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Rapid Determination of Milk Components and Detection of Adulteration Using Fourier Transform Infrared Technology

Ivan Von Mendenhall
Utah State University

RAPID DETERMINATION OF MILK COMPONENTS AND DETECTION OF ADULTERATION USING FOURIER TRANSFORM INFRARED TECHNOLOGY

by

Ivan Von Mendenhall

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Ivan V. Mendenhall

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ABSTRACT

Rapid Determination of Milk Components and Detection of Adulteration Using Fourier Transform Infrared Technology

by

Ivan V. Mendenhall, Doctor of Philosophy

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Major Professor: Dr. Rodney J. Brown
Department: Nutrition and Food Sciences

Absorption bands responding to changes in fat, protein, and lactose concentrations in milk were determined. The effects of milk fat variation and lipolysis on the infrared spectrum were studied.

Absorbances from 1283 to 1100 cm^{-1} correlated with fat, protein, and lactose concentration and showed a low response to milk fat variation and lipolysis. A Fourier transform infrared spectrometer equipped with an attenuated total internal reflectance cell was calibrated using these absorption bands, partial least squares statistics, and milk samples from herds in Minnesota. When the fat, protein, and lactose concentrations in these samples were predicted, the standard deviations of difference (reference - infrared) were .22, .06, and .02%. When the fat, protein, and lactose concentrations in a separate set of samples from herds in California were predicted, the standard deviations of difference were 1.23, .10, and .07%. Substitution of a 15 μm pathlength transmission cell for the attenuated total internal reflectance cell changed the standard deviations of difference to .07, .11, and .06% in the calibration (Minnesota) samples and .09, .10, and .16% in the validation (California) samples.

Infrared spectroscopy was used to measure whey powder in an adulterated sample of nonfat dry milk. Mixtures of nonfat dry milk containing whey powder at various

concentrations were analyzed using absorption bands between 1400 and 1200 cm^{-1} in the infrared spectrum. There was a strong correlation ($r > .99$) between predicted and measured concentrations of whey powder in adulterated samples. Accuracy was not affected by processing conditions, source of nonfat dry milk, and origin of whey powder.

A rapid method for detecting soybean oil in process cheese was developed. The infrared spectrum of each sample was collected using an accessory designed for analysis of solid samples. A linear relationship fit (= .98) when the ratio of absorbance at 2957 and 2852 cm^{-1} was plotted versus percent adulteration.

(121 pages)

PART 1. QUANTITATIVE INFRARED ANALYSIS: A REVIEW

THEORY OF QUANTITATIVE INFRARED ANALYSIS

Traditionally, multicomponent quantitative analysis required separation of components of interest, usually by a chromatographic method, prior to component measurement. These types of analyses are time consuming (2). Infrared absorption spectroscopy is primarily used to characterize compounds in a qualitative analysis. Recently, multicomponent quantitative analysis by infrared absorption has become an attractive alternative to traditional methods, due mainly to the proliferation of computers interfaced to or built into new instruments. These computers can record, sort, transform, and do complex operations on large amounts of data in short times (3). The major advantage of using infrared methods instead of chromatographic methods is that the components of a system do not have to be separated before analysis. This reduces analysis time, and that can be translated into cost savings (2).

The relationship between light absorbed by a species and its concentration is the Beer-Lambert law:

$$A = \log(I_o/I) = a\ b\ c \qquad [1]$$

where A is absorbance, I_o is intensity of radiation incident on the sample, I is intensity of radiation incident on the detector, a is absorptivity, b is pathlength, and c is concentration of the analyte. If we assume radiation emitted by the source is monochromatic, and a constant pathlength is used, equation [1] can be expressed as

$$A = k\ c \qquad [2]$$

where k is the proportionality constant. Normally in a laboratory, samples do not contain a single component but multiple components of interest. To allow for multiple components, the Beer-Lambert law must be expanded (2). Equation [3] is an expanded form of the Beer-Lambert law which accounts for the possible interference between components (band overlap).

$$A_1 = k_{11}\, c_1 + k_{12}\, c_2 + \ldots\ldots\ldots + k_{1n}\, c_n$$

$$A_2 = k_{21}\, c_1 + k_{22}\, c_2 + \ldots\ldots\ldots + k_{2n}\, c_n \qquad [3]$$

$$A_n = k_{n1}\, c_1 + k_{n2}\, c_2 + \ldots\ldots\ldots + k_{nn}\, c_n$$

A_i is absorbance at the i^{th} analytical frequency, k_{ij} is the proportionality constant, and c_j is concentration of the j^{th} component. This set of equations can be more conveniently expressed in matrix form. In matrix notation, the Beer-Lambert law can be expressed as

$$\mathbf{A} = \mathbf{K}\,\mathbf{C} \qquad [4]$$

A is a matrix of calibration spectra with dimensions $n \times i$ where n is the number of calibration standards and i is the number of analytical frequencies. **C** is a matrix of component concentrations with dimensions $n \times j$ where j is the number of chemical components of interest. **K** is a matrix of proportionality constants with dimensions $j \times i$. Several methods are available for calibration of the instrument and prediction of the components of interest in unknown samples. These methods are based on the assumption that there is a linear relationship between absorbance and component concentration (5).

STATISTICAL METHODS OF MULTIVARIATE QUANTITATIVE ANALYSIS

The K-matrix Method

The classical least squares method, better known as the K-matrix method, is based on the assumptions that absorbance at each frequency is proportional to component concentrations and error in spectral absorbances is responsible for model error (5). During calibration, spectra are collected for standard solutions and the **K** matrix is solved for using the following equation:

$$\mathbf{K} = \mathbf{A}\,\mathbf{C}'\,(\mathbf{C}\mathbf{C}')^{-1} \qquad [5]$$

Once the **K** matrix is determined, the concentrations of components of interest in an unknown sample may be solved for by using the following equation:

$$\mathbf{c} = (\mathbf{K}'\,\mathbf{K})^{-1}\,\mathbf{K}'\,\mathbf{a} \qquad [6]$$

where **a** is the spectrum of the unknown sample and **K** is from equation [5] (5). The K-matrix method has advantages and disadvantages. The model allows for overdetermination of the number of wavelengths used without having to increase the number of calibration samples (5). Nonzero intercepts can be incorporated into the model, to approximate deviations from the Beer-Lambert law, by adding a column to the **K** matrix and a row of 1's to the **C** matrix (1). The K-matrix method requires two matrix inversions, increasing the possibility of round-off error by the computer (6). Also, if the concentrations of components of interest sum to a constant, it is not possible to use a nonzero intercept because the **C** matrix becomes singular, therefore noninvertable (2). Finally, all interfering chemical components in the spectral region of interest need to be known and their concentration included in the calibration (5).

The P-matrix Method

Inverse least squares, or the P-matrix method, models the concentration as a function of absorbance:

$$\mathbf{C} = \mathbf{P}\,\mathbf{A} \qquad [7]$$

where **C** and **A** are as in the K-matrix method and **P** is a matrix with dimensions $i \times j$. During calibration, the **P** matrix is determined by the following equation:

$$\mathbf{P} = \mathbf{C}\,\mathbf{A}'\,(\mathbf{A}\,\mathbf{A}')^{-1} \qquad [8]$$

The **P** matrix may be used directly in equation [7] to predict the concentrations of components of interest in unknown samples. Like the K-matrix, this method also has advantages and disadvantages. Use of the P-matrix method requires only one matrix inversion, thereby minimizing round-off error by the computer (2). Unlike the K-matrix method where least squares regression minimizes error in absorbance, the P-matrix method minimizes the error in concentration (5). Adding a column to the P-matrix and a row of 1's to the bottom of the **A** matrix allows for a nonzero intercept in the P-matrix method (1). Analyses based on this model are invariant with respect to the number of chemical components in the analysis, so presence of an impurity in the calibration samples does not affect the analysis even if the concentration is unknown (5). The main disadvantage of the P-matrix method is that the number of calibration samples has to be equal to or greater than the number of frequencies used (2). Also colinearity problems (the near linear relationships between absorbance at multiple frequencies) can become significant and degrade precision when the number of frequencies becomes too large (5).

Principal component regression (PCR) and partial least squares (PLS) analysis are methods that combine the full spectrum advantages of the K-matrix method with the ability to do the analysis one chemical component at a time as in the P-matrix method (5).

Principal Component Regression

In the PCR model, the **A** matrix in equation [7] undergoes a singular value decomposition resulting in formation of three matrices:

$$\mathbf{A} = \mathbf{U}\,\mathbf{S}\,\mathbf{V}' \qquad [9]$$

where **V'** is an orthogonal matrix with rows containing principal components of the calibration spectra and dimensions equal to the number of analytical frequencies; **U** is an orthogonal matrix, its rows containing the "factor loadings" (the linear combination of principal components that form the original matrix **A**) and having dimensions equal to the number of calibration samples; and **S** is a nonnegative diagonal matrix with dimensions equal to the number of calibration spectra. The values in **S**, when squared, represent the contribution of each principal component to the variance of the spectra in **A** (4). The **P** matrix in equation [7] may be solved for by the equation:

$$\mathbf{P} = \mathbf{V}\,\mathbf{S}^{-1}\,\mathbf{U}'\,\mathbf{C} \qquad [10]$$

Once the **P** matrix is determined, the concentration of components in an unknown sample may be solved for by:

$$\mathbf{c} = \mathbf{a}\,\mathbf{P} \qquad [11]$$

When using PCR analysis, it is important to retain only principal components which are useful for prediction and discard those principal components with smaller singular values, which are sensitive to noise. The number of principal components retained is the "rank" of the model. The optimum rank is determined by a cross-validation procedure (9).

Partial Least Squares Analysis

The PLS model described by Martens and Jenson (8) is similar to the PCR model. An unknown spectrum is expressed as a linear combination of the principal components of the calibration spectra, but in the PLS model, the principal components are chosen because they are correlated with the concentration information. This forces factors with

high relevance for the chemical data that may be ignored as "noise" in the PCR model into the solution (8). In the PLS model the **A** matrix in equation [7] is decomposed:

$$\mathbf{A} = \mathbf{T}\,\mathbf{B} + \mathbf{E} \qquad [12]$$

where **T** is a matrix of the orthogonal component vectors of the **A** matrix (latent variables), with dimensions $r \times i$ where r is the rank of the model; **B** is a matrix of factor loadings with dimensions $n \times r$; and matrix **E** contains the residual error in fitting the matrix **A** to r latent variables (4). The **C** matrix in equation [7] is also decomposed into a similar matrix equation:

$$\mathbf{C} = \mathbf{U}\,\mathbf{P} + \mathbf{F} \qquad [13]$$

where **U** is a matrix of latent variables with dimensions $n \times r$. **P** is a matrix of factor loadings with dimensions $j \times r$; **F** contains the residual error in fitting the matrix C with r factors (4). Calibration is done by iteratively estimating a latent variable that will optimally predict concentrations of the components of interest. All spectral and chemical data is then projected onto this latent variable, and a new latent variable representing a linear combination of spectral residuals after the first projection is iteratively estimated. This second latent variable is orthogonal to the first and optimally predicts the residuals of the chemical data after the first projection. This process continues until the fit of the model is optimized. The number of latent variables chosen corresponds to the rank of the model. Prediction is done by first fitting the spectral data of an unknown sample to the calibration model, yielding spectral lack-of-fit data and estimates of the underlying PLS factors. From these factors the chemical data is predicted (7).

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PART 2. INFRARED ANALYSIS OF MILK: A REVIEW

DEVELOPMENT OF INFRARED ANALYSIS OF MILK

The components of milk that normally determine its worth are fat, protein, and lactose. Dairy product manufacturers need specific proportions of these components in product formulations to make a product acceptable to consumers, meet federal regulations, and maximize yield. So, the ability to measure these components rapidly and accurately is important. Historically, chemical methods were the standards of the industry for making these measurements. Chemical methods are still used in laboratories and smaller manufacturing plants. These methods are also used as references for calibration of infrared instruments. Some disadvantages of chemical methods are long analysis times and expense in time and reagents. Ramm (25) reported that in the early 1960s there was a large increase in demand for milk testing that prompted instrument manufacturers to develop faster, cheaper methods for measuring the components of milk. Of the methods investigated, infrared absorption spectroscopy proved most successful.

Early Development Work

Infrared absorption spectroscopy of dairy products presented difficulties not encountered in other applications of the technique. The water in milk absorbs strongly in the infrared and can mask the spectral components of interest. Water dissolves conventional sample cell materials like sodium chloride. Another problem arises from the nonhomogeneous nature of milk that results in loss of transmitted energy because of scattering. For these reasons, work in infrared absorption spectroscopy of dairy products until the early 1960s was confined to the KBr pressed disc method for dried milk and butter (13).

In 1961, Goulden (14) published a method for the quantitative analysis of milk by infrared absorption. Samples of milk were first homogenized then analyzed in a double beam spectrometer. By filling the reference cell with water and subtracting its

spectrum from the sample spectrum, the spectra of components of interest were unmasked. However, absorption of water was so intense in both beams that only a small amount of radiation reached the detector, resulting in maximum optical densities of about .2 for a cell pathlength of 50 μm. Little improvement was made by increasing the pathlength because the reduced solvent transmission required an increase in the monochromator slit width that decreased the resolution and increased the stray radiation. Homogenization before analysis decreased the diameter of the fat globules to a mean of 1 μm. This significantly reduced radiation scattering that happens when particles have a diameter range equivalent to the wavelength range of the incident radiation (2 to 15 μm). The spectra had absorption peaks near 1724, 1538, and 1042 cm^{-1} (5.8, 6.5, and 9.6 μm) that could be mainly attributed to fat, protein, and lactose. Fat and lactose concentrations were estimated directly from the 1724 cm^{-1} (termed "fat A") and 1042 cm^{-1} bands. Goulden found that fat concentration affects the protein absorption band requiring corrections for changes in fat concentration when measuring protein. Twenty different samples from three different breeds of cows in different stages of lactation were analyzed. Standard deviations of 3% or less were seen for each of the components.

Infrared Milk Analyzer (IRMA)

In 1964, an infrared milk analyzer (IRMA) was developed. This instrument had a built-in homogenizer and could measure the fat, protein, lactose, and solids-not-fat (SNF) in a 30 ml sample in less than 1 min. The optical design of the IRMA was as follows: Radiation emitted from the source was split into two beams. One beam passed through the sample cell and one through the reference cell. With reciprocating mirrors, the beams were alternately focused at the entrance slit of a monochromator that consisted of a diffraction grating and prism dispersing unit. Selected wavelengths of light exited the monochromator, passed through a semiconductor filter to remove stray

radiation, and fell onto a thermocouple that converted the alternating radiant energy into an alternating electromotive force. This small voltage was amplified and drove an optical comb attenuator into the reference beam until equilibrium between the reference and sample beam was reached. A potentiometer on the shaft of the optical comb attenuator converted the beam attenuation into a corresponding d.c. voltage that was measured by a digital voltmeter or other suitable output device (16).

The homogenizer and double beam design alleviated scattering of radiation and masking of spectral components of interest by intense water absorption. This instrument also has a set of optical filters that when placed in the sample beam, simulate the absorption of fat, protein, or lactose. This allows the accuracy of calibration and the stability of the instrument between calibrations to be verified. Preliminary testing was done on 24 milk samples representing four different breeds of cows. The standard deviations of difference between chemical and infrared values for fat, protein, lactose, and SNF were .10, .10, .10, and .25%.

Goulden (15) and Biggs (1) explained several factors that affected the performance of the IRMA. The efficiency of the homogenizer affects the fat signal. Lower efficiencies that result in larger fat globules cause an increase in the fat signal as expected. Once the homogenization pressure reaches 3,000 p.s.i., multiple homogenizations fail to further decrease the signal. The temperature difference between sample and reference cell also affects component signals. This effect is about .1% transmission for each degree Celsius temperature difference. A heat exchanger, placed in line after the homogenizer, equilibrates the temperature of the incoming milk with the temperature of the water in the reference cell, minimizing these effects.

Interferences by milk components affect the performance of IRMA. A change in the percentage of one milk component, all other solid components remaining the same, is equivalent to a change in water concentration. Displacement of water results in a decreased signal, so the net absorptivities of interfering components are mostly

negative. The effect of fat absorbance at the protein wavelength proved to be the only significant source of interference. This is corrected by using the already determined fat percentage to add to or subtract an appropriate amount from the protein reading.

Biggs (2) published the results of a study on the precision and accuracy of the infrared milk analyzer. The values determined with IRMA for fat, protein, lactose, and SNF were compared with Mojonnier, semi-micro Kjeldahl, polarimetry, and USDA lactometer methods. Mean differences of .01% or less and standard deviations of difference between means of duplicate tests of .03% were reported for fat, protein, and lactose. Mean differences of .015% and standard deviations of difference of .09% were reported for SNF.

The Milkoscan 300 and 203

In 1978, Biggs (3) published the results of a study on estimation of fat, protein, and lactose using a new infrared instrument, the Milkoscan (models 300 and 203) produced by A/S N. Foss Electric, Denmark. The model 300 analyzes fat and protein at a rate of 300 samples/h. The model 203 analyzes fat, protein, and lactose at a rate of 200 samples/h.

The Milkoscan instruments operate on the same principle as the IRMA except for a few design changes to reduce the errors associated with water displacement and scattering by fat globules. Instead of using a reference cell for subtracting the effects of water, a single sample cell is used and the sample is analyzed with two different wavebands; a primary or sample waveband at which there is high absorptivity by the measured component, and a secondary waveband where the opposite is true. With this single cell approach, water concentration is constant in the two beams for each individual sample. If the water absorptivities are different at the two wavebands, there is an interference effect proportional to the magnitude of this absorptivity difference, to the amount of interfering component, and to its specific volume. The manufacturer

anticipated these interference effects and used them to correct the instrument signals electronically. Another effect, light scattering by fat and protein particles, is compensated for by equivalent scattering in the reference beam.

This instrument also uses optical filters instead of a diffraction grating for selection of analytical and reference wavelengths. The advantages of filters over diffraction gratings are that they simplify the optical design, enabling the instrument to operate at a higher energy throughput that is critical in aqueous systems. Filter transmission characteristics are difficult to reproduce, stray energy may be transmitted, and transmission characteristics may change with temperature.

Biggs (3) used six instruments located in six different laboratories. Calibrations were done independently. Thirty six milk samples were pre-analyzed by accepted standard methods then analyzed by Milkoscan. Error components were estimated by the statistical methods advocated by Youden (34). Results from pairs of samples at similar levels of component concentration are used to calculate estimates of precision and systematic errors also differences between the average amount found and the average amount present. Precision errors for fat were .033% for the first and .022% for the second of duplicate tests. Precision errors for protein were .021% for both tests. Systematic errors for fat were .17% with calibrations based on reference analysis at each laboratory but decreased to .044% with calibrations by reference analysis from one laboratory. Systematic errors of .067% for protein decreased to .03% when calibrations were based on a common reference analysis. Standard errors of estimate for lactose were .034 and .033%.

The Multispec Instrument

The next instrument evaluated by Biggs (4) was the Multispec manufactured by Multispec, Ltd, Wheldrake, UK. The Multispec instrument uses the same double beam in wavelength, single cell system as the Milkoscan instruments. Sample and reference

optical filters are also used instead of a diffraction grating. High pressure double stage homogenization (3,000 to 4,000 p.s.i.) and a variable tilt fat sample filter reduce scattering of energy from fat globules and errors resulting from shifts in the wavelength of fat absorption (3). A sealed optical console, thermostatic temperature control of console, homogenizer, and sample cell, and electronic correction for cross interference effects are standard.

Milk samples including homogenized and unhomogenized herd milks, individual cow milks, and packaged milks were analyzed in duplicate by the Multispec on two different days, three days apart. Reference analysis for fat, protein, and lactose was by Mojonnier, semi-micro Kjeldahl, and polarimetry. For statistical analysis of the data, results were separated into four groups: a set of calibration samples, pre-homogenized herd milks, individual cow milk, and packaged retail milk. Both individual group data and combined data were analyzed.

For fat analysis, within day mean differences between duplicate estimates were all .01% or less and between day mean differences averaged .025%. Standard deviations of difference for both within and between day estimates were nearly all less than .02%, averaging .014% for grouped data and .016% for the combined data. For the grouped data, mean values for Multispec results varied from .003 to .032% higher than the mean standard results, and on the second day from .019% lower to .008% higher. Standard deviations of difference averaged .016% for packaged milk, .035% for unhomogenized calibration milk, .04% for homogenized herd milk, and .06% for individual cow milk. For the combined data, the Multispec results averaged .018% higher than the standards on the first day and .007% lower than the standards on the second day, with an average standard deviation of difference of .041%. Sample cell purging efficiency for fat, estimated by alternately measuring fat in samples of milk and water, was 99.5%. The effect of sample temperature was negligible within the temperature range of 35 to 40°C.

For protein analysis, within and between-day mean differences averaged .003 and

.009%. Standard deviations of difference averaged .015% for both within and between-day comparisons. For comparisons between Multispec and reference results, Multispec means for the combined data averaged .015 and .006% lower than the means for the standards on the first and second day. Standard deviations of difference between Multispec and standard results averaged .02% for individual cow milk, .037% for unhomogenized calibration milk, .043% for homogenized herd milk, and .041% for packaged milk that combine to give an average standard deviation of difference of .035%. Purging efficiency for protein was 98.8% and variations in sample temperature between 35 and 40°C had a negligible effect on the results.

For lactose analysis, within and between-day mean differences between duplicate estimates averaged .003% and .007% for grouped data and .003% or less for combined data. Standard deviations of difference were .02% for both within and between-day differences. Mean differences between Multispec and reference results averaged .005% for unhomogenized calibration milks and .002% for homogenized herd milks. Mean values for individual cow milks averaged .04% higher than the references and mean values for packaged milks averaged .18% lower than references. Standard deviations of difference were .03% for individual cow milk and .04% for other types. Purging efficiency for lactose was 98.1%. Samples analyzed at 45°C showed a slight sample temperature effect.

Performance Specifications

In 1979, Biggs (5) published performance specifications for infrared milk analyzers. These were given in response to a recommendation from subcommittee C of the Association of Official Analytical Chemists (AOAC) who suggested that a set of performance specifications be written to alleviate the need for evaluating and approving many individual instruments used for an approved method. Instruments meeting these specifications would automatically comply with the requirements of the approved

method. Maximum limits of .02% are recommended for precision of infrared analysis of fat, protein, and lactose, and .04% for total solids. Maximum systematic errors of .06% for fat, protein, and lactose, and .12% for total solids are required when instrumental results are compared with results of specified AOAC methods.

The Milkoscan 100 Series

In 1980, Van de Voort (32) evaluated a new Milkoscan instrument, the Milkoscan 104. This instrument represented a second generation of the single cell dual wavelength instruments first introduced in 1975. Several improvements were incorporated into this instrument. The servo motor, optical comb attenuator mechanism was replaced with an electronic ratio system. The number of mirrors was reduced from nine to two. Other changes were a thermostatically controlled filter housing, relocated chopper, and improved filters and detector.

This instrument was evaluated using herd, individual cow, commercial, and composite random milk samples. Mean differences and standard deviations of difference were .02% and $\pm$ .02% for reproducibility and .05% and $\pm$ .06% for accuracy. Van de Voort found that variation in the average molecular weight of milk fat is the primary reason for the noncorrespondence between infrared and chemical methods.

A New Fat Wavelength

Sjaunja (28) studied the effects of variation of molecular weight and unsaturation in milk fat. He used a Multispec instrument equipped with a filter for analysis of fat at 2941 to 2857 cm^{-1} (3.4 to 3.5 μm, termed "fat B") besides filters for the conventional wavelengths used for fat, protein, and lactose analysis. This band results from vibration of the carbon-hydrogen bonds in fatty acids. Sjaunja found that fat analysis at the fat A wavelength is sensitive to change in refractive index of milk owing to changes in the chain length and saturation level of fatty acids. This sensitivity to variation in

refractive index accounted for 27% of the total variation in analysis difference between infrared and standard methods at the fat A wavelength and 3% at the fat B wavelength. The standard deviation of difference of milk fat analysis at fat B was similar to that at fat A (SD = .083 and .084%). When a combination of the two wavelengths was used, the standard deviation decreased to .075%. This slight increase in accuracy is accomplished at the expense of increased analysis time because four wavelengths are used instead of three.

The Milkoscan 605

In 1985, Sjaunja and Andersson (31) reported the evaluation of another new infrared milk analyzer, the Milkoscan 605. This instrument is a single cell, dual wavelength analyzer with an optical system similar to the Milkoscan 104. Changes made in the model 605 were modifications to the infrared detector, addition of a filter wheel that holds five pairs of filters, and an improved pumping unit and homogenizer. The model 605 has a microprocessor that converts primary signals of infrared absorption to correspond to concentrations of milk components. The microprocessor allows intercorrection factors, linearity corrections, slopes, and intercepts to be controlled as numerical values. Some critical points in the instrument (flow pressure, temperature, and sample volume) are monitored by the microprocessor and rinsing and zero-setting routines are controlled. The instrument has programs for automatic calibration, linearity setting, and intercorrection factor setting.

The instrument was evaluated using milk samples from 30 individual cows and 30 herds of two different breeds (Swedish Red and White and Swedish Freisan). The samples were preserved with .02% bronopol and analyzed in duplicate with the Milkoscan 605 for fat (at both 5.7 and 3.5 μm), protein, and lactose. Reference analysis for fat, protein, and lactose was by Rose-Gottlieb, Kjeldahl, and Luff-Schoorl methods. Standard deviations between duplicate infrared analyses were .006, .005, .008, and .001

for fat A (5.7 μm), fat B (3.5 μm), protein, and lactose. Standard deviations of difference between infrared and reference methods were .095, .057, .056, .048, and .061 for fat A, fat B, fat A+B, protein, and lactose.

All wavelengths had transfer errors (purging efficiency) of less than 1% per unit component concentration. Protein and lactose had larger transfer errors than fat. The analytical effect of temperature variations was small. Between 30 and 47°C, the error was within ± .02%.

The citric acid content of milk influenced all the infrared results. The dependence was estimated as an increase of between .005 and .009% in the fat A, fat B, and protein results as citric acid increased by .01%. Lactose results decreased by .008%. Because the normal range of citric acid in cow milk is ± .05% (10), the analytical error in practice is small but the differences in concentration of citric acid in milk from different species can cause systematic analytical errors if the infrared determinations are based on calibration with cow milk samples.

Future Prospects for Infrared Analysis of Milk

The trend in infrared milk analyzers is toward simplified, robust optics, and computerization. This improves the stability and reliability of measurements and ease and accuracy of calibration. These advances in fixed filter spectroscopy have not overcome the limits imposed by the small number of sensors (filter bands) available (17, 31), nor have they overcome interference by changing levels of saturation in milk fat from one sample to the next or the effects of lipolysis on milk fat. Although fixed filter infrared absorption still has desirable features, other procedures that allow for the use of many more wavelengths should be adopted for testing of dairy products.

Fourier transform infrared (FTIR) spectrometry has many advantages over fixed filter methods. With FTIR, measurements at many wavelengths can be made simultaneously. FTIR measurements are narrow bands of the spectrum instead of the

broad bands of filter measurements. With added wavelengths available, estimates of sample composition can be made more accurately. The large number of measurements possible in a short time allows more powerful data processing methods to be used. Any number or combination of readings can be used to measure any component. Handling of this large amount of data is no longer a problem. With the aid of a computer, a FTIR instrument can consider variables like saturation level of fat, chain length of fatty acids and lipolysis of fat so they do not interfere with accurate measurements.

In summary, application of instruments such as FTIR to dairy product analysis will let us to do a better job of measuring the components we now measure and to measure other components as they become important (7).

THE EFFECTS OF SATURATION AND CHAINLENGTH OF MILK FAT ON INFRARED ANALYSIS OF MILK

Because infrared analysis is an indirect method, it is not surprising that chemical changes in the component of interest can cause errors in measurement of concentration. Early researchers in infrared milk analysis realized this and studied the effects of compositional changes on instrument accuracy.

Milk protein concentration, as determined by infrared, is nearly proportional to the weight of milk proteins since the amino acid composition is nearly constant. Variations in amino acid composition, if they did occur, would have a similar effect on infrared and Kjeldahl (reference) methods (6). Milk fat composition, on the other hand, is affected by factors like feed, breed, stage of lactation, season, and mastitis. (29).

Feeding Studies

Early investigation of the effects of change in fatty acid composition on the infrared fat A signal (5.73 μm) was feeding studies. Dunkley et al. (8) fed one group of six cows (group C) a conventional hay ration and another group of 6 cows (group P) a formaldehyde protected sunflower-soybean supplement. Samples were collected bi-weekly over 12 wk for a total of 36 samples from each group. Fat, protein, and lactose were measured chemically by the Babcock, Udy dye-binding, and polarimetric methods. The fatty acid composition of each sample was determined by gas chromatography. Each sample was also analyzed with an IRMA infrared milk analyzer. Mean differences (chemical - infrared) for the group C cows were .09, .02, and .06% for fat, protein, and lactose. Mean differences for group P cows were .46, -.08, and .23%, the fat result being significantly different by a Students t test. The expected increase in linoleic acid (C18:2) in group P cows was accompanied by an increase in stearic (C18:0) and oleic (C18:1) acid concentrations for a total increase in

concentration of 18-carbon fatty acids of 38.5%. The significant difference between Babcock and infrared fat analysis in group P cows was attributed to the increase in molecular weight of the fatty acids.

Franke et al. (11) did a similar experiment using protected tallow as a supplement. Three groups of 7 cows (basal, medium, and high) were fed 0, 15, and 30% protected tallow supplements. Samples were collected every 3 wk for the first 15 wk post-partum for each group of cows. The mean differences for cows in the basal, medium and high groups were: for fat; .01, .29, and .30%; for protein; .08, -.02, and .03%; and for lactose; .05, .25, and .21%. The differences between the fat analysis related to the treatments were significantly different from zero and were correlated with the mean molecular weight of the fatty acids. For protein, there was little difference between the analysis related to the treatments and the differences did not correlate with mean molecular weight of the fatty acids. For lactose, feeding protected tallow caused a significant difference only for the high group and correlations with mean molecular weight were not significant.

In a later paper by Franke et al. (12) a 30% whole cottonseed diet caused a significant difference between infrared and Babcock results and the difference was correlated with mean molecular weight of fatty acids. The significance of this experiment is that samples were collected only after 14 and 15 wk of treatment to remove the effect of changes in fatty acid composition in early lactation (24). For each group, distributions were not as broad as the ranges of mean molecular weight in the protected tallow study. The effect on the infrared measurement of fat was the same.

In these studies, supplementation of the diet with protected fats caused a decrease in the infrared fat readings relative to the reference method. This can be explained because infrared radiation at the fat A wavelength measures the number of ester linkages present (ca. 3/molecule), effectively measuring the molecular concentration of the fat. Chemical methods for fat determination generally measure the weight

concentration. Changes in the mean molecular weight of fat caused by changes in fatty acid composition will cause variation between the chemical and infrared methods (32).

Relation of the Error at the Fat A Wavelength to Refractive Index

Analytical error at the fat A wavelength has been attributed to changes in refractive index of the milk caused by variation in mean molecular weight of the fat (19, 27, 29, 32). The refractive index of milk fat increases with an increasing number of carbon atoms in the fatty acid chain and with higher unsaturation. Kerkhof-Mogot et al. (20) postulated that based on a refractive index range of 1.4524 to 1.4566 for milk at 40°C, corresponding to refractometer numbers of 40 and 46, and milk with a fat content of 4%, deviations of .06% fat per refractometer number unit would be seen. The fatty acid composition of 34 samples of milk fat was determined by gas chromatography and the mean molecular weight of each sample was calculated. The relationship between mean molecular weight and the respective refractive index was determined. Based on the confidence limits of the slope, the effect was estimated to be $\leq$ -.05% fat per refractometer number unit. Only 65% of the variation in mean molecular mass was explained by differences in refractive index. It is not a matter of variation of refractive index but of variation in mean molecular mass that causes the difference between chemical and infrared (fat A) determination of fat content.

Sjaunja (29) analyzed milk samples from 89 cows (of three different breeds) weekly for 7 mo. Reference analysis for fat, protein, and lactose was by Gerber, Kjeldahl, and Luff-Schoorl methods. Infrared analysis of each sample was by a Milkoscan 104. The refractive index and saponification number were also determined for each sample. Standard deviations of difference for fat and protein were .110 and .052%. Fat content was underestimated at high refractive index and overestimated at low refractive index. The variation in refractive index accounted for 28% of the total variation in analysis difference and the variation in average molecular weight accounted

for only 7%. This contradicts the findings of Kerkhof-Mogot et al. (20). Variation in the average molecular weight of the milk fat probably would have accounted for more of the deviation between the methods had the infrared method (using fat A) been sensitive only to variations in fatty acid chainlength. Also, the average molecular weight accounting for less of the variation than the refractive index might be explained by poor accuracy when measuring the average molecular weight and correlation between light scattering effects owing to poor homogenization and the refractive index (29). The effects of breed, individual cow, stage of lactation, and somatic cell count all had significant effects on fat analysis by the infrared method with stage of lactation the most pronounced.

Effects of Fatty Acid Variation on the Fat B Wavelength

Nexo et al. (23) suggested measurement of fat at the fat B wavelength to overcome the error in infrared fat determination at the fat A wavelength. Infrared absorbance at fat B, caused by stretching vibrations of C-H bonds, is more closely related to mass than the fat A wavelength (20).

Mills et al. (22) evaluated the C-H stretch region for estimation of fat in aqueous fat emulsions. Ten emulsions were prepared from the following natural fats and oils: refined and bleached coconut, corn, palm, peanut, and soy bean oils; slightly, moderately, and highly saturated soy bean oils; cold pressed olive oil, and butter oil. Each emulsion was analyzed for fat content by the Mojonnier method and for fatty acid composition by gas-liquid chromatography. The iodine and saponification numbers of each sample were calculated from the chromatography data. Each sample was also analyzed with a Multispec infrared milk analyzer at both the fat A and fat B wavelengths. Regressions were done to relate the absorbance at fat A, fat B, and fat A + fat B to the concentration of fat in each sample. A Students t test for homogeneity of regression compared slopes of individual fat samples to see if a single

calibration could be used for all types of fat.

When using the fat A wavelength, 8 of the 10 slopes were estimates of a common slope with butter and coconut oils being significantly different. The 8 fat samples with a common slope all had a similar molecular weight (ca. 200) and when used in a linear regression to predict fat concentration, gave a standard deviation of difference of .060%.

When the slopes were compared using the fat B data, homogeneity was seen for butter, palm, and coconut oils. Although these samples differed in iodine number (saturation), the expected variation owing to saturation was offset by converse differences in mean molecular weight. In soybean oils, where the mean molecular weights were similar but degree of hydrogenation differed, there was a decrease in signal as a function unsaturation. This decrease can be explained because the C-H bonds next to carbon double bonds exhibit a depressed absorbance (6). When the fat B data was used in a linear regression to predict fat concentration, the standard deviation of difference was .20%. This value decreased to .076% by including the iodine number in the regression. Use of the fat A + fat B data and the iodine number in the regression decreased the standard deviation of difference to .027%.

Sjaunja (28) compared the accuracy of fat A, fat B, and fat A + fat B in the determination of fat content in milk samples collected from 50 cows (of three different breeds) once a week for 7 wk. Accuracies, expressed as standard deviations of difference between chemical and infrared methods were .084, .083, and .075% for fat A, fat B, and fat A + fat B wavelengths. Although fat determination at fat B appeared to be independent of refractive index, accuracy did not improve. When 30 analyses were done on a milk sample of average fat content, the residual standard deviation was .036% when using the fat B wavelength compared to .007% when using the fat A wavelength suggesting a lower repeatability at the fat B wavelength. Another consideration that must be taken into account when using the fat B wavelength for fat

determination is that since protein and lactose absorb at this wavelength, they must be simultaneously determined and corrected for (20, 28), increasing analysis time.

Eastridge et al. (9) studied the effects of feeding a high fat diet on infrared analysis of milk using the fat A and fat A + B methods. Diets supplemented with calcium soap, tallow, and yellow grease were compared to control groups and no significant difference in fat concentration as determined by Babcock, fat A, or fat A + B methods was seen. Fatty acid analysis showed that stage of lactation has a greater effect on mean molecular weight of fatty acids than does feeding of fat confirming the findings of Sjaunja (29).

THE EFFECTS OF LIPOLYSIS ON INFRARED ANALYSIS OF MILK

Milk collected for infrared analysis is subject to conditions during sampling and transport that could lead to varying degrees of lipolysis of the milk fat (33).

Sjaunja (30) found that as the free fatty acid level of milk increases, the fat A signal decreases by .025%/mmol of free fatty acid. Because the absorption at fat A is characteristic of ester carbonyl groups, a decrease in the number of ester groups caused by enzymatic hydrolysis is accompanied by a decrease in absorbance (33). The fat B signal, the protein signal, and the lactose signal all increased steadily at a rate of .033, .010, and .010%/mmol of free fatty acid. Suggested reasons for the increase in signal at fat B are hydrogen bonding between water and released fatty acids, dimerization of free fatty acids, and absorption by the CH_2 groups on the glycerol part of the molecule all of which absorb at this wavelength (6). The increase in the protein signal is attributed to an absorption of the carboxylate anion of soluble free fatty acids at 1563 cm^{-1} (20, 30). Sjaunja and Andersson confirmed these findings in a later paper (31). Robertson et al. (26) and Grappin and Jeunet (18) published similar findings.

Kerkhof-Mogot et al. (20), Kyla-Siurola and Antila (21), and Van de Voort et al. (33) published similar findings with the exception that they found the fat B signal is independent of lipolysis.

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PART 3. ALTERNATIVE WAVELENGTHS FOR THE DETERMINATION OF FAT, PROTEIN, AND LACTOSE IN MILK

ABSTRACT

Absorption bands from 1283 to 1100 cm^{-1} correlated with fat, protein, and lactose concentrations and show a low response to fat variation and lipolysis A Fourier transform infrared spectrometer equipped with an attenuated total internal reflectance cell was calibrated using these absorption bands, partial least squares statistics, and a set of standard milk samples from herds in Minnesota. When the fat, protein, and lactose concentrations in the set of samples used to calibrate the instrument were predicted, the standard deviations of difference were .22, .06, and .02%. When the fat, protein, and lactose concentrations in a separate set of standard samples from herds in California were predicted using the generated calibration, the standard deviations of difference were 1.23, .10, and .07%. Substitution of a 15 μm pathlength transmission cell for the attenuated total internal reflectance cell decreased the standard deviations of difference of fat, protein, and lactose prediction to .07, .11, and .06% in the calibration samples and .09, .10, and .16% in the separate set of samples.

INTRODUCTION

Goulden (10) first used infrared radiation to measure fat, protein, and lactose in milk. The measurement of fat is based on absorbance at 1724 cm^{-1} (fat A wavelength) by ester carbonyl groups of fat molecules. Protein measurement is based on absorbance at 1538 cm^{-1} by peptide bonds of protein molecules, and lactose measurement is based on absorbance at 1042 cm^{-1} by hydroxyl groups of lactose molecules.

Absorbance at the fat A wavelength measures the molecular concentration of the fat. Most chemical methods for fat determination measure the weight concentration (25). Changes in the mean molecular weight of fat caused by feed, breed, stage of lactation, season, and mastitis will cause variation between the chemical and infrared methods (22). This was shown in feeding studies where an increase in mean molecular weight of fatty acids when cows were fed a protected fat supplement caused the infrared method to underestimate the chemically determined fat concentration (5, 6, 7).

Nexo et al. (18) suggested measurement of fat at 2865 cm^{-1} (fat B wavelength) as an alternative to the fat A wavelength. Absorbance at the fat B wavelength, caused by stretching vibrations of C-H bonds, is more related to mass than the fat A wavelength (14). Absorption at the fat B wavelength is affected by variation in saturation of fatty acids caused by a decrease in absorbance by C-H groups next to carbon double bonds (17).

Sjaunja (21) compared measurement of fat in milk using fat A, fat B, and fat A + B. Milk was collected from 50 cows (three different breeds) once a week for 7 wk. Standard deviations of difference between chemical and infrared methods were .084, .083, and .075%. When 30 analyses were done on a milk sample of average fat content, the residual standard deviation was .036% when using fat B compared to .007% when using fat A suggesting a lower repeatability at fat B. When using fat B, protein and lactose must be measured and corrected for because both components

absorb at this wavelength (21). This results in increased analysis time.

Milk collected for infrared analysis is subject to conditions during sampling and transport that lead to varying degrees of lipolysis (26).

Sjaunja (23) found that as the free fatty acid level of milk increases, the fat A signal decreases by .025%/mmol of free fatty acid. Because absorption at fat A is characteristic of ester carbonyl groups, a decrease in the number of ester groups caused by enzymatic hydrolysis is accompanied by a decrease in absorbance (26). The fat A, protein, and lactose signals all increased at a rate of .033, .010, and .010%/mmol of free fatty acid. Suggested reasons for the increase in signal at fat B are hydrogen bonding between water and released fatty acids, dimerization of free fatty acids, and absorption by the CH_2 groups on the glycerol part of the molecule (3). The increase in the protein signal is attributed to absorption by the carboxylate anion of soluble free fatty acids at 1563 cm^{-1} (14, 23). Sjaunja and Andersson confirmed these findings in a later paper (24). Robertson et al. (19) and Grappin and Jeunet (11) published similar findings. Kerkhof-Mogot et al. (14), Kyla-Siurola and Antila (15), and Van de Voort et al. (26) published similar findings except they found that the fat B signal is independent of lipolysis.

The objective of this study was to find a set of absorption bands that respond to changes in fat, protein, and lactose concentration and exhibit a low response to variation in saturation and molecular weight of fatty acids and lipolysis. We then evaluated the ability of these absorption bands to measure the fat, protein, and lactose in milk samples.

MATERIALS AND METHODS

Preparation of Samples Used to Find Absorption Bands That Respond to Changes in Fat and Protein Concentration

Fresh milk was collected from five herds and combined for a total volume of 10 L. Seventeen milliliters of bronopol preservative was added to the milk and the milk was kept overnight at 4°C to allow creaming. The following day the milk was separated into four fractions. The skim layer was siphoned away from the cream layer. Parts of the naturally skimmed milk and cream were retained to make up two of the four fractions. Another part of the naturally skimmed milk was mechanically skimmed with a small electric separator to reduce the fat content to < .1%. This skim milk was then ultra filtered to a 2× concentration with a Filtron GP 163 ultrafiltration instrument using a 10,000 molecular weight cut-off membrane. The retentate and ultrafiltrate were the remaining two fractions. A sample of each of the four fractions was collected for fat and protein analysis. Table 1 shows the results of the fat and protein analysis.

TABLE 1. Results of fat and protein analysis on four milk fractions.

Fraction	Fat by the Gerber method (g/L)	Protein by the Amido black method (g/kg)
Naturally skimmed milk	5.0	30.7
Cream	168.0	10.9
2× Retentate	1.0	60.6
Ultrafiltrate	0	.3

Twenty five 100 ml samples were prepared from the four fractions. The samples were designed for a fat range of 20 to 55 g/L and a protein range of 22 to 42 g/kg. Reference methods for fat and protein were Gerber and Amido black. Table 2 lists the average fat

and protein concentration of each sample.

TABLE 2. The average fat and protein concentration of each sample prepared from the four fractions.

Sample	Fat Concentration (g/L)	Protein concentration (g/kg)
11	21.15	23.50
12	19.95	28.15
13	20.60	33.30
14	20.50	38.60
15	20.75	44.00
21	29.95	24.55
22	30.95	28.90
23	30.40	34.00
24	30.55	39.45
25	30.50	44.70
31	39.25	25.30
32	39.75	29.65
33	39.70	34.90
34	39.80	40.20
35	39.50	45.30
41	48.00	25.80
42	48.00	30.50
43	48.15	35.65
44	48.25	41.05
45	49.00	45.90
51	58.00	26.30
52	57.75	31.45
53	57.55	36.35
54	58.00	42.00
55	57.55	46.95

Table 2 shows that the concentration of fat and protein in each sample allowed comparison of several samples where one component remained as constant as possible and the other varied.

Each sample was scanned 32 times at 4 cm^{-1} resolution from 3000 to 800 cm^{-1} using a Nicolet model 740 Fourier transform infrared (FTIR) spectrometer (Nicolet Instrument Corp., Madison, WI) equipped with a deuterated triglycine sulfate detector.

The sampling apparatus was manufactured by Delta Instruments, Holland, and consisted of a two-stage homogenizer and a sampling cell with calcium flouride windows and a 17 μm pathlength. The samples were preheated to 40°C and temperature control points (homogenizer and sample cell) were also regulated at 40°C. The 32 scans were averaged to produce the spectrum of each sample. The spectrum of distilled water was the background.

Preparation of Samples Used to Find Absorption Bands That Respond to Changes in Lactose Concentration.

Five samples were prepared with lactose concentrations varying from 1 to 5%. The samples were prepared by weighing the desired amount of α-lactose monohydrate (Mallinckrodt Chemical Works, St. Louis, MO.) and dissolving it in 15 ml of distilled water. The samples were held overnight at room temperature to allow equilibration between the α and β forms of the sugar. Each sample was scanned 64 times at 4 cm^{-1} resolution from 3000 to 800 cm^{-1} using a Digilab FTS-7 FTIR spectrometer (Bio-Rad, Digilab Division, Cambridge, MA) equipped with a deuterated triglycine sulfate detector. The 64 scans were averaged to produce the final spectrum of each sample. The sampling accessory was an attenuated total internal reflectance (ATR) cell (Buck Scientific Inc., East Norwalk, CT) with a zinc selenide crystal. No temperature control device was used but the temperature of the optical bench was 30°C during the experiment.

A blank cell was the background and the spectrum of water was subtracted from each sample spectrum. Subtraction was done by first multiplying the spectrum of water by a subtraction factor corresponding to the percent water in the milk sample and then subtracting this modified water spectrum from the spectrum of the sample.

Preparation of Samples Used to Find Absorption Bands with a Low Response to Variation in Saturation and Chainlength

Fatty acids (99% pure by capillary gas chromatography) were purchased from Sigma Chemical Co., St. Louis, MO. Reagent alcohol (HPLC grade) was purchased from Fisher Scientific, Fair Lawn, NJ.

Saturation effects were studied using solutions of n-octadecanoate (C18:0), cis-Δ^9-octadecenoate (C18:1), cis, cis-Δ^9, Δ^{12}-octadecadienoate (C18:2), and cis, cis, cis-Δ^9, Δ^{12}, Δ^{15}-octadecatrienoate (C18:3), prepared by dissolving .2000 g of fatty acid in 5 ml of reagent alcohol for a final concentration of 4.82%. These samples were selected because of their identical number of carbon atoms and varying degree of saturation. Solutions of n-decanoate (C10:0), n-dodecanoate (C12:0), n-tetradecanoate (C14:0), n-hexadecanoate (C16:0), and n-octadecanoate (C18:0) were prepared and analyzed in the same manner to study the effects of chainlength variation. These samples were selected because of their varying number of carbon atoms and identical degrees of saturation. Each sample was analyzed with the Digilab FTIR using parameters identical to those used for the lactose solutions. The background spectrum was a blank sample cell. The spectrum of reagent alcohol was subtracted from each sample spectrum by first multiplying the spectrum of reagent alcohol by .9518 (the fraction of reagent alcohol in each solution) and then subtracting this modified spectrum from the spectrum of each sample.

The fatty acids used in this experiment are commonly found in the triglycerides of milk.

Preparation of Samples Used to Find Absorption Bands with a Low Response to Lipolysis

Pasteurized, homogenized, 2% milk from the dairy processing plant at Utah State University was used. Lipase (Type VII-S from Candida cylindracea) was purchased

from Sigma Chemical Co., St. Louis, MO. The Digilab FTIR spectrometer, equipped with an ATR sample cell, was used to record the infrared spectra.

Lipase enzyme (.0011 g) was dissolved in 50 ml of distilled water. This solution was placed in the sample cell and 64 scans at 4 cm^{-1} resolution were averaged to produce the background spectrum. The sample cell was then cleaned, dried, and filled with 5 ml of a solution prepared by dissolving .0011 g of lipase enzyme in 50 ml of 2% pasteurized homogenized milk. Sixty four scans at 4 cm^{-1} resolution were collected and averaged every minute for 1 h as lipolysis occurred. The temperature of the optical bench was 30°C during the experiment.

Calibration Samples

Milk standard samples were purchased from Dairy Quality Control Institute (DQCI), St. Paul, MN and Michelson Laboratories, Commerce, CA. The samples were herd milks collected in Minnesota and California. These laboratories collect samples from their suppliers every week. Fat, protein, and lactose are determined by Babcock, Kjeldahl, and high performance liquid chromatography (note: Michelson Laboratories, Inc. measures lactose by infrared analysis which is an approved reference method for lactose in California). The samples are then shipped to milk analysis laboratories where they are used to calibrate infrared instruments. Table 3 shows the fat, protein, and lactose concentrations in each sample as measured by the reference methods. The variation of component concentrations in a set of samples reflects the expected variation due to feed, breed, and stage of lactation.

Each sample was incubated in a water bath at 40°C for 5 min and homogenized with a Milkoscan homogenizer (A/S N. Foss Electric, Denmark) before analysis. The samples were then scanned 64 times at 4 cm^{-1} resolution using the Digilab FTS-7 FTIR spectrometer equipped with a deuterated triglycine sulfate detector.

TABLE 3. Fat, protein, and lactose content in standard samples as determined by reference methods (D means samples from DQCI and M samples from Michelson Labs).

Sample	Fat (%)	Protein (%)	Lactose (%)
D1	2.49	3.28	4.84
D2	3.22	3.32	4.77
D3	3.46	3.14	5.86
D4	3.70	3.19	4.82
D5	3.84	3.16	4.82
D6	4.64	3.73	4.76
D7	3.18	3.15	4.85
D8	3.68	3.10	4.81
D9	3.81	3.32	4.75
D10	4.11	3.26	4.84
D11	4.75	3.30	4.85
D12	5.86	3.87	4.71
M1	5.35	3.28	4.89
M2	2.42	3.24	4.98
M3	3.73	3.15	4.92
M4	3.27	3.32	5.05
M5	3.72	3.31	5.10
M6	3.96	3.26	4.93
M7	3.98	3.41	4.94
M8	3.93	3.40	4.98
M9	4.50	3.59	5.09
M10	5.14	4.26	4.88
M11	4.90	4.16	4.98

The 64 scans were averaged to produce the final spectrum of each sample. No temperature control device was used but the temperature of the optical bench was 30°C during the experiment. Calibration was by a PLS software package provided by Digilab.

RESULTS AND DISCUSSION

Spectral data was transformed to ASCII format using software provided by Digilab. This data was sent to a Vax computer where statistical analysis software (SAS Institute, Inc., Cary, NC) was used to compute the statistics.

Effects of Fat Concentration on the Infrared Spectrum of Milk

Figure 1 shows the spectra of five milk samples (samples 11, 21, 31, 41, and 51 from Table 2) with varying fat concentration and minimal variation (26.30 to 23.50 g/kg for protein) in protein and lactose concentration. Although the percent lactose was not measured, no variation was seen at 1042 cm^{-1} which is used for lactose measurement. Figure 2 shows a plot of the correlation coefficient of absorbance and fat concentration versus wavenumber for these samples. Regions of the spectrum with correlation coefficients greater than .90 were 2994 to 2825 cm^{-1}, 1799 to 1724 cm^{-1}, 1471 to 1451 cm^{-1}, 1284 to 1103 cm^{-1}, and 860 to 859 cm^{-1}.

Effects of Protein Concentration on the Infrared Spectrum of Milk

Figure 3 shows the spectra of five milk samples (samples 31, 32, 33, 34, and 35 from Table 2) with varying protein concentration and minimal variation (39.80 to 39.25 g/l for fat) in fat and lactose concentrations. The correlation coefficient for absorbance and protein concentration was calculated at each wavenumber and a graph of correlation coefficient versus wavenumber is shown in Figure 4. Coefficients greater than .90 were seen at 3030 to 2500 cm^{-1}, 1698 to 1656 cm^{-1}, 1621 to 1038 cm^{-1}, 1017 to 990 cm^{-1}, and 850 to 841 cm^{-1}.

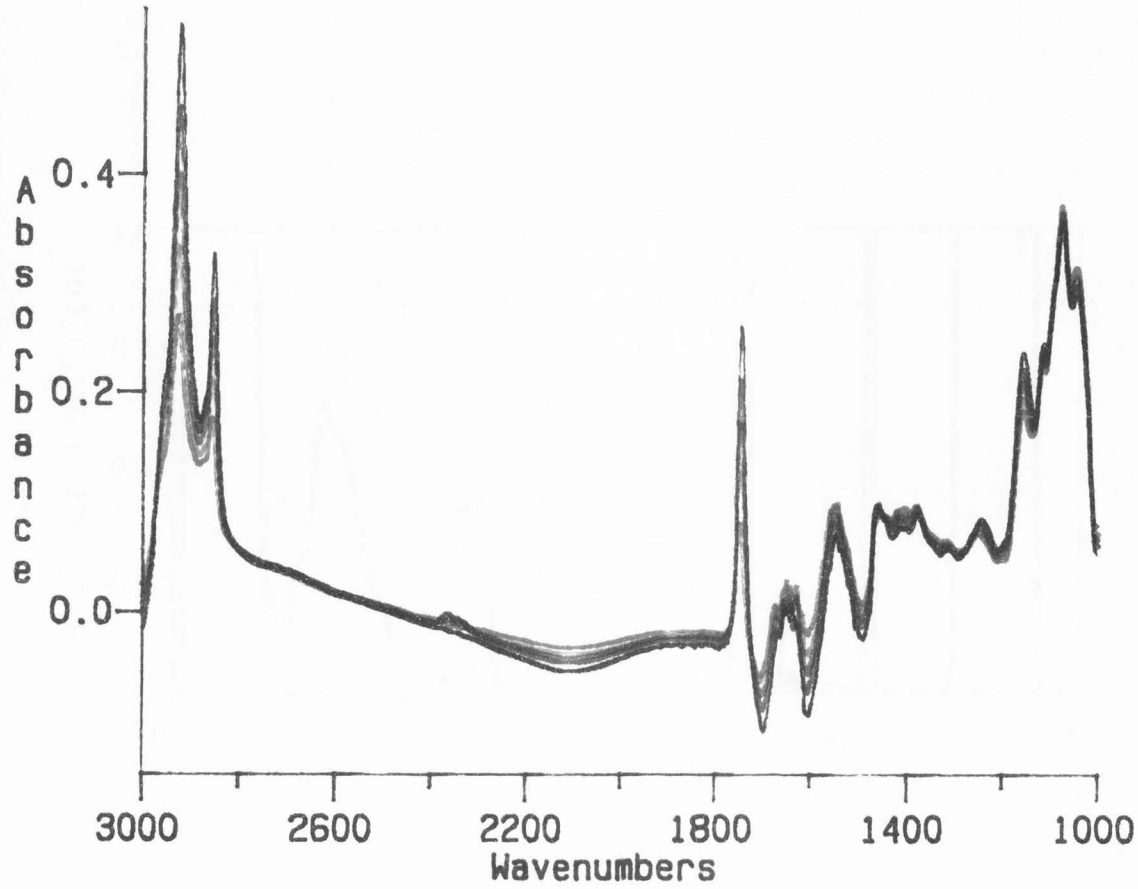

Figure 1. The effect of varying fat concentration (protein and lactose held constant) on the infrared spectrum of milk (spectra are keyed as follows: red = 21.15, yellow = 29.95, green = 39.25, blue = 48.00, and black = 58.00 g fat /L).

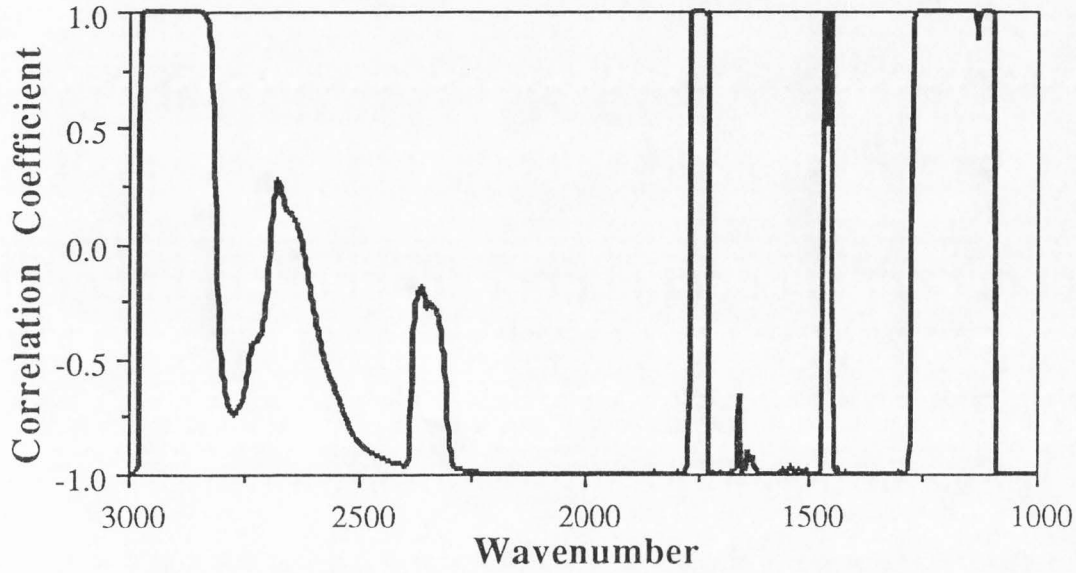

Figure 2. The correlation of absorbance and fat concentration at each wavenumber in the infrared spectrum of milk.

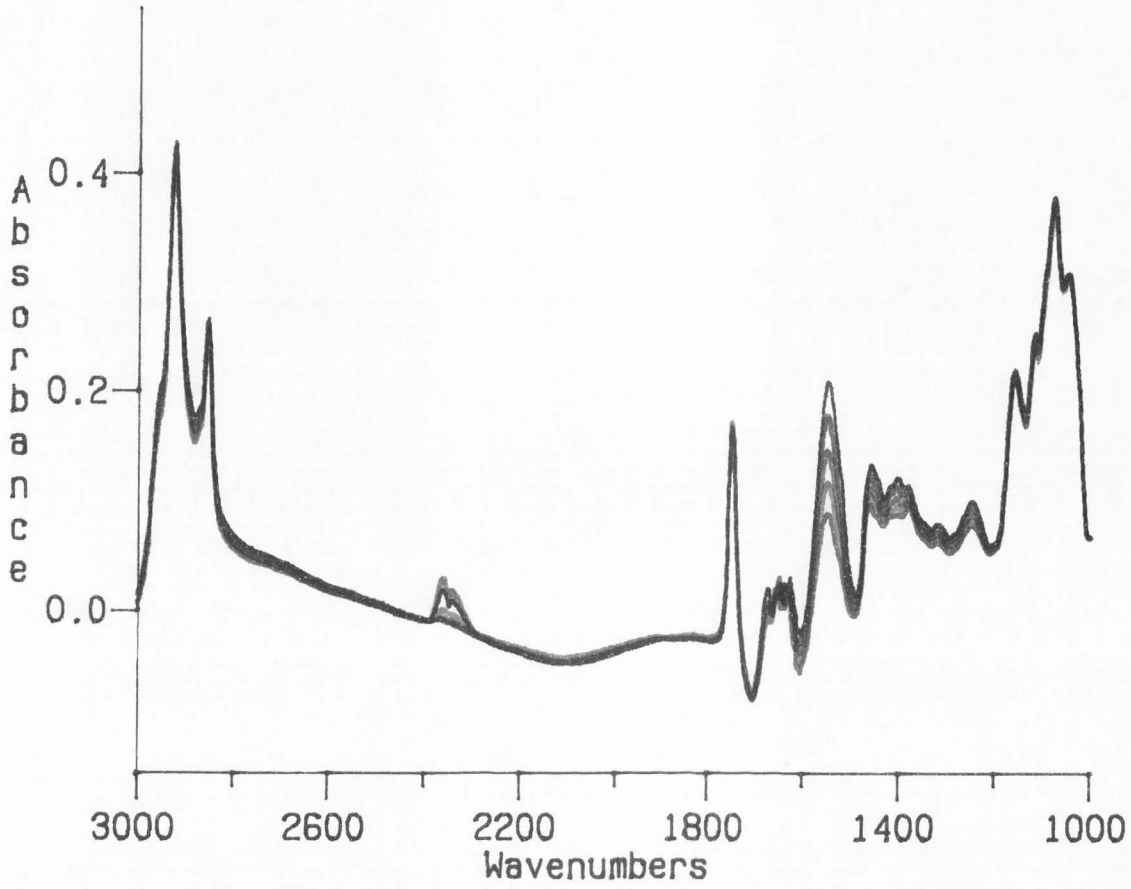

Figure 3. The effect of varying protein concentration (fat and lactose held constant) on the infrared spectrum of milk (spectra are keyed as follows: red = 25.30, yellow = 29.65, green = 34.90, blue = 40.20, and black = 45.30 g protein/kg).

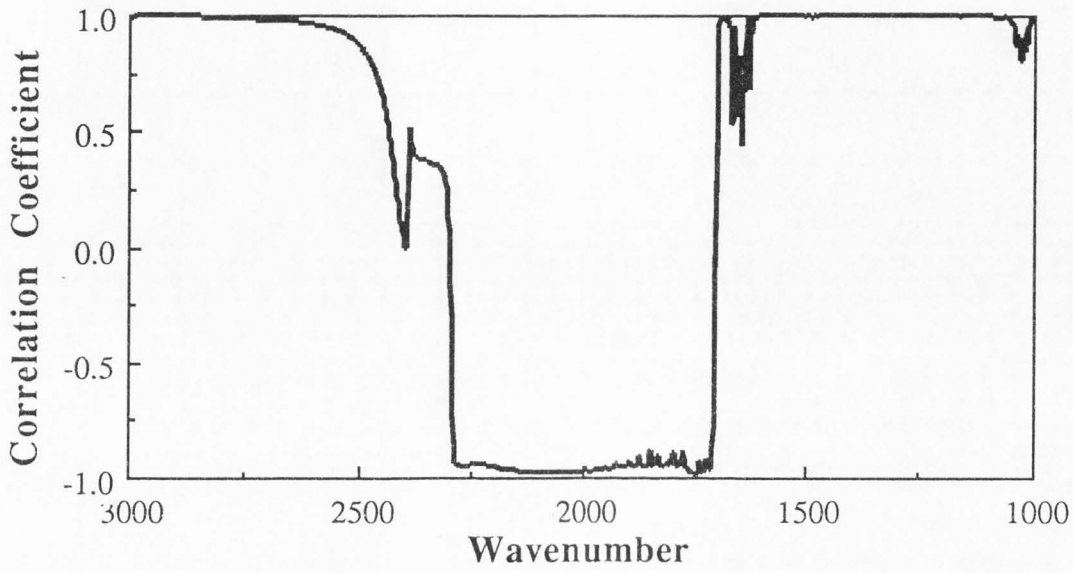

Figure 4. The correlation of absorbance and protein concentration at each wavenumber in the infrared spectrum of milk.

Effects of Lactose Concentration on the Infrared Spectrum of Milk

Figure 5 shows the spectra of five lactose solutions with concentration varying from 1 to 5%. Figure 6 shows a graph of correlation coefficient for absorbance and lactose concentration versus wavenumber. Absorption bands with correlation coefficients greater than .90 were 3145 to 2551 cm^{-1}, 2262 to 1992 cm^{-1}, and 1776 to 833 cm^{-1}.

Effects of Saturation and Chainlength of Fatty Acids

Figure 7 shows the spectra of fatty acids used to study saturation effects. The standard deviation of absorbance was calculated at each wavenumber and plotted versus wavenumber in Figure 8. High variation was seen in the fat B and lactose regions of the spectrum. As the number of double bonds increased the fat B signal decreased, which agrees with the findings of Mills (17).

Figure 9 shows the spectra of fatty acids used to study chainlength effects. The standard deviation of absorbance was calculated at each wavenumber and plotted versus wavenumber in Figure 10. High variation was seen in the fat A and in the lactose regions of the spectrum. As chainlength of the fatty acids increased, the fat A signal decreased which agrees with the literature (5, 6, 7, 22). In this experiment, absorption in the fat A region of the spectrum was not caused by ester carbonyl groups but by undissociated carboxyl groups of fatty acids that absorb at 1724 cm^{-1} (20). The same effect would be expected had triglycerides been used. Variation at the lactose wavenumber (1042 cm^{-1}) caused by both saturation and chainlength effects may be a characteristic of fatty acids and not triglycerides. Long chain fatty acid absorption bands that disappear upon esterification have been found in this region of the spectrum (1).

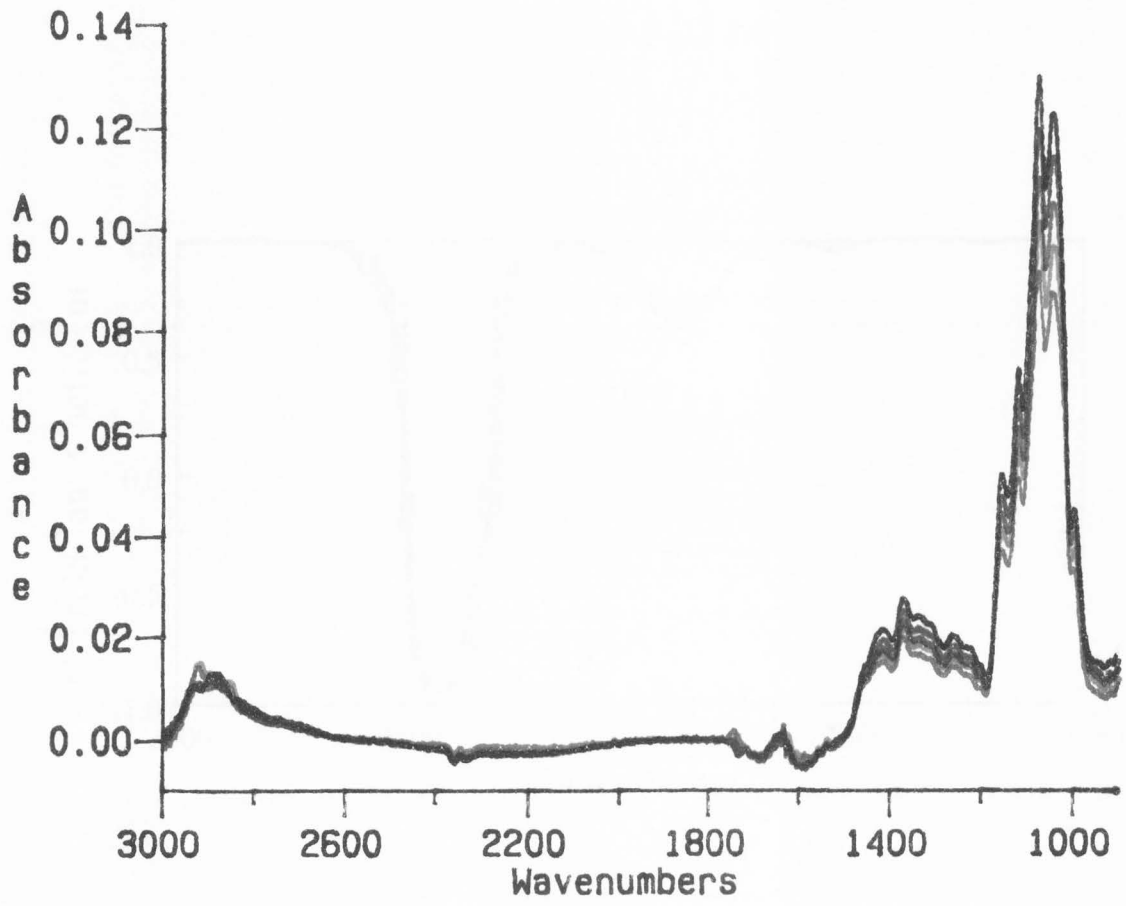

Figure 5. The effects of varying lactose concentration (in solutions of lactose) on the infrared spectrum of lactose (the spectra are keyed as follows: red = 1.00, yellow = 2.00, green = 3.00, blue = 4.00, and black = 5.00% wt/wt lactose).

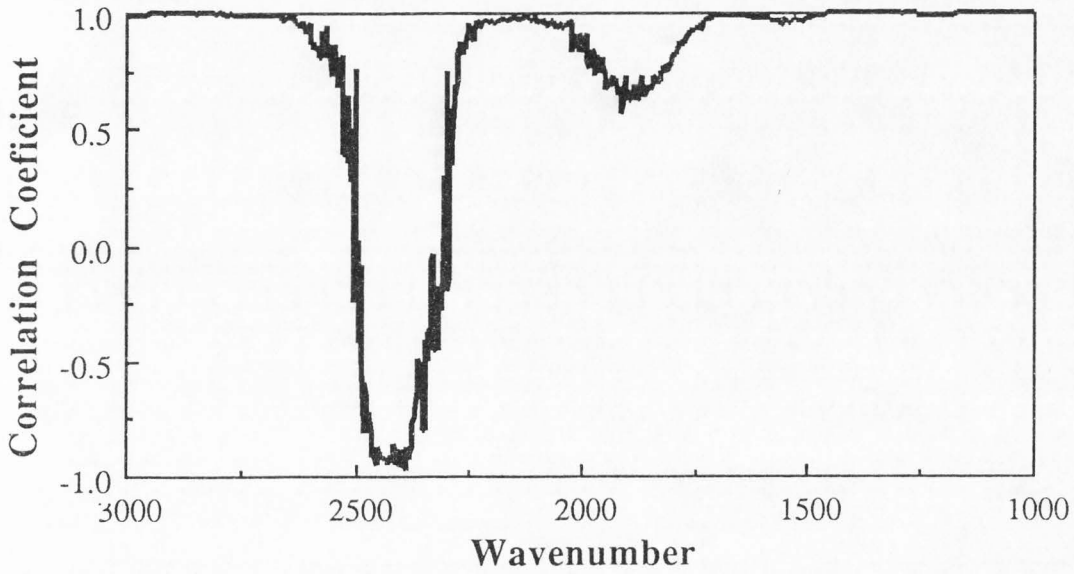

Figure 6. The correlation of absorbance and lactose concentration at each wavenumber in the infrared spectrum of lactose.

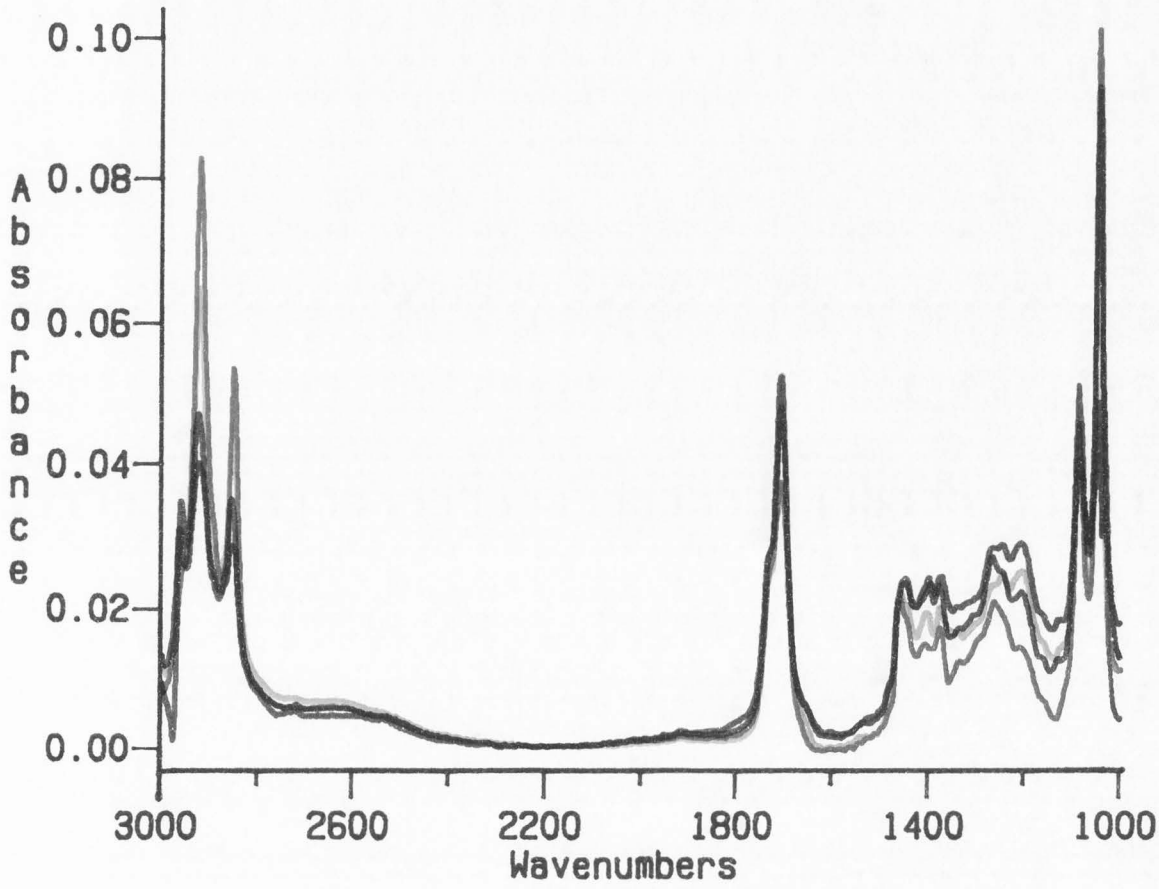

Figure 7. The effects of saturation on the infrared spectrum of the series of 18 carbon fatty acids (the spectra are keyed as follows: red = C18:0, yellow = C18:1, green = C18:2, and blue = C18:3).

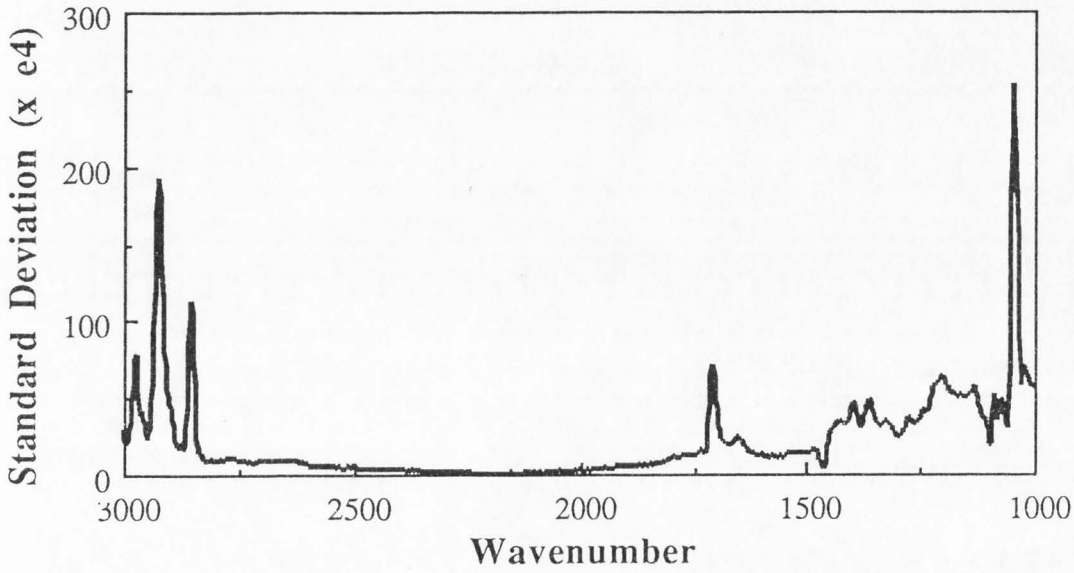

Figure 8. A plot of standard deviation of absorbances versus wavenumber for the 18 carbon fatty acids C18:0, C18:1, C18:2, and C18:3.

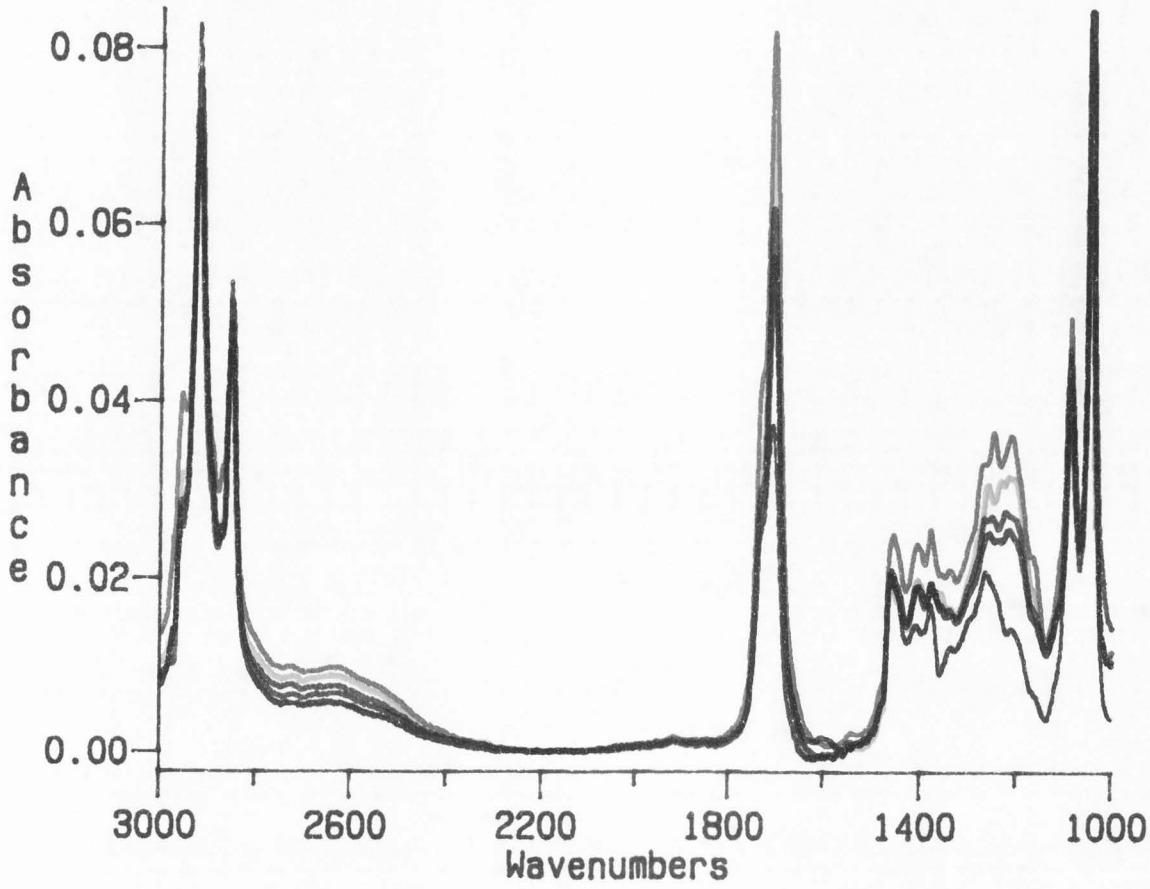

Figure 9. The effects of chainlength on the infrared spectrum of a series of fatty acids (the spectra are keyed as follows: red = C10:0, yellow = C12:0, green = C14:0, blue = C16:0, and black = C18:0).

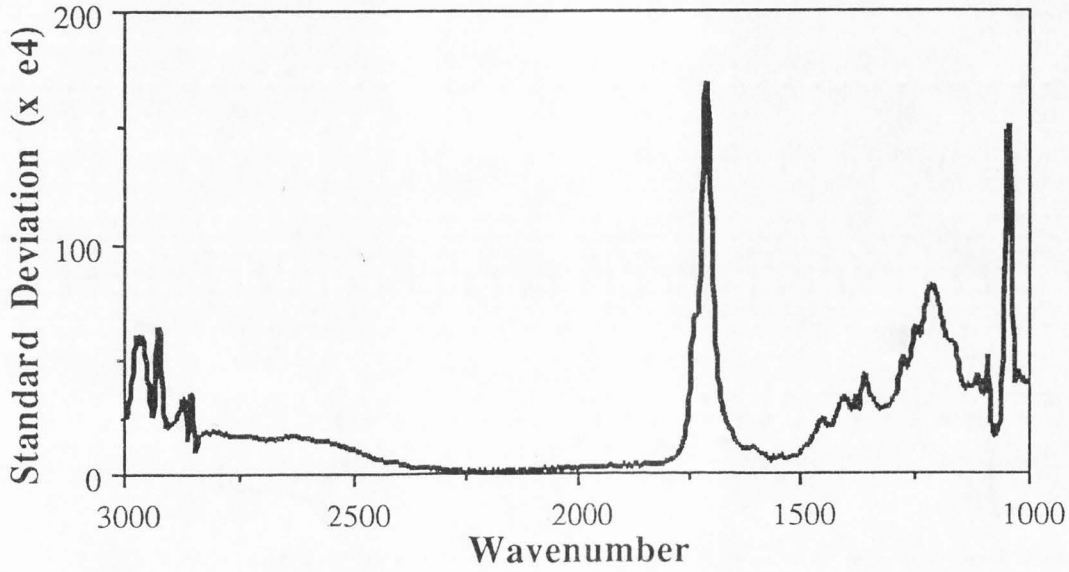

Figure 10. A plot of standard deviation of absorbances versus wavenumber for a series of fatty acids (C10:0, C12:0, C14:0, C16:0, and C18:0).

Franke et al. (6, 7) found that the effect of rations, causing variation in chainlength and saturation, on lactose readings was not significant, and fat concentration had more of an effect than fat composition which agrees with the findings of Grappin and Collin (12).

Absorption bands from 2801 to 1066 cm^{-1} and 1034 to 800 cm^{-1} showed a low response to saturation variation. Absorption bands from 3333 to 1795 cm^{-1}, 1580 to 1088 cm^{-1}, and 1034 to 800 cm^{-1} showed a low response to molecular weight variation.

Effects of Lipolysis on the Infrared Spectrum of Milk

Figure 11 shows five spectra collected at intervals as lipolysis occurred. The standard deviation of absorbance was calculated at each wavenumber and plotted versus wavenumber in Figure 12. Absorption bands with a low response to lipolysis were 2725 to 1795 cm^{-1} and 1391 to 942 cm^{-1}.

Figure 13 shows the fat B region of the five spectra in Figure 11. As the free fatty acid concentration increased, the signal also increased confirming the findings of Sjaunja (23), Robertson et al. (19), and Grappin and Jeunet (11).

Figure 14 shows the fat A region of the five spectra in Figure 11. The signal decreased as the free fatty acid concentration increased. The fat A signal is caused by absorption of ester carbonyl groups. As lipolysis occurs, a molecule of glycerol and three fatty acid molecules are formed. The lower molecular weight fatty acids are soluble in the aqueous phase of the milk and at the normal pH of milk are predominantly in the ionized COO^- form (23). When ionization happens, the absorption band in the fat A region disappears and a new band appears at 1563 cm^{-1} characteristic of COO^- groups (20). Figure 15 shows this band (at 1563 cm^{-1}) that appears as a shoulder to the protein absorption band at 1538 cm^{-1}.

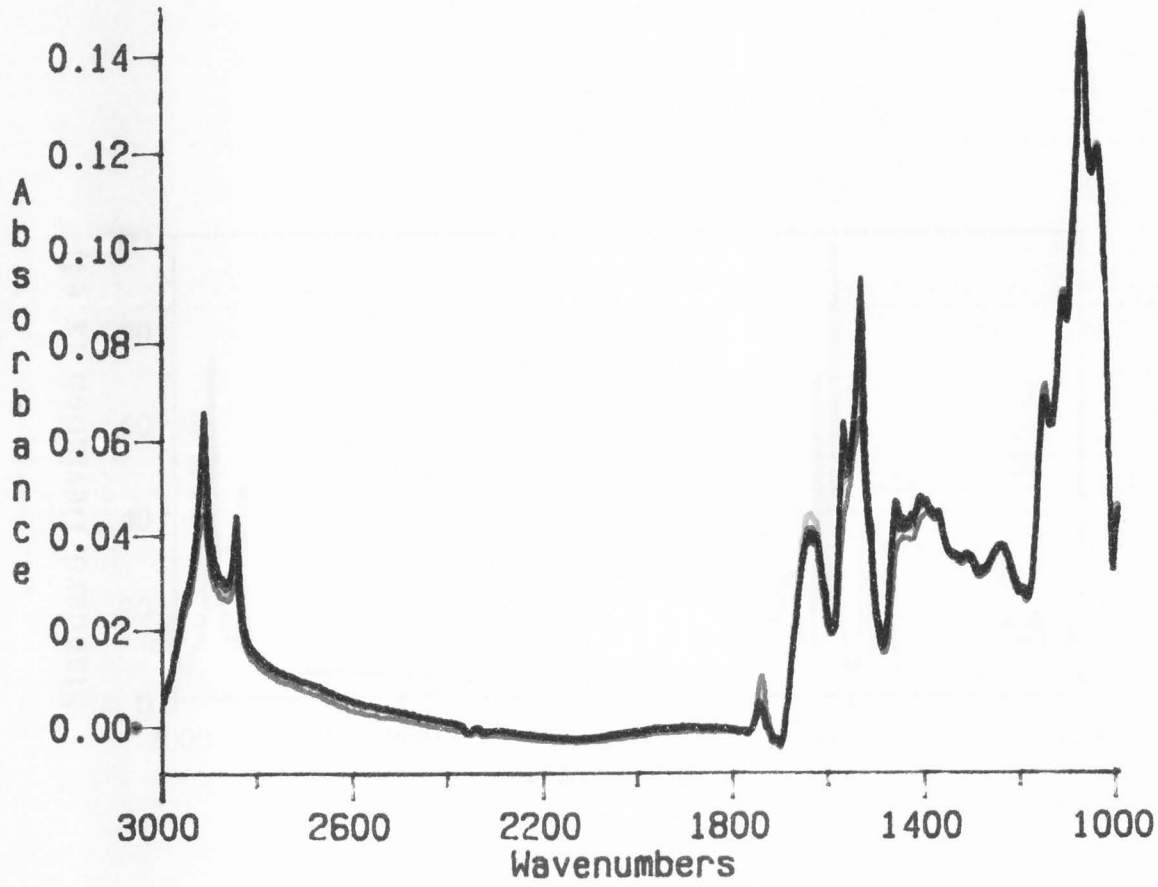

Figure 11. The effects of lipolysis on the infrared spectrum of milk (the spectra are keyed as follows: red = 13, yellow = 25, green = 37, blue = 49, and black = 60 min after enzyme addition).

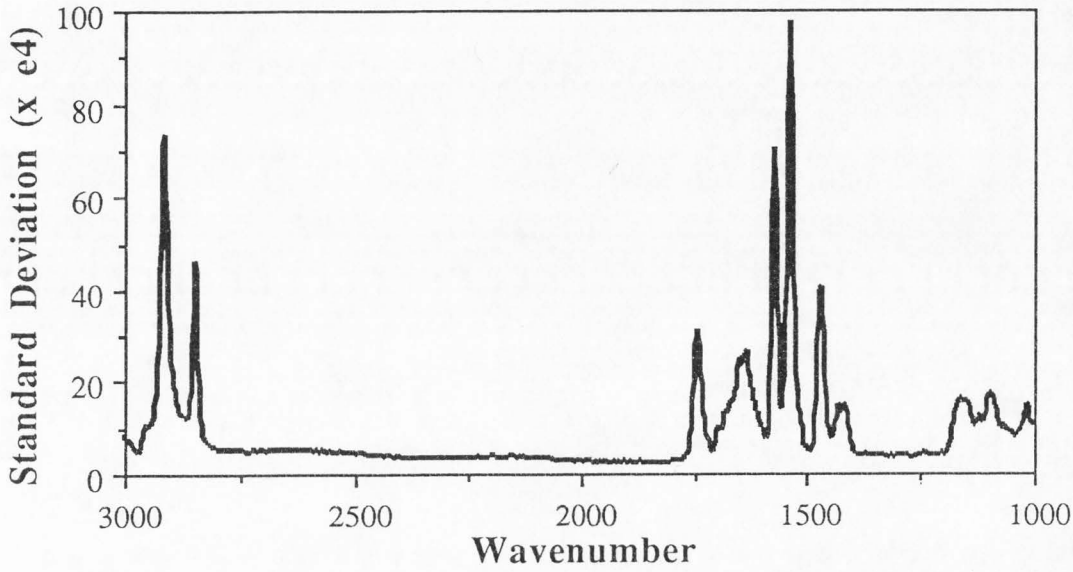

Figure 12. A plot of standard deviation of absorbances (caused by lipolysis) versus wavenumber in a milk sample.

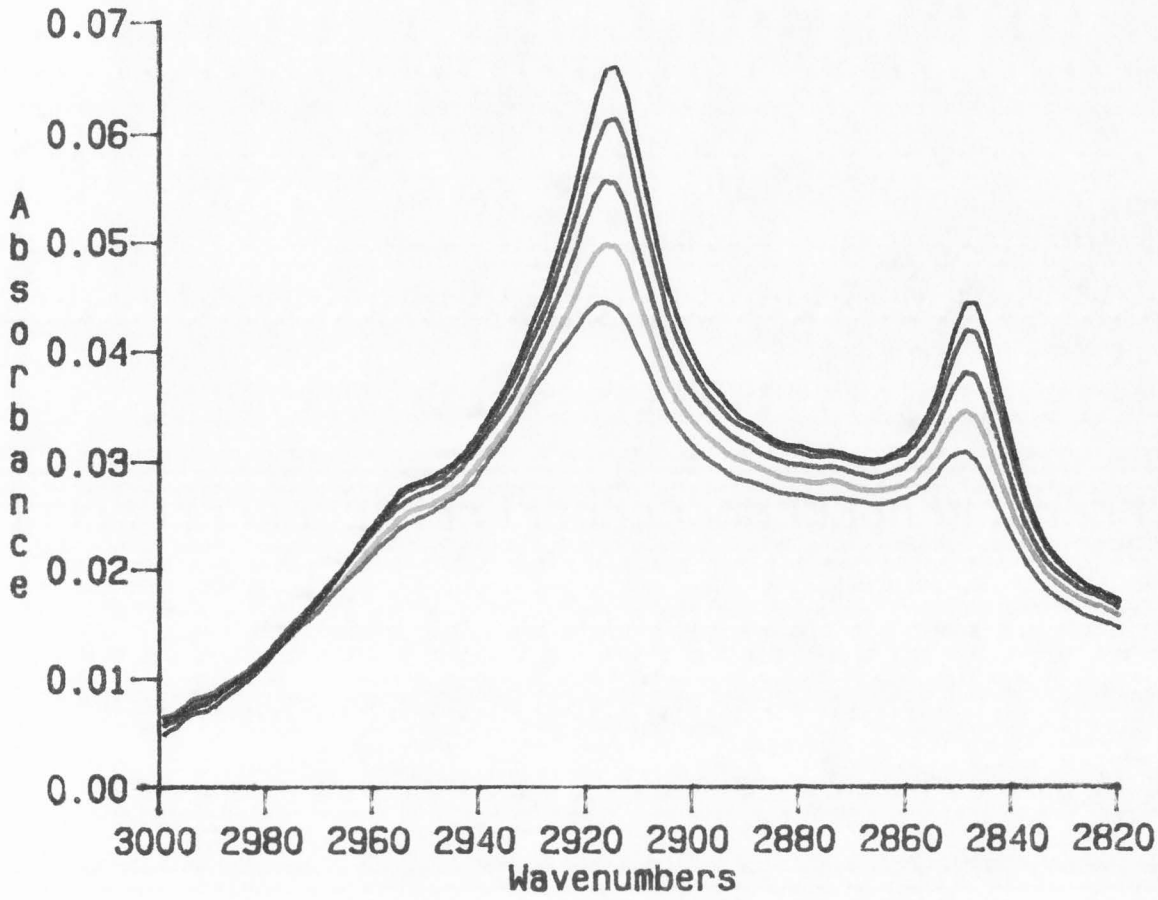

Figure 13. The effects of lipolysis on the "fat B" region of the infrared spectrum of milk (the spectra are keyed as follows: red = 13, yellow = 25, green = 37, blue = 49, and black = 60 min after enzyme addition).

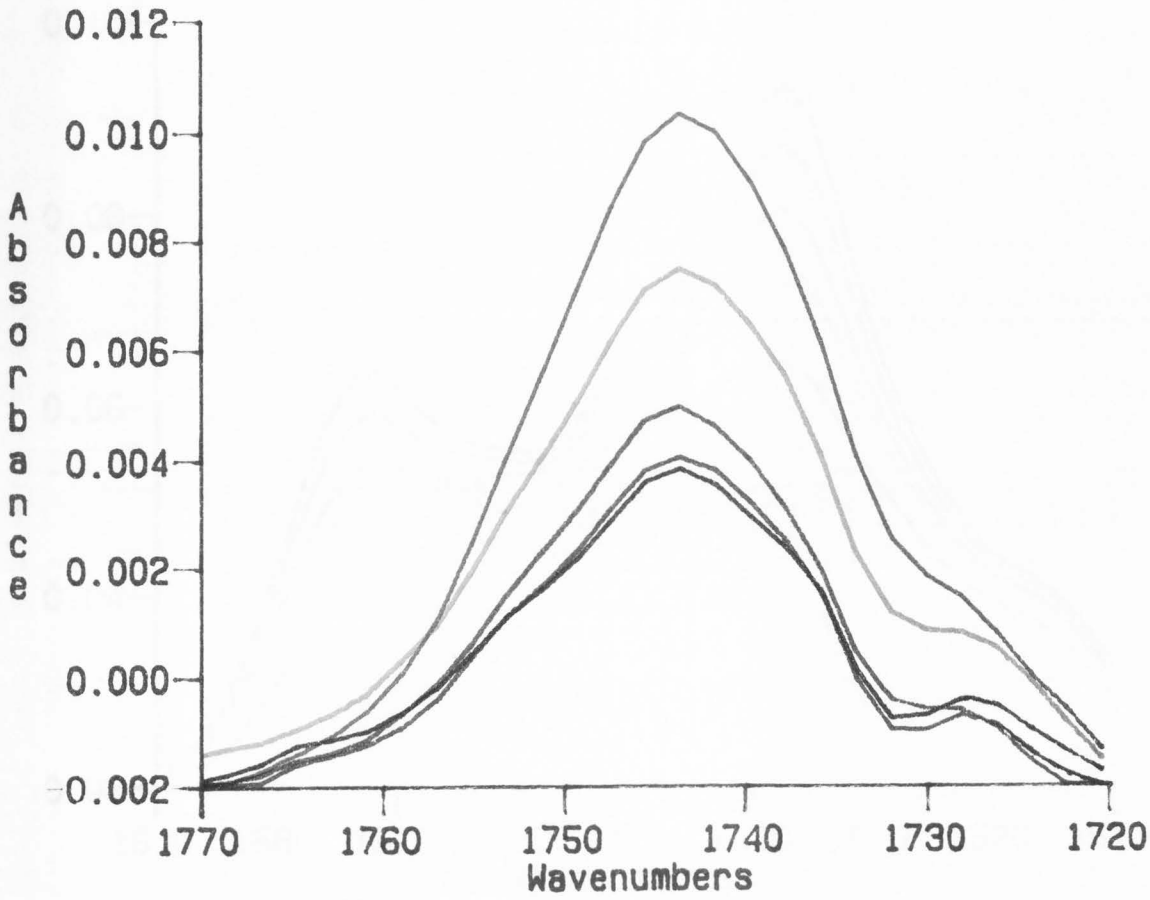

Figure 14. The effects of lipolysis on the "fat A" region of the infrared spectrum of milk (the spectra are keyed as follows: red = 13, yellow = 25, green = 37, blue = 49, and black = 60 min after enzyme addition).

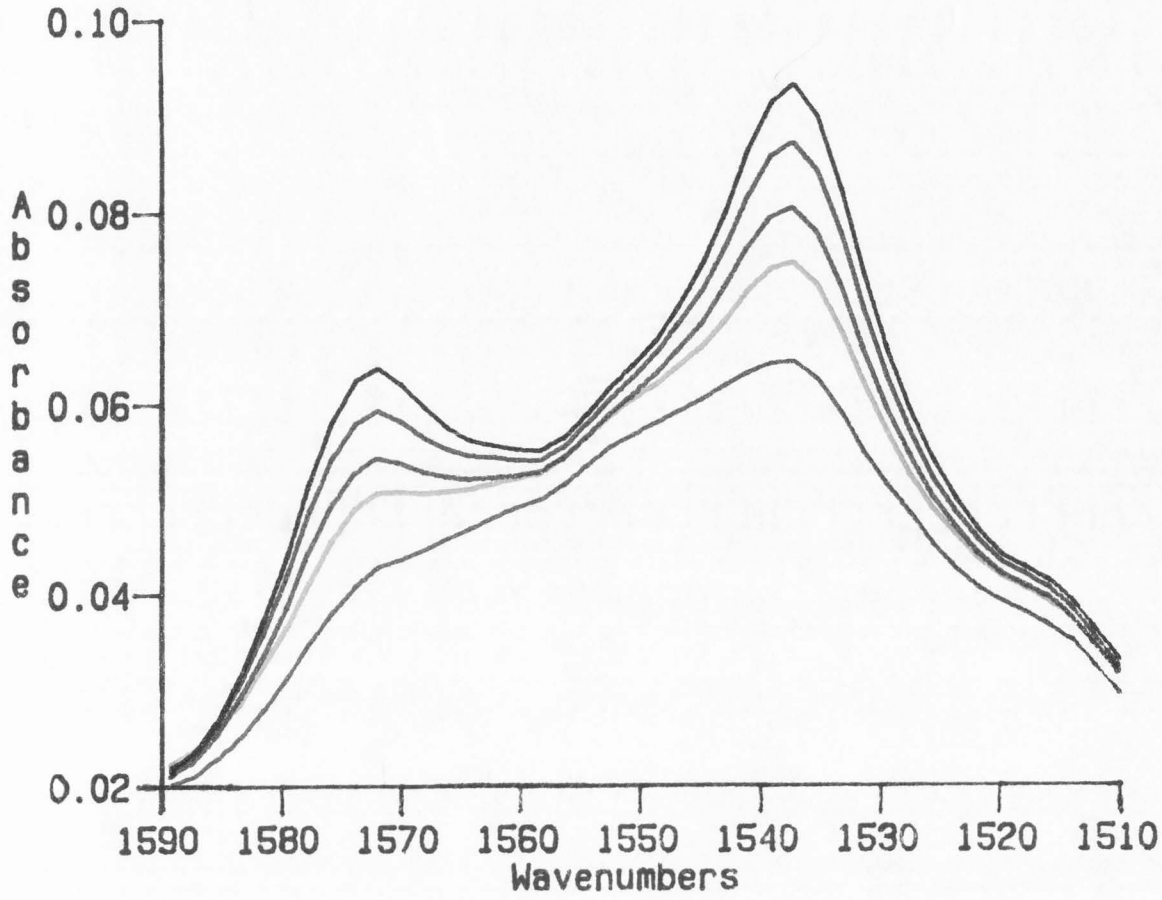

Figure 15. The effects of lipolysis on the protein (amide II) absorbance region of the infrared spectrum of milk (the spectra are keyed as follows: red = 13, yellow = 25, green = 37, blue = 49, and black = 60 min after enzyme addition).

Figure 15 shows that as lipolysis happens, the protein signal increases. In the literature, this increase in signal is attributed to absorption by ionized carboxyl groups of free fatty acids (23, 26). Our results show the ionized carboxyl group absorption band (at 1563 cm^{-1}) and the protein band (at 1538 cm^{-1}) are separate. We suspected that proteolytic activity of the enzyme caused the increase in absorption at the protein signal. When the experiment was repeated using skim milk, no increase in signal was seen (Figure 16). The reason for increase in absorbance at 1538 cm^{-1} remains to be determined.

Figure 17 shows that absorbance in the lactose region of the spectrum is unaffected by lipolysis. The results of this experiment show that with the exception of lactose, all the absorption bands used in modern infrared milk analyzers are affected by lipolysis.

Selection of Absorption Bands and Calibration Procedures

Absorption bands from 1283 to 1100 cm^{-1} respond to changes in fat, protein, and lactose concentration and show a low response to variation in saturation and mean molecular weight and lipolysis.

Many statistical methods were considered for incorporating the information in these absorption bands into a meaningful calibration. A method was desired that would allow us to overdetermine the calibration with respect to the number of absorption bands and calibration samples. Overdetermination decreases any systematic or random error in the calibration spectra thereby increasing the robustness of the calibration (9). Overdetermination using the K-matrix and P-matrix methods is described in the literature (4, 13). The K-matrix or classical least squares method allows for overdetermination of absorption bands and calibration samples, but all interfering chemical components in the spectral region of interest need to be known and their concentration included in the calibration.

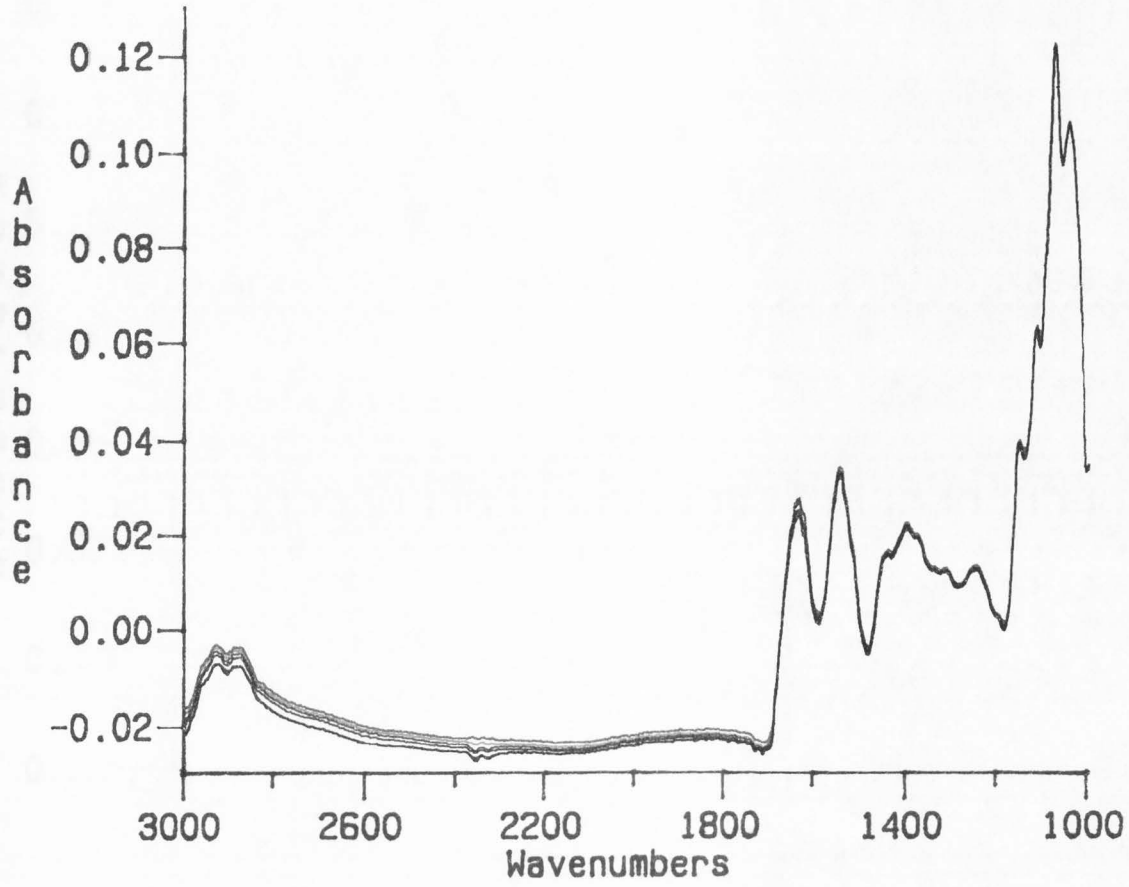

Figure 16. The effects of lipolysis on the infrared spectrum of skim milk (the spectra are keyed as follows: red = 9, yellow = 13, green = 25, blue = 37, and black = 49 min after enzyme addition).

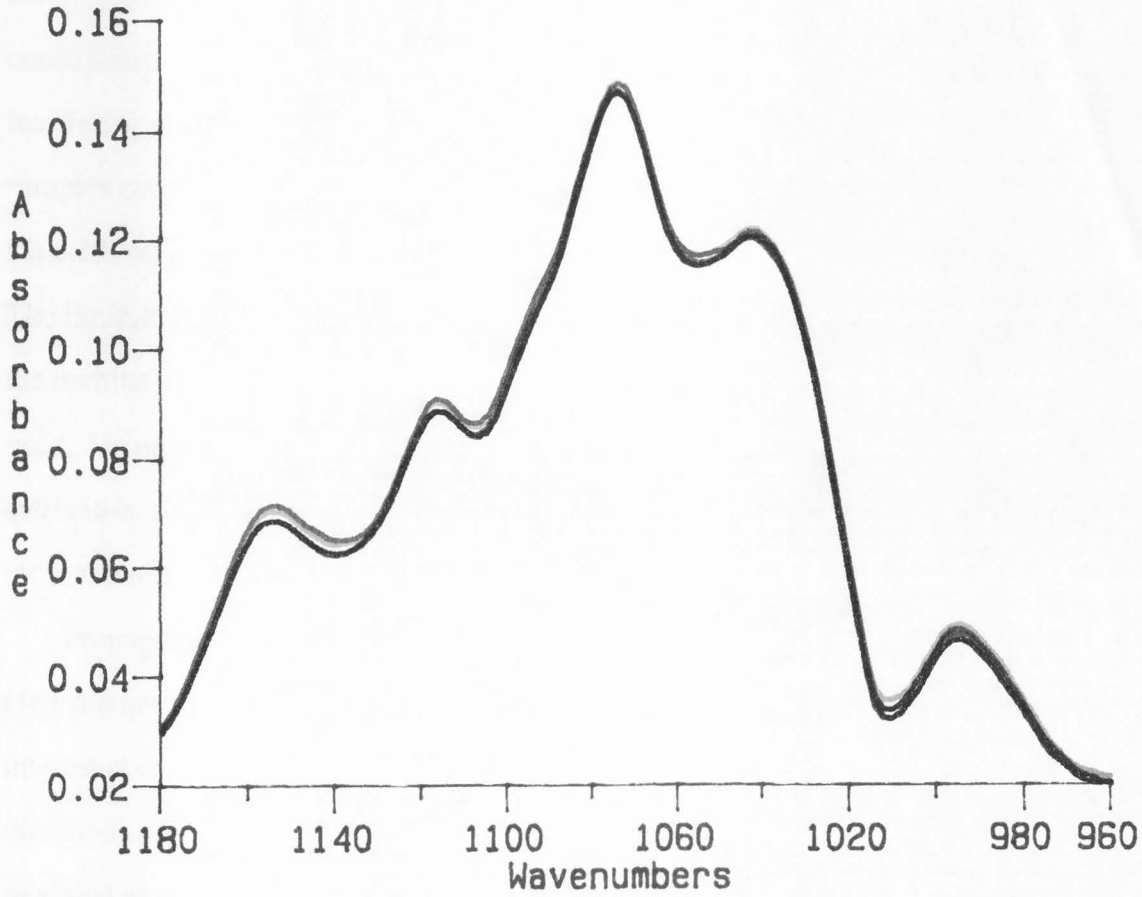

Figure 17. The effects of lipolysis on the lactose absorbance region of the infrared spectrum of milk (the spectra are keyed as follows: red = 13, yellow = 25, green = 37, blue = 49, and black = 60 min after enzyme addition).

The K-matrix method is well suited to solutions of pure components of known concentration instead of a biological solution like milk that contains many minor constituents. The P-matrix or inverse least squares method can accommodate interfering chemical components (impurities). To discriminate against impurities, samples containing the impurities at various concentration levels must be included in the calibration set, though the impurity concentrations themselves need not be known. The limitation of the P-matrix method that makes it unsuitable for our application is that the number of calibration samples must be greater than the number of absorption bands used. Using all 94 data points would require the preparation and analysis of at least 95 calibration samples. To make the P-matrix method more practical, the limitation of more references than frequencies must be overcome.

Principal component regression (PCR) (8) or partial least squares regression (PLS) (16), are used for this purpose. In both cases, the information in the spectral region of interest is compressed into a smaller number of factors by computing the orthogonal directions of the maximum variance in the spectral data. Unknown spectra are then modeled as linear combinations of these factors. In the PCR model, a factor may not contain any information about the components of interest but may be dominated by matrix effects for example (9). In the PLS model, concentration information is used so that only factors that are correlated with component concentrations are selected. This forces factors with high relevance for the chemical data, that may be ignored as noise in the PCR model, into the solution (16). We used the PLS method in our experiment because of these advantages.

The PLS algorithm requires assignment of a rank to the calibration. The rank of a calibration is the number of latent factors used to model the concentrations of components of interest in unknown samples. Assignment of a rank must be done carefully because the number of factors selected must be large enough to estimate the concentrations of components of interest and small enough to avoid including latent

factors composed largely of noise in the model.

Once a rank is assigned, concentrations of components of interest in unknown samples are estimated using the generated calibration model. For this experiment, a rank of 5 was assigned to the model. The samples from DQCI were used to calibrate the instrument. When infrared milk analyzers are evaluated, the results reported in the literature are based on prediction of component concentrations in samples used to generate the calibration, Table 4 summarizes the results when the fat, protein and lactose concentration in the samples used to calibrate the instrument were predicted.

TABLE 4. Predicted fat, protein, and lactose content in samples (DQCI) used to generate the calibration (SDD = standard deviation of the difference between reference and infrared methods).

Sample	Predicted Fat (% wt/wt)	(R-I)	Predicted Protein (% wt/wt)	(R-I)	Predicted Lactose (% wt/wt)	(R-I)
1	2.57	.08	3.26	-.02	4.85	.01
2	3.59	.37	3.22	-.10	4.81	.04
3	3.19	-.27	3.18	.04	4.83	-.03
4	3.61	-.09	3.20	.01	4.80	-.02
5	3.65	-.19	3.07	-.09	4.83	.01
6	4.62	-.02	3.69	-.04	4.77	.01
7	3.02	-.16	3.17	.02	4.83	-.02
8	3.64	-.04	3.17	.07	4.80	-.01
9	4.25	.44	3.37	.05	4.78	.03
10	4.23	.12	3.35	.09	4.83	-.01
11	4.70	-.05	3.28	-.02	4.85	.00
12	5.67	-.19	3.86	-.01	4.70	-.01
	SDD	**.22**	**SDD**	**.06**	**SDD**	**.02**

Table 5 summarizes the results when the fat, protein and lactose concentrations in the California samples were predicted.

TABLE 5. Predicted fat, protein, and lactose content in the California samples (Michelson Laboratories).

Sample	Predicted Fat (% wt/wt)	(R-I)	Predicted Protein (% wt/wt)	(R-I)	Predicted Lactose (% wt/wt)	(R-I)
1	3.91	-1.44	3.36	.08	4.81	- .08
2	3.23	.81	3.20	- .04	4.79	- .19
3	3.30	- .43	3.12	- .03	4.81	- .11
4	3.97	.70	3.17	- .15	4.79	- .26
5	2.78	- .94	3.24	- .07	4.83	- .27
6	4.03	.07	3.28	.02	4.79	- .14
7	3.40	- .58	3.26	- .15	4.79	- .15
8	5.73	1.80	3.50	.10	4.76	- .22
9	3.23	-1.27	3.52	- .07	4.82	- .27
10	6.48	1.34	4.05	- .21	4.69	- .19
11	6.94	2.04	3.97	- .19	4.67	- .31
	SDD	**1.23**	**SDD**	**.10**	**SDD**	**.07**

The standard deviation of the difference (SDD) is a commonly used measure of accuracy of the infrared method. The Association of Official Analytical Chemist's (AOAC) specification for accuracy of infrared milk analyzers is a SDD of not greater than .06 for each component, based on the samples used for calibration (2).

The bias in estimation of lactose in the Michelson samples was probably caused by the higher average lactose concentration in these samples relative to the calibration samples. The SDD for fat prediction in the Michelson samples is far from satisfactory.

Further investigation showed that absorbance of water in this region of the spectrum affected error in fat prediction and had little effect on estimation of protein and lactose. We attempted to compensate for water contribution by subtracting the spectrum of water from each sample until the absorbance at 2083.33 cm^{-1} (an unsaturated water absorption band that does not overlap a component absorption band) became zero in the resultant spectrum. However, some of the modified spectra appeared to have abnormalities caused by either over-subtraction or under-subtraction of water and when the calibration and prediction scheme outlined previously was done using these spectra, SDD's for fat, protein, and lactose were 1.25, .09, and .07%.

These observations led us to believe that the large error in fat prediction was a direct result of our inability to accurately and reproducibly subtract water. We optimized the water subtraction procedure by doing a linear regression for each sample using fat prediction error as the independent variable and the percentage of water subtracted from the sample spectrum (water subtraction factor) as the dependent variable. When the spectra obtained by subtracting the percentage of water corresponding to zero fat prediction error were used in the calibration and prediction scheme, the SDD for fat decreased to .02% and the SDD for protein and lactose did not change appreciably. Our original intent in optimizing the water subtraction factor was to correlate the optimized factors with some measurable component in the milk samples but we found no correlation. We speculated that preferential adsorption of fat onto the surface of the crystal in the ATR cell was causing our problem with fat prediction.

Sets of milk standard samples (similar to those used in previous experiments) were purchased from the same suppliers and the experiment was repeated using a transmission cell with calcium flouride windows and a 15 μm pathlength. The water subtraction approach (using the absorption band at 2083.33 cm^{-1}) showed no improvement in fat prediction. When the sample spectra were used in the calibration and prediction scheme without water subtraction, the standard deviations of difference

for fat, protein, and lactose prediction were .06, .11, and .05% in the calibration samples (Table 6) and .09, .10, and .16% in the separate set of samples (Table 7).

TABLE 6. Predicted fat, protein, and lactose content in samples used to generate the calibration. A transmission cell was used in this experiment.

Sample	Predicted Fat (% wt/wt)	(R-I)	Predicted Protein (% wt/wt)	(R-I)	Predicted Lactose (% wt/wt)	(R-I)
1	3.45	-.01	3.15	.07	4.90	.09
2	3.93	.10	3.32	.11	4.81	.02
3	4.01	.01	3.29	-.07	4.67	-.01
4	3.24	-.12	3.19	-.19	4.77	-.05
5	3.62	.08	3.46	.18	4.88	.06
6	3.91	.01	3.20	.05	4.78	-.06
7	5.84	-.02	3.68	.04	4.84	.04
8	4.63	.04	4.24	-.01	4.92	-.04
9	4.45	-.02	3.37	-.14	5.03	-.08
10	4.65	-.07	4.09	-.04	4.91	.03
	SDD	**.07**	**SDD**	**.11**	**SDD**	**.06**

TABLE 7. Predicted fat, protein, and lactose content in a set of samples used to validate the calibration. A transmission cell was used in this experiment.

Sample	Predicted Fat (% wt/wt)	(R-I)	Predicted Protein (% wt/wt)	(R-I)	Predicted Lactose (% wt/wt)	(R-I)
1	3.58	-.04	3.14	-.10	4.84	.00
2	4.79	.01	3.92	-.15	4.79	.04
3	3.85	.13	3.19	-.09	4.81	.03
4	3.60	-.15	3.19	-.04	4.81	-.39
5	3.25	-.03	3.17	-.07	4.84	-.20
6	3.72	.10	3.19	-.02	4.90	-.18
7	4.04	.03	3.51	.15	4.93	-.06
	SDD	**.09**	**SDD**	**.10**	**SDD**	**.16**

CONCLUSIONS

We conclude that there is information available in the infrrared spectrum for predicting the fat, protein, and lactose concentrations in milk. Current milk analysis technology uses only small pieces of this information.

Although variation caused by changes in saturation and chainlength of fatty acids was seen in all regions of the spectrum, the variation at the fatt A and fat B absorption bands is high compared to all others.

Absorption bands from 1283 to 1100 cm^{-1} allow us to accurately predict the fat, protein, and lactose concentrations in a diverse set of herd millk samples when appropriate sampling techniques are used. This instrumental method is as rapid as conventional filter instruments and has the additional advantage of minimizing errors caused by milk fat variation and lipolysis.

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PART 4. SPECTROSCOPIC DETERMINATION OF WHEY PROTEIN CONCENTRATE POWDER IN NONFAT DRY MILK

ABSTRACT

Infrared spectroscopy was used to measure the level of whey protein concentrate in an adulterated sample of NDM. Three samples of NDM (including high and low-heat processed samples) and three whey protein concentrate powders with protein and lactose concentrations similar to those in NDM (34% protein and 50% lactose) were obtained from various sources. One hundred and thirty five blends of NDM containing various concentrations of whey protein concentrate were analyzed with spectral information between 1400 and 1200 cm^{-1}. There was a strong correlation ($r > .99$) between predicted and measured concentrations of whey protein in adulterated samples. Accuracy was not affected by processing conditions, source of NDM, and origin of whey protein concentrate powder.

INTRODUCTION

Increased cheese production has increased the quantity of whey produced. In the past, whey was commonly dumped, a practice deterred by high sewage treatment costs that encouraged cheese manufacturers to find alternative, preferably profitable, uses for whey. Whey protein concentrates (WPC) are ingredients in a variety of processed foods. Commercial applications of WPC are limited compared to NDM, so WPC is less expensive than NDM (5). This makes it attractive to sell blends WPC/NDM blends labeled as NDM in violation of the Code of Federal Regulations (3). Measuring protein or lactose content of suspect samples will not detect and quantify WPC added to NDM. WPC can be manufactured to contain the same concentrations of protein and lactose as NDM.

Haarland and Ashworth (6) developed a turbidimetric method for estimating the amount of nondenatured whey protein in NDM to determine the baking quality of NDM solids. This method was later modified by Leighton (9). However, Basch et al. (2) found both the Haarland and Ashworth and Leighton methods inadequate to measure whey protein in WPC/NDM blends. Olieman and van den Bedem (12) determined rennet whey solids (levels greater than .8%) in skim milk powder by measuring glycomacropeptide isolated by high performance liquid chromatography (HPLC). This procedure cannot detect added whey powder produced by the direct acidification of cheese milk (5). Greenberg (5) described an amino acid analysis method that could detect added WPC at levels greater than 10%. The method works equally well with acid or sweet (rennet) whey and is not affected by heat treatment of the skim milk. Basch et al. (2) used an electrophoretic method to measure WPC (levels greater than 15%) added to NDM. Hill et al. (7) detected added WPC (levels greater than 10%) based on the difference in concentration of sulfhydryl groups in NDM and WPC.

These methods require substantial time for either preparation or analysis. Infrared spectroscopy has been widely used to quantitatively measure components in mixtures. It is rapid, nondestructive, and does not require that components be separated before measurement. The increased signal -to-noise ratio and computing ability achievable with commercially available Fourier transform infrared instruments has overcome the major disadvantages once associated with infrared methods.

Proteins have three characteristic absorbances in the infrared spectrum. Two of these, the amide I (ca. 1600 to 1700 cm^{-1}) and the amide III (ca. 1200 to 1400 cm^{-1}) absorbance bands are sensitive to the polypeptide backbone conformation and have been used to study the secondary structure of proteins (14). The sensitivity to secondary structure of proteins means that these absorption bands might be able to distinguish between proteins (11). The amide I band is more intense, but its overlap with an intense water deformation band at 1645 cm^{-1} means the spectrum of water must be subtracted or D_2O must be used as the solvent. Algorithms developed to subtract water from spectra (4, 13) often do not give reproducible results. D_2O solvent causes a shift in amide I vibrations and precludes the study of biologically intact systems (4). The amide III band, although less intense than the amide I band, has been used successfully to study the secondary structure of proteins (1, 8) and is not overlapped by water absorptions. For these reasons, we selected the amide III absorption band to detect adulteration of NDM with WPC.

MATERIALS AND METHODS

Three NDM and three WPC samples were used in the experiment. Two of the NDM samples (low-heat and high-heat processed) were from the American Dairy Products Institute, Chicago, IL. The third NDM sample was a commercially available low-heat processed sample from California Milk Producers, Artesia, CA. WPC powders with protein and lactose concentrations similar to those in NDM (34% protein and 50% lactose) were from Dairyland Products Inc., Savage, MN; Davisco International, Inc., St. Peter, MN; and Foremost Whey Products, Baraboo, WI.

Twelve calibration standards were prepared by combining one NDM sample with one WPC sample. The concentration of WPC powder in the standards ranged from .99 to 36.59% (dry basis).

Five blends of WPC and NDM (1.26, 5.00, 9.99, 18.00, and 33.00% WPC) were prepared for each possible combination of a NDM sample with a WPC sample for a total of 45 blends. Three repetitions of this preparation scheme resulted in a total of 135 blends. On day-1, the calibration standards and 45 of the blends (repetition 1) were analyzed. Blends in the other two repetitions were analyzed on separate days. Each sample was reconstituted on the day it was analyzed with 20 ml of distilled water for a total solids concentration of 10%.

The analysis was done by scanning blends sixty four times at 4 cm^{-1} resolution from 1400 to 1200 cm^{-1} using a Digilab FTS-7 FTIR spectrometer equipped with a deuterated triglycine sulfate detector. The 64 scans were averaged and ratioed against the spectrum of a blank sample cell to produce the final spectrum of each blend. The sampling accessory used was an attenuated total reflectance (ATR) cell with a zinc selenide crystal.

Calibration of the FTIR was done using partial least squares statistics (PLS) in a software package provided by Digilab, Cambridge MA. The PLS method has been

described by Martens and Jenson (10). Information contained in the spectral region of interest is compressed into a smaller number of factors by computing the orthogonal directions of the maximum variance in the spectral data. Unknown spectra are then modeled as linear combinations of these factors. Factors correlated with concentration information are selected for the model, thereby forcing minor factors with high relevance for the chemical data into the solution (10).

Calibration of the FTIR using PLS statistics is a two stage process. First, concentration and absorbance information from a set of standard samples is used to show the instrument what response to expect from given percentages of each component of interest. Then, the generated calibration is used to predict concentrations of components of interest in a separate set of standard samples called the validation set. The rank (number of factors used in the algorithm) of the model is varied and the process is repeated to minimize the difference between true and predicted concentrations in the validation set. For this experiment, the optimal calibration resulted from using a rank of 4. This calibration was then used to predict concentration of WPC in the 135 blends.

A split plot model was used in the analysis of variance. WPC, NDM, and repetition were the whole plot and concentration was the subplot. The variable of interest was the relative difference between the predicted and true concentrations of WPC in each blend.

RESULTS AND DISCUSSION

Spectra (1400 to 1200 cm^{-1}) of typical NDM and WPC samples are shown in Figures 18 and 19. The different absorbance characteristics of caseins and whey proteins in this region of the spectrum allow quantitative measurement of WPC in a blend of WPC and NDM.

Inspection of the results from prediction of WPC in the blends showed a bias in the data within repetitions as well as the presence of suspected outliers. The bias was attributed to instrument drift during the 8 h required for scanning of each replication set (45 samples). To compensate, the observations for the five samples of varying concentration in each WPC/NDM blend were normalized to a mean of zero. Six of the 135 values were farther than 3 SD away from the mean relative difference for their concentration groups. The corresponding values in the two replications not containing the outlier blend were averaged and used to replace each outlier and 6 degrees of freedom were removed from the error term in the analysis of variance (Table 8).

A diagram of predicted versus true concentration for the 135 blends is shown in Figure 20. The correlation coefficient for the means (n = 27) plotted in Figure 20 was $r > .99$. Variability decreased as concentration of WPC increased. In the analysis of variance (Table 8), concentration was the only significant variable $\alpha \leq .05$. When a Fishers least significant difference (LSD) test at $\alpha \leq .05$ was applied to the mean relative difference at each concentration, the 1.26% concentration was significantly different from the concentrations $\geq 5.00\%$.

TABLE 8. Analysis of variance table used to determine the treatment effects in the whey protein concentrate adulteration experiment.

Source	df	Mean Squares	F Ratio	Prob > F
Repetition	2	1.9020	.5776	> .25
NDM	2	.5355	.1620	> .25
WPC	2	2.9100	.8837	> .25
NDM × WPC	4	4.8662	1.4777	> .25
Whole plot error	16	3.2931		
CONC	4	59.7180	11.8022	<.001
CONC × NDM	8	1.1375	.2248	> .25
CONC × WPC	8	7.3111	1.4449	.25 > *P* > .10
CONC × NDM × WPC	16	6.6377	1.3118	.25 > *P* > .10
Sub plot error	66*	5.0599		
Total	134	6.1901		

WPC = source of whey protein concentrate, NDM = source of nonfat dry milk, CONC = concentration.

* six degrees of freedom removed for the six estimated values.

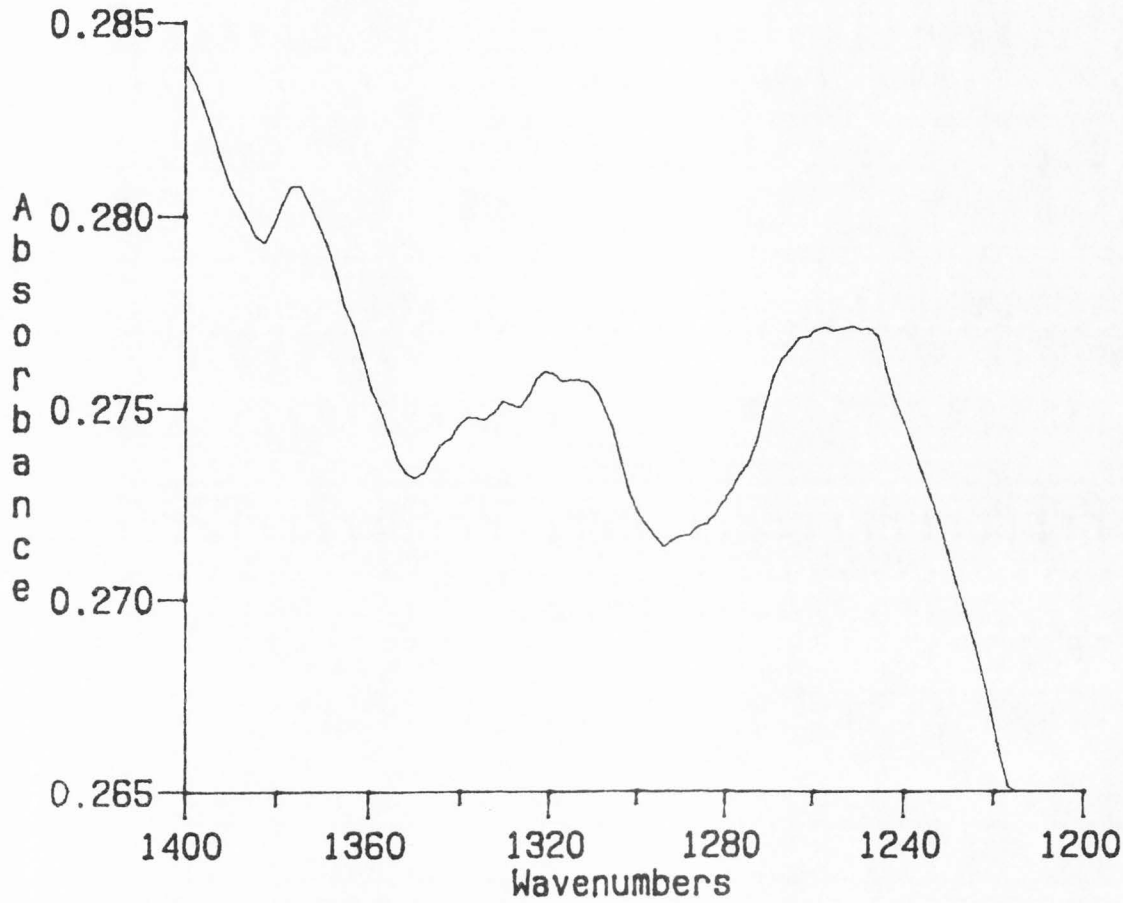

Figure 18. The spectrum (1400 to 1200 cm^{-1}) of a nonfat dry milk sample.

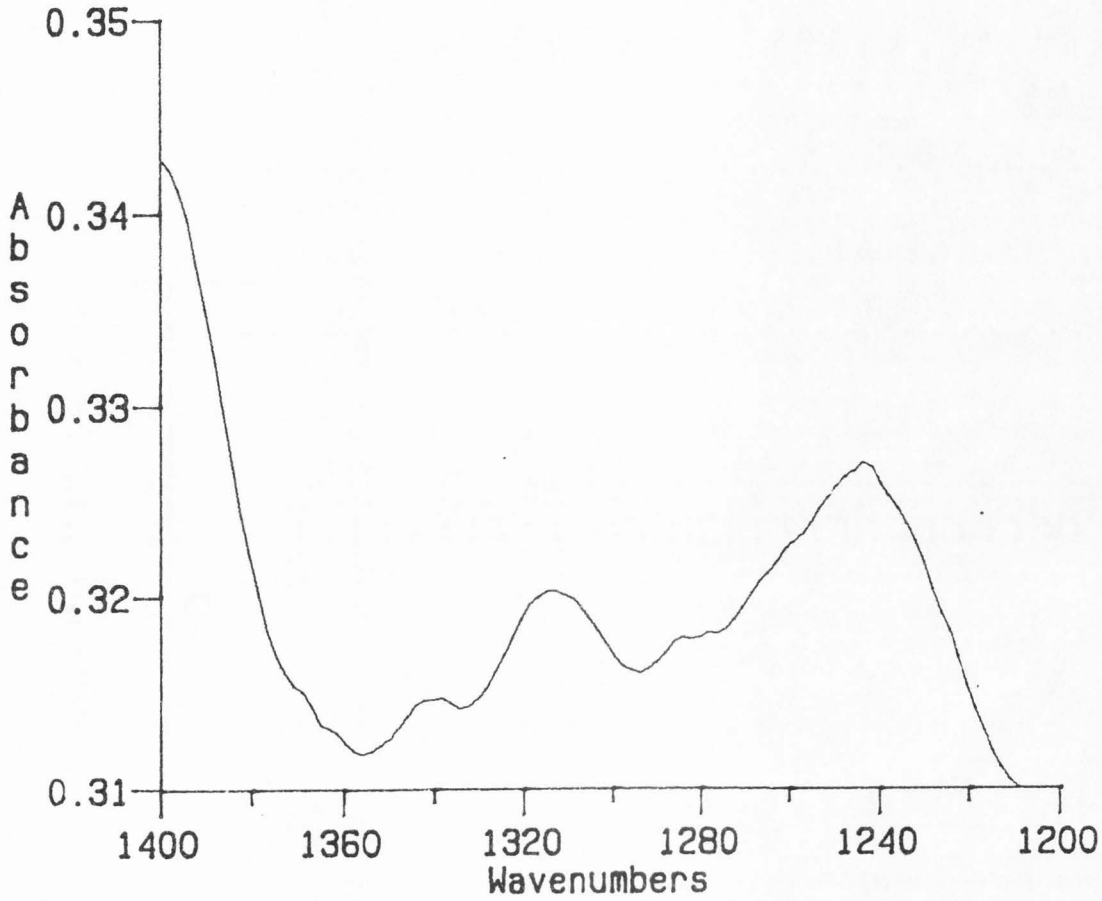

Figure 19. The spectrum (1400 to 1200 cm^{-1}) of a whey protein concentrate sample.

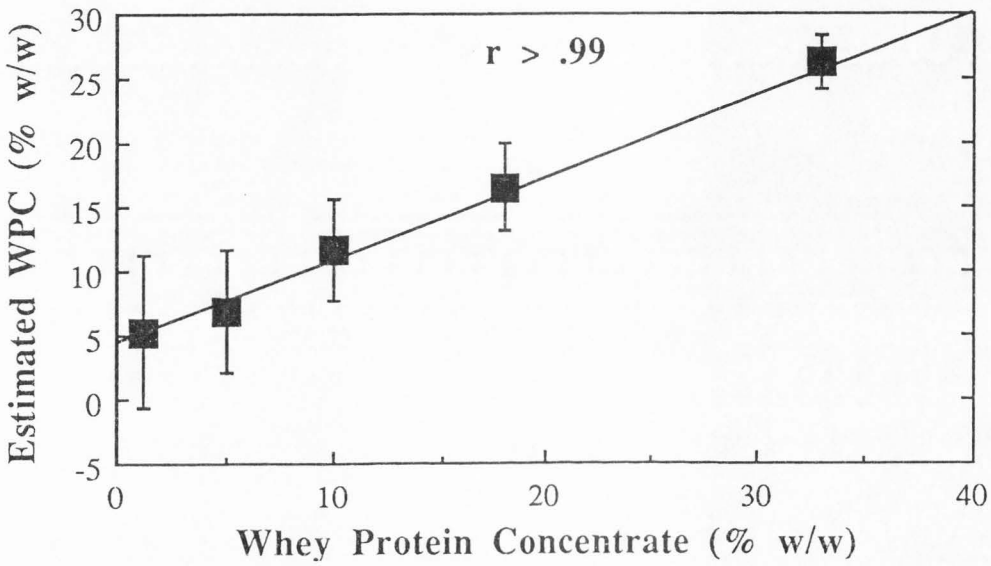

Figure 20. A scatter plot of true whey protein concentrate (WPC) concentration versus predicted WPC concentration for the 135 blends used in the experiment.

CONCLUSIONS

This method enabled us to predict the concentration of WPC in NDM with a correlation between measured and predicted WPC concentrations of $r > .99$. The method is independent of processing conditions or source of NDM and origin of WPC powder. Rapidity of this method is its main advantage. Calibration of the instrument required 1 h to complete. Once the calibration was completed, the turnover time for analysis of individual samples including analysis and cell cleaning and drying was 5 min.

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PART 5. DETECTION OF A NONDAIRY FAT IN PROCESS CHEESE

ABSTRACT

A rapid method for detecting partially hydrogenated soybean oil in process cheese was developed. Ten samples were prepared by combining various portions of an imitation process cheese made from partially hydrogenated soybean oil with real process cheese. The infrared spectrum of each sample was collected with a Fourier transform infrared spectrometer equipped with an attenuated total reflectance sample cell designed specifically for the analysis of solid samples. Use of this sample cell eliminated all sample preparation that was previously required for infrared spectroscopy of food materials. A linear relationship ($r = .9801$) was seen when the ratio of absorbance at 2957 cm^{-1} and 2852 cm^{-1} was plotted versus the percent adulteration.

INTRODUCTION

Adding vegetable fat to dairy products is a common form of food adulteration. The following spectroscopic methods are proposed to detect this type of adulteration.

Bartlet and Chapman (3) found that the ratio of infrared absorption by isolated trans double bonds at 967 cm^{-1} to absorption by cis-trans conjugated double bonds at 948 cm^{-1} is constant in butter fat. One hundred and eighty nine samples of butter fat were collected for 1 yr. A differential spectrum of a 4% solution of the fat in carbon tetrachloride was recorded using a 4% solution of pure butterfat in the reference beam. The percentage transmittance readings at 967 cm^{-1} and 948 cm^{-1} were subtracted from the readings at 920 cm^{-1} and plotted as the abscissa and ordinate of a graph (4). Ninety nine percent confidence limits were assigned to the line on the graph. Absorbance ratios fall outside of the confidence limits for samples adulterated with as little as 7% of various partially hydrogenated nondairy fats that contain predominantly isolated trans double bonds.

De Ruig (4) reported the ability to detect addition of commercial margarines, partially hydrogenated fats, and beef tallow to pure butter fat at the 5% level using a modification of the Bartlett-Chapman method. The modification is the use of pure sample and reference instead of a 4% solution in carbon tetrachloride.

Other infrared absorption bands that have potential for detecting adulteration are those from 3030 cm^{-1} to 2857 cm^{-1} that characterize various C-H absorptions; an olefinic C-H absorption occurring at 3030 cm^{-1} and aliphatic absorptions at 2941 cm^{-1} and 2857 cm^{-1}. Arnold and Hartung (2) reported a correlation ($r = .98$) of the iodine number of various food fats and oils with the ratio of absorbance at 3030 cm^{-1} to that at 2857 cm^{-1}. This relationship is valid for unprocessed fats and oils. However, hydrogenation of fats and oils has the effect of reducing the infrared ratio more than expected because trans double bonds result in less olefinic C-H absorption than cis

double bonds. The authors concluded that a prediction equation is valid only for a specific type of hydrogenated oil.

Anderson et al. (1) reported a linear correlation (r = .99) between unsaturation in various unprocessed fats and oils (including several milkfat samples) and the absorbance at 3030 cm^{-1} when differential infrared spectra are collected using a completely saturated substance (tristearin) in the reference beam. Hydrogenated samples deviate from the regression line confirming the findings of Arnold and Hartung (2).

The purpose of this experiment was to develop a simplified spectroscopic method for measuring addition of vegetable fat to dairy products.

MATERIALS AND METHODS

Pasteurized process American cheese and an imitation process cheese made with partially hydrogenated soybean oil were purchased from the dairy case in a local supermarket for use in this experiment. Eleven samples were prepared by combining various portions of the imitation process cheese with the real process cheese. The amount of imitation process cheese in the samples ranged from 0 to 100% in increments of 10%.

Each sample was prepared by first weighing the appropriate amounts of each cheese, blending the mixture, melting the mixture with continuous stirring, and pressing until cool to form a homogeneous slice of process cheese.

The infrared spectrum of each cheese slice was collected as follows. The cheese slice was placed on the surface of a zinc selenide crystal in a Contact Sampler™ sampling accessory manufactured by Spectra-Tech Inc. Stamford, CT and held against the crystal with a pressure device. The sampling accessory was placed in the optical bench of a Digilab FTS-7 (Bio-Rad, Digilab Division, Cambridge, MA) Fourier transform infrared spectrometer equipped with a deuterated triglycine sulfate detector. Sixty four scans of each cheese slice collected at 2 cm^{-1} resolution were averaged to produce the final spectrum. The Contact Sampler™ is a horizontal attenuated total reflectance (ATR) accessory. In the ATR technique, infrared radiation reflects through a crystal of high refractive index. At each reflection point, an evanescent wave penetrates into the sample that is in contact with one or more faces of the crystal. The penetration of the evanescent wave provides a short, reproducible pathlength.

RESULTS AND DISCUSSION

The spectra of samples containing 0, 50, and 100% imitation process cheese (Figures 21, 22, and 23) show how the ratio of absorbances at 2957 cm^{-1} and 2852 cm^{-1} is directly related to the amount of imitation process cheese in each sample. This is expected because the addition of imitation process cheese increases the mean saturation level of the fat in each sample proportionately. An abnormally high absorbance ratio was seen for the sample containing 20% imitation process cheese. We attributed this to inadequate mixing of the sample and discarded it from our sample set.

When a least squares line was fitted to the remaining ten samples (Figure 24), a correlation coefficient of .98 was seen that compares well with the correlation reported by Arnold and Hartung (2).

Absorbances at 967 cm^{-1} and 948 cm^{-1} were too weak to determine if the method of Bartlet and Chapman (3) would be useful in this application..

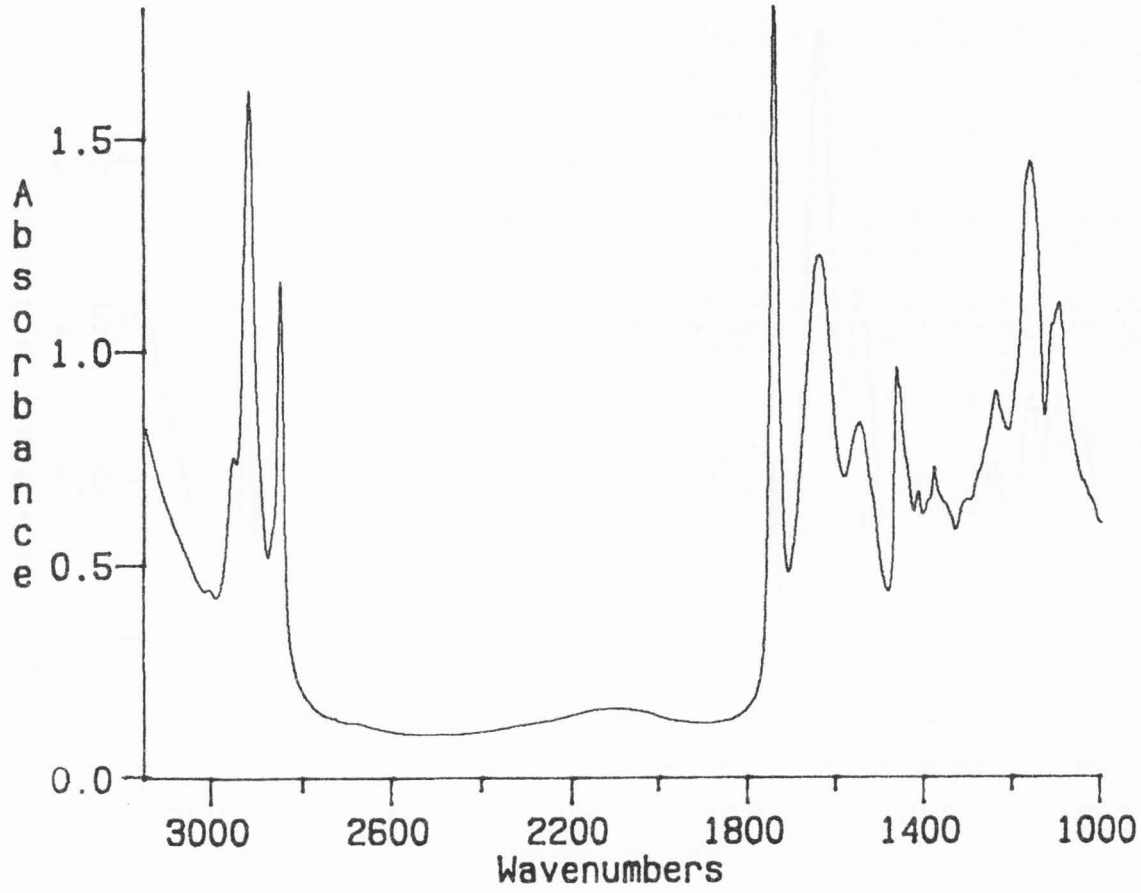

Figure 21. The infrared spectrum of a process cheese sample containing no imitation cheese.

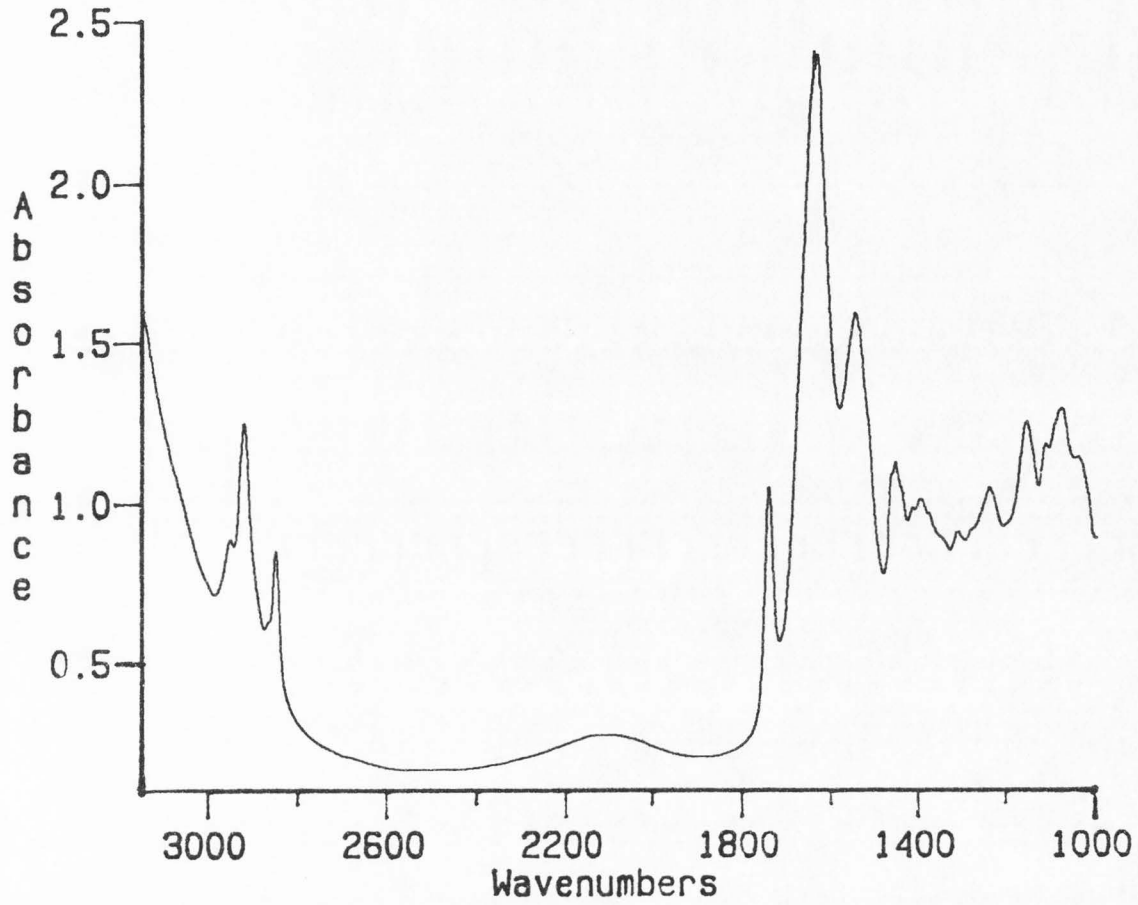

Figure 22. The infrared spectrum of a process cheese sample containing 50% imitation cheese.

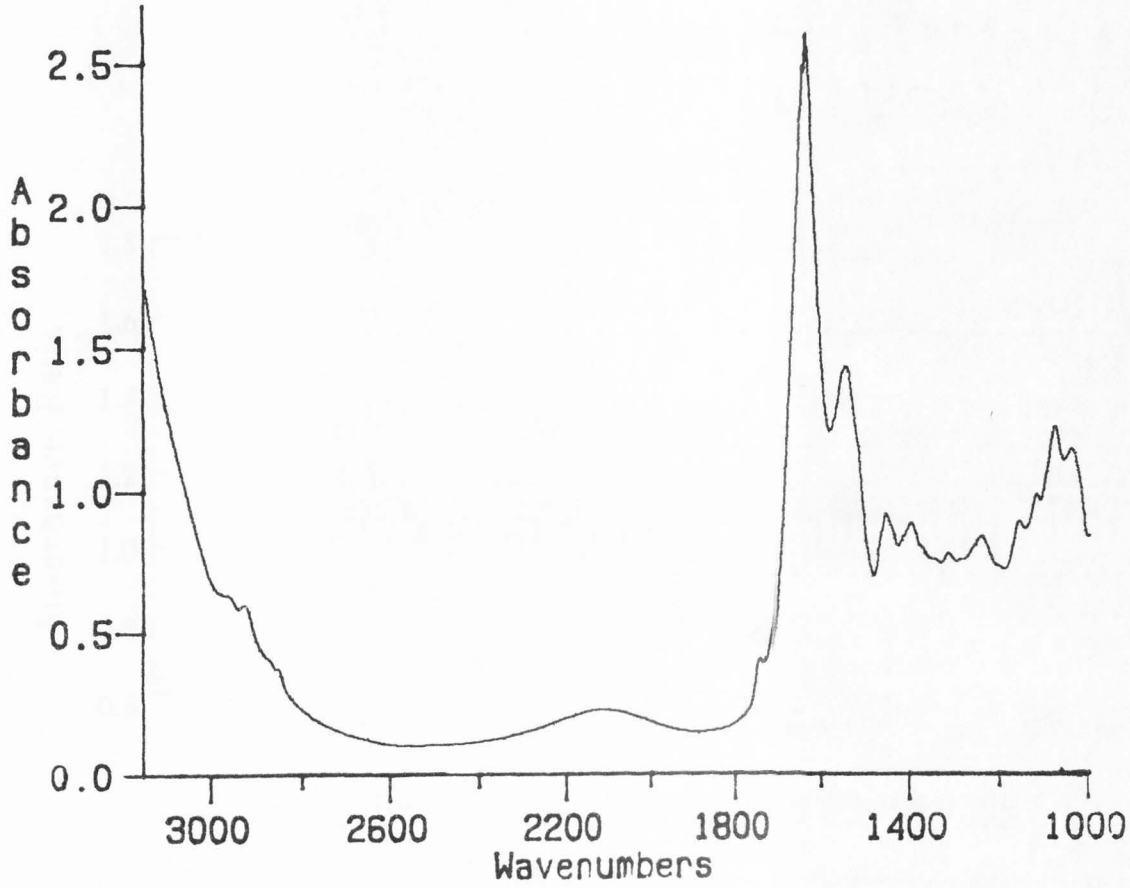

Figure 23. The infrared spectrum of an imitation process cheese.

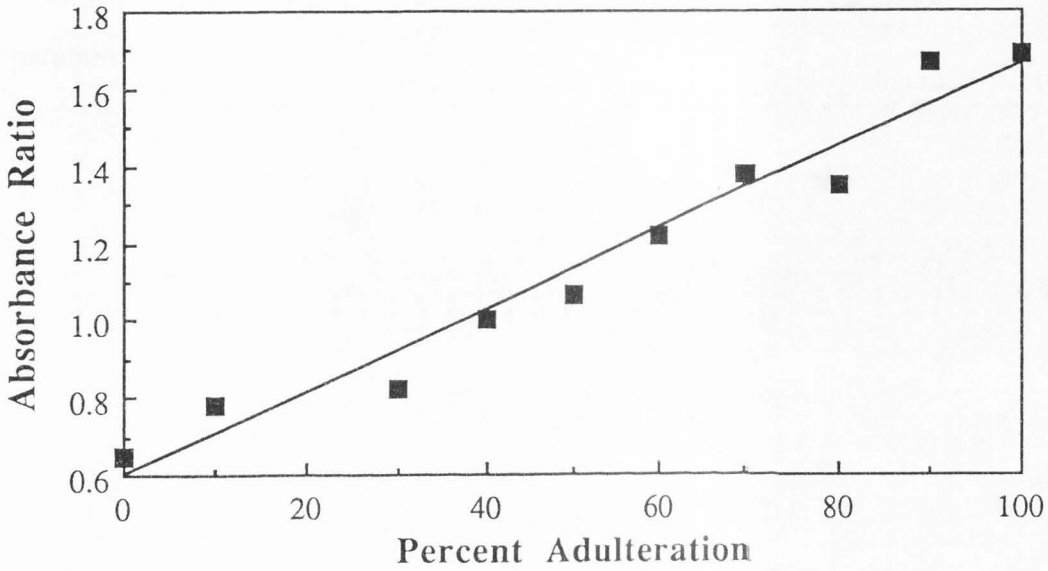

Figure 24. A plot of percent adulteration (with imitation cheese) versus the ratio of absorbance at 2957 cm^{-1} and 2852 cm^{-1}.

CONCLUSIONS

The infrared method described by Arnold and Hartung (Arnold and Hartung 1971) for determining the saturation level of fats and oils was easily adapted to the direct determination of partially hydrogenated soybean oil in pasteurized process American cheese. The Contact Sampler™ allowed us to get good results with no sample preparation.

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GENERAL SUMMARY

Information is available in the infrared spectrum for predicting the fat, protein, and lactose concentrations in milk. Current milk analysis technology uses only small pieces of this information.

Although variation caused by changes in saturation and chainlength of fatty acids was seen in all regions of the spectrum, the variation at the fat A and fat B absorption bands is high compared to all others.

Absorption bands from 1283 to 1100 cm^{-1} allow us to accurately predict the fat, protein, and lactose concentrations in a diverse set of herd milk samples when appropriate sampling techniques are used. This instrumental method is as rapid as conventional filter instruments and has the additional advantage of minimizing errors caused by milk fat variation and lipolysis.

Infrared spectroscopy coupled with multivariate statistical methods is a valuable tool for rapidly detecting adulteration of dairy products. Whey protein concentrate powder was detected in nonfat dry milk at concentrations as low as 5%. The method is independent of processing conditions of nonfat dry milk and origin of whey protein concentrate powder.

The infrared method described by Arnold and Hartung (2) for determining the saturation level of fats and oils was easily adapted to the direct determination of partially hydrogenated soybean oil in pasteurized process American cheese. The Contact Sampler™ allowed us to get good results with no sample preparation.

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VITA

Ivan V. Mendenhall

Candidate for the Degree of

Doctor of Philosophy

Dissertation: Rapid Determination of Milk Components and Detection of Adulteration Using Fourier Transform Infrared Technology

Major Field: Nutrition and Food Sciences

Education:

B. S. Utah State University, Logan, Utah. Major in Chemistry, minor in Japanese. 1987.

Ph.D. Utah State University, Logan, Utah, Nutrition and Food Sciences. 1991.

Experience:

Senior Research Technician, Utah State University, Logan (1988-1991). Planned and conducted research focused on improving existing methods and developing new methods for the analysis of dairy products using Fourier transform infrared spectroscopy.

Visiting Researcher, Institute Nationale Researches Agricole, Poligny, France (Summer of 1989). Assisted in the development of a method to monitor milk coagulation using Fourier transform near infrared spectroscopy.

Engineer Assistant, Morton Thiokol, Inc. Brigham City, Utah (1987-1988). Developed and implemented contamination controls in the manufacture of critical solid rocket motor parts. Evaluated and implemented instrumentation for environmental monitoring of "clean room" manufacturing areas.

Teaching assistant, Utah State University, Logan, Utah (1986-1987). Supervised general chemistry laboratories and tutored students enrolled in general chemistry courses.

Quality Control Technician, Con Agri, Inc., Hyrum, Utah (1983-1984). Analyzed beef by-products for protein content and beef tallow for purity.

Professional Memberships:

American Dairy Science Association (ADSA)
Agricultural and Food Chemistry (AGFD) division of Am. Chem. Soc.
Institute of Food Technologists (IFT)

Honors:

Gold Key National Honor Society

Presentations

Mendenhall, I. V. and R. J. Brown. 1990. Alternative wavelengths for infrared measurement of fat, protein, and lactose in milk. Presented at the 85th Annual American Dairy Science Association Meetings. Raleigh, NC.

Mendenhall, I. V. and R. J. Brown. 1991. Spectroscopic determination of whey protein concentrate powder in nonfat dry milk. Presented at the 201st National Meeting of the American Chemical Society. Atlanta, GA.

Personal Data:

Born 11 May 1961, Logan, Utah
Married Deana Farnsworth, 3 June 1983
Two children: Lindsay age 5, Jordan age 2