STUDYING MILK COAGULATION KINETICS WITH LASER SCANNING
CONFOCAL MICROSCOPY, IMAGE PROCESSING, AND COMPUTATIONAL
MODELING

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ABSTRACT

STUDYING MILK COAGULATION KINETICS WITH LASER SCANNING CONFOCAL MICROSCOPY, IMAGE PROCESSING, AND COMPUTATIONAL MODELING

Richard Joseph Hennessy

The kinetics of milk coagulation are complex and still not well understood. A deeper understanding of coagulation and the impact of the relevant factors would aid in both cheese manufacturing and also in determining the nutritional benefits of dairy products. A method using confocal microscopy was developed to follow the movement of milk fat globules and the formation of a milk protein network during the enzyme-induced coagulation of milk. Image processing methods were then used to quantify the rate of coagulation. It was found that the texture of the protein network is an indicator of the current status of the milk gelation, and hence can be used to monitor the coagulation process. The imaging experiment was performed on milk gels with different concentrations of the coagulation enzyme, chymosin. Rheological measurements were taken using free oscillation rheometry to validate the imaging results. Both methods showed an inverse relationship between rennet concentration and the coagulation time.

The results from the imaging study were used to create a computational model, which created simulated images of coagulating milk. The simulated images were then analyzed using the same image analysis algorithm. The temporal protein network texture behavior in the simulated images followed the same pattern as the protein texture in the confocal imaging data. The model was developed with temperature and rennet concentration as user inputs so that it could be implemented as a predictive tool for milk coagulation.

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I. INTRODUCTION

1.1 Motivation

The kinetics of milk coagulation are complex and still not well understood. A deeper understanding of coagulation and the impact of the relevant factors would aid in both cheese manufacturing and also in determining the nutritional benefits of dairy products (Karlsson, 2007). Empirically, milk coagulation has been widely studied (Alexander, 1924; Mellema, 2002; Mateo, 2009). The rate and extent of coagulation depends on several factors, such as firmness of gel to cutting, surface area of the curd, pressure, pH, temperature, and the composition of the milk, which can vary both biologically and seasonally (Carlson, 1987; Carlson, 1985; Arias, 2000). Development of a 2-dimensional optical method with high spatial and temporal resolution would allow us to study in detail the gelation kinetics of milk (Auty, 1999). A deeper understanding of the milk coagulation kinetics and the relevant factors that can affect the process will help dairy product manufactures predict the important coagulation parameters based on inputs such as temperature, pH, enzyme concentration, and the source of the milk. Understanding the kinetics of milk coagulation will also lead to a better understanding of how nutrition is extracted from dairy products during digestion. This information can then be incorporated into a computer model of milk coagulation, which can be used as a predictive tool.

1.2 Current Methods

Current methods of monitoring the coagulation of milk include free oscillation rheometry (FOR), traditional rheometry, texture analysis, NIR and MIR spectroscopy, and machine vision.

Free Oscillation Rheometry

The current state-of-the-art non-optical method for studying milk gelation kinetics is based on free oscillation rheometry. This principle was discovered by Prof. Leif Bohlin and Dr. Mats Ranby, and has also been extensively used for the analysis of blood coagulation (Tynngard, 2008). Oscillating a sample to determine its visco-elastic properties is a well-known principle. Traditional rheometry uses an oscillating cup with a bob submerged into the sample. As the measuring cup oscillates, the elasticity of the sample will transfer to the submerged bob. Elasticity can then be measured depending on how the bob moves compared to the cup. Figure 1 below shows a schematic of a traditional rheometer.

Figure 1. Schematic of a traditional rheometer. The red part represents the motor and the blue part represents the bob.

The sample is oscillated in its cup by a motor (red). As elasticity increases, the moving cup will start to affect the bob (blue) inserted in the sample, and the bob will start to move with the cup, but it will lag behind the movement of the cup. (Trkova, 2004). When elasticity finally becomes so high that it would represent a solid material, the two pendulums will move completely in parallel (Tynngard, 2008). The movement of the cup relative to the bob is used to measure the elasticity.

The ReoRox G2, which was used in this study, utilizes a slightly different method. Free Oscillation Rheometry (FOR) can measure both elasticity and viscosity from one single graph. The FOR principle uses an oscillating movement, too, but it also

uses a torsion wire system to set a sample into oscillation. A magnet pulls back the measuring head connected to a torsion wire. On release, the torsion wire will set the cup into free oscillation and its movement is recorded by an optical detector. From the recorded oscillation graph, both elasticity and viscosity for the sample are calculated.

Figure 2, illustrates the FOR mechanism.

Figure 2. Schematic of a rheometer using the free oscillation rheometry technique.

The viscosity and elasticity of a sample are calculated by analyzing the frequency change and amplitude damping of the oscillating sample. Every third second, the system records 15 points of the magnitude of the oscillations. Viscosity is calculated from the amplitude damping of a scan (Tynngard, 2008). Figure 3, below, shows an example of amplitude damping.

Figure 3. Example of the output from the ReoRox G2 showing a decrease in amplitude used to calculated viscosity

In a low viscous material, like water, the cup will oscillate without setting much liquid in movement. Only a thin layer next to the cup will be affected, giving a minimum amount of damping. While for a high viscosity material, like oil, a larger part of the liquid sample in the cup will be forced to move which will slow down the oscillation faster.

A sample's elasticity is calculated from the frequency change of the oscillation (Tynngard, 2008), but it requires a bob that is submerged into the sample. The coagulating sample will work as a flexible media between the cup and the bob, and for every stretch out of the gel, a repelling force is created on the return path. The stronger the elasticity is, the higher the repelling force is created, and a high increase in frequency is obtained.

Figure 4. Sample output displayed on ReoRox software. The pink plot is elasticity and the blue plot it viscosity.

The graph above describes the typical elasticity curve of a coagulating sample. When the coagulating process starts, first viscosity increases, which is shown in the blue plot. The coagulum building up on the surfaces of the cup and the bob adds weight to the cup which will slow down its oscillation. However, the gap of liquid prevents an increase in elasticity. In rheological terms, this is sometimes described as the visco-elastic phase, since the sample is both elastic and viscous at the same time. As the sample coagulates, the gap between the cup and bob will finally be closed, and elasticity in the sample is enabled (Tynngard, 2008). At this point, frequency will start to increase, since the elasticity will have a repelling force on the bob. This point is referred to as the start of gap load.

NIR and MIR Spectroscopy

NIR and MIR spectroscopy combined with chemometric analysis techniques have been used to both predict and measure the coagula properties of milk. NIR radiation is defined as the wavelength region from 750 to 2,500 nm lying between the visible light and the infrared light (Woodcock, 2008). NIR spectroscopy is a physical, non-destructive, high-precision technology requiring minimal or no sample preparation. A typical NIR food spectrum has two dominant and broad peaks located near 1,440 and 1,930 nm (Cattaneo, 2005). These peaks are due to water and are responsible for some typical complications encountered in chemometric analysis. Effects of hydrogen bonding and sample temperature are also found to affect the reliability of NIR spectroscopic results (Cattaneo, 2005). The main disadvantage of NIR spectroscopy is its weak sensitivity to minor constituents such as salt and water-soluble nitrogen (Fagan, 2009). The sensitivity limit is about 0.1% for most constituents. NIR spectroscopy has traditionally been applied for the measurement of compositional parameters of food products (Gonzalez-Martin, 2009). However, it can also be used for the determination of complex properties such as texture and sensory attributes (Fagan, 2007; Anderson, 2005; Herbert, 2000).

MIR spectroscopy is the measurement of the wavelength and intensity of the absorption of the mid-infrared range (2,500 – 50,000 nm) by a sample. The MIR range is sufficiently energetic to excite molecular vibrations to higher energy levels. The wavelength of MIR absorption bands are characteristic of specific types of chemical bonds, and the main application of MIR spectroscopy is the identification of organic and organometallic molecules (Woodcock, 2008; Fagan, 2006). Its application to quantitative

studies has increased during the last decade. Particular advantages of MIR spectroscopy are speed of measurement, moderate instrument cost, and the relative ease of sample presentation. MIR spectroscopy has become more accessible to food samples after the introduction of attenuated total reflection sample presentation systems (De Marchi, 2009).

Machine Vision Techniques

Machine vision techniques involve robust on-line image capture systems in conjunctions with a variety of approaches to image processing. Researchers have found that image processing using a threshold technique had potential for monitoring syneresis (Everard, 2007). Image texture analysis, including first-order gray level statistics, gray level co-occurrence matrix, and fractal dimension have also been used to monitor dairy processing techniques (Fagan, 2008). The fractal dimension technique has been found to be useful for controlling curd moisture content in cheese making (Horne, 1989; Horne, 1987).

Two studies used the observation that during cheese manufacture the color of milk proceeds from a continuous white mass before cutting to a mixture of white particles in a mostly clear yellowish whey (Mateo, 2009; Everard, 2009). Direct observation also reveals that light scattered by whey becomes increasingly yellow in hue as syneresis progresses. Based on these two observations, they were able to gain valuable information about syneresis kinetics by taking the ratio of the white and yellow areas calculated by processing the images obtained after gel cutting.

1.3 Project Goals

The main goal of this project is to develop a novel optical technique to characterize and study the kinetics of milk coagulation. Fluorescence confocal microscopy and image analysis techniques will be used to follow the mobility of particles through the milk coagulation process as they are confined and incorporated into a gel network. All results will be validated using a rheometer to monitor the evolution of rheological properties. This information will then be used to help create a computational model, which can predict the coagulation rate based on different input variables, such as pH, temperature, and rennet concentration.

Despite intensive research effort, now spanning many decades, there is still no definitive overall description of the kinetics of milk gel formation which would allow prediction of the cutting time from knowledge of milk composition and treatment (De Marchi, 2009). It is only recently that we have realized the gelation process can be treated as a continuum and that it was mainly the constraints of earlier theories that artificially divided the process and confined studies to particular stages of the gelation process (Esteves, 2001). In rheometry, we now have the instrumentation to directly measure gelation curves (Trkova, 2004). By combining rheological methods able to accurately measure the changing mechanical properties of coagulating milk and imaging techniques able to measure the changing structural characteristics, we hope to create a more complete description of milk coagulation kinetics.

II. BACKGROUND INFORMATION

2.1 Milk Constituents

To understand the coagulation reaction of milk, we must look more closely at the individual components of milk to discern their role, particularly the fat, protein, and minerals. Figure six, below, shows the percentages of the main constituents in milk.

Figure 5. A pie chart showing the percentage each main constituent in milk represents.

Fat

Fat exists in natural milk as small globules surrounded by membrane proteins and in a size range dependent on the individual cow. The fat in milk is what produces the flavor, smell, and body in cheeses. Unless the milk is homogenized, which is what you would find at a grocery store, the fat globules are trapped in the protein network created in gel formation. This is one reason that the size of the fat globules is important in determining the coagulation properties of the milk (Auty, 2001).

Protein

There are two types of protein that can be found in milk: the globular whey proteins, which are soluble in the serum phase, and the caseins which exist in a stable

colloidal suspension of aggregates known as casein micelles (Muller-Renaud, 2004).

Cheese making exploits the destabilizing mechanisms nature has built into this colloidal system by using the natural enzyme chymosin to hydrolyze the κ -casein and induce the destabilization of the casein micelle system to form a gel (Herbert, 1999). The properties of the caseins play a major role in the gelation kinetics (Schmidt, 1982).

Milk Clotting Enzymes

Milk clotting enzymes were obtained originally by extraction from the stomachs of calf and adult bovine rennets. Rennet is a complex of enzymes produced in any mammalian stomach to digest the mother's milk. Concerns in the 1960s that world cheese production had increased to such an extent that the production of rennet products derived from animal tissue would be insufficient to meet future demand led to the development of alternative products. In the late 1980s, recombinant DNA technology was used to clone the gene for chymosin, the main clotting component of calf rennet. *E. Coli*, *Aspergillus nige* and *Kluveromyces lactis* were used as host organisms. The chymosin products generated now are referred to as fermentation produced chymosin (Castillo, 2006).

Chymosin is a gastric proteinase which is secreted in the abomasal mucosa of new-born mammals during the first days of life (Syme, 2002). It is the main clotting enzyme in calf rennet. The fermentation-produced chymosins have a high ratio of milk clotting to general proteolytic activity and no significant difference in cheese yield have been reported between recombinant chymosin and calf rennet.

2.2 Rennet-Induced Coagulation of Milk

Casein gels can be prepared by adding rennet enzyme. With the addition of rennet, the stability of the dispersion of casein particles is lost. As a result, aggregation takes place, eventually leading to the formation of a space-spanning structure (Lodaite, 2000). The particles in rennet-induced casein gels are para-casein micelles. These are the casein micelles that have been submitted to rennet action. A casein micelle is an approximately spherical particle formed by association of casein molecules, possibly with one intermediate structural level, called sub-micelles (Blair, 1971). At the surface of casein micelles, a hairy layer of the hydrophilic parts of k-casein accounts for a steric stabilization of the casein dispersion. The main active component in chymosin induces aggregation, and consequently gelation, by cutting this hairy layer (Sandra, 2007). Figure 7 shows a drawing of a casein micelle and casein submicelle. Notice the “hairy” layer.

Figure 6. A drawing of the casein micelle and the casein submicelle (Schmidt, 1982).

2.3 Measurement of the Milk Clotting Time

The easiest and most common way to measure milk clotting time is the visible observation of coagulating milk in a rotating tube (Carlson, 1985). The time taken for the milk to appear as a gel is defined as the rennet coagulation time. Figure 8 shows a rennet mixture being added to milk. The importance of the rennet coagulation time has been determined by the number of different techniques tested over the years (Konuklar, 2002; Tan, 2007; Hardy, 1981; Ustunol, 1991). Many of the techniques described have also been developed as research tools to study the influence of variables such as temperature, pH, and milk composition and treatment. The most useful of these techniques are those where the behavior of the variables can be predicted by mathematical models based on descriptions of the reaction mechanisms (Hyslop, 1996; Guinee, 1997).

Figure 7. Rennet being added to milk.

2.4 Simple Kinetic Model of Rennet-Induced Coagulation

The earliest attempt to describe the kinetics of the clotting process was made in the 1870s by Storch and Segeleke. Their model simply stated that the clotting time was inversely related to the concentration of rennet used to clot the milk. A further refinement was postulated by Holter in 1932 and rearranged by Foltmann in 1959 to give the equation:

$$RCT = \frac{k}{[E]} + A \quad (1)$$

where k is a constant describing the coagulation rate, A is a constant describing the clotting time for an infinitely high enzyme concentration, $[E]$ is the enzyme concentration, and RCT is the rennet coagulation time. This relationship is purely empirical, but it is an important relationship which has to be satisfied by any more descriptive mechanistic model, even if only over a restricted range of enzyme concentration and RCT values (Blair, 1971).

2.5 Development of Rheological Properties During Rennet Coagulation

One way to measure gel formation is to monitor the evolution of rheological properties. Dynamic rheology applies an oscillatory shear stress and measures the response from the developing gel. The measurement yields the elastic modulus, which is a measure of the energy stored per oscillation cycle and reflects how the sample behaves as an elastic solid, and the viscous modulus which is a measure of the energy dissipated per cycle and indicates how much the sample behaves as a viscous liquid (Tynngard, 2008). The shear moduli are defined as follows:

$$G' = \frac{\tau_0}{\gamma_0} \cos \delta \quad (2)$$

$$G'' = \frac{\tau_0}{\gamma_0} \sin \delta \quad (3)$$

$$\tan \delta = \frac{G''}{G'} \quad (4)$$

where τ_0 is the shear stress, γ_0 is the strain, G' is the elastic modulus, G'' is the viscous modulus, and δ is the phase angle of the applied stress or strain. This means that when δ is greater than 45 degrees, the viscous component dominates, whereas when δ is less than 45 degrees, the sample appears more like an elastic solid. A rennet-induced milk gel is described as a visco-elastic solid. In rheological terms, the critical gelation point is often taken as the time at which the elastic modulus exceeds the viscous modulus (Karlsson, 2007).

Free oscillation rheometry (FOR) is able to measure both elasticity and viscosity from a single graph. The FOR principle utilizes an oscillating movement using a torsion wire system to set a sample into oscillation. A magnet pulls back the measuring head connected to a torsion wire. On release, the torsion wire will set the cup in free oscillation and its movement is recorded by an optical detector. From the recorded oscillation graph, elasticity and viscosity can be calculated. The viscosity and elasticity of a sample is calculated by analyzing the frequency change and amplitude dampening of the oscillating sample (Tynngard, 2007).

2.6 Modeling the Gel-Firming Kinetics

There have been many attempts to model the growth of the gel firmness with time. These range from the purely empirical to those rooted in some kinetic mechanism.

Tested simply on their ability to fit the observed growth curves, some are more successful than others which fail to reproduce important features. Others have no theoretical basis, which makes them less useful as predictive tools.

Scott Blair and Burnett proposed the following empirical model:

$$G(t) = G_{\infty} \exp\left(\frac{-\tau}{(t - t_g)}\right) \quad (5)$$

to describe the increase in shear modulus (G) with time beyond the gel point occurring at t_g , τ being a constant characteristic of the sample and determined by fitting and G^{∞} being the value of G when $t = \infty$ (Blair, 1971). In favor of this model is the observation immediately following the gel point that it reaches a maximum rate of firmness and tends to plateau as t tends to infinity. Against it is that the model is purely empirical, which makes prediction of the dependence of reaction variables nearly impossible.

Another model, developed by Tuszynski, assumes that the shape of the growth curve is similar to that for simple autocatalytic reactions. This model proposes that the growth kinetics can be fit to:

$$\frac{dG}{dt^*} = kG(G_{\infty} - G) \quad (6)$$

where $t^* = t - t_g$, G_{∞} is the value of the shear modulus at $t = \infty$ and k is the rate constant for the process, and is found by fitting. Again, the model provides no indication how changes in reaction variables will influence the gel-firming kinetics (Carlson, 1987).

Douillard proposed a model where the rate of change of shear modulus with time followed first order kinetics:

$$\frac{dG}{dt^*} = k(G_\infty - G) \quad (7)$$

$$t^* \geq 0 \text{ or } t \geq t_g$$

this equation can be integrated to give:

$$G = G_\infty[1 - \exp(-kt^*)] \quad (8)$$

with the initial condition that $G = 0$ at $t = t_g$.

This equation has been successfully used in several studies on the rennet coagulation of milk. Fitting curves to an nth-order reaction equation shows that the Douillard equation above gives the best fit (Carlson, 1987). Further demonstrations show that the rate parameter, k , has a power-law dependence on enzyme concentration with an exponent of 0.8. Assuming that ‘gel’ reacts with ‘polymer’ in the sol phase and that ‘gel’ does not cross-link by itself, shows that the concentration of gel is proportional to $1 - \exp(-\alpha t)$. Carlson thus reached the important conclusion that beyond the gel point, most of the growth in gel firmness arises as a result of aggregation between the infinite cluster and the smaller clusters and micelles in the sol phase (Carlson, 1987).

The Douillard equation also emerges as a limiting case of the Carlson model shown below. The Douillard equation does not reproduce the acceleratory phase immediately after the gel point, which means further refinements to it are required. Some of these are to be found in the Carlson Model:

$$G = G_\infty \left[1 - \left(\frac{k_l}{k_l - k_f} \right) \exp(-k_f t^*) + \left(\frac{k_l}{k_l - k_f} \right) \exp(-k_l t^*) \right] \quad (9)$$

where $t^* = t - t_g$, k_1 is the rate constant for the creation of ‘active’ sites and k_f is the rate constant for the destruction of these sites as they are incorporated into the gel network. Essentially, the model shows active sites being created on micelles, which then go on to react with one another in forming bonds in the gel network (Carlson, 1987). Both reactions, activation and destruction, are considered first-order processes. Activation requires the enzymatic hydrolysis of κ -casein, and therefore k_1 is proportional to the enzyme concentration. That the site destruction reaction should also be a first-order process is an empirical observation.

Application of the Carlson model to gel firming curves has given excellent fits with very low standard errors. This confirms the findings of Esteves (2001) et al. who compared its performance to those of the Scott Blair and Douillard models. Further efforts must be directed to more extensive tests of the Carlson model and more quantitative testing of the model would also be helpful in determining whether the many parameters involved possess realistic values or whether they are merely ‘best fits’.

2.7 Fractal Models of Rennet-Induced Milk Gels

Fractal theories have been applied to the flocculation of casein particles (Horne, 1989; Dalgleish, 1988). Aggregates can be considered fractal if their geometry is scale invariant which means that their structure is similar when viewed over a large range of magnifications. This means that the emphasis of the fractal concept is on structure, which makes it an ideal analytical tool for the analysis of images. It is a mathematical description of the distribution of a particle cluster or network in space. Various models

have been used to predict gel or cluster properties based on that fractal organization (Horne, 1987).

The number of particles in an aggregate or cluster (N_p) is given by:

$$N_p = \left(\frac{R}{a}\right)^{D_f} \quad (10)$$

where R is the radius of the flow, a is the primary particle size and D_f is the fractal dimension. The fractal dimension is usually a non-integer and is always less than the geometric or dimension of three. Since the number of particles that could be present in a close-packed cluster is given by:

$$N_c = \left(\frac{R}{a}\right)^3 \quad (11)$$

the volume fraction of the cluster is given by:

$$\varphi_{cluster} = \frac{N_p}{N_c} = \left(\frac{R}{a}\right)^{D_f-3} \quad (12)$$

The average volume fraction decreases as the cluster grows. When it reduces to the volume fraction of particles in the system, φ_0 , the clusters fill the total space available and the gel is formed (Horne, 1989).

2.8 Milk Processing and Gel Formation

Milk coagulation by rennet can be influenced by a number of processing treatments applied to the milk. The gel formation characteristics of high-pressure and heat-treated milk have been studied extensively in the last decade (Arias, 2000; De Marchi, 2009; Castillo, 2006; Gatti, 1995).

High Pressure

High pressure treatment influences the coagulation properties of milk indirectly through a number of effects on milk proteins. These effects include a reduction in size of the casein micelles, denaturation of β -lactoglobulin and possible interaction of β -lactoglobulin with micellar k-casein. Gel firmness can be improved by high-pressure treatment of milk through an increased recovery of whey proteins and moisture content (Arias, 2000).

Treatment of milk at pressures of up to 200 MPa for 30 min reduces the rennet coagulation time, while higher pressures, up to 600 MPa, results in rennet coagulation times similar to those of untreated milk. The effect of pressure treatment on micelle size in skim milk is temperature-dependent. Pressure treatment of milk at 4 °C causes a reduction in micelle size, at 20 °C resulted in no change and at 40 °C causes an increase in micelle size (Arias, 2000).

Heat Treatment

Heat treatment of milk results in a number of changes in physico-chemical properties. These include denaturation of whey proteins, the interactions between the denatured whey proteins and the casein micelles and the conversion of soluble calcium to the colloidal state. Milk that has been heated at a temperature in excess of pasteurization has poor renneting and gel formation characteristics.

Thermal denaturation of β -lactoglobulin is known to affect the coagulation properties of milk (Castillo, 2006). It has been shown that heating milk affects the clotting process by slowing or inhibiting the primary phase of rennet action as k-casein- β -

lactoglobulin cross-linking reduces the susceptibility of k-casein to hydrolysis by chymosin. The rennet coagulation properties of heated milk can be partially restored by acidification of heated milks to pH values below 6.2, or acidification of heated milk to low pH values (~5.5) followed by reneutralization to 6.7 (Castillo, 2006).

2.9 Confocal Microscopy of Dairy Products

Confocal microscopy is an optical imaging technique used to increase optical resolution and contrast of an image by using point illumination and a spatial pinhole to eliminate out of focus light in samples that are thicker than the focal plane. It enables the reconstruction of three-dimensional structures from the obtained images (Hassan, 2002). A confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal. Since only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution is much better than that of wide-field microscopes. Since only one point in the sample is illuminated at a time, imaging requires scanning over a regular raster in the sample (Herbert, 1999). A schematic of a confocal microscope can be seen below.

Figure 8. Schematic illustrating how confocal laser scanning microscopy works (Nikon Microscopy U)

Confocal scanning laser microscopy (CSLM) has widely been used for the study of food microstructure (Hassan, 2002; Herbert, 1999; Auty, 1999; Auty, 2001; Fenoul, 2008; Lopez, 2007; Pugnali, 2005; Westerman, 2009). Of the many fluorescent probes available for conventional fluorescence microscopy, relatively few have been used for CSLM of foods. Nile Blue and Nile Red have been used in conjunction with CSLM to localize fat in various food products including fat spreads, cheeses, and chocolate (Herbert, 1999). Nile Blue has also been used to localize starch in cereal products and milk proteins in dairy products when excited at 633nm. Improved image acquisition rates have facilitated the study of dynamic events by CSLM. Full exploitation of dynamic

CSLM relies on the development of staining methods capable of localizing specific structural components while minimizing sample disturbance. The rapid assessment of the distribution of the principal components of food products is of great benefit to food technologists seeking to develop novel products (Herbert, 1999).

2.10 Dynamic Confocal Scanning Laser Microscopy

Food texture is largely influenced by the microstructure of the individual components and their relationship to each other. Proteins and fats are important structural elements in a wide variety of foods, influencing texture and flavor release. The ability to form gels is a fundamental functional property of many proteins, particularly in products such as cheese and yoghurt. Recently, improved image acquisition rates and high-efficiency detectors have facilitated the study of real time dynamic events by CSLM. However, there have been very few published papers showing CSLM data of the gelation process. A combination of dynamic confocal imaging of the gelation process and image analysis techniques based on theoretical models of gelation kinetics would lead to a greater understanding of the milk gelation process (Auty, 1999).

2.11 Image Texture Analysis

Pixels are basic components of images. Two kinds of information are contained in each pixel: the brightness value and the location of the pixel. The former is the color feature and features extracted from the latter are considered size of shape features. Texture is another important image feature that corresponds to both brightness value and pixel location (Zheng, 2006). As texture is an important image feature for describing

properties of objects in images, which are partitioned from other parts in images according to certain significant criteria, it has been introduced into a wide range of applications, such as metal surface analysis, textile characterization, cell recognition and counting, ultrasonic image processing, and food qualities evaluation (Zheng, 2006). Texture of images reflects changes of intensity values of pixels, which might contain information about the geometric structure of objects. In food images, texture can reflect cellular structure and can be used as an indicator of food quality. For example, texture can be used to reveal the tenderness of beef. Texture features can be classified into four categories: 1) statistical texture; 2) structural texture; 3) model-based texture; and 4) transform based texture.

The gray level co-occurrence matrix is one of the most widely used statistical texture analysis methods, in which texture features are extracted by some statistical approaches from the co-occurrence matrix. Before building the matrix, the direction of the pixel pairs and the distance between the pixel pairs need to be chosen. The direction can be selected from 0, 45, 90, and 135 degrees, while distance depends on the resolution of the texture. The matrix is constructed by counting the number of pixel pairs with the grey value k and l at the specified directions and distances. From the co-occurrence matrix, texture properties can be extracted (Fagan, 2008).

In fractal model-based texture analysis methods, surface intensity of an image is obtained by plotting coordinates of pixels against their grey level values in the z -axis. Fractal texture of images can then be calculated by determining the fractal dimension (FD) calculated from the following power-law scaling:

$$L(\varepsilon) \approx C \varepsilon^{1-FD} \quad (13)$$

where $L(\epsilon)$ is the unit measurement such as perimeter, area, and brightness difference while ϵ is the scale used and C is a constant for a certain fractal (Zheng, 2006). The fractal dimension can be calculated by obtaining the value of $(1 - FD)$, which is call the Hurst coefficient and can be calculated from the slope of the least-square linear regression of the logarithmic plot of $L(\epsilon)/C$ versus ϵ (Zheng, 2006).

Transform-based texture analysis is performed by convolving the image with a mask that transforms the image from the spatial domain to the object domain, which means that after the convolution mask, objects of images such as edges, spots, lines, or combinations of all of these can be revealed after the mask (Zheng, 2006). One popular mask is the Sobel operator, which is an effective edge-detecting operator. Once the image is transformed into and edge map, the values of the edge intensities can be used to quantify the texture of the image. Another transform based texture analysis method is to perform the Fourier transform of the image. Working in frequency space can be used to perform certain image measurements while reducing the computation effort significantly compared to the spatial space. Transform based texture analysis will be the method used for image texture analysis in this study.

2.12 Significance to Potential Medical Applications

The similarities between blood clotting and milk coagulation point to the possibility of using the methods described in this study to characterize the coagulation properties of milk to also characterize the clotting properties of blood (Blair, 1971). Currently, the rheological methods used to characterize the coagulation properties of milk used in this study are being used to study the clotting behavior of blood (Tynngard,

2008). By performing similar experiments where the blood is imaged during the clotting process could also provide valuable information about the kinetics of blood clotting.

Similar to milk coagulation, blood clotting involves a protein component, fibrinogen. Proteins in blood plasma, called coagulation factors or clotting factors, respond in a complex cascade to form fibrin strands which strengthen the platelet plug. Using confocal microscopy to image the process of blood coagulation would allow us to gain deeper understanding of the structural changes that occur during the blood coagulation process.

III. MATERIALS AND METHODS

3.1 Dynamic Confocal Scanning Laser Microscopy to Study Milk Gelation

The following procedure describes the process used to image milk gel formation and the use of image processing and statistical techniques used for data analysis.

3.1.1 Milk and Rennet Mixtures

Raw, full-fat milk sample were obtained from the Cal-Poly dairy herd. The rennet, chymosin was obtained from Chr. Hansen, Inc. (Milwaukee, WI). A standardized solution of 100% fermentation produced chymosin was used. The name of the product is CHY-MAX extra (P/N 73863) and contains chymosin in a salt brine solution with sodium benzoate added as a preservative along with caramel color. It has a minimum activity of 600 international milk clotting units/ml (IMCU/ml).

Six different rennet-milk mixtures were made with the following concentrations of chymosin: 0.0 μ l/ml, 0.2 μ l/ml, 0.4 μ l/ml, 0.6 μ l/ml, 0.8 μ l/ml, and 1.0 μ l/ml. Table 1 below summarizes the different mixtures.

Table 1. Different rennet-milk mixtures used in experiment

SAMPLE	Milk Volume	Chymosin Volume	[Chymosin]
1	20ml	0 μ l	0ul/ml
2	20ml	4 μ l	0.2ul/ml
3	20ml	8 μ l	0.4ul/ml
4	20ml	12 μ l	0.6ul/ml
5	20ml	16 μ l	0.8ul/ml
6	20ml	20 μ l	1.0ul/ml

3.1.2 Fluorescent Dyes

All fluorescent dyes were obtained from Sigma-Aldrich Ireland (Tallaght, Dublin 24, Ireland). A solution of Nile Red was made using acetone as a solvent with a concentration of 0.02g/l. A solution of Fast Green FCF was made using distilled water as a solvent with a concentration of 0.1g/l. The two solutions were mixed together in equal parts to create a solution for dual labeling of both fat and protein. Nile Red was used for staining fat and Fast Green FCF was used for staining protein. The excitation wavelengths for Nile Red and Fast Green FCF are 488 and 633nm, respectively.

3.1.3 Imaging Procedure

All microscopy experiments were carried out using an Olympus IX81 Fluoview 1000 laser scanning confocal microscope 20X, 1.30 N.A. air objective. This microscope is inverted, motorized and fully automated. It has three fiber-optic coupled lasers, two galvanometer scanning mirrors for x-y-scanning, a motorized stage and a photomultiplier detector (Figure 9).

Figure 9. Olympus IX81 Fluoview 1000 confocal microscope used for dynamic imaging of the milk coagulation process (Olympus)

An effective lateral resolution of 0.15 μ m and effective axial resolution of 0.58 μ m can be expected from this setup. The confocal microscope was setup to image using two separate channels: one with an excitation wavelength of 488nm and another with an excitation wavelength of 640nm. The 488nm excitation channel was used to image Nile Red and the 640 and the 640nm excitation channel was used to image Fast Green FCF. The microscope was configured to capture 640x640 pixel images.

After the initial setup of the confocal microscope, the sample had to be prepared. Twenty ml of milk were placed in a beaker, allowed to reach room temperature, and a micro pipette was used to mix the specified amount of chymosin into the milk. The milk was mixed with the chymosin using a magnetic stir plate. Using a pipette, 400 μ l of the milk-chymosin mixture was placed in a single chamber in an 8 well Lab-Tek II chamber slide (LAB-TEK, Naperville, IL, P/N 154941). Next, 100 μ l of the Nile Red and Fast Green FCF mixture were also added the chamber and mixed into solution with the milk. Wide field illumination was used to focus the confocal microscope, and then the

microscope was turned on for imaging. The gain for each channel was adjusted to maximize contrast, and then images were captured every 15 seconds for 30 minutes, with each image taking 1.5 seconds to capture, giving a total of 120 images per sample. Nile Red was chosen to be labeled as red and Fast Green FCF was chosen to be labeled as green. The images were exported as .bmp files and saved on an external hard drive. The figure below shows the procedure used to image coagulating milk. Figure 10 shows the progression of steps used during the imaging procedure.

Figure 10. A flowchart describing the imaging procedure for the dynamic milk coagulation imaging

3.1.4 Image Analysis

MATLAB 7.11.0 (Natick, MA) was used for all image analysis. First the images were loaded into MATLAB one at a time as 24bit RGB images. RGB images consists of three layered images, each representing the intensity for the different color components,

red, green, and blue. The red layer was extracted from each image and was used to create a 3 dimensional stack of images called 'fat', giving a 640x640x120 matrix. Next, the green layer was extracted from each image and was used to create a 3 dimensional stack of images called 'protein', giving another 640x640x120 matrix. The two matrices were analyzed separately to characterize the coagulation properties of the milk. Figure 11 shows an image from the confocal microscope. This image was taken 30 minutes into the coagulation process.

Figure 11. Single image of coagulating milk from the confocal microscope. The green shows the protein network of casein and the red shows the fat globules.

Figure 12 shows the extraction of the green color layer from the image in Figure 11. This layer represents the protein network and is used later to calculate the texture of the protein network. Figure 13 shows the extracted red layer, which represents the location of fat globules in the coagulating milk. When recombined, Figures 12 and 13 will create the original image shown in Figure 12.

Figure 12. The green color channel extracted from the original image and converted to a grayscale image. This represents the protein network.

Figure 13. The red color channel extracted from the original image and converted to a grayscale image. This represents the fat globules.

The matrix labeled 'fat' was analyzed by quantifying the motion of the fat particles from one image to the next. This was done by first taking the absolute difference between two successive images and creating another matrix labeled 'fat_diff', giving a 640x640x119 matrix. When there is movement from one image to the next, there will be values displayed on the difference image, but when there is no movement, the difference image will be almost entirely zero. An example of a difference image can be seen in figure 14. This difference image is from early in the coagulation process, so there are many spots of high values on the image, which represent movement of the fat globules.

Because each moving globule leaves positive and negative values, the absolute value of this image is analyzed.

Figure 14. This is an example of a difference image created by subtracting the current fat globule image from the previous fat globule image. The absolute value of this image is then taken and the mean value is calculated to quantify the movement.

To account for photobleaching, the photo chemical destruction of a fluorophore, the mean intensity of each image was calculated. The assumption that any decrease in intensity from one image to the next occurred because of photobleaching was used to account for results in the ‘fat_diff’ data occurring from photo bleaching. The following equations below describe the process used to calculate the fat movement curve:

$$PB_i = |mean(Im_{i+1}) - mean(Im_i)| \quad (14)$$

$$D_i = \text{mean}(|Im_{i+1} - Im_i|) \quad (15)$$

$$M_i = D_i - PB_i \quad (16)$$

where PB is the difference in intensity between successive images from photobleaching, D is the difference between images which is used to quantify the movement of the fat globules, and M is the final value used to quantify fat globule movement where photobleaching effects have been accounted for. The string of values for M is then saved for further analysis to characterize the coagulation properties of the milk.

To analyze the development of the protein structure as the milk coagulates, the matrix labeled ‘protein’ was analyzed. All images in the matrix were first contrast stretched to account for any photobleaching effects. Contrast stretching adjusts the values in the image so that the entire dynamic range is covered. The following equation describes the process for contrast stretching:

$$s = (r - c) \left(\frac{b - a}{d - c} \right) + a \quad (17)$$

where a is the upper limit of the intensity values, b is the lower limit of the intensity values, c is the lower limit of the values in the image and d is the upper limit of the values in the image. Then for each pixel, the original value r is mapped to output value s. Contrast stretching is performed to normalize all images so that any affects from photobleaching are accounted for.

Next, the protein structure is quantified by calculating the texture of the images. There are many ways to calculate the texture of an image, as discussed in section 2.10. The method used in this study was a transform based method. The images were transformed by convolving them with the Sobel operator. Convolution is a mathematical

operation on two functions, producing a third function that is typically viewed as a modified version of the original function. Below is the Sobel operator.

$$\mathbf{G}_y = \begin{bmatrix} -1 & -2 & -1 \\ 0 & 0 & 0 \\ +1 & +2 & +1 \end{bmatrix} * \mathbf{A} \quad \text{and} \quad \mathbf{G}_x = \begin{bmatrix} -1 & 0 & +1 \\ -2 & 0 & +2 \\ -1 & 0 & +1 \end{bmatrix} * \mathbf{A} \quad (18)$$

Convolution is performed by dragging the Sobel operator over the image so that it covers every pixel. The transformed image is created by multiplying all the numbers in the Sobel operator by the values they cover in the original image then adding them up to create a single number. The operator is then moved over to the next pixel. This number represents one pixel located where the center of the Sobel operator is. The two different Sobel operator are used to get transformations in the x and y directions. These are then added together to calculate the total gradient. In simplified terms, the operator calculates the gradient of the image intensity at each point. The result therefore shows how “abruptly” or “smoothly” the image changes at that point. So an image that is completely uniform will contain all zeros, but a rough image will contain large values in the transformed image. Once the gradient image is calculated, the mean of the entire image is calculated to quantify the entire texture of the image. This is done for all images in the time sequence and the values are saved for further processing. The equations below describe the process:

$$G = \sqrt{G_x^2 + G_y^2} \quad (19)$$

$$T_i = \text{mean}(G_i) \quad (20)$$

where G_x is the gradient in the x-direction, G_y is the gradient in the y-direction, G is the overall magnitude of the gradient, and T_i is the texture value for each individual image.

Figures 15 and 16 show calculated gradient images. Figure 15 is from early in the coagulation process, and therefore is a smooth image. Figure 16 is towards the end of the coagulation process, and thus, is much rougher.

Figure 15. This is the gradient image created using the Sobel operator. This is from early in the coagulation process, as you can see the image is relatively smooth.

Figure 16. This is the gradient image created using the Sobel operator. This is from late in the coagulation process, as you can see the image is relatively rough.

Figure 17 summarizes the image analysis process.

Figure 17. Summary of image analysis

3.1.5 Statistical Analysis and Fitting Data to Models

After image analysis, the fat movement data and the protein texture data were plotted and analyzed separately.

First, the fat globule movement values (M_i) were plotted vs. time and then the average value for the last 20 data points was subtracted from all the fat globule movement values. This was done so that the values would eventually decrease to zero when there was no movement. Because of the noise inherent in the imaging setup, the difference between successive frames would never reach zero on its own. Next, the values were fit to the following equation:

$$M = \beta e^{-\alpha t} \quad (21)$$

where M is the value of fat globule movement from the fit, β is a fitted variable representing the initial fat globule movement, α is another variable representing the rate of decrease in fat globule movement, and t is the time in minutes. The Levenberg-Marquardt algorithm for nonlinear least squares was used to fit the data to equation 21. The Levenberg-Marquardt algorithm works by interpolating between the Gauss-Newton algorithm and the method of gradient descent. The Levenberg-Marquardt algorithm is more robust than the Gauss-Newton algorithm, which means that in many cases it finds a solution even if it starts very far off the final minimum.

To test the accuracy of the described method, the temperature and the concentration constants were omitted from the fitting equation, and the fat globule rate constant was calculated. Because the rennet concentration and temperature were known, we expected to see an increase in the rate constant with an increase in the rennet concentration.

After the fat globule movement rate data was analyzed, the protein texture results were analyzed. First the data was plotted as a function of time. Initially, as the fat globules move towards the surface of the milk, the texture of the protein matrix decreases, and after a period of time the protein structure begins to form as the milk coagulates, causing an increase in the texture of the protein matrix. To characterize the rate of protein matrix formation, the time between the minimum protein texture value, which occurs when the fat is moving towards the surface, and the time of fastest increase in the protein texture is found. To do this, first the data was filtered with an averaging filter of length five and the gradient of the filtered data was calculated. Using the gradient of the filtered data, the first zero was found and assumed to be the minimum of the protein texture. Next, the maximum of the gradient of the filtered data was found and assumed to be the point of the fastest protein texture increase. The time between these two points was calculated and used to characterize the protein matrix formation rate of the milk. Also, the value for the maximum rate of texture increase was calculated and used to characterize the protein matrix formation.

3.2 Validation of Results with Rheological Measurements

The ReoRox G2 (MediRox, Nykoping, Sweden) was used for all rheological measurements (Figure 18). The principles of the ReoRox are described in section 1.2. Raw milk was heated in the incubation chamber at 37 °C for twenty minutes. A rennet solution was created and different concentrations of chymosin were added to the milk. The same concentration values used in the dynamic confocal microscopy experiment were also used for the rheological measurements. The ReoRox program was opened and

1ml of the milk rennet solution was added to the sample holder and placed in the measurement chamber of the ReoRox. The sample button was pressed to begin measurements. The ReoRox G2 has four measurement chambers, allowing simultaneous measurement of four different samples. Each sample was measured for 30 minutes, and each concentration of milk-rennet mixture was measured four separate times. The elasticity data was saved and exported to Excel for further analysis.

Figure 18. The ReoRox G2 system (MediRox)

3.3 Computer Model of Milk Coagulation

To create a predictive tool for the milk gelation process, a computer model of the coagulation process was developed using MATLAB. The purpose of the model was to

create a simulated time-lapsed video similar to the results from the confocal microscope. To do this, two separate images were created: one representing the fat globules, and the other representing the protein network.

To model the movement of the fat globules, Brownian dynamics were used. The following equations were used to describe the movement of the fat particles:

$$D = \frac{k_b T}{6\pi\mu r} \quad (22)$$

$$kick = randn\sqrt{2Ddt} \quad (23)$$

where D is the Stokes-Einstein diffusivity, k_b is the Boltzman Constant, T is the temperature in kelvin, μ is the viscosity, r is the globule radius, randn is a random number selected from a normal distribution of standard deviation of 1, dt is the time step size, and kick is the movement of the fat globule during one time step. In order to create a 2D model, the kick was calculated in both the x and y directions. The image was populated with 2000 fat globules, and each fat globule was given a different random kick in the x and y directions for each time step. In order to model the gel firming kinetics, the viscosity was increased with time according to the following function:

$$\mu = \mu_0 e^{a*t*dt} \quad (24)$$

where μ is the viscosity, μ_0 is the initial viscosity, a is the coagulation rate constant, t is the time step, and dt is the size of the times steps. The exponential increase in viscosity led to a slowing of the movement of the fat globules, and the rate of the slowing was determined by the rate constant, a. Also, the size of the fat globules is decreased with time to simulate the decrease in fat globule size that is seen during the confocal imaging. This simulates the movement of the fat globules towards the surface of the milk.

To model the protein network during milk coagulation, a simulated image was created using the following function:

$$P_i = + \left(1 - e^{-\frac{atdt}{100}}\right) randn(FOV) \quad (25)$$

where P_i represents the current protein network image, P_{i-1} represents the previous protein network image, a represents the coagulation rate constant, t represents the current time step, dt represents the length of the time steps, and $randn(FOV)$ represents an image composed of random numbers sampled from a normal distribution with standard deviation of 1. FOV is the size of the image in pixels. As time increases, the protein network images goes from a constant image, to one with increasing variation and texture.

The two simulated images of fat and protein are then overlaid to create the final simulated time-lapsed movie. The figure below shows the simulated images at six different time points.

Figure 19. Output of milk coagulation computer simulation.

The simulated images were then analyzed using the same image processing algorithm used to analyze the images from the confocal microscope. This allows a comparison of the rate constant chosen for the model and the time parameters computed by the algorithm, which could then be used to correlate the rate constant to chymosin concentration. The following figures are examples of the plots provided by the image analysis algorithm used on the simulated images.

Figure 20. Output of image analysis algorithm applied to the simulated images from the computational model showing the fat globule movement plotted against time.

Figure 21. Output of image analysis algorithm applied to the simulated images from the computational model showing the protein network texture plotted against time.

IV. RESULTS

4.1 Dynamic Confocal Scanning Laser Microscopy of Milk Coagulation

The following six figures below show the raw images at six different time intervals: 1) 0 min; 2) 1 min; 3) 3 min; 4) 6 min; 5) 15min; and 6) 30 min. Also included are the plots generated from the image analysis algorithms and the related best fit curves and important parameters that can be used to characterize the coagulation rates. The first plot shows the movement of the fat globules over time. The exponential decay curve is fit to the data and the coefficients used in the fit are also displayed on the plot. The second plot shows the texture of the protein matrix over time. The green 'x' represents the minimum of the texture plot and the red 'x' represents the maximum rate of increase for the texture. The time between these two points is overlaid on the plot. Also, the maximum slope is calculated and this value is shown on the plot. The green 'x' on the

first plot corresponds to the same time as the green 'x' on the second plot, and the red 'x' on the first plot represent the value where the movement is ten times less than the movement value at the green 'x'.

Figure 22 shows the progression of images from a sample where no enzyme was added. Notice that the fat globule rapidly move out of focus and decrease in size and the protein network remains as a smooth surface. Figures 23-27 show the progression of images with each figure having a higher enzyme concentration than the last. Notice that at higher enzyme concentrations, the protein network forms a rough structure more rapidly, and also, the fat globules are trapped in the protein network before they can move to the surface of the sample. Below the images are the plots of the fat globule movement and the protein network texture. Various parameters, described in Table 2, are calculated automatically and displayed on each plot.

Figure 22. Data and analysis plot from 0 $\mu\text{l/ml}$ chymosin concentration. The top of the figure shows six images from the raw confocal data at different time intervals. Notices the texture of the protein does not increase with time, since there is no chymosin in this sample. The plot on the bottom left shows the fat globule movement plotted against time and the plot on the bottom right shows the protein texture plotted against time.

Figure 23. Data and analysis plot from 0.2μl/ml chymosin concentration. The top of the figure shows six images from the raw confocal data at different time intervals. The plot on the bottom left shows the fat globule movement plotted against time and the plot on the bottom right shows the protein texture plotted against time.

Figure 24. Data and analysis plot from 0.4μl/ml chymosin concentration. The top of the figure shows six images from the raw confocal data at different time intervals. The plot on the bottom left shows the fat globule movement plotted against time and the plot on the bottom right shows the protein texture plotted against time.

Figure 25. Data and analysis plot from 0.6µl/ml chymosin concentration. The top of the figure shows six images from the raw confocal data at different time intervals. The plot on the bottom left shows the fat globule movement plotted against time and the plot on the bottom right shows the protein texture plotted against time.

Figure 26. Data and analysis plot from 0.8 μl/ml chymosin concentration. The top of the figure shows six images from the raw confocal data at different time intervals. The plot on the bottom left shows the fat globule movement plotted against time and the plot on the bottom right shows the protein texture plotted against time.

Figure 27. Data and analysis plot from 1.0µl/ml chymosin concentration. The top of the figure shows six images from the raw confocal data at different time intervals. The plot on the bottom left shows the fat globule movement plotted against time and the plot on the bottom right shows the protein texture plotted against time.

The following table summarizes the different parameters found in each plot and describes how they were found and what they represent.

Table 2. Different parameters calculated from image analysis algorithm

Protein Formation Time (min)	The time between the minimum protein network texture and the maximum rate of increase in texture
Max protein formation rate (1/min)	The rate of maximum increase for protein network texture
Fat movement rate constant (1/min)	The fitting parameter alpha in the decreasing exponential function used to fit fat movement

The protein formation time, max protein formation rate, and the fat movement rate were all plotted on an error bar plot against the chymosin concentration. This can be seen in figures 28 through 30. This was done to help illustrate which parameters were best suited to characterize the milk coagulation kinetics.

Protein Formation Time vs. Chymosin Concentration

Figure 28. Error bar plot of the protein formation time vs the chymosin concentration. Each data point on the plot is the average from five sample (n=5)

Max Protein Formation Slope vs. Chymosin Concentration

Figure 29. Error bar plot of the max protein formation slope vs the chymosin concentration. Each data point on the plot is the average from five sample (n=5)

Figure 30. Error bar plot of the fat movement rat constant vs the chymosin concentration. Each data point on the plot is the average from five sample (n=5)

4.2 Rheological Measurements of Milk Coagulation

Rheological measurements were collected using the ReoRox G2. Elasticity was plotted over time for 30 min. A sample plot for each chymosin concentration can be seen below. For each concentration level, four different samples were tested, giving a total of 24 plots. The blue line on each plot represents the viscosity. The viscosity plot is to be used to determine when the milk begins to coagulate. The yellow plot represents the elasticity of the coagulating milk. Notice the sample begins to exhibit elasticity soon after the onset of coagulation. Figures 31-35 show outputs from each difference rennet concentration.

Figure 31. 0.2ul/ml sample. ReoRox output plot showing the viscosity (blue) and the elasticity (yellow) vs time.

Figure 32. 0.4ul/ml sample. ReoRox output plot showing the viscosity (blue) and the elasticity (yellow) vs time.

Figure 33. 0.6ul/ml sample. ReoRox output plot showing the viscosity (blue) and the elasticity (yellow) vs time.

Figure 34. 0.8ul/ml sample. ReoRox output plot showing the viscosity (blue) and the elasticity (yellow) vs time.

Figure 35. 1.0ul/ml sample. ReoRox output plot showing the viscosity (blue) and the elasticity (yellow) vs time.

To characterize the coagulation properties, the maximum slope in each elasticity curve was calculated. The results can be seen below in the error bar plot.

Figure 36. Error bar plot showing the max elasticity slope vs chymosin concentration. Each point on the plot is the mean of four samples (n=4)

4.3 Computer Model of Coagulation

Figure 37 shows the relationship between the chosen coagulation rate constant, a , for the computer model of milk coagulation and the coagulation time found by the analysis program. The time used for this plot comes from the protein network texture and is defined as the time between the minimum texture value and the time of fastest increase for the texture. These results can then be used to correlate the model coagulation rate constant to the chymosin concentration, which will allow the prediction of coagulation properties based on the chymosin concentration.

Figure 37. Plot showing the protein formation time of the simulated images vs. the coagulation rate constant chosen for the simulation.

V. DISCUSSION

The objective of this study was to develop a method using confocal microscopy to study the kinetics of milk coagulation, and then validate the new methods using existing rheological methods, and lastly use the collected data to develop a computational model to aid in the understanding in milk coagulation kinetics. Rennet-induced coagulating milk was observed using laser scanning confocal microscopy to create time lapsed videos showing the coagulation process. Image processing techniques were used to quantify the confocal microscopy results and these results were compared to rheological data collected using free oscillation rheometry. The rheological data validated the confocal microscopy results by showing an increased chymosin concentration correlated to a faster increase in elasticity. We hypothesized that the increased protein network structure, characterized by the texture, is responsible for the increase in elasticity. Initially, the protein appeared as a fine dispersion of small particles. After the onset of coagulation the casein particles began to aggregate, eventually forming a three-dimensional network at the onset of gelation. Fat droplets appeared as red circular regions bounded by a protein-rich coating of variable thickness. After 30 minutes, the renneted milk appeared as a continuous protein network with occluded fat droplets and moisture. Comparing these results to the rheological measurements showed that changes in the protein microstructure were accompanied by increases in the elasticity up to the end of the experiment. Figure 38 shows a plot of the plot of the max elasticity slope divided by the inverse of the protein formation time versus the chymosin concentration. This is then fit to a horizontal line, where the height of the line represents the calibration factor needed to convert the inverse of the protein formation time to a max elasticity value.

Figure 38. Plot of the max elasticity slope times the protein formation time. This is done to show a conversion factor between the two parameters.

Figure 38 shows how it is possible to convert from the max elasticity slope from the rheological data to the protein formation time found using confocal microscopy. Notice a low mean squared error, which means there is a strong correlation between the two sets of data. The following equation can be used for conversion:

$$MES = \frac{2.8314}{PFT} \quad (13)$$

where MES is the max elasticity slope and PFT is the protein formation time.

The major goal in kinetic studies is to establish a rate equation which attempts to describe the velocity of a reaction in terms of experimentally measurable parameters. Typically, the experimentally measurable parameters are concentrations of products or reactants during the reaction. Enzyme kinetics can then be used to analyze the results. This is often done by creating a Lineweaver-Burk plot, which plot the inverse of the maximum reaction rate versus the substrate concentration. While this study is interested in determining the rate of an enzymatic reaction, we are not measuring the concentration

of reactants and products, but instead we are measuring the development of the structure and mechanical properties.

Previous studies using confocal microscopy to study coagulating milk have been limited to only staining for protein, and lack quantitative analysis of the images (Auty, 1999). By applying image processing techniques to the confocal microscopy images of milk coagulation, we were able to extract quantitative data that allows easy characterization of the system. The image processing algorithm was able to quantify the coagulation process by extracting three different parameters: 1) The protein formation time; 2) The max protein formation rate; and 3) the fat movement rate constant. The protein formation time is defined as the time between the minimum texture value for the protein network and the time of fastest increase in the texture of the protein network. Plots used to find these parameters can be seen in the results section. You will notice an initial decrease in the texture value. This is due to the expulsion of the fat globules to the surface of the coagulating milk, which leaves the protein network as a smooth surface. Then, as the protein network begins to form, the texture increases. The max protein formation rate is defined as the value of the maximum slope in the plot showing the protein network texture. The fat movement rate constant is the fitting parameter α in the decreasing exponential used to fit the fat globule movement data.

Plotting the values of the three different parameters against the chymosin concentration made it clear which parameter was best suited to describe the milk coagulation. The protein formation time was found to work best for distinguishing between the different chymosin concentrations. The main reason this parameter worked best is that it isn't depended on the raw values of the data, but rather on the time

relationship between the data. Since milk clotting time is traditionally described in units of time, it also makes this parameter especially useful. The other two parameters can easily be affected by the acquisition parameters of the confocal microscope. Changing the laser power or the PMT sensitivity will change the brightness of the images, which can cause the algorithm to infer a different slope, or different rate of movement for the fat globules. Since milk clotting time is traditionally described in units of time, it also makes this parameter especially useful for describing the coagulation properties of a renneted milk. Also, this method using microscopy and image analysis was able to follow the mobility of particles through the gel point, which is when the milk turns from a viscous to an elastic material, as they are confined and incorporated into a gel network. This showed that the protein rearrangement process dominates beyond the gel point.

In order to validate the results obtained from the confocal microscopy experiments, rheological measurements of renneted milk were obtained using the ReoRox G2 rheometer, which uses the free oscillation rheometry technique to measure both viscosity and elasticity. It has been shown the structural rearrangements affect the rheological properties of rennet-induced milk gels. Most experimental results in the studies on rearrangements have been obtained for casein gels, mostly by making use of rheometry, permeability, and microscopy (Auty, 1999; Carlson, 1985; Fagan, 2006). As the dynamics of ageing gels are caused by a thermodynamic instability, theories on phase separation would apply (Hyslop, 1996). Many particle or emulsion gels can be described as gels made up of viscoelastic particles (Karlsson, 2007). In general, the rate of aging of such gels is determined by the rate at which particle conformations change from open to more compact (Lodaite, 2000). Unfortunately, the characteristics of the initial

composition of casein gels are difficult to quantify. Some research has been done to show that the initial stages of the compaction behavior of casein gels can be quantified using fractal scaling models which are applied to the results of rheological measurements and give information on the geometric structure of the particle network in terms of strand properties and the sizes of the compact building blocks (Horne, 1989). The changes in structure and rheology of rennet-induced casein gels during ageing can be explained in terms of specific rearrangements. While other research has shown this can be explained by fractal models, this study suggests the rearrangements can be described using image texture analysis. Comparison between the rheological data and the imaging data shows that an increase in the protein network texture corresponds to an increase of the casein gel elasticity. A comparison of figures 28 and 36 show that an increase in chymosin concentration can be correlated to both a decrease in the protein formation time and an increase in the maximum rate of elasticity increase. This result supports the hypothesis that the protein network formation is responsible for the change in rheological properties of milk during the coagulation process.

Numerous studies have shown the application of image texture for the evaluation of food (Zheng, 2006). Texture is an important image feature and has been applied greatly in the food industry for quality evaluation and inspection. Image texture is able to account for both pixel values and their spatial relationship in a single value, allowing a large amount of information to be condensed. In this study, a transform based texture analysis was performed. This technique has been applied to various foods, including meat, fruit, and vegetables. We were able to quantify the current level of protein network formation in coagulating milk by calculating a transform based texture value. This

transformation was performed by convolution with a Sobel operator, which finds the gradient image. The mean value of the gradient image was then used as the texture parameter. One similar study used image texture analysis for online determination of curd moisture and whey solids in laboratory-scale stirred cheese vat. As a rapid, consistent, nondestructive, and objective tool, computer vision has been successfully established as a technique for quality inspection of food products, including cheese. They concluded that a fractal dimension analysis was the best for predicting curd moisture and the level of solids in whey during syneresis. However, they did not study the effectiveness of convolution based transform technique used in this study. In order to better understand and predict the effect of chymosin concentration on the development of the protein microstructure, a computation model was developed.

In this study, a model was developed to create images similar to the confocal images captured. Random variables were used to model both the movement of the fat particles and the formation of the protein network. To model the movement of the fat globules, Brownian motion was assumed and the viscosity of the milk gel increased with time exponentially, with a rate constant defined by the user. This rapid increase in viscosity eventually leads to the immobilization of the fat globules. Also, the size of the fat globules was decreased with time to simulate the fat moving towards the surface of the gel. This can be observed in the raw confocal data. In the model output for fat globule movement shown in figure 20, you will notice jumps in the fat globule movement values. These occur from the discrete decreases in the fat globule size that were meant to simulate the movement of fat globules to the surface of the milk. Because of these jumps, the fat globule model was not considered to successfully simulate the actual movement of

the fat globules. To model the protein network, an image composed of random number from a normal distribution was created and added to the previous protein network image. The standard deviation of the random numbers in the image increased at rate which was controlled by the same user defined constant that was used to control the increase in viscosity. The image of the protein network was then scaled to that any pixel above 0.1 was considered the maximum possible value (255) and any number below -0.1 was considered the minimum possible value (0). The images of the protein network and the fat globules were then overlaid and a video of the simulated images was recorded. The same image processing algorithm used to process the confocal data was then used to analyze the simulated images. The initial decrease in size and the dispersion of the fat globules created an initial decrease in the texture value of the protein network, and eventually the addition of randomness to the image caused the texture to rise rapidly at first, and then level off. Figure 37 shows the relationship between the coagulation time and the user defined rate parameter. Notice this follows a similar curve to the plot showing the relationship between chymosin concentration and coagulation except it's on a different scale. A constant was built into the model so that the user could input a chymosin concentration instead of an arbitrary rate constant. This allows the model to work as a predictive tool to see how the concentration of chymosin can change the coagulation time.

VI. CONCLUSION

A better understanding of the coagulation kinetics of milk would aid in both the production of dairy products and also in determining the nutritional benefit of dairy products. In this study, a novel technique using confocal microscopy and image processing methods was developed to image coagulating milk, and extract the coagulation properties from the obtained image. The results from the imaging were validated using existing rheological measurements by showing an increase in chymosin concentration decreases the concentration time. The findings also suggest that the rearrangement of the proteins is responsible for the changes in the rheological properties. Further work is necessary to confirm this suggestion. Using the imaging data, a computational model that simulates the images taken using the confocal was taken. Currently, the user can input both temperature and chymosin concentration into the model to create a series of simulated images. To validate the model, the images were analyzed using the same image analysis program used to analyze the confocal images. It was found that the model responded to an increase in chymosin concentration the same way the real milk did. Because of the accuracy of the model, the mathematics used to describe the simulated images may lead to better understanding of the mechanisms responsible for milk coagulation. Currently, the model would work as a tool for predicting coagula properties, but further work is necessary to include additional variables that must be considered during the production of dairy products.

REFERENCES

- Alexander, Jerome. "Notes and Correspondence-The Rennin Coagulation of Milk." *Industrial & Engineering Chemistry* 16.9 (1924): 974. Print.
- Andersen, Charlotte M., Mette Vishart, and Vibeke K. Holm. "Application of Fluorescence Spectroscopy in the Evaluation of Light-Induced Oxidation in Cheese." *Journal of Agricultural and Food Chemistry* 53.26 (2005): 9985-992. Print.
- Arias, M., R. Lopez-Fandino, and A. Olano. "Influence of PH on the Effects of High Pressure on Milk Proteins." *Milchwissenschaft* 53 (2000): 191-94. Print.
- Auty, M. A., M. A. Fenelon, C. Mullins, and D. M. Mulvihill. "Dynamic Confocal Scanning Laser Microscopy Methods for Studying Milk Protein Gelation and Cheese Meltin." *Scanning* 21 (1999): 299-304. Print.
- Auty, Mark A. E., Myra Twomey, Timothy P. Guinee, and Daniel M. Mulvihill. "Development and Application of Confocal Scanning Laser Microscopy Methods for Studying the Distribution of Fat and Protein in Selected Dairy Products." *Journal of Dairy Research* 68.03 (2001). Print.
- Blair, G. W. Scott. "A Simple Model to Describe the Kinetics of the Coagulation of Casein (milk) and Fibrin (blood)." *Rheologica Acta* 10.3 (1971): 316-18. Print.
- Carlson, Alfred, Charles G. Hill, and Norman F. Olson. "Kinetics of Milk Coagulation: III. Mathematical Modeling of the Kinetics of Curd Formation following Enzymatic Hydrolysis of κ -casein?parameter Estimation." *Biotechnology and Bioengineering* 29.5 (1987): 601-11. Print.
- Carlson, Alfred. "Kinetics of Gel Forming in Enzyme Coagulated Milk." *Biotechnology Progress* 1.1 (1985): 46-52. Print.
- Castillo, M., J. Lucey, and F. Payne. "The Effect of Temperature and Inoculum Concentration on Rheological and Light Scatter Properties of Milk Coagulated by a Combination of Bacterial Fermentation and Chymosin. Cottage Cheese-type Gels." *International Dairy Journal* 16.2 (2006): 131-46. Print.
- Cattaneo, T., C. Giardina, N. Sinelli, M. Riva, and R. Giangiacomo. "Application of FT-NIR and FT-IR Spectroscopy to Study the Shelf-life of Crescenza Cheese." *International Dairy Journal* 15.6-9 (2005): 693-700. Print.
- Dalgleish, D. "A Geometrical Model to Describe the Initial Aggregation of Partly Renneted Casein Micelles." *Journal of Colloid and Interface Science* 123.1 (1988): 80-84. Print.
- De Marchi, M., C. C. Fagan, C. P. O'Donnell, A. Cecchinato, R. Dal Zotto, M. Cassandro, M. Penasa, and G. Bittante. "Prediction of Coagulation Properties, Titratable Acidity, and PH of Bovine Milk Using Mid-infrared Spectroscopy." *Journal of Dairy Science* 92.1 (2009): 423-32. Print.

- Dogariu, A., J. Uozumi, and T. Asakura. "Ballistic Propagation of Light through Fractal Gels." *Waves in Random Media* 4.1 (1994): 1-6. Print.
- Esteves, Cristina L. C., John A. Lucey, and Euclides M. V. Pires. "Mathematical Modelling of the Formation of Rennet-induced Gels by Plant Coagulants and Chymosin." *Journal of Dairy Research* 68.03 (2001). Print.
- Everard, C. D., D. J. O'Callaghan, C. C. Fagan, C. P. O'Donnell, M. Castillo, and F. A. Payne. "Computer Vision and Color Measurement Techniques for Inline Monitoring of Cheese Curd Syneresis." *Journal of Dairy Science* 90.7 (2007): 3162-170. Print.
- Everard, C.D., D.J. O'Callaghan, M.J. Mateo, M. Castillo, F.A. Payne, and C.P. O'Donnell. "The Use of Colour Parameters Derived from an Online Fibre-optic Sensor to Monitor Curd Syneresis during Cheese Making." *Journal of Food Engineering* 94.1 (2009): 1-6. Print.
- Fagan, C. C., C. Everard, C. M. O'Donnell, G. Downey, E. Sheenan, C. Delahunty, and D. O'Callaghan. "Evaluating Mid-infrared Spectroscopy as a New Technique for Predicting Sensory Attributes of Processed Cheeses." *J. Dairy Sci.* 90 (2007): 1122-132. Print.
- Fagan, C., C. Odonnell, P. Cullen, and C. Brennan. "The Effect of Dietary Fibre Inclusion on Milk Coagulation Kinetics." *Journal of Food Engineering* 77.2 (2006): 261-68. Print.
- Fagan, C.C., C.-J. Du, C.P. O'Donnell, M. Castillo, C.D. Everard, D.J. O'Callaghan, and F.A. Payne. "Application of Image Texture Analysis for Online Determination of Curd Moisture and Whey Solids in a Laboratory-Scale Stirred Cheese Vat." *Journal of Food Science* 73.6 (2008): E250-258. Print.
- Fagan, Colette C., Manuel Castillo, Donal J. O'Callaghan, Fred A. Payne, and Colm P. O'Donnell. "Visible-near Infrared Spectroscopy Sensor for Predicting Curd and Whey Composition during Cheese Processing." *Sensing and Instrumentation for Food Quality and Safety* 3.1 (2009): 62-69. Print.
- Fenoul, F., M. Le Denmat, F. Hamdi, G. Cuvelier, and C. Michon. "Technical Note: Confocal Scanning Laser Microscopy and Quantitative Image Analysis: Application to Cream Cheese Microstructure Investigation." *Journal of Dairy Science* 91.4 (2008): 1325-333. Print.
- Gallier, Sophie, Derek Gragson, Rafael Jimenez-Flores, and David Everett. "Surface Characterization of Bovine Milk Phospholipid Monolayers by Langmuir Isotherms and Microscopic Techniques." *J. Agric. Food Chem* (2011). Print.
- Gatti, Carlos, and Miryam Pires. "Effect of Monovalent Cations on the Kinetics of Renneted Milk Coagulation." *Journal of Dairy Research* 62.04 (1995): 667. Print.

- González-Martín, I., J.M. Hernández-Hierro, A. Vivar-Quintana, I. Revilla, and C. González-Pérez. "The Application of near Infrared Spectroscopy Technology and a Remote Reflectance Fibre-optic Probe for the Determination of Peptides in Cheeses (cow's, Ewe's and Goat's) with Different Ripening times." *Food Chemistry* 114.4 (2009): 1564-569. Print.
- Guillemin, Hervé, Ioan Cristian Trelea, Daniel Picque, Bruno Perret, Thomas Cattenoz, and Georges Corrieu. "An Optical Method to Monitor Casein Particle Size Distribution in Whey." *Le Lait* 86.5 (2006): 359-72. Print.
- Guinee, Timothy P., Claire B. Gorry, Donal J. O'Callaghan, Brendan T. O'Kennedy, Niamh O'Brie, and Mark A. Fenelon. "The Effects of Composition and Some Processing Treatments on the Rennet Coagulation Properties of Milk." *International Journal of Dairy Technology* 50.3 (1997): 99-106. Print.
- Hardy, Joël, and Jacques Fanni. "Application of Reflection Photometry to the Measurement of Milk Coagulation." *Journal of Food Science* 46.6 (1981): 1956-957. Print.
- Hassan, A.N., J.F. Frank, and M. Corredig. "Microstructure of Feta Cheese Made Using Different Cultures as Determined by Confocal Scanning Laser Microscopy." *Journal of Food Science* 67.7 (2002): 2750-753. Print.
- Herbert, Sophie, Brigitte Bouchet, Alain Riaublanc, Eric Dufour, and Daniel J. Gallant. "Multiple Fluorescence Labelling of Proteins, Lipids and Whey in Dairy Products Using Confocal Microscopy." *Le Lait* 79.6 (1999): 567-75. Print.
- Herbert, Sophie, Nadine Mouhous Riou, Marie Franoise Devaux, Alain Riaublanc, Brigitte Bouchet, Daniel J. Gallant, and Eric Dufour. "Monitoring the Identity and the Structure of Soft Cheeses by Fluorescence Spectroscopy." *Le Lait* 80.6 (2000): 621-34. Print.
- Horne, David S. "Application of Fractal Concepts to the Study of Caseinate Aggregation Phenomena." *Journal of Dairy Research* 56.03 (1989): 535. Print.
- Horne, David S. "Determination of the Fractal Dimension Using Turbidimetric Techniques. Application to Aggregating Protein Systems." *Faraday Discussions of the Chemical Society* 83 (1987): 259. Print.
- Hyslop, Douglas B., and Karsten B. Qvist. "Application of Numerical Analysis to a Number of Models for Chymosin-induced Coagulation of Casein Micelles." *Journal of Dairy Research* 63.02 (1996): 223. Print.
- Karlsson, A., R. Ipsen, and Y. Ardo. "Rheological Properties and Microstructure during Rennet Induced Coagulation of UF Concentrated Skim Milk." *International Dairy Journal* 17.6 (2007): 674-82. Print.
- Konuklar, G. "Rennet-Induced Milk Coagulation by Continuous Steady Shear Stress." *Journal of Colloid and Interface Science* 250.1 (2002): 149-58. Print.

- Lodaite, Kristina, Karin Ostergren, Marie Paulsson, and Petr Dejmek. "One-dimensional Syneresis of Rennet-induced Gels." *International Dairy Journal* 10 (2000): 829-34. Print.
- Lopez, C., B. Camier, and J. Gassi. "Development of the Milk Fat Microstructure during the Manufacture and Ripening of Emmental Cheese Observed by Confocal Laser Scanning Microscopy." *International Dairy Journal* 17.3 (2007): 235-47. Print.
- Mateo, M. J., D. J. O'Callaghan, and C. P. O'Donnell. "Comparison between Red-green-blue Imaging and Visible-near Infrared Reflectance as Potential Process Analytical Tool for Monitoring Syneresis." *J. Dairy Sci.* 93 (2009): 1882-889. Print.
- Mateo, M. J., D. J. O'Callaghan, C. D. Everard, M. Castillo, F. A. Payne, and C. P. O'Donnell. "Validation of a Curd-syneresis Sensor over a Range of Milk Composition and Process Parameters." *Journal of Dairy Science* 92.11 (2009): 5386-395. Print.
- Mellema, M. "Effects of Structural Rearrangements on the Rheology of Rennet-induced Casein Particle Gels." *Advances in Colloid and Interface Science* 98.1 (2002): 25-50. Print.
- Muller-Renaud, Stéphanie, Didier Dupont, and Philippe Dulieu. "Quantification of β -Casein in Milk and Cheese Using an Optical Immunosensor." *Journal of Agricultural and Food Chemistry* 52.4 (2004): 659-64. Print.
- Pugnaloni, L., L. Matiamerino, and E. Dickinson. "Microstructure of Acid-induced Caseinate Gels Containing Sucrose: Quantification from Confocal Microscopy and Image Analysis." *Colloids and Surfaces B: Biointerfaces* 42.3-4 (2005): 211-17. Print.
- Sandra, S., Marcela Alexander, and Douglas G. Dalgleish. "The Rennet Coagulation Mechanism of Skim Milk as Observed by Transmission Diffusing Wave Spectroscopy." *Journal of Colloid and Interface Science* 308.2 (2007): 364-73. Print.
- Schmidt, D.G., "Association of caseins and casein micelle structure." *Developments in Dairy Chemistry* 1982 Vol. 1 pp. 61-86.
- Syme, Christopher D., Ewan W. Blanch, Carl Holt, Ross Jakes, Michel Goedert, Lutz Hecht, and Laurence D. Barron. "A Raman Optical Activity Study of Rheomorphism in Caseins, Synucleins and Tau." *European Journal of Biochemistry* 269.1 (2002): 148-56. Print.
- Tan, Y.L., A. Ye, H. Singh, and Y. Hemar. "Effects Of Biopolymer Addition On The Dynamic Rheology And Microstructure Of Renneted Skim Milk Systems." *Journal of Texture Studies* 38.3 (2007): 404-22. Print.
- Trkova, J. "Influence of Protein Concentration on Rheological Properties of Carrageenan Gels in Milk." *International Dairy Journal* 14.4 (2004): 337-43. Print.
- Tunick, Michael H., and Diane Van Hekken. "Torsion Analysis Of Fresh And Aged Cheeses." *Journal of Texture Studies* 34.2 (2003): 219-29. Print.

Tynngård, Nahreen, Tomas Lindahl, Sofia Ramstrom, Tuulia Raf, Olof Rugarn, and Gosta Berlin. "Free Oscillation Rheometry Detects Changes in Clot Properties in Pregnancy and Thrombocytopenia." *Platelets* 19.5 (2008): 373-78. Print.

Ustunol, Z., C. L. Hicks, and F. A. Payne. "Diffuse Reflectance Profiles of Eight Milk-Clotting Enzyme Preparations." *Journal of Food Science* 56.2 (1991): 411-20. Print.

Westermann, Signe, Dagmar A. Brüggemann, Karsten Olsen, and Leif H. Skibsted. "Spatial Distribution of Light-induced Lipid Oxidation in Semi-hard Yellow Cheese as Detected by Confocal Microscopy." *Food Chemistry* 116.3 (2009): 756-60. Print.

Woodcock, Tony, Colette C. Fagan, Colm P. O'Donnell, and Gerard Downey. "Application of Near and Mid-Infrared Spectroscopy to Determine Cheese Quality and Authenticity." *Food Bioprocess Technol* 1 (2008): 117-29. Print.

Zheng, C., D. Sun, and L. Zheng. "Recent Applications of Image Texture for Evaluation of Food Qualities—a Review." *Trends in Food Science & Technology* 17.3 (2006): 113-28. Print.

APPENDIX

```
% syneresis.m
% Ricky Hennessy
% 2/11/2011

%% Choose Directory
clear all, close all
DirName = uigetdir;
cd(DirName)
fileListing = dir(cd);

%% Create Movie
h = waitbar(0,'Analyzing Coagulation Process...');
for i = 1:length(fileListing) - 2;
    filename = fileListing(i + 2).name;
    image = imread(filename);
    image = imresize(image,.7);
    imshow(image)
    F(i) = getframe;
    fat(:,:,i) = double(medfilt2(image(:,:,1),[3 3]));
    protein(:,:,i) = double(medfilt2(image(:,:,2),[3 3]));
    waitbar(i/((length(fileListing)-2))),
end
close(h)
[Nx Ny Nz] = size(fat);

%% Calculate fatrate
for i = 1:Nz-1
    Im1 = fat(:,:,i);
    Im2 = fat(:,:,i+1);
    ImDiff = abs(Im2 - Im1);
    IntChange(i) = mean(Im1(:)) - mean(Im2(:));
    if IntChange(i) < 0
        IntChange(i) = 0;
    end
    fatrate(i) = mean(ImDiff(:)) - IntChange(i);
end
fatrate = fatrate - min(fatrate);
fatrate = fatrate(2:end-1);
fatrate = fatrate - mean(fatrate(end-20:end));

%% Calculate protrate
for i = 1:Nz-1
    Im = protein(:,:,i);
    Im = Im - min(Im(:));
```

```

    Im = double(Im * (255/max(Im(:))));
    [Gx Gy] = gradient(Im);
    G = sqrt(Gx.^2 + Gy.^2);
    protrate(i) = mean(G(:));
end

%% Plot protrate
M = 5;
protrateF = filter(ones(1,M)/M,1,protrate);
protrateF = protrateF(M+1:end);
protrateD = diff(protrateF);
j = 0;
for i = 1:(length(protrateD)-1)
    if protrateD(i) <= 0 && protrateD(i+1) >= 0
        j = j + 1;
        zz(j) = i;
    end
end
[Y I] = max(protrateD);
t = (0:length(protrate)-1)*(30/Nz);
figure(1)
plot(t,protrate,'o');
title('Texture of Protein Matrix in Coagulating Milk','fontsize',16)
xlabel('Time (min)')
ylabel('Texture of Protein Matrix (Unitless)')
hold on
plot(t(zz(1)+M),protrate(zz(1)+M),'gx','MarkerSize',16,'linewidth',3);
plot(t(I+M),protrate(I+M),'rx','MarkerSize',16,'linewidth',3);
TIME = t(I+M) - t(zz(1)+M);
if protrate(zz(1)+M) > protrate(I+M)
    TIME = inf;
end
if I < 6
    L = I - 1;
else
    L = 5;
end
slopepts = protrateF((I-L):(I+5));
ts = (((I-L):(I+5))+M)/4;
modelFun = @(C,t) C(1)*ts + C(2);
startingVals = [1 1];
coefEsts = nlinfit(ts, slopepts, modelFun, startingVals);
xgrid = ts;
line(xgrid, modelFun(coefEsts, xgrid), 'Color','k','linewidth',4);

loc = axis;

```

```

text(15,loc(4)*.8, xlabel(['TIME = ', num2str(TIME), ' min']), 'fontsize', 16)
text(15,loc(4)*.6, xlabel(['SLOPE = ', num2str(coefEsts(1)), ' (1/min)']), 'fontsize', 16)
hold off

```

```

%% Plot fatrate
t = (0:length(fatrate)-1)*(30/Nz);
modelFun = @(C,t) C(1) * exp(-C(2) * t);
startingVals = [0.5 0.001];
coefEsts = nlinfit(t, fatrate, modelFun, startingVals);
yy = find(fatrate < (fatrate(zz(1)+M))/10);
figure(2)
plot(t, fatrate, 'o');
xgrid = t;
line(xgrid, modelFun(coefEsts, xgrid), 'Color', 'k', 'linewidth', 2);
title('Movement of Fat Particles in Coagulating Milk', 'fontsize', 16)
xlabel('Time (min)')
ylabel('Movement of Fat Particles (Unitless)')
legend('Raw Data', 'Fit', 'Location', 'NorthEast')
hold on
plot(t(zz(1)+M), fatrate(zz(1)+M), 'gx', 'MarkerSize', 16, 'linewidth', 3);
plot(t(yy(1)), fatrate(yy(1)), 'rx', 'MarkerSize', 16, 'linewidth', 3);
TIME = t(yy(1)) - t(zz(1)+M);
loc = axis;
text(20, loc(4)*.8, xlabel(['TIME = ', num2str(TIME), ' min']), 'fontsize', 16)
text(20, loc(4)*.7, xlabel('Movement = beta*e^(-alpha*t)'), 'fontsize', 12)
text(20, loc(4)*.6, xlabel(['alpha = ', num2str(coefEsts(2))]), 'fontsize', 12)
text(20, loc(4)*.5, xlabel(['beta = ', num2str(coefEsts(1))]), 'fontsize', 12)
hold off

```

```

%% Movie2AVI
% movie2avi(F, 'movie')

```

```

% milkcoag.m
% Ricky Hennessy
% 4/20/2011

```

```

close all, clear all

```

```

%% User Inputs
n = 120;           % Number of Time Steps
np = 2000;         % Number of Fat Globules
t = 30;            % Total Time of Simulation (min)
r = 1e-6;          % Radius of Fat Globules (m)

```



```

vi = 1e-3;           % Initial Viscosity (Pa.s)
T = 298;             % Temperature (K)
FOV = 500;           % Field of View (Pixels)
a = 1e-2;            % Coagulation Rate (1/s)
size = 15;           % Initial Size of Plotting Globules
sizerate = .01;       % Rate of size decrease [0 1]
green = zeros(64,3);
green(:,2) = linspace(0,1,64);

%% Constants
kb = 1.3802e-23;      % Boltzman Constant (J/K)
dt = (t*60)/n;        % Time Step (s)

%% Initial Location of Fat Globules
x = rand(1,np) * FOV;
y = rand(1,np) * FOV;
prot = zeros(FOV, FOV);

%% Simulation

for i = 1:n
    prot = prot - (1 - exp(-a*i*dt*.01))*randn(FOV,FOV);
    vis = vi * exp(a*(i-1)*dt);
    D = (kb*T) / (6*pi*vis*r);
    kick = (FOV/(1e-5)) * sqrt(2*D*dt);
    x = x + kick * randn(1,np);
    y = y + kick * randn(1,np);
    figure(1)
    imagesc(prot,[-2 2])
    colormap(green)
    hold on
    plot(x,y,'r','markersize',size)
    hold off
    H(i) = getframe;
    size = size - size * sizerate;
    axis([0 FOV 0 FOV])
end

```