THE PREDICTION OF MILK FAT GLOBULE SIZE DISTRIBUTION USING MID-INFRARED

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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August 2016

ABSTRACT

Mid-infrared (**MIR**) milk analyzers provide rapid, cost effective and direct determination of milk components. However, the accuracy of MIR readings can be impacted by homogenization efficiency. Poor homogenization fails to break fat globules to smaller sizes. Large fat globules increase light scattering, leading to an inaccurate estimation of milk components. There is a need for an efficient and accurate method built into the MIR milk analyzer that could be used to warn the instrument operator that the homogenizer is near failure and needs to be replaced to ensure quality of results.

Our first objective was to develop partial least squares (**PLS**) models using data from MIR spectra to predict the particle size of milk fat globules and validate the models. PLS models were developed and validated for predicting the following parameters: particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3]. The accuracy of the prediction models provide an alternative for routine quality assurance to monitor the daily average of the particle size performance and determine if the homogenizer within a MIR milk analyzer is near the failure level and needs to be replaced. This will help maintain repeatable and accurate milk testing.

Our second objective was to determine the impact of homogenization efficiency on accuracy and repeatability of MIR predicted fat, true protein, and anhydrous lactose determination given by traditional filter models versus PLS prediction models. Repeatability and accuracy were determined by conducting 17 sequential MIR readings on externally homogenized and unhomogenized milk using five in-line MIR homogenizers with different performance. Variation in the homogenizer performance

on unhomogenized milks had a much larger impact on accuracy of component testing than on repeatability. The increase of particle size distribution d(0.9) due to poor homogenization impacted fat tests the most and lactose tests the least. The accuracy of both traditional filter models and PLS models was influenced by poor homogenization.

BIOGRAPHICAL SKETCH

Larissa Vieira Di Marzo was born in March of 1988 in Sao Paulo, Brazil. After graduating in 2005 from her high school, Colegio Cecilia Cacapava Conde, she attended one year preparatory course for her B. S. In 2007, she began her B. S. studies in food engineering at the University of Sao Paulo (USP), Brazil. During 2007 she performed undergraduate research at the laboratory of functional genomics at USP and was awarded at the 17th International Symposium of Undergraduate Research from USP with honorable mention and a travel to University of Porto, Portugal, were she presented her project. In 2008, she performed undergraduate dairy science research at the laboratory of technology of animal products at USP. After two years working with research she had the opportunity to spend one year working in United States. In the first semester of that year she conducted research at the Animal Science Research Center at University of Missouri, MO. In the second semester she conducted a project at the Northeast Dairy Foods Research Center at Cornell University, NY. This project was select as the best reference method developed by an undergraduate student and she received the Ines Cereijo TDRM AOAC International Student Award 2012. During 2012, Larissa completed one year internship at the department of the Milk Business at Dannon Company in Pocos de Caldas, Brazil. During her internship, she realized that she could make contributions to the dairy industry through development of new analytical tools that will support more efficient and sustainable production of milk at the farm and improve control of dairy product manufacturing processes and safety of dairy foods.

Larissa graduated from college in 2013 and enrolled at Cornell University in the M. S. program in food science in the fall of that year. At Cornell, she received the 2016

WNY IFT awards in recognition of her outstanding achievements in Food Science. She also participated of the IFTSA & MARS product development team and was engaged in Christian organizations around campus. When she graduates she will continue her studies with a doctoral training to develop measurement tools in milk analysis that will help improve dairy farm management and quality control.

I dedicate this work to God, who is my refuge, my strength, and helped me through all the steps of this journey.

ACKNOWLEDGMENTS

First, I would like to express my deepest appreciation to my advisor, Dr. David Barbano, for all the time dedicated towards tutoring and encouraging me and for inspiring me to become a better researcher each day.

I want to extend my gratitude to Dr. Michael Van Amburgh, professor of the Department of Animal Science, for guiding me in my minor studies.

I am grateful to the Northeast Dairy Foods Research Center, and the Cornell University Department of Food Science for funding my research.

I would like to thank Chassidy Coon, Michelle Billota, and Sara Bova of Cornell University for their technical assistance.

I thank my fellow labmate Michael Adams for assisting me through these past years.

I also thank my dearest friend Fabiana Duarte for all friendship and time spent helping me during this journey.

I would like to give special thanks to my parents, Luiz Carlos and Eliete Di Marzo, for all love, support, advices, and for being so present in my life even when we are physically distant. I thank you for never measuring effort to help me. I also thank my sister Tamires Di Marzo, and my brother Rodrigo Di Marzo, for all love and friendship.

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LIST OF ABBREVIATIONS

ANCOVA.....Analysis of covariance

CLS.....Classical least squares

EDTA.....Ethylenediamine tetraacetate

FTIR.....Fourier transform infrared

FPRESS.....F-Test predicted residual sum of squares

ILS.....Inverse least squares

MD.....Mean difference

MIR.....Mid-infrared

PLS.....Partial least squares

PRESS.....Predicted residual sum of squares

RO.....Reverse osmosis

RPD.....Residual prediction deviation

RSD.....Relative standard deviation of the difference

SAS.....Statistical analysis system

SDD.....Standard deviation of the difference

SECV.....Standard error of cross validation

SEM.....Scanning electron microscopy

TEM.....Transmission electron microscopy

CHAPTER ONE

INTRODUCTION

Overview of Milk Components Analysis

Reference Chemistry for Mid-Infrared Calibration. Annually, in the United States, millions of pounds of milk are produced and tests are performed daily for the determination of main components (i.e., fat, true protein, and other solids). The test results are used by processors to determine the payment of dairy farmers, and by the farmers for dairy herd management (Lynch et al., 2004; Barbano and Lynch, 2006). Considering the large volume of milk produced daily, a large number of tests need to be performed, yet the chemical methods for each component are very time consuming and not practical. Mid-infrared (**MIR**) milk analyzers have been used and have positively impacted dairy industries and farmers by providing rapid, cost effective and direct determination of milk components (Barbano and Clark, 1989; Lynch et al., 2004; Lynch et al., 2006; Adams and Barbano, 2015).

The MIR milk analyzers are used as secondary test and the instruments need to be calibrated by chemical reference methods (Lynch et al., 2006; Wojciechowski et al., 2016). The following AOACI (2000) chemical reference methods currently used for analysis of main components of milk are: modified Mojonnier ether extraction for fat (AOAC method 989.05), Kjeldahl for true protein (AOAC method 991.22), oven drying for total solids (AOAC method 990.20), and enzyme analysis for anhydrous lactose (AOAC method 2006.06) modified to measure lactose by weight instead of volume (Lynch et al., 2007; Wojciechowski et al., 2016). In recent years a set of 14 modified

milks was developed and optimized targeting the improvement of analytical performance of chemical reference methods used for calibration of MIR milk analyzers, resulting on the improvement of MIR accuracy (Wojciechowski et al., 2016).

Milko-Testers. The determination of milk fat based on the measurement of light scattering was first presented by Haugaard and Pettinati (1959). In this method, milk was homogenized to ensure uniform fat globule size distribution. Coherent scattering was eliminated by diluting milk sample with sodium (tetra) ethylenediamine tetraacetate (**EDTA**) solution. The turbidity caused by the casein micelles was eliminated by a calcium chelating agent, enabling the use of visible light (600 cm^{-1}). Since turbidity caused by the casein micelles was eliminated, the method ensured that light scattering was exclusively due to fat globules in milk. The remaining factors that impact light scattering were fat percentage and average size of the fat globules. Therefore, two independent measurements were necessary to solve for one or both of these quantities. Detailed theory behind this method have been discussed by Haugaard and Pettinati (1959).

In 1964, the first instrument designed to quantify the fat content of milk by the measurement of scattered light was produced by Foss Electric (Hillerod, Denmark) and was called the Milko-tester (Shipe, 1969). The Milko-tester worked based on the principle described above, with the difference that fat percentage reading was directly measured by the amount of light passing through a photocell and registered on a calibrated galvanometer (Shipe, 1969). In 1969, a collaborative study compared the Milko-tester (Foss Electric, Hillerod, Denmark) to the Babcock test, which is used as one of the reference methods for milk fat determination (Shipe, 1969). Based on the

results presented in this collaborative study the Foss Milko-tester was approved as AOAC official indirect method for determination of fat content in raw, unhomogenized milk (Shipe, 1969; Shipe 1972; Shipe and Senyk, 1973). A series of other collaborative studies were made: Shipe (1972) compared Babcock, Mojonnier, and Foss Milko-tester; Shipe and Senyk (1973) compared Babcock and Foss Milko-tester. These studies also compared different models of Milko-testers: Mark II, Mark III and Automatic (MTA). Later, a collaborative study to determine the reliability of the Mark III Industrial Model Milko-tester was developed. This model was designed to analyze unhomogenized and homogenized milk samples (Shipe and Senyk, 1975).

The critical factor for Milko-tester measurements was the size of milk fat globules, which is dependent of homogenizer efficiency. The homogenizer efficiency used to be checked by comparing the milk fat content after two homogenizations of the same milk. If the homogenizer had poor performance, it was expected a change on the readings after the second homogenization (Shipe, 1969).

Evolution of Mid-Infrared Milk Analyzers. The determination of milk components using MIR analysis is based on the principle that each specific chemical bond absorbs MIR energy at a specific wavelength, and the measurement of the intensities of the absorption peaks makes it possible to quantify milk components (Goulden, 1964; Biggs, 1967; Biggs et al., 1987). Molecules are made up by group of atoms chemically bonded together. When the MIR energy passes through milk, the MIR energy is absorbed by the chemical bonds, which moves the bond to an excited vibrational state. The absorption of energy from the light beam reduces the energy measure compared to the light beam energy without a sample. The intensity of the light

absorbed (i.e., signal amplitude) is read by the detector and recorded as function of frequency. The MIR spectra gives information about the chemical bonds present in the sample. Moreover, it makes possible to quantify a chemical component based on the quantity of absorbed radiation by each chemical bond. According to the Lambert Beer's law, there is a direct relationship between absorbed radiation and concentration of the milk component being measured (Biggs et al., 1987). The MIR frequencies from 1000 to 4000 cm^{-1} is commonly used for measurement of milk components (Agnet, 1998).

Characteristics of the Mid-Infrared Optical Bench. MIR instruments have used a prism, grating, and finally optical interference filters to isolate specific wavelengths from the MIR light. A prism or diffraction grating were used in early research based scanning MIR spectrophotometers to produce the full spectrum of a sample and generally were not designed for speed or for analysis of aqueous samples. Aqueous samples were a challenge because the typical cuvettes for liquid samples had sodium chloride windows (i.e., transparent to MIR light) and the optical path within the instrument was protected from variation in moisture content in the ambient room air. In the early evolution of MIR analysis of aqueous samples, calcium fluoride cuvette windows were developed (i.e., relatively transparent to MIR light with low solubility in water) and sealed optical benches with desiccant inside were added to the MIR spectrophotometer. However, scanning MIR spectrophotometers were too slow (5 to 20 minutes per sample) for high speed analysis of large numbers of samples and therefore optical filters for specific analytical purposes were developed.

Optical interference filters (Figure 1.1) are circular disks made of crystals layers attached to a filter wheel that moves, in sequence, each filter into the light path, allowing

a band of wavelengths of light to pass through the milk sample (Kaylegian et al., 2009). Most optical filter-based MIR milk analyzers used four sample filters: fat B (carbon-hydrogen stretch, 3.48 µm), fat A (carbonyl stretch, 5.72 µm), protein (amide stretch, 6.47 µm), and lactose (hydroxyl stretch, 9.61 µm) in combination with four reference filters: fat B (3.6 µm), fat A (5.6 µm), protein (6.7 µm), and lactose (7.7 µm) (Biggs et al., 1987; Smith et al., 1993; Lynch et a., 2006). The reference filters reduce the effect of water absorption and light scattering which are strong in the wavelength region between 2 to 10 µm (Goulden, 1964; Biggs et al., 1987).

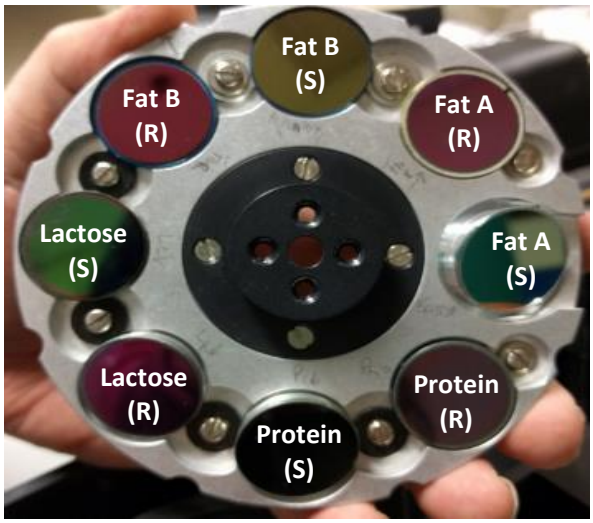

Figure 1.1. Illustration of the optical interference filter wheel with fat B, fat A, protein, and lactose sample (S) and reference (R) filters.

The absorption difference between sample and reference wavelength pair for both the milk sample and the zeroing solution are determined (Biggs et al., 1987). Because the optical filters varied slightly in their transmission characteristics (Smith et al., 1995) from one to the next the mean wavelength and bandwidth of the filters varied from instrument to instrument. The alignment and tilt of the optical filter in the filter

wheel also influence the mean wavelength and bandwidth produced by the filter (Smith et al., 1995). This created a challenge because the intercorrection factors need to cancel out water displacement effects and the effects of background variation in the concentration of other milk components had to be determined for each instrument and limited the accuracy and instrument to instrument agreement in test results.

To ensure that the MIR meets minimum mechanical and electrical performance standards that will allow it to obtain accurate results, a precalibration evaluation is performed before calibration (Barbano and Clark, 1989; Lynch et al., 2006). The precalibration evaluation is performed by the following steps: (1) flow system check, (2) homogenization efficiency, (3) water repeatability, (4) zero shift, (5) linearity, (6) primary slope, (7) milk repeatability, (8) purging efficiency, and (9) intercorrection factors. Detailed information about each precalibration step was given by Barbano and Clark (1989) and Lynch et al. (2006). If the MIR passes the evaluation, then the accuracy of the test results should be dependent on the accuracy of the chemical reference tests and the ability of the MIR operator to handle and mix the samples properly (Barbano and Clark, 1989; Lynch et al., 2006).

The most currently used version of MIR milk analyzer is the Fourier Transform Infrared (**FTIR**), which uses an interferometer to generate, within a few seconds, a complete MIR spectra of each sample. There are 3 different manufacturers of FTIR milk analyzers, two manufacturers use a Michelson interferometer and the third uses a wishbone interferometer design.

The general design of the Michelson interferometer is shown in Figure 1.2 and consists of a MIR light source (#1), a beam splitter (#2), two mirrors: one fixed (#3) and another that can move along an axis that is perpendicular to its plane (#4), a sample cell (#5), and a detector (#6).

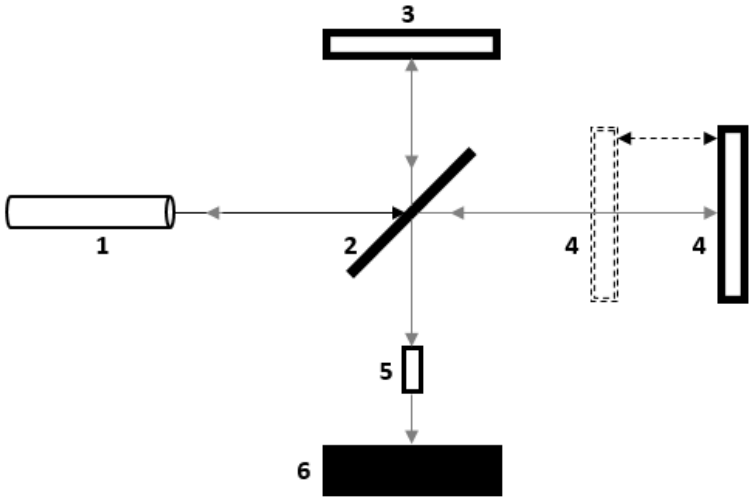

Figure 1.2. Schematic diagram of the Michelson interferometer: (1) MIR light source, (2) beam splitter, (3) fixed mirror, (4) movable mirror, (5) sample cell, and (6) detector.

The full spectrum of MIR light beam passes through a beam splitter. The beam splitter is used to divide the MIR light into two paths: one partially reflected to the fixed mirror (#3) and another partially transmitted to the movable mirror (#4). The two beams of different path length (one fixed and one variable) produce constructive and destructive interference as a result of the path difference generated by the position of the two mirrors. The light beams return to the beam splitter, recombine, pass through the sample cell (#5) and reach the detector (#6). The variation in energy intensities of the MIR light sensed by the detector at each frequency (f) is recorded as a function of

path difference of the beams generated by the movable mirror (Van de Voort, 1992; Griffiths and Haseth, 2007). In practice, an interferogram is collected on the blank (i.e., zeroing solution) first. Fourier transformation is used to convert this interferogram into the blank transmission spectra (S_o). Next, an interferogram is collected on the milk sample. Fourier transformation is used to convert this interferogram into the milk sample transmission spectra (S). The blank transmission spectra and the milk sample transmission spectra are used to calculate the absorbance spectra giving as final result by the MIR using the following equation: $A(f) = \log [S_o(f) / S(f)]$ (Agnet, 1998). The accuracy of the results (and the wavelength determination) given by an instrument using Michelson interferometer is dependent on the alignment of the fixed mirror in relation to the movable mirror and the maintenance of an exact angle of the movable mirror in relation to the plane of the beam splitter (Griffiths and Haseth, 2007). A reference solution (e.g., equalizer) is used to check the accuracy of wavelength calibration. Furthermore, the resolution of the spectra depends of the design (i.e., distance of travel of the moving mirror) of the optical bench. Many other design characteristics (i.e., uniformity of energy output from the light source across the range of wavelengths, distance from cuvette to the detector, control of moisture in the light path, total length of the light path, etc.) of the optical bench are important.

The Bomem interferometer is shown in Figure 1.3 and consists of a MIR light source (#1), a beam splitter (#2), fixed mirrors (#3) attached to the end of the arms of a wishbone (#4), a sample cell (#5), and a detector (#6). The Bomem interferometer works based on the same interferometry principles previously described. Because both mirrors

move together and the distance between the mirrors is fixed the accuracy of wavelength calibration is constant in the wishbone design and no equalizer solution is required.

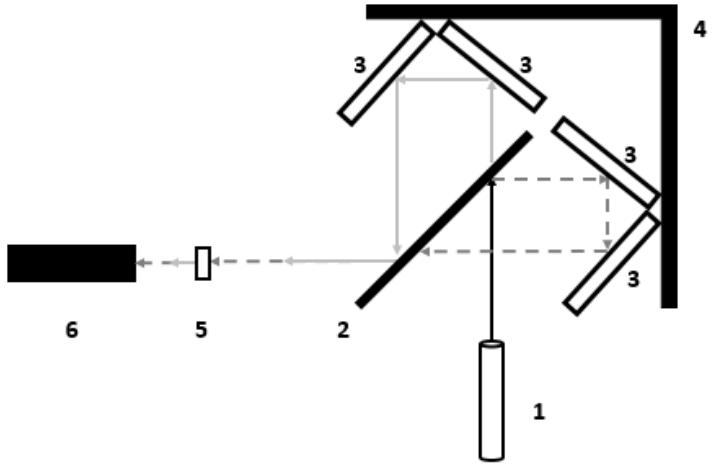

Figure 1.3. Schematic diagram of the Bomem interferometer: (1) MIR light source, (2) beam splitter, (3) fixed mirrors, (4) wishbone, (5) sample cell, and (6) detector.

Mid-Infrared Optical and Virtual Filters. Data from the MIR absorbance spectra produced by a Fourier transform MIR spectrophotometer can be used to simulate a fixed optical filter MIR instrument by digitally creating virtual filters with center wavelength and bandwidth of sample and reference selected based on the same general wavelengths used for optical interference filter instruments (Lynch et al., 2006, Kaylegian et al., 2009). The advantage of virtual filters over optical filters is that virtual filters are created in the instrument software and are exactly the same from one instrument to the next. Kaylegian et al. (2009) determined the optimum center wavelengths and bandwidths for virtual fat B, fat A, protein and lactose measurements on milk for an optical system with an 8 cm^{-1} resolution. The virtual filters allow the use of the same intercorrection factors from instrument to instrument when primary slope

of the uncorrected signal is set (Barbano and Clark, 1989) in a one-to-one relationship with change in the concentration of the milk component being measured (Kaylegian et al., 2009).

Homogenization and Mid-Infrared Milk Analysis. A laboratory homogenizer made it possible for homogenized emulsions to be analyzed by a MIR milk analyzer (Phipps 1960, 1975). Today, all MIR milk analyzers have an internal homogenizer that breaks the native fat globules to smaller sizes (Goulden, 1961; Biggs, 1967; Biggs et al., 1987). The main reason why fat globules need to be reduced to smaller sizes is that large fat globules scatter light, leading to an inaccurate estimate of fat, true protein and lactose content of milk (Barbano and Clark, 1989; Smith et al. 1993). Furthermore, the MIR energy passing through inefficiently homogenized milk can be distorted by Christiansen light scattering effect, which causes the shift of the apparent wavelength of maximum absorption by the carbonyl and carbon-hydrogen groups to a longer wavelength affecting the accuracy of MIR readings (Goulden, 1961). This shift in wavelength or light absorbance may also have a negative impact on the accuracy of the determination of the concentration of the major components of milk. In order to minimize light scattering and guarantee the reliability of quantitative absorption measurements, the size of large fat globules in milk needs to be reduced to less than 1/3 of the shortest wavelength at which the measurement is made. Fat B is the shortest wavelength (3.48 μm) used for fat analysis, therefore the fat globule size distribution d(0.9) should be less than 1.16 μm (Goulden, 1961; Goulden, 1964; Smith et al., 1993).

Overview of Milk Homogenization

The Milk Fat Globule and Homogenization. Milk fat globules synthesis takes place in each mammary secretory cell that surround the lumen of the alveoli within the mammary gland (Patton and Keenan, 1975). In the alveolus, each lumen is a receptor of milk secreted by the lactating cells surrounding it and all lumens are connected by a network of ducts that ends up taking the milk to the teat cistern (Figure 1.4). Each secretory cell has an arterial blood capillary that supplies substrates for milk synthesis (Patton and Keenan, 1975).

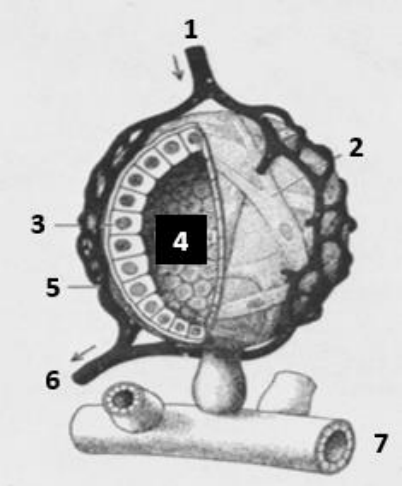

Figure 1.4. Illustration of the main structures of the alveolus: (1) arterial blood, (2) myoepithelial cell, (3) lactating cell, (4) lumen, (5) capillaries, (6) venous blood, and (7) duct. Adapted from Patton and Keenan (1975).

Milk fat is mostly triacylglycerol (about 97 to 98%), which are synthesized from glycerol and fatty acids (Keenan and Mather, 2006). About 60% of the fatty acids are synthesized within the mammary secretory cell from the components acetate and beta

hydroxybutyrate which have their origins in the rumen (Keenan and Mather, 2006). This produces the de novo fatty acids $C_{4:0}$ to $C_{14:0,}$ $C_{14:1}$ and about half of $C_{16:0,}$ $C_{16:1}$ (i.e., mixed origin fatty acids) for a cow that is in positive energy balance. The remaining 40% of the fatty acids (i.e., preformed fatty acids) in milk fat come into the mammary secretory cell preformed from diet or adipose. The preformed fatty acids comprise about half of $C_{16:0,}$ $C_{16:1}$ and the longer chain length fatty acids (Keenan and Mather, 2006). Fat globules formed in the cytosol of each secretory cell and move to the inner surface of the plasma membrane that faces inward to the lumen of the alveoli. The fat droplet pushes through the cell membrane and becomes enveloped by the plasma membrane of the secretory cell as it is expelled from the lactating cell into the lumen of the alveoli.

The membrane surrounding milk fat globules has three layers (Patton and Keenan, 1975; Keenan and Mather, 2006). The first layer existed in the cell prior to secretion. The other two layers are the inner surface of the plasma membrane and outer surface of the plasma membrane (i.e., exposed surface on the milk fat globule), that are super-imposed on the fat globule surface at secretion (Patton and Keenan, 1975). The membrane composition is different from the milk fat or the milk plasma. The composition of the natural milk fat globule membrane is protein, phospholipids, cerebrosides, and cholesterol. Traces of monoglycerides and free fatty acids are also present (Walstra et at., 2005). The natural fat globule membrane prevents fat globules from coalescence and protects the inside of the globules against lipoprotein lipase enzyme activity. Once the interfacial tension between fat globule and milk plasma is very low, the enzyme cannot penetrate the membrane (Mulder and Walstra, 1974; Walstra et at., 2005).

During milk homogenization, milk fat globule size is reduced. The milk specific surface area before homogenization is about 3 m^2/g and after homogenization the specific surface area increases to about 15 m^2/g. Therefore, homogenization results in a 5 to 10 fold increase of the milk fat-plasma interfacial surface area. This creates an uncovered oil-water interface (i.e., surface of the new formed fat globules that lacks protein), and protein from the plasma is adsorbed onto the surface of the new formed fat globules (Mulder and Walstra, 1974) to stabilize the interface and reduce interfacial tension. Depending of the proportion of fat to protein in milk, the surface of the new formed fat globules may lack protein, and the small fat globules may easily come together to share protein at their interface forming clusters (Mulder and Walstra, 1974; Walstra et at., 2005).

Commercial Homogenizers. The recommended milk temperature for commercial milk homogenization is between 60 to 75°C, which achieves breakage of milk fat into smaller fat globules and reduces the tendency of fat globules to aggregate and rise to the top of container of fluid milk (Trout, 1950; Walstra et at., 2005). If the temperature of homogenization of milk is below the melting point of milk fat (i.e., < 40°C), fat will be in the solid state resulting in incomplete fat dispersion and ineffective homogenization (Trout, 1950; Bylund, 1995). From a milk quality perspective for consumer products, a temperature between 60 to 75°C inactivates the lipases activity and avoids release of free fatty acids and off-flavor development (Walstra et al., 2005). In a typical commercial homogenizer, a high pressure positive displacement pump forces heated milk through a narrow gap in the homogenizer valve (Mulder and Walstra, 1974; Phipps, 1985). As the milk is forced through the gap of the 1st stage homogenizer

valve at high pressure, the linear velocity of the milk and shear forces increase. Often the high velocity milk is projected against a surface to create high turbulence and more shear leading to reduction in fat globule size (Mulder and Walstra, 1974; Walstra et al., 2005). The high pressure positive displacement pump can be equipped with a single piston or with multi-pistons (3, 5 or 7). In a single piston homogenizer (Figure 1.5), the homogenizer valves open and close with every stroke of the piston and the flow pressure goes from zero to the set pressure for that stage and back to zero as the valve opens and closes (Figure 1.6A).

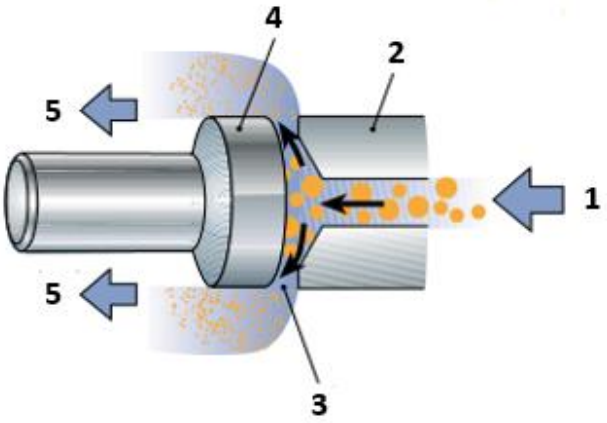

Figure 1.5. Illustration of the high pressure positive displacement pump of a commercial homogenizer equipped with a single piston: (1) unhomogenized milk, (2) seat, (3) narrow gap, (4) piston, (5) homogenized milk. Adapted from Bylund (1995).

The multi-pistons are operated intentionally out of phase to achieve a constant applied pressure, continuous flow of milk and uniform homogenization. The higher the number of pistons, the more the pressure fluctuations are minimized (Phipps, 1985) and the homogenizer valves are running open continuously with relatively constant pressure

decrease across the gap (Figure 1.6B). In this way, the valve wear is minimized and consistent particle size is achieved (Walstra, 1975).

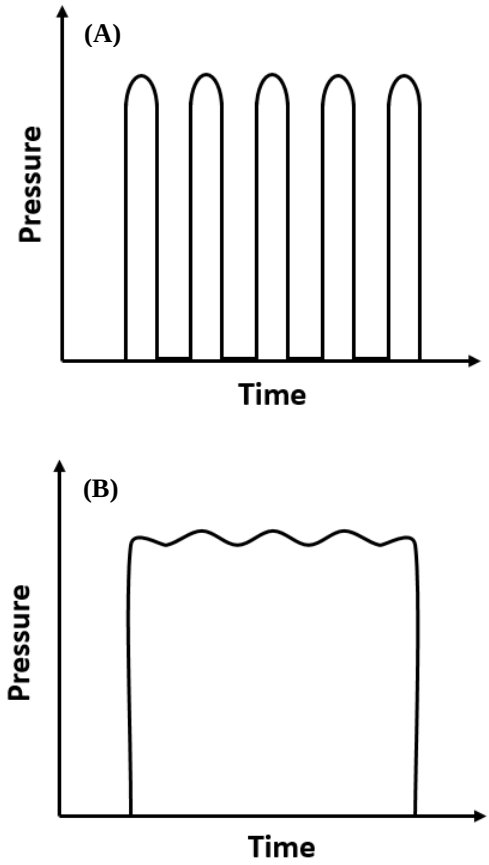

Figure 1.6. Comparison of pressure fluctuations on 1st stage of milk homogenization when the commercial homogenizer is equipped with (A) a single piston versus (B) multi-pistons.

Typical homogenization of pasteurized fluid milk is done with a 2-stage homogenizer with a 1st stage pressure of 20 MPa and 2nd stage pressure of 5 MPa (Walstra et al., 2005) with a multi-pistons homogenizer. The 2nd valve should always operate at lower pressure (i.e. 20% of the total pressure) (Walstra, 1975). The function of the high pressure 1st stage is to break fat globules to smaller sizes. The newly formed

small fat globules are no longer exclusively covered with the original milk fat globule membrane. Instead, they are also covered with protein adsorbed from the milk plasma (Walstra et al., 2005). In the turbulent environment created by velocity of the milk and shear forces in the milk exiting the 1st stage, the small fat globules may start to collide before they are completely covered with protein, leading to fat globule coalescence and clustering after the 1st stage (Mulder and Walstra, 1974). The function of the low pressure 2nd stage is to break up the fat globule clusters (Walstra, 1975; Phipps, 1985; Walstra et al., 2005). At the 2nd stage the pressure is low, so that the new surface created is insignificant and new clusters are not formed (Mulder and Walstra, 1974). Enough time needs to be given for the newly formed fat globules to cluster after milk passes through the 1st stage valve so that the 2nd stage valve will be able to fulfill its role in breaking clusters (Walstra et al., 2005). The typical fat globule size distribution d(0.9) in a commercially homogenized milk is about 1.2 to 1.8 μm (Caplan and Barbano, 2013).

Mid-Infrared Homogenizers. The MIR homogenizer designs are different than a commercial homogenizer. There are two types of MIR designs: one with the spring in the milk flow and one with the springs outside the milk flow. All homogenizers within MIR milk analyzers are single piston homogenizers, so the pressure across the homogenizer stages is going from zero to full pressure and back to zero with every pump stroke during the pumping of each milk sample. Some MIR homogenizer designs that have the springs in the milk flow are very different than commercial homogenizers. This design of homogenizer has been used by Multispec (no longer in business), Bentley Instruments (Chaska, Minnesota), and Delta Instruments (Drachten, Netherlands). This

type of homogenizer includes the 2-stages connected in series within a single homogenizer housing, which is mounted in-line the MIR. The 1st and 2nd stage springs are different in strength with a pressure of 15 MPa on 1st stage and 3 MPa on 2nd stage. Heated milk (about 40°C) is pumped through the homogenizer. This is a much lower temperature than commercial homogenization and if the raw milk sample remains for any period of time at 40°C, there will be rapid lipolysis and release of free fatty acids (Wiking and Dickow, 2013).

A 2-stage homogenizer designed with springs outside the milk flow has two separate homogenizer valve housings connected in series in the MIR milk analyzer. Heated milk is pumped through the homogenizer and reaches the 1st stage seat, the 1st stage ball and the piston. The high pressure operating against the 1st stage spring opens a narrow gap between the seat and the ball, allowing milk to flow through the 1st stage of homogenization and exit to the 2nd stage. Next, milk flows to the 2nd homogenizer housing. At this 2nd stage an equal pressure (i.e., 10 MPa on each stage) is applied against the spring to open the gap between the seat and the ball in the Foss Electric (Hillerod, Denmark) model FT 6000 and FT 120 infrared milk analyzers. In the newer Foss Electric model FT+ the 1st stage pressure is higher than the 2nd stage pressure. The same is true in the Delta Instruments CombiScope FTIR 600/300 Hp.

In both homogenizer designs, the seats and balls open and close with every pump stroke, resulting in variation in the applied pressure and intermittent flow of milk. Mechanical failures of seats, balls, and springs over time are more likely to happen with this rapid opening and closing during pumping of each sample. This may result in differences in homogenization efficiency from one pump stroke to the next which will

influence repeatability. The reality is that only a portion of homogenized milk from a single pump stroke is actually scanned in the instrument's cuvette to produce a spectra for the milk sample. We must assume that variation in homogenization efficiency from one pump stroke to the next is small.

Tests Available to Evaluate Mid-Infrared Homogenizer Efficiency

Recycle Test. In the recycle test, unhomogenized milk is run through a MIR milk analyzer, the readings are recorded, and the homogenized milk is collected from the instrument outlet tube. Next, the collected homogenized milk is rerun through the instrument and the readings are recorded. This test was developed to evaluate homogenizer performance of Milko-testers (Shipe, 1969), but the same test was applied to MIR milk analyzers when they became available. If the difference between the average MIR readings for the fat test on the unhomogenized milk and the instrument homogenized milk is < 0.05% for a milk containing a mass fraction of 3.5% milk fat, then the homogenizer meets minimum performance standards (AOACI, 2000; IDF, 2000). The method does not specify if this should be done with the fat A (C=O stretch) or fat B (symmetrical C-H stretch) measurements. Although the recycle test is very rapid and practical, the weakness of this test is that if the instrument is not performing homogenization at all (i.e., the homogenizer is very bad), then it will produce the same readings before and after homogenization and will pass the evaluation (Barbano and Clark, 1989; Smith et al., 1993; Lynch et al., 2006).

Electron Microscopy. This method is subdivided into two types: scanning electron microscopy (**SEM**) and transmission electron microscopy (**TEM**). The study of milk fat globule size distribution falls into the TEM, specifically the freeze-fracturing

technique. In the freeze-fracturing technique, milk is quickly frozen, fractured at -130 to -100°C and replicated (Kaláb, 1981). The fracturing can be done by placing frozen milk in a holder. In this case, a thin-walled metal tube with the lower part attached to the freeze-fracturing table cooled to the desired temperature is used (Kaláb, 1981). The upper part of the metal tube is knocked off by a mechanical arm after the desired temperature and vacuum are achieved (Kaláb, 1981). The fractured surface is replicated with platinum and reinforced with carbon. Another methods of operation were described in details by Kaláb (1981). Despite the electron microscopy extends the resolution of optical microscopy of 0.5 μm to 1 nm, making it possible to study in detail the physical properties of milk components, it is not a practical method for routine measurement of particle size because it has a high cost of equipment and it is difficult to collect enough images and measure particle size distribution from photographic images (Kaláb, 1993).

Particle Size Analysis. This method evaluates homogenizer efficiency using forward laser light scattering to determine fat globule size distribution after unhomogenized milk is homogenized through the MIR milk analyzer (Lynch et al., 2006). Laser light scattering uses Mie theory to calculate the particle size distribution, assuming a volume equivalent sphere model. Mie theory predicts scattering intensity as a function of the angle at which light is scattered at the point of interaction with a spherical particle (Horvath, 2009). The milk fat globule size distribution reported by laser light scattering particle size analyzers is based on the volume of the sphere (i.e., according to the volume of each fat globule present in the sample a number of different particle size distribution parameters are calculated). The parameter reported as particle size distribution d(0.5) is the median of volume distribution (i.e., half of the total fat

globules volume in the sample comes from particles with diameter smaller than the d(0.5) value and half of the total fat globules volume in the sample comes from particles with diameter larger than the d(0.5) value). The parameter reported as particle size distribution d(0.9) indicates that 90% of the total fat globules volume in the sample comes from particles with diameter that lies below the d(0.9) value. The surface volume mean diameter D[3,2] and volume moment mean diameter D[4,3] are calculated from the particle size distribution (Allen, 1990). The surface volume mean diameter D[3,2], also known as Sauter mean diameter, is calculated using the equation: $x_{SV} = \sum x^3 dN / \sum x^2 dN$, where x = average particle diameter lying in the size range x_i to x_n (μm) and dN = the percentage of the total number of particles lying in the size range x_i to x_n. The volume moment mean diameter D[4,3], also known as De Broucker mean diameter, is calculated using the equation: $x_{VM} = \sum x^4 dN / \sum x^3 dN$, where x = average particle diameter lying in the size range x_i to x_n (μm) and dN = the percentage of the total number of particles lying in the size range x_i to x_n (Allen, 1990). In this test, if the particle size distribution d(0.9) of the milk homogenized through the MIR is larger than 1.7 μm, then the homogenizer is deteriorating and needs to be replaced (Smith et al., 1995). Particle size analysis is very accurate, but is less practical to perform daily.

Partial Least Squares Prediction Models

What is Partial Least Squares? Partial least squares (**PLS**) regression is a statistical modeling tool that has been used for quantitative prediction of chemical component concentration from MIR spectra. It combines the advantages of the classical least squares (**CLS**) and the inverse least squares (**ILS**) methods to overcome the limitations of both (Haaland and Thomas, 1988).

The advantage of the CLS method is that it considers all the frequencies of the complete spectral region being analyzed to determine concentration of components instead of consider only the frequency of the maximum peak. The number of frequencies used in the spectra is dependent on the resolution of the optical system (i.e., 16 cm^{-1}, 8 cm^{-1}, 4 cm^{-1}, etc.). The large number of frequencies (i.e., data points) results in high precision of measurements. But the weakness is that all chemical components of a sample in the spectral region used for analysis need to be known (Haaland and Thomas, 1988).

The ILS advantage is that the analysis of the quantitative spectra can be performed even if only one component is known. The disadvantage of this method is that it is limited to a small number of spectrum frequencies because the inverted matrix used in the calculations must have dimensions equal to the number of frequencies, and because the precision of the results can be impacted by collinearity when the number of frequencies becomes too large (Haaland and Thomas, 1988). In milk, there is collinearity of changes in fat and protein.

The PLS is a method that uses the complete spectral region to determine concentration of components and can be used even if all chemical components of a samples are not known. Detailed information about each least squares method and theory behind it was given by Haaland and Thomas (1988).

How does Partial Least Squares Work? The PLS modeling process assumes that there is a relationship between absorbance and component concentrations. It has two steps: prediction model development and external sample validation. In the prediction model development step, the relationship between the MIR absorbance

spectra and milk component concentrations is estimated from a set of reference milk samples with spectral and concentration outliers identified and removed. In this step, the PLS model is constructed using the following equation: $Y = \beta_0 + \beta_1 X_1 + \cdots + \beta_k X_k + e$, where Y = response, X = MIR spectral absorbance, β = beta coefficients, and e = error term. The estimation of beta coefficients is based on the observed Y values and PLS scores for the optimum number of PLS factors and indicates which X absorbance are contributing to the modeling of the response Y (Janik et al., 2007). Cross validation (cyclic leaving out 1 sample at a time) is applied for determining the number of factors used for each model. The number of factors selected for each prediction model is generally the number of factors that produce the minimum standard error of cross validation (**SECV**). Adding more factors to the model starts modeling in noise and may increase the SECV (Haaland and Thomas, 1988). In the external sample validation step the developed model is used to predict the component concentration from the "unknown" milk sample spectrum and results are statistically compared to the analytical reference test (Haaland and Thomas, 1988).

Research Objectives

Homogenization of each milk sample is an important step included in a MIR milk analyzer. It impacts repeatability (Smith et al., 1994) and accuracy of the MIR results, and consequently impacts milk payment. After homogenization, the fat globule size distribution d(0.9) of milk should be less than 1/3 of the wavelength at which the measurement is made, to minimize light scattering caused by large milk fat globules. Ideally, the fat globule size distribution d(0.9) of milk should be less than 1/3 of fat B wavelength (i.e., d(0.9) < 1.16 μm). Fat B is the shortest wavelength used for fat analysis

(Goulden, 1964; Smith et al., 1993). The challenge is that there is a range of fat globule sizes in homogenized milk, so both the mean and skew of the size distribution are important with respect to light scattering. The d(0.9) is an index of the fat globule diameter above which 10% of the volume of the fat resides, even if the total mean diameter is < 1.16 µm. If d(0.9) gets larger than 1.7µm, then the homogenizer has deteriorated and should be replaced (Smith et al, 1995, Lynch et al., 2006). From the perspective of the laboratory technician running a MIR milk analyzer, a method running on the instrument that would produce information to warn the instrument operator that the homogenizer is near failure is needed. Furthermore, in the literature there is no specific information comparing the impact of variation in homogenization efficiency on the accuracy of major milk component prediction by MIR using traditional filter versus PLS models.

Our objectives were: 1) to develop PLS models using data from Fourier Transform MIR spectra to predict the particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3] of milk fat globules and validate the PLS models performance; and 2) to determine the impact of MIR homogenizer efficiency on accuracy and repeatability of Fourier Transform MIR predicted fat, true protein, and anhydrous lactose determination given by traditional filter and PLS prediction models.

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CHAPTER TWO

PREDICTION OF FAT GLOBULE PARTICLE SIZE IN HOMOGENIZED MILK USING FOURIER TRANSFORM MID-INFRARED

ABSTRACT

Our objective was to develop partial least squares models using data from Fourier Transform mid-infrared (MIR) spectra to predict the particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3] of milk fat globules and validate the models. The goal of the study was to produce a method built into the MIR milk analyzer that could be used to warn the instrument operator that the homogenizer is near failure and needs to be replaced to ensure quality of results. Five homogenizers with different homogenization efficiency were used to homogenize pasteurized modified unhomogenized milks and farm raw bulk tank milks. Homogenized milks were collected from the homogenizer outlet and then run through a MIR milk analyzer without an in-line homogenizer to collect a MIR spectra. A separate portion of each homogenized milk was analyzed with a laser light scattering particle size analyzer to obtain reference values. The study was replicated 3 times with 3 independent sets of modified milks and farm raw bulk tank milks. Validation of the models was done with a set of 34 milks that were not used in the model development. Partial least squares regression models were developed and validated for predicting the following parameters from MIR spectra: milk fat globule particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume

moment mean diameter D[4,3]. The basis for the ability to model particle size distribution of milk fat emulsions was hypothesized to be the result of the PLS modeling detecting absorbance shifts in MIR spectra of milk fat due to the Christiansen effect. The independent sample validation of particle size prediction methods found that there was more variation in d(0.9) and D[4,3] predictions than the d(0.5) and D[3,2] predictions relative to laser light scattering reference values and this may be due to variation in particle size among different pump strokes. The accuracy of the d(0.9) prediction for routine quality assurance to determine if a homogenizer within a MIR milk analyzer was near the failure level (i.e., d(0.9) > 1.7 μm) and needed to be replaced is fit-for-purpose. The daily average particle size performance (i.e., d(0.9)) of a homogenizer based on the mean for the day could be used for monitoring homogenizer performance.

INTRODUCTION

Annually, in the United States, millions of pounds of milk are produced and tested daily for the determination of the concentration of the main components (i.e., protein, fat and other solids). The results are used by processors to determine the payment of dairy farmers, and by the farmers for dairy herd management (Lynch et al., 2004; Barbano and Lynch, 2006). Mid-infrared (MIR) milk analyzers have been used and have positively impacted dairy industries and farmers by providing rapid, cost effective and direct determination of milk components (Barbano and Clark, 1989; Lynch et al., 2004; Lynch et al., 2006; Adams and Barbano, 2015).

A laboratory homogenizer made it possible for homogenized emulsions to be analyzed by a MIR milk analyzer (Phipps 1960, 1975). Today, all MIR milk analyzers have an internal homogenizer that breaks the native fat globules to smaller sizes (Goulden, 1961; Biggs, 1967; Biggs et al., 1987). The main reason why fat globules need to be reduced to smaller sizes is that large fat globules increase light scattering, leading to an inaccurate estimate of fat, protein and lactose content of milk (Barbano and Clark, 1989; Smith et al. 1993). Furthermore, large fat globules can also lead to the Christiansen light scattering effect (Goulden, 1964; Smith et al., 1993), which causes a change of in the refraction of light at wavelengths near maximum absorption by the carbonyl and carbon-hydrogen groups. The Christiansen effect causes a shift in the apparent wavelength of maximum light absorption to a longer wavelength. This effect can be reduced by decreasing the fat globule size distribution d(0.9). Ideally, after homogenization, the fat globule size distribution d(0.9) of milk should be less than 1/3 of the wavelength of fat B (3.48 μm), which is the shortest wavelength used for fat analysis (Goulden, 1964; Smith et al., 1993). Different types of homogenizers have different efficiency. At the same pressure, a single stage homogenizer will be less efficient than a double stage homogenizer. Other factors that may impact homogenizer efficiency are: milk temperature, pump speed, pump stroke length, fat content, and time of usage (Goulden and Phipps, 1964; Walstra and Jenness, 1984). The deterioration of a homogenizer's mechanical components over time may be difficult for the operator to detect and will have a negative impact on analytical repeatability and accuracy (Lynch et al., 2006).

Various tests to evaluate homogenizer efficiency have been used over the years. One test is called the "recycle test" where an unhomogenized milk is run through a MIR milk analyzer, the readings are recorded, and the homogenized milk is collected from the instrument outlet tube. Next, the collected homogenized milk is rerun through the instrument and the readings are recorded. If the difference in readings for the fat test on the unhomogenized milk and the instrument homogenized milk is <0.05%, then the homogenizer meets minimum performance standards (AOAC, 2000; IDF, 2000). As the homogenizer performance decreases the difference between the two results gets larger, but as the homogenizer performance continues to get worse across time, the difference in results becomes <0.05% again, but the homogenizer is not getting better. Thus, a homogenizer that does not homogenize at all or very poorly will pass the evaluation (Barbano and Clark, 1989; Smith et al., 1993; Lynch et al., 2006). Therefore, the recycle test, is easy to preform, but because of this weakness, the test is not very good.

Another method to evaluate homogenizer efficiency is the determination of milk fat globule size distribution using a laser light scattering particle size analyzer after unhomogenized milk is homogenized through the MIR milk analyzer (Lynch et al., 2006). Laser light scattering uses Mie theory to calculate the particle size distribution, assuming a volume equivalent sphere model. Mie theory predicts scattering intensity as a function of the angle at which light is scattered at the point of interaction with a spherical particle (Horvath, 2009).

The milk fat globule size distribution d(0.9) reported by laser light scattering particle size analyzers is based on the volume of the sphere (i.e., according to the volume of each fat globule present in the sample the instrument calculates a diameter). The

parameter reported as particle size distribution d(0.5) is the median of volume distribution (i.e., half of the total fat globules volume in the sample comes from particles with diameter smaller than the d(0.5) value and half of the total fat globules volume in the sample comes from particles with diameter larger than the d(0.5) value). The parameter reported as particle size distribution d(0.9) indicates that 90% of the total fat globules volume in the sample comes from particles with diameter that lies below the d(0.9) value. The surface volume mean diameter D[3,2] and volume moment mean diameter D[4,3] are calculated from the particle size distribution (Allen, 1990). The surface volume mean diameter D[3,2], also known as Sauter mean diameter, is calculated using the equation: $x_{SV} = \sum x^3 dN / \sum x^2 dN$, where x = average particle diameter lying in the size range x_i to x_n (μm) and dN = the percentage of the total number of particles lying in the size range x_i to x_n. The volume moment mean diameter D[4,3], also known as De Broucker mean diameter, is calculated using the equation: $x_{VM} = \sum x^4 dN / \sum x^3 dN$, where x = average particle diameter lying in the size range x_i to x_n (μm) and dN = the percentage of the total number of particles lying in the size range x_i to x_n (Allen, 1990).

Particle size of the milk produced by a homogenizer within a MIR should result in a mean fat globule size distribution d(0.9) < 1.7 μm. If d(0.9) ≥ 1.7 μm, then the homogenizer performance has deteriorated and should be replaced (Smith et al, 1995, Lynch et al., 2006). Our objective was to develop models using data from Fourier Transform MIR spectra to predict the particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3] of milk fat globules and validate the PLS models performance. The goal of the study was

to produce a method built into the MIR milk analyzer that could be used to warn the instrument operator that the homogenizer is near failure and needs to be replaced to ensure quality of results.

MATERIALS AND METHODS

Experimental Design

Five homogenizers with different homogenization efficiency (i.e., produced different milk globule size distributions) were used to homogenize two types of milk samples sets. The different types of homogenizers will be explained later in the paper. The first type of milk sample set contained 12 pasteurized preserved modified unhomogenized milks ranging from 1.0 to 5.7% fat as described by Kaylegian et al. (2006), and the second set contained 12 different preserved farm raw bulk tank milks obtained from the USDA Federal Milk Market Laboratory (Cleveland, OH) that ranged in fat from about 2.6 to 5.6%. All milks were preserved with bronopol (Microtabs II, Advanced Instruments, Norwood, MA) at 0.01% m/m concentration as described by Barbano et al. (2010).

Milks were run through each homogenizer at 40 to 42^{o}C. Two different pumping systems were used, one for homogenizers from Delta Instruments HomoScope (Model HU-3.0, Delta Instruments, Drachten, Netherlands) and a modified Milkoscan 104 pumping system for homogenizers from Foss Electric (Hillerod, Denmark). All homogenized milks were collected from the homogenizer outlet and then pumped through a MIR milk analyzer without an in-line homogenizer (LactoScope FTIR Advanced (FTA), Delta Instrument, Drachten, Netherlands). The in-line homogenizer

was replaced with a reverse inlet valve in the flow system of a Delta Instruments model FTA and a MIR spectrum was collected for every homogenized milk. Separate portions of the same homogenized milks were analyzed with a particle size analyzer (Mastersizer 2000, model MS2000; Malvern Instruments, Worcestershire, United Kingdom). The study was replicated 3 times with 3 independent sets of modified milks and farm raw bulk tank milks. The spectra from the total population of milks were analyzed using PLS regression analysis with Grams A/I PLSIQ Version 7.00 software (Thermo Fisher Scientific Inc., Waltham, MA 02454) to develop PLS prediction models for particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3] of milk fat globules in homogenized milks. The PLS models were developed in two steps: 1) prediction model development, where the relationship between the MIR absorbance spectra and response [d(0.5), d(0.9), D[3,2], and D[4,3]] was estimated from the set of reference samples; 2) external sample validation, where the prediction model was used to predict the particle size of each "unknown" milk from the sample spectra in comparison to laser light scattering reference test values on each milk (Haaland and Thomas, 1988). The external validation of the accuracy of the PLS models was determined using a set of 34 farm raw bulk tank milks that were not part of the population of milks used to develop the PLS prediction models.

Homogenizers Used in the Study

Different homogenizers were tested to select the ones that would produce a wide range of particle size distribution d(0.9) that might be encountered when the performance of a homogenizer within a MIR milk analyzer deteriorates. Each

homogenizer that was going to be tested was placed either into the Delta HomoScope or into the modified MilkoScan 104. Unhomogenized milk was warmed up to 42°C and run through the system. Homogenized milk was collected at the outlet and it was run through the particle size analyzer to verify the homogenizer given particle size distribution d(0.9). Based on the d(0.9) values five homogenizers were selected to produce homogenized milk with the following expected average d(0.9) values: 1.2, 1.4, 1.6, 1.8, 2.5 μm. One 2-stage MIR homogenizer from Foss Electric was selected. Four different 2-stage MIR homogenizers from Delta Instruments were selected. The homogenization systems were not attached to a MIR milk analyzer and were designed only to produce a population of milks with a wide range of milk fat globule size distribution d(0.9) for the purpose of prediction model development.

Homogenization of Milks

On each test day, a water bath (Model 406015, AO Scientific Instruments, Division of Warner-Lambert Technologies, Keene, NH, United States) was used to heat water to 42°C. An Erlenmeyer flask containing 0.01% (v/v) Triton-X 100 surfactant solution (G000071020, Delta Instruments, Drachten, Netherlands), 0.5% (w/v) nonfoaming Stella anionic cleaning solution (336446, Foss Electric, Hillerod, Denmark) and two homogenizers were all placed in the water bath. The homogenizers were tempered to 42°C so they would quickly come to temperature equilibrium when attached to the pumping system. The first six milk samples were placed in the water bath and warmed to 42°C, while the other milk samples were placed in a bin containing crushed ice and were placed into the water bath one at a time as soon as another sample was taken out to be homogenized. The first milk was mixed by inversion, the

temperature was checked and the milk was run through the homogenizer and collected at the outlet in a clear 90 mL vial (CPP03EDM-CL, Capitol Plastic Products, 1030 Riverfront Center, Amsterdam, NY). The collected homogenized milk was mixed by inversion and a portion was poured into a clear 15 mL vial (CPP500-Cornell, Capitol Plastic Products, 1030 Riverfront Center, Amsterdam, NY) and placed in crushed ice for rapid cooling and then refrigerated at 4°C for later analysis with laser light scattering. The homogenized milk left into the 90 mL vial was analyzed using a MIR milk analyzer that did not contain a homogenizer in the flow system. This procedure was repeated for all samples of the two sets, and in between each sample the homogenizer was flushed with distilled water at 42°C. At the end of each run the homogenizer was cleaned by running 150 mL of reverse osmosis (**RO**) water, 150 mL of Stella and 150 mL of Triton-X 100 solution, and then the homogenizer was detached from the pumping system. The next homogenizer that was in the water bath was connected to the pumping system and the procedure was repeated. The same unhomogenized milks were pumped through all five different homogenizers. The order in which the homogenizer heads were used to produce homogenized milks was different within each replicate.

With each of the 5 homogenizers, a set of 12 different modified milk (Kaylegian et al., 2006) samples ranging from about 1.0 to 5.7% fat were homogenized and a set of 12 individual farm raw bulk tank milks were homogenized to produce 120 homogenized milks. This was replicated 3 times in different weeks using a different set of modified milks and producer milks in each replicate to produce spectra from 360 homogenized milks for PLS models development.

Mid-Infrared Analysis

MIR analysis of the 360 homogenized milks described above was performed with the LactoScope FTIR Advanced (FTA) milk analyzer equipped with a BMX optical bench (ABB Bomem, Montreal, Canada). A CaF_2 cuvette (36 µm) was used. For collection of the MIR spectra for milk samples, the LactoScope FTA was operated at a spectral resolution of 8 cm^{-1} (digital resolution of 3.85 cm^{-1}) collecting averages of 16 scans per analysis (using standard double-sided forward-backward interferogram acquisition, cosine apodization and phase correction for Fourier transformation yielding an energy spectrum). The average of 16 scans was transformed into an absorption spectrum by calculation of the -log ratio with reference to the spectrum of zero liquid (demineralized water containing 0.01% triton) and storing the absorption spectrum for milk. The instrument homogenizer was replaced with a reverse inlet valve that had exactly the same shape as an in-line homogenizer but did not contain balls, seats or springs normally present in a working homogenizer. For collection of the MIR spectra for milk samples, the instrument was cleaned using Decon 90 (Decon Laboratories Ltd., E. Sussex, UK). Spectra were collected for each milk using 3 pumping cycles to flush externally homogenized milk through the flow system with the reverse inlet valve and 3 measure cycles without pumping to collect spectra. The range of wavenumbers in the spectra was from 400 cm^{-1} to 4000 cm^{-1}. These spectra in combination with the Malvern Mastersizer 2000 reference particle size values were analyzed using the PLS routines of the Grams A/I PLSIQ Version 7.00 software (Thermo Fisher Scientific Inc., Waltham, MA 02454) to calculate the beta coefficients for PLS prediction models designed to predict fat globule size based on the MIR spectra. Data obtained by the MIR and particle

size analysis were used to develop 4 PLS statistical models: particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3] of milk fat globules in homogenized milk.

Particle Size Analysis

Milks were analyzed using a Malvern Mastersizer 2000 (Malvern Instruments, Worcestershire, United Kingdom), software version 5.4. Milks at 42°C were dispersed in RO water at 42°C to ensure that the milk fat was liquid and globules were spherical. The refractive index for the particles (i.e., milk fat) at 42°C was set at 1.458 and 1.33 for the suspending media (water 42°C) for the red laser (forward light scattering). The particle size model was set as general purpose and particle shape was set to spherical. The measure time for sample and background was set at 5 s with 5000 snaps. A light obscuration range limit was set to fall with a range of 7 to 9%, with 3 measurement cycles per sample with zero time delay between measurements.

The Mastersizer 2000 has both a red (forward scatter for larger particles, e.g., fat globules) and a blue laser (side scattering for smaller particles, e.g., casein micelles). In our work, only the particle size distribution of the fat globules was of interest. To eliminate the contribution of casein micelles from the particle size distribution, the data kill function of the Mastersizer 2000 software was set up to include only the particles in the size range from 0.195 μm to 18.32 μm. While there may be some small naturally occurring fat droplets in raw milk < 0.195 μm in diameter that are not a product of homogenization, those fat droplets were not of interest and they have very little impact on the volume based particle size distribution of fat. Most of the casein micelles are < 0.195 μm in diameter so using the data kill function was a practical way to exclude the

contribution of the casein micelles. This approach was developed by comparing the particle size results from a Mastersizer E with the Mastersizer 2000 on the same homogenized and unhomogenized milk samples. The Mastersizer E has only a red laser and has no side scatter detectors.

Before each sample analysis, the flow system was rinsed with RO water at 45°C and drained three times to ensure that the cell was warm, and then 45°C RO water was added to fill the system and recirculated to remove air. The recirculation pump was set to 2250 rpm. Vials of homogenized milks were heated to 42°C in a water bath (Narco, model 220-A, National appliance company, Portland, Oregon), mixed by inversion and added to recirculating RO water in the sample dispersion unit until the laser obscuration was in the range of 7 to 9% , and after that the analysis was started. Once all the samples were run, the system was rinsed three times with RO water, 2% Contrad-70 solution was added to the dispersion unit and left for 15 min with the pump set to 1750 rpm. Then, the solution was drained and the system was rinsed with 45°C RO water until there was no more foam. The following measured parameters from Malvern Mastersizer 2000 were used: particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3] of milk fat globules in homogenized milk.

Development of the Partial Least Squares Statistical Models

Removal of Outliers. Of the total 360 modified milks and farm raw bulk tank milks, the data set for d(0.5) and d(0.9) model development had 11 and 3 milk samples removed, respectively, which were spectral outliers. The D[3,2] model had 12 spectral outliers removed, and the D[4,3] model had 16 spectral outliers removed during the

iterative modeling process. Criteria for removal of outliers was based on Mahalanobis distances.

Calculation of Partial Least Squares Models. The PLS models for prediction of d(0.5), d(0.9), D[3,2], and D[4,3] were calculated using the following spectral ranges: 3000 to 2750, 1800 to 1700 and 1585 to 1000 cm^{-1}. Data were not mean centered and a baseline correction was not used. Cross validation (cyclic leaving out 1 sample at a time) was applied for determining the number of factors used for each model. Spectral and concentration outlier samples were identified, removed from the data set and then the PLS modeling was repeated with outliers removed. The number of factors selected for each final prediction model was the number of factors that produced an F-Test predicted residual sum of squares (**FPRESS**) of approximately 0.57, which will be near the minimum standard error of cross validation (SECV). Adding more factors to the model starts modeling in noise and may increase the SECV (Haaland and Thomas, 1988). The PLS prediction models were developed using the following equation: $Y = \beta_0 + \beta_1 X_1 + \cdots + \beta_k X_k + e$, where Y = response [i.e., d(0.5), d(0.9), D[3,2], and D[4,3] values], X = MIR spectral absorbance, β = beta coefficients, and e = error term. The estimation of beta coefficients is based on the observed Y values and PLS scores for the optimum number of PLS factors and represents the correlation between Y and each of the X absorbances (Janik et al., 2007). PLS models for d(0.5), d(0.9), D[3,2], and D[4,3] were developed and installed into the Delta Instruments FTIRScope Advanced software to enable the MIR to output particle size data directly for each milk tested.

Performance of the Partial Least Squares Models

Slope and Intercept Adjustment. The PLS models for MIR prediction of d(0.5), d(0.9), D[3,2], and D[4,3] were slope and intercept adjusted using MIR calibration milks (samples 7 to 14 from modified milk sets). Three homogenizers with different homogenization efficiency were used to homogenize each of the 8 modified milk samples. Each homogenizer was inserted in the Delta Instruments LactoScope FTIR Advanced (FTA) and each milk sample was analyzed 3 times to produce 3 MIR predictions of the 4 particle size parameters for each milk. There are multiple pump strokes through the homogenizer in one milk analysis cycle of the instrument. The milk in the cuvette that is used for production of the spectra is the milk from a portion of one pump stroke (about 0.42 grams), while the milk collected from one pumping cycle for particle size analysis represents many pump strokes during that pumping cycle. Homogenization efficiency may vary slightly from one pump stroke to the next with the same milk. The instrument homogenized milk from the second and third pumping/analysis cycle was collected from the instrument's outlet tube and analyzed using the Malvern Mastersizer 2000 to produce particle size reference values d(0.5), d(0.9), D[3,2], and D[4,3] for each milk. The mean difference (**MD**), standard deviation of the difference (**SDD**), and relative standard deviation of the difference (i.e., coefficient of variation) (**RSD**) were calculated for the d(0.5), d(0.9), D[3,2], and D[4,3]. A linear regression equation for each predicted particle size parameter was calculated by plotting reference values given by Malvern Mastersizer 2000 as a function of MIR predicted values (X-axis) using Excel 2016 (Microsoft, Redmond, WA). These linear regression equations provided the slope and intercept adjustment of each

predicted particle size parameter. Each model parameter was slope and intercepted adjusted in the FTIRScope Advanced Version 1.23 (Delta Instruments, Drachten, Netherlands) software of the MIR instrument.

Validation of the Models. After adjustment of slope and intercept, the performance of the PLS models for MIR prediction of d(0.5), d(0.9), D[3,2], and D[4,3] was conducted with 34 individual farm raw bulk tank milks (12 from Cleveland, Ohio; 12 from Dallas, Texas; and 10 from Lebanon, New Hampshire) that were not part of the population of milks used to develop the PLS prediction models. Three homogenizers with different homogenization efficiency were used to homogenize each of the 34 milks, resulting in 102 samples to validate each PLS model. The homogenizers were connected in-line within the Delta Instruments LactoScope FTIR Advanced (FTA) and each sample was analyzed in triplicate (i.e., 3 pumping cycles). The instrument homogenized milk from the second and third pumping cycles was collected separately from the instrument's outlet tube and analyzed by the Malvern Mastersizer 2000 as described above. A value for each sample for the d(0.5), d(0.9), D[3,2], and D[4,3] of milk fat globule was predicted by the MIR. The mean of reference values, mean of MIR predicted values, MD between MIR predicted and reference values, SDD, and RSD were calculated for the d(0.5), d(0.9), D[3,2], and D[4,3] PLS models. To determine if the mean of the reference values was different from the mean of the instrument predicted values a t-test was performed using SAS (SAS Version 8.02, 2011, SAS Institute Inc., Cary, NC).

RESULTS

Developed Partial Least Squares Models

The number of samples, mean of Malvern Mastersizer 2000 reference values, standard deviation, and minimum and maximum values used for development of each model are shown in Table 2.1. The range of d(0.9) values and the corresponding related particle size parameters used for the modeling covers the critical range values (1.0 to 3.4 μm) that needs to be measured to determine if a homogenizer within a MIR milk analyzer has adequate performance to ensure repeatable and accurate data for other milk components measured by a MIR milk analyzer (Smith et al., 1994; Smith et al., 1995; and Lynch et al., 2006). In the USDA Federal Milk Marketing Orders a d(0.9) value of 1.7 μm is used as practical decision value to replace a homogenizer in a MIR milk analyzer (Smith et al., 1995; Lynch et al., 2006). New homogenizers are expected to produce a d(0.9) of milk homogenized at 40^{o}C of $\leq$ 1.5 μm.

A 12 factor model was selected as the optimum model for prediction of d(0.5) and D[3,2] and a 10 factor model was selected as the optimum model for prediction of d(0.9) and D[4,3] (Table 2.1). The SECV, R-square, F-Ratio predicted residual sum of squares (PRESS), F-Test (FPRESS), and residual prediction deviation (**RPD**) values [i.e., ratio of standard deviation of final population of reference samples (i.e., with outliers removed) used to develop PLS prediction models to standard error of cross validation] for each PLS model are shown in Table 2.1. The higher the RPD value, the greater the probability of an accurate prediction of an unknown sample. If the SECV is large compared to the SD, resulting in a small RPD, the PLS model is considered less robust. A RPD value between 2 and 5 indicates that the calibration may be useful for

screening purposes (i.e., qualitative determination) (Williams, 2010). The RPD and R-square for the d(0.5), d(0.9) and D[3,2] models are near 3 and 0.9, respectively, while the D[4,3] has a lower RPD and R-square (Table 2.1).

Table 2.1. Number of samples, mean of reference values, standard deviation (SD), minimum and maximum values of final population of samples (i.e., with outliers removed) used to develop PLS prediction models. Number of factors, standard error of cross validation (SECV), R-square, F-Ratio predicted residual sum of squares (PRESS), F-Test (FPRESS), and residual prediction deviation (RPD) of the calibration step for the PLS models to predict particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3] from the MIR spectra of homogenized milk samples.

	MIR PLS prediction models			
	d(0.5)	d(0.9)	D[3,2]	D[4,3]
Number of samples	349	357	348	344
Mean (μm)	0.57	1.53	0.47	0.80
SD (μm)	0.10	0.33	0.06	0.16
Minimum (μm)	0.32	1.03	0.34	0.50
Maximum (μm)	0.88	3.36	0.67	1.44
Number of factors	12	10	12	10
SECV	0.03	0.12	0.02	0.09
R-square	0.93	0.88	0.92	0.68
F-Ratio (PRESS)	1.03	1.01	1.03	1.01
F-Test (FPRESS)	0.60	0.54	0.61	0.53
RPD	3.73	2.83	3.44	1.75

The optimized MIR sample and reference center wavenumbers for fat, protein, and lactose measurements in milk are: 2851 and 2812 cm^{-1} (fat B – carbon hydrogen stretch); 1748 and 1791 cm^{-1} (fat A – carbonyl stretch); 1541 and 1491 cm^{-1} (protein – amide stretch); and 1048 and 1293 cm^{-1} (lactose – hydroxyl stretch), respectively

(Kaylegian et al., 2009). For the development of PLS prediction models, three spectral regions were used: 3000 to 2750 cm^{-1}, 1800 to 1700 cm^{-1}, and 1585 to 1000 cm^{-1}. Beta coefficients for the PLS models for prediction of d(0.5), d(0.9), D[3,2], and D[4,4,3] were plotted as a function of wavenumber (cm^{-1})**,** as shown in Figures 2.1 and 2.2.

The beta coefficients for the d(0.5) and D[3,2] models are very similar in the wavenumber region between 3000 to 2750 cm^{-1} (Figure 2.1A) and between 1800 to 1700 cm^{-1}, and 1585 to 1000 cm^{-1} (Figure 2.2A). The beta coefficients for the d(0.9) and D[4,3] models have large values in the wavenumber regions between 3000 to 2750 cm^{-1} (Figure 2.1B), particularly at center wavelength 2851 cm^{-1} that is used for the prediction of fat B (C-H stretch) content of milk and between 1800 to 1700 cm^{-1}, and 1585 to 1000 cm^{-1}, particularly at 1748 cm^{-1} that is used for the prediction of fat A (C=O stretch) content of milk (Figure 2.2B). The regions of the spectra where the beta coefficients are large are the areas that contain the most information to predict milk fat globule particle size distribution. This is consistent with the fact that poor homogenization performance within a MIR milk analyzer influences the accuracy of milk fat test.

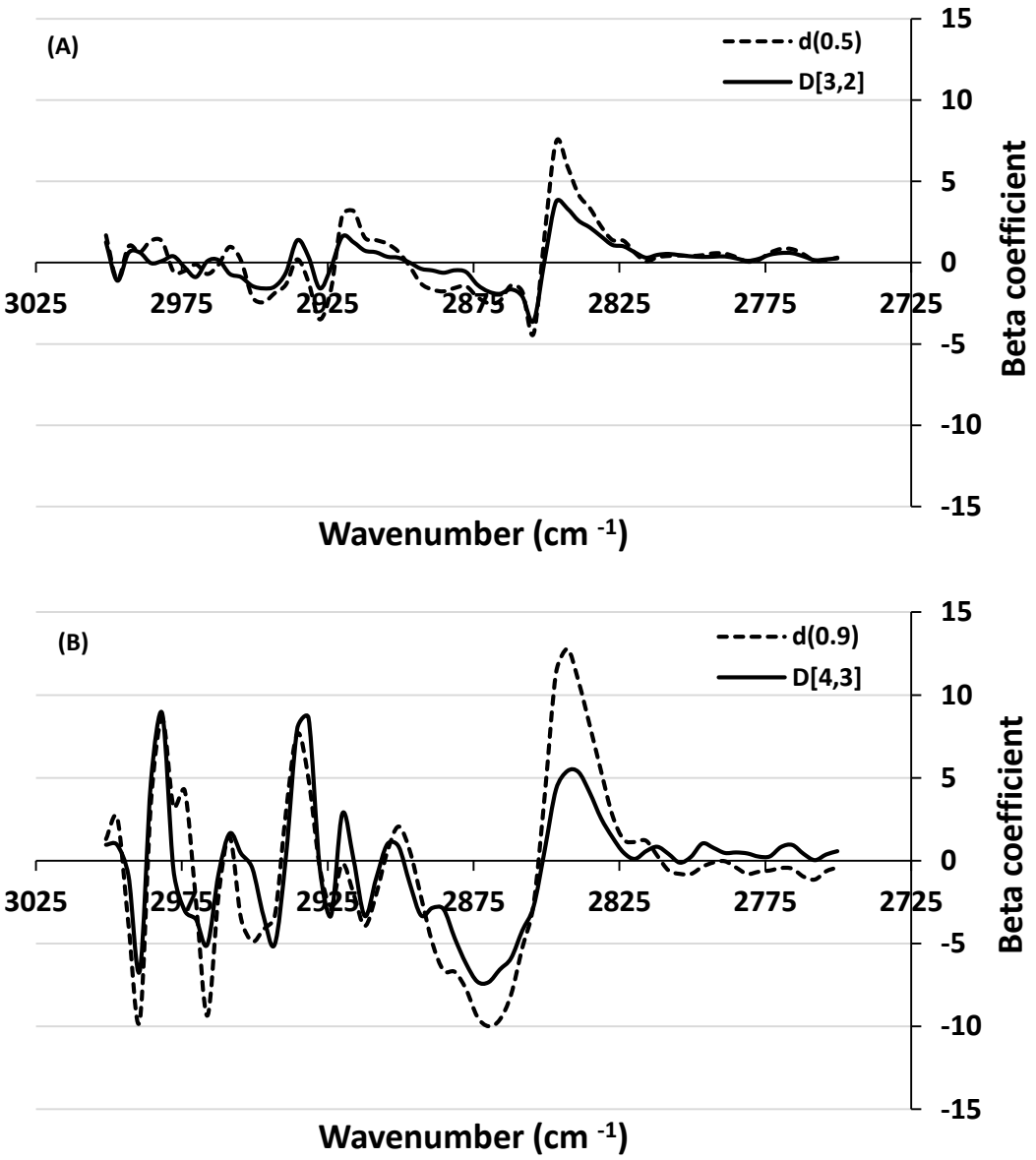

Figure 2.1. Beta coefficient plotted as a function of wavenumber (cm^{-1}) in spectral region of 3000 to 2750 cm^{-1} for the PLS prediction models: (A) particle size distribution d(0.5) and surface volume mean diameter D[3,2], and (B) particle size distribution d(0.9) and volume moment mean diameter D[4,3] of milk fat globules in homogenized milk.

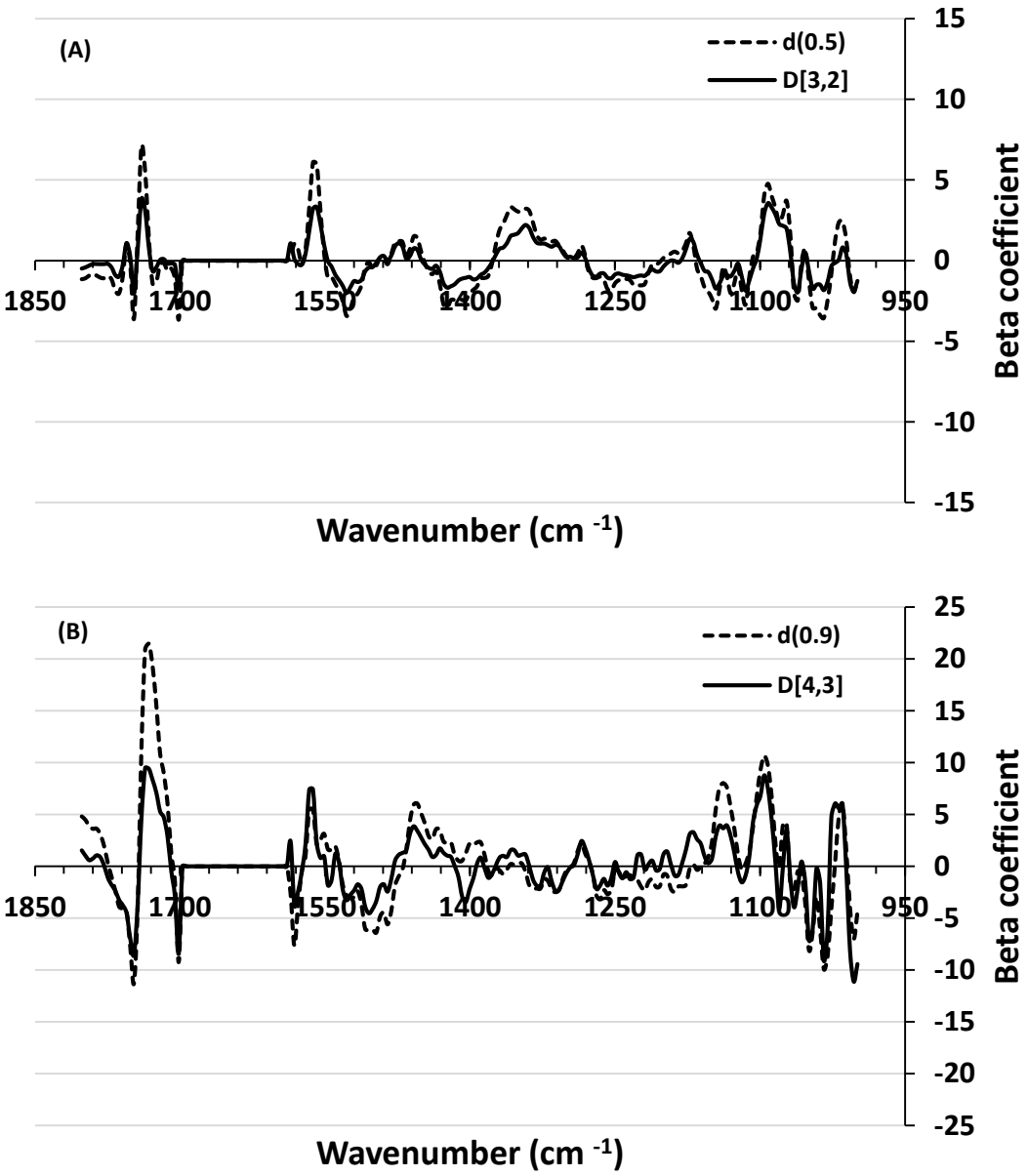

Figure 2.2. Beta coefficient plotted as a function of wavenumber (cm^{-1}) in spectral region of 1800 to 1700 cm^{-1} and 1585 to 1000 cm^{-1} for the PLS prediction models: (A) particle size distribution d(0.5) and surface volume mean diameter D[3,2], and (B) particle size distribution d(0.9) and volume moment mean diameter D[4,3] of milk fat globules in homogenized milk.

Performance of Developed Partial Least Squares Models

Slope and Intercept Adjustment. A new PLS model prediction output starts with a secondary slope and intercept of 1 and 0, respectively. Comparison of mean d(0.5), d(0.9), D[3,2], and D[4,3] reference values versus MIR predicted values, for milks 7 to 14 in the modified milk calibration set before and after slope and intercept adjustment, are shown in Table 2.2. After slope and intercept adjustment the MD between reference and MIR was zero for all predicted parameters and the SDD and RSD of calibration adjustment are shown in Table 2.2. The magnitude of the slope and intercept adjustment was the largest on the d(0.9) prediction. After adjustment, the RSD decreased from 8.8 to about 5.5%. The prediction model for volume mean diameter D[4,3] had the highest RSD.

Validation of the Models. The slope and intercept adjustments (Table 2.2) were applied to the MIR predicted values for the independent validation samples to determine the performance of the MIR particle size prediction models. The comparison of the d(0.5), d(0.9), D[3,2], and D[4,3] reference values versus MIR predicted values are shown in Table 2.3. For real-time evaluation of homogenizer performance in a MIR milk analyzer, the prediction of d(0.9) is the most important parameter related to analytical performance of the MIR milk analyzer (Lynch et al., 2006). There was good agreement on average of laser light scattering particle size reference values and MIR estimates of the four particle size distribution parameters (Table 2.3).

Table 2.2. Comparison of slope, intercept, R-square, mean of reference values, mean of MIR predicted values, mean difference (MD) between MIR predicted and reference values, standard deviation of the difference (SDD), and relative standard deviation (RSD) of modified milk calibration set (samples 7-14) used as the calibration set for the PLS models to predict particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3] from the MIR spectra of homogenized milk samples, before and after slope and intercept adjustment.

Calibration Adjustment	Parameter	Slope	Intercept	R-square	Reference	MIR Predicted	MD	SDD	RSD
Before	d(0.5)	1.00	0.00	0.93	0.69	0.66	-0.031	0.029	4.23
After	d(0.5)	0.96	0.06	0.95	0.69	0.69	0.000	0.029	4.17
Before	d(0.9)	1.00	0.00	0.88	1.71	1.84	0.129	0.151	8.81
After	d(0.9)	0.71	0.41	0.90	1.71	1.71	0.000	0.094	5.48
Before	D[3,2]	1.00	0.00	0.92	0.53	0.52	-0.014	0.021	3.95
After	D[3,2]	0.93	0.05	0.92	0.53	0.53	0.000	0.021	3.84
Before	D[4,3]	1.00	0.00	0.68	0.91	0.92	0.014	0.082	9.01
After	D[4,3]	0.82	0.15	0.73	0.91	0.91	0.000	0.077	8.46

MD = mean of MIR predicted minus mean of reference.
RSD = (SDD/mean reference) x 10

Table 2.3. Comparison of mean of reference values, mean of MIR predicted values, mean difference (MD) between MIR predicted and reference values, standard deviation of the differences (SDD) between reference and predicted, and relative standard deviation (RSD) of validation step of the PLS models performance evaluation.

		MIR			
Parameter	Reference	Predicted	MD	SDD	RSD
d(0.5)	0.658[a]	0.634[b]	-0.024	0.036	5.47
d(0.9)	1.626[a]	1.645[a]	0.019	0.148	9.10
D[3,2]	0.516[a]	0.503[b]	-0.012	0.022	4.32
D[4,3]	0.868[a]	0.838[b]	-0.029	0.110	12.67

MD = mean of MIR predicted minus mean of reference
RSD = (SDD/mean reference) x 100
[ab] Means not sharing a common superscript differ ($P < 0.05$)

The mean MIR predicted values for d(0.5), D[3,2], D[4,3] were lower ($P < 0.05$) than laser light scattering values, while no difference in the MIR predicted d(0.9) versus laser light scattering was detected. All mean differences were small (i.e., 0.03 µm). The RSD for the predictions of d(0.9) and D[4,3] were larger than for the d(0.5) and D[3,2], indicating more sample-to-sample analytical variation.

DISCUSSION

What did the partial least squares modeling see in the spectra to predict particle size?

The beta coefficient plots contain the spectral information that drives the PLS model predictions and indicates the correlation between the response Y [d(0.5), d(0.9), D[3,2], and D[4,3] values] and each of the X absorbance (i.e., indicate which X absorbance are contributing to the modeling of the response Y) (Janik et al., 2007).

Large beta coefficients in the wavenumber region between 2855 to 2845 cm^{-1} (Figure 1B) and between 1740 to 1710 cm^{-1} (Figure 2.2B) for the d(0.9) and D[4,3] models, indicate that there is a strong correlation between the infrared light absorbance present in those regions of the MIR spectra and the estimate of [d(0.9) and D[4,3]]. To determine what was happening in those regions of the spectra, MIR absorbance spectra were plotted as a function of wavenumber (cm^{-1}) for sample 12 (5% fat and 2.4% true protein) of the modified milk set, replicate 1, homogenized using five different homogenizers to produce milk with different particle size distributions d(0.5) and d(0.9) in spectral region 3000 to 2750 cm^{-1} (Figure 2.3) and 1800 to 1700 cm^{-1} (Figure 2.4). Spectra of other samples exhibited similar behavior.

At low magnification there was nothing outstanding in the MIR absorbance spectra in the wavenumber region 3000 to 2750 cm^{-1} (Figure 2.3A) and 1800 to 1700 cm^{-1} (Figure 2.4A), but if we zoom in the region of absorbance between 2860 to 2850 cm^{-1} (Figure 2.3B), and 1750 to 1740 (Figure 2.4B) we observe that there was a systematic shift in the region of absorbance of fat B and fat A, respectively.

According to Goulden (1964) and Smith et al. (1993), large fat globules can lead to the Christiansen effect, which is a change of the refractive index of milk fat at wavelengths near maximum absorption by the carbonyl and carbon-hydrogen groups. This shifts the apparent wavelength of maximum light absorption to a longer wavelength. The Christiansen effect is observed in Figures 2.3B and 2.4B. We conclude that systematic variation in the spectra due to the Christiansen effect enabled the PLS models to predict particle size distribution from the MIR spectra.

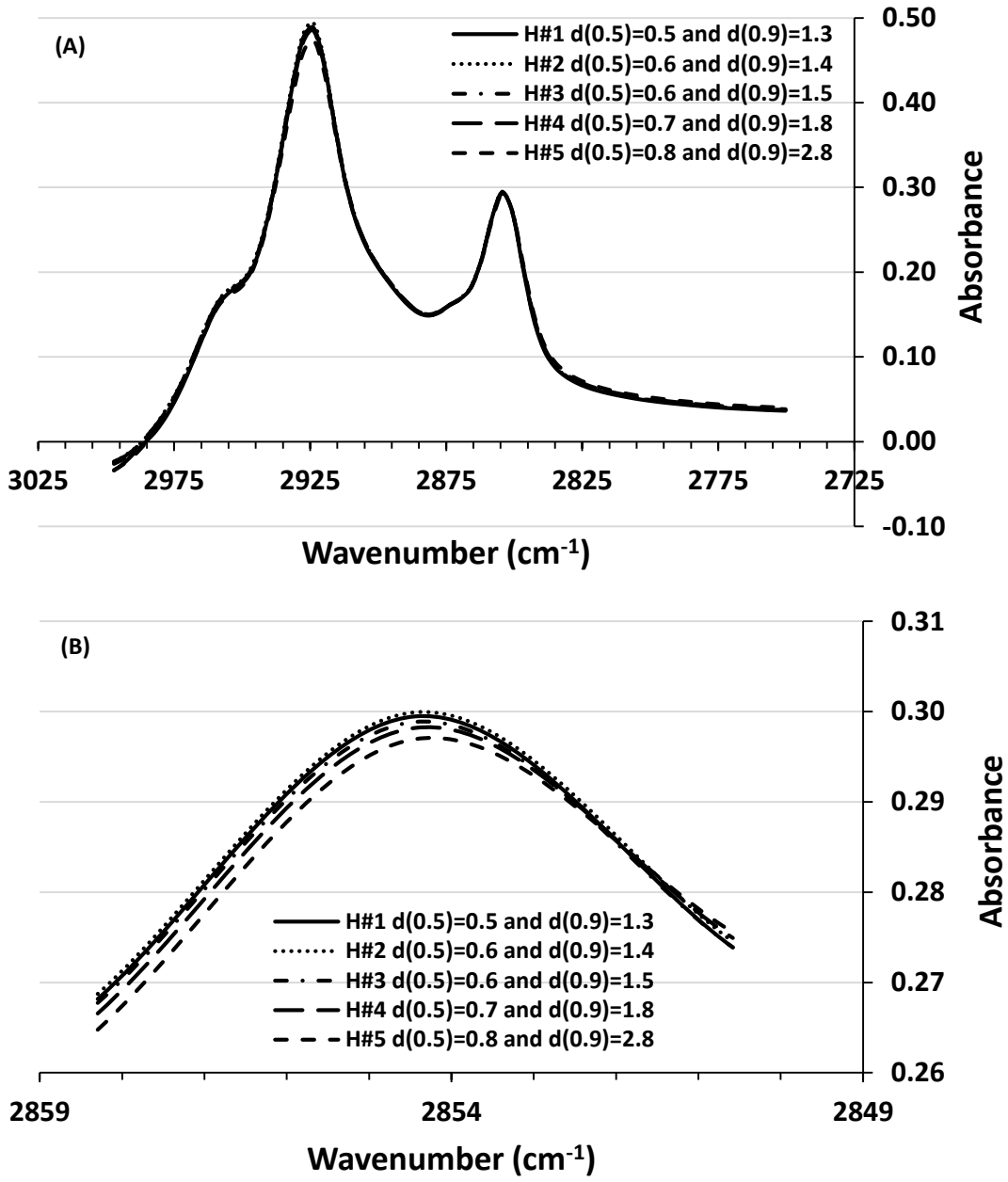

Figure 2.3. MIR absorbance spectra plotted as a function of wavenumber (cm^{-1}) for sample 12 (5% fat and 2.4% true protein) of the modified milk set, replicate 1, homogenized with five different homogenizers (H#1 to H#5) producing milks with different particle size distributions d(0.5) and d(0.9) in spectral region of: (A) 3000 to 2750 cm^{-1} and (B) 2860 to 2850 cm^{-1}.

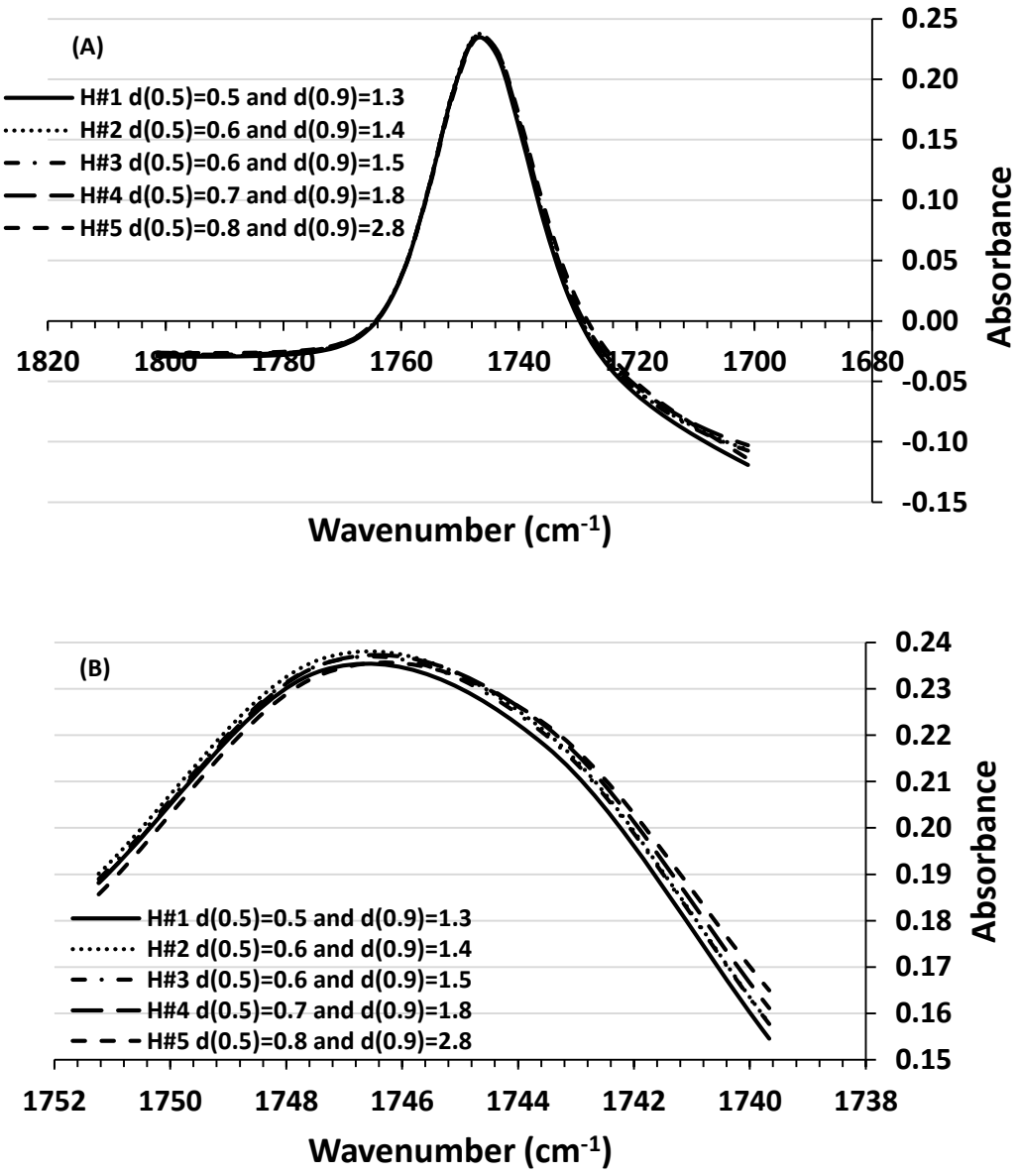

Figure 2.4. MIR absorbance spectra plotted as a function of wavenumber (cm^{-1}) for sample 12 (5% fat and 2.4% true protein) of the modified milk set, replicate 1, homogenized with five different homogenizers (H#1 to H#5) producing milks with different particle size distributions d(05) and d(0.9) in spectral region of: (A) 1800 to 1700 cm^{-1} and (B) 1750 to 1740 cm^{-1}.

What could be the cause of the larger sample-to-sample variation of the d(0.9) and D[4,3]?

In the model development phase, the milk in the cuvette and the milk tested by laser light scattering were identical because the homogenization was done with a homogenizer external to the MIR and there was no homogenizer in the MIR flow system. This approach should produce MIR prediction models that would produce accurate results. Both for slope and intercept adjustment of the model data output and for validation the situation was different because the homogenization step took place in-line within the instrument's flow system, not external to the system. As indicated previously, the volume of instrument homogenized milk (about 2 drops) in the cuvette is a very small portion of the total milk that passed through the homogenizer during the measurement cycle. For slope and intercept adjustment and for validation, the reference test was conducted on the total volume of milk from the measurement cycle (i.e., many pump strokes) while the milk spectra for that milk is only from a portion of one pump stroke. It is likely that there is pump stroke to pump stroke variation in the homogenization efficiency. Thus, in the case of the real time analysis, there is random variation from pump stroke to pump stroke in the particle size produced by the homogenizer. This would lead to accurate predictions on average of many samples but larger sample-to-sample variation. It is likely that the variation in the particle size distribution produced from one pump stroke to the next will be larger as the homogenizer becomes less efficient (i.e., produces larger particle size). This may be why the larger RSD are observed for the d(0.9) and D[4,3], while the average d(0.9) and D[4,3] for a large number of validation samples had good agreement of overall mean

values (Table 2.3). This may lead to the decrease in milk fat analytical repeatability reported for less efficient homogenizers (Smith et al., 1994) and may also impact the accuracy of fat predictions by both traditional filter models and PLS prediction models of fat content of milk.

Another factor that may have caused the larger sample-to-sample variation in estimated d(0.9) and D[4,3] is the influence of variation from sample-to-sample in fatty acid chain length and unsaturation on MIR results, as described by Kaylegian et al. (2009). As fatty acid chain length increases, an increase in absorbance is expected at the sample center wavelength for fat B and fat A (Kaylegian et al., 2009). Furthermore, as the unsaturation of fatty acids increases, a decrease in absorbance is expected at the fat B sample and reference center wavelengths and an increase in light absorbance is expected for the fat A sample wavelength (Kaylegian et al., 2009). This variation in fatty acid chain length and unsaturation causes changes in absorbance in the region of the spectra where the beta coefficients are large for particle size prediction and this variation is not related to variation in particle size.

CONCLUSIONS

Partial least squares regression models for predicting the particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3] of milk fat globules in homogenized milk from a MIR milk spectra were developed and validated. The basis for the ability to model particle size distribution of milk fat emulsions was hypothesized to be the result of the PLS modeling detecting absorbance shifts in MIR spectra of milk fat due to the Christiansen

effect. The independent sample validation of particle size prediction methods found that there was more variation in d(0.9) and D[4,3] predictions than the d(0.5) and D[3,2] predictions relative to laser light scattering reference values and this may be due to variation in particle size among different pump strokes. The accuracy of the d(0.9) prediction for routine quality assurance to determine if a homogenizer within a MIR milk analyzer was near the failure level (i.e., d(0.9) > 1.7 μm) and needed to be replaced is fit-for-purpose. The daily average particle size performance (i.e., d(0.9)) of a homogenizer based on the mean for the day could be used for monitoring homogenizer performance.

ACKNOWLEDGMENTS

The authors thank the USDA Federal Milk Markets laboratories of Cleveland, OH; Dallas, TX; and Lebanon, NH for their collaboration. The authors thank the Northeast Dairy Foods Research Center (Ithaca, NY) and Delta Instruments (Drachten, Netherlands) for support of this research. The technical assistance of Chassidy Coon, Michelle Billota, and Sara Bova of Cornell University and the service staff of Delta Instruments was greatly appreciated.

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CHAPTER THREE

IMPACT OF HOMOGENIZER PERFORMANCE ON ACCURACY AND REPEATABILITY OF MID-INFRARED PREDICTED VALUES FOR MAJOR MILK COMPONENTS

ABSTRACT

Our objective was to determine the impact of mid-infrared (MIR) homogenizer efficiency on accuracy and repeatability of Fourier Transform MIR predicted fat, true protein, and anhydrous lactose determination given by traditional filter and partial least squares (PLS) prediction models. Five homogenizers with different homogenization performance based on laser light scattering particle size analysis were used. Repeatability and accuracy were determined by conducting 17 sequential readings on milk homogenized externally to the instrument (i.e., control) and unhomogenized milk. Milk component predictions on externally homogenized milks were impacted by variation in homogenizer performance, but the magnitude of impacts were small (i.e., < 0.025%) when milks were pumped through both efficient and inefficient homogenizers within a MIR milk analyzer. Variation in the in-line MIR homogenizer performance on unhomogenized milks had a much larger impact on accuracy of component testing than on repeatability. The increase of particle size distribution d(0.9) from 1.35 to 3.03 μm due to poor homogenization impacted fat tests the most: traditional filter based fat B (- 0.165%), traditional filter based fat A (- 0.074%), and fat PLS (- 0.078%), at a d(0.9) of 3.03 μm. Variation in homogenization efficiency also impacted traditional filter based

true protein test (+ 0.012%), true protein PLS prediction (-0.107%), and traditional filter based anhydrous lactose test (+ 0.027%), at a d(0.9) of 3.03μm. Effects of variation in homogenization on anhydrous lactose PLS predictions were small. The accuracy of both traditional filter models and PLS models was influenced by poor homogenization. The value of 1.7 μm for a d(0.9) used by the USDA Federal Milk Market laboratories as a criteria to make the decision to replace the homogenizer in a MIR milk analyzer appears to be a reasonable limit, given the magnitude of impact on the accuracy of fat tests. In the future as new PLS models are developed to measure other components in milk, the sensitivity of the accuracy of the predictions of these models to factors such as variation of homogenizer performance should be determined as part of the ruggedness testing during PLS model development.

INTRODUCTION

Mid-infrared (MIR) milk analysis is based on the principle that each specific chemical bond absorbs MIR energy at a specific wavelength, and the measurement of the intensities of the absorption peaks makes it possible to quantify milk components (Goulden, 1964; Biggs, 1967; Biggs et al., 1987). The MIR energy passing through inefficiently homogenized milk can be distorted by the Christiansen light scattering effect, which causes a shift in the apparent wavelength of maximum absorption by the carbonyl and carbon-hydrogen groups to a longer wavelength affecting the accuracy of MIR readings (Goulden, 1961). This shift in wavelength or light absorbance may have a negative impact on the accuracy of the determination of the concentration of the major components of milk. As a result, quality assurance programs for MIR milk analysis

often include a test to determine if the homogenizer in a MIR milk analyzer is working properly (Lynch et al., 2006). The systematic shift in the absorbance spectra due to the Christiansen effect enables PLS prediction of particle size distribution d(0.9) from the Fourier transform MIR spectra as an alternative method to determine if the homogenizer within a MIR milk analyzer needs to be replaced (Di Marzo, 2016) instead of having an external laboratory run laser light scattering particle size analysis on milk homogenized by each instrument.

Commercial Homogenization of Milk. The recommended milk temperature for commercial milk homogenization is between 60 to 75°C, which achieves breakage of milk fat into smaller fat globules and reduce the tendency of fat globules to aggregate and rise to the top of container of fluid milk (Trout, 1950; Walstra et at., 2005). If the temperature of homogenization of milk is below the melting point of milk fat (i.e., < 40°C), fat will be in the solid state resulting in incomplete fat dispersion and ineffective homogenization (Trout, 1950; Bylund, 1995). In a typical commercial homogenizer, a high pressure positive displacement pump forces heated milk through a narrow gap in the homogenizer valve (Mulder and Walstra, 1974; Phipps, 1985). As the milk is forced through the gap at high pressure, the linear velocity of the milk and shear forces increase. Often the high velocity milk is projected against a surface to create high turbulence and more shear leading to reduction in fat globule size (Mulder and Walstra, 1974; Walstra et al., 2005). The high pressure positive displacement pump can be equipped with a single piston or with multi-pistons (3, 5 or 7). In a single piston homogenizer, the valves open and close with every stroke of the piston and the flow pressure goes from zero to the set pressure for that stage back to zero as the valve opens and closes. The multi-

pistons are operated intentionally out of phase to achieve a constant applied pressure, continuous flow of milk and uniform homogenization. The higher the number of pistons, the more the pressure fluctuations are minimized (Phipps, 1985) and the homogenizer valves are running open continuously with relatively constant pressure decrease across the gap. In this way, the valve wear is minimized and consistent particle size is achieved (Walstra, 1975).

Typical homogenization of pasteurized fluid milk is done with a 2-stage homogenizer with a 1st stage pressure of 20 MPa and 2nd stage pressure of 5 MPa (Walstra et al., 2005). The second valve should always operate at lower pressure (i.e. 20% of the total pressure) (Walstra, 1975). The function of the high pressure 1st stage is to break fat globules to smaller sizes. The newly formed small fat globules are no longer exclusively covered with the original milk fat globule membrane. Instead, they are also covered with protein adsorbed from the milk plasma (Walstra et al., 2005). In the turbulent environment created by velocity of the milk and shear forces in the milk exiting the 1st stage, the small fat globules may start to collide before they are completely covered with protein, leading to fat globule coalescence (Mulder and Walstra, 1974). The 1st stage creates up to 10 fold increase of the milk fat-plasma interfacial surface area. If the surface of the new formed fat globules lacks protein, the small fat globules may easily come together to share protein at their interface forming clusters (Mulder and Walstra, 1974). The function of the low pressure 2nd stage is to break up the fat globule clusters (Walstra, 1975; Phipps, 1985; Walstra et al., 2005). At the 2nd stage the pressure is low, so that the new surface created is insignificant and new clusters are not formed (Mulder and Walstra, 1974). Enough time needs to be given for the newly

formed fat globules to cluster after milk passes through the 1st stage valve so that the 2nd stage valve will be able to fulfill its role in breaking clusters (Walstra et al., 2005). The typical fat globule size distribution d(0.9) in a commercially homogenized milk is about 1.2 to 1.8 µm (Caplan and Barbano, 2013).

Homogenization in a Mid-Infrared Milk Analyzer. The MIR homogenizer designs are slightly different than a commercial homogenizer. All homogenizers within MIR milk analyzers are single piston homogenizers, so the pressure across the homogenizer stages is going from zero to full pressure and back to zero with every pump stroke during the pumping of a single milk sample. Some MIR homogenizer designs have the springs in the milk flow and this is different than commercial homogenizers. A 2-stage homogenizer with the springs in the milk flow is shown in Figure 3.1. This design of homogenizer has been used by Multispec (no longer in business), Bentley Instruments (Chaska, Minnesota), and Delta Instruments (Drachten, Netherlands). This type of homogenizer includes the 2 stages connected in series within a single homogenizer housing, which is mounted in the MIR as shown in Figure 3.1A. In Figure 3.1B the internal parts of the homogenizer (#1) are shown. The strength of the 1st (#8) and 2nd stage (#13) springs are different (Figure 3.1B). Heated milk (about 40°C) is pumped through the homogenizer and reaches the 1st stage seat (#4) and the ball (#7). Higher milk temperatures are not used in MIR milk analyzers because of the negative impact of high milk temperatures on the cuvette. The high milk pressure (15 MPa) operating against the spring (#8) forces the ball off the seat and opens a narrow gap between the seat and the ball, completing the 1st stage of homogenization. Then, milk flows through the spring (#8) and reaches the seat (#10) and the ball (#12). In the 2nd

stage a lower pressure (3 MPa) is need against the spring (#13) to open the gap between the seat and the ball, thus there is a flow pressure decrease between stage 1 and 2. Homogenized milk passes through the spring (#13) and flows to the cuvette in the MIR flow system.

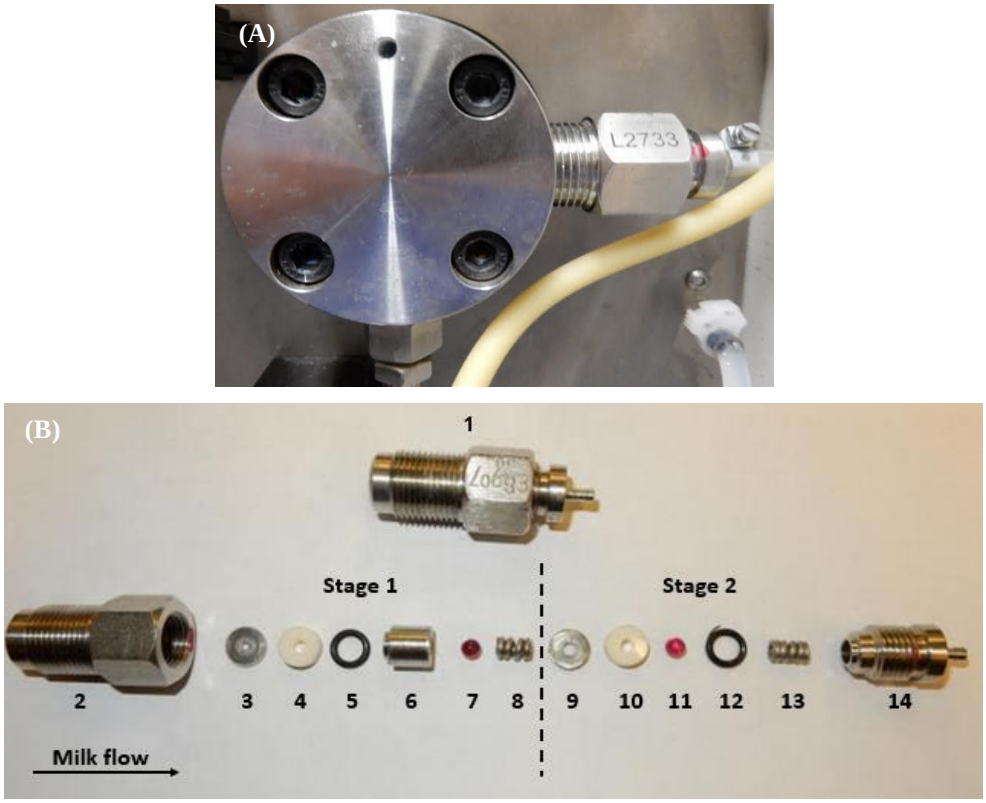

Figure 3.1. A 2-stage homogenizer design with the springs in the milk flow: (A) homogenizer mounted in the instrument, and (B) parts of the 2 stages of the homogenizer: (1) assembled homogenizer, (2) homogenizer housing, (3) 1st stage support disc, (4) 1st stage seat, (5) O-ring, (6) cylindrical spring guide, (7) 1st stage ruby ball, (8) 1st stage spring, (9) 2nd stage support disc, (10) 2nd stage seat, (11) O-ring, (12) 2nd stage ruby ball, (13) 2nd stage spring, (14) homogenizer outlet cap.

A 2-stage homogenizer designed with springs outside the milk flow is shown in Figure 3.2. This type of homogenizer has two separate homogenizer valve housings connected in series in the MIR (Figure 3.2A). In Figure 3.2B the parts of one homogenizer stage housing (#1) are shown. In this type of homogenizer, the 2 stages are identical and set at equal pressures. Heated milk is pumped through the homogenizer and reaches the seat (#3), the ball (#4) and the piston (#9) (Figure 3.2B).

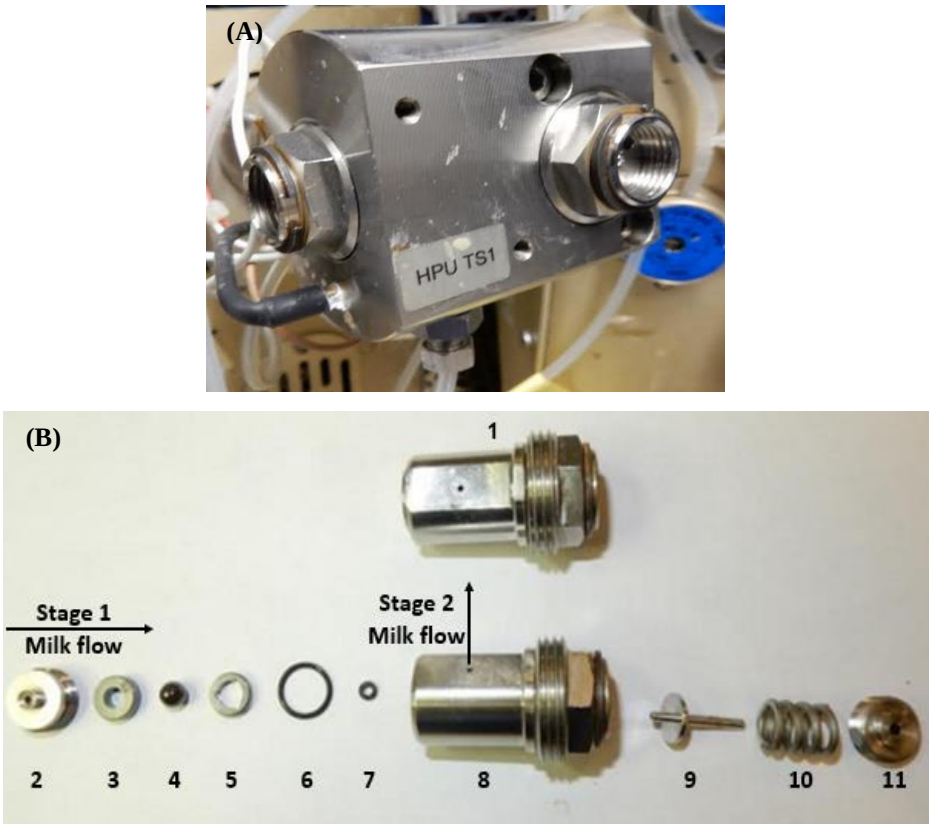

Figure 3.2. A 2-stage homogenizer design with springs outside the milk flow: (A) homogenizer mounted in the instrument with 2 identical homogenizer stages, and (B) parts of one of the 2 stages of the homogenizer: (1) 1st stage homogenizer valve assembled, (2) screw in end cap, (3) 1st stage seat, (4) 1st stage ball, (5) aligner to keep ball centered on seat, (6) O-ring, (7) O-ring around piston that puts pressure on the ball, (8) 1st stage homogenizer housing, (9) 1st stage piston, (10) 1st stage spring, (11) 1st stage end cap that is turned in to compress the 1st stage spring to apply the appropriate loading pressure of the piston against the 1st stage ball.

The high pressure operating against the spring (#10) opens a narrow gap between the seat and the ball, allowing milk to flow through the 1st stage of homogenization and exit to the 2nd stage. Next, milk flows to the 2nd homogenizer housing. At this 2nd stage an equal pressure (i.e., 10 MPa on each stage) is applied against the spring to open the gap between the seat and the ball in the Foss Electric (Hillerod, Denmark) model FT 6000 and FT 120 MIR milk analyzers. In the newer Foss model FT+ the 1st stage pressure is higher than the 2nd stage pressure. In both homogenizer designs, the seats and balls open and close with every pump stroke, resulting in variation in the applied pressure and intermittent flow of milk. Mechanical failures of seats, balls, and springs over time are more likely to happen with this rapid opening and closing during pumping of each sample. This may result in differences in homogenization efficiency from one pump stroke to the next which will influence repeatability. The reality is that only a portion of homogenized milk from a single pump stroke is actually scanned in the cuvette.

Homogenization efficiency can impact MIR repeatability, accuracy, and consequently impact milk payment. In the literature there is no specific information comparing the impact of variation in homogenization efficiency on the accuracy of major milk component prediction by MIR using traditional filter versus PLS models. Our objective was to determine the impact of MIR homogenizer efficiency on accuracy and repeatability of Fourier Transform MIR predicted fat, true protein, and anhydrous lactose determination given by traditional filter and PLS prediction models.

MATERIALS AND METHODS

Experimental Design

Five in-line 2-stage MIR homogenizers (Delta Instruments, Drachten, Netherlands) with different homogenization efficiency (i.e., produced different milk fat globule size distributions) were used to homogenize unpreserved, pasteurized, externally homogenized whole milk and unhomogenized whole milk (Cornell Dairy, Ithaca, NY). Prior to the experiment, the slopes and intercepts for fat, protein and lactose predictions were adjusted using modified milk calibration samples (Kaylegian et al. 2006) with a homogenizer that produced homogenized milk with fat globule size distribution d(0.9) of about 1.28 µm. Each homogenizer used in the study was connected in-line to a MIR milk analyzer (LactoScope FTIR Advanced (FTA), Delta Instruments, Drachten, Netherlands), and 18 component test predictions for traditional filter based fat B, traditional filter based fat A, fat PLS, traditional filter based true protein, true protein PLS, traditional filter based anhydrous lactose, and anhydrous lactose PLS were collected for both externally homogenized and unhomogenized milk samples at 40 to 42^{o}C. Homogenized milks were collected from the MIR outlet tube and then analyzed with a laser light scattering particle size analyzer (Mastersizer 2000, model MS2000; Malvern Instruments, Worcestershire, United Kingdom). Both externally homogenized and unhomogenized milks were run through all five different homogenizers. Repeatability and accuracy of fat, true protein, and anhydrous lactose determination using traditional filter and PLS prediction models were evaluated.

Evaluation of Repeatability

The repeatability test was performed with the LactoScope FTIR Advanced (FTA) milk analyzer equipped with a BMX optical bench (ABB Bomem, Montreal, Canada). A CaF_2 cuvette (36 µm) was used with a fixed virtual filter calibration approach (Kaylegian et al. 2006). Precalibration was performed according to the procedures described by Lynch et al. (2006), and modified milks were used to adjust slope and intercept of fat, protein and lactose predictions (Kaylegian et al., 2006). Traditional virtual filter models used the optimized filter wavelengths determined by Kaylegian et al., 2009 for fat A, fat B, protein and lactose and the gain (i.e., scale factor) and intercorrection factors used in the current study are summarized in Table 3.1. In addition, PLS models were also used to predict fat, true protein, and anhydrous lactose using Delta Instruments PLS model parameter numbers 9507, 9508, and 9509, respectively. On test day, unpreserved, pasteurized, externally homogenized and unhomogenized whole milks were provided by the Cornell University Dairy (Ithaca, NY) and split into clear 90 mL vials (Capitol Plastic Products, 1030 Riverfront Center, Amsterdam, NY). Homogenized and unhomogenized milks and 0.01% (volume/volume) Triton-X 100 surfactant solution (G000071020, Delta Instruments, Drachten, Netherlands) were warmed to 42 $\pm$ 1°C using a water bath (GCA Corporation, Chicago, IL). The five different homogenizers were tempered to 42°C in the same water bath so they would quickly come to temperature equilibrium when attached to the MIR system.

Table 3.1. Sample and reference center wavenumbers (cm^{-1}), bandwidths, powers, scales, offsets, and intercorrection factors used in MIR milk analyzer.

Wavenumber (cm^{-1})										Intercorrection factors			
Sample					Reference								
Center	Bandwidth	Power	Scale	Offset	Center	Bandwidth	Power	Scale	Offset	Fat B	Lactose	Protein	Fat A
2851	26	0	34.810	0	2812	24	0	-34.810	0	1.000	-0.149	-0.053	0.000
1048	20	0	17.947	0	1293	14	0	-17.947	0	0.045	1.000	0.022	0.000
1541	20	0	22.256	0	1491	12	0	-22.256	0	0.066	0.051	1.000	0.000
1748	16	0	22.171	0	1791	16	0	-22.171	0	0.000	0.029	0.023	1.000

The first homogenizer was connected to the MIR system and the instrument was cleaned using Decon 90 (Decon Laboratories Ltd., E. Sussex, UK) and adjusted to read zero using a 0.01% (volume/volume) Triton-X 100 solution. The first vial of homogenized milk was mixed by inversion, the temperature was checked and the milk was pumped through the MIR system, 3 corrected readings were collected, and the MIR homogenized milk was collected at the instrument's outlet tube in a clear 60 mL vial (Capitol Plastic Products, 1030 Riverfront Center, Amsterdam, NY). The MIR homogenized milk collected into the 60 mL vial was analyzed by laser light scattering particle size analyzer (Mastersizer 2000, model MS2000; Malvern Instruments, Worcestershire, United Kingdom) using data kill function set up to exclude particle smaller than 0.195 μm to remove casein micelles from final results as described by Di Marzo (2016). This procedure was performed for 6 vials of homogenized milk, followed by 6 vials of unhomogenized milk, resulting in a total of 18 corrected readings for the homogenized samples, and 18 corrected readings for the unhomogenized samples, for each homogenizer. The first of the 18 readings was discarded to avoid carry over from the Triton-X 100 solution. In between the change of homogenized milk to unhomogenized milk the instrument flow system was rinsed with 0.01% (volume/volume) Triton-X 100 surfactant solution. At the end of the test the MIR flow system was rinsed, cleaned, zeroed, the homogenizer was changed, and the procedure described above was repeated. The mean of the MIR predicted values, standard deviation (SD) and range were calculated for traditional filter based fat B, traditional filter based fat A, fat PLS, traditional filter based true protein, true protein PLS, traditional filter based anhydrous lactose, and anhydrous lactose PLS.

Impact of Homogenizer Performance on Accuracy of Mid-Infrared Readings

Data collected during the repeatability test was used to evaluate the impact of change in homogenization performance on accuracy of MIR predicted values given by traditional filter and PLS prediction models. The mean of 17 MIR readings calculated for fat B (traditional filter), fat A (traditional filter), fat PLS, true protein (traditional filter), true protein PLS, anhydrous lactose (traditional filter), and anhydrous lactose PLS was used to calculate the residual difference between homogenizers for unhomogenized milks. The calculation was done as follows: Residual difference (%) = (mean MIR predicted component using test homogenizer) – (mean MIR predicted component using reference homogenizer), where MIR predicted component = fat B (traditional filter), fat A (traditional filter), fat PLS, true protein (traditional filter), true protein PLS, anhydrous lactose (traditional filter), and anhydrous lactose PLS; test homogenizer = 1, 2, 3, 4, 5; and the reference homogenizer was the homogenizer that had the best homogenization performance (i.e., lowest d(0.9)). Residual difference of corrected readings for the unhomogenized milks were plotted (Y) as a function of laser light scattering particle size reference values (X) for each MIR parameter to help visualize and determine the impact of variation in homogenizer performance on accuracy of MIR predicted values for major milk components.

Statistical Analysis

A PROC GLM LMEANS analysis was performed using SAS (SAS, Version 8.02, 2011, SAS Institute Inc., Cary, NC) to determine if the means for MIR predicted fat B (traditional filter), fat A (traditional filter), fat PLS, true protein (traditional filter), true protein PLS, anhydrous lactose (traditional filter), anhydrous lactose PLS, and

particle size distribution d(0.9) were different among the 5 homogenizers, for externally homogenized milks and unhomogenized milks.

An ANCOVA test was performed using JMP (JMP, Version Pro 12, SAS Institute Inc., Cary, NC) to determine if there were differences ($P < 0.05$) in the slopes of the regression lines among MIR predicted fat B and fat A using traditional filter models (Kaylegian et al., 2009) versus PLS predicted fat, predicted true protein using a traditional filter model (Kaylegian et al., 2009) versus PLS predicted true protein, and predicted anhydrous lactose using a traditional filter model (Kaylegian et al., 2009) versus PLS predicted anhydrous lactose plotted as a function of reference particle size distribution d(0.9).

RESULTS

Impact of Homogenizer Performance on Accuracy of Mid-Infrared Readings

Externally Homogenized Milks. The impact of variation in homogenizer performance on the MIR predicted values for major milk components of externally homogenized milks are shown in Table 3.2. If well homogenized milk (i.e., d(0.9) = 1.12 μm) is pumped through homogenizers with very different homogenization performance, what happens to the d(0.9) and the predicted component values?

Table 3.2. Mean fat globule size distribution d(0.9) and mean component (%), standard deviation (SD), and range of 17 MIR readings for traditional filter fat B, traditional filter fat A, fat PLS, traditional filter true protein, true protein PLS (T. protein PLS), traditional filter anhydrous lactose (An. lactose), and anhydrous lactose PLS (An. lactose PLS) on homogenized and unhomogenized whole milks using 5 different homogenizers.

		Homogenized				Unhomogenized			
Parameter	Homo-genizer	d(0.9) (µm)	Component (%)	SD	Range	d(0.9) (µm)	Component (%)	SD	Range
Fat B	1	0.98^{c}	3.856^{a}	0.004	0.015	1.35^{d}	3.764^{a}	0.005	0.017
Fat B	2	1.01^{cb}	3.851^{b}	0.006	0.022	1.34^{d}	3.755^{b}	0.006	0.023
Fat B	3	1.04^{b}	3.847^{c}	0.004	0.015	1.82^{c}	3.718^{c}	0.007	0.022
Fat B	4	1.02^{b}	3.842^{d}	0.006	0.022	2.53^{b}	3.667^{d}	0.018	0.052
Fat B	5	1.11^{a}	3.831^{e}	0.005	0.017	3.03^{a}	3.599^{e}	0.007	0.026
Fat A	1	0.98^{c}	3.802^{a}	0.002	0.009	1.35^{d}	3.718^{a}	0.004	0.017
Fat A	2	1.01^{cb}	3.800^{a}	0.003	0.010	1.34^{d}	3.711^{b}	0.004	0.015
Fat A	3	1.04^{b}	3.800^{b}	0.002	0.009	1.82^{c}	3.692^{c}	0.005	0.019
Fat A	4	1.02^{b}	3.787^{c}	0.004	0.013	2.53^{b}	3.667^{d}	0.010	0.033
Fat A	5	1.11^{a}	3.782^{d}	0.003	0.009	3.03^{a}	3.644^{e}	0.007	0.026
Fat PLS	1	0.98^{c}	3.840^{a}	0.009	0.032	1.35^{d}	3.743^{a}	0.011	0.040
Fat PLS	2	1.01^{cb}	3.827^{bc}	0.007	0.024	1.34^{d}	3.731^{b}	0.007	0.029
Fat PLS	3	1.04^{b}	3.830^{b}	0.008	0.029	1.82^{c}	3.714^{c}	0.013	0.055
Fat PLS	4	1.02^{b}	3.822^{cd}	0.008	0.029	2.53^{b}	3.694^{d}	0.014	0.050
Fat PLS	5	1.11^{a}	3.820^{d}	0.009	0.027	3.03^{a}	3.665^{e}	0.011	0.041

True protein	1	0.98[c]	3.017[b]	0.003	0.010	1.35[d]	3.008[d]	0.003	0.015
True protein	2	1.01[cb]	3.014[c]	0.003	0.011	1.34[d]	3.008[d]	0.004	0.017
True protein	3	1.04[b]	3.020[a]	0.005	0.015	1.82[c]	3.017[b]	0.004	0.014
True protein	4	1.02[b]	3.015[c]	0.004	0.015	2.53[b]	3.013[c]	0.005	0.017
True protein	5	1.11[a]	3.015[bc]	0.003	0.011	3.03[a]	3.020[a]	0.004	0.013
T. protein PLS	1	0.98[c]	3.023[a]	0.006	0.019	1.35[d]	3.003[a]	0.006	0.016
T. protein PLS	2	1.01[cb]	3.025[a]	0.004	0.012	1.34[d]	3.006[a]	0.003	0.013
T. protein PLS	3	1.04[b]	3.025[a]	0.006	0.023	1.82[c]	2.989[b]	0.003	0.012
T. protein PLS	4	1.02[b]	3.023[a]	0.005	0.018	2.53[b]	2.927[c]	0.016	0.061
T. protein PLS	5	1.11[a]	3.017[b]	0.005	0.015	3.03[a]	2.896[d]	0.004	0.014
An. lactose	1	0.98[c]	4.611[c]	0.006	0.023	1.35[d]	4.597[c]	0.008	0.033
An. lactose	2	1.01[cb]	4.621[a]	0.004	0.020	1.34[d]	4.601[c]	0.006	0.024
An. lactose	3	1.04[b]	4.617[b]	0.005	0.017	1.82[c]	4.608[b]	0.008	0.034
An. lactose	4	1.02[b]	4.617[b]	0.005	0.020	2.53[b]	4.619[a]	0.007	0.026
An. lactose	5	1.11[a]	4.622[a]	0.004	0.015	3.03[a]	4.624[a]	0.007	0.023
An. lactose PLS	1	0.98[c]	4.602[b]	0.004	0.010	1.35[d]	4.597[b]	0.006	0.020
An. lactose PLS	2	1.01[cb]	4.606[a]	0.005	0.010	1.34[d]	4.596[b]	0.005	0.010
An. lactose PLS	3	1.04[b]	4.604[ab]	0.005	0.010	1.82[c]	4.596[b]	0.005	0.010
An. lactose PLS	4	1.02[b]	4.602[b]	0.006	0.020	2.53[b]	4.602[a]	0.006	0.020
An. lactose PLS	5	1.11[a]	4.604[ab]	0.005	0.010	3.03[a]	4.599[ab]	0.006	0.020

[abcde] Means not sharing a common superscript differ ($P < 0.05$)

The in-line instrument homogenization of externally homogenized milk produced very little change (i.e., 0.98 to 1.11 μm) in d(0.9) values (Table 3.2). Component predictions on externally homogenized milks were impacted ($P < 0.05$) by variation in homogenizer performance, but the magnitude of impacts were small (i.e., < 0.025%).

Unhomogenized Milks. Typically in payment testing, raw unhomogenized milk (i.e., d(0.9) = about 6 to 7 μm) is analyzed and the homogenizer within the instrument flow system is expected to reduce particle size distribution d(0.9) to less than 1.7 μm. The homogenization of unhomogenized milks by the same five in-line MIR homogenizers as above produced five d(0.9) levels from 1.35 to 3.03 μm, which would include homogenizers with acceptable and unacceptable (i.e., d(0.9) > 1.7 μm) homogenization performance (Smith et al., 1995; Lynch et al., 2006). No difference was detected in d(0.9) between homogenizers 1 and 2, but homogenizers 3, 4, and 5 were different ($P < 0.05$) from each other and from homogenizers 1 and 2. Accuracy of component predictions on unhomogenized milks were impacted more by variation in homogenizer performance than for externally homogenized milk (Table 3.2). Poor homogenization impacted the accuracy of fat predictions ($P < 0.05$) at all d(0.9) levels. The increase of particle size distribution d(0.9) with poor homogenization produced lower ($P < 0.05$) fat tests: traditional filter based fat B (- 0.165%), traditional filter based fat A (- 0.074%) , and fat PLS (- 0.078%), at a d(0.9) of 3.03 μm (Table 3.2). Variation in homogenization efficiency produced a slightly higher (0.012%) traditional filter based true protein test ($P < 0.05$) and a 0.107% lower ($P < 0.05$) true protein PLS prediction at a d(0.9) of 3.03 μm. For traditional filter based anhydrous lactose test, the

poor homogenization efficiency produced a 0.027% higher anhydrous lactose at a d(0.9) of 2.53 and 3.03 μm (Table 3.2), with the impact of variation in homogenization on accuracy of anhydrous lactose test being lower than for fat or true protein. This due to the fact that the wavelengths used for lactose measurement (9.61 μm) are much longer than for fat B (3.48 μm), fat A (5.72 μm) and protein (6.47 μm) (Goulden, 1964; Smith et al., 1993). After homogenization, the fat globule size distribution d(0.9) of milk should be less than 1/3 of the wavelength at which the measurement is made, to minimize light scattering caused by large milk fat globules. Ideally, the fat globule size distribution d(0.9) of milk should be less than 1/3 of fat B wavelength (i.e., d(0.9) < 1.16 μm). Fat B is the shortest wavelength used for fat analysis (Goulden, 1964; Smith et al., 1993). For anhydrous lactose, the impact of light scattering on MIR measurements would be larger with the milk fat globule size distribution d(0.9) larger than 3.20 μm. In general, repeatability (i.e., SD) was smaller for externally homogenized milk than for unhomogenized milks (Table 3.2).

Homogenization Efficiency: Traditional Filter versus Partial Least Squares Models. There is no specific information in the literature on the impact of variation in homogenization efficiency on the predictions of the major milk components by traditional filter versus PLS models. The slopes of the regression lines for MIR traditional filter model (Kaylegian et al., 2009) predicted fat B and fat PLS plotted as a function of reference particle size distribution (Figure 3.3A) were different ($P < 0.05$). On the contrary, no difference was detected ($P > 0.05$) in the slope of the regression lines for MIR traditional filter model predicted fat A and fat PLS plotted as a function of reference particle size distribution (Figure 3.3A). According to Smith et al. (1994),

fat B may be more affected by the poor homogenization performance than fat A because of its shorter wavelength.

The slopes of the regression lines for traditional filter model (Kaylegian et al., 2009) MIR predicted true protein and true protein PLS (Figure 3.3B) and for traditional filter model MIR predicted anhydrous lactose and anhydrous lactose PLS (Figure 3.3C) as a function of reference particle size distribution were different ($P < 0.05$). In the case of true protein, the specific PLS model used in the present study was much more sensitive to variation in homogenizer performance than the traditional filter model and the impact on accuracy of the true protein test at a d(0.9) of 3.03 μm was relatively large. The anhydrous lactose traditional filter model results were impacted more than the anhydrous lactose predicted with a PLS model, but the impact of homogenization efficiency on lactose was much smaller than on fat and true protein.

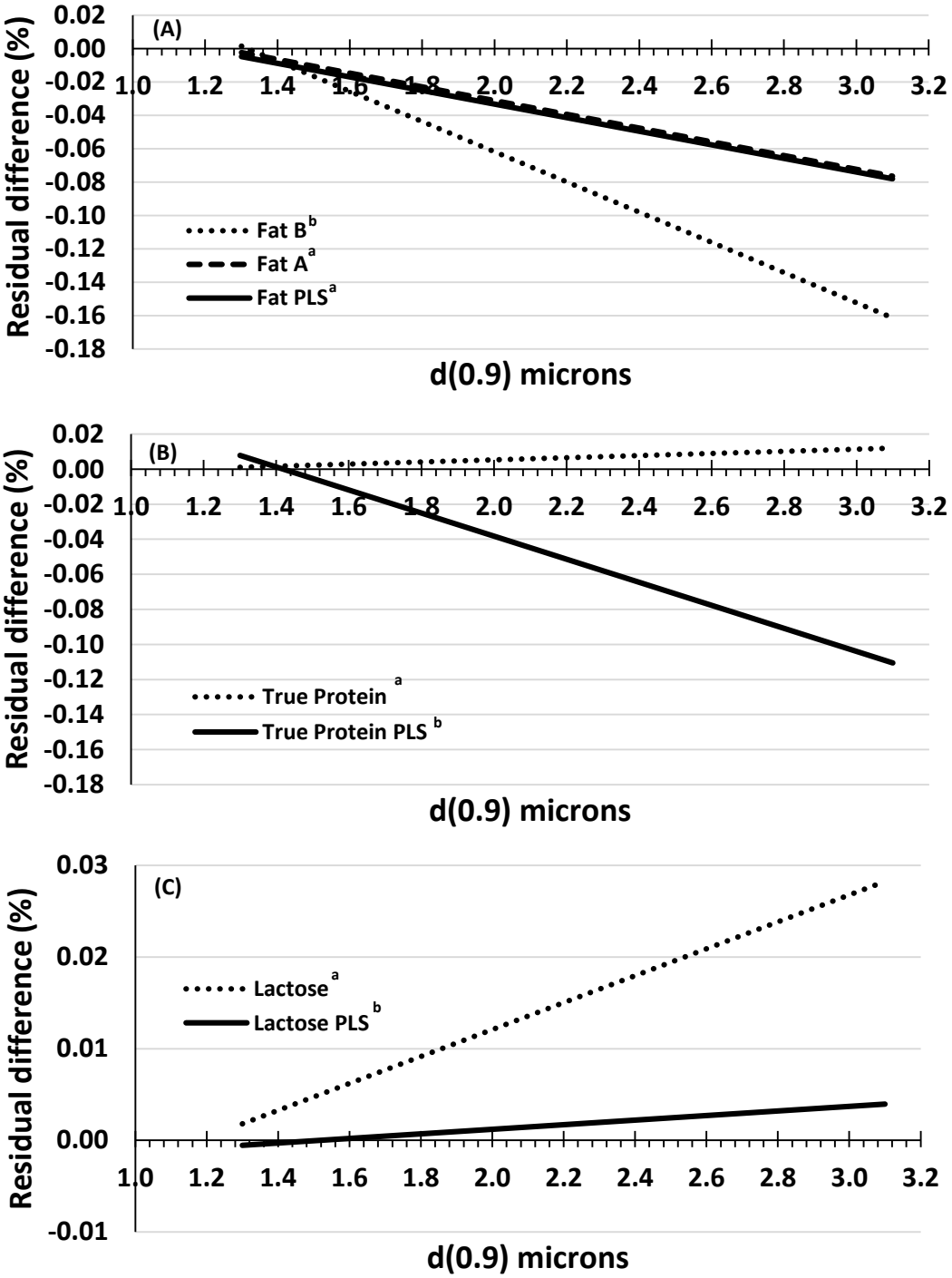

Figure 3.3. MIR predicted residual difference for (A) fat B (traditional filter), fat A (traditional filter), and fat PLS, (B) true protein (traditional filter) and true protein PLS, and (C) anhydrous lactose (traditional filter), and anhydrous lactose PLS, plotted as a function of fat globule size distribution d(0.9). Residual difference (%) = (mean MIR predicted component using test with homogenizer 1, 2, 3, 4, and 5) – (mean MIR predicted component using homogenizer 1 (i.e., the best homogenizer). ^{ab} – slopes of lines with different superscripts differ ($P < 0.05$).

DISCUSSION

Methods for Homogenizer Evaluation. Precalibration tests are currently used to detect if the MIR homogenizer is working properly (Barbano and Clark, 1989; Lynch et al., 2006). In the "recycle test", if the difference between the average MIR readings for fat test on the unhomogenized milk and instrument homogenized milk is > 0.05% for a milk containing a mass fraction of 3.5% milk fat, the homogenizer fail homogenization efficiency (IDF, 2000). Although this method is very rapid and practical, a MIR with a very poor homogenizer will pass the evaluation, as the readings before and after homogenization will be the same (Barbano and Clark, 1989, Smith et al., 1993; Lynch et al., 2006). Another method to track homogenizer efficiency is by checking the MIR absorbance at wavenumber 3750 cm^{-1}. As particle size increases when the same unhomogenized milk is being homogenized by a deteriorated homogenizer, light scattering increases and absorbance at 3750 cm^{-1} increases. The increase of light scattering will lead to a pronounced rise in base-line at shorter wavelengths, where the fat globule sizes are of the same order as the wavelength (Goulden, 1964). The limitation of this measure is that sample to sample variation in concentration of fat, protein, and lactose also impact the absorbance at 3750 cm^{-1}, so unless you test the same sample over a period of months and compare readings, the results are difficult to interpret. Absorbance at 3750 cm^{-1} is useful to compare the relative homogenization efficiency among different homogenizers on the same milk sample. The test to evaluate homogenization efficiency that is more accurate, but is less practical to perform daily, is the laser light scattering particle size analysis to determine milk fat globule size distribution d(0.9) after unhomogenized milk is homogenized

within the MIR (Lynch et al., 2006). If d(0.9) of the milk homogenized through the MIR is larger than 1.7 μm, then the homogenizer is deteriorating and needs to be replaced (Smith et al., 1995). This approach is used on a monthly basis to evaluate homogenizer performance by the USDA Federal Milk Market laboratories where instrument homogenized milks are sent to central testing laboratory for a laser light scattering test to determine if the d(0.9) is < 1.7 μm.

Recently, a MIR partial least squares d(0.9) prediction model was developed (Di Marzo, 2016) as an alternative method for use in routine quality assurance to determine if the homogenizer within a MIR milk analyzer was near the failure level (i.e., d(0.9) > 1.7 μm). The advantage of this method is that the homogenizer performance can be monitored daily using the mean d(0.9) given by each MIR instrument each day. Additionally, the method can be used for milk with a wide range of fat content (Di Marzo, 2016).

Causes of Homogenizer Failure. The deterioration of a homogenizer's mechanical components over time needs to be detected by the MIR operator to minimize a negative impact on analytical accuracy and repeatability. What causes MIR milk analyzers to have poor homogenization efficiency? There are three common problems for a 2-stage homogenizer designed with springs in the milk flow. First, if the coil spring has a rough spot on the end that is in direct contact with the ball it can chip the ball (Figure 3.4) preventing it to form perfect closure against the seat (Trout, 1950; Walstra et al., 2005).

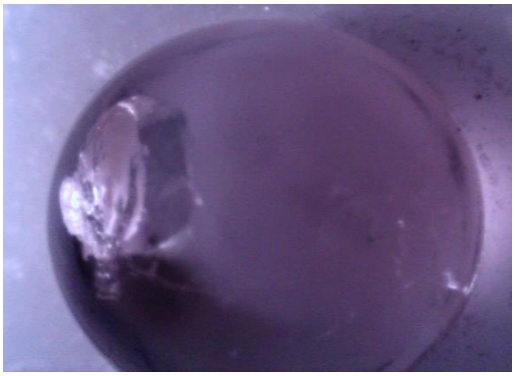

Figure 3.4. Common failure of a 2-stage homogenizer design with the springs in the milk flow: chip of the sapphire ball caused by a spring with rough end.

On this type of homogenizer, the ball rotates, so the same area is not always contacting the seat, and the homogenizer performance may oscillate between getting better and worse depending on whether the place on the ball with the chip is contact with the seat. Second, because the spring is in direct contact with milk, pieces of foreign material present in milk and ball chips can get stuck in the spring impacting homogenization performance and purging efficiency (Walstra et al., 2005). Third, if the spring is bent it can cause an unequal opening between the ball and the seat. When these things happen it is common to hear a high pitched squeaking noise produced by the homogenizer. If the bent spring is allowing the opening between the seat and ball to happen only on one side, accumulation of material between ball and the seat will diminish homogenization performance.

For a 2-stage homogenizer designed with springs outside the milk flow, two problems are common. First, it is possible to see a wear ring (Figure 3.5A) form on the ball over time where the ball makes contact with the seat. This happens as consequence of a pulsing single piston pump forcing milk against the ball, resulting in repeated

opening the narrow gap between the ball and the seat and bringing the ball to its original position against the seat when pressure is not applied (Wenrich, 1946; Trout, 1950; Walstra et al., 2005). In this type of homogenizer it appears that the balls do not rotate, so the same area on the ball is always opening and closing against the seat. A localized erosion of the ball lets milk to flow through this point with a lower pressure decrease across the seat and will cause poor homogenizer performance. Second, damage to the seat occurs on the edge that contacts the ball, leading to the accumulation of material between the ball and seat (Figure 3.5B).

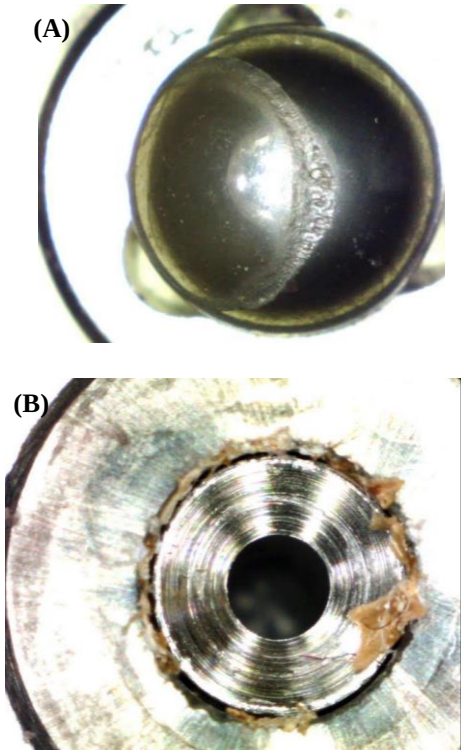

Figure 3.5. The failures of mechanical components of a 2-stage homogenizer with springs outside the milk flow: (A) wear ring formed on the ball at the point where the ball contacts the seat, and (B) showing the accumulation of material on the seat that will not allow the ball and seat to form a tight seal when the valve closes.

Fat Globule Particle Size Based Decision to Replace a Homogenizer. Is the fat globule size distribution d(0.9) value currently used as criteria to replace homogenizer reasonable? The USDA Federal Milk Market laboratories uses a d(0.9) value of 1.7 μm as a criteria to make the decision to replace the homogenizer in a MIR milk analyzer (Smith et al., 1995; Lynch et al., 2006). A d(0.9) of 1.8 μm impacted the accuracy of MIR fat readings ($P < 0.05$), lowering the results for traditional filter based fat B (-0.046%), traditional filter based fat A (-0.026%), and fat PLS (-0.028%) and SD of repeatability gets larger (Table 3.2). New commercial homogenizers for MIR milk analyzers are capable of producing a d(0.9) in the range of 1.1 to 1.5 μm and the time of use of homogenizer before it reaches a d(0.9) of 1.7 μm is usually 6 months or longer based on experience in the USDA Federal Milk Markets. Thus, a d(0.9) of 1.7 μm has been used in practice and appears to be a reasonable limit, given the magnitude of impact on the accuracy of fat test results.

Impact of Homogenization Efficiency: Traditional Filter versus Partial Least Squares Models. The impact of variation in homogenizer performance on the results of traditional filter model predicted values for main components are well established (Smith et al., 1995) and the detail of standard optimum sample and reference wavelengths, setting primary slope, approximate intercorrection factors are well understood and documented in the literature. The situation with respect to the impact of variation in homogenizer performance on the predicted values for PLS models is not so clear, as shown in Figure 3.3. Users need to recognize that the structure of each PLS model for prediction of the same milk component is unique and dependent on a number of factors (data preprocessing before modeling, base line correction, mean centering,

scaling, ranges of wavelengths used, outlier removal, optical bench resolution, etc.) and most importantly the exact population of samples (i.e., MIR spectra) used for the PLS modeling. All PLS models are not the same. Thus, 2 different PLS models developed for the same model of MIR milk analyzer may have different analytical performance characteristic and also different sensitivities to external factors such as variation in homogenization or variation in type or concentration of preservative system (i.e., both the active and inert ingredients). Generally, a laboratory that is using a PLS model has no tools to diagnose these performance characteristics of PLS models and in general the instrument manufacturers do not provide this type of information. At best, a user (or researcher using results from PLS model) should know and report the equipment manufacturer PLS model identification and version number used for production of results used in the study. Imagine doing a research study to determine the impact of management practice or feeding technique on milk fat production and have the results of the same study come out significantly different depending on which PLS model was used to analyze the same spectra. Documentation and reporting of the specific manufacturer's identification of PLS model and version used is warranted.

In future, as new PLS models are developed to measure other components in milk (e.g., fatty acids, citrate, etc.), the sensitivity of their prediction accuracy to factors such as variation of homogenizer performance should be determined as part of the ruggedness testing during the PLS model development process. PLS prediction models for other milk components not addressed in this paper may be more or less sensitive to light scattering of fat globules depending on the specific wavelengths used in each PLS model and magnitude of the beta coefficients used at those wavelengths.

CONCLUSIONS

Repeatability and accuracy of Fourier Transform MIR predicted fat, true protein, and anhydrous lactose given by traditional filter and PLS prediction models were determined. Component predictions on externally homogenized milks were impacted by variation in homogenizer performance, but the magnitude of impacts were small (i.e., < 0.025%) when milks were pumped through both efficient and inefficient homogenizers within a MIR analyzer. Variation in the in-line MIR homogenizer performance on unhomogenized milks had a much larger impact on accuracy of component testing than on repeatability. The increase of particle size distribution d(0.9) from 1.35 to 3.03 μm due to poor homogenization impacted fat tests the most: traditional filter based fat B (- 0.165%), traditional filter based fat A (- 0.074%), and fat PLS (- 0.078%), at a d(0.9) of 3.03 μm. Variation in homogenization efficiency also impacted traditional filter based true protein test (+ 0.012%), true protein PLS prediction (- 0.107%), and traditional filter based anhydrous lactose test (+ 0.027%), at a d(0.9) of 3.03 μm. Effects of variation in homogenization on anhydrous lactose PLS predictions were small. The accuracy of both traditional filter models and PLS models was influenced by poor homogenization. The value of 1.7 μm for a d(0.9) used by the USDA Federal Milk Market laboratories as a criteria to make the decision to replace the homogenizer in a MIR milk analyzer appears to be a reasonable limit, given the magnitude of impact on the accuracy of fat test. In the future as new PLS models are developed to measure other components in milk, the sensitivity of the accuracy of the predictions of these models to factors such as variation of homogenizer performance should be determined as part of the ruggedness testing during PLS model development.

ACKNOWLEDGMENTS

The authors thank the Northeast Dairy Foods Research Center (Ithaca, NY). The technical assistance of Chassidy Coon, Michelle Billota, and Sara Bova of Cornell University and the service staff of Delta Instruments was greatly appreciated.

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CHAPTER FOUR

CONCLUSIONS AND FUTURE WORK

Partial least squares regression models for predicting the particle size distributions d(0.5) and d(0.9), surface volume mean diameter D[3,2], and volume moment mean diameter D[4,3] of milk fat globules from a MIR milk spectra were developed and validated. The basis for the ability to model particle size distribution of milk fat emulsions was hypothesized to be the result of the PLS modeling detecting absorbance shifts in MIR spectra of milk fat due to the Christiansen effect. The independent sample validation of particle size prediction methods found that there was more variation in d(0.9) and D[4,3] predictions than the d(0.5) and D[3,2] predictions relative to laser light scattering reference values and this may be due to variation in particle size among different pump strokes. The accuracy of the d(0.9) prediction for routine quality assurance to determine if a homogenizer within a MIR milk analyzer was near the failure level (i.e., d(0.9) > 1.7 μm) and needed to be replaced is fit-for-purpose. The daily average particle size performance (i.e., d(0.9)) of a homogenizer based on the mean for the day could be used for monitoring homogenizer performance.

Repeatability and accuracy of Fourier Transform MIR predicted fat, true protein, and anhydrous lactose given by traditional filter and PLS prediction models were determined. Component predictions on externally homogenized milks were impacted by variation in homogenizer performance, but the magnitude of impacts were small (i.e., < 0.025%) when milks were pumped through both efficient and inefficient homogenizers within a MIR analyzer. Variation in the in-line MIR homogenizer

performance on unhomogenized milks had a much larger impact on accuracy of component testing than on repeatability. The increase of particle size distribution d(0.9) from 1.35 to 3.03 μm due to poor homogenization impacted fat tests the most: traditional filter based fat B (- 0.165%), traditional filter based fat A (- 0.074%), and fat PLS (- 0.078%), at a d(0.9) of 3.03 μm. Variation in homogenization efficiency also impacted traditional filter based true protein test (+ 0.012%), true protein PLS prediction (- 0.107%), and traditional filter based anhydrous lactose test (+ 0.027%), at a d(0.9) of 3.03 μm. Effects of variation in homogenization on anhydrous lactose PLS predictions were small. The accuracy of both traditional filter models and PLS models was influenced by poor homogenization. The value of 1.7 μm for a d(0.9) used by the USDA Federal Milk Market laboratories as a criteria to make the decision to replace the homogenizer in a MIR milk analyzer appears to be a reasonable limit, given the magnitude of impact on the accuracy of fat, protein and lactose tests.

In the future, the d(0.9) model could be used as an alternative method in routine quality assurance to determine if the homogenizer within a MIR milk analyzer is near the failure level when testing milk. The advantage of this method is that the homogenizer performance can be monitored daily using the mean d(0.9) given by each MIR instrument each day. Additionally, determination of the impact of variation in milk fat globule size distribution d(0.9) on the accuracy of newly developed MIR PLS models should be part of the ruggedness testing of newly developed PLS models. Monitoring and better control of homogenizers within MIR milk analyzers will improve the accuracy of milk payment testing.