental part of this dissertation, an in depth analysis was performed related to the effect of various kinds of phospholipids which are characterized by different molecular structure and chemical composition, on the heat stability of whey protein containing emulsions and solutions.

Firstly, dialkyl- and lysophospholipids distinguished by different alkyl chain length and polarity of the head group were incorporated in different concentrations in RCM emulsions, which were heated for 20 min at 121°C. Viscosity, particle size distribution and protein recovery measurements revealed that the addition of lysophosphatidylcholine (LPC) can increase the heat stability of milk emulsions to a great extent and the effect is proportional to the concentration of LPC added for all three LPC species studied. However, the more pronounced stabilizing effect of the C16-LPC and especially C18-LPC was evident, whereas in the case of C14-LPC addition the effect was less significant. This could be attributed to the increased hydrophobicity and hence surface activity of the longest LPC (i.e. stearyl). A higher extent of interaction with the hydrophobic residues of the denatured whey proteins and/or protein aggregates upon heating can be speculated, hence preventing further aggregation and providing better heat stability, as compared to the shorter analogues, which are more hydrophilic as can be deduced from their larger CMC values.

In a next step, the effect of two anionic dialkyl-phospholipids (DMPG and DPPG) as well as of two zwitterionic dialkyl-phospholipids (DMPC and DPPC) was tested. Similarly, when the longer alkyl chain phospholipids DPPG and DPPC were used, even at relatively low concentrations, a significant decrease in the aggregation tendency was observed, whereas the shorter DMPC and DMPG resulted in a less significant decrease of aggregation. Upon addition of 0.1 % of all dialkyl phospholipids and especially DPPC and DPPG, the aggregation tendency was minimized, resulting in RCM with a liquid structure. However, in the case of the two anionic (PG) phospholipids, the beneficial effect was lost upon further increase of the added concentration, giving rise to a significant increase of both viscosity and particle size, a fact which confirms the importance of the phospholipid polar head group on the heat coagulation of RCM emulsions.

Overall, and taking into account that high-heat SMP, which was the source of milk solids non-fat in all previous experiments with RCM emulsions, contains denatured whey

proteins and whey protein aggregates, we can speculate that these aggregates will constitute the structural basis of further aggregation and gelation when the system is subjected to intense heating at elevated temperatures. Therefore, based on the heat-stabilizing effect of phospholipids which were incorporated into the RCM emulsions prior to heating, we can hypothesize that phospholipids form complexes with the already denatured whey proteins, or with the protein aggregates, preventing or limiting the continuation of aggregation and hence the development of heat coagulated emulsions. However, the effect of phospholipids was proved to be strongly affected by their molecular structure, as determined by their polar head group, alkyl chain length and number of alkyl chains. From the results obtained in chapters 4, 5 and 6, we can conclude that phospholipids with longer alkyl chain length are characterized by a stronger heat-stabilizing effect as compared to their shorter analogues. This can be attributed to the increased hydrophobicity and therefore the higher tendency to interact with the denatured whey proteins and/or the protein aggregates. In addition, the polar group of the incorporated phospholipids, seems to play a decisive role on the extent of heat coagulation. Whereas the zwitterionic lyso- and dialkylphospholipids had a heat-stabilizing effect which was proportional to the added concentration, the anionic phospholipids were characterized by an optimum concentration above which the beneficial effect was lost. This particular behavior could be explained based on the fact that upon utilization of anionic phospholipids at low concentrations, specific binding takes place. During this stage, electrostatic interactions with specific binding sites of the protein occur which lead to an overall increase of the net negative charge of the protein, which prevents coagulation phenomena due to the increased repulsive forces. Furthermore, hydrophobic interactions between the chains of the surfactant and the hydrophobic residues of the whey proteins take place, preventing further complexation of whey proteins aggregates. Upon higher concentrations, all binding sites may become saturated, whereby the protein becomes completely unfolded and further aggregation may take place by hydrophobic interaction. Finally, the effect of the number of phospholipids alkyl chains was a subject of investigation. Whereas in chapter 4, the effect of hydrolyzed phospholipids seemed to be much more effective by increasing the heat stability of RCM emulsions, in chapter 6, no significant differences were noticed when LPC and dialkyl phospholipids were utilized. Based on these results, we can speculate that the better heat-stabilizing effect of hydrolyzed phospholipids in chapter 4

can be attributed to the fatty acids produced, which is in line with the heat-stabilizing effect of sodium laurate or sodium dodecyl sulphate as described by Tran Le et al., (2011b). Whereas protein displacement by small-molecular weight surfactants (such as phospholipids) has been described in literature, it is worth mentioning that there was no indication of protein displacement from the interface was found in the experimental conditions used, where the protein concentrations were much higher compared to the phospholipids concentrations.

Based on the fact that the whey protein fraction is the heat sensitive fraction of dairy protein, the effect of phospholipid molecular structure on its interaction with whey proteins in aqueous solutions was investigated. For these experiments, a β -lg enriched native WPI was selected so that both the effect on denaturation as well as aggregation and gelation could be observed.

CD experiments were performed to assess the extent of secondary structural changes of β -lg upon incubation in the presence of anionic (DMPG, DPPG, DMPA, and DPPA) and zwitterionic (DMPC, DPPC and C14-LPC) phospholipids, whereas high-resolution H-NMR has been utilized to investigate the whey protein-phospholipid interaction into more detail. Upon DMPG addition, a significant unfolding effect at room temperature was noticed, whereas DMPA, DPPA and DPPG only revealed a downward shift of the ellipticity at 45°C. Therefore, it was concluded that whey protein-phospholipid interaction was highly favored at temperatures above the phospholipid's phase transition temperature T_m . On the other hand, the zwitterionic DMPC, DPPC and C14-LPC did not induce any significant whey protein conformational changes at both room temperature and 45°C, which indicates that zwitterionic phospholipids either do not interact with whey proteins or at least do not induce structural modifications upon sorption. Changes in ellipticity were only noticed after incubation at 90°C which, however, was due to thermal unfolding of protein. These observations indicate that the polar head group of phospholipids largely affects its interaction with globular proteins.

NMR measurements revealed that the fatty acyl chain resonances in anionic phospholipids (DMPG and DPPG) as well as in myristoyl LPC shifted to a less polar environment upon addition of whey protein, whereas the hydrophilic parts were hardly affected. Consequently, it was concluded that the protein-phospholipid interaction

occurred mainly by hydrophobic interaction. This hypothesis was further sustained by STD-NMR experiments. Comparison of the CD and NMR data obtained in the presence of myristoyl LPC clearly indicated that the lack of protein structural reorganization could not be ascribed to a lack of interaction with this monoalkyl zwitterionic phospholipid. On the other hand, the NMR spectra of zwitterionic dialkyl phospholipids were not affected by whey protein addition, which indicates that these phospholipids prefer interaction with other phospholipids rather than with whey proteins. In addition, diffusion NMR clearly showed that in the experimental conditions used, phospholipid molecules could interact with proteins, whereas protein binding to phospholipid vesicles did not occur.

The results of the last experimental part showed that the interaction between phospholipids and whey proteins does not require whey protein denaturation, whereas a temperature above the phospholipid T_m is needed to have enough flexibility. Whereas for anionic phospholipids, this interaction resulted into structural changes, zwitterionic phospholipids did not introduce any structural protein rearrangement.

Overall, and based on our results, the conclusions and the possible mechanisms based on the results of this dissertation can be summarized as followed:

- a. Phospholipids interact with whey proteins:
 - Both with native (i.e. below denaturation temperature) and denatured (i.e. preheated).
 - Anionics induce denaturation
 - Zwitterionics do not affect the protein's secondary structure
- b. Phospholipids prevent (further) whey protein aggregation/gelation and also minimize additional protein deposition at the oil/water interface.
- c. Excessive concentrations of anionic phospholipids have decreased heat stability: apparently the enhanced unfolding makes the proteins more sensitive towards further aggregation.

Thermal unfolding (upon heating native proteins or soluble already denatured proteins), exposes reactive groups such as thiol groups and/or hydrophobic patches which favor association reactions by either thiol-disulphide interaction or hydrophobic attraction. Surface active molecules, such as mono- and dialkylphospholipids can cover

these (especially hydrophobic) reactive sites and hence prevent or minimize aggregation. However, anionic surfactants promote unfolding and hence can also favor aggregation (at higher concentration). Hereby, it has to be kept in mind that heat treatment has multiple effects and can also induce casein dissociation from casein micelles as well as calcium ion release from the colloidal calcium phosphate present. The latter is highly determined by pH. Hence, (prevention of) heat coagulation is known to be strongly pH dependent. Considering this aspect, it is important to mention that all experiments on RCM were performed at the natural pH (i.e. 6.5), whereby the results obtained cannot be directly extrapolated to other pH conditions.

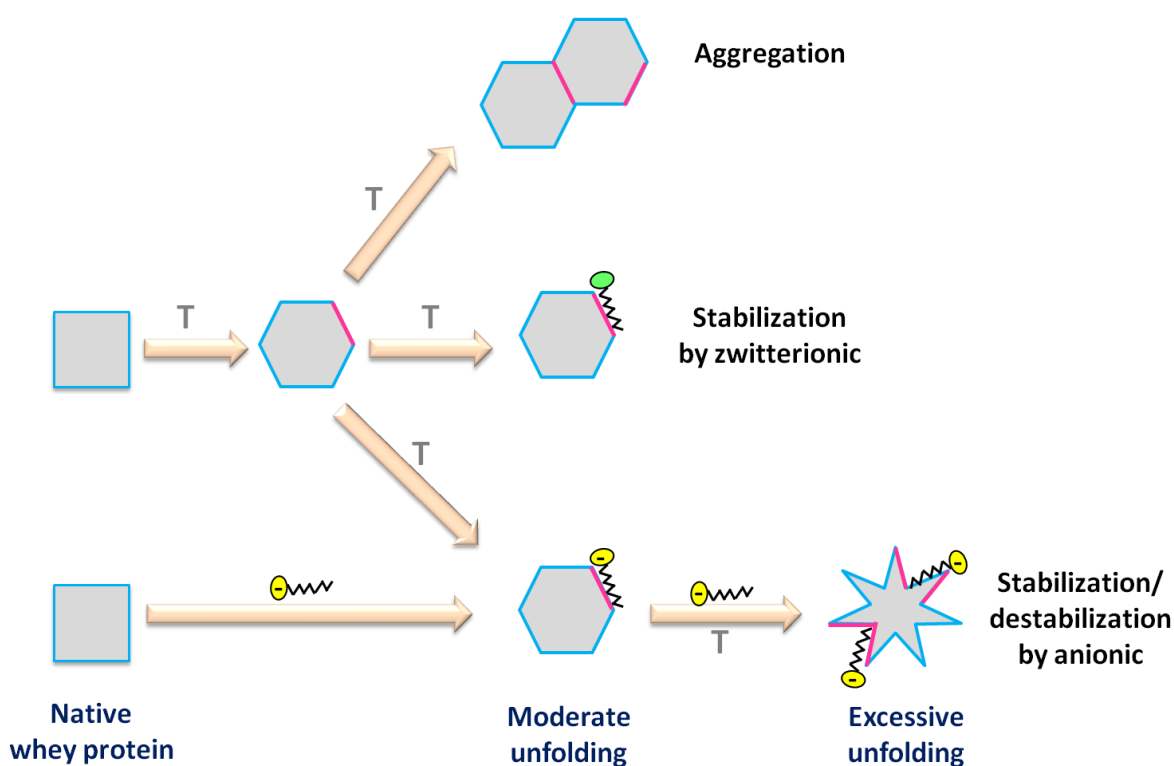


Figure 8.1. Schematic representation of the possible mechanisms, based on the results of this thesis, focusing on the whey protein fraction and the added phospholipids. The pink lines represent the reactive groups of the whey proteins.

From the largely different effect of different phospholipids, it follows that the functional properties of phospholipid mixtures as present in lecithins, strongly depend on their phospholipid composition, which opens interesting perspectives for optimization.

8.2. Further perspectives

This thesis analyzed into depth the main parameters which influence the heat stability of whey protein emulsions, including the heating temperature and the heating time, the homogenization pressure as well as the protein and oil content. In addition, with the aim to produce a product which is desirable by the consumer, phospholipid-containing ingredients such as sunflower lecithin or dairy by-products were utilized resulting in the manufacture of recombined concentrated milk emulsions with decreased coagulation, after their treatment at elevated temperatures. In a further step, different types of purified phospholipids were added directly into recombined concentrated milk samples or they were mixed with whey protein isolate solutions, not only to investigate which kinds of phospholipids are more efficient in increasing the heat stability of milk emulsions, but also to clarify the exact mechanisms which lead to this contribution.

Further research would be useful in order to collect some more information to build a more complete picture regarding the heat-induced coagulation of concentrated milk and the effect of phospholipids against its stimulation. In our research, we reached to the conclusion that heating at elevated temperatures and for prolonged time causes heat coagulation of recombined concentrated milk, whereas in the presence of surface active molecules, heat coagulation is inhibited or sometimes prevented. It would be very interesting to be able to assess the exact time at which distinct (rheological) changes take place in both the absence and presence of phospholipids or phospholipid containing ingredients. Hereby, the development of a direct, but objective technique to evaluate the HCT would surely provide an added value. Traditionally, the HCT is determined by visual inspection upon controlled heating. The application of more recent measurement techniques, such as diffusing wave spectroscopy (DWS), could enable the in-situ and on-line quantification of heat coagulation.

The beneficial effect of phospholipid enriched dairy by-products against heat coagulation is described in chapter 4, which was attributed to the increased presence of MFGM, material rich in phospholipids and membrane proteins that migrates to the buttermilk during butter production. A very interesting approach would be the isolation and purification of MFGM, for example by microfiltration (Morin et al., 2007), and its utilization as ingredient with heat-stabilizing properties. The purified MFGM material can possibly be used in smaller concentrations compared to the ones which were used

for SBP and CRP. In addition, the final product would acquire an attribute which is more appealing towards the consumer since no exogenous ingredients are used, but also due to the fact that the MFGM is acknowledged as a food component with health benefits.

The results of chapter 5 already demonstrated that enzymatic hydrolysis of sunflower lecithin, using phospholipase A1, prior to its supplementation in recombined concentrated milk emulsions had remarkable heat-stabilizing properties. It seems worthwhile to evaluate whether phospholipase treatment of phospholipid enriched dairy by-products or even of skim milk powder as such, can have a beneficial effect against heat coagulation. In addition, an interesting future approach would be to check the effect of the fatty acids which are liberated upon the enzymatic hydrolysis of sunflower lecithin.

The results of chapter 6 revealed that anionic phospholipids are characterized by an optimal concentration above which their beneficial effect towards heat-induced coagulation is weakened. However, with our experiments, we could not identify the exact concentration during which the behavior of anionic phospholipids is reversed, and this can constitute a very interesting study for the near future.

In this dissertation, heat coagulation is described as a phenomenon which takes place due to whey protein denaturation and subsequent interaction with other denatured whey proteins and/or with caseins. These interactions occur as a result of the accessible hydrophobic residues and the thiol group which were previously enclosed in the interior of whey protein globular structure. The addition of phospholipids showed that heat coagulation can be controlled by the hydrophobic interactions which take place between the hydrophobic sites of the unfolded whey proteins and the added phospholipids (anionic), hence hindering the hydrophobic binding of denatured whey proteins with themselves. However, it would be very interesting to understand to what extent heat coagulation depends on the disulfide bonding between the denatured whey proteins and κ -caseins. For that purpose, it would be interesting to investigate to what extent derivatisation of the free thiol group of the whey proteins, which might be realized by reagents, such as Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), inhibits heat coagulation by avoiding the complex formation with κ -caseins.

The focal point of this research is based on the contribution of polar lipids on the heat induced changes in concentrated milk systems, by mainly highlighting the modifications which take place on the whey protein fraction, in the presence or not of different kinds of phospholipids or phospholipid containing ingredients. This is due to the fact that whey proteins constitute the protein fraction that has been extensively studied due to its sensitivity at high temperatures. For that reason, in our study, this was the aspect which was considered that it largely affects the intensity of heat coagulation. However, we cannot exclude the fact that casein micelles, which constitute an essential milk ingredient, can also contribute to the instability of milk upon heat treatment (de Kort et al., 2012). For that reason, some future experiments focusing on the heat coagulation of casein micelles containing solutions would be very interesting and would give a broader and further complete picture related to the heat stability of milk. Micellar casein isolate solutions can be prepared and treated at high temperature and experiments including protein solubility and rheology, before and after heating, would give an insight regarding the degree of heat coagulation of casein micelles. In addition, solutions containing different ratios of whey protein isolate and micellar casein isolate can also provide some further information related to the heat-induced modifications which take place in solutions which contain both milk proteins. Finally, some tests (e.g. NMR) involving micellar casein isolate solutions in the presence of phospholipids, will provide very useful details regarding potential interactions which may take place between them during heating.

Chapter 9

SUMMARY

Summary

During the last decades, several researchers tried to clarify and fully explain the exact mechanism of the heat-induced coagulation which occurs during heating milk at elevated temperatures. Based on literature data, this phenomenon is mainly a consequence of heat denaturation and unfolding of the globular whey protein fraction of milk, which results in the exposure of the previously buried hydrophobic and free thiol groups. As the denaturation process proceeds, several interactions start to take place leading to the formation of a product with modified organoleptic and physicochemical characteristics. The most important interactions which are believed to take place during heating milk at high temperatures are the interactions between denatured whey proteins with themselves and/or the complex formation of denatured whey proteins (or whey protein aggregates) with casein micelles. The heat-induced network between milk proteins is essentially built due to the hydrophobic bonding between the denatured whey proteins, as well as the thiol-disulfide exchange between the denatured whey proteins with themselves and/or with the κ -caseins.

In order to be able to apply high temperatures and at the same time to decrease the degree of protein complexation, the application of surface active molecules (surfactants) has been proposed. Previous studies described that the effect of surfactants on the heat stability of whey protein containing emulsions is based on the protein displacement or interaction with proteins bound on the oil interface, and/or interaction with free proteins in solution. In the first chapter, the literature related to the importance of the heat treatment in dairy industry is described, along with the consequences that this process exerts to the final product. Attention is mainly paid to the modifications which occur during milk processing, including high temperatures, pH alterations, homogenization pressure and milk composition, whereas a particular interest is given to the nature of the heat-induced interactions which arise when heating is performed at temperatures higher than 60°C. In addition, this chapter provides general information for the mechanisms and capacity of the surface active molecules as heat stabilizers of milk emulsions.

The experimental part of this doctoral dissertation covers three main parts. The objective of the first part was to give an overview regarding the main factors which can drastically affect the tendency of heat coagulation of RCM emulsions.

The first and most important factor which was examined included the temperature, as well as the period during which the emulsions were treated. A temperature-controlled oil bath was used, and the temperature in the interior of the samples was monitored in order to be aware of the exact time which was needed for the samples to acquire the desired temperature (i.e. 121°C). In addition, the positioning of the samples in the oil bath and the cooling medium (air versus water), were some of the parameters which were tested. The information obtained from this chapter was used to develop and optimize a heating method which allowed us to investigate in depth the degree of heat coagulation of RCM emulsions. In a next step, RCM were heated within a temperature range of 80-121°C and for 10-120 min. The extent of heat coagulation was tested as a function of the protein content and the pressure during microfluidisation. The results revealed a gradual increase of heat coagulation in emulsions containing a higher content of protein and homogenized at the highest pressure (560 bar), whereas lower pressures were proven to be more effective by means of heat stability.

The second part of this project, which dealt with the utilization of surface active molecules for improving the heat stability of milk emulsions, consisted of two chapters. Chapter 4 described the effect of two phospholipid enriched dairy by-products on the heat stability of RCM: CRP originating from the production of butter oil from dairy cream and SBP. The results from chapter 4 showed that the addition of either SBP or CRP exercised a heat-stabilizing effect, largely preventing the heat-induced increase in both viscosity and particle size, showing that these by-products are very promising ingredients in case of milk emulsions that are subjected to severe heating, since they allow heat stabilization without utilization of exogenous ingredients, such as soybean lecithins or alternative surfactants. Chapter 5 analyzed the heat-stabilizing effect of sunflower lecithin, a byproduct from the degumming process of crude sunflower seed oils. The main justification for sunflower lecithin utilization emerges from its classification as a non-GMO product and it can therefore be considered as an attractive replacement for soybean lecithin.

The results showed that lecithin addition in RCM emulsions demonstrated a significant decrease in coagulation tendency with the effect being directly proportional to the added concentration. Moreover, sunflower lecithin was enzymatically hydrolyzed, using a phospholipase A1, aiming to the production of lysophospholipids. The experimental

data proved that the degree of enzymatic hydrolysis can play a decisive role on the heat stability of whey protein containing emulsions and solutions: an optimum hydrolysis time of 20 min was observed in our experimental set-up, above which the beneficial heat-stabilizing effect of hydrolyzed lecithin addition was reduced.

In the third and final part of this research, the effect of the phospholipids molecular structure and chemistry on the heat stability of whey protein containing emulsions and solutions was analytically examined. The aim of this part was to investigate whether there are any interactions which take place between phospholipids and whey proteins that may prevent the emergence of heat-induced coagulation phenomena. Furthermore, we wanted to investigate whether the structure of the incorporated phospholipid plays a decisive role on the heat stability. For that reason, in chapter 6, the effect of different kinds of purified phospholipids was examined, after their incorporation prior to homogenization and at different concentrations in RCM emulsions. The effect of heating was evaluated by analyzing the particle size and the rheological properties of RCM before and after heating at 121°C, as well as by determining the residual amount of protein in the continuous phase. The results revealed that the addition of either lyso- or dialkylphospholipids can increase the heat stability of milk emulsions to a great extent. In addition, this study proved that the phospholipid's polar and hydrophobic characteristics play a decisive role on the tendency of heat coagulation. Chapter 7 provided an insight into the association mechanisms which take place between whey proteins and various phospholipids. Whereas Circular Dichroism was used to reveal information related to the secondary structure of whey protein, high-resolution diffusion- and STD-NMR measurements were applied to study the interaction mechanism between the protein and the phospholipids in more depth.

The phospholipids which were used were characterized by different polarity, as well as by different length of the hydrophobic tail, and hence phase transition temperature (T_m), whereas protein-phospholipid mixtures were incubated both above and below the phospholipid T_m . The results showed that anionic phospholipids can promote whey protein unfolding at temperatures above their T_m but also at room temperature, whereas the zwitterionic polar lipids did not induce any significant whey protein conformational changes. The combination of techniques indicated that the absence of protein structural

rearrangements was not due to the absence of interactions, but was rather due to the fact that the interactions did not have any effect on the protein's structure.

Finally in chapter 8, conclusions from the results attained throughout the whole doctoral thesis were stated. Along with these, suggestions for future work were given based on the experimental data as well as information in the literature.

Chapter 10

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Curriculum Vitae

Personal information

Full name: Kasinos Marios

E-mail: m_kasinos@hotmail.com

Nationality: Cypriot

Date of Birth: August 30, 1981

Gender: Male

Education

September 2009 - October 2014: Doctoral Researcher-Assistant

University of Ghent

PhD in Applied Biological Sciences, Faculty of Bioscience Engineering, Department of Applied Analytical and Physical Chemistry, Particle and Interfacial Technology Group (PaInT).

Key Modules: Research, emulsions, surfactants, colloidal chemistry, dairy science, lecturing, conferences, supervision of Master thesis students, doctoral schools training.

September 2007 - June 2009: Master of Food Science and Technology

Interuniversity program between University of Ghent and Katholieke Universiteit Leuven

Key Modules: Food processing, food colloids, food marketing, food microbiology, HACCP, food chemistry, biochemistry, nutrition and dietetics.

September 2001 - September 2006: Bachelor and Master in Agriculture and Agricultural Biotechnology

Agricultural University of Athens

Key Modules: Molecular biology, biochemistry, genetics, microbiology, entomology, zoology, biotechnology of plants, animals and microorganisms.

Publications

- Kasinos, M., R. R. Karbakhsh, R. R., & Van der Meeren, P. (2014). Sensitivity analysis of a small-volume objective heat stability evaluation test for recombined concentrated milk. *International Journal of Dairy Technology*. In press.
- Kasinos, M., Mukarukundo F., De Beuf K., & Van der Meeren, P. (2014). Anionic and zwitterionic phospholipids differently affect the heat coagulation of recombined concentrated milk emulsions. *International Dairy Journal*, *39*, 131-138.
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Conferences

- Aesean Food Conference 2009, Bandar Seri Begawan, Brunei (Poster presentation)
- Food Colloids 2010, Grenada, Spain (Poster presentation)
- ILPS Phospholipid Congress 2011, Rotterdam, The Netherlands (Oral presentation)
- Food Colloids 2012, Copenhagen, Denmark (Poster presentation)
- NIZO Dairy Conference 2013, Papendal, The Netherlands (Poster presentation)
- ILPS Lecithin Short Course, 2014, Ghent, Belgium (Oral presentation)
- III Reunión Interdisciplinaria de Tecnología y Procesos Químicos Los Cocos, Córdoba, Argentina (Oral presentation)

Master thesis students supervision

Pham Thi Tuyet Mai, 2011-2012: Influence of composition and processing on the heat stability of recombined evaporated milk.

Mukarukundo Francine, 2012-2013: Heat stabilizing properties of phospholipids enriched dairy by-products.

Arnout Declerck, 2013-2014: Invloed van formulatieparameters op de fysicochemische stabiliteit van caseinaat-gestabiliseerde olie-in-water emulsies.

Rafat Karbakhsh Ravari, 2013-2014.

