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Increasing the Retention of Lipid-Soluble Components in a Curd Matrix

Megan Tippetts
Utah State University

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INCREASING THE RETENTION OF LIPID-SOLUBLE COMPONENTS

IN A CURD MATRIX

by

Megan Tippetts

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Nutrition, Dietetics, and Food Sciences

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UTAH STATE UNIVERSITY
Logan, Utah

2011
ABSTRACT

Increasing the Retention of Lipid-Soluble Components in a Curd Matrix

by

Megan Tippetts, Doctor of Philosophy
Utah State University, 2011

Retention of lipid-soluble components can be increased in a curd matrix by using emulsions rather than the direct addition of fortified oil. The retention of vitamin D$_3$ was increased in a model system with fortified emulsions (emulsifier: dairy proteins) 96-97% compared to 62-72% control. Retention of fortified emulsions (78%) remained greater than the control (58%) in small-batch Cheddar cheese. Bilayer emulsions were evaluated to increase retention of lipid-soluble components even further. Physicochemical characteristics of the bilayer emulsions were evaluated prior to curd inclusion. Nonfat dry milk (NDM) was used as the primary emulsifier at 1 wt% dairy proteins. Polysaccharides (t-carrageenan, low-methoxyl [LMp] and high-methoxyl pectin) and gelatin were secondary layers. Secondary emulsions formulation was 2.5 wt% oil, 0.5 wt% protein, and 0.2 wt% secondary biopolymer. Emulsions were adjusted to pH 3, 5, and 7 after homogenization. Factors that influence stability are biopolymer concentration, droplet size/distribution/charge ($\zeta$-potential), and viscosity. t-Carrageenan
was the most stable, independent of pH, of all the emulsions. This increased stability was a consequence of the affinity of the protein layer and \( \iota \)-carrageenan through the additional homogenization step. LMp was also stable at pH 7 due to calcium bridging, which correlates with the increased viscosity. The microstructure of the emulsions was examined using scanning electron microscopy. A strong correlation was found between emulsion instability and the presence of thick webbing, due to excess biopolymer, as seen in the micrographs. Stable emulsions were likely to have distinct droplets without a thick web. The exception was gelatin (pH 3), which still had individual droplets but was unstable due to depletion flocculation.

The retention of lipid-soluble substances using secondary emulsion was evaluated in a model curd matrix between primary (50:50 fortified: non-fortified oil) and secondary (100% fortified oil) emulsions. There was no significant difference \((\alpha=0.05)\) in retention of “fortified” oil between primary and secondary emulsions; however, the same fortification level was obtained using secondary emulsions using half the oil. Curd made with 0.01M CaCl\(_2\) had overall lower retention than curd with no additional calcium. Secondary emulsions could be used to fortify various gel matrices (e.g., curd, yogurt, and tofu). Marketing possibilities are endless after preliminary evaluation.
PUBLIC ABSTRACT

Increasing the Retention of Lipid-Soluble Components in a Curd Matrix

Megan Tippetts, under the direction of Dr. Silvana Martini at Utah State University, proposes to demonstrate that it is possible to increase the retention of lipid-soluble components (e.g. vitamin D) in a curd matrix (e.g. Cheddar cheese). This project was coordinated with the Western Dairy Center for the initial phase of the project.

The initial phase used vitamin D₃ fortified emulsions stabilized with a dairy-based emulsifier. The objective was to determine if vitamin D₃ delivered in an emulsion form to the milk before cheesemake would have higher retention in the cheese versus vitamin D₃ oil added directly to the milk. The addition of vitamin D₃ in an emulsified form to milk before cheesemaking resulted indeed in a higher retention in Cheddar cheese independently of the dairy-based emulsifier used with a retention of approximately 78%.

The second phase of this research was to develop secondary emulsions to increase the retention of lipid-soluble compounds even further. Secondary emulsions are formed by a primary emulsion with the addition of a polar secondary emulsifier layer. The hypothesis is that the secondary emulsifier will interact with the milk proteins during cheesemaking and increase the retention of the fortified oil phase. Secondary emulsions formulated in this research were more stable that the primary emulsions. The same retention of a lipid-soluble component was achieved compared to the primary emulsion, but with half the oil. The use of emulsions to fortify foods could be used in other curd matrices (e.g. yogurt and tofu) and has the potential to be used in other food products.
ACKNOWLEDGMENTS

I would like to thank my advisor, Silvana Martini, PhD, for taking me on as her first doctoral student and guiding me throughout the process. I’d also like to thank (in alphabetical order) the other members of my committee, Stacey Hills, Ph.D., Donald McMahon, Ph.D., Ilka Nemere, Ph.D., and Marie Walsh, Ph.D., for their professionalism, insights, and support. Also, I’d like to thank my mom and dad for taking all my calls.

Additionally, I’d like to thank David Irish, Rebekah Kerr, Nicholas Chiew, Rebekah Lance, and Alicia Martin for making cheese (and model cheese) with me, you helped keep it fun. Thanks to F. K. Shen for her assistance with the SEM portion of the research. The pictures turned out beautifully. Thanks to Dr. Broadbent’s lab for allowing me to use the UVP and letting me stay for as long as it took. Finally, thanks to all my friends and family who supported me throughout.

Megan Tippetts
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### LIST OF SYMBOLS, NOTATIONS, AND DEFINITIONS

**Abbreviation Key**

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<th>Definition</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>α-casein</td>
<td>Alpha-casein</td>
</tr>
<tr>
<td>α-Lb</td>
<td>Alpha-lactobumin</td>
</tr>
<tr>
<td>β-casein</td>
<td>Beta-casein</td>
</tr>
<tr>
<td>β-Lg</td>
<td>Beta-lactoglobulin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaCN</td>
<td>Calcium caseinate</td>
</tr>
<tr>
<td>HMp</td>
<td>High methoxyl pectin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>ι-carr</td>
<td>Iota-carrageenan</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>κ-carrageenan</td>
<td>Kappa-carrageenan</td>
</tr>
<tr>
<td>λ-carrageenan</td>
<td>Lamda-carrageenan</td>
</tr>
<tr>
<td>LMp</td>
<td>Low methoxyl pectin</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>MFG</td>
<td>Milk fat globule</td>
</tr>
<tr>
<td>mm/d</td>
<td>Millimeter/day</td>
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<tr>
<td>mV</td>
<td>Millivolts</td>
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<tr>
<td>Abbreviation</td>
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<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NR</td>
<td>Nile red</td>
</tr>
<tr>
<td>NDM</td>
<td>Nonfat dry milk</td>
</tr>
<tr>
<td>$n_i$</td>
<td>Number of droplets of $d_i$</td>
</tr>
<tr>
<td>$\omega$</td>
<td>Oil-in-water</td>
</tr>
<tr>
<td>$\omega$-3</td>
<td>Omega-3 fatty acids</td>
</tr>
<tr>
<td>Pa.s</td>
<td>Pascal.second</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>NaCN</td>
<td>Sodium caseinate</td>
</tr>
<tr>
<td>SBO</td>
<td>Soybean oil</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity</td>
</tr>
<tr>
<td>$d_{3,2}$</td>
<td>Volume-surface mean diameter</td>
</tr>
<tr>
<td>WPC</td>
<td>Whey protein concentrate</td>
</tr>
<tr>
<td>WPI</td>
<td>Whey protein isolate</td>
</tr>
<tr>
<td>$\zeta$-potential</td>
<td>Zeta-potential</td>
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CHAPTER 1

INTRODUCTION AND OBJECTIVES

Vitamin D is an essential vitamin that is synthesized when the body is exposed to sunlight or it is consumed through fortified foods and supplements. Vitamin D deficiency is on the rise, as people are not being exposed to sunlight or consuming sufficient amounts of vitamin D. Having a deficiency in vitamin D is linked with an increased risk of diabetes mellitus, cancer, autoimmune disorders, osteoporosis (Calvo, Whiting, & Barton, 2004), and childhood rickets (Rani & Shaw, 2001; Tomashek et al., 2001). Currently, adequate intake of vitamin D as defined by the Food and Nutrition Board is between 400-800 IU/d; depending on age, and assuming no vitamin D synthesis due to sun exposure. The tolerable upper limit (TUL) intake is 4,000 IU/d for individuals older than 9 yr (National Institute of Health, 2011). Consumers are able to obtain their daily dose of vitamin D in their milk; however, milk consumption has been on the decline (Banville, Vuillemand, & Lacroix, 2000). Cheese consumption, on the other hand, is on the rise (Upreti, Mistry, & Warthesen, 2002), but cheese is currently unfortified. It is, therefore, an ideal candidate to fortify with vitamin D.

Previous research on the fortification of cheese with vitamin D$_3$ has been performed where the vitamin D was dissolved in propylene glycol and incorporated in an emulsion with polysorbate 80 (a synthetic surfactant) as the emulsifier (Banville et al., 2000; Kazmi, Vieth, & Rousseau, 2007; Wagner et al., 2008). Retention of vitamin D was shown to be above 90% in these two studies which incorporated approximately 500-1,000 IU/g of cheese, which is approximately 14,000-28,000 IU/serving (3.5-7x the TUL)
and not an accurate representation of what the fortification level would be in the cheese. Although previous studies have shown vitamin D can be retained in cheese, the main source was not “label friendly,” as they used synthetic emulsifiers and a propylene glycol solvent. The term “label friendly” usually refers to the use of ingredients that are familiar to the consumer rather than unknown chemical components. As consumers increasingly desire (indeed, demand) “label friendliness” (Guzey & McClements, 2006a) other alternatives should be evaluated. These alternatives would include dairy protein emulsifiers, and polysaccharides derived from seaweed (carrageenan), citrus (pectin), or animal (gelatin)—all ingredients consumers would be familiar with.

Fortification of lipid-soluble components in a cheese matrix is possible, as seen from previous research. However, complete retention is sometimes difficult (Banville et al., 2000) since lipid-soluble components are usually partially lost with the milk fat in the whey (Fagan, Castillo, Payne, O'Donnell, & O'Callaghan, 2007). Emulsions might be able to improve retention as the lipid-soluble components would be protected from exiting with the fat. Emulsions could potentially interact with the caseins due to the emulsifier layer at the oil/water interface in the curd and be a better carrier for lipid-soluble components into a curd matrix (vitamin D being just the first of many such components) than a hydrophobic oil, which would isolate itself during the heating of the milk for cheesemake and not be evenly distributed throughout the curd and more likely to leave with the whey. Another possibility is multilayer emulsions, which are formulated with a primary layer of ionic emulsifier, usually a protein, which adsorbs to the lipid
droplets during homogenization. An oppositely charged polyelectrolyte is added to the system which adsorbs to the droplet surfaces and produces an emulsion of droplets coated with a two-layer interface. Though the level of stability varies slightly depending on the pH of the system, the charged surface of such emulsions does maintain stability (Guzey & McClements, 2007). Much research has been done on multilayer emulsions (Dickinson & Pawlowsky, 1997; Gu, Decker, & McClements, 2004a, 2004b; 2005a, 2005b; Guzey & McClements, 2006b; Guzey et al., 2007); however, none of the research has utilized a curd matrix. In addition, most of the previous research has used purified proteins (e.g, β-lactoglobulin, sodium caseinate, caseins) as emulsifiers and the use of a complex system such as nonfat dry milk powder (NDM) has not been tested. Nonfat dry milk powder can be used as a primary emulsifier as it is composed of caseins and whey proteins. The system will be evaluated at pH 3, 5, and 7 to compare the stability of the primary emulsion above, at, and below its isoelectric point (pH =5), as to evaluate the impact of a secondary layer to the emulsion droplets. To understand the stability of an emulsion the ζ-potential, droplet size and distribution, and viscosity can be measured. The latter is rarely if ever measured, which could lead to an increased understanding of the interactions between layers and between droplets on stability. Also, scanning electron microscopy has not been done for multilayer emulsions, which can give a visual representation of how the fat droplets interact and change according to pH. After understanding the physicochemical characteristics of emulsions, retention then needs to be evaluated in a model curd matrix. Instead of evaluating one specific lipid soluble
component, Nile red can be used as a model lipid-soluble substance incorporated in the lipid phase to represent a “fortified” emulsion. As Nile red excites at wavelengths 515-560 nm and emits at >590 nm (Greenspan, Mayer, & Fowler, 1985) using ultraviolet light with an ethidium bromide filter, it is possible to excite a sample and evaluate the intensity as compared to the primary emulsion. The differences can then be evaluated to see if one emulsion (bilayer) is better or worse than the primary emulsion. This can be done in the model curd system at various levels of calcium, which may or may not affect the retention of the emulsion.

Finally, one of the final outcomes of applied research is to apply the results in an industrial setting. The product’s marketability can be evaluated, which can be done through a strengths-weaknesses-opportunities-threats (SWOT) analysis (Coman & Ronen, 2009). First an internal scrutiny is done on the product (SW) and then an environmental scanning is done to identify O & T to the product (Coman & Ronen, 2009). The SWOT analysis aids in defining the marketing strategy of the product. One part of the strategy is to determine who the primary target market should be. That is who would incorporate the emulsions into their production processes.

**Hypothesis**

Dairy-based emulsions can be formulated using different protein/polysaccharide combinations and can be used to improve the retention of lipid-soluble components in a cheese matrix, which can be utilized in the marketplace.
Objectives

1. Determine the retention of vitamin D, a lipid soluble substance, in cheese systems by using oil-in-water emulsions in full-fat and low-fat cheese.
   a. Analyze the retention of dairy protein emulsions to oil-based lipid-soluble components in a model cheese curd using different emulsifiers
   b. Confirm the retention of vitamin D obtained in a model cheese curd system using a small scale Cheddar cheese production.

2. Create bi-layer emulsions to be used as novel delivery matrices to improve the retention of lipid soluble components in a model cheese system.
   a. Optimization of emulsion bi-layer formulation.

3. Evaluate the microstructure of primary and bi-layer emulsions using scanning electron microscopy to enhance the understanding of destabilization kinetics as a function of secondary layer type and pH.

4. Evaluate the retention of lipid soluble components using a dyed bi-layer emulsion in a model cheese system with ultraviolet emission imaging.

5. Assess the marketability of one bi-layer emulsion.

Rationale

Though much research has been done on retention of vitamin D in cheese, there is a need to broaden the use of natural and commercially available ingredients to improve the stability and efficiency of dairy-based delivery matrices. The current project is a preliminary study of how emulsions might be retained in a curd system (using vitamin D
as an example), which could then be applied to other lipid soluble components such as flavors, lipid-soluble vitamins/nutrients, and ω-3 rich oils (not just to incorporate them, but also to protect them from oxidation through the cheese-making process). Multilayer emulsions have been found to be more stable over time as compared to primary emulsions (one layer of emulsifier) (Gu et al., 2005a,b). This benefit suggests that multilayer emulsions can be used to create low-fat dressings, sauces, dips, and desserts. In addition, the viscosity of the emulsions used in this research will be measured to evaluate the effect of multilayer emulsion formation on possible textural changes associated with this novel processing condition.

References


CHAPTER 2
LITERATURE REVIEW

1. Introduction

This literature review will commence with an overview of what emulsions are and where they are found in everyday foods. Next, emulsifiers will be discussed as they are needed for the emulsion to remain homogenous rather than destabilize into the initial two phases (i.e., lipid and aqueous). Then, a more focused look at proteins and polysaccharides specific to the research done for this dissertation will be presented. After that, the methods of analysis will be addressed to explain how emulsions are analyzed for stability and physicochemical characteristics (i.e., viscosity, droplet size and distribution, and $\zeta$-potential). Finally, an application of how emulsions can be utilized will be covered.

2. Emulsions

Emulsions are formed by two immiscible phases (i.e., lipid and aqueous). When the two phases are blended, they rapidly separate into a top lipid layer (lower density) and a bottom aqueous layer (higher density); contact between the two phases is thermodynamically unfavorable without an additional component (i.e., stabilizer/emulsifier) at the oil/water interface to keep one phase suspended in the other (McClements, 2005, 2007; Rousseau, 2000). There are two main types of emulsions: 1) oil-in-water (o/w), which is when the lipid phase is dispersed throughout the aqueous
phase; and 2) water-in-oil (w/o), which is when the aqueous phase is dispersed throughout the lipid phase. Some common examples of o/w emulsions in foods are milk, cream, salad dressing, mayonnaise, soups, sauces, and dips; examples of w/o emulsions in foods are butter and margarine (Guzey & McClements, 2006).

The study of food emulsions is extensive as emulsion stability is of great importance as a good indication of the product’s shelf-life. There are many environmental factors that can affect the stability of an emulsion such as ionic strength, pH, and temperature during processing and/or storage (Mun, Cho, Decker, & McClements, 2008). Most studies of emulsions are done with model systems, which consist of fundamental components (i.e., oil, water, and emulsifier) which are submitted to various environmental stresses (e.g., pH, ionic strength, and heat treatment). However, food emulsions are more complex as the aqueous phase may contain other soluble components such as sugars, salts, acids, bases, alcohols, surfactants, proteins, and polysaccharides. The lipid phase may consist of lipid-soluble components such as mono-, di-, and triacylglycerols, free fatty acids, sterols, and vitamins. The combination thereof leads to a complex interfacial region, which could consist of a mixture of surface-active components such as protein, polysaccharides, phospholipids, surfactants, and alcohols (McClements, 2005). Using complex ingredients commonly available in the food industry such as nonfat dry milk powder which consists of proteins (caseins and whey), lactose, minerals (i.e., calcium, magnesium, phosphorus, potassium, sodium, and
zinc), and vitamins (e.g., vitamin A, B_6, and C, thiamine, riboflavin, niacin, pantothentic acid, and folacin) adds new variables that can affect emulsion stability.

3. Stability

As mentioned above, an emulsion’s stability is indicative of its shelf-life, if the product is an emulsion (e.g., salad dressing), or only needs to be stable for a shorter duration as it is an intermediate step (e.g., cake batters). Emulsion stability is used to describe the resistance of an emulsion to changes in its properties over time, and the slower the properties change the more stable the emulsion (McClements, 2005). There are multiple physicochemical mechanisms that might be responsible for the destabilization of the emulsion such as creaming, sedimentation, flocculation, and coalescence.

3.1. Creaming and sedimentation

Creaming and sedimentation are both forms of gravitational separation. This means that the droplets in the emulsion have a different density to that of the continuous phase which surrounds the droplets (McClements, 2005). Creaming refers to the droplets that have a lower density than the surrounding liquid and sedimentation refers to droplets with a higher density than the surrounding liquid. For example, soybean oil (at room temperature) has a lower density than water, which means the o/w emulsion would cream during destabilization; however, for a water-in-oil emulsion the water phase, being the droplets surrounded by oil, would sediment out.
The way to gain a basic understanding of a system and how it might destabilize would be to apply Stokes law (Eq. 1).

\[ v_{\text{Stokes}} = \frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1} \]  

[1]

where \( v_{\text{Stokes}} \) is the rate of creaming, and the sign determines which direction the droplets move with positive moving up and negative moving down (sediment). The densities (\( \rho \)) are for the continuous phase (1) and the droplets (2). The viscosity (\( \eta \)) is of the continuous phase and the radius (\( r \)) is for the droplets. For model systems, a way to define a stable emulsion is one that creams less than 1 mm/day (McClements, 2005), whereas this might not be stable enough for an emulsion to be stored for 6 months.

3.2. Flocculation

Flocculation is when the droplets in an emulsion associate with each other but maintain their individual integrities (McClements, 2005). Associated droplets are defined as a floc, and the floc has a larger radius than an individual droplet. The new radius can then be plugged into equation 1 and the rate of creaming is increased, and flocculation can be detrimental to emulsion stability.

3.2.1. Bridging flocculation

Bridging flocculation is when a biopolymer (e.g. protein or polysaccharide) adsorbs either directly to the surface of two or more droplets or to another adsorbed
emulsifier molecule on the droplets (McClements, 2005). This is done through hydrophobic or electrostatic interactions. Also, bridging flocculation occurs when there is insufficient biopolymer to cover all the droplets, which is why interactions occur with two or more droplets (Dickinson, Golding & Povey, 1997) at sufficiently high concentrations there is sufficient biopolymer to completely cover the surfaces of the droplets (McClements, 2005) and the destabilization mechanism is no longer bridging flocculation.

3.2.2. Depletion flocculation

Depletion flocculation is when there is a presence of non-adsorbing colloidal particles (e.g., biopolymers) in the continuous phase of an emulsion, which increases attractive forces between droplets due to an osmotic effect from the exclusion of the excess colloidal particles from the narrow region surrounding the droplets (McClements, 2005). The amount of attractive forces between droplets can be correlated with the concentration of non-adsorbed colloidal particles, as the concentration increases so due the attractive forces and when the attractive force becomes large enough it can overcome the repulsive interactions between the droplets causing flocculation (McClements, 2005).

3.3. Coalescence

Coalescence is when two or more droplets merge together to form a single larger droplet (McClements, 2005). This process is due to the Laplace pressure being locally high, and the oil will flow to lower pressure sites (Fredrick, Walstra & Dewettinck,
2010). The process occurs to create the most thermodynamically stable state for the emulsion, which involves decreasing the contact area between the oil and water phases (McClements, 2005). The new droplet assumes a spherical shape as this shape yields the lowest Laplace pressure (Fredrick et al., 2010). The process occurs in emulsions with the presence of an emulsifier, and there is insufficient emulsifier to cover the droplets. When the exposed surface area (that not covered by emulsifier) comes in close proximity of another exposed droplet surface coalescence can occur; or if the film (emulsifier layer) is broken from being stretched or torn due to high shear or a large stress and the droplets are close coalescence is possible (Fredrick et al., 2010; McClements, 2005).

3.3.1 Partial coalescence

Partial coalescence occurs when an oil droplet is penetrated by a solid fat crystal in another droplet. The oil from the punctured droplet surrounds the fat crystal as that is the most thermodynamically stable state and an irregularly shaped aggregate is formed versus the standard sphere (McClements, 2005).

4. Emulsifiers

To increase the stability of an emulsion the use of an emulsifier is required. Examples of emulsifiers are low molecular weight surfactants (e.g., sugar esters and polysorbates), phospholipids (e.g, lecithin), proteins (e.g., sodium caseinate and \(\beta\)-lactoglobulin), and polysaccharides (e.g., pectin and carrageenans), all of which are amphiphilic molecules (McClements, 2005). That is, they are able to interact with both
the lipid and aqueous phases. Each amphiphilic molecule has a hydrophobic tail (or patches) that adsorbs to the lipid phase, and a hydrophilic head (or patches) that interacts with the aqueous phase (Guzey & McClements, 2006; Ogawa, Decker, & McClements, 2003). During homogenization, the emulsifier adsorbs to the interface of the newly formed oil droplets and lowers the interfacial tension. At appropriate concentrations, the emulsifier is able to improve the stability of the emulsion by delaying droplet aggregation through repulsive forces and the formation of a protective interfacial membrane which is resistant to rupture and has low surface tension (Surh, Decker, & McClements, 2006).

Although a wide variety of synthetic and natural emulsifiers (such as low molecular weight surfactants, phospholipids, and biopolymers) are considered GRAS (generally recognized as safe) for utilization in food emulsions, there is an increasing trend to replace synthetic emulsifiers with natural ones (Guzey et al., 2006; Surh et al., 2006). By incorporating only natural ingredients, the range of options becomes limited, especially for emulsifiers. The emulsifiers must stabilize emulsions over a wide range of environmental stresses, be compatible with the other ingredients, easy to use and cost effective (Guzey et al., 2006; Mun et al., 2008).

The emulsifiers utilized in this research are all natural and consist of milk proteins (caseins and whey proteins), gelatin, and polysaccharides (i.e., pectin and carrageenan), all of which are from animal or plant sources.
5. Proteins

Proteins are extracted from various natural resources and are used as emulsifiers as they are able to facilitate emulsion formation, improve stability and produce desirable physicochemical properties in o/w emulsions (Surh et al., 2006). Proteins consist of amino acid sequences and, depending on the sequence, form a variety of different structures such as random coil, fibrous, and globular, which are adopted to minimize the overall free energy of the system (Matalanis, Jones, & McClements, 2011). Proteins are amphiphilic and therefore have areas that are hydrophobic and hydrophilic; the arrangement of these different areas in the protein determines the type of protein folding and structure. Due to their amphiphilic nature, proteins are known for their surface activity which has a major role in the formation and stabilization of emulsions by a combination of electrostatic and steric interactions (Gancz, Alexander, & Corredig, 2006; Patino & Pilosof, 2011). The interactions made by a protein with surrounding molecules are dependent on the charge of the system. Proteins have a net positive charge at pH values below the isoelectric point (pI) of the protein, a neutral overall charge at pH values equal to the pI, and a net negative charge at pH values above the pI (Matalanis et al., 2011). It should be noted that even at its pI a protein still has regions which are positively and negatively charged and can still interact with other molecules through electrostatic (attractive and repulsive) interactions (Matalanis et al., 2011).

The proteins used in this study are milk proteins, which consist of casein and whey proteins, and gelatin. Their characteristics are described below.
5.1 Dairy proteins

Though emulsification properties are the only focus of this research, milk proteins are also known for their water binding, foaming, whipping, and gelation properties. These different functional properties position them as ideal ingredients to be used in a myriad of food products (Singh, 2011). For example, with hydrophobic and hydrophilic areas proteins are able to bind water and fat, which in conjunction with emulsification and gelation, create comminuted meat products (Zayas, 1997). Also, the properties of milk proteins are able to coagulate under appropriate conditions leading to the development of cheese (Zayas, 1997). Understanding the function properties of proteins in model systems can be used as indicators or predictors of how they might function in real food systems (Zayas, 1997).

Milk proteins consist of 80% caseins (αs1, αs2, β, κ) and 20% whey proteins, which are mainly β-lactoglobulin, α-lactobumin, and bovine serum albumin (Corredig, Sharafbafi, & Kristo, 2011). It has been reported that there is competitive adsorption between the caseins and the whey proteins at the lipid interface during homogenization of skim milk solutions (Dickinson, 1997). There is a preferential adsorption of the casein fraction, though casein micelles complicate the adsorption behavior of the individual caseins (Dickinson, 1997). The concentration of protein in milk is approximately 3.4%. A difference in protein concentration in an emulsion could affect the adsorption of the proteins; the ratio of protein to lipid content would also impact which proteins would adsorb to the lipid surface. The incorporation of milk proteins into oil-in-water
emulsions not only can affect stability but also has an impact on the oxidative stability, especially for polyunsaturated oils (Singh, 2011).

5.1.1 Caseins

Caseins ($\alpha_s1$, $\alpha_s2$, $\beta$, $\kappa$) are phosphoproteins, which are flexible molecules (rheomorphic) and amphiphilic (Singh, 2011). A casein monomer on an oil-water interface may be regarded as a complex linear copolymer giving an entangled monolayer of flexible chains having some sequences of segments at indirect contact with the surface (trains) and loops in the aqueous phase (Dickinson, 1997). Individually concentrated caseins interact with each other, forming micelles, to protect their hydrophobic areas, which are not uniformly dispersed along the peptide chain, on the inside of the micelle and the hydrophilic areas on the outside interacting with the aqueous phase (Singh, 2011). The concentration of the casein monomers can be correlated to a critical micelle concentration (CMC), which is when the monomers shift from being individually solvated (very low concentrations) to aggregating into micelles and becoming a mix of free monomers and aggregates of varying sizes (Leclerc & Calmettes, 1997). The CMC is also influenced by temperature; $\beta$-casein has a CMC of 0.05 wt% at 40°C, but is predominately in monomer form below 15 °C (O’Connel, Brinberg, & de Kruif, 2003). Casein micelles have an average diameter in the range of 100-300 nm (Matalanis et al., 2011) and contain small amounts of calcium (bound mostly to $\alpha$ and $\beta$-caseins), phosphate, magnesium, and citrate (Singh, 2011). Being flexible makes caseins excellent emulsifiers, as they possess limited tertiary structure and little secondary
structure, and they easily unfold at the interface (Zayas, 1997). McMahon and Oommen (2008) report that the secondary structure should be considered as being intrinsically unstructured proteins rather than the more commonly viewed reference of a random coiled structure.

When the various caseins interact with each other they form casein micelles, which are a system of particles of colloidal size held together and organized by means of noncovalent intermolecular binding interactions (McMahon & Oommen, 2008). Casein micelles have a net zero charge close to pH 4.9 (Tuinier, Rolin, & de Kruif, 2002). One proposed model indicates that micelles are formed via calcium phosphate bridges of multiple submicelles, which are spherical aggregates of several casein monomers connected together by hydrophobic and electrostatic interactions (Langendorff, Cuvelier, Launay, & Parker, 1997). The hydrophilic areas of the casein submicelles are orientated towards the exterior, with κ-caseins located at the periphery as they have a hydrophilic C-terminal end interacting with the continuous aqueous phase. The multiple κ-caseins form a “hairy” layer around the micelle and give colloidal stability (Langendorff et al., 1997) against calcium precipitation (Singh, 2011), though when micelles are subjected to high pressures such as with homogenization they are disrupted (Zayas, 1997).

There is a positively charged area on κ-casein between residues 97-112, which is absent from α and β-caseins, even at pH above the pI of caseins (Langendorff et al., 1997). This area is possibly the point of interaction between a positive patch on the casein micelle and anionic polysaccharides when the protein has an overall net-negative
charge. Though the interior of the micelles are inaccessible to large molecules, as the polyelectrolyte layer of κ-casein attracts or repel molecules to or from it through electrostatic interactions, small molecules (e.g., β-Lg, β-casein, polyphenols, rennet or some enzymes) are able to access the internal structure (Corredig et al., 2011).

On the other hand, McMahon and Oommen (2008) describe the network as an interlocking lattice model of the casein micelle supramolecule. Calcium phosphate nanoclusters maintain the integrity of the supramicelle and are integral in the synthesis of casein micelles (which make up the supramicelle). The calcium phosphate nanoclusters are presumed to be located at the interlocking sites of the casein micelles and are binding sites for other caseins as well. The lattice model is therefore a linked group of caseins with calcium phosphates as the junction point for multiple chains to link at one point. McMahon’s model agrees with the predominance of κ-casein at the periphery as terminal molecules, however the lattice structure is irregular in nature and supports an open structure of the casein micelle, which explains how it can be such a flexible micelle rather than rigid.

Besides the pure forms of the caseins, caseinate can be formed (e.g., sodium caseinate [NaCN] and calcium caseinate [CaCN]) by modifying caseins through multiple steps as reported by Singh (2011). This process includes the acidified extraction of the casein and the solubilization of the acid casein in solutions of NaOH or Ca(OH)_2 to neutralize the casein curd.
A common emulsifier used in the food industry is NaCN as it reacts rapidly during homogenization to establish a thick, sterically stabilized layer that protects the oil droplets from destabilizing and from auto-oxidation (Richards, Golding, Mijesundera, & Lundin, 2011). In solution, NaCN forms aggregates or sub-micelles at concentrations above the CMC as there is a high proportion of hydrophobic amino acid side chains throughout the primary structure (Dickinson & Golding, 1997). Concentrations of 2% or below of NaCN in o/w emulsion is advised as higher concentrations lead to depletion flocculation (Dickinson et al., 1997; Singh, 2011). However, by adding calcium, it is possible to eliminate the effects of depletion flocculation (Singh, 2011). NaCN is considered a better alternative to emulsion stability than whey proteins since caseins tend to be more heat stable and are less likely to denature when heat is applied.

5.1.2 Whey proteins

Whey proteins represent approximately 20% of milk proteins; approximately 50% of the whey proteins are β-lactoglobulin (β-Lg), which is a globular protein, of 18,000 Da and contains two disulfide bonds (Singh, 2011). The other types of whey proteins are constituted by α-lactalbumin (α-Lb) (~21%), bovine serum albumin, and immunoglobulins. Dairy practices have found numerous uses for whey proteins and these have become a functional ingredient in the form of whey protein concentrate (WPC), whey protein isolate and whey hydrolytes (Zayas, 1997). The protein concentration in WPC contains between 35 to 80% of protein and the functionality of WPC depends on how the whey was processed (e.g., heat treatment, filtration process).
(Zayas, 1997). β-lactoglobulin has a compact tertiary structure and an orderly secondary structure as a dimer at the pH of milk (Dickinson, 1997). The typical globular structure of β-Lg has a high hydrophobicity in the protein core. A moderate heat treatment is recommended to promote partial unfolding of the globular structure and improve its emulsifying capacity (Zayas, 1997). Without a heat treatment, β-Lg still absorbs to the interface; however, it takes longer to reorganize itself to the lipid’s surface, and some proteins do not have the time to reorganize as other molecules adsorb to the surface and pack the β-Lg in place (Dickinson, 1997). This means that adsorption of β-Lg (as to whether it will retain its original structure or reorganize to cover the oil droplet) is dependent on concentration of protein in the system. β-Lg may also contribute to increased viscosity over time as disulfide bonds are formed between molecules (Zayas, 1997) from thiol-disulfide interchange reactions between droplets leading to droplet aggregation (Kim, Decker, & McClements, 2002). The other main whey protein α-Lb is a rigid globular protein which is able to stabilize o/w emulsions; however, α-Lb does not emulsify nearly as much oil as β-Lg; its emulsion capacity is 120 ml oil/g protein vs. 185 ml oil/g for β-Lg, (Zayas, 1997).

5.2 Gelatin

Gelatin (Figure 2.1) is known primarily for its thermoreversible gelation behavior, but it also has many other functional applications such as water-holding capacity, thickening, colloid stabilization, whipping and emulsification (Dickinson & Lopez, 2001). Gelatin is a derivative of animal collagen (e.g., pig, cow or fish) of relatively high
molecular weight (Surh et al., 2006). It is prepared by sweltering animal tissues in the presence of an acid or an alkali (Taherian, Britten, Sabik, & Fustier, 2011). Gelatin is not a truly homogenous substance as it consists of a considerable number of constituents which differ in aggregation and flocculation from solution at various temperatures, though gelatin can be fractionated to then flocculate within a more narrow temperature range (Straup, 1931). There are two types of gelatin: Type A has a pI of approximately 7-9, while type B has a pI of approximately 5 (Taherian et al., 2011). This means that type B would follow the same trend as milk proteins when pH is compared, while Type A would be positively charged at higher pH values and might interact more with milk proteins at a higher pH. The benefit of adding type A gelatin to o/w emulsions would be to increase oxidative stability as the gelatin would be positive at high pH and would be able to repel iron ions from the oil droplet surface (Surh et al., 2006; Taherian et al., 2011).

Figure 2.1. Gelatin (Chaplin, 2011) a linear protein with many glycine residues (almost every third), prolines and 4-hydroxyproline residues.
6. Polysaccharides

Polysaccharides are either homo-polysaccharides which consist of one monosaccharide, or hetero-polysaccharides, which consist of multiple types of monosaccharides. Polysaccharides differ in terms of type, number, sequence, and bonding of monosaccharides within their polymer chain (Matalanis et al., 2011). Functional properties such as solubility, thickening, gelation, and emulsification of polysaccharides are determined by the molecular differences among them (Matalanis et al., 2011). When working with food emulsions, the effect of polysaccharides on emulsification properties has been widely studied since these molecules are added to improve texture and stability (Gancz et al., 2006). Long-term stability of emulsions can be enhanced with the addition of polysaccharides to control the rheology and network structure of the continuous phase, and to retard phase separation and gravity–induced creaming (Patino & Pilosof, 2011). A critical concern when using polysaccharides in food products is the concentration of the ingredient, as the properties of the final food product depend on the interactions between the polysaccharide and other macromolecules in the system (Corredig et al., 2011).

The polysaccharides used in this research are t-carrageenan and pectin (low methoxyl and high methoxyl).

6.1 Carrageenan

Carrageenans are commonly used as functional ingredients especially in the preparation of milk gels and in the stabilization of milk-based products such as ice cream,
milk beverages, and puddings (Corredig et al., 2011; Langendorff et al., 1997). \(\kappa\)-
Carrageenan (Figure 2.2), which is extracted from red seaweed, is a di-sulphated linear
polysaccharide, with a base chain of D-galactose and 3,6-andhydro-D-galactose; in
aqueous solutions it can undergo a reversible temperature-dependent coil to helix
transition through its sulfate groups and 3,6-andhydro-D-galactose ring, and can gel in
the presence of cations (e.g., calcium) which bridge the chains of carrageenan (Corredig
et al., 2011; Dickinson & Pawlowsky, 1997). \(\kappa\)-Carrageenan also interacts with proteins
at almost any pH (>2) through ionic interactions, even when both polymers are anionic,
because the sulfate groups on the polysaccharide have attractive electrostatic interactions
with the cationic patches on the protein (e.g., the hydrophilic C-terminal end of \(\kappa\)-casein)
(Gu, Decker, & McClements, 2004b).

\[\text{Figure 2.2. } \kappa\text{-Carrageenan (CyberColloids, n.d.a), a linear anionic polysaccharide, with two sulfate groups per two sugar molecules.}\]
6.2 Pectin

Pectins (Figure 2.3) are polysaccharides with a galacturonic acid backbone, which is esterified with methoxyl groups (Gancz, Alexander, & Corredig, 2005). Depending on the degree of esterification (DE), pectins can be classified into high methoxyl (>50% DE) or low methoxyl (<50% DE) pectin (HMP and LMP, respectively). Pectins are extracted from citrus, apples or beets (Corredig et al., 2011) and are widely used in dairy products for texture and stabilization as the pectin interacts with the casein through calcium ion bridges preventing aggregation through electrostatic repulsive forces and steric interactions, as in acidic milk drinks (Kouame, Bohoua, Assemand, Tano, & Kouame, 2010). Interactions between the milk proteins and pectin occur below the pI of the milk proteins as they become positively charged and can interact with anionic pectin through attractive electrostatic forces (Sergersen et al., 2007). Kouame et al. (2010) also report that at neutral pH, LMP does not form complexes with the casein micelles as both biopolymers are anionic at that pH; however, Dickinson (1998) does report that LMP is able to form stabilizing gels with calcium at neutral pH.

Figure 2.3. Pectin chain (OSU, 2011). If more than 50% of the carboxyl groups are methylated then it is high-methoxyl pectin (above), if less than 50% then low-methoxyl pectin.
Without calcium to bridge them at pH 6.7, each biopolymer (pectin and casein micelles) become isolated as no interactions occur, each having a net-negative charge, and attractive electrostatic forces between micelles occur and exclude the pectin molecules causing phase separation via depletion flocculation (Maroziene & de Kruif, 2000).

HMp, on the other hand, though able to interact with casein at low pH (<4), has low affinity and areas of HMp that do not connect with the milk proteins, indicating that when added to a emulsion stabilized with milk proteins the suspended droplets are kept dispersed by steric interactions (Sergersen et al., 2007). At pH between 4.5 and 5, near the pI of milk proteins, HMp adsorbs to the protein by electrostatic attractive forces and stabilizes milk protein emulsions through the formation of a three dimensional network (Corredig et al., 2011).

7. **Multilayer emulsions**

Multilayer emulsions are composed of a primary emulsion stabilized using an ionic emulsifier which interacts through electrostatic interactions with an oppositely charged polyelectrolyte that adsorbs to the droplet surface producing a secondary layer. This process is called electrostatic deposition and can be repeated multiple times (Guzey & McClements, 2006). Electrostatic deposition can be achieved by directly combining oppositely charged molecules of two biopolymers by attractive electrostatic interactions. For example, the primary layer has a net negative charge that means that the secondary layer needs to have a net positive charge. The secondary layer must have sufficient net
positive charge not only to bind to the primary layer, but also to keep the dispersed phases suspended through repulsive electrostatic interactions. An alternate way to induce electrostatic deposition is by adding a secondary biopolymer to the primary emulsion with the same net charge. Then by adjusting the pH, the secondary biopolymer will adsorb to the primary emulsion uniformly (preventing aggregation of droplets) as the net charge of the primary emulsion becomes oppositely charged (Guzey & McClements, 2006). The attraction between the layers is dependent on the pH of the system, which determines the charge of the biopolymers and therefore the strength of the interactions between layers (Guzey & McClements, 2007); the ionic strength of the solution also alters the interactions between layers.

Most multilayer emulsions have used proteins as the primary emulsifier with a polysaccharides as the secondary polyelectrolyte. Proteins have the ability to form a strong adsorbed layer at the o/w interface, while polysaccharides are used secondarily for their texture, rheological, and stability characteristics (Dickinson et al., 1997; Gancz et al., 2005). Multilayer emulsions are being researched to develop new foods or improve the shelf life of existing products; however, the interactions between proteins and polysaccharides must be controlled as the interactions might also cause aggregation and creaming (Gancz et al., 2005; Guzey & McClements, 2006, 2007). The possibilities of usage are extensive for protein/polysaccharide multilayer emulsions such as delivery systems suitable for encapsulating, protecting and delivering lipophilic functional components in the food and pharmaceutical industries (e.g., flavors, colors, oil-soluble
vitamins, conjugated linoleic acid, nutraceuticals, and ω-3 rich oils) (Hu, Li, Decker, Xiao, & McClements, 2011; Matalanis et al., 2011). It is also important that the emulsions are made from food-grade ingredients, are economical, and use reliable processing operations (Matalanis et al., 2011). Guzey and McClements (2006) point out that the initial cost of producing multilayer emulsions might be high due to additional ingredients and a new processing condition. Producers may be able to recoup costs from reduced loss of faulty emulsifiers or sell the product at a higher cost due to improved functionality and shelf life. However, in the end, multilayer emulsions might be the solution to creating highly stable emulsions which are resistant to multiple environmental stresses. The following subsections review the multilayer emulsion studies of the various proteins and polysaccharides which were used in this research, that is, how gelatin, τ-carrageenan, HMp and LMp have been combined with a protein to form multilayer emulsions.

### 7.1 Gelatin multilayer emulsions

Gelatin has been used as a secondary layer and a tertiary layer in multilayer emulsions (Gu, Decker, & McClements, 2005a; Taherian et al., 2011). Taherian et al. (2011) analyzed fish gelatin as a secondary layer, with whey protein isolate (WPI) as the primary emulsion. Taherian’s research compared the multilayer emulsion to primary emulsions made with the individual proteins. This was one of the only groups that homogenized (at the same pressure) both layers rather than waiting for electrostatic deposition. They compared results at pH 3.4 and 6.8, reporting that the multilayer
emulsion was more stable at both pH values than the individual proteins alone, and that
the viscosity increased over time, and the \( \zeta \)-potential favored the net charge of the WPI.
Gu, Decker, and McClements (2005a) studied multilayer emulsions with gelatin as the
tertiary layer. Unlike Taherian, Gu used electrostatic deposition, which meant that each
layer added to the primary emulsion took approximately 24 hr for complete deposition.
The secondary layer used by these authors was \( \iota \)-carrageenan. Gu et al. (2005a) reported
that with gelatin as the tertiary layer, emulsion stability is strongly dependent on the pH
of the solution. Stable tertiary layers were formed at pH 6; however, at pH 8 the \( \iota \)-
carrageenan did not adsorb to the primary emulsion and gelatin became the secondary
layer; also, the multilayers were more stable when the ionic strength of the emulsion was
increased.

7.2 \( \iota \)-Carrageenan multilayer emulsions

While studying tertiary layers Gu et al. (2005a) reported the stability of secondary
emulsions formulated with \( \beta \)-Lg and \( \iota \)-carrageenan secondary layer and found that stable
emulsions were obtained at pH 4-6, but not at pH 7-8, where there was also no difference
in \( \zeta \)-potential between the primary and secondary emulsions. These results correlated
with their research done on the effect of pH and \( \iota \)-carrageenan concentration to the
stability of \( \beta \)-Lg primary emulsions (Gu et al., 2004b), with pH 6 being the most stable,
independent of carrageenan concentration (0-0.15wt%). For all the other pH values, the
emulsions destabilized at concentrations greater than 0.08%. These authors also studied
the effect of various carrageenans (τ, λ, and κ) at pH 3, 5, and 6 at concentrations between 0 to 0.15% with 5% corn oil and 0.5% β-Lg primary layer (Gu, Decker, and McClements, 2005b). For τ-carrageenan, the results were the same as the ones published in 2004, with high stability at pH 5 and 6; even pH 3 was more stable at only 50% creaming versus the 90% in 2004 (though, this was due to the measurement being taken after 24h vs. 1 wk). As for κ and λ-carrageenans (after 24 h), emulsions formulated with these polysaccharides were more unstable at pH 5 (any concentration) and 6 (> 0.05 wt% carrageenan) than τ-carrageenan. As for pH 3, λ-carrageenan was the most stable and κ-carrageenan was the least, which was interesting as the difference in ζ-potential was the greatest with κ-carrageenan, indicating a higher amount of adsorption. Gu, Decker, and McClements (2004a) also evaluated the impact of thermal processing (30-90°C) on τ-carrageenan secondary emulsion with and without 150 mM NaCl. They reported that τ-carrageenan emulsions were stable for the range of temperatures without the addition of salt, and with salt at temperatures below 60°C. However, when the temperature increased above 60 °C, τ-carrageenan desorbed from the droplets and the β-Lg denatured and aggregated. Interestingly, when the primary emulsifier was changed to bovine serum albumin there was a weak electrostatic attractive interaction occurring at pH 6, and the emulsions are the most stable without the τ-carrageenan secondary layer (Dickinson et al., 1997). This finding indicates that a change in protein can affect the interactions of the system, which could increase or decrease stability.
In summary, all of the multilayer emulsions formulated with t-carrageenan using electrostatic deposition resulted in unstable emulsions at pH 7 and 3.

7.3 Pectin multilayer emulsions

Li et al. (2010) studied the efficiency of multi-layer emulsions to increase the stability of the emulsion, protect the components from degradation, and to release encapsulated components when specific environmental factors are introduced. They studied a combination of β-Lg, chitosan, and pectin or alginate for the emulsifier layers. The emulsions had a final concentration of 0.5 wt% oil, 0.05% β-Lg, 0.025% chitosan, and 0-0.25% pectin/alginate. Secondary emulsions formulated with chitosan were stable at concentrations of 0.05 to 0.08 wt%; 0.05% was used in the formulation of the tertiary emulsions at pH values of 4.5-5.5. After the addition of the tertiary layer, pectin tertiary emulsions were less charged (lower ζ-potential) than alginates; however, both were stable at concentrations between 0.1-0.15% at pH 5.5. These authors state that stability is dependent on the charge of the outer biopolymer layer, which is useful in the design of encapsulated and delivery systems.

7.3.1 HMp

Dickinson, Semenova, Antipova, and Pelan (1998) compared bi-layer emulsions of singular caseins (i.e., αs1, β and sodium caseinate) with HMp as the secondary layer at pH 7.0 and 5.5. They found that at pH 7 an emulsion with 11% oil, 0.6% NaCN had increased stability as the creaming decreased from 80% to less than 20% after 3 d at
ambient temperature when the HMp concentration increased. Also at pH 7, the viscosity (40% oil, 2% protein, 0.01M NaCl) increased as the amount of pectin increased and exhibited a shear-thinning (pseudoplastical fluid) behavior (Dickinson et al., 1998). These authors suggest that the increase in viscosity is a consequence of the interactions between HMp and casein, and depletion flocculation of the droplets. For pH 5.5, though the viscosity still increased with pectin addition, it was not to the magnitude of that at pH 7, and the destabilization of the emulsions was still evident. Guzey and McClements (2007) evaluated the impact of HMp secondary layer on β-Lg emulsions with respect to ionic strength and pH. The emulsions were 0.1 wt% corn oil, 0.05wt% β-Lg, and 0.02wt% pectin. The pectin was incorporated before and after the adjustment in ionic strength. When NaCl was added before pectin, the droplet size in the emulsion increased, which indicated that salt inhibits pectin adsorption to the surface of the primary emulsion thereby giving droplets time to aggregate, creating flocs with an outer layer of pectin. Unfortunately, though the researchers mention taking stability measurements, they did not include them in their findings nor explain why the results were not included.

7.3.2 LMP

Hu et al. (2011) evaluated the stability of lipid droplets coated by biopolymer nano-laminated coatings. Emulsions with two to four layers made of casein, pectin, and chitosan in various orders were made. Total oil content was 0.25%. This group found that casein emulsions were stable at pH 3, 6 and 7 after 24 h. With the addition of pectin as the secondary layer, the emulsions were stable from pH 3-7; with pectin as the
quaternary layer, emulsions were stable from pH 4 to 7. However, when pectin was the tertiary layer, the stability was poor at all pH values (3-7); again these were measured only after 24h. This study showed that though it is possible to add layers, stability is not dependent on just the final layer, but on how the interfacial layer is made (i.e., which layer is first, second, etc.).

8. Methods of Analysis

8.1 ζ-potential

Many emulsifiers, such as the proteins and polysaccharides which are used in this study, are ionic or are capable of being ionized. The electrical characteristics depend on the number and type of ionizable groups on the molecule (McClements, 2005). Another component to the net charge of a system is the amount of other ionic substances in the solution, which might include multivalent mineral ions (e.g., Ca$^{2+}$, Cu$^{2+}$, or Fe$^{3+}$) or other polyelectrolytes. The magnitude of the electrical charge on the emulsion droplets can be altered by the adsorption of these ionic substances that can change the sign of the charge or act as bridges between droplets. All of this affects the ζ-potential, including the presence of ions which aren’t adsorbed to the droplet, but alter the ionic strength of the solution (McClements, 2005). ζ-potential is the electrical charge associated with the movement of a droplet in an electrical field (McClements, 2005). The ζ-potential indicates the net charge of the emulsion. An absolute increase in the net charge from neutral usually indicates the droplets are able to repulse each other through electrostatic
interactions and therefore maintain stability. When the polysaccharide/protein adsorbs to the droplet surface, there is a change in the droplet’s charge. When there is no change to ζ-potential, the assumption is that there has been no absorption (Gu et al., 2005a). Though the ζ-potential is a good gauge to compare the net charge of emulsions, it should not be relied on as the sole indicator of stability for complex systems. Also, when the addition of a secondary layer does not change the ζ-potential of the system, but the emulsion becomes more or less stable, then the droplet charge of the emulsion is not stabilizing the emulsion, but some other reaction (e.g., calcium bridging) is stabilizing or destabilizing the emulsion.

8.2 Droplet size and distribution

One of the most important factors influencing emulsion properties is droplet size. To obtain good physical stability it is usually important to formulate emulsions with small droplets (below 1 μm) by high pressure homogenization (Dickinson, 1997) since droplet size can impact the shelf life, appearance, texture and flavor of an emulsion (McClements, 2005). Real food emulsions always contain a distribution of droplet sizes, which creates a more complicated system. The distribution range of droplet sizes for an emulsion can sometimes be critical to know, while at other times it is sufficient to have the average droplet diameter (McClements, 2005). A common way to report the diameter measurement is as the volume-surface mean diameter ($d_{3,2}$) using Eq. 2; or the volume fraction-length mean diameter ($d_{4,3}$) as in Eq. 3 (McClements, 2005). The use of $d_{3,2}$ is useful in calculating the total surface area of droplets in an emulsion when the mean
diameter of the droplets and the volume fraction of the dispersed phase are available; \( d_{4,3} \), on the other hand, is more sensitive to the presence of large particles and hence flocculation (McClements, 2005).

\[
d_{3,2} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}
\]

\[
d_{4,3} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}
\]

where \( n_i \) is the number of particles in each size-class per unit volume of the emulsion and \( d_i \) is the particle size diameter in each size-class.

When emulsions are formulated with insufficient emulsifier or when there has been extensive droplet flocculation a bimodal distribution of droplets can be obtained. When this occurs it is better to report the distribution than the average, as part of the picture would be lost (McClements, 2005). By evaluating the droplet size over time it is possible to understand how the emulsion is destabilizing. If there is no change in droplet size, but there is destabilization, then it is possible to interpret the results as droplet flocculation; however, if the droplet size increases with respect to time, then the destabilization mechanism is most likely coalescence, and insufficient emulsifier to cover the droplets was probably responsible for this phenomenon. Another item to take into consideration is oiling off. Instead of droplets becoming a larger droplet, the oil is released into solution and migrates to the top, forming an oil layer (Guzey & McClements, 2006). This occurs when there is insufficient emulsifier to cover the droplets; they coalesce which eventually leads to “oiling off” (McClements, 2005).
8.3 **Viscosity**

Food emulsions exhibit a wide range of rheological behaviors as they are compositionally and structurally complex materials (McClements, 2005). The viscosity of emulsions can be low for fluids such as milk and fruit beverages, to viscoelastic for gels such as yogurt and desserts, or solid for products such as butter and margarine (McClements, 2005). Moreover, it is possible to obtain a wide range of viscosities for liquid food emulsions. Fluid emulsions, like milk, have low viscosities, while that of double cream is highly viscous. Emulsions commonly exhibit shear-thinning behavior, which means they are pseudoplastic fluids. Pseudoplastic fluids have the most common non-ideal behavior flow; that is, the apparent viscosity decreases when the shear rate is increased. The decrease in viscosity occurs for a variety of reasons; for example, the spatial distribution of the particle may be altered by the shear field, non-spherical particles may become aligned with the flow field, solvent molecules bound to the particles may be removed, or flocs may be deformed and disrupted (McClements, 2005).

8.4 **Scanning electron microscopy**

Scanning electron microscopy (SEM) is performed by raster scanning a sample using magnetic fields to deflect an electron beam (Russ, 2005). Sample information is obtained by multiple signals (i.e., secondary electrons, various elemental X-ray intensities, etc.) collected by different detectors and digitized separately, and then converted into a pixilated image (Russ, 2005). Bermudez-Aguirre, Mawson, and Barbosa-Canovas (2008) evaluated the microstructure of milk fat globules membranes
(MFGM) in whole milk. The study compared heated milk to thermosonicated milk at 63°C. The surface of the milk fat globule (MFG) was roughened after thermosonication, as the ultrasound waves disintegrated the MFGM and released triacylglycerides. The structure of the milk after sonication showed smaller fat globules (<1µm) and a granular surface, which was caused by the interaction between MFG and casein micelles. Ayala-Hernandez, Goff, and Corredig (2008) looked at the relationship between milk proteins and exopolysaccaride (EPS), which is produced by lactic acid bacteria in fermented media (milk permeate and buttermilk media). The media is pasteurized at 63 °C for 30 min and inoculated with 2% *L. Lactis ssp. cremoris* JFR1 and the procedure for sample preparation and immobilization was performed as described by Martin, Goff, Smith, and Dalgleigh (2006). They observed thin filament strands that connected the bacteria to the proteins. The EPS were clearly intertwined within the protein matrix. When SEM was performed in fermented buttermilk, there was an aggregation of EPS, protein and bacteria (pH 5.8), combined with a mesh-like network. In comparison, milk permeate had aggregates of whey proteins (multiple spheres connected), with stands of EPS. The EPS strands increased as the protein concentration increased.

9. Application

Multilayer emulsions can be used as fortified emulsions with lipid-soluble substances (e.g., vitamin D) which can be incorporated in different food systems such as beverages, milk, and/or cheese, to name a few. One possibility would be to add vitamin D to cheese as a promising solution to increase vitamin D consumption of the population.
and decrease vitamin D deficiency in society. The consumption of milk has declined over the years, and the incorporation of cheese into the diet has increased (Kazmi, Vieth, & Rousseau, 2007), which makes cheese a good alternative as a fortified food. The incorporation of vitamin D in a cheese matrix has been studied by many different groups of researchers. The first study was reported by Banville, Vuillemard, and Lacroix (2000), whose goal was to compare different methods for fortifying Cheddar cheese with vitamin D$_3$; Vitex D, an o/w emulsion with the vitamin solubilized in propylene glycol and polysorbate 80 (synthetic surfactant) as the emulsifier vs. crystalline vitamin D$_3$ in the milk fat vs. Vitex D in liposomes o/w/o solution (not homogenized)—liposomes encapsulated the vitex D). Banville incorporated vitamin D$_3$ at a concentration of 400 IU/liter of milk; the percent recovery of vitamin D$_3$ in the curd was 42.7 Vitex D, 40.5% crystalline vitamin D$_3$, 61.5% liposomes. The next group of researchers that evaluated fortification of dairy products with vitamin D was Kazmi et al. (2007), who researched the retention of vitamin D in cheese, yogurt and ice cream using Vitex D, Vitex D homogenized in butteroil, and crystalline vitamin D$_3$ (dissolved in ethanol and added to butteroil). The goal of Kazmi’s research was to develop ways to fortifying non-beverage dairy products with vitamin D at a lab-scale. Vitamin D concentration in the milk was 100,000 IU/kg milk or 50,000 IU/kg of milk. Considering a 10% yield during the cheese making process, the final amount of vitamin D in the cheese was 500-1000 IU/g of curd so 14,000-28,000 IU/serving. The total recovery of vitamin D in the whey and curd was $95.4 \pm 1.7\%$ for the crystalline vitamin D and $97 \pm 3.1\%$ for the emulsified one. Kazmi
comments on the high concentration in the samples for their HPLC process as they only sampled 1g at a time. Though understandable, the high concentration does not show if the same results would concur with lower concentrations. Wagner et al. (2008) evaluated the retention of vitamin D₃ in full and low-fat Cheddar cheese. The objective of Wagner’s study was to evaluate whether the retention of vitamin D was affected by the size of the manufacturing facility. These authors used Vitex D at a concentration of ~1000 IU/g for a 28,000 IU/serving. The recovery of vitamin D₃ in the cheese was 91% for full-fat and 55% for low-fat cheese. Note that the US upper tolerable limit for vitamin D₃ is 4,000 IU/day (National Institute of Health, 2011). Kazmi et al. (2007) used these high amounts so that HPLC analysis would only require 1 gram of the sample. Wagner et al. (2008) used Kazmi’s HPLC procedure and therefore fortified to the same levels.

The addition of an emulsion, which is primarily made of the same ingredients as the milk (with the added benefit of being a functional or key component to the cheese milk), would decrease the number of ingredients on the label and yet increase the value of the product. The emulsions used in this study can be incorporated not only into a cheese matrix to add functional ingredients, but also include oil-soluble flavors which might be of interest to artisan cheese makes to enhance their unique cheeses. An emulsion would be able to disperse a flavor compound throughout the curd, immediately, which would alter the flavor components differently than a rub. The emulsion could also be incorporated into salad dressings, dips, sauces, and beverages to alter the texture, add
functional lipid-soluble ingredients, etc. as they have different stabilities and various pH and texture.

Vitamin D is an essential vitamin (Figure 2.4). Vitamin D deficiency results in childhood rickets (Rani & Shaw, 2001; Tomashek et al., 2001) and the increased risk of chronic diseases, such as diabetes mellitus, cancer, autoimmune disorders and osteoporosis (Calvo, Whiting, & Barton, 2004). The body synthesizes vitamin D when exposed to sunlight (UVB radiation), and in today’s society, another major source is through supplements and fortified foods in the form of vitamin D$_2$ (ergocalciferol) or D$_3$ (cholecalciferol) (Holick et al., 2008). The amount of sunlight needed varies depending on age, skin, location, use of suntan lotion, etc., but 100 IU of D$_2$ is sufficient to avoid rickets (Holick, 2004). Armas, Hollis, and Heaney (2004) determined that vitamin D$_2$ was less effective than D$_3$; however, a more recent study (Holick et al., 2008) reported that the two were equally effective in maintaining serum 25-hydroxyvitamin D levels. Both studies indicate that vitamin D$_3$ is effective.

**Figure 2.4.** Chemical structures of vitamin D$_2$ (A) and vitamin D$_3$ (B) (Cyberlipid, n.d.b)
References


CHAPTER 3
FORTIFICATION OF CHEESE WITH VITAMIN D₃ USING DAIRY PROTEIN EMULSIONS AS DELIVERY SYSTEMS¹

Interpretive Summary

The objective of this study was to fortify cheese with vitamin D₃ and increase its retention using different dairy protein-based oil-in-water emulsions. Vitamin D₃ was incorporated as part of the oil phase of an oil-in-water emulsion, which was then mixed into the milk prior to making cheese. Vitamin D₃ retention in cheese curd formulated with the vitamin D₃ emulsions increased compared to the retention obtained using a lipid form of vitamin D₃. The retention of vitamin D₃ observed in the emulsion-fortified curds was not affected by the type of protein used during emulsion formulation.

Abstract

Vitamin D is an essential vitamin that is synthesized when the body is exposed to sunlight or after the consumption of fortified foods and supplements. The purpose of this research was to increase the retention of vitamin D₃ in Cheddar cheese by incorporating it as part of an oil-in-water emulsion using a milk protein emulsifier. Four oil-in-water vitamin D emulsions were made using sodium caseinate, calcium caseinate, nonfat dry milk, and whey protein and their use in making vitamin D fortified cheese curd was compared to using commercial vitamin D₃ oil using a model system.

¹ Coauthored with Silvana Martini, Carl Brothersen, and Donald J. McMahon
Significantly more vitamin D₃ ($P < 0.05$) was retained in the curd when using the emulsified vitamin D₃ than the control. No significant differences ($\alpha = 0.05$) were observed in the retention of vitamin D₃ between the different emulsifiers used. Mean vitamin D₃ retention in cheese curd was 96% when the emulsions were added to either whole or skim milk, compared to using the control fortified oil which gave mean retentions of only 71% and 64% when added to whole milk and skim milk, respectively, in a model system. Similar improvement in retention was achieved when cheese was made from whole and reduced fat milk. When sufficient vitamin D₃ was added to produce cheese containing a target level of approximately 280 IU per 28-g serving, retention was greater when the vitamin D was added to milk after emulsifying with nonfat dry milk than when using vitamin D₃ oil directly. Only 58% ± 3% of the vitamin D fortified oil was retained in full fat Cheddar cheese, while 78% ± 8% and 74% ± 1% was retained using the vitamin D emulsion in full fat and reduced fat Cheddar cheese, respectively.

**Introduction**

Vitamin D is an essential vitamin that is synthesized when the body is exposed to sunlight. However, the major source of vitamin D in modern society is through the consumption of fortified foods and supplements. In today’s environment, health organizations such as the US Food and Drug Administration are becoming more aware of the link between vitamin D deficiency and the increased risk of chronic diseases, such as diabetes mellitus, cancer, autoimmune disorders and osteoporosis (Calvo et al., 2004).
Recent studies in the United States and United Kingdom (Rani and Shaw 2001; Tomashek et al., 2001) have shown an increase in childhood rickets due to vitamin D deficiency suggesting that insufficient vitamin D is being ingested or synthesized.

In certain areas and cultures (e.g., veiled Arab Danish women) where the population is not exposed to sufficient sunlight, 85% of women have severe vitamin D deficiencies (Weaver and Fleet, 2004). Other sectors of the population that have limited capacity of vitamin D synthesis through cutaneous production are those who live in northern latitudes during winter, those who protect their skin from UVB rays, and elderly subjects and/or dark-skinned individuals (Weaver and Fleet, 2004; Calvo et al., 2004).

Current adequate intake of vitamin D as defined by the Food and Nutrition Board (FNB) at the Institute of Medicine (Indiana, US) is between 400-800 IU/d (depending on age, and assuming no vitamin D synthesis due to sun exposure) and the tolerable upper limit (UL) intake is 4,000 IU/d for all ages (National Institute of Health, 2011).

United States and Canadian populations are dependent on fortified foods and supplementation for their vitamin D needs during times when there is a lack of sunlight and subsequent ultraviolet-B absorption (Calvo et al., 2004). Vitamin D is present in some food sources such as eggs, mushrooms, and fish. Naturally occurring vitamin D levels in egg yolks and mushrooms are inconsistent. Fatty fish is also a natural source of vitamin D, with salmon being the most commonly consumed in the US. Other sources, such as liver and other organ meats have high vitamin D levels but are not consumed regularly. For many people vitamin D fortified milk has been the major source of dietary
vitamin D (Banville et al., 2000) but milk consumption has declined over the past years. In contrast, cheese consumption has increased; suggesting cheese may be a good alternative for vitamin D fortification. It is therefore important to evaluate the amount of vitamin D that can be retained in cheese during manufacture and aging (Banville et al., 2000).

Banville et al. (2000) studied the effect of vitamin D$_3$ delivery matrix on its retention in Cheddar cheese. Vitamin D$_3$ entrapped in multilamellar liposomes increased retention by approximately 20% (62% retention) compared to a water-soluble emulsion (i.e., oil-in-water (o/w) emulsion), or when crystallized vitamin D was mixed in the cream prior to addition to the milk (~41% retention). Kazmi et al. (2007) reported that when making vitamin D fortified cheese in a small-scale using either a pre-dissolved crystalline or a pre-emulsified form of vitamin D$_3$ (500 to 1,000 IU/g of cheese), the retention was approximately 90% for both forms of vitamin D$_3$. Finally, Wagner et al. (2008) studied the fortification of whole and low-fat cheeses. They found that retention was similar to that found by Kazmi for whole-fat cheese (91%), but they reported a lower retention of vitamin D in low-fat cheese with less than 55% retention; and they incorporated approximately 1,000 IU/g of cheese. The previous studies used a vitamin D$_3$ o/w emulsion with polysorbate 80 as the emulsifier and the vitamin D was dissolved in propylene glycol.

The objective of this study was to determine the retention of vitamin D$_3$ in full fat and low-fat curd model systems using oil-in-water emulsions formulated with different
dairy protein powders as emulsifiers (sodium and calcium caseinates, NDM, and whey protein concentrate), to verify the results within a Cheddar cheese system with a standard serving of vitamin D (200 to 400 IU/28 g serving), and to compare the retention of vitamin D$_3$ obtained in full fat and reduced fat cheese with that of a commercially available non-emulsified vitamin D$_3$ fortified oil.

**Materials and methods**

**Emulsion Formulation**

Oil-in-water (o/w) emulsions were made in triplicate using 4 protein powders: NDM (36% protein; Foster Farms Dairy, Modesto, CA), whey protein concentrate (WPC) (80% protein; Grande CIG, Lomira, WI), calcium caseinate (CaCN) (94.2% protein; Erie Foods International, Inc. Rochelle, IL) and sodium caseinate (NaCN) (94% protein; Erie Foods International, Inc.). The oil phase was a 1:1 by weight mixture of vitamin D$_3$ (10$^6$ IU/g of oil; BASF Corp, Florham Park, NJ) and soybean oil (Bunge Limited, White Plains, NY). The o/w emulsions consisted of 50 g/kg oil in a pH 7 0.01M Na$_2$HPO$_4$ solution containing 20 g/kg protein and 2 g/kg Microgard® 730 cultured dextrose, (Danisco, Bakersfield, CA) as an antimicrobial. Protein content of dairy powders was determined from the fact sheet the company provided with each powder and adjusted to 20g protein/kg of emulsion as necessary. Emulsions were blended using high shear force (Ultra Turrax:IKA T18 basic, IKA, Wilmington NC) for 1 min at 18,000 rpm and then homogenized with a microfluidizer (Model M-110S, Microfluidics Newton,
MA) (17.4 ± 1.6 MPa) for one pass. A commercial vitamin D₃ oil (Tate & Lyle, VS-AD200, Sycamore, IL) containing 40,000 IU/g was used as a control. The amount of vitamin D₃ in the fortified emulsions, control, fortified milk, cheese, whey, and curd was quantified as described below.

**Emulsion Physicochemical Stability**

Each emulsion (5 to 7 ml) was placed into individual flat bottom test tubes (Sci Tec Inc., Sandyhook, CT) and stability was measured daily for a week using a TurbiScan 2000 (Sci Tec Inc., Sandyhook, CT) in backscattering mode (Mengual et al., 1999). Readings were obtained every 40 μm of the length of the tube to characterize emulsion homogeneity. Repeating the measurement of backscattered light along the sample length over time produces a super-imposable emulsion fingerprint, which characterizes the stability or instability of the sample (i.e., the more identical the readings, the more stable the system) (Mengual et al., 1999; Tippetts and Martini 2009).

**Model Cheesemaking**

Pasteurized (72 °C for 15 s) skim and whole milk (non-homogenized) were obtained from the Gary Haight Richardson Dairy Products Laboratory (Utah State University, Logan, UT). Milk was stored at 5 °C before use. The milk was heated to 39 °C and cooled to 31 °C to restore coagulation properties inhibited by cooling (Qvist, 1979). Four hundred milliliters of milk was fortified with 400 μl of emulsion or 250 μl of non-emulsified control vitamin D₃ fortified oil (Tate & Lyle, VS-AD200, Sycamore, IL)
and homogenized with an Omni International 5000 (general laboratory homogenizer) (Omni International, Kennesaw, GA) for 30 s. The target level of vitamin D₃ was 25 IU/ml of milk.

Two hundred-milliliter portions of fortified milk were placed in 250-ml polycarbonate Nalgene centrifuge bottles (VWR, West Chester, PA). Then 4 g of glucono-δ-lactone and 0.4 ml of double strength (~650 International milk clotting units/ml) chymosin rennet (Maxiren DS; DSM Food Specialties, Parsippany, NJ) were added and incubated for 30 min at 31 °C to allow coagulum formation. The coagulum was manually cut using a metal spatula into 16 segments and then centrifuged at 1,000 g at 25 °C for 30 min. After centrifuging, the supernatant whey was decanted and weighed and curd was weighed. Approximately 100 g of whey and more than 5 g of curd were retained and frozen for vitamin D₃ analysis (AOAC, 2007) from a certified laboratory (O’Neal Scientific Services, Inc., MO). Each sample was performed in triplicate.

**Small Scale Cheesemaking**

Pasteurized (72 °C for 15 s), non-homogenized, and cooled milk (reduced fat [2%] and whole) was obtained from the Gary Haight Richardson Dairy Products Laboratory and made into cheese over 2 d (in triplicate) using 13.6 kg of milk per vat using a completely randomized design. Milk was pre-heated to 39 °C then cooled to 31 °C during which time 400 µl of NDM vitamin D₃ emulsion formulated as described above, was added to each vat. The NDM emulsifier was chosen for comparing the retention of vitamin D fortified o/w emulsions to the non-emulsified commercial vitamin
D₃ in full and reduced fat Cheddar cheese. The fortified emulsion was added at a concentration of 400 IU per serving (28 g of curd), which is within current guidelines (National Institute of Health, 2011). Control samples were fortified with 250 µl of non-emulsified commercial vitamin D₃ fortified oil (Tate & Lyle, VS-AD200, Sycamore, IL). Milk samples were taken at this point for vitamin D₃ quantification. After 5 min, 2.0 g of frozen pellets of *Lactococcus lactis* ssp. *lactis/cremoris* starter culture (DVS 850; CHR Hansen, Milwaukee, WI) was added as starter, then 1.6 ml of CaCl₂ (35% solution, supplier etc) and 0.7 ml of single strength annatto color (Nelson-Jameson) were added. After 30 min of ripening, double strength (~650 International milk clotting units/ml) diluted chymosin rennet (1:20 rennet: cold water) (Maxiren DS; DSM Food Specialties, Parsippany, NJ) was added, the milk was allowed to coagulate and the curd was cut after 30 min using wire harps (0.64 cm spacing). The curd was allowed to heal for 5 min and then stirred for 25 min. The curd was then heated to 39 °C over 25 min and then stirred for an additional 35 min. The whey was drained when the curd reached a pH of 6.3. The curd was dry stirred until it reached pH 5.5. A whey sample was taken for analysis prior to salting. Then the curd was salted (40 g NaCl), hooped, and pressed at 241 kPa overnight, vacuum packaged and stored at 4 °C. A portion of each cheese sample was grated with a microplane grater to homogenize the sample, vacuum packed, frozen, and sent for vitamin D quantification in duplicate.
The percent of IUs in the cheese was calculated as in Eq. 1.

\[
\left( \frac{\text{IU}_{\text{curd}}}{g_{\text{curd}}} \times \frac{\text{IU}_{\text{d.m}}}{g_{\text{d.m}}} \times 100 \right) = \%\text{IU}_{\text{curd/d.m}} \]

where IU\text{curd} is the amount of IUs of vitamin D in the curd, g\text{curd} is weight of the curd, IU\text{d.m} is the calculated value of how many IUs (vitamin D) were in the milk, g\text{d.m} is the weight of the milk, and \%\text{IU}_{\text{curd/d.m}} is the percent of IUs in the curd with respect to how many IUs were calculated to be in the milk.

**Statistical Analysis**

Cheese making trials were performed in triplicate. Data reported are the mean and standard error values calculated from the replicates. Significant differences were analyzed using a two- or one-way ANOVA test, as appropriate, and Bonferroni and LSD post-tests (\( \alpha = 0.05 \)). Statistical analysis was performed using Graph Pad software (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, www.graphpad.com) and SAS 9.1 TS Level 1M3 XP PRO platform. All emulsion treatments were compared to a control which utilized the non-emulsified commercial source of vitamin D.

**Results and Discussion**

**Emulsion Stability**

The stability of o/w emulsions formulated with the 4 different emulsifiers (i.e., NDM, WPC, CaCN and NaCN) is given in Figure 3.1.
Figure 3.1. Effect of emulsifier on the stability mean ± SD (change in height (mm) of serum level) of oil-in-water emulsions over one week: □, NDM; ▲, WPC; V, NaCN; ♦, CaCN. (n=4)

Emulsions had a rate of destabilization of approximately 0.6 to 0.7 mm/d and were considered stable as the rate was less than 1 mm/d (McClements, 2004). Emulsions formulated with WPC as emulsifier had a significantly (P<0.05) higher phase separation at d 7 than emulsions formulated with NDM or NaCN as emulsifiers. NDM, initially was more stable than the other emulsifiers; however, by d 7 was not significantly more stable than NaCN.

Model Cheese Making

The amounts of whey and curd obtained during the model process on average were 162 ± 2g and 42 ± 1g for full fat curd and 185 ± 1g and 20 ± 1g for low-fat curd.
The amount of curd obtained in the model cheese making using whole milk is higher (~20%) than the amount obtained during a standard cheese making process (~10%) due to the higher moisture content of the curd.

The higher moisture content in the model system is due to lack of pressing of the curd, which occurs during a standard cheese make process. The lower curd yield observed for the skim milk samples is due to the lack of fat (Mistry, 2001). No significant differences were found between treatments in the amount of whey and curd within a given milk type. Therefore, the type of emulsifier used did not impact the curd yield.

**Vitamin D Retention in Model System**

Mean vitamin D contents of the milk as well as the amount of vitamin D obtained in the whey and curd are shown in Table 3.1. Significantly less ($P < 0.05$) amount of vitamin D was obtained in the whey when using the emulsified treatments compared to the amount of vitamin D obtained in the whey when the model cheese curd was made with the addition of the non-emulsified control. Curds obtained with the emulsified vitamin D contained approximately 50% more vitamin D than the control curd (96% to 98% compared to 62% to 71%, respectively). Vitamin D retention was not significantly different ($\alpha = 0.05$) between curd model systems formulated with whole and skim milk. This is consistent with Kazmi et al. (2007) who studied vitamin D fortification of a Cheddar cheese-like system.
Table 3.1. Mean (± SE) amount of Vitamin D$_3$ added to whole and skim milk as either an unemulsified control, or emulsified using nonfat dry milk (NDM), whey protein concentrate (WPC), sodium caseinate (NaCN) or calcium caseinate (CaCN) and its partitioning between whey and curd produced using model cheese making, n= 3

<table>
<thead>
<tr>
<th>Emulsion</th>
<th>Milk</th>
<th>Whey</th>
<th>Curd</th>
<th>Whey + Curd</th>
<th>Recovery$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IU(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole Milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5,025 ± 451$^a$</td>
<td>1,152 ± 190$^b$</td>
<td>2,914 ± 286$^b$</td>
<td>4,066 ± 311$^a$</td>
<td>71.5 ± 2.9$^b$</td>
</tr>
<tr>
<td>NDM</td>
<td>4,771 ± 304$^a$</td>
<td>151 ± 51$^a$</td>
<td>4,418 ± 462$^a$</td>
<td>4,569 ± 448$^a$</td>
<td>96.6 ± 1.2$^a$</td>
</tr>
<tr>
<td>WPC</td>
<td>5,659 ± 552$^a$</td>
<td>164 ± 13$^a$</td>
<td>4,496 ± 205$^a$</td>
<td>4,660 ± 192$^a$</td>
<td>96.4 ± 0.4$^a$</td>
</tr>
<tr>
<td>NaCN</td>
<td>5,173 ± 527$^a$</td>
<td>110 ± 22$^a$</td>
<td>4,180 ± 300$^a$</td>
<td>4,290 ± 288$^a$</td>
<td>97.4 ± 0.6$^a$</td>
</tr>
<tr>
<td>CaCN</td>
<td>4,680 ± 192$^a$</td>
<td>113 ± 8$^a$</td>
<td>4,294 ± 252$^a$</td>
<td>4,407 ± 245$^a$</td>
<td>97.4 ± 0.3$^a$</td>
</tr>
<tr>
<td>Skim Milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5,219 ± 611$^a$</td>
<td>1,349 ± 214$^b$</td>
<td>2,214 ± 171$^b$</td>
<td>3,562 ± 302$^a$</td>
<td>62.4 ± 3.6$^b$</td>
</tr>
<tr>
<td>NDM</td>
<td>5,992 ± 187$^a$</td>
<td>99 ± 13$^a$</td>
<td>4,252 ± 140$^a$</td>
<td>4,351 ± 127$^a$</td>
<td>97.7 ± 0.4$^a$</td>
</tr>
<tr>
<td>WPC</td>
<td>5,292 ± 653$^a$</td>
<td>152 ± 27$^a$</td>
<td>3,948 ± 548$^a$</td>
<td>4,100 ± 574$^a$</td>
<td>96.3 ± 0.2$^a$</td>
</tr>
<tr>
<td>NaCN</td>
<td>5,096 ± 361$^a$</td>
<td>172 ± 21$^a$</td>
<td>4,589 ± 76$^a$</td>
<td>4,761 ± 61$^a$</td>
<td>96.4 ± 0.5$^a$</td>
</tr>
<tr>
<td>CaCN</td>
<td>4,967 ± 456$^a$</td>
<td>140 ± 7$^a$</td>
<td>4,398 ± 139$^a$</td>
<td>4,538 ± 146$^a$</td>
<td>96.9 ± 0.1$^a$</td>
</tr>
</tbody>
</table>

$^1$Recovery = curd/(whey + curd)*100.

$^{ab}$Means with a common letter in the same column were not significantly different ($\alpha = 0.05$).
**Vitamin D Retention in Cheddar Cheese**

Nonfat dry milk was used as the emulsifier for this experiment as the emulsion had the best stability over the first 48 h and no differences on vitamin D retention were observed as a function of emulsifier used as previously discussed (Table 3.1). The proximate analyses of the cheeses obtained are summarized in Table 3.2. The yields obtained for the whole and reduced cheeses were approximately 9% and 7%, respectively.

Retention of vitamin D₃ was not significantly different \((P < 0.05)\) between cheeses made from whole or reduced fat milk (Table 3.3). The similarity of retention between milk types might be attributed to the interaction between the protein emulsifier and the casein curd matrix.

**Table 3.2.** Proximate analysis of Cheddar cheese made with whole and reduced fat milk (n=3). Control is whole-fat Cheddar cheese with fortified vitamin D oil.

<table>
<thead>
<tr>
<th>Milk</th>
<th>Mass (kg)</th>
<th>Fat (%)</th>
<th>pH</th>
<th>Moisture (%)</th>
<th>Salt (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>1.23 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.3 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.45 ± 0.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>36.2 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.00 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduced</td>
<td>0.97 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.0 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.50 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.8 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.46 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>1.23 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.7 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.27 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.1 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.54 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-b</sup>Means within a column with different superscripts are significantly different \((\alpha = 0.05)\)
Table 3.3. Summary of the Mean ± SE of vitamin D₃ (IU) retention in small-scale curd (c) and calculated vitamin D₃ in milk (d.m) and the percent retained in the curd versus the total in the d.m (recovery). (n=3)

<table>
<thead>
<tr>
<th>Milk</th>
<th>Curd</th>
<th>Milk (d.m)</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>7,887 ± 450ᵃ</td>
<td>10,147 ± 67ᵃ</td>
<td>78 ± 8ᵃ</td>
</tr>
<tr>
<td>Reduced</td>
<td>7,580 ± 93ᵃ</td>
<td>10,213 ± 67ᵃ</td>
<td>74 ± 1ᵃ</td>
</tr>
<tr>
<td>Control</td>
<td>5,902 ± 192ᵇ</td>
<td>10,175 ± 0ᵃ</td>
<td>58 ± 3ᵇ</td>
</tr>
</tbody>
</table>

ᵃᵇ Means within a column with different superscripts are significantly different (α = 0.05)

Whereas, direct addition of vitamin D₃ oil, the oil would migrate to the milk fat, and then be lost in the whey. The significantly (P < 0.05) greater retention of the emulsions to that of the control indicates that there is an increase in reactions between the emulsions and the curd than with the oil just being trapped within the curd matrix.

The retention of vitamin D reported in this paper are lower (78 ± 8 % and 74 ± 1 %) than those of Kazmi et al. (2007), which was 90.3 ± 3.5 % for an approximate 10% yield with 39.2% ± 0.3% moisture for their Cheddar cheese-like samples; however, our model system showed a 40% higher retention than observed by Wagner et al. (2008) with low-fat curd fortified with high concentrations of vitamin D. The amount of vitamin D initially added to milk between studies was drastically different. The amount of vitamin D for small batch cheese of 5 to 10 IU/g or 280 IU/serving was significantly lower than that of Kazmi et al. (2007) (500 to 1,000 IU/g or 14,000 to 28,000 IU/serving) or Wagner et al. (2008) (~900 IU/g). The difference in concentration of vitamin D could be
significant between the two tests, as our model results concurred with Kazmi's results; our results having a high retention (~97%) for all emulsion samples (Table 3-2) and contained approximately 250 IU/g. The decrease in concentration of the vitamin D might have led to a higher IU variance in the cheeses, which might explain the discrepancy between the studies. Also, with a larger amount of whey (~12kg), and a decrease in the concentration of vitamin D it would be difficult to obtain an accurate sampling of the vitamin D in the whey. This might explain the non-existence of vitamin D for Banville et al. (2000) in the whey, and the excessive IU amounts for the control’s whey vitamin D content in this current study (~7,000 IU), which was more than 68% of what was added and did not correlate with the IU found in the curd. The amount of vitamin D in the cheese is important as manufactures would like to create a functional product without wasting the functional ingredient, or being accused of excessive levels (>4,000 IU/serving, the tolerable upper intake limit (National Institute of Health, 2011). The concentration of vitamin D needs to be considered as studies with amounts of vitamin D above the daily upper tolerable limit might not have the same results within the daily recommended allowance, as discovered in this study.

**Conclusion**

This research indicated that oil-in-water emulsions formulated with a milk protein emulsifier can be used to improve the retention of vitamin D in full and reduced fat cheeses. The use of dairy proteins as the emulsifier instead of polysorbate 80, would also increase the use of dairy ingredients versus synthetic additives. The retention of vitamin
D in cheese curd is improved by incorporating vitamin D as part of an emulsion using milk proteins as the emulsifier prior to adding it to the milk. With retentions of 96% to 98% in a model system and 74% to 78% in reduced and full fat cheese in a small batch Cheddar cheese system. Though, the type of protein powder (NDM, WPC, or caseinates) used to formulate the emulsion did not affect the retention of vitamin D in the curd, the use of a dairy protein emulsifier was better than incorporating the fortified oil alone. However, this study demonstrates the need to assess fortification at applied levels rather than extreme levels if the fortification is to be of use in industry.

References


CHAPTER 4
INFLUENCE OF τ–CARRAGEEenan, PECTIN, AND GELATIN ON THE PHYSICOCHEMICAL PROPERTIES AND STABILITY OF MILK PROTEIN-STABILIZED EMULSIONS²

Abstract

This study evaluated the stability of bi-layer emulsions as a function of secondary layer composition and pH. Primary emulsions were formulated with 5% soybean oil, 1% protein from nonfat dry milk powder as emulsifier and τ-carrageenan (τ-carr), low methoxylpectin (LMp), high methoxylpectin (HMp), or gelatin as secondary layers. ζ-potential values increased for each emulsion as the pH decreased from 7 to 3, with τ-carr emulsions being consistently more negatively charged than the primary emulsion and significantly more stable. ζ-Potential values were not always correlated to emulsion stability. Gelatin secondary emulsions formulated at pH 3 and HMp secondary emulsions formulated at pH 7 were unstable due to the presence of depletion flocculation. In addition, the stability of LMp secondary emulsions at pH 7 might be due to an increased viscosity in the system. The stability of bi-layer emulsions in this research is driven by the presence of depletion flocculation as previously show by others, droplet charge, droplet size and distribution and viscosity.

² Coauthored with Silvana Martini
**Practical Application**

This research has practical applications for industry. The use of everyday ingredients (nonfat dry milk powder, gelatin, pectin, and carrageenan), which are understood and accepted by the average consumer, creates label-friendly products that are the wave of the future. Stable emulsions can be formed using these ingredients at various pH values. Understanding the stability and how pH impacts the physicochemical characteristics and stability of these emulsions will enable manufactures to use ordinary ingredients to create products that are more healthful to the consumer (e.g., low-fat dressings, sauces, dips, and beverages) without necessitating additional consumer education.

**Introduction**

Multi-layer emulsions are new delivery systems with unique encapsulation and release properties that can be used to improve the stability of food systems (Dickinson and Pawlowsky 1997; Gu and others 2005a, 2005b; Guzey and McClements 2006a; Mun and others 2008; Li and others 2010; Vladisavljevic and McClements 2010; Hu and others 2011). Multi-layer emulsions are obtained by combining oppositely charged components in the oil/water interface through a process called electrostatic deposition. In general, the addition of a secondary layer increases emulsion stability over a wide range of processing conditions (e.g., pH, ionic strength, and temperature) (McClements 2005a; Mun and others 2005; Guzey and McClements 2006b). Previous studies evaluated the effect of pH (Dickinson and Pawlowsky 1997; Dickinson and others 1998; Gu and others
2004a, 2004b), secondary layer type (Gu and others 2005a), concentration (Dickinson and Pawlowsky 1997), and ionic strength (Gu and others 2004a, 2004b; Guzey and McClements 2007) on the stability, particle size, and ζ-potential value of the primary and multilayered emulsions (Dickinson and Lopez 2001; Ogawa and others 2003; Gancz and others 2006). Specific proteins (e.g., β-Lg, BSA, caseins, sodium caseinate) were used in these studies and their interaction with the secondary layer (e.g. carrageenan, pectin, gelatin) were evaluated. The stability of these systems was highly dependent on the chemical nature of the molecules forming the different layers. These studies provide an excellent background regarding the fundamental science responsible for the formation of multilayered emulsions. However, the use of a more complex emulsifier, such as nonfat dry milk, which is a common ingredient available to the food industry, has not been tested in the formation of multilayered emulsions. Some research has been performed using milk solutions (3.4% of protein) with polysaccharides (Tuinier and others 1999; Tuinier and de Kruif 1999; Acero-Lopez and others 2010). The milk studies showed depletion flocculation occurs in the presence of high-methoxyl pectin or exocellular polysaccharides. The biopolymers segregated into casein-rich and polysaccharide-rich phases and did not interact; however, the addition of κ-carrageenan reduced the rate of destabilization between pectin and casein phase separation. In addition, the effect of emulsion viscosity on the stability of multilayered emulsions formulated with nonfat dry milk as emulsifier was not reported in the scientific literature.
The objective of this study was to formulate bi-layer oil-in-water (o/w) emulsions using soybean oil as the oil phase and nonfat dry milk powder as emulsifier. The secondary layer was \( \epsilon \)-carrageenan, low or high methoxyl pectin, or gelatin. The effect of a secondary layer on the emulsion’s physicochemical properties (i.e., stability, \( \zeta \)-potential, viscosity, and droplet size and distribution) as a function of pH was evaluated. The goal of this study is to provide evidence that \( \zeta \)-potential values and droplet size and distribution are not the only parameters that define stability of bi-layer emulsions stability.

**Materials and Methods**

*Primary emulsion:* Oil-in-water emulsions were formulated with 5 wt% soybean oil (SBO) (Western Family Inc., Madison, WI). One percent protein (1 wt%) from nonfat dry milk (NDM) powder was used as emulsifier based on being 36% protein. NDM also includes 1.5% lactose, 0.2% ash, and approximately 0.4% calcium, 0.3% phosphorus, 0.05% potassium, and 0.01% sodium (Foster Farms Dairy, Modesto, CA, U.S.A.). The NDM was dispersed in a 5mM phosphate buffer solution (pH 7) and stirred for 30 min at ambient temperature; and 0.2% Microgard\textsuperscript{®} 730 (Danisco, Madison, WI, U.S.A.) was added as an antimicrobial.

*Emulsion preparation:* The lipid and aqueous phases were mixed using an Ultra Turrax (IKA T18 basic, Wilmington, NC, U.S.A.) at 18,000 rpm for 1 min, then immediately put through a Microfluidics microfluidizer processor (Model M-110S, Newton, MA) at 17.4 \pm 1.6 MPa for 3 cycles.
Secondary emulsion: Independent solutions of 0.4 wt% of t-carregeenan (t-carr), low-methoxyl pectin (LMP), high-methoxyl pectin (HMP) (TicGums, White Marsh, MD, U.S.A.) and gelatin (Kraft Foods Global, IL, U.S.A.) were prepared in 5mM phosphate buffer solution (pH 7) and heated to 50°C for 30 min. The primary emulsion and secondary solution in a 50:50 weight ratio were mixed using a modification of the method reported by Taherian and others (2011): Ultra Turrax was used for 30 seconds at 18,000 rpm followed by two cycles through the microfluidizer at 17.4 ± 1.6 MPa. The final oil content in these secondary emulsions was 2.5%, with 0.5 wt% protein and 0.2% secondary polysaccharide or protein. A control emulsion with 2.5% SBO without a secondary layer was also formulated.

pH: After formulation, the pH of the primary and secondary emulsions was changed to 7, 5 and 3 (± 0.1) using 0.1 M HCl or NaOH. The pH values were chosen at neutral pH, near the isoelectric point (pI) of the milk proteins (4.6-5.2), and below the pI of the milk proteins to determine if a secondary layer could improve the stability at pH values where the primary emulsion would be prone to instability.

ζ-potential: Primary and secondary emulsions were added to 5mM phosphate buffer solutions at 0.5 wt% to avoid light scattering during measurement. The buffer was changed to pH 3 and 5 (± 0.1) using 0.1 M HCl. Five readings were taken of each sample using a Zeta-meter System 3.0+ (Zeta-Meter Inc., Staunton, VA, U.S.A.). Details on the use of this technique can be found in Wu (2007).
Physicochemical stability: Five to seven milliliters of the emulsions were placed in a test tube designed for the TurbiScan 2000 (Sci-Tec Inc., Sandyhook, CT, U.S.A.). Samples were stored at 5° C and readings were taken daily over the span of a week according to Tippetts and Martini (2009).

Droplet size analysis: Droplet size distributions for emulsions were determined using Beckman Coulter particle characterization equipment (LS20 Version 3.19, Beckman Coulter Inc., Brea, CA, U.S.A.) (Xu and di Guida, 2003). The distribution is reported as volume percent with respect to the droplet’s diameter. The diameter measurement is reported as the volume-surface mean diameter \( d_{3,2} \) using equation 1 where \( n_i \) is the number of droplets of diameter \( d_i \) (McClements 2005b.)

\[
\frac{1}{d_{3,2}} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}
\]

Rheology measurements: The viscosity of the samples was measured using a magnetic bearing rheometer (TA Instruments, AR-G2, Wilmington, DE, U.S.A.) with a standard sized concentric aluminum cylinder. The flow procedure (5 °C) was set at a shear rate range from 0.001 to 300.0 s\(^{-1}\). The viscosity of the samples was measured on days 0, 3, and 7 for stable emulsions and days 0 and 3 for unstable emulsions.

Statistical analysis: Experiments were performed in duplicate. Data reported are the means and standard deviations calculated from the replicates. Significant differences were analyzed using a two- or one-way ANOVA test, as appropriate, and Bonferroni and LSD post-tests (\( \alpha = 0.05 \)). Statistical analysis was performed using Graph Pad software.
Results and Discussion

ζ-potential: Figure 1 shows ζ-potential values of primary and secondary emulsions as a function of pH. The ζ-potential of the primary emulsion was significantly affected (p<0.05) by the pH of the emulsion (-27.7 ± 2.4 mV; -17.4 ± 3.0 mV; and 21.5 ± 1.3 mV for pH 7, 5, and 3, respectively). These values do not differ significantly from those of the aqueous solution (NDM solution prior to homogenization) used to prepare the emulsion (-23.0 ± 2.7, -19.4 ± 6.6, and 27.0 ± 5.6 mV for pH 7, 5, and 3, respectively). The net electrical charge was negative at pH 7, being above the isoelectric point (pI) of milk proteins (pI ~4.6-5.2) and became positive as the emulsion’s pH decreased to values below the pI of milk proteins (pH = 3). The ζ-potential values of each secondary emulsion exhibited a similar significant decrease (p<0.05) with increasing pH except for low-methoxyl pectin (LMp) and gelatin, where no difference in ζ-potential were observed between pH 5 and 7 for each emulsion. The ζ-potential of primary emulsions are similar to the ones reported by other authors (Anema and Klostermeyer 1996; Wade and Beattie 1997; Michalski and others 2001; Surh and others 2006).
Figure 4.1. \(\zeta\)-potential of primary and secondary emulsions (mean ± SE; n=5). □: primary; ▲: t-carrageenan; ▼: Low-methoxyl pectin; ◆: high-methoxyl pectin; and ●: gelatin. Different superscripts indicate significant difference (p<0.01) at each pH between emulsions (lower case) and for each emulsion between pH values (upper case).

The \(\zeta\)-potential values of gelatin secondary emulsions at pH 7 and 5 (zero and 3.2 ± 3.4 mV, respectively) are significantly (p<0.001) higher than the ones observed for primary emulsions. Gelatin (pI = 5) solutions used to formulate the secondary emulsions have a net zero charge at pH 7 and 5 (\(\zeta\)-potential = 0) and have a \(\zeta\)-potential of 22.6 ± 4.0 mV at pH 3. The lack of charge in the gelatin protein solution at pH 7 and 5 results in a partial neutralization of the \(\zeta\)-potential of the emulsion when gelatin is added to the primary emulsion with values increasing from -27.7 ± 2.4 mV; -17.4 ± 3.0 mV for the
primary emulsion to 0 and 3.2 ± 3.4 mV for the secondary emulsion formulated with gelatin for pH 7 and 5, respectively. These results suggest that electrostatic interactions for protein-protein layers (gelatin and milk proteins) are formed between the positively and negatively charged areas on the two proteins. Fewer interactions between the two proteins occurred at pH 3 as both proteins are positively charged, and no significant difference was found between the primary and secondary emulsion’s ζ-potential.

The addition of HMp as a secondary layer resulted in a significant decrease (p<0.05) in the ζ-potential at pH 3 (8.0 ± 2.3 mV) compared to the primary emulsion. However, as the pH increased to 5 and 7, the ζ-potential was not significantly different from the primary emulsion, indicating negligible adsorption took place. The ζ-potential of the HMp solutions are -14.2 ± 1.4, -9.9 ± 1.5, and -4.3 ± 1.8 mV for pH 7, 5, and 3, respectively. When the HMp emulsion is adjusted to pH 3, electrostatic interactions between the HMp, with a net negative charge, and the protein, with a net positive charge, decrease the ζ-potential for the secondary emulsion in a significant manner (p<0.05).

The ζ-potential of ι-carrageenan (ι-carr) secondary emulsion was significantly lower (p<0.001) than the primary emulsion at each pH (-43.3 ± 3.2, -36.3 ± 2.4, and zero mV for pH 7, 5, and 3, respectively). The ι-carr secondary emulsions have a cumulative ζ-potential value of the primary and ι-carr solution (-23.2 ± 4.3, -19.9 ± 4.8, and -19.2 ± 4.2 mV for pH 7, 5, and 3, respectively). These results are in agreement with the ones reported by Gu and others (2004a).
As previously mentioned, at pH 5 and 7, the t-carr solution and the primary emulsion are negatively charged; therefore, no interactions among the t-carr and the milk proteins are expected. Our results suggest that the electrostatic interactions observed in the secondary emulsions formulated at pH 5 and 7 are a consequence of interactions formed between the positively charged areas on the protein with the anionic polysaccharide; that is between the –NH$_3^+$ (milk proteins) and –OSO$_3^-$ (t-carr) groups (Dickinson 1998). Gu and others (2004a) reported a lack of interaction between t-carr and β-Lg at pH 7. The increased interaction observed in our research might be due to the second homogenization step used to formulate the secondary emulsions. In particular, the secondary homogenization step might increase the absorption of the secondary layer by exposing more cationic areas of the flexible caseins (Singh 2011) due to the additional pressure to interact with the anionic polysaccharides and thereby influencing the absorptivity and stability of the emulsion. This could explain the increase in the ζ-potential for the secondary emulsions here, when there is no change in ζ-potential for Gu and others (2004a).

The ζ-potential of LMp secondary emulsions at pH 5 (-32.9 ± 3.0 mV) was significantly (p<0.001) lower than the primary emulsion suggesting that there is an interaction between the two layers for these emulsions. As described by Dickinson (1998) the anionic groups (–COO$^-$) of the pectins (HMp and LMp) interact with positive patches (–NH$_3^+$) present in the first layer of emulsifier formed by the milk proteins. No significant differences were found in the ζ-potential values of the LMp secondary
emulsions and the primary emulsions at pH 3 and 7. The calcium (divalent cation) in the system at pH 7 became a bridge between carboxyl groups on the pectin strands. At pH 3, the calcium would then be competitive with the positively charged proteins to interact with the anionic groups on pectin. At neither pH would the pectin be interacting with the proteins, which is why the ζ-potential values did not change significantly from the primary emulsion.

Physicochemical stability: Figure 4.2 shows the phase separation kinetics of primary, control, and secondary emulsions stored for 7 days at 5 °C as a function of pH. The amount of phase separation is defined by the change in height (mm) of the aqueous layer. As shown in Figure 4.2A, the primary emulsion was stable at pH 7 with a rate of destabilization of 0.51 ± 0.09 mm/d. The average destabilization rates for the primary emulsion at pH 5 and 3 are 2.57 ± 0.46 and 1.87 ± 0.20 mm/d, respectively. The primary emulsions were less stable as the pH approached the pI of the milk proteins due to protein aggregation from the reduction in electrostatic repulsion, as is seen in the reduction in ζ-potential (Figure 4.1).

The control emulsion (Figure 4.2B) is less stable than the primary emulsion with a destabilization rate of 1.13 ± 0.07, 2.76 ± 0.46 and 2.18 ± 0.50 mm/d for pH 7, 5 and 3, respectively. Lower stability was attributed to the lower oil content in the emulsions, which can increase the rate of phase separation (Tippetts and Martini 2009).
Figure 4.2. Change (Δ) in height (mean ± SE; n = 5) of the aqueous phase of the destabilized emulsions for A: primary emulsion; B: control; C: gelatin; D: HMp, E: t-carr and F: LMp, secondary emulsions at pH 3: ■; 5: ▲ and 7: ▼.
The gelatin secondary emulsion (Figure 4.2C) stability declined as the pH decreases from 7 to 3 (rate of destabilization of $0.70 \pm 0.03$ mm/d, $2.70 \pm 0.74$, and $3.36 \pm 0.90$ mm/d for pH 7, 5, and 3 respectively). These stability results cannot be predicted by the $\zeta$-potential values presented in Figure 4.1 since secondary emulsions formulated with gelatin at pH 7 are significantly more stable that the ones formulated a pH 5 and 3 even though the $\zeta$-potential values at pH 7 is zero. Similarly, secondary emulsions formulated with gelatin at pH 3, which had $\zeta$-potential values of approximately -18 mV (Figure 4.1) are the least stable as shown in Figure 4-2C.

While most of the secondary emulsions are stable at pH 7, HMp (Figure 4.2D) is not. This secondary emulsion destabilized at a rate of $4.38 \pm 0.43$ mm/d. Even though the $\zeta$-potential of the primary, HMp, and LMP are not significantly different at pH 7, their phase separation kinetics are. At high pH (>4), the electrostatic interactions between HMp and the milk proteins become weaker (Figure 4.1) as the pH increases and free HMp molecules might trigger depletion flocculation events (Dickinson and others 1998; Gancz and others 2006) resulting in highly unstable secondary emulsions at pH 7 and 5 (destabilization rate of $2.54 \pm 0.88$ mm/d). The high stability of HMp secondary emulsions at pH 3 (destabilization rate: $0.63 \pm 0.25$ mm/d) is an expected result due to the $\zeta$-potential difference between the primary and secondary emulsions as observed in Figure 4.1 suggesting interactions between milk proteins as they become positively charged and form complexes with the anionic pectin (Gancz and others 2005).
Secondary emulsions with the greatest stability, independent of pH, are formulated with t-carr (Figure 4.2E), which also had significantly lower ζ-potential values, indicating electrostatic attraction between the two layers. These emulsions are significantly (p<0.001) more stable at pH 5 and pH 3 than all the other emulsions with rates of destabilization of 0.07 ± 0.07, 0.05 ± 0.05 and 0.20 ± 0.06 mm/d for pH 7, 5, and 3, respectively. The increased stability might be due to the sulfate groups present on the carrageenan, which remain ionized at all practical pH values (Damodaran and others 2008).

Similar to the gelatin secondary emulsions, LMP secondary emulsions (Figure 2F), which are stable at pH 7 (0.2 ± 0.09 mm/d), become increasingly unstable as the pH decreases.

Droplet size distributions: Figures 4.3 to 4.5 show the droplet size distribution based on volume percent of droplets as a function of droplet diameter at pH 7 (Figure 4.3), 5 (Figure 4.4), and 3 (Figure 4.5) and as a function of storage time (days 0, 3, and 7). No significant (p > 0.05) differences were found in the droplet size diameter (d3,2) over time and between emulsions for emulsions at pH 7.

The average d3,2 for the primary emulsion was 1.14 ± 0.17 μm. The control, gelatin, HMP, t-carr, and LMP secondary emulsions have a d3,2 of 1.01 ± 0.09; 0.99 ± 07; 1.08 ± 0.08; 1.15 ± 0.10; and 0.98 ± 0.07 μm, respectively. The distributions at pH 7 (Figure 4.3) also have little deviation over time.
Figure 4.3. Mean droplet size diameter (µm; n = 3) distribution with respect to percentage of the volume (Vol%) at a given diameter at pH 7 for primary (A), control (B), gelatin (C), HMp (D), τ-carr (E) and LMp (F) emulsions for days 0: ■; 3: ▲ and 7: ▼.
**Figure 4.4.** Mean droplet size diameter (µm; n = 3) distribution with respect to percentage of the volume (Vol%) at a given diameter at pH 5 for primary (A), control (B), gelatin (C), HMp (D), i-carr (E) and LMp (F) emulsions for days 0: ■; 3: ▲ and 7: ▼.
Figure 4.5. Mean droplet size diameter ($\mu$m; n = 3) distribution with respect to percentage of the volume (Vol%) at a given diameter at pH 3 for primary (A), control (B), gelatin (C), HMp (D), t-carr (E) and LMp (F) emulsions for days 0: ■; 3: ▲ and 7: ▼.
One noticeable exception is the control emulsion (Figure 4.3B) and the gelatin secondary emulsion (Figure 4.3 C), where droplets with larger diameters can be observed after 7 days of storage. However, this progression did not significantly affect $d_{3.2}$ values. It is interesting to note that even though HMP secondary emulsion had a low $d_{3.2}$ value and was not significantly different from the other emulsions, it is significantly less stable (Figure 4.2 D and 4.3 D), which was previously explained by the presence of depletion flocculation (Gancz and others 2006).

Droplet size distributions at pH 5 were broader than those observed at pH 7 and in some cases bi-modal distributions were observed (Figure 4.4). At pH 5, emulsions are very close to the pI of the milk proteins and therefore, droplets are more likely to coalesce and form larger droplets. Table 4.1 reports $d_{3.2}$ values for these emulsions which were significantly ($p<0.05$) larger than the ones observed for emulsion formulated at pH 7 for the primary, control, and gelatin emulsions. For gelatin the $d_{3.2}$ at pH 5 is significantly ($p<0.001$) greater than the ones obtained at pH 3 and 7, which can be explained by the lack of charge in the droplets at pH 5 (Figure 4.1) as both proteins are close to the pI. A slightly broader distribution is observed for the primary, control and LMP secondary emulsions. Though LMP and gelatin secondary emulsions’ $d_{3.2}$ are significantly larger than the rest, only t-carr is stable. As previously discussed, this stability is probably due to the sulfate groups present in the t-carr, causing electrostatic repulsions between droplets (Damodaran and others 2008).
### Table 4.1. Droplet size diameter mean ($d_{3,2} \pm SD$ in µm) at pH 5 for day 0, 3, and 7 for primary, control, and secondary emulsions (gelatin, HMp, t-carr, and LMp)

<table>
<thead>
<tr>
<th>Emulsion</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>$7.21 \pm 1.75^a$</td>
<td>$6.12 \pm 3.04^a$</td>
<td>$6.53 \pm 3.12^a$</td>
</tr>
<tr>
<td>Control</td>
<td>$11.99 \pm 1.72^a$</td>
<td>$11.27 \pm 2.08^a$</td>
<td>$11.43 \pm 3.21^a$</td>
</tr>
<tr>
<td>Gelatin</td>
<td>$54.74 \pm 11.42^b$</td>
<td>$57.47 \pm 9.29^b$</td>
<td>$56.94 \pm 12.84^{bc}$</td>
</tr>
<tr>
<td>HMp</td>
<td>$5.46 \pm 3.35^a$</td>
<td>$4.96 \pm 3.52^a$</td>
<td>$4.19 \pm 1.80^a$</td>
</tr>
<tr>
<td>t-carr</td>
<td>$4.18 \pm 0.70^a$</td>
<td>$7.95 \pm 5.20^a$</td>
<td>$7.53 \pm 1.01^{ab}$</td>
</tr>
<tr>
<td>LMp</td>
<td>$67.89 \pm 41.40^b$</td>
<td>$60.09 \pm 52.49^b$</td>
<td>$82.59 \pm 91.51^c$</td>
</tr>
</tbody>
</table>

Superscripts indicate significant differences ($p<0.05$) among columns

Droplet size distributions observed at pH 3 were similar to the ones described for pH 5, with broader distributions for the primary, control, t-carr and LMp secondary emulsions (Figure 4.5 A-B, E-F). The primary and control emulsions both had greater $d_{3,2}$ (Table 4.2) compared to those at pH 5 as did secondary emulsions formulated with t-carr and LMp. On the other hand, the droplet size distribution of gelatin and HMp secondary emulsions is shifted toward smaller sizes compared to the distributions at pH 5 (Figure 4.5 C-D and Figure 4.4 C-D). The $d_{3,2}$ at pH 3 (Table 4.2) for gelatin is slightly larger than the one observed at pH 7 but significantly smaller than reported at pH 5; nevertheless the emulsion is more unstable at pH 3 (Figure 4.2). Like HMp, the high instability of gelatin at pH 3 might be due to excess gelatin in the media due to the lack of
interaction between the proteins (Figure 4.1) triggering depletion flocculation. HMp secondary emulsion’s droplet size of is not significantly different between pH 3 and 5; however, the stability of the emulsion at pH 3 is significantly greater (p<0.001), which correlates with the difference between the primary and HMp secondary emulsion’s ζ-potential (Figure 4.1). It is interesting to note that though the $d_{3,2}$ for t-carr secondary emulsion at pH 3 is greater than at pH 5, and even greater than HMp at pH 3, this secondary emulsion (t-carr) is significantly more stable. As previously discussed, this increased stability of t-carr secondary emulsions at different pH values might be due to the many sulfate groups, and correlates with the large ζ-potential differences between the primary and secondary emulsions reported in Figure 4.1.

Table 4.2. Droplet size diameter mean ($d_{3,2} \pm SD$ in µm) at pH 3 for day 0, 3, and 7 for primary, control, and secondary emulsions (gelatin, HMp, t-carr, and LMp)

<table>
<thead>
<tr>
<th>Emulsion</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>8.88 ± 3.23$^{ab}$</td>
<td>9.27 ± 2.82$^{ab}$</td>
<td>9.44 ± 3.24$^{ab}$</td>
</tr>
<tr>
<td>Control</td>
<td>10.08 ± 0.92$^a$</td>
<td>12.64 ± 2.88$^a$</td>
<td>11.75 ± 2.79$^a$</td>
</tr>
<tr>
<td>Gelatin</td>
<td>6.91 ± 1.02$^{ab}$</td>
<td>5.46 ± 1.65$^b$</td>
<td>5.66 ± 0.96$^b$</td>
</tr>
<tr>
<td>HMp</td>
<td>4.30 ± 1.33$^b$</td>
<td>4.56 ± 1.43$^b$</td>
<td>3.24 ± 0.36$^b$</td>
</tr>
<tr>
<td>t-carr</td>
<td>12.14 ± 2.50$^a$</td>
<td>9.68 ± 5.08$^{ab}$</td>
<td>13.83 ± 4.34$^a$</td>
</tr>
<tr>
<td>LMp</td>
<td>53.93 ± 33.38$^a$</td>
<td>98.73 ± 59.56$^c$</td>
<td>143.46 ± 175.26$^c$</td>
</tr>
</tbody>
</table>

Superscripts indicate significant differences (p<0.05) among columns.
**Viscosity:** Figure 4.6 shows the viscosity of the emulsions as a function of time and pH at a shear rate of 0.1 s⁻¹. All emulsions showed shear-thinning behavior.

Viscosity of the primary emulsions (Figure 4.6A) at pH 7 did not significantly change over time (0.01 ± 0.00 Pa.s). The viscosity increased significantly (p<0.05) by day 3 for pH 5 (2.66 ± 2.18 Pa.s) and 3 (3.04 ± 1.03 Pa.s), due to creaming. The same trends can be seen for the control emulsion (Figure 4.6B), which at pH 7 has an average viscosity of 0.01 ± 0.00 Pa.s. By day 3, the viscosity of the control emulsions for pH 3 and 5 (0.82 ± 0.08 Pa.s; 0.63 ± 0.12 Pa.s, respectively) was significantly (p<0.05) greater than the initial day. The control emulsions were less viscous than the primary emulsions due to less oil in the emulsions.

At pH 7 the average viscosity for gelatin secondary emulsions throughout the week was 0.02 ± 0.01 Pa.s. The viscosity was significantly greater (p<0.001) on day 3 at pH 5 and remained low at pH 3. The higher viscosity observed at pH 5 correlates with the large droplet sizes (Figure 4.4C; Tables 4.1 and 4.2) of gelatin secondary emulsions. Then as the droplet size and distribution decreased at pH 3, viscosity decreased as well.

HMp secondary emulsion (Figure 4.6D), became significantly (p<0.01) more viscous at pH 3 by day 7 (0.73 ± 0.30 Pa.s). HMp, at pH 3, is able to form a complex with caseins resulting in the emulsion being stabilized by steric interactions (Dickinson 1998). The stability of these emulsions (Figure 4.2D) might be due to the steric repulsive forces between particles, causing the slight increase in viscosity observed during storage.
Figure 4.6. Mean ± SE (n=3) of viscosity (η) of the emulsion (cream layer of unstable emulsions) for A: primary emulsion; B: control; C: gelatin, D: HMP, E: ι-carr and F: LMP, secondary emulsions. Day 0: □; Day 3: ■; and Day 7: □. Superscripts indicate significant difference (p<0.05) at each pH between days.
The t-carr secondary emulsions were stable from pH 7 to 3, and no significant differences were found in the viscosities as a function of time for pH 3 (1.73 ± 0.18 Pa.s) and 7 (0.64 ± 0.53 Pa.s). Interestingly, when emulsions were formulated at pH 5 the viscosity increased during storage reaching 8.44 ± 4.00 Pa.s for day 7. From the ζ-potential values, the polysaccharide did adsorb to the primary layer at each pH, and interestingly near the pI of the proteins, the interactions increased as the emulsion was able to stay stable, and yet became more viscous.

LMp secondary emulsions were the only emulsions that showed a decrease in viscosity values during storage time (pH 7). The initial viscosity (8.24 ± 1.19 Pa.s) of LMp was significantly (p<0.05) higher than the following days (~4.29 ± 0.11 Pa.s). This might be due to the emulsion rearranging itself into a more thermodynamically stable matrix. Considering that the ζ-potential of LMp secondary emulsions at pH 5 and 7 are not significantly different, the higher viscosity value observed for LMp secondary emulsions formulated at pH 7 indicates different reactions occur for the increased stability of the emulsions than compared to the ones formulated at pH 5 (Figure 4.2). Even with the low concentrations of LMp (0.2%) used to form the secondary emulsion, viscosity plays an important role on their stability. One possibility of the increased stability is the presence of divalent calcium in the aqueous solution, which is able to bind to two pectin strands by the carboxyl groups and create a matrix, which could hold the primary emulsion in suspension. This would account for the lack of difference in the ζ-potential between the primary and secondary emulsions, the stability of the emulsion, and
the increase in viscosity. Further research should be performed to systematically evaluate the effect of LMP concentration, emulsion droplet size on the viscosity, and stability of the system.

**Conclusion**

This research shows that droplet charge, measured by $\zeta$-potential, can be responsible for emulsion stability, as shown for $\iota$-carr secondary emulsions; while emulsion’s viscosity might be a main indicator of an emulsion’s stability as is in the case of LMP at pH 7, differentiating emulsions stabilized by charge vs. physicochemical interactions. The inclusion of a complex system for creating emulsions led to stable emulsions, which had previously been cited as unstable for a given pH (e.g. $\iota$-carr at pH 3 and 7; LMP at pH7). This could be due to either the interaction between components used and/or to the processing conditions used to form the emulsions (secondary emulsions being homogenized immediately vs. electrostatic deposition over time). In other words, a complex system might be more effective under certain conditions than a singular whey protein or caseinate in creating a stable emulsion. Further systematic studies need to be performed to evaluate the interaction between caseins and whey proteins and processing conditions on the stability of bi-layer emulsions. In all cases, this study proved that, depending on the individual components and the processing conditions used, secondary layers created with NDM are able to improve the stability of primary emulsions, which can help in the development of novel structures and textures of interest to the food industry.
References


CHAPTER 5

FAT DROPLET MICROSTRUCTURE OF PROTEIN/POLYSACCHARIDE OR PROTEIN/PROTEIN BI-LAYER EMULSIONS AS A FUNCTION OF PH

Abstract

The microstructure of fat droplets of bi-layer emulsions was studied as a function of pH (i.e. 7, 5, and 3) using scanning electron microscopy. The bi-layer emulsions consisted of a primary emulsion: 5 wt% soybean oil (SBO) in a 1% protein (nonfat dry milk) aqueous solution. The secondary layer was t-carrageenan, high- (HMp), low (LMP)-methoxyl pectin, or gelatin. The secondary emulsions consisted of 2.5% SBO, 0.5% protein, and 0.2% polysaccharide or protein. The microstructure of primary emulsions changed from individual droplets to aggregated droplets as the pH of the system decreased from 7 to 3. Gelatin secondary emulsions were stable at pH 7 with defined droplets and became unstable at pH 5 and 3. The destabilization mechanisms at pH 5 and 3 were different: at pH 5 there is complete aggregation of protein due to their proximity to the isoelectric point; and at pH 3 the droplets are perfectly separated, suggesting that at this pH, when the net charge is positive, the destabilization is mainly due to depletion flocculation. HMp secondary emulsions shift from being stable (individual droplets) at pH 3 to being unstable (extensive webbing between droplets) at pH 7. The t-carrageenan secondary emulsions are stable at each pH and the individual droplet microstructure is minimally altered as the pH changes, though there is a fine

Coauthored with Silvana Martini and F.K. Shen
webbing at pH 3. LMp secondary emulsions shift from being stable (individual droplets) at pH 7 to being unstable (extensive webbing between droplets) at pH 3. The microstructure of bi-layer emulsions can aid in interpreting the destabilization mechanisms at a given pH.

1. Introduction

Numerous emulsion-based food products are stabilized using a combination of proteins and polysaccharides, which contribute to textural properties and the shelf-life of the product (Gancz, Alexander, & Corredig, 2006). In addition, these molecules can be used to produce multilayered emulsions with novel encapsulation and release properties (Gu, Decker, & McClements, 2005a). Multilayer emulsions usually have an initial ionic protein layer, as proteins are more apt than polysaccharides in producing small emulsion droplets at low concentrations (McClements, 2004) and have the ability to form strong adsorbed layers at the oil-water interface, which inhibit droplet coalescence (Galazka, Dickinson, & Ledward, 1999). The second layer is formed by adding an oppositely charged ionic polysaccharide. Polysaccharides are used as they stabilize emulsions in a wider range of environmental conditions (e.g., pH ionic strength, temperature, etc.) (McClements, 2004) and improve their texture (Gancz et al., 2006). The addition of polysaccharides might also modify the rheology of the dispersed phase affecting stability by flocculation and by changing the creaming behavior (Galazka et al., 1999; Gancz et al., 2006).
Much research has been done to determine the effects of the interactions between proteins and polysaccharides. High methoxyl pectin (HMP) has been combined with whey or casein proteins. Studies show that HMP adsorbs better at acidic pH than at neutral pH, which affects stability, droplet size and distribution (Dickinson, Semenova, Antipova, & Pelan, 1998; Gancz, Alexander, and Corredig, 2005, 2006). \(\tau\)-Carrageenan (\(\tau\)-carr) has been studied with bovine serum albumin (BS), \(\beta\)-lactoglobulin (\(\beta\)-Lg) with and without gelatin (Dickinson and Pawlowsky, 1997; Gu, Decker, and McClements, 2004a, 2004b, 2005a, 2005b). \(\tau\)-Carr weakly interacts with BSA and \(\beta\)-Lg at pH 7 and above, but the interactions become stronger as the pH decreases. Weak interactions have been correlated with unstable emulsions, stronger interactions lead to greater stability (pH 5, 6); however, strong adsorption can also lead to unstable emulsions (pH 3). The same trends were seen between \(\tau\)-carr (secondary) and gelatin (tertiary) multilayer emulsions. Gelatin has also been used as a secondary layer in whey protein emulsions and was shown to increase emulsion stability at pH 3.4 and 6.8 (Taherian, Britten, Sabik, & Fustier, 2011). And Hu, Li, Decker, Xiao, and McClements (2011) looked into a combination of sodium caseinate and LMp and found it possible to create stable emulsions between pH 3-7 for at least 24h. These studies have been based on the measurement of \(\zeta\)-potential values and the consequent stability of the emulsion. However, inspection at the microstructural level has never been performed.

The objective of this research was to understand the changes in microstructure of the fat droplets in bi-layer emulsions with respect to pH adjustment as seen with scanning
electron microscopy (SEM) to provide a better understanding regarding the microstructural characteristics of the emulsions that are related to their stability.

2. Materials and Methods

2.1. Primary emulsion

Oil-in-water (o/w) emulsions were formulated with 5 wt% soybean oil (SBO) (Western Family Inc., Madison, WI, U.S.A.). 1 wt% protein of nonfat dry milk (NDM) powder (Foster Farms Dairy, Modesto, CA, U.S.A.) was dispersed in a 5mM phosphate buffer solution (pH 7); and 0.2% Microgard® 730 (Danisco, Madison, WI, U.S.A.) was added as an antimicrobial.

2.1.1. Emulsion Preparation

The lipid and aqueous phases were mixed using an Ultra Turrax (IKA T18 basic, Wilmington, NC, U.S.A.) at 18,000 rpm for 1 min, then immediately put through a Microfluidics Microfluidizer Processor (Model M-110S, Newton, MA) at 17.4 ± 1.6 MPa for 3 cycles.

2.1.2. Secondary emulsions

Independent solutions of 0.4 wt% of t-carregeanan (t-carr), low-methoxyl pectin (LMP), high-methoxyl pectin (HMP) (TicGums, White Marsh, MD, U.S.A.) and gelatin (Kraft Foods Global, IL, U.S.A.) were prepared in 5mM phosphate buffer solution (pH 7) and heated to 50°C for 30 min. The primary emulsion and secondary solution in a 50:50...
weight ratio were mixed: Ultra Turrax for 30 seconds at 18,000 rpm followed by 2 cycles through the microfluidizer at 17.4 ± 1.6 MPa. The final oil content in these secondary emulsions was 2.5%, with 0.5 wt% protein and 0.2% secondary polysaccharide or protein. An emulsion with 2.5% SBO (control) without a secondary layer was also formulated.

2.2. pH

After homogenization, the pH of the primary and secondary emulsions was changed to 7, 5 and 3 (± 0.1) using 0.1 M HCl or NaOH. The pH values were chosen at neutral pH, near the isoelectric point (pI) of the milk proteins (4.6-5.2), and below the pI of the milk proteins to determine if a secondary layer could improve the stability at pH values where the primary emulsion would be prone to instability.

2.3. Physicochemical stability

Five to seven mL of an emulsion was placed in a test tube designed for the TurbiScan 2000 (Sci-Tec Inc., Sandyhook, CT, U.S.A.). Samples were stored at 5° C and scans were taken daily over the span of a week. TurbiScan measurements of emulsion stability has previously been discussed by Tippetts and Martini (2009).

2.4. Scanning Electron Microscopy (SEM)

Emulsion samples were placed in disposable 15 mL sterile polypropylene test tubes and centrifuged at 1500 rpm for 10 min at 10 °C. The supernatant fat layer of the sample was transferred to disposable 2 mL sterile plastic microcentrifuge tubes. The
supernatant fat layer of the sample was fixed with 2% buffered glutaraldehyde in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7) for 24 h at 4 °C. After fixation, a small amount of sample was transferred onto a glass cover slip coated with L-lysine. The sample was then rinsed three times with 0.1 M HEPES buffer for 5 min each time. The post fixing was conducted by reaction with 0.4 M HEPES containing 1% osmium tetroxide at 4°C for 24 h. The sample was then washed 3 times with HEPES buffer (0.1M) for 10 min each time. Dehydration of samples was achieved in a series of ethanol solutions (50%, 70%, 95%, and 100%). The sample was washed 2 times for 10 min each; the last solution (100% ethanol) was repeated 3 times. A second dehydration process with hexamethyldisilazane (HMDS) consists of consecutive contact (15 min each time) between the samples and ethanol/HMDS solutions at different ratios (2:1, 1:1, 1:2); the last solution (HMDS alone) was repeated 3 times. Air drying was conducted by leaving the last HMDS sample solution evaporating in a fume hood overnight. Samples were mounted on aluminum stubs and sputtered with a layer of 15-nm gold before being introduced into the Hitachi S4000 scanning electron microscope. The images were obtained at an acceleration voltage of 20 kV.

2.5. Statistical analysis

Experiments were performed in duplicate. Data reported are the means and standard deviations calculated from the replicates. Significant differences were analyzed using a two- or one-way ANOVA test, as appropriate, and Bonferroni and LSD post-tests (α = 0.05). Statistical analysis was performed using Graph Pad software (GraphPad Prism.
3. Results and Discussion

3.1. Stability

Phase separation kinetics at a pH of 7, 5, and 3 for primary, control, and secondary emulsions stored over 7 days at 5 °C were reported in Chapter 4 and summarized in Figure 5.1. At pH 7 (Figure 5.1A), the primary and most secondary emulsions were stable, that is they destabilized at a rate less than 1mm/day (McClements, 2004). In particular, emulsions destabilized at a rate of 0.51 ± 0.09 mm/d for the primary emulsions; 0.70 ± 0.03 mm/d for gelatin; 0.07 ± 0.07 mm/d for t-carr; and 0.2 ± 0.09 for LMP secondary emulsions. HMP secondary emulsion and the control emulsion were not stable with destabilization rates of 4.38 ± 0.43 and 1.13 ± 0.07 mm/d, respectively. The discrepancy with HMP was interesting as there was no difference in the droplet diameter mean (d_{3,2}) between emulsions (~1 µm, Chapter 4). The lack of change in d_{3,2} values suggests that the destabilization of the HMP secondary emulsions was likely due to reversible flocculation rather than coalescence. It is also interesting to note that t-carr and LMP secondary emulsions were significantly (p<0.05) more stable after day 3 than the primary emulsion, which had twice the amount of oil. Usually, lower concentrations of oil lead to faster destabilization rates (Tippetts & Martini, 2009), therefore, the addition of a polysaccharide had a stabilizing effect on the emulsion.
Figure 5.1. Mean ± SE of the change in height of the serum phase of the primary (□); Control (▲); Gelatin (▼); HMp (●); ι-carr (●); and LMp (■) emulsions at pH 7 (A), pH 5 (B), and pH 3 (C) over a 7 d period (n=5)
At pH 5 (Figure 5.1B), which is within the range of the isoelectric point (pI) of dairy proteins (~4.6-5.2), the destabilization rate increased for each emulsion except that of t-carr secondary emulsion. The destabilization rate of t-carr was 0.05 ± 0.05 mm/d and was significantly (p<0.001) more stable than the other emulsions that had destabilization rates ranging from 1.36 to 2.76 mm/d. The t-carr secondary emulsion remained stable as the sulfate groups remained ionized and the droplets stayed suspended due to repulsive electrostatic interactions. LMp secondary emulsion and the control emulsion both had completely destabilized by day 1, whereas the other emulsion continued to destabilize over time.

When the pH of the emulsion was adjusted to 3 (Figure 5.1 C), the destabilization rate increased for gelatin and LMp secondary emulsions (3.36 ± 0.90 and 3.01 ± 0.30 mm/d, respectively) and they were more unstable than the primary and control emulsions. However, HMp became more stable with a destabilization rate of 0.63 ± 0.25 mm/d. The increase in stability is probably due to the increased interaction between the positively charged areas on the protein, which can interact with the anionic polysaccharide, covering the droplets in an HMp coating (Gancz et al., 2005). The t-carr secondary emulsions were the most stable at pH 3 with a rate of destabilization of 0.20 ± 0.06 mm/d, as the polysaccharide still was anionic due to the sulfate groups present on the carrageenan (Damodaran, Parkin, & Fennema, 2008), although the emulsion was less stable than at pH 5 and 7 as creaming had begun by the end of the week.
3.2. Scanning electron microscopy (SEM)

Scanning electron microscopy was performed for the primary, control and secondary emulsions to understand the destabilization mechanisms of these systems (Figures 5.2 to 5.4). Figure 5.2 shows the microstructure of the emulsions at pH 7 at two different magnifications. Similar trends were observed at both magnification levels for the stable emulsions (i.e., primary, control, gelatin, t-carr, and LMP) described in Figure 5.2 (pH = 7). Spherical and individual droplets are observed without any webbing between droplets in Figure 5.2 A-F, I-L. It is interesting to compare the microscopic structure of the primary emulsion (Figure 5.2 A-B) to that of fat globules in whole milk as seen in the work done by Bermudez-Aguirre, Mawson, and Barbosa-Canovas (2008). When the milk fat globule has not been homogenized the coating is similar to that of the primary emulsion (Figure 5.2 A and B), which is composed of soybean oil and disrupted caseins and globular whey proteins. The control emulsion with half the amount of oil, but the same amount of protein (Figure 5.2 C and D) also has well defined droplets with rougher surfaces probably due to unattached protein. The control emulsion is slightly less stable than the primary, even though both had an average droplet $d_{3,2}$ of $1.14 \pm 0.03 \mu m$ and viscosity of $0.007 \pm 0.003$ Pa.s (Chapter 4). Gelatin secondary emulsion (Figure 5.2 E and F) also has defined droplets; however, there is slight webbing surrounding the droplets and interconnecting them. At higher magnification, it would appear that some of the droplets deflated or were ruptured.
Figure 5.2. SEM images of centrifuged emulsion droplet surfaces of primary (A-B), control (C-D), gelatin (E-F), HMP (G-H), 1-carr (I-J), and LMP (K-L) emulsions at 20.0kVx 6-10K magnification (left) and x18-20K magnification (right) at pH 7. The scale bar is for 5.00 µm (left) and 1.5-1.6 µm (right)
In any case, the gelatin secondary emulsion is as stable as the primary emulsion, which would correlate with gelatin having similar droplet size and viscosity as the primary and control emulsions. Though, it is interesting to note that the gelatin secondary emulsions have a $\zeta$-potential of zero as compared $-27.7 \pm 2.4$ mV for the primary emulsion (Chapter 4), which indicates that the gelatin was adsorbed to the surface of the primary emulsion. This stability, with a neutral charge, is due to gelatin having optimal emulsifying properties at its pI (Zayas, 1997) unlike the caseins and whey proteins, which are more stable at pH values away from their pI.

On the other hand, the HMp secondary emulsions (Figure 5.2 G and H) are not stable. HMp is a mass with the fat droplets almost completely surrounded by a matrix of polysaccharide. At pH 7, little interaction between HMp solution and the primary emulsion occurred as both were negatively charged ($\zeta$-potential values of $-14.2 \pm 1.4$ and $-27.4 \pm 2.4$mV, respectively). The mesh around the droplets might indicate depletion flocculation, which occurs with unattached HMp molecules (Dickinson et al., 1998; Gancz et al., 2005). In comparison the two most stable emulsions are $\tau$-carr and LMp secondary emulsions (Figure 5.2 I and L). For $\tau$-carr secondary emulsions (Figure 5.2 I and J) the image is similar to the primary and control emulsions, with well-defined droplets. The difference in stability is that the $\tau$-carr emulsions have a greater negative charge ($-42.3 \pm 3.2$ mV) than the primary emulsions (Chapter 4), due to the sulfate groups, which increase the electrostatic interactions (repulsive) in keeping the droplets separated. On the other hand, the LMp secondary emulsions (Figure 5.2 K and L) which
have the same characteristics of the other stable emulsions are not stabilized by the same mechanism as they do not interact with the caseins (Dickinson, 1998) as there is no absorbance to the primary emulsion. Dickinson does mention that LMp stability could be due to calcium cations bridging the LMp and creating a matrix, which is not necessarily clear in the figure, but does correlate with the increased viscosity of the emulsion (8.2 ± 1.2 Pa.s) as compared to the primary emulsion’s viscosity (0.01 ± 0.00 Pa.s) (Chapter 4).

With the exception of t-carr secondary emulsion, primary, control, and secondary emulsions become unstable at pH 5. This is an expected result due to the poor emulsifying properties of dairy proteins at this pH which is very close to the proteins’ isoelectric point (pI). At this pH proteins begin to aggregate as they lose their charge and hydrophobic interactions occur. The aggregation is very evident with the primary and control emulsions (Figure 5.3 A-D) since no secondary layer is present in these emulsions and protein-protein interactions are promoted. In comparison to emulsions formulated at pH 7, the proteins surrounding the droplets have bulked and clumped, creating a thick web of proteins between fat droplets. As the samples were centrifuged and dehydrated any protein that was still soluble in the aqueous phase is not seen. The images are of the precipitated droplets in a web of proteins that are interacting through hydrophobic interactions. When gelatin, which also has a pI close to 5, was used as the secondary layer droplet aggregation was also observed (Figure 5.3 E and F) and a tight web of free protein is observed in Figure 5.3 E. In addition, when a higher magnification is used (20K x 25K) tight interactions between droplets are seen with several overlapping
droplets (Figure 5.3 F). This behavior might also be due to bridging between droplets due to protein-protein interactions. On the other hand, as the pH drops from 7 to 5, HMp secondary emulsion droplets became more individual and the matrix that was seen at pH 7 is not in evidence at pH 5. Large deposits are seen on the fat droplets (Figure 5.3 H), which appear to be more localized than those of more stable emulsions (which appear to be rough, but not with significant protrusions). These findings suggest that the instability of the HMp secondary emulsions at pH 7 might be caused by the matrix observed by the SEM in Figure 5.2 G and H. This matrix is almost lacking in the emulsions at pH 5 and the stability of these emulsions is significantly improved (Figure 5.1). This matrix, described in Figure 5.2 and 5.3 might be caused by free HMp that does not interact with the protein layer in the primary emulsion as evidenced by ζ-potential values of -18.2 ± 1.6 and -18.0 ± 3.0mV, respectively. The γ-carr secondary emulsion droplets, though still stable, did start to build a slight lattice matrix (different than seen in the primary, control, and gelatin emulsions as it appears more lace-like than web-like), suggesting that the sulfate bonds keep the droplets from destabilizing by interacting with the calcium in the system and forming lace-like threads between droplets. The increase in aggregation between droplets might also explain the increase in viscosity (Chapter 4), which was greater than any other emulsion at pH 5 (8.4 ± 4.0 Pa.s vs. <2.5 Pa.s, respectively).
Figure 5.3. SEM images of centrifuged emulsion droplet surfaces of: primary (A-B), control (C-D), gelatin (E-F), HMP (G-H), t-carr (I-J), and LMP (K-L) emulsions at 20.0kV x 6K magnification (left) and x20-25K magnification (right) at pH 5. The scale bar is for 3-5.00 µm (left) and 1.2-1.5 µm (right).
LMP secondary emulsion at pH 5 (Figure 5.3 K-L) has a similar appearance as gelatin (Figure 5.3 E), though the web matrix appears to be longer chains than the short stubby branches on the gelatin, which might be a result of the different chemical composition of the secondary layer (protein vs. polysaccharide).

The emulsion interactions as determined by SEM are reported in Figure 5.4 for pH 3. At lower magnification the primary emulsion (Figure 5.4 A and B) has formed a network of proteins, connecting the fat droplets with thick strands; while the control emulsions (Figure 5.4 C and D) appear to be individual droplets. At higher magnification, the surface of the primary emulsions are still connected by strands of protein and have a rough surface; the control droplets, though, are more dense, have a rougher surface, and appear to be linked by a coarse mesh (Figure 5.4 D). The coarse surface would indicate more interactions happening between the droplets and surrounding materials than observed at pH 7 (Figure 5.2 A-D) and less than at pH 5 (Figure 5.3 A-D). The decrease in webbing and increase in individual droplets from pH 5 indicate the droplets have more repulsive electrostatic interactions at pH 3 than near their pI, which is also observed in the improved rate of destabilization from pH 5 to 3 from 2.6 to 1.9 mm/d. However, though there is an increase in repulsive interactions, it is not sufficient to stabilize the emulsion as the absolute net charge of the emulsion at pH 3 (\(\pm 2.1\) mV) is not different from that at pH 5 (\(\pm 3.0\) mV), meaning the emulsion would need to have a greater net charge to become stable given the emulsifier.
Gelatin secondary emulsion at pH 3 (Figure 5.4 E and F) have independent and smooth droplets (as compared to the control) but with a few structures attached to the droplet wall. These structures are probably formed by gelatin molecules that did not attach efficiently to the primary layer. The lack of interaction (or the inefficient interaction) between the primary and secondary layer for these emulsions is demonstrated by the lack of change in the $\zeta$-potential values with values reported of $18.7 \pm 2.5$ and $20.6 \pm 2.1$ mV for the gelatin secondary emulsion and the primary emulsion, respectively (Chapter 4). Instability for these secondary emulsions is then due to depletion flocculation, as the gelatin do not adsorb to the primary emulsion the excess gelatin does not form a webbing as it remained with the aqueous phase than separated out with the lipid phase for fixing for SEM.

HMp secondary emulsions (Figure 5.4 G and H) are individual droplets with little to no webbing, and the emulsion is stable as the anionic polysaccharide interacts with the positive patches on the protein and are able to keep the droplets suspended. The $\lambda$-carr secondary emulsion still is made of individual droplets (Figure 5.4 I and J), though the web matrix has increased in density from that at pH 5 the viscosity is less at pH 3 ($1.6 \pm 0.6$ Pa.s) than at pH 5 ($2.4 \pm 1.5$ Pa.s) (Chapter 4). The increase in viscosity at pH 5 is possibly due to an increase in calcium bridging between $\lambda$-carr chains, as there are less repulsive electrostatic forces than at pH 3 and 7 when the protein net charge is neutral.
Figure 5.4. SEM images of centrifuged emulsion droplet surfaces of primary (A-B), control (C-D), gelatin (E-F), HMP (G-H), t-carr (I-J), and LMP (K-L) emulsions at 20.0kVx 6-10K magnification (left) and x18-20K magnification (right) at pH 3. The scale bar is for 3-5.00 µm (left) and 1.5 µm (right).
At pH 3, though there are positive electrostatic forces, there is still some calcium bridging between the sulfate groups, which is shown in the lace-like lattice between droplets of 1-carr chains maintaining the stability of the emulsion; however, the viscosity is lower. At pH 7, there is sufficient negative charge to interact with the calcium without bridging to occur. Finally, as HMp secondary emulsions lost the webbing and became more stable, LMp secondary emulsions (Figure 5.4 K-L) gained webbing, and the polysaccharide matrix increased as the pH decreased causing the emulsion to become more and more unstable.

4. Conclusions

This research showed that the microstructure of the emulsions, together with emulsion net charge and viscosity can be used to explain their stability. When the emulsions became unstable, a network of protein or polysaccharide had formed causing the destabilization of the emulsion to various degrees. In some cases, the excess polysaccharide/protein might interact among them and create a molecular network that maintains droplets suspended which stabilizes the emulsion (gelatin at pH 7 and 1-carr at pH 3). These SEM images also show excess polysaccharide molecules responsible for depletion flocculation and emulsion instability due to lack of molecular interaction between primary and secondary layers. These are the first results that show molecular events responsible for emulsions’ stability at the nanoscale level. These findings will help in the understanding of how protein/polysaccharide bilayer emulsions are impacted by pH, which can then be utilized in creating more stable emulsions.
References


CHAPTER 6

DETERMINATION OF LIPID RETENTION IN A CURD MATRIX WITH NILE RED AND UVP IMAGING

Abstract

The purpose of this research was to develop a method to evaluate the retention of lipid-soluble compounds in cheese using a model system for curd formation. Nile red was used as the lipid-soluble compound and added to the oil phase of a 5% oil-in-water emulsion. The retention of Nile red in the curd was evaluated as a function of emulsion type (primary vs. secondary emulsion), pH (3, 5 and 7), and calcium content (0, 0.004, and 0.01M CaCl$_2$) in the media. Nile red-fortified emulsions were added to a milk protein concentrate dispersion with a final protein content of 3.4%. A model cheesemaking process was used to obtain curd samples and the fluorescence of Nile red retained in the curd was quantified using ultraviolet light. Curds with different amounts of Nile red were obtained to develop a calibration curve. A linear correlation was determined between the intensity of Nile red in the curd and its concentration. This method was used to evaluate the effect of bi-layers emulsions on the retention of Nile red in a curd system. Nile red emulsions were formulated with different secondary layers (i.e. gelatin, low and high methoxy pectin, and t-carrageenan). No significant differences ($\alpha = 0.05$) in the retention of Nile red in the curd between emulsions and pH values were observed. Significant differences ($p < 0.05$) were found in the retention of Nile red as a

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Footnote: 4 Coauthored with Silvana Martini
function of calcium addition. This research shows that the retention of lipid-soluble substances in cheese can be quantified using a rapid-bench-top method instead of more time consuming methods (e.g, HPLC).

1. Introduction

Vitamin D is one of the lipid-soluble components that has received significant attention during the last 10 years. Vitamin D is a fat-soluble vitamin produced endogenously when ultraviolet rays from sunlight contact the skin and trigger vitamin D synthesis (National Institute of Health, 2011). However, exposure to sufficient sunlight is variable. Though naturally available in some foods such as cod liver oil, salmon, and tuna, vitamin D is also added to foods such as orange juice, milk, and yogurt and is also available as a supplement. Concerns about incorporating enough vitamin D in the diet is important as it plays a role in bone metabolism and cell growth and most individuals do not intake the recommended daily allowance of 600 IU/day (National Institute of Health, 2011; Wagner et al., 2008). Researchers are evaluating the possibility of fortifying more food sources with vitamin D as the consumption of milk, a main source of vitamin D, is decreasing (Kazmi, Vieth, & Rousseau, 2007; Upreti, Mistry, & Warthesen, 2002; Wagner et al., 2008). The consumption of cheese, on the other hand, has increased steadily since 1980 (Upreti et al., 2002). For this reason, cheese has been a focus of fortification, as it is a universal consumption product. Cheddar cheese is a good candidate for vitamin D fortification since it only has traces of lactose and is therefore available to lactose intolerant individuals (Banville, Vuillemard, & Lacroix, 2000).
Other important lipid-soluble components are other vitamins such as A, E, and K, ω-3 rich oils, nutraceuticals, flavors, colors, etc. which can all enhance the product’s nutritional value. Vitamin A is needed for vision, immune function, bone remodeling, growth, etc. (Haskell & Brown, 1999). A common fortification combination is vitamin D and A. Vitamin E (α-tocopherol) is a lipid-soluble antioxidant, which has been shown to inhibit oxidation of low-density lipoproteins (LDL) and the proliferation of smooth-muscle cells invitro, decreases the rate of ischemic heart disease (Stampfer et al., 1993; Stephens et al., 1996). Besides vitamins, other functional ingredients include ω-3 fatty acids (e.g., eicosapentaenoic acid, EPA, docosahexaenoic acid, DHA, and conjugated linoleic acid, CLA), which are known to protect against heart disease, improve brain and eye function in infants, and reduce susceptibility to mental illness in adults (Taherian, Britten, Sabik, & Fustier, 2011). Being able to incorporate all of these functional lipid or lipid-soluble compounds is important, because it can increase the value of foods that consumers normally include in their diet without having to buy supplements. Flavor is also important in creating or improving product lines and is understood to be the perception of volatile compounds released from food while eating; volatile compounds are mostly hydrophobic and are more effective when being added to the lipid phase (Jo & Ahn, 1999). In addition, flavor also includes the taste perceptions such as sweet and salt and chemical feeling factors such as heat and cooling (Meilgaard, Civille, & Carr, 1999). These other lipid soluble components could be incorporated individually or in
combination to a lipid phase of an emulsion and then added to milk to make cheese, increasing the functional properties and hence the value of the cheese.

Calcium content could influence the retention of the added components in a curd matrix. Previous research shows that the addition of calcium during cheese make can reduce the time of coagulation, and low calcium concentrations (4.10 mM CaCl$_2$) increase gel firmness (Lucey & Fox, 1993; McMahon, Brown, Richardson, & Ernstrom, 1984). To obtain maximum yield and quality of curd there is an optimum concentration of calcium chloride and firmness of the curd which is dependent on the temperature to cut the curds and moisture content (Fagan, Castillo, Payne, O'Donnell, & O'Callaghan, 2007).

Previous research (Chapter 3) has shown that the retention of vitamin D, a lipid-soluble component in cheese, can be improved by using oil-in-water emulsions as delivery matrices. The use of bilayer emulsions, which usually have an ionic protein primary layer to surround the oil droplets, and then an oppositely charged ionic polysaccharide layer (McClements, 2005), might be an ideal way to incorporate the functional lipid component. The charge on the droplets could interact with the caseins as the curd forms and be entrapped within the curd matrix rather than possibly exiting with some of the fat in the whey. There is a need to evaluate whether the type of emulsion used during cheese fortification affects the retention of lipid-soluble substances. To address this need, the purpose of this research was to develop a bench top method to
quantify the retention of a lipid-soluble compound in a curd matrix as a function of delivery matrix, pH (of the emulsion) and calcium content in the media.

2. Materials and Methods

2.1. Primary emulsion materials

Oil-in-water (o/w) emulsions were formulated with 5 wt% soybean oil (SBO) (Western Family Inc., Madison, WI). 1 wt% protein (emulsifier) of nonfat dry milk (NDM) powder (Foster Farms Dairy, Modesto, CA, U.S.A.) was dispersed in a 5mM phosphate buffer solution (pH 7); and 0.2% Microgard® 730 (Danisco, Madison, WI, U.S.A.) was added as an antimicrobial.

2.1.1. Nile red addition

Nile red (Sigma-Alderich Corp., St. Louis, MO, USA) was added to the SBO to represent lipid-soluble substances. A saturated solution of Nile red in SBO was prepared by dissolving 4.4 to 6.0 mg of Nile Red in 15 ml of SBO. The suspension was mixed and left overnight at 5 °C to allow saturation of SBO with Nile red. The Nile red SBO (NL-SBO) suspension was then centrifuged at 4,000g for 10 min to separate the excess Nile red. The NL-SBO solution was then used to make the 5 wt % o/w emulsions.

2.2. Emulsion Preparation

The lipid and aqueous phases were mixed using an Ultra Turrax (IKA T18 basic, Wilmington, NC) at 18,000 rpm for 1 min, then immediately put through a Microfluidics
Microfluidizer Processor (Model M-110S, Newton, MA) at 17.4 ± 1.6 MPa for three cycles.

2.3. Secondary emulsions

Independent solutions of 0.4 wt% of 1-carregeanan (1-carr), low-methoxyl pectin (LMP), high-methoxyl pectin (HMP) (TicGums, White Marsh, MD) and gelatin (Kraft Foods Global, IL) were prepared in 5mM phosphate buffer solution (pH 7) and heated to 50°C for 30 min. The primary emulsion and secondary solution in a 50:50 weight ratio were mixed using an Ultra Turrax for 30 seconds at 18,000 rpm followed by 2 cycles through the microfluidizer at 17.4 ± 1.6 MPa. The final oil content in these secondary emulsions was 2.5% NL-SBO, with 0.5 wt% protein and 0.2% secondary polysaccharide or protein. These were compared with a primary emulsion that had a 5% oil phase (50:50 SBO:NR-SBO) and a control (100 NR-SBO) with 1% protein.

2.4. pH

After homogenization, the pH of the primary and secondary emulsions was changed to 7, 5 and 3 (± 0.1) using 0.1 M HCl or NaOH. The pH values were chosen above the isoelectric point (pI), near the pI of the milk proteins (4.6-5.2), and below the pI of the milk proteins to determine if the addition of a secondary layer could improve the retention of the emulsion in a curd matrix.
2.5. Model Cheesemaking

Milk solutions were made of milk protein concentrate at 80% protein (MPC80, ID Milk Products Inc., Jerome, ID) for a final protein content of 3.4%. Three milk solutions were made: control (no added calcium), 0.004M CaCl$_2$, and 0.01M CaCl$_2$ (Mallinckrodt, Paris, KY). The solution was stirred overnight at 5 °C before use for complete dispersion of MPC80 and CaCl$_2$.

The milk solution was heated to 39 °C and cooled to 31 °C to restore coagulation properties inhibited by cooling (Qvist, 1979). Two hundred-milliliter portions of milk were placed in 250-ml polycarbonate Nalgene centrifuge bottles (VWR, West Chester, PA) and 5 ml of NR-SBO emulsions were added, and blended with an Ultra Turrax for 30 seconds. Then 4 g of glucono-δ-lactone and 0.4 ml of diluted chymosin rennet (Maxiren DS; DSM Food Specialties, Parsippany, NJ) containing 1.8 international milk clotting units/ml were added and incubated for 30 min at 31 °C to allow coagulum formation. The coagulum was manually cut using a metal spatula nine times, and then centrifuged at 1,000g at 25 °C for 30 min. After centrifuging, the curd was separated out, packed in bags, and stored at 4°C overnight prior to analysis.

2.6. Standard curve

A standard curve was made with different concentrations of NL-SBO in a primary emulsion. NL-SBO was put into the emulsion at 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 g of the total 2.5 g of oil phase in the 50g emulsion solution. After emulsification through the microfluidizer, 5 ml of the Nile red emulsion were placed into 200 ml of reconstituted
milk protein concentrate at 80% protein (MPC80), with a total protein content of 3.36% (which is standard in milk) in distilled water to make curd (as described above for model cheese). MPC80 was used to have a milk solution that could be kept consistent between batches of cheese rather than the variability in whole milk. After the curd was made, the curd samples were stored overnight at 5°C and UVP analysis was performed on the following day.

2.7 Ultra Violet Pictures (UVP)

UVP (Ultra-Violet Products Ltd., CA) is an image acquisition method that can be used in samples that have been spiked with a compound that excites under UV conditions. Nile red excites at 515-560 nm and emits at >590 nm (Greenspan, Mayer, & Fowler, 1985). UVP was used to quantify the retention of Nile red in the curd using a UV filter for ethidiun bromide (which excites at 605 nm).

Curds obtained from the model cheesemaking process (Section 2.5.) were collected and broken up using a spatula and bowl. A portion of the broken curd was placed in the center of a mold (22x22 mm, 0.08 mm thick) in the UVP darkroom. A piece of parafilm was placed over the curd and it was smoothed down using a cylindrical stir rod to fit in the mold, creating a uniform and homogeneous sample. The parafilm was taken off the sample, and the mold was removed. After the sample was enclosed in the UVP darkroom, the UV light was turned on and a picture was taken at high intensity, using the ethidium bromide option for color.
2.7.1. *Image J analysis*

Sample images obtained with the ethium bromide color option were analyzed using ImageJ for red intensity. The sample selection tool was used to ensure that only the sample area was analyzed using the Macro: RGB histogram. The red intensity histogram of the curd sample was analyzed. From the information obtained, the mean red intensity was used to generate a calibration curve for Nile red retention in the cheese making process using a model system.

2.8. *Scanning Electron Microscopy (SEM)*

LMp secondary emulsion samples were placed in disposable 15 mL sterile polypropylene test tubes and centrifuged at 1500 rpm for 10 min at 10 °C. The supernatant fat layer of the sample was transferred to disposable 2 mL sterile plastic microcentrifuge tubes. The supernatant fat layer of the sample was fixed with 2% buffered glutaraldehyde in 0.1 M HEPES buffer (PH 7) for 24 h at 4 °C. After fixation, a small amount of sample was transferred onto a glass cover slip coated with L-lysine. The sample was then rinsed three times with 0.1 M HEPES buffer for 5 min each time. The post fixing was conducted by reaction with 0.4 M HEPES containing 1% osmium tetroxide at 4°C for 24 h. The sample was then washed 3 times with HEPES buffer (0.1M) for 10 min each time. Dehydration of samples was achieved in a series of ethanol solutions (50%, 70%, 95%, and 100%). The sample was washed 2 times for 10 min each; the last solution (100% ethanol) was repeated 3 times. A second dehydration process with hexamethyldisilazane (HMDS) consists of consecutive contact (15 min each time)
between the samples and ethanol/HMDS solutions at different ratios (2:1, 1:1, 1:2); the last solution (HMDS alone) was repeated 3 times. Air drying conducted by leaving the last HMDS sample solution evaporating in a fume hood overnight. Samples were mounted on aluminum stubs and sputtered with a layer of 15-nm gold before introduced into the Hitachi S4000 scanning electron microscope. The images were obtained at an acceleration voltage of 20 kV (Chapter 5).

2.9. Statistical Analysis

Experimental design was done as a whole plot, subplot-subplot design. Each plot was a replicate (a replicate was a day) at a given pH with one subplot being the secondary layer (treatment) of the emulsions, and second subplot being the CaCl$_2$ concentration of the milk solution. The milk solutions were randomized as to which was done first, second, and third. The treatments were randomized as to which were created first through fourth. Six replicates at each pH were done. The primary emulsion with no addition of CaCl$_2$ ($p_0$) was used to normalize the data. Each treatment’s intensity was divided by the intensity of $p_0$, which gave a ratio of how much more or less Nile red the curd had retained versus the $p_0$. Data reported are the means and standard deviations. Significant differences were analyzed using Graph Pad software (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com) and SAS 9.2 TS Level 1M3 XP_PRO platform (Cary, NC).
3. Results and Discussion

3.1. Standard Curve

The increase in red intensity in the curd as a function of Nile red concentration is represented visually in Figure 6.1. There is a small amount of background noise, which is represented by the sample without Nile red added (Figure 6.1 A, a). As the Nile red concentration increases incrementally from 20 to 100% in the oil phase the red intensity does also. The red intensity was quantified using the UVP as mentioned in the materials and methods section and the data is reported in Figure 6.2. A significant linear correlation (α = 0.05) was found between the red intensity in the curd and the amount of Nile red added in the emulsion, suggesting that this is a good method to evaluate the retention of a lipid-soluble substance in cheese using a model cheesemaking method.

![Figure 6.1](image)

Figure 6.1. Ultraviolet (UV) images of curd made with MPC80 and emulsions containing NL-SBO. UV images A-F are without the ethidium bromide filter, and images a-f are with the ethidium bromide filter. The concentration of NR goes from 0 (A/a) to 100% (F/f) in increments of 20%.
Figure 6.2. Standard curve of red intensity (mean ± SE; n=3; ethidium bromide filter) with respect to the percent of NL-SBO in the emulsion

3.2. Secondary layer Nile Red retention comparison

The ratio of red intensity between all emulsions and the primary emulsion with and without calcium (0.004 M and 0.01 M) is reported in Table 6.1 for pH values of 7, 5, and 3. No significant differences ($\alpha = 0.05$) were found between the primary and secondary emulsion at pH 7, 5, or 3. Neither was there significant difference between red intensity between pH values.
Table 6.1. Red intensity ratio (mean ± SD) of primary (no CaCl$_2$) to each treatment and CaCl$_2$ content at pH 7, 5, and 3. No significance was seen between secondary emulsions treatments and pH; however a significant difference (p<0.05) was determined between concentrations of CaCl$_2$. Columns with the same superscript letter are not significantly different ($\alpha=0.05$) n=6, n=90 for pooled data.

<table>
<thead>
<tr>
<th>pH 7</th>
<th>Treatment</th>
<th>0</th>
<th>0.004 M</th>
<th>0.01 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>1.00 ± 0.00</td>
<td>0.93 ± 0.13</td>
<td>0.85 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>1.08 ± 0.08</td>
<td>1.01 ± 0.23</td>
<td>0.91 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>HMP</td>
<td>1.00 ± 0.09</td>
<td>1.04 ± 0.23</td>
<td>0.89 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>t-carr</td>
<td>0.99 ± 0.12</td>
<td>1.01 ± 0.17</td>
<td>1.03 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>LMP</td>
<td>1.01 ± 0.06</td>
<td>1.02 ± 0.10</td>
<td>1.01 ± 0.33</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 5</th>
<th>Treatment</th>
<th>0</th>
<th>0.004 M</th>
<th>0.01 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>1.00 ± 0.00</td>
<td>0.98 ± 0.16</td>
<td>0.91 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.99 ± 0.07</td>
<td>0.97 ± 0.09</td>
<td>0.98 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>HMP</td>
<td>0.99 ± 0.04</td>
<td>0.98 ± 0.08</td>
<td>0.98 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>t-carr</td>
<td>1.02 ± 0.06</td>
<td>0.98 ± 0.09</td>
<td>0.99 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>LMP</td>
<td>0.97 ± 0.06</td>
<td>0.93 ± 0.08</td>
<td>0.87 ± 0.12</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 3</th>
<th>Treatment</th>
<th>0</th>
<th>0.004 M</th>
<th>0.01 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>1.00 ± 0.00</td>
<td>0.97 ± 0.10</td>
<td>0.91 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>1.04 ± 0.13</td>
<td>1.01 ± 0.11</td>
<td>0.99 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>HMP</td>
<td>1.06 ± 0.17</td>
<td>1.02 ± 0.11</td>
<td>0.87 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>t-carr</td>
<td>1.00 ± 0.08</td>
<td>0.94 ± 0.20</td>
<td>0.89 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>LMP</td>
<td>1.01 ± 0.17</td>
<td>0.85 ± 0.14</td>
<td>0.88 ± 0.11</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pool</th>
<th>Milk</th>
<th>0$^a$</th>
<th>0.004M$^{ab}$</th>
<th>0.01M$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>1.02 ± 0.09</td>
<td>0.98 ± 0.14</td>
<td>0.93 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>1.02 ± 0.01</td>
<td>0.98 ± 0.01</td>
<td>0.93 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>
It is interesting to note here that the primary emulsion had twice the amount of oil as the secondary emulsions. It is possible to add a secondary emulsion to the cheese making process with half the oil and still retain the same amount of lipid-soluble substance in the curd.

The retention of the secondary emulsions at an equivalent intensity in the curd is interesting as the emulsions do not exhibit the same trends in stability. In previous research, it was seen that at pH 7, each emulsion was stable (destabilization rate <1 mm/d, McClements, 2005) for the purposes of this research except for HMp, which destabilized rapidly (4.4 ± 0.1 mm/d), even though all of the droplets were approximately 1.0 µm (Chapter 4). As the pH decreased the droplet size increased to approximately 5-10 µm at pH 5, especially for LMP and gelatin with droplet sizes of approximately 60 µm, and at pH 3 to approximately 5-12 µm, with LMP at approximately 90 µm (Chapter 4). The stability was correlated with the droplet size, since LMP was highly unstable at pH 5 and 3, as was gelatin. HMp, however, increased in stability as the pH dropped and at pH 3 with an average droplet size of approximately 4 µm and destabilization rate of 0.6 ± 0.3 mm/d (Chapter 4).

The most stable of all the emulsions were those made with κ-carrageenan, which had a destabilization rate of 0.2 ± 0.1 mm/d at pH 3 and even less (~0.06 mm/d) for pH 5 and 7; κ-carrageenan droplet size increased over the pH adjustment to being approximately 12 µm at pH 3, which might explain the faster destabilization rate at pH 3.
Retention of lipid-soluble components has been limited. Other studies have looked at the retention of fortified emulsions in cheese versus direct oil addition. No differences were observed by Kazmi et al. (2007) when they compared the incorporation of vitamin D as oil and emulsion at 500-1,000 IU/g of curd. The vitamin D had retentions over 90% in the model curd as analyzed by HPLC. Ye et al. (2009) fortified processed cheese with fish oil. The fish oil in an emulsion was less oxidized as compared to fish oil being directly added to the cheese over a five week time as measured by lipid oxidation and sensory techniques. However, both the oil and the emulsion were dependent on concentration of fish oil; higher amounts of fish oil could be added in the emulsified form (i.e., emulsion vs. oil: 20g vs. 10g of fish oil per kg of cheese at 1 wk; 10g vs. 5g by wk 5). Barrett, Porter, Marando, and Chinachoti (2011) fortified performance bars with fish and flaxseed oils in emulsified form with different antioxidants, and found that the quality was significantly lower than that of the control (no fortification), which indicated that the antioxidants did not work and the addition of the emulsion to the performance bar altered the flavor and firmness attributes detrimentally. McCowen et al. (2010) fortified yogurt with DHA fortified emulsions to increase individuals ω-3 levels. The fortification worked; however, sensory was not discussed nor the retention of the emulsion in the yogurt. Though it is important to first solve the issue of flavor, eventually the retention of the ω-3 rich oils will need to be evaluated. If combined with other components within an emulsion such as to reduce
oxidation through the addition of antioxidants (such as vitamin E), it might be beneficial to evaluate the retention through preliminary studies using Nile red fortification.

Significant differences (p<0.05) in red intensity between the overall intensity of milk types with no additional calcium (1.02 ± 0.02) and with 0.01M CaCl₂ (0.93 ± 0.02) were found. In general, the retention of a Nile red decreased as the CaCl₂ concentration increased. The amount of curd obtained with no additional calcium was on average 17.1 ± 0.9 g. When 0.004 M CaCl₂ was used, 20.6 ± 1.2 g of curd was obtained, while 23.1 ± 0.6 g of curd was obtained when using 0.01M CaCl₂. The difference in the amount of curd created during the model cheesemaking system corroborates the fact that the calcium addition affects the extensiveness of the gel network formed during cheesemaking (McMahon et al., 1984). Calcium plays an important role in curd formation by providing linkages within casein micelles and the connecting of casein micelles (Joshi, Muthukumarappan, & Dave, 2003). If too much calcium is added the gel network formed could become too tightly knit. In our model process, higher concentration of 0.01M CaCl₂ produced a grainier curd, so even though the curd formation was quick, after being cut the curd was granular versus a more knit curd network seen at 0.004M CaCl₂ and curd with no additional CaCl₂. The difference could be correlated between the emulsions and the intensities found in the 0.01M CaCl₂ system. The ι-carrageenan and LMP emulsions had on average a higher retention in the curd at 0.01M CaCl₂, which might be associated with the increased negative net charge (~ -43 and -30 mV, respectively) from having many anionic groups (-OSO₃⁻ and –COO⁻).
respectively) at pH 7 that could interact with the calcium and the proteins, becoming more intricately bound in the curd matrix (Chapter 4). At pH 5 and 3, the retention of Nile red using LMP secondary emulsions slightly decreased with CaCl₂ concentration. This could be related to the low stability of these secondary emulsions as reported in Chapter 4. The emulsion had large droplets and a lower intensity range (0.75-0.99). The combination might indicate non-uniformity of the samples throughout the curd.

To correlate how the emulsions might have interacted with the curd, scanning electron microscopy (SEM) images of LMP secondary emulsions are shown in Figure 6.3. When LMP secondary emulsions are stable (pH 7), small droplets ($d_{3,2} = 0.92 \pm 0.01 \mu m$) are observed and highly anionic droplets ($\zeta$-potential values of $-30.4 \pm 3.1 mV$) are unique and individual (Figure 6.3 A).

Figure 6-3. LMP secondary emulsion images obtained using scanning electron microscopy at pH 7 (A), pH 5 (B) and pH 3 (C)
The same was observed for ɩ-carrageenan at pH 7. However, as the pH of the emulsion decreased to pH 5 and then 3, the droplet size increased and the stability of the emulsions decreased dramatically for LMp secondary emulsions. A webbing of polysaccharide and protein occurred at pH 5 and 3 (Figure 6.3 B and C). This was due to the milk proteins losing charge as the pH of the system reached the pI of the caseins.

The emulsion was already unstable and interconnections between droplets forming, so that when added to the cheese milk and acidified that there were fewer connections available to make between the LMp secondary emulsion and the casein curd matrix. The retention of a lipid-soluble substance depends on the amount of calcium in the media and the net charge and stability of the emulsion.

4. Conclusion

This research shows that quantification of lipid-soluble components can be performed using ultraviolet imaging. The concentration of lipid-soluble components can be retained in the curd in secondary emulsions at the same levels of primary emulsion, but with half the oil. This method might be applied to other curd substances such as tofu, yogurt, or other coagulated products to quantify the amount of lipid phase retained in the matrix when using emulsions as delivery materials. Also, interactions between the emulsion and the curd matrix might be explained by how the emulsion is destabilized and how that might affect the retention in a curd matrix.
References


CHAPTER 7

PROTEIN/\(\pi\)-CARRAGEEANAN (\(\pi\)) OIL-IN-WATER EMULSIONS

1. Introduction

Previous chapters have explained the research involved with developing bi-layer emulsions and their possible uses in the food industry (Chapters 4-6). This chapter takes the next step and outlines the process of moving from the research realm to the marketing arena.

Protein/\(\pi\)-carrageenan (\(\pi\)) oil-in-water emulsions also known as \(\pi\) (pi)-emulsions are a new way to fortify food products with fat-soluble vitamins (i.e., A, D, E, and K) or flavors (e.g., hazelnut, orange liqueur, or hot chili). \(\pi\)-emulsions are all natural. The lipid-soluble component will be solubilized in the oil phase, which could consist of unsaturated fatty acid oils such as soybean, olive, canola, sunflower, etc., which have low melting points and would not crystallize at refrigeration temperatures after emulsification. The emulsifiers consist of milk proteins (casein and whey proteins from nonfat dry milk), and \(\pi\)-carrageenan, which is obtained from red seaweed. The objective is to create an emulsion with 2.5% of oil. The oil phase contains the functional component of the emulsion that can be a vitamin or flavor. The oil phase makes it possible to add highly concentrated components to cheese, sauces, and soups and provides protection to the functional ingredient improving its retention in the food matrix. A small amount of \(\pi\) emulsions will be very efficient at the incorporation of the
functional component since it is composed of micro-sized droplets, which can easily be dispersed throughout the product.

The objective of this chapter is to examine possible uses of $\pi$-emulsions and different marketing strategies.

2. Process

A mission statement relevant to $\pi$-emulsions must be determined as it would define the purpose of the product and is a tangible proclamation within the organization of how $\pi$-emulsions would be viewed by employees and by the outside world (Palmer & Short, 2008). $\pi$-emulsions mission statement includes how the product is to be viewed by its target market, the need for $\pi$-emulsions, how the need is to be addressed, and what principles or beliefs guide the work.

SWOT analysis is the breaking down of a product into the strengths (S), weaknesses (W), opportunities (O) and threats (T) that might affect the product or a firm. The SWOT analysis is a scrutiny of the internal strengths and weaknesses of the product/firm followed by the investigation of external opportunities and threats (Coman & Ronen, 2009). Understanding the strengths of the product/firm enables the company to seek markets and opportunities and identify potential events that could threaten the value or position of the product (Coman & Ronen, 2009).

Target market is used to evaluate the target population for a specific product, in this case $\pi$-emulsions.
3. Mission Statement

The purpose of π-emulsions is to take the use of vitamins and flavors to the next level: bringing fortification of products with unique flavor combinations (with or without additional nutrients) to manufacturers. The ultimate objective is to increase the quality and possibilities of food products, dairy in particular, by fortifying them with essential nutrients (with or without expansive possibilities of flavorings) using high quality ingredients for consumer friendly labeling.

4. SWOT Analysis (Table 7.1)

Table 7.1. Summary of strengths, weaknesses, opportunities, and threats (SWOT) associated with the production and use of π-emulsions.

<table>
<thead>
<tr>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Versatile</td>
<td>*Allergens</td>
</tr>
<tr>
<td>*Functional</td>
<td>*Unknown product</td>
</tr>
<tr>
<td>*Stable</td>
<td>*Shelf-life</td>
</tr>
<tr>
<td>*Dairy based</td>
<td>*Unknown flavor</td>
</tr>
<tr>
<td>*All natural</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Opportunities</th>
<th>Threats</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Consumer demand for products that are:</td>
<td>*Competition</td>
</tr>
<tr>
<td>Nutrient-rich</td>
<td>*Belief in functional components</td>
</tr>
<tr>
<td>Low-fat</td>
<td>*Cost of implementation</td>
</tr>
</tbody>
</table>
**Strengths:** The strengths of \( \pi \)-emulsions which make them of product value and therefore marketable include the following:

1. **Versatile:** \( \pi \)-emulsions can be made to fit a food producer’s need. These emulsions can be used to fortify products with lipid-soluble substances such as vitamins and/or flavors. As initial testing suggests (Chapter 4), oil-soluble nutrients and flavors could be applied to numerous product lines.

2. **Functional:** \( \pi \)-emulsions are formed by small oil droplets dispersed in a continuous aqueous phase (Chapter 4). This is an ideal characteristic to easily disperse the emulsions throughout another fluid system while maintaining the integrity of the droplets and their protected lipid-soluble substances and obtaining a homogeneous product in terms of texture and nutritional characteristics. For example, \( \pi \)-emulsions would be added prior to making cheese, yogurt, ice cream, etc. to ensure homogeneity.

3. **Stable:** \( \pi \)-emulsions are stable for a pH range of 7 to 3 and temperature range of 4°C to 39°C (Chapters 4 and 6). The emulsion needs to be stored at refrigeration temperature. Also, from observation the emulsion was stable for ~6 months at 4°C, giving it a reasonable shelf-life for a dairy based emulsion.

4. **Dairy based:** \( \pi \)-emulsions are dairy-based, with nonfat dry milk as the aqueous phase, which includes casein and whey proteins for the emulsifier, and calcium and other minerals (Chapter 4), which would be an excellent way to fortify other dairy systems such as soups, sauces, dips, desserts, and beverages.
Natural: all main ingredients of π-emulsions are of animal or plant origin, which would be a bonus for the “label friendly” consumer (Guzey & McClements, 2006).

Weaknesses: The following weaknesses of π-emulsions indicate possible challenges associated with the product:

1. Allergens: π-emulsions are made from nonfat dry milk, which means they contain dairy proteins. As other like products, they cannot be consumed by individuals with milk protein allergies and a warning label would be required (Pele, Brohee, Anklam, & Van Hengel, 2007).

2. Unknown product: π-emulsions would be a new product on the market. The producer needs to be educated on the versatility and the benefit of adding π-emulsions to their products for fortification and/or flavoring. The home consumer would benefit from advertising focused on the basic use and benefits of π-emulsions and how π-emulsions would make their lives better (Kitt & Strater, 2008).

3. Unknown shelf-life: currently, shelf-life beyond 6 months is unknown. Determining shelf-life can be a benefit for manufactures, since consumers consider shelf-life as important as taste and nutrition during the decision making process to purchase (Sen & Block, 2009). Further shelf life studies need to be performed such as microbial testing, and stability towards phase separation for exact time frames.

4. Unknown flavor: the addition of flavors has yet to be studied, and therefore tests of how the emulsion with lipid-soluble flavors would alter the flavor of cheese or yogurt are unknown. Also, research into the level of intensity required to impart the desired
contrast in flavor to the original product (e.g., cheese, yogurt, sauce, dip, etc.) must be conducted.

Opportunities: the following opportunities illustrate possibilities of applying \( \pi \)-emulsions in the market place:

1. Consumers now have expectations of more nutrient-rich foods (Bagel, 2004). \( \pi \)-emulsions could easily be incorporated into any dairy system as a functional ingredient, which would add value to the consumer.

2. Consumers also have expectations of low-fat foods (Wansink & Chandon, 2006). The addition of \( \pi \)-emulsions can be used as a fat replacer for products such as salad dressings, sauces and dips to create low-fat alternatives with the same textural properties. It would be possible to replace some milk fat in complete dairy systems (e.g., yogurt) and yet retain the sensory aspects.

Threats: possible threats associated with \( \pi \)-emulsions are minimal but include:

1. Intense competition in the food industry. There are large numbers of players, which intensifies the rivalry (Datamonitor, 2011a). Competition to \( \pi \)-emulsions would be vitamins/extracts/flavored oils/flavor emulsions (e.g., LorAnn Oils) currently on the market. The fragrance and flavor market is around $11-$12 billion and the five largest companies represent 65-70% of the global market, which increases competitiveness (Datamonitor, 2006). Some brands are owned by large food manufacturers and others are small privately owned companies (Datamonitor, 2011b).
π-emulsions need to be shown to be more efficient and effective at flavor/nutrient dispersal throughout the product at or below the cost of the competition.

2. Belief in functional components for food systems by the consumer. Consumers may or may not believe in the health claims of functional foods, which impacts their decision to purchase products containing the “value-added” components (Naylor, Droms, & Haws, 2009).

3. Costs for implementation (Bettencourt & Bettencourt, 2011). The challenge for corporate management would be to identify and evaluate any changes to machinery, storage, marketing, and distribution that would be required.

5. Target Market

Although the potential for global marketing is a possibility, for the purpose of this paper only two markets will be addressed.

5.1 Primary Market

Manufacturers of dairy products, who want to increase nutritional value and/or add product lines with a variety of flavors, are the primary market. For example, artisan cheese makers could add functional ingredients to make a healthier cheese or add flavor notes to their cheese, creating new and exciting combinations to expand the appeal of cheese in the market. By incorporating π-emulsions, the loss of the functional component would be minimized, increasing cost-effectiveness an objective of suppliers (Christiansen, 2011). Also, manufactures could incorporate these ingredients into other
dairy products, such as yogurt smoothies, sour cream, etc. Suppliers of \( \pi \)-emulsions are committed to expanding facilities to meet the demands of the manufacturer expectations (Christiansen, 2011).

\( \pi \)-emulsions can potentially be used as a base for new low-fat (more unsaturated fat than saturated fat) products. The shelf-life might also be increased with the added stability of \( \iota \)-carrageenan in the product. Fortified \( \pi \)-emulsions with \( \omega-3 \) rich oil would have a longer shelf-life to off-flavors as the oxidizing compounds would first react with the \( \iota \)-carrageenan and protein levels before oxidizing the \( \omega-3 \) unsaturated fatty acids. The pH of these emulsions would likely be acidic so as the net-charge around the droplets would be positive and thereby decrease interaction with oxidizing agents such as iron and copper. The suppliers of \( \pi \)-emulsions are dedicated to investigating both nutritional claims and applications (Christiansen, 2011).

5.2 Secondary Market

The home chef. A little \( \pi \) will go a long way. The addition of flavorants increases the pleasure of food, especially for the those above the age of 60, whose ability to taste has diminished (Boczko & McKeon, 2010). \( \pi \)-emulsions could mimic flavors that are normally found only in restaurants. Being made of natural ingredients \( \pi \)-emulsions appeal to consumers because they have clean label friendly statements (Jacobsen, 2011).
Consumers are attracted to flavor variety, which indicates that line extensions offering perceived variety are a value-add (Pullgadda & Ross, 2010). Manufacturers of π-emulsions should advertise their range of possibilities under one name with multiple options (e.g., various flavor and/or nutrient combinations).

Emulsions could also be freeze-dried and sold as powders, which would reduce storage and transportation costs. The shelf-life would be increased as the water activity would be minimal, and storage in amber glass bottles would decrease oxidation. The powder could either be added to flavor baked goods or dairy items or used as a sprinkle of flavor to be added directly on top of items (e.g., yogurt or beverages such as milk or hot chocolate, coffee, or protein shakes). The product could be sold in small packets to be used as a flavoring versus a creamer or supplement (Johnsen, 2006).

6. Conclusion

This is a new product that can expand the fortification of foods by emulsions. Although further testing on flavor components and texture needs to be performed prior to flavor line production, incorporating fat-soluble vitamins into the emulsion is ready now to increase product value.

References


CHAPTER 8
CONCLUSIONS

The retention of vitamin D was improved by fortifying the cheese milk with an emulsion (78% ± 8%) versus the direct addition of vitamin D oil (58% ± 3%) for full fat Cheddar cheese. The concentration added in this study was at 5-10 IU/g (140-280 IU/serving), and the retention was higher than the ones reported by Banville, Vuillemard, and Lacroix (2000), who added approximately 4 IU/g of cheese and had retentions of 41% to 62% depending on the type of fortification (fortified oil, emulsion, or emulsion in oil). The values of retention in our study are lower than those seen by Kazmi, Vieth, and Rousseau (2007) and Wagner et al. (2008) who observed retentions of approximately 90% for both their model Cheddar cheese-like system and their manufacturer’s scale Cheddar cheese. However, the concentration of vitamin D used for the latter’s research was between 500-1000 IU/g (14-28,000 IU/serving), which is greater than the upper tolerable limit (4000 IU/d) as recommended by the National Institute of Health (2011).

This study shows that the use of dairy proteins from nonfat dry milk (NDM) as the emulsifier results in a better retention if vitamin D than using emulsions formulated with polysorbate 80 emulsifier. The improved retention was also observed in comparing the retention of fortified emulsions incorporated into low-fat curd. Wagner et al. (2008) reported a approximately 55% retention of low-fat curd; however, this study with a model system at similar concentrations (900 to 250 IU/g, respectively) had a retention of near 97%. The discrepancy between the two studies might be due to the emulsifier used. The
dairy proteins might interact more with the caseins forming the curd matrix that the polysorbate 80, which would entrap more fortified emulsion and increase the retention.

This study shows that dairy based emulsions are good carriers for lipid-soluble components that need to be incorporated into a curd matrix.

The hypothesis that the proteins were interacting with the curd matrix led to analyzing emulsions with bi-layers. Both the primary and secondary layers had a net charge on them, and the study was to determine if the interactions between emulsion and curd matrix could be increased either with a change in the outer layer (polysaccharide/protein) or the charge of the outer layer (positive/negative). The protein content of the emulsifier (NDM) was altered from 2% protein in the vitamin D experiment to 1% protein in the bilayer emulsion study. The rate of destabilization remained approximately at 0.6 mm/d at 5% oil concentration for both amounts of protein. The decrease in protein concentration for the bilayer study decreased the NDM used which improved the production of the emulsion.

The addition of the secondary layer to the emulsion led to significant changes in stability as the pH of the solution was altered. The 1-carrageenan (1-carr) secondary emulsions had the greatest stability, independent of pH, which was due to the sulfate groups remaining ionized at pH >2 (Damodaran, Parkin, & Fennema, 2008). The least stable 1-carr secondary emulsions were obtained at pH 3 with a destabilization rate of 0.2 mm/d, which was still better than HMp secondary emulsions of 0.63 mm/d. The stability of the 1-carr secondary emulsions is the opposite of what was observed in other studies at
pH 7 and 3 (Gu, Decker, & McClements, 2004, 2005), which were highly unstable (50-90% creaming). This might be a consequence of the high pressure homogenization conditions used in this study. While previous studies formulated the emulsions using electrostatic deposition over time, our research used high pressure homogenization which forces interactions between the two interfacial layers in the emulsions. This was also in evidence for secondary emulsions made with LMp, which were stable at pH 7.

Dickinson (1998) reported that there is negligible interactions between LMp and dairy proteins at pH 7, which corresponds with the similar ζ-potential values, which would indicate that there would not be a change in stability. However, there is an increase in stability for LMp secondary emulsions compared to the primary emulsion, and the better stability is probably due to the calcium in the system from the NDM, which can link the pectin groups together and create a matrix keeping the primary emulsion suspended (Dickinson, 1998). This is corroborated by the increased viscosity of the system via the pectin connections versus the primary emulsions stability.

The interactions between oil droplets of the primary and secondary emulsions were observed using SEM. Few studies have examined the microstructure of emulsions, though milk has been studied in conjunction with polysaccharides (Acero-Lopez, Alexander, & Corredig, 2010; Bermudez-Aguirre, Mawson, & Barbosa-Canovas, 2008). This research found a good correlation between instability and the amount of interactions between the droplets. Stable emulsions were obtained (<1mm/d) (McClements, 2005) when droplets were well defined and isolated without the presence of an interconnecting
web. The exception was gelatin at pH 3, where the droplets were independent and there was not a webbing observed; however, this was correlated with the fact that the gelatin did not adsorb to the primary emulsion as all the proteins were positively charged and the instability was due to depletion flocculation, which kept the droplets as individual entities but forced the serum out inducing phase separation. The interactions and stability of secondary emulsions was needed to correlate how the emulsion might react with the curd matrix.

The retention of the lipid phase of an o/w emulsion in the curd matrix was quantified using ultraviolet imaging. Ultraviolet light excited the Nile red in the curd and quantified the retention of oil in the curd. Though there was no significant difference between retentions of primary and secondary emulsions, the study did show that it was possible to retain the same amount of the oil of interest (e.g., lipid-soluble vitamin, flavor, etc.) with half the amount of oil. Also, there was a trend of increased retention of 1-carrageenan and LMp secondary emulsions at pH 7 at 0.01M CaCl₂. The increased retention might have to do with the interactions between the highly anionic polysaccharide and divalent cation (Ca²⁺) and the dairy proteins. These two samples were the exception as the rest of the emulsions at various pH had an overall trend to have less retention in the curd as the calcium concentration increased to 0.01M than the curd with no additional calcium.

Future research could be done on the quantification of fortified o/w emulsions in different complexes (i.e., yogurt, tofu, gelatin, etc.) at different levels of concentration.
Also, sensory analysis is needed to determine the textural properties of the emulsions and how they might benefit another system such as being a fat substitute. Secondary layers with a net positive charge at pH 7 would be interesting to investigate, as they might interact even more with the curd matrix as the pH drops from 6.8 to 5.4 over the course of cheese make.

References


APPENDICES
APPENDIX A

STATISTICS FOR CHAPTER 3

Table A 1. ANOVA table for curd (g) of bench-top curd with whole and skim milk for different treatments (NDM, WPC, NaCN, and CaCN)

<table>
<thead>
<tr>
<th>Source</th>
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<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Block</td>
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<td>86.824827</td>
<td>43.412413</td>
<td>1.89</td>
<td>0.1794</td>
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<td>Milk</td>
<td>1</td>
<td>3598.6082</td>
<td>3598.6082</td>
<td>156.9</td>
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<tr>
<td>treat</td>
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<td>31.618047</td>
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<td>0.34</td>
<td>0.8442</td>
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<tr>
<td>Milk*treat</td>
<td>4</td>
<td>18.420487</td>
<td>4.605122</td>
<td>0.2</td>
<td>0.9346</td>
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</table>

Table A 2. ANOVA table for whey (g) of bench-top curd with whole and skim milk for different treatments (NDM, WPC, NaCN, and CaCN)

<table>
<thead>
<tr>
<th>Source</th>
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<th>Sum of Squares</th>
<th>Mean Square</th>
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<th>p-value</th>
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<td>2.68</td>
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<tr>
<td>Milk</td>
<td>1</td>
<td>4035.6401</td>
<td>4035.6401</td>
<td>140.21</td>
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<td>4</td>
<td>40.757153</td>
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<td>0.8379</td>
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<td>Milk*treat</td>
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<td>15.1861</td>
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<td>0.9687</td>
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Table A 3. ANOVA table for total IU curd of bench-top curd with whole and skim milk for different treatments (NDM, WPC, NaCN, and CaCN)

<table>
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<tr>
<th>Source</th>
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<th>p-value</th>
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<td>Block</td>
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<td>732.55</td>
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<td>243358.33</td>
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<tr>
<td>treat</td>
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<td>3732391.3</td>
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<td>&lt;.0001</td>
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<tr>
<td>Milk*treat</td>
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<td>1249050.7</td>
<td>312262.68</td>
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<td>0.3939</td>
</tr>
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</table>
Table A 4. ANOVA table for total IU whey of bench-top curd with whole and skim milk for different treatments (NDM, WPC, NaCN, and CaCN)

<table>
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<th>p-value</th>
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<td>46532.676</td>
<td>23266.338</td>
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<tr>
<td>Milk</td>
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<td>14727.442</td>
<td>14727.442</td>
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</tr>
<tr>
<td>treat</td>
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<td>5950044.5</td>
<td>1487511.1</td>
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<td>&lt;.0001</td>
</tr>
</tbody>
</table>

| Milk*treat  | 4  | 54347.463      | 13586.866   | 0.74 | 0.5765  |

Table A 5. ANOVA table for total IU whey+curd of bench-top curd with whole and skim milk for different treatments (NDM, WPC, NaCN, and CaCN)

<table>
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<th>Mean Square</th>
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<th>p-value</th>
</tr>
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<td>0.9134</td>
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<tr>
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<td>138351.98</td>
<td>138351.98</td>
<td>0.45</td>
<td>0.5124</td>
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<tr>
<td>treat</td>
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<td>2064826.2</td>
<td>516206.56</td>
<td>1.67</td>
<td>0.2014</td>
</tr>
</tbody>
</table>

| Milk*treat  | 4  | 1140782.2      | 285195.54   | 0.92 | 0.4734  |

Table A 6. ANOVA table for Percent IU in curd per (whey+curd) of bench-top curd with whole and skim milk for different treatments (NDM, WPC, NaCN, and CaCN)

<table>
<thead>
<tr>
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<th>p-value</th>
</tr>
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<td>4.120779</td>
<td>2.060389</td>
<td>0.27</td>
<td>0.7672</td>
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<tr>
<td>Milk</td>
<td>1</td>
<td>27.624307</td>
<td>27.624307</td>
<td>3.61</td>
<td>0.0737</td>
</tr>
<tr>
<td>treat</td>
<td>4</td>
<td>4308.0586</td>
<td>1077.0146</td>
<td>140.57</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

| Milk*treat  | 4  | 99.955333      | 24.988833   | 3.26 | 0.0354  |
Table A 7. ANOVA table for total IU in the milk of bench-top curd with whole and skim milk for different treatments (NDM, WPC, NaCN, and CaCN)

<table>
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<tr>
<th>Source</th>
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<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
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<tr>
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<td>2</td>
<td>565.7965</td>
<td>282.89825</td>
<td>1.11</td>
<td>0.3506</td>
</tr>
<tr>
<td>Milk</td>
<td>1</td>
<td>397.58632</td>
<td>397.58632</td>
<td>1.56</td>
<td>0.2273</td>
</tr>
<tr>
<td>treat</td>
<td>4</td>
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<td>1433.8001</td>
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<tr>
<td>Milk*treat</td>
<td>4</td>
<td>901.11112</td>
<td>225.27778</td>
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</table>

Table A 8. ANOVA table for percent fat in the cheese (full vs. reduced fat)

<table>
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<th>p-value</th>
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<tr>
<td>Milk</td>
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<td>220.7</td>
<td>110.3</td>
<td>0.0009</td>
</tr>
<tr>
<td>Residual</td>
<td>6</td>
<td>23.83</td>
<td>3.972</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>244.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table A 9. ANOVA table for percent moisture in the cheese (full vs. reduced fat)

<table>
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<th>Mean Square</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>2</td>
<td>0.9074</td>
<td>0.4537</td>
<td>0.7638</td>
</tr>
<tr>
<td>Residual</td>
<td>6</td>
<td>9.653</td>
<td>1.609</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>10.56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table A 10. ANOVA table for final weight of cheese (full vs. reduced fat)

<table>
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<tr>
<th>Source</th>
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<td>Milk</td>
<td>2</td>
<td>0.1203</td>
<td>0.06016</td>
<td>P&lt;0.0001</td>
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<tr>
<td>Residual</td>
<td>6</td>
<td>0.004668</td>
<td>0.000778</td>
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<tr>
<td>Total</td>
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<td>0.125</td>
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</table>
Table A11. ANOVA table for pH of the cheese (full vs. reduced fat)

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<tr>
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<tr>
<td>Milk</td>
<td>2</td>
<td>0.09056</td>
<td>0.04528</td>
<td>0.0393</td>
</tr>
<tr>
<td>Residual</td>
<td>6</td>
<td>0.04667</td>
<td>0.007778</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>0.1372</td>
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</tbody>
</table>

Table A12. ANOVA table for percent salt in the cheese (full vs. reduced fat)

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<tbody>
<tr>
<td>Milk</td>
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<td>1.27</td>
<td>0.6348</td>
<td>0.0817</td>
</tr>
<tr>
<td>Residual</td>
<td>6</td>
<td>0.9735</td>
<td>0.1623</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>2.243</td>
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</tr>
</tbody>
</table>

Table A13. ANOVA table for IU in curd in small-scale cheese (full, reduced, control)

<table>
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<th>p-value</th>
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<tr>
<td>rep</td>
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<td>256200</td>
<td>128100</td>
<td>0.4151</td>
<td>0.6858</td>
</tr>
<tr>
<td>milk</td>
<td>2</td>
<td>6854000</td>
<td>3427000</td>
<td>11.11</td>
<td>0.0233</td>
</tr>
<tr>
<td>Residual</td>
<td>4</td>
<td>1234000</td>
<td>308600</td>
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</tr>
</tbody>
</table>

Table A14. ANOVA table for theoretical amount of vitamin D IU in milk in small-scale cheese (full, reduced, control)

<table>
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<th>p-value</th>
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<tr>
<td>rep</td>
<td>2</td>
<td>26670</td>
<td>13330</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>milk</td>
<td>2</td>
<td>6717</td>
<td>3358</td>
<td>0.5038</td>
<td>0.6381</td>
</tr>
<tr>
<td>Residual</td>
<td>4</td>
<td>26670</td>
<td>6667</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A 15. ANOVA table for percent IU in curd per IU in milk with theoretical amount of vitamin D in small-scale cheese (full, reduced, control)

<table>
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<th>p-value</th>
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<td>rep</td>
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<td>57.56</td>
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<td>680.2</td>
<td>340.1</td>
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<td>0.0261</td>
</tr>
<tr>
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<td>4</td>
<td>131.1</td>
<td>32.78</td>
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</table>
APPENDIX B

STATISTICS FOR CHAPTER 4

Table B 1. ANOVA table for \( \zeta \)-potential of primary and secondary emulsions with respect to pH

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
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<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \zeta )-potential * pH</td>
<td>8</td>
<td>2311</td>
<td>288.9</td>
<td>31.67 &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>( \zeta )-potential</td>
<td>4</td>
<td>9138</td>
<td>2284</td>
<td>250.4 &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>2</td>
<td>22310</td>
<td>11160</td>
<td>1223 &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>60</td>
<td>547.4</td>
<td>9.123</td>
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</table>

Table B 2. ANOVA table for change in height (mm) of serum for primary, control, and secondary emulsions at pH 7

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<tbody>
<tr>
<td>Emulsion*Day</td>
<td>35</td>
<td>123.5</td>
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<td>8.918 &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Emulsion</td>
<td>5</td>
<td>273.7</td>
<td>54.75</td>
<td>138.4 &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>7</td>
<td>291.1</td>
<td>41.58</td>
<td>105.1 &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>168</td>
<td>66.47</td>
<td>0.3956</td>
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</tbody>
</table>

Table B 3. ANOVA table for change in height (mm) of serum for primary, control, and secondary emulsions at pH 5

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>Emulsion*Day</td>
<td>35</td>
<td>1080</td>
<td>30.87</td>
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<td>Emulsion</td>
<td>5</td>
<td>5505</td>
<td>1101</td>
<td>73.19 &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>7</td>
<td>3603</td>
<td>514.7</td>
<td>34.21 &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>168</td>
<td>2527</td>
<td>15.04</td>
<td></td>
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</tr>
</tbody>
</table>
Table B 4. ANOVA table for change in height (mm) of serum for primary, control, and secondary emulsions at pH 3

<table>
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<th>Source</th>
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<tbody>
<tr>
<td>Emulsion*Day</td>
<td>35</td>
<td>1296</td>
<td>37.02</td>
<td>3.01</td>
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<tr>
<td>Emulsion</td>
<td>5</td>
<td>8398</td>
<td>1680</td>
<td>136.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Day</td>
<td>7</td>
<td>3041</td>
<td>434.4</td>
<td>35.34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>168</td>
<td>2065</td>
<td>12.29</td>
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</tr>
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</table>

Table B 5. ANOVA table for droplet size (µm) for primary, control, and secondary emulsions at pH 7

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<tr>
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<td>0.09426</td>
<td>0.009426</td>
<td>0.8175</td>
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<td>Day</td>
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<td>0.02493</td>
<td>0.01247</td>
<td>1.081</td>
<td>0.3546</td>
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<tr>
<td>Emulsion</td>
<td>5</td>
<td>0.2083</td>
<td>0.04166</td>
<td>3.613</td>
<td>0.0135</td>
</tr>
<tr>
<td>Residual</td>
<td>25</td>
<td>0.2883</td>
<td>0.01153</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table B 6. ANOVA table for droplet size (µm) for primary, control, and secondary emulsions at pH 5

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion*Day</td>
<td>10</td>
<td>643</td>
<td>64.3</td>
<td>0.1558</td>
<td>0.9983</td>
</tr>
<tr>
<td>Day</td>
<td>2</td>
<td>120.6</td>
<td>60.32</td>
<td>0.1461</td>
<td>0.8645</td>
</tr>
<tr>
<td>Emulsion</td>
<td>5</td>
<td>35620</td>
<td>7124</td>
<td>17.26</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>44</td>
<td>18160</td>
<td>412.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table B 7. ANOVA table for droplet size (µm) for primary, control, and secondary emulsions at pH 3

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion*Day</td>
<td>8</td>
<td>39.37</td>
<td>4.922</td>
<td>0.7085</td>
<td>0.682</td>
</tr>
<tr>
<td>Day</td>
<td>2</td>
<td>1.593</td>
<td>0.7967</td>
<td>0.1147</td>
<td>0.892</td>
</tr>
<tr>
<td>Emulsion</td>
<td>4</td>
<td>399.2</td>
<td>99.81</td>
<td>14.37</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>34</td>
<td>236.2</td>
<td>6.946</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table B 8. ANOVA table for viscosity (\( \eta \)) for primary, control, and secondary emulsions at pH 7

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion*Day</td>
<td>8</td>
<td>20.72</td>
<td>2.589</td>
<td>21.3</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Emulsion</td>
<td>4</td>
<td>143.7</td>
<td>35.92</td>
<td>295.5</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Day</td>
<td>2</td>
<td>2.862</td>
<td>1.431</td>
<td>11.77</td>
<td>0.0007</td>
</tr>
<tr>
<td>Residual</td>
<td>16</td>
<td>1.945</td>
<td>0.1216</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table B 9. ANOVA table for viscosity (\( \eta \)) for primary, control, and secondary emulsions at pH 5

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion*Day</td>
<td>5</td>
<td>4.834</td>
<td>0.9667</td>
<td>0.988</td>
<td>0.4537</td>
</tr>
<tr>
<td>Emulsion</td>
<td>5</td>
<td>25.76</td>
<td>5.153</td>
<td>5.266</td>
<td>0.0042</td>
</tr>
<tr>
<td>Day</td>
<td>1</td>
<td>5.856</td>
<td>5.856</td>
<td>5.985</td>
<td>0.0256</td>
</tr>
<tr>
<td>Residual</td>
<td>17</td>
<td>16.63</td>
<td>0.9785</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table B 10. ANOVA table for viscosity (\( \eta \)) for primary, control, and secondary emulsions at pH 3

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion*Day</td>
<td>5</td>
<td>5.342</td>
<td>1.068</td>
<td>1.134</td>
<td>0.3907</td>
</tr>
<tr>
<td>Emulsion</td>
<td>5</td>
<td>15.34</td>
<td>3.067</td>
<td>3.256</td>
<td>0.04</td>
</tr>
<tr>
<td>Day</td>
<td>1</td>
<td>5.096</td>
<td>5.096</td>
<td>5.41</td>
<td>0.0368</td>
</tr>
<tr>
<td>Residual</td>
<td>13</td>
<td>12.25</td>
<td>0.942</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX C

STATISTICS FOR CHAPTER 5

### Table C 1. ANOVA table for change in height (mm) of serum for primary, control, and secondary emulsions at pH 7

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion*Day</td>
<td>35</td>
<td>123.5</td>
<td>3.528</td>
<td>8.918</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Emulsion</td>
<td>5</td>
<td>273.7</td>
<td>54.75</td>
<td>138.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Day</td>
<td>7</td>
<td>291.1</td>
<td>41.58</td>
<td>105.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>168</td>
<td>66.47</td>
<td>0.3956</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table C 2. ANOVA table for change in height (mm) of serum for primary, control, and secondary emulsions at pH 5

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion*Day</td>
<td>35</td>
<td>1080</td>
<td>30.87</td>
<td>2.052</td>
<td>0.0014</td>
</tr>
<tr>
<td>Emulsion</td>
<td>5</td>
<td>5505</td>
<td>1101</td>
<td>73.19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Day</td>
<td>7</td>
<td>3603</td>
<td>514.7</td>
<td>34.21</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>168</td>
<td>2527</td>
<td>15.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table C 3. ANOVA table for change in height (mm) of serum for primary, control, and secondary emulsions at pH 3

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion*Day</td>
<td>35</td>
<td>1296</td>
<td>37.02</td>
<td>3.011</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Emulsion</td>
<td>5</td>
<td>8398</td>
<td>1680</td>
<td>136.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Day</td>
<td>7</td>
<td>3041</td>
<td>434.4</td>
<td>35.34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>168</td>
<td>2065</td>
<td>12.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX D

STATISTICS FOR CHAPTER 6

**Table D 1.** Covariance estimates for Nile red intensity in the curd for pH (7, 5, 3), emulsion treatments (primary, gelatin, HMp, t-carr, and LMp), for milk with CaCl2 concentrations of 0, 0.004M and 0.01M

<table>
<thead>
<tr>
<th>Covariance Parameter</th>
<th>Subject</th>
<th>Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block</td>
<td>replicate</td>
<td>0</td>
</tr>
<tr>
<td>Block*pH</td>
<td>replicate</td>
<td>0.00154</td>
</tr>
<tr>
<td>Block<em>pH</em>Treat</td>
<td>replicate</td>
<td>0.00165</td>
</tr>
<tr>
<td>Block<em>pH</em>milk</td>
<td>replicate</td>
<td>0.00702</td>
</tr>
<tr>
<td>Residual</td>
<td></td>
<td>0.01132</td>
</tr>
</tbody>
</table>

**Table D 2.** Fit statistics\(^a\) for Nile red intensity in the curd for pH (7, 5, 3), emulsion treatments (primary, gelatin, HMp, t-carr, and LMp), for milk with CaCl2 concentrations of 0, 0.004M and 0.01M

<table>
<thead>
<tr>
<th>Fit Statistics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2 Res Log Likelihood</td>
<td>-196.7</td>
</tr>
<tr>
<td>AIC</td>
<td>-188.7</td>
</tr>
<tr>
<td>AICC</td>
<td>-188.5</td>
</tr>
<tr>
<td>BIC</td>
<td>-189.6</td>
</tr>
</tbody>
</table>

\(^a\)Autoregressive moving average structure (1,1). AIC = Akaike information criterion; AICC = Finite-population corrected AIC; BIC = Schwarz’s Bayesian information criterion

**Table D 3.** Type 3 tests of fixed effects (ANOVA) for Nile red intensity in the curd for pH (7, 5, 3), emulsion treatments (primary, gelatin, HMp, t-carr, and LMp), for milk with CaCl2 concentrations of 0, 0.004M and 0.01M

<table>
<thead>
<tr>
<th>Effect</th>
<th>df</th>
<th>Den DF</th>
<th>F Value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>2</td>
<td>15</td>
<td>0.49</td>
<td>0.6242</td>
</tr>
<tr>
<td>Treat</td>
<td>4</td>
<td>60</td>
<td>2.10</td>
<td>0.0926</td>
</tr>
<tr>
<td>pH*Treat</td>
<td>8</td>
<td>60</td>
<td>1.47</td>
<td>0.1861</td>
</tr>
<tr>
<td>milk</td>
<td>2</td>
<td>30</td>
<td>3.48</td>
<td>0.0437</td>
</tr>
<tr>
<td>pH*milk</td>
<td>4</td>
<td>30</td>
<td>0.26</td>
<td>0.8990</td>
</tr>
<tr>
<td>Treat*milk</td>
<td>8</td>
<td>120</td>
<td>0.74</td>
<td>0.6601</td>
</tr>
<tr>
<td>pH<em>Treat</em>milk</td>
<td>16</td>
<td>120</td>
<td>0.97</td>
<td>0.4905</td>
</tr>
</tbody>
</table>
APPENDIX E

PERMISSION-TO-USE LETTERS

Megan,

Please feel free to use the paper referenced here in your dissertation.

Regards,
Carl

Carl Brothersen
Associate Director
Western Dairy Center
8700 Old Main Hill
Logan, UT 84322-8700
phone: 435-797-3466
tax: 435-797-2379
e-mail: carl.brothersen@usu.edu

"In wilderness is the preservation of the world." Henry David Thoreau.

On Jan 13, 2012, at 11:10 AM, Megan Tippets wrote:

January 13, 2012

Dear Carl Brothersen,

I am in the process of preparing my dissertation in the NDFS department at Utah State University. I hope to complete in the spring of 2012.

I am requesting your permission-to-use Chapter 3: Fortification of Cheese with Vitamin D3 using Dairy Protein Emulsions as Delivery Systems (see attached document) in my dissertation.

I will include acknowledgments in the chapter as a footnote.

Please indicate your approval of this request by signing in the space provided. If you have any questions, please reply to this email.

I hope you will be able to reply immediately.

Thank you for your cooperation,

——

I hereby give permission to Megan Tippets to print the requested material in her dissertation.
I hereby give permission to Megan Tippett to print the above requested material in her dissertation.

Signed

Fen-Ann K. Shen

---

From: Megan Tippett [nutmeg2002@yahoo.com]
Sent: Wednesday, January 11, 2012 6:47 PM
To: Fen-Ann Shen
Subject: permission to use letter

January 11, 2012

Dear F.K. Shen,

I am in the process of preparing my dissertation in the NDFS department at Utah State University. I hope to complete in the spring of 2012.

I am requesting your permission to use Chapter 5: FAT DROPLET MICROSTRUCTURE OF PROTEIN/POLYSACCHARIDE OR PROTEIN/PROTEIN BI-LAYER EMULSIONS AS A FUNCTION OF PH. I will include acknowledgments in the chapter as a footnote.

Please indicate your approval of this request by signing in the space provided. If you have any questions, please reply to this email.

I hope you will be able to reply immediately.

Thank you for your cooperation.

I hereby give permission to Megan Tippett to print the above requested material in her dissertation.

Signed
CURRICULUM VITAE

MEGAN TIPPETTS

EDUCATION
PhD FOOD SCIENCE, Utah State University, Logan, UT, 2008-present
Research and Teaching Assistantships:
~ Research: Study the retention of vitamin D fortified emulsions in cheese
~ NFS Experimental Foods Course Teacher's Assistant (Spring 2008 and 2009)
~ Sensory assistant and panelist for various sensory studies (2006-present)
Awards:
~ 2011 USU Dissertation Fellowship
~ 1st Place ID Milk Processors Association (2008, 2011) for innovative dairy products
~ 2009 Honored Student Award: American Oil Chemists' Society
~ 2nd Place ID Milk Processors Association (2010) for an innovative dairy product
Memberships:
~ Member of American Oil Chemists' Society (AOCS) (2006-present)
~ Member of American Association of Candy Technology (AACT) (2007-present)
~ Member of the USU Food Product Development Team 2007-2011

MS FOOD SCIENCE, Utah State University, Logan, UT, 2008
Research and Teaching Assistantships:
~ Research: Study the effect of oil content and processing conditions on oil-in-water emulsions
~ NFS Food Sensory Course Teacher's Assistant (Spring 2008)
Awards:
~ Third Place: IFT Bonneville Section Student Research Poster Contest (2008)
~ Recipient, Ghandi Scholarship (2006-07; 2007-8)
~ Recipient, Seeley-Hinkley Scholarship (2006-07)

BS CHEMICAL ENGINEERING, University of Utah, Salt Lake City, UT, 2002
~ Recipient, Isaacson Scholarship (2000-01; 2001-02)
~ Team Leader of the first ChemE Car team for the University of Utah
~ Team Leader for brochure and website for the 2000 Rocky Mountain Regional AIChE conference. Increased attendance by 60% with professional quality advertising and informational packets.

BA FRENCH, University of Utah, Salt Lake City, Utah, 1998
~ French Tutorial in Rouen, France (1998)
~ Study Abroad in Neufchatel, Switzerland (1995)

ARTICLES


PROFESSIONAL EXPERIENCE
NESTLÉ PTC MARYSVILLE, Marysville, OH
“provides solutions for good-tasting food beverages, as well as services, that bring nutrition, health and wellness to consumers.”
Intern (2010-2011)
~ Analyzed oil samples:
  o Oxidation
  o Crystallization and melting parameters
  o Solid fat content profiles
~ Worked on specifications and trained others to work on company program.

WATSON PHARMECEUTICAL, Salt Lake City, UT
“… dedicated to being a leading provider of pharmaceutical products.”
~ Created chemical inventory program, increased efficiency for chemical availability and ordering
~ Increased efficiency, over 50% of quality control tests where completed prior to due date

UNITED STATES AIR FORCE, Albuquerque, NM
The mission of the 377th Air Base Wing is to provide world-class nuclear surety, expeditionary forces, and support to base operations.

Bioenvironmental Engineer, Officer in Charge (OIC) - Industrial Hygiene Element (2005 - 2006)
~ Chief Bioenvironmental Engineer of deployable/contingency Preventive Aerospace Medicine Unit Type Code (UTC) for Kirtland Air Force Base (AFB)
~ Lead 1 civilian, 2 officers, 17 enlisted in 5 industrial hygiene teams
~ Directed operational risk management of occupational activities for 250+ industrial workplaces

Accomplishments:
~ Received Honorable Discharge from US Air Force in January 2006
~ Awarded the Air Force Commendation Medal for Outstanding Achievement at Kirtland AFB
~ Recognized as Outstanding Performer by Chief Goral of the Pentagon during Operational Readiness Exercise November 2005—only 2 were honored out of 150+ participants

Bioenvironmental Engineer, OIC Environmental Protection Element (2003 - 2005)
~ Chief Bioenvironmental Engineer at Manas Air Base, Kyrgyzstan (summer 2004) for OPERATION ENDURING FREEDOM
~ Led 1 civilian, 2 NCOs, and 1 Airman, and $165K of contractor support
~ Ran sampling efforts; ensured compliance with Environmental Protection Agency (EPA) laws and regulations

Accomplishments:
~ Awarded the Air Force Commendation Medal for Outstanding Achievement during OPERATION ENDURING FREEDOM, summer 2004
~ Awarded Company Grade Officer of the Year (2004) for Aerospace Medicine Squadron
~ Awarded Company Grade Officer of the Quarter for Medical Group, 3rd quarter 2004
BD MEDICAL SYSTEMS, Sandy, UT
To "Optimize the Infusion Therapy Process Wherever Care is Delivered"
- Completed protocol on temperature/humidity chamber, new equipment used to age samples
- Assembled prototypes for various new designs of catheters
- Tested prototypes using Standard Operating Procedures (SOPs) for various physical properties