Use of Fourier transform infrared (FTIR) spectroscopy to determine the health-promoting index (HPI) of cow's milk

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Use of Fourier transform infrared (FTIR) spectroscopy to determine the health-promoting index (HPI) of cow’s milk

by

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ABSTRACT

Fourier transform infrared (FTIR) spectroscopy steadily has been gaining popularity for the analysis of milk components including total fat, protein, lactose, and ketones. To date, there have been few descriptions of the use of FTIR spectroscopy for the determination of milk fatty acid composition.

The first objective of this thesis was to develop a method utilizing FTIR spectroscopy for prediction of the health-promoting index (HPI) of milk. The HPI is known to affect the relative softness of butter (Bobe et al., 2003); so, this model could be used to predict which cows’ milk could be designated for generation of niche products. To accomplish this objective, milk was collected from 281 cows for analysis. One set of samples was esterified into butyl esters for analysis via gas chromatography (GC), and the lipid was extracted from another set to analyze via FTIR spectroscopy. The GC-derived values and FTIR spectra were used to make predictive models by using the partial least squares (PLS) procedure of SAS. The models subsequently were used to generate predictions of milk composition by using FTIR spectra from three additional sets of 135 cows. The predictive model generated for HPI did not generate values for data not included in the predictive model satisfactorily (mean = 0.266, root mean-squared error of prediction (RMSEP) = 0.122); so, predictive models were created for additional milk fatty acid combinations. The predictive models generated estimates for several milk composition parameters relatively well. For example, the model generated to determine the ratio of unsaturated to saturated fatty acids is able to predict with a mean = 0.347 and RMSEP = 0.096. With further development, the use of FTIR
spectroscopy to determine milk fatty acid composition could become an essential part of milk analysis protocols.

The second objective of this study was to develop a method utilizing FTIR spectroscopy to determine fatty acid composition of milk that could be commercially marketed. At this time, this objective has not been achieved because the O-H stretching vibrations of water absorb nearly all of the photons present in the wavelengths at which the C-H and alkene bonds of fatty acids absorb energy. To counteract the interference of the O-H stretching vibrations at these wavelengths, it was necessary to remove water from raw milk samples before analysis. Several potential methods to eliminate water were investigated and a modification of the Folch method (Folch and Stanley, 1957) was chosen. The Folch method produces accurate and reproducible results (Avalli and Contarini, 2005, Zhang et al., 2008), but takes several hours per sample to complete as well as requiring the use of chloroform, which has multiple potential negative human health effects. Further investigation is needed to develop a method that will eliminate water from samples quickly and in a manner that does not pose risk to human health.

**Key words**: dairy cow, fatty acid composition, Fourier transform infrared spectroscopy, partial least squares analysis, health-promoting index
1.1 Thesis Organization

This thesis is presented as one complete paper with abstract, introduction, materials and methods, results, discussion, conclusion, acknowledgement, and references for submission to the *Journal of Dairy Science*. The title of the paper is: “Use of Fourier transform infrared (FTIR) spectroscopy to determine milk fatty acid composition.” The thesis was prepared from research conducted to partly fulfill the requirements for a Master of Science degree. The data indicate that FTIR spectroscopy is able to predict several parameters of the fatty acid (FA) composition of milk relatively well but predicts other parameters of milk FA composition poorly. The paper is preceded by a literature review with references and is followed by a general discussion and recommendations for future research. Appendices are included to present preliminary research and data not presented elsewhere in this work.

1.2 Literature Review

The FA content of milk affects several aspects of milk products, including flavor, appearance, spreadability of butter, and potential effect on human health. Consumption of dairy products with a high proportion of saturated FA is believed to have negative effects on cardiovascular disease (CVD) risk factors such as serum total and LDL-cholesterol (Grundy and Vega, 1988). In 1991, an atherogenic index (AI) was proposed by Ulbricht and Southgate to relate lipids to dietary risk for cardiovascular disease. The AI is defined as the sum of the proportion of lauric and palmitic acids plus four times the proportion of myristic...
acid in the milk fat divided by the sum of the proportion of total unsaturated FA (Ulbricht and Southgate, 1991). Chen et al. (2004) proposed that the inverse of the AI be renamed “health-promoting index” (HPI) to more correctly represent the potential positive effects of a high unsaturated: saturated FA ratio on health. I choose to use the HPI terminology throughout the thesis.

Aside from its effect on CVD risk factors, a low HPI also is associated with hardness and poor spreadability of butter at refrigeration temperature (Edmondson et al., 1974; Ashes et al., 1997). Bobe et al. (2003) showed that milk from cows with a higher unsaturated milk FA concentration produced butter samples with a higher HPI and were more spreadable, softer, and less adhesive. Prior to this study, it was believed that cow dietary nutrition was the primary factor influencing milk FA composition (Palmquist et al., 1993). However, Bobe et al. (2003) were able to show a phenotypic variation in the milk FA profile of milk produced by cows fed the same diet. Cows with high milk HPI values had a 15% lower proportion of saturated FA and a 44% higher proportion of monounsaturated fatty acids (MUFA) present in their milk fat than cows with low milk HPI values. Cows with greater HPI scores also were believed to have lower proportion of total FA derived from de novo fatty acid synthesis because there was a higher proportion of C6:0 to C16:0 and a higher proportion of conjugated linoleic acid (CLA). Furthermore, this study and studies of others (Middaugh et al., 1988; Stegeman et al., 1992; Baer et al., 2001; Ramaswamy et al., 2001) showed that differences in FA composition does not affect the flavor of the butter produced from milk with greater HPI scores.

To date, scientists lack a convenient, quick, and reliable method for determination of the HPI score of milk. The creation of such an instrument would aid dairy farmers in
determining the FA composition of the milk produced by their cows. They then could separate their herds into subgroups based upon relative HPI scores. Milk from the high-HPI group then could be collected separately from the milk from cows with mid- or low-HPI scores and designated for processing into more spreadable, less adhesive, “healthy” butter or other “healthy” dairy products.

Gas chromatography (GC) methods traditionally have been used to determine the FA composition of milk via column retention times of the individual FA. This method is very reliable but requires expensive equipment and training of personnel to use the equipment and software. Columns have limited lifetimes and require continual maintenance and replacement. Programs used to provide resolution between saturated and unsaturated FA present in samples take around 80 minutes per sample. Gas chromatographic analysis also requires a butylation or methylation step to prepare the lipids for analysis, which results in additional sample preparation time. Furthermore, the high temperatures used for preparation of methyl or butyl esters can result in the loss of volatile fatty acids (VFA). Water and oxygen can effectively render a column useless; so, care must be taken to ensure that samples do not contain any water and that the column is continually flushed with gasses such as helium, hydrogen, and compressed air, all of which must be replaced periodically. All of these reasons demonstrate that the development of an alternative method of analysis is vital for the practical application of the HPI. One proposed alternative to GC for the determination of HPI is Fourier transform infrared (FTIR) spectroscopy.

Infrared (IR) spectroscopy works by expressing vibrational modes of covalent bonds in molecules and assists in the quantification of any component present in a sample that absorbs IR radiation. FTIR spectroscopy is a fast, nondestructive, and easy method for milk
analysis that allows simultaneous measurement of several different components of milk (Etzion et al., 2004). Furthermore, FTIR spectrometers require little maintenance as compared with gas chromatographs. There is only one moving part: the moving mirror. The sample cells and crystals are virtually maintenance free. To decrease background noise, carbon dioxide is flushed through the sample chamber, but the amount of gas needed is minimal and flow-through devices eliminate the need to open and close the sample chamber. Once the machine is set up correctly, flow-through devices also significantly decrease the time needed per sample analysis. Each scan takes about one second, and several scans can be added together to ratio out random noise (signal averaging). FTIR spectroscopy has been used in combination with other spectroscopic methods to determine the concentrations of various components present in milk. With the use of an algorithm or model relating milk fat composition to HPI, an instrument utilizing this technology could be used to assist dairy farmers in sorting herds into subgroups on the basis of relative HPI. Fourier transform infrared spectroscopy is not without its flaws – individual FA cannot be separated without some other sample preparation and the instrument itself is expensive, but many testing laboratories already utilize the technology in devices such as the MilkoScan™ instruments produced by FOSS Analytical (Eden Prairie, MN).

**Milk composition:**

The main composition of Holstein milk includes water, casein, protein, fat globules, lactose, and ash. It has been reported that between 97 and 98% of milk fat is in the form of triacylglycerol (TG). Phospholipids, cholesterol, and cholesteryl esters, diacylglycerols, monoacylglycerols, free FA, and the fat-soluble vitamins A, D, E, and K make up the remaining 2-3%. In whole milk, 57% of the milk fat is composed of saturated FA, 25% is
composed of MUFA, and 9% is composed of polyunsaturated fatty acids (PUFA). Of these, the main saturated fatty acids are palmitic, stearic, and myristic acids. Oleic acid is the main MUFA in milk fat. Trans fatty acids are present in very small amounts in milk and dairy foods because of bihydrogenation of dietary lipids in the rumen of ruminants, and the amount present in a serving of dairy foods is less than the U.S. Food and Drug Administration’s (FDA) labeling threshold of 0.5 g per serving. The major trans fatty acid in milk fat is vaccenic acid, some of which is converted to conjugated linoleic acid (CLA), which has been associated with numerous health benefits (Miller et al., 2007).

**Health implications of “healthier” milk products:**

Diets high in saturated fatty have been shown cause an increase in risk factors for cardiovascular disease (CVD) such as serum total and low density lipoprotein (LDL)-cholesterol (Grundy and Vega, 1988). Replacement of dietary fat rich in saturated fatty acids by carbohydrates has been established as one method of decreasing circulating LDL-cholesterol, but it can also result in reduction of high density lipoprotein (HDL)-cholesterol and/or increase of serum triacylglycerol (Katan, 1997, Katan et al., 1997). Dairy fats are known to be naturally high in saturated fatty acids such as myristic and palmitic acids, but altering the quality of fat consumed by replacement of saturated fatty acids with mono- (MUFA) or poly- (PUFA) unsaturated fatty acids can improve serum lipid profiles (Grundy and Vega, 1988). Poppitt et al. (2002) substituted a natural butterfat that was modified by bovine feeding practices to contain a higher proportion of MUFA and PUFA in place of saturated fatty acids into the diets of healthy male volunteers. They also fed a non-modified butterfat as a control. Diets were controlled for total fat, cholesterol, carbohydrates, fiber, and micronutrients such as salt, potassium, and calcium. Half of the total fat in the diet was
provided by butterfat (20% of the total energy) and was scaled to total energy intake and body weight for each subject. Total cholesterol and LDL-cholesterol were decreased significantly in subjects receiving the modified butterfat compared with control, although there was a significant decrease relative to baseline measurements within both treatments. The modified butterfat also decreased LDL-cholesterol in comparison to baseline measurements, but LDL-cholesterol was not statistically different from baseline in the control group. There was no significant difference in HDL-cholesterol between treatments or for the modified butterfat group in comparison to baseline values, but there was a significant decrease in HDL-cholesterol in the control group. Circulating TG concentrations were decreased for both treatments, but no statistical difference existed between treatments. By using criteria set by the MR FIT study (Law et al., 1994), CVD and stroke risk would decrease by up to 27 and 24%, respectively, by using the modified butterfat.

**Methods traditionally used for milk fatty acid composition analysis**

Several methods have been used to analyze milk fatty acid composition. These methods include the use of fatty acid methyl esters (FAME) and GC (Ulberth and Henninger, 1994; Kadegowda, 2008), fatty acid butyl esters and GC (Bobe et al., 2003; Bobe et al., 2007), reverse-phased high-performance liquid chromatography (HPLC; Robinson and MacGibbon, 1998b), and argentation thin-layer chromatography (Ag-TLC; Robinson and MacGibbon, 1998a, Ulberth and Haider, 1992; Kadegowda, 2008).

Descriptions of GC and FTIR spectroscopy are provided in the introduction to this work. Another method that has been used to analyze the fatty acid composition of milk fat is reverse-phased HPLC (Robinson and MacGibbon, 1998b). The methodology behind reverse-phased HPLC is that compounds stick to a reverse-phased HPLC column in a highly aqueous
mobile phase and then are eluted from the column with the highly organic mobile phase. Compounds are separated on the basis of their hydrophobic character. The HPLC requires two pumps and columns packed with silica particles in most cases. It requires two solvents, an aqueous solvent (usually water, but in this case, milk) and an organic solvent. The column must be re-equilibrated to the high aqueous solvent before another analysis can be performed. This equilibration requires about 20 minutes, depending on column length, flow rate, and hydrophobicity of the sample. The temperature of the column must be tightly controlled, and, when a column is left in a highly aqueous solution for up to 10 hours, the analysis will give irreproducible results (Guzzetta, 2007). These qualities all combine to equal much sample preparation time before analysis of the sample even begins. The sample is eluted into several tubes on the basis of column retention time and a chromatogram is generated that reflects the separation of individual fatty acids (Robinson and MacGibbon, 1998b).

Argentation thin-layer chromatography requires the preparation of an Ag-TLC plate by impregnation of the solid phase with silver ions. A mobile phase needs to be determined and varied with the nature of the sample being analyzed because a stronger mobile phase is required if the sample is high in oleic, linoleic, or linolenic acid groups. The method also requires an eluent of 1:1 chloroform/toluene and a development tank. The sample is incubated in the development tank for about 1 hour over a distance of about 18 cm and drying under a ventilated hood for at least one hour. The resultant bands can be analyzed by FAME analysis (Robinson and MacGibbon, 1998a).

**Milk fat analyzers available in the current market**

FOSS Analytical offers several instruments for the analysis of milk components. The MilkoScan™ FT2, MilkoScan™ FT120, MilkoScan™ Minor, MilkoScan™ 4000,
MilkoScan™ 50, and MilkoScan™ FT6000 are all examples of instruments that are currently available for the analysis of several milk components, including milk fat. These instruments quantify total milk fat but do not quantify individual fatty acid composition. FOSS Analytical also offers in-line products including the Process Analytics™ Dairy and ProcesScan™ FT, which are able to analyze milk in real-time for fat, protein, lactose, solids, and other components (FOSS Analytical, 2008).

NETCO produces several instruments including the Milk Analyzer, Electronic Milk Fat Tester, Milko Muneem, and Milk Fat Tester. All of these instruments utilize infrared spectroscopy in their analysis of milk components.

The Acudairy produced by Analytical Technology, Inc. - USA is another example of an infrared laboratory instrument. It can analyze up to 250 samples per hour for fat, protein, and lactose content.

Milkotester, Ltd. produces ultrasonic milk analyzers. The products that this company offers include the Master Mini, Master PS, Master S, Master Plus PS, and the Master Plus S. Milkotester, Ltd. also offers the Master Easy, a portable ultrasonic milk analyzer. All of these models analyze raw milk samples in approximately 60-90 seconds and measure several parameters including “fatness.”

There are several other milk analyzers available on the market, but regardless of the technology utilized by the various analyzers, none of the available technologies assess milk fat fatty acid composition. They are all capable of analyzing milk for total milk fat percentage, but with the escalating concern over the effect of an increased ratio of saturated to unsaturated fatty acids on CVD risk (Ulbricht and Southgate, 1991; Haug et al., 2007),
dairy producers will benefit by the use of technology that predicts fatty acid composition as well as total fat content of milk.

**Fourier transform infrared spectroscopy**

Fourier transform infrared spectroscopy is a non-dispersive method of infrared spectroscopy. The original infrared spectrometers were “dispersive,” meaning that the instruments separated individual frequencies of energy emitted from the infrared source via the use of a prism or grating. After passing through a sample, the amount of energy at each frequency is measured by a detector and the results are represented by a spectrum of intensity versus frequency. This method takes a considerable amount of time, and FTIR spectroscopy was created in part to overcome this disadvantage.

Fourier transform infrared spectroscopy measures all infrared frequencies simultaneously via the use of an interferometer. The sample can be analyzed via either transmittance or reflectance mode. Transmission FTIR spectroscopy was the method employed during this study. Briefly, a silica carbide rod serves as the radiation source. Photons released from the radiation source are directed into the interferometer, where they contact a beamsplitter, which is a semireflecting device. The beamsplitter “splits” the beam of photons in two. One half of the IR beam is transmitted to a fixed mirror and the other half is reflected to a moving mirror. After contacting and being reflected by the two mirrors, the beams are recombined at the beamsplitter. An interference pattern is produced that passes through the sample and is focused on the detector. An interferogram is created that contains information over the entire IR region to which the detector is responsive. The interferogram is converted from an intensity versus time within the mirror scan spectrum to the final IR
spectrum showing intensity versus frequency by a mathematical operation known as Fourier transformation (Hsu, 1997).

Advantages of FTIR spectroscopy over other methods are summarized in part in the “Introduction” section. Further advantages include greater optical throughput because of decreased time per sampling and precise measurement without the need for external calibration. Infrared spectroscopy allows positive identification, or qualitative analysis, of different kinds of material as well as quantitative analysis via the use of modern software algorithms to analyze the area of the peaks in the spectra produced by the spectrometer (Thermo Nicolet Co., 2001).

Goal of the presented research:

My goal was to locate an IR region by using FTIR spectroscopy that represents the difference between saturated and unsaturated fatty acids present in milk samples. Preliminary research with pure fatty acids (see Appendix A3a) and literature searches (Rodriguez-Saona, 2006) indicated that the window between 3000 and 2830 cm\(^{-1}\) would best serve this purpose. Further preliminary research with pure fatty acids (see Appendix A3b) revealed a peak at 3010 cm\(^{-1}\) present only in samples that contained one or more unsaturated bonds. In raw milk samples, the peak at 3010 cm\(^{-1}\) was masked by the presence of water. The decision was made, therefore, to eliminate this interference by preparing the samples by using a modified Folch method (Folch and Stanley, 1957). Carbon disulfide was selected as the solvent to use for reconstitution of fatty acids derived from the milk samples for FTIR analysis because preliminary research indicated that C=S bond stretching did not interfere at the absorbance value of interest (see Appendix A1).

Previous use of FTIR spectroscopy for determination of milk composition:
Sugars:

The alternating least squares method (ALS) was used by Hansen et al. (1999) to resolve FTIR spectral data from a dairy batch process where lactose is enzymatically hydrolyzed to glucose and galactose without the need for reference analyses. The FTIR absorbance spectra in this study were calculated by using a water background, resulting in slightly negative absorbances, which were used in a variation of the Beer-Lambert law, $C = XA^+$, where $C$ = matrix containing the concentrations corresponding to each spectrum, $X$ = landscape containing the spectra in its rows, and $A^+$ = pseudoinverse of $A$, where $A$ is a matrix containing the linear combination of the pure constituent spectra. They found that an ALS with three components gave stable and reasonably pure spectra, but the correlation to the actual lactose concentration in the milk samples was poor. An ALS with four components, however, gave a more linear relationship to the lactose reference results, without using the reference results during optimization. One drawback to using the ALS method is that the ALS concentrations are in arbitrary units; so, only relative process changes could be detected. However, lactose reference results are not needed to create an optimal model for predicting the lactose concentration of milk samples.

Acetone:

Acetone, acetoacetate, and β-hydroxybutyrate (BHBA) concentrations are high in the blood of cows suffering from ketosis, and, furthermore, there is a strong correlation between the concentration of acetone in milk and the ketone bodies in the blood of ketotic cows (Steger et al., 1972). Therefore, there is a strong incentive to develop a fast, easy method for determining the acetone concentration in milk. Hansen (1999) used FTIR spectroscopy in combination with multivariate calibration to quantify acetone in bovine milk. Absorbance
spectra for milk in this study were produced by dividing the spectra by a water spectrum and performing log transformation on the results. However, when FTIR spectroscopy was used to determine acetone concentrations in milk, differentiating between ketotic and possibly ketotic cows was difficult; so, other methods of analysis such as a flow injection analysis (FIA)-based method is required to be able to use this method for purposes other than simple screening.

Heuer et al. (2001) later used FTIR spectroscopy in combination with partial least squares (PLS) regression to predict the concentration of acetone in milk. The spectra produced by this method also were compared with results from a gas chromatographic head space method. When using a threshold of 0.4 to 1 mM for subclinical ketosis, PLS predictions resulted in 95 to 100% sensitivity and 96 to 100% specificity. The authors were able to estimate acetone in milk with a precision of 0.21 mM by FTIR spectroscopy by using a spectral range of 1450 to 1200 cm$^{-1}$ and second derivative spectra.

Another study conducted by de Roos et al. (2007) utilized a MilkoScan™ FT6000 (FOSS Analytical A/S, Hillerød, Denmark) to evaluate FTIR predictions for ketone bodies in milk including acetone, acetoacetate, and BHBA. Concentrations of each ketone body were determined by chemical methods using segmented flow analysis. Calibration models for FTIR analysis for each of the ketone bodies were created by using normal and log-transformed data, no variable selection method, and very simple principal variables. The proportion of acetoacetate present in the milk was small enough that it was not used for the FTIR predictive model. The correlation between chemical method results and predicted concentrations via FTIR spectroscopy for acetate and BHBA was found to be $R^2 = 0.80$. It
was concluded from this study that FTIR predictions for acetate and BHBA were valuable for screening cows for subclinical ketosis and could serve in the evaluation of herd health status.

**Protein:**

Fat and casein are the main milk components contributing to cheese yield (Sorensen et al., 2003). Casein is the limiting factor, and the casein fraction is more important than the crude protein (CP) or true protein fractions for increasing cheese yield. Prior to this study, direct casein measurements had not been possible using the rapid, inexpensive methods available for total protein content determination because they used high throughput IR spectrometers with fixed optical filters. In this study, casein determination was based on the Rowland principle in which casein is defined as the proteins that precipitate at pH 4-6. The authors used the direct method for determining casein concentration from the nitrogen content of the precipitate because it was slightly more precise in preliminary studies. They also used a PLS regression algorithm to correlate spectral data with reference data. In the end, this study resulted in a 30% increase in the accuracy of casein content determination by direct FTIR spectroscopy in comparison to FTIR-determined CP.

Etzion et al. (2004) used FTIR spectroscopy in combination with 3 different methods: simple band integration, PLS, and neural networks, to determine protein concentration in raw cow milk after water subtraction. Simple integration gave very poor results, whereas PLS and neural network methods gave much better results. The PLS method gave prediction errors of approximately 0.22% protein, whereas the neural network method gave 0.20% prediction errors when based on principal component analysis scores alone, and 0.08% prediction errors with the inclusion of lactose and fat concentrations.
In 2002, van der Ven et al. used FTIR spectroscopy in combination with multivariate data analysis techniques to analyze whey and casein hydrolysates. Proteins often are hydrolyzed to decrease allergenicity, to achieve dietary requirements, or to improve functional properties, but this hydroxylation often leads to changes in solubility, viscosity, taste, emulsion and foam forming, and emulsion and foam stability. Although spectroscopic methods previously had been used to study protein secondary structure and for qualitative and quantitative determination of proteins in solution, hydrolysates had not been examined by FTIR spectroscopy prior to the van der Ven et al. (2002) study. Their goal was to utilize the FTIR spectra of casein and whey hydrolysates to determine bitterness, solubility, and emulsion and foam properties. Using FTIR spectroscopy, the authors were able to differentiate between hydrolysates made from different protein sources and enzyme classes, which was not possible with size exclusion chromatography and reversed-phase chromatography. The FTIR spectra were used to create a correlation between predicted and measured bitterness scores of whey and casein hydrolysates made with neutral and alkaline enzymes ($R^2 = 0.79$) but the authors were unable to find a correlation for hydrolysates made with acidic enzymes because they were treated with sodium hydroxide prior to sensory testing to mask the acid taste, which may have influenced the bitterness perception. They were able to predict emulsion-forming ability and emulsion stability for the hydrolysates with $R^2 = 0.89$ and 0.71, respectively. However, predicting foam-forming ability and foam stability was tricky. A good model was obtained for combined casein and whey hydrolysates ($R^2 = 0.75$) and for whey hydrolysates alone, but no correlation could be formed between FTIR spectra and foam-forming ability for casein. Furthermore, there was a poor correlation between FTIR spectra and foam stability for whey hydrolysates, but a good correlation was
found between FTIR spectra and foam stability for casein hydrolysates. Ultimately, FTIR spectroscopy in combination with multivariate data analysis was used successfully to correlate FTIR spectra with various functional properties of whey and casein hydrolysates. Some of these correlations could not be predicted by previous methods, and the speed and ease of use of FTIR spectroscopy might be a good substitute for more labor intensive methods.

**Fat:**

Lefier et al. (1996) were able to examine raw milk without separating the individual components of the milk. Among other goals, this study sought to compare the ability of FTIR spectroscopy and multivariate analysis to measure fat in raw milk and to compare the analytical performance of and calibration procedures for FTIR and conventional filter-based milk analyzers. They utilized several spectral windows to calibrate the FTIR instrument and eliminate interference from water vapor and carbon dioxide. The window between 3000 and 2830 cm\(^{-1}\) (3.33 to 3.53 µm) is dominated by the stretching vibrations of CH\(_3\), CH\(_2\), and CH groups present in fatty acids (Lefier et al., 1996). This window contains what has been traditionally coined as the “fat B” wavelength (3.48 µm) (Kaylegian, 2007). The C=O rotation and stretching vibration of the ester groups of fat is represented by the window between 1800 and 1680 cm\(^{-1}\) (5.55 to 5.95 µm) (Lefier et al, 1996). This window contains what has been traditionally coined as the “fat A” wavelength (5.73 µm) (Kaylegian, 2007). Finally, in raw milk, a “mixed region” containing information about proteins and fatty acids is found between 1500 and 1300 cm\(^{-1}\) (6.66 to 7.69 µm). When single calibrations were performed in the Lefier et al. study, the standard deviation for fat measurement was much higher with the filter-based instrument than with the FTIR instrument (Lefier et al., 1996),
lending support to the idea that the milk fat data obtained from FTIR instruments is highly accurate and reproducible while requiring little external calibration.

Ulberth and Haider (1992) used FTIR spectroscopy to quantify trans fatty acids in butter. Standard infrared spectroscopic procedures were known to be accurate for the assessment of moderate or high concentrations of trans fatty acids but not for the assessment of low concentrations of trans fatty acids. The authors’ goal therefore was to develop a technique to better analyze trans fatty acids when present in samples in low concentration. Fatty acid methyl esters were formed from the melted butter samples and analyzed by FTIR spectroscopy in a variable length sampling cell with NaCl windows. Triacylglycerols also were measured as a solution in carbon disulfide. Background correction was found to lead to well resolved Gaussian-shaped peaks for both sets of samples. This study demonstrated that FTIR spectroscopy with background correction was well-suited for the analysis of samples with low concentrations of trans fatty acids in TG.

**The partial least squares procedure:**

The partial least squares procedure of SAS/STAT was used in this study for generation of predictive models for various milk fatty acid combinations. The partial least squares method is a general statistical method that was created originally by Herman Wold in 1966 for modeling “paths” of causal relation between a number of “blocks” of variables. The PLS procedure in SAS/STAT software is different from this general definition in that it only fits predictive PLS models. The PLS procedure uses one “block” of predictors and one “block” of responses. When the predictors used in this model are highly correlated, the PLS procedure aims to account for the variation in the predictors. The assumption made by the methods used by the PLS procedure is that directions in the predictors space that are well
sampled should provide a better prediction for new observations which is achieved by
extracting successive linear combinations of the predictors that address one or both of two
goals: explaining response variation and explaining predictor variation. The method of PLS
attempts to balance these objectives by searching for factors that explain both response and

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CHAPTER II
USE OF FOURIER INFRARED (FTIR) SPECTROSCOPY TO DETERMINE MILK FATTY ACID COMPOSITION

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ABSTRACT

Fourier transform infrared (FTIR) spectroscopy steadily has been gaining popularity for the analysis of milk components including total fat, protein, lactose, and ketones. To date, there have been few descriptions of the use of FTIR spectroscopy for the determination of milk fatty acid composition. The original goal of the study was to develop a method for prediction of the health-promoting index (HPI) of milk. The HPI is known to affect the relative softness of butter (Bobé et al., 2003); so, this model could be used to predict which cows’ milk could be designated for generation of niche products. To accomplish this objective, milk was collected from 281 cows for analysis. One set of samples was esterified into butyl esters for analysis via gas chromatography (GC), and another was extracted to analyze via FTIR spectroscopy. The GC-derived values and FTIR spectra were used together to make a predictive model for HPI by using the partial least squares (PLS) procedure of SAS. The model subsequently was used to generate predictions of HPI by using FTIR spectra from three additional sets of 135 cows. The predictive model generated for HPI did not generate values for data not included in the predictive model satisfactorily (mean = 0.266, root mean-squared error of prediction (RMSEP) = 0.122); so, predictive models were created for additional milk fatty acid combinations. The predictive models generated estimates for several milk composition parameters relatively well. For example, the model generated to determine the ratio of unsaturated to saturated fatty acids is able to predict with a mean = 0.347 and RMSEP = 0.096. With further development, the use of FTIR spectroscopy to determine milk fatty acid composition could become an essential part of milk analysis protocols.
(Key words: dairy cow, fatty acid composition, Fourier transform infrared spectroscopy, partial least squares analysis, health promoting index)

Abbreviation key: GC = gas chromatography, FTIR = Fourier transform infrared, PLS = partial least squares, HPI = health-promoting index, RMSEP = root mean-squared error of prediction

INTRODUCTION

The fatty acid (FA) content of butter affects its taste, spreadability, and potential effect on health (Bobe et al., 2003). Consumption of dairy products with a high proportion of saturated FA is believed to have negative effects on cardiovascular disease (CVD) risk factors such as serum total and LDL-cholesterol (Grundy and Vega, 1988). In 1991, an atherogenic index (AI) was proposed by Ulbricht and Southgate to relate lipids to dietary risk for cardiovascular disease. The AI is defined as the sum of the proportion of lauric and palmitic acids plus four times myristic acid in the milk fat divided by the proportion of total unsaturated FA (Ulbricht and Southgate, 1991). To promote a more positive image of dairy products, Chen et al. (2004) proposed using health-promoting index (HPI), which is the inverse of AI. Bobe et al. (2003) showed that milk from cows with a higher unsaturated milk FA concentration produced butter samples with a lower AI and were more spreadable, softer, and less adhesive. Cows with high milk HPI values had a 15% lower proportion of saturated FA and a 44% higher proportion of MUFA present in their milk fat than cows with low milk HPI values. Furthermore, this study and studies of others (Middaugh et al., 1988; Stegeman et al., 1992; Baer et al., 2001; Ramaswamy et al., 2001) showed that differences in FA composition does not affect the flavor of the butter produced from milk with lower AI scores.
To date, scientists lack a convenient, quick, and reliable method for determination of the FA composition of milk. The development of such a method would aid dairy farmers in determining the FA composition of the milk produced by their cows. They then could separate their herds into subgroups based upon relative HPI scores. Milk from the high-HPI group then could be collected separately from the milk from cows with mid- or low-HPI scores and designated for processing into more spreadable, less adhesive, “healthy” butter or other “healthy” dairy products.

Infrared (IR) spectroscopy works by expressing vibrational modes of covalent bonds in molecules and assists in the quantification of any component present in a sample that absorbs IR radiation. Fourier transform infrared (FTIR) spectroscopy is a fast, nondestructive, and easy method for milk analysis that allows simultaneous measurement of several different components of milk (Etzion et al., 2004). FTIR spectroscopy has been used in combination with other spectroscopic methods to determine the concentrations of various components present in milk; and, with the use of an algorithm or method of relating milk fat composition to HPI, this technology could be used to assist dairy farmers in sorting herds into subgroups based on relative HPI. Moreover, dairy testing laboratories could incorporate this method into their current technology of assaying milk composition of individual cows for dairy producers.

MATERIALS AND METHODS

Experimental Design and Sample Collection

Milk samples (~50 mL each) were collected from 281 Holstein cows from the Iowa State Dairy Farm in Ankeny, IA on July 24, 2006. Milk samples (~50 mL each) were collected on three more occasions (May 25, 2007, June 8, 2007, and June 25, 2007) from 135
cows from the original collection that were lactating during the three subsequent sample collections. The maximal recommended length of storage for refrigerated (4°C) milk is 72 hours. At -20°C, the maximal recommended length of storage is 12 months, and at -70°C milk theoretically can be stored indefinitely (Jensen, 1995). At -20°C, biolytic activity slowly continues, but at -70°C, biolytic activity is virtually stopped; so, long-term storage at -70°C is preferred. However, upon freezing, some proteins are denatured and there are changes in the nature of the mineral complex of milk. More importantly, the lipid globule emulsion is destabilized (Jensen, 1995). Therefore, it was desirable to extract lipids from the milk samples in this study in as short of time after collection as possible. Lipids were stored at 4°C and extracted in triplicate from individual cows’ milk within four days of collection by using a modification of the Folch method (Folch and Stanley, 1957). When frothing became a problem, the shaking step was decreased to 30 min. The samples were dried under heat (50°C) and nitrogen gas.

After drying, one triplicate of extracted lipid from each sample was weighed and resuspended to 10% total lipid in carbon disulfide. Samples were analyzed by using a 0.05 mm pathlength CaF$_2$ cell (International Crystal Laboratories, NJ) and a Bruker IFS66v FT-IR/FT-Raman spectrometer (Bruker Optics Inc., Billerica, MA). The resultant raw data were analyzed by using Opus Version 4.2 Spectroscopic Software (Bruker Optics Inc., Billerica, MA).

Another triplicate of extracted lipid from each sample was weighed and resuspended in chloroform and esterified into butyl esters. Briefly, 10 mg lipid was dried in tubes under nitrogen and heat. Redistilled 1-Butanol (650 µL) was added to the lipid, and 100 µL of acetyl chloride was added while vortexing. The tubes were purged with nitrogen, capped, and
heated at 60°C for 30 min. Three milliliters of 4% (w/v) K\textsubscript{2}CO\textsubscript{3} and 1 mL of hexanes were added, and the tubes were purged with nitrogen and capped. The samples then were vortexed for 20 seconds and spun in a swinging-bucket centrifuge (IEC Model UV or IEC Model K; International Equipment Co., Boston, MA) for 10 min at 3000 x g. The aqueous (bottom) layer was aspirated off, 3 mL of millipure water was added, and the samples were vortexed for 30 seconds. They then were spun again for 10 min at 3000 x g, and the organic layer was aspirated and put into GC vials. The vials were purged with nitrogen and capped. The butyl esters were analyzed by gas chromatography by using a Varian 3350 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA) equipped with a split/splitless injector, a flame ionization detector, and a SP\textsuperscript{TM}-2560 fused silica capillary column (100 m x 0.25 mm x 0.2 µm thickness; Supelco, Bellefonte, PA). The injection split was set at 50:1, and head pressure was set at 46 psi. Helium was used as the gas vector. Injector and detector temperatures were set at 240°C. For determination of butyl esters, oven temperature was held at 80°C for 5 min immediately after injection of the sample, increased at 3°C/min until it reached 165°C, held for 10 min at this temperature, and increased at 5°C/min to 240°C, which was held for 16 min. The total time per sample was 74 min. Identification of FA butyl ester peaks was based upon retention times obtained for butyl esters prepared from FA standards. The HPI was calculated from the weight percentages. Assignment of peaks was verified for 10 samples by using an Agilent 6890 gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA) with a 7683 injector and the same column and method listed above and a Micromass GC-TOF Mass Spectrometer (Waters Associates, Milford, MA). The libraries used for assignment of peaks were the 7\textsuperscript{th} edition of the Wiley Registry of Mass

**Statistical Analyses**

Data for milk composition were analyzed by using the partial least squares (PLS) procedure of SAS Version 9.1.3 Service Pack 3 (2003). The GC-derived data collected from the initial 281 cows were used as the block of predictors, and the FTIR spectra were used as the block of responses. FTIR spectra from the additional three sets of 135 cows were used by the procedure to predict values that were compared with GC-derived data. The root mean-squared error of prediction (RMSEP) then was calculated for each predicted model to determine how well the predicted values matched the GC-derived values.

**RESULTS AND DISCUSSION**

The original goal of the study was to determine whether FTIR spectroscopy could accurately and rapidly predict HPI. Fourier transform infrared spectroscopy previously has been used to predict sugar content (Hansen et al., 1999), acetone content (Hansen, 1999, Steger et al., 1972, Heuer et al., 2001, de Roos et al., 2007), protein content (Sorensen et al., 2003, Etzion et al., 2004, van der Ven et al., 2002), and total fat percentage (Lefier et al., 1996, Kaylegian, 2007) of milk. To date, there have been few published works that describe the use of FTIR spectroscopy to predict the fatty acid composition of milk (Ulberth and Haider, 1992).

Previous literature (Lefier et al., 1996, Kaylegian, 2007) identified the presence of several regions in the infrared spectrum that correspond to stretching vibrations and rotations of various bonds found within fatty acids. The window between 3000 and 2830 cm$^{-1}$ (3.33 to 3.53 µm) is dominated by the stretching vibrations of -CH$_3$, -CH$_2$, and -CH groups (Lefier et
This window contains what has been traditionally coined as the “fat B” wavelength (3.48 µm) (Kaylegian, 2007). The C=O rotation and stretching vibration of the ester groups of fat are represented by the window between 1800 and 1680 cm$^{-1}$ (5.55 to 5.95 µm) (Lefier et al, 1996). This window contains what has been traditionally coined as the “fat A” wavelength (5.73 µm) (Kaylegian, 2007). Finally, in raw milk, a “mixed region” containing information about proteins and fatty acids is found between 1500 and 1300 cm$^{-1}$ (6.66 to 7.69 µm).

Ulberth and Haider (1992) used FTIR spectroscopy to quantify trans fatty acids in butter. This is one of the few examples of studies in which FTIR spectroscopy was used to predict fatty acid parameters of milk. Fatty acid methyl esters were formed from melted butter samples and analyzed by FTIR spectroscopy. Triacylglycerols also were measured as a solution in carbon disulfide. The study of Ulberth and Haider (1992) demonstrated that FTIR spectroscopy was well-suited for the analysis of trans fatty acids in TG.

In this experiment, milk lipid extraction was conducted using a modified Folch method (Knock, 2007). Instead of 2 g of finely chopped tissue, 4 g of milk were weighed out for extraction. The lipids were then analyzed using FTIR spectroscopy and the PLS procedure of SAS (2003). The PLS procedure was applied to the data from the first set of cows to create a predictive model for HPI. The correlation between GC-predicted HPI and model-predicted HPI was relatively high (mean = 0.302, RMSEP = 0.041; Fig. 1). However, the model did not predict samples that were not included in the original model as well as expected (mean = 0.266, RMSEP = 0.122; Fig. 2). As a result of the poor prediction of HPI, the study was expanded to determine if FTIR spectroscopy and the PLS procedure could be used to predict other combinations of milk FA. Table 1 lists means and RMSEP values for
each predicted parameter. Although unsuccessful when used to predict the HPI of milk, FTIR spectroscopy can be used for the prediction of other fatty acid parameters of milk with some degree of success.

The models created for each parameter predicted values for the data included in the model that correlated well with the GC-determined values, which was expected because those data are the basis for the models. None of the models predicted the FA parameters of interest not used in the models as well as the models predicted the values from which they were created, but several parameters were predicted relatively well. For example, the model predicted the weight percentage of unsaturated FA (Fig. 3), the weight percentage of long-chain unsaturated FA (16 to 18 carbons in length), the weight percentage of monounsaturated fatty acids (MUFA), the ratio of MUFA to total saturated FA, and the ratio of unsaturated to total saturated FA reasonably well (see Table 1 for mean values and RMSEP values). The model created to predict the proportion of saturated FA overestimated the GC-derived values, but the values appear to cluster around a 1:1 line with a non-zero intercept (Fig. 4).

The models created by the PLS procedure did not predict several of the parameters to a satisfactory degree. Those models poorly predicted the weight percentage of medium-chain saturated FA (8 to 14 carbons in length), the weight percentage of long-chain saturated FA (16 to 18 carbons in length), the ratio of C\textsubscript{16:0} to C\textsubscript{14:0}, the ratio of C\textsubscript{18:1} to C\textsubscript{18:0}, the ratio of C\textsubscript{16:1} to C\textsubscript{16:0}, the ratio of C\textsubscript{14:1} to C\textsubscript{14:0}, the weight percentage of polyunsaturated fatty acids (PUFA), and the ratio of PUFA to saturated FA. The ratio of C\textsubscript{16:0} to C\textsubscript{14:0} is representative of the activity of elongase and the ratios of C\textsubscript{18:1} to C\textsubscript{18:0}, C\textsubscript{16:1} to C\textsubscript{16:0} and C\textsubscript{14:1} to C\textsubscript{14:0} represent the activity of stearoyl-coenzyme A desaturase, that is, the enzyme that produces...
medium- and long-chain unsaturated FA (Mele et al., 2007). The activity of these enzymes was not able to be predicted by the models created by PLS.

The use of FTIR spectroscopy for the determination of fatty acid parameters of milk was met with some degree of success. Previously FTIR spectroscopy has been used to determine milk constituents such as sugar content, protein content, acetone content, and total fat percentage as well as quantifying the *trans* fatty acid composition of milk samples. The work presented in this paper demonstrates that FTIR spectroscopy in combination with the PLS procedures of SAS can accurately generate predictions of several milk fatty acid parameters.

**CONCLUSIONS**

Fourier transform infrared spectroscopy currently is used in the dairy industry for determination of several milk constituents including total fat percentage, but to date there have been few published accounts of an attempt to use FTIR spectroscopy to determine fatty acid composition of milk. Presented here is the use of FTIR spectroscopy in combination with the PLS procedure to predict several milk fatty acid components. We showed that the combination of FTIR spectroscopy and the PLS procedure can predict the weight percentage of unsaturated FA, the weight percentage of long-chain unsaturated FA (16 to 18 carbons in length), the weight percentage of MUFA, the ratio of MUFA to total saturated FA, and the ratio of unsaturated to total saturated FA reasonably well.

**ACKNOWLEDGMENTS**

The authors would like to thank the managers and employees of the Iowa State University Dairy Farm (Ankeny, IA), in particular J. Dedrick, for the care of the cows and
assistance in obtaining research data and milk samples and S. Lund and P. Dixon for assistance with statistics.

REFERENCES


Table 1. Descriptive parameters of models created by the partial least squares procedure performed on Fourier transform infrared spectra of lipids derived from raw milk samples.

<table>
<thead>
<tr>
<th>Fatty acid (FA) parameter of interest</th>
<th>RMSEP&lt;sup&gt;4&lt;/sup&gt; of data used in PLS model</th>
<th>Mean value of data used in PLS model&lt;sup&gt;2,5&lt;/sup&gt;</th>
<th>RMSEP of data not included in PLS model</th>
<th>Mean value of data not included in PLS model&lt;sup&gt;3,6&lt;/sup&gt;</th>
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<tr>
<td>HPI</td>
<td>0.041</td>
<td>0.302</td>
<td>0.122</td>
<td>0.266</td>
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<tr>
<td>Unsaturated FA</td>
<td>1.185</td>
<td>27.006</td>
<td>3.832</td>
<td>22.938</td>
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<td>Saturated FA</td>
<td>1.817</td>
<td>72.934</td>
<td>9.432</td>
<td>66.790</td>
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<td>Medium-chain saturated FA</td>
<td>2.908</td>
<td>21.627</td>
<td>6.300</td>
<td>23.750</td>
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<td>Long-chain saturated FA</td>
<td>1.393</td>
<td>46.949</td>
<td>8.631</td>
<td>38.354</td>
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<tr>
<td>Long-chain unsaturated FA</td>
<td>1.841</td>
<td>25.772</td>
<td>4.980</td>
<td>21.484</td>
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<tr>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;/C&lt;sub&gt;14:0&lt;/sub&gt;</td>
<td>0.322</td>
<td>2.674</td>
<td>0.950</td>
<td>2.114</td>
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<td>C&lt;sub&gt;18:1&lt;/sub&gt;/C&lt;sub&gt;18:0&lt;/sub&gt;</td>
<td>0.213</td>
<td>1.939</td>
<td>0.751</td>
<td>1.893</td>
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<tr>
<td>C&lt;sub&gt;16:1&lt;/sub&gt;/C&lt;sub&gt;16:0&lt;/sub&gt;</td>
<td>0.010</td>
<td>0.049</td>
<td>0.016</td>
<td>0.052</td>
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<tr>
<td>14:1/14:0</td>
<td>0.023</td>
<td>0.093</td>
<td>0.050</td>
<td>0.102</td>
</tr>
<tr>
<td>PUFA</td>
<td>0.355</td>
<td>2.479</td>
<td>0.595</td>
<td>2.475</td>
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<tr>
<td>MUFA</td>
<td>1.661</td>
<td>24.539</td>
<td>4.164</td>
<td>20.382</td>
</tr>
<tr>
<td>PUFA / saturated FA</td>
<td>0.005</td>
<td>0.034</td>
<td>0.014</td>
<td>0.037</td>
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<tr>
<td>MUFA / saturated FA</td>
<td>0.035</td>
<td>0.341</td>
<td>0.081</td>
<td>0.309</td>
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<tr>
<td>Unsaturated FA / saturated FA</td>
<td>0.036</td>
<td>0.375</td>
<td>0.096</td>
<td>0.347</td>
</tr>
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</table>

1 Fatty acid parameter of interest: Unsaturated FA = sum of the proportion of (C<sub>14:1</sub> + C<sub>16:1</sub> + C<sub>18:1</sub> + C<sub>18:2</sub> + C<sub>18:3n3</sub> + C<sub>18:3n6</sub>); HPI = proportion of total unsaturated FA / sum of the proportion of lauric and palmitic acids plus four times myristic acid; Saturated FA = sum of the proportion of (C<sub>6:0</sub> + C<sub>8:0</sub> + C<sub>12:0</sub> + C<sub>13:0</sub> + C<sub>14:0</sub> + C<sub>15:0</sub> + C<sub>16:0</sub> + C<sub>17:0</sub> + C<sub>18:0</sub>); Medium-chain saturated FA = sum of the proportion of (C<sub>8:0</sub> + C<sub>10:0</sub> + C<sub>11:0</sub> + C<sub>12:0</sub> + C<sub>13:0</sub> + C<sub>14:0</sub>); Long-chain saturated FA = sum of the proportion of (C<sub>16:0</sub> + C<sub>17:0</sub> + C<sub>18:0</sub>); Long-chain unsaturated FA = sum of the proportion of (C<sub>16:1</sub> + C<sub>18:1</sub> + C<sub>18:2</sub> + C<sub>18:3n3</sub> + C<sub>18:3n6</sub>); PUFA = sum of the proportion of (C<sub>18:2</sub> + C<sub>18:3n3</sub> + C<sub>18:3n6</sub>); MUFA = sum of the proportion of (C<sub>14:1</sub> + C<sub>16:1</sub> + C<sub>18:1</sub>)

2 Mean value of data included in PLS model: mean of gas chromatography (GC)-determined values

3 Mean value of data not included in PLS model: mean of GC-determined values

4 RMSEP: root mean-squared error of prediction

5 For data used in the PLS model, n = 265, with the exception of C<sub>14:0</sub> / C<sub>14:0</sub>, where n = 263 because the proportion of C<sub>14:1</sub> was not large enough to measure in 2 samples

6 For data not included in but predicted by the PLS model, n = 398.
Figure 1: Relationship of spectrscopically determined and chemically determined health-promoting index (HPI = (sum of proportion of total MUFA and PUFA) / (sum of proportion of C\textsubscript{12:0} + C\textsubscript{16:0} + 4 x sum of proportion of C\textsubscript{14:0})). n=265. RMSEP = 0.041.
Figure 2: Relationship of partial least squares (PLS) model-predicted values of unknown sample composition and chemically determined health-promoting index (HPI = (sum of proportion of total MUFA and PUFA) / (sum of proportion of $C_{12:0}$ + sum of proportion of $C_{16:0}$ + 4 x sum of proportion of $C_{14:0}$)). n=265. RMSEP = 0.122.
**Figure 3**: Relationship of partial least squares (PLS) model-predicted values of unknown sample composition and chemically determined proportion of unsaturated fatty acids. n=398. RMSEP = 3.832.
Figure 4: Relationship of partial least squares (PLS) model-predicted values of unknown sample composition and chemically determined proportion of saturated fatty acids. n=398. RMSEP = 9.432.
CHAPTER III
GENERAL CONCLUSIONS

General Discussion

The research presented in this thesis demonstrates the use of FTIR spectroscopy for the prediction of the fatty acid composition of milk. Fourier transform infrared spectroscopy has been used to predict sugar content (Hansen et al., 1999), acetone content (Hansen, 1999, Steger et al., 1972, Heuer et al., 2001, de Roos et al., 2007), protein content (Sorensen et al., 2003, Etzion et al., 2004, van der Ven et al., 2002), and total fat percentage (Lefier et al., 1996, Kaylegian, 2007) of milk. To date, there have been few published works that describe the use of FTIR spectroscopy to demonstrate the fatty acid composition of milk.

There were ultimately two goals of the project presented in this thesis. The first was to determine whether FTIR spectroscopy could be used to determine the fatty acid composition of milk, specifically the HPI. The second was to develop a procedure that could be marketed commercially for the determination of the fatty acid composition of milk. The first goal was met with some success, as shown in Table 1. The second goal of developing a procedure to predict the fatty acid composition of milk that can be marketed commercially was not achieved.

Fourier transform infrared spectroscopy previously has been used to predict sugar content (Hansen et al., 1999), acetone content (Hansen, 1999, Steger et al., 1972, Heuer et al., 2001, de Roos et al., 2007), protein content (Sorensen et al., 2003, Etzion et al., 2004, van der Ven et al., 2002), and total fat percentage (Lefier et al., 1996, Kaylegian, 2007) of milk. However, there have been few descriptions of the use of FTIR spectroscopy for the determination of milk fatty acid composition. One example is a study in which Ulberth and
Haider (1992) used FTIR spectroscopy to quantify trans fatty acids in butter. Fatty acid methyl esters were formed from melted butter samples and analyzed by FTIR spectroscopy. Triacylglycerols also were measured as a solution in carbon disulfide. The study of Ulberth and Haider (1992) demonstrated that FTIR spectroscopy was well-suited for the analysis of trans fatty acids in TG.

One objective of the study was to establish whether or not FTIR spectroscopy could be used to accurately and rapidly determine the fatty acid composition of milk, and in particular, the HPI. To accomplish this objective, milk was collected from 281 cows for analysis. One set of samples was esterified into butyl esters for analysis via GC, and the lipid was extracted from another set to analyze via FTIR spectroscopy. The GC-derived values and FTIR spectra were used to make predictive models by using the partial least squares (PLS) procedure of SAS. Subsequently the models were used to generate predictions of milk composition by using FTIR spectra from three additional sets of 135 cows. The predictive model generated for HPI did not generate values for data not included in the predictive model satisfactorily (mean = 0.266, root mean-squared error of prediction (RMSEP) = 0.122); so, predictive models were created for additional milk fatty acid combinations. The predictive models generated estimates for several milk composition parameters relatively well. Table 1 lists means and RMSEP values for each predicted parameter. Although unsuccessful when used to predict the HPI of milk, FTIR spectroscopy can be used for the prediction of other fatty acid parameters of milk with some degree of success. With further development, the use of FTIR spectroscopy to determine milk fatty acid composition could become an essential part of milk analysis protocols.
The models created for each parameter predicted values for the data included in the model that correlated well with the GC-determined values, which was expected because those data are the basis for the models. None of the models predicted the FA parameters of interest not used in the models as well as the models predicted the values from which they were created, but several parameters were predicted relatively well. For example, the model predicted the weight percentage of unsaturated FA (Fig. 3), the weight percentage of long-chain unsaturated FA (16 to 18 carbons in length), the weight percentage of monounsaturated fatty acids (MUFA), the ratio of MUFA to total saturated FA, and the ratio of unsaturated to total saturated FA reasonably well (see Table 1 for mean values and RMSEP values).

The models created by the PLS procedure did not predict several of the parameters to a satisfactory degree. Those models poorly predicted the weight percentage of medium-chain saturated FA (8 to 14 carbons in length), the weight percentage of long-chain saturated FA (16 to 18 carbons in length), the ratio of C\textsubscript{16:0} to C\textsubscript{14:0}, the ratio of C\textsubscript{18:1} to C\textsubscript{18:0}, the ratio of C\textsubscript{16:1} to C\textsubscript{16:0}, the ratio of C\textsubscript{14:1} to C\textsubscript{14:0}, the weight percentage of polyunsaturated fatty acids (PUFA), and the ratio of PUFA to saturated FA. The ratio of C\textsubscript{16:0} to C\textsubscript{14:0} is representative of the activity of elongase and the ratios of C\textsubscript{18:1} to C\textsubscript{18:0}, C\textsubscript{16:1} to C\textsubscript{16:0} and C\textsubscript{14:1} to C\textsubscript{14:0} represent the activity of stearoyl-coenzyme A desaturase, that is, the enzyme that produces medium- and long-chain unsaturated FA (Mele et al., 2007). The activity of these enzymes was not able to be predicted by the models created by PLS.

The use of FTIR spectroscopy for the determination of fatty acid parameters of milk was met with some degree of success. Previously FTIR spectroscopy has been used to determine milk constituents such as sugar content, protein content, acetone content, and total fat percentage as well as quantifying the \textit{trans} fatty acid composition of milk samples. The
work presented in this paper demonstrates that FTIR spectroscopy performed on milk samples with water removed in combination with the PLS procedures of SAS can accurately generate predictions of several milk fatty acid parameters.

Fourier transform infrared spectroscopy currently is used in the dairy industry for determination of several milk constituents including total fat percentage, but to date there have been few published accounts of an attempt to use FTIR spectroscopy to determine fatty acid composition of milk. Presented here is the use of FTIR spectroscopy in combination with the PLS procedure to predict several milk fatty acid components. We showed that the combination of FTIR spectroscopy and the PLS procedure can predict the weight percentage of unsaturated FA, the weight percentage of long-chain unsaturated FA (16 to 18 carbons in length), the weight percentage of MUFA, the ratio of MUFA to total saturated FA, and the ratio of unsaturated to total saturated FA reasonably well.

The second objective of this study was to develop a method utilizing FTIR spectroscopy to determine fatty acid composition of milk that could be commercially marketed. At this time, this objective has not been achieved because the O-H stretching vibrations of water absorb nearly all of the photons present in the wavelengths at which the C-H and alkene bonds of fatty acids absorb energy. To counteract the interference of the O-H stretching vibrations at these wavelengths, it was necessary to remove water from raw milk samples before analysis. Several potential methods to eliminate water were investigated and a modification of the Folch method (Folch and Stanley, 1957) was chosen. The Folch method produces accurate and reproducible results (Avalli and Contarini, 2005, Zhang et al., 2008), but takes several hours per sample to complete as well as requiring the use of chloroform, which has multiple potential negative human health effects. Further investigation is needed to
develop a method that will eliminate water from samples quickly and in a manner that does not pose risk to human health.

**Recommendations for Future Research**

There are a few areas of research that still need to be addressed. To assess the wavelengths of the FTIR spectra representing the unsaturated fatty acid bonds, sample preparation was required to remove water from the samples. The modified Folch method (Folch and Stanley, 1957, Appendix A13) was selected to address this problem because it is a proven method in our laboratory for the accurate and reproducible extraction of lipid from samples (Zhang et al., 2008). The modified Folch method, however, takes several hours per sample to perform and involves the use of chloroform, which has multiple potential negative human health effects. Furthermore, chloroform contains a C-H vibration that interferes in the region of alkene vibration because of fatty acid skeleton carbons (Appendix 1A); so, the lipid portion that is isolated from samples must be dried under nitrogen conditions and reconstituted in a different solvent. In this study, the selected solvent was carbon disulfide (CS$_2$), which, among other human health concerns, is nephrotoxic, hepatotoxic, and a central nervous system toxin. Carbon disulfide is also a flammable liquid. Both solvents require personal protection including the use of a respirator or fumigation hood (Fisher Scientific, 2004, Fisher Scientific, 2008). Because of the multiple health hazards and concern over proper disposal of chloroform and CS$_2$, it is desirable to find an alternative method for sample preparation.

To make the technology for the determination of milk fatty acid composition marketable, an alternate method from the one used in this work should be pursued. In this study, a liquid spectrophotometer cell (International Crystal Laboratories, NJ), transmission
FTIR spectroscopy, and a DTGS detector were used for the analysis of lipid dissolved in CS$_2$. After spectroscopic analysis was already underway, the use of alternative FTIR methods was discussed (McClelland, Personal communication, Veysey, Personal communication). Of noteworthy consideration are diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS), photoacoustic spectroscopy (PAS; McClelland et al., 2002), and the use of a standard reflectance detector. The added bonus of using DRIFTS or PAS is that sample preparation would involve no chemicals and has the potential to be much less time consuming than the Folch method (Folch and Stanley, 1957). The samples would simply need to be desiccated in some way before analysis to remove the interference of water. Because of time and budget constraints, these methods could not be investigated further for this work.

References


Veysey, S. 2008. Personal communication. Chemical Instruments Services Lab, Iowa State University, Ames, IA.

APPENDIX A: PRELIMINARY WORK

Figures are included if:

i. They are relevant to the decision not to use raw milk
ii. They are relevant to the decision to use the modified Folch method
iii. They are relevant to the decision to use carbon disulfide (CS$_2$) for reconstitution of lipids

1. Deciding on a solvent
   a. Chloroform – large absorption peak in area of interest (~3000cm$^{-1}$)

Figure A1a. FTIR spectrum of chloroform
b. Carbon disulfide – works very well (no absorption peak in area of interest (~3000\,cm^{-1})

![Figure A1b. FTIR spectrum of carbon disulfide](image)

Concerns: Carbon disulfide has a nephrotoxic and hepatotoxic effects as well as being a central nervous system toxin. Prolonged or repeated exposure to carbon disulfide can produce target organ damage as well as other health risks. It is also a flammable solvent.

c. Deuterated chloroform – has potential (no peak in area of interest because the C-H bond does not exist), but is expensive as a solvent
   i. Potential use for extraction – perhaps solid phase extraction?
   ii. Folch method uses 14 mL of chloroform + ~5 more during filtering – if wasn’t so expensive, may be able to justify using it and not drying down prior to FTIR analysis

2. Oils
   a. In each of chloroform and CS\textsubscript{2} (the spectrum of chloroform is subtracted from the oils dissolved in chloroform and the spectrum of carbon disulfide is subtracted from oils dissolved in carbon disulfide)
i. Grapeseed oil – high in PUFA

Figure A2a. FTIR spectrum of grapeseed oil in chloroform

Figure A2b. FTIR spectrum of grapeseed oil in carbon disulfide
ii. “Esencial plus” oil – high in MUFA

Figure A2c. FTIR spectrum of “Esencial plus” oil in chloroform

Figure A2d. FTIR spectrum of “Esencial plus” oil in carbon disulfide
iii. Crisco – contains trans FA

Figure A2e. FTIR spectrum of Crisco in chloroform

Figure A2f. FTIR spectrum of Crisco in carbon disulfide

Conclusions: Figures A2a – A2f demonstrate the presence of a peak at \( \sim 3010 \text{cm}^{-1} \). This peak was investigated further by investigating individual fatty acid standards dissolved in carbon disulfide.
3. FA standards
   a. 2% lipid in CS$_2$ (the spectrum of carbon disulfide has been subtracted from each of these spectra)
      i. Lauric acid ($C_{12:0}$)

![Figure A3a. FTIR spectrum of lauric acid](image)

ii. Myristic acid ($C_{14:0}$)

![Figure A3b. FTIR spectrum of myristic acid](image)

iii. Palmitic acid ($C_{16:0}$)
iv. Stearic acid (C\textsubscript{18:0})
v. Oleic acid (C\textsubscript{18:1})

![Figure A3e. FTIR spectrum of oleic acid](image)

vi. Linoleic acid (C\textsubscript{18:2})

![Figure A3f. FTIR spectrum of linoleic acid](image)
vii. Linolenic acid (C\textsubscript{18:3})

![Figure A3g. FTIR spectrum of linolenic acid](image)

viii. Trilinolenin

![Figure A3h. FTIR spectrum of trilinolenin](image)
ix. Trilaurin

Figure A3i. FTIR spectrum of trilaurin

x. Trimonstirin

Figure A3j. FTIR spectrum of trimyristin
xi. Tripalmitin

![FTIR spectrum of tripalmitin](image)

xii. Tristearin

![FTIR spectrum of tristearin](image)

b. Peak at 3010 cm\(^{-1}\) was verified and only occurred in samples with unsaturated bonds (Figs. A3e, A3f, A3g, and A3h)
4. Whole milk
The ultimate goal was to significantly decrease the amount of sample preparation and total duration of time needed to acquire information about the fatty acid composition of milk.

a. Used raw milk in 0.05 mm CaF$_2$ cell – problems with water absorption around 3000 cm$^{-1}$

![Figure A4a. FTIR spectrum of whole milk](image)
5. Skim milk
   a. Skim milk

\[ \text{Figure A5a. FTIR spectrum of skim milk} \]

b. Skim milk (the spectrum of water has been subtracted from the spectrum of skim milk)

\[ \text{Figure A5b. FTIR spectrum of skim milk (water spectrum subtracted)} \]

6. Skim milk or whole milk + grapeseed oil (the spectrum of skim milk has been subtracted from these spectra)
   a. 0.28 g oil, dilute to 10 mL with CS\(_2\) for 2% PUFA
b. 0.56 g oil, dilute to 10 mL with CS₂ for 4% PUFA
Conclusions: The subtraction of the milk spectrum from samples including milk and an added percentage of grapeseed oil does not yield satisfactory results. The addition of grapeseed oil to skim milk and whole milk does not yield satisfactory results.

7. Water
   a. Used double distilled water in spectrophotometric cell to determine the FTIR spectrum of water
Conclusions: The O-H stretch of water uses up almost all of the photons around 3010 cm⁻¹.

8. Attenuated total reflectance (ATR) FTIR
   a. Water via ATR-FTIR

Figure A10a. Attenuated total reflectance FTIR spectrum of water
b. Whole milk via ATR-FTIR

Figure A10b. FTIR attenuated total reflectance (ATR) spectrum of raw milk

c. Whole milk via ATR (the spectrum of water has been subtracted from the spectrum of whole milk)

Figure A10c. ATR-FTIR spectrum of raw milk
d. Conclusions: Removes problem from water absorption in raw milk around 3010 cm⁻¹, but does not completely solve problems with using raw milk. This method needs further investigation before being implemented on a large scale basis.

9. Reproducibility
   a. Transmittance
      i. Take 4-5 measurements immediately after loading cell into machine – much variation
      ii. t=15 min – basically all measurements after this one are stable
      iii. t=30 min
      iv. t=60 min
      v. t=90 min
      vi. t=105 min
   b. ATR – very reproducible over a 15-minute test period

Conclusions: FTIR spectroscopy, whether transmittance mode or reflectance mode, is highly reproducible.

10. Modified Folch method – tried with 1 g, 2 g, and 4 g milk. Got greatest quantity of lipid at 4 g without overloading the ability of chloroform to dissolve the lipid.
Procedure:
   a. Measure 4 g of milk into tubes
   b. Add 17.0 mL of methanol:water (3.5:1) to each tube
   c. Vortex 15 seconds
   d. Add 6.5 mL of chloroform
   e. Vortex 15 seconds
   f. Shake on wrist-action shaker for 1 hour
   g. Add 7.5 mL of chloroform
   h. Add 7.5 mL of aqueous 0.37% KCl solution
   i. Invert tubes GENTLY three times (DO NOT SHAKE)
   j. Centrifuge 20 min at 500 x g
   k. Aspirate and discard top aqueous layer immediately after removing from centrifuge
   l. Add 10 mL of aqueous 0.37% KCl solution
   m. Invert tubes GENTLY three times (DO NOT SHAKE)
   n. Centrifuge 20 min at 500 x g
   o. Aspirate and discard top aqueous layer immediately after removing from centrifuge
   p. Filter samples directly into scintillation vials (pre-weighed) using a Buchner funnel and a suction flask
   q. Use new glass filter paper for (4.25 cm Whatman) for each sample. The pattern side should be up.
   r. Carefully remove vial with tweezers and cap it.
   s. Discard the filter paper and wipe out funnel with tissue or a kimwipe. Rinse with 2 to 3 mL of chloroform
t. Place full scintillation vials into the concentrator (with caps removed) to evaporate the chloroform. Close the concentrator lid and turn on the heat set to 50°C. SLOWLY turn on N₂ in the fume hood. The samples take approximately 2 ½ hours to dry.

u. After the samples have been dried down, remove them from the concentrator and replace their lids. Let them cool to room temperature.

v. Weigh vials to obtain lipid weight.

11. Bronolab-W II Liquid Preservative and Broad Spectrum Microtabs™
The Dairy Herd Information Association (DHIA) utilizes Bronolab-W II Liquid Preservative (D&F Control Systems, Inc, Dublin, CA) and Broad Spectrum Microtabs™ (D&F Control Systems, Inc, Dublin, CA) to preserve milk samples that will be analyzed with infrared instruments, so each was added to separate 20 mL samples of whole milk and analyzed via FTIR spectroscopy.

a. Obtained Bronolab-W II Liquid Preservative and Broad Spectrum Microtabs™ from Dairy Services Lab in Dubuque, IA.

b. Did not dissolve in CS₂

c. Added to whole milk, did Folch method and FTIR

Conclusions: The effect of the addition of these preservatives on the determination of the fatty acid composition of milk could not be determined due to interference by the O-H stretch of water in the area containing information about the alkene vibrational stretching of unsaturated fatty acids. The addition of these preservatives should not interfere with the determination of the fatty acid profile of lipids purified from milk samples because it is not lipid soluble.
APPENDIX B: ADDITIONAL UNSUCCESSFUL PROTOCOLS

1. Carbon disulfide extractions
   a. 2 g milk added to 10 mL CS$_2$
      i. Does not dissolve- separates back into layers
   b. Centrifuge off milk fat, dissolve in CS$_2$
      i. 10 min at 7000 x g in Micro7 centrifuge (Fisher)
      ii. Another 5 min at 7000 x g
      iii. Total of 0.2791 g milk cake collected from 9 mL milk, added 5 mL of CS$_2$ – did not go into solution
          1. Sonicated for ~15 min in sonicating water bath – did not dissolve
          2. Decanted off the liquid, spun at 7000 x g for 5 min
          3. Collect liquid portion to analyze
      i. 8 mL milk into 2 mL CS$_2$
      ii. Spin down 12 mL milk (12 min at 9100 x g), collect milk fat, add 10 mL CS$_2$ to milk fat, homogenize, collect CS$_2$ fraction
      iii. Sonicate 15 min, spin down (12 min at 9100 x g), collect milk fat, add 10 mL CS$_2$
      iv. Sonicate 15 min, add CS$_2$, shake, collect CS$_2$ fraction (5 mL milk, 5 mL CS$_2$)
      v. Homogenize milk 10 min, add CS$_2$ (7 mL milk, 5 mL CS$_2$)

2. Solid phase extraction
   The use of solid phase extraction (SPE) was briefly examined for the isolation of lipids from raw milk. Because of time and budget constraints, this technique was unable to be explored further.
   a. Alltech Associates Silica Extract-Clean™ Columns: 5000 mg bed weight, 8.0 mL column size (Part number 209202)

3. Sodium sulfate
   Anhydrous sodium sulfate is a relatively innocuous chemical (MSDS) that can be used to removing traces of water from organic solutions. The use of sodium sulfate for removing water from raw milk was investigated by adding sodium sulfate to 2 mL of milk until the mixture obtained a sticky powder texture. Sodium sulfate was added to the mixture in an attempt to dissolve the lipid-soluble components and decanted for analysis via FTIR spectroscopy.
   Procedure:
   a. Measure out 1 g milk, add sodium sulfate until forms a cake, rinse with CS$_2$ and collect CS$_2$ portion
   Conclusions: Worked well for extraction of some fatty acids, but reproducibility, practicality and ease of use are in question.

4. Skim milk or whole milk + Esencial plus oil
   a. 0.29 g oil, dilute to 10 mL with CS$_2$ for 2% MUFA
b. 0.59 g oil, dilute to 10 mL with CS₂ for 2% MUFA

Conclusions: The subtraction of the milk spectrum from samples including milk and an added percentage of Esencial plus oil does not yield satisfactory results. The addition of Esencial plus oil to skim milk and whole milk does not yield satisfactory results.

5. Skim milk + FA standard
   a. Did not work well- FA standards are oils, immiscible in aqueous solutions
      i. Tried heating milk plus FA standard in sonication bath – did not work
      ii. Tried shaking up – FA standard just floated to top of milk

Conclusions: The addition of a fatty acid standard to milk cannot be used for the assessment of fatty acid composition of milk.

6. Liver lipid Folch method
   Procedure:
   a. Weigh accurately 4 g of milk into a screw-top 25 x 150 mm extraction tube
   b. Add 10 mL chloroform: methanol and sonicate for 30 seconds
   c. Cap tubes tightly, vortex, and place tubes on wrist-action shaker for 60 min
   d. Add 4 mL of water, invert 3 times
   e. Centrifuge 20 min at 500 x g
   f. Aspirate off the water-methanol layer and discard
   g. Filter samples directly into scintillation vials (pre-weighed) using a Buchner funnel and a suction flask
   h. Use new glass filter paper for (4.25 cm Whatman) for each sample. The pattern side should be up.
   i. Carefully remove vial with tweezers and cap it.
   j. Discard the filter paper and wipe out funnel with tissue or a kimwipe. Rinse with 2 to 3 mL of chloroform
   k. Place full scintillation vials into the concentrator (with caps removed) to evaporate the chloroform. Close the concentrator lid and turn on the heat set to 50°C. SLOWLY turn on N₂ in the fume hood. The samples take approximately 2 ½ hours to dry.
   l. After the samples have been dried down, remove them from the concentrator and replace their lids. Let them cool to room temperature.
   m. Weigh vials to obtain lipid weight.

Conclusions: Had problems with fat cake formation and separation into layers.

7. Abbreviated Folch – is analogous to A13 with the subtraction of steps c, e, f, k, l, and m.
   Procedure:
   a. Measure 4 g of milk into tubes
   b. Add 17.0 mL of methanol:water (3.5:1) to each tube
   c. Vortex 15 seconds
   d. Shake on wrist-action shaker for 1 hour
e. Add 7.5 mL of chloroform
f. Add 7.5 mL of aqueous 0.37% KCl solution
g. Invert tubes GENTLY three times (DO NOT SHAKE)
h. Invert tubes GENTLY three times (DO NOT SHAKE)
i. Centrifuge 20 min at 500 x g
j. Aspirate and discard top aqueous layer immediately after removing from centrifuge
k. Filter samples directly into scintillation vials (pre-weighed) using a Buchner funnel and a suction flask
l. Use new glass filter paper for (4.25 cm Whatman) for each sample. The pattern side should be up.
m. Carefully remove vial with tweezers and cap it.
n. Discard the filter paper and wipe out funnel with tissue or a kimwipe. Rinse with 2 to 3 mL of chloroform
o. Place full scintillation vials into the concentrator (with caps removed) to evaporate the chloroform. Close the concentrator lid and turn on the heat set to 50°C. SLOWLY turn on N₂ in the fume hood. The samples take approximately 2 ½ hours to dry.
p. After the samples have been dried down, remove them from the concentrator and replace their lids. Let them cool to room temperature.
q. Weigh vials to obtain lipid weight.

Notes about method:
- At filter step, got cloudy chloroform that cleared after sitting for several hours – believe KCl salt was unable to separate from chloroform layer
- Adding another rinse step defeats the purpose of shortening down the full Folch method
- Wrist-action shaker for 30 min at half force worked better (prevented foaming of milk)
APPENDIX C: ADDITIONAL FIGURES

Figure 1: Relationship of spectroscopically determined and chemically determined weight percentage of unsaturated fatty acids ($C_{14:1} + C_{16:1} + C_{18:1} + C_{18:2} + C_{18:3n3} + C_{18:3n6}$). n=265. Root mean-squared error of prediction (RMSEP) = 1.185.

Figure 2: Use of model derived from data in Fig. 1 to predict composition of milk samples. n=398. RMSEP = 3.832.
Figure 3: Relationship of spectroscopically determined and chemically determined health-promoting index (HPI = sum of proportion of C_{12:0} + sum of proportion of C_{16:0} + 4 x sum of proportion of C_{14:0} / sum of proportion of total MUFA and PUFA). n=265. RMSEP = 0.041.

Figure 4: Use of model derived from data in Fig. 3 to predict composition of milk samples. n=398. RMSEP = 0.122.
Figure 5: Relationship of spectroscopically determined and chemically determined weight percentage of saturated fatty acids. n=265. RMSEP = 1.817.

Figure 6: Use of model derived from data in Fig. 5 to predict composition of milk samples. n=398. RMSEP = 9.432.
Figure 7: Relationship of spectroscopically determined and chemically determined weight percentage of medium-chain saturated fatty acids (medium-chain = C\textsubscript{8:0} + C\textsubscript{10:0} + C\textsubscript{11:0} + C\textsubscript{12:0} + C\textsubscript{13:0} + C\textsubscript{14:0}). n=265. RMSEP = 2.908.

Figure 8: Use of model derived from data in Fig. 7 to predict composition of milk samples. n=398. RMSEP = 6.300.
Figure 9: Relationship of spectroscopically determined and chemically determined weight percentage of long chain fatty acids ($C_{16:0} + C_{17:0} + C_{18:0}$). n=265. RMSEP = 1.393.

Figure 10: Use of model derived from data in Fig. 9 to predict composition of milk samples. n=398. RMSEP = 8.631.
Figure 11: Relationship of spectroscopically determined and chemically determined weight percentage of long-chain unsaturated fatty acids ($C_{16:1} + C_{18:1} + C_{18:2} + C_{18:3\text{n3}} + C_{18:3\text{n6}}$). $n=265$. RMSEP = 1.841.

Figure 12: Use of model derived from data in Fig. 11 to predict composition of milk samples. $n=398$. RMSEP = 4.980.
Figure 13: Relationship of spectroscopically determined and chemically determined ratio of $\text{C}_{16:0}$ to $\text{C}_{14:0}$ fatty acids. $n=265$. RMSEP = 0.322.

Figure 14: Use of model derived from data in Fig. 13 to predict composition of milk samples. $n=398$. RMSEP = 0.950.
Figure 15: Relationship of spectroscopically determined and chemically determined ratio of $C_{18:1}$ to $C_{18:0}$ fatty acids. $n=265$. RMSEP = 0.213.

Figure 16: Use of model derived from data in Fig. 15 to predict composition of milk samples. $n=398$. RMSEP = 0.751.
Figure 17: Relationship of spectroscopically determined and chemically determined ratio of \( \text{C}_{16:1} \) to \( \text{C}_{16:0} \) fatty acids. \( n=265 \). \( \text{RMSEP} = 0.010 \).

Figure 18: Use of model derived from data in Fig. 17 to predict composition of milk samples. \( n=398 \). \( \text{RMSEP} = 0.016 \).
Figure 19: Relationship of spectroscopically determined and chemically determined ratio of C_{14:1} to C_{14:0} fatty acids. n=263. RMSEP = 0.023.

Figure 20: Use of model derived from data in Fig. 19 to predict composition of milk samples. n=398. RMSEP = 0.050.
Figure 21: Relationship of spectroscopically determined and chemically determined weight percentage of PUFA ($C_{18:2} + C_{18:3n3} + C_{18:3n6}$). $n=265$. RMSEP = 0.355.

Figure 22: Use of model derived from data in Fig. 21 to predict composition of milk samples. $n=398$. RMSEP = 0.595.
Figure 23: Relationship of spectroscopically determined and chemically determined weight percentage of MUFA ($C_{14:1} + C_{16:1} + C_{18:1}$). $n=265$. RMSEP = 1.661.

Figure 24: Use of model derived from data in Fig. 23 to predict composition of milk samples. $n=398$. RMSEP = 4.164.
Figure 25: Relationship of spectroscopically determined and chemically determined ratio of PUFA to saturated fatty acids. n=263. RMSEP = 0.005.

Figure 26: Use of model derived from data in Fig. 25 to predict composition of milk samples. n=398. RMSEP = 0.014.
Figure 27: Relationship of spectroscopically determined and chemically determined ratio of MUFA to saturated fatty acids. n=265. RMSEP = 0.0347.

Figure 28: Use of model derived from data in Fig. 27 to predict composition of milk samples. n=398. RMSEP = 0.081.
Figure 29: Relationship of spectroscopically determined and chemically determined ratio of unsaturated to saturated fatty acids. n=265. RMSEP = 0.036.

Figure 30: Use of model derived from data in Fig. 29 to predict composition of milk samples. n=398. RMSEP = 0.096.
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