OPTIMIZING SAMPLE PREPARATION AND SCANNING METHODS FOR COMPONENT ANALYSIS OF RAW MILK BY FOURIER TRANSFORM NEAR INFRARED SPECTROSCOPY

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Last but not least, thanks to my family, Kelsie, and Kirby for always being by my side and for understanding my drive towards perfection.

Dedication

I dedicate this thesis to all my nieces and nephews as an example of what hard work can get you.

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1 Introduction

Evaluation of raw milk for components such as fat, protein, and casein is important prior to cheese manufacturing so the process can be standardized. In cheese-making, standardization of milk on the basis of protein, especially the casein proteins, is important for maintaining end-product quality and for predicting cheese yields (Sørensen and Jepsen, 1998; Downey et al., 2005; Woodcock and Fagan, 2008).

General analytical benchtop methods, such as the Kjeldahl procedure for protein measurement and Mojonnier method for fat analysis, are time and resource consuming and require the operator to have extensive technical training. The concept of using spectroscopy, specifically light in the mid-infrared region, to analyze component quantities has solved most of the issues associated with the traditional benchtop methods. Mid-infrared spectroscopy (MIRS) provides simultaneous analysis of multiple components quickly, but the cost of the instrument and associated maintenance is very expensive (Rodriguez-Otero et al., 1997; Tague and Chimenti, 2012). Small manufacturers cannot afford the price of current spectroscopic methods and do not have the flexibility to evaluate finished cheese for its components, while on-farm processors are often limited in their ability to test their own milk or products. Alternative spectroscopic techniques like near-infrared spectroscopy (NIRS) with the flexibility to evaluate both liquids and solids would greatly benefit cheesemakers.

NIRS offers many different advantages in that it is very fast, can be used to analyze both solids and liquids, and has functional on-line measurement capabilities. Most importantly, near-infrared (NIR) instruments are cheaper compared to mid-infrared (MIR) techniques because of their simpler design and lack of an attached homogenizer (BÜCHI Labortechnik AG, 2010; Tague and Chimenti, 2012). While NIRS has found practical use in the grain and pharmaceutical industries, there still remains an issue with its application in the dairy industry as made clear by Siesler et al. (2008).

One challenge to NIRS use in dairy products has been the poor calibration quality for fat in raw milk. This is thought to be due to the presence of large fat globules in milk, which causes excessive light scattering as infrared energy interacts with a sample during analysis. As a result, predictions of component quantities become inaccurate. Limiting the effects of the fat component in milk is possible by using standard homogenization processes as explained in previous studies (Barbano and Clark, 1989; Thiebaud et al., 2003; Hayes et al., 2005); however, the cost associated with these makes standard homogenization impractical. Additionally, exploring different methods for presenting the sample to infrared instruments could theoretically improve milk analysis (Wetzel, 1983; Ilari et al., 1988; Reich, 2005).

The overall objective of this research was to optimize different sample preparation and presentation techniques to give a more accurate prediction of raw milk components by NIRS. The literature review contains relevant information concerning the analysis of samples by NIRS, which includes a review of NIR instrument components, a discussion of chemometrics and calibration development, and a comparison of different spectroscopic methods of analysis. Also included is an overview of the milkfat globule and different homogenization treatments as they apply to NIRS and the dairy industry. The introduction, materials and methods, results and discussion, and conclusion consider the effects of various homogenization methods on fat particle size distribution and effects of homogenization and sample presentation methods on calibration quality.

2 Review of Literature

2.1 Near-Infrared Spectroscopy (NIRS)

In its most basic form, spectroscopy can serve as a quality analysis tool through the production, measurement, and analysis of spectra resulting from electromagnetic radiation interacting with matter (Siesler et al., 2008). This approach can be used to activate the vibrational motion of atoms within a molecule leading to an enhanced view

of the intermolecular forces among them. These intermolecular forces can then be interpreted by the analyst using the electromagnetic spectrum to identify organic compounds and functional groups (specifically, O-H, C-O, C-H, N-H) based on their unique banding absorption patterns and intensity of absorption to find the relative concentration within the entire sample (Thygesen et al., 2005; Sathyanarayana, 2007).

Improvements in precision and ease of methodology compared to other exhaustive quality control techniques (GC, HPLC, NMR, MS) has led to the widespread use of vibrational spectroscopy in the chemical, pharmaceutical, petrochemical, polymer, cosmetic, food, and agricultural industries (Kizil et al., 2002; Siesler et al., 2008). With respect to the food industry, near-infrared spectroscopy (NIRS) is viewed by some analysts as a more appealing option compared to Raman and infrared (IR), two historically common methods of spectroscopy, because of its high quantitative analytical ability due to its lower absorption coefficients (Benito et al., 2008).

In samples analyzed by NIRS, wavelengths of light between the visible and mid-IR regions (780-2,500 nm) are absorbed by molecules causing stretching and bending amongst chemical bonds. Absorption bands in the NIR region of the electromagnetic spectrum are mostly represented by overtones and the mid-IR region combination vibrations (Wetzel, 1983). Combination vibrations occur when two molecules make a simultaneous transition to a higher state of energy ($v = 0$ to $v = 1$) following the absorption of a photon (Diem, 1994). Overtones occur when a molecule makes a transition from the ground state ($v = 0$) directly to the second (or third) excited state ($v =$ 2 or 3) upon the absorption of a photon. Unfortunately, this direct transition reflects the action of a harmonic oscillator, which is prohibited following the selection rules of quantum mechanics (Diem, 1994; McQuarrie and Simon, 1997). This transition state must instead be simplified as a non-oscillating harmonic oscillator whose molecular vibration sequence becomes nothing more than an approximation, thus, limiting the ability to measure how much light can be absorbed by the sample. This limited

measurement results in weak absorption bands, which, along with broad combination vibration bands, are difficult to interpret (Sun, 2009). However, these approximations are starting to improve.

The adoption of NIRS as a reliable method of analysis during the past 25 years stems from improvements made in optical fibers, computers and chemometrics, and in the conversion of chemical data into relevant information (Wetzel, 1983; Hirschfeld, 1984; McClure, 1994). The first use of NIRS was in agriculture to measure moisture in grain (Norris, 1964). It was then adapted for use to measure protein, fat, and moisture in foods (Davies and Grant, 1987; Gunasekaran and Irudayaraj, 2001), view differences in wine based on vintage year (Cozzolino et al., 2003), detect and quantify changes in both olive oil and beverages due to adulteration (Paradkar et al., 2002; Kasemsumran et al., 2005; Pontes et al., 2006; Cen and He, 2007), as well as to identify textural attributes in fruit and vegetables more recently (Nicolai et al., 2007). As the use of NIRS in the food industry continues to expand; the need for system requirements to become more adaptable and feasible also increases. In the dairy industry, on-line measurement of product quality using spectroscopy is a key feature that many companies utilize for simultaneous analysis of moisture, protein, fat, casein, and lactose. With NIRS, rapid determination of these components, as well as other intact materials in dairy products, can become a reality.

The application and challenges of using NIRS in the dairy industry will be explored in this literature review. Additionally, methods for overcoming these obstacles, a description of the major components of the NIR spectrometer, as well as the mechanics behind spectral analysis including an evaluation of the advantages and disadvantages of the three major types of vibrational spectroscopy is also considered.

2.1.1 Comparison of Raman, Mid-Infrared (MIR), and NIR Spectroscopy

Raman Spectroscopy

Raman spectroscopy includes the broad frequency range of 2,500-200,000 nm and utilizes an energy scattering technique that provides high structural selectivity for components in a sample (Siesler et al., 2008). The higher wavelengths in this region are considered valuable since they may contain information regarding the crystalline lattice, which is a characteristic used for rapid identification of samples with this arrangement (Siesler et al., 2008). Traditionally, Raman spectroscopy has been used in academia, but its recognition in industry becomes more prevalent when combined with Fourier Transformation- a mathematical technique used to improve the ability to interpret spectra that will be explored in a later section. Use in industry is practical for Raman spectroscopy because it requires just a small sample size, no sample preparation, and can be used for on-line testing (Burns and Ciurczak, 2008)

Disadvantages for Raman spectroscopy are caused mostly by its energy source, which is a laser that emits monochromatic radiation in the UV-VIS range (400-780 nm). This emitted energy varies in frequency more than in MIR or NIR and can excite sample molecules to energy states much higher than the vibrational level (Siesler et al., 2008). From here, the energy returns as fluorescence to either the ground state or the first excited energy state. Problems arise because as fluorescence increases, there is a decrease in scattering efficiency since additives or impurities are superimposed on the spectrum and can overwhelm the signal (Burns and Ciurczak, 2008). Safety issues with the radiation emitting laser also limit Raman's use in industry (Siesler et al., 2008). Meanwhile, the cost for a Raman spectrometer is the most expensive when compared to MIR and NIR. Estimates from $175,000-$250,000 are common because of the high cost of the excitation laser and detector (Tague and Chimenti, 2012). Also, associated maintenance of these two items can be pricey due to short laser lifetime and replacement costs. On the positive side, there is little need for a technical user to use complicated statistical modeling to extract information since spectral characteristics are easier to interpret. (Tague and Chimenti, 2012)

Mid-Infrared Spectroscopy (MIRS)

MIRS covers a more specific frequency range (2,500-25,000 nm) than does Raman spectroscopy, and uses polychromatic radiation from an LED source made of silicon carbide (Siesler et al., 2008). Also, unlike Raman, MIRS takes advantage of a frequency-specific absorption technique for detection, which leads to high structural selectivity and spectra containing fundamental bands with sharp, distinctive peaks that are ripe for interpretation (Chung et al., 1999; Burns and Ciurczak, 2008). Easy spectral interpretation, complete automation, small sample sizes, and relatively rapid sampling procedures (up to 400 samples/h) has led to MIRS being widely used in industries such as dairy because it can quantitatively measure multiple components (fat, protein, lactose, etc.) concurrently (Rodriguez-Otero et al., 1997). MIRS has also been recognized as an official method by IDF, APHA, and AOAC. Calibrations of infrared spectrometers for the determination of fat, protein, and lactose in raw milk are based on standards set in IDF International Standard 141C:2000 (International Dairy Federation (IDF), 2000). For example, the standard error of predicted values (SEP) for total protein, fat, and lactose must be within 0.02-0.10 g of the actual quantities (IDF, 2000; Luginbuhl, 2002).

While MIRS has seemingly become a widely accepted industry tool, there have been a number of disadvantages found by users. Analysis of intact components, milk powders, and other non-liquid dairy products presents a challenge since much of the emitted light gets absorbed causing a reduction in the frequency range and in the instrument's ability to retrieve a signal (Rodriguez-Otero et al., 1997). Even if a strong signal can be recovered, regular maintenance of the MIR instrument is required to ensure the signal is properly converted into useable information. Such upkeep includes: maintenance of precalibration to limit spectral drift, maintaining calibrations for sample components using costly, time-consuming methods, and maintaining efficiency of the homogenizer or other sample preparation instruments that may be attached to the spectrometer (Barbano and Clark, 1989). Probably the biggest drawbacks for MIRS lie in its inability to be used for on-line applications and its cost. Mid-IR fibers (composed

of chalcogenide or silver chloride) make up the transmission window of the sample cell and are very expensive. Furthermore, the added cost of calibrations, built-in homogenizers, or other attached sample preparation methods bring the overall cost of MIRS to $75,000-$125,000 (Schoenfuss, 2012; Tague and Chimenti, 2012).

NIR Spectroscopy (NIRS)

NIRS has four principle advantages: speed, easy sample preparation, simultaneous measurement of multiple properties from one spectrum, and no degradation of sample during measurement (McClure, 1994; Rodriguez-Otero et al., 1997; Blanco and Villarroya, 2002; Siesler et al., 2008). Samples can be measured and spectra produced in less than ten seconds (BÜCHI Labortechnik AG, 2010). The speed of the overall process, including sample preparation, is minimal since solid samples can typically be presented to NIR instruments as is. Liquid samples can be measured in the lab, at-line, or on-line with the help of quartz light fiber optics. Once samples are measured, enough information is contained within spectra since there are multiple scans, which create numerous data points. This becomes practical when multiple properties need to be measured at once. Finally, any sample measured by the NIR instrument can be returned to its original batch since there is no degradation by solvents or by any chemical reagents typically added during sample preparation for spectroscopy.

When cost is considered, NIRS is the cheapest among the three primary vibrational methods ($45,000-$60,000) (BÜCHI Labortechnik AG, 2010; Schoenfuss, 2012; Tague and Chimenti, 2012). The sample holder (made from glass and quartz), light source (tungsten halogen), laser (12 VDC HeNe, wavelength at 632.992 nm) and detector are generally cheaper options. Additionally, the lack of sample preparation methods makes the NIR instrument a better alternative to MIR.

The primary disadvantage is that calibrations are required for each component to be measured (McClure, 1994). When using NIR for quantitative analysis, calibrations

are generated by using complex statistical models (since they are based on overtone and combinations of vibration bands). The data sets also need to cover a wide variation in samples if calibrations are to be robust. If the calibration model is not a good representation of this variation, then analysis becomes inaccurate and model transfer between instruments becomes difficult. Another disadvantage lies in the interpretation of broad combination and overtone peaks in NIR spectra resulting in poor structural selectivity (Siesler et al., 2008). However, this disadvantage can be improved with pretreatments such as normalizations and derivatives and using chemometric software to improve analysis (BÜCHI Labortechnik AG, 2007).

Some NIR users argue that even pretreatments cannot help with the detection of components present at levels below 0.1% (Burns and Ciurczak, 2008; Siesler et al., 2008), but repeated verification of minor constituents using wet chemistry methods has been shown to increase the sensitivity of detection (Davies and Grant, 1987; McClure, 1994; Rodriguez-Otero et al., 1997). Additionally, as instrumentation and methods for chemometric analysis improves, reductions in signal-interference factors such as background noise, light scattering, and overlapping absorption peaks become more realistic and sensitivity will increase.

2.1.2 FT-NIR Spectrometer Components

The physical components of NIR spectrometers have continued evolving since they were first used in 1964 (Norris). However, there still exist four primary steps necessary to develop the spectrum from which data can be interpreted, including: generating a light source, splitting the light beam, sample detection, and signal processing. Each of these steps can be seen in Figure 1.

Generating a Light Source

A tungsten halogen lamp (**1**), which is capable of providing energy in the near-infrared region for an extended time, is used in most NIR spectrometers (Cen and He,

2007). Like most white light sources, the halogen lamp generates a large amount of heat during its short operational lifespan (~6,000 h) making it necessary for the spectrometer to have two bulbs in order to avoid deviations in spectral output caused by lamp fluctuations during measurement sequences (BÜCHI Labortechnik AG, 2010). In addition to a source of light being generated, a laser beam can also be used within the spectrometer to generate a constant beam of energy to serve as a wavelength reference. The laser helps to ensure reproducibility and accuracy by limiting the interference factor from the instrument on the light path (BÜCHI Labortechnik AG, 2010).

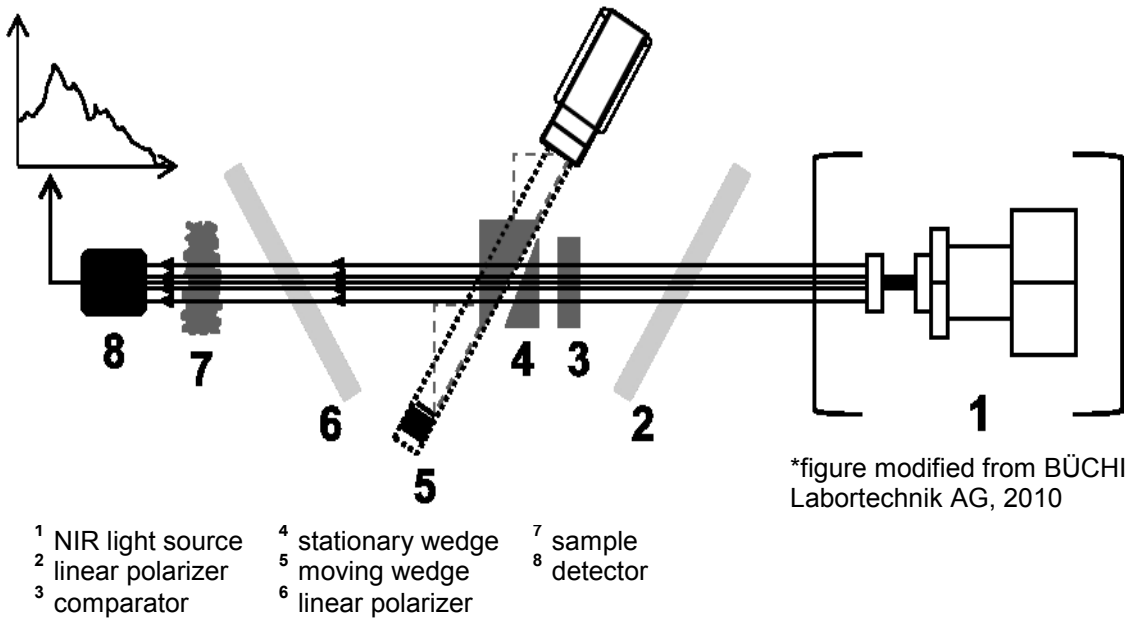

Figure 1. The internal diagram of a NIRFlex N-500 Fourier Transformation Near-Infrared (FT-NIR) spectrometer*.

Splitting the Light Beam

Once the light has been emitted from the halogen source, it passes through a linear polarizer (**2**) creating a well-defined beam of polarized light more fit for characterization. From here, the light is split into two defined orthogonal paths by a

comparator (**3**). One beam gets reflected off a small stationary wedge (**4**) while the other by a moving wedge (**5**) attached to a linear drive shaft within the spectrometer. The individual movement and geometric placement of the wedges helps in creating a thicker light path that ultimately helps increase the sensitivity of the instrument while also increasing the signal to noise ratio (Jackson, 2002; BÜCHI Labortechnik AG, 2010). The reflected light beams hit a second polarizer (**6**), which combines them into a single path with varying intensities (now referred to as an inferogram). This process (Figure 1) is the basis for a Michelson inferometer, a commonly used set of techniques for extracting data from electromagnetic waves (Marshall and Verdun, 1990; Jackson, 2002; Meyer et al., 2006). However, the inclusion of a moveable wedge, as illustrated in Figure 1, requires a Fourier transform to be made with the data to obtain a spectrum. This movable wedge is used to widen the light path so that more of the sample can be analyzed, which radically increases instrument sensitivity (Griffiths and de Haseth, 1986).

Sample Detection

Sample detection (**8**) within NIR spectrometers varies significantly with the sample (**7**) being analyzed and the mode of detection. Samples vary in their optical properties, so the type of sample presentation is very important. In principle, light interacts with particles, and the added energy creates an excited vibrational state within the chemical bonds of the material, which leads to a change in its spectral characteristics at different wavelengths (Nicolai, et al., 2007; Sathyanarayana, 2007). The classic method for analyzing materials is through diffuse reflectance, as seen in Figure 2. This method involves light entering the sample media, encountering an individual boundary (i.e. a powder granule), and getting refracted before being diffusely reflected back at random angles to the detector (Wetzel, 1983; Barnes et al., 1989; Cen and He, 2007; BÜCHI Labortechnik AG, 2010).

figure modified
from Wetzel, 1983

Figure 2. Light interacting with sample components through diffuse reflection

Diffuse reflection is best modeled by Kubelka and Munk (Equation 2.1), whereby diffuse reflectance of an infinitely thick sample (R_∞) is related to K, the molar absorption constant, and S, the scattering coefficient (Kortum, 1969) (Davies and Grant, 1987):

$$\frac{K}{S} = \frac{(1 - R_\infty)^2}{2R_\infty} \tag{2.1}$$

Most of the light during diffuse reflectance is inevitably scattered throughout the sample while interacting with a number of different particle boundaries before exiting the media. This scattering can lead to spectra with non-uniform baselines and changing offsets. Furthermore, scattering effects are affected by varying sample thickness (Wetzel, 1983), particle size (Wetzel, 1983; Reich, 2005), or smoothness of sample surface (Ilari et al., 1988). Petri dishes or other similar sampling cups are typically used for improved control of sample thickness when solids and other non-translucent materials are analyzed by diffuse reflectance.

Liquid samples and translucent materials, on the other hand, are more commonly analyzed with transmission or transflectance and can occur either off-line or at the production line with the help of a fiber optic probe (Figure 3). In transmission, energy

passes through the quartz cuvette or sample cup with quartz windows containing the liquid at a defined pathlength and the transmitted light contains the spectral information as seen in Figure 4 (Stuart, 2004; Siesler et al., 2008).

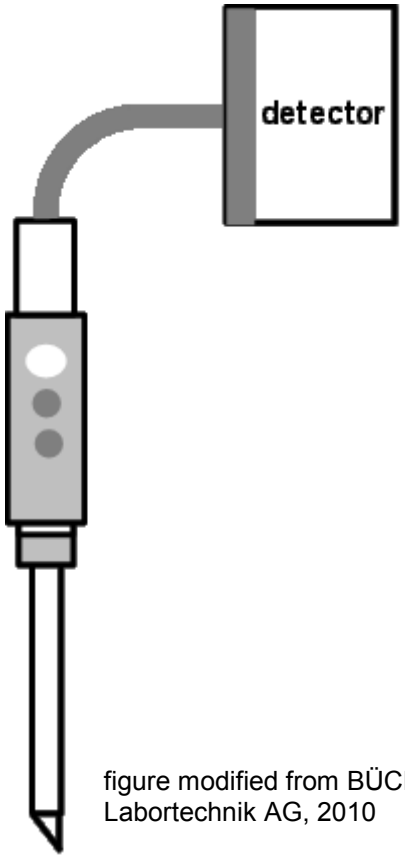

Figure 3. Fiber optic probe used for rapid at-line measurement of samples

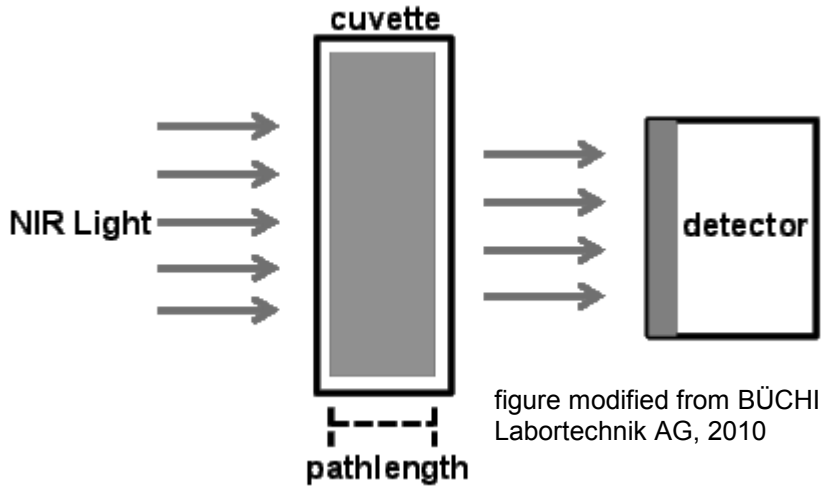

Figure 4. Light interacting with a sample during transmission mode

Transflectance is set-up similarly to both transmission and diffuse reflection in that light passes through the sample as a single pathway and then is reflected off a diffuse reflector to pass through the sample once more (Figure 5) (Ritchie, 2009; BÜCHI Labortechnik AG, 2010). A petri dish or standard sample holder is practical in this method. Additionally, the use of an NIR spectrometer flow cell adaptor powered by a peristaltic pump can be used to mimic an on-line measurement of a liquid sample by the transflectance method (Figure 6) (BÜCHI Labortechnik AG, 2010).

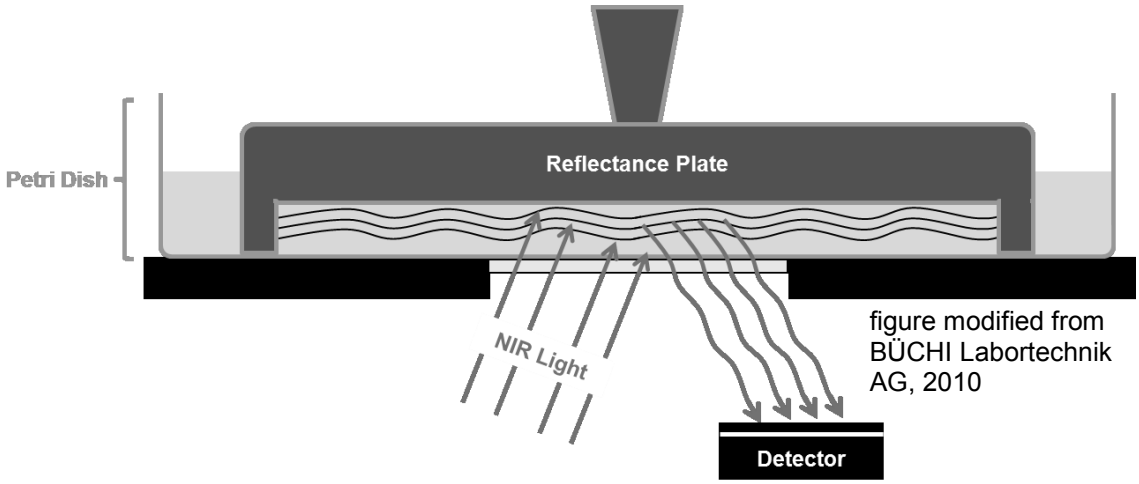

Figure 5. Analysis of a sample in a petri dish using the transflectance method

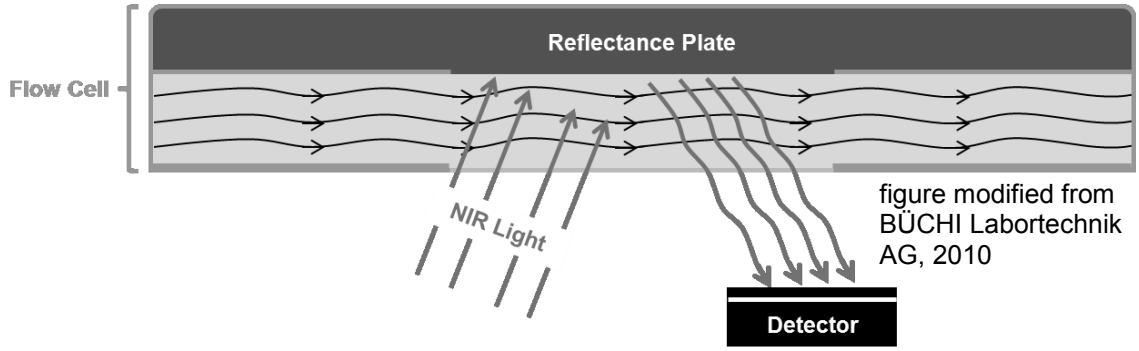

Figure 6. Analysis of a sample flowing through the detector in transflectance mode

Regardless of the detection method, a sample detector made of silicon, lead sulfide, or indium gallium arsenide (InGaAs) is standardized for the NIR spectrometer. Silicon and lead sulfide detectors are inexpensive, but silicon detectors have the advantage of being faster and more compact, while the lead sulfide material provides better signal-to-noise properties and higher wavelength sensitivity (Reich, 2005; Siesler

et al., 2008). InGaAs detectors are optimal for NIR measurement combining both speed and size with better signal and sensitivity; however, the cost of this detector reflects its characteristics (Siesler et al., 2008; BÜCHI Labortechnik AG, 2010).

The placement of the detector near the area of sample measurement is equally important to the type of material it is made from. It is to the manufacturer's benefit to place the detector where the most reflected and transmitted light can be taken in. This is done to maximize the signal and to average the light gathered from all directions and angles, as it may have been scattered during reflectance (Wetzel, 1983). The detector acquires the signal and translates it into absorbance (*A*) values relative to a standard reference. In reflectance mode, *A* is measured as *log 1/R*, whereas *A* is *log 1/T* during transmittance.

Signal Processing

A computer attached to the spectrometer has software with the ability to acquire data, pretreat the signal, transfer the data from analog to digital, and perform numerical analysis. With the help of Fourier transformation, spectral quality has been dramatically improved and data processing made easier (Griffiths and de Haseth, 1986; Stuart, 2004). Using the transformed spectra, analysts are able to optimize parameters on the spectrometer, such as wavelength regions of interest, number and frequency of scans, and modes of detection to maximize the signal to noise ratio and to improve overall precision and reproducibility of experiments.

2.1.3 Chemometrics

The resulting spectrum from NIR measurements often includes broad peaks with only minor shifts in the shape of absorption bands. For this reason, the only way to understand these complex spectra is through the use of mathematical models in chemometric software. Chemometrics is a method for processing, evaluating, and interpreting chemical data (Massart et al., 1997; Vandeginste et al., 1998). With

Chemometrics, the goal is to find statistical correlations among acquired spectral data and known property values (obtained from lab analysis) for samples in the calibration set. If correlations occur through repeated measurements then prediction of quantity, quality, or identification for unknown samples is possible. There exist two key steps in chemometrics: spectral data pre-processing and development of calibrations for both qualitative and quantitative description.

Spectral Data Pre-processing

NIR spectral data includes many variations in the absorption bands. While some of these variations are caused by changes in the physical and chemical properties of the sample there are still other effects such as background instrument noise, light scattering, and overlapping absorption bands responsible for the majority of the variation (BÜCHI Labortechnik AG, 2007). Pretreating the spectra mathematically transforms the signal to limit unwanted characteristics and to improve the overall spectral quality. Two of the most common pretreatments applied to spectra include scatter-corrective methods and spectral derivatives (Rinnan et al., 2009).

Scatter correction mainly involves three techniques (standard normal variation 'SNV', multiplicative scatter correction 'MSC', and normalization) capable of minimizing the physical differences among samples caused by scattering light and limiting baseline shifts. In MSC, flaws resulting from undesirable scattering effects are removed before further data analysis (Figure 7). For this reason, it is commonly used in applications of diffuse spectroscopy (Naes et al., 2002). MSC considers each individual spectrum rather than an average, and the model is

$$x_{ik} = a_i + b_i \bar{x}_k + e_{ik} \quad (i = 1,\ldots, \mathrm{N};\ \mathrm{k} = 1,\ldots, \mathrm{K}) \tag{2.2}$$

where i is the sample (spectrum) number and k is the wavelength number. a_i and b_i are constants representing additive and multiplicative effects, respectively, for spectrum i (Martens et al., 1983; Geladi et al., 1985). These unknown coefficients need to be

calculated for each spectrum using all or at least a portion of the *K* spectral measurements before they can be used for the final MSC transform (2.4).

$$\bar{x}_k = \frac{1}{N}\sum_{i=1}^{N} x_{ik} \tag{2.3}$$

$\bar{x}_k$, is the mean or average for samples at the *k*th wavelength while e_{ik} is the error associated with all other unwanted effects on the spectrum not modeled by additive or multiplicative constants. The known coefficient, $\hat{a}_i$ is subtracted from x_{ik} and the result is divided by $\hat{b}_i$ in the MSC transform, x_{ik}^* (Martens et al., 1983); Geladi et al., 1985).

$$x_{ik}^* = (x_{ik} - \hat{a}_i)/\hat{b}_i \tag{2.4}$$

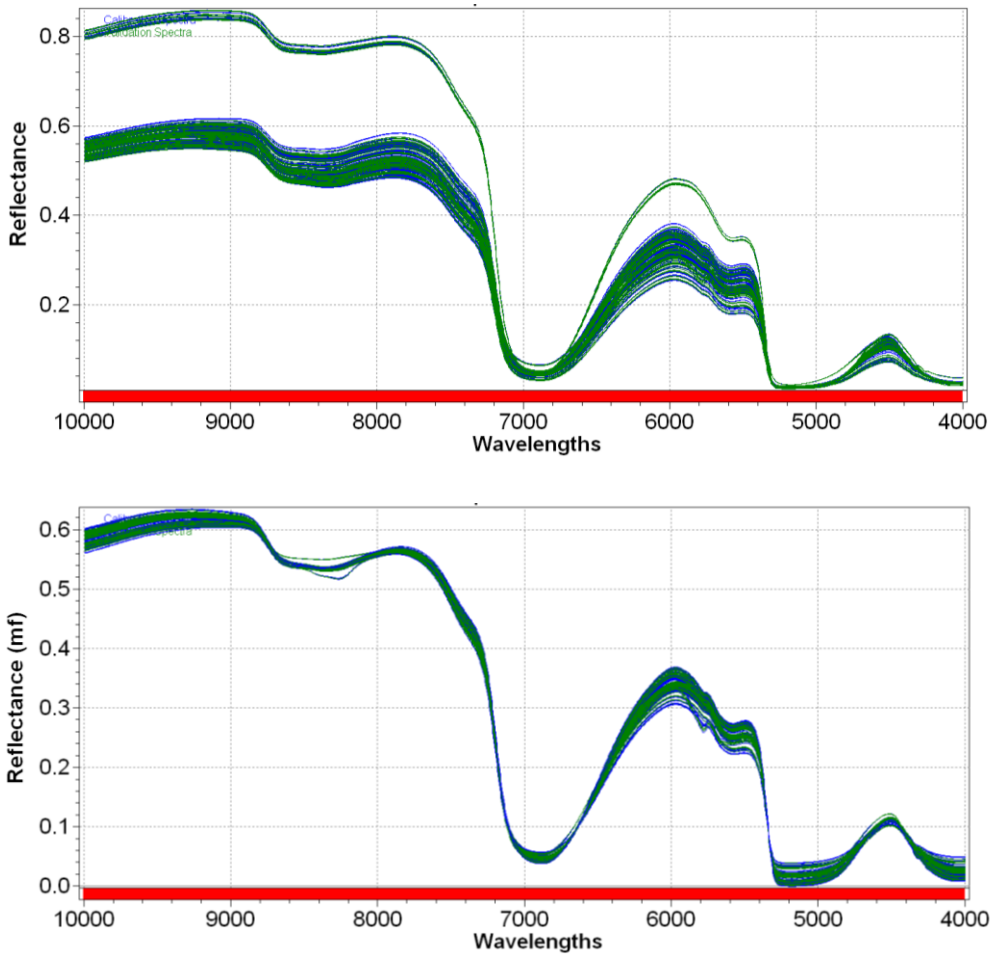

Figure 7. NIR spectra for raw milk before (*top*) and after (*bottom*) MSC transformation

The model for adjusting spectra using SNV and normalization is very similar to the traditional MSC method. If x_{ik} represents the same ith sample for the spectrum measurement at the kth wavelength then the transformation becomes

$$x_{ik}^{*} = (x_{ik} - m_i)/s_i \qquad (2.5)$$

where m_i is the mean of the K spectral measurements for sample i and s_i is the standard deviation of the same K measurements (Barnes et al., 1989; Naes et al., 2002). The main

difference in methods is SNV uses the mean of measurements from the spectrum of interest instead of using the mean from all samples at a particular wavelength. For normalization corrections, m_i is set equal to zero (Rinnan et al., 2009). Some reports indicate that SNV and normalization procedures are more sensitive to spectral noise (Rinnan et al., 2009); however, there is a general agreement in most sources that MSC and SNV/normalization corrections can be used interchangeably (Barnes et al., 1989; Naes et al., 2002; Rinnan et al., 2009).

Derivatives are another way of reducing baseline effects and emphasizing smaller absorption peaks. First derivatives can be used to calculate the slope of any mathematical function, i.e. infrared spectrum, which are represented by either positive or negative lobes in the derivative. This can be seen in Figure 8 where at the peak of the spectrum the slope is zero so the first derivative is a horizontal line at zero. Note the baseline of the derivative line in Figure 8 has been reduced to zero because the derivative of any constant is zero, which demonstrates the function's ability to offset spectrum (Smith, 2009).

Second order derivatives, which are derivatives of first derivatives, can also be applied to spectra to remove both additive and multiplicative effects as well as to measure concentration of sample material. It measures the direction of the curvature of a function by looking at the function's slope. Figure 9 displays a second derivative curve whereby a change in direction from positive to negative to positive in the derivative line represents the concave down feature of the function. The maximum of the function occurs at the second derivative's minimum. For this reason, second derivatives are useful in peak picking and identifying functional peaks (Smith, 2009). They are also useful for separating overlapping peaks and shoulders because these will have different slopes in the spectrum (Rinnan et al., 2009; Smith, 2009). Similar to first derivative functions, the baseline in Figure 9 is at zero since it is represented by a constant, which has no slope and subsequently no change in slope.

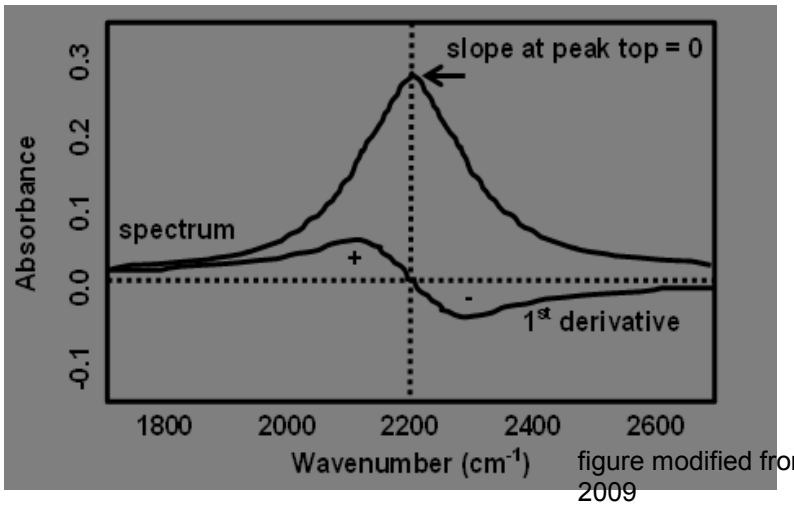

figure modified from Smith, 2009

Figure 8. Top: Part of a spectrum of a sample plotted in absorbance. Bottom: The first derivative of this spectrum. The first derivative is zero at the spectrum maxima

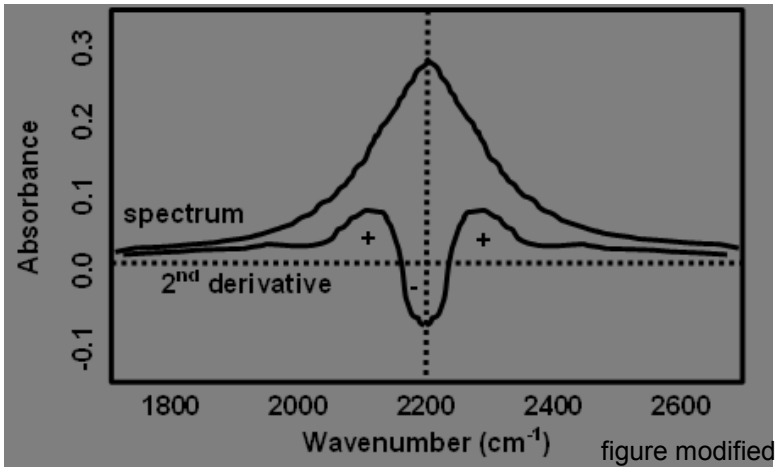

figure modified from Smith, 2009

Figure 9. Top: Part of a spectrum of a sample plotted in absorbance. Bottom: The second derivative of this spectrum

Arguably one of the more attractive features in the use of second derivatives is their ability to predict material concentrations in quantitative calibrations. This is possible since the area of the features within a second derivative curve is directly

proportional to the material concentration represented by the absorbance peak at a given wavelength (Smith, 2009).

The pitfall to using derivatives is there is more noise in the transformed spectra. This problem can be adjusted by applying a simultaneous smoothing technique, which reduces the noise, but may broaden some peaks (Savitzky and Golay, 1964). The Savitzky-Golay derivative model is one example of how this combined approach works.

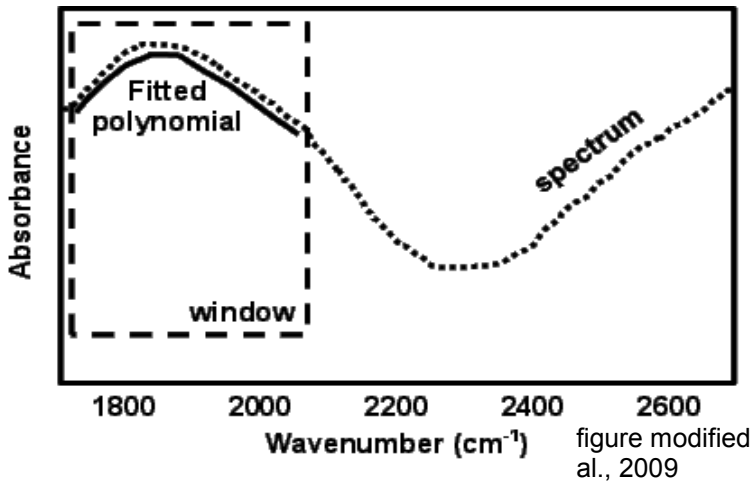

figure modified from Rinnan et al., 2009

Figure 10. The Savitzky-Golay smoothing technique. A 7-point window and second-order polynomial is used for smoothing

In the Savitzky-Golay method (Figure 10), a window is placed around a certain region of the spectrum with the center point being at the wavelength of interest. Within this window, a low-ordered polynomial is fit to the data points using least (residual) squares method (Savitzky and Golay, 1964; Naes et al., 2002; Rinnan et al., 2009). The number of wavelengths this window spans is determined by the analyst, but typically ranges from three to nine "points". Using a polynomial to represent the data not only smooths the spectrum, but also provides a novel evaluation of the line's derivatives. The highest order derivative that can be calculated is based on the degree of the polynomial

used for fitting (i.e. third derivative can be calculated for a third order equation) (Rinnan et al., 2009). Once the derivatives in this region have been calculated, the window is moved along the curve and centered on the next wavelength of interest. The process is repeated until all points have been considered.

As was mentioned previously, broadening of peaks is one inherent problem with some smoothing techniques. This is caused by using a high number of points inside the window described in the Savitzky-Golay method, which rounds off peaks and troughs (Naes et al., 2002). However, too few points in the window will cause increased noise once the derivatives have been calculated. Therefore, it is important for users to optimize both the number of points to include in the window along with the degree of the fitted polynomial without over-processing the spectra into nonsense.

Qualitative Calibration Development

Qualitative calibrations involve identification testing for different chemical substances and separation of different qualities of the same material. This is accomplished by scanning samples and comparing their absorption spectra to a database of reference spectra. The combination of positive matching along with negative correlations for characteristics that should not be present is the essence of qualitative measurements (BÜCHI Labortechnik AG, 2007; Ritchie, 2009). Two main methods for qualitative calibration include Cluster Analysis and SIMCA (Soft Independent Modeling of Class Analogy). Both of these methods utilize Principle Component Analysis (PCA), a statistical evaluation tool that compresses redundant segments of spectral data into a few key components representative of the entire spectra (Esbensen and Geladi, 1987).

Cluster analysis describes techniques involving the grouping of objects displaying similar and predefined traits into specified regions (Massart and Kaufman, 1983; Bratchell, 1989). While there are many methods describing different cluster analysis techniques, almost all can be summarized using a multidimensional plane displaying the

location of groupings relative to one another as seen in Figure 11. From here, the grouping of objects based on certain characteristics may be visibly confirmed and outliers can be identified. One traditional method involves the nearby neighbor concept whereby objects that are close to one another are more similar than those far away (Hastie et al., 2009). Additionally, there are more complex models such as those that employ statistics and Gaussian distribution to more clearly define objects as belonging to a cluster (Xu et al., 1998). Overall, cluster analysis depends heavily on the data, purpose for analysis, and how the results are interpreted (Bratchell, 1989).

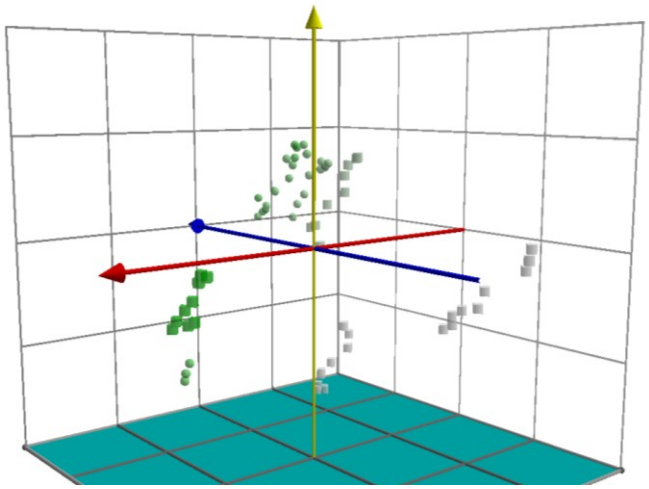

Figure 11. A cluster analysis tool used to give a multi-dimensional view of groupings relative to one another in qualitative analysis

On the other hand, SIMCA is more precise than cluster analysis since PCA analysis occurs for individual groups of the data. Only a number of principle components are retained in each class to represent the variation within that group set (Wold et al., 1977, 1983; Jolliffe, 2005). The retained principle components are then used in the group-specific model so new observations can be classified more precisely on a best fit basis (Wold et al., 1977, 1983). Deciding on the number of principle components is the main challenge for users. Too few can alter the information contained within the group

while too many principle components weaken the signal-to-noise ratio (Wold et al., 1977, 1983). Cross-validation helps decide on the number of principle components by purposely leaving out parts of the data within a group while using PCA. Then, using minimal principle components, omitted data are predicted and repeatedly substituted into the group until the principle component model gives the smallest prediction error. The optimum number of principle components used for this model is necessary for producing the highest signal-to-noise ratio (Wold et al., 1983; BÜCHI Labortechnik AG, 2007). With a known prediction model, qualitative calibrations can be validated during the measurement of a new spectrum by ensuring the resulting residual is lower than the allowed tolerance. Additionally, validation can be checked if the spectrum is within an allowable distance to a particular cluster and if the identity of the substance matches the identity of the substance used for calibration assembly (BÜCHI Labortechnik AG, 2007).

Quantitative Calibration Development

Quantitative calibrations are used to measure concentration levels of components within a sample by developing methods that interpret the intensities of response signals in the near-infrared spectra. Previous studies have shown NIRS being capable of measuring concentrations (dry matter, ash, protein, fat, microbial populations, hydroxyl number, etc.) (Davies and Grant, 1987; Rodriguez-Otero et al., 1997; Gunasekaran and Irudayaraj, 2001), physical components (stiffness, internal damage, texture, density, and viscosity) (Lammertyn et al.1998; Clark et al., 2003; Nicolai et al., 2007), and even predicting sensory attributes (Mehinagic et al., 2004). Three algorithms commonly used in quantitative analysis include: multiple linear regression (MLR), partial least squares regression (PLSR), and principle component regression (PCR).

The basis for MLR is linear regression; an approach to modeling a dataset by examining the relationship between dependent and independent variables using a line of best fit. MLR is explained by considering the effect of more than one independent variable. In vibrational spectroscopy, the relationship between spectral responses and

certain physical or chemical properties of the sample is of interest (Ritchie, 2009). Wavelengths used as the independent variable in calibrations can confirm both the presence and quantity of an attribute using the regression equation. Approximately two-thirds of data sets should be used to develop the prediction or "calibration equation", and the remaining one-third of data should be placed into the validation set for confirmation of a linear relationship (BÜCHI Labortechnik AG, 2007). The goodness of fit between the calibration and validation curves can be examined using the square of the correlation coefficient (r^2) along with standard deviation values (*sdev*) and standard error of prediction (*SEP*). An $r^2 = 1.0$ indicates that a prediction line perfectly fits the data while *sdev* and *SEP* should be low to indicate a small deviation from the mean values of the validation curve (Fisher, 1922; Cox, 1972). MLR has had many uses in statistical analysis, and its use has evolved beyond that of normal independent variables being used as predictors of physical and chemical properties.

Partial least squares regression (PLSR) is a non-restrictive extension of MLR. In PLSR, the prediction functions are represented by factors, which are direct linear combinations of the original *X* (indicator or observable) and *Y* variables (Wold, 1966; de Jong, 1993). It can be considered a nonrestrictive model since the number of factors produced can exceed the maximum number of combinations of *X* and *Y* variables; thus, allowing the model to be used even when the predictor variables exceed the number of observations (de Jong, 1993). Additionally, PLSR is applied when there is limited information about the population or measurement scale. Although PLSR is quite flexible in its use, a certain amount of information regarding the relationship between the observable variable and its predicted counterpart must be known so the regression model can be relied upon (Wold et al., 2001; Haenlein and Kaplan, 2004).

Estimation of PLSR and MLR are far from ideal at times because of the inherent problem caused by collinearity. Multicollinearity occurs when independent variables are highly correlated through a linear relationship (Liu et al., 2003). So, when independent

variables are used to calculate predictive factors, or they themselves are used as predictors, error is introduced into the regression equation since multivariate models typically treat independent variables as being uniquely different (Farrar and Glauber, 1967; Liu et al., 2003).

PCR is an extension of analysis by PLSR; however, collinearity does not affect the end result. This is because the dependent variable is based on the principle components representing the independent variables instead of being fitted with just the independent variables during regression. As was mentioned previously, PCA combines correlated independent variables into principle components, which are also independent and uncorrelated to one another (Esbensen and Geladi, 1987; Liu et al., 2003). The PCA method makes the variable set more fit for precise estimation since only a subset of the principle components is used (Jolliffe, 1982). Regression equations are then built on these uncorrelated principle components until an equation of best fit is found (i.e. one with maximum r^2 and minimum *sdev and SEP*) (Draper and Smith, 1981; Liu et al., 2003). The linear regression equation is built on the equation of best fit in the final step.

Like many spectrometric methods, NIRS requires maintenance of calibration standards to not only achieve accurate predictions of both qualitative and quantitative sample properties, but also limit spectral drift caused by the instrument. This is especially important for food products where NIR may be used to validate sample components claimed on ingredient lists, verify the absence of adulterated materials, or confirm temperatures reached during thermal processing. Verification of accurate calibrations may even be requested during state and federal level audits. If the prediction of components fails because of instrument or product variation, calibrations need to be updated or transferred (Cogdill et al., 2005). Calibration transfer describes a mathematical process where models or data are transformed so they become compatible with multiple instruments. The difficulty in transferring calibrations is caused by the need for both instruments to be identical in terms of their design, wavelength region, and

method of measurement; however, meeting this criteria does not always ensure successful transfer (Sun, 2009).

While the problem of calibration transfer still remains for MIR instruments, there have been a number of improvements in the NIR instrument design in recent years to assist with instrument-to-instrument compatibility. These include: diode lasers for wavelength correction, internal reference standards, reoccurring system suitability tests, and charge-coupled devices (CCD) paired among similar instruments in the assembling factory (Sun, 2009; BÜCHI Labortechnik AG, 2010). Additionally, methods for incorporating instrument variation into calibrations in anticipation of eventual instrument-to-instrument transfer also exist.

2.1.4 Factors Affecting Calibration Development

Data pre-processing is often used to "clean up" spectra before developing qualitative and quantitative calibrations; however, there are some factors inherent in the sample which can continue to cause issues while developing NIR calibrations. These factors include particle size, sample thickness, strong absorption of light by the water component, and sample temperature. Unless accounted for during analysis, any single factor could alter the spectral characteristics leading to a poor prediction of component quantities.

Particle Size and Sample Thickness

Amongst the few factors hindering the application of NIR, variance in sample particle size probably carries the most weight, especially during analysis by reflection methods. Recall equation 2.1, where diffuse reflection of an infinitely thick sample (R_∞) is dependent upon a scattering coefficient, S (Kortum, 1969). This indicates that reflection measurements may be inconsistent given variable particle size differences. In a review of NIRS applied to industry, Wetzel reported that minimizing sample particle size (to "a few micrometers in diameter") and minimizing particle size distribution produces

sufficient scattering, which will provide a good representation of the sample since more particles can interact with the beam of light (1983).

In addition to particle size affecting spectral quality, the sample thickness can also influence the intensity of signal absorption bands. Thicker samples contain a larger number of molecules along the light path which can absorb and interact with the incident energy; thus, producing stronger absorption bands. For diffuse reflectance (equation 2.1), the intensity of reflected light increases until reflectance of a sample with optical thickness (R_∞) is reached. Thicknesses can range from 1 mm to 3 cm depending on the type of samples being analyzed by reflectance methods (Burns and Ciurczak, 2008). Sample width is typically much smaller for the transmittance method of analysis.

Heterogeneous materials like milk, cheese, and other dairy products provide a unique problem to NIR due to the natural size variation and size distribution of the milkfat globule within the sample matrix (Smith et al., 1994; Laporte and Paquin, 1999). In Figure 12, incoming light is absorbed and scattered by the dairy sample during diffuse reflectance. Excessive scattering can lead to a loss of light as the penetration depth of thicker samples is increased. Considering these effects makes it necessary to use averages from repeated measurements during development of calibration data.

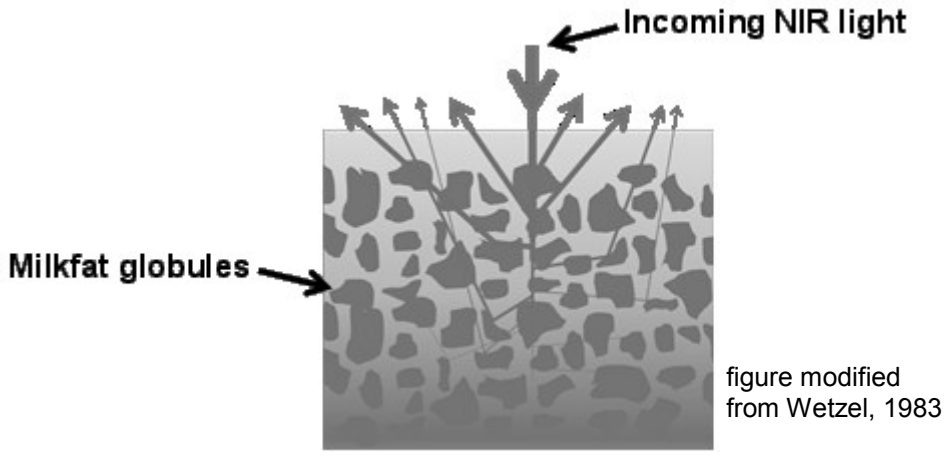

Figure 12. Excessive scattering caused by a large fat globule distribution during NIR analysis of dairy products. Light can be lost if sample thickness is too large

Water

Water is known to be a strong absorber of light in the infrared region, including that which is NIR, and NIR spectra for samples of high moisture (> 80%) are often dominated by broad water bands. This is caused by the influence of solutes like ions, organic monomers, and polymers that bind to water through hydrogen bonding (Buening-Pfaue, 2003). Since hydrogen bonds are typically very weak, the absorption of light relies more on the hydration potential of the surrounding solutes leading to shifts at both lower and higher wavelengths. While the effect of hydrogen bonding may increase the difficulty of interpretation of NIR spectra, it has not completely limited the use of NIRS in food analysis. Water was among the first properties to be measured using NIRS as it was typically measured in low moisture products like soybeans, rice and wheat flours (Downey and Byrne, 1983; Davies and Wright, 1984; Davies and Grant, 1987). The analysis of water in high moisture products like cheese became more common in the mid 1980's after Frank and Birth (1982) began a trend of analyzing composition of different cheeses by NIR methods. Subsequent studies by Weaver (1984), Frankhuizen and van

der Veen (1985), and Pierce and Wehling (1988, 1994) continued looking at moisture in cheese products among other components (i.e. protein and fat).

Sample Temperature

The effect of sample temperature on spectral variation has been well documented for the analysis of different foods by NIR (Iwamoto and Kawano, 1986, 1995; Maeda et al., 1995; Mitsumoto et al., 2000; Buening-Pfaue, 2003). Specifically, the effect of temperature on the hydrogen-bonded water structure is of interest because it causes shifts in absorption bands to either higher or lower wavelengths based on water molecules forming or breaking hydrogen bonds or undergoing changes in motion (Buening-Pfaue, 2003). The shifts also depend on the "species" of water, including: free water molecules, molecules with one group participating in hydrogen bonding, and molecules with two OH groups involved with hydrogen bonds (Iwamoto et al., 1995).

The effect of temperature on hydrogen-bonded water molecules also influences the repeatability of NIR measurements for high moisture foods. Unanticipated hydration or dehydration effects during sample handling can influence the bonding of water molecules to solutes; thus, creating changes in shifts and intensities of absorption bands in the NIR spectrum (Buening-Pfaue, 2003). To compensate for these effects, repeatability files containing spectra of the same sample measured repeatedly under different conditions are gathered and added to calculations during calibration development (Tillmann and Paul, 1998). This technique minimizes systematic differences for not only temperature, but also for different NIR instruments, which allows for transferable calibrations- a problem that has traditionally limited the expanded use of NIRS in the food industry (Westerhaus, 1990).

2.2 NIRS in the Dairy Industry

Analysis of milk and milk products has become a remarkable qualitative and quantitative application of NIRS (Pierce and Wheling, 1994; Rodriguez-Otero et al.,

1997). NIRS, along with MIRS and Raman spectroscopy, have taken the place of other time and labor exhausting chemical methods, such as Babcock and Mojonnier methods for fat analysis, Kjeldahl for protein, and high-performance liquid chromatography or polarimetry for lactose analysis (van de Voort and Ismail, 1991). Outside of these key components for milk products, there are a number of other attributes capable of being measured by NIRS. These include: measuring moisture, solids-non-fat, and fat in butter (Hermida et al., 2001) and measuring adulteration of milk by foreign fats (Sato et al., 1990) and milk powder by melamine (Lu et al., 2009). NIRS can also serve as an important analysis tool during cheese production, which will be further explored in the following section.

2.2.1 Use of NIRS in Cheese Production

The use of NIRS in the dairy industry has continued expanding with the optimization of measuring techniques, such as the fiber optic probe and solids module attachments. The fiber optic probe allows for rapid, non-destructive measurement of processed cheese samples during on-line analysis (Čurda and Kukačková, 2004), while the solids module can be used for analysis of both artisan and processed cheese samples in at-line and laboratory settings (McQueen et al., 1995; Rodriguez-Otero et al., 1995; Čurda and Kukačková, 2000). NIRS has most commonly been used to measure finished product component quantities such as moisture (Wheling and Pierce, 1988), total solids (Rodriguez-Otero et al., 1995), fat (Pierce and Wheling, 1994), protein (Rodriguez-Otero et al., 1995), and lactose (Lee et al., 1997). To understand the importance of NIRS for hard cheese manufacturers, one must first have a basic understanding of the production process. The following description of hard cheese production has been summarized from Walstra et al. (2006).

Depending on the cheese to be made, milk may be pasteurized, or at least mildly heated, to reduce spoilage organisms and improve the environment for starter cultures to grow. Once the milk has been heat treated and cooled back to a temperature more

suitable for starter bacteria to grow, cultures and adjunct bacteria are added. Ripening follows shortly after, increasing the pH and adding flavor. The enzyme rennet is added to catalyze the conversion of casein to para-casein, which coagulates in the presence of calcium ions to form the curd. The remaining milk protein is whey. The cheese product is allowed to ferment until pH = 6.4 before being cut and heated once more to assist in whey separation. The curd continues to ferment during a "cheddaring" stage until pH = 5.1-5.5. Here, whey continues separating out as the curd mats tightly together. In the last few steps of cheese production, the curd is salted, pressed into cheese blocks, aged, and packaged for selling.

The casein component in raw milk obviously plays an important role when it comes to cheese production. Using NIRS to measure the quantity of casein in the incoming milk supply would allow manufacturers to predict the amount of curd formed based on the amount of rennet added. In terms of maintaining product quality, NIRS can be used throughout many stages of cheese production. It could be used to monitor milk coagulation, degree of syneresis, cheese ripening, and to measure certain sensory properties (Sørensen and Jepsen, 1998; Downey et al., 2005; Woodcock and Fagan, 2008). However, the primary issue with using at-line or laboratory NIRS methods is that curd composition changes as soon as the sample is cut from the batch. Whey separates from the curd causing changes in moisture that can drastically affect the NIR spectral signal. Mateo and colleagues have recognized this problem and have successfully performed a trio of on-line experiments using an NIR sensor and sight glass mounted on a vat containing curd as a means of monitoring syneresis (2009). Calibration models produced promising results in this experiment, and further validation is required before it can be applied in the cheese industry (Mateo et al., 2009). Maintaining finished product quality is also key for processed cheese manufacturers as their profits are often driven by managing the variability in natural cheese. One such variable to manage is intact casein.

Many studies have examined the effect of intact casein content on the functionality of process cheese (Meyer, 1973; Shimp, 1985; Kapoor and Metzger, 2008). Recent research by Gold Peg Int. shows that even a 1% change in intact casein content can cause an 8-11% change in viscosity, firmness, and fracture stress (2012). This is significant given intact casein levels can vary from 0-18% in natural cheese ingredients, based on the level of aging (Kapoor and Metzger, 2008; Gold Peg Int., 2012). It could be possible to measure intact casein content of natural cheese products using NIRS and the attached solids module as a way for better management of process cheese quality; however, few studies supporting this application are available.

While the application of NIRS has expanded to include analysis of cheese products, there still is one major issue preventing it from being widely accepted in the dairy industry. This issue involves the fat component in milk; specifically, the large size variation of the fat globule. Recall from section 2.1.4 that particle size directly affects NIR light scattering, which can lead to poor spectral quality and inaccurate predictions for component analysis. A review of the milkfat globule and its surrounding membrane is discussed in the following section.

2.2.2 Milkfat Globule (MFG) and Milkfat Globule Membrane (MFGM)

The average size of fat globules in unhomogenized milk is 3.5 μm (Walstra, 1975; Laporte and Paquin, 1999) and the size distribution ranges from 0.1-15 μm (Wiking et al., 2004; Huppertz and Kelly, 2006). It is not unusual for cows' milk to contain $>10^{10}$ fat globules per mL (Huppertz and Kelly, 2006). Further analysis by Walstra revealed three sub-classes for the size distribution of fat molecules within milk (1969): 'small particles' (0.10-1.5 μm), making up 80% of the total number of fat particles, but only ~3% of the total weight, 'medium particles' (1.5-7.0 μm) , making up ~95% of the weight, and large particles (7-15 μm), making up just 2% of the total weight (Huppertz and Kelly, 2006). The size of fat globules has been shown to decrease within the later lactation phase (Walstra, 1969) and also as fat content is increased (Wiking et al., 2003;

Huppertz and Kelly, 2006). Because of light scattering effects, the size of fat globules is a direct problem for spectroscopic analysis of dairy products; however, a wide variation in particle size can also lead to undesired compositional changes causing more issues in analysis.

Composition of Milkfat Globule and MFGM

The milkfat globule is secreted from the epithelial cells of mammals (Jensen et al., 1991). The lipid in the globule is mainly comprised of triglycerides and is surrounded by an outer membrane composed of phospholipids, with included proteins, glycoproteins, enzymes (xanthine oxidoreductase and alkaline phosphatase). The membrane is the original membrane from the milk secretory cell, and is pinched around the lipid as it leaves the cell. An organizational view of the membrane in Figure 13 shows a bilayer structure in which a dense protein coat lies next to the inner part of the membrane (facing the triglyceride core), and a region of phospholipids forms the inner part of the membrane (Keenan and Mather, 2006; Park, 2009). In addition to providing physical stability for the fat globule, the MFGM is also responsible for many of the interactions between fat and milk serum involving highly reactive enzymes (Park, 2009). Therefore, the membrane surface area and enzymes contained within must both be considered when reviewing problems associated with the properties of dairy products.

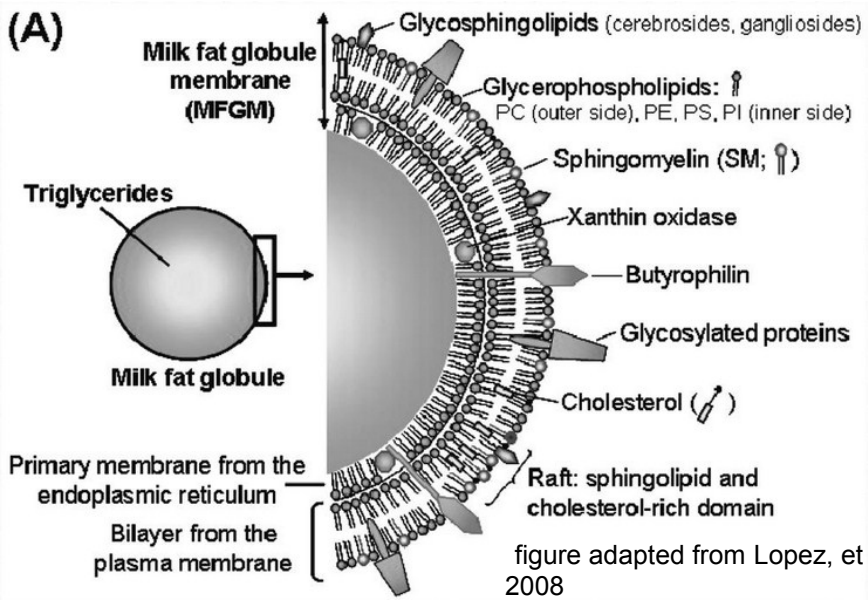

Figure 13. An illustration of the structure of the milkfat globule membrane

Partial Coalescence

An increase in fat globule size increases the amount of partial coalescence (Huppertz and Kelly, 2006). In spectroscopy, coalescence of any kind is problematic since it is represents a heterogeneous mixture, which will not result in an accurate measurement of the sample. Partial coalescence in milk can occur for a number of reasons. Among those reasons, larger fat globules tend to have larger fat crystals protruding from the MFGM. At cold temperatures, these crystals can penetrate the film between two globules, leading to the conjunction and formation of an irregularly shaped aggregate (Van Boekel and Walstra, 1981). A layer of cream is then formed at the top of milk samples since the lipids comprising it have a lower density than the surrounding milk plasma (0.994 g/mL and 1.033 g/mL, respectively) (Jensen, 1995).

Cold Agglutination

Cold agglutination represents another method of coalescence and eventual separation of fat. While there has been numerous attempts to explain how cold

agglutination is possible (Koops et al., 1966; Huppertz and Kelly, 2006), Euber and Brunner were the ones who identified three necessary components responsible for spontaneous creaming (1984): 1) a milkfat globule, 2) a component unstable in heat, such as the immunoglobulin (IgM) protein found within the globulin fraction of milk, and 3) the 'skim milk membrane' (SMM), a component that is unstable following homogenization. IgM acts as the agglutinin binding both SMM and the fat globule through carbohydrate interactions, whereas SMM, consisting of mainly lipoproteins, binds solely with IgM (Euber and Brunner, 1984). Although IgM is a more suitable binding partner, its ability to cluster fat globules is limited. According to Euber and Brunner, SMM forms a larger cluster network with fat globules by using its ability to crosslink (1984). Additionally, the ionic strength, dielectric constant, pH, and the temperature of the surrounding milk serum are some environmental factors capable of increasing the binding of IgM to fat particles and SMM (Euber and Brunner, 1984; Huppertz and Kelly, 2006).

Effect of Temperature on MFG and MFGM Composition

A temperature change in the medium surrounding fat globules can have a profound effect on the physical nature of dairy products. Cooling milk (2-4 °C) causes a release of 10-15% of phospholipids in the MFGM, which are then absorbed by the plasma portion (Koops and Tarassuk, 1959; Patton et al., 1980; Huppertz and Kelly, 2006). It also causes a release of xanthine oxidoreductase and copper from the membrane to the plasma region (Mulder and Walstra, 1974). All of these factors contribute to a change in milk composition and onset of rancidity as reported by King (1962) and others (Richardson and Guss, 1965). Freezing and subsequent thawing can lead to excessive clumping in dairy cream products (Mulder and Walstra, 1974), but this physical effect is not as prominent in milk that was stored frozen for three months. There is, however, a noticeable increase in rancidity, which indicates that a change in the MFGM composition has occurred (Babcock et al., 1946).

Heating dairy products also affects the physical and chemical properties. The quantity of protein attached to casein micelles and fat globules increases as whey protein, specifically β-lactoglobulin (Corredig and Dalgleish, 1998), gets denatured and attaches to K-casein and the MFGM through a disulfide bond (Houlihan et al., 1992; Lee and Sherbon, 2002). The added whey protein results in fat globules becoming more involved in acid and enzyme mediated coagulation processes, which affects the rheological properties of fermented milk and cheese products (Lee and Sherbon, 2002). Further studies have shown even minimal heating (45-50 °C) leads to ~50% of total protein loss from the MFGM, which could be a result of the lipid core melting and rearranging (Ye et al., 2002). For raw milk, heating decreases the rate of cream formation since the added protein on the membrane caused an increase in the density of the fat globule complex, thus, preventing it from rising to the plasma surface (Huppertz and Kelly, 2006). Even though this coagulation may not always be visible, it is important to remember that even slight changes in sample composition will have an impact on spectroscopic measurements.

2.2.3 Effect of Homogenization on Milk Composition

The MFGM is fairly inefficient when it comes to protecting the particle against mechanical forces such as shearing, cavitation, or turbulence. The effects on MFG composition are especially profound if the lipid is in a liquid state, as it is prior to homogenization where the liquid milk is often pre-warmed (≥ 40 °C). Homogenization is a technique often utilized in the dairy industry to reduce MFG particle size as a method for delaying the separation of the cream layer during shelf life in products. The creaming delay is observed as a result of a reduction in fat globule size and adsorption of various proteins on the MFG surface, which leads to a denser particle that resists rising (Fox et al., 1960; Walstra,1975; Huppertz and Kelly, 2006). The molecule also resists spontaneous creaming since the fat crystals within the globule are smaller, and resists cold agglutination since the active component that is unstable following homogenization has been disrupted. During standard homogenization processing, liquid milk is forced

through a small orifice at pressures between 10-20 MPa, which stresses fat globules to a point where they split into smaller particles with diameters 0.75-0.85 μm (Laporte and Paquin, 1999; IDF, 2000). Valve homogenizers, particularly those with multiple valves, are among the most commonly used in the dairy industry (Figure 14). Walstra was among the first to have identified key factors that influence the extent of particle size reduction in homogenization (1975). Among these, the pressure exerted on the sample and the design of the equipment used during homogenization likely has the greatest influence.

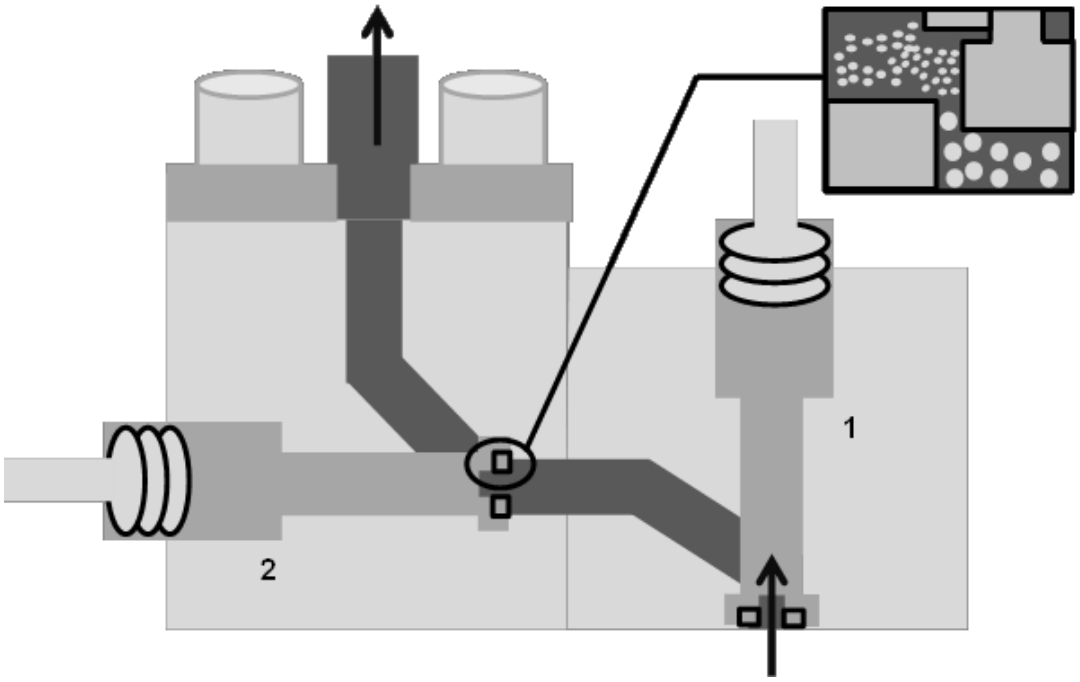

Figure 14. An internal diagram of the operation of a standard two-stage homogenizer showing the first (**1**) and second (**2**) stages

High Pressure Homogenizers

Advances in homogenization technology for improved efficiency include high pressure valve homogenizers (100-300 MPa), microfluidizers, ultrasonicators, and

compact tissue homogenizers. High pressure homogenizers operate mostly in the same way as regular valve homogenizers, but with higher pressures being applied. An intensifier powered by a hydraulic pump, as described by Thiebaud and colleagues, is added to the stream to increase the flow rate of the milk as it passes through a single high pressure ceramic valve assembly (Thiebaud et al., 2003). High pressure treatments can significantly reduce microbial populations, modify milk coagulation properties, and create a more narrow size distribution for fat particles compared to standard pressure homogenization (Thiebaud et al., 2003; Hayes et al., 2005).

Microfluidization

Microfluidizers serve as another method for high pressure homogenization. In this process, liquid milk gets split into two streams by different microchannels and is recombined in a chamber when the two jet streams slam into one another causing disruption of MFG by cavitation (Paquin, 1999). The pressure in this system can range between 100-300 MPa and is solely controlled by the equipment design (Paquin, 1999). Signficant changes in MFG and MFGM composition occur when using this high of pressure, leading to reduced particle sizes and very narrow size distributions. However, the cost of Microfluidizers reflect their high efficiency. A recent internet search of used benchtop homogenizers for dairy applications showed that prices range from $10,000-$32,000.

Sonication

Ultrasonication has been identified as another "emerging technology" used to increase the efficiency of processing, provide safer foods, increase shelf stability, and to improve food functionality, especially in products like milk (Butz and Taucher, 2002; Ashokkumar et al., 2010). It operates on the basis of cavitation of microbubbles, i.e. fat globules in milk, caused by strong internal vibrations produced from ultrasonic waves (Ashokkumar et al., 2010). Ultrasound, which describes sound waves above detected at high frequencies by people (>18 kHz), is transmitted to microbubbles causing them to

repeatedly grow and collapse until they exceed their cavitation limit and can no longer grow. Sonication has proven to be a useful tool in reducing particle sizes in whey protein concentrate solutions (Ashokkumar et al., 2010) as well as reducing fat globule size and size distribution in liquid milk (Ertugay et al., 2004). Although sonicators have proven to be efficient, the emittance of ultrasound creates an ear-piercing, high-pitched sound that is discomforting to anyone in the nearby lab space. Water-resistant, see-through sound enclosures are available to limit this problem (Qsonica, 2012). The cost of benchtop sonicators depends on their power capabiltiies and attachments, but most fall in the $1,500-$6,000 range, which contributes to their increasingly popular use in food systems (Fisher Scientific, 2012).

High Shear Tissue Homogenization

Recent advances in homogenization technology have focused more on cost efficieny by using high-speed mixing to create emulsions. One of these high speed options is an Ultra-Turrax® tissue homogenizer tube disperser, which sells for $500-$800 (IKA, 2012). This homogenization method utilizes a rotor-stator design to create flow and maximize shear in the sample without compromising the sample due to heat degradation (IKA, 2012). The extremely compact tube disperser has a variable speed setting (300-6,000 rpm), quiet noise output (50 dB), and can achieve significant particle shearing in less than five minutes (Ding and Shah, 2009; IKA, 2012). Its application has primarily been in academia as a tool for reducing the size of encapsulated probacteria and in emulsion science for creating solutions of animal feed and peanut butter (Sharman and Gilbert, 1991), apples (Hercegova et al., 2005), and even blue cheese (Pataky and Schoenfuss, 2012). Overall, the use of Ultra-Turrax® tube disperser for particle size reduction in dairy products has not been studied, but due to its lower cost and ease of use compared to other homogenization techniques, there is little doubt for expanded use of the tube disperser in the future.

Evaluating Homogenization Efficiency by Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a way of measuring particle size distribution of emulsions, colloids, polymers, proteins, nanoparticles, and micelles (Berne and Pecora, 1976). The operating principle of DLS relies on fluctuations in scattered light as a result of a laser beam interacting with a sample. As seen in Figure 15, light is scattered at a variety of angles, θ, by differently sized sample particles and captured by surrounding photon detectors. Particle size analyzers, such as the Mastersizer® (Malvern Instruments), take "snap-shots" of the scattering pattern at a moment in time while the sample is flowing through the instrument. Multiple snap-shots (~2,000) are taken very quickly and averaged to get a good representation of the overall sample (Malvern Instruments, 1997). Once the measurement sequence is complete, the spectral data is interpreted in terms of particle size distribution using either the Fraunhofer approximation or Mie theory.

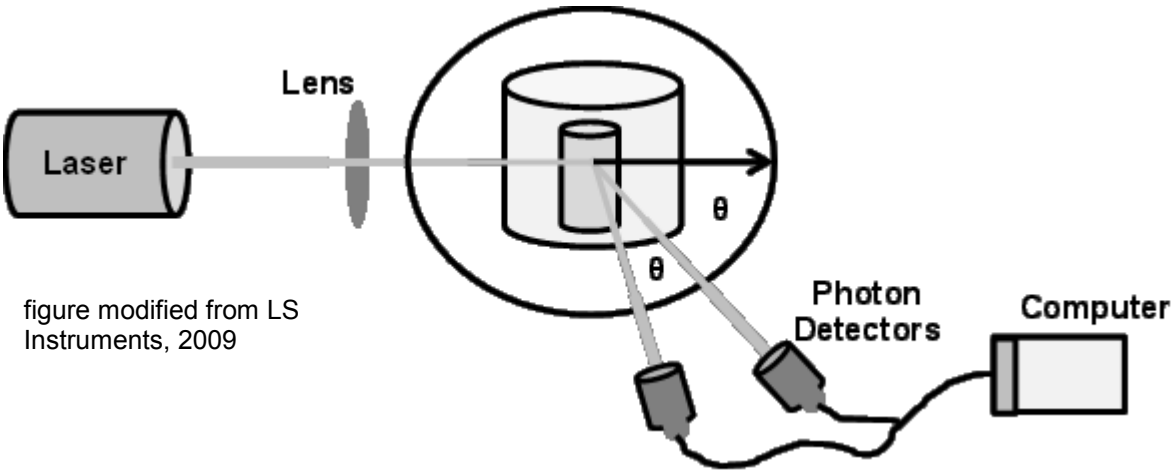

Figure 15. The internal diagram of a DLS instrument showing the basic operation. Light is scattered at different angles, θ, and gathered by photon detectors surrounding the sample

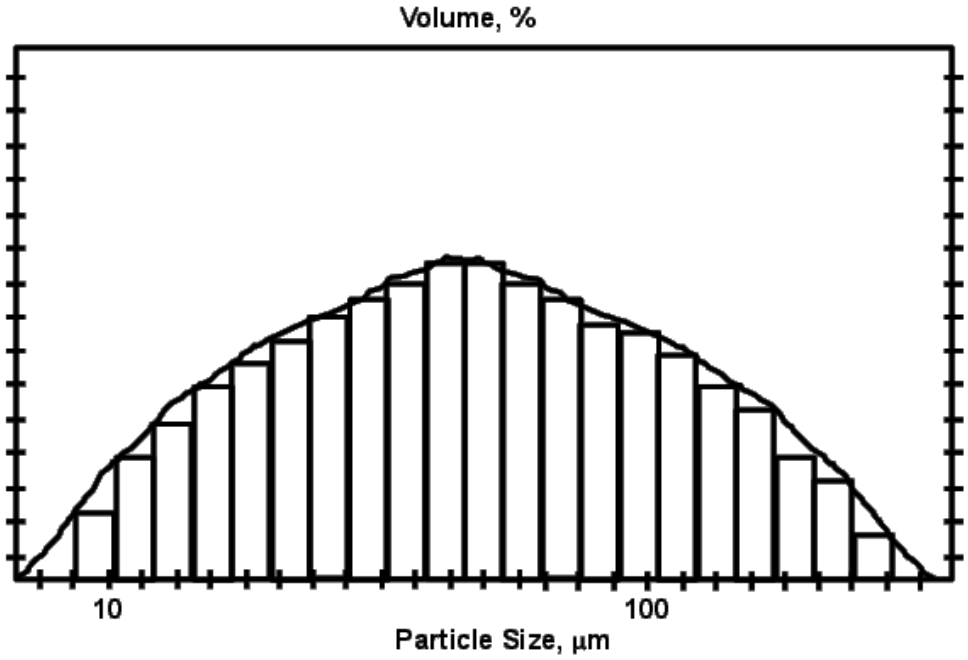

Figure 16. Reflected light patterns in DLS are converted into a distribution of particle sizes viewed as a histogram or smoothed curve.

The Fraunhofer approximation considers the particle shape as a solid, opaque disk whose size is known. The primary downfall of this technique is its limited ability to fully describe the nature of the materials to be analyzed. It often over-estimates and under-estimates the scattering efficiency of very small particles, resulting in a shift of the average article size distribution (Malvern Instruments, 1997; Vargas-Ubera et al., 2007). In contrast, the Mie theory is more accurate as it can predict scattering patterns based on spherical particles (Vargas-Ubera et al., 2007). It also considers light passing through certain particles and the possible adsorption of some light (Malvern Instruments, 1997). Although more accurate, the Mie theory requires the user to assume information about certain characteristics such as the particle and surrounding medium refractive indexes. The refractive index (RI) is a number that describes how light moves through and reflects off certain materials. It is dependent on both the solvent and particle in solution. Milk, for example, can be described as milk fat (RI: 1.46) in water (RI: 1.33) (Malvern

Instruments, 1997). The RI for water is used since it makes up the majority of milk composition (~87%). The reason fat is used instead of a protein component is because of the greater size of fat globules, which scatter light more strongly. This is especially true for raw milk, which has a yellow tint because of the carotene component in the large fat particle (Mulder and Walstra, 1974). Most particle size instruments can then use the refractive indexes in combination with analysis models (i.e. polydisperse, multimodal, monomodal) to interpret the reflected light patterns as a distribution of particle sizes as seen in Figure 16.

Homogenization Cluster Formation

Homogenization may be capable of delaying complete fat separation, but there will still be the natural tendency for globules to cluster and partially coalesce. The formation of homogenization clusters occurs when casein micelles attach to globules as a result of there being not enough MFGM components to cover the additional globule surface area created during processing (Darling and Butcher, 1978; Lee and Sherbon, 2002). Once attached, the casein molecule acts as a crosslinking agent by joining onto other fat globules with available surface area (Ogden et al., 1976; Darling and Butcher, 1978). These homogenized milk clusters have an effect on heat coagulation and rennet coagulation, which negatively affects the stability of whole milk and cheese production, respectively (Sweetsur and Muir, 1983; Huppertz and Kelly, 2006). While these effects can be minimized by multiple passes through homogenization systems or by using multiple valve assemblies, they are sometimes desired for their acid-coagulation properties in products like yogurt. The organization of milk components, both before and after homogenization, can drastically effect dairy product production; thus, analysis of incoming milk by NIRS offers a rapid means for not only determining milk quality, but also for predicting end-product quality.

Homogenization and Infrared Analysis

Knowing how homogenization affects particle size and size variation has played an important role in improving infrared analysis of milk components. Barbano and Clark (1989) confirmed the need for efficient homogenization prior to infrared analysis as a way to increase the accuracy of results by decreasing the effect of light scattering caused by large fat globules. This study was based on results from Grappin and Jeunet (1976) and van de Voort (1980) who found that decreasing MFG diameter below 3 μm and 2 μm, respectively, limited the amount of light scattering. Furthermore, Remillard and colleagues (1993) demonstrated that calibrations developed using mid-infrared analysis of non-homogenized raw milk were not adequate for fat analysis (SEP = 0.13) of homogenized milk due to variations in fat globule size. As dairy research began to shift towards using NIRS for analysis of milk components in the late 1990's, there was still a focus on the relationship between homogenization and improved calibration accuracy. These results have been summarized in Table 1, and further discussed in the following paragraph.

Laporte and Paquin (1999) considered homogenized milk and unhomogenized milk during transmittance analysis of fat, protein, and casein. While there was an improvement in calibrations for nitrogeneous components when both types of samples were considered, analysis of fat became less accurate. This was explained by the large variation in fat globule size in the sample set, which led to spectral variation. Subsequent studies involving in-line analysis of unhomogenized milk samples showed that accurate predictions of sample components are possible (Schmilovitch et al., 2000), especially if certain spectral regions and sample thicknesses are considered during measurement (Tsenkova et al., 1999; Woo et al., 2002). The research focus of in-line analysis by NIRS has continued to the present day, but with the addition of combined analysis of short wave NIR with the visible region (400 - 1000 nm) (Bogomolov et al., 2012). Additionally, a recent study involving a newly designed in-line NIR measuring tool was

used for fat, protein, lactose, and urea analysis by diffuse reflectance (Melfsen et al., 2012). Results from both reports were promising.

Table 1. Standard error of prediction (SEP) and cross-validation (SECV) values for components in raw milk obtained in recent studies using NIRS

	SEP (%)		SEP (%)		SECV (%)
Author, Year	[1]Laporte and Paquin, 1999	[2]Melfsen et al., 2012	[1,3]Bogomolov et al., 2012	[1]Woo et al., 2002	[1]Tsenkova et al., 1999
	Unhomogenized/ Homogenized		Unhomogenized		
Fat	0.05/0.07	0.09	0.05	0.06	0.11
Protein	0.09/0.06	0.05	0.03	0.10	0.10
Lactose	[4]-	0.06	-	0.10	0.08
Casein	0.07/0.06	-	-	-	-
Urea	-	0.18	-	-	-

[1]analysis performed using transmittance method
[2]analysis performed using diffuse reflectance method
[3]study combined short wave NIR with the visible region
[4]component was not measured

Components in unhomogenized milk have been predicted more accurately lately; however, the models for these predictions are based on manipulative mathematical transformations and corrections applied to the original dataset as a way to minimize unwanted effects such as excessive light scattering caused by large fat globules. The problem arises when datasets become nonsensical as a result of excessive pre-processing, which is why various homogenization methods still need to be considered as practical solutions to make NIRS a more viable option for analysis of dairy products.

3 Reducing Milkfat Particle Size by Homogenization

3.1 Introduction

Evaluation of raw milk for components such as fat, protein, and casein is important during cheese manufacturing and when processing other dairy products. In cheese-making, standardization of milk on the basis of protein, especially the casein proteins, is important for ensuring consistent finished product quality and for predicting cheese yields. High quality and robust analytical procedures are critical for the successful identification of components and their quantities, and while sample analysis is often most emphasized when it comes to the analytical method, there exist three other important steps: sampling, sample preservation, and sample preparation (Mitra and Brukh, 2003; Luthria, 2006). Sample preparation is particularly important during analysis of dairy products because it can be used to reduce potential interferences caused by the milkfat globule (MFG) during spectroscopic measurement.

Homogenization is one important method of sample preparation that has been used by the dairy industry to prepare samples for infrared analysis. Knowing how homogenization affects particle size and size variation has played an important role in improving infrared analysis of milk components. Barbano and Clark (1989) confirmed the need for efficient homogenization prior to mid-infrared analysis as a way to increase the accuracy of results by decreasing the effect of light scattering caused by large fat globules. Homogenization is often used because it can reduce MFG particle size from 3.5 μm (Walstra, 1975; Laporte and Paquin, 1999) to 0.75-0.85 μm (Laporte and Paquin, 1999; IDF, 2000). Additionally, homogenization reduces the range of MFG size distribution.

During standard homogenization, liquid milk is forced through a small orifice at pressures between 10-20 MPa, which stresses fat globules to a point where they split into smaller particles. Valve homogenizers, particularly those with multiple valves, are among the most commonly used by dairy manufacturers (Figure 17). Small, two-stage

valve homogenizers are included in the design of mid-infrared analyzers and are recognized by the International Dairy Federation (IDF) as a standard procedure for dairy product analysis (2000). Walstra was among the first to have identified key factors that influence the extent of particle size reduction in homogenization; among these, the pressure exerted on the sample and the design of the equipment used during homogenization likely has the greatest influence (Walstra, 1975).

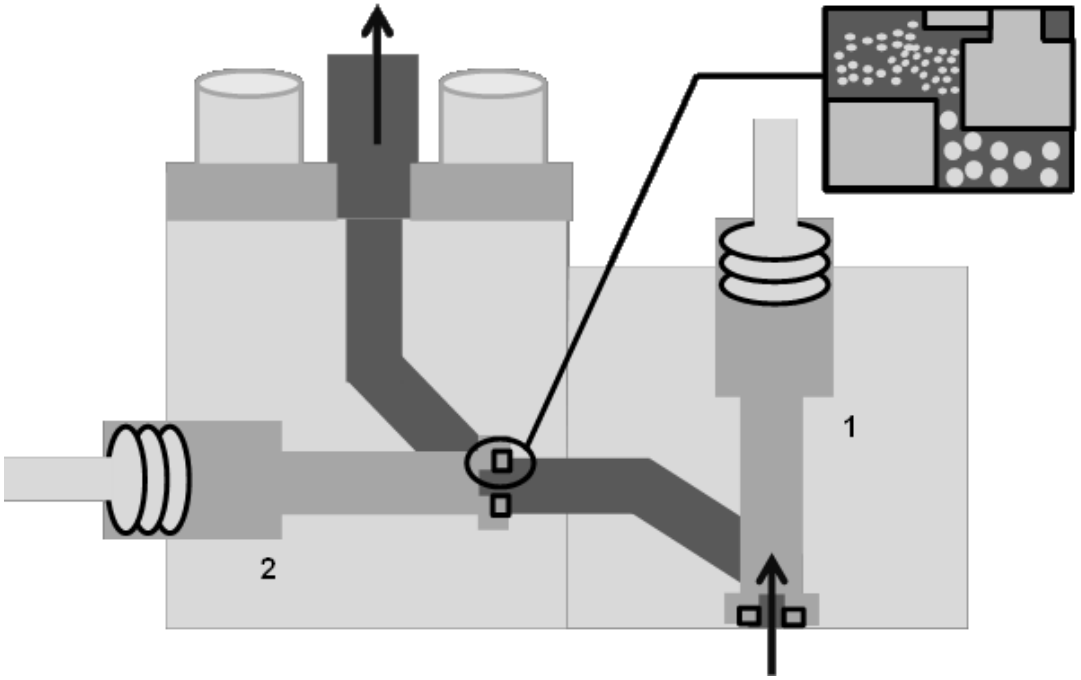

Figure 17. An internal diagram of the operation of a standard two-stage homogenizer showing the first (1) and second (2) stages

Although standard valve homogenizers may be efficient in reducing MFG size, they are impractical for use as a sample preparation step during the analytical procedure because they are expensive and require regular maintenance to maintain efficiency (Barbano and Clark, 1989). Recent advances in homogenization technology have focused more on cost efficiency and include techniques utilizing ultrasonic waves and high-speed mixing to create stabilized emulsions. If these alternative methods could be

optimized to reduce MFG sizes in raw milk, they could be incorporated as a sample preparations step prior to dairy product analysis

Ultrasonication has been adapted for use in the food industry to increase processing efficiency, provide safer foods, increase shelf stability, and improve food functionality, especially in products like milk (Butz and Taucher, 2002; Ashokkumar et al., 2010). It operates on the basis of cavitation of microbubbles, i.e. fat globules in milk, caused by strong internal vibrations produced from ultrasonic waves (Ashokkumar et al., 2010). Ultrasound waves are transmitted to microbubbles causing them to repeatedly grow and collapse until they exceed their cavitation limit and can no longer grow. Sonication has proven to be a useful tool in reducing particle sizes in whey protein concentrate solutions (Ashokkumar et al., 2010) as well as in reducing fat globule size and size distribution in liquid milk (Ertugay et al., 2004). The cost of benchtop sonicators also makes them favorable for use in sample preparation since most fall in the $1,500- $6,000 range, depending on the instrument's power capabilities and attachments (Fisher Scientific, 2012).

Another alternative method to be explored for sample preparation is the Ultra-Turrax tube disperser (IKA Works, Wilmington, NC). This high-seed mixer utilizes a rotor-stator design to create flow and maximize shear in the sample without compromising it due to heat degradation (IKA, 2012). The extremely compact Ultra-Turrax tube disperser has a variable speed setting (300-6,000 rpm), quiet noise output (50 decibel, dB), low cost ($500-$800) (IKA, 2012), and can achieve significant particle shearing in less than five minutes (Ding and Shah, 2009). Its application has primarily been in academia as a tool for reducing the size of encapsulated probacteria and in emulsion science for creating solutions of animal feed and peanut butter (Sharman and Gilbert, 1991), apples (Hercegova et al., 2005), and even blue cheese (Pataky and Schoenfuss, 2012). Overall, the use of the Ultra-Turrax tube disperser as a sample preparation step for dairy product analysis has not been studied, but because of the need

for robust and high quality analytical procedures, there could be an opportunity for expanded application of this method.

In the current study, laboratory particle size reduction methods were optimized for sample preparation. Specifically, two-stage valve homogenization, sonication, and the Ultra-Turrax tube disperser were compared based on their efficiency in reducing average particle size and size distribution. The processing time and sample temperature were also considered. The objective for this research was to evaluate whether sufficient particle size and size distribution could be achieved using cost-efficient sonication and tube dispersion methods. It is anticipated that this research will assist dairy manufacturers with sample preparation during ingredient and finished product analysis to increase production efficiency.

3.2 Materials and Methods

While the materials and methods have been described below, the expanded methods, including those describing the operation of the homogenization processes and particle size analyzer are in section A.1.1 of the Appendix.

3.2.1 Materials

Raw bovine skim milk (Skim) (DQCI, Mounds View, MN) and raw bovine whole milk obtained from stock at the University of Minnesota dairy barn (UMN) (UMN, Falcon Heights, MN), Autumnwood Farm (Atmwd) (Forest Lake, MN), and Arthurst Farm (BrnSws) (Plato, MN) was used for a portion of sampling. Raw bovine whole milk (UMN, Falcon Heights, MN) supplemented at different levels with both 70% milk protein concentrate (MPC) (Idaho Milk Products, Jerome, ID) and whole milk powder (WMP) (Nestlé, Vevey, Switzerland) was also used as samples.

3.2.2 Experimental Design and Preparation of Raw Materials

The study was designed as a randomized complete block to be repeated for each type of milk sampled. The randomized complete block design has four fixed

homogenization treatments (Ultra-Turrax tube dispersion, sonication, two-stage homogenization and no homogenization) and two response variables (particle size diameter and particle size distribution) measured immediately following homogenization.

Preparation of Raw Whole Milk

Raw whole milk samples were collected fresh daily and stored at 4 °C until preparations for homogenization began. For tube dispersion, two-stage homogenization, and no homogenization, samples were removed from refrigeration 30 min prior to treatment application and heated to 38 °C using a water bath. Samples designated for sonication were kept at 4 °C until treatment application.

Preparation of Raw Skim Milk

Raw whole milk (DQCI, Mounds View, MN) was separated into cream and skim milk in the Joseph J. Warthesen Food Processing Center at the University of Minnesota (St. Paul, MN) using a Westfalia Milk Separator MP 1254 (Centrico, INC, Englewood, New Jersey). Immediately following separation, skim milk samples were stored at -30 °C for 14 days until analysis could occur. Samples were thawed using a warm water bath and stored (4 °C) prior to homogenization. Samples were prepared for homogenization similarly to raw whole milk samples.

Preparation of High Fat Raw Whole Milk Samples

Dry whole milk fortified with iron, zinc sulfate, and vitamins A, C, and D_3 (Nestlé, Vevey, Switzerland) was added to heated (38 °C) raw whole milk (UMN, Falcon Heights, MN) in quantities that would increase the original level of fat by 40%. The supplemented milk was then gently shaken for 2 min and prepared for homogenization similarly to raw whole and skim milk samples.

Preparation of Protein Supplemented Raw Whole Milk

Milk protein concentrate 70% (Idaho Milk Products, Jerome, ID) was added to heated (38 °C) raw whole milk (UMN, Falcon Heights, MN) in quantities that would increase the original level of protein by 150%. The protein supplemented milk was gently shaken for 2 min and prepared for homogenization similarly to raw whole and skim milk samples.

3.2.3 Preliminary Experiments to Determine Homogenization Conditions

Ultra-Turrax tube disperser

Six preliminary trials took place to determine optimum homogenization conditions (sample temperature during processing, Ultra-Turrax tube disperser speed setting, and time of mixing) based on maximum particle size reduction in raw whole milk samples. For the Ultra-Turrax tube disperser preliminary trials (Figure 17), there was a 5-7 °C rise in temperature, which would not have been enough to affect the molecular composition of milk samples. Early trials indicated a rise in particle size diameter with increasing mixing time at constant speed (2,100 rpm), which was measured using a digital tachometer. Milk for these trials had been stored at 4 °C prior to homogenization, thus, producing ideal conditions for milk fat agglomeration during mixing, which explained the rise in particle size diameter with increased duration of mixing. To overcome this issue, samples were pre-heated to 38 °C to ensure the milk fat was entirely melted. Additionally, the Ultra-Turrax tube disperser speed setting was adjusted to 5,100 rpm to increase the shearing effect on the MFG membrane. The mixing time selected was 3.5 min based on preliminary trials (Figure 18) showing a rapid decrease in particle size during the first 2 min and a more gradual decline during the last 1.5 min. Particle sizes were not effectively reduced after 3.5 min.

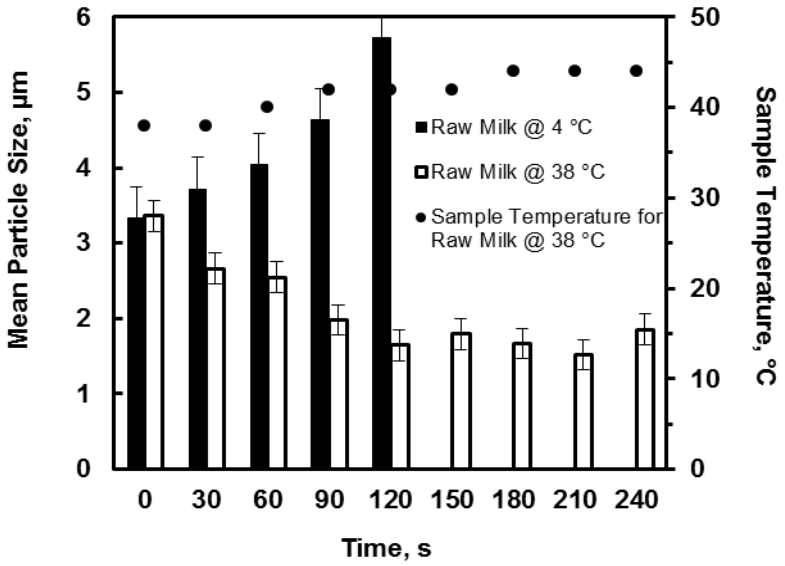

Figure 18. Mean particle size and sample temperature as a function of timed Ultra-Turrax tube dispersion for raw whole milk samples at 4 °C and 38 °C. Sample temperature was initially 38 °C

Sonication

Five preliminary trials were performed to determine the optimum sonication conditions (sample temperature during processing and time of sonication) resulting in the greatest particle size reduction for raw whole milk samples (25 mL). Initial selections of the amplitude setting (65%) and microtip probe size (0.125 in) were based on prior experience. During sonication, there was a ~35 °C rise in temperature, which is capable of disrupting molecular composition of milk samples. To avoid this disruption, samples were kept in an ice bath throughout sonication; however, a reduction in particle size diameter was not observed with increasing sonicating time. Particle size diameter only decreased significantly with increasing sample temperatures. To compromise, a cold water bath was used instead to keep the temperature of the sample from increasing above 38 °C. The sonicating time selected was 12 min based on preliminary trials showing a steady decrease in particle size through 10 min (Figure 19). After 12 min of sonicating

samples in a cold water bath, temperatures began exceeding 38 °C and no further decrease in particle size diameters was found.

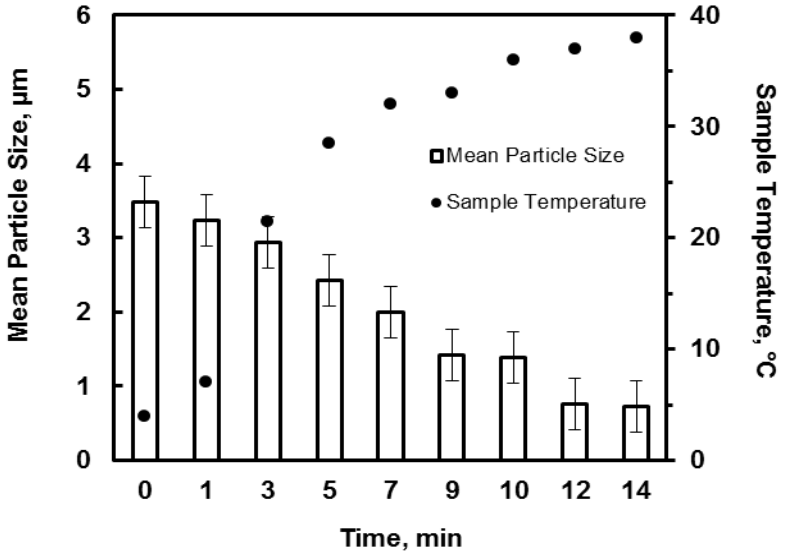

Figure 19. Mean particle size and sample temperature as a function of timed sonication for raw whole milk samples. Sample temperature was initially 4 °C

Two-Stage Valve Homogenization

Six preliminary trials were needed to ensure an acceptable reduction in raw whole milk particle size could be achieved using a two-stage laboratory valve homogenizer. Initial pressure settings, which are typical for large scale valve homogenizers, were selected for the first stage (10.3 MPa) and second stage (3.4 MPa) homogenizing valves. Average raw whole milk particle size was not effectively reduced at these pressures (Figure 20). For this reason, the first and second stage valves were adjusted (16.5 MPa and 6.2 MPa, respectively) resulting in particle sizes that were smaller in Figure 20, yet not quite in the particle size range of industrial processed whole milk (0.75-0.85 µm) (Laporte and Paquin, 1999; IDF, 2000) due to the inefficiency of our homogenizer

compared to other studies. Additionally, the outlet temperature of milk samples was monitored throughout processing and increased approximately 5 °C.

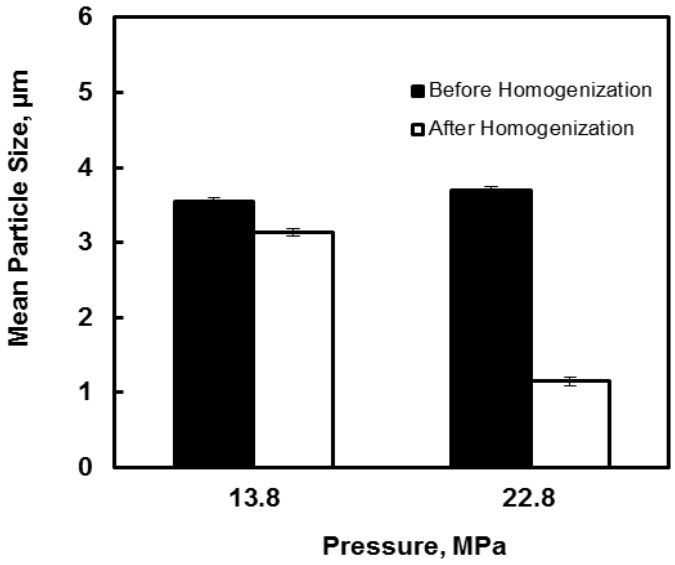

Figure 20. Mean particle size as a function of pressure in two-stage valve homogenization of raw whole milk before and after processing

3.2.4 Methods

Ultra-Turrax tube disperser

Aliquots of milk (15 mL) in centrifuge tubes were removed from a water bath (38 °C) and gently shaken prior to being poured into DT-20 mL tubes with rotor-stator elements (IKA Works, INC, Wilmington, NC). Tissue homogenization (2,100 RPM) occurred in 1 min intervals on an Ultra-Turrax tube disperser workstation (IKA Works, INC, Wilmington, NC) for 3.5 min. After each minute of mixing, milk samples were poured between the rotor-stator tubes and the original centrifuge tubes to ensure thorough mixing. Upon completion of tissue homogenization, milk samples were returned to their original centrifuge tubes and placed in a water bath (38 °C).

Sonication

Aliquots of milk (25 mL) in centrifuge tubes were removed from refrigeration (4 °C) and gently shaken prior to sonication in time mode using a Model 150E Ultrasonic Dismembrator (Fisher Scientific, Pittsburg, PA) with a 0.125 in diameter tapered microtip probe (EDP No. 109-122-1065) at 65% amplitude for 12 min. Sonication occurred while samples were in their respective centrifuge tubes and submersed in a cold water bath to ensure sample temperatures of ≤40 °C. Following sonication, milk samples were returned to storage at 4 °C.

Two-Stage Homogenization

Samples containing approximately 500 mL of heated raw milk (50-55 °C) were homogenized using a standard two-stage NS 1001L2K Panda- Laboratory Homogenizer (GEA Process Engineering INC, Hudson, WI) operating at 22.8 MPa (1^{st} Stage: 16.5 MPa, 2^{nd} Stage: 6.2 MPa). Homogenized samples were allowed to cool to room temperature until particle size analysis occurred

3.2.5 Particle Size Diameter and Distribution

All milk samples were kept in centrifuge tubes at room temperature for 30 min prior to particle size analysis. Particles were examined by laser light scattering using a Mastersizer S (Malvern Instruments, Malvern, UK) with a He/Ne laser (633 nm), range lens (300 mm), and an active beam length of 2.4 mm. Milk (refractive index, RI: 1.46) was dispersed in distilled water (RI: 1.33) and pumped through the particle size analyzer using a Small Volume Sample Dispersion Unit 2023 (Malvern Instruments, Malvern, UK). Malvern Instruments software uses the Mie theory to produce a polydisperse model displaying particle size distribution and the average diameter size.

3.2.6 Statistical Analysis

Responses for average particle size diameter and size distribution were analyzed to assess the effects of the homogenization methods by conducting a one-way analysis of

variance (ANOVA) for each lot of milk using RStudio version 0.95.262 (RStudio, Boston, MA). Based on these ANOVAs, pairwise comparisons were presented using Fisher's least significant difference testing (R Development Core Team, 2012).

3.3 Results and Discussion

The results and discussion are described in this section. The expanded results, including statistical analysis and supplementary tables are in section A.1.2 of the Appendix.

3.3.1 Particle Size

All homogenization methods had a significant effect on particle size for all lots as illustrated in Figure 21 and Table 2. Particle size was most effectively reduced using sonication, and the range of particle sizes achieved after sonication was similar to other studies for industrial processed milk (Laporte and Paquin, 1999; IDF, 2000). In contrast, the Ultra-Turrax tube disperser was not as effective, but was still capable of reducing particle size by ~50% for most lots.

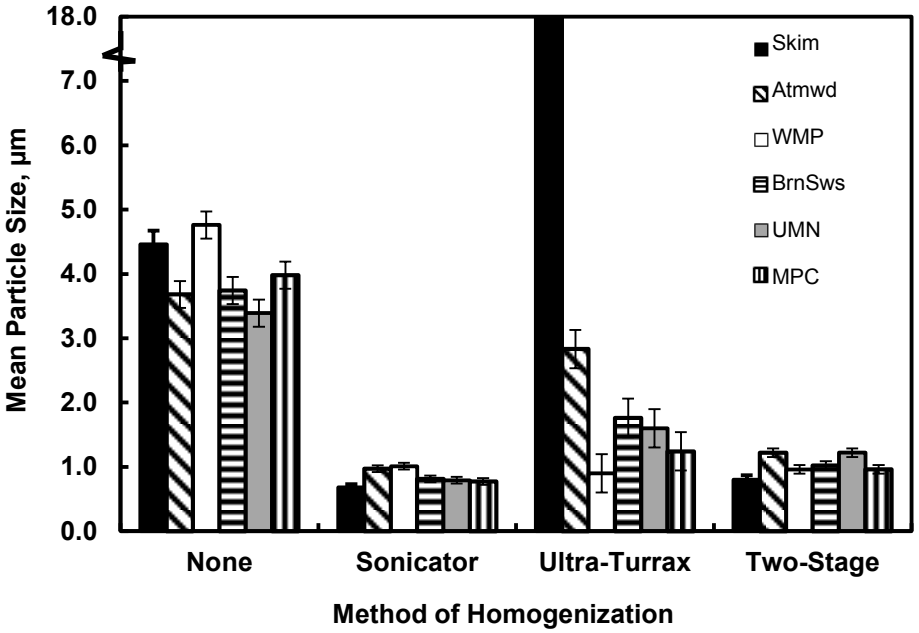

Figure 21. Mean particle sizes of raw milk after four homogenization treatments. Measurements included milk samples (n=24) from each of six lots: skim milk (Skim), raw milk from Autumnwood Farm (Atmwd), raw milk fortified with whole milk powder (WMP), raw milk from Arthurst Farm (BrnSws), raw milk from the University of Minnesota dairy (UMN), raw milk fortified with milk protein concentrate (MPC)

Table 2. Mean particle sizes of raw milk for each homogenization treatment assessed by conducting a standard analysis of variance using pairwise comparison, and corrected with Fisher's LSD tests for differences. Responses for each lot were analyzed independently from one another

	Milk Lot						
[1]Method	[2]Skim	[3]Atmwd	[4]WMP	[5]BrnSws	[6]UMN	[7]MPC	SD among sample replicates (n=24)
None	4.46[a]	3.68[a]	4.76[a]	3.74[a]	3.39[a]	3.98[a]	0.52
Sonicator	0.68[b]	0.97[b]	1.01[b]	0.81[b]	0.79[b]	0.77[b]	0.13
Ultra-Turrax	17.46[c]	2.83[c]	0.90[b]	1.76[c]	1.60[c]	1.24[c]	0.73
Two-Stage	0.80[b]	1.22[d]	0.96[b]	1.02[d]	1.22[d]	0.96[b]	0.16

[1]Method = Method of Homogenization
[2]Skim = Skim milk
[3]Atmwd = Raw milk from Autumwood Farm
[4]WMP = Raw milk fortified with whole milk powder
[5]BrnSws = Raw milk from Arthurst Farm
[6]UMN = Raw milk from University of Minnesota Dairy
[7]MPC = Raw milk fortified with milk protein concentrate
[a-d]Treatments with different letters differ significantly within columns ($p < 0.05$)

The observed results were likely caused by multiple mechanical forces acting on the MFG during two of the more effective methods, sonication and two-stage homogenization. Pressure gradients in both of these systems caused cavitation of particles while agitation also would have led to a size reduction through particle shearing. In contrast, the Ultra-Turrax tube disperser did not contain any pressure changes and only would have reduced MFG size by shearing. Particle size reduction could have also been affected by an increase in sample temperature during processing, which would have changed homogenization efficiency. Sample temperature increased from 4 °C to 38 °C during sonication (Figure 19) because of mechanical oscillations causing friction within the liquid. For two-stage valve homogenization, raw milk samples were pre-heated to a higher temperature (50 °C) compared to other methods, which could have increased the

efficiency of homogenization. Samples for the Ultra-Turrax tube disperser were also pre-heated (38 °C), but only a slight increase in temperature (5 °C) occurred during processing (Figure 18). These results have been summarized in Table 3.

Table 3. A summary of processing factors and four methods of homogenization affecting mean particle size reduction in raw milk

[1]Method	Mechanical Forces	Sample Temperature (°C) (pre-processing)	[2]Sample ΔT (°C)	[3]Particle Size Reduction (%)
Sonicator	Cavitation, Shearing	4	35	79
Ultra-Turrax	Shearing	38	6	32
Two-Stage	Cavitation, Shearing	50	5	74

[1]Method = Method of Homogenization
[2]Change in temperature measured after processing
[3] Average particle size reduction values considering all lots

In skim milk processed by the Ultra-Turrax tube disperser, the average particle size was much larger (~18.0 μm) than before the samples were processed (~4.5 μm). Since the majority of fat had been removed from samples during the separation process, the larger particles observed could have been protein aggregates that formed as a result of freezing the skim milk samples or more likely after tube dispersion had damaged the protein surface. This aggregation would have led to samples containing a large size distribution, which is seen in the illustration for particle size distribution (Figure 22).

3.3.2 Particle Size Distribution

Similar to the results for mean particle size, sonication was the most effective method of homogenization for reducing the distribution of particle sizes (Figure 22). Although the decrease in size distribution was not as extreme compared to the reductions in particle size, the effect of homogenization on all lots was still significant (Table 4).

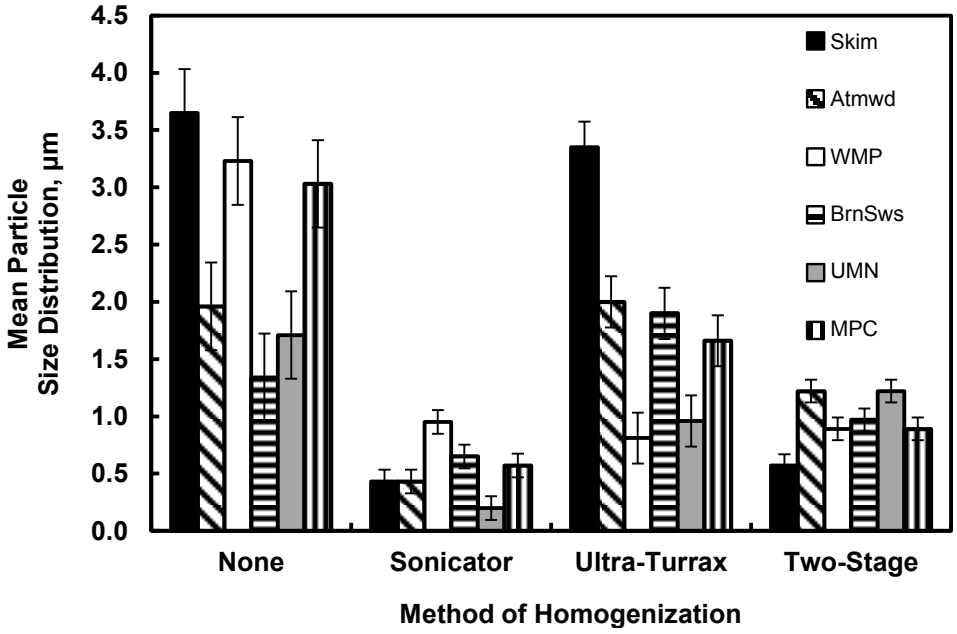

Figure 22. Mean particle size distributions of raw milk after four homogenization treatments. Measurements included milk samples (n=24) from each of six lots: skim milk (Skim), raw milk from Autumnwood Farm (Atmwd), raw milk fortified with whole milk powder (WMP), raw milk from Arthurst Farm (BrnSws), raw milk from the University of Minnesota farm (UMN), raw milk fortified with milk protein concentrate (MPC)

Table 4. Mean particle size distributions of raw milk for each homogenization treatment assessed by conducting a standard analysis of variance using pairwise comparison, and corrected with Fisher's LSD tests for differences. Responses for each lot were analyzed independently from one another

	Milk Lot						
[1]Method	[2]Skim	[3]Atmwd	[4]WMP	[5]BrnSws	[6]UMN	[7]MPC	SD among sample replicates (n=24)
None	3.65[a]	1.96[a]	3.23[a]	1.34[a]	1.71[a]	3.03[a]	0.94
Sonicator	0.43[b]	0.43[b]	0.95[b]	0.65[b]	0.20[b]	0.57[bc]	0.25
Ultra-Turrax	3.35[c]	2.00[c]	0.81[b]	1.90[c]	0.96[c]	1.66[c]	0.55
Two-Stage	0.57[b]	1.22[d]	0.89[b]	0.97[d]	1.22[d]	0.89[b]	0.24

[1]Method = Method of Homogenization
[2]Skim = Skim milk
[3]Atmwd = Raw milk from Autumwood Farm
[4]WMP = Raw milk fortified with whole milk powder
[5]BrnSws = Raw milk from Arthurst Farm
[6]UMN = Raw milk from University of Minnesota Dairy
[7]MPC = Raw milk fortified with milk protein concentrate
[a-d]Treatments with different letters differ significantly within columns ($p < 0.05$)

The Ultra-Turrax tube disperser was inconsistent in reducing particle size distribution. It was effective for reducing fat particle size in raw milk fortified with both whole milk powder and milk protein concentrate, and in raw milk from the University of Minnesota dairy; however, the size distribution was made worse for raw milk obtained from Arthurst and Autumnwood dairies. Since both particle size and particle size distribution results are correlated with one another, the lack of multiple mechanical forces acting on the MFG or differences in sample temperature could explain why sonication and two-stage homogenization were better methods of processing. However, this reasoning does not explain the inconsistent results seen for the Ultra-Turrax tube disperser method. One possible explanation is that milk from Arthurst and Autumnwood dairies contained a higher percentage of conjugated linoleic acid in the overall fat

content. This would likely have been caused by cows grazing pasture instead of a forage or grain diet (Dhiman et al., 1999, 2005) as is the case with livestock at the University of Minnesota dairy. Because of the inclusion of conjugated linoleic acid, the melting point of the fat component in these samples would have been lower, and there would have been a wider array of particle sizes after processing since only a portion of the fat would be affected by Ultra-Turrax tube dispersion.

3.4 Conclusions

Three homogenization methods (sonication, Ultra-Turrax tube dispersion, two-stage valve) were used to reduce particle size and particle size distribution for the fat component in raw milk. Excellent particle size reduction was observed after sonication and two-stage homogenization, but size reduction using the Ultra-Turrax tube dispersing was not as good. For reducing particle size distribution, sonication was best and two-stage homogenization did not perform as well. Meanwhile, tube dispersion gave mixed results, which could be attributed to fewer mechanical forces acting on the fat component during processing and to type of fat in milk. Sonication is the recommended method for reducing particle size and size distribution prior to analysis of raw milk ingredients and product attributes; however, the quicker, more cost-efficient tube dispersion method could give promising results in some applications.

4 The Effect of Homogenization and Sample Presentation on the Prediction of Component Quantities for Raw Milk

4.1 Introduction

Evaluation of raw milk for components such as fat, protein, and casein is important prior to cheese manufacturing so the process can be standardized. In cheese-making, standardization of milk on the basis of protein, especially the casein proteins, is

important for maintaining end-product quality and for predicting cheese yields (Sørensen and Jepsen, 1998; Downey et al., 2005; Woodcock and Fagan, 2008).

General analytical benchtop methods, such as the Kjeldahl procedure for protein measurement and Mojonnier method for fat analysis, are time and resource consuming and require the operator to have extensive technical training. The concept of using spectroscopy, specifically light in the mid-infrared region, to analyze component quantities has resolved most of the issues associated with the traditional benchtop methods. Analysis of milk components using mid-infrared spectroscopy (MIRS) has become a standardized method with calibrations that must have specific reproducibility limits set by the International Dairy Federation (IDF), the International Organization for Standardization (ISO), the International Committee for Animal Recording (ICAR), and the Arbeitsgemeinschaft Deutscher Rinderzüchter (ADR). The recommendations for reproducibility limits (*R*) for laboratory, at-line, and in-line milk analyzers have been summarized by Melfsen and colleagues (2012) and have been reproduced in Table 5.

Table 5. Reproducibility limits (*R*) for analysis of milk (laboratory, at-line, and in-line recommendations)[1], and percentage of NIR prediction residuals (RES) equal to or below *R* ($RES_{\leq R}$, %)[2]

Property	Laboratory IDF/ISO		Laboratory ICAR/ADR		At-line		In-line	
	R	$RES_{\leq R}$ (%)	*R*	$RES_{\leq R}$ (%)	*R*	$RES_{\leq R}$ (%)	*R*	$RES_{\leq R}$ (%)
Fat (%)	0.13*	92.75	0.07"	73.28	0.14"	93.89	0.17"	95.80
Protein (%)	0.10*	95.42	0.07"	87.79	0.14"	98.47	0.17"	99.62
Lactose (%)			0.07"	80.92	0.14"	95.04	0.17"	98.47
Urea (mg/L)			25^{+}	84.35	50’	97.33	62.50’	100

[1]Symbols represent the references corresponding to the value of *R*: (*) indicates value according to IDF standard 141C:2000 (IDF, 2000) and ISO standard 9622 (ISO, 1999; SD of fat: 0.045%; SD of protein: 0.035%); (+) indicates ADR recommendation 1:13 (ADR, 2002); (“) indicates the ICAR standard (ICAR, 2010); (‘) indicates value according to the ICAR (2010) and ADR (2002) recommendations
[2]Reproduced from Melfsen, Hartung, and Haeussermann (2012)

Although MIRS can provide simultaneous analysis of multiple components quickly, the cost of the instrument and associated maintenance is very expensive (Rodriguez-Otero et al., 1997; Tague and Chimenti, 2012). Raman spectroscopy is another method used for analysis, but the laser-emitting monochromatic radiation source has led to issues with signal detection and expensive hardware (Burns and Ciurczak, 2008; Siesler et al., 2008). Small manufacturers cannot afford the price of current spectroscopic methods and do not have the flexibility to evaluate finished cheese for its components, while on-farm processors are often limited in their ability to test their own milk or products. Alternative spectroscopic techniques like near-infrared spectroscopy (NIRS) with the flexibility to evaluate both liquids and solids would greatly benefit dairy producers.

In samples analyzed by NIRS, wavelengths of light between the visible and mid-IR regions (780-2,500 nm) are absorbed by molecules causing stretching and bending amongst chemical bonds. Absorption bands in the NIR region of the electromagnetic spectrum are mostly represented by overtones and the mid-IR region combination vibrations (Wetzel, 1983). Interpreting the NIR spectra can be difficult because of both weak and broad signals (Siesler et al., 2008); however, approximations for the sample's molecular vibration sequence have improved. Technical advances in optical fibers, computers and chemometrics, and in the conversion of chemical data into relevant information have allowed analysts to better detect and predict chemical components and their quantities (Wetzel, 1983; Hirschfeld, 1984; McClure, 1994).

NIRS offers many different advantages in that it is very fast, can be used to analyze both solids and liquids, and has functional on-line measurement capabilities. Most importantly, NIR instruments are cheaper compared to other spectroscopic techniques because of their simpler design and lack of an attached homogenizer (BÜCHI Labortechnik AG, 2010; Tague and Chimenti, 2012). While NIRS has found practical use

in the grain and pharmaceutical industries, there are still challenges with its application in the dairy industry (Siesler et al., 2008).

The restrictive factor for NIRS use in dairy products is thought to be the presence of large fat globules in raw milk, which causes excessive light scattering as NIR energy interacts with a sample during analysis. The phenomenon of light scattering by particles varying in size leads to poor spectral quality and inaccurate predictions during component analysis. These effects have been well-documented (Kortum, 1969; Wetzel, 1983; Davies and Grant, 1987). If particle size and size distribution can be reduced, component analysis could be improved. Additionally with smaller component sizes, there would be a good representation of the sample since more particles could interact with the beam of light (Wetzel, 1983).

Limiting the effects of the fat component in milk by reducing particle size and distribution is possible using homogenization during sample preparation. Small, two-stage valve homogenizers are included in the design of MIR analyzers to reduce the effects of the fat component prior to spectroscopic analysis, but there are costs associated with their maintenance and efficiency (Barbano and Clark, 1989; Thiebaud et al., 2003; Hayes et al., 2005). Cost-efficient methods involving sonication and high speed mixing are more suitable as sample preparation methods for smaller dairy manufacturers to analyze dairy products. If these alternative homogenization methods can be optimized for sample preparation to improve calibration quality, then predictions for component quantities will be more accurate.

Another way for improving the calibration quality could be to optimize different methods for presenting raw milk for NIR analysis (Wetzel, 1983; Ilari et al., 1988; Reich, 2005). Liquid samples are typically analyzed by NIRS using transflectance, which involves light passing through the sample as a single pathway and reflecting off a diffusion plate to pass through the sample once more (Ritchie, 2009; BÜCHI Labortechnik AG, 2010). While a petri dish or other standard sample holder is most

practical for this method, it is often criticized because only an aliquot of the entire sample is analyzed (Figure 23). Alternatively, liquid can be pumped through a flow cell in the detector, which mimics the on-line measurement of a liquid sample and allows for better overall sample representation (Figure 24) (BÜCHI Labortechnik AG, 2010). The flow cell could also be used without pumping during static measurement, serving as a combination of the two aforementioned methods.

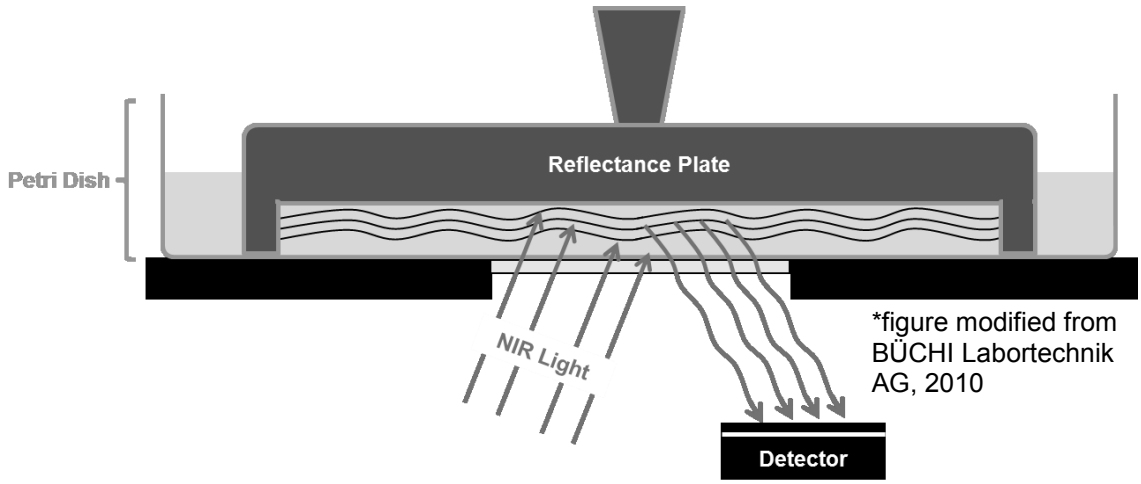

Figure 23. Analysis of a sample in a petri dish using the transflectance method*

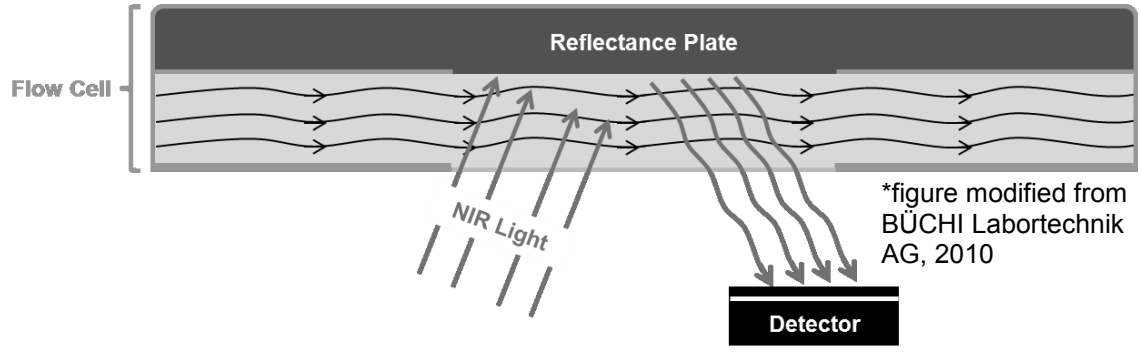

Figure 24. Analysis of a sample flowing through the detector in transflectance mode*

In the current study, the objective was to optimize different sample preparation and presentation techniques to give a more accurate prediction of raw milk components by NIRS. Alternative processing methods using an Ultrasonic Dismembrator and an Ultra-Turrax tube disperser, a novel tissue homogenizer, were compared with a standard two-stage valve benchtop homogenizer. Three sample presentation methods were also optimized to improve calibration quality. Raw milk was presented for NIR analysis using a petri dish and a flow cell technique with both static and dynamic flow. Given the many ways to improve NIR calibration quality using different sample preparation and presentation methods, we hypothesized that standard two-stage valve homogenization combined with dynamic flow cell measurement would give the best predictions for milk components. It is anticipated that this research will assist smaller dairy manufacturers in maintaining product quality and predicting product yields by providing a practical solution for improved NIR analysis.

4.2 Materials and Methods

The materials and methods are described in this section. The expanded methods, including those describing the operation of the NIRFlex® N-500, are in section A.2 of the Appendix.

4.2.1 Materials

Raw bovine skim milk (Skim) (DQCI, Mounds View, MN) and raw bovine whole milk obtained from stock at the University of Minnesota dairy barn (UMN) (UMN, Falcon Heights, MN), Autumnwood Farm (Atmwd) (Forest Lake, MN), and Arthurst Farm (BrnSws) (Plato, MN) was used for a portion of sampling. Raw bovine whole milk (UMN, Falcon Heights, MN) supplemented at with both 70% milk protein concentrate (MPC) (Idaho Milk Products, Jerome, ID) and whole milk powder (WMP) (Nestlé, Vevey, Switzerland) was also used as samples.

4.2.2 Experimental Design

The study was designed as a randomized complete block to be repeated for each lot of milk sampled. The experimental design consisted of a full 4 x 3 factorial design with four homogenization treatments (Ultra-Turrax tube dispersing, sonication, two-stage homogenization and no homogenization) and three NIRS presentation methods (petri dish with transflectance cover, flow cell with static flow, and flow cell with dynamic flow), totaling 12 treatments.

4.2.3 Sample Preparation for FT-NIR Analysis

Samples for each of the four homogenization treatments were prepared following the procedures in section 3.2.3 and 3.2.4. Samples varying in fat, protein, moisture, and ash content were collected from six different lots to give a range of properties necessary to establish robust calibrations. Overall, 144 samples were processed for each treatment.

4.2.4 Sample Presentation for FT-NIR Analysis

Samples were presented for component analysis by NIRS using three different presentation techniques: petri dish with transflectance cover, flow cell with static flow, and flow cell with dynamic flow. Overall, 576 samples were analyzed by NIR for each presentation method.

Petri Dish with Transflectance Cover

Each raw milk sample (approximately 7 ± 2 mL) was scanned by an FT-NIR spectrophotometer (NIRFlex N-500 polarization interferometer with attached N510 Flex Solids, BÜCHI Labortechnik AG, Switzerland) in the NIR region of 1,000-2,500 nm in transflectance mode with a triplicate measurement using a glass petri dish with a transflectance cover. Milk samples were gently agitated between each sequence in triplicate. The recorded spectrum of each sample was averaged from 32 repeated instrumental scans. The absorbance was recorded as $1/R$, where R represents reflectance

of light. Triplicate spectral data were averaged in order to approximate actual spectral data for each sample. All scanning was performed at 20 °C.

Static Analysis by FT-NIR with Flow Cell Application

Samples of raw milk (15 mL, 20 °C) were scanned by an FT-NIR spectrophotometer in the NIR region of 1,000-2,500 nm in transflectance mode with a triplicate measurement using a Variable Flow Mini-Pump (Control Company, Friendswood, TX). Milk samples were agitated using the pump between each sequence in triplicate. A quick rinse with distilled water of the pump's tubing was done before each new sample. The recorded spectrum of each sample was averaged from 32 repeated instrumental scans. Triplicate spectral data were averaged in order to approximate actual spectral data for each sample. All scanning was performed at 20 °C.

Dynamic Analysis by FT-NIR with Flow Cell Application

Raw Milk samples (15 mL, 20 °C) were scanned by the FT-NIR spectrophotometer similarly to samples during static analysis by FT-NIR, but with continuous circulation using the peristaltic pump operating at 22.5 mL min^{-1}. Distilled water was used to rinse the pump's tubing after each sample. The recorded spectrum of each sample was averaged from 160 repeated instrumental scans (not in triplicate measurement). All scanning was performed at 20 °C.

4.2.5 Chemical Analysis

Chemical analysis for the properties of total protein, casein, fat, moisture, and ash were performed in duplicate so that each spectrum would have a reference value on which the calibration could be built. Each property value is an average of duplicate measurements. Using the acquired dataset, a two-way analysis of variance (ANOVA) was performed, and based on these ANOVAs, pairwise comparisons were presented using Fisher's least significant difference testing. All statistical tests were computed using RStudio version 0.95.262 (RStudio, Boston, MA).

Total Protein

Total protein (% total nitrogen *x* 6.38) of all milk samples was determined using Official Method 991.20 (AOAC International, 2006) based on the Kjeldahl method with the following modifications: Kjeldahl tablets, Mercury and Selenium free (2 x 2.5 g) (Fisher Scientific Inc., Fairlawn, NJ) were used in place of the catalyst solution, distilled water (50 mL) was added to the digestion flasks upon cooling, 32% NaOH (100 mL) was added to the digestion flask during distillation of raw whole milk samples, 32% NaOH (115 mL) was added to the digestion flask during distillation of raw skim milk samples.

Casein

Casein (% casein nitrogen *x* 6.38) protein of all milk samples was determined using Official Method 998.06 (AOAC International, 2006) based on casein precipitation from milk at pH 4.6 and Kjeldahl determination of protein content via AOAC 991.20 (AOAC International, 2006) with the following modifications: Kjeldahl tablets, Mercury and Selenium free (1 x 2.5 g) (Fisher Scientific Inc., Fairlawn, NJ) were used in place of the catalyst solution, distilled water (50 mL) was added to the digestion flasks upon cooling, 32% NaOH (100 mL) was added to the digestion flask during distillation of all raw milk samples.

Total Fat

Total fat was determined using Official Method 989.05 (AOAC International, 2006) based on the Mojonnier method with three ether extractions and the use of a Mojonnier Milk Tester (Mojonnier Bros Co., Chicago, IL). The only modification to this method was the substitution of NH_4OH with 32% NaOH (1.5 mL).

Moisture

Moisture content of raw milk samples was determined using a modified Official Method 990.19 (AOAC International, 2006) with a 5.0-5.5 g sample and a drying oven on "low" setting (95 °C) for the pre-drying stage. Pre-drying took place until there was

no free movement of liquid milk (~ 1 h). Samples were dried to a constant weight at 100 °C and 0.683 m mercury for 4 h.

Ash

Ash was determined by muffle furnace following Bulletin 916, Standards for Grades of Dry Milk (American Dairy Products Institute, 2002). The only modifications were the use of 5.0-5.5 g of sample and a pre-drying stage with a drying oven on "low" setting (95 °C) for 4 h.

4.2.6 NIR Spectra Processing and Analysis

Acquired NIR spectra were evaluated with the chemometric program, NIRCal v5.2 (BÜCHI Labortechnik AG, 2007). The original spectra in the wavelengths from 1,000-2,500 nm were pretreated using the Savitzky-Golay first derivative smoothing technique with 9 points followed by Standard Normal Variate (SNV) normalization. Models were developed from the absorbance data of all raw milk samples using partial least squares regression (PLSR) analysis based on six principal components. Outlying spectra were not used for modeling.

For dynamic analysis, approximately 1/3 of all spectra from the sample set were placed into validation while the remaining spectra were used for the calibration set. In milk samples analyzed in triplicate by both the static and petri dish methods, each set of three spectra for a given sample was placed either entirely in the calibration set or entirely in the validation set. Overall, approximately 1/3 of all spectra were used for the validation set while the remaining spectra were placed in the calibration set. Additionally, spectra representing extreme property values were placed into the calibration set for all analysis.

In total, 60 calibration curves were developed based on 12 possible treatment combinations for each of the 5 measured properties. Calibrations were compared to one another based on their overall quality.

Different statistical parameters were used in order to assess the calibration quality after each set of treatments. The calibration quality value (Q-value) is a specific index used to give the overall quality of a NIR calibration (BÜCHI Labortechnik AG, 2007). The closer the value is to 1, the better the calibration. Calibrations with Q-values above 0.5 are useable, but those above 0.75 are considered robust (BÜCHI Labortechnik AG, 2007). The highest Q-value was used as the primary criteria for deciding the best calibration model in this study. Q-value accounts for more common quality parameters such as the standard error of calibration (SEC) and the standard error of prediction (SEP) and the squared coefficient of correlation for each of the calibration and validation procedures (R^2_{cal} and R^2_{val}, respectively).

SEC and SEP describe the magnitude of the standard deviation for calibration and validation procedures, respectively (Burns and Ciurczak, 2008). Furthermore, R^2_{cal} and R^2_{val} were recorded to determine the amount of variation in the data. These two values are traditionally modeled by the calibration equation as a total fraction of 1.0.

Overall, responses for Q-value, SEC, SEP, R^2_{cal}, and R^2_{val} were all analyzed to assess the effects of the homogenization and presentation methods for each measured property (i.e. fat, moisture, casein). For ease of comparison, a multiplicative inverse was applied for Q-value and SEP raw data. A two-way analysis of variance (ANOVA)) was performed, and based on these ANOVAs, pairwise comparisons were presented using Fisher's least significant difference testing. The aforementioned statistical tests were computed using RStudio version 0.95.262 (RStudio, Boston, MA).

4.3 Results and Discussion

The results and discussion are described in this section. The expanded results, including statistical analysis and supplementary tables are in section A.2.2 of the Appendix.

4.3.1 Chemical Analysis

The results of chemical analysis for the properties of fat, total protein, casein, ash, and moisture for raw milk samples used in the study is seen in Figures 25a-e, respectively. Overall, there was a wide variation of composition among samples from different lots, which was caused by both different breeds and lactation stages of cow herds used in the study and by varying levels of fortification using whole milk powder (WMP) and milk protein concentrate (MPC). Skim milk samples were very low in fat and higher in moisture content, as expected. For whole raw milk samples without fortification, all properties, besides ash, were statistically different. The amount of ash was highest for samples from the University of Minnesota (UMN) and Autumnwood Farm (Atmwd) lots among whole milk samples, which could have been caused by factors such as the breed, lactation stage, or feed ration. Furthermore, fortified milk samples contained not only the most ash, but also the most total protein and casein. This was anticipated based on label information for the milk protein concentrate (MPC) ingredient (fat 1.2%, lactose 15%, ash 7%) and whole milk powder (WMP) ingredient, which had added iron, zinc sulfate, and vitamins A, C, and D_3.

Because of the large number of samples used for calibration development, it was important for samples to contain a large range of property values to enhance the robustness of measurements by near-infrared analysis. The range of property values for different samples used in the calibration and validation sets was nearly identical during development and would likely affect future measurements.

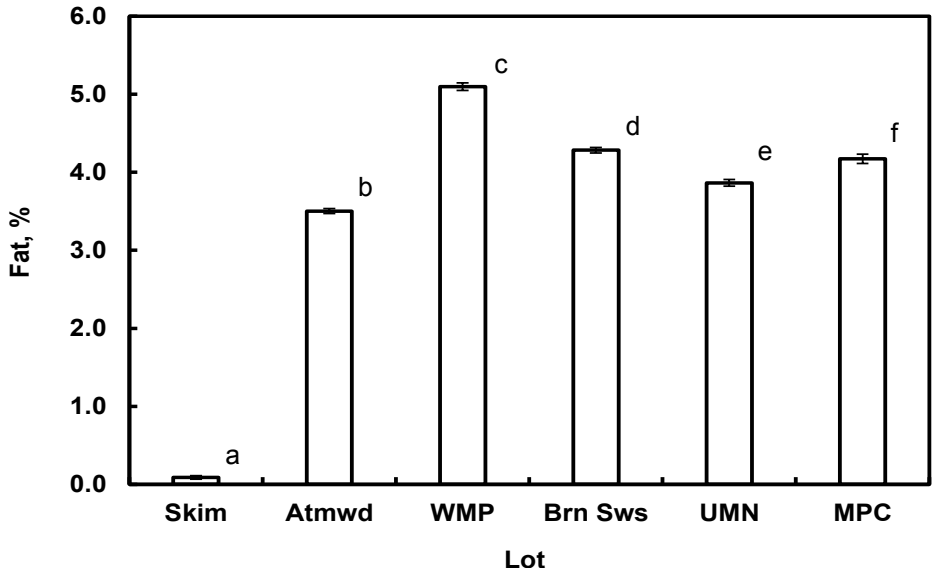

Figure 25a. Mean fat content (on wet basis) and confidence limits (95%) of raw milk samples (n=24) from each of six lots: skim milk (Skim), raw milk from Autumnwood Farm (Atmwd), raw milk fortified with whole milk powder (WMP), raw milk from Arthurst Farm (BrnSws), raw milk from the University of Minnesota farm (UMN), raw milk fortified with milk protein concentrate (MPC). Means with different letters are significantly different ($p < 0.05$)

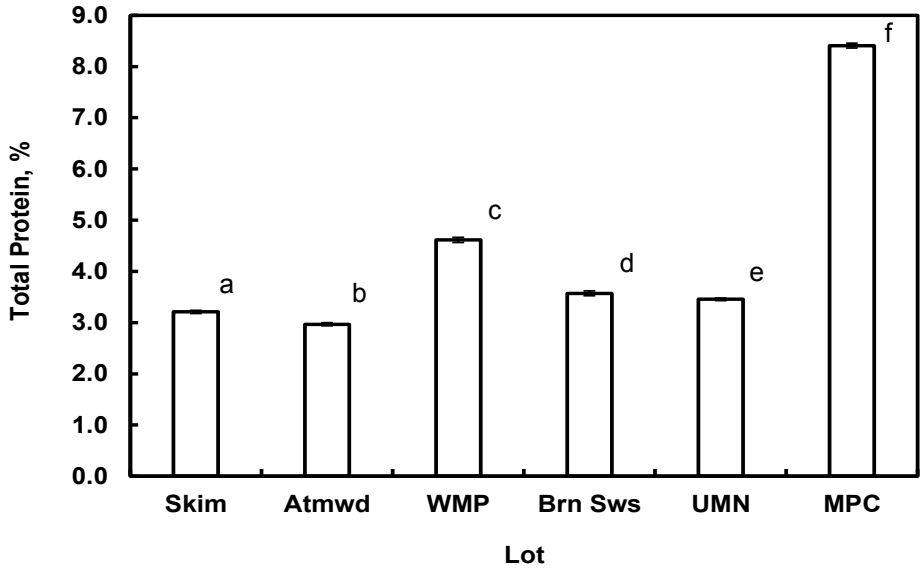

Figure 25b. Mean total protein content and confidence limits (95%) of raw milk samples (n=24) from each of six lots: skim milk (Skim), raw milk from Autumnwood Farm (Atmwd), raw milk fortified with whole milk powder (WMP), raw milk from Arthurst Farm (BrnSws), raw milk from the University of Minnesota farm (UMN), raw milk fortified with milk protein concentrate (MPC). Means with different letters are significantly different ($p < 0.05$)

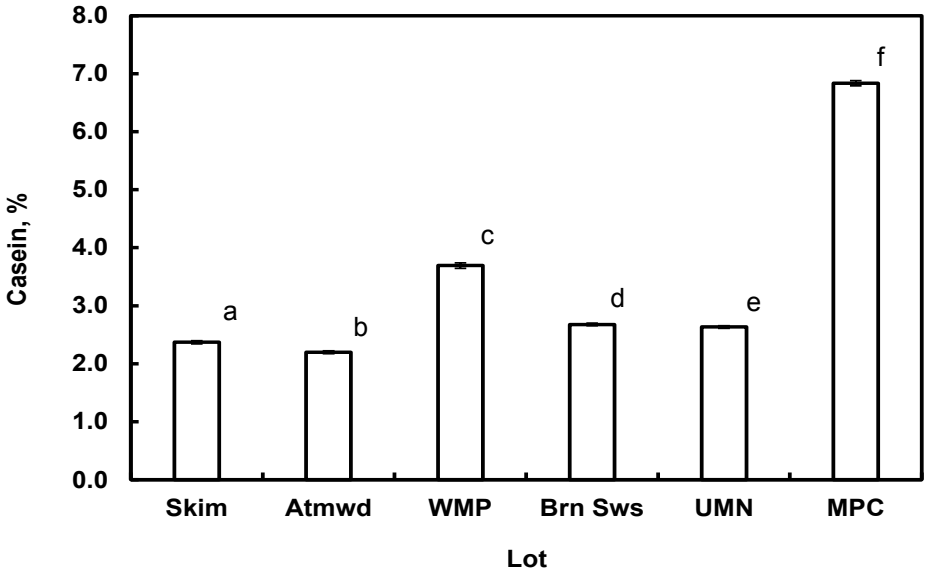

Figure 25c. Mean casein content and confidence limits (95%) of raw milk samples (n=24) from each of six lots: skim milk (Skim), raw milk from Autumnwood Farm (Atmwd), raw milk fortified with whole milk powder (WMP), raw milk from Arthurst Farm (BrnSws), raw milk from the University of Minnesota farm (UMN), raw milk fortified with milk protein concentrate (MPC). Means with different letters are significantly different ($p < 0.05$)

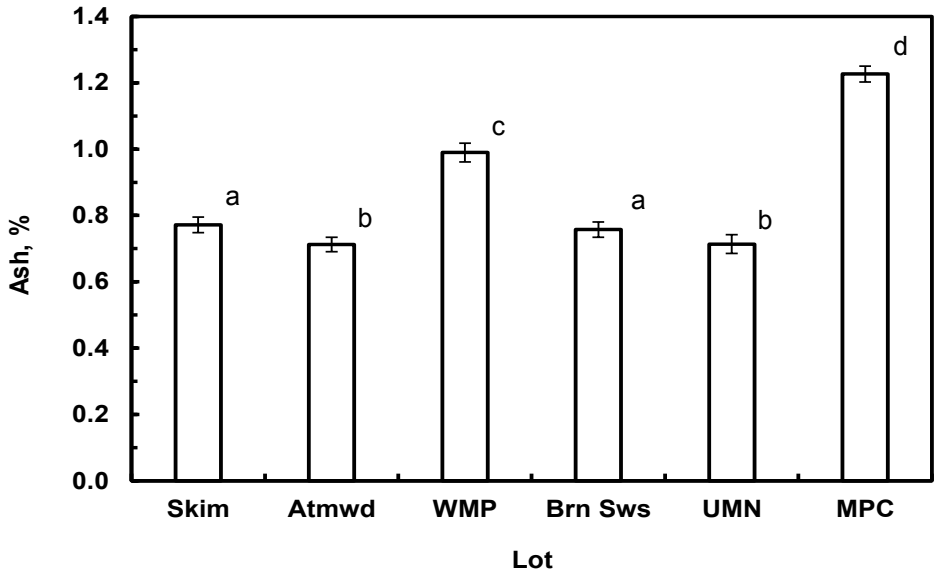

Figure 25d. Mean ash content (on wet basis) and confidence limits (95%) of raw milk samples (n=24) from each of six lots: skim milk (Skim), raw milk from Autumnwood Farm (Atmwd), raw milk fortified with whole milk powder (WMP), raw milk from Arthurst Farm (BrnSws), raw milk from the University of Minnesota farm (UMN), raw milk fortified with milk protein concentrate (MPC). Means with different letters are significantly different ($p < 0.05$)

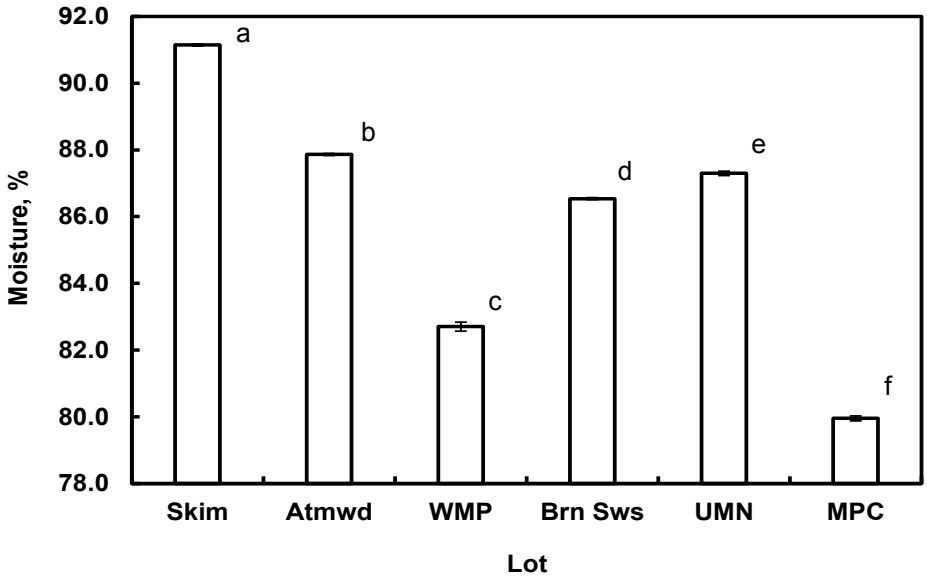

Figure 25e. Mean moisture content and confidence limits (95%) of raw milk samples (n=24) from each of six lots: skim milk (Skim), raw milk from Autumnwood Farm (Atmwd), raw milk fortified with whole milk powder (WMP), raw milk from Arthurst Farm (BrnSws), raw milk from the University of Minnesota farm (UMN), raw milk fortified with milk protein concentrate (MPC). Means with different letters are significantly different ($p < 0.05$)

4.3.2 Homogenization Effects on Calibration Quality

Homogenization did not improve calibration quality for the properties measured in raw milk samples (Table 6). The average Q-values representing calibrations for unhomogenized milk samples were similar to those for Ultra-Turrax tube dispersed and sonicated samples for all measured properties, and were higher compared to two-stage homogenized samples for total protein, casein, and moisture calibrations.

Table 6. Treatment means for parameters representing calibration quality for each measured property based on different homogenization methods[1,2]

Property (%)	Homogenization[3]	Q-value[4]	Calibration (n=96)		Validation (n=48)	
			SEC	R^2_{cal}	SEP[5]	R^2_{val}
Fat	None	0.880[a]	0.011[a]	0.990[a]	0.020[a]	0.971[a]
	Sonicator	0.862[a]	0.012[a]	0.988[a]	0.017[a]	0.976[a]
	Ultra-Turrax	0.810[a]	0.011[a]	0.989[a]	0.021[a]	0.971[a]
	Two-Stage	0.766[a]	0.002[b]	0.995[b]	0.044[a]	0.996[b]
Total Protein	None	0.894[a]	0.009[a]	0.996[a]	0.013[a]	0.993[a]
	Sonicator	0.888[a]	0.009[a]	0.994[a]	0.016[a]	0.992[a]
	Ultra-Turrax	0.902[a]	0.010[a]	0.993[a]	0.016[a]	0.992[a]
	Two-Stage	0.604[b]	0.001[b]	0.997[b]	0.013[a]	0.992[a]
Casein	None	0.933[a]	0.006[a]	0.997[a]	0.008[a]	0.996[a]
	Sonicator	0.887[a]	0.006[ab]	0.997[a]	0.011[a]	0.992[b]
	Ultra-Turrax	0.918[a]	0.007[a]	0.995[a]	0.011[a]	0.993[ab]
	Two-Stage	0.641[b]	0.001[b]	0.999[b]	0.074[b]	0.993[ab]
Ash	None	0.911[a]	0.010[a]	0.982[a]	0.003[a]	0.974[a]
	Sonicator	0.910[a]	0.011[a]	0.980[a]	0.003[a]	0.976[a]
	Ultra-Turrax	0.916[a]	0.012[a]	0.984[a]	0.002[a]	0.980[a]
	Two-Stage	0.806[a]	0.001[b]	0.999[b]	0.006[b]	0.979[a]
Moisture	None	0.896[a]	0.017[a]	0.996[a]	0.025[a]	0.992[a]
	Sonicator	0.892[a]	0.014[ab]	0.997[a]	0.022[a]	0.995[a]
	Ultra-Turrax	0.855[a]	0.017[a]	0.995[a]	0.021[a]	0.995[a]
	Two-Stage	0.345[b]	0.003[b]	0.999[b]	0.116[b]	0.988[a]

[1]SEC = standard error of calibration; R^2_{cal} = coefficient of correlation of calibration; SEP = standard error of prediction; R^2_{val} = coefficient of correlation of validation
[2]Treatments with different superscript letters differ significantly within columns for each property ($p < 0.05$)
[3]Homogenization = method of homogenization
[4,5]Treatment means have been transformed back to the measured scale

The reason for two-stage homogenization producing lower quality calibrations could be explained by variations in chemical composition as a result of whey protein denaturation during the pre-heating step prior to processing. Milk temperature had been increased to ~55 °C, which, according to past studies, would have been enough to initiate the denaturation sequence (Hillier and Lyster, 1979; Mulvihill and Donovan, 1987).

Overall, most calibrations were very good and could be used to predict accurate quantities based on Q-value ≥ 0.75 (BÜCHI Labortechnik AG, 2007), and those with smaller Q-values, aside from the moisture calibration for two-stage homogenized samples, could still be used with limited accuracy (Q-value ≥ 0.50). SEP represents the standard deviation for the residuals of the spectra set due to differencesbetween the actual component quanities (measured by wet chemistry methods) and the NIR predicted vales for samples outside the calibration set (Burns and Ciurczak, 2008). In comparison to related studies for unhomogenized milk analysis (Table 1), SEP was noticeably lower for most properties during analysis. Laporte and Paquin reported SEP of 0.05, 0.09, and 0.07 for fat, protein, and casein (1999). Woo (2002) and Melfsen (2012) both achieved good calibrations for fat (0.06 and 0.09, respectively) and for protein (0.10, and 0.05). For this study, SEP for fat (0.020), protein (0.013), and casein (0.008) calibrations for unhomogenized milk samples were exceptionally low. Furthermore, except for casein and moisture calibrations for two-stage homogenization, all the SEP values obtained met the upper limit IDF standard of 0.06% (IDF, 2000)

As shown in Table 6, the calibration responses were not improved by homogenization treatments, yet, the overall quality of calibrations was still very good. One possible explanation for this result could be that the sample temperature we used

during NIR analysis (20 °C) was in the middle range of temperatures used by other sources. In the aforementioned studies, NIR spectra were gathered from milk samples at or near 40 °C (Melfsen et al. (2012) performed NIR analysis of milk, 38 °C, immediately following milking); while, in another study by Bogomolov et al. (2012), comparatively low results for SEP in fat (0.05) and protein (0.03) were observed for calibrations of unhomogenized raw milk at 25 °C. On the other hand, NIR technician specialists have reported that milk at refrigeration temperatures (4 °C) has only been analyzed with below-average results (BÜCHI Labortechnik AG, personal communication, January 10, 2012). Physical difference such as density could affect light scattering properties at lower temperatures, while changes in chemical composition caused by melted milkfat or partially-denatured whey would likely affect the spectral signal at higher sample temperatures. Although some reasonable conclusions can be made from these observations, additional research regarding the optimization of sample temperature for milk analysis by NIRS is still needed.

4.3.3 Sample Presentation and Calibration Quality

Calibration quality for the NIR analysis of raw milk was not affected by different presentation methods. Based on the results from Table 7, both static and dynamic flow cell methods were similar to one another and to the petri dish with transflectance cover method for all properties, aside from the calibration for moisture using the petri dish with transflectance cover (Q-value = 0.508). The calibration for moisture in raw milk resulted in a less accurate calibration due in part to the evaporation of water from the petri dish to the surrounding atmosphere before and during measurement. The open design of the petri dish method in comparison to the enclosed flow cell would have allowed moisture to escape from the sample; thus, altering the chemical composition more as the measurement progressed.

Table 7. Treatment means for parameters representing calibration quality for each measured property based on different presentation methods[1,2]

Property (%)	Presentation[3]	Q-value[4]	Calibration (n=96)		Validation (n=48)	
			SEC	R^2_{cal}	SEP[5]	R^2_{val}
Fat	Static	0.865[a]	0.007[a]	0.991[a]	0.020[a]	0.979[a]
	Dynamic	0.808[a]	0.015[a]	0.990[a]	0.031[a]	0.969[a]
	Petri Dish w/ TC	0.821[a]	0.008[a]	0.992[a]	0.019[a]	0.981[a]
Total Protein	Static	0.842[a]	0.006[a]	0.996[a]	0.010[a]	0.993[a]
	Dynamic	0.895[a]	0.013[a]	0.994[a]	0.021[a]	0.994[a]
	Petri Dish w/ TC	0.758[a]	0.006[a]	0.996[a]	0.018[a]	0.990[a]
Casein	Static	0.869[a]	0.008[a]	0.997[a]	0.010[a]	0.995[a]
	Dynamic	0.921[a]	0.004[a]	0.997[a]	0.013[a]	0.996[a]
	Petri Dish w/ TC	0.780[a]	0.004[a]	0.997[a]	0.013[a]	0.989[a]
Ash	Static	0.922[a]	0.001[a]	0.985[a]	0.002[a]	0.979[a]
	Dynamic	0.927[a]	0.002[a]	0.987[a]	0.003[a]	0.984[a]
	Petri Dish w/ TC	0.840[a]	0.001[a]	0.984[a]	0.003[a]	0.970[a]
Moisture	Static	0.851[a]	0.009[a]	0.997[a]	0.024[a]	0.996[a]
	Dynamic	0.870[a]	0.022[b]	0.996[a]	0.027[a]	0.995[a]
	Petri Dish w/ TC	0.508[b]	0.012[a]	0.996[a]	0.030[a]	0.988[a]

[1]SEC = standard error of calibration; R^2_{cal} = coefficient of correlation of calibration; SEP = standard error of prediction; R^2_{val} = coefficient of correlation of validation
[2]Letters within columns differ significantly for each property ($p < 0.05$)
[3]Presentation = method of presentation; Static = flow cell with static analysis; Dynamic = flow cell with dynamic analysis; Petri Dish w/ TC = petri dish with transflectance cover
[4,5]Treatment meansr have been transformed back to the measured scale

The observed calibrations can nearly all be used with a high level of accuracy (i.e. Q-value ≥ 0.75); however, further analysis of the Q-value for each scanning method shows the petri dish with transflectance cover produced calibrations near the lower limit of the 95% confidence interval compared to either of the flow cell techniques. The reason for this could again be attributed to the design of the petri dish scanning method. Recall Figure 5, which describes NIR light passing through a fixed sample width created by a transflectance cover as part of analysis by transflectance. Spectral signal quality could be diminished if light escapes from the petri dish as a result of scattering effects between particles in the sample. An overview of the petri dish with transflectance cover in Figure 26 shows the transflectance cover supported by a non-continuous base that would allow light to escape through the open sides. As a result, the detector would have received a weaker signal as evident by the quality of the calibrations observed for each property.

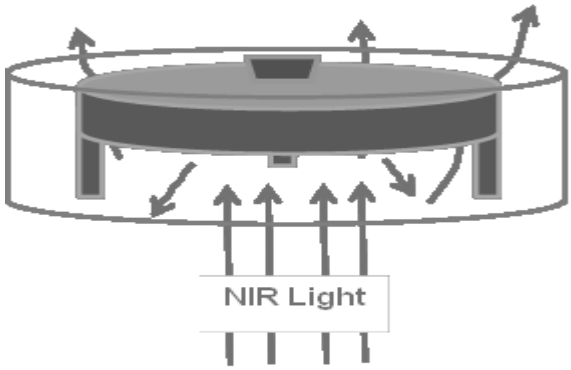

Figure 26. The petri dish with transflectance cover method. The non-continuous base of the transflectance cover allows light to escape

4.4 Conclusions

Sample preparation and scanning methods for NIRS were optimized to improve the accuracy of prediction for raw milk properties. Sample preparation using two alternative homogenization techniques (sonication and Ultra-Turrax tube dispersion) and

standard homogenization (two-stage valve) did not improve calibration quality. Calibrations developed from milk prepared by two-stage valve homogenization were less accurate because of whey protein denaturation causing changes in chemical composition. Results also showed that sample temperatures of ~20 °C may be beneficial for producing very accurate predictions of component quantities. As for the presentation of samples for NIR analysis, neither the flow cell (static or dynamic) nor the petri dish with transflectance cover was better in terms of calibration quality. All methods were capable of producing high quality calibrations; however, further analysis showed that flaws in the design of the petri dish with transflectance cover could lead to a loss of moisture or NIR light, which could affect the accuracy of predictions in component analysis. In conclusion, properties in an unknown sample of raw milk at 20 °C can be most accurately predicted in the unhomogenized state using static or dynamic flow cell methods for NIR scanning.

5 Concluding Remarks

Sample preparation using sonication, Ultra-Turrax tube dispersing, and two-stage valve homogenization was optimized to improve the prediction of component quantities in raw milk using NIRS. The successful reduction of particle size and size distribution of the fat component during all sample preparation methods was not an early indicator of improved calibration quality. All observed calibrations could be used with a high level of accuracy. Those for unhomogenized samples were similar in quality to calibrations prepared using sonication and Ultra-Turrax tube dispersion, but not for two-stage valve homogenization, which gave mixed results because of chemical changes that occurred as a result of preheating the milk before processing. Furthermore, the method of scanning using either the flow cell for static and dynamic analysis or petri dish with transflectance cover had no effect on the quality of analysis. The lack of differences in calibrations could have been affected by the lower sample temperature (20 °C) used during NIR

analysis compared to other studies, but additional testing for temperature affects is necessary. Overall, it was demonstrated that no sample preparation step is necessary for improving NIRS calibrations and that analysis using flow cell or petri dish scanning methods does not differ for milk at room temperature.

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7 Appendix

A.1 Reducing Milkfat Particle Size by Homogenization

A.1.1 Expanded Methods

Ultra-Turrax®

Materials and Equipment

- Raw milk
- 15 mL centrifuge tube
- Ultra-Turrax® Tube disperser Workstation
- 20 mL Rotor Stator Disperser Tube (DT-20)
- Water bath (38 °C)
- Thermometer

Procedure

1. Turn on small water bath and set to 38 °C or about setting "5" on temperature control
2. While water bath is heating, pour milk (~15 mL) into 15 mL centrifuge tube and submerse in water bath. Allow sample to reach 38 °C
3. Set-up Ultra-Turrax® Tube disperser Workstation by connecting the power supply and pressing the power button.
4. Set speed setting to "7" (about 2,100 RPM), and timer for 3.5 min
5. Pour milk from centrifuge tube into Disperser Tube and tightly screw on cap.

   Notice Ultra-Turrax® is not meant to be used with liquids at temperatures > 40 °C

6. Lock the Disperser Tube into the workstation by turning clockwise and press start.

   Notice If the workstation does not immediately start it will automatically try reversing direction 3x in order to free any obstruction. If the instrument does not start, power down the Ultra-Turrax® and restart it once more. If the instrument still will not start, power down and clean any debris using water and paper towels before restarting once more.

7. After mixing is complete, return sample to original centrifuge tube and store sample at room temperature.

   Notice Do not tap Disperser Tube on benchtop! This could damage the rotor-stator mechanism!

Sonication

Materials and Equipment

- Raw milk
- 50 mL centrifuge tube
- Model 150E Ultrasonic Dismembrator with a 0.125 in Diameter Tapered Microtip Probe
- 1 Ringstand
- 2 3-pronged clamps with holders
- 1 500 mL beaker with ice water
- 4 °C Cooler
- Thermometer

Procedure

1. Pour milk (~25 mL) into 50 mL centrifuge tube and place into 4 °C cooler. Allow sample to reach 4 °C.

2. Set-up sonicator in secluded room by connecting the power supply and sonicating horn. Using a 3-pronged clamp, secure the sonicating horn in place above where the sample will be placed. After powering up, set for "timed sonication" at 65% amplitude for 12 min.

   Notice Do not damage the sonicating horn by hitting or dropping on benchtop surface!

3. Remove cap from sample centrifuge tube and secure in place below sonicating horn using 3-pronged clamp. Allow the bottom half of centrifuge tube to be submersed into ice water bath.

4. Lower the sonicating horn into the sample tube (~ 1 in. from bottom) and begin sonication by pressing "start".

   Notice Sonicators emit ear-damaging noise so make sure to limit exposure!

5. Once finished, record the sample temperature immediately, and place cap onto sample tube.

6. Store sample at room temperature

Two-Stage Homogenization

Materials and Equipment

- Raw milk
- 500 mL glass milk bottle
- NS 1001L2K Panda- Laboratory Homogenizer
- 1 Funnel

- 1 Rubber hose (1 in. diameter, 1 ft. in length)
- 2 hose clamps
- 1 50 mL centrifuge tube
- DI Water
- Water bath (50 °C)
- Thermometer

Procedure

1. Pour milk (~500 mL) into 500 mL glass storage bottle and place into warm water bath. Allow sample to reach 50 °C.

2. Set-up two-stage homogenizer by attaching the funnel to the rubber hose using a hose clamp and securing the assembly to the inlet spout with another hose clamp. Attach the inlet spout to the bottom of the instrument (beneath the 1st stage valve) using a gasket and steel clamp.

3. Next, attach the steel outlet spout to the side of the instrument (near the 2nd stage valve) using a gasket and steel clamp.

4. Fill rubber hose and funnel with DI water.

5. Connect the power supply and turn on the instrument using the red switch. Quickly begin squeezing the U-shaped portion of the rubber hose to push water into the inlet valve to begin flow.

   Notice Do not let the DI water level drop below the funnel, otherwise flow will stop!

6. Once water starts appearing at the outlet spout, turn the 2nd stage valve clockwise until indicator reads 900 psi (62 bar). Similarly, turn the 1st stage valve until pressure is 3,300 psi (230 bar).

7. Allow water level to nearly fall below funnel and quickly pour pre-heated milk sample into the funnel.

8. Wait ~1 min before collecting homogenized milk from outlet spout with 50 mL centrifuge tube.

9. Once the sample has been collected, reduce pressure in both valves until indicator is "0". Store milk at room temperature.

10. With homogenizer still running, flush system with water then ~20% v/v alkaline cleaner, and then ~20% v/v sanitizer to prevent filth and corrosion.

11. Carefully clean removable parts by hand with similar order of solutions as in previous step.

Malvern Particle Size Analyzer

Materials and Equipment

- Homogenized raw milk in centrifuge tube
- 3 mL pipette
- Mastersizer Particle Size Analyzer with a He/Ne laser (633 nm), range lens (300 mm), and an active beam length of 2.4 mm.
- Small Volume Sample Dispersion Unit 2023
- DI Water

Procedure

1. Set-up Mastersizer by turning both the instrument and laser on (red button and key switch near end of instrument)

   Notice Allow at least 15 min for laser to warm-up after starting!

2. Allow milk to equilibrate to room temperature (~20 °C) prior to analysis

3. Set-up analysis using Malvern software.

    a. Control→Setup All

    b. Range, 300F: 0.5-900 µm

    c. Sample Unit: MS1- Small Volume Dispersion Unit

    d. Analysis Model: Polydisperse

    e. Kill Data channels: Low- 0, High- 0

    f. Beam Length: 2.4 mm

    g. Particle Density: 1.000 g/cm^3

    h. Measurement: Use oil (RI: 1.46) in DI water (RI: 1.33)

4. Fill dispersion unit with DI Water. Turn on dispersion pump and set at 2,300 RPM

    Notice Do not run pump without water in dispersion unit chamber!

5. Select "Measurement Sequence"

    a. Label sample using text box→Next

    b. Laser power needs to be > 75% → Next

    c. Obscuration approximately = 0.0%

        Notice If obscuration exceeds 3%, flush dispersion unit chamber and flow lines with DI water. If obscuration is still not close to "0", a wash cycle consisting of a 20% v/v alkaline detergent may be used followed by a 20% v/v sanitizer to remove any filth. Refill chamber with fresh DI water.

d. Using a pipette, carefully add milk sample to dispersion chamber while it is flowing and watch for obscuration value to reach 15-25% → Next →OK

6. Once the instrument has finished scanning the sample, a polydisperse model will be generated by the software. Record necessary values.

7. When finished, flush the dispersion chamber with DI water 3x and turn off the laser and Mastersizer.

A.1.2 Expanded Results

Table A.1.1. Mean particle sizes of four homogenization methods measured for 24 samples (n=24) within six lots of milk

	Mean Particle Size, µm					
[1]Method	[2]Skim	[3]Atmwd	[4]WMP	[5]BrnSws	[6]UMN	[7]MPC
None	4.46	3.68	4.76	3.74	3.39	3.98
Sonicator	0.68	0.97	1.01	0.81	0.79	0.77
Ultra-Turrax	8.00	2.83	0.90	1.76	1.60	1.24
Two-Stage	0.80	1.22	0.96	1.02	1.22	0.96

[1]Method = Method of Homogenization
[2]Skim = Skim milk
[3]Atmwd = Raw milk from Autumwood Farm
[4]WMP = Raw milk fortified with whole milk powder
[5]BrnSws = Raw milk from Arthurst Farm
[6]UMN = Raw milk from University of Minnesota Dairy
[7]MPC = Raw milk fortified with milk protein concentrate

In this study, there were two effects capable of influencing both particle size and particle size distribution. The method of homogenization was considered the main effect, while the lot of milk was considered a secondary effect. Time of sampling was initially treated as a covariate, but was evaluated as being insignificant and not included during individual analysis within each lot of milk. In the ideal set-up, only the homogenization

method would significantly affect the main responses. Each lot was treated individually for the analysis that followed, which included individual analysis of variance for 24 bottles (n=24) for each lot (Tables A.1.2 and A.1.4) and Fisher's LSD tests (Tables 2 and 4) in section 3.3.

Table A.1.2 Individual analysis of variance for mean particle size, one for each of the six lots of milk with four methods of homogenization as the main effect

Sources of Variation	Degree of Freedom	Sum of Squares					
		[2]Skim	[3]Atmwd	[4]WMP	[5]BrnSws	[6]UMN	[7]MPC
Rep	23	178.60	1.41	14.49	0.15	0.10	4.62
[1]Method	3	4745.4*	121.3*	260.8*	128.98*	93.88*	163.43*
Error	69	551	3.871	37.718	0.551	0.305	14.573

* = significant at 95% confidence interval
[1]Method = Method of Homogenization
[2]Skim = Skim milk
[3]Atmwd = Raw milk from Autumwood Farm
[4]WMP = Raw milk fortified with whole milk powder
[5]BrnSws = Raw milk from Arthurst Farm
[6]UMN = Raw milk from University of Minnesota Dairy
[7]MPC = Raw milk fortified with milk protein concentrate

Table A.1.3. Mean particle size distributions of four homogenization methods measured for 24 samples (n=24) within six lots of milk

	Particle Size Distribution, μm					
[1]Method	[2]Skim	[3]Atmwd	[4]WMP	[5]BrnSws	[6]UMN	[7]MPC
None	3.65	1.96	3.23	1.34	1.71	3.03
Sonicator	0.43	0.43	0.95	0.65	0.20	0.57
Ultra-Turrax	3.35	2.00	0.81	1.90	0.96	1.66
Two-Stage	0.57	1.22	0.89	0.96	1.22	0.89

[1]Method = Method of Homogenization
[2]Skim = Skim milk
[3]Atmwd = Raw milk from Autumwood Farm
[4]WMP = Raw milk fortified with whole milk powder
[5]BrnSws = Raw milk from Arthurst Farm
[6]UMN = Raw milk from University of Minnesota Dairy
[7]MPC = Raw milk fortified with milk protein concentrate

Table A.1.4. Individual analysis of variance for particle size distribution, one for each of the six lots of milk with four methods of homogenization as the main effect

Sources of Variation	Degree of Freedom	Sum of Squares					
		[2]Skim	[3]Atmwd	[4]WMP	[5]BrnSws	[6]UMN	[7]MPC
Rep	23	33.502	2.920	10.402	0.212	0.151	11.133
[1]Method	3	217.751*	53.239*	99.569*	20.819*	28.455*	86.852*
Error	69	71.542	5.605	26.263	1.044	0.630	25.096

* = significant at 95% confidence interval
[1]Method = Method of Homogenization
[2]Skim = Skim milk
[3]Atmwd = Raw milk from Autumwood Farm
[4]WMP = Raw milk fortified with whole milk powder
[5]BrnSws = Raw milk from Arthurst Farm
[6]UMN = Raw milk from University of Minnesota Dairy
[7]MPC = Raw milk fortified with milk protein concentrate

In general, as particle size was decreased, milk became more visibly white as the yellow tint had faded during homogenization. This white color is caused by the scattering of light by the dispersed phase of fat globules, casein micelles, and colloidal

calcium phosphate, as described by Chandan in the Handbook of Food Products Manufacturing (2006). As milk was homogenized, there was an increase in the number and total volume of fat globules, resulting in a whiter colored sample.

Raw skim milk samples appeared to have more foam formation following both Ultra-Turrax® and sonication as a result of intense agitation causing protein surfaces to interact with one another. The stability of this foam depends on two factors, according to Chandan (2006). The first involves a lowering of surface tension, which allowed surface active components to spread and gather to form a film. Second, the film's elastic properties must be strong enough to prevent attachment of gas cells. Furthermore, the presence of fat would reduce surface tension by a physical effect (Chandan, 2006).

A.2 The Effect of Homogenization and Sample Presentation on the Prediction of Component Quantities for Raw Milk

A.2.1 Expanded Methods

NIRFlex® N-500

Creating an application for spectra collection

1. Open NIRWare Management Console
2. Click "Application Designer" in the left index pane, then click on "New" to open the New Application designer window
3. Fill in the open fields, including type of application (quantification), the measurement cell add-on, SOP text (which will display the directions for performing the measurement), and any other fields.
4. Click the Save icon to save the application to the database
5. Open the created application in the index pane by clicking on the + symbol

6. Select the subheading “Operator Configuration” to see additional settings for editing

7. Begin editing by choosing “Show Advanced Settings” from the icon toolbar at the top.

8. To begin choosing fields for editing, you must place the application into editor’s mode by selecting the “Edit Data Set” icon on the right side of the icon toolbar at the top.

9. Make necessary changes to the additional fields. Changing number of measurement sequences from 1 to 3 is a common change in order to increase robustness of calibrations.

10. Click the save icon from the toolbar.

Measuring spectra of reference samples

1. Turn on spectrometer using switch in the back of the instrument

   Notice Allow ~10 min for instrument to be detected by computer before moving on to next step as indicated by green light on top of spectrometer

2. Start the “Operator” software

3. Click the “Select Application” icon and choose the application created in the previous step

   Notice Applications can still be used in the “created idle” state if the user is logged on as “administrator”. Applications in the “Approved Idle” state cannot have their settings changed.

4. Once the application is loaded into the software, the user can name the batch and subsequent samples to be taken

Notice If an error appears stating "the current measurement cell does not match the measurement cell in the settings of the chosen application" first, check to see if the correct measurement cell add-on has been placed on the spectrometer, then reload the application. Second, try rotating the measurement cell add-on 180°, and then reload the application.

5. A system suitability test (SST) will automatically start once the first sample has been loaded and the green start button has been clicked

6. Periodic measurements of internal and external references will take place during sample analysis. It is important to follow the on-screen instructions during this time. The external reference should always be measured automatically first.

   Notice Intensity values (y-axis) for the external and internal reference spectra should have a maxima ≥ 100,000 and ≥ 125,000, respectively

7. All spectra (including the external reference) are displayed in one window. To have a closer look at sample spectrum, deactivate the check box for "External Reference" below the graphic

Analyzing samples using the flow cell add-on

1. Place the flow cell add-on onto the spectrometer making sure either ends of the rubber tubing are submersed into a beaker of DI water

2. While the application for flow cell static (FCstatic-TJR) or flow cell dynamic (FCdynamic-TJR) is loading, pump water through the flow cells using the peristaltic pump on a high speed setting (#7). Allow this to continue for ~2 minutes to flush out any debris that may have accumulated in the flow cell during storage

3. Set-up the "in" rubber tube inside of a centrifuge tube containing the sample. Place the "out" end into a waste beaker. Turn on the pump so the sample pushes

the water in the tubing to the waster beaker. Allow this to continue for ~30 seconds

4. If the sample needs to be reused later, or if there is only a small amount of sample to begin with, place the outlet into the centrifuge tube so the sample recycles.

5. (Static measurement only) Make sure the flow cell pump is off when starting the measurement. Start analysis.

   (Dynamic measurement only) Make sure the flow cell pump is on (low speed setting, #5, 22.5 ml/min) when starting the measurement. Start analysis.

   Notice An external measurement will most likely take place before the first sample measurement begins. The external sample holder and reference will need to be placed on the spectrometer- so carefully remove the flow cell add-on and proceed with the external reference measurement

6. (Static measurement only) After each individual measurement sequence, turn on the pump and circulate for ~10 seconds to get a good representation of the overall sample

7. Once the sample has been measured, flush the flow cell tubing with a suitable cleaning agent to ensure no residue is left behind, which could cause mold formation. If more samples of the same batch are to be collected, flush the tubing with DI water.

<u>Analyzing samples using the petri dish add-on</u>

1. Place the petri dish add-on onto the spectrometer

2. While the application for the petri dish with transflectance cover method is loading, place 4-7 mL of the sample into a glass petri dish. Carefully lower the

transflectance cover on top of the sample ensuring no air bubbles are trapped by viewing through the bottom of the dish.

3. Set the petri dish with sample onto the spectrometer and click “start”.

   Notice An external measurement will most likely take place before the first sample measurement begins. The external reference will need to be placed onto the sample holder

4. After each individual measurement sequence, lift the transflectance cover off the petri dish and gently agitate the sample. Place the transflectance cover back on ensuring no air pockets.

5. Once the sample has been measured, clean the petri dish and transflectance cover with a suitable cleaning agent to ensure no residue is left behind, which could cause mold formation. If more samples of the same batch are to be collected, rinse each with DI water and dry.

<u>Defining properties and property (reference) values</u>

1. Open NIRWare Management Console and select “Sample Management” from the left index pane

2. Select “Properties” and click on “new property” to create the property of interest

3. Define the new property and fill-in necessary fields and click the “save” icon

4. Next, begin matching up samples to properties by selecting “samples” from the left index pane beneath “Sample Management”

5. Sort the list of samples by their application name, then select those for which you want to define property values for by holding Ctrl for multiple selection

6. Click “open existing data set” icon in the top left corner to create a new sample set

7. Assign properties (created in step 3) by selecting them from the “available properties” list and clicking the right directional arrow

8. The properties should then appear in the “samples” window with empty box that can be filled in with the property values for each sample

9. Once finished with inserting values, click the “save” icon

Creating a calibration in NIRCal

Setting up a project for editing in NIRCal

1. Upload desired spectra into a new project (see section on **Uploading spectra in NIRCal**)

2. Once spectra have uploaded, select which property you wish to put into calibration by highlighting properties in the NIR-Explorer window*

    a. Right click on the unwanted properties and select “remove from set→calibration”

    b. Repeat for any unwanted properties*

    Notice If no properties exist you must create at least one before proceeding with editing (see section on **Adding a property to a spectra set or to a new project in NIRCal**

    **It’s best to run just one property for each calibration

3. Now, you must put the newly added property into calibration

    a. First, create a copy of the “unnamed calibration” within the sub-folder of “calibrations” in the NIR-Explorer window

    b. Highlight “calibrations” and right click on “unnamed”, which should have a red dot by its title since it is considered “active”

c. Create a copy*

   Notice Creating a copy of the calibration is necessary because when you save the file to the C: drive, it freezes the current calibration making it unable to be edited; creating a copy calibration and setting it as "active" allows the user to edit the spectra

d. Rename the copy with the same name as the property you recently added

e. Set the newly named calibration as active by right clicking and choosing "set as active"

4. The newly added property should be set as the only property "into calibration". Check this by highlighting "properties" and verifying there is just one green push pin image next to the property you want to run the calibration

5. Select "CLU" for identification of differences in spectra or select either "PCR" or "PLS" for quantification of spectra if you wish to create a calibration curve*. These options are available in the toolbar at the top of the NIRCal workspace

   Notice Both PCR and PLS can also be used for simple identification of spectra differences; however the default plots displayed primarily focus on parameters affecting the calibration and validation curves

6. Select "calibration overview plots" ("O") from the same toolbar

7. Click "yes" when asked about having the software automatically creating both c-set and v-set

8. Proceed with viewing plots and modifying c and v-sets in the sections on either **Performing Quantification** or on **Performing Identification** depending on which method of analysis you chose in step 5).

Uploading spectra in NIRCal

1. Log onto NIRCal and select "Create a new project"

2. With the new project window active, select "Search and Import Spectra from Database"

3. Select desired spectra by clicking and holding ctrl key (to select multiple spectra) or using the shift key

4. Spectra will upload after selecting the green check mark

Adding a property to a spectra set or to a new project in NIRCal

1. Upload desired spectra into a new project

2. Save the file to the C: drive as .nir before adding properties by selecting File→Export→Project*

   Notice Generally, it's best to work on a .nir file that has been saved on the C: drive instead of working on a project out of the database since there are often editing restrictions in place once the file has been saved to the database

3. Open the .nir file (either by dragging and dropping into an empty NIRCal workspace or by right clicking and selecting "open"

4. In the NIR-Explorer window, right click on properties and choose "add"→click "ok" to add 1 property. Rename the newly added property

5. Characteristics need to be assigned to the newly created property so that prediction plots can be generated and compared to the original data

   a. Highlight "properties" within the NIR-Explorer window and right click to select "table original", which brings up a grid table*

      Notice If the software doesn't allow you to select "table original", it may be necessary to select "PCR", "PLS", or "CLU" first from the toolbar at the top of the NIRCal workspace

   b. If this property is meant to be used for identification of differences between spectra, then type either "1" (representing positive) or "0" (representing negative) in the grid box. Either of these values should correspond to the samples in the first column

   c. If this property is meant for quantification to create a calibration curve, insert values into the grid box to uniquely identify the treatment for a sample or group of samples

Close out of "table original" window when finished

Performing Quantification

1. Plots will have already been generated by the software after automatically assigning the c and v-sets when selecting "calibration overview plots" or "O"

2. The easiest way to achieve the best calibration curve is by running the automated "Calibration Wizard" under "Wizard" in the toolbar, but before this can be done two things must occur first:

    a) All scans/spectra of an individual sample must be entirely placed in **either** the c-set **or** the v-set. The software **does not** do this for you when it automatically generates the c and v-sets

    b) Pretreatments must be performed to help the user better understand the different plots as well as to give a more precise calibration when running the "Calibration Wizard". The following are some of the pretreatments that can be applied in order of importance:

        i) Normalizations and Derivatives

            - Normalizations are common when analyzing all samples (particularly those that are liquids) on the NIR in order to eliminate general variability caused by both the instrument and sampling effects. The list of options is under the toolbar heading "Calibration"→"Pretreatments"→"Normalization"

            - Derivatives are useful for identifying general spectra differences (1st Taylor Series) in the Pretreated Spectra and Regression Coefficients Plot or for identifying specific stretches in a sample's spectra that may differ from other samples (2nd Taylor Series). Options are available under "Calibration"→"Pretreatments"→"Derivatives"

            - Select "calibration overview plots" to refresh the plots after you've chosen the necessary pretreatment*

Notice There exists an "undo" button under "Calibration→Pretreatments", which must be selected before applying another series of pretreatments

ii) Predicted Property vs. Original Property Plot

- Select 2/3 of the data points representing sample spectra to be used for the c-set by pressing the "s" key (notice a change in the cursor image) and clicking and dragging around the points of interest*

  Notice Press "z" key at any time to return cursor to magnifying glass

- Next, right click and add the selected points to the calibration. If these points were initially used in the validation set you will need to right click and choose remove from validation so that each sample set is either entirely in the c or v-set*

  Notice Extreme sample sets (of either high or low values) should be used for calibration to define the limits of acceptance

- Repeat for the data points to be assigned to the validation (~1/3)

- Remove any spectra that appear irrelevant or as outliers by highlighting the points and removing from both the v-set and c-set

- Select "calibration overview plots" or "O" to refresh the plots after you've chosen the necessary pretreatment*

iii) V-Set Press

- Used to minimize error while determining calibration curve by reducing the Principle Components (PC's) in the v-set

- Open the v-set press window and look for one of the first minimum's that occur in the graph, most likely occurring in the lower range of PC's (2-5)

- Press the "q" key to change the cursor, then click and drag a box around the values in the x-axis that are greater than the PC value where the minimum occurs

- Right click and select "remove selection→from calibration PC's" to reduce the number of PC's*

  Notice 90% of the information for each spectra set is contained within the first PC as it has the largest impact when determining the calibration/validation. Precision improves only slightly when there is >1 PC

- Select "calibration overview plots" to refresh the plots after you've chosen the necessary pretreatment*

iv) Removing Pretreated Spectra Noise Region

- Remove excess noise in the spectra range, which occurs at higher wavelengths

- Select the "Pretreated Spectra" window and press the "q" key to see a change in your cursor

- Left click and drag a box around the x-axis corresponding to the noise region

- Right click and choose "remove selection→from calibration"

- Select “calibration overview plots” to refresh the plots after you’ve chosen the necessary pretreatment*

3. Once the pretreatments and new settings have all been applied, run the calibration wizard by selecting “Wizard→Calibration Wizard”. Be sure to choose the correct description of your sample (liquid, solid, gas, paste, etc.) and calibration behavior (usually “as precise as possible”). Click “OK”

4. The 5 best calibrations with the highest Q –values are found in the list in the NIR-Explorer window under “calibrations”*

   Notice To improve the Q-value and associated standard deviations in the calibration and validation sets, open the “Predicted Property vs. Original Property” window and continue adding or removing groups of spectra into and out of the c and v-sets. Remember to click “calibration overview plots” to refresh all the plots once changes have been made.

5. Choose the “save” feature once you are finished. **Remember to always save the project to the C: drive!**

Performing Identification

1. Plots will have already been generated by the software after automatically assigning the c and v-sets when selecting “calibration overview plots” or “O”

2. Pretreatments must be performed to help the user better understand the different plots. Normalizations and Derivatives are two of the more common pretreatments

   a. Normalizations are common when analyzing all samples (particularly those that are liquids) on the NIR in order to eliminate general variability caused by both the instrument and sampling effects. The list of options is under the toolbar heading “Calibration”→”Pretreatments”→”Normalization”

   b. Derivatives are useful for identifying general spectra differences (1st Taylor Series) in the Pretreated Spectra and Regression Coefficients Plot or for identifying specific stretches in a sample’s spectra that may differ from other samples (2nd Taylor Series). Options are available under “Calibration”→”Pretreatments”→”Derivatives”

c. Select “calibration overview plots” to refresh the plots after you’ve chosen the necessary pretreatment*

Notice There exists an “undo” button under “Calibration→Pretreatments”, which must be selected before applying another series of pretreatments

3. Look at different plots to begin understanding what differences exist for the samples

a. Scores vs. Scores (“Graphics→scores→2D”)

i) Change PC(x):1 and PC(y):2 to PC(x):1 and PC(y):1 to focus only on the 1st Principle Components since this is where 90% of the spectra information lies

ii) Click on the white space in the plot and select the “r” key

iii) Look for separation or clustering between groups of spectra by holding the cursor over each data symbol for identification*

Notice Looking at the Scores vs. Scores in 3D also serves as a good visual image to see clustering effects between groups of spectra

b. Pretreated Spectra

i) Look for dominating peaks such as H_2O (5200-5500 wavelengths), protein (4200-4600), and fat (5500-5800)

- could indicate whether samples were over-contaminated with water

- the more water there is in the sample means the temperature of the sample was easier to alter during the actual scan, thus, skewing results

ii) Choose one of these dominant peaks to zoom in and visually see if there was separation between the different spectra (which should be isolated by color), or to see if clusters of spectra can be considered one-in-the-same*

Notice Press the "f" key to zoom back to a fitted view

c. Loadings ("Graphics→Loadings")

i) Before examining the Loadings graph, it's important to apply a different pretreatment. The 2nd Derivative Taylor Series should be used in combination with one of the Normalization pretreatments*

Notice 2nd Derivative Taylor Series focuses on maximums and minimums of peaks instead of inflection points, which is used when applying the 1st Derivative Taylor Series

ii) The Loadings chart, when compared or laid over the Pretreated Spectra plot tells you what the actual differences in the spectra are. A straight horizontal line at 0 represents no difference, but any deviation from 0 represents a difference in measured samples

iii) In the toolbar, click on "Modules→chemical bonding", which turns on a feature that allows for identification of chemical stretches when the cursor is placed over an area of the chart in the

Pretreated Spectra. The different stretches are displayed at the bottom of the NIRCal workspace window

4. Choose the "save" feature once you are finished. **Remember to always save the project to the C: drive!!**

Integrating a calibration into an application

1. Open the NIRWare Management Console software and click on "open" from the "Application Designer" option in the left index pane
2. Select the application that had been created in the section Creating an application for spectra collection
3. Click the "copy dataset" icon from the list of icons in the top toolbar

   Notice Remember, if the application had been placed into the "Approved idle" state then no changes can be made. This is why a copy needs to be made. If the status had been left at "Created idle" then the following steps can be performed in the original application
4. Before making any changes to the copied (or original) application, the "Edit data set" icon needs to be selected from the top toolbar
5. Once in edit mode, the application name and description can be changed
6. Next, make sure the number of measurement sequences is 1 within the "Operator Configuration" settings. Click on the "save icon"
7. Select "Properties" and then "new" within the application from the index pane
8. Click on the down arrow symbol in the "Assigned calibration" field and set a check mark in front of the calibration to use. Select the green arrow to confirm the addition of this calibration

Notice This should be one of the calibrations created and saved in the last step of **Performing Quantification** in Creating a calibration in NIRCal

9. Repeat for each property that is to be measured by the application and click the "save" icon

10. This application can now be used by the Administrator regardless of state, or it can be restricted for operator use by changing the state to "Approved" in the top icon toolbar

Total Protein

Objective

The purpose was to determine the total amount of protein in raw milk samples.

Materials and Equipment

- Kjeldahl digestion flasks (20)
- Kjeldahl digestion/distillation equipment
- Titration equipment
- Sulfuric Acid (95-98%) (25 mL x # *of samples)*
- 0.1 N sulfuric acid (~1 L)
- Kjeldahl tablets (mercury and selenium-free)
- DI Water (50 mL x # *of samples*)
- Sodium hydroxide (32%) (100 mL x # *of samples*)
- Boric Acid (4%) (50 mL x # *of samples*)
- Methyl red indicator

Procedure- Preparation

1. Gently agitate milk to ensure homogeneity
2. Warm milk to 38 °C
3. Weigh milk (5 mL) into digestion tubes
4. Place Kjeldahl tablets (2 x 2.5 g) into each digestion tube
5. Carefully add 25 mL sulfuric acid to each digestion tube inside hood
6. Prepare two blanks with a Kjeldahl tablet and sulfuric acid
7. Place tube rack above digester and press start just once (digestion temperature ~420 °C)

Procedure- Digestion

1. Upon "ready" indicator, turn on water to condenser in back of hood space
2. Lower the digestion tube rack and top manifold onto the heated wells
3. Press start once more and make sure the scrubbing device turns on
4. Once finished, allow samples to cool on heating block, and turn off power supply once fuming ends
5. Add DI water (50 mL) to each digestion tube once cooled

Procedure- Distillation

1. Plug in and start distill unit
2. Ensure full DI water and sodium hydroxide containers
3. Turn on condensing water behind distillation unit

4. Once the light and sound indicate the unit has been preheated, add ~50 mL to an empty digestion tube and secure it to the distill unit
5. Press start to begin 5 min distillation to flush the lines and equipment
6. When finished, remove digestion tube with safety gloves
7. Load the first sample digestion tube
8. Add 50 mL of boric acid to the receiving flask (250 mL) and add two drops of methyl red indicator
9. Dispense sodium hydroxide (100-115 mL) by briefly pressing the reagent button (~7.4 on reagent timer display). The sample should turn yellow-light yellow
10. Press start to begin the 5 min distillation. Repeat with all samples

Procedure-Titration

1. Turn on the titrator using the switch in the back
2. Calibrate the pH meter (refer to quick manual near titrator unit)
3. Set up autotitrator with 0.1 N sulfuric acid
4. Flush burette lines with the above reagent to ensure no residual reagents are left in the lines from previous titrations
5. Set the method settings to "5" and proceed to titrate samples to pH = 5.2

   Notice The max volume to be dispensed setting should be >50 mL in case nitrogen content of samples is high

Expressing Results

Calculate the total protein content of the sample using the following equation

% total protein = % nitrogen x 6.38

$$\% \text{ nitrogen} = \frac{1.4007(V_n - V_b)(N_{acid})}{M_n}$$

where

V_n = volume of acid used to titrate sample, *n* (mL)

V_b = volume of acid used to titrate blank sample (mL)

M_n = mass of sample (g)

N_{acid} = normality of acid used for titration (mol/L)

Casein

Objective

The purpose was to determine the amount of casein in raw milk samples

Materials and Equipment

- All materials and equipment needed for total protein analysis
- Sodium acetate solution (1 M/L) (50 mL)
- Acetic acid solution (10% v/v) (50 mL)
- Buffer solution (sodium acetate solution/acetic acid solution) (100 mL)
- Filter paper (Whatman No. 1, 15 cm, nitrogen-free)
- Funnel, ceramic
- Receiving flask (500 mL) with vacuum attached

Procedure-Preparation

1. Gently agitate milk to ensure homogeneity
2. Warm sample to 38 °C
3. Weigh sample (5 mL) into Kjeldahl flask
4. Add DI water (70 mL) to each flask
5. Add acetic acid solution (0.75 mL) to flask and gently agitate
6. Allow precipitant to settle for 10 min as indicated by pH of 4.6
7. Add sodium acetate solution (0.75 mL) to flask and gently agitate
8. Pour mixture from flask through filter paper and funnel into receiving flask under vacuum. Let drain completely
9. Meanwhile, add buffer solution (30 mL) to Kjeldahl flask and gently agitate
10. Pour mixture through same filter paper and combine filtrates
11. Repeat with another buffer solution rinse
12. Remove filter paper and allow to slightly dry before twisting closed and placing back into original Kjeldahl flask
13. Repeat with remaining samples and proceed with digestion as in total protein analysis
14. Digest and distill at least one blank sample containing filter paper, sulfuric acid, and a Kjeldahl tablet

Expressing Results

Calculate the casein content of the sample using the following equation

% casein = % casein nitrogen x 6.38

$$\% \text{ casein nitrogen} = \frac{1.4007(V_n - V_b)(N_{acid})}{M_n}$$

where

V_n = volume of acid used to titrate sample, n (mL)

V_b = volume of acid used to titrate blank sample (mL)

M_n = mass of sample (g)

N_{acid} = normality of acid used for titration (mol/L)

Fat

Objective

The purpose was to determine the percentage of fat in raw milk samples

Materials and Equipment

- Weighing dishes (8.5-9.5 cm diameter and 4.5-5.5 cm tall)
- Vacuum oven- Vacuum oven must be capable of maintaining a temperature between 70-75□C at >20 in vacuum.
- Desiccator
- Mojonnier-type fat extraction flasks
- Corks (natural cork stoppers, appropriate size to fit fat extraction flask)
- Raw milk samples
- Sodium Hydroxide (32%) (1.5 mL)
- Phenolphthalein indicator
- Ethyl alcohol (95%) (15 mL)
- Diethyl ether, certified ACS, BHT stabilized (55 mL)

- Petroleum ether (55 mL)
- Fume hood

Procedure- Preparation of Weighing Dishes

1. Number clean weighing dishes
2. Pre-dry in a vacuum oven maintained at 25.5 inches Hg for 2 hours at 70-75 °C
3. Remove from vacuum oven and put in a desiccator and cool to room temperature

Procedure- Preparation of Sample

1. Weigh sample (10 g) into the lower bulb of a Mojonnier flask
2. Add ammonium hydroxide (1.5 mL) to the sample and mix thoroughly in the small bulb of the flask
3. Add 3 drops of phenolphthalein indicator to help see the interface between aqueous and ether layers
4. For the first extraction, add ethyl alcohol (10 mL), stopper with a cork, and shake the flask for 15 seconds
5. Add ethyl ether (25 mL), replace cork, and shake flask very vigorously for 1 min, releasing built-up pressure by loosening the cork as necessary
6. Add petroleum ether (25 mL), replace cork, and repeat vigorous shaking for 1 min
7. Place flasks in sample holder and use Mojonnier centrifuge to create separation of ether and aqueous layers
8. Remove the cork and carefully decant the ether solution into the weighing dish. When the ether solution is decanted into the dish, be careful not to pour over any

suspended solids or aqueous phase. Ether can be evaporated in the hood while the second evaporation is being conducted

9. Carry out a second extraction by repeating steps 4-8, except add 5 mL ethyl alcohol instead of 10 mL, and add 15 mL each of ethyl ether and petroleum ether instead of 25 mL portions. Rinse the inside of the neck of the extraction flask when adding the ethers. If the interface of the aqueous and ether layers is below the neck of the flask after resting, add water to bring the level approximately halfway up the neck to enable the final decantation of ether to be as complete as possible. Add the water slowly down the inside surface of the flask so that there is minimum disturbance of the separation

10. Carry out a third extraction without the addition of ethanol and by repeating steps 4-8. Again, only use 15 mL of ethyl ether and 15 mL of petroleum ether. Rinse the inside of the extraction flask when adding the ethers. If necessary, raise the interface by the addition of water

11. Completely evaporate solvents collected in the weighing dish in a fume hood

12. Dry weighing dishes with extracted fat to a constant weight in a vacuum oven at 70-75 °C at 25.5 inches of Hg for 2 hours. Also insert two blank weighing dishes into vacuum oven

13. Remove weighing dishes from oven and place in a desiccator to cool to room temperature

14. Weigh dry, cooled dishes to the nearest 0.1 mg

Expressing results

Calculate the fat content of the sample using the following equation

$$W_f = \frac{(M_1 - M_2) - B}{M_0}$$

where

W_f = the percentage of fat by mass of the sample

M_0 = the mass (g) of the sample

M_1 = the mass (g) of the weighing dish and the extracted fat

M_2 = the mass (g) of the empty sample weighing dish

B = the mass (g) of any dry residue in the blank weighing dish, which is calculated by subtracting the weight of the empty blank weighing dish from the weight of the weighing dish and any extracted residues

Moisture

Objective

The purpose was to determine the moisture of raw milk samples.

Materials and Equipment

- Raw milk samples
- Vacuum oven (100 °C)
- Forced air oven on "low" setting (95 °C) (used for pre-drying samples)
- Vacuum drying oven (100 °C)
- Desiccator
- Weighing pans (metal, round, flat-bottomed, 5 cm in diameter)

Procedure- Preparing weighing dishes

1. Preheat vacuum oven to 100 °C

2. Label aluminum, 65 mm diameter weighing dishes with sample ID's and blanks
3. Dry aluminum weighing dishes in the oven/dryer at 100 °C for one hour and store in desiccator at room temperature until ready for use

Procedure- Preparing samples

1. Gently agitate milk to ensure homogeneity
2. Warm milk to 38 °C
3. Weigh milk (5.0-5.5 g) into weighing pans, analyze in duplicate. Additionally, create two blank weigh dishes
4. Place aluminum dishes in forced air oven on "low" (95 °C) for 4 hours, or until no free movement of milk can be seen
5. Next, place aluminum dishes in the 100 °C vacuum oven
6. Dry to constant weight under 0.683 m of Hg for 4 h
7. Admit dried air into the oven during drying, about 117 on gauge, dried by passing through a solid desiccant of indicating calcium sulfate
8. Stop vacuum pump and carefully allow dried air into the oven
9. Remove the weighing pans from the oven and place in a desiccator. Cool to room temperature
10. Weigh the cooled, dried samples

Expressing results

Calculate the moisture content of the sample using the following equation

$$\% \text{ moisture (w.b.)} = \frac{(M_0 - M_1)}{M_0} x100$$

where

M_0 = the initial mass (g) of the sample

M_1 = the mass (g) of the dried sample

Ash

Objective

The purpose was to determine the total ash content of raw milk samples.

Materials and Equipment

- Muffle furnace (550 °C)
- Forced air oven on "low setting" (95 °C) (used for pre-drying samples)
- Crucibles, porcelain (50 mL)
- Desiccator
- Raw milk samples
- Hydrochloric acid
- Nitric acid

Procedure

1. Record the weight of each crucible when empty. Be sure to include a blank crucible. Perform in duplicate
2. Gently agitate milk to ensure homogeneity
3. Warm milk to 38 °C

4. Weigh milk (5.0-5.5 g) into crucibles, analyzing in duplicate
5. Place crucibles and contents into forced air oven on low setting (95 °C) for 4 hours, or until no free movement of milk can be seen
6. Next, place crucibles with contents in muffle furnace for 12 h. Draw a picture of the orientation of the crucibles in the furnace in case the labels disappear
7. Turn off the furnace and allow samples to cool. Do not open the furnace door
8. Check for a drop in temperature with a thermometer after ~8 h
9. Remove the samples from the furnace when the temperature of the furnace is ~100 °C
10. Transfer the crucible and contents with tongs to a desiccator in the order that they were in the furnace
11. Weigh the ash after it has cooled to room temperature in a desiccator

<u>Expressing results</u>

Calculate the ash content of the sample using the following equation

$$\% \text{ ash (w.b.)} = \frac{M_1}{M_0} x100$$

where

M_0 = the initial mass (g) of the sample

M_1 = the mass (g) of the dried ash

A.2.2 Expanded Results

Chemical Analysis

Table A.2.1. Individual analysis of variance for each chemical property with lot sampled (Lot) as the main effect

Sources of Variation	Degree of Freedom	Fat	Total Protein	Casein	Ash	Moisture
Reps in Lot	47	1.84*	0.88	0.73*	0.06	9.40
Lot	5	737.4*	1015.5*	744.6*	10.2*	383.5*
Error	235	5.56	5.28	2.41	0.32	33.50

* = significant at 95% confidence interval

The results from the individual analysis of variance for fat, total protein, casein, ash, and moisture can be seen in Table A.2.1. Samples from different lots differed significantly at the 95% confidence interval for all chemical properties, which is not surprising given that raw whole milk gathered from different lots would have been produced from cows of different breeds or lactation stages. Skim milk had the majority of its fat content removed causing an increase in the overall moisture content. Meanwhile, samples fortified with whole milk powder (WMP) and milk protein concentrate (MPC) contained lower overall moisture and had higher fat and total protein/casein content, respectively. Ash was also higher for fortified samples. The results from Fisher's LSD test for each property can be viewed in their respective figures (Figures 25a-e).

Table A.2.2a. Combined analysis of variance for each measured response with method of homogenization and presentation method as the main effects. Calculated using data in Tables 6, section 4.3.2, and Table 7, section 4.3.3.

		P-value				
Source of Variation	Degree of Freedom	Q-value	SEC	R^2_{cal}	SEP	R^2_{val}
Property	4	0.0849	2e-12***	6e-9***	5e-3***	9e-8***
[1]Homogenization (*H*)	3	2e-9***	1e-8***	3e-6***	3e-7***	0.5006
[2]Presentation (*P*)	2	0.0057*	3e-6***	0.2452	2e-2***	0.0733
H x *P*	5	0.0047*	0.4124	0.5263	3e-3***	0.0681

[1]Homogenization = Method of Homogenization
[2]Presentation = Method of Presentation
* = significant above 95% confidence interval
*** = significant above 98% confidence interval

Table A.2.2b. Combined analysis of variance for Q-value and SEP after a multiplicative inverse transformation of the original values. Homogenization and presentation methods were treated as main effects

		P-value	
Source of Variation	Degree of Freedom	$Q\text{-value}^{-1}$	SEP^{-1}
Property	4	0.3000	< 2e-16***
[1]Homogenization (*H*)	3	7e-4***	2e-3***
[2]Presentation (*P*)	2	0.1363	6e-3***
H x *P*	5	0.0952	0.4962

[1]Homogenization = Method of Homogenization
[2]Presentation = Method of Presentation
* = significant above 95% confidence interval
*** = significant above 98% confidence interval

During statistical analysis, there were three effects (property, method of homogenization *H*, and presentation, *P*) capable of influencing the following caibration quality parameters: Q-value, SEC, R^2_{cal},SEP, R^2_{val}. The method of homogenization and presentation method were considered the main effects, while the properties measured were secondary effects. Since analysis would be performed separately for each property, it was desired for the interaction between the two main effects to be insignificant so the results could be viewed and discussed independently. However, Table A.2.2a shows the interaction term (*H* x *P*) is significant for Q-value and SEP. A multiplicative inverse transformation of the original data for these two measurements was necessary to remove the significance in the interaction term (Table A.2.2b). (More common transformations such as log and square root functions were ineffective).

The transformed Q-value and SEP data (and original data for SEC, R^2_{cal}, and R^2_{val}) was used in Tables A.2.3 and A.2.4 for the individual analysis of variance based on each property. Also, the transformed data had been applied during analysis of pairwise comparisons by Fisher's LSD tests (Table 6, section 4.3.2, and Table 7, section 4.3.3). For reporting and comparison of treatment means in both Table 6 (section 4.3.2) and Table 7 (section 4.3.3), Q-value and SEP were back-transformed while maintaining the 95% confidence intervals so that results from Fisher's LSD tests would still hold true.

Table A.2.3. Individual analysis of variance for Q-value^{-1}, SEC, R^2_{cal}, SEP, and R^2_{val} for each of the five properties measured with methods of homogenization as the main effect

Property (%)	[1]Sum of Squares for Homogenization Methods				
	Q-value^{-1}	Calibration		Validation	
		SEC	R^2_{cal}	SEP^{-1}	R^2_{val}
Fat	0.0429	1.41e-4	1.89e-4	1543	9.09e-4*
Total Protein	0.4759*	1.07e-4	5.80e-5	696	5.07e-6
Casein	0.3592*	4.41e-5	2.51e-5*	16050*	2.43e-5
Ash	0.0342	4.72e-6*	5.57e-4*	89611*	7.48e-5
Moisture	5.113	2.69e-4	3.28e-5*	2180*	6.81e-5

[1]Calculated using 3 and 7 degrees of freedom for the homogenization method main effect and for error, respectively
* = significant above 95% confidence interval

Table A.2.4. Individual analysis of variance for Q-value^{-1} , SEC, R^2_{cal}, SEP, and R^2_{val} for each of the five properties measured with presentation methods as the main effect

Property (%)	[1]Sum of Squares for Presentation Methods				
	Q-value^{-1}	Calibration		Validation	
		SEC	R^2_{cal}	SEP^{-1}	R^2_{val}
Fat	0.0132	1.30e-4*	9.37e-6	880	2.88e-4
Total Protein	0.0760	1.12e-4	6.42e-6	5740	2.79e-5
Casein	0.0719	3.85e-5	8.18e-7	1775	1.08e-4
Ash	0.0304	1.55e-6	2.07e-5	26303	3.66e-4
Moisture	1.6473	2.88e-4*	4.59e-6*	154	1.33e-4

[1]Calculated using 2 and 8 degrees of freedom for the presentation method main effect and for error, respectively
* = significant above 95% confidence interval